Larval development and fatty acid composition of *Ostrea edulis* (L.) fed four different single diets from conditioning to pre-settlement

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Abstract :

Survival, growth and fatty acid composition of *Ostrea edulis* larvae (L.) fed four different single species, microalgal diets, *Tisochrysis lutea* (*T*)*, Chaetoceros neogracile* (*Cg*), *Skeletonema marinoi* (*Sm*) or *Tetraselmis suecica* (*Ts*) from broodstock to pre-settlement, were studied. Lower larval growth (5.5 µm to 6.5 µm/d) was recorded in progeny continuously fed single *S. marinoi* or *T. suecica*, whereas good growth was achieved with single *T. lutea* (7.8 µm/d). Larvae, originated from broodstock receiving *Sm* or *Ts*, exhibited growth compensation when fed a bispecific balanced diet (*TCg*). This did not occur when broodstock and larvae were fed *Cg* or *T,* for which single or mixed diets led to similar larval growth. Furthermore, survival was high (>90%) regardless of microalgal diet, except for larvae fed from broodstock to pre-settlement *T* (53%) or *Ts* (2%). There were significant differences in 20:5 (n-3) and 22: 6 (n-3) contents in polar and neutral fractions of *O. edulis* expelled larvae dependent on broodstock diet, as well as throughout larval development, but no clear trend was apparent when comparing fatty acid (FA) relative composition of both fractions of *O. edulis* larvae fed different diets at release or prior to settlement. In contrast, such correlation occurred when FA was expressed in absolute content but exclusively for larvae-fed single diets and was particularly noticeable between 22: 6 (n-3) and growth and survival. In the present work, broodstock nutritional deficiencies have been revealed in *O. edulis* progeny, compensated thereafter by feeding the larvae a mixed diet, and in this balanced condition, no obvious relation with larval development indicators was found with main fatty acid contents.

Keywords : broodstock conditioning, diet, larval development, *Ostrea edulis*

Introduction

Broodstock conditioning is an important step in the molluscan hatchery process. Indeed, conditioning allows the extension of the reproductive period (Helm, Bourne & Lovatelli, 2004) and leads generally to better gamete quality (Utting & Millican, 1997). The importance of temperature in molluscan reproduction has been thoroughly described (e.g. Mann, 1979; Fabioux, Huvet, Le Souchu, Le Pennec & Pouvreau, 2005; Maneiro, Silva, Pazos, Sánchez & Pérez-Parallé, 2017a), but the role of nutrition in quantitative aspects of reproduction is being recognized increasingly (e.g. Enriquez-Diaz, Pouvreau, Chavez-Villalba & Le Pennec, 2009). In *O. edulis*, a 6% daily ration has been shown to be highly effective for broodstock conditioning (Utting, 1993), and the importance of food quality based on different microalgal diets was pointed out by Millican and Helm (1994). Since that pioneering work, only a few dedicated studies have been carried out (e.g. Berntsson, Jonsson, Wängberg & Carlsson. 1998; Maneiro, Pérez-Parallé, Silva, Sánchez & Pazos, 2017b). Indeed research on *O. edulis* reproduction was shelved following *Marteilia refringens* and *Bonamia ostreae* disease outbreaks from 1970-1980 that caused a rapid decline in the flat oyster industry in France (Buestel, Ropert, Prou & Goulletquer, 2009) and throughout Europe (Laing, Walker & Areal, 2005; Bromley, McGonigle, Ashton & Roberts, 2016). However, *O. edulis* remains an emblematic species in Brittany (North West of France), with an annual production of 1000 - 1500 tons (Robert, Sanchez, Pérez-Parallé, Ponis, Kamermans & O'Mahoney, 2013). This interest increased quite recently because of high oyster *C. gigas* juvenile mortalities (e.g. Petton, Pernet, Robert & Boudry, 2013; Azema, Lamy, Boudry, Renault, Travers & Degremont, 2017). Accordingly, we revisited flat oyster feeding requirements during conditioning and showed that, based

upon ecophysiological and biochemical studies, some microalgae more effectively supported gametogenesis (González-Araya, Quéau, Quéré, Moal & Robert, 2011; González-Araya, Lebrun, Quéré & Robert, 2012a). Specificall, *Chaetoceros neogracile* and *Skeletonema marinoi* were ingested and assimilated well; whereas, *Tetraselmis suecica* was poorly absorbed with no significant enrichment in the main fatty acids and sterols in any tissue at the end of a 6 week feeding experiment. *Tisochrysis lutea* was found to be moderately effective as a diet (assimilation efficiency = 20%) and a highly efficient source of essential lipids (e.g 22:5 (n-6)) (González-Araya et al., 2011). On the other hand, larval development has shown to be related to the type of food delivered during conditioning (Berntsson et al., 1997), with apparent but inconsistent compensation when larvae are fed a mixed diet (González-Araya et al., 2012b). Moreover, several studies (Helm, Holland, Utting & East, 1991, Jonsson, Berntsson, André & Wängberg, 1999) have suggested that some PUFAs are essential for survival and development of *O. edulis* larvae as demonstrated for *C. gigas* larvae (da Costa, Robert, Quéré, Wikfors & Soudant, 2015).

Such interactions between broodstock diets and subsequent larval development were addressed in the present work by feeding *O. edulis* larvae the same single diets as broodstock and comparing larval performance to larvae fed a bispecific diet used as reference for oyster development in our laboratory (Rico Villa, Pouvreau & Robert, 2009; Petton, Le Souchu, Mingant & Robert, 2009). We hypothesised that broodstock nutritional deficiencies could be revealed and compensated at larval development by feeding the larvae a mixed, balanced diet.

Materials and methods

Broodstock conditioning and larval rearing

A total of 360 18-month-old flat oysters (≈ 5 cm length and 0.5 g flesh dry weight) originating from Brittany (Bay of Cancale, France) were distributed homogeneously in translucent, 50-L tanks (30 oysters per tank for an equivalent biomass: 1kg total weight and 16 g dry flesh weight). Triplicate tanks were set up for each of the four single species diets tested. Seawater was maintained at 19 °C in a flow-through system at a flow rate of 12 L h^{-1}, and the oysters were fed continuously by means of a peristaltic pump, at 900 µm^3 µL^{-1} per feeding condition to limit pseudofaeces deposition (González-Araya et al., 2011). Seawater at ambient salinity (34 g L^{-1}) was filtered on 1-µm polypropylene filter media following UV treatment. Four microalgal diets were delivered during broodstock conditioning (40 days): *Tisochrysis lutea* (formely named *Isochrysis* affinis *galbana* or *T-Iso*: CCAP 927/14 = *T*), *Chaetoceros neogracile* (UTEX LB2658 = *Cg*), *Skeletonema marinoi* (CCAP 66/4 = *Sm*), or *Tetraselmis suecica* (CCAP 1077/3= *Ts*). Microalgal size, growth, biochemical profiles, and other characteristics have been already detailed in exponential and stationary phases (Robert et al., 2004; González-Araya et al., 2011). Accordingly, only a brief outline will be given here with total fatty acid compositions, expressed as relative and absolute contents (Table 1). From each treatment, released larvae were distributed in 5-L, translucent, methacrylate cylinders and reared in flow-through conditions with UV treatment (González-Araya et al., 2012 b). Larval expulsion was asynchronous, but broodstock fed different diets released first batches of larvae within 10 days meaning that they were not reared simultaneously but in a closed window-time (April). Larvae at a density of 5 mL^{-1} were

distributed, in triplicate for each larval diet, in seawater at 22 °C and 34 g L^{-1} salinity. Larvae were fed continuously the same single-species, microalgal diets as delivered to broodstock at 1,500 µm^3 µL^{-1} equivalent *T. lutea* volume (≈ 40 cells) or the mixed diet *T. lutea* plus *C. neogracile* (*TCg*: v/v), which has been shown to be an effective bi-specific diet for *Crassostrea gigas* larvae (Rico-Villa, Le Coz, Mingant & Robert, 2006; Ben Kheder, Quéré, Moal & Robert, 2010). Each broodstock diet was split into treatments with two larval diets, *i.e.,* 8 different larval conditions were assessed: **B***T***L***T*, **B***T***L***TCg*, **B***Cg***L***Cg*, **B***Cg***L***TCg*, **B***Sm***L***Sm*, **B***Sm***L***TCg*, **B***Ts***L***Ts*, and **B***Ts***L***TCg*. Thus, the designation **B***T***L***T* means that broodstock (**B**) was fed *T. lutea (T)* and larvae (**L**) expelled from them were also fed *T. lutea*; whereas, **B***Sm***L***TCg* means that broodstock was fed *S. marinoi* and larvae expelled from them were fed *T. lutea* plus *C. neogracile* (*TCg*).

Larval length and survival were quantified on days 3, 6, 9 and 11 under the light microscope by use of an image analysis technique (WinImager 2.0 and Imaq Vision Builder 6.0 software for image capture and treatment, respectively) and direct counting of empty shells for dead larvae estimation. The number of pediveligers ready to set (competent larvae possessing an eyespot) was estimated at the end of the experiment, on day 11.

Biochemical procedures

About 10 000 larvae were sampled, on each larval treatment, at 0 (released larvae) and 11 days (end of larval rearing); the samples were collected by filtration on pre-ignited (at 450° C) GF/D filter pads (3 µm porosity). For lipid extraction, the filter pads were placed separately in tubes containing a mixture of chloroform-methanol (2:1, v/v) with

0.01 wt% butylated hydroxytoluene (BHT) as antioxidant, closed under nitrogen, shaken and frozen at -20°C.

An aliquot of the sample (chloroform-methanol mixture) was evaporated to dryness, with a known amount of 23:0 fatty acid (as internal standard) and lipids were recovered with three washings of 500 µl each of $CHCl_3$-MeOH (98:2, v/v). This was placed on top of silica gel microcolum (30 x 5 mm ID, Kieselgel, 70-230 mesh (Merk), previously heated to 450° C and deactivated with 5% water (Marty, Delaunay, Moal & Samain, 1992). The neutral lipids were eluted with 10 ml of $CHCl_3$-MeOH (98:2) and the polar lipids were recovered with 10 ml of MeOH. The neutral and polar fractions were taken for methylation and analysis by chromatography.

Statistical procedures

Analysis of variance (ANOVA) was used to test for treatment effects. Statistical analyses were performed using STATISTICA software (version 8.0). Significant differences were detected between the means at the 5% threshold and a posteriori multiple comparison test between the means (Tukeys' test), after transformation of percentage data by the function [arcsin (racine x i/100)].

Results

Larval growth

The size of expelled larvae ranged from 172 µm (± 2.1) to 179 µm (± 2.7) with no significant difference when oysters were fed either diatoms or *T. lutea*. In contrast,

newly-released larvae were significantly smaller (p< 0.01) when broodstock were fed *T. suecica* (165 μm ± 3.5).

Larvae originating from broodstock fed *S. marinoi* and fed a similar diet had lower growth (Fig. 1A), with a daily length increment of ≈ 5.5 μm d^{-1} (Fig. 2A). The supply of a mixed larval diet (*TCg*) improved growth somewhat (Fig. 1B), with daily growth of 6.0 μm d^{-1} (Fig. 2A). Similarly larvae fed *T. suecica* as broodstock exhibited relatively good growth until day 7 (Fig. 1A), with a daily length increment of 6 μm d^{-1} (Fig. 2A); whereas, a noticeable improvement in larval development was recorded when these larvae were fed mixed diet *TCg* (Fig. 1B), leading to a daily length increment of ≈ 8.0 μm d^{-1} (Fig. 2A). Larvae originated from broodstock fed *T. lutea* or *C. neogracile* exhibited the highest growth when fed broodstock-based diets (Fig. 1A), with 7.5 and 7.1 μm d^{-1} daily length increments, respectively (Fig. 2A), but no significant differences (p>0.05) were found when larvae were fed mixed diets (Fig 1B), with daily length increments of 8.3 and 7.8μm d^{-1} respectively (Fig. 2A).

Larval survival

Under most experimental conditions, larval survival was high, > 90% on day 11, except when larvae and broodstock were fed single *T. lutea* (53%) or *T. suecica* (2% : Fig. 2B). For this last condition, mortalities took place progressively with moderate larval survival on day 3 (40%) increasing to (100%) on day 7; whereas, larvae fed *T. lutea* exhibited low mortalities until day 11 when mortality was sudden.

Larval competence

No clear relationship between diet and competence to metamorphosis wa found (Fig. 2C). At the end of the larval rearing period (day 11), 25% of pediveligers were eyed when larvae originating from broodstock fed *T* or *Ts,* were fed *TCg*. However, eyed larvae accounted for 46-50% of the total in larvae originating from broodstock fed *C. neogracile* or *S. marinoi*, respectively (Fig. 2C).

Biochemical composition

The biochemical contents of the four microalgae used here as food f230or *O. edulis* have been described in a previous work reporting on broodstock conditioning (González-Araya et al., 2010), so only a brief outline will be given here. With 10.2% of total fatty acids, *T. lutea* was rich in 22:6 (n-3) (DHA) but very poor (0.33%) in 20:5(n-3) (EPA). With ≈ 22-23% of total fatty acids, *C. neogracile* and *S. marinoï* were rich in 20:5 (n-3) but relatively poor (1.1%) to moderate (4.4%) in 22:6 (n-3). With 30.8% *T. suecica* was rich in 16:0 (*vs* 6.7-11.1% for the other species). In contrast, with only 0.23% *T. suecica* almost lacked 22:6 (n-3); whereas, it contained moderate amount (4.4%) of 20:5 (n-3).

16:0, 20:5 (n-3) and 22:6 (n-3) were the dominant fatty acids in *O. edulis* larvae with values varying from 6.7% to 21.6% in polar fraction (Table 2) and 3.3% to 23.3% in neutral fraction (Table 4) regardless larval stage or diet.

At release, a similar relative amount of 16:0 in the polar fraction was recorded in larvae regardless broodstock diet, with values fluctuating from 12.3% to 13.7% (Table 2). In contrast, when expressed in absolute content, the larvae originating from broodstock fed

S. marinoi exhibited a significantly lower concentration (p<0.05) with 0.7 ng larva^{-1} *vs* 1.1-1.6 ng larva^{-1} for the other diets (Table 3). Similarly, significant differences (p<0.05) in 16:0 relative content were found in neutral fraction of expelled larvae, with values varying from 14% to 23.3%; accordingly, broodstock diet interfered with initial biochemical composition of larval storage (Table 4). Such effects were also found when fatty acid composition was expressed in absolute content with, however, no differences (p<0.05) in initial larval biochemical content when broodstock were fed *T. lutea* or *T. suecica* (Table 5). Despite initial stores, no apparent enrichment in 16:0 occurred during *O. edulis* larval development because 16:0 relative content remained quite similar (p> 0.05), with values varying from 10.6 to 15.9% in larval polar fraction (Table 2). In contrast, there was an overall decrease in this FA in the larval neutral fraction throughout larval development regardless of diet (Table 4).

There were significant differences (p<0.05) in 20:5 (n-3) relative and absolute contents within *O. edulis* expelled larvae dependent upon broodstock diet, with values fluctuating from 6.7% to 19.1% and from 0.7 to 2.4 ng larva^{-1} in the polar fraction (Tables 2, 3) and from 3.3% to 15.8% and 3.5 to 25.1 ng larva^{-1} in the neutral fraction (Tables 4, 5). Thus, larvae originating from broodstock fed *C. neogracile* or *S. marinoi* exhibited the highest EPA concentration in both fractions (polar: 19.1% and 17.1% (Table 2); neutral: 15.8% and 14.7% (Table 4) respectively). When expressed as absolute FA content, this was the case only for *C. neogracile,* with 2.4 ng larva^{-1} and 25.1 ng larva^{-1} in the polar and neutral fractions, respectively (Tables 3, 5). In contrast, progeny originating from broodstock fed *T. lutea* showed the lowest relative contents (6.7% and 3.3%, respectively, in polar and neutral fractions: Tables 2, 4); a finding confirmed when FA composition was expressed as absolute content for the neutral fraction (Table 5) but not for the polar fraction (Table 3). Surprisingly the initial content

of expelled larvae originated form broodstock fed *T. suecica* exhibited relatively high percentage content of 20:5 (n-3) in both fractions (15.2 and 11.9% respectively in polar and neutral fractions: Tables 2, 4), which was confirmed for the absolute content (1.2 ng larva^{-1} and 9.3 ng larva^{-1} respectively in polar and neutral fractions: Tables 3, 5). In both fractions, 20:5 (n-3) concentration increased significantly ($p<0.05$) in larvae fed *T. lutea* that had originated from broodstock also fed *T. lutea* (15.9% *vs* 6.7% and 18.1% *vs* 3.3%, respectively in polar and neutral fractions: Tables 2, 4). This enhancement was more acute when FA composition was expressed in absolute content (13.3 *vs* 0.7 ng larva^{-1} and 333.5 *vs* 3.5 ng larva^{-1} respectively in polar and neutral fractions: Tables 3, 5) A similar finding was obtained with broodstock and larvae solely fed *C. neogracile,* with EPA larval relative content increasing to 25.7% *vs* 19.1% and 24.2% *vs* 15.8%, respectively, in polar and neutral fractions: Tables 2, 4); an acute enhancement in the neutral fraction was also found when expressed as absolute FA content (235.8 ng larva^{-1} *vs* 25.1 ng larva^{-1} (Table 5).

With $R^2 = 0.10$, a non-significant ($p>0.05$) linear relationship between mean daily growth and EPA relative content was found, regardless of larval diet. In contrast, when expressed in absolute FA content, a relationship with growth occurred for larvae fed single diets ($R^2 = 0.51$: Fig. 3A) but did not exist for larvae fed mixed diets ($R^2 = 0.03$: Table 6 and Fig. 3C). Similar, general trends were confirmed for the other larval development descriptors (e.g., competence: Table 6 and Fig. 3 B, D).

There were significant differences ($p<0.05$) in 22:6 (n-3) content of *O. edulis* expelled larvae between broodstock diet treatments, with values fluctuating from 12.5% to 21.6% in the polar fraction (Table 2) and from 8.0% to 15.5% in the neutral fraction (Table 4), which was confirmed when FA composition was expressed as absolute content for the neutral fraction (Table 5) but not for the polar fraction (Table 3). Larvae

originating from broodstock fed *T. lutea* or *T. suecica* exhibited the highest DHA concentrations in both fractions (polar: 21.6% and 20.2% (Table 2); neutral: 15.5% and 13.6% (Table 4) respectively). However such a relationship was only confirmed for *T. lutea* when biochemical composition was expressed in absolute content in the neutral fraction (Table 5). With the exception of larvae expelled from broodstock fed *S. marinoi*, 22:6 (n-3) concentration decreased significantly ($p<0.05$) in both fractions throughout larval development, regardless of larval diet (Tables 2, 4); whereas, no clear trend could be established when FA composition of larvae was expressed as absolute content (Tables 3, 5).

With $R^2 = 0.09$, a non-significant ($p>0.05$) linear relation between mean daily growth and DHA relative content was found. In contrast, when expressed in absolute FA content, a significant relationship with growth was found for larvae fed single diets ($R^2 = 0.82$: Fig. 3A), but not for larvae fed mixed diets ($R^2 = 0.03$: Table 6 and Fig. 3C). A similar, general trend was confirmed for the other larval development descriptors and specifically for survival ($R^2 = 0.97$ *vs* 0.19 : Table 6).

Diets had lower influence on 20:4(n-6) (ARA) content of expelled larvae, in both fractions (polar: 3.7% to 4.7% (Table 2); neutral: 1.0% to 2.4% (Table 4),) as well as throughout larval development during which significant ($p<0.05$) decreases occurred for larvae fed *C. neogracile* or *T. suecica* (Tables 2, 4). This trend was not confirmed when FA composition was expressed in absolute content (Tables 3, 5).

With $R^2 = 0.02$, no linear relationship between mean daily growth and DHA relative content was found. In contrast, when expressed as absolute FA content, a relationship with growth was found for larvae fed single diets ($R^2 = 0.74$: Fig. 3A), but did not exist for larvae fed mixed diets ($R^2 = 0.00$: Table 6 and Fig. 3C). Similar, general trends were

confirmed for the other larval development descriptors, and for survival ($R^2 = 0.82$ *vs* 0.15 : Table 6).

Discussion

Live microalgae are essential for successful larval performance, especially bivalve metamorphosis that relies on energy reserves stored during two developmental stages (Labarta, Fernandez-Reiriz & Perez-Camacho, 1999). The first stage corresponds to embryonic development wherein endogenous reserves are provided by the egg (Bayne, 1973), and the second stage prior to metamorphosis, which depends on reserves stored during all of larval life. The latest depends on the food value of algal diets supplied to growing larvae combined with endogenous reserves (Whyte, Bourne & Ginther, 1990). The nutritional value of different live microalgae is determined by ingestibility (cell size/shape), digestibility, and nutrient content (Brown, Jeffrey, Vilkman & Dunstan, 1997). The nutritional role in bivalve nutrition has been emphasized for essential polyunsaturated fatty acids (PUFAs), especially the n-3 fatty acids eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) necessary for successful growth and development (Langdon & Waldock, 1981; Thompson, Guo & Harrison, 1996; Martinez-Fernandez, Acosta-Salmon & Southgate, 2006). These lipids play important structural roles in regulating cell-membrane fluidity and other cellular functions (Hazel, Williams, Livermore, Mozingo, 1991; Hall, Parrish & Thompson, 2002). In general, bivalves have limited or no ability to synthesize PUFAs or to elongate shorter-chained compounds (da Costa et al., 2016) but require these for growth and development (Waldock & Holland, 1984; Whyte, Bourne, & Hodgson, 1989; Chu

& Greaves, 1991; Marty, Delaunay, Moal & Samain, 1992; Delaunay, Marty, Moal & Samain, 1993). Although the effects of nutrition on larval growth and survival have been studied extensively in a wide range of molluscs (see review of Marshall, McKinley & Pearce, 2012), the effects of food on broodstock and subsequent larval development performances have received less attention.

In the present work, initial larval size was related to diet quality expressed as the type of microalgae delivered during broodstock conditioning. Thus, when larval growth relies on energetic reserves stored during embryogenesis, larvae released from broodstock fed single *T. suecica* were probably poorly enriched compared to larvae from broodstock fed other microalgal diets. Such larval size variability upon release (from 178 to 186 µm), related to broodstock diets, have been already reported (Berntsson et al., 1997) with, however, no statistical differences between treatments. This result may have been attributable to the richness of natural seston in incoming seawater. Indeed in the reported experiment, unfed oysters released larvae of similar size to those expelled by fed oysters. In contrast, in our experiment seawater was 1-µm filtered and UV-treated, meaning that there was no apparent bias in our feeding conditions. However, no clear trend could be detectable in fatty acid composition of newly released larvae originated from broodstock fed *T. suecica*, when analysing specific fatty acids such as DHA, with initial content as high as that recorded in progeny originated for broodstock fed *T. lutea* (20 *vs* 22% and 1.6 *vs* 2.2 ng larva^{-1} for relative and absolute contents respectively). A transfer from original oyster reserve (stored before conditioning) might explain that compensation because *T. suecica* lacked DHA (0.23%) and has been shown to be poorly assimilated in gonad and other tissues during *O. edulis* broodstock conditioning (González-Araya et al., 2011). The situation was similar when considering FA ratios as (n-3)/(n-6), 22:6/20:5, 22:5/20:4 that did not reveal any outliers nor explanatory values.

No differences in larval growth were found when larvae were fed single or mixed diets, originated from broodstock previously fed *T. lutea* or *C. neogracile*, with no clear trend in main relative fatty acids composition. Moreover, when fed *T. lutea*, *O. edulis* larval growth was quite similar to that reported with *Isochrysis galbana*, a closely-related haptophyte (9 μm d^{-1}: Ferreiro, Perez-Camacho, Labarta, Beiras, Planas & Fernandez-Reiriz, 1990). In contrast, larvae originating from broodstock fed *S. marinoi* showed lower growth regardless of larval diet, single or mixed. In this case, relative content of 20:5 (n-3) remained constant from release to metamorphosis except when larvae were fed single *S. marinoi* but decreased significantly, in both polar and neutral fractions, when mixed larval diet *TCg* was delivered. This result contrasts with a previous work (Enright, Newkirk, Craigie & Castell, 1986) that classified *S. costatum* (named now *S. marinoi*) as the second highest-ranked diatom for *O. edulis* growth (juvenile). Such apparent contradiction can be explained by the size of this microalga and the stage of bivalve development at which this diatom was delivered. Indeed, *S. marinoi* is a colonial diatom formed of spherical to cylindrical cells joined in chains. Colonies can be longer than 60 μm (Sauriau & Baud, 1994), but in 300-L batch cultures in Argenton, colonies are usually composed of 2 to 4 cells, and the chains rarely exceed 10-15 μm in length (Robert, unpublished data). Accordingly, this phytoplankton size is more appropriate for older bivalve stages than larvae (Robert & Gérard, 1999). When fed *S. marinoi*, *O. edulis* larval performance was, nevertheless, more-closely in agreement with that reported by Ferreiro et al. (1990), who classified this diatom as the worst tested microalga, supporting *O. edulis* larval growth of only 0.4 μm d^{-1} (12- fold lower than in the present work). On the other hand, larvae fed the mixed diet and originating from broodstock fed *T. suecica* showed surprisingly similar growth from day 7 as larvae released by broodstock previously fed *T. lutea* or *C. neogracile*. Larvae fed solely *T.*

suecica exhibited lower growth, reaching however 6 µm d^{-1} (*vs* 7.3 µm d^{-1} for the mixed larval diet). This relatively good larval growth was, however, counterbalanced by very low larval survival (2%) that did not allow biochemical analysis. These results contrast with those reported by Berntsson et al. (1998) for broodstock fed single *T. suecica* that induced higher larval growth (4.5 µm d^{-1}) than larvae originated from broodstock fed *T. suecica* plus *C. calcitrans* (1.7 µm d^{-1}).

No relationships were found between larval and growth main fatty acids when they were expressed in relative content. In contrast, a positive correlation was found with DHA absolute content and growth for single larval diets, regardless of microalgae species; whereas such correlation was not obvious for mixed larval diets. A similar correlation with DHA relative content of released *O. edulis* larvae and larval growth has been already reported (Berntsson et al., 1997) and should explained 50% of the variation in growth rate among broodstock. In contrast these authors did not find any correlation between EPA and growth as pointed out in this present work.

Generally, in the present work larval survival was high (≥ 90%), except for larvae fed *T. suecica* and originating from broodstock also fed *T. suecica* (2%). Our results partially agree with those of Fereiro et al (1990) who reported large survival fluctuations between *O. edulis* larval batches, fed *T. suecica*, from 0 to 100%, with, relatively good growth though (8.7 µm d^{-1}). When broodstock were fed *T. suecica* and released larvae were fed our mixed diet, larval survival was similar to that reported by Berntsson et al. (1998), with survival ≥ 90%. When continuously fed single *T. suecica* from broodstock through the entire larval life, deficiencies might occur, leading to high larval mortality. We noted a similar trend for *T. lutea* with, however, higher survival (53%) but no clear relation with main relative fatty acid content was established. Lastly, competence to metamorphose did not reveal clearly the influence of larval diet when comparing the

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effects of each microalgae. However positive correlations were found with EPA absolute contents and eyed larval percentage, with single larval diets regardless of the species; such a correlation was not significant for mixed larval diets. Larvae continuously fed *S. marinoi*, from broodstock to pre-settlement, exhibited a low level of competence, which is in agreement with corresponding data on growth. A similar trend occurred with larvae fed single *T. suecica*, which is in agreement with data on survival. Moreover, except for *T. lutea*, mixed diets (TC_g) led to higher larval competence, which is partially in agreement with our previous work (González-Araya et al., 2012).

When analysing the overall *O. edulis* development performance, larvae fed single *T. lutea* from broodstock to pre-settlement exhibited deficiencies which were expressed in 50% mortality. Larvae fed *S. marinoi* showed lower growth and competence clearly related to cell size, which was unlikely to have been consumed efficiently this early stage. Lastly, when larvae were fed *T. suecica*, severe nutritional deficiencies occurred, leading to the death of the entire larval population. Regardless of broodstock feeding, however, these potential deficits seem to be mitigated when expelled larvae are fed TC_g, which globally led to better larval performances.

Conclusion

The present work confirmed that some broodstock nutritional deficiencies, originating from broodstock conditioning, could be compensated at early stage development when larvae are fed a mixed, balanced diet. Although larvae fed *T. suecica* from broodstock to pre-settlement exhibited high mortalities and moderate growth, those receiving a bispecific diet displayed good larval performance. Such partial improvement also

occurred with *T lutea* for larval survival. However, to accurately understand the relationships between biochemical transfers from diets to broodstock, from broodstock to initial larvae, and from diets to larvae in development, a more complex experimental design should be carried out. It should be based on single, efficient diets characterized by different fatty acids and should include a starved control to specify the contributions of broodstock and larval reserves. Moreover it would also include metamorphosis determination, because, in contrast with *C. gigas*, *O edulis* larval growth is insufficient to discriminate the effects of diets. Some evidence was found, as mortality data revealed severe deficiencies that could not be related to biochemical composition because insufficient live larvae remained for analysis. Such an experiment will be reported in a forthcoming paper.

Acknowledgments

References

Azema P., Lamy J.B., Boudry P., Renault T., Travers, M.A., Degremont L. (2017) Genetic parameters of resistance to *Vibrio aestuarianus*, and OsHV-1 infections in the Pacific oyster, *Crassostrea gigas*, at three different life stages. *Genetic Selection evolution* **49**. https://doi.org/10.1186/s12711-017-0297-2

Bayne B.L. (1973). Aspects of the metabolism of *Mytilus edulis* during starvation. *Netherland Journal of Sea Research* **7**, 399-441.

Ben Kheder R., Quéré, C., Moal J., Robert R. (2010). Effect of nutrition on *C. gigas* larval development and the evolution of physiological indices. Part A: quantitative and qualitative diet effects. *Aquaculture* **305**, 165-173. https://doi.org/10.1016/j.aquaculture.2010.04.022

Berntsson K.M., Jonsson P.R., Wängberg S.Å., Carlsson A.S. (1998). Effects of broodstock diets on fatty acid composition, survival and growth rates in larvae of the European flat oyster, *Ostrea edulis*. *Aquaculture* **154**, 139-153. https://doi.org/10.1016/S0044-8486(97)00041-0.

Bromley C., McGonigle C., Ashton E.C., Roberts D. (2016). Bad moves: Pros and cons of moving oysters - A case study of global translocations of *Ostrea edulis* Linnaeus, 1758 (Mollusca: Bivalvia). *Ocean & Coastal Management* **122**, 103-115. http://dx.doi.org/10.1016/j.ocecoaman.2015.12.012

Brown M.R., Jeffrey S.W., Vilkman J.K., Dunstan, G.A. (1997). Nutritional properties of microalgae for mariculture. *Aquaculture* **151**, 315-331. https://doi.org/10.1016/S0044-8486(96)01501-3

Buestel D., Ropert M., Prou J., Goulletquer P. (2009). History, status, and future of oyster culture in France. *Journal of Shellfish Research* **28**, 813-820. https://doi.org/10.2983/035.028.0410

Chu F.L.E., Greaves J. (1991). Metabolism of palmitic linoleic and linolenic acids in adult oysters, *Crassostrea virginica*. *Marine Biology* **110**, 229-236. https://doi.org/10.1007/BF01313708

Delaunay F., Marty Y., Moal J., Samain J.F. (1993). The effect of monospecific algal diets on growth and fatty acid composition of *Pecten maximus* (L.) larvae ; *Journal of Experimental Marine Biology and Ecology* **173**, 163-179. https://doi.org/10.1016/0022-0981(93)90051-O

da Costa F., Robert R., Quéré C., Wikfors G., Soudant P. (2015). Essential fatty acid assimilation and synthesis in larvae of the bivalve *Crassostrea gigas*. *Lipids* **50**, 503-511. https://doi.org/10.1007/s11745-015-4006-z

Enright C.T., Newkirk G.F., Craigie J.S., Castell J.D. (1986). Evaluation of phytoplankton as diets for juvenile *Ostrea edulis* L. *Journal of Experimental Marine Biology and Ecology* **96**, 1-13. https://doi.org/10.1016/0022-0981(86)90009-2

Enriquez-Diaz M., Pouvreau S., Chavez-Villalba J., Le Pennec M. (2009). Gametogenesis, reproductive investment, and spawning behavior of the Pacific giant oyster *Crassostrea gigas*: evidence of an environment-dependent strategy. *Aquaculture International* **17**, 491-506. https://doi.org/10.1007/s10499-008-9219-1

Fabioux C., Huvet A., Le Souchu P., Le Pennec M., Pouvreau S. (2005). Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. *Aquaculture* **250**, 458-470. https://doi.org/10.1016/j.aquaculture.2005.02.038

Ferreiro M.J., Perez-Camacho A., Labarta U., Beiras R., Planas M., Fernandez-Reiriz M.J. (1990). Changes in the biochemical composition of *Ostrea edulis* larvae fed on

different food regimes. *Marine Biology* **106**, 395-401. https://doi.org/10.1007/BF01344318

González-Araya R., Quéau I., Quéré C., Moal J., Robert R. (2011). A physiological and biochemical approach to selecting the ideal diet for *Ostrea edulis* (L.) broodstock conditioning (Part A). *Aquaculture Research* **42**, 710-726. 10.1111/j.1365-2109.2010.02731.x

González-Araya R., Lebrun L., Quéré C., Robert R. (2012a). The selection of the ideal diet for *Ostrea edulis* (L.) broodstock conditioning (part B). *Aquaculture* **362-363**, 55-66. https://doi.org/10.1016/j.aquaculture.2012.06.029

González Araya R., Petton B., Mingant C., Robert R. (2012b). Influence of diet assemblage on *Ostrea edulis* broodstock conditioning and subsequent larval development. *Aquaculture* **364-365**, 272-280. http://dx.doi.org/10.1016/j.aquaculture.2012.08.036

Hall, J.M., Parrish, C.C., Thompson, R?J., 2002. Eicosapentaeonic acid regulates scallop (*Placopecten magellanicus*) membrane fluidity in response to cold. *Biology Bulletin* **202**, 201-203. https://doi.org/10.2307/1543469

Hazel J.R., Williams E.E., Livermore R., Mozingo N. (1991). Thermal adaption in biological membranes: functional significance of changes in phospholipid molecular species composition. *Lipids* **26**, 277-282. https://doi.org/10.1007/BF02537137

Helm M.M., Holland D.L., Utting S.D., East J. (1991). Fatty acid composition of early non-feeding larvae of the European flat oyster, *Ostrea edulis* L. *Journal of Marine Biological Association UK* **71**, 691-705.https://doi.org/10.1017/S0025315400053248

Helm M.M., Bourne N., Lovatelli A. (2004). Hatchery culture of bivalves, a practical manual. FAO Fisheries Technical Paper, No.471 FAO, Rome, Italy, 177pp.

Jonsson P. R., Berntsson K.M., André C., Wängberg S.-Å. (1999). Larval growth and settlement of the European oyster (*Ostrea edulis*) as a function of food quality measured as fatty acid composition. *Marine Biology* **143**, 559-570. https://doi.org/10.1007/s002270050571

Labarta U., Fernandez-Reiriz M.J., Perez-Camacho A. (1999). Energy, biochemical substrates and growth in the larval development, metamorphosis and postlarvae of *Ostrea edulis*. *Journal of Experimental Marine Biology and Ecology* **238**, 225-242. https://doi.org/10.1016/S0022-0981(98)00171-3

Laing I., Walker P., Areal F. (2005). A feasibility study of native oyster (*Ostrea edulis*) stock regeneration in the United Kingdom. CARD Project FC1016, Native oyster Stock Regeneration – A review of Biological, Technical and Economic Feasibility, 95 pp.

Langdon C.J., Waldock M.J. (1981). The effect of algal and artificial diets on the growth and fatty acid composition of *Crassostrea gigas* spat. *Journal of Marine Biological Association UK* **61**, 431-448. https://doi: 10.1017/S0025315400047056

Maneiro V., Silva A., Pazos A.J., Sánchez J.L., Pérez-Parallé M.L. (2017a). Effects of temperature and photoperiod on the conditioning of the flat oyster (*Ostrea edulis* L.) in autumn. *Aquaculture Research*, **48**, 4554-4562. https://doi:10.1111/are.13280

Maneiro V., Pérez-Parallé M.L., Silva A., Sánchez J.L., Pazos A.J. (2017b). Conditioning of the European flat oyster (*Ostrea edulis*, Linnaeus 1758): effect of food ration. *Aquaculture Research*, **48**, 4363-4370. https://doi:10.1111/are.13259

Mann R. (1979). Some biochemical and physiological aspects of growth and gametogenesis in *Crassostrea gigas* and *Ostrea edulis* grown at sustained elevated temperatures. *Journal of Marine Biological Association UK* **59**, 546-559. https://doi.org/10.1017/S0025315400046208

Marshall R., Mc Kinley S., Pearce M. C. (2012). Effects of nutrition on larval growtg and survival in bivalves. *Review in Aquaculture* **2**, 33-55. doi: https://10.1111/j.1753-5131.2010.01022.x

Martinez-Fernandez, E., Acosta-Salmon H., Southgate P.C. (2006). The nutritional value of seven species of tropical microalgae for black-lip pearl oyster (*Pinctada margaritifera*, L) larvae. *Aquaculture* **257**, 491-503. https://doi.org/10.1016/j.aquaculture.2006.03.022

Marty Y., Delaunay F., Moal J., Samain J.F. (1992). Changes in the fatty acid composition of *Pecten maximus* (L.) during larval development. *Journal of Experimental Marine Biology and Ecology* **163**, 221–234. https://doi.org/10.1016/0022-0981(92)90051-B

Millican P.F., Helm M.M. (1994). Effects of nutrition on larvae production in the European flat oyster, *Ostrea edulis*. *Aquaculture* **123**, 83-94. https://doi.org/10.1016/0044-8486(94)90121-X

Petton B., Pernet F., Robert R., Boudry P. (2013). Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. *Aquaculture Environment Interactions* **3**, 257-273. https://doi.org/10.3354/aei00070

Petton B., Le Souchu P., Mingant C., Robert R. (2009). Determining hydrobiological larval rearing parameters for *Crassostrea gigas* in small volume flow-through containers. LARVI '09 – Fish & shellfish larviculture symposium., 07-10 September 2009, Ghent, Belgium, Book of Abstract, Special Publication EAS, No 38, 333-334.

Rico-Villa B., Le Coz J.R., Mingant C., Robert R. (2006). Influence of phytoplankton diet mixtures on microalgae consumption, larval development and settlement of the

Pacific oyster *Crassostrea gigas* (Thunberg). *Aquaculture* **256**, 377-388. https://doi.org/10.1016/j.aquaculture.2006.02.015

Rico Villa B., Pouvreau S., Robert R. (2009). Influence of food density and temperature on ingestion, growth and settlement of Pacific oyster larvae *Crassostrea gigas*. *Aquaculture* **287**, 395-401. https://doi.org/10.1016/j.aquaculture.2008.10.054

Robert R., Sanchez J.L., Pérez-Parallé L., Ponis E., Kamermans P., O'Mahoney M. (2013). A glimpse of the mollusc industry in Europe. *Aquaculture Europe* **38**, 5-11.

Robert R., Gérard A. (1999). Bivalve hatchery technology: the current situation for the Pacific oyster *Crassostrea gigas* and the scallop *Pecten maximus* in France. *Aquatic Living Resources* **12**, 121-130. https://doi.org/10.1016/S0990-7440(99)80021-7

Robert R., Chrétiennot-Dinet M.J., Kaas R., Martin-Jézéquel V., Moal J., Le Coz J.R., Nicolas J.L., Bernard E., Connan J.P., Le Dean L., Gourrierec G., Leroy B., Quéré C. (2004). Amélioration des productions phytoplanctoniques en écloserie de mollusques: caractérisation des microalgues fourrage, *RI DRV/RA*-**2004-05**, 149pp.

Sauriau P.G., Baud J.P. (1994). Artificial filament breakage of the diatom *Skeletonema costatum* intended for mollusk aquaculture. *Aquaculture* **123**, 69-81. https://doi.org/10.1016/0044-8486(94)90120-1

Thompson P.A., Guo M., Harrison P.J. (1996). Nutritional value of diets that vary in fatty acid composition for larval pacific oysters (*Crassostrea gigas*). *Aquaculture* **143**, 379-391. https://doi.org/10.1016/0044-8486(96)01277-X

Utting S.D. (1993). Procedures for maintenance and hatchery-conditioning of bivalve broodstocks. *World Aquaculture*, Technical Report, 78-82.

Utting S.D., Millican P.F. (1997). Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability. *Aquaculture* **155**, 45-54. https://doi.org/10.1016/S0044-8486(97)00108-7

Waldock M.J., Holland D.L. (1984). Fatty acid metabolism in young oysters, *Crassostrea gigas*: polyunsaturated fatty acids. *Lipids* **19**, 332-336. https://doi.org/10.1007/BF02534783

Whyte J.N.C., Bourne N., Hodgson C.A. (1989). Influence of algal diets on biochemical composition and energy reserves in *Patinopecten yessoensis* (Jay) larvae. *Aquaculture* **78**, 333-347. https://doi.org/10.1016/0044-8486(89)90110-5

Whyte J.N.C., Bourne N., Ginther N.G. (1990). Biochemical and energy changes during embryogenesis in the rock scallop *Crassadoma gigantea*. *Marine Biology* **106**, 239-244. https://doi.org/10.1007/BF01314806
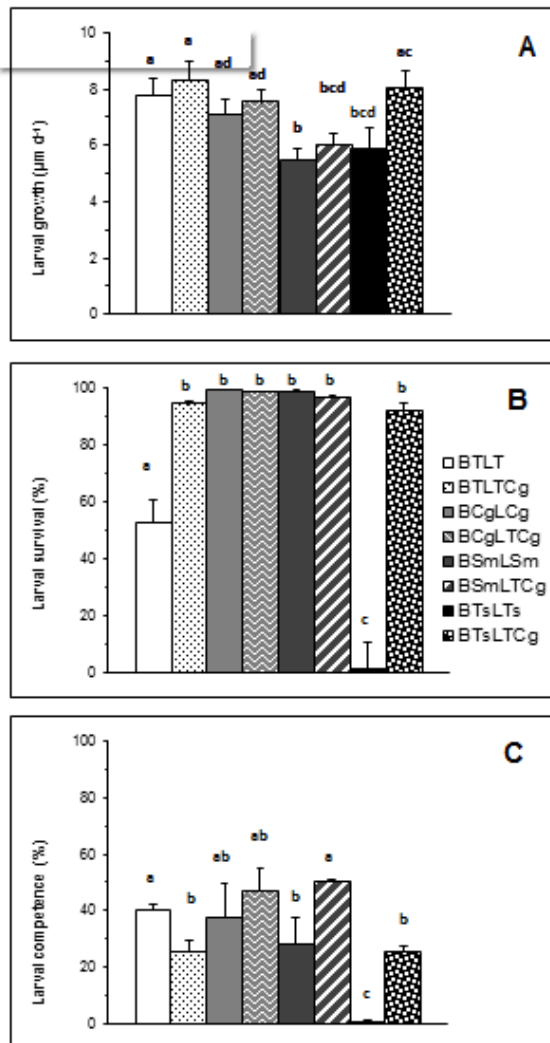
Figures

Figure 1. Mean growth and standard deviation of *O. edulis* larvae fed different single diets: T*: Tisochrysis lutea*, Cg*: Chaetoceros neogracile*, Sm: *Skeletonema marinoi* and Ts*: Tetraselmis suecica* (A) or fed bispecific diet TCg: *T. lutea + C. neogracile* (B), from release (day 0) to day 11. Larvae were originated from parents fed similar single diets (T, Cg, Sm or Ts) during broodstock conditioning.
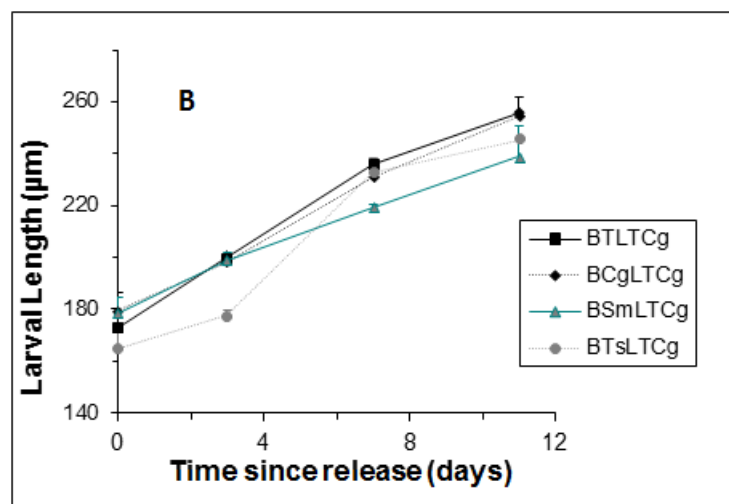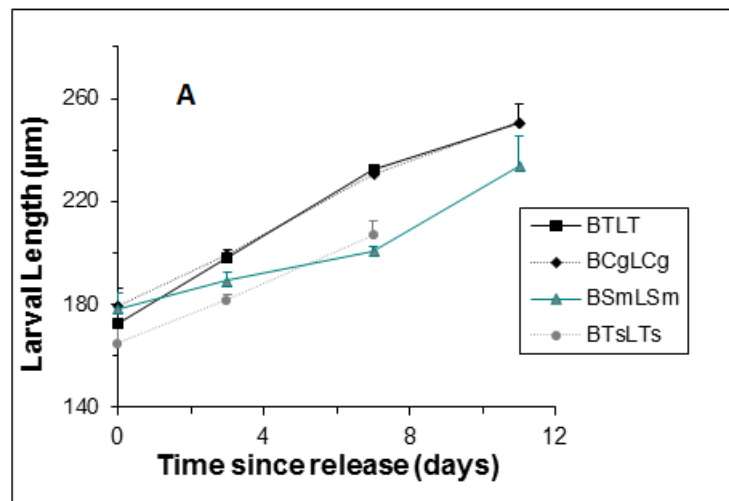
Figure 2. Mean daily growth (A), survival (B) and larval competence (C) (± S.D.) of *O. edulis* larvae (**L**) fed different single diets: T: *Tisochysis lutea*, Cg: *C. neogracile*, Sm: *S. marinoi* and Ts: *T. suecica* or bi-specific diet TCg: *T. lutea + C. neogracile*, on day 11. Larvae were originated from parents fed similar single diets (T, Cg, Sm or Ts) during broodstock conditioning (**B**).

Significant differences (p<0.05) among treatments are denoted by different letters.

Figure 3. Regression analysis of larval development indicators and main fatty acids (AA, EPA, DHA) absolute contents in 11d-old *O. edulis*: **A** daily growth of larvae fed three different single diets (*Tisochrysis lutea*: solid line *Chaetoceros neogracile*: broken line and *Skeletonema marinoï:* dotted line); **B** daily growth of larvae fed mixed diet *T. lutea* plus *C. neogracile*; **C** larval competence of larvae (% eyed larvae) fed three different single diets (*T. lutea*: solid line *C. neogracile*: broken line and *S. marinoï:* dotted line); **D** competence of larvae fed mixed diet *T. lutea* plus *C. neogracile*. Such regression was not evaluated for survival due to low differences between most of the conditions (> 90%).