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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

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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 precedently 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

119 **2.1. Oyster conditioning**

120

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

139

140 2.2. Biochemical parameters and condition index

141 Each month, shell weight and flesh wet weight of 10 oysters were measured after widthdrawal of
142 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°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

191 FSC and low SSC, while small agranulocytes have low FSC and SSC. Thus, the three sub-
192 populations were distinguished according to their size and cell complexity (granularity). Total
193 hemocyte, granulocyte, hyalinocyte, and small agranulocyte concentrations estimated from the
194 flow rate measurement of the flow-cytometer (Marie et al., 1999) as all samples were run for 30
195 sec. Results were expressed as number of cells per ml. Small agranulocyte concentrations are not
196 presented in this report because they represented only a small proportion of the total hemocyte
197 count and are considered to possess little activity (Lambert et al., 2003).
198
199 2.3.3. Functionnal parameters
200 These parameters were measured on pool of hemolymph. For each sampling date and each
201 condition (4 and 12% diet, "R" and "S"), hemolymph from at least five animals was pooled and
202 analyses were ran on three pools of five individuals.
203
204 2.3.3.1. Phagocytosis
205 An aliquot of 100 µL pooled hemolymph, diluted with 100 µL of FSSW, was mixed with 30 µL
206 of YG, 2.0-µm fluoresbrite microspheres, diluted to 2% in FSSW (Polysciences, Eppelheim,
207 Germany). After 120 minutes of incubation at 18°C, hemocytes were fixed with 230 µL of a 6%
208 formalin solution and analysed at 500-530 nm by flow cytometry to detect hemocytes containing
209 fluorescent beads. The percentage of phagocytic cells was defined as the percentage of hemocytes
210 that had engulfed three or more beads (Delaporte et al., 2003).
211
212
213 2.3.3.2. Adhesion assay

214 Hemocyte adhesion assays were performed according to the procedure reported previously
215 (Delaporte et al., 2006a), adapted from another study (Choquet et al., 2003). Briefly, a 100µL
216 aliquot of pooled hemolymph was allowed to adhere in an 24-well microplate, either in sterile
217 seawater or in seawater with Vibrio sp. S322 (50 bacteria/ hemocyte), a strain known for its
218 pathogenecity to bivalve larvae (Nicolas et al., 1996). After three hours of incubation, non-
219 adhering cells were fixed in 6% formalin solution and stained using SYBR Green I (final
220 concentration, 1/1,000 in DMSO) and then detected and counted by flow-cytometry. Results are
221 expressed as the percentage of adhering hemocytes incubated with FSSW or bacteria, relative to
222 the initial hemocyte count.
223
224 2.3.3.3. Reactive oxygen species production
225 Reactive oxygen species (ROS) production by untreated hemocytes was measured using 2'7'-
226 dichlorofluorescein diacetate, DCFH-DA (Lambert et al., 2003). A 100 µL aliquot of pooled
227 hemolymph was diluted with 300 µl of FSSW. Four µL of the DCFH-DA solution (final
228 concentration of 0.01 mM) was added to each tube maintained on ice. Tubes were then incubated
229 at 18°C for 120 minutes. After the incubation period, DCF fluorescence, quantitatively related to
230 the ROS production of untreated hemocytes, was measured at 500-530 nm by flow-cytometry.
231 Results are expressed as the mean geometric fluorescence (in arbitrary units, AU) detected in
232 each hemocyte sub-population.
233
234 **2.4. Statistical analysis**
235 Three-way, multifactor analysis of variance was performed to compare biochemical and
236 hemocyte parameters (independent variables) according to diet, phenotype (summer mortality
237 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

261 Condition index was significantly affected by food level (Figure 2, MANOVA, $p<0.0001$), but
262 not by oyster phenotype or sampling date. Oysters fed the 12% diet had a higher mean condition
263 index than those fed the 4% diet. However, it should be noted that condition index of summer
264 mortality-susceptible oysters fed the 12% diet dropped significantly (only in July) compared to
265 corresponding, resistant oysters. This selective drop in condition index resulted in significant
266 interactions between time and diet ($P<0.05$) and between time and phenotype ($P<0.01$).
267
268 **3.4. Biochemical composition**
269 Carbohydrate content of oysters was significantly affected by oyster phenotype (Figure 3A,
270 $p<0.05$), with "S" oyster families having a higher mean carbohydrate content compared to "R"
271 oysters. Also, a significantly-higher carbohydrate content was reported in oysters fed the 12%
272 diet, compared to oysters fed the 4% diet ($p<0.05$). Carbohydrate content was also greatly
273 affected by sampling date ($p<0.0001$), decreasing steadily from May to August. Finally, a
274 significant interaction was noted between diet and time.
275 A significantly-higher mean lipid content was observed in "S" oysters compared to "R" oysters
276 (Figure 3B, $p<0.01$). Total lipid content was also significantly affected by the dietary
277 conditioning ($p<0.0001$). Oysters fed the 12% diet contained a higher lipid content than those fed
278 the 4% diet. As with carbohydrate content, total lipid content changed significantly over time
279 ($p<0.05$); total lipid content increased during the course of the experiment. Note, however, that
280 lipid content of "S" oysters fed both 4 and 12% diets dropped temporarily in July, but this drop in
281 lipid occurred one month later for "R" oysters. Interaction between time and phenotype was the
282 only significant interaction for the lipid content and was likely attributable to this drop in July for
283 the "S" oyster families.
284

285 **3.5. Hemocyte viability**

286 No significant difference was observed between dietary treatments, nor between summer
287 mortality-susceptible and -resistant oyster families. Percentages of alive hemocytes ranged from
288 95.8% to 99% during the course of the experiment (data not shown).

289

290 **3.6. Total hemocyte, granulocyte and hyalinocyte concentrations**

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

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

303

304 **3.7. Phagocytosis**

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

308

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 succesive 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.

347 Possible relationships between differential survival and physiological parameters (reproduction,
348 energy allocation, immunology) were explored and discussed hereafter.
349
350 Reproductive parameters and mortality :
351 As previously described (Enriquez-Diaz, 2004; Delaporte et al., 2006a), high trophic condition
352 (12% of algal dry weight per oyster dry weight) resulted in a higher gonad development than low
353 trophic condition (4%). Data showed a higher lipid content in "S" oysters compared to "R"
354 oysters, mainly during the first part of vitellogenesis until June. Gonad development estimation
355 using lipid content has been shown several times to be a good indicator of maturation level
356 (Deslous-Paoli et al., 1981; Soletchnik et al., 1999; Soletchnik et al., 2002) and was found to be
357 in good agreement with gonad area estimation using quantitative histological techniques in
358 controlled conditions (Delaporte et al., 2006a). Thus, this result suggests that the gametogenic
359 process may depend upon the oyster phenotype. "S" oysters invested more in reproduction than
360 "R" oysters under the same trophic conditions. Differential reproduction effort could explain, at
361 least partially, the difference in mortality observed not only between "S" and "R" oysters, but
362 also between oysters fed 4% and 12% diets. Indeed, experimental infections by *V. splendidus*
363 resulted in a higher mortality in oyster fed the 12% diet than those fed 4% (Gay, 2004).
364 The difference of mortality between "R" and "S" oysters accelerated after July at the end of
365 vitellogenesis. During this period, increases in turbidity were observed in the tank by continuous
366 optical records using the MAREL system (Bourles, 2004) that are likely to correspond with
367 gamete emission. These turbidity increases were more frequently observed for "S" oysters fed the
368 12% diet, compared to "R" oysters fed the same diet (data not shown). These gamete emissions
369 were confirmed in July by the lower condition index and lipid content in "S" oysters compared to
370 "R" oysters when both groups were fed the 12% diet. However, the CI drop does not correspond

371 to a massive spawning, but rather to more frequent, partial spawnings in “S” oysters. Thus, when
372 considering the entire gametogenic process, the “S” oyster families produced a more intense
373 reproductive effort than “R” oysters, as previously shown for the first generation of selected
374 oysters reared in the field at Fort Espagnol (Morbihan, France) (Enriquez-Diaz, 2004). Partial
375 spawnings were also observed by histological analysis in fully ripe “S” oysters in the field
376 (Enriquez-Diaz, 2004). Thus, it is suggested that high reproductive effort and partial spawnings
377 may contribute to enhancing the susceptibility of “S” oysters to summer mortality and/or
378 infection. It is speculated that spawning events, dependent upon gonad maturity, may provide
379 organic matter in the palleal cavity, thus favoring vibrio proliferation and increasing the infection
380 risk, as shown in experimental trials (F. Le Roux , personal communication).
381
382 Energetic parameters and mortality:
383 In previous investigations, summer mortality was generally observed in field surveys and
384 laboratory experiments when glycogen content was at its lowest level and oysters were fully
385 ripened (Koganezawa, 1974; Mori, 1979; Perdue et al., 1981). In the present study, glycogen
386 drastically decreased during the gametogenic process, strengthening all previous studies stating
387 that there is a drastic energy consumption during reproductive processes in oysters (Mori et al.,
388 1965; Perdue and Erickson, 1984; Ruiz et al., 1992; Berthelin et al., 2000; Delaporte et al.,
389 2006a). No relationship, however, could be established between the lowest observed level of
390 glycogen, or the rate of glycogen decrease, and the difference in mortality observed according to
391 food level or oyster phenotype. From this lack of a direct relationship, it appeared that low
392 glycogen only indicates an energy imbalance during gametogenesis, and that energy acquisition
393 during this period depends mostly upon food supply. These data do not, however, provide
394 information on carbohydrate fluxes. Survival differences between oyster phenotypes could,

395 instead, be related to the mechanisms of energy acquisition and/or expenditure, which may vary
396 in efficiency between “S” and “R” oysters. Indeed, it has been reported that “S” oysters had a
397 lower, compared to “R” oysters, expression of genes coding for glucose 6P production
398 (Hexokinase, phosphoglucomutase, and phosphoenol-pyruvatecarboxy kinase) in July, just before
399 the mortality event (Samain et al., in press). In *Drosophyla melanogaster*, a selective mutation in
400 one of the key genes in the energetic pathway can alter flux in the pathway to glycogen synthesis
401 (Verrelli and Eanes, 2000). Polymorphism studies on these genes are ongoing to detect possible
402 detrimental alleles. Other effectors, such as stress (Tanguy et al., 2006) or infection, can lead to
403 similar metabolic pathway perturbation. More work investigating these aspects is needed.
404
405 Hemocyte parameters and mortality :
406 One of the most striking results in hemocyte parameters was the clear difference between “R”
407 and “S” oysters, in term of reactive oxygen species (ROS) production by untreated hemocytes. In
408 May and June, hyalinocytes and granulocytes of “S” oysters had a much higher ROS production
409 than those of “R” oysters, whatever the dietary treatment. In a previous field study, it was also
410 observed that after 4 months of rearing, 7 months old “S” oysters had a significantly higher ROS
411 production than “R” oysters, whatever the rearing site, Normandy, Brittany or Charentes
412 (Lambert et al., in press). Thus, results obtained for ROS production in experimental conditions
413 with G2 “R” and “S” oysters are in good agreement with those obtained in the field with G1 “R”
414 and “S” oysters. The differences in ROS production observed here in May and June disappeared
415 in July when mortality rate was maximal, suggesting that the high ROS level observed in May-
416 June in “S” oysters may anticipate a major physiological disturbance/stress. In the literature,
417 modulation of ROS production has been associated with various biological events such as acute
418 stress (Lacoste et al., 2002), experimental infection by *V. aestuarianus* (Labreuche et al., 2006b;

419 Labreuche et al., 2006a) or dietary modification (Hégaret et al., 2004; Delaporte et al., 2006a;
420 Delaporte et al., 2006b) in oysters, but also with changes in metabolic activities associated with
421 detoxification and respiration processes in other biological models (Sheehan and Power, 1999;
422 Batandier et al., 2002; Cardenas et al., 2004; Keller et al., 2004; Manduzio et al., 2005).
423 Moreover, in a review on reproductive strategy and survival (Heininger, 2002), the authors cited
424 several studies suggesting a link between reproduction and oxidative stress. Reproduction
425 increases energy expenditure and nutrient metabolism and results in higher mitochondrial activity
426 and ROS production. Unless the antioxidant defences also increase, reproduction can enhance the
427 susceptibility to oxidative stress. Previous works (Taub et al., 1999) have shown that a mutation
428 of a catalase gene affected life span in the worm *Caenorhabditis elegans.* According to Heininger
429 (2002) slowed aging in a species is the feature of a better resistance to oxidative stress.
430 According to the above literature, reproductive activity may be considered as a physiological
431 stress. The difference in reproductive activity between “S” and “R” oysters may, thus, result in
432 the observed difference in ROS production. In a previous experimental study (Samain et al., in
433 press), “S” oysters, after a 8 days temperature increase from 13°C to19°C and high food supply,
434 consistently showed more Hsp 70 (stress related proteins) in gills and lower catalase activities
435 than “R” oysters, possibly resulting from higher vitellogenic activity in “S” oysters compared to
436 “R” oysters. In another invertebrate model, Landis and Tower (2005) observed that Hsp70
437 expression during aging of *Drosophila* is up-regulated in response to oxidative stress, obtained
438 for example by null-mutation in either superoxide dismutase (SOD) or catalase gene. Thus, we
439 speculate that temperature and food increase in our experiment led to a higher reproductive
440 activity for “S” oysters, compared to “R”, possibly resulting in higher ROS production associated
441 with lower detoxification. We cannot, however, exclude the possibility that selection may have
442 occurred for some enzymes involved in ROS production (NADPH-oxidase, NO synthase) and/or

443 detoxification (superoxide dismutase, catalase, gluthation peroxidase). With a suppression
444 substractive hybridization experiment (SSH) performed between “R” and “S” families during a
445 summer mortality event (Huvet et al., 2004), a few of the more-frequently, differentially-
446 expressed genes during several summer mortality events with different age classes of oysters
447 were identified. Among these genes, a cavortin-like gene was shown to be more induced in
448 mantle-gonad tissue from “R” oysters than from “S” oysters. This gene was later characterized as
449 a hemocyte *Cg* SOD gene (Gonzalez et al., 2005) The expression of this gene is thought to
450 contribute to reactive oxygen species detoxification. These early results suggest that “R” and “S”
451 oysters could possibly be differenciated by antioxidant capacities.
452
453 Among the other hemocyte parameters, only hyalinocyte counts of “S” oysters were found to
454 increase to a higher level than those of “R” oysters in July, concomitantly with a rise in
455 mortality., Such a significant rapid increase of THC was also observed 3 and 5 days post-
456 infection in *V. aestuarianus* strain 01/32 injected animals compared to sterile seawater injected
457 animals (Labreuche et al., 2006b). Together *V. aestuarianus* and *V. splendidus* constitute the two
458 major vibrio species found in moribund oysters in the field and are thought to be involved in
459 summer mortality (Lacoste et al., 2001; Gay et al., 2004; Garnier et al., in press). So, the above
460 results tend to support the hypothesis that the difference in mortality measured between the two
461 survival phenotypes in July could possibly result at the end from a differential infection occuring
462 between “R” and “S” oyster.
463
464 In conclusion, the present study showed that parameters associated to reproduction and hemocyte
465 activities can be significantly different in oyster phenotypes “R” *vs* “S” with no or little
466 interaction with food level. During active gametogenesis, “S” oysters developed more lipid

467 stores, presumably associated with gonad tissue, than “R” oysters. Higher gonad development
468 and partial spawning of “S” oysters are thought to increase their pathogen and/or mortality
469 susceptibility. Our data confirm and further document the genetic correlation between
470 reproductive effort and survival reported in *C. gigas* by Ernande et al. (2004).
471 “S” oysters also had hemocytes with significantly-higher ROS production than “R” oysters.
472 Further research is necessary, however, to attribute the change in ROS production to the
473 difference in reproductive activity or differencial gene selection or expression in ROS
474 detoxification-related processes. Although a relationship between hemocyte ROS production and
475 oyster mortality appeared in the present study, it was difficult to establish; nevertheless,
476 numerous examples in literature can be cited where ROS production is related to survival.
477 At the end of the reproductive process, a differential increase of hemocyte number between “R”
478 and “S” oysters may provide a window for infection for a short period of time, as indicated in
479 previous observations during experimental infection studies.
480 Relationships between ROS production, partial spawning, and infection of highly-mature oysters
481 should be continued to be documented. On-going investigations on gene expression,
482 polymorphism of candidate genes and QTL mapping will certainly contribute to better understand
483 the genetic and functional differences between “S” and “R” oysters and, more specifically, the
484 origin of excess ROS production in “S” oysters. Development of infection models and specific
485 probes for pathogen identification in oyster cellular structure are in preparation to better assess
486 the infection dynamics.
487
488
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711 **Table 1**: Summary of the three-way analysis of variance comparing biochemical and hemocyte parameters (independent variables)
712 according to diet, phenotype (resistant R and susceptible S), and sampling date and their interactions during the experiment. *: $p<0.05$;
713 **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$; NS: non significant.

	Main effects					**Interactions**			
Variables	Time effect	Diet effect		Phenotype effect		Time vs Diet	Time vs Phenotype	Diet vs Phenotype	Time vs Diet vs Phenotype
Condition index	NS	****	12% > 4%	NS	-	*	**	NS	NS
Carbohydrates (mg/g DW)	****	*	12% > 4%	*	S > R	*	NS	NS	NS
Lipids (mg/g DW)	*	****	12% > 4%	**	S > R	NS	***	NS	NS
Hemocyte counts (cells/ml)	****	NS	-	NS	-	NS	NS	NS	NS
Hyalinocyte counts (cells/ml)	****	*	12% > 4%	*	S > R	NS	NS	NS	NS
Granulocyte counts (cells/ml)	***	NS	-	NS	-	NS	NS	NS	NS
Hemocyte mortality (%)	****	NS	-	NS	-	NS	NS	NS	NS
Phagocytosis (%)	****	NS	-	NS	-	NS	NS	NS	NS
Adhesion (%, with FSSW)	**	NS	-	NS	-	NS	NS	NS	NS
Adhesion (%, with *Vibrio* S322)	***	NS	-	NS	-	*	NS	NS	NS
ROS production in hyalinocytes	NS	NS	-	***	S > R	NS	NS	NS	NS
ROS production in granulocytes	*	NS	-	**	S > R	NS	*	NS	NS

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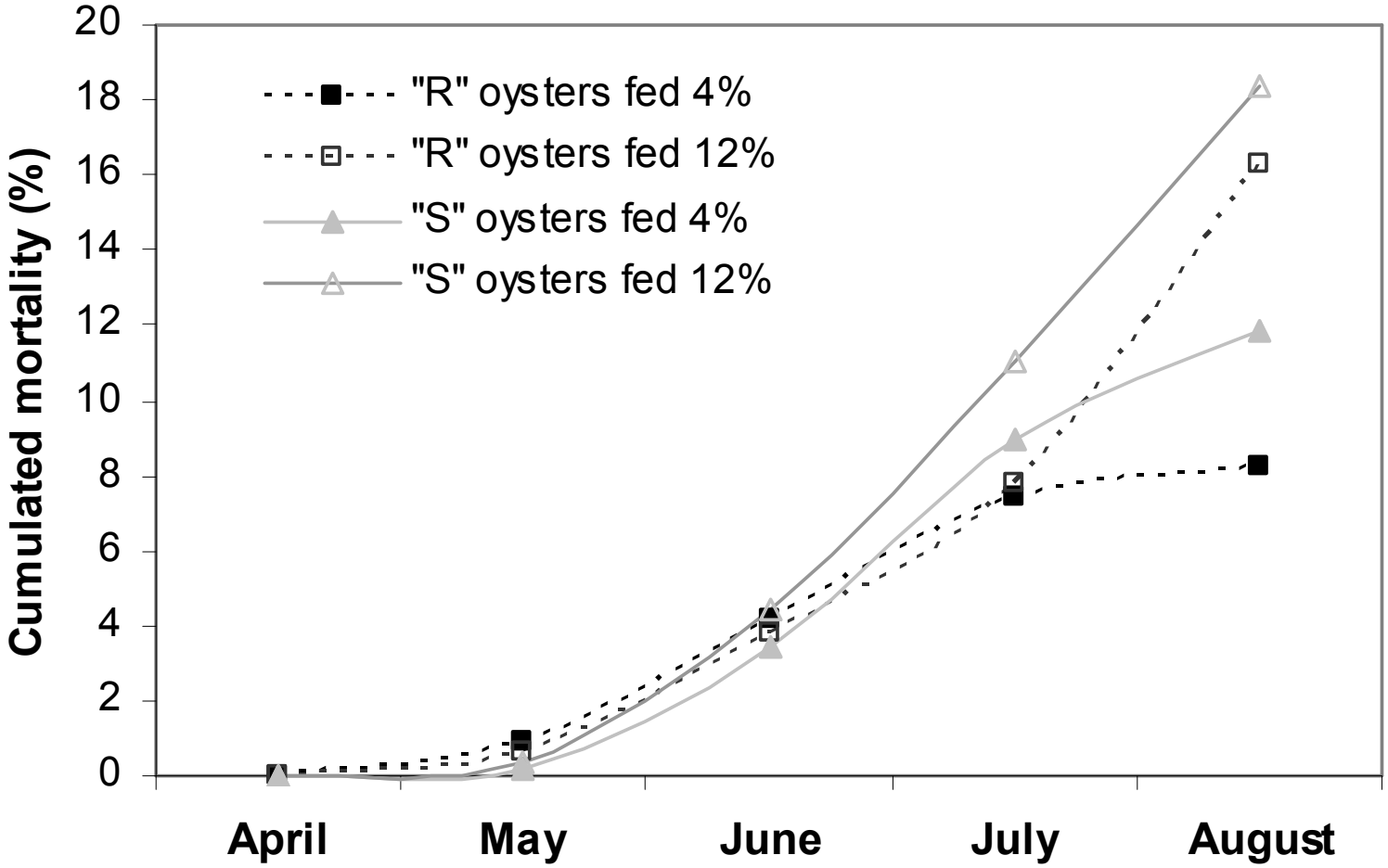
718 **Fig. 1**: Cumulative mortality percentages of susceptible "S" and resistant "R" oysters fed the 4
719 and 12% diets.
720 **Fig. 2** : Condition index of susceptible "S" and resistant "R" oyster families fed two dietary
721 rations (4 or 12% of algal dry weight/ oyster dry weight, daily) under controlled conditions
722 (Mean ± S.D., n=10). Condition index of oysters fed the12% ration was significantly higher than
723 that of oysters fed the 4% ration ($P<0.001$). There was no significant effect of sampling date or
724 phenotype.
725 **Fig. 3**: Carbohydrate (A) and lipid (B) contents of susceptible "S" and resistant "R" oyster
726 families fed two dietary rations (4 or 12% of algal dry weight/ oyster dry weight, daily) under
727 controlled conditions. Results are expressed as mg carbohydrates (A) and total lipids (B) per mg
728 oyster dry weight (Mean ± S.D., n=10). Carbohydrate and lipid contents were significantly higher
729 in oyster fed the 12 diet than those fed the 4% diet ($P<0.05$ and $P<0.001$, respectively).
730 Carbohydrate and lipid contents of susceptible "S" oyster families were significantly higher than
731 those of resistant "R" oyster families ($P<0.05$ and $P<0.01$, respectively).
732 **Fig. 4**: Granulocyte (A) and hyalinocyte (B) concentrations of susceptible "S" and resistant "R"
733 oyster families reared under controlled conditions, regardless of the dietary rations. Results are
734 expressed as cells ml^{-1} (Mean ± S.D., n= 20. Hyalinocyte concentration is significantly higher in
735 "S" oysters than in "R" oysters.
736 **Fig. 5**: Phagocytic activity of hemocytes from susceptible "S" and resistant "R" oyster families
737 reared under controlled conditions, regardless of the dietary rations. Results are expressed as
738 percentage of hemocytes that have engulfed three beads and more (Mean ± S.D., n= 6).
739 **Fig. 6A and 6B**: Adhesion of hemocytes incubated with filtered sterile seawater, FSSW (A) and
740 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).

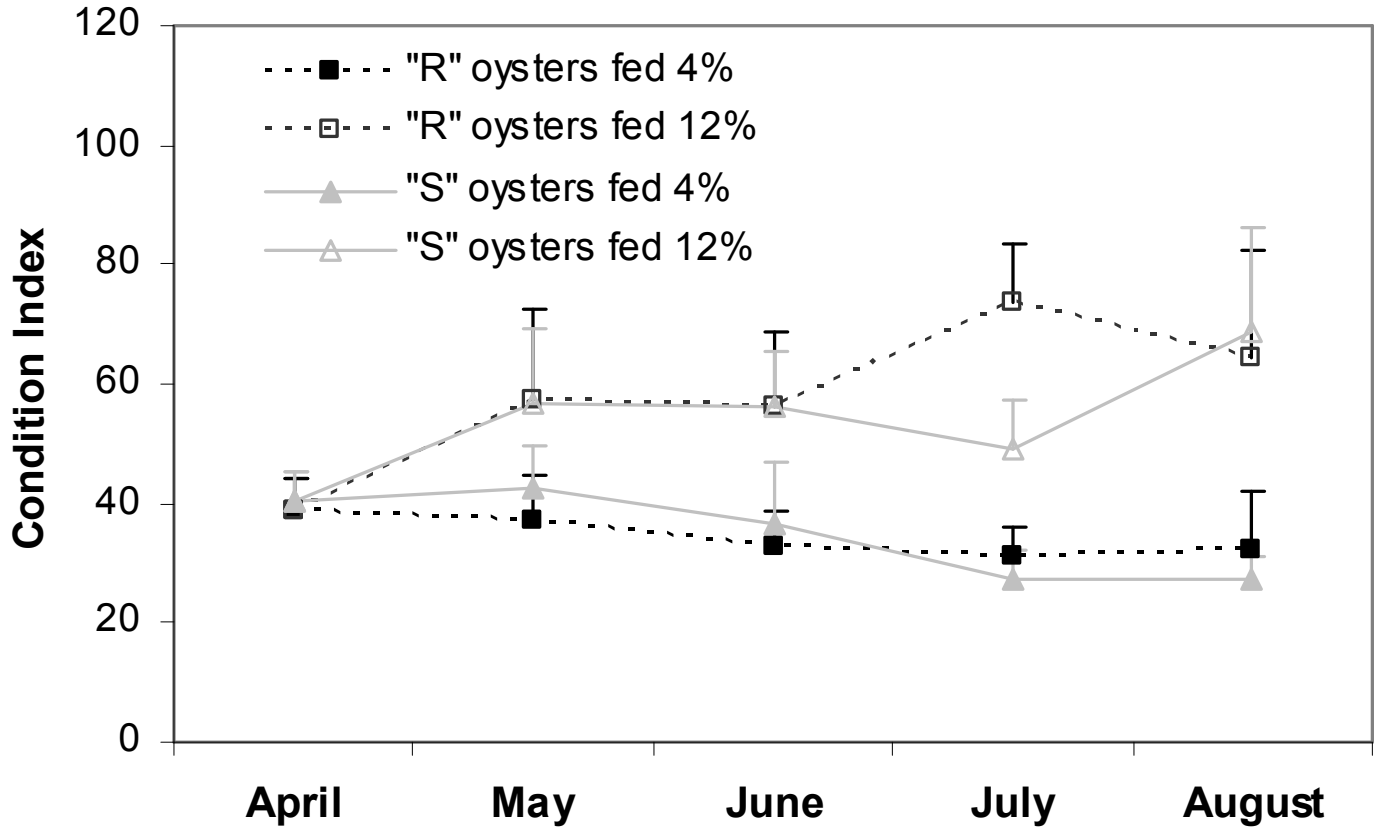
747



748

749 **Figure 1**

750

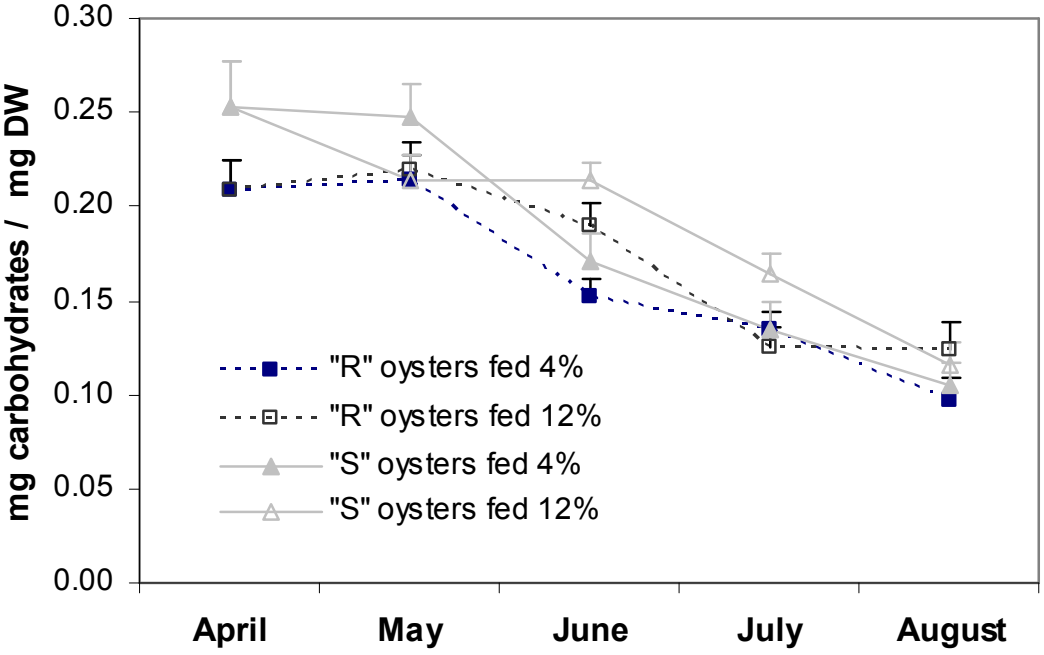


751

752

753 **Figure 2**

754 **A**



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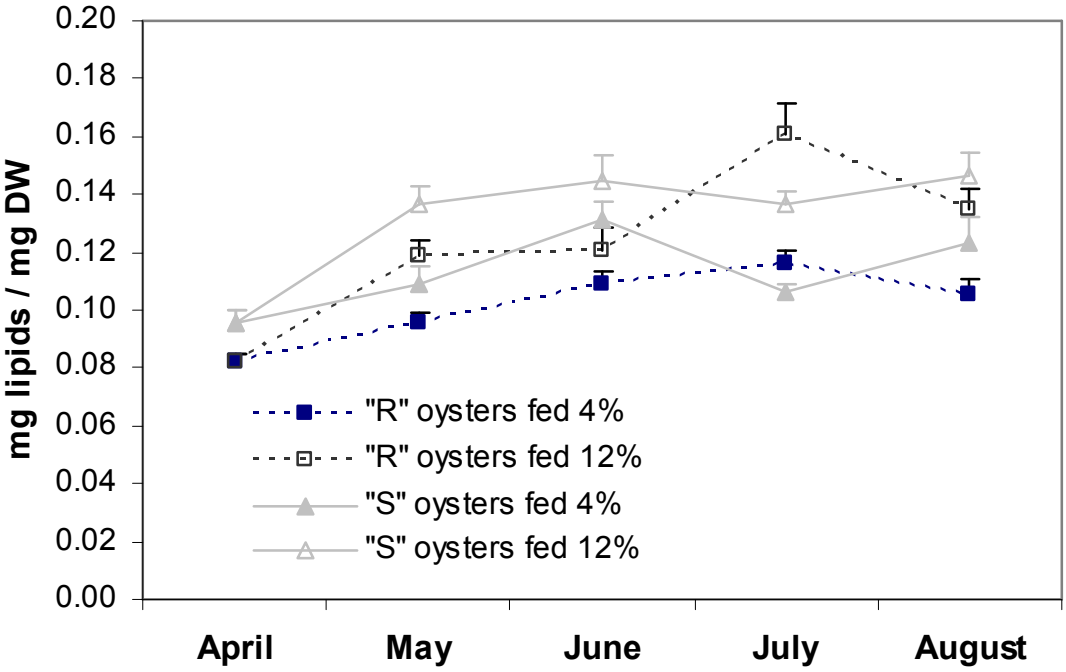
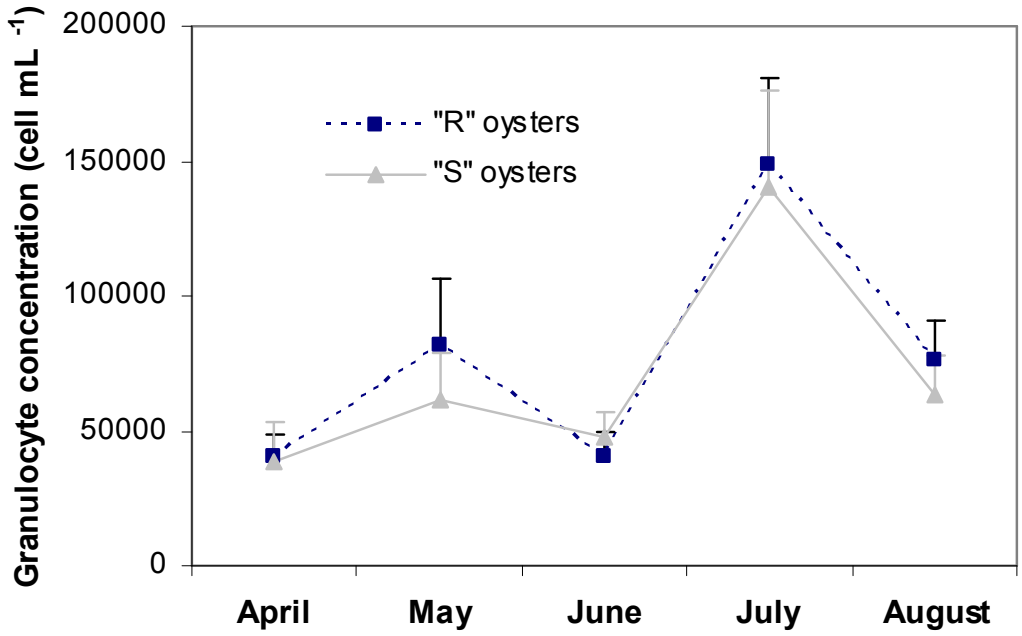
756 **B**



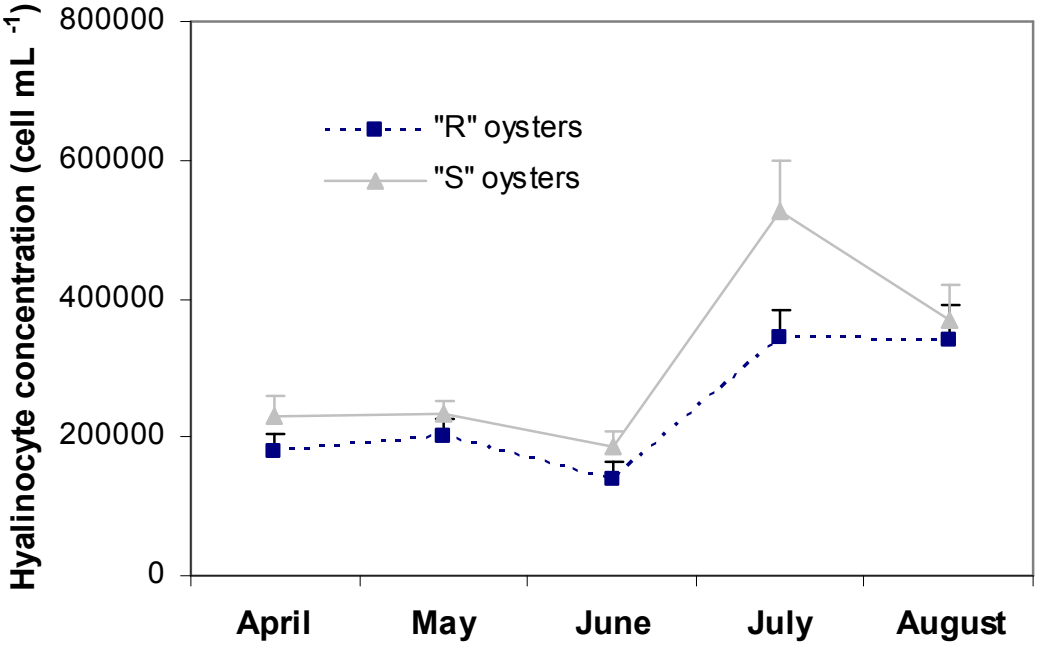
757
758 **Figure 3A and 3B**

759 **A**



760

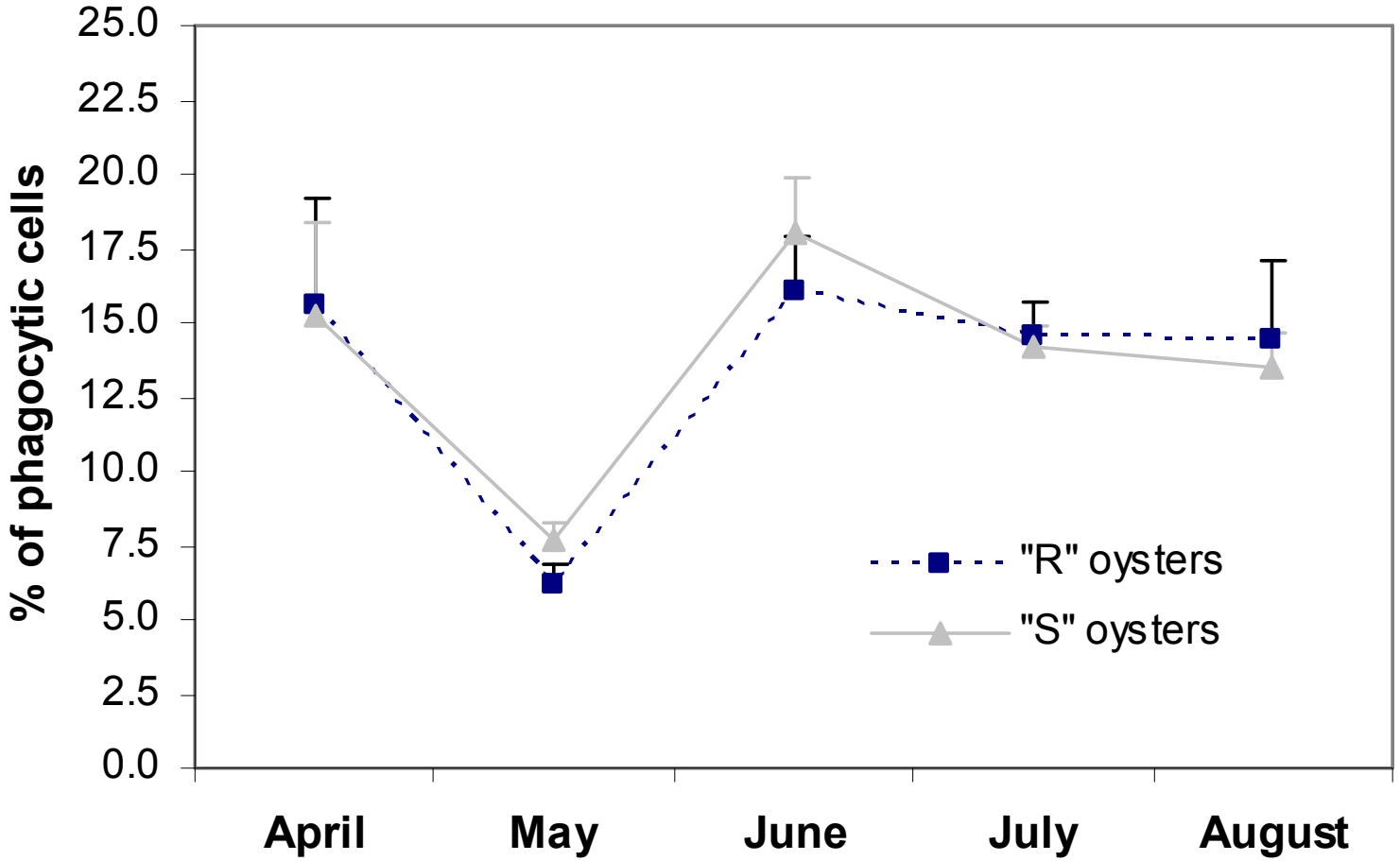
761 **B**



762

763 **Figure 4A and 4B**

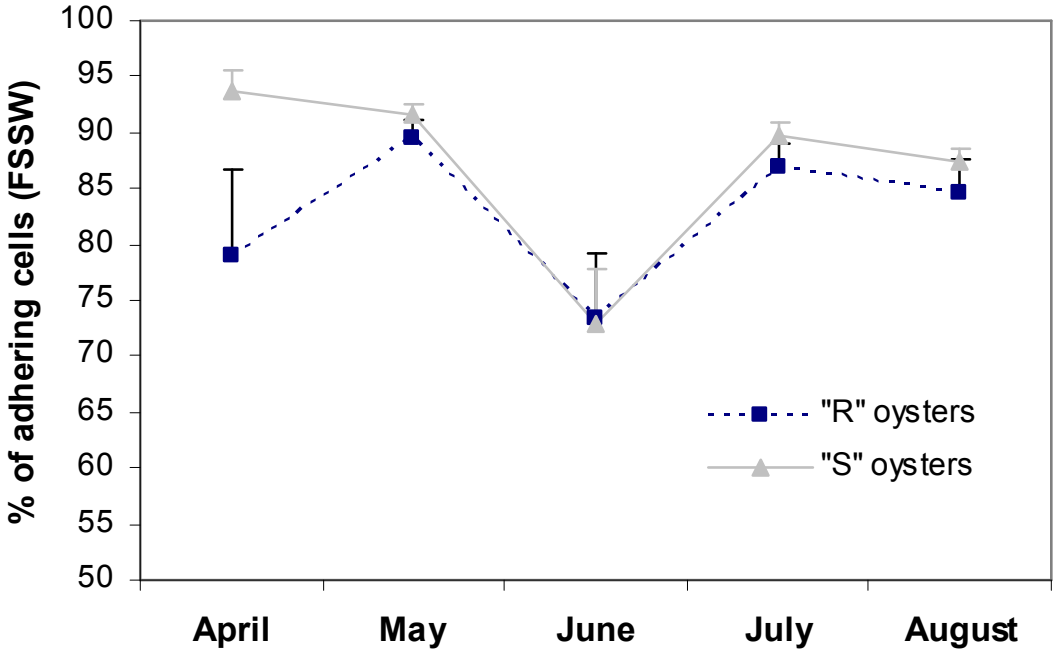
764



765

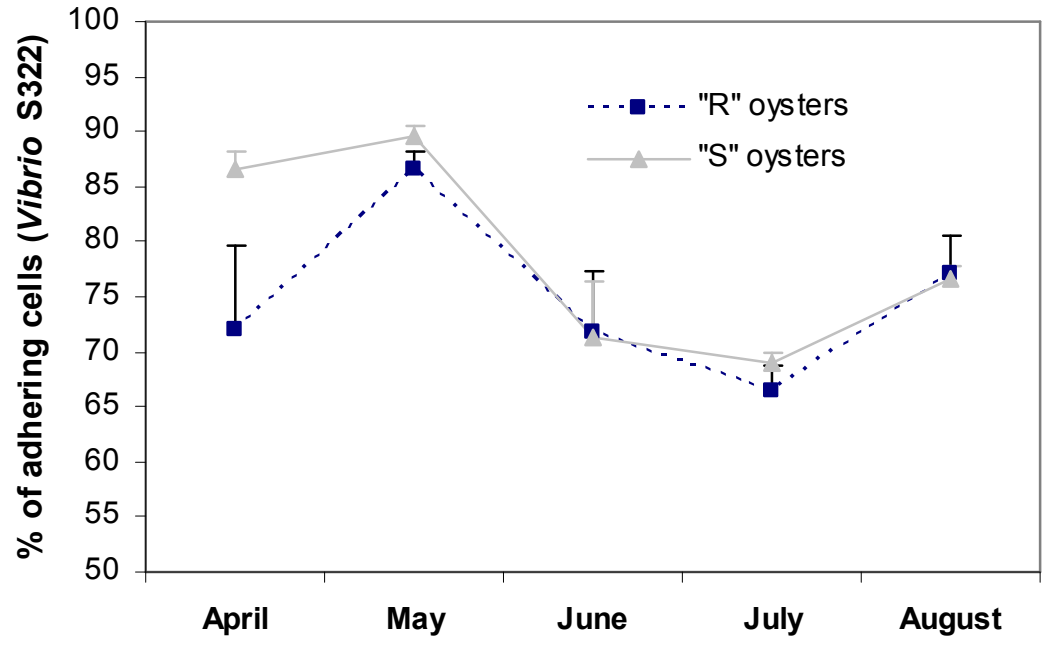
766 **Figure 5**

767 **A**



768

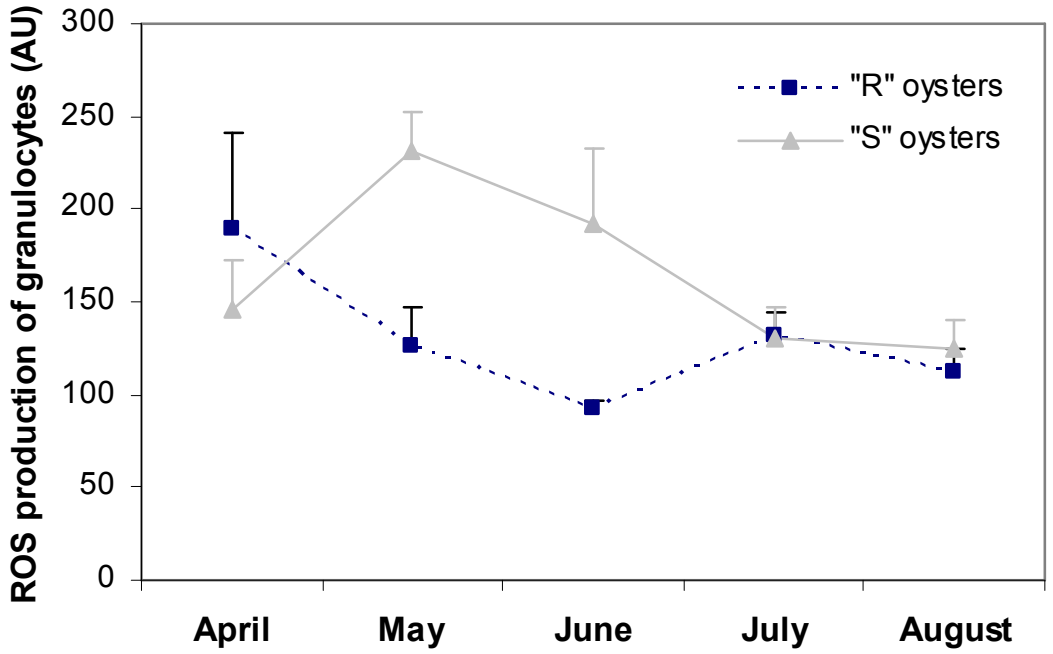
769 **B**



770

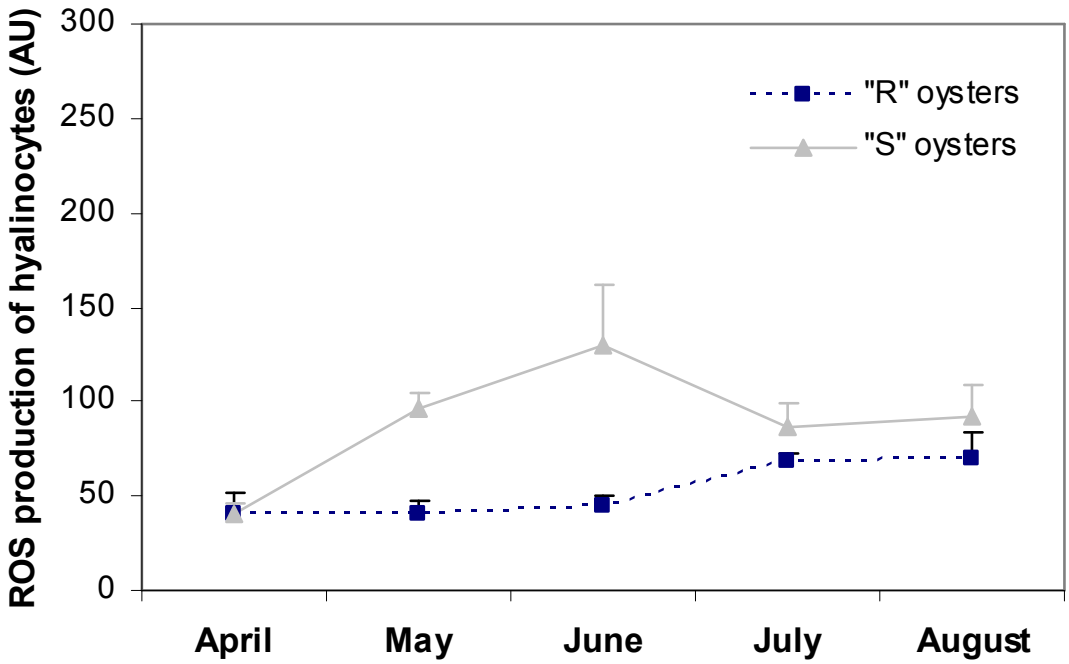
771 **Figure 6A and 6B**

772 **A**



773

774 **B**



775

776 **Figure 7A and 7B**