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Effect of *Rhodomonas salina* addition to a standard hatchery diet during the early ontogeny of the scallop *Pecten maximus*

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Abstract:

The main objective of this study was to identify algal diets that maximize the survival and growth and alter the biochemical content of *Pecten maximus* larvae with the aim of improving metamorphosis. We also evaluated the potential of the cryptophyceae *Rhodomonas salina* as a food source for these larvae. Two flagellates, *Isochrysis aff. galbana* (T) and *Pavlova lutheri* (P), and two diatoms, *Chaetoceros gracilis* (C) or *Skeletonema costatum* (S), were tested as two ternary diets, namely PTC and PTS. PTC and PTS were compared with diets that also included *R. salina* (R). The addition of *R. salina* and the replacement of *C. gracilis* by *S. costatum* in the traditional hatchery diet seem to be two interesting alternatives for increasing the productivity of larval scallop culture and improving the metamorphosis rate. With these two diets, larval growth increased and metamorphosis was observed to occur more rapidly. Moreover, our results showed that the addition of *R. salina* significantly improved the overall condition of the larvae by promoting an increase in organic matter and total lipids. This accumulation of lipids during ontogeny seems to promote larvae to grow and to complete metamorphosis more rapidly than with the other diets. The level of polyunsaturated fatty acids in the algae could also at least partially explain the results obtained, since the PUFA level of *C. gracilis* was about half those of *S. costatum* and *R. salina*.

Keywords: Scallop; Nutrition; Metamorphosis; Energetic content; Algal fatty acids and sterols

Introduction

The success of larval scallop culture and the recruitment of postlarvae in natural populations depends on the accumulation of endogenous energy reserves to support larval development and the metamorphic period. Metamorphosis consists of a series of events involving major tissue reorganization to transit from a pelagic to a benthic way of life. During this period, bivalve larvae are limited in their ability to feed on exogenous particulates and rely on endogenous sources of energy. Neutral lipids and proteins account for >80% of the energy budget of metamorphosing bivalves; carbohydrates are usually considered to be of minor importance (Holland & Spencer 1973, Gallager et al. 1986, Whyte et al. 1992, Videla et al. 1998, García-Esquivel et al. 2001). However, these findings do not rule out the potential of dietary carbohydrate in sustaining the energetic needs of bivalve larvae during early ontogeny. Indeed, larvae of the scallop *Patinopecten yessoensis* fed a diet rich in carbohydrate exhibited a higher nutritional condition compared to those fed low carbohydrate diets (Whyte et al. 1989).

Microalgal polysaccharides are of nutritional significance since the digestive efficiency of marine invertebrates depends on the type of polysaccharide ingested (Kristensen 1972). For instance, one of the main digestive enzymes of *Pecten maximus* larvae is amylase, suggesting that starch is likely a preferred carbohydrate for this species. *Rhodomonas salina* is known to be rich in starch. Shumway et al. (1985) showed that ingested *R. salina* were absorbed in a majority of the bivalves they studied. Some preliminary results showed that *Pecten maximus* larvae fed *R. salina* exhibited an increase (by 1.8 times) in organic matter compared to those fed other diets (Robert et al.

1994). Although the use of *R. salina* in a routine diet for different invertebrate species or for juvenile bivalves has been previously reported (Enright et al. 1986, Brown et al. 1998, McCausland 1999), the potential of *R. salina* to improve the growth rate and metamorphosis in larval bivalves has not yet been investigated.

The objectives of the current research were (1) to optimize the classical ternary algal diets used in *P. maximus* hatchery practices with the aim of maximizing larval survival and growth and improving biochemical content before settlement, and (2) to evaluate the addition of the cryptophyceae *R. salina* to the larval diet and to examine the related larval performance during metamorphosis.

Material and methods

Animal maintenance and algal production

Adult scallops, *Pecten maximus* (about 150 g whole wet weight), were collected from the Bay of Brest and kept in a flow-through seawater system at 15°C for 30 days and continuously fed with a mixture of *Isochrysis aff. galbana*, *Pavlova lutheri* and *Chaetoceros gracilis* at the Écloserie du Tinduff near Brest (Northern Brittany, France). Scallops were induced to mass spawn by thermal shock following the method of Gruffydd & Beaumont (1970). Two days after fertilization, D-stage larvae were collected on a 45 µm square mesh screen. Five days after fertilization, scallops were transferred to IFREMER's experimental hatchery located in Argenton, France. Nutritional experiments consisting of 5 treatments were conducted in triplicate in 2-L glass beakers at an initial density of 7 larvae·ml^{-1}. Larvae were reared in a temperature-controlled room at 19-20°C

97 in 1-µm cartridge-filtered seawater at 34 ppt. Water was renewed every 2 or 3 days and
98 bacterial growth was limited by the addition of the antibiotic thiamphenicol at 8 $mg \cdot L^{-1}$.
99 Larval cultures were maintained until metamorphosis (21 days post fertilization, dpf),
100 when survivors were harvested for biochemical analysis.
101
102 Microalgae were cultured in 18-L carboys with 1-µm filtered sterilized seawater enriched
103 with Conway medium and silicate (40 $mg \cdot L^{-1}$) for diatoms. Algae were maintained at
104 ~20°C under continuous illumination from cool-white fluorescence lights at a light
105 intensity of 1500 lux and were mixed with aeration (air:CO_2 = 98.5:1.5%). Diatoms were
106 produced in batch and harvested in late exponential growth phase. Other species were
107 produced following a continuous method and harvested the day before utilization (Robert
108 et al. 1996a).
109
110 *Experimental design*
111 Two flagellates, *Isochrysis aff. galbana* (T) and *Pavlova lutheri* (P), and two diatoms,
112 *Chaetoceros gracilis* (C) and *Skeletonema costatum* (S), were tested as two standard
113 hatchery diets, namely PTC and PTS. These diets were compared to a new diet that
114 included *R. salina* (R), namely PTCR. Larvae were fed once a day with a daily ration of
115 one of three diets: 1:1:1 PTC or PTS at 45 $cells \cdot \mu l^{-1}$ (~1.1 $ng \cdot \mu l^{-1}$ dry weight, dw), or
116 1:1:1:1 PTCR at 60 $cells \cdot \mu l^{-1}$ (~3.3 $ng \cdot \mu l^{-1}$ dw). Two additional control diets were tested
117 to account for the higher biomass of the PTCR diet: PTC*C, which consisted of a daily
118 ration of 165 $cells \cdot \mu l^{-1}$ of PTC (~3.3 $ng \cdot \mu l^{-1}$ dw), and PTC*G, which consisted of an
119 increasing daily ration of PTC, starting with 45 and increasing to 165 $cells \cdot \mu l^{-1}$ (~1.1 to

120 ~3.3 ng·µl^{-1}dw from 5 to 21 dpf). The latter treatment was used to avoid an over
121 saturation of the larval culture with algae at the beginning of the experiment. Algal cells
122 were visually examined with a dissecting microscope at 40 x and counted using a
123 haemocytometer cell before feeding scallop larvae.
124
125 *Growth and survival measurements*
126 Growth and survival were estimated every 2 or 3 days until metamorphosis (21 dpf) for
127 each experimental treatment (n=3 for each of the 5 diets). During the larval cycle, larvae
128 were collected on a 45-µm mesh and mortality was assessed by counting a sample of
129 approximately 250 individuals. Mortality is expressed as a cumulative number of empty
130 shells based on the total number of shells. From hatching to the beginning of the
131 experiment (2 to 5 dpf respectively), only ca. 10% mortality occurred. Shell growth was
132 calculated on a sample of 50 to 90 larvae per treatment (n=3×5) using shell length
133 measurements (anterior-posterior distance) made with image analysis software (SXM for
134 MAC). Live larvae were recognized by the appearance of the double ring at the margin of
135 the shell, corresponding to a peripheral groove on which the dissoconch shell will be
136 attached (Doroudi et al. 1999). This criterion was used to assess the number of larvae that
137 began metamorphosis (Gerard et al. 1989).
138
139 *Ash-free dry weight*
140 The ash-free dry weight of larvae was estimated to evaluate their level of organic matter.
141 Larvae were ground, dried at 80°C for 24h to obtain the dry weight, and then heated at

450°C for 4h to determine ash weight; a Mettler M3 microbalance was used. The organic matter was calculated from the difference between the dry matter and the ash weight.

Biochemical analysis

Biochemical analyses of microalgae were carried out in triplicate for each separate microalgal culture of *C. gracilis*, *S. costatum* and *R. salina*. Since the flagellates *I. galbana* and *P. lutheri* were common to all three diets and their biochemical contents are given in several publications (Delauney et al. 1993, Soudant et al. 1996, 2000, Brown et al. 1997), they were not characterized. Microalgal cultures (5 to 15 ml) were filtered onto 25 mm Whatman GF/F filters pre-combusted at 450°C. The filters were placed in 15 ml glass tubes containing 6 ml of a mixture of chloroform and methanol (2:1, v:v) with 0.01% butylated hydroxytoluene (BHT) as an antioxidant, closed under nitrogen, shaken, and frozen at -20°C for lipid extraction following the Folch procedure (Folch et al. 1957). Multiple procedure blanks were prepared. Prior to lipid extraction, samples were sonicated for 10 min in chloroform-methanol (2:1; v/v) using a sonicating bath at 5°C. Lipid extracts were evaporated to dryness and recovered with three 500-µl washings of 98:2 chloroform:methanol.

Total lipids were transesterified under nitrogen using BF_3/CH_3OH for 10 minutes at 100°C. Fatty acid methyl esters (FAME) were analyzed using a gas chromatograph equipped with an on-column injector, a DB-Wax (30 m x 0.25 mm; 0.25 µm film thickness) capillary column and a flame ionization detector. Hydrogen was used as the

gas carrier. Fatty acids were identified by comparing their retention time with standards and quantified with tricosanoic acid (23:0) as an internal standard.

Sterols were obtained from the neutral lipid fraction as previously described (Marty et al. 1992). Neutral lipids were transesterified using sodium methoxyde (MeONa) at ambient temperature for 90 minutes. This method allows the protection of certain phytosterols such as 24-methylene cholesterol. Esterified sterols were analyzed using a gas chromatograph equipped with an on-column injector and a Restek Rtx65 fused silica capillary column (15 m x 0.25 mm; 0.25 µm film thickness). Hydrogen was used as the gas carrier. Esterified sterol fractions were identified by comparing their retention times with standards and were quantified with cholestane as an internal standard.

One sample of 21-day-old larvae (ca. 6000-11000 ind.) was harvested from each tank onto 25 mm Whatman GF/F filters pre-combusted at 450°C. Lipids from scallop larvae were extracted for total lipid quantification as previously described for micoalgae. Subsamples were used for colorimetric determinations of proteins (Lowry et al. 1951) and carbohydrates (Dubois et al. 1956). Larvae were rinsed with 10 ml of 37% ammonium formate then homogenized in 1 ml of nanopure water before analysis. Colorimetric assays were performed on a Uvikon® spectrophotometer.

Statistical analysis

Survivorship patterns were compared using the Life Test procedure from SAS 8.02 (SAS Institute Inc. 1999-2001). Multiple analyses of variance (MANOVAs) were conducted to

187 determine differences in fatty acid and sterol profiles among algal species. MANOVAs
188 on the fatty acid profiles considered total fatty acids (TFA), monosaturated fatty acids
189 (MUFA), polyunsaturated fatty acids (PUFA), EPA, DHA, and AA as dependent
190 variables A two-way analysis of variance (ANOVA) was conducted to determine
191 differences in shell length and occurrence of double rings as a function of diet and day. A
192 one-way ANOVA was performed to determine differences in organic matter as a function
193 of dietary treatment. A one-way MANOVA was performed to determine differences in
194 absolute ($ng \cdot larva^{-1}$) and relative (mass % of OM) concentrations of protein, lipid and
195 carbohydrate in 21-day-old scallops. Where differences were detected, least-square
196 differences (LSD) multiple comparison tests were used to determine which means were
197 significantly different. Residuals were screened for normality using the expected normal
198 probability plot and further tested using the Kolmogorov-Smirnov test. Homogeneity of
199 variance-covariance matrices was tested using the Levene test. Percentages were arcsine
200 square-root transformed to achieve homogeneity of variances. Variance analyses were
201 carried out using SPSS 13.0 for Windows (Chicago, IL). The significance value for all
202 analyses was set at $P<0.05$.
203
204 **Results**
205 *Microalgal lipid composition*
206 Total fatty acids (TFA, in $pg \cdot cell^{-1}$) in *R. salina* were 2.2 and 2.5 times higher than those
207 observed in *C. gracilis* and *S. costatum* respectively (Table 1). The diatom *C. gracilis*
208 was lower in polyunsaturated fatty acids (PUFA) compared to *S. costatum* (by 1.9 times)
209 and *R. salina* (by 2.2 times). Indeed, *C. gracilis* was characterized by elevated levels of

saturated (SFA) and monounsaturated (MUFA) fatty acids, with these two fatty acid classes constituting ~72% of the TFA. The long chain PUFAs 20:5n-3 (EPA), 22:6n-3 (DHA) and 20:4n-6 (AA) collectively contributed 48.9, 36.8 and 24.2% of the total PUFA found in *C. gracilis, S. costatum* and *R. salina* respectively. The major long chain PUFA in *C. gracilis* was EPA while AA and DHA remained low. *S. costatum* was characterized by a high level of EPA, an intermediate level of DHA and a low level of AA. Finally, *R. salina* exhibited the lowest level of EPA, an intermediate level of DHA and the highest level of AA. As a consequence, the DHA/EPA ratio varied from 0.11 in *C. gracilis* and 0.38 in *S. costatum* to 0.75 in *R. salina.*

Total sterol concentration (in $fg \cdot cell^{-1}$) in *R. salina* was ~2 times lower than those observed in *S. costatum* and *C. gracilis* (Table 2). High sterol concentrations were characteristic of the microalgal species. For instance, *C. gracilis* had a high level of cholesterol and fucosterol and low levels of 24-methylene cholesterol, iso-fucosterol and brassicasterol. In contrast, *S. costatum* exhibited a high level of 24-methylene cholesterol, intermediate levels of campesterol and cholesterol, and low levels of β-sitosterol and desmosterol. Finally, *R. salina* showed two major sterols, brassicasterol (> 90% of total sterol) and cholesterol.

Scallop performance

Diet and day showed an interaction in their effect on the shell length of scallop larvae (Table 3 and Fig. 1). Larvae offered PTS and PTCR exhibited greater shell lengths than those fed all other diets ($P<0.001$), and the PTS and PTCR diets showed similar growth

233 (P=0.081). These results are reflected by the growth rates, with values of 7.29±0.25 and
234 6.94±0.33 µm day^{-1} for PTS and PTCR compared to 5.04±0.53, 4.69±0.43 and 3.88±0.16
235 µm day^{-1} for PTC, PTCC and PTCG. No dietary effect was detected on scallop survival
236 (Lifetest procedure, P=0.966). At the end of the experiment, cumulative survival varied
237 between ~70 to 80% (Fig. 2). The occurrence of double rings varied as a function of day
238 and diet (Table 3 and Fig. 3). The first double rings appeared at around 15 dpf in larvae
239 fed the PTCR diet. The highest percentage of double rings was recorded at 17 dpf for the
240 PTCR diet compared to the PTS diet (P<0.05); larvae from the PTC diet had no double
241 rings at all. At 19 and 21 dpf, scallops fed PTCR and PTS were at a similar stage of
242 development (P=0.981) and showed a higher level (~5×) of double rings than scallops fed
243 the other diets (P<0.003). Occurrences of double rings were similar between larvae fed
244 PTC, PTC*G and PTC*C (P=0.862). Finally, dissoconchs were first observed after 19
245 dpf in cultures fed PTCR and only after 21 dpf for those fed PTS. Cultures fed other diets
246 did not exhibit any dissoconchs during the experiment. Therefore, PTCR and PTS
247 promoted higher growth rates and better metamorphic yields than the other diets tested.
248
249 *Biochemical content*
250 The ash-free dry weight of 21 dpf larvae varied between ~350 and 650 ng·larva^{-1},
251 depending on the dietary treatment (P=0.002). Indeed, the top performing larvae fed
252 PTCR and PTS exhibited ash-free dry weights nearly twice as high as larvae fed all other
253 dietary treatments (P<0.011; Fig. 4). The absolute contents (amount per larva) of lipids
254 and proteins in 21 dpf larvae were significantly influenced by the diet while carbohydrate
255 content was not (Table 4). Indeed, larvae fed the top-performing PTCR and PTS diets

showed an increase (~1.8×) in their lipid and protein contents compared to those fed other diets (Table 5, $P<0.001$). Larvae fed PTCR had higher lipid levels than larvae fed PTS ($P=0.003$); both these diets had lipid levels that were significantly higher than other diets ($P<0.010$). Larvae fed PTCR and PTS had similar proteins levels ($P=0.566$) that were again significantly higher than other diets. Relative concentrations of lipids, proteins and carbohydrates were ~32, 63 and 5% of OM, respectively (Fig. 5). Therefore, differences in the ash-free dry weight were mainly attributable to lipids and proteins since carbohydrates were scarce in 21 dpf larvae.

Discussion

Based on growth rate, on the biochemical content of larvae and on metamorphic yield (estimated by the double ring rate), our results showed that PTCR and PTS were the best performing diets, followed by PTC and then PTC*C and PTC*G, for the culture of scallop *Pecten maximus* larvae. Larvae fed PTS or PTCR showed higher organic matter and total lipid and protein contents than larvae fed PTC. Moreover, larvae fed the PTCR diet had a higher total lipid and organic matter content and underwent metamorphosis earlier compared to larvae fed the PTS diet. Our results show that the addition of *R. salina* significantly improved the condition of the larvae by promoting the enrichment of organic matter through proteins and total lipids. The starch content in the *R. salina* cells (~40% of total carbohydrates; present study) seemed beneficial to *P. maximus* larvae compared to others algal species; carbohydrate levels in diatoms (*C. gracilis* and *S. costatum*) and prymnesiophytes (*I. galbana* and *P. lutheri*) are generally under 15% (Brown et al. 1997). These carbohydrates could provide an energy source for metabolic

279 demand during larval development. It may also be converted to and accumulated as
280 lipids. This high level of organic matter in the form of proteins and lipids stored in
281 organisms with shells of about the same size at metamorphosis seems to promote larvae
282 to complete metamorphosis more rapidly. The biomass provided by the PTCR diet was
283 approximately three times higher than that of the PTC diet (3.3 compared to 1.1 $ng \cdot \mu l^{-1}$
284 dw) and was amplified by the larger volume of *R. salina,* which has cell sizes of 11.9 to
285 12.8 µm in diameter (Schiopu et al. 2006). In addition to the extra biomass provided by
286 the addition of *R. salina* to the traditional larvae diet composed of *Pavlova lutheri*,
287 *Isochrysis aff. galbana* and *Chaetoceros gracilis*, we observed a significant effect of *R.*
288 *salina* on larval scallop culture. Larvae fed the adjusted PTC diet (on the basis of algal
289 dry weight), that is, with a continuous or gradual addition of algae (PTC*C and PTC*G)
290 to obtain biomasses similar to the PTCR diet, did not show superior growth or higher
291 biochemical content than larvae fed the PTC diet. In fact, lower growth was observed
292 with these diets even though there was a higher availability of organic matter. These
293 results suggest a negative effect of a high concentration of small algal cells on the feeding
294 activity of scallop larvae, as has been reported for mussel larvae (Sprung 1984, 1989),
295 probably due to the saturation of the particulate filtration system. Lu and Blake (1996)
296 observed that *I. galbana* cell concentrations higher than 40 $cells \cdot \mu l^{-1}$ saturated the
297 ingestion rate of larval bay scallops, *Argopecten irradians*. In our experiment, we did not
298 evaluate whether the 165 $cells \cdot \mu l^{-1}$ of the PTC*C and PTC*G diets decreased the
299 ingestion rate of larvae, but these high algal densities did not increase scallop larva
300 mortality. The increased feed concentrations may cause water quality problems due to
301 bacteria proliferation. Nevertheless, the use of antibiotics to ensure the success of *P.*

302 *maximus* larval culture seems to limit this problem, as no more mortality was observed in
303 these treatments. The use of antibiotics at a concentration of 8 $mg{\cdot}L^{-1}$ has been
304 demonstrated to be beneficial for the survival of scallop larvae with no impact on the
305 growth rate (Nicolas et al. 1996, Robert et al. 1996). The quantitative advantage for larval
306 growth with *R. salina* in the algal diet may have resulted from the high level of digestible
307 organic matter available. The results suggest a good ingestion and retention ability as
308 well as efficient assimilation of this large microalgae by the *P. maximus* scallop larvae, as
309 has already been demonstrated for adult sea scallops, *Placopecten magellanicus*
310 (Shumway et al. 1985).
311
312 Qualitative differences between diets can contribute to performance. The PTS diet, which
313 provided less algal dry weight than the PTCR diet, showed better growth than the PTC
314 diet from day 12 until the end of the experiment and a growth pattern similar to PTCR at
315 the end of the larval cycle (between 17 and 21 dpf). The PUFA levels in our diets could
316 explain at least partially the growth and metamorphic results. Indeed, the PUFA level of
317 *C. gracilis* in the PTC diet was half that of *S. costatum* in PTS and also lower, but to a
318 lesser extent, than *R. salina* in the PTCR diet. Lipids, and more particularly the essential
319 polyunsaturated fatty acids (PUFA), are generally considered to be the most important
320 constituent of the algal diet for bivalve larvae (Whyte et al. 1989, Delaunay et al. 1993,
321 Soudant et al. 1998, Nevejan et al. 2003, Pernet & Tremblay 2004).
322
323 However, the success of the PTS diet is not in accordance with a previous study, where
324 poor ingestion of *S. costatum* in a PTS diet was observed and related to lower growth

compared to *P. maximus* larvae fed with *Chaetoceros calcitrans* (Soudant et al. 1998). According to published results on the biochemical compositions of microalgae species, the polyunsaturated fatty acid profile of *S. costatum* is similar to that of *C. gracilis,* showing a predominance of EPA with low levels of DHA, very low levels of AA and low variability between strains (Brown et al. 1989, Volkman et al. 1989, Dunstan et al. 1993). However, in our experiment, the DHA percentage composition was five-fold higher in *S. costatum* compared to *C. gracilis*. It has been previously observed that *P. maximus* larvae fed on diatoms only had a good growth rate but failed at metamorphosis (Delaunay et al. 1993). This was attributed to a high DHA requirement during this period of active changes in morphology and membrane building, as an EPA/DHA ratio of about 2 was highly controlled in polar lipids of *Pecten maximus* larvae. A higher percentage of DHA in the PTS diet would meet such a requirement better than the PTC diet and would improve the growth and success of *P. maximus* larval metamorphosis; survival was similar with other diets, as has been observed in *Crassostrea gigas* larvae (Volkman et al. 1989) and spat (Thompson & Harrisson 1992). These high levels of DHA in *S. costatum* compared to values reported in the literature would reflect culture conditions. Many studies indicate that diatoms submitted to nutritive stress will significantly increase their lipid levels (Dunstan 1993, Von Elert 2002, Pernet et al. 2003). However, high DHA levels may also be associated with a specific strain, which seems to be the case in our study. The good performance obtained when PTC was supplemented with *R. salina* in the PTCR diet could also result from a higher DHA/EPA ratio compared to the diet consisting of *C. gracilis* alone.

348 We observed that saturated fatty acids (SFA) seemed not to be a predominant factor
349 influencing the growth of *P. maximus* larvae: the SFA in *C. gracilis,* included in the PTC
350 diet, were twice as high as those from *S. costatum*. SFA provide more energy via β-
351 oxidation than PUFA, and our results contradict an earlier study showing a superiority of
352 diets containing higher amounts of SFA for *Placopecten magellanicus* and *Crassostrea*
353 *gigas* larvae (Langdon & Waldock 1981). It is possible that the SFA effect can be
354 detected when other limiting factors are optimized.
355
356 The sterol composition of microalgae could be important in determining the nutritional
357 value for bivalves (Wikfors et al. 1996). In our study, we observed that the total amount
358 of sterol was twice as high in *C. gracilis* and *S. costatum* compared to *R. salina*.
359 Furthermore, the cholesterol concentration in *C. gracilis* was more than twice that in *S.*
360 *costatum* and 5 times higher than concentrations in *R. salina*. Thus, in the diets studied,
361 the level of sterols and more specifically cholesterol seemed not to be limiting for larval
362 growth. Nevertheless, we observed that *S. costatum* sterol is composed mainly of 24-
363 methylene cholesterol (nearly 42%) and that *R. salina* is very rich in brassicasterol (90%),
364 two sterol classes used by oyster spat for the bioconversion of cholesterol (Soudant et al.
365 2000). Marine bivalves contain complex mixtures of C_{26} to C_{30} sterols (cholesterol =
366 C_{27}), with each molecular species being characterized by a planar ring system with a 3β-
367 hydroxyl group and a side chain of varying length. Furthermore, bivalves have a
368 negligible capacity for the biosynthesis or conversion of sterols, although they are
369 capable of selectively incorporating certain sterols (Soudant et al. 1996, Knauer et al.

1999). In *Pecten maximus,* the preferential incorporation of cholesterol over other sterols has been previously observed (Soudant et al. 1998).

In conclusion, our results suggest that a more efficient diet to improve larval growth of *P. maximus* and to attain metamorphosis more rapidly would be obtained by the addition of *R. salina* to a traditional hatchery diet composed of *I. galbana*, *P. lutheri* and *C. gracilis*. We also observed that the replacement of *C. gracilis* by *S. costatum* in a diet including *I. galbana* and *P. lutheri* seemed to improve larval growth of *P. maximus*. However, this last result seems to be associated with a specific *Skeletonema costatum* strain and particular culture conditions. Nevertheless, further investigations are being undertaken to better understand the effect of the addition *R. salina* on the kinetics of fatty acids, lipid classes, proteins and carbohydrates during larval development and for metamorphosis success and spat competency.

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References

Brown MR, Jeffrey SW, Garland CD (1989) Nutritional aspects of microalgae used in aquaculture: A literature review. Report 205, CSIRO Marine Laboratories

393 Brown MR, Jeffrey SW, Volkman JK, Dunstan GA (1997) Nutritional properties of
394 microalgae for mariculture. Aquaculture 15:315-331
395 Brown MR, McCausland MA, Kowalski K (1998) The nutritional value of four
396 Australian microalgal strains fed to Pacific oyster *Crassostrea gigas* spat.
397 Aquaculture 165:281-293
398 Delaunay F, Marty Y, Moal J, Samain J-F (1993) The effect of monospecific algal diets
399 on growth and fatty acid composition of *Pecten maximus* (L.) larvae. J. Exp. Mar.
400 Biol. Ecol. 173:163-179
401 Doroudi MS, Southgate PC, Mayer RJ (1999) Growth and survival of blackslip pearl
402 oyster larvae fed different densities of microalgae. Aquacul. Int. 7:179-187
403 Dubois M, Gillies KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for
404 the determination of carbohydrates and related substances. Anal. Chem. 28:350-
405 356
406 Dunstan G (1993) Changes in the lipid composition and maximization of the
407 polyunsaturated fatty acid content of three microalgae grown in mass culture. J.
408 Appl. Phycol. 5:71-83
409 Dunstan GA, Volkman JK, Barrett SM, Leroi J-M, Jeffrey SW (1993) Essential
410 polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae).
411 Phytochem. 35:155-161
412 Enright CT, Newkirk GF, Craigie JS, Castell JD (1986) Evaluation of phytoplankton as
413 diets for juvenile *Ostrea edulis* L. J. Exp. Mar. Biol. Ecol. 96:1-13
414 Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and
415 purification of total lipids from animal tissues. J. Biol. Chem. 226:497

416 Gallager SM, Mann R, Sasaki GC (1986) Lipid as an index of growth and viability in
417 three species of bivalve larvae. Aquaculture 56:81-103
418 Garcia-Esquivel Z, Bricelj VM, Gonzalez-Gomez MA (2001) Physiological basis for
419 energy demands and early postlarval mortality in the Pacific oyster, *Crassostrea*
420 *gigas*. J. Exp. Mar. Biol. Ecol. 263:77-103
421 Gerard A, Salaun M, Tritar S (1989) Critères de compétences des larves à la
422 métamorphose chez *Pecten maximus*. Haliotis 19:373-380
423 Gruffydd LD, Beaumont AR (1970) Determination of the optimum concentration of eggs
424 and spermatozoa for the production of normal larvae in *Pecten maximus*. Helgol.
425 Wiss. Meeresunters 20: 486-497
426 Holland DL, Spencer BE (1973) Biochemical changes in fed and starved oysters, *Ostrea*
427 *edulis* L., during larval development, metamorphisis and early spat growth. J.
428 Mar. Biol. Ass. U.K. 53:287-298
429 Knauer J, Barrett SM, Volkman JK, Southgate PC (1999) Assimilation of dietary
430 phytosterols by Pacific oyster *Crassostrea gigas* spat. Aquacul. Nut. 5:257-267
431 Kristensen JH (1972) Carbohydrases of some marine invertebrates with notes on their
432 food and on the natural occurrence of the carbohydrates studied. Mar. Biol.
433 14:130-142
434 Langdon CJ, Waldock MI (1981) The effect of algal and artificial diets on the growth and
435 fatty acid compostion of *Crassostrea gigas* spat. J. Mar. Biol. Ass. U.K. 61:431-
436 440
437 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the
438 Folin phenol reagent. J. Biol. Chem. 193:265-275

439 Lu YT, Blake NJ (1996) Optimum concentrations of *Isochrysis galbana* for growth of
440 larval and juvenile bay scallops, *Argopecten irradians concentricus* (Say). J.
441 Shellfish Res. 15:635-643
442 Marty Y, Delaunay F, Moal J, Samain J-F (1992) Changes in the fatty acid composition
443 of *Pecten maximus* (L.) during larval development. J. Exp. Mar. Biol. Ecol.
444 163:221-234
445 McCausland MA, Brown MR, Barrett SM, Diemar JA, Heasman MP (1999) Evaluation
446 of live microalgae and microalgal pastes as supplementary food for juvenile
447 Pacific oysters (*Crassostrea gigas*). Aquaculture 174:323-342
448 Nevejan N, Saez I, Gajardo G, Sorgeloos P (2003) Energy vs. essential fatty acids: what
449 do scallop larvae (*Argopecten purpuratus*) need most? Comp. Biochem. Physiol.
450 134B:599-613
451 Nicolas J-L, Corre S, Gauthier G, Robert R, Ansquer D (1996) Bacterial problems
452 associated with scallop *Pecten maximus* larval culture. Dis. Aquat. Org. 27:67-76
453 Pernet F, Tremblay R (2004) Effect of varying levels of dietary essential fatty acid during
454 early ontogeny of the sea scallop *Placopecten magellanicus*. J. Exp. Mar. Biol.
455 Ecol. 310:73-86
456 Pernet F, Tremblay R, Demers E, Roussy M (2003) Variation of lipid class and fatty acid
457 composition of *Chaetoceros muelleri* and *Isochrysis* sp. grown in a
458 semicontinuous system. Aquaculture 221:393-406
459 Robert R, Moal J, Campillo M-J, Daniel JY (1994) The food value of starch rich
460 flagellates for *Pecten maximus* (Linné) larvae. Preliminary results. Haliotis
461 23:169-171

462 Robert R, Moal J, Miner P, Quéré C, Connan JP, Daniel JY, Le Coz JR, Samain JF,
463 Mazuret M (1996a) Microalgae biomass from photobioreactor as food for fish and
464 shellfish larvae (MANTA). European Community Program, final report. A1R1-
465 CT92-0286, pp. 68-91
466 Robert R, Miner P, Nicolas J-L (1996b) Mortality control of scallop larvae in the
467 hatchery. Aquacul. Inter. 4:305-313
468 Schiopu D, George SB, Castell J (2006) Ingestion rates and dietary lipids affect growth
469 and fatty acid composition of *Dendraster excentricus* larvae. J. Exp. Mar. Biol.
470 Ecol. 328:47-75
471 Shumway SE, Cucci TL, Newell RIE, Yentsch CM (1985) Particle selection, ingestion,
472 and absorption in filter-feeding bivalves. J. Exp. Mar. Biol. Ecol. 91:77-92
473 Soudant P, Marty Y, Moal J, Masski H, Samain JF (1998) Fatty acid composition of
474 polar lipid classes during larval development of scallop *Pecten maximus* (L.).
475 Comp. Biochem. Physiol. 121A:279-288
476 Soudant P, Marty Y, Moal J, Robert R, Quere C, Le Coz JR, Samain JF (1996) Effect of
477 food fatty acid and sterol quality on *Pecten maximus* gonad composition and
478 reproduction process. Aquaculture 143:361-378
479 Soudant P, Sanles MV, Quere C, Le Coz JR, Marty Y, Moal J, Samain JF, Sorgeloos P
480 (2000) The use of lipid emulsions for sterol supplementation of spat of the Pacific
481 oyster, *Crassostrea gigas*. Aquaculture 184:315-326
482 Sprung M (1984) Physiological energetics of mussel larvae (*Mytilus edulis*). II. Food
483 uptake. Mar. Ecol. Prog. Ser. 17:295-305

484 Sprung M (1989) Field and laboratory observations of *Dreissena polymorpha* larvae:
485 abundance, growth, mortality and food demands. Arch. Hydrobiol. 115:537-561
486 Thompson PA, Harrisson PJ (1992) Effects of monospecific algal diets of varying
487 biochemical composition on the growth and survival of Pacific oyster
488 (*Crassostrea gigas*) larvae. Mar. Biol. 113:645-654
489 Von Elert E (2002) Determination of limiting polyunsaturated fatty acids in *Daphnia*
490 *galeata* using a new method to enrich food algae with single fatty acids. Limnol.
491 Oceanogr. 47:1764-1773
492 Videla JA, Chaparro OR, Thompson RJ, Concha II (1998) Role of biochemical energy
493 reserves in the metamorphosis and early juvenile development of the oyster
494 *Ostrea chilensis*. Mar. Biol. 132:635-640
495 Volkman JK, Jeffrey SW, Nichols PD, Rogers GI, Garland CD (1989) Fatty acid and
496 lipid composition of 10 species of microalgae used in mariculture. J. Exp. Mar.
497 Biol. Ecol. 128:219-240
498 Wikfors GH, Patterson GW, Ghosh P, Lewin RA, Smith BC, Alix JH (1996) Growth of
499 post-set oysters, *Crassostrea virginica*, on high-lipid strains of algal flagellates
500 *Tetraselmis* spp. Aquaculture 143:411-419
501 Whyte JNC, Bourne N, Ginther NG, Hodgson CA (1992) Compositional changes in the
502 larva to juvenile development of the scallop *Crassadoma gigantea* (Gray). J. Exp.
503 Mar. Biol. Ecol. 163:13-29
504 Whyte JNC, Bourne N, Hodgson CA (1989) Influence of algal diets on biochemical
505 composition and energy reserves in *Patinopecten yessoensis* (Jay) larvae.
506 Aquaculture 1989:333-347

Table 1. Fatty acid composition of the diatoms *Chaetoceros gracilis* (CHGRA) and *Skeletonema costatum* (SKEL) and the flagellate *Rhodomonas salina* (RHODO), expressed as % of total fatty acids. Also shown are the n-3/n-6, DHA/EPA and EPA/AA ratios as well as the total fatty acids (TFA; pg·cell^{-1}).

Fatty acid	**CHGRA**	**SKEL**	**RHODO**
14:0	16.38	14.39	15.48
16:0	24.85	6.84	20.17
18:0	3.77	0.93	1.46
Σ SFA	**45.45**	**22.27**	**37.11**
16:1n-9	-	0.08	-
16:1n-7	25.31	13.94	1.11
16:1n-13t	0.61	1.14	0.52
18:1n-9	0.24	1.14	11.79
18:1n-7	0.96	1.14	1.68
Σ MUFA	**27.13**	**18.43**	**15.61**
16:2n-7	2.54	1.55	-
16:2n-4	1.93	2.53	-
16:3n-4	5.59	20.55	-
18:2n-6	0.18	2.46	15.01
18:3n-6	0.46	0.07	3.27
18:3n-3	-	0.99	7.98
18:4n-3	0.72	3.75	7.80
20:4n-6 (AA)	0.1	0.27	2.98
20:5n-3 (EPA)	10.71	13.80	4.78
22:6n-3 (DHA)	1.14	5.30	3.58
Σ PUFA	**24.31**	**52.64**	**46.77**
Σ n-3	**13.11**	**24.57**	**24.68**
Σ n-6	**1.09**	**3.35**	**22.06**
n-3/n-6	12.03	7.33	1.12
DHA/EPA	0.11	0.38	0.75
EPA/AA	107.1	51.1	1.60
TFA (pg·cell^{-1})	5.17	4.53	11.34

511 **Table 2.** Sterol compositions of the diatoms *Chaetoceros gracilis* (CHGRA) and
512 *Skeletonema costatum* (SKEL) and the flagellate *Rhodomonas salina* (RHODO),
513 expressed as % of total sterols.
514

Sterol	**CHGRA**	**SKEL**	**RHODO**
Cholesterol	47.21	20.04	9.71
Brassicasterol	1.31	-	90.29
Desmosterol	-	4.04	-
Campesterol	-	28.04	-
24-methylene cholesterol	8.40	41.56	-
Stigmasterol	-	-	-
β-sitosterol	-	6.33	-
Fucosterol	38.18	-	-
Iso-fucosterol	4.90	-	-
Total sterols ($fg \cdot cell^{-1}$)	107.68	98.22	55.68

515

516 **Table 3**. Summary of ANOVAs showing the effect of day and diet on (a) shell length and
517 (b) occurrence of double rings.
518

Source of variation	df	MS	*F*-value	*p*
(a) Shell length				
Day	6	1.1×10^{4}	446	<0.001
Diet	4	2.5×10^{3}	99	<0.001
Day*Diet	24	256	10	<0.001
Error	70	25		
Corrected total	104			
(b) Double ring*				
Day	1	0.478	239	<0.001
Diet	4	0.553	277	<0.001
Day*Diet	4	1.6×10^{-3}	0.818	0.529
Error	20	1.9×10^{-3}		
Corrected total	29			

519 *Arcsinus transformation for double ring occurrence was applied to normalize the data.
520

521 **Table 4.** Summary of MANOVAs showing the effect of diets on lipids, proteins and
522 carbohydrates at (a) absolute (ng larva^{-1}) and (b) relative (%) values.
523

Diet	df	MS	*F*-value	*p*
(a) Absolute concentration*				
Lipids	4	7.03×10^{-2}	44.1	<0.001
Proteins	4	4.84×10^{-2}	19.3	<0.001
Carbohydrates	4	1.35×10^{-2}	1.8	0.198
Error lipids	10	1.59×10^{-3}		
Error proteins	10	2.51×10^{-3}		
Error carbohydrates	10	7.36×10^{-3}		
Corrected total lipids	14			
Corrected total proteins	14			
Corrected total carbohydrates	14			
(b) Relative concentration*				
Lipids	4	1.86×10^{-3}	7.3	0.005
Proteins	4	1.84×10^{-3}	5.5	0.013
Carbohydrates	4	2.88×10^{-3}	3.9	0.057
Error lipids	10	2.52×10^{-4}		
Error proteins	10	3.36×10^{-4}		
Error carbohydrates	10	7.39×10^{-4}		
Corrected total lipids	14			
Corrected total proteins	14			
Corrected total carbohydrates	14			

524 *Logarithmic and arcsinus transformations, respectively, were applied on absolute (ng
525 larva^{-1}) and relative (%) values to normalize the data.

526 **Table 5**. The biochemical content of larvae (ng larva^{-1}) at 21 dpf fed with PTC, PTC*G,
527 PTC*C, PTCR and PTS (mean ± SD). See text for diet abbreviations.
528

	PTC	PTC*G	PTC*C	PTCR	PTS
Lipid	78.5 ± 7.6^{a}	67.2 ± 2.3^{a}	59.6 ± 6.6^{a}	141.5 ± 13.0^{b}	106.4 ± 10.5^{c}
Protein	135.6 ± 10.8^{a}	131.4 ± 4.5^{a}	122.6 ± 16.3^{a}	234.7 ± 38.9^{b}	221.0 ± 26.8^{b}
Carbohydrate	15.3 ± 1.1^{a}	12.0 ± 0.6^{a}	11.6 ± 2.6^{a}	18.2 ± 4.6^{a}	15.8 ± 3.0^{a}

529 Values (±SD) with different superscript letters are significantly different (P<0.05).
530
531

532 **FIGURE LEGENDS**

533 Fig. 1. Shell length of *Pecten maximus* larvae fed different diets as a function of the day
534 post fertilization (mean ± SD; n=3 replicate tanks per diet). See text for diet
535 abbreviations.

536 Fig. 2. Survival of *Pecten maximus* larvae fed different diets as a function of the day post
537 fertilization (mean ± SD; n=3 replicate tanks per diet). See text for diet
538 abbreviations.

539 Fig. 3. Occurrence of double rings in *Pecten maximus* larvae fed PTC, PTC*G, PTC*C,
540 PTCR or PTS a function of the number of days post fertilization (mean ± SD; n=3
541 replicate tanks per diet). Different letters indicate significant differences. See text
542 for diet abbreviations.

543 Fig. 4. Ash-free dry weight (ng larvae^{-1}) of *Pecten maximus* larvae fed PTC, PTC*G,
544 PTC*C, PTCR, or PTS at 21 days post fertilization (mean ± SD; n=3 replicate
545 tanks per diet). Different letters indicate significant differences between diets. See
546 text for diet abbreviations.

547 Fig. 5. Relative concentrations of lipids, proteins and carbohydrates of *Pecten maximus*
548 larvae fed PTC, PTC*G, PTC*C, PTCR, and PTS at 21 days post fertilization
549 (mean ± SD, n=3 replicate tanks). See text for diet abbreviations.

550

Figure(s)

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FIG 1

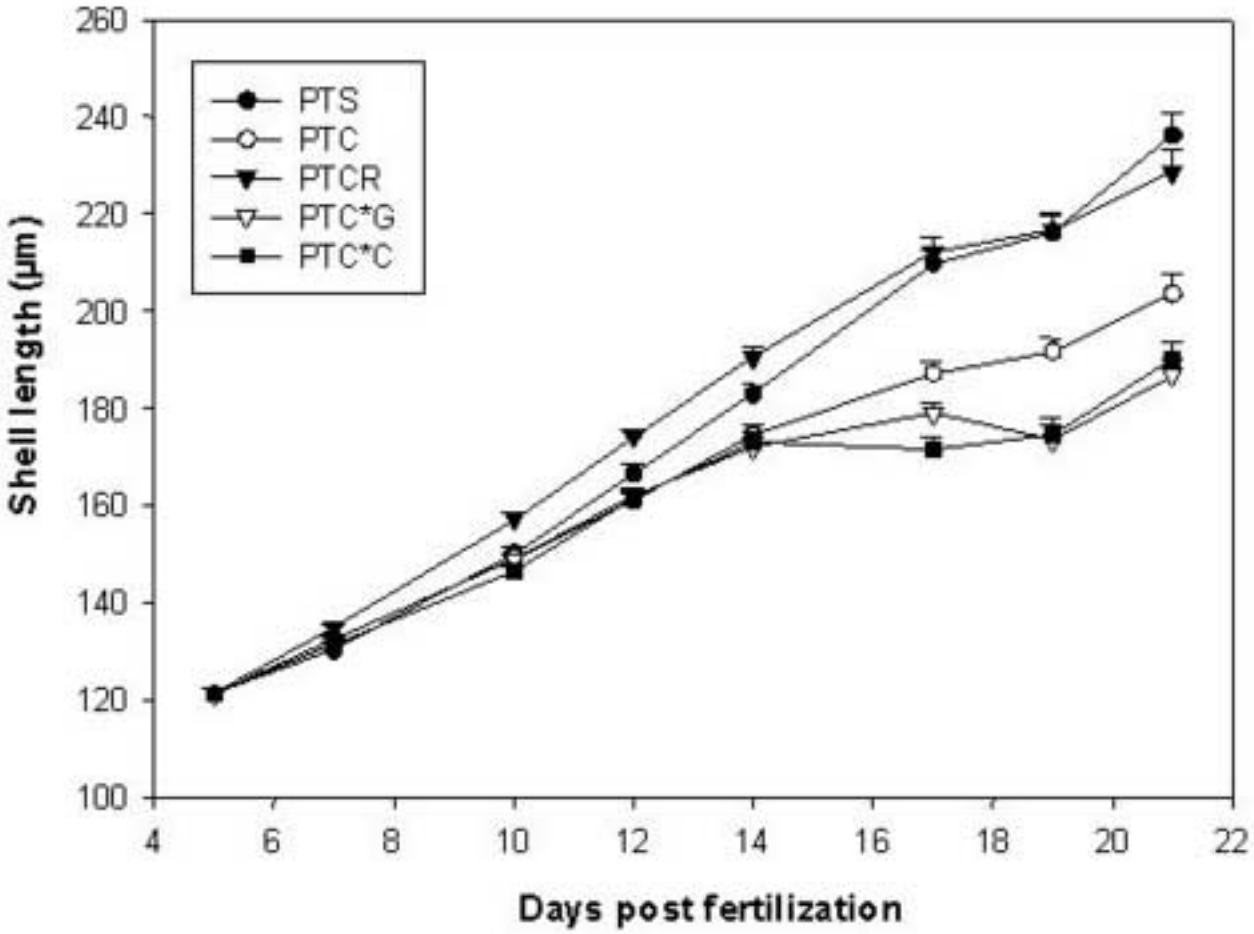

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FIG 2

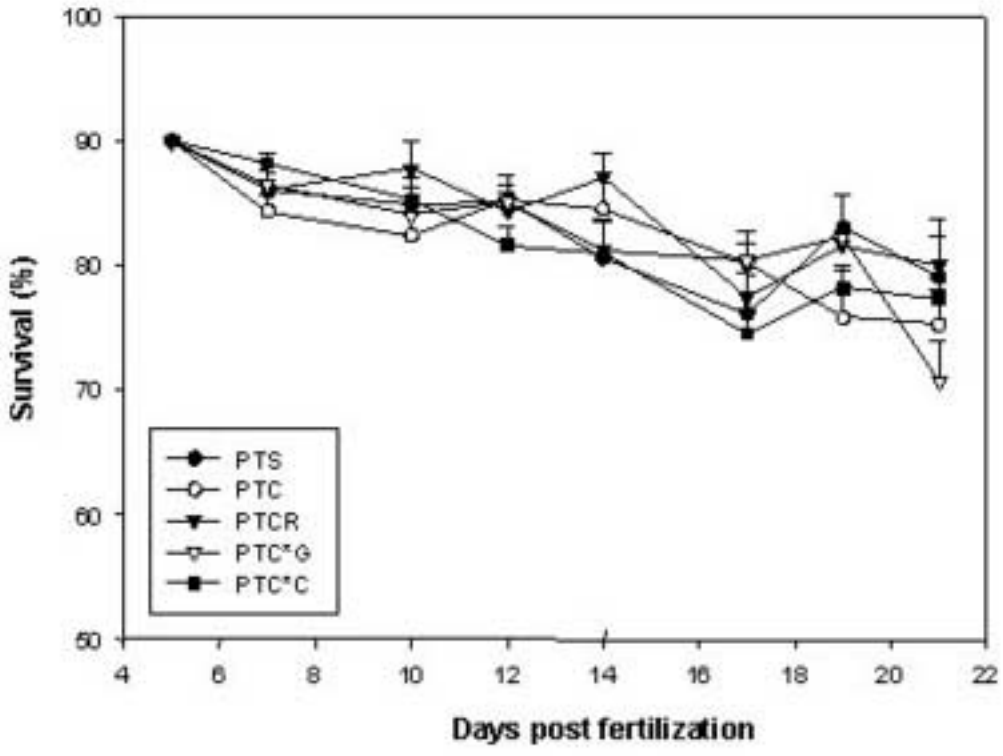

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Fig. 3

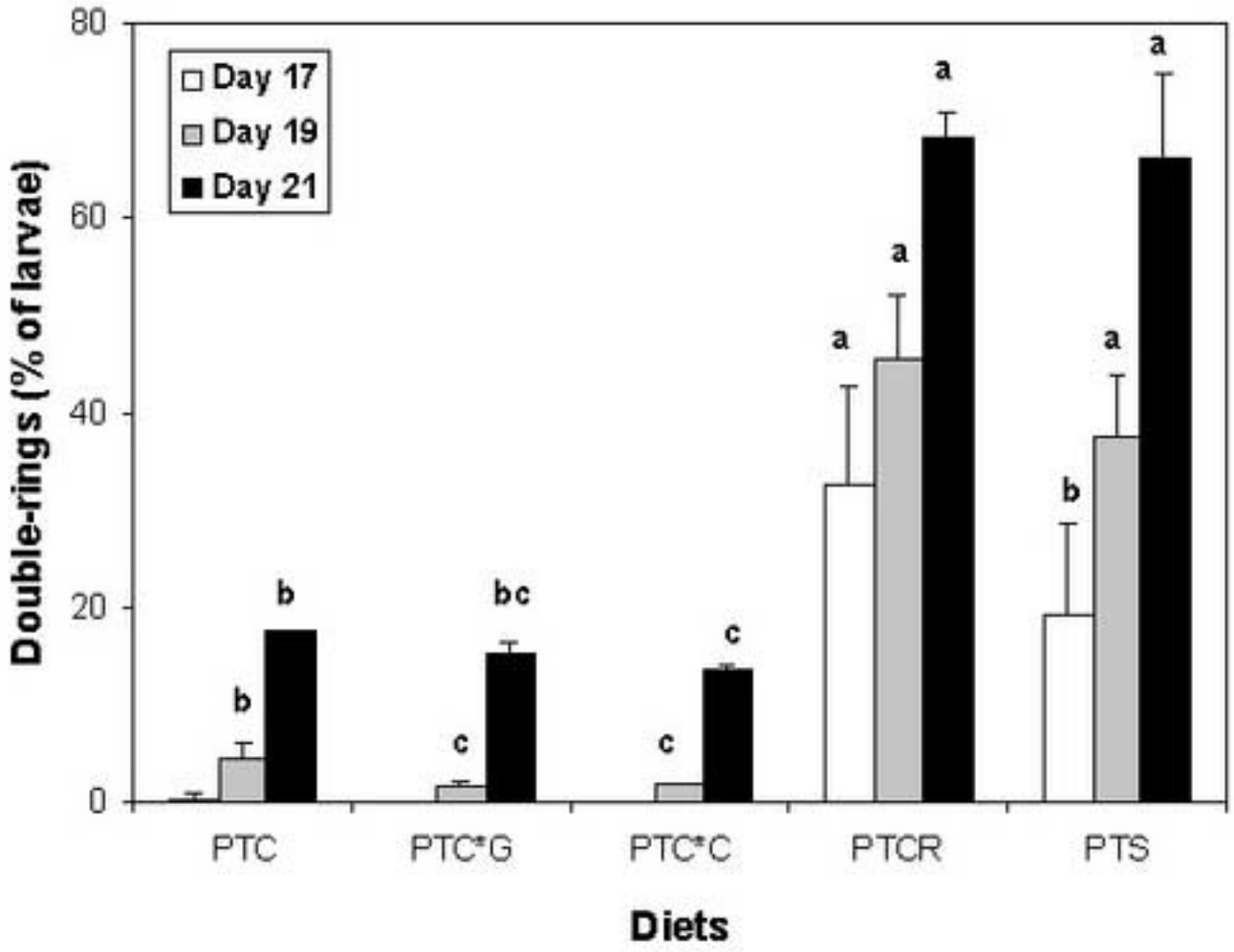

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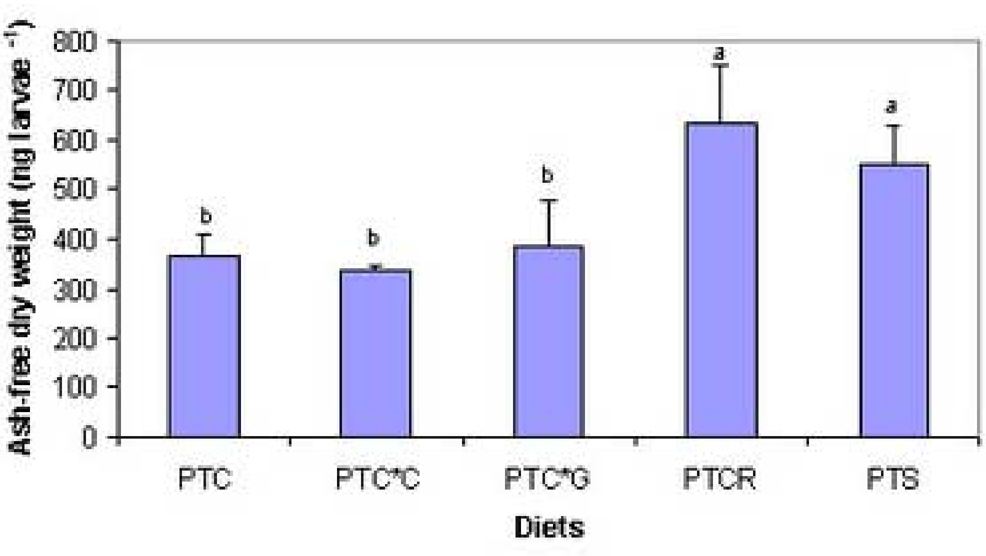
Fig 4.



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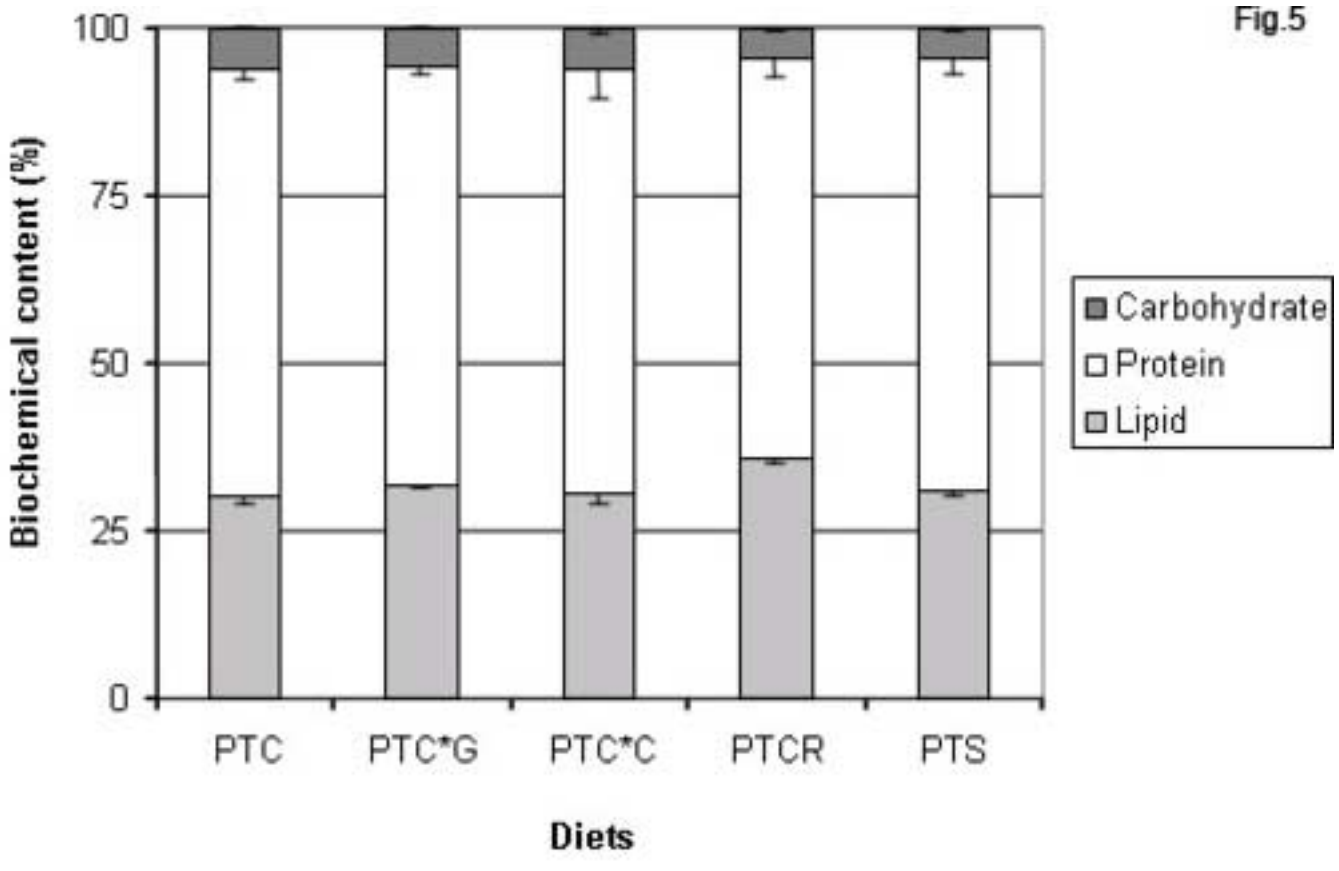

Fig.5

Ms. Ref. No.: AQUA-D-06-00612

Title: Effect of *Rhodomonas salina* addition to a standard hatchery diet during the early ontogeny of the scallop *Pecten maximus*

As requested, all comments of the two reviewers have been included. The details of our modifications are described below following the reviewers' comments.

Reviewer #1:

1. p. 2, line 45-47. The statement "This high level of organic matter in the form of lipids seems to promote larvae to grow and to complete metamorphosis more rapidly than with the other diets" -seems to imply that this (R. salina) diet contained more lipid, when in fact it was the larvae. The accumlation of lipid was likely to have resulted as a consequence of better growth with the R.salina diet, with the larvae accumulating lipid during ontogeny. This point needs to made clear; currently it is a little confusing.

   We agree and therefore modified the sentence for: Moreover, our results showed that the addition of *R. salina* significantly improved the overall condition of the larvae by promoting an increase in organic matter and total lipids. This accumulation of lipids during ontogeny seems to promote larvae to grow and to complete metamorphosis more rapidly than with the other diets.

2. p. 4, line 88: size of broodstock scallops, if available.

   The sizes are not available, but we have added wet weight.

3. p. 4, lines 91 and 92: More information would be useful on the process for thermal shock of broodstock (i.e. temperature change and time) - or reference to another paper where it is cited.

   A reference to Gruffydd & Beaumont (1970) has been added.

4. p. 5, lines 103-104: please state light intensity and culture volume

   Information added.

5. p. 12, lines 265-266: A value of carbohydrate of ˜40% is cited for R. salina from the study, but no other data for this or other species is given in the paper. For example, how does this 40% compare with other species examined ?

   Information from the literature has been added: "…carbohydrate levels in diatoms (*C. gracilis* and *S. costatum*) and prymnesiophytes (*I. galbana* and *P. lutheri*) are generally under 15% (Brown et al. 1997).

6. p. 25 line 501: "larve" should read "larva"

   Done.

7. p. 26 line 503: "larve" should read "larva"

   Done.

8. Fig 1: y axis of graph, "lenght" should read "length"

   Done.

9. Fig. 4: y axis of graph, "larvae" should read "larva"
Done.

Reviewer #2:
General: Several times throughout the ms the full latin name (ie. Rhodomonas salina) is written, even after it has been written in full earlier in the chapter (ie pg2 - line 39, pg12 - line 273, 274, 275,276). Please correct and shorten (ie. R. salina and similar).
Done.

Pg1 - line 29: Keywords: perhaps fatty acids or algal fatty acids could be included?
Done.

Pg2 - line 33-36 Abstract: I think first sentence should be re-phrased, as it seems a bit long.
Done.

– line 45: Consider using the word "increase" instead of "enrichment" as the meaning seems more appropriate. Also, consider using "higher" instead of "high" as it is not really shown that this is a high value in general, although it was the highest in your work.
Done.

Pg3 - line 55: It is not clear to me what the authors mean by "Metamorphosis is a stressful series…" Consider deleting "stressful"
Done.

Pg4 - line 76: I suggest that "recently" is changed to "previously", as the reports from 1986 to 1999 hardly can be called recent.
Done.

Pg4 - line 91: I was very disturbed to read "Male and females" concerning the spawning of Pecten maximus as this species is a functional hermaphrodite. Surely, the co-authors from France must know this and cannot have read the manuscript thoroughly.
Corrected.

- line 93: was there any particular reason for using the 21 <mu>m mesh size to collect veliger larvae? Normally a 45 <mu>m mesh size is used.
This was an error and is now corrected.

-line 95: number of replicate beakers should be mentioned here.
Done.

Pg5 - line 99: the abbreviation for "days post fertilization", dpf, should rather be moved to the beginning of the chapter Material and methods", and used consistently thereafter (it is not on pg. 10, line 235 and pg 11 line 236).
Done.

- line 105: consider using similar ratio signs for both gases and volumes, ie "air:CO2=98.5:1.5%" (or similar).

Done.

- line 113: It is interesting to know if the daily ration of feed was distributed once or twice a day. Please add the information.

Done.

- lines 114-115: It is very unclear why the total number of cells fed to the larvae was not the same for all diets. This would have excluded one factor to consider when interpreting the results. Neither the cell number nor the dry weight of the feed rations is similar, and this is very hard to understand. In my opinion this is one of the serious weaknesses of the work. Normally an algal cell concentration higher than 20-40 cells/<mu>l will be saturating for scallop larval ingestion rate (ie. Lu and Blake, 1997). This means that increasing cell concentration above this will finally cause an overload of particles for larval feeding, and hence, decrease ingestion rate. The concentration of 165 cells/<mu>l may or may not cause the ingestion rate to decrease. Also, the increased feed concentrations causes water quality problems even for as short term as 2-3 days. I strongly feel that possible effects of different cell concentrations in your diets should be included in the discussion.

The two additional control diets (PTC*C and PTC*G) were tested to account for the higher biomass of the PTCR diet, but with the larger cells of *R. salina*, it was impossible to test the effect of similar biomass without increasing the cell concentration. The differences in cell concentration and biomass of the diets are already discussed (Page 13, line 293-300) in relation to the saturation of the filtration system. But as suggested, results from the Lu and Blake study have been added and discussed.

Pg6 - line 125: mention here that samples were taken from all larval units (n=3 for each diet).

Done.

- line 126: consider changing "recovered" to "collect" as recover rather means to "get well".

Done.

- line 131: add how many individuals were measured from each larval unit (n=?).

Done.

- line 137: from your description of methods, the organic matter is ash-free dry weight and I suggest you use that expression. - line 139: add the duration of heating at 450C (hours?)

Done.

Pg7 - line 145-146: Even if PT was similar for all diets, I believe that you should have described the quality (FA) of each diet instead of only the species that were different from diet to diet. Then you would have seen the actual ratio of differences in fatty acids between the diets. Now, even if R have a double amount of a fatty acid compared to C, it does not necessarily give the same picture for the diet because the ratio of the species (as % of total number of cells) differs between the diets (ie. In PTCR R is ¼ of the total cells, in PTC C is 1/3 of the total cells).

As flagellates were not sampled, it is not possible to add these analyses. However, we added in this section that the biochemical content of these flagellates has been presented in the

literature (Delauney et al. 1993, Soudant et al. 1996, Brown et al. 1997, Soudant et al. 2000).

- line 146: what was the number of replicates for the algal samples?
Three; this has been added to the text.

- line 147: the size (mm?) for the GF/F filters?
25 mm; this has been added to the text.

- line 148: the total volume of the glass tubes? And the volume used of the chloroform:methanol mix? - line 153: Do you mean that samples were evaporated to dryness, or actually the lipids? Also, was the ratio of chloroform:methanol again based on volume (v:v)? Please add this to your text.
Done.

Pg8 - line 172: Consider changing the word "Pools" to "Samples", and was only one sample collected from each tank? Please add in text - line 173: the size of the GF/F filter (mm?) - line 176: How many ml of ammonium formate was used to rinse each filter with larvae? - line 181-189 Statistics: What statistics were used for larval fatty acids?
This information has been added in the text.

Pg9 - Line 198-199 Results: The first sentence can be deleted as the same thing is explained in Material and Methods. - line 200: in parenthesis it says that TFA is given as ng/cell, but in the table 1 it says pg/cell. Correct what is wrong.
Done and corrected for pg/cell.

Pg10 - line 213: Only one sterol conc. is given for R. salina, so it should all be in singular ("concentration" and "was")
Corrected.

- line 215: unless all microalgal spp are characterized by high sterol concentrations, a "the" must be put in front of "microalgal species" to get the meaning "the three spp you have used here".
Corrected.

- line 223-225 Scallop performance: the shell height is given in Figure 1, but it could be interesting to know the mean growth rate as well. It always indicates if the larval groups seemed healthy or not. Perhaps just given in the text. - line 227-228: Figure 2 gives % survival, and so this is what it should be referred to in the text as well (it says "cumulative mortality").
Done.

Pg11 - line 241-254: I suggest as earlier that you use the term ash-free dry weight instead of OM, and also change "scallop" with "larvae" in this paragraph. The word "concentration" should be changed to "content" as you describe the amount per individual. It is also confusing to read about % differences on absolute values. Perhaps it could be given in parts?
Modifications made as requested.

Pg12 - line 264: delete "global" - line 265: change "contained" to "content" - line 267:

"developmental metabolism" is unclear to me. May need to rephrase. - line 272: is the volume of R.salina really "very large"? Not compared to larger algae, so perhaps you could rephrase it to "larger" as it was larger than the other algae in your study (but they are actually small algae).

Done.

Pg.13 - line283-285: the effect of high concentrations (up to 165 cells/ml) of algal cells on larvae is mentioned for your study, but you should also mention the level of concentration that was reported by Sprung (1984, 1989). I am not convinced that reports for adults are interesting in this context, as the feeding and ingestion varies from larvae to adults. I suggest that you find more references concerning bivalve larvae and rather delete the information about adults. - line 287-288: consider to change "resulted from" to "may have resulted from" as the results are only indicative. Also, I would not call 60 cells/<mu>l "low cells concentration", as indicated by a rather large number of reports about the cultivation of P. maximus larvae (ie Robert et al.). - line 293: Delete "Other" as there are no other "qualitative differences" to refer to.

Done.

Pg14 -line 313: you should add "only" after "diatoms" as the reference by Delaunay et al. is about monospecific diets.

Done.

Discussion:
In addition to expend the discussion about the possible effect of different cell concentrations for the different diets, the use of antibiotics should also be mentioned. Adding antibiotics to larval units of small volumes may seem necessary to ensure larval survival. However, have you investigated if the antibiotic affects the larvae in any way?

We did not investigate the effects of antibiotics in this study; however, it has been discussed in literature. This text has been added at the end of the first paragraph of the discussion:

"The increased feed concentrations may cause water quality problem by bacteria proliferation. Nevertheless, the use of antibiotics to ensure the success of *P. maximus* larval culture seems to limit this problem, as no more mortality was observed in these treatments. The use of antibiotics at a concentration of 8 $mg \cdot L^{-1}$ has been demonstrated to be beneficial for the survival of scallop larvae with no impact on the growth rate (Nicolas et al. 1996, Robert et al. 1996)."

Pg18 - line 413 References: Knauer et al. 1998 cannot be found in the text. Delete if that is the case.

Done.

Tables.
Pg 22 Table 1: In addition to what is noted in the table text, there are also 3 ratios (n-3/n-6 etc.) and TFA as pg/cell. Should be included in the table text.

Done.

Pg25 Table 4: -line 498: after "absolute" I suggest you add (ng larvae-1) and similar after "relative", instead of at the bottom of the table.
- line 498 and 501: change "concentrations" to "values"

Done.

Pg26 Table 5: - line 502: Delete "Quantitative proportion of" - line 503: move 1) (ng larvae-1) and 2) "at day 21", to after "larvae" in line 502. Also, rather add "means + SD" on this line

Done.

Figure legends.

Pg27 - line 509: delete "on"- line 509-510: Add "note that both axes do not begin at 0". - line 511: Is survival given as % of day 4 number? Please explain. Delete "on". Add "note that both axes do not begin at 0"- line 513: What is occurrence of double rings given as a % of? Please explain. - line 521: Also the relative concentrations of lipids, etc - as % of what?

Done. The survival rate beginning at 90% represents the mortality observed between hatching and the beginning of the nutritional experiment after transfer to the IFREMER hatchery, as described in the material and methods section (line 131-132).

FIGURES

Fig 3. Y-axis title should be of similar format as #1 and #2 (ie. Double-rings (% of ?)).

Done.

Fig 4. Y-axis title should be of similar format as #1 and #2 (Ash-free dry weight (ng larvae-1)

Done.

Fig 5. Y-axis should have the "%" at the end of the title (Biochemical content (% of ?))

Done.