Effects of dietary microalgae on growth, survival and fatty acid composition of sea urchin *Paracentrotus lividus* throughout larval development.

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Abstract

This study investigated the growth, survival and fatty acid composition of sea urchin *Paracentrotus lividus* larvae fed four microalgal diets: *Cricosphaera elongata*, *Pleurochrysis carterae*, *Tetraselmis suecica* and *Dunaliella tertiolecta* (control). Larvae were successfully raised to competence for metamorphosis when fed *C. elongata*, *P. carterae* and *D. tertiolecta* diets but significant differences were found in survival rate and development. Larvae fed *C. elongata* showed 3 times higher survival and 20 % faster development than larvae fed the other two microalgae diets that supported development. In contrast, *T. suecica* failed to fully support development and larvae stalled at the four arms stage for more than 30 days. The urchin larvae could accumulate long-chain polyunsaturated fatty acids (LC-PUFA) such as docosahexaenoate (DHA; 22:6n-3), eicosapentaenoate (EPA; 20:5n-3) and arachidonate (ARA; 20:4n-6), either by assimilation and retention of dietary fatty acids, and/or synthesis from α-linolenic acid 18:3n-3 and linoleic acid 18:2n-6. Moreover, an accumulation of n-3 LC-PUFA and higher EPA/DHA and EPA/ARA ratios appeared to be associated with improved larval performance. The results indicate that live microalgae species, with appropriate fatty acid profiles are able to improve *P. lividus* larval performance, ultimately increasing hatchery profitability.

Key words: *Paracentrotus lividus*, sea urchin, larvae, nutrition, microalgae, fatty acids

1. INTRODUCTION

Sea urchin gonads (roe) are considered a delicacy worldwide and especially in Japan where they are called “uni” and valued as sushi (Lawrence, 2001). As natural stocks are declining due to fishing pressure (Boudouresque and Verlaque, 2001; Andrew et al., 2002), mariculture has an important role to play to supply high quality seeds, juveniles and adults (Guidetti et al., 2004; Pais et al., 2007). Due to its high roe content, the purple sea urchin *Paracentrotus lividus* (Lamarck, 1816) has been identified as an ideal candidate to satisfy increasing demand in Europe but wild stocks generally cannot sustain a fishery (Watson and Stokes, 2000). *P. lividus* is widely distributed in south-western Europe, and reaches the northern limits of its range in Scotland. As there are only a few isolated populations in Scotland, a fishery is not viable and so a fully-farmed approach is the only option for its production. The first commercial production of this species in the UK started at the Ardtoe Marine Laboratory (AML) in 2007.

Research on sea urchin was initiated 100 years ago (Koehler, 1883) and more recently studies have focused on larval feeding (Fenaux et al., 1985; Pedrotti and Fenaux, 1992; Fenaux , 1994; George et al., 2004; Liu et al., 2007), culture methods for both larvae (Leighton, 1995; Kelly et al., 2000) and adults (Grosjean et al., 1998). However, many aspects of *P. lividus* biology including control of sexual maturation and larvae and adults nutritional requirement still require investigation to support a successful intensive commercial production. Importantly, most research trials performed to date have used laboratory scale culture methods that often result in survival rates far greater than those achieved by the industry and so are not always applicable to commercial scale ventures (Fenaux et al., 1985; Pedrotti and Fenaux, 1992; Kelly et al., 2000; George et al. 2004; Liu et al., 2007).

High mortalities during larviculture of marine fish and shellfish remain a major bottleneck in aquaculture that limits production (Dhert et al., 2001). Larval nutrition and the provision of optimal feeds are known to be a key factor in overcoming these problems

(Rainuzzo et al., 1997). It is well known that marine organisms require certain essential fatty acids (EFA), specifically n-3 and n-6 polyunsaturated fatty acids (PUFA), for their normal development. Many studies have shown that the absolute and relative amounts of dietary EFA have direct effects on larval development and survival of aquaculture species (Coutteau et al., 1997; Sargent et al., 1999, 2002; Tocher, 2003). Furthermore, each echinoderm species has specific dietary requirements and may require specific PUFA at given developmental stages (Cook et al., 2000; Castell et al., 2004). The importance of PUFA, especially linolenic acid (18:3n-3) and the long-chain PUFA (LC-PUFA), docosahexaenoic acid (DHA; 22:6n-3), has been demonstrated in the sand dollar *Dendraster excentricus* larvae where dietary provision of these fatty acids led to better survival and growth (Schiopu et al., 2006).

The use of artificial feeds has been investigated in *P. lividus*. The aim was to enhance larvae performance (survival and growth) through the provision of higher levels of total lipid (energy) and specific EFA, including DHA, eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6), than those present in the control diet *Dunaliella tertiolecta* (Liu et al., 2007). Results from that trial indicated that the artificial feed could support development, but larvae showed better growth performance when fed *D. tertiolecta,* mainly due to poor acceptance of the artificial diet. Nonetheless, the fatty acid profile of *D. tertiolecta* appears to be suboptimal as it contains only a very low level of EPA and essentially no DHA (Kelly et al., 2000, Liu et al., 2007) that could lead to nutritional deficiencies in the larvae. Artificial feeds that are commercially available to date are not likely to support the needs of sea urchin hatcheries due to a number of reasons including palatability issues, as larvae have the ability to select feed prior to ingestion (Liu et al., 2007), lack of information on diet formulations, prohibitive costs of producing feeds with very fine particles (micro-encapsulated) for a relatively small market. For these reasons *D. tertiolecta* is the main live feed microalgae used extensively for the rearing of all echinoderms larval stages. However, previous studies suggested that other microalgae species including

Cricosphaera elongata and *Pleurochrysis carterae* could be used for sea urchin larviculture (Pedrotti and Fenaux, 1992).

The present study revealed, for the first time, the evolution of fatty acid profile and the effects of dietary fatty acid composition on urchin larval fatty acid composition throughout development, it also provides a possible solution to improve production output of commercial hatchery. To this end, we compared sea urchin larvae growth performance when fed different live microalgae species that are easy to culture and readily accepted by the larvae, and with more balanced fatty acid profiles, especially, LC-PUFA. The hypothesis being that these microalgae with more appropriate levels and ratios of LC-PUFA will better support development and enhance growth of *P. lividus* larvae. Furthermore, this work will help to further understand physiological requirements of echinoplutei and enable optimisation of *P. lividus* larval rearing protocols to increase hatchery profitability.

2. MATERIALS AND METHODS

2.1. General methods

Three year old *P. lividus*, raised at the Ardtoe Marine Laboratory (AML; 56N 46' - 5W 52'), and fed on brown algae *Palmaria palmata*, *Laminaria digitata* and *Saccharina lattisima* (20:40:40, wet weight) over the culture period, were induced to spawn in February 2010 by injection of 1 M KCl (40 µl per g of body weight) into the coelom via the peristomial membrane. Three females (51.0 ± 1.3 g) and three males (49.0 ± 1.3 g) were spawned. Each female spawned approximately 2 million eggs that were fertilized by adding few drops of diluted sperm. Fertilization rate, assessed 2 h post fertilization, was 98.5 ± 1.0 %. The fertilized eggs were left to hatch in static seawater without aeration for 24 h in the dark. Hatching rate was 85.0 ± 1.0 %.

Seawater used during the process of spawning, hatching, and larval rearing was filtered (4 µm) and UV treated, and room temperature was maintained at 18 ± 2 °C throughout

the larval cultivation period. Larvae were stocked at a density of 4 per ml in 80 L conical plastic tanks and cultivated in aerated static water in continuous light. A complete water exchange and thorough cleaning of the tanks was carried out every 3rd day. Age at competence was defined as the number of degree-days required for at least 75 % of the larvae fed each treatment to reach competence for settlement, which was considered achieved when the rudiment was equal in size or larger than the stomach. As temperature differences between treatments could affect larval rate of development Degree days (ºC d^{-1}) unit was used to compare age at competence. The larval culture methods were adapted from Kelly et al. (2000).

2.2. Experimental diets

The experimental design involved four triplicated treatments. *Dunaliella tertiolecta* (7µm Equivalent Spherical Diameter, 180µm^3 Volume) (control), *Tetraselmis suecica* (7µm ESD; 180µm^3 Volume), *Pleurochrysis carterae* (8.9µm ESD; 380µm^3 Volume) and *Cricosphaera elongata* (8.9µm ESD; 380µm^3 Volume) were used as diets. The first three species were sourced from the Culture Collection for Algae and Protozoa (CCAP: Oban, Scotland) while the last was isolated at Dip.Te.Ris (Genova University, Italy) and shipped to AML. Microalgae cultures were grown in 100 L polyethylene bags in sterilized seawater enriched with the f/2 medium. Meta-silicates were added to media for *C. elongata* and *P. carterae*. Microalgae during their exponential growth phase were fed to the larvae every 3rd day and ration was standardized between treatments according to microalgae species cell size. For larvae with two, three and four pairs of arms, the daily feeding rate was 1500, 4500, and 7500 cells ml^{-1} respectively when using *D. tertiolecta* or *T. suecica*, as adapted from Kelly et al. (2000) and Jimmy et al. (2003), and 750, 2250 and 3250 cells ml^{-1} respectively when using *C. elongata* or *P. carterae*. Microalgae culture densities were determined by spectrophotometry (DR/2000 Direct Reading Spectrophotometer, Hach Lange Ltd, UK) using standard

concentration curves for each species prepared using light microscopy and cell counting via hemocytometer. Accuracy was checked via regression analysis (R-sq ≥0.8). Whilst particle counting is often used in small scale trials to assess microalgae culture density, spectrophotometry is widely used in commercial aquaculture and has been considered of good precision when compared with other commonly used methods including cell counting via hemocytometer (Butterwick et al., 1982).

The equation used to calculate feed ration was: Volume of algae given = (number of algae cells x rearing volume) / Algae culture concentration.

2.3. Larval growth, morphology and survival

Larval morphology can be drastically influenced by feeding regime (Kelly et al., 2000; Strathmann et al., 1992; Fenaux et al., 1994) and therefore the major larval body features were measured. At 5, 11, 17 and 23 days post fertilisation (DPF), 20 larvae were randomly sampled from each rearing tank and larval length, body width and post-oral arm length were measured using an image analysis software (Image Pro Plus^{TM}, Media Cybernectics, Silver Spring, Maryland, USA). The development of additional larval arms was also recorded. Larval survival was assessed volumetrically every 3rd day and when competence for settlement was achieved by at least 75 % of the larvae in each rearing tank.

2.4. Lipid and fatty acid analyses

Microalgae samples (20 x 10^6 cells) were collected by filtration onto a GF/F filter (Whatman Ltd, Maidstone, UK) before being placed in glass vials containing 5 ml of chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. Algal culture concentration was assessed as described above and volume filtered recorded to assess the number of cells present in each sample. Urchin eggs were collected from the five gonopores immediately after spawning using a pipette and placed in glass vials

containing 5 ml chloroform/methanol plus BHT as above. Urchin larvae samples (about 1200 larvae) from each replicate at each developmental stage (pyramid, 4 arms, 6 arms, 8 arms and rudiment) were collected after filtration through a 40 μm sieve and stored in glass vials as above. All samples were stored at -20 °C for 5 weeks prior to analyses.

Total lipid of microalgae, urchin eggs and larvae was extracted following the method of Folch et al. (1957). Samples were homogenized in the chloroform/methanol (as above) using a tissue disrupter (Ultra Turax™, IKA Werke Gmbh & Co. KG, Staufen, Germany), and 1 ml 0.88 % KCl was added and the homogenates mixed before centrifugation at 600 g for 5 min (Jouan C412, Pegasus Scientific Inc., Rockville, USA). The upper aqueous phase was aspirated and the solvent evaporated under a stream of oxygen-free nitrogen (OFN). Lipid content was determined gravimetrically after desiccation overnight. The total lipid extracts were re-dissolved at a concentration of 10 mg/ml in chloroform/methanol (2:1, v/v) plus BHT.

Fatty acid compositions of total lipid were determined by gas chromatography according to Christie (2003). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification at 50 ^{o}C for 16 h with extraction and purification by thin-layer chromatography as described previously (Tocher and Harvie, 1988). The FAME were separated and quantified by gas-liquid chromatography using a Fisons 8160 instrument (ThermoFisher, UK) equipped with a 30 m x 0.32 mm i.d., 0.25 μm capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection. Hydrogen was used as carrier gas and temperature programming was from 50 to 150 ^{o}C at 40 ^{o}C min^{-1} and then to 230 ^{o}C at 2.0 ^{o}C min^{-1}. Individual methyl esters 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 FAME quantified through a comparison with a heptadecanoic acid (17:0) internal standard.

2.5. Statistical analysis

All analyses were carried out using the statistical package of Minitab 15.0 (Minitab Ltd., UK). Larval morphometric measurements were analysed using a General Linear Model (Zar, 1999). Normality and homogeneity of variance were improved where necessary by either log or reciprocal transformations. Development of arms and rudiment, age at competence and fatty acids were analysed using one-way ANOVA followed by the Tukey multiple comparison test to assess where significant differences occurred. Survival rates (described as a percentage) were analysed using analysis of proportions where 95 % confidence limits were calculated for the respective proportions (Fowler and Cohen, 1987). When the upper and lower confidence limits of the respective proportions were not found to overlap the proportions were considered to be statistically different at the 5 % level ($p = 0.05$).

3. RESULTS

3.1. Lipid content and fatty acid composition of microalgae

The total lipid (TL) content varied between the four microalgal diets. *D. tertiolecta* had the highest TL content (0.14 mg per million cells) followed by *C. elongata* (0.09 mg per million cells), by *T. suecica* (0.06 mg per million cells) and by *P. carterae* (0.05 mg per million cells). *C. elongata* contained the highest proportion of total PUFAs, followed by *P. carterae*, *T. suecica* and *D. tertiolecta* (Table 1). Similarly, total n-3 and n-6 PUFA were highest in *C. elongata*, followed by *P. carterae*, *D. tertiolecta* and *T. suecica*. However with regard to LC-PUFAs, *T. suecica* showed the highest proportion of EPA followed by *C. elongata* and *P. carterae*, with *D. tertiolecta* showing only a very low level. In contrast, *P. carterae* was rich in DHA followed by *C. elongata*, with *T. suecica* and *D. tertiolecta* showing low levels. *D. tertiolecta* on the other hand showed the highest proportion of ARA, followed by *T. suecica*, whereas both *P. carterae* and *C. elongata* were devoid of this fatty acid (Table 1).

3.2. Survival and age at competence

Sea urchin larvae fed *C. elongata* showed significantly higher survival at 24 DPF and competence (14.4 %) compared with larvae fed *P. carterae* (5.7 %) or *D. tertiolecta* (5.2 %) (Fig. 1). Competence for settlement was first reached by larvae fed *C. elongata* (462 ± 30 ºC d^{-1}), followed by larvae fed *P. carterae* (519 ± 30 ºC d^{-1}) and by larvae fed *D. tertiolecta* (578 ± 29 ºC d^{-1})(data not shown). Sea urchin larvae fed on *T. suecica* never reached competence and after 600 ºC d^{-1} were still at the four arms stage (data not presented).

3.3. Growth and development

The sea urchin larvae growth rate was reflected in larval body indices, such as post-oral arm length, larval length and body width (Fig. 2 a, b and c). Post-oral arm length did not increase in larvae fed *T. suecica* and it was significantly smaller than all the other treatments at 11 and 17 DPF. Post-oral arm length in urchin larvae fed *D. tertiolecta*, *P. carterae* and *C. elongata* increased over the first 11 DPF, and decreased thereafter. Arm shortening continued in larvae fed *D. tertiolecta*, *P. carterae* and *C. elongata* and at competence the arms of larvae fed these treatments were significantly shorter than in larvae fed *T. suecica* (Fig. 2a).

Larval length increased over the first 11 DPF when significant differences were observed between treatments with larvae fed *P. carterae* significantly longer than larvae fed the other treatments, and larvae fed *D. tertiolecta* and *C. elongata* significantly longer than larvae fed *T. suecica* (Fig. 2b). Length of larvae fed *P. carterae*, *C. elongata* and *D. tertiolecta* decreased between 11 and 23 DPF but, at the end of the trial, they were still significantly longer than larvae fed *T. suecica* (Fig. 2b).

No significant difference in larval width was observed between larvae fed *D. tertiolecta*, *P. carterae* and *C. elongata* up to 11 DPF (Fig. 2c). However, from 17 DPF onwards significant differences between treatments were observed. Larvae fed *C. elongata* were the

largest, followed by *P. carterae*, *D. tertiolecta* and *T. suecica*. No significant change in body width was observed in larvae fed *T. suecica* treatment during the whole period of larval culture (Fig. 2c).

The timing of development of new pairs of arms and rudiment varied between urchin larvae fed the different microalgae treatments (Fig. 3). As shown above for age at competence, larvae fed *C. elongata* and *P. carterae* had the fastest rate of development, with a greater average percentage of larvae having the 3rd pair of arms by 11 DPF and the rudiment by 23 DPF compared to urchin larvae fed *D. tertiolecta*. By 17 DPF, 100 % of the larvae in the *C. elongata* and *P. carterae* treatments had developed the 3rd pair of arms compared with an average of 63.3 % in the *D. tertiolecta* treatment. A significant difference in rudiment development was observed at 23 DPF when 100 % and 85 % of the larvae in *C. elongata* and *P. carterae* treatments, respectively, had developed rudiment, whereas only 40 % of the larvae with rudiment was observed in larvae fed *D. tertiolecta*. Larvae fed *T. suecica* showed shrinkage of the stomach from 10 DPF onwards and did not develop the third pair of arms or show any signs of rudiment development.

3.4. Lipid content and fatty acid composition of *P. lividus* larvae

Total lipid content of the urchin larvae increased during the trial period regardless of the dietary treatment, however no significant differences were observed until the rudiment stage (Fig. 4). By the end of the trial larvae fed *T. suecica* had significantly lower lipid content compared with the other treatments. Significant differences between *C. elongata*, *P. carterae* and *D. tertiolecta* treatments were observed at the rudiment stage with lipid content in larvae fed *C. elongata* being significantly higher than larvae fed the other two treatments.

The fatty acid profiles of the urchin larvae throughout development showed the clear effects of the onset of exogenous feeding (4 arms stage) and differences between treatments increased from the six arms stage onwards (Fig. 5, 6, Tab. 2). Total saturated and

monounsaturated fatty acids of larvae fed *C. elongata*, *P. carterae* and *D. tertiolecta* showed a slight increment during embryonic development (from eggs to pyramid stage). From onset of feeding onwards however they showed significant decreasing trends regardless of the treatment (Fig. 5 a, b).

Relative abundance of total n-3 PUFA decreased during embryonic development, but increased from the pyramid stage onwards when larval body structures begin to form (Fig. 6a). Significant differences were observed between treatments from the 8 arms stage onwards when larvae fed *C. elongata* showed the highest relative n-3 PUFA content followed by *P. carterae* and *D. tertiolecta* treatments. At 8 arms, larvae fed *C. elongata* still showed the highest n-3 PUFA content but now followed by larvae fed *D. tertiolecta* and then *P. carterae*. Total n-6 PUFA relative abundance did not change significantly up to the 8 arms stage when larvae fed *P. carterae* showed significantly higher n-6 PUFA content than *D. tertiolecta* and *C. elongata* (Fig. 6b). At the rudiment stage *D. tertiolecta* and *P. carterae* fed larvae showed higher n-6 PUFA content than *C. elongata*.

The proportion of EPA in the larvae decreased significantly during embryonic development, but increased after onset of feeding to reach, at 6 arms stage, comparable levels to eggs (Table 2). Until the 6 arms stage, no significant differences were observed between treatments, whereas at the 8 arms stage larvae fed *C. elongata* showed higher EPA levels than larvae fed *D. tertiolecta,* and larvae fed *P. carterae* showed no significant difference with either *C. elongata* or *D. tertiolecta* treatments. At competence for settlement, the level of EPA in larvae fed *C. elongata* and *D. tertiolecta* were significantly higher than larvae fed *P. carterae*. The proportion of ARA in larvae also decreased during embryonic development, but only increased slightly after initiation of exogenous feeding (Table 2). The only significant difference between treatments was observed at competence, when larvae fed *D. tertiolecta* had higher ARA than larvae fed *C. elongata* and *P. carterae*, reflecting the algae fatty acid profiles. The DHA content was negligible in urchin eggs but its proportion increased during

all phases of development in larvae fed *C. elongata* and *P. carterae* whereas it declined after onset of exogenous feeding in larvae fed *D. tertiolecta* (Table 2).

Ratios between EPA/ARA and DHA/EPA in larvae also varied between dietary treatments. The EPA/ARA ratio increased during larval development in all treatments although it started to increase earlier in larvae fed *C. elongata* (4 arms stage) than in larvae fed *P. carterae* (6 arms stage) and *D. tertiolecta* (8 arms stage) treatments. Therefore, at competence, this ratio was higher in larvae fed *C. elongata* and *P. carterae* than in larvae fed *D. tertiolecta*. The DHA/EPA ratio was very low in the eggs and increased in larvae fed *C. elongata* and *P. carterae* up to 4 arms stage, but it did not change significantly thereafter. A different trend was observed for this ratio in larvae fed *D. tertiolecta* where it peaked at the pyramid stage to then sharply decrease during larval development (Table 2).

4. **DISCUSSION**

4.1. *Effect of dietary treatments on larval growth, morphology and survival*

The age at competence observed in the present trial was greater than previously observed (Gosselin and Jangoux, 1996; Kelly et al., 2000; Liu et al., 2007), but within the timeframe of previous observations at AML when using out of season broodstock. The different dietary microalgae showed significant effects on development that may be attributable to the nutritional properties of the diets. Thus, development was more rapid in larvae fed *C. elongata*, followed by *P. carterae* and *D. tertiolecta* and this was also reflected in the appearance of the third pair of arms and rudiment. The shorter cycle of larvae fed the *C. elongata* treatment was reflected in a significantly higher survival. At a hatchery level, higher survival and a shorter culture period means significant economic advantages such as increased juvenile output and savings in labor, space and consumables. More specifically, the major practical implications of this result was the increased number of juveniles produced by the hatchery (50,000 competent larvae per rearing tank versus 16,000 usually obtained with the

standard rearing protocol) and in a 20% shorter time, allowing for a better economic return when *C. elongata* was used as larval feed. Although this result brings considerable advantages it is still significantly lower than what is reported in smaller scale trials, suggesting that better larval survival could be achievable reducing, for instance, stocking density. However, production output relies on numbers of individuals produced per cycle and not on percentage of survived individuals. Commercial operations, in fact, often trades survival percentage for overall production output and are therefore reluctant in reducing stocking densities.

Significant differences in post-oral arm length and larval length during development were observed between larvae fed the dietary treatments. However, by the end of the trial, there were no significant differences between those treatments that supported development but they all were significantly different from larvae fed *T. suecica*. Morphometric data and their trend over time shown in the present study agrees well with previous observations (Liu et al., 2007).

Shortening of the post-oral arms was suggested to be a sign of over-feeding (Kelly et al., 2000), or homeostatic response of the larvae to allocate energy towards earlier metamorphosis when food was abundant (Strathmann et al., 1992; Fenaux et al., 1994). Moreover, programmed cell death (PCD) process was identified in the sea urchin *Hemicentrotus pulcherrimus* arms concomitantly with metamorphosis (Sato et al., 2006). It seems possible, although not yet known, that PCD might already be active during late larval developmental stages and be responsible for the observed arms shortening. These suggestions led to the conclusion that the observed general shortening of arms during development can probably be explained as a natural phenomenon accompanying larval development. For this reason, total larval length and post-oral arm length cannot be considered as good indicators of larvae growth or condition. On the contrary, larval body width in the *C. elongata* treatment was significantly greater than all the other treatments and this correlated well with the overall better performance (higher survival and faster development) of larvae fed this diet. We

therefore suggest the latter as a more reliable body feature for the evaluation of growth and general larvae condition.

This study also shows that a majority of the *P. lividus* larvae fed *T. suecica* did not develop the 3rd pair of arms by the end of the experimental period, whereas the larvae fed *all the other treatments* had already achieved competence by this time. Larvae fed *T. suecica* also showed a significantly reduced body width compared to larvae fed the other diets. Although most larvae had settled by 27 DPF the experiment was continued until 31 DPF to allow extra time for the development of larvae in the *T. suecica* treatment, but no sign of further development in this treatment group was observed. This suggests that either the nutritional value of *T. suecica* was insufficient for normal development or that the larvae were unable to feed effectively on this microalga. Observations of stomach content and feeding activity indicated that larvae had consumed *T. suecica* cells but signs of stomach shrinkage occurred from 10 DPF onwards as previously reported for *P. lividus* larvae fed commercial algae paste that contained 20 % of *T. suecica* (Liu et al., 2007). These observations suggest that this microalgae species may disrupt digestive functions possibly resulting in some damage to the stomach. Nonetheless, signs of starvation, such as elongation of the arms (Miner, 2005), were not observed and larvae were able to survive for a long time without developing beyond the four arms stage. This has previously been observed by Liu et al. (2007), but direct correlation with a specific component of the diet (*T. suecica*) was not reported.

4.2. Larval development and fatty acid composition

The four microalgae diets used in the present trial differed in both lipid contents and fatty acid profiles. Although *D. tertiolecta* showed the highest total lipid content, it had only very low levels of the n-3 LC-PUFA, DHA and EPA, whereas these EFA were fairly abundant in the other microalgae. However, *D. tertiolecta* showed the highest percentage of ARA, which was not detected in either *C. elongata* or *P. carterae* and only in small amounts in *T. suecica.*

P. carterae showed the highest proportions of total n-3 LC-PUFA and DHA with a high DHA/EPA ratio. In contrast, *T. suecica* and *C. elongata* both showed higher EPA levels with low DHA/EPA ratios. Therefore it was surprising that *T. suecica* and *C. elongata* showed such contrasting effects on larval development. This suggested that larvae perform better when fed microalgae that show a low DHA/EPA (high EPA) but with low levels of ARA. Although it is difficult to compare fatty acids profile of microalgae between studies due to the influence of culture conditions, comparable fatty acid profiles were reported for *D. tertiolecta* (Liu et al., 2007; Mendoza Guzman et al., 2011), *T. suecica* (Gonzales-Arays et al., 2011; Mendoza Guzman et al., 2011), and *P. carterae* (Kato et al., 1996).

It has been shown that the ratio between specific LC-PUFA (DHA/EPA and EPA/ARA) is as important, or more, than their absolute levels (Reitan et al., 2003; Schiopu et al. 2006; Liu et al., 2007). However contrasting results were reported for sea urchins as Schiopu et al. (2006) reported that larvae of *D. excentricus* develop better when fed a higher ratio of DHA/EPA whilst Liu et al. (2007) suggested that *P. lividus* larvae develop better when fed lower DHA/EPA ratio. In the present study, larvae performed better when their dietary DHA/EPA ratio was lower and EPA/ARA higher, confirming the observation of Liu et al. (2007) and highlighting a possible species-specific response to dietary EFA proportions. High EPA alone did not support development. Whether low DHA or high ARA could be the limiting factor in *T. suecica* is not clear. *P. carterae* had a high DHA content and DHA/EPA ratio when compared to *C. elongata*, but also had significantly lower lipid content that may also affect its nutritional quality for urchin larvae. Therefore the present results suggest that high dietary lipid content and n-3 LC-PUFA, with low DHA/EPA and high EPA/ARA ratios were the best combination for promoting growth, development and survival of *P. lividus* larvae. Of course, other physical or biochemical characteristics of *C. elongata* other than lipid content and fatty acid composition, such as protein and micronutrient content, may also be

important factors in promoting larval growth and survival. Further research in this area is therefore required.

The fatty acid profiles of the urchin larvae throughout embryonic development (eggs to pyramid) may indicated that specific fatty acids are consumed during embryonic development. This appears to be a consequence of the maternal urchin diets as the macroalgae used are generally rich in EPA and ARA and deficient in DHA (Jamienson and Reid, 1972), which was reflected in the egg fatty acid composition. As a consequence, EPA and ARA could have been relatively more utilized for energy (oxidation), although EPA may also be converted (desaturated and elongated) to DHA during embryogenesis. Conversely, it is also possible that the reduced proportions of EPA and ARA in the pyramid stage could be a consequence of increased proportions of saturated and monounsaturated fatty acids due to possible increased lipogenic activity.

In contrast, the relative proportions of LC-PUFA generally increased during larval development. This accumulation may be a direct result of exogenous feeding and selective retention of LC-PUFA relative to saturated and monounsaturated fatty acids whose proportions declined during larval development probably reflecting preferential oxidation. Detailed study of the levels of LC-PUFA in the *P. lividus* larvae fed the various microalgae feeds, which differed in LC-PUFA compositions, revealed some interesting suggestions on endogenous fatty acid metabolism of the larvae and notably conversion of dietary fatty acids and possibly biosynthesis from precursors. The data from larvae fed *D. tertiolecta*, showing substantial increased EPA, despite relatively low dietary EPA, suggest biosynthesis of EPA from the high level of dietary 18:3n-3. The activity of 18:3n-3 to EPA pathway in *P. lividus* larvae was supported by the fact that ARA levels increased in larvae fed all microalgae feeds, including *C. elongata* and *P. carterae* that had essentially no ARA, suggesting active biosynthesis of ARA from 18:2n-6 and other n-6 precursors (18:3n-6/20:3n-6). The elongation and desaturation of 18:2n-6 and 18:3n-3 to ARA and EPA, respectively, was also

observed in the larvae of *D. excentricus* (Schiopu et al., 2006), juvenile *Strongylocentrotus droebachiensis* (Castell et al., 2004), and to some extent in the adult sea urchin *P. miliaris* (Bell et al., 2000; Pantazis et al., 2000). Although several digestive enzymes have been found in echinoderm larvae, such as peptidase (Doyle, 1956), esterase (Ryberg, 1973), and alkaline phosphatase (Evola-Maltese, 1957), there have only been indirect reports of desaturase activity in adult sea urchins (*P. miliaris*) to date (Bell et al., 2000). Further study in this area is required to better understand sea urchin nutrition and lipid metabolism. However, the decreasing level of DHA in the larvae fed *D. tertiolecta* reflects the low dietary input of DHA and suggests little conversion of EPA to DHA. Thus the increasing levels of DHA in the larvae fed both *C. elongata* and *P. carterae* must reflect selective retention of dietary DHA rather than conversion of dietary EPA, which was also accumulated in larvae fed these microalgae. This appears to be supported by the DHA/EPA data as larvae fed *C. elongata* and *P. carterae* maintained relatively high ratios throughout development, while it sharply decreased in larvae fed *D. tertiolecta* supporting the conclusion that DHA levels in *P. lividus* larvae were more dependent upon dietary DHA than biosynthesis from EPA.

Among LC-PUFA, the n-3 series was particularly used during embryonic development and then accumulated in larvae tissues during development. Similar or slightly higher levels of n-3 LC-PUFA were found in larvae at competence compared with the respective diets, which indicated that larvae actively accumulated these fatty acids from the feed and retained them in their tissues. This observation is in contrast to that observed in previous trials (Liu et al., 2007) where the opposite trend was observed.

Liu et al. (2007) also suggested that higher level of 20:3n-3 and 20:4n-3 in the larvae was beneficial for larval development, however no correlation between these fatty acids and performance of the larvae was found in the present study. Larvae fed *C. elongata* that showed the best survival and greatest body width had contents of 3.5 % and 4.1 % for these fatty acids respectively, whereas larvae fed *D. tertiolecta* had the worst performance but the highest

20:3n-3 content and an intermediate 20:4n-3 content, and larvae fed *P. carterae* showed intermediate survival and body width but with the lowest contents of 20:3n-3 and 20:4n-3. Our data on fatty acid profile of the early development stages differ from that reported by Gago et al., (2009). However, the only likely comparable treatment used in that study was the "wild" diet but, unfortunately, details of that diet were not reported. Although it is likely that adult *P. lividus* were fed wild *Laminaria sp.,* it would be different in its nutritional properties from the diet used in the present study due to seasonal effects as the previous study was carried out in summer whereas the present study was performed over winter. A previous study conducted on *P. lividus* fed wild *Laminaria sp.* in winter, and spawned in winter (out of season) confirmed our data (Kreissig, 2009). Moreover EFA content of late larval stages in the present study agreed well with that reported for larvae fed *D. tertiolecta* (Liu et al., 2007).

5. **CONCLUSION**

Normal development of *P. lividus* larvae was observed in *Cricosphaera elongata*, *Pleurochrisys carterae* and *Dunaliella tertiolecta* treatments. Larvae fed *Tetraselmis suecica* did not show any sign of development and stalled at the four arms stage, and also showed signs of stomach shrinkage although no culture crash was observed until 31 DPF. The mechanisms regulating this highly plastic response to adverse conditions remain to be identified. The data suggest that relatively high dietary lipid content and n-3 LC-PUFA, with low DHA/EPA and high EPA/ARA ratios were the best combination for promoting growth, development and survival of *P. lividus* larvae, and so microalgae species or blends that can supply this combination would be preferable feeds.

Although other nutritional qualities of the test diets such as protein and/or amino acid, vitamins and mineral contents and compositions will vary between live feeds and almost certainly play important roles in larval nutrition, it was beyond the scope of the present work to measure and quantify all potential factors affecting echinoplutei performances.

Nonetheless, the present data clearly show increased production output and a shorter production cycle when *C. elongata* is used as larval diet providing production cost reduction and increased revenue thus increasing hatchery profitability.

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Table 1. Fatty acid profiles of microalgae live feeds. Values are given as % of total fatty acids. [1]Total **s**aturated contain 15:0 and 22:0; [2]predominantly 16:1n-7; [3]predominantly 20:1n-9; [4]Totals contain 20:2n-6. ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentanoic acid; PUFA, polyunsaturated fatty acid.

Table 2. Essential fatty acids and relative ratios of *P. lividus* larvae fed different diets during development. Data are expressed as mean ± SD (n=3)

Figure legends

Figure 1. Survival rate of *P. lividus* larvae fed *Pleurochrysis*, *Dunaliella* and *Cricosphaera* diets. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences.

Figure 2. Larval length (a), Post-oral arm length (b) and Body width (c) of sea urchin larvae fed different microalgae diets from 5 to 23 days after fertilization. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences between treatments at each time point.

Figure 3. The appearance of third pair of arms and larval rudiment for *P. lividus* larvae fed different diets. Data are expressed as mean ± SD (n=3). Superscripts indicate significant differences between treatment at each time point.

Figure 4. Total lipid content of *P. lividus* larvae fed four microalgae diets throughout development. Data are expressed as mean ± SD (n=3). Asterisks indicate significant differences between treatments.

Figure 5. Percentage of total saturated (A) and total monounsaturated (B) fatty acids of *P. lividus* larvae fed different diets throughout development. Values are given as percentage of total fatty acids (mean ± SD, n=3).

Figure 6. Total n-3 (A) and n-6 PUFA (B) contents of *P. lividus* larvae fed different diets throughout development. Values are given as percentage of total fatty acids (mean ± SD, n=3). Superscripts indicate significant differences between treatments at each time point.

Table 1

	D. tertiolecta	*P. carterae*	*C. elongata*	*T. suecica*
14:0	0.8	0.6	0.3	0.5
16:0	18.6	17.4	21.6	20.9
18:0	6.8	1.8	2.0	0.5
20:0	0.4	2.2	5.0	0.2
∑ saturated[1]	30.1	22.2	29.4	22.3
16:1[2]	5.5	7.6	3.8	9.4
18:1n-9	7.7	5.8	3.9	12.4
18:1n-7	1.5	0.9	1.5	1.6
20:1[3]	0.9	0.3	1.3	2.3
22:1n-11	0.3	0.2	0.0	0.1
24:1n-9	0.3	0.0	0.2	0
∑ monounsaturated	16.4	14.8	10.7	25.8
18:2n-6	4.5	14.4	8.0	4.6
18:3n-6	3.2	1.8	1.0	1.2
20:3n-6	0.7	0.0	2.6	0.1
20:4n-6	0.9	0.1	0.2	1.1
22:5n-6	0.0	0.1	0.6	0.0
∑ n-6 PUFA[4]	9.1	16.6	12.7	7.1
18:3n-3	28.3	9.8	8.3	11.9
18:4n-3	1.2	16.9	16.9	7.3
20:4n-3	0.0	0.0	0.2	0.5
20:5n-3	0.7	3.5	3.5	7.5
22:5n-3	0.0	0.2	0.1	0.1
22:6n-3	1.4	8.6	10.3	0.6
∑ n-3 PUFA	31.7	38.9	39.5	28.0
16:2	1.3	4.8	3.4	1.0
16:3	2.3	0.4	2.3	1.6
16:4	8.9	0.5	0.5	12.0
Total C16 PUFA	12.4	5.8	6.2	14.6
Total PUFA	45.2	61.3	58.4	49.7
EPA/ARA	0.8	31.5	16.1	6.8
DHA/EPA	1.9	2.5	2.9	0.1

Table 2

Fatty acid	EPA			ARA			DHA			EPA/ARA			DHA/EPA		
Treatment	*C. elongata*	*P. carterae*	*D. tertiolecta*	*C. elongata*	*P. carterae*	*D. tertiolecta*	*C. elongata*	*P. carterae*	*D. tertiolecta*	*C. elongata*	*P. carterae*	*D. tertiolecta*	*C. elongata*	*P. carterae*	*D. tertiolecta*
Egg	10.7 ±0.9	10.7 ±0.9	10.7 ±0.9	8.6 ±1.1	8.6 ±1.1	8.6 ±1.1	0.3 ±0.2	0.3 ±0.2	0.3 ±0.2	1.2	1.2	1.2	0.03	0.03	0.03
Pyramid	2.9 ±1.5	2.2 ±1	1.9 ±0.9	1.7 ±0.6	1.2 ±0.5	1.0 ±0.9	1.6 ±0.7	1.5 ±1.2	2.9 ±0.5	1.7	1.8	1.8	0.6	0.7	1.5
4 arms	2.6 ±0.9	3.2 ±0.6	3.5 ±0.4	1.7 ±0.6	1.7 ±0.7	2.0 ±0.8	2.3 ±1.2	2.9 ±0.7	2.9 ±0.9	1.6	1.8	1.7	0.9	0.9	0.8
6 arms	9.0 ±1.1	7.1 ±2.5	7.9 ±1.6	3.4 ±0.2	4.1 ±1.3	4.3 ±0.2	**6.0 ±0.5a**	**4.9 ±1.7ab**	**1.9 ±0.1b**	2.6	1.7	1.8	0.7	0.7	0.2
8 arms	**12.6 ±1.1a**	**9.1 ±0.8ab**	**6.0 ±0.5b**	3.9 ±0.1	3.9 ±0.1	4.3 ±0.2	**8.6 ±0.3a**	**7.1 ±0.2b**	**1.5 ±0.2c**	3.3	2.3	1.4	0.7	0.8	0.3
Rudiment	**13.4±1.1a**	**9.13 ±0.6b**	**12.3±1.6a**	**3.0±0.4a**	**2.9±0.3a**	**5.2±0.2b**	**8.0 ±0.7a**	**7.1 ±0.2a**	**0.7 ±0.1b**	4.5	3.1	2.4	0.6	0.8	0.05

Fig. 1.

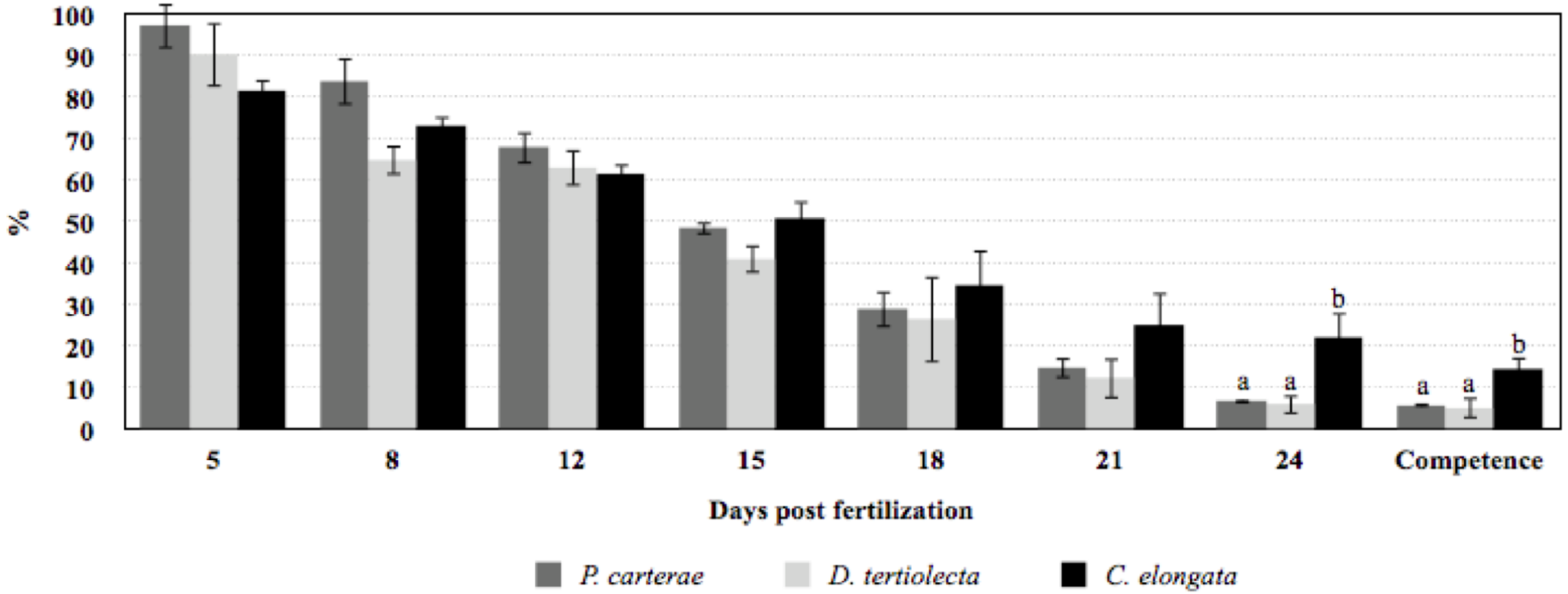

100
90
80
70
60
50
40
30
20
10
0
%
a
a
b
a
a
b
5
8
12
15
18
21
24
Competence
Days post fertilization
P. carterae
D. tertiolecta
C. elongata

Fig. 2.

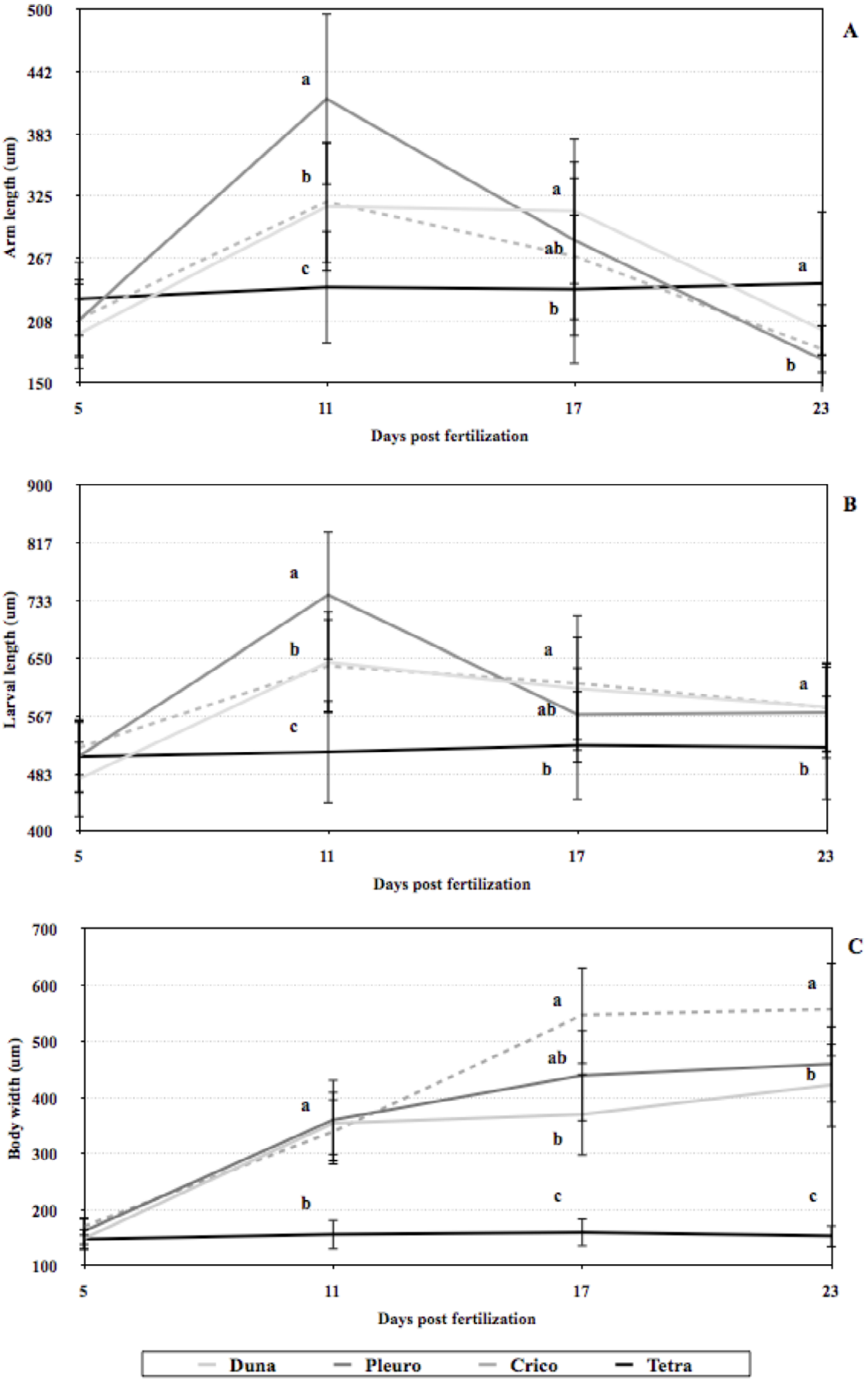

A
Arm length (um)
500
442
383
325
267
208
150
a
b
c
a
ab
b
a
b
5
11
17
23
Days post fertilization
B
Larval length (um)
900
817
733
650
567
483
400
a
b
c
a
ab
b
a
b
5
11
17
23
Days post fertilization
C
Body width (um)
700
600
500
400
300
200
100
a
b
b
a
ab
b
c
a
c
5
11
17
23
Days post fertilization
Duna
Pleuro
Crico
Tetra

Fig. 3

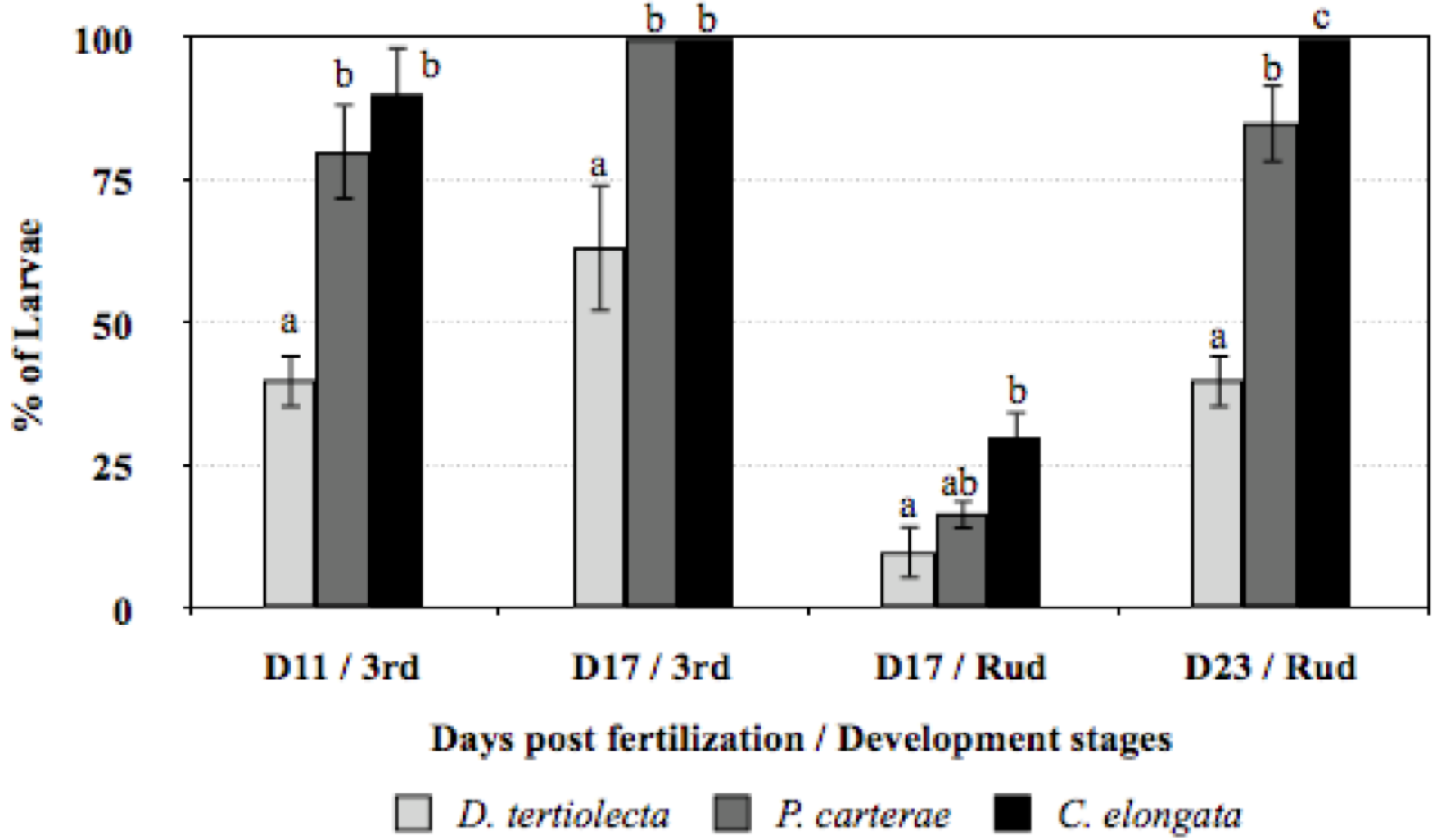

100
75
50
25
0
% of Larvae
a
b
b
a
b
b
a
ab
b
a
b
c
D11 / 3rd
D17 / 3rd
D17 / Rud
D23 / Rud
Days post fertilization / Development stages
D. tertiolecta
P. carterae
C. elongata

Fig. 4.

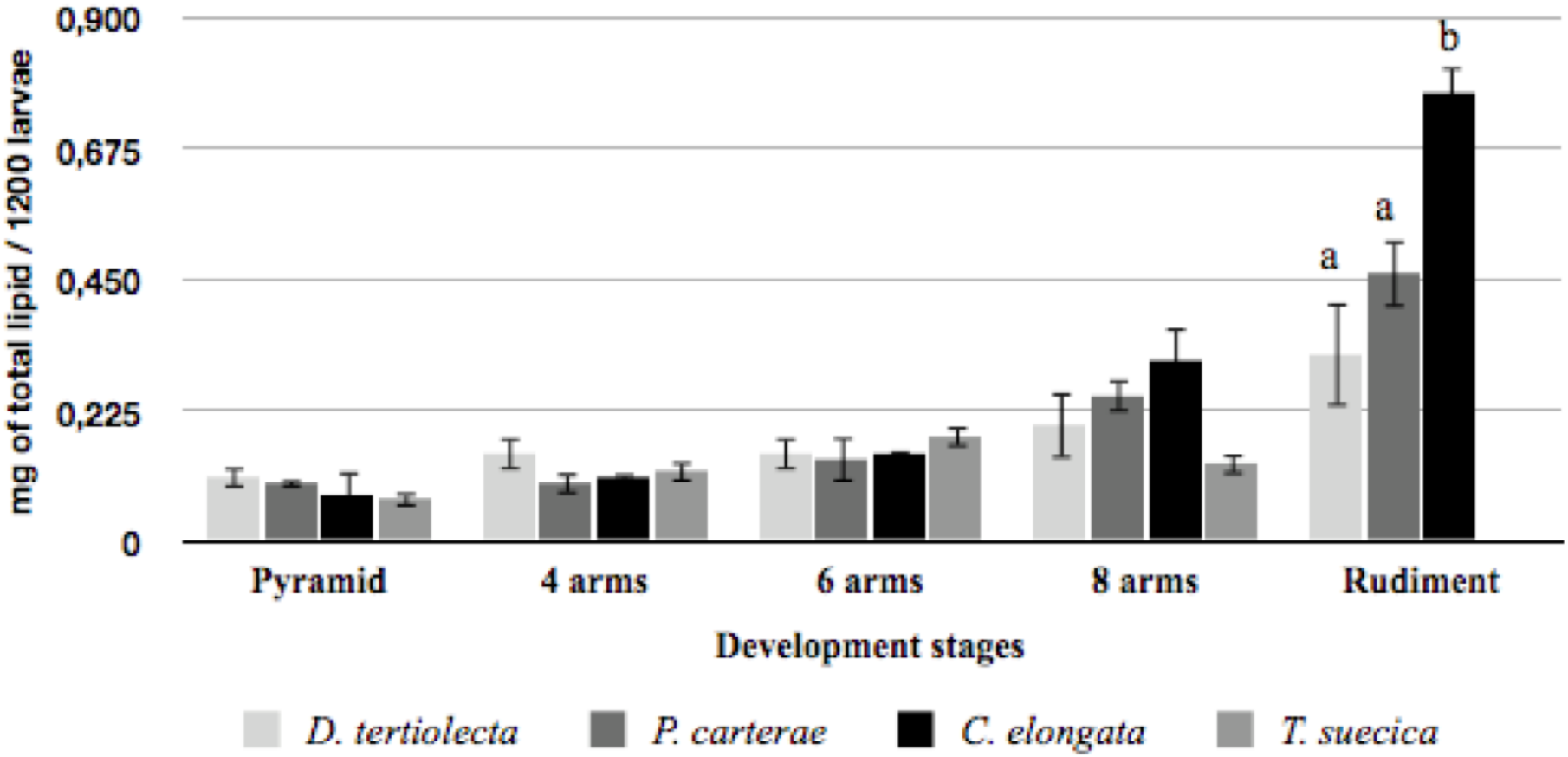

0,900
0,675
0,450
0,225
0
mg of total lipid / 1200 larvae
a
a
b
Pyramid
4 arms
6 arms
8 arms
Rudiment
Development stages
D. tertiolecta
P. carterae
C. elongata
T. suecica

Fig. 5.

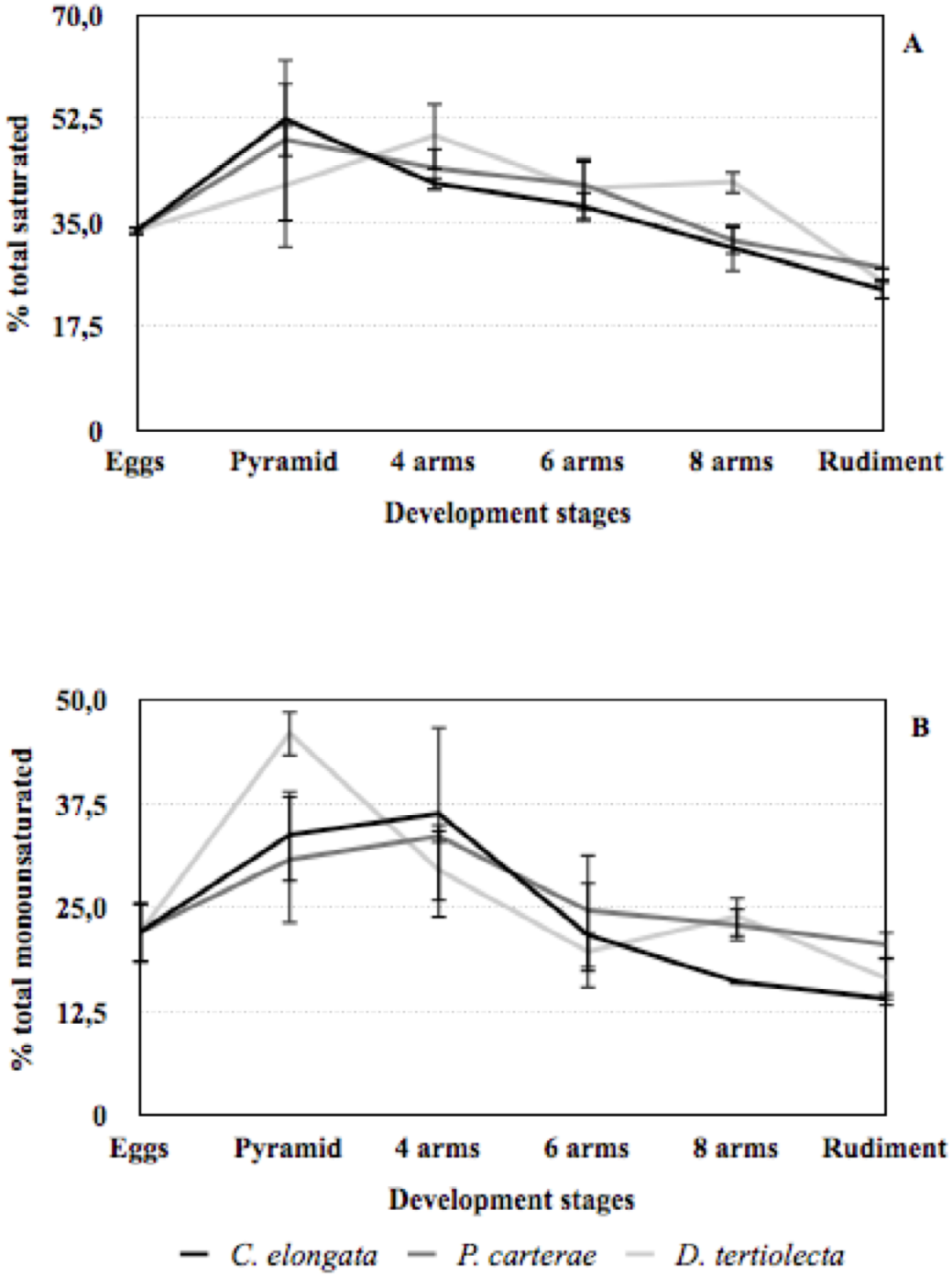
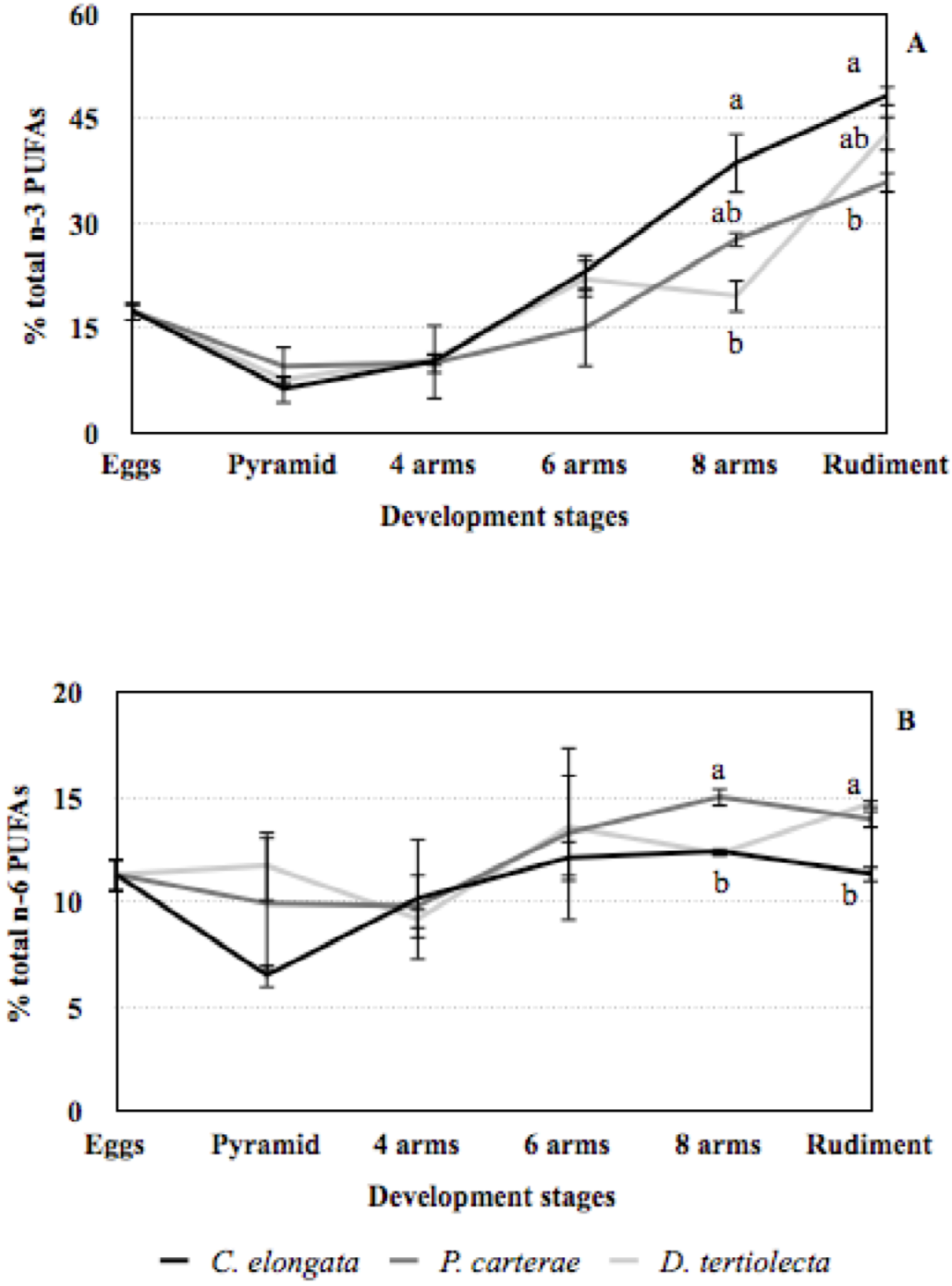

A
70,0
52,5
35,0
17,5
0
% total saturated
Eggs
Pyramid
4 arms
6 arms
8 arms
Rudiment
Development stages
B
50,0
37,5
25,0
12,5
0
% total monounsaturated
Eggs
Pyramid
4 arms
6 arms
8 arms
Rudiment
Development stages
C. elongata
P. carterae
D. tertiolecta

Fig. 6.


A
% total n-3 PUFAs
60
45
30
15
0
a
a
ab
ab
b
b
Eggs
Pyramid
4 arms
6 arms
8 arms
Rudiment
Development stages
B
% total n-6 PUFAs
20
15
10
5
0
a
a
b
b
Eggs
Pyramid
4 arms
6 arms
8 arms
Rudiment
Development stages
C. elongata
P. carterae
D. tertiolecta