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Essential Fatty Acid Assimilation and Synthesis in Larvae of the Bivalve *Crassostrea gigas*

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Abstract :

Essential fatty acids (EFA) are important for bivalve larval survival and growth. The purpose of this study was to quantitatively assess for the first time through a mass-balance approach dietary EFA incorporation and synthesis within *Crassostrea gigas* larvae. A first experiment was carried out using two microalgae, *Tisochrysis lutea* (T) and *Chaetoceros neogracile* (Cg), as mono- and bi-specific diets. A second experiment using a similar design was performed to confirm and extend the results obtained in the first. Flow-through larval rearing was used for accurate control of food supply and measurement of ingestion. Non-methylene-interrupted fatty acids were synthetized from precursors supplied in the diet: 16:1n-7 and 18:1n-9, mediated by Δ5 desaturase. Moreover, this Δ5 desaturase presumably allowed larvae to convert 20:3n-6 and 20:4n-3 to 20:4n-6 and 20:5n-3, respectively, when the product EFA were poorly or not supplied in the diet, as when larvae were fed T exclusively. Under our experimental conditions, none of the diets induced 22:6n-3 synthesis; however, 22:6n-3 incorporation into larval tissues occurred selectively under non-limiting dietary supply to maintain optimal levels in the larvae. This combination of flow-through larval rearing and biochemical analysis of FA levels could be applied to additional dietary experiments to precisely define optimal levels of EFA supply.

Abbreviations

CG-MS	Gas chromatography-mass spectrometry
Cg	*Chaetoceros neogracile*
DMOX	2-alkenyl-4,4-dimethyloxazoline
EFA	Essential fatty acid(s)
FA	Fatty acid(s)
Fad	Fatty acyl desaturase
FAME	Fatty acid methyl ester(s)
NMIFA	Non-methylene-interrupted fatty acid(s)
PAR	Photosynthetically active radiation
PUFA	Polyunsaturated fatty acid(s)
PVC	Polyvinyl chloride
T	*Tisochrysis lutea*

Keywords : Fatty acid, Larvae, Lipids, Metabolism, Oyster, Synthesis

1. Introduction

Essential, n-3 fatty acids (EFA) are important for larval survival and growth in bivalve mollusks [1]. Among them, eicosapentaenoic acid (20:5n-3) fulfills roles both as an energy source and as a precursor of eicosanoids; whereas, docosahexaenoic acid (22:6n-3) is involved in maintaining membrane structures and functions [2, 3]. Moreover, the n-6 arachidonic acid (20:4n-6) has been identified as affecting growth and survival during larval and postlarval stages [4, 5]. Generally it is accepted that the capability for EFA synthesis in bivalves is absent or insufficient to meet nutritional needs, and therefore EFA must be supplied exogenously to sustain optimal survival and growth (for review see Knauer and Southgate [6]).

Despite a consensus that long-chain n-3 PUFAs are essential in the diets of bivalve mollusks, some metabolic modifications have been found in some species. A marked ability to elongate 18:2n-6 to 20:2n-6, without however, desaturation to 20:4n-6, has been reported in *Mesodesma mactroides* [7]. Similarly, elongation of 18:2n-6 to 20:2n-6 occurred in *Crassostrea virginica* [8]. These authors, however, recorded desaturation of 18:2n-6 only in one, single oyster [8]. Lastly, *C. gigas* juveniles can elongate and desaturate 18:3n-3 fatty acid to 20:5n-3 and 22:6n-3, but only at rates insufficient to sustain optimal growth [9]. These previous studies were conducted using radiolabelled fatty-acid precursors. Other studies using descriptive approaches suggested some capabilities to elongate and desaturate polyunsaturated fatty acids (PUFA) for EFA synthesis [10, 11], but these remain to be confirmed. To our knowledge, no study had demonstrated *in vivo* EFA synthesis definitively in bivalve larvae. Because of these uncertainties, optimal dietary EFA supply cannot yet be established properly, as this would require quantitative assessment of metabolic abilities in larvae.

The flow-through (open) system, wherein a continuous replacement of water avoids the build-up of organic matter and oxygen deficiency, allows the maintenance of a constant food density in a culture tank [12-14] and an accurate determination of microalgal consumption by larvae. By controlling larval food quantity available, it is possible to vary nutritional quality of the diet, i.e., by varying mixes or physiological condition of living microalgae, independently. Diet quality effects upon larval development can be assessed, as well as the incorporation of individual biochemical microalgal components within the larvae, using a mass-balance approach. In the present study, we used two microalgae to vary EFA dietary supply: *Tisochrysis lutea* (formely known as *Isochrysis* affinis *galbana* or T-*Iso*) and *Chaetoceros neogracile. T. lutea* is rich in 22:6n-3; whereas, *C. neogracile* is rich in 20:5n-3 and also contains greater quantities of 20:4n-6 than *T. lutea* [15]. This innovative approach to assessing incorporation of fatty acids, particularly EFA, in mollusks was used to investigate fatty acid metabolism, conversion, and synthesis in *C. gigas* larvae.

2. Material and methods

2.1. Microalgae culture

Two different microalgae were tested as mono- and bi-specific diets: *Tisochrysis lutea* (named in the present paper T, volumetric size, 45 μm^3, strain CCAP 927/14) and *Chaetoceros neogracile* (named in the present paper Cg, 77 μm^3, strain UTEX LB2658). Microalgae were grown in 6-L glass carboys at 20-23°C under continuous

illumination (180-220 µmol photons m^{-2} s^{-1} PAR) provided by cool-white fluorescent tubes. Seawater (salinity 34-35‰) was 1-µm filtered, enriched with sterilized Conway medium (400 mg $NaNO_3$) [16] and then autoclaved. A 3% CO_2-air mixture was supplied to support growth and to maintain the pH within a range of 7.5-8.1. For culture of the diatom *C. neogracile*, sodium metasilicate (40 mg L^{-1}) was added as a silica source, and salinity was reduced to 25‰ by addition of distilled water. Microalgae were harvested at the late-logarithmic phase (after 3-4 days).

2.2. Broodstock conditioning and spawning and larval culture

Broodstock were collected from Aber Benoit (Brittany, France) and transferred to Argenton hatchery facilities for conditioning. Individuals were placed in open-flow tanks at 19ºC where they were maintained for 2 months with a daily supply of a mixed diet of *T. lutea* and *C. neogracile* equivalent to 6% of oyster dry weight in dry weight of algae per day. Gonads of the broodstock were stripped for gamete collection, using 11 males and 7 females. After counting, fertilization was performed at a ratio of 50 spermatozoa per oocyte. Two hours later, the embryos were incubated in cylindro-conical tanks in 1-µm-filtered seawater at 22ºC. After 48 hours of incubation, the percentage of D-larvae was determined. Veligers were transferred to 5-L, translucent, methacrylate cylinders and reared, in triplicate, in a flow-through system as described by Gonzalez-Araya et al. [12]. A continuous seawater flow of 0.87 ml min^{-1} was provided from the bottom of each experimental tank (100% tank water renewal per hour). Each diet was delivered by pumping from a reservoir, which was cleaned and filled with the appropriate feed daily, directly to the larval tanks down the seawater line. Seawater was 1-µm-filtered and UV-treated. Temperature was maintained at 25°C using a thermo-regulated automatic valve, and ambient salinity was 34‰. In each tank, aeration, provided from the bottom to maximize circulation of water, was set at 30 ml min^{-1}. The outlet of each tank was equipped with a beveled polyvinyl chloride (PVC) pipe as a sieve to prevent larvae from escaping. Mesh sieve sizes of 40, 60 and 80 µm were used at the beginning of the experiment, on days 6 or 7, and day 10, respectively.

Two experiments were performed to study fatty acid assimilation by *C. gigas* larvae and to determine optimal levels optimal levels of dietary EFA for *C. gigas* larvae. In the first, the objectives were tackled using two single diets (T or Cg) and bi-specific diet (TCg). In this experiment, all groups of larvae were reared until they reached competency (19 days post-fertilization for the lowest group). A second, follow-up trial using only two diets (T and TCg) was performed to confirm and validate results observed in the first experiment (synthesis of 20:4n-6 and 20:5n-3 in larvae fed T). Experiment 2 lasted 15 days (i.e. until larvae fed TCg reached competency). Each experimental treatment consisted of three replicate tanks of larvae. Regardless to diet delivered, larvae were kept in a constant concentration of 1,500 μm^3 μl^{-1} of microalgae at the exit of the rearing tanks, corresponding to ≈ 40 algal cells μl^{-1} (equivalent *T. lutea* [17]). Seawater at the inlet and outlet of each experimental tank was sampled twice a day to determine phytoplankton consumption. Cell counts were done using an electronic particle counter (Multisizer 3) equipped with a 100-µm aperture tube. Larvae were reared at a density of 100 and 50 larvae ml^{-1} for experiments 1 and 2, respectively, both densities leading to equivalent larval survival and growth in such flow-through system [18].

2.3. Biochemical analysis

Samples of each diet (250-300 x 10^6 cells) were collected during all larval culture (diets were temporally replicated, n = 5 and n = 7 for experiments 1 and 2, respectively). Thus, food composition data represent means and variances of fatty acid measurements throughout larval rearing. Each larval replicate for all treatments was sampled on day 2 (D-shaped larvae, start of feeding), on day 8, and when the positive control (TCg) reached the pediveliger stage (day 16 or 15, for experiments 1 and 2, respectively). In the first experiment, larval samples also were collected when larvae fed other diets reached the pediveliger stage (day 18 or 19, for larvae fed T or Cg, respectively). Samples were washed with 3.5% ammonium formate to remove salt and collected on 450ºC pre-combusted GF/F glass fiber filters (Whatman, diameter 47 mm). Lipids were extracted in 6 ml chloroform-methanol (2:1, v/v) according to Folch et al. [19], sealed under nitrogen, and stored at -20°C.

The neutral and polar lipids of the larvae were separated on a silica-gel microcolumn (50 mm x 5 mm; Kieselgel Merck, 70-230 µm mesh) previously heated to 450°C, deactivated with 6% water as described by Soudant et al. [20], and analyzed following the method described by Marty et al. [2]. Neutral and polar lipids were eluted with 10 ml $CHCl_3$-MeOH (98:2, v/v) and 10 ml MeOH, respectively. Fatty acids (FA) of microalgae were quantified relative to total lipids. The saturated fatty acid 23:0 (10 µl) was added as an internal standard for FA quantitative measurements. Samples were evaporated under nitrogen and transesterified with 1 ml of BF_3-MeOH (10%) for 10 min at 95ºC [21]. After cooling, 1 ml of hexane and 1 ml of water were added to each sample vial which were agitated and centrifuged. The organic upper phase containing fatty acid methyl esters (FAMEs) was collected and cleaned with 1 ml of water that was eliminated thereafter. FAMEs were recovered and analyzed by gas chromatography using a GC (HP 6890) with auto-sampler equipped with a capillary column (JW DB wax, 30 m length x 0.25 mm i.d. x 0.25 µm film thickness), with a cool, on-column injector at 60ºC and a FID detector at 300ºC. The carrier gas was H_2, at a constant flow of 2 ml min^{-1}. FAMEs were identified and quantified by means of a standard 37-component FAME mix and other standard mixtures from marine bivalves combining analysis on polar and non-polar columns [20]. Non-methylene-interrupted (NMI) fatty acids $20{:}2_{\Delta5,11}$, $22{:}2_{\Delta7,13}$, $20{:}2_{\Delta5,13}$ and $22{:}2_{\Delta7,15}$ (also designated 20:2i, 22:2i, 20:2j and 22:2j in the literature) were identified in oyster lipids based upon retention times and further confirmed by GC-MS after DMOX derivation (data not shown).

2.4. Data treatment and statistical analysis

In the present work, we focused on fatty acid metabolism between day 8 and days 15-16 because the first 5-6 days of larval life in *C. gigas* correspond to a period during which larvae feeding changes from lecitotrophic to exotrophic. During this period exogenous feeding is low (during the gradual activation of digestive enzymes in early larvae) and larvae are still relying upon the energy reserves contained within the embryo itself [22]. We also report essential fatty acid (EFA) incorporation during the entire larval phase, between day 2 and competence for metamorphosis (Experiment 1: day 16 for TCg; day 18 for T and day 19 for Cg; and experiment 2: day 15 for TCg) to determine EFA requirements.

We compared the amount of each ingested fatty acid (pmol $larvae^{-1}$) with the incorporated counterpart by larvae between two sampling dates. For each experiment,

FA ingestion was calculated using mean FA composition for each diet (n = 5 or n = 7 for experiments 1 or 2, respectively) and microalgal consumption for each diet treatment. When a fatty acid was present in larvae but absent from the diet, we assumed that this fatty acid is of endogenous origin (through biosynthesis or bioconversion of another dietary fatty acid). When the accumulated amount of a fatty acid within larvae was above 100% of the ingested amount, we assumed that this fatty acid had both exogenous and endogenous origins.

Data normality first was evaluated using the Shapiro-Wilk test, and then one-way analysis of variance (ANOVA) for significant differences was performed using STATISTICA software (Stat Soft, Inc., Tulka, OK, USA, version 12). Homogeneity of variances was checked by means of the Barlett test. When necessary, *post hoc* analyses with the LSD test were applied. Percentage data were arcsine-transformed to normalize variance [23]. Differences were considered statistically significant if $P \leq 0.05$.

3. Results

3.1. Experiment 1

Analysis of fatty acids in larvae and in each diet fed to larvae revealed evidence of both exogenous and endogenous sources in the larvae. Larvae synthetized higher quantities of 20:1n-7 than 20:1n-9, regardless of diet (Fig. 1A). A higher capacity for *de novo* synthesis of non-methylene-interrupted fatty acids was observed for 22:2i in larvae fed T and TCg; whereas, higher values were recorded for 22:2j in larvae receiving TCg (11.8 pmol $larva^{-1}$). Synthesis of 22:2j from precursors also appeared to be efficient in larvae fed Cg solely (7.0 pmol $larva^{-1}$) (Fig. 1A).

Low incorporation of 18:2n-6 and 18:3n-6 compared to fatty acid ingestion was found under all experimental conditions (Table 1). Incorporation of 20:2n-6 was higher as compared to its supply in larvae fed T (0.7 *vs* 1.1 pmol $larva^{-1}$; Table 1), and concomitantly, 20:4n-6 content in larvae fed this diet surpassed 5.1 times the quantity supplied in the diet (0.8 *vs* 1.1 pmol $larva^{-1}$ of fatty acid incorporation *vs* ingestion: Table 1).

Incorporation of 18:4n-3 was moderate regardless of diet. High accumulation of 20:4n-3 and 20:5n-3 (7.5 and 1.9-fold above the dietary supply, respectively) was observed in the 20:4n-3 and 20:5n-3-very low diet (T) (Table 1). Conversely, larvae fed Cg and TCg diets that were rich in 20:5n-3, incorporated 20:5n-3 in lower amounts than ingested as 20:5n-3 supply in the diets increased (28% and 38%, respectively: Table 1). Larvae receiving Cg, however, showed high capacity to elongate 20:5n-3 into 22:5n-3 (incorporation was 8-fold ingestion: Table 1). Larvae fed Cg, which was poor in 22:6n-3, exhibited the highest incorporation of 22:6n-3 compared to dietary supply (71% of the 22:6n-3 supplied in the diet Cg was incorporated by larvae; whereas, larvae fed T and TCg accumulated only 44-52% of dietary 22:6n-3: Table 1).

3.2. Experiment 2

Incorporation of 16:1n-7 and 18:1n-9 remained moderate for both T and TCg diets (Table 2). In contrast, 18:1n-7 was incorporated measurably by larvae, specifically

when fed TCg (48.6 *vs* 28.0 pmol larva^{-1} incorporated *vs* ingested, respectively: Table 2). Reaching 16.4 pmol larva^{-1}, high synthesis of 20:1n-7 from precursors occurred in larvae fed TCg (Fig. 1B). NMIs accumulated in larvae fed all diets, but 22:2j synthesis was particularly high for those fed TCg (≈ 18.4 pmol larva^{-1}) (Fig. 1B).

Modest incorporation of 18:2n-6 and 18:3n-6 was observed in larvae fed T or TCg (only 29-31% of the amount ingested was incorporated: Table 2). Synthesis of 20:2n-6 and 20:4n-6 was observed in larvae fed both diets, with, however, higher activity recorded in T-fed larvae (Table 2). In contrast, higher incorporation of 22:5n-3 was observed in larvae receiving TCg (Table 2).

The n-3 precursors of C20-22 n-3 PUFA, 18:3n-3 and 18:4n-3, were moderately incorporated (19-22%) (Table 2). Larvae fed T accumulated 20:5n-3 at a high rate (incorporation was 2-fold dietary supply: Table 2). Larvae incorporated only half of the 22:5n-6 and 22:6n-3 ingested by larvae regardless of diet (Table 2).

3.3. Fatty acid incorporation by competent larvae

In experiment 1, pediveliger larvae fed T synthetized 20:4n-6 and 20:5n-3 from precursor fatty acids (based upon differences in FA content between competent larvae at day 18 and D larvae at day 2), with 5.8 and 1.8 fold more 20:4n-6 and 20:5n-3, respectively, than dietary supplies (Fig. 2). Only 50% of 22:6n-3 supplied was incorporated in larvae fed T (Fig. 2). Larvae fed Cg achieved competence on day 19 in experiment 1. They incorporated 82, 39, and 76% of ingested 20:4n-6, 20:5n-3, and 22:6n-3 respectively (Fig. 2). Incorporation of 20:4n-6, 20:5n-3, and 22:6n-3 in larvae receiving TCg (experiment 1) reached 101, 38 and 45% of the amount ingested, respectively, from day 2 to 16, when larvae achieved competence (Fig. 2). In experiment 2, pediveliger larvae fed TCg synthetized 20:4n-6 (10.6 *vs* 7.0 pmol larva^{-1} incorporated *vs* ingested, respectively, between day 2 and competence on day 15: Fig. 2). Conversely, incorporation of 20:5n-3 and 22:6n-3 represented only 56% and 53% of the amount ingested by larvae (Fig. 2).

4. Discussion

To our knowledge, this is the first study of fatty acid incorporation in a larval mollusk using a flow-through, larval-rearing system. This system permits accurate quantification of algal cells, and their component fatty acids, consumed by the larvae. Using a mass-balance approach, assimilation of individual EFA can be calculated accurately, and optimal levels of dietary EFA can be determined.

Non-methylene-interrupted (NMI) fatty acid synthesis from structurally-related precursors was observed in our study, as previously demonstrated in another bivalve species by Zhukova [24, 25]. MUFAs supplied in the diet, 16:1n-7 and 18:1n-9, were elongated to 20:1n-7 and 20:1n-9 and subsequently desaturated by a Δ5 desaturase into 20:2$_{\Delta5,13}$ and 20:2$_{\Delta5,11}$. The products were further elongated to 22:2$_{\Delta7,15}$ and 22:2$_{\Delta7,13}$, respectively. Consequently, Δ5 desaturase appears to be active in *C. gigas* larvae under certain conditions, such as during the synthesis of NMI or in response to nutritional shortages. Indeed, we observed 20:4n-6 and 20:5n-3 synthesis in larvae fed

T in both experiments; this diet was very low in 20:4n-6 and 20:5n-3. Moreover, in the second experiment, larvae fed TCg also synthesized 20:4n-6. These EFA were synthesized by desaturation of 20:3n-6 and 20:4n-3 to respectively 20:4n-6 and 20:5n-3, leading us to propose putative Δ5 desaturase activity in *C. gigas* larvae. A Δ5-like desaturase was identified in other molluscan species, such as *Octopus vulgaris* [26], *Haliotis discus hannai* [27] and *Chlamys nobilis* [28]. A single fatty-acid desaturase (Fad)-like gene (www.ncbi.nlm.nih.gov/protein/EKC30965) has been found in the *C. gigas* genome. Although the putative synthesis of 20:4n-6 and 20:5n-3 from precursor FAs suggests that these PUFAs cannot be considered strictly as EFA for *C. gigas*, biosynthetic activity (if precursors are available in the diet) may not be sufficient to promote optimal larval performance.

As mentioned above, Δ5 desaturase activity requires 20:3n-6 and 20:4n-3 as substrates, and these FAs are absent or present in trace amounts in most microalgae species used currently to feed larval bivalves in the hatchery. Regarding the n-3 series of FAs, we found in larvae fed T appreciable 20:4n-3 content in polar (0.5% for both experiments %) or neutral lipid fractions (0.8 and 0.7% for experiment 1 and 2, respectively %), although this FA was scarce in this microalga. This PUFA appears to be a product of elongation of 18:4n-3. The low incorporation observed for 18:4n-3 (20-23%) by larvae may be partially explained by metabolic conversion to n-3, 20C PUFA. Similarly, the low absorption values of 18:3n-3 may be attributable to the role of this FA as a precursor for longer-chain, n-3 PUFAs. De Moreno et al. [7] reported that 18:3n-3 can be desaturated at the Δ6 position into 18:4n-3 and elongated to 20:4n-3, but no biosynthesis of 20:5n-3 and 22:6n-3 occurred in the clam *Mesodesma mactroides*. Waldock and Holland [9], however, detected small amounts of ^{14}C-labelled 20:5n-3 and 22:6n-3 in *C. gigas* juveniles fed *Dunaliela tertiolecta*, which did not provide precursors other than 18:3n-3. From our results, we propose a metabolic sequence in which 18:4n-3 is elongated to 20:4n-3 and then desaturated at the Δ5 position to 20:5n-3. An alternative sequence that can be postulated is 18:3n-3 elongation to 20:3n-3 and desaturation to 20:4n-3 by a Δ8. Pathways using 18:4n-3 and 18:3n-3 as dietary precursors are not mutually exclusive. We speculate that the dominance of one pathway or the other may depend upon proportions of the two precursors in the diet; in T 18:4n-3 is more abundant than 18:3n-3. Moreover, as synthesis of 20:4n-3 from 18:4n-3 involves only one elongation reaction, it is thought to be more energy efficient than combine elongation and desaturation of dietary 18:3n-3.

Regarding n-6 compounds, the FA 20:3n-6 was found in low proportions in larvae but in higher proportions in larvae fed T. The diet T supplied large quantities of 18:2n-6, with this precursor provided by the diet in sufficient quantity to support the creation of longer-chain, n-6 PUFAs. Our quantitative data suggest that the precursor 18:2n-6 was elongated to 20:2n-6 in larvae fed T. Similarly, De Moreno et al. [7] demonstrated that radiolabeled 18:2n-6 was incorporated into 20:2n-6. Accordingly, we postulate putative Δ8 desaturase activity in the larvae converting 20:2n-6 to 20:3n-6. The FA 18:2n-6 also may be a substrate of putative Δ6 desaturase to produce 18:3n-6, which could be elongated further to 20:3n-6. As 18:3n-6 is also present in T, however, elongation of dietary 18:3n-6 to 20:3n-6 may also marginally contribute to 20:4n-6 synthesis.

Overall, we postulate putative Δ6 and Δ8 desaturase activities on n-3 and n-6 precursors, but our quantitative data do not allow us to definitively discriminate which pathways are favored for i) n-3 series, 18:3n-3 → 20:3n-3 → 20:4n-3 *vs* 18:4n-3 → 20:4n-3 and for ii) n-6 series, 18:2n-6 → 20:2n-6 →20:3n-6 *vs* 18:3n-6 → 20:3n-6. Recently, a fatty acyl desaturase (Fad) with Δ8 activity was isolated in the scallop *Chlamys nobilis* and functionally characterized in recombinant yeast showing that this Fad could desaturate exogenously-added PUFA 20:3n-3 and 20:2n-6 to 20:4n-3 and

20:3n-6, respectively [29]. Nevertheless, we assumed that larvae would metabolize preferentially most abundant precursors using most energy cost-efficient pathways.

The synthesis of 20:4n-6 and 20:5n-3 from precursors observed in larvae fed T highlights the importance of both FAs for *C. gigas* larval development. The possibility that 20:5n-3 was synthetized *de novo* to fuel larval development appears highly unlikely, because the energetic cost of producing this FA is higher than using SFA and/or MUFAs for energy purposes. Both 20:4n-6 and 20:5n-3 are important precursors for eicosanoid synthesis, particularly prostaglandins. Eicosanoids formed from 20:5n-3 are less biologically active than those derived from 20:4n-6 [30]. An unbalanced 20:5n-3/20:4n-6 ratio may lead to harmful effects, as 20:5n-3 competitively inhibits the formation of eicosanoids from 20:4n-6 [31]. The importance of 20:5n-3 in the oyster has been related to immunity [32]. In light of observed synthesis of 20:5n-3 from precursor FA, it is impossible to exclude the possibility of other roles of this n-3 PUFA which remain to be determined. When 20:5n-3 is provided in more than sufficient quantity in the diet, as with the Cg diet, we assumed production of 20:5n-3 from dietary precursors is reduced or inactive. Only 20% of ingested 20:5n-3 was incorporated into larvae fed Cg. Surplus ingested 20:5n-3 may be allocated to: 1), an energy source; and/or 2) substrate to produce 22:5n-3, which surpassed by 7.2 times the dietary supply to compensate low level of 22:6n-3 in Cg. Similarly, larvae fed TCg synthetized 22:5n-3 (120% of dietary supply) in response to an excess of 20:5n-3 delivered with Cg in the diet.

In the present study, the highest incorporation of 22:6n-3 was observed in larvae fed Cg (76% of the amount supplied in the diet during all of larval development) because of low 22:6n-3 supply within this diatom. Sixteen-day-old larvae reared on 22:6n-3-sufficient diets exhibited 45-50% incorporation of 22:6n-3 compared to ingestion. Thus, it appears that larvae were able to modulate 22:6n-3 incorporation when the diet is not limited in this FA to maintain optimal levels in membrane lipids. This EFA plays a structural role, as it is retained selectively during starvation [33]. With diets deficient in this FA, larvae appear to very efficiently incorporate 22:6n-3, as was apparent for larvae fed Cg. Incorporation of 22:6n-3 detected in our study under limiting 22:6n-3 supply is in accordance with organic-matter assimilation efficiency reported for bivalves, which is approximately 70% [34]. Indeed, 22:6n-3 was not synthetized, *de novo* or from precursors, under any of the experimental conditions used herein. Pronounced elongation of 20:5n-3 to 22:5n-3 was found in larvae fed Cg, but desaturation to 22:6n-3 was not observed. Such an increase in 22:5n-3 may take place to compensate for insufficient dietary supply of 22:6n-3. Our data suggest that a Δ4 desaturase enzyme may not exist in Pacific oyster larvae, or at least was inactive in the present study.

As mentioned, the present study revealed different degrees of synthesis of PUFAs from various precursors mediated by a Fad with Δ5 activity in larvae. For instance, this putative Δ5 desaturase participated in 20:4n-6, 20:5n-3, and NMI synthesis in larvae fed T, with preferential 20:5n-3 synthesis over 20:4n-6. Sargent et al. [35] stated that Δ5 and Δ6 desaturating activities were regulated by competitive substrate inhibition, with preference for longer FA chains and a higher unsaturation level. These authors established a desaturase-activity hierarchy among FAs: 22:6n-3 > 20:5n-3 > 20:4n-6 > 18:3n-3 > 18:2n-6 > 18:1 > 16:1. On the other hand, larvae fed Cg and TCg synthesized only NMI, as 20:4n-6 and EPA seemed to be provided in sufficient quantities by the diet to meet larval requirements. Complex regulatory mechanisms appear to be involved in the control of PUFA levels in Pacific oyster larvae, which changes our concept of what constitutes an “essential” fatty acid in the diet.

Use of the flow-through larval rearing technique, which provides accurate algal consumption data, has allowed quantification of incorporation of individual fatty acids in Pacific oyster larvae. Future studies using similar methodology should be carried out using a greater number of dietary conditions to determine EFA requirements and limitation in bivalve larvae, taking into account that some synthesis of EPA is possible. Our findings suggest the putative synthesis of 20:4n-6 and 20:5n-3 from precursor FA, mediated by the enzyme Δ5 desaturase when these EFA are insufficient in the diet. In addition, putative Δ6 and Δ8 desaturase activities are proposed. Larvae were not capable, however, of synthesizing 22:6n-3 from shorter-chain precursors under the dietary conditions used in the present study. The *C. gigas* genome appears to contain a single fatty-acid desaturase (Fad)-like gene (http://www.ncbi.nlm.nih.gov/protein/EKC30965), which includes the integral-membrane enzymes: Δ4, Δ5, Δ6, Δ8, Δ8-sphingolipid, and Δ11 desaturases found in vertebrates, higher plants, fungi, and bacteria. Further studies on desaturase activities with molecular and enzymatic approaches are necessary to confirm activities and determine which desaturating capabilities are present in *C. gigas* and how dietary and physicochemical factors are controlling them.

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Tables

Table 1 Fatty acid ingestion and incorporation (expressed in pmol larva^{-1}) by *Crassostrea gigas* larvae between day 8 and day 16 in experiment 1 (mean ± S.D.).

Fatty acid	Ingestion			Incorporation		
	T	Cg	TCg	T	Cg	TCg
16:1n-7	34.8 ± 2.9^{c}	512.4 ± 14.1^{a}	415.4 ± 15.6^{b}	8.1 ± 1.5^{c}	25.6 ± 5.8^{b}	39.2 ± 2.7^{a}
18:1n-9	118.8 ± 9.8^{a}	12.8 ± 0.4^{b}	131.3 ± 4.9^{a}	23.3 ± 4.7^{a}	1.4 ± 0.4^{b}	17.2 ± 2.6^{a}
18:1n-7	21.5 ± 1.8^{b}	16.4 ± 0.4^{c}	34.6 ± 1.3^{a}	14.5 ± 2.7^{b}	17.1 ± 3.5^{b}	32.1 ± 1.5^{a}
18:2n-6	37.2 ± 3.1^{a}	9.7 ± 0.3^{c}	33.3 ± 1.2^{b}	9.8 ± 1.8^{a}	2.5 ± 0.5^{b}	9.8 ± 0.6^{a}
18:3n-6	4.1 ± 0.3^{b}	4.4 ± 0.1^{b}	8.9 ± 0.3^{a}	1.0 ± 0.2^{b}	0.6 ± 0.1^{c}	1.3 ± 0.0^{a}
20:2n-6	0.7 ± 0.1^{b}	0.5 ± 0.0^{c}	1.1 ± 0.0^{a}	1.1 ± 0.2^{a}	0.2 ± 0.1^{c}	0.7 ± 0.1^{b}
20:3n-6	0.8 ± 0.1^{c}	1.1 ± 0.0^{b}	1.9 ± 0.1^{a}	0.6 ± 0.1^{a}	0.2 ± 0.0^{b}	0.6 ± 0.1^{c}
20:4n-6	0.8 ± 0.1^{c}	4.6 ± 0.1^{b}	5.2 ± 0.2^{a}	4.1 ± 0.5^{a}	2.8 ± 0.6^{c}	5.3 ± 0.3^{a}
22:5n-6	14.6 ± 1.2^{a}	0.1 ± 0.0^{c}	12.7 ± 0.5^{b}	8.3 ± 1.1^{a}	0.2 ± 0.0^{b}	7.1 ± 1.6^{a}
18:3n-3	60.4 ± 5.0^{a}	1.2 ± 0.0^{b}	61.3 ± 2.3^{a}	13.1 ± 2.3^{a}	0.8 ± 0.2^{c}	9.6 ± 0.2^{b}
18:4n-3	139.6 ± 11.5^{a}	4.0 ± 0.1^{b}	152.0 ± 5.7^{a}	31.8 ± 6.0^{a}	0.4 ± 0.3^{b}	25.9 ± 0.6^{a}
20:3n-3	0.9 ± 0.1^{a}	0.0 ± 0.0^{b}	0.9 ± 0.0^{a}	0.7 ± 0.1^{a}	0.0 ± 0.0^{c}	0.4 ± 0.0^{a}
20:4n-3	0.3 ± 0.0^{c}	1.4 ± 0.0^{b}	2.0 ± 0.1^{a}	2.0 ± 0.4^{a}	0.4 ± 0.1^{b}	1.6 ± 0.4^{a}
20:5n-3	3.8 ± 0.3^{c}	127.5 ± 3.5^{a}	87.0 ± 3.3^{b}	7.0 ± 1.1^{b}	35.5 ± 5.7^{a}	32.9 ± 1.1^{a}
22:5n-3	1.1 ± 0.1^{b}	0.2 ± 0.0^{c}	1.2 ± 0.0^{a}	0.8 ± 0.1^{b}	1.8 ± 0.3^{a}	1.5 ± 0.3^{a}
22:6n-3	88.4 ± 7.3^{a}	6.9 ± 0.2^{b}	96.9 ± 3.6^{a}	45.4 ± 6.8^{a}	4.9 ± 1.7^{b}	42.6 ± 9.5^{a}

Values with same letters in the same row for ingestion and incorporation are not significantly different at $P > 0.05$.

Table 2 Fatty acid ingestion and incorporation (expressed in pmol larva^{-1}) by *Crassostrea gigas* larvae between day 8 and day 15 in experiment 2 (mean ± S.D.).

Fatty acid	Ingestion		Incorporation	
	T	TCg	T	TCg
16:1n-7	22.5 ± 3.5^{b}	337.6 ± 20.8^{a}	3.8 ± 0.9^{b}	47.7 ± 4.7^{a}
18:1n-9	66.3 ± 10.4^{b}	129.0 ± 8.0^{a}	11.5 ± 2.4^{b}	21.1 ± 1.7^{a}
18:1n-7	8.8 ± 1.4^{b}	28.0 ± 1.7^{a}	7.1 ± 1.4^{b}	48.6 ± 2.5^{a}
18:2n-6	24.3 ± 3.8^{b}	55.1 ± 3.4^{a}	7.0 ± 1.1^{b}	17.0 ± 0.9^{a}
18:3n-6	2.8 ± 0.4^{b}	11.4 ± 0.7^{a}	0.8 ± 0.1^{b}	3.3 ± 0.2^{a}
20:2n-6	0.4 ± 0.1^{b}	1.1 ± 0.1^{a}	0.8 ± 0.1^{b}	1.3 ± 0.0^{a}
20:3n-6	0.8 ± 0.1^{b}	1.6 ± 0.1^{a}	0.4 ± 0.1^{b}	1.0 ± 0.0^{a}
20:4n-6	1.0 ± 0.2^{b}	6.9 ± 0.4^{a}	2.8 ± 0.1^{b}	10.2 ± 0.9^{a}
22:5n-6	9.9 ± 1.6^{b}	18.4 ± 1.1^{a}	4.9 ± 0.7^{b}	10.4 ± 1.0^{a}
18:3n-3	31.6 ± 5.0^{b}	57.5 ± 3.5^{a}	6.5 ± 1.6^{b}	12.8 ± 0.3^{a}
18:4n-3	78.5 ± 12.3^{b}	157.4 ± 9.7^{a}	14.8 ± 3.9^{b}	31.6 ± 2.1^{a}
20:3n-3	0.5 ± 0.1^{b}	1.3 ± 0.1^{a}	0.2 ± 0.1^{b}	0.5 ± 0.0^{a}
20:4n-3	0.2 ± 0.0^{b}	1.9 ± 0.1^{a}	1.0 ± 0.2^{b}	1.6 ± 0.0^{a}
20:5n-3	2.5 ± 0.4^{b}	117.8 ± 7.3^{a}	4.9 ± 0.2^{b}	64.7 ± 3.2^{a}
22:5n-3	1.0 ± 0.2^{b}	2.1 ± 0.1^{a}	0.6 ± 0.0^{b}	2.9 ± 0.2^{a}
22:6n-3	59.6 ± 9.3^{b}	115.1 ± 7.1^{a}	26.7 ± 3.6^{b}	57.2 ± 4.9^{a}

Values with same letters in the same row for ingestion and incorporation are not significantly different at $P > 0.05$.

Figures

Fig. 1 Synthesis of non-methylene interrupted fatty acids and their precursors by *Crassostrea gigas* larvae between day 8 and day 15-16 (mean ± S.D.). **A** Experiment 1. **B** Experiment 2.

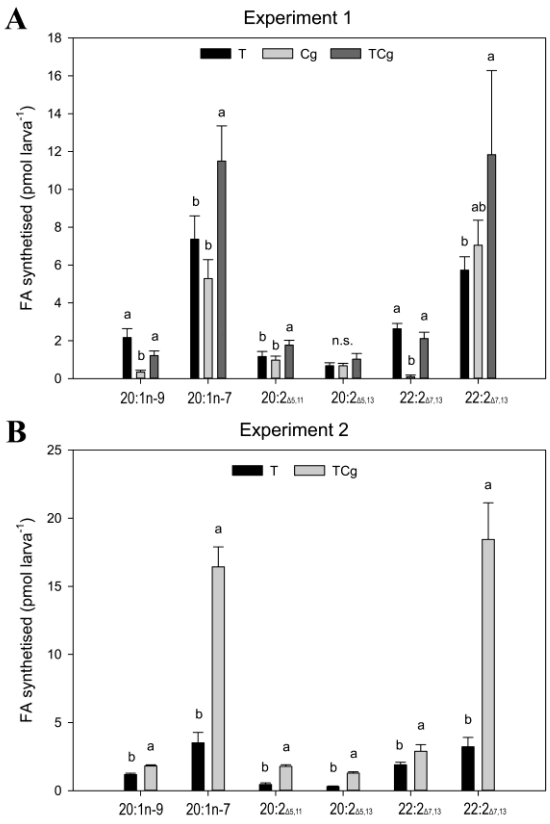

Fig. 2 Essential fatty acid ingestion and incorporation by *Crassostrea gigas* larvae between day 2 and competent larvae (mean ± S.D.) in experiments 1 and 2. **A** 20:4n-6. **B** 20:5n-3. **C** 22:6n-3.