



# The role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*)

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## Abstract

The paramyxean protozoan, *Marteilia sydneyi*, is the etiological agent of QX disease in Sydney rock oysters (*Saccostrea glomerata*). QX disease affects the farming of oysters in Queensland and on five rivers in New South Wales (NSW), Australia. Disease outbreaks occur during summer months (January to April) and are associated with high mortality rates (up to 98%), which limit the growing out period for oyster production. This study investigated the relationship between oyster host defense systems and QX disease. Oysters from the same brood stock were harvested from QX prone and QX free growing areas over the course of the 2000–2001 outbreak. A variety of parameters—infection intensity in the digestive diverticulum, total hemolymph protein content, condition index and phenoloxidase (PO) activity—were measured in these oysters. Phenoloxidase activity, which is often associated with host defense, was significantly suppressed in oysters from the QX prone area when compared to those from the QX free river. There was also a strict negative correlation between phenoloxidase activity and the intensity of parasitic infection ( $p = 0.0038$ ). In contrast, neither total protein content nor condition indexes differed between oysters from the QX prone and QX free rivers. These data suggest that inhibition of prophenoloxidase cascade may facilitate lethal infection by *M. sydneyi*.

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## 1. Introduction

This study investigates the host–parasite relationship between Sydney rock oysters, *Saccostrea glomerata* (formerly *S. commercialis*; Buroker et al., 1979; Anderson and Adlard, 1994), and the paramyxean parasite, *Marteilia sydneyi*, which causes fatal QX disease. QX disease was first detected in *S. glomerata* from Moreton Bay, in the northern Australian state of Queensland during 1972 (Wolf, 1972). It has since spread extensively in Queensland and has been detected in five rivers in the adjacent state of New South Wales (NSW) (Adlard and Ernst, 1995; Roubal et al., 1988). Mortality rates in QX-affected estuaries of up to 98% occur during summer months (January to April) and the impact of this disease has been so great in some areas that oyster farming has been abandoned.

Prior to 2002, the only stages of the *M. sydneyi* life cycle that had been characterized were the presporulation and sporulation stages, which occur within oyster digestive glands (Perkins and Wolf, 1976). However, a very recent study by Kleeman et al. (2002) has now thoroughly defined the life cycle of *M. sydneyi* within a variety of oyster tissues. Despite this new information, *M. sydneyi* has never been found outside its oyster hosts and the parasite's mode of infection has not been resolved.

This lack of relevant data has prevented effective management of QX disease in endemic areas. Current management regimes rely only on the removal of oysters from the water in QX prone estuaries during the infective period. An attempt is being made by NSW Fisheries to develop QX disease resistant oysters by interbreeding the rare survivors of infection that reach market size. However, this breeding program is currently “on-hold” due to continuing problems with large scale hatchery production of spat, even though mortality rates among “resistant” strains are reported to have declined (Dr. John Nell, NSW Fisheries, personal communication). Despite its dramatic effect on the viability of oyster farming in infected estuaries and the absence of effective management strategies, there is little existing information about physiological effects on oysters that might lead to the establishment of lethal infection by *M. sydneyi*.

The premise of our investigation is that *M. sydneyi* must evade or suppress the host defense systems of oysters in order to establish infection. This possibility—that pathological infection depends on immunosuppression or evasion—has already been tested in a number of other shelled mollusks. For instance, in the Eastern oyster, *Crassostrea virginica*, formation of reactive oxygen intermediates (ROI) and phagocytic activity is suppressed by live *Perkinsus marinus*, even though both ROI and phagocytosis are stimulated by exposure to non-viable parasites. These studies suggest that *P. marinus* avoids phagocytosis by either suppressing cellular activity via the release of an inhibitory molecule or by preventing molecular recognition processes that precede phagocytic activity (Anderson, 1999; Ford and Ashton-Alcox, 1998).

Similar forms of immunosuppression have been shown to affect the prophenolox- idase-activating (proPO) system. The proPO system is a proteolytic cascade that contributes to both defensive responses and pigmentation in a variety of animals including shelled mollusks. Activation of the proenzyme, proPO, by serine proteases

initiates the *o*-hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine and the oxidation of phenols to quinones. These quinones polymerize to form the pigment, melanin (Sritunyalucksana and Söderhäll, 2000; Aroca et al., 1990). Various intermediates of the proPO cascade contribute to defensive responses such as phagocytosis, nodule formation and melanotic encapsulation (Marmaras et al., 1996; Söderhäll et al., 1993; Aspán et al., 1995; Lanz et al., 1993; Gollas-Galván et al., 1999; Sung et al., 1998).

The ability of pathogens to suppress the proPO cascade has been demonstrated in a number of species. Richards and Edwards (2000) have found that hemolymph fractions from parasitized larvae of the insect, *Lacania oleracea*, had significantly lower phenoloxidase (PO) activities relative to those from non-parasitized individuals. Infection, according to their report, was facilitated by specific inhibitory factors released from parasites, which prevent the activation of the proPO cascade. Similarly, Shelby et al. (2000) showed that parasitized larvae of another insect, *Heliothis virescens*, lacked two key enzymes of the proPO cascade, phenoloxidase and dopachrome isomerase, in their plasma.

Here, we test whether inhibition of the proPO system in Sydney rock oysters is associated with QX disease. The data show that PO activities are suppressed during a typical infection cycle, implying that immunosuppression of oysters may allow *M. sydneyi* to establish lethal infections.

## 2. Materials and methods

### 2.1. Oysters

Naïve Sydney rock oysters were grown on trays in Porto Bay, Hawkesbury River, NSW, Australia (33°34'S, 151°15'E). This area has never been affected by QX disease. Two thousand of these naïve oysters were transplanted in November 2000 to Neverfail Bay, Georges River (34°01'S, 151°10'E), an area that has been subjected to annual outbreaks of QX disease since 1996. A further 100 oysters from Porto Bay were transplanted to Neverfail Bay in March 2001. Groups of five to six oysters were collected for analysis on a regular basis over the period of December 2000 to May 2001 from both Porto and Neverfail Bays.

### 2.2. Calculation of condition indexes

Oysters were cleaned and air-dried before being weighed. Once hemolymph had been collected (see below), oysters were shucked so that valves and internal tissues could be weighed separately. Valves were air-dried for at least one hour before weighing. The condition index (CI), a value used by other workers to gauge the general “health” of oysters, was then calculated as follows (Smith et al., 2000):

$$\text{CI} = \text{wet tissue weight} \div (\text{weight prior to shucking} - \text{air dried shell weight})$$

### 2.3. Collection of hemolymph

Oysters shells were notched near the adductor muscle sinus and hemolymph was collected through a 22-gauge needle into a 5-ml syringe. Hemolymph was immediately transferred to microcentrifuge tubes and held on ice.

### 2.4. Tissue preservation and assessment of infection intensity

After weighing, tissues from shucked oysters were placed in a 10-fold excess of 4% (v/v) formaldehyde in sterile filtered seawater (FSW, 0.45 µm filtration). Tissues were incubated in this fixative for 24 h at 4 °C and then washed three times for 2 h per wash in FSW. After washing, tissue samples were stored in 50% ethanol at 4 °C.

Infection intensity was determined by homogenizing 100 mg of digestive glands that had been dissected from fixed tissue in a Dounce homogenizer containing 1 ml FSW. A total of 100 µl of the homogenate was diluted with 900 µl of FSW before the frequency of *M. sydneyi* was determined using a hemocytometer. The identification of *M. sydneyi* was based on the descriptions of Wolf (1972) and Perkins and Wolf (1976).

### 2.5. Protein determination

Total hemolymph protein concentrations were determined by the Bradford method using a Bio-Rad Protein Assay kit (Bio-Rad, Castle Hill, NSW) with bovine serum albumin as a standard.

### 2.6. Phenoloxidase assay

Phenoloxidase activity was assayed spectrophotometrically to quantify the formation of melanochrome from L-3,4-dihydroxyphenylalanine (L-DOPA). The assay used here was modified from the methods of Gollas-Galván et al. (1997) and Deaton et al. (1999). Briefly, oyster hemolymph (100 µl) was incubated with 100 µl L-DOPA (4 mg/ml in FSW, ICN, Irvine, CA, USA) for 3 h in 96-well flat bottom tissue culture plates (Sarstadt, Technology Park, SA, Australia). Absorbance was then measured at 490 nm on a microplate reader. Triplicate wells were analyzed for each oyster and data were adjusted by subtracting absorbance values for wells containing L-DOPA alone.

### 2.7. Statistical analysis

Data analysis was performed using Analyse-it software (version 1.65, Analyse-it, England). Correlations between different parameters were quantified using Pearson correlation analysis and were verified by linear regression analysis. One- and two-way analyses of variance (ANOVA) were also performed to test the significance of interactions between dependent factors (protein content, PO activity and condition index) and independent factors (river and date). Either the Scheffé or Tukey's methods at 99% confidence levels were used to verify the source of variation identified by one-way ANOVA.

### 3. Results

#### 3.1. Onset and intensity of *M. sydneyi* infection

Two-cell plasmodia of *M. sydneyi* were first detected during the last week of January 2001 in the hemolymph of oysters placed in the Georges River during November 2000. Infection in the digestive gland was first detected 2 weeks after it was first identified in the hemolymph (Fig. 1). At this time, plasmodia were observed undergoing sporulation to form 8 to 16 sporonts per plasmodium. The intensity of infection in the tissue increased continuously until the end of March. By May, infection intensity had decreased due to progressive mortality of infected individuals. The mortality rate of the oysters over the period February 2001 to May 2001 was 98%. *M. sydneyi* was never detected in oysters that remained in the Hawkesbury River over the infection period.

#### 3.2. PO activities during the infection period

PO levels among oysters transferred to Georges River in November 2000 declined gradually during the period when *M. sydneyi* sporonts were evident in digestive glands (early February until the end of March) (Fig. 2). They then increased slightly over the period April–May, at the same time that infection intensity decreased as a result of mortality among infected individuals. Pearson and linear least squares regression analysis indicated that PO levels were significantly negatively correlated with parasite infection intensity (Fig. 3,  $r^2=0.19$ ,  $p=0.0038$ ).

A rapid decline in PO activity was evident among oysters transplanted from the Hawkesbury River on 15 March 2001, during the period of maximum infection intensity (Fig. 2). Within 1 week of their transfer to the Georges River, *M. sydneyi* were detected in hemolymph of the transplanted oysters and their PO levels had decreased to a third of the activity evident among oysters that remained in the Hawkesbury River. These suppressed

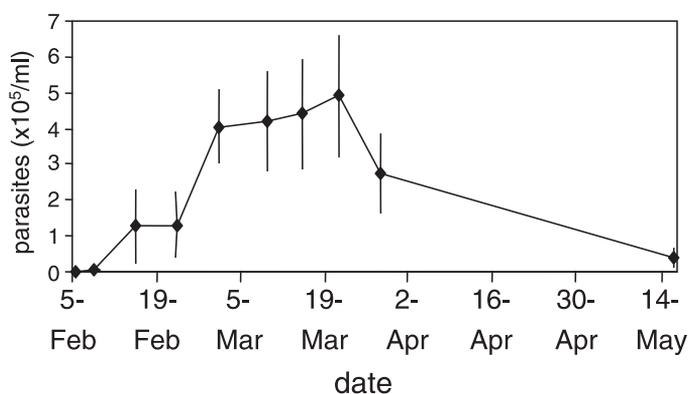


Fig. 1. Intensity of *M. sydneyi* infection (parasites  $\times 10^5/\text{ml}$ ) in the digestive diverticulae of Sydney rock oysters at various times during the period 5 February 2001 to 16 May 2001. Bars represent standard error,  $n=6$ .

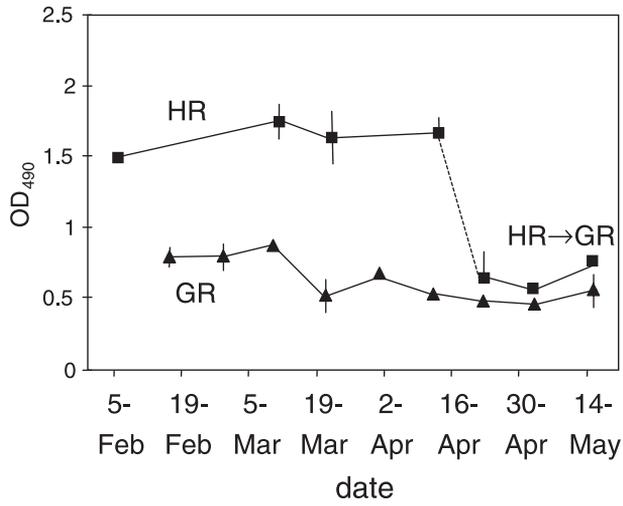


Fig. 2. Phenoloxidase activities (OD<sub>490</sub>) measured at various times during the period 5 February 2001 to 16 May 2001 in Sydney rock oysters (OD<sub>490</sub>) from Georges River (GR), Hawkesbury River (HR) and among oysters transferred from HR to GR on 15 March 2001 (HR → GR). Bars represent standard error, *n* = 6.

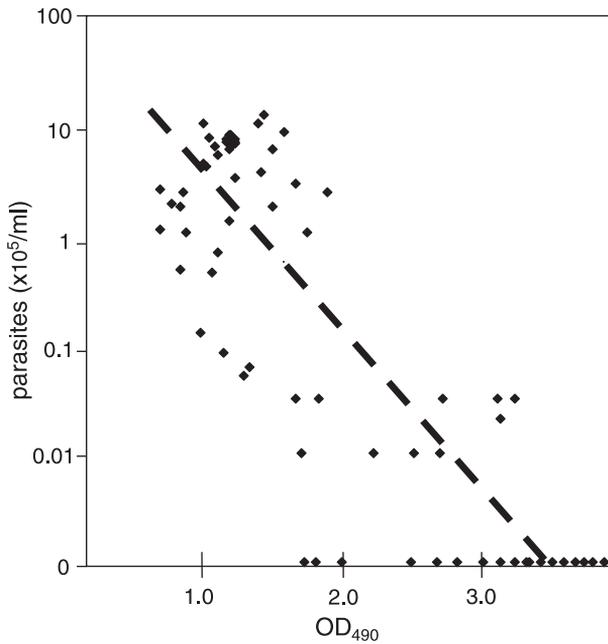


Fig. 3. Linear regression analysis of infection intensity (parasites × 10<sup>5</sup>/ml) versus phenoloxidase activities (OD<sub>490</sub>) of Sydney rock oysters in the Georges River at various times during the period of 5 February 2001 to 16 May 2001. *r*<sup>2</sup> = 0.19, *p* = 0.0038, *n* = 60.

levels of PO activity were similar to those of oysters that had been transplanted to the Georges River in November 2000.

The PO activities of oysters that remained in the Hawkesbury River were significantly higher than those of transplanted oysters throughout the period 5 February to 15 March (Fig. 2). Hence, over the course of the infection period, one-way ANOVA of PO activities in the Georges and Hawkesbury Rivers revealed that variation in PO activity over time was only significant ( $p < 0.0001$ ) in the Georges River. Similarly, two-way ANOVA, which simultaneously tested PO activities against both date and river, indicated that variation in PO activity was statistically significant ( $p < 0.0001$ ) only in the Georges River after the onset of *M. sydneyi* infection.

### 3.3. Variation in total protein content of hemolymph

The total protein concentrations of hemolymph from oysters transplanted to the Georges River in November 2000 also decreased over the infection period (Fig. 4). However, unlike PO activity, Hawkesbury River oysters exhibited a similar gradual decrease in protein concentration over the same time period, except for 22 February, at which time protein concentrations increased as the result of a spawning event. One-way ANOVA indicated that oysters in both rivers had a significant decline in protein content over the course of the infection period ( $p = 0.0002$ ). However, two-way ANOVA demonstrated that total hemolymph protein content did not differ significantly between oysters from the Georges River

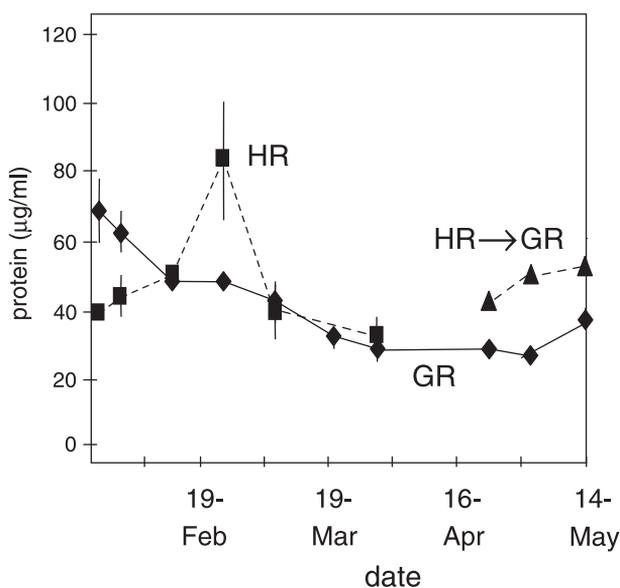


Fig. 4. Total protein concentrations ( $\mu\text{g/ml}$ ) of Sydney rock oyster hemolymph at various times during the period 5 February 2001 to 16 May 2001. Bars represent standard error,  $n = 6$ . Hawkesbury River (HR), Georges River (GR), oysters transplanted from HR to GR on 15 March 2001 (HR  $\rightarrow$  GR).

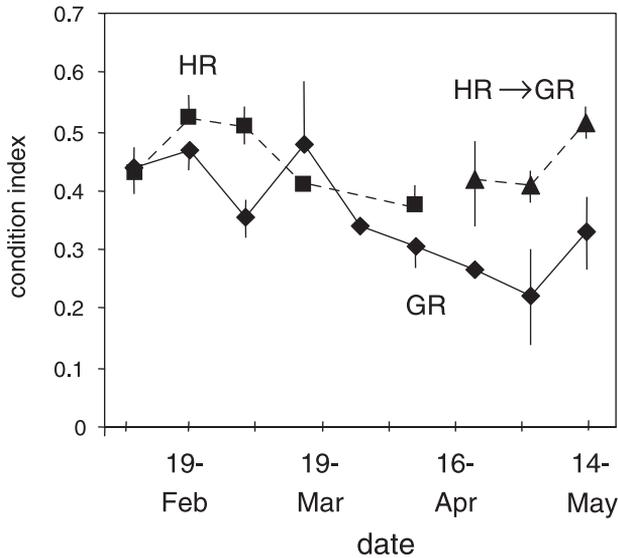


Fig. 5. Condition index for Sydney rock oysters at various times during the period 5 February 2001 to 16 May 2001. Bars represent standard error,  $n=6$ . Hawkesbury River (HR), Georges River (GR), oysters transplanted from HR to GR on 15 March 2001 (HR → GR).

and Hawkesbury River ( $p=0.6936$ ). The total protein contents of hemolymph from oysters transplanted to the Georges River on 15 March were comparable to the protein levels found in oysters from the Hawkesbury River.

#### 3.4. Variation in condition index

Condition indexes decreased gradually over the period February–March 2001 among oysters transplanted to Georges River in November 2000 and among those that remained in the Hawkesbury River (Fig. 5). The condition index values did not vary significantly ( $p>0.05$ ) between these two groups of oysters. The second group of transplanted oysters (transferred on 15 March 2001) had similar condition indexes to those of oysters transplanted to the Georges River prior to the infection period, and to Hawkesbury River oysters (Fig. 5).

## 4. Discussion

The data presented here indicate that *M. sydneyi* infection may be initiated in Sydney rock oysters by suppression of the host's proPO system. Parasitic infection was first detected in the hemolymph of oysters in the Georges River during late January 2001 and became evident in digestive glands during the second week of February. From then, the intensity of infection increased continually in the digestive gland reaching its highest levels in late March. It was also evident that uncontrolled infections could be rapidly established

among oysters transplanted to the Georges River at the height of the infection period (March 2001).

Plasmodia, the earliest defined stage of *M. sydneyi* life cycle, were first detected in the oyster hemolymph then spread rapidly to the digestive diverticulum. Sporonts, the only known post-plasmodial form of *M. sydneyi*, were never detected in the hemolymph. They were only found in the digestive diverticulum after plasmodia were detected in this organ. These observations agree with those of Perkins and Wolf (1976).

The ability to accurately determine the onset and intensity of *M. sydneyi* infection has allowed us to identify a strong association between infection and PO activity. In other systems, the proPO cascade acts as a defensive response, and PO activity is often up-regulated at the initiation of parasitic infection (Söderhäll and Cerenius, 1998). However, such up-regulation was not evident during the present study. At the onset of *M. sydneyi* infection in digestive diverticulae, the PO activity of oysters in the QX prone Georges River were substantially lower than those from a QX free location (Hawkesbury River). Even as the intensity of infection rose, PO activity remained impeded, so that there was a significant negative correlation between PO activity and infection intensity throughout the infection period.

The difference in PO activity between the Georges and Hawkesbury Rivers is consistent with the active suppression of the PO cascade in the Georges River. This was best demonstrated among oysters transplanted to the Georges River at the height of the infection period (March 2001). PO levels in these oysters dropped significantly within a week of their transfer, at the same time that *M. sydneyi* infection was first detected.

The decline of proPO activity associated with the onset of infection in the digestive diverticulum is unlikely to be the result of variation in oyster life cycles. PO activities for naïve Hawkesbury River oysters, which were from the same breeding stock as those transplanted to the Georges River, did not decrease during the infection period.

Even though PO activity was continuously suppressed in the Georges River relative to the Hawkesbury River, other physiological parameters tested in this study did not differ significantly between those two growing areas. Both total hemolymph protein content and condition index declined equally in oysters from the Hawkesbury River and those transplanted to the Georges River before the infection period. Moreover, these parameters did not differ between Hawkesbury River oysters and those transplanted to the Georges River at the height of the infective season, even though the latter were rapidly infected by *M. sydneyi*.

The absence of parasite associated effects on total hemolymph protein content and condition index during *M. sydneyi* infection indicates that the observed decreases in PO activity were the result of specific immunosuppression rather than general morbidity associated with pathogenesis. To explain this immunosuppression, we are currently testing two hypotheses. The first suggests that *M. sydneyi* itself releases serine proteases or serine protease inhibitors that interfere with the proteolytic activation of the proPO system. Such immunosuppressive mechanisms are evident in other oyster diseases. For instance, serine proteases have been isolated from *P. marinus*, a pathogenic parasite that causes heavy losses in Eastern oyster populations in the USA (Faisal et al., 1998).

The alternative hypothesis is that some as yet unknown environmental parameter is responsible for the suppression of the proPO system, allowing for the opportunistic

infection of immunosuppressed hosts by *M. sydneyi*. The current study showed that a variety of general water quality parameters (temperature, pH, dissolved oxygen and salinity) do not differ significantly between the QX-prone (Georges) and QX-free (Hawkesbury) rivers (data not shown). However, this does not discount the existence of some other environmental factor that inhibits PO.

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