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Contents

Breeding Sydney rock oysters and its effects on resilience

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Abstract

Winter mortality and QX disease have had severe impact on the Sydney Rock Oyster industry. A selection program was established in the '90s to breed lines that are resistant to these diseases. Selection was also undertaken to enhance growth and collectively this has had positive effects on resilience to environmental factors such as ocean acidification. This paper provides an overview of the work that has been done to elucidate the genetic basis of resilience in the breeding lines and how it might be used in the future.

Introduction

The Sydney rock oyster Industry

Sydney rock oysters (SRO), *Saccostrea glomerata*, are a native species common from the NSW-Victoria border to northern Queensland (Lamprell and Healy, 1998). SRO were once found in large sub-tidal beds in many estuaries along the eastern seaboard and were heavily exploited following European settlement. SRO were both a food source for aboriginal communities and early settlers and were later used for the supply of lime for construction. In the late 1800s, depletion of the beds led to cultivation of the species and for almost a century SRO were the most important cultivated mollusc in Australia. The vast majority of SRO production occurs in NSW, although a small industry operates in southern Queensland and production has occurred in southern Western Australia.

Traditionally, seed supply to the NSW industry was derived from natural catch. Farmers deploy sticks and other collecting devices in estuarine areas where the larvae aggregate and settle,

usually in summer and autumn. These collectors are then taken to other areas, sometimes other estuaries, for growout. Today, approximately 80% of the seed required for SRO production still comes from natural catch, but increasingly more of the industry's total seed demand is being met by hatcheries. The major incentive for the adoption of hatchery produced seed has been the development of selectively bred stocks.

Historically, SRO production peaked in the mid 1970s at around 10,000 tonnes, but today production has fallen to approximately 40% of that peak and is valued at A$30 million per annum (NSW Department of Primary Industries, 2013). The fall in production has been attributed to a number of factors, but key among them has been the spread of disease and competition from the introduced faster growing Pacific oyster (*Crassostrea gigas*). SRO are comparatively slow growing, taking over 3 years on average to reach "plate" size at 50 g, whereas Pacific oysters of a similar size can be produced in less than 12 months in NSW.

Disease in Sydney rock oysters

Sydney rock oysters are affected by a number of diseases, with the most significant in terms of their impact on oyster cultivation being QX ("Queensland Unknown") and winter mortality (WM). QX is caused by a haplosporidian parasite, *Marteilia sydneyi*. Infection generally occurs in summer although losses are protracted and mortality can continue for many months. This disease came to prominence in the mid to late 1970s in southern Queensland and northern NSW, before it then became established in estuaries further south. In 1994, QX disease was first detected in the Georges River (Adlard and Ernst, 1995), where within three years it halted all SRO production. Subsequent to this outbreak a survey of the remaining major NSW oyster producing estuaries confirmed that *M. sydneyi* was present in oysters in all but one estuary tested across the state (Adlard and Wesche, 2005), even though outbreaks of QX disease had never been recorded in these estuaries. In 2004, QX occurred for the first time in the Hawkesbury River with devastating effects, such that production of SRO almost ceased within 12 months. Estimates of the impact of QX vary, but at the time of the initial outbreaks the Georges and Hawkesbury Rivers together were responsible for almost 20% of NSW's oyster production. The continued spread of QX disease outbreaks remains a significant threat to the SRO industry.

WM has a long history in NSW with records extending back to the early 20th century. Unlike QX, the impact of WM tends to be more variable from year to year, although losses exceeding 60% have been reported. The disease occurs in winter and spring in estuaries in central and southern NSW. Losses are greatest in "dry" years at high salinity sites where older oysters cultured lower in the water column suffer the highest mortality levels (Smith *et al*., 2000). The disease was originally attributed to the haplosporidian parasite, *Bonamia roughleyi,* however recent work has called this into question. *B. roughleyi* is rarely seen in clinical cases of WM and the very low prevalence of this organism relative to histological changes has led to the conclusion it is not responsible for this disease (Spiers *et al.*, 2013). Another aetiological agent and/or a confluence of environmental factors is a more likely cause of WM.

Breeding Sydney rock oysters

A selective breeding program for SRO commenced in 1990. Based originally on mass selection, the program targeted faster growth and WM disease resistance before it was expanded to include QX disease resistance in 1997 (Nell, 2003). Initially, four breeding lines were established at three sites in both Port Stephens and the Georges River (Fig. 1). After the advent of QX disease in the Georges River in 1994, the three original sites were replaced with Quibray Bay, Woolooware Bay and Lime Kiln Bar. These locations were selected on the basis of their differing exposure to winter mortality or QX disease. In the Georges River, WM typically affects sites in the lower, more oceanic, regions of the estuary (e.g. Quibray Bay), whereas QX disease typically occurs in the upper reaches (e.g. Lime Kiln Bar). Woolooware Bay is found between these two locations and is affected by both WM and QX disease.

To date, seven generations of mass selected oysters have been produced, with the most recent generations still undergoing performance evaluation. After five generations of selection at Port Stephens, lines of oysters were produced that were 36% heavier than non-selected controls, and that could reach market size 10 months faster than the expected 38 months (Nell, 2006). Similar results were achieved in the Georges River in the absence of QX disease, but when present, QX disease severely stunted the growth of surviving control oysters, further exaggerating the growth advantage of the selected lines (Nell, 2006). By the third generation, losses resulting from WM in the Quibray Bay selected line were reduced by half (22% vs. 46%), whereas the Lime Kiln Bar line could be grown to market size in 2 yr at QX-affected sites with minimal losses (22% vs. 80% in comparison with non-selected controls (Nell and Perkins, 2006)). Background unexplained mortality for SRO ranges from 10–20% over a 2- to 3-y growing period, and so this represents full resistance; however, Nell and Perkins (2006) noted that substantial losses were incurred if the resistant lines were exposed to QX disease for a second season. Similar results with respect to WM and QX resistance were obtained in the Georges River for fourth generation selected oysters (Dove *et al*., 2013a). Losses caused by QX were 28% in selected lines after one season of exposure compared to 97% in non-selected controls. Mortality of WM selected oysters at Quibray Bay was 23% versus 52% in non-selected controls.

The WM and QX resistance of fourth generation selected oysters was also assessed in Merimbula Lake and Hawkesbury River, respectively to investigate genotype and environment interactions and to assure farmers that survival and growth measured in the Georges River was transferable to other key SRO farming locations (Dove *et al.*, 2013b). In Merimbula Lake WM selected oysters were significantly heavier and had significantly lower mortality compared to non-selected oysters. Likewise in the Hawkesbury River, QX resistant oysters where significantly heavier and had a mortality level of 21.7% compared to 80.1% in non-selected oysters. Furthermore, growth performance was comparable with that measured in the Georges River (Dove *et al*., 2013b).

Although the mass selection program continues, it has been rationalized to allow for additional pedigreed family lines to be created. The four Port Stephens lines were amalgamated and held at two locations. Meanwhile, the Woolooware Bay and Lime Kiln Bar lines have been com-

bined in an effort to increase dual resistance. The combined line, the Quibray Bay line, and a new QX line that was established in 2005 have now been moved from the upper to the lower reaches of the Georges River to expose oysters to both QX and winter mortality disease to attempt to develop further their resistance and value to the SRO industry.

So far a total of 119 pedigreed SRO families have been produced (Fig. 1) from a variety of stocks including individuals from the mass selected lines. The first 30 families were produced in 2007 on the basis of their phenoloxidase phenotypes, a potential marker of QX resistance (Bezemer *et al*., 2006), and were deployed in the Georges River to elucidate further the role of the phenoloxidase enzyme cascade in QX disease resistance. In 2008, 27 families were created from within-line crosses (Fig. 1) and their performance was assessed while being exposed to winter mortality and QX disease in the Georges River. In 2010, 32 families were created from wild stocks with the first F2 generation being created in 2011. Performance assessments of the pedigreed families were initially based largely on growth and survival, with meat yield assessed at the time of harvest. Following the creation of the 2010 families, performance criteria were expanded to include ongoing monitoring of condition index and shell shape. Preliminary observations of selected lines found differences in their reproductive/physiological responses to environmental conditions (Bayne *et al*., 1999; O'Connor and Dove, 2009). A comparison of the responses of progeny of the 5th generation oysters selected for fast growth and non-selected, wild-caught oysters with hatchery conditioning found marked differences in both condition indices and reproductive capability (O'Connor and Dove, 2009) that were later confirmed in field trials at 3 farming sites in NSW (Dove and O'Connor, 2012). Observations by oyster growers farming selectively bred lines are that selected oysters condition at a different rate and to a different extent compared with naturally (or wild-) caught oysters grown under similar conditions.

General resilience

Under cultivation, oysters face a broad range of stressors, which include a variety of diseases, natural environmental perturbations, anthropogenic impacts and stressors resulting from cultivation practices. Observations accumulated during the field assessment of selectively bred stock within the selective breeding program and from the use of families to address specific research questions, have led to a number of findings pertinent to the resilience of selectively bred SRO. In particular, observations of the performance of selectively bred stock in the face of other diseases and the impacts of climate change variables, such as ocean acidification, have suggested resilience to stressors other than the specific traits for which the oysters were selected.

In the absence of a laboratory infection model, performance assessment of disease resistant oysters has been based on their deployment to estuaries throughout NSW and southern Queensland. This has shown that regardless of location or disease status of the estuary, it is uncommon for mortality in disease resistant lines to exceed that of unselected controls. While trials in the Georges River have suggested that selection for resistance to either QX or WM

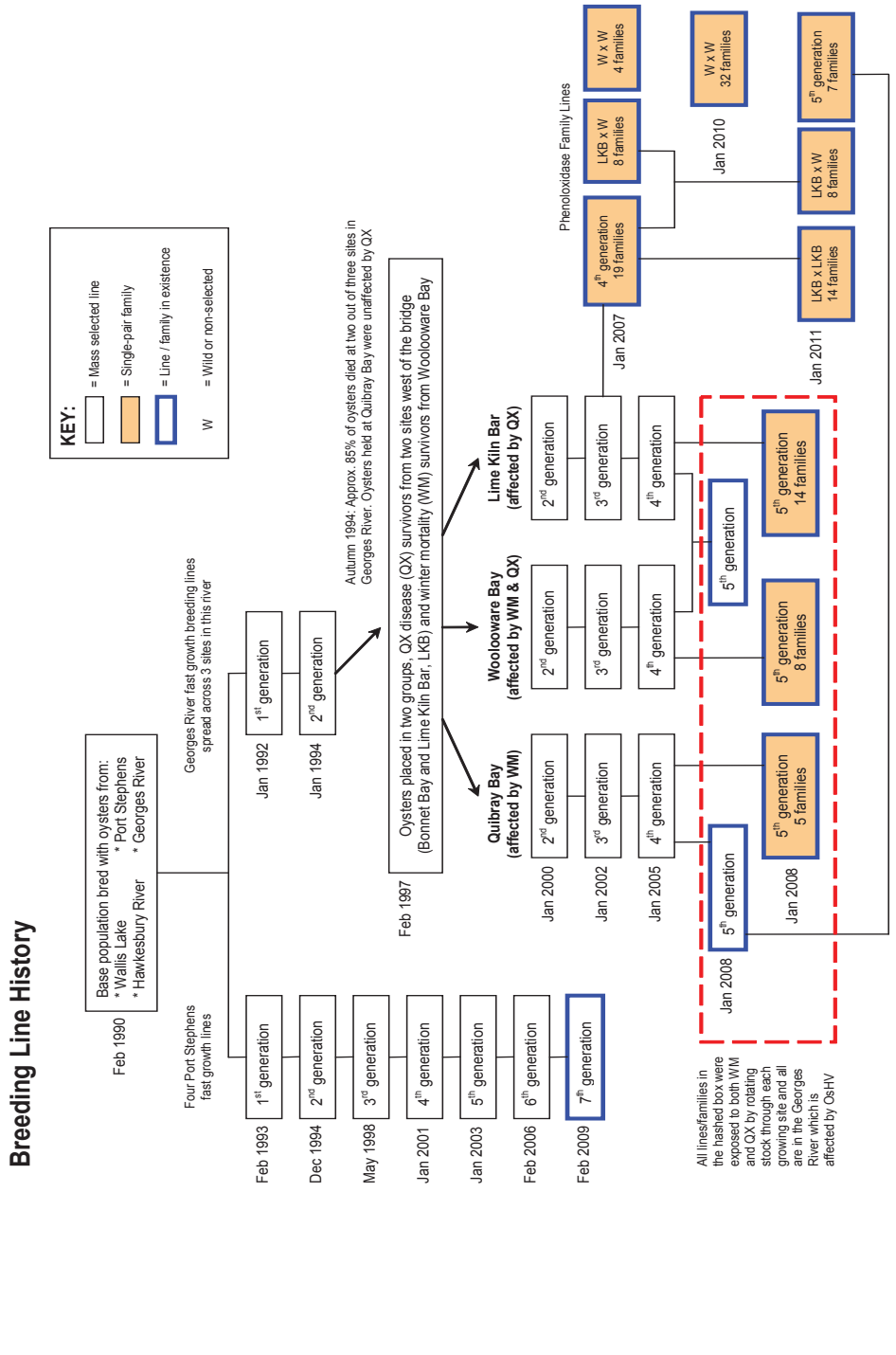

Figure 1. An overview of the Sydney rock oyster breeding program

has not inferred a benefit against the other, examples do exist where these lines have shown improved survival in the face of other diseases. Green *et al.* (2008) found that in the absence of QX disease, QX-resistant lines had significantly higher survival than wild-caught controls (0% vs. 32% mortality), and attributed the most likely cause of mortality in controls to disseminating hemocytic neoplasia, a contagious and often fatal condition reported in molluscs. Despite debate over the cause of WM and the degree to which, *B. roughleyi* is present and contributing, WM resistant oysters show greater survival irrespective of the presence or absence of *B. roughleyi* during WM outbreaks.

With respect to climate change resilience, ocean acidification is predicted to fundamentally alter marine ecosystems. Elevations in atmospheric carbon dioxide (CO_2) are causing oceans to acidify in a process known as ocean acidification (IPCC, 2013). Much of the anthropogenic CO_2 that is released into the atmosphere is absorbed by the oceans, which lead to changes in ocean chemistry. Internationally evidence suggests that ocean acidification will have devastating impacts on marine calcifying organisms, especially molluscs, with the potential to harm aquaculture industries (Gazeau *et al*., 2013).

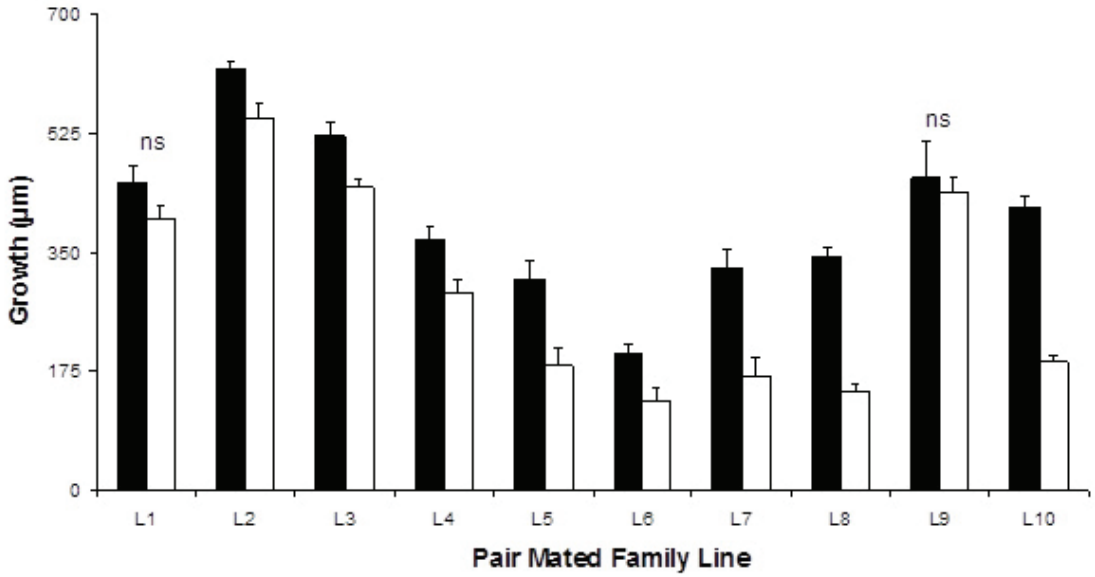

Figure 2. Mean shell growth of pair mated families created from the mass selected lines of Saccostrea glomerata spat after four days at ambient (375 ppm) and elevated (1000 ppm) partial pressure of CO_2 (pCO_2) at 26 °C and salinity 35 ppt, n = 3. Black bars represent growth at 375 ppm; White bars represent growth at 1000 ppm. Error bars (SE). pCO_2 x line df = 9, MS = 6696.67, F = 4.32, P < 0.001

Early studies found that wild populations of the SRO were extremely vulnerable to ocean acidification. Larvae and spat (oysters less than 25 mm long), of the SRO were smaller in size, suffered mortality and took longer to develop when reared in acidified seawater (Parker *et al*., 2009, 2010, 2011; Fig. 2). The impacts were even greater when temperature was increased with 100% mortality of larvae after only two days (Parker *et al*., 2010; Fig. 3).

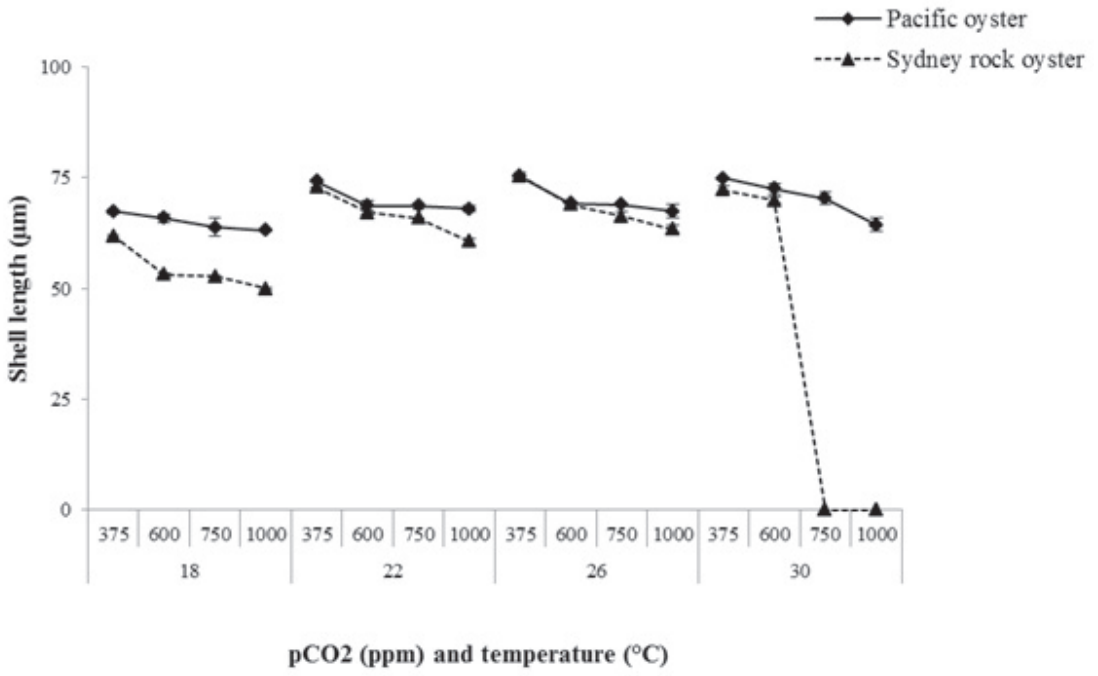

Figure 3. Mean shell length of larvae of Saccostrea glomerata and Crassostrea gigas after 48 h in the partial pressure of CO_2 (pCO_2) (375, 600, 750, 1000 ppm) and temperature (18, 22, 26, 30 °C) treatments. 375 ppm is the concentration of CO_2 in ambient seawater, while 1000 ppm CO_2 is the concentration of CO_2 expected at the end of this century. Species x pCO_2 x temperature df = 9, MS = 4.81, 4.18, P < 0.01

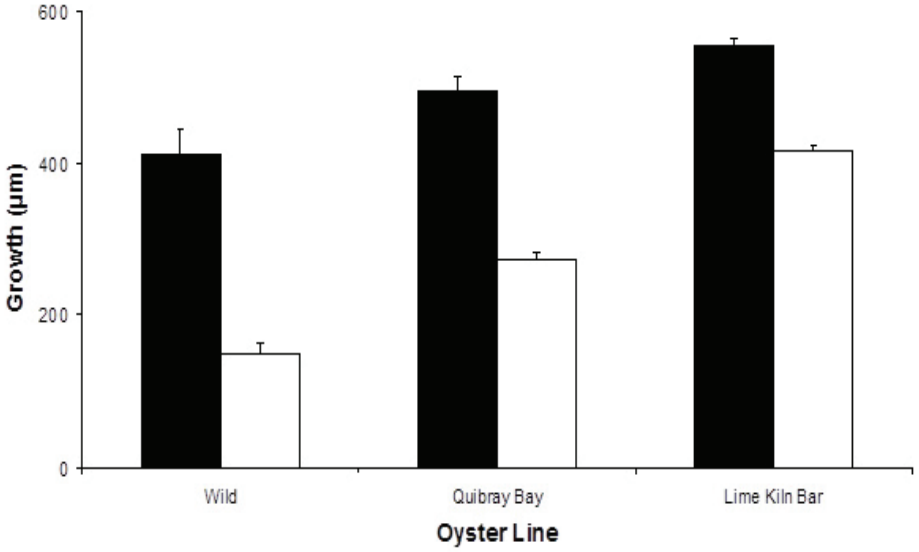

Figure 4. Mean shell growth of mass selected lines of Saccostrea glomerata spat after four days at ambient (375 ppm, black bars) and elevated (1000 ppm, white bars) partial pressure of CO_2 (pCO_2); 26 °C, 35 ppt, n = 3. Error bars (SE). pCO_2 x Line df = 2, MS = 4160.19, F = 4.33, P < 0.05

A key mechanism involved in the resilience of the selectively bred lines to ocean acidification is the capacity of these lines to increase their standard metabolic rate. Adults of the QX resistant line were able to increase their standard metabolic rate by 16.5% relative to the wild population when exposed to acidified seawater (Parker et al., 2012). The QX resistant line also had a better capacity to regulate their acid-base balance than the wild population during exposure to acidified seawater. The hemolymph, the equivalent of blood in invertebrates, pH of the wild population was reduced by 0.45 pH units compared to only 0.32 pH units in the QX resistant line when exposed to acidified seawater.

In 2010, a program to breed for climate change resilience in SRO commenced. Adults of a wild population and a QX resistant line were exposed to acidified seawater during reproductive conditioning and their resulting offspring were collected and exposed to the same acidified conditions. After one generation, the impact of acidified seawater on the growth of larval of the wild population was significantly reduced and the growth of larvae from the QX-resistant line was not affected (Parker *et al.*, 2012; Fig. 5). When these first generation offspring grew into adults, they were better able to regulate their acid-base balance. These "climate proofed" SRO breeding lines provide the aquaculture industry with a resource on which to base future studies.

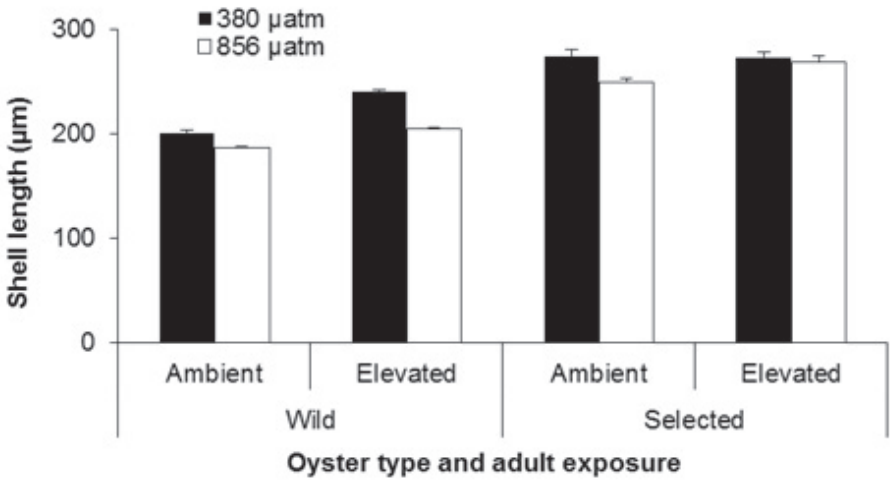

Figure 5. Size of larvae exposed to ambient (380 µatm) and elevated (856 µatm) partial pressure of CO_2 (pCO_2) at 19 days from parents that were exposed to ambient and elevated concentrations of pCO_2. pCO_2 df = 1, MS = 3012.74, F = 12.99, P < 0.01; Line df = 1, MS = 2410.58, F = 10.39, P < 0.01; Adult exposure P = ns

The basis for resilience in oysters and future research

As the Sydney rock oyster breeding program has matured and improvements in certain traits have become apparent, the interest and capacity to investigate the basis for improvements have increased. In particular the rise of the "omics" (proteomics, transcriptomics, genomics etc.) has offered new tools to explore the physiological mechanisms underlying responses and traits.

In SRO, this research began with proteomics and the elucidation of the role of the phenoloxidase (PO) cascade and particular isoforms of phenoloxidase in QX resistance. Initial studies on the role of PO in QX disease resistance arose from field based experiments, which demonstrated that inhibition of PO enzymatic activity in oysters occurred immediately prior to the onset of *M. sydneyi* infection resulting in QX outbreaks (Peters and Raftos, 2003). Such inhibition of PO activity was apparent in estuaries where QX outbreaks were common, but not in QX free areas. PO is a critical component of the oyster immune system and it has been shown to be involved in phagocytic defensive responses against *M. sydneyi* in healthy oysters (Aladaileh *et al*., 2007a,b; Butt and Raftos, 2008, Kuchel *et al*., 2010). Hence it was postulated that the inhibition of PO activity associated with QX disease outbreaks resulted in decreased immunological competence among oysters and increase susceptibility to uncontrolled *M. sydneyi* infection. This hypothesis was supported by subsequent analyses, which showed that oysters bred for QX disease resistance differed from non-selected (susceptible) oysters in both PO enzymatic activity and the expression of particular electrophoretic isoforms of PO (Newton *et al*., 2004; Bezemer *et al*., 2006). Similar work on single pair mated families of SRO found a strong

association between one isoform of PO and QX disease associated mortality (Kan and Raftos, unpublished data). Other evidence suggests that the suppression of PO activity associated with QX disease outbreaks results from environmental stress, such as low salinity after rainfall. Both field (Peters and Raftos, 2003; Butt *et al*., 2007) and laboratory-based (Butt *et al*., 2006; 2008; Butt and Raftos, 2007) studies indicate that low salinity and other types of stress inhibit PO activity, allowing *M. sydneyi* to proliferate and cause QX disease in SRO.

These data suggested that PO could be a robust target for marker assisted selection to breed QX disease resistant SRO. However, a number of factors mitigate against sole reliance on PO as a marker for selective breeding. The methods used to identify the PO isoforms associated with QX disease resistance and susceptibility are relatively cumbersome and unreliable, primarily because *S. glomerata* PO has still not been characterised at the level of protein or nucleotide sequence. More importantly, subsequent work has shown that many other factors in addition to PO contribute to QX disease resistance (Green *et al*., 2009; Simonian *et al*., 2009). Simonian *et al*. (2009) used two dimensional electrophoresis (2DE) to identify six different proteins, including superoxide dismutase (SOD)-like molecules that were differentially expressed in QX disease resistant SRO. Similarly, an expressed sequence tag analysis by Green *et al*. (2009) detected four differentially expressed genes (again including SOD-like genes) in QX resistant oysters compared to a non-selected line. More extensive proteomic analyses now suggest that more than 20 proteins may be involved in QX disease resistance (Thompson *et al*., unpublished data). The range of genes and protein identified in these studies fits an emerging pattern of response in oysters to a range of different environmental stresses, including disease (Raftos *et al*., 2014). Numerous studies have shown that stress in oysters, including infectious disease, primarily affects mitochondrial energy metabolism with downstream effects on cellular stress responses, antioxidant enzymes and the cytoskeleton. Many genes in these subcellular pathways have already been implicated in resistance to QX disease (Green *et al*., 2009; Simonian *et al*., 2009). The involvement of these systems in QX disease resistance may also help to explain the enhanced metabolic activity identified in QX resistance oysters during studies of their resilience to ocean acidification (Parker *et al*., 2012 as described above).

The obvious involvement of many genes in QX disease resistance means that effective marker assisted approaches to selective breeding will have to rely on the simultaneous characterisation of multiple genetic factors in the parents and offspring of selective breeding lines. This will rely on a genome-wide understanding of heritable differences between QX disease resistant and susceptible oysters. To meet this need for additional data, we are currently undertaking comprehensive RNAseq next generation nucleotide sequencing and shotgun proteomic comparisons of disease resistant and susceptible SRO to generate a complete list of genes that are associated with resistance to QX and WM. This list will form the basis of a multigene approach to marker assisted selection where parental generations will be selected based on their expression of numerous disease resistance genes.

Summary

The SRO industry has survived for over a century. Following steady growth through to the 1970s a range of factors including disease and competition from other faster growing species has seen significant declines in production. To combat this decline a breeding program began in 1990 that now supplies approximately 20% of industry seed supply. SRO stocks are available that show significantly improved resistance to the two major SRO diseases. Observations of general performance of these lines have shown evidence of increased resilience in the face of several other environmental stressors that were not specifically chosen as traits for selection. Selectively bred SRO have been observed to have higher general survival and to be able to maintain greater growth under adverse pH and temperature regimes. Studies have identified a suite of genes that are affected during specific disease outbreaks and evidence is emerging suggesting that many of these genes are involved in a broad range of stress responses. The immediate future for SRO breeding will involve transitioning the breeding program from one based on mass selection to a pedigreed family program and trials have begun to investigate the potential for marker assisted selection.

Acknowledgements

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