Journal of Invertebrate Pathology
February 2011, Volume 106, Issue 2, Pages 179-191
http://dx.doi.org/10.1016/j.jip.2010.09.003

Responses of diploid and triploid Pacific oysters *Crassostrea gigas* to Vibrio infection in relation to their reproductive status

S. De Decker[a], J. Normand[a], D. Saulnier[a], F. Pernet[b], S. Castagnet[a] and P. Boudry[c, *]

[a] Ifremer, Laboratoire de Génétique et Pathologie, Ave. Mus de Loup, Ronce-Les-Bains, 17390 La Tremblade, France
[b] Ifremer, Laboratoire Environnement Ressources Languedoc-Roussillon, Pôle Mer et Lagunes, Bd Jean Monnet, BP 171, 34203 Sète Cedex, France
[c] Ifremer, Laboratoire de Physiologie des Invertébrés, Technopôle Brest Iroise, BP 70, 29280 Plouzané, France

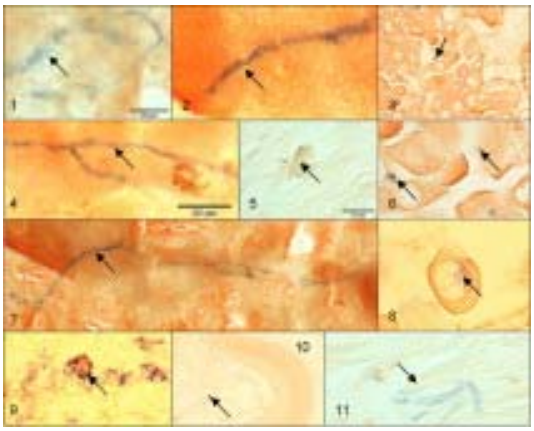
*: Corresponding author : P. Boudry, email address: Pierre.Boudry@ifremer.fr

Abstract:

Several *Vibrio* species are known to be pathogenic to the Pacific oyster *Crassostrea gigas*. Survival varies according to pathogen exposure and high mortality events usually occur in summer during gametogenesis. In order to study the effects of gametogenetic status and ploidy (a factor known to affect reproduction allocation in oysters) on vibriosis survival, we conducted two successive experiments. Our results demonstrate that a common bath challenge with pathogenic *Vibrio splendidus* and *Vibrio aestuarianus* on a mixture of mature, spawning and non-mature oysters can lead to significant mortality. Previous bath challenges, which were done using only non-mature oysters, had not produced mortality. Immunohistochemical analyses showed the affinity of *Vibrio* for gonadic tissues, highlighting the importance of sexual maturity for vibriosis infection processes in oysters. Mortality rate results showed poor repeatability between tanks, however, in this bath challenge. We then tested a standardized and repeatable injection protocol using two different doses of the same combination of two *Vibrio* species on related diploid and triploid oysters at four different times over a year. Statistical analyses of mortality kinetics over a 6-day period after injection revealed that active gametogenesis periods correspond to higher susceptibility to vibriosis and that there is a significant interaction of this seasonal effect with ploidy. However, no significant advantage of triploidy was observed. Triploid oysters even showed lower survival than diploid counterparts in winter. Results are discussed in relation to differing energy allocation patterns between diploid and triploid Pacific oysters.

Graphical abstract

Immunohistochemistry revealed that Vibrio showed affinity for gonadic tissues.



Research highlights

► A bath challenge with pathogenic *Vibrio* on mature oysters led to significant mortality. ► Immunohistochemistry revealed that *Vibrio* showed affinity for gonadic tissues. ► Active gametogenesis periods correspond to high susceptibility to experimental vibriosis. ► Triploid oysters did not show higher resistance than diploid to experimental vibriosis.

Keywords: Experimental vibriosis; *Crassostrea gigas*; *Vibrio*; Survival distribution function; Triploidy; Reproductive effort

1. Introduction

For many years, significant mortalities of Pacific oyster *Crassostrea gigas* have been reported during the summer period. These seasonal mortalities produce no specific clinical signs of disease and affect both juveniles and adults, but are usually greater among young individuals (i.e. ~~–s~~eed" or ~~–s~~pat"). This is notably the case in France, where seed mortalities have become a serious threat to the oyster industry. The aetiology of this phenomenon is clearly multifactorial, resulting from complex interactions between pathogenic agents, environmental factors and the genetic and physiological status of the oysters (Samain and McCombie, 2008). Two main types of pathogenic agents, the herpes virus OsHV-1 (Renault et al., 1995) and bacteria of the genus *Vibrio* (*Vibrio splendidus* and *Vibrio aestuarianus*), have been detected in dying oysters during summer mortality episodes (Garnier et al., 2007; Gay et al., 2004; Labreuche et al., 2006b; Lacoste et al., 2001; Le Roux et al., 2002; Saulnier et al., 2009). For *Vibrio*, a four-year epidemiological survey (2003-2007) aiming to identify bacteria pathogenic to *C. gigas* confirmed the high prevalence of *Vibrio splendidus* and *Vibrio aestuarianus* bacterial strains associated with mortality events in France (Saulnier *et al.*, 2010). The virulence of the *Vibrio* strains isolated during mortality outbreaks has also been assessed using a controlled experimental infection approach (Saulnier *et al.*, 2010).

Besides virulence of pathogenic agents, the variability of oyster physiological status, which is under the control of environmental conditions and genetic characteristics, probably explains much of the variability observed in severity of summer mortality episodes (Samain and McCombie, 2008). Indeed, allocation to each of the main physiological functions (i.e. growth, reproduction and survival) is known to covary, generating physiological and/or genetic trade-offs (Kooijman, 2000; Stearns, 1976). Thus, high reproductive effort for *C. gigas*, which can account for 55 % of the annual energy budget (Van der Meer, 2006), was expected to result in an increase in mortality. A reproduction-survival trade-off was demonstrated using selective breeding experiments (Beattie et al., 1980; Boudry et al., 2008; Perdue et al., 1981). The proposed explanation was that high sexual maturation activity could lead to physiological weakness (Mori, 1979), and that this would result in an immune depression (Duchemin et al., 2007; Li et al., 2009b; Pouvreau and Lepennec, 2006; Soletchnik et al., 1997). Moreover, some environmental factors are known to trigger gonadic tissue development. High food availability (Chavez-Villalba et al., 2003) and warmer seawater temperature (Fabioux et al.,

2005) have been shown to increase reproductive allocation. Due to the trade-off between reproductive effort and survival mentioned above, these factors have also been shown to be associated with higher summer mortality (Pouvreau et al., 2003; Soletchnik et al., 2005; Soletchnik et al., 1999; Soletchnik et al., 2007). Finally, the sign of the genetic correlations between summer survival and reproductive allocation have also been shown to vary according to food abundance (Ernande et al., 2004), demonstrating the primary importance of exogenous factors in controlling energy allocation to different physiological functions and their covariation.

In oysters, triploidy induction is the most common genetic method to enhance production yield through phenotypic improvement (Piferrer et al., 2009). Triploidy affects allocation to physiological functions in oysters and a wide-range of other species. Most importantly, triploid Pacific oysters commonly exhibit a reduced but variable reproductive effort (Normand et al., 2009) and, in parallel, exhibit faster growth than diploid individuals (Allen and Downing, 1986; Nell and Perkins, 2005). Triploid oysters have also been seen to have higher resistance to summer mortality (Boudry et al., 2008), although contradictory performances have also been reported for triploid summer survival (Cheney et al., 2000). It should, however, be noted that only a limited number of studies have examined physiological performances of triploid molluscs, meaning that little is known about actual energetic allocation to different functions. The improved growth and survival of triploid oysters has mainly been interpreted as an indirect effect of triploidy through energetic reallocation from gonadic development to somatic growth and resistance (Allen and Downing, 1986; Allen and Downing, 1990; Garnier-Géré et al., 2002). An alternative hypothesis explains the improvement of survival in triploid oysters as a result of a superior energy budget irrespective of energetic re-allocation processes (Hawkins *et al.*, 1994)

The present study aimed to investigate the relationships between ploidy, survival and reproductive allocation, in oysters challenged with pathogenic *Vibrio*. First, a non-invasive experimental infection protocol coupled with specific immunohistochemical analysis was used to localize pathogenic *Vibrio* in oyster organs and to test for its affinity for gonadic tissue. Second, susceptibility to vibriosis and gonad development were studied over an annual reproductive cycle on related diploid and triploid oysters using a reproducible experimental co-infection protocol and two pathogenic *Vibrio* (*V. splendidus and V. aestuarianus*) (Saulnier *et al.*, 2010). Outcomes are discussed in terms of resource allocation and life-history

121 theory. The results offer some insight into the oyster summer mortality phenomenon and,
122 more generally, show how host-bacteria interactions can mitigate infectious diseases in
123 bivalves.
124

2. Material and methods

2. 1. First experiment – Bath challenge and immunohistochemical analysis

2. 1. 1. Animals studied and reproductive conditioning

One-year-old diploid *C. gigas* oysters were purchased from natural recruitment in the Bassin de Marennes-Oléron (Charente-Maritime, France). Half of these oysters were maintained for two months indoors, using the same temperature and food availability conditions as those found in the field (Ifremer, La Tremblade). The second half of the oysters were matured for two months indoors, using controlled temperature (20 °C) to induce gonadic maturation. Two different sets of oysters were obtained within this second group: spawning and mature (reproductive status was checked visually). Three types of oysters were thus obtained from the common initial population: 1. spawning, 2. mature and 3. non mature animals. All oysters were maintained at 16 °C for two days before the bath challenge (described below) in order to induce spawning events in the ripe individuals when they were transferred to the experimental tanks at 20 °C.

2. 1. 2. Bath challenge

A bath infection challenge with *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041 strains was first carried out on diploid oysters displaying contrasted sexual maturity. Groups of 3 to 5 oysters of each type (1. spawning, 2. mature, 3. non mature animals) were placed together in 10-litre tanks, after shell tagging for identification. Four tanks were set up for the bath challenge and three other tanks were prepared in the same way as an infection control. The combination of LGP32-GFP and 02/041 strains was produced by mixing suspensions of each bacterium at equal concentration (1:1 v/v), as described in Saulnier *et al.* (2010), and adding the mixture to the UV-treated seawater of each tank at a concentration of 10^6 CFU/ml (CFU: Colony Forming Unit) established by absorbance determination at 600 nm wavelength and by plate counting method on Marine Agar (Difco) using appropriate dilutions. Oysters were immerged in contaminated seawater at 18 °C for 40 h in non-circulating aerated conditions. They were then abundantly rinsed with UV-treated seawater and transferred to new tanks filled with fresh UV-treated seawater. Control oysters were treated in the same manner as

bath-infected ones, replacing the *Vibrio* inoculum with artificial seawater. Spawning events, which affected oysters at the spawning stage, were induced by thermal stress at 20°C in all tanks; they were observed in both contaminated and control tanks 1-2 h post-transfer, with strong contractions of the oyster muscle and gametes visible in the water. Thereafter, oyster mortality was monitored daily for 2 weeks. Any dead oysters were removed from each tank daily, individually labelled and fixed in Davidson's fluid (30 % filtered seawater, 30 % ethanol 95 %, 20 % formaldehyde 36 %, 10 % glycerol and 10 % acetic acid) for immunohistochemical analysis.

2. 1. 3. Immunohistochemical analysis

Because the LGP32-GFP mutated strain also has a gene coding chloramphenicol acetyl transferase (CAT), responsible for resistance to chloramphenicol, this last gene product was targeted to immuno-localize LGP32-GFP by immunohistochemistry. This mutant *V. splendidus* strain was provided by F. Le Roux. A commercial polyclonal sheep antibody against CAT type 1 and labelled with digoxigenin antigen was purchased from Roche. Before use, the specificity of the polyclonal antibody solution was increased by *in vitro* absorption on a wild LGP32 strain bacterial suspension.

A few dead mature animals sampled during bath immersion were fixed in Davidson's fluid for 24 h and transferred to 70 % ethanol before being dehydrated in alcohol-xylene series and embedded in paraffin wax. All steps were performed at room temperature. Sections of 5 µm were mounted on silane-prep slides (Sigma), dewaxed, rehydrated, and successively incubated for 30 min in blocking reagent (Roche) prepared in 1X Tris-buffered saline solution (TBS), 30 min with anti-CAT antibody (1 µg/ml) prepared in blocking reagent TBS solution, and 30 min with anti-Digoxigenin Fab fragments antibody conjugated with alkaline phosphatase (Roche) and diluted in TBS. Slides were washed twice with TBS between each step. Immuno-detection was performed with alkaline phosphatase chromogen BCIP-NBT (Sigma) prepared in an alkaline phosphatase buffer (Tris-base 100 mM pH 9, $MgCl_2$ 50 mM, NaCl 100 mM) during one hour of incubation. Slides were then counter-stained with Bismarck brown, mounted and observed under white light microscopy.

2. 1. 4. Statistical analysis

A χ^2 test was performed to analyse significant differences of survival rates obtained after the bath challenge comparing with those obtained after the control bath.

2. 2. Second experiment – Injection challenges

2. 2. 1. Animals studied

The origin of the oysters used in this part of the study and the rearing procedures used are described in detail by Normand *et al.* (2009). Briefly, diploid (“2n”) and triploid (“3nCB” and “3nDT”) groups were produced in June 2006 by crosses between diploid genitors or between diploid and tetraploid genitors. The 3nCB oysters were obtained by chemically-induced retention of polar body II (Gérard et al., 1999) on diploid × diploid crosses, while 3nDT oysters were obtained by crossing diploid females with tetraploid males (Guo et al., 1996). One diploid female, 16 diploid males and 16 tetraploid males were used to generate the corresponding progenies. This scheme of crosses allowed genetic differences between triploids and diploids to be minimized by using common genitors for the three groups as far as possible. Ploidy of these progenies was assessed by flow cytometry on two samples of 100 individuals per group, using DAPI staining of whole DNA content of the nucleus (Allen, 1983). Triploidy was thus confirmed to be over 95 % successful for both 3nCB and 3nDT groups.

All half-sib families were grown in the same zootechnical facilities and rearing site in order to homogenize the environmental conditions among groups. Oyster rearing was conducted following standard hatchery and nursery procedures, after which oysters were transferred to plastic mesh bags, attached to off-bottom iron tables following the local farming practices in the Bassin de Marennes Oléron (Charente-Maritime, France). All three groups were monitored monthly throughout the rearing and experimental period. No significant mortality was ever observed in the oyster bags in the intertidal area during the period of challenge studies in the laboratory with animals of the same groups.

2. 2. 2. Experimental design of the injection challenges

Three preliminary injection experiments were performed on sampled oysters from the three groups during April 2007 in order to determine appropriate injection doses for a DL50.

Four successive experimental challenges were performed starting on May 10th 2007 (date A), June 26th 2007 (date B), August 29th 2007 (date C) and January 21st 2008 (date D), corresponding to different reproductive stages in the diploid oysters (Table 1). For each challenge, 42 to 69 (mean number = 57) randomly sampled oysters from each group were placed in thermo-controlled aerated seawater in order to acclimate them to the seawater temperature at 20 °C over five days before transfer to the infection room. Subsequently, 100 µL of a mix of virulent *Vibrio* suspension were injected into the adductor muscle of the oysters. The challenge procedures are described in Saulnier *et al.* (2010). In the present study, the two different virulent *Vibrio* strains belonging to both species frequently detected during oyster mortality outbreaks - *Vibrio splendidus* LGP32-GFP strain and *Vibrio aestuarianus* 02/041 strain (Garnier et al., 2007) - were used in equal concentration because of their synergistic effect (Saulnier *et al.*, 2010). At each date, the pre-determined high dose and low dose were used, corresponding respectively to 10^7 and 10^6 total CFU injected per individual, as defined in the three preliminary injection experiments (see above). Negative controls consisted of a group of 20 to 36 oysters injected with sterile artificial seawater. At least three tanks were used per date, group and dose as experimental structure replicates (16 to 21 oysters per tank). Mortality was monitored twice a day and newly dead oysters were removed from each tank over a 6-day period. Moribund animals were checked for infection by LGP32-GFP and 02/041 pathogenic strains using classical bacteriological analysis on haemolymph samples, and specific molecular-based-diagnostic tests (Saulnier et al., 2007).

2. 2. 3. Histological characterization

Qualitative (reproductive stage, see Table 2) and quantitative (gonadic occupation, GO) monitoring of oyster reproductive effort was performed on subsamples of the experimental groups. A mean of 40 individual oysters were analysed per date and group. Histological slides of *C. gigas* tissues were analysed following the method described in Normand *et al.* (2009) to obtain the area fractions of gonadic tissue (GA) and the whole visceral mass area (WVMA). Reproductive effort was estimated by gonadic occupation (GO) as: GO (%) = GA x 100 / WVMA. Reproductive stage of diploid oysters was determined following a qualitative classification (5 stages from 0 to 4) adapted from Mann (1979) and Lango-Reynoso (2000). The reproductive stages of triploid oysters are not presented here due to the lack of an appropriate classification scale (Normand et al., 2009).

2. 2. 4. Statistical analysis

All statistical analyses were done with SAS (Statistical Analysis Software, V.9).

For each of the *Vibrio* injected oysters (n=1245), we modelled the time from injection to death. We eliminated those animals that had not died by the last assessment, 6 days post-injection time from this analysis.

Considering the entire data set, we were first interested in modelling the effects of the categorical predicators and their interactions on the survival functions. We built a general model including the following variables: experimental infection date (dates: A, B, C et D), experimental group (groups: 2n, 3nDT, 3nCB), treatment (injection doses: higher or lower) and experimental structure replicate (at least 3 replicates by date, group and dose). We used Cox proportional hazard model to perform regression analysis of survival data, employing the TPHREG procedure (Proportional Hazard Regression) that specifically allowed us to quantitatively examine the relationship between the survival kinetics of oysters, categorical variables and their interactions. We also used a contrast statement in order to test for effects of ploidy level (i.e. diploid versus triploid) and triploidy induction method (i.e. 3nCB versus 3nDT). The complete model first included all fixed effects and interaction terms between date, group, treatment and replicate. We then performed classical backward elimination procedure to obtain the final reduced model: Survival = date + group + treatment + date × group + date × treatment.

This analysis detected highly significant interactions between infection date, group, and treatment. We therefore performed univariate analysis using the non-parametric Kaplan-Meier method to test for differential performances among groups at the same date or within groups throughout the sampling period (Kaplan and Meier, 1958). We used the LIFETEST procedure to compute non-parametric estimates of the survival distribution function and generate mean survival time estimators. Equivalences between the survival curves within groups and among groups using the same infection dose were tested with Wilcoxon tests.

For the investigation of group and date effects on gonadic occupation, we verified the homogeneity of variances using a Levene test (UNIVARIATE procedure) and values were squared-transformed to ensure homoscedasticity. An analysis of variance model (GLM procedure) (Littel et al., 2002) was finally built as: GOt = date + group + date × group.

Due to a significant interaction between date and group, we tested for date effect within group using a LS-means statement. We decided to exclude the values for gonadic occupation

293 measured at date D, because gonadic occupation was null in all sampled individuals at this
294 time.
295
296

3. Results

3. 1. Bath challenge and immunohistochemical analysis

3. 1. 1. Mortality rates

Mean survival at 13 days post-infection after 40 h bath challenge is presented in Fig. 1A. No significant mortality had occurred in control tanks and survival was very significantly lower in bath challenged tanks than in control tanks ($\chi^2 = 27.62$, $p < .0001$). To our knowledge, this is the first time that experimental vibriosis infection using a bath protocol efficiently induced any mortality in *C. gigas*. A high variability of survival rates was found for bath challenge conditions, with one tank showing quite low mortality. Survival rates measured in the four tanks were 15 %, 29 %, 36 % and 78 %. Furthermore, we found no significant differences in mortality with reproductive status ($\chi^2 = 1.18$, $p = 0.5551$; survival rates of 31%, 42 % and 45 % for spawning, mature and non mature individuals, respectively).

3. 1. 2. *V. splendidus* LGP32-GFP strain localization in gonadic tissues

Immunohistochemical analysis revealed that the mutant *V. splendidus* LGP32-GFP strain had infected different kinds of tissues in several oysters that died 7 days post-infection after 40 h of contact with this strain by bath challenge (Fig. 1B). In contrast, no labelling was observed in control animals, confirming the specificity of the diagnostic test. Numerous bacteria were detected for the first time in the gonadic tissue, but also in the adductor muscle, conjunctive tissue or digestive gland, with bacterial cells found alone or organized in foci (Fig. 1B, pictures 1, 2, 3, 4, 6, 7 and 8).

3. 2. The four injection challenges

3. 2. 1. Reproductive status of oysters during the four injection experiments

Histological analysis allowed us to determine the sexual maturation stages and gonadic occupation at the time of each experimental infection (Table 2) and to compare reproductive status of diploid and triploid oysters.

At the time of the first infection (A), the majority of individuals in the diploid group had initiated their reproductive maturation and presented early stages of gonadic development, with 7 % of individuals in stage 1 and 90 % in stage 2 (Fig. 2A); mean gonadic occupation was 34 %. Most triploid oysters (about 90 %) also presented immature gonadic tissues, with a lower - but significant - GO (16 %) compared with diploid individuals (Fig. 2B).

At the time of the second infection (B), all diploid oysters were ripe, i.e. in stage 3, reaching complete maturation of their gonadic tissues and ready to spawn (Fig. 2A). Their mean GO was significantly higher than at the time of infection A (68 %) ($p < 0.0001$). Triploid oysters presented a similar gonadic occupation as at infection A ($p = 0.1302$) (Fig. 2B).

At the third infection (C), about 25 % of diploid oysters were still in stage 3 while a larger proportion (about 50 %) were in the post-spawning stage (stage 4) (Fig. 2A). Moreover, the remaining quarter showed no development of gonadic tissues at all (stage 0), suggesting that they had spawned a few weeks before sampling (Fig. 2A). As a result, the mean GO of diploid oysters was lower than at the time of infection B (49 %) ($p < 0.0001$). A few triploid individuals still showed some gonadic maturation at date C with low mean GO (for 3nDT group, $p < 0.0001$ and for 3nCB group, $p = 0.0034$), indicating that most oysters of the triploid groups underwent resorption of gonadic tissues.

Finally, as expected for the winter period, no sexual activity was observed at the time of infection D (100 % of individuals being in stage 0) (Fig. 2A).

Overall, clear reproductive differences were visible between diploid and triploid oysters at infections A, B and C, while no difference was observed during the resting stage when infection D was performed. The two types of triploid (3nCB and 3nDT) showed similar reproductive status over the sampling dates, except at infection C were GO was higher for 3nDT than for 3nCB (3 % versus 11% respectively).

3. 2. 2. Injection dose characterization

Two infection doses flanking the LD50 were determined in three preliminary experiments and used for the four injection experiments. The lower dose, corresponding to the injection of a mix of LGP32 and 02/041 strains at 5×10^5 CFU oyster^{-1} for each strain, gave a mean survival rate of 76 ± 15 %, whereas the higher dose, corresponding to the injection of a mix of LGP32 and 02/041 strains at a dose of 5×10^6 CFU oyster^{-1} for each strain, gave a mean survival rate of 35 ± 20 %. This dose determination allowed us to control the degree of severity of the challenge and to obtain analyzable variable survival rates.

365

366 3. 2. 3. Experimental infection monitoring

367

368 No mortality occurred in the control tanks in any of the experiments (data not shown).
369 Mortality in the inoculated treatments was therefore considered to be due to the *Vibrio* mix.
370 Moreover, molecular and classical bacteriological diagnostic tests confirmed the *Vibrio*
371 infection with a high concentration, up to 10^6 CFU/ml, of the inoculated *Vibrio* found in
372 individual haemolymph samples.

373

374 3. 2. 4. Survival of the injection challenge

375

376 3. 2. 4. 1. The Proportional Hazard model with categorical covariates: multivariate approach

377

378 The contrast analysis done using the TPHREG procedure detected a significant differentiation
379 between diploid and triploid groups ($\chi^2 = 6.12$, $p = 0.013$) but did not detect any effect of
380 triploidy induction method ($\chi^2 = 0.22$, $p = 0.640$) (Table 3).
381 Global analysis revealed a very significant treatment effect ($\chi^2_1 = 50.77$, $p < .0001$), which is
382 due to the lower survival obtained with the higher injection dose. Date and group effects were
383 also significant (respectively $\chi^2_3 = 10.24$, $p = 0.017$; $\chi^2_2 = 6.17$, $p = 0.046$), as was their
384 interaction ($\chi^2_5 = 11.44$, $p = 0.043$) and the interaction between date and treatment ($\chi^2_3 = 8.68$,
385 $p = 0.034$). Consequently, we analysed survival functions with a univariate approach in order
386 to further describe the variability of response to experimental vibriosis.

387

388 3. 2. 4. 2. Kaplan-Meier estimates of survival functions: univariate approach

389

390 For each treatment, the survival distribution function, which represents the proportion of
391 animals still alive each day of the period following injection, was characterized by the
392 product-limit method (Kaplan and Meier, 1958), and plotted from the day of injection until
393 the end of the test (Fig. 3).

394

395 The survival kinetics comparisons within groups and their mean survival times are presented
396 in Fig. 3 and Figs 4A & 4B, respectively.
397 For the lower dose and for 2n oysters, the mean survival time calculated at the date B was
398 substantially lower than at the other dates, even though the survival kinetics obtained were not

399 statistically different between infection dates (date A: 5.3 ± 0.2 days, date B: 4.9 ± 0.2 days,
400 date C: 5.3 ± 0.2 days, date D: 5.3 ± 0.1 days) (Fig. 4A). At the higher dose, the mean
401 survival time gradually rose from date A to date D, with increasing values from 3.4 ± 0.2 days
402 (date A) to 4.1 ± 0.1 days (date D). For this dose, survival kinetics of diploid oysters obtained
403 at date D were significantly different from those obtained at dates A ($p = 0.0108$) and B ($p =$
404 0.0447) and similar to the one obtained at date C (although C was similar to those obtained at
405 the dates A and B) (Fig. 4B).
406 In the same way, the survival kinetics obtained within 3nDT oysters were not statistically
407 different from each other at the lower dose, with a minimum mean survival time at date A
408 reaching 4.6 ± 0.1 days. The mean survival times calculated at dates B, C and D were 5.2 ±
409 0.2 days, 5.4 ± 0.2 days and 5.0 ± 0.1 days, respectively (Fig. 4A). At the higher dose,
410 survival kinetics within 3nDT at the date B were significantly different to those observed at
411 dates C ($p = 0.0022$) and D ($p = 0.0022$), which were similar to each other. The survival
412 distribution function obtained at date A is similar to those obtained at dates B, C and D. The
413 mean survival times calculated at dates C (3.7 ± 0.2 days) and D (3.4 ± 0.2 days) was higher
414 than those calculated at dates A (2.9 ± 0.2 days) and B (2.6 ± 0.2 days) (Fig. 4B).
415 The same pattern of survival distribution functions was observed at both doses for the 3nCB
416 group, and the kinetic obtained at date C for the higher dose was significantly different from
417 those calculated at date B ($p < 0.0001$) and date D ($p = 0.0002$) (Figs 4A & 4B). The
418 maximum survival times for both doses were found at date C (lower dose: 5.9 ± 0.05 days;
419 higher dose: 4.6 ± 0.2 days). At date B, survival reached 5.2 ± 0.2 days at the lower dose and
420 3.2 ± 0.2 days at the higher dose, while at date D it was 4.9 ± 0.2 days at the lower dose and
421 3.4 ± 0.2 days at the higher dose (Fig. 4B).
422
423 Comparisons of survival kinetics among groups and mean survival times are presented in Figs
424 3, 4A & 4B.
425 The survival kinetics obtained for 2n and 3nDT groups at date A were statistically similar to
426 each other for both doses (lower dose: $p = 0.6119$; higher dose: $p = 0.1298$). However, the
427 mean survival time obtained for the 2n group (5.3 ± 0.2 days at the lower dose and 3.4 ± 0.2
428 days at the higher dose) was higher than that obtained for the 3nDT group (4.6 ± 0.1 days at
429 the lower dose and 2.9 ± 0.2 days at the higher dose) (Figs 4A & 4B).
430 At date B, the 3nDT group presented a lower mean survival time than the 2n group at the
431 higher dose ($p = 0.053$) (2n: 3.5 ± 0.2 days, 3nDT: 2.6 ± 0.2 days, 3nCB: 3.2 ± 0.2 days)

whereas the other survival functions obtained for the three groups are alike for both doses (Figs 4A & 4B).

In experimental infection C, the 3nCB group survival functions were significantly different from those of the 2n (lower dose: $p = 0.0230$, higher dose: $p = 0.0325$) and 3nDT groups (lower dose: $p = 0.0046$; higher dose: $p = 0.0023$). Mean survival times for the 3nCB group at this date were 5.9 ± 0.05 days after the lower injection dose and 4.6 ± 0.2 days after the higher injection dose (Figs 4A & 4B). The survival functions obtained at both doses for the 2n group (lower dose: 5.3 ± 0.2 days; higher dose: 3.9 ± 0.2 days) were similar to those obtained for the 3nDT group (lower dose: 5.4 ± 0.1 days; higher dose: 3.7 ± 0.2 days).

At date D, the 2n group survival function obtained at the higher dose was significantly different from the triploid groups (2n *vs.* 3nCB group: $p = 0.0241$; 2n *vs.* 3nDT group: $p = 0.0114$), and the 2n mean survival time (4.1 ± 0.2 days) was higher than those of 3nDT (3.4 ± 0.2 days) and 3nCB (3.4 ± 0.2 days). At this date, the same order was obtained at the lower dose, with 2n mean survival time at 5.3 ± 0.2 days, 3nDT at 5.0 ± 0.2 days and 3nCB at 4.9 ± 0.2 days (Figs 4A & 4B).

4. Discussion

4. 1. Potential and limitations of bath challenges *versus* injection

Experimental infections of aquatic animals using viruses, bacteria or parasites can be performed to fulfil different objectives: to estimate the efficiency of preventive treatments, study routes of infection, test the resistance of selected lines (Gomez-Leon et al., 2008; Goyard et al., 2008; Lallias et al., 2008) or different ploidy groups (Ching et al., 2009; O'Flynn et al., 1997), and to assess host factors linked to susceptibility (Goarant et al., 1998; Goarant et al., 2006b; Labreuche et al., 2006a). For bacteria, experimental infections are also often used to explore pathogenicity (Gay *et al.*, 2004; Goarant *et al.*, 2006b), coupled with phenotypic and/or molecular characterization of virulence factors. Such experiments are used in epidemiological studies to assess the dynamics of infectious diseases (Goarant et al., 2006a; Guisande et al., 2008; Saulnier et al., 2000).

The best testing method may be co-habitation, bath or injection challenge, depending on objectives, analytical plan and biological characteristics of hosts and pathogens. In the case of *V. splendidus* or *V. aestuarianus* and Pacific oyster *C. gigas*, the bath method led to no vibriosis-induced mortality in groups of non sexually mature oysters (Labreuche *et al.*, 2006a) and personal observations). As this type of protocol leads to significant mortality in other species (Farto et al., 2003; Kettunen and Fjalestad, 2006; Løvoll et al., 2009; Planas et al., 2005; Travers et al., 2008), the negative results obtained with *C. gigas* and *Vibrio* indicate the complex nature of infection processes in this model. However, the success of our bath experiment, carried out using sexually mature oysters led, for the first time, to significant mortality compared with controls, highlighting the importance of the stage of sexual maturity for *Vibrio* infection processes in oysters. However, our bath challenge protocol showed poor repeatability between tanks. It appeared that this method did not create a common challenge to all animals, probably due to the multiplicity of events which could have occurred between the time the water was contaminated and the moment when the *Vibrio* entered the oyster. Moreover, we cannot exclude the possibility that the artificial reproduction conditioning might have had an effect on immune-physiological status and susceptibility to vibriosis of tested oysters. Nonetheless, this bath protocol could be used to map the route of *Vibrio* infection on both spatial and temporal scales. The observed success of our bath protocol in

presence of mature and spawning animals led us to focus on the links between reproduction and survival to vibriosis on related diploid and triploid oysters at different stages of their reproductive cycle, using a standardized injection protocol.

Considering the implication of *Vibrio splendidus* and *V. aestuarianus* in mortality events of the Pacific oyster *C. gigas*, a reproducible experimental co-infection model was recently developed in order to provide an integrative tool for the better understanding of summer mortality (Saulnier *et al.*, 2010). In the present study, experimental infections were carried out paying particular attention to the reproducibility of all controllable parameters: preparation of the inocula, injection procedures, density, seawater quality, tanks, temperature, aeration, mortality survey specification. We ensured that all animals were at equal risk of being infected by using the same amount of bacterial inoculum at the four different dates over the year. Moreover, the fact that no mortality was observed in control tanks during any of the injection experiments strongly suggests that the mortality in *Vibrio* challenge tanks was due to the injected *Vibrio* mix. Furthermore, we verified with the plate-counting method and molecular diagnostic tests that haemolymph samples from some freshly dead oysters harboured dominant bacteria belonging to the same species as those injected (data not shown), fulfilling one of the Koch's postulates and confirming the *Vibrio* infection was the cause of death. Besides these verifications, the clear dose effect, observed at each date, confirmed *Vibrio* infection. In the light of these results, we can be confident that experimental injections were performed under as uniform conditions as possible, allowing an objective comparison of the four sampling dates.

Nevertheless, although survival of control oysters injected with sterile artificial seawater was not affected (i.e. 100 % survival rates observed at each condition), it is obvious that this procedure of experimental infection is invasive and bypasses the natural means of entrance of virulent *Vibrio* into the host tissues and, in this way, bypasses the first lines of defence. However, even though this injection procedure does not mimic the natural route of infection, it allowed experimental co-infection to be tested in a way that led to repeatable vibriosis mortality results. This allowed us to further investigate to importance of reproductive status, modulated by ploidy and time, on resistance to vibriosis.

Lastly, summer mortality under natural conditions arises from a complex interaction of factors (Samain and McCombie, 2008) that we cannot claim to have reproduced under

experimental conditions. We worked under controlled experimental conditions to assess the effect of sexual maturity and ploidy, both of which appear to be relevant. It should also be noted also that we cannot totally exclude the implication of other factors that covaried over time with the reproductive status of the studied oysters.

4. 2. Statistical analysis of survival kinetics

The final general Cox model using TPHREG procedures (Cox, 1972) allowed us to quantitatively examine the overall relationship between susceptibility of challenged oysters and several chosen factors. The power and originality of this procedure lies in its use of categorical variables and interactions. It computes statistical tests using realistic degrees of freedom and is therefore the procedure best suited to our data, although it is still in the testing phase. Similar results were obtained using the more classical PHREG procedure (data not shown). This type of analysis has rarely been used on aquatic animals, although it is widely applied in medical statistics as it provides the hazard ratio – or the risk of death – assessment, according to treatment and prognostic variables (Arriagada et al., 2009; Kwok et al., 2010).
These analyses confirmed the value of this kind of experimental challenge tool, indicating that it could be adapted and integrated into multidisciplinary studies on shellfish mortality events and used to identify host factors and interactions that influence bacterial virulence and aggravate infectious disease.
In further studies, growth could be monitored to improve the interpretation of variability of survival, although no clear differences in growth pattern were detected between groups. Similar trends were observed regarding summer mortality of oysters in the field (Dégremont *et al.*, 2005). The inclusion of individual quantitative phenotypic data describing sexual maturation or somatic growth in the general model would allow the relationship between survival and reproductive effort to be assessed at an individual scale. Such data is not easy to obtain, as non-destructive methods to quantify reproductive status of oysters are still in development (Davenel *et al.*, 2006).

4. 3. Relation between reproductive status, triploidy and survival of *Vibrio* challenges

4. 3. 1. Sexual maturity increases oyster susceptibility to vibriosis

As already mentioned, the present study, examining the reaction of sexually mature oysters to bath challenge, revealed significant mortality. Even though the *Vibrio* entry route remains to be identified, numerous *V. splendidus*-GFP bacteria were specifically detected in gonadic tissues, using the specific immunohistochemical method (Fig. 1B). The organisation in foci reinforces the hypothesis that *Vibrio* has an affinity for this kind of tissue. Gonadic tissue could constitute both a facilitated route of entrance and a highly nutritive tissue, favourable to *Vibrio* infection and establishment. Following this hypothesis, *Vibrio* infection could be considerably amplified by high gonadic tissue weight, which can account for up to 86 % of the individual body weight at maturity (Deslous-Paoli and Héral, 1988; Enríquez-Díaz *et al.*, 2009). Even though mortality rates were variable among tanks, this new result underlines the involvement of sexual maturity in oyster susceptibility to vibriosis; more precisely the transmissibility of *Vibrio* within oysters through gonadic tissue or between oysters *via* spawning. Nevertheless, considering the complexity of infection processes, we cannot use such an approach to define the precise mechanism that compromises the immune responses of *C. gigas*. However, it is interesting to note that extracellular proteases, e.g. metalloproteases, could facilitate the invasion process in gonadic tissue due to their proteolytic activity. Metalloproteases are (1.) known to be involved in tissue invasion and the modulation and/or destruction of host defence (Travis et al., 1995); (2.) described in both of the studied strains of *V. splendidus* and *V. aestuarianus* pathogenic to *C. gigas*, as essential determinants of virulence (Binesse et al., 2008; Labreuche et al., 2006b); and (3.) are a common feature of pathogenic bacteria strains associated with mortality events in *C. gigas* reared in France (Saulnier *et al.*, 2010). The affinity of the bacteria for gonadic tissues underlined in the present study should probably be considered as an aggravating factor that acts in synergy with energetic re-allocation from survival to reproduction to increase oyster vulnerability to vibriosis.

The significant variations in resistance to experimental infections over the reproductive cycle could be linked to sexual maturation. One major trend is the increase of survival at date C compared with dates A and B, observed for all the three groups and both treatments. Considering the trade-off between reproduction and survival in *C. gigas*, it is tempting to correlate this date effect with reproductive status and interpret this variation of susceptibility as an effect of the sexual maturation on survival performances. Diploid individuals are indeed known to make a high energetic allocation to reproductive tissues, (estimated at 55 % of the annual energy budget) (Van der Meer, 2006). Such a reproductive effort could result in physiological weakness that prevents oysters from responding efficiently to vibriosis

585 infection; this has been widely evoked as a part of the explanation for summer mortality of
586 cultured oysters in France (Delaporte et al., 2006; Duchemin et al., 2007; Gagnaire et al.,
587 2006; Li et al., 2009a; Pouvreau and Lepennec, 2006; Soletchnik et al., 1997). Interestingly,
588 lower phagocytic activity and adhesive capacity of oyster haemocytes were reported during
589 the reproductive season (Delaporte et al., 2006; Gagnaire et al., 2006; Li et al., 2009b).
590 Furthermore, energy balance probably did not explain all costs at the mature stage. The very
591 high gonad development in *C. gigas* may result in mechanical difficulties, as gonad size and
592 density probably affect haemolymph circulation (Tran et al., 2008). We suspect that the
593 observed decrease in resistance to experimental infection during the period of active
594 gonadogenesis is due to both the energetic and mechanic disturbance caused by gonad
595 formation.
596 This interpretation is mainly based on the observed covariation between mean group GO and
597 survival at any given time, although alternative experimental approaches could be used to test
598 for a causal relationship between gonad development dynamics and increasing susceptibility
599 to summer mortality. The production of divergent lines for reproductive allocation (Normand,
600 2009) or resistance to summer mortalities (Dégremont et al., 2007) could also constitute
601 interesting material to further investigate this point, especially as a genetic correlation has
602 been observed between these two traits (Ernande et al., 2004; Huvet et al., 2010).
603
604 4. 3. 2. Triploid responses to experimental vibriosis: indications of sexual maturation costs
605
606 Considering the whole sampling period and both triploid groups, we did not observe higher
607 survival in triploid individuals than in diploid ones. This result leads to the conclusion that the
608 reduction of gonadic development in triploid oysters does not lead to a significant
609 improvement of resistance to experimental vibriosis. Under natural conditions, previous
610 studies reported variable results on the relative resistance of diploid and triploid oysters to
611 summer mortality (Allen and Downing, 1986; Cheney et al., 2000; Gagnaire et al., 2006;
612 Garnier-Géré et al., 2002). As triploid oysters often show better growth than diploids (Allen
613 and Downing, 1986; Nell and Perkins, 2005), this is commonly presumed to be mainly related
614 to energy reallocation from gonadic development to somatic growth.
615
616 In our second experiment, at date C, both types of triploid oysters showed a similar higher
617 resistance to experimental vibriosis compared with diploids. This result was not repeated on
618 the other dates, suggesting that triploid oysters might have reduced resistance during the

reproductive period, possibly due to some sort of reproductive cost. This hypothesis is supported by the fact that histological analysis revealed a significant development of gonadic tissue in both triploid groups, indicating a significant allocation to reproduction in these individuals, as reported in some previous studies (Allen and Downing, 1990; Normand et al., 2009). Additionally, it is probable that the measurements of gonadic occupation did not fully reflect the amount of energy devoted to reproduction in triploid oysters, notably because they have the tendency to resorb reproductive tissue before reaching full maturity (Allen and Downing, 1990; Normand et al., 2008; Shpigel et al., 1992). Clearly, such spontaneous atresia tends to disconnect the measured GO at a given time from the true allocation to reproduction, which would be underestimated in this case.

Moreover, histological observations of atresic tissues in diploid and triploid oysters typically showed massive haemocyte infiltrations, which could be interpreted as an inflammatory phenomenon that could also lead to excess costs, independent of energetic allocation to reproduction (Delaporte et al., 2007). Knowing that such reproductive tissue degeneration processes are far more common in triploid than in diploid oysters (Allen and Downing, 1990; Shpigel et al., 1992), the disturbed reproductive tissue development in triploid oysters might result in some additional underestimated costs. It appears difficult in this context to precisely estimate the energetic allocation to reproduction in triploid individuals, a practical problem that was already pointed out in a previous study (Normand et al., 2009). Our experimental approach of estimating allocation to reproduction by direct measure of gonad development and maturity and measuring the costs paid by physiological covariates like survival goes some way to offering a solution to this problem.

4. 3. 3. Evidence for winter depression in triploid oysters

Ultimately, the two triploid groups appeared more susceptible to experimental vibriosis infection at date D (resting period) than at date C, while this was not the case for the diploid oysters, which showed a gradual increase in survival during the autumn and winter period. This suggests an intrinsic weakness of triploid oysters at a time when diploid survival remains unaffected by the costs of reproductive activity. Following this hypothesis, another physiological function could be acting as an energetic sink in triploid oysters, meaning that these individuals require more resources to survive. Such a hypothesis, of an extra maintenance cost in triploid oysters during the winter season, could also explain the recurrent

reports of high autumnal weight loss in these individuals or unexpected consumption of carbohydrate reserves (Allen and Downing, 1986; Lambert et al., 2008). In fact, some evidence already suggests that induced triploidy could lead to general detrimental effects on growth and survival. In fish, triploid individuals are generally surpassed by their diploid counterparts until they gain an advantage from their reduced reproductive development (Piferrer et al., 2009). Growth and survival is slightly depressed in triploid salmonids, but they usually overtake diploid individuals when the latter reach sexual maturity (Chourrout et al., 1986; Quillet et al., 1988). Experimental *Vibrio* challenges conducted on diploid and triploid Chinook salmon also illustrate reduced performance of triploid individuals (Ching et al., 2009).

However, previous studies have proposed that improved growth in triploid molluscs could be explained by an increase in net energy budget (Hawkins et al., 2000; Hawkins et al., 1994; Wang et al., 2002), a phenomenon independent of energetic re-allocation from reproduction to other functions. Such results have been thought to be due to increased heterozygosity in polyploid individuals, suggesting that triploidy could confer an advantage *per se* on growth and survival through the increase of intra-individual allelic diversity and the optimisation of energy balance (Hawkins et al., 2000). Other studies found conflicting results, with very few differences in energy budget detected between diploid and triploid Sydney rock oysters (Kesarcodi-Watson et al., 2001a; Kesarcodi-Watson et al., 2001b) or clams (Mason et al., 1988). These authors explained the observed difference in growth rates as the result of re-allocation from reproduction. At present, the hypothesis of a *per se* triploid survival advantage appears doubtful in molluscs.

5. Conclusion

To summarize, we propose a scenario linking our experimental observations with the summer mortality episodes observed under natural environmental conditions. Working at different dates over a year on oysters issued from one controllable experimental production and reared under common environmental condition allowed us to better document the effect of reproduction on oyster resistance to *Vibrio* challenge. During the period of active sexual maturation, survival performances in oysters challenged with experimental vibriosis were lower than those recorded after spawning. This suggests that the individual resistance to a pathogenic threat varies through the year as a result of a physiological trade-off between

reproduction and survival. We suspect that such a trade-off is primarily made up of energetic components, but is also influenced by additional components that are independent of energetic allocation. Previously published studies have already pointed out the fact that this summer period of physiological weakness probably coincides with a period when exogenous stresses (temperature, pollutants) (Samain and McCombie, 2008) and pathogenic pressures (Thompson et al., 2006) are exacerbated. The conjunction of increased susceptibility of the host and environmental and pathogenic stressors results in the mass mortalities observed in reared stocks of *C. gigas*.

We found that despite their reduced reproductive tissue development, triploid individuals did not show better performances than diploid ones when considering the entire period of the study. This could be due to underestimated costs of reproductive activity for triploid oysters. Reduced triploid survival performances in winter also suggest an intrinsic depression in triploid performance compared with diploids, potentially compensated during the reproductive period. In this case, although winter would correspond to a period of physiological weakness in triploid oysters, this probably does not lead to such heavy mortality as that observed in summer because the pathogen pressure is lower.

Acknowledgements

We are grateful to the hatchery teams at the La Tremblade and Bouin Ifremer stations for their help with the production of the oysters used in the present study. We thank Dr. Frédérique Le Roux (Harvard Medical School, formerly at Institut Pasteur) for providing the *Vibrio splendidus* LGP32 mutated strain expressing a resistance to chloramphenicol antibiotic and a Green Fluorescent Protein. Thanks also to Patrick Soletchnik and Nicolas Taris for their analytical contribution and to Philippe Haffner and Emmanuelle Omnes for their technical help. We are particularly grateful to Ismaël Bernard for his help and support on theoretical ecophysiology background, Ronan Becheler for writing suggestions, Yann Reynaud for critical reading of the manuscript and Helen McCombie for help with the English.

715

716 **References**

717

718 Allen, S. K., 1983. Flow cytometry: assaying experimental polyploidy fish and shellfish.
719 Aquaculture. 33**,** 317-328.

720 Allen, S. K., Downing, S. L., 1986. Performance of triploid Pacific oysters, *Crassostrea gigas*
721 (Thunberg). I. Survival, growth, glycogen content, and sexual maturation in yearlings.
722 J. Exp. Mar. Biol. Ecol. 102**,** 197-208.

723 Allen, S. K., Downing, S. L., 1990. Performance of triploid Pacific oysters, *Crassostrea*
724 *gigas*: Gametogenesis. Can. J. Fish. Aquat. Sci. 47**,** 1213-1222.

725 Arriagada, R., Dunant, A., Pignon, J.-P., Bergman, B., Chabowski, M., Grunenwald, D.,
726 Kozlowski, M., Le Pechoux, C., Pirker, R., Pinel, M.-I. S., Tarayre, M., Le Chevalier,
727 T., 2009. Long-Term Results of the International Adjuvant Lung Cancer Trial
728 Evaluating Adjuvant Cisplatin-Based Chemotherapy in Resected Lung Cancer. J. Clin.
729 Oncol. 28**,** 35-42.

730 Beattie, J. H., Chew, K. K., Hershberger, W. K., 1980. Differential survival of selected strains
731 of Pacific oysters (*Crassostrea gigas*) during summer mortality. Proc. Natl. Shellfish
732 Assoc. 70**,** 184-189.

733 Binesse, J., Delsert, C., Saulnier, D., Champomier-Verges, M. C., Zagorec, M., Munier-
734 Lehmann, H., Mazel, D., Le Roux, F., 2008. The metalloprotease Vsm is the main
735 toxic factor for *Vibrio splendidus* extracellular products. Appl. Environ. Microbiol. 74**,**
736 7108-17.

737 Boudry, P., Dégremont, L., Haffray, P., The genetic basis of summer mortality in Pacific
738 oyster and potential for improving survival by selective breeding in France. In: J. F.
739 Samain, H. McCombie, (Eds.), Summer mortality of Pacific oyster *Crassostrea gigas*.
740 The Morest Project, Editions Ifremer/Quae, Paris, 2008, pp. 153-196.

741 Chavez-Villalba, J., Cochard, J.-C., Le Pennec, M., Barret, J., Enriquez-Diaz, M., Caceres-
742 Martinez, C., 2003. Effects of temperature and feeding regimes on gametogenesis and
743 larval production in the oyster *Crassostrea gigas*. J. Shellfish Res. 22**,** 721-731.

744 Cheney, D. P., MacDonald, B. F., Elston, R. A., 2000. Summer mortality of Pacific oysters,
745 *Crassostrea gigas* (Thunberg): initial findings on multiple environmental stressors in
746 Puget Sound, Washington, 1998. J. Shellfish Res. 19**,** 353-359.

747 Ching, B., Jamieson, S., Heath, J. W., Heath, D. D., Hubberstey, A., 2009. Transcriptional
748 differences between triploid and diploid Chinook salmon (*Oncorhynchus tshawytscha*)
749 during live *Vibrio anguillarum* challenge. Heredity. 104**,** 224.
750 Chourrout, D., Chevassus, B., Krieg, F., Happe, A., Burger, G., Renard, P., 1986. Production
751 of second generation triploid and tetraploid rainbow trout by mating tetraploid males
752 and diploid females — Potential of tetraploid fish. Theor. Appl. Genet. 72**,** 193-206.
753 Cox, D. R., 1972. Regression Models and Life-Tables. J. R. Stat. Soc. 34**,** 187-220.
754 Davenel, A., Quellec, S., Pouvreau, S., 2006. Noninvasive characterization of gonad
755 maturation and determination of the sex of Pacific oysters by MRI. Magn. Reson.
756 Imaging. 24**,** 1103.
757 Dégremont, L., Bédier, E., Soletchnik, P., Ropert, M., Huvet, A., Moal, J., Samain, J.-F.,
758 Boudry, P., 2005. Relative importance of family, site, and field placement timing on
759 survival, growth, and yield of hatchery-produced Pacific oyster spat (*Crassostrea*
760 *gigas*). Aquaculture. 249**,** 213-229.
761 Dégremont, L., Ernande, B., Bédier, E., Boudry, P., 2007. Summer mortality of hatchery-
762 produced Pacific oyster spat (*Crassostrea gigas*). I. Estimation of genetic parameters
763 for survival and growth. Aquaculture. 262**,** 41-53.
764 Delaporte, M., Soudant, P., Lambert, C., Jegaden, M., Moal, J., Pouvreau, S., Dégremont, L.,
765 Boudry, P., Samain, J.-F., 2007. Characterisation of physiological and immunological
766 differences between Pacific oysters (*Crassostrea gigas*) genetically selected for high
767 or low survival to summer mortalities and fed different rations under controlled
768 conditions. J. Exp. Mar. Biol. Ecol. 353**,** 45-57.
769 Delaporte, M., Soudant, P., Lambert, C., Moal, J., Pouvreau, S., Samain, J.-F., 2006. Impact
770 of food availability on energy storage and defense related hemocyte parameters of the
771 Pacific oyster *Crassostrea gigas* during an experimental reproductive cycle.
772 Aquaculture. 254**,** 571-582.
773 Deslous-Paoli, J.-M., Héral, M., 1988. Biochemical composition and energy value of
774 *Crassostrea gigas* (Thunberg) cultured in the bay of Marennes-Oléron. Aquat. Living
775 Resour. 1**,** 239-249.
776 Duchemin, M. B., Fournier, M., Auffret, M., 2007. Seasonal variations of immune parameters
777 in diploid and triploid Pacific oysters, *Crassostrea gigas* (Thunberg). Aquaculture.
778 264**,** 73-81.
779 Enríquez-Díaz, M., Pouvreau, S., Chávez-Villalba, J., Le Pennec, M., 2009. Gametogenesis,
780 reproductive investment, and spawning behavior of the Pacific giant oyster

781 *Crassostrea gigas*: evidence of an environment-dependent strategy. Aquacult. Int. 17**,**
782 491.
783 Ernande, B., Boudry, P., Clobert, J., Haure, J., 2004. Plasticity in resource allocation based
784 life history traits in the Pacific oyster, *Crassostrea gigas*. I. Spatial variation in food
785 abundance. J. Evol. Biol. 17**,** 342-356.
786 Fabioux, C., Huvet, A., Le Souchu, P., Le Pennec, M., Pouvreau, S., 2005. Temperature and
787 photoperiod drive *Crassostrea gigas* reproductive internal clock. Aquaculture. 250**,**
788 458-470.
789 Farto, R., Armada, S. P., Montes, M., Guisande, J. A., Pérez, M. J., Nieto, T. P., 2003. *Vibrio*
790 *lentus* associated with diseased wild octopus (*Octopus vulgaris*). J. Invertebr. Pathol.
791 83**,** 149-156.
792 Gagnaire, B., Soletchnik, P., Madec, P., Geairon, P., Le Moine, O., Renault, T., 2006. Diploid
793 and triploid Pacific oysters, *Crassostrea gigas* (Thunberg), reared at two heights
794 above sediment in Marennes-Oleron Basin, France: Difference in mortality, sexual
795 maturation and hemocyte parameters. Aquaculture. 254**,** 606-616.
796 Garnier-Géré, P. H., Naciri-Graven, Y., Bougrier, S., Magoulas, A., Héral, M., Kotoulas, G.,
797 Hawkins, A., Gérard, A., 2002. Influences of triploidy, parentage and genetic diversity
798 on growth of the Pacific oyster *Crassostrea gigas* reared in contrasting natural
799 environments. Mol. Ecol. 11**,** 1499-1514.
800 Garnier, M., Labreuche, Y., Garcia, C., Robert, M., Nicolas, J. L., 2007. Evidence for the
801 involvement of pathogenic bacteria in summer mortalities of the Pacific oyster
802 *Crassostrea gigas*. Microb. Ecol. 53**,** 187-196.
803 Gay, M., Berthe, F. C., Le Roux, F., 2004. Screening of *Vibrio* isolates to develop an
804 experimental infection model in the Pacific oyster *Crassostrea gigas*. Dis. Aquat. Org.
805 59**,** 49-56.
806 Gérard, A., Ledu, C., Phélipot, P., Naciri-Graven, Y., 1999. The induction of MI and MII
807 triploids in the Pacific oyster *Crassostrea gigas* with 6-DMAP or CB. Aquaculture.
808 174**,** 229-242.
809 Goarant, C., Ansquer, D., Herlin, J., Domalain, D., Imbert, F., De Decker, S., 2006a.
810 "Summer Syndrome" in *Litopenaeus stylirostris* in New Caledonia: Pathology and
811 epidemiology of the etiological agent, *Vibrio nigripulchritudo*. Aquaculture. 253**,** 105-
812 113.

Goarant, C., Régnier, F., Brizard, R., Marteau, A.-L., 1998. Acquisition of susceptibility to *Vibrio penaeicida* in *Penaeus stylirostris* postlarvae and juveniles. Aquaculture. 169**,** 291-296.

Goarant, C., Reynaud, Y., Ansquer, D., De Decker, S., Saulnier, D., Roux, F. l., 2006b. Molecular epidemiology of *Vibrio nigripulchritudo*, a pathogen of cultured penaeid shrimp (*Litopenaeus stylirostris*) in New Caledonia. Syst. Appl. Microbiol. 29**,** 570-580.

Gomez-Leon, J., Villamil, L., Salger, S. A., Sallum, R. H., Remacha-Trivino, A., Leavitt, D. F., Gomez-Chiarri, M., 2008. Survival of eastern oysters *Crassostrea virginica* from three lines following experimental challenge with bacterial pathogens. Dis. Aquat. Org. 79**,** 95-105.

Goyard, E., Goarant, C., Ansquer, D., Brun, P., de Decker, S., Dufour, R., Galinié, C., Peignon, J.-M., Pham, D., Vourey, E., Harache, Y., Patrois, J., 2008. Cross breeding of different domesticated lines as a simple way for genetic improvement in small aquaculture industries: Heterosis and inbreeding effects on growth and survival rates of the Pacific blue shrimp *Penaeus (Litopenaeus) stylirostris*. Aquaculture. 278**,** 43-50.

Guisande, J. A., Perez Lago, E., Prado, S., Nieto, T. P., Farto Seguin, R., 2008. Genotypic diversity of culturable *Vibrio* species associated with the culture of oysters and clams in Galicia and screening of their pathogenic potential. J. Shellfish Res. 27**,** 801-809.

Guo, X., DeBrosse, G. A., Allen, S. K., 1996. All-triploid Pacific oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. Aquaculture. 142**,** 149-161.

Hawkins, A., Magoulas, A., Héral, M., Bougrier, S., Naciri-Graven, Y., Day, A. J., Kotoulas, G., 2000. Separate effects of triploidy, parentage and genomic diversity upon feeding behaviour, metabolic efficiency and net energy balance in the Pacific oyster *Crassostrea gigas*. Genet. Res. 76**,** 273-284.

Hawkins, A. J. S., Day, A. J., Gérard, A., Naciri, Y., Ledu, C., Bayne, B. L., Héral, M., 1994. A genetic and metabolic basis for faster growth among triploids induced by blocking meiosis I but not meiosis II in the larviparous European flat oyster, *Ostrea edulis L.* J. Exp. Mar. Biol. Ecol. 184**,** 21-40.

Huvet, A., Normand, J., Fleury, E., Quillien, V., Fabioux, C., Boudry, P., 2010. Reproductive effort of Pacific oysters: A trait associated with susceptibility to summer mortality. Aquaculture. 304**,** 95-99.

Kaplan, E. L., Meier, P., 1958. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc. 53**,** 457-481.

Kesarcodi-Watson, A., Klumpp, D. W., Lucas, J. S., 2001a. Comparative feeding and physiological energetics in diploid and triploid Sydney rock oysters (*Saccostrea commercialis*): II. Influences of food concentration and tissue energy distribution. Aquaculture. 203**,** 195.

Kesarcodi-Watson, A., Lucas, J. S., Klumpp, D. W., 2001b. Comparative feeding and physiological energetics of diploid and triploid Sydney rock oysters, *Saccostrea commercialis*: I. Effects of oyster size. Aquaculture. 203**,** 177.

Kettunen, A., Fjalestad, K. T., 2006. Resistance to vibriosis in Atlantic cod (*Gadus morhua L.*): First challenge test results. Aquaculture. 258**,** 263-269.

Kooijman, S. A. L. M., 2000. Dynamic energy and mass budgets in biological systems. Cambridge University Press, Cambridge, UK.

Kwok, J., Langevin, S. M., Argiris, A., Grandis, J. R., Gooding, W. E., Taioli, E., 2010. The impact of health insurance status on the survival of patients with head and neck cancer. Cancer. 116**,** 476-485.

Labreuche, Y., Lambert, C., Soudant, P., Boulo, V., Huvet, A., Nicolas, J. L., 2006a. Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. Microbes Infect. 8**,** 2715-24.

Labreuche, Y., Soudant, P., Goncalves, M., Lambert, C., Nicolas, J. L., 2006b. Effects of extracellular products from the pathogenic *Vibrio aestuarianus* strain 01/32 on lethality and cellular immune responses of the oyster *Crassostrea gigas*. Dev. Comp. Immunol. 30**,** 367-79.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gelebart, F., Cordevant, C., Lange, M., Poulet, S. A., 2001. A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). Dis. Aquat. Org. 46**,** 139-45.

Lallias, D., Arzul, I., Heurtebise, S., Ferrand, S., Chollet, B., Robert, M., Beaumont, A. R., Boudry, P., Morga, B., Lapègue, S., 2008. Bonamia ostreae-induced mortalities in one-year old European flat oysters *Ostrea edulis*: experimental infection by cohabitation challenge. Aquat. Living Resour. 21**,** 423-439.

Lambert, C., Moal, J., Le Moullac, G., Pouvreau, S., Les risques associés à la physiologie de l'huître en période de reproduction. In: J. Samain, H. McCombie, (Eds.), Mortalités

879 estivales de l'huître creuse *Crassostrea gigas*. Défi Morest, Ifremer/Quae, Paris, 2008,
880 pp. 153-196.
881 Lango-Reynoso, F., Chávez-Villalba, J., Cochard, J. C., Le Pennec, M., 2000. Oocyte size, a
882 means to evaluate the gametogenic development of the Pacific oyster, *Crassostrea*
883 *gigas* (Thunberg). Aquaculture. 190**,** 183-199.
884 Le Roux, F., Gay, M., Lambert, C., Waechter, M., Poubalanne, S., Chollet, B., Nicolas, J. L.,
885 Berthe, F., 2002. Comparative analysis of *Vibrio splendidus*-related strains isolated
886 during *Crassostrea gigas* mortality events. Aquat. Living Resour. 15**,** 251-258.
887 Li, Y., Qin, J. G., Li, X., Benkendorff, K., 2009a. Monthly variation of condition index,
888 energy reserves and antibacterial activity in Pacific oysters, *Crassostrea gigas*, in
889 Stansbury (South Australia). Aquaculture. 286**,** 64-71.
890 Li, Y., Qin, J. G., Li, X., Benkendorff, K., 2009b. Spawning-dependent stress response to
891 food deprivation in Pacific oyster *Crassostrea gigas*. Aquaculture. 286**,** 309-317.
892 Littel, R., Stroup, W., Freund, J., 2002. SAS for Linear Models, Fourth Edition., Cary, NC,
893 USA.
894 Løvoll, M., Wiik-Nielsen, C. R., Tunsjø, H. S., Colquhoun, D., Lunder, T., Sørum, H., Grove,
895 S., 2009. Atlantic salmon bath challenged with *Moritella viscosa* - Pathogen invasion
896 and host response. Fish Shellfish Immunol. 26**,** 877-884.
897 Mann, R., 1979. Some biochemical and physiological aspects of growth and gametogenesis in
898 *Crassostrea gigas* and *Ostrea edulis* grown at sustained elevated temperature. J. Mar.
899 Biol. Assoc. U.K. 59**,** 95-110.
900 Mason, K. M., Shumway, S. E., Allen, S. K. J., Hidu, H., 1988. Induced triploidy in the soft-
901 shelled clam *Mya arenaria*: energetic implications. Mar. Biol. 98**,** 519-582.
902 Mori, K., 1979. Effects of artificial eutrophication on the metabolism of the Japanese oyster
903 *Crassostrea gigas*. Mar. Biol. 53**,** 361-369.
904 Nell, J. A., Perkins, B., 2005. Studies on triploid oysters in Australia: farming potential of all-
905 triploid Pacific oysters, *Crassostrea gigas* (Thunberg), in Port Stephens, New South
906 Wales, Australia. Aquac. Res. 36**,** 530-536.
907 Normand, J., Déterminismes génétiques de l'allocation à la reproduction chez les huîtres
908 creuses (*Crassostrea gigas*) triploïdes. Laboratoire de Génétique et Pathologie,
909 IFREMER. Université de La Rochelle, France, 2009, pp. 224.
910 Normand, J., Ernande, B., Haure, J., McCombie, H., Boudry, P., 2009. Reproductive effort
911 and growth in *Crassostrea gigas*: comparison of young diploid and triploid oysters
912 issued from natural crosses or chemical induction. Aquat. Biol. 7**,** 229-241.

913 Normand, J., Le Pennec, M., Boudry, P., 2008. Comparative histological study of
914 gametogenesis in diploid and triploid Pacific oysters (*Crassostrea gigas*) reared in an
915 estuarine farming site in France during the 2003 heatwave. Aquaculture. 282**,** 124-
916 129.
917 O'Flynn, F. M., McGeachy, S. A., Friars, G. W., Benfey, T. J., Bailey, J. K., 1997.
918 Comparisons of cultured triploid and diploid Atlantic salmon (*Salmo salar* L.). ICES
919 J. Mar. Sci. 54**,** 1160-1165.
920 Perdue, J. A., Beattie, J. H., Kenneth, K. C., 1981. Some relationships between gametogenetic
921 cycle and summer mortality phenomenon in the Pacific oyster (*Crassostrea gigas*) in
922 Washington State. J. Shellfish Res. 1**,** 9-16.
923 Piferrer, F., Beaumont, A., Falguière, J.-C., Flajshans, M., Haffray, P., Colombo, L., 2009.
924 Polyploid fish and shellfish: Production, biology and applications to aquaculture for
925 performance improvement and genetic containment. Aquaculture. 293**,** 125-156.
926 Planas, M., Perez-Lorenzo, M., Antonio Vazquez, J., Pintado, J., 2005. A model for
927 experimental infections with *Vibrio* (*Listonella*) anguillarum in first feeding turbot
928 (*Scophthalmus maximus L.*) larvae under hatchery conditions. Aquaculture. 250**,** 232-
929 243.
930 Pouvreau, S., Enriquez-Diaz, M., Le Souchu, P., Connan, J. P., Le Roy, B., Mingant, C.,
931 Moal, J., Delaporte, M., Le Coz, J. R., Samain, J. F., Reproduction, bioenergetic and
932 summer mortality of *Crassostrea gigas*: experimental approach. In: N. S. Association,
933 (Ed.), Annual Meeting of The National Shellfisheries Association, Vol. 22, New-
934 Orleans, LA (USA), 2003, pp. 351.
935 Pouvreau, S., Lepennec, M., 2006. Ecophysiology of the reproduction of cupped oyster,
936 *Crassostrea gigas*. IFREMER.
937 Quillet, E., Chevassus, B., Blanc, J., Krieg, F., Chourrout, D., 1988. Performances of auto and
938 allotriploids in salmonids. I. Survival and growth in fresh water farming. Aquat.
939 Living Resour. 1**,** 29-43.
940 Renault, T., Le Deuff, R., Cochennec, N., Chollet, B., Maffart, P., 1995. Herpes-like viruses
941 associated with high mortality levels in larvae and spat of Pacific oysters, *Crassostrea*
942 *gigas*: A comparative study, the thermal effects on virus detection in hatchery-reared
943 larvae, reproduction of the disease in axenic larvae. Vet. Res. 26**,** 539-543.
944 Samain, J. F., McCombie, H., 2008. Summer mortality of Pacific oyster *Crassostrea gigas*.
945 The Morest Project. 379p.

946 Saulnier, D., De Decker, S., Haffner, P., 2009. Real-time PCR assay for rapid detection and
947 quantification of *Vibrio aestuarianus* in oyster and seawater: A useful tool for
948 epidemiologic studies. J. Microbiol. Methods. 77**,** 191-197.
949 Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., Garcia, C., 2010. A large-
950 scale epidemiological study to identify bacteria pathogenic to Pacific oyster
951 *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity.
952 Microb. Ecol. 59**,** 787-798.
953 Saulnier, D., De Decker, S., Haffner, P., Duperthuy, M., Le Roux, F., Development of
954 diagnostic tools for the detection of *Vibrio* species and pathogenic strains associated
955 with mortalities of cultured pacific cupped oyster *Crassostrea gigas* in France. Vibrio
956 2007 meeting, Pasteur Institute, Paris (France), 2007, pp. 191.
957 Saulnier, D., Haffner, P., Goarant, C., Levy, P., Ansquer, D., 2000. Experimental infection
958 models for shrimp vibriosis studies: a review. Aquaculture. 191**,** 133-144.
959 Shpigel, M., Barber, B. J., Mann, R., 1992. Effects of elevated temperature on growth,
960 gametogenesis, physiology, and biochemical composition in diploid and triploid
961 Pacific oysters, *Crassostrea gigas* Thunberg. J. Exp. Mar. Biol. Ecol. 161**,** 15.
962 Soletchnik, P., Lambert, C., Costil, K., 2005. Summer mortality of *Crassostrea gigas*
963 (Thunberg) in relation to environmental rearing conditions. J. Shellfish Res. 4**,** 197-
964 207.
965 Soletchnik, P., Le Moine, O., Faury, N., Razet, D., Geairon, P., Goulletquer, P., 1999.
966 Summer mortality of the oyster in the Bay Marennes-Oléron: Spatial variability of
967 environment and biology using a geographical information system (GIS). Aquat.
968 Living Resour. 12**,** 131-143.
969 Soletchnik, P., Razet, D., Geairon, P., Faury, N., Goulletquer, P., 1997. Ecophysiologie de la
970 maturation sexuelle et de la ponte de l'huître creuse *Crassostrea gigas*: réponses
971 métaboliques (respiration) et alimentaires (filtration, absorption) en fonction des
972 différents stades de maturation. Aquat. Living Resour. 10**,** 177-185.
973 Soletchnik, P., Ropert, M., Mazurie, J., Fleury, P. G., Le Coz, F., 2007. Relationships
974 between oyster mortality patterns and environmental data from monitoring databases
975 along the coasts of France. Aquaculture. 271**,** 384-400.
976 Stearns, S. C., 1976. Life-history tactics: A review of the ideas. Q. Rev. Biol. 51**,** 3-47.
977 Thompson, F. L., Austin, B., Swings, J., 2006. The biology of Vibrios. American Society for
978 Microbiology Press, Washington, D. C.

Tran, D., Massabuau, J.-C., Vercelli, C., 2008. Influence of sex and spawning status on oxygen consumption and blood oxygenation status in oysters *Crassostrea gigas* cultured in a Mediterranean lagoon (Thau, France). Aquaculture. 277**,** 58.

Travers, M.-A., Basuyaux, O., Le Goic, N., Huchette, S., Nicolas, J.-L., Koken, M., Paillard, C., 2008. Influence of temperature and spawning effort on *Haliotis tuberculata* mortalities caused by *Vibrio harveyi*: an example of emerging vibriosis linked to global warming. Glob. Chang. Biol. 15**,** 1365-1376.

Travis, J., Potempa, J., Maeda, H., 1995. Are bacterial proteinases pathogenic factors? Trends Microbiol. 3**,** 405-407.

Van der Meer, J., 2006. An introduction to Dynamic Energy Budget (DEB) models with special emphasis on parameter estimation. J. Sea Res. 56**,** 85.

Wang, Z., Guo, X., Allen, S. K., Wang, R., 2002. Heterozygosity and body size in triploid Pacific oysters, *Crassostrea gigas* Thunberg, produced from meiosis II inhibition and tetraploids. Aquaculture. 204**,** 337.

997
998 **Table 1**

999 Characteristics of experimental infection challenges (infection date, injected dose, number of
1000 animals transferred to the infection room). CFU: Colony Forming Unit, SASW: Sterile
1001 Artificial Sea Water.

1002

Infection	Injected dose (CFU/ind.)	Number of animals injected		
		2n	3nDT	3nCB
A 10-May-07	10^7	63	53	not tested
	10^6	62	54	
	SASW	26	32	
B 26-Jun-07	10^7	48	49	48
	10^6	48	49	49
	SASW	20	20	20
C 29-Aug-07	10^7	60	60	56
	10^6	61	63	59
	SASW	20	20	20
D 21-Jan-08	10^7	69	68	42
	10^6	67	68	49
	SASW	36	36	21

1003

1004

Table 2

Reproductive scale for *Crassostrea gigas* based on Mann (1979) and Lango-Reynoso *et al.* (2000).

Stage	Histological description
0 (resting stage)	No trace of sexual development; follicles are non-existent or elongated and consist of undifferentiated germinal epithelium
1 (early growth stage)	Follicles are small and isolated with numerous spermatogonia or oogonia
2 (late growth stage)	Follicles are actively developing with primary gametocytes and some free (secondary) spermatozoa and oocytes
3 (mature)	Near ripe or ripe follicles, densely packed with maturing gametes; presence of mature gametes
4 (spawning and reabsorption stages)	Follicles distended, numerous gametes remain

Table 3

Cox regression model results using the TPHREG procedure.

Variables	DDL	χ^2	Pr > χ^2
Date	3	10.24	0.017
Group	2	6.17	0.046
Treatment	1	50.77	< .0001
Date × Group	5	11.44	0.043
Date × Treatment	3	8.68	0.034
Results of the contrast statement			
2n vs 3n	1	6.12	0.013
3nCB vs 3nDT	1	0.22	0.640

Fig. 1

A. Mean (± SE) survival rates (%) obtained 13 days post-infection after 40 h exposure to *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041 strains by bath immersion. Results are given according to the reproductive status of the tested oysters. A χ^2 test was performed to analyse significant differences of survival rates obtained after bath challenge.

B. *V. splendidus* LGP32-GFP mutated strain infecting *Crassostrea gigas*. Immunohistochemical analysis of (1,2,3,4,6,7,8) gonadic tissue, (5,11) adductor muscle and (9,10) conjunctive tissue of oysters that had died during the 7 days following the 40h exposure to *V. splendidus* LGP32 and *V. aestuarianus* 02/041 strains by bath immersion.

Fig. 2

A. Variation in proportion of sexual maturity stages for diploid individuals tested at the four *Vibrio* injection challenges (A, B, C, D) using the reproductive scale for *Crassostrea gigas* based on Mann (1979) and Lango-Reynoso *et al.* (2000) (see Table 1).

B. Mean gonadic occupation (GO %) measurements (± SE) made on 2n, 3nDT and 3nCB individuals tested at the four *Vibrio* injection challenges (A, B, C, D). Different letters indicate significant differences among and within groups (ANOVA, $p<0.001$). *"nt"* means "not tested".

Fig. 3

Kaplan-Meier estimate of the survival distribution function obtained each day in the three tanks used per treatment, for the three groups injected with the two injection doses. The lower dose is represented by a dotted line (**…**), the higher dose (**-**) by a solid line.

Fig. 4

Mean (± SE) survival times calculated from the Kaplan-Meier survival distribution functions in the three tanks used per date (A, B, C, D) for the three groups, 2n, 3nDT and 3nCB, injected with the lower dose **(A)** and the higher dose **(B)** of *Vibrio* strains. *"nt"* means "not tested". Comparisons between survival curves were performed for each dose with the Wilcoxon test: i. the equivalence between the survival curves among groups at a common infection date is represented by Arabic numerals, ii. the equivalence between the survival curves within groups (each group separately, 2n, 3nDT or 3nCB) is represented by letters.

Fig. 1 A

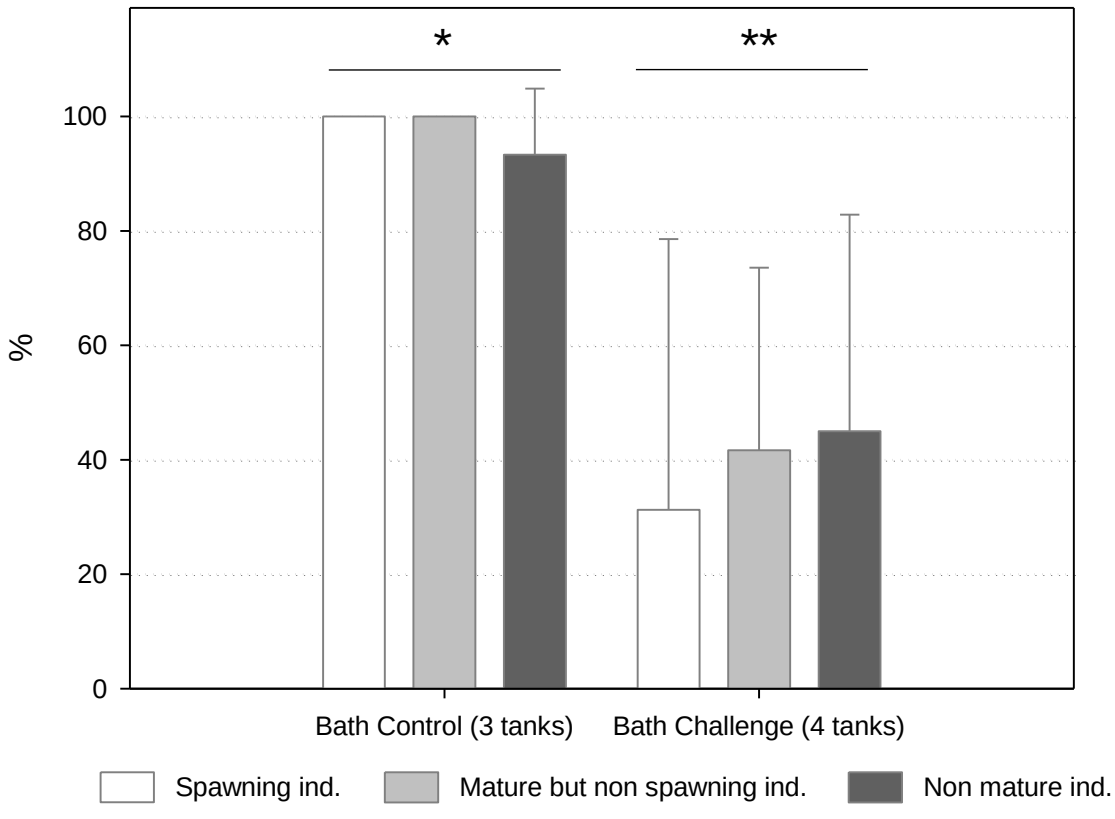

*
**
100
80
60
40
20
0
%
Bath Control (3 tanks)
Bath Challenge (4 tanks)
Spawning ind.
Mature but non spawning ind.
Non mature ind.

Fig. 1 B

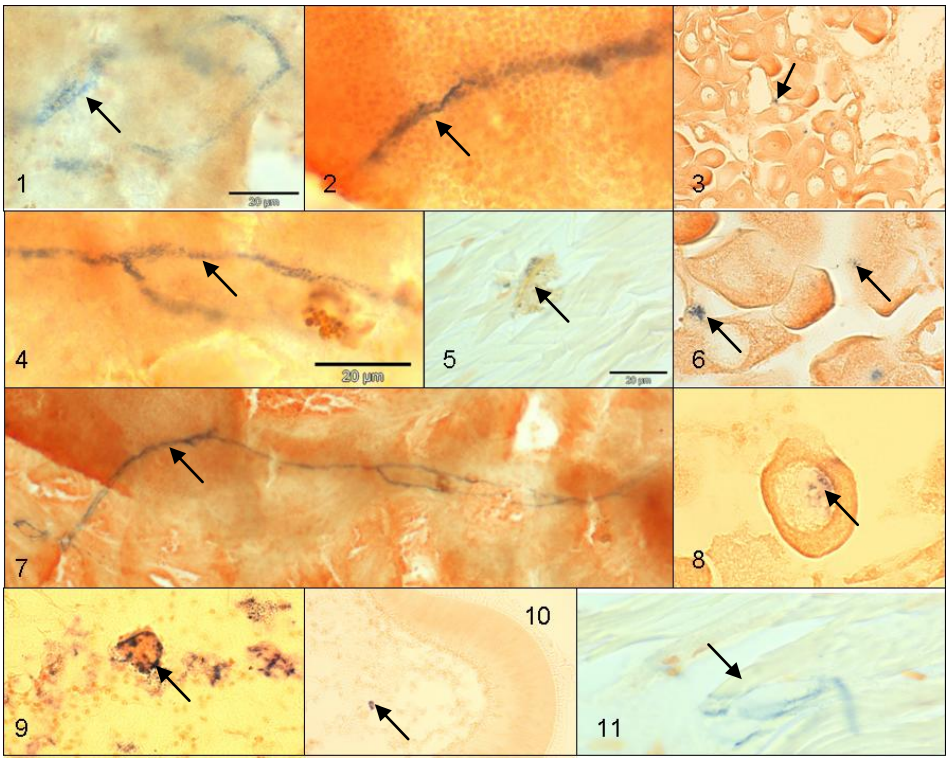


Fig. 2 A

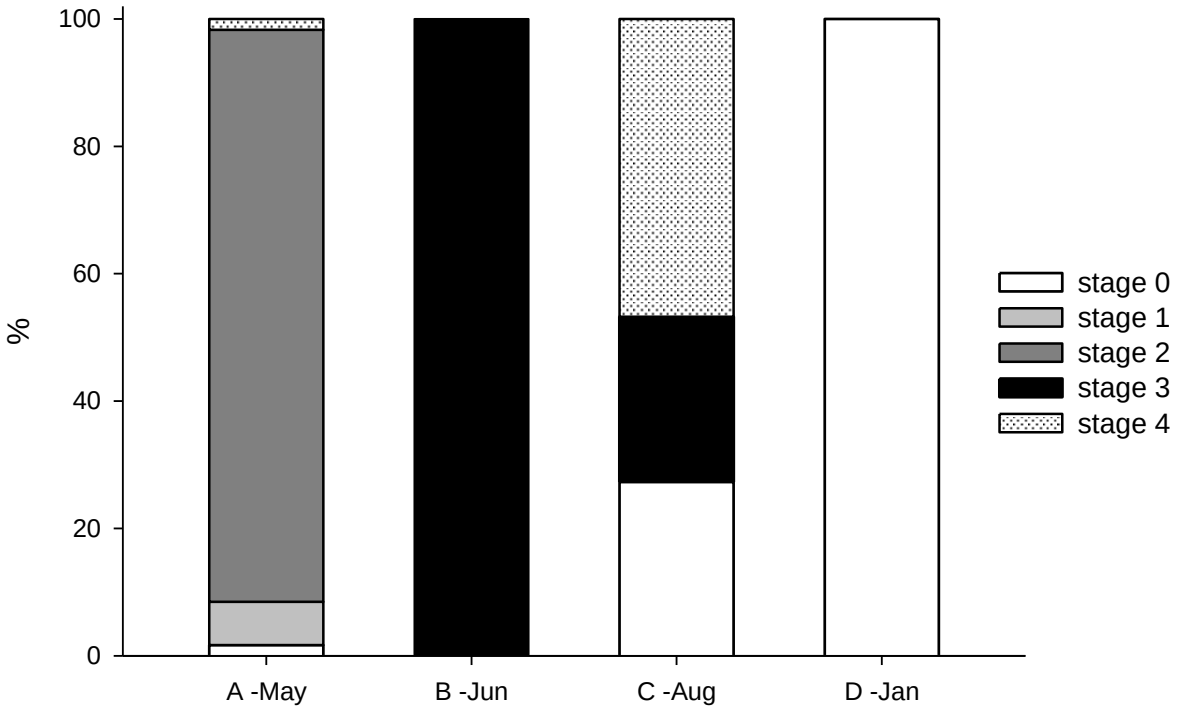

100
80
60
40
20
0
%
A -May
B -Jun
C -Aug
D -Jan
stage 0
stage 1
stage 2
stage 3
stage 4

Fig. 2 B

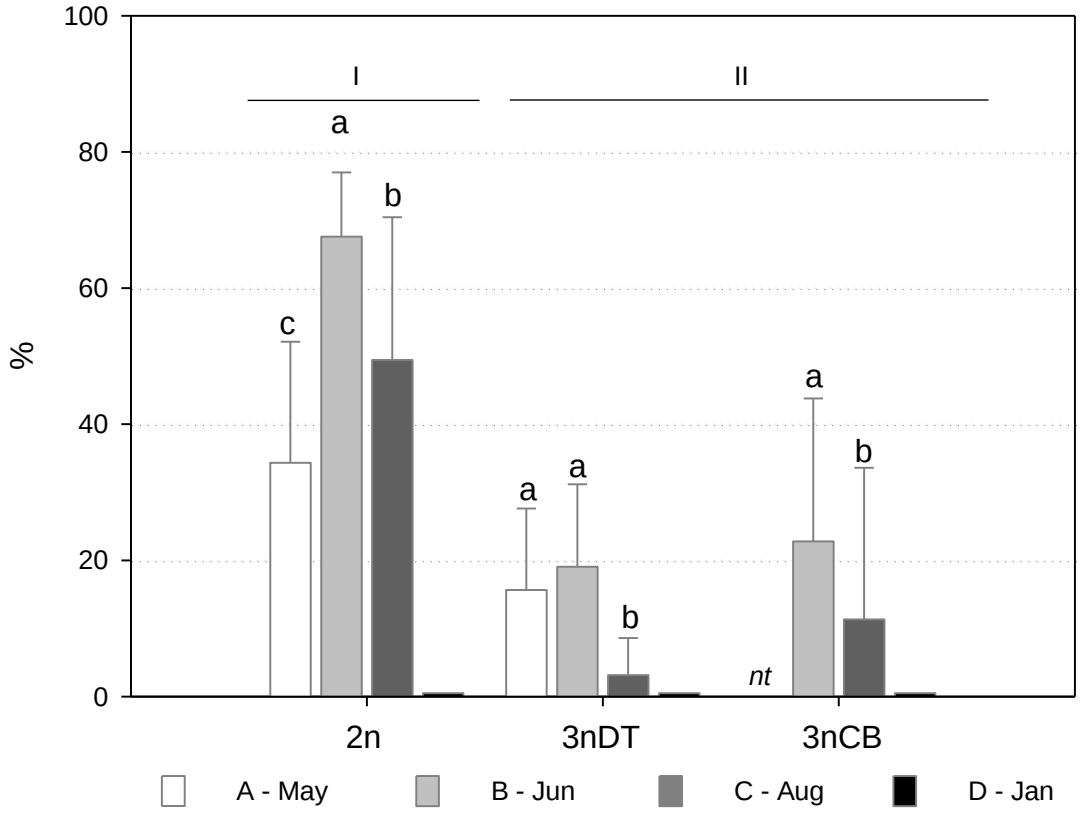

100
80
60
40
20
0
%
I
II
a
b
c
a
a
b
a
b
nt
2n
3nDT
3nCB
A - May
B - Jun
C - Aug
D - Jan

Fig. 3

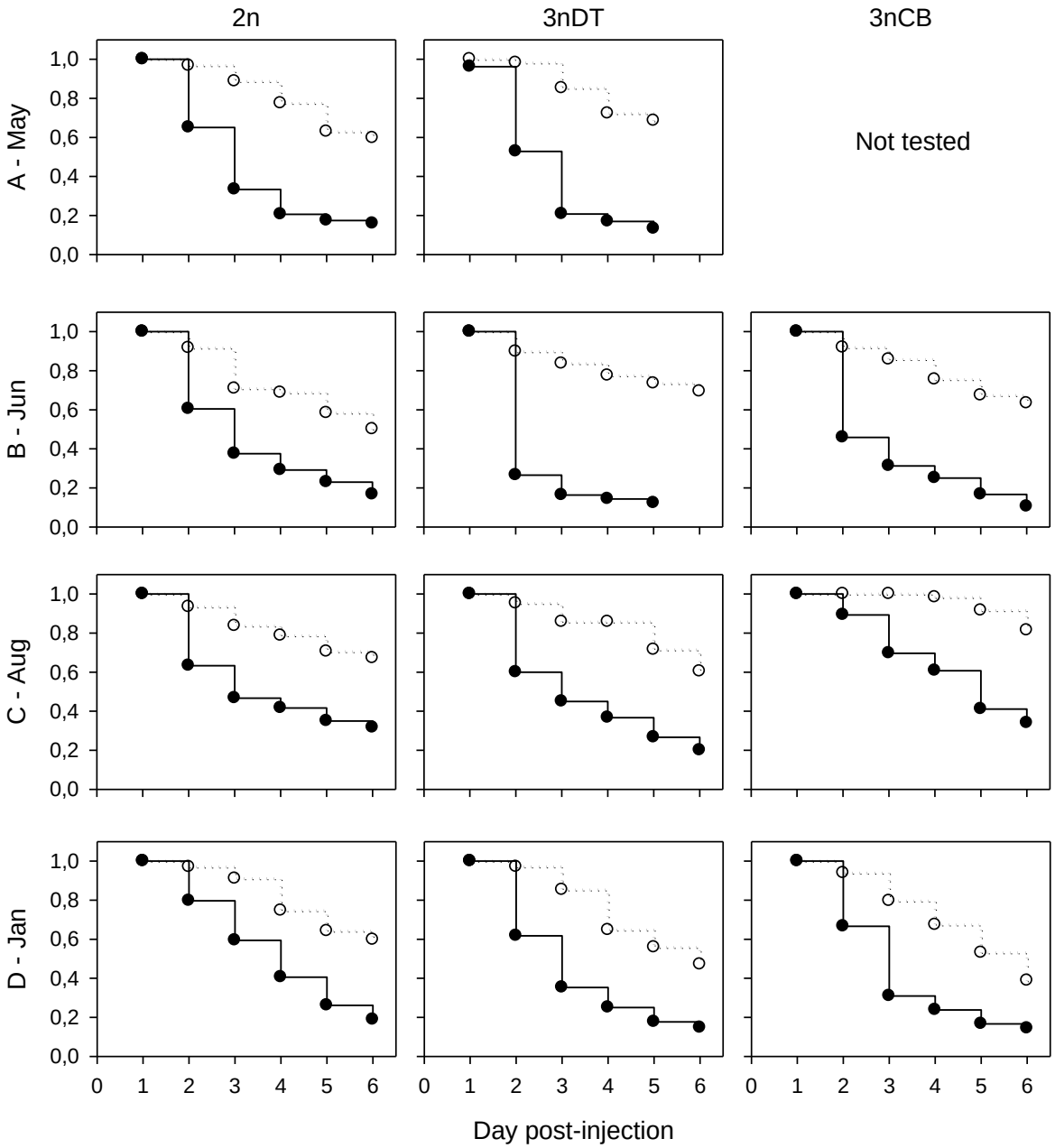

2n
3nDT
3nCB
Not tested
A - May
B - Jun
C - Aug
D - Jan
0,0
0,2
0,4
0,6
0,8
1,0
0
1
2
3
4
5
6
Day post-injection

Fig. 4 A

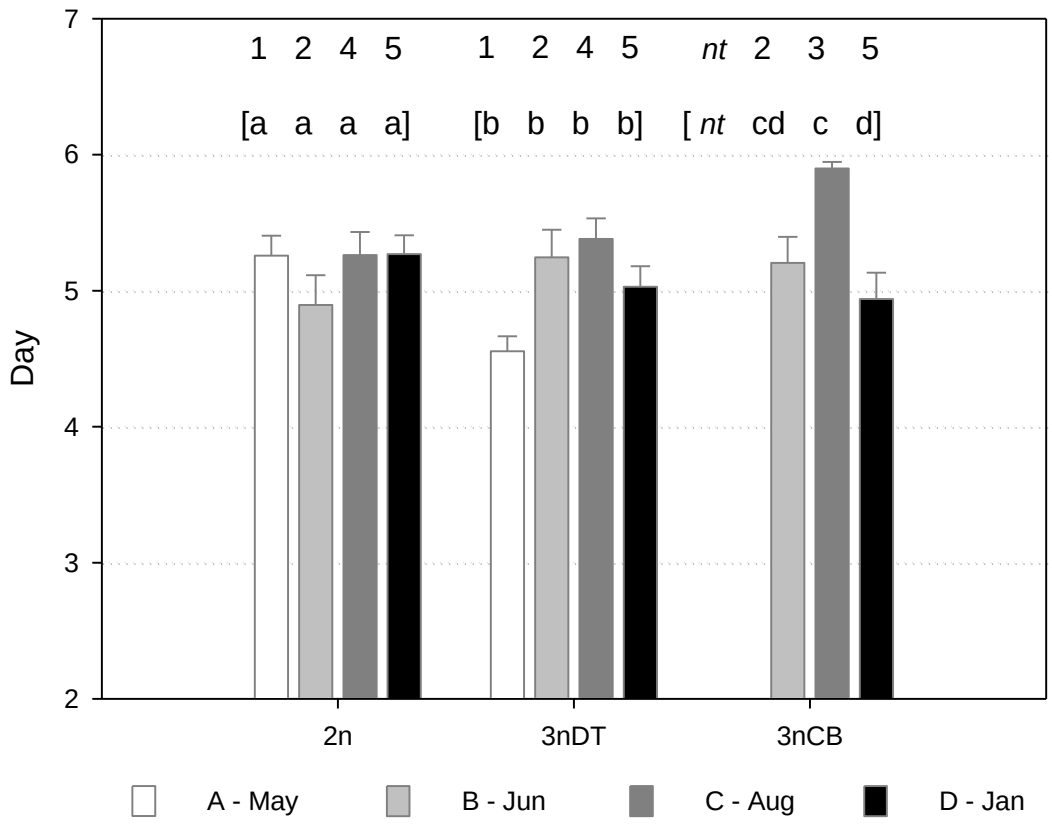
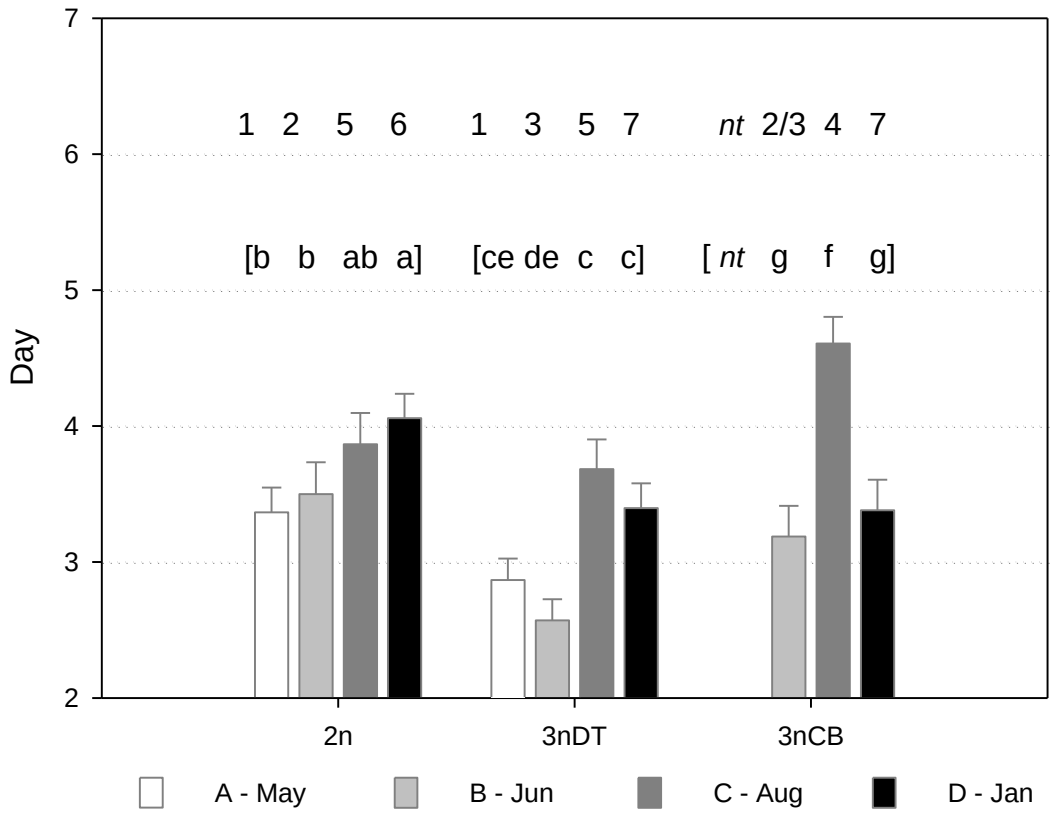

1 2 4 5 1 2 4 5 nt 2 3 5
[a a a a] [b b b b] [nt cd c d]
Day
7
6
5
4
3
2
2n
3nDT
3nCB
A - May
B - Jun
C - Aug
D - Jan

Fig. 4 B


7
6
5
4
3
2
Day
1 2 5 6 1 3 5 7 nt 2/3 4 7
[b b ab a] [ce de c c] [nt g f g]
2n
3nDT
3nCB
A - May
B - Jun
C - Aug
D - Jan