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Breeding for QX disease resistance negatively selects one form of the defensive enzyme, phenoloxidase, in Sydney rock oysters

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Abstract

QX disease in Sydney rock oysters (*Saccostrea glomerata*) is caused by the paramyxean protozoan, *Marteilia sydneyi*. Disease outbreaks occur during summer (January to May) and can result in up to 95% mortality. The New South Wales Department of Primary Industries has been selectively breeding *S. glomerata* for resistance to QX disease since 1996. Previous work suggests that this breeding program has specifically affected the defensive phenoloxidase enzyme system of oysters. The current study more thoroughly characterises the effect of selection on the different forms of phenoloxidase found in oyster populations. Native polyacrylamide gel electrophoresis (native-PAGE) identified five discrete types of phenoloxidase in non-selected (wild type) and fourth generation QX disease resistant (QXR_4) oysters. One electrophoretically distinct form of phenoloxidase, PO^b, is significantly less frequent in resistant oysters when compared to the wild type population. The frequency of PO^b also decreased in both the wild type and QXR_4 populations over the course of a QX disease outbreak. This suggests that possession of PO^b makes oysters susceptible to QX disease and that breeding for resistance has resulted in negative selection against this form of phenoloxidase.

Keywords: *Saccostrea glomerata*; Phenoloxidase; QX disease; *Marteilia sydneyi*; Oysters

1. Introduction

Since the early 1970s, the Sydney rock oyster industry in the eastern Australian states of New South Wales (NSW) and Queensland has been seriously affected by outbreaks of QX disease [1]. QX is an infectious disease mediated by the protozoan parasite, *Marteilia sydneyi* [2]. Epizootics now affect a number of estuaries in NSW including one of the

Abbreviations: CR, native (not farmed) oysters from the Clarence River; DPI, Department of Primary Industries; FSW, filtered seawater; 4-HA, hydroquinone monomethyl ether; MAC, marine anticoagulant; MBTH, 3-methyl-2-benzothiazolinone hydrazone; native-PAGE, native polyacrylamide gel electrophoresis; NSW, New South Wales; QXR_4, fourth generation oysters bred for QX disease resistance.

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State's most productive growing areas. Mortality rates of up to 95% have been reported during some outbreaks. *M. sydneyi* infection generally occurs in mid-summer (January–February) and may be associated with heavy rainfall [3–5].

Peters and Raftos [6] have shown that *M. sydneyi* infects oysters after their immune system, notably the phenoloxidase cascade, has been suppressed. Phenoloxidase is a bifunctional enzyme that is critical to host defence and wound repair in many invertebrates [6–10]. The enzyme has both monophenolase and *o*-diphenolase activities that contribute to a cascade of biochemical reactions transforming tyrosine-based substrates into the pigment, melanin [11–13]. Melanin and a number of intermediate metabolites in the phenoloxidase pathway have immunological functions including antimicrobial activity. Peters and Raftos [6] concluded that suppression of phenoloxidase activity decreases the ability of oysters to control *M. sydneyi*, leading to the development of QX as an opportunistic disease. That conclusion is supported by recent evidence, which shows that *M. sydneyi* is present in many growing areas where QX disease outbreaks do not occur (R. Adlard, unpublished data).

There are currently no farming practices that can control QX and the disease continues to spread [6]. Hence, the development of disease resistant oysters has been made a priority by the NSW Department of Primary Industries (DPI). This government authority has been interbreeding survivors of QX disease outbreaks in the Georges River, Sydney, since 1996. Selective breeding for disease resistance reduced mortality by 22% in the second generation [14], and by almost 40% after the third generation of selection (J. Nell, unpublished data).

Similar mass selection programs have been used to generate resistance to MSX disease in the American eastern oyster, *Crassostrea virginica* [15,16] and to Bonamiasis in the European flat oyster, *Ostrea edulis* [17,18]. In *C. virginica*, strains selected for MSX resistance seem to limit infection by increasing the number of haemocytes in their haemolymph to heal lesions, remove debris and repair tissue [19]. In contrast, selectively bred *O. edulis* have decreased frequencies of hyalinocytes in their haemolymph. These cells may be a target for intracellular infection by *Bonamia ostreae*, so decreases in their frequency could make oysters less susceptible to disease [20].

Despite the success of selective breeding, the process used to obtain broodstock has significant disadvantages. Survivors of disease outbreaks are chosen for interbreeding without understanding what characteristics allowed them to survive or whether those characteristics are heritable. Individuals that remained uninfected by chance may also be included in the broodstock. To remedy these problems, we are investigating markers of QX disease resistance that can be incorporated into DPI's selective breeding program. Our data indicate that breeding for QX resistance has affected the phenoloxidase system of oysters. Newton et al. [21] found that third generation QX disease resistant (QXR_3) oysters had significantly higher phenoloxidase activities than the wild type population, and that QXR_3 and wild type oysters expressed different forms of phenoloxidase.

On the basis of these results, the authors suggested that increased disease resistance in the QXR_3 population was due to selection for different types of phenoloxidase [21]. However, the precise nature of this selection could not be resolved because of the small sample sizes tested and the limited resolution of the native-PAGE system used to identify different types of phenoloxidase. The current study more thoroughly characterises the relationship between different forms of phenoloxidase and QX disease resistance by increasing the sensitivity of the native-PAGE system and by analysing more oysters.

2. Materials and methods

2.1. Sydney rock oysters

Three populations of *S. glomerata*, designated wild type, CR and QXR_4, were used. Oysters were approximately three years old at the time of collection. Wild type oysters were collected from commercial oyster leases in Porto Bay on the Hawkesbury River, NSW (33°34′42″ S, 151°13′40″ E), where they were grown from wild caught spat. At the time of this study, the Hawkesbury River had never experienced an outbreak of QX disease and so oysters collected from this site had never undergone selection for QX disease resistance.

QXR_4 oysters were the fourth generation of oysters bred for QX disease resistance in the Georges River (34°00′ S, 151°10′ E). The Georges River has suffered annual outbreaks of QX disease since 1994. Oysters were grown in trays at Lime Kiln Bar (33°59′08″ S, 151°03′10″ E) and were transferred to Neverfail Bay (33°59′41″ S, 151°04′21″ E) during 2003.

CR oysters were native (not farmed) animals collected from the Clarence River in northern NSW (29°29′10″, 153°47′60″). This population has survived annual outbreaks of QX disease since 1976, and so has undergone substantial natural selection for QX disease resistance. CR oysters were kindly collected by Ben Perkins (NSW DPI).

Oysters were equilibrated for up to four weeks in aerated 50 l aquaria filled with seawater collected from the Georges River. They were maintained at a constant temperature of 23 °C and were fed with Aquasonic invertebrate food supplement (5 ml/200 l water) at regular intervals.

2.2. Exposure of oysters to QX disease

Two hundred wild type oysters were transplanted to Neverfail Bay on the Georges River in December, 2003. These oysters joined a group of more than 300 QXR_4 oysters, which had been in Neverfail Bay since June, 2003. To define the duration of the 2004 QX epizootic, groups of five to six wild type oysters were sampled at regular intervals between January and May, 2004. These oysters were tested for phenoloxidase enzyme activity and *M. sydneyi* infection intensity. Infection intensity was determined by dissecting 100 mg of fresh tissue from the gills and digestive glands of *S. glomerata*. This tissue was homogenised in 1 ml of filtered seawater (FSW; 0.45 μM filter) in a Dounce homogeniser. The homogenates were diluted 1:10 in FSW and *M. sydneyi* sporonts were stained with KOVA (Hycor Biomedical Inc., California, USA) so that their frequency could be calculated using a haemocytometer according to the method of Peters and Raftos [6]. The identification of *M. sydneyi* was based on previous descriptions [22,23].

2.3. Collection of haemolymph

Oysters were removed from aquaria 30 min prior to haemolymph extraction so that excess seawater could drain from their mantle cavities. A small hole was pierced in the shell hinge and haemolymph (2 ml) was extracted from the foot sinus using a 22-guage needle fitted to a 5 ml syringe. After haemolymph was withdrawn, samples were immediately transferred to 10 ml polypropylene tubes and held on ice.

2.4. Native-PAGE

Electrophoretically distinct forms of phenoloxidase were detected by native-PAGE using a Mini-PROTEAN II vertical slab gel system (BioRad, Reagents Park, NSW, Australia). Haemolymph (2 ml) was centrifuged for 5 min at 2500 × *g* (4 °C). The supernatant was then discarded and the haemocytes were re-suspended in 50 μl 2% v/v Nonidet-P40 (NP-40; Fluka, Switzerland) in 10 mM Tris−HCl (pH 7.0). Samples were then centrifuged at 5000 × *g* for 15 s to remove cellular debris. Forty μl of the lysate was then mixed with 10 μl 0.35 M Tris−HCl containing 10% v/v glycerol (pH 6.8) before being applied to 8% native-PAGE gels (Gradipore, Frenchs Forest, NSW, Australia). Gels were electrophoresed in 0.03 M Tris−HCl containing 0.187 M glycine (pH 8.3) for 3 h at 110 V (4 °C). After electrophoresis, gels were stained for phenoloxidase enzyme activity with 20 mM hydroquinone (1,4-benzenediol; Sigma Aldrich, Sydney, NSW, Australia; 15 min) in FSW containing 5 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH; Sigma Aldrich), before being rinsed with distilled water [21].

2.5. Phenoloxidase enzyme activity assay

Haemolymph (100 μl per well) was added in triplicate to 96-well microtest plates (Sarstedt, Technology Park, Adelaide, South Australia). Samples were incubated with 100 μl hydroquinone monomethyl ether (4-HA; 5 mM in FSW; Fluka, Switzerland) containing 1 mM MBTH. Colour development was quantified spectrophotometrically at 490 nm using a microplate spectrophotometer (BioRad). Absorbance was measured immediately after the addition of substrates (0 time-point). Plates were then incubated at 22 °C and an additional absorbance measurement was made after 60 min. Negative controls were included to correct for spontaneous hydrolysis of substrates. Initial (0 time-point) readings were subtracted from measurements made after 60 min to determine the change in absorbance. Data from triplicate wells were averaged to provide enzyme activities for individual oysters.

2.6. Statistical analyses

Data were analysed using SPSS Version 12.0 for Windows (Microsoft, North Ryde, NSW, Australia). Pearson's or contingency Chi-square analyses were used to compare the frequencies of phenoloxidase types in different oyster populations [24]. One-way analysis of variance was performed to test the significance of differences in phenoloxidase activity and infection intensity. Differences were considered to be significant if $P < 0.05$.

3. Results

3.1. Five types of phenoloxidase were detected by native-PAGE

Native-PAGE identified five electrophoretically distinct forms of phenoloxidase (designated PO^a to PO^e) among the 273 wild type, QXR_4 and CR oysters phenotyped during this study (Fig. 1). The different forms of phenoloxidase occurred in individual oysters either singly or in pairs. No oysters expressed more than two forms of phenoloxidase. All possible combinations of bands (i.e. PO^a alone, PO^a/PO^b, PO^a/PO^c, etc.) were identified, except for PO^e alone, PO^c/PO^e and PO^d/PO^e.

3.2. Frequencies of the different forms of phenoloxidase differ between populations

Fig. 2 shows the frequencies of the different forms of phenoloxidase in wild type, CR and QXR_4 oysters tested prior to the 2004 QX disease outbreak. PO^b was almost twice as common among wild type oysters when compared with the QXR_4 population ($\chi^2 = 9.19$, $P < 0.05$, $df = 1$). In contrast, PO^d was over four times more frequent in the QXR_4 oysters than in the wild type population (Fig. 2; $\chi^2 = 34.72$, $P < 0.05$, $df = 1$). The frequencies of PO^a ($\chi^2 = 1.60$, $P > 0.05$, $df = 1$) and PO^c ($\chi^2 = 3.57$, $P > 0.05$, $df = 1$) were slightly, but not significantly, higher in QXR_4 oysters when compared to wild types.

A similar pattern was evident among CR oysters (Fig. 2). PO^b occurred almost twice as frequently among wild type oysters than in the CR population ($\chi^2 = 6.07$, $P < 0.05$, $df = 1$). The decreased frequency of PO^b in CR oysters was compensated by significantly higher frequencies of both PO^a ($\chi^2 = 6.40$, $P < 0.05$, $df = 1$) and PO^d ($\chi^2 = 5.14$, $P < 0.05$, $df = 1$). The frequency of PO^c ($\chi^2 = 0.02$, $P > 0.05$, $df = 1$) did not differ significantly between the CR and wild type populations.

3.3. PO^b is less frequent among survivors of a QX disease outbreak

M. sydneyi was first detected during February in the gills of wild type oysters that had been transplanted to the Georges River in December, 2003 (Fig. 3B). After two weeks, plasmodial stages were observed undergoing sporulation in the digestive glands to form 8 to 16 sporonts per sporozoont (Fig. 3A). Oysters sampled during this period were symptomatic of parasitic infection, displaying gill lesions and shrunken body tissues. Infection intensity increased

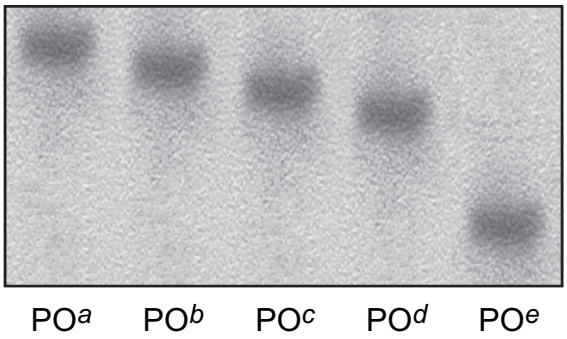

Fig. 1. Native-PAGE of the five different forms of phenoloxidase (PO^a–PO^e) that were identified in wild type, CR and QXR_4 oysters. In individual oysters, these forms appear either singly or in pairs.

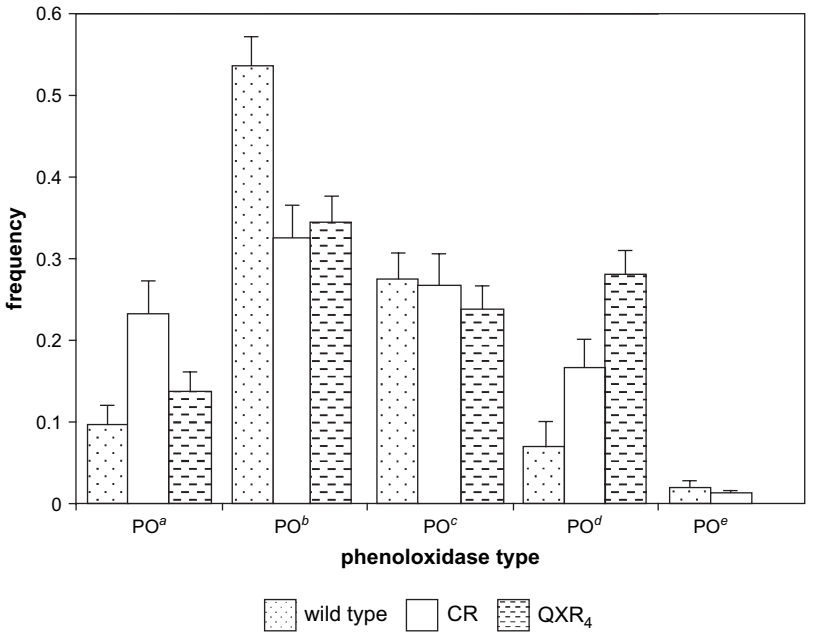

Fig. 2. Frequencies of oysters expressing five different forms of phenoloxidase (PO^a–PO^e) detected by native-PAGE in wild type ($n = 109$), QXR_4 ($n = 109$) and non-farmed, CR ($n = 60$) oysters. Bars = binomial standard error.

significantly until April (Fig. 3B; January 30 vs. April 5, $P < 0.05$), when 10% mortality was recorded among wild type oysters. The increase in infection intensity was preceded by a decrease in phenoloxidase enzyme activity, which occurred in late January and early February (Fig. 3C). By the time *M. sydneyi* was first detected in oysters, their phenoloxidase activities were approximately one quarter that of oysters tested on January 30 (January 30 vs February 12, $P < 0.05$). By early May, 50% of wild type oysters and 28% of QXR_4 oysters in the Georges River had died. Mortality did not increase further during May. Infection intensity decreased substantially after April 5, reflecting the preferential survival of uninfected individuals. Based on these data, the period encompassing the 2004 epizootic was defined as January 30 to May 7, 2004.

Native-PAGE revealed that PO^b was significantly less frequent among the 48 QXR_4 oysters that survived the 2004 outbreak compared with those analysed before exposure to disease (Fig. 4). Thirty four percent of QXR_4 oysters tested before the outbreak carried PO^b, compared to 26% of QXR_4 oysters that survived the epizootic ($\chi^2 = 3.9$, $P < 0.05$, $df = 1$). The loss of PO^b from the QXR_4 population was partially compensated by a 1.5 fold increase in the frequency of PO^c ($\chi^2 = 2.1$, $P < 0.05$, $df = 1$). A small increase was also observed in the frequency of PO^d in QXR_4 oysters tested after QX-selection.

A similar pattern was evident among wild type oysters. Only 18% of wild type oysters that survived the 2004 outbreak expressed PO^b, compared with 58% of wild type oysters tested prior to the infective period (Fig. 4; $\chi^2 = 5.4$, $P < 0.05$, $df = 1$). The frequencies of PO^a and PO^c increased significantly among wild type oysters after QX-selection ($P < 0.05$, $df = 1$).

3.4. QXR_4 oysters express two forms of phenoloxidase more frequently than wild type oysters

In addition to the loss of PO^b, a significant difference was evident between wild type and QXR_4 oysters in the frequency of individuals that expressed two, as opposed to one form of phenoloxidase. This difference was apparent for all combinations of phenoloxidase types, not just those containing PO^b (Table 1). Fig. 5 shows that 55% of QXR_4 oysters expressed two forms of phenoloxidase, compared to 12% of wild type oysters ($\chi^2 = 81.9$, $P < 0.05$, $df = 1$). However, this difference was only evident when QXR_4 oysters were tested before the 2004 epizootic. The frequency of QXR_4 oysters bearing two forms of phenoloxidase fell from 55% before January 30 to 15% among

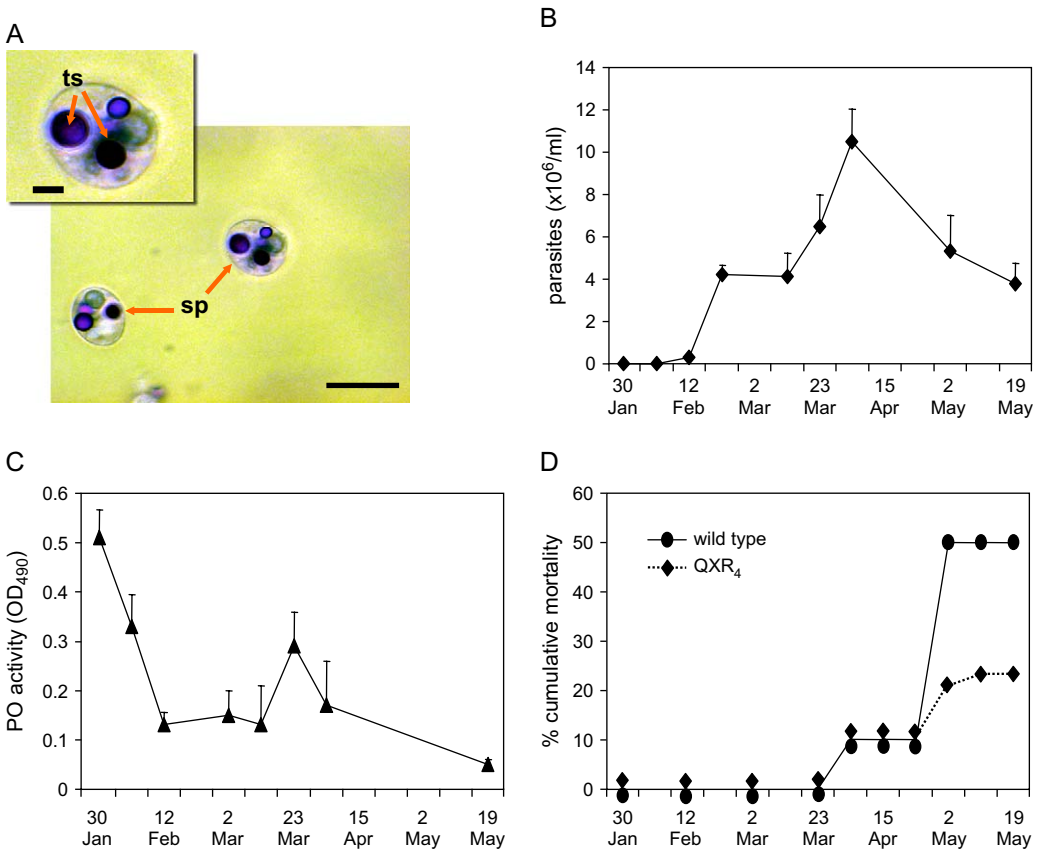

Fig. 3. (A) Mature sporonts (Sp) of *M. sydneyi* detected in the digestive diverticulum of a wild type Sydney rock oyster that had been exposed to the 2004 QX disease outbreak in the Georges River. Inset – high magnification of an individual sporont containing tricellular spores (ts). (B) *M. sydneyi* infection intensity in wild type oysters at various times during Jan–May, 2004. $n \geq 5$, bars = SEM. (C) Phenoloxidase activities of wild type oysters tested between Jan–May, 2004. n ≥ 5, bars = SEM. (D) Cumulative mortality among QXR_4 and wild type oysters during Jan–May, 2004.

individuals tested after May 7 (Fig. 5). As a result, the frequency of QXR_4 oysters expressing two forms of phenoloxidase after the outbreak did not differ significantly from that of the wild type or CR populations ($P > 0.05$).

4. Discussion

QX disease threatens the future of the Sydney rock oyster industry in Australia. In the past 2 years, severe QX disease epizootics have resulted in the collapse of oyster production on the Hawkesbury River. This had been the second largest farming estuary in Australia, producing over 12 million rock oysters in 2001/02. The sudden appearance of QX disease in the Hawkesbury system is significant because of the history of QX disease. Oyster production on another river near Sydney, the Georges River, has now been abandoned due to repeated QX outbreaks. It is likely that without the introduction of effective disease management practices, the recent QX outbreaks on the Hawkesbury River will result in the abandonment of rock oyster cultivation there as well.

The spread of QX has added impetus to the disease resistance breeding program being undertaken by NSW DPI. In the absence of other control practices, management of epizootics now relies on the deployment of disease resistant, QXR, oysters. In April 2005, the NSW government announced a rescue package that will provide farmers on the Hawkesbury River with funds to purchase hatchery reared QXR oysters. However, the QXR strain still only yields about 50% survival per outbreak (J. Nell, unpublished data), a level that is unlikely to be commercially viable in the long term. Our research is being conducted to identify heritable molecular markers that can be used to increase the

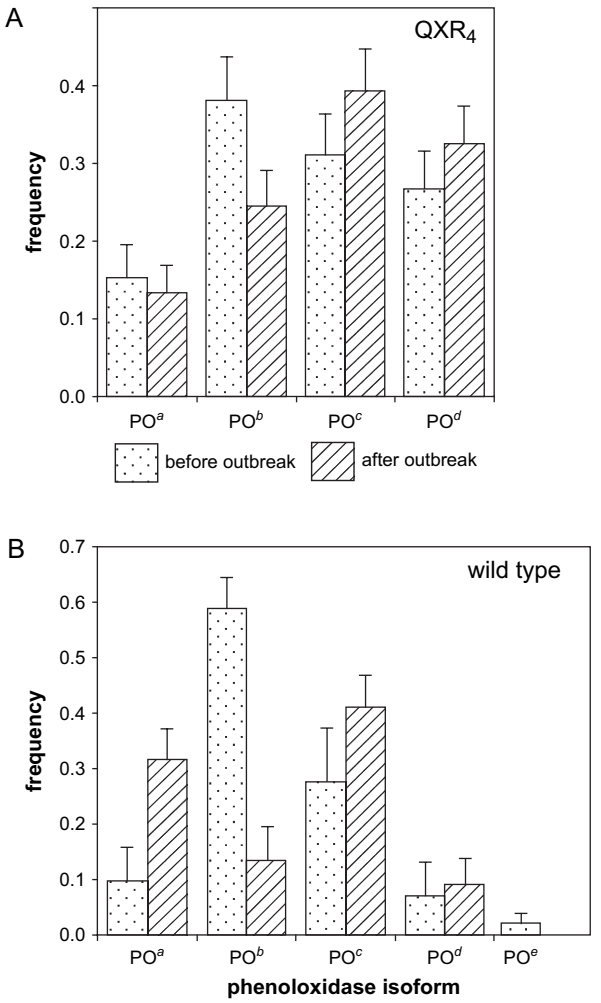

Fig. 4. Frequencies of oysters expressing the five electrophoretically distinct forms of phenoloxidase (PO^a to PO^e) observed among (A) QXR_4 ($n = 109$ before outbreak, $n = 48$ after outbreak) or (B) wild type ($n = 109$ before outbreak, $n = 11$ after outbreak) oysters tested before and after the populations had passed through the 2004 QX disease outbreak in the Georges River. PO^e was not detected in the QXR_4 population. Bars = binomial standard error.

effectiveness of selection for QX disease resistance. In the current study, we tested the hypothesis that selection for QX resistance has affected the phenoloxidase system of *S. glomerata*.

Native-PAGE identified five discrete forms of phenoloxidase in wild type, CR and QXR_4 oysters. Individual oysters expressed either one or two of these forms. Using a similar electrophoretic technique, Newton et al. [21] identified just two types of phenoloxidase in wild type and third generation disease resistant (QXR_3) oysters. The native-PAGE protocol used in previous analyses was optimised in the current study by improving sample preparation and staining techniques. This allowed us to increase resolution and identify additional forms of phenoloxidase. Moreover, only fifty QXR_3 oysters were examined in the earlier study, whilst a total of 273 individuals were successfully typed in the current report.

Chi-squared analyses indicated that the QXR_4 and wild type populations differed significantly in the frequency with which they expressed the PO^b form of phenoloxidase. PO^b was 20% less frequent in QXR_4 oysters than in wild type individuals. A similar deficit of PO^b, relative to wild types, was evident among native oysters from the Clarence River that had undergone substantial natural selection for QX disease resistance. These data suggest that breeding for QX disease resistance has resulted in the negative selection of PO^b. It is unlikely that the differences in the frequency of PO^b between wild type and resistant oysters reflect random genetic drift or inbreeding rather than the

Table 1
Frequencies of phenoloxidase phenotypes among wild type and QXR_4 Sydney rock oysters tested prior to the 2004 QX disease outbreak

Phenoloxidase phenotype	No. individuals		χ^2
	Wild type	QXR_4	
PO^a	9	6	1.5
PO^b	54	18	72.0
PO^c	29	14	16.1
PO^d	4	11	4.5
PO^a/PO^b	1	8	6.1
PO^a/PO^c	0	5	5.0
PO^a/PO^d	1	5	3.2
PO^a/PO^e	1	0	1.0
$PO^{b/}PO^c$	0	8	8.0
PO^b/PO^d	4	23	15.7
PO^b/PO^e	4	0	4.0
$PO^{c/}PO^d$	2	11	7.4
Total	109	109	144.4, $P < 0.001$

Individual χ^2 values were calculated for each genotype.

specific effects of selective breeding. An analysis of 14 allozyme loci by English et al. [25] found no evidence for inbreeding among QXR oysters, nor genetic drift between populations.

To confirm that the loss of PO^b is associated with QX disease resistance, the phenoloxidase profiles of both wild type and QXR_4 oysters were re-tested after the 2004 QX epizootic in the Georges River so that they could be compared to data collected before the outbreak. Three separate parameters were used to determine when the 2004 outbreak began and ended. In addition to cumulative mortality data, parasite cell counts were employed to gauge the intensity of infection and phenoloxidase enzyme activity was used as a measure of disease susceptibility. *M. sydneyi* was first detected during mid-February, 2004, in the gills of wild type oysters transferred to the Georges River in December, 2003. Maximum infection intensity occurred in late March, 2004. These results are consistent with previous analyses. Peters and Raftos [6] found that infection during the 2001 and 2002 epizootics began in February and increased continuously until the end of March.

Phenoloxidase enzyme activity provided a complementary measure of the onset of QX disease. Previous accounts of the QX infective cycle have shown that phenoloxidase activities decrease immediately before *M. sydneyi* infections are established [4,6]. In this study, a five-fold decrease in phenoloxidase activity was observed between January 30 and February 12, 2004, immediately prior to the detection of *M. sydneyi* in the gills of oysters. For the duration of the epizootic, phenoloxidase activity remained significantly lower than on January 30, before the outbreak. The combination of phenoloxidase activity and infection intensity data indicate that the 2004 Georges River epizootic started in February, with infection reaching a maximum around March 30. Cumulative mortality peaked among both wild

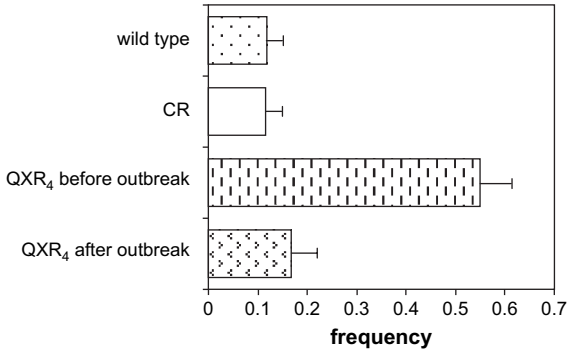

Fig. 5. Frequencies of oysters from the wild type ($n = 109$), CR ($n = 60$) and QXR_4 populations expressing two forms of phenoloxidase. Wild type and CR oysters were tested prior to January, 2004. QXR_4 were tested before January 30, 2004 ($n = 109$) and after May 7, 2004 ($n = 48$). Bars = binomial standard errors.

type and QXR_4 oysters in early May. Hence, oysters collected before February and after early May were compared to determine the effect of QX disease on phenoloxidase before and after selection.

PO^b was four times less frequent in wild type oysters tested after the 2004 epizootic compared with those phenotyped before the outbreak. A less substantial but still significant loss of PO^b was evident among QXR_4 oysters. PO^b was 10% less common in QXR_4 oysters that survived the epizootic relative to QXR_4 individuals analyzed before the onset of disease. The decrease in PO^b frequency among wild type oysters may have been greater than that observed in QXR_4 oysters because PO^b had already been depleted from the QXR_4 population by four generations of selective breeding.

Loss of the PO^b phenoloxidase isoform was associated with compensatory increases in the frequency of PO^c or PO^d in the QXR_4 population and PO^a and PO^c in wild type oysters. This suggests that replacement of PO^b during QX-selection is a random occurrence and that none of the phenoloxidase types identified in this study could be described as providing ''resistance'' to QX disease. Instead, it is clear that the PO^b isoform is associated with susceptibility.

In addition to the loss of PO^b, oysters expressing two, as opposed to one form of phenoloxidase were more frequent in the QXR_4 population than in wild types. However, the higher frequency of oysters with two types of phenoloxidase was not associated with disease resistance. The proportion of QXR_4 oysters expressing two forms of the enzyme decreased to levels equivalent to those of the wild type oysters after the population had passed through the 2004 QX disease outbreak. Moreover, the frequency of individuals with two forms of phenoloxidase in the CR population, which has been subjected to intensive natural selection for QX resistance, did not differ significantly from that of wild type oysters. We suspect that the higher proportion of oysters with two forms of phenoloxidase in the QXR_4 population reflects the different mating systems that gave rise to the different populations. Wild type and CR oysters were the progeny of broad spawning in the wild, whilst QXR_4 oysters were produced by cross fertilisation of defined parental broodstocks mated under controlled conditions. Broad spawning, particularly amongst oysters, has been shown to result in extremely small effective population sizes that distort phenotype frequencies in populations [26–28]. Despite this, the loss of oysters with two forms of phenoloxidase after the QXR_4 population had passed through the 2004 QX disease outbreak cannot yet be explained and is under investigation.

Unlike differences in the frequency of oysters expressing two types of phenoloxidase, the loss of PO^b is directly related to selection for QX resistance. PO^b may represent a powerful molecular marker that can be used for the further development of QX disease resistant oysters. However, we still do not have any information about the genetic origin of the five electrophoretically distinct forms of phenoloxidase identified in this study or their heritability. Abundant evidence from other systems suggests that rapid acquisition of high level resistance resulting from intense selection of the type seen in QXR oysters often results from changes to just one gene [29]. Hence, we are now undertaking a Mendelian genetic analysis to test whether PO^b represents an allele of a single phenoloxidase gene that can be used as the target for further selective breeding.

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