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Aquaculture
April 2006; 254(1-4) : 571-582
http://dx.doi.org/10.1016/j.aquaculture.2005.10.006
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Impact of food availability on energy storage and defense related hemocyte parameters of the Pacific oyster Crassostrea gigas during an experimental reproductive cycle

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Abstract: The aim of this study was to test the effect of food quantity on energy storage and defense capacities of oysters during a reproductive cycle. One-year-old Crassostrea gigas oysters were fed two different dietary rations (4% and 12% of oyster dry weight in algal dry weight per day) in controlled experimental conditions over an annual cycle. Oyster dry weights, carbohydrate and lipid contents, energetic adenylate charge, and hemocyte parameters of oysters were significantly affected by reproductive processes related to seasonal temperature variation and, to a lesser extent, by the dietary rations. Energy parameters decreased during gametogenesis as gonads developed then increased during the gonad resorption phase. The additional energy provided to oysters fed the 12% diet compared to oysters fed the 4% diet was allocated mainly to the development of more gonad tissue. Regardless of diet, hemocyte concentrations were also seasonally affected. Hemocyte concentrations were low during gametogenesis and significantly increased during the gonadal resorption and tissue restoration phase. Phagocytic activity and adhesive capacity of hemocytes were temporarily inhibited during gametogenesis and were at their lowest levels in June. Oysters fed the 12% diet had significantly higher hemocyte concentrations and lower phagocytosis activity and reactive oxygen species production compared to those fed the 4% diet.

Keywords: *Crassostrea gigas*; Energy storage; Hemocyte parameters; Phagocytosis; Reproduction; Reactive oxygen species production

1. Introduction

Since 1991, summer mortalities affecting both juveniles and adults of the Pacific oyster, Crassostrea gigas, have been reported in French sea farms (Goulletquer *et al.*, 1998). These mortalities are generally associated with temperatures > 20°C (Beattie *et al.*, 1980; Soletchnik *et al.*, 1999, 2003) and coincide with the period of sexual ripeness (Perdue *et al.*, 1981). Because high temperature and sexual ripeness are associated with low energetic status, it could be hypothesized that food availability affects oyster fitness during the reproductive cycle.
Food availability is known to be an important factor for bivalve development, affecting broodstock energy reserves, duration of the maturation process, fecundity, quality and quantity of eggs, and larval development (Berntsson *et al.*, 1997; Utting and Millican, 1997; Hendricks *et al.*, 2003). Food availability is also a critical environmental factor, along with temperature, for gametogenesis. In general, a temperature threshold (above 7-10°C) has to be reached before gametogenesis is initiated (Deslous-Paoli and Héral, 1988; Ruiz *et al.*, 1992), and then after initiation, the rate at which gametogenesis proceeds depends on temperature and food availability. The high energetic cost of reproductive processes was suspected to result in immuno-depression and thus to be involved in summer mortality events by making oysters more susceptible to opportunistic pathogens and environmental stress (Pouvreau *et al.*, 2003).
Generally, it is believed that the capability of oysters to react to diseases, injuries or parasite infections depends upon their defense system. Hemocytes are considered the main cellular mediators of the defense system in bivalves (Cheng, 1996). Hemocytes are responsible for recognition, phagocytosis, and elimination of non-self particles by microbicidal activities (Pipe, 1992; Cheng, 1996; Chu, 2000). Many authors have shown that environmental factors modulate hemocyte capabilities of bivalves (Fisher *et al.*, 1989; Santarém *et al.*, 1994; Fisher *et al.*, 1996; Carballal *et al.*, 1998; Chu, 2000; Oliver *et al.*, 2001; Soudant *et al.*, 2004). It is generally thought that hemocyte activities vary seasonally among geographic location, habitat and according to the physiological conditions of the oysters (including disease). Nevertheless, the complexity of the natural environment makes it difficult to distinguish the impact of different environmental parameters (e.g. food availability, water temperature, salinity, pollution, presence of pathogens) and physiological status (e.g. sexual cycle, disease status) on the health and survival of bivalves.
The purpose of this study was to assess the impact of two dietary rations on biochemical composition and hemocyte parameters (hemocyte concentrations, phagocytic activity, adhesive capacity, viability and reactive oxygen species production of hemocytes) of oysters over an annual cycle by rearing oysters under controlled temperature and photoperiod conditions in an experimental hatchery. Results were evaluated with reference to a histological study (Enriquez-Diaz, 2004) performed in parallel to the present study to assess the reproductive status.

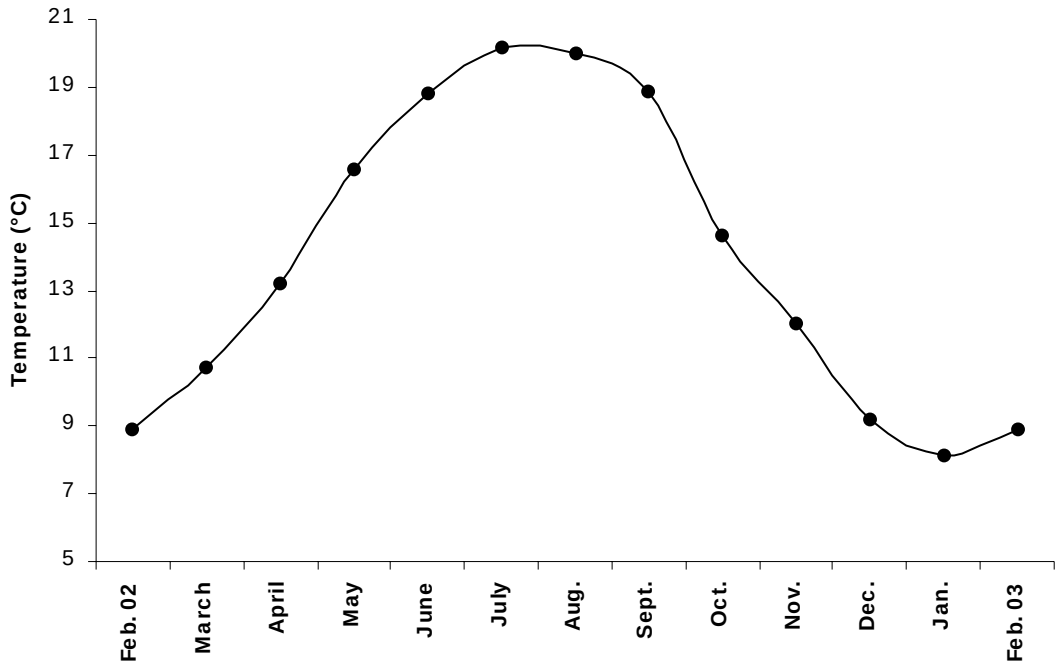
2. Materials and Methods

2.1. ***Oyster dietary treatments***

Oysters were produced in 2001 in the IFREMER hatchery at La Tremblade (Charente, France) from 30 wild broodstock oysters collected in the Marennes-Oléron Bay. Spat were reared at the IFREMER station in Bouin (Vendée, France), and then juveniles were held in the marine ponds of Marennes during the winter of 2002. One-year-old oysters were maintained at the IFREMER shellfish laboratory in Argenton (Finistère, France) from February 2002 to February 2003, in 700-L raceways with 20µm-filtered running seawater. Oysters were fed a mixed diet of three microalgae T-Iso (Isochrysis aff. galbana, clone Tahiti), Chaetoceros calcitrans, and Tetraselmis chui provided in equal biomass proportions. Occasionally, difficulties were encountered in

producing Chaetoceros calcitrans, making it necessary to replace this diatom with another, Skeletonema costatum. The algal daily ration was maintained at 4% of oyster dry weight in algal dry weight per day for the first group (4% diet), and 12% for the second (12% diet). During the experiment, the annual average photoperiod and temperature cycle of Marennes-Oléron was applied artificially, and spawning was induced mid-July (Figure 1). Tanks and oysters were cleaned twice a week. From February 2002 to February 2003, fifteen oysters were sampled monthly for biochemical analysis, except in January 2003. Twenty oysters were sampled in April, June, July, August and October to characterize oyster hemocyte parameters during the reproductive period. On these dates, three pools of five or six individuals were used in order to have enough hemolymph for all hemocyte assays. For the other sampling dates (March, May, September, November and December), only hemocyte sub-population concentrations and viability were followed in 10 individual oysters as hemolymph collected from an individual oyster is generally not sufficient to process all the hemocyte parameters.

Figure 1: Temperature cycle applied during the conditioning corresponds to the mean of temperature reported in Marennes-Oléron over the last ten years.



2.2. Oyster dry weight

At each sampling date, whole-oyster wet flesh weight of 15 oysters was measured individually, and soft tissue samples were combined in 3 pools of five animals each. Pools were then frozen and stored in liquid nitrogen (-196°C) for later biochemical analyses. Pooled tissues were ground with a Dangoumeau homogeniser. A known amount of wet homogenate (about 1g) from each pool was placed in a pre-weighed aluminium cup for 48h at 80°C. Aluminium cups were then weighed again for a dry weight measurement. Finally, for each indidvidual oyster contributing to a pool, individual whole-oyster dry flesh weight was back-calculated from the whole-oyster wet

flesh weight based on a dry weight to wet weight ratio measured on 1 g of pooled wet homogenate. Results are expressed as the mean of the 15 calculated individual whole-oyster dry flesh weights for each dietary ration.

2.3. Biochemical composition

For total lipid, protein and carbohydrate analyses, 600 mg of the above wet homogenate was re-suspended with 3 ml of distilled water, and then divided into three aliquots (200 µl for carbohydrate and protein analysis and 400µl for total lipid analysis). 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 and evaporated under a nitrogen stream; the 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. Results are expressed as mg of carbohydrate, lipids and protein per mg of oyster dry flesh weight for each pool.

2.4. Adenylate energy charge

Adenylate Energy Charge (AEC) analyses were conducted on the same wet homogenate used for dry weight measurement and biochemical analyses according to Moal *et al.* (1989). Briefly, nucleotides were extracted from 200 mg of the oyster powder prepared above with 2 ml of trichloro-acetic acid (TCA), neutralized with 1.2 ml of amine freon (trioctylamine/trifluoro-trichloro-ethane, v:v, 1:5). Extracted nucleotides were analysed by high-performance liquid chromatography on a reverse phase column with a counter-ion (tributylamine). AEC was calculated as follows: (ATP + 0.5 ADP)/(ATP + ADP +AMP), a value that varies between 0 and 1. AEC represents an estimation of the “instantaneous” cellular energy available for oysters. In healthy animals, AEC varies between 0.8-0.9; with partial stress the values drops to 0.5 (Ivanovici, 1980).

2.5. Hemolymph sampling

Hemolymph was withdrawn from individual oysters using a 1 ml plastic syringe fitted with a 25-gauge needle via a notch adjacent to the adductor muscle. Shells of oyster were notched a day before withdrawing hemolymph to let oysters recover from the stress induced by the notch and also to naturally clear the notch from small shell debris. All hemolymph samples were stored individually in micro-tubes held on ice. Individual samples were examined microscopically for contamination (sperm, ovocytes, algae, debris). As recommended by FCM manufacturer samples were filtered through 80µm mesh prior to analysis in order to eliminate potential large debris (> 80 µm) which could potentially clump the flow cytometer.

2.6. Measurements of hemocyte parameters by flow cytometry

The characterisation of hemocyte type, number and functions were determined using a FACScalibur (BD Biosciences, San Jose, CA USA) flow cytometer equipped with a 488 nm argon laser. Methods to measure hemocyte parameters are described hereafter.

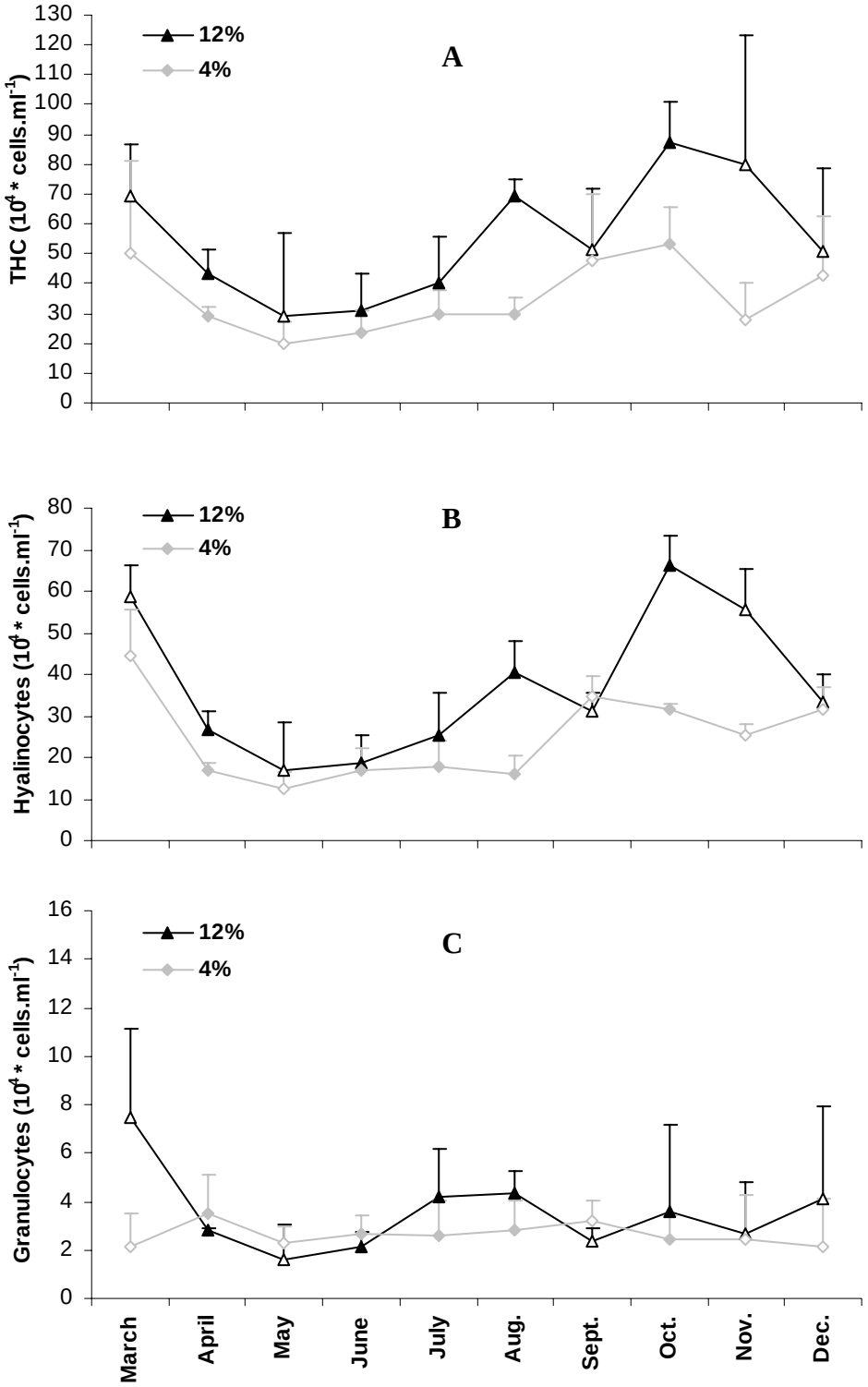
2.6.1. Hemocyte concentrations

100 µl of each hemolymph sample was fixed by adding 300 µl of a 4% formalin solution in filtered sterile seawater (FSSW). Samples were incubated with SYBR Green I (Molecular probes, 10X final concentration), a nucleic acid specific dye, in darkness at room temperature for 30 minutes before flow cytometry analysis. SYBR Green fluorescence was measured at 500-530 nm by the flow cytometer.

All SYBR Green stained cells were visualised on a Forward Scatter height (FSC, size) and Side Scatter height (SSC, cell complexity) cytogram, allowing identification of hemocyte sub-populations. Granulocytes are characterised by high FSC and high SSC, hyalinocytes by high FSC and low SSC, while agranulocytes have low FSC and SSC. Thus, the three sub-populations were distinguished according to their size and cell complexity (granularity). Total hemocyte, granulocyte and hyalinocyte concentrations were expressed as number of cells per ml. These concentrations were estimated from the flow rate measurement of the flow-cytometer as all samples were ran for 30 sec. Agranulocyte concentrations were not presented in this study as they represented only a small proportion of the total hemocytes and are considered to possess little activity (Lambert *et al.*, 2003).

Data from individual and pooled samples are presented all together on Figure 5A, B and C in order to visualize hemocyte sub-population concentrations over the entire 12-month experiment. However statistical analyses on individual and pooled oysters were performed separately in this study.

Figure 5: Total hemocyte concentration (A), hyalinocyte (B) and granulocyte (C) concentrations (in 10^4 cell.ml^{-1}) of oysters fed two dietary rations (4% and 12% of algal dry weight/oyster dry weight) during an average temperature cycle (similar to Marennes-Oléron, France) under hatchery controlled conditions (mean ± SD; n=3 pools in April, June, July, August, October, filled symbols and n=10 individuals in March, May, September, November and December, unfilled symbols).



2.6.2. Hemocyte viability

Hemocyte viability was assayed according to Delaporte *et al.* (2003). Briefly, 150 µl of hemolymph from each pool was transferred into a tube containing 150 µl of anti-aggregant solution. Ten minutes before flow cytometry analysis, samples were incubated with propidium iodide (PI, final concentration of 20 $\mu g.ml^{-1}$), a fluorescent dye specific for nucleic acids which permeates membranes of dead cells only. The PI fluorescence of dead cells was measured at 550-600 nm. The percentage of viable hemocytes was calculated by the percentage of hemocytes not showing PI fluorescence relative to total hemocyte counts.

2.6.3. Phagocytosis assays

One hundred µl of hemolymph of each pool diluted with FSSW (1:1, v:v) **was** brought into contact with fluorescent beads (fluoresbrite microspheres YG 2.0 microns, polysciences, Eppelheim, Germany) in a micro-tube at a final concentration of 0.3% of the commercial suspension. After 60 minutes of incubation at 18°C, hemocytes were fixed with 230 µl of 6% formalin solution, and analysed at 500-530 nm by flow cytometry to detect hemocytes containing fluorescent beads. The phagocytic activity of hemocytes was estimated as the percentage of hemocytes that had engulfed two beads or more.

2.6.4. Adhesive capacity

The adhesive capacity of C. gigas hemocytes was assessed by modifying the procedure of Choquet *et al.* (2003). For this assay, the Vibrio sp. strain S322 was used as a pathogenic challenge for the oyster hemocytes. This strain was demonstrated to be pathogenic for larvae of the scallop Pecten maximus and the oysters Crassostrea gigas and Ostrea edulis by Nicolas *et al.* (1996). The Vibrio sp. S322 suspension was prepared as described by Lambert *et al.* (2003) after 24h of culture in Zobell 2216E (Difco) medium.

For each dietary ration, two 100 µl sub-samples of hemolymph from each pool were distributed into a 24 well-microplate. Then, 100 µl of FSSW were added to the first sub-sample (as a control) and 100 µl of a Vibrio sp. S322 suspension (50 cells/hemocyte) to the second. Simultaneously, a third 100 µl sub-sample was directly fixed in a flow cytometer tube by adding 300 µl of a 4% formalin solution in FSSW to estimate the hemocyte concentration at the beginning of the assay. After three hours of incubation at 18°C, hemocytes in the microplate were fixed by addition of 200 µl of 6% formalin solution. The supernatant containing non adhesive cells was filtered and transferred into a tube for flow cytometry. Cells from the supernatants and the cells fixed in 4% formalin solution from the beginning were incubated with SYBR Green I (10X final concentration) for 30 minutes in darkness at room temperature. Cell concentrations were then evaluated by flow cytometry as described above. Results are expressed as the percentage of adhering hemocytes with or without bacteria, relative to the initial hemocyte concentration.

2.7.5. Reactive oxygen species production

The reactive oxygen species (ROS) production of hemocytes was measured following a method from Bass *et al.* (1983) adapted to C. gigas by Lambert *et al.* (2003) and using 2'7'-dichlorofluorescein diacetate (DCFH-DA). For each sample, aliquots of 150 µl pooled hemolymph were diluted with FSSW (1:1, v:v) into a tube maintained on ice. DCFH-DA (final concentration of 0.01 mM) was added and tubes were incubated at 18°C. After 120 minutes of incubation, DCF fluorescence, quantitatively related to the ROS production of hemocytes without any stimulation (basal level), was measured at 500-530 nm by flow cytometry. Results are expressed as the mean DCF fluorescence (expressed in arbitrary units, A.U.) of the different

hemocyte sub-populations.

2.8. Statistical analysis

Two-way analysis of variance (2-way ANOVA) was performed for all biochemical and immune parameters using STATGRAPHICS Plus 5.1 statistical software (Manugistics, Inc., Rockville, MD, USA) to test the effect of dietary ration and sampling date. When a significant effect ($p<0.05$) of sampling date was found, Multiple Range Test (Fisher's least significant difference, LSD) was used to determine which means were significantly different from which others. Percentage data were transformed (arcsin of the square root) before ANOVA, but are presented in figures as untransformed percentage values.

3. Results

Statistical results from the 2-way ANOVA performed on all biochemical and immune parameters were summarized in Table 1. The most significant results are described hereafter.

3.1 Oyster dry weight

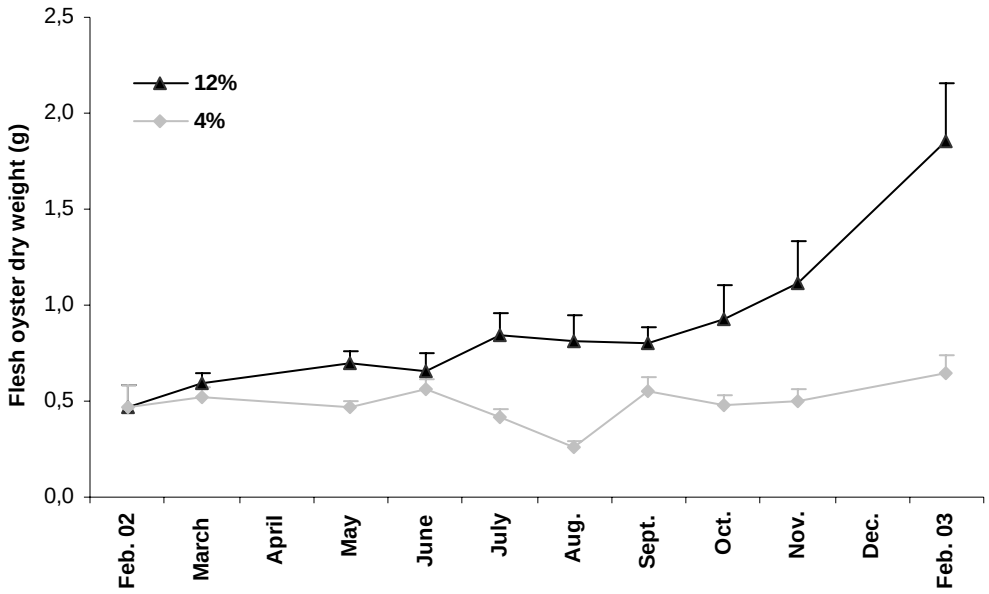
Mean dry weight (DW) of oysters fed the 12% diet was significantly higher than that of oysters fed the 4% diet during the entire experiment (Figure 2, 2-way ANOVA, $p<0.0001$). DW of oysters fed the 12% diet increased from February to July, then stayed at a constant level during summer, and increased again from September-October 2002 to February 2003. At the end of the experiment, mean DW of oysters fed the 12% diet increased, almost three fold from the beginning of the conditioning in February 2002 (0.47) to February 2003 (1.86). By contrast, mean DW of oysters fed the 4% diet was quite stable (except a short drop in August) and little increase from 0.47 to 0.64.

Table 1: Summary of the 2-way ANOVA performed on biochemical parameters, hemocyte concentrations and hemocyte activities.

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

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

Figure 2: Dry weight of oysters fed two dietary rations (4% and 12% of algal dry weight/oyster dry weight) during an average temperature cycle (similar to Marennes-Oléron, France) under hatchery controlled conditions (mean ± SE, n=15).



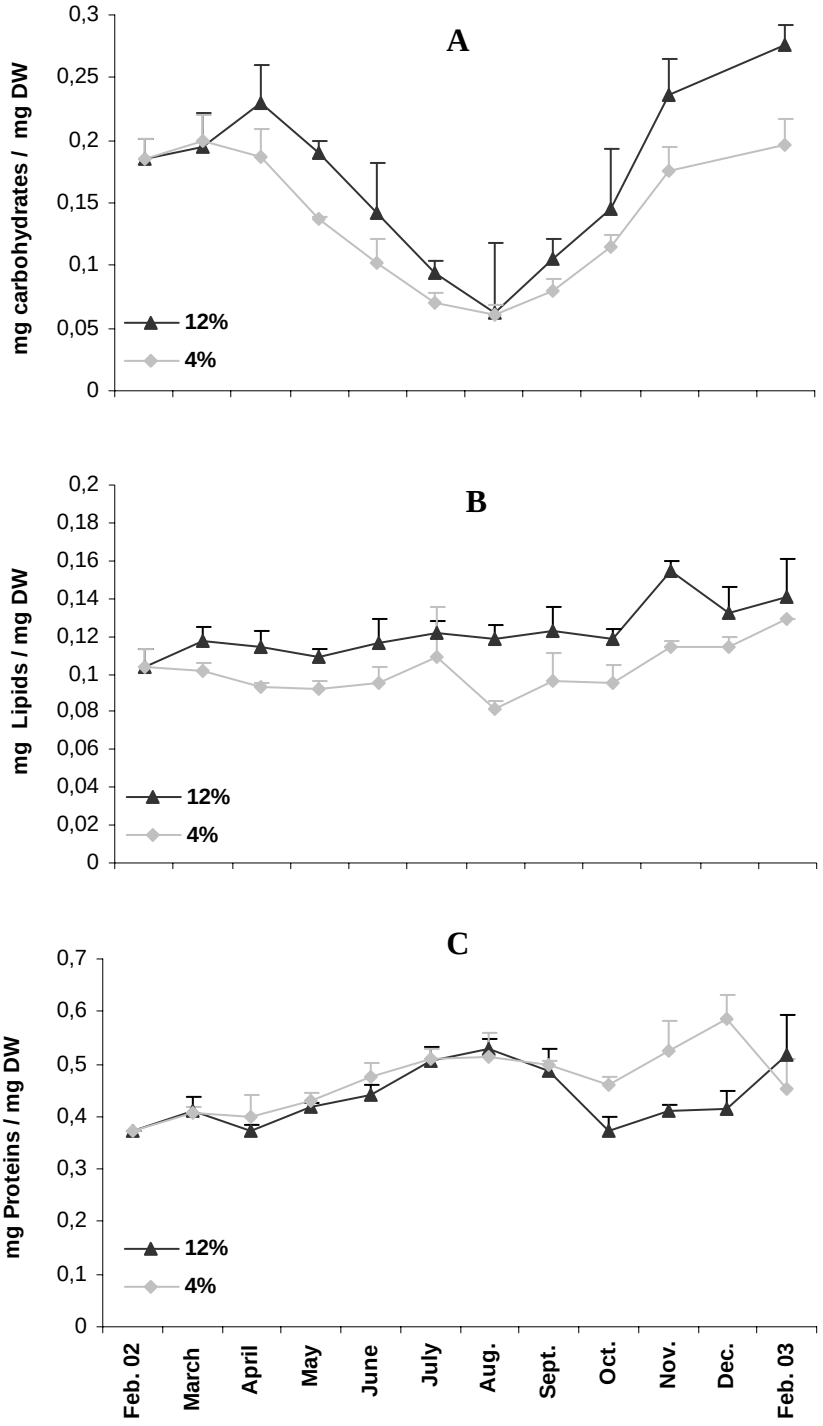
3.2. Total carbohydrate

Oysters fed the 12% diet had a significantly higher carbohydrate content than those fed the 4% diet (Figure 3A, 2-way ANOVA, $p<0.0001$). Carbohydrate content dropped from 0.23 mg and 0.19 mg in April for oysters, respectively, fed the 12% and 4% diet to 0.06 mg per mg of oyster DW in August for both dietary treatments. During the fall, feeding oysters with the 12% diet resulted in a faster and more intense restoration of carbohydrate content than the 4% feeding. Also, carbohydrate content was observed to be negatively correlated with temperature ($R^2 = 0.64$, $p<0.0001$).

3.3. Total lipids

Over the entire conditioning period, oysters fed the 12% diet had a significantly higher lipid content than those fed the 4% diet (Figure 3B, 2-way ANOVA, $p<0.0001$). Also, lipid content of oysters fed both dietary rations slowly increased from 0.10 mg per mg of oyster DW in February 2002 to 0.13 and 0.14 mg per mg of oyster DW in oysters fed 4% and 12% diets, respectively, at the end of the experiment.

Figure 3: Total carbohydrate (A), lipid (B) and protein (C) contents of oysters fed two dietary rations (4% and 12% of algal dry weight/oyster dry weight) during an average temperature cycle (similar to Marennes-Oléron, France) under hatchery controlled conditions (mean ± SD, n=3). Results are expressed as mg of carbohydrates, lipids and protein per mg of oyster dry weight.



3.4. Proteins

Oysters fed the 4% and 12% diet had similar protein contents over the whole experiment (Figure 3C, 2-way ANOVA, $p>0.05$) although protein content tended to be higher for oysters fed the 4% diet than those fed the 12% diet from October to December. Over the whole experiment, protein content increased steadily from 0.37 mg per mg of oyster DW in February 2002 to 0.52 mg per mg of oyster DW in February 2003 (2-way ANOVA, $p<0.001$).

3.5. Adenylate energy charge

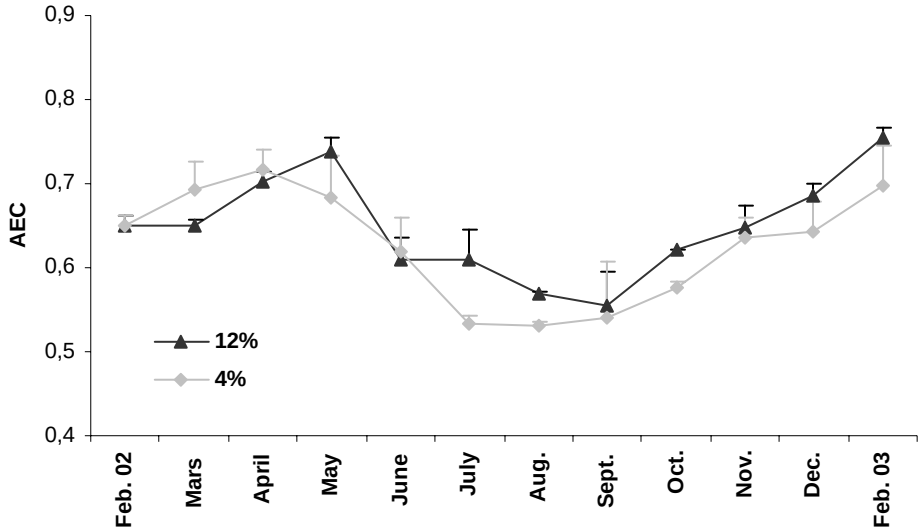
Mean AEC of oysters fed the 12% diet was significantly higher than that of oysters fed the 4% diet over the entire experiment (Figure 4, 2-way ANOVA, $p<0.01$). Changes of AEC can be divided in three phases for both dietary rations: (1) an increase until April and May for oysters fed both the 4% and 12% diet, (2) a decrease until September, with the lowest values in July for oysters fed the 4% diet and in September for those fed the 12% diet, (3) a parallel increase for the two diets until the end of the experiment in February 2003, reaching maximal values of 0.70 and 0.75 for oysters fed the 4% and 12% diets, respectively. Moreover, changes of AEC during the experiment paralleled changes in total carbohydrate. A significant and strong correlation between AEC and total carbohydrate content was observed ($p<0.0001$, $R^2 = 0.64$). AEC (as carbohydrate content) was also negatively correlated with sea water temperature ($R^2 = 0.32$, $p<0.01$).

3.6. Total, hyalinocyte and granulocyte concentrations

Total hemocyte and hyalinocyte (the largest hemocyte sub-population) concentrations were significantly affected by the dietary treatments while granulocyte concentration was not. Indeed, oyster fed the 12% diet had significantly higher total hemocyte and hyalinocyte counts than those fed the 4% diet (2-way ANOVA, $p<0.01$) when statistics were performed on pooled data.

Total hemocyte and hyalinocyte concentrations exhibited similar seasonal variations (2-way ANOVA, $p<0.0001$) while granulocyte concentration remained low and stable. Total hemocyte and hyalinocyte concentrations were high in March, decreased in April and then remained low from May to July. Then, both highly increased for oysters fed the 12% diet (to a lesser extent for oysters fed the 4% diet) and reached a maximum level in October. Finally, interactions between sample dates and dietary rations were found to be significant (2-way ANOVA, $p<0.05$) for the total hemocyte and hyalinocyte concentration.

Figure 4: Adenylate energy charge of oysters fed two dietary rations (4% and 12% of algal dry weight/oyster dry weight) during an average temperature cycle (similar to Marennes-Oléron, France) under hatchery controlled conditions (mean ± SD, n=3).



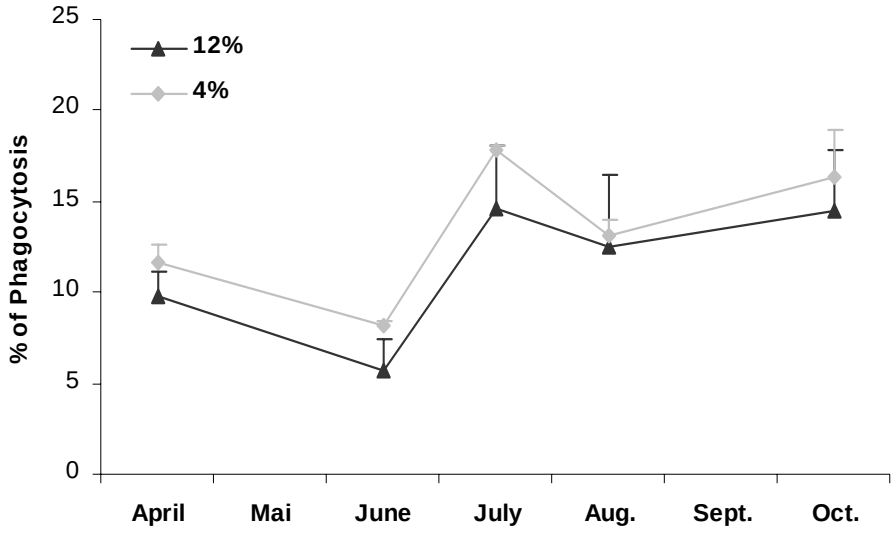
3.7. Hemocyte viability

During the experiment, hemocyte viability was not affected by dietary conditioning nor sampling date (data not shown, 2-way ANOVA, $p>0.05$). High viability percentages were observed with an average of 92.5% of viable cells in hemolymph samples (i.e. 7.5% dead cells).

3.8. Phagocytic activity

Over the entire experiment oysters fed the 4% diet had a significantly higher phagocytic activity than those fed the 12% diet (Figure 6, 2-way ANOVA, $p<0.05$). This activity varied significantly according to the sampling dates (2-way ANOVA, $p<0.0001$). An initial rapid decrease of the phagocytic activity from 10.6% in April to 6.9% in June was observed, followed by a sharp increase reaching 16.2% in July. Phagocytic activity then remained stable until October.

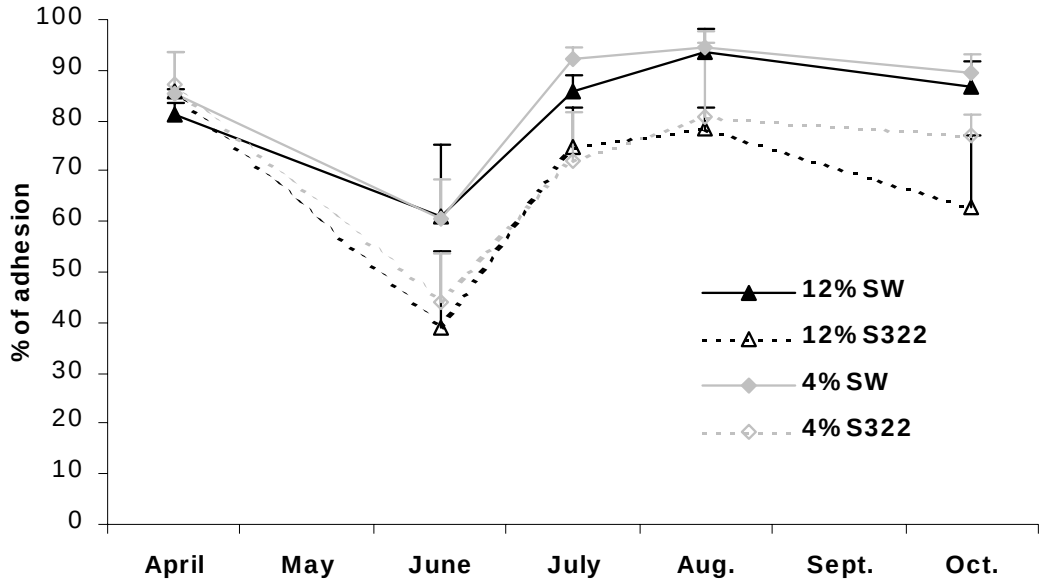
Figure 6: Phagocytic activity of hemocytes (expressed as percentage of hemocytes that have engulfed two beads and more) of oysters fed two dietary rations (4% and 12% of algal dry weight/oyster dry weight) during an average temperature cycle (similar to Marennes-Oléron, France) under hatchery controlled conditions (mean ± SD, n=3)



3.9. Adhesive capacity

Over the entire experiment, the adhesive capacity of hemocytes incubated with seawater or with the Vibrio sp. S322 was not significantly affected by the dietary rations (Figure 7, 2-way ANOVA, $p>0.05$). However, an effect of sampling date was detected for hemocytes incubated with or without the bacteria. Adhesive capacity of hemocytes in June (40% and 60% with and without the Vibrio sp. S322, respectively) was significantly lower than in April, July, August and October (an average of 77% and 88% with and without the Vibrio sp. S322, respectively) (2-way ANOVA, $p<0.0001$).

Figure 7 : Adhesive capacity of hemocytes incubated 3 hours with sterile seawater and in presence of the Vibrio sp. S322 after oysters were fed two dietary rations (4% and 12% of algal dry weight/oyster dry weight) during an average temperature cycle (similar to Marennes-Oléron, France) under hatchery controlled conditions (mean ± SD, n=3).



3.10. Reactive oxygen species production

Reactive oxygen species (ROS) production of hemocyte sub-populations after 120 minutes in FSSW was slightly affected by the dietary conditioning (Table 2). When all sampling dates were considered, mean granulocyte ROS production of oysters fed the 12% diet was significantly higher than the ROS production of oysters fed the 4% diet (2-way ANOVA, $p<0.05$). This was not the case for hyalinocytes (2-way ANOVA, $p>0.05$). ROS production of both hemocyte sub-populations was also seasonally affected ($p<0.0001$), mainly because of the high ROS production measured in April (371 A.U. for granulocytes and 306 A.U. for hyalinocytes), which was significantly higher than that measured at other sampling dates. From June to October, the ROS production of both hemocyte sub-populations was stable, with an average of 161 A.U. for granulocytes and 178 A.U. for hyalinocytes.

Table 2 : Reactive oxygen species production of hemocytes (expressed in Arbitrary Units; n=3; mean ± SD) from oysters fed two dietary rations (4% and 12% of algal dry weight/oyster dry weight) during an average temperature cycle (similar to Marennes-Oléron, France) under hatchery controlled conditions. Small letters indicated difference between sampling dates (Fisher's least significant difference).

		April	June	July	August	October
Granulocytes	**12%**	388 ± 41 ^{b}	161 ± 24 ^{ac}	155 ± 28 ^{a}	177 ± 43 ^{c}	194 ± 41 ^{ac}
	4%	364 ± 67 ^{b}	153 ± 5 ^{ac}	135 ± 19 ^{a}	182 ± 37 ^{c}	129 ± 30 ^{ac}
Hyalinocytes	**12%**	287 ± 5 ^{b}	166 ± 19 ^{a}	197 ± 22 ^{a}	172 ± 49 ^{a}	212 ± 29 ^{a}
	4%	330 ± 6 ^{b}	187 ± 49 ^{a}	168 ± 32 ^{a}	164 ± 2 ^{a}	155 ± 34 ^{a}

4. Discussion

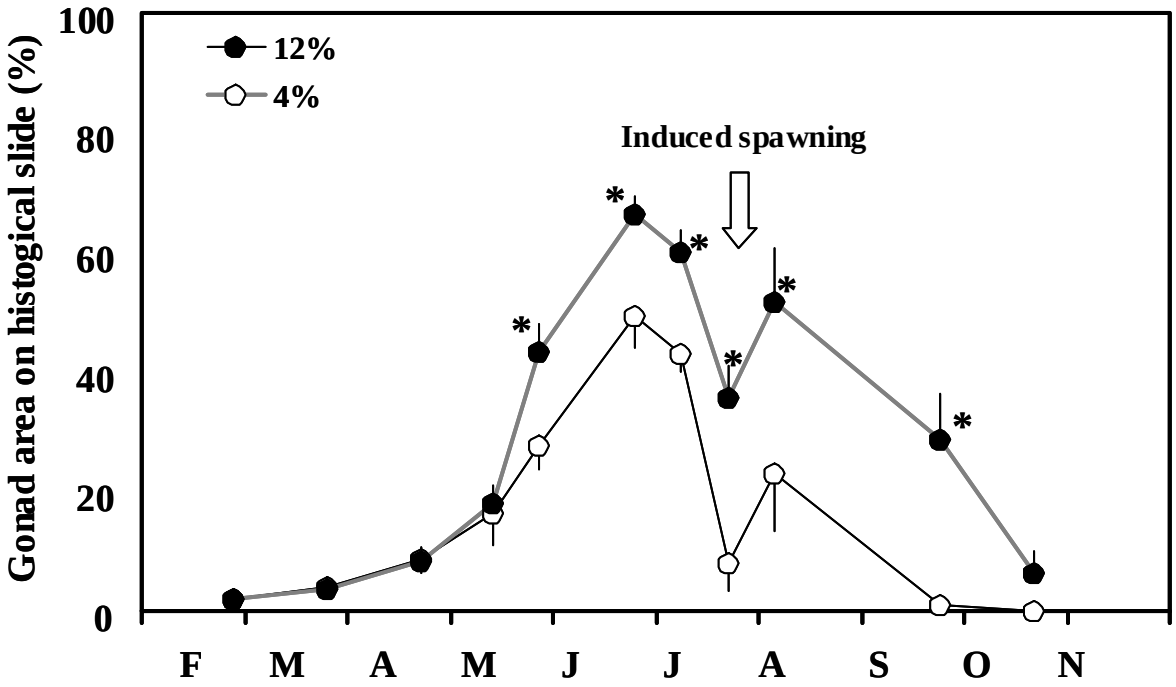
The present study tested the impact of food supply on the biochemical composition and hemocyte parameters of oysters over an annual cycle under experimentally-controlled conditions. A histological study was simultaneously performed by Enriquez-Diaz (2004), so discussion of the biochemical and hemocyte changes during reproductive process is possible. Combined, these data will contribute to a better understanding of the interactions between energetic, hemocyte parameters and reproductive processes.

4.1. Changes related to the reproductive processes

Irrespective of dietary rations, changes in all energetic and hemocyte parameters can be separated into two successive phases related to reproductive processes: a gametogenesis phase and a growth phase including glycogen storage and somatic growth.

Measures of energy status, such as the carbohydrate content and adenylate energy charge (AEC), revealed that there was a significant consumption of energy reserves from May to August and then a restoration of this compartment from October to February. Changes in these two parameters were significantly inversely correlated with seawater temperature in the rearing tanks. It is well known that increase and utilization of carbohydrate storage is a result of the balance between food supply and energy demands of two successive biological processes, reproduction and growth, both of which are temperature dependent. So, the depletion of carbohydrate content and AEC from May to August is considered to be a consequence of utilization of reserves for the gametogenic processes taking place during this period (Moal *et al.*, 1991 a and b). This result is in agreement with studies of Mori *et al.* (1965), Perdue and Erickson (1984), Ruiz *et al.* (1992) and more recently Li *et al.* (2000) and Berthelin *et al.* (2000). Thereafter, there is a restoration of the energy reserves of oysters in the fall (September-November). Histological analyses of Enriquez-Diaz (2004) (Figure 8) confirmed that those changes were related to the balance between the reproductive process, energy storage, and growth.

Figure 8 : **Importance of gonad area of oysters conditioned in same conditions than those of the present study and estimated on histological slide expressed as the percentage of gonad area in the visceral area (Enriquez-Diaz, 2004).**



Oysters had low hemocyte concentrations from April to July, which increased steadily to their highest concentrations in October. These changes were driven primarily by changes in concentration of hyalinocytes and agreed with the study of Fisher *et al.* (1996) on the seasonal hematological variability of eastern oysters, Crassostrea virginica. These authors observed a decline in hemocyte concentrations through the summer, reaching their lowest level in August and coinciding with the highest water temperature and active spawning by the oysters. In the present study, the lowest hemocyte concentration also corresponded with the highest water temperature, but not with active spawning of all oysters as only those fed 4% spawned completely in July (Enriquez-Diaz, 2004). The high hemocyte concentrations in September and October observed by Fisher et *al.* (1996) were associated to a decreasing temperature and to the end of spawning. We also proposed that more hemocytes may be generated in fall for gamete resorption and restructuring of gonad tissue. This concurred with histological observations implicating hemocytes in gamete resorption and storage tissue restoration reported by Mathieu *et al.* (2003).

Intriguingly, other studies on oysters, C. virginica (Chu and La Peyre, 1993; Volety *et al.*, 1999; Chu, 2000), revealed a higher hemocyte concentration during summer than during winter. A higher hemocyte concentration in summer has also been observed for other species such as the blue mussel Mytilus galloprovincialis (Carballal *et al.*, 1998) and the Manila clam Ruditapes philippinarum (Soudant *et al.*, 2004). It is possible that the contrasting results are related to the presence of pathogens. Indeed, an increase of circulating hemocytes was often reported in bivalves infected with pathogens (Ford *et al.*, 1993; Paillard *et al.*, 1996; Carballal *et al.*, 1998;

Ordas *et al.*, 2000; Cochennec-Laureau *et al.*, 2003).
With regard to hemocyte activities, the lowest phagocytic activity was reported in June when temperatures reached 19°C and when oysters had low energy reserves and exhibited maximum gonad growth (Enriquez-Diaz, 2004). This occurred simultaneously with a loss of adhesive capacity, accentuated when challenged in vitro with Vibrio sp. S322. Interestingly, a similar pattern was observed for diploid and triploid oysters reared in fields (P. Soletchnik and B. Gagnaire, personnal communications). As the decrease of phagocytic activities was stronger in diploid oysters than in triploid oysters, the authors argued that when gonads of diploid oysters are ripened, oysters have to insure maintenance of the gonad tissue and, thus, can not spare energy for hemocyte activities such as phagocytosis. Nevertheless triploids which did not invest energy in reproduction also exhibited a decrease in June. At this point, it is unclear what exogenous and endogenous parameters may be related to the loss of phagocytic activity, as changes in phagocytic activity were not correlated with either maximum seawater temperature or minimum energy content (as the lowest energy contents and the highest temperature were recorded in August in this study). The relationship between hormonal factors and hemocyte activities during oyster reproductive processes should be investigated, as hormones like estradiol and progesterone were demonstrated to inhibit phagocytosis activity of macrophages in the common carp Cyprinus carpio (Yamaguchi *et al.*, 2001; Watanuki *et al.*, 2002).
Although the initial decrease in ROS production from April to June could be linked with the decrease of phagocytosis observed during the same period, the ROS production remained low thereafter until the end of the experiment, while phagocytosis increased. In natural populations of C. virginica, Fisher *et al.* (1996) reported seasonal variation of ROS production with a decrease from spring to summer, followed by an increase during the fall and winter months. These authors suggested that ROS production can be related to temperature variation, but was not clearly linked to the reproductive cycle of oysters. In the present study, no relationships could be established between ROS production with either reproduction processes or temperature variation.

4.2. Changes related to dietary rations
In the present study, dry weights (DW) of oysters fed the 12% diet increased over the whole experiment while the dry weights of oysters fed the 4% diet was quite stable. This suggested that energy supply was sufficient to allow oysters to grow despite the energy cost of the gametogenic process. However, changes in carbohydrate content and AEC between oysters fed the two dietary rations indicated that energy reserves decreased during gametogenesis (from May to August) irrespective of the diet ration (12%, 4%). The lowest AEC values reached in August indicated partially detrimental conditions according to Ivanovici (1980). The additional food provided by the 12% diet appeared as insufficient as the 4% diet to compensate for the energy reserve decrease (carbohydrate and AEC) observed during gametogenesis. The additional energy provided by the 12% diet was mostly invested in gametogenesis as corroborated by the higher lipid content in oysters fed the 12% diet compared to those fed the 4% diet. Increase in lipid content is generally associated with gonad development. Enriquez-Diaz (2004) also showed histologically that, at the maximum period of gametogenesis (mid-summer), the percentage of gonad area of oysters fed the 12% diet was 66% while it was only 49% for oysters fed the 4% diet (Figure 8). Agreement between biochemical and histological data shows that the reproductive effort depends on the dietary rations. Thus, during the reproductive period, the more oysters are fed, the more energy they devote to reproductive processes. Such a reproductive effort for oysters fed the 12% diet compared to oysters fed the 4% diet may indeed be considered as more physiologically stressful. Indeed, at this date, it was observed that the scope for growth of oysters was more deeply negative for oysters fed the 12% diet than those fed the 4% diet

(Enriquez-Diaz, 2004). This led us to suggest that these oysters may have insufficient energy to invest in their immune system and fight against bacteria infection during gametogenesis. This may explain the higher susceptibility of oysters fed the 12% diet observed in the experimental bacterial infection with Vibrio lentus which was performed in July 2002 (Pouvreau *et al.*, 2003). Thereafter, during the second phase of the experiment (August to the end), the additional food inputs was heavily invested in growth and energy storage.

Hemocyte parameters were also affected by the dietary rations but to a lesser extent compared to the seasonal effect. Oysters fed the 12% diet had a significantly higher hemocyte concentration than those fed the 4% diet. It is possible that feeding oysters with the 12% diet allowed them to invest some energy in processes such as hemocyte production especially during the gonad resorption and restructuring phase.

During the experiment, oysters fed the 12% diet had slightly lower phagocytic activity. The lower hemocyte activity was unrelated to energy content as carbohydrate content and AEC values were significantly higher for oysters fed the 12% diet during the entire experiment than oysters fed the 4% diet. Phagocytic activity was possibly more affected by reproductive effort, as oysters fed the 4% diet transferred less energy in the development of gonad tissue compared to those fed the 12% diet. Statistical analysis also revealed a higher ROS production of granulocytes for oysters fed the 12% diet, compared to oysters fed the 4% diet. This concurred with recent results of Hégaret *et al.* (2004) showing a higher values of ROS production for granulocytes of oysters fed the 50% diet than those fed a 10% diet.

5. Conclusion

Through the present experiment, we demonstrated that the hemocyte parameters of oysters were more influenced by reproduction related to seasonal temperature variation than by food availability. Active gametogenesis in May-June resulted not only in a reduction of energy reserves, but in a reduction of number of circulating hemocytes (mostly hyalinocytes), adhesion capacity and phagocytic activity. Additionally, oysters fed the 12% diet, which demonstrated the highest reproductive effort, have lower phagocytic activity and ROS production than oysters fed the 4% diet. This led us to conclude that hemocyte functions may be perturbed by high reproductive effort.

6. Acknowledgements

We are grateful to Dr Lionel Dégremont and co-workers for the hatchery production of the oysters used in the present study. We want also to thank our colleagues of the Argenton hatchery for taking care of oysters during this one year conditioning and Dr J.L. Nicolas for supplying the Vibrio sp. S322. The authors would like to thank Dr Gary H. Wikfors, NOAA Fisheries, Northeast Fisheries Center (Connecticut, USA) and Dr Vincent G. Encomio, Florida Gulf Coast University (Florida, USA), for the critical review of this manuscript. This research was a part of the MOREST project supported by a grant from IFREMER, the Région Bretagne, Région Normandie, Région Pays de Loire, Région Poitou-Charente and the Conseil Général du Calvados. Contribution N°965 of the IUEM, European Institute for Marine Studies (Brest, France).

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