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Effect of a mono-specific algal diet on immune functions in two bivalve species – *Crassostrea gigas* and *Ruditapes philippinarum*

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Summary

The impact of diets upon the fatty acid composition of haemocyte polar lipids and consequently upon immune parameters has been tested in the oyster *Crassostrea gigas* and the clam *Ruditapes philippinarum*. Oysters and clams were fed each of three cultured algae: *Chaetoceros calcitrans*, which is rich in 20:5(n-3) and 20:4(n-6) and poor in 22:6(n-3) fatty acids; T-*Iso* (*Isochrysis* sp.), which is rich in 22:6(n-3) and deficient in 20:5(n-3) and 20:4(n-6); and *Tetraselmis suecica*, which is deficient in 22:6(n-3) and contains only small amounts of 20:5(n-3) and 20:4(n-6).

Fatty acid composition of haemocyte polar lipids was greatly affected by the diet. Oysters and clams fed *C. calcitrans* maintained a higher proportion of 20:5(n-3) and 20:4(n-6) in their haemocyte polar lipids, while these polyunsaturated fatty acids decreased drastically for animals fed T-*Iso*. However, the T-*Iso* diet maintained 22:6(n-3) in haemocyte polar lipids of both species. Higher 20:5(n-3) and 20:4(n-6) contents in diets appeared to have a positive effect upon total haemocyte count, granulocyte percentage, phagocytic rate and oxidative activity of clam haemocytes. Similarly, a positive effect of 20:5(n-3) on oxidative activity of oyster haemocytes was observed but to a lesser extent than in clams. Interestingly, when oyster haemocytes are submitted to a stressful condition, a positive effect of a higher dietary 22:6(n-3) content on the phagocytic rate was noticed.

Key words: bivalve, immunology, nutrition, haemocyte membrane, oxidative activity, phagocytosis, polyunsaturated fatty acids, *Crassostrea gigas*, *Ruditapes philippinarum*.

Introduction

The internal defence system of molluscs consists of both cellular and humoral immunity. The haemocytes, which are the most important cells involved in internal defence, circulate within an open vascular system across all epithelial boundaries and in extrapallial fluids (Paillard et al., 1996; Allam and Paillard, 1998). Several reviews summarize the knowledge about types and functions of mollusc haemocytes (Fisher, 1986; Auffret, 1988; Cheng, 1996; Ford and Tripp, 1996; Chu, 2000). Bivalve haemocytes can be classified using the diversity of their morphology and functions (Cheng, 1996; Chu, 2000). Despite the vast information published, some disagreements remain concerning the number of haemocyte sub-populations. Undoubtedly, these discrepancies arise because of differences in definition among researchers as well as variability with location, season, health status among individuals and methodology. There are, however, some general similarities in cell types among species, and authors generally agree that there are at least two cell types: granulocytes and hyalinocytes (or agranulocytes). Xue (1998) observed the presence of three haemocyte sub-populations for the European flat oyster *Ostrea edulis*: granulocytes, large hyalinocytes and small agranulocytes. These three sub-populations were previously described in *O. edulis* by Auffret (1988) and Chagot (1989). Similar cell types were also noted in the Eastern oyster *Crassostrea virginica* (Ford and Ashton-Alcox, 1993; Cheng, 1996), the carpet-shell clam *Ruditapes decussatus* (López et al., 1997) and Manila clam *Ruditapes philippinarum* (Oubella et al., 1993, 1996; Cima et al., 2000) and the blue mussel *Mytilus edulis* (Pipe et al., 1997). In these studies, the determination and classification of haemocytes were based upon morphology and staining characteristics using light microscopy. To date, studies using flow cytometry to analyse mollusc haemocytes remain scarce, although flow cytometry allows rapid analyses of cell morphology and functions using fluorescent markers.

A few authors have used flow cytometry in the study of haemocyte populations in *C. virginica* (Ford and Ashton-Alcox, 1993; Ashton-Alcox and Ford, 1998), *O. edulis* and Pacific oyster *Crassostrea gigas* (Xue, 1998; Xue et al., 2001). This technique also allows the characterization of haemocyte functions. In bivalves, the first studies on haemocyte activities using flow cytometry were reported by Xue (1998) for *O. edulis* and *C. gigas*. More recently, different tools have been developed for *C. gigas* (Lambert et al., in press) and *R.*

philippinarum and *R. decussatus* (Allam et al., 2001). Two main haemocyte functions can be evaluated using this technique: phagocytosis, which represents probably the most investigated functional aspect of haemocyte activity (Cheng, 1996), and the 'oxidative burst'. Generation of reactive oxygen species at the end of the oxidative burst process has been observed in several bivalves such as *C. gigas* (Bachère et al., 1991; Greger et al., 1995), *C. virginica* (Anderson et al., 1992), *O. edulis* (Hervio et al., 1989; Bachère et al., 1991), *M. edulis* (Pipe, 1992; Noël et al., 1993) and Mediterranean mussel *Mytilus galloprovincialis* (Arumugan et al., 2000) but has not been detected in clam species such as *R. decussatus* (López et al., 1994) and hard shell clam *Mercenaria mercenaria* (Cheng et al., 1975). Also, production of nitrite oxide, another active oxygen intermediate, has been recently demonstrated in *C. gigas* (Nakayama and Maruyama, 1998), *M. edulis* (Ottaviani et al., 1993), *M. galloprovincialis* (Arumugan et al., 2000) and *R. decussatus* (Taffala et al., 2003). Nitrite oxide production is known to be involved in killing foreign microorganisms such as parasites, bacteria and viruses (Moncada et al., 1991; Bogdan, 1997).

Many studies have shown that the quality of the algal diet affects the growth and development of molluscs such as the great scallop *Pecten maximus* (Delaunay et al., 1993; Soudant et al., 1996a, 1998), *C. gigas* (Knauer and Southgate, 1997; Soudant et al., 1999, 2000), *O. edulis* (Berntsson et al., 1997), *R. decussatus* (Albentosa et al., 1996; Fernández-Reiriz et al., 1998, 1999) and *R. philippinarum* (Caers et al., 1999). Dietary conditioning is also important in the resistance of the catfish *Ictalurus punctatus* to a stressful condition (Lingenfelser et al., 1995). Moreover, in vertebrates it is well established that the composition and function of immune cells are influenced by the fatty acid composition of the diet (Peterson et al., 1998; Lennartz, 1999; De Pablo et al., 2000; Sasaki et al., 2000; Kelley and Rudolph, 2000; Calder, 2001). In bivalves, at present, the relationship between immunity and nutrition remains unexplored. The purpose of this study is to assess the impact of mono-algal dietary conditioning upon the fatty acid composition of haemocyte membranes and the immune functions of two species – *C. gigas* and *R. philippinarum* – using flow cytometry. Different immune parameters were measured: haemocyte concentration, viability rate, phagocytic activity, oxidative activity and adhesive capacity.

Materials and methods

Broodstock conditioning

Juveniles of *Crassostrea gigas* Thunberg 1793 were provided by a French commercial hatchery (SATMAR, Lannilis, France) and grown out in Aber Wrach (Finistère, France). Eighteen-month-old oysters reached an average live mass of 25 g. Clams *Ruditapes philippinarum* Adams and Reeve 1850 were collected in the bay of Marennes-Oléron (Charente, France). In March 2001, oysters and clams were maintained in trays that were placed in a 700-litre tank equipped with an air–water lift. They were acclimatized to experimental temperature 12 days prior to the dietary conditioning treatment. Temperature was raised by 1°C per day to a final temperature of 17°C. During the acclimatization period, oysters and clams were fed daily a mixed diet of T-*Iso* (*Isochrysis* sp.) and *Chaetoceros calcitrans*.

After the acclimatization period, oysters and clams were divided randomly and distributed into three tanks. Three mono-algal diets were used to feed animals for two months. Tanks and animals were cleaned weekly. The microalgal species were selected on the basis of their known nutritional quality, especially their polyunsaturated fatty acid composition: *C. calcitrans* is rich in 20:5(n-3) and 20:4(n-6) and deficient in 22:6(n-3); T-*Iso* is rich in 22:6(n-3) and deficient in 20:5(n-3) and 20:4(n-6); and *T. suecica* is deficient in 22:6(n-3) and contained only small amounts of 20:5(n-3) and 20:4(n-6) (Table 1). The daily algal supply was established to provide an equivalent dry mass (DM) supply of each alga: i.e. 0.6×10^9 cells animal^{-1} day^{-1} of *C. calcitrans* and T-*Iso* (DM=20 pg cell^{-1}) and 0.06×10^9 cells animal^{-1} day^{-1} of *T. suecica* (DM=200 pg cell^{-1}). During the experiment, animals were sampled at the initial point and after four weeks and eight weeks of conditioning. At each sampling, three pools of five animals were used for immune analysis, biochemical analysis and condition index measurements and three other pools were used for lipid analysis. Before the sampling, animals were maintained unfed for 24 h. This protocol was adopted to provide sufficient time to remove any algal cells from the haemolymph, which may have interfered with assays.

Table 1. *Fatty acid composition of algae used in the experiment, expressed as percentage of total lipids*

Fatty acid composition	*C. calcitrans*	*T. suecica*	T-*Iso*
14:0	23.0±2.6	0.5±0.1	26.5±3.0
16:0	5.7±1.5	24.9±3.6	11.2±2.5
18:0	0.5±0.1	0.6±0.1	0.5±0.2
16:1(n-7)	20.6±2.5	0.1±0.3	5.5±1.8
18:1(n-9)	0.6±0.1	13.8±7.6	11.2±1.8
18:1(n-7)	0.5±0.1	1.5±0.3	0.7±0.2
16:2(n-7)	1.9±2.5	0.1±0.2	0.1±0.1
16:3(n-4)	10±1.7	0	0.1±0
18:2(n-6)	0.7±0.1	5.7±1.2	7.1±2.7
18:3(n-3)	0	10.5±0.9	5.7±0.9
18:4(n-3)	0.7±0.2	10.7±3.3	12.5±0.9
20:4(n-6)	2.0±0.5	0.5±0.1	0.1±0.0
20:5(n-3)	17.8±2.7	5.4±1.1	0.4±0.1
22:5(n-6)	0.1±0.1	1.5±2.0	2.1±0.4
22:5(n-3)	0.1±0	0.1±0.1	0.1±0.1
22:6(n-3)	1.3±0.2	0.1±0.0	7.8±0.9
Total saturated	29.8±3.2	26.1±3.7	39±4.7
Total monounsaturated	25.5±2.3	20.1±8.3	18.5±2.9
Total polyunsaturated	39.9±4.8	53.2±8.1	40.8±3.1
(n-3)/(n-6)	5.6	4.2	3.0
22:6(n-3)/20:5(n-3)	0.1	0	22.5
22:5(n-6)/20:4(n-6)	0	4.2	19.5

Values are means ± S.D. (N=3).

Condition index

At the start and end of the experiment, the condition index of oysters and clams was measured according to Lawrence and Scott (1982). This index is related to physiological processes (growth or decrease in biological reserve). The condition index was calculated following the formula: dry flesh mass / (total mass – shell mass)×1000.

Haemolymph sampling

Haemolymph samples were withdrawn from the adductor muscle using a 1 ml plastic syringe fitted with a 25-gauge needle. For oyster samples, a notch was made in the shell two days before the bleeding in order to access the muscle without damaging the mantle. Haemolymph samples were stored individually in Eppendorf tubes held on ice. Individual samples were observed under optical microscope to control the quality of the haemolymph bleeding. Haemolymph was then filtered through 80 μm and five individual samples were pooled. Three pools were prepared for each treatment and each species.

Biochemical analysis (lipid, protein and carbohydrate)

Biochemical analyses were performed on three pools of five animals frozen in liquid nitrogen at the beginning and at the end of the experiment. Each pool was ground with a Dangoumeau homogeniser at –180°C and sub-sampled for total lipid, protein and carbohydrate analysis. Samples for dry mass were placed in pre-weighed aluminium cups, dried for 48 h at 80°C and then weighed. Briefly, total lipid contents were estimated according to Bligh and Dyer (1959) after extraction in a dichloromethane–ethanol–water mixture. The purified extract was placed in a pre-weighed Teflon cup, the organic phase was evaporated under a nitrogen stream and the total lipid content was estimated by weighing. Carbohydrate and protein contents were measured colorimetrically as described by Dubois et al. (1956) and Lowry et al. (1951), respectively.

Extraction and preparation of algae and haemocytes for fatty acid analysis

Algae

Algal samples for each diet were filtered onto pre-ignited (450°C) GF/F filters. The filters were then placed in tubes containing a 2:1 v/v mixture of chloroform–methanol and frozen at –20°C under nitrogen. Fatty acid analyses were performed on total lipids.

Haemocytes

Three pools of 4 ml of haemolymph were collected for each treatment and each species. Haemolymph was filtered (60 μm) and centrifuged at 463 ***g*** min^{-1} for 10 min. Supernatant was eliminated and 2 ml of chloroform–methanol mixture (2:1 v/v) was added to the haemocyte pellet. The tube was then sealed under nitrogen and stored at –20°C.

Polar and neutral lipid separation

Neutral and polar lipids of the haemocyte lipid extract were purified on a silica gel microcolumn according to Marty et al. (1992). Briefly, an aliquot of the sample (chloroform–methanol mixture) was evaporated to dryness, and lipids were recovered with three washings of 500 μl each of chloroform–methanol mixture (98:2 v/v). Lipids were placed on top of a silica gel microcolumn [30×5 mm i.d.; Kieselgel; 70–230 mesh (Merck); previously heated to 450°C and deactivated with 5% water]. The neutral lipids were eluted with 10 ml of chloroform–methanol mixture (98:2 v/v). The polar lipids were recovered with 15 ml of methanol. A known amount of 23:0 fatty acid, as internal standard, was added in both neutral and polar fractions.

Esterification

Total lipids of algae and the neutral and polar lipids of haemocytes were transesterified by 10 wt % boron trifluoride/methanol (Metcalfe and Schmitz, 1961) and analysed according to the method described by Marty et al. (1992).

Fatty acid analysis

Briefly, the fatty acid methyl esters (FAME) were analysed in a gas chromatograph with an on-column injector, a DB-Wax (30 m×0.25 mm; 0.25 μm film thickness) capillary column and a flame ionization detector. Hydrogen was used as the carrier gas. The fatty acids were identified by comparing their retention times with those of standards and were checked using a non-polar column (CP Sil 8 CB; 30 m×0.25 mm; 0.25 μm film thickness).

It is generally accepted that polar lipids, mainly comprising phospholipids, provide a good approximation of cell membrane lipids (Soudant et al., 1995, 1997, 1998). Moreover, the neutral lipid fraction always represented less than 10% of the total lipids in haemocytes of both species. Consequently, only the fatty acid composition of the polar lipid fraction from haemocyte lipid extract is presented in this study.

Measurements of the immunological parameters by flow cytometry

Instrumentation

The characterisation of haemocytes was performed on a FACScalibur flow cytometer (Becton-Dickinson, San Diego, CA, USA) equipped with a 488 nm argon laser. The light scattered by the particles indicated their size (FSC, Forward SCatter height) and internal complexity (SSC, Size SCatter height). Fluorescence, whether autofluorescence or from fluorescent dyes, is measured by a specific detector: FL1 for green dye (500–530 nm), FL2 for orange dye (550–600 nm) and FL3 for red dye (>630 nm).

Chemicals and buffer

Anti-aggregant solution for bivalve haemocytes (AASH) was prepared according to Auffret and Oubella (1995): 2.5% NaCl, 1.5% EDTA in 100 mmol l^{-1} phosphate buffer, pH 7.4.

Propidium iodide (IP; Sigma, St Quentin Fallavier, France) working solution: 1 mg IP was dissolved in 1 ml of distilled water, divided into aliquot parts and stored at –20°C.

Cytochalasine B (CK; Sigma) working solution: 1 mg CK was dissolved in 1 ml 100% ethanol, divided into aliquot parts and stored at –20°C.

Phorbol myristate acetate (PMA; Sigma) working solution: 1 mg PMA was dissolved in 1 ml dimethyl sulfoxide (DMSO; 99.5% min), divided into aliquot parts and stored at –20°C.

2′7′-dichlorofluorescein diacetate (DCFH-DA; Sigma) stock solution: a 10 mmol l^{-1} DCFH-DA solution was prepared in DMSO and then diluted to 10% in filtered sterile seawater (FSSW) (working solution 1 mmol l^{-1}).

All the above-described working solutions were used at 1% final concentration (v/v) in all the immunological assays.

Beads: Fluoresbrite microspheres YG 2.0 microns (Polysciences, Eppelheim, Germany) were diluted to 2% in FSSW, divided into portions and stored at 4°C.

Haemocyte viability, total haemocyte and sub-population counts

These three parameters were measured on the same sub-sample. 150 μl sub-samples from each of the three pools previously made were transferred into a tube containing 150 μl anti-aggregant solution (AASH), stored on ice.

Ten minutes before flow cytometry analysis, samples were incubated with IP at a final concentration of 20 μg ml^{-1}. IP is a fluorescent DNA/RNA-specific dye that only permeates through the membranes of dead cells and stains the nucleic acid. The IP fluorescence was measurable on the FL2 detector of the flow cytometer. Mortality rate of haemocytes was calculated by the percentage of haemocytes showing fluorescence; however, results are presented as percentage of viable haemocytes.

The haemocyte concentration was evaluated on the same sub-sample according to the flow rate and the number of counted events during the time of the analysis (usually 30 s). The flow rate was measured daily and showed little variation from day to day (below 3%).

On the oyster cytograms, the identification of haemocyte sub-populations was possible. Three sub-populations could be distinguished by their FSC and SSC: granulocytes, hyalinocytes and small agranulocytes. For the clams, the sub-populations were too close on the cytogram, which prevented their counting determination. Thus, the distinction of forms, numbers and activities of each haemocyte sub-population was possible only for oysters.

Phagocytosis assays

At the end of the experiment, phagocytic activity of haemocytes was tested using fluorescent beads for three conditions of analysis: without pre-incubation and after a pre-incubation of two hours at 18°C or 30°C. These conditions were applied to explore how haemocytes react to a waiting period.

For the first condition, a 100 μl sub-sample of haemolymph, primarily diluted with 100 μl of FSSW, was brought into contact with 30 μl of the working solution of fluorescent beads in Eppendorf tubes. Another equivalent tube was prepared with cytochalasine B (CK), an inhibitor of phagocytic activity, at the final concentration of 10 μg ml^{-1}. Then, after 60 min of incubation at 18°C, haemocytes were fixed with a 6% formalin solution and analysed by flow cytometry (FL1). The paired tubes, with or without CK, were run one after the other. The percentage of fluorescent cells in the tube without CK is attributable to phagocytosis or adhesion whereas the percentage of fluorescent cells in the tube with CK corresponds to adhesion but not to phagocytosis. Phagocytosis rate (true phagocytosis) was thus calculated as the difference in percentage between these paired tubes.

For the second and third conditions, haemocytes were processed as above but were submitted to a pre-incubation of two hours at 18°C or 30°C before the addition of beads.

During the phagocytosis assay, no haemocyte distinctions between sub-populations were realised because of probable changes in haemocyte morphology characteristics (FSC and SSC) caused by the incorporation of fluorescent beads.

Oxidative activity

The method was adapted from Bass et al. (1983) and used 2′7′-dichlorofluorescin diacetate (DCFH-DA). A 150 μl sub-sample of haemolymph was diluted (+150 μl) with FSSW. Simultaneously, another 150 μl sub-sample of haemolymph from the same pooled sample was mixed with 150 μl of FSSW and 3 μl of PMA at the final concentration of 10 μg ml^{-1}. 3 μl of the DCFH-DA working solution was added to each tube maintained on ice to yield a final concentration of 10 μmol l^{-1}. Then, tubes were incubated with DCFH-DA for 20 min at one of two temperatures (18°C or 30°C) in the presence or absence of PMA. During incubation with haemocytes, DCFH-DA diffused into the cells, where the acetate groups (–DA) are removed by esterase, thereby trapping DCFH within the cells. The intracellular DCFH, a non-fluorescent fluorescein analogue, was oxidized to highly fluorescent 2′7′-dichlorofluorescein (DCF; emitted fluorescence at 530 nm) by haemocytes. Intracellular DCFH oxidation is quantitatively related to the oxidative activity of the haemocyte and primarily mediated by H_2O_2 but also by other oxidant molecules such as superoxide anion, peroxyl radical and peroxynitrite anion (Haugland, 2002). DCF production was measured by evaluating the green fluorescence on the FL1 detector (maximal detection wavelength: 550 nm) of the flow cytometer (laser excitation wavelength: 488 nm). The oxidative activity corresponds to the mean geometric fluorescence detected in the total haemocyte population and is expressed in arbitrary units (A.U.).

For oysters, the number and activity of each haemocyte sub-population could be distinguished. Thus, results of the DCFH oxidation are presented for each haemocyte sub-population.

Adhesive capacity

For this assay, two incubation temperatures were tested: 18°C and 30°C. For each temperature, a 150 μl sub-sample of haemolymph from each pool was pipetted into a 24-well microplate while a second sub-sample was pipetted directly

into a tube containing 150 µl of 6% formalin solution. After three hours of incubation, haemocytes in the microplate were fixed by addition of 150 µl of 6% formalin solution. The supernatant was then filtered on an 80 µm sieve and transferred into a tube for flow cytometer analysis. The count of haemocytes not adhering was calculated as the percentage of haemocytes in the supernatant, relative to the total haemocyte count in the control condition (directly fixed) for each temperature of incubation.

Statistical analysis

To compare the dietary conditioning, one-way analysis of variance (ANOVA) was performed for all immune parameters using Statview (SAS Institute, Inc., Cary, NC, USA). Percentage data were transformed (arcsin of the square root) before ANOVA but are presented in figures and tables as untransformed percentage values. To test the temperature incubation effect, PMA effect and 2-month conditioning (initial *vs* final), a *t*-test was performed using Statview.

Results

Composition of the mono-specific algal diet

During the experiment, the lipid profile of each alga was determined (Table 1). The three algae used have a specific polyunsaturated fatty acid (PUFA) composition, especially for essential fatty acids 20:5(n-3), 22:6(n-3) and 20:4(n-6). *Chaetoceros calcitrans* had little 22:6(n-3) (1.3%) but was rich in 20:5(n-3) (17.8%) and 20:4(n-6) (2.0%). By contrast, T-*Iso* was poor in 20:5(n-3) (0.4%) and 20:4(n-6) (0.1%) but was characterized by its high 22:6(n-3) content (7.8%). The third algae used, *Tetraselmis suecica*, contained trace amounts of 22:6(n-3) (0.1%) and small amounts of 20:5(n-3) (5.4%) and 20:4(n-6) (0.5%).

Fatty acid composition of haemocyte polar lipids of C. gigas and R. philippinarum

As mentioned earlier, polar lipids, mainly comprising phospholipids, provide a good approximation of cell membrane lipids. Thus, only the fatty acid composition of polar lipids of haemocytes is presented in the Results section and discussed in regards to immune parameters in the Discussion. During the experiment, the fatty acid profile of haemocyte polar lipids (membrane lipids) was greatly influenced by the dietary conditioning for both species (Table 2).

The fatty acid composition of haemocyte polar lipids of both bivalve species fed *C. calcitrans* contained a higher content of 20:5(n-3) and 20:4(n-6) than those fed the two other diets. Moreover, the proportion of these fatty acids was maintained or slightly increased in haemocyte polar lipids during the 8 weeks of conditioning. Concomitantly, the 22:6(n-3) PUFA content decreased significantly during the dietary conditioning for both species fed *C. calcitrans*. Results obtained with T-*Iso* diet showed an opposite trend. 22:6(n-3) and 20:4(n-6) levels in haemocyte polar lipids were maintained whereas 20:5(n-3) levels decreased. Finally, haemocyte polar lipids of oysters and clams fed *T. suecica* presented an intermediate level of 20:5(n-3), 20:4(n-6) and 22:6(n-3) content compared with the two other dietary treatments. Meanwhile, proportions of fatty acid classes were little affected by the dietary treatment. Only the total polyunsaturated fatty acid proportion in haemocytes of oysters fed T-*Iso* was significantly different from those observed in haemocytes of *T. suecica*-fed oysters.

Mortality

At the end of the experiment, high mortality was observed in oysters fed *T. suecica* (42%), while lower mortality was found in oysters fed the two other algal diets: 31% for T-*Iso*

Table 2. *Essential polyunsaturated fatty acid in the polar lipid fraction of membrane haemocytes, expressed as percentage of total lipids, at the start and after eight weeks of conditioning*

		After 8 weeks		
	Initial	*C. calcitrans*	*T. suecica*	T-*Iso*
Oysters				
20:4(n-6)	5.6±1.2	7.5±1.6^{b}	4.5±0.1^{a,*}	4.4±0.9^{a,*}
20:5(n-3)	20.2±3.1	17.9±5.0^{a}	12.3±4.1^{a}	9.7±2.1^{a,*}
22:6(n-3)	19.5±4.9	5.0±1.1^{a,*}	8.0±5.1^{a,*}	18.2±0.7^{b}
Total saturated	20.4±10.2	30.4±6.2^{a,*}	35.5±1.5^{a,*}	25.7±5.9^{a}
Total monounsaturated	12.2±5.22	17.6±2.2^{a}	16.1±1.6^{a}	17.1±0.6^{a}
Total polyunsaturated	60.1±12.4	45.5±2.0^{a,b}	43.0±1.6^{a,*}	50.9±4.2^{b}
Clams				
20:4(n-6)	5.2±0.8	10.4±1.3^{b,*}	6.6±0.4^{a}	5.0±0.4^{a}
20:5(n-3)	6.7±0.2	11.1±0.3^{a,*}	6.4±0.3^{b}	1.6±0.6^{c,*}
22:6(n-3)	22.6±1.8	9.7±0.6^{a,*}	14.8±1.2^{b}	21.3±1.7^{c}
Total saturated	23.9±5.9	23.9±0.2^{a}	20.8±1.6^{a}	22.3±3.7^{a}
Total monounsaturated	12.8±1.1	16.7± 2.2^{a}	14.7±0.9^{a}	16.8±1.2^{a,*}
Total polyunsaturated	54.3±5.6	50.9±2.7^{a}	54.7±1.6^{a}	54.8±2.1^{a}

Different lower-case letters indicate significant difference between dietary treatments (mean ± S.D.; N=3; ANOVA P<0.01 for clams and P<0.05 for oysters). Asterisks indicate significant difference between a dietary treatment and initial at P<0.05.

Table 3. *Condition index and biochemical composition of oysters and clams at the start and after eight weeks of conditioning*

	Initial	After 8 weeks		
		C. calcitrans	*T. suecica*	T-*Iso*
Oysters				
Condition index	66.4±5.0	82.6±19.3[a,*]	48.9±26.6[b]	61.8±21.7[c]
Lipid	8.9±1.9	10.8±0.8[a]	8.1±0.5[b]	9.1±0.9[a,b]
Carbohydrate	7.0±1.1	10.2±2.1[a]	4.0±0.2[b,*]	7.2±1.2[a]
Protein	46.5±6.4	46.2±1.0[a]	50.86±3.5[a]	45.7±1.1[a]
Clams				
Condition index	71.8±5.2	79.0±21.5[a]	46.2±10.4[b,*]	82.4±12.7[a]
Lipid	5.2±0.5	6.7±1.8[a,b]	6.3±0.3[a,*]	7.7±0.4[b,*]
Carbohydrate	6.7±1.9	6.2±2.2[a,b]	4.5±0.5[a]	7.1±0.8[b]
Protein	42.8±1.5	45.9±5.7[a]	53.7±3.4[a,*]	48.8±2.3[a,*]

Total lipids, carbohydrate and protein content are expressed as percentage of dry mass. Different lower-case letters indicate significant difference between dietary treatments (mean ± S.D.; N=3; ANOVA P<0.001 for condition index and P<0.05 for biochemical composition). Asterisks indicate significant difference between a dietary treatment and initial at P<0.05.

and 26.6% for *C. calcitrans*. These mortality rates were high compared with those of clams, for which mortality was 0%, 1% and 2.3% for the *C. calcitrans*, T-*Iso* and *T. suecica* diets, respectively.

Condition index (CI)

Results showed that oysters and clams fed *T. suecica* had a lower CI than those fed *C. calcitrans* or T-*Iso* (Table 3; P<0.001). T-*Iso* maintained the physiological status of oysters and clams during the experiment, while only oysters fed *C. calcitrans* showed a significant increase in CI at the end of the experiment (P<0.05).

Biochemical composition

Results of biochemical composition measurements for both bivalve species showed that oysters and clams fed *T. suecica* were more affected by 8 weeks of feeding than those fed the other two diets, especially for carbohydrate content (Table 3; P<0.05). *T. suecica* feeding led to a decrease in carbohydrate content compared with the initial value, while *C. calcitrans* and T-*Iso* supported an increase or maintenance of this component. Lipid content was also affected by the dietary conditioning. Oysters and clams fed *T. suecica* had the lowest lipid content compared with those fed other diets. By contrast, protein content appeared to be the most stable component. Neither dietary conditioning nor the duration of the conditioning affected the protein content of oysters and clams significantly.

Total haemocyte count and viability of haemocytes

At the end of the experiment, total haemocyte count (THC) of clams fed *C. calcitrans* was higher compared with the two other treatments, but only significantly compared with T-*Iso* (Table 4; P<0.05). For the oysters, no significant difference was observed between dietary conditions. However, THC decreased with *T. suecica* and T-*Iso* diets compared with initial values.

Viability of haemocytes of both species was not significantly affected by the dietary treatment.

Adhesion

The adhesion capacity of haemocytes was not affected by the nutritional conditions (Table 4). However, haemocytes lost their capacity for adhesion with increasing temperature. More haemocytes were found in the supernatant at an incubation of 30°C compared with an incubation of 18°C for both species (P<0.01). The results also showed that the adhesive capacity of clam haemocytes was significantly higher than that of oyster haemocytes (P<0.001).

Haemocyte sub-populations in oysters

Percentages of oyster haemocyte sub-populations at the end

Table 4. *Total haemocyte count (THC), viability rate of haemocytes (measured with propidium iodide) and adhesion rate of oyster and clam haemocytes after two hours of incubation at 18°C and 30°C*

	Initial	T=8 weeks		
		C. calcitrans	*T. suecica*	T-*Iso*
THC (10^5 cells ml^{-1})				
Oysters	6,4±2.2	5,8±1.0[a]	3,7±1.1[a]	3,7±1.0[a]
Clams	5,9±1.2	14±2.5[a,*]	9,6±5.5[a,b]	8±1.2[b]
Viability (% of live cells)				
Oysters	97.2±1.5	95.9±0.8[a]	96.7±1.3[a]	96.1±0.9[a]
Clams	97±0.8	96.4±0.5[a]	97.4±0.8[a]	95.2±1.5[a]
Adhesion (% of adhered cells)				
18°C				
Oysters	ND	91.8±0.5[a]	91.4±2.7[a]	90.6±1.1[a]
Clams	ND	98.9±0.9[a]	98.5±1.2[a]	99.0±0.4[a]
30°C				
Oysters	ND	89.4±0.4[a]	89.6±0.9[a]	88.5±1.9[a]
Clams	ND	98.0±0.7[a]	96.7±0.7[a]	96.7±1.1[a]

Different lower-case letters indicate significant difference between dietary treatments (mean ± S.D.; N=3; ANOVA P<0.05 for THC and viability and P>0.05 for adhesion). ND, not determined. Asterisks indicate significant difference between a dietary treatment and initial at P<0.05.

Table 5. *Percentage of haemocyte sub-populations in haemolymph samples of oysters measured with propidium iodide at the end of the experiment*

	C. calcitrans	*T. suecica*	T-*Iso*
Granulocytes	16.8±2.5[a]	14.7±3.9[b]	14.9±3.7[b]
Hyalinocytes	69.9±2.9[a]	72.1±1.5[b]	73.6±3.7[c]
Small agranular cells	13.3±0.5[a]	13.1±2.5[a]	11.5±2.2[a]

Different lower-case letters indicate significant difference between dietary treatments (mean ± S.D.; N=3; ANOVA P<0.01).

of the experiment are presented in Table 5. The diet affected the proportion of granulocyte and hyalinocyte sub-populations significantly (P<0.01). The percentage of granulocytes of oysters fed *C. calcitrans* was significantly higher than for oysters fed *T. suecica* or T-*Iso*, and the highest percentage of hyalinocytes (73.6%) was found with the T-*Iso* dietary conditioning. The small agranular cell fraction was not affected by any diet; the mean percentage of this fraction was ~13.1%.

Phagocytosis assay

A significant effect of the dietary treatment was shown on the phagocytic rate of clam haemocytes when analysed without pre-incubation (Fig. 1; P<0.01). Haemocytes of clams fed *C. calcitrans* had a higher phagocytic rate (21.7%) than those of clams fed *T. suecica* and T-*Iso* algae (14.7% and 8.8%, respectively). However, when haemocytes were subjected to a two-hour pre-incubation at 18°C or 30°C, the dietary effect disappeared.

In the same analysis, no difference between dietary conditions was observed for oysters without pre-incubation (Fig. 2). A mean phagocytic rate of 29.2% was measured. However, when haemocytes were subjected to two hours of pre-incubation at 18°C, their phagocytic activity dropped to a mean of 12.8% for oysters fed *C. calcitrans* and *T. suecica*. However, this activity was maintained at 20.9% for oysters fed T-*Iso*, a significantly higher value compared with the two other

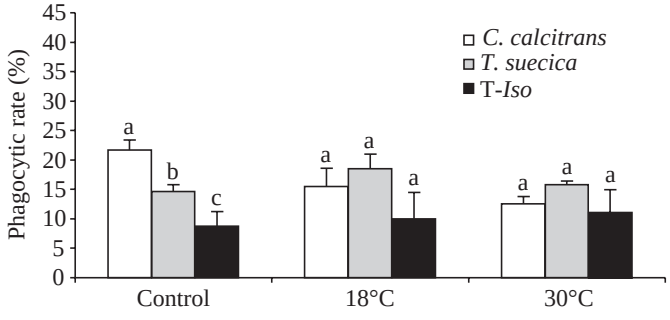

Fig. 1. Phagocytic rate of clam (*Ruditapes philippinarum*) haemocytes submitted to three conditions: control (without pre-incubation) and after a pre-incubation of two hours at 18°C or 30°C. Different lower-case letters indicate significant difference between dietary treatments (mean ± S.D.; N=3; ANOVA P<0.01).

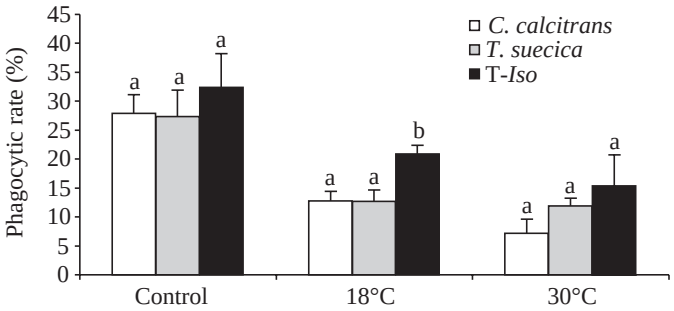

Fig. 2. Phagocytic rate of oyster (*Crassostrea gigas*) haemocytes submitted to three conditions: control (without pre-incubation) and after a pre-incubation of two hours at 18°C or 30°C. Different lower-case letters indicate significant difference between dietary treatments (mean ± S.D.; N=3; ANOVA P<0.01).

diets. The results, although not statistically significant between dietary treatments, were similar after the 30°C pre-incubation.

Oxidative activity

Haemocytes of clams fed *C. calcitrans* were the most reactive for both 18°C and 30°C incubation temperatures (Fig. 3; P<0.001). At 18°C, a mean of 149 A.U. was measured, whereas for *T. suecica* and T-*Iso* the intensities of DCFH oxidation were lower (93 A.U. and 78 A.U., respectively). At 30°C, the DCFH oxidation of haemocytes from clams fed with *C. calcitrans* was 220 A.U., compared with a mean of 118 A.U. and 111 A.U. for clams fed *T. suecica* and T-*Iso*, respectively. The increase of DCFH oxidation from 18°C to 30°C was significant only for *C. calcitrans* and T-*Iso* (P<0.01).

For oysters, the DCF fluorescence level of both granulocyte and hyalinocyte sub-populations was analysed (Fig. 4). Small agranulocytes demonstrated only low oxidative activity. The oxidative activity seemed to be similar for both sub-populations when comparing the means. Nevertheless, the dietary conditioning and the temperature of incubation affected the activity of sub-populations. At 18°C, granulocytes as well as hyalinocytes of T-*Iso*-fed oysters were significantly less active (P<0.05) than those of *C. calcitrans* and *T. suecica* conditioning. After incubation at 30°C, haemocyte sub-populations of oysters fed *C. calcitrans* were significantly the most active (165 A.U. and 156 A.U. for granulocytes and hyalinocytes, respectively). No effect of the temperature of incubation was noted on the haemocyte sub-population activity of oysters fed *C. calcitrans* and T-*Iso* (P>0.05). Nevertheless, oxidative activity was noted to be lower at 30°C than at 18°C for hyalinocytes of *T. suecica*-fed oysters.

Finally, stimulation by PMA had no measurable effect upon the oxidation of DCFH by haemocytes for both species.

Proportion of haemocyte sub-populations incubated with DCFH-DA

When incubated with DCFH-DA, it was interesting to note that granulocytes of oysters fed the *C. calcitrans* diet were

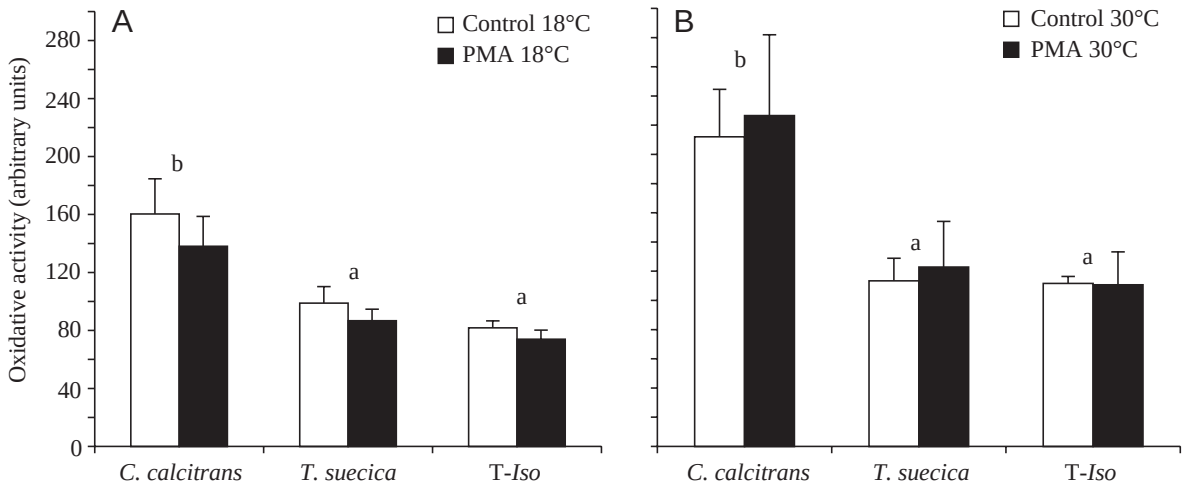

Fig. 3. DCFH activity of clam haemocytes after 20 min of incubation with phorbol myristate acetate (PMA) and without PMA (control) at (A) 18°C or (B) 30°C. Different lower-case letters indicate significant difference between dietary treatments (mean ± S.D.; N=3; ANOVA P<0.01). No effect of PMA stimulation was noted.

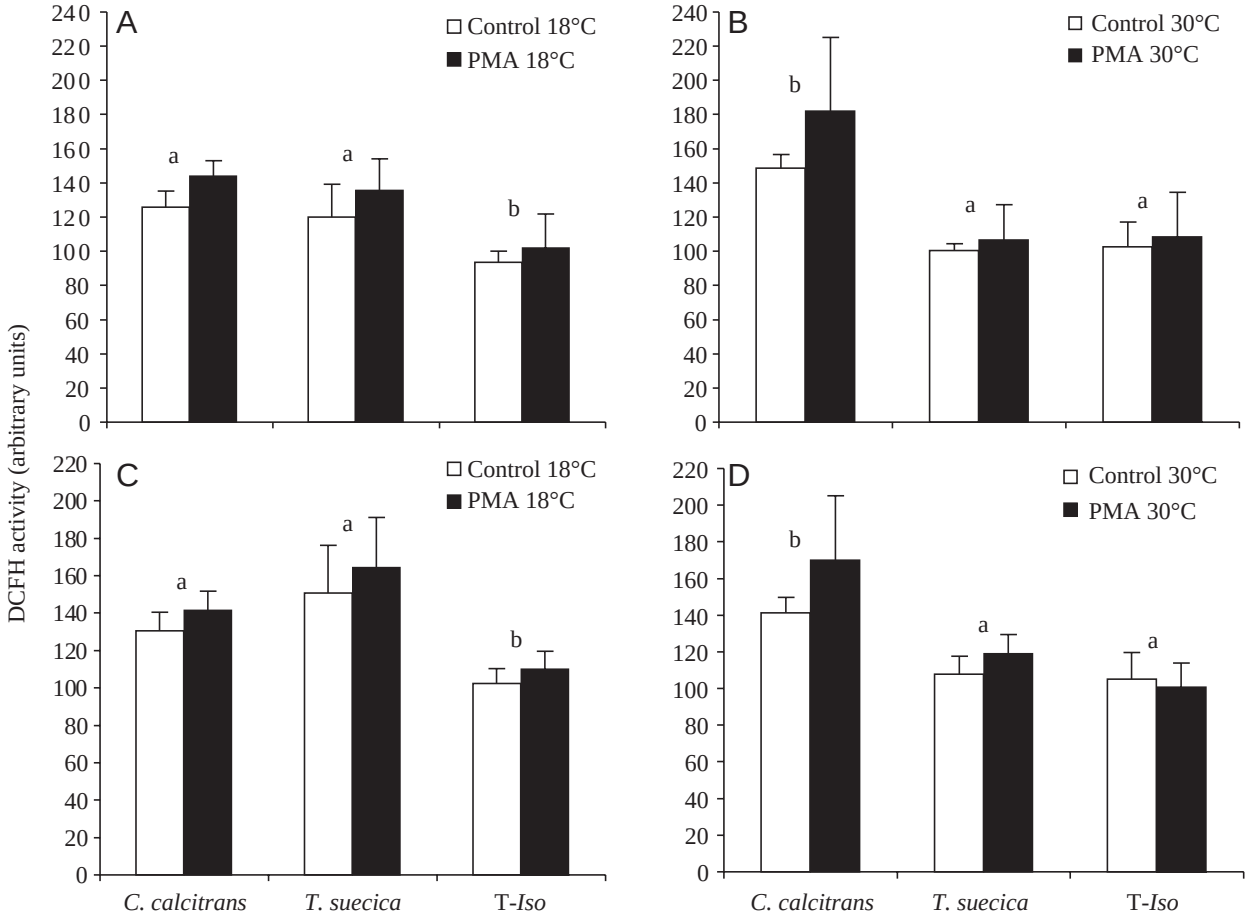

Fig. 4. 2′7′-dichlorofluorescein (DCFH) activity of oyster haemocyte sub-populations after 20 min of incubation with phorbol myristate acetate (PMA) aand without PMA (control) at different temperatures: (A) granulocyte sub-population at 18°C; (B) granulocyte sub-population at 30°C; (C) hyalinocyte sub-population at 18°C; (D) hyalinocyte sub-population at 30°C. Different lower-case letters indicate significant difference between dietary treatments (mean ± S.D.; N=3; ANOVA P<0.05). No effect of PMA stimulation was noted.

present in higher concentrations than in oysters fed the two other diets (Table 6; $P<0.01$) as observed in oyster haemocytes incubated with PI. With the same diet, a significant decrease of granulocyte counts (approximately 50%) after PMA stimulation was observed while hyalinocyte and agranulocyte counts remained stable. For the *T. suecica* and T-*Iso* diets, we also observed a decrease of granulocytes after PMA stimulation, but this was not statistically significant. Thus, the dietary effect upon the proportion of haemocyte sub-populations disappeared after PMA stimulation.

Discussion

The aim of this study was to test the influence of dietary conditioning on the fatty acid profile of haemocytes and consequently on immune parameters of two bivalve species, the oyster *C. gigas* and the clam *R. philippinarum*. Three algal mono-specific diets were selected from available strains in the hatchery according to their specific PUFA composition (Table 1). Although the vitamin and amino acid contents may vary between mariculture microalgae species, it is established that bivalve requirements for the above compounds are covered whatever the utilised species (Brown, 1991; Brown and Miller, 1992; Seguineau et al., 1996), Consequently, the PUFA composition was considered as the most determining factor of the nutritive quality of the dietary treatments, and the amino acid and vitamin compositions were considered as minor determining factors.

Condition index (CI) results showed that both shellfish species were affected by the dietary conditioning. The decrease of CI and carbohydrate content observed for clams and oysters fed *T. suecica* and also the high mortality of oysters fed this diet indicated that this diet had a poor nutritional value. This result is in agreement with previous studies that have shown that *T. suecica* is a poor-to-moderate food for different bivalves: *O. edulis* (Laing and Millican, 1986), *Saccostrea commercialis* (Nell and O'Connor, 1991; O'Connor et al., 1992) and *Venerupis pullastra* (Albentosa et al., 1993). T-*Iso* and *C. calcitrans* appeared to have a higher nutritive value than *T. suecica*, with an increase or a maintenance of the CI and carbohydrate content compared with initial values. Moreover, for oysters, the highest CI and energy storage were observed with *C. calcitrans*, while for clams it was observed with T-*Iso*. This could be explained by specific requirements or differential capacity for filtration and assimilation in the two species. Difference in physiology may also account for the higher mortalities observed for oysters.

This is the first time that the fatty acid composition of immune cells of *C. gigas* and *R. philippinarum* has been analysed. The fatty acid composition of polar lipids is considered to provide a good approximation of cell membrane lipids (Soudant et al., 1995, 1997, 1998).

At the beginning of the experiment, oyster and clam haemocyte membranes contained similar amounts of 22:6(n-3) (19.5% and 22.6%, respectively) and 20:4(n-6) (5.6 and 5.2%, respectively). Conversely, they had different 20:5(n-3) contents (20.2% and 6.7%, respectively). The observed fatty acid composition was in agreement with the results of Helm and Laing (1987) and Fernández-Rieriz et al. (1998).

During the experiment, the fatty acid composition of haemocyte polar lipids was greatly influenced by the dietary conditioning. According to the fatty acid profile of the diets, oysters and clams fed *C. calcitrans* slightly increased or maintained their proportions of 20:5(n-3) and 20:4(n-6) in their polar lipids, while a reduction in these PUFA was noted for animals fed T-*Iso* algae. However, T-*Iso* algae, which provided high amounts of 22:6(n-3), allowed the maintenance of this PUFA in the haemocyte membranes of animals fed this diet. This impact of the lipid profile of the diet on haemocyte polar lipids agrees with other observations reported in other tissues or in larvae (Delaunay et al., 1993; Berntsson et al., 1997; Soudant et al., 1996b, 1997, 1999).

Morphological and functional parameters of *C. gigas* and *R. philippinarum* were concomitantly measured using flow cytometry. Putative granulocytes, hyalinocytes and a third population considered to be small agranular cells can be

Table 6. *Concentration (10^5 cells ml^{-1}) of haemocyte sub-populations in* C. gigas *hemolymph samples during DCFH-DA analysis*

	18°C			30°C		
	C. calcitrans	*T. suecica*	T-*Iso*	*C. calcitrans*	*T. suecica*	T-*Iso*
Control condition						
Granulocytes	$1.0\pm0.05^{b,A}$	$0.6\pm0.3^{a,b,A}$	$0.5\pm0.2^{a,A}$	$1.1\pm0.04^{b,A}$	$0.6\pm0.2^{a,A}$	$0.5\pm0.2^{a,A}$
Hyalinocytes	$2.0\pm0.2^{a,A}$	$1.4\pm0.2^{a,A}$	$1.5\pm0.2^{a,A}$	$2.3\pm0.2^{b,A}$	$1.5\pm0.3^{a,A}$	$1.6\pm0.2^{a,A}$
Small agranular cells	$1.1\pm0.1^{a,A}$	$0.8\pm0.2^{a,A}$	$0.9\pm0.3^{a,A}$	$1.1\pm0.1^{a,A}$	$0.9\pm0.3^{a,A}$	$0.9\pm0.2^{a,A}$
With PMA						
Granulocytes	$0.4\pm0.1^{a,B}$	$0.5\pm0.2^{a,A}$	$0.3\pm0.1^{a,A}$	$0.5\pm0.1^{a,B}$	$0.4\pm0.1^{a,A}$	$0.3\pm0.1^{a,A}$
Hyalinocytes	$1.6\pm0.3^{a,A}$	$1.3\pm0.4^{a,A}$	$1.2\pm0.1^{a,A}$	$1.8\pm0.4^{a,A}$	$1.3\pm0.2^{a,A}$	$1.3\pm0.1^{a,A}$
Small agranular cells	$0.9\pm0.1^{a,A}$	$0.9\pm0.3^{a,A}$	$0.8\pm0.2^{a,A}$	$0.8\pm0.1^{a,A}$	$0.8\pm0.2^{a,A}$	$0.7\pm0.2^{a,A}$

DCFH-DA, 2′7′-dichlorofluorescein diacetate; PMA, phorbol myristate acetate.

Different lower-case letters indicate significant difference between dietary treatments. Different upper-case letters indicate a significant effect of the analysis conditions (control *vs* PMA; mean ± S.D., $N=3$, ANOVA $P<0.01$ for dietary treatment; *t*-test $P<0.05$ for the PMA effect).

separated on oyster cytograms. However, as Ashton-Alcox and Ford (1998) discussed, we cannot distinguish large granulocytes from small granulocytes. Nevertheless, the percentages of the two main sub-populations were in agreement with those found in the literature for *C. virginica* (Ashton-Alcox and Ford, 1998), *C. gigas* and *O. edulis* (Auffret, 1988; Chagot, 1989; Xue et al., 2001). Oyster haemocyte sub-populations are comprised on average of 71.8% hyalinocytes, 15.5% granulocytes and 12.6% agranular cells. Total haemocyte counts (THC) of oysters were also stable and in the same range of values as those reported by Ashton-Alcox and Ford (1998) for *C. virginica* using the same technique. By contrast, an increase of clam THC was observed at the end of the experiment, as compared with the initial sampling. Nevertheless, our THC values were similar to those reported by Ordás et al. (2000) but lower than those found in the same species by Oubella et al. (1996) and Allam et al. (2000, 2001).

Concerning haemocyte activities, the range of phagocytosis index using fluorescent latex beads obtained in the present study agrees with those found in the literature. Our values are similar to those of Cima et al. (2000) and Xue (1998) for clams and oysters, respectively. Regarding the oxidative activity, PMA did not significantly induce DCFH oxidation by haemocytes of either species, although it did increase the production of reactive oxygen species measured using chemiluminescence in oyster or mussel haemocytes (Nakayama and Maruyama, 1998; Arumugan et al., 2000; Torreilles et al., 1996). This apparent contradiction with the published papers on oysters could be explained by the fact that haemocytes in our study were withdrawn without using an anti-aggregant solution and may already have been in an excited state. Thus, as suggested by Lambert et al. (in press), it seems that the oxidative activity level was so high initially that it was not possible to further increase it with PMA. We also noted an intriguing decrease of granulocytes in the differential haemocyte counts after PMA stimulation. The loss of granulocyte counts for oysters fed *C. calcitrans* diet was around 50%, whereas a decrease of 10–20% in the two other haemocyte sub-population counts was observed. The significance of this observation is unclear. It is suggested that the loss of granulocytes by PMA corresponds to a degranulation of this haemocyte sub-population after stimulation. This result was confirmed with a loss of complexity (SSC values) for granulocytes (data not shown).

Haematological parameters as well as phagocytosis and oxidative activities of clam haemocytes were more affected than those of oyster haemocytes by dietary treatment. Our results showed that *C. calcitrans* had a positive impact on the total haemocyte count, granulocyte percentage and defence-related activities. Concurrently, the lowest total haemocyte count, phagocytosis and oxidative activities of haemocytes were found with clams fed T-*Iso*. Higher levels of 20:5(n-3) and, to a lesser extent, 20:4(n-6) in haemocyte polar lipids of clams fed *C. calcitrans* compared with the other diets appeared to be important for these immune parameters. It is interesting to note that higher phagocytosis and oxidative activities paralleled with a higher percentage of granulocytes. The latter sub-population is thought to represent the most active cells in bivalve defence (Cheng, 1996). The importance of 20:5(n-3) in immune functions could be explained by its putative energetic role in bivalves as proposed by Delaunay (1992) and Soudant (1995). We can suggest that haemocytes that have accumulated 20:5(n-3) can be more active during phagocytosis and metabolic processes because they have more energy and capacity to respond to the stimulation. Concomitantly, the positive effect of 20:4(n-6) on phagocytosis can also be paralleled to the positive effects proposed by Lennartz (1999) for vertebrates. This author suggested that the phagocytic activity was related to the 20:4(n-6) produced by phospholipase A2 (PLA2) in macrophages. 20:4(n-6)/PLA2 may facilitate membrane fusion, thereby mediating membrane movement during phagocytosis. Moreover, the 20:4(n-6) positive effect on oxidative activity can be related to the role of this fatty acid as a precursor of 4-series leukotrienes and 2-series prostaglandins (Calder, 2001). These eicosanoids have different effects on vertebrate immunology. In a review, Calder (2001) underlined that the leukotriene LTB4, a potent chemotactic agent for leukocytes, induced the release of lysosomal enzymes, increased vascular permeability and enhanced generation of reactive oxygen species. But this relationship observed in vertebrates must be taken with caution. Indeed, membranes of immune cells of vertebrates contain a large amount of 20:4(n-6) (10–20%) compared with 22:6(n-3) and 20:5(n-3) (less than 4%) (Calder, 2001), whereas bivalve membrane lipids are predominated by 22:6(n-3) and 20:5(n-3) (Soudant et al., 1996b, 1997, 1998, 1999; present study). Nevertheless, at the end of experiment, haemocyte membrane lipids of *C. calcitrans*-fed clams contained similar amounts of 20:4(n-6) (10.4%) to those of human immune cells. So, the higher oxidative activity obtained for haemocytes of clams fed *C. calcitrans* seems to agree with the role of 20:4(n-6) as a precursor of leukotrienes in reactive oxygen species production.

In the oyster, the impact of the dietary conditioning was less clear than it was for clams. As for clams, oxidative activity can be associated with the high content of 20:5(n-3) and 20:4(n-6) in haemocyte polar lipids of oysters fed *C. calcitrans*. However, the 22:6(n-3) content of T-*Iso* appeared to be more important for the phagocytic activity of oyster haemocytes. This dietary effect was only significant after application of a stress on haemocytes (pre-incubation for two hours at 18°C or 30°C). This relationship observed with 22:6(n-3) may be attributable to the potential positive effect of this fatty acid in stress responses (Kanazawa, 1995; Utting and Millican, 1995). The different response in phagocytosis compared with clams can underline a species-specific immune response. It is also interesting to underline that variations in 20:5(n-3) levels between treatments in clam haemocyte lipids (1–11%) were much greater than those observed in oyster haemocyte lipids (10–18%). On the contrary, variations in both 22:6(n-3) and 20:4(n-6) according to the dietary treatment in both species were of a similar range.

In conclusion, a relationship between nutrition and health status seems to exist in bivalves, as in vertebrates. Although other biochemical compounds may vary concomitantly, the fatty acid composition of mono-specific algal diets appears, by modifying membrane lipid composition, to affect membrane functions of oyster and clam haemocytes. Nevertheless, the effect of each fatty acid has not been clearly determined. Experiments with artificial diet are needed to distinguish the individual importance of each fatty acid in the cellular immune functions of bivalves.

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