The correlation of attributes of egg source with growth, shape, survival and development in larvae of the temperate sea cucumber *Australostichopus mollis*

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Abstract

The proportion of larvae completing the larval cycle and contributing to subsequent generations can be affected by earlier impacts on egg source or maternal origin. Consequently, during the larval cycle a suite of larval attributes that are impacted on exogenously may be correlated with attributes of egg source. For the temperate sea cucumber *Australostichopus mollis* egg size and egg numbers from four females were correlated with an index of larval viability, which was the proportion surviving, multiplied by the proportion in late auricularia. This survival viability index for larvae from each female was weakly associated with the mean number of hyaline spheres appearing in the folds of the ciliated band. Furthermore, for larvae from each female the ciliated band length to larval length ratio was correlated with the proportion of embryos with features characteristic of normal development, a measure of reproductive success. Although overall shape did not differ between larvae from different females the rate of change in larval shape did. However, growth and development did not differ between larvae from different females and were not correlated with egg numbers and egg size. Growth and development appeared decoupled from any effects of egg source and its association with reproductive success, the rate of change in shape characteristics, or the survival viability index. Larvae that appear competent as expressed in phenotype are not necessarily representative of attributes related to egg source.

Introduction

In aspidochirote sea cucumbers, relative to the number of gametes produced during spawning there is considerable variation in the numbers of larvae surviving the entire larval period and being competent to complete the larval cycle (James et al. 1994; Ito 1995; Ramofafia et al. 1995; Martinez and Richmond 1998; Battaglene et al. 1999; Mercier et al. 2000; Morgan 2000; Asha and Muthiah 2002; James 2004; Mercier et al. 2004; Pitt et al. 2004; Wang and Chen 2004; Sui 2004; Liu et al. 2004; Giraspy and Ivy 2005, 2006; Laximinarayana 2005; Morgan 2008a, b and c). This may occur because of the interaction between attributes related to fertilisation, reproductive success and maternal origin or egg source, and its subsequent impact on larval growth, shape, survival and development.

Variation in size of larvae may be related to the food source of parents and its impact on egg quality and subsequent growth during the larval cycle. In earlier studies egg size was correlated with larval size in *Strongylocentrotus droebachensis* and a number of other echinoderms (McEdward 1986). For instance, in *S. droebachensis* larger eggs have been correlated with animals from a food-rich site, resulting in larvae that increased in size (Bertram and Strathmann 1998). Conversely, in another study no relationship was found between egg size and food rations in captivity for *S. droebachensis* and no difference in larval size was observed (Meidel et al. 1999). The allocation of resources to reproduction does not necessarily correlate with changes in egg size (McEdward and Carson 1987) as individuals may produce more, smaller eggs.

Variation in the shape of larvae has also been related to egg size and implicated as reflecting differences in maternal origin. Variation of larval shape in echinoderms such as the sea urchins *Arbacia lixula* and *S. droebachensis* and the sea star *Pisaster ochraceus* has been related to maternal origin (George et al. 1990; Bertram and Strathmann 1998; George 1999; Meidel et al. 1999). However, for *S. droebachensis* variation in shape as a result of maternal origin was small compared to developmental plasticity in response to food availability (Bertram and Strathmann 1998). Changes in the shape of larvae in relation to maternal origin have also been related to differences in egg quality, either due to different diets in captivity or different habi-

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tats *in situ* (McEdward and Carson 1987; George et al. 1990; George 1999). However, egg size was not correlated with larval shape and changes in ciliated band length in *S. droebachensis* and a number of other echinoderms (McEdward 1986).

Variation in survival and the viability of larvae may reflect differences in maternal origin and reproductive success. Recent studies have determined that fertilisation history affects reproductive success or 'the fraction of a female's eggs that are fertilised' (Levitan 2005). For the sea urchin *Strongylocentrotus franciscanus* differences between sexes in the intensity of sexual selection were dependant on mate density. Despite multiple paternities the variance in reproductive success was lower in males and higher in females (Levitan 2005). In the sea urchins *Heliocidaris erythrogramma* and *Holopneustes purpurescens* mating order influenced the quantity and quality of offspring sired by competing males (Marshall et al. 2004). Differences in egg size between females and its relationship with fertilisation meant that fertilisation history influenced the size distribution of offspring. First spawning males produced larger fitter offspring.

Variation in the development of larvae has also been correlated with differences in egg size and related to differences in maternal origin. For instance, in *S. droebachensis* smaller eggs resulted in delayed development during early larval stages (Sinervo and McEdward 1988), while in another study on *S. droebachensis* larger eggs were correlated with larvae that had a shorter larval period even though development was not affected (Bertram and Strathmann 1998). In *S. droebachensis* the rate of metamorphosis was faster and larvae metamorphosed sooner when originating from parents fed an enriched diet even though there was no effect on growth rate or size at metamorphosis (Meidel et al. 1999).

Previous studies on the effects of egg source on larval growth, shape, survival and development and its implications for larval competency in sea cucumbers are limited. The period of time in captivity has been implicated in affecting egg quality in the sea cucumber *Holothuria scabra* because of a change in broodstock condition (Morgan 2000). Hatch rates and numbers of eggs spawned from *H. scabra* were reduced for broodstock kept in captivity for extended periods of time (Morgan 2000). However, subsequent effects on larval growth and development were not quantified. The size and shape of larvae from *Actinopyga echinites* has been found to differ between ditheothreitol-induced (DTT-induced) shedding of oocytes and resulting larvae, which were smaller, and ovary-induced shedding of oocytes and resulting larvae (Chen et al. 1991). DTT-induced larvae in the mid- to late auricularia larval stages were morphologically different, having an asymmetrical shape (Chen et al. 1991). Without determining the relationship between suites of larval attributes and egg source it is difficult to determine the contribution of parentage to the ability of larvae to complete the larval cycle.

The reproductive season of *Australostichopus mollis* occurs over a period of approximately four months, in summer from November to February (Sewell 1992; Archer 1996; Morgan 2008a). It is hypothesised that for individual females the quality of embryos is related to attributes of egg source such as egg numbers and egg size, which is then expressed as variation in larval phenotype. Changes in growth, shape, survival and development of larvae should relate to egg source and affect the ability of larvae to complete the larval cycle. Three questions were posed in this study: (i) Was there a relationship between egg source and the growth and shape of larvae? (ii) Were larval survival and development related to differences in egg source? (iii) Was there any relationship between the appearance of hyaline spheres prior to metamorphosis and egg source?

Methods

Larval source

Larvae of *Australostichopus mollis* were obtained from broodstock collected in the field once every two weeks and spawned in the hatchery (Morgan 2008a). Spawned eggs from four females were kept separate and fertilised with freshly spawned sperm mixed together from four males. Fertilised eggs were then rinsed and left in a 40 L tub of 1 µm filtered UV-sterilised seawater to hatch (Morgan 2008b). Attributes of egg source were measured and included broodstock weight, egg size from biopsies prior to spawning, numbers of eggs spawned, and the proportion of embryos developing with features characteristic of 'normal' development (see Morgan 2008b). Approximately 1000 ± 100 'normal' early auricularia larvae were counted out for each jar three days after fertilisation. Larvae were concentrated in a 60 mm mesh sieve for counting and used in experiments at an initial concentration of approximately 1 larva ml^{-1}. Larval age was subsequently referred to as time from first feeding.

Larval culture

A windscreen wiper motor was used in conjunction with a variable voltage transformer (1 to 12 volts) to power a paddle stirrer. Glass preserving jars containing 1 L of 1 µm filtered UV-sterilised seawater were used for experiments. Experiments were conducted in a light dark cycle (L:D) of 16:8 hours at a temperature of 20 ± 1°C. The diatom *Chaetoceros muelleri* was cultured in Gillard's F2 medium and

during log phase growth fed to larvae at 2000 cells ml^{-1} day^{-1} once a day. There were three replicate jars for larvae from each female.

Every second day 90% of the seawater was siphoned out of each jar using a siphon hose and a 60 mm mesh cup to prevent larvae being extracted. Larvae were removed for morphological measurements by pipetting 5 to 10 mL of seawater containing 15 to 40 larvae into a petri dish. The remaining larvae contained in the seawater in the jars were then drained into a clean jar and 1 mm UV-sterilised seawater added to fill the jar up to 1 l.

Larval growth and development

Larval development was defined by the development of the left and right somatocoel and the axohydrocoel (Smiley 1986; Archer 1996; Morgan 2001; Sewell and McEuen 2002; Morgan 2008c). On days 1, 3, 7, 12, 18 and 22 from first feeding (three days post fertilisation) 10 larvae were measured for growth and development from the 15 to 40 removed from each jar. Measurements were made using a compound microscope and eyepiece micrometer at ´100 magnification. Total length, width, posterior length (post-oral hood length), posterior gut (posterior end to the front of the gut), lateral maximum (posterior end to maximum width), lateral depth (maximum depth of the posterior fold), mouth length, mouth width, gut length, gut width, left somatocoel length, and axohydrocoel length were measured and the number of hyaline spheres counted (see Morgan 2008c).

Data analysis

Changes in total length of larvae from each female over successive days were analysed for each experiment. A Tukeys test for a significant difference between means with the Kramer adjustment for multiple comparisons was used to compare means. A one-way analysis of variance was used for the total numbers of surviving larvae counted in each jar at the end of each experiment. Counts were corrected for removal of larvae for measurements. An index of larval viability was also calculated by multiplying the proportion surviving by the proportion in late auricularia. This gave a value for the proportion of larvae likely to complete the larval cycle.

Mixed model analysis of variance in SAS version 8 was used for morphometric variables measured for larvae in each experiment. Effects tested were FEMALE, FEMALE x DAY, REPLICATE within (FEMALE) and DAY x REPLICATE within (FEMALE). Ratios of morphometric variables were analysed simultaneously by reducing the 9 morphometric ratios to their principal components using principal component analysis (PCA) (see Morgan 2008c). Analysis of variance was performed on the first principal component.

Scale-free length ratios were used, as the first principal component in size dependant analysis represents an unknown number of allometrically related shape variables (James and McCulloch 1990; George 1999; Morgan 2008c). Log transformations of length ratio data did not significantly improve normality so the original data was adhered to. For the PCA of length ratios least squares (LS) means were used to compare differences between treatments across days in experiments.

The length of the ciliated band of larvae was calculated for larvae from each female. For each female, four larvae were photographed using a digital camera mounted on a compound microscope. Images were then analysed using SigmaScan Pro software (see Morgan 2008c). The ratio of ciliated band length to all length variables for larvae that were photographed was then compared between females using PCA analysis on day 12.

Discriminant function analysis was used to examine all morphometric variables simultaneously for length ratio and ciliated band length ratio data to determine variables that best reflected differences between larvae from different females. This is used to estimate randomly how efficiently larvae could be discriminated as having come from different females. For each experiment a discriminant function was calculated and each individual larva classified as coming from a particular female.

A log-linear model was used to quantify differences in the numbers of larvae in different stages of development between females. A three-way contingency table was constructed where treatments were FEMALE, DAY (1, 3, 7, 12, 18 and 22) and STAGE (early, mid-, late auricularia). Exclusion of the term 'DAY' in the model would mean accounting for only the summed differences in stages across females in experiments. No information on variation through time could be determined for each stage using this approach.

Results

Larval attributes and egg source

Females with a higher proportion of viable embryos ('normal'), having features characteristic of development, were correlated (0.50) on day 12 with larvae that had a longer ciliated band relative to total larval length. For females F1 and F2, 90% and 80% of embryos respectively were considered to have features characteristic of normal development compared to 65% for F3 and F4.

Larvae with a higher survival viability index were correlated with females that produced more eggs (0.64) and had a larger egg size (0.80). Females F1 and F2 produced 195,000 ± 40,100 and 88,000 ± 20,600 eggs respectively compared to 31,000 ± 1,900 and 62,000 ± 15,300 from F3 and F4 (mean ± SE). In F1 and F2 egg size was 120 ± 17 and 124 ± 23 µm respectively compared to 101 ± 17 and 111 ± 20 µm for F3 and F4 (mean ± SD). No difference in wet weight of broodstock existed for larvae from different females ($p > 0.05$).

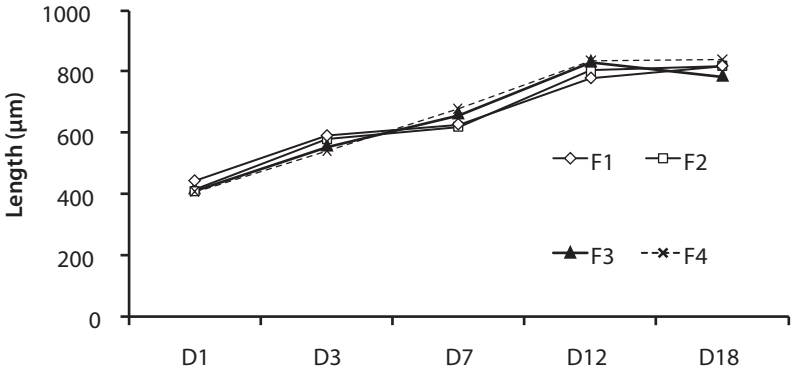

Figure 1. Growth in total length (µm; mean ± SE) of larvae from females where day = day 1 from first feeding (larval age = plus 3 days). F1, F2, F3 and F4 = female number. Larvae were fed 2000 cells ml^{-1} day^{-1} of the diatom *Chaetoceros muelleri*.

Larval growth

Larvae from all four females grew at a similar rate through to day 18, reaching a maximum of 841 ± 22 µm for F3 and a minimum of 781 ± 28 µm (mean ± SE) in F4 ($p > 0.05$; Fig. 1). Larvae on days 7, 12 and 18 were longer than larvae on day 3 and larvae on days 12 and 18 were longer than larvae on day 7 ($p < 0.01$; Tukey-Kramer).

Larval shape

Principal component 1 (PCA1) of length ratio variables accounted for 30% of variation in the data. Differences between females were attributed to the ratios of length to gut length and mouth length to mouth width in PCA1 and gut length to gut width and gut length to lateral maximum in PCA2. Discriminant function analysis showed there was only a 25% to 50% chance of correctly identifying a larva when chosen at random as belonging to any particular female. For PCA1 overall there was no difference in shape characteristics ($p > 0.01$; Fig. 2). However, the rate of change in shape differed as larvae from F1 and F3 tended to retain shorter more rounded guts and mouths for longer, relative to other larval structures as time progressed ($p < 0.01$; LS means). Larvae from F2 and F4 tended to have longer, narrower guts and mouths earlier and were wider and had more lateral folding in the ciliated band earlier.

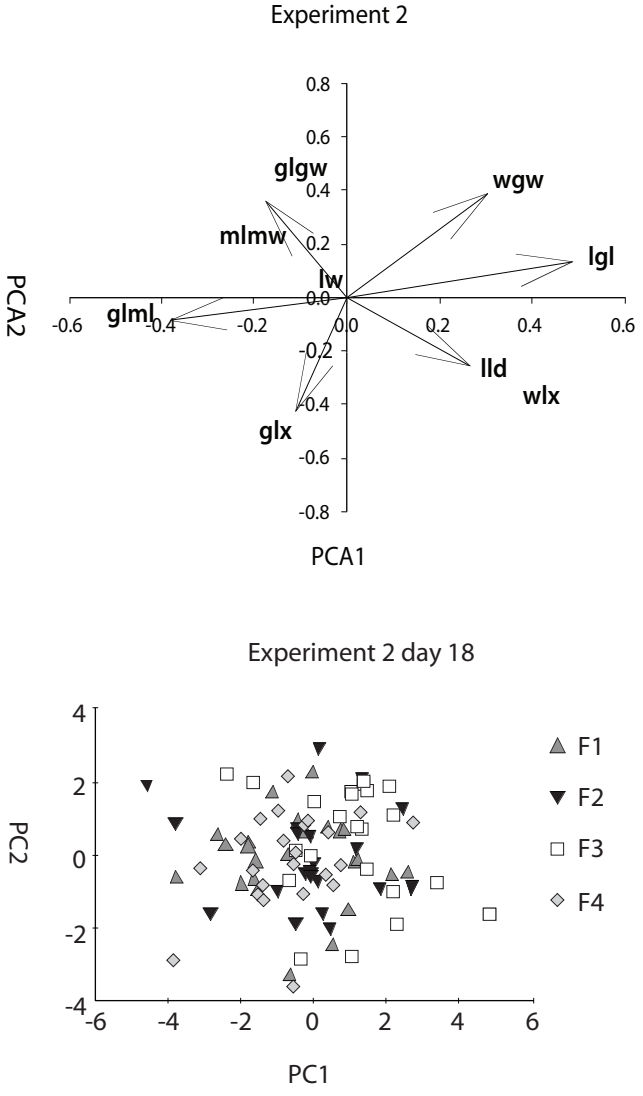

Figure 2. Principal components analysis (PCA) plot of length ratio variables. lw = length/width; glgw = gut length/gut width ; mlmw = mouth length/mouth width; lgl = length/gut length; wgw = width/gut width; glml = gut length/mouth length; glx = gut length/lateral max; wlx = width/lateral max; lld = length/lateral depth.

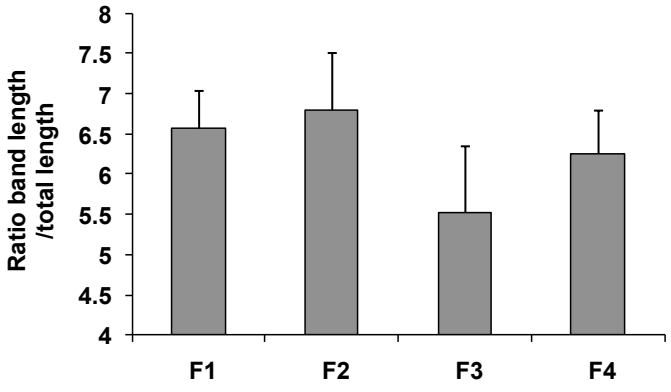

Figure 3. Ratio of ciliated band length to total length for larvae from females F1, F2, F3 and F4 on day 12 (mean ± SE; n = 4 larvae per female).

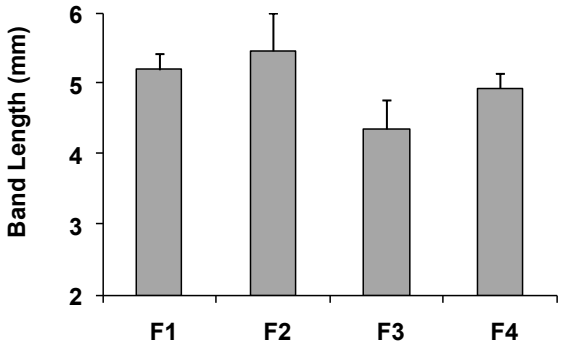

Figure 4. Ciliated band length for larvae from females F1, F2, F3 and F4 on day 12 (mean ± SE; n = 4 larvae per female).

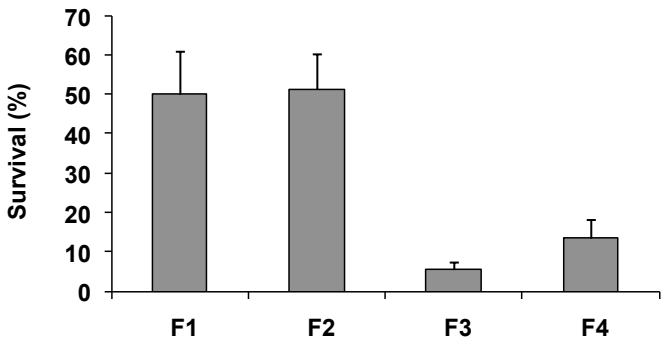

Figure 5. Survival (%) of larvae from females F1, F2, F3 and F4 (mean ± SE) fed 2000 cells ml^{-1} day^{-1} of the diatom *Chaetoceros muelleri*.

For ciliated band length ratios PCA1 accounted for 83% of variation between females. Most of the variation was explained by changes in band length to larval length and band length to larval width, similar to the weighting of variables contributing to treatment effects observed for length ratios. When all ciliated band length ratio variables were considered simultaneously there was only a 0% to 12.5% chance of incorrectly identifying larvae as belonging to a female other than that from which it originated. PCA1 of band length ratios differed between females ($p < 0.01$; LS means; Fig. 3). Overall, larvae from females F1 and F2 had a total band length of 5.2 ± 0.23 and 5.5 ± 0.54 respectively while larvae from F3 and F4 had a total band length of 4.4 ± 0.41 and 4.9 ± 0.21 (mean ± SE) respectively ($p < 0.01$; Fig. 4).

Survival and larval viability

The highest survival was recorded in larvae from females F1 and F2 at 50.0% ± 11% and 51.7% ± 9% (mean ± SE) respectively compared to 5.5% ± 2% and 13.5% ± 4.7% for F3 and F4 respectively ($p < 0.01$; Tukeys; Fig. 5). However, larvae less likely to reach late auricularia and successfully metamorphose may survive long periods of time in cultures. Survival was adjusted for numbers of larvae reaching late auricularia by multiplying the proportion surviving by the proportion in late auricularia to give an index of larval viability or larval success. For F1 and F2, 40% and 54% respectively were likely to complete metamorphosis compared to 3.6% and 11% for F3 and F4.

Development

Overall there was no difference in development between larvae from different females ($Chi^2 > 0.01$). The mid-auricularia stage appeared on day 7 and there was a relatively low proportion of mid-auricularia occurring from day 7 to day 12 (Fig. 6). A large number of remaining larvae were late auricularia by day 18 for all females. However, the rate of development differed between larvae from different females across days ($Chi^2 < 0.01$). Furthermore, for late auricularia the number of hyaline spheres in the folds of the ciliated band did not differ between females ($p > 0.05$). This was also reflected in the proportion of remaining larvae that were metamorphosing (Fig. 7). For larvae from females F3 and F4 the rate of transition to late auricularia over day 12

was faster and on day 22 a higher proportion was observed metamorphosing sooner ($p < 0.01$).

Discussion

Larval growth and shape

No correlation was found between growth and egg source for larvae from different females. Furthermore, differences in shape characteristics between larvae obtained from different females were limited. However, band length to larval length ratios were distinguished between larvae from different females. Ratios were also correlated with the proportion of embryos that had features characteristic of 'normal' development. The use of ciliated band length to length ratios to determine differences between larvae from different females and their relationship to early development and attributes of egg source may have some merit and requires further investigation.

Other studies have found inconsistencies in the relationship between egg size and larval shape. Starved larvae from small eggs of *Pisaster ochraceus* were wider than starved or fed larvae from large eggs, representing an increase in the length of the ciliated band (George 1999). Larvae of *Strongylocentrotus droebachensis* were larger in high food rations for eggs originating from low broodstock rations in captivity even though no difference in egg size was found (Meidel et al. 1999).

Alternatively, larvae from *S. droebachensis* originating from large eggs at a shallow food-rich site were larger in both high and low food rations but the trend was more evident in low food rations (Bertram and Strathmann 1998). However, both size and shape of larvae from *S. droebachensis*, *S. purpuratus*, *S. franciscanus*, *Dendraster excentricus*, *Heterocentrotus mammillatus*, *Colobocentrotus atratus* and *Tripneustes gratilla* were not correlated with egg size (McEdward 1986).

Survival and larval viability

The survival viability index used as a measure of larval competence was correlated with both egg size and egg numbers, distinguishing between larvae from different females. Effects of maternal nutrition on eggs and offspring are common even for animals that lack parental care and include performance and survival (Bertram and Strathmann 1998). Survival, measured as the ability to complete the larval cycle relative to the number of larvae in the late auricularia stage, was higher for females that produced more eggs with a larger egg size. Furthermore, this viability index was weakly associated with the mean number of spheres in the folds of the ciliated band prior to metamorphosis.

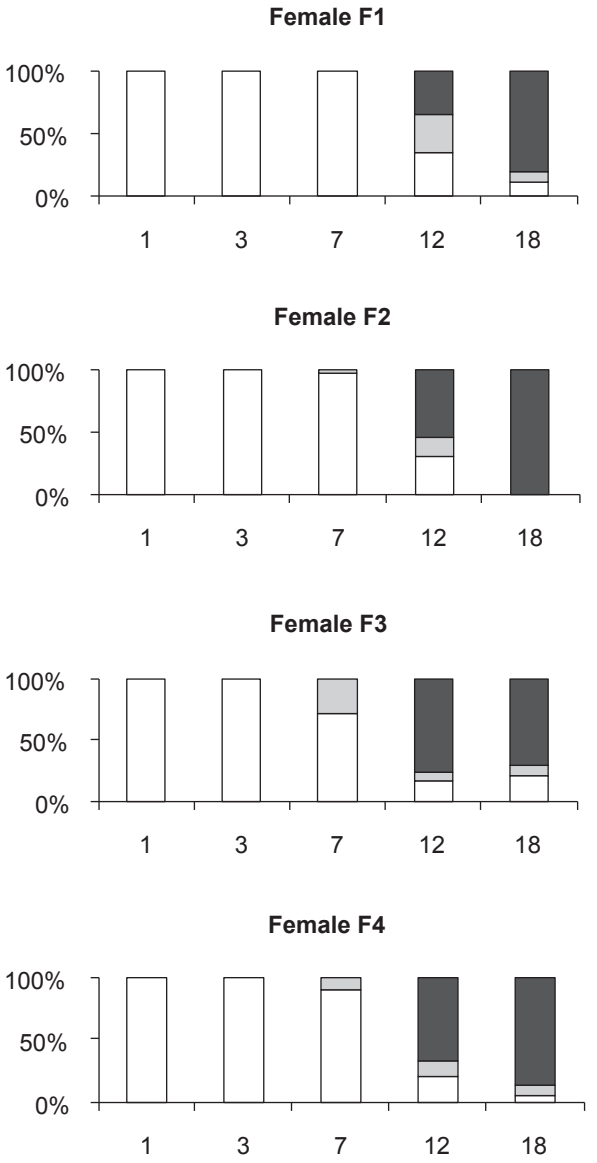

Figure 6. Summary of development of larvae from different females where day 1 = day 1 from first feeding (larval age = plus 3 days). X axis = days; Y axis = proportion of larvae in each stage (%). White = early; Grey = mid-; Black = late auricularia larval stage.

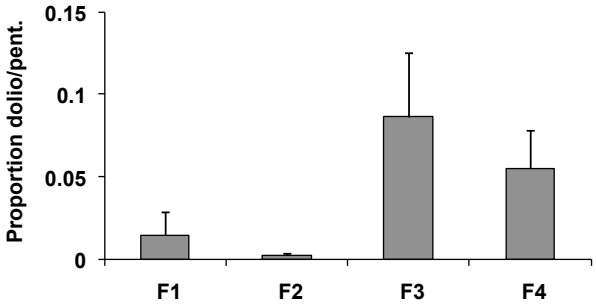

Figure 7. Proportion of surviving larvae on day 22 from females F1, F2, F3 and F4 that were either doliolaria or pentacula (mean ± SE).

Incorporation of a survival viability index in addition to measures of reproductive success (see Levitan 2005) is a way of determining downstream effects on the contribution of parentage (Marshall et al. 2004; Evans and Marshall 2005) to the fraction of eggs fertilised. A change in egg size has been correlated with larval viability in the sea urchins *Strongylocentrotus droebachensis* and *S. purpuratus* (Sinervo and McEdward 1988). When a decrease in egg size resulted in smaller larvae with simpler body forms, there was slower development through early feeding, and the effects of egg size were restricted to the early larval stages (Sinervo and McEdward 1988).

Larval development

No overall difference in development was observed for larvae from different females but the rate at which development proceeded differed. However, as with growth, development appeared decoupled from any effects of maternal origin and was not related to attributes of egg source. For example larvae from female F4 developed at a similar rate as those from F2 yet F4 had a significantly reduced survival viability index, numbers of eggs, and egg size. A direct relationship does not always exist between egg content and/or egg size and larval development as was found for the starfish *Solaster stimpsoni* and in other studies on the sea urchin *S. droebachensis* (McEdward and Carson 1987; Bertram and Strathmann 1998; Meidel et al. 1999). In high food rations for broodstock of *Strongylocentrotus droebachensis* the rate of larval metamorphosis was higher and larvae metamorphosed sooner (Meidel et al. 1999).

In another study the strong correlation of larval development of the seastar *Pisaster ochraceus* with maternal origin could only be distinguished during the middle phase of the larval cycle (George 1999). In the present study the rate of transition to late auricularia was reflected in a higher proportion metamorphosing sooner for larvae from different females. However, the appearance of hyaline spheres in larvae had limited correlation with attributes of egg source. Furthermore, there was no difference in numbers of hyaline spheres between larvae from different females.

Previous studies have determined that these hyaline spheres were not essential for metamorphosis as larvae have been observed settling and surviving subsequent development without them (Smiley 1986; McEuen and Chia 1991; Dautov and Kashenko 1995; Dautov 1997; Sewell and McEuen 2002). However, hyaline spheres may be needed to fuel metamorphosis by providing a reservoir to meet the structural requirements of morphogenic changes during metamorphosis, increasing the probability of survivorship post-settlement (see Sewell and McEuen 2002). Consequently, the rate of larval stage transition and the relationship between mean number of spheres and the survival viability index may act in synergy, reflecting differences in the contribution of egg source to the larval life cycle.

Conclusion

In conclusion early development in embryos appeared related to later differences in ciliated band length to larval length ratios for larvae from different females. Furthermore, the larval survival viability index appeared correlated with egg source, represented by egg numbers and egg size. However, attributes of egg source appeared decoupled from subsequent growth and development. A limited correlation between attributes of egg source and phenotype existed for larvae from different females. These attributes should be examined further to determine causal relationships.

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