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Effects of feed supply on Pacific oyster larvae grown at high salinity



Prepared for PIRSA Marine Biosecurity

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Kathryn Wiltshire, Simon Bryars and Xiaoxu Li

SARDI Aquatic Sciences,
PO Box 120, Henley Beach, SA 5022



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South Australian Research and Development Institute

SARDI Aquatic Sciences
2 Hamra Avenue
West Beach SA 5024

Telephone: (08) 8207 5400

Facsimile: (08) 8207 5406

<http://www.sardi.sa.gov.au>

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Author(s): Kathryn Wiltshire, Simon Bryars and Xiaoxu Li

Reviewers: Steven Clarke, Mandee Theil, Michael Sierp

Approved by: Jason Tanner



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EXECUTIVE SUMMARY

This study investigates possible ecophysiological factors that may be limiting the establishment of wild Pacific oyster populations in South Australian waters. A literature review conducted on previous studies of Pacific oyster larvae and adults identified that salinity and food availability are possible factors restricting larval development and hence subsequent wild settlement. High salinity (>35‰) and low food availability (0-4mg/l Chlorophyll *a*) are typical in oyster farming regions in SA. However, knowledge is lacking on the impacts of reduced feed rations and of salinity >35‰ on Pacific oyster larval growth and survival. This knowledge is required in order to better assess the risk of wild populations establishing in South Australian waters.

In order to determine the impacts of food limitation on larval growth under the high salinity conditions (i.e. $\geq 35\text{‰}$) typical of South Australian waters, growth and survival of Pacific oyster larvae under four feeding regimes were evaluated in the laboratory over a 21-day culture period. The feed levels were: typical hatchery feeding rate of 100 000 algal cells ml⁻¹ (High feed), Medium feed of 10 000 cells ml⁻¹, Low feed of 1 000 cells ml⁻¹ and Nil feed added. In all cases the feed was a 1:1 mix of *Isochrysis galbana* and *Chaetoceros calcitrans* algal cells, which are frequently used by industry for rearing larval oysters. Larval oysters were maintained at a typical summer water temperature of 22.8°C and 10µm filtered natural seawater at ambient salinity of 38.8‰ was used. Larvae were obtained by spawning naturally conditioned oysters sourced from Coffin Bay.

Mortality of larvae offered Nil or Low feed was significantly greater than for High feed, with <1% of the former surviving to day 22 of the experiment, while 18% of the High feed group survived. Average survival in Medium feed larvae was 4% but this was not significantly different to that in other feed treatments. Despite possibly improved survival, the Medium feed larvae did not grow significantly more than either of the Nil or Low feed larvae. The High feed larvae grew more than twice as fast as any of the other groups and reached a significantly larger size at day 16.

These results suggest that given sufficient feed, larvae spawned from farmed oysters are able to grow and survive at a salinity and temperature typical of South Australian waters in summer. However a decreased food supply greatly reduces growth and survival. Assuming linear growth, the High feed group of Pacific oyster larvae could reach an average size at which they are competent to metamorphose at around 44 days, while larvae in other groups would take over 100 days to reach the same average size. In addition to the higher mortality due to reduced food availability, a prolonged larval period also increases the risk of natural predation and possibly also of advection away from suitable sites for settlement and subsequent growth. Natural phytoplankton levels in South Australia are relatively low, but greatly variable. Therefore, limited Pacific oyster larvae would be expected to survive through to settlement in South Australian waters under typical conditions. However, periods of increased feed supply do occur in South

Australian oyster growing areas, particularly over summer, and the likelihood and quantity of oyster settlement could increase during such times.

1 INTRODUCTION

The Pacific oyster, *Crassostrea gigas*, has been widely and successfully cultivated both in its native Japan and in many other regions of the world (Olsen 1994; Shatkin et al. 1997; Ruesink et al. 2005). Pacific oysters have established self-sustaining wild populations in many of the regions to which they have been introduced, including several areas around Australia (Medcof & Wolf 1975; Ayres 1991; Dinamani 1991; Diederich 2005, 2006; Rajagopal et al. 2005).

In South Australia, spat settlement has been recorded at a number of locations over several seasons, but the extent of such settlement is relatively limited and those oysters that do establish seem to be controlled by predation (Hone 1993, 1996; Vandeppeer 1995; Madigan & Clarke 1998). This limited establishment is despite Pacific oysters having been cultivated in South Australia for several decades (Olsen 1994) and in many regions (Hone 1993, 1996; Madigan & Clarke 1998; Wear et al. 2004). It is likely that environmental conditions in South Australian waters are limiting the success of *C. gigas* larval development, and thus reducing the likelihood and quantity of settlement, but it is unclear exactly which factors are important.

One notable difference between South Australia and regions where Pacific oysters have established is the relatively high salinity of South Australian waters. While a maximum of 30‰ is considered optimal for *C. gigas* larval development (Helm & Millican 1977; Nell & Holliday 1988), salinities of 35-40‰ are common in oyster farming areas of South Australia (Madigan & Clarke 1998). Originally it was believed that this high salinity would prevent larval development entirely (Medcof and Wolf 1975; Grove-Jones 1985; Olsen 1994), however, since wild oysters do occur, albeit in limited numbers, this is clearly not the case. It is still possible that high salinity may be one factor limiting the success of larval Pacific Oysters within South Australian waters, however, this does not explain the occurrence of wild oysters in some areas while none have been found in other regions of similar salinity (Hone 1993; Madigan & Clarke 1998).

Another potentially limiting factor is the relatively low feed supply available, with Chlorophyll *a* levels in South Australian oyster growing areas being typically 0-4mg/l; considerably lower than the optimum for *C. gigas* larvae of >12 mg/l (Hone 1993). While optimum values for *C. gigas* larval development have been investigated, less is known about the limits of their tolerances and the effects of adverse environmental conditions on growth and survival of larvae. More knowledge of these factors is required in order to determine the potential for wild populations to establish or spread within South Australia and elsewhere. In particular, a literature review by Wiltshire (2007) identified that knowledge of the impacts of higher than optimal salinity, and of reduced feed on larval development, were lacking.

The only studies to investigate effects of salinities $\geq 35\text{‰}$ showed that salinities $>30\text{‰}$, and particularly $>35\text{‰}$, significantly reduced larval growth but had no discernable impact on

mortality of fed larvae over 6-7 days (Nell & Holliday 1988; His et al. 1989). However, when food was withheld, adverse effects of salinity on growth were greater, and mortality increased (His et al. 1989). Biochemical modelling also suggests that when food is limiting, the range of temperature and salinity over which successful development occurs will be reduced (Bochenek et al. 2001). Therefore, it is possible that food limitation is more critical for larvae grown under higher salinity conditions and that these factors could act in concert to restrict larval development. Conversely, when food supply is good, this may allow development under environmental conditions that would otherwise not be suitable (Bochenek et al. 2001; Hofmann et al. 2004).

In this study, Pacific oyster larvae cultured under ambient salinity (~39‰) and controlled temperature (~23°C) conditions were offered four levels of feed from nil to a typical hatchery feeding regime of 100 000 algal cells ml⁻¹. Our objective was to determine how food supply affects the growth and survival of larvae under environmental conditions that are typical of South Australian waters during the likely spawning season (late spring through summer). The larval performances at this relatively high salinity were assessed over a period longer than has previously been investigated.

2 METHODS

Adult farmed Pacific oysters (13 female and 15 male) in spawning condition were obtained from Port Douglas, Coffin Bay, SA at the end of November 2007. In the laboratory, five genetically distinct batches of fertilised eggs were obtained by manually stripping gametes from three males and three females per batch. The two largest females contributed eggs to two batches each. Eggs and sperm were mixed in 2-litre glass beakers using 10µm filtered natural seawater at 23°C. After 30 minutes fertilisation, the eggs were washed with filtered seawater. Eggs from each batch were stocked in separate 20-litre tanks at a density of approximately 55 eggs per ml.

At 30 hours post fertilisation, 150 000 D-stage larvae from each larval batch were assigned to each of four feed treatments and transferred to 15-litre hatchery tanks, giving five replicates per treatment. Tanks were filled with filtered natural seawater with a salinity of 38.8 ± 0.5 ‰, and were located in a controlled environment room that maintained water temperature at 22.8 ± 1.3 °C (mean \pm s.d.). Feed was first added to all fed treatments when D-stage larvae were placed in the tanks (day 1) and subsequently 3 times per week following water changes. In all cases a 1:1 ratio of *Isochrysis galbana* and *Chaetoceros calcitrans* algal cells, a diet previously shown to give good growth rates (Rico-Villa et al. 2006), was used. The Nil feed treatment received no added feed. The High feed treatment received 100 000 cells ml⁻¹, ie 50 000 cells ml⁻¹ each of *Isochrysis galbana* and *Chaetoceros calcitrans*, which represents a typical hatchery feeding level and the same feeding concentration and frequency, as well as the same algal mix, as used in several previous studies (eg.

Helm & Millican 1977; His et al. 1989; Laing 1995). The two other feed treatments were: Medium feed (10 000 cells ml⁻¹) and Low feed (1 000 cells ml⁻¹).

Water changes involved a complete exchange of water with larvae screened onto 35µm mesh. Larvae were resuspended in 300ml and a subsample of known volume collected for each tank at each sampling time. A 1ml subsample (approximately 500 larvae) was collected on days 1, 4, 6, 9, and 12 and a 2ml subsample (approximately 1000 larvae) on days 16, 19 and 22. Samples were preserved by adding 1-2 drops of Lugol iodine solution (110g Potassium iodide, 50g crystalline iodine and 100ml glacial acetic acid per litre in distilled water).

Preserved larvae were observed and photographed under a compound microscope. For samples from days 9, 12, 16, 19 and 22, numbers of empty shells in a random sample of at least 100 larvae were counted as a measure of mortality. For day 1 samples, 30 larvae from each larval batch were photographed. For all other samples, a maximum of 10 non-empty shells from each tank where “live” (non-empty) larvae were found were photographed.

Photographs were analysed using *ImageJ* (U.S. National Institute of Health, Bethesda, MD) image analysis software to determine Feret (maximum calliper) diameter as a measure of size for each of the photographed larvae. Where at least five non-empty larvae were found and photographed, size was averaged for that tank. Tanks where less than five non-empty individuals were found were not included in the analyses.

Analysis of results was carried out in *SPSS 16.0 for Windows* (SPSS Inc., Chicago, IL). Prior to analysis, % survival at day 21 data were arcsine transformed (Zar 1996). A two-way analysis of variance (ANOVA) with feed as a fixed factor and larval batch as a random factor was used to compare survival to day 21 between groups. Post Hoc tests with Bonferroni corrections applied were carried out where significant differences were found to determine groupings.

Analysis of covariance (ANCOVA) with day as covariate, feed as a fixed factor and batch as a random factor was used to compare larval size over the experimental period. The interaction term feed*day was included to test whether feed treatments exhibited significantly different growth rates. Where the feed*day interaction term was found to be significant, two-way ANOVAs (again considering feed and batch) were performed to compare daily growth rates and final size. Average daily growth rate to the end of the culture period was determined for each tank as the difference between final size and day 1 size of the relevant larval batch, divided by the number of days. Post hoc tests with Bonferroni corrections applied were then carried out where significant differences were found to determine groupings.

To estimate the comparative length of the larval period between feed levels, it was assumed that growth rates would remain constant for each group. The growth rate (calculated as above) was used to calculate the days required for larvae to reach an average size of 280µm. This size

was chosen as it is regarded as the minimum size at which *C. gigas* larvae are competent to metamorphose (Bochenek et al. 2001; Rico-Villa et al. 2006).

3 RESULTS

3.1 Growth

On day 12 and after day 16, less than five non-empty shells were found in many Nil and Low feed larval samples leading to insufficient data being available to enable a comparison of larval size between treatments after day 16. Therefore, only size data for days 4, 6, 9 and 16 was used in the analyses of size and growth.

ANCOVA demonstrated that the interaction of day*feed was highly significant ($F_{(3,74)}=55.809$, $p<0.001$), indicating that larvae within the different feed treatments grew at significantly different rates. This was confirmed by ANOVAs that demonstrated significant differences between both growth rate and size at day 16 for the different feed levels (Table 1). Larval batch did not have a significant effect on either parameter (Table 1).

Table 1. ANOVA results for growth rate and size (Feret diameter) at day 16.

Parameter	df	F	Sig.
Size			
Feed*	3	17.217	<0.001
Batch	4	0.563	0.695
Error	10		
Growth rate			
Feed*	3	17.217	<0.001
Batch	4	0.556	0.669
Error	10		

*significant difference

Table 2. Pairwise comparisons of size at day 16 with Bonferroni corrections applied

Comparison	Mean difference	p
Nil vs Low feed	-11.239	1.000
Nil vs Med feed	-2.863	1.000
Nil vs High feed*	-51.972	<0.001
Low vs Med feed	8.376	1.000
Low vs High feed*	-40.732	0.003
Med vs High feed*	-49.109	<0.001

*significant difference

At day 16 the average size of High feed larvae was 142 μ m, which was significant greater than the size reached by larvae in the other feed treatments. In fact, differences in size and growth rate between the Nil, Low and Medium feed groups were highly non-significant ($p=1.000$; Tables 2,3), with larvae in these three treatments reaching an average size of 94 μ m at day 16 (Fig. 1). Although differences were not significant, there did appear to be marginally better growth in Low feed than Nil feed larvae, however, Medium feed larvae grew less than Low feed larvae. Possibly

this was related to differences in survival; Low feed larvae suffered greater mortality early in the culture period than Medium feed larvae (Fig. 3) so the feed ration (i.e. number of algal cells) available per larva would have been greater than if more larvae survived. However, the feed ration should still have been higher for Medium than Low feed larvae given the ten-fold greater feed concentration used. Since insufficient “live” larvae were found in two of the Low feed tanks the average size for this group was determined from only three measurements, and it cannot be ruled out that the slightly large size observed was due to chance. The growth rate of larvae given a typical hatchery feed regime (High feed) was more than double that of any of the other feed treatment groups (Fig. 2).

Table 3. Pairwise comparisons of daily growth rate to day 16 with Bonferroni corrections applied

Comparison	Mean difference	p
Nil vs Low feed	-0.706	1.000
Nil vs Med feed	-0.191	1.000
Nil vs High feed*	-3.465	<0.001
Low vs Med feed	0.515	1.000
Low vs High feed*	-2.759	0.002
Med vs High feed*	-3.274	<0.001

*significant difference

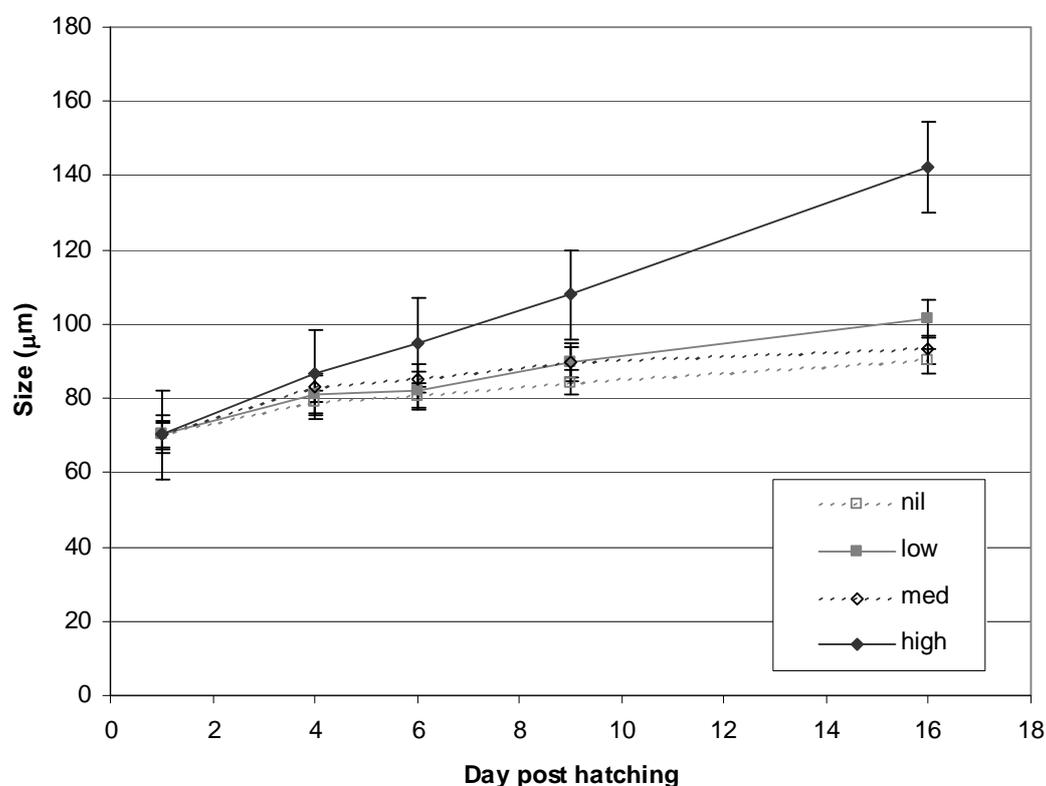


Figure 1. Average larval size (Feret diameter) from day 1 to day 16 under four feeding levels. Error bars represent \pm s.e. ($n=5$ for days 4 to 16, except low feed day 16, $n=3$). Average size at day 1 for all treatments was $70.2 \pm 1.04 \mu\text{m}$ ($n = 20$).

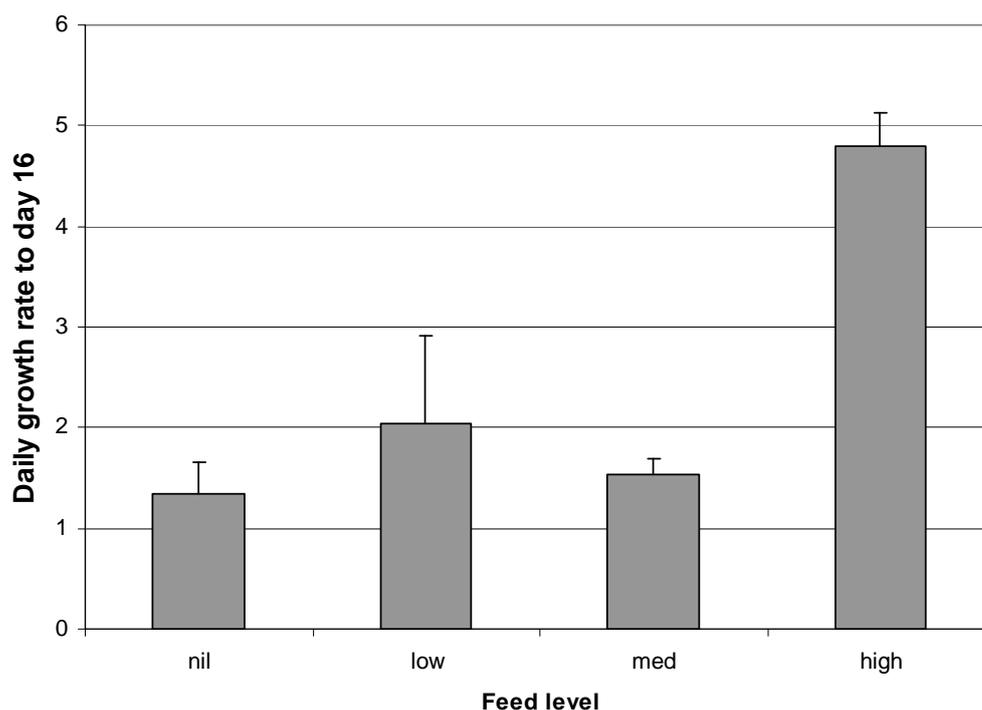


Figure 2. Average daily growth rate to day 16. Error bars represent s.e. (n=5, except low feed, n=3).

If growth throughout the larval period is assumed to be linear and equal to that at day 16, then larvae in the High feed group would be expected to reach an average minimum size suitable for metamorphosis at day 44. Larvae from the other feed treatments would reach the same average size after a minimum of 103 and average of 133 days (Table 4).

Table 4. Comparison of length of larval period between feed groups assuming constant linear growth, based on growth rate to day 16.

Group	Initial size	Daily growth rate	Days to reach 280µm
High feed	70.2	4.80	43.7
Medium feed	70.2	1.53	137.1
Low feed	70.2	2.04	102.8
Nil feed	70.2	1.34	156.6

3.2 Survival

There was no evidence of mortality in samples of newly hatched D-stage larvae (day 1) or at day 4. At day 6 some empty shells were noted in four of the five Low feed tanks, three each of the Nil and Medium feed tanks and two of the High feed tanks, but these were not enumerated. However, by day 9, when empty shells were counted, the Nil and Low feed groups had suffered >50% mortality. By day 22 <1% of larvae in these two treatments survived, while 4% of the Medium feed and 18% of the High feed larvae survived (Fig. 3). Two-way ANOVA showed that batch did not have a significant effect on survival ($F_{(4,12)}=0.791$, $p=0.553$), while effects of feed were significant ($F_{(3,12)}=6.639$, $p=0.007$). Given the high variability in survival within treatments the difference in survival to day 22 between High and Medium feed groups was not found to be

significant, but survival in the Nil and Low feed groups was significantly lower than in the High feed group (Table 5, Fig. 4).

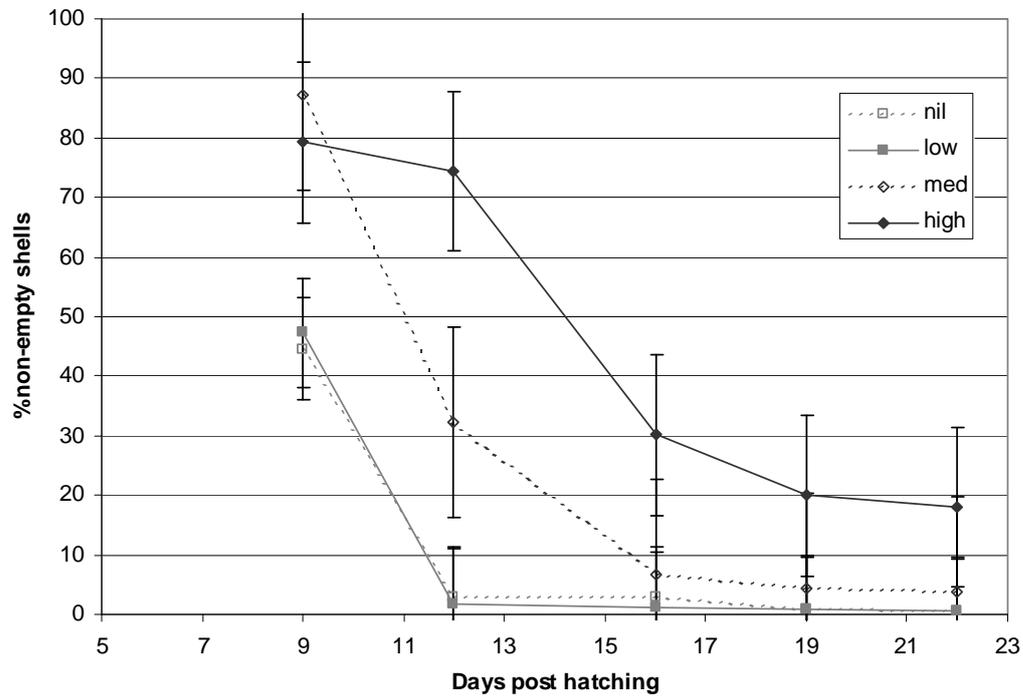


Figure 3. Average % survival from day 9 to day 22. Error bars represent \pm s.e. (n=5). No mortality was evident at day 1 or day 4. Mortality at day 6 was not evaluated.

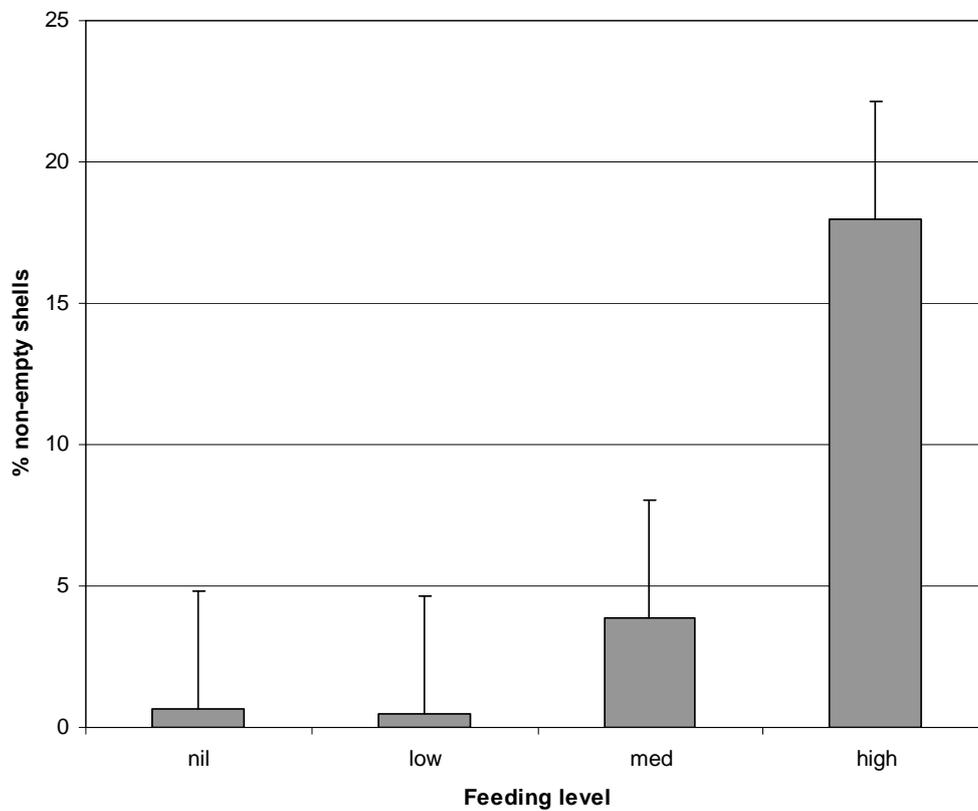


Figure 4. Average % Survival (defined as percentage of non-empty shells) at day 22. Error bars represent s.e. (n=5).

Table 5. Pairwise comparisons of survival between feed levels with Bonferroni corrections applied.

Comparison	Mean difference	p
Nil vs Low feed	0.003729	1.000
Nil vs Med feed	-0.110412	1.000
Nil vs High feed*	-0.329567	0.007
Low vs Med feed	-0.114141	1.000
Low vs High feed*	-0.333296	0.006
Med vs High feed	-0.219155	0.110

*significant difference

4 DISCUSSION

Despite the obvious importance of nutrition for larval growth and survival, few previous studies have investigated the impacts of a reduced feed supply on Pacific oyster larvae, particularly under possibly adverse conditions of salinity, i.e. at or above the reported optimum of 30‰ (Wiltshire 2007). The results presented here show that feed supply has a profound effect on both growth and survival of *C. gigas* larvae, and provide further evidence that, given sufficient feed, the higher than optimal salinity (>30‰) typical of South Australian waters does not prevent larval growth and survival. It should be noted that the larvae used in this experiment were obtained from adults grown in and acclimated to South Australian conditions. While the specific conditions the adults were exposed to leading up to this experiment are unknown, salinity in Coffin Bay often exceeds 40‰ and salinities above 50‰ have been recorded (Hone 1996). Thorough studies of acclimation have not been carried out, but both Helm & Millican (1977) and Muranaka & Lannan (1984) showed that larvae from adults kept at a higher salinity (30‰) fared better than those from adults kept at lower (15-20‰) salinity when raised at 30‰.

Previous studies carried out at salinities between 25 and 30 ‰, showed that Pacific oyster larvae are able to withstand and recover from short periods of starvation (His & Seaman 1992) and may be able to tolerate periods of >17 days without feeding (Moran & Manahan 2004). As Moran & Manahan (2004) note, withholding feed for a short period does not necessarily result in starvation since oyster larvae are able to utilise both bacteria and dissolved organic matter as an energy source to maintain metabolism (Fankboner & De Burgh 1978; Wellborn & Manahan 1990, Douillet 1993a,b). In the current study both bacteria and dissolved organic matter would have been available for oyster larvae in addition to the algal feed added, and this explains the fact that even unfed larvae grew to a certain extent. What is more pertinent is that the addition of up to 10 000 algal cells ml⁻¹ (Medium feed treatment) did not have any significant positive effect on growth, although it may marginally improve survival.

The addition of 100 000 cells ml⁻¹ did significantly improve growth, and extrapolation of daily growth rate data suggests that the larval period would be considerably shorter for larvae at this

feed level than for any of the other feed treatments (44 days compared with a minimum of 103 days). These estimates of larval period are made only to illustrate the impact that reduced growth may have on larval period, and are unlikely to reflect the true length of the larval period under these feeding conditions. These estimates assume a linear growth rate when, in fact, growth should be exponential where feed is not limiting (Helm & Millican 1977; Bochenek et al. 2001). Conversely, growth in food-limited larvae could be expected to level off, since as larvae grow, the proportion of their energy needs that can be supplied by bacteria and dissolved organic matter reduces (Douillet 1993b). Hence it is likely that the difference in larval period between different feed levels may be even greater than estimated. Additionally, given the high mortality of the Nil, Low and Medium feed groups it is unlikely larvae at these feed levels would survive a prolonged larval period.

Larval survival in cultured oysters is highly variable and has been shown to have a large genetic component (Lannan 1980). In the current experiment, larval batch did not have a significant effect on survival; although there was large variability between survival in different tanks, there was no clear pattern related to batch. Despite this variability, there was a significant difference in mortality evident between feed treatments. In particular, survival in the Nil and Low fed oyster larvae cultures was very low, with < 1% surviving to 21 days. The addition of 10 000 cells ml⁻¹ (Medium feed) led to a marginal improvement in survival to 4%, although this was not significantly different to survival in Nil or Low feed larvae. However, even should this be a true difference in survival, it is still unlikely to be sufficient to allow survival through to settlement at this feed level when the prolonged larval period due to slow growth is also considered. As well as the mortality due to feed level shown here, a longer larval period can be expected to lead to greater natural mortality due to predation (Bochenek et al. 2001) and may also increase the chance that larvae are advected by tides and currents away from sites suitable for settlement and subsequent growth (Arakawa 1990). Additionally, it has been shown that even larvae of a suitable size for metamorphosis may not settle successfully if they are lacking in biochemical reserves, as may be the case where food is limiting (Laing 1995; Laing & Earl 1998; Bochenek et al. 2001; Powell et al. 2002).

It is clear from these results that a high level of feed is needed to support growth of oyster larvae, however, it is problematic to relate this directly to natural algal cell concentrations or Chlorophyll measurements. This is due to the fact that feed quality is also a very important factor in determining larval growth and survival (Powell et al. 2002), and different algal diets lead to vastly different growth rates (Thompson & Harrison 1992; Rico-Villa et al. 2006). The feed value of natural plankton assemblages is largely unknown (Thompson & Harrison 1992; Douillet 1993b) and the overall importance of bacterial and dissolved organic feed sources in the wild is difficult to determine (Douillet 1993b). Natural food availability in many areas where wild oysters occur is often below that found to be required in laboratory studies (Douillet 1993a,b).

Nonetheless, we found that greater phytoplankton availability lead to faster growth and better survival, so differences in phytoplankton availability in the field could have a large impact on the numbers of Pacific oyster larvae successfully surviving through to settlement.

Chlorophyll *a* concentrations in South Australian oyster farming regions measured by Hone (1993) were low (0-4mg/l) compared with the optimum value of >12mg/l suggested for this species (Coleman 1986; Hone 1993). However, data from SASQAP (http://www.pir.sa.gov.au/aquaculture/monitoring_and_assessment/sasqap; unpublished historical data) show that phytoplankton concentrations vary widely with location, season and year. Concentrations are generally highest in summer when oyster larvae are likely to occur but, for the same site, concentration may vary by several orders of magnitude from one month to the next, and also between years for the same month. Given the results of our study, it is possible that a period of considerably higher than usual phytoplankton availability could significantly increase the chance of successful *C. gigas* larval development and subsequent settlement. However, in periods and regions where feed is limiting, successful settlement appears unlikely.

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