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Lipid and protein utilization during lecithotrophic development in the asteroid *Stegnaster inflatus*, with a review of larval provisioning in lecithotrophic echinoderms

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ABSTRACT: The eggs of echinoderms with lecithotrophic development provide structural and nutritional materials (lipids, proteins) to complete development without any external sources of nutrition. Previous studies have suggested that a relatively large proportion of the maternal lipid reserves remain after settlement to provision the early post-metamorphic juvenile. Here we examined lipid and protein utilization during lecithotrophic development of the asterinid starfish *Stegnaster inflatus* which has large (~400 µm diameter), negatively buoyant eggs. *S. inflatus* produces eggs with a large amount of lipid (mean ± SD: 2047 ± 315 ng egg^{-1}), with 26% structural lipids and 74% energetic lipids dominated by diacylglycerol ether (DAGE; 66%). Similar amounts of protein were present in the egg (mean ± SD: 2143 ± 157 ng egg^{-1}), with a lipid:protein ratio of 0.96. Approximately 80% of the egg protein is used prior to settlement. In contrast, 51.0% of the energetic lipids provided in the egg, and 40.3% of the DAGE remain for provisioning of the early juvenile. A review of lecithotrophic development in echinoderms reveals that asteroid, echinoid and ophiuroid species have an excess of 50% of the maternal lipids remaining in the settled post-larva. When considering maternal investment in offspring for lecithotrophic developers, we need to consider energetic use during the short dispersal period, as well as provisioning for post-metamorphic early juvenile life.

KEY WORDS: Asterinidae · Echinodermata · Non-feeding larva · Demersal larva · Larval buoyancy · Lipid · Protein · Maternal provisioning

1. INTRODUCTION

Marine invertebrates with pelagic development, in general, achieve metamorphosis either through a feeding larva derived from a 'small egg' (planktotrophy) or a non-feeding larva derived from a 'larger egg' (lecithotrophy). Characterization of 'small' and 'large' eggs differs between phyla, and there can be considerable overlap in the egg sizes associated with each mode of development, within taxa and in marine invertebrates as a whole (Jaeckle 1995, Sewell & Young 1997, Moran & McAlister 2009, Marshall et al. 2012, 2018a, Moran et al. 2013).

Detailed study of the eggs of planktotrophic and lecithotrophic species has also revealed differences in biochemical composition, with lecithotrophic species having a higher lipid content than planktotrophs relative to egg size (Jaeckle 1995, McEdward & Morgan 2001, Sewell & Manahan 2001, Moran et al. 2013, Falkner et al. 2015, Peters-Didier & Sewell 2017). In the particular case of echinoderms, the large eggs of species with lecithotrophic development are not simply scaled up versions of the eggs of species with planktotrophic development, as there is also a modification in the primary energetic lipid class from triacylglycerol (TAG) to diacylglycerol ether (DAGE);

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this pattern is evident in Asteroidea, Ophiuroidea and Echinoidea (Prowse et al. 2009, Falkner et al. 2015, Byrne & Sewell 2019, Davidson et al. 2019). Additionally, there is a dichotomy in biochemical composition with respect to the location of development: pelagic lecithotrophs generally have positively buoyant eggs, with a high lipid:protein ratio, while species with benthic or brooded development usually have negatively buoyant eggs, with a more equal investment of lipid and protein (Emlet 1994, Prowse et al. 2008, Montgomery et al. 2017).

While it was initially thought that the large lipid stores in lecithotrophic echinoderms were used to fuel larval development to the juvenile stage in the absence of feeding (e.g. Mortensen 1921, Thorson 1950), there is increasing evidence that a relatively large proportion of the maternal lipid reserves remains after settlement to provision the early juvenile (Emlet & Hoegh-Guldberg 1997, Villinski et al. 2002, Falkner et al. 2015, Byrne & Sewell 2019). Here, we focus on lecithotrophic development in the asterinid *Stegnaster inflatus*, a cushion star found in the shallow subtidal throughout New Zealand (Zamora et al. 2019). This species has a large egg, ca. 400 µm in diameter (Prowse et al. 2009, Zamora et al. 2019), hatches from a gastrula to form a brachiolaria larva with limited swimming capability, begins settlement at 8 d and completes metamorphosis within 12 d (Zamora et al. 2019). In contrast to other asterinids with a free-living brachiolaria larva, the eggs of *S. inflatus* are negatively buoyant with the indication that development in this species occurs on or near the benthos (Zamora et al. 2019). Previous research by Prowse et al. (2009) identified large reserves of DAGE and small amounts of TAG in the egg. Here we describe the use of the maternal energetic reserves of lipids and proteins during development of *S. inflatus* and provide further evidence that most of the maternal lipid reserves in echinoderms with lecithotrophic development are reserved for post-metamorphic development.

2. MATERIALS AND METHODS

2.1. Larval cultures

Mature *Stegnaster inflatus* were collected from Army Bay, Whangaparaoa Peninsula, New Zealand (36° 35′ 52″ S, 174° 48′ 55″ E), during the austral summer of 2015, transported in buckets with seawater and transferred to aerated plastic tanks (L × W × H: 87 × 60 × 29 cm, containing an air stone) with flowing seawater within an environmentally controlled (18–20°C) recirculating seawater facility in the School of Biological Sciences, University of Auckland. Adults were fed small porcelain crabs *Petrolisthes novaezealandiae* for 1–2 d before obtaining gametes using 1-MA (10^{-4} M in filtered seawater) induction from 3 females (see Zamora et al. 2019 for full details).

The eggs of 3 females and the sperm from 1 male were used to provide embryos for larval cultures separated by source female. Cultures were set up in 4 l plastic tanks (N = 3 tanks per female; total 9 tanks) filled with 1 µm filtered seawater, fitted with paddle stirrers and maintained in a temperature-controlled room at 18–20°C; henceforth we refer to these cultures by their biological source (i.e. Females 1–3). The initial embryo density was 1 embryo ml^{-1}. Half of the seawater was carefully replaced by reverse filtration (50 µm Nitex screen) every 2 d until the juvenile stage was achieved (ca. 2 wk post-fertilization).

The embryos were sampled for lipid and protein quantification at definitive points in development: fertilized embryo, blastula (18 h post-fertilization [hpf]), gastrula (26 hpf), early brachiolaria (EB: 42 hpf), late brachiolaria (LB: 120 hpf), settlement (192 hpf post fertilization) and post-settlement juveniles (288 hpf). A full description of all morphological stages is provided by Zamora et al. (2019). Individuals of each stage (20 ind. $replicate^{-1}$) were collected from each larval culture using a transfer pipette, placed in a 1.5 ml Eppendorf tube, centrifuged to allow removal of seawater and stored at −80°C for later analysis. Initially we collected 3 replicate tubes from each larval culture at each sampling time for protein and lipid analysis. However, sampling for the larval description (Zamora et al. 2019) and larval mortality reduced numbers available for biochemical sampling, and the larval numbers per replicate decreased from the LB stage to 6–15 for lipids and to 5 individuals for protein. In the settlement and juvenile stages, only the progeny of 2 females were available for the lipid samples (Females 1 and 2), and in the protein samples, the progeny of 2 females were represented in LB and only 1 female for the settlement and juvenile stages.

2.2. Lipid analysis

Lipids were extracted from frozen samples following the protocols developed by Holland & Gabbott (1971), with modifications by Sewell (2005) for echinoderms, and the minor difference that we used

methanol and chloroform from the LiChrosolv Hypergrade for LC-MS range (Merck Millipore). In brief, samples were sonicated in 250 µl of MilliQ water for 15–20 s at 10 kHz using an ultrasonic disintegrator (Sanyo Soniprep 150), and lipids were extracted as described in detail by Sewell (2005) with ketone as an internal standard. Lipid extracts were stored at –20°C until analysis.

Lipid class quantification was performed in an Iatroscan MK-6s thin layer chromatography/flame ionization detection (TLC/FID) system and silica gel S-III chromarods as described by Sewell (2005). The lipid extracted from each sample was dried down in a stream of N_2 gas, and 10 µl of chloroform were added (Gilson positive displacement pipette) to re-dissolve the lipids. Aliquots of 1 µl of the re-dissolved lipid extract from each sample were spotted on 2 duplicate chromarods using a Drummond Micro-dispenser fitted with precision glass bores (± 1%). Each rack of 10 chromarods thus included 4 samples; the remaining rods were used as a blank to monitor contamination of development solvents, and the last chromarod was spotted with a dilution of a composite of highly purified lipid standards (99%) to detect possible peak position shifts due to external conditions (Peters-Didier & Sewell 2017). Chromarods were developed using a 2-stage development process based on Parrish (1987) as described in detail by Peters-Didier & Sewell (2017), and chromatograms were recorded using AZUR 4.6 software (Datalys).

Lipid class quantification was based on quadratic regressions from multilevel calibration curves (<1 to 5 µg) using highly purified lipid standards (99%) dissolved in chloroform (Sewell 2005). Standards represented the structural lipids: phospholipid (PL), sterol (ST), acetone-mobile polar lipids (AMPL); neutral energetic lipids: aliphatic hydrocarbon (AH), wax ester (WE), triacylglycerol (TAG), free fatty acid (FFA); and the internal standard ketone. We used a TAG calibration curve to quantify the amounts of the primary energetic lipid DAGE in *S. inflatus* due to the unavailability of a commercial DAGE standard. Use of a TAG standard curve in Iatroscan TLC/FID is appropriate because the FID is essentially a carbon counter (Holm 1999) and the change in effective carbon number resulting from the substitution of 1 ether bond in DAGE for 1 ester bond in TAG is negligible for molecules of this size (Jorgensen et al. 1990); calibration curves using a custom-made squalene standard for DAGE provided by Dr. P. Nichols (CSIRO) and Dr. P. Virtue (University of Tasmania) also showed no difference in slope parameters between TAG or DAGE (Prowse et al. 2009, Falkner et al. 2015). The dominance of DAGE as the primary energetic lipid in *S. inflatus* was corroborated, as in Prowse et al. (2009), by spiking the chromarods with small amounts of shark liver oil lipids extracted from Good Health 100 mg capsules, which contain 25% DAGE by weight.

2.3. Protein quantification

Total soluble protein was determined from samples homogenized and sonicated in a Tris buffer (20 mM, pH = 7.9) containing EDTA (5 mM), NaCl (130 mM) and a protease inhibitor cocktail (cOmplete™ Mini, Roche) at the recommended concentration. The sample homogenate was shaken on ice for 15 min, centrifuged at 18 000 × *g* (4°C for 20 min), and the supernatant retained. Total soluble protein was measured at 562 nm using a Micro BCA™ Protein Assay Kit (Pierce) and a microplate reader (BioRad) following Prowse et al. (2008), and a calibration curve using bovine serum albumin (BSA, $r^2 > 0.991$).

2.4. Statistical analyses

All statistical analyses were performed using the permutational multivariate analysis of variance (PERMANOVA) add-on in Primer 7.0.13 (Primer-E, Quest Research) on Euclidean distance matrices; this approach results in a classical univariate *F*-statistic, but where the p-values are obtained by permutation, and therefore avoids the assumption of normality in traditional ANOVA (Anderson 2017). Differences between the eggs from the different females were analysed using 1-way PERMANOVA, with unrestricted permutation of raw data and 9999 permutations. Lipid class and protein utilization was examined during the period of larval development until settlement (i.e. 0 to 192 h) using a 2-way PERMANOVA (factors: female, time), permutation of residuals under a reduced model and 9999 permutations. In a separate analysis, we compared post-settlement changes in lipid and protein by comparing samples at settlement (192 h) with juvenile samples (288 h).

3. RESULTS

3.1. Egg biochemistry

The eggs of *Stegnaster inflatus* are spherical, with a mean ± SD diameter of 390 ± 15 µm (N = 30,

Zamora et al. 2019) and a mean total lipid content of 2047 ± 315 ng egg^{-1} (N = 3 females). There was no significant difference in total lipid content between the 3 females (pseudo-$F_{2,6}$ = 1.67, p = 0.2576). With an egg volume of 31.06 nl, the total lipid density in *S. inflatus* is 65.90 ng nl^{-1}.

S. inflatus eggs contained 6 lipid classes: the energetic lipids AH, DAGE and FFA; and the structural lipids ST, PL and AMPL. In sample runs with eggs, we generally saw clear separation of the PL and AMPL peaks, while in later developmental stages there was only a single combined PL+AMPL peak. For quantification, we have presented information on the 4 separate lipid classes (AH, DAGE, FFA, ST) plus a combined PL+AMPL peak.

Egg lipids were dominated by DAGE (mean ± SD: 1348 ± 240 ng egg^{-1}, N = 3 females), comprising 68.4, 62.7 and 66.1% of lipid content, depending on female (Fig. 1). Small amounts of AH and FFA were also present in the eggs, resulting in the energetic lipids comprising 73.8% of the total lipid. The remaining structural lipids, PL+AMPL and ST, made up the remainder of the egg (Fig. 1). To facilitate future comparative analyses in echinoderms, full lipid information (ng, proportion) is provided in Table A1 in the Appendix.

The mean total protein content in the eggs of *S. inflatus* was 2143 ± 157 ng egg^{-1} (mean ± SD, N = 3 females), with no significant difference in protein content between females (pseudo-$F_{2,6}$ = 3.21, p = 0.1169). Protein density in the eggs was 68.98 ng nl^{-1}, with a lipid:protein ratio of 0.96.

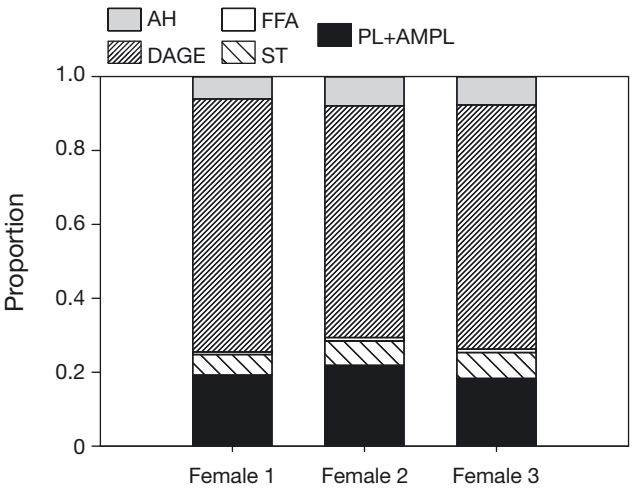

Fig. 1. Proportional contribution of lipid classes in the eggs from 3 female *Stegnaster inflatus*. PL+AMPL: phospholipid and acetone-mobile polar lipids; ST: sterol; FFA: free fatty acid; DAGE: diacylglycerol ether; AH: aliphatic hydrocarbon

3.2. Lipid utilization during development

The total lipid content in *S. inflatus* significantly decreased during larval development (Table 1A, Fig. 2A), with pairwise tests showing a significant difference between fertilized egg and EB, a pattern driven by energetic lipids (Fig. 2B), and specifically by DAGE (Table 1A, Fig. 3A), which was reduced by 32.6%. For the remaining energetic lipids, FFA showed a significant but small increase during larval development, while AH showed no significant change between egg and settlement (Table 1A, Fig. 3C,E). Structural lipids showed an initial decline from egg to EB, and then remained relatively stable from LB until settlement (Table 1A, Fig. 2C). The component structural lipid classes, PL+AMPL and ST, showed variable patterns depending on the female that was the source for the cultures (PL+AMPL: significant interaction term female×time, Table 1A, Fig. 3B) or increased in amounts between EB and LB and remained constant until settlement (ST: Table 1A, Fig. 3D).

The statistical results in Table 1A show lipid use during development using the cultures derived from Females 1–3. In Female 3, due to sampling for larval descriptions (Zamora et al. 2019) and mortality for unknown reasons, we did not have sufficient material to sample the settlement or juvenile stages. To ensure that this female did not have embryos/larvae with an unusual lipid profile, we repeated analysis of lipid use over time with Females 1 and 2 only (Table 1B). The only change was the significance of 1 pairwise test (structural lipid), suggesting that patterns in lipid utilization over time were not biased by the inclusion of Female 3.

Between settlement and the juvenile stage at 288 hpf, there was a significant increase in total, energetic and structural lipids (Table 1C, Fig. 2), although there were also significant differences between individuals sourced from the 2 remaining cultures (interaction terms, Table 1C). Energetic lipid increases were driven by large increases in FFA and AH in juveniles derived from Female 1, and to a lesser extent from Female 2 (Fig. 3C,E). There was no significant change in DAGE between settlement and the juvenile stage (Table 1C). The same pattern was observed in the structural lipids, PL+AMPL and ST, with larger lipid amounts in juveniles derived from Female 1 (Figs. 2C & 3B,D).

Notably, however, 55.0% of the total lipid, 51.0% of the energetic lipid and 40.3% of the DAGE invested by female *S. inflatus* in the egg remained at settlement for use by the juvenile.

Table 1. Utilization of lipid classes in cultures of *Stegnaster inflatus* analysed using 2-way PERMANOVA (factors: female, time). Pseudo-*F* values are presented for each factor and the interaction term for (A) the period of larval development (to 192 h) for cultures derived from all 3 females, (B) the period of larval development (to 192 h) for Females 1 and 2 only, and (C) the post-settlement period (192–288 h) for Females 1 and 2 only. Significant results are shown in **bold**. Pairwise tests are simplified to consider the main developmental stages: embryos (E), early brachiolaria (EB), late brachiolaria (LB) and settlement (S). Lipid classes are DAGE: diacylglycerol ether; FFA: free fatty acid; AH: aliphatic hydrocarbon; PL: phospholipid; AMPL: acetone-mobile polar lipid; ST: sterol

(A) Larval development: E to S for Females 1–3

	Female		Time		Female×Time		Pairwise time
	$F_{2,33}$	p	$F_{5,33}$	p	$F_{9,33}$	p	
Total	9.58	**0.0006**	12.43	**0.0001**	1.96	0.0781	E>EB>LB=S
Energetic	11.73	**0.0003**	14.753	**0.0001**	1.87	0.0941	E>EB>LB=S
DAGE	13.80	**0.0001**	19.47	**0.0001**	1.85	0.0979	E>EB>LB=S
FFA	2.70	0.0834	4.74	**0.0023**	1.41	0.2247	E=EB<LB=S
AH	0.18	0.8523	1.86	0.1177	0.79	0.6488	–
Structural	1.90	0.1712	7.03	**0.0001**	1.75	0.1171	E>EB<LB=S
PL+AMPL	4.26	**0.0237**	14.83	**0.0001**	2.25	**0.0407**	Sig. interaction
ST	1.09	0.3519	3.30	**0.0147**	0.73	0.6909	E>EB<LB=S

(B) Larval development: E to S for Females 1 and 2

	Female		Time		Female×Time		Pairwise time
	$F_{1,24}$	p	$F_{5,24}$	p	$F_{5,24}$	p	
Total	11.89	**0.0014**	12.99	**0.0001**	2.21	0.0836	E>EB>LB=S
Energetic	16.58	**0.0006**	16.27	**0.0001**	2.08	0.099	E>EB>LB=S
DAGE	20.59	**0.0001**	21.43	**0.0001**	1.93	0.1334	E>EB>LB=S
FFA	4.08	0.0524	3.90	**0.01**	1.39	0.2543	E=EB<LB=S
AH	0.14	0.7224	0.86	0.5434	0.93	0.4983	–
Structural	0.93	0.3499	7.21	**0.0002**	1.25	0.3193	E>EB=LB=S
PL+AMPL	6.79	**0.0136**	19.54	**0.0001**	1.25	0.3127	E>EB=LB>S
ST	0.91	0.3581	3.05	**0.0274**	0.65	0.6688	E>EB<LB=S

(C) Post-settlement: S and J juvenile for Females 1 and 2

	Female		Time		Female×Time	
	$F_{1,8}$	p	$F_{1,8}$	p	$F_{1,8}$	p
Total	3.93	0.0812	19.69	**0.0072**	13.47	**0.0054**
Energetic	4.95	0.0613	13.08	**0.0139**	12.49	**0.0058**
DAGE	4.23	0.0724	2.27	0.1762	7.36	**0.0241**
FFA	14.43	**0.0077**	29.24	**0.0034**	42.96	**0.0025**
AH	3.42	0.1086	21.76	**0.0082**	11.39	**0.0062**
Structural	5.36	**0.0475**	41.19	**0.0021**	20.56	**0.0033**
PL	5.58	**0.0489**	41.75	**0.0023**	10.05	**0.014**
ST	3.54	0.0862	27.76	**0.0022**	22.35	**0.0035**

3.3. Protein utilization during development

Protein samples from all 3 cultures were only available from egg until EB (Fig. 4A). Analysis of this part of development showed a significant difference in protein content over time (pseudo-$F_{3,24}$ = 33.45, p = 0.0001), and between females ($F_{2,24}$ = 3.64, p = 0.042), but with a non-significant interaction term ($F_{6,24}$ = 2.11, p = 0.094). The decrease in protein content was 55.5% between the egg and the EB larva at 42 hpf (Fig. 4A). Low sample numbers prevented detailed examination of protein content in later developmental stages, but during the LB and settlement stages, protein content appeared to stabilize in the region of ca. 20% of the protein content of the egg (Fig. 4A).

The differential use of lipid and protein in larval development in *S. inflatus* can be visualised using

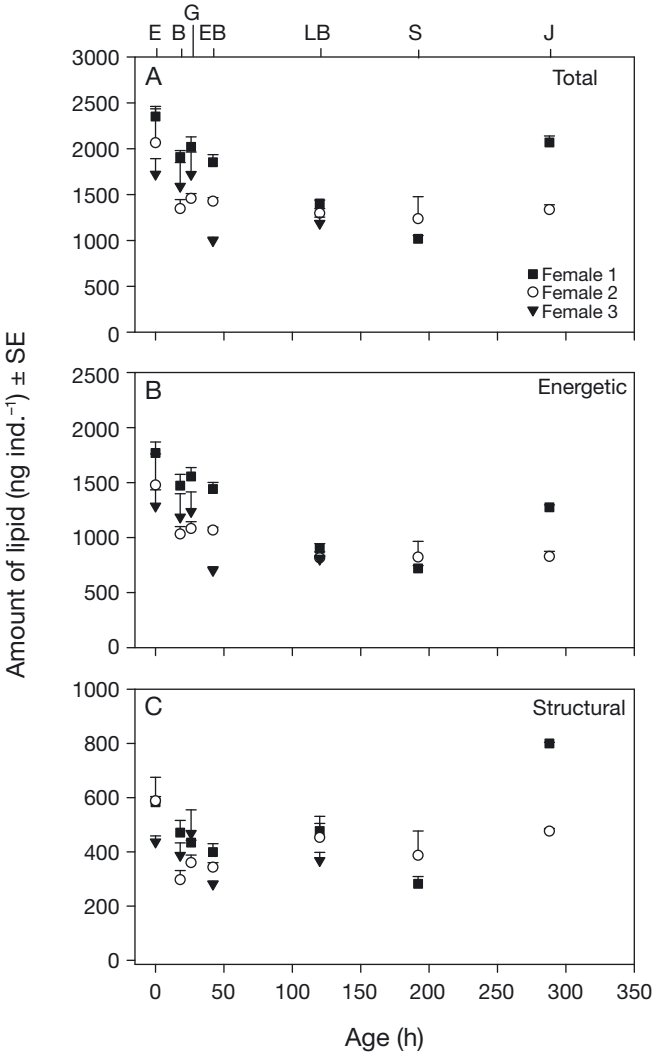

Fig. 2. Change in (A) total, (B) energetic (sum of DAGE, FFA, AH; abbreviations defined in Fig. 1) and (C) structural (sum of PL+AMPL and ST) lipids during development of *Stegnaster inflatus*. Mean values shown for each female (= culture) separately. Error bars (SE) shown in positive direction only to improve clarity. Upper frame of Panel A shows developmental stages, where E: embryo; B: blastula; G: gastrula; EB: early brachiolaria; LB: late brachiolaria; S: settlement; J: juvenile

the lipid:protein ratio (Fig. 4B). Starting from a ratio close to 1 (0.96), the ratio steadily increased to reach 2.75 at the LB stage (Fig. 4B). The energy used for larval development from egg to settlement was 919 ng total lipid and 1858 ng total protein. If we use the values from Gnaiger (1983) to calculate energetic equivalents (protein = 24.0 kJ g^{-1}; lipid = 39.5 kJ g^{-1}), then *S. inflatus* consumes 44.6 mJ of energy from protein and 36.3 mJ from lipid during the 8 d of larval development.

4. DISCUSSION

4.1. Maternal investment

The large egg (~400 µm) of *Stegnaster inflatus* is provisioned with large amounts of lipid and protein, as previously observed in other lecithotrophic echinoderms (Shilling & Manahan 1994, Villinski et al. 2002, Bryan 2004, Prowse et al. 2008, 2009, Falkner et al. 2015, Byrne & Sewell 2019, Davidson et al. 2019). The 3 females in this study had similar levels of maternal investment in lipid and protein, but with values of DAGE ranging from 1139 to 1610 ng, slightly lower than the mean reported by Prowse et al. (2009, their Fig. 2) of ca. 1950 ng DAGE. We also did not detect the small levels of TAG seen by Prowse et al. (2009, ~95 ng), but there were differences in methodology (e.g. Prowse et al. 2009 spotted 1 µl of a concentrated lipid extract, we spotted all of an extract from a small number of eggs), and this may also be a result of differences in maternal investment between collection locations and years (Marshall et al. 2018a,b).

Stegnaster is a sister taxon to the genus *Meridiastra* (Byrne 2013, Zamora et al. 2019), a genus which also exhibits pelagic lecithotrophy and for which we have comparative information on lipid and protein investment in the egg (Prowse et al. 2008, 2009). In making this comparison we should first note that the energetic lipids described by Prowse et al. (2008) as TAG were later confirmed to be DAGE (Prowse et al. 2009), the energetic lipid found in *S. inflatus*. *M. oriens*, *M. calcar* and *M. gunnii* all have a similar egg size to *S. inflatus* (399–431 µm) but have almost 3× the provisioning of DAGE (1348 ng compared to 4703–7403 ng) and almost twice the lipid density (*S. inflatus* = 66.13 ng nl^{-1} compared to 132–176 ng nl^{-1} for *Meridiastra*; Prowse et al. 2008). The total protein content in the eggs of *S. inflatus* was 2143 ng egg^{-1}, similar in value to that for *Meridiastra* species with pelagic lecithotrophic development (*M. oriens*: 2359 ng egg^{-1}; *M. calcar*: 3103 ng egg^{-1}, *M. gunnii*: 3018 ng egg^{-1}; Prowse et al. 2008). Accordingly, the protein density in *S. inflatus* (68.98 ng nl^{-1}) is also very similar to the 3 species of *Meridiastra* (range 71–83 ng nl^{-1}).

Differences in maternal provisioning might be related to development time, which is relatively short in *S. inflatus* (8 d), but only a slightly shorter than in *M. calcar* and *M. gunnii* with settlement in 11–12 d (M. Byrne pers. obs.). Alternatively, high lipid provisioning in the genus *Meridiastra* might be related to egg buoyancy, with lipid:protein ratios >2 in all 3 species: 2.98 in the positively buoyant egg of *M. gunnii*, and

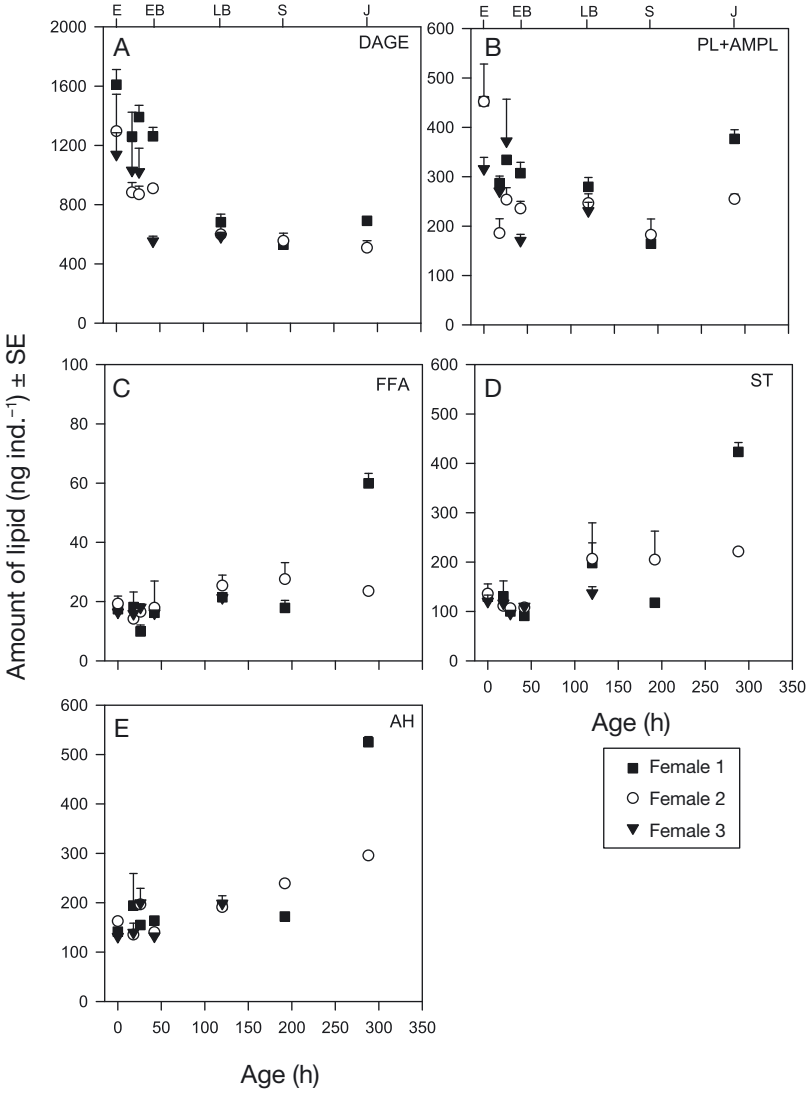

Fig. 3. Lipid class composition (mean ± SE) during development (stages as defined in Fig. 2) of *Stegnaster inflatus*. (A,C,E) Energetic lipids (DAGE, FFA, AH; abbreviations defined in Fig. 1); (B,D) structural lipids (PL+AMPL, ST). Error bars shown in positive direction only to improve clarity

2.62 and 2.15 in the neutral-negatively buoyant eggs of *M. oriens* and *M. calcar*, respectively (Prowse et al. 2008). The strongly negatively buoyant eggs of *S. inflatus* with less lipid and more protein (lipid:protein ratio of 0.96), make them more similar to eggs from the more distantly related *Parvulastra exigua,* which has a lipid:protein ratio of 0.8 and benthic development (Byrne 1995, Prowse et al. 2008). Lower lipid provisioning in *S. inflatus* may also partly explain the close association of brachiolaria larvae with the bottom of the culture container and the high sinking rates in all stages of larval development (Zamora et al. 2019). Although the brachiolaria of *S. inflatus* has the body profile typical of the planktonic and positively buoyant brachiolaria of other asterinids (Byrne 2013), they naturally sink and are likely to be demersal larvae (Zamora et al. 2019) or may develop on the substratum in a manner similar to the benthic brachiolaria of *Tosia neossia* (Naughton & O'Hara 2009).

4.2. Lipid and protein utilization during development

S. inflatus larvae use about half of the energetic lipids invested in the egg during development, with over 51% of the energetic lipid reserves, primarily DAGE, remaining at settlement for use by the post-metamorphic juvenile. A combination of sampling for morphological descriptions (Zamora et al. 2019), and loss of 1 culture meant that we had low numbers of metamorphosed larvae and 4 d old juveniles. Additionally, significant interaction terms for female×time in all lipid classes and in protein content after settlement means that the post-settlement changes seen here are only indicative. However, both remaining cultures showed increases in protein and structural lipids expected from post-settlement growth, and increases in FFA, and AH, the latter probably related to increased pigmentation as this lipid class includes carotenoids (Volkman & Nichols 1991), which are present in adult starfish (Fox & Scheer 1941, de Nicola 1956) and in echinoderm eggs and larvae (Montgomery et al. 2017). While low sample sizes prevent us from making an accurate assessment of protein use during development (only a single female culture providing limited replicates), we estimate that 80% of the initial protein investment in the egg is used, with the lipid: protein ratio of the larvae increasing to >4 just before settlement.

Few comparative studies of biochemical use during lecithotrophic development in echinoderms have considered the full developmental period from egg/embryo until settlement. Information on total lipid, total protein or both in the egg and settled juvenile stages is available from representatives of 3 classes of echino-

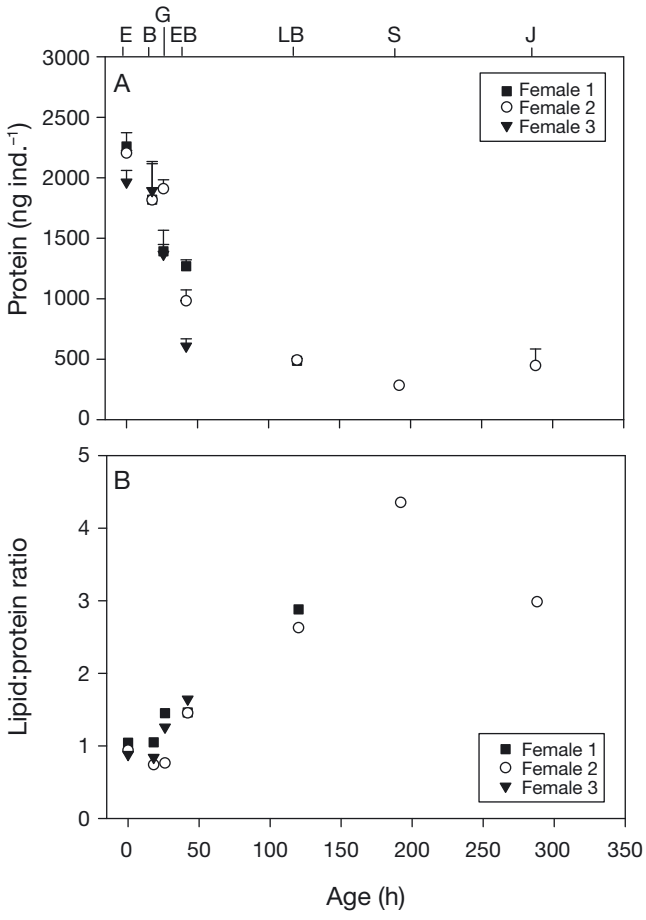

Fig. 4. Change in (A) protein content and (B) lipid:protein ratio during development (stages as defined in Fig. 2) of *Stegnaster inflatus*. Error bars (SE) shown in positive direction only to improve clarity

derms, with varying larval morphology (Table 2). Four out of 5 of these studies also have information on the utilization rates of different lipid classes, and allow separation of the energetic lipid classes, such as DAGE, FFA and WE, from the structural lipids. All of these studies show that large amounts of lipid, mainly DAGE, and variable amounts of protein remain at settlement to support the post-metamorphic juvenile (Table 2).

The other asteroid available for comparison to *S. inflatus* is the goniasterid *Mediaster aequalis*; both species are in the Order Valvatida. This species has an egg size exceeding 1 mm and is provisioned with orders of magnitude higher amounts of lipid (134.5 µg) and protein (12.2 µg), and with a lipid:protein ratio of 11.02 (Bryan 2004). *M. aequalis* will delay metamorphosis until it receives a specific tubeworm settlement cue (Birkeland et al. 1971), but is competent to settle from Day 35. Total protein increases over the course of development, but in competent larvae, 51.5% of the total lipid remained (Day 35), and when the experiment ended at Day 76, completely metamorphosed larvae retained 31.9% of the initial lipid investment in the egg (Table 2).

Similarly, large percentages of maternal investment are retained in the post-larval stage of 2 ophiuroids, the amphilepidid *Ophionereis schayeri* and the clarkcomid *Clarkcoma pulchra,* and 1 echinoid, the camarodontid *Heliocidaris erthyrogramma* with lecithotrophic development (Table 2). In these species, over 65% of the DAGE, ca. 69% of the energetic lipids and, in the ophiuroids, over 70% of the protein remains at settlement for use by the post-metamorphic juvenile.

Hints as to the generality of these findings in other echinoderms with lecithotrophic larvae are revealed in studies that examine changes in ash-free dry weight (AFDW) during the full period of development. Moreno & Hoegh-Guldberg (1999) compared the pelagic lecithotroph *Meridiastra calcar* and the benthic lecithotroph *Parvulastra exigua*. *M. calcar* lost 37–39% of egg AFDW during development to the juvenile (i.e. retained 61–63% of the egg AFDW), while in *P. exigua*, the mean AFDW of settled juveniles was higher than, but not significantly different from, that of the eggs.

Proceeding to marine invertebrates more broadly, Moran & Manahan (2003) showed that lecithotrophic larvae of the abalone *Haliotis fulgens* retained 73.7% of the protein and 48.1% of the primary energetic lipid TAG provisioned in the egg at settlement. Similarly, the palaemonid shrimp *Palaemonetes zariquieyi* has an abbreviated, non-feeding larval development comprising only 3 zoeal stages. Between zoea 1 and the juvenile, 53% of the lipids are used (i.e. 47% remain), with 36.4% of the primary energetic lipid TAG remaining at the end of larval life (Urzúa et al. 2013).

Thus, the quantitative data presented and reviewed here strongly support the suggestion of numerous authors (e.g. Emlet & Hoegh-Guldberg 1997, Moreno & Hoegh-Guldberg 1999, Byrne & Cerra 2000, Alcorn & Allen 2009, Pernet et al. 2012, Marshall et al. 2018a) that marine invertebrate species with large initial egg sizes are provisioning their eggs not only for the period of lecithotrophic development, but are retaining considerable biochemical reserves for the juvenile phase of the life cycle.

Observations of changes in amounts of lipid and protein during development are also important in relation to the 2 phases of movement in pelagic larvae: away from the spawning site on the benthos into the pelagic zone, and the return journey to the benthos at settlement (Allen et al. 2018). Therefore, not only the kinetics of change of maternal lipid and protein reserves, but also how these changes influence

Table 2. Comparative information on lipid and protein composition of lecithotrophic echinoderms. Lipid classes are DAGE: diacylglycerol ether; FFA: free fatty acid; TAG: triacylglycerol; WE: wax ester; ME: methyl ester; –: no data available

Species (larval type)	Egg size (µm)	Egg Total lipid (ng)	Egg Total protein (ng)	Settlement Total lipid (ng)	Settlement Total protein (ng)	% remaining Total lipid	% remaining Total protein	Energetic lipid Egg	Energetic lipid Juvenile	% remaining	Source, energetic lipid class
Asteroids											
Stegnaster inflatus (brachiolaria)	390	2054	2143	1128	(284)[a]	54.9	13.3	1511	771	51.0	This study, DAGE
								18	23	128.3	FFA
								1529	794	51.9	All (DAGE, FFA)
Mediaster aequilus (brachiolaria)	1000–1200	138600	12100	72400	25300	51.5[b]	209.1[b]	–	–	–	Bryan (2004)
				44200	15760	31.9[c]	130.2[c]	–	–	–	Bryan (2004)
Ophiuroids											
Ophionereis schayeri (reduced ophiopluteus)	248	–	1087	–	885	–	81.4	953	634	81.4	Falkner (2007), DAGE[4]
								41	55	134.1	WE
								994	689	69.3	All (DAGE + WE)
Clarkcoma pulchra (vitellaria)	290	–	1688	–	1249	–	74.0	2085	1466	70.3	Falkner (2007), DAGE[d]
								400	252	63.0	WE + ME
								2485	1718	69.0	All (DAGE, WE, ME)
Echinoids											
Heliocidaris erythrogramma	390–405	6255	–	4356	–	67.6	–	4585	3036	66.2	Byrne & Sewell (2019), DAGE
								974	721	74.0	TAG + WE
								5559	3757	67.6	All (DAGE, TAG, WE)

[a]Based on N = 1 female; [b]Values for Day 35 when larvae can be induced to metamorphose; note protein increases during development; [c]Values for complete metamorphosis; note protein increases through development; [d]Reported as TAG by Falkner (2007), later identified as DAGE by Falkner et al. (2015)

buoyancy and the plankton–benthos transition need to be considered. Chia et al. (1984, p. 1207) noted that larvae with particularly large amounts of lipids, such as asteroids with lecithotrophic development 'often float at the surface until the lipid yolk reserves are depleted'. Changes from positively buoyant embryos to negatively buoyant larvae prior to settlement have been described in *Pteraster tesselatus* (McEdward 1992) and *Mediaster aequalis* (Bryan 2004). Some lecithotrophic species undergo morphological changes to compensate for positive buoyancy. For example, when transitioning to the benthos, the sea urchin *Heliocidaris erythrogramma* develops an epaulette-like ciliary band, enabling downward swimming to counter the buoyancy of the remaining DAGE reserves (Byrne et al. 2001, Byrne & Sewell 2019; M. Byrne pers. obs.).

Here in *S. inflatus* we had a somewhat different situation, as the fertilized eggs were initially negatively buoyant with a sinking rate of 0.16 cm s^{-1} (Zamora et al. 2019). Although containing large amounts of DAGE with a specific gravity <1 (0.89, Lewis 1970), the large amounts of protein (lipid:protein ratio 0.96) and other, as yet unknown, components with specific gravities >1 have combined to produce a negatively buoyant embryo. In the period between fertilized egg and EB (42 hpf) the rate of loss of lipid was about half that of protein (lipid: 355 ng d^{-1}; protein: 680 ng d^{-1}), the lipid:protein ratio increased by about 50% (0.96 to 1.5), yet there was continued loss of buoyancy as evident in an increased sinking rate (to 0.22 cm s^{-1}; Zamora et al. 2019). We did not measure sinking rates in LB, but the lipid:protein ratio continued to rise to near 3 prior to settlement, while at this time larvae were observed using their adhesive discs to attach to the bottom of culture vessels (Zamora et al. 2019). The lipid: protein ratio, in this species at least, does not reflect changes in sinking rates or behaviour prior to settlement. We suggest that future studies should take the next step and correlate biochemical

changes with direct measurements of larval density (g ml^{-1}) or specific gravity (Lowndes 1944, Pennington & Emlet 1986), or indirect measures derived from sinking rates (Kelman & Emlet 1999). This might provide a better understanding of the ecological importance of larval biochemistry and changing buoyancy prior to settlement.

Does the retention of large energetic reserves at settlement improve juvenile quality in species with lecithotrophic development or influence juvenile size? To address these questions experimentally requires manipulation of the energetic reserves at the start or during the larval period. Altering the egg energetic/biochemical composition at the beginning of larval development has been done with 2 approaches. The first is to collect females from 'favourable' versus 'unfavourable' sites where the egg quality varies due to differing maternal nutritive state or to alter egg 'quality' experimentally using feeding regimes with different amounts or food quality. For example, George (1994) showed that females of the brooding lecithotroph *Leptasterias epichlora* from an exposed 'favourable' site were larger, and produced larger eggs with higher protein content and more juveniles with a higher protein content than the sheltered 'unfavourable' site. Experimental manipulation of egg energetics through use of feeding regimes has been undertaken with small-egg echinoderms with planktotrophic development (e.g. George 1994, Meidel et al. 1999), but we are not aware of similar studies in lecithotrophic species.

The second approach is to use techniques from experimental embryology to produce eggs with reduced provisioning, through blastomere manipulations or removing lipids from the embryo after fertilization. Blastomere manipulations have been used primarily in echinoderm species with planktotrophic development (reviewed by Alcorn & Allen 2009, Allen 2012), with a single study in the facultative planktotroph *Clypeaster rosaceus* (eggs 266–274 µm; Allen et al. 2006). Alternatively, in cases where the egg lipids are extruded into the blastocoel and are thus extracellular, these lipids can be experimentally removed from blastulae using short periods of centrifugation (Emlet & Hoegh-Guldberg 1997, Hoegh-Guldberg & Emlet 1997). In *H. erythrogramma*, centrifugation resulted in the reduction of ca. 40% of the egg volume and ca. 50% of the organic mass. Lipid reduction did not affect larval viability, metabolic rate or time to metamorphosis but did reduce buoyancy and resulted in smaller, shorter-lived juveniles (Emlet & Hoegh-Guldberg 1997). If *S. inflatus* has extracellular lipids in the blastula, this experimental approach might be usefully applied to link biochemical composition, buoyancy and post-settlement juvenile growth and survival.

An alternative approach is to manipulate the length of the lecithotrophic larval period so that more of the maternal reserves are used prior to settlement. For example, Wendt (1998) used bright lights to extend the pelagic duration of embryos from the bryozoan *Bugula neritina* from 1 to 24 h. Extended larval swimming (1 vs. 24 h) significantly decreased the larval carbon content (Wendt 2000) and reduced post-settlement colony growth and reproductive output (Wendt 1998). Similar experiments using light to delay metamorphosis in the ascidian *Diplosoma listerianum* showed similar carry-over effects, with extended swimming larvae having significantly reduced energetic content (Bennett & Marshall 2005), and the initial settled zooids having smaller feeding structures and an overall reduction in colony growth rates (Marshall et al. 2003). In *Mediaster aequalis,* Bryan (2004) extended larval life by withholding the settlement cue (non-polar extracts from tubes of the polychaete *Phyllochaetopterus prolifica*), and documented larval biochemical composition, but did not consider carry-over effects to the juvenile stage.

More generally, within a range of species and developmental modes, larger offspring tend to have better survival and higher rates of post-metamorphic growth than smaller offspring (see review by Marshall et al. 2018a). Recent research suggests that this may be due to differential use of energy reserves to complete metamorphosis; smaller offspring use ca. 47% of their energy reserves compared to ca. 22% in larger offspring (Pettersen et al. 2015). The energy-advantage of larger offspring, whether within a species or across developmental modes, is an important area for future research.

The next steps in our *Stegnaster* research will be to extend our energetic measurements further into the juvenile phase to examine carry-over or latent effects (Pechenik 2018), and to examine the effect of food level (starvation, feeding) on post-settlement juvenile survival and growth. The short larval life of *S. inflatus*, and the detailed data on maternal provisioning provided here, make it an ideal species for future investigation of how larval and juvenile energetics can influence post-settlement life.

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Appendix.

Table A1. Egg data from the 3 female *Stegnaster inflatus*: (A) ng lipid, (B) proportion of lipid. Values based on N = 3 replicate measurements of 20–30 eggs per female. Lipid classes are defined in Fig. 1

(A)	AH	DAGE	FFA	ST	PL+AMPL	Total
Female 1	141.49	1610.09	17.44	130.10	452.77	2351.87
Female 2	162.48	1295.96	19.28	136.02	452.03	2065.77
Female 3	131.39	1138.94	16.40	120.25	316.02	1723.01
Mean	145.12	1348.33	17.71	128.79	406.94	2046.89
(B)	AH	DAGE	FFA	ST	PL+AMPL	Total
Female 1	0.0602	0.6846	0.0074	0.0553	0.1925	1
Female 2	0.0787	0.6273	0.0093	0.0658	0.2188	1
Female 3	0.0763	0.6610	0.0095	0.0698	0.1834	1
Mean	0.0717	0.6577	0.0088	0.0636	0.1982	

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