

Immune response of the short neck clam *Paphia malabarica* to salinity stress using flow cytometry

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Short title: Immunological response of short neck clam to salinity stress...

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Abstract

Paphia malabarica is a predominant and commercially important bivalve in India, persistently challenged by wavering salinity in a monsoon-influenced estuary. To examine the organism's immunological response under such a condition we challenged *P. malabarica* with different salinities (0, 5, 15, 25 and 35) for varied periods using a two-way experimental approach (in vitro and in vivo). This is the first study to report the response of *P. malabarica* hemocytes to salinity stress from a monsoon-influenced estuary on the southwest coast of India. Evaluation of total hemocytes count, mortality, lysosomal content, reactive oxygen species production, phagocytic and esterase activity was carried out using flow cytometric analysis. In both the experimental conditions, hemocyte parameters were significantly compromised at lower salinities (0 and 5) with an evident immuno-salinity tolerance range of 15-35. The damaging impact of 0 and 5 salinities on hemocyte function intensified with a longer exposure period, indicating that prolonged exposure to low salinity could be detrimental to bivalve wellness if they are pushed beyond their tolerance range which is usually observed during the monsoon. Further studies should focus on the interactive effect of salinity tagged with different stressors influencing biology of *P. malabarica*.

Keywords: *Paphia malabarica*, Hemocytes, Immunological parameters, Estuary, Salinity, Flow cytometry

1. Introduction

Salinity is a key environmental factor modifying functional responses in marine environment, which in turn influences growth and wellness of marine molluscs (Wang et al., 2012a). Role of salinity in the physiological functioning of bivalves affecting filtration rate (Riisgård et al., 2013), cardiac activity (Bakhmet et al., 2005; Braby 2006), respiration (Stickle and Sabourin, 1979), physiological energetics (Gardner and Thompson, 2001), protein content in tissue (de Bravo, 2003), size and growth rate (Westerbom et al., 2002) has been studied earlier. It is well known that monsoon-affected estuarine environment is subjected to monsoonal flushing and cleaning by tidal waves. An influx of fresh water in the receiving estuaries has a considerable impact on the organisms inhabiting the area (Kimmerer, 2002). Under heavy rainfall events, dilution of salinity is inevitable and it might take many days to restore the environment to previous ambient conditions depending upon the intensity of rainfall. Since molluscs mostly prevail in estuarine coastal habitat, they are subjected to such extreme salinity variations.

The short neck clam *Paphia malabarica* (Chemnitz 1782), one of the most commercially valuable and widely distributed venerid clam species on the southwest coast of India (Appukuttan, 1996; Nagvenkar et al., 2014), is exploited mainly for its meat as well as shell resource in industrial applications. During the southwest monsoon, central-west coast of India is profoundly influenced by precipitation leading to increased freshwater influx and land run off in coastal waters. In estuarine condition, apart from the monsoonal salinity straining effect, natural bacterial load, including potentially pathogenic species, release of toxicants and industrial discharge bring about disruption and destruction of vitality. To endure in such dynamic unpropitious conditions, bivalves rely on innate immunity.

Invertebrate's free circulating blood cells are the primary mediators of immunity, which shield the organism through deleterious conditions. The bivalve immune system is comprised of humoral and cellular components which support the organism against parasites, disease and environmental stresses (Cheng, 1981). Hemocytes are the circulating cells of hemolymph, playing a cardinal role in the bivalve immune system (Adema, 1991) and physiological process (Donaghy et al., 2009). Hemocytes contribute to the immune-response by executing activities such as clump formation, encapsulation of potential threat, phagocytosis, nodule formation and conciliation of cytotoxicity (Allam and Raftos, 2015; Perez and Fontanetti, 2011). Hemocyte functionality has been used as an index for bivalve health under different stressful conditions such as salinity, temperature, anoxia, pH, pollutants, pharmaceutical products and nano materials (Gagnaire et al., 2006a, 2006b;

Gagne et al., 2006; Huang et al., 2016; Sui et al., 2016; Wang et al., 2011, 2012b, 2014; Wu et al., 2016; Yu et al., 2010). The characterization of immunological parameters of hemocytes in respect to their environments proves to be essential in understanding the cell-mediated response to environmental as well as pathological stresses (Donaghy et al., 2012). Previous studies have reported the consequential role of salinity on hemocyte functions of bivalves resulting in a compromised immune state (Matozzo and Marin, 2011 and references therein). For instance, Wang et al. (2012b) reported compromised immune functionality of *Perna viridis* under hyposalinity conditions; lower salinities altered phagocytic activity, esterase activity, hemocyte viability in the Pacific oyster *Crassostrea gigas* (Gagnaire et al., 2006a); Rodrick (2008) demonstrated impairment of hemocyte function under hyposaline treatments. Bussel et al. (2008) also found that salinity could bring about a significant reduction in eosinophilic hemocytes and phagocytic capacity in the mussel *Mytilus edulis*. In unraveling such hemocytic response, flow cytometry techniques have been entirely instrumental and productive in past studies. It has been of prime importance for hemocyte characterization as well as understanding invertebrate ecological immunology (Hégaret et al., 2003a, 2003b; Wang et al., 2012b, 2012c). However, no literature is available on immune functionality of *P. malabarica* under salinity stress.

Moreover, while conducting observational studies on *P. malabarica* in a monsoon-influenced Betul estuary (study area of our current investigation), we witnessed a change in salinity level from 35 to 20 with exceptionally low values (as low as 5) during monsoonal flushing. High mortality of *P. malabarica* population was also evident during this period. Wang et al. (2012b) also observed summer mortality in mussel *P. viridis*, which was attributed to low salinity and hypoxia.

In view of this, we assessed the immune response of *P. malabarica* to varying salinity conditions using flow cytometry (FCM). Investigating salinity stress on *P. malabarica* immune system can provide valuable insights into the immune-salinity tolerance range and might be useful in elucidating causes for their increased mortality during the monsoon. In vitro experiments were carried out to investigate the immediate response of hemocytes to varying salinity. Since this might not be a simulation of natural conditions, an in vivo approach was also used to understand the hemocyte response when they are housed within the body. Most of the studies on bivalve immunology are carried out in vitro or in vivo but rarely both experimental approaches are employed simultaneously which was addressed in our study by undertaking the two-way experimental approach. To the best of our knowledge this is the first study from the Indian region.

2. Methods

Clams (shell length: 3.5-4 cm; wet weight: 21.56 ± 1.34 g) were collected manually three times (80-100 individuals per sampling) during the month of May-June 2016 from Sal river (35 salinity) $15^{\circ}08'38.9''\text{N}$ $73^{\circ}57'10.2''\text{E}$, Betul, South Goa, India (Fig.1). The clams were immediately transferred to cage tubs receiving a daily change of seawater (35 salinity) collected from the sampling site and fed with the laboratory maintained *Chaetoceros calcitrans* (3×10^9 cells/L). Water clarity and mortality of clams was monitored every day. The clams were acclimatized to laboratory conditions for 5 days before starting the experiments.

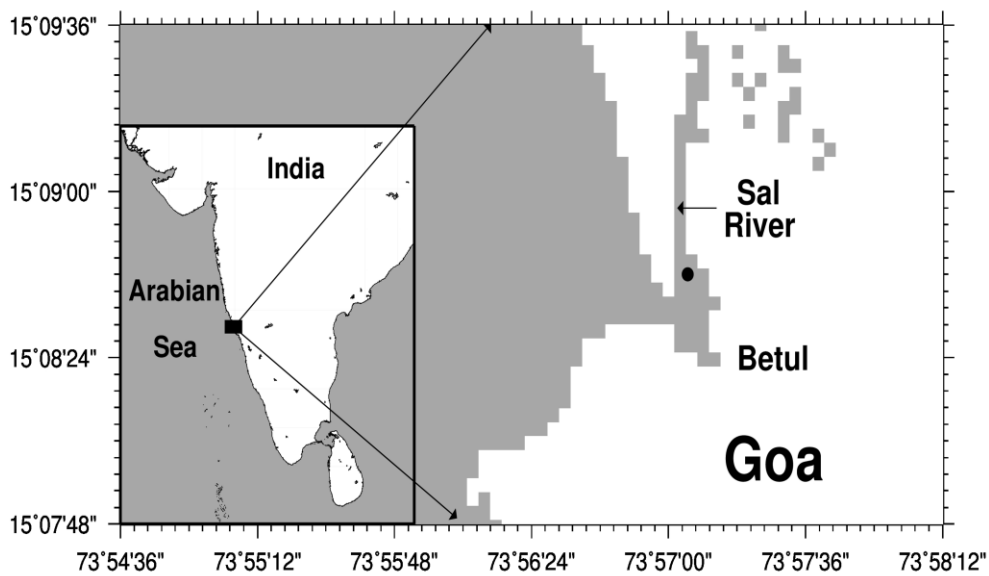


Fig. 1. Location of study area

2.1. *In vitro* Experiment

Hemolymph was collected from the posterior adductor muscle of clam using a 3ml sterile syringe (needle size 0.55x25 mm). 350 μl of hemolymph was extracted from each individual and kept on ice to minimize aggregation of hemocytes. Hemolymph samples were pooled from 20 clams and equally divided by volume into five sterile 5 ml polypropylene round-bottom tubes for salinity treatments. In each tube, hemocyte concentration was determined using a flow cytometer. Different salinities (0, 5, 15, 25 and 35) were achieved by dilution of seawater (autoclaved and filtered) with Milli-Q water using ATAGO refractometer. The experimental setup was adopted as described by Gagnaire et al. (2006a) with a slight modification where, the centrifugation step was eliminated as it was observed that centrifugation resulted in mortality of hemocytes to some extent. Therefore, hemolymph in each tube was diluted (1:2 in filtered autoclaved sea water) with varying salinity sea

water and incubated at 15 °C (Gagnaire et al., 2006a) in Panasonic (MIR-154-PE) cooled incubator. FCM analyses were carried out after 2 and 5 h of incubation. All the analyses were completed within 2 h. The experiments were performed in triplicates (n=3).

2.2. *In vivo Experiment*

In the laboratory, 12 clams were placed in each of the five tanks (15 L), which were filled with 12 L of water with different salinities (0, 5, 15, 25 and 35) achieved by dilution of filtered sea water (Whatman GF/F filters, Pore size 0.7µm) with fresh water collected from the sampling site. The tanks were inoculated with *C. calcitrans* (3×10^9 cells/L) and maintained at room temperature. Feed and water were replenished every day along with the removal of fecal pellets. Mortality of clams was monitored daily. For analysis, randomly four clams were removed from each of the salinity treatment tank on day 1, 4 and 6; hemolymph was extracted and hemocytes were analyzed using FCM for their immuno-parameters. The experiment was repeated twice using two different batches. In each batch, analyses were carried out on two separate pools of hemolymph obtained from four clams of the same treatment [$n = 2$ (batches) X 2 (pooled replicates) = 4]. All the analyses were done immediately (within 2 h from the time of extraction) to minimize post extraction time induced stress.

2.3. *Flow Cytometry Analysis*

A BD FACS-VERSE flow cytometer (BD Biosciences) equipped with a blue laser (Wavelength: 488 nm Optical power: 20 mW) was used to carry out flow cytometer analyses. For positioning hemocyte (3-15µm) events on forward scattering (FSC)-side scattering (SSC) cytogram, the forward scatter detector and side scatter detector were set to 70V-253V, respectively. The FSC threshold level was set to 4200 for exclusion of cell debris and bacterial events of the hemolymph samples. The selection of fluorescence channels was based upon the immune parameters monitored; hemocyte counts, lysosomal content, reactive oxygen species production and esterase activity fluorescence were detected at FITC-channel (530/30), and mortality at PE-channel (575/26). For each sample, minimum 10,000 events were recorded and the flow rate was kept medium for all the hemocyte parameter analysis. The data were processed with BD FACSuite software version 1.0.5 (Becton Dickinson).

2.3.1. *Total hemocyte counts (THC)*

Sub-sample of hemolymph was diluted by adding antiaggregant modified Alsever's solution (1:1 ratio), as described in Bachère et al. (1988). To this, SYBR green nuclear stain (1/1000 of stock

solution) was added and incubated in the dark for 30 min before FCM analysis at 22 ± 2 °C (Donaghy et al., 2012). Green fluorescence emission from nuclear stained positive cells was recorded using FITC-channel (Fig. 2A) and gated on FSC-SSC dot plots. The hemocyte count is expressed as cells/ml.

2.3.2. Hemocyte mortality (HM)

Hemolymph was diluted with antiaggregant modified Alsever's solution (1:1 ratio). Propidium iodide (PI; 10 µg/ml, Molecular Probes) was added to the sample and incubated in the dark for 30 min at 22 ± 2 °C (Donaghy et al., 2012). Samples were analyzed on FCM and PI positive cell fluorescence (Fig. 2B) was captured and gated on the PE-channel histogram. Mortality of hemocytes is expressed in terms of percentage (%).

2.3.3. Lysosomal content (LC)

Lyso Tracker Green (Molecular Probes, final concentration 1 µM) was added to the hemolymph sub-sample and incubated for 40 min at 22 ± 2 °C (Donaghy et al., 2012). Intracellular lysosomal content (equivalent to Lyso Tracker fluorescence observed) was analyzed on FCM by capturing green emitted fluorescence (Fig. 2C) with the help of the FITC-channel histogram. Lysosomal content is expressed as mean values of arbitrary units (A.U.) of emitted fluorescence.

2.3.4. Reactive oxygen species (ROS)

Carboxy-DCFDA (Molecular Probes) was added to hemolymph sub-samples (final concentration of 0.01 mM) and incubated in the dark for 30 min at 22 ± 2 °C (Donaghy et al., 2012). ROS production (Fig. 2D) was analyzed on FCM and emitted fluorescence is expressed in terms of mean values of A.U. on FITC-channel histogram.

2.3.5. Esterase activity (EA)

Fluorescein diacetate (FDA, Sigma) was added to hemolymph sub-sample with a final concentration of 2 µM and incubated in the dark for 20 min at 22 ± 2 °C; adapted with some modification from Gagnaire et al. (2006b). Esterase activity (Fig. 2E) was analyzed by capturing the emitted fluorescence on FITC-channel histogram and expressed in terms of mean values of A.U.

2.3.6. Phagocytosis (PG)

Phagocytic activity was carried out by incubation of hemolymph sub-sample with Fluoresbrite 1.0 µm Microspheres (Polysciences 2.7% solids-latex, 1:50 hemocyte bead ratio) in the dark conditions at 22 ± 2 °C for 60 min. Phagocytic cells (Fig. 2F) were considered as those which

had ingested more than three beads to normalize the attachment possibilities. Cells were gated on the FITC-channel histogram; phagocytic cells were differentiated from non-phagocytic cells using fluorescence peaks of beads on FITC-channel histogram and are expressed in terms of percentage (%) (Delaporte et al., 2003).

2.4. *Microscopic examination*

To ensure the functioning of different immune-parametric protocols, hemocyte population was sorted using FCM and microscopic observations were carried out. The gated population of hemocytes was sorted with a two-way purity cell sorting technique using BD FACS Aria II flow cytometer (BD Biosciences) equipped with 480 and 633 nm laser sources. The sorted samples were transferred (100 μ l) immediately on the cavity slide and observed under an Olympus BX51 light-fluorescent microscope equipped with an Olympus DP71 digital imaging system and Image-Pro software 6.2. The photomicrographs of hemocytes are presented in Fig. 2.

2.5. *Statistical analysis*

Prior to analysis all percentage values were converted into r angular arcsinus $\sqrt{\%}$ (% of positive cells) and checked for normality and homogeneity. To evaluate salinity and time effects on the immune parameters, two-way analysis of variance (ANOVA) was performed using SPSS 16.0 version. Subsequently, pairwise comparison was performed at a significance level of ≤ 0.05 for evaluating interactive effect of salinity at each fixed time and vice-versa.

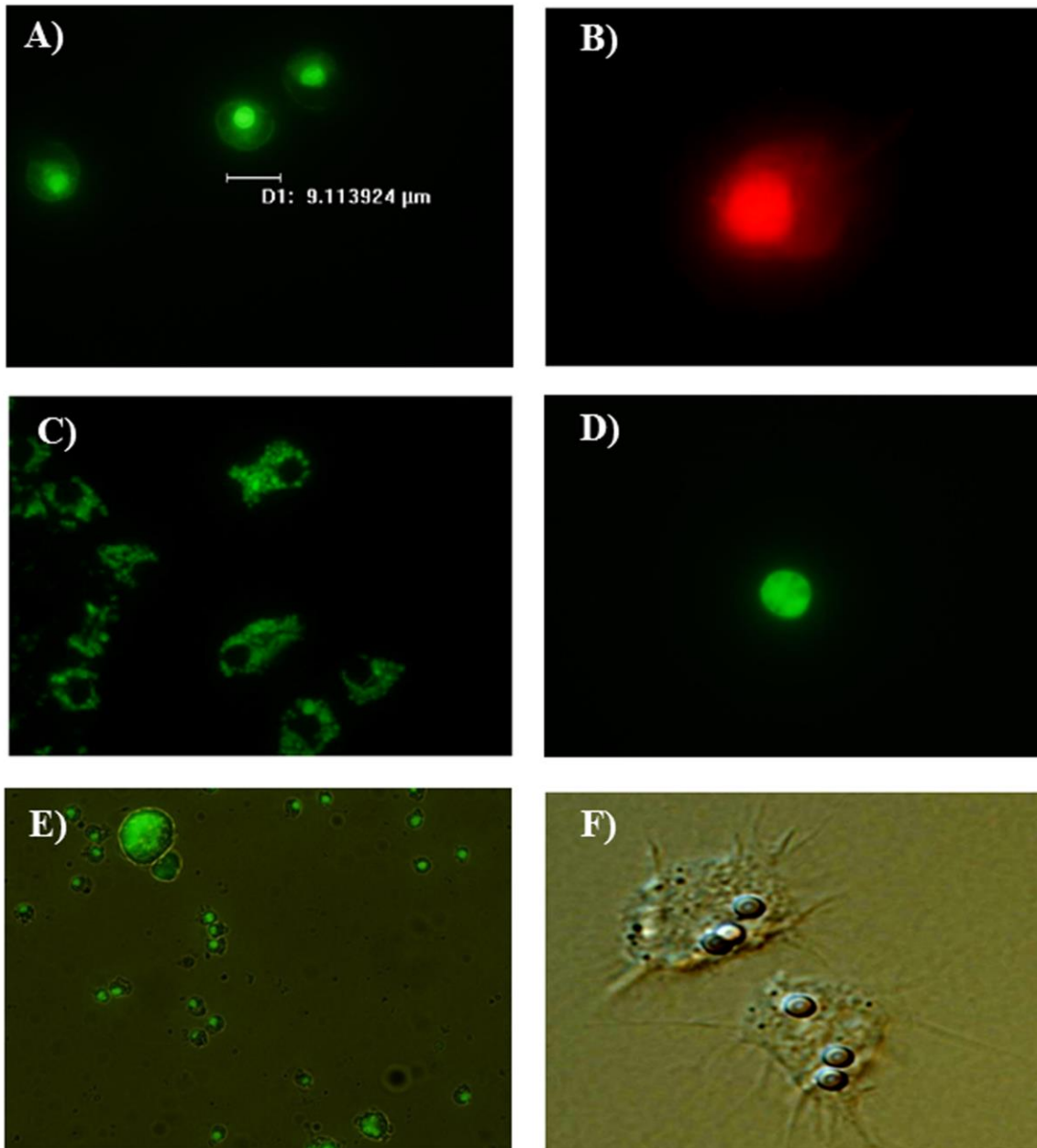


Fig. 2. Microscopic examination of *P. malabarica* hemocytes **A)** SYBR green stained hemocytes for THC, **B)** Hemocyte stained with PI for HM, **C)** Hemocytes stained with LYSO Tracker Green for LC, **D)** Hemocyte stained with Carboxy-DCFDA for ROS, **E)** Hemocytes stained with FDA for EA, and **F)** Beads phagocytosed by hemocytes (PG).

3. Results

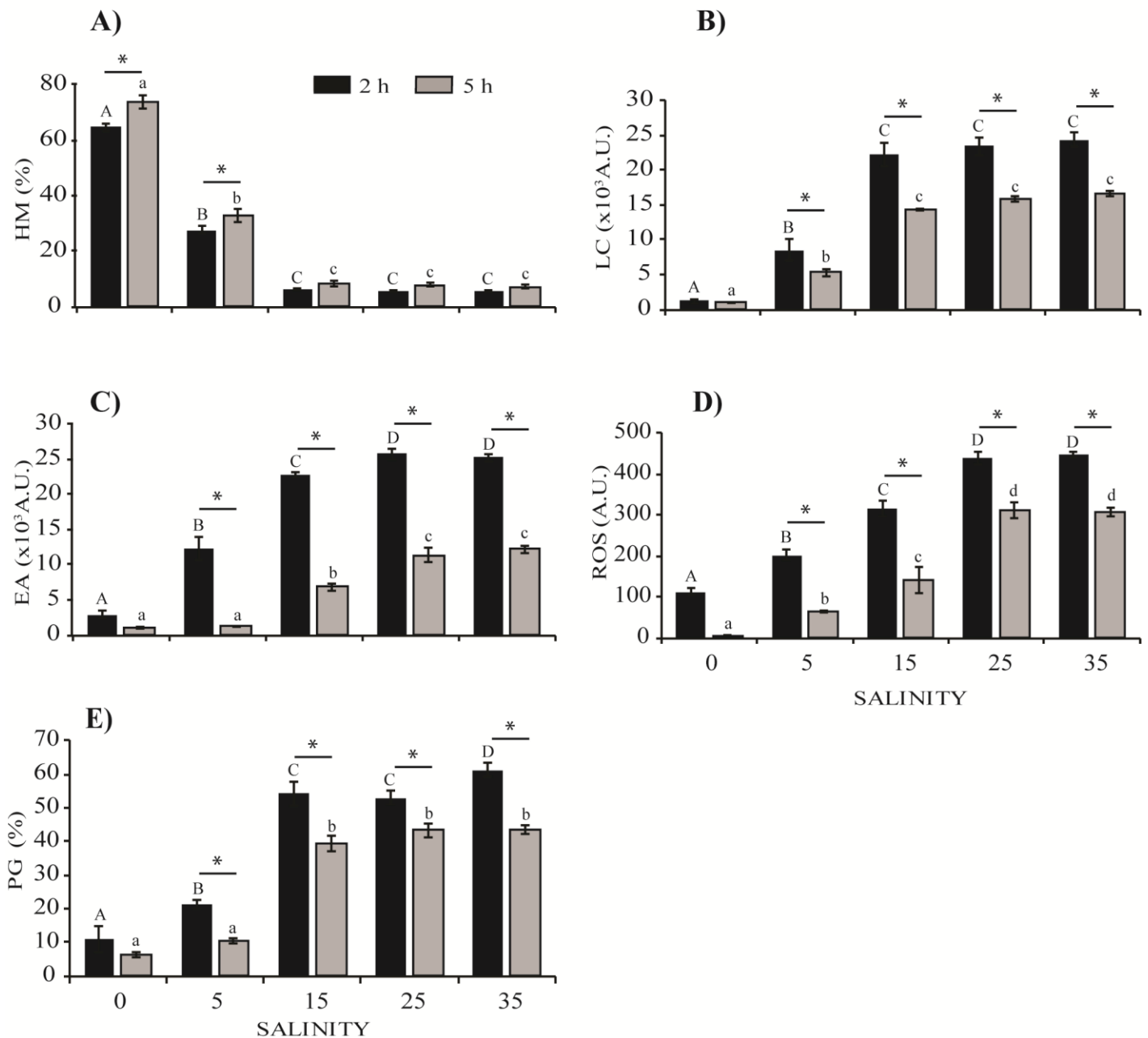
3.1. *In vitro* experiments

Overall, salinity and exposure period had a significant effect on hemocyte functions (Fig. 3). The compromised immune response of hemocytes was observed at 0 and 5, with not much difference among 15, 25 and 35 salinities. The ruptured hemocytes were evident only in 0 and 5 salinities. The

overall response of hemocytes diminished with the time. Two-way ANOVA results for the effects of salinity, time and their interactions on the immune responses of *P. malabarica* are shown in Fig. 3 and Table 1.

The mortality of hemocytes was significantly altered by salinity and exposure period (Fig. 3A). After 2 h, mortality of hemocytes at 0 salinity was significantly higher (64.53 ± 1.60 %) followed by 5 (27 ± 1.50 %), whereas no significant difference among 15 (6.22 ± 0.19 %), 25 (5.63 ± 0.17 %) and 35 (5.43 ± 0.30 %) salinities was observed. After 5 h of exposure, hemocyte mortality was significantly higher at 0 (73.61 ± 2.33 %) and 5 (32.94 ± 2.51 %).

In contrast to mortality, lysosomal content decreased at lower salinities and with time of exposure (Fig. 3B), being lowest in 0 salinity (1206 ± 294.04 A.U.) preceded by 5 (8460 ± 1523.90 A.U.) after



2 h. The lysosomal content of hemocytes was significantly higher and similar among 15 (22285.33 ± 1617.72 A.U.), 25 (23407 ± 1177.22 A.U.) and 35 (24374.33 ± 989.41 A.U.) salinities. A significantly lower lysosomal content was observed after 5 h as compared to 2 h in all salinities except for 0.

Fig. 3. Comparison using two-way ANOVA for in vitro experiments; Effect of salinity (0, 5, 15, 25 and 35) at each fixed time (2 h represented by ‘capital’ letters, 5 h represented by ‘small’ letters) and

effect of time (represented by ‘*’) at each fixed salinity level, on **A**) Hemocyte mortality (HM) expressed in %, **B**) Lysosomal content (LC) of hemocytes expressed in A.U, **C**) Esterase activity (EA) of hemocytes expressed in A.U., **D**) Reactive oxygen species (ROS) production by hemocytes expressed in A.U., and **E**) Phagocytosis (PG) by hemocytes expressed in %. Values are mean \pm standard deviation. Different letters denote significant differences among salinity treatments; asterisk indicates significant effect of time at each fixed salinity treatments ($p < 0.05$).

A decreasing trend was also noted with esterase activity of hemocytes (Fig. 3C); after 2 h, low esterase activity was observed in 0 (2907 ± 438.04 A.U.) and 5 salinities (12271 ± 1648.09 A.U.), whereas 15, 25 and 35 salinities showed higher activity. Hemocyte esterase activity between 25 (25736.67 ± 850.53 A.U.) and 35 (25276 ± 530.51 A.U.) salinities showed no significant difference. After 5 h exposure, esterase activity weakened in all the salinity treatments.

A significant alteration in ROS production by hemocytes was observed with salinity and exposure time (Fig. 3D). After 2 h, least ROS production was observed at 0 salinity (111.33 ± 8.50 A.U.) preceded by 5 (202.33 ± 11.37 A.U.) and 15 (315.66 ± 19.85 A.U.) with no significant difference between 25 and 35 salinities. After 5 h exposure, ROS production was significantly lower in all the treatments except 0.

Salinity and exposure time significantly changed phagocytic activity of hemocytes (Fig. 3E). Phagocytic activity was found least in 0 salinity (10.83 ± 3.85 %) preceded by 5 (21.2 ± 1.42 %) after 2 h. The highest activity was observed at 35 (61.2 ± 1.92 %) with no significant difference between 15 and 25 salinities. After 5 h exposure, phagocytic activity diminished significantly in all the salinity treatments except 0.

3.2. *In vivo* experiments

After acclimatization, on day 1 neither feeding nor fecal pellets were observed at 0, 5 and 15 salinities, whereas at 25 and 35, the clams were active throughout the experiment with active feeding and fecal pellet production. A quick withdrawal of the siphons and foot was also noticed at the slightest disturbance. Subsequently, on day 2, feeding was resumed at salinity 15. After day 2,

the mortality of clams was observed only at 0 and 5 salinities with no survival differences among rest of the salinities (15, 25 and 35) (no values for day 6 at 0 and 5 salinities because of clam's mortality). On day 4, gaped valves, absence of feeding and fecal pellets with very slow withdrawal of the siphons and foot in response to disturbance were observed in clams maintained at 0 and 5 salinity treatments. The effects of salinity, time and their interactions on immune parameters are summarized in Fig. 4 and Table 1.

Hemocytes mortality on day 1 was considerably higher in 0 (12.65 ± 1.77 %) and 5 salinities (11.41 ± 0.35 %) than the rest (Fig. 4A). On day 4, all salinity treatments showed a significant increase in mortality compared to day 1. At salinity 15, mortality increased further on day 6, whereas no significant difference was observed at a salinity of 25 and 35 throughout the experiment.

Lysosomal content was significantly affected with lower salinities and longer exposure period (Fig. 4B). On day 1, values were low at 0 salinity (2088.25 ± 112.25 A.U.) preceded by 5 (5694.75 ± 302.75 A.U.) and 15 (10135.75 ± 24.75 A.U.), whereas 25 and 35 salinities showed no significant difference in the activity. Subsequently on day 4, lysosomal content decreased in all salinities with no significant changes observed between 15 and 25 on day 6. For salinity 35, an increase in the activity was observed.

Clams exposed to lower salinities showed a significant reduction in esterase activity (Fig. 4C). Day 1 observed very low esterase activity in 0 (3036.75 ± 118.25 A.U.) and 5 salinities (2453 ± 98 A.U.). The esterase activity was relatively higher at 35 and 25 salinities followed by 15. Esterase activity decreased on day 4 at all the salinities except for 25 and 35. A further drop in esterase activity was observed on day 6 at 15, 25 and 35 salinities.

After exposure to salinity for varying period, hemocytes ROS production modified significantly (Fig. 4D). On day 1, ROS production was significantly lower at 0 (242.5 ± 6.5 A.U.) and 5 salinities (419.75 ± 14.25 A.U.) than the rest; ROS production at 15, 25 and 35 salinities did not vary significantly. A further decrease in the ROS production was evident in 0 and 5 salinities on

day 4, whereas at a salinity of 25 and 35, it didn't vary significantly. On day 6, the lowest value of ROS production was observed at salinity 15 (420.5 ± 8 A.U.). On day 1, 4 and 6 no significant differences in values were found in 25 and 35 salinities.

Phagocytic activity was less at lower salinities and decreased with the days of exposure (Fig. 4E). Day 1 showed low values in 0 salinity (26.38 ± 0.48 %) preceded by 5 (32.85 ± 0.18 %) and 15 (45.26 ± 0.12 %). No significant difference was observed between 25 and 35 salinities. Compared to day 1, day 4 values were relatively low for 0, 5 and 15 salinities, whereas no significant difference was observed in 25 and 35. In comparison with day 4 and 6, the activity did not vary significantly for a salinity of 15, 25 and 35.

Total hemocyte counts on day 1 did not vary significantly irrespective of salinity treatments (Fig. 4F). A significant reduction in THC was observed on day 4 at 0 and 5 salinities, whereas at 15, 25 and 35 salinities, no significant changes were observed. Compared to day 1 and 4, THC values on day 6 showed no significant difference at 15, 25 and 35 salinities (Fig. 4F).

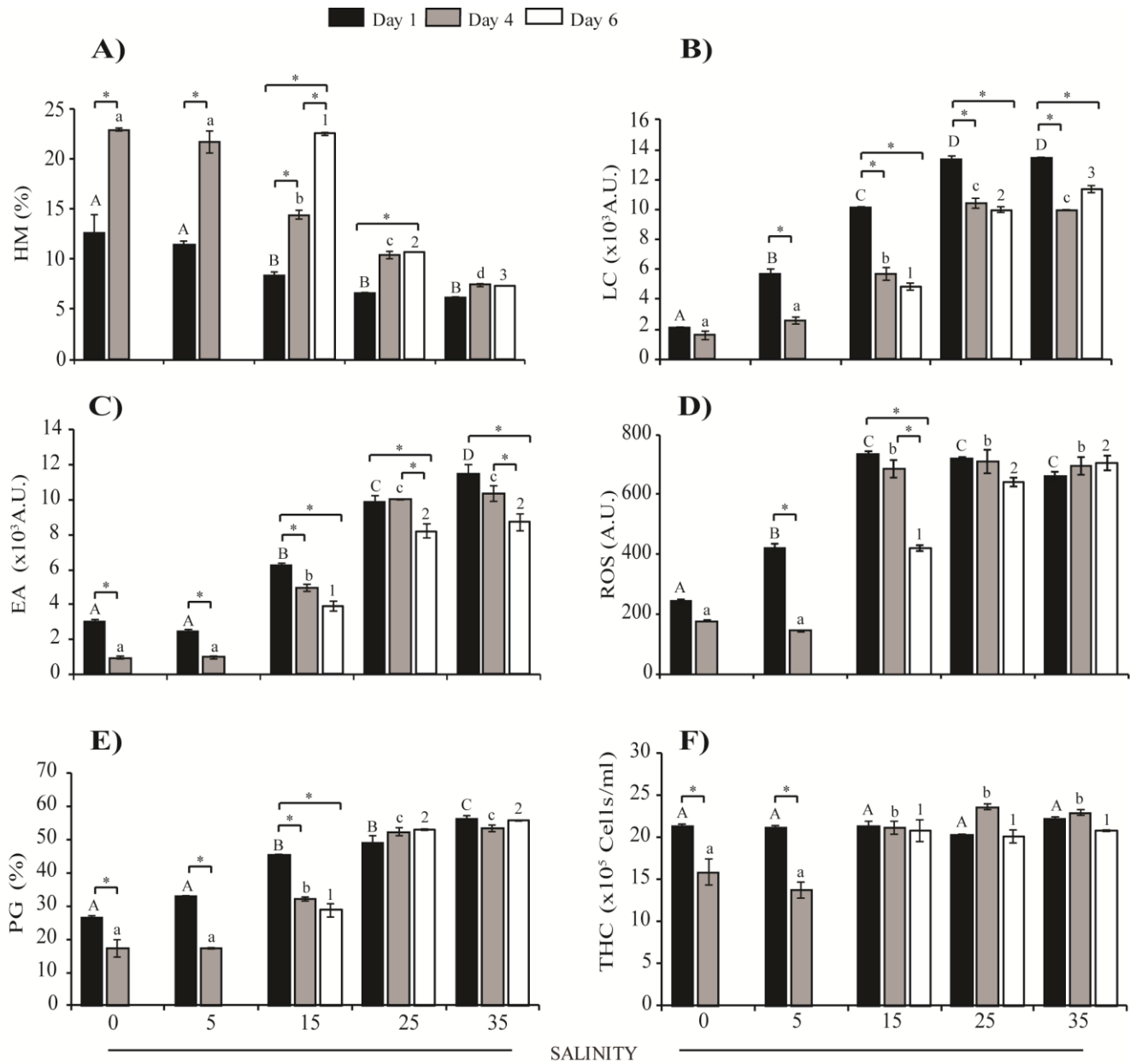


Fig. 4. Comparison using two-way ANOVA for in vivo experiments; Effect of salinity (0, 5, 15, 25 and 35) at each fixed day (Day 1 represented by ‘capital’ letters, Day 4 represented by ‘small’ letters and, Day 6 represented by numbers) and effect of time (represented by ‘*’) at each fixed salinity level, on **A)** Hemocyte mortality (HM) expressed in %, **B)** Lysosomal content (LC) of hemocytes expressed in A.U., **C)** Esterase activity (EA) of hemocytes expressed in A.U., **D)** Reactive oxygen species (ROS) production by hemocytes expressed in A.U., **E)** Phagocytosis (PG) by hemocytes expressed in %, and **F)** Total hemocyte counts (THC) expressed in Cells/ml. Values are mean \pm standard error. Different numbers and letters denote significant differences among salinity treatments; asterisk indicates significant effect of time at each fixed salinity treatments ($p < 0.05$).

Table 1 Two-way ANOVA showing the interactive effects of salinity and time on Hemocyte mortality (HM), Lysosomal content (LC), Esterase activity (EA), Reactive oxygen species (ROS), Phagocytosis (PG), and Total hemocyte counts (THC) of *P. malabarica*. Significant difference ($p < 0.05$).

Hemocyte parameters	In vitro				In vivo		
	Factors	df	F	p	df	F	p
HM	Salinity	4	1.972	0.000	4	170.011	0.000
	Time	1	59.803	0.000	2	473.373	0.000
	Salinity & Time	4	11.847	0.008	8	349.259	0.000
LC	Salinity	4	582.471	0.000	4	1.203	0.000
	Time	1	270.388	0.000	2	335.09	0.000
	Salinity & Time	4	22.665	0.000	8	21.818	0.000
EA	Salinity	4	592.320	0.000	4	917.650	0.000
	Time	1	1.685	0.000	2	126.427	0.000
	Salinity & Time	4	82.902	0.000	8	3.029	0.008
ROS	Salinity	4	516.647	0.000	4	341.798	0.000
	Time	1	596.323	0.000	2	78.287	0.000
	Salinity & Time	4	3.793	0.019	8	14.869	0.000
PG	Salinity	4	484.998	0.000	4	737.110	0.000
	Time	1	212.620	0.000	2	164.621	0.000
	Salinity & Time	4	10.201	0.000	8	43.321	0.000
THC	Salinity	-	-	-	4	218.087	0.000
	Time	-	-	-	2	297.234	0.000
	Salinity & Time	-	-	-	8	88.218	0.000

Discussion

Organisms inhabiting an estuarine condition have varied tolerance and response range. They can counter-attack the stress-effect up to their threshold range of tolerance, but as the stressor extends beyond the acceptable range it can negatively impact the organism. Apart from inducing metabolic adjustments (Wang et al., 2012a), changing salinity has been reported to alter the immunological response of bivalves including hemocyte concentration, volume, phagocytosis, superoxide dismutase and lysosomal activity (Bussell et al., 2008; Fisher et al., 1987; Gagnaire et al., 2006a; Matozzo et al., 2007; Monari et al., 2007; Reid et al., 2003; Rodrick, 2008; Wang et al.,

2012b). Osmotic stress also leads to an additional expenditure of energy for maintaining osmotic balance in the hemolymph (Harris and Aladin, 1997). Moreover, an increase in the occurrence of high rainfall subsequently leading to high flow events in rivers and estuaries has been predicted as one of the consequences of global climate change (Booij, 2005).

Under heavy rainfall conditions, *P. malabarica* is more vulnerable to diametric salinity stress resulting in high possibility of immune-modulation which was clearly exhibited in the present investigation. The hemocyte functions of *P. malabarica* diminished at lower salinities (0 and 5) in both i.e. in vitro and in vivo and the effect intensified with duration of exposure. Among 15, 25 and 35 salinities, insignificant variation in immunological parameters indicated the immuno-tolerance range of 15-35. The experimental significance of in vitro cell exposure was to remove the shielding or buffering effect if any, provided by the body during osmotic adjustments (Harris and Aladin, 1997; Shumway, 1977), thus allowing us to capture the immediate response of hemocytes to a stressor. Although it does not mimic the natural conditions, but the resultant response of hemocytes was no different than the results obtained by in vivo exposure (except for THC as it was assessed only under in-vivo approach). However, despite the same pattern, in vitro hematological values were different and mostly higher than in vivo and this could be attributed to direct or immediate response of hemocytes to the stressor. These kind of studies are very useful to comprehend the response of an organism at the cellular level. Ram Mohan and Velayudhan (1998) reported that *P. malabarica* could endure salinity range of 12 to 40. Mohite (2009) also observed tolerance of *P. malabarica* to salinity as low as 14 during the summer, while the tolerance level reduced to a salinity of 5.6 during the monsoon.

Changes in immunological response under lower salinities can be attributed to osmotic stress (Pipe and Coles, 1995), circulation of hemocytes into the connective tissue (Cajaraville et al., 1996), and the physiological response generated by reduced respiration (Wang et al., 2012b). In the current study, mortality of clams, as well as hemocytes, was observed at lower salinities. This finding is parallel to Gagnaire et al. (2006a) observation in which they found an increase in *C. gigas* hemocytes mortality as well as the organism at lower salinity. Clams mortality could be attributed to osmotic stress resulting in compromised functioning of hemocytes. Cell mortality is primarily caused by necrotic or apoptotic processes (Hengartner, 2000). Under hyposaline conditions (0 and 5), in vitro treatment witnessed ruptured hemocytes potentially in response to osmotic shock (Wang et al., 2012b) whereas, in vivo exposure exhibited behavioral response of valve closure in clams, as a consequence of osmotic stress. This kind of isolation response to external stress was also observed in the mussels to falling seawater concentration (Berger and Kharazova, 1997; Davenport, 1979),

coupled with the energetic cost of adaptation to changing external salinity (Sadok et al., 1997). In such isolated conditions, autophagy is upregulated by breaking down proteins and organelles thus recycling products into protein synthesis and other energy producing pathways for temporary self-sustenance (Moore, 2006). Thus, closure of valves brings out the survival option for an organism in undesirable environmental conditions such as salinity stress or air exposure.

The hemocyte concentration is very well linked to external environmental factors, including salinity (Wang et al., 2011, 2012b, 2014) and treated as a sensitive tool for assessment of bivalve conditions in a given environment. It was suggested that acute salinity alteration inflict inhibitory role on hemocytes circulation and locomotion (Fisher, 1988). In this study, the alteration in hemocyte counts evident on day 4 at lower salinities (0 and 5) can be ascribed to infiltration of hemocytes in different organs. Similar results have been also observed in other bivalves. Bussell et al. (2008) found a significant lowering of hemocytes count in mussel *M. edulis* kept at lower salinities than ambient salinity (32); Jauzein et al. (2013) witnessed a reduction in hemocytes counts at lower salinities in Venus clams. They suggested an alteration in THC might be caused by osmotic stress and elevated infiltration of hemocytes in different organs as a part of the inflammatory response and for optimizing nutrient distribution, therefore, altering the ability to survive in lowering salinity conditions. Wang et al. (2012b) considered a decrease of THC in *P. viridis* due to cell lysis, diapedesis, and osmosis of water between the external water medium hemolymph for regulation of osmolality. Oliver et al. (2008) also found a decrease in THC in *M. edulis*, after rapid reduction of salinity caused by rainfall. They reasoned it with a drop in heart pumping rate, resulting into less pumping of hemocytes into peripheral organs such as adductor muscles. This could be another possible reason for the lowered THC in the current investigation as hemocytes were sampled from the posterior adductor muscles of *P. malabarica*.

Alterations in the lysosomal content of hemocyte have been used as a proxy for understanding hemocyte lysosomal pathology (Lowe et al., 1995) as well as effect of abiotic stressors (Sui et al., 2016; Wang et al., 2012b). Wang et al. (2012b) observed a decrease in the extension of the lysosomal compartment due to hyposalinity. In this study, the lysosomal content was very low at 0 and 5 salinities, and with long-exposure time, a further decrease in the content was evident which could be linked to mortality of hemocytes (Gagnaire et al., 2006a, 2006b), eventually thinning out the ability of an organism to fight against pathogens. Moore and Gelder (1985) demonstrated an increase in the activity of hydrolytic enzymes or lysozyme including acid phosphatase, beta-glucuronidase, beta-N-acetylglucosaminidase, acid-beta-galactosidase after phagocytosis of the dinoflagellate *Isochrysis galbana*. These enzymes are secreted during

phagocytosis (Cheng et al., 1975) and are involved in the degradation of glycoprotein complexes thus proving their role in destruction of phagocytosed organisms (Moore and Gelder, 1985).

Lysosomal compartments of hemocytes are able to release lysozyme and ROS (Cheng, 1983; Austin and Paynter, 1995). Release of reactive oxygen species by hemocytes is a critical oxidative defense of the innate immune response (Anderson, 1994) by which potential pathogens are destroyed followed by phagocytic action. Many authors have pointed out the sensitivity of respiratory burst for assessing environmental salinity stress. Wang et al. (2012b) and Cheng et al. (2004) found reduced ROS production at lower salinities in *P. viridis* and *Haliotis diversicolor*, respectively. The present study too indicated impact of lower salinities (0 and 5) as well as longer-exposure time on lowering of ROS production in *P. malabarica*. This decreasing response can be linked to high mortality rate, morbidity, and enzymes responsible for ROS production. In mussels, lower ROS production by hemocytes was linked to inhibition of enzymes, which are responsible for the production of ROS (Lushchack, 2011).

Phagocytosis is a crucial aspect of the internal defense mechanism in mollusks against pathogens and foreign materials (Wang et al., 2012b). Phagocytic activity along with humoral components such as antimicrobial peptides, lysozyme, and other defense factors help in the obliteration of the phagocytosed target. Bussell et al. (2008) found a significant weakening of phagocytosis in *M. edulis* kept at lower salinities than ambient salinity. In the current investigation, very low phagocytic activity was observed at 0 and 5 salinities (in vitro as well as in vivo) and with longer exposure time a further reduction in the activity was observed. Under hyposaline conditions, Wang et al. (2012b) also witnessed modulated phagocytic activity in *P. viridis*. Cheng et al. (2004) found decreased phagocytic activity in *H. diversicolor* at salinities lower than control. Low phagocytosis can be elucidated with high mortality of the cells and morbidity. Kuchel et al. (2010) observed a reduction in phagocytic activity of *Pinctada imbricate* hemocytes, which was supported with either osmotic stress or increased infiltration of hemocytes into connective tissues and organs. Gagnaire et al. (2006a) also reported reduced phagocytic activity in oyster *C. gigas* under low salinities. However, alteration in phagocytic response to salinity could be species specific (Wang et al., 2012b).

Esterases comprise of hydrolases that catalyze the cleavage as well as the formation of ester bonds, playing an immunological role in the bivalve defense system (Lehtonen et al., 2006). Environmental stresses such as salinity and temperature can alter the esterase activity (Pretti and Cognetti-Varriale, 2001; Wang et al., 2012b). The present study showed very low levels of esterase

activity at lower salinities which was expected as there was a high hemocyte mortality (Gagnaire, 2006a). In hyposaline conditions, Wang et al. (2012b) and Gagnaire et al. (2006a) also reported lowered esterase activity in *P. viridis* and *C. gigas*, respectively.

There is a high possibility of translation of weak immune parameters into a debilitated state of the organism, and could be one of the reasons of clam's augmented monsoonal mortality as witnessed in the present study area. Lower salinity can inflict immune-compromised state in bivalves by the diminution of immune-competence, making them more susceptible to bacterial infection and disease development (Rodrick, 2008; Reid et al., 2003). Moreover, diametrical stress in marine environment tends to affect the physiological as well as immunological state of bivalves interactively rather than in isolation (Bussell et al., 2008; Wang et al., 2012b). Recent studies have experimentally proven the interactive effect of abiotic stresses on bivalves. For instance, short-term hypoxia and low pH lowered the immunological response of thick shell mussel *Mytilus coruscus* (Sui et al., 2016); long term interactive effect of low pH and high temperature compromised the immune response of *M. coruscus* (Wu et al., 2016); hypoxia and nano titanium oxide synergistically affected the immune response of *P. viridis* (Wang et al., 2014); interactive effect of chronic hypoxia and hypo salinity reduced the anti-predatory response in *P. viridis* (Wang et al., 2012a); hypoxia and high temperature combinedly affected the immune functionality of *P. viridis* (Wang et al., 2011). In view of this, we propose the importance and necessity of studying the interactive effect of salinity along with other environmental stresses on *P. malabarica*.

5. Conclusions

The present study accounts for the immunological response of *P. malabarica* from a monsoon-influenced tropical estuary using the two-way experimental approach which is also the first report from the south-west coast of India. The investigation emphasized the role of hemocytes as investigatory cells for ascertaining salinity stress in the estuarine habitat. In accordance with the results, acute and prolonged lower salinity changes led to altered immune surveillance in *P. malabarica*. The two-way approach confirmed a negative relationship between *P. malabarica* immune functionality to hyposalinity with an immuno-salinity tolerance range of 15-35. Even though in vitro conditions do not represent natural environment conditions, it surely assisted us to construe the biology of cells and their immediate interaction under stress when they are not housed inside the body. The diminishing response of hemocyte parameters in lower salinities could be attributed to salinity driven osmotic stress; establishing a tradeoff between energetic cost and survival under adverse conditions. As an organism has to make a choice between survivability and expenditure of

energy for self-sustenance under isolation response, which could be linked to lowering metabolism resulting in compromised lower immune-functions. The compromised immune functions can be translated into increased pathogenic susceptibility and alteration in growth rate leading to hampered survival ability. Also, prolonged exposure to salinity stress seems to be detrimental to bivalve physiological and immunological functioning, which could be the possible reason for their elevated mortality in a monsoon influenced estuary. This finding is very important in understanding the immune competence of *P. malabarica* in an estuarine condition which is subjected not only to salinity variation, but other ambient stressors too. Different biotic and abiotic factors tagged with anthropogenic activities could be detrimental to bivalve's health leading to a cascading effect on the higher trophic level. Therefore, further studies should consider the synergistic effect of different stressors on *P. malabarica* for unraveling biological consequences on the bivalve, subsequently influencing trophodynamics in estuarine conditions.

Conflict of interest

Both the authors declare no conflict of interest.

Submission declaration

Both the authors declare that the presented work has not been published previously and it is not under consideration for publication elsewhere.

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