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Immunosuppressive effects of environmental stressors on immunological function in *Pinctada imbricata*

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ABSTRACT

This study assessed the effects of mechanical agitation, hypo-saline conditions, and exposure to the air on the Akoya pearl oyster, *Pinctada imbricata*, focusing specifically on the immunological activity of haemocytes. Both phagocytosis and phenoloxidase activity decreased significantly when oysters were exposed to all three stressors. Transient decreases were also evident in total haemocyte counts after mechanical stress and exposure to air, while significant increases in total haemocyte counts were evident after exposure to low salinity. Acid phosphatase activity increased significantly when oysters were exposed to air. The frequency of granulocytes in the haemolymph increased significantly when oysters were stressed by hypo-saline conditions, whilst the relative frequency of granulocytes did not differ significantly after mechanical agitation or exposure to air. The total protein content of haemolymph increased significantly when oysters were stressed by mechanical agitation and low salinity. These results suggest that fluctuations in environmental conditions affect circulating haemocytes and their cytochemistry, and that the different immunological parameters tested were influenced uniquely according to the type of stressor.

1. Introduction

The successful cultivation of any marine organism is dependent upon a stable relationship with their environment. Fluctuations in environmental conditions can significantly affect homeostasis and lead to physiological stress [38]. Typical stressors associated with aquaculture include hypo- and hyper-thermia, declines in dissolved oxygen content (hypoxia), mechanical agitation, fluctuations in salinity and exposure to air resulting in desiccation and anoxia [4,7,8,16,17]. Many of these stressors have been shown to compromise immune function [27]. A number of studies have demonstrated that extended periods of stress can lead to immunosuppression, which can result in opportunistic infections [12,15,33].

Until recently, the Akoya pearl oyster formed the basis of a highly lucrative pearling industry in Japan. Between 1993 and 1996, productivity declined significantly from 118 000 kg to 63 000 kg [28]. It was postulated that a combination of factors facilitated the proliferation of disease, including environmental fluctuations and overcrowding [35]. Due to these declines in the Akoya market, Australian companies, in conjunction with Japanese investors, are developing an Akoya pearling industry in Port Stephens, NSW, Australia [28]. In order to develop a successful industry, it is imperative to discern optimal farming conditions, as well as those that may compromise productivity. For this reason, our study will focus on the physiological implications of environmental stress on *Pinctada imbricata*, specifically investigating key immunological parameters.

In marine bivalves, immunological defence is based on innate responses that are mediated by both cellular and humoral factors [32]. Cellular defence includes haemocyte-mediated responses such as coagulation, encapsulation, phagocytosis, phagolysosomal activity and the production of reactive oxygen species (ROS; superoxide anion, hydrogen peroxide and hydroxyl radical [2,6,30].

Granulocytes and hyalinocytes represent the two main types of immunocompetent haemocytes in oysters [34]. Granulocytes are highly defensive haemocytes containing hydrolytic enzymes that assist in the intracellular killing of pathogens and represent the cellular aspect of internal defence [2]. Contrasting to this, hyalinocytes contain few to no cytoplasmic granules and are said to have a “silky” appearance [20,39]. For this reason, granulocytes are

Abbreviations: two-way ANOVA, two-way analyses of variance; DIC, differential interference microscopy; FSW, filtered seawater; MBTH, 3-methyl-2-benzothiazoline hydrozone; NSW DPI, New South Wales Department of Primary Industries; PO, phenoloxidase; PBS, phosphate buffered saline; ROS, reactive oxygen species; SEM, standard error of the mean; TEM, transmission electron microscope; THC, total haemocyte counts; 4-HA, hydroquinine monomethyl ether.

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considered to be more immunologically active than hyalinocytes [2].

Oysters are filter feeders and represent a class of marine invertebrates which are osmo- and thermo-conformers [40]. Although the ocean is relatively stable, coastline and estuarine waters do vary in both temperature and salinity. This dependence on their external environment makes oysters highly vulnerable to these environmental fluctuations, which can result in physiological stress [4,7,8,17]. Recent studies have shown that hypo-saline conditions (25 ± 1 ppt) decrease haemocyte abundance in the mussel *Mytilus galloprovincialis* [26], and inhibit the activity of intracellular phenoloxidase in Sydney rock oysters, *Saccostrea glomerata* (*S. glomerata*; [7]. High water temperatures (23–27 °C) have also been shown to inhibit haemocyte locomotion and adhesion in the eastern oyster *Crassostrea virginica* (*C. virginica*; [18] as well as resulting in a decrease in haemocyte abundance and phagocytic activity in the clam *Ruditapes philippinarum* [3,17,40]).

Fluctuations in ambient water temperature and salinity have not only been shown to compromise immunological activity but they have also been implicated with increased parasite/pathogen loads by creating a more suitable environment for their survival and reproduction [27]. In 1982, Haskin et al. investigated the density gradient of the pathogen *Haplosporidium nelsoni* (MSX disease) in Delaware Bay (USA). They found disease proliferation to be directly correlated with salinity [19]. At low salinities (9–18 ppt) the parasite was found to enter *C. virginica*, but its development was severely retarded [19]. Similar studies by Butt et al. [7] showed that salinity significantly affects the presence and infection rates of *S. glomerata* with *Marteilia sydneyi* (QX disease). Increased infection was correlated with a decrease in the defensive enzyme phenoloxidase, associated with a decline in salinity [7].

The current study examines the effects of low salinity, mechanical agitation and exposure to air on the haemocytes of the Akoya pearl oyster, *P. imbricata*. These stressors were selected as typical perturbations experienced by pearl oysters farmed for aquaculture.

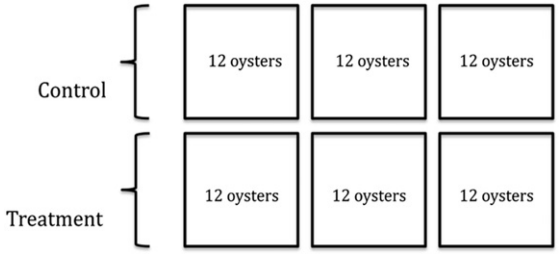
2. Materials and methods

2.1. Animals

Adult *P. imbricata* (50–80 mm in dorso-ventral height) were supplied by Port Stephens Fisheries Center, New South Wales (32°44′S, 152°08′E). Oysters were housed at the Sydney Institute of Marine Science (SIMS) and acclimatised for at least 14 days in temperature-controlled (22 °C, 45 L) flow through aquaria. The oysters were fed every two days with M-1 diet (Aquasonic Pty Ltd, Wauchope, NSW).

2.2. Experimental design

Three different types of environmental stressors were tested; low salinity (25 ppt), mechanical agitation (10 min, 300 rpm) and exposure to air (2 h periods every 24 h). To test the effects of altered salinity, oysters were transferred to closed aquaria containing seawater from Sydney harbour that had been diluted to 25 ppt with Milli-Q water for the duration of the experiment. Salinity was measured using a TPS WP-81 water quality meter (Enviroequip, Brisbane, Australia). Hypo-saline conditions chosen for exposure were based upon fluctuations recorded in the Port Stephens region (Wayne O'Connor pers. com.). Mechanical stress was simulated by placing oysters on a MS1 Minishaker (Crown, Scientific, NSW, Australia) and agitating them for 10 min at 300 rpm every 24 h. Once the oysters had undergone mechanical agitation they were returned to the tanks. In order to test air exposure treatment oysters were removed from the aquaria and placed on the lab bench every 24 h for 2 h. After the oysters were stressed, they were returned to the tanks. A total of 6 tanks housed 12 oysters each, three control tanks (36 oysters in total) and 3 treatment tanks (36 oysters in total; see diagram below). For each time point (0, 24, 72 & 120 h) three oysters were randomly selected from each of the three tanks corresponding to the treatment, totalling 9 oysters per time point for both control and for treatment. Partial water changes (50% or 25 L) were performed on closed tanks every 24 h. This experimental design was replicated for each stressor tested.



2.3. Sampling

Groups of 9 oysters ($N = 9$) from treatment and control tanks were removed from the aquaria 0, 24, 72 and 120 h after the beginning of each experiment (totaling 72 oysters). They were placed on absorbent towel for 10 min prior to haemolymph extraction to drain excess water from their mantle cavities. Haemolymph was extracted from each oyster by rupturing the pericardial cavity located near the adductor muscle using a 27-gauge needle fitted to a 1-mL syringe. Whole haemolymph was immediately transferred to 1.5 mL microcentrifuge tubes and held on ice before being stored at −80 °C. Haemolymph samples used to examine phagocytosis were overlaid onto acid–alcohol washed slides with adherent yeast. Cells collected for total and differential haemocyte counts were fixed in 4% paraformaldehyde.

2.4. Differential haemocyte counts

In order to differentiate between *P. imbricata* blood cells, haemocytes were stained with the Romanowsky stain, Giemsa/May-Grünwald [2]. Thirty microlitres of whole haemolymph was spotted onto acid–alcohol cleaned slides. Cells were allowed to adhere for 25 min in a humid chamber. After adhesion, samples were fixed for 20 min with paraformaldehyde (4% w/v in FSW; Sigma–Aldrich, Castle Hill, NSW). The slides were then immersed in May-Grünwald stain for 6 min. The cells were counterstained with Giemsa for a further 30 min and subsequently washed in Phosphate Buffered Saline (PBS; 136 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, adjusted to pH 7.5). Slides were air-dried and mounted with Ultramount No. 7 (Fronine Laboratory Supplies, Taren Point, NSW) and examined using Olympus BH-2 microscope. One hundred haemocytes were counted per oyster and classified as either granulocytes or hyalinocytes by the scheme of Cheng [10,11]. Although both hyalinocyte and granulocyte abundance were assessed, we have only presented data relating to granulocyte abundance as they play a more significant role in host defence [20].

2.5. Phagocytosis assay

Congo-red-stained yeast (*Saccharomyces cerevisiae*; Sigma–Aldrich), were used as target cells to test the phagocytic capacity of haemocytes. Two hundred and 50 mg of yeast in 5 ml FSW were mixed with 5 ml 0.8% Congo red prepared in FSW. The yeast were then autoclaved at 90 °C for 20 min. The suspensions were