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Mechanical Stress Induces Neuroendocrine and Immune Responses of Sea Cucumber (*Apostichopus japonicus*)

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Abstract Grading procedure in routine sea cucumber hatchery production is thought to affect juvenile sea cucumber immunological response. The present study investigated the impact of a 3-min mechanical perturbation mimicking the grading procedure on neuroendocrine and immune parameters of the sea cucumber *Apostichopus japonicus*. During the application of stress, concentrations of noradrenaline and dopamine in coelomic fluid increased significantly, indicating that the mechanical perturbation resulted in a transient state of stress in sea cucumbers. Coelomocytes concentration in coelomic fluid increased transiently after the beginning of stressing, and reached the maximum in 1 h. Whereas, coelomocytes phagocytosis at 3 min, superoxide anion production from 3 min to 0.5 h, acid phosphatase activity at 0.5 h, and phenoloxidase activity from 3 min to 0.5 h were all significantly down-regulated. All of the immune parameters recovered to baseline levels after the experiment was conducted for 8 h, and an immunostimulation occurred after the stress considering the phagocytosis and acid phosphatase activity. The results suggested that, as in other marine invertebrates, neuroendocrine/immune connections exist in sea cucumber *A. japonicus*. Mechanical stress can elicit a profound influence on sea cucumber neuroendocrine system. Neuroendocrine messengers act in turn to modulate the immunity functions. Therefore, these effects should be considered for developing better husbandry procedures.

Key words *Apostichopus japonicus*; mechanical stress; neuroendocrine; immune response

1 Introduction

Sea cucumber, *Apostichopus japonicus*, is one of the most important species intensively cultured in China. Due to the continuously increasing demand for dried sea cucumber and overexploitation of natural resource, mass production of sea cucumber juveniles and their culture have evolved as a prosperous fraction of aquaculture sector in north China. The total number of juveniles produced in 2013 reached 73 billions (DOF, 2014).

Size grading is routinely practiced in commercial sea cucumber juvenile production in China, during which sea cucumbers are forced to pass through a sieve and thus they are separated by size and stocked separately. Such practice is believed to be able to enhance the growth of individuals in small size, avoid size variation (Dong *et al*., 2010), and facilitate feeding and harvesting. However, grading may stress sea cucumbers seriously.

Early studies have demonstrated that environmental stressors, such as contaminants, rapid shift of temperature and salinity, hypoxia and mechanical perturbation, reduce the growth of marine invertebrates, suppress their immune system and increase their disease susceptibility (Chu and Hale, 1994; Cheng *et al*., 2004; Liu and Chen, 2004; Chang *et al*., 2009; Hooper *et al*., 2011). When marine invertebrates experience stresses, their sympathetic nervous system receive stimulation and release catecholamines (adrenaline, noradrenaline and dopamine) which then serve as neuroendocrine messengers, diverting bioenergetics resources from certain processes such as reproduction, growth and immunity to specific physiological functions that help them maintain homeostasis. Therefore, mass culture of marine animals requires a better understanding of stress physiology, so that husbandry practices can be optimized to reduce stress incurrence. Unfortunately, the neuroendocrine response and immune change of sea cucumber induced by grading process have

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not been investigated.

In invertebrates, immune response is based on both cellular and humoral factors (Schmid-Hempel, 2003). Coelomocytes, locating mainly in the coelomic cavities, are the effector cells of echinoderm immune system (Coteur *et al*., 2004). Similar to their immune system homologues, haemocytes in molluscs, coelomocytes are capable of carrying out phagocytosis, encapsulation and intercellular respiratory burst, avoiding invasion of microbes (Gu *et al*., 2010). For humoral defense of sea cucumber, the phenolxidase (PO) system is an important immune defense mechanism. PO activity has been detected in coelomic fluid and circulating coelomocytes of *Isostichopus badionotus* (Klanian, 2013) and *A. japonicus* (Zhang *et al*., 2010; Zhao *et al*., 2011; Liu *et al*., 2012).

The immune response of an animal can be assessed by measuring changes of immune variables. In this study, the response of fundamental immune components of sea cucumbers to acute mechanical stress was evaluated, aiming to optimize husbandry practice in the seedling raising of sea cucumber.

2 Materials and Methods

2.1 Animals

Juvenile sea cucumbers *A. japonicus* (5.09 g ± 0.14 g) were collected from a commercial farm in Qingdao, China, and acclimatized one month in three 500 L cylindrical tanks (200 animals each tank) with filtered, aerated water (14–15℃). During acclimation, sea cucumbers were fed *ad libitum* with a diet (7% protein and 3% lipid) consisting of sea mud and *Sargassum thunbergii* powder, and seawater was changed once a day.

2.2 Stress

In total, 140 sea cucumbers were taken from one acclimation tank and put into a wooden box (40 cm × 40 cm × 8 cm) with a plastic mesh with holes (0.8 cm in diameter) at bottom. The sieve was shaken by hand at a rate of approximately 2 times per second in seawater. Juveniles were just submerged in seawater and curled into spheres during shaking. After 3 min of simulated grading, sea cucumbers were allowed to recover in their original tank. Twenty individuals were selected randomly for testing at eight time points (0 min, 3 min, 0.5 h, 1 h, 2 h, 4 h, 8 h and 16 h). The shaking time of 3 min was selected because it is usually adopted for grading practice. Experiments were repeated three times. No sea cucumber died during experiments.

2.3 Sample Collection

Coelomic fluid was withdrawn from individuals each using a 2 mL sterile syringe through body wall. The procedure was finished within 20 s to minimize the effect of sampling on immune response. Coelomic fluid from 5 sea cucumbers was pooled, yielding 2 mL of coelomic fluid. Coelomocytes were immediately counted 4 times using a haemocytometer and calculated as cells per mL coelomic fluid. For the measurement of phagocytosis and intracellular superoxide anion production, 200 μL of coelomic fluid was removed and the coelomocyte concentration was rapidly adjusted to 10^6 cells mL^{-1} with anticoagulant solution (0.02 mol L^{-1} EGTA, 0.48 mol L^{-1} NaCl, 0.019 mol L^{-1} KCl, 0.068 mol L^{-1} Tri-HCl, pH 7.6) (Gu *et al*., 2010). The remaining coelomic fluid was stored at −80℃ for enzyme activity and catecholamines quantity analyses.

2.4 Phagocytosis Assay

Phagocytosis assay was performed according to Ballarin *et al*. (2003) with some modifications. In brief, 100 microliters coelomic fluid containing 10^6 cells mL^{-1} in anticoagulant solution was placed on a glass slide. The coelomocytes were left to adhere for 30 min in a moist incubation chamber before rinsing with anticoagulant solution and adding 100 μL yeast (*Saccharomyces cerevisiae*) suspension in FSW (yeast/coelomocyte ratio= 100:1). After a further 60 min incubation, the glass slides were repeatedly dipped in FSW to remove uningested yeast cells and coelomocytes were fixed for 30 min at 4℃ in a solution of 1% saccharose and 1% glutaraldehyde in FSW, washed in phosphate-buffered saline (0.03 mol L^{-1} KCl, 1.37 mol L^{-1} NaCl, 0.065 mol L^{-1} Na_2HPO_4, 0.015 mol L^{-1} KH_2PO_4) and stained using 5% Giemsa solution in distilled water. The Giemsa solution was removed after 10 min and coverslips were placed over the slide. Three counts of 200 cells were made under a Nikon 80i light microscope from each slide and the percentage of coelomocytes with ingested yeast cells was then calculated.

2.5 Measurement of Intracellular Superoxide Anion Production

Intracellular superoxide anion production was measured according to the method described early (Bussell *et al*., 2008). One hundred microliters of coelomocytes suspension and equal volume of nitroblue tetrazolium (NBT, Sigma) solution (2 mg mL^{-1} in Tris/HCl buffer containing 2% NaCl, pH 7.6) were added to triplicate wells of a 96 well plate for each sample point. The remaining wells were filled with 100 μL of NBT and 100 μL of Tris/HCl as negative controls. The plate was incubated in dark for 1 h, centrifuged at 120 r min^{-1} for 10 min with the supernatant discarded. The cells were washed twice with Tris-HCl (pH 7.6) and fixed with 100 μL of methanol for 10 min. After fixation, the plate was centrifuged at 300 r min^{-1} with supernatant removed and cells air-dried. The cells were then carefully rinsed five times with 100 μL of 50% methanol before adding 120 μL of 2 mol L^{-1} KOH and 140 μL of DMSO. The optical density (OD) value at 620 nm was measured on a microplate reader spectrophotometer and the results were expressed as OD value/10^6 cells mL^{-1}.

2.6 Acid Phosphatase Activity

Frozen coelomic fluid was thawed at 4℃, disrupted with an ultrasonic cell disruptor, centrifuged at 4000 r min^{-1} and 4℃ for 10 min. Supernatant was collected for

acid phosphatase (ACP) activity assay using a commercial kit (Nanjing Jiancheng, China) (Zhao *et al*., 2011). One unit of ACP activity was defined as the degradation of 1 mg phenol per 100 mL coelomic fluid at 37℃ within 30 min.

2.7 Phenoloxidase Activity

Samples were prepared as described in Section 2.6. Phenoloxidase (PO) activity was measured spectrophotometrically using L-3, 4-dihydroxyphenylalanine (L-DOPA; Sigma, USA) as substrate and trypsin (Sigma, USA) as elicitor according to Klanian (2013). Briefly, 50 μL of coelomic fluid and 50 μL of 0.1% trypsin in cacodylate buffer (CAC, 10 mmol L^{-1} sodium cacodylate, 10 mmol L^{-1} $CaCl_2$, pH 7.0) were added to triplicate wells of a 96 well plate for each sample point, incubated at 25℃ for 20 min, and then 50 μL of L-DOPA (0.3% in CAC buffer) was added. Optical density was measured at 490 nm. One unit of enzyme activity was expressed as an increase in absorbance of 0.001 min^{-1}.

2.8 Measurement of Catecholamines

Catecholamines, including noradrenaline (NA) and dopamine (DOP), in coelomic fluid were quantified using HPLC with electrochemical detection as Qu *et al*. (2009) described. Coelomic fluid was thawed at 4℃ in refrigerator, centrifuged at 600 r min^{-1} and 4℃ for 10 min to remove the cellular debris. 500 μL of coelomic fluid was placed into a clean Eppendorf tube, to which 500 μL of TRIS buffer (1.5 mol L^{-1}, pH 8.6, containing 0.07 mol L^{-1} EDTA), 50 μL of 5 nmol L^{-1} sodium metabisulfite, 100 μL of 10 ng mL^{-1} internal standard-dihydroxybenzylamine (DHBA) and 10 mg acid-washed alumina were added. The mixture was agitated for 15 min, centrifuged at 1000 r min^{-1} for 2 min and the supernatant was discarded. The alumina was washed with distilled water and then centrifuged for 2 min at 1000 r min^{-1} before removal of the supernatant. Then, 100 μL 0.2 mol L^{-1} acetic acid was added to the alumina, mixed for 10 min and centrifuged for 2 min at 1000 r min^{-1}. The supernatant was carefully collected, stored at −20℃ and analysed within 2 weeks. Catecholamines were separated on an Agilent XDB C18 column (150 mm × 4.6 mm, 5 μm) with a mobile phase (50 mmol L^{-1} citric acid, 0.05 mmol L^{-1} EDTA, 50 mmol L^{-1} sodium dihydrogen-phosphate, 3 mmol L^{-1} sodium chloride, 0.4 mmol L^{-1} octanesulfonic acid, and 5% methanol, pH 3.0) at a flow rate of 1 mL min^{-1}. An Agilent ESA electrochemical detector was used at 0.7 V with sensitivity of 5 nAFS.

2.9 Statistical Analysis

Data are expressed as mean ± SD ($n = 3$). One-way analysis of variance (ANOVA) was performed to compare data among sample points. The homogeneity of data were tested before the analysis and the difference was significant if $P \leq 0.05$. When the ANOVA detected a significant difference, multiple comparison test of Tukey was carried out. The analyses were performed using SPSS 17.0 software.

3 Results

3.1 Catecholamines

A 3 min mechanical stress significantly changed NA and DOP concentrations in sea cucumber coelomic fluid. NA (Fig.1a) and DOP (Fig.1b) concentrations changed in the same way over time. During exposure to the mechanical stressor, noradrenaline and dopamine concentration increased ($P < 0.05$) to seven- and three-folds of the basal value, respectively. After stress, NA and DOP concentrations decreased rapidly, returning to the basal values 1 h after beginning of experiment.

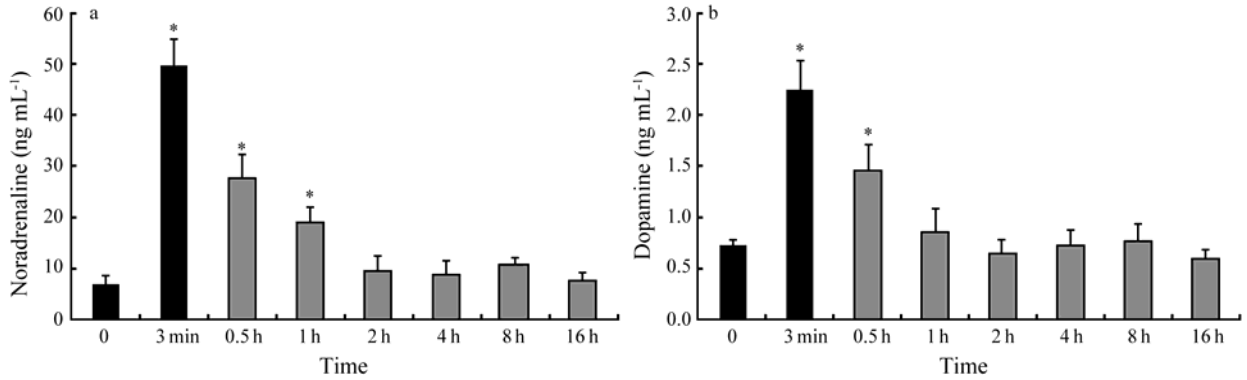

Fig.1 Effect of a 3-min mechanical stress on noradrenaline (a) and dopamine (b) concentrations in coelomic fluid of sea cucumber *A. japonicus*. Black bars represent values recorded during stress and hatched bars represent values recorded after stress. The concentration is expressed as mean ± SE ($n = 12$). Asterisks denote significant differences from basal values (sample time 0; $P < 0.05$).

3.2 Coelomocytes Concentration in Coelomic Fluid

Coelomocytes concentration in coelomic fluid showed a non-significant increase during application of the stress, from 8.97×10^6 cells mL^{-1} at the beginning to 10.23×10^6 cells mL^{-1} at the end (Fig.2a). After stress, it increased significantly ($P < 0.05$) and reached a maximum of 16.16×10^6 cells mL^{-1} at 1 h after the onset of stress and then

began to decrease and return to the initial level in 3 h.

3.3 Phagocytosis Activity

As indicated in Fig.2b, a significant ($P<0.05$) decrease, from 14.86% to 5.48%, in the percentage of phagocytic cells was observed during stress. Following stress the percentage of phagocytic cells tended to increase significantly ($P<0.05$) in 1 h and then decreased to roughly the same value (12.79%) as beginning of experiment in one hour.

3.4 Measurement of Intracellular Superoxide Anion Production

Intracellular superoxide anion production significantly decreased ($P<0.01$) from 0.206 OD values to 0.108 OD values per 10^6 cells mL^{-1} in 3 min stress (Fig.2c). Intracellular superoxide anion production began to increase after stress and returned to the initial value in 1 h.

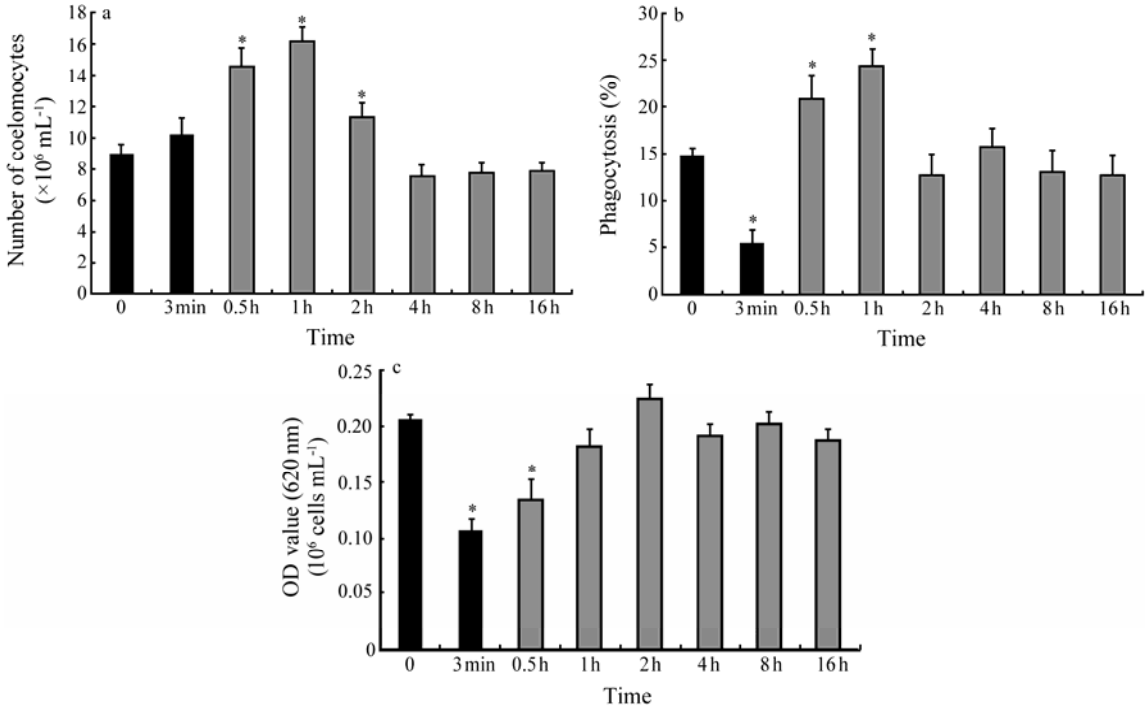

Fig.2 Effect of a 3-min mechanical stress on (a) coelomocytes concentration, (b) phagocytosis activity and (c) the production of intracellular superoxide anion in coelomic fluid of *A. japonicus*. Black bars represent values recorded during stress and hatched bars represent values recorded after the stress. Each bar represents mean ± SE ($n=12$). Asterisks denote significant ($P<0.05$) differences from basal values (sample time 0).

3.5 ACP Activity

At the start point of the experiment, ACP activity was around 3.59 U per 100 mL coelomic fluid (Fig.3a). During stress, ACP activity decreased slightly. When stress ended, ACP activity decreased significantly ($P<0.05$) and reached a minimum of 2.05 U per 100 mL coelomic fluid in 0.5 h. After that, it began to increase to 4.41 U per 100 mL coelomic fluid at 4 h, which was significantly higher than the initial value, and then decreased to initial value in 4 h.

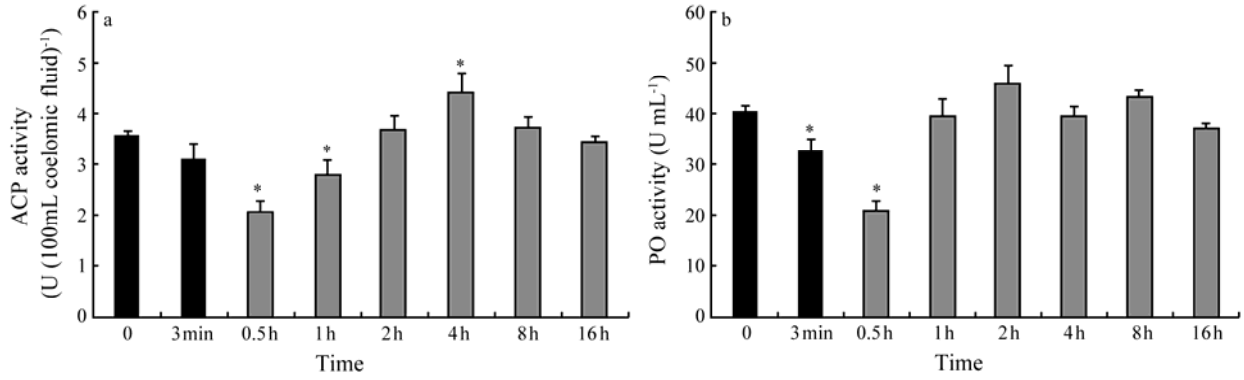

Fig.3 Effect of a 3-min mechanical stress on (a) acid phosphatase (ACP) and (b) phenoloxidase (PO) activity in coelomic fluid of *A. japonicus*. Black bars represent values recorded during the stress period and hatched bars represent values recorded after the disturbance. Each bar represents mean ± SE ($n=12$). Asterisks denote significant ($P<0.05$) differences from basal values (sample time 0).

3.6 PO Activity

PO activity decreased significantly in 0.5 h after the beginning of stress, returned to initial value in 1 h and remained unchanged until the end of experiment.

4 Discussion

The release of neurohormones such as glucocorticoids and catecholamines is a primary response to stress in vertebrates (Chrousos and Gold, 1992; Ottaviani and Franceschi, 1996). There are increasing evidences that this neuroendocrine response system also exists in aquatic invertebrates (Lacoste *et al*., 2001a, 2001b; Pequeux *et al*., 2002; Qu *et al*., 2009; Simon *et al*., 2010). Previous studies have demonstrated that *A. japonicus* changes activities of catecholamines in response to temperature and salinity variations during aestivation (Wang *et al*., 2008). The present study also proved that NA and DOP concentrations increased significantly in coelomic fluid when sea cucumber encountered a mechanical stress. Presumably, neuroendocrine stress-response axis has been preserved during the course of evolution.

In molluscs, a decline of the number of circulating haemocytes was identified after the start of a mechanical stress (Lacoste *et al*., 2002; Malham *et al*., 2002, 2003). Noradrenaline and dopamine injections were also demonstrated to decrease the haemocyte count in hemolymph in white shrimp *Litopenaeus vannamei* (Cheng *et al*., 2006; Pan *et al*., 2011). In contrast, there was no reduction in coelomocytes concentration during application of the stress in our experiment was found. According to a study in starfish, the coelomic epithelium was identified as one of the sources of new coelomocytes, which releases coelomocytes upon LPS injection (Holm *et al*., 2008). It is possible that mechanical stress also induced proliferation of coelomocytes in coelomic epithelium, and then coelomocytes entered coelomic fluid. The following decrease in the number of coelomocytes in coelomic fluid is proposed to be the result of coelomocytes fixation in tissues (Chang *et al*., 2011). An alternative suggestion is that during stress, immunoactive coelomocytes migrated from connective tissues into coelomic fluid. However, the reasons for this phenomenon remained to be unknown.

In invertebrates, phagocytosis represents an important mechanism of host defense (Bayne, 1990). During phagocytosis of coelomocytes, several reactive oxygen species (ROS) are produced, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen. All these short-living compounds can be directly toxic to pathogens (Roch, 1999). ROS production by phagocytes may be an important mechanism of antimicrobial protection of holothurians (Dolmatova *et al*., 2003). In the present study, phagocytosis activity and intracellular superoxide anion production decreased within 5 min after the beginning of stress. A similar phenomenon is observed in abalone *Haliotis tuberculata* (Malham *et al*., 2003), oyster *Crassostrea gigas* (Lacoste *et al*., 2002) and oyster *Pinctada imbricata* (Kuchel *et al*., 2010) subjected to a mechanical stressor. Conversely, Malham *et al*. (2002) observed an increase in phagocytosis activity and intracellular superoxide production in the octopus *Eledone cirrhosa* during an air exposure. These facts suggest that the effects of stress hormones on invertebrate immune function are diverse, and usually depend on the type of the stressor, the animal species considered and the physiological condition of the animal (Husmann *et al*., 2011; Adamo, 2012). The reduction in phagocytosis activity and superoxide anion production observed in disturbed sea cucumber may be due to the β-adrenergic receptors expressed by coelomocytes, which permit noradrenaline to exert a dose-dependent inhibitory effect on these coelomocyte functions (Lacoste *et al*., 2001c, 2001d, 2001e).

Stresses were reported to depress the activity of enzyme involved in immune defense in many marine invertebrates. PO activity was inhibited in oysters exposed to handling practices (Kuchel *et al*., 2012). A significant decrease in acid phosphatase activity was observed in Venus clams *Chamelea gallina* exposed to anoxic stress (Pampanin *et al*., 2002). In addition, noradrenaline injections inhibited acid phosphatase activity in *S. glomerata* (Aladaileh *et al*., 2008). Dopamine injection resulted in a significant decrease in PO activity (Pan *et al*., 2011). The decline in the activity of ACP and PO maybe the result of several events, *e.g*., change in lysosomal integrity, reduction in the frequency of defensive coelomocytes and depression of proPO-activating enzyme and PO activity by NA (Pampanin *et al*., 2002; Chang *et al*., 2011; Kuchel *et al*., 2012).

Importantly, the fact that immunological functions are down-regulated during and after the mechanical disturbance could be highly detrimental to the sea cucumbers. Previous study has shown that oysters infected with the oyster pathogen *Vibrio splendidus* carried higher bacterial loads and experienced higher mortalities following a 15-min mechanical disturbance than infected unstressed animals (Lacoste *et al*., 2001a). The farmers anecdotally reported mechanical stress linked with disease outbreaks and mortality and reduced growth in sea cucumbers. Thus, it seems that mechanical stress depresses the immunity of sea cucumber and augments the susceptibility of the animal to certain pathogens. Furthermore, interactive effects of various stresses might also depress the immunity and disease-resistance ability of sea cucumbers. Wang *et al*. (2012) reported that there were interactive effects of hypoxic and hyposaline stresses on immune responses in mussel *Perna viridis*. During farming, various stresses were bound to challenge the immune system of sea cucumbers. It is possible that mechanical stress increases the susceptibility of sea cucumbers to other forms of stress such as acute temperature or salinity stress. Further work should aim at testing this hypothesis.

In conclusion, the present study demonstrated that mechanical stress induced quantifiable alterations in neuroendocrine and immune response in *A. japonicus*. Concentrations of noradrenaline and dopamine in coelomic fluid

elevated significantly during stress, and returned to the baseline levels at 2 h. All immune parameters investigated were significantly down-regulated by stress, except coelomocyte concentration, which increased transiently after the beginning of the stress. All of the immune parameters had recovered to baseline levels by 8h after the start of experiment. To further understand the neuroimmune processes in *A. japonicus* exposed to environmental stressors, more studies should be conducted on adrenergic receptors involved in stress-induced immune changes in these animals.

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