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Growth and kinetics of lipids and fatty acids of the clam *Venerupis pullastra* during larval development and postlarvae

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Abstract

This study examines the larval development, metamorphosis and postlarval stage of *Venerupis pullastra* in relation to growth, lipids content and fatty acid composition, specifically those believed to be essential for most bivalves (i.e. 20:5n-3 and 22:6n-3). Clam larvae were fed with two species of microalgae supplied individually or mixed –*Isochrysis galbana* and *Tetraselmis suecica*–species normally used in bivalve hatcheries. Larvae fed with *T. suecica* showed a progressive accumulation of lipids and fatty acids but did not survive to metamorphosis. Contrarily, larvae fed with *I. galbana* or mixed diet showed a progressive decline in lipids and essential fatty acids (20:5n-3 and 22:6n-3) from the pediveliger stage onwards, and a survival rate of 95% until the start of metamorphosis. The lower content in n-6 and the absence of 22:6n-3 in *T. suecica* diet might contribute to the massive mortality observed for larvae fed with this diet. That diet seems to fail in the supply of some particular nutrient that allows energetic transformation of reserves for growth and metamorphosis. Nevertheless, larvae fed on mixture diet showed higher weight growth values at postlarval stage than those larvae fed on *I. galbana* diet.

KEY WORDS: clam, fatty acids, growth, larval development, lipid's kinetic, microalgal diets, *Venerupis pullastra*

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Introduction

Larval development and survival is determined by the energy reserves stored during two stages of development. One corresponds to embryonic development and is mainly governed by the endogenous reserves supplied to the eggs from the parents (Bayne 1973). The next stage is a previous period until metamorphosis when stored energy reserves are essential, and depends on the feed value of the diets supplemented for larval growth (Whyte *et al*. 1989, 1990).

Recently, an in-depth revision of lipid metabolism, Sewell (2005), has discussed the importance of the lipid matrix during bivalve larval development. Utting (1986) and Whyte *et al*. (1989, 1990), on the other hand, noted the importance of the protein content of the diet for strong larval growth up to metamorphosis, as well as the requirement of a diet sufficiently balanced in proteins, lipids and carbohydrates. Labarta *et al*. (1999) evaluated the growth and processes of energy acquisition in *Ostrea edulis* during larval development, as well as the role of lipids, proteins and carbohydrates from an energetic and structural perspective, and showed that lipids were the main source of metabolic energy for *O. edulis* throughout larval development.

The importance of lipids as a dietary requirement has been extensively studied for many species of bivalves over recent decades (Albentosa *et al*. 1994, 1996; Caers *et al*. 1998; Fernández-Reiríz *et al*. 1999; Soudant *et al*. 1999; Pernet *et al*. 2006). The fatty acid composition has been described for some bivalve species (Watanabe & Ackman 1974; Holland 1978; Waldock & Holland 1984; Hendriks *et al*. 2003; Milke *et al*. 2004). Nonetheless knowledge of fatty acid composition in *V. pullastra* during larval and postlarval development is lacking.

Existing results showed that long-chain (n-3) and (n-6) PUFA were important for mollusc larvae (Delaunay *et al*. 1993; Leonardos & Lucas 2000), similar to many marine species. These criteria, as well as acceptability and digestibility, may help explain their nutritional value (Albentosa *et al*. 1994, 1996; Fernández-Reiríz *et al*. 1999).

Waldock & Holland (1984) investigated the metabolism of fatty acids in *Crassostrea gigas* juveniles. This author pointed out that *C. gigas* has some capacity for elongating and

desaturating n-3 fatty acids to produce n-3HUFA, although too low to sustain optimum growth. The same results were obtained by Chu & Greaves (1991) for *Crassostrea virginica* where ^{14}C-labelled 20:5n-3 and 22:6n-3 were not detected from labelled 18:3n-3. These findings are comparable to those observed by Albentosa *et al*. (1994, 1996) in *V. pullastra* spat and *Ruditapes decussatus* spat.

The requirement for certain fatty acids appears to be species dependent. *Tapes semidecussatus* and *Mercenaria mercenaria* require 22:6n-3, while *Crassostrea* sp. shows a fundamental requirement for 20:5n-3 (Helm & Laing 1987). Both 20:5n-3 and/or 22:6n-3 can meet the bivalve requirements for n-3 PUFA (Fernández-Reiríz *et al*. 1999). Nonetheless, Pernet *et al*. (2007) reported that the mussel seemed better able that the oyster to selectively incorporate 20:5n-3 fatty acid.

The NMID fatty acids are preferably found in the polar lipids of mollusks (Ackman & Hooper 1973; Irazu *et al*. 1984; Kraffe *et al*. 2004). Pathways for the biosynthesis of 20:2NMI and 22:2NMI fatty acids have been reported in the bivalve mollusks *Scapharca broughtoni* and *Mytilus edulis* (Zhukova 1991). These results indicated that mollusks have active fatty acid elongation and desaturation systems that allow synthesis of these NMI fatty acids. The NMIDs, specifically 20:2NMID, were observed in similar amounts to some PUFAs in the larval development stage of *O. edulis*, but became relatively less important from the onset of metamorphosis. Furthermore, these acids were only present in residual quantities during the postlarval stage (Labarta *et al*. 1999).

This study investigates the larval development, metamorphosis and postlarval stage of *V. pullastra* in relation to growth, kinetic response of lipids and the fatty acid composition, with regard to three experimental diets.

Materials and methods

Larval cultivation

Venerupis pullastra (L.) larvae were obtained from broodstock conditioned at the Instituto Español de Oceanografía (A Coruña, NW Spain). Spawning and larval culture were carried out following Pérez-Camacho *et al*. (1977). Larvae were maintained for 34 days until attaining the postlarval stage.

After 2 days of incubation of the eggs in 100 L glass fibre containers, D-veliger larvae stage was attained, showing a mean length of 98.6 μm. These larvae were transferred to 400 L tanks maintained at 18 °C with filtered water (1 μm) and 50 cells μL^{-1} of *I. galbana* clon T-ISO as diet concentration. The water and food was renewed every 2 days. After 11 days under these conditions, the larvae reached a mean length of 176.8 μm (umbonate larvae), which allowed them to capture cells with the size of *Tetraselmis suecica* (about 7.64 μm). At this point, the experiment with different diets was initiated.

Two tanks for each experimental diet were deployed. The experiment was carried out within 100 L fibre glass tanks with filtered sea water (1 μm) at 18 °C with a larval density of five larvae mL^{-1}. The larvae were fed with two species of microalgae (*I. galbana* and *T. suecica*) supplied individually (100 cells μL^{-1} for *I. galbana* and its equivalent volume, 10 cells μL^{-1} for *T. suecica*) or mixed (50 cells μL^{-1} *I. galbana* and five cells μL^{-1} for *T. suecica*). The water and food were renewed every 2 days.

Larval samples for biochemical analysis were taken at day 2 (D larva), day 13 (umbonate larvae) day 17 (pediveliger larvae), day 22 (start of metamorphosis) and day 34 (postlarvae).

Size was determined using a binocular microscope with ocular micrometer (model SMZ-10, Nikon Instruments Europe, Amstelveen, The Netherlands). Individual dry weights (DW) were measured on glass microfibre filters (Cat Nº 1825-025, Whatman International Ltd, Maidstone, UK) after washing the larvae with distilled water and dried in an oven at 110 °C for 3 h. Organic weight (OW) was determined by the difference between the dry and ashed weight following combustion (450 °C for 4 h). The weight was measured using an electronic microbalance (model M3P; Sartorius AG, Goettingen, Germany).

Analysis of lipids and fatty acids

Lipids were first extracted with chloroform:methanol (1:2; VWR International S.A.S., Briare, France) and, after centrifugation (3246 ***g***), the precipitate was re-extracted with chloroform:methanol (2:1). Both supernatants were subsequently washed with chloroform:methanol:water (8:4:3) as described previously (Fernández-Reiríz *et al*. 1989). The solvents contained 0.05% butylated hydroxytoluene (Merck Schuchardt OHG, Hochenbrunn, Germany). To quantify total lipids, the method described by Marsh & Weinstein (1966) was used with a tripalmitine standard (Sigma Aldrich Inc., Buchs, Switzerland). Samples were stored under nitrogen at –70 °C until further processing. The results of lipids were transformed into their energy equivalent (kJ ind^{-1} 10^{-6}) following Beukema & De Bruin (1979).

Fatty acids from total lipids were trans-esterified to methyl esters with methanolic hydrogen chloride (VWR

International S.A.S., Briare, France) following Christie (1982). The acids were subsequently analysed on a gas chromatograph (model 8500; Perkin-Elmer Inc., MA, USA) equipped with a fused silica capillary column (30-m length, 0.25 mm i.d.; model SP-2330, Supelco, PA, USA) and a PTV cold injector (Perkin-Elmer Inc.) operated in the solvent elimination mode. The injector temperature was 275 °C and the column temperature was increased from 140 to 210 °C at a rate of 1.0 °C min^{-1}, with N_2 carrier gas (0.069 Pa = 10 psi). Non-adecanoic acid (Sigma-Aldrich Inc., Buchs, Switzerland) was used as an internal standard and a response factor was calculated for each fatty acid for quantitative analyses. A combination of analytical procedures (GC-MS; gas chromatograph model HP5890 and mass detector model 5971, Agilent Technologies Inc., CA, USA) was required for conclusive structure determination of non-methylene-interrupted dienoic (NMID) fatty acids.

Statistical analysis

Homogeneity of variance was tested with the Bartlett test. When non-homogeneity, data were modified using logarithmic transformation. The differences between means of growth and lipid content over time were analysed using ANOVA and a Tukey test at a significance level of $P < 0.05$ (Snedecor & Cochran 1980; Zar 1984). Correlations between clam growth and fatty acid contents were examined by Pearson's correlation coefficients.

Results

Growth and survival

The survival rate of larvae fed with *I. galbana* and the mixture of *I. galbana* and *T. suecica* was 95% until the start of metamorphosis (day 22). The larvae fed exclusively with *T. suecica* had a mortality rate above 40% at day 22, which increased to 100% over the following two days, at which point the culture was ended.

The D larvae displayed significantly lower lengths and dry weights (98.6 ± 3.92 µm and 0.12 ± 0.04 µg) than the umbonate larvae (176.8 ± 5.92 µm and 0.53 ± 0.10 µg; ANOVA, $P < 0.001$). Significant changes were also observed in organic (0.03 ± 0.00 and 0.19 ± 0.00 µg OW for D and umbonate larvae respectively; ANOVA, $P < 0.001$) and lipidic content (0.05 ± 0.02 and 0.08 ± 0.03 µg µg DW^{-1} for D and umbonate larvae, respectively; ANOVA, $P < 0.001$).

Differences in growth parameters were observed between diets in some stages (Table 1; ANOVA, $P < 0.05$). Larvae fed

Table 1 Average and standard deviation of growth parameters (size, dry weight (DW) and organic weight (OW)) and lipidic content (expressed in dry weight and organic weight basis and in individual content in weight (µg ind^{-1}) and in energetic equivalents (KJ $ind^{-1}10^{-6}$)) during *Venerupis pullastra* larvae development

	I. galbana		*I. galbana*			*T. suecica*			Mixture		
	2 days	13 days	17 days	22 days	34 days	17 days	22 days		17 days	22 days	34 days
	D Larvae	Umbonate	Pediveliger	Metamorphosis	Postlarvae	Pediveliger	Metamorphosis		Pediveliger	Metamorphosis	Postlarvae
Size (µm)	98.6 ± 3.92	176.8 ± 5.92	208.8 ± 5.80^{a}	246.5 ± 0.35^{d}	539.0 ± 14.1^{g}	210.1 ± 7.92^{a}	230.1 ± 0.89^{e}		213.1 ± 1.56^{a}	241.5 ± 4.45^{d}	548.9 ± 21.2^{g}
DW (µg)	0.12 ± 0.04	0.53 ± 0.10	0.87 ± 0.23^{a}	1.37 ± 0.04^{d}	14.6 ± 1.90^{g}	0.74 ± 0.14^{b}	1.11 ± 0.09^{e}		0.95 ± 0.02^{a}	1.44 ± 0.17^{d}	21.9 ± 2,22^{h}
OW (µg)	0.03 ± 0.00	0.19 ± 0.00	0.34 ± 0.09^{a}	0.51 ± 0.01^{d}	4.53 ± 0.40^{g}	0.27 ± 0.04^{b}	0.35 ± 0.07^{e}		0.39 ± 0.01^{a}	0.46 ± 0.15^{d}	5.67 ± 0.57^{h}
Lipids											
µg µg DW^{-1}	0.05 ± 0.02	0.08 ± 0.03	0.09 ± 0.01^{a}	0.06 ± 0.03^{d}	0.03 ± 0.02^{g}	0.08 ± 0.02^{a}	0.14 ± 0.01^{b}		0.08 ± 0.01^{a}	0.06 ± 0.00^{d}	0.03 ± 0.02^{g}
µg µg OW^{-1}	0.19 ± 0.04	0.21 ± 0.05	0.23 ± 0.02^{a}	0.16 ± 0.09^{d}	0.09 ± 0.07^{g}	0.22 ± 0.08^{a}	0.44 ± 0.04^{b}		0.20 ± 0.03^{a}	0.21 ± 0.07^{d}	0.13 ± 0.06^{g}
µg ind^{-1}	0.01 ± 0.00	0.04 ± 0.00	0.08 ± 0.03^{a}	0.08 ± 0.03^{d}	0.39 ± 0.14^{g}	0.06 ± 0.02^{a}	0.15 ± 0.02^{b}		0.08 ± 0.01^{a}	0.09 ± 0.01^{d}	0.68 ± 0.09^{h}
kJ ind^{-1} 10^{-6}	0.21 ± 0.01	1.37 ± 0.01	2.72 ± 0.93^{a}	2.87 ± 1.18^{d}	13.5 ± 5.13^{g}	2.16 ± 0.81^{a}	5.29 ± 0.69^{b}		2.67 ± 0.28^{a}	3.27 ± 0.23^{d}	23.8 ± 3.19^{h}

Different letters represent significant differences (ANOVA; $P < 0.05$) between experimental diets in each larval stage [pediveliger (a, b, c), metamorphosis (d, e, f) and postlarvae (g, h)].

on *T. suecica* showed significant lower weight values than the other diets at day 17 whereas no differences in length were observed between diets (Table 1; ANOVA ~~$P < 0.05$~~). At the onset of metamorphosis (day 22), larvae fed with *T. suecica* showed significantly lower shell length and dry weight (230.1 ± 0.89 μm and 1.11 ± 0.09 μg; ANOVA $P < 0.05$) than larvae fed with *I. galbana* (246.55 ± 0.35 μm and a weight of 1.37 ± 0.04 μg) or the mixture diet 241.5 ± 4.45 μm and 1.44 ± 0.17 μg). After metamorphosis, the postlarvae fed with *I. galbana* presented similar lengths than those fed on mixture diet (539.0 ± 14.1 and 548.9 ± 81.2 μm, respectively) but lower weight values (14.6 ± 1.90 and 21.9 ± 2.22 μg for *I. galbana* and mixture diets, respectively; ANOVA $P < 0.05$).

The highest increase in growth rates (length or weight) were observed between metamorphosis and postlarval stage (Fig. 1). Nonetheless, no significant differences were detected in length or weight growth rates between diets in any of the larval stages (Fig. 1).

The lipid content of larvae fed with *T. suecica* increased over the 22 day experimental period (onset of the metamorphosis). However, with the other diets the lipid content showed the maxima values at the pediveliger stage and a decrease onwards, showing the lowest content in the postlarval stage (day 34; ANOVA $P < 0.05$). In the pediveliger stage, the energy content of the lipids was similar (ANOVA; $P > 0.05$) in the larvae fed with the three diets (~2.5 kJ ind^{-1} 10^{-6}, Table 1). At the onset of metamorphosis, the larvae fed with *T. suecica* showed significantly higher lipid content (5.3 kJ ind^{-1} 10^{-6}, ANOVA, $P < 0.05$) although they did not survive metamorphosis. The largest lipid content (ANOVA, $P < 0.001$) in the postlarval stage was found in the larvae fed with the mixed diet (23.8 kJ ind^{-1} 10^{-6}) due to their higher weight values (Table 1).

Equations were derived to describe the evolution of lipid content, dry and organic weight in their energetic equivalents (kJ g^{-1}~~, dry weight, basis,~~ Fig. 2) from the onset of the dietary experience. Larvae fed with *T. suecica* showed a linear or exponential increase along the development in lipid content and weight values (Fig. 2; Appendix 1) whereas larvae fed with *I. galbana* or the mixed diet showed a maximum in the pediveliger stage and a progressive decline in lipid content and weight (Fig. 2; Appendix 1).

Fatty acids

Fatty acid composition of the diets

Table 2 shows the composition of fatty acids of the three different experimental diets.

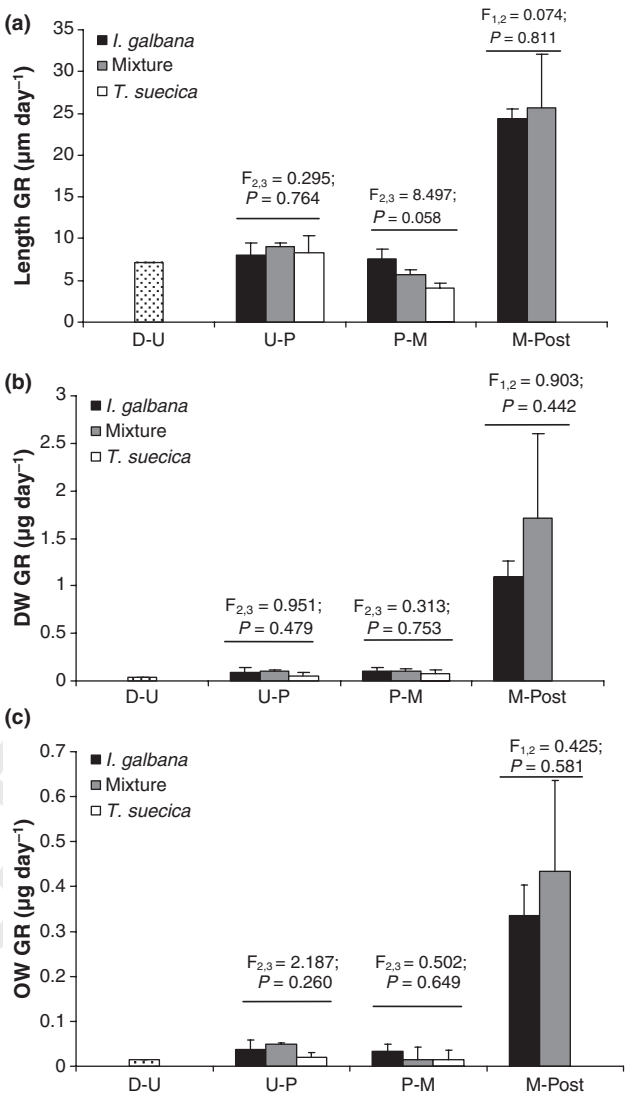

Figure 1 Length growth rate (μm day^{-1}) (a), dry weight growth rate (μg day^{-1}) (b) and organic growth rate (μg day^{-1}) (c) over *Venerupis pullastra* larval development (D–U: growth rate from veliger D to umbonate veliger; U–P: growth rate from umbonate veliger to pediveliger; P–M: growth rate from pediveliger to metamorphosis, M-Post: growth rate from metamorphosis to postarvae) with ANOVA results for differences between diets.

The main fatty acids found in *I. galbana* diet were 14:0, 16:0, 18:0, 18:1n-9, 18:4n-3 and 22:6n-3. The total fatty acid content was 148.9 μg mg~~$^{-1}$~~ DW~~$^{-1}$~~ (64.8, 34.5 and 49.6 μg mg~~$^{-1}$~~ DW~~$^{-1}$~~ for saturated, monoenoic and polyenoic fatty acids, respectively). The content of n-6 fatty acids was 11.3 μg mg~~$^{-1}$~~ DW~~$^{-1}$~~ and 38.1 μg mg~~$^{-1}$~~ DW~~$^{-1}$~~ for n-3 PUFA. The n-3:n-6 and n-6:n-3 ratios were 3.4 and 0.3, respectively.

In *T. suecica* diet the main fatty acids recorded were 16:0, 18:0, 18:1n-9, 18:3n-3, and 20:5n-3. The total fatty acids

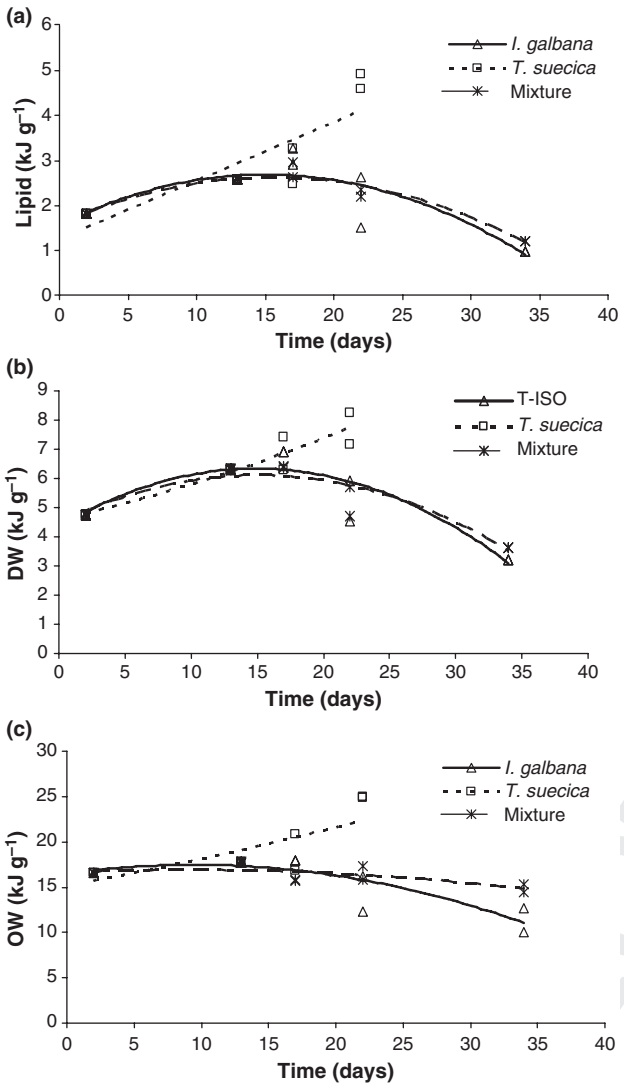

Figure 2 Fits on the evolution of lipid content (a) dry (b) and organic weight (c) (expressed in energy equivalents (kJ g^{-1}) during larval development of *Venerupis pullastra* fed with different diets.

content was three times lower than in the other experimental diets, 45.1 µg mg DW-1 (18.2, 15.5 and 11.4 µg mg DW-1 for saturated, monoenoic and polyenoic fatty acids, respectively). n-6 fatty acid content was 1.88 µg mg DW-1 and for n-3 PUFA was 9.5 µg mg DW^{-1}. The n-3:n-6 ratio was 5.1, and 0.2 for n-6:n-3.

The main fatty acids found in the diet composed of *I. galbana* + *T. suecica* were 16:0, 18:0, 18:1n-9, 18:4n-3, 18:3n-3, 14:0, 20:5n-3 and 22:6n-3. The main groups were saturated fatty acids (60.2 µg mg DW^{-1}), polyunsaturated fatty acids (54.6 µg mg DW^{-1}) and monounsaturated fatty acids (28.6 µg mg DW^{-1}). The n-3PUFAs content was 9.5 µg mg DW^{-1}. The n-3:n-6 ratio was 2.9, and 0.5 for n-6:n-3.

Fatty acid composition of the clam

Table 3 shows the main fatty acids content and groups of fatty acids (µg mg DW^{-1}) along the larval development with different experimental diets.

From a developmental point of view, we observed for some fatty acids (16:0 and 18:0) a progressive increase until metamorphosis with a sharp decline in postlarvae stage for the three experimental diets. In the case of 18:4n-3, 20:2NMID and 22:6n-3, we observed an opposite trend, with a progressive decrease for the three experimental diets until postlarvae stage, but with significant lower values in larvae fed with *T. suecica* (Table 3; ANOVA, $P < 0.05$). Another group of fatty acids (18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3 and 22:4n-6) showed the latter trend for *I. galbana* and mixture diets but a continuous accumulation in larvae fed on *T. suecica* (Table 3; ANOVA, $P < 0.05$). With regard to 20:4n-6, although we observed the later trend, changes in content during development were not significant for any experimental diet.

From a diet point of view, the 20:4n-6 content at pediveliger stage was significantly higher in larvae fed on *T. suecica*. Similarly the 20:5n-3 was significantly higher in larvae fed on *T. suecica*, whereas the 22:6n-3 was higher in the larvae fed with *I. galbana* and mixed diets in all the developmental stages (Table 3; ANOVA, $P < 0.05$).

The larvae fed with *T. suecica* showed a progressive increase on the total fatty acids content over development ($P < 0.05$), reaching significantly greater quantities at metamorphosis (Table 3; ANOVA, $P < 0.05$). In the other two diets the total fatty acid content decreased significantly during the development ($P < 0.05$), reaching postlarval stage with the lowest content. The behavior described above was also observed for the saturated, monoenoic and polyenoic fatty acids (Table 3).

Relationship between growth and fatty acids

No significant correlation was observed between weight growth values and dietary fatty acids (data not shown).

Correlation analysis between fatty acids content in larvae and weight growth during larval and postlarval development of *V. pullastra* revealed various positive and negative correlations with fatty acids or with their ratios (Table 4).

In the first larval stages (D and umbonates larvae; larvae fed with *I. galbana)* larval weight was positively related to

some fatty acids (among others 22:6n-3, n3:n6 ratio and total fatty acids content; Table 4A) and negatively related to 18:0, 20:5n-3, 20:2NMID, Σ Saturated and Σ n-11. In the successive developmental stages (from pediveliger to postlarvae) all the significant correlations indicated a negative relationship between weight growth of larvae fed with *I. galbana* and the content of main fatty acids (including 20:5n-3, 20:2NMID and 22:6n-3; Table 4B). Nonetheless, growth of larvae fed with *T. suecica*, showed positive relationships with six fatty acids and only one negative correlation with 18:4n-3 content (Table 4C). With the mixture diet, weight growth were significantly and negatively related to five fatty acids, none of which were the essential fatty acids 20:5n-3 or 22:6n-3, whereas a significant relationship was observed with the 20:2NMID content (Table 4D).

Table 2 Fatty acid composition of experimental diets

	I. galbana	*T. suecica*	Mixture
	μg mg DW^{-1}	μg mg DW $^{-1}$	μg mg DW $^{-1}$
14:0	21.3 ± 0.00	0.81 ± 0.02	8.10 ± 0.31
15:0	1.42 ± 0.01	0.30 ± 0.00	0.41 ± 0.02
16:0	25.4 ± 0.40	12.3 ± 0.11	27.5 ± 0.61
16:1n-9	2.11 ± 0.00	0.80 ± 0.01	1.04 ± 0.12
16:1n-7	4.10 ± 0.03	0.90 ± 0.03	4.10 ± 0.11
16:4n-3	0.23 ± 0.01	nd	nd
17:0	1.40 ± 0.02	0.71 ± 0.02	1.71 ± 0.20
17:1n-7	0.50 ± 0.01	nd	nd
18:0	14.8 ± 0.20	3.73 ± 0.11	22.5 ± 0.62
18:1n-9	26.2 ± 0.31	12.1 ± 0.20	21.1 ± 0.51
18:1n-7	1.70 ± 0.01	1.04 ± 0.01	2.32 ± 0.10
18:2n-6	4.11 ± 0.01	1.72 ± 0.12	8.10 ± 0.31
18:3n-6	0.40 ± 0.00	nd	1.61 ± 0.20
18:3n-3	4.80 ± 0.03	6.10 ± 0.31	12.0 ± 0.81
18:4n-3	17.4 ± 0.02	nd	19.2 ± 0.51
18:5n-3	1.61 ± 0.21	0.41 ± 0.00	nd
20:0	0.60 ± 0.00	0.13 ± 0.01	nd
20:1n-9	nd	0.72 ± 0.02	nd
20:3n-6	3.50 ± 0.10	nd	0.92 ± 0.03
20:4n-6	1.20 ± 0,91	0.22 ± 0.10	0.71 ± 0.02
20:4n-3	1.10 ± 0.20	nd	nd
20:5n-3	0.83 ± 0.01	2.90 ± 0.03	5.60 ± 0.31
22:5n-6	2.21 ± 0,01	nd	1.31 ± 0.12
22:5n-3	0.42 ± 0.02	nd	nd
22:6n-3	11.8 ± 0.10	nd	3.91 ± 0.30
Σ Saturated	64.8	18.2	60.2
Σ Monoenoic	34.5	15.5	28.6
Σ Polyenoic	49.6	11.4	54.6
Σ Total FA	148.9	45.1	143.4
Σn-3	38.1	9.51	40.3
Σn-6	11.3	1.92	13.9
Σn-7	6.20	1.89	6.40
Σn-9	28.3	13.6	22.1
Σn-3 PUFA	13.0	2.90	9.51
n-3:n-6	3.37	5.10	2.89
n-6:n-3	0.30	0.20	0.34

Discussion

In general, bivalves fed with multi-specific microalgal diets show higher growth than those fed with mono-specific diets (Albentosa *et al.* 1993; Milke *et al.* 2004). In the present study, diets comprised of *I. galbana* and the mixture diets (*I. galbana* and *T. suecica*) showed higher growth values in length and weight during larval development of *V. pullastra* than those fed with *T. suecica*. Furthermore, only these diets led to survival past metamorphosis to the postlarval stage. Larvae fed with mixture diet showed higher growth values at postlarval stage than larvae fed on *I. galbana* diet.

Numerous studies have examined the nutritional value of microalgal species for bivalve mollusc culture (Webb & Chu 1983; Ferreiro *et al.* 1990; Albentosa *et al.* 1993, 1996; Delaunay *et al.* 1993; Fernández-Reiríz *et al.* 1998, 2006; Pérez-Camacho *et al.* 1998; Milke *et al.* 2004). The size and cellular volume of *T. suecica* is greater than *I. galbana* (7.64 μm and 249.85 μm^3 compared to 4.0 μm and 35.07 μm^3, respectively). However, both can be efficiently retained by bivalve filtration system (Albentosa *et al.* 1993, 1996). The digestibility of the microalgal cells may be another key factor for growth. Romberger & Epifanio (1981) reported 10 times lower assimilation efficiency of *T. suecica* than *I. galbana* cells by *C. virgínica*.

In our study, significant relationships were observed between weight growth and fatty acid composition of larvae, but no correlation was observed between growth and dietary fatty acids. In agreement, Leonardos & Lucas (2000) observed significant correlation between certain larval fatty acids (i.e. n-3 fatty acids) and growth in *M. edulis* larvae. However, the latter could not be extrapolated directly to similar relationships between dietary fatty acids and larval growth (Leonardos & Lucas 2000).

Despite of the nutritional importance of the n-3 group that includes 20:5n-3 and 22:6n-3, Pearson's correlation only showed significant negative correlation with growth and n-3 group when larvae were cultivated with *I. galbana*. This diet was used from the beginning of the experimentation. However, feeding with the other two diets was initiated at the umbonate phase which can suggest that fatty acids are not only transferred but accumulated in the food web. Results also showed a negative correlation between growth and the 20:2NMID fatty acid content in larvae fed with *I. galbana* and mixed diet. Latter results suggest that this fatty acid have a significant role in determining the weight of the larvae *V. pullastra* despite of their low content. Although little is known about the function of the NMID fatty acids, the pathways for their biosynthesis in mollusc have been

Table 3 Fatty acid composition (average ± SD expressed in μg mg DW^{-1}) during *Venerupis pullastra* larval development

	I. galbana		*I. galbana*			*T. suecica*		Mixture		
	2 days	13 days	17 days	22 days	34 days	17 days	22 days	17 days	22 days	34 days
Fatty acid	D Larvae	Umbonate	Pediveliger	Metamorphosis	Postlarvae	Pediveliger	Metamorphosis	Pediveliger	Metamorphosis	Postlarvae
14:0	1.52 ± 0.10^{1}	2.58 ± 0.09^{2}	3.23 ± 0.10^{3a}	3.27 ± 0.00^{4d}	1.86 ± 0.00^{5g}	1.45 ± 0.09^{3b}	1.59 ± 0.06^{3e}	2.43 ± 0.19^{2c}	2.14 ± 0.13^{3f}	1.30 ± 0.37^{4h}
15:0	0.45 ± 0.04	0.27 ± 0.02	0.29 ± 0.04	0.29 ± 0.00	0.19 ± 0.00	0.27 ± 0.02	0.32 ± 0.07	0.29 ± 0.02	0.25 ± 0.05	0.15 ± 0.06
16:0	9.18 ± 0.29^{1}	8.63 ± 0.49^{1}	9.77 ± 0.29^{3a}	10.6 ± 0.00^{4d}	5.14 ± 0.00^{5g}	11.3 ± 0.49^{3b}	12.7 ± 0.49^{4e}	10.2 ± 0.26^{3a}	10.8 ± 0.25^{4d}	4.91 ± 1.00^{5h}
16:1n9	1.38 ± 0.17	0.64 ± 0.13	0.76 ± 0.17	0.87 ± 0.00	0.46 ± 0.00	1.49 ± 0.13	1.39 ± 0.06	1.08 ± 0.16	1.14 ± 0.09	0.41 ± 0.14
16:1n7	2.03 ± 0.05	1.53 ± 0.03	1.39 ± 0.05	1.32 ± 0.00	0.77 ± 0.00	1.14 ± 0.03	1.22 ± 0.02	1.25 ± 0.05	1.16 ± 0.10	0.58 ± 0.14
17:0	0.52 ± 0.07	0.25 ± 0.01	0.33 ± 0.07	0.33 ± 0.00	0.17 ± 0.00	0.33 ± 0.01	0.38 ± 0.03	0.29 ± 0.03	0.42 ± 0.21	0.14 ± 0.03
17:1n7	1.99 ± 0.37	1.01 ± 0.07	0.83 ± 0.37	0.84 ± 0.00	0.61 ± 0.00	0.53 ± 0.07	0.88 ± 0.18	1.01 ± 0.11	0.53 ± 0.21	0.76 ± 0.10
18:0	3.75 ± 0.07^{1}	2.67 ± 0.12^{2}	3.18 ± 0.07^{3a}	3.92 ± 0.00^{4d}	2.30 ± 0.00^{5g}	2.99 ± 0.12^{3a}	4.15 ± 0.25^{3d}	3.12 ± 0.14^{3a}	3.41 ± 0.40^{4e}	2.15 ± 0.38^{5h}
18:1n9	2.76 ± 0.36^{1}	8.73 ± 0.14^{2}	6.06 ± 0.36^{3a}	5.01 ± 0.00^{4d}	3.13 ± 0.00^{5g}	10.22 ± 0.14^{3b}	10.3 ± 0.66^{3e}	7.92 ± 0.19^{3c}	6.17 ± 0.81^{4f}	3.28 ± 0.02^{5h}
18:1n7	1.82 ± 0.10	2.57 ± 0.02	2.67 ± 0.10	2.50 ± 0.00	1.16 ± 0.00	2.52 ± 0.02	2.51 ± 0.10	2.63 ± 0.01	2.26 ± 0.37	1.06 ± 0.13
18:2n6	0,62 ± 0.09^{1}	1.11 ± 0.05^{2}	1.13 ± 0.09^{2a}	1.06 ± 0.00^{3b}	0.76 ± 0.00^{4g}	0.92 ± 0.05^{3b}	1.03 ± 0.02^{3d}	1.05 ± 0.05^{2a}	0.85 ± 0.03^{3d}	0.65 ± 0.10^{4h}
18:2n4	0.38 ± 0.00	0.10 ± 0.09	0.10 ± 0.00	0.11 ± 0.00	0.08 ± 0.00	0.06 ± 0.09	0.13 ± 0.18	0.18 ± 0.01	0.17 ± 0.08	0.08 ± 0.01
18:3n6	0.14 ± 0.06	0.38 ± 0.05	0.62 ± 0.06	0.58 ± 0.00	0.27 ± 0.00	0.59 ± 0.05	0.54 ± 0.15	0.62 ± 0.06	0.60 ± 0.15	0.27 ± 0.02
18:3n3	0.18 ± 0.27^{1}	1.84 ± 0.05^{2}	1.62 ± 0.27^{2a}	1.51 ± 0.00^{3d}	1.04 ± 0.00^{4g}	3.02 ± 0.05^{3b}	3.20 ± 0.07^{4e}	2.47 ± 0.24^{3c}	1.56 ± 0.10^{4d}	1.15 ± 0.35^{5h}
18:4n3	0.31 ± 1.04^{1}	5.26 ± 0.19^{2}	5.50 ± 1.04^{2a}	3.74 ± 0.00^{3d}	2.31 ± 0.00^{4g}	2.22 ± 0.19^{3b}	1.75 ± 0.09^{3e}	4.79 ± 0.43^{3a}	1.75 ± 0.01^{4e}	2.21 ± 0.70^{5h}
20:1n11	0.41 ± .0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:1n9	0.56 ± 0.09	1.29 ± 0.00	1.12 ± 0.09	1.03 ± 0.00	0.66 ± 0.00	1.57 ± 0.00	1.75 ± 0.01	1.46 ± 0.06	1.28 ± 0.12	0.76 ± 0.15
20:1n7	0.50 ± 0.02	0.18 ± 0.00	0.20 ± 0.02	0.22 ± 0.00	0.11 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.03	0.18 ± 0.02	0.11 ± 0.02
20:2n6	0.49 ± 0.04	0.45 ± 0.01	0.52 ± 0.04	0.50 ± 0.00	0.48 ± 0.00	0.44 ± 0.01	0.48 ± 0.02	0.53 ± 0.07	0.45 ± 0.01	0.41 ± 0.09
20:3n6	0.82 ± 0.05	0.77 ± 0.04	0.71 ± 0.05	0.96 ± 0.00	0.85 ± 0.00	0.77 ± 0.04	1.16 ± 0.07	0.76 ± 0.03	0.73 ± 0.16	0.70 ± 0.01
20:4n6	0.79 ± 0.13^{1}	0.49 ± 0.01^{2}	0.57 ± 0.13^{3a}	0.45 ± 0.00^{3d}	0.32 ± 0.00^{3g}	0.62 ± 0.01^{3a}	0.74 ± 0.05^{3e}	0.67 ± 0.04^{3a}	0.51 ± 0.01^{3d}	0.38 ± 0.09^{3g}
20:4n3	0.47 ± 0.04	0.00 ± 0.01	0.97 ± 0.04	0.82 ± 0.00	0.56 ± 0.00	1.02 ± 0.01	1.48 ± 0.18	1.08 ± 0.04	0.75 ± 0.02	0.74 ± 0.25
20:5n3	2.96 ± 0.04^{1}	0.56 ± 0.28^{2}	0.42 ± 0.04^{2a}	0.38 ± 0.00^{3d}	0.24 ± 0.00^{4g}	2.22 ± 0.28^{3b}	2.81 ± 0.02^{3e}	1.46 ± 0.14^{3c}	0.80 ± 0.05^{4f}	0.71 ± 0.17^{5h}
22:1n9	0.18 ± 0.04	0.31 ± 0.06	0.36 ± 0.04	0.32 ± 0.00	0.35 ± 0.00	0.34 ± 0.06	0.52 ± 0.21	0.50 ± 0.18	0.26 ± 0.04	0.28 ± 0.05
20:2NMID	0.94 ± 0.02^{1}	0.39 ± 0.02^{2}	0.43 ± 0.02^{3ab}	0.45 ± 0.00^{4d}	0.25 ± 0.00^{5g}	0.37 ± 0.02^{2a}	0.00 ± 0.00^{3e}	0.48 ± 0.01^{3b}	0.35 ± 0.04^{4f}	0.10 ± 0.14^{5h}
22:3n9	0.30 ± 0.01	0.61 ± 0.16	0.15 ± 0.01	0.80 ± 0.00	0.41 ± 0.00	0.49 ± 0.16	1.13 ± 0.06	0.79 ± 0.04	0.18 ± 0.03	0.57 ± 0.26
22:3n6	0.14 ± 0.33	0.00 ± 0.32	0.39 ± 0.33	0.19 ± 0.00	0.29 ± 0.00	0.23 ± 0.32	0.16 ± 0.22	0.10 ± 0.15	0.89 ± 0.18	0.00 ± 0.00
22:4n6	0.72 ± 0.12^{1}	0.41 ± 0.09^{2}	0.39 ± 0.12^{2a}	0.42 ± 0.00^{3d}	0.30 ± 0.00^{4g}	0.30 ± 0.09^{2b}	0.50 ± 0.19^{3d}	0.48 ± 0.07^{2c}	0.22 ± 0.10^{3e}	0.17 ± 0.06^{4h}
22:5n6	0.44 ± 0.21	1.32 ± 0.05	1.59 ± 0.21	1.53 ± 0.00	1.24 ± 0.00	0.77 ± 0.05	0.89 ± 0.02	1.38 ± 0.04	0.80 ± 0.01	0.94 ± 0.28
22:5n3	0.59 ± 0.16	0.20 ± 0.05	0.28 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.18 ± 0.05	0.00 ± 0.00	0.16 ± 0.10	0.21 ± 0.01	0.00 ± 0.00
22:6n3	3.74 ± 1.61^{1}	7.25 ± 0.20^{2}	7.06 ± 1.61^{2a}	4.89 ± 0.00^{3d}	3.14 ± 0.00^{4g}	2.52 ± 0.20^{3b}	1.95 ± 0.10^{3e}	5.88 ± 0.38^{3c}	2.22 ± 0.09^{4f}	3.12 ± 0.44^{5h}
Σ Saturated	15.4 ± 0.32^{1}	14.4 ± 0.51^{2}	16.8 ± 0.32^{3a}	18.4 ± 0.00^{4d}	9.66 ± 0.00^{5g}	16.4 ± 0.51^{3a}	19.2 ± 0.55^{4e}	16.3 ± 0.36^{3a}	17.00 ± 0.53^{4f}	8.64 ± 1.14^{5h}
Σ Monoenoic	11.6 ± 0.57^{1}	16.3 ± 0.22^{2}	13.4 ± 0.57^{3a}	12.1 ± 0.00^{4d}	7.26 ± 0.00^{5g}	17.8 ± 0.22^{3b}	18.6 ± 0.72^{4e}	16.0 ± 0.33^{2c}	12.9 ± 0.93^{3f}	7.24 ± 0.30^{4g}
Σ Polyenoic	14.0 ± 1.99 ^{1}	21.1 ± 0.56^{2}	22.4 ± 1.99^{2a}	18.4 ± 0.00^{3d}	12.5 ± 0.00^{4g}	16.7 ± 0.56^{3b}	18.00 ± 0.46^{4e}	22.9 ± 0.68^{2a}	13.0 ± 0.34^{3f}	12.2 ± 1.04^{4g}
Σn-3	8.26 ± 1.94	15.1 ± 0.40	15.8 ± 1.94	11.33 ± 0.00	7.28 ± 0.00	11.17 ± 0.40	11.2 ± 0.24	15.8 ± 0.65	7.28 ± 0.14	7.92 ± 0.95
Σn-6	4.16 ± 0.45	4.92 ± 0.34	5.91 ± 0.45	5.69 ± 0.00	4.51 ± 0.00	4.64 ± 0.34	5.51 ± 0.34	5.61 ± 0.20	5.06 ± 0.30	3.50 ± 0.33
Σn-7	6.34 ± 0.39	5.29 ± 0.08	5.08 ± 0.39	4.88 ± 0.00	2.66 ± 0.00	4.19 ± 0.08	4.61 ± 0.21	5.08 ± 0.12	4.12 ± 0.44	2.50 ± 0.22
Σn-9	5.17 ± 0.42	11.6 ± 0.26	8.45 ± 0.42	8.03 ± 0.00	5.00 ± 0.00	14.10 ± 0.26	15.1 ± 0.69	11.8 ± 0.31	9.03 ± 0.82	5.31 ± 0.33
Σn-11	0.41 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Σ PUFA n-3	8.08 ± 1.92^{1}	13.3 ± 0.40^{2}	14.2 ± 1.92^{2a}	9.82 ± 0.00^{3d}	6.24 ± 0.00^{4g}	8.15 ± 0.40^{3b}	7.98 ± 0.22^{3e}	13.4 ± 0.60^{2a}	5.72 ± 0.10^{3f}	6.77 ± 0.88^{4h}

Table 3 (Continued)

	I. galbana		*I. galbana*			*T. suecica*		Mixture		
	2 days	13 days	17 days	22 days	34 days	17 days	22 days	17 days	22 days	34 days
Fatty acid	D Larvae	Umbonate	Pediveliger	Metamorphosis	Postlarvae	Pediveliger	Metamorphosis	Pediveliger	Metamorphosis	Postlarvae
n-3/n-6	1.99 ± 0.39[1]	3.07 ± 0.20[2]	2.68 ± 0.39[3a]	1.99 ± 0.00[4d]	1.61 ± 0.00[5g]	2.41 ± 0.20[3b]	2.03 ± 0.13[3e]	2.82 ± 0.15[3a]	1.44 ± 0.09[4f]	2.26 ± 0.34[5h]
n-6/n-3	0.51 ± 0.07[1]	0.33 ± 0.01[2]	0.37 ± 0.02 [3a]	0.50 ± 0.00[4d]	0.62 ± 0.00[5g]	0.42 ± 0.02[3a]	0.49 ± 0.02[3d]	0.35 ± 0.00[3a]	0.69 ± 0.03[4e]	0.44 ± 0.01[5h]
NMID	0.94 ± 0.02	0.39 ± 0.02	0.43 ± 0.02	0.45 ± 0.00	0.25 ± 0.00	0.37 ± 0.02	0.00 ± 0.00	0.48 ± 0.01	0.35 ± 0.04	0.10 ± 0.14
Total FA	66.4 ± 2.95[1]	90.0 ± 0.98[2]	88.3 ± 2.95[2a]	79.3 ± 0.00[3d]	49.2 ± 0.00[4g]	85.4 ± 0.98[3a]	92.1 ± 1.31[4e]	94.0 ± 1.13[3b]	68.8 ± 1.50[4f]	47.4 ± 1.91[5g]

FA, total fatty acid; ~~NMID, non-methylene interrupted dienoic fatty acid~~; PUFA, polyunsaturated fatty acid.
Different numbers represent significant differences (ANOVA; $P < 0.05$) in fatty acid content between developmental stages fed with the same experimental diet. Different letters represent significant differences (ANOVA; $P < 0.05$) in fatty acids content between experimental diets in each larval stage [pediveliger (a, b, c), metamorphosis (d, e, f) and postlarvae (g, h)].

established (Zhukova 1991) and, since this group of fatty acids are not detected in the algal diets may have been entirely synthesized by the *V. pullatra* larvae.

Alkanani *et al.* (2007) working with adults of *M. edulis* showed that although n-3 were significantly correlated with growth, stepwise regression did not find n-3 in combination with other variables to be an important growth predictor. However, the stepwise regression showed that 20:2NMID fatty acid explained the major percentage of the variance of the mussel growth and consequently this fatty acid is considered as a major predictor for mussel growth. The survival during larval development may depend on the ability to develop new structures, including shells, while reserves are being consumed (Labarta *et al.* 1999; Veniot *et al.* 2003). The absence of 20:2NMID could be related to the failure of metamorphosis in larvae fed with *T. suecica*.

Larvae fed with *T. suecica* reached day 17 (pediveliger stage) with an intermediate size compared to the other two diets and dry weight values significantly lower than larvae fed with *I. galbana* and mixed diet. At day 22, majority of larvae are close to metamorphosis or it has been already initiated.

Table 4 Pearson's correlation coefficient between larval fatty acids and weight growth values (μg DW indiv^{-1}) for *V. pullastra*. (A) before the onset of the experimental diets and for larvae fed with ~~*Isochrysis*~~ (B), ~~*Tetraselmis*~~ (C) or Mixture (D) diets

	I. galbana (A)	*I. galbana* (B)	*T. suecica* (C)	Mixture (D)
Fatty acid	DW	DW	DW	DW
14:0	0.992**	−0.991**	0.997**	−0.886*
16:0	ns	−0.978**	0.964*	−0.903*
18:0	−0.989*	−0.869*	ns	−0.883*
18:1n-9	0.994**	−0.938**	0.974*	−0.850*
18:2n-6	0.984*	−0.982**	0.951*	ns
18:3n-3	0.987*	−0.982**	0.957*	ns
18:4n-3	0.995**	−0.848*	−0.980*	ns
20:4n-6	ns	ns	ns	ns
20:5n-3	−0.999**	−0.976**	ns	ns
20:2NMID	−0.986*	−0.985**	ns	−0.864*
22:4n-6	ns	−0.955**	ns	ns
22:6n-3	0.998**	−0.849*	ns	ns
Σ Saturated	−0.998**	−0.972**	ns	−0.904*
Σ Monoenoic	1.000**	−0.978**	ns	−0.860*
Σ Polyenoic	1.000**	−0.923**	0.959*	ns
Σn-3PUFA	1.000**	−0.850*	ns	ns
Σn-3	~~Ns~~	−0.824*	ns	ns
Σn-6	~~Ns~~	−0.914*	ns	ns
Σn-7	~~Ns~~	−0.974**	ns	ns
Σn-9	0.994**	−0.979**	ns	ns
Σn-11	−1.000**	ns	ns	ns
Σn-3/Σn-6	1.000**	ns	ns	ns
Total fatty acids	0.998**	−0.974**	ns	ns

*$P < 0.05$; **$P < 0.001$; ns, no significant.

At this point, larvae fed with *T. suecica* showed lower weight growth that those fed with *I. galbana* and mixed diet but higher lipid content (Table 1). Similarly, total fatty acids content showed higher values for larvae fed with *T. suecica*, although this diet showed the lowest content in total fatty acids. The latter could indicate that larvae fed with *T. suecica* were lacking of some particular nutrient that prevent an adequate utilization of energetic stores in growth, whereas larvae fed with *I. galbana* and mixed diet utilized their reserves to increase their growth and development.

The dietary fatty acids profiles were comparables to previously described results (Albentosa *et al*. 1994). In agreement with Soudant *et al*. (1999), we observed that dietary fatty acids composition influences the fatty acid profile of the larvae *V. pullastra*, as highlighted the significant differences observed between diets in the content of different groups of fatty acids (Table 3). These results suggest a limited capacity for *de novo* synthesis of long-chain PUFA in bivalves, as was previously reported (Delaunay *et al*. 1993; Caers *et al*. 2003).

PUFAs stored during larval development are used during metamorphosis to provide the energetic requirements for the synthesis of new structures (Delaunay *et al*. 1993). Beside other nutrients, lack of 22:6n-3 fatty acid in *T. suecica* diet might contribute to the higher mortality observed for larvae fed on the latter diet. Delaunay *et al*. (1993) reported that this fatty acid is partially replaced by 20:5n-3 in polar lipids of larvae fed on *Chaetoceros calcitrans* with no apparent negative effects on growth. Nonetheless fewer pediveliger larvae were able to settle in comparison to those which accumulated primarily 22:6n-3 (Delaunay *et al*. 1993). Other deficient fatty acids in *T. suecica* diet, were long-chain n-6PUFA. Delaunay *et al*. (1993) showed that *Pecten maximus* larvae need also n-6PUFA as previously demonstrated for adult oysters (Trider & Castell 1980). Although no significant relationships between the 20:4n-6 content and growth was observed, the important metabolic role of 20:4n-6 fatty acid as a precursor of prostaglandins (Smith & Murphy 2003) may result in a high turnover and requirement for this fatty acid.

In summary, the higher content of 20:5n-3 in *T. suecica* diet compared to the other diets apparently was not enough to compensate the absence of ~~20:6n-3.~~ In addition, the lower n3:n6 ratio pointed out deficiencies in the n-6 group, also important for larval growth. Those dietary deficiencies might prevent an adequate use of energetic reserves that were continuously accumulated in larvae and consequently preclude an adequate development and survival. Nonetheless when *T. suecica* is combined with *I. galbana* diet in mixture diet, nutritional deficiencies might be compensated as pointed out the survival rate. In addition, larvae fed on mixture diet reached postlarval stage with weight growth values higher than those fed on *I. galbana*, as was expected for multi-specific algal diets (Albentosa *et al*. 1993; Milke *et al*. 2004).

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Appendix 1

Equations to describe the evolution of dry weight (DW), organic weight (OW) and lipid content, expressed in their

energy equivalents (kJ g^{-1}) during larval development for the three experimental diets.

T. suecica

DW (kJ g^{-1}) = $4.5523e^{0.024\ time}$ ($r^2 = 0.91$, $P < 0.001$)

OW (kJ g^{-1}) = $15.168e^{0.018\ time}$ ($r^2 = 0.63$, $P < 0.001$)

Lipids (kJ g^{-1}) = 0.1305 time + 1.2443 ($r^2 = 0.76$, $P < 0.01$)

I. galbana

DW (kJ g^{-1}) = $-0.0089\ time^2 + 0.2659\ time + 4.3537$ ($r^2 = 0.91$, $P < 0.001$)

OW (kJ g^{-1}) = $-0.0107\ time^2 + 0.2027\ time + 16.501$ ($r^2 = 0.74$, $P < 0.001$)

Lipids (kJ g^{-1}) = $-0.005\ time^2 + 0.1492\ time + 1.5688$ ($r^2 = 0.77$, $P < 0.001$)

Mixture

DW (kJ g^{-1}) = $-0.0072\ time^2 + 0.2183\ time + 4.4689$ ($r^2 = 0.91$, $P < 0.001$)

OW (kJ g^{-1}) = $-0.0034\ time^2 + 0.0614\ time + 16.626$ ($r^2 = 0.52$, $P < 0.001$)

Lipids (kJ g^{-1}) = $-0.043\ time^2 + 0.1336\ time + 1.5857$ ($r^2 = 0.94$, $P < 0.001$)

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1 It is a survival percentage. It is not possible to transform in g kg^{-1}
2 Waltham
3 Santa Clara

Changes to introduce:

1- In page 4 line 3 change "(Table 1; ANOVA P<0.05)" into "(Table 1)"
2- In page 4 line 36 change "(KJ g^{-1}, dry weight, basis, Fig. 2)" into "(KJ g^{-1}, Fig. 2)
3- In page 4 second column line 3, 4 and 6 change "DW-1" into "DW^{-1}"
4- In Figure 2 legend (page 5) change "weight (c) (expressed in energy" into "weight (c) expressed in energy"
5- In page 5 line 39 and 41 change "DW-1" into "DW^{-1}"
6- In Table 3 foot note (page 8) change "NMID, non-methhylene interrupted dienoic fatty aci" into "NMID, non-methylene interrupted dienoic fatty acid"
7- In Table 4 legend change "*Isochrysis* (B), *Tetraselmis* (C)" into "*I. galbana* (B), *T. suecica* (C)"
8- In table 4 change "Ns" in captital letters into "ns"
9- In page 9 line 43 change "20:6n-3" into "22:6n-3"
10- In the Acknowledgements section, change "This work was funded by MEC. AGL2004-07023-C02-02/ACU" into "This work was funded by CIRCLE (Climate Impact Research Coordination for a larger Europe) 08MDS0184402PR.

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