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# Reproductive effort and growth in *Crassostrea gigas*: comparison of young diploid and triploid oysters issued from natural crosses or chemical induction

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ABSTRACT: Early reproductive effort and growth were measured in 3 groups of the Pacific oyster *Crassostrea gigas*: 1 diploid group and 2 triploid groups resulting from either chemical induction (3nCB) or crosses between tetraploid and diploid parents (3nDT). Oysters were reared under intensive nursery conditions and sampled when 5 mo old. Reproductive effort was estimated by cross-sectional area measurements of the visceral mass (i.e. gonadic occupation) and maturation stage was assessed by qualitative histology. As expected, comparison of the reproductive patterns of these 3 groups revealed a lower reproductive effort in triploid individuals relative to diploid. However, gonadic occupation in triploid oysters was higher than expected in both 3nCB and 3nDT groups, as the gonadic occupation was 47% of that in diploids at the sampling date. Our results suggest that, despite much lower mature gamete production in triploid oysters relative to diploid, their reproductive effort can be significant, even in young individuals. Additionally, a significant relationship was observed between gender and body mass within each group and for gonadic occupation in the diploid group, suggesting that there is a link between sex and fitness-related traits in *C. gigas*.

KEY WORDS: *Crassostrea gigas* · Gametogenesis · Oyster · Triploidy



## INTRODUCTION

Polyploidization, the acquisition of more than 2 sets of chromosomes, is recognized as a significant factor in the evolution of eukaryotes (Otto & Whitton 2000). Polyploidy impacts the genotype and phenotype at different levels (Comai 2005). In the long term, additional chromosome sets provide opportunities for genome evolution, such as neofunctionalization and silencing, as well as organizational modifications such as intergenomic chromosomal exchanges or saltational reorganization (see Wendel 2000 for review). Polyploidization also induces direct changes by perturbing cellular architecture, as increasing the DNA content of a cell usually increases its volume (Melaragno et al. 1993). The augmentation of genetic material and gene copies obviously has implications that change heterozygosity and lead to additive (Johnson et al. 2007) and non-additive (Auger et al. 2005) effects on gene expression and resulting phenotypic traits, probably due to disruptions of regulatory pathways and epigenetic instability (Auger et al. 2005). By these means, polyploidy has played an important role in plant evolution (Soltis & Soltis 1999, Otto & Whitton 2000, Paterson 2005), though it appears to have occurred more rarely in animal evolution (Orr 1990, but see Mable 2004).

Triploidy is a special case of ploidy status because its genomic imbalance leads to very low fertility, such that

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it is often associated with sterility and has been proposed as a method to confine non-native cultivated species (Hulata 2001) or reduce the impact of escapes (Piferrer et al. 2009). In fact, triploidy does not always result in an evolutionary dead-end, especially when accompanied by clonal reproduction as in some plants. Chromosome doubling at meiosis may restore fertility and promote the fixation of fully fertile autotetraploid individuals (Husband 2004).

Economically beneficial characteristics of polyploidy have led triploidy to be used in a wide range of farmed plants and aquatic animals to improve their production, notably in bivalve molluscs (Beaumont & Fairbrother 1991, Nell 2002, Piferrer et al. 2009), where artificial induction is made possible by characteristics of meiosis timing in their female gametes. Methods inhibiting polar body expulsion using cytochalasin B or 6-DMAP (Gérard et al. 1999) were developed first for triploid induction and allowed diploid–triploid comparisons to be made (e.g. for oysters see Allen & Downing 1986, Kesarcodi-Watson et al. 2001, Mallia et al. 2006; for mussels see Beaumont et al. 1995, Brake et al. 2004; for clams see Guo & Allen 1994a; for scallops see Tabarini 1984, Allen et al. 1986, Racotta et al. 2008). More recently, the production of tetraploid oysters (Guo & Allen 1994b, Eudeline et al. 2000a, McCombie et al. 2005b) has led to a large increase in the aquaculture of triploid oysters produced by mating tetraploid with diploid parents (Guo et al. 1996). Although the relative performances of diploids and triploids vary according to environmental conditions (Nell 2002, Piferrer et al. 2009), triploid oysters often show faster growth (e.g. Allen & Downing 1986, Nell & Perkins 2005), better survival (Boudry et al. 2008) and a strong—but variable—diminution of gonad development when compared with their diploid counterparts (Allen & Downing 1990, Normand et al. 2008, for a review of various enhancements of oyster yield offered by triploidization see Nell 2002). The effect of triploidy on reproductive effort is of particular interest because it leads to more constant flesh quality through the year, whereas gametogenesis strongly reduces the marketability of diploid oysters during their reproductive period (Allen & Downing 1990). Interestingly, the availability of tetraploid oysters has also offered new perspectives in terms of selective breeding strategies (McCombie et al. 2005a,b) and comparison between triploid individuals obtained by different methods (Eudeline 2004).

Their minimal reproductive effort means that triploid individuals are considered as worthwhile models for ecophysiological investigations (Honkoop 2003), as well as ecotoxicological (Amiard et al. 2004, Marie et al. 2006) or immunological (Gagnaire et al. 2006) studies. This is notably the case in the Pacific oyster *Crassostrea gigas*, a species in which reproduction strongly influences the annual energy budget (Deslous-Paoli & Héral 1988, Pouvreau et al. 2006).

Despite the advantages they offer, triploid Pacific oysters are, in fact, only partially sterile (Allen & Downing 1990, Normand et al. 2008). Their allocation to reproduction is not low enough to consider them as non-reproductive (i.e. zero gametogenesis controls) and may be detrimental to their saleability for human consumption and risky for their use in genetic confinement. Numerous hypotheses have been suggested to explain the difference in growth and survival between diploid and triploid bivalves, including polyploid gigantism (Guo & Allen 1994a), heterozygosity (Stanley et al. 1984, Hawkins et al. 2000), gene dosage (Zouros et al. 1996) and, of course, sterility (Allen & Downing 1986), which would allow energy reallocation from gonadic development to somatic growth. However, probably due to the relative technical difficulty of quantifying reproductive allocation in oysters, this factor has seldom been recorded when comparing growth performance between diploid and triploid oysters (e.g. Garnier-Géré et al. 2002). Moreover, classical methods employed to measure gametogenesis in diploids tend to underestimate the amount of energy devoted to reproduction in triploids, due to intrinsic characteristics of these individuals like retardation of gametogenesis and incomplete maturation of germ cells (Normand et al. 2008). Finally, resource allocation-based life-history traits such as growth, survival and reproduction are correlated in *Crassostrea gigas* (Ernande 2001), and triploidy affects most, if not, all of these traits, making comparisons between diploid and triploid individuals for any one of these traits difficult to interpret. As a result, despite the large number of studies dedicated to the characterization of the physiological performance of triploid Pacific oysters, some basic knowledge concerning the quantification of their energetic allocation to reproduction and sex determinism and the relation between growth and reproduction is still lacking.

In the present study, reproduction and growth were compared between diploid and 2 different types of triploid oyster, using 5 mo old oysters reared in intensive nursery conditions. These triploids were produced either by polar body II (PBII) retention or by crossing diploid females and tetraploid males. An examination of these different types of triploid oyster was judged necessary because of their fundamental differences, which could influence their growth and reproductive characteristics, e.g. chemical induction by polar body retention leads to triploidy from the addition of 2 chromosome sets from the mother and 1 from the father, while the progeny of a diploid × tetraploid cross will have 1 set from the mother and 2 from the father. The

use of triploid individuals from PB retention is a practical means by which to compare performances of diploids and triploids from the same breeders (i.e. avoiding potential effects due to genetic determinism rather than an effect of triploidy on the phenotypic traits studied), but the physiology of chemically induced triploid oysters may be disturbed by chemical treatment independently from triploidy (Goulletquer et al. 1996). Chemical production of triploid oysters has declined in the last decade with the growing use of tetraploid genitors and public unpopularity of the use of chemicals on foodstuffs (Guo & Allen 1994b). Diploid × tetraploid crosses also provide higher yields of triploid oysters, which has led to an overall expansion of triploid oysters in the shellfish industry and, thus, in the environment.

We focused on young individuals so as to minimize potential bias from the expected differential energy storage dynamics between diploid and triploid oysters. Reproduction was assessed using qualitative and semi-quantitative histological measures, and growth was evaluated by the weight of individuals at the end of the experiment (at 5 mo of age). Our aim was to quantify the reproductive difference between individuals of the 3 groups in relation to their sex and body weight.

## MATERIALS AND METHODS

**Production of biological material and rearing conditions.** In April 2006, following a maturation period under standard conditions (Fabioux et al. 2005) in the Ifremer experimental hatchery (La Tremblade, Charente Maritime, France), 12 diploid female oysters, 16 diploid males and 16 tetraploid males were randomly chosen to be crossed to produce 3 groups of individuals for study. Genitors were strip-spawned individually and concentrations of gametes estimated using Thoma or Malassez slides coupled to an image processing system (Samba Technologies). This procedure allowed us to balance the gametic contribution between genitors.

Oocytes from each of the 12 females were then divided into 2 pools and fertilized at a ratio of 100 spermatozoa per oocyte by pooled sperm of either the 16 tetraploid or the 16 diploid males. The crosses with tetraploid males produced a first triploid group (named '3nDT' as in Wang et al. 1999). Embryos resulting from crosses with diploid males were subsequently divided into 2 groups of equal size. One was treated with cytochalasin B to induce the retention of the 2nd polar body, thus producing a second triploid group by chemical induction (named '3nCB' as in Wang et al. 1999) following the method described in (Gérard et al. 1999), while the other was allowed to develop normally and produced a diploid group (2n). The 2n and 3nCB groups were thus bred from exactly the same genitors, and the 3nDT group from the same females but different (tetraploid) males. This crossing design allowed us to minimize genetic differences between triploid individuals and diploid controls by using the same breeders as much as possible to produce the 3 groups.

Embryos from different females and groups were reared separately in thirty-six 30 l larval rearing tanks until 2 d post-fertilization, when we selected the progenies of the 6 females presenting the highest triploidization success in the 3nCB group. Progenies from these same 6 females were then also chosen in the other groups (2n and 3nDT). Within each group, the progenies of these 6 females were then mixed together and the pooled progenies reared in three 150 l tanks until metamorphosis and settlement. Larval rearing and settlement procedures were conducted as described in Ernande et al. (2003). The newly settled spat were grown in a micronursery for 1 mo up to 2 mm length and were then transferred to the Ifremer experimental nursery in Bouin (Vendée, France), where they were reared in identical 50 cm diameter mesh-bottomed tubs in a single concrete tank with a seawater upwelling system (Bacher & Baud 1992), ensuring common rearing conditions between groups. *Skeletonema costatum* was fed to oysters ad libitum ($0.5 \times 10^9$ cells $l^{-1}$). Survival was monitored throughout the rearing period. Finally, 320 randomly chosen 5 mo old oysters were sampled from each group. Studied groups were not replicated but rearing facilities and experimental procedures were identical between them, and also to those reported by Ernande et al. (2003), for which no significant heterogeneity between rearing structures was observed.

**Triploidy determination.** Induction success was verified by flow cytometry using DAPI staining of the total DNA content of the nucleus (Chaiton & Allen 1985, in Nell 2002). In 2 d old larvae, ploidy verification tests were performed on pools of 100 individuals (estimated after counting on a squared slide), whereas analyses were performed individually for spat. Ploidy level was checked twice during the rearing period and a final time on the sampling day by testing more than 100 randomly chosen individuals each time.

**Body mass.** The soft flesh weight of all the individuals sampled for the present study was weighed to 0.01 g precision. Flesh was superficially dried by swabbing with soft absorbent paper.

**Histology.** Qualitative and quantitative measures of reproductive effort were acquired following the method described in Enriquez-Diaz (2004). Briefly, the whole visceral mass of each oyster was separated from peripheral tissues (i.e. mainly the gills, mantle and adductor muscle), individually labelled and fixed in Davidson's fluid. A transverse cross-section was then

realized precisely through the middle of the visceral mass, dehydrated in an ascending alcohol series, wax embedded, sliced at 5 µm and put on a standard histological slide. The slides were then stained with hematoxylin & eosin solutions.

**Qualitative analysis of reproductive stage.** Reproductive stage was determined following a qualitative classification (5 stages: 0 to 4) adapted from Mann (1979) and Lango-Reynoso et al. (2000) (Table 1). For triploid asynchronous hermaphrodites that presented several cohorts of germ cells, 2 different cases were met:

(1) One gender was clearly dominant and exhibited maturing germ cells, whereas the cohort of germ cells of the opposite gender was marginal and often atresic. In this case the reproductive stage was then determined for the dominant cohort only.

(2) Cohorts of male or female germ cells seemed to be maturing simultaneously, therefore indicating a case of apparently 'true' hermaphroditism. As this condition was very seldom found during the present study (3 out of 898 individuals determined for sex), we decided to exclude these individuals from the data set to simplify the analyses.

The slides were first examined for gender determination and classification of reproductive stages. Depending on the presence or absence or male and/or female germline cells, individuals were classified as either male, female, hermaphrodite (asynchronous) or sexually undifferentiated.

**Quantitative analysis of reproductive effort.** As the second step of our evaluation of reproduction, the slides were scanned with a digital camera attached to a microscope and treated using image analysis software (Imaq Vision Builder, National Instruments). Reproductive effort was estimated by gonadic occupation (GO, %), measured as the ratio between the gonad area (GA) and the whole visceral mass area (WVMA) following the relationship presented in Enriquez-Diaz (2004):

$$GO = (GA \times 100)/WVMA$$

Table 1. Classification of reproductive stages based on Mann (1979) and Lango-Reynoso et al. (2000)

| Stage | Histological description |
|---|---|
| 0. Resting | No trace of sexual development; follicles are non-existent or elongated and consist of undifferentiated germinal epithelium |
| 1. Early growth | Follicles are small and isolated with numerous spermatogonia or oogonia |
| 2. Late growth | 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 reabsorbtion | Follicles distended; numerous gametes remain |

**Statistical analysis.** The effects of the ploidy level (2n versus 3n) and of the triploidy induction method (3nDT versus 3nCB) on the frequencies of sex classes and maturity stages were analysed using generalized linear models (procGENMOD with contrast statement, SAS), considering the induction method effect as nested within the ploidy effect (Littell et al. 2002). The comparison between groups for gender classes or maturity stages was realized using model adjustments for multinomial distributions, whereas class-by-class comparisons were performed considering binomial distributions.

Linear models (procGLM, SAS) were also used to analyze normally distributed data on reproductive effort (log[GO+1]) and final soft flesh weight (FW) (Littell et al. 2002). In both cases, differences between ploidy groups and sexual dimorphisms were tested by between-group ANOVA with the experimental group, gender and their interaction as fixed effects (Littell et al. 2002). To further investigate sexual dimorphism, we then performed ANOVAs (procGLM, SAS) with a gender effect within each ploidy group. Significance levels of the differences between different factor levels (ploidy group and gender) from the ANOVAs were tested by pair using the LSmeans option (procGLM, SAS) (Littell et al. 2002). The sexually undifferentiated individuals were excluded from this particular analysis as they could not be classed as belonging to any gender class.

We intentionally decided to avoid using fresh mass as an explanatory covariate for reproductive effort because this trait integrates the gonadic as well as the somatic weight.

## RESULTS

### Proportion of triploid individuals in the 2 triploid groups

Across the 12 parental females used in our crossing scheme, cytochalasin B induction led to 40–100% triploidy depending on half-sib family. We selected and mixed together the 6 best female progenies, in which triploidy induction was >95%, to make the 3nCB group. At the spat stage (5 mo after fertilization), the triploidy rate in the 3nCB (n = 110) and 3nDT (n = 115) groups was 95.5 and 97.4%, respectively. The remaining oysters consisted of 3.6 and 1.7% diploids in

the 3nCB and 3nDT groups, respectively. Additionally, 1 mosaic individual was observed in each of these groups.

### Sexual maturity

The classification of individuals by their reproductive stage (Table 1) showed a highly significant difference in the frequency distribution of individuals across different stages between diploid and triploid oysters (2n versus 3n for all reproductive stages: $\chi^2 = 260.7$, $p < 0.0001$) (Table 2). Between the 2 groups of triploid oysters, the difference was more limited but still significant (3nCB versus 3nDT for all reproductive stages: $\chi^2 = 4.7$, $p = 0.03$) (Table 2). More precisely, triploids seemed to present a relative delay in their gonadogenesis dynamics compared to diploids (Table 2). Indeed, a few individuals in the triploid groups showed no development of gonadic tissues at all (Stage 0: 7.1 and 2.4 % for groups 3nCB and 3nDT, respectively), whereas all diploid individuals had initiated gonadic maturation (Stage 0: 0 %). After 5 mo of intensive rearing, most of the oysters in the triploid groups were at precocious stages of gonadic development and frequencies of individuals in Stages 1 and 2 were significantly superior to those in the corresponding diploid sample (2n versus 3n: Stage 1, $\chi^2 = 15.88$, $p < 0.0001$; Stage 2, $\chi^2 = 237.43$, $p < 0.0001$) (Table 2). In contrast, diploid spat were mainly reproductively mature, with 80.9 % of individuals in Stage 3. Only a few individuals from the triploid groups (3nCB: 4.2 %; 3nDT: 0.4 % [1 individual]) achieved complete maturation of their gonadic tissues (2n versus 3n: Stage 3, $\chi^2 = 111.27$, $p < 0.0001$) (Table 2). There was little apparent difference between the 3nCB and 3nDT groups, except for the occurrence of Stage 2, which was clearly higher in the 3nDT group (3nCB versus 3nDT: Stage 2, $\chi^2 = 15.19$, $p < 0.0001$) (Table 2).

### Sex determinism

Percentages of individuals belonging to the different gender classes showed a significant difference between ploidy levels (2n versus 3n for all gender classes: $\chi^2 = 4.82$, $p = 0.03$), mainly due to the higher occurrence of hermaphrodites in triploid groups. In contrast, triploidy induction method did not appear to have a significant influence on the frequency of individuals in different gender classes (3nCB versus 3nDT: $\chi^2 = 2.27$, $p = 0.13$) (see Table 2). Sex ratio was clearly unbalanced overall, as male was the dominant gender for all ploidy groups (82.7, 80.4 and 73.7 % in groups 2n, 3nCB and 3nDT, respectively). Hermaphrodites were significantly more abundant in both 3nDT and 3nCB groups than in the diploid group (10.7 and 9.7 % for groups 3nCB and 3nDT, respectively, versus 1.8 % for group 2n; 2n versus 3n: $\chi^2 = 14.98$, $p = 0.01$) (Table 2).

Females appeared to be scarcer in the 3nCB group than in the 3nDT group (8.9 versus 16.6 %, respectively; 3nCB versus 3nDT: $\chi^2 = 5.96$, $p = 0.01$) but there was no difference between diploid and triploid groups (2n versus 3n: $\chi^2 = 1.59$, $p = 0.21$) (Table 2).

### Effects of ploidy level, triploidy induction method and gender on reproductive effort

The 2 triploid groups showed an average GO 2.1 times lower than their diploid relatives (Fig. 1). Mean (±SD) GO was 25.2 ± 11.8, 11.8 ± 7.8 and 11.8 ± 7 % in groups 2n, 3nCB and 3nDT, respectively (Fig. 1). Between-group ANOVA showed a significant effect of group ($F_{2,704} = 18.94$, $p < 0.0001$) and the interaction between gender and group ($F_{4,704} = 4.65$, $p = 0.001$), but a non-significant effect of gender class ($F_{2,704} = 0.34$, $p = 0.71$) on GO (Table 3). Pairwise comparisons between groups showed significant differ-

Table 2. $\chi^2$ tests performed on maturity stages and gender classes

| Stage/gender | 2n | | 3nCB | | 3nDT | | 2n vs. 3n | | 3nCB vs. 3nDT | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | % | N | % | N | % | $\chi^2$ | p | $\chi^2$ | p |
| Stage 0 | 0 | 0 | 17 | 7.1 | 6 | 2.4 | – | – | 5.59 | 0.01 |
| Stage 1 | 2 | 0.7 | 35 | 14.6 | 22 | 8.7 | 15.88 | <0.0001 | 4.09 | 0.04 |
| Stage 2 | 52 | 18.4 | 178 | 74.2 | 223 | 88.1 | 237.43 | <0.0001 | 15.19 | <0.0001 |
| Stage 3 | 229 | 80.9 | 10 | 4.2 | 1 | 0.4 | 111.27 | <0.0001 | 5.17 | 0.02 |
| Stage 4 | 0 | 0 | 0 | 0 | 1 | 0.4 | – | – | – | – |
| All stages | 283 | – | 240 | – | 253 | – | 260.7 | <0.0001 | 4.7 | 0.03 |
| Males | 234 | 82.7 | 180 | 80.4 | 182 | 73.7 | 3.21 | 0.07 | 0.59 | 0.09 |
| Females | 44 | 15.5 | 20 | 8.9 | 41 | 16.6 | 1.59 | 0.21 | 5.96 | 0.01 |
| Hermaphrodites | 5 | 1.8 | 24 | 10.7 | 24 | 9.7 | 14.98 | 0.01 | 0.04 | 0.70 |
| All genders | 283 | – | 224 | – | 247 | – | 4.82 | 0.03 | 2.27 | 0.13 |

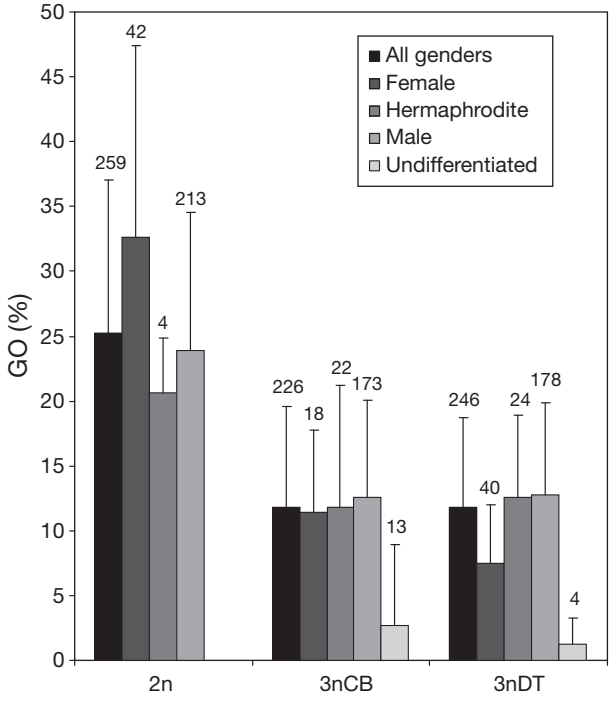


Fig. 1. *Crassostrea gigas*. Mean gonadic occupation (GO; % of visceral mass area) by gender and group. Error bars: + SD; numbers above error bars: N. See 'Materials and methods—production of biological material and rearing conditions' for definition of groups

entiation of the 2n group from each of the 3n groups separately (2n – 3nCB: $p < 0.0001$; 2n – 3nDT: $p < 0.0001$), but no differences in GO between the 2 triploid groups. Pairwise comparisons were then performed within groups to discriminate the effects of the different genders.

Within the diploid group, no significant effect of gender was observed on GO ($F_{2, 256} = 2.13$, $p = 0.12$), although a slight but significant ($p = 0.04$) difference in reproductive effort was found between females and males (Table 4). Female diploid oysters exhibited a mean reproductive effort 1.4 times higher than males (32.6 versus 23.8%) (Fig. 1).

When considering the 3nCB group, no significant effect of gender was found either in the overall ANOVA ($F_{2, 209} = 0.06$, $p = 0.94$) or using pairwise comparisons. In the 3nDT group, however, gender did have a highly significant effect on reproductive effort ($F_{2, 239} = 9.25$, $p = 0.0001$) (Table 4). Within-group comparisons showed a significantly higher GO in 3nDT males and hermaphrodites compared with 3nDT females (12.8 and 12.5 versus 7.5%, respectively) (Fig. 1). Unlike diploid females, triploid females did not seem to present a higher GO than other sexually differentiated individuals of their group in either case.

Table 3. Between-group ANOVA (fixed effects) on reproductive effort, and pairwise comparisons between factor levels. GO: gonadic occupation. log(GO+1) = Gender + Group + Group × Gender

| Source | ANOVA | | |
|---|---|---|---|
| | df | *F* | p |
| Group | 2 | 18.94 | <0.0001 |
| Gender | 2 | 0.34 | 0.71 |
| Group × Gender | 4 | 4.65 | 0.001 |
| Residuals | 704 | | |
| Group | LS means (pairwise comparisons) | | |
| | Log(GO+1) | 2n | 3nCB |
| 2n | 3.14 | | |
| 3nCB | 2.38 | <0.0001 | |
| 3nDT | 2.31 | <0.0001 | 0.507 |

Table 4. Within-group ANOVA on reproductive effort, and pairwise comparisons between factor levels. GO: gonadic occupation; Herm.: hermaphrodite. log(GO+1) = Gender

| Group | ANOVA | | | | LS means (pairwise comparisons) | | | |
|---|---|---|---|---|---|---|---|---|
| | Source | df | *F* | p | Gender | Log(GO+1) | Female | Herm. |
| 2n | Gender | 2, 256 | 2.13 | 0.12 | Female | 3.31 | | |
| | | | | | Herm. | 3.06 | 0.523 | |
| | | | | | Male | 3.05 | 0.040 | 0.979 |
| 3nCB | Gender | 2, 209 | 0.06 | 0.94 | Female | 2.39 | | |
| | | | | | Herm. | 2.34 | 0.841 | |
| | | | | | Male | 2.40 | 0.958 | 0.735 |
| 3nDT | Gender | 2, 239 | 9.25 | 0.0001 | Female | 2.01 | | |
| | | | | | Herm. | 2.48 | 0.003 | |
| | | | | | Male | 2.45 | <0.0001 | 0.884 |

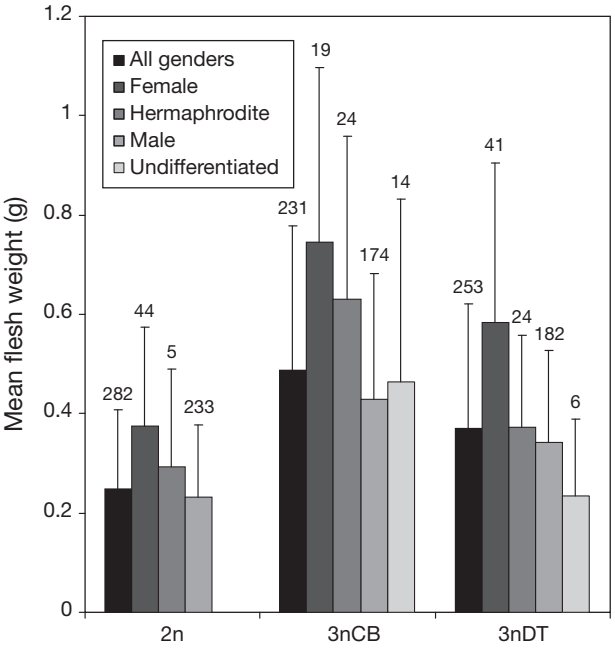


Fig. 2. *Crassostrea gigas*. Mean flesh weight (g) by gender and group. Error bars: +SD; numbers above error bars: N. See 'Materials and methods—production of biological material and rearing conditions' for definition of groups

### Effects of ploidy level, triploidy induction method and gender on final weight

Triploid oysters presented higher soft flesh weight than diploids at the sampling date (Fig. 2): 3nCB individuals were the heaviest (mean weight = 0.49 ± 0.29 g), followed by 3nDT (0.37 ± 0.25 g) and then 2n individuals (0.25 ± 0.16 g). Weight was significantly affected by ploidy group ($F_{2,743}$ = 33.73, p < 0.0001), gender ($F_{2,743}$ = 50.69, p < 0.0001) and their interaction ($F_{4,743}$ = 4.21, p = 0.002) (Table 5). Comparisons of soft flesh weight by group and gender showed the same trend for all 3 groups (Fig. 2): females were the largest individuals, followed by hermaphrodites and males. Females were indeed significantly heavier than males (p > 0.0001) (Table 6) in all 3 groups, and significantly heavier than hermaphrodites (p = 0.001) in the 3nDT group. Hermaphrodites presented mean weights intermediate between males and females, and were only significantly different from males (p = 0.001) in the 3nCB group (Fig. 2, Table 6).

Table 5. Between-group ANOVA (fixed effects) on body weight, and pairwise comparisons between factor levels. BW: body weight. BW = Gender + Group + Group × Gender

| ANOVA | | | |
|---|---|---|---|
| Source | df | *F* | p |
| Group | 2 | 33.73 | <0.0001 |
| Gender | 2 | 50.69 | <0.0001 |
| Group × Gender | 4 | 4.21 | 0.002 |
| Residuals | 743 | | |

| LS means (pairwise comparisons) | | | |
|---|---|---|---|
| Group | BW (g) | 2n | 3nCB |
| 2n | 0.30 | | |
| 3nCB | 0.61 | <0.0001 | |
| 3nDT | 0.43 | 0.001 | <0.0001 |

## DISCUSSION

### Triploidization

We were particularly careful during the present study to work using triploid groups presenting a high proportion of triploid individuals, as the methods used rarely produce 100% triploidy. We minimized the presence of diploid individuals in the cytochalasin B-induced group by making treatments on fertilised

Table 6. Within-group ANOVA on body weight, and pairwise comparisons between factor levels. BW: body weight; Herm.: hermaphrodite. BW = Gender

| Group | ANOVA | | | | LS means (pairwise comparisons) | | | |
|---|---|---|---|---|---|---|---|---|
| | Source | df | *F* | p | Gender | BW | Female | Herm. |
| 2n | Gender | 2/279 | 16.05 | <0.0001 | Female | 0.38 | | |
| | | | | | Herm. | 0.29 | 0.266 | |
| | | | | | Male | 0.23 | <0.0001 | 0.376 |
| 3nCB | Gender | 2/220 | 18.12 | 0.94 | Female | 0.75 | | |
| | | | | | Herm. | 0.65 | 0.212 | |
| | | | | | Male | 0.43 | <0.0001 | 0.001 |
| 3nDT | Gender | 2/244 | 21.125 | <0.0001 | Female | 0.58 | | |
| | | | | | Herm. | 0.37 | 0.001 | |
| | | | | | Male | 0.34 | <0.0001 | 0.531 |

oocytes from individual females, keeping the maternal half-sib families separate initially, then selecting the best ones with the highest percentage triploidy. In a similar way, Eudeline et al. (2000b) optimized tetraploidy induction using oocytes from individual females rather than making an oocyte pool from a number of females. As a result of our procedure, the 2 triploid groups that we studied exhibited a very high (>96%) percentage of triploid individuals; the remainder were diploids or mosaics. Incidental diploids could result from ploidy reversion (Allen et al. 1999), inter-batch contamination or because cytochalasin B induction is often not totally effective (Gérard et al. 1999). Mosaics are more likely to be due to a fundamental instability of artificial triploid animals (Allen & Guo 1996, Allen et al. 1999). To have a precise understanding of the nature of such exceptions, a more in-depth study requiring chromosome spreads is needed, as chromosome counting is currently the reference method for studying chromosome number anomalies in oysters (e.g. Wang et al. 1999, McCombie et al. 2005a, Batista et al. 2007), and flow cytometry is unlikely to detect small variations in chromosome number. For the purposes of the present study on the comparison of reproductive effort and growth between the 3 studied groups, the small proportion of non-diploid individuals can only have had a very limited effect, as the number of 3n oysters of both groups showing reproductive development far exceeds the non-triploid percentage detected. The high frequency and degree of reproductive development that we observed in the triploid groups cannot therefore be attributed to the limited presence of diploids.

### Gonadogenesis in diploid and triploid spat

The most striking result of our work was the very high proportion of reproductively maturing spat. For the diploid group, all individuals appeared to have initiated their gonadic maturation, despite being only 5 mo old, with a corresponding mean soft flesh mass of 0.25 g. This very small weight at sexual maturity is lower than the estimated 0.4 g minimum proposed by Pouvreau et al. (2006) and suggests that no minimal size or age is required for Pacific oyster to initiate gonadic maturation. In other words, it may well be that metamorphosis and sexual maturation coincide, so that adult traits are fixed after metamorphosis, but the high availability of nutrients in intensive nursery conditions probably lead to an enhancement of such precocious gonadogenesis.

Surprisingly, and despite the partial sterility of triploid oysters, the rate of maturing individuals was very high in the 2 triploid groups (92.9 and 97.6% for groups 3nCB and 3nDT, respectively). Gonadic occupation in the 2 triploid groups was only 47% less than in the diploid group. This particular result strongly suggests that reproductive effort (i.e. the proportion of energy allocated to reproduction) (Todd & Havenhand 1983) was far from negligible in these triploid oysters and is consistent with previously published studies employing gonadic occupation to compare gonad development between diploid and triploid oysters (Allen & Downing 1986, Shpigel et al. 1992). On the other hand, other studies have shown that the production of fully mature gametes in triploid Pacific oysters is greatly reduced compared to diploids, i.e. between 2 and 13% of diploid female oocyte production (Guo & Allen 1994c, Gong et al. 2004, respectively).

This suggests that in triploid oysters, gonadic occupation is not closely related to eventual gamete numbers, because only a fraction of the germinal cells appeared to mature to the gametic stage. Moreover, these individuals are known to undergo spontaneous reproductive tissue resorption events (Allen & Downing 1990, Shpigel et al. 1992) that could also result in differences between diploid and triploid temporal dynamics of gonad maturation (the relationship between gonad development at precocious stages, atresia and gamete production is more extensively discussed in the next paragraph). Further study of triploid oysters should therefore be conducted throughout the reproductive cycle to better describe the sexual maturation of these individuals, including the aspects related to temporal dynamic of gonad maturation.

Under our experimental conditions, reproductive effort was high in triploid oysters at the beginning of gametogenesis, reaching half the level of diploid ones. This result suggests that energy allocation to reproduction in triploid Pacific oysters can be significant, even though the ultimate production of mature gametes remains much lower. Further comparative assessment of oyster energy budgets should be performed throughout the reproductive cycle to better describe the temporal dynamics of gonad maturation in triploid oysters and estimate the costs of building gonadic tissue in diploid and triploid oysters. This would require closer monitoring of the different temporal dynamics of gonad evolution in diploid and triploid individuals and the energy yield of reproductive tissue resorbed through atresy for reallocation to other functions.

Moreover, gonadic occupation and the temporal dynamics of gonad maturation in Pacific oysters are highly sensitive to variation in environmental conditions, which has been demonstrated for diploid individuals (Chavez-Villalba et al. 2003, Fabioux et al. 2005). Environmental variation probably also contributes to variability of reproductive effort estimators published for triploid Pacific oysters (Allen & Downing 1986, Shpi-

gel et al. 1992, Guo & Allen 1994c, Gong et al. 2004). Further comparative studies of triploid versus diploid performances should be made under different environmental conditions (i.e. contrasted rearing sites) to fully describe the reproductive capacities of these oysters.

### Disturbance of gametogenesis in triploid oysters

Another interesting result is the clear difference between diploid and triploid individuals in terms of quantitative and qualitative reproductive parameters. In addition to the reduction of gonadic occupation, there was a higher occurrence of hermaphrodites and individuals at precocious maturity stages in the triploid groups. The 2 groups of triploid oysters were obviously more similar to one another than either was to the diploid group, despite the common parents used for the 3nCB and 2n groups. The method of triploid induction used (chemical induction or mating between diploid and tetraploid broodstock) may lead to genetic differences at several levels, as mentioned in previous studies: unbalanced contribution of parental genetic values (Blanc et al. 2005), higher heterozygosity of 3nDT individuals (Hawkins et al. 2000), effects of unintentional selection during the production of tetraploid oysters (i.e. selection of fertile triploid females, Guo & Allen 1994b), as well as long-term toxic effects of chemical cytoblocking molecules such as cytochalasin B (Goulletquer et al. 1996). The effect of triploidy on early reproduction appears, however, to be very similar whatever the induction method used. The frequently observed minimal fertility of triploid individuals is a general pattern, directly related to the ploidy level and already mentioned for numerous species in both plants and animals, which seems to be independent of other indirect effects. One causal factor of the infertility of triploid individuals is related to chromosomal pairing, segregation during meiosis and the fact that homologous chromosomes fail to synapse due to genomic imbalance (Allen et al. 1986, Crane & Sleeper 1989, Otto & Whitton 2000, Maldonado-Amparo et al. 2004). This is consistent with the results of the present and some previous studies (Allen & Downing 1990, Normand et al. 2008), all of which show that the difficulty met by triploid Pacific oysters in developing gonadic tissues occurs in the final stage of gametogenesis. High ratios of individuals presenting stages at least as developmentally advanced as maturing spermatogonias or oogonias (Stages 1 and 2: 92.9% and 97.6% for groups 3nCB and 3nDT, respectively) were indeed observed, while individuals showing fully mature gonadic tissues were almost absent in triploid groups (Stage 3: 4.1, 0.4 and 80.9% for groups 3nCB, 3nDT and 2n, respectively). Such irregular gonadogenesis was revealed by our histological observations of asynchronous maturation of different cohorts of germ cells, characterized by the high ratios of asynchronous hermaphrodites. We also noticed marginal, but recurrent, atresic gametes (results not shown) while another cohort of germ cells was still maturing. In contrast, maturation in diploid oysters appears to be a continuous phenomenon that leads to homogeneous and mature gonadic tissue. A hypothesis of the dynamics of gonadic maturation in triploid oysters is that they may develop a first cohort of germ cells that reaches the gonial stage, but then face problems at meiosis that prevent complete maturation of most of the gonias. The oysters then halt their gametogenesis but then initiate a new maturation episode with a new cohort of germ cells, sometimes accompanied by a change in gender (described as asynchronous hermaphroditism) and atresy of the original germinal products.

Problems of chromosome pairing probably represent the main reason why gamete production and zygote viability remain haphazard in triploid animals, but do not lead to a complete inhibition of gametogenesis. It appears clear that a variable proportion of gonias do manage to mature beyond the premeiotic stage to reach the gametic stage, as demonstrated by the high occurrence of Stage 2 in the triploid groups of the present study. This differential ability across germ cells from the same individual to fully mature remains intriguing, especially considering the apparent difficulty for trivalent homologous chromosomes to associate in synapsis (see Crane & Sleeper 1989 for a study of meiotic chromosome association in polyploids, McKee 2004 for a review of homologous pairing, and Feitsma et al. 2007 for a case study of chromosome pairing problems), but this difficulty has been noticed for most species in which polyploidy has been induced (Allen et al. 1986, Maldonado-Amparo et al. 2004, Cal et al. 2006).

### Sex determinism and sexual dimorphisms in diploid and triploid spat

One unexpected result of the present study was the slight, but significantly higher, gonadic occupation of diploid females compared to males. This result is of interest as it is, to our knowledge, the first time that sexual dimorphism (not protandry, which is a different phenomenon well known in bivalves) has been reported for reproductive effort in Pacific oysters, a preliminary study having shown no difference (Ernande et al. 2004). A study of individual temporal dynamics of gametogenesis is now needed to test whether male and female *Crassostrea gigas* mature at the same speed. Conversely, the reverse gender effect on gonadic occupation appeared in 3nDT oysters of the present

study as males and hermaphrodites had larger gonads than females. In all cases, the enhanced reproductive effort in diploid females (1.4×) compared with diploid males was sufficiently significant that we looked more closely at this particular aspect to examine its implications for growth and fitness. *C. gigas* is commonly described as a protandric dioecious mollusc, in which most individuals are males during the first reproductive event, whereas latter seasons of maturation usually show an increasing proportion of females (Galstoff 1964). Nevertheless, the relative influence of genetic versus environmental (potentially mediated by physiological state) factors in determining the ability of a single individual to shift from one gender to another remains uncertain. In general, molluscs exhibit a large variety of sex systems as well as various mechanisms for sex determination (review in Yusa 2007). For *C. gigas*, it was presumed that sex determinism is mainly controlled by environmental factors (Amemiya 1929, Galstoff 1964), but Guo et al. (1998) proposed genetic determinism of sex in oysters on the basis of observed sex ratio variation between families. In a recent study, Baghurst & Mitchell (2002) found that males and females presented different growth rates. Following Guo et al. (1998), they interpreted this difference as the tendency of genetically determined females to exhibit higher growth rates than males, rather than the tendency of fast-growing individuals to be more susceptible to mature as females.

Induced triploidy can offer an experimental way to study the genetic basis of sex determination because of the parental imbalance caused by this manipulation. In fish, triploidization has been widely used as a way to control sex ratio and produce monogender populations (review in Devlin & Nagahama 2002). Triploidy also impacts sex ratio in some bivalve species: triploid *Mya arenaria* and *Argopecten ventriculosus* are found to be only females (Allen et al. 1986, Ruiz-Verdugo et al. 2000), whereas triploid *Mytilus edulis* are only males (Brake et al. 2004). In the present study, no significant difference in sex ratio was observed between the 3 studied multi-family groups. This absence of direct impact of triploidy on sex ratio in *Crassostrea gigas* has also been mentioned in previous studies (Allen & Downing 1990, Normand et al. 2008) and suggests that genetic effects related to parental dosage on sex determinism are weak in this species.

From an evolutionary point of view, protandry in dioecious species is often explained by the size advantage hypothesis (Ghiselin 1969). Under this hypothesis, the reproductive success of females is limited by their total size, whereas the males' ability to fertilise eggs is less dependent on size. This discrepancy results from the much higher number of spermatozoa that can be produced by a male compared with the number of oocytes produced by a female of equal size, and the correlation between the number of eggs produced and a female's size. In the present study, the relationship between gender and final soft flesh weight were clear: females had the heaviest soft flesh weight, followed by hermaphrodites and finally by males, which were the smallest. This may reflect a sex determination strategy in *Crassostrea gigas* that is partly based on individual status. Well-nourished oysters with a high energy budget or large energy stores would preferentially develop female gonads, whereas slow-growing individuals would channel their reproductive effort to male gonads, thus optimising the number of gametes produced. Following this hypothesis, asynchronous hermaphrodites, like those we observed, would be oysters of intermediate condition that began to initiate gonadic maturation for a given gender, and then changed their sexual function. This hypothesis is consistent with our data and with the results of Baghurst & Mitchell (2002), though it might diverge from their own hypothesis of genetic determinism. Our results could be interpreted as additional evidence for the importance of environmental control of sex determinism in the Pacific oyster, notably mediated via individual energy budget. Genetic factors could indeed play a role via their effects on energy allocation and growth. The fact that sexually undifferentiated individuals in triploid groups also exhibited lower body weights could also be considered an indication of energy-mediated sex determinism in *C. gigas*. We hypothesize that gonadic development is under the control of the energy budget, and is thus related to both growth and reproduction. Under this hypothesis, temperature (Fabioux et al. 2005) and food availability (Ernande et al. 2004) are the 2 major factors controlling gonadic development, with a threshold level of stored energy that needs be reached to initiate gonadogenesis in spat. This threshold energy density level: (1) would be higher in triploid than in diploid individuals (as both triploid groups usually exhibited far more undifferentiated individuals than diploid ones) and (2) may be positively correlated with the probability of an individual performing gametogenesis as a female for either ploidy group (as better nourished individuals appear to mature as females).

Our results could also have implications for selective breeding for improved growth in Pacific oyster once the level of genetic influence is understood. More light could be shed on this aspect by looking at the influence of parentage by using genetic markers. If individuals that exhibit female gender in their first years of maturation are intrinsically faster-growing oysters, the sex ratio of families could be considered as a way to select fast-growing families. By a similar strategy, individual selection of the young females in a population could also lead to faster-growing progenies.

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