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Effects of different dietary microalgae on survival, growth, settlement and fatty acid composition of blue mussel (*Mytilus galloprovincialis*) larvae

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ABSTRACT

The diatom *Chaetoceros calcitrans* is a major component of many bivalve hatcheries, yet it is expensive and notoriously difficult to culture on a commercial scale. In an attempt to reduce dependence on the diatom *C. calcitrans*, mussel larvae (*Mytilus galloprovincialis*) were subjected to feeding experiments which altered levels of the diatom under controlled hatchery conditions. Growth, survival and settlement success of mussel larvae were determined in response to five mixed algal diets in which the relative contributions of *C. calcitrans* was varied over the experimental period (30 days). Fatty acid profiles of the larvae and algal diets were also assessed. The exclusion of *C. calcitrans* from the diet had no significant differences on larval growth and only minor differences in total fatty acid content were found between treatments. Fatty acid analysis revealed that larval survival was strongly influenced by the proportions of dietary docosahexaenoic acid (DHA), while settlement was positively correlated with higher ratios of the n-3 long-chained polyunsaturated fatty acid (n-3 LC-PUFA) (namely, DHA and eicosapentaenoic acid, EPA), to the n-6 LC-PUFA (arachidonic acid, ARA). Despite similar relative and absolute n-3 LC-PUFA levels in the larvae under different dietary treatments, the larvae receiving high levels of *C. calcitrans* performed significantly better in terms of survival and settlement success. These results indicate that the (DHA + EPA)/ARA ratio is a key factor in determining larval performance, rather than the total amount of these fatty acids.

1. Introduction

Mussels are one of the most widely farmed aquaculture species, with global production in 2006 estimated at 1.8 million tonnes and valued at more than $1.2 billion USD (FAO, 2008). Blue mussel species belong to the genus *Mytilus* (*M. edulis*, *M. galloprovincialis* and *M. trossulus*), and are now one of the most widely cultured groups worldwide, with the major species of interest in Australia identified as the non-indigenous *M. galloprovincialis* (Mather et al., 2009). *Mytilus* species possess high potential for further aquaculture development; historically the mussel industry has relied, and still relies substantially, on wild recruitment to seed mussel ropes. However, patterns of natural recruitment are highly variable from year to year and can be influenced by a number of environmental and biological factors (Dobretsov and Wahl, 2008; Marshall et al., 2010). In response to managing environmental constraints of wild stock, and a growing demand for mussel products, the development of bivalve hatchery technology has provided the aquaculture industry with an alternative reliable source of mussel spat in an otherwise highly variable production trade (Camacho et al., 1995). However, significant production problems still remain. The most stringent limitations include rearing bivalves to post-larval stages to complete the life cycle, the mass-production of microalgae for feeding and optimising environmental parameters for larvae (Robert and Gerard, 1999).

The lack of parental investment in offspring results in the production of a large number of small larvae to compensate for starvation and other mortality factors which dramatically reduce survival rates in the wild (Jorgensen, 1981; Thorson, 1950). It also necessitates a larval 'feeding' stage where there is a high demand for exogenous food sources. This life stage signifies the importance of optimising larvae nutrition as a crucial aspect of overall mussel physiological ecology, and hence, hatchery operations (Widdows, 1991). Microalgae are the primary food source used in aquaculture as live feeds for all growth stages of bivalves (Brown et al., 1997). Culturing live algae as feed in hatcheries is necessary considering the limited success of artificial feeds (Laing, 1987). However, consistent algae biomass production can be labour-intensive and costly; generally accounting for 30% of total seed-production costs (Rivero-Rodriguez et al., 2007). Meeting the specific diet requirements of bivalve larvae depends not only on concentration, but also composition of feed (Baldwin and Newell, 1995a). It is now recognised that the nutritional value of a multi-species algal diet is superior to a monoculture, whereby combinations

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of different algal species is thought to impart synergistic effects on mussel growth (Galley et al., 2009; Stromgren and Cary, 1984; Walne, 1970).

Feeding mixed diets of at least one type of flagellate and one type of diatom has been shown to produce optimal growth and development of bivalve larvae (Galley et al., 2009; Laing, 1991; Martinez-Fernandez and Southgate, 2007). Consistently commercially reliable species used in hatcheries worldwide typically include the flagellates: *Isochrysis galbana* and *Pavlova lutheri*; and the diatom *Chaetoceros calcitrans;* all of which have good nutritional properties as feed for many aquaculture organisms (Gouda et al., 2006; Smith and Chanley, 1975). *C. calcitrans* is considered one of the most suitable strains of microalgae as feed for bivalve larvae (Brown and Robert, 2002), not only because of its biochemical composition, but also its small size, digestibility and absence of toxins. However, it has proven difficult to culture in sizeable quantities necessary for large scale hatchery operations, possibly as a result of light limitation (Watson et al., 2004) and contamination (Laing, 1991), which subsequently compromises its suitability for commercial applications. Thus, it has been necessary for some mussel hatcheries to supplement *C. calcitrans* with other diatoms, such as *Chaetoceros muelleri*. However, research into the nutritional value of this species as a dependable source of microalgae feed for mussel larvae is lacking.

Lipid content and composition is a critical factor in larval development during early life stages and for settlement (Jonsson et al., 1999; Levine and Sulkin, 1984). By investigating a range of larval health indicators in response to changes in microalgae feed, specific relationships between biochemical composition and larval health indicators can be detected. As with all other aquatic animals, lipids are the principal source of energy for bivalve larvae, and within the dietary lipid fraction, essential fatty acids (EFA) are paramount for growth and development (Marshall et al., 2010). Normal aquatic larval growth and development necessitates three types of long-chained polyunsaturated fatty acids (LC-PUFA) in the diet; docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) (Sargent et al., 1997). High dietary levels of DHA have been shown as necessary for larval neural development in marine fish, while EPA and ARA play crucial functions as the precursors of hormone-like eicosanoids, which serve important roles in a multitude of physiological functions (Tocher, 2003).

Despite their importance in larval development, little information is available on EFA requirements for bivalves, and in particular bivalve larvae (Marshall et al., 2010). It has been reported that in general mussel larvae possess limited fatty acid elongase and desaturase activity, thus suggesting that dietary LC-PUFA (namely EPA, DHA and ARA) certainly play a major role (Knauer and Southgate, 1999; Milke et al., 2004; Ponis et al., 2006; Seguineau et al., 2005). However, the actual relative importance and potential interactions of these fatty acids in mussel larvae nutrition is yet to be well established, and primarily based on speculative observations (Knauer and Southgate, 1999). So far it is generally understood, primarily in other aquatic larval species, that the balance between EFA has crucial effects on larval health (Pernet and Tremblay, 2004). Eicosanoid production is a function of cellular levels of ARA, relative to EPA, and an imbalance between these LC-PUFA can be detrimental (Tocher, 2003). Nevertheless, there is paucity of information with respect to the required proportions of these LC-PUFA, specific for the diet of blue mussel larvae. Similarly, very little is known of the roles and possible interactions of other dietary fatty acid and fatty acid classes.

The present study determines the effect of different diets on the survival, growth, settlement and fatty acid composition of hatchery reared larvae of the blue mussel *M. galloprovincialis*. Analyses of algae and larvae tissue lipid composition are used to determine the relationship between fatty acids composition of the diets and larval performance. These findings provide crucial information on the nutritional requirements of hatchery reared *M. galloprovincialis* larvae and the optimisation of feeding practices that maximise larvae yield and minimise costs in aquaculture hatcheries.

2. Materials and methods

2.1. Spawning and larvae production

All experiments were carried out at the Department of Primary Industries mussel hatchery Queenscliff, (VIC, Australia). Samples of approximately 100 wild mussel broodstock (*M. galloprovincialis*) for each experiment were supplied by a local mussel farm, Werribee (VIC, Australia). Broodstock were grown from natural spatfall harvested from the same region and were spawned on the same day as collection. Two spawning runs were carried out during the experimental period, the first in July 2009 and the second in September, 2009.

Spawning was induced by increased water flow, light intensity, and thermal shock, attained by progressively increasing seawater temperature from 16 to 21°°C over approximately 1 h to trigger release of gametes. Males and females were identified at the time of gamete release and isolated into individual spawning chambers. Gametes from same sex individuals were then pooled (from approximately 50 males and 50 females) and sperm and egg concentration were assessed from several replicate counts made using a Beckman coulter counter which were then diluted to a sperm: egg ratio of 7:1 as per standard hatchery practices. Fertilisations were carried out in four replicate 20-l containers and eggs were rinsed with UV-treated, 1 μm filtered seawater approximately 30 min after sperm and eggs were mixed together. All developing embryos were transferred to 4000-l incubation tanks within 3 h, with observations made regularly to ensure that fertilisation progressed normally. Embryos were fed at 30 h, and collected onto 45 μm sieves at 48 h. Selective larval grading ensured only those larger than 60 μm (shell length) were kept for rearing purposes.

2.2. Rearing facilities

At day two, larvae were introduced into 25 2-l tanks at approximately 20 larvae·ml^{-1}. Larval rearing involved a closed culture system with gently aerated UV-treated 1 μm filtered seawater maintained at approximately 16 °C and completely renewed every 2nd day over the duration of each experiment. Each feeding experiment (see details below) was carried out over a period of approximately 4–5 weeks (28 and 33 days, for experiments 1 and 2 respectively) with larvae fed twice per day. Larvae were reared from D-stage larvae (first larval stage) through to pediveliger (competent for settlement) during this experimental period. The second experiment also included settlement observations over a three day period.

2.3. Feeding experiments

Two separate feeding experiments were carried out during each of the spawning runs. For each feeding experiment five diets were compared, with five replicates per treatment. Microalgae used in the feeding experiments were cultured onsite with the initial culture purchased from a commercial supplier (CSIRO Collection of Living Microalgae Centre, TAS, Australia). Four species were cultured: *I. galbana, P. lutheri, C. calcitrans* and *C. muelleri*. All species were maintained in a series of 400-l bags (except for *C. calcitrans* which is difficult to culture on a large scale and was therefore grown in 20-l carboys). Cultures were grown in UV-treated, 1 μm filtered seawater with temperature kept at 16 °C and pH maintained at 8.0–8.2 using CO_2 bubbled into the culture and controlled by a pH probe.

A preliminary study (experiment 1) was undertaken to determine the optimum feed density provided to larvae throughout development and to validate that the experimental setup was comparable with

hatchery standards. The feeding experiment investigated five algae concentrations based on cell numbers of 0 cells ml^{-1}, 7500 cells ml^{-1}, 15,000 cells ml^{-1}, 30,000 cells ml^{-1} and 60,000 cells ml^{-1}, with larvae reared in a closed system over 28 days. Larvae were fed a standard hatchery diet with the same algal composition consisting of 66% flagellates (*I. galbana* and *P. lutheri*) and 33% diatoms consisting of either *C. calcitrans* (fed throughout the entire larval development) or *C. muelleri* (fed only at night after day 14).

For experiment 2, the algal cell concentration was standardised to 60,000 cells ml^{-1} based on the results from experiment 1. Larvae were fed five different diets which varied in diatom composition, with gradual sequential replacement of *C. calcitrans* by *C. muelleri* fed to the larvae over the experimental period (Fig. 1). The control diet was based on standard hatchery protocol (treatment 1 — T1) and consisted of 66% combined *I. galbana* and *P. lutheri* and 33% *C. calcitrans*. After day 14, at night, *C. calcitrans* was replaced by *C. muelleri*. Treatment 2 (T2) comprised of an identical diet regime as the control group however, at day 21, *C. calcitrans* was replaced with *C. muelleri* only. Treatment 3 (T3) followed the same regime; however, *C. muelleri* replaced *C. calcitrans* completely after day 14. Treatment 4 (T4) diet altered the control regime from day 1 with larvae fed a combination of *C. calcitrans* (day) and *C. muelleri* (night) until day 14 where *C. muelleri* was fed exclusively. Treatment 5 (T5) feed regime involved the complete removal of *C. calcitrans* from the larval diet, with replacement by *C. muelleri* (day and night) over the entire 30 day experiment.

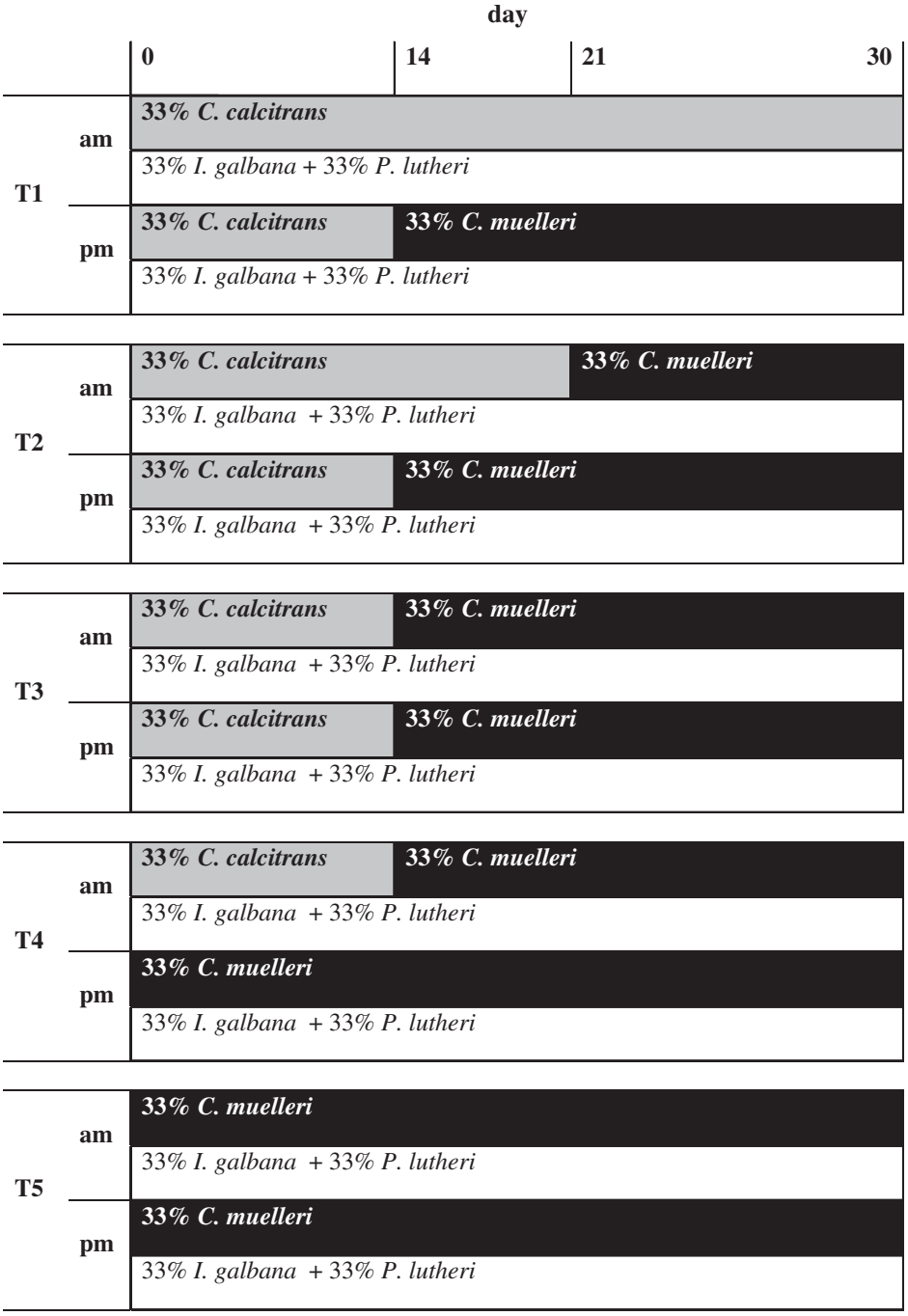

Fig. 1. Diet regimes of *M. galloprovincialis* larvae fed different compositions of diatoms; *C. calcitrans*, and *C. muelleri*. T1 = treatment 1 (standard hatchery operations); T2 = treatment 2; T3 = treatment 3; T4 = treatment 4; and T5 = treatment 5.

Hatchery production of bivalve larvae often utilise flow-through systems where fresh microalgae are continually renewed to maintain constant feed density (Helm et al., 2004). The experimental design used in this study was a closed system. However, to ensure that constant feed levels were maintained in the closed system, algal cell concentrations in each tank were measured using a Beckman Coulter Multisizer 3 to record residual cell levels, and to determine the amount of algae feed required twice daily. Residual algal counts were based on a single coulter counter count after extensive testing showed the coulter counter to have provided a very precise and accurate estimate of residual algal levels. The total amount of algal cells administered during the entire feeding experiment was then adjusted accordingly. As a result, algae supply was not a limiting factor. In addition, larval health response variables (see below) were routinely recorded such that larval condition in the closed system was analogous to that of hatchery standards. Seawater was changed and tanks cleaned with chlorine and sodium thiosulfate once every 2 days. Larvae were sieved using a 60 μm screen to ensure no undeveloped or dead larvae were screened out. When large amounts of fouling were observed (specifically tanks with high feed concentrations), a 500 μm screen was used to remove by-products.

2.4. Measures of growth, mortality and lipid content

Ten larvae from each replicate tank were sampled every second day to record shell length (antero-posterior axis parallel to the hinge). Photographs were taken under a compound microscope using a Marlin F-033 C to assess developmental stage and lipid content (as a visual lipid score). The visual lipid score involved microscopic observations of lipid globules within the larvae body to quantify lipids in individual larvae. This provided a visual representation of total body lipid. A rating from 0 (no lipid) to 3 (high amount of lipid) was assigned to each sample, based on the overall size and quantity of lipid globules present. The sum of all scores for each replicate was then pooled and each treatment was ranked in comparison to other treatments. A similar method was used by Castell and Mann (1994) involving the quantification of lipid content (however, the use of lipid-specific fluorescent stain was not used in this study) to visually estimate the proportion of lipid content in oyster larvae.

At the end of each experiment, 20 larvae from each replicate were sampled to determine final shell length, lipid content (visual lipid score) and developmental stage. Larval mortality was estimated at the end of the experiments by sampling approximately 100 larvae and counting translucent shells (i.e. those shells containing no living tissue) through microscopic observations. Experiment 2 was extended to include settlement rates. Fifteen downweller containers were used to contain a pooled sample of approximately 100 larvae from each treatment (replicates combined). The downwellers were then placed in a 240-l tank with seawater kept at 16 °C and feeding density maintained at 1.0×10^5 cells ml^{-1}. Hoses were placed in each container to produce a water flow system and stimulate settlement onto the mesh substrates, as demonstrated by Dobretsov and Wahl (2008). Water was exchanged and the tank cleaned every 2 days. After 3 days, the larvae were collected and microscopic observations of attachment to the substrate recorded.

Relative and total fatty acid content was quantitatively determined through fatty acid analysis for both larvae and the algal feed. As samples of mussel larvae were too small to quantify total lipid gravimetrically, quantitative fatty acid analysis was used in addition to the visual lipid score to estimate total lipid. Samples of centrifuged algae and larvae were stored at −80 °C and freeze–dried before extraction. Lipid and fatty acid analysis preparation was performed as described by Palmeri et al. (2007). Lipid extraction was carried out using dichloromethane–methanol (2:1 ratio) according to Folch et al. (1957), with chloroform substituted by dichloromethane, and fatty acids were esterified into methyl esters using the acid catalysed methylation method (Christie, 2003). The internal standard was 23:0 (Sigma-Aldrich, Inc., St. Louis, MO, USA) and fatty acid methyl esters were isolated and identified using a Shimadzu GC 17A (Shimadzu, Chiyoda-ku, Tokyo, Japan) equipped with an Omegawax 250 capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness, Supelco, Bellefonte, PA, USA), a flame ionisation detector (FID), a Shimadzu AOC-20i auto injector, and a split injection system (split ratio 50:1). The temperature program was 150–180 °C at 3 °C min^{-1}, then from 180 to 250 °C at 2.5 °C min^{-1} and held at 250 °C for 10 min. The carrier gas was helium at 1.0 ml min^{-1}, at a constant flow. Each of the fatty acids was identified relative to known external standards. The resulting peaks were then corrected by the theoretical relative FID response factors and quantified relative to the internal standard.

2.5. Statistical analysis

Data was tested for assumptions of normality and homogeneity of variances, with the use of a normal probability plot and Levene's test, respectively. All data sets met the assumption of the parametric tests unless otherwise stated. In those cases where the data did not meet the assumptions of normality and equal variance, non-parametric tests were used to test for significant differences between treatment groups. All statistical tests were carried out with the use of statistical software package, Minitab® (V15).

A one-way analysis of variance (ANOVA) was used to test for significant differences between diets for each response variable (growth, mortality, eyespot, settlements and fatty acid composition). Where any significant differences were observed between treatments ($p<0.05$), the effects of diet on the response variables were evaluated using a post-hoc Tukey's multiple comparison test. Statistical analysis of lipid ranked data (visual lipid score) was carried out using a non-parametric Kruskal–Wallis test for both experiments and its equivalent parametric test, a one-way ANOVA (when assumptions were met) as suggested by Quinn and Keough (2002) to determine differences between means for days 20 to 30. Where significant differences existed, both Mann–Whitney–Wilcoxon and Tukey's pairwise comparison tests were performed, respectively. Regression analysis was used to determine the relationships between fatty acid composition of each algal diet and the final fatty acid composition of the larval tissue and larval fitness response variables in Experiment 2. Only those relationships that were significant ($p<0.05$) are reported.

3. Results

3.1. Feeding experiment 1: algal feed density

Strong positive correlations were found between feed density and all response variables tested over the concentration range used (Table 1). The highest algae cell density of 60,000 cells ml^{-1} resulted in the greatest shell growth, survival, eyespot development and visual lipid score. Survival of larvae fed 60,000 cells ml^{-1} was about 35.7% higher than the starved treatment group. The overall performance of larvae fed 60,000 cells ml^{-1} in the experimental setup was similar to that of previous data recorded in the commercial hatchery. Thus, the highest algal density feed was adopted for all subsequent feeding experiments.

3.2. Feeding experiment 2: algal feed composition

Larval mortality, visual lipid score and larval settlement varied significantly between treatments, however, no significant effects of diet treatment on either growth or development of eyespots were detected (Table 2). The complete replacement of *C. calcitrans* with *C. muelleri* (T5) produced significantly higher mortality rates ($F_{4,20}=4.26$, $P=0.012$) compared with diets that had progressively higher levels of *C. calcitrans*. A Tukey's pairwise comparison test indicated significant

Table 1
Response variables tested (mean ± SEM) of *M. galloprovincialis* larvae fed diets of different feed density in Experiment 1.

Feeding regimen (cells ml^{-1})	n	0	7500	15,000	30,000	60,000
Response variable (mean ± SE)						
Growth as Δ in size (μm)	20	$15.43^{a} \pm 0.42$	$66.93^{b} \pm 2.76$	$96.48^{c} \pm 3.14$	$138.68^{d} \pm 5.23$	$172.43^{e} \pm 1.32$
Mortality %	100	$41.87^{c} \pm 6.62$	$16.73^{b} \pm 1.01$	$6.88^{a} \pm 0.62$	$8.37^{ab} \pm 0.84$	$6.13^{a} \pm 0.37$
Eyespot % day 28	20	$0.00^{a} \pm 0.00$	$0.19^{b} \pm 0.12$	$12.62^{c} \pm 0.10$	$80.23^{d} \pm 1.81$	$89.72^{e} \pm 1.09$
Visual lipid score day 20	20	$10.00^{a} \pm 0.00$	$20.00^{b} \pm 0.00$	$20.00^{b} \pm 0.00$	$27.33^{c} \pm 0.88$	$33.00^{d} \pm 0.58$
Visual lipid score day 22	20	$10.00^{a} \pm 0.00$	$20.00^{b} \pm 0.00$	$23.33^{c} \pm 0.33$	$20.33^{b} \pm 0.33$	$23.67^{c} \pm 0.67$
Visual lipid score day 24	20	$10.00^{a} \pm 0.00$	$19.33^{b} \pm 0.67$	$27.00^{bc} \pm 1.53$	$27.67^{c} \pm 1.86$	$32.67^{c} \pm 2.91$
Visual lipid score day 26	20	$10.00^{a} \pm 0.00$	$20.00^{b} \pm 0.00$	$23.00^{b} \pm 2.08$	$28.33^{c} \pm 1.20$	$36.00^{d} \pm 0.58$

Different letters in same row indicate significant differences (one-way ANOVA, $\alpha = 0.05$, a<b<c<d).

Table 2
Response variables tested (mean ± SEM) of *M. galloprovincialis* larvae fed diets of different feed composition in Experiment 2.

Feeding regimen	T1	T2	T3	T4	T5
Response variable (mean ± SE)					
Growth (μm)	181.85 ± 2.78	181.00 ± 7.44	186.25 ± 2.23	188.95 ± 0.96	193.55 ± 1.63
Mortality %	$2.07^{a} \pm 0.27$	$3.06^{a} \pm 0.61$	$5.94^{ab} \pm 3.06$	$3.30^{a} \pm 1.00$	$12.35^{b} \pm 3.40$
Eyespot % day 18	56.00 ± 5.10	46.00 ± 12.50	50.00 ± 7.07	58.00 ± 4.90	62.00 ± 9.70
Visual lipid score day 20	$30.00^{b} \pm 1.00$	$30.33^{b} \pm 0.88$	$28.67^{b} \pm 0.88$	$27.67^{ab} \pm 1.20$	$23.33^{a} \pm 0.88$
Visual lipid score day 30	$37.33^{b} \pm 0.88$	$33.00^{a} \pm 1.53$	$32.33^{a} \pm 0.33$	$32.00^{a} \pm 0.58$	$31.00^{a} \pm 0.58$
Settlement %	$39.00^{b} \pm 15.40$	$17.84^{ab} \pm 3.11$	$21.55^{ab} \pm 5.22$	$7.52^{a} \pm 1.96$	$5.22^{a} \pm 1.47$

Different letters in same row indicate significant differences (one-way ANOVA, $\alpha = 0.05$, a<b<c<d). Refer to Fig. 1 for treatment descriptions.

differences between T1, T2, T4 and T5 ($P<0.05$). Visual lipid score was highest for the diet with *C. calcitrans* fed throughout the entire experiment (T1) and lowest for the diet with no *C. calcitrans* at both day 20 ($F_{4,14} = 8.31$, $P = 0.003$) and day 30 ($F_{4,14} = 7.76$, $P = 0.004$) of larval development. Settlement was also positively affected by diets high in *C. calcitrans*. Settlement success was significantly higher in those treatments fed *C. calcitrans* for a longer duration of the experiment ($F_{4,20} = 4.44$, $P = 0.025$). A post-hoc pairwise comparison test indicated T1 (39.0 ± 15.4) had higher settlement rates than T4 and T5, 7.52 ± 1.96 and 5.22 ± 1.47, respectively (Tukey's test, $P<0.05$).

The total algae administered (number of algae cells per larvae) over the entire experimental period are reported in Table 3. As expected, no differences were recorded for the total amount of algae and for the two flagellates (*P. lutheri* and *I. galbana*) provided to mussel larvae during the entire experiment. However, the amounts of *C. calcitrans* were progressively reduced from T1 to T5, and accordingly replaced by *C. muelleri*, as per the designed experimental protocol.

3.3. Fatty acid composition of algae

A total of 32 fatty acids were isolated and identified in the four algae species tested with significant differences in lipid content and fatty acid make-up detected between species (Table 4). Flagellates (*P. lutheri* and *I. galbana*) showed significantly higher overall total lipid content and total fatty acid (mg/g algae dry weight), while total fatty acid per g of lipid (mg/g lipid) was higher in diatoms (*C. muelleri* and *C.calcitrans*). However, no major variations in major fatty acid classes were observed (Table 4). Polyunsaturated fatty acids (PUFA) comprised most of the fatty acids found in the algae (~36%) followed by saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), (~35% and ~28%, respectively). Within the long chain polyunsaturated fatty acid (LC-PUFA) class, ARA (20:4n-6), EPA (20:5n-3) and DHA (22:6n-3) all showed variation in each of the algae species, both % wet weight total fatty acid and as nanogram per algal cell. There was a significant difference in ARA with higher proportions noted in *C. muelleri* (5.68%), however, quantitatively per algal cell, *C. calcitrans* showed significantly higher levels (164 ng). *P. lutheri* and *C. calcitrans* also displayed relatively high amounts of EPA as proportions and as per algal cell than the other species, while DHA showed higher levels in the flagellates, *P. lutheri* and *I. galbana* for both percentages of wet weight and per algal cell, than the diatoms *C. calcitrans* and *C. muelleri*.

3.4. Fatty acid composition of larvae

No overall significant differences in either total fatty acid (mg/g of larvae dry weight) or major classes of fatty acids in larvae were detected, irrespective of diet, with only significant differences noted for 18:0, 20:4n-6 (ARA) and 22:4n-6 (Table 5). The major SFA identified in the larvae were 16:0 which constituted approximately 17% of all fatty acids across treatments (no significant differences; $P>0.05$), followed by 14:0 and 18:0. The latter (18:0) showed significant differences between treatments ($F_{4,20} = 3.57$, $P = 0.024$) where T5 had larger amounts of the SFA, 18:0, than T1. Overall SFA contributed 30.37% ± 0.32 of total larvae fatty acids. A one-way

Table 3
Total algae administered (algal cells per larvae) over the experimental period.

Feeding regimen	T1	T2	T3	T4	T5
Total algae cells administered per larvae (mean ± SE)					
I. galbana	1328.7 ± 53.9	1234 ± 105	1261.6 ± 63.1	1417.6 ± 58.5	1446.2 ± 48.7
P. lutheri	1328.7 ± 53.9	1234 ± 105	1261.6 ± 63.1	1417.6 ± 58.5	1446.2 ± 48.7
C. calcitrans	$894.5^{d} \pm 37.6$	$563.7^{c} \pm 55.5$	$384.5^{b} \pm 29.0$	$249.5^{b} \pm 26.7$	$0.0^{a} \pm 0.0$
C. muelleri	$434.2^{a} \pm 17.1$	$669.9^{b} \pm 50.7$	$877.0^{c} \pm 50.3$	$1168.1^{d} \pm 32.2$	$1446.2^{e} \pm 48.7$

Different letters in same row indicate significant differences (one-way ANOVA, $\alpha = 0.05$, a<b<c<d).

Table 4
Fatty acid profile of microalgae used in Experiment 2. Values expressed as % w/w of total fatty acid of algae (ng/cell).

	P. lutheri	*I. galbana*	*C. calcitrans*	*C. muelleri*
8:0	0.19 (5)	0.14 (16)	0.09 (6)	0.24 (3)
10:0	0.34 (9)	0.19 (21)	0.23 (15)	0.29 (4)
12:0	0.06 (1)	0.06 (7)	0.10 (6)	0.07 (1)
14:0	12.84 (332)	22.28 (2467)	22.16 (1490)	15.74 (195)
14:1n-5	0.34 (9)	0.19 (22)	0.22 (15)	0.32 (4)
15:0	0.28 (7)	0.40 (44)	0.53 (36)	0.81 (10)
16:0	13.51 (350)	13.92 (1542)	16.35 (1099)	15.73 (195)
16:1n-7	12.78 (331)	4.33 (480)	28.20 (1896)	39.28 (486)
16:2n-4	1.36 (35)	0.39 (44)	3.94 (265)	1.46 (18)
16:3n-4	0.69 (18)	0.20 (22)	0.32 (22)	0.08 (1)
16:3n-3	0.20 (5)	0.25 (27)	0.48 (32)	0.11 (1)
18:0	0.43 (11)	0.60 (67)	0.53 (36)	1.49 (18)
18:1n-9	0.78 (20)	18.39 (2037)	1.24 (84)	0.69 (9)
18:1n-7	1.83 (47)	1.74 (192)	0.55 (37)	0.51 (6)
18:2n-6	0.51 (13)	6.59 (730)	0.50 (34)	0.61 (7)
18:3n-6	0.33 (8)	0.45 (49)	0.21 (14)	0.91 (11)
18:3n-4	0.21 (5)	0.07 (8)	0.04 (2)	TR (0)
18:3n-3	1.07 (28)	5.09 (564)	0.53 (36)	0.28 (3)
18:4n-3	7.07 (183)	9.08 (1006)	0.52 (35)	0.41 (5)
20:0	TR (0)	0.33 (37)	TR (0)	0.15 (2)
20:1	TR (0)	0.31 (34)	0.08 (6)	0.04 (0)
20:2n-6	0.06 (2)	0.10 (11)	0.05 (4)	0.05 (1)
20:4n-6 (ARA)	0.43 (11)	0.19 (21)	2.44 (164)	5.68 (70)
20:4n-3	0.04 (1)	0.05 (5)	0.06 (4)	0.04 (1)
20:5n-3 (EPA)	33.22 (860)	0.62 (69)	17.41 (1171)	12.95 (160)
22:0	0.09 (2)	0.40 (44)	0.37 (25)	0.22 (3)
22:1	0.12 (3)	0.27 (30)	0.09 (6)	TR (0)
22:2n-6	TR (0)	0.03 (3)	TR (0)	TR (0)
22:4n-6	0.61 (16)	1.69 (187)	0.84 (56)	0.06 (1)
22:5n-3	0.04 (1)	0.04 (4)	0.07 (5)	0.04 (0)
24:0	0.08 (2)	0.14 (16)	0.27 (18)	0.49 (6)
22:6n-3 (DHA)	10.50 (272)	11.48 (1272)	1.58 (106)	1.25 (15)
SFA	27.80	38.46	40.63	35.23
MUFA	15.86	25.23	30.39	40.84
PUFA	56.34	36.31	28.98	23.93
n-6 PUFA	1.94	9.04	4.04	7.31
n-3 PUFA	52.14	26.60	20.64	15.08
n-6 LC-PUFA	1.11	2.01	3.33	5.79
n-3 LC-PUFA	43.80	12.19	19.11	14.28
Lipid % (on dry weight)	21.47	21.12	15.30	18.47
Total FA (mg/g of lipid)	405.98	513.10	587.70	592.50
Total FA (mg/g of algae dry weight)	87.20	108.20	89.82	109.57

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids and LC-PUFA: long chain polyunsaturated fatty acids. TR = trace amounts.

ANOVA also revealed that the variability of the MUFA between treatments was low ($p>0.05$). MUFA constituted 30.86% ± 1.11, with C16:1n-7 comprising approximately 16% of all fatty acids. PUFA were the most abundant source of fatty acids in the mussel larvae, constituting 38.78% ± 0.69. No significant differences were noted for n-6 LC-PUFA. However, ARA showed considerable variability with higher levels in T2, T3, T4 than T1 ($F_{4,20}=9.20$, $P<0.0001$). Similarly, 22:4n-6 was significantly affected by treatment. No significant differences were noted for n-3 LC-PUFA, and EPA and DHA composed approximately of 10% and 9% of fatty acids, respectively. T1 was found to have the lowest content of ARA, yet conversely, also the highest content of DHA, yet no variabilities of significance were detected ($p>0.05$). One-way ANOVA also showed variation in 22:4n-6 between feeding regimes with considerably higher levels in T2 than T5 ($F_{4,20}=3.16$, $P=0.036$).

Despite the relatively limited amounts of significantly different results observed in the larvae body fatty acid composition, several interesting trends of fatty acid and fatty acid classes of the larvae were observed in relation to the diet treatment. In fact, all possible correlations and linear relationships amongst the individual fatty or fatty acid class measured in the body of larvae under the different treatments and the actual total amount of that fatty acid or fatty acid class administered during the entire experiment were compared. Almost no relationships between the total fatty acid administered via the combination of the four algae and the fatty acid composition of larvae were observed, whilst strong relationships between the fatty acid derived from the two diatoms, *C. calcitrans* and *C. muelleri* were noted. For example, coefficient of determination (R^2) values higher than 0.5 were found for: SFA, MUFA, 20:4n-6, 22:4n-6, 22:6n-3 and n-3 LC-PUFA from *C. calcitrans,* and SFA, 20:4n-6, 22:4n-6 and 22:6n-3 provided from *C. muelleri*. Only SFA provided in algae of *P. lutheri* and *I. galbana* was detected as significantly related to that found in the larvae tissue.

3.5. Role of fatty acid composition on mortality and settlement

Regression analysis revealed significant relationships between levels of the n-3 and n-6 LC-PUFA (specifically, EPA, DHA and ARA) in the larvae tissue and some response variables tested. In particular, DHA content had a strong positive relationship with survival of mussel larvae. Approximately 97% of the variation in mortality at the end of the 30 day experiment was explained by differences in the amount of DHA in the body ($R^2=0.97$, $F_{1,3}=107.74$, $P=0.002$) (Fig. 2). Higher mortality was observed in treatments rich in *C. muelleri* (and hence, deficient in *C. calcitrans*), and consequent modification of DHA content of larvae. Additionally, significant relationships between larval SFA and MUFA contents and mortalities were detected (Fig. 2), where an increase in SFA was closely correlated with decreased mortality ($R^2=0.86$, $F_{1,3}=18.5$, $P=0.023$), while an inverse relationship was detected for MUFA ($R^2=0.91$, $F_{1,3}=32.5$, $P=0.011$).

The balance between n-3 and n-6 LC-PUFA in the larvae tissue also proved important for viability. Larvae with higher EFA ratios of (EPA + DHA)/ARA showed a significant capability for increased settlement rate, where 80% of the variation between treatments was explained by the EFA ratio in the larval tissue ($R^2=0.80$, $F_{1,3}=12.14$, $P=0.040$) (Fig. 3). Algae composition affected larval competency such that diets low in *C. calcitrans* showed a marked decrease in combined EPA and DHA, and thus, only approximately 5% settlement was observed at day 33.

4. Discussion

Achieving optimal algal composition for bivalve feed which produces favourable traits has been the aim of extensive nutritional studies for many aquaculture species (Brown and Robert, 2002; Gouda et al., 2006; Leonardos and Lucas, 2000a). However, the often unreliable and time consuming production of microalgae is presently a major issue in bivalve hatcheries (Robert and Gerard, 1999). Experiments performed in this study were carried out to improve hatchery efficiency by evaluating the nutritional importance of *C. calcitrans*, the culture of which has proven to be a negative impact on overall hatchery efficiency and management costs. This study clearly shows that the algal composition of the diet can significantly affect the performance of hatchery reared blue mussel larvae. The removal of *C. calcitrans* from the diet significantly increased mortality and lowered settlement success, however no differences in growth or total fatty acid content between the diets was observed. The analysis of larval fatty acid composition revealed that the absence of *C. calcitrans* in the diet was detrimental to larval health, especially since EFA ratios within the larvae tissue are closely related to mortality rates and settlement success, both of which are crucial to producing market product adult mussels, and hence, of central importance to hatchery operations.

The fatty acid profiles of cultured microalgae were comparable to those previously reported (Brown et al., 1997; Leonardos and Lucas, 2000b; Volkman et al., 1989), however, biochemical composition has been shown to vary considerably, depending on culture conditions (Thompson et al., 1996). Unexpectedly, fatty acid analysis revealed no

Table 5
Fatty acid profile (% w/w of total fatty acid) (mean ± SEM) of *Mytilus galloprovincialis* larvae fed the five different feeding regimes.

	T1	T2	T3	T4	T5
8:0	0.03 ± 0.03	0.22 ± 0.07	0.06 ± 0.06	0.11 ± 0.07	0.21 ± 0.09
10:0	0.89 ± 0.11	0.90 ± 0.09	0.71 ± 0.06	0.80 ± 0.04	0.99 ± 0.06
12:0	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	0.05 ± 0.03	0.03 ± 0.03
14:0	6.93 ± 1.54	6.80 ± 0.56	7.11 ± 0.15	6.76 ± 0.13	4.62 ± 1.69
14:1n-5	0.03 ± 0.03	0.12 ± 0.07	0.04 ± 0.04	0.08 ± 0.04	0.04 ± 0.04
15:0	0.50 ± 0.06	0.32 ± 0.10	0.55 ± 0.11	0.40 ± 0.08	0.48 ± 0.06
16:0	17.70 ± 0.52	17.11 ± 0.19	16.20 ± 0.33	16.98 ± 0.28	17.11 ± 0.48
16:1n-7	15.58 ± 0.44	15.76 ± 0.58	16.55 ± 0.68	15.83 ± 0.49	18.52 ± 1.22
16:2n-4	0.59 ± 0.20	0.66 ± 0.13	0.70 ± 0.11	0.37 ± 0.15	0.86 ± 0.12
16:3n-4	0.58 ± 0.16	0.30 ± 0.14	0.49 ± 0.07	0.30 ± 0.13	0.53 ± 0.19
16:3n-3	8.20 ± 0.58	7.95 ± 0.26	7.76 ± 0.58	8.66 ± 0.40	7.38 ± 0.72
18:0	3.92 ± 0.12^{a}	4.72 ± 0.28^{ab}	4.81 ± 0.14^{ab}	4.39 ± 0.17^{ab}	4.92 ± 0.30^{b}
18:1n-9	5.33 ± 0.19	6.45 ± 0.23	5.19 ± 0.35	5.45 ± 0.58	5.66 ± 0.83
18:1n-7	3.77 ± 0.08	3.84 ± 0.18	4.09 ± 0.23	3.96 ± 0.21	4.29 ± 0.31
18:2n-6	2.77 ± 0.15	2.49 ± 0.18	2.56 ± 0.12	2.76 ± 0.15	2.56 ± 0.14
18:3n-6	0.15 ± 0.11	0.19 ± 0.08	0.10 ± 0.07	0.16 ± 0.08	0.32 ± 0.13
18:3n-4	0.04 ± 0.03	0.10 ± 0.07	0.16 ± 0.12	0.13 ± 0.07	0.06 ± 0.06
18:3n-3	1.44 ± 0.07	1.28 ± 0.15	1.55 ± 0.18	1.23 ± 0.32	1.41 ± 0.10
18:4n-3	2.03 ± 0.10	1.62 ± 0.27	1.71 ± 0.11	1.80 ± 0.15	2.10 ± 0.22
20:0	0.80 ± 0.21	0.73 ± 0.36	0.90 ± 0.06	0.55 ± 0.21	0.72 ± 0.29
20:1	2.92 ± 0.27	2.87 ± 0.47	2.95 ± 0.33	2.91 ± 0.14	2.60 ± 0.23
20:2n-6	0.10 ± 0.04	0.11 ± 0.07	0.07 ± 0.03	0.07 ± 0.04	0.04 ± 0.04
20:4n-6 (ARA)	2.38 ± 0.17^{a}	3.60 ± 0.33^{b}	3.77 ± 0.21^{b}	4.14 ± 0.15^{b}	3.85 ± 0.22^{b}
20:4n-3	0.05 ± 0.03	0.03 ± 0.03	0.07 ± 0.03	0.06 ± 0.04	0.03 ± 0.03
20:5n-3 (EPA)	10.36 ± 0.25	9.38 ± 0.29	9.38 ± 0.25	9.68 ± 0.27	10.08 ± 0.30
22:0	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.07 ± 0.03	0.06 ± 0.03
22:1	2.13 ± 0.55	1.60 ± 0.70	2.50 ± 0.24	1.80 ± 0.67	1.41 ± 0.56
22:2n-6	0.09 ± 0.04	0.05 ± 0.03	0.09 ± 0.04	0.11 ± 0.07	0.04 ± 0.04
22:4n-6	1.30 ± 0.21^{ab}	1.51 ± 0.25^{b}	0.94 ± 0.04^{ab}	1.10 ± 0.12^{ab}	0.72 ± 0.16^{a}
22:5n-3	0.12 ± 0.04	0.35 ± 0.14	0.23 ± 0.10	0.27 ± 0.03	0.22 ± 0.06
24:0	0.14 ± 0.05	0.08 ± 0.03	0.11 ± 0.03	0.15 ± 0.07	0.09 ± 0.07
22:6n-3 (DHA)	9.08 ± 0.31	8.81 ± 0.13	8.62 ± 0.20	8.89 ± 0.15	8.06 ± 0.33
SFA	30.95 ± 2.51	30.92 ± 2.44	30.49 ± 2.33	30.26 ± 2.41	29.22 ± 2.38
MUFA	29.76 ± 2.46	30.65 ± 2.52	31.33 ± 2.39	30.02 ± 2.51	32.52 ± 3.01
PUFA	39.28 ± 1.56	38.43 ± 1.47	38.18 ± 1.46	39.72 ± 1.55	38.26 ± 1.46
n-6 PUFA	6.79 ± 0.54	7.95 ± 0.66	7.52 ± 0.70	8.34 ± 0.76	7.52 ± 0.71
n-3 PUFA	31.28 ± 2.03	29.42 ± 1.91	29.31 ± 1.87	30.57 ± 1.99	29.29 ± 1.87
n-6 LC-PUFA	3.87 ± 0.49	5.27 ± 0.74	4.87 ± 0.78	5.42 ± 0.86	4.65 ± 0.81
n-3 LC-PUFA	19.61 ± 2.50	18.57 ± 2.30	18.30 ± 2.29	18.89 ± 2.36	18.39 ± 2.34
Total FA (mg/g of larvae dry weight)	30.62 ± 1.50	31.05 ± 1.32	30.16 ± 1.58	27.48 ± 0.55	34.45 ± 3.16

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids and LC-PUFA: long chain polyunsaturated fatty acids. Different letters in same row indicate significant differences (one-way ANOVA, $\alpha = 0.05$, a<b<c<d), $n = 5$.

variation in overall total fatty acid content of the mussel larvae, despite the visual lipid score revealing significant differences. The visual scoring of lipid content was highly variable and somewhat subjective and thus caution should be used in the interpretation of such data. Further validation of this technique is required to determine if it reflects real differences in lipid content in larvae. Nevertheless, relative individual fatty acids of the larvae differed significantly between treatments, and are likely to reflect the fatty acid composition of the algae species being preferentially consumed (Budge et al., 2001; Rouillon and Navarro, 2003). As such, the predominant influence of diatoms on fatty acid composition of the larvae tissue clearly suggests the existence of selective utilisation of *C. calcitrans*, followed by *C. muelleri*. In contrast, the fatty acid composition of *I. galbana* and *P. lutheri* appear to make little contribution towards the final larval body composition (low R^2 values), which suggests that flagellates are not actively consumed, at least when diatoms are also provided, under these experimental conditions. This conclusion is consistent with a previous report on *M. edulis* adults where diatoms were selectively digested in the gut compared with flagellates (Rouillon and Navarro, 2003).

According to Chuecas and Riley (1969) and Murphy et al. (2003), algal dietary fatty acids possess a high n-3 LC-PUFA content which is generally reflected in the composition of bivalve larvae tissue (Jonsson et al., 1999) as demonstrated for specific LC-PUFA in *C. calcitrans* and *C. muelleri*. These include the biologically important 22:6n-3 (DHA), 20:5n-3 (EPA) and 20:4n-6 (ARA) which account for a significant proportion of PUFA in the larvae tissue. Such findings are confirmed in a report by Freites et al. (2002) which found DHA, EPA and ARA represented 56–58% of total PUFA in adult mussels. The relative proportions of these important LC-PUFA in the tissue are a crucial factor of larval health, and are highly dependent on phytoplankton species consumed (Budge and Parrish, 1998; Freites et al., 2002). As such, the relationship between LC-PUFA and larval performance will assist with clarifying the nutritional biochemical requirements of mussel larvae.

Phytoplankton species used in the diet strongly influences the survival of mussel larvae to progress into the adult life stage (Baldwin and Newell, 1995b; Gallager et al., 1986; Tang et al., 2006). In the second feeding experiment, variation between mortality rates and diet was clearly evident where larvae reared on a diet high in *C. calcitrans* produced higher larval survival than those on the *C. muelleri* based diet. The favourable effect of a mixed species diet consisting of *P. lutheri* or *I. galbana* in conjunction with *C. calcitrans* upon bivalve survival is comparable with other reports by Helm and Laing (1987) and Volkman et al. (1989). It is likely these species synergistically contribute the dietary fatty acid supplements required for survival through to the adult life stage.

DHA levels in algal tissue samples were found to be relatively low for both *C. calcitrans* and *C. muelleri*, consistent with levels reported by Delaunay et al. (1993) and Martinez-Fernandez et al. (2006). However, *C. calcitrans* contained significantly higher levels of EPA (Whyte et al., 1990). A distinct correlation between bodily DHA

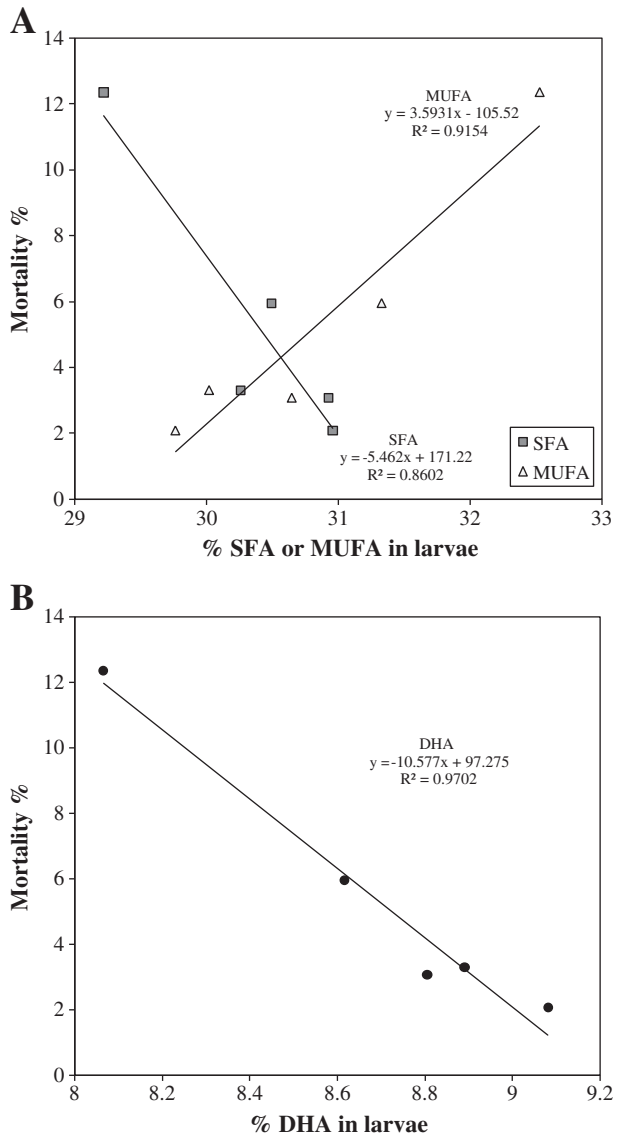

Fig. 2. Relationship between SFA ($R^2 = 0.86$, $F_{1,3} = 18.5$, $P = 0.023$), MUFA ($R^2 = 0.91$, $F_{1,3} = 32.5$, $P = 0.011$) (A) and DHA ($R^2 = 0.97$, $F_{1,3} = 107.74$, $P = 0.002$) and (B) level (% w/w of total fatty acids) in the body and mortality of *M. galloprovincialis* larvae.

content and survival shows the importance of DHA for larval health, which is demonstrated by an observed increase from treatments rich in *C. calcitrans* and lower in *C. muelleri*. Pernet and Tremblay (2004) produced similar results in the sea scallop, where a diet which included *C. muelleri* produced moderate larval survival (compared with diets excluding the diatom), and which was suggested to be the result of a dietary deficiency in DHA. Fatty acid groups detected in the larvae tissue were also found to show a significant correlation with larval survival, where mortality proportionally increased with MUFA and decreased with higher SFA. This was unexpected, given a paucity of information in research to support the importance of SFA and MUFA for bivalve larvae survival. However, a report by Tocher (2003) revealed the physiological importance of these fatty acids, where the MUFA products formed from the desaturation of some SFA possess lower melting temperatures. Hence conversion from SFA to MUFA products can alter the viscosity of the cell membranes and, hence, cellular activities, which may have caused detrimental effects on larval survival.

The ability of mussel larvae to undergo successful settlement is a result of many interrelating factors, with microalgae playing a key role, both as a chemical cue for attachment to a substrate (Dobretsov and Wahl, 2008) and as a dietary requirement necessary for complete pediveliger development (Phillips, 2002). The amount of *C. muelleri* present in the diet was found to explain a large proportion of the variability in the recruitment of larvae to successfully metamorphose to the adult life stage. This result is supported by Jonsson et al. (1999) where a significant variation between settlement rates was explained by differences in algal diet fatty acid composition.

Fatty acids EPA and ARA both serve as the precursors of the important hormone-like eicosanoids (Sargent et al., 1999). However, the eicosanoids derived by the two fatty acids are differentially active, and as such, a specified balance is required for optimal eicosanoids functioning. The (EPA + DHA)/ARA ratio of the diets, and the consequent body fatty acid composition, showed a direct correlation with the ability of mussel larvae to undergo settlement. These findings are consistent with Jonsson et al. (1999), who found a significant relationship between n-3 LC-PUFA and settlement success in the European oyster. *C. calcitrans* was found to have considerably higher levels of EPA in comparison to *C. muelleri* as reported by Helm and Laing (1987), however, there were no marked differences in the amount found in the larvae tissue composition across all treatments. It is plausible that any deficiency of EPA in diets lacking *C. calcitrans* may have been adequately supplemented by *P. lutheri* which contains high levels of the fatty acid. Also, only minor discrepancies in the DHA levels between treatments and diatom species were noted, suggesting that n-3 LC-PUFA did not exclusively play a role in the discrepancies observed between treatments.

Conversely, fatty acid analysis found *C. calcitrans* contained considerable levels of ARA per cell, however, as a % of total fatty acid, *C. muelleri* contained significantly high amounts of ARA, subsequently providing larvae with considerably higher proportions of n-6 LC-PUFA, which serve as a crucial component of bivalve nutrition (Milke et al., 2004). However, an overall increase in the percentage of ARA observed in larvae across diets with progressively higher *C. muelleri* corresponded with significantly reduced settlement. At the end of the experiment, higher growth rates were observed in treatments receiving increased levels of *C. muelleri*, such as T4 and T5, however results were not significant. Non-significant results of growth rates between multispecies diets consisting of *I. galbana* with either *C. muelleri* or *C. calcitrans* were also noted by Galley et al. (2009). As a highly active compound, ARA may have played a role to enhance the growth of larvae, as previously proposed by Milke et al. (2008) for sea scallop postlarvae. However, high levels of ARA, relative to other EFA

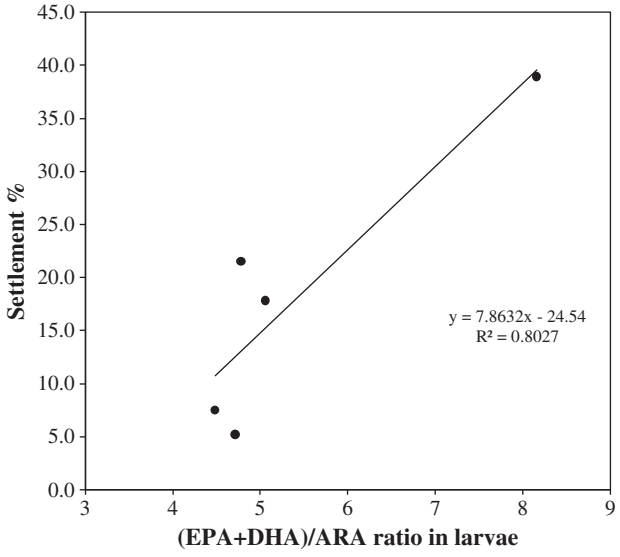

Fig. 3. Relationship between (EPA + DHA)/ARA (% w/w of total fatty acids) ratio in the body and settlement rate of *M. galloprovincialis* larvae ($R^2 = 0.80$, $F_{1,3} = 12.14$, $P = 0.040$).

generate production of more biologically active (or proinflammatory) eicosanoids in the larvae which may be harmful to early larval developmental stages (Rowley et al., 2005; Sargent et al., 1999). From these results, it is proposed that subsequent increased physiological responses may have impeded larvae competency at the pediveliger (settlement) stage. According to Helm and Laing (1987), observed differences in the development between treatments may not be solely due to the presence or absence of essential components in the diet, but in the equilibrium between them. Results therefore suggest that it is the ratio between n-3 LC-PUFA/n-6 LC-PUFA in microalgae that plays a significant role in fulfilling the dietary requirements of viable bivalve larvae, rather than their individual fatty acids, as demonstrated by Rico-Villa et al. (2006) with imbalances in EFA producing poor settlement. Consequently, the present findings have further highlighted the importance of determining a correct ratio of dietary components, such as DHA, EPA and ARA, to identifying a suitable mixed microalgae species diet.

The ability of *C.calcitrans* to support larval growth and development is clearly demonstrated in larvae under the standard hatchery diet (T1), producing the highest lipid content (visual lipid score), survival and settlement rates. Similar results emphasising the importance of *C. calcitrans* in bivalve nutrition have been well documented in previous studies (Gouda et al., 2006; Krichnavaruk et al., 2007; Marshall et al., 2010; Rivero-Rodriguez et al., 2007; Volkman et al., 1989). As such, the absence of growth variation between treatments was unexpected, considering the emphasis of growth as a widely regarded indication of bivalve larvae health (Bayne, 1965; Epifanio, 1979; Sprung, 1984; Stromgren and Cary, 1984), with larval growth generally believed to increase with diet diversity (Webb and Chu, 1983). Bivalve growth is largely a function of food supply (Helm and Millican, 1977; Stromgren and Cary, 1984). Therefore, it is likely that the high mortality observed in T5 of Experiment 2 caused higher algal feed concentration per larvae, and as a result, may have masked the effect of any deficiencies present in the diet. This may serve as the rationale for a lack of expected discrepancies between growth rates with other diet treatments, as well as any functional relationship between diet composition and larval growth that may have otherwise been detected. Contrary to widely held views, larvae growth may not reveal subtle, but nevertheless important, differences in the nutritional quality of algae diet, as shown by this study. As such, measures of shell growth alone can be a misleading indicator of larval condition. Rather, a holistic approach incorporating a range of response variables is required when defining the nutritional value of algae species and their affects on larval performance.

5. Conclusion

The results of the feeding experiments clearly demonstrate how alterations in microalgae species composition can produce variation in mortality and settlement rates, although, no difference in growth was supported. It was concluded that *C. calcitrans* as principal diatom in conjunction with *P. lutheri* and *I. galbana* constituted a nutritionally superior diet, which was further supported by the apparent selective larval feeding of *C. calcitrans* as the primary diatom, compared with *C. muelleri*. A clear correlation between fatty acid composition in the diet and in the tissues of *M. galloprovincialis* larvae was found with DHA level highly influential for survival. However, altering diet can affect the balance between fatty acid ratios of n-3 LC-PUFA (EPA and DHA) to n-6 LC-PUFA (ARA) which can in turn alter physiological functions of larvae, such as ability to undergo successful settlement and metamorphosis. The inclusion of *C. muelleri* in the diet appears to increase ARA levels in the body, relative to other EFA, causing a direct negative impact on settlement. In contrast, *C. calcitrans* contributes a relatively propitious balance of n-3 and n-6 to the diet, producing a moderating effect to readjust the EFA ratio in the diet, such that development is improved. It is clear that blue mussel cannot utilise *C. muelleri* with the same efficiency of *C. calcitrans* at such early stages of development.

The full implications of this study suggest that it is necessary to find an alga species with a similar fatty acid profile to *C. calcitrans*, particularly a species characterised by lower ARA content compared to *C. muelleri*, which is more robust, such that it can be successfully cultured on a large scale. Further research on potential algal species as a diatom substitute supplied to blue mussel larvae is highly warranted such that nutritional value is optimised, and culture practices are enhanced. In the meantime, further research is required to improve and refine culture methods for *C. calcitrans*.

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