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# Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs

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**Abstract**

Heterotrophically grown algae and fungal biomass and their residual materials from an industrial oil extraction process were used as components in marine larval and broodstock diets. *Crypthecodinium* sp. phospholipid extract and meal, used to enrich rotifers and *Artemia* nauplii, produced higher levels of DHA and higher DHA/EPA ratios in these zooplankters than *Schizochytrium* sp. algal whole cell preparation or fish oil-based emulsion. The improved enrichment resulted in enhanced growth of Atlantic halibut larvae, whereas several other marine larvae species (sea bream, European sea bass and striped bass) respond almost equally to all enrichment materials. In addition, a 60% replacement of menhaden oil with algal oil and meal in striped bass broodstock diets resulted in a similar growth increase to that obtained with standard commercial diets. Striped bass broodstock fish diets supplemented with an arachidonic acid (ArA)-rich oil obtained from heterotrophically grown fungi, *Mortierella alpina*, was shown to have significant benefits on the hatching rate of larvae. These findings demonstrate the potential of single cell heterotrophs as a partial substitute or replacement for fish-based ingredients in aquaculture diets.
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*Keywords:* Algae; Heterotrophs; Enrichment diet; Broodstock diet; Essential fatty acids

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## 1. Introduction

Currently, fish meal and fish oil are the main ingredients in finfish and marine shrimp feeds. Together they provide a good balance of high quality protein (amino acid composition) and lipids (containing long chain $n-3$ polyunsaturated fatty acids) in a highly digestible energy dense form. Studies have shown that diets containing fish-based ingredients have generally performed better in terms of growth and feed efficiency than diets containing alternative plant-based sources (Moyano et al., 1992; Webster et al., 1992; Gallagher, 1994; Kikuchi, 1999). However, as a result of a decreasing supply of fishery byproducts and concerns over its quality, the aquaculture industry is now actively investigating alternatives nutrient sources (Naylor et al., 2000).

Considerable interest and research have been devoted to developing unicellular organisms such as yeast, molds, bacteria, microalgae and fungi as additives to aquaculture feeds. Several studies have demonstrated that the partial replacement of fish-based ingredients with yeast and bacteria did not compromise fish survival, growth, or disease resistance (Anon, 1977; Dabrowski, 1982; Dabrowski et al., 1985; Murray and Marchant, 1986). A major advantage in the use of unicellular organisms is that the technology exists to produce industrial quantities under controlled and environmentally safe conditions. Moreover, the composition of many microorganisms can be manipulated to produce higher levels of protein and lipid, by enrichment with specific essential amino acids or fatty acids (Kangas et al., 1982; Tan and Johns, 1991; Sanchez et al., 1995; Day and Tsavalos, 1996). In fact, oil extracts from algae containing long-chain polyunsaturated fatty acids (LC-PUFA) are already in use as nutritional supplements in human infant formulas (reviewed in Cohen et al., 1995). Although a number of studies have reported the inclusion of photosynthetic microalgae in aquaculture feeds (Day et al., 1990; Zhou et al., 1991; Laing and Millican, 1992; Day and Tsavalos, 1996; Langden and Onal, 1999), this approach has not found commercial application to replace fish-based ingredients, mostly due to their high production costs and culture inefficiency (Borowitzka, 1992; Chaumont, 1993; Wilkinson, 1998). A cost-effective alternative is to use heterotrophic algae grown in conventional fermentors (Day et al., 1991; Gladue and Maxey, 1994). This type of algal culture is carried out under controlled conditions using an organic carbon source (i.e. glucose) to provide both carbon and energy for growth.

The heterotrophic growth approach has several distinct advantages over phototrophic culture. The major advantage is that heterotrophic culture does not need the investment in lighting and electricity necessary in phototrophic algal growth. The production of heterotrophic algal biomass, which is carried out according to existing standardized technology, can cost less than US\$5 $kg^{-1}$ (Gladue and Maxey, 1994), whereas phototrophic algae production can be two orders of magnitude higher (Benemann, 1992). Moreover, heterotrophic culture produces higher cell densities ($>75$ g $l^{-1}$ DW, Running et al., 1994) in readily available large-scale growth vessels (up to $500 \times 10^3$ l), which markedly reduces the harvesting effort.

In a series of studies, we tested the use of heterotrophically grown microalgae and its extracted oil as sources of nutrients and essential fatty acids for rotifers and *Artemia*, which were then used in fish larvae as well as in formulated broodstock diets. These sources can be particularly rich in docosahexaenoic acid (DHA) and arachidonic acid (ArA), which are

required for larval growth and survival as well as contributing to egg and sperm quality when included in broodstock diets.

## 2. Materials and methods

### 2.1. Algal sources

Heterotrophicaly grown algal-based materials included the following: spray-dried preparations of whole cells (WC-*Cr*), defatted cells (DC-*Cr*), DHA-rich triacylglycerol (TAG-*Cr*) and phospholipid (PL-*Cr*) extractions of *Crypthecodinium* sp., whole cells (WC-*Cl*) of *Chlorella* sp., and an ArA-rich phospholipid extract (PL-*Mo*) of the fungus, *Mortierella alpina*. All algal meals and their residual materials originated from an industrial fermentation oil extraction process performed by Martek Bioscience (Columbia, MD, USA). Prior to the oil extraction, *Crypthecodinium* sp. cells were broken by mechanical shear forces and enzyme degradation, the resulting biomass then spray-dried and the crude oil extracted with hexane using standard industrial oil extraction protocol. Residual PL-*Cr* materials were products of down stream refining and winterizing processes of the algal crude oil extract. The lipid and fatty acid composition of these materials are presented in Table 1. In addition, two enrichment mixes containing spray-dried WC-*Cl* and PL-*Cr* (50:50), or WC-*Cr* and PL-*Mo* 87.5:12.5) were also prepared. A mix of 4% alginic acid, 1% ascorbic acid, 1% α-tocopherol and 2% silicon-based anti-foaming agent (all from Sigma, St. Louis, MO) were added (w/w basis) to each of the spray-dried algal materials. The effect of these algal and fungal sources on the nutritional status of the live food and fish larvae were compared with the commercial product Algamac-2000, a spray-dried whole cell preparation of heterotrophically grown *Schizochytrium* sp. (Bio-Marine, Hawthorne, CA, USA) or DHA-Selco, a fish oil-based emulsion (INVE Aquaculture, Dendermonde, Belgium).

### 2.2. Broodstock diet

The effects of heterotrophically grown algal-based sources were tested in the diet of striped bass, *Morone saxatilis*, broodstock at the Aquaculture Research Center of the Center of Marine Biotechnology (COMB, Baltimore, MD, USA). Three diets, identical in protein, lipid and energy contents and containing 8–11% of DC-*Cr*, were prepared. They differed,

Table 1
Lipid (%DW), DHA, EPA and AA (% of total fatty acids) composition of the algal[1] and fungal[2] materials

| | TAG-*Cr*[1] | PL-*Cr*[1] | PL-*Mo*[2] | WC-*Cr*[1] | DC-*Cr*[1] |
|---|---|---|---|---|---|
| Lipid | 100 | 57.4 | 55.4 | 23.4 | 12 |
| DHA | 40 | 48 | N.D. | 51 | 50 |
| EPA | <0.5 | <0.5 | N.D. | <0.5 | <0.5 |
| AA | N.D. | N.D. | 56 | N.D. | N.D. |

TAG-*Cr*: DHA-rich triacylglycerols extract of *Crypthecodinium* sp.; PL-*Cr*: DHA-rich phospholipids extract of *Crypthecodinium* sp.; PL-*Mo*: ArA-rich phospholipids extract of *Mortierella* sp.; WC-*Cr*: whole *Crypthecodinium* sp. cells; DC-*Cr*: defatted *Crypthecodinium* sp. cells: N.D.: not detectable.

however, in their levels of PL-*Cr* and PL-*Mo*. Diets-1 and -2 were supplemented with 2% and 4% of PL-*Cr*, respectively, while Diet-3 was supplemented with 4% PL-*Cr* and 5% PL-*Mo*. A commercially available grow-out diet for striped bass (Moore-Clark, Bellingham, WA, USA) was used as the control diet (Diet-4). Diet formulation, proximate analysis and selected fatty acid and amino acid compositions are presented in Tables 2 and 3.

After the fish were biopsied for sex, each diet was fed to one group of 15 fish (5 males and 10 females, 3–6 kg each) in a 4-$m^3$ circular tank. Each tank was supplied with 4–6 psu synthetic seawater (15 °C) recirculated through a drum-filter and bio-filter system and exposed to natural photoperiod. Fish were fed a ration of 15 g $kg^{-1}$ BW $day^{-1}$, starting 1 month before spawning and continued throughout the year until the next spawning season. Average female and male weights in each dietary treatment at the beginning of feeding trails and at the beginning of the second spawning season are given in Table 4.

All fish were again biopsied at the beginning of the second spawning season. One mature female from each treatment reaching a final stage of gonadal development (oocyte diameter >800 μm) was implanted with controlled-release implants containing a GnRH analog (50 μg $kg^{-1}$ BW, Mylonas et al., 1998). Two males, from each treatment, were also induced by implanting with similar GnRH implants (20 μg $kg^{-1}$ BW). The induced fish were placed in a separate spawning tank, under similar water conditions as the original tank, and then the water temperature increased overnight to 17 °C. Fish commenced spawning ca. 48 h following spawning induction. A similar procedure was repeated during the second spawning season until three different females from each dietary treatment had spawn successfully. Each spawning tank was equipped with a net basket attached to the overflow for collecting eggs, which were harvested every few hours, and their total wet biomass recorded. The eggs were then placed in 60-l incubation tanks (100 eggs $l^{-1}$) equipped with

Table 2
Formulation of striped bass broodstock experimental diets

| Ingredients (% dry weight) | Diet-1 | Diet-2 | Diet-3 |
|---|---|---|---|
| Squid meal (75% protein) | 40 | 40 | 40 |
| Soy proteins extract (47% protein) | 20 | 20 | 20 |
| Anchovy meal (64% protein) | 8 | 8 | 8 |
| Meat meal (50% protein) | 5 | 5 | 5 |
| Menhaden oil | 8 | 6 | 3 |
| DC-*Cr* | 11 | 11 | 8 |
| PL-*Cr* | 2 | 4 | 5 |
| PL-*Mo* | 0 | 0 | 5 |
| Dicalcium phosphate | 1.5 | 1.5 | 1.5 |
| Choline chloride | 0.5 | 0.5 | 0.5 |
| Vitamin premix | 0.5 | 0.5 | 0.5 |
| Mineral premix | 1 | 1 | 1 |
| L-methionine | 0.5 | 0.5 | 0.5 |
| L-lysine | 1 | 1 | 1 |
| L-arginine | 0.2 | 0.2 | 0.2 |
| Ascorbic acid | 0.2 | 0.2 | 0.2 |
| α-Tocopherol | 0.2 | 0.2 | 0.2 |

Formulation is based on weight percentages. Squid meal was obtained from APC, Ames, IA, USA. All ingredients were of industrial grade. Diets were extruded (10-mm pellet size) at Integral Fish Foods, Grand Junction, CO, USA.

Table 3
Composition of the striped bass broodstock experimental and control diets

| | Diet-1 | Diet-2 | Diet-3 | Diet-4 |
|---|---|---|---|---|
| *Proximate composition (% dry weight)* | | | | |
| Protein | 50 | 50 | 51 | 42 |
| Fat | 14 | 12 | 11 | 26 |
| Fiber | 3 | 3 | 4 | 2 |
| Ash | 9 | 9 | 10 | 8 |
| Moisture | 10 | 12 | 13 | 7 |
| Peroxide value | 0.8 | 0.1 | 0.1 | 1.0 |
| *Essential amino acid composition (% dry weight)* | | | | |
| Methionine | 1.5 | 1.4 | 1.5 | 1.5 |
| Cystine | 0.4 | 0.4 | 0.5 | 0.5 |
| Lysine | 4.0 | 4.1 | 4.1 | 2.4 |
| Phenylalanine | 2.0 | 2.1 | 1.8 | 1.9 |
| Leucine | 3.7 | 4.0 | 3.3 | 4.0 |
| Isoleucine | 1.9 | 2.0 | 1.4 | 1.6 |
| Threonine | 2.1 | 2.2 | 2.1 | 1.9 |
| Valine | 2.1 | 2.4 | 1.3 | 2.0 |
| Histidine | 1.3 | 1.3 | 1.5 | 1.1 |
| Arginine | 3.6 | 3.7 | 3.6 | 2.5 |
| *Fatty acid composition (% dry weight)* | | | | |
| 14:0 | 0.4 | 0.3 | 0.3 | 0.9 |
| 16:0 | 2.6 | 2.1 | 1.4 | 4.4 |
| 16:1 | 0.9 | 0.7 | 0.5 | 2.3 |
| 18:0 | 0.8 | 0.7 | 0.4 | 1.8 |
| 18:1 | 3.7 | 2.2 | 1.5 | 5.7 |
| 18:2$n-6$ | 0.4 | 0.5 | 0.5 | 0.7 |
| 18:3$n-3$ | 0.1 | 0.2 | 0.1 | 0.8 |
| 20:4$n-6$ | 0.2 | 0.3 | 1.8 | 0.4 |
| 20:5$n-3$ | 0.8 | 0.7 | 0.7 | 2.9 |
| 22:6$n-3$ | 1.7 | 3.6 | 3.7 | 1.7 |
| DHA/EPA/ArA | 10:5:1 | 10:2:1 | 10:2:5 | 10:17:2 |

Essential amino acid composition in all diets was similar except lysine, Diets-1, -2 and -3 contained about 4% lysine while Diet-4 contained only 2.4%. Peroxide values expressed in meq $kg^{-1}$. Diet DHA/EPA/ArA ratio represents the proportion between the three fatty acids (relative to 10 parts of DHA). Proximate and amino acid analyses, and peroxide value were performed at New Jersey Feed Laboratory, Trenton, NJ, USA. Fatty acid analysis was performed at the Center of Marine Biotechnology (COMB).

gentle aeration having a 150% daily water exchange. A sample of viable eggs from each spawning female was taken, washed in distilled water and kept at $-80$ °C for later lipid and fatty acid analysis.

### 2.3. Enrichment feeds for rotifers and Artemia nauplii

A series of enrichment experiments were performed at COMB in which the effect of WC-*Cr*, PL-*Cr* and a mix of both materials (WC-*Cr*/PL-*Cr*) on rotifer and *Artemia* nauplii survival, lipid content and DHA incorporation were evaluated. The enrichment efficiency

of these lipid sources to increase the DHA content and DHA/EPA ratios in rotifers and *Artemia* was compared with widely used commercial enrichment products containing either a heterotrophically grown microalgae (*Schizochytrium* sp., Algamac-2000) or a fish oil-based emulsion (DHA-Selco).

Decapsulated *Artemia* nauplii (premium grade, Sanders Brine Shrimp, Ogden, UT) were hatched and kept at room temperature for 6–8 h until they reached instar-II stage (complete development of digestive system) before being transferred to the enrichment medium. *Artemia* nauplii were enriched for 16 h at a density of $200 \times 10^3$ nauplii $l^{-1}$ in 20 practical salinity units (psu) artificial seawater (28 ± 1 °C). Two rations of 0.3 g $l^{-1}$ of each enrichment diet were fed at times 0 and 8 h.

Rotifers, *Brachionus plicatilis* (Florida Aqua Farms, Dade City, FL), were cultured in either 30- or 100-l conical tanks, at 20 psu artificial seawater (24 ± 1 °C), and at a density of $200–600 \times 10^3$ rotifers $l^{-1}$, and fed 0.5 g WC-*Cl* per $10^6$ rotifers $day^{-1}$. During enrichment, the rotifers were fed their respective diets for 8 h at a density of $500 \times 10^3$ rotifers $l^{-1}$ at 20 psu artificial seawater (28 ± 1 °C). Two rations of 0.05 or 0.1 g $l^{-1}$ of each enrichment diet were fed at times 0 and 4 h. Density of rotifers and *Artemia* nauplii in each enrichment container was recorded at the beginning and termination of the enrichment (after 8 and 16 h, respectively). Rotifer and *Artemia* survival, as a result of their enrichment diets, were recorded and samples of these zooplankters collected were washed well in distilled water before being stored at − 80 °C for later lipid and fatty acid analysis.

### 2.4. Larvae rearing

Striped bass, *Morone saxatilis*, larvae obtained from spawns of Diet-4 fed broodstock were raised on rotifers, *B. plicatilis* (2–5 rotifers $ml^{-1}$). Seventeen-day-old post-hatched larvae were randomly distributed in eighteen 60-l rearing tanks (160 larvae $tank^{-1}$) and grown as described in Harel et al. (2000). Newly hatched *Artemia* nauplii were fed in one ration to the larvae from days 17–24 at a concentration of 200–400 nauplii $l^{-1}$, while the rotifer concentration in the tanks was reduced gradually to complete removal by day 24. Larvae were then fed twice daily *Artemia* nauplii, previously enriched with either PL-*Cr*, Algamac-2000 or emulsified olive oil. The feeding experiment was terminated on day 46 where samples of 40 larvae were washed with distilled water, blotted dry, and lyophilized to constant weight. The dry weight of individual larvae was determined (Mettler UMT2 ± 0.1 mg, Toledo, Switzerland) and the samples then stored at − 80 °C until analysis for lipid and fatty acids composition.

Gilthead sea bream, *Sparus aurata*, and European sea bass, *Dicentrarchus labrax*, larvae originated from spawns of locally maintained broodstock at the National Center for Maricylture (NCM, Eilat, Israel) were stocked over 3 days with 2-day-old larvae (40,000 larvae $tank^{-1}$). Sea bream larvae from days 3 to 20 were fed twice daily rotifers, *B. rotundiformis*, that were previously enriched on first phototrophically grown *Nannochloropsis* sp. and then on either Algamac-2000 or PL-*Cr*. Rotifer and *Artemia* enrichments and larval feeding were carried out at NCM as described in Koven et al. (2001). Sea bream larval growth as a function of enrichment materials was recorded on day 34. European sea bass larvae were maintained in darkness until day 8 when they then started feeding on non-enriched *Artemia* nauplii until day 11. From days 11 to 21, the larvae were fed twice daily

under low illumination (approximately 100 lx) Algamac-2000- or PL-*Cr*-enriched *Artemia* nauplii.

The rearing system for Atlantic halibut, *Hippoglossus hippoglossus*, larvae was composed of two 1500-l circular tanks having a flat bottom and dark-colored walls. These tanks were supplied with continuous freshly filtered and temperature controlled seawater (12 ± 1 °C, 32 ‰). The tanks were stocked with newly hatched larvae (7 larvae $l^{-1}$), from spawns of locally maintained broodstock (at AKVAFORSK, Sunndalsora, Norway). Live photosynthetic algae, *Tetraselmis* sp., were added three times daily to the larvae culture medium, at a density of 20,000 cells $ml^{-1}$. During the first 10 days, the larvae were fed three times daily with *Artemia* nauplii enriched for 16 h with DHA-Selco or PL-*Cr*. From days 10 to 90, the larvae were fed a mixture of young (24-h post-hatch) and old (72-h post-hatch) enriched *Artemia*. These enrichments were carried out for 16 h in 1700-l conical tanks containing well-aerated seawater (25 ‰) at 27 ± 1 °C and stocked with up to 200 nauplii $ml^{-1}$. Both young and old *Artemia* nauplii were fed one ration (0.3 g $l^{-1}$) of their respective enrichment media at the beginning of the enrichment period and another ration after 12 h. Old *Artemia* were obtained by culturing the nauplii in a suspension of extra fine fish meal diet (10 mg $l^{-1}$). A sample of 40 halibut larvae were weighed at the end of the feeding experiment (day 90), and visually checked for the completion of pigmentation and eye migration. Larvae were then lyophilized for 48 h and kept at −80 °C for later lipid and fatty acid analysis.

### 2.5. Lipid and fatty acid analysis

Lipid and fatty acid analysis of rotifer and *Artemia* nauplii enrichments, striped bass and halibut larvae were carried out at COMB as described previously (Harel et al., 2000). Lipid and fatty acid analysis of the sea bream and European sea bass larvae and their enriched rotifers and *Artemia* feeds were carried out at NCM as described previously (Koven et al., 2001).

### 2.6. Data analysis

All data are reported as means ± S.E.M. Following testing for homogeneity of variance, analysis of variance was performed (Stat view 4.5, Abacus Concepts Berkley, CA, 1992) to determine differences between treatment means of survival, growth and fatty acid percentage. Survival and fatty acids percentages were normalized by arcsine transformation prior to analysis. When significant differences between the means were detected, a Bonferroni multiple comparison test was applied. Differences were considered significant at the $P < 0.05$ levels.

## 3. Results

### 3.1. Striped bass broodstock

In general, fish fed the control diet maintained body weight during the first month of feeding, while fish fed the experimental diets lost ca. 10% of their initial weight, possibly

as a result of their adaptation to the new diets. However, after 12 months of feeding, weights within each sex group of fish fed Diets-1, -3 and -4 were similar, whereas fish fed Diet-2 grew significantly slower ($P<0.05$, Table 4). Males fed Diet-2 grew marginally during the 12-month feeding period, and females only gained 0.4 kg as compared to over 1-kg weight gain in females fed Diets-1, 3- and -4. The fact that the control diet (Diet-4) contained 26.2% lipid while Diets-1, -2 and -3 contained 16.7% did not appear to influence the final weight gain.

The greatest number of fish with developed gonads was found in the control diet fed group. All males fed Diet-4 were spermiating, by the beginning of the second spawning season, and most Diets-4 and -1 fed females developed ripe gonads (final maturation stage—FMS, oocyte diameter >800 μm). This compared to only 20–40% spermiating males in Diets-1, -2-and 3 and 50–60% FMS females in Diets-2 and -3 fed groups (Table 5). Fish fed Diet-2 performed poorest in terms of weight gain and reproductive development.

### 3.2. Fecundity and egg hatching rate

The average egg biomass per 1-kg BW female and egg hatching rate (%) are presented in Table 5. Egg production was not significantly affected by dietary treatments; however, the hatching rate of eggs from fish fed Diet-3 (high DHA and high ArA) was almost three-fold higher ($P<0.05$) than the control Diet-4. The eggs of fish fed Diet-1 (low in DHA) contained the lowest DHA levels (10% TFA) and were mostly unfertilized or died shortly after spawning, whereas eggs from all the other three dietary treatments have significantly ($P<0.05$) higher DHA content (ca. 15% TFA). The level of ArA in eggs from broodstock fed Diet-3 was markedly higher (2.5% TFA) than eggs from fish fed Diets-1,-2 and -4 (0.3% TFA). Dietary treatments also affected the relative ratios of egg DHA, EPA and ArA (DHA/EPA/ArA, Table 5).

### 3.3. DHA/EPA/ArA enrichment efficiency of rotifers and *Artemia nauplii*

The effect of WC-*Cr*, PL-*Cr*, WC-*Cr*/PL-*Mo* and WC-*Cl*/PL-*Cr* enrichment preparations, in comparison with commercially available materials, on lipid, DHA and ArA

Table 4
Weight gain of striped bass males and females fed with algal meal derived diets

| Diet | Sex | Initial weight (kg) | Weight (kg) after: | |
|---|---|---|---|---|
| | | | 1 month | 12 months |
| Diet-1 | male | $3.3 \pm 0.7$ | $3.2 \pm 0.4$ | $4.2 \pm 0.8^{A,B}$ |
| | female | $5.0 \pm 0.6$ | $4.6 \pm 1.0$ | $6.0 \pm 0.3^{A}$ |
| Diet-2 | male | $3.6 \pm 0.4$ | $3.2 \pm 0.4$ | $3.7 \pm 0.4^{B}$ |
| | female | $4.8 \pm 0.7$ | $4.5 \pm 0.5$ | $5.2 \pm 0.3^{B}$ |
| Diet-3 | male | $4.0 \pm 0.4$ | $3.5 \pm 0.5$ | $4.3 \pm 0.2^{A,B}$ |
| | female | $4.9 \pm 0.8$ | $4.6 \pm 0.6$ | $5.8 \pm 0.1^{A,B}$ |
| Diet-4 | male | $3.9 \pm 0.4$ | $4.1 \pm 0.3$ | $4.8 \pm 0.5^{A}$ |
| | female | $5.1 \pm 0.7$ | $5.7 \pm 0.6$ | $6.2 \pm 0.4^{A}$ |

Values in each sex group sharing the same superscript are not significantly different ($P>0.05$). Values are means ± S.E.M. for 10 females and 5 males.

Table 5
The effects of algal meal derived diets on striped bass broodstock gonadal development, fecundity, and egg hatching rate and fatty acid composition

| Diet | Spermiating males (%) | FMS females (%) | Egg biomass (g $kg^{-1}$ BW) | Hatching rate (%) | Egg DHA/EPA/ArA | |
|---|---|---|---|---|---|---|
| | | | | | Content | Ratio |
| Diet-1 | 40 | 100 | $280 \pm 50^{A}$ | No Hatch | 10/8/0.3 | 10:8:0.3 |
| Diet-2 | 20 | 50 | $400 \pm 90^{A}$ | $15 \pm 5.4^{A,B}$ | 14/8/0.3 | 10:6:0.2 |
| Diet-3 | 40 | 60 | $290 \pm 20^{A}$ | $22 \pm 8.5^{A}$ | 16/7/2.5 | 10:5:1.5 |
| Diet-4 | 100 | 80 | $260 \pm 50^{A}$ | $8.1 \pm 5.9^{B}$ | 15/8/0.3 | 10:5:0.2 |

Gonadal development represents the percentage of spermiating males and females at the final maturation stage (FMS, oocyte diameter >800μm) in each dietary treatment. Values for egg biomass (g eggs per kg female BW) and hatching rate are the mean ± S.E.M. of triplicate spawns of three different females within each dietary treatment. Dietary treatments having the same superscript are not significantly different ($n=3$, $P>0.05$). Egg DHA/EPA/ArA contents are presented in percentage of total fatty acids, and the ratio represents the proportion between the three fatty acids (relative to 10 parts of DHA) in the egg total lipids extract.

accumulation in rotifers and *Artemia* nauplii is shown in Table 6. Enrichment concentrations for rotifers were established at 0.05 g $l^{-1}$ since preliminary tests have shown that survival reduced severely if rotifers exposed to higher PL-*Cr* levels (49.7% after 8 h of enrichment at 0.1 g $l^{-1}$). Rotifer survival at 0.05 g $l^{-1}$ enrichment level was high in all enrichment treatments (78–90%) with the exception of PL-*Cr* enrichment where survival was slightly lower (75.7%, $P<0.05$). Olive oil, DHA-Selco and WC-*Cl*/PL-*Cr* delivered the lowest levels of DHA to the rotifers, whereas Algamac-2000, PL-*Cr*, WC-*Cr* and WC-*Cr*/PL-*Mo* were effective in increasing the percentage of DHA in rotifers fatty acids to over 20%. However, the highest DHA/EPA ratios were obtained in rotifers fed with PL-*Cr* and WC-*Cr* diets. Moreover, the inclusion of 12.5% PL-*Mo* with WC-*Cr* diet was sufficient to elevate ArA content in the rotifers up to 10-fold of their initial content.

Over 75% of the *Artemia* survived after the 16-h enrichment period, with no significant deleterious effects by the enrichment sources. During enrichment, the size of *Artemia* nauplii increased from 620 μm in length at instar-II to ca. 900 μm after 16 h regardless of dietary treatment ($P>0.05$). The greatest increase in lipid content was observed in PL-*Cr*-enriched *Artemia*, whereas WC-*Cr* and Algamac-2000 enrichments resulted in lower lipid accumulations ($P<0.05$). Furthermore, the phospholipid/triacylglycerol ratio in PL-*Cr*-enriched *Artemia* was significantly higher than the other treatments ($P<0.05$). PL-*Cr* and WC-*Cl*/PL-*Cr* treatments were associated with the greatest accumulation of DHA in the nauplii (17.2 ± 1.0 and 19.5 ± 0.8% of TFA, respectively), whereas Algamac-2000- and DHA-Selco-enriched *Artemia* has significantly lower levels (7.4 ± 0.2 and 10.8 ± 0.8% of TFA, respectively). The high DHA incorporation in *Artemia* fed the WC-*Cr* or PL-*Cr* diets was also reflected in the significantly higher ratios of DHA/EPA (over >2.5), compared to those of Algamac-2000- and DHA-Selco-enriched *Artemia* (1.1 and 1.2, respectively). The addition of 12.5% PL-*Mo* in the WC-*Cr* diet increased the *Artemia* ArA content 3–4-fold over their initial content, without a significant reduction in the accumulation of DHA or EPA ($P>0.05$).

Table 6
Lipid and DHA, EPA and ArA accumulation in rotifers and *Artemia* nauplii fed with fish oil- and algal-based enrichment preparations

| | DHA-Selco | Algamac-2000 | PL-*Cr* | WC-*Cl*/PL-*Cr* | WC-*Cr* | WC-*Cr*/PL-*Mo* | Olive oil |
|---|---|---|---|---|---|---|---|
| *Rotifers* | | | | | | | |
| Survival (%) | $78.9 \pm 8.0^{A,B}$ | $81.7 \pm 3.8^{A,B}$ | $75.7 \pm 3.8^{B}$ | $78.2 \pm 6.4^{A,B}$ | $89.9 \pm 5.8^{A}$ | $86.8 \pm 2.2^{A}$ | $86.6 \pm 4.3^{A}$ |
| Lipid (%DW) | $19.4 \pm 0.5^{B,C}$ | $20.3 \pm 0.4^{B}$ | $19.9 \pm 0.8^{B}$ | $22.2 \pm 0.6^{A}$ | $17.4 \pm 0.6^{D,E}$ | $18.6 \pm 0.8^{C,D}$ | $16.4 \pm 1.0^{E}$ |
| ArA (%TFA) | $0.9 \pm 0.1^{C}$ | $1.5 \pm 0.1^{B}$ | $0.6 \pm 0.1^{D}$ | $0.9 \pm 0.1^{C}$ | $0.5 \pm 0.1^{D}$ | $7.1 \pm 0.3^{A}$ | $0.6 \pm 0.4^{D}$ |
| EPA (%TFA) | $6.6 \pm 0.1^{A}$ | $3.3 \pm 0.4^{B}$ | $2.9 \pm 0.2^{B}$ | $3.5 \pm 0.3^{B}$ | $2.3 \pm 0.2^{C}$ | $3.2 \pm 0.2^{B}$ | $2.5 \pm 0.3^{C}$ |
| DHA (%TFA) | $8.8 \pm 0.6^{D}$ | $23.4 \pm 0.7^{A}$ | $24.5 \pm 1.1^{A}$ | $10.5 \pm 0.4^{C}$ | $22.3 \pm 1.0^{A,B}$ | $21.5 \pm 0.2^{B}$ | $1.1 \pm 0.6^{E}$ |
| DHA/EPA/ArA | 10:7:1 | 10:5:1 | 10:1:0 | 10:3:1 | 10:1:0 | 10:2:3 | 10:25:5 |
| *Artemia* | | | | | | | |
| Survival (%) | $81.6 \pm 2.5^{A}$ | $82.3 \pm 4^{A}$ | $79.5 \pm 10.7^{A}$ | $85.6 \pm 3.0^{A}$ | $81.1 \pm 2^{A}$ | $87.4 \pm 3.5^{A}$ | $84.8 \pm 1.4^{A}$ |
| Lipid (%DW) | $22.6 \pm 1.7^{B}$ | $19.3 \pm 0.3^{C}$ | $25.3 \pm 0.7^{B}$ | $28.9 \pm 1.3^{A}$ | $17.7 \pm 1.2^{C,D}$ | $19.0 \pm 0.6^{C}$ | $15.6 \pm 1.0^{D}$ |
| ArA (%TFA) | $2.2 \pm 0.2^{C}$ | $3.4 \pm 0.6^{B}$ | $2.8 \pm 0.2^{B,C}$ | $3.1 \pm 0.3^{B}$ | $2.1 \pm 0.3^{C}$ | $7.9 \pm 0.3^{A}$ | $3.6 \pm 0.6^{B}$ |
| EPA (%TFA) | $9.3 \pm 0.3^{A}$ | $5.6 \pm 0.2^{B}$ | $4.4 \pm 0.1^{C}$ | $4.8 \pm 0.2^{C}$ | $4.7 \pm 0.2^{C}$ | $3.7 \pm 0.1^{D}$ | $4.1 \pm 0.4^{C,D}$ |
| DHA (%TFA) | $10.8 \pm 0.8^{C}$ | $7.4 \pm 0.2^{D}$ | $17.2 \pm 1.0^{A}$ | $19.5 \pm 0.8^{A}$ | $12.5 \pm 0.9^{B}$ | $10.6 \pm 0.2^{C}$ | N.D |
| DHA/EPA/AA | 10:9:2 | 10:8:5 | 10:2:2 | 10:3:2 | 10:4:2 | 10:4:8 | 0:1:1 |
| PL/TAG | $0.51 \pm 0.07^{B}$ | $0.48 \pm 0.07^{B}$ | $0.68 \pm 0.04^{A}$ | $0.58 \pm 0.06^{A,B}$ | $0.47 \pm 0.06^{B}$ | $0.51 \pm 0.04^{B}$ | $0.48 \pm 0.02^{B}$ |

Rotifers were enriched with two portions of 0.05 g $l^{-1}$ each every 4 h, at a density of $500 \times 10^3$ rotifers $l^{-1}$ and 20 psu artificial sea water (28 ± 1 °C). *Artemia* were enriched with two portions of 0.3 g $l^{-1}$ each every 8 h, at a density of $200 \times 10^3$ nauplii $l^{-1}$. WC-*Cl*/PL-*Cr* is a spray-dried mix containing 50% WC-*Cl* with 50% PL-*Cr*. WC-*Cr*/PL-*Mo* is a spray-dried mix of 87.5% WC-*Cr* with 12.5% PL-*Mo*. DHA/EPA/ArA represents the proportion between the three fatty acids (relative to 10 parts of DHA). PL/TAG represents the ratio between *Artemia* phospholipids (PL) and triacylglycerols (TAG). Fatty acid levels are presented in percentage of total fatty acids (%TFA). Values are the mean ± S.E.M. of triplicate enrichments ($n = 3$). Values with different superscripts indicate significant difference between enrichment treatments ($P > 0.05$). N.D.: not detectable.

### 3.4. Larval growth

The growth of 17- to 46-day-old striped bass larvae fed DHA- and ArA-deficient *Artemia* was the lowest ($P < 0.05$) among all experimental groups (105 ± 10% DW gain, Table 7). In contrast, the growth of larvae fed PL-*Cr*-enriched *Artemia* showed the greatest DW gain (175 ± 23%). Not surprisingly, DHA level in larvae fed DHA- and ArA-deficient *Artemia* was low and nearly equivalent to their EPA and ArA levels (Table 7), while the

Table 7
Effect of fish oil- and algal-based enrichment materials for rotifers and *Artemia* on final weight and DHA, EPA, ArA accumulation in striped bass, European sea bass, gilthead sea bream and Atlantic halibut larvae

| | Olive oil | Algamac-2000 | PL-*Cr* | DHA-Selco |
|---|---|---|---|---|
| *Striped bass* | | | | |
| Final weight (mg DW) | $19.6 \pm 1.9^{B}$ | $24.4 \pm 1.6^{A}$ | $26.4 \pm 6.1^{A}$ | |
| ArA (%TFA) | $2.3 \pm 0.1$ | $3.6 \pm 0.1$ | $2.9 \pm 0.1$ | |
| EPA (%TFA) | $3.4 \pm 0.7$ | $6.1 \pm 0.2$ | $5.6 \pm 0.1$ | |
| DHA (%TFA) | $2.8 \pm 0.2$ | $11.1 \pm 0.3$ | $13.8 \pm 0.6$ | |
| Larvae DHA/EPA/ArA | 10:11:8 | 10:5:3 | 10:4:2 | |
| *European sea bass* | | | | |
| Final weight (mg DW) | | $4.1 \pm 0.1^{A}$ | $3.4 \pm 0.2^{B}$ | |
| ArA (%TFA) | | $4.2 \pm 0.1$ | $1.6 \pm 0.0$ | |
| EPA (%TFA) | | $6.2 \pm 0.1$ | $6.7 \pm 0.1$ | |
| DHA (%TFA) | | $15.9 \pm 0.1$ | $20.1 \pm 0.4$ | |
| Larvae DHA/EPA/ArA | | 10:4:3 | 10:3:1 | |
| *Sea bream* | | | | |
| Final weight (mg DW) | | $1.7 \pm 0.1$ | $1.6 \pm 0.2$ | |
| ArA (%TFA) | | $4.5 \pm 0.1$ | $1.8 \pm 0.1$ | |
| EPA (%TFA) | | $6.1 \pm 0.0$ | $7.4 \pm 0.3$ | |
| DHA (%TFA) | | $13.7 \pm 0.3$ | $17.5 \pm 0.7$ | |
| Larvae DHA/EPA/ArA | | 10:4:3 | 10:4:1 | |
| Rotifers DHA/EPA/ArA | | 10:2:1 | 10:2.5:1 | |
| *Artemia* DHA/EPA/ArA | | 10:4:2 | 10:3:0.5 | |
| *Atlantic halibut* | | | | |
| Final weight (mg WW) | | | 520 | 280 |
| ArA (%TFA) | | | 1.7 | 1.8 |
| EPA (%TFA) | | | 9.8 | 11.6 |
| DHA (%TFA) | | | 15.9 | 13.0 |
| Larvae DHA/EPA/ArA | | | 10:6:1 | 10:9:1 |
| *Artemia* 24-h DHA/EPA/ArA | | | 10:2:0.1 | 10:8:2 |
| *Artemia* 72-h DHA/EPA/ArA | | | 10:4:0.5 | 10:7:2 |

Composition of the live feeds for striped bass larvae is presented in Table 6. Striped bass experiment and fatty acid analysis were carried out at COMB; sea bream and European sea bass experiments and fatty analysis were carried out and NCM; Atlantic halibut experiment was carried out at AKVAFORSK and the fatty acid analysis at COMB. Fatty acid levels expressed in percentage of total fatty acids (%TFA). Growth for striped bass, sea bream and European sea bass is expressed in mg dry weight (mg DW) and for Atlantic halibut in mg wet weight (mg WW).

DHA levels in larvae fed DHA-enriched *Artemia* were two-fold higher than EPA and about three-fold higher than ArA.

The effect of rotifer and *Artemia* enrichment with either Algamac-2000 or PL-*Cr* on sea bream larval growth was not significantly different ($1.7 \pm 0.1$ and $1.6 \pm 0.2$ mg DW larvae, respectively, $P > 0.05$, Table 7). In experiments with 11- to 36-day-old European sea bass larvae, a growth advantage ($P < 0.05$) was demonstrated in larvae fed *Artemia* enriched with Algamac-2000 compared to PL-*Cr* ($4.1 \pm 0.1$ and $3.4 \pm 0.2$ mg final DW, respec-

tively, $P < 0.05$). This result may reflect a nutritional requirement for $n-6$ HUFA by this species, which was satisfied by the high docosapentaenoic acid (DPA, 22:5$n-6$) level in Algamac-2000.

Atlantic halibut larvae fed PL-*Cr*-enriched *Artemia* were almost two-fold larger than larvae fed DHA-Selco-enriched *Artemia* (Table 7). Moreover, 83% of the PL-*Cr* fed halibut larvae fully completed their pigmentation and eye migration process compared to 50% of the DHA-Selco fed larvae (statistical analysis was not performed in this experiment because of the small number of tank replicates). The DHA-Selco-enriched *Artemia* provided the larvae with almost equal amounts of DHA and EPA, whereas PL-*Cr*-enriched *Artemia* provided over 60% more DHA than EPA. Both DHA-Selco and PL-Cr fed larvae accumulated similar amounts of ArA, respecting the natural ArA levels in the *Artemia*.

## 4. Discussion

Heterotrophically grown algal and fungal supplemented diets are highly effective in delivering essential fatty acids either through larval live food enrichment or directly through the fish diet. The commercial viability of this new lipid source, however, will depend on production costs and its competitiveness with fish-based products. The current price of DHA-rich algal dry biomass (*Crypthecodinium* sp. or *Schizochytrium* sp.) is about US\$25 $kg^{-1}$, whereas menhaden oil costs about US\$2.50 $kg^{-1}$. Yet, a true comparison should include the efficiency of the material to deliver essential nutrients to the fish, such as $n-3$ or $n-6$ PUFA. The level of DHA in *Crypthecodinium* sp. meal is an order of magnitude higher than menhaden oil; therefore, on a weight basis, the amount of algal meal required to deliver sufficient levels of DHA would be much less than that of fish oil. Furthermore, increased demand and as a result the production capacity may bring the price of heterotrophic algae even more closer to the optimal cost of US\$5 $kg^{-1}$ (Gladue and Maxey, 1994).

Fish oil is used in fish feed mainly because it offers a range of fatty acid classes, including the long chain $n-3$ PUFA, that contribute to the energy, growth and reproductive demands of the fish. However, the level of $n-3$ PUFA in fish oil varies depending on species, extraction procedure and storage conditions. In fact, standard available fish oils (menhaden, anchovy, herring, etc.) do not offer sufficient levels of DHA or DHA/EPA ratios to completely satisfy the nutritional demands for reproduction and larval growth (Harel et al., 1994, 2000; Sargent et al., 1997). Consequently, marine oils where the DHA levels are particularly high due to its origin in specific fish tissues (cod liver oil, tuna orbital oil), or through special extraction procedures (silage, cold acetone) have been recommended in broodstock diets and larval rearing enrichment preparations (McEvoy and Sargent, 1999; Sargent et al., 1999). However, the availability of these high DHA-containing oils is limited and often prohibitively expensive to produce. In addition, diets formulated with standard available fish oils are usually low in ArA. This is relevant in light of the increasing body of evidence attesting to the species-specific requirement of this essential fatty acid (Castell et al., 1994; Bessonart et al., 1999; Harel et al., 2000; Koven et al., 2001).

In the present study, results indicate that ArA supplemented diet improves egg hatching rate. ArA probably acts as a stress-alleviating mediator in the broodstock fish that were maintained in small close recirculated tanks and in artificial seawater (4–6 psu). These stressful conditions were possibly responsible for the low hatching rate of eggs from all DHA and commercial diets fed fish (8–15% hatching of total spawn). Although, increasing ArA dietary levels during reproduction could reduce the associated stress effects, it may be as essential to also provide better living conditions for the fish since hatching rate of striped bass eggs should be over 62% (Woods and Sullivan, 1993; Spade and Bristow, 1999). Higher ArA dietary levels may be also required during other periods of environmental and developmental changes. In support of this, we have previously shown that striped bass larvae required ArA supplemented diet, especially during osmotic challenge (Harel et al., 2000). Recent studies on seabream suggested a correlation between dietary ArA and cortisol and ionic regulation following a rapid drop in seawater salinity (Koven, personal communication). Similarly, an ArA requirement has been demonstrated in Atlantic salmon undergoing parr–smolt transformation and osmoregulation activities of their body fluids (Bell et al., 1997).

The results of the present study show that a spray-dried mix of WC-*Cr* with 12.5% PL-*Mo* efficiently delivered both DHA and ArA to the live prey of larvae. Rotifers and *Artemia* enriched with these preparations provide DHA/EPA/ArA ratios of 10:2:3 and 10:4:8, respectively, compared to only a minor amount of ArA in zooplankters fed the WC-*Cr*. However, rotifers were more efficient than *Artemia* in absorbing dietary DHA but less so incorporating ArA, suggesting that rotifers may require a lower DHA/ArA ratio in their enrichment than the *Artemia* to supply sufficient levels of these fatty acids. It is worthwhile noting that nutritional requirements for essential fatty acids may vary between species and developmental stages. For example, in white bass larvae, the optimal DHA/ArA and EPA/ArA dietary ratios were established at 2:1 and 1:1, respectively (Harel et al., 2000). This contrasts with flat fish larvae such as turbot and Atlantic halibut which require much higher ratios of over 10:1 (Castell et al., 1994; Bell et al., 1995; Sargent et al., 1997, 1999). An additional consideration is the likelihood of DHA deficiency in vision and other neural tissues in larvae fed fish oil-based diet, as has been observed in Atlantic halibut larvae eyes fed *Artemia* enriched even with DHA-fortified fish-oil (McEvoy et al., 1998). Using spray-dried mixes of different sources of algal meals and oils, this adjustment can be easily made to deliver optimal quantities and ratios or when a specific requirement may appear.

It is doubtful whether available fish oils would be able to satisfy these divergent DHA/EPA/ArA needs of larvae and broodstock from the increasing number of farmed marine teleosts. On the other hand, by using established fermentation techniques, lipids from heterotrophic algal groups, such as chrysophytes, cryptophytes and dinoflagellates, can produce high levels of EPA, DHA and ArA (Cohen et al., 1995; Behrens and Kyle, 1996; Apt and Behrens, 1999). Consequently, combinations of these alga strains can offer a broader range of fatty acids to meet more effectively the species-specific dietary requirements. Examples are the algal species, *Schizochytrium* sp. and *Crypthecodinium cohnii*, which can produce high levels (up to 50%) of DHA (Barclay and Zeller, 1996; Behrens and Kyle, 1996), while the dried biomass from *Nitzschia* sp. (Boswell et al., 1992), *Nanochloropsis* (Sukenik, 1991) and *Navicula* sp. (Tan and Johns, 1996) can offer a rich source

of EPA (Kyle et al., 1989; Apt and Behrens, 1999). Moreover, extracted oils from the heterotrophic fungi, *Mortierella* sp., which contain up to 54% ArA can be readily produced and combined with other heterotrophic products.

Another potential use of heterotrophic algal preparations that could be very useful in hatcheries of marine finfish, shrimp and oysters is to partially substitute or supplement a live algae diet of the larvae. The value of live algae supplement to the culture medium of many marine larvae is well established (Reitan et al., 1993; Harel et al., 1998; Harel and Place, 1999). However, live photosynthetic algae production is costly and inconsistent and may represents up to 30% of the cost of larval production (Coutteau and Sorgeloos, 1992). The partial replacement of up to 40% living algal supplements using spray-dried heterotrophically grown algal biomass have been reported for a number of marine organisms, such as mussel, *Mytilus galloprouincialis* (Davis and Campbell, 1998; Langden and Onal, 1999), juvenile bivalve molluscs (Laing and Millican, 1992) and juvenile clams, *Tapes semidecussata* (Boieng, 1997).

In conclusion, the heterotrophic, large-scale production of algal biomass represents a potential high quality substitute for fish-based ingredients in aquaculture feeds. Specifically, algal whole cell preparations and algal and fungal oil extracts could be superior alternative sources for DHA, EPA and ArA enrichment products in a wide variety of fish larval feeds and in broodstock diets. The current production levels of heterotrophic algae are insufficient to replace the worldwide use of fish byproducts in aquaculture. On the other hand, the natural fishery is a dwindling resource that can vary in both quantity and quality, and where supplies and processing costs are likely to rise. These are compelling reasons to turn to heterotrophic production as a cost-effective and stable source of marine type lipids in fish feeds and for the enrichment of the live food.

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