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Changes in biochemical and hemocyte parameters of the Pacific oysters *Crassostrea gigas* fed T-*Iso* supplemented with lipid emulsions rich in eicosapentaenoic acid

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Abstract:

The aim of this study was to assess the effect of dietary eicosapentaenoic acid (20:5n-3) on hemocyte parameters such as hemocyte concentration, phagocytosis, and non-stimulated reactive oxygen species (ROS) production in Pacific oysters *Crassostrea gigas*, as well on proximate biochemical and fatty acid compositions. One-year-old oysters (*C. gigas*) were fed T-*Isochrysis aff. galbana* (T-*Iso*), which is low in 20:5n-3, either alone or with supplements of a lipid emulsion rich in 20:5n-3 at 1%, 10% or 50% (dry weight of the algal ration) for up to 7 weeks. Changes in gill fatty acid composition demonstrated that the lipid emulsion was well ingested by oysters during the dietary conditioning. Biochemical analysis indicated that oysters fed supplements of 50% and, to a lesser extent, 10% lipid emulsions had a higher total lipid content compared with oysters fed other diets, suggesting a more advanced reproductive status for the oysters fed high doses of lipid emulsion. Moreover, some oysters in these two treatment groups spawned during the last three weeks of the seven-week feeding experiment. Lipid supplements had a significant influence on hemocyte concentration, phagocytic index and non-stimulated hemocyte ROS production. After 4 weeks, highest hemocyte concentrations were found in oysters fed on a supplement of 50% lipid emulsion compared with those fed on other diets but the hemocytes derived from these oysters had the lowest short-term phagocytic index. After 7 weeks of dietary conditioning, the ROS production in non-stimulated hemocytes of oysters fed 10% and 50% lipid emulsion declined. These results suggested that 20:5n-3, and perhaps its eicosanoid metabolites, affected oyster hemocyte functions; however, the reproductive status of oysters may also have interfered with the 20:5n-3 dietary effect.

Keywords: Bivalves; *Crassostrea gigas*; Eicosapentaenoic acid (20:5n-3); Hemocytes; Immunology; Polyunsaturated fatty acids

Introduction

The impact of nutrition, especially dietary long chain n-3 essential polyunsaturated fatty acids (n-3 PUFAs) has been extensively studied in human medical research. Fish oil, which is rich in n-3 PUFAs, has been shown to be an immuno-modulator for a number of human immune functions, including ex vivo lymphocyte proliferation, cytotoxic T lymphocyte and natural killer cell activities, and production of cytokines in human and laboratory animals (see Calder, 2001; Harbige, 2003 for review). Similar immuno-modulatory effects were also observed in fish (Waagbø et al., 1993; Kiron et al., 1995; Lingenfelser et al., 1995; Thompson et al., 1996). Lingenfelser et al. (1995) demonstrated that macrophage phagocytic activity of the channel catfish Ictalurus punctatus depends on the fatty acid composition of the diet and also the rearing temperature condition. Their results suggested that macrophage n-3 PUFA enrichment, by feeding fish with menhaden oil, might allow maintenance of macrophage membrane fluidity and increase phagocytic activity at 18°C while it was not beneficial when fish were reared at 25°C (Lingenfelser et al., 1995).

Several studies have reported the importance of lipids in bivalve nutrition. The long chain n-3 PUFAs, eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), and the long chain n-6 PUFA, arachidonic acid (20:4n-6), are believed to be essential to bivalve development (De Moreno et al., 1976; Chu and Greaves, 1991; Jonsson et al., 1999). Consequently, algal diets have to provide those essential PUFAs (Langdon and Waldock, 1981; Chu and Webb, 1984; Knauer and Southgate, 1997; Jonsson et al., 1999). Thus, algal diets composed of mixed species are often used in hatcheries because they provide a greater range of potentially essential lipids (Enright et al., 1986; Laing and Millican, 1986; Albentosa et al., 1993; Brown et al., 1998).

Artificial particle types, such as microcapsules (Langdon and Waldock, 1981; Langdon and Siegfried, 1984; Numaguchi and Nell, 1991), liposomes (Parker and Selivonchick, 1986), or lipid microspheres (Heras et al., 1994) containing essential PUFAs have been developed as substitutes or supplements for live algal diets. Several studies reported the effect of supplements of essential PUFAs using artificial diets on growth and development of several bivalve species: Chilean-Peruvian scallop Argopecten purpuratus larvae (Nevejan et al., 2003), Manila clam Ruditapes philippinarum (Caers et al., 2000b) and Pacific oyster C. gigas spat (Langdon and Waldock, 1981; Caers et al., 2000a et b), juvenile sea scallops Placopecten magellanicus (Coutteau et al., 1996), adult C. gigas (Robinson, 1992; Uriarte et al., 2004) and adult European flat oysters Ostrea edulis (Heras et al., 1994). Although, they are still not used routinely in hatcheries, artificial particles can offer a practical solution to reduce PUFA deficiencies in hatchery-reared molluscs. Lipid emulsions have also been described as effective carriers of essential fatty acids for bivalve nutrition.

The relationship between nutrition and immune responses in bivalves has been recently investigated. Delaporte et al. (2003) have demonstrated that Pacific oysters C. gigas and Manila clams R. philippinarum fed the 20:5n-3 rich alga (17.8%), Chaetoceros calcitrans, showed higher hemocyte concentration, reactive oxygen species production and phagocytic activity than animals fed T-Isochrysis aff. galbana, clone Tahitian (T-Iso) or Tetraselmis suecica. Both T-Iso and T. suecica contain only small amounts of 20:5n-3 (0.4 and 5.4% respectively). These authors argued that difference in the quantity in 20:5n-3, 22:6n-3 and 20:4n-6 among algae might be implicated in the changes in these hemocyte parameters as demonstrated for vertebrates and fishes fed on different oils. Similarly, Hégaret et al. (2004) demonstrated that the quality of the algal diet affected certain hemocyte parameters of Eastern oysters C. virginica. Phagocytic activity of oysters fed Skeletonema costatum was higher than that of oysters fed T. chui or a mixed diet (containing both algal species) when oysters were subjected to a temperature increase. Nonetheless, no specific PUFA could be clearly identified as being responsible for those changes.

The objective of the present study was to assess the impact of dietary 20:5n-3 supplements on hemocyte concentrations, hemocyte viability, phagocytosis and non-stimulated reactive oxygen species production of Pacific oysters C. gigas. Oysters were fed on T-Iso alga with or without supplements of a lipid emulsion rich in 20:5n-3 and oysters from different treatments were sampled for measurement of hemocyte parameters, proximate biochemical composition, condition index after 0, 2, 4 and 7 weeks.

Materials and Methods

Lipid emulsions

The lipid emulsion was prepared with EPAX 4510 TG (Polaris, Pleuven, France) - a marine oil mixture of triglycerides (TAG) rich in 20:5n-3 (eicosapentaenoic acid, 45%) and 22:6n-3 (docosahexaenoic acid, 10%) according to the method described by Pernet et al. (2004) except that 5% w/w Span 40 and 0.02% w/w ethoxyquin (antioxidant) were added to the TAG and no Sudan Red was used as a marker.

Oyster dietary treatments and sampling procedures

Oysters were fed experimental diets at the Hatfield Marine Science Center (Newport, Oregon, USA). One-year-old Pacific oysters Crassostrea gigas (Thunberg 1793) were acclimated at 17°C in ambient seawater (30 ‰ salinity) for two weeks prior to the start of the experiment, and reached a mean total live weight of 24 g. During the acclimation period, oysters were fed T-Iso. T-Iso was chosen as the algal diet because it contains low levels of 20:5n-3 (1.9%). After the acclimation period, oysters were randomly divided and distributed into twelve 60 l tanks (3 replicate tanks per treatment) continuously supplied with filtered (0.2 µm) seawater at 36 exchanges per day. Thereafter, oysters were fed either T-Iso alone or T-Iso supplemented with different levels of a lipid emulsion for seven weeks (mid February- April 2003). The daily algal ration (algal dry weight) was adjusted each week to equal 5% oyster dry tissue weight. Lipid emulsion supplements were added at 0%, 1%, 10%, or 50% of the dry weight of the T-Iso algal ration. T-Iso and lipid supplements were continuously added to tanks via peristaltic pumps. During the last three weeks of the seven-week feeding experiment, spawning was observed in tanks of the oysters fed on supplements of 10 and 50% w/w lipid emulsion.
Oysters were sampled at 0, 2, 4 and 7 weeks of dietary conditioning. At each sampling, 13 oysters from each replicate tank were sampled and sent to the Virginia Institute of Marine Science, Gloucester Point, Virginia, USA. Upon arrival, oysters were notched, maintained unfed for 24h in 30 ‰ seawater at 17°C. Hemolymph was then sampled for immune assays. A day after hemolymph withdrawal, gills from five oysters and the whole body of the eight remaining oysters from each replicate tank were frozen at -80°C, freeze dried and stored at -20°C for fatty acid and proximal biochemical analysis.

Biochemical analysis

Biochemical analyses were performed on pooled freeze-dried tissue samples of eight animals from each replicate tank. Pooled tissue samples were ground. Then 100 mg of dry powder was hydrated with 3 ml of distilled water and divided into three aliquots for total lipid, protein and carbohydrate analyses. Total lipid content was determined according to Bligh and Dyer (1959) after extraction with a mixture of dichloromethane-ethanol-water. Carbohydrate and protein contents were measured colorimetrically as described by Dubois et al. (1956) and Lowry et al. (1951), respectively. Results are expressed as mg of carbohydrates, lipids and proteins per mg of oyster dry tissue weight.

Lipid and fatty acid analysis

Total lipid extraction

The changes in fatty acid composition of hemocytes and gill tissues due to dietary conditioning were previously observed to be similar (Delaporte, 2005); therefore, fatty acid analyses were performed on gills rather than hemocytes because of insufficient materials of the latter for lipid analysis. Freeze-dried gill tissues of the 5 oysters from each replicate tank were pooled and ground together to form a powder. Aliquots of 30 mg of freeze-dried gill powder were rehydrated in 800 µl of distilled water then extracted successively with two aliquots of 3 ml of chloroform-methanol solutions (1:2 and 2:1, v:v). The lipid extract was stored at -20°C under nitrogen for later lipid analysis. To analyze fatty acid composition of gill neutral and polar lipids, total gill lipid extracts were separated into neutral and polar lipids using a Silica gel micro-column according to the method described by Marty et al. (1992).

T-Iso and emulsion samples were filtered onto pre-ashed (450°C) GF/F filters. The filters were then placed in tubes containing 3 ml of methanol. The methanol was evaporated to dryness and replaced with 6 ml of a mixture of chloroform-methanol (2:1, v:v) for complete lipid extraction. Thereafter, algal

and emulsion lipid extracts were stored at - 20°C under a nitrogen atmosphere until analysis was conducted. Fatty acid analyses were performed on total lipid extracts.

Fatty acid trans-esterification

The gill neutral and polar lipids as well as total lipid extracts from algae and lipid emulsions were transesterified with 10% (w:w) boron trifluoride/methanol (Metcalfe and Schmitz, 1961) as described by Delaporte et al. (2005). Gill total fatty acid content (neutral + polar lipids) was expressed as µg of fatty acids (FAs) per mg of gill dry weight. Fatty acid composition was expressed as weight percentage of the total FAs. Since polar lipids are generally the dominant lipids in gill tissues (Allen and Conley, 1982; Delaporte et al., 2005), only fatty acid composition of the polar lipid fraction of gill lipid extracts was calculated.

Hemolymph sampling

Hemolymph was withdrawn from individual oysters via a notch adjacent to the adductor muscle using a 1 ml plastic syringe fitted with a 25-gauge needle. Shells of oysters were notched a day before hemolymph withdrawal to allow oysters to recover from the stress induced by notching and to eliminate shell debris. All hemolymph samples were stored individually in micro-tubes at 0°C and examined microscopically for any contamination with debris or gametes. Hemolymph samples of five to six oysters from each replicate tank were pooled in order to have enough hemolymph for all hemocyte assays. Three replicate tanks per dietary treatment were analysed for each hemocyte assay.

Phagocytosis assay

Hemocyte phagocytic activity was assayed according to the method of Chu and La Peyre (1993). Briefly, a 20 µl aliquot of pooled hemolymph from each replicate tank was added to a glass slide and allowed to adhere and form a hemocyte monolayer at room temperature for 30 minutes. A 20 µl aliquot of zymosan suspension (1 mg/ml) in filtered sterile seawater (FSSW) was added to the hemocyte monolayer and incubated for one hour at room temperature. After the incubation period, slides were rinsed with FSSW and hemocytes were fixed with Dietrich' fixative and stained with Hemal-Stains I and II (Hemal Stain Co., Inc., Danbury, CT) for later counting using a light microscope at 400X. Phagocytic activity was expressed as a phagocytic index (i.e. ratio of number of hemocytes with zymosan / total number of hemocytes).

Measurements of differential hemocyte counts, hemocyte viability and reactive oxygen species production using flow cytometry

Total and differential hemocyte counts, hemocyte viability and reactive oxygen species (ROS) production were determined using a Beckman Coulter® EPICS® Altra™ flow cytometer (Miami, FL, USA) connected to a computer using Expo32 as acquisition software. Optical alignment and stability were monitored daily using 10µm diameter fluorescent flow-Count fluorospheres (Coulter PN6605359). As recommended by FCM manufacturer all pooled hemolymph samples were filtered through a 80µm mesh prior to analysis to eliminate large debris (> 80 µm).

Total and differential hemocyte concentrations were determined as described by Delaporte et al. (2006a). Briefly, 100 µl pooled hemolymph from each replicate tank were fixed with 300 µl of a 4% (v:v) formalin solution made up in FSSW. Hemocyte DNA was stained with SYBR Green I (Molecular probes, 10X final concentration), for 30 minutes and incubated in darkness at room temperature prior to analysis. SYBR Green I is a nucleic acid specific dye staining both live and dead cells. SYBR Green stained hemocytes were detected at 500-530 nm (yellow-green fluorescence) by the flow cytometer.
Based on the Forward Scatter (FSC) and Side Scatter (SSC) measurement, which are respectively proportional to the size and the complexity of the cells, differential hemocyte sub-populations were discriminated: granulocytes (high FSC, high SSC), hyalinocytes (high FSC, low SSC) and small agranulocytes (low FSC, low SSC). Total hemocyte, granulocyte and hyalinocyte concentrations were expressed as number of cells per ml. Agranulocyte concentrations were not calculated as they represented only a small proportion of the total hemocytes and are considered to have little activity (Lambert et al., 2003).

Hemocyte viability was assayed according to Delaporte et al. (2003). Briefly, aliquots of 150 µl pooled hemolymph from each replicate tank were filtered and transferred to a tube containing an anti-aggregating solution for bivalve hemocytes prepared according to Auffret and Oubella (1995). Ten minutes before flow cytometry analysis, samples were incubated with propidium iodide (final concentration 20 $\mu g.ml^{-1}$) and measured at 550-600 nm. Propidium iodide is a fluorescent DNA/RNA-specific dye, which only permeates through membranes of dead cells. Percentage of viable hemocytes was calculated as the percentage of hemocytes not showing PI fluorescence relative to total hemocyte counts.

ROS production in non-stimulated hemocytes was measured following the method of Lambert et al. (2003) employing 2'7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is widely used to measure generation of ROS in mammalian neutrophils and macrophages (Vowells et al., 1995) and in snail hemocytes (Hahn et al., 2000). Oxidation of DCFH-DA by ROS leads to fluorescent product, which can be quantified using flow cytometer (Lambert et al., 2003).
To measure ROS in non-stimulated hemocytes, 150 µl of pooled hemolymph from each replicate tank were first diluted with 150 µl of FSSW in tube and maintained on ice. Then, DCFH-DA (final concentration of 0.01 mM) was added and tubes were incubated at 18°C. After 120 and 180 minutes incubation, DCF fluorescence was measured to determine ROS production in hemocytes and was expressed in fluorescent arbitrary units (A.U). Only results of ROS production after 180 minutes of incubation were presented as difference among dietary treatments was only noted at this incubation time.

Statistical analysis

Two-way analysis of variance (2-way ANOVA) was performed to test the effects of dietary treatments and sampling times on all hemocyte and biochemical parameters using STATGRAPHICS Plus 5.1 statistical software (Manugistics, Inc., Rockville, MD, USA). When a significant effect ($p<0.05$) of dietary treatment or sampling times was found, Multiple Range Test (Fisher's least significant difference, LSD) was used to determine the significance of differences among means. Percentage data were transformed (arcsin square root) before ANOVA, but presented in figures and tables as non-transformed percentages.

Results

Fatty acid (FA) composition of T-Iso alga and lipid emulsions

T-Iso alga was rich in 18:4n-3 (18.6%), 22:6n-3 (18.0%), 18:1n-9 (12%), 14:0 (11.2%) and 16:0 (10.4%); but, it was low in 20:5n-3 (1.9%) and deficient in 20:4n-6 (0.2%) (Table 2). The lipid emulsion contained high levels of 20:5n-3 (40.9%) and 22:6n-3 (9.4%). The three fatty acids, 20:4n-6, 20:1n-9 and 18:0, which were present at very low levels in T-Iso, 0.2%, 0.1%, and 0.5%, respectively, were found in fairly high proportions in the lipid emulsion (2.2%, 3.7% and 3.9% respectively). The ratio of n-3/n-6 fatty acids was also higher in the lipid emulsion (13.9) than in T-Iso (8.3).

Fatty acid composition of gill polar lipids

The fatty acid composition of gill polar lipids changed significantly after 2 weeks of dietary conditioning and the change intensified toward the end of the experiment (Table 3; 2-way ANOVA, $p<0.0001$). During the whole experiment, 20:5n-3 content was maintained in gills of oysters fed 50% lipid emulsion, but this fatty acid decreased significantly in gills of oysters fed the other diets (2 way-ANOVA, $p<0.0001$). Consequently, at the end of the experiment, 20:5n-3 content of gill polar lipids was positively correlated with the levels of lipid supplements. While the 20:4n-6 content of gill polar lipids of oysters fed T-Iso alone or supplemented with 1% lipid emulsion was stable over the duration of the experiment; 20:4n-6 content of oysters fed 10% and 50% lipid emulsion increased (2-way ANOVA, $p<0.0001$) to become significantly higher than those of oysters fed T-Iso alone or T-Iso supplemented with 1% lipid emulsion at the end of the experiment (2-way ANOVA, $p<0.0001$). As a consequence, 20:5n-3/20:4n-6 ratio decreased more quickly in gills of oyster fed T-Iso alone and 1% lipid emulsion than in those fed 10% and 50% lipid emulsion.
At the end of the experiment, some other individual fatty acids in gill polar lipids also changed significantly according to dietary treatment, including 18:0, 18:2n-6, 18:3n-3, 22:5n-6, 22:5n-3 and

22:6n-3 (Table 3). The 18:2n-6, 18:3n-3, 22:5n-6 and 22:6n-3 contents of gill polar lipids was significantly higher in oysters fed T-Iso alone and supplemented with 1% lipid emulsion than those fed 10% and 50% lipid emulsion, whereas 18:0 and 22:5n-3 contents increased significantly in oysters fed 10% and 50% lipid emulsion. However, the total proportion of saturated, mono- and polyunsaturated fatty acids in gill polar lipids did not change due to dietary treatment (2-way ANOVA, $p>0.05$).

Oyster dry tissue weight

There were no significant differences in oyster dry tissue weight (DW) among dietary treatments (Table 4; 2-way ANOVA, $p>0.05$), but DW of oysters significantly decreased in the first 2 weeks of the experiment, especially for oysters fed T-Iso supplemented with 1% and 10% lipid emulsion (2-way ANOVA, $p< 0.0001$). By the end of dietary conditioning, oysters regained their original dry weight.

Biochemical composition

No differences in carbohydrate content were observed among dietary treatments (Figure 1A, 2-way ANOVA, $p<0.05$). However, oysters fed T-Iso alone had the highest carbohydrate content (0.20 mg mg^{-1} of DW) at the end of the experiment and those supplemented with 50% lipid emulsion had the lowest (0.15 mg mg^{-1} of DW). A significant time effect was also observed for carbohydrate content and it was mainly due to an initial decrease in carbohydrate content after the first 2 weeks of feeding (2-way ANOVA, $p<0.0001$), followed by an increase to higher levels at the end of the experiment.
Total lipid content in oysters was significantly influenced by diet (2-way ANOVA, $p<0.0001$). Oysters fed 50% lipid emulsion had higher lipid content compared with oysters fed the other diets (Figure 1B, 2-way ANOVA, $p<0.0001$), especially after 4 weeks of conditioning. However, by the end of the experiment, the dietary treatment effect disappeared. The total lipid content of oysters fed T-Iso alone, or supplemented with 1% and 10% lipid emulsion reached the same level as that of oysters fed T-Iso supplemented with 50% lipid emulsion.
Dietary treatment had no effect on protein content (Figure 1C, 2-way ANOVA, $p>0.05$), but a significant time effect was evident (2-way ANOVA, $p<0.01$) with higher protein content in oysters after 2 weeks and 4 weeks of dietary conditioning compared to those at the beginning and end of the experiment.

Hemocyte concentrations

Total hemocyte and hyalinocyte (the main hemocyte population) concentrations were significantly affected by dietary treatment (Figure 2A and B, 2-way ANOVA, $p<0.001$).
Oysters fed a supplement of 10% and 50% lipid emulsion had higher hemocyte and hyalinocyte concentrations than those fed T-Iso supplemented with 1% of lipid emulsion or T-Iso alone (Figure 2A and B; 2-way ANOVA, $p<0.001$), especially after 4 weeks of dietary conditioning. Moreover, a significant time effect was evident for total hemocyte, granulocyte and hyalinocyte concentrations (2-way ANOVA, $p<0.0001$). Total hemocyte and hyalinocyte concentrations slightly but significantly decreased after the first two weeks, then significantly increased after 4 weeks and remained at the same levels throughout the experiment. The granulocyte concentrations followed a similar trend except that granulocyte concentration remained the same after the first two weeks of feeding (Figure 2C).

Hemocyte viability

Hemocyte viability was not affected by dietary conditioning (2-way ANOVA, $p>0.05$), but a significant time effect was detected (2-way ANOVA, $p<0.001$). Hemocyte viability was significantly lower after 4 weeks of dietary conditioning (89.1%) compared to an average of 92.3% of viability in hemolymph samples (i.e. 7.7% dead cells) collected at other sampling times (data not shown).

Phagocytosis assay

Phagocytic index was significantly affected by the dietary conditioning (Figure 3; 2-way ANOVA, $p<0.01$). Differences were most apparent among treatments after 4 weeks of dietary conditioning; hemocytes of oysters fed 1% lipid emulsion had the highest phagocytic index (0.7), followed by those fed T-Iso alone; whereas, the phagocytic index of hemocytes of oysters fed 10% and 50% lipid emulsion were low (0.2). A significant time effect was observed (2-way ANOVA, $p<0.0001$). The

phagocytic index of oysters fed T-Iso alone or T-Iso supplemented with 1% lipid emulsion increased significantly from 0.2 to 0.6, after 4 weeks of feeding. However, the phagocytic index in oysters fed 10 and 50% lipid emulsion took longer to increase but eventually reached similar values to those of other treatments after 7 weeks of conditioning.

Reactive oxygen species (ROS) production in non-stimulated hemocytes

ROS production in non-stimulated granulocytes and hyalinocytes was significantly different among dietary treatments (2 way-ANOVA, $p<0.01$ and 0.05 respectively) and among sampling times (2-way ANOVA, $p<0.0001$) with an 180 min incubation assay (Figure 4). Differences were primary noted at the end of the experiment after 7 weeks of conditioning. Granulocytes and hyalinocytes of oysters fed T-Iso alone and supplemented with 1% lipid emulsion had higher ROS (average of 160 A.U. and 110 A.U. respectively) compared to those of oysters fed 10% and 50% lipid emulsion (30.5 A.U. and 72 A.U., respectively).
Intriguingly, significant morphological changes were observed in granulocytes and hyalinocytes from oysters fed 10% and 50% lipid emulsion at the end of the experiment. A significant decrease of size and complexity of granulocytes was reported between the 4 and 7 week of dietary treatment (Figure 5, 1-way ANOVA, $p<0.0001$ respectively). Similar changes were noticed for hyalinocytes (data not shown).

Discussion

Impact of dietary conditioning on fatty acid profile of gill polar lipids

Oysters supplemented with 10% and 50% lipid emulsion maintained 20:5n-3 and increased 20:4n-6 contents. Apparently oysters consumed and metabolized the lipid emulsion and supplementary feeding oysters with 10% and 50% lipid emulsion compensated for a deficiency of 20:5n-3 in T-Iso. The alga T-Iso contained very low levels of 20:5n-3 and 20:4n-6, 1.9% and 0.2% respectively. At the end of the experiment, the highest level of 20:5n-3 (15.3%) in gill polar lipids was in the group fed T-Iso supplemented with 50% lipid emulsion while the 20:5n-3 content in oysters fed T-Iso alone almost halved, from the initial 14.8% to 7.5%. Caers et al. (2000a, 2002) achieved a similar pattern of enrichment for 22:6(n-3) by feeding oyster spat or broodstock a 22:6n-3 deficient microalgae (T. suecica or Dunalliella tertiolecta, respectively) supplemented with 50% lipid emulsion rich in 22:6n-3.

Impact of dietary conditioning on biochemical composition of oysters

High doses of lipid supplements (10% and 50% emulsion) appeared to accelerate and/or increase gonadal development as lipid contents in oysters fed 10% or 50% emulsion were higher than in oysters fed T-Iso alone or supplemented with 1% lipid emulsion after 4 and 7 weeks feeding. Increased reproductive effort in oysters fed on 10% and 50% lipid emulsions was also indicated by spontaneous spawning of these oysters during the 4 to 7 week dietary conditioning period. The effect of high doses of lipid emulsion on gametogenesis is possibly related to the additional dietary energy provided by the lipid emulsion. Enriquez-Diaz (2004) and Delaporte et al. (2006a) demonstrated that at the stage of active gametogenesis an increase of food supply from 4 to 12% algal dry weight per oyster dry tissue weight in C. gigas was almost exclusively invested in gonadal development and little devoted to energy storage. Soudant et al. (1999) observed in a field experiment that gametogenesis of C. gigas was associated with preferential accumulation of 20:5n-3 in gonad tissues. Lipid emulsions rich in 22:6n-3 was previously used to enhance gametogenesis of Argopecten purpuratus (Caers et al., 1999, 2002). Furthermore, addition of n-3 PUFA-enriched lipid emulsion to a microalgal diet during broodstock conditioning of the baltic tellinid *Macoma balthica* resulted in greater fecundity and response to spawning induction than broodstock receiving a standard algal diet (Hendricks et al., 2003).

Impact of dietary conditioning on hemocyte parameters

In the present study, differences in hemocyte parameters due to dietary treatment were observed. The increase in hemocyte concentration, mainly due to an increase in hyalinocytes, in oysters supplemented with 50% lipid emulsion appeared to be similar to that observed with R. philippinarum and C. gigas after 8 weeks of feeding on C. calcitrans, which contains higher levels of 20:5n-3 (17.8%)

and 20:4n-6 (2.0%) compared to T-Iso or T. suecica (Delaporte et al., 2003). However, it is difficult to attribute this increase in hemocyte concentration only to the 20:5n-3 PUFA since a concomitant increase of 20:4n-6 was also noticed during the experiment. Indeed, Delaporte et al. (2006b) demonstrated that a dietary supply of 20:4n-6 to oysters also favoured an increase of hemocyte concentration. The later decrease of hemocyte concentration from 4 to 7 weeks in the treatment group supplemented with 50% lipid emulsion is possibly a result of spawning in some of the oysters before hemolymph sampling. Cho and Jeong (2005) observed a decrease in hemocyte concentration in oysters that have been induced to spawn before hemolymph withdrawal. Indeed, more investigations are needed to specifically understand the effect of dietary 20:5n-3 on changes in oyster hemocyte concentrations, independently of the reproduction process.

After 4 weeks, phagocytic activity of oysters fed 0, or 1% lipid emulsion increased drastically while those of oysters fed 10% and 50% lipid emulsion remained low. This observation is similar to those reported for humans. Eicosapentaenoic acid (20:5n-3) was shown to inhibit phagocytic activity of human neutrophils (Sipka et al., 1996; Kew et al., 2004) and leukocytes (Virella et al., 1989), and natural killer cell activity (Peterson et al., 1998; Thies et al., 2001). Nevertheless, the putative inhibitory effect of 20:5n-3 on phagocytic activity disappeared at the end of the experiment although oysters still maintained a similar level of 20:5(n-3) in the gill polar lipids. This may be due to the increase in the content of other long chain PUFAs, especially 20:4n-6 in hemocyte membranes, indicated by the increase of 20:4n-6 in gill lipids of these oysters. The effect of 20:4n-6 and its metabolite (leukotriene LTB_4) in increasing phagocytic activity has been described in vertebrate studies (Bailie et al., 1996; Mancuso et al., 1998; Lennartz, 1999). Also, Delaporte et al. (2006b) showed a temporary increase of phagocytic activity in oysters provided with 20:4n-6 supplementation. Moreover, the balance between n-3 and n-6 FAs, especially between 20:5n-3 and 20:4n-6, might have affected oyster hemocyte activity (e.g., such as phagocytic activity). Horrobin et al. (2002) and Harbige (2003) reported that for vertebrates, an optimal balance between n-3 and n-6 FA, may ensure effective immunological responses against pathogens.

Additionally, the reproductive processes in oysters might have affected hemocyte phagocytic activity since spawning occurred in some of the oysters supplemented with 10% or 50% lipid emulsion. Indeed, Delaporte et al. (2006a) showed a decrease of hemocyte phagocytic activity in C. gigas during gametogenesis. Hormonal changes related to reproductive processes may affect defence mechanisms of maturing animals. Yamaguchi et al. (2001) and Watanuki et al. (2002) reported in vitro and in vivo inhibition of phagocytic activity in carp Cyprinus carpio by estradiol, progesterone and 11-ketotestoterone. Thus, the cause of variation in phagocytic activity during the course of the dietary conditioning is not clearly understood and remains to be investigated.

The observed significant dietary effect on non-stimulated ROS production by hemocytes after 180 minutes of incubation at the end of the experiment may reflect a differential competence of hemocytes to produce ROS. Morphological changes (relative size and complexity of cells i.e. respectively the FSC and SSC measured by FCM) were observed in hemocyte populations of oysters fed 10% and 50% of lipid emulsion concomitantly with the decrease ROS production between the two measurements (120 and 180 min; data not shown). It is not known whether or not the differences in non-stimulated hemocyte ROS production and in cell morphology are really due to changes in fatty acid composition of the hemocyte membranes. In vertebrates, n-3 PUFAs have been showed to have a negative effect on ROS production (Fisher et al., 1990; Varming et al., 1995; Calder, 2001) while 20:4n-6 and its eicosanoid metabolites positively modulated NADPH-oxydase activity (Lennartz, 1999; Mazières et al., 1999; Calder, 2001). The later activity is involved in the synthesis of superoxide anion. Thus, we speculate that changes in ROS production in non-stimulated hemocytes may be a result of the change in the ratio 20:5n-3 / 20:4n-6 or in eicosanoids derived from both PUFAs. However, further study is required to identify the exact causes responsible for variation in all the studied hemocyte parameters during this dietary conditioning.

Conclusion

In conclusion, this study demonstrated that high amounts of 20:5n-3 PUFAs supplied by lipid emulsion appeared to enhance hemocyte concentration, but had a temporary suppressive effect on phagocytosis activity and non-stimulated ROS production. It is possible that 20:5n-3 may affect hemocyte parameters by changing membrane properties or by acting as a competitive inhibitor of 20:4n-6 for eicosanoid production and NADPH-oxydase activation. Reproduction processes in oysters might have also interfered with the dietary treatment effect on the measured hemocyte parameters in the present study. Future study should be directed to (1) discriminate the specific effects of 20:5n-3

and 20:4n-6 and interaction effects between these two fatty acids on immune functions of oysters and to (2) distinguish dietary from reproductive effects.

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Table 1: Fatty acid composition of T-Iso and the lipid emulsion used in the experiment. Results are expressed as percentage of total fatty acids (n=3, Mean ± SD).

	T-Iso	**Emulsion**
14:0	11.2 ± 1.4	0.4 ± 0.0
16:0	10.4 ± 0.8	6.3 ± 0.0
18:0	0.5 ± 0.1	**3.9 ± 0.1**
16:1n-7	3.3± 0.2	1.4 ± 0.1
18:1n-9	12.0 ± 0.3	7.5 ± 0.1
18:1n-7	1.3 ± 0.2	4.5 ± 0.0
20:1n-9	0.1 ± 0.0	**3.7 ± 0.1**
18:2n-6	3.4 ± 0.3	0.9 ± 0.0
18:3n-3	6.2 ± 0.5	0.7 ± 0.0
18:4n-3	18.6 ± 1.5	3.2 ± 0.0
20:4n-6	0.2 ± 0.0	**2.2 ± 0.0**
20:5n-3	1.9± 0.3	**40.9 ± 0.0**
22:5n-6	1.9 ± 0.2	0.3 ± 0.0
22:5n-3	0.3 ± 0.0	2.4 ± 0.0
22:6n-3	18.0 ± 1.6	9.4 ± 0.0
Total SFAs	22.5 ± 1.2	12.0 ± 0.2
Total MUFAs	18.0 ± 0.5	21.8 ± 0.0
Total PUFAs	59.2 ± 1.4	66.1 ± 0.2
n-3/n-6	8.3 ± 0.2	13.9 ± 0.2
22:6n-3/20:5n-3	9.6 ± 2.2	0.2 ± 0.0
22:5n-6/20:4n-6	10.8± 2.1	0.1 ± 0.0

SFAs = Saturated Fatty Acids, MUFAs = Mono-unsaturated Fatty Acids, PUFAs = Polyunsaturated Fatty Acids

Table 2: Summary of the 2-way ANOVA performed on biochemical and hemocyte parameters.

	Statistical effects		
	Dietary treatment	**Sampling date**	**Interaction**
Parameters			
Biochemical parameters			
Oyster dry weight (DW)	NS	****	NS
Carbohydrate content (mg/mg DW)	NS	****	NS
Lipid content (mg/mg DW)	****	****	*
Protein content (mg/mg DW)	NS	**	NS
Hemocyte concentrations			
Total hemocyte concentration (cells/mL)	***	****	NS
Hyalinocyte concentration (cells/mL)	***	****	NS
Granulocyte concentration (cells/mL)	NS	****	NS
Viability (%)	NS	***	NS
Hemocyte activities			
Phagocytic activity	**	****	NS
ROS production of granulocytes (A.U., 120 min.)	NS	NS	NS
ROS production of hyalinocytes (A.U., 120 min.)	NS	*	NS
ROS production of granulocytes (A.U., 180 min.)	**	****	*
ROS production of hyalinocytes (A.U., 180 min)	*	****	**

NS = non significant, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, NA = non available

Table 3 : Fatty acid composition of gill polar lipids (expressed as weight percentage of total fatty acids of the fraction), total FAs (expressed as µg of FAs per mg of gill DW) and percentage of polar lipids (corresponding to the proportion of FAs associated to the polar lipid fraction) of C. gigas during dietary conditioning. Small letters indicate significant differences among dietary treatments (ANOVA, mean ± SD).

		After 2 weeks				After 4 weeks				After 7 weeks			
	Initial	**0%**	**1%**	**10%**	**50%**	**0%**	**1%**	**10%**	**50%**	**0%**	**1%**	**10%**	**50%**
	(n=6)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)
14:0	0.6 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	1.1 ± 0.1^{a}	1.0 ± 0.1^{ab}	0.9 ± 0.1^{bc}	0.7 ± 0.1^{c}	1.2 ± 0.1^{a}	1.4 ± 0.4^{a}	1.3 ± 0.3^{a}	0.7 ± 0.1^{b}
16:0	9.9 ± 0.3	9.6 ± 0.3	10.1 ± 0.2	9.8 ± 0.5	9.7 ± 0.2	10.0 ± 0.5	10.5 ± 0.3	10.3 ± 0.5	10.5 ± 0.4	10.7 ± 1.0	11.9 ± 1.1	11.7 ± 0.3	11.1 ± 0.4
18:0	5.4 ± 0.4	5.0 ± 0.2	5.1 ± 0.3	5.6 ± 0.0	5.0 ± 0.4	5.0 ± 0.1^{a}	5.0 ± 0.2^{a}	5.4 ± 0.3^{ab}	5.7 ± 0.4^{b}	3.4 ± 0.2^{a}	4.0 ± 0.0^{ab}	4.2 ± 0.3^{b}	5.5 ± 0.1^{c}
16:1n-7	1.3 ± 0.4	1.7 ± 0.7	1.7 ± 1.0	1.5 ± 0.7	1.6 ± 0.2	1.7 ± 0.5	1.8 ± 0.3	0.9 ± 0.3	1.3 ± 0.2	1.9 ± 0.9	2.4 ± 0.5	2.2 ± 0.2	1.3 ± 0.9
18:1n-9	1.8 ± 0.1	2.5 ± 0.4	2.1 ± 0.2	2.2 ± 0.3	2.4 ± 0.1	2.7 ± 0.2	2.9 ± 0.2	2.6 ± 0.2	2.7 ± 0.3	3.5 ± 0.1	3.6 ± 0.1	3.4 ± 0.2	3.0 ± 0.2
18:1n-7	5.2 ± 0.2	5.6 ± 0.3^{a}	5.0 ± 0.4^{b}	5.4 ± 0.2^{ab}	6.2 ± 0.2^{c}	4.7 ± 0.4^{a}	4.8 ± 0.1^{a}	5.3 ± 0.2^{b}	5.8 ± 0.2^{b}	4.8 ± 0.1^{a}	4.4 ± 0.6^{a}	5.1 ± 0.0^{a}	5.7 ± 0.2^{b}
20:1n-11	3.3 ± 0.3	3.2 ± 0.3^{a}	3.3 ± 0.3^{a}	3.5 ± 0.1^{a}	2.7 ± 0.2^{b}	3.1 ± 0.2	3.0 ± 0.2	3.0 ± 0.3	2.8 ± 0.1	2.5 ± 0.3	2.6 ± 0.1	2.6 ± 0.0	2.5 ± 0.1
20:1n-9	1.6 ± 0.2	2.2 ± 0.3^{ab}	1.9 ± 0.2^{a}	2.3 ± 0.1^{b}	2.9 ± 0.1^{c}	2.7 ± 0.4	2.9 ± 0.1	3.5 ± 0.0	3.4 ± 0.1	3.0 ± 0.2	3.1 ± 0.3	3.3 ± 0.0	3.6 ± 0.1
20:1n-7	5.4 ± 1.5	6.1 ± 0.2	6.2 ± 0.3	6.2 ± 0.3	5.6 ± 0.2	6.2 ± 0.3^{a}	5.9 ± 0.0^{a}	6.0 ± 0.2^{a}	5.3 ± 0.2^{b}	5.2 ± 0.3	5.2 ± 0.2	4.9 ± 0.2	5.3 ± 0.4
18:2n-6	0.6 ± 0.1	0.8 ± 0.1^{a}	0.7 ± 0.0^{b}	0.6 ± 0.0^{b}	0.6 ± 0.0^{b}	0.8 ± 0.0^{a}	0.8 ± 0.1^{a}	0.7 ± 0.1^{b}	0.5 ± 0.0^{c}	1.1 ± 0.1^{a}	1.0 ± 0.1^{a}	0.8 ± 0.0^{b}	0.5 ± 0.0^{c}
18:3n-3	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.6 ± 0.0^{a}	0.6 ± 0.1^{a}	0.5 ± 0.0^{b}	0.3 ± 0.0^{c}
18:4n-3	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	0.9 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.6 ± 0.6	1.5 ± 0.1	1.2 ± 0.3	1.0 ± 0.4
20:2i	0.8 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.0^{a}	0.4 ± 0.1^{b}	0.5 ± 0.0^{b}	0.4 ± 0.0^{b}	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
20:2j	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.1^{a}	0.4 ± 0.0^{b}	0.6 ± 0.2	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0
20:2n-6	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
20:4n-6	3.8 ± 0.1	3.9 ± 0.2^{a}	4.0 ± 0.0^{a}	4.1 ± 0.1^{ab}	4.3 ± 0.2^{b}	4.2 ± 0.0^{a}	4.3 ± 0.0^{a}	4.9 ± 0.2^{b}	4.9 ± 0.2^{b}	4.0 ± 0.2^{a}	3.9 ± 0.1^{a}	4.8 ± 0.1^{b}	5.5 ± 0.6^{c}
20:5n-3	14.8 ± 1.4	12.3 ± 1.4^{a}	13.8 ± 0.4^{a}	13.9 ± 0.7^{a}	15.8 ± 0.6^{b}	10.0 ± 0.6^{a}	9.9 ± 0.7^{a}	12.4 ± 0.6^{b}	15.5 ± 0.7^{c}	7.5 ± 0.3^{a}	9.3 ± 0.1^{b}	11.6 ± 0.4^{c}	15.3 ± 0.4^{d}
22:2i	2.0 ± 0.2	2.5 ± 0.3	2.2 ± 0.2	2.6 ± 0.2	2.7 ± 0.2	3.2 ± 0.2^{a}	3.4 ± 0.2^{a}	3.8 ± 0.2^{b}	3.4 ± 0.1^{a}	3.9 ± 0.4	3.9 ± 0.6	4.0 ± 0.1	3.8 ± 0.1
22:2j	11.0 ± 0.8	10.7 ± 0.5	10.7 ± 0.8	11.2 ± 0.3	9.7 ± 0.6	10.7 ± 0.5^{a}	10.3 ± 0.2^{a}	9.9 ± 0.5^{a}	8.5 ± 0.5^{b}	8.6 ± 0.7	8.6 ± 0.6	7.7 ± 0.4	7.4 ±0.5
22:5n-6	1.9 ± 0.4	2.5 ± 0.2^{a}	2.2 ± 0.1^{ab}	2.1 ± 0.2^{b}	1.8 ± 0.0^{c}	2.9 ± 0.2^{a}	2.9 ± 0.2^{a}	2.5 ± 0.0^{b}	1.8 ± 0.1^{c}	3.6 ± 0.5^{a}	3.1 ± 0.0^{ab}	2.7 ± 0.1^{b}	1.7 ± 0.1^{c}
22:5n-3	1.5 ± 0.1	1.2 ± 0.1^{a}	1.3 ± 0.1^{a}	1.3 ± 0.1^{a}	1.8 ± 0.1^{b}	1.0 ± 0.2^{a}	1.1 ± 0.1^{a}	1.4 ± 0.1^{b}	2.0 ± 0.1^{c}	0.8 ± 0.0^{a}	0.9 ± 0.1^{a}	1.3 ± 0.0^{b}	2.0 ± 0.2^{c}
22:6n-3	19.3 ± 1.2	19.0 ± 0.4^{a}	18.4 ± 0.8^{a}	17.0 ± 0.4^{b}	16.7 ± 0.4^{b}	19.5 ± 0.3^{a}	18.8 ± 0.6^{a}	17.7 ± 0.5^{b}	16.2 ± 0.6^{c}	20.4 ± 1.8^{a}	19.3 ± 0.1^{a}	18.1 ± 0.6^{ab}	16.0 ± 0.6^{b}

SFAs	18.6 ± 0.3	17.4 ± 0.3	17.9 ± 0.6	18.2 ± 0.5	17.2 ± 0.2	18.3± 0.8	18.6 ± 0.5	18.5 ± 0.8	18.5 ± 0.6	17.2 ± 1.3	19.3 ± 1.4	18.9 ± 0.4	19.0 ± 0.7
MUFAs	20.2 ± 1.7	23.5 ± 1.8	22.4 ± 1.8	23.0 ± 1.4	23.4 ± 0.4	23.5 ± 1.2	23.9 ± 0.7	22.3 ± 1.1	23.3 ± 0.8	23.1 ± 1.9	23.5 ± 0.4	24.0 ± 0.4	23.7 ± 2.1
PUFAs	60.2 ± 1.5	58.2 ± 1.7	58.6 ± 2.5	57.8 ± 1.5	58.7 ± 0.4	57.4 ± 0.4	56.5 ± 0.4	58.5 ± 0.2	57.6 ± 1.3	59.0 ± 3.4	56.3 ± 1.2	56.1 ± 0.0	56.9 ± 2.5
Total n-6	7.2 ± 0.5	8.3 ± 0.5	8.1 ± 0.1	7.9 ± 0.5	7.7 ± 0.2	9.1 ± 0.2 [a]	9.2 ± 0.2 [a]	9.3 ± 0.3 [a]	8.4 ± 0.2 [b]	10.1 ± 0.6	9.9 ± 0.2	9.7 ± 0.6	9.2 ± 0.5
Total n-3	38.6 ± 1.6	35.2 ± 1.9	36.2 ± 1.5	34.7 ± 1.6	37.3 ± 0.8	33.2 ± 0.7 [ab]	32.6 ± 0.2 [a]	34.3 ± 0.5 [b]	36.3 ± 1.1 [c]	35.1 ± 4.4	32.8 ± 0.3	33.5 ± 1.2	35.4 ± 1.5
n-3/n-6	5.4 ± 0.5	4.2 ± 0.5	4.5 ± 0.2	4.4 ± 0.4	4.9 ± 0.1	3.7 ± 0.1 [a]	3.6 ± 0.1 [a]	3.7 ± 0.1 [a]	4.3 ± 0.1 [b]	3.5 ± 0.6 [a]	3.3 ± 0.0 [a]	3.5 ± 0.3 [a]	3.9 ± 0.1 [b]
20:5n-3/20:4n-6	3.8 ± 0.4	3.1 ± 0.3 [a]	3.4 ± 0.1 [ab]	3.4 ± 0.1 [ab]	3.7 ± 0.2 [b]	2.4 ± 0.2 [a]	2.3 ± 0.1 [a]	2.5 ± 0.0 [a]	3.2 ± 0.1 [b]	1.9 ± 0.2 [a]	2.4 ± 0.1 [b]	2.4 ± 0.1 [b]	2.8 ± 0.2 [c]
Total FAs	35.2 ± 5.8	26.8 ± 0.6 [a]	32.3 ± 5.4 [ab]	33.3 ± 5.0 [ab]	39.4 ± 4.5 [b]	28.1 ± 3.0 [a]	27.0 ± 0.4 [a]	32.8 ± 1.2 [b]	44.7 ± 2.9 [c]	31.9 ± 5.3	32.6 ± 1.5	33.8 ± 2.8	38.5 ± 6.6
% polar lipids	69.4 ± 8.4	69.7 ± 2.6	64.3 ± 1.5	69.1 ± 4.3	64.6 ± 3.0	64.7 ± 2.0	68.1 ± 1.7	68.7 ± 3.8	59.5 ± 8.6	71.7 ± 5.5	65.9 ± 6.1	65.5 ± 0.6	71.9 ± 8.3

SFAs = Total Saturated Fatty Acids; MUFAs = Total Monounsaturated Fatty Acids; PUFAs = Total Polyunsaturated Fatty Acids; Non Methylene Interrupted Fatty Acids: 20:2i = 20:2Δ5,11 ; 22:2i = 22:2Δ7,13 ; 20:2j = 20:2Δ5,13; 22:2j = 22:2Δ7,15 ; DW: dry weight.

Table 4 : Oyster tissue dry weight (expressed in g, n=8, Mean ± SD) of oysters fed T-Iso supplemented with lipid emulsion at 0%, 1%, 10% and 50% of algal dry weight. Small letters indicate significant difference between dietary treatments.

		Initial	2 weeks	4 weeks	7 weeks
Oyster dry weight	**0%**	0.51 ± 0.15^{ab}	0.46 ± 0.16^{ab}	0.40 ± 0.13^{a}	0.56 ± 0.14^{b}
	1%	0.55 ± 0.15^{a}	0.41 ± 0.17^{b}	0.43 ± 0.11^{b}	0.58 ± 0.17^{a}
	10%	0.57 ± 0.21^{a}	0.42 ± 0.17^{b}	0.47 ± 0.17^{b}	0.55 ± 0.18^{a}
	50%	0.52 ± 0.20^{a}	0.48 ± 0.14^{a}	0.49 ± 0.18^{a}	0.51 ± 0.15^{a}

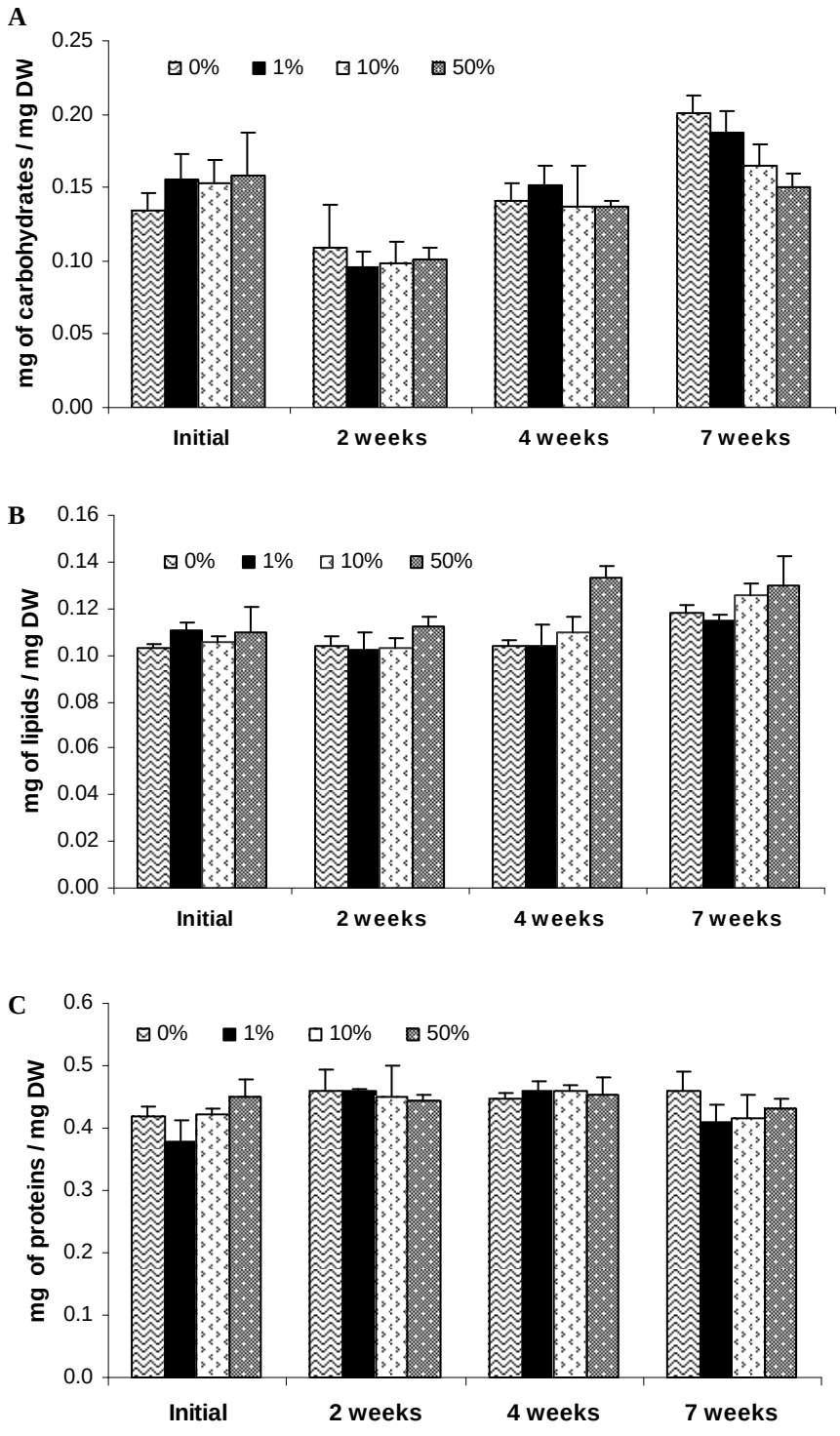

Fig.1: Total carbohydrate (A), lipid (B) and protein (C) contents of oysters fed T-Iso alone or T-Iso supplemented with 1%, 10% and 50% lipid emulsion. Results are expressed as mg of carbohydrates, lipids and proteins per mg of oyster dry weight (n=3, mean ± S.D.).

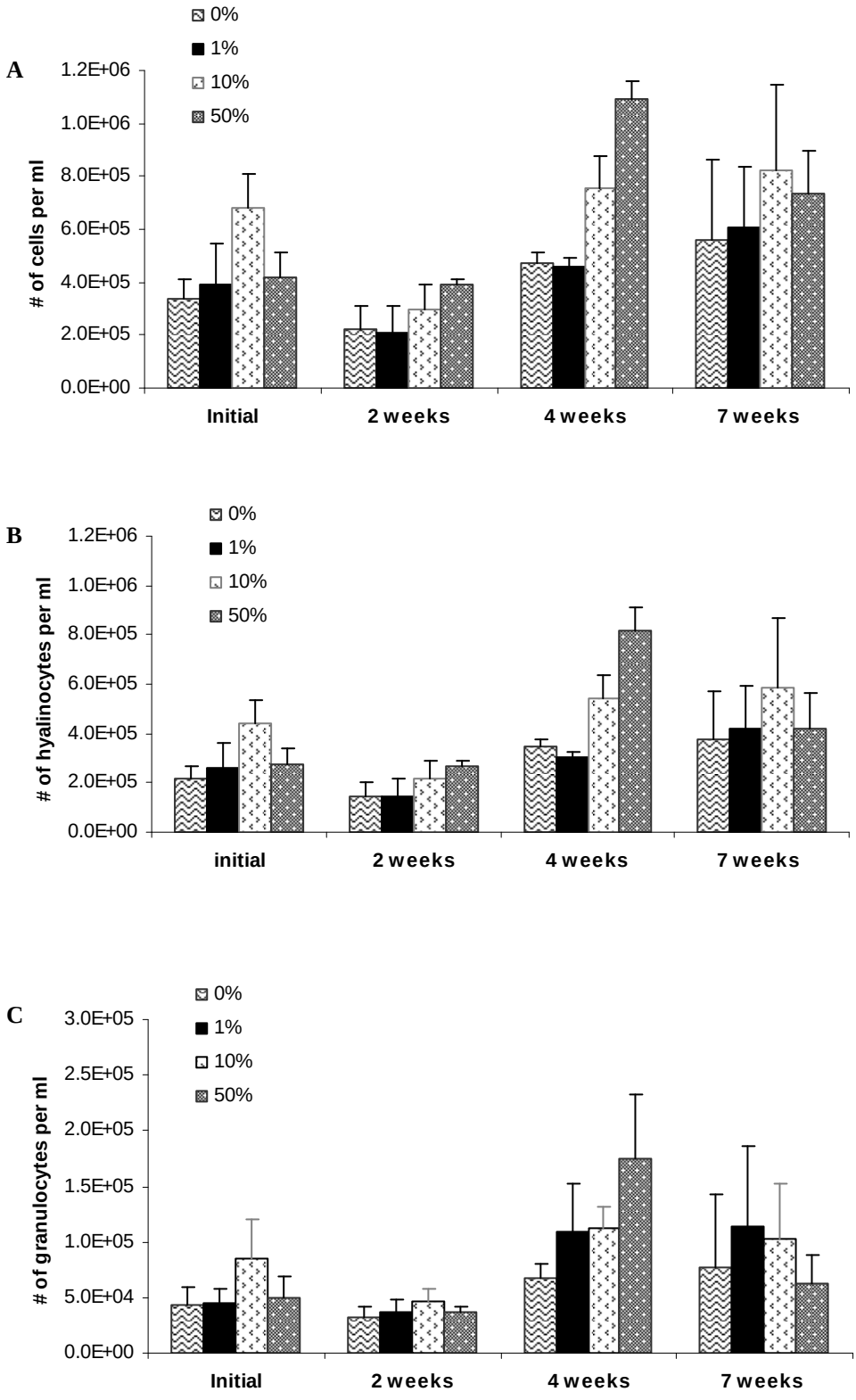

Fig. 2: Total hemocyte (A), hyalinocyte (B) and granulocyte (C) concentrations in hemolymph samples from oysters fed T-Iso alone or T-Iso supplemented with 1%, 10% and 50% lipid emulsion (n=3, mean ± S.D.).

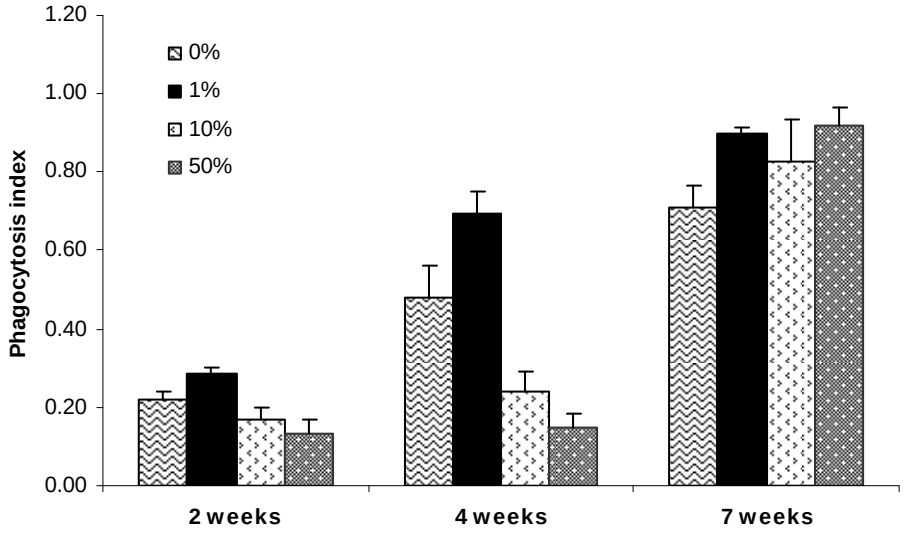

Fig. 3: Phagocytic index of hemocytes of oysters fed T-Iso alone or T-Iso supplemented with 1%, 10% and 50% lipid emulsion. Phagocytic index corresponds to the ratio between the number of hemocytes that engulfed zymosan particles and the total number of hemocytes (n=3, mean ± S.D.).

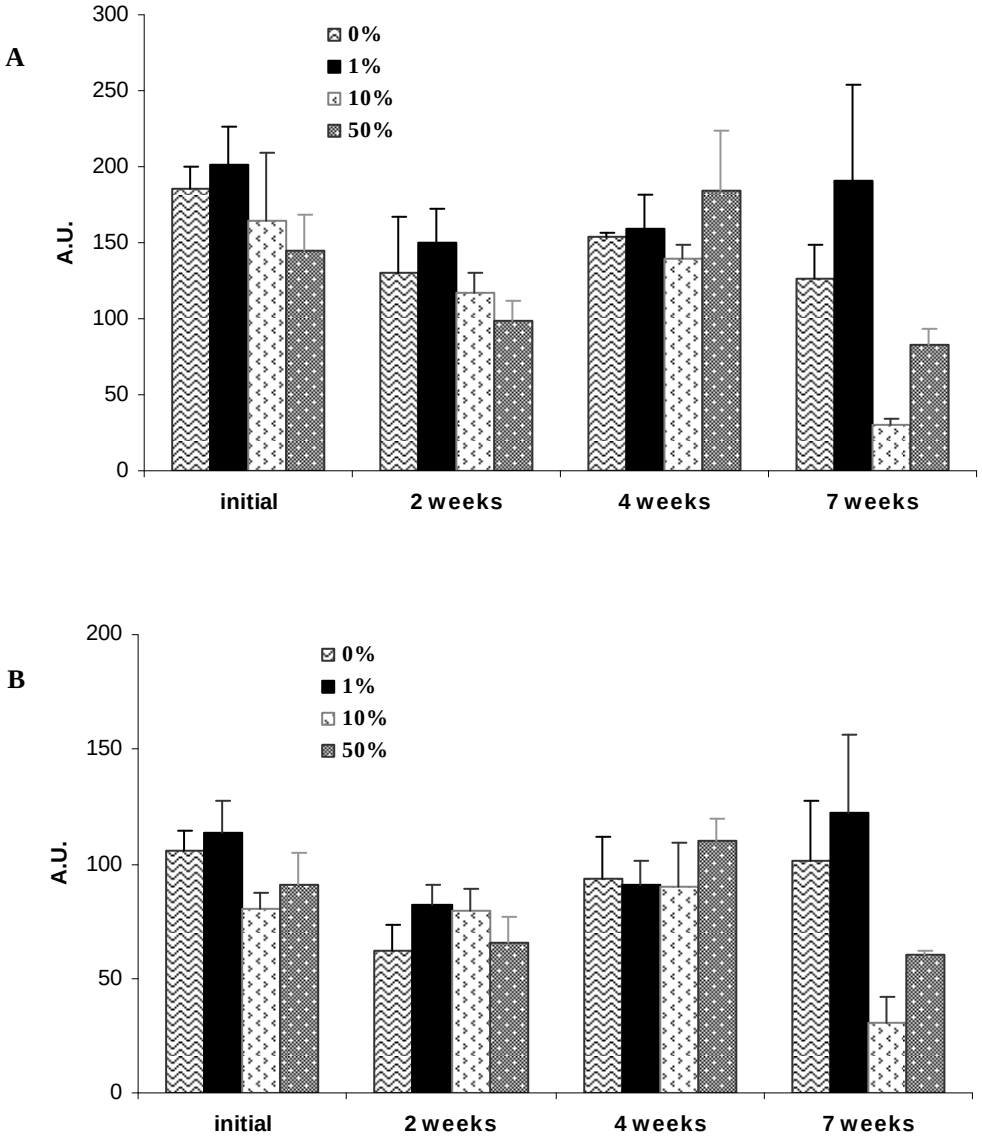

Fig. 4 : Reactive oxygen species production of non-stimulated hemocytes : granulocytes (A) and hyalinocytes (B) of oysters fed T-Iso alone or T-Iso supplemented with 1%, 10% and 50% lipid emulsion after 180 minutes of incubation with DCFH-DA in filtered sterile sea water, expressed in Arbitrary Units (A.U.) (n=3, mean ± S.D.).

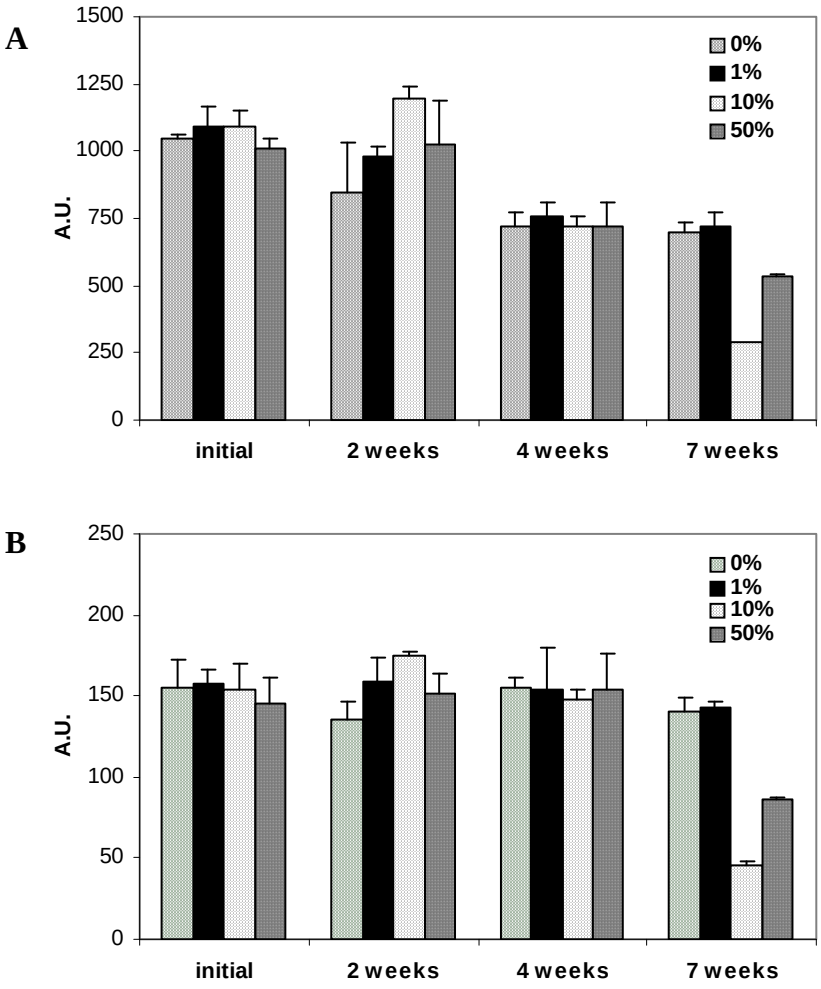

Fig. 5 : Morphological changes of granulocytes after 180 minutes of incubation with DCFH-DA in filtered sterile sea water. Relative complexity (A) and size (B) in Arbitary Units (A.U.) (n=3, mean ± S.D.).