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PII: S1095-6433(12)00517-X
DOI: doi: 10.1016/j.cbpa.2012.11.010
Reference: CBA 9449

To appear in: *Comparative Biochemistry and Physiology, Part A*

Received date: 11 September 2012
Revised date: 14 November 2012
Accepted date: 15 November 2012

Please cite this article as: Carboni, Stefano, Hughes, Adam D., Atack, Tim, Tocher, Douglas R., Migaud, Herve, Fatty acid profiles during gametogenesis in sea urchin (*Paracentrotus lividus*): Effects of dietary inputs on gonad, egg and embryo profiles, *Comparative Biochemistry and Physiology, Part A* (2012), doi: 10.1016/j.cbpa.2012.11.010

CBP ms.21453 Revised – part A

Fatty acid profiles during gametogenesis in sea urchin (*Paracentrotus lividus*): Effects of dietary inputs on gonad, egg and embryo profiles

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Abstract

The effects of dietary fatty acids on the composition of *Paracentrotus lividus* gonads were investigated to determine whether dietary inputs affect their relative abundance during gametogenesis. Egg and embryo FA compositions were compared with that of mature gonads to understand how maternal FA are transferred to the offspring. Urchins were fed an experimental pellet diet in comparison to brown kelp (*Laminaria digitata*). FA profiles of diets, gonads, eggs and embryos revealed the presence in gonads of FA that were absent in the diets and/or higher contents of some long-chain polyunsaturated fatty acids (LC-PUFA). Moreover, some unusual FA, such as non-methylene interrupted (NMI), were found in gonads, eggs and embryos, but not in the diets, suggesting that *P. lividus* may be capable of synthesizing these FA and accumulating them in the eggs. A description of gonad FA profiles during gametogenesis is reported for the first time and data suggest that eicosapentaenoic and docosahexaenoic acids are accumulated during gametogenesis, while arachidonic acid is highly regulated and is the only LC-PUFA clearly accumulated into the eggs along with NMI. Further studies are required to determine if maternal provisioning of FA has the potential to influence sea urchin production outputs and increase hatchery profitability.

Key words: *Paracentrotus lividus*, sea urchin, nutrition, fatty acids, diets, gonads.

1. Introduction

Larvae of marine organisms initiate their development supported by nutrients provided by the egg. In species that have planktotrophic feeding larvae, nutrients within the egg fuel development of the feeding larva. In invertebrates, including echinoderms with indirect development, the feeding stage is achieved only after the differentiation of the digestive tract, ciliary feeding apparatus and enzyme systems (Gallager et al., 1986; Strathmann et al., 1992; Pernet et al., 2004), and the duration of the facultative feeding period varies among species and with the availability of maternal provisions (Byrne et al., 2008). Maternal provisioning of nutrients, including essential fatty acids, is therefore important for the normal development of the embryo and plays an important role in offspring performance.

In echinoids the duration of development and general larval condition are also strongly influenced by environmental factors (Fenaux et al., 1994; Miller and Emlet, 1999; Schiopu et al., 2006; Liu et al., 2007). In favorable conditions, development time is shortened, minimizing the duration of the vulnerable planktonic stage usually characterized by high mortality (López et al., 1998; Lamare and Barker, 1999; Liu et al., 2007). In addition to supporting the planktonic stage, nutrients accumulated by the larvae provide energy for the metamorphosis and development of the early juvenile (George et al., 1997; Moran and Emlet, 2001; Schiopu et al., 2006). The size, growth and survival of early juveniles are in fact strongly influenced by the nutrients accumulated and stored by the larvae (Vaïtilingon et al., 2001; Pechenik, 2006; Pernet et al., 2006). Thus in echinoids, larval culture conditions affect metamorphic success and juvenile performance.

Triacylglycerol (TAG) is the major lipid class supplying energy to fuel larval development in many echinoderm and mollusc eggs (Podolsky et al., 1994; Sewell and Manahan, 2001; Villinski et al., 2002; Sewell, 2005; Prowse et al., 2008). TAGs are metabolized during pre-feeding development, while egg phospholipid (PL) and protein remain relatively stable as these nutrients are used primarily as structural components of the developing larval body (George et al., 1997; Sewell, 2005; Meyer et al., 2007; Prowse et al., 2008). As major components of most lipids, fatty acids (FA) have functional roles as a source of metabolic energy (as in TAGs), as structural components (as in membrane PL), and as precursors of bioactive molecules (Sargent et al., 2002; Tocher, 2003). In particular, long-chain polyunsaturated fatty acids (LC-PUFA) such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20:4n-6) have important physiological functions. In growing larvae LC-PUFA can act as ligands for transcription factors and nuclear receptors, influencing gene expression (Izquierdo and Koven, 2011). Furthermore, it has been shown that LC-PUFA, through cyclooxygenase and lipoxygenase-derived eicosanoids, regulate cortisol production by modulating ACTH-stimulated inter-renal cells in sea

bream, *Sparus aurata*, possibly playing a role in stress response (Ganga et al., 2006). Dietary ARA, EPA and DHA compete for acylation and incorporation into the PL membrane of cells and also as substrates for the eicosanoid enzyme systems (Bell et al., 1991a, b). Therefore, the overall impact of LC-PUFA on larval physiology is directly related to the level and ratio of these compounds in tissue phospholipids. For these reasons, these FA are the main focus of the present work.

Fatty acid composition of gonads, eggs or larvae have been described in several echinoid species such as *Paracentrotus lividus*, *Psammechinus miliaris*, *Strongylocentrotus droebachiensis*, *Dendraster excentricus* and *Lythechinus variegatus* (Cook et al., 2000; George et al., 2000; Castell et al., 2004; Hughes et al., 2006; Schiopu et al., 2006; Gago et al., 2009; Suckling et al., 2011; Carboni et al., 2012). It is generally recognized that FA compositions of sea urchin gonads reflect dietary inputs although reproductive status could alter relative FA abundance in *P. lividus* (Hughes et al., 2005, Martinez-Pita et al., 2010). However, a complete description of the FA profile of each gametogenic stage is not currently available. Marine vertebrates cannot synthesize PUFA de novo although they can have limited ability to further elongate and desaturate dietary PUFA (Sargent et al., 2002). It has been suggested that PUFA desaturase and elongase activities may also be present in some marine invertebrates such as sea urchin adults (Cook et al., 2000; Bell et al., 2001; Castell *et al.*, 2004) and larvae (Schiopu et al., 2006; Liu et al., 2007; Carboni et al., 2012).

Most studies on *P. lividus* nutrition have focused on gonadal index (GI) improvement or gonads' flavor and/or color enhancement for human consumption (Shpigel et al., 2006; Symonds et al., 2007). In contrast, there are few available data on the effects of dietary FA on maternal provisioning to *P. lividus* larva. The aims of the present work were 1) to determine the effects of broodstock diet on gonad fatty acid composition during the various gametogenic stages, and 2) to evaluate how egg and embryo fatty acid composition were influenced by the maternal diet.

2. Materials and methods

2.1 Culture conditions and experimental design

After a starvation period of three weeks, 200 individuals of *Paracentrotus lividus* (27.4 mm ± 0.3 test diameter) reared at the Ardtoe Marine Laboratory (AML, Argyll, Scotland; 56N 46' / 5W 52') were randomly divided into four groups corresponding to two treatments in duplicate (50 individuals/group). One experimental pelletized diet (Table 1 for ingredients), provided by the Scottish Association for Marine Science (Pellet diet, P), and a diet of fresh brown algae (*Laminaria digitata*, kelp diet, K) were fed to adult *P. lividus* over a period of three months (May - July 2011). Urchins were kept under ambient temperature and photoperiod within plastic baskets suspended in 100 L tanks with water exchange set at 1 L min^{-1}. Temperature, recorded daily, rose gradually from 9 to 16 °C and day-length increased from 15 h in May to 17 h in June 2011. The urchins were fed

daily and, every third day, uneaten pellets were siphoned from the bottom of the tank. As the urchins were kept on suspended baskets, crumbles of uneaten pellets were depositing on the bottom of the tank and therefore were not available to the individuals. Only fresh pellets were therefore ingested during the trial period. Uneaten kelp was also removed as required and replaced with fresh fronds.

At the end of the trial, individuals from each replicate were induced to spawn by injection of 1 M KCl (40 μL per g of body mass) into the coelom via the peristomial membrane (Kelly et al., 2000; Liu et al., 2007; Carboni et al., 2012) and gametes from three females and three males from each replicate were collected and mixed before fertilization. Eggs were fertilized by addition of 10 mL of diluted sperm. Fertilization rate was assessed under the microscope 2 h post fertilization. Fertilized eggs were incubated in static seawater without aeration for 24 h in the dark (Liu et al., 2007). Hatching rate was estimated for each batch as the proportion of swimming larvae (counted volumetrically) over the number of incubated eggs. A proportion of the offspring from each treatment (approximately 100,000 larvae per replicate) were reared in isolation in triplicate glass tanks of 2 L volume. Developing embryos from each treatment were sampled 48 hours post-fertilization for fatty acid analyses. Seawater used during the process of artificial fertilization, egg incubation and embryos rearing was filtered (4 μm) and UV treated, and water temperature was maintained at 18±2 °C throughout the cultivation period.

2.2 Proximate composition

Diets were grounded prior to determination of proximate composition, moisture and ash contents according to standard protocols (AOAC, 2000). Crude protein contents were measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, UK) according to Lynch and Barbano (1999). Crude lipid contents were determined using the Soxhlet method according to standard procedures (AOAC, 2000) with extraction in petroleum ether at 120 ^{o}C (Avanti Soxtec 2050 Auto Extraction apparatus, Foss, Warrington, UK). Fibres were analysed using the Foss fibrecap system with 1 g samples being de-fatted with petroleum ether then boiled for 30 min in 350 mL 1.25 % sulphuric acid and washed in boiling distilled water. Samples were then boiled for another 30 min in 350 mL 1.25 % NaOH and washed again in water. Samples were incinerated at 600 °C for 4 h and finally re-weighed. Dietary fibre content was calculated as a percentage of the initial weight of the sample. Energy content of the diets was measured by bomb calorimetry using a Parr 6200 calorimeter according to standard procedures. The carbohydrate content was calculated as the difference between dry weight and the sum of protein, lipid, ash and fibre.

2.3 Histology

At the beginning of the feeding trial (Day 0) and every 30 days, five sea urchins per replicate were cut outside the peristomial membrane and gonads separated from the other organs, blotted dry with paper towel. Samples were stored in 10% neutral buffered formalin and then dehydrated, embedded in paraffin and sectioned at 5 μm. Three branches from each individual were analyzed in order to confirm synchronization between branches. The sections were stained with haematoxylin and eosin (H/E) and analyzed under the binocular microscope (Olympus, BH2). Gametogenic stages were identified according to Byrne (1990). The two remaining branches from each individual were stored at -20 °C in chloroform/methanol (2:1 by vol.) containing 0.01 % butylated hydroxytoluene (BHT) for lipid extraction and fatty acid analysis.

2.4 Total lipid and fatty acid contents and composition

After dissection, gonadal samples from each individual were independently stored in 5 mL chloroform/methanol (2:1 by vol.) containing 0.01 % butylated hydroxytoluene (BHT) as an antioxidant at -20 °C prior to analyses. Gonad samples from each individual were analyzed separately. FA profiles of gonad samples from individuals showing the same gametogenic stage within each treatment were averaged to describe the FA profiles characteristic of each observed gametogenic stage.

Urchin eggs were collected from the five gonopores immediately after spawning using a pipette and stored in glass vials as above. Embryos from each replicate were collected by filtration onto a GF/F filter (Whatman Ltd, Maidstone, UK) before being placed in glass vials and stored as above.

Total lipids were extracted and quantified according to Folch et al. (1957). Fatty acid compositions were determined by gas chromatography of FA methyl esters (FAMEs) essentially according to Christie (2003) all as described in detail by Carboni *et al*. (2012). Individual FAMEs were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19), and FAMEs quantified through comparison with a heptadecanoic acid (17:0) internal standard.

2.5 Statistical analysis

Statistical analysis was performed with MINITAB ® version 15.0. Normality and homogeneity of variance were confirmed using Kolmogorov-Smirnov test. Gonadal LC-PUFA contents were compared using a General Linear Model (GLM) with all interactions being analysed by Tukey post hoc test to identify significant differences (Table 2 for GLM outputs). Egg, embryo and diet LC-PUFA, expressed as percentages of total fatty acids, and moisture, ash, carbohydrate, fiber, protein and lipid as well as fertilization and hatching rates were arcsine transformed and analysed by one-way ANOVA. The non-parametric multivariate analysis ANOSIM (Analysis of similarities) was

used to identify significant differences in FA profiles between gametogenic stages in the gonads, eggs and embryos. SIMPER (Similarity percentage) test was used to identify which FAs were primarily responsible for the observed differences (Clarke and Warwick, 1994). Data were untransformed and Euclidian distance was used as the metric. In all cases, significant differences were determined at $p < 0.05$.

3. Results

3.1 Diets composition

The proximate compositions of the two diets showed significant differences for all the measured parameters. Moisture ($F=422$; $p= 0.002$), ash ($F=225$; $p=0.004$) and carbohydrate ($F=140$; $p=0.007$) contents were higher in the Kelp diet whilst its lipid ($F= 680$; $p=0.001$), protein ($F=57,6$; $p=0.02$) and energy ($F=1265$; $p=0.001$) contents were lower (Table 3). Moreover, multivariate analysis revealed that there was a significant difference in overall FA signature of the diets ($p=0.01$), FA compositions are shown in Table 3. Monounsaturated FAs were the most represented group in the Pellet diet, although 18:2n-6 was the most abundant FA in this diet accounting for the vast majority of n-6 PUFA. In the Kelp diet, n-3 PUFA was the most abundant class with EPA and 18:4n-3 being the most represented FA. ARA and EPA contents were significantly higher in Kelp than in the Pellet diet (ARA: $F=70.3$ $p=0.01$; EPA: $F=63.2$ $p=0.01$) whilst no DHA was found in Kelp. Therefore, only individuals fed the Pellet diet received dietary DHA input. The differences in EPA, DHA and ARA contents between the diets were reflected in the relative proportion of these LC-PUFAs with the EPA/ARA ratio being significantly higher in the Pellet diet than in Kelp, whilst the absence of DHA in the Kelp diet did not allow for DHA/EPA ratio calculation for this diet.

3.2 Fertilization and hatching rates

The average fertilization rate was 98 ± 1% with no significant difference between treatments. The average hatching rate was 85 ± 2% and no significant differences between treatments were observed. These values are similar to those observed in previous studies where the same spawning trigger was used (Liu et al., 2007).

3.3 Histology

Both diets supported development of ovaries and testes over the trial period with no significant differences between treatments and, at the end of the trial, all induced-individuals produced viable gametes as demonstrated by the high fertilization rate obtained in both treatments. As no significant difference between treatments was recorded, histology data from individuals in the two treatment groups were pooled in order to describe gametogenesis of the entire population under the

experimental conditions. At the beginning of the trial the majority of individuals (90%) were found to be at the recovery stage (stage I) with a few individuals still in the spent stage (stage VI). At day 30 the population was less homogeneous and 10 % of the individuals were still at stage I, whereas the rest were equally divided between stage II and III. By day 60 most of the urchin population (75 %) was in stage III with the remainder at stage II and at day 90 only 30 % were still at stage III while 70 % progressed to stage IV (Fig. 1).

3.4 Fatty acid composition of eggs, embryos and gonads during gametogenesis

ANOSIM analysis revealed significant differences between treatments when all the tissues (gonads, egg and embryos) were pooled ($P = 0.01$), and this is clearly shown in the nMDS plot in Fig. 2. Nonetheless, when tissues within each treatment were analyzed by one-way ANOSIM followed by pair-wise test, only overall FA signatures of gonads and embryos within the Pellet diet were found to be significantly different ($P = 0.02$). The SIMPER test showed that the only LC-PUFA involved in the observed difference was ARA, which was clearly accumulated in the eggs and further retained during embryo development in the Pellet treatment (Table 4). Moreover EPA was significantly higher in eggs and embryos produced by urchins fed Kelp than in those derived from the broodstock fed the Pellet diet, whilst the opposite was true for DHA, following a common pattern observed in the gonads. Interestingly 20:2 and 20:3 NMI FA were also accumulated in the eggs from urchins fed both dietary treatments although eggs and embryos derived from the Pellet treatment had significantly higher 20:2 and 20:3 NMI compared with those of the Kelp treatment (Table 4).

As significant differences between males and females were not observed, individuals of both genders were pooled according to gametogenic stage. Fatty acid profiles of all gametogenic stages observed during the trial are shown in Table 5. One-way ANOSIM for FA signatures of gonads at different maturity stages highlighted a significant difference between Stage I and all the other stages for urchins in both treatments, however the only significant difference beyond this point was observed between Stage I and Stage III in gonads of urchins fed the Kelp diet. The SIMPER test is of particular interest in this case as it highlighted a continual increment of saturated FA (14:0) as gametogenesis progressed in gonads of urchin fed Kelp whilst it did not change significantly in urchins fed the Pellet diet (Table 5). Interestingly, the FA primarily responsible for the observed differences between maturity stages were not the same in the two treatments: 18:1n-9 for Kelp diet and18:2n-6 for Pellet diet. More specifically 18:1n-9 significantly decreased after Stage I in the Kelp treatment whereas it increased in the Pellet treatment, and 18:2n-6 significantly increased after Stage I in the Pellet treatment whereas it did not change in urchins fed the Kelp treatment.

As for LC-PUFA, EPA increased significantly during gametogenesis in urchins fed Kelp whilst its relative content decreased significantly in urchins fed the Pellet diet establishing a major difference

between the two treatments at gametogenic Stage II, III and IV (Fig. 3a). DHA exhibited an opposite pattern, significantly increasing in gonads of urchins fed the Pellet diet, whilst it did not change in gonads of urchins fed Kelp (Fig. 3b). Despite being absent in the Pellet diet, ARA content in the gonads of urchins fed the two treatments was not significantly different and it did not change during gametogenesis (Table 5).

4. Discussion and conclusions

Artificial feeds (diets) must be developed to substitute wild collected macroalgae for *P. lividus* aquaculture to become commercially and environmentally sustainable. Early studies investigated diet formulations with higher protein, lipid and energy contents compared to natural macroalgae via inclusion of protein-rich fish or plant meals and fish oil. These diets were able to promote good somatic and gonadal growth. Nonetheless, no data are currently available on the effects of broodstock diets on the FA profiles of the different gametogenic stages of *P. lividus* gonads, and on the maternal provisioning of fatty acids to eggs and embryos.

During the present trial, gametogenesis was not affected by differences in the diets suggesting that environmental conditions such as temperature and photoperiod regulate this process as long as minimal nutritional requirements are met. Stage V gonads were not observed and this might be due to the relatively low maximum temperature (16 °C) achieved during this trial. This is supported by the fact that natural spawning was never observed during commercial production at AML, suggesting that a higher daily average temperature is required to meet the Effective Accumulated Temperature (EAT) to trigger natural spawning as previously suggested (Liu *et al*., 2002).

In animals capable of converting 18-carbon PUFA to LC-PUFA, the first step is the action of fatty acyl Δ6-desaturase, which converts 18:2n-6 to 18:3n-6 and 18:3n-3 to 18:4n-3 (Tocher, 2003). The second step in the LC-PUFA biosynthesis pathway is the action of fatty acyl elongase, which converts 18:3n-6 to 20:3n-6 and 18:4n-3 to 20:4n-3. These elongated products are then further desaturated (Δ5 desaturase) to ARA and EPA, respectively. EPA is also substrate for further elongation and desaturation to produce DHA. The presence in the gonads of some fatty acids that are not detected in the diets such as 18:3n-6 and 20:3n-6, not found in the Pellet diet, or 22:5n-3 and DHA, not found in the Kelp diet, or the much higher content of ARA in the gonads of individuals fed the Pellet diet than in the diet itself, suggests that sea urchin may have the ability to synthesize LC-PUFA. The positive correlation between dietary inputs of FA substrates (18:2n-6 and 18:3n-3) of the LC-PUFA biosynthesis pathway and their content in the gonads clearly shows that tissue levels of these two fatty acids are influenced by their contents in the diets. The very nature of a multistep enzymatic pathway where the fatty acid product of one step becomes substrate for the following step makes it difficult to be conclusive about endogenous metabolism based simply on

relationships between tissue levels of the fatty acids. This is further complicated in the present study, by significant dietary inputs of some of the pathway intermediates such as 18:4n-3, 20:4n-3 or indeed EPA.

The fact that NMI FA are not present in either of the diets used in the present study also suggests that *P. lividus* may be capable of synthesizing these fatty acids. Cook et al. (2000) and Castell et al. (2004) found similar NMI FA in *P. miliaris* and *S. droebachiensis* and both suggested that these species of sea urchin were capable of *de novo* synthesis of NMI FA. Moreover Zhukova (1986, 1991) used ^{14}C-labeled acetate to show that mussels were capable of *de novo* synthesis of the same NMI FA identified in sea urchins. Our data also showed that NMI FAs are selectively accumulated in the eggs and embryos suggesting that they may be important for larval development. Results of the present experiment agree with a relationship between NMI FA and essential FA previously suggested for *S. droebachiensis* for which, under specific conditions, primitive taxa such as echinoids could use NMI FA to provide physiological functions commonly associated with LC-PUFA (Castell *et al*., 2004; Gonzalez-Duran *et al*., 2008). Indeed, the ability to biosynthesize 20:3 NMI FA from the precursor 18:2n-6 and to substitute ARA in the membrane phospholipids may represent an important advantage for organisms exposed to fluctuating temperatures which requires membrane fluidity adaptations. Indeed, it was shown that the unusual double bond position in 20:3 NMI FA causes a melting point shift of about 10 °C lower (Zakhartsev et al., 1998; Pirini et al., 2007) and this could partly explain the wide geographical distribution of *P. lividus*.

The significant differences in 20:2 and 20:3 NMI FA in eggs and embryos observed between dietary treatments might be explained by the higher levels in the Pellet diet of their precursors (20:1n-9 and 18:2n-6), which are converted via Δ5 desaturase into 20:2 and 20:3 NMI FA respectively, as suggested by Zhukova (1991).

In stage I gonads of urchins fed the Kelp diet, 18:1n-9 was present although it decreased during gametogenesis and its contents in eggs and embryos were lower than those of urchins fed the Pellet diet. This could be explained by elongation of 18:1n-9 to 20:1n-9 which, in turn, might be converted to 20:2 NMI if the metabolic pathway identified by Zhokova (1991) in bivalves was also operating in sea urchin. On the other hand, 18:2n-6 is a precursor for both 20:3 NMI and ARA (Barnathan, 2009) and both of these FA were not present in the Pellet diet. However, it is not possible to be conclusive on how this FA was utilized, as end products of both pathways were present in the gonads, eggs and embryos of urchins fed the Pellet diet.

In conclusion, the present study provides, for the first time, a detailed description of the evolution of fatty acid profiles of *P. lividus* gonads during gametogenesis. Although no definitive conclusions can be made, it seems that, among LC-PUFA, EPA and DHA are primarily accumulated during gametogenesis when available in the diet. In contrast, ARA appears to be more constant throughout

gametogenesis and more independent of dietary input. ARA is the only LC-PUFA clearly accumulated in the eggs along with NMI FA.

As already suggested by Gago *et al.* (2009), we confirmed that FA profiles of sea urchin eggs and embryos can be controlled through broodstock nutrition. This could play a role in the development of new feeds and protocols for first feeding of sea urchin larvae. Further studies on the effects of maternal provisioning of LC-PUFA on larvae performance are required to determine if broodstock nutrition has indeed the potential to be used to influence sea urchin production output. Moreover, it is important to confirm the capacity of sea urchins for endogenous production of LC-PUFA and if so, identify and functionally characterize the genes involved in LC-PUFA production. Finally, in depth investigation should be carried out to better understand NMI FA metabolism and interactions between these quantitatively minor FA and LC-PUFA in echinoids species.

Acknowledgments. This work was supported by the EU FP7 project "ENRICH", University of Stirling and Ardtoe Marine Laboratory. Special thanks go to Mariachiara Chiantore at Dip.Ter.Ris of Genova University, James Dick and Deborah Faichney of the Nutrition group at the Institute of Aquaculture, Stirling University, to all AML staff and to Bridie Grant. Special thanks go to the anonymous reviewers who helped improving the manuscript.

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Legends

Figure 1. Gametogenic stages of the individuals under experimental conditions as observed during the trial period (n=2, 5 individuals/replicate/time point). Individuals from the two treatments were pooled.

Figure 2. Non-metric Multi Dimensional Scale (nMDS) plot for fatty acid composition of sea urchin mature gonads (n=2; 3 individuals/replicate), egg (n=2; 3 females/replicate) and embryos (n=3, approximately 5000 embryos/replicate) in the two treatments.

Figure 3. EPA (a) and DHA (b) content in gonads of urchins fed kelp or pellet diets throughout gametogenesis. Values of EPA and DHA are given as percentage of total fatty acids (mean ± SD, n=2; 10 individuals/treatment/stage). Superscripts indicated significant differences between treatments.

Table 1. Pellet diet ingredients

Ingredients %	
Soybean meal	21.27
Wheat meal	22.85
Canola meal	21.27
Potato starch	19.84
Gelatine	5.60
Sodium alginate	2.24
Linseed oil	2.24
Lecithin (plant based)	2.24
Vitamin premix	0.56
Mineral premix	0.34
Inositol	0.01
Stabilised vitamin C	0.09
Paradigmox (antioxidant)	0.22
Algro®	1.25

Table 2. GLM outputs for analyses done on EPA and DHA data showing degrees of freedom, F and P values.

Source	**EPA**			**DHA**		
	DF	**F**	**P**	**DF**	**F**	**P**
Time	3	0.90	0.453	3	4.17	0.015
Treat	1	84.63	0.000	1	51.93	0.000
Time*Treat	3	27.20	0.000	3	10.29	0.000
Rep (Treat)	2	0.03	0.973	2	1.68	0.204

SS and MS were ≤0.01

Table 3: Each fatty acid is expressed as a percentage of total fatty acids (mean ± SD, n=3). Ash, carbohydrates, fibre, protein and lipids are expressed as a percentage of the diet dry weight. Energy content is given per gram of diet dry weight. Asterisks indicate non-detected values and superscripts indicate significant differences between treatments.

Fatty Acids	Kelp	Pellet
14:0	6.7 (±1.3)	2.0 (± 0.3)
16:0	16.0 (±2.0)	15.5 (±1.0)
18:0	*	[a]3.4 (±0.1)
∑ saturated	23.9 (±3.5)	21.6 (±1.3)
16:1n-9	2.8 (±0.2)	*
16:1n-7	2.1 (±0.2)	3.4 (±0.01)
18:1n-9	[b]14.0 (±1.2)	[a]19.1 (±0.6)
18:1n-7	*	[a]4.1 (±0.03)
20:1n-9	*	[a]4.7 (±0.9)
22:1n-11	*	[a]4.4 (±0.1)
∑ monounsaturated	20.9 (±1.6)	38.4 (±1.0)
18:2n-6	[b]5.6 (±0.5)	[a]26.9 (±0.3)
18:3n-6	0.8 (± 0.1)	*
20:3n-6	0.4 (± 0.0)	*
20:4n-6 (ARA)	[a]9.3 (±1.5)	*
∑ n-6 PUFA 1	16.2 (±2.1)	27.3 (±0.3)
18:3n-3	6.2 (±0.5)	3.5 (±0.1)
18:4n-3	[a]11.4 (±1.7)	[b]0.9 (±0.1)
20:5n-3 (EPA)	[a]13.8 (±1.9)	[b]2.9 (±0.3)
22:5n-3	*	0.6 (±0.04)
22:6n-3 (DHA)	*	[a]4.2 (±0.2)
∑ n-3 PUFA	32.0 (±4.2)	12.4 (±0.1)
Total C16 PUFA	20.9 (±1.6)	19.3 (±1.1)
Total PUFA	48.3 (±6.3)	40.0 (±0.3)
EPA/ARA	1.5 (±0.04)	13.2 (±2.2)
DHA/EPA	*	1.5 (± 0.2)
Moisture %	[a]75.4 (± 0.08)	[b]10.2 (± 3.72)
Ash %	[a]5.7 (± 0.01)	[b]3.6 (± 0.04)
Carbohydrate %	[a]79.3 (± 0.2)	[b]67.2 (± 1.02)
Fibre %	[a]2.9 (± 0.06)	[b]2.0 (± 0.05)
Protein %	[b]11.9 (± 1.2)	[a]22.4 (± 0.07)
Lipid %	[b]0.2 (± 0.02)	[a]4.8 (± 0.04)
Energy (KJ/g)	[b]3.1 (± 0.01)	[a]16.1 (± 0.19)

Table 4:LC-PUFAs and NMI FAs in mature gonads, egg and embryos. Each FA is expressed as percentage of total fatty acids. Superscripts indicate significant differences between treatments within tissues (mean ± SD, n=2).

Diet	**Kelp**			**Pellet**		
Sample	**Gonad**	**Egg**	**Embryos**	**Gonad**	**Egg**	**Embryos**
ARA	^{a}7.4±1.0	^{a}10.3±0.8	^{a}11.3±1.6	^{a}6.9±1.0	^{a}11.6±0.7	^{a}13.0±1.2
EPA	^{a}12.4±0.9	^{a}12.6±0.9	^{a}12.0±2.0	^{b}5.8±1.2	^{b}5.9±0.2	^{b}6.6±0.7
DHA	^{b}0.2±0.1	^{b}0.6±0.1	^{b}0.8±0.2	^{a}2.8±0.4	^{a}3.1±0.04	^{a}3.0±0.4
20:2 NMI	^{b}4.9±0.8	^{b}6.5±0.01	^{b}6.0±0.2	^{a}6.7±0.6	^{a}7.9±0.1	^{a}7.8±0.1
20:3 NMI	^{b}3.9±0.6	^{b}5.8±0.8	^{b}5.2±0.3	^{a}6.6±1.4	^{a}8.5±0.2	^{a}8.0±0.5

Table 5: Fatty acid profiles of the gonads during gametogenesis of urchins fed the two diets. Each fatty acid is expressed as a percentage of total fatty acids (mean ± SD; n=2; 10 individuals/treatment/stage). Asterisks indicate non-detected values. Samples were taken at 30 days intervals. Superscripts indicate significant differences between stages within treatments (mean ± SD, n=2)

Diet	**Kelp**				**Pellet**			
Fatty Acids	**Stage I**	**Stage II**	**Stage III**	**Stage IV**	**Stage I**	**Stage II**	**Stage III**	**Stage IV**
14:0	^{b}5.7 (± 2.0)	^{a}11.4 (± 1.2)	^{a}10.8 (± 4.6)	^{a}13.7 (± 2.1)	10.5 (± 2.0)	7.1 (± 2.8)	7.4 (± 2.1)	8.1 (± 1.4)
16:0	14.7 (± 3.2)	14.2 (± 0.5)	14.1 (± 2.4)	15.1 (± 1.1)	14.4 (± 2.2)	13.2 (± 2.7)	13.6 (± 1.5)	15.7 (± 1.2)
18:0	^{a}4.4 (± 1.3)	2.6 (± 0.2)	2.5 (± 1.3)	^{b}2.3 (± 0.3)	2.1 (± 0.5)	2.6 (± 0.6)	2.4 (± 0.4)	2.4 (± 0.3)
∑ saturated	26.4 (± 5.5)	29.2 (± 1.7)	28.5 (± 5.7)	32.1 (± 2.9)	28.5 (± 3.6)	23.8 (± 5.0)	24.1 (± 3.2)	26.9 (± 1.8)
16:1n-7	3.2 (± 0.5)	3.2 (± 0.5)	3.5 (± 1.2)	4.0 (± 0.8)	4.8 (± 1.9)	3.5 (± 1.3)	3.2 (± 0.9)	3.3 (± 0.7)
18:1n-9	7.2 (± 3.2)	3.2 (± 0.5)	4.0 (± 1.3)	4.2 (± 0.6)	3.6 (± 0.9)	7.3 (± 0.8)	6.3 (± 0.9)	6.1 (± 0.3)
18:1n-7	2.2 (± 0.5)	2.3 (± 0.2)	2.3 (± 0.3)	2.3 (± 0.1)	2.7 (± 0.9)	2.6 (± 0.4)	2.8 (± 0.4)	2.9 (± 0.1)
20:1n-11	5.5 (± 1.7)	4.0 (± 0.7)	4.2 (± 1.0)	3.8 (± 0.5)	3.8 (± 2.2)	3.9 (± 0.4)	3.9 (± 0.5)	3.6 (± 0.4)
20:1n-9	5.4 (± 0.8)	6.3 (± 0.4)	6.4 (± 1.0)	6.4 (± 1.1)	6.1 (± 0.6)	6.5 (± 0.7)	6.4 (± 0.6)	5.9 (± 0.9)
22:1n-9	2.9 (± 0.8)	2.6 (± 0.2)	2.8 (± 0.8)	2.7 (± 0.3)	3.6 (± 0.4)	2.3 (± 0.6)	2.2 (± 0.4)	1.8 (± 0.1)
∑ monounsaturated	29.5 (± 5.2)	22.4 (± 1.4)	23.3 (± 1.9)	23.6 (± 0.5)	24.7 (± 4.6)	27.1 (± 1.9)	25.8 (± 1.3)	24.8 (± 1.1)
18:2n-6	1.9 (± 1.0)	1.9 (± 0.9)	3.0 (± 2.4)	1.8 (± 0.3)	^{b}2.3 (± 1.5)	^{a}10.9 (± 2.6)	^{a}10.1 (± 2.3)	^{a}10.7 (± 1.0)
18:3n-6	0.1 (± 0.1)	0.2 (± 0.0)	0.3 (± 0.1)	0.2 (± 0.0)	0.3 (± 0.1)	0.1 (± 0.0)	0.1 (± 0.0)	0.1 (± 0.0)
20:3n-6	0.4 (± 0.4)	0.6 (± 0.3)	0.6 (± 0.3)	0.5 (± 0.1)	^{b}0.3 (± 0.1)	^{ab}0.8 (± 0.3)	^{b}1.3 (± 0.4)	^{b}1.7 (± 0.5)
20:4n-6	9.8 (± 3.3)	8.5 (± 0.8)	8.4 (± 2.0)	7.4 (± 1.3)	9.3 (± 2.1)	6.8 (± 1.9)	7.3 (± 1.4)	7.0 (± 0.3)

∑ n-6 PUFA	12.4 (± 4.0)	11.4 (± 1.8)	13.0 (± 4.1)	10.0 (± 1.5)	12.4 (± 2.3)	18.8 (± 3.8)	19.6 (± 3.0)	19.6 (± 0.9)
18:3n-3	1.2 (± 0.4)	2.2 (± 0.3)	1.8 (± 0.5)	2.0 (± 0.2)	2.0 (± 0.5)	1.9 (± 0.3)	1.8 (± 0.3)	1.7 (± 0.3)
18:4n-3	[b]2.1 (± 1.1)	[a]5.1 (± 0.3)	[ab]3.7 (± 1.9)	[ab]4.2 (± 0.9)	[a]4.1 (± 1.1)	[b]1.4 (± 0.9)	[b]1.3 (± 0.7)	[b]1.0 (± 0.8)
20:5n-3	[b]9.0 (± 0.5)	[a]12.8 (± 0.5)	[a]11.6 (± 2.0)	[a]12.6 (± 1.0)	[a]11.6 (± 1.5)	[b]7.1 (± 0.4)	[b]6.9 (± 1.3)	[b]5.8 (± 1.2)
22:5n-3	0.4 (± 0.2)	0.3 (± 0.1)	0.4 (± 0.3)	0.4 (± 0.2)	0.4 (± 0.1)	0.7 (± 0.4)	0.6 (± 0.2)	0.5 (± 0.1)
22:6n-3	*	*	*	*	*	2.6 (± 1.0)	2.6 (± 0.8)	2.8 (± 0.5)
∑ n-3 PUFA	15.8 (± 3.4)	23.2 (± 0.8)	20.5 (± 3.8)	21.4 (± 0.7)	20.4 (± 2.8)	14.6 (± 0.5)	14.3 (± 1.9)	12.7 (± 2.0)
Total PUFA	37.3 (± 6.2)	44.9 (± 2.5)	44.3 (± 3.6)	41.3 (± 2.3)	43.2 (± 2.3)	46.2 (± 6.2)	47.2 (± 3.1)	45.5 (± 0.5)
18:0 DMA	6.1(± 2.1)	3.2 (± 0.6)	3.5 (± 1.5)	3.0 (± 0.5)	3.2 (± 2.0)	2.6 (± 0.6)	2.7 (± 0.7)	2.2 (± 0.4)
20:2 NMI	4.5 (± 1.1)	5.3 (± 0.7)	5.3 (± 0.9)	5.2 (± 0.8)	5.8 (± 1.9)	6.5 (± 0.7)	6.4 (± 0.5)	6.8 (± 0.6)
20:3 NMI	3.8 (± 1.8)	4.4 (± 0.4)	5.0 (± 1.8)	4.2 (± 0.5)	4.0 (± 0.9)	5.9 (± 2.4)	6.4 (± 1.7)	6.4 (± 1.3)

Figure 1

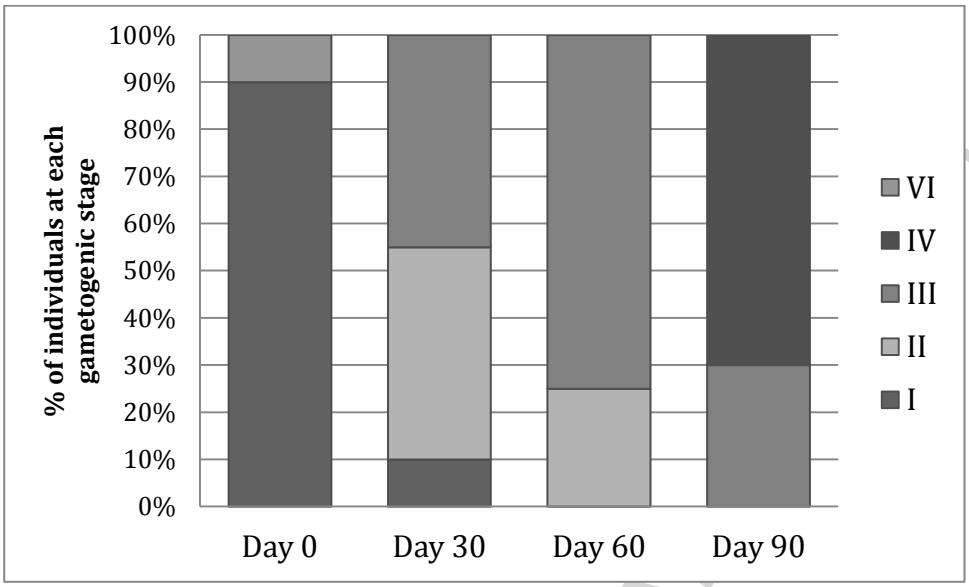

Figure 2

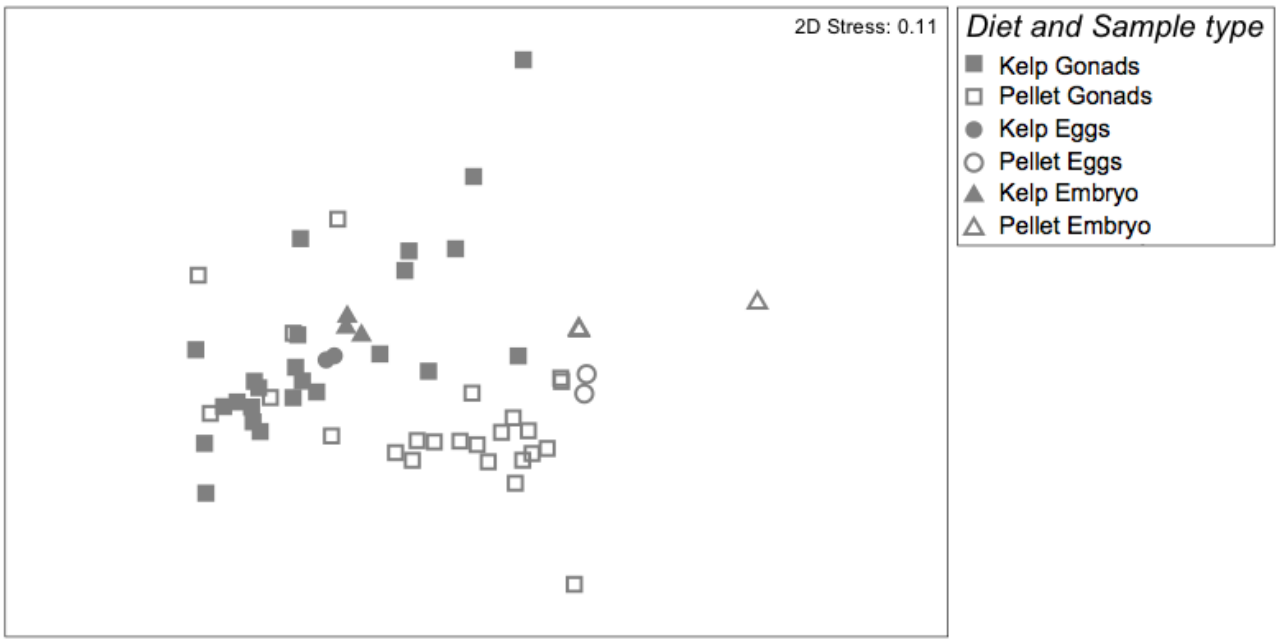

Figure 3a

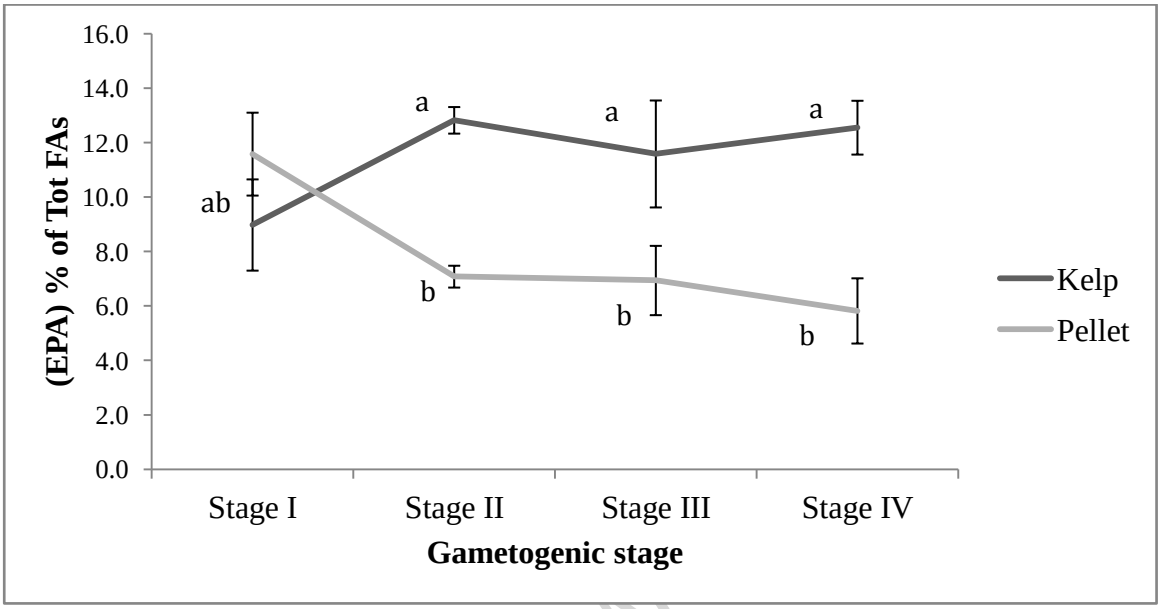

Figure 3b