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The effect of low salinity on phenoloxidase activity in the Sydney rock oyster, *Saccostrea glomerata*

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Abstract

This study tested the effects of low salinity on phenoloxidase activity in Sydney rock oysters (*Saccostrea glomerata*). Phenoloxidase is a key component of the immune system in *S. glomerata*. Previously, we have shown that decreased phenoloxidase activity is associated with susceptibility to fatal QX disease. In the current study, laboratory-based experiments were used to identify factors that contribute to decreased phenoloxidase activity. We found that exposing oysters to water collected from a QX-prone oyster growing area after heavy rain significantly inhibited their phenoloxidase activities. Similar inhibition was evident when oysters were held in oceanic water with artificially lowered salinities. These results suggest that exposure to low salinity decreases phenoloxidase activity. Field trials that exploited a natural salinity gradient in the Georges River, Sydney, supported this conclusion. The phenoloxidase activities of oysters transplanted to up-river sites that had low salinities were significantly lower than those of oysters held at seaward sites with higher salinities. All of these data implicate low salinity as a key environmental stressor that is associated with inhibition of the *S. glomerata* immune system.

Keywords: Environment; Immune; *Marteilia sydneyi*; Oysters; Phenoloxidase; Salinity

1. Introduction

Sydney rock oyster farming is one of Australia's largest aquaculture industries. However, production levels have fallen by over 40% since the 1970s (Heasman et al., 2000). Decreased production has resulted from competition with other oyster species and high levels of mortality caused by two infectious diseases, winter mortality and QX disease (Nell, 2003).

QX disease is mediated by a paramyxean protozoan, *Marteilia sydneyi* (Adlard and Ogburn, 2003; Anderson et al., 1995). Outbreaks of the disease now occur frequently in a number of growing areas on the east coast of Australia, including the Georges River, Sydney (Adlard and Ernst, 1995). Up to 98% of oysters die during QX epizootics.

Recently, we have shown that susceptibility to QX disease is associated with decreased phenoloxidase

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activity in oysters from QX-prone growing areas (Peters and Raftos, 2003). Phenoloxidase is a key component of the oyster immune system. It catalyses a cascade of oxidation reactions that convert tyrosine-based substrates to phenols and quinones, leading to the formation of the pigment, melanin. Melanin is an important component of capsules that sequester invasive pathogens. Intermediates of the cascade also recruit cellular defenses such as phagocytosis (Peters and Raftos, 2003; Soderhall et al., 1994) or act as fungistatic and antibacterial agents (Sorrentino et al., 2002).

In a previous study, we found that the transplantation of oysters from QX-free areas to QX-prone sites resulted in the rapid inhibition of phenoloxidase activity, followed shortly after by *M. sydneyi* infection (Peters and Raftos, 2003). The data indicated that *M. sydneyi* infection itself is not responsible for the inhibition of phenoloxidase activity. Instead, it seems that some other environmental stressor causes the decrease in phenoloxidase activity, and that *M. sydneyi* infection occurs as a result of this immunosuppression.

Similar relationships between environmental stress and decreased immune competency have been identified in a number of marine bivalves including the edible mussel, *Mytilus edulis* (Grundy et al., 1996; Pipe et al., 1997, 1999), and the oysters *Crassostrea gigas* (Lacoste et al., 2002) and *Crassostrea virginica* (Anderson et al., 1996; Chu and Hale, 1994; Fisher et al., 1999). Recently, Lacoste et al. (2002) identified a molecular mechanism that links immune function and stress in oysters. They found that oysters can mount neuroendocrine stress reactions that are similar to the catecholamine-based systems of vertebrates (Lacoste et al., 2001b,c). Release of the hormone, noradrenaline, during stress reactions in oysters inhibits defense responses like phagocytosis and the production of reactive oxygen intermediates (Lacoste et al., 2001a). This suggests that disease resistance in oysters can be inhibited by adaptive changes to their physiology that occur in response to deleterious environmental conditions.

In Sydney rock oysters, a link between disease and environmental stress is supported by circumstantial evidence that associates QX disease susceptibility with environmental perturbation. The sporadic geographical distribution of QX-prone estuaries indicates that environmental conditions within individual estuaries dictate disease susceptibility (Adlard and Wesche, 2004). Similarly, QX disease outbreaks do not occur every year in all affected estuaries, suggesting that environmental conditions must be optimal before epizootics can occur. *M. sydneyi* has also been discovered in many estuaries that have never experienced outbreaks of QX disease. This implies that an (some) additional factor(s), other than the presence of the parasite, contributes to disease outbreaks (Adlard, personal communication).

There have already been some attempts to identify environmental factors that are associated with QX disease susceptibility. Early evidence suggested that QX outbreaks only occur after heavy rain (Haysom, 1978; Lester, 1986). This led a number of workers to test whether QX disease is triggered by decreased environmental pH associated with runoff from acid sulphate soils. Wesche (1995) showed that the severity of QX outbreaks may be dependent upon pH induced stress. However, Anderson et al. (1995) found that *M. sydneyi* infection still occurred in the absence of major pH fluctuations.

The lack of information about environmental parameters that contribute to QX disease outbreaks provided the impetus for the current study. Here, we test whether exposure to low salinity can explain the decrease in phenoloxidase activity that is associated with QX susceptibility.

2. Materials and methods

2.1. Oyster collecting sites

Saccostrea glomerata that had been grown from single seed culture for approximately three years were collected from Porto Bay (33°34′42″ S, 151°13′40″ E) on the Hawkesbury River, NSW, Australia. They were kindly supplied by Kevin and Sue Buie of K and S Buie Oyster Farms. Over the duration of this study (January, 2003 to April, 2004), the Hawkesbury River was considered to be free of *M. sydneyi* (Adlard and Wesche, 2004). Since then there has been a severe outbreak of QX disease in the upper reaches of the Hawkesbury River (Callinan and McOrrie, 2004). However, *M. sydneyi* has still not been detected in the area from which oysters used in this study were

collected (Porto Bay; R. Adlard, Queensland Museum, personal communication).

2.2. Laboratory exposures

Oysters from the Hawkesbury River were placed in aquaria (maximum of 8 oysters per aquarium) containing 20-L oceanic seawater collected from Little Manly, Sydney (33°48′S, 151°17′E), or water from Neverfail Bay on the Georges River, Sydney (33°59′41″ S, 151°04′21″ E). The Georges River has suffered severe QX disease outbreaks annually since 1994 (Nell, 2003). Water from the Georges River was collected before or after heavy rain. Heavy rain was defined as >50 mm precipitation falling on the Georges River catchment over three days. Less than 10 mm of precipitation had to fall on the catchment over the preceding fortnight before water was classified as being collected before heavy rain. Rainfall data were obtained from the Bureau of Meteorology, Sydney. In one series of experiments, oysters were exposed to oceanic seawater (collected from Little Manly,) that had been diluted (20% to 100% v/v) with Milli-Q distilled water (Millipore, North Ryde, NSW) to provide salinities ranging from 7 to 34 ppt.

Aquaria were maintained at a constant temperature of 22 °C and oysters were fed at regular intervals with Reef-Nature invertebrate food (5 mL/200 L of water, Aquasonic, Ingleburn, NSW). Salinity and pH were measured in each aquarium using an Horiba U-10 Water Quality Checker (Kyoto, Japan).

A minimum of four replicate aquaria were used per treatment. At least 6 oysters were held in each aquarium for 12 days before their PO activities were measured.

2.3. Field trials

Two field trials were conducted during October, 2003 and February, 2004. Oysters were transferred in mesh bags to five sites along a 5 km stretch of the Georges River upstream from the river mouth. Starting from the most seaward, those sites were: Quibray Bay (34°00′S, 151°11′E) Shipwrights Bay (34°01′28″ S, 151°06′23″ E), Neverfail Bay (33°59′41″ S, 151°04′21″ E), Lime Kiln Bay (33°59′08″ S, 151°03′10″ E) and Salt Pan Creek (33°58′26″ S, 151°03′35″ E). Three bags of oysters were tethered at each site. Each bag contained 8 oysters. Bags were fixed to posts located just above the low tide mark so that all oysters were out of the water for a short period at each tide. Oysters were held at each site for 12 days before being tested for PO activity.

2.4. Water quality data

Water quality data collected at Salt Pan Creek on the Georges River over the period January, 2003 to April, 2004 was kindly provided by Sydney Water (Sydney). The parameters measured were conductivity, temperature, dissolved oxygen, pH, turbidity, total nitrogen and total phosphates. Salinity was calculated from conductivity and temperature data by the method of Clesceri et al. (1998). Temperature, pH and salinity were measured weekly at other sites on the Georges River using an Horiba U-10 Water Quality Checker.

2.5. Phenoloxidase assay

Oysters were allowed to air dry before up to 2 mL haemolymph was withdrawn using 22-gauge needles fitted to 5-mL syringes. A notch was made at the shell hinge and haemolymph was withdrawn from the pericardial cavity. Haemolymph was immediately placed in polypropylene tubes and held on ice. It was centrifuged for 5 min (5000×g, 4 °C) so that the supernatant (cell free haemolymph, CFH) could be collected.

One hundred microlitres of CFH was added per well to 96-well flat bottom microtitre plates (Sarstadt, Technology Park, SA). One hundred microlitres of the phenoloxidase substrate, hydroquinine monomethyl ether (4-HA; Fluka, Switzerland; 5 mM in filtered seawater) containing the chromogenic nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH, Sigma Aldrich, Castle Hill, NSW; 1 mM), was also added to each well (Dicko, 2002). Filtered seawater (FSW) was prepared by ultra-filtration through 0.45-μm filters (Millipore). Absorbance was measured at 490.0 nm using a Model 550 microplate spectrophotometer (Bio-Rad, Regents Park, NSW) immediately after the addition of substrates. Plates were then incubated at 22 °C for 1 h before a second absorbance reading was made. Initial (0 time-point) readings were

subtracted from measurements made after 60 min so that the change in absorbance per minute (ΔOD_{490}/min) could be calculated. Data were adjusted to take into account absorbance in wells containing phenoloxidase substrates in the absence of CFH (negative controls) and the total protein content of CFH. Protein concentrations in CFH were measured using a Bio-Rad Protein Assay kit with a standard curve generated with bovine serum albumin. Phenoloxidase activities are presented as the change in absorbance per minute per μg protein (ΔOD_{490}/min/μg protein).

2.6. Data analysis

Data were analysed using SPSS v.11 software (Microsoft, Sydney, NSW). Tests for normalcy and Levene's test for homogeneity of variance were carried out on all data. Alpha values were adjusted to eliminate potential errors associated with any heterogeneous variance. Two-way analysis of variance (ANOVA) was used to establish whether significant differences existed between aquaria or bags within a given site or treatment (Kerr et al., 2002; Morgan and Griego, 1998). Tukey's test was used during post hoc analysis to establish where significant differences occurred within data sets (Underwood, 1997). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Effects on phenoloxidase activity of water collected before and after rain

Oysters exposed to water collected after heavy rain had lower phenoloxidase activities than those held in water collected before rainfall. This was demonstrated in two independent experiments using water collected at different times (Fig. 1). The first of these experiments compared the phenoloxidase activities of oysters before and after exposure (Fig. 1A). It showed that phenoloxidase activity in oysters exposed to water collected after rain was decreased to almost half of that evident among oysters tested before exposure and those held in water collected before heavy rain (ANOVA, $df = 2$, $p < 0.05$).

Similar results were obtained in the second experiment, which compared the phenoloxidase activities of oysters held in water collected from the Georges River before and after heavy rain with those of oysters held in oceanic seawater from Little Manly (Fig. 1B). The mean phenoloxidase activity of oysters exposed to water collected after heavy rain was less than half that of oysters maintained in oceanic seawater and approximately one third of that among oysters exposed to water collected prior to rainfall (ANOVA, $df = 2$, $p < 0.001$).

In both experiments, water collected from the Georges River after heavy rain had substantially lower salinity than water collected before rain and oceanic seawater (Fig. 1). Other water quality parameters, including pH, turbidity and tempera-

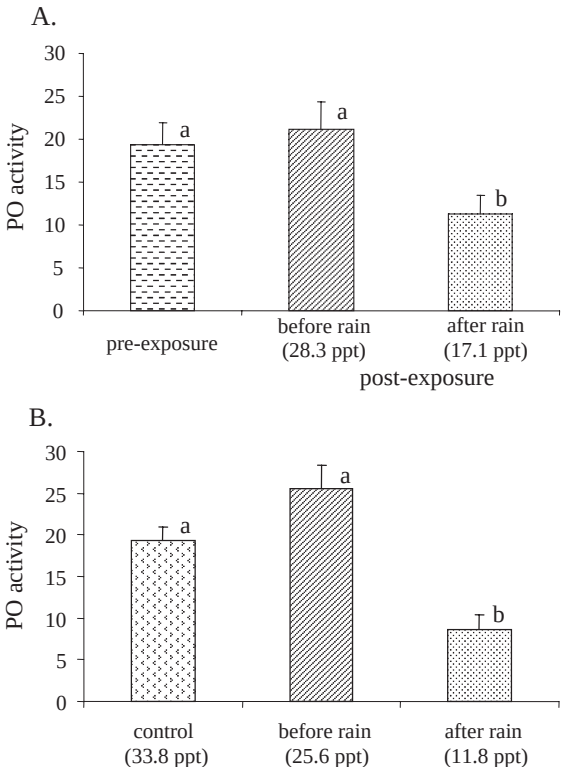

Fig. 1. Mean phenoloxidase activities (PO, ΔOD_{490}/min/μg protein $\times 10^6$) of oysters exposed to water collected from the Georges River before and after heavy rainfall events, or to oceanic seawater collected from Little Manly (control). Phenoloxidase activities were measured either before or after exposure to the different water treatments. (A) Water collected between March 9, 2003 and April 20, 2003. (B) Water collected between March 14, 2004 and April 30, 2004. Like letters indicate comparisons that did not differ significantly ($p > 0.05$), $n \geq 36$, bars = 1 S.E.M.

ture, did not differ between treatments (data not shown).

3.2. Effects on phenoloxidase activity of artificially lowered salinity

Exposing oysters to oceanic seawater diluted with distilled water substantially decreased phenoloxidase activity (Fig. 2). Oysters held in 40% v/v seawater (13.5 ppt) had mean phenoloxidase activities that were half those of oysters maintained in 100% seawater (33.8 ppt; ANOVA, $df=2$, $p<0.001$). Dilution of seawater to 20% v/v caused phenoloxidase activity to fall by 80% relative to undiluted seawater, and by 60% compared to 40% v/v seawater (ANOVA, $df=2$, $p<0.001$).

3.3. Effects of exposure to natural salinity gradients on phenoloxidase activity

In both field trials, differences in phenoloxidase activities among oysters transplanted to the Georges River were dependent upon the location at which oysters were held along the river (Fig. 3). In the first trial, mean phenoloxidase activity among oysters held at Neverfail Bay was 40% higher than in oysters transplanted to Lime Kiln Bay and Salt Pan Creek (Fig. 3A, Tukey's test, $p<0.05$). The salinity of water at Neverfail Bay (25.7 ppt) was also greater than that of Lime Kiln Bay (21.4 ppt) and Salt Pan Creek (19.9 ppt).

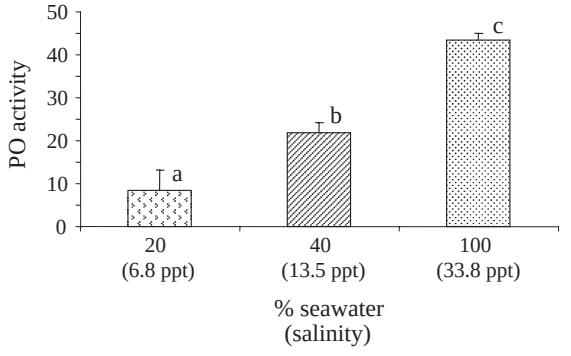

Fig. 2. The effect of artificially altered salinity (% v/v seawater, ppt in brackets) on the mean phenoloxidase activities (PO, ΔOD_{490}/min/μg protein $\times 10^6$) of oysters. Salinity was lowered by adding distilled water to oceanic seawater collected from Little Manly. Like letters indicate comparisons that did not differ significantly ($p>0.05$), $n\geq 36$, bars = 1 S.E.M.

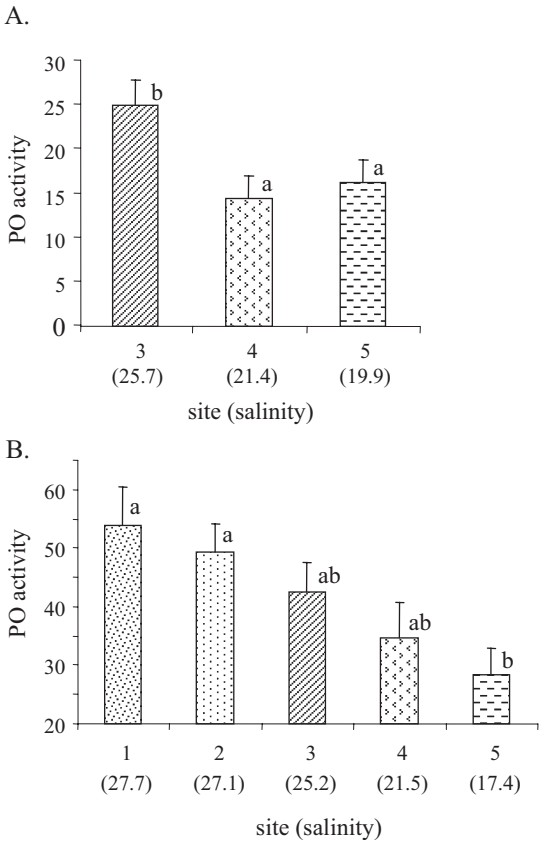

Fig. 3. Mean phenoloxidase activities (PO, ΔOD_{490}/min/μg protein $\times 10^6$) of oysters that had been transplanted to different sites along the Georges River during (A) October, 2003 or (B) February, 2004. Sites are numbered progressively from sea-ward to inland (1 — Quibray Bay; 2 — Shipwrights Bay; 3 — Neverfail Bay; 4 — Lime Kiln Bar; 5 — Salt Pan Creek). Like letters indicate comparisons that did not differ significantly ($p>0.05$), $n=18$, bars = 1 S.E.M.

Similar results were obtained during the second field trial (Fig. 3B). Phenoloxidase activity decreased in up-river locations that had less saline waters. In this experiment, the most seaward sites, Quibray Bay (27.7 ± 1.34 ppt) and Shipwrights Bay (27.1 ± 0.31 ppt), had significantly higher phenoloxidase activities than Salt Pan Creek, the most inland site (Tukey's test, $p<0.05$). The PO activity of oysters held at sites with intermediate salinities (Neverfail Bay, 25.3 ± 0.53 ppt and Lime Kiln Bay, 20.9 ± 0.79 ppt) did not differ significantly from other sites. However, they contrib-

uted to an obvious trend of decreasing PO activity with low salinity.

4. Discussion

Anecdotal evidence from oyster farmers has often associated QX disease outbreaks with heavy summer rainfall. In a previous study, it was shown that QX outbreaks correspond with the inhibition of PO activity among oysters. Hence, we began the current investigation by testing the effects of water collected from the Georges River before and after heavy rainfall events on the PO activities of *S. glomerata*. Phenoloxidase activity was significantly inhibited among oysters held in water collected after rain. In contrast, there was very little change in the PO activities of oysters maintained in water collected prior to rain. This suggested that PO activity was reduced by some component of water affected by heavy rainfall. The measurement of various generic water quality parameters (salinity, pH, temperature, turbidity, dissolved oxygen, total nitrogen and total phosphorous) showed that salinity was the only environmental factor that differed substantially between water collected before and after rain. Hence salinity became the focus for further investigation. In the current study, the effects of low salinity on PO activity were further investigated by testing the effect of artificially altered salinities. Historical water quality data show that salinity in the Georges River can fall to less than 10 ppt after heavy rain. The exposure of oysters to artificially lowered salinities within this range significantly decreased dose-dependent PO activity.

Similar effects of low salinity on immune function have been identified in other aquatic invertebrates, including the freshwater prawn, *Macrobranchium rosenbergii*, (Cheng and Chen, 2000, 2002), a number of estuarine shrimp species (Le Moullac and Haffner, 2000; Perazzolo et al., 2002; Vargas-Albores et al., 1998) and various species of abalone (Chen and Chen, 2000; Martello et al., 2000). The immune functions monitored in these other studies included total haemocyte counts, phenoloxidase activity, protein concentrations and phagocytic activity. A number of other environmental factors, such as the concentration of various pollutants, have also been shown to inhibit immune function in a variety of marine bivalves (Chu and Hale, 1994; Coles et al., 1994; Fisher et al., 1999; Grundy et al., 1996; Pipe et al., 1999).

Having identified the effects of low salinity under controlled laboratory conditions, we then tested whether similar inhibition occurred in a natural salinity gradient. Two field trials were undertaken to exploit the salinity gradient that is known to occur in the Georges River. Both trials showed that oysters kept at seaward sites had significantly higher phenoloxidase activities than oysters maintained at up-river (inland) sites. This corresponded with substantial differences in salinity between seaward and up-river locations. Importantly, none of the other water quality parameters measured (temperature, pH and turbidity) differed significantly between sites.

The relationship between low salinity and PO suppression complements previous work which showed that PO levels decrease prior to QX disease outbreaks in the Georges River (Peters and Raftos, 2003). When combined, the data suggest that a link exists between salinity and QX disease outbreaks. Such relationships have been identified in oysters before. Two separate studies on eastern oysters (*Crassostrea virginica*) have shown that external stressors can affect disease susceptibility. Fisher et al. (1999) found that the intensity of *Perkinsus marinus* infection was increased in oysters that had been exposed to tributyltin relative to unexposed oysters. Similarly, Chu and Hale (1994) showed that oysters exposed to a range of organic pollutants had increased susceptibility to *P. marinus* infection. Recent studies of the abalone, *Haliotis diversicolor supertexta*, have also shown that decreased immune competence and increased susceptibility to bacterial infection result from exposure to a range of environmental factors, including low salinity (Cheng et al., 2004a,b,c,d).

Although our data provide strong evidence for an association between low salinity and QX susceptibility, that link cannot be demonstrated directly. Laboratory based infection experiments cannot be undertaken because *M. sydneyi* has never been isolated outside of its oyster host and oyster to oyster transmission does not take place (Kleeman and Adlard, 2000; Wesche et al., 1999). Despite this inability to directly test the link between low salinity and disease susceptibility, such an association is supported by historical data from the Georges River. A nationwide drought in Australia meant that very little

precipitation fell across the Georges River catchment during summer and early autumn of 2003. As a result, salinity in the river remained at levels that were consistently higher than in the four previous years. 2003 was also the first year since 1996 that no major QX disease outbreak occurred in the Georges River (unpublished data). Data for numerous other water quality parameters revealed no differences between 2003 and previous years in factors other than salinity during the key infection period. This suggests that the absence of a QX disease outbreak in 2003 may have been due to the consistently higher salinities during that period.

If the association between low salinity and QX disease proves to be robust it will provide the first evidence that could contribute to the effective management of QX disease. However, detailed investigation of other environmental stressors will also be important. Although it is clearly implicated in disease susceptibility by this study, salinity may not be alone in its ability to affect immune function in *S. glomerata*.

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