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Influence of one selected *Tisochrysis lutea* strain rich in lipids on *Crassostrea gigas* larval development and biochemical composition

Da Costa F. [1, 2], Petton Bruno [1], Mingant Christian [1], Bougaran Gaël [3], Rouxel Catherine [3], Quéré Claudie [1], Wikfors Gary H. [4], Soudant Philippe [5], Robert René [1, 6, *]

[1] Ifremer, Laboratoire des sciences de l'Environnement Marin (UMR 6539, LEMAR), Plouzané, France
[2] Novostrea Bretagne, Sarzeau, France
[3] Ifremer, Laboratoire Physiologie et Biotechnologies des Algues, Nantes Cedex 3, France
[4] Northeast Fisheries Science Center, NMFS, NOAA, Milford CT, USA
[5] Laboratoire des Sciences de l'Environnement Marin (UMR 6539, LEMAR), IUEM/UBO, Plouzané, France
[6] Ifremer, Unité Littoral, Centre Bretagne - ZI de la Pointe du Diable, Plouzane, France

* Corresponding author : René Robert, email address : rene.robert@ifremer.fr

Abstract :

Effects of a remarkably high overall lipid *Tisochrysis lutea* strain (T+) upon gross biochemical composition, fatty acid (FA), sterol and lipid class composition of *Crassostrea gigas* larvae were evaluated and compared with a normal strain of *Tisochrysis lutea* (T) and the diatom *Chaetoceros neogracile* (Cg). In a first experiment, the influence of different single diets (T, T+ and Cg) and a bispecific diet (TCg) was studied, whereas, effects of monospecific diets (T and T+) and bispecific diets (TCg and T+Cg) were evaluated in a second experiment. The strain T+ was very rich in triglycerides (TAG: 93–95% of total neutral lipids), saturated FA (45%), monounsaturated FA (31–33%) and total fatty acids (4.0–4.7 pg cell^{-1}). Larval oyster survival and growth rate were positively correlated with 18:1n-7 and 20:1n-7, in storage lipids (SL), and negatively related to 14:0, 18:1n-9, 20:1n-9, 20:4n-6 and trans-22-dehydrocholesterol in membrane lipids (ML). Surprisingly, only the essential fatty acid 20:5n-3 in SL was correlated positively with larval survival. Correlations suggest that physiological disruption by overabundance of TAG, FFA and certain fatty acids in larvae fed T+ was largely responsible for the poor performance of these larvae. 'High-lipid' strains of microalgae, without regard to qualitative lipid composition, do not always improve bivalve larval performance.

Keywords : larvae, lipids, oyster, *Tisochrysis lutea*

1. Introduction

Nutrition is the major factor influencing larval bivalve survival and growth, as reviewed comprehensively by Marshall and co-authors (2010). In bivalve hatcheries, microalgal production is expensive and may represent 50% or more of hatchery operation costs (Coutteau & Sorgeloos 1992; Borowitzka 1997). Currently, constant supply of living microalgae remains the only food source for bivalve larval rearing under controlled conditions as no substitute feeds have been developed (Muller-Feuga *et al.* 2003; Spolaore *et al.* 2006). For example, *Macoma balthica* larvae fed *Isochrysis* sp. showed higher larval growth than larvae fed this microalga supplemented with lipid enrichment emulsions (DHA/EPA) (Hendriks *et al.* 2003). Overall, alternative feeds, such as microcapsules and emulsions, are known to be poorly assimilated (Seguineau *et al.* 2005). Accordingly, the search new microalgal strains for bivalve rearing that meet all nutritional requirements of bivalves is of utmost importance. Mutated microalgae seem to be a promising way of modifying nutritional value to cover bivalve nutritional requirements. The criteria for selecting a suitable algal diet for bivalve larvae are based upon morphology (especially size), ease of culture, absence of toxicity, and ability of the larvae to trap, ingest, digest, and assimilate the algae (Marshall *et al.* 2010). Beyond ingestion and digestion, food value is mainly determined by biochemical composition of microalgae. Regarding biochemical quality of microalgae, many studies have shown that carbohydrate, protein and lipid quantity do not explain differences in nutritional value of microalgae (Brown *et al.* 1989; Robert & Trintignac 1997). Lipid quality composition is considered as the most determining factor of nutritional quality of diets; whereas, amino acid, vitamin and mineral compositions are considered as minor determining factors (Delaporte *et al.* 2003).

The beneficial effect of lipid-rich diets upon larval growth is well supported in the literature for *Crassostrea gigas* (Powell *et al.* 2002), *Argopecten purpuratus* (Nevejan *et al.* 2003) and *Pecten maximus* (Delaunay *et al.* 1992; Tremblay *et al.* 2007). Moreover, diets rich in lipids also promote higher larval competence in *C. gigas* and *P. maximus* larvae (Haws *et al.* 1993; Tremblay *et al.* 2007). Triacylglycerols (TAG) were identified as a main source of energy for *C. gigas* larvae, because they are readily available in microalgae (Ben Kheder *et al.* 2010). Essential fatty acids (EFAs), particularly the n-3 fatty acids eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), are important for larval survival and growth (Rico-Villa *et al.* 2006) because they are major membrane components (Hendriks *et al.* 2003) and possible modulators of membrane function (Palacios *et al.* 2005). DHA is involved in maintaining a suitable membrane structure; whereas, 20:5n-3 fulfills a role as both an energy source and as a precursor of eicosanoids (Howard & Stanley 1999; Whyte *et al.* 1990, 1991; Marty *et al.* 1992). Oversupply of 20:5n-3 to bivalves has not been reported, but this has been reported to induce malformations in the sea bass *Dicentrarchus labrax* larvae (Gisbert *et al.* 2005). The n-6 fatty acids docosapentaenoic acid (22:5n-6) and arachidonic acid (20:4n-6) also influence survival and growth during scallop larval and postlarval stages (Pernet *et al.* 2005; Milke *et al.* 2006). The sterol composition of microalgae could also be important in determining the nutritional value for bivalves (Wikfors *et al.* 1996). Literature in bivalve larval nutrition has focused mainly on 20:5n-3 and 22:6n-3; whereas, studies investigating 20:4n-6, 22:5n-6, or sterols remain scarce (Soudant *et al.* 1998a, b; Pernet *et al.* 2005).

Biochemical and fatty acid composition of a given species of phytoplankton can be modified according to its growth phase (Fernández-Reiriz *et al.* 1989; Brown *et al.* 1993), culture system (Ponis *et al.* 2006), and growing conditions, i.e. nutrients, temperature, irradiance and light quality (Wikfors *et al.* 1984; Mortensen *et al.* 1988;

Sukenik & Wahnon 1991). Manipulation of algae may be a relatively simple way to improve feed composition without culturing several phytoplankton species, but instead focusing on strains that are known to be ingested and digested (Marshall *et al.* 2010). *Tisochrysis lutea* (formely *Isochysis* affinis *galbana* or T-*Iso:* Bendif *et al.* 2013) is a microalga used widely as live food in bivalve hatcheries because of its advantageous fatty acid spectrum and its relative ease of culture (Ewart & Pruder 1981; Napolitano *et al.* 1990). Protein accumulation is enhanced during the exponential phase in *T. lutea;* whereas, carbohydrates and lipids are mainly stored during the stationary phase (Brown *et al.* 1993). Moreover, strains of this species may differ in nutritional quality (Wikfors & Patterson 1994), i.e. in biochemical composition and nutritional value to invertebrates (Brown *et al.* 1997). Bougaran and co-workers (2012) performed a mutation-selection procedure and obtained a strain of *T. lutea* especially rich in lipids (T+). This strain, when harvested in stationary phase and was cultured under nitrogen limitation has increased TAG storage and maintained 22:6n-3 content. This was expected to be beneficial for bivalve larval development because the energy content is increased without sacrificing 22:6n-3.

The development of a flow-through larval-rearing technique in different volumes (Rico-Villa *et al.* 2008; Gonzalez-Araya *et al.* 2012) has allowed the definition and standardization of the quantitative needs for *C. gigas* larvae at a given rearing temperature (Rico-Villa *et al.* 2009). This rearing technique enabled investigation of nutritional quality of microalgae and larval development while maintaining a constant dietary supply, i.e., larvae truly are fed *ad libitum*. Using this new rearing method, the aim of this study was to investigate the effect of *T. lutea* lipid enrichment upon *Crassostrea gigas* larval performance with the practical goal of improving bivalve larval feeding in the hatchery.

2. Materials and methods

2.1. Microalgal culture

Three different microalgae were tested as mono- and bi-specific diets: 1) *Tisochrysis lutea* (named here T, volumetric size, 45 μm^3, strain CCAP 927/14), 2) *T. lutea* strain rich in lipids (named here T+, 77 μm^3, strain CCAP 927/17), and 3) *Chaetoceros neogracile* (named here Cg, 77 μm^3, UTEX LB2658). Microalgae were grown in 6-L glass carboys at 20-23 °C under continuous illumination (180-220 µmol photons m^{-2} s^{-1}) provided by cool-white fluorescent tubes. Seawater (salinity 34-35 PSU) was filtered to 1 µm, enriched with sterilized Conway medium (400 mg $NaNO_3$) (Walne 1966) and then autoclaved. T+ was grown in seawater enriched with sterilized Conway medium poor in nitrogen (200 mg $NaNO_3$). A 3% CO_2-air mixture was supplied to support growth and to maintain the pH within a range of 7.5-8.1. For culture of the diatom *C. neogracile*, sodium metasilicate (40 mg L^{-1}) was added as a silica source and salinity was reduced to 25 g L^{-1} by addition of distilled water. T and Cg were harvested at the late-logarithmic phase (after 3-4 days), and T+ was harvested in stationary phase (after 10-11 days).

2.2. Broodstock conditioning, larval culture, and measurements

Broodstock were collected from Aber Benoit (Brittany, France) and transferred to Argenton hatchery facilities for conditioning. Individuals were maintained in open, flow-through tanks at 19 ºC for 2 months with a daily supply of a mixed diet of *T. lutea* and *C. neogracile* equivalent to 6% of oyster dry weight daily. Broodstock gonads were stripped for gamete collection, using 11 males and 7 females. After counting,

fertilization was performed at a ratio of 50 spermatozoa per oocyte. Two hours later, the embryos were incubated in 150-L cylindro-conical tanks in seawater filtered to 1 µm and UV- treated at 22 ºC. After 48 hours of incubation, the percentage of D-larvae was determined microscopically. Veligers were transferred to 5-L, translucent, methacrylate cylinders and reared, in triplicate, in flow-through as described by Gonzalez-Araya *et al.* (2012). A continuous, filtered, UV-treated seawater flow of 0.87 ml min^{-1} was provided from the bottom of each experimental tank (100% tank water renewal h^{-1}). Each diet was delivered by pumping from a reservoir, which was cleaned and filled with the appropriate feed daily. Temperature was maintained at 25 °C using a thermo-regulated automatic valve, and ambient salinity was 34 PSU. In each tank, aeration, provided from the bottom to maximize circulation of water within the tank, was set at 30 ml min^{-1}. The outlet of each tank was equipped with a beveled PVC pipe as a sieve to prevent larvae from escaping. Mesh sieve sizes of 40, 60 or 80-µm were used at the beginning of the experiment, on day 6 or 7, and 10, respectively.

Larvae were fed different single- and bi-specific microalgal diets at a constant concentration of 1,500 μm^3 μl^{-1} at the exit of the rearing tanks, corresponding to ≈ 40 algal cells μl^{-1} (equivalent *T. lutea*: Rico-Villa *et al.* 2010). For each replicate tank, phytoplankton consumption was evaluated to establish any potential relationship between larval growth, algal cell ingestion, and biochemical transfer from microalgae to larvae. Twice a day, inlet and outlet water was sampled from each experimental tank. Cell concentrations at inlet and outlet were quantified using an electronic particle counter (MULTISIZER™ 3 COULTER COUNTER®, Beckman Coulter, Inc., Pasadena, CA, USA) equipped with a 100-µm aperture tube. Cell consumption was calculated as the difference in μm^3 or microalgal cells between inlet and outlet of each tank to estimate the number of cells removed by larvae from suspension. Seawater flow rate also was measured twice a day to assess cell consumption per day. A sub-sample of 1 ml was collected every other day from each larval replicate for survival and larval length determination. Survival was estimated by counting larvae under the light microscope. Larval length was determined using an image analysis technique (WinImager 2.0 and Imaq Vision Builder 6.0 software (National Instruments, Austin, Texas, USA) for image capture and treatment, respectively).

When at least 50% of the larvae had developed the pigmented inclusion known as the eye-spot, and were thus considered as “competent” (ready to metamorphose), each larval population was transferred to a PVC container with a 125-µm nylon mesh base and kept in raceway tanks. Larvae were counted, and percentages of competent larvae were determined. Larvae were spread in each tray bottom, provided with shell chips as settlement substrate, and fed the same diets as used for larval culture. After 15 days, metamorphosis success was determined by sieving post-set through a 400-µm mesh sieve and counting the number of remaining spat. Percentage of metamorphosis was calculated as the number of live spat divided by the initial number of larvae ready to metamorphose.

2.3. Experimental trials

The food value of *T. lutea* strain T+ to support *C. gigas* larval development was evaluated in two independent experiments. In the first, the influence of single-species diets (T, T+, Cg) upon larval survival, growth, and metamorphosis was studied. The bi-specific diet TCg was the positive control, and unfed larvae were the negative control. Larval density was set at 100 larvae ml^{-1} s.

A second, follow-up experiment was performed to confirm the poor oyster larval performances recorded with T+ and to determine if T+ nutritional value could be

improved by combining it with a diatom (T+Cg). The controls were similar to those in Exp. 1. Larvae were reared at a density of 50 ml^{-1}; no differences in *C. gigas* larval performances at 50 or 100 larvae ml^{-1} have been shown previously in the flow-through system used here (Petton *et al.* 2009).

2.4. Biochemical analysis

Samples of each microalgal diet (250-300 x 10^6 cells) were collected during larval culture (n = 5 and n = 7 for experiments 1 and 2, respectively). Each larval replicate for all treatments was sampled on day 2 (D-shaped larvae, start of feeding; ≈ 200,000 larvae per sample), on day 8 (≈ 50,000 larvae per sample), and when the positive control reached the pediveliger stage (days 16 and 15, for experiments 1 and 2, respectively; ≈ 20,000 larvae per sample). In the first experiment, larval samples also were collected when larvae fed other diets reached the pediveliger stage (day 18/19; ≈ 15,000 larvae per sample). During the first experiment, samples for analysis of proximate biochemical composition (total lipids, proteins and carbohydrates) of microalgae and larvae were collected directly in graduated flasks, washed with 3.5% ammonium formate to remove salts, centrifuged, and the supernatant was removed. All samples were stored at -20 ºC. Samples of algae and larvae for lipid analysis (fatty acids, sterols and lipid classes for both experiments) were collected on 450 ºC pre-combusted GF/F glass-fiber filters (Whatman, diameter 47 mm) and washed with 3.5% ammonium formate. Lipids were extracted in 6 ml chloroform-methanol (2:1, v/v) according to Folch *et al.* (1957) and stored at -20 °C under nitrogen.

Samples for proximate biochemical composition analysis were suspended in 2 ml of distilled water and homogenized. Then, aliquots for the different analysis were distributed in test tubes. Carbohydrates were analyzed following the Dubois *et al.* (1956) method using glucose as the standard. After samples were hydrolyzed in 1 N NaOH at room temperature for 1 h, total protein content was assayed using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) in microplates and read at a wavelength of 750 nm with an Bio-Tek Synergy HT microplate reader (BioTek Instruments, Inc. , Winooski, VT, USA) using BSA as the standard. Total lipids were quantified gravimetrically by the Bligh & Dyer (1959) method. Dry and ash free weights were determined with a 300 µl aliquot of the first fraction (2 ml) distributed in pre-weighed capsules dried at 80 ºC for 48 h and subsequently combusted at 450 ºC for 5 h.

The neutral and polar lipids of the larvae were separated on a silica gel micro-column (30 mm x 5 mm; Kieselgel Merck, 70-230 µm mesh) heated to 450 °C, deactivated with 6% water as described by Soudant *et al.* (1995), and analyzed following the method described by Marty *et al.* (1992). Fatty acids (FA) of microalgae were identified without separation on silica gel micro column. The fatty acid 23:0 (10 µl) was added as an internal standard for FA quantitative measurements. Samples were evaporated under nitrogen and transesterified with 1 ml of BF_3-MeOH (10%) for 10 min at 95 ºC (Metcalfe & Schmitz 1961). After cooling, 1 ml of hexane and 1 ml of water were added to each sample vial, and vials were agitated and centrifuged. The organic, upper phase containing fatty acid methyl esters (FAMEs) was collected and cleaned with 1 ml of water that was eliminated thereafter. FAMEs were recovered and analyzed by gas chromatography using a GC (HP 6890; HP, Wilmington, DE, USA) with an auto-sampler equipped with a capillary column (JW DB wax, 30 m length x 0.25 mm i.d. x 0.25 µm film thickness), and on-column injector at 60 ºC and a FID detector at 300 ºC. The carrier gas was H_2, at a constant flow of 2 ml min^{-1}. FAMEs were identified and quantified by means of a standard 37-component FAME mix and other known standard mixtures from marine bivalves (Soudant *et al.* 1995).

Sterols of microalgae and larvae were hydrolyzed with sodium methoxide (MeONa) for 90 min at room temperature as described in Soudant *et al.* (2000). The sterols were extracted in hexane and injected directly into the gas chromatograph Chrompak CP 9002 (Varian Inc., Walnut Creek, CA, USA) equipped with a Restek RTX65 fused silica capillary column (15 m x 0.25 mm, 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 ºC. The sterols were identified by comparison of their retention times with standards, and cholestane was used as an internal standard.

Lipid class composition was determined by HPTLC using 200×100 mm silica-gel plates (Silicagel 60, Merck) according to Soudant *et al.* (1999). Silica plates were subjected to a first elution to eliminate impurities with a solution of hexane: diethylether (1:1, v/v) for neutral lipids (NL) and a solution of methyl-acetate: isopropanol: chloroform: methanol: KCl 0.25% (10:10:10:4:3.6; v/v) for polar lipids (PL). The chromatographic plates were activated for 30 min at 120 ºC. The samples were placed on the plates with an automated sampler designed for TLC (Automatic TLC sampler 4 and software WinCATS, CAMAG, Muttenz, Switzerland). Neutral lipids were separated with two successive developments, first with a solution of hexane: diethyl ether: acetic acid (20:5:0.5; v/v) and, after drying, followed by development in a hexane: diethyl ether (97:3; v/v) solution. Phospholipids were separated by development with a solution of methyl-acetate: isopropanol: chloroform: methanol: KCl 0.25% (10:10:10:4:3.6; v/v). Plates were developed with a 3% $CuSO_4$ and 8% H_3PO_4 solution heated to 160 °C for 20 min. The developed plates were visualized with a Scanner-Densitometer, equipped with a monochromatic 370 nm bulb, and lipid classes were identified and quantified comparing band intensity between standards and samples using winCATS software. Commercial and lab-made standards employed for the identification and quantification of lipid classes; sterols (ST), alcohols (AL), alkenones 1 and 2 (ALK 1 and 2), free fatty acids (FFA), triacylglycerol (TAG), glyceryl ethers (GE) and sterol esters (StE) for neutral lipids; and lysophosphatidyl-choline (LPC), sphingomyelin (Sm), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cardiolipin (Ca) for phospholipids.

2.5. Statistical analysis

Data normality was first evaluated using the Shapiro-Wilk test, and then one-way analysis of variance (ANOVA) for significant differences was performed using STATISTICA software (Stat Soft, Inc., Tulka, OK, USA, version 12). Homogeneity of variances was checked by means of the Barlett test. When necessary, post hoc analyses with the LSD test were applied. Percentage data were arcsine-transformed to normalize variance (Sokal & Rohlf 1995). Differences were considered statistically significant if $P \leq 0.05$.

FA in larval lipid reserves, as well as FA and sterols in larval lipid membranes, were integrated in a principal component analysis (PCA) to explore variable distribution using the STATISTICA software (Stat Soft, Inc., Tulka, OK, USA, version 12). Further, to investigate relationships between FA in lipid reserves and FA and sterols in larval lipid membranes (as independent variables) with larval survival and growth rate (as dependent variables), Pearson correlation coefficients were calculated using STATISTICA software. Larvae *vs* microalgae comparisons were made on experiment-specific algal data.

3. Results

3.1. Biochemical composition of the diet

The T+ diet contained higher relative content of lipids (692 g Kg^{-1}) and lower quantity of total protein (124 g Kg^{-1}) than T (481 and 297 g Kg^{-1}, respectively). Furthermore, different relative lipid contents were found between T+ (962 g Kg^{-1}) and Cg (548 g Kg^{-1}) that contrasted with similar total lipid amounts on a per-cell basis (11.2 *vs* 10.9 pg $cell^{-1}$). Protein content was 3-4 fold lower in T+ than in Cg (124 g Kg^{-1} and 2.0 pg $cell^{-1}$ *vs* 368 g Kg^{-1} and 7.3 pg $cell^{-1}$: Table 1). The lowest relative carbohydrate content (85 g Kg^{-1}) was found in Cg (Table 1). Compared to the other diets, higher significant ash content (24%) was found in the diatom Cg ($P < 0.05$: Table 1).

Neutral lipid class, FA, and sterol compositions of the diets from experiment 1 and 2 were pooled in Table 2 after checking with ANOVAs that there were no differences in composition within the same diet between experiments 1 and 2. The selected strain T+ was extremely rich in triglycerides (TAG), which accounted for 94% of total neutral lipids (NL) (2.6 pg $cell^{-1}$) (Table 2). This TAG accumulation was 4 and 17-fold higher than in T when expressed respectively as relative (94 *vs* 25%; Table 2) and as absolute values (0.2 *vs* 2.6 pg $cell^{-1}$). A high relative content of TAG also was found in Cg (79%). The relative content of sterols was higher in diets containing Cg (alone or in combination with T and T+: 15, 10 and 9%, respectively; Table 2). Sterol (ST) relative content was higher in T than T+ (5.2 *vs* 2.4%), although its absolute content showed opposite patterns (8.1 *vs* 64.0 fg $cell^{-1}$). Quantification of individual polar lipid (PL) classes in the microalgae was unfeasible because several polar lipid classes co-eluted and failed to align with known standards.

The selected strain T+ was 3.6-fold richer in total fatty acids (TFA) as measured by GC-FID than T, 4.3 *vs* 1.3 pg $cell^{-1}$, respectively (Table 2). The strain T+ showed higher proportions of total saturated fatty acids (SFAs) and total monounsaturated fatty acids (MUFAs), representing together 80% of TFA, mainly comprised of 14:0 (32%) and 18:1n-9 (28%). The strain T was richer in 18:4n-3 (15%) and 22:6n-3 (12%), but contained similar amount of 16:0 as T+ (13-14%: Table 2). In contrast, the diets including the diatom *C. neogracile* (Cg, TCg and T+Cg) were richer in 16:0 (16-24%), 16:1n-7 (11-38%), 20:5n-3 (5-10%) (Table 2).

Brassicasterol was the unique or main sterol found in T and T+, respectively (Table 2). Small amounts of cholesterol and 24-methylene-cholesterol were also detected (about 1%) in T+. A higher diversity of sterol composition was observed in Cg, which predominantly contained cholesterol (54%) and fucosterol (33%) and at a lesser extent 24-methylene-cholesterol and isofucosterol (Table 2). The bi-specific diet TCg contained similar proportions of cholesterol and brassicasterol (38-39%); whereas,, T+Cg contained a 4-fold higher relative proportion of brassicasterol (67%) compared to cholesterol (17%) (Table 2). A much higher total alkenone content was recorded in T compared to T+, reflecting significant differences in ALK1 (48.5 and 0.8% of total neutral lipids, respectively) and ALK2 relative values (18 and 0.4% of total neutral lipids, respectively) (Table 2).

3.2. Larval food consumption

Experiment 1

On day 16, larvae fed standard strain T consumed 3-fold more algae than those fed T+, the latter showing high inter-tank variability (Fig. 1A). Similar cumulative algal consumption (250-260 x 10^3 algae $larva^{-1}$) was needed to achieve competence in the larvae fed Cg and TCg; larvae fed the bi-specific diet were, however, competent 3 days earlier than those fed Cg alone (Fig. 1A; Table 3). In contrast, larvae fed T reached the pediveliger stage after ingesting of 343 x 10^3 algae $larva^{-1}$ by day 18 (Table 3).

Experiment 2

On day 8, regardless of diet, cumulative algal consumption remained low, with the weakest feeding observed in larvae fed T+ (Fig. 1B). Larvae fed TCg exhibited the highest overall microalgal uptake during the first and second weeks ($P < 0.05$: Fig. 1B). Although larvae fed TCg reached competence on day 15, with an overall consumption of ≈ 320 x 10^3 algae $larva^{-1}$, larvae receiving the unialgal diet T consumed more algae (≈ 425 x 10^3 algae $larva^{-1}$, day 19) to reach the pediveliger stage on day 19 (Table 3).

3.3. Larval survival and growth

Experiment 1

On day 16, survival was similar for larvae fed Cg or TCg (≈ 60%: Fig. 2A); whereas, with only 4% survival, most of larvae fed T+ were dead on day 16, which was significantly lower than unfed larvae (16%).

The best feed, in terms of larval growth, was the bi-specific diet TCg (16 µm d^{-1}) and the lowest was T+ (≈ 2 µm d^{-1}), which was only slightly higher than unfed (≈ 1 µm d^{-1}) (Fig. 2B). Similar growth, ≈ 12 µm d^{-1} was observed for mono-specific diets T and Cg (Fig. 2B).

Larvae fed TCg reached the pediveliger stage on day 16, but no larvae “ready to set” were found in treatments receiving single-species diets, including T+ (Table 3). Surprisingly, metamorphosis success was similar regardless of diet, except for larvae that received T+ that never completed metamorphosis (Table 3).

The integration of all contributors to larval performance is represented by the final yield (Table 3), corresponding to the number of settled postlarvae/initial number of D larvae. Both mono-specific diets exhibited similar final yields (3%); whereas, TCg led at 21% of final yield.

Experiment 2

The positive control TCg again was the best diet, supporting the highest larval survival (≈ 81 %) and growth (≈ 19 µm d^{-1}) (Fig. 3A). Larvae fed exclusively *T. lutea* (T) exhibited higher survival (≈ 60%) and growth (≈ 13 µm d^{-1}) than those receiving T+Cg (≈ 45% and ≈ 8.5 µm d^{-1}, respectively) (Fig. 3A and C). On day 8, the single diet T+ supported poor survival (≈ 45%) and growth (to ≈ 96 µm) (Fig. 3A and B). After collecting the larvae for biochemical analysis, the number of remaining larvae was insufficient to continue this treatment. Larvae fed TCg were competent to settle on day 15; whereas, larvae receiving T achieved morphological competence 4 days later (Table 3). Both metamorphosis (≈ 60%) and final yield (≈ 52%) were highest in larvae

fed TCg (Table 3). On day 15, larvae fed TCg were sacrificed for biochemical analysis, so neither competence nor settlement could be achieved thereafter.

3.4. Biochemical composition of the larvae

Experiment 1

D-shaped larvae (day 2, initial) were composed mainly of proteins (564 g Kg^{-1} of organic matter, OM) and lipids (401 564 g Kg^{-1} OM) (Table 4).

On day 16, larvae fed TCg had doubled in dry weight compared to those receiving T or Cg ($P < 0.05$), although the percentage of ash was similar among dietary treatments (62-66%: Table 4). Similar relative contents of proteins (760-780 g Kg^{-1}) and lipids (175-192 g Kg^{-1}) were observed in all fed larvae ($P > 0.05$: Table 4). Slight differences between fed and unfed larvae were found in carbohydrate content, varying from 44 to 48 g Kg^{-1} (Table 4). Biochemical data for larvae subjected to T+ were not reported because low survival impeded sampling.

The highest contents in total neutral lipids (Total NL) were found in larvae fed T+ and TCg (135 to 187 ng $larva^{-1}$); whereas, the lowest values (0.8 ng $larva^{-1}$) were recorded in unfed larvae (Table 5). Larvae fed T+ contained a higher mean amount of total NL than those fed T (170 *vs* 119 ng $larva^{-1}$: Table 5) despite their smaller size (104 *vs* 216 µm: Fig. 2B). The lowest relative percentage of NL (%NL) with respect to total lipids was observed in unfed larvae (18%). The highest %NL was recorded in larvae fed T+ (78%). Similar relative %NL values were found in larvae fed Cg and TCg (30-35%) (Table 5). Similar proportions of TAG were found in larvae fed TCg and T (85-88%: Table 5); this relative proportion remained constant until larvae reached the pediveliger stage on days 16 and 19 respectively (Table 3). In contrast, larvae fed T+ exhibited the lowest proportion of TAG among fed larvae (67%: Table 5).

The highest proportions of free fatty acids (FFA) were observed in unfed larvae and T+-fed larvae (32% and 26%, respectively: Table 5).

When they were not fed, larvae mainly retained sterol (ST) (56%: Table 5). Larvae fed T+ showed the lowest relative content of ST (5%), whereas larvae receiving Cg exhibited the highest (18%: Table 5). Larvae fed T+ showed the highest TAG/ST ratio (17; Table 5). The highest proportion of total polar lipids (PL) was observed in unfed larvae (82%: Table 6). Larvae fed Cg (alone or in combination with T) contained significantly higher proportions of PL (54 to 70%) compared to the other fed larvae (Table 6). Pre-feeding D-shaped larvae mainly contained phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) in decreasing order of proportion (≈ 40, 30 and 20%) (Table 6). Compared to fed larvae, unfed larvae contained significantly lower PC (19%) and higher PE (37%: Table 6). On day 16, except those fed T+, larvae subjected to different microalgal diets contained similar proportions of the different polar lipid classes compared to larvae on day 2 (initial). On day 16, larvae fed T+ exhibited higher contents of PI (31%) and PS (12%) and a lower proportion of PE (18%) than initial larvae (Table 6).

Fatty acids in neutral lipids of the larvae clearly reflected fatty acid composition of the diets. Larvae fed T incorporated mainly 22:6n-3 in PUFA reserves (15%) leading to an increase in percentage of n-3 PUFAs (38% *vs* 23% initially: Table 7). Larvae fed T+ incorporated mainly n-9 MUFAs in reserves (31% *vs* 8% initially: Table 7), specifically 18:1n-9 (26%). FA composition of larvae fed Cg was dominated by 16:1n-7 (21%), 16:0 (19%) and 20:5n-3 (15%: Table 7). Larvae fed the mixed diet TCg exhibited an

intermediate FA composition attributable to the more diverse FA contributions from T and Cg, resulting in an equal relative proportion of 20:5n-3 and 22:6n-3 (7%: Table 7). In contrast, unfed larvae stored mainly SFAs (51% *vs* 40% initially) and retained MUFAs (24% *vs* 26% initially) (Table 7). In larvae fed T, Cg, or TCg, the n-3/n-6 ratio was above 4; whereas, n-3/n-6 ratio was below 2 for those fed T+ (Table 7). Larvae fed T mainly incorporated 22:6n-3 in membrane lipids (21% *vs* 19% initially); whereas, larvae fed Cg mainly incorporated 20:5n-3 in membrane lipids (22% *vs* 11% in D larvae) (Table 8). When fed TCg the relative contents of neither 22:6n-3 nor 20:5n-3 changed (respectively 17.4% *vs* 18.5% initially and 10% *vs* 11% originally). The proportions of non-methylene interrupted (NMI) fatty acids and total dimethyl acetyls (DMA) in larvae fed T+ were much lower (1.6% and 2.4% respectively) than in the other dietary treatments (Table 8). Larvae fed T+ mainly incorporated MUFAs in membranes (37% *vs* 17% initially), especially 18:1n-9 (13%) and 18:1n-7 (7%). Larvae given T+ showed the lowest n-3/n-6 ratio (1.9).

At the start of the experiment, pre-feeding D-shaped larvae mainly contained brassiscasterol and cholesterol (33-34%) and to a lesser extent desmosterol, 24-methylene-cholesterol and stigmasterol (7-8%) (Table 9). On day 16, the sterol composition of the larvae was influenced by that of the diet. For example, larvae fed T and T+ contained high relative contents of brassicasterol, 78 and 63% respectively. A similar relative proportion of cholesterol was observed in larvae fed both diets (6-7%), although cholesterol was not supplied by T or T+. A slight increase in relative content of cholesterol was found in larvae fed Cg (58% on day 16 *vs* 33.5% initially: Table 9) as compared to the proportion of this sterol supplied by the diet (54%: Table 2). On day 16, the relative proportion of fucosterol in larvae fed Cg was 3-fold higher than originally (17.3% *vs* 5.9% respectively: Table 9), which clearly reflected the high content of this sterol in the diet (32%: Table 2). The sterol composition of larvae fed TCg was dominated by brassicasterol and cholesterol in equivalent proportions (38%); whereas, unfed larvae preferentially retained cholesterol (63%) and to a lesser extent brassicasterol (24%).

Experiment 2

Highest content of total neutral lipids (NL) were found in larvae fed TCg on day 15 (373 ng larva^{-1}: Table 5). Similar proportions of NL were, however, recorded in all fed larvae on day 15, varying from 57 to 61%. D-shaped larvae mainly contained triacylglycerols (TAG: 87%) and to a lesser extent sterols (ST: 10%). Regardless of diet, relative contents of phosphatidylethanolamine (PE), cardiolopine (CL), and polar lipids (% PL) in larvae remained constant (24 to 25%, 7 to 8%, 40 to 43%, respectively: Table 6). Percentage of PI (19-23%) and PS (~ 8%) varied marginally, but significantly, according to diet (Table 6).

Fatty acid composition of neutral lipids (NL) of 15-day-old larvae reflected dietary composition (Table 10). For instance, the high level of DHA (22:6n-3) supplied by T (Table 2) was found in NL of larvae fed T (18% *vs* 8% initially: Table 7). Similarly, larvae fed T+ exhibited high levels of total MUFAs (33% *vs* 24% initially: Table 7), and especially n-9 MUFAs (27% *vs* 7% initially) mainly attributable to the high supply of 18:1n-9 by this diet (28%: Table 2). Larvae fed the mixed diet TCg showed the highest mean relative content of 20:5n-3 on day 15 (11%) (Table 7).

Unfed larvae showed a high proportion of saturated fatty acids (SFAs) in NL (74% *vs* 36% initially: Table 7). A higher proportion of total, non-methylene interrupted fatty acids (∑ NMI) was observed in larvae fed bi-specific diets TCg (0.9%) and T+Cg (0.8%) than in larvae fed T alone (Table 7); whereas, the ratio of 22:6n-3/20:5n-3 was

lower in larvae fed diets containing Cg (0.5-0.6) compared to those receiving T (7.3: Table 7).

Regardless of diet, the relative content of total PUFAs in larval polar lipids (PL) remained quite stable on day 15, with values ranging from 47 to 51% (Table 8). Larvae fed TCg or T+Cg maintained overall similar amounts of 22:6n-3 (14-16%), 20:5n-3 (8-10%) and 20:4n-6 (2-3%) in membranes on day 15 (Table 8).Cholesterol relative content decreased in larvae fed T (6%) compared to D-shaped larvae (36%) (Table 9). Other sterols present in minor proportions (1-2%) were tDehydrocholesterol, desmosterol, campesterol, 24-methylene-cholesterol and stigmasterol (Table 9). The main sterol detected in larvae fed TCg was cholesterol (41%); whereas, brassicasterol was the most representative sterol in larvae fed T+Cg (47%) (Table 9).

Relationships between growth, survival and FA and sterol composition in grouped data

A higher growth rate in larvae fed TCg was observed in experiment 2 compared to experiment 1 (18.9 *vs* 15.7 µm day^{-1}). Those larvae receiving T showed similar growth rates in both experiments. Survival also was lower in experiment 1 than in experiment 2 in larvae fed T and TCg. Larvae fed T+ showed similar growth and survival on day 8 between experiments. Both metamorphosis and final yield were higher in larvae fed T and TCg in experiment 2 than in experiment 1.

To better discriminate FA requirements and metabolism of larvae and dietary influence upon FA composition in NL (reserves), a Principal Component Analysis (PCA) was performed. Survival and growth rate were included as supplementary variables, which were not used for the extraction of principal components, but were mapped into the coordinate system (factor structure) determined from the variables and cases selected for the analysis. By this method, FA profiles of larvae associated with high survival and growth rate were inferred from proximity in the correlation circle. Moreover, distribution of cases in PCA factor plots (i.e. larvae fed different diets) allows the determination of relationships between larvae subject to different diets deduced from proximity in factors plots. PCA for FA in NL extracted 3 components with eigenvalues higher than 1.0, which explained 95.5% of the variability in the original data. The first principal component (PC1) explained 65.7% of the combined variance and the second component (PC2) 21.7%. A correlation circle was plotted using PC1 and PC2 as axes to separate the individual data according to larvae subjected to the different dietary treatments (Fig. 4A). Survival and growth rate were grouped with 18:0 and 20:1n-7, but opposed to 14:0 and 18:1n-9 (Fig. 4A). Further, larvae were distributed in the factorial plane according to diets (Fig. 4B). Larvae fed Cg were located in the positive side of PC1; whereas, T and T+ were located in the negative side of PC1 (Fig. 4B). Larvae fed T and T+ were discriminated on PC2. Correlation analysis was performed to relate FA composition in NL to survival and growth rate. We present only the significant correlations with a p value lower than 0.01. Survival and growth rate were positively related to 18:1n-7 (0.64 and 0.61, respectively) and 20:1n-7 (0.57 and 0.71, respectively). A significant positive correlation was observed between 20:5n-3 (0.55) and survival. In contrast, survival and growth rate were negatively related to 14:0 (-0.67 and -0.77, respectively) and 18:1n-9 (-0.73 and -0.82, respectively: Fig. 5A).

Three components with eigenvalues higher than 1.0 were extracted in PCA for FA and sterols in membranes. These components together explained 90.3% of the variability in the original data; PC1 and PC2 explained 46.3% and 36.8% of the combined variance respectively (Fig. 6A). Survival and growth rate were grouped with 20:1n-7, 20:1n-11, 16:0, 18:0, 18:0dma, 20:1dma and 22:2j, and opposed to 14:0, 18:1n-9, 18:2n-6, 20:4n-6 and trans-22-dehydrocholesterol (Fig. 6A). Larvae fed T were placed in the

positive side of PC1 and the negative side of PC2, opposed to those fed Cg (negative side of PC1 and positive side of PC2: Fig. 6B). Larvae fed T+ were located in the positive side of PC1 and PC2 (Fig. 6B).Survival and growth rate were negatively correlated with 14:0 (-0.61 and -0.76, respectively), 18:1n-9 (-0.68 and -0.83, respectively: Fig. 5B), 20:1n-9 (-0.65 and -0.61, respectively), 20:4n-6 (-0.61 and -0.80, respectively) and trans-22-dehydrocholesterol (-0.63 and -0.73, respectively) in larval membrane lipids. In contrast, larval survival and growth rate were positively related to 16:0 (0.56 and 0.66, respectively), 20:1n-7 (0.65 and 0.88, respectively: Fig. 5C), 22:2j (0.70 and 0.83, respectively: Fig. 5D), 20:1dma (0.709 and 0.748, respectively) and desmosterol (0.635 and 0.58, respectively) in larval membranes. Cholesterol was positively related to survival (0.51). Positive correlations were also observed between growth rate and 18:0 (0.57), 20:1n-11 (0.69) and 18:0dma (0.63).

4. Discussion

In the present study the strain rich in lipids (T+) was characterized from a biochemical point of view and found to be a poor choice for supporting larval development of *C. gigas*. Biochemical composition of microalgae, especially lipid quantity and quality, is an important factor influencing larval survival and growth (reviewed in Marshall *et al.* 2010). All microalgal diets tested in the present study differed significantly in nutritional value for Pacific oyster larvae. The mixed diet TCg produced the best survival and growth of oyster larvae, but the mono-specific diets T or Cg also supported fairly good survival and growth, i.e., metamorphosis competence was achieved with both dietary treatments, and metamorphosis occurred. In contrast, T+ yielded poor results in larval survival and growth, and competence was not reached.

T+ alone was ingested poorly by the larvae, although its diameter (5.0-5.7 µm) was similar to Cg, which was ingested readily. All species used in this study were in the size range reported to be ingested by mollusk larvae (i.e. 2-10 µm) (Palmer & Williams 1980). Consequently, algal size likely is not the reason for the poor value of T+ as a larval feed.

Microalgae may be a vector of bacteria, including *Vibrio* spp., to larvae. The influence of total heterotrophic bacteria or *Vibrio* spp. in the microalgae supplied as food upon survival and growth of larvae was considered to be negligible, as no differences were observed between diet treatments (data not presented). Accordingly, the most reasonable explanation for the low value of T+ as food for oyster larvae is intrinsic to the algal strain.

In the first experiment, similar larval survival and growth were observed when larvae were fed T alone or Cg. In the second experiment, when T+ was combined with Cg, growth was lower than in larvae fed T solely (8.6 *vs* 12.6 µm d^{-1}). Moreover, larvae fed T+Cg had significantly reduced survival and higher inter-tank variability compared to T; therefore, it can be concluded that adding T+ to Cg diminished the food value of Cg. Growth of *C. gigas* larvae has been shown to be related inversely to protein content in the diet (Utting 1986; Thompson & Harrison 1992). Protein is well known to be an essential dietary component for oyster larvae, as it is for all grazers and predators, but microalgae that are exceptionally high in protein tend to be low in lipid, which would appear to be the reason for the negative correlation between dietary protein and larval growth. Biochemically-based models suggest that low protein diets reduce larval growth but allow greater storage of lipid, resulting in increased metamorphic success (Powell *et al.* 2002). Hoffman *et al.* (1994) proposed that the optimal ratio of dietary protein to the sum of lipid and carbohydrate (P:L+C) was in the range of 1.1 to 1.4 over a range of food concentrations in their biochemical-based model of *C. gigas* larval

development and growth. In our study, dietary P:L+C ratios were lower than 0.7, even in the dietary treatments that supported high larval survival and growth (i.e. TCg), making difficult a comparison between our analytical data and outputs of simulations from the cited, biochemically-based models. Neither growth nor survival of *Pinctada margaritifera* larvae was correlated with the P:L+C ratio (Martínez-Fernández *et al.* 2006); instead, early development of *P. margaritifera* larvae was correlated positively with dietary lipid, protein, and carbohydrates (Martínez-Fernández *et al.* 2006). Thompson *et al.* (1993) found that low protein content in *Isochrysis galbana* and *C. neogracile* (1.4-2.8 pg cell^{-1}), which were similar to total protein content observed in T+ in the present study, did not impede *C. gigas* larval development. In fact, the bi-specific T+Cg diet, which should have greater protein content than T because of the higher protein content of Cg (data not analyzed in the present study), exhibited lower value as food for *C. gigas* larvae than T in experiment 2. Further, in experiment 1, survival of larvae fed T+ was lower than survival of unfed larvae, suggesting not just a deficiency, but interference with metabolism by T+.

Carbohydrates may play a part in the optimal utilization of other reserves, such as protein and lipids in bivalve larvae (Haws *et al.* 1993). In our study, no significant differences were found, either in terms of total carbohydrate content (3.0 pg cell^{-1}) or as relative content (17-22% OM), between diets T, T+ and TCg (Table 1). Similar relative contents (% OM) were found in larvae on day 16, with a slightly lower proportion in those larvae with *C. neogracile* in the diet (Cg and TCg). Accordingly, carbohydrates in the diets appear to have had minimal influence upon larval performance in our study.

Brown *et al.* (1997) reported similar essential amino acid composition in microalgae and in *C. gigas* larvae. Several authors pinpointed that amino acid composition of microalgae is not crucial in determining the growth response of bivalves (Webb & Chu 1983; Enright *et al.* 1986; Brown *et al.* 1989; Brown 1991). Although vitamin contents may vary between microalgae, it is established that vitamin requirements in bivalves are satisfied regardless the species used for aquaculture purposes (Brown & Miller, 1992; Brown & Farmer 1994; Seguineau *et al.* 1996; Brown *et al.* 1999). Fabregas & Herrero (1986) found little variation in trace metal composition in different mariculture microalgae species. Minerals and vitamins are known to be incorporated from seawater and diets (Samain *et al.* 1992; Wang *et al.* 1995; Reinfelder *et al.* 1997). In fact, when microalgae were supplied to larvae we also provided the minerals and vitamins present in microalgal culture medium, thus reducing the variability in these components between dietary treatments. Accordingly, only a small portion, if any, of the nutritional quality of the microalgal diets can be attributed to amino acids, vitamins or minerals and we can consider that nutritional quality of microalgae was mainly attributable to lipid quality.

Leonardos & Lucas (2000) stated that dietary fatty acid content appears to be the main factor influencing algal nutritional value, even though dietary protein and carbohydrate contents may also modify microalgal value as food. This may be the case of T+ in our study; we cannot discard the possibility that protein content may be insufficient for larval survival and growth, but we consider that imbalanced neutral lipid class and fatty acid composition of T+ may play a more important role in determining the poor nutritional effectiveness of T+. The microalgae T+ and Cg exhibited similar total lipid (TL) content (11 pg cell^{-1}); T+ exhibited, however, a higher TL relative content. TAG accounted for 27% of TL in T+ in experiment 1; whereas, TAG was only 5% in Cg (data not presented). Accordingly, the low food value of T+ may be attributed to an imbalanced composition of lipid classes, rather than TL quantity. The high TAG quantities in T+ may supply excess energy to larvae, although not meeting other specific nutritional requirements.

The excessive TAG supply of T+ (17-fold higher than T: Table 2) led to the accumulation of large quantities of TAG in the larvae fed this microalga on day 16 compared to T (113 *vs* 104 ng larva^{-1}: Table 5), although T+-fed larvae were smaller than T-fed larvae (104 *vs* 216 µm: Fig. 2B; Experiment 1). Various authors have indicated that the level of TAG stored in bivalve larvae generally is considered to be an indicator of potential growth (Waldock & Nascimento 1979; Delaunay *et al.* 1993). High levels of TAG microspheres in the diet (20 and 50%), however, significantly decreased *Mytilus* sp. larval survival (Pernet *et al.* 2004). Excess TAG stores, therefore, may disturb larval metabolism; most of the supplementary energy delivered with T+ was not metabolized and allocated for growth, but rather stored in larval tissues.

TAGs are the main larval energy reserves; content is partially dependent upon larval size, so TAG content must be normalized. On the other hand, as sterols are integrated into structural membranes, they increase with the membrane surface as larvae grow. Accordingly, membrane sterols represent a reference, weighted according to lipid reserves (Fraser 1989). The TAG/ST ratio previously was used as a larval-quality index in several studies (e.g. Fraser 1989; Pernet *et al.* 2003; Ben Kheder *et al.* 2010). In our study, we observed a rise in TAG/ST index in larvae fed T+ on day 16 compared with larvae fed other diets. Ben Kheder *et al.* (2010) observed TAG/ST values ranging from 6 to 9 in *C. gigas* larvae fed standard and high rations of T-*Iso* and *Chaetoceros calcitrans* forma *pumilum*, similar to our results in larvae fed T, Cg and TCg. To our knowledge, a TAG/ST index as high as that observed with T+ in the present study has not been reported previously. We postulate that the elevated TAG/ST ratio in larvae fed T+ may be an indicator of an imbalance between energy reserves and membrane structures in the larvae.

We observed that the high levels of TAG in larvae fed T+ on day 16 in experiment 1 were coincident with high levels of free fatty acids (FFA: Table 5). High relative content of FFA also was found in unfed larvae on day 16. High levels of FFA were reported in larvae of *Mytilus* sp. fed TAG microspheres (Pernet *et al.* 2004). These results suggest that larval catabolism of TAG is a mechanism activated to eliminate excess. Indeed, FFAs can be direct products of TAG degradation by lipases (Derewenda 1994). Consequently, as T+ is rich in SFAs and MUFAs, TAG catabolism in larvae may result in high levels of calorie-rich FAs, specifically 14:0, 18:1n-9 and 16:0. FFA content also can be stimulated by stress (Minick & Chavin 1972). We postulate that increased levels of FFAs in larvae fed T+ may be caused by metabolic stress arising from excess dietary TAG; these high levels may imbalance larval metabolism as elevated FFAs are known have a deleterious effect in a variety of animals (Takagi *et al.* 1982).

Correlation analysis showed that 14:0 and 18:1n-9 in neutral (NL) and polar lipids (PL) of the larvae are associated with poor larval survival and growth rate. This is in agreement with PCAs in NL and PL that also showed that 14:0 and 18:1n-9 were opposed to survival and growth rate. Both FAs were supplied in large quantities by T+ (1.0-1.4 pg alga^{-1}). Thompson *et al.* (1993, 1996) demonstrated that the sum of 14:0+16:0 in the diet was positively correlated with larval growth in *C. gigas.* A threshold for 14:0 supplied in the diet may exist, however, or the combination of certain FAs in the diet may interact, producing deleterious effects.

The relative content of 20:4n-6 in PL of the larvae was negatively correlated with survival and growth rate in our study. In addition, 20:4n-6 in PL was opposed to survival and growth rate in PCA. The importance of 20:4n-6 in invertebrate species is possibly attributable to roles in eicosanoid production and stress response (Howard & Stanley 1999). High 20:4n-6 levels in the larvae, relative to 20:5n-3 and 22:6n-3, generate production of more proinflammatory eicosanoids, which may be harmful to early larval developmental stages (Sargent *et al.* 1999; Rowley *et al.* 2005). Low

settlement was reported in *Mytilus galloprovincialis* when the (20:5n-3+22:6n-3)/ 20:4n-6 ratio in larvae was low (Pettersen *et al.* 2010). Similarly, this ratio was low in NL and PL in larvae fed T+ compared to larvae fed other diets in the present study. In contrast, several studies have suggested that 20:4n-6 deficiency, rather than overabundance, has detrimental effects upon survival and resistance to pathogens in bivalves (e.g. Pernet *et al.* 2005). For example, low survival and growth of *Placopecten magellanicus* larvae was observed with a diet low in 20:4n-6 (0.41%) (Pernet & Tremblay 2004). The MUFAs 18:1n-7 in NL and 20:1n-7 in NL and PL were positively correlated with survival and growth rate and also placed close to survival and growth rate in PCAs. Under our experimental conditions, 20:1n-7 may have an energetic or structural role, depending upon the lipid class, as postulated by Sargent & Whittle (1981) and Ackman (1983). Moreover, 20:1n-7 may be stored temporally in NL as a reservoir for eventual incorporation within membranes, or this FA may serve as precursor for the j series of NMI FAs. Although little is known about NMI FA functions in bivalve larvae, numerous studies agree on their structural and functional roles in biological membranes (Paradis & Ackman 1977; Klingensmith 1982; Pirini *et al.* 2007). Kraffe *et al.* (2004) proposed important membrane properties for the NMI FAs, such as phase transition temperature, membrane fluidity, or activity of membrane-bound proteins. On day 16, high levels of NMI FAs were incorporated within larval membrane lipids (7.9-8.6%), except for those larvae fed T+ (1.6%: Table 8). The importance of 22:2j, which is derived from 20:1n-7, can be highlighted in PL because a positive correlation with larval survival and growth rate was observed and it also plotted close to them in the PCA. Consequently, n-7 MUFAs may play an important role in *C. gigas* larval nutrition as precursors of the series j of NMI FAs incorporated in larval membranes. The low proportion of NMI FAs in larvae fed T+ may be caused by competition between n-7 and n-9 for Δ5 desaturase, as n-9 MUFA precursors were supplied in high amounts in larvae fed T+.

Overall low levels of NMI FAs in PL of larvae fed T+ on day 16 compared to the other fed regimes were observed; in addition, a low proportion of 20:1n-11 was observed in larvae fed T+ compared to the other groups of larvae. The latter MUFA in PL was correlated positively with larval growth rate and it was close to growth in the PCA. Kraffe *et al.* (2004) suggested that this MUFA probably is biosynthesized in bivalves by a Δ9 desaturase acting on 20:0, as this desaturase also participates in the synthesis of NMI precursors obtained by Δ9 desaturation and elongation of 16:0 and 18:0 (Zhukova 1991). The FA 20:1n-11 also has been recorded in tissues of other mollusks (Kraffe *et al.* 2004) and in hemocytes of clams and oysters (Le Grand *et al.* 2011), wherein 20:1n-11 and NMI FAs varied in a parallel manner. Le Grand *et al.* (2011) speculated that the association of these FAs with plasmalogens in hemocytes may be related to resistance to abiotic and biotic stresses.

It is now well known that 20:5n-3 and 22:6n-3 are essential FAs for oysters, as previously shown by Langdon & Waldock (1981). In the present study, however, a positive correlation between 20:5n-3 in NL and survival was the only statistical relationship detected for either of these essential lipids. For instance, the quantity of both EFAs supplied by T+ might not be limiting for survival and growth of the larvae as this alga supplied 1.8- and 1.6-fold more 20:5n-3 and 22:6n-3, respectively, than T. Larvae fed T achieved competence on day 18, which suggests that EFAs were not limiting at the lower quantities supplied by T. These observations strengthen the argument that poor larval performance on T+ diets is a consequence of metabolic disruption, rather than nutritional deficiency.

Sterols are required for the maintenance of cellular membrane fluidity (Dowhan & Bogdanov 2002). Larvae fed TCg on day 16 exhibited similar proportions of brassicasterol and cholesterol in membrane lipids (37-39%). Cholesterol appeared to be mainly supplied by Cg; whereas, T provided only brassicasterol. We consider it

unlikely that the low levels of cholesterol in T+ limited larval survival and growth, because T, which also lacked this sterol, promoted growth and competence in *C. gigas* larvae, but the time needed to achieve competence was longer than in larvae fed TCg. Positive correlations between larval survival and growth rate and desmosterol were observed. Moreover, cholesterol was the only dietary sterol positively correlated to larval survival. Indeed, cholesterol is considered to be an essential sterol for mollusks (Soudant *et al.* 1996b). The positive correlation observed for desmosterol may be linked to its role as an intermediate in phytosterol bioconversion to cholesterol (Knauer *et al.* 1998).

Our study showed that diet modulated larval PL class composition. Sixteen-day-old larvae fed T, Cg, and TCg mainly retained PC (35-38%), PE (29-31%) and PI (19-25%) in decreasing order of quantity (Table 6). Larvae fed T+ exhibited a higher relative content of PI (30%). The high 20:4n-6 proportions quantified in PL of larvae fed T+ may be found in PI, as Soudant *et al.* (1996a, 1998a) reported that 20:4n-6 was the main PUFA in PI. This PL is implicated in a variety of cell functions, such as apoptosis, cell metabolism, and gene expression (McPhail 2002). The high relative contents of PI and 20:4n-6 in larvae fed T+ may result in altered cellular metabolism. Larvae fed T+ showed lower relative content of PE (18%) compared to the other diets. It is generally accepted that PE is mainly composed of PE-plasmalogens in bivalve mollusks (Soudant *et al.* 1995; Kraffe *et al.* 2004). The lower PE relative content in larvae fed T+ is in good agreement with previous observations of low DMA (18:0dma and 20:1dma), 20:1n-11 and NMI FAs in larvae fed T+. 20:1dma was positively correlated with survival and growth rate and placed close to survival and growth in the PCA,; whereas, 18:0dma was positively correlated with only growth rate. These compounds originate from transmethylation of 1-alkenyl chains of plasmalogens found in high proportions in membrane lipids of mollusks (Joseph 1982; Kraffe *et al.* 2004). Nevertheless, information concerning the biological functions of plasmalogens is still limited. Reduced proportions of plasmalogens in larvae fed T+ may reflect a response to oxidative stress, as plasmalogens are thought to play an antioxidant role in membranes, stopping the spread of the lipid peroxidation cascade (Leβig & Fuchs 2009; Le Grand *et al.* 2013). Le Grand *et al.* (2013) stated that plasmalogens, 20:1n-11, and NMI FAs synthesis rates may be interdependent. NMI FAs are thought to be implicated in the cellular response to oxidative stress (Barnathan 2009). In these diene FAs, the double bonds are separated by more than one methyl group, making them less sensitive to oxidation than other PUFAs with the same number of insaturations (Kaneniwa *et al.* 1988). Similarly, 20:1n-11, with only one double bond, is expected to be less sensitive to oxidation than PUFAs (Halliwell 1999). Le Grand *et al.* (2013) hypothesized that, among plasmalogens, the vinyl-ether linkage of PL containing 20:1n-11 or NMI FAs could act as a preferential ROS oxidation target, leading to a lower proportion of plasmalogens under disrupted conditions (i.e. neoplastic cells).

In the present study, a Principal Component Analysis (PCA) was carried out to explore variable distribution (FA in larval lipid reserves; and FA and sterols in larval lipid membranes). A similar multivariate analysis was used previously in adults to compare the FA profiles of different organs (Caers *et al.* 1999), to discriminate dietary and species influences upon the FA composition of gill polar lipids (Delaporte *et al.* 2005), and in larvae to evaluate the influence of lipid classes and FA profiles upon larval stages (Sánchez-Lazo & Martínez-Pita 2012), and to discriminate FA and sterol profiles according to diets and/or metamorphosis competence (Rico-Villa *et al.* 2006). Nevertheless, none of these studies used larval performance characters (survival and growth rate) as supplementary variables to relate them to specific FA and sterol compositions in larvae. In addition, we used correlation analysis to investigate relationships between FAs and sterols in larval lipid reserves and membranes with larval survival and growth rate. Results achieved with both statistical methods fit quite

well, as FA and sterols with highly significant positive or negative correlations to larval survival and growth rate were plotted near or opposed to them on PCA plots, respectively. The combination of both statistical approaches could be an effective strategy to better understand FA and sterol metabolism and dynamics in bivalve hatchery studies.

5. Conclusion

This study clearly demonstrated poor performance of *C. gigas* larvae fed the lipid-rich *Tisochrysis lutea* strain (T+) grown under nitrogen limitation and harvested at stationary phase. Too high-TAG, SFA and MUFA supplied by T+ may be responsible for the poor survival and growth of larvae receiving this microalga. The unbalanced biochemical composition of the larvae fed T+ (i.e. high TAG stores, high FFA, etc.) may have resulted in disruption of metabolic pathways, especially those involved in lipid and eicosanoid processing, during larval development. This study shows the deleterious effects of very-high dietary supply of 18:1n-9, 18:2n-6 and 20:4n-6 to *C. gigas* larvae, suggesting a requirement for relatively high availability n-3 over n-6 PUFAs and n-7 over n-9 MUFAs. Further research is needed to investigate the cellular metabolism involved in responses to specific lipids in *C. gigas* larvae fed different microalgae.

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Figures

Figure 1. Cumulative phytoplankton consumption during larval development of *Crassostrea gigas* larvae fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, expressed as mean ± S.D. A. Experiment 1. B. Experiment 2. Values with same letter are not significantly different at $P > 0.05$ among treatments within a day.

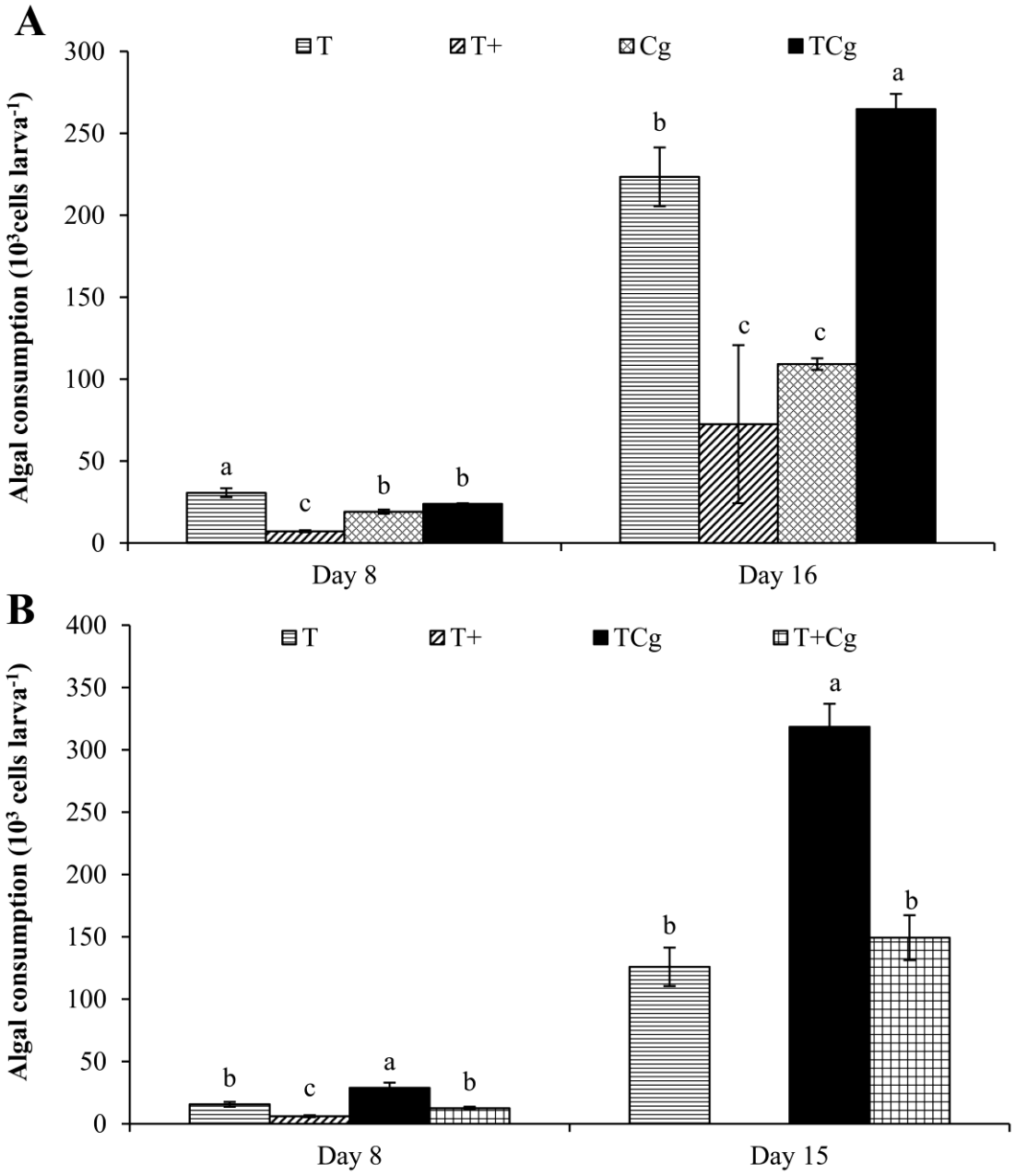

Figure 2. Survival and growth in length of *Crassostrea gigas* larvae fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, expressed as mean ± S.D (Experiment 1). A. Mean survival (%). B. Shell length (µm). Values with same letters are not significantly different at $P > 0.05$ among treatments within a day. Initial larval shell length on day 2 = 76.6 ± 2.7 µm.

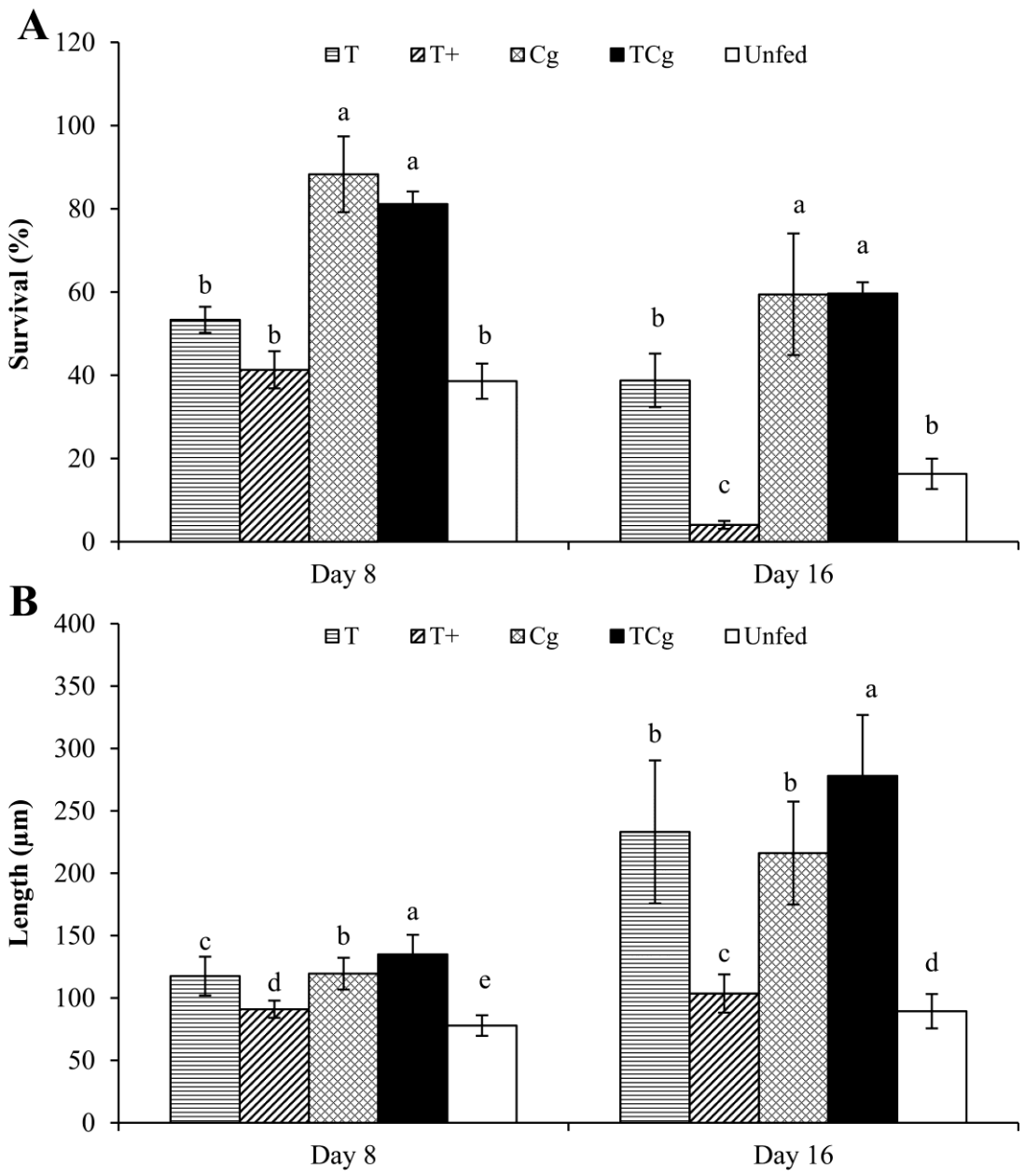

Figure 3. Survival and growth in length of *Crassostrea gigas* larvae fed different mono- or pluri-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, expressed as mean ± SD (Experiment 2). A. Mean survival (%). B. Shell length (µm). Values with same letters are not significant at $P > 0.05$ among treatments within a day. Initial larval shell length on day 2 = 72.71 ± 0.86 µm.

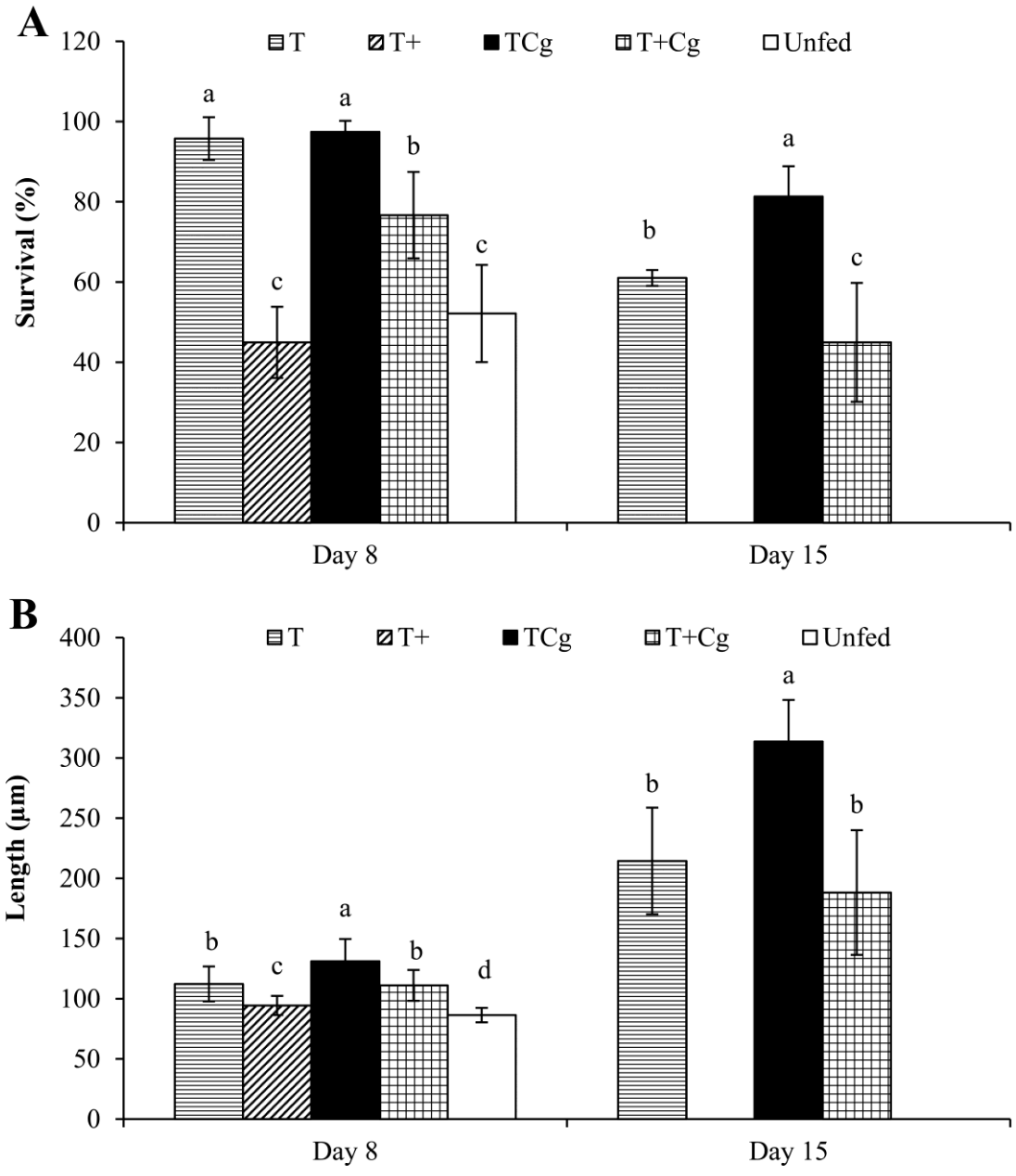

Figure 4. PCA plot of FA variables in NL, growth rate and survival on day 15-16 (grouped data from experiment 1 and 2) according to the two principal components 1 and 2. A. Correlation circle. B. Factorial plan.

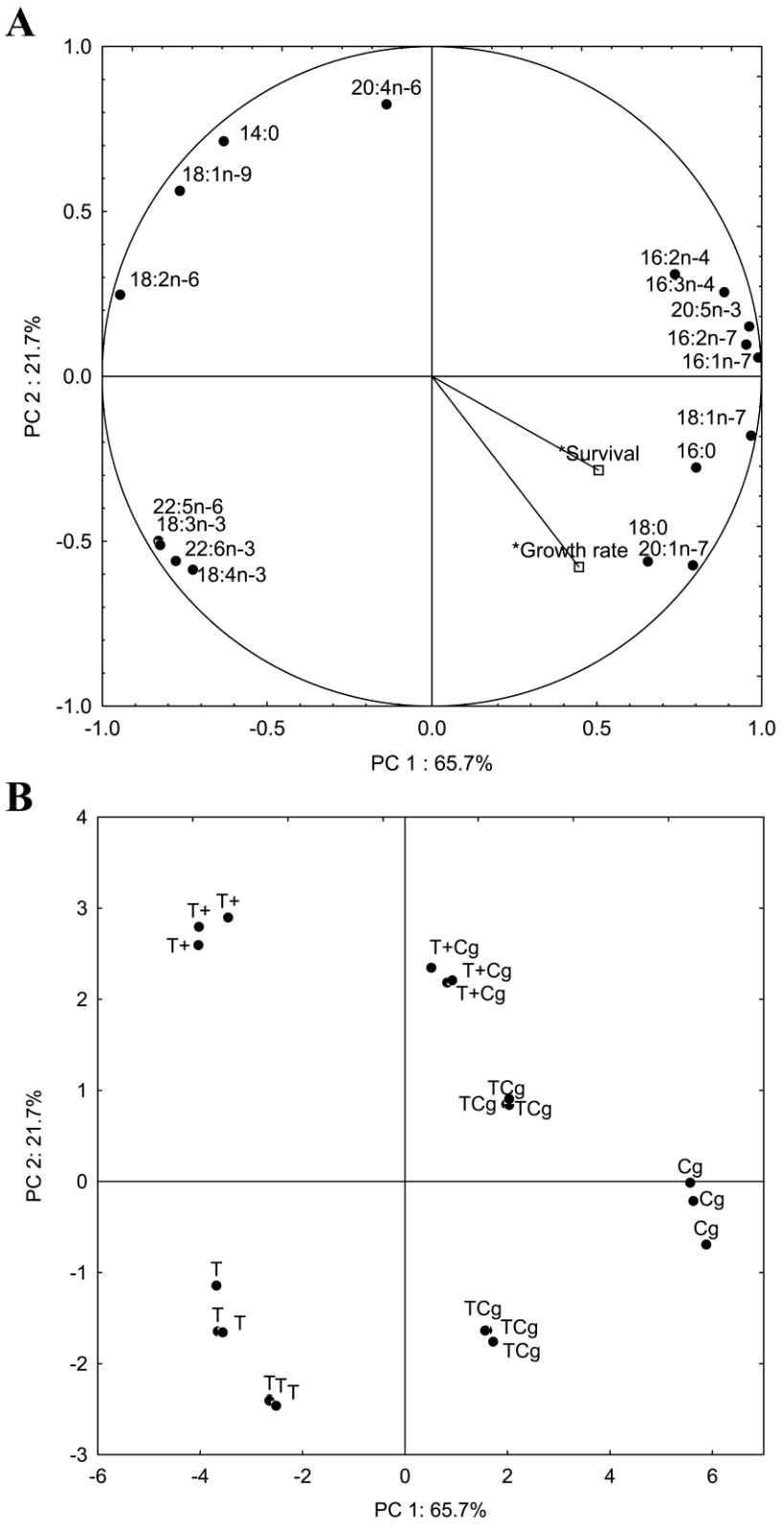

Figure 5. Relationship between FA of the larvae on day 15-16 and growth rate (µm day^{-1}; grouped data from experiments 1 and 2). A. 18:1n-9 in reserve lipids. B. 18:1n-9 in membrane lipids. C. 20:1n-7 in membrane lipids. D. 22:2j in membrane lipids.

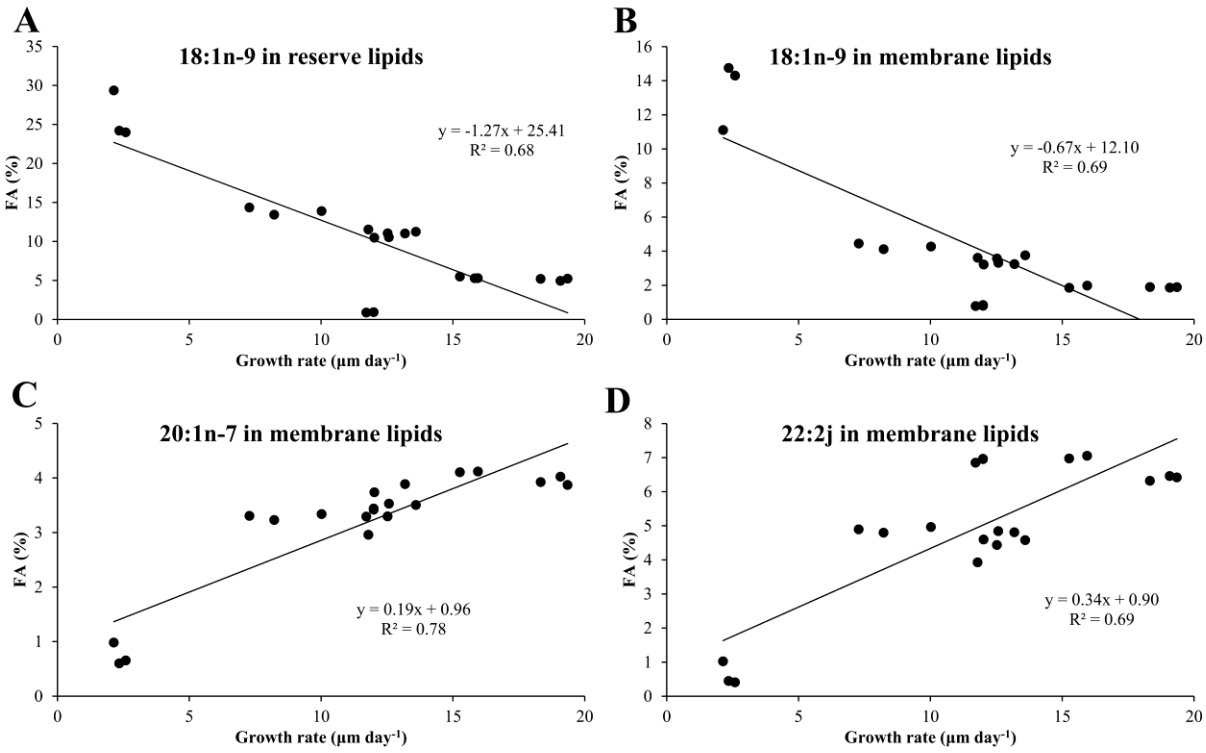

Figure 6. PCA plot of FA and sterol variables in membranes, growth rate and survival on day 15-16 (grouped data from experiments 1 and 2) according to the two principal components 1 and 2. A. Correlation circle. B. Factorial plan. tDehyd: tDehydrocholesterol; Brass: brassicasterol; Chol: cholesterol; Desm: desmosterol; 24-meth: 24-methylene-cholesterol.

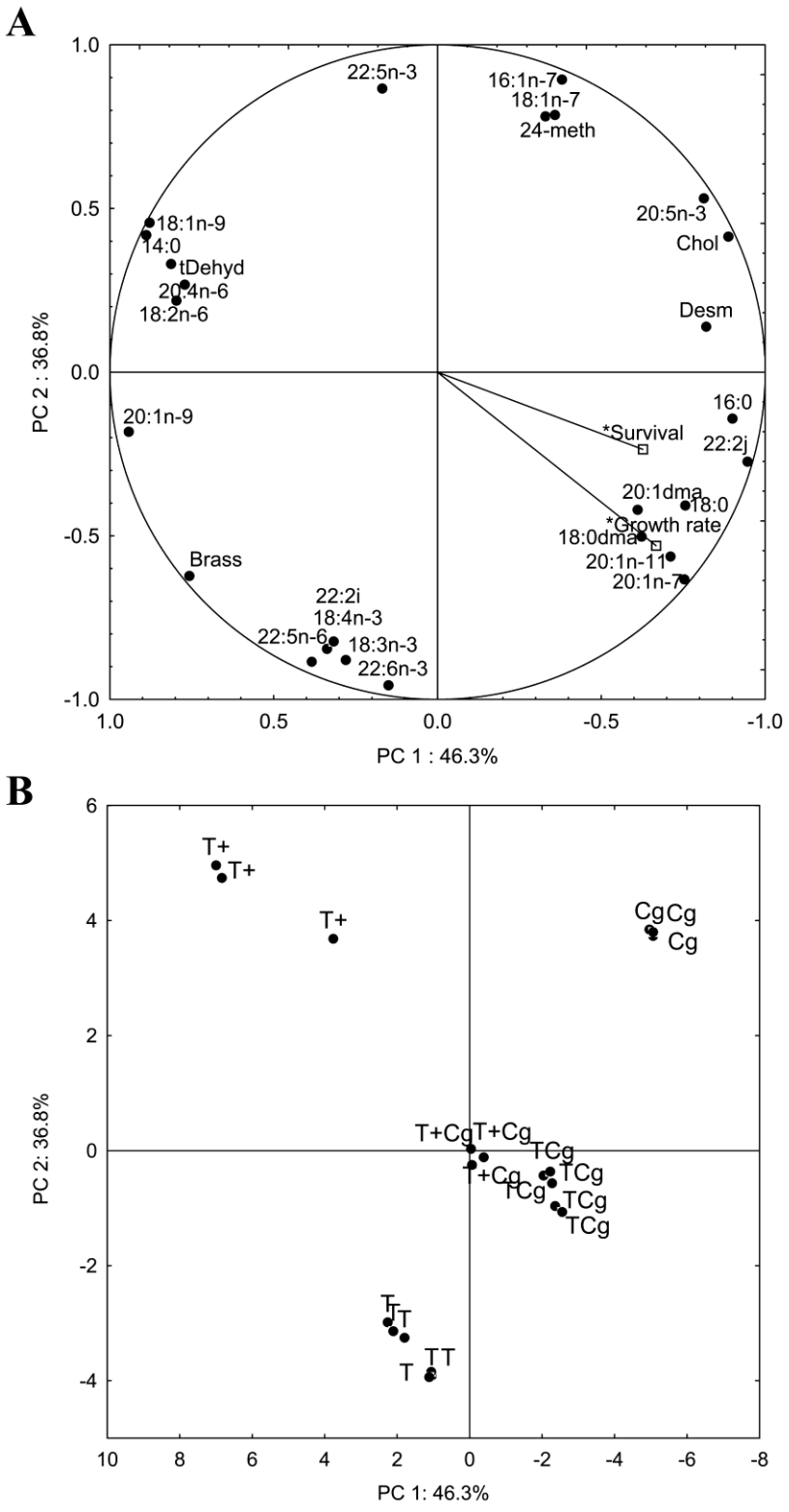

Table 1 Dry and organic weight, ash content and gross composition of the diets in experiment 1, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile* (mean ± S.D., n = 5). OM: organic matter.

	T	T+	Cg	TCg
Dry weight (pg cell^{-1})	12.3 ± 2.5^{b}	17.1 ± 3.7^{a}	16.7 ± 4.2^{a}	13.0 ± 0.6^{ab}
Organic weight (pg cell^{-1})	10.7 ± 1.9	12.7 ± 7.5	11.7 ± 3.2	12.0 ± 3.4
Ash (% dry weight)	12.7 ± 4.0^{b}	12.9 ± 1.9^{b}	23.8 ± 3.7^{a}	13.5 ± 0.6^{b}
Lipids (g Kg^{-1} OM)	481.8 ± 38.7^{b}	691.8 ± 61.1^{a}	547.5 ± 26.3^{b}	490.0 ± 64.9^{b}
Proteins (g Kg^{-1} OM)	296.7 ± 51.4^{b}	124.3 ± 16.6^{c}	367.9 ± 12.0^{a}	367.9 ± 12.0^{a}
Carbohydrates (g Kg^{-1} OM)	225.3 ± 35.9^{a}	168.9 ± 44.7^{a}	84.5 ± 24.9^{b}	167.5 ± 68.4^{a}

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

Table 2 Fatty acid, sterol and neutral lipid class composition of the total lipids (relative content) of the different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, expressed as the mean relative content (wt% of total fatty acids or sterols ± S.D., n = 12 for grouped data of experiments 1 and 2). Summary values are also reported as concentration, rather than percent, at the end of each grouping. ∑ SFA: Total saturated fatty acids; ∑ MUFA: Total monounsaturated fatty acids; ∑ PUFA: Total polyunsaturated fatty acids; AL: Alcohols; ALK1: alkenones 1; ALK2: alkenones 2; FA: Fatty acid; FFA: free fatty acids; ST: sterols; TAG: triacylglycerol.

FA	T	T+	Cg	TCg	T+Cg
14:0	17.8 ± 3.0	31.6 ± 1.0	8.3 ± 0.9	14.2 ± 1.9	25.9 ± 2.1
16:0	13.0 ± 1.3	13.9 ± 0.7	24.4 ± 1.4	15.8 ± 3.4	15.9 ± 2.5
18:0	0.5 ± 0.4	0.4 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	0.5 ± 0.1
16:1n-9	0.9 ± 0.5	0.4 ± 0.1	0.9 ± 0.5	0.9 ± 0.4	0.5 ± 0.3
16:1n-7	3.6 ± 0.5	2.2 ± 0.2	37.8 ± 2.9	17.4 ± 3.0	11.2 ± 1.6
18:1n-9	12.6 ± 1.9	27.6 ± 0.9	1.0 ± 0.1	6.9 ± 1.0	19.2 ± 1.4
18:1n-7	1.9 ± 0.4	1.5 ± 0.3	1.0 ± 0.2	1.6 ± 0.4	1.5 ± 0.4
16:2n-7	0.4 ± 0.1	0.1 ± 0.0	2.2 ± 0.3	1.4 ± 0.2	0.9 ± 0.2
16:2n-4	0.7 ± 0.1	0.2 ± 0.1	1.5 ± 0.3	1.7 ± 0.8	1.5 ± 0.6
16:3n-4	0.1 ± 0.1	0.0 ± 0.0	5.3 ± 0.8	4.1 ± 1.1	3.4 ± 0.6
18:2n-6	4.3 ± 1.1	5.8 ± 0.7	0.9 ± 0.2	2.7 ± 0.8	3.7 ± 1.6
18:3n-6	0.5 ± 0.2	0.1 ± 0.0	0.4 ± 0.1	0.5 ± 0.2	0.3 ± 0.1
18:3n-3	6.1 ± 0.9	3.0 ± 0.3	0.1 ± 0.0	3.1 ± 0.6	2.0 ± 0.3
18:4n-3	14.7 ± 1.3	6.3 ± 0.5	0.3 ± 0.1	8.0 ± 0.8	4.8 ± 0.7
18:5n-3	2.0 ± 0.6	0.2 ± 0.1	0.0 ± 0.0	1.2 ± 0.4	0.2 ± 0.1
20:4n-6	0.2 ± 0.1^{c}	0.3 ± 0.0^{b}	0.3 ± 0.1^{ab}	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}
20:5n-3	0.5 ± 0.1^{d}	0.2 ± 0.0^{d}	9.5 ± 2.8^{a}	6.2 ± 1.6^{b}	4.5 ± 1.0^{c}
22:5n-6	2.0 ± 0.2	0.9 ± 0.1	0.0 ± 0.0	1.1 ± 0.1	0.7 ± 0.2
22:6n-3	12.3 ± 1.3^{a}	5.6 ± 0.5^{c}	0.6 ± 0.2^{e}	6.6 ± 0.9^{b}	3.8 ± 2.0^{d}
∑ SFAs	33.2 ± 2.6^{c}	46.7 ± 1.6^{a}	34.6 ± 1.9^{c}	31.8 ± 3.2^{c}	43.4 ± 4.4^{b}
∑ MUFAs	20.4 ± 2.3	33.0 ± 0.8^{b}	43.2 ± 2.2^{a}	28.4 ± 3.6^{c}	31.2 ± 6.9^{b}
∑ n-9	13.9 ± 2.2	28.6 ± 0.9	2.1 ± 0.6	7.8 ± 1.4	17.4 ± 7.2
∑ n-7	6.2 ± 0.6	4.4 ± 0.5	39.5 ± 1.9	19.5 ± 3.1	13.1 ± 1.6
∑ PUFAs	45.0 ± 1.7^{a}	20.4 ± 3.5^{d}	22.2 ± 3.5^{cd}	38.1 ± 4.9^{b}	23.7 ± 3.2^{c}
∑ n-4	0.9 ± 0.4	0.3 ± 0.1	6.9 ± 1.0	5.9 ± 1.8	4.9 ± 1.2
∑ n-6	7.1 ± 1.7	3.9 ± 3.3	3.3 ± 1.2	4.8 ± 1.0	1.7 ± 0.4
∑ n-3	36.3 ± 2.4	16.0 ± 1.4	11.0 ± 3.1	25.4 ± 3.1	15.8 ± 3.0
n-3/n-6	5.6 ± 2.2	7.3 ± 3.9	3.7 ± 1.6	5.2 ± 0.9	9.4 ± 2.0
22:6/20:5	26.2 ± 4.9	22.5 ± 1.2	0.1 ± 0.0	1.1 ± 0.4	0.9 ± 0.5
22:5/20:4	16.1 ± 6.4	3.6 ± 0.4	0.2 ± 0.1	3.5 ± 1.0	1.7 ± 0.3
Total FA (fg cell^{-1})	1361.9 ± 156.4^{c}	4333.1 ± 752.9^{a}	3814.3 ± 1035.8^{ab}	1730.0 ± 254.4^{c}	3700.2 ± 576.6^{b}
Sterols					
Cholesterol	0.0 ± 0.0	1.0 ± 2.0	54.2 ± 0.9	39.5 ± 11.1	17.4 ± 2.2
Brassicasterol	100.0 ± 0.0	97.8 ± 3.7	0.0 ± 0.0	38.1 ± 8.4	66.7 ± 4.0
24-methylene-cholesterol	0.0 ± 0.0	1.3 ± 2.2	8.4 ± 1.9	6.3 ± 1.9	5.6 ± 1.2
Fucosterol	0.0 ± 0.0	0.0 ± 0.0	32.8 ± 2.2	18.7 ± 4.7	9.5 ± 1.5
Isofucosterol	0.0 ± 0.0	0.0 ± 0.0	4.5 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
Total Sterols (fg cell^{-1})	17.2 ± 2.6^{c}	139.9 ± 23.3^{a}	131.8 ± 32.7^{a}	48.5 ± 16.5^{b}	129.2 ± 18.6^{a}
Total Alkenones (fg cell^{-1})	323.2 ± 98.1^{a}	119.7 ± 20.5^{b}	0.0 ± 0.0^{d}	292.0 ± 90.5^{a}	71.8 ± 8.6^{c}
Neutral lipid classes					
TAG	24.8 ± 7.1^{e}	94.0 ± 1.5^{a}	79.4 ± 7.2^{c}	31.3 ± 4.2^{d}	83.5 ± 3.8^{b}
FFA	2.1 ± 0.6^{c}	1.1 ± 0.4^{d}	4.6 ± 2.2^{a}	3.0 ± 2.1^{c}	4.2 ± 1.1^{b}
AL	1.4 ± 0.5^{c}	1.3 ± 1.0^{c}	1.4 ± 0.7^{bc}	2.7 ± 1.7^{a}	2.4 ± 0.6^{ab}
ST	5.2 ± 3.1^{c}	2.4 ± 1.0^{d}	14.5 ± 4.7^{a}	10.4 ± 5.0^{b}	8.6 ± 2.9^{b}
ALK1	48.5 ± 8.4^{a}	0.8 ± 0.3^{c}	0.0 ± 0.0^{d}	33.8 ± 10.6^{b}	0.7 ± 0.2^{c}
ALK2	18.1 ± 6.9^{a}	0.4 ± 0.1^{b}	0.0 ± 0.0^{c}	14.4 ± 4.9^{a}	0.5 ± 0.1^{b}

Total Neutral Lipids (fg cell^{-1})	626.3 ± 169.2^{b}	2803.7 ± 458.2^{a}	701.2 ± 172.9^{b}	629.0 ± 191.9^{b}	2176.1 ± 463.8^{a}

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

Table 3 Mean percentage ± S.D. of pediveligers ready to settle at the end of the rearing period, mean percentage of metamorphosis ± S.D. 12 and 15 days later for experiment 1 and 2, respectively; and final yield percentage ± S.D. of *Crassostrea gigas* larvae fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids, and Cg = *Chaetoceros neogracile.* Data are expressed as percentage of total larvae numbered at the last sampling.

Diet	Morphological competence (%)			Metamorphosis (%)	Final yield (%)
	Day 15-16	Day 18	Day 19		
Experiment 1					
T	13.4 ± 4.1[b]	60.5 ± 3.6[a]	n.d.	33.3 ± 2.7[a]	3.2 ± 1.7[b]
T+	0.0 ± 0.0[d]	n.d.	n.d.	n.d.	n.d.
Cg	0.6 ± 0.5[c]	34.0 ± 4.4[b]	61.9 ± 2.0	20.5 ± 6.7[a]	3.3 ± 0.0[b]
TCg	69.6 ± 2.8[a]	n.d.	n.d.	37.7 ± 8.9[a]	20.9 ± 1.1[a]
Experiment 2					
T	0.0 ± 0.0[c]	n.d.	54.4 ± 9.0	30.6 ± 6.8[b]	8.2 ± 1.4[b]
T+	n.d.	n.d.	n.d.	n.d.	n.d.
TCg	89.0 ± 4.0[a]	n.d.	n.d.	60.2 ± 2.5[a]	52.0 ± 10.0[a]
T+Cg	3.2 ± 2.6[b]	n.d.	n.d.	n.d.	n.d.

n.d. no data. For each of the experiments values with same letters in the same column and day are not significantly different at $P > 0.05$.

Table 4 Dry and organic weight, ash content and gross composition of *Crassostrea gigas* larvae (experiment 1) fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, on days 2 (initial) and 16 (mean ± S.D., n = 3). OM: organic matter.

		Dry weight (µg larva^{-1})	Organic weight (µg larva^{-1})	Ash (% dry weight)	Lipid (g Kg^{-1} OM)	Protein (g Kg^{-1} OM)	Carbohydrates (g Kg^{-1} OM)
Initial		0.1 ± 0.0	0.0 ± 0.0	72.1 ± 0.9	401.2 ± 105.7	564.3 ± 101.4	34.4 ± 6.3
Day 16	T	2.9 ± 0.4^{b}	1.1 ± 0.1^{b}	61.8 ± 2.5^{b}	191.7 ± 9.3^{b}	760.1 ± 12.8^{a}	48.2 ± 4.1^{a}
	T+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Cg	2.4 ± 0.3^{b}	0.8 ± 0.1^{c}	65.9 ± 0.7^{b}	182.5 ± 16.1^{b}	773.8 ± 14.8^{a}	43.8 ± 5.8^{ab}
	TCg	5.5 ± 0.6^{a}	2.0 ± 0.1^{a}	63.2 ± 1.1^{b}	175.1 ± 5.6^{b}	780.4 ± 5.4^{a}	44.6 ± 1.7^{ab}
	Unfed	0.4 ± 0.0^{d}	0.1 ± 0.0^{d}	80.3 ± 4.0^{a}	551.3 ± 87.0^{a}	402.4 ± 80.7^{b}	46.3 ± 8.3^{a}

n.d. no data. Values with same letters in the same column are not significantly different at $P > 0.05$.

Table 5 Neutral lipid classes of *Crassostrea gigas* larvae fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, on days 2 (initial) and 15-16, expressed as the mean relative content (wt% of total neutral lipid classes ± S.D., n = 3). Total neutral lipids were calculated as the sum of quantified neutral lipid classes. AL: Alcohols; ALK1: alkenones 1; ALK2: alkenones 2; FFA: free fatty acids; ST: sterols; TAG: triacylglycerol; %NL: percentage calculated as %NL/(NL+PL).

		TAG	FFA	AL	ST	ALK1	ALK2	TAG/ST	Total NL (ng larva^{-1})	%NL
Experiment 1										
Initial		71.7 ± 1.9	19.1 ± 1.8	0.0 ± 0.0	9.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	7.8 ± 0.4	3.2 ± 0.0	49.6 ± 1.1
Day 16	T	87.7 ± 1.0^{a}	0.8 ± 0.5^{b}	0.8 ± 0.1^{b}	8.9 ± 0.5^{c}	1.4 ± 0.1^{b}	0.5 ± 0.0^{a}	9.9 ± 0.6^{b}	119.0 ± 16.8^{ab}	54.9 ± 1.7^{b}
	T+	66.7 ± 8.3^{d}	26.1 ± 7.1^{a}	1.0 ± 0.4^{b}	5.0 ± 1.5^{d}	1.0 ± 0.2^{c}	0.2 ± 0.1^{b}	17.0 ± 0.1^{a}	169.6 ± 37.1^{a}	78.3 ± 4.8^{a}
	Cg	77.8 ± 1.0^{c}	3.3 ± 2.4^{b}	0.5 ± 0.2^{b}	18.4 ± 1.4^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	4.2 ± 0.3^{d}	44.0 ± 10.7^{c}	30.3 ± 7.5^{cd}
	TCg	84.7 ± 0.2^{ab}	1.2 ± 0.4^{b}	0.4 ± 0.0^{b}	11.5 ± 0.4^{c}	1.9 ± 0.2^{a}	0.2 ± 0.0^{b}	7.3 ± 0.2^{c}	187.3 ± 7.7^{a}	34.9 ± 0.7^{c}
	Unfed	0.0 ± 0.0^{e}	31.8 ± 9.8^{a}	12.3 ± 2.3^{a}	55.9 ± 10.9^{a}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{e}	0.8 ± 0.3^{d}	17.7 ±2.1^{d}
Experiment 2										
Initial		87.2 ± 0.5	3.0 ± 0.5	0.0 ± 0.0	9.9 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	8.8 ± 0.2	3.3 ± 0.3	50.4 ± 2.7
Day 15	T	87.4 ± 0.4^{a}	1.8 ± 0.4^{a}	0.8 ± 0.3^{a}	8.0 ± 0.3^{b}	1.3 ± 0.2^{a}	0.8 ± 0.2^{a}	11.0 ± 0.5^{a}	105.2 ± 18.0^{b}	59.6 ± 1.4^{a}
	TCg	88.5 ± 1.4^{a}	1.2 ± 0.3^{a}	0.7 ± 0.2^{a}	8.2 ± 0.9^{b}	1.1 ± 0.2^{a}	0.4 ± 0.0^{a}	10.9 ± 1.3^{a}	372.8 ± 6.0^{a}	60.4 ± 2.0^{a}
	T+Cg	86.8 ± 1.5^{a}	2.7 ± 1.1^{a}	0.0 ± 0.0^{b}	9.9 ± 0.4^{a}	0.4 ± 0.1^{b}	0.3 ± 0.1^{b}	8.8 ± 0.5^{b}	91.9 ± 3.9^{b}	56.8 ± 2.0^{a}

For each of the experiments values with same letters in the same column are not significantly different at $P > 0.05$.

Table 6 Polar lipid classes of *Crassostrea gigas* larvae fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, on days 2 (initial) and 15-16, expressed as the mean relative content (wt% of total polar lipid classes ± S.D., n = 3). PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; PE: phosphatidylethanolamine; CL: cardiolipin; %PL: percentage calculated as %PL/(NL+PL).

		PE	PI	CL	PS	PC	Total PL (ng larva^{-1})	%PL
Experiment 1								
Initial		29.6 ± 0.4	21.6 ± 1.2	5.0 ± 0.4	6.7 ± 0.3	37.1 ± 1.3	3.2 ± 0.1	50.4 ±1.1
Day 16	T	29.4 ± 0.6^{a}	20.3 ± 0.6^{b}	7.5 ± 0.1^{a}	7.6 ± 0.1^{ab}	35.3 ± 0.6^{a}	97.2 ± 6.7^{b}	45.1 ± 1.7^{c}
	T+	17.8 ± 9.1^{b}	30.8 ± 5.5^{a}	6.4 ± 1.3^{ab}	12.0 ± 3.0^{a}	33.0 ± 3.1^{a}	34.8 ± 11.3^{c}	21.7 ± 4.8^{d}
	Cg	31.3 ± 1.4^{a}	19.2 ± 0.2^{b}	6.7 ± 0.7^{ab}	7.7 ± 0.3^{ab}	35.2 ± 1.6^{a}	101.6 ± 11.5^{b}	69.7 ± 7.5^{b}
	TCg	29.2 ± 0.5^{a}	24.6 ± 0.7^{a}	3.3 ± 0.2^{c}	4.5 ± 0.3^{b}	38.4 ± 0.7^{a}	349.0 ± 14.9^{a}	65.1 ± 0.7^{b}
	Unfed	36.7 ± 9.3^{a}	25.2 ± 9.4^{a}	9.6 ± 2.6^{a}	9.9 ± 2.7^{a}	18.5 ± 4.6^{b}	3.8 ± 1.3^{d}	82.3 ± 2.1^{a}
Experiment 2								
Initial		26.3 ± 0.6	18.3 ± 0.3	6.4 ± 0.7	8.1 ± 0.6	41.0 ± 0.6	3.3 ± 0.5	49.6 ± 2.7
Day 15	T	25.0 ± 0.8^{a}	23.3 ± 1.9^{a}	7.3 ± 0.4^{a}	7.6 ± 0.2^{b}	36.9 ± 1.5^{a}	71.7 ± 14.5^{b}	40.4 ± 1.4^{a}
	TCg	25.2 ± 1.0^{a}	19.2 ± 0.5^{b}	7.1 ± 0.3^{a}	8.3 ± 0.4^{a}	40.3 ± 2.0^{a}	244.4 ± 20.2^{a}	39.6 ± 2.0^{a}
	T+Cg	24.1 ± 0.6^{a}	21.6 ± 2.2^{b}	7.9 ± 0.6^{a}	8.1 ± 0.2^{ab}	38.3 ± 2.2^{a}	69.9 ± 3.0^{b}	43.2 ± 2.0^{a}

For each of the experiments values with same letters in the same column are not significantly different at $P > 0.05$.

Table 7 Fatty acid composition of neutral lipids of *Crassostrea gigas* larvae fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, on days 2 (initial) and 15-16, expressed as the mean relative content (wt% of total FA in neutral lipid fraction ± S.D., n = 3). ∑ SFA: Total saturated fatty acids; ∑ DMA: Total dimethyl acetals; ∑ MUFA: Total monounsaturated fatty acids; ∑ PUFA: Total polyunsaturated fatty acids; ∑ NMI: Total non-methylene interrupted-fatty acids.

Fatty acid	Experiment 1						Experiment 2			
	Initial	Day 16					Initial	Day 15		
		T	T+	Cg	TCg	Unfed		T	TCg	T+Cg
20:4n-6	1.2 ± 0.0	0.7 ± 0.1^{cd}	1.1 ± 0.1^{b}	0.8 ± 0.0^{c}	0.5 ± 0.0^{d}	2.7 ± 0.2^{a}	1.3 ± 0.1	0.9 ± 0.1^{b}	1.0 ± 0.0^{a}	1.1 ± 0.1^{a}
20:5n-3	7.2 ± 0.1	1.9 ± 0.0^{c}	1.7 ± 0.1^{c}	15.4 ± 0.7^{a}	6.7 ± 0.2^{b}	1.8 ± 0.8^{c}	9.3 ± 0.3	2.4 ± 0.4^{c}	11.3 ± 0.1^{a}	8.5 ± 0.5^{b}
22:6n-3	9.1 ± 0.1	15.2 ± 0.6^{a}	6.9 ± 0.4^{b}	0.9 ± 0.0^{c}	7.0 ± 0.1^{b}	0.6 ± 0.5^{c}	8.3 ± 0.1	17.5 ± 0.2^{a}	6.4 ± 0.1^{b}	4.4 ± 0.13^{c}
∑ SFAs	36.9 ± 0.5	25.2 ± 0.6^{c}	30.4 ± 1.0^{b}	28.9 ± 1.6^{b}	29.5 ± 1.5^{b}	51.3 ± 3.5^{a}	36.1 ± 0.9	23.8 ± 0.4^{b}	23.0 ± 0.3^{c}	30.6 ± 0.4^{a}
∑ DMAs	0.3 ± 0.0	0.3 ± 0.0^{b}	0.2 ± 0.0^{b}	0.6 ± 0.0^{bc}	0.5 ± 0.0^{b}	1.8 ± 0.9^{a}	0.5 ± 0.2	0.3 ± 0.0^{b}	0.3 ± 0.1^{b}	0.5 ± 0.1^{a}
∑ MUFAs	26.0 ± 0.1	23.7 ± 0.5^{c}	38.1 ± 3.1^{a}	38.2 ± 0.9^{a}	30.7 ± 0.5^{b}	24.2 ± 0.6^{c}	24.3 ± 0.5	21.3 ± 0.1^{c}	29.3 ± 0.3^{b}	33.5 ± 0.3^{a}
∑ n-9	7.9 ± 0.1	12.4 ± 0.3^{b}	31.0 ± 2.5^{a}	1.6 ± 0.1^{d}	6.3 ± 0.1^{c}	10.7 ± 0.8^{b}	6.8 ± 0.1	12.4 ± 0.3^{b}	5.9 ± 0.1^{c}	15.1 ± 0.5^{a}
∑ n-7	16.2 ± 0.2	9.5 ± 0.2^{c}	6.2 ± 0.2^{d}	33.2 ± 0.7^{a}	21.9 ± 0.3^{b}	10.2 ± 0.9^{c}	15.4 ± 0.1	7.9 ± 0.3^{c}	22.1 ± 0.3^{a}	17.3 ± 0.7^{b}
∑ PUFAs	36.2 ± 0.4	49.5 ± 0.6^{a}	29.7 ± 2.5^{c}	30.1 ± 0.8^{c}	37.2 ± 1.1^{b}	19.8 ± 1.3^{d}	38.7 ± 0.5	53.9 ± 0.4^{a}	46.8 ± 0.1^{b}	34.0 ± 0.3^{c}
∑ n-4	1.0 ± 0.0	0.6 ± 0.0^{c}	0.3 ± 0.0^{d}	5.9 ± 0.1^{a}	3.4 ± 0.2^{b}	0.1 ± 0.0^{d}	1.4 ± 0.2	0.9 ± 0.1^{c}	8.4 ± 0.1^{a}	5.5 ± 0.5^{b}
∑ n-6	6.9 ± 0.1	8.8 ± 0.1^{b}	9.8 ± 0.9^{a}	2.7 ± 0.1^{d}	5.2 ± 0.1^{c}	9.4 ± 0.7^{ab}	6.0 ± 0.2	10.8 ± 0.3^{a}	6.5 ± 0.1^{b}	6.5 ± 0.3^{b}
∑ n-3	23.0 ± 0.2	38.4 ± 0.6^{a}	19.2 ± 1.6^{c}	17.7 ± 0.8^{c}	25.7 ± 0.7^{b}	4.9 ± 0.5^{d}	25.6 ± 0.4	40.8 ± 0.4^{a}	29.2 ± 0.1^{b}	19.5 ± 0.3^{c}
∑ NMI	4.1 ± 0.1	1.0 ± 0.1^{cd}	0.2 ± 0.1^{d}	1.9 ± 0.1^{b}	1.3 ± 0.1^{bc}	3.3 ± 0.9^{a}	5.0 ± 0.5	0.7 ± 0.1^{b}	0.9 ± 0.0^{a}	0.8 ± 0.0^{a}
n-3/n-6	3.4 ± 0.0	4.4 ± 0.1^{c}	2.0 ± 0.0^{d}	6.5 ± 0.2^{a}	5.0 ± 0.1^{b}	0.5 ± 0.0^{e}	4.3 ± 0.2	3.8 ± 0.1^{b}	4.5 ± 0.1^{a}	3.0 ± 0.2^{c}
22:6/20:5	1.3 ± 0.0	8.1 ± 0.2^{a}	4.2 ± 0.5^{b}	0.1 ± 0.0^{d}	1.1 ± 0.0^{c}	0.4 ± 0.3^{d}	0.9 ± 0.0	7.3 ± 0.9^{a}	0.6 ± 0.0^{b}	0.5 ± 0.0^{b}
22:5/20:4	1.3 ± 0.1	3.9 ± 0.3^{a}	1.3 ± 0.2^{c}	0.1 ± 0.0^{d}	2.2 ± 0.0^{b}	0.3 ± 0.2^{d}	0.9 ± 0.0	3.3 ± 0.3^{a}	1.1 ± 0.0^{b}	0.6 ± 0.0^{c}
ng larva^{-1}	2.0 ± 0.2	61.2 ± 10.6^{ab}	85.6 ± 36.7^{a}	34.4 ± 7.0^{bc}	84.0 ± 9.1^{a}	0.5 ± 0.2^{c}	2.6 ± 0.6	30.2 ± 5.0^{b}	100.0 ± 9.9^{a}	43.4 ± 9.5^{b}

For each of the experiments values with same letters in the same row are not significantly different at $P > 0.05$.

Table 8 Fatty acid composition of polar lipids of *Crassostrea gigas* larvae fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, on days 2 (initial) and 15-16, expressed as the mean relative content (wt% of total FA in polar lipid fraction ± S.D., n = 3). ∑ SFA: Total saturated fatty acids; ∑ DMA: Total dimethyl acetals; ∑ MUFA: Total monounsaturated fatty acids; ∑ PUFA: Total polyunsaturated fatty acids; ∑ NMI: Total non-methylene-interrupted-fatty acids.

Fatty acid	Experiment 1						Experiment 2			
	Initial	Day 16					Initial	Day 15		
		T	T+	Cg	TCg	Unfed		T	TCg	T+Cg
20:4n-6	3.6 ± 0.1	2.5 ± 0.1^{b}	3.4 ± 0.3^{b}	2.3 ± 0.1^{b}	2.6 ± 0.6^{b}	5.8 ± 1.6^{a}	3.8 ± 0.1	2.7 ± 0.3^{ab}	2.4 ± 0.0^{b}	3.1 ± 0.2^{a}
20:5n-3	11.0 ± 0.3	3.4 ± 0.1^{d}	4.4 ± 0.3^{d}	21.7 ± 0.3^{a}	9.9 ± 0.9^{b}	6.6 ± 2.0^{c}	12.2 ± 0.6	3.9 ± 0.3^{b}	10.0 ± 0.2^{a}	10.1 ± 0.6^{a}
22:6n-3	18.5 ± 0.8	20.0 ± 0.3^{a}	8.9 ± 1.6^{c}	5.9 ± 0.0^{d}	17.4 ± 2.7^{a}	13.5 ± 1.1^{b}	16.7 ± 0.9	20.3 ± 0.6^{a}	15.2 ± 0.1^{b}	14.5 ± 0.2^{b}
∑ SFAs	22.9 ± 2.2	18.5 ± 0.5^{b}	24.0 ± 1.3^{a}	17.8 ± 0.0^{b}	19.2 ± 0.6^{b}	23.8 ± 4.9^{a}	21.9 ± 1.1	19.6 ± 0.7^{a}	18.7 ± 0.3^{b}	18.6 ± 0.3^{b}
∑ DMAs	8.5 ± 0.5	11.7 ± 0.7^{a}	2.4 ± 0.7^{c}	11.3 ± 0.5^{ab}	6.2 ± 0.4^{c}	9.3 ± 0.6^{bc}	10.9 ± 0.5	10.4 ± 1.7^{a}	12.2 ± 0.7^{a}	11.2 ± 0.6^{a}
∑ MUFAs	16.7 ± 0.5	19.8 ± 0.6^{c}	36.9 ± 2.2^{a}	23.2 ± 0.3^{b}	21.3 ± 0.3^{bc}	22.5 ± 1.4^{b}	15.6 ± 1.1	18.5 ± 0.6^{c}	20.2 ± 0.2^{b}	22.2 ± 0.6^{a}
∑ n-9	3.3 ± 0.2	5.3 ± 0.3^{b}	22.6 ± 5.0^{a}	2.0 ± 0.1^{b}	3.0 ± 0.1^{b}	5.0 ± 0.4^{b}	2.7 ± 0.3	5.4 ± 0.2^{b}	3.3 ± 0.1^{c}	6.2 ± 0.2^{a}
∑ n-7	10.5 ± 0.3	9.6 ± 0.3^{d}	11.6 ± 1.9^{bc}	15.8 ± 0.2^{a}	12.6 ± 0.9^{b}	10.2 ± 0.8^{cd}	9.3 ± 0.3	9.4 ± 0.7^{c}	13.5 ± 0.1^{a}	11.7 ± 0.3^{b}
∑ PUFAs	51.7 ± 1.7	49.6 ± 0.9^{ab}	30.5 ± 0.9^{c}	46.0 ± 0.4^{ab}	51.2 ± 2.5^{a}	44.6 ± 6.1^{b}	51.3 ± 1.1	51.1 ± 1.1^{a}	48.7 ± 0.4^{b}	47.0 ± 1.0^{b}
∑ n-4	0.2 ± 0.0	0.2 ± 0.0^{bc}	0.1 ± 0.0^{bc}	0.9 ± 0.0^{a}	0.5 ± 0.0^{b}	0.1 ± 0.1^{c}	0.3 ± 0.0	0.7 ± 0.1^{b}	1.3 ± 0.1^{a}	1.2 ± 0.1^{a}
∑ n-6	9.8 ± 0.3	9.3 ± 0.1^{b}	9.9 ± 0.9^{b}	5.0 ± 0.4^{c}	8.4 ± 0.5^{b}	12.9 ± 2.3^{a}	8.2 ± 0.2	10.6 ± 0.4^{a}	8.4 ± 0.0^{c}	8.9 ± 0.2^{b}
∑ n-3	32.8 ± 1.2	32.3 ± 1.0^{a}	18.6 ± 0.9^{c}	31.7 ± 0.4^{a}	33.3 ± 2.7^{a}	23.7± 3.5^{b}	33.0 ± 1.1	32.2 ± 0.7^{a}	30.6 ± 0.4^{b}	28.9 ± 0.9^{c}
∑ NMI	8.9 ± 0.3	7.9 ± 0.2^{a}	1.6 ± 0.7^{b}	8.1 ± 0.3^{a}	8.6 ± 0.8^{a}	7.9 ± 0.3^{a}	9.7 ± 0.4	7.5 ± 0.3^{b}	8.2 ± 0.1^{a}	7.8 ± 0.2^{ab}
n-3/n-6	3.4 ± 0.0	3.5 ± 0.1^{b}	1.9 ± 0.2^{c}	6.3 ± 0.1^{a}	4.0 ± 0.6^{b}	1.8 ± 0.1^{c}	4.0 ± 0.1	3.1 ± 0.1^{c}	3.7 ± 0.0^{a}	3.3 ± 0.1^{b}
22:6/20:5	1.7 ± 0.0	5.8 ± 0.2^{a}	2.0 ± 0.2^{b}	0.3 ± 0.0^{c}	1.8 ± 0.4^{b}	2.1 ± 0.4^{b}	1.4 ± 0.0	5.2 ± 0.3^{a}	1.5 ± 0.0^{b}	1.4 ± 0.1^{b}
22:5/20:4	1.0 ± 0.0	1.5 ± 0.1^{a}	0.6 ± 0.1^{b}	0.1 ± 0.0^{c}	1.3 ± 0.4^{a}	0.4 ± 0.1	0.7 ± 0.0	1.5 ± 0.1^{a}	1.2 ± 0.0^{b}	0.9 ± 0.1^{c}
ng larva^{-1}	1.1 ± 0.1	40.6 ± 4.4^{b}	14.5 ± 3.4^{d}	32.9 ± 5.6^{c}	65.8 ± 1.4^{a}	1.3 ± 0.6^{e}	1.5 ± 0.1	26.8 ± 2.6^{b}	95.2 ± 13.5^{a}	35.2 ± 9.5^{b}

For each of the experiments values with same letters in the same row are not significant at $P > 0.05$.

Table 9 Relative content of sterols of *Crassostrea gigas* larvae fed different mono- or bi-specific diets, with T = *Tisochrysis lutea* (clone T-*Iso*), T+ = T-*Iso* rich in lipids and Cg = *Chaetoceros neogracile*, on days 2 and 15-16 (wt% of total sterols ± S.D., n = 3).

	Experiment 1						Experiment 2 (day 15)			
	Initial	Day 16					Initial	Day 15		
		T	T+	Cg	TCg	Unfed		T	TCg	T+Cg
Norcholesterol	n.d.	n.d.	1.8 ± 1.0	n.d.	n.d.	n.d.	2.7 ± 0.2	n.d.	n.d.	n.d.
tDehydrocholesterol	n.d.	n.d.	1.4 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cDehydrocholesterol	1.7 ± 0.1	1.8 ± 0.1^{b}	3.4 ± 0.8^{a}	0.9 ± 0.2^{c}	0.6 ± 0.1^{cd}	n.d.	3.9 ± 0.4	1.4 ± 0.3^{a}	0.6 ± 0.0^{b}	0.7 ± 0.1^{b}
Dihydrocholesterol	n.d.	0.5 ± 0.1^{b}	n.d.	1.6 ± 0.3^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	0.8 ± 0.2
Cholesterol	33.5 ± 0.3	5.8 ± 0.1^{c}	6.7 ± 2.0^{c}	58.2 ± 1.1^{a}	38.7 ± 0.8^{b}	62.9 ± 17.3^{a}	36.4 ± 1.5	6.2 ± 1.5^{c}	41.2 ± 0.6^{a}	32.3 ± 1.0^{b}
Brassicasterol	34.4 ± 0.3	78.2 ± 1.0^{a}	62.9 ± 4.7^{b}	2.4 ± 0.2^{e}	37.3 ± 0.8^{c}	23.8 ± 1.6^{d}	25.6 ± 1.7	83.7 ± 2.7^{a}	37.7 ± 0.2^{c}	46.8 ± 0.3^{b}
Desmosterol	7.3 ± 0.4	1.8 ± 0.0^{c}	0.9 ± 0.0^{d}	7.9 ± 0.2^{a}	6.9 ± 0.1^{b}	n.d.	8.2 ± 0.8	2.7 ± 0.7^{c}	9.4 ± 0.5^{a}	7.7 ± 0.8^{b}
Campesterol	n.d.	1.1 ± 0.1^{b}	2.5 ± 0.1^{a}	0.2 ± 0.0^{d}	0.7 ± 0.1^{c}	n.d.	n.d.	1.2 ± 0.1^{a}	n.d.	0.7 ± 0.1^{a}
24-methylene-cholesterol	7.3 ± 0.1	2.1 ± 0.2^{d}	5.2 ± 0.3^{c}	8.3 ± 0.2^{b}	4.8 ± 0.4^{c}	12.2 ± 2.6^{a}	10.2 ± 0.6	2.2 ± 1.2^{a}	2.3 ± 0.1^{a}	2.7 ± 0.3^{a}
Stigmasterol	7.6 ± 0.1	1.6 ± 0.3^{b}	2.4 ± 0.4^{a}	n.d.	n.d.	n.d.	5.6 ± 1.4	2.6 ± 1.8^{a}	1.4 ± 0.1^{a}	1.7 ± 0.5^{a}
4α-methylporiferasterol	n.d.	2.7 ± 0.1^{a}	2.3 ± 0.2^{b}	0.7 ± 0.2^{d}	1.1 ± 0.2^{c}	n.d.	n.d.	n.d.	0.6 ± 0.3^{a}	0.5 ± 0.1^{a}
β-sitosterol	2.1 ± 0.4	2.5 ± 0.2^{c}	5.7 ± 1.3^{b}	1.1 ± 0.1^{d}	1.5 ± 0.3^{cd}	10.5 ± 0.4^{a}	2.6 ± 0.8	n.d.	0.5 ± 0.0^{b}	1.1 ± 0.4^{a}
Fucosterol	5.9 ± 0.1	1.6 ± 0.0^{d}	2.1 ± 0.1^{c}	17.3 ± 0.2^{a}	7.7 ± 0.1^{b}	n.d.	3.2 ± 0.6	n.d.	6.5 ± 0.1^{a}	4.4 ± 0.4^{b}
Isofucosterol	n.d.	0.4 ± 0.0^{d}	2.7 ± 0.2^{a}	1.6 ± 0.0^{b}	0.7 ± 0.1^{c}	n.d.	1.1 ± 0.9	n.d.	n.d.	0.8 ± 0.3
ng larva^{-1}	0.3 ± 0.0	9.5 ± 2.0^{b}	9.7 ± 3.8^{b}	7.5 ± 1.2^{b}	18.7 ± 1.3^{a}	0.2 ± 0.0^{c}	0.3 ± 0.0	6.5 ± 0.5^{b}	23.9 ± 2.9^{a}	8.6 ± 0.6^{b}

n.d.: not detected. For each of the experiments values with same letters in the same row are not significantly different at $P > 0.05$.