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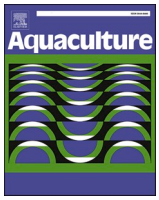

Using combinations of microalgae to condition European flat oyster (*Ostrea edulis*) broodstock and feed the larvae: Effects on reproduction, larval production and development

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ABSTRACT

Hatchery production of European flat oyster (*Ostrea edulis*) is erratic and is dependent upon broodstock conditioning and larval rearing conditions, including nutrition at both steps. In this study, the combined effects of broodstock and larval diets upon larval growth, survival, competence and metamorphosis and fatty acid and sterol compositions were studied. Four-year-old broodstock were conditioned in 700-L flow-through tanks (50 oysters per tank) at 19 °C and subjected to three dietary conditions in duplicate during 19 weeks. Two mixed diets were supplied continuously at an equivalent of 2 billion cells per day per oyster: *Rhodomonas salina* + *Chaetoceros neogracile* (Rs + C) or *R. salina* + *Thalassiosira weissflogii* (Rs + Tw), and the third condition was unfed. Samples of microalgae, released larvae (day 0), and larvae at day 9 were collected, and fatty acids (FAs) in neutral (NL) and polar lipids (PL) and sterols were analysed. Larval collection was continuous to estimate numbers of larvae released, which was dependent upon broodstock diet. Oysters fed Rs + C doubled the number of larvae compared to those receiving Rs + Tw - 47.8 compared to 23.1 million larvae, respectively. FAs in NL and PL, as well as sterol composition of released larvae, differed significantly depending upon broodstock diet. Larvae were reared in 5-L cylinders in a flow-through system at 22 °C. Larvae were subsequently fed different single and mixed microalgal diets in triplicate, incorporating *Tisochrysis lutea* (T), *C. neogracile* (C), and *Diacronema lutheri* (D), at a constant concentration of 1500 μm^3 μL^{-1} at the outlet of the rearing tank. Larval growth responded to both broodstock and larval diets; whereas, larval survival, competence, and settlement were influenced by both factors and their interactions. Broodstock receiving Rs + C produced larvae that grew faster (5.6–10.8 $\mu m\ day^{-1}$) than larvae from the other broodstock dietary regimes (4.6–9.6 $\mu m\ day^{-1}$). Larvae from fed broodstock (Rs + C or Rs + Tw) receiving the bi-specific diet T + C exhibited the highest growth rate (9.5–10.8 $\mu m\ day^{-1}$); whereas, those fed the single diet *D. lutheri* exhibited low growth and competence (4.6–6.1 $\mu m\ day^{-1}$; 4%) on day 9. Fed larvae originating from broodstock fed Rs + C exhibited better survival (92–97%) than those released by broodstock fed Rs + Tw (70–93%). Except for those supplied with T, fed larvae exhibited higher settlement when originating from broodstock receiving Rs + C. On day 9, FA and sterol compositions of larvae reflected the biochemical content of the larval diets. Overall, the best results were observed in larvae fed T + C originating from broodstock fed Rs + C.

1. Introduction

The European flat oyster (*Ostrea edulis*) has substantial commercial value in Europe. *O. edulis* global production (aquaculture + fisheries) dropped significantly in the last decades, from 23,510 t in 1970 to 2746 t in 2019 (FAO, 2022). At the end of the 1970s, two successive disease outbreaks associated with the parasites *Marteilia refringens* and *Bonamia ostreae* decimated *O. edulis* populations. Another driver of *O. edulis*

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population decline was overfishing (Laing et al., 2005). In the past, *O. edulis* was fished and cultivated mainly as human food, but nowadays there is an increasing number of active projects in Europe using *O. edulis* in the ecological restoration of habitats (Pogoda et al., 2019). Consequently, there is a high demand for hatchery *O. edulis* seed production that currently is unmet (Robert et al., 2013).

Broodstock conditioning is a major step in the operation of bivalve hatcheries. Maturation of broodstock is under control of various factors, such as temperature, salinity, and photoperiod, as well as food quantity and quality; temperature and food are thought to be most important (Mann, 1979; Newell et al., 1982). In the recent years, optimal parameters of temperature, photoperiod, food ration, and water renewal have been established for *O. edulis* broodstock conditioning. For instance, the combination of a gradual increase in temperature from 14 to 18 °C together with a photoperiod of 16 h light:8 h dark induces *O. edulis* larval release after four weeks of conditioning in winter or ten weeks in autumn (Maneiro et al., 2016, 2017b).

O. edulis is a larviparous species in which the brooding period lasts 7–10 days (Orton, 1936). Fecundity and initial larval quality have been mainly related to the effect of food on *O. edulis* broodstock conditioning (Helm et al., 1991; Millican and Helm, 1994; Berntsson et al., 1997). The quantitative diet has been found to range from 3 to 6% of oyster dry weight in algae per day (Maneiro et al., 2017a, 2020) depending on water flow rate; whereas the qualitative aspect of the diet is more complex. Indeed, the influence of different microalgae on gonad development, biochemical composition, and larval production of *O. edulis* broodstock has already been investigated since the pioneers works of Frolov and Pankov (1992) and Millican and Helm (1994). Thus, based on single diets comparison, using concomitantly, physiological, biochemical and biological approaches, González-Araya et al. (2011) recommended the addition of *Skeletonema marinoi* or *Chaetoceros neogracile* during *O. edulis* broodstock conditioning; whereas in a second similar study, González-Araya et al. (2012b) advised the use of *Rhodomonas salina* or *Thalassiosira weissflogii*. On the other hand, González-Araya et al. (2012a) reported higher fecundity and a more consistent larval release by *O. edulis* broodstock fed a mixed diet of *R. salina* + *T. weissflogii* compared to each of single diet. The recommendation of a mixed diet for *O. edulis* broodstock conditioning was accordingly established. However, the best mixed diet assemblage has still to be found and the combination of *R. salina* to *C. neogracile* or *S. marinoi*, need to be investigated.

On the other hand, it is well known that food quality has a major influence upon the development of larvae and juvenile oysters (da Costa et al., 2016; Laing and Millican, 1986; Rico-Villa et al., 2006). The literature attributes key nutritional roles in bivalve nutrition to the essential fatty acids (EFAs), particularly the n-3 fatty acids (FA) eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA), and the n-6 FA arachidonic acid (20:4n-6, ARA) (Berntsson et al., 1997; Rico-Villa et al., 2006), and the essential sterol, cholesterol (da Costa et al., 2016). *Tisochrysis lutea* is rich in DHA, and the diatom *Chaetoceros* sp. are rich in EPA and cholesterol, whereas, *Pavlova* (now *Diacronema*) *lutheri* is relatively well balanced in both fatty acids. Some diatoms have high nutritional value for oysters, such as juvenile Pacific oyster *Magallana gigas* (formerly known as *Crassostrea gigas*) and *O. edulis* (Enright et al., 1986; Knuckey et al., 2002); however, both *T. lutea* and *D. lutheri* supported poor larval growth and survival in *M. gigas* (Helm and Laing, 1987; Rico-Villa et al., 2006). Growth of bivalve larvae and juveniles is, however, often improved when the diet consists of mixed microalgae (Epifanio, 1979). This enhanced growth is attributed to the supply of complementary essential nutrients brought by each component as well as higher food uptake (Rico-Villa et al., 2006).

The diet used for feeding oyster larvae in the hatchery at Argenton (North Brittany) consists of a mixture of two flagellates (*D. lutheri* and *T. lutea*) and the diatom *Chaetoceros calcitrans* forma *pumilum*. Such diet was previously established for rearing the King scallop *Pecten maximus* larvae (Delaunay et al., 1993) with success but the use of this mixed diet for *O. edulis* larvae remains empirical.

The present work contributes to new knowledge on the responses of *O. edulis* larvae to different microalgal diets, and includes incorporation of data relating to the effects of broodstock conditioning on larval production, survival and growth.

2. Material and methods

2.1. Conditioning and larval rearing

A total of 300 four-year-old *O. edulis*, *Bonamia* and *Marteilia* free, originating from Norway were conditioned in spring (conditioning started late March 2009). Oyster broodstock were distributed in 6 flow-through tanks of 700-L (50 individuals per tank) with seawater filtered through a 1-μm cotton bag filter followed by UV treatment, at 19 °C and permanent light. Seawater inflow was regulated at 2 L min^{-1} with a flowmeter. Tanks were drained and cleaned daily and treated once a week with a bactericide/fungicide/virucide solution.

Three experimental conditions were tested in duplicate. Oysters were fed continuously an equivalent of 2 billion cells per day per oyster during 19 weeks. Two quantitatively-equivalent diets were used for conditioning broodstock: Rs + C = *Rhodomonas salina* (Rs: strain CCAP 978/24, mean volumetric size of 160 μm^3, mean dry weight 130 pg $cell^{-1}$) + *Chaetoceros neogracile* (C: strain UTEX LB 2658, 80 μm^3, 70 pg $cell^{-1}$) or Rs + Tw = *R. salina* + *Thalassiosira weissflogii* (Tw: strain CCAP 1085/3, 900 μm^3, 250 pg $cell^{-1}$). The Rs + Tw diet had been used successfully for *O. edulis* conditioning (González-Araya et al., 2012a) and was accordingly used in the present work as control. Additional experiments had shown highest absorption efficiencies and transfer of essential dietary components in oysters fed the flagellate *R. salina* and the diatom *C. neogracile* (González-Araya et al., 2012b) and was delivered here as a new mixed diet. To estimate the effects of initial oyster reserves (built in the natural surroundings before transfer to the laboratory), upon reproductive development and subsequent larval performance, a third batch of *O. edulis* was given no supplemental cultured microalgae (unfed). Broodstock were fed continuously by means of a peristaltic pump that mixed the algae with filtered seawater at the inlet of each tank for each feeding condition. Expelled larvae were collected from the water surface by larval collectors equipped with 100-μm mesh placed in the outflows of each tank. Larval collectors were inspected daily from week 6 until the end of conditioning on week 19 when no more larvae were expelled in any of the conditions. When detected in each tank, released larvae were collected and counted at each "spawning" (expelled larvae). Released larvae (week 8 for those from unfed broodstock and week 9 from those issued from fed broodstock) were transferred to a 500-mL measuring cylinder. Filtered seawater was added to a known volume. With an automatic pipette set at 100 μL, 5 replicates were sampled, while agitating the contents of the measuring cylinder with a suitable diameter perforated plunger. Larvae were counted using a light microscope. When a large release of larvae was recorded (~1 million) in a single spawning event from each broodstock experimental condition (i.e. sufficient number of larvae was obtained to set up the larval feeding trial), larvae were distributed in 5-L translucent methacrylate cylinders, reared in flow-through, with 1-μm-cartridge filtered and UV-treated seawater, in triplicate as previously detailed (González-Araya et al., 2012a). Larvae were held at a density of 5 larvae mL^{-1}, 22 °C and ambient salinity (34 ppt) with no photoperiod control. Three different microalgae were tested as mono-, bi- and pluri-specific larval diets: *Tisochrysis lutea* (named T, volumetric size, 46 μm^3, strain CCAP 927/14), *C. neogracile* (named C, 80 μm^3, UTEX LB 2658) and *Diacronema lutheri* (formerly known as *Pavlova lutheri*, named here D, 50 μm^3, strain CCAP 931/1). Larvae were fed at a constant concentration of 1500 μm^3 μL^{-1} at the outlet of the rearing tank, corresponding to ~40 algal cells μL^{-1} (equivalent *T. lutea*: Rico-Villa et al., 2010). The microbiological status of the larvae at release was evaluated to screen for pathogens. The number of *Vibrio* spp. per larva at release was

determined by plating on thiosulphate-citrate-bile-sucrose (TCBS) medium; if this load was >1 colony forming unit (CFU) per larva, thereafter the entire feeding trial was abandoned.

Few larvae were lost during larval rearing; accordingly, larval performance variables reported here relate to the entire population. Larval length and survival were estimated on days 3 and 6 by sampling 10-mL of seawater containing larvae ($n \geq 100$) from each cylinder and counting under a light microscope using image analysis (WinImager 2.0 and Imaq Vision Builder 6.0 software for images capture and treatment, respectively). On day 9, cylinders were drained, larvae were retained on a sieve, and the entire larval population was transferred in a 100-mL test tube. Filtered seawater was added to a known volume. With an automatic pipette set at 50 µL, 5 replicate subsamples were taken of the contents while agitating the contents of the test tube with a perforated plunger. Larvae were counted using a light microscope to determine growth in length and survival (n ≥ 100). Data collected on day 9 only are reported in the present paper.

When >50% of the larvae had developed the eye-spot in at least two of the larval dietary conditions (on day 9), and were thus considered as "competent," each larval population was transferred to a PVC container with a 125-µm nylon mesh and kept in a 150-L raceway tank. Larvae were distributed at a density of 0.6–1.3 larvae mL^{-1}, or 4–8 postlarvae cm^{-2} (supposing 100% settlement), in each bottom tray and provided with calibrated oyster shellfish chips (400 µm) as settlement substrate. A continuous, 1-µm filtered by cotton bag and UV-treated, seawater flow of 9 L h^{-1} was provided continuously by a down-weller system. Temperature were set at 22 °C, and ambient salinity was 34 ppm. All larvae, irrespective of the larval dietary treatment, were fed the same diet continuously (T + C + D). One week later, the experiment was ended, and metamorphosis was estimated by determining the number of remaining larvae in suspension (absence of dissoconch). Because unfed larvae did not show any competence after 9 days of rearing, they were not included in the metamorphosis trial.

2.2. Biochemical analysis

For all experimental conditions, microalgal feeding was stopped at the beginning of the sampling day before larval samples were collected. Larvae were drained and rinsed with 1-µm-cartridge-filtered and UV-treated seawater. Duplicate larval samples, 5×10^4 D-larvae for each nutritional broodstock condition (Rs + C; Rs + Tw and unfed) at day 0 (released larvae), and duplicate samples of 10^4 larvae from each larval diet condition (T, C, D, T + C, T + D, C + D and T + C + D) originating from broodstock fed Rs + C or Rs + Tw at day 9, were recovered on a pre-ignited (overnight at 450 °C) GF/D, glass-fibre filter (Whatman, diameter 47 mm, 3 µm porosity) by filtration and washed with 3.5% ammonium formate. Each filter with larvae was placed in a glass vial containing a mixture of chloroform-methanol (2:1, *v*/v) according to Folch et al. (1957), sealed under N_2, and stored at −20 °C for up to 6 months before analysis.

For microalgal fatty acid (FA) and sterol analyses, 50-mL triplicate samples of microalgal suspension were filtered following similar harvest methods to those used for larvae.

Neutral and polar lipids were separated on a Silica gel micro-column according to Marty et al. (1992). An aliquot of lipid extract was evaporated to dryness and recovered with three washings of 500 µL each of chloroform–methanol mixture (98:2, v/v). The sample was placed on top of a silica gel microcolumn (30 × 5 mm I.D. Kieselgel, 70–230 mesh, Merck), previously heated to 450 °C and deactivated with 5 wt% water according to Soudant et al. (1995). The neutral lipids (NL) were first eluted with 10 mL of chloroform–methanol mixture (98:2, v/v). The polar lipids (PL) then were recovered with 15 mL of methanol. A known amount of 23:0 fatty acid, as an internal standard, was added to neutral and polar fractions. Both fractions were evaporated to dryness, re-suspended with 1 mL of a chloroform–methanol mixture (2:1, v/v) and stored under nitrogen at −20 °C until analysis.

After evaporation to dryness, each lipid extract or fraction was transesterified with 10% BF_3 (*w*/w) in methanol for 15 min at 95–100 °C (Metcalfe and Schmitz, 1961). After cooling, the fatty acid methyl esters (FAMEs) were extracted with hexane according to the method described by Marty et al. (1992). Separation of FAMEs was carried out on a gas chromatography (GC) system (HP 6890) equipped with a flame ionization detector, an on-column injector and a DBWAX capillary column (J & W, 25 m × 0.32 mm; 0.2 Am film thickness). The column was temperature-programmed from 60 to 150 °C at 30 °C/min and 150 to 220 °C at 2 °C/min. Hydrogen was used as the carrier gas at 2.0 mL. min^{-1}. Identification of FAMEs was based upon comparison of retention times with those of authentic standards and confirmed by gas liquid chromatograph-mass spectrometry (GC–MS). 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 4,4-Dimethyloxazolazine (DMOX) derivation (data not shown). Fatty-acid peaks were integrated and analysed using HP chemstation software. Total fatty acid content (polar+neutral lipids) per larva was expressed as ng of FAME per larvae. Fatty acid composition was expressed as absolute content (ng total fatty acid per larva) and relative content (weight percent of the total fatty acids of each fraction).

Sterols were analysed using the method described by Soudant et al. (2000) after transesterification with sodium methoxide (MeONa) in a Chrompack CP9002 gas chromatograph Varian Inc., Walnut Creek, CA, USA) equipped with a Restek Rtx64 fused silica capillary column (15 m × 0.25 mm x 0.25 µm film thickness) using an on-column injection system. Hydrogen was used as the carrier gas with a temperature gradient from 160 to 280 °C. The sterols were identified by comparison of retention times with standards, and cholestane was used as an internal standard.

2.3. Biochemical composition of the diets

Fatty acid (FA) and sterol profiles of microalgal diets to feed broodstock and larvae were determined (Table 1). *R. salina* had similar contents of 20:5n-3 and 22:6n-3 (8–10%) and exhibited higher contents of 20:4n-6 (2%) compared to the other microalgae used in the present study (0–1%; Table 1). *T. weissflogii* and *C. neogracile* were poor in 22:6n-3 (1–4%) and 20:4n-6 (0–1%) but rich in 20:5n-3 (20–23%; Table 1). *R. salina* was characterized by high contents of 18:2n-6 (18%), 18:3n-3 (12%) and 18:4n-3 (14%; Table 1). *T. weissflogii* and *C. neogracile* were rich in 16:1n-7 and 16:3n-4 (Table 1). *T. lutea* was rich in 14:0, 18:1n-9, 18:2n-6, 18:4n-3 and 22:6n-3 (Table 1). *D. lutheri* was rich in 20:5n-3, 16:0, 16:1n-7 and 22:6n-3 (Table 1).

Brassicasterol was the main sterol in *R. salina* and *T. lutea* (97–99%; Table 1). *T. weissflogii* contained mainly 24-methyl-cholesterol (79%) and in a lesser extent isofucosterol (11%; Table 1). Cholesterol was the main sterol in *C. neogracile* (51%; Table 1). This diatom also contained a high proportion of fucosterol (37%; Table 1). *D. lutheri* was characterized by a high diversity of sterols. It contained methylpavlovol (36%), ethylpavlovol (16%), stigmasterol (16%), methylporiferasterol (14%) and β-sitosterol (12%; Table 1).

2.4. Statistical analysis

Data were analysed using STATISTICA software (version 12; Stat Soft, Inc., Tulka, OK, USA). Data normality was first evaluated using the Shapiro-Wilk test. One-way analyses of variance (ANOVA) were used to test the effects of larval diet on growth rate, survival, competence, settlement, fatty acid and sterol composition of the larvae. Two-way ANOVA was used to test the combined effects of broodstock nutritional diets and larval diets upon larval growth rate, survival, competence, and settlement. When necessary in one- and two-way ANOVAs, post hoc analyses with the Least Significant Difference (LSD) test were applied. Percentage data were transformed by the function [arcsin

Table 1
Fatty acid and sterol composition of the total lipids (relative content) of the microalgae used as diets expressed as the mean relative content (wt% of total fatty acids or sterols ± SD, $n = 3$). Summary values are also reported as concentration, rather than percentage, at the end of each grouping.

Fatty acid	*R. salina*	*T. weissflogii*	*T. lutea*	*C. neogracile*	*D. lutheri*
14:0	7.3 ± 2.9	7.9 ± 1.2	19.0 ± 8.8	10.1 ± 1.0	10.0 ± 0.6
16:0	13.6 ± 2.2	13.6 ± 0.9	9.4 ± 0.2	11.1 ± 0.7	19.5 ± 2.2
18:0	0.5 ± 0.2	0.0 ± 0.0	0.2 ± 0.5	0.0 ± 0.0	0.4 ± 0.1
16:1n-9	1.1 ± 0.2	0.0 ± 0.0	0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
16:1n-7	0.7 ± 0.4	20.1 ± 3.3	5.1 ± 0.4	25.4 ± 0.6	16.3 ± 4.2
18:1n-9	1.3 ± 0.6	0.0 ± 0.0	12.1 ± 0.5	0.8 ± 0.2	1.3 ± 0.3
18:1n-7	2.0 ± 0.3	1.2 ± 0.1	1.2 ± 0.1	0.5 ± 0.1	1.7 ± 0.0
16:2n-4	0.1 ± 0.0	5.9 ± 0.9	0.5 ± 0.1	3.3 ± 0.4	0.5 ± 0.1
16:3n-4	0.0 ± 0.0	18.0 ± 1.4	0.3 ± 0.2	11.6 ± 2.2	0.1 ± 0.0
18:2n-6	18.0 ± 4.0	0.6 ± 0.0	11.6 ± 3.5	1.2 ± 0.4	2.4 ± 0.3
18:3n-6	3.8 ± 1.6	0.3 ± 0.0	1.3 ± 0.3	1.1 ± 0.0	1.7 ± 0.3
18:3n-3	11.5 ± 2.5	0.6 ± 0.1	6.1 ± 0.6	0.2 ± 0.0	1.6 ± 0.4
18:4n-3	13.7 ± 1.6	1.6 ± 0.0	12.1 ± 3.2	0.9 ± 0.1	6.6 ± 0.6
20:4n-6	2.4 ± 0.2	0.2 ± 0.0	0.2 ± 0.2	1.4 ± 0.1	0.5 ± 0.4
20:5n-3	9.5 ± 0.9	20.4 ± 4.6	0.3 ± 0.2	22.7 ± 0.1	23.4 ± 2.1
22:5n-6	0.2 ± 0.2	0.0 ± 0.0	1.7 ± 1.0	0.0 ± 0.0	1.0 ± 0.3
22:6n-3	8.2 ± 2.6	3.6 ± 0.6	10.2 ± 2.5	1.1 ± 0.5	10.8 ± 0.3
Σ SFA	21.4 ± 2.3	21.5 ± 1.0	29.5 ± 9.4	22.0 ± 0.5	34.7 ± 2.0
Σ MUFA	8.4 ± 1.3	22.5 ± 5.9	20.5 ± 1.2	28.2 ± 0.2	19.9 ± 4.3
Σ n-9	2.5 ± 0.7	0.1 ± 0.0	12.9 ± 0.3	0.9 ± 0.3	1.4 ± 0.1
Σ n-7	3.1 ± 0.3	21.6 ± 2.2	7.3 ± 1.1	26.4 ± 0.5	18.7 ± 4.2
Σ PUFA	68.7 ± 6.3	52.9 ± 6.3	48.4 ± 8.0	47.1 ± 1.4	49.4 ± 2.9
Σ n-4	0.1 ± 0.4	23.8 ± 1.6	0.8 ± 0.4	15.0 ± 1.8	0.6 ± 0.1
Σ n-6	24.6 ± 5.5	1.0 ± 0.3	15.2 ± 7.2	4.0 ± 0.6	5.9 ± 0.8
Σ n-3	44.0 ± 4.1	26.4 ± 2.6	32.1 ± 0.7	25.3 ± 0.7	42.8 ± 2.3
n-3/n-6	1.8 ± 0.6	25.0 ± 2.8	2.1 ± 1.9	6.3 ± 1.4	7.2 ± 0.5
22:6/20:5	0.9 ± 0.3	0.2 ± 0.0	31.1 ± 6.7	0.1 ± 0.0	0.5 ± 0.0
Total FA (fg cell^{-1})	26,081.7 ± 13,746.9	5874.2 ± 708.7	1367.6 ± 518.8	1848.9 ± 87.3	2672.7 ± 636.6
Sterols					
Cholesterol	0.4 ± 0.3	4.4 ± 0.8	0.7 ± 0.1	50.9 ± 2.0	0.4 ± 0.1
Brassicasterol	97.3 ± 0.5	0.0 ± 0.0	99.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Desmosterol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.1
Campesterol	0.3 ± 0.2	3.2 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	2.9 ± 0.1
24-methylene cholesterol	0.0 ± 0.0	78.6 ± 1.1	0.0 ± 0.0	6.6 ± 2.0	0.0 ± 0.0
Stigmasterol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	16.4 ± 2.0
4α-methylporiferasterol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	13.8 ± 0.9
β-sitosterol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	11.6 ± 1.4
Fucosterol	0.0 ± 0.0	3.0 ± 0.0	0.0 ± 0.0	37.3 ± 0.6	0.0 ± 0.0
Isofucosterol	0.0 ± 0.0	11.2 ± 0.8	0.0 ± 0.0	5.2 ± 0.7	0.0 ± 0.0
Methylpavlovol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	36.1 ± 1.9
Ethylpavlovol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	16.4 ± 1.4
Total Sterols (fg cell^{-1})	434.3 ± 193.7	1685.5 ± 449.1	83.7 ± 7.7	103.4 ± 13.2	806.2 ± 57.6

Σ SFA, Total saturated fatty acids; Σ MUFA, Total monounsaturated fatty acids; Σ PUFA, Total polyunsaturated fatty acids.

(racine x i/100)] to normalize variance (Sokal and Rohlf, 2000). Differences were considered statistically significant if $P \leq 0.05$.

The relative FA and sterol content (%) of the microalgal diet supplied to oyster larvae was compared with the relative FA in NL and PL and sterol composition in the competent larvae on day 9 by a similarity percentage analysis (SIMPER) to determine the relationship between FA and sterol profiles of the microalgae and those of the larvae. Larval composition data of both broodstock diets (Rs + C and Rs + Tw) were pooled for each larval diet. Data of FA and sterol percentages of the samples were logarithmically (log[x + 1]) transformed. Only the FA and sterols that cumulatively contributed up to 80% of the dissimilarities recorded were selected to identify the differences in the FA and sterol profile between competent larvae and microalgae fed (Clarke and Gorley, 2006). SIMPER analyses were performed using PRIMER (Quest Research Limited, Albany, Auckland, New Zealand, version 5).

To compare the distribution of feeding treatments (broodstock and larval diets) according to FA composition in NL (reserves) and composition of membranes (FA in PL and sterols) in larvae, a principal component analysis (PCA) was performed using the STATISTICA software (version 12; Stat Soft, Inc.). The distribution of feeding treatments in PCA factor plots (i.e. newly released larvae from different bloodstock diets (day 0 larvae) and larvae originating from different bloodstock diets after 9 days of feeding different larval diets) provided insight into relationships between larvae from different dietary treatments, deduced from proximity in factor plots.

Further, to investigate relationships between FA in lipid reserves and FA and sterols in membranes of the larvae on day 9 originating from broodstock fed Rs + C or Rs + Tw (as independent variables) with growth rate, survival, competence and settlement (as dependent variables), stepwise multiple linear regression using a backward elimination technique was calculated using STATISTICA software. Few variables explained variability in larval performance in the model (variables that met the 0.05 significance level).

3. Results

3.1. Oyster fecundity

Broodstock fed Rs + Tw and unfed broodstock started to release larvae on week 4; whereas, broodstrock fed Rs + C started during week 6 (Fig. 1). Fed broodstock released larvae nearly all weeks of conditioning until week 19, but unfed broodstock released larvae only until week 12 (Fig. 1). Broodstock fed Rs + C released up to 7.0 million larvae per week; whereas, the total numbers of larvae released per week recorded in the other treatments were lower (5.5 and 3.6 million larvae released per week for broodstock fed Rs + Tw and unfed, respectively). Broodstock fed Rs + C released a total of 47.8×10^6 larvae, while broodstock fed Rs + Tw released only half that number of larvae (23.1×10^6 larvae).

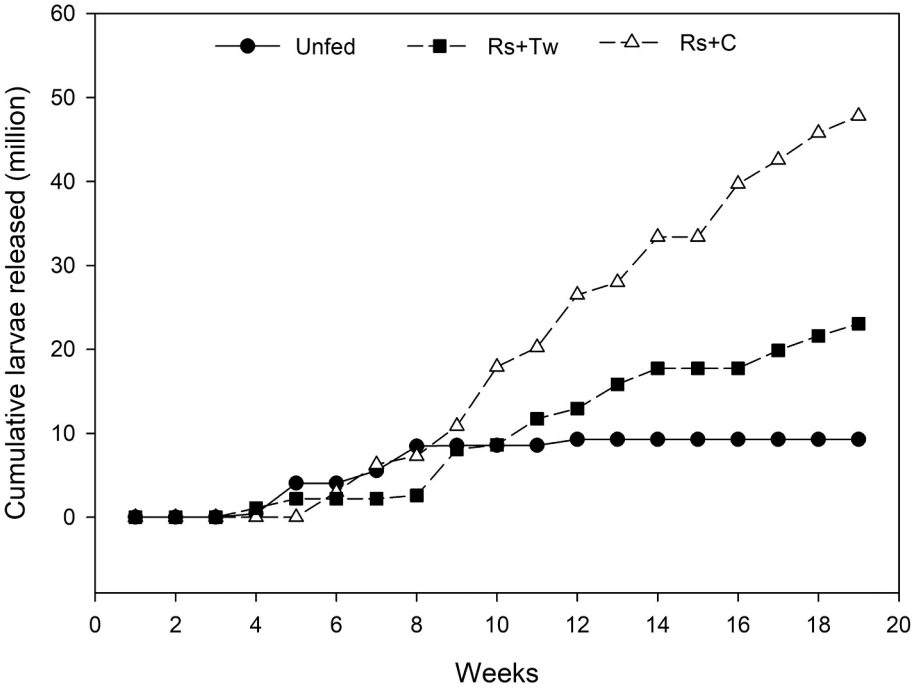

Fig. 1. Cumulative number of larvae released (in millions) by broodstock fed *Rhodomonas salina* + *Thalassisosira weissflogii* (**Rs** + **Tw**), *Rhodomonas salina* + *Chaetoceros neogracile* (**Rs** + **C**) and unfed broodstock.

Unfed broodstock released only 9.3 × 10^6 larvae, and among them a higher proportion of dead larvae was observed (8.9% vs ~ 4% in fed broodstock).

3.2. Effects on larval performance

Mean initial size of larvae released by unfed broodstock was slightly greater (172 μm) than larvae originating from fed broodstock, irrespective of diet (167–168 μm).

Growth rate was significantly affected by both broodstock and larval diet (2-way-ANOVA, $P = 0.002$ and $P = 0.000$, respectively). Fed larvae originating from unfed broodstock were able to grow normally (6.1–9.3 μm day^{-1}; Fig. 2A) at a similar growth rate to larvae from broodstock receiving Rs + Tw diets. Broodstock receiving Rs + C diet, however, produced larvae that grew significantly faster than larvae from the other two groups of broodstock.

Larvae fed the single diet of *D. lutheri* exhibited low growth (4.6 to 6.1 μm d^{-1}; Fig. 2A) and <4% competence on day 9 (Fig. 3A). In contrast, regardless of broodstock diet, unfed larvae showed poor growth (0.1 to 0.4 μm day^{-1}; Fig. 2A). Larvae from fed broodstock receiving the mixed T + C diet exhibited the highest growth rate (9.5–11.0 μm day^{-1}; Fig. 2A).

Larval survival was significantly affected by both broodstock and larval diets and the interaction of both factors (2-way-ANOVA, $P = 0.000$). Fed larvae from unfed broodstock exhibited significantly lower survival than larvae receiving the same diet but originating from fed broodstock, except for larvae fed T or D, which exhibited similar survival to those from broodstock fed Rs + Tw (Fig. 2B). Unfed larvae from unfed broodstock were more resilient (93% survival) than unfed larvae released by fed broodstock (0% survival; Fig. 2B). Fed larvae from parents receiving Rs + C showed significantly better survival (92–97%) than those released from parents fed Rs + Tw, wherein survival was more variable (70–93%: Fig. 2B).

A significant effect of broodstock and larval diet, and the interaction of both factors, was observed upon larval competence (2-way-ANOVA, $P = 0.000$). The lowest competence in larvae fed T + C (4% on day 9) was observed in larvae from unfed parents; whereas, the highest was found in larvae fed T + C from broodstock fed Rs + C and Rs + Tw (82%, Fig. 3A).

Broodstock and larval diet and the interaction of both factors significantly affected settlement rate (2-way-ANOVA, $P = 0.000$). Larvae fed T + C showed the highest metamorphosis rate, varying from 29 to 56% depending upon parental broodstock diet (Fig. 3B). When *D. lutheri* was included in a bi-specific diet, metamorphosis was unimproved (metamorphosis rate of 32–44%; Fig. 3B). Metamorphosis was lowest in larvae fed *D. lutheri* (0–6%: Fig. 3B). Moreover, *D. lutheri* depressed metamorphosis when combined with T or C in the diet during larval development. Except for larvae fed T, higher metamorphosis rate was achieved in larvae from broodstock fed Rs + C (Fig. 3B).

3.3. Effects of broodstock diet on released oyster larval composition

FA profiles in neutral lipids (NL) of released larvae were influenced by dietary conditions of broodstock (Table 2). Larvae from broodstock fed Rs + Tw were richer in saturated fatty acids (SFA, 41% of total fatty acids (TFA) in NL), mainly in 16:0, than the other conditions (22–27% of TFA in NL, Table 2). Larvae originating from broodstock fed Rs + C were characterized by higher relative contents of 16:1n-7, 18:1n-7, 18:2n-6, and 20:5n-3 in NL; whereas, larvae from broodstock fed Rs + Tw exhibited higher relative contents of 18:1n-9, 20:1n-7, 18:3n-3, and 18:4n-3 in NL (Table 2). Larvae from unfed broodstock showed a higher proportion of 22:6n-3 and non-methylene-interrupted (NMI) FA in PL than larvae from fed parents (22% and 10% vs 10% and 7%, respectively; Table 2). Larvae released by each broodstock group differed significantly in TFA content (total NL + PL), Rs + C > Unfed > Rs + Tw (21 > 15 > 11 ng of TFA $larva^{-1}$; $P < 0.05$, one-way-ANOVA).

Cholesterol and desmosterol levels were higher in larvae released by broodstock fed Rs + C than in larvae originating from broodstock fed Rs + Tw (50 and 6% vs 29 and 3%, respectively; Table 3). In contrast, larvae released by parents fed Rs + Tw contained more 24-methylene cholesterol and campesterol than larvae originating from broodstock fed Rs + C (Table 3). Larvae from broodstock fed Rs + Tw did not contain fucosterol and isofucosterol (Table 3). Larvae originating from unfed broodstock mainly contained cholesterol (33%), brassicasterol

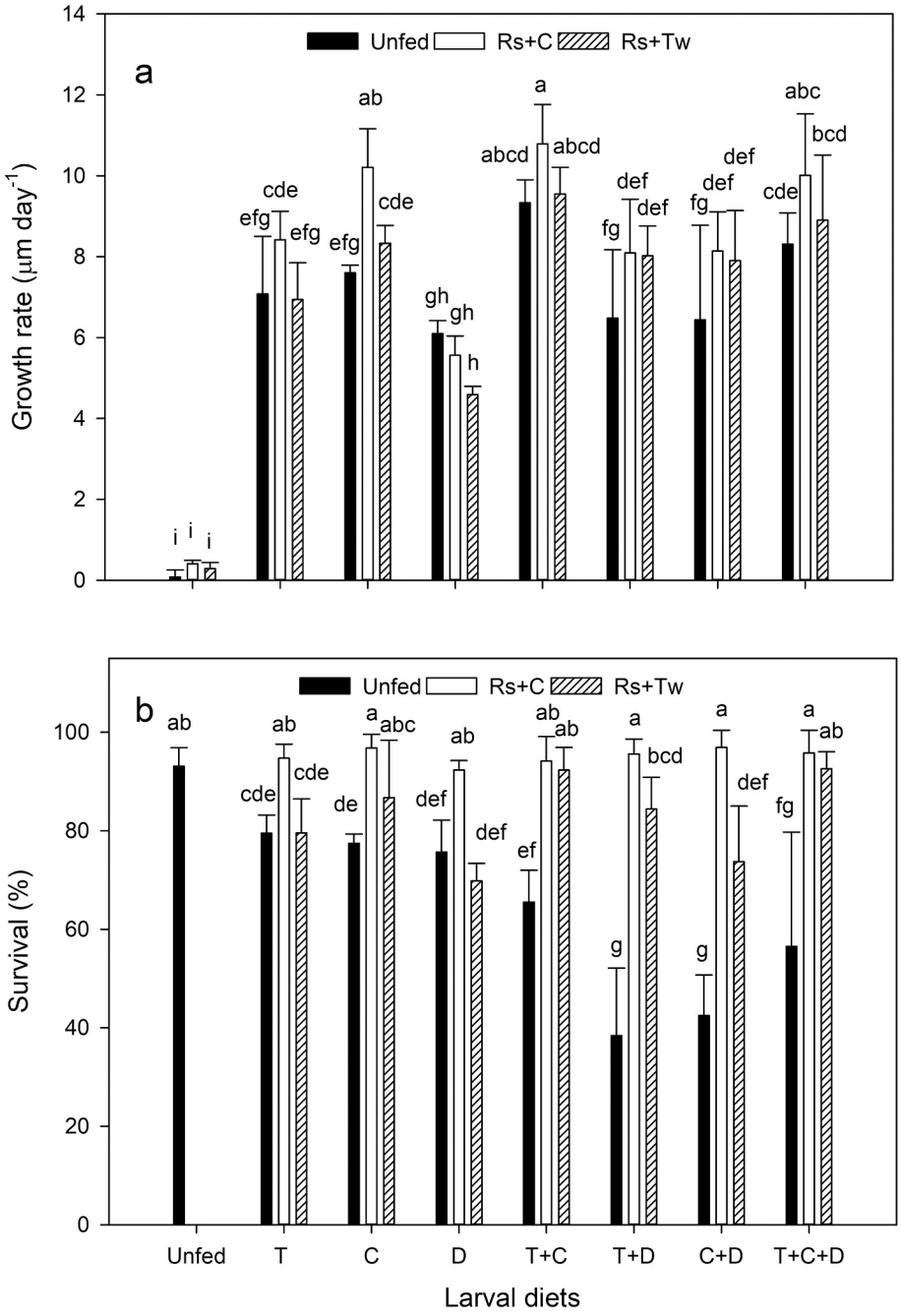

Fig. 2. Growth rate (a, µm day^{-1}) and survival (b, %) on day 9 of larvae fed *Tisochrysis lutea* (**T**), *Chaetoceros neogracile* (**C**), *Diacronema lutheri* (**D**), bi-specific diet **T** + **C**, **T** + **D**, **C** + **D** and multi-specific diet **T** + **C** + **D** originating from *O. edulis* broodstock previously fed *Rhodomonas salina* + *Chaetoceros neogracile* (**Rs** + **C**) and *Rhodomonas salina* + *Thalassisosira weissflogii* (**Rs** + **Tw**) or unfed. Values with same letter are not significantly different at $P > 0.05$.

(18%) and 24-methylene cholesterol (12%; Table 3). The highest storage of total sterols was observed in larvae originating from broodstock fed Rs + C; whereas, the lowest content was found in larvae released by broodstock fed Rs + Tw (Table 3). Larvae from broodstock feeding conditions greatly differed in cholesterol content per larva, with contents of 1.1, 0.5 and 0.4 ng larva^{-1} for broodstock fed Rs + C, starved, or fed Rs + Tw, respectively. The same trend was observed for total sterols per larva (Table 3).

3.4. Effects of microalgal diets on competent larval composition

Competent larvae produced by broodstock fed on both Rs + C and Rs + Tw diets showed FA composition in NL similar to those of the larval diet (Tables 4 and 5). For example, the FA profile in NL of larvae receiving T as single diet was similar to the FA composition of the microalga *T. lutea* (SIMPER, dissimilarity 14.0%, Supplementary table S1), but this FA profile differed from the FA composition of the other two microalgae used as larval feed in this study (SIMPER, dissimilarity 36.3–45.8%). The same pattern was observed when the FA relative contents in NL of larvae fed mono-specific diets of C and D were compared to the FA profile of the microalgae *C. neogracile* or *D. lutheri*, respectively (Supplementary table S1). Larvae originating from broodstock fed both Rs + C and Rs + Tw diets, receiving C and D in single or mixed diets, contained high proportions of n-7 MUFA, mainly 16:1n-7; whereas, larvae fed T stored high levels of 18:1n-9 (Tables 4 and 5). Competent larvae fed T showed high percentages of 18:2n-6, 18:3n-3,

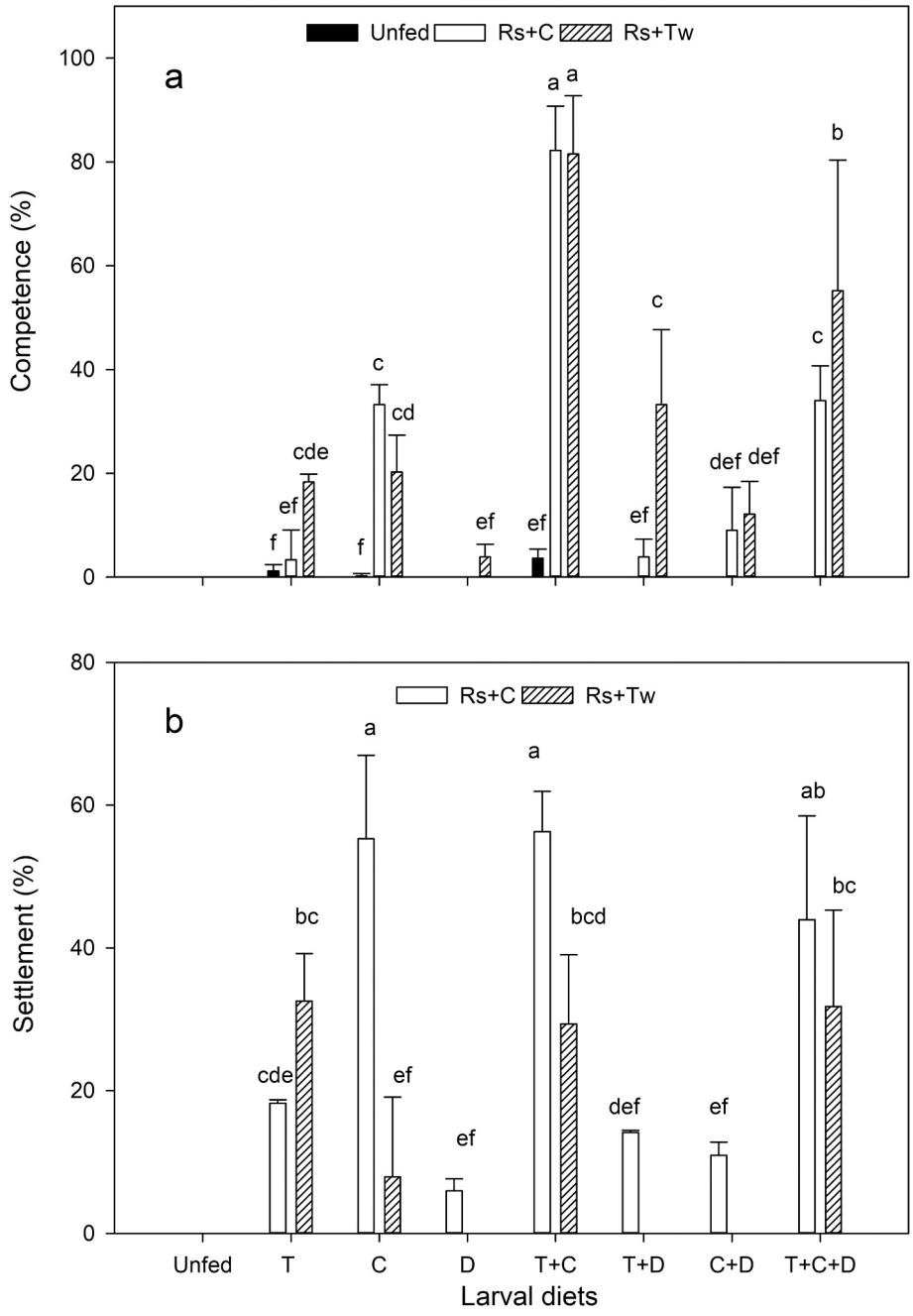

Fig. 3. Competence (a, %) and settlement (b, %) on day 9 (both) of larvae fed *Tisochrysis lutea* (**T**), *Chaetoceros neogracile* (**C**), *Diacronema lutheri* (**D**), bi-specific diet **T** + **C**, **T** + **D**, **C** + **D** and multi-specific diet **T** + **C** + **D** originating from *O. edulis* broodstock previously fed *Rhodomonas salina* + *Chaetoceros neogracile* (**Rs** + **C**) and *Rhodomonas salina* + *Thalassisosira weissflogii* (**Rs** + **Tw**) or unfed. Values with same letter are not significantly different at $P > 0.05$.

18:4n-3, and 22:6n-3 in NL, exhibiting the highest proportion of PUFA in NL (Tables 4 and 5). Larvae receiving C showed high levels of 20:5n-3, but larvae fed D contained moderate levels of both 20:5n-3 and 22:6n-3 (Tables 4 and 5). The highest relative contents of 20:4n-6 were observed in larvae fed the single diets C or D (Tables 4 and 5).

Except for larvae fed *T. lutea* in mono- and pluri-specific diets, FA composition in PL of competent larvae did not reflect clearly FA profiles of the microalgae (Supplementary table S1). Larvae receiving T incorporated in PL significantly higher percentages of 18:1n-9, 18:2n-6, 18:4n-3, and 22:6n-3 than the larvae receiving other microalgae (Tables 6 and 7). Competent larvae fed C were enriched in 20:5n-3 in PL; whereas, larvae receiving D incorporated balanced amounts of 20:5n-3 and 22:6n-3 in PL (Tables 6 and 7). The highest n-3/n-6 ratio was observed in larvae fed C (Tables 6 and 7).

Sterol composition of competent larvae reflected the sterol composition of the larval diets, regardless of the broodstock diet (Tables 8 and 9, Supplementary table S1). For example, larvae fed single diets and the corresponding microalgae showed similar sterol profiles (SIMPER, dissimilarity 36.2–49.5%, Supplementary table S1). Competent larvae fed T were enriched mainly in brassicasterol (69–78%) in membranes; whereas, larvae fed C mainly incorporated cholesterol (59–70%) (Tables 8 and 9). Surprisingly, neither methylpavlovol nor ethylpavlovol were found in the membranes of larvae fed D (Tables 8 and 9), even when these sterols were supplied in the diet (Table 1). The uptake of 4α-methyl poriferasterol was particularly low in larvae fed diets containing *D. lutheri* (Tables 8 and 9), even though this alga contained 14% of this

Table 2
Total neutral and polar fatty acids composition in released larvae of *Ostrea edulis* expressed in relative contents (weight % of total neutral and polar fatty acids ± S.D., $n = 2$), obtained from broodstock previously fed *Rhodomonas salina* + *Chaetoceros neogracile* (Rs + C), fed *Rhodomonas salina* + *Thalassiosira weissflogii* (Rs + Tw) or unfed.

	Neutral lipids			Polar lipids		
Fatty acid	**Unfed**	**Rs + C**	**Rs + Tw**	**Unfed**	**Rs + C**	**Rs + Tw**
14:0	2.3 ± 0.1^{b}	3.9 ± 0.0^{a}	5.0 ± 0.7^{a}	0.3 ± 0.0^{B}	1.2 ± 0.3^{A}	1.1 ± 0.3^{A}
16:0	15.2 ± 0.1^{b}	18.9 ± 0.1^{b}	29.4 ± 2.7^{a}	10.3 ± 0.3^{B}	14.1 ± 1.5^{A}	13.0 ± 0.8^{AB}
18:0	4.7 ± 0.2^{b}	3.7 ± 0.1^{b}	6.8 ± 0.7^{a}	4.3 ± 0.1	5.2 ± 0.0	4.8 ± 0.5
16:1n-7	2.9 ± 0.0^{b}	5.7 ± 0.1^{a}	0.0 ± 0.0^{c}	1.7 ± 0.0^{B}	2.6 ± 0.0^{A}	2.1 ± 0.2^{B}
18:1n-9	3.3 ± 0.1^{a}	1.1 ± 0.0^{b}	2.3 ± 0.0^{c}	1.9 ± 0.0^{A}	0.6 ± 0.1^{C}	1.2 ± 0.2^{B}
18:1n-7	2.5 ± 0.0	6.2 ± 0.0	3.5 ± 4.9	0.8 ± 1.1^{C}	3.7 ± 0.4^{A}	2.4 ± 0.1^{B}
20:1n-7	4.3 ± 0.1^{b}	4.6 ± 0.1^{b}	7.3 ± 0.1^{a}	3.4 ± 0.0^{C}	5.4 ± 0.1^{A}	4.4 ± 0.1^{B}
16:4n-1	4.1 ± 0.3^{a}	3.0 ± 0.3^{a}	0.4 ± 0.2^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:2n-6	0.6 ± 0.4^{b}	4.2 ± 0.0^{a}	0.0 ± 0.0^{c}	0.4 ± 0.0^{C}	3.4 ± 0.2^{A}	2.2 ± 0.1^{B}
18:3n-3	1.1 ± 0.0^{c}	2.5 ± 0.0^{b}	3.1 ± 0.1^{a}	0.8 ± 0.0^{B}	1.3 ± 0.1^{A}	1.3 ± 0.1^{A}
18:4n-3	1.3 ± 0.0^{b}	1.5 ± 0.0^{b}	2.0 ± 0.1^{a}	0.6 ± 0.0	0.5 ± 0.1	0.7 ± 0.0
20:2NMI	1.3 ± 0.0^{a}	1.1 ± 0.0^{b}	1.4 ± 0.1^{a}	0.7 ± 0.1	1.3 ± 0.5	0.8 ± 0.0
20:4n-6	2.0 ± 0.1^{b}	2.3 ± 0.0^{a}	2.5 ± 0.0^{a}	3.8 ± 0.1^{B}	5.2 ± 0.0^{A}	3.9 ± 0.2^{B}
20:4n-3	0.4 ± 0.0^{a}	0.3 ± 0.0^{b}	0.4 ± 0.0^{a}	0.2 ± 0.0^{A}	0.0 ± 0.1^{C}	0.1 ± 0.0^{B}
20:5n-3	7.5 ± 0.1^{c}	14.3 ± 0.3^{a}	11.6 ± 0.3^{b}	11.0 ± 0.2^{C}	22.9 ± 3.8^{A}	15.6 ± 1.1^{B}
22:2NMI	8.7 ± 0.3^{a}	5.7 ± 0.2^{b}	6.0 ± 0.2^{b}	8.5 ± 0.1	9.8 ± 1.8	10.0 ± 0.2
22:5n-6	0.4 ± 0.5	0.2 ± 0.0	0.0 ± 0.0	1.1 ± 0.0^{A}	0.3 ± 0.0^{B}	0.5 ± 0.0^{B}
22:5n-3	1.1 ± 0.0^{a}	0.8 ± 0.1^{b}	0.6 ± 0.1^{b}	1.6 ± 0.0^{A}	1.3 ± 0.0^{B}	1.4 ± 0.1^{A}
22:6n-3	21.5 ± 0.0^{a}	9.6 ± 0.2^{b}	10.0 ± 0.1^{b}	23.1 ± 0.4^{A}	10.0 ± 0.5^{C}	15.7 ± 0.3^{B}
∑ SFA	22.2 ± 0.2^{c}	26.5 ± 0.0^{b}	41.3 ± 4.2^{a}	14.9 ± 0.4	17.9 ± 2.5	18.8 ± 1.6
∑ MUFA	18.8 ± 0.2	20.3 ± 0.4	14.9 ± 4.9	13.7 ± 0.9^{AB}	16.1 ± 1.1^{A}	10.2 ± 0.3^{C}
∑.n-9	4.1 ± 0.1^{a}	1.9 ± 0.7^{c}	3.1 ± 0.0^{b}	2.4 ± 0.0^{A}	2.2 ± 0.1^{B}	1.2 ± 0.2^{C}
∑ n-7	9.7 ± 0.1	16.5 ± 0.2	10.8 ± 5.0	5.9 ± 1.1^{B}	10.5 ± 1.4^{A}	9.1 ± 0.0^{A}
∑ PUFA	54.1 ± 0.3^{a}	50.4 ± 1.4^{b}	43.7 ± 0.9^{c}	56.0 ± 0.4	53.7 ± 1.2	48.5 ± 1.4
∑ n-6	4.0 ± 0.8^{b}	8.6 ± 0.4^{a}	6.0 ± 0.5^{b}	6.3 ± 0.1^{B}	10.6 ± 0.1^{A}	6.8 ± 0.7^{B}
∑ n-3	35.4 ± 0.0^{a}	30.3 ± 0.7^{b}	28.6 ± 0.2^{b}	39.8 ± 0.3^{A}	31.7 ± 3.2^{B}	34.9 ± 0.8^{A}
∑ NMI	10.0 ± 0.2^{a}	6.8 ± 0.2^{b}	7.3 ± 0.2^{b}	9.2 ± 0.0	11.1 ± 2.3	6.8 ± 0.2
n-3/n-6	9.0 ± 1.7^{a}	3.5 ± 0.1^{b}	4.8 ± 0.4^{b}	6.3 ± 0.1^{A}	3.0 ± 0.3^{C}	5.1 ± 0.3^{B}
22:6/20:5	2.9 ± 0.0^{a}	0.7 ± 0.0^{b}	0.9 ± 0.0^{b}	2.1 ± 0.1^{A}	0.2 ± 0.3^{C}	1.0 ± 0.1^{B}
22:5/20:4	0.2 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	0.3 ± 0.0^{A}	0.1 ± 0.0^{B}	0.1 ± 0.0^{B}

Σ SFA, Total saturated fatty acids; Σ MUFA, Total monounsaturated fatty acids; Σ PUFA, Total polyunsaturated fatty acids; Σ NMI, Total non-methylene-interrupted fatty acids. Values with same lower case letters in the same row are not significantly different at $P > 0.05$ for NL and values with the same capital letters in the same row are not significantly different at $P > 0.05$ for PL.

Table 3
Main sterols expressed in relative content (weight % of sterol ± S.D., $n = 2$) and in total quantity (ng larva^{-1}) in released larvae of *Ostrea edulis*, obtained from broodstock previously fed *Rhodomonas salina* + *Chaetoceros neogracile* (Rs + C), fed *Rhodomonas salina* + *Thalassiosira weissflogii* (Rs + Tw) or unfed. Values with same letters in the same row are not significantly different at $P > 0.05$.

Sterols	**Unfed**	**Rs + C**	**Rs + Tw**
Norcholesterol	4.9 ± 0.0^{a}	1.0 ± 0.1^{c}	2.5 ± 0.1^{b}
cDehydrocholesterol	2.4 ± 0.1^{a}	0.5 ± 0.0^{c}	1.0 ± 0.0^{b}
tDehydrocholesterol	8.0 ± 0.0^{a}	2.5 ± 0.0^{c}	4.3 ± 0.0^{b}
Dihydrocholesterol	0.7 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Cholesterol	33.1 ± 0.3^{b}	49.9 ± 0.7^{a}	29.1 ± 0.5^{c}
Brassicasterol	17.9 ± 0.2^{b}	23.6 ± 0.0^{a}	26.7 ± 2.8^{a}
Desmosterol	2.1 ± 0.1^{c}	5.7 ± 0.2^{a}	3.0 ± 0.1^{b}
Campesterol	3.8 ± 0.1^{a}	2.1 ± 0.1^{c}	10.0 ± 0.2^{a}
24-methylene cholesterol	11.9 ± 0.1^{b}	5.0 ± 0.4^{c}	15.8 ± 0.1^{a}
Stigmasterol	3.8 ± 0.5^{a}	1.6 ± 0.5^{b}	2.9 ± 0.5^{ab}
4α-methylporiferasterol	1.1 ± 0.1^{a}	0.6 ± 0.1^{b}	1.0 ± 0.2^{ab}
β-sitosterol	5.9 ± 0.3^{a}	1.5 ± 0.1^{c}	2.9 ± 0.2^{b}
Fucosterol	1.3 ± 0.1^{b}	5.0 ± 0.1^{a}	0.0 ± 0.0^{c}
Isofucosterol	3.0 ± 0.1^{a}	1.0 ± 0.1^{b}	0.0 ± 0.0^{c}
Total Sterols (ng larva^{-1})	1.6 ± 0.0^{b}	2.3 ± 0.1^{a}	1.3 ± 0.1^{c}

sterol (Table 1). The level of campesterol in larvae fed *D. lutheri* (10–12%; Tables 8 and 9), irrespective of broodstock diet, was very high considering the level in *D. lutheri* (3%; Table 1).

3.5. Relationships between larval performance and FA and sterol composition of competent larvae

Stepwise multiple linear regression showed that 75.5% of the variance in growth rate was explained by the relative amounts of the FA in NL 14:0, 18:1n-7, and 20:5n-3 (Supplementary table S2). The FA 18:3n-3 in NL showed a negative coefficient in the predictive model for survival; whereas, 20:4n-3 in NL showed a positive coefficient. Larval competence was associated with SFA, n-7 and n-9 MUFAs, 18:2n-6, and non-methylene-interrupted (NMI) FA in NL (92.0% of the variance was explained with this model). Settlement was associated with 18:1n-7 and 20:5n-3 in NL, providing a descriptive model with but a low percentage of the variance explained (44%).

Growth rate variance was explained by the composition of membranes (FA in PL and sterol composition), specifically implicating 20:1n-7, 18:2n-6, 20:4n-6, 22:2NMI, and campesterol (Supplementary table S2). The relative amounts of 20:1n-7 and 22:2NMI explained 69.9% of the variance in larval survival. The model describing larval competence rate was influenced by 18:1n-9, 18:2n-6, 20:4n-6, cholesterol and 24-methylenecholesterol, which together accounted for 92% of the variance. A high percentage of the variance was explained in the predictive model of settlement, which included the SFA 18:0, the 20:1n-7, the n-6 PUFA 20:4n-6, the 22:2NMI, and the sterols 24-methylenecholesterol and stigmasterol.

Principal Component Analysis (PCA) for FA in NL extracted 3 components with eigenvalues higher than 1.0, which explained 86.7% of the variability in the original data. Newly-released larvae (day 0) from starved broodstock and broodstock fed Rs + Tw were located in close proximity; whereas, newly-released larvae from broodstock fed Rs + C were located close to 9-day-old-larvae from broodstock fed Rs + C (Fig. 4A). Larvae fed T, regardless of broodstock diet, formed one group, another group included larvae fed T + C, T + D and T + C + D, and a more diffuse group included larvae fed D, C and C + D (Fig. 4A).

PCA for larval membranes (FA in PL and sterols) extracted 6 components with eigenvalues higher than 1.0, which explained 89.9% of the variability in the original data. The first principal component (PC1) explained 36.3% of the combined variance and the second component (PC2) explained 21.3% (Fig. 4B). Newly released larvae formed a homogeneous group (Fig. 4B). Irrespective of broodstock diet (Rs + C or Rs + Tw), larvae were separated according to larval diets (Fig. 4B). Larvae

Table 4
Total neutral fatty acids composition in *Ostrea edulis* larvae at day 9, obtained from broodstock previously fed *Rhodomonas salina* + *Chaetoceros neogracile* (Rs + C), and fed mono, bi and multi-specific diets expressed in relative contents (weight % of total neutral fatty acids ± S.D., n = 2). **T**: *Tisochrysis lutea*; **C**: *Chaetoceros neogracile*; **D**: *Diacronema lutheri*; **T + C**: *T. lutea* + *C. neogracile*; **T + D**: *T. lutea* + *D. lutheri*; **C + D**: *D. lutheri* + *C. neogracile*; **T + C + D**: *T. lutea* + *C. neogracile* + *D. lutheri*. Values with same letters in the same row are not significantly different at $P > 0.05$.

	Oyster larval diets						
	T	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
14:0	9.2 ± 0.3^{a}	5.7 ± 1.1^{b}	3.0 ± 0.5^{c}	8.6 ± 0.1^{a}	8.2 ± 0.1^{a}	5.8 ± 0.1^{b}	7.9 ± 0.0^{a}
16:0	9.5 ± 0.4^{c}	11.6 ± 1.8^{abc}	13.9 ± 2.1^{a}	10.7 ± 0.1^{bc}	13.3 ± 0.0^{a}	13.9 ± 0.2^{a}	12.4 ± 0.3^{ab}
18:0	2.1 ± 0.2^{c}	2.8 ± 0.8^{bc}	4.1 ± 0.6^{a}	2.3 ± 0.1^{c}	2.9 ± 0.1^{bc}	3.2 ± 0.0^{ab}	2.7 ± 0.2^{bc}
16:1n-7	3.3 ± 0.3^{d}	12.2 ± 1.6^{a}	8.0 ± 1.2^{c}	8.7 ± 0.1^{bc}	7.0 ± 0.5^{c}	13.3 ± 0.0^{a}	9.9 ± 0.4^{b}
18:1n-9	11.7 ± 0.1^{a}	1.0 ± 0.6^{e}	2.1 ± 0.3^{d}	5.7 ± 0.2^{c}	8.2 ± 0.1^{b}	1.2 ± 0.0^{e}	5.1 ± 0.0^{c}
18:1n-7	3.6 ± 0.1^{d}	9.7 ± 1.2^{ab}	6.4 ± 0.9^{c}	6.7 ± 0.0^{c}	6.7 ± 0.2^{c}	10.6 ± 0.1^{a}	8.3 ± 0.3^{b}
20:1n-7	1.8 ± 0.0^{e}	3.2 ± 0.5^{bc}	3.8 ± 0.5^{ab}	2.4 ± 0.0^{d}	3.0 ± 0.1^{c}	3.9 ± 0.0^{a}	2.9 ± 0.1^{cd}
16:2n-7	0.0 ± 0.0^{b}	2.2 ± 1.0^{a}	0.8 ± 0.9^{ab}	1.6 ± 0.0^{a}	0.0 ± 0.0^{b}	1.2 ± 1.7^{ab}	1.3 ± 0.8^{ab}
16:2n-6	0.6 ± 0.1	0.4 ± 0.5	0.7 ± 1.1	0.0 ± 0.0	0.3 ± 0.2	1.1 ± 1.6	0.3 ± 0.4
16:2n-4	0.2 ± 0.0^{b}	1.8 ± 0.9^{a}	0.1 ± 0.1^{b}	1.5 ± 0.0^{a}	0.2 ± 0.0^{b}	1.5 ± 0.2^{a}	1.2 ± 0.1^{a}
16:3n-4	0.0 ± 0.0^{e}	4.6 ± 2.1^{a}	0.3 ± 0.1^{d}	3.6 ± 0.2^{ab}	0.0 ± 0.0^{e}	2.7 ± 0.0^{bc}	2.0 ± 0.0^{c}
16:4n-1	0.8 ± 0.0^{bc}	1.7 ± 0.9^{ab}	3.5 ± 0.1^{a}	0.4 ± 0.6^{c}	1.3 ± 0.1^{ab}	1.5 ± 0.0^{ab}	1.3 ± 0.1^{ab}
18:2n-6	11.8 ± 0.1^{a}	0.8 ± 0.4^{e}	1.5 ± 0.2^{d}	6.3 ± 0.0^{c}	7.7 ± 0.2^{b}	0.3 ± 0.0^{f}	5.3 ± 0.0^{c}
18:3n-3	6.1 ± 0.2^{a}	0.3 ± 0.1^{f}	0.4 ± 0.1^{e}	2.8 ± 0.0^{c}	3.4 ± 0.0^{b}	0.3 ± 0.0^{ef}	2.1 ± 0.1^{d}
18:4n-3	10.5 ± 0.2^{a}	0.9 ± 0.1^{e}	1.0 ± 0.2^{e}	5.2 ± 0.1^{b}	5.6 ± 0.0^{b}	1.6 ± 0.0^{d}	4.2 ± 0.1^{c}
20:2NMI	0.5 ± 0.0^{e}	0.8 ± 0.1^{bc}	0.9 ± 0.1^{a}	0.5 ± 0.0^{de}	0.7 ± 0.0^{c}	0.9 ± 0.0^{ab}	0.6 ± 0.0^{cd}
20:4n-6	0.8 ± 0.0^{c}	1.2 ± 0.1^{a}	1.2 ± 0.2^{a}	0.9 ± 0.1^{bc}	0.9 ± 0.0^{bc}	1.1 ± 0.0^{ab}	0.8 ± 0.0^{bc}
20:4n-3	0.5 ± 0.0^{a}	0.2 ± 0.0^{d}	0.2 ± 0.0^{d}	0.3 ± 0.0^{c}	0.3 ± 0.0^{b}	0.2 ± 0.0^{d}	0.3 ± 0.0^{c}
20:5n-3	1.5 ± 0.1^{d}	22.8 ± 4.1^{a}	12.5 ± 2.3^{b}	14.5 ± 0.3^{b}	7.7 ± 0.1^{c}	20.2 ± 0.2^{a}	14.3 ± 0.3^{b}
22:2NMI	1.5 ± 0.0^{cd}	2.7 ± 1.1^{b}	4.7 ± 0.5^{a}	1.2 ± 0.0^{d}	2.4 ± 0.1^{bc}	3.0 ± 0.1^{b}	2.0 ± 0.0^{bc}
22:5n-6	2.9 ± 0.0^{a}	0.4 ± 0.5^{d}	1.3 ± 0.3^{bc}	1.1 ± 0.0^{bc}	2.2 ± 0.0^{ab}	0.5 ± 0.0^{cd}	1.2 ± 0.0^{bc}
22:5n-3	0.2 ± 0.0^{c}	0.4 ± 0.1^{b}	0.6 ± 0.1^{a}	0.2 ± 0.0^{c}	0.3 ± 0.0^{c}	0.4 ± 0.0^{b}	0.3 ± 0.0^{c}
22:6n-3	14.4 ± 0.1^{a}	4.2 ± 3.3^{c}	9.7 ± 2.3^{ab}	6.3 ± 0.0^{bc}	12.9 ± 0.1^{a}	5.7 ± 0.0^{bc}	7.5 ± 0.1^{bc}
∑ SFA	20.8 ± 0.4^{b}	20.1 ± 1.5^{b}	21.0 ± 3.2^{b}	21.5 ± 0.0^{ab}	24.4 ± 0.0^{a}	22.9 ± 0.4^{ab}	22.9 ± 0.5^{ab}
∑ MUFA	22.4 ± 0.3^{c}	27.8 ± 1.8^{ab}	21.8 ± 3.0^{c}	25.5 ± 0.4^{b}	26.3 ± 0.1^{b}	30.2 ± 0.0^{a}	27.5 ± 0.1^{ab}
∑.n-9	12.9 ± 0.3^{a}	1.7 ± 0.6^{e}	2.6 ± 0.6^{d}	6.0 ± 0.3^{c}	8.7 ± 0.1^{b}	1.8 ± 0.1^{e}	5.5 ± 0.3^{c}
∑ n-7	9.0 ± 0.0^{d}	25.2 ± 2.4^{a}	18.3 ± 2.7^{bc}	18.3 ± 0.0^{bc}	16.7 ± 0.2^{c}	27.8 ± 0.1^{a}	21.2 ± 0.2^{b}
∑ PUFA	55.7 ± 0.6^{a}	49.7 ± 1.0^{b}	43.4 ± 6.0^{c}	51.3 ± 0.9^{b}	48.8 ± 0.6^{b}	45.7 ± 0.6^{bc}	48.6 ± 0.1^{b}
∑ n-4	0.3 ± 0.0^{c}	8.2 ± 3.7^{a}	0.7 ± 0.3^{c}	7.4 ± 0.3^{ab}	0.3 ± 0.0^{c}	5.5 ± 0.3^{ab}	4.6 ± 0.1^{b}
∑ n-6							

Table 4 (*continued*)

	Oyster larval diets						
	T	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
	18.3 ± 0.2^{a}	4.5 ± 1.8^{d}	7.2 ± 1.6^{cd}	10.1 ± 0.1^{b}	13.1 ± 0.0^{b}	4.5 ± 1.5^{d}	9.4 ± 0.6^{bc}
∑ n-3	34.4 ± 0.4^{a}	29.7 ± 0.3^{ab}	25.6 ± 4.9^{b}	30.1 ± 0.1^{a}	31.1 ± 0.3^{a}	29.2 ± 0.0^{ab}	29.3 ± 0.3^{ab}
∑ NMI	2.0 ± 0.0^{d}	3.4 ± 1.3^{bc}	5.7 ± 0.6^{a}	1.7 ± 0.0^{d}	3.1 ± 0.1^{bc}	3.9 ± 0.1^{b}	2.6 ± 0.0^{cd}
n-3/n-6	1.9 ± 0.0^{b}	7.8 ± 2.0^{a}	3.6 ± 0.1^{ab}	3.0 ± 0.0^{b}	2.4 ± 0.0^{b}	6.9 ± 2.3^{a}	3.1 ± 0.2^{b}
22:6/20:5	9.3 ± 0.4^{a}	0.3 ± 0.3^{e}	0.8 ± 0.0^{c}	0.4 ± 0.0^{d}	1.7 ± 0.0^{b}	0.3 ± 0.0^{de}	0.5 ± 0.0^{cd}
22:5/20:4	3.9 ± 0.0^{a}	0.3 ± 0.4^{e}	1.0 ± 0.0^{cd}	1.3 ± 0.1^{bcd}	2.4 ± 0.0^{ab}	0.5 ± 0.0^{de}	1.4 ± 0.0^{bc}

Σ SFA, Total saturated fatty acids; Σ MUFA, Total monounsaturated fatty acids; Σ PUFA, Total polyunsaturated fatty acids; Σ NMI, Total non-methylene-interrupted fatty acids.

fed mono-specific diets (T, C and D) were located far away from each other, representing a triangle in the x-y axis (Fig. 4B).

4. Discussion

4.1. Effects of broodstock diet

Our results showed that the quantity of larvae released by *O edulis* broodstock was affected by broodstock nutrition. Broodstock fed Rs + C released more larvae (48 million) than broodstock fed Rs + Tw (23 million) or unfed broodstock (9 million). This result confirms previous studies. Indeed, *O. edulis* broodstock showed differences in fecundity per female, doubling the number of released larvae per female by broodstock fed the mixed diet Rs + Tw at 6% dry weight (0.39 million larvae) compared to the single diets *R. salina* or *T. weissflogii* (0.16–0.28 million larvae) (González-Araya et al., 2012a). Similarly, Millican and Helm (1994) reported that larval production in *O. edulis* was affected by the microalgal species delivered, obtaining the poorest result with the single diet *Dunalliela tertiolecta*. To explain such results, Utting and Millican (1997) hypothesized that bivalve broodstock control fecundity to maintain a consistent lipid level. The present work does not support this supposition. Indeed, differences in total fatty acid (TFA) content in NL + PL of released larvae originating from broodstock fed different diets, were found, with nearly 2-fold more TFA in larvae released by broodstock fed Rs + C (21 ng larva^{-1}) than larvae expelled by broodstock fed Rs + Tw (11 ng larva^{-1}). Furthermore, larvae released by unfed broodstock showed intermediate values of TFA (15 ng larva^{-1}). TFA in PL can be influenced by the size of the oyster and subsequent number and size of the cells (Blanchier and Boucaud-Camou, 1984). As no difference in the initial size of the larvae released by broodstock fed different diets was found, we expect that larvae from different origins have similar amounts of TFA in PL (membranes). Consequently, we may expect that the differences in TFA may be attributed to the stored reserves in the form of FA in NL. Data suggest that under our experimental conditions *O. edulis* broodstock adapted fecundity and lipid reserves of the released larvae depending upon broodstock diet.

Despite *T. weissflogii* containing much more FA than *C. neogracile*, TFA contents in larvae from broodstock fed Rs + C became higher than in larvae from broodstock fed Rs + Tw. This apparently surprisingly result may be related to differences in algal ingestion. It has been shown previously that *O. edulis* exhibited higher ingestion rates of *C. neogracile* than *T. weissflogii*, and higher transfer of fatty acids and sterols was observed in broodstock receiving *C. neogracile* (González-Araya et al., 2011, 2012b). This physiological process may explain the higher allocation of biochemical reserves to gametes and subsequently to released larvae by broodstock fed a diet containing *C. neogracile*.

Table 5
Total neutral fatty acids composition in *Ostrea edulis* larvae at day 9, obtained from broodstock previously fed *Rhodomonas salina* + *Thalassiosira weissflogii* (Rs + Tw), and fed mono, bi and multi-specific diets expressed in relative contents (weight % of total neutral fatty acids ± S.D., n = 2). **T**: *Tisochrysis lutea*; **C**: *Chaetoceros neogracile*; **D**: *Diacronema lutheri*; **T + C**: *T. lutea* + *C. neogracile*; **T + D**: *T. lutea* + *D. lutheri*; **C + D**: *C. neogracile* + *D. lutheri*; **T + C + D**: *T. lutea* + *C. neogracile* + *D. lutheri*. Values with same letters in the same row are not significantly different at $P > 0.05$.

	Oyster larval diets						
Fatty acid	**T**	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
14:0	11.3 ± 0.3^{a}	6.7 ± 0.4^{bcd}	6.2 ± 0.4^{cd}	9.5 ± 0.4^{ab}	9.4 ± 0.1^{ab}	5.6 ± 2.7^{d}	8.7 ± 0.2^{abc}
16:0	10.1 ± 0.0^{b}	12.8 ± 3.0^{b}	19.8 ± 1.0^{a}	11.5 ± 0.6^{b}	14.0 ± 0.3^{b}	11.6 ± 3.8^{b}	12.8 ± 0.3^{b}
18:0	2.0 ± 0.0^{b}	3.0 ± 0.7^{b}	4.5 ± 0.1^{a}	2.6 ± 0.3^{b}	5.5 ± 1.1^{a}	2.5 ± 0.4^{b}	2.8 ± 0.0^{b}
16:1n-7	3.8 ± 0.1^{e}	15.2 ± 0.2^{b}	14.8 ± 0.0^{b}	10.6 ± 0.0^{c}	9.1 ± 0.9^{d}	16.3 ± 0.2^{a}	11.0 ± 0.2^{c}
18:1n-9	12.7 ± 0.2^{a}	1.0 ± 0.6^{d}	2.4 ± 0.1^{c}	5.7 ± 0.3^{b}	6.7 ± 0.9^{b}	0.8 ± 0.1^{d}	5.1 ± 0.2^{b}
18:1n-7	4.1 ± 0.0^{c}	12.8 ± 0.1^{a}	13.0 ± 0.6^{a}	7.7 ± 0.4^{b}	7.1 ± 0.7^{b}	14.6 ± 2.4^{a}	8.2 ± 0.0^{b}
20:1n-7	1.5 ± 0.1^{d}	3.2 ± 0.1^{ab}	3.4 ± 0.0^{a}	2.3 ± 0.1^{c}	2.6 ± 0.7^{bc}	2.8 ± 0.3^{abc}	2.8 ± 0.0^{abc}
16:2n-7	0.5 ± 0.0^{ab}	2.3 ± 1.1^{a}	0.0 ± 0.0^{b}	1.8 ± 0.0^{a}	0.6 ± 0.9^{ab}	2.2 ± 0.2^{a}	1.1 ± 1.6^{ab}
16:2n-6	0.0 ± 0.0^{b}	0.1 ± 0.2^{ab}	0.6 ± 0.2^{a}	0.0 ± 0.0^{b}	0.4 ± 0.6^{ab}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
16:2n-4	0.3 ± 0.0^{ab}	1.9 ± 0.9^{a}	0.0 ± 0.0^{b}	0.8 ± 1.1^{ab}	0.7 ± 0.2^{ab}	1.9 ± 0.1^{a}	1.2 ± 0.2^{a}
16:3n-4	0.0 ± 0.0^{d}	4.3 ± 2.0^{a}	0.0 ± 0.0^{d}	3.3 ± 0.0^{ab}	0.6 ± 0.0^{c}	3.1 ± 0.2^{ab}	1.9 ± 0.1^{b}
16:4n-1	0.2 ± 0.0^{b}	0.2 ± 0.1^{b}	0.0 ± 0.0^{c}	0.2 ± 0.0^{b}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	1.3 ± 0.2^{a}
18:2n-6	13.2 ± 0.0^{a}	1.1 ± 0.6^{d}	2.6 ± 0.1^{c}	6.4 ± 0.1^{b}	6.2 ± 1.2^{b}	1.2 ± 0.1^{d}	5.5 ± 0.2^{b}
18:3n-3	6.0 ± 0.0^{a}	0.4 ± 0.2^{cd}	0.8 ± 0.0^{c}	2.6 ± 0.0^{b}	2.5 ± 0.5^{b}	0.1 ± 0.2^{d}	1.9 ± 0.2^{b}
18:4n-3	10.7 ± 0.1^{a}	1.1 ± 0.7^{d}	2.7 ± 0.1^{c}	5.0 ± 0.1^{b}	4.8 ± 0.8^{b}	1.4 ± 0.0^{d}	4.2 ± 0.2^{b}
20:2NMI	0.2 ± 0.1	0.6 ± 0.1	0.2 ± 0.3	0.6 ± 0.1	0.8 ± 0.5	0.2 ± 0.3	0.6 ± 0.0
20:4n-6	0.6 ± 0.0^{e}	1.0 ± 0.1^{a}	0.9 ± 0.0^{b}	0.8 ± 0.1^{bc}	0.7 ± 0.1^{de}	0.8 ± 0.0^{cd}	0.8 ± 0.0^{bc}
20:4n-3	0.4 ± 0.0^{a}	0.2 ± 0.1^{d}	0.0 ± 0.0^{e}	0.3 ± 0.0^{bc}	0.3 ± 0.0^{b}	0.0 ± 0.0^{e}	0.2 ± 0.0^{cd}
20:5n-3	1.1 ± 0.0^{d}	22.4 ± 3.4^{a}	15.7 ± 0.6^{b}	14.3 ± 0.1^{b}	8.8 ± 1.5^{c}	25.8 ± 6.6^{a}	13.2 ± 0.2^{bc}
22:2NMI	0.7 ± 0.0^{d}	1.4 ± 0.0^{b}	1.5 ± 0.0^{b}	0.8 ± 0.1^{d}	1.2 ± 0.1^{c}	1.4 ± 0.1^{bc}	2.0 ± 0.1^{a}
22:5n-6	2.2 ± 0.0^{a}	0.2 ± 0.3^{c}	0.9 ± 0.0^{b}	0.9 ± 0.0^{b}	1.2 ± 0.2^{ab}	0.1 ± 0.2^{c}	1.2 ± 0.0^{ab}
22:5n-3	0.2 ± 0.0^{ab}	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.2 ± 0.0^{ab}	0.2 ± 0.0^{ab}	0.1 ± 0.1^{b}	0.2 ± 0.0^{ab}
22:6n-3	11.5 ± 0.0^{a}	2.7 ± 2.2^{c}	7.6 ± 0.3^{ab}	5.0 ± 0.0^{bc}	7.0 ± 1.5^{b}	3.6 ± 0.1^{c}	7.9 ± 0.3^{ab}
∑ SFA	23.5 ± 0.3^{abc}	22.5 ± 3.3^{bc}	30.5 ± 1.5^{a}	23.6 ± 1.4^{abc}	28.9 ± 0.6^{ab}	19.8 ± 7.0^{c}	24.2 ± 0.1^{abc}
∑ MUFA	24.0 ± 0.3^{d}	33.4 ± 0.2^{ab}	33.6 ± 0.9^{b}	28.1 ± 0.8^{c}	28.1 ± 0.1^{c}	34.8 ± 2.1^{a}	28.5 ± 0.2^{c}
∑ n-9	13.6 ± 0.2^{a}	1.5 ± 0.4^{d}	2.4 ± 0.1^{c}	6.0 ± 0.5^{b}	7.1 ± 1.0^{b}	0.9 ± 0.2^{d}	5.3 ± 0.2^{b}
∑ n-7	9.9 ± 0.0^{e}	31.2 ± 0.2^{ab}	31.2 ± 0.6^{b}	21.0 ± 0.2^{c}	20.3 ± 1.2^{d}	33.8 ± 2.5^{a}	22.3 ± 0.1^{c}
∑ PUFA	51.3 ± 0.1^{a}	42.6 ± 3.8^{b}	35.2 ± 0.9^{b}	46.7 ± 1.1^{ab}	39.9 ± 3.9^{b}	44.8 ± 4.7^{ab}	46.6 ± 0.5^{ab}
∑ n-4	0.4 ± 0.0^{de}	7.1 ± 3.3^{a}	0.0 ± 0.0^{e}	5.9 ± 1.2^{ab}	1.8 ± 0.2^{cd}	6.5 ± 0.3^{ab}	4.2 ± 0.0^{bc}
∑ n-6							

Table 5 (*continued*)

	Oyster larval diets						
Fatty acid	**T**	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
	18.4 ± 0.0^{a}	3.4 ± 1.2^{d}	6.1 ± 0.2^{c}	9.5 ± 0.2^{b}	10.0 ± 1.8^{b}	3.1 ± 0.8^{d}	9.0 ± 0.2^{b}
∑ n-3	30.8 ± 0.1^{ab}	27.5 ± 0.4^{ab}	27.5 ± 1.0^{ab}	27.9 ± 0.2^{ab}	24.9 ± 3.5^{b}	31.4 ± 6.2^{a}	28.3 ± 1.1^{ab}
∑ NMI	0.9 ± 0.1^{d}	2.0 ± 0.1^{b}	1.7 ± 0.3^{bc}	1.3 ± 0.0^{c}	2.1 ± 0.4^{b}	1.6 ± 0.2^{bc}	2.6 ± 0.0^{a}
n-3/n-6	1.7 ± 0.0^{b}	9.2 ± 2.2^{a}	4.5 ± 0.0^{b}	2.9 ± 0.1^{b}	2.5 ± 0.1^{b}	10.9 ± 4.8^{a}	3.1 ± 0.0^{b}
22:6/20:5	10.5 ± 0.0^{a}	0.2 ± 0.2^{e}	0.5 ± 0.0^{cd}	0.3 ± 0.0^{d}	0.8 ± 0.0^{b}	0.1 ± 0.0^{e}	0.6 ± 0.0^{bc}
22:5/20:4	3.8 ± 0.3^{a}	0.3 ± 0.4^{c}	1.0 ± 0.0^{b}	1.1 ± 0.1^{b}	1.8 ± 0.1^{b}	0.2 ± 0.3^{c}	1.5 ± 0.1^{b}

Σ SFA, Total saturated fatty acids; Σ MUFA, Total monounsaturated fatty acids; Σ PUFA, Total polyunsaturated fatty acids; Σ NMI, Total non-methylene-interrupted fatty acids.

The present study showed that *O. edulis* broodstock nutrition has a predominant influence in subsequent larval growth, survival, larval competence, and metamorphosis success. These results agree with those reported in *O. edulis* larvae fed different diets from broodstock fed mono- and bi-specific diets (González-Araya et al., 2012a). In the present work, larvae from parents fed Rs + Tw, exhibited similar growth rates to larvae from unfed broodstock, but lower growth rate than larvae from broodstock fed Rs + C. Larvae from unfed broodstock receiving bi- and pluri-specifics larvae diets exhibited lower survival than those from fed broodstock. These results contrasted with those of Millican and Helm (1994) and Berntsson et al. (1997), who reported that larvae released by unfed *O. edulis* performed similarly in terms of growth and mortality to those produced by fed broodstock. Neither of these studies reported long-term effects of broodstock nutrition upon larval competence or settlement rate. In the present work, larvae from starved parents did not achieve setting competence. This may suggest that stored reserves in larvae released from unfed broodstock may lack essential components or enough energy reserves to support complete larval development.

Surprisingly, unfed larvae released from broodstock fed both microalgal diets did not survive at day 9; whereas, unfed larvae from unfed parents exhibited a high survival rate (93%) after 9 days of rearing. González-Araya et al. (2012a) reported also low survival (<1%) of unfed larvae from fed broodstock. *O. edulis* is a larviparous species in which larvae undergo endo- and mixotrophic stages during the period of life in the pallial cavity of the mother oyster (Labarta et al., 1999). Exogenic feeding may start even inside the pallial cavity, as food particles were found in the digestive system of the larvae before larval release (Labarta et al., 1999). We hypothesize that the higher survival found in the present study in unfed larvae released by unfed broodstock may be explained by lower metabolic rate, and therefore energy consumption, compared to unfed larvae originated from fed broodstock. Unfed larvae from unfed broodstock had not been previously exposed to food in the pallial cavity of the brooding females and therefore digestive enzymes may have not been activated as a consequence of microalgal ingestion. In contrast, unfed larvae from fed broodstock may have started feeding in the pallial cavity of the females before they were released and, therefore, their digestive enzymes may have been activated. Our interpretation is that once they were released they had a higher metabolic activity, and because they were reared without any food supply, they depleted their stored reserves faster.

The differences in larval performance observed in our study may be explained by differences in biochemical composition of released larvae attributable to broodstock nutrition. Initial growth rate of newly released *O. edulis* larvae correlates well with neutral lipid content at liberation (Helm et al., 1973; Holland and Spencer, 1973). Triacylglycerols (TAG) are an important source of energy for bivalve

Table 6
Total polar fatty acids composition in *Ostrea edulis* larvae at day 9, obtained from broodstock previously fed *Rhodomonas salina* + *Chaetoceros neogracile* (Rs + C), and fed mono, bi and multi-specific diets expressed in relative contents (weight % of total neutral fatty acids ± S.D., $n = 2$). **T**: *Tisochrysis lutea*; **C**: *Chaetoceros neogracile*; **D**: *Diacronema lutheri*; **T + C**: *T. lutea* + *C. neogracile*; **T + D**: *T. lutea* + *D. lutheri*; **C + D**: *D. lutheri* + *C. neogracile*; **T + C + D**: *T. lutea* + *C. neogracile* + *D. lutheri*. Values with same letters in the same row are not significantly different at $P > 0.05$.

	Oyster larval diets						
Fatty acid	**T**	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
14:0	4.7 ± 0.2	2.8 ± 0.0	4.3 ± 3.4	2.9 ± 0.1	3.4 ± 0.1	2.6 ± 0.1	3.4 ± 0.2
16:0	14.7 ± 1.2	14.8 ± 0.1	16.1 ± 2.8	13.7 ± 0.3	14.4 ± 0.3	16.1 ± 0.2	15.8 ± 0.7
18:0	4.0 ± 0.5^{b}	4.7 ± 0.1^{ab}	5.2 ± 1.0^{a}	4.2 ± 0.1^{ab}	4.1 ± 0.4^{b}	5.0 ± 0.0^{ab}	4.3 ± 0.0^{ab}
18:1n-9	5.7 ± 0.4^{a}	0.7 ± 0.1^{d}	2.5 ± 2.0^{bc}	2.6 ± 0.1^{b}	3.3 ± 0.0^{ab}	0.9 ± 0.0^{cd}	2.4 ± 0.0^{bc}
18:1n-7	2.7 ± 0.0^{ab}	5.9 ± 0.1^{a}	2.2 ± 3.0^{b}	3.5 ± 0.1^{ab}	3.7 ± 0.0^{ab}	5.3 ± 0.1^{ab}	4.3 ± 0.3^{ab}
20:1n-7	2.9 ± 0.1^{c}	5.9 ± 0.0^{a}	3.2 ± 1.7^{bc}	4.8 ± 0.2^{ab}	4.3 ± 0.1^{abc}	5.8 ± 0.1^{a}	4.5 ± 0.5^{abc}
16:2n-6	0.9 ± 0.1^{a}	0.8 ± 0.1^{a}	0.0 ± 0.0^{b}	0.2 ± 0.3^{ab}	0.5 ± 0.2^{a}	0.0 ± 0.0^{b}	0.3 ± 0.4^{ab}
16:2n-4	0.0 ± 0.0^{b}	0.5 ± 0.0^{a}	0.1 ± 0.1^{b}	0.3 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
16:3n-4	0.0 ± 0.0^{b}	0.4 ± 0.0^{a}	0.0 ± 0.0^{b}	0.3 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.1 ± 0.1^{b}
18:2n-6	6.8 ± 0.1^{a}	0.2 ± 0.0^{c}	0.0 ± 0.0^{c}	4.2 ± 0.0^{ab}	4.3 ± 0.1^{ab}	0.5 ± 0.8^{c}	3.5 ± 0.1^{b}
18:3n-3	2.6 ± 0.1^{a}	0.4 ± 0.0^{d}	0.4 ± 0.0^{d}	1.5 ± 0.1^{b}	1.5 ± 0.1^{b}	0.4 ± 0.0^{d}	1.2 ± 0.1^{c}
18:4n-3	3.0 ± 0.1^{a}	0.4 ± 0.0^{e}	0.5 ± 0.2^{de}	1.4 ± 0.0^{bc}	1.6 ± 0.1^{b}	0.6 ± 0.1^{d}	1.2 ± 0.0^{c}
20:2NMI	0.0 ± 0.0^{c}	1.1 ± 0.0^{ab}	3.2 ± 3.0^{a}	0.8 ± 0.0^{abc}	0.9 ± 0.1^{abc}	1.2 ± 0.1^{ab}	0.4 ± 0.6^{bc}
20:4n-6	3.0 ± 0.2	2.7 ± 0.0	2.3 ± 1.1	2.4 ± 0.2	2.7 ± 0.1	2.8 ± 0.1	2.5 ± 0.0
20:4n-3	0.3 ± 0.0^{a}	0.1 ± 0.1^{ab}	0.1 ± 0.1^{ab}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.0 ± 0.0^{b}	0.1 ± 0.1^{ab}
20:5n-3	4.4 ± 0.2^{d}	28.2 ± 0.2^{a}	10.8 ± 4.8^{c}	16.2 ± 0.5^{b}	10.4 ± 0.4^{c}	23.2 ± 0.7^{a}	16.4 ± 0.6^{b}
22:2NMI	5.6 ± 0.4^{b}	8.3 ± 0.1^{a}	7.1 ± 2.2^{ab}	7.5 ± 0.4^{ab}	7.4 ± 0.3^{ab}	8.7 ± 0.1^{a}	7.4 ± 0.3^{ab}
22:5n-6	4.5 ± 0.3^{a}	0.1 ± 0.1^{e}	1.6 ± 0.7^{cd}	2.8 ± 0.1^{b}	3.4 ± 0.2^{ab}	1.0 ± 0.0^{d}	2.5 ± 0.0^{bc}
22:5n-3	0.5 ± 0.0^{e}	1.4 ± 0.0^{a}	1.0 ± 0.3^{bc}	0.8 ± 0.0^{d}	0.6 ± 0.0^{de}	1.1 ± 0.0^{b}	0.8 ± 0.0^{cd}
22:6n-3	17.9 ± 1.3^{a}	3.9 ± 0.0^{d}	10.3 ± 3.8^{bc}	12.1 ± 0.2^{bc}	17.4 ± 0.1^{a}	9.1 ± 0.2^{c}	13.8 ± 0.4^{ab}
∑ SFA	23.4 ± 2.0	22.3 ± 0.0	25.6 ± 7.2	20.8 ± 0.2	21.9 ± 0.8	23.7 ± 0.3	23.5 ± 0.9
∑ MUFA	11.3 ± 0.5	12.4 ± 0.0	10.0 ± 4.6	12.7 ± 2.3	11.4 ± 0.1	12.1 ± 0.2	11.3 ± 0.2
∑ n-9	5.7 ± 0.4^{a}	0.7 ± 0.1^{d}	2.5 ± 2.0^{bc}	2.6 ± 0.1^{b}	3.3 ± 0.0^{ab}	0.9 ± 0.0^{cd}	2.4 ± 0.0^{bc}
∑ n-7	5.6 ± 0.1	11.8 ± 0.1	7.5 ± 6.6	10.2 ± 2.3	8.0 ± 0.1	11.2 ± 0.2	8.9 ± 0.2
∑ PUFA	49.5 ± 2.3^{a}	48.6 ± 0.1^{a}	37.3 ± 9.6^{b}	50.8 ± 1.8^{a}	51.0 ± 0.9^{a}	48.6 ± 0.4^{a}	50.2 ± 0.5^{a}
∑ n-4	0.0 ± 0.0^{b}	1.0 ± 0.0^{a}	0.1 ± 0.1^{b}	0.6 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.1 ± 0.1^{b}
∑ n-6	15.2 ± 0.5^{a}	3.7 ± 0.1^{c}	3.9 ± 1.8^{c}	9.6 ± 0.6^{b}	11.0 ± 0.6^{b}	4.4 ± 0.8^{c}	8.8 ± 0.5^{b}
∑ n-3	28.6 ± 1.5^{ab}	34.4 ± 0.2^{a}	23.0 ± 8.7^{b}	32.3 ± 0.7^{a}	31.7 ± 0.5^{a}	34.4 ± 1.0^{a}	33.5 ± 1.1^{a}
∑ NMI	5.6 ± 0.4^{d}	9.5 ± 0.1^{ab}	10.3 ± 0.8^{a}	8.3 ± 0.5^{bc}	8.2 ± 0.2^{bc}	9.8 ± 0.2^{a}	7.8 ± 0.9^{c}
n-3/n-6	1.9 ± 0.0^{d}	9.2 ± 0.1^{a}	6.0 ± 0.6^{b}	3.4 ± 0.1^{c}	2.9 ± 0.1^{c}	8.0 ± 1.7^{a}	3.8 ± 0.1^{c}
22:6/20:5	4.1 ± 0.1^{a}	0.1 ± 0.0^{g}	1.0 ± 0.1^{c}	0.7 ± 0.0^{e}	1.7 ± 0.1^{b}	0.4 ± 0.0^{f}	0.8 ± 0.0^{d}

Table 6 (*continued*)

	Oyster larval diets						
Fatty acid	**T**	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
22:5/20:4	1.5 ± 0.0^{a}	0.0 ± 0.0^{e}	0.7 ± 0.0^{c}	1.2 ± 0.0^{b}	1.3 ± 0.0^{ab}	0.4 ± 0.0^{d}	1.0 ± 0.0^{b}

Σ SFA, Total saturated fatty acids; Σ MUFA, Total monounsaturated fatty acids; Σ PUFA, Total polyunsaturated fatty acids; Σ NMI, Total non-methylene-interrupted fatty acids.

larvae, especially in periods of low food availability or during starvation (Ben Kheder et al., 2010a, 2010b; Gallager et al., 1986). As aforementioned, larvae released from unfed broodstock showed lower TFA content than larvae released from broodstock fed Rs + C and higher TFA content than larvae originated from broodstock fed Rs + Tw. We may expect a lower supply of TAG in released larvae originating from unfed parents. In the present study, we quantified neither total lipid reserves nor neutral lipid classes; however, certain fatty acids, such as SFA and 20:5n-3 have roles as energy sources to fuel embryogenesis and larval development (da Costa et al., 2012; Whyte et al., 1990, 1991). Our data showed that *O. edulis* larvae from unfed broodstock had a lower proportion of 20:5n-3 and total SFA in NL and lower proportion of 20:5n-3 in PL than larvae released from parents fed microalgal mixtures. Accordingly, we hypothesize that reduced energy supply in the form of reserve lipids may have a negative effect upon *O. edulis* larval survival and metamorphosis success. Larvae from unfed parents showed higher levels of 22:6n-3 in NL and PL than larvae from fed broodstock. These differences in PUFA profiles in NL and PL of larvae at release may be explained by differential parental nutrition. Helm et al. (1991) suggested that PUFAs in PL of larvae originated from fatty acids stored by parents prior to or during early stages of oogenesis; whereas, PUFAs in NL of larvae reflected broodstock diet during later stages of oocyte development. Differences in levels of 20:5n-3 and 22:6n-3 may have a profound effect upon the development of larvae from *O. edulis* broodstock (Helm et al., 1991). In fact, it was demonstrated that levels of 22:6n-3 and total PUFA in *O. edulis* larvae at release are positively related to growth rate (Berntsson et al., 1997). These authors also suggested the essentiality of 20:5n-3 as the combination of it with 22:6n-3 explained 90% of the relationship between PUFA and growth rate.

Unfed broodstock released larvae with higher levels of NMI FA in NL compared to fed broodstock. NMI FA might have been synthetized de novo from desaturation and elongation of 18:1n-9 and 16:1n-7 (Barnathan, 2009; da Costa et al., 2015; Zhukova, 1991). The biological function of NMI FA is unclear (Barnathan, 2009), although accumulation in PL suggests that NMI FA have structural and functional roles in membranes (Paradis and Ackman, 1977; Pirini et al., 2007). NMI FA have an unusual unsaturation pattern that confers upon cell membranes higher resistance to oxidative processes and microbial lipases than other PUFAs (Barnathan, 2009). Generally, these compounds increased in membranes (polar lipids) of starved bivalves (Caers et al., 1999; Thompson and Harrison, 1992; Ventrella et al., 2013). Strikingly, NMI FA were also found in high proportion in NL of *O. edulis* larvae released from unfed broodstock. Larvae of the grooved razor shell *Solen marginatus* also stored high quantities of NMI FA in NL and PL (da Costa et al., 2011). MNI FA in NL of bivalve larvae may serve as temporary reservoirs for latter transfer of NMI FA to PL (da Costa et al., 2011, 2012). Our results suggest that NMI FA seemed to be synthetized in addition to PUFA, as in previous studies in clams (Caers et al., 1999; Fernández-Reiriz et al., 2017).

Larvae from broodstock fed the diet Rs + C exhibited higher growth rate, larval survival, competence, and settlement rate than larvae from broodstock fed Rs + Tw. Larvae from parents fed Rs + C contained a higher percentage of 20:5n-3 and PUFA in NL and showed higher percentages of 20:4n-6, 20:5n-3, and cholesterol, and less 22:6n-3, in membranes than larvae from parents fed Rs + Tw. The 20:5n-3 FA fulfils

Table 7
Total polar fatty acids composition in *Ostrea edulis* larvae at day 9, obtained from broodstock previously fed *Rhodomonas salina* + *Thalassiosira weissflogii* (Rs + Tw), and fed mono, bi and multi-specific diets expressed in relative contents (weight % of total neutral fatty acids ± S.D., n = 2). **T**: *Tisochrysis lutea*; **C**: *Chaetoceros neogracile*; **D**: *Diacronema lutheri*; **T + C**: *T. lutea* + *C. neogracile*; **T + D**: *T. lutea* + *D. lutheri*; **C + D**: *C. neogracile* + *D. lutheri*; **T + C + D**: *T. lutea* + *C. neogracile* + *D. lutheri*. Values with same letters in the same row are not significantly different at $P > 0.05$.

	Oyster larval diets						
Fatty acid	**T**	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
14:0	3.3 ± 0.1^{ab}	3.5 ± 0.1^{ab}	2.5 ± 0.1^{c}	3.4 ± 0.0^{ab}	3.4 ± 0.1^{ab}	3.2 ± 0.1^{ab}	3.7 ± 0.1^{a}
16:0	15.9 ± 1.5^{c}	13.9 ± 0.8^{ab}	17.2 ± 1.3^{a}	14.2 ± 0.1^{bc}	15.2 ± 0.5^{bc}	16.7 ± 0.6^{a}	15.2 ± 0.6^{abc}
18:0	3.9 ± 0.3	4.6 ± 0.1	4.2 ± 0.4	4.3 ± 0.3	4.8 ± 0.4	5.0 ± 1.0	4.0 ± 0.1
16:1n-7	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	2.9 ± 0.2^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	1.3 ± 1.9^{ab}
18:1n-9	3.4 ± 1.2^{a}	0.8 ± 0.0^{b}	1.1 ± 0.0^{b}	2.6 ± 0.5^{a}	2.6 ± 0.5^{a}	1.3 ± 0.5^{b}	2.6 ± 0.1^{a}
18:1n-7	3.1 ± 1.3^{b}	6.0 ± 0.1^{a}	5.0 ± 0.4^{a}	3.5 ± 0.1^{b}	3.5 ± 0.1^{b}	5.7 ± 0.5^{a}	4.4 ± 0.2^{ab}
20:1n-7	3.7 ± 0.3^{d}	4.7 ± 0.4^{ab}	3.8 ± 0.3^{cd}	4.1 ± 0.3^{bcd}	4.3 ± 0.3^{abc}	4.8 ± 0.1^{a}	4.4 ± 0.2^{abc}
16:2n-6	0.6 ± 0.1^{a}	0.8 ± 0.2^{a}	0.8 ± 0.1^{a}	0.0 ± 0.0^{c}	0.4 ± 0.0^{ab}	0.6 ± 0.1^{a}	0.2 ± 0.3^{bc}
18:2n-6	4.4 ± 2.1^{a}	0.0 ± 0.0^{cd}	1.4 ± 0.1^{bc}	3.7 ± 0.2^{ab}	3.8 ± 0.0^{ab}	0.7 ± 0.9^{cd}	3.9 ± 0.2^{ab}
18:3n-3	2.0 ± 0.6^{a}	0.7 ± 0.1^{c}	0.6 ± 0.1^{c}	1.4 ± 0.1^{ab}	1.4 ± 0.1^{ab}	0.7 ± 0.2^{c}	1.3 ± 0.0^{b}
18:4n-3	1.8 ± 0.7^{a}	0.3 ± 0.0^{c}	0.7 ± 0.0^{b}	1.4 ± 0.2^{a}	1.4 ± 0.2^{a}	0.6 ± 0.1^{bc}	1.4 ± 0.0^{a}
20:2NMI	0.7 ± 0.1	0.9 ± 0.2	0.6 ± 0.6	0.8 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.2
20:4n-6	2.7 ± 0.1^{a}	2.5 ± 0.2^{ab}	2.2 ± 0.0^{c}	2.2 ± 0.1^{c}	2.4 ± 0.2^{bc}	2.3 ± 0.0^{bc}	2.3 ± 0.0^{bc}
20:4n-3	0.2 ± 0.1^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.0 ± 0.0^{b}	0.1 ± 0.0^{a}
20:5n-3	9.7 ± 8.3^{c}	27.7 ± 0.4^{a}	18.4 ± 0.9^{ab}	13.1 ± 2.3^{bc}	14.0 ± 2.7^{bc}	21.9 ± 1.5^{ab}	15.6 ± 0.3^{bc}
22:2NMI	7.8 ± 0.2^{c}	8.7 ± 0.9^{ab}	8.8 ± 0.0^{ab}	8.0 ± 0.3^{bc}	8.0 ± 0.3^{bc}	9.1 ± 0.2^{a}	7.6 ± 0.0^{c}
22:5n-6	3.6 ± 1.7^{a}	0.0 ± 0.0^{d}	1.9 ± 0.0^{bc}	2.8 ± 0.1^{ab}	2.9 ± 0.2^{ab}	1.0 ± 0.1^{c}	2.6 ± 0.0^{ab}
22:5n-3	0.7 ± 0.3^{b}	1.4 ± 0.0^{a}	0.9 ± 0.1^{b}	0.7 ± 0.1^{b}	0.7 ± 0.1^{b}	1.0 ± 0.0^{ab}	0.7 ± 0.0^{b}
22:6n-3	16.4 ± 5.5^{a}	4.9 ± 0.3^{c}	14.7 ± 0.1^{ab}	14.8 ± 1.7^{ab}	15.5 ± 1.9^{a}	10.2 ± 0.5^{b}	13.8 ± 0.5^{ab}
∑ SFA	20.5 ± 1.9^{c}	23.9 ± 0.8^{ab}	24.0 ± 1.1^{ab}	21.6 ± 0.2^{bc}	22.3 ± 1.0^{abc}	25.0 ± 1.7^{a}	22.9 ± 0.8^{abc}
∑ MUFA	13.6 ± 0.3^{cd}	14.6 ± 0.5^{bcd}	12.5 ± 0.1^{d}	16.1 ± 0.4^{a}	13.6 ± 0.6^{cd}	14.6 ± 0.0^{bc}	15.4 ± 1.9^{ab}
∑ n-9	4.9 ± 1.1^{a}	2.5 ± 0.1^{b}	1.3 ± 0.3^{b}	3.7 ± 0.3^{a}	3.2 ± 0.6^{a}	2.3 ± 0.5^{b}	3.1 ± 0.1^{a}
∑ n-7	6.8 ± 1.7^{c}	10.7 ± 0.5^{a}	8.9 ± 0.1^{abc}	10.5 ± 0.5^{a}	7.8 ± 0.3^{bc}	10.5 ± 0.6^{a}	10.2 ± 1.9^{ab}
∑ PUFA	55.0 ± 1.8^{ab}	52.0 ± 1.2^{b}	54.4 ± 0.1^{ab}	54.6 ± 0.1^{ab}	56.2 ± 0.5^{a}	53.4 ± 1.7^{ab}	56.0 ± 0.7^{ab}
∑ n-4	0.3 ± 0.4^{ab}	1.1 ± 0.0^{a}	0.0 ± 0.0^{b}	0.5 ± 0.7^{ab}	0.4 ± 0.5^{ab}	0.9 ± 0.0^{a}	0.5 ± 0.2^{ab}
∑ n-6	13.2 ± 4.0^{a}	4.8 ± 0.2^{d}	7.8 ± 0.1^{bc}	10.2 ± 0.4^{ab}	11.1 ± 0.1^{a}	5.6 ± 0.5^{cd}	10.6 ± 0.6^{ab}
∑ n-3	33.0 ± 1.5^{c}	36.5 ± 0.4^{a}	37.2 ± 0.4^{a}	34.4 ± 0.5^{bc}	35.9 ± 0.2^{abc}	36.7 ± 1.9^{ab}	36.1 ± 0.3^{abc}
∑ NMI							

Table 7 (*continued*)

	Oyster larval diets						
Fatty acid	**T**	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
n-3/n-6	8.5 ± 0.3^{c}	9.6 ± 0.7^{ab}	9.4 ± 0.6^{abc}	8.8 ± 0.4^{bc}	8.8 ± 0.3^{bc}	10.2 ± 0.2^{a}	8.6 ± 0.2^{c}
22:6/20:5	3.4 ± 2.0^{c}	7.6 ± 0.2^{a}	4.8 ± 0.0^{b}	3.4 ± 0.2^{c}	3.2 ± 0.0^{c}	6.6 ± 0.9^{b}	3.4 ± 0.2^{c}
22:5/20:4	4.0 ± 1.8^{a}	0.2 ± 0.0^{b}	0.8 ± 0.0^{ab}	1.2 ± 0.3^{ab}	1.1 ± 0.4^{ab}	0.5 ± 0.0^{b}	0.9 ± 0.0^{ab}
	1.3 ± 0.6^{a}	0.0 ± 0.0^{c}	0.9 ± 0.0^{a}	1.3 ± 0.1^{a}	1.2 ± 0.2^{a}	0.4 ± 0.0^{b}	1.1 ± 0.0^{a}

Σ SFA, Total saturated fatty acids; Σ MUFA, Total monounsaturated fatty acids; Σ PUFA, Total polyunsaturated fatty acids; Σ NMI, Total non-methylene-interrupted fatty acids.

a role as both an energy source and as a precursor of eicosanoids (Whyte et al., 1990, 1991; Marty et al., 1992). The higher 20:5n-3 content in stored reserves of newly-released larvae may provide a higher initial energy supply, and this may explain the higher performance of larvae from broodstock fed Rs + C. The observed differences in larval performance between broodstock fed Rs + C and Rs + Tw may be explained by the higher cholesterol content in larvae produced by parents receiving *C. neogracile* in the diet. *O. edulis* larvae from broodstock fed Rs + C exhibited higher cholesterol content (1.1 ng larva^{-1}) than larvae released by broodstock fed Rs + Tw or starved (0.4–0.5 ng larva^{-1}). Cholesterol content in released larvae was maintained at a certain level when cholesterol dietary supply to broodstock was low, as in broodstock fed Rs + Tw, or absent, as in unfed broodstock. This may be explained by the essentiality of this sterol for molluscs during early phases of development and gametogenesis, as previously suggested by Soudant et al. (1996). These authors found that cholesterol level in female gonads of *Pecten maximus* maintained a certain level, even when dietary supply was low, as when scallops were fed *T. lutea* (Soudant et al., 1996).

4.2. Effects of larval diets

Larval performance (i.e., growth rate, survival, larval competence, and settlement) was also affected by larval diets. *D. lutheri* led to the lowest *O. edulis* larval performance (growth and metamorphosis) when used as single diet and had no additive food value when associated to other microalgae (*T. lutea* and/or *C. neogracile*). These results agree with those reported by Rico-Villa et al. (2006) for the Pacific oyster *M. gigas* larvae fed mono- and bi-specific diets including *D. lutheri*. In contrast, Ferreiro et al. (1990) reported good growth and high percentage of eyespot in larvae released from broodstock matured in a raft in wild conditions when fed a unialgal diet of *D. lutheri*. The same authors, in a second trial using hatchery-conditioned broodstock, observed low performance of larvae fed *D. lutheri*. This may suggest that the high growth and morphological competence observed in the first experiment was attributable to higher nutritional quality of larvae released by wild broodstock. Jonsson et al. (1999) reported a growth rate of *O. edulis* larvae fed *D. lutheri* of 6.4 μm day^{-1} in a 12-day larval-rearing experiment, which was higher than what we observed for larvae fed *D. lutheri* originating from fed broodstock (4.6–5.6 μm day^{-1}). In other bivalve groups, such as scallops, *D. lutheri* has been successfully used, e.g., with the King scallop *Pecten maximus* (Delaunay et al., 1993) or the Australian scallop *Pecten fumatus* larvae (Heasman et al., 1996).

When used as single diet, the diatom *C. neogracile* led to generally better performance of larvae (growth and metamorphosis) than the single diet *T. lutea*. This was also observed in the growth, survival and competence of *O. edulis* larvae fed *C. neogracile*, which showed nearly the same results as when combined with *T. lutea* (González-Araya et al., 2012a). No differences in *O. edulis* larval growth were found when larvae were fed single *T. lutea* or *C. neogracile* or a mixed diet containing *T. lutea* and *C. neogracile*, when larval source was broodstock previously fed

Table 8
Main relative sterol composition in *Ostrea edulis* larvae at day 9 obtained from broodstock previously fed *Rhodomonas salina* + *Chaetoceros neogracile* (Rs + C), and fed mono-, bi- and pluri-specific diets, expressed in mean relative contents (weight % of total sterols ± S.D., n = 2). **T**: *Tisochrysis lutea*; **C**: *Chaetoceros neogracile*; **D**: *Diacronema lutheri*; **T + C**: *T. lutea* + *C. neogracile*; **T + D**: *T. lutea* + *D. lutheri*; **C + D**: *D. lutheri* + *C. neogracile*; **T + C + D**: *T. lutea* + *C. neogracile* + *D. lutheri*. Values with same letters in the same row are not significantly different at $P > 0.05$.

	Oyster larval diets						
	T	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
Cholesterol	16.4 ± 0.6^{e}	58.8 ± 15.5^{a}	24.4 ± 2.2^{de}	49.4 ± 0.0^{ab}	16.9 ± 0.1^{e}	40.7 ± 0.6^{bc}	31.7 ± 0.2^{cd}
Brassicasterol	69.1 ± 0.9^{a}	4.3 ± 0.1^{e}	5.4 ± 1.9^{e}	32.0 ± 0.1^{b}	22.6 ± 0.4^{c}	3.8 ± 0.2^{e}	16.9 ± 0.4^{d}
Desmosterol	4.8 ± 0.5^{b}	5.8 ± 0.8^{ab}	5.6 ± 1.9^{ab}	7.6 ± 1.1^{a}	4.4 ± 0.2^{b}	5.5 ± 0.2^{ab}	4.9 ± 0.1^{b}
Campesterol	3.1 ± 1.0^{c}	5.3 ± 2.4^{bc}	10.5 ± 0.3^{a}	0.5 ± 0.6^{d}	9.1 ± 0.2^{ab}	7.6 ± 0.1^{ab}	7.1 ± 0.1^{ab}
24-methylene cholesterol	2.1 ± 0.0^{a}	2.7 ± 1.3^{a}	0.0 ± 0.0^{b}	2.8 ± 0.1^{a}	2.4 ± 0.0^{a}	2.7 ± 0.0^{a}	2.8 ± 0.1^{a}
Stigmasterol	4.5 ± 0.0^{d}	0.9 ± 0.1^{f}	21.7 ± 1.0^{a}	1.3 ± 0.0^{e}	18.7 ± 0.4^{b}	15.2 ± 0.8^{c}	14.2 ± 0.0^{c}
4α-methylporiferasterol	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	3.4 ± 0.0^{a}	0.0 ± 0.0^{c}	2.1 ± 0.1^{b}	2.0 ± 0.0^{b}	2.1 ± 0.1^{b}
β-sitosterol	0.0 ± 0.0^{e}	1.0 ± 0.0^{d}	30.7 ± 3.5^{a}	1.7 ± 0.0^{d}	21.6 ± 0.4^{b}	19.4 ± 0.2^{b}	16.5 ± 0.1^{c}
Fucosterol	0.0 ± 0.0^{c}	7.1 ± 3.4^{a}	0.0 ± 0.0^{c}	4.0 ± 0.3^{b}	2.2 ± 0.1^{b}	3.2 ± 0.1^{b}	3.3 ± 0.2^{b}
Isofucosterol	0.0 ± 0.0^{b}	0.7 ± 0.3^{a}	0.0 ± 0.0^{b}	0.7 ± 0.2^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.4 ± 0.6^{a}
Methylpavlovol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ethylpavlovol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total (ng larva^{-1})	4.7 ± 0.4^{e}	12.0 ± 0.4^{b}	2.7 ± 0.4^{f}	14.2 ± 0.2^{a}	9.3 ± 0.2^{c}	7.0 ± 1.4^{d}	9.3 ± 1.8^{c}

Table 9
Main relative sterol composition in *Ostrea edulis* larvae at day 9 obtained from broodstock previously fed *Rhodomonas salina* + *Thalassiosira weissflogii* (Rs + Tw), and fed mono, bi and multi-specific diets expressed in relative contents (weight % of total neutral fatty acids ± S.D., n = 2). **T**: *Tisochrysis lutea*; **C**: *Chaetoceros neogracile*; **D**: *Diacronema lutheri*; **T + C**: *T. lutea* + *C. neogracile*; **T + D**: *T. lutea* + *D. lutheri*; **C + D**: *C. neogracile* + *D. lutheri*; **T + C + D**: *T. lutea* + *C. neogracile* + *D. lutheri*. Values with same letters in the same row are not significantly different at $P > 0.05$.

	Oyster larval diets						
	T	**C**	**D**	**T + C**	**T + D**	**C + D**	**T + C + D**
Cholesterol	11.8 ± 0.2^{f}	69.9 ± 2.2^{a}	13.3 ± 0.5^{f}	48.2 ± 0.2^{b}	19.3 ± 0.0^{e}	36.6 ± 0.4^{c}	27.6 ± 0.1^{d}
Brassicasterol	78.3 ± 0.5^{a}	5.4 ± 0.6^{e}	5.1 ± 0.3^{e}	32.8 ± 0.1^{b}	23.7 ± 0.0^{c}	4.7 ± 0.1^{e}	19.8 ± 0.2^{d}
Desmosterol	2.1 ± 0.4^{c}	6.0 ± 0.1^{a}	4.2 ± 0.3^{b}	5.5 ± 0.2^{a}	4.1 ± 0.0^{b}	5.7 ± 0.2^{a}	4.7 ± 0.0^{b}
Campesterol	2.6 ± 0.4^{d}	1.8 ± 0.0^{e}	12.4 ± 0.1^{a}	2.1 ± 0.4^{de}	9.1 ± 0.0^{b}	7.5 ± 0.1^{c}	7.7 ± 0.1^{c}
24-methylene cholesterol	2.4 ± 0.2^{d}	5.7 ± 1.0^{a}	3.4 ± 0.1^{bc}	3.7 ± 0.1^{bc}	2.9 ± 0.0^{cd}	4.0 ± 0.0^{b}	3.2 ± 0.0^{bc}
Stigmasterol	2.7 ± 0.2^{e}	0.0 ± 0.0^{g}	18.8 ± 0.2^{a}	1.6 ± 0.1^{f}	15.5 ± 0.0^{b}	12.8 ± 0.7^{d}	13.9 ± 0.2^{c}
4α-methylporiferasterol	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	3.0 ± 0.4^{a}	0.0 ± 0.0^{d}	2.5 ± 0.0^{b}	2.1 ± 0.1^{bc}	2.2 ± 0.1^{bc}
β-sitosterol	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	37.6 ± 0.6^{a}	1.3 ± 0.2^{d}	20.8 ± 0.0^{b}	20.2 ± 0.2^{b}	18.1 ± 0.1^{c}
Fucosterol	0.0 ± 0.0^{e}	10.6 ± 0.1^{a}	2.2 ± 0.6^{d}	4.7 ± 0.1^{b}	2.0 ± 0.0^{d}	5.3 ± 0.0^{b}	2.8 ± 0.0^{c}
Isofucosterol	0.0 ± 0.0^{b}	0.5 ± 0.8^{ab}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	1.1 ± 0.1^{a}	0.0 ± 0.0^{b}
Methylpavlovol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ethylpavlovol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total (ng larva^{-1})	7.4 ± 1.6^{cd}	8.0 ± 1.0^{cd}	5.0 ± 0.1^{e}	11.0 ± 1.1^{ab}	5.7 ± 0.0^{de}	9.0 ± 0.8^{bc}	11.8 ± 0.2^{a}

T. lutea or *C. neogracile* (González-Araya and Robert, 2018). In contrast, Rico-Villa et al. (2006) reported that the combination of *T. lutea* with *C. calcitrans* f. *pumilum* yielded better larval performance results in *M. gigas* than the diatom alone. In contrast, other authors reported negative effects of *C. calcitrans* upon *O. edulis* larval growth (Jonsson et al., 1999).

Each microalga that was tested in larval diets exhibited distinctive FA and sterol profiles. For example, *T. lutea* was rich in 22:6n-3 and brassicasterol, *C. neogracile* contained mainly 20:5n-3 and cholesterol, and *D. lutheri* supplied 20:5n-3, and to a lesser extent 22:6n-3, and methylpavlovol and ethylpavlovol. FA and sterol compositions of the larvae were influenced by larval diet supplied, as can be observed in PCA plots. The PUFAs 20:5n-3 and 22:6n-3 are implicated as essential FAs for oysters, as previously reported by Langdon and Waldock (1981). In the present work, a high percentage of 20:5n-3 in NL of the larvae at day 9 was related to high growth of the larvae and settlement success. González-Araya and Robert (2018) found a positive correlation between 20:5n-3 absolute content in larvae at day 11 with growth rate for mono-specific larval diets. The EFA 20:5n-3 has a role as an energy source to fuel embryogenesis and larval development (da Costa et al., 2012; Whyte et al., 1990, 1991). Accordingly, the supply of 20:5n-3 by dietary *C. neogracile* and/or *D. lutheri* may have contributed to the energy necessary for larval growth and to fuel metamorphosis. Surprisingly, despite the importance of 22:6n-3 in membrane structure, no relationship was found with this FA and larval performance indicators, as has been reported in previous studies (Berntsson et al., 1997; González-Araya and Robert, 2018). This may be related to constant, ideal temperature conditions under which larvae were reared.

The PUFA 20:4n-6 plays important roles in eicosanoid production and stress response in invertebrate species (Howard and Stanley, 1999). In the present study, a multiple regression analysis showed that the relative content of 20:4n-6 in PL of the larvae at day 9 exhibited a negative correlation with growth rate and settlement of *O. edulis* larvae, but in contrast a positive link with competence.

The stepwise multiple regression identified the important roles of n-7 MUFA in NL and PL of the larvae at day 9 in this study. The 18:1n-7 in NL contributed significantly with a negative coefficient to explain the variation in growth rate, competence, and settlement rate models. This disagrees with the positive correlation of 18:1n-7 found in *M. gigas* larvae (da Costa et al., 2016). Surprisingly, 16:1n-7 and 20:1n-7 in NL were positively correlated with larval competence. A higher proportion of 20:1n-7 in PL was associated with an increase in growth and settlement rates of *O. edulis* larvae. The positive correlation observed for 20:1n-7 in NL and PL is supported by the results reported by da Costa et al. (2016), who found a positive correlation between this FA relative content in NL and PL with *M. gigas* larval growth and survival. Our findings may confirm important energetic or structural roles of 20:1n-7 suggested by Sargent and Whittle (1981) and Ackman (1983). These three n-7 MUFA belong to the same biosynthetic pathway and may serve as precursors for the j series of NMI FAs. Thus, the larvae may have

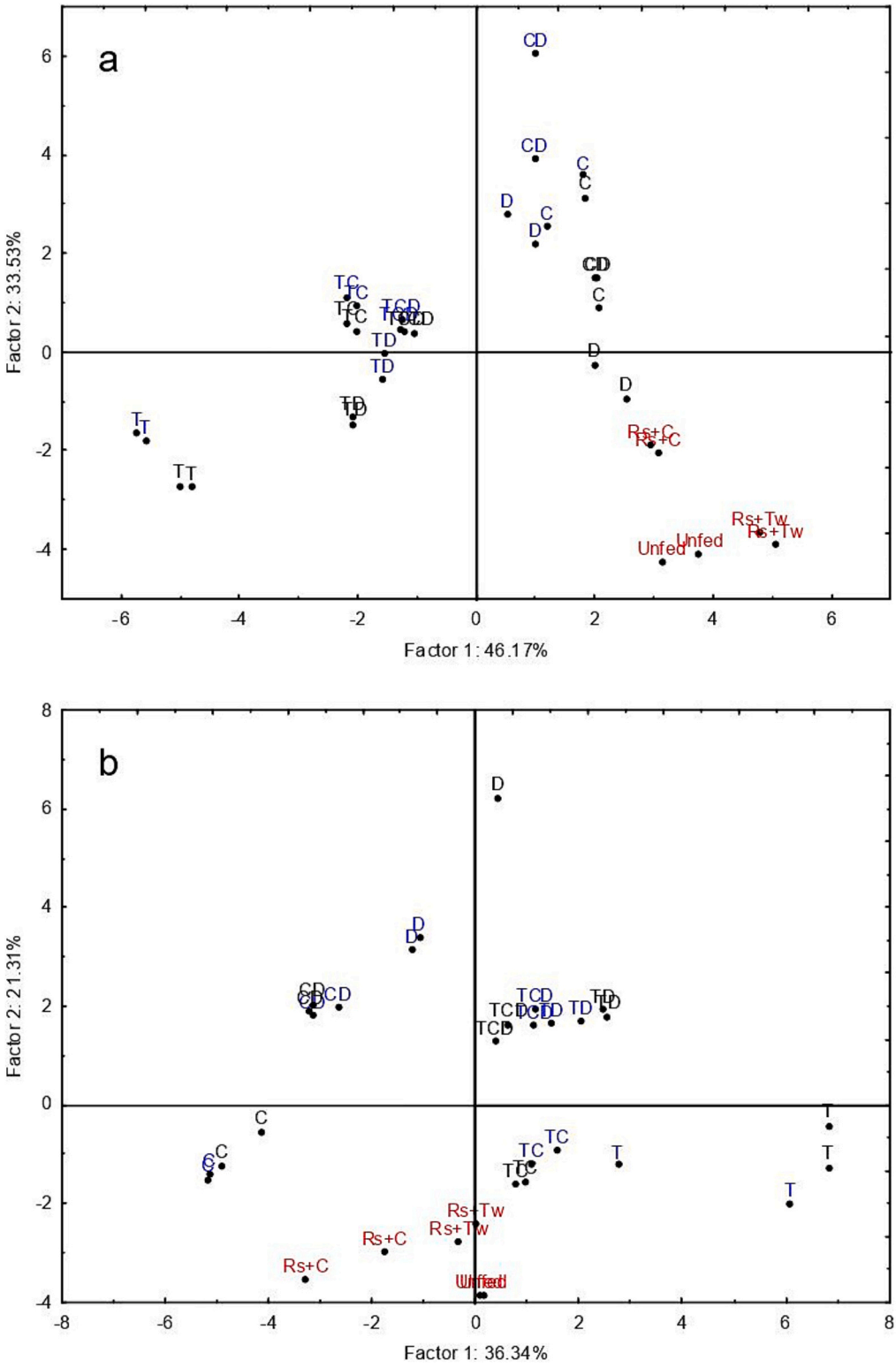

Fig. 4. Factorial plan of PCA plot of larval composition of newly released larvae obtained from broodstock subject to diets (marqued in red; *Rhodomonas salina* + *Thalassiosira weissflogii* (Rs + Tw), *Rhodomonas salina* + *Chaetoceros neogracile* (Rs + C) and unfed) and 9-day-old larvae originated from broodstock fed Rs + Tw (in blue) and Rs + C (in black) subject to different mono-, bi- and pluri-specific larval diets containing **T**: *Tisochrysis lutea*; **C**: *Chaetoceros neogracile* and **D**: *Diacronema lutheri*. a. Reserve lipids (FA in NL). b. Membrane lipids (FA in PL and sterols). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

regulated by unknown internal and external factors the bioconversion of the large quantities of 16:1n-7 supplied with the diet by *D. lutheri* and *C. neogracile*.

It is well known that sterols play a variety of roles in living organisms, acting as structural components of cell membranes, steroid hormones, and vitamin D precursors (Soudant et al., 2000). In our study, the relative sterol composition of 9-day-old larvae was significantly influenced by larval diet. For instance, *O. edulis* larvae fed T + C accumulated more cholesterol (48–49%) than brassicasterol (32–33%) on day 9. Cholesterol was mainly supplied by *C. neogracile*; whereas, *T. lutea* provided only brassicasterol. Cholesterol is considered to be an essential sterol for molluscs (Soudant et al., 1996). In the present study, a positive effect of cholesterol was observed in competence in the multiple linear regression. In *M. gigas* larvae, cholesterol was positively correlated with survival (da Costa et al., 2016).

The results of the multiple linear regression showed a negative effect

of campesterol in the predictive model of growth rate and negative effects of stigmasterol and 24-methylene-cholesterol in the settlement model. These negative effects may be related to the low performance the larvae receiving mono- and bi-specific diets containing *D. lutheri*, which supplied large proportions of stigmasterol (16%) and minor relative contents of campesterol (3%) that were found in larval membranes.

The sterol profile of *D. lutheri* included methylpavlovol and ethylpavlovol, which are specific to pavlovophytes (Véron et al., 1996). Surprisingly, methylpavlovol and ethylpavlovol were not incorporated at all by *O. edulis* larvae after 9 days of feeding and 4α-methyl poriferasterol was poorly incorporated. Methylpavlovol and ethylpavlovol were not accumulated in *O. edulis* broodstock tissues when *D. lutheri* was fed as a mono-specific diet (González-Araya et al., 2012b). Soudant et al. (1998) also observed low incorporation of methylpavlovol, ethylpavlovol and 4α-methyl poriferasterol by *Pecten maximus* larvae fed a mixed diet including *D. lutheri*. These authors suggested that *P. maximus* larvae may negatively select these sterols by a putative mechanism of structural recognition. The sterols (methylpavlovol, ethylpavlovol and 4α-methyl poriferasterol) have a methyl group in C4 and lack a double bond in C5 (Soudant et al., 1998). These structural features in common in these sterols may explain rejection by *O. edulis* larvae. On the contrary, the preferential accumulation of stigmasterol and β-sitosterol by *O. edulis* larvae fed diets containing *D. lutheri* indicated that this alga was ingested and digested.

5. Conclusions

This study highlights the importance of both broodstock and larval microalgal diets in *O. edulis* larval performance in terms of larval growth, survival, competence, and settlement rate. *O. edulis* broodstock fed a mixed diet composed of *R. salina* + *C. neogracile* (Rs + C) exhibited higher fecundity than broodstock receiving the other mixed diet or starved. The larvae released by each broodstock group differed in fatty acid composition in NL and PL and in sterols. In general, larvae from broodstock fed Rs + C yielded better growth, survival, competence, and settlement rate. Larval diets significantly affected fatty acid and sterol composition of larvae on day 9. The best results were observed in larvae fed T + C from broodstock fed Rs + C, which suggests the importance of including the diatom *C. neogracile* in broodstock and larval diets as a source of the essential fatty acid 20:5n-3 (EPA, eicosapentaenoic fatty acid) and the sterol cholesterol. Further research should focus on the determination of the optimal requirements of fatty acids and sterols of both broodstock and larvae of *O. edulis*.

CRediT authorship contribution statement

Fiz da Costa: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Ricardo González-Araya:** Investigation, Writing – review & editing. **René Robert:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739302.

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