THE EFFECTS OF DIET, TEMPERATURE AND SALINITY ON SURVIVAL OF LARVAE OF THE FLUTED GIANT CLAM, *TRIDACNA SQUAMOSA*

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Abstract *Giant clams have been cultured for decades, yet few formal studies have examined their reproduction and early life history. Here we present two experiments that provide baseline information on the effects of micro-algal feeding, temperature and salinity on fertilization success and development of the fluted giant clam,* Tridacna squamosa*. The effect of different micro-algae feeds, i.e.* Tetraselmis suecica *(CS-187),* Chaetoceros mulleri *(CS-176), and yeast, on veliger survival was tested. A mixed-algal diet of 1:1 v/v* T. suecica + C. mulleri + *yeast resulted in approximately double larval survival by 24 h but no significant differences were identified between the uni-algal and mixed-algal diets at 48 h. Temperature and salinity were examined using a 2 × 2 design; with temperatures of ~22.5°C and ~29.5°C, and salinities of 27‰ and 30‰. At ~29.5°C fertilization success was significantly greater than at ~22.5°C, but higher temperatures were detrimental to the development of trochophores. There were no significant differences in either embryo or trochophore numbers for the salinities tested. The results indicate that both micro-algal diet and temperature can affect* T. squamosa *larval initiation and development; knowledge that can be used to improve their mariculture.*

Keywords *Algal particulate food, mariculture, giant clam,* Tridacna*, larval biology, Singapore*

INTRODUCTION

Giant clams (Bivalvia: Tridacnidae) are found in the shallow coral reefs of the Indo-Pacific. They possess filter-feeding gills but also obtain nutrition supplements from photosynthetic products produced by zooxanthellae (*Symbiodinium* sp.) embedded within their mantle tissues (Fitt, 1988). Giant clams are protandric hermaphrodites (Rosewater, 1965; Ellis, 1998) but, unlike other hermaphroditic bivalves, they first spawn sperm then eggs. Previous investigations of larval ecology in giant clams have focused primarily on rearing of the larvae for mariculture (e.g. Jameson, 1976; Heslinga *et al*., 1984; Copland & Lucas, 1988) as well as related topics such as nutritional ecology, growth rates and physiology of the larvae and juveniles (e.g. Fitt & Trench, 1981; Crawford *et al*., 1986; Klumpp & Lucas, 1994; Elfwing *et al*., 2003). Most of these examined *Tridacna gigas*, *T. terovoa*, *T. derasa*, *T. maxima* and *Hippopus hippopus*, whereas *T. squamosa* remains relatively poorly studied. LaBarbera (1974, 1975), Beckvar (1981), Fitt & Trench (1981) and Foyle *et al.* (1997) have described the spawning, early post-larval development, calcification of larval shell and acquisition of zooxanthellae in *T. squamosa*. However, for this species, there is very little information available on the optimal conditions for fertilization success and efficacy of different micro-algal feeds.

In many parts of Southeast Asia, giant clams are an important commercial food source; they also play a range of ecological roles, for instance, by acting as nurseries and refugia to various other reef organisms (Mingoa-Licuanan & Gomez, 2002). Over the last few decades, traditional harvesting practices and degradation of their natural environments due to destructive fishing practices have led to a decline in wild giant clam numbers (Mingoa-Licuanan & Gomez, 2002; Tan & Yasin, 2003). To satisfy the commercial market, as well as to replenish depleted natural stocks, numerous mariculture research and restoration projects are presently operating in the Indo-Pacific region (e.g. Heslinga *et al.*, 1984; Crawford *et al*., 1986). In Bolinao, Philippines, for instance, there exists an extremely successful *T. gigas* mariculture programme that provides giant clams for both trade and conservation efforts (Alcala & Alcazar, 1987; Gomez & Minogoa-Licuanan, 2006).

Coral reefs in Singapore used to host five species of giant clam, i.e. *T. gigas*, *T. squamosa*, *T. maxima*, *T. crocea* and *H. hippopus* (Rosewater, 1965) but recent surveys indicate that *H. hippopus*

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is now absent and *T. maxima* is locally functionally extinct (Guest *et al*., 2008; Neo & Todd 2012b). This present status can be attributed to massive coastal development and reclamation projects that have resulted in the destruction or degradation of many coral reefs in Singapore (Hilton & Manning, 1995; Chou, 1996). *Tridacna squamosa* is one of the more common species, yet even these are found only at very low densities and are concentrated on just one reef (Guest *et al*., 2008). In order to prevent further decline of this iconic invertebrate there exists an ongoing *T. squamosa* restocking effort that involves *ex situ* spawning and rearing of young clams. To enhance the mariculture aspect of this programme, we examined: (i) the effect of feeding micro-algae on veliger larval survival and (ii) the combined effects of temperature and salinity on fertilization of embryos and trochophores.

Materials and Methods

Spawnings were conducted at the Tropical Marine Science Institute (TMSI) on St John's Island (1°13´N, 103°50´E), Singapore. Mature *T. squamosa* brood stock was removed from a local reef (Raffles Lighthouse; 1°09'N, 103°44'E) located 13.7 km southwest of St John's Island. One-meter wide square tanks (depth = 0.7 m) were used for spawning; these were located in a large naturally-lit shed and were supplied with 1 µm-filtered, UV-treated seawater with a temperature of ~22.5°C and salinity of 30‰. Two different sets of clams were spawned on two different occasions: 14 August 2007 (for the feeding study) and 29 August 2007 (for the temperature × salinity study); four clams the first time and five the second time. For both spawnings, the mature giant clams were induced by injecting 2.0 ml of 20 µM concentration serotonin solution (crystalline 5-hydroxytryptamine, creatine sulfate complex, Sigma-Aldrich Pte Ltd, Product No. H7752–1G) into the gonads via the mantle tissue beside the excurrent siphon region (Braley, 1985). When each clam released sperm (after ~30 mins), the sperm-suspension was collected in separate 10 l buckets and diluted to give a density of ~10,000 sperm ml^{-1} (as determined from counts using a Neubauer haemocytometer). Each clam was then rinsed by transferring them among a series of tanks containing clean seawater. When eggs were released, the egg-suspension was collected in a fresh bucket. The unfertilized eggs were then washed on a 22 µm plankton screen. Density was approximately 22 eggs ml^{-1} for the first spawning and 14 eggs ml^{-1} for the second spawning (as determined from counts using a Bogorov tray).

Effect of micro-algal diets on larvae survival Prior to the feeding trials, fertilization of eggs and sperm took place in funnel tanks, i.e. cylindrical tanks with conical bottoms (diameter = 0.5 m, depth = 1.1 m). An egg-sperm ratio of 1:50 was used for fertilization as this is known to reduce polyspermy (Neo *et al.,* 2011). Four funnel tanks were each filled with 200 l of 1 µm-filtered UV-treated seawater, 10 l of eggs suspension (~220,000 eggs) and 1.1 l of sperm suspension (~10,000 sperm ml^{-1}). The resultant suspension of eggs and sperm was observed under an inverted microscope for signs of healthy cell divisions, which occurred after approximately 3 to 4 h. The developing embryos were left in the same tanks under a 12:12 h light/dark lighting regime with gentle aeration provided through air stones. To remove dead embryos, larvae were washed daily through 300 µm sieves with 1µm-filtered, UV-treated seawater.

Four feeding diet treatments (microalgae originating from the CSIRO collection) were prepared: *Tetraselmis suecica* (CS-187); *Chaetoceros mulleri* (CS-176); 1:1 v/v *T. suecica* + *C. mulleri*; and 1:1 v/v *T. suecica* + *C. mulleri* + yeast (*Saccharomyces cerevisiae*). For a period of three days prior to the experiment the microalgae were cultured in 1000 ml flasks using algal culture media (Braley, 1992: 64) at ~22.6°C under a 12:12 h light/dark lighting regime with gentle aeration provided through glass Pasteur pipettes (1 mm apertures). For all treatments, the concentration of each treatment was maintained at ~10,000 cells ml^{-1} (Fitt *et al*., 1984) and only algae cultures in the logarithmic phase of growth were used. An over-abundance of food was provided, i.e. although the biomass was different among the feed types, there was always enough to satiate the larvae (Fitt *et al*., 1984). Prior to each feeding, algae suspensions were filtered with a 50 µm plankton screen to remove cell clumps, counted using a Neubauer haemocytometer, and diluted to the required density in a 1000 ml solution. Yeast (0.1 mg) was first dissolved in 100 ml of deionized water and then 20 ml of the mixture was added to the 1000 ml of diet feed. To each 1000 ml beaker, 300 ml of feed suspension was added, and the total volume

was made up to 800 ml with 1 µm-filtered seawater and gently aerated.

From the larvae stock stored in the funnel tanks, healthy four-day old veliger larvae were filtered out using a 50 µm plankton screen washed with 1 µm-filtered seawater. In each of twelve 800 ml beakers, 400 veligers (culture density = 8 veligers per 10 ml) were added before being fed one of the four diets (× three replicates each = 12 beakers). After 24 h, seawater was changed and 300 ml of zooxanthellae (*Symbiodinium* sp. at ~10,000 cells ml^{-1}) mixture was introduced so the cells could become established in the guts of the veligers (Fitt, 1988). Zooxanthellae were extracted from a piece (400 mm^2) of an adult clam's mantle by homogenizing the tissue in a blender to release the cells; the mixture was then filtered using a 50 µm plankton screen and washed with 1 µm-filtered seawater. After another 12 h, seawater was exchanged and the veligers again were fed with 300 ml of the treatment diets. Two samples of 10 ml from each beaker were taken (after thorough agitation) after 24 h (immediately before the introduction of zooxanthellae), 36 h (immediately before being fed) and 48 h. Healthy veligers were counted.

The combined effect of temperature and salinity on the fertilization of embryos A 2 × 2 experiment design was used to test the combined effects of temperature (~22.5°C and ~29.5°C) and salinity (27‰ and 30‰) on fertilization of eggs. Five replicates of each of the four treatments were prepared. Different salinities were achieved by diluting 30‰, 1 µm-filtered and UV-treated stock seawater with deionized water; these were then maintained within ± 1 ‰ of the desired levels. An egg-sperm ratio of 1:50 was also used here: 70 µl of sperm-suspension (containing ~10,000 sperm ml^{-1}) was added to 300 ml egg-suspension (with ~14 eggs ml^{-1}) in 500 ml flasks and gently mixed. The flasks were placed in indoor facilities with combined natural and artificial lighting. Ambient air temperatures were recorded continuously throughout the experiments at five-minute intervals with temperature loggers (32K Waterproof StowAway TidbiT temperature loggers; ONSET Computer Corporation) placed near the flasks. The 22.6°C (±0.2°C) flasks were kept at ambient temperature in an air-conditioned indoor facility whereas the 29.3°C (±0.3°C) flasks were maintained at ambient temperature in a non-air-conditioned facility. After 3 h and 24 h, two samples of 2 ml from each flask were extracted (after thorough agitation). The numbers of fertilized eggs, undeveloped embryos, live and dead trochophores were counted as described earlier.

DATA ANALYSIS

A two-factor repeated measures ANOVA was used to test the effect of micro-algal diets on the survival of larvae with time. Two-factor ANOVA and *post-hoc* Tukey HSD tests were used to determine the combined effects of temperature and salinity on survivorship of embryos and trochophores. All data fulfilled assumptions of normality, sphericity and homogeneity of variances except those for the micro-algal diets, which failed the sphericity test. In this case, the adjusted univariate H–F Epsilon statistic was used. Statistical analyses were conducted on JMP IN 5.1, STATISTICA 5.0 (Stat-Soft) and GMAV 5.

RESULTS

Effect of micro-algal diets on larvae survival Straight-hinge D-veliger larvae were observed from 24 h to 40 h after fertilization. It was noted that higher mortality occurred after the introduction of feeds (including zooxanthellae inoculation); bacterial proliferation was observed and the veligers exuded internal granular material (Neo *et al.*, 2011). Two-factor, repeated measures ANOVA indicated significant differences among the micro-algal diets and among the times of feeding, but the interaction between time and diet was also significant (Table 1). *Tetraselmis suecica* + *C. mulleri*

Table 1 Two-factor, repeated measures analysis of variance comparing mean numbers of live *Tridacna squamosa* larvae among the four micro-algal diet treatments and time (n=3).

Source of variation	Effect	df	MS	F ratio	P
Diets	Among diets	*3*	0.1374	6.1640	0.01781
Time	Among times	*2*	0.1876	4.1490	0.03535
	Diet × time	*6*	0.1389	3.0717	0.03392

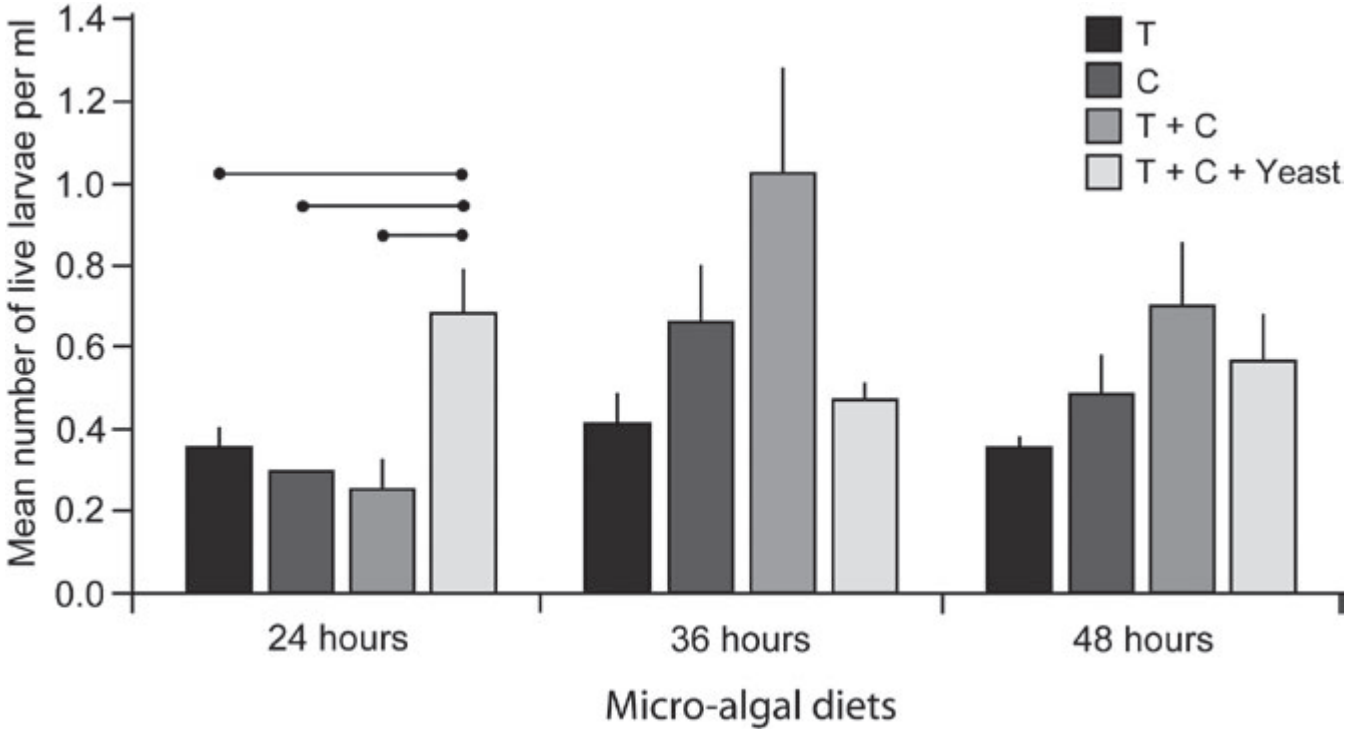

Figure 1 Effect of diet treatments on mean number of live *Tridacna squamosa* larvae at 24 h, 36 h and 48 h. Error bars indicate standard error. Treatments that are significantly different (Tukey HSD test; $P<0.05$) are indicated by black circles linked by horizontal lines. T = *Tetraselmis suecica* (CS-187). C = *Chaetoceros mulleri* (CS-176).

+ yeast diet resulted in the greatest survival of *T. squamosa* veligers at 24 h compared to all other diet treatments (Fig. 1). Overall, 85.42% of larvae survived after 24 h when provided with *T. suecica* + *C. mulleri* + yeast diet. After 36 h and 48 h of feeding, however, Tukey HSD showed no significant differences among diets on the mean number of live larvae.

The combined effects of temperature and salinity on the fertilization of embryos Temperature had a significant effect on the survivorship of live embryos and trochophores, whereas salinity had no effect at either larval stage (Table 2). There were significantly more embryos after 3 h in the ~29.5°C treatment compared to ~22.5°C (Fig. 2); after 3 h at ~29.5°C the fertilization success in 27‰ and 30‰ salinity was 88.6% and 99.3% survival respectively, compared to 61.4% and 58.6% after 3 h at ~22.5°C. Conversely, more trochophores (after 24 h) were found in the ~22.5°C treatments compared to ~29.5°C. That is, after 24 h at ~29.5°C the percentage survival in 27‰ and 30‰ salinity was 13.9% and 3.6%, respectively, compared to 46.8% and 32.5% after 24 h at ~22.5°C.

DISCUSSION

Most previous research on giant clams has focused on their growth and survival in mariculture (Beckvar, 1981; Heslinga *et al*., 1984) and farming of giant clams for re-stocking or trade is often successful (Lucas, 1994). Nevertheless, even though clams release huge numbers of gametes at each spawning, they experience high larval mortality before completing metamorphosis (Alcazar & Solis, 1986; Ellis, 1998). The present study provides new data on the conditions required for successful fertilization and early development of *T. squamosa* embryos and other larval stages. This information should help ensure greater survival and a higher proportion of healthy *T. squamosa* larvae for mariculture applications.

Feeding is known to affect the survival of bivalve larvae (e.g. Milke *et al*., 2006; Gouda *et al*., 2006). Algal strains used previously in giant clam aquaculture, i.e. *Isochrysis galbana*, *C. mulleri* and *Tetraselmis* sp. (Fitt *et al*., 1984; Estacion *et al*., 1986) have enhanced larval survivorship in the species tested compared to unfed larvae (Ellis, 1998). Fitt *et al.* (1984) concurred that unfed veligers demonstrated initial high growth

Table 2 Two-factor analysis of variance test comparing mean number of *Tridacna squamosa* embryos (a) and trochophores (b) among the temperature (n=2) and salinity (n=2) treatments.

Source of variation	df	MS	F	P
(a) Development of embryos				
Temperature	1	112.8125	19.7754	0.0005
Salinity	1	1.5125	0.2651	0.6137
Temperature × Salinity	1	4.5125	0.7910	0.3870
Residual	16	5.7047		
Cochran's test: C = 0.5294				
(b) Development of trochophores				
Temperature	1	1.3528	28.5254	0.0001
Salinity	1	0.1769	3.7307	0.0713
Temperature × Salinity	1	0.0012	0.0248	0.8769
Residual	16	0.0474		
Cochran's test: C = 0.6090				

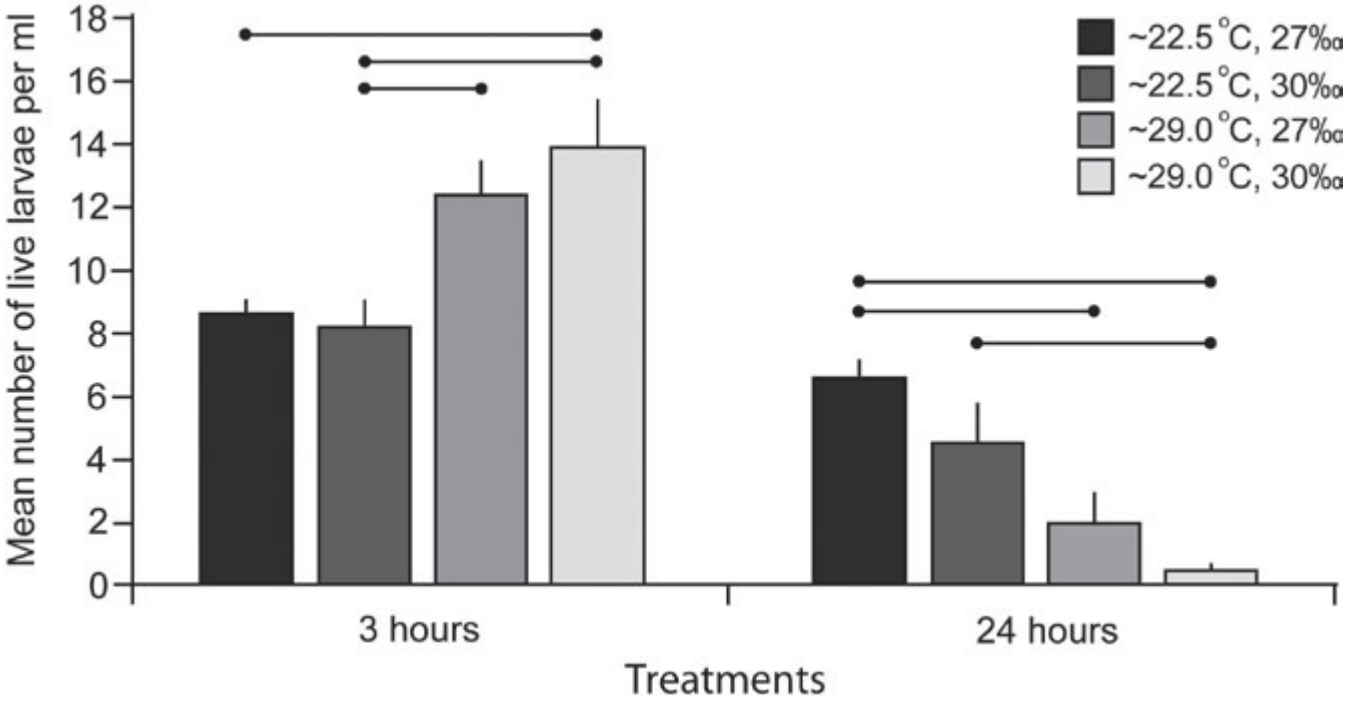

Figure 2 Effects of combined temperatures and salinities on the mean number of *Tridacna squamosa* embryos at 3 h and trochophores at 24 h. Error bars indicate standard error. Treatments that are significantly different (Tukey HSD test; P<0.05) are indicated by black circles linked by horizontal lines.

rates during the veliger stage, after which growth rates declined to zero and mortality increased markedly. In contrast, the fed veligers provided with particulate food had significantly higher survivorship and lower mortality than controls. Here, we tested *C. mulleri* (CS-176), *T. suecica* (CS-187), and yeast. Yeast was used as a nutritional supplement for the overall diet treatment as it usually contains a higher level of quality protein compared to micro-algae, but lacks the polyunsaturated fatty acids (Robert & Trintignac, 1997; Brown *et al.*, 1996). It has been used in other bivalve rearing, such as hard clam, *Mercenaria mercenaria*, aquaculture (Coutteau *et al*., 1994; Brown *et al*., 1996). For giant clams in general, the developmental stage is very short, i.e. just seven days before settlement, therefore the veligers were evaluated for only a short

period of time (two days). Furthermore, as larvae develop and metamorphose, they acquire zooxanthellae and changes in nutritional requirements are expected (Boidron-Métairon, 1995). For example, findings from Grice & Bell (1998) indicated that ammonium input prior to 5 mm size resulted in negative growth of giant clams, but increased growth of clams in their later life. In the present study, the number of live *T. squamosa* veliger larvae was significantly greater when fed with a mixture of *T. suecica* + *C. mulleri* + yeast during the first 24 h; but there were no significant effects of diet on live larvae numbers at 36 h and 48 h. Earlier work has reported that quantity and quality of micro-algae lipid composition are essential in optimizing bivalves' larval growth (e.g. Brown, 2002). The ability of bivalves to synthesize essential fatty acids (e.g. eicosapentanoic, EPA, arachidonic, ARA, and docosahexaenoic, DHA, acids) is limited; therefore, higher lipid compositions in micro-algal diets are associated with faster development of larvae (Soudant *et al*., 2000).

Generally, bivalves fed with a mixed-algal diet exhibit higher survival rates than those fed a unialgal diet as the former gives a better balance of nutrients and mitigates against differences in digestibility (Webb & Chu, 1983; Rico-Villa *et al.*, 2006). The size of micro-algae may also affect the ease of filtering by bivalve larvae (Boidron-Métairon, 1995), for example, mussel and scallop larvae naturally feeding on small (<10 µm) phytoplankton (Raby *et al.*, 1997). *Chaetoceros mulleri* is characterized by a high proportion of EPA (20:5n-3) with low levels of ARA (20:4n-6) and DHA (22:6n-3), and *T. suecica* is characterized by a lack of DHA and low levels of ARA and EPA (Rivero-Rodríguez *et al.*, 2007). As yeast contained higher protein levels, the addition of yeast enhances nutritional value of the diet (Coutteau *et al.*, 1994; Brown *et al.*, 1996). As all three polyunsaturated fatty acids are necessary to improve survival of bivalve larvae, the combination of *C. mulleri* and *T. suecica* with yeast appears to be beneficial for the very early stages of larval development and growth in *T. squamosa* but beyond 24 h, based on a qualitative interpretation of Fig. 1, a diet of just *C. mulleri* and *T. suecica* may be sufficient to increase trochophore survival.

Testing the combined effects of temperature and salinity on survival provides a better indication of larval responses to environmental conditions than examining their effects separately (Kinne, 1964). We found significant differences in *T. squamosa* larval survival between the two temperature regimes tested. During the initial stages of fertilization at ~29.5°C, the high percentage of live embryos could be due to the relatively large proportion of successful egg-sperm contacts that can be attributed to the high kinetic energies of eggs and sperm, which are temperature-dependent (Styan, 1998). However, exposure to ~29.5°C resulted in almost total mortality at 24 h, indicating that *T. squamosa* trochophores were less tolerant to high temperatures. Raised temperatures are known to improve the initial fertilization process, but lower temperature enhances survival. Manipulative studies by Manoj Nair & Appukuttan (2003) confirmed that the thermal optimum for *Perna viridis* larval development, growth and survival occurred at 31ºC, but total mortality was reported after 24 h exposure to 33ºC and 35ºC. Development of *T. squamosa* trochophores in cultured environments was greater at ~22.5°C, similar to findings of studies on the Asian date mussel, *Musculista senhousia* (Semenikhina *et al.*, 2008). Even though survival rate and development of *T. gigas* larvae are known to decrease with reduced salinities (20‰ and 25‰) (Blidberg, 2004), no such pattern was found here as salinity had no significant effect on the number of living embryos. However, the survival of trochophores was higher at 27‰ than at 30‰, suggesting *T. squamosa* embryos may have some degree of salinity tolerance, but not the trochophores.

Populations of giant clams are declining across the Indo-Pacific (Lucas, 1994) and more conservation efforts are necessary to ensure the survival of these charismatic animals. Although there have been successful cases of giant clam farming (Gomez & Mingoa-Licuanan, 2006), better techniques for mariculture can further benefit both commercial and conservation endeavours. The findings presented here highlight the importance of providing micro-algal diet to improve veliger survivorship, and emphasise the role of temperature in enhancing larval initiation and development. In summary, the fertilization and development of *T. squamosa* can be enhanced by providing a mixed-algal diet and ensuring that water temperature is approximately 22.5°C.

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