Original Article

Immune Response of Lion's paw Scallop *Nodipecten subnodosus* (Sowerby, 1835) Challenged with *Vibrio alginolyticus*

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Abstract

Immune response of Lion's paw scallop *Nodipecten subnodosus* to the challenge of *Vibrio alginolyticus* strain APSA2 was studied through the characterization of the activity of lysosomal enzymes and hemocyte size and number. Samples were obtained as hemocyte lysate supernatant (HLS) and plasma, after 6 and 24 hours, and 3, 6, and 10 days after challenge. The enzymatic activity was tested by colorimetric and lysoplate assay techniques. Between sampling times, significant differences in enzymatic activity determined by the colorimetric technique were found. The enzymatic activity was significantly higher in HLS than in plasma; in addition more enzymes were detected in HLS as compared with plasma. The enzymes with higher activity were leucyl arylamidase and esterase in both, HLS and plasma. Lysozyme-like activity did not show significant differences between the sampling times. However, in plasma the lysozyme-like activity was significantly higher than in HLS, suggesting that lysozyme was released from hemocytes into plasma as a first response to the challenge. A significantly negative correlation was found between protein concentration in plasma and in HLS. Based on the findings reported here, we can affirm that hemocytes and lysosomal enzymes are two of the mechanisms that this species has to fight bacterial attacks.

Keywords: hemocyte, hydrolytic enzymes, *Nodipecten subnodosus*, pectinid

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บทคัดย่อ

การตอบสนองทางภูมิคุ้มกันของหอยแครงตีนสิงโต *Nodipecten subnodosus* (Sowerby, 1835) ต่อเชื้อ *Vibrio alginolyticus*

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การศึกษาการตอบสนองทางภูมิคุ้มกันของหอยแครงตีนสิงโต *Nodipecten subnodosus* (Sowerby, 1835) ต่อเชื้อ *Vibrio alginolyticus* สายพันธุ์ APSA2 โดยพิจารณาจากกิจกรรมของไลโซโซมอลเอนไซม์ ขนาดและจำนวนของเซลล์เม็ดเลือด โดยทำการเก็บตัวอย่างจาก hemocyte lysate supernatant (HLS) และพลาสม่า ในเวลา 6 และ 24 ชั่วโมง และ 3 6 และ 10 วัน หลังจากการท้าทายด้วยเชื้อ การวัดกิจกรรมของเอนไซม์ทำโดยการวัดด้วยวิธี colorimetric และ lysoplate assay ผลการศึกษาพบว่ากิจกรรมของเอนไซม์ที่วัดด้วยวิธี colorimetric มีความแตกต่างกันเมื่อเก็บตัวอย่างที่เวลาต่างกัน และยังพบว่ากิจกรรมของเอนไซม์ใน HLS มีค่าสูงกว่าในพลาสม่าอย่างมีนัยสำคัญทางสถิติ นอกจากนั้นยังพบด้วยว่าใน HLS มีจำนวนเอนไซม์หลากชนิดว่าในพลาสม่า โดยเอนไซม์ที่พบว่ามีกิจกรรมสูงทั้งใน HLS และในพลาสม่า ได้แก่ leucyl arylamidase และ esterase ในขณะที่กิจกรรมของไลโซโซมอลเอนไซม์ ไม่พบว่ามีความแตกต่างกันเมื่อเก็บตัวอย่างที่เวลาต่างกัน อย่างไรก็ตามพบว่ากิจกรรมของไลโซโซมอลเอนไซม์ในพลาสม่ามีค่ามากกว่าใน HLS อย่างมีนัยสำคัญทางสถิติ เป็นข้อบ่งชี้ว่าไลโซไซม์อาจถูกหลั่งจากเซลล์เม็ดเลือดเข้าสู่พลาสม่า แสดงถึงการตอบสนองแรกต่อการท้าทายของเชื้อ นอกจากนั้นยังพบว่ามีความสัมพันธ์ในเชิงลบระหว่างระดับโปรตีนในพลาสม่ากับใน HLS จากผลการศึกษาดังกล่าวนักวิจัยเชื่อว่ากลไกในการตอบสนองของหอยชนิดนี้ต่อการติดเชื้อแบคทีเรีย อาศัยเซลล์เม็ดเลือดและไลโซโซมอลเอนไซม์

คำสำคัญ: เซลล์เม็ดเลือด ไฮโดรไลติกเอนไซม์ หอยแครงตีนสิงโต *Nodipecten subnodosus* pectinid

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Introduction

The Lion's paw scallop, *Nodipecten subnodosus* (Sowerby, 1835), constitutes an important fishery resource in Baja California Sur, México; however, its abundance has decreased throughout its distribution range. Due to the taste and size of its adductor muscle together with its high growth rate, nowadays several research institutions and private enterprises are attempting to develop a suitable culture technology (Koch et al., 2005). However, this activity is being carried out without proper scientific bases about its biology, so the outcomes are not entirely satisfactory far. Diseases are among the main issues that the culture of pectinids is currently facing, as these are difficult to detect in natural environment, so many of them have been scarcely studied (Barbosa et al., 2001). The incidence of bacteria of the genus *Vibrio*, which causes massive illness and mortality, is regarded as the most common sanitary problem found in culture systems of mollusc larvae (Beaz-Hidalgo et al., 2010; Romalde and Barja, 2010).

The mollusc's physiological defense system consists of both cellular and humoral immunity. Hemocytes are the most important cells involved in internal defense, circulating within an open vascular system across all epithelial boundaries and in extrapallial fluids (Allam and Paillard, 1998; Tiscar and Mosca, 2004; Lambert et al., 2007). For this reason, descriptive parameters such as concentration or size of hemocytes can provide information about cell division processes or infection (Lambert et al., 2007).

Enzymatic activity in the hemocytes' lysosomes has been studied as an indicator of immune capacity in many bivalve species such as *Crassostrea gigas* (Luna-González et al., 2004), *C. virginica* (Tiscar and Mosca, 2004; Romalde and Barja, 2010), *Mercenaria mercenaria* (Tiscar and Mosca, 2004; Buggé et al., 2007; Romalde and Barja, 2010), *Mya arenaria* (Pichaud et al., 2008), *Mytilus galloprovincialis* (Carballal et al., 1997; Torreilles et al., 1997) and *Tiostrea chilensis* (Cabello et al., 2005). In bivalves, hydrolytic enzymes participate in the elimination and degradation of microorganisms within and outside hemocytes (Luna-González et al., 2002; Buggé et al., 2007) where they can modify the molecular conformation of the cell membrane, thus facilitating its recognition and phagocytosis (Tiscar and Mosca, 2004; Romalde and Barja, 2010). The activity of lysosomal enzymes has been demonstrated in *C. gigas* and *Ostrea edulis* (Xue and Renault, 2000), *C. virginica, Corbicula japonica, M. arenaria, M. edulis* and *M. mercenaria* (Tiscar and Mosca 2004; Buggé et al., 2007), and *M. galloprovincialis* (Cajaraville et al., 1995). Thus, understanding the immune response of *N. subnodosus* to bacterial infections can facilitate the design of disease-control strategies in cultures of this species.

The aim of this work was to characterize the immune response of *N. subnodosus*, the Lion's paw scallop, by quantifying circulating cells and variations in the concentration of hydrolytic enzymes after being challenged with the bacterium *V. alginolyticus*, strain APSA2.

Materials and Methods

Experimental animals: Adult clams, ranging between 7 and 10 cm in height, from a suspended culture system located at the Guerrero Negro lagoon, BCS, Mexico, were used. Study specimens were acclimated for 10 days in 20-l plastic tanks containing filtered (5 µm) sea water and constant aeration. Food consisted in a mixture of *Chaetoceros gracilis* and *Tetracelmis* sp. (2:1).

Vibrio alginolyticus (APSA2): The bacterial strain (*V. alginolyticus* APSA2) used was provided by the Laboratory of Microbial Ecology at the Centro de Investigaciones Biológicas del Noroeste, SC. The strain was isolated from culture tanks of white shrimp post-larvae (*Litopenaeus vannamei*) at the aquaculture company Acuacultores de La Paz, SA. This strain is pathogenic to larvae of *Argopecten ventricosus, Atrina maura* and *N. subnodosus* (Luna-González et al., 2002). The strain was kept in Luria-Bertani Broth (LBB) containing 3% NaCl and 15% (v/v) glycerol, and stored at -80^{0}C. To determine the microbial count (colony forming units, CFU), the strain was grown in Trypticase Soy Broth (TSB, Difco) for 24 hours at 30^{0}C. The bacteria culture was centrifuged at 8000 x g for 10 min at room temperature and the cell pellet was re-suspended in one milliliter of sterile distilled water with 2.5% NaCl. Then, the bacterial suspension was adjusted to an optical density of one in a spectrophotometer (Thermo Spectronic Genesys 2, Thermo Scientific) at 580 nm. The serial dilution method was used to determine the microbial count (CFU/ml) in the bacterial suspension.

Median lethal dose (LD50): Prior to the assay, it was necessary to determine the LD50 of *V. alginolyticus.* To this end, five lots of eight randomly chosen, previously acclimated *N. subnodosus* specimens, were used. Each lot was infected by injecting the adductor muscle with one of the following concentrations of *V. alginolyticus:* 1x10^2, 1x10^3, 1x10^4, 1x10^5 and 1x10^6 CFU/organism. 12 specimens injected with saline solution (3% NaCl) were used as a control. Specimens were followed up for ten days; symptoms of Vibriosis and daily survival were recorded. The LD50 was calculated by using Probit analysis (Finney, 1952) with StatFi version 2009® (AnalystSoft Inc).

Specimen infection: Fifty healthy clams were used for the assay. Thirty of them were infected with a sublethal dose of bacteria by injecting the adductor muscle; the remaining 20 were injected with saline solution as control. All the specimens were kept for ten days in 20-l plastic tanks containing filtered sea water, under constant aeration, and were fed a mixture of *Ch. gracilis* and *Tetracelmis* sp. (2:1).

Hemolymph collection: Sampling took place at different times (6, 24 hours, and 3, 6, 10 days) after infection. On each occasion, six infected and four control specimens were randomly chosen. A 3 ml sample of hemolymph was drawn from each organism, by puncturing the hemolymphatic sinus of the adductor muscle with a sterile 5 ml plastic syringe fitted with a 21G x 32 mm needle. In order to prevent hemolymph coagulation after extraction, the syringe was previously rinsed with a cold 5% sodium oxalate solution in isotonic saline solution (450 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3) (Garcia-Carreño et al., 2008). Samples were immediately transferred to previously cooled, sterile Eppendorf tubes, which were kept cold throughout the manipulation.

Hemocyte quantification and measurement: Hemocytes were counted in a Neubauer chamber, from a 50 µl hemolymph sample drawn from either infected or control organisms. The hemolymph sample was diluted 1:1 (v/v) in a 4% formalin solution (Luna-González et al., 2004) to prevent the agglutination of hemocytes. Hemocytes were measured from digital photographs of hemolymph samples. Fifty hemocytes from each specimen were measured using the Sigma Scan Pro (V 5.0) software. The outline of each hemocyte was manually drawn and the lengths of the major and minor axes were measured by the software. Hemocyte diameter was calculated as the average length of both axes.

Separation of plasma and hemocytes: Immediately after extraction, hemolymph samples were refrigerated centrifuged at 500 x g for 10 min, separating the plasma in the supernatant and hemocytes in the pellet. Once separated, hemocytes were re-suspended and washed twice in previously cooled tris saline buffer (50 mM Tris; 400 mM NaCl, pH 7.5) by centrifuging as above. Finally, hemocytes were re-suspended in 2 ml of Tris–HCl buffer (250 mM Tris, pH 6.5).

Preparation of the hemocyte lysate supernatant (HLS): Hemocytes were lysed by subjecting them to two freezing-thawing cycles. The HLS was obtained by centrifuging the suspension in a refrigerated centrifuge at 20,500 x g for 30 min and discarding the cellular debris pellet.

Activity of hydrolytic enzymes: Samples from each time point (6, 24 hours, and 3, 6, 10 days) were pooled to make 4 ml samples of either plasma or HLS. Enzymatic activity was detected by means of the commercial kit API ZYM (BioMérieux, France), which has been successfully used in bivalve molluscs (Carballal et al., 1997; López et al., 1997; Luna-González et al., 2002, 2004). Duplicate measurements were taken using two reactive strips in each case (including control samples), adding 65 µl to each of the 20 wells followed by incubation at 37^0C for 4 hours. Afterwards, and once the strips reached room temperature, kit reagents A and B were added and readings were taken after 5-10 min under natural lighting. The intensity of the reaction was assessed based on the color scale provided by the manufacturer and transformed to nM of hydrolyzed substrate. Enzymatic activity was expressed in units representing the amount of hydrolyzed substrate in nM/mg of protein.

Detection of lysozyme-like activity: Agarose plates were used to detect lysozyme-like activity in plasma and hemocytes. A 4 mg/ml solution of the lyophilized bacteria *Micrococcus luteus* (Sigma) was prepared. One milliliter of this solution was taken and 14 ml of a 1% agarose solution prepared with Tris-HCl buffer (50 mM, pH 5.2) (previously kept at 40^0C) were added. The mixture was poured into Petri dishes (135 mm diameter, 15 mm depth). Once solidified, eight 6 mm-diameter wells were excavated in each Petri dish. Each well was added with 30 µl of each sample pool or control per time point. Four replicates were made (four wells each). In addition, diluted saliva (1:9 in saline solution 0.1% NaCl) was used as a positive control. After 24 hours of incubation at room temperature, the diameter of the lysis halo was measured (0.1 mm= 1 U). Results were expressed as units per milligram of protein (U/mg of protein). Protein concentration (plasma and HLS samples) was determined with Bradford's method (Campa-Córdova et al., 2002), using a commercial reagent (Sigma) and bovine albumin serum (Sigma) as a standard.

Statistical analysis: A one-way ANOVA was used to compare the variables recorded for each control between sampling times (five levels: 6, 24 hours, and 3, 6, 10 days). As no significant difference was found (p<0.05), all the results were pooled as one single control for subsequent analyses. Similarly, a one-way ANOVA was used to compare clam size between sampling times (six levels: control, 6, 24 hours, and 3, 6, 10 days) and, as no significant difference was found, a potential effect of clam size on the results was discarded. One-way ANOVA followed by Tukey's HSD tests were applied to compare the variables recorded between sampling times (six levels: control, 6, 24 hours, and 3, 6, 10 days). Also, correlation analyses between the diameter and the number of hemocytes, and between the lysozyme concentration and the hemocyte number were done. A significant level (α) of 0.05 was used for all the analysis.

Results

Sublethal dose: The control group and the groups infected with the lower bacterial concentrations ($1x10^2$ up to $1x10^4$ CFU/ml) showed a 100% survival throughout the assay. The lot infected with $1x10^5$ CFU/ml showed 87.5% mortality after 24 hours, and remained unchanged for the remaining of the assay. The lot infected with the highest bacteria concentration ($1x10^6$ CFU/ml) was the most severely affected: It showed a 75, 62.5, and 0% of survival after 24, 48, and 72 hours, respectively. The LD50 was 470,088 CFU/organism. However, the bacterial concentration of $1x10^5$ CFU/ml was considered as the sublethal dose to force an immune response having low mortality. The main symptoms observed in the moribund organisms included an abundant production of mucus in the mantle and a massive detachment of gill and adductor muscle.

Hemocyte quantification: Significant differences (p<0.05) were found in the number of hemocytes/ml in the control group and in the groups examined at different sampling times (Fig 1). Although no significant difference was found with respect to the control group ($8.29\pm0.78x10^6$ cells/ml), the average number of hemocytes 24 hours after the start of the assay was significantly higher (p=0.01; $12.34\pm0.96x10^6$ cells/ml) as compared to the number after 6 hours ($3.8\pm0.96x10^6$ cells/ml) and after 6 days (p=0.001; $4.05\pm0.87x10^6$ cells/ml). At the end of the assay (10 days), the number of hemocytes ($9.65\pm1.0x10^6$ cells/ml) increased significantly as compared to the number found after 6 hours (p=0.029), and 6 days (p=0.036).

Hemocyte diameter: Significant differences (p<0.05) were found in the hemocyte diameter when the control group was compared with the groups examined at different sampling times (Fig 1). Six hours after the start of the assay, the hemocyte diameter was significantly larger (p= 0.000; 16.15 ± 0.19 µm) than that of the control (15.4 ± 0.10 µm), decreasing significantly (p=0.001) after 24 hours (15.1 ± 0.19 µm). At the end of the assay, the hemocyte diameter was similar (15.3 ± 0.23 µm) to that in the control group.

Enzymatic activity: Hydrolytic enzyme activity was determined with the kit API ZYM, which did not reveal any clear trend. Out of the 19 enzymes that the kit can determine, 11 types of hydrolytic enzymes were found in HLS (Table 1). Significant differences were found both in enzymatic activity between sampling times (p=0.000) and in the activity of different types of enzymes (p=0.004). The enzymes with the highest activity in HLS were leucyl arylamidase and esterase. On the other hand, only 6 types of hydrolytic enzymes were found in plasma (Table 2). Significant differences were also found in the concentration of different types of enzymes

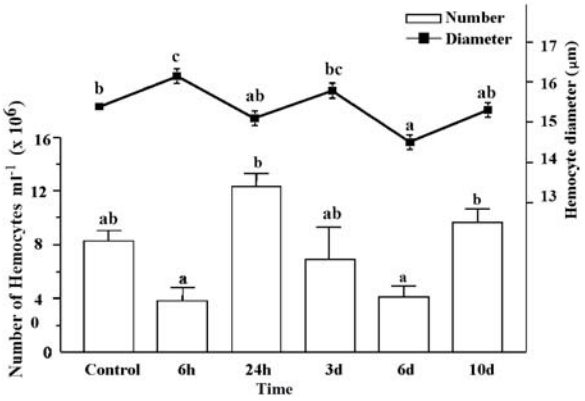

Figure 1 Number and diameter of hemocytes throughout the assay. For each variable, means not sharing the same superscript are significantly different. Bars correspond to standard deviations.

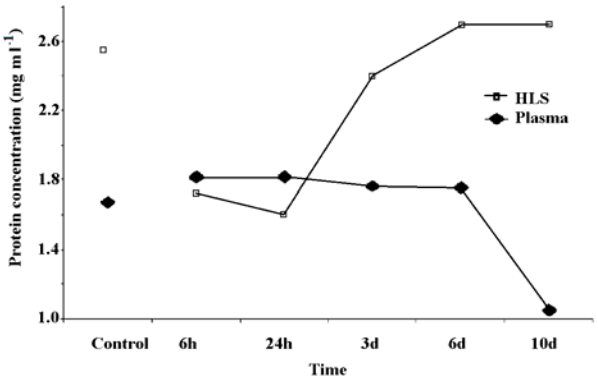

Figure 2 Protein concentration in plasma and HLS of *Nodipecten subnodosus* for control and at different times after infection with *Vibrio alginolyticus* APSA2.

(p=0.000), as well as between sampling times (p=0.000). As with HLS, the enzymes with the highest hydrolytic activity in plasma were esterase and leucyl arylamidase. Enzymes that either did not vary significantly or were not present in the assay included all glycosidases and most proteases, with the exception of leucyl arylamidase.

Lysozyme-like activity: No significant differences were found in the concentration of lysozyme between sampling times, neither in HLS (p=0.05) nor in plasma (p=0.05) (Tables 1 and 2). However, a noticeable increase (2.25 times) in lysozyme concentration was observed in HLS between 6 and 24 hours after the start of the assay. Lysozyme concentration in plasma 24 hours after the start of the assay was lower (4510 U/mg of protein) than the concentration observed 6 hours after infection (5302 U/mg of protein). On the other hand, the lysozyme concentration in plasma was significantly higher (p=0.000) than in HLS.

Protein determination: Figure 2 shows the variations in protein concentration observed in HLS and plasma of control and infected specimens throughout the experiment. A significant negative correlation (r=-0.54, p<0.05) was found between protein concentration in plasma and in HLS. As the assay proceeded, protein concentration in plasma tended to decrease, while it increased in HLS. Protein concentration in plasma decreased from the start (1.8199 mg/ml) until the end (1.0547 mg/ml) of the assay. The highest concentrations were observed in HLS by the end of the assay (between 6-10 days). Protein concentration increased from the lowest value recorded (1.6027 mg/ml) 24 hours after the infection, until the sixth day (2.6988 mg/ml).

Table 1 Enzymatic activity (means±SD) using the API ZYM kit (U hydrolysed substrate in nM/mg protein) and the lysoplate assay (U/mg of protein) detection in hemocyte lysate of *Nodipecten subnodosus* at different times from infection with *Vibrio algynolitucus*. For lysozyme, the diluted saliva (positive control) had an activity of 18000±769.33 U/mg of protein.

Enzymes	Control	6 hours	24 hours	3 days	6 days	10 days
Phosphatases						
Acid phosphatase	2.0±0.1	2.9±0.0	3.1±0.0	2.1±0.0	1.8±0.0	2.7±1.3
Naphthol phosphohydrolase	3.4±2.3	2.9±0.0	3.1±0.0	2.1±0.0	3.7±0.0	1.9±0.0
Alkaline phosphatase	2.1±0.6	1.4±0.0	1.6±0.0	1.6±0.7	0.9±0.0	1.9±0.0
Esterases						
Esterase (C1)	3.3±2.5	2.9±0.0	12.5±0.0	4.2±0.0	1.8±0.0	5.5±2.6
Lipases						
Lipase esterase (C8)	0.0±0.0	2.9±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Proteases						
Leucyl arylamidase	15.4±0.1	23.2±0.0	24.9±0.0	16.7±0.0	14.8±0.0	14.8±0.0
Valyl arylamidase	1.5±0.4	1.4±0.0	1.6±0.0	0.0±0.0	1.4±0.6	1.4±0.6
Cystyl arylamidase	5.2±0.9	2.2±1.0	0.0±0.0	1.0±0.0	1.4±0.6	1.4±0.6
α-chymotrypsin	0.6±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.9±0.0
Glycosidases						
β-galactosidase	1.8±0.1	1.4±0.0	3.1±0.0	1.0±0.0	1.4±0.6	0.9±0.0
β-glucuronidase	0.5±0.1	0.0±0.0	1.6±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Lysozyme	3134.7±48.6	1348.8±10.3	3028.5±24.7	1717.6±13.9	2481.8±20.4	2796.7±16.0

Table 2 Enzymatic activity (means±SD) using the API ZYM kit (U hydrolysed substrate in nM/mg protein) and the lysoplate assay (U/mg of protein) detection in plasma of *Nodipecten subnodosus* at different times from infection with *Vibrio algynolitucus*. For lysozyme, the diluted saliva (positive control) had an activity of 18000±769.33 U/mg of protein

Enzymes	Control	6 hours	24 hours	3 days	6 days	10 days
Phosphatases						
Acid phosphatase	3.9±0.8	0.0±0.0	1.4±0.0	0.0±0.0	1.4±0.0	0.0±0.0
Naphthol phosphohydrolase	1.3±1.0	1.4±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.4±0.0
Alkaline phosphatase	4.8±0.0	0.0±0.0	0.0±0.0	22.6±0.0	0.0±0.0	0.0±0.0
Esterases						
Esterase (C1)	11.6±5.4	11.0±0.0	6.9±5.8	5.7±0.0	14.2±4.0	9.5±0.0
Lipases						
Lipase esterase (C8)	2.4±0.4	0.0±0.0	1.4±0.0	0.0±0.0	0.0±0.0	9.5±0.0
Proteases						
Leucyl arylamidase	8.2±1.0	6.9±5.8	16.5±0.0	5.7±0.0	4.3±2.0	7.1±3.3
Valyl arylamidase	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Cystyl arylamidase	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
α-chymotrypsin	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Glycosidases						
β-galactosidase	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
β-glucuronidase	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Lysozyme	3774.7±22.9	5302.1±32.1	4510.4±10.1	5173.2±23.9	6129.8±40.4	4913.0±12.0

Discussion

In the assay conducted to determine the sublethal dose, the infection was controlled in almost all the bacterial concentrations tested. However, the highest concentration used (10^6 CFU/ml) was clearly beyond the immune response of *N. subnodosus*, leading to total mortality 72 hours after the start of the assay.

In invertebrates, one of the earliest signs of the immune response to an aggression is the variation in the number of circulating cells (Montes et al., 1997; Tiscar and Mosca, 2004; Hannam et al., 2010) although this variation sometimes resulted from air exposure (Malagoli et al., 2007), reduced nutritional activities (Donaghy et al., 2009), salinity (Malagoli et al., 2007; Matozzo et al., 2007), and temperature (Yu et al., 2009). Hemocytes constitute the first line of defense against pathogens and foreign particles. In this study, after 6 hours of the start of the assay a decreased number of hemocytes were observed as compared with the number in the control group (3.8±0.96 vs 8.3±0.78). Although this reduction was not statistically significant (p>0.05), it could be indicative of a defense response against bacteria, as hemocytes could have been lysed (Tiscar and Mosca, 2004; Buggé et al., 2007; Romalde and Barja, 2010), or some infiltration towards the tissues could have occurred in order to repair potential injuries (Barber, 2004; Tiscar and Mosca, 2004; Beaz-Hidalgo et al., 2010). Afterwards, the number of hemocytes increased significantly (p<0.05) after 24 hours of infection, compared to the number observed before (12.3±0.96 vs. 8.3±0.78). This number increase could be due to hemocyte hypersynthesis (Montes et al., 1997; Barber, 2004; Souza and Andrade, 2006) as an immune response to the infection aimed at keeping the immunological capability at an optimum level. A sudden rise in the number of hemocytes in response to an infection has been observed in other species such as *Biomphalaria glabrata* (Souza and Andrade, 2006), *C. virginica* (Barber, 2004; Beaz-Hidalgo et al., 2010; Romalde and Barja, 2010) and *Tapes semidecussatus* (Montes et al., 1997).

The variation of the size and internal complexity of hemocytes could be due to an external stress such as sudden algal blooms (Pierce and Henry, 2008), pollution (Donaghy et al., 2010; Hannam et al., 2010), temperature increase (Hegaret, 2003), and vibriosis (Araya et al., 2009). In this study, after 24 hours, the average size of hemocytes decreased significantly, relative to the size after 6 hours (p<0.05, 16.1±0.19 vs 15.1±0.19 μm), and along with a significant increase (p<0.05) in hemocyte number. These variations in size can be regarded as a new generation of cells produced by the organism in order to fight the infection, given that cell size increases as cells mature.

With regard to the determination of enzymatic activity, only 11 active enzymes were detected in HLS and 6 in plasma (out of the 19 that API ZYM kit detects). Higher enzyme concentrations were observed in HLS than in plasma. This difference has been previously reported for other species (*A. ventricusu, A. maura, C. virginica, C. gigas*) (Xue and Renault, 2000; Luna-González et al., 2004; Tiscar and Mosca, 2004; Romalde and Barja, 2010). A likely explanation for the presence of higher enzyme concentrations in HLS is that hemocytes synthesize these enzymes and, when their number and maturity increase, the concentration of enzymes also increases (Cheng, 1992). In addition, one of the strategies of bivalves to fight pathogens is the release of enzymes into the plasma by hemocyte degranulation and lysis (Tiscar and Mosca, 2004; Romalde and Barja, 2010) so that, once released, enzymes are capable of acting against pathogens.

In this study, the same enzymes detected in plasma were also found in HLS, so it can be assumed that all the enzymes detected in the plasma of *N. subnodosus* were produced by hemocytes. In this respect, it has been reported that some hydrolytic enzymes found in plasma likely originate in sites other than hemocytes, such as the digestive gland or

the intestine (Xue and Renault, 2000); however, this could not be confirmed in this work.

The enzymes found at higher concentrations, both in plasma and in HLS, were leucyl arylamidase and esterase. The presence of those enzymes has been reported by other authors (Carballal et al., 1997; López et al., 1997; Xue and Renault, 2000; Luna-González et al., 2004). Besides, the leucyl arylamidase and esterase are known to be in charge of bacterial recognition and degradation (Luna-González et al., 2004), so it can be assumed that these might represent the main mechanism of bacterial control in *N. subnodosus*.

On the other hand, the highest lysozyme concentrations were found in plasma, contrasting with the results from the quantification of the hydrolytic enzymes by API ZYM kit. This could be due to the fact that this enzyme was the first to be released by hemocytes, as lysozyme is known to be released into the plasma upon stimulation, either by infection or by any other foreign agent (Luna-González et al., 2002). In addition, lysozyme is a non-specific humoral component of the invertebrate immune system that functions as an antibacterial protein (Tiscar and Mosca, 2004; Buggé et al., 2007). However, some studies of indirect infections have found a higher lysozyme concentration in HLS than in plasma (Cronin et al., 2001; Luna-González et al., 2004). A direct infection produces a much faster and more evident immune response resulting in the release of lysozyme, which does not occur with an indirect infection.

In our study, a significant negative correlation was found between protein concentration in plasma and in HLS. As the assay proceeded, protein concentration in plasma tended to decrease, while it increased in HLS. Protein content is related to immune response in invertebrates. Downs et al. (2001) related increased protein content after exposure to immunostimulants to the protective effect of the immune system in grass shrimp, *Palaemonetes pugio*, against potential pathogens. Campa-Córdova et al. (2002) found a significant increase in protein content in *L. vannamei* hemocytes after exposure to 0.5 mg/ml of β-glucans.

Based on the findings reported here, we can affirm that the increase in hemocyte number and the release of enzymes into the plasma are two of the mechanisms that this species has to fight bacterial attacks. We can state that *N. subnodosus* possesses an efficient immune response against *V. alginolyticus* APSA2.

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References

Allam, B. and Paillard, C. 1998. Defense factors in clam extrapallial fluids. Dis Aquat Org. 33: 123-128.

Araya, M.T., Siah, A., Mateo, D.R., Markham, F., McKenna, P., Johnson, G.R. and Berthe, F.C.J. 2009. Morphological and molecular effects of *Vibrio splendidus* on hemocytes of softshell clams, *Mya arenaria*. J Shellfish Res. 28(4): 751-758.

Beaz-Hidalgo, R., Balboa, S., Romalde, J.L. and Figueras, M.J. 2010. Diversity and pathogenecity of *Vibrio* species in cultured bivalve mollusks. Environmen Microbiol Rep. 2(1): 34-43.

Barber, B.J. 2004. Neoplastic diseases of commercially important marine bivalves. Aquat Living Resour. 17(4): 449-466.

Barbosa, V., Luna-González, A., Aguirre-Guzmán, G., Riquelme, C. and Ascencio-Valle, F. 2001. Enfermedades microbianas de pectínidos cultivados en Iberoamérica. In: Los Moluscos Pectínidos de Iberoamérica: Ciencia y Acuicultura. A.N. Maeda-Martínez (ed). Editorial Limusa: 325-342.

Buggé, D.M., Hégaret, H., Wikfors, G.H. and Allam, B. 2007. Oxidative burst in hard clam (*Mercenaria mercenaria*) haemocytes. Fish Shellfish Immunol. 23(1): 188-196.

Cabello, A.E., Espejo, R.T. and Romero, J. 2005. Tracing *Vibrio parahaemolyticus* in oysters (*Tiostrea chilensis*) using a green fluorescent protein tag. J Exp Mar Biol Ecol. 27(2): 157-166.

Cajaraville, M.P., Pal, S.G. and Robledo, Y. 1995. Light and electron microscopical localization of lysosomal acid hidrolases in bivalve haemocytes by enzyme cytochemistry. Acta Histochem Cytochem. 28: 409-416.

Campa-Córdova, A.I., Hernández-Saavedra, N.Y., de Philippis, R. and Ascencio, F. 2002. Generation of superoxide anion and SOD activityin haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to β-glucan and sulphated polysaccharide. Fish Shellfish Immunol. 12: 353-366.

Carballal, M.J., López, C., Azevedo, C. and Villalba, A. 1997. Enzymes involved in defense function of haemocytes of mussel *Mytilus galloprovincialis*. J Invertebr Pathol. 70: 96-105.

Cheng, T.C. 1992. Selective induction of release of hydrolase from *Crassostrea virginica* haemocytes by certain bacteria. J Invertebr Pathol. 59: 197-200.

Cronin, M.A., Culloty, S.C. and Mulcahy, M.F. 2001. Lysozyme activity and protein concentration in the haemolymph of the flat oyster *Ostrea edulis*. Fish Shellfish Immunol. 11: 611-622.

Donaghy, L., Lambert, C., Choi, K.S. and Soudant, P. 2009. Hemocytes of the carpet shell clam

(*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): Current knowledge and future prospects. Aquaculture 297(1-4): 10-24.

Donaghy, L., Hong, H.K., Lee, H.J., Jun, J.C., Park Y.J. and Choi, K.S. 2010. Hemocyte parameters of the Pacific oyster *Crassostrea gigas* a year after the Hebei Spirit oil spill off the west coast of Korea. Helgoland Mar Res. 64: 349-355.

Downs, C., Fauth, J.E. and Woodley, C.M. 2001. Assessing the health of grass shrimp (*Palaemonetes pugio*) exposed to natural and anthropogenic stressors: A molecular biomarker system. Mar Biotechnol. 3: 380-397.

Finney, D.J. 1952. Probit Analysis. Cambridge University Press. Cambridge, England. 350 pp.

Garcia-Carreño, F.L., Cota, K., and Navarrete del Toro, M.A. 2008. Phenoloxidase activity of hemocyanin in white leg shrimp *Penaeus vannamei*: conversion, characterization of catalytic properties, and role in postmortem melanosis. J Agric Food Chem. 56: 6454–6459.

Hannam, M.L., Bamber, S.D., Galloway, T.S., Moody, A.J. and Jones, M.B. 2010. Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*. Chemosphere 78: 779-784.

Hegaret, H., Wikfors, G.H. and Soudant, P. 2003. Flow-cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation I. Haemocyte types and morphology. J Exp Mar Bio Ecol. 293(2): 237-248.

Koch, V., Mazon-Suastegui, J.M., Sinsel, F., Robles Mungaray, M., and Dunn, D. 2005. Lion's paw scallop (*Nodipecten subnodosus*, Sowerby 1835) aquaculture in Bahía Magdalena, México: effects of population density and season on juvenile growth and mortality. Aquaculture Res. 36(5): 505-512.

Lambert, C., Soudan, P., Dégremont, L., Delaporte, M., Moal, J., Boundry, P., Jean, F., Huvet, A. and Samain, J.F. 2007. Haemocyte characteristics in families of oyster, *Crassostrea gigas*, selected for differential survival during summer and reared in three sites. Aquaculture 270: 276-288.

López, C., Carballal, M.J., Azevedo, C. and Villalba, A. 1997. Enzyme characterization of the circulating haemocytes of the carpet shell clam, *Ruditapes decussatus* (Mollusca: Bivalvia). Fish Shellfish Immunol. 7: 595-608.

Luna-González, A., Maeda-Martínez, A.N., Sainz, J.C. and Ascencio-Valle, F. 2002. Comparative susceptibility of veliger larvae of four bivalve molluscs to a *Vibrio alginolyticus* strain. Dis Aquat Org. 49: 221-226.

Luna-González, A., Maeda-Martínez, A.N., Ascencio-Valle, F. and Robles-Mungaray, M. 2004. Ontogenetic variations of hydrolytic enzymes in the Pacific oyster *Crassostrea gigas*. Fish Shellfish Immunol. 16: 287-294.

Malagoli, D., Casarini, L., Sacchi, S. and Ottaviani, E. 2007. Stress and immune response in the mussel *Mytilus galloprovincialis*. Fish Shellfish Immunol. 23(1): 171-177.

Matozzo, V., Monari, M., Foschi, J., Serrazanetti, G.P., Cattani, O. and Marin, M.G. 2007. Efects of salinity on the clam *Chamelea gallina*. Part I: alterations in immune responses. Mar Biol. 151(3): 1051-1058.

Montes, J.F., Del Río, J.A., Durfort, M. and García-Valero, J. 1997. The protozoan parasite *Perkinsus atlanticus* elicits a unique defensive response in the clam *Tapes semidecussatus*. Parasitology 114: 339-349.

Pichaud, N., Pellerin, J., Fournier, M., Gauthier-Clerc, S., Rioux, P., and Pelletier, E. 2008. Oxidative stress and immunologic responses following a dietary exposure to PAHs in *Mya arenaria.* Chem Cent J. 2: 23-34.

Pierce, R.H. and Henry, M.S. 2008. Harmful algal toxins of the Florida red tide (*Karenia brevis*): Natural chemical stressors in south Florida coastal ecosystems. Ecotoxicology 17(7): 623-631.

Romalde, J.L. and Barja, J.L. 2010. Bacteria in molluscs: Good and bad guys. In: Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. A. Mendez-Vilas (ed). Formatex Research Center: 136-147.

Souza, S.S. and Andrade, Z. 2006. On the origin of the *Biomphalaria glabrata* hemocytes. Mem Inst Oswaldo Cruz. 101(1): 213-218.

Tiscar, P.G. and Mosca, F. 2004. Defense mechanisms in farmed marine molluscs. Vet Res Commun. 28(1): 57-62.

Torreilles, J., Guèrin , M.C. and Roch, P. 1997. Peroxidase-release associated with phagocytosis in *Mytilus galloprovincialis* haemocytes. Dev Comp Immunol. 21: 267-275.

Xue, Q. and Renault, Y.T. 2000. Enzymatic activities in European flat oyster, *Ostrea edulis*, and pacific oyster, *Crassostrea gigas*, hemolymph. J Invertebr Pathol. 76: 155-163.

Yu, J.H., Song, J.H., Choi, M.C. and Park, S.W. 2009. Effects of water temperature change on immune function in surf clams, *Mactra veneriformis* (Bivalvia: Mactridae). J Invertebr Pathol. 102(1): 30-35.