Vol. 304: 133–142, 2005 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published December 8

Utilization of lipids during early development of the sea urchin *Evechinus chloroticus*

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ABSTRACT: Detailed studies of lipid utilization during bivalve development have shown that lipids are important at 2 critical periods: embryogenesis and metamorphosis. Using the Iatroscan TLC/FID system I examined lipid class utilization during development of *Evechinus chloroticus* to determine whether lipids were also important for an echinoderm at these times. Eggs of *E. chloroticus* contained 34.41 ng of lipid, primarily polar lipids (52.0 %) and triglyceride (29.4 %). To determine whether there was a different pattern of lipid utilization between larvae reared in the presence or absence of particulate food, larvae were either fed 6000 cells ml^{-1} *Dunaliella* every 2 d or starved. While there was no change in the amount of total, structural or energy storage lipids over time, there was a significant difference in the amount of structural lipids between Fed and Starved treatments. This was related to the continuing development of Fed larvae and cessation of development of Starved larvae at the 4-arm pluteus stage. In both treatments, triglycerides were rapidly utilized from the early 4-arm echinopluteus to the late 4-arm larva with fully developed arms and gut. Another neutral lipid, free fatty acid, accumulated in the 8-arm echinopluteus stage of the Fed larvae. This suggests that lipid stored during the planktonic phase, in combination with the proximate constituents of the larval body, provides the energy for the metamorphic and perimetamorphic periods in echinoderm development. Thus sea urchins, as in bivalves, appear to have 2 critical periods for lipid use during development.

KEY WORDS: Sea urchin · Echinoid · *Evechinus* · Development · Larva · Lipid · Triglyceride · Free fatty acid

INTRODUCTION

The importance of lipids as an energy source for development of marine invertebrates has long been recognized. Holland (1978) concluded, based primarily on studies with crustaceans and bivalves, that 'there is now a large body of evidence that lipid rather than carbohydrate is the major metabolic energy reserve in most marine animals. This is true for... almost all the larvae of benthic marine invertebrates so far studied' (p. 118). In the 30 yr since this review there has been a continued focus on lipid metabolism during development of crustaceans and bivalves. Of these taxa, the development of bivalves represents the pattern observed for the majority of planktotrophic marine invertebrates: a life cycle involving an extended free-swimming period after embryo development, the collection of particulate food with a larval feeding structure, and settlement and metamorphosis at the end of larval life.

Development of bivalve molluscs has 3 stages: Stage 1 includes cleavage, gastrulation and embryogenesis to the prodissoconch I larva; Stage 2 involves a period of growth as the prodissoconch II shell is deposited and the velum and visceral mass increase in size; and Stage 3 which starts with the development of the foot and primary gill filaments and is terminated by settlement and metamorphosis of the young adult (Bayne 1971, 1972, 1976a). Stages 1 and 3 are periods of intense morphogenetic activity, when new organs are developed and there is little increase in size. Stage 2 represents a period of minimal development of new organs (Bayne 1971, 1972, Bayne et al. 1975). The greatest reliance on stored energy reserves is in Stages 1 and 3. The energy for Stage 1 is provided to the embryo by

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the adult during vitellogenesis (Bayne et al. 1975, Bayne 1976a, Whyte et al. 1990), and for Stage 3 by feeding during the planktonic phase (Bayne 1972, 1976a, Whyte et al. 1989, 1990).

While both protein and carbohydrate are important energy reserves during development, studies on hatchery-reared bivalves have provided experimental evidence that energy stores of lipid are used in Stage 1 during embryogenesis (e.g. Bayne et al. 1975, Bayne 1976a, Gallager & Mann 1986, Gallager et al. 1986, Whyte et al. 1987, 1990, Lu et al. 1999, Pernet et al. 2003a, Moran & Manahan 2004) and Stage 3 during metamorphosis (e.g. Holland & Spencer 1973, Bayne 1976a, Gabbott 1976, Holland 1978, Gallager & Mann 1986, Gallager et al. 1986, Whyte et al. 1989, Videla et al. 1998, Labarta et al. 1999, Lu et al. 1999, Pernet et al. 2003b). Biochemically-based models for growth and development of larval *Crassostrea gigas*, which include neutral lipid as a parameter, suggest that the fraction of larvae that complete metamorphosis is dependent on the initial egg size (lipid content) and the ability to store lipid for metamorphosis (e.g. Bochenek et al. 2001, Powell et al. 2004). This provides further support for the importance of lipids at 2 critical periods (embryogenesis, metamorphosis) in bivalve development.

The 2 critical stages for stored lipid reserves during larval development in bivalves both occur at times in the life cycle when the feeding larva lacks a functional mechanism for particulate feeding and is in an essentially non-feeding phase. Not discounting the importance of dissolved organic matter during development (DOM: Manahan et al. 1982, Manahan 1990), lipids are important prior to formation of the larval velum and during the transition between velar and gill feeding at metamorphosis (Bayne 1976b, Sastry 1979). It is of interest to examine if there are 2 critical stages for lipid utilization in other taxa with planktotrophic larvae and similar non-feeding periods.

Lipid utilization in sea urchin development has been the focus of numerous studies (e.g. see review by Czihak 1975). Early studies found a decline in total lipid content during development (e.g. Ephrussi & Rapkine 1928, Ephrussi 1933, Hayes 1938, Öhman 1944), suggesting that lipids were utilized as an energy source and/or as the precursor of other compounds (Mohri 1964). Later research suggested that total lipid levels did not decrease prior to hatching, but that there was a decrease from the swimming blastula to the pluteus stage (Mohri 1964, Hoshi & Nagai 1970), primarily due to the loss of triglycerides (Kozhina et al. 1978, Yasumasu et al. 1984, Podolsky et al. 1994). Additional information on the role of lipids during sea urchin development has been provided by studies on the change in biochemical composition, including total lipids, during the embryonic or larval stages (Fenaux et al. 1985, 1992, George et al. 1990, 1997, Shilling & Manahan 1990).

In this study I examined lipid utilization during embryo and larval development in the sea urchin *Evechinus chloroticus*. There were 2 major aims: first, to ascertain whether the 2 critical stages of lipid utilization occur in sea urchin larvae and second, to determine whether the pattern of lipid utilization varies with the availability of particulate food.

MATERIALS AND METHODS

Larval culture. *Evechinus chloroticus* were collected by snorkel from Matheson's Bay (36° 18.1′ S, 174° 48.65′ E) in the Hauraki Gulf, New Zealand, and then transported to the Leigh Marine Laboratory and maintained in flowing seawater tanks (2.1 × 0.87 m, 0.18 m deep). Gametes were obtained by intracoelomic injection of ca. 3 ml of 0.55M KCl. Eggs from 6 females were collected by inversion over separate 100 ml beakers of 1 µm filtered UV-sterilized seawater (FSW) and pooled in a glass beaker with a final volume of 600 ml. Sperm from 4 males was collected 'dry' and combined in a diluted sperm suspension for fertilizing the eggs. Gamete quality (sperm motility, shape and appearance of eggs) of both the male and female gametes was checked microscopically prior to fertilization. Fertilization success of the embryos used in the culture was >95%. Before setting up the larval cultures, a volume of fertilized eggs (Day 0) was concentrated in a 50 ml Falcon tube, the concentration determined, and ~500 eggs pipetted into 12 replicate Eppendorf tubes. After brief centrifugation the seawater was removed and the samples frozen at –80°C until lipid analysis.

Larval cultures were established in three 100 l plastic barrels containing ca. 90 l of FSW. Cultures were housed in a hatchery at 20 ± 1°C with a 16:8 h light:dark cycle. Air-stones were placed at the bottom of the barrel to provide gentle water movement throughout the cultures. The initial stocking density was 10 embryos per ml to allow for large-scale sampling of the early embryo stages for lipid analysis.

At Day 3, when the larvae were early 4-arm echinoplutei (Lamare & Barker 1999, Sewell et al. 2004), the cultures were assigned to one of 2 treatments: Fed or Starved.

Fed: Two of the 100 l barrels of larvae were assigned to the Fed treatment and larvae were fed 6000 cells ml^{-1} of *Dunaliella tertiolecta* every 2 d (ration based on Lamare & Barker 1999 and Sewell et al. 2004). Algae were reared in the hatchery in Guillard's F/2 medium under constant fluorescent illumination at 20°C and added in the exponential growth phase.

Starved: The third 100 l barrel was assigned to the Starved treatment and given no algal food. Because the cultures were open to the atmosphere and the gametes were not obtained under sterile conditions, the cultures may have been inoculated with other microorganisms. Consequently, cultures with no algal food may have received some organic material during the course of the experiment.

Every 2 d, coinciding with feeding of the Fed treatment, 30 to 40% of the water in each of the 100 l barrels (2 Fed treatments, 1 Starved) was removed by reverse filtration through a 60 µm mesh, the volume replaced with FSW and the Fed treatments received 6000 cells ml^{-1} of *Dunaliella tertiolecta*. Every 6 to 8 d the entire contents of each barrel was gently filtered onto a 60 µm mesh and the larvae stored in a 20 l bucket while the barrel was cleaned (scrubbed to remove the biofilm, cleaned with a high-pressure freshwater hose, rinsed with seawater) and refilled with FSW. After the larvae had been gently rinsed they were returned to the barrel and the Fed treatment received 6000 cells ml^{-1} of *D. tertiolecta*.

To examine changes in lipid utilization with development, a sample was removed from each treatment every 2 to 3 d (alternating between the 2 Fed containers), the larvae were concentrated and ~500 embryos/larvae pipetted into an Eppendorf tube. After brief centrifugation the seawater was removed, and the samples were frozen at −80°C until lipid analysis; 7 to 12 Eppendorf tubes were taken at each sampling for the Fed and Starved treatments.

Lipid analysis. Lipid was extracted from frozen egg, embryo and larval samples following the method of Holland & Gabbott (1971) with minor modifications. Sample homogenates were prepared by the addition of 250 µl of ultrapure water to the Eppendorf tube and ultrasonication with a Sanyo Soniprep 150 fitted with an exponential probe for 15 to 20 s. The sonicate was transferred with a drawn Pasteur pipette to a 1 ml glass V-vial (Wheaton) containing 25 µl of the internal standard in chloroform. This 'internal standard' is technically a 'surrogate spike' which is not used directly for quantitation (Keith et al. 1983). The amount of internal standard measured in the final concentrate provides an estimate of lipid recovery (Parrish 1987). Ketone was used as an internal standard as natural concentrations are low in marine tissues (Delmas et al. 1984, Parrish & Ackman 1985). This lipid does not co-elute with any other lipid classes in the development system used and is suitable in marine invertebrate tissues provided there is not a large wax ester peak (Parrish 1987). No wax ester peak was observed in preliminary analysis of the lipid classes in *Evechinus chloroticus* fertilized eggs or larvae.

Chloroform (100 µl) and methanol (250 µl) were added to the V-vials so that the final ratio of water:chloroform:methanol was 2:1:2 (Bligh & Dyer 1959). After vigorous shaking for ca. 5 min, the V-vials were centrifuged at 1000 RPM for 5 min at room temperature. Both the aqueous and chloroform fractions were transferred to a clean glass V-vial, leaving the solid non-lipid material (NLM) behind. An additional 250 µl water and 250 µl chloroform were added, the vial shaken for a further 1 to 2 min, and re-centrifuged at 1000 RPM for 5 min at room temperature. Most (90%) of the upper water and methanol fraction was removed with a Pasteur pipette and discarded, with little disturbance to the interface between the aqueous and chloroform layers. The lower chloroform layer (ca. 375 µl = [100 + 250 added in extraction] + 25 µl from internal standard) was transferred to a third V-vial using a 200 µl Eppendorf pipette with a chloroform-rinsed tip. Immediately before spotting, the lipid extract was dried down in a stream of N_2 gas and 10 µl of chloroform added using a Gilson positive displacement pipette. All solvents used in lipid extraction were HPLC-grade and all V-vials used in the extraction process were cleaned with 3× methanol and 3× chloroform washes as recommended by Parrish (1999).

The amount of each lipid class in the lipid extract was quantified using an Iatroscan Mark V^{new} TLC/FID system and silica gel S-III Chromarods following the protocols defined by Parrish (1997, 1999) with 2 minor modifications. As only a small volume of sample was spotted on the Chromarods no solvent focusing was required (for details see Delmas et al. 1984, Parrish 1987, 1999, Bergen et al. 2000). Instead of drying in the Iatroscan, the Chromarods were dried for 5 min in a Rod Dryer TK-8 (Iatron Laboratories) at 100°C. The protocol developed by Parrish (1987, 1999), which makes use of the partial scanning facility of the Iatroscan, allows separation of all the lipid classes of a single sample and ensures that any NLM, which can lead to overestimation of the phospholipid peak (Fraser et al. 1985), remains at the origin (Parrish 1997, 1999). After ensuring that the Chromarods were clean, 1 µl of sample was applied to each Chromarod with a fixed volume Drummond Microdispenser fitted with Drummond Precision Glass Bores (volumetric tolerance of ±1%); 4 separate samples were processed on each run (2 replicate Chromarods of each sample; total 8 rods). The remaining 2 Chromarods were used to run a mid-level concentration of the composite lipid standard (see below) to determine the relative standard deviation (RSD) of the response factors for that set of Chromarods from the calibration curve (see Bergen et al. 2000) and a blank (unspotted) Chromarod to test for contamination of the developing solvents.

Chromarods were developed in the Parrish (1999) triple development system which resulted in 3 chromatograms (for an example see Fig. 1). Development

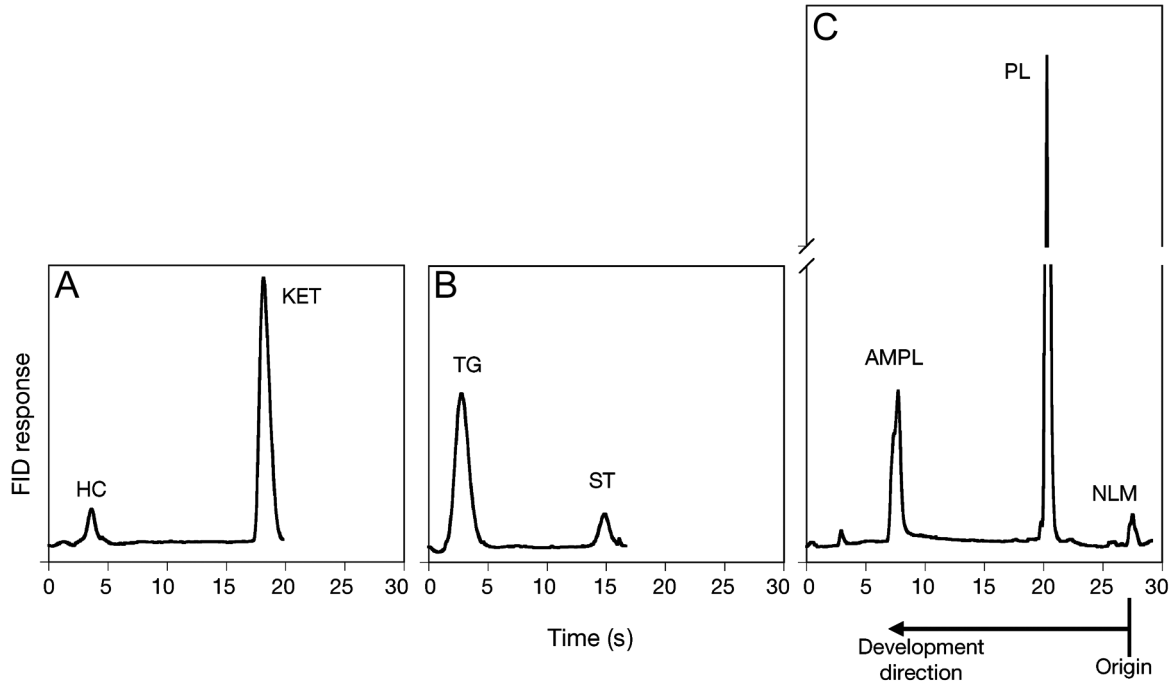

Fig. 1. *Evechinus chloroticus*. TLC/FID chromatograms of lipids extracted from fertilized eggs using the Parrish (1999) triple development. The 3 panels are the chromatograms resulting from the 3 scans of the Chromarods: (A) Hexane: diethyl ether: formic acid (98.95:1:0.05), 25 min, 5 min dry, and redeveloped for 20 min. Partial scan on Iatroscan (PPS25). (B) Hexane: diethyl ether: formic acid (79:20:1) for 40 min. Partial scan on Iatroscan (PPS40). (C) 100% acetone for 15 min, 2 × 10 min in chloroform: methanol:water (5:4:1). Full scan. *x*-axis is time (s); *y*-axis shows FID response to the same relative scale; note axis break in (C). As each lipid class has a different FID response, peak areas cannot be directly compared between different lipid classes. HC: aliphatic hydrocarbon; KET: ketone internal standard; TG: triglyceride; ST: cholesterol; AMPL: acetone-mobile polar lipids; PL: phospholipid; NLM: non-lipid material

solutions, preparations of the developing tank and timings were as in Parrish (1999, see his Fig. 1.4), with the exception that the partial scan after the second development had the Partial Scan Mode (PPS) set to 40 to ensure that an initially unidentified peak was retained in the third chromatogram. Chromarods were run in the Iatroscan with a 30 s scan and settings of 2000 ml min^{-1} O_2 and 160 ml min^{-1} H_2. Chromarods were burnt from the solvent front towards the origin in Fig. 1C at the NLM peak. Data were collected with an SES-Chromstar PC-board and the peaks quantified using SES-Chromstar version 4.10 (SES Analysesysteme).

Quantification of the lipid per sample was based on multilevel calibration curves generated for each lipid class on each rack of 10 Chromarods. Rods were calibrated with an 8-component composite standard made from highly purified lipid standards (99%) in HPLC-grade chloroform and stored under nitrogen at –20°C (Parrish 1987, 1999, Bergen et al. 2000). The lipid classes were phospholipid (PL: L-α-phosphoditylcholine), free sterol (ST: cholesterol), fatty alcohol (ALC: 1-hexadecanol), free fatty acid (FFA: palmitic acid), triglyceride (TG: tripalmitin), ketone (KET: 3-hexadecanone), wax ester (WE: lauryl acid myristyl ester), and aliphatic hydrocarbon (HC: nonadecane). Standards were purchased from ICN Biomedicals. (FFA), Acros Organics (HC) or SIGMA (all others). As no purified monoacylglycerol standard was available at the time of sample analysis I assumed, as did Delmas et al. (1984), that AMPL (acetone-mobile phospholipid) had a similar FID response to the PL calibrated with L-α-phosphoditylcholine. The composite standard covered the range of lipid concentrations in embryo/larval tissue (range 0.063 to 1.923 µg $µl^{-1}$). As at low loadings (<1 to 5 µg) the FID response is curved, quadratic (second order polynomial) regressions were used as recommended by Delmas et al. (1984), Parrish & Ackman (1985) and Parrish (1987). Peak areas for the calibration curves were based on the mean of 2 to 3 separate Chromarods; r^2 values were >0.995 for all lipid classes.

At each time point, and for each treatment (Fed, Starved), lipids were extracted and analyzed with the Iatroscan for 3 replicate Eppendorf tubes of ~500 ind. (fertilized eggs, embryos, or larvae). The concentrations of each lipid class per sample were determined based on the percent recovery of the internal standard and the calibration curve appropriate for each lipid class on each rack of Chromarods. Total lipid was cal-

culated by summing the amounts (ng) of PL, AMPL, ST, TG, FFA and HC for each sample.

Differences between the Fed and Starved treat-ments and changes in amounts of each lipid class with time were analyzed with 2-way ANOVA (factors: Fed or Starved, Time) using SAS version 9.0. Assumptions of ANOVA (normality, homogeneity of variance) were checked using Shapiro-Wilks test and residual analysis respectively. All analyses were conducted on untransformed data.

RESULTS

Lipid classes in eggs

Five lipid classes were observed in fertilized eggs (Fig. 1), with a total lipid content of 34.41 ng per egg (Table 1). The total lipid in the egg was dominated by TG (29.4%), with lesser amounts of the neutral lipids, HC (13.1%) and ST (5.5%, Table 1). The remainder of the egg (52.0 ± 1.7%) is made up of polar lipids (PL + AMPL; Fig. 1, Table 1). The AMPL separated in the acetone development include glycolipids, pigments, and any remaining neutral lipids from the PL (Parrish 1987). This peak is often named 'chloroplast lipids' because the peak may contain pigments and chloroplast-associated galactosyl-lipids, but it can also include monoacylglycerols (Striby et al. 1999). As there are no chloroplasts in animal cells, and spikes with 1-monopalmitoyl-rac-glycerol standards confirmed the presence of monoacylglycerols, it is assumed that AMPL, which shows minor peak splitting (Fig. 1), represents a combination of glycolipids and monoacylglycerols. The NLM left at the origin (Fig. 1) is presumed to consist of traces of protein, carbohydrate and humic material if present (Parrish 1987) and could not be quantified.

Lipid utilization during development

Lipid content was quantified during the first 21 d of development (Fig. 2). By Day 2, the larvae have developed to the early 4-arm echinopluteus with postoral and anterolateral

Table 1. *Evechinus chloroticus*. Lipid class analysis of fertilized eggs using Iatroscan TLC/FID. Data are mean ± SE of 3 independent samples of 500 fertilized eggs. nd = not detectable

	Amount per egg (ng)	% of egg
Phospholipid + acetone-mobile polar lipid (PL + AMPL)	18.05 ± 2.98	52.0 ± 1.7
Cholesterol (ST)	1.84 ± 0.45	5.5 ± 1.6
Triglyceride (TG)	10.04 ± 2.69	29.4 ± 7.3
Free fatty acid (FFA)	nd	nd
Aliphatic hydrocarbon (HC)	4.48 ± 2.78	13.1 ± 8.5
Total	34.41 ± 4.57	100

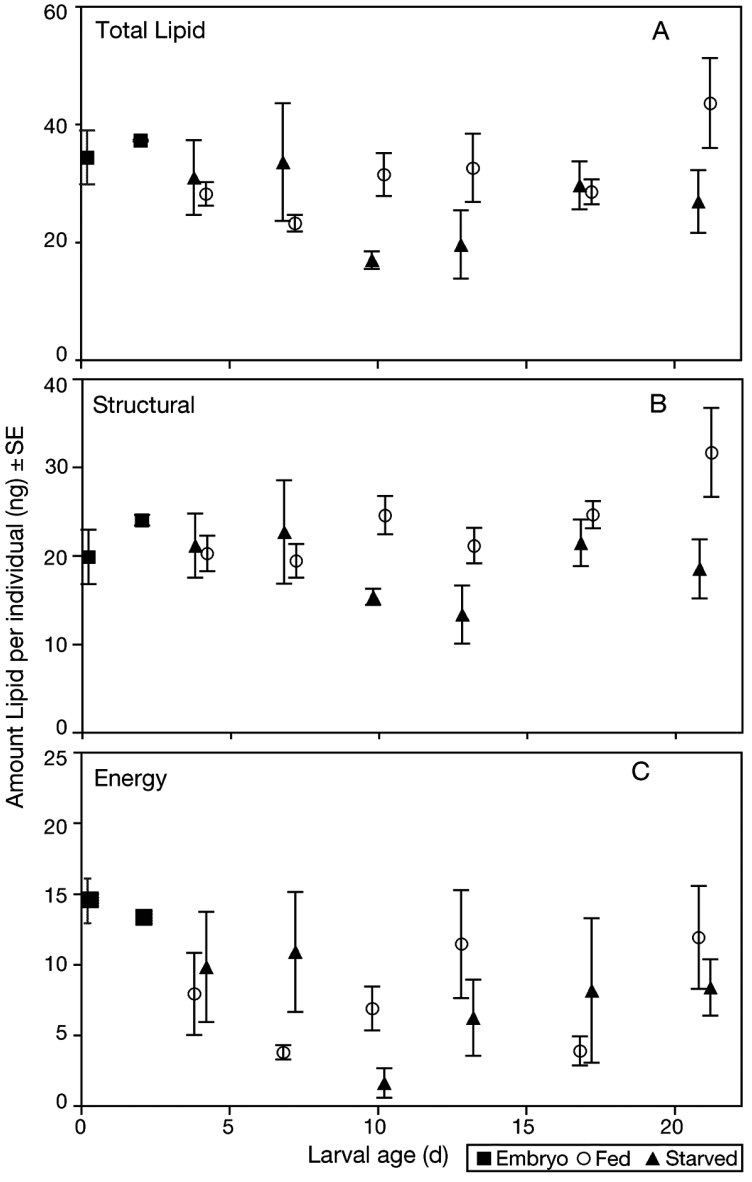

Fig. 2. *Evechinus chloroticus*. Patterns of lipid utilization during development. (A) Total lipid: sum of all lipid classes; (B) structural lipids: polar lipids (PL + AMPL) and ST; (C) energy lipids: includes TG, FFA + HC. Data are mean ± SE; N = 3. Note that embryo (Day 0), and Fed/Starved treatments have been offset on each sample day for clarity

arms present, but not at full length. Between Days 2 and 6, larvae from both Fed and Starved treatments continued to increase the length of the postoral and anterolateral arms and developed an obvious gut after Day 4. Fed larvae continued to develop through the 6- and 8-arm pluteus stages after Day 12, with some larvae showing early rudiment formation by Day 21. In contrast, the surviving Starved larvae did not develop past the 4-arm stage (for details see Sewell et al. 2004).

During the first 2 d of development there were no changes in the total, structural, or energy storage lipids (Days 0 to 2: t = –0.61, –1.32, 0.76, respectively; df = 4, all ns; Fig. 2). After the feeding treatments began at Day 3, the total lipid content of larvae did not change from Day 4 to 21 and there was no significant difference between the Fed and Starved treatments (Fig. 2, Table 2). Similarly, the energy storage lipids showed no significant differences between feeding treatments or among days (Fig. 2, Table 2). In contrast, there was a significant difference in structural lipid content between Fed and Starved treatments, but not among days (Fig. 2, Table 2). Fed larvae had a higher structural lipid content (overall mean ± SE, N = 18: Fed = 23.64 ± 1.36 ng, Starved = 18.78 ± 1.42 ng), a result of the continuing development in the Fed larvae to the 8-arm stage.

Detailed information on changes in lipid class composition reveals a clearer picture of patterns of lipid utilization during development (Fig. 3). The greatest change occurs in the amount of TG. Although there was no significant difference in TG content between Days 0 and 2 (t = –0.16, df = 4, ns), TG declined rapidly between the early (Day 2) and fully-formed 4-arm larvae with gut (Days 4 to 6). The 2-way ANOVA shows that Fed and Starved larvae have different patterns in TG utilization over time (significant Day × Food interaction, Fig. 2, Table 2). Fed larvae show higher levels of TG until Day 13, while the amount of TG is <1 ng ind^{-1} for the Starved larvae after the 4-arm pluteus stage is reached at Day 6 (Fig. 3). As the

Table 2. *Evechinus chloroticus*. Summary table of 2-way ANOVAs for factors Day (Days 4 to 21) and Food (Fed, Starved) for both lipid composition and separate lipid classes. Total lipid: sum of all lipid classes; structural lipids: (PL + AMPL) + ST; energy lipids: TG + FFA + HC. See Table 1 for lipid class abbreviations. Analyses based on untransformed amounts of lipid per individual (ng). Significant results highlighted in **bold**

	Day (df = 5, 24)		Food (df = 1, 24)		Day × Food (df = 5, 24)	
	F	p	*F*	p	*F*	p
Total lipid	1.13	ns	3.52	ns	2.12	ns
Structural lipid	1.53	ns	**7.52**	**<0.05**	2.10	ns
Energy lipid	0.98	ns	0.0005	ns	1.46	ns
PL + AMPL	1.32	ns	**5.18**	**<0.05**	1.98	ns
ST	**3.18**	**<0.05**	**16.10**	**<0.001**	1.36	ns
TG	**25.00**	**<0.0001**	**18.76**	**<0.001**	**10.78**	**<0.0001**
FFA	**12.21**	**<0.0001**	3.50	ns	**12.44**	**<0.0001**
HC	0.68	ns	0.60	ns	0.56	ns

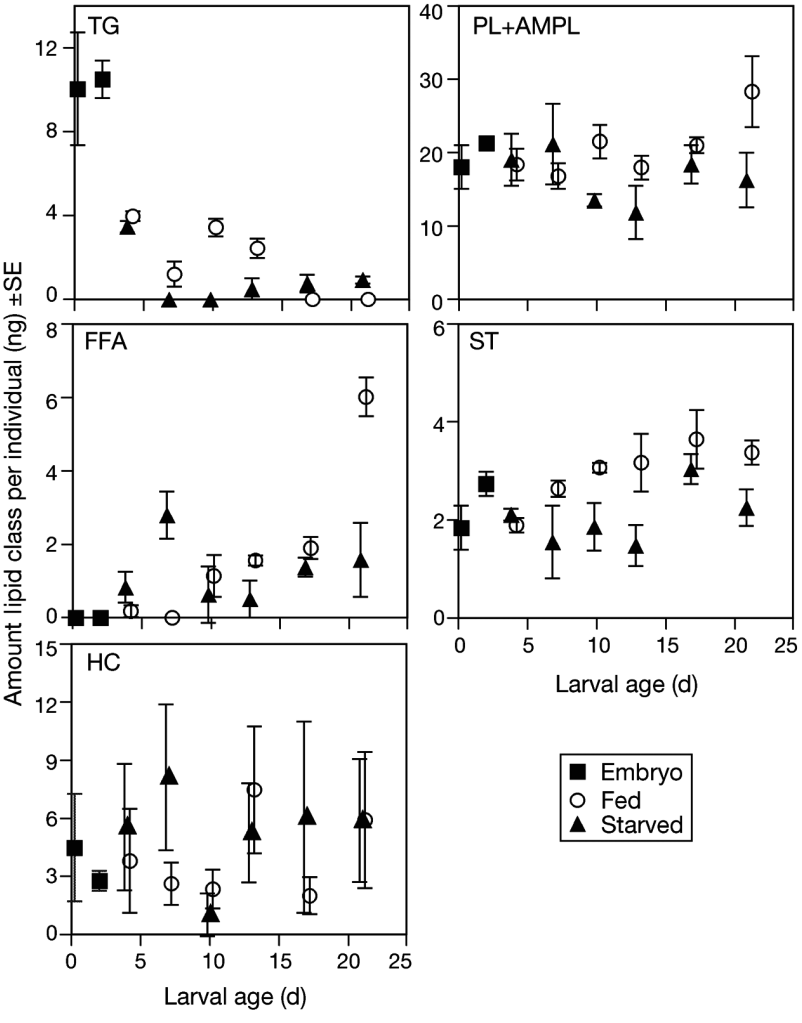

Fig. 3. *Evechinus chloroticus*. Changes in amounts of each lipid class during development in the Fed and Starved treatments. Left column shows the energy lipids TG, FFA and HC. Right column shows the structural lipids PL + AMPL and ST. Data are mean ± SE; N = 3. Note that embryo (Day 0), and Fed/Starved treatments have been offset on each sample day for clarity

F-ratio for the day main effect is substantially larger than the *F*-ratio for the Day × Food interaction (Table 2) the test for differences in TG content can be interpreted between days, in addition to interpreting the interaction. The TG content at Day 4 was significantly higher than all other days (Tukey's *a posteriori* testing, $\alpha = 0.05$, overall mean Day 4 ± SE, N = 6: 3.72 ± 0.20 ng).

FFA were not detectable in larvae until Day 4 (Fig. 3). The significant Day × Food interaction term emphasizes the differing patterns of FFA utilization between Fed and Starved larvae (Table 2). The Starved larvae have an early peak in FFA content at Day 7, when the TG has fallen to non-detectable levels, and then have low levels (< 2 ng ind.$^{-1}$) for the remainder of development. The Fed larvae, in contrast, show gradually increasing levels of FFA with time (Fig. 3). In particular, Tukey's *a posteriori* testing ($\alpha = 0.01$) showed that the Day 21 Fed larvae had a significantly higher FFA content than all other Day × Food combinations (mean 'Day 21 × Fed' ± SE, N = 3: 6.02 ± 0.47 ng).

In contrast to the patterns seen in TG and FFA, there was no significant difference in HC content between feeding treatments or day (Fig. 3, Table 2). This lipid class had the highest variability among samples (SE > 40% of mean in all larval measurements, Fig. 3) and, as a result, contributes to the high variability seen in the sum of the energy storage lipids (TG, FFA, HC; Fig. 2, Table 2) during development.

The 2 structural lipid classes (polar lipids [PL + AMPL] and ST) generally show similar patterns with development, with no significant differences in PL + AMPL ($t = -1.05$, df = 4, ns) and ST ($t = -1.72$, df = 4, ns) in the period between fertilized egg and early 4-arm plutei (Day 2, Fig. 3). There is, however, a significant difference in PL + AMPL and ST content between feeding treatments after Day 4 (Fig. 3, Table 2). The Fed larvae, which continue to develop through the 6-arm and 8-arm plutei, have higher content of both lipid classes than the Starved larvae, which under these culture conditions do not develop past the 4-arm stage (PL + AMPL: overall mean ± SE, N = 18: Fed 20.68 ± 1.28 ng, Starved 16.74 ± 1.39 ng; ST: Fed 2.97 ± 0.19 ng, Starved 2.05 ± 0.19 ng). For ST content there is also a significant difference with time (Table 2). However the Tukey *a posteriori* groupings ($\alpha = 0.05$) show considerable overlap (Group A = Days 10, 13, 17, 21; Group B = Days 4, 7, 10, 13, 21).

DISCUSSION

Lipid utilization during sea urchin development

The eggs of *Evechinus chloroticus* have a similar lipid content and lipid class composition to that described for other echinoids (see Isono & Isono 1975; their Table 1). The neutral lipids are dominated by TG and ST, and the polar lipids include both PL and glycolipids (AMPL). Direct comparisons to previous studies are difficult due to differences in extraction and separation of lipid classes, but the presence of glycolipids (Chelomin & Svetashev 1978, Kozhina et al. 1978) and the dominance of TG as the energy storage lipid has been described in the eggs of other planktotrophic echinoids: *Arbacia punctulata* (Metzman et al. 1978), *Strongylocentrotus intermedius* (Chelomin & Svetashev 1978, Kozhina et al. 1978), *Hemicentrotus pulcherrimus* (Deguchi et al. 1979, Yokota et al. 1993) and *Anthocidaris crassispina* (Yasumasu et al. 1984).

Villinski et al. (2002) reported both TG and WE as energy storage lipids in eggs of 4 species of planktotrophic echinoids. The WE to TG ratio ranged from 0.085 in *Heliocidaris tuberculata* to 0.85 in *Eucidaris tribuloides* (Villinski et al. 2002). WE have not been reported in previous studies of planktotrophic echinoids (Chelomin & Svetashev 1978, Kozhina et al. 1978, Metzman et al. 1978, Deguchi et al. 1979, Yasumasu et al. 1984, Yokota et al. 1993) and were not seen in the small eggs (87 µm, Franke et al. 2002) of *Evechinus chloroticus*. If WE were present in detectable amounts in *E. chloroticus*, the peak would have been to the left of the KET peak in the first chromatogram (Fig. 1, see Parrish 1987, 1999). Further Iatroscan analysis of the eggs of planktotrophic echinoids using a triple-development system that separates all lipid classes (Parrish 1987, 1999), is required to clarify whether WE are a general feature of echinoid eggs and part of the 'ancestral oogenic strategy' proposed by Villinski et al. (2002, p. 1767) for sea urchins and starfish.

The Iatroscan TLC/FID system has allowed re-examination of the decrease in total lipid content during sea urchin development (e.g. Ephrussi & Rapkine 1928, Ephrussi 1933, Hayes 1938, Öhman 1944, Mohri 1964, Hoshi & Nagai 1970) and provides further evidence that TG are the lipid class fueling early larval development in planktotrophic echinoids (*Strongylocentrotus intermedius*, Kozhina et al. 1978; *Anthocidaris crassispina*, Yasumasu et al. 1984; *Sterechinus neumayeri*, Podolsky et al. 1994). With detailed knowledge of the concomitant changes in the neutral/energy and polar/structural lipids during embryo and larval development in *Evechinus chloroticus*, previous studies on the biochemical composition of sea urchin larvae can be interpreted more fully.

Studies of lipid content during sea urchin larval development have usually measured total lipid using either the colorimetric sulphophosphovanillin method (Fenaux et al. 1985, 1992, George et al. 1990, 1997) or spectrophometrically after charring (Shilling & Manahan 1990). During early development of *Strongylocen-*

trotus purpuratus there is a decrease in total lipid between the prism stage (Day 2) and the 4-arm pluteus (Day 4, Shilling & Manahan 1990) which, as shown here for *Evechinus chloroticus*, is likely due to utilization of maternally-derived energy storage lipids. Studies on *Paracentrotus lividus* over the first week of development (Fenaux et al. 1992) have shown a stable total lipid content, similar to that observed here, where the decreases in TG are balanced by structural lipid changes early in development. There is also a large increase in total lipid content of fed larvae of *P. lividus* (Fenaux et al. 1985, George et al. 1997), *Arbacia lixula* (George et al. 1990, 1997) and *Encope michelini* (George et al. 1997) just prior to metamorphosis. Although our measurements did not extend to this stage of development (Day 23, Walker 1984, Lamare & Barker 1999, Sewell et al. 2004), it is probable that the increase in total lipid is the result of increases in structural lipids resulting from growth of the juvenile rudiment and perhaps to changes to the neutral lipids as seen in *E. chloroticus*.

Do echinoids have 2 critical periods for lipid utilization?

In *Evechinus chloroticus* the maternally-derived neutral lipid reserves (TG) show a great decline in both Fed and Starved treatments immediately after formation of the early 4-arm pluteus and during the period of rapid growth of the postoral and anterolateral arms and formation of the larval gut (Lamare & Barker 1999, Sewell et al. 2004). Fed and Starved larvae show a different pattern of TG utilization through development, with only trace amounts of TG present in either treatment after Day 13.

The rapid decline in TG levels during early echinopluteus development has also been observed in *Anthocidaris crassispina* (Yasumasu et al. 1984) and in *Sterechinus neumayeri* (Podolsky et al. 1994). In *A. crassispina*, TG levels decrease greatly between the gastrula and the prism stage, with a low level of TG present in the 48 h old pluteus (Yasumasu et al. 1984). *Sterechinus neumayeri*, with considerably slower development, shows a similar decline in TG levels between the gastrula and late 4-arm pluteus stage (38 d, Podolsky et al. 1994). Thus, in 3 species of echinoids (*A. crassispina*, *S. neumayeri*, *Evechinus chloroticus*) TG are used after gastrulation and during the development of the feeding stage larva, equivalent to the Stage 1 critical step in bivalve molluscs (Bayne 1971, 1972, 1976a).

In contrast to bivalve molluscs, where stores of TG (Gallager et al. 1986, Pernet et al. 2003b) are used to fuel the metamorphic transition to the juvenile (Stage 3 critical step in bivalves, Bayne 1971, 1972, 1976a), the evidence presented here suggests a different neutral lipid, FFA, may be important in fueling metamorphosis in Fed *Evechinus chloroticus*. There was, however, no evidence of the storage of TG during the period of planktonic feeding and prior to rudiment formation in *E. chloroticus*. As the lipid analyses involve extraction of the entire larva, any TG present would have been detected.

Neutral lipid stores to be used in bivalve metamorphosis accumulate in the digestive gland and associated organs during the planktonic stage (2) of bivalve development (Bayne 1976b, Elston 1980, Gallager et al. 1986). This lipid is used to provide energy during the non-feeding period between disintegration of the velum and formation of a functional gill (2 to 8 d, Bayne 1976b, Sastry 1979, Gallager et al. 1986). In echinoids, where metamorphosis involves the eversion of the echinoid rudiment, the collapse of the larval body and the resorption of most of the larval tissues (Chia & Burke 1978, Burke 1989, McEdward & Miner 2001a), there is also a non-feeding period where the benthic postlarva does not have a functional mouth and anus (7 to 10 d, Chia & Burke 1978, Miller & Emlet 1999, Vaïtilingon et al. 2001).

While the source of energy for echinoids during the perimetamorphic period (from larval competence, through metamorphosis and the endotrophic postlarval life until the appearance of the exotrophic juvenile; Gosselin & Jangoux 1998) is not specifically known, it is generally thought that newly settled individuals must 'live off maternal reserves or food acquired during larval life' (McEdward & Miner 2001b, p. 69). A potential source of energy for the perimetamorphic period is the larval tissue (epidermis, pre-oral-lobe, digestive system) resorbed during metamorphosis (Chia & Burke 1978), and the protein, lipid (polar and/or neutral) and carbohydrate contained within. Evidence from *Dendraster excentricus* suggests that a significant lipid contribution may come from lipid droplets that accumulate in the columnal cells of the larval stomach during development (Chia & Burke 1978, Reitzel et al. 2004).

In conclusion, the research presented here for *Evechinus chloroticus* and previous studies (Yasumasu et al. 1984, Podolsky et al. 1994) suggest that neutral lipids are important in echinoids during embryogenesis, after gastrulation and during growth of the full-size 4-arm echinopluteus (equivalent to Stage 1 in bivalves). Although the full period of metamorphosis and settlement was not studied in the current experiments, it is predicted that the energy for metamorphosis and the perimetamorphic period (equivalent to Stage 3 in bivalves) may be provided by a combination of neutral lipids present in the larva, such as the FFA seen in *E. chloroticus*, with a contribution from other biochemical components of the resorbed larval body.

Acknowledgements. Thanks to the Director, J. Montgomery, and staff of the Leigh Marine Laboratory, where this research was conducted; to A. Morgan for assistance in the hatchery; and to M. J. Cameron who, with a School of Biological Sciences Summer Studentship, provided assistance in the culture phase of the experiments. Thanks to C. Parrish for assistance in identifying the unknown peak, M. Byrne for helpful discussions on echinoderm development, and K. Ruggiero for statistical advice and comments on the manuscript. The comments of 3 anonymous reviewers and the contributing editor, J. Lawrence, significantly improved the final version of the manuscript. Research funds were provided to M.A.S. from the University of Auckland Research Fund (Grant No: 3601170).

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Editorial responsibility: John Lawrence (Contributing Editor), Tampa, Florida, USA

Submitted: December 24, 2004; Accepted: June 28, 2005
Proofs received from author(s): November 1, 2005