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Influence of diet assemblage on *Ostrea edulis* broodstock conditioning and subsequent larval development

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Abstract:

In contrast with the Japanese oyster *Crassostrea gigas* and the Manila clam *Ruditapes philippinarum*, *Ostrea edulis* seed production in the hatchery has been reported to be erratic, with sudden and unexplained larval and post-metamorphosis mortalities. Fecundity and initial larval quality have been related to broodstock conditioning, but effects on larval development and metamorphosis remain poorly understood. In addition, molluscan larval mortalities have been often associated with bacterial contamination and flow-through techniques may help to overcome this problem. Both aspects have been considered in the present work. *O. edulis* broodstock were conditioned at 19 °C and fed three different microalgal diets. Two were single-species diets: *Rhodomonas salina* (R_s) or *Thalassiosira weissflogii* (T_w) and the third was a combination of both species (R_sT_w: 50/50 in equivalent cell volume). Mean fecundity, expressed as mean number of larvae released by oysters fed different diets, was 0.16, 0.28 and 0.39 million, respectively; whereas, mean larval size at release differed significantly from 174 to 181 µm. Moreover, when broodstock were fed combined assemblage (R_sT_w), larval release occurred more consistently. Larvae were subsequently fed two different diets over an 11-day period: *Chaetoceros gracilis* solely (C_g) or a bi-specific assemblage (T: *Isochrysis affinis galbana* plus C_g). Larval growth ranged from 5.5 to 7.4 µm d^{-1} for larvae fed C_g and was generally higher (8.1 µm d^{-1}) in larvae fed the mixed diet TC_g. On day 11, larval survival and competence ranged from 50 to 75% and 40 to 70% respectively, these results being closely related to broodstock nutrition. On day 18 the larval settlement ranged from 1 to 60%. When analyzing overall performance, from fecundity to settlement, best results were obtained with broodstock, fed the bi-specific diet (R_sT_w), which released numerous larvae over a short period with satisfactory larval development and high metamorphosis, and these larvae also fed bi-specific diet, TC_g.

Highlights

► We study the role of three microalgal diets on flat oyster broodstock conditioning. ► The influence of three other different diets is determined on larval development. ► R_sT_w leads to the highest *O. edulis* fecundity ► TC_g leads to the highest *O. edulis* settlement. ► Best overall performances are obtained with mixed diets from broodstock to larvae.

Keywords : *Ostrea edulis* ; Feeding ; Broodstock conditioning ; Larval development ; Flow-through

1. Introduction

From the 70's and 80's two successive diseases affected *O. edulis* production in Brittany (main area in France for its culture) and the population dropped from 20,000 tons to 1,000 – 1,500 tons y^{-1} nowadays (Buestel et al., 2009). Despite several attempts to control marteiliosis and bonamiosis in natural surroundings (Grizel, 1985) or eradicate its effects through modified husbandry (Le Bec et al., 1991; Robert et al., 1991), introduction of exotic flat oysters (*e.g. O. puelchana*: Pascual et al., 1991), and genetic improvement (Naciri-Graven et al., 1999), the flat oyster population has never recovered.

This situation was quite similar for most countries in Europe (Laing et al., 2005) and, in this context, except in some limited free disease areas (*e.g*. Scotland, North Ireland, Norway, Denmark) flat oyster farming consists in improving oyster growth before the fateful limit of 3 years old or equivalent size and, accordingly, *O. edulis* production in Europe is constrained.

However, progress has been made in breeding for diseases resistance including new genetic tools (Lalias et al., 2009; Morga et al., 2011). Currently, a selective breeding program is, accordingly a possibility to enhance flat oyster farming. Such targeted genetic orientation, however, will not be feasible until the difficulty inherent to a lack of fully reliable methods in hatchery for this species is overcome. Indeed in the hatchery, unexplained mortalities have often been reported during larval rearing on day 8 and post-settlement (anonymous, 2004; Bédier, 2004; Laing et al., 2005). Hatchery methods are now relatively well known for many mollusks (*e.g. Crassostrea gigas*: Utting and Spencer, 1991, *Ruditapes philippinarum*: Helm and Pellizato, 1990*, Mercenaria mercenaria*: Castagna and Kraeuter, 1981). Despite indisputable know-how, mainly due to pioneers work (Walne, 1974), the state of the art in hatchery rearing of *O. edulis* remains clearly insufficient to support reliable seed production, probably because of a lack of updated, detailed knowledge of the biology of this species. Compared to oviparous and dioecious species such as *C. gigas* and *R. decussatus/philippinarum*, the flat oyster is larviparous. Fecundity and initial larval quality have been related clearly to broodstock conditioning, mainly food delivery (Helm et al., 1991; Millican and Helm; 1994; Utting and Millican, 1997) but further effects on larval development and metamorphosis are poorly known. Maternal effects on metamorphosis have been reported with *O. chilensis* which incubates larvae for a very long period (Wilson et al., 1996); whereas in *O. edulis*, such parental effects have been shown to affect only larval growth and survival (Berntsson et al., 1997). The present work will contribute to this scope by focusing on the effects of food on *O. edulis* broodstock conditioning and subsequent larval development, including metamorphosis.

Moreover molluscan larval mortalities have often been associated with bacterial contamination, specifically to vibrios (Elston, 1984, Thompson et al., 2003; Estes et al., 2004). Widely described for Pectinidae (Riquelme et al., 1996; Lambert et al., 1999), and cupped oysters *Crassostrea* spp. (Elston and Leibovitz, 1980; Jeffries, 1982; Sugumar et al., 1998; Elston et al., 2008), vibriosis has recently been shown to affect *O. edulis* as well (Prado et al., 2005) confirming previous results on the same species (Jeffries, 1983). It is well known that scallops are very sensitive to vibrio infection (Nicolas et al., 1996) which led to the use of antibiotics, as a preventive measure, to limit larval mortalities (Robert et al., 1995). Such practices are not sustainable, and Norwegian researchers have partly overcome this problem by developing 5,000-L flow-through larval rearing technique (Magnesen et al., 2006). To study the ecophysiological requirements of mollusks with low fertility (1-2 million oocytes in *O. edulis) vs* 10-20 in *P. maximus* in hatchery conditions (Le Pennec et al., 1998) a 5-L container was designed, and a new, flow-through larval-rearing configuration was developed for *O. edulis.*

2. Material and methods

Microalgae and diet composition

Four different microalgal species were used in the present study: two large species for broodstock conditioning, *Rhodomonas salina* (R_s: volumetric size ≈ 200 µm^3, dry weight 60 pg cell^{-1}, strain CCAP 978/24) and *Thalassiosira weissflogii* (T_w: 950 µm^3, 250 pg cell^{-1}, CCAP 1077/5) and two small species for larval rearing, *Isochrysis* affinis *galbana* (T: 40 µm^3, 12 pg cell^{-1}, CCAP 927/14 also named T-Iso) and *Chaetoceros gracilis* (C_g: 80 µm^3, 25 pg cell^{-1}, UTEX LB2658).
O. *edulis* broodstock conditioning was assessed using three microalgal diets in duplicate: two single-species, *R*. *salina* with a daily ration of 10^9 cells oyster^{-1}or *T*. *weissflogii* (0.25×10^9 day^{-1} oyster^{-1}) and the bi-specific combination of *R*. *salina* plus *T*. *weissflogii* (50/50 equivalent cell volume). Because feeding in the inhalant chamber occurs in *O*. *chilensis* during motherhood (Chaparro et al., 2001, 2006), 10% of T-Iso supplemented all diets during the second month of conditioning.
O. *edulis* larval development was achieved using mono (*C*. *gracilis*) or bi-specific diets (T-Iso plus *C*. *gracilis*) delivered to maintain continuously 25 cells (± 5) per each larva, as recommended for *C*. *gigas* larvae (Rico Villa et al., 2009); whereas unfed larvae were used as a negative control. Additionally in a single experiment, a batch of larvae was also fed T-Iso to elucidate its role in larval development excluding metamorphosis.

Broodstock conditioning

Three hundred flat oysters from the same location were dredged from the Bay of Brest (Brittany, France) in March 2007. After scrubbing shells free of fouling organisms and debris before stocking in experimental structures at the Ifremer experimental station of Argenton (Brittany, France), oysters were pre-conditioned to experimental temperature (19 °C) by a daily gradual increase of 1 °C during 10 days. Seawater temperature was maintained at 19 °C by means of regulated thermal floodgates. Thereafter 50 2 year-old oysters were distributed homogeneously (70 g ± 20 mean whole weight, 1.7 g ± 0.6 mean meat dry weight, 70 mm ± 8 mean length) in each duplicate tank (2 m length x 0.5 m width x 0.2 m depth) per experimental condition. Continuous flow of 1-µm filtered-seawater was delivered from the top at a constant flow rate of 40 L h^{-1} (40% renewal h^{-1}). Daily rations of 6% dry weight microalgae (mg) per oyster meat (g) were provided to broodstock by peristaltic pumps that mixed the algae with filtered seawater at the inlet of each tank. All tank outlets were secured with a 100-µm mesh sieve to prevent larvae from escaping. *O*. *edulis* broodstock conditioning continued over a 4 month-period from February to May 2007.
Seawater at inlet and outlet of each experimental tank was sampled twice a day (morning and afternoon) and phytoplankton counts were made using an electronic particles counter (Multisizer 3 equipped with a 100-µm aperture tube). Grazing was expressed in number of cells removed from suspension oyster^{-1} d^{-1} or in µm^3 oyster^{-1} d^{-1}. For each experimental condition, fecundity was assessed as the number of released larvae over the considered period.

Larval rearing

When detected, expelled larvae were counted and measured for length. When a large release of larvae was recorded (≥ 1 million), larval rearing was set up in a dedicated flow-through cylindrical system inspired by the Cawthron design (King et al., 2005). Made from 3 mm, transparent Polymethyl methacrylate, six 5-l tanks (104 cm height

and 9 cm diameter) were self-supported on a 12-mm PVC table of 150 cm length (Fig. 1a). The outlet of each tank was equipped with a 32 mm PVC pipe connected within the tank to a bevelled, 100-µm sieve to prevent larvae from escaping. Each microalga was delivered by pumping from a reservoir into each inlet pipe through a 4-mm translucent, flexible line (Fig. 1b). Each larval rearing tank received from the top 1-µm-filtered phytoplankton-enriched seawater through a secondary line with flow controlled by a flow-meter (one per tank: Fig. 1c). In each larval tank, aeration was provided from the bottom and was maintained at 0.5 L min^{-1} using a 4-mm PVC valve (Fig. 1). Lastly the outlet of each cylinder was connected to a 6-mm tube allowing complete tank draining. For each broodstock origin (diet) and when a sufficient number of released larvae was collected, larvae were reared in those flow through units at a density of 5 larvae ml^{-1} with seawater flow maintained at 1.3 L h^{-1} (≈ 6 renewals day^{-1}). Seawater temperature/salinity was maintained at 22 °C/34 ppt according to Robert et al. (1988). Each broodstock diet was split into treatments with three larval diets, i.e., 9 different larval conditions were assessed: **B***R*$_s$**L**S, **B***R*$_s$**L***C*$_g$, **B***R*$_s$**L**T*C*$_g$, **B***T*$_w$**L**S, **B***T*$_w$**L***C*$_g$, **B***T*$_w$**L**T*C*$_g$, **B***R*$_s$*T*$_w$**L**S, **B***R*$_s$*T*$_w$**L***C*$_g$ and **B***R*$_s$*T*$_w$**L**T*C*$_g$. Moreover a high number of released larvae, originating from broodstock fed $R_sT_{w,}$ allowed to set up an additional feeding condition and accordingly a batch of larvae was fed T. Iso alone. Thus, the designation **B***R*$_s$**L**S means that broodstock was fed *R. salina* and larvae from them were starved; whereas, **B***R*$_s$*T*$_w$**L**T*C*$_g$ means that broodstock was fed *R*. *salina* plus *T*. *weissflogii*, and larvae from them were fed T-Iso plus *C*. *gracilis*.

Phytoplankton cell density was assessed twice a day at inlet and outlet of each experimental rearing tank, and adjustments were made to stabilize cell count around each larva at 25 cells (± 5) or 1,000 $µm^3$ equivalent T-Iso, values reported as an effective algal-cell density for *C*. *gigas* larval development (Rico-Villa et al., 2009).

In each rearing tank, larvae were collected by siphoning subsamples and larval shell length was measured on days 0 (released), 3, 7 and 11 using image analysis (Image vision Builder version 6.0). Survival was estimated on day 11 on the entire larval population collected by draining, mixing thoroughly, and counting a sub-sample under the light microscope. The number of larvae ready to set (competent larvae showing presence of large eyespots and active foot) was estimated prior to a selective grading on 200-µm mesh. Pediveligers > 280 µm were distributed at an initial density varying from 0.3 to 0.7 larvae ml^{-1} in 30-L tanks containing plastic disks as cultch (settlement material) (Rico-Villa et al., 2006). They were fed the bi-specific diet (T*C*$_g$: 50/50, v/v) thereafter, post-set, were maintained in a flow through system (9 L h^{-1}; 30% h^{-1} seawater renewal) for an additional week. The number of eyed pediveligers selected was, however, limited and we only obtained sufficient numbers of post-set for duplicate treatments fed bi-specific diets from broodstock to larvae.

Analytical procedures

Growth and cell size of microalgae cultures were estimated using a Multisizer 3, and for dry weight determinations, 50 ml of culture were harvested and centrifuged (3,200 g, 10min); pellets then were rinsed with 20 ml of 0.5 M ammonium formate, re-centrifuged and placed in pre-weighed tin capsules. Dry weight and ash were measured after heating at 80 °C (overnight) and 450 °C (4h) respectively. Gross composition analysis followed methods of Lowry et al. (1951) for proteins, Bligh and Dyer (1959) for lipids and Dubois et al. (1956) for carbohydrates. Fatty acids were analyzed following methods described by Marty et al. (1992) with 23:0 as an internal standard. Fatty acid composition was expressed as the relative contents of the total fatty acids of the entire fraction.

Statistical analysis

Data were analyzed using STATISTICA software (version 8.0). One-way analyses of variance (ANOVA) were used to test the effects of larval diet on larval length, survival and competence. Two-way analysis of variance was used to test the combined effects of broodstock nutritional diets and larval diets on larval length. When needed, data transformation (arcsin[sqr(X/100)]) to respect homogeneity of residues distribution (Sokal and Rohlf, 2001) was made. Differences of mean were assessed using *a posteriori* Tukey's tests.

3. Results

Broodstock conditioning

Consumption by oyster

Grazing was estimated daily. When expressed in cells number per oyster per day, grazing clearly was related to the type of food delivered during conditioning, with maximum values of 0.52×10^9 ($\pm 0.10 \times 10^9$, on day 19), 1.1×10^9 ($\pm 0.1 \times 10^9$, on day 31) and 2.2×10^9 ($\pm 0.4 \times 10^9$, on day 35) for T_w, R_sT_w, and R_s respectively. When expressed in volume (μm^3 oyster^{-1} d^{-1}), similar trends were observed with optimal values of 120×10^9 ($\pm 6.7 \times 10^9$, on day 19), 180×10^9 ($\pm 15 \times 10^9$, on day 31) and 220×10^9 ($\pm 72 \times 10^9$, on day 35) for T_w, R_sT_w and R_s respectively. Whatever the diet, a general decrease in grazing was detected from day 35 until the end of the experiment, but this trend was difficult to quantify because of data overlap. To obtain a clearer overall view of microalgae uptake changes, data were accordingly stacked and expressed on a weekly basis (Fig. 2). With this visualization, grazing, expressed as cells number oyster^{-1} wk^{-1}, showed a steady decrease from week 6 until the end of the experiment for broodstock fed R_s alone or the mixture R_sT_w, with values decreasing from 1-1.2 $\times 10^9$ to 0.2×10^9 (six fold decrease). In contrast, grazing seemed to be almost constant when broodstock were fed T_w (Fig. 2a); however a decline occurred when grazing was expressed as cells volume oyster^{-1} d^{-1} (Fig. 2b) with values decreasing from 85×10^9 to 5×10^9 (17 fold decrease).

Oyster fecundity

As previously done with grazing, fecundity data were stacked and expressed on a weekly basis. For broodstock fed R_s alone, the first larval release occurred on week 4; whereas fecundity increased from 0.5-1, on week 6, to 6×10^6 larvae, on week 10, simultaneously with a grazing-rate decrease (Fig. 3a). Similar relationships between larval release and grazing decrease were also found with the other feeding conditions: T_w (Fig. 3b) alone or the mixture R_sT_w (Fig. 3c). The greatest number of larvae released occurred with flat oysters fed R_sT_w with a mean number of 0.39 million ± 0.04 per oyster with precocious (week 3) and steadier expulsion. The lowest number of released larvae was recorded for broodstock fed R_s alone with a mean number of 0.16 million ± 0.007 per oyster; whereas, brooders fed T_w exhibited intermediate performances (0.28 million ± 0.12) with the last expulsion occurring on week 11 (Fig. 3b).

Larval development

Size at release and biochemical composition

Initial larval size was related to broodstock nutrition, with mean lengths of 174 µm (± 1), 176 µm (± 2) and 181 µm (± 1) for larvae originating from broodstock oysters fed R_sT_w, T_w and R_s respectively, with these differences being significant (*2*, *5.5*, *p = 0.004*). Tukeys' tests, however, revealed significant differences only between T_w and R_sT_w. In contrast, no differences in gross biochemical composition of newly-released larvae were recorded, with proteins varying from 13.0 to 16.2% (*2, 5.1, p = 0.051*), carbohydrates from ≈ 1 to 1.2% (*2*, *2.1*, *p = 0.2*) and lipids from 2.9 to 4.0% (*2*, *4.2*, *p = 0.07*) (Table 1).

The algal species used differed in several aspects of fatty acid composition. *R*. *salina* exhibited relative high content of 20:5(n-3) with 10% and was also rich in 22:6(n-3) with 8%; whereas, 20:4(n-6) represented 3% of total fatty acids (Table 2). In contrast, *T*. *weissflogii* showed lower amount of 22:6(n-3) and 20:4(n-6) with 4% and 0.2% respectively, but this diatom was particularly rich in 20:5(n-3) reaching 20% of total fatty acids. On the other hand, *R*. *salina* was characterized by a high content of 18:2(n-6), 18:3(n-3), 18:4(n-3) representing 18%, 12% and 14% respectively; whereas, *T*. *weissflogii* was poor in these three fatty acids with 1-2%. An opposite trend occurred with 16:3(n-4) and 16:1(n-4), which both were 18% in *T*. *weissflogii*; whereas, these fatty acids did not exceed 1% in *R*. *salina* (Table 2).

Despite these differences in the fatty acid composition of the diet, newly- released *O*. *edulis* larvae were very similar in PUFA contents with no significant differences between larvae originated from broodstock fed different diet ($p<0.05$): Table 2.

Larval growth

Regardless of broodstock diet, larvae were released in sufficient quantity in week 8 and were reared under two different feeding conditions; unfed larvae were used as negative controls.

The effects of broodstock diets (R_s, T_w, R_sT_w) on larvae combined with the effects of larval diet (C_g, TC_g) on larval growth (expressed as total daily growth rate from D1 to D11) highlighted a predominant influence of initial breeders nutrition (*2*, *9.3*, *p = 0.004)* but no influence of larva nutrition (*1, 0.15*, *p = 0.70)* as well as interaction effects (broodstock diet ***** larval diet: (*2*, *0.2*, *p = 0.80).* Regardless of the experimental conditions unfed larvae did not grow significantly; at the end of the experiment larval length only reached 175-180 µm (Figs. 4a, 4b, 4c). In contrast, larvae originating from broodstock fed R_s exhibited the highest growth of 7 µm d^{-1} leading to a final length of 258 µm (Fig. 4 a). Larvae fed *C*. *gracilis* alone or the bi-specific diet TC_g showed similar growth (*p =0.012*) (Figs. 4b, 4c) with the exception of larvae originating from broodstock fed R_s during the first week ($p <0.01$) (Fig. 4 a). Under these latter conditions, the mixed larval diet improved larval growth significantly, but this difference disappeared thereafter (Fig. 4a). When the larvae originating from broodstock fed R_sT_w, were provided solely T-Iso, larval growth was depressed with 4.8 µm d^{-1}, leading to a final size of 226 µm (Fig. 4c).

Larval survival

Survival was assessed at the end of larval rearing, on day 11. Low survival was observed for unfed larvae (<1%), regardless of broodstock diet. Overall, larvae originating from broodstock fed R_s or T_w exhibited higher survival from 58 to 74% for larvae fed *C. gracilis* or TC_g (Fig. 5a). The lowest survival, 50 to 55% was recorded for larvae released by broodstock fed R_sT_w, regardless of larval feeding (Fig. 5a). The combined effects of broodstock and larval diets (which excluded T-Iso) on larval survival revealed a predominant influence of nutrition during conditioning (*2*, *6.91*, *p =*

0.002) these differences being significant only between **B**Rs**L**TC_g and **B**Rs**L**C_g *(p = 0.003*) and **B**Rs**L**TC_g and **B**T$_w$**L**C_g *(p = 0.002).*

Larval competence and metamorphosis

When fed the mixed larval diet TC_g, compared to single-species diet C_g, larvae exhibited higher competence (60-70%) (Fig. 5b). The combined effects of diets on broodstock and larvae upon larval competence (which excluded T-Iso) revealed a predominant influence of broodstock nutrition (*2*, *757.6*, *p<0.0001*), and a significant influence of larval nutrition (*1*, *126.6, p<0.0001).* In contrast, no interaction effects (broodstock diet x larval diet) were noted *(2*, *0.7*, *p = 0.49).* When fed T-Iso, however, larval competence represented 75% of that achieved with *C*. *gracilis* in larvae released by parents fed R_sT_w (31% *vs* 41%).
Whatever the broodstock diet, higher settlement was recorded when larvae were fed the mixed larval diet TC_g (25-60%: Fig. 5c), but metamorphosis performance was poor (1.3%) when broodstock were fed *R. salina* and larvae *C*. *gracilis* (Fig. 5c). Settlement ranged accordingly from 1.3 to 60% but the experimental design was incomplete to allow overall statistical data treatment. Thus, the effect of broodstock diet on larval settlement is difficult to confirm, although feeding broodstock *R*. *salina* led to similar larval metamorphosis (≈ 26%) as in broodstock fed *C*. *gracilis* (≈ 30%) which is only half the value recorded when broodstock received the mixed diet (≈ 60%). Similarly, when considering parents fed single-species microalgal diets, overall mean post-larval yield (from initial larval release to the end of settlement) was ≈ 7.5% for larvae fed single diets *vs* 28% for those fed bi-specific diet.

4. Discussion

It is difficult to differentiate the role of adult reserves from that of additional food. For the cupped oyster *C. gigas*, which has external fertilization and larval development, additional food for broodstock appears to be of less importance than the initial content of glycogen reserves before conditioning (Cannuel and Beninger, 2007). In some cases, food availability favors growth and maintenance rather than reproduction (Donaldson, 1991); whereas, Muranaka and Lannan (1984) found that the fecundity of *C*. *gigas* broodstock was 60% greater when fed an algal food supplement rather than starved. Such apparent contrasting results can be explained by trials reported by Chávez-Villalba et al. (2003) who showed that a 6% (dry weight of algae per dry meat oyster weight) addition per day of equal quantities of *Chaetoceros calcitrans* and T-Iso had a positive effect on *C*. *gigas* fertility, but these effects were closely related to the season and the amount of initial reserves in oysters at the beginning of conditioning. Thus, in spring, the mean fertility (number of eggs per female) of fed broodstock originating from six different French oyster areas was 5.23 million (± 2.91) *vs* 0.77 million (± 1.21) for unfed groups, while in summer fertility reached 30.74 million (± 12.05) *vs* 8.11 million (± 7.20), respectively, for fed and unfed oysters. Gamete viability, however, did not differ significantly between conditions, nor did larval growth when larvae were fed a mixed diet (Chávez-Villalba et al., 2003).
On the other hand, Devauchelle and Mingant (1991) reported that the fecundity of the hermaphroditic scallop *P. maximus,* at similar gametogenic stages, decreased with feeding levels. Gonad activity suffered first at lower feeding levels, but only in the female part of the gonad. No eggs were obtained after 28 and 45 days of conditioning when scallops were kept unfed; whereas, fecundity increased by 8 to 25% with a food ration of 3×10^9 cells animal^{-1} day^{-1} and by 30-60% with 14×10^9 cells animal^{-1} day^{-1}. Similar results were reported for *P. fumatus*, showing that gonad condition and egg

production improved as feeding rates increased from 12.5 to 100 satiation (equivalent to 0.75 to 6 x 10^9 cells $scallop^{-1}$ day^{-1} respectively) at all test temperatures in the 12-21 °C range (Heasman et al., 1996).

The genus *Ostrea* is larviparous; accordingly broodstock must sustain egg development, embryogenesis and larval growth in the gill cavity for at least 1 week (Walne, 1974). Starving females of the Chilean oyster *Ostrea chilensis* induced low spat growth and survival (Wilson et al., 1996); whereas, *O*. *edulis* broodstock receiving *T*. *suecica*- enriched seawater produced earlier broods with subsequent larval growth and settlement improvement (Helm et al., 1973). Moreover, an algal diet representing 3 to 6% of the initial meat weight of oysters (dry weight to dry weight) per day increased larval production in *O*. *edulis* as did the type of algae delivered, the poorest result being obtained with the single diet *Dunaliella tertiolecta* (Millican and Helm, 1994).

In the present work, when fed three different diets - *Rhodomonas salina*, *Thalassiosira weissflogii* and mixed *R. salina* plus *T weissflogii* at 6% dry weight -, *O*. *edulis* broodstock did not show any specific diet preference during the first month of experiment, grazing rate being quite similar for all diets delivered. Beyond day 35, microalgae uptake decreased sharply and similarly for all diets until day 50. Such grazing depression could be explained by larval incubation in brachial chamber as reported in *O*. *chilensis* by Chaparro et al. (2001) who linked larval inactivity and water circulation interference in the pallial cavity. Furthermore, *O*. *edulis* fecundity development (expressed as number and intensity of released larvae) presented an antagonistic relationship to food consumption, with obvious “pre-spawning” activity and large fecundity differences depending upon broodstock diet: 0.16 million larvae per oyster were released for $\mathbf{B}R_S$, 0.28 million for $\mathbf{B}T_w$ and 0.39 million for $\mathbf{B}R_ST_w$. When compared to similar age (2-yr-old oyster), 0.5 million larvae released per oyster was reported by Walne (1974), which is in agreement with values found in the present work for broodstock fed the bi-specific diet (≈ 0.4 million) as well as oysters fed single diets (≈ 0.2 million).

Larval survival ranged from 50 to 75% with a significant effect of broodstock feeding leading to differences between single and bi-specific diets but no clear trend emerged as already reported by Helm (1969, 1977) or Bertsson et al. (1997) (Table 3). In static culture, higher survival has been reported (95%: Helm 1969, Ferreiro et al., 1990), but no information is given on the use of antibiotics. Antibiotics utilization is, however, suspected as this was previously recommended (Walne, 1966). In a more recent work, the use of erythromycin was shown to be essential to survival of larvae in French experimental and commercial hatcheries employing stagnant-water methods (Bédier, 2004). Flow through larval rearing techniques were developed accordingly, and this practice was highly successful with *C. gigas*, leading to survival > 90% in 150 L (Rico-Villa et al., 2008) or in similar 5-L containers (Petton et al., 2009a). For *O*. *edulis*, the lowest larval survival reported here probably is attributable to poor optimization of the techniques, particularly the direction of food-enriched seawater flow that has shown to be more efficient from bottom to top for *C. gigas* larvae (Petton et al., 2009b).

Generally, a multi-specific microalgal diet is beneficial to mollusk larval development (*e.g.* Robert and Gérard, 1999, Rico-Villa et al., 2006; Marshall et al., 2010). A successful mixed-algal diet must be composed of two or three species with high nutritive values, often combining diatoms and flagellates (Robert and Gérard, 1999). In *O*. *edulis*, mixed-algal species diets support generally better growth and competence than single-species diets (Helm et al., 2004, Table 3), and the results reported here showed that this ascertainment is true for T- Iso (Fig. 4c). In contrast, the difference in larval growth between the bi-specific diet TCg and *C*. *gracilis* is slim, regardless of broodstock diet. Indeed the additive effect of T-Iso for flat-oyster larval growth is slight, but high for metamorphosis as already shown for the cupped oyster *C*. *gigas* (Rico-Villa et al., 2006). This generalization disagrees, however with the work of Jonnson et al. (1999) on *O*. *edulis* larvae for which a negative effect on growth of *C. calcitrans* was reported. High fluctuations in diatom quality may be suspected because batches of *C.*

calcitrans varied at harvest from 3 x 10^6 cells ml^{-1} to 7 x 10^6 cells ml^{-1} (Jonnson et al., 1999).
In the present study, larval competence ranged from 30 to 70% with a clear effect of larval nutrition and a positive influence of the bi-specific diet *vs* single-species diet, as already pointed out with *C*. *gigas* (Rico-Villa et al., 2006: Marshall et al., 2010). Such differences also occurred for settlement; single-species larval diets led to lower metamorphosis performances (8%) compared to larval bispecific diets (28%), as well as for broodstock diet, for which bi-specific diets led to 60% settlement (*vs* 30%).
Initial larval proximate condition (proteins, lipids, carbohydrates), however, did not reveal any differences. On the other hand, it has been shown that neutral lipids in the form of triacylglycerol (TAG), is an important source of energy for bivalve larvae, especially in periods of low food availability or during starvation (Gallager et al., 1986; Whyte et al., 1992; Ben Kheder et al., 2010 a, b). More precisely, essential fatty acids (EFAs), particularly omega-3 fatty acids eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are important to bivalve-larval growth and development (Langdon and Waldock, 1981) because they are major membrane components (Hendriks et al., 2003) and possible modulators of membrane function (Palacios et al., 2005). DHA is, indeed, involved in maintaining suitable structure of membranes; whereas, EPA has a role as energy source and as a precursor of eicosanoids. In addition, the omega-6 fatty acids docosapentaenoic acid (22:5n-6, DPA) and arachidonic acid (20:4n-6, AA) have been identified as fatty acids affecting growth and survival of larval and postlarval stages (Pernet et al., 2005; Milke et al., 2006, 2008). Yet, initial *O. edulis* fatty acid composition did not reveal any differences and, larval initial potential seemed to be similar.
The present work confirms that one critical factor in conditioning *O*. spp. is feeding in terms of the species of microalgae delivered and/or ration (Wilson et al., 1996; Utting and Millican, 1997; Chapparo et al., 2006; Dunphy et al., 2006). “Natural” food has however proved to be better for broodstock conditioning than hatchery controlled feeding (Helm et al., 1991; Millican and Helm, 1994) and progress in this field is accordingly expected. To better understand how microalgae are mobilized to support reproduction, studies will have to be carried out to include ingestion, assimilation and biochemical allocation to gonads.

Conclusion

When *Ostrea edulis* broodstock are fed *Rhodomonas salina* or *Thalassiosira weissflogii*, lower fecundity is recorded than when broodstock are fed a mix of the two species.
When fed the mixed diet (T-Iso plus *Chaetoceros gracilis*), development of larvae is overall better than with single-species microalgal diets.
C. gracilis plays an important role in *O*. *edulis* larvae growth but addition of T-Iso improves significantly percentage metamorphosis.
Broodstock diet has a clear influence on *O*. *edulis* larval development, including metamorphosis.

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Table 1: Proteins, carbohydrates and total lipids, expressed in % of dry weight (± S.D., n = 3), in *Ostrea edulis* released larvae originated from broodstocks fed *R*. *salina* (R_s), *T*. *weissflogii* (T_w), *R*. *salina* plus *T*. *weissflogii* (R_sT_w).
Same letter in the same line indicates no significant differences

	R_s		T_w		R_sT_w	
Proteins	[a] 13.00	(1.73)	[a] 16.23	(1.27)	[a] 15.30	(0.53)
Carbohydrates	[b] 0.96	(0.06)	[b] 1.15	(0.10)	[b] 1.18	(0.22)
Lipids	[c] 2.88	(0.12)	[c] 4.02	(0.66)	[c] 3.80	(0.57)

Table 2
Total fatty acids composition in *R. salina*, *T. weissflogii* microalgae and *O. edulis* (L.) released larvae originated from broodstock fed *R. salina* (R_s), *T. weissflogii* + *R. salina* (T_wR_s) and *T. weissflogii* (T_w), expressed in % of total fatty acids (± S.D.; n = 3).

Fatty acids	Microalgae				Larvae					
	R. salina		*T. weissflogii*		R_s		T_wR_s		T_w	
14:0	1.79	(0.57)	24.97	(2.81)	1.29	(0.01)	1.56	(0.12)	1.86	(0.26)
16:0	0.86	(0.31)	0.18	(0.01)	12.28	(1.14)	13.65	(0.32)	15.73	(0.97)
18:0	0.54	(0.23)	0.00	(0.00)	5.39	(0.90)	4.48	(0.18)	4.27	(0.65)
16:1 (n−9)	1.12	(0.22)	0.00	(0.00)	0.29	(0.01)	0.23	(0.06)	0.19	(0.07)
16:1 (n−7)	0.74	(0.36)	20.14	(3.25)	1.05	(0.12)	3.15	(0.96)	5.03	(1.32)
18:1 (n−9)	1.26	(0.56)	0.00	(0.00)	1.85	(0.17)	1.77	(0.22)	2.03	(0.19)
18:1 (n−7)	2.04	(0.32)	1.18	(0.08)	1.62	(0.30)	3.38	(0.53)	4.08	(0.19)
16:2 (n−7)	0.00	(0.00)	1.66	(0.05)	0.18	(0.20)	0.32	(0.28)	0.38	(0.33)
16:2 (n−4)	0.09	(0.01)	5.85	(0.90)	0.01	(0.01)	0.14	(0.05)	0.21	(0.09)
16:3 (n−4)	0.00	(0.05)	17.95	(1.44)	0.00	(0.00)	0.05	(0.01)	0.07	(0.06)
18:2 (n−6)	18.04	(4.03)	0.57	(0.01)	3.07	(0.16)	2.64	(0.57)	1.89	(0.67)
18:3 (n−6)	3.76	(1.61)	0.27	(0.01)	0.20	(0.05)	0.21	(0.06)	0.11	(0.05)
18:3 (n−3)	11.51	(2.46)	0.59	(0.09)	1.59	(0.12)	1.39	(0.34)	0.89	(0.41)
18:4 (n−3)	13.66	(1.58)	1.54	(0.01)	1.35	(0.30)	1.36	(0.21)	1.02	(0.28)
20:4 (n−6)	2.41	(0.20)	0.22	(0.01)	5.03	(0.03)	3.78	(0.13)	3.25	(0.25)
20:5 (n−3) (EPA)	9.51	(0.85)	20.43	(4.56)	9.25	(0.95)	10.97	(0.76)	11.72	(0.78)
22:5 (n−6)	0.20	(0.21)	0.00	(0.00)	0.84	(0.19)	0.73	(0.15)	0.64	(0.07)
22:6 (n−3) (DHA)	8.18	(2.64)	3.60	(0.55)	17.72	(2.04)	16.85	(3.16)	15.01	(2.11)
Sum MUFA	8.35	(1.29)	22.47	(5.89)	11.30	(0.57)	15.04	(1.53)	16.96	(1.10)
Sum (n−9)	2.48	(0.67)	0.09	(0.01)	3.05	(0.31)	2.66	(0.37)	2.57	(0.14)
Sum (n−7)	3.13	(0.29)	21.58	(2.22)	5.98	(0.36)	10.33	(1.80)	12.84	(0.96)
Sum PUFA	68.74	(6.28)	52.86	(6.33)	50.33	(3.23)	48.38	(1.91)	45.28	(0.43)
Sum (n−4)	0.09	(0.40)	23.80	(1.57)	0.11	(0.09)	0.49	(0.14)	0.65	(0.22)
Sum (n−6)	24.55	(5.49)	1.05	(0.26)	10.66	(0.18)	8.35	(0.43)	6.76	(0.67)
Sum (n−3)	44.02	(4.07)	26.35	(2.62)	33.23	(3.21)	33.01	(2.03)	30.84	(0.62)
(n−3) / (n−6)	1.79	(0.57)	24.97	(2.81)	3.12	(0.25)	3.98	(0.34)	4.60	(0.53)
DHA / EPA	0.86	(0.31)	0.18	(0.01)	1.92	(0.03)	1.56	(0.41)	1.29	(0.26)

Table 3
Review of larval development performances and survival in closed-flow system related to temperature, larval density and nutritional conditions, P: *Pavlova lutheri*, I: *Isochrysis galbana*, Ph: *Phaeodactylum tricornutum*, Sk: *Skeletonema costatum* (*marinoi*), Ts: *Tetraselmis suecica*, Cc: *Chaetoceros calcitrans*, Rh: *Rhodomonas sp.*, Ti: *Isochrysis affinis galbana* (clone T-Iso), Cg: *Chaetoceros gracilis*, Ps: *Platymonas* (*Tetraselmis*) *suecica*, Pp: *Pseudoisochrysis paradoxa*, Ir: *Imantonia rotunda*, nd: not determined.

T °C	Density (larva ml^{-1})	Nutritional condition	Growth rate (% or µm d^{-1})	Survival (%)	Competence rate (%)	Metamorphosis rate (%)	Final size (µm)	Rearing period (days)	Source
22–24	0.625	I, Ts, Cc, I + Ts, I + Cc, Ts + Cc, I + Ts + Cc	Nd	83, 90, 93, 91, 98, 98	9, 26, 36, 42, 31, 59, 64	Nd	278, 286, 286, 296, 293, 296, 311	8	Helm, 1969
Nd	2.67	I + Ts	Nd	65.5	Nd	Nd	297	12	Holland and Spencer, 1973
Nd	1.75	P, I, Ph, Sk	51, 52, 52.2%	93, 93, 96, 79	Nd	Nd	295, 295, 295, 200	11	Ferreiro et al., 1990
20	1	I, P, I + P, I + Cc, I + P + Cc	8.5, 2.8, 6.4, 9.7, 5.4 µm d^{-1}	Nd	Nd	25, 0, 30, 0, 0	303, 279, 318, 234, 266	12	Jonsson et al., 1999
16	Nd	I + Ts	Nd	Nd	Nd	Nd	304	17	Labarta et al., 1999
22–24	0.175	I, Ts, I + Ts	47, 50, 57%	88, 87, 92	Nd	18, 22, 40	261, 267, 279	11	Helm, 1977
26	Nd	I, Ph, P, Ps, Pp, Cc, Ir	40, 46, 28, 28, 32, 40, 35%	53, 58, 46, 75, 36, 57, 37	Nd	Nd	260, 270, 255, 255, 245, 260, 250	8	Wilson, 1978
Nd	1.75	Cc, Ts, P, Rh	54, 62, 45, 44%	97, 99, 5, 90	58, 66, 10, 1	Nd	287, 296, 265, 264	12	Ferreiro et al., 1990
22	4.5	I	55%	30	24–79	Nd	285	10	Walne, 1966
20	2.75	Sk + Ti + P	95%	15	Nd	Nd	299	14	Glize, 1994
21	4.5	Ti + Cg	11 µm d^{-1}	84	Nd	Nd	302	13	Coatanea et al., 1994
21	2	Ts, I + Ts, Ts + Cc	4.45, 5.32, 1.67 µm d^{-1}	75, 52, 72	Nd	Nd	219, 221, 204	12	Berntsson et al., 1997

Figure 1. (a) Overview of 5 L cylinder for *Ostrea edulis* larval rearing, (b) details of inlet and outlet of phytoplankton enriched seawater and (c) overview of mesh sieve in larval tank.

Figure 2. Weekly consumption in *Ostrea edulis* (L.) broodstock, fed single *R. salina* diet (**B***R*$_s$), single *T. weissflogii* diet (**B***T**w***) and bi-specific diet **B***R*$_s$*T*$_w$, expressed as billion of cells oyster^{-1} d^{-1} (a) and billion of µm^3 oyster^{-1} d^{-1} (b).

Figure 3. Weekly evolution of grazing (billion µm^3 oyster^{-1}) and fecundity of *Ostrea edulis* (L.) broodstock fed: (a) *R*. *salina* (*R*$_s$), (b) *T*. *weissflogii* (*T*$_w$), (c) *R*. *salina* plus *T*. *weissflogii* (*R*$_s$*T*$_w$).

Figure 4. Larval growth (µm) for larvae fed *Isochrysis affinis galbana* (T), *Chaetoceros gracilis* (Cg), bi-specific diet T*C*$_g$ and originated from *Ostrea edulis* (L.) broodstocks previously fed: (a) *R*. *salina* (**B***R*$_s$), (b) *T*. *weissflogii* (**B***T*$_w$), (c) *R*. *salina* plus *T*. *weissflogii* (**B***R*$_s$*T*$_w$).

Figure 5. Survival (a), competence (b) and metamorphosis (c) of *Ostrea edulis* (L.) larvae starved or fed *Chaetoceros gracilis* (*C*$_g$) or *Isochrysis aff. galbana* + *Chaetoceros gracilis* (T*C*$_g$), and originated from oysters fed *R*. *salina* (**B***R*$_s$), *T. weissflogii* (**B***T*$_w$), *R*. *salina* + *T*. *weissflogii* (**B***R*$_s$*T*$_w$).
N.A.: Data not achieved due to technical failure.

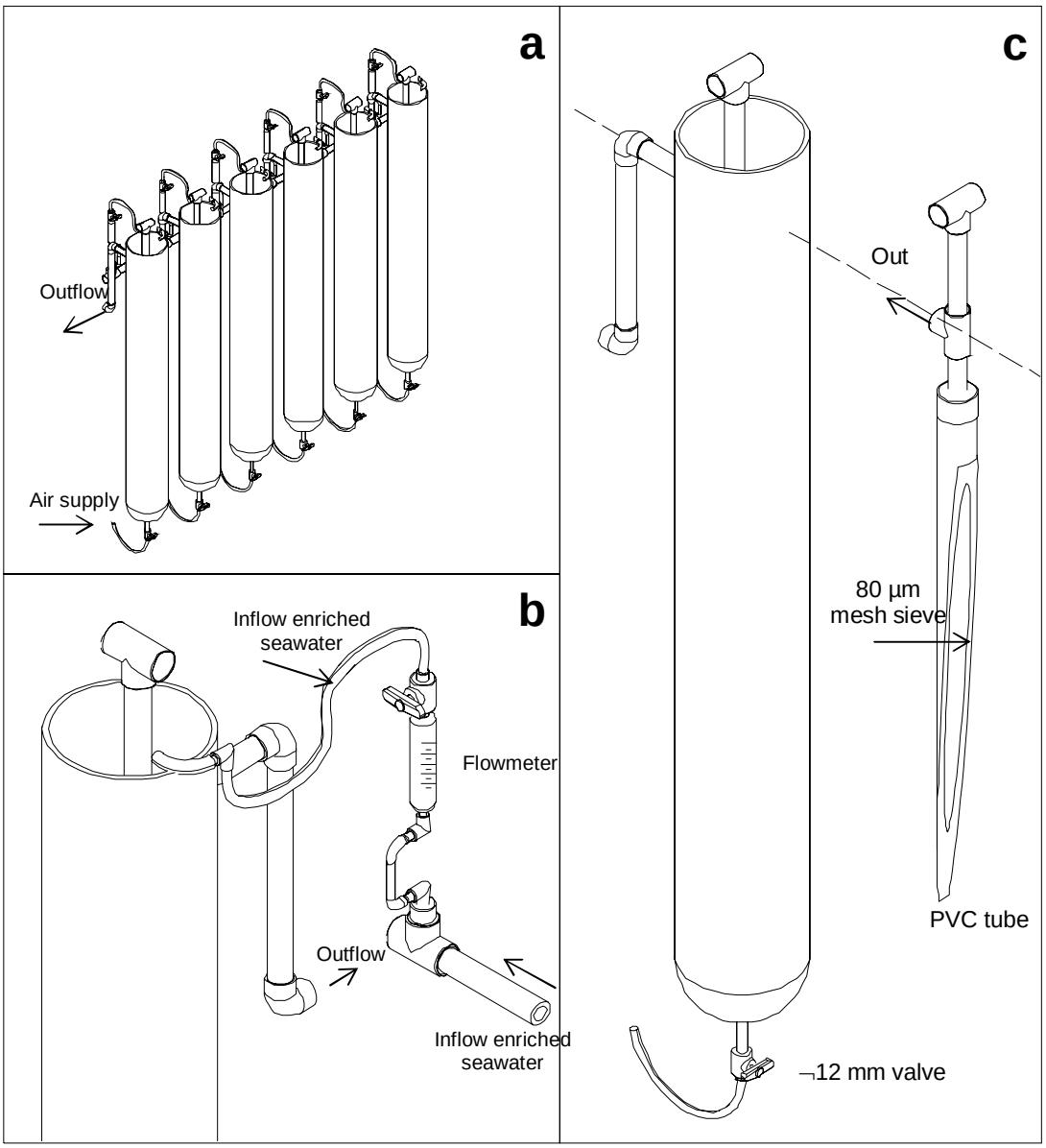

a
Outflow
Air supply
b
Inflow enriched seawater
Flowmeter
Outflow
Inflow enriched seawater
c
Out
80 µm mesh sieve
PVC tube
¬12 mm valve

Figure 1

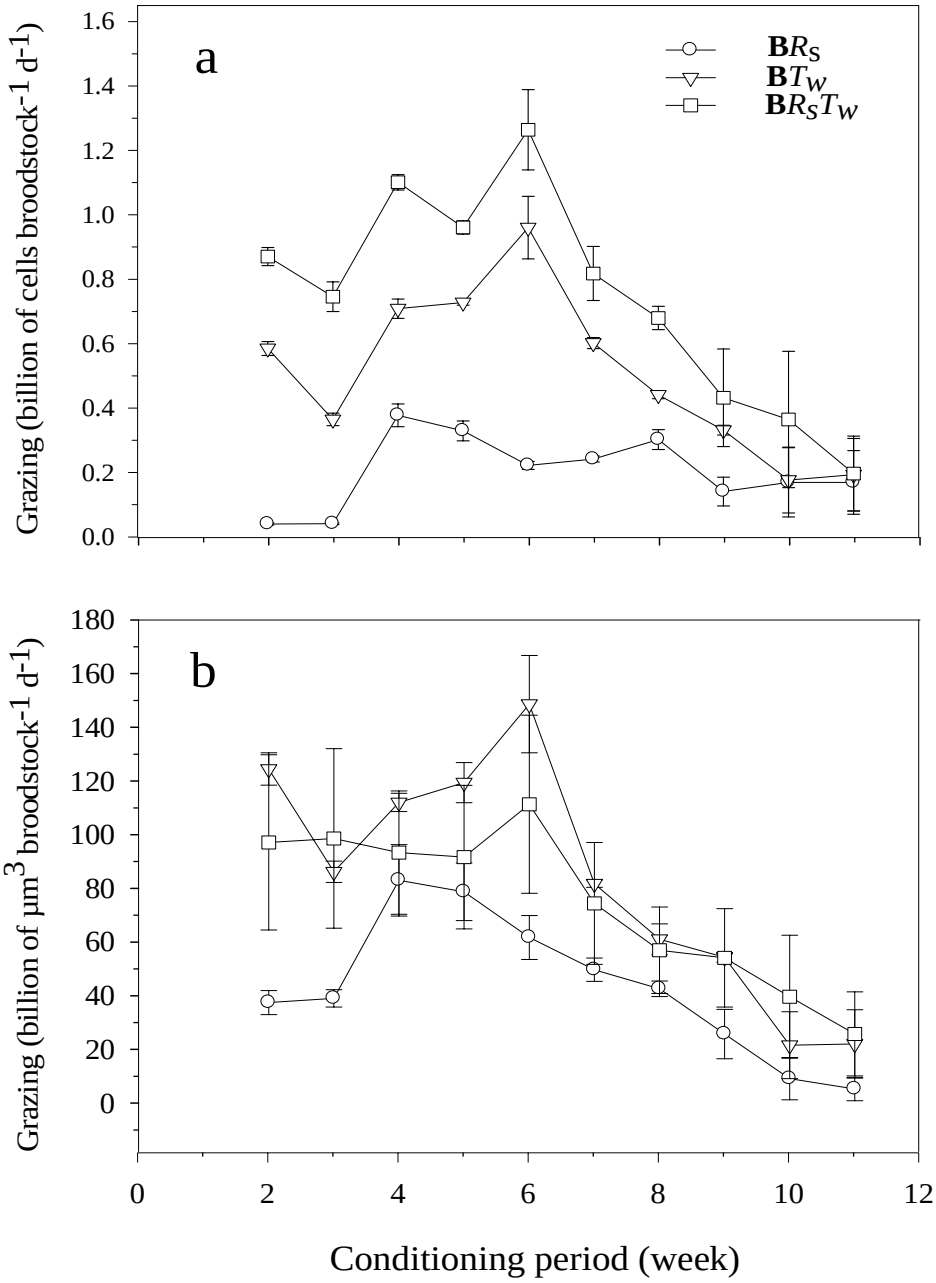

a
b
$\mathbf{B}R_S$
$\mathbf{B}T_W$
$\mathbf{B}R_ST_W$
Grazing (billion of cells broodstock^{-1} d^{-1})
Grazing (billion of µm^3 broodstock^{-1} d^{-1})
Conditioning period (week)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
20
40
60
80
100
120
140
160
180
0
2
4
6
8
10
12

Figure 2

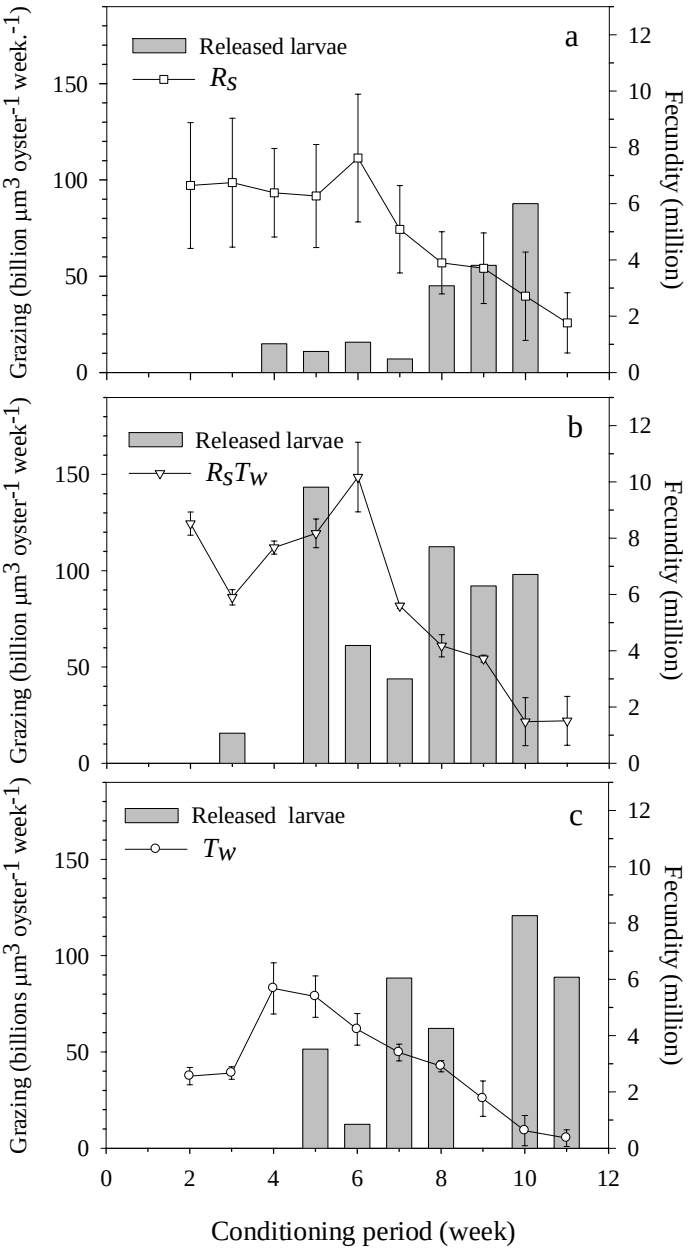
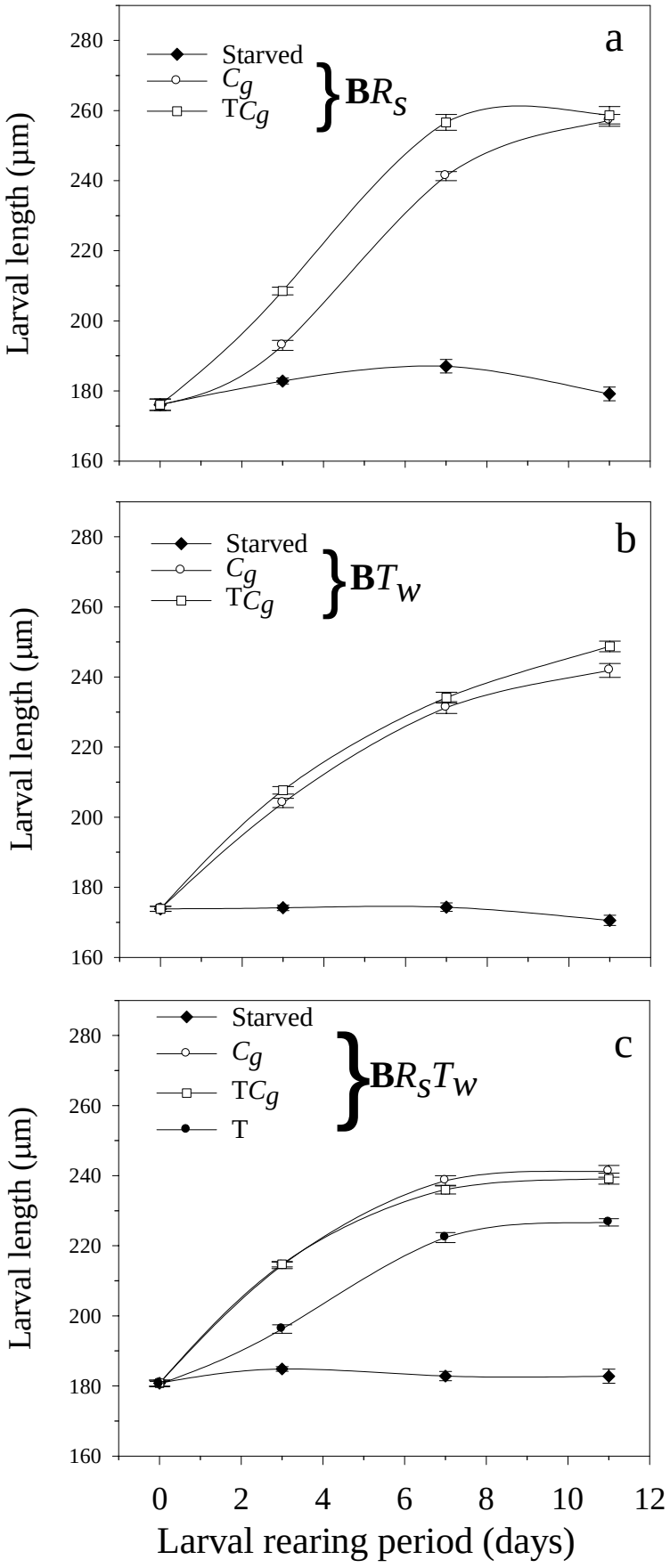

a
Released larvae
R_S
b
Released larvae
$R_S T_W$
c
Released larvae
T_W
Grazing (billion µm3 oyster-1 week.-1)
Grazing (billion µm3 oyster-1 week-1)
Grazing (billions µm3 oyster-1 week-1)
Fecundity (million)
Conditioning period (week)

Figure 3

Figure 4


a
Starved
C_g
TC_g
BR_S
b
Starved
C_g
TC_g
BT_W
c
Starved
C_g
TC_g
T
B$R_S T_W$
Larval length (μm)
160
180
200
220
240
260
280
0
2
4
6
8
10
12
Larval rearing period (days)

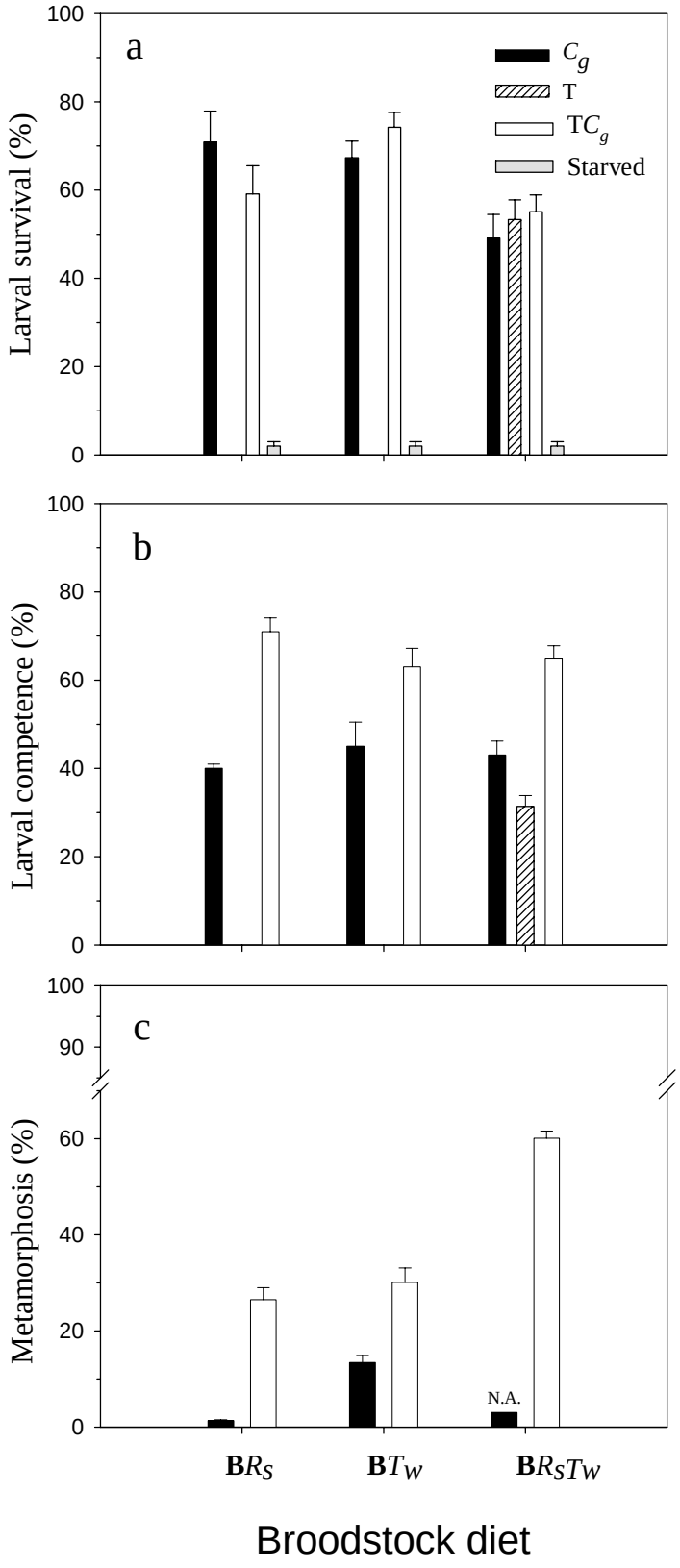

a
b
c
Larval survival (%)
Larval competence (%)
Metamorphosis (%)
100
80
60
40
20
0
90
C_g
T
TC_g
Starved
N.A.
BR_S
BT_W
BR_STW
Broodstock diet

Figure 5