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Three-stage lipid dynamics during development of planktotrophic echinoderm larvae

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ABSTRACT: The eggs of marine species with planktotrophic development must contain, at a minimum, sufficient material for production of a larva that can then sequester additional materials to grow and metamorphose successfully. In echinoderms, lipids perform crucial energy storage and structural functions during larval construction, but their roles during later development and metamorphosis are poorly understood. We investigated lipid-class depletion in early development and accumulation in late development and a lipid nutritional condition index (energetic lipid:sterol ratio) from the egg to the juvenile in the sea star *Patiriella regularis* and the sea urchin *Heliocidaris tuberculata*. Three phases were identified: (1) rapid depletion of energetic lipids during embryogenesis and the facultative feeding period (between feeding competence and exhaustion of energetic lipid reserves), (2) larval growth with no improvement in lipid nutritional condition, and (3) rapid lipid accumulation in advanced larvae prior to metamorphosis. Maternally derived energetic lipids were depleted more slowly in fed than unfed larvae but were still exhausted quickly. *Patiriella regularis* improved their lipid condition index during Phase 3 by accumulating energetic lipid (triacylglycerol [TAG], diacylglycerol ether [DAGE]) reserves that were then partially used to fuel settlement and metamorphosis. In contrast, *Heliocidaris tuberculata* did not accumulate TAG or DAGE during this phase, suggesting that metamorphosis is fuelled by other reserves, which we hypothesize may be phospholipids.

KEY WORDS: Larval development · Maternal provisioning · Juveniles · Sea stars · Sea urchins

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INTRODUCTION

Many marine invertebrates develop through a planktonic larval phase prior to a dramatic restructuring at metamorphosis and the production of a juvenile. Species with little maternal investment per offspring typically spawn many small eggs with development through feeding (planktotrophic) larvae (Emlet et al. 1987, Jaeckle 1995, Sewell & Young 1997, Sewell 2005, Prowse et al. 2008, Moran & McAlister 2009, Moran et al. 2013, Falkner et al. 2015). At a minimum, the eggs of species with planktotrophic development must contain sufficient material for the production of an exotrophic larva capable of feeding in the plankton. Of the egg components used to fuel larval construction and development, lipids appear to be more important than proteins or carbohydrates (Holland 1978, Jaeckle 1995, Moran & Manahan 2003).

Within the Echinodermata, species with planktotrophic development have eggs that are dominated by proteins and structural lipids (e.g. phospholipids) and which also contain a small amount of energy-storage lipids (Sewell 2005, Meyer et al. 2007, Prowse et al. 2008, 2009, Whitehill & Moran 2012, McAlister & Moran 2013, Falkner et al. 2015). The latter are mainly triacylglycerol (TAG), but may also include other lipids such as diacylglycerol ether (DAGE), ali-

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phatic hydrocarbon and the wax and methyl esters (Sewell 2005, Falkner et al. 2006, Meyer et al. 2007, Byrne et al. 2008a,b, Prowse et al. 2008, 2009, Whitehill & Moran 2012, McAlister & Moran 2013). These energetic lipid classes (see Marangoni 2017) play a major role in fuelling larval construction (Sewell 2005, Meyer et al. 2007, Byrne et al. 2008b, Prowse et al. 2008).

Once the larval digestive tract has differentiated, planktotrophic larvae enter the facultative feeding period (FFP), during which they do not require food to continue development because maternal provisions are still available (Herrera et al. 1996, Reitzel et al. 2005). The exhaustion of energetic lipid reserves in unfed larvae appears to mark the end of the FFP (Miner et al. 2005, Byrne et al. 2008b). Feeding during the FFP is beneficial as it allows larvae to maintain maternally derived energetic lipid reserves for longer (Byrne et al. 2008a) and may have positive downstream effects on developmental duration and the condition of post-metamorphic juveniles (Reitzel et al. 2005).

Beyond the FFP, the successful recruitment of planktotrophic larvae relies on their ability to accumulate energy reserves to fuel metamorphosis and the perimetamorphic period, during which the juvenile digestive tract forms (Fenaux et al. 1988, George et al. 1990b, Vaïtilingon et al. 2001, Pernet et al. 2006). Late-stage asteroid and echinoid larvae possess substantial lipid reserves (Fenaux et al. 1985, George et al. 1990a, 1997, Byrne et al. 2008a). Much of this lipid is deposited in the stomach epithelium, where it can be stored for metamorphosis and/or the perimetamorphic period (Chia & Burke 1978, Reitzel et al. 2004, Byrne et al. 2008a). Competent settlement stage larvae of the echinoids *Strongylocentrotus droebachiensis* and *Tripneustes gratilla*, and several marine bivalves, accumulate significant stores of TAG, the same lipid class that dominates their eggs, which are thought to support metamorphosis (Gallager et al. 1986, Bochenek et al. 2001, Villinski et al. 2002, Pernet et al. 2004, Powell et al. 2004, Byrne et al. 2008a).

The dynamics of lipid-class use during the prefeeding stage and accumulation during the feeding larval stage has not been characterised through development of echinoderms with planktotrophic larvae. We used 2 echinoderm species with feeding larvae—the sea star *Patiriella regularis* (165 µm diameter egg) and the sea urchin *Heliocidaris tuberculata* (95 µm diameter egg) (Raff & Byrne 2006, Prowse et al. 2008)—to investigate the depletion and sequestration of energetic and structural lipid classes throughout development to the juvenile. In addition, we used a nutritional condition index (energetic lipid:sterol content), as previously used in crustacean, bivalve and fish larvae, which is independent of organism size because sterol is largely cell membrane cholesterol (Fraser 1989, Håkanson 1989, Harding & Fraser 1999, Pernet et al. 2003a, Tremblay et al. 2007, Burke et al. 2008). Using this index, together with data for individual lipid classes, we tracked the nutritional condition of the larvae of *P. regularis* and *H. tuberculata* through development to determine the ability of feeding larvae to improve their nutritional condition prior to metamorphosis. We addressed 4 questions. (1) When are lipids utilized and sequestered by planktotrophic echinoderm larva as they develop from fertilization to metamorphosis? (2) Are energy-storage lipids depleted more slowly in fed than starved larvae, as documented for larvae of other sea urchins (Sewell 2005, Meyer et al. 2007, Byrne et al. 2008b, McAlister & Moran 2013)? (3) What classes of energy-storage lipids are accrued by feeding larvae late in development? (4) How does the nutritional condition of larvae change as they develop?

MATERIALS AND METHODS

Spawning, egg sampling and fertilization

Patiriella regularis and *Heliocidaris tuberculata* were collected in Hobart, Tasmania (42° 53′ 15″ S, 147° 20′ 20″ E) and Chowder Bay, Sydney, New South Wales (33° 50′ 29″ S, 151° 15′ 11″ E), respectively. Dissected *P. regularis* ovaries (2 females) were placed in 10^{-5} M 1-methyladenine (1-MA) in 1-µm filtered seawater (FSW) to induce egg maturation and ovulation. *H. tuberculata* (3 females) were induced to spawn with an intracoelomic injection of 1–2 ml 0.5 M KCl. All eggs were rinsed 3 times with FSW before sampling and fertilisation. Three samples of eggs from each female (n = 600 and 700 eggs per sample for *P. regularis* and *H. tuberculata*, respectively) were collected from egg suspensions of a known concentration, as determined using a Sedgewick-Rafter counting chamber. The egg samples were placed in microcentrifuge tubes, briefly centrifuged, the excess seawater removed, and stored at –80°C until analysis.

Feeding larvae to metamorphosis—*P. regularis*

Eggs from the 2 females were each split between three 600 ml culture jars of FSW and fertilized with sperm pooled from 3 males (~10^5 sperm ml^{-1}). The embryos (15 ml^{-1}) were reared at 18–19°C. Water

changes (90%) were conducted daily by reverse filtration. Embryo and larval samples were collected from each jar (i.e. 3 samples per female) as described above at 24 h (hatched gastrula), 48 h (developing bipinnaria) and 72 h (early bipinnaria).

Feeding-stage bipinnaria (3 d) from each female were transferred into two 20 l plastic containers (1.5 larvae ml^{-1}). One culture was fed the diatom *Chaetoceros muelleri* (20 000 cells ml^{-1}) every second day, and the other was not fed. As the 1-µm FSW was not sterilized, this unfed culture may have had small quantities of food such as microorganisms. The cultures were aerated by gentle bubbling to keep the larvae in suspension, and water changes (90%) were conducted every 2–4 d. For the fed cultures, water changes were carried out just before feeding. Larvae were sampled (3 microcentrifuge tubes from each culture) for lipid analysis on Days 7 (bipinnaria), 23 (late bipinnaria) and 35 (brachiolaria). On Days 7 and 23, the number of larvae collected per sample was 600 and 220, respectively. For the final sample on Day 35, the number of larvae collected per sample from the fed and unfed cultures was 50 and 280, respectively.

To examine the energetics of metamorphosis, eggs from a third *P. regularis* were fertilized with sperm pooled from several males. The larvae were reared in a single 20 l container and fed (as above) to the brachiolaria stage (30 d). They were then placed in eight 200 ml glass culture dishes (n = 100 larvae per culture), fed (as above) with water change every 2 d. By Day 47, most larvae were competent to settle, as indicated by the presence of a well-developed juvenile rudiment and searching behaviour. Competent larvae were collected for lipid analysis (n = 25 larvae from 4 dishes). To induce settlement, another set of competent larvae were transferred to four 200 ml glass dishes (n = 25 larvae per dish) containing fragments of coralline algae. At Day 53, juveniles were collected (n = 25 juveniles per dish). Juveniles were defined as benthic, mobile individuals (2–3 d post-settlement). As these juveniles originated from a single female parent, the mean lipid data for the 4 dishes are presented, but were not analysed statistically.

Feeding larvae to metamorphosis—*H. tuberculata*

Eggs from the 3 females were each split between three 600 ml culture jars of FSW and fertilized with sperm pooled from 3 males (~10^5 sperm ml^{-1}). The embryos (15 ml^{-1}) were reared at 18–19°C with daily water changes (90%). Embryo and larval samples were collected from each jar (3 samples per female per time point) at 17 h (hatched gastrulae) and 48 h (early 2-armed echinopluteus) (as above). On Day 3, feeding echinoplutei from each female were placed in six 2 l glass beakers at a density of 10 ml^{-1} (6 beakers per female, 18 beakers total). For each female, the larvae in 3 beakers were fed (*C. muelleri*, 20 000 cells ml^{-1} every 2 d) and those in the other 3 beakers were not fed. The cultures were gently stirred using an automated paddling system. Larvae were sampled from each beaker for lipid analysis (as above) on Days 4 (4-armed larvae), 12 (6-armed larvae), 29 (8-armed larvae) and 43 (rudiment-stage larvae). On Days 4, 12 and 29, 700 individuals were collected per sample. Depending on availability, on Day 43, 20–34 larvae were collected. Unfed larvae were sampled on Day 4 and 12 (500 per sample), after which time the unfed cultures (from Female 1 only) exhibited developmental arrest and mortality.

On Day 43, rudiment-stage larvae (n = 20) from each of the 3 beakers were placed in individual 10 ml wells in 6-well plastic culture dishes containing a small piece of coralline algae to induce settlement. Metamorphosis occurred over the following week. Juveniles were defined as benthic, mobile individuals (2–3 d post-settlement). Juveniles (Day 49) from each female were pooled (9–15 juveniles per female, n = 3 females) to give sufficient material for lipid analysis.

Lipid extraction and analyses

Lipid was extracted from frozen egg samples as described in Prowse et al. (2008). Total lipid extracts were dissolved in a known volume of chloroform (5–30 µl). Lipid classes in *P. regularis* samples were separated, identified and quantified using an Iatroscan Mark V^{new} Thin-Layer Chromatography/Flame Ionization Detection (TLC/FID) system and the triple-stage protocol recommended by Parrish (1999) with the modifications of Sewell (2005). The third stage of the Parrish (1999) method separates 2 classes of polar lipid, acetone mobile polar lipids and phospholipid, from non-lipid material (NLM) present in the sample. As the results obtained for *P. regularis* showed that NLM was negligible, only the first 2 stages were used for analysis of *H. tuberculata* samples. Total polar lipid (phospholipid + acetone mobile polar lipid) was determined for each sample. The polar lipids are dominated by phospholipid, the major constituent of cell membrane, and defined as structural lipids. Identification of lipid classes was achieved by comparison

with lipid standards. Details of the different classes are provided in Marangoni (2017).

Quantification of lipids was achieved using calibration curves produced for each lipid class on a rack of 10 chromarods. A single DAGE + TAG peak was quantified using the calibration curve for TAG (Prowse et al. 2009). The total energetic lipid per individual was calculated as the sum of aliphatic hydrocarbon, DAGE + TAG and the methyl and wax esters if present. A lipid nutritional condition index was calculated as the ratio of energetic lipid to sterol content.

The DAGE + TAG peak in the chromatograms of the larvae of *P. regularis* was investigated in 1 sample of brachiolaria (Day 47), and juveniles (Day 53) (n = 25 for each stage) were examined using TLC/FID after a 1-stage hexanediethyl ether (96:4 v/v) development (Phleger et al. 1997, Nelson et al. 1999, Nichols et al. 2001, Prowse et al. 2009).

Larval morphometry and histochemistry

Fed larvae of *P. regularis* (Days 7, 23, 35) and *H. tuberculata* (Days 12, 29) (n = 5 species^{-1} d^{-1}) were placed in a 1.5 ml tube and fixed with 1 drop of 10% formalin. These larvae were photographed and measured using image analysis software (ImageJ) as in previous studies of asteroid and echinoid larvae (George 1999, Sewell et al. 2004, Wolfe et al. 2015). For *P. regularis*, larval length was measured as the distance between the anterior and posterior ends of the larvae. For *H. tuberculata*, larval length was calculated by summing the lengths of the postoral arm rod and the body rod.

Advanced *P. regularis* larvae (Day 30, n = 10), were stained with the fluorochrome Nile Red to identify sites of neutral lipid storage. When bound to neutral lipids, Nile Red fluoresces yellow under blue light excitation (Carman et al. 1991). To stain larvae, 5 µl of a 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, placed on a glass slide and photographed using an Olympus BX60 epifluorescence microscope with 488 nm excitation. Unstained larvae were used as controls.

Statistical analyses

For the 2 fed cultures of *P. regularis*, a subsampled repeated-measures ANOVA (RMANOVA) (Quinn & Keough 2002) was used to examine temporal changes in 4 variables (energetic lipid, sterol, polar lipid and lipid nutritional condition index). This design was employed because larvae from each *P. regularis* female were subsampled from a single culture. For each variable, 3 planned comparisons (*t*-tests) were made between (1) eggs and bipinnaria (Day 7), (2) bipinnaria and late bipinnaria (Day 23) and (3) late bipinnaria and brachiolaria (Day 35).

Data for changes in 4 variables (energetic lipid, sterol, polar lipid and lipid condition index) for the fed cultures of *H. tuberculata* reared to rudiment-stage larvae (Day 43) were analysed by RMANOVA. These analyses included female as a random, between-subjects factor, cultures as the subjects and time as the repeated measure. To guard against potential violation of the sphericity assumption, p-values for the female × time interaction effect were corrected by using Greenhouse-Geisser adjusted df. One *H. tuberculata* culture failed by Day 5, leaving missing cells for all variables at the remaining 3 sampling times. Lipid-class data were also only partially obtained for one other *H. tuberculata* sample. Missing values were imputed using the closest match method recommended for repeated-measure designs (see Elliott & Hawthorne 2005); specifically, data for 3 missing samples from the *H. tuberculata* culture that failed by Day 5 were imputed with lipid-class data from the closest matching culture as judged by RMSE. The df for the time × culture (female) interaction term were decreased by the number of imputed values (prior to Greenhouse-Geisser adjustment) and the p-value for the female × time interaction terms calculated accordingly. For each variable, 3 planned comparisons (*t*-tests) were made between (1) eggs and early 6-arm larvae (Day 12), (2) early 6-arm and early 8-arm larvae (Day 29), and (3) early 8-arm and rudiment-stage larvae (Day 43).

To determine when fed and unfed larvae of *H. tuberculata* diverged in lipid-class content, a 1-way ANOVA with 4 levels (unfed larvae of Female 1 and fed larvae of Females 1, 2 and 3) was used for data obtained from the fed and unfed cultures at Days 4 and 12. A single planned contrast (*t*-test) was used to compare the unfed culture with the mean of the fed cultures at these sampling times. Prior to all ANOVAs, the assumptions (normality, homogeneity of group variances) were checked using residual analysis (Quinn & Keough 2002); data were log (x + 1) transformed as required to meet these assumptions.

RESULTS

Egg lipid profiles

The eggs of *Patiriella regularis* (165 µm diameter) contained far more lipid than those of *Heliocidaris tuberculata* (95 µm diameter) (140 and 28.6 ng, respectively), reflecting their larger size. Structural lipids dominated the eggs of both species (Fig. 1). Energetic lipids contributed 33.7 and 35.3% of total egg lipid in *P. regularis* and *H. tuberculata*, respectively (Fig. 1). The combined DAGE + TAG peak dominated energetic lipid reserves in both species, accounting for 93.4 (*P. regularis*) and 96.3% (*H. tuberculata*) of egg energetic lipid (Fig. 1). The eggs of *P. regularis* contained low levels of methyl ester (Fig. 1), while *H. tuberculata* eggs contained trace levels of wax ester (not shown). Proportions of the lipid groups (aliphatic hydrocarbon, DAGE + TAG, sterol, polar lipid) were similar in the eggs of both species (Fig. 1).

P. regularis

Lipid content of the fed bipinnaria larvae of *P. regularis* (Fig. 2) decreased rapidly over the first 7 d (Fig. 3), while the unfed larvae exhibited developmental arrest. In fed larvae (mean ± SE length = 529 ± 13 µm, n = 5) (Fig. 2a) the egg stores of energetic lipid were all but exhausted, being reduced from 47.2 ng egg^{-1} to 0.7 ng ind.$^{-1}$ (Table 1, Fig. 3a). There were also significant decreases in sterol and polar lipid over the first 7 d (sterol: 5.3 to 2.8 ng ind.$^{-1}$; polar lipid: 87.6 to 43.0 ng ind.$^{-1}$) (Table 1, Fig. 3b,c). The loss of energetic lipid exceeded that of sterol, so that the original egg lipid condition index of 8.96 was reduced to 0.26 (Table 1, Fig. 3d).

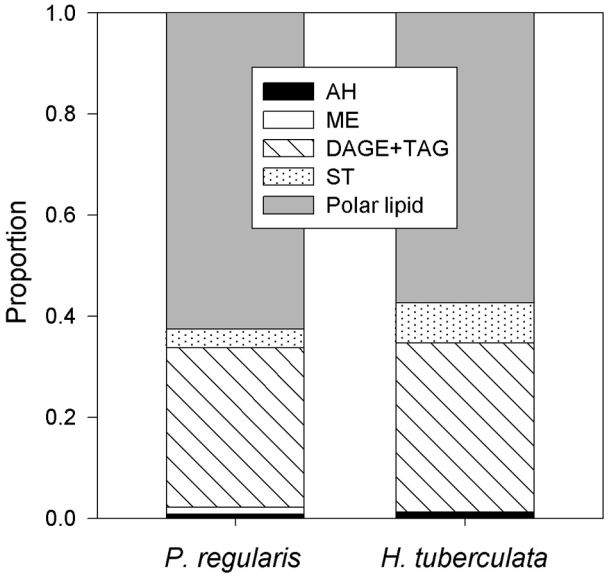

Fig. 1. *Patiriella regularis* and *Heliocidaris tuberculata*. Proportional contribution of lipid classes to total egg lipid content. AH: aliphatic hydrocarbon; DAGE: diacylglycerol ether; ME: methyl ester; ST: sterol; TAG: triacylglycerol

In fed larvae, the content of energetic lipid, sterol and polar lipid increased significantly to the late bipinnaria stage (Day 23) (Table 1, Fig. 3b,c), although there was no significant change in the larval lipid condition index (Table 1). These larvae had a mean (±SE) length of 642 ± 44 µm (n = 5) (Fig. 2b) and continued to grow, reaching a mean length of 1067 ± 20 µm (n = 5) by the brachiolaria stage (Day 35) (Fig. 2c). The larvae accumulated more energetic lipid than sterol and thereby improved their lipid condition index from 0.26 to 6.59 (Table 1, Fig. 3). Brachiolaria (Day 35) from the 2 females contained a mean total lipid content of 629 ng, including 171 ng energetic lipid, 26 ng sterol and 432 ng polar lipid. When viewed under blue light, brachiolaria (Day 35) stained with Nile Red fluoresced bright yellow in the brachiolaria arms, the ciliated band and stomach, indicating the presence of neutral lipids in these regions (Fig. 2d).

The competent brachiolaria of *P. regularis* (Day 47) had a mean (±SE) total lipid content of 865 ± 29.6 ng (n = 4), including 121 ± 12.3 ng (n = 4) energetic lipid, 40 ± 1.4 ng (n = 4) sterol and 704 ± 26.1 ng (n = 4) polar lipid (Fig. 4). Following metamorphosis, juvenile *P. regularis* contained a mean total lipid content of 720 ± 48 ng (n = 4), including 69 ± 6.8 ng (n = 4) energetic lipid, 46 ± 2.0 ng (n = 4) sterol and 605 ± 4.1 ng (n = 4) polar lipid (Fig. 4). Thus, metamorphosis in the offspring of the single female resulted in a drop in the lipid nutritional condition index from a mean (±SE) of 2.89 ± 0.27 (n = 4) in competent larvae to 1.38 ± 0.13 (n = 4) by the juvenile stage. Separation of the DAGE + TAG peak into its constituent components demonstrated that the DAGE contribution was similar for competent brachiolaria (31% of the combined peak) and juveniles (39%).

H. tuberculata

By Day 12, fed *H. tuberculata* larvae (mean ± SE length = 555 ± 16 µm, n = 5) (Fig. 5a) had 6 arms; the postoral and anterolateral arms and the posterodorsal arms were just beginning to grow (Fig. 5a). Energetic lipids were rapidly depleted during early devel-

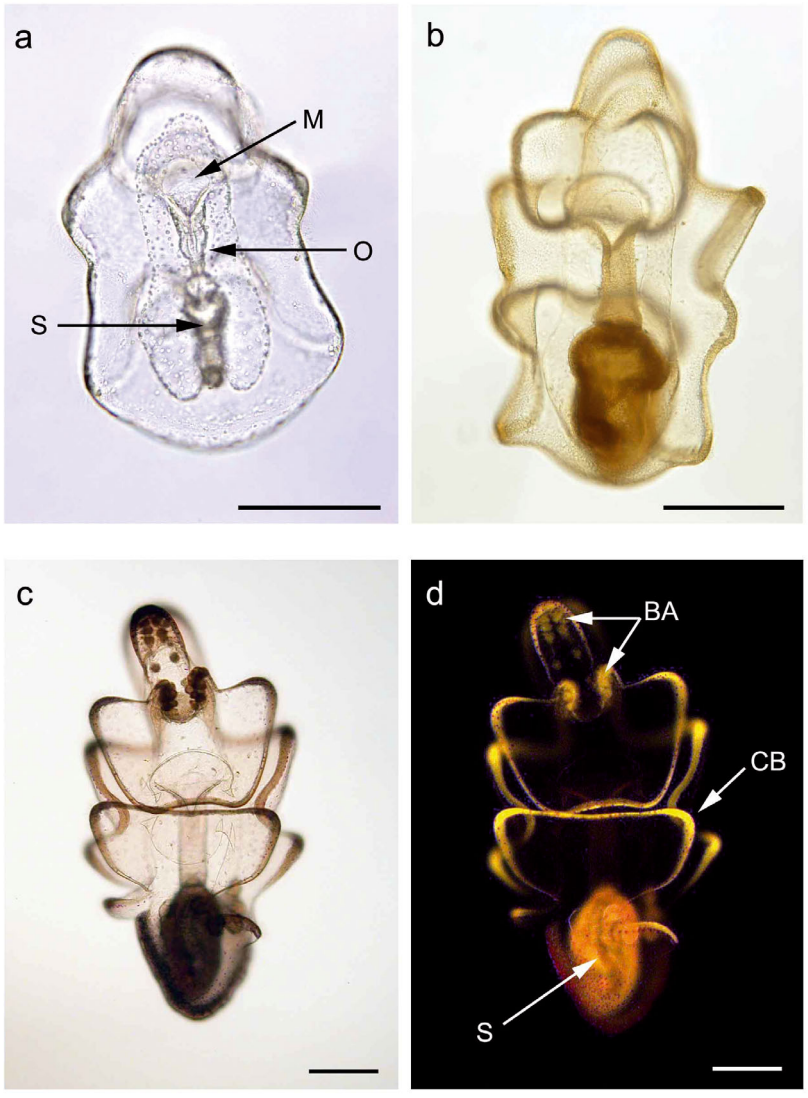

Fig. 2. *Patiriella regularis* fed larvae. (a) Day 7, bipinnaria; (b) Day 23, late bipinnaria; (c) Day 35, brachiolaria; (d) the same brachiolaria as in (c) stained with Nile Red and viewed under blue light excitation. BA: brachiolaria arms; CB: ciliated band; M: mouth; O: oesophagus; S: stomach. Scale bars = 200 µm

opment of fed *H. tuberculata* larvae, and by Day 12 the DAGE + TAG peak was below the detection limit (Fig. 6a, Table 2). Larval content of structural lipids increased significantly over this period (Table 2, Fig. 6b,c). Sterol content increased from 2.3 to 3.9 ng ind.$^{-1}$ and polar lipid content increased from 16.4 to 35.4 ng ind.$^{-1}$. Due to the depletion of energetic lipid reserves and the rise in sterol content, the lipid condition index was reduced from 47.0 in the egg to 0.07 at Day 12 (Table 2, Fig. 6c). The DAGE + TAG peak remained undetectable in fed larvae for the remainder of the experiment. Trace levels of aliphatic hydrocarbon were detected in advanced larvae. There were no subsequent changes in energetic lipid content or the lipid condition index (Table 2, Fig. 6a,d).

By Day 29, fed cultures contained early 8-arm larvae (mean ± SE length = 650 ± 18 µm, n = 5). The posterodorsal arms were well developed and the pre-oral arms were forming (Fig. 5b). Between the early 6-arm larva and the early 8-arm stage (Fig. 5c), larval content of the structural lipid classes did not change (Table 2, Fig. 6b,c). Rudiment-stage larvae (Day 43) began to resorb their arms (Fig. 5d). Larval content of sterol and polar lipid increased up to this stage (sterol: 3.2 to 26 ng ind.$^{-1}$; polar lipid: 28.2 to 148.1 ng ind.$^{-1}$) (Table 2, Fig. 6b,c). Following exposure to a settlement cue (coralline algae), the larvae settled and metamorphosed over the following week (Fig. 5e,f). Juveniles (Day 49) began their benthic existence with 396 ng ind.$^{-1}$ of total lipid, which was almost entirely sterol and polar lipid (Fig. 6). The lipid condition index of the juveniles was only 0.001. There were no significant differences between the offspring of the 3 females in lipid content or lipid nutritional condition index (Table 2, Fig. 6).

Starved larvae of *H. tuberculata* had undetectable DAGE + TAG at early 4-arm larva (Day 4) when this peak was still present for fed larvae. At this time, the energetic lipid content of fed larvae was 0.28 ng ind.$^{-1}$ and exceeded the 0.02 ng ind.$^{-1}$ present in unfed larvae at 4 d (*t*-test, t_8 = 12.5, p < 0.001) (Fig. 6a). By Day 12, unfed larvae arrested development and their sterol and polar lipid content was significantly lower than that of fed larvae (*t*-tests, t_7 = 5.9 and 6.2, p = 0.001 and p < 0.001, respectively) (Fig. 6b,c).

DISCUSSION

This study of the dynamics of lipid classes over development to the juvenile stage in echinoderms with planktotrophic larvae shows that changes in energetic and structural lipids can be divided into 3 distinct phases: (1) early rapid depletion of energetic lipids; (2) larval growth with no improvement in lipid

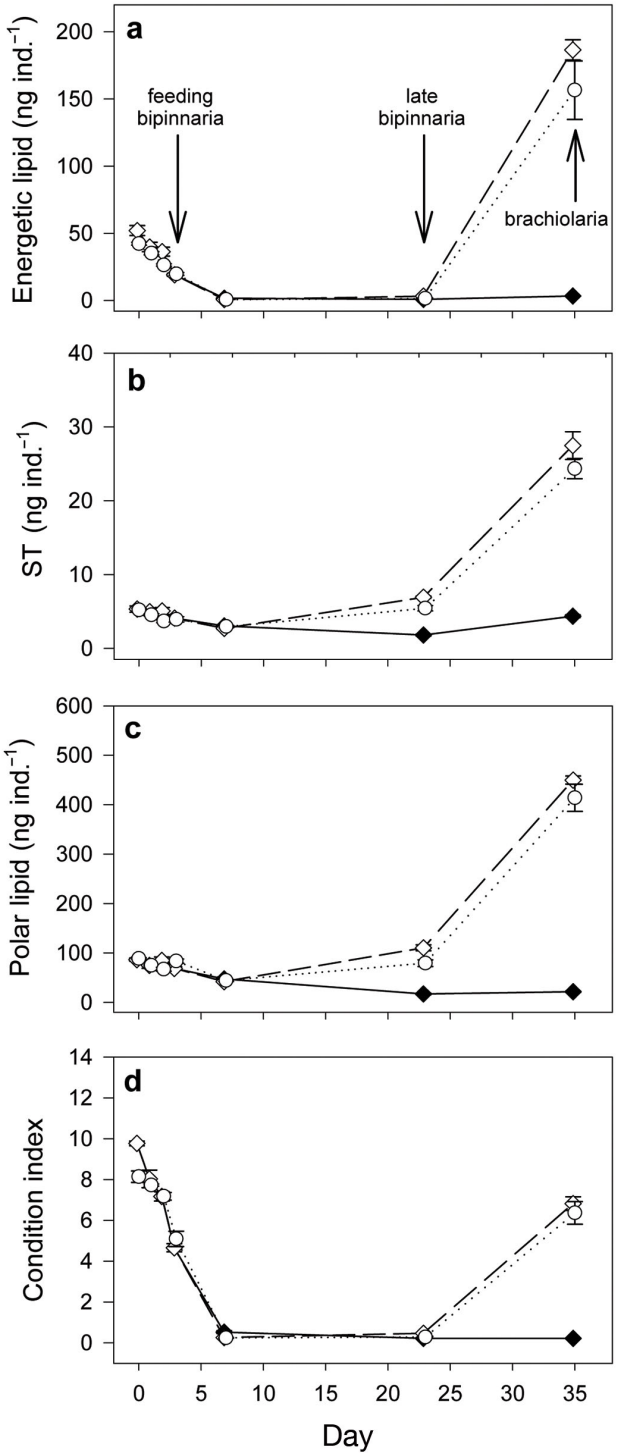

Fig. 3. *Patiriella regularis*. (a) Energetic lipid, (b) sterol (ST) and (c) polar lipid content per offspring from egg to the brachiolaria stage. (d) Lipid condition index of the same offspring, energetic lipid:sterol ratio. Arrows to larval stages in (a) apply in all panels to fed larvae only. Error bars (mean ± SE, n = 3 samples from the same culture after 3 d) are obscured by symbols in some cases. ◆: unfed offspring of Female 1; ◇: fed offspring of Female 1; ○: fed offspring of Female 2

nutritional condition; and (3) accumulation of lipids in advanced larvae prior to metamorphosis. This is a modification of the 2-stage hypothesis of Sewell (2005) as it incudes a period of 'stasis' between the periods of lipid depletion and lipid accumulation.

Phase 1. Rapid depletion of energetic lipids during embryogenesis and the FFP

The offspring of *Patiriella regularis* and *Heliocidaris tuberculata* quickly utilized their maternal TAG/DAGE lipid reserves, as found for other asteroids and echinoids with planktotrophic development (Byrne & Cerra 2000, Sewell 2005, Meyer et al. 2007, Byrne et al. 2008b, Prowse et al. 2008, Moran & McAlister 2009, Whitehill & Moran 2012, Moran et al. 2013). This reflects the need to fuel the intense morphogenetic activity associated with the production of a functional feeding larva (Sewell 2005, Moran & Allen 2007).

Energetic lipid content (TAG) decreased significantly more slowly in fed than unfed larvae of *H. tuberculata*, as also seen in *Evechinus chloroticus* and *Tripneustes gratilla* (Sewell 2005, Byrne et al. 2008b). Fed echinoplutei may maintain a buffer of energetic lipid to guard against a temporally variable food supply (Byrne et al. 2008b). This buffer, however, is short-lived, as shown here for *P. regularis* and *H. tuberculata* and previously for *Evechinus chloroticus* (Sewell 2005). It appears that early larvae maximize growth to achieve feeding competence at the expense of preserving energetic lipid stores.

Phase 2. Larval growth with little improvement in nutritional condition

Once energetic lipid reserves were depleted, fed larvae of *P. regularis* and *H. tuberculata* developed more slowly and accumulated little or no lipid. For *P. regularis*, only small amounts of energetic and structural lipid were accumulated between the bipinnaria (Day 7) and late bipinnaria (Day 23), and the lipid nutritional condition index of larvae did not change. The increase in structural lipid content reflected the substantial increase in larval size over this period from 529 to 642 µm in length. For *H. tuberculata*, no significant change was detected in any lipid class or in the lipid condition index from the early 6-arm (Day 12) to early 8-arm (Day 29) larval stages, despite the addition of one extra pair of arms and the expected increase in feeding efficiency. Similarly, there is little

Table 1. *Patiriella regularis*. Summary of the subsampled RMANOVA used to examine changes in offspring lipid class content and lipid nutritional condition index (CI) over time. The full design is illustrated for offspring energetic lipid content: F = female; T = time; F × T = female × time interaction; C = culture, nested within F × T; S = sample nested within culture and F × T. The effect of time has been tested by calculating the *F*-ratio using the mean square of the F × T interaction effect as the denominator (Quinn & Keough 2002). The results of planned contrasts (*t*-tests) between certain sampling times are also shown. Significant results are highlighted in **bold**

Variable	Source of variation	df	$F_{6,6}$	p	Egg vs. 7 d		7 d vs. 23 d		23 d vs. 35 d	
					t_6	p	t_6	p	t_6	p
Energetic lipid	F	1	No test							
	T	6	287.84	**0.001**	25.02	**0.001**	91	**0.003**	29.7	**0.001**
	F × T	6	No test							
	C(F × T)	0								
	S(C(F × T))	28								
	Residual	0								
Sterol	Time	6,6	132.06	**0.001**	6.28	**0.001**	7.99	**0.001**	16.70	**0.001**
Polar lipid	Time	6,6	63.34	**0.001**	5.54	**0.001**	6.02	**0.001**	12.05	**0.001**
CI	Time	6,6	486.12	**0.001**	36.05	**0.001**	1.59	0.163	29.73	**0.001**

change in total lipid content of other feeding echinoid larvae in the period immediately following larval construction (Cellario & George 1990, George et al. 1997, Sewell 2005, Whitehill & Moran 2012). As the lipid condition index of neither *P. regularis* nor *H. tuberculata* improved during this stage, energy derived from planktonic food is likely to be used for growth and maintenance rather than being processed for storage.

Phase 3. Accumulation of lipids in advanced larvae prior to metamorphosis

The larvae of *P. regularis* grew substantially and accumulated large amounts of structural lipid (sterol and polar lipid) during the brachiolaria larval stage. Brachiolaria larvae also deposited energetic lipid (TAG + DAGE) and improved their lipid condition index, indicating that increases in energetic lipid content were not simply due to increased larval size. Conspicuous increases in the total lipid content of planktotrophic echinoid larvae occur just prior to metamorphosis (Fenaux et al. 1985, George et al. 1990a, 1997, Byrne et al. 2008a). Fluorescence microscopy revealed that the stomach of advanced *P. regularis* brachiolaria serves as a site of storage of neutral lipid, similar to that indicated by histological sections of late-stage echinoid larvae (Chia & Burke 1978, Reitzel et al. 2004, Byrne et al. 2008a). Competent echinoplutei of *Strongylocentrotus droebachiensis* and *Tripneustes gratilla* contain significant TAG and phospholipid deposits (Villinski et al. 2002, Byrne et al. 2008a) and those of *Evechinus chloroticus* and *Strongylocentrotus purpuratus* accumulate free fatty acid (Sewell 2005, Meyer et al. 2007).

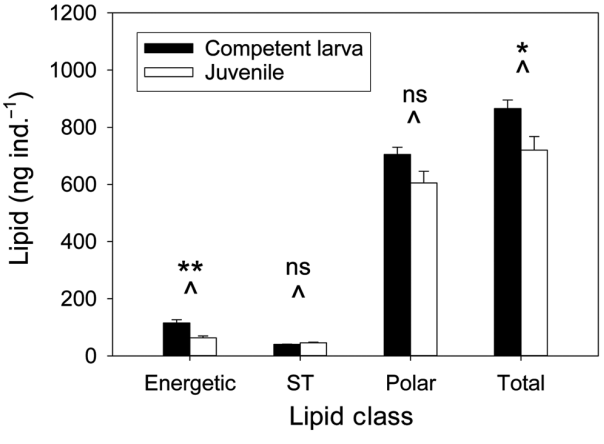

Fig. 4. *Patiriella regularis*. Lipid-class content per offspring and lipid condition index for competent larvae and juveniles from a single *P. regularis* female. Error bars are mean ± SE (n = 4). ST: sterol. ns: not significant; *p < 0.05; **p < 0.01

The ability for advanced larvae of *P. regularis* to improve their lipid nutritional condition is likely to be important in the success of the early postlarval stages (Byrne 2008a). Once larvae are close to their maximum size, improvements in feeding ability and/or a reduced growth rate of larval structures (e.g. ciliary band) may allow more energy to be diverted to storage in the gut wall as a prelude to metamorphosis.

In *P. regularis*, the process of metamorphosis is likely to be metabolically expensive since energetic lipid content declined by 43% over this period, although some of this decline may be due to basal

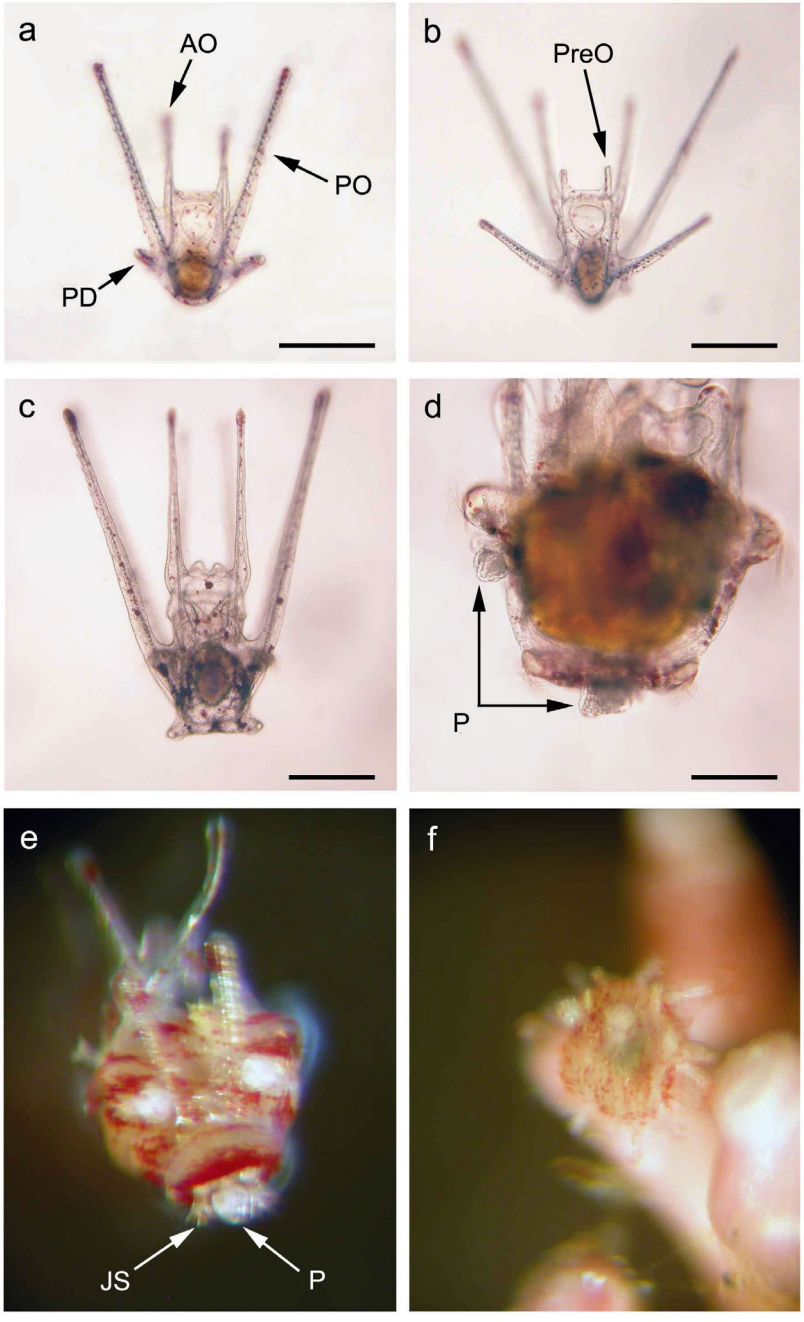

Fig. 5. *Heliocidaris tuberculata* fed larvae. (a) Day 12, early 6-arm larva; (b) Day 29, early 8-arm larva; (c) Day 36, 8-arm larva; (d) Day 43, rudiment-stage larva; (e) Day 46, settling larva; (f) Day 49, juvenile sea urchin. AO: antero-lateral arm; PO: postoral arm; PD: posterodorsal arm; PreO: preoral arm; P: pedicellariae; JS: juvenile spines. Scale bars = (a–c) 200 µm; (d) 100 µm

maintenance. Newly metamorphosed *P. regularis* juveniles retained 69 ng ind.$^{-1}$ of energetic lipid (~representing 10% of total lipid) that is presumably used to fuel subsequent growth and development. These energetic reserves may be particularly important during the perimetamorphic period, before the juveniles are capable of independent feeding (Gosselin & Jangoux 1998). There was no change in polar lipid or sterol content over the course of metamorphosis.

The larvae of *H. tuberculata* accumulated structural lipid between the early 8-arm and the rudiment stage larva and, in contrast to *P. regularis*, did not sequester energetic lipid reserves prior to metamorphosis. The low lipid nutritional index of competent *H. tuberculata* larvae and juveniles may indicate either that the algal diet was suboptimal, or that there may be a role for non-energetic lipid classes, such as phospholipids, in fuelling the last of the 3 larval phases, as described for the larva to juvenile transition in lobsters (Jeffs et al. 2002). Support for the role of phospholipids as an energy source in *H. tuberculata* is the finding that spermatozoa of several sea urchin species use phospholipid to produce fatty acids to fuel swimming (Mita & Nakamura 1998). Future work on *H. tuberculata* might investigate this hypothesis by measuring the activity of phospholipases during the final phase of larval development.

If such differences, as we see here for lipid dynamics in the larvae of *P. regularis* and *H. tuberculata*, are present in the natural environment, this will likely contribute to variation in mortality in the plankton and after metamorphosis, as observed in other invertebrate taxa (Pechenik et al. 1998, Moran & Emlet 2001, Phillips 2002, 2004, Marshall & Keough 2004). Larval experience and ability to accrue nutrients to support the early juvenile may also influence size at metamorphosis (Pechenik et al. 1998, Emlet & Sadro 2006, Byrne et al. 2008a). The differing capacity of the larvae of the 2 species investigated here to accrue energetic lipid reserves for the juvenile, long after the egg energetic lipid reserves are exhausted, shows that use of egg size as a proxy for offspring size (e.g. Marshall et al. 2012) has its limitations.

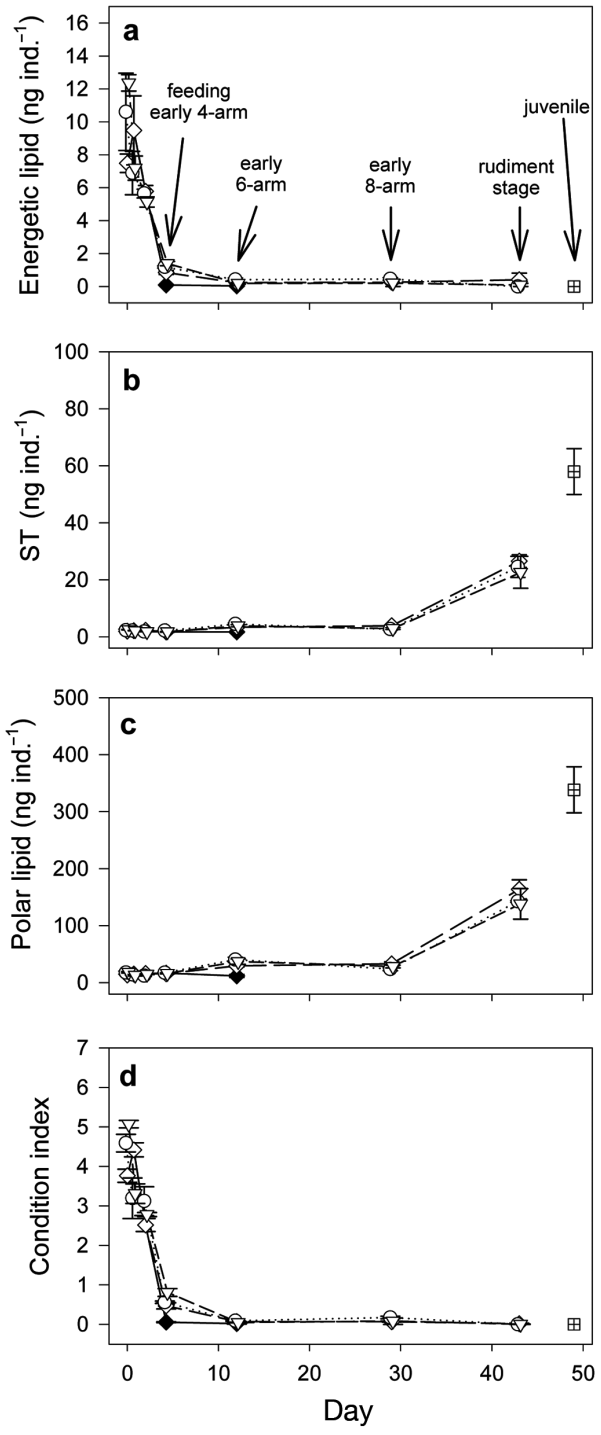

Fig. 6. *Heliocidaris tuberculata*. (a) Energetic lipid, (b) sterol (ST) and (c) polar lipid content per offspring from egg to juvenile. (d) Lipid condition index of the same offspring, energetic lipid:sterol ratio. Labels in (a) apply in other panels to fed larvae only. (◆) unfed offspring of Female 1; (◇) fed offspring of Female 1; (○,▽) fed offspring of Females 2 and 3, respectively. Error bars (mean ± SE, n = 3 samples per female, except n = 1 per female for juveniles) are obscured by symbols in some cases. (⊞) pooled sample of juveniles (n = 3 females)

Energetic lipids accrued by the larvae of *P. regularis*

Adult echinoderms, including those investigated here, are apparently capable of de novo DAGE synthesis as this lipid class is relatively rare in marine food webs and yet is deposited in their eggs (Oudejans & van der Sluis 1979, Falk-Petersen & Sargent 1982, Hayashi & Kishimura 1997, Prowse et al. 2009). It appears that the feeding larvae of *P. regularis* are also capable of DAGE synthesis as the *Chaetoceros muelleri* algae fed to them are not a source of this lipid class (Pernet et al. 2003b). Both DAGE and TAG fuel embryogenesis, metamorphosis and early juvenile development in *P. regularis*.

Energetic lipids played a critical role during the embryogenesis of the feeding larvae of *P. regularis* and *H. tuberculata*, but the role of lipids in metamorphosis is less clear and is likely to vary with phylogeny and larval experience (e.g. food supply). Further biochemical studies are required to elucidate how morphogenesis during this crucial life history transition is fuelled in echinoderms with planktotrophic development and whether phospholipids may also be used as an energy source in this phylum.

Following depletion of maternally derived energy storage lipids for larval construction, the larvae of *P. regularis* and *H. tuberculata* fed, grew and developed for weeks without accruing significant stores of energetic lipid. There appears to be an imperative for early larvae to grow quickly and increase their food-capturing ability rather than to immediately accumulate storage energy for later development (Young et al. 1989, McEdward 1997). For feeding echinoderm larvae, developmental duration is reduced in high food environments (Fenaux et al. 1994, Reitzel et al. 2004, 2005, Sewell et al. 2004), up to a limit (see Pedrotti & Fenaux 1993, Wolfe et al. 2015). It is not known whether planktonic food supply can be sufficiently high to allow feeding larvae to maintain a significant energetic buffer throughout their development. However, the striking phenotypic plasticity of many planktotrophic echinoderm larvae in the length of their feeding apparatus (ciliary band) in response to environmental food levels with heterochronic acceleration of rudiment formation in favourable nutrient conditions (George 1999, Soars et al. 2009, Wolfe et al. 2015) indicates the potential for fine tuning of lipid dynamics.

The larvae of *P. regularis* sequestered energetic lipid prior to metamorphosis, indicating the ability to take advantage of food up to the point of benthic transition. This may explain the resistance to starva-

Table 2. *Heliocidaris tuberculata*. Summary of RMANOVA for offspring lipid class content and lipid nutritional condition index (CI) over the course of development to the rudiment stage at 43 d. The analyses included 1 random, between-subjects factor (female = F), with cultures as the subjects and time (T) as the within-subjects factor. Greenhouse-Geisser adjusted degrees of freedom (GG-df) were used for the F × T interaction effects. The results of planned contrasts (*t*-tests) between certain sampling times are also shown. Significant results are highlighted in **bold**

Variable	Source of variation	df	GG-df	*F*	p	Egg vs. 12 d		12 d vs. 29 d		29 d vs. 43 d	
						t_{12}	p	t_{12}	p	t_{12}	p
Energetic lipid	F	2,6	–	0.17	0.847						
	T	6,12	–	153.2	**0.001**	18.6	**0.001**	0.07	0.944	1.2	0.256
	F × T	12,32	5.7,15	1.67	0.198						
Sterol	F	2,6	–	0.56	0.600						
	T	6,12	–	196.7	**0.001**	5.08	**0.001**	2.18	0.050	23.0	**0.001**
	F × T	12,32	5.7,15	1.47	0.250						
Polar lipid	F	2,6	–	0.10	0.906						
	T	6,12	–	114.12	**0.001**	6.63	**0.001**	1.81	0.096	15.0	**0.001**
	F × T	12,32	6.3,16	2.34	0.077						
CI	F	2,6	–	0.59	0.584						
	T	6,12	–	204.73	**0.001**	21.8	0.001	0.33	0.750	1.2	0.269
	F × T	12,32	9,13.2	3.29	0.039						

tion exhibited by advanced feeding larvae of other asteroids (Olson & Olson 1989, Allison 1994). Given that energy-storage lipids are sequestered before metamorphosis in multiple echinoderm species (Villinski et al. 2002, Sewell 2005, Meyer et al. 2007, Byrne et al. 2008a), the timing of food pulses may be a critical factor in determining juvenile condition and performance (Phillips 2004). The feeding larvae of some species, particularly the 'boom and bust' echinoderms (e.g. *Acanthaster planci*, *Asterias amurensis*), are likely to be highly opportunistic, with food pulses late in development influencing population increase through greater numbers completing metamorphosis (Uthicke et al. 2009, Wolfe et al. 2015, 2017). To test importance of Phase 3 (lipid accumulation) and whether food pulses occurring late in development might significantly improve the nutritional condition of juveniles, larvae should be reared to an advanced stage and then subjected to different food treatments (Pechenik et al. 1996a,b).

The ecological consequences of the larval to juvenile nutritive condition need to be addressed in future research. Of particular interest will be comparison between *H. tuberculata*, where low levels of energetic lipids were found prior to metamorphosis, and our previous work on *T. gratilla* where high TAG levels remain in the juveniles (Byrne et al. 2008b). Our proposal that *H. tuberculata* might be using phospholipids to fuel metamorphosis is presented as a working hypothesis, but may require a careful reassessment of what we think we know about lipid dynamics during planktotrophic development of echinoderms.

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