Characterisation of physiological and immunological differences between Pacific oysters (Crassostrea gigas) genetically selected for high or low survival to summer mortalities and fed different rations under controlled conditions

Maryse Delaporte\textsuperscript{a}, Philippe Soudant\textsuperscript{b, *}, Christophe Lambert\textsuperscript{b}, Marine Jegaden\textsuperscript{b}, Jeanne Moala\textsuperscript{a}, Stéphane Pouvreau\textsuperscript{a}, Lionel Dégremont\textsuperscript{c}, Pierre Boudry\textsuperscript{c}, Jean-François Samain\textsuperscript{a}

\textsuperscript{a}Laboratoire de Physiologie des Invertébrés, centre IFREMER de Brest, BP 70, 29280 Plouzané, France.
\textsuperscript{b}Laboratoire des Sciences de l’Environnement Marin, UM R 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Place Copernic, Technopôle Brest-Iroise, 29280 Plouzané, France.
\textsuperscript{c}Laboratoire de Génétique et Pathologie, IFREMER, 17390 La Tremblade, France.

*: Corresponding author : P. Soudant, phone: + 33 (0) 2 98 49 86 23, fax.: + 33 (0) 2 98 49 16 86 45, email address : Philippe.Soudant@univ-brest.fr

Abstract:

Within the framework of a national scientific program named “MORtalités ESTivales de l’huître creuse Crassostrea gigas” (MOREST), a family-based experiment was developed to study the genetic basis of resistance to summer mortality in the Pacific oyster, Crassostrea gigas. As part of the MOREST project, the second generation of three resistant families and two susceptible families were chosen and pooled into two respective groups: “R” and “S”. These two groups of oysters were conditioned for 6 months on two food levels (4% and 12% of oyster soft-tissue dry weight in algal dry weight per day) with a temperature gradient that mimicked the Marennes–Oléron natural cycle during the oyster reproductive period. Oyster mortality remained low for the first two months, but then rapidly increased in July when seawater temperature reached 19 °C and above. Mortality was higher in “S” oysters than in “R” oysters, and also higher in oysters fed the 12% diet than those fed 4%, resulting in a decreasing, relative order in cumulative mortality as follows; 12% “S” > 12% “R” > 4% “S” > 4% “R”.

Although the observed mortality rates were lower than those previously observed in the field, the mortality differential between “R” and “S” oysters was similar. Gonadal development, estimated by tissue lipid content, followed a relative order yielding a direct, positive relationship between reproductive effort and mortality as we reported previously by quantitative histology. Regarding hemocyte parameters, one of the most striking observations was that reactive oxygen species (ROS) production was significantly higher in “S” oysters than in “R” oysters in May and June, regardless of food level. The absence of known environmental stress under these experimental conditions suggests that the ROS increase in “S” oyster could be related to their higher reproductive activity. Finally, a higher increase in hyalinocyte counts was observed for “S” oysters, compared to “R” oysters, in July, just before mortality. Taken together, our results suggest an association of genetically based resistance to summer mortality, reproductive strategy and hemocyte parameters.

Keywords: Crassostrea gigas; Genetic selection; Hemocyte parameters; Reactive oxygen species (ROS); Reproduction; Summer mortality
1. Introduction

Summer mortalities of the Pacific oyster, *Crassostrea gigas*, were first reported in the 1940s in Japan (Koganezawa, 1974), in the late 1950s on west coast of North America (Glude, 1974; Koganezawa, 1974; Cheney et al., 2000), and in early 1980s in France (Goulletquer et al., 1998). These seasonal mortalities affect both adults and juveniles, with no specific clinical signs of disease.

To date, some pathogenic agents have been detected and isolated during summer-mortality events (Elston et al., 1987; Friedman and Hedrick, 1991; Lacoste et al., 2001; Le Roux et al., 2002; Waechter et al., 2002; Gay et al., 2004; Garnier et al., in press), but these organisms have not been clearly and systematically implicated in mortalities. One common feature of these summer-mortality events is that they are associated with at least one of the following parameters: high trophic conditions, elevated summer temperatures, and coincidence with the period of sexual ripeness in oysters (Soletchnik et al., 1999; Soletchnik et al., 2003; Soletchnik et al., 2005). Only a few experimental studies, however, have confirmed this contention (Lipovsky and Chew, 1972; Perdue et al., 1981). The high energetic cost associated with reproduction, combined with high summer temperatures, was hypothesized to weaken the oysters and make them more susceptible to opportunistic pathogens (Perdue et al, 1981, Koganezawa, 1974). Findings from MOREST, a national multidisciplinary program initiated in France in 2001, show that other environmental and potentially-stressful factors associated with rain, aquaculture practices, and sediment quality also seemed to be related to oyster summer mortality (Soletchnik et al., 2003; Soletchnik et al., 2005). Moreover, summer mortality was found to be linked, to some extent, to genetic variability in oysters (Beattie et al., 1980; Hershberger et al., 1984; Ernande et al., 2004). During the MOREST project, bi-parental families were bred in the hatchery following a half-sib nested design and deployed in three rearing sites (Ronce, Rivière d’Auray and Baie des Veys) during the summer...
2001. At the end of the summer period, family had the largest variance-component for survival (46%) (Dégremont et al., 2005). Heritability of spat survival was estimated to be very high (Dégremont et al., 2007). In 2002, families selected for high (called “R” for resistant) or low (“S” for susceptible) survival were used to produce a second generation which tested in the field under similar conditions as the previous year. In October, the mortality of the “R” oysters was 2%, 12% and 6% in Ronce, Rivière d’Auray, and Baie des Veys sites, respectively, but consistently higher, 23%, 42% and 32% for the “S” oysters. Once again, second generation family represented the largest variance (61%), and this second field experiment confirmed that survival is a highly heritable trait (Dégremont, 2003). Other family-based, selective-breeding programs also have shown high broad-sense heritability for survival in C. gigas (Evans and Langdon, 2006) and C. virginica (Dégremont, personal communication) and realized heritability for yield, a parameter combining survival and growth, in C. gigas on the US West Coast (Langdon et al., 2003). Clearly a significant genetic effect was observed in the complex summer mortality phenomenon.

Little information is available, however, on the physiological basis of divergent selection for “S” vs “R” oysters. Within the framework of MOREST, several field and laboratory studies were performed to compare various biological parameters in “R” and “S” oyster families, or groups of families, to explain survival differences (Samain et al., in press). As mentioned before, the high energetic cost associated with reproduction, combined with high summer temperatures and other possible stresses, is suspected to weaken the oysters and make them more susceptible to opportunistic pathogens. As capability of an oyster to react to diseases, injuries or parasite infestation depends upon innate, humoral and cellular defence mechanisms (Cheng, 2000; Chu, 2000), it appears pertinent to assess whether or not survival traits include better immune responses.
One approach to assessing immune responses of oysters is to measure hemocyte parameters (descriptive and functional). Indeed, hemocytes are considered to be the main cellular mediators of the defence system in bivalves (Volety and Chu, 1995; Cheng, 1996), responsible for recognition, phagocytosis, and elimination of non-self particles by microbicidal activities (Pipe, 1992; Cheng, 2000; Chu, 2000). Recently, we reported that some hemocyte activities (phagocytosis, adhesion) decreased during gametogenesis, especially when gonads approach ripeness (Delaporte et al., 2006a; Gagnaire et al., 2006). Other studies (Enriquez-Diaz, 2004) demonstrated by histological analysis that “S” families from the first generation exhibited earlier and higher gonad development than “R” families when reared together in Rivière d’Auray (France).

In the present study, the objective was to assess whether or not different survival of summer mortalities is related to reproductive, energetic, or immune status evaluated by quantifying biochemical and hemocyte parameters. These parameters were assessed on a subsample of animals from a group of three “R” families and a group of two “S” families produced by divergent selection and evaluated in the field, as reported above. These groups were compared in experimental conditions during the period of active reproduction (from April to August 2003). To exacerbate any putative difference in reproductive strategy between “R” and “S” oysters, and thus assess interactions between reproduction and survival phenotype, oysters of both “R” and “S” groups were fed two levels of food (4% and 12% of oyster dry weight in algal dry weight per day).

2. Materials and Methods
2.1. Oyster conditioning

Second generation (G2) of summer mortality-susceptible “S” and -resistant “R” oyster families were produced in 2002 in the IFREMER hatchery at La Tremblade (Charente, France) from broodstocks selected based upon the survival phenotype in 2001 (Dégremont et al., 2003). From each selected F1 family, 25 females and 25 males were used as parents to produce a F2 family. Spat of G2 “S” and “R” families were reared at the IFREMER station in Bouin (Vendée, France), a cold-water site, to prevent summer mortality, and then kept in a commercial hatchery in Normandy (France) during the winter period of 2002-2003. In March 2003, one-year-old oysters from three second generation resistant families and two second generation susceptible families were combined to constitute one stock of resistant oysters and one of susceptible oysters. Each stock was separated in two 700-L raceways to be fed 4% and 12% of oyster dry weight in algal dry weight per day (termed as 4% and 12% diets) from April to August 2003 at the IFREMER experimental hatchery in Argenton (Finistère, France). The algal diet consisted of a mixture of four micro-algae: T-Iso (*Isochrysis affinis galbana*, clone Tahiti), *Chaetoceros calcitrans*, *Skeletonema costatum* and *Tetraselmis chui* provided in equal biomass proportions. During the dietary conditioning, the annual average of photoperiod and temperature cycle of Marennes-Oléron was applied, as described by Delaporte et al. (2006a). Tanks and oysters were cleaned daily, and oyster mortality was monitored. Each month from April to August, ten oysters were sampled from each group to analyse the biochemical and hemocyte parameters.

2.2. Biochemical parameters and condition index

Each month, shell weight and flesh wet weight of 10 oysters were measured after withdrawal of hemolymph for hemocyte parameter analysis described below. Individual animals were frozen in
liquid nitrogen (-196°C) and ground with a Dangoumeau homogeniser; the resulting homogenate was stored at -80°C for latter biochemical analysis. To assess whole, oyster-flesh dry weight, a known amount of the above homogenate was weighed in a pre-weighed aluminium cup, dried for 48h at 80°C and then weighed again. A dry weight / wet weight ratio was estimated from these measurements and used to back-calculate individual whole, oyster-flesh dry weight. Condition index of individual oysters was then calculated as described previously (Walne and Mann, 1975), following the formula: dry flesh weight / dry shell weight X 1000.

Biochemical analyses on homogenates (stored at -80°C) of 10 individual oysters were performed as previously described (Delaporte et al., 2006a). Total lipid content was estimated according to (Bligh and Dyer, 1959) and carbohydrate content was measured colorimetrically (Dubois et al., 1956). Carbohydrate and lipid contents were expressed as mg of lipid or carbohydrate per mg of oyster dry weight.

2.3. Measurements of hemocyte parameters by flow cytometry

Characterisation of hemocyte sub-populations, number and functions were performed using a FACScalibur (BD Biosciences, San Jose, CA USA) flow cytometer equipped with a 488 nm argon laser. As recommended by FCM manufacturer, all samples were filtered through 80µm mesh prior to analysis to eliminate any large debris (> 80 µm) which could potentially clog the flow cytometer. Methods for measuring hemocyte parameters are described hereafter.

2.3.1. Hemolymph sampling

Hemolymph was withdrawn from individual oysters using a 1 ml plastic syringe fitted with a 25-gauge needle inserted through a notch made adjacent to the adductor muscle just prior to
bleeding. All hemolymph samples were examined microscopically to check for contamination (e.g., gametes, tissue debris) and then stored in micro-tubes held on ice.

Two kinds of hemocyte parameters were evaluated on hemolymph: descriptive parameters (hemocyte viability and total and hemocyte sub-population concentrations), and functional ones (phagocytosis, adhesion assay and reactive oxygen species (ROS) production). Analyses were done as described below.

2.3.2. Descriptive parameters: Hemocyte viability, total and hemocyte sub-population concentration

These parameters were measured individually on 10 hemolymph samples, for each sampling date and each condition (4 and 12% diet, R and S). An aliquot of 100 µl of individual hemolymph was transferred into a tube containing a mixture of Anti-Aggregant Solution for Hemocytes, AASH (Auffret and Oubella, 1995) and filtered sterile seawater (FSSW), 200 µl and 100 µl respectively. Hemocyte DNA was stained with two fluorescent DNA/RNA specific dyes, SYBR Green I (Molecular probes, Eugene, Oregon, USA, 1/1000 of the DMSO commercial solution), and propidium iodide (PI, Sigma, St Quentin Fallavier, France, final concentration of 10 µg ml$^{-1}$) in the dark at room temperature ($20^\circ$C) for 60 minutes before flow-cytometric analysis. SYBR Green I permeates both dead and live cells, while PI permeates only through membranes of dead cells. SYBR Green and PI fluorescences were measured at 500-530 nm (green) and at 550-600 nm (red), respectively, by flow-cytometry. Thus, by counting the cells stained by PI and cells stained by SYBR Green, it was possible to estimate the percentage of viable cells in each sample. 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 small agranulocytes have low FSC and SSC. Thus, the three sub-populations were distinguished according to their size and cell complexity (granularity). Total hemocyte, granulocyte, hyalinocyte, and small agranulocyte concentrations estimated from the flow rate measurement of the flow-cytometer (Marie et al., 1999) as all samples were run for 30 sec. Results were expressed as number of cells per ml. Small agranulocyte concentrations are not presented in this report because they represented only a small proportion of the total hemocyte count and are considered to possess little activity (Lambert et al., 2003).

2.3.3. Functionnal parameters

These parameters were measured on pool of hemolymph. For each sampling date and each condition (4 and 12% diet, “R” and “S”), hemolymph from at least five animals was pooled and analyses were ran on three pools of five individuals.

2.3.3.1. Phagocytosis

An aliquot of 100 µL pooled hemolymph, diluted with 100 µL of FSSW, was mixed with 30 µL of YG, 2.0-µm fluoresbrite microspheres, diluted to 2% in FSSW (Polysciences, Eppelheim, Germany). After 120 minutes of incubation at 18°C, hemocytes were fixed with 230 µL of a 6% formalin solution and analysed at 500-530 nm by flow cytometry to detect hemocytes containing fluorescent beads. The percentage of phagocytic cells was defined as the percentage of hemocytes that had engulfed three or more beads (Delaporte et al., 2003).

2.3.3.2. Adhesion assay
Hemocyte adhesion assays were performed according to the procedure reported previously (Delaporte et al., 2006a), adapted from another study (Choquet et al., 2003). Briefly, a 100µL aliquot of pooled hemolymph was allowed to adhere in an 24-well microplate, either in sterile seawater or in seawater with *Vibrio* sp. S322 (50 bacteria/ hemocyte), a strain known for its pathogenicity to bivalve larvae (Nicolas et al., 1996). After three hours of incubation, non-adhering cells were fixed in 6% formalin solution and stained using SYBR Green I (final concentration, 1/1,000 in DMSO) and then detected and counted by flow-cytometry. Results are expressed as the percentage of adhering hemocytes incubated with FSSW or bacteria, relative to the initial hemocyte count.

2.3.3.3. Reactive oxygen species production

Reactive oxygen species (ROS) production by untreated hemocytes was measured using 2'7'-dichlorofluorescein diacetate, DCFH-DA (Lambert et al., 2003). A 100 µL aliquot of pooled hemolymph was diluted with 300 µl of FSSW. Four µL of the DCFH-DA solution (final concentration of 0.01 mM) was added to each tube maintained on ice. Tubes were then incubated at 18°C for 120 minutes. After the incubation period, DCF fluorescence, quantitatively related to the ROS production of untreated hemocytes, was measured at 500-530 nm by flow-cytometry. Results are expressed as the mean geometric fluorescence (in arbitrary units, AU) detected in each hemocyte sub-population.

2.4. Statistical analysis

Three-way, multifactor analysis of variance was performed to compare biochemical and hemocyte parameters (independent variables) according to diet, phenotype (summer mortality susceptible and resistant oysters), and sampling date. Percentage data were transformed (as
arcsine of the square root) before MANOVA, but are presented in figures and tables as untransformed percentages. The method used to discriminate between the means was Fisher’s least significant difference (LSD) procedure. Results were deemed significant at $p<0.05$. All statistical analyses were performed using STATGRAPHICS Plus 5.1 (Manugistics, Rockville, USA).

3. Results

All statistical analyses are summarized in Table 1. Immediately apparent is a significant difference between “R” and “S” oyster phenotypes, for biochemical parameters (carbohydrate and lipid contents) and for hemocyte parameters (hyalinocyte counts and ROS production of hyalinocytes and granulocytes). Details are presented below.

3.1. Oyster mortality

Cumulative mortality was monitored throughout the experiment. Cumulative mortality remained below 5% during the first two months. A rapid increase in mortality was noticed in July, up to a maximum of 19% (Figure 1), resulting in differences in cumulative mortality according to dietary treatment and oyster phenotype. Oysters fed the 12% diet exhibited higher mortalities than those fed the 4% diet, while “S” oysters showed higher mortalities than “R” oysters. Consequently, a gradient in oyster cumulative mortality was observed as follows: 12% “S” oysters > 12% “R” oysters > 4% “S” oysters > 4% “R” oysters, from July until the end of the experiment.

3.2. Condition index
Condition index was significantly affected by food level (Figure 2, MANOVA, p<0.0001), but
not by oyster phenotype or sampling date. Oysters fed the 12% diet had a higher mean condition
index than those fed the 4% diet. However, it should be noted that condition index of summer
mortality-susceptible oysters fed the 12% diet dropped significantly (only in July) compared to
corresponding, resistant oysters. This selective drop in condition index resulted in significant
interactions between time and diet (P<0.05) and between time and phenotype (P<0.01).

3.4. Biochemical composition

Carbohydrate content of oysters was significantly affected by oyster phenotype (Figure 3A,
p<0.05), with “S” oyster families having a higher mean carbohydrate content compared to “R”
oysters. Also, a significantly-higher carbohydrate content was reported in oysters fed the 12%
diet, compared to oysters fed the 4% diet (p<0.05). Carbohydrate content was also greatly
affected by sampling date (p<0.0001), decreasing steadily from May to August. Finally, a
significant interaction was noted between diet and time.

A significantly-higher mean lipid content was observed in “S” oysters compared to “R” oysters
(Figure 3B, p<0.01). Total lipid content was also significantly affected by the dietary
conditioning (p<0.0001). Oysters fed the 12% diet contained a higher lipid content than those fed
the 4% diet. As with carbohydrate content, total lipid content changed significantly over time
(p<0.05); total lipid content increased during the course of the experiment. Note, however, that
lipid content of “S” oysters fed both 4 and 12% diets dropped temporarily in July, but this drop in
lipid occurred one month later for “R” oysters. Interaction between time and phenotype was the
only significant interaction for the lipid content and was likely attributable to this drop in July for
the “S” oyster families.
3.5. Hemocyte viability

No significant difference was observed between dietary treatments, nor between summer mortality-susceptible and resistant oyster families. Percentages of alive hemocytes ranged from 95.8% to 99% during the course of the experiment (data not shown).

3.6. Total hemocyte, granulocyte and hyalinocyte concentrations

As total hemocyte concentration was mostly represented by hyalinocytes and granulocytes, only changes in these hemocyte sub-population concentrations of “R” and “S” oysters fed the two dietary conditionings are detailed below.

Counts of hyalinocytes, which represented the predominant hemocyte sub-population, were significantly different in the two oyster phenotypes (Figure 4B, p>0.05) and were affected by dietary conditioning as well (Table 1, p<0.0001). Granulocyte counts were not affected by either factor (diet or oyster phenotype). During the entire experiment, “S” oysters contained more circulating hyalinocytes than “R” oysters, especially in July. Feeding oysters the 12% diet resulted in a higher hyalinocyte count than in oysters fed the 4% diet (Table 1). Neither oyster phenotype nor dietary treatment affected total hemocyte or granulocyte concentrations. The most significant changes in total hemocyte, hyalinocyte and granulocyte counts were related to the sampling date; mainly there was an appreciable increase in both cell types in July.

3.7. Phagocytosis

Results showed that neither phenotype nor dietary effects were observed (Table 1). Phagocytosis varied significantly between sampling dates (Figure 5) with the lowest phagocytic activity in May and the highest in June (p<0.0001).
3.8. Adhesion capacity

As with hemocyte phagocytic activity, neither phenotype nor diet affected adhesion capacity of hemocytes, incubated with FSSW or with *Vibrio* sp. S322. We note the large variations in this parameter (Table 1).

3.9. Reactive oxygen species production

Production of reactive oxygen species (ROS) by untreated hemocytes was greatly influenced by oyster phenotype (Table 1) for both hyalinocyte and granulocyte sub-populations (MANOVA, p<0.001 and p<0.01 respectively). ROS production was higher in untreated hemocytes from “S” oysters than in “R” oysters in May and June (Figure 7A and B). There was no diet effect on ROS production of either hemocyte sub-population. The observed, statistically-significant interaction between time and phenotype for granulocyte ROS production indicates that this activity had a different temporal pattern, according to oyster phenotype, during the experiment.
4. Discussion

Within the framework of France’s national program on oyster summer mortality, MOREST, a family-based genetic experiment was developed to demonstrate that oysters can be selected on their survival. The ‘survival’ trait was shown to be highly heritable (Dégremont et al., 2007) and the difference in survival between “R” and “S” selected oysters was confirmed over several successive generations. Although survival performance is highly heritable, the physiological basis of summer-mortality resistance is especially difficult to assess in the field, as all possibly-contributing environmental factors can not be easily and exhaustively measured. Therefore, the present study was undertaken to measure the physiological and survival performances of summer mortality resistant “R” and susceptible “S” oysters under controlled, experimental conditions.

Several laboratory experiments indicated that broodstock conditioning over 19°C with high food supply often resulted in progressive mortality (Samain, unpubl. oyster-conditioning trials). This mortality was often associated to the presence of *Vibrio aestuarianus* which has been isolated recurrently from moribund oysters in hatcheries (Garnier et al., in press).

In the present study, mortality of three resistant and two susceptible families of second generation G2 was monitored under controlled, experimental conditions with two food rations. Oyster mortality remained low for 2 months (April, May), but then began to rise in June, and then increased rapidly in mid-July when seawater temperature was above 19°C. As expected, “S” oysters experienced higher mortality than “R” oysters. The difference in mortality rate between “S” and “R” oysters in the present study was in good agreement, although at a lesser extent, with that observed for the same “S” and “R” groups of oysters in field conditions when water temperature reached the same critical threshold, 19°C (Soletchnik et al., 2003; Soletchnik et al., 2005). This observation showed thus that survival trait of selected oysters can also be detected under controlled conditions.
Possible relationships between differential survival and physiological parameters (reproduction, energy allocation, immunology) were explored and discussed hereafter.

Reproductive parameters and mortality:

As previously described (Enriquez-Diaz, 2004; Delaporte et al., 2006a), high trophic condition (12% of algal dry weight per oyster dry weight) resulted in a higher gonad development than low trophic condition (4%). Data showed a higher lipid content in “S” oysters compared to “R” oysters, mainly during the first part of vitellogenesis until June. Gonad development estimation using lipid content has been shown several times to be a good indicator of maturation level (Deslous-Paoli et al., 1981; Soletchnik et al., 1999; Soletchnik et al., 2002) and was found to be in good agreement with gonad area estimation using quantitative histological techniques in controlled conditions (Delaporte et al., 2006a). Thus, this result suggests that the gametogenic process may depend upon the oyster phenotype. “S” oysters invested more in reproduction than “R” oysters under the same trophic conditions. Differential reproduction effort could explain, at least partially, the difference in mortality observed not only between “S” and “R” oysters, but also between oysters fed 4% and 12% diets. Indeed, experimental infections by *V. splendidus* resulted in a higher mortality in oyster fed the 12% diet than those fed 4% (Gay, 2004).

The difference of mortality between “R” and “S” oysters accelerated after July at the end of vitellogenesis. During this period, increases in turbidity were observed in the tank by continuous optical records using the MAREL system (Bourles, 2004) that are likely to correspond with gamete emission. These turbidity increases were more frequently observed for “S” oysters fed the 12% diet, compared to “R” oysters fed the same diet (data not shown). These gamete emissions were confirmed in July by the lower condition index and lipid content in “S” oysters compared to “R” oysters when both groups were fed the 12% diet. However, the CI drop does not correspond...
to a massive spawning, but rather to more frequent, partial spawnings in “S” oysters. Thus, when considering the entire gametogenic process, the “S” oyster families produced a more intense reproductive effort than “R” oysters, as previously shown for the first generation of selected oysters reared in the field at Fort Espagnol (Morbihan, France) (Enriquez-Diaz, 2004). Partial spawnings were also observed by histological analysis in fully ripe “S” oysters in the field (Enriquez-Diaz, 2004). Thus, it is suggested that high reproductive effort and partial spawnings may contribute to enhancing the susceptibility of “S” oysters to summer mortality and/or infection. It is speculated that spawning events, dependent upon gonad maturity, may provide organic matter in the palleal cavity, thus favoring vibrio proliferation and increasing the infection risk, as shown in experimental trials (F. Le Roux, personal communication).

Energetic parameters and mortality:

In previous investigations, summer mortality was generally observed in field surveys and laboratory experiments when glycogen content was at its lowest level and oysters were fully ripened (Koganezawa, 1974; Mori, 1979; Perdue et al., 1981). In the present study, glycogen drastically decreased during the gametogenic process, strengthening all previous studies stating that there is a drastic energy consumption during reproductive processes in oysters (Mori et al., 1965; Perdue and Erickson, 1984; Ruiz et al., 1992; Berthelin et al., 2000; Delaporte et al., 2006a). No relationship, however, could be established between the lowest observed level of glycogen, or the rate of glycogen decrease, and the difference in mortality observed according to food level or oyster phenotype. From this lack of a direct relationship, it appeared that low glycogen only indicates an energy imbalance during gametogenesis, and that energy acquisition during this period depends mostly upon food supply. These data do not, however, provide information on carbohydrate fluxes. Survival differences between oyster phenotypes could,
instead, be related to the mechanisms of energy acquisition and/or expenditure, which may vary in efficiency between “S” and “R” oysters. Indeed, it has been reported that “S” oysters had a lower, compared to “R” oysters, expression of genes coding for glucose 6P production (Hexokinase, phosphoglucomutase, and phosphoenol-pyruvatecarboxy kinase) in July, just before the mortality event (Samain et al., in press). In *Drosophyla melanogaster*, a selective mutation in one of the key genes in the energetic pathway can alter flux in the pathway to glycogen synthesis (Verrelli and Eanes, 2000). Polymorphism studies on these genes are ongoing to detect possible detrimental alleles. Other effectors, such as stress (Tanguy et al., 2006) or infection, can lead to similar metabolic pathway perturbation. More work investigating these aspects is needed.

Hemocyte parameters and mortality:

One of the most striking results in hemocyte parameters was the clear difference between “R” and “S” oysters, in term of reactive oxygen species (ROS) production by untreated hemocytes. In May and June, hyalinocytes and granulocytes of “S” oysters had a much higher ROS production than those of “R” oysters, whatever the dietary treatment. In a previous field study, it was also observed that after 4 months of rearing, 7 months old “S” oysters had a significantly higher ROS production than “R” oysters, whatever the rearing site, Normandy, Brittany or Charentes (Lambert et al., in press). Thus, results obtained for ROS production in experimental conditions with G2 “R” and “S” oysters are in good agreement with those obtained in the field with G1 “R” and “S” oysters. The differences in ROS production observed here in May and June disappeared in July when mortality rate was maximal, suggesting that the high ROS level observed in May-June in “S” oysters may anticipate a major physiological disturbance/stress. In the literature, modulation of ROS production has been associated with various biological events such as acute stress (Lacoste et al., 2002), experimental infection by *V. aestuarianus* (Labreuche et al., 2006b;
or dietary modification (Hégaret et al., 2004; Delaporte et al., 2006a; Delaporte et al., 2006b) in oysters, but also with changes in metabolic activities associated with detoxification and respiration processes in other biological models (Sheehan and Power, 1999; Batandier et al., 2002; Cardenas et al., 2004; Keller et al., 2004; Manduzio et al., 2005). Moreover, in a review on reproductive strategy and survival (Heininger, 2002), the authors cited several studies suggesting a link between reproduction and oxidative stress. Reproduction increases energy expenditure and nutrient metabolism and results in higher mitochondrial activity and ROS production. Unless the antioxidant defences also increase, reproduction can enhance the susceptibility to oxidative stress. Previous works (Taub et al., 1999) have shown that a mutation of a catalase gene affected life span in the worm Caenorhabditis elegans. According to Heininger (2002) slowed aging in a species is the feature of a better resistance to oxidative stress. According to the above literature, reproductive activity may be considered as a physiological stress. The difference in reproductive activity between “S” and “R” oysters may, thus, result in the observed difference in ROS production. In a previous experimental study (Samain et al., in press), “S” oysters, after a 8 days temperature increase from 13°C to19°C and high food supply, consistently showed more Hsp 70 (stress related proteins) in gills and lower catalase activities than “R” oysters, possibly resulting from higher vitellogenic activity in “S” oysters compared to “R” oysters. In another invertebrate model, Landis and Tower (2005) observed that Hsp70 expression during aging of Drosophila is up-regulated in response to oxidative stress, obtained for example by null-mutation in either superoxide dismutase (SOD) or catalase gene. Thus, we speculate that temperature and food increase in our experiment led to a higher reproductive activity for “S” oysters, compared to “R”, possibly resulting in higher ROS production associated with lower detoxification. We cannot, however, exclude the possibility that selection may have occurred for some enzymes involved in ROS production (NADPH-oxidase, NO synthase) and/or
detoxification (superoxide dismutase, catalase, glutathion peroxidase). With a suppression
substractive hybridization experiment (SSH) performed between “R” and “S” families during a
summer mortality event (Huvet et al., 2004), a few of the more-frequently, differentially-
expressed genes during several summer mortality events with different age classes of oysters
were identified. Among these genes, a cavortin-like gene was shown to be more induced in
mantle-gonad tissue from “R” oysters than from “S” oysters. This gene was later characterized as
a hemocyte Cg SOD gene (Gonzalez et al., 2005). The expression of this gene is thought to
contribute to reactive oxygen species detoxification. These early results suggest that “R” and “S”
oysters could possibly be differenciated by antioxidant capacities.

Among the other hemocyte parameters, only hyalinocyte counts of “S” oysters were found to
increase to a higher level than those of “R” oysters in July, concomitantly with a rise in
mortality. Such a significant rapid increase of THC was also observed 3 and 5 days post-
infection in V. aestuarianus strain 01/32 injected animals compared to sterile seawater injected
animals (Labreuche et al., 2006b). Together V. aestuarianus and V. splendidus constitute the two
major vibrio species found in moribund oysters in the field and are thought to be involved in
summer mortality (Lacoste et al., 2001; Gay et al., 2004; Garnier et al., in press). So, the above
results tend to support the hypothesis that the difference in mortality measured between the two
survival phenotypes in July could possibly result at the end from a differential infection occurring
between “R” and “S” oyster.

In conclusion, the present study showed that parameters associated to reproduction and hemocyte
activities can be significantly different in oyster phenotypes “R” vs “S” with no or little
interaction with food level. During active gametogenesis, “S” oysters developed more lipid
stores, presumably associated with gonad tissue, than “R” oysters. Higher gonad development and partial spawning of “S” oysters are thought to increase their pathogen and/or mortality susceptibility. Our data confirm and further document the genetic correlation between reproductive effort and survival reported in *C. gigas* by Ernande et al. (2004).

“S” oysters also had hemocytes with significantly-higher ROS production than “R” oysters. Further research is necessary, however, to attribute the change in ROS production to the difference in reproductive activity or differential gene selection or expression in ROS detoxification-related processes. Although a relationship between hemocyte ROS production and oyster mortality appeared in the present study, it was difficult to establish; nevertheless, numerous examples in literature can be cited where ROS production is related to survival.

At the end of the reproductive process, a differential increase of hemocyte number between “R” and “S” oysters may provide a window for infection for a short period of time, as indicated in previous observations during experimental infection studies.

Relationships between ROS production, partial spawning, and infection of highly-mature oysters should be continued to be documented. On-going investigations on gene expression, polymorphism of candidate genes and QTL mapping will certainly contribute to better understand the genetic and functional differences between “S” and “R” oysters and, more specifically, the origin of excess ROS production in “S” oysters. Development of infection models and specific probes for pathogen identification in oyster cellular structure are in preparation to better assess the infection dynamics.

Acknowledgements
We are grateful to technicians from La Tremblade and Bouin for the spat production of the oysters used in the present study and our colleagues of the Argenton hatchery for taking care of oysters during the experimental conditioning. 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, the Conseil Général du Calvados and European Community (IFOP). Sincere thanks are due to Gary H. Wikfors for English corrections. Contribution N°xxx of the IUEM, European Institute for Marine Studies (Brest, France)

References


mortality in the Pacific oyster (Crassostrea gigas) and its relationship with physiological, immunological characteristics and infection processes. Aquaculture in press.


Table 1: Summary of the three-way analysis of variance comparing biochemical and hemocyte parameters (independent variables) according to diet, phenotype (resistant R and susceptible S), and sampling date and their interactions during the experiment. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; NS: non significant.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Main effects</th>
<th>Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time effect</td>
<td>Diet effect</td>
</tr>
<tr>
<td>Condition index</td>
<td>NS</td>
<td>****</td>
</tr>
<tr>
<td>Carbohydrates (mg/g DW)</td>
<td>****</td>
<td>*</td>
</tr>
<tr>
<td>Lipids (mg/g DW)</td>
<td>*</td>
<td>****</td>
</tr>
<tr>
<td>Hemocyte counts (cells/ml)</td>
<td>****</td>
<td>NS</td>
</tr>
<tr>
<td>Hyalinoocyte counts (cells/ml)</td>
<td>****</td>
<td>*</td>
</tr>
<tr>
<td>Granulocyte counts (cells/ml)</td>
<td>***</td>
<td>NS</td>
</tr>
<tr>
<td>Hemocyte mortality (%)</td>
<td>****</td>
<td>NS</td>
</tr>
<tr>
<td>Phagocytosis (%)</td>
<td>****</td>
<td>NS</td>
</tr>
<tr>
<td>Adhesion (%), with FSSW</td>
<td>**</td>
<td>NS</td>
</tr>
<tr>
<td>Adhesion (%), with Vibrio S322</td>
<td>***</td>
<td>NS</td>
</tr>
<tr>
<td>ROS production in hyalinocytes</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ROS production in granulocytes</td>
<td>*</td>
<td>NS</td>
</tr>
</tbody>
</table>
Fig. 1: Cumulative mortality percentages of susceptible “S” and resistant “R” oysters fed the 4 and 12% diets.

Fig. 2: Condition index of susceptible “S” and resistant “R” oyster families fed two dietary rations (4 or 12% of algal dry weight/ oyster dry weight, daily) under controlled conditions (Mean ± S.D., n=10). Condition index of oysters fed the 12% ration was significantly higher than that of oysters fed the 4% ration (P<0.001). There was no significant effect of sampling date or phenotype.

Fig. 3: Carbohydrate (A) and lipid (B) contents of susceptible “S” and resistant “R” oyster families fed two dietary rations (4 or 12% of algal dry weight/ oyster dry weight, daily) under controlled conditions. Results are expressed as mg carbohydrates (A) and total lipids (B) per mg oyster dry weight (Mean ± S.D., n=10). Carbohydrate and lipid contents were significantly higher in oyster fed the 12 diet than those fed the 4% diet (P<0.05 and P<0.001, respectively). Carbohydrate and lipid contents of susceptible “S” oyster families were significantly higher than those of resistant “R” oyster families (P<0.05 and P<0.01, respectively).

Fig. 4: Granulocyte (A) and hyalinocyte (B) concentrations of susceptible “S” and resistant “R” oyster families reared under controlled conditions, regardless of the dietary rations. Results are expressed as cells ml⁻¹ (Mean ± S.D., n= 20. Hyalinocyte concentration is significantly higher in “S” oysters than in “R” oysters.

Fig. 5: Phagocytic activity of hemocytes from susceptible “S” and resistant “R” oyster families reared under controlled conditions, regardless of the dietary rations. Results are expressed as percentage of hemocytes that have engulfed three beads and more (Mean ± S.D., n= 6).

Fig. 6A and 6B: Adhesion of hemocytes incubated with filtered sterile seawater, FSSW (A) and with Vibrio sp. S322 at 50 cells per hemocyte (B). Hemocytes were sampled from susceptible “S”
and resistant “R” oyster families reared under controlled conditions, regardless of the dietary rations. Results are expressed in percentage of adhering cells (Mean ± S.D., n=6).

**Fig. 7A and 7B:** Granulocyte(A) and hyalinocyte(B) ROS production of hemocytes from susceptible “S” and resistant “R” oyster families reared under controlled conditions, regardless of the dietary rations. Results are presented as the mean DCF fluorescence (quantitatively related to ROS production) expressed in arbitrary units, AU (Mean ± S.D., n=6).
Figure 1

Cumulated mortality (%)

April May June July August

"R" oysters fed 4%
"R" oysters fed 12%
"S" oysters fed 4%
"S" oysters fed 12%
Figure 2
Figure 3A and 3B
Figure 4A and 4B
Figure 5

% of phagocytic cells

April May June July August

"R" oysters
"S" oysters
Figure 6A and 6B
Figure 7A and 7B