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The effects of stocking density and ration on survival and growth of winged pearl oyster (*Pteria penguin*) larvae fed commercially available micro-algae concentrates

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ABSTRACT

Commercially available micro-algae concentrates have been successfully used as an alternative to live micro-algae as a food source during routine larval culture of the winged pearl oyster, *Pteria penguin*. This supports the development of simplified hatchery facilities and larval rearing protocols that are more appropriate to Pacific island nations. An optimal feeding regime based on these products that also accounts for larval stocking density is yet to be developed. Two experiments were conducted at a commercial pearl oyster hatchery facility in the Kingdom of Tonga to examine the combined effects of stocking density and ration on survival and growth of both D-stage (from 1 to 8 days post-fertilsation) and umbo-stage (from 8 to 17 days) *P. penguin* larvae. Both experiments used a factorial design combining three larval stocking densities (D-stage: 2, 6 & 10 larvae mL^{-1}; umbo-stage: 1, 3 & 5 larvae mL^{-1}) and three rations (D-stage: 5, 10 & 15 cells mL^{-1}; umbo-stage: 10, 15 & 20 cells mL^{-1}). Survival during D-stage was significantly improved in aquaria stocked below 10 larvae mL^{-1}, whereby a density of 6 mL^{-1} maximised larval production. An intermediate ration of 10×10^3 cells mL^{-1} maximised both survival and growth during D-stage. Increasing the initial stocking density of umbo-stage larvae from 1 to 3 mL^{-1} resulted in significant reductions in both survival and growth. Growth of umbo-stage larvae stocked at a density of 1 mL^{-1} increased significantly when ration remained below 20×10^3 cells mL^{-1}. The results of this study provide a basis for optimised hatchery culture protocols for *P. penguin* that are more appropriate to Pacific island nations.

1. Introduction

Hatchery propagation has become increasingly important as a source of oysters for the cultured pearl industry and is now a necessity in regions where the collection of wild spat no longer provides adequate numbers of oysters to sustain half-pearl (mabè) production on a commercial scale (Southgate, 2008). The near exhaustion of *Pteria penguin* populations in Japan and China has meant that commercial half-pearl production from this species is now dependent on hatchery propagation (Liang et al., 2001; Yu et al., 2004; Southgate et al., 2008), which potentially ensures a consistent supply of oysters and allows selection for commercially desirable genetic traits such as increased growth rate, disease tolerance and improved pearl quality (Evans et al., 1995).

Despite the advantages of hatchery production, it is often beyond the capability of many developing countries in the Pacific, where the technical resources and skilled personnel required for successful hatchery operation are often lacking. Production of appropriate quantities of high quality live micro-algae as a larval food source is a common bottleneck due to the requirement for dedicated facilities, specialised equipment and technical oversight (Couteau and Sorgeloos, 1992; Helm et al., 2004). A large proportion of the infrastructure and costs associated with establishing a pearl oyster hatchery in the Pacific are attributed to micro-algae culture (Ito, 1999).

Recent years have seen increased availability of commercially available 'off-the-shelf' food products that are designed to replace or supplement live aquaculture foods including micro-algae. A

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number of these products, such as dried and concentrated micro-algae, have been developed specifically for bivalves, and prior studies have assessed the potential of such products for replacing live micro-algae for pearl oyster larvae (e.g. Southgate et al., 1998; Teitelbaum and Ngaluafe, 2008). Various species of phototrophically grown, highly concentrated marine micro-algae are now available commercially and have potential in many aspects of bivalve production, including hatchery culture (Reed and Henry, 2014). These products are now used routinely as the sole food source during hatchery production of the winged pearl oyster, *Pteria penguin*, in Tonga, and have greatly simplified hatchery procedures (Southgate et al., 2016).

Micro-algal concentrates are composed of intact but non-viable, non-motile cells (Reed and Henry, 2014) that are negatively buoyant. As such, micro-algae concentrates behave differently to live micro-algae when introduced to larval rearing tanks (Heasman et al., 2000) and optimised feeding regimes may be different even if the nutritional content of the food source is similar. Ration must allow access to an appropriate level of nutrition to support larval development, without causing reduced water quality resulting from the decomposition of unconsumed feed (Loosanoff and Davis, 1963; Doroudi et al., 1999). The efficacy of ration varies according to larval density (Doroudi and Southgate, 2000; Liu et al., 2010), therefore an understanding of the specific interactions between these two factors is required to achieve efficient larval production and to minimise food waste. This study therefore, investigated the effects of stocking density and ration on survival and growth of both D-stage and umbo-stage *P. penguin* larvae fed commercially available micro-algae concentrates.

2. Materials and methods

2.1. Larval production

This study was conducted at the Aquaculture Facility of the Ministry of Agriculture and Food, Forests and Fisheries (MAFFF) at Sopu in the Kingdom of Tonga (21°07′21″S; 175°13′36″W). Fifty *Pteria penguin* brood-stock were collected from a long-line located 500 m offshore and cleaned, before being induced to spawn via repeated air exposure (Victor et al., 2001). Broodstock were placed in a shallow spawning tank containing UV-treated 1 μm filtered sea water (FSW) for 1 h, before tanks were completely drained, leaving the oysters exposed to direct sunlight for 10 min. The tank was then refilled with FSW at a temperature of 28° C and the oysters left undisturbed for 30 min. This process was repeated twice before several males began to release sperm, prompting the females to spawn 3–5 min later.

Once spawning began, the procedures for achieving fertilisation followed the standard methods described for pearl oysters (Southgate, 2008). Fertilised eggs were rinsed onto a mesh sieve to remove excess spermatozoa and then placed into static incubation tanks containing gently aerated 1 μm FSW at a stocking density of 50 eggs mL^{-1} (Southgate et al., 1998; Doroudi and Southgate, 2003). Embryonic development was monitored using an optical microscope (200X). After an incubation period of 22 h, the shelled D-stage larvae that had developed were washed and counted, and the required number of larvae were distributed into the aquaria used for Experiment 1. D-stage larvae that were not used in Experiment 1 were cultured for a further 8 days in 1,000 L tanks using typical methods for *P. penguin* larvae (Southgate et al., 2016) and then used in Experiment 2.

Aquaria in both experiments were filled with UV-treated 1 μm FSW and underwent a complete water exchange every two days, in accordance with typical culture methods for *P. penguin* larvae (Southgate et al., 2016). Aquaria were held in a temperature controlled water bath at 28 °C throughout the experiments. Each aquarium had a removable lid and was gently aerated through a glass tube inserted from the top.

2.2. Micro-algae concentrates

Larvae were fed commercially available micro-algae concentrates from the Instant Algae® range (Reed Mariculture Inc., San Jose, CA, USA) in both experiments. The products used in this study were mono cultured *Isochrysis* sp. ("Isochrysis 1800®"), mono-cultured *Pavlova* sp. ("Pavlova 1800®"), and a third product composed of a mixture of *Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissflogii* and *Tetraselmis* sp. ("Shellfish Diet 1800®"). All were obtained from an Australian distributor of the products and were stored in their original bottles in a refrigerator at 4 °C for the duration of the study. Prior to use, a 5 mL aliquot of each concentrate was added to 2 L of FSW in a separate container and gently hand-shaken to disperse the micro-algae cells. The cell densities in resulting suspensions were determined using a haemocytometer and the volume needed to provide the required ration for each aquarium was dispensed through a 20 μm mesh sieve to remove or break up any clumps of micro-algae cells that may have been present.

2.3. Experiment 1

Experiment 1 assessed the combined effects of stocking density and ration on survival and growth of D-stage *P. penguin* larvae from 1 to 8 days post-fertilisation. The mean antero-posterior shell measurement (APM) of 50 randomly selected D-stage larvae at the start of the experiment was 81.0 ± 3.8 μm. Larvae were stocked into 5 L aquaria at densities of 2, 6 and 10 larvae mL^{-1} and fed a 1:1 ratio (based on cell count) of "Isochrysis 1800®" and "Pavlova 1800®" at a ration of either 5, 10 or 15×10^3 cells mL^{-1} once daily. The combined effects of larval density and ration each at 3 levels with 3 replicates required a total of 27 experimental aquaria.

2.4. Experiment 2

Experiment 2 examined the effects of stocking density and ration on survival and growth of umbo-stage *P. penguin* larvae from 8 to 17 days post-fertilisation. Prior to reaching umbo-stage, larvae were raised according to the intermediate conditions applied in Experiment 1 (i.e. density = 6 larvae mL^{-1}, ration = 10×10^3 cells mL^{-1}). The APM of 50 randomly selected umbo-stage larvae at the start of the experiment was 105.9 ± 10.7 μm. Larvae were stocked into 5 L aquaria at densities of 1, 3 and 5 larvae mL^{-1} and fed a 1:1:1 ratio of "Isochrysis 1800®", "Pavlova 1800®" and "Shellfish Diet 1800®" at a ration of either 10, 15 or 20×10^3 cells mL^{-1} once daily. The resulting cell count of the feed used constituted approximately 43% *Isochrysis* sp., 40% *Pavlova* sp., 10% *Thalassiosira weissflogii* and 7% *Tetraselmis* sp. The combined effects of larval density and ration each at 3 levels with 3 replicates required a total of 27 experimental aquaria.

2.5. Sampling and data analysis

At the end of each experiment, the contents of individual aquaria were collected on a 25 μm mesh screen and washed into separate 50 mL vials containing 4% formaldehyde solution in buffered seawater. Survival and growth were estimated by removing triplicate 1 mL sub-samples from each 50 mL vial and examining the contents using an optical microscope (200X). The number of surviving larvae were counted and the shell length (APM) of 20 random individuals was measured. Relative larval production, or yield mL^{-1}, was calculated by multiplying initial density by mean survival. The normality of raw data was assessed using probability plots of residual

values and proportional data were square-root arcsine transformed prior to analysis. Significant differences between treatment means were determined by two-way ANOVA and post-hoc Tukey tests ($\alpha = 0.05$).

3. Results

3.1. Experiment 1

A two-way ANOVA examining the effects of stocking density and ration on survival (%) of D-stage *P. penguin* larvae revealed no significant interaction between the two factors. Overall mean survival improved ($F = 61.456$, $df = 2$, $p < 0.001$) from 27.3% to 54.1% when stocking density was decreased from 10 to 6 larvae mL^{-1}, but a further decrease in density did not significantly improve survival any further. At stocking densities greater than 2 larvae mL^{-1}, mean survival significantly improved ($F = 3.180$, $df = 2$, $p = 0.029$) when using an intermediate ration of 10×10 cells mL^{-1} rather than a high ration of 15×10 cells mL^{-1} (Fig. 1A). The lowest larval density of 2 larvae mL^{-1} yielded a significant ($F = 43.097$, $df = 2$, $p < 0.001$) reduction in larval production, that was 35% and 42%, respectively, that at densities of 6 and 10 larvae mL^{-1} (Fig. 1B).

There was a significant interaction ($F = 9.07$, $df = 4$, $p < 0.001$) between the effects of stocking density and ration on shell length of D-stage *P. penguin*, indicating that the effect of ration differed according to stocking density. When considered independently, density had an overall significant effect on shell length ($F = 25.22$, $df = 2$, $p < 0.001$), which increased by 3.7% from an average of 101.4 μm to 105.2 μm when stocking density was reduced from 10 to 6 larvae mL^{-1}, and by a further 2.6% to 107.9 μm when stocking density was reduced to 2 larvae mL^{-1}. Ration had the largest effect on larval size at a low stocking density of 2 larvae mL^{-1}, whereby growth was reduced ($F = 10.147$, $df = 2$, $p = 0.002$) by an average of 7% when applying a high ration of 15×10 cells mL^{-1} (Fig. 1C). Conversely, at the highest stocking density of 10 larvae mL^{-1}, a ration of 15×10 cells mL^{-1} resulted in a significant increase ($F = 4.307$, $df = 2$, $p = 0.015$) in shell length when compared to the lowest ration (Fig. 1C).

3.2. Experiment 2

A significant ($F = 4.479$, $df = 4$, $p = 0.003$) interaction between stocking density and ration on survival (%) of umbo-stage *P. penguin* larvae indicated that ration affected survival differently according to stocking density. Density had an overall significant effect on survival ($F = 212.110$, $df = 2$, $p < 0.001$), whereby average survival increased from 1.4% to 9.8% when stocking density was reduced from 5 to 3 larvae mL^{-1}, and then further improved to 35.3% with a reduction in density to 1 larvae mL^{-1}. At stocking densities greater than 1 larvae mL^{-1}, mean survival significantly improved ($F = 6.750$, $df = 2$, $p = 0.003$) when ration was increased above 10×10 cells mL^{-1} (Fig. 2A), however, increasing density from 1 to 3 larvae mL^{-1} did not significantly affect production of larvae (Fig. 2B).

There was no significant interaction between the effects of stocking density and ration on the shell length of umbo-stage *P. penguin* larvae. Density had an overall significant effect on growth ($F = 206.835$, $df = 2$, $p < 0.001$), whereby mean APM increased by 15.6% from 155.6 μm to 179.8 μm when density was reduced from 5 to 3 larvae mL^{-1}, and by a further 12.1% to 201.5 μm when density was reduced to 1 larvae mL^{-1}. Ration had a significant effect on larval size only when stocking density was low, whereby a high ration of 20×10^3 cells mL^{-1} resulted in a 7% decrease in shell length when compared to lower rations ($F = 6.576$, $df = 2$, $p = 0.002$) (Fig. 2C).

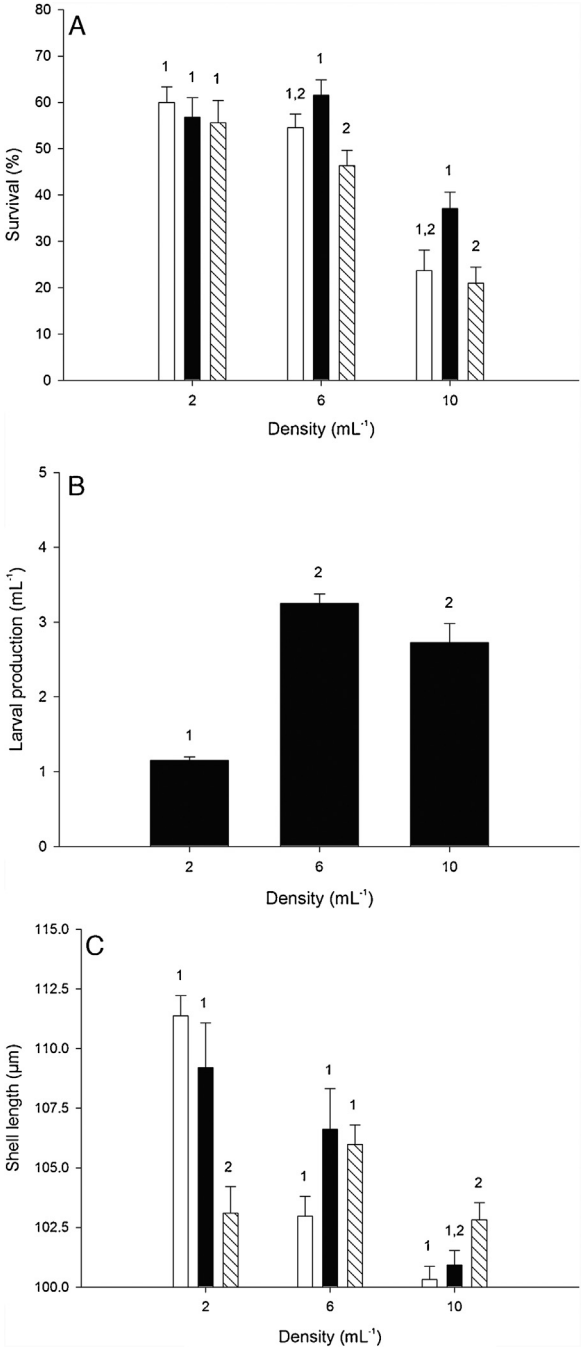

Fig. 1. (A) Mean (± SE) survival (%) of *Pteria penguin* larvae during D-stage (days 1–8) at three levels of stocking density and ration (5 □, 10 ■ and 15 ▨ algae cells mL-1); (B) mean larval production per volume of culture medium at three levels of stocking density; (C) mean antero-posterior shell measurement (APM) at three levels of stocking density and feed ration. Superscript numbers represent Tukey groupings within individual density treatments.

4. Discussion

This study is not the first to assess alternatives to live micro-algae as a food source for pearl oyster larvae. Much of the prior research in this field has focussed on the black-lip pearl oyster *Pinctada margaritifera*, where substitution of 25–50% of a live micro-algae diet with dried micro-algae (*Tetraselmis* sp.) was reported without significantly reducing survival or growth of D-stage larvae (Southgate et al., 1998; Doroudi et al., 2002). The use of yeast-based products in conjunction with live micro-algae has been shown

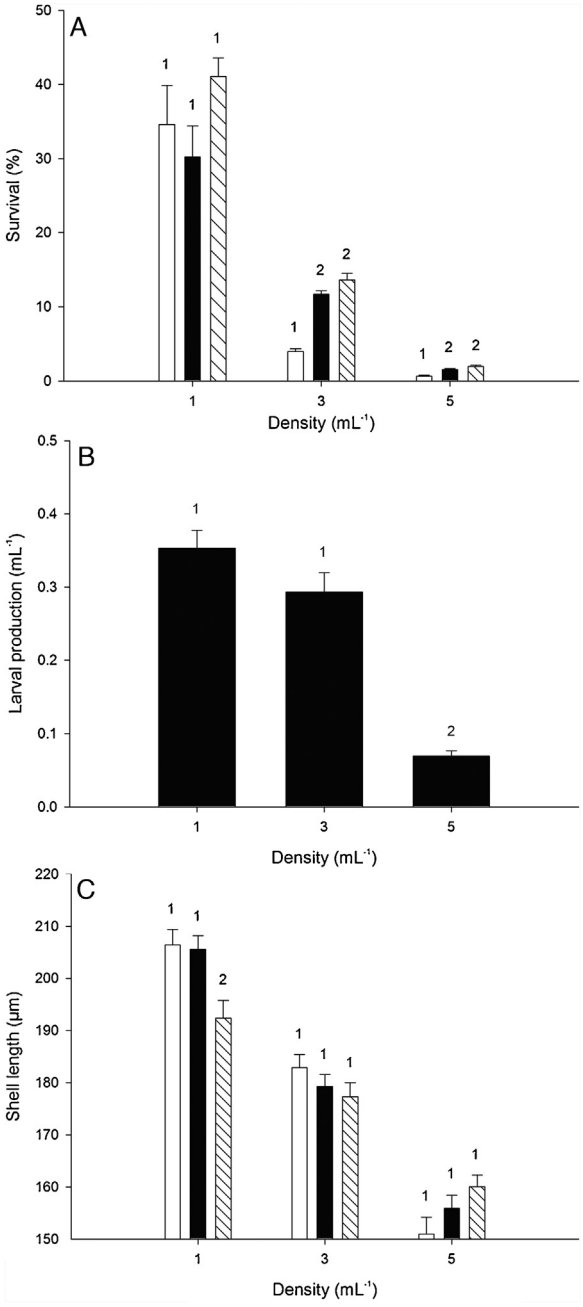

Fig. 2. (A) Mean (± SE) survival (%) of *Pteria penguin* larvae during umbo-stage (days 8–17) at three levels of stocking density and ration (5 □, 10 ■ and 15 ▨ algae cells mL^{-1}); (B) mean larval production per volume of culture medium at three levels of stocking density; (C) mean antero-posterior shell measurement (APM) at three levels of stocking density and ration. Superscript numbers represent Tukey groupings within individual density treatments.

to boost the growth rate of umbo-stage *P. margaritifera* larvae (Southgate et al., 1998). Such products are generally most effective when used as partial substitutes for live micro-algae and the need for micro-algae production by hatcheries remains. More recently, however, use of commercially available micro-algae concentrates for significant partial replacement (Teitelbaum and Ngaluafe, 2008) and total replacement (Southgate et al., 2016) of live micro-algae has been reported for *Pteria penguin* larvae. The results of the current study allow further development of a feeding protocol for *P. penguin* larvae based on these products. Such development is particularly important in regions where the culture of live micro-algae as a food source for pearl oyster larvae is technically prohibitive.

Stocking density was found to be more influential on survival of *P. penguin* larvae than ration. Survival of D-Stage larvae remained above 50% with an increase in stocking density from 2 to 6 larvae mL^{-1}, however, a further increase in density to 10 mL^{-1} caused a reduction in survival to approximately 27%. Moreover, survival of umbo-stage larvae decreased from 35% to less than 10% when larval density was raised above 1 mL^{-1}. The survival rates at higher densities for both D-stage and umbo-stage *P. penguin* larvae seen in this study reflect those observed previously during commercial production (Southgate et al. 2016). Increases in density have been correlated with elevated bacterial loading during larval culture of the pearl oyster *Pinctada fucata* (Subhashi et al., 2007). Subhashi et al. (2007) found six bacterial isolates within *P. fucata* rearing tanks that caused high mortality, five of these were *Vibrio* spp., which are known to be particularly dangerous for bivalve molluscs (DiSalvo et al., 1978) including pearl oysters (Jones, 2007), and capable of causing prolific mortality during hatchery production (Sainz-Hernández and Maeda-Martínez, 2005).

Mean growth rates at the lowest density treatments during both D-stage and umbo-stage development were consistent with those observed for *P. penguin* larvae in previous studies (Wassnig and Southgate, 2012; Southgate et al., 2016). Increased stocking density impacted on growth resulting in larvae with significantly smaller mean antero-posterior shell length during both stages of larval development. Growth of larvae in tanks with a high stocking density remained relatively low even when ration was increased, supporting the notion that factors other than access to food limit growth at high stocking density (Liu et al., 2006). Elevated density triggers a corresponding increase in the collision rate between swimming larvae causing retraction of the velum and cessation of feeding, as well as longer term energy loss associated with shell repair (Cragg, 1980; Sprung, 1984; Liu et al., 2006). Higher larval density may also result in rapid accumulation of toxic metabolites that can be detrimental to both immune function and growth (Sprung, 1984; Yan et al., 2006; Raghavan and Gopinathan, 2008).

It is often assumed that larval survival at a given density is largely a function of food availability (Helm and Millican, 1977). The results of this study and of previous research conducted with *P. margaritifera* larvae (Doroudi and Southgate, 2000), suggest that ration had a minimal effect on survival of D-stage pearl oyster larvae. This may be related to a partial reliance of younger larvae on endogenous energy reserves being the primary determinant of larval viability, rather than exogenous food sources (Bayne, 1983; Fraser, 1989; Doroudi and Southgate, 2003).

Doroudi and Southgate (2000) reported that survival of umbo-stage *P. margaritifera* larvae decreased with increasing ration of live micro-algae within the range of 2–30 × 10^3 cells mL^{-1}, regardless of stocking density. This trend was thought to result from the decomposition of uneaten food in higher ration treatments causing elevated levels of dangerous bacteria. In contrast, the results of this study show that at stocking densities greater than 1 larvae mL^{-1}, increasing ration from 10 × 10^3 cells mL^{-1} to 20 × 10^3 cells mL^{-1} improved mean survival of umbo-stage *P. penguin* larvae. The ability of umbo-stage *P. penguin* larvae to survive at higher rations is likely to facilitate recruitment across a wider ecological range encompassing near shore turbid environments (Milione and Southgate, 2012), whereas *Pinctada* spp. are restricted to the oligotrophic waters of off-shore reefs (Wada and Tëmkin, 2008).

Doroudi et al. (1999) demonstrated that the ration of live micro-algae required to maximise survival of *P. margaritifera* larvae (10 × 10^3 cells mL^{-1}) had to be doubled to maximise growth rate. Maximum growth of D-stage and umbo-stage *P. margaritifera* larvae occurs at live micro-algae rations of ~8 × 10^3 cells mL^{-1} and 25 × 10^3 cells mL^{-1}, respectively, regardless of changes in larval density between 1 and 5 larvae mL^{-1} (Doroudi et al., 1999). In contrast, the effects of ration on growth of *P. penguin* larvae appear

to be somewhat density dependent. Ration had the most marked effect on growth of both D-stage and umbo-stage larvae at low densities (D-stage: 2 mL^{-1}; umbo-stage: 1 mL^{-1}), whereby a high ration (D-stage: 15×10^3 cells mL^{-1}; umbo-stage: 20×10^3 cells mL^{-1}) resulted in a significant reduction in shell length. This may result from decomposition of uneaten micro-algae generating less favourable culture conditions that negatively impact larval growth rate (Loosanoff and Davis, 1963; Sprung, 1984; Liu et al., 2006). Therefore, while there is evidence to support the notion that *P. penguin* survive well at high rations, the results of this study suggest that there is a negative impact on larval development.

The results of this study allow further development of a feeding protocol for hatchery raised *P. penguin* larvae based on commercially available micro-algae concentrates. It is recommended that *P. penguin* hatcheries adopt a stocking density of 6 larvae mL^{-1} during D-stage to maximise larval production per unit volume of culture medium whilst incurring only a slight reduction in growth rate. A ration of 10×10^3 cells mL^{-1} optimised mean survival and growth of D-stage larvae when stocked at 6 mL^{-1}. Increasing density of umbo-stage larvae from 1 to 3 mL^{-1} resulted in a marked reduction of both survival and growth, and is therefore not recommended for efficient hatchery production. At a low density of 1 mL^{-1} for umbo-stage larvae, ration should be maintained at 10×10^3 cells mL^{-1} to minimise food costs and avoid reduced larval growth at higher feed rations ($\geq 15 \times 10^3$ cells mL^{-1}). A considerable proportion of the infrastructure and cost associated with establishing pearl oyster hatcheries in the Pacific is attributed to live micro-algae culture (Ito, 1999). The use of commercially available micro-algae concentrates to replace live micro-algae supports development of simplified, affordable hatchery facilities and larval rearing protocols that are more appropriate to Pacific island nations.

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