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Influences of triploidy, parentage and genetic diversity on growth of the Pacific oyster *Crassostrea gigas* reared in contrasting natural environments

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Abstract

An increasing number of hypotheses are being proposed to explain the faster growth potential of triploids in molluscs, including their partial sterility or their higher heterozygosity compared to diploids. Triploid advantage however, remains controversial for poorer sites, because of a potential trade-off with survival. These questions were addressed in *Crassostrea gigas* by deploying meiosis II triploids and their diploid siblings from a single mass spawning of three males and seven females, in two contrasting locations for their trophic resources. One hundred and fifty individuals were sampled at each site after nine months, measured for weight and biochemical composition, and genotyped using three microsatellite and seven allozyme loci. Higher performance was observed at the fast-growing site for all traits except shell weight, and triploids had greater weights and biochemical contents than diploids at harvest. Triploids also grew faster at the poorer site, and showed similar survival rates to diploids at both sites. Triploids had significantly higher average allozyme and microsatellite diversity. However, they performed better for a wide range of individual heterozygosity values, arguing for an advantage of the triploid state *per se*, that could be due to positive effects on growth of both sterility of triploids with subsequent resource re-allocation and possible faster transcription with three copies of each gene. Despite evidence of very low or no inbreeding in the diploid sample, positive associations between individual allozyme diversity and growth were detected, which explained little but significant amounts of phenotypic variation. These associations were interpreted as direct effects of allozymes, either alone or including epistatic interactions with other loci. In addition, measures of individual distance (*mean-d*^{2}) specific to microsatellites, were negatively correlated with growth in diploids, indicating possible effects of outbreeding depression between more distant genomes of parents from distinct populations.

Keywords: Allozyme diversity, *Crassostrea gigas*, growth, *mean-d*^{2}, microsatellite, triploidy

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††This article is dedicated to S. B. who died tragically in 2001.

Introduction

Triploidy is now widely used to obtain faster growth in shellfish (see Beaumont & Fairbrother 1991 for a review). Several hypotheses have been proposed to explain the higher growth rate of triploids compared to diploids.

Physiological hypotheses include the effect of the observed sterility of triploids, which would divert more metabolic flux to growth (Allen *et al*. 1986; Hand & Nell 1999), and an increased volume of polyploid cells (Guo & Allen 1994). Among the genetic hypotheses, the most common one is based on the higher expected heterozygosity of triploids, because of the higher probability that triploids possess two or three different alleles per locus (Allendorf & Leary 1984; Guo *et al*. 1992), thus resulting in 'heterosis' effects (Mitton & Grant 1984; Zouros & Foltz 1987; Hedgecock *et al*. 1996), and positive influences upon feeding rate, absorption efficiency and/or growth efficiency (Hawkins & Day 1999; Hawkins *et al*. 2000). The 'gene dose' hypothesis suggests that triploids may perform better because of a potential for faster transcription due to the presence of three copies of the same genes (Magoulas *et al*. 2000). Higher performance of triploids may also result from a depressed growth of diploids because of partial and progressive loss of chromosomes, exposing recessive deleterious mutations with a higher probability (Zouros *et al*. 1996). Moreover, triploids may have a greater stress resistance than diploids, both sterility and higher heterozygosity in triploids being associated with lower metabolic energy costs, thus more energy being available for growth or survival under stressful conditions (Hawkins & Day 1996; Hawkins 1996). However, breeders have questioned whether triploids grow faster or survive longer under suboptimal conditions, with some data suggesting lower survival (Stanley *et al*. 1984; see also Maguire *et al*. 1995 in one site out of three). More data are required to help us understand fully the comparative advantages of inducing triploids for on-growing in different environments.

Genetic markers, first with allozymes, and now with more informative markers such as microsatellites, have proved useful tools for marine organisms in breeding programmes (Naciri-Graven *et al*. 2000) and population genetics (Bierne *et al*. 1998; Launey & Hedgecock 2001), with applications for ploidy and parentage identification (Magoulas *et al*. 2000), mating system analyses and genetic diversity assessments (Huvet *et al*. 2000; Lemaire *et al*. 2000). Particularly among marine species, a large number of studies have reported positive associations between allozymic genetic diversity and fitness-related traits (Britten 1996). The interpretation of these associations is still a matter of controversy as it has proven difficult to distinguish between alternative explanations that either entail direct effects of allozyme loci on fitness variation in the 'direct overdominance' hypothesis (Mitton 1993), or indirect effects through correlations of allozymes, then considered neutral, with fitness genes in the 'associative overdominance' hypothesis (Ohta 1971; Charlesworth 1991). In this second hypothesis, genotypic correlations can be due either to linkage disequilibrium in small populations (Hill & Robertson 1968), or to identity disequilibrium created by inbreeding in particular mating systems and population structures (Weir & Cockerham 1973; David 1999; Bierne *et al*. 2000). Comparing those associations with genetic diversity estimated on either allozymes or microsatellites, that are neutral *a priori*, can help to test alternative explanations of an observed heterozygote advantage.

This paper describes part of a collaborative EEC study that aimed at establishing the genetic and physiological advantages of inducing triploidy in *Crassostrea gigas*. We report how growth and biochemical content were monitored in 300 diploid and triploid siblings from a single mass spawning, elsewhere genotyped for eight allozymes and three microsatellites (Magoulas *et al*. 2000), in order to study the effects of triploidy, parentage, and heterozygosity under different environmental conditions. Performance was compared in two contrasting bays, with a view to confirming any advantages of triploidy in both food-rich and food-poor environments. The second objective was to test whether genetic diversity, estimated with either allozymes or microsatellites at individual and family levels, could be used to help predict fitness-related performance and provide arguments for the genetic hypotheses of triploid possible advantage. Our experimental design controlled for environmental site, ploidy and parentage factors, as already performed in past studies (Davis 1989; Nell *et al*. 1994; Maguire *et al*. 1995). But this is the first study where (i) individual estimates of genetic diversity and fitness-related data were jointly recorded on all individuals, and (ii) those data were combined and the different effects were adjusted in generalized linear models for more rigorous statistical resolution and testing of possible associations.

Materials and methods

Genotype rearing, triploidy induction and determination

The overall programme of triploidy induction, rearing of juvenile and adult animals, allozyme and microsatellite analyses that were undertaken in *Crassostrea gigas* has been detailed in two recent papers (Hawkins *et al*. 2000; Magoulas *et al*. 2000). We only summarize here information relevant to understanding the context of the growth experiments. In April 1994, three male and seven female *C. gigas* oysters originating from the Seudre river (Atlantic Coast, France), were sexually matured and artificially mass-crossed in an open system of circulating sea water. Sperm and oocytes were stripped from the gonads and treated for inducing triploidy according to Gérard *et al*. (1999), a subsample of the same mass spawning being kept untreated and used as control. Only meiosis II (MII) triploids that were produced at a good rate were used in the present experiment. The percentage of triploids was assessed using image analysis at the larval, post-larval and

adult stages following Gérard *et al*. (1994), and it was preliminarily shown to decrease slowly, from 92% at the larval stage to 86.6% in September 1994, and to stabilize thereafter (86% in March 1995, on 234 individuals). Therefore, it was decided to determine the triploidy of each animal at harvest.

Field growth experiments and studied traits

Two different sites were chosen to compare growth under contrasting conditions. Marennes-Oléron (Charente Maritime, France) is known to be a slow-growing (oligotrophic) site, whereas Thau Lagoon (Hérault, France) is a fast-growing (eutrophic) site. An extensive monitoring conducted by IFREMER (REMORA network) has indeed shown that Marennes-Oléron is the poorest site in France among the 45 studied, whereas the Thau lagoon is ranked among the three best sites (Goulletquer 1998). At each site, 150 MII putative triploids and 150 diploid siblings were labelled before deployment. Three mesh bags were used at Marennes-Oléron bay for each ploidy class with about 50 oysters per bag. No difference in growth was previously recorded at that site between oysters grown at densities of 300 or 600 animals per bag (Ph. Goulletquer, unpublished results). Competition effects between individuals were therefore assumed to be negligible within bags. Three suspended ropes were used for each ploidy class at Thau Lagoon following normal practice there, which also meant a low density and reduced competition.

Oysters were harvested in January 1996 when they reached a range of weights between 50 and 70 g, corresponding to the third and fourth categories and to 80% of commercialized oysters in Marennes-Oléron. Survival was calculated in each ploidy class at each site as the number of surviving oysters against the number of oysters initially deployed (150) and percentage comparisons were tested with binomial tests (ε-statistics; Schwartz 1991). To maximize the chance of detecting correlations between heterozygosity and growth, the final sampling of animals was stratified by including the 12–13 heaviest and the 12–13 lightest oysters in each ploidy class and at each site, which were combined with a further 50 oysters that had been chosen at random, giving a total of 302 sampled individuals. Total, dry shell and dry tissue weights were recorded on all selected individuals. Animals were killed, their flesh was homogenized (after small samples were taken for allozyme and microsatellite analyses), weighed and freeze-dried for 72 h and weighed again. Condition indices were defined as (dry tissue weight + 1000)/dry shell weight. Approximately 10 mg of homogenized dry tissue was used to measure the proximate biochemical composition. Proteins were analysed following the procedure of Lowry *et al*. (1951). Carbohydrate and glycogen contents were determined according to Dubois *et al*. (1956). Lipids were extracted and purified according to Blight & Dyer (1959), and then analysed as described by Marsh & Weinstein (1966).

Estimation of genetic diversity

Microsatellite and allozyme analyses, detection of refractory diploids — that did not present significant weight differences with true diploids and thus pooled together — and the unambiguous identification of both parents for all but seven animals using highly polymorphic microsatellite loci have all been reported by Magoulas *et al*. (2000). A total of 294 offspring were finally included in this study and the numbers of individuals observed by site, ploidy class, male and female parentage are reported below in Tables 1 and 4. The experimental design is unbalanced, with smaller contributions from some parents (M2, F2, F4 and F6), but numbers of individuals were largely sufficient to test the effects of sites, ploidy levels and genotypes (see following section).

Individual heterozygosity (H), the proportion of heterozygous loci, and allelic diversity (D), used for distinguishing between triploids having either two or three different alleles were computed as in Magoulas *et al*. (2000). Both H and D were computed on allozymes (H_A, D_A) and microsatellites (H_M, D_M). A third measure of genetic diversity was used, that is specific to microsatellites and assumes a 'stepwise' mutation model (Valdes *et al*. 1993), with alleles differing from each other by one or more repeats. The squared difference in repeat units between two alleles at a locus (d^2) is expected to be related to their time since coalescence (Coulson *et al*. 1998) and to be a useful indicator of the fitness consequences of inbreeding and outbreeding (Pemberton *et al*. 1999). It was computed across the three scored microsatellite loci for diploids as mean-$d^2 = 1/3\,\Sigma_i(l_a - l_b)^2$, l_a and l_b being the lengths in repeat units of alleles a and b at locus i. For triploids, mean-d^2 was computed as mean-$d^2 = 1/3\,\Sigma_i[(l_a - l_b)^2 + (l_a - l_c)^2 + (l_b - l_c)^2]/3$, l_c being the length in repeat unit for the third allele.

Statistical analyses

Pearson correlation coefficients were computed between family means of all traits within each of the four combinations of site and ploidy level (Sokal & Rohlf 1995), and they were used to design groups of characters. Sites, ploidy levels and genotypic male and female classes were tested for all quantitative traits as main effects in analyses of variance (ANOVA) using the generalized linear models (GLM) procedure from the SAS statistical package (SAS Institute 1988). As local competition effects between individuals were considered negligible, individuals were considered as independent experimental units. The stratified sampling including the heaviest and the lightest individuals ensured the maximum range of variation to be

Table 1 Means for growth and biochemical traits measured on 294 oysters

	MO 2N	MO 3N	*t*-test 2N–3N within MO	*t*-test 2N–3N within TL	TL 2N	TL 3N	*t*-test MO–TL within 2N	*t*-test MO–TL within 3N
Number of individuals	93	55			88	58		
Survival§	95.3	92.7	ns‡	ns‡	97.3	96.7	ns‡	ns‡
Weight 95 (g)	15.0 ± 1.1	15.0 ± 1.4	ns	*	15.7 ± 1.1	17.5 ± 1.4	ns	*
Weight 96 (g)	46.9 ± 3.0	62.9 ± 5.2	***	***	55.7 ± 3.3	76.6 + 6.5¶	***	**
Dry shell weight (g)	32.2 ± 2.1	43.1 ± 3.5	***	***	32.4 ± 2.0	46.5 ± 4.0	ns	ns
Dry tissue weight (g)	0.97 ± 0.1	1.36 ± 0.2	***	***	1.32 ± 0.1	1.85 ± 0.2	***	***
Condition index	29.9 ± 1.1	30.7 ± 1.4	ns	ns	40.9 ± 1.9	39.5 ± 2.4	***	***
Protein content (mg)	313.9 ± 25.0	435.7 ± 50.6	***	***	407.7 ± 29.5	559.6 ± 60.8	***	**
			NP test	NP test			NP test	NP test
Lipids content (mg)	108.3 (56.5)	156.0 (87.1)	†††	†††	127.7 (59.0)	201.3 (129.0)	†	ns
Carbohydrate content (mg)	91.0 (67.4)	175.2 (115.7)	†††	†††	157.3 (80.6)	228.7 (139.2)	†††	†
Glycogen content (mg)	33.1 (24.5)	72.3 (53.7)	†††	†††	73.6 (59.6)	120.6 (92.4)	†††	†††
Protein %	32.6 (3.7)	32.0 (4.1)	ns	ns	30.9 (2.5)	30.7 (4.1)	†††	ns
Lipids %	11.3 (4.1)	11.6 (4.0)	ns	ns	9.8 (3.8)	11.2 (5.7)	†	ns
Carbohydrate %	9.2 (4.4)	12.8 (5.8)	†††	ns	11.9 (4.1)	12.6 (5.3)	†††	ns
Glycogen %	3.6 (2.3)	5.3 (3.0)	†††	ns	5.5 (3.5)	6.6 (3.6)	†††	ns

95% confidence intervals are given for traits for which normality can be assumed within samples, standard deviations are given in brackets for the other traits.
MO, Marennes-Oléron; TL, Thau lagoon; 2N, diploids (including refractory triploids); 3N, true triploids.
Probabilities associated with Student *t*-test for mean comparisons between ploidy levels or between sites: ns for $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
Probabilities associated with non-parametric (Mann–Whitney) tests for mean comparisons between ploidy levels and between sites: ns for $P > 0.05$, $†P < 0.05$, $††P < 0.01$, $†††P < 0.001$.
‡Probabilities associated with a binomial test for percentage comparisons.
§Computed on 150 individuals for each ploidy class at each site.
¶Calculated on 57 individuals instead of 58.

represented for each combination of site and ploidy level, thus significant effects for site and ploidy could be considered with confidence. The possible increase of imbalance between male and female contributions was accounted for by the models used.

Since all traits except initial weight showed heteroscedasticity between classes (using Bartlett's tests; Sokal & Rohlf 1995) and non-normality of residuals (using Shapiro–Wilk's tests; SAS Institute 1988) various transformations were tried following the recommendations of Kung (1988) and Sokal & Rohlf (1995), until homoscedasticity and normality of residuals were observed. Suitable transformations were found for all traits. The complete model was the following:

$$Y_{ijklm} = \mu + S_i + P_j + S_i^*P_j + M_k + F_l + S_i^*M_k + S_i^*F_l + P_j^*M_k + P_j^*F_l + M_k^*F_l + (\text{higher order interactions effects between three effects}) + e_{ijklm} \quad (1)$$

where μ is the general mean, Y_{ijklm} is the growth measurement for the m^{th} individual of the j^{th} ploidy class (tested with principal effect P_j), in the i^{th} site (tested with effect S_i), and whose parents are male k and female l (respectively tested by effects M_k and F_l). $S_i^*P_j$, $S_i^*M_k$, $S_i^*F_l$, $P_j^*M_k$, $P_j^*F_l$ and $M_k^*F_l$ are the interaction effects between all four principal effects and e_{ijklm} is the residual. Type III sums of squares (SSQ_{III}) allowed to account for the imbalance in the experimental design (SAS Institute 1988), and they are adjusted by definition to all effects in the model. Ploidy, site and male and female effects were all considered as fixed effects because of their small number of levels. Optimal models were found by dropping sequentially the nonsignificant effects with the lowest SSQ_{III}, and reciprocally, the model selected was checked by a forward addition of effects.

Relationships between quantitative traits and genotypic diversity were investigated with similar generalized linear models to those above but with the combined use of individual diversity measures as covariates in nested analyses of covariance. Growth variation was adjusted to the different controlled factors in the experiment, thus increasing the efficiency in detecting any potential association with diversity. Associations were tested within each combination of ploidy and site and separately within groups of individuals pooled by female or male parentage. For example, the model for testing the effect of diversity within each female, for

Table 2 Summary of phenotypic correlations between growth and biochemical traits family means for each combination of ploidy level (2N, diploids and 3N, triploids) and site (MO, Marennes-Oléron and TL, Thau Lagoon)

Groups and traits	Group 1	Group 2	Group 3
Group 1 Dry shell weight Dry tissue weight Weight in 1996 Protein content	All strong and positive 23 out of 24 significant 23 between 0.8 and 1 Range (0.7 to 0.9)	All positive 39 out of 64 significant 43 between 0.6 and 0.9 Range (0.2 to 0.9)	Mostly negative 12 out of 64 significant 21 between −0.8 and −0.5 Range (−0.8 to 0.4)
Group 2 Weight in 1995 Lipids content Carbohydrate content Glycogen content Condition index		All positive 8 out of 24 significant 9 between 0.6 and 0.8 Range (0.0 to 0.8)	No general trend 9 out of 64 significant 39 between −0.3 and 0.3 Range (−0.8 to 0.9)
Group 3 Protein % Lipids % Carbohydrate % Glycogen %			No general trend 1 out of 24 significant 14 between −0.2 and 0.2 Range (−0.4 to 0.7)

a trait for which the optimal model only included the four main effects (site, ploidy, male and female), is the following:

$$Y_{ijklm} = \mu + S_i + P_j + M_k + F_l + (\alpha_l D_m) + e'_{ijklm} \quad (2)$$

where Y_{ijklm}, S_i, P_j, M_k and F_l are as in model (1), D_m is the *individual* diversity measure and α_l the coefficients of regression estimated within each female l after adjustment for the female effect F_l (l index) and all the other effects, e'_{ijklm} is the residual of the model that includes the regression residual. Similar models were declared using diversity or mean-d^2 as individual covariates within ploidy levels, female or male parents. A classical analysis of covariance only performing the regression of the residuals of the ANOVA onto the covariates (Sokal & Rohlf 1995) was shown in comparison. In order to analyse the potential effect of variation in inbreeding levels of the different families, adjusted family effects for fitness-related traits were estimated separately within diploids and triploids, and plotted against D_A and D_M as well as mean-d^2. The adjusted family means for these diversity measures were also used as covariates for predicting family effects in the following generalized linear models applied separately for diploids and triploids:

$$Y_{ijkl} = \mu + S_i + (\beta D_{jk} + r_{jk}) + e''_{ijkl} \quad (3)$$

with the same notations as in model (1) ($\beta D_{jk} + r_{jk}$) is the family effect defined by the different crosses between male j and female k parents, that is split into one regression term βD_{jk} on the adjusted family mean diversity covariate D_{jk}, and the residual of the regression r_{jk}, e''_{ijkl} being the residual of the model.

Results

Survival

Survival rates estimated on a total of 600 individuals raised in farmed conditions in the two different environments were adjusted according to the resulting numbers of individuals found in each combination of site and ploidy levels, that took account of refractory diploids. They ranged between 92.7% and 97.3% (Table 1). Differences in survival were not significant either between ploidy classes at each site or between sites [94.3% at Marennes-Oléron (MO) and 97.0% at Thau Lagoon (TL), $P < 0.10$].

Phenotypic correlations

Phenotypic correlations were computed at the family mean level for each combination of ploidy and site. For each correlation computed, the corresponding graph was checked for homogeneity. All analysed traits could be clustered in three different groups according to absolute correlation values and significance levels (Table 2). Although fairly large differences were sometimes observed, there was no general trend in correlation coefficients from the poorer to the richer site, or from the diploids to the triploids.

Contribution of ploidy, site and parentage to the variation of growth and biochemical traits

Using transformed variables, optimal models of ANOVA were found for all traits using sites, ploidy, female parentage and male parentage as main effects (Table 3). These models explained between 20% and 41% of total

Table 3 Analyses of variance on growth and biochemical traits

Phenotypic traits	Transformation	Sources of variation†					R^2‡
		Site	Ploidy	Site × Ploidy	Female	Male	
May 1995							
Weight	—	1.2*	ns	ns	4.1*	2.5**	8.3
January 1996							
Weight	Log	6.6***	15.6***	ns	9.0***	3.6***	34.8
Dry shell weight	Square root	ns	19.6***	ns	9.4***	3.6***	32.6
Dry tissue weight	Log	11.8***	10.9***	ns	12.4***	3.7***	38.8
Protein content	Cubic root	8.6***	11.3***	ns	12.3***	3.6***	35.8
Lipids content	Log	2.4**	8.6***	ns	6.8***	1.8*	19.6
Carbohydrate content	Log	13.0***	11.2***	1*	4.8**	ns	30.0
Glycogen content	Cubic root	12.8***	12.4***	ns	ns	ns	25.2
Condition index	Square root	32.9***	ns	ns	8.3***	ns	41.2
Protein %	Square root	4.3***	ns	ns	ns	ns	4.3
Lipids %	Square root	2.0**	ns	ns	ns	ns	2.0
Carbohydrate %	Square root	3.4**	4.0***	2.0**	ns	ns	9.4
Glycogen %	Square root	6.0***	5.3***	ns	ns	ns	11.3
Allozyme heterozygosity (H_A)	—	1.2**	17.0***	ns	19.2***	4.7***	42.1
Allozyme diversity (D_A)	—	ns(9%)	24.2***	2.0*	16.2***	3.1***	45.3
Microsatellite heterozygosity (H_M)	—	ns	1.9*	ns	ns	4.0**	5.9
Microsatellite diversity (D_M)	—	ns	65.0***	ns	ns	ns	65.0
Mean-d^2§	Square root	2.6*	ns	ns	ns(10%)	8.9***	13.3§

ns for $P > 0.05$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

†Proportion of total variation explained (in % of Total Sums of Squares) for each effect and associated significance level.

‡Indicative coefficient of determination for each effect computed as ratio of type I Sums of Squares (SSQ), which are additive in unbalanced designs in contrast to adjusted type III SSQ (SAS Institute 1988) onto total SSQ.

§For this trait, a significant Site × Male effect was found ($P < 0.05$), which explained 1.8% of total variation.

variation in sums of squares (SSQ) for growth and biochemical traits, and a much smaller amount of variation for biochemical composition expressed as percentages (between 2% and 11%, Table 3). Only two significant interactions were found between site and ploidy for carbohydrate content and carbohydrate percentage. No significant higher order interaction between any main source of variation was found for all the traits considered. In general, oysters performed better in TL than in MO, and triploids were heavier than diploids in both sites (Table 1).

At the start of growth monitoring in May 1995, there was no overall effect of triploidy (Table 3), although a small difference could be detected when considering diploids and triploids at TL only (Table 1), and a small site effect was detected, mainly due to differences among triploids (Tables 1 and 3). In groups 1 and 2, site and ploidy effects were highly significant for all traits, except the site effect for dry shell weight (DSW) and the ploidy effect for condition index (CI) (Table 3). The site effect was significant for all traits of group 3, although explaining a smaller part of the total variation (Table 3). Interaction effects between site and ploidy were detected for carbohydrate content (CC) and carbohydrate percentage (CP) only but explained a very small amount of total variation (between 1% and 2%).

At the end of growth monitoring, the effect of female parentage was significant for all growth traits and chemical contents except glycogen content (Table 3), explaining between 5% and 12% of total variation. Male effect was also significant for all growth traits except CI, as well as for protein and lipid content, but was associated with a lower amount of SSQ variation (1.8% to 3.6%). For the traits that showed significant differences between females or males, offspring from parents F5 and F2 performed significantly better than other females' progenies, and males M3 and M1 progenies remained superior to M2 offspring, as already observed for initial weight.

Analyses of variation for diversity measures

Differences between ploidy levels, site, male and female were also tested for all diversity measures (Table 3). Normality of residuals was verified for all measures except microsatellite heterozygosity (H_M) and mean-d^2. Mean-d^2 residuals were normalized with a square-root transformation, but no transformation was found for H_M that was the least informative measure, most individuals being heterozygous for the three microsatellite loci (Table 3). On the other hand, microsatellite allelic

Table 4 Means and standard deviations for three parameters of diversity and groups of individuals associated with levels of variation in the experiment. Sites are as in **Table 1**

Level of variation	No.*	Allozyme diversity (D_A)		Microsatellite diversity (D_M)		$\sqrt{(\text{mean-}d^2)}$†	
		Mean	SD‡	Mean	SD	Mean	SD
Females							
F1	65	0.36	0.15	1.04	0.27	7.94	3.43
F2	12	0.55	0.17	1.32	0.39	8.07	2.06
F3	150	0.59	0.25	1.19	0.40	6.96	2.47
F4	5	0.40	0.18	1.10	0.22	6.97	1.94
F5	45	0.43	0.15	1.25	0.30	7.28	2.44
F6	8	0.26	0.07	1.00	0.18	8.75	4.81
F7	9	0.46	0.11	1.11	0.24	8.05	3.65
Males							
M1	52	0.37	0.19	1.21	0.29	9.14	3.95
M2	14	0.29	0.13	0.98	0.16	8.39	2.87
M3	228	0.54	0.23	1.16	0.38	6.88	2.29
Ploidy							
Diploids	181	0.41	0.185	0.94	0.14	7.19	3.34
Triploids	113	0.64	0.22	1.53	0.30	7.62	1.65
Site							
MO	148	0.51	0.22	1.16	0.33	7.79	3.10
TL	146	0.49	0.24	1.16	0.38	6.90	2.43

*Number of individuals for each sample in each level of variation.
†Square root of mean-d^2.
‡SD, standard deviation.

diversity (D_M) ranged from 0.5 to 2 (including seven possible values) and 65% of its total variation in sums of squares (SSQ_T) was attributed to differences between ploidy classes, triploids being significantly more polymorphic on average than diploids but with overlapping ranges (Table 4). Allozyme heterozygosity and allelic diversity (H_A and D_A) showed similar patterns of variation, with significant effects of ploidy, male parentage and female parentage, together explaining over 40% of SSQ_T. Although a smaller amount of mean-d^2 variation was explained by ANOVA, the male effect was two to three times more important than for classical diversity measures (Table 3). Consequently, mean-d^2 discriminated male parentage better than heterozygosity or diversity (see also Table 4). The reverse was observed for female parentage, that was best discriminated by allozyme heterozygosity and diversity.

Phenotypic correlations between H_A and D_A were all equal or greater than 0.9 for each combination of site and ploidy level (data not shown). H_M or D_M were not, in any sample, significantly correlated to H_A or D_A, with one exception for D_M and D_A, that showed a positive correlation within triploids at TL ($r = +0.47$, $P < 0.01$). Mean-d^2 was not correlated to any measure estimated with allozymes, but was weakly and positively related to H_M at MO and to D_M (except for diploids at MO).

Associations between individual phenotypic variation and genotypic diversity

D_A, D_M and mean-d^2 were chosen as individual covariates because of the different information on diversity that they were likely to bring, based on the type of loci (coding, noncoding), their patterns of variation in ANOVA and correlations with the other measures presented above. Similarly, analyses were performed for dry shell weight (DSW), glycogen content (GC) and CP, as traits representative of groups 1, 2 and 3, respectively. As carbohydrate percentage (CP) did not present any significant association in adjusted models with D_A, D_M and mean-d^2, results are only detailed for DSW and GC (Table 5).

Overall, a significant and positive association was observed between D_A and DSW, after having adjusted the variation in DSW for all effects controlled in the experiment (model *a*, Table 5). This positive association could be observed when grouping individuals either by site and ploidy levels (model *b*), by male (model *c*), or by female (model *d*), since all but one regression coefficients were positive, and seven out of 12 were significant. However, these associations explained very little additional variation (R-squares ranging from 1.6% to 3.3% in Table 5). A similar pattern was observed when considering the relationships between GC and D_A, with positive regression coefficients in most groups and similar levels of variation explained.

Table 5 Classical analysis of covariance (model *a*) and nested analyses of covariance (models *b* to *d*) testing the association between diversity estimated as individual diversity for allozymes and microsatellites (D_A and D_M) or mean-d^2 and two quantitative traits representative of groups 1 and 2: dry shell weight (DSW), and glycogen content (GC); phenotypic correlations are given in the last two columns

Models	Groups of individuals	Covariate	F-test probability		Significance and sign of regression coefficients		Optimal R^2 increase (%)		Phenotypic correlations	
			DSW	GC	DSW	GC	DSW	GC	DSW	GC
Model *a*		D_A	**	ns	$+^{**}$	$+^{ns}$	1.6	ns	+0.27***	+0.17**
	complete	D_M	ns	ns	$+^{ns}$	$+^{ns}$	ns	ns	+0.38***	+0.30***
	sample	mean-d^2	ns	*	$-^{ns}$	$-^{*}$	ns	1.5	ns	−0.17**
Within sites and ploidies (model *b*)	MO-2N	D_A	*	*	$+^{**}$	$+^{*}$	2.5	2.6	ns	ns
	MO-3N				$+^{ns}$	$-^{ns}$			ns	ns
	TL-2N				$+^{**}$	$+^{ns}$			+0.25*	ns
	TL-3N				$+^{ns}$	$-^{ns}$			ns	−0.29*
	MO-2N	D_M	ns	ns	$+^{ns}$	$+^{ns}$	ns	ns	ns	ns
	MO-3N				$+^{ns}$	$+^{ns}$				
	TL-2N				$+^{ns}$	$+^{ns}$				
	TL-3N				$+^{ns}$	$-^{ns}$				
	MO-2N	mean-d^2	ns	ns	$-^{ns}$	$-^{ns}$	ns	ns	−0.21*	ns
	MO-3N				$+^{ns}$	$-^{ns}$			ns	ns
	TL-2N				$-^{ns}$	$-^{ns}$			ns	ns
	TL-3N				$+^{ns}$	$-^{ns}$			ns	ns
Within males (model *c*)	M1	D_A	*	ns	$+^{*}$	$+^{ns}$	2.0	ns	+0.34*	+0.36**
	M2				$-^{ns}$	$+^{ns}$			ns	ns
	M3				$+^{*}$	$+^{ns}$			+0.26**	+0.15*
	M1	D_M	ns	ns	$+^{*}$	$+^{ns}$	ns	ns	+0.65***	+0.55***
	M2				$+^{ns}$	$-^{ns}$			ns	ns
	M3				$+^{ns}$	$+^{ns}$			+0.33**	+0.27*
	M1	mean-d^2	ns	*	$-^{ns}$	$-^{ns}$	ns	2.5	ns	−0.34*
	M2				$-^{ns}$	$-^{*}$			ns	−0.70**
	M3				$-^{ns}$	$-^{*}$			ns	ns
Within females (model *d*)	F1	D_A	*	*	$+^{**}$	$+^{**}$	3.3	4.0	+0.58**	+0.29*
	F2				$+^{ns}$	$+^{*}$			ns	ns
	F3				$+^{*}$	$+^{ns}$			+0.34***	+0.18*
	F5				$+^{ns}$	$+^{**}$			+0.37*	+0.35*
	F1	D_M	ns	ns	$+^{ns}$	$+^{ns}$	ns	ns	+0.32**	ns
	F2				$-^{ns}$	$+^{ns}$			ns	+0.65*
	F3				$+^{ns}$	$+^{ns}$			+0.38***	+0.23**
	F5				$+^{*}$	$+^{ns}$			+0.57***	+0.56***
	F1	mean-d^2	ns	*	$-^{ns}$	$-^{ns}$	ns	4.5	ns	−0.29*
	F2				$+^{ns}$	$+^{ns}$			ns	ns
	F3				$-^{ns}$	$-^{*}$			ns	ns
	F5				$+^{ns}$	$+^{ns}$			ns	ns

MO, Marennes-Oléron; TL, Thau lagoon; 2N, diploids; 3N, triploids; M, male; and F, female.
ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Associations were significant within diploids at MO (models *b*, Table 5), and within three out of four females (model *d*) that had a number of offspring ranging from 12 to 65.

No significant association was found overall between D_M and any of these two traits. We report in Table 5 the only two positive and significant correlations that were found for DSW within male M1 and female F5 (in models *c* and *d*). Using mean-d^2 as individual covariate, no significant association was observed with DSW (models *a* to *d*), but regression coefficients were negative for all levels

Table 6 Factorial regressions testing diversity (D_A and mean-d^2) estimated within diploids and within triploids for offspring from the same family (female × male cross), and used as family covariates

Diversity used as family covariate	F-test significance and sign of regression		Proportion of family sums of squares explained		Corresponding correlation value and significance	
	DSW	GC	DSW	GC	DSW	GC
Within diploids						
D_A	ns(+)	ns(+)	ns	ns	0.31^{ns}	0.23^{ns}
mean-d^2	*(–)	ns(–)	13.1%	ns	-0.23^{ns}	-0.07^{ns}
Within triploids						
D_A	***(–)	*(–)	35.1%	15.5%	0.34^{ns}	-0.38^{ns}
mean-d^2	ns(–)	ns(–)	ns	ns	-0.39^{ns}	-0.41^{ns}

DSW and GC are as in **Table 5**.
ns, not significant at $P < 5\%$, *significant at $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.
†See also Figures 1, 2 and 3.

involving diploids and the MO site, and within the three males (Table 5). However, for GC, negative and significant associations were found with mean-d^2 in the analysis of covariance (model *a*), within males M2 and M3 (model *c*) and within female F3 (model *d*). Comparing the phenotypic correlations estimated on individual data with the associations obtained using different models of nested covariance analyses illustrates the interest of the former analyses (Table 5). Indeed, positive correlations in most groups of individuals between D_M and DSW could no longer be observed after adjustment to principal model effects as they were due essentially to the effect of triploidy (see above the ANOVA for D_M in Table 4). On the other hand, the adjustment allowed for significant associations to be detected between diversity D_A and DSW or GC, within diploids at MO and TL for example, that could not be observed on raw data.

Associations between family phenotypic variation and average genotypic diversity

Generalized linear modelling of DSW and GC were performed, using diversities (D_A, D_M and mean-d^2) as family covariates within diploids or triploids (Table 6). Family effects for DSW and GC were not significantly associated with D_A whereas they were negatively and significantly associated within triploids. Using mean-d^2 as family covariate, a negative association was observed within both diploids and triploids, but it was only significant for DSW within diploids. Associations between site-adjusted family means for DSW and GC were also plotted against D_A, D_M and mean-d^2, that were averaged for each family and adjusted to the site effect (Figs 1, 2, 3). Best fitting curves, weighting each family by its number of individuals, were found for diploids and triploids. In all cases, linear or second-degree polynomial adjustments gave the best fits among those tested (exponential, logarithm or power). Within diploids, positive but non-significant associations were observed between DSW and D_A ($R^2 = 5.9\%$, Fig. 1a) or between GC and D_A ($R^2 = 8.9\%$, Fig. 1b). In contrast, polynomial fitting curves were found within triploids for DSW and GC that explained, respectively, 63.4% and 58.1% of the variation (Fig. 1a,b). For both traits, an optimum range of intermediate diversity values seems to be observed for the best phenotypes, with a trend towards less productive family averaged phenotypes for higher diversities, consistently with the negative associations found for triploids (Table 6). Comparing diploids and triploids for 11 families for which data were available, significant differences (4/11) were for better triploids (Fig. 1a). With D_M, no significant pattern was found for both traits and both ploidy levels (Fig. 2a,b). In diploids, this could be explained by the high heterozygosity level recorded ($D_M = 1$) on most individuals, that did not allow discrimination between families. Among the few families that had D_M differences, the trends were however, clearly negative. Adjustments for DSW and GC as a function of mean-d^2 were all not significant, explaining no more than a few per cent of variation, except in the case of DSW and mean-d^2 within diploids (Fig. 3, $R^2 = 13.1\%$).

Discussion

Survival

Difference in survival between diploids and triploids has been controversial, with some authors reporting a higher survival for triploids than diploids (Allen & Downing 1986; Matthiessen & Davis 1992), and colleagues reporting the opposite (Stanley *et al*. 1984). In the present experiment, survival rates were very high for both diploids and triploids, with similar survival between each ploidy class at both locations. This is consistent with observations of

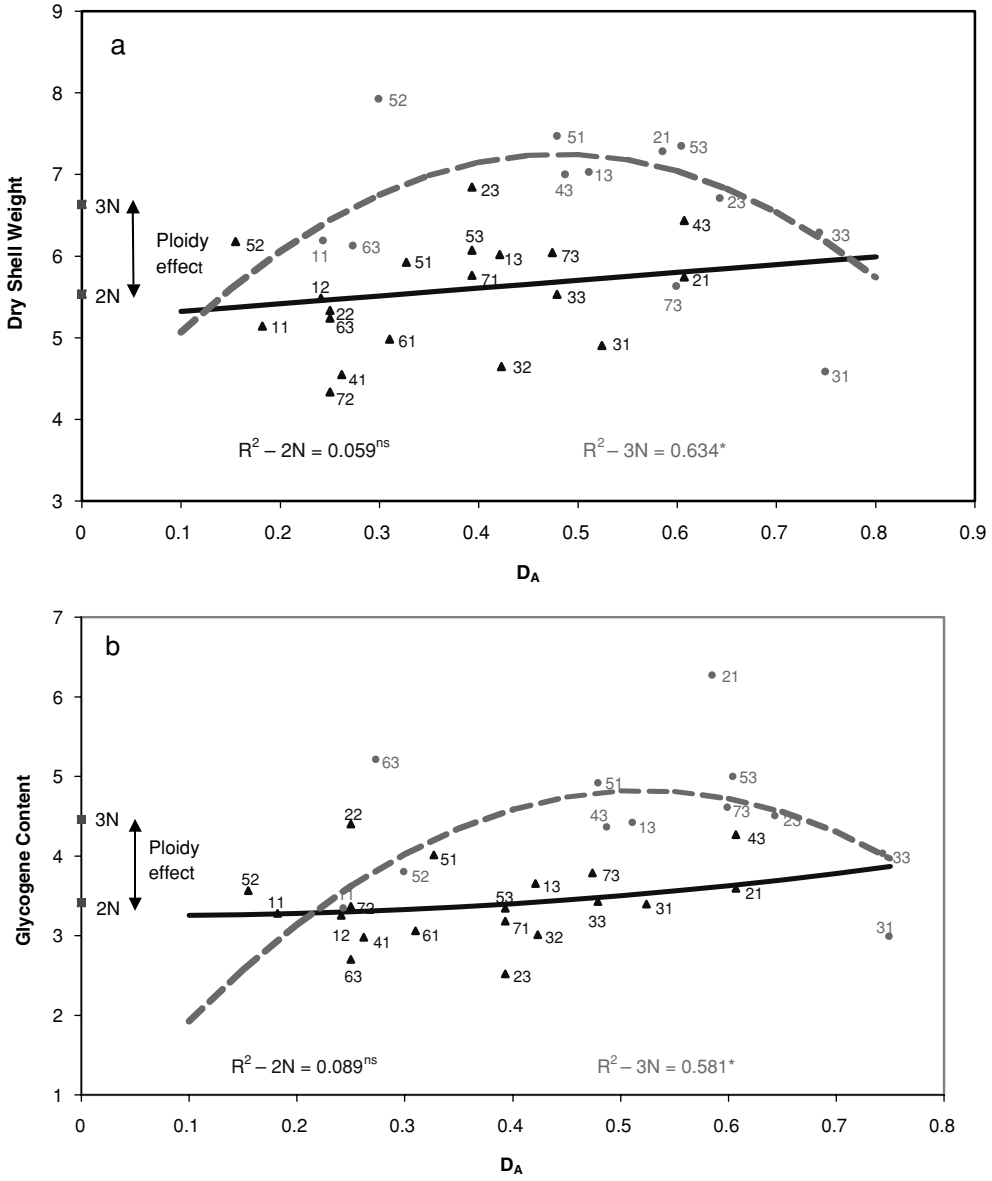

Fig. 1 Plots of adjusted family means between allozyme diversity and dry shell weight (a) or glycogen content (b), and R^2 of corresponding linear or polynomial adjustments for the relationships. Continuous line, adjustment for diploids; dotted line, adjustment for triploids; 2N, diploids; 3N, triploids; ▲, family means within diploids; ●, family means within triploids; ■, ploidy means. The family identification number is given beside each spot as female identification number followed by male identification number.

Nell *et al*. (1994) on *Saccostrea commercialis*, another shellfish where triploidy has been investigated for farming, and other studies comparing growth between diploids and triploids within the same genus as *Crassostrea gigas* (e.g. Maguire *et al*. 1995). An explanation for this high survival rate would be that the main mortality has occurred before deployment through genetic load purging, and specifically during the larval stage, in agreement with the results of Downing & Allen (1987) showing that once the D-larval stage had been reached, triploids exhibit similar mortality rates to diploids.

Ploidy and site effects on growth and biochemical composition

Variations in observed growth and biochemical contents at harvest were well explained (20–40%) by the four controlled factors: site, ploidy, male and female parentage. In general, site and ploidy together accounted for more than half of this variation, oysters performing better at the richest site, and triploids being heavier than diploids at both sites. In contrast, relative biochemical compositions were very stable between any factor levels, and only small

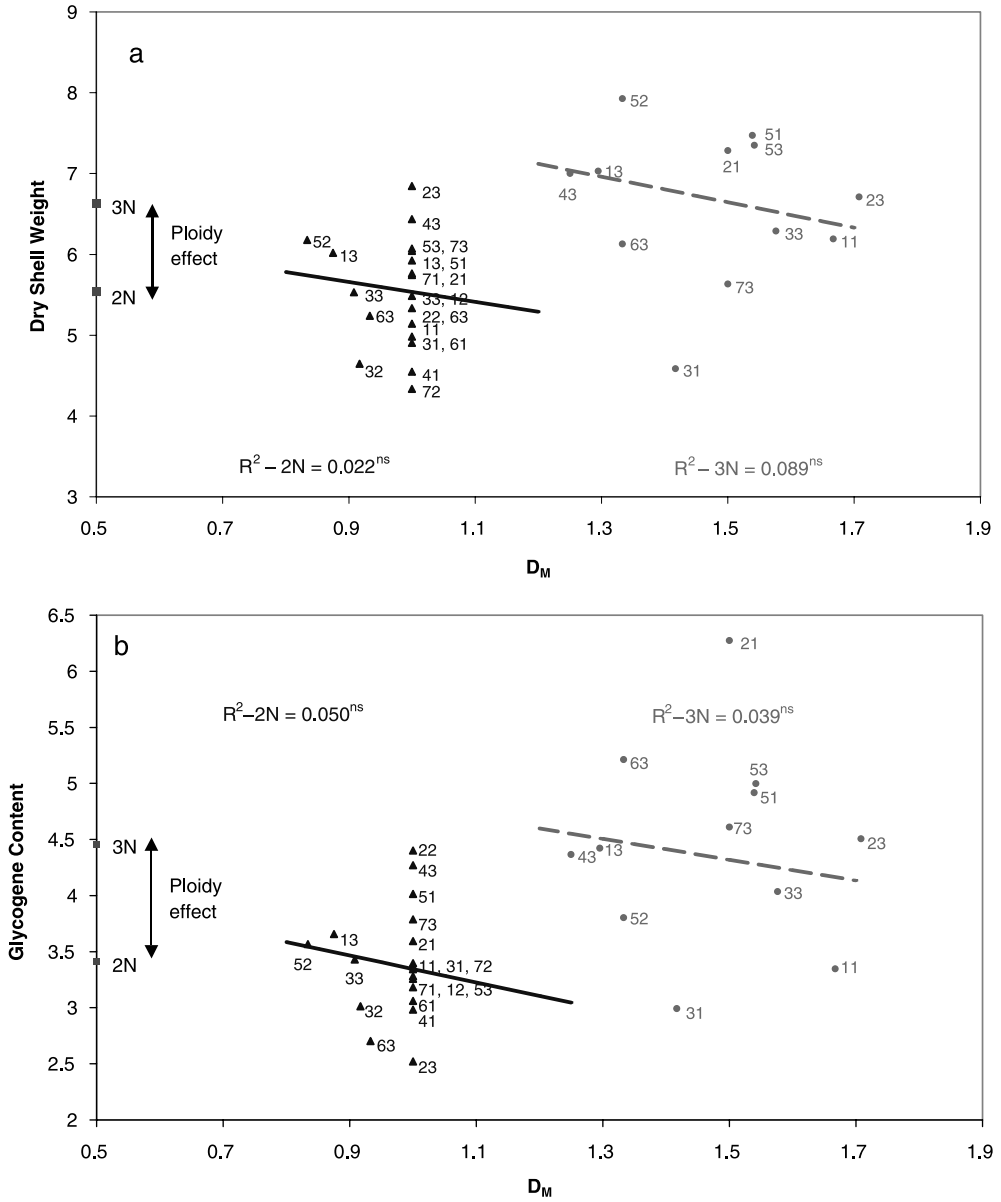

Fig. 2 Plots of adjusted family means between microsatellite diversity and dry shell weight (a) or glycogen content (b). R^2, symbols for fitting curves and for spots are as in Fig. 1.

differences could be detected, mostly between sites for diploids.

Significantly faster soft-tissue growth and higher biochemical contents of both carbohydrate and glycogen were observed in TL than in Marennes-Oléron MO. This suggests differences in food availability and acquisition rates between the two environments. MO is an over-exploited estuarine ecosystem, with high quantities of suspended seston that are often of low organic content. In contrast, TL is a typical lagoon ecosystem, where more nutritious seston is usually available, albeit at lower concentrations. Moreover, an absence of tides in TL means that oysters can feed continuously (Goulletquer 1998). Higher average temperatures at TL might also have influenced growth positively, according to results of Davis (1989) showing that triploids reared in two sites of similar productivity grew faster where temperature was higher (20 °C vs. 16 °C on average).

It is notable that percentages of glycogen and carbohydrate were higher in triploids at both sites, but this effect was significant only at the poorest and coldest site (Table 4). This could be interpreted either as faster accumulation or reduced utilization of carbohydrates, or both. Carbohydrates, and more specifically glycogen, represent the oysters' energy reserves. In a high carrying capacity ecosystem such as TL, triploids accumulated on average 45% more carbohydrates and 63% more glycogen than diploids, and

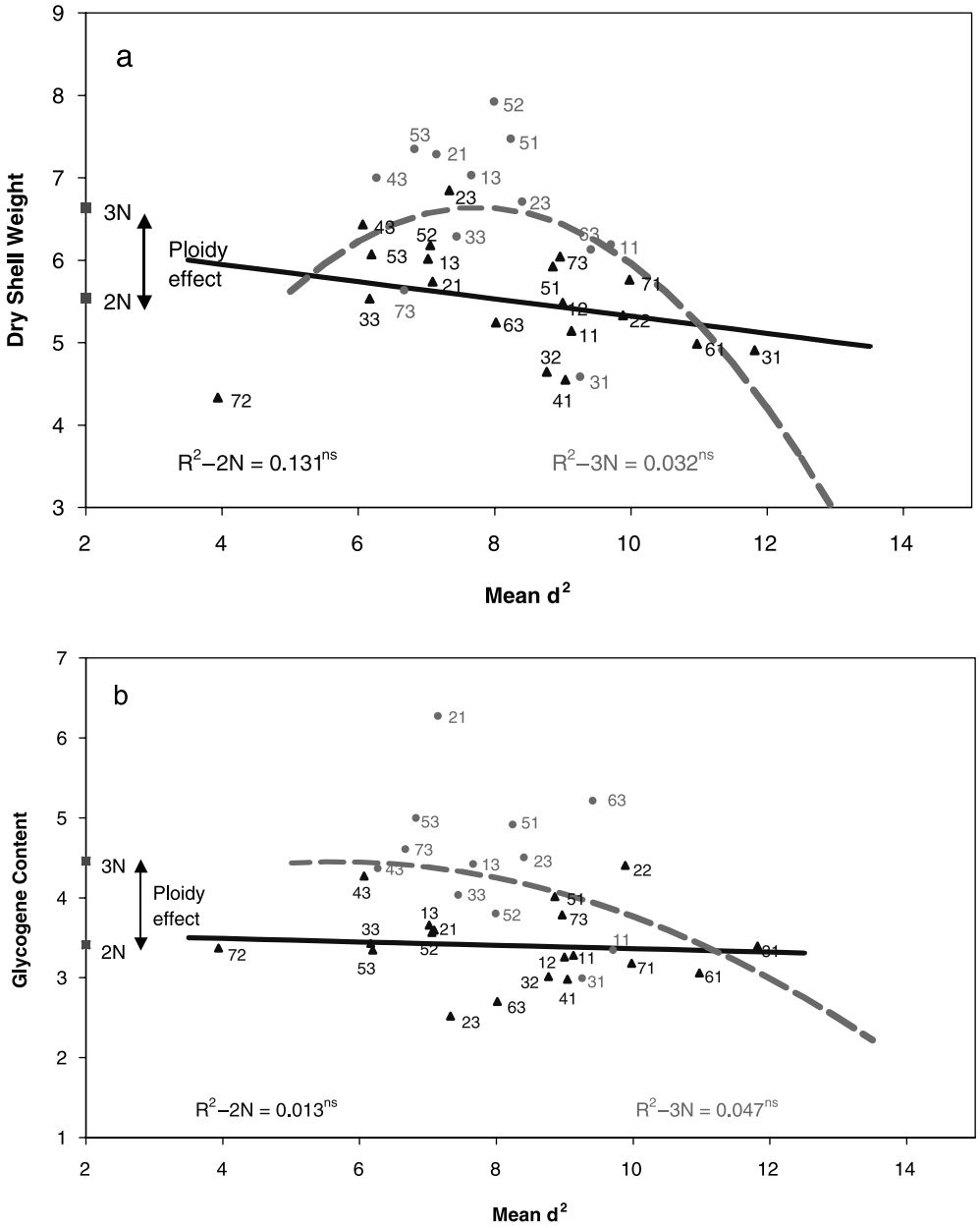

Fig. 3 Plots of adjusted family means between mean-d^2 and dry shell weight (a) or glycogen content (b). R^2, symbols for fitting curves and for spots are as in Fig. 1.

both ploidy classes showed good winter growth. In a low carrying capacity environment such as MO, where less food is available during winter, oysters have to use their energy reserves. By accumulating 93% more carbohydrates and 118% more glycogen than diploids, carbohydrate percentages in triploids from MO reached similar levels to those in TL. Thus in poorer or more stressful conditions, triploids are expected to resist winter starvation better than diploids, and to show fewer associated spring and summer mortalities. This is of interest for oyster farming and breeding, and consistent with previous results of greater protein and carbohydrate contents of triploids reared under stressful conditions (Shpigel *et al*. 1992).

Association between growth and individual or family heterozygosity in diploids

Using the allozyme diversity D_A as an individual covariate, significant and positive relationships were found between D_A and both DSW and GC within different samples of diploid individuals. These associations were not due to a possible influence of age (as in David *et al*. 1995), for

individuals were produced from the same mass spawning. However, they explained a small percentage (2.5–4%) of total variation, and were not significant at the family level, although still positive.

If a consensus exists for the reality of a weak but consistent positive association in marine bivalves (Britten 1996), no satisfactory conclusions have been reached concerning its genetic basis. Following David *et al*. (1995), alternative hypotheses have been linked to either 'local' or 'general' effects. 'Local' effects would encompass direct effects of the allozymes studied or indirect effects due to 'associative overdominance' through linkage with deleterious alleles of genes responsible for increased fitness or to more complex metabolic models including epistasis with those linked genes (Hawkins *et al*. 1989). Associative overdominance could also relate to 'general' effects assuming that the studied loci reflect genomic diversity, and that the correlation would be caused by inbreeding, or associated with a particular population structure.

There is evidence of very low levels or absence of inbreeding in the material studied here. Magoulas *et al*. (2000) have observed significant heterozygote excesses in five out of the 20 single locus tests performed on the same sample within diploids, in accordance with a reduction of the genetic load during larval stages (Bierne *et al*. 1998; Launey & Hedgecock 2001). Low inbreeding is also supported by the fact that microsatellite loci are heterozygous in most individuals, which explains why a diversity measure like D_M was not discriminant among diploids. In a situation where inbreeding is absent or undetectable, the positive and significant associations between individual allozyme diversity and both DSW and GC, might be viewed as an indication of direct effects of allozyme loci on fitness (see also David & Jarne 1997). According to the regression coefficients values among diploids at both sites (1.14 at MO and 1.33 at TL in model *b* from Table 5), the heterozygotes' advantage was stronger in the richer environment, while the opposite may have been expected if the association was an effect of inbreeding on fitness.

Comparison of findings for allozymes and microsatellites, that are truly neutral *a priori*, can help in the discussion of alternative hypotheses for growth–diversity associations. The high polymorphism of microsatellites was valued by estimating mean-d^2, a diversity measure that would reflect the coalescence time for alleles carried by an individual at one locus and the average time since coalescence between two gametes for many loci, assuming a stepwise mutation model (Pemberton *et al*. 1999). In contrast to the positive associations observed with D_A at the individual level, negative relationships were observed between growth performance and mean-d^2 within different samples of diploids. A negative and significant association was also detected at the family level, thus families of diploids with higher mean-d^2 showed on average significantly lower performances for DSW, with a higher proportion of variation explained than when considering individual relationships. These results may indicate intraspecific 'outbreeding depression', that would negatively affect offspring produced by gametes carrying relatively more distant genomes. Outbreeding depression could be caused in F_1 hybrids by disruptions in local adaptation due to genes by environment interaction or to negative epistatic interactions, or could originate in F_2 crosses from the break-up of coadapted groups of genes conferring higher fitness in particular environments (Templeton 1986), recombination breaking down beneficial combinations of alleles (recombinational load) (e.g. Edmands 1999). Therefore, such an outbreeding depression is only understandable if the parents come from different populations or species (with effects in F_1 crosses) or if they are themselves the result of crossings between different populations (with effects in F_2 crosses). One hypothesis would be that some of the sampled parents belong to *Crassostrea angulata* that has been shown to intercross with *C. gigas* (Huvet *et al*. 2001). This is however, poorly supported by the fact that all the western populations of *C. angulata* are restricted to South Spain and Morocco (Huvet *et al*. 2000; P. Boudry personal communication). One alternative explanation is that the parents used in this study were not from the Seudre locality only but originated also from other localities. This is possible, given that oyster commercial management often leads farmers to obtain material from different localities in France, and that significant differentiation for the same microsatellites has been observed in France between different populations of *C. gigas* (Huvet *et al*. 2000). By qualitatively comparing the data from the Marennes-Oléron (Seudre) sample from Huvet *et al*. (2000) and this study, we observed differences in allele sizes in both samples that are very unlikely to be due to differences in protocols alone. Different origins are also consistent with the observation that mean-d^2 was very efficient at differentiating among male parents. In the limited number of studies that have compared microsatellites mean-d^2 and allozyme diversity, correlations are positive but triangular, a high heterozygosity corresponding to either a very high or very low mean-d^2 (Pemberton *et al*. 1999). In this study, we showed that mean-d^2 was not correlated with allozyme diversity among individuals. Hedrick *et al*. (2001) recently challenged mean-d^2 as a valuable diversity measure, showing that it was not a good predictor of either inbreeding and outbreeding in a population of wolves with known kinship over a few generations. Their experimental data are however very different to those in this study, with a smaller number of individuals (30), a mean and a range for mean-d^2 approximately 10 times smaller than in our case, and the result that, in their analysis, mean-d^2 was not more informative than microsatellite heterozygosity. This is consistent with the occurrence of

inbreeding in the study by Hedrick *et al.* (2001), in contrast to evidence in this work. Furthermore, according to its definition, mean-d^2 might be more adequate when used for comparing ancestry between individuals of more distant populations, as originally proposed by Coulson *et al*. (1998).

Origin and variance of triploid advantage

Triploidy significantly increased performance for all absolute measures of growth at both sites, similarly to previous findings in *C. gigas* (Shpigel *et al*. 1992; Maguire *et al*. 1995; Hand & Nell 1999). Concerning the possible origins of triploid advantage mentioned in the introduction, we investigated the effects of higher heterozygosity, but our results are also relevant to the gene dosage and the sterility hypothesis. Sterility of triploid molluscs is now a well-known phenomenon (Beaumont & Fairbrother 1991), even if it may not be complete (Allen & Downing 1990). Sterility was not measured in our experiment, but differences in weight between diploids and triploids appeared between deployment in May 1995 and January 1996, after reproductive spawning whilst in the natural environment. This would be consistent with an effect of full or partial sterility in triploids.

Average levels of heterozygosity and allelic diversity were significantly higher in MII triploids than in their diploid siblings, in agreement with theoretical and experimental results (Allendorf & Leary 1984; Hawkins *et al*. 1994; Magoulas *et al*. 2000; on the same material). However, the range of variation of genetic diversity among triploids largely overlapped that observed among diploids. Triploids with relatively lower diversity could be found that out-performed diploids. This suggests an advantage of the triploid state *per se* whatever the diversity of the individual. More precisely, based on plots between family means for growth and diversity measures, polynomial adjustments showed that there is a range of lower to intermediate values of diversity for which triploids are always better than diploids. This triploid advantage thus seems to be independent from the diversity level of individuals and would be consistent with either the positive effect of gene dosage on growth or an advantage of triploids due to sterility that would affect subsequent growth. Without additional information on triploid sterility, it is not possible to conclude in our case and more data are required to establish fully any genetic dosage effects.

Assuming some outbreeding depression in progenies from diploid parents showing high mean-d^2 raises the question of the observation of similar effects in the corresponding triploid families. Across triploid families, there was a trend towards lower performance at higher diversity values, especially for DSW. This negative effect was tested significant in regressions of triploid family effects on D_A in generalized linear models for both DSW and GC. A reason why these effects were observed with D_A but not with mean-d^2 at the triploid level might be that at the highest diversity values, D_A is representative also of a high genetic distance between the parental genomes forming the triploids. This is not the case for mean-d^2 values, as they might be biased down, both because of the double contribution of the female parents in triploids that lowers the average mean-d^2 estimate of the family, and also because female showed a smaller range of mean-d^2 values than male parents.

In general, our findings demonstrate that due to their different nature, allozymes and microsatellites reveal different mechanisms underlying the fitness-related consequences of genomic interactions through mating. Allozyme diversity indicates a small advantage of being more heterozygous within a common genetic background, whilst mean-d^2 suggests that it is only the case as long as parents have not been isolated for so long that alleles are not too different. Altogether, our results suggest the influence of both direct effects of allozymes as in David & Jarne (1997) without excluding more complex epistatic interactions with other loci, and possible effects of outbreeding depression. This later hypothesis, in conjunction with the absence of inbreeding, would be more consistent with the occurrence of parents from different populations in our sample. We have no evidence of small-scale differentiation having occurred in our sampling location, but recent studies show that it may be possible in particular situations with adequate protocols (Hare & Avise 1996; David *et al*. 1997). On a temporal scale also, if population subdivision was due to a large variance in reproductive success and thus to small effective size (e.g. Hedgecock 1994), it would generally imply the existence of inbreeding, unlike in our case. Moreover, this study reveals that the triploid advantage exists whether the site is poor or rich, and for a large range of individual heterozygosity, suggesting effects of both gene dosage and sterility, and the need for additional experiments to understand better the relative importance of both factors.

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This reseach forms part of an EEC program (AIR1-CT92-0168) coordinated by Anthony Hawkins (PML, UK), who shares with Maurice Héral and the late Serge Bougrier (Ifremer CREMA, France) an interest and deep experience in mollusc ecophysiology. This program has been conducted in collaboration with André Gérard (Ifremer, France) who has long worked on mollusc cytogenetics, as well as with Antonios Magoulas and Georgios Kotoulas (IMBC, Creete) who have special interests in the population genetics of marine species. Pauline Garnier-Géré and Yamama Naciri Graven are quantitative geneticists. They share common interests in statistical analyses, now working on plant population genetics and phylogeography.
