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Stress and immune response to bacterial LPS in the sea urchin *Paracentrotus lividus* (Lamarck, 1816)

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ABSTRACT

The immune system of the sea urchin species *Paracentrotus lividus* is highly complex and, as yet, poorly understood. *P. lividus* coelomocytes mediate immune response through phagocytosis and encapsulation of non-self particles, in addition to the production of antimicrobial molecules. Despite this understanding, details of exactly how these processes occur and the mechanisms which drive them are still in need of clarification. In this study, we show how the bacterial lipopolysaccharides (LPS) is able to induce a stress response which increases the levels of the heat shock proteins HSP70 and HSP90 only a few hours after treatment. This study also shows that LPS treatment increases the expression of the β-thymosin-derivated protein paracentrin, the precursor of antimicrobial peptides.

1. Introduction

In spite of the ostensible simpleness of the body structure of echinoderms, and, in particular, of sea urchins, their immune system is far from being well understood; it would seem to be specialized in performing a variety of functions. Echinoderm immune cells are a heterogeneous population, both at the morphological and at the functional level and their profile can vary from one sea urchin species to another in terms of morphology, abundance, size, roles and physiology. Sea urchin coelomocytes, the cells that circulate in the coelomic fluid, can be divided into four subpopulations: phagocytes, vibratile cells, colourless and red spherule cells, as described for *Strongylocentrotus purpuratus* [1–3] and for *Paracentrotus lividus* [4–6]. Coelomocytes can mediate immune responses through cytolysis [4,7], phagocytosis [8,9], encapsulation of non-self particles [3,10], graft rejection [3,11] and through the production of antimicrobial molecules [12–18]. The sea urchin immune system also includes multiple sets of lectins, proteins with different antimicrobial activities, Toll-like receptors [19,20] and associated signalling proteins [21]. In all likelihood, further components are still in need of description. For example, it is known that in echinoderms and, in particular, in the class Holothuroidea [22], the stress pathway is involved in the pathogen recognition process, however, nothing is still clear about the role of heat shock proteins in the sea urchin *P. lividus* during a bacterial infection.

HSPs proteins are ubiquitous and highly conserved stress proteins which play an important role in the response to potentially deleterious stress conditions, also acting to prevent cell toxicity and death by protecting cells and tissues against damage. In terms of apparent molecular mass, HSPs can be classified into five groups: HSP20-40, 60, 70, 90 and 100.

HSPs were first described as stress proteins appearing after a heat shock; however, we now know that they participate in a number of intracellular processes; acting, for example, as protein folding chaperones [23,24], involved in the protection against apoptosis [25], in inflammation [26,27], and in mediating extracellular effects on immune-active cells [28]. HSPs have the capacity to prevent inappropriate protein aggregation, to facilitate the gain of function of re-assembled proteins, and to mediate the transport of irreversibly damaged proteins to degradative organelles and proteasomes [29,30]. HSPs improve cellular survival by repairing denatured proteins, dissociating initial loose protein aggregates and ensuring the correct folding and translocation of proteins [24]. These functions are also important in physiological processes during de novo protein synthesis, as well as in the folding of nascent polypeptides and transport [31]. While they can mediate intercellular signalling, HSPs are also capable of binding to adjacent cells and of initiating signal transduction [32,33]. HSPs can be

Abbreviations: HSP, heat shock protein; LPS, Lipopolysaccharides; HPLT, hours post LPS treatment; aCF, artificial coelomic fluid; PA, phagocytic cells; CSC, colourless spherule cells; RSC, red spherule cells; VC, vibratile cells; QPCR, quantitative polymerase chain reaction

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viewed as survival proteins, possessing an intrinsic ability to confer protection against most apoptotic stimuli [34,35]. Amongst HSPs, the highly conserved HSP70 is present in low levels in many cells and tissues, and is upregulated by stress [36]. HSP70 is involved in the folding of nascent proteins and in the refolding of mature proteins [37,38], and in the translocation of proteins from one compartment to another in the cell [39]. Furthermore, they also influence the activities of intracellular signalling molecules involved in mitosis and inflammation. Helmbrecht et al. (2000) demonstrated that, in dividing cells, HSP70 was found associated with important cell cycle regulatory proteins as scaffold-promoting agents in complexes of mitogen-activated signalling proteins [40]. During the inflammatory response, HSP70 contributes to the processing and presentation of the antigen [41–44].

Furthermore, we also studied the production of the antimicrobial factor β-thymosin vertebrate, produced in response to the infection process. Thymosins are secreted by the thymus and were initially found in the calf thymus as lymphocytopoietic factors [45]. They play an important regulatory role in the immune, neuroendocrine and reproductive systems of the body [45–47]. Composed by small acidic polypeptides with molecular weights ranging from 1 to 15 kDa, they are classified into three groups: thymosin alpha, characterized by immunomodulatory effects [48]; beta and gamma, an actin-binding protein family with a variety of functions such as the stimulation of angiogenesis, the enhancement of wound and scarring healing and the regulation of the immune system [49,50]. Amongst these three groups, thymosin beta is highly conserved and consists of 40–44 amino acid residues; it has been widely detected in both vertebrates and invertebrates [51,52]. In invertebrates, β-4 thymosin was found to be involved in the immune defence system through the augmentation of haemocyte production [53] or, more in general, through haemocyte homeostasis [54]. In echinoderms, β-thymosin and, in particular, their derivate Paracentrin 1, a short peptide, was found to exhibit antimicrobial and antibiofilm activities [17,55–57]. However, not enough is known about the role of β-thymosin in *Paracentrotus lividus*.

Based upon these considerations, in the present study we studied the physiological response of *Paracentrotus lividus* after challenge with LPS. In particular, we examined the possible involvement of both cytoplasmic and mitochondrial HSPs in immune response and their modulation on a temporal scale. We also examined whether β-thymosin production is modulated following an infection process or whether it is constitutively expressed and present at detectable levels in coelomocytes ready to exert their function.

2. Materials and methods

2.1. Animals

A sample of 40 healthy, adult individuals of the sea urchin *P. lividus* were collected from the waters off Palermo, along the north coast of Sicily, at a depth of 5–10 m and lying near a *Posidonia oceanica* seagrass meadow. The animals were maintained at 15 °C in an aerated 110-L glass aquarium with filtered seawater and under a 10 h/14 h light/dark cycle. Seawater was prepared using Instant Ocean Sea Salt (Mentor, OH, USA) dissolved in deionized water corrected for salinity (38–39‰) and pH (8.1 ± 0.1). A small volume of water (10–20 L) was changed weekly, and the animals were fed once a week with commercial invertebrate food (Azoo, Taikong Corp., Taiwan).

2.2. Treatment of animals with LPS

Commercial *Escherichia coli* (055:B5) lipopolysaccharide (LPS) was resuspended in artificial coelomic fluid (aCF) (10 mM $CaCl_2$; 14 mM KCl; 50 mM $MgCl_2$; 398 mM NaCl; 1.7 mM Na_2HCO_3; 25 mM Na_2SO_4) as suggested by Terwilliger [58]. Aliquots of LPS solution at a final concentration of 2 μg/mL of coelomic fluid, were injected into the coelomic cavity of different adult specimens of *P. lividus* through the peristomal membrane to stimulate an infection with gram-negative bacteria. Control individuals were injected with aCF alone, without LPS. Subsequently, the CF (4 mL) was carefully collected from the oral region through the peristomal membrane by syringe, preloaded with the same volume of an isosmotic anticoagulant solution (ISO–EDTA; 0.5 M NaCl, 20 mM Tris-HCl, and 30 mM EDTA; pH 7.4), at 1, 3, 6, 24-h post LPS treatment (HPLT). Cells were washed by centrifugation (900 × *g* for 10 min at 4 °C), suspended in ISO–EDTA and counted using a Burker hemocytometer. Coelomocytes were then aliquoted in tubes at a density of 1×10^7 cells mL^{-1} and stored at 4 °C until further use.

2.3. Total coelomocyte count (TCC) and differential coelomocyte counts (DCC)

Total coelomocyte count (TCC) was performed using a Burker hemocytometer under a light microscope at 1000× magnifications (Leica DMLB equipped with a digital camera Leica DC 200, Germany). Count values were the average number of coelomocytes observed in 30 microscopic fields for each animal (~1000 coelomocytes). Dead cells were evaluated, using the eosin-y exclusion test (0.5% in ISO–EDTA), and excluded from the total and relative counting.

To evaluate the differential coelomocyte count (DCC), coelomocyte smear preparations were examined and the cells were classified as phagocytic cells (PC), colourless spherule cells (CSC), red spherule cells (RSC) and vibratile cells (VC), according to Matranga et al. (2006) [59], Smith et al. (2010) [60] and Arizza et al. (2013) [8]. Each coelomocyte subpopulation, at the different time steps, was normalized with the relative control subpopulation whose value was set to 1.

2.4. Coelomocyte lysate supernatant (CLS)

Coelomocytes suspended in ISO–EDTA were centrifuged, the supernatant was removed and the resulting pellet was lysed in RIPA buffer (50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) in the presence of an anti-protease cocktail (pepstatin A, E−64, bestatin, leupeptin, aprotinin, and AEBSF 0.1% final concentration) (Sigma), incubated on ice for 20 min, then sonicated (Branson, Model B15, Danbury, CT) at 4 °C for 20s (1 pulse/s, 70% duty cycle) and finally centrifuged at 27,000 × *g* for 20 min at 4 °C to remove any debris. Protein concentrations were determined by means of Quibit fluorimetry (Quibit 2.0 Fluorometer), and sample aliquots were stored at −80 °C until further use.

2.5. Extraction of RNA from coelomocytes (CLS)

Total RNA from control and treated immune cells was extracted using the Direct-Zol RNA Miniprep Kit according to the manufacturer's instructions (Zymo research) and quantified using a bio-photometer (Quibit 2.0 Fluorometer). The RNA quality was verified through agarose gel electrophoresis at 1.5%.

2.6. Real-time PCR (RT-PCR)

Quantification of gene expression was performed using QuantiTect SYBR Green RT-PCR kit as described in the manufacturer's manual (Qiagen). The 18S gene [61] was used as the internal reference. The primer sequences for HSP90, HSP70, HSP60, HSP56 [62] and β-Thymosin used in RT-PCR analysis are shown in Table 1. The RT-PCR was run as follows: Reverse transcription 1 × cycle at 50 °C for 30 min, PCR initial activation step at 95 °C for 15 min and 40 cycles, denaturation 15 s 95 °C, annealing at 50 °C (HSP90 and β-Thymosin) or 60 °C (HSP70, HSP60 and HSP56) and 30 s at 72 °C for extension.

2.7. Electrophoresis on SDS polyacrylamide gel (SDS-PAGE)

Procedures for ten percent sodium dodecyl

Table 1
Primer sequences for HSP90, HSP70, HSP60, HSP56 [62] and β-Thymosin used in RT-PCR analysis.

Hsp90	Forward 5'	CCGATGCTTTGGACAAGATTC - 3'
	Reverse 5'	ATGTCAGCCTTGGTCATTCC - 3'
Hsp70	Forward 5'	CAGAACCACGCCCAGCTATG - 3'
	Reverse 5'	GCTTGGATGCTACTATCGTTG - 3'
Hsp60	Forward 5'	GAATATCCAGTGTACTCCGAC - 3'
	Reverse 5'	GCATCAGCTAAGAGGTCAAC - 3'
Hsp56	Forward 5'	GGAGCTATGCTAAGGACATC - 3'
	Reverse 5'	CTACAGCCTTAGCGACAGTG - 3'
β-Thymosin	Forward 5'	CGACAAACCAGACGTCAGC - 3'
	Reverse 5'	CGGTCTTCTCCTGCTCAATG - 3'

sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed according to Laemmli (1970) [63]. In brief, this involved the following: after determining the total protein contents of CLS, 10 µg of protein from each sample were added to the sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol 10% SDS, 0.05% bromophenol blue, SB) containing 5% β-mercaptoethanol. The mixed samples were then denatured for 10 min at 100 °C. Proteins obtained were compared with those of standard proteins (SDS-PAGE Colour Prestained Protein Standard, Broad range, Euroclone) so as to calculate the molecular weight of each band.

2.8. Immunoblotting

After SDS-PAGE migration, the proteins pattern was transferred to a nitrocellulose sheet (Hybond-ECL Amersham Pharmacia Biotech) according to Towbin et al. (1979) [64], using the Bio-Rad *Trans*-Blot SD Semi Dry Transfer Cell appliance. Immunoblots were performed according to Celi et al. (2012) [65]. Nitrocellulose sheets were soaked for 1 h at 20 °C with 5% bovine serum albumin (BSA) in 10 mM Tris-Base, 150 mM NaCl, pH 7.4, 0.1% Tween 20 (TBS-T) and were then incubated overnight at 4 °C in a humid chamber with gentle stirring, in a solution containing the appropriate *anti*-HSP antibody: *anti*-HSP90 monoclonal antibody produced in mice (Sigma Aldrich) 1:1000 in 3% BSA in TBS-T; *anti*-HSP70 monoclonal antibody produced in mice (Sigma Aldrich) 1:1000 in 3% BSA in TBS-T for CLS. After incubation, membranes were incubated for 1 h at 20 °C with a diluted solution of the secondary antibody anti-mouse IgG conjugated with alkaline phosphatase (Sigma Aldrich) (1:10000 anti-mouse IgG in TBS-T) in order to highlight the presence of HSP70 and HSP90. Immunoreactivity was then demonstrated using a mixture of BCPI-NBT (Sigma Aldrich). Densitometric analysis of the band intensities was performed using Image-J software through the evaluation of the stained protein band intensities as integrated optical density values (IDVs). Integrated density corresponds to the sum of the values of the pixels in the image or selection and is equivalent to the product of area and mean gray value. In each experiment, the control value was set as 1 and the treated values were normalized to the control.

2.9. Dot blot test

Ten micrograms of protein from each sample were applied to a nitrocellulose filter pre-soaked in PBS and air dried for 15 min. The filter was blocked in 1% BSA in PBS for 30 min; then, the membrane was incubated overnight at 4 °C, in a humid chamber with gentle stirring, with a diluted solution (1:500 in 3% BSA-TBS-T) of rabbit anti-Thymosin β10 polyclonal antibody (Santa Cruz); after incubation and TBS washing, membrane was incubated at 20 °C with a diluted solution (1:5000 in TBS-T) of the secondary antibody donkey anti-rabbit IgG (Sigma Aldrich), conjugated with alkaline phosphatase, for 1.5 h. After washes, the filter was treated with the appropriate mixture of BCPI-NBT, as described for immunoblotting. Stained dots on a white background indicated positive results. The densitometric analysis of the band intensities was performed using Image-J software.

2.10. Statistical analysis

A total of 12 untreated (control) and 12 treated specimens (3 animals for each time point) of sea urchins were used. The experiments were performed at least in triplicate. For immune cells counts, immunoblotting and dot blot test, the significance of the results obtained was assessed through the Student's t-test, with differences being considered significant at $p < 0.05$. Statistical analysis was performed on TCC and qPCR values obtained using the one-way ANOVA analysis of variance test followed by Tukey's multiple comparison test using R statistical software (http://www.r-project.org).

3. Results

3.1. Coelomocyte cells counts

To estimate variations in the number of coelomocyte populations after LPS treatment, both the total coelomocyte count (TCC) (Fig. 1) and the differential coelomocyte count (DCC) (Fig. 2) were monitored at different experimental times.

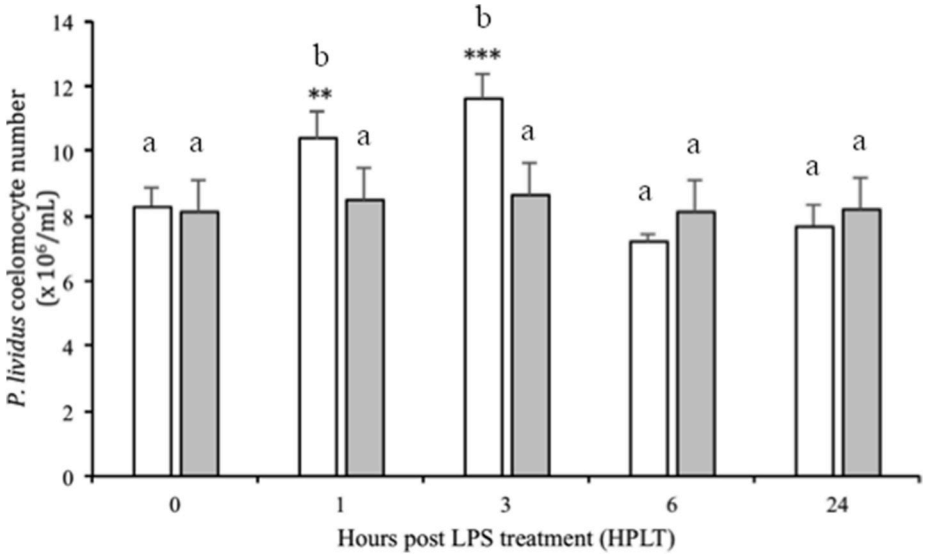

Fig. 1. Total coelomocyte count.
Total coelomocyte count of *P. lividus* specimen treated (□) or untreated (■) with LPS solution. Data values were the average numbers of coelomocytes observed in 30 microscopic fields for each animal (~1000 cells) using a Burker hemocytometer, at 0, 1, 3, 6 and 24 HPLT; ** = $p < 0.01$; *** = $p < 0.001$. Distinct letters show significant differences ($p < 0.05$). Error bars represent standard deviation.

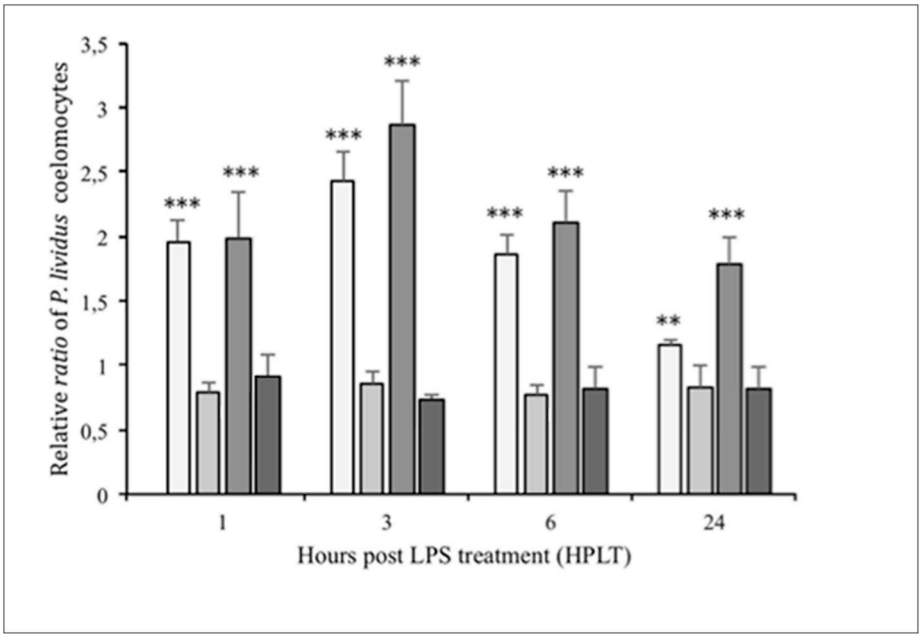

Fig. 2. LPS coelomocytes subpopulation modulation.
Effect of LPS treatment on the relative number of phagocytic cells (□), colourless spherule cells (▩), red spherule cells (■) and vibratile cells (■) of *Paracentrotus lividus* coelomocyte subpopulations compared to their relative untreated control group (aCF alone without LPS). *** = $p < 0.001$; ** = $p < 0.01$. Error bars represent standard deviation.

The number of coelomocytes in untreated *P. lividus* from 0 to 24 HPLT was similar to that reported by Arizza et al. (2013) ($8.1 \pm 0.65 \times 10^6$/mL). LPS treatment significantly increased the number of coelomocytes after the first hour of treatment ($10.4 \pm 0.74 \times 10^6$/mL, $p < 0.01$), reaching a maximum at 3 HPLT, when values were $11.6 \pm 0.65 \times 10^6$/mL ($p < 0.001$). For subsequent treatments, 6 and 24 HPLT, values obtained did not significantly differ from the untreated controls. The Anova Test was also performed among different time points. The test revealed that the TCC significantly changed at 1 and 3 HPLT compared to the other time points. To determine variations in the composition of the coelomocytes, the *P. lividus* coelomocyte subpopulations were compared.

Fig. 2 shows variations in coelomocyte populations after LPS treatment at different hour post-injection. The number of PCs increased significantly ($p < 0.001$) at 1 HPLT, when the value increased to 1.96 ± 0.16, reaching its highest value at 3 HLPT (2.43 ± 0.22). Differences in RSC values were always statistically significant. Their value differed significantly ($p < 0.001$) from the control set in each timepoint and they reached their highest value at 3 HPLT. Ratio values of colourless spherulocytes (CSC) to vibratile cells (VC) populations at 1, 3, 6 and 24 HPLT did not differ significantly when compared with the control.

3.2. Analysis of HSP and β-thymosin RNAs expression after LPS treatment

Real time PCRs were performed on RNAs extracted from *P. lividus* coelomocytes treated with 200 µL of LPS solution at 2 µg/mL and the expression of HSP70, HSP90, HSP56, HSP60 and β-thymosin genes was analysed.

In particular, HSP70 gene expression (Fig. 3) increased significantly ($p < 0.001$) following LPS from 1 to 24 HPLT, compared to 0 HPLT, and was found to reach maximum expression at 1 HPLT (5.48 ± 0.33). At 3, 6 and 24 HPLT, HSP70 gene expression was found to be less intensive than that observed after the first hour of treatment; however, it was still significantly $p < 0.001$ different from 0 HPLT. The gene expression of HSP90 post LPS treatment was found to increase significantly ($p < 0.001$) at 1 HPLT (2.9 ± 0.66), reaching a maximum at 3 HPLT (3.52 ± 0.42) and decreasing to a value of 1.67 ± 0.34 at 6 HPLT (Fig. 4). Furthermore, qPCR at 24 HPLT showed no statistically significant differences in HSP90 gene expression compared to 0 HPLT. HSP56 and HSP60 Real Time PCRs (Fig. 5) did not show any statistically significant differences in the heat shock protein gene expression at 1, 3, 6 and 24 HPLT compared to 0 HPLT. Modulation of β-thymosin gene expression induced by LPS treatment (Fig. 6) showed a statistically significant increase in mRNA expression levels at 1 HPLT, and a peak of expression at 3 HPLT. Furthermore, at 6 and 24 HPLT, mRNA levels were found to have reverted to control values.

3.3. Expression analysis of HSP proteins in coelomocytes after LPS treatment

Stress response in sea urchin coelomocytes following LPS treatment was also monitored through Western-blotting assays of HSP70 and HSP90. The equal loading of protein samples (10 µg) was also confirmed through Red Ponceau staining (Data not show). Expression of HSP70 was evaluated by immunoblot with specific mouse *anti*-HSP70 Abs and densitometric analysis of these bands clearly indicated a significant increase ($p < 0.001$) in the protein expression from 1 HPLT to 24 HPLT. In particular, the assay showed an HSP70 expression peak 3 h after LPS treatment (Fig. 7).

Expression of HSP90 was evaluated through immunoblot with mouse *anti*-HSP90 Abs. Specific antibodies cross-reacted with a 90 kDa band of the coelomocyte protein electrophoretic pattern. In treated samples, band intensity gradually increased to a statistically significant peak at 3 h post LPS treatment ($p < 0.001$). The experiment also showed that HSP90 expression levels returned to control values after 24 h (Fig. 8).

3.4. Expression of β-thymosin protein

The immune response following LPS treatment was also evaluated through the expression of antimicrobial factors. In particular, we analysed β-thymosin protein expression through dot-blot assays. Experiments showed a statistically significant ($p < 0.001$) increase in antimicrobial protein expression at 3 HPLT, which is still present at 6 HPLT. Tests also showed that β-thymosin protein expression levels returned to control values after 24 h (Fig. 9).

4. Discussion

Successful survival of invertebrates is mainly linked to a variety of innate defence pathways, which act against microbial pathogens. The

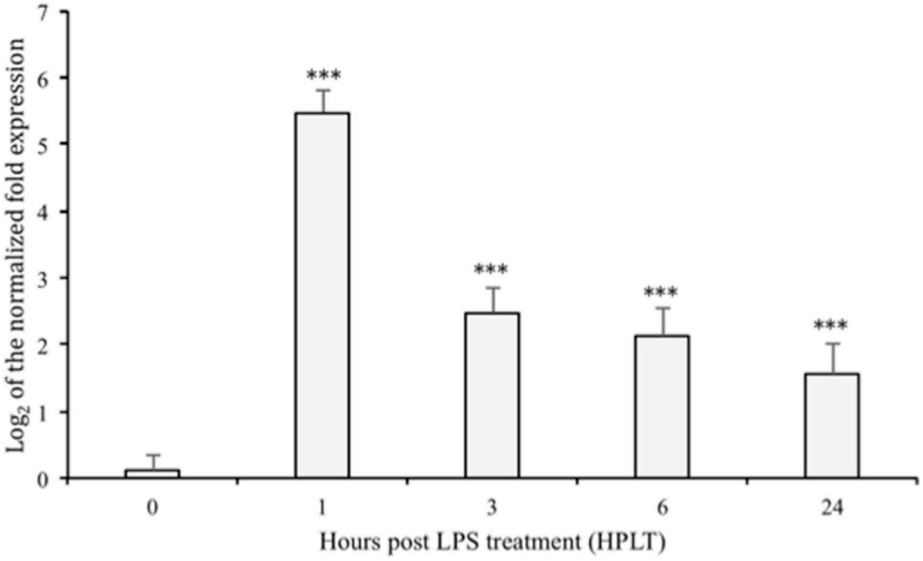

Fig. 3. Real time PCR analysis of HSP70 expression.
Expression of HSP70 evaluated by Real Time PCRs. RT-PCR was performed using RNAs extracted from the *P. lividus* specimens following LPS treatment at 1, 3, 6 and 24 h. Data are Log_2 of the normalized fold expression compared to the relative untreated group (aCF alone without LPS). Mean values were significantly different according to R statisitc method *** = $p < 0.001$. Error bars represent standard deviation.

echinoderm immune system, and in particular that of *Paracentrotus lividus*, is highly efficient in contrasting injurious agent invasion through cellular and humoral responses; a number of homologous and analogous components are also found in other invertebrates and vertebrates alike. It is, in fact, their key position in the evolutionary tree (as invertebrate deuterostomes, thus sharing a common evolutionary branch with vertebrates) that makes the study of their immune system an extremely interesting and exciting field. The immune response, mediated by coelomocytes, is able to activate cellular processes, such as encapsulation, phagocytosis and cytotoxicity [66], and humoral responses, mainly due to molecules like lectins, cytokines and profilins, complement and antimicrobial peptides.

In the present study, we described the molecular and cellular response of *P. lividus* coelomocytes after LPS injection into the coelomic cavity at different exposure times. LPS is a major surface component of gram-negative bacteria and, in the same way as occurs in higher vertebrates, LPS has proved to be a powerful activator in several invertebrate deuterostomes, suggesting that they possess cellular cell surface receptors that bind LPS and, subsequently, bacteria. In the tunicate *Ciona intestinalis*, LPS activates an inflammatory-like reaction that in turn sets in motion numerous immune mechanisms, both at humoral and at cellular level [67–69]. LPS treatment of the tunicate *Halocynthia roretzi* activates haemocyte secretion and the release of haemagglutinin [70]. Smith et al. (1996) demonstrated that the presence of bacteria, allogenic stimulation and injury activated the coelomocytes of the sea urchin *Strongylocentrotus purpuratus* to produce a typical gene response [71]. One gene family which is highly up-regulated in response to immune challenge is the Sp185/333 family [72–76]. The sea cucumber *Parastichopus californica* can neutralize *in vitro* LPS in 4 h [77]. Moreover, Smith and Davidson (1994) measured the elevations in the profiling transcript, whose functions are linked to mechanisms employed by coelomocytes to eliminate microbes: mechanisms include phagocytosis, secretion, degranulation and clot formation [78]. Clow et al. (2000) showed a dramatic increase in amounts of SpC3 in the CF in response to LPS treatment [79].

The response of *P. lividus* to LPS injection into the coelomic cavity appears to be a multifaceted phenomenon including a) an increase in total circulating coelomocytes, b) an increase in phagocytic cells, (c) an increase in expression of HSP70 and 90 and (d) an increase in β-thymosin. On the contrary, HSP56 and 60 seemed to be unresponsive to LPS treatment.

Coelomocytes can be detected in different proportions depending on the state of health of the sea urchin and on the type of stress and immune pressures it has been subjected to Refs. [2,59].

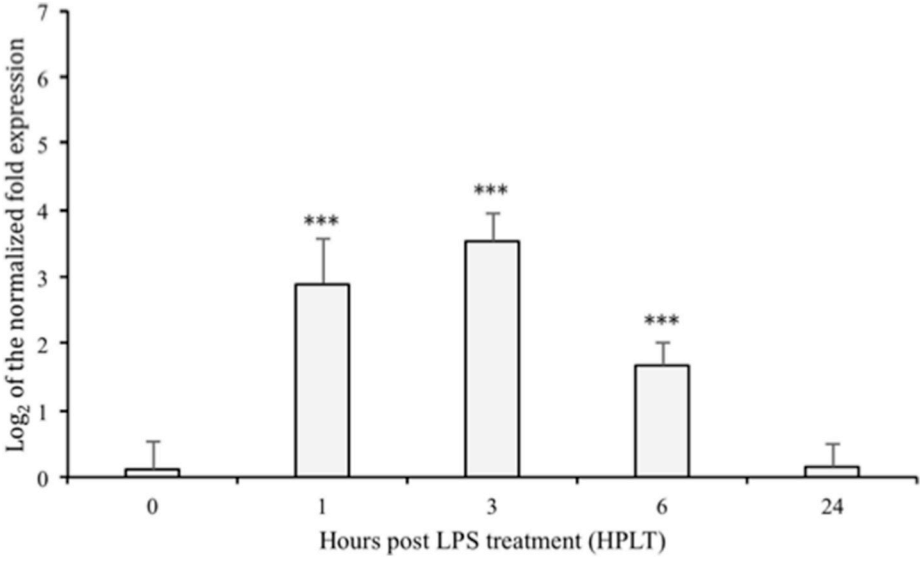

Fig. 4. Real time PCR analysis of HSP90 expression.
Expression of HSP90 evaluated by Real Time PCRs. RT-PCR were performed using RNAs extracted from *P. lividus* specimens following LPS treatment at 1, 3, 6 and 24 h. Data are Log_2 of the normalized fold expression compared to the relative untreated group (aCF alone without LPS). Mean values were significantly different according to R statisitc method *** = $p < 0.001$. Error bars represent standard deviation.

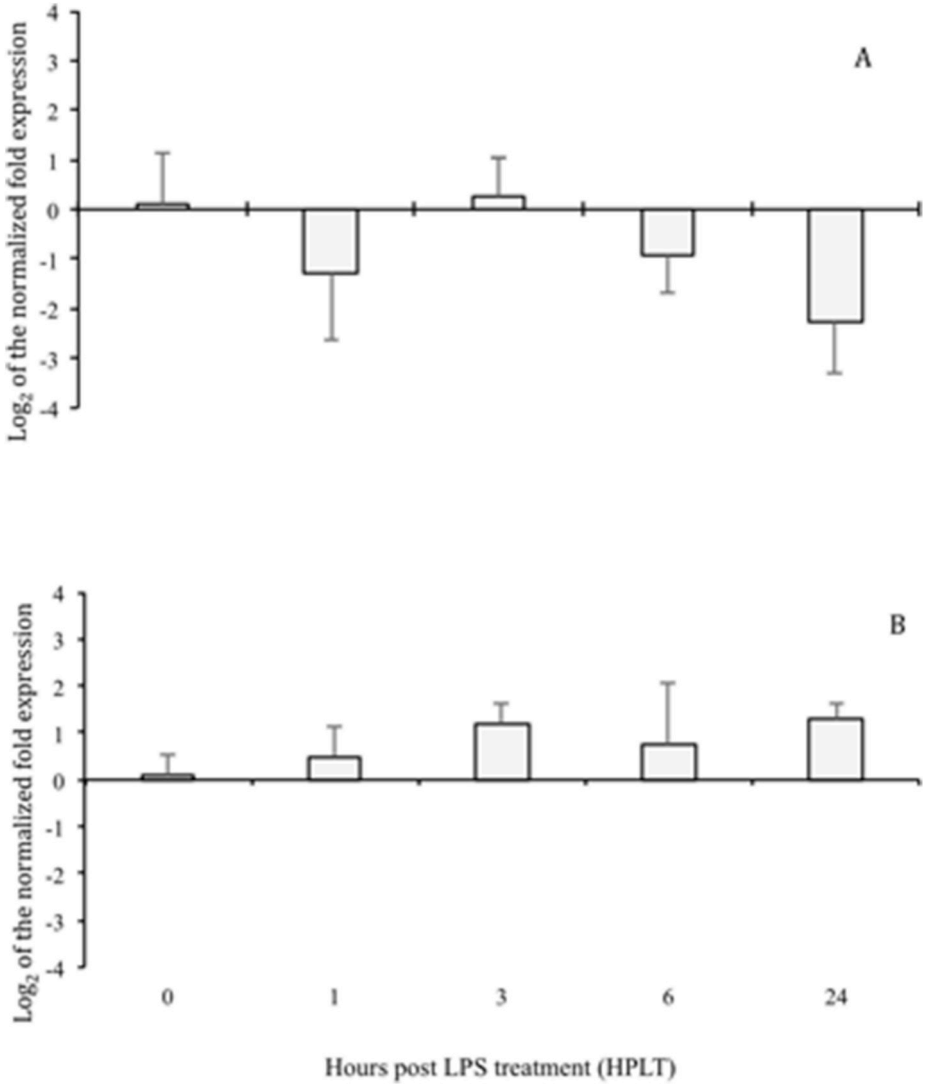

Fig. 5. Real time PCR analysis of HSP56 and HSP60 expression.
Expression of HSP56 (A) and HSP60 (B) evaluated by Real Time PCRs. RT-PCR was performed using RNAs extracted from *P. lividus* specimens following LPS treatment at 1, 3, 6 and 24 h. Data are Log_2 of the normalized fold expression compared to the relative untreated group (aCF alone without LPS). Error bars represent standard deviation.

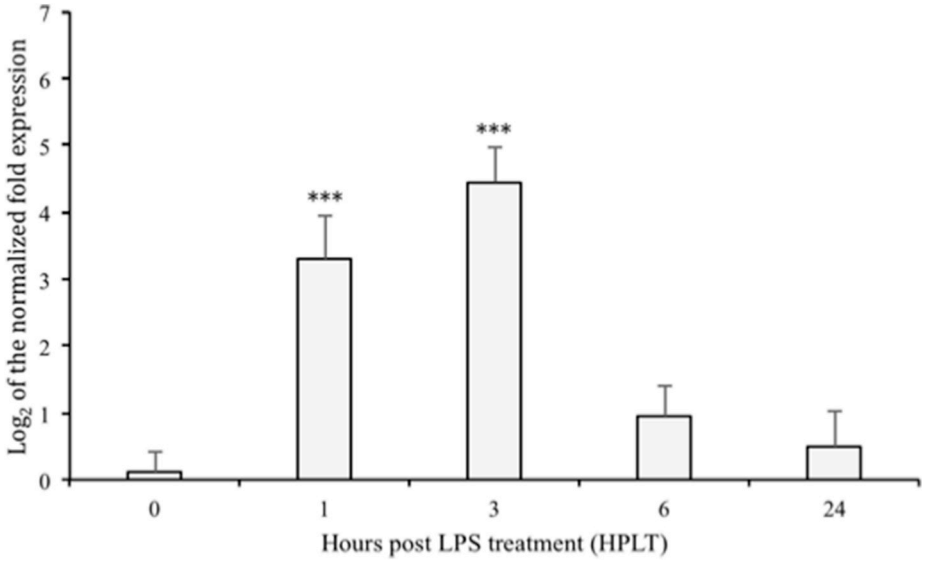

Fig. 6. Real time PCR analysis of β-thymosin expression.
Expression of β-thymosin evaluated by Real Time PCRs. RT-PCR was performed using RNAs extracted from *P. lividus* specimens following LPS treatment at 1, 3, 6 and 24 h. Data are Log_2 of the normalized fold expression compared to the relative untreated group (aCF alone without LPS). Mean values were significantly different according to R statisitc method *** = $p < 0.001$. Error bars represent standard deviation.

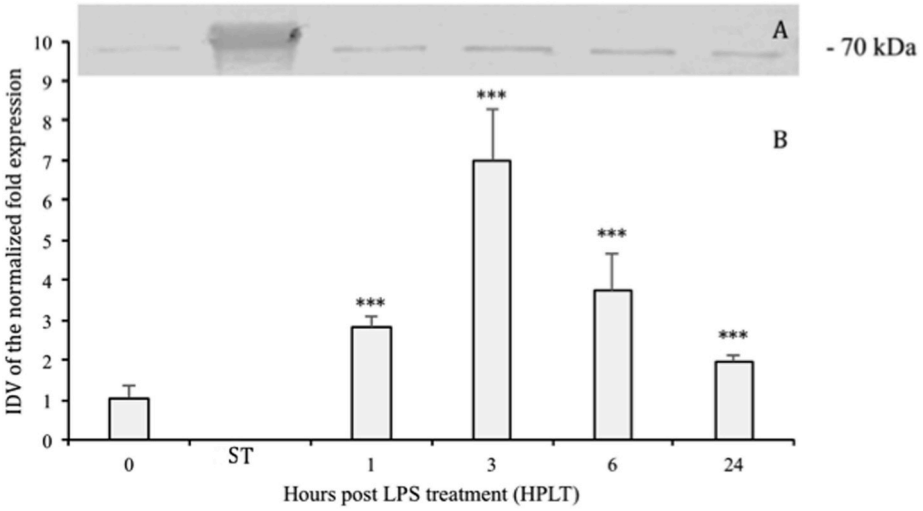

Fig. 7. HSP70 Immunoblot.
A: A representative immunoblot performed on coelomocyte protein extracts at 1, 3, 6 and 24 HPLT, marked with specific mouse monoclonal *anti*-HSP70 antibody. B: Intensities of the stained protein bands were evaluated as integrated optical density values (IDVs). The histogram represents mean increase calculated from band intensity values at different times (0–24 HPLT) and those of the controls (*** = P < 0.001). Error bars represent standard deviation. ST = Standard.

Our experiments showed a significant increase (compared to the control) in the total number of coelomocytes, starting from 1 HPLT, reaching a peak at 3 HPLT and returning to control values at 6 HPLT. In particular the increase of the total coelomocyte number at 1 and 3 HPLT is in accordance with other studies demonstrating, in echinoderms like *S. neumayeri*, *S. purpuratus* and *A. Rubens*, the innate immune activation and the increase of the cell proliferation gene expression due to LPS treatment [10,80,81]. Furthermore, Gonzales- Aravena showed, in *S. neumayeri*, a similar trend of the TCC at 6 and 24 HPLT in which the number of coelomocytes decrease to control values. Because the innate immune activation induces an oxidative stress by ROS production and demands a high energetic cost that generates a metabolic depression in order to spare energy [82], we can speculate that these are the hypothetical reasons why, in *P. lividus*, we found the decrease in the total coelomocyte number after 6 HPLT.

The increase in total circulating coelomocytes recorded at treatment start (1–3 HPLT) could be also an indicator of immediate inflammatory-like response, which aims to eliminate injurious agents. Indeed, increases seemed to be due to an increase in two coelomocyte types in particular, defined by Matranga et al. (2005) as fast-moving cells: (i) PCs and (ii) RSCs, which peaked at 3 HPLT [5]. PCs are involved in a series of immune reactions and they are able to phagocytize, encapsulate and, under immunological stimulus, express immune-related genes (including complement homologues, a C-type lectin and a homologue of NFkB [83]). RSCs contain echinochrome A, a naphthoquinone which gives the cells their characteristic red colour. Echinochrome A is degranulated in the presence of bacteria or stress conditions [84,85] and has antimicrobial properties against both Gram-positive and Gram-negative bacteria [13,14,86].

RSCs accumulate around injuries and sites of infection [11,84], suggesting that these cells and echinochrome A play a role in the immune response of adult sea urchins. Furthermore, RSCs have been shown to increase during environmental impacts, such as the presence of pollutants, as shown by Matranga et al. (2000) [87].

The effect of LPS treatment was further investigated at the molecular level, and we found, for the first time in these animals, that LPS treatment induced over-expression of the cytoplasmic stress proteins HSP 70 and HSP 90, both at the transcriptional (Figs. 3 and 4) and at the translational level (Figs. 8 and 9, respectively).

Previous studies on HSPs in echinoderms have demonstrated that HSPs levels can increase in response to exposure to numerous environmental “stress” conditions, such as regeneration processes, wound healing, temperature changes, pollution, acidosis, low salinity [88] and hypoxia [89–95]. Furthermore, it has been shown that HSP70 could play a role as a promoter of cell division; in fact, a rapid up-regulation of HSP70 found in sea star coelomocytes post-amputation could be interpreted as a prerequisite for subsequent regenerative capability [96]. Indeed, molecular approaches to the analysis of echinoderm regeneration have suggested the involvement of HSP70 as well as of growth factors [93]. It seems from the literature that HSP70 may play a role in eliciting cell division in other animals [97].

Among the echinoids, *P. lividus* showed a basal level of HSP70 that

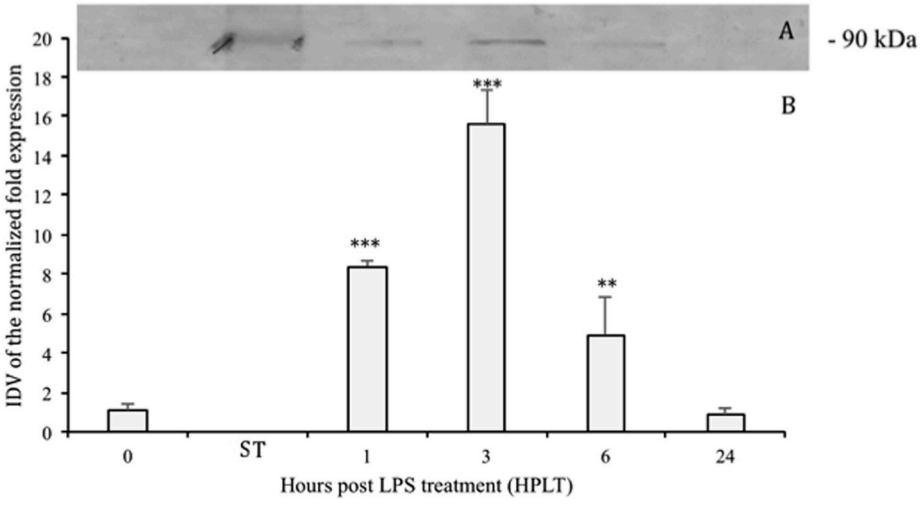

Fig. 8. HSP90 Immunoblot.
A: A representative immunoblot performed on coelomocyte protein extracts at 1, 3, 6 and 24 HPLT, marked with specific mouse monoclonal *anti*-HSP90 antibody. B: Intensities of the stained protein bands were evaluated as integrated optical density values (IDVs). The histogram represents the mean increase calculated from band intensity values at different times (0–24 HPLT) and those of the controls (*** = P < 0.001; ** = P < 0.01). Error bars represent standard deviation. ST = Standard.

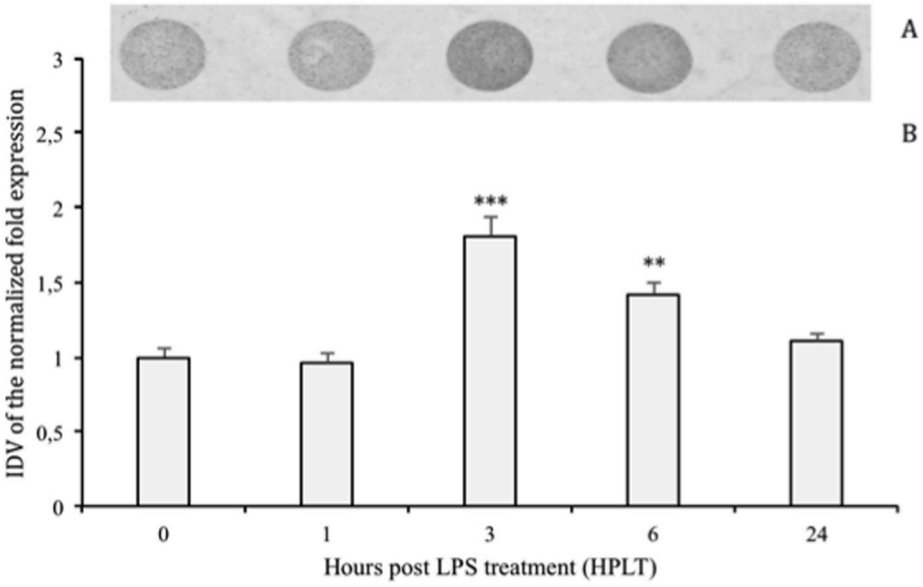

Fig. 9. β-Thymosin DOT-BLOT.
A: A representative dot-blot performed on coelomocyte protein extracts at 1, 3, 6 and 24 HPLT, marked with specific rabbit monoclonal *anti*-β-Thymosin antibody. B: Stained protein dot intensities were evaluated as integrated optical density values (IDVs). The histogram represents the mean increase calculated from band intensity values at different times (0–24 HPLT) and those of the controls (*** = $P < 0.001$; ** = $P < 0.01$). Error bars represent standard deviation.

can be modulated in response to environmental conditions. An overexpression of the HSP70 protein was found in coelomocytes from temperature-stressed urchins [87]. The same responses were obtained from coelomocytes treated with cold, acidic and heavy metal treatments [98]. Furthermore, HSP70 was upregulated in embryos exposed to UV-B at the stage of the mesenchyme blastula. These results confirm the involvement of HSP70 in all stress-response mechanisms.

Our results showed that LPS activated the stress pathway with the up-regulation of HSPs levels in treated *P. lividus* specimens. In particular, the RNA levels of HSP 70 showed a peak of expression at 1HPLT (Fig. 3) and a statistically significant increase in the relative protein level at 1HPLT that reach the maximum value at 3HPLT (Fig. 7). The RNA levels of HSP 90 showed greater values at 1 and 3HPLT (Fig. 4) and a peak of protein expression at 3HPLT (Fig. 8). These results seem to suggest that LPS is able to induce an early increase of HSP 70 and 90 both at transcriptional and translational level. These results suggest the existence of an interconnection between the stress pathway and the innate immune response through the modulation of the HSP proteins.

It has been suggested that HSPs could function as potent activators of the innate immune system [99,100]. It has been demonstrated that HSPs occur at basic levels of expression, increasing after injury and sepsis. In mammals, a number of studies have shown that bacteria or bacterial products induced the expression of stress proteins in host cells [101–103]. Ramaglia et al. (2004) showed that HSP90 expression and the tissue-specific HSP90 response were upregulated during bacterial infection in the western painted turtle *Chrysemys picta bellii*, highlighting the role of HSP90 in immunopathological events in reptiles. In the malaria vector *Anopheles gambiae*, for example, several HSPs were found to be upregulated after utilizing heat-inactivated *Salmonella typhimurium* and *Staphylococcus aureus* [104,105]. Furthermore, it has been shown that HSP70 levels increase in haemocytes of the bay scallop *Argopecten irradians* up to 16 h after infection with *Vibrio anguillarum* [106,107]. HSP70 was also upregulated in the midgut of *Rhodnius prolixus* (vector of the protozoan parasite *Trypanosoma cruzi* and responsible for trypanosomiasis in humans) after immunization by microinjection with *Escherichia coli* and *Micrococcus luteus* [108]. Basu et al. (2001) and Calderwood et al. (2007b) showed that HSP60, HSP70, and HSP90 can stimulate the innate immune response, eliciting nonspecific cytokine and chemokine secretion from cells of the mammalian innate immune system, to upregulate costimulatory molecules and to activate antigen-presenting cells (APCs), in particular, dendritic cells (DCs), via a number of receptors [32,109].

Therefore, activation of the expression of HSPs in *P. lividus* after LPS treatment could be related to both activation of the immune system and protection from the hazardous activity of molecules produced by the immune mechanism itself [104]. As a result of bacterial infection, generation of reactive oxygen species ROS [110] occurs, which could result in protein denaturation or proteotoxicity [111]. The accumulation of denatured proteins in the host cell might trigger HSP expression. Upregulation of HSPs is conceivably a protective mechanism: HSPs may bind to damaged or misfolded proteins to restore their original structure [104].

Based on studies of the modulation of cytosolic HSPs, mitochondrial HSP56 and HSP60 did not show any molecular modulation following LPS treatment. Previous reports have shown that HSP60 and HSP56 protein levels increased after manganese and cadmium exposure in *P. lividus* embryos due to toxicity effects that interfere with various interdependent mitochondrial functions, such as oxidative stress, calcium homeostasis deregulation and direct inhibition of electron chain complexes [112]. Our results suggest that sea urchin early response to a hypothetical Gram-negative infection, represented by LPS treatment, does not involve the activation of the mitochondrial chaperone stress pathway, which, however, is highly active during xenobiotic stress.

The biological effects of host defence mechanisms, in response to LPS challenge in *P. lividus,* not only activate stress protein pathways but also involve antimicrobial peptides, leading to an increase, for instance, in the amount of antimicrobial β-thymosin, both at the RNA and at the protein level. Antimicrobial activity of β-thymosin against *E. coli, Bacillus subtilis and Candida albicans* has been reported for *Crassostrea gigas* [113]. We previously demonstrated that the < 5 kDa fraction of coelomocyte acid extract has antimicrobial and antistaphylococcal biofilm formation activity [56]. This specific activity was mainly due to Paracentrin 1 (a specific fragment of 11 amino acids that is derived from β-thymosin [17]), which is responsible for antimicrobial activity and biofilm formation inhibition of the staphylococcal and *Pseudomonas aeruginosa* strains [16]. In the present study, we show, for the first time in *P. lividus*, that LPS treatment could induce a specific level increase of both the RNA and protein components of β-thymosin, with an expression peak 3 h after exposure. Furthermore, the initial increase in antimicrobial protein levels (3 HPLT) is delayed compared to the initial increase in HSP levels (1 HPLT). Similar results were described by Diamond et al. (1996) who demonstrated that tracheal epithelial cells expressed a gene for a peptide antibiotic when exposed to LPS [114]. In *Limulus polyphemus*, antimicrobial peptides are stored in haemocyte granules and released into the plasma upon stimulation by microbial substances, such as lipopolysaccharides and β-glucans [115,116].

5. Conclusions

The results emerging from this study represent a further important step in the study of the defence mechanisms that *P. lividus* are able to implement in response to stressful stimuli. We have shown how bacterial LPS constitutes both a microbial threat and a stress that can activate a number of defence mechanisms, both at the cellular level, by increasing the population of specific coelomocytes and at the molecular level, by up-regulating heat shock proteins HSP70 and HSP90 and increasing the concentration of β-thymosin, a peptide from which Paracentrin 1 is derived.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.017.

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