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ORIGINAL PAPER

Maternal provisioning for larvae and larval provisioning for juveniles in the toxopneustid sea urchin *Tripneustes gratilla*

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Abstract Lipid and protein biochemistry of eggs (84 µm in diameter), embryos and early larvae of the tropical echinoid *Tripneustes gratilla* (Linnaeus 1758) were quantified to determine how maternal provisions are used to fuel development of the echinopluteus. The eggs contained a mean of 30.82 ng lipid and 87.32 ng protein. Energetic lipids were the major lipid component (55.52% of total lipid) with the major class being triglyceride (TG: mean 15.9 ng, 51.58% of total). Structural lipid was dominated by phospholipid (PL: mean 11.18 ng, 36.26% of total). Early embryogenesis was not a major drain on egg energetic lipid and protein. Development of the functional feeding larva used ca. 50% of initial egg energetic lipid and most of this was TG. Maternal TG was still present in the 8-day echinoplutei and it was estimated that this energetic lipid would be depleted in unfed larvae by day 10. There was no change in PL. In a separate experiment lipid biochemistry of rudiment stage larvae and early developing juveniles were quantified to determine how lipids are used during metamorphosis. Fed larvae accumulated lipid (mean 275.49 ng) with TG and PL being the major energetic and structural lipids, respectively. Larval lipid stores were not appreciably depleted by metamorphosis and so were available for the early benthic stage juvenile. Juveniles started their benthic existence with 314 ng total lipid (TG: mean 46.84 ng, 14.9% of total, PL: mean 137.51 ng, 43.67% of total). Nile Red histochemistry and histology showed that the stomach serves as a nutrient storage organ and, that lipid stores accrued by larvae sustain developing juveniles for up to 4 days post settlement. Triglyceride supported both non-feeding stages of development and the prefeeding larval and perimetamorphic benthic stage. In this first study of lipid stores in settlement stage echinoderm larvae, we show that *T. gratilla* larvae sequester the same major energetic lipid (TG) to support the early juvenile that the female parent provided them to fuel early development.

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Introduction

The larvae of marine invertebrates initiate their development supported by a quotient of nutrients provided by the egg. In species with feeding (planktotrophic) larvae these nutrients fuel development of the functional feeding larva. Differentiation of the digestive tract, enzyme systems and ciliary feeding apparatus are required to achieve the feeding stage and metamorphosis depends on the energy gleaned from planktonic food (Gallager et al. 1986; Strathmann et al. 1992; Pernet et al. 2004, 2006). The duration of the facultative feeding period, the period during which larvae

can develop without exogenous food, varies among species and with the availability of maternal provisions (Miner et al. 2005; Byrne et al. 2008). Duration of development and larval condition are strongly influenced by environmental conditions (Fenaux et al. 1994; Miller and Emlet 1999; Schiopu et al. 2006). In favourable nutritive conditions development time is shortened, reducing the duration of the vulnerable planktonic stage during which mortality is high (López et al. 1998; Lamare and Barker 1999). In addition to supporting the planktonic stage, nutrients accrued by larvae fuel metamorphosis and development of the early benthic juvenile (Fenaux et al. 1985; George et al. 1990, 1997; Pechenik et al. 1998; Moran and Emlet 2001; Emlet and Sadro 2006; Schiopu et al. 2006). The size, growth and performance of early juveniles are strongly influenced by the nutrient stores sequestered by the larvae (Miller and Emlet 1999; Vaïtilingon et al. 2001; Emlet and Sadro 2006; Pechenik 2006; Pernet et al. 2006). Thus, larval experience affects metamorphic success and juvenile performance.

Per-offspring maternal investment is an integral part of life-history theory with a plethora of models developed to examine the relationship between egg energy and the production and quality of offspring (Vance 1973; Marshall and Keough 2006; McEdward and Miner 2006). Despite the intense interest in the tradeoffs between egg size, fecundity, mortality and planktonic duration, empirical data on how maternal provisions are used in development and the energetics of metamorphosis and the early juvenile are scarce. Many echinoderms and molluscs use egg triglyceride as the major energy lipid to fuel larval development (Podolsky et al. 1994; Jaeckle 1995; Sewell and Manahan 2001; Villinski et al. 2002; Moran and Manahan 2003; Sewell 2005; Falkner et al. 2006; Meyer et al. 2007; Prowse et al. 2008). Triglycerides are metabolised during prefeeding development, while egg structural lipids (e.g. phospholipid) and protein remain relatively stable as these nutrients are used as materials to construct the larval body (George et al. 1997; Sewell 2005; Meyer et al. 2007; Prowse et al. 2008). Carbohydrates are a minor energetic component (1–3%) of echinoderm eggs (Jaeckle 1995).

For echinoderms, data on lipid class content egg^{-1} are available for two echinoids, one ophiuroid and two asteroids with planktotrophic larvae, through the use of thin layer chromatography-flame ionisation detection (TLC-FID) (Podolsky et al. 1994; Sewell 2005; Falkner et al. 2006; Meyer et al. 2007; Prowse et al. 2008). This method allows class specific quantification of energy storage and structural lipids (Parrish 1987, 1999). Here we present details of maternal provisioning in the pan tropical Indo-West Pacific echinoid *Tripneustes gratilla* using TLC-FID. A recent genetic study confirmed that this is a single panmictic species with one of the widest distributions known for the Echinoidea (Lessios et al. 2003).

Tripneustes species are ecologically important, especially in sea grass habitats and are a food source for island communities (Koike et al. 1987; Scheibling and Mladenov 1987; Juinio-Menez et al. 1998; Vaïtilingon et al. 2003; Lawrence and Agatsuma 2007). *T. gratilla* is a boom and bust species with short-lived annual populations (Ebert 1982; Dafni 1992; Lawrence and Agatsuma 2007). The rapid growth (60 mm in diameter in 5 months) of *T. gratilla* has prompted interest in its energetics and potential for aquaculture (Vaïtilingon 2004; Lawrence and Bazhin 1998; Dworjanyn et al. 2007; Dworjanyn and Pirozzi 2008).

Tripneustes gratilla spawns small eggs (85–90 µm in diameter) and, depending on rearing temperature, and food supply, development takes15–52 days (Chen and Run 1988; Juinio-Menez et al. 1998; Shimabukuro 1991; Vaïtilingon et al. 2005; Dworjanyn and Pirozzi 2008). To determine how nutrients are used in development, we quantified the egg nutrients (lipid classes and protein) available to the pre-feeding embryos of *T. gratilla*, the utilisation of egg reserves as development proceeded to the echinopluteus, and the lipid content of settlement stage larvae and juveniles. In separate larval rearing experiments we investigated two ontogenetic time periods: (1) early development to the well-developed 4-arm larval stage and, (2) late development encompassing the rudiment stage larva, metamorphosis and the early developing juvenile. This is the first study of stored lipid in settlement stage echinoplutei and utilisation of these lipids through metamorphosis to the early juvenile. We compared the nature of lipid reserves provided by the maternal parent to the developing larva and those provided by the larva to the developing juvenile.

Materials and methods

Specimen collection and egg samples

Tripneustes gratilla were collected from Camp Cove (33°50′S; 151°16′E), South West Rocks (30°53′S; 153°02′E) and Port Stephens (32°41.40′S; 152°03′E), New South Wales, Australia in November–May, 2003–2004. They were induced to spawn by injection of 1–2 ml of 0.5 M KCl. Egg diameters ($n = 30$) from one female were calculated from digital images using Image J software (NIH). Replicate egg samples for lipid ($n = 3$) and protein ($n = 3$) analyses were collected from each of the four females. Eggs were placed in microcentrifuge tubes, centrifuged briefly and seawater was removed with a pipette. Samples were placed on ice and stored at −80°C until analysis. Egg numbers were determined by egg counts in aliquots of an egg suspension with known concentrations. Samples for lipid and protein analysis contained 700–800 and 2,000 eggs, respectively.

Fertilisation and development

Experiment 1 investigated the dynamics of maternal lipid utilisation to the initial functional feeding larva (4 days) and well-developed feeding larva (8 days). One male and one female were used to ensure that the larvae were full siblings as in previous studies (Phillips 2002; Miner and Vonesh 2004; Sewell et al. 2004). Therefore the results should not be influenced by selection for different maternal investment in different genotypes (Miles et al. 2007). The eggs were placed in filtered (1.0 µm) seawater (FSW) and fertilised (10^6 sperm $ml^{-1)}$ at 19–20°C. After a rinse in FSW to remove excess sperm they were examined to ensure that at least 90% were fertilised.

The embryos were placed in 3 × 500 ml beakers with FSW and on day 3 were allocated to 3 × 20 l cultures with and aeration using air pumps in series. They were reared at 19–20°C at initial densities of 5 larvae ml^{-1} and were not fed. This density was required to have sufficient numbers of larvae for repeated sampling for lipid and protein analysis. The FSW was changed after hatching and at every sampling point. Two samples were collected as above from each culture on days 2, 3, 4, 5, 6, and 8 post-fertilisation, one each for lipid (800 larvae) and protein (2,000 larvae) analyses, and stored at −80°C prior to analysis. This culture was terminated on day 9.

Experiment 2 investigated the lipid content of rudiment stage larvae and early juveniles. *T. gratilla* larvae were reared at 25°C in an aerated 125 l tank (4 larvae ml^{-1}) to the rudiment stage. They were fed *Chaetoceros muelleri* initially 3 × 10^4 cells ml^{-1} increasing to 4 × 10^4 cells ml^{-1} at later stages. The majority of the FSW was changed daily with addition of food each time. At the rudiment stage (30 days), samples (n = 3) of 60 larvae were placed in tubes and stored at −80°C for lipid analysis. The remaining larvae were transferred to three 400 ml beakers with FSW to induce settlement and metamorphosis by introduction of scrapings of a natural biofilm developed on Perspex plates in flow-through tanks (Dworjanyn and Pirozzi 2008). The FSW in the beakers was exchanged by reverse filtration. To compare lipid use from the rudiment stage larva to the juvenile stage we sampled juveniles promptly after metamorphosis. Because metamorphosis was asynchronous, individual beakers did not provide enough juveniles at any one time for separate samples. As a result, juveniles were collected as they became available in all beakers using a glass pipette. Three samples of 25 juveniles were collected in tubes and stored at −80°C for lipid analysis.

Lipid extraction and analysis

Lipid was extracted from the frozen egg, embryo, larval and juvenile samples following Holland and Gabbott (1971) with minor modifications (Sewell 2005). Each sample was homogenised briefly (15 s) in ultra-pure milliQ water using a sonicator (Sanyo Soniprep 150). Lipids were obtained using a chloroform/methanol extraction protocol (Sewell 2005). The final chloroform layer containing the sample lipids was isolated, dried down in a stream of N_2 gas and redissolved in a known volume of chloroform. A ketone internal standard was added prior to extraction to estimate lipid recovery (Sewell 2005; Falkner et al. 2006, Prowse et al. 2008). Lipid classes were identified and quantified using the Iatroscan Mark V^{new} TLC/FID system and silica gel S-III Chromarods following the protocols of Parrish (1999). Portions (1–3 µl) of each sample were spotted on to two Chromarods and lipids separated chromatographically using a 3-stage development process (Parrish 1999). After each development, a portion of every Chromarod was analysed via hydrogen flame ionisation detection resulting in three chromatograms per Chromarod. Calibration curves (quadratic regressions) from serial dilutions of a composite lipid standard were used to quantify lipids per sample (Sewell 2005).

Protein extraction and quantification

Protein was extracted from the frozen egg and larval samples following the method of Imagawa et al. (2004) with modifications. The samples were thawed on ice and cooled lysis buffer (20 mM Tris-HCl; 130 mM NaCl; 5 mM EDTA) and 1% protease inhibitor and 1% Triton-X were added. The sample was homogenised with a probe sonicator for 15–20 s and incubated on ice for 15 min on a flat bed shaker. The sample was then centrifuged. The supernatant containing the extracted proteins (minus the overlying lipid layer) was retained and stored at −80°C until analysis. Two protein measurements were made for each sample using the Micro BCA protein Assay Kit (Pierce) following the manufacturer's instructions. Serial dilutions of bovine serum albumen (BSA) were used to construct a standard curve to determine total protein content in the samples. The samples were diluted in deionised water (1:60) and pipetted (150 µl) into a microplate well to which 150 µl of the working solution (Micro BCA Reagent A, B, C) was also added. The plate was briefly shaken and incubated at 60°C for 1 h. Protein content was assayed colorimetrically at 550 nm wavelength using a Bio-Rad plate reader. The average of the two measures was calculated for each sample.

Statistical analyses

The assumption of homogeneity of group variances of data from Experiment 1 was examined by plots of residuals against group means (Quinn and Keough 2002). Temporal changes in the major energetic lipid, triglyceride (TG), and

the major structural lipid, phospholipid (PL) larva^{-1} were examined with one-way analysis of variance (ANOVA) with sampling time as a fixed factor. We investigated depletion of egg TG and protein in early embryos with a planned comparison contrasting egg versus 2-day embryos. Linear regression analysis was used to estimate the time at which maternal TG reserves are exhausted in unfed *T. gratilla* larvae using data from the onset of feeding competence day 4 to day 8. Protein data on days 4 and 8 were examined in a planned contrast.

Larval histochemistry and histology

Whole mount histochemistry (Nile Red) and histology (plastic sections) were used to document lipid storage in feeding larvae, rudiment stage larvae, post-larvae and juveniles. The larvae used were from cultures separate from those used for biochemistry. For Nile Red histochemistry, larvae were reared for 3 weeks as described for Experiment 2 and lipid distribution was examined in 12-day and 21-day larvae ($n = 10$ per age). Unstained larvae were used as controls. Under blue light excitation, when bound to neutral lipids (Carman et al. 1991), this fluorochrome fluoresces bright yellow. To stain larvae, 5 µl of the Nile Red stock solution (1.0 mg ml^{-1} acetone) was added to 4 ml of FSW (1:800 dilution). Live larvae were placed in the solution in the dark for 1 h, rinsed in FSW and were photographed using an Olympus BX60 epifluorescence microscope using 488 nm excitation.

To determine the location of the lipid in larval and juvenile tissues we used archived plastic sections of *T. gratilla* larvae reared (60 l tanks, 1 larva ml^{-1}) at 24°C and fed *Phaeodactylum tricornutum* (6×10^4 cells ml^{-1}) at an aquaculture facility (Aqua-lab) in Toliara, Madagascar (see Vaïtilingon et al. 2003). Metamorphosis was induced using coralline algae. Rudiment stage larvae and juveniles, 1, 4 and 8 days post settlement, were fixed in 2.5% glutaraldehyde in FSW, dehydrated and embedded in plastic (Spurr's) resin. Sections (1.0 µm) were stained with toluidine blue and photographed using an Olympus DP10 digital camera.

Results

Egg lipid class and protein content

The eggs of *T. gratilla* (mean 84.4 µm in diameter, SE = 0.73) contained five lipid classes (Table 1, Fig. 1), two energy storage lipids: aliphatic hydrocarbon (AH) and triglyceride (TG) and three structural lipids: acetone-mobile polar lipids (AMPL), phospholipid (PL) and sterol (ST). Total lipid (mean 30.82 ng egg^{-1}) did not differ among the eggs from four females (ANOVA, $F_{(3,8)} = 0.96$, $P = 0.46$).

Table 1 Total lipid, lipid classes and protein in the eggs of *T gratilla*

Measurement	Mean (SE) (ng)	% of total lipid
Total lipid	30.82 (1.04)	100.00
Energetic lipids		
AH	1.22 (0.28)	3.95
TG	15.90 (0.55)	51.58
Structural lipids		
AMPL	1.52 (0.16)	4.92
PL	11.18 (0.58)	36.26
ST	1.01 (0.11)	3.29
Protein	87.32 (2.65)	

Means (SE) were calculated from lipid and protein data obtained for the eggs of four females. Energy storage lipids: *AH* aliphatic hydrocarbon, *TG* triglyceride; Structural lipids: *AMPL* acetone-mobile polar lipid, *PL* phospholipid, *ST* sterol

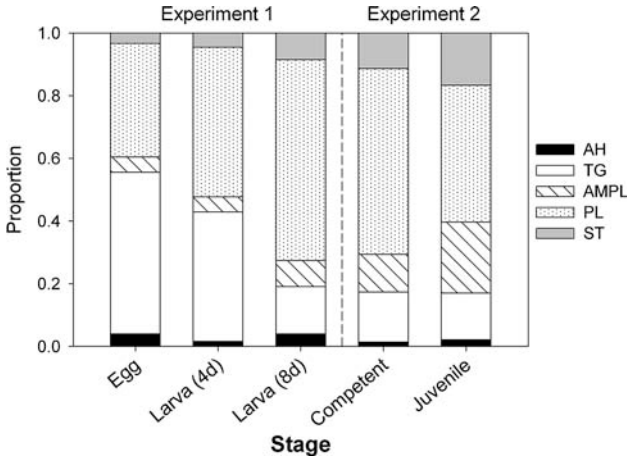

Fig. 1 Histograms showing proportion of the five lipid classes in eggs and unfed early 4-day and 8-day *T. gratilla* larvae (Experiment 1) and rudiment stage fed larvae and juveniles (Experiment 2). Triglyceride (TG) is used in development of the echinopluteus which is dominated by phospholipid (PL). *AH* aliphatic hydrocarbon, *AMPL* acetone-mobile polar lipid, *ST* sterol

Energy storage lipids dominated (55.58% of total) with TG being the most important energy lipid (Table 1, Fig. 1). Phospholipid was the major structural lipid (Table 1, Fig. 1). There were no differences between the TG (ANOVA, $F_{(3,8)} = 2.81$, $P = 0.11$) and PL (ANOVA, $F_{(3,8)} = 0.44$, $P = 0.73$) contents of the eggs from four females. The other lipids (AH, AMPL, ST) were minor components (Table 1, Fig. 1). The eggs of *T. gratilla* had a mean total protein content of 87.32 ng (Table 1) and this did not differ among females (ANOVA, $F_{(3,8)} = 0.96$, $P = 0.46$).

Development and histology

By 3.5 days the digestive tract and the ciliary band had differentiated and the larvae were competent to feed. Unfed larvae reared to day 9 had pale or transparent stomachs

(Fig. 2a–c), while in fed larvae, the stomach pigment increased with age (Fig. 2d). The stomach was dark brown in rudiment stage larvae (Fig. 2e).

The stomach wall of larvae stained with Nile Red fluoresced bright yellow under blue (488 nm wavelength) excitation (Fig. 2f), indicating the presence of neutral lipids. The body wall fluoresced orange-red indicating the presence of polar lipids that would have largely been in cell membranes. Unstained larvae were weakly fluorescent under blue light (data not shown). Lipid droplets with refractile optics were present in the stomach wall (Fig. 2d).

Sections of rudiment stage larvae show an abundance of lipid spheres in the stomach epithelium (Fig. 3a). These lipid stores were also present in the stomach of developing juveniles and were largely depleted by day 4 post settlement (Fig. 3b, c). There was no evidence of the lipid by day 8 post settlement (Fig. 3 d).

Experiment 1: use of maternal lipid and protein in development of the echinopluteus

Utilisation of egg lipid to the early functional feeding larval stage (4 days) and the well-developed 4-arm (8 days) echinopluteus is shown in Figs. 1, 4 and 5. Egg TG decreased during the experiment to day 8 (ANOVA, $F_{(6,14)} = 48.67$, $P < 0.001$; Figs. 1, 5), but not in pre-feeding embryos (egg-vs-2 d; $t_{14} = 0.78$, $P = 0.44$). Development of the feeding larva utilised approximately 50% of egg TG by day 4 (Fig. 5). Loss of TG from day 4 was estimated by the equation $\text{TG} = -1.48 \times \text{time} + 14.46$ ($r2 = 0.933$, ANOVA, $F_{(1,10)} = 139.8$, $P < 0.001$) indicating that these reserves would be exhausted by 9.8 days post-fertilisation. In contrast, phospholipids remained constant throughout development (ANOVA, $F_{(6,14)} = 0.93$, $P = 0.50$; Fig. 5). Total protein differed between sampling times (ANOVA, $F_{(6,14)} = 5.43$, $P = 0.004$; Fig. 4). Egg protein did not change in prefeeding embryos (egg-vs-2 days; $t_{14} = 1.13$, $P = 0.28$), but increased between days 4 and 8 ($t_{14} = 4.46$, $P < 0.001$).

Experiment 2: lipid in fed rudiment stage larvae and juveniles

Growth of feeding larvae is fuelled by phytoplankton and the larvae of *T. gratilla* increased their energy content by accumulating lipid stores in their tissues (Figs. 1, 2d, e, 5). Rudiment stage larvae had a total mean lipid content of 275.49 ng (SE = 36.64, $n = 3$) dominated by TG (mean 44.0 ng, SE = 2.69, $n = 3$) and PL (mean 163.31 ng, SE = 32.92, $n = 3$) (Figs. 1, 5). The juveniles started their benthic existence with 314.88 ng total lipid, including 46.84 ng TG (SE = 8.40, $n = 3$) (14.91% of total) and 137.51 ng PL (SE = 16.13, $n = 3$) (43.67% of total) (Figs. 1, 5).

Discussion

As found here for *Tripneustes gratilla*, triglyceride (TG) is the major energetic source in the eggs to support early development in sea urchins with planktotrophic larvae (Yasumasu et al. 1984; Podolsky et al. 1994; George et al.

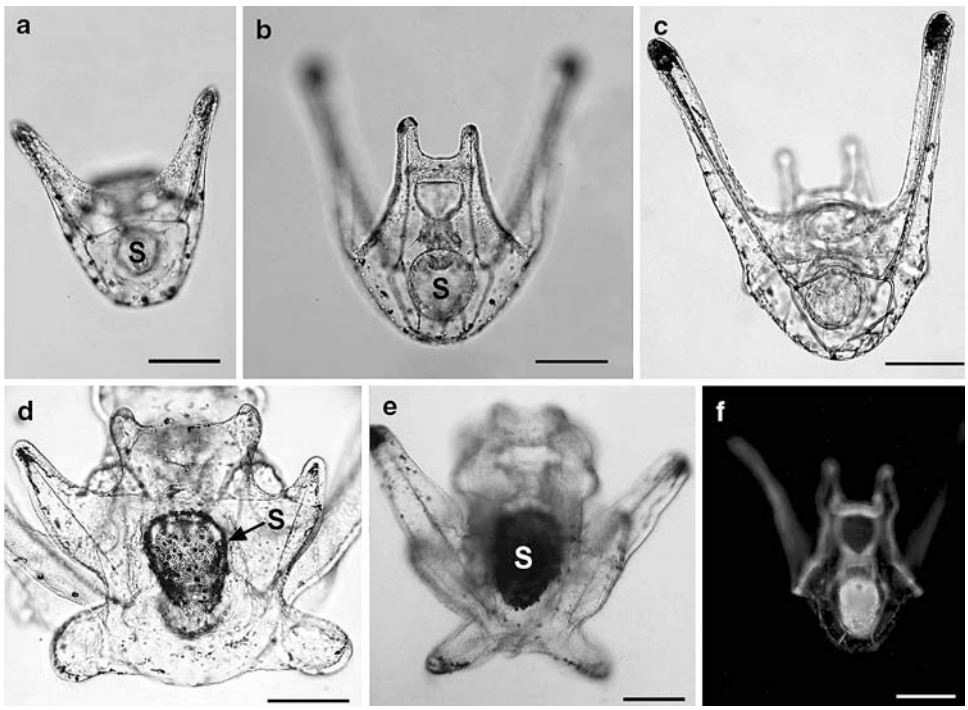

Fig. 2 *Tripneustes gratilla* larvae: unfed larvae from Experiment 1, fed larvae from Experiment 2. **a** early 2-arm functional feeding larva at 4 days post-fertilisation. **b** 5 d larva. **c** Well-developed 4-arm larva at 9 days at termination of the experiment. Note transparent stomach of unfed larvae. **d** 8-arm 18-day larva with pigment (*dark spots*) and lipid droplets (*transparent spheres*) in the stomach wall. **e** 30-day larva with opaque brown stomach. **f** Nile Red stained larva (12 days) has a bright yellow stomach under blue excitation. *S* stomach. *Scale bars* 100 µm

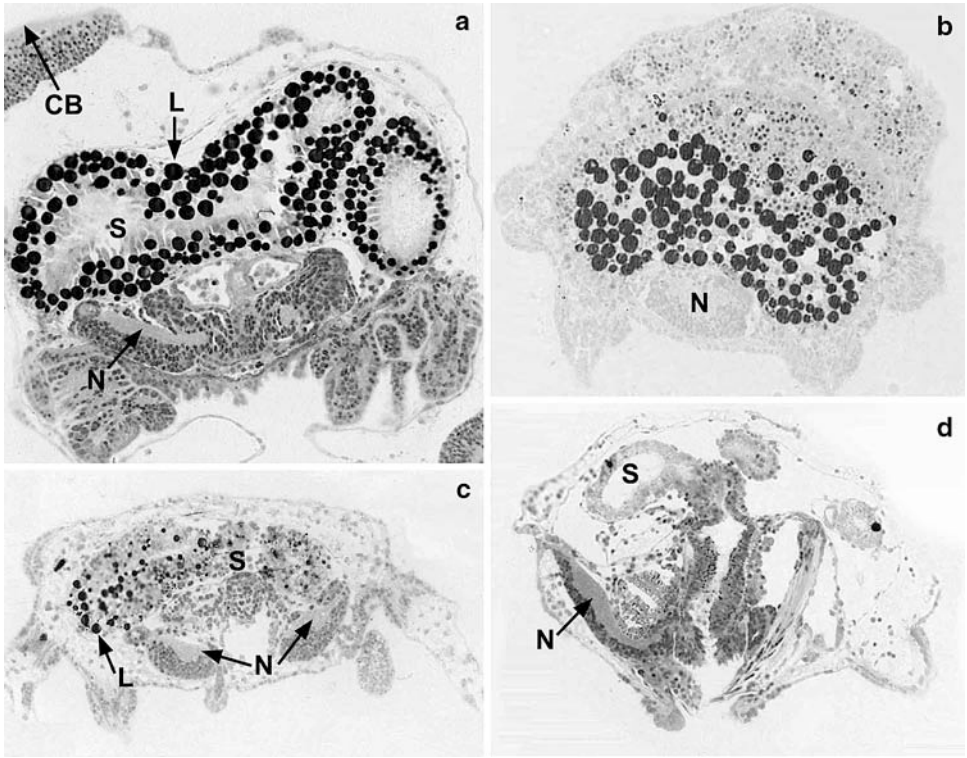

Fig. 3 Histological sections of *T. gratilla.* **a** Juvenile rudiment in an advanced larva showing accumulation of lipid spheres (L) in the stomach epithelium. **b** Developing juvenile 1-day post settlement, the stomach is filled with lipid spheres. **c** Juvenile 4 days post settlement, lipid spheres (L) are still evident. **d** Juvenile 8 days post settlement, lipid spheres are not evident. *CB* ciliated band, *N* nerve, *S* stomach. *Scale bars* 50 μm

1997; Villinski et al. 2002; Sewell 2005; Meyer et al. 2007). Across nine species in seven echinoid families, TG is the most important energetic source to fuel development of the functional feeding echinopluteus (Metzman et al. 1978; Kozhina et al. 1978; Yasumasu et al. 1984; Yokota et al. 1993; Podolsky et al. 1994; Villinski et al. 2002; Sewell 2005; Meyer et al. 2007). Maternal TG provisions are also known to fuel development of the feeding larvae of asteroids and ophiuroids (Villinski et al. 2002; Falkner et al. 2006; Prowse et al. 2008). The phospholipid (PL) and protein content remained relatively constant to the early feeding larval stage indicating that these egg reserves are largely used to provide structural compounds in the developing larva, but not to fuel development, as found for other echinoids (Podolsky et al. 1994; George et al. 1997; Sewell 2005; Meyer et al. 2007). The increase in protein in unfed larvae from day 4 to day 8 coincided with an increase in the surface area of the arms and may be due to uptake of dissolved organic molecules across the epithelium (Shilling and Manahan 1990), although the magnitude of increase is higher than might be expected.

In contrast to the results obtained in a previous study of lipids in the eggs of planktotrophic echinoids (Villinski et al. 2002), wax esters were not present in the eggs of *T. gratilla,* nor are they present in the eggs of *Evechinus chloroticus* (Sewell 2005) or *Strongylocentrotus purpuratus* (Meyer et al. 2007). This study and those by Sewell (2005) and Meyer et al. (2007) used the TLC/FID method, which provides detailed information on lipid class types whereas Villinski et al. (2002), who reported wax esters in *S. purpuratus*, used conventional TLC. We suggest that the presence of wax esters in the eggs of echinoids with planktotrophic development may not be correct.

At 84 μm diameter, the eggs of *T. gratilla* are similar to those of other echinoderms with planktotrophic larvae (Sewell and Young 1997) and, while egg nutrient types present are similar to those of other species, their proportions differed. A comparison with species for which we have TLC/FID data (Table 2), several of which occur at the same latitude as *T. gratilla*, shows that this echinoid has a high proportion of energetic lipid in its eggs and a large TG component. This may contribute to the comparatively long facultative feeding period of *T. gratilla* larvae (Byrne et al. 2008). If TG is a particularly good energy source for echinoid development, this might contribute to the robust characteristics of *T. gratilla* larvae in artificial culture (Juinio-Menez et al. 1998: Lawrence and Bazhin 1998). The notable success of *T. gratilla* in commercial culture may be facilitated in part by its maternal provisioning strategy (Review, Lawrence and Bazhin 1998).

The levels of maternal TG did not change in early development of *T. gratilla* indicating that early embryogenesis (cleavage, blastulation, gastrulation, hatching) was not costly with regard to energetic lipids. By comparison, a major proportion of maternal energetic lipid reserves (ca. 50%) was used during development of the feeding larva by day 4 post-fertilisation, similar to that found for *E. chloroticus* and *S. purpuratus* (Sewell 2005; Meyer et al. 2007).

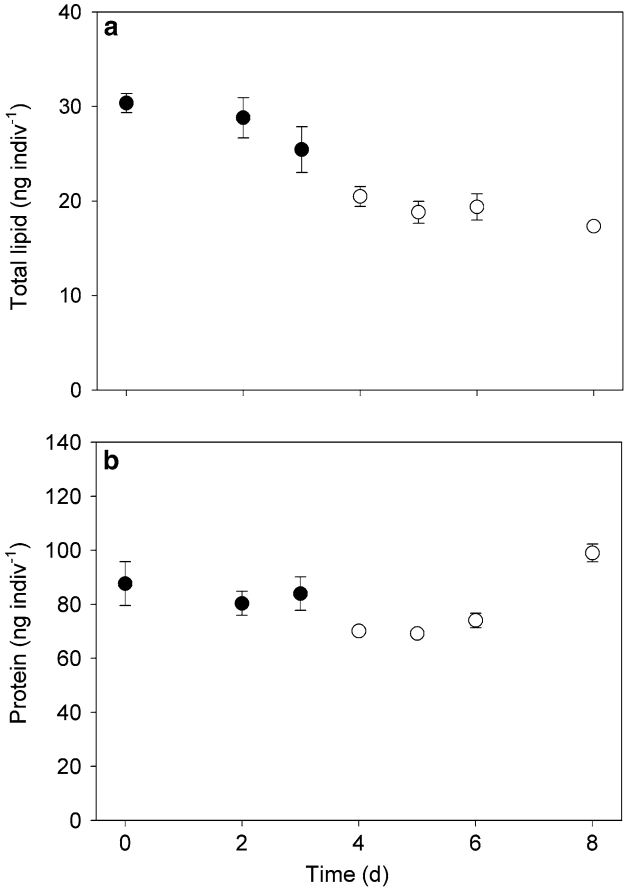

Fig. 4 Maternal total lipid (**a**) and protein (**b**) stores embryo^{-1} larva^{-1} in development of *T. gratilla* during the prefeeding (*filled circles*) and feeding stages (*open circles*) to day 8 post-fertilisation. Larvae were not fed. ($n = 3 \pm$ SE)

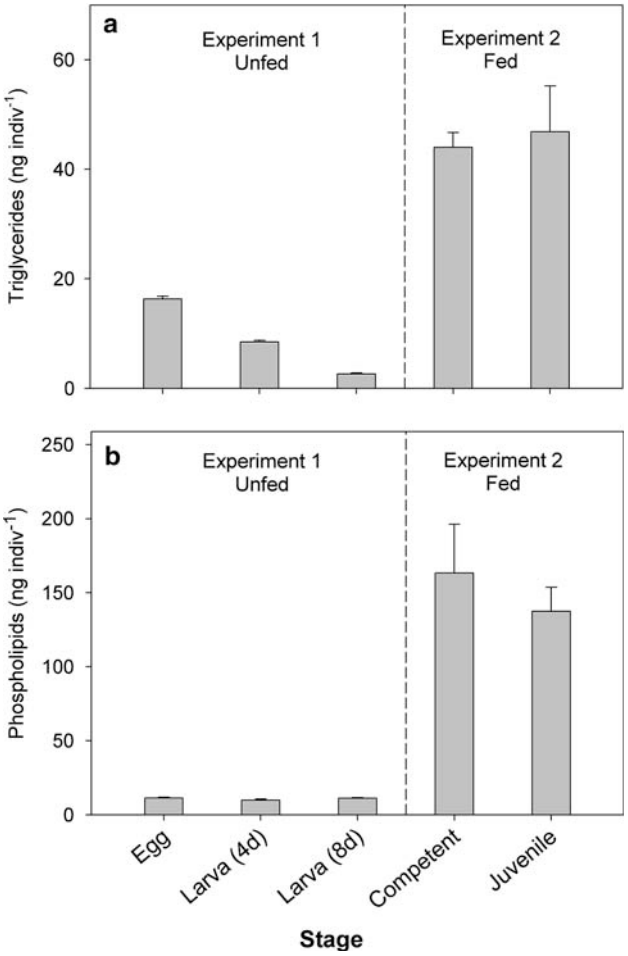

Fig. 5 Histograms of TG and PL content individual^{-1} in the egg, early (4 days) and well-developed (8 days) unfed echinoplutei from Experiment 1 and rudiment stage larvae and newly settled developing juveniles from Experiment 2. Data for Experiment 1 are mean ± SE from three replicates. For Experiment 2 the data are mean ± SE from three subsamples

Maternally derived TG was still present in 8-day echinoplutei of *T. gratilla* and it appears that this energetic lipid is not fully exhausted until approximately 10 days post-fertilisation. Ten-day-old unfed larvae of *S. purpuratus* and *E. chloroticus* have undetectable (Meyer et al. 2007) or low levels (<2 ng) of maternal TG (Sewell 2005).

During their development, the larvae of *T. gratilla* utilised the energy provided by the phytoplankton food and accumulated lipid stores. Late echinoplutei of *Paracentrotus lividus*, *Arbacia lixula*, *Encope michelini* and *Dendraster excentricus* also accrue lipids from phytoplankton with a conspicuous increase in their total lipid content just prior to metamorphosis (Fenaux et al. 1985; George et al. 1990, 1997; Schiopu et al. 2006). Phospholipids dominated the lipid biochemistry of advanced *T. gratilla* larvae. These are associated with cell and organelle membranes and so the increase in the proportion of this lipid class with larval growth is likely to be due to the increase in the epithelial cell surface area in larger, more advanced larvae.

Triglyceride was the major energy storage lipid in rudiment stage larvae of *T. gratilla*; the same energetic lipid stored by pre-metamorphic bivalve larvae (Gallager et al. 1986; Bochenek et al. 2001; Pernet et al. 2004, 2006; Powell et al. 2004). In contrast *E. chloroticus* larvae reared to the 8-arm stage (20 days) on *Dunaliella salina* accumulated free fatty acid (FFA) and *S. purpuratus* larvae reared to the 6-arm stage (10 days) on *Rhodomonas* sp. accumulated FFA and hydrocarbons (Sewell 2005; Meyer et al. 2007). It is not known what lipids these echinoids accumulate in rudiment stage larvae nor do we know what lipids the larvae used for histology accumulated from *Phaeodactylum tricornutum*. Differences in the type and quantity of lipid accumulated by feeding larvae are diet related (Schiopu et al. 2006; Liu et al. 2007). The important finding of the present study is that TG fuelled the two non-feeding stages at either end of development of *T. gratilla*; the prefeeding larval and perimetamorphic benthic stages. *T. gratilla* larvae sequestered the same major energetic lipid class from their *Chaetoceros muelleri* diet to sustain the early benthic

Table 2 Maternally derived lipid and protein content in the eggs of echinoid, ophiuroid and asteroid echinoderms with planktotrophic development as determined by Iatroscan TLC/FID studies

Species	Egg diam (μm)	Proportion energetic lipid in total lipid (%)	Proportion TG in total lipid (%)	Protein content ng egg^{-1}
Tripneustes gratilla[a]	84	55.5	51.5	87.30
Evechinus chloroticus[b]	83	42.0	29.4	74.84
Sterechinus neumayeri[c]	180	60.0	60.0	280.0
Ophionereis fasciata[d]	103	38.0	31.0	24.00
Patiriella regularis[e]	165	36.4	34.0	317.2
Meridiastra mortenseni[e]	239	38.2	36.3	889.1

[a] This study, [b]Sewell 2005; unpub. Data, [c]Podolsky et al. 1994; McClintock and Baker 1997, [d]Falkner et al. 2006, [e]Prowse et al. 2008

juvenile that the female parent provided to fuel early development.

Nile Red fluorescence and histology showed that the energetic lipids accumulated by fed *T. gratilla* larvae are stored in the stomach, as for other echinoderm larvae (Chia and Burke 1978: Byrne et al. 2003; Reitzel et al. 2004). Nile Red is used to document the location of lipids in bivalve, crustacean and echinoid larvae (Hentschel and Emlet 2000; Phillips 2002; Reitzel et al. 2004). Rudiment stage *T. gratilla* larvae had conspicuous lipid spheres in the stomach epithelium and free in the lumen. The stomach is the only echinoid larval organ retained through metamorphosis. It serves as the anlage for the juvenile gut and so provides a nutritive bridge between the two life stages.

Although *T. gratilla* reared in laboratory conditions accrue substantial energetic stores, the nutritive condition of echinoplutei and other echinoderm larvae in the plankton is variable (Review, Olson and Olson 1989). Wild-caught echinoplutei of *Paracentrotus lividus* had the long-armed profile of nutrient limited larvae (Fenaux et al. 1994). In contrast, the size of the rudiment in wild echinoplutei of *Strongylocentrotus* species indicated that the larvae were not nutrient limited and newly settled juveniles had substantial nutritive reserves apparently accrued by the larva (Miller and Emlet 1999).

Although we only had one source of rudiment stage larvae and juveniles for lipid biochemistry, the weight of evidence from three independent sources of data (biochemistry, Nile Red staining, histology) shows that feeding larvae accrue lipids from phytoplankton and that a considerable proportion of these reserves are available for the perimetamorphic period during which early juveniles become competent to feed. It can take 7–20 days for the juvenile echinoid digestive tract to differentiate post settlement, marking the end of the perimetamorphic period (Chia and Burke 1978; Gosselin and Jangoux 1998; Vaïtilingon et al. 2001). The processes associated with settlement, resorption of the larval body and differentiation of the juvenile in *T. gratilla* were not costly with respect to larval TG stores. Larval effort to produce an energetically expensive offspring bridges the transitional metamorphic stage. The results obtained for *T. gratilla* contrast with those reported for scallops where TG reserves are significantly depleted by the physiological requirements of metamorphosis (Pernet et al. 2006).

Tripneustes gratilla larvae increased their energy content to the benefit of the benthic juvenile stage. Offspring fitness in this echinoid may be largely mediated by larval investment in juvenile structures rather than by egg size, similar to that suggested for other echinoids (Strathmann et al. 1992; Hart 1995; Miller and Emlet 1999). In *T. gratilla* and other marine invertebrates with small eggs and planktotrophic development, larval experience is the primary determinant of juvenile fitness (Miller and Emlet 1999; Pernet et al. 2006; Schiopu et al. 2006). This link between larval experience and juvenile fitness is reported for many invertebrate taxa, but is not incorporated into current egg size evolution models (Pechenik 2006; Podolsky and Moran 2006).

Regardless of developmental mode (planktotrophy, lecithotrophy), there appears to be selection to provision the early juvenile echinoderm either through accumulation of nutrients by feeding larvae or through hypertrophic deposition of nutrients in eggs (Herrera et al. 1996; Byrne et al. 1999; Byrne and Cerra 2000; Villinski et al. 2002; Prowse et al. 2008, present study). It would be interesting to compare the nutritive profile of the rudiment stage larvae of closely related species that start life from small and large eggs to determine if a particular suite of nutrients is favoured for the early benthic life stage. Echinoids with facultative planktotrophy appear to have a mixed strategy (Emlet 1986; Hart 1996; Allen et al. 2006) and the nutritive profile of their eggs would provide insights into evolutionary changes in oogenesis selected for at the transition between planktotrophy and lecithotrophy.

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