Early Effect of Inbreeding as Revealed by Microsatellite Analyses on *Ostrea edulis* Larvae

Nicolas Bierne,*,† Sophie Launey,† Yamama Naciri-Graven†,1 and François Bonhomme*

**Laboratoire Génome et Populations, Centre National de la Recerche Scientifique UPR 9060, Université Montpellier II, F-34095 Montpellier, France, and †Laboratoire Génétique, Aquaculture et Pathologie, Institut Français pour la Recherche et l'Exploitation de la Mer (Ifremer), F-17390 La Tremblade, France*

Manuscript received July 16, 1997
Accepted for publication November 18, 1997

ABSTRACT

This paper reports new experimental evidence on the effect of inbreeding on growth and survival in the early developmental phase of a marine bivalve, the flat oyster *Ostrea edulis*. Two crosses between full sibs were analyzed using four microsatellite markers. Samples of 96 individuals were taken just after spawning (day 1), at the end of the larval stage before metamorphosis (day 10) and at the postlarval stage (day 70). Significant departure from Mendelian expectation was observed at two loci in the first cross and two loci in the second. Departure from 1:1 segregation occured in one parent of the first cross at three loci and genotypic selection, which resulted in highly significant heterozygote excesses, was recorded at three out of four loci in cross C1 and at two out of three loci in cross C2. Across the four markers, there were similar significant excesses of multilocus heterozygosity, and significant multilocus heterozygosity–growth correlations were recorded for both crosses at all stages. These results suggest that microsatellite markers, often assumed to be neutral, cosegregated with fitness-associated genes, the number of which is estimated to be between 15 and 38 in the whole genome, and that there is a potentially high genetic load in *Ostrea edulis* genome. This load provides a genetic basis for heterosis in marine bivalves.

MOST marine bivalves are characterized by high fecundity, large population sizes, external fertilization with broadcast spawning, and extensive larval dispersal. They should thus illustrate the canonical model of population genetics known as panmixia. Nevertheless, numerous studies of electrophoretic variation in natural populations have revealed a general trend toward heterozygote deficiencies relative to Hardy-Weinberg equilibrium (HWE; reviewed in Zouros and Foltz 1984; Blanc and Bonhomme 1986; Gaffney *et al.* 1990; Beaumont 1991). Just as puzzling is the observation sometimes made in the same species, that multilocus heterozygosity (MLH) for allozymes is positively correlated with fitness-related traits in juveniles or adults, such as growth, viability, or physiological traits (Zouros *et al.* 1980; Koehn and Shumway 1982; Koehn and Gaffney 1984; Hawkins *et al.* 1989; Gaffney *et al.* 1990; Zouros and Pogson 1994). Classically, the studies of bivalve genetics have discussed separately the departure from HWE on one side and the MLH–fitness correlation on the other side, trying to reconcile both afterwards. Various combinations of hypotheses have been put forward to account for one or the other of these apparently contradictory trends. These hypotheses refer to very different biological phenomena, such as the existence of typing artifacts, null alleles, partial aneuploidy, genomic imprinting, deleterious genes, population substructuring, or partial inbreeding.

Apart from electrophoretic problems, such as null alleles (Foltz 1986; Katoh and Foltz 1988; Gaffney 1994), two classes of arguments have been invoked to explain heterozygote deficiencies and heterozygosity–fitness correlations: (1) The first class involves selection acting directly on allozyme genotypes. Heterozygote deficiencies could be caused by selection against heterozygotes during the larval phase (Zouros and Foltz 1984; Mallet *et al.* 1985; Singh and Green 1984; Blanc and Bonhomme 1986; Hawkins *et al.* 1989; Gaffney 1990), whereas heterotic effects (MLH–fitness relationship) could be the result of direct overdominance at allozyme loci at the adult stage (Koehn and Shumway 1982; Zouros *et al.* 1983; Koehn *et al.* 1988; Mitton 1993; Zouros and Pogson 1994). These models consider that the metabolism of sessile invertebrates is an important element of their adaptation to the fluctuating external conditions, and that the neutral status of allozymes can thus be questioned; (2) On the other hand, the second class of arguments states that allozyme polymorphism is neutral but indirectly reflects variation at fitness loci through genetic correlations promoted by some deviations from HWE. These models, therefore, put emphasis on the reproductive biology and population dynamics of marine bivalves. Several independent observations have indeed challenged the idea that marine organisms occur in large, homogeneous, randomly mating popula-

Corresponding author: François Bonhomme, Laboratoire Génome et Populations, Station Méditerranéenne de l'Environnement Littoral, 1 quai de la Daurade, 34200 Sète, France.
E-mail: bonhomme@crit.univ-montp2.fr

[1] *Present address:* Institut de Zoologie et d'Ecologie Animale (IZEA), Université de Lausanne CH-1015 Lausanne/Dorigny, Switzerland.

tions. Large variation in reproductive success leading to effective population sizes several orders of magnitude below census numbers (Hedgecock 1994; Avise 1994), as well as recruitment variations caused either by food availability (Peterman and Bradford 1987) or by fluctuations in the physical transportation of larvae (Roughgarden *et al.* 1988), could create the conditions for local genetic differentiation between cohorts (David *et al.* 1997a,b). Heterozygote deficiencies could be directly generated by nonrandom mating or within-sample structure (Wahlund effect). Reproductive biology features, such as partial inbreeding, may also create indirect correlations between heterozygosity and fitness (Ohta 1971; Strobeck 1979; Charlesworth 1991; Zouros *et al.* 1980; Zouros 1993). These correlations may be of two kinds: (1) Small instantaneous effective population sizes may promote gametic disequilibria between marker loci and overdominant or deleterious recessive alleles. In this case, allozyme loci will reflect variation at dominant or overdominant fitness loci localized in their chromosomal vicinity (local effect). (2) Partial inbreeding may generate variation in overall genomic heterozygosity among individuals so that homozygosity at any locus will correlate with inbreeding depression (general effects; David *et al.* 1995). Direct overdominance at allozyme loci may certainly be considered as a local effect (the chromosomal vicinity being in this case reduced to the locus itself), which is therefore difficult to distinguish from associative overdominance caused by gametic disequilibria.

An in-depth review of the above-mentioned studies would reveal two things. First, that data on the genetic processes that happen during the larval and metamorphic phases (during which most of the developmental processes take place) are conspicuously absent, and second, that although dramatic inbreeding depression has been reported in the laboratory, the extent and distribution of genetic load, two crucial parameters to be tested to assess the pertinence of the "general effect" hypotheses, are still largely unexplored (but see review by David *et al.* 1995).

Screening the larval stage has not proven to be easily feasible using allozyme electrophoresis techniques (but see Hu *et al.* 1992), but PCR techniques afford a sufficiently sensitive method. Genotyping of mussel larvae (*Mytilus edulis*) was performed by Côrte-Real *et al.* (1994) on an intron-length polymorphism at a single locus to test the inheritance of this marker. In the same way, Hu and Foltz (1996) have characterized the inheritance of single copy nuclear DNA (scnDNA) polymorphism in the juvenile oysters *Crassostrea virginica*. If allozyme loci are the causative agents of the MLH–fitness correlation ("selection" hypothesis; Zouros and Pogson 1994), no relationship should be found for a set of unlinked, strictly neutral markers. An opposite trend should, however, be observed if allozyme loci act as markers of larger portions of the genome ("associative overdominance" hypothesis; Ohta 1971; Zouros *et al.* 1980). In the only study implying both type of markers available to date, Pogson and Zouros (1994) found an MLH–growth correlation with seven allozyme loci, while a set of eight nuclear RFLP loci failed to produce a significant correlation. As concluded by these authors, other analyses have to be performed before a general conclusion can be drawn. In the same study, heterozygote deficiencies were found, although smaller than generally observed, at the anonymous nuclear DNA markers. A similar result was also obtained for some scnDNA loci by Foltz and Hu (1996) in the American oyster *C. virginica*, although this was interpreted as artifactual by Hare *et al.* (1996).

The microsatellite loci recently developed in the flat oyster *Ostrea edulis* (Naciri *et al.* 1995) has allowed us to carry out a first study on controlled crosses in this species to address some of the questions mentioned above. The aim of the present work was to develop a multilocus analysis on single larvae to check for the existence of early heterozygote deficits and, at the same time, to test the occurrence of MLH–fitness correlation at an early stage of the life cycle. Preliminary assays of inheritance (Naciri *et al.* 1995) and unpublished data on natural populations of the flat oyster for two microsatellite loci suggested the occurrence of possible artifacts caused by PCR techniques and high mutation rates. We analyzed the segregation of these markers before and after settlement of progenies from controlled pair matings, which eliminates some of the alternative hypotheses proposed before. Because allozymic data on pair crosses have usually failed to exhibit an MLH–fitness correlation (Beaumont *et al.* 1983; Beaumont 1991; Gaffney and Scott 1984; Alvarez *et al.* 1989), and especially because the variability at microsatellite loci in natural populations of the flat oyster is such that it is nearly impossible to find multihomozygous genotypes in progenies of crosses between unrelated wild animals (unpublished data), we analyzed the larval progenies of two crosses between full sibs from a preceding wild intercross at four microsatellite loci.

Doing so allows us also to address the second gap in the literature concerning genetic load, since inbreeding at the fullsib level is expected to reveal the effect of homozygosity of quite large chromosomal fragments around each marker locus (Strauss 1986). Recent work on the Pacific oyster *C. gigas* (McGoldrick and Hedgecock 1997) has shown that fixation and segregation of allozyme markers in adults from inbred lines deviate from neutral expectations, revealing a strong and probably epistatic selection for viability. In the present study, we extend this analysis to early life history stages, using presumably neutral DNA markers. By measuring the sizes of larvae and scoring their genotype in samples taken at several time intervals, we test the occurrence of an MLH–growth correlation at this stage of the life cycle. Furthermore, we discuss from our results the in-

tensity of the genetic load and its genomic distribution, as well as the potential consequences of early larval selection on the observed genotypic distribution in natural populations.

MATERIAL AND METHODS

Pair matings: In March 1995, pair matings were obtained between wild animals of the French Atlantic coast near Quiberon Bay and animals from inbred lines that were selected for a resistance to *Bonamia ostreae* at the experimental IFREMER hatchery of La Tremblade (Charente Maritime, France). Since *O. edulis* is a larviparous species, fertilization takes place in the brood chamber of female oysters, and larvae are nursed for a whole week before being released into the seawater. For this reason, in vitro fertilization is very difficult to achieve. In February 1996, fullsibs from two different pair matings were placed by pair in 10 aquariums with a continuous flow of filtered seawater at 18°, a modified photoperiod (8–14 hr of light during the experiment duration), and a surplus system to collect larvae in 100-μm sieves. Oysters were induced to spawn by thermal shocks (up to 30°). Because of this technique, it was impossible to distinguish male from female.

Collection and treatment of larvae: For each cross, larvae were transferred to a 30-liter classical larval cylindrical growing pond just after spawning. A first sample of 96 larvae was then taken and preserved in pure alcohol (day 1). A second sample was taken at the end of the larval stage (day 10), before settlement. A last series of samples was obtained at the postlarval stage (day 70). Individual larvae (days 1 and 10) were measured on their largest diameter using a Nikon (Garden City; NY) profile projector and then collected in 15 μl alcohol using a micropipette (Gilson, Villiers-le-Bel, France) and transferred to 0.5-ml sterile microfuge tubes. Before being measured, spat (day 70) were also weighed with a precision scale. Since the shape of spat is usually uneven at this stage, weights were expected to be a better estimator of growth.

DNA extraction, PCR procedures, and electrophoresis: Because of the small amounts of tissue that were available, the Chelex extraction method (modified from Singer-Sam *et al.* 1989) was used. Alcohol was evaporated at room temperature and then 400 μl of 5% chelating resin (Chelex, Bio-Rad, Richmond, CA) and 5 μl of proteinase K were added to each sample tube. The mixtures were shaken and heated overnight in a stove at 55°, then vortexed, heated again at 100° for 15 min, vortexed a second time, and centrifuged at 10,000 *g* for 2 min. The supernatant was stored at −20° until it was used as a template for PCR. The same protocol was used for spat DNA extraction, but the supernatant was diluted 10 times in 5% Chelex. Five microliters of PCR mixture containing 0.5 μM of each primer (one labeled with ^{33}P), 2 mM $MgCl_2$, 0.2 mm of each dNTP, and 0.25 unit of Red Goldstar DNA polymerase (Eurogentec, Liège, Belgium) were added to 10 μl of DNA solution during the first 2 min–94° denaturing stage of the PCR program (hot start). Thirty PCR cycles (30 sec at 94° for 1 min at the optimum hybridization temperature, 1 min at 72°) were run in a Crocodile III thermocycler (Appligène, Strasbourg, France). PCR products were electrophoresed on 10% PAGE gels (acrylamide:bisacrylamide, 29:1, 7 M urea) using 0.5× TBE (Tris-borate EDTA) buffer. Results were visualized by autoradiography after exposing the dry gel overnight.

Amplifying two different loci during the same PCR reaction can sometimes generate artifacts such as null alleles. Loci were therefore amplified individually in this study. Electrophoresis of two or more loci differing in size, however, were performed. Four loci [namely *Oedu*.B0 (Naciri *et al.* 1995), *Oedu*.J12, *Oedu*.O9, and *Oedu*.T5 (S. Launey, unpublished data)] among the five loci tested were used in this study.

Segregation distortions: We observed two types of segregations. The first type corresponds to the case where both parents were heterozygous for the same two alleles, and the second one corresponds to the case where both parents shared only one or no allele in common. First, we analyzed genotypic proportions using G tests for goodness-of-fit: we tested for a 1:2:1 ratio in type 1 segregation and for a 1:1:1:1 ratio in type 2 segregation. In the case of type 2 loci, we were also able to test the deviation from a 1:1 segregation of alleles in each parent. Heterogeneity G test (Gh) values from a replicated G test were calculated to examine temporal heterogeneity for both genotypic or allelic proportions (Sokal and Rohlf 1996). When no significant heterogeneity was found across sampling times, data were pooled to obtain a more powerful test, the pooled G test (Gp test; Sokal and Rohlf 1996). The significance levels of G, Gh, and Gp tests were Bonferroni adjusted. When contrasting results were obtained for Gh or Gp tests, Fisher's procedure was used to combine probabilities at each locus to test for overall significance (Sokal and Rohlf 1996).

Linkage disequilibrium: Linkage between loci was examined by calculating the recombination rate (θ) for the most probable parental allelic association, and θ was tested against the null hypothesis θ = 0.5 by a two-tailed binomial exact test.

Single-locus heterozygosities: Deviations from the expected heterozygote frequencies were estimated as:

$$D = H_{obs} / H_{exp} - 1, \quad (1)$$

where H_{obs} is the number of observed heterozygotes, and H_{exp} is the number of expected heterozygotes under Mendelian segregation). *D* values were tested using G test for goodness-of-fit to a 1:1 ratio for type 1 crosses and to a 1:3 ratio for type 2 crosses. Once again, if no significant temporal heterogeneity was found using a Gh test, data were pooled and analyzed using a Gp test.

Allele frequencies in the natural population: Microsatellite allele frequencies were obtained for a natural population from Quiberon Bay by S. Launey (unpublished data). These data allowed us to test whether there was a link between homozygote deficiencies in the two crosses and allele frequencies in natural populations, as suggested by McGoldrick and Hedgecock (1997) on allozyme analyses.

MLH distribution: Expected MLH distributions were computed on the basis of expected single-locus heterozygosities. Observed and expected distributions were compared using a G test for goodness-of-fit and were tested for temporal heterogeneity (Gh test).

MLH–growth correlations: To facilitate comparisons among samples, larval shell lengths and $\log_e$ (spat weights) were expressed in standard deviation units from the sample mean (standardized deviates). Locus-specific effects of heterozygosity on growth were expressed as:

$$d = M_{SHet} - M_{SHom}, \quad (2)$$

where M_{SHet} and M_{SHom} are the mean standardized sizes of heterozygotes and homozygotes respectively (Gaffney 1990). The effect of heterozygosity *vs.* homozygosity was tested using a one fixed effect model. *P* values for the different tests were combined stage by stage according to Fishers procedure. Each sample was sorted by size and grouped into size quartiles, ranging from smallest (quartile I) to largest (quartile IV), according to the method of Gaffney (1990). MLH was plotted on size quartiles, and the relationship between the two parameters was expressed as a product–moment correlation.

RESULTS

Spawning and choice of crosses: Five parental pairs effectively produced offspring out of the 10 that were monitored. Parents were genotyped at *Oedu*.B0, *Oedu*.J12, *Oedu*.O9, and *Oedu*.T5 microsatellite loci. The parents of cross C1 had at least one allele in common at the four loci, and the parents of cross C2 had at least one allele in common at three out of the four loci. These two crosses were chosen because they had the largest possible number of MLH ranks in their progenies. The difficulty of finding alleles in common between full sibs suggests that the polymorphism of microsatellite loci in the original population is high.

PCR results: Ninety-six four-locus analyses were performed at each stage for the two crosses, and an average yield of 80% was obtained (cross C1 day 10: 86%, day 70: 78%; cross C2 day 1: 78%, day 10: 80%, day 70: 80%). Most of the remaining 20% individuals amplified no loci, and only very few PCR amplified one, two, or three loci. The DNA yield from a single individual was enough to perform up to 20 amplifications at day 1, 40 at day 10, and >1000 at day 70.

Linkage analysis: A recombination rate significantly different from 0.5 is observed between loci *Oedu*.O9 and *Oedu*.J12 (cross C1 day 10: $\theta = 0.13$, $P < 10^{-5}$; day 70: $\theta = 0.31$, $P < 10^{-5}$; cross C2 day 10: $\theta = 0.42$, $P = 0.023$; day 70: $\theta = 0.34$, $P < 10^{-5}$). Note the surprising lower recombination rate for cross C1 at day 10, a stage that exhibits Mendelian proportions according to G tests. Linkage can bias the MLH distribution study because it modifies the expected number of double homozygotes and double heterozygotes. This was not a problem for cross C2, however, because locus *Oedu*.J12 exhibits no homozygotes and was not taken into account for the MLH study. For cross C1, the linkage gives rise to an equal excess of double heterozygotes and double homozygotes, so effect on the MLH distribution is not a nuisance. Tests on MLH were also performed for C1 without *Oedu*.O9 and without *Oedu*.J12, yielding similar results.

Segregation analyses: *Cross C1:* Segregation analyses are presented in Table 1. Unfortunately, the day 1 sample was not available, so the earliest results are for day 10. At this stage, no significant departure from Mendelian expectation was observed at the experimentwise level ($\alpha = 0.00625$) although *Oedu*.T5 showed a significant result at the single-test level ($P = 0.0117$, Table 1A). At day 70, three loci (*Oedu*.O9, *Oedu*.B0, and *Oedu*.J12) showed significant departures from Mendelian expectations at the single-test level ($\alpha = 0.05$), but only *Oedu*.J12 remained significant at the experimentwise level. No temporal heterogeneity between stages was detected using Gh tests for the four loci ($P > 0.05$). As a result, data were pooled, and Gp tests were all significant at the 5% level, and two of them remained significant at the experimentwise level ($\alpha = 0.0125$, *Oedu*.O9 and *Oedu*.T5). Gp-associated probabilities were combined following Fisher's procedure to test the overall significance of the departure from Mendelian expectation. Because *Oedu*.O9 and *Oedu*.J12 were shown to be linked, the test was computed on three loci, excluding the one or the other locus. Both tests were significant at the 0.1% level, indicating that the null hypothesis of Mendelian proportions over stages and loci can be rejected firmly. The large polymorphism of microsatellites allowed us to test for allelic proportions in the two parents for three out of the four loci (Table 1B, *Oedu*.O9, *Oedu*.B0, and *Oedu*.T5). For parent 1 at day 10, an experimentwise-significant departure from the 1:1 ratio was observed for *Oedu*.T9 ($P = 0.0023$). At day 70, significant segregation distortions were observed for *Oedu*.O9 and *Oedu*.B0 ($\alpha = 0.05$), but the distortion remained significant at the experimentwise level ($\alpha = 0.0167$) for the latter locus only. Since no heterogeneity was detected between stages (Gh, $P > 0.05$), data were pooled, and the three Gp tests appeared to be significant at the experimentwise level ($\alpha = 0.0167$). On the other hand, parent 2 showed no significant departure from a 1:1 ratio ($P > 0.05$ for G, Gh, and Gp tests).

Cross C2: Segregation analyses are presented in Table 2. Unfortunately, *Oedu*.J12 was not amplified at day 1 for technical reasons. No significant departure from Mendelian proportions was detected at day 1 ($P > 0.05$). At day 10, *Oedu*.O9 and *Oedu*.B0 showed significant departures from Mendelian expectation at the single-test level, but none of them remained significant at the experimentwise level ($\alpha = 0.0045$, $P = 0.0153$ and 0.0241, respectively). At day 70, the same two loci showed significant departures from Mendelian proportions, but only *Oedu*.B0 remained significant at the experimentwise level ($\alpha = 0.0045$, $P = 0.0271$ and 5.5×10^{-5}, respectively). Although some differences were observed between stages for at least two loci, only Gh for *Oedu*.B0 was significant ($\alpha = 0.0125$, $P = 0.0006$). Data were pooled for the three other loci, and *Oedu*.O9 showed an overall and significant departure from Mendelian proportions at the experimentwise level ($\alpha = 0.0167$, $P = 0.0003$). Allele segregation was tested in both parents for *Oedu*.O9, *Oedu*.T5, and *Oedu*.J12 (Table 2B). No significant departure from a 1:1 ratio was detected in both parents, except for parent 2 at day 10 and *Oedu*.J12 locus, for which the G test was significant at the single-test level ($P = 0.0433$), but no more significant at the experimentwise level ($\alpha = 0.00625$). No heterogeneity was found between stages, and Gp tests remained nonsignificant in any case for both parents.

Heterozygotes excesses and MLH distributions *Cross C1:* Heterozygote excesses are present at all loci for cross C1 because D is always positive (Table 3). These excesses are not significant at day 10 according to G tests, but they become significant later on at the experimentwise level ($\alpha = 0.00625$) for loci *Oedu*.O9 and *Oedu*.J12, and significant at the single-test level ($\alpha = 0.05$) for the

TABLE 1

Segregation statistics for cross C1

A. Analysis of genotypic proportions

Loci	Progeny genotypes	Day 10 (n = 86) Genotype frequencies	Day 10 (n = 86) G test	Day 70 (n = 78) Genotype frequencies	Day 70 (n = 78) G test	Global analyses: Temporal heterogeneity	Global analyses: Pooled test
Oedu.09	*158/158*	0.198		0.115			
	158/160	0.221	G = 3.122	0.256	G = 10.468	Gh = 2.16	Gp = 11.43
	168/158	0.325	NS	0.359	P = 0.015	NS	***P*** = **0.0096**
	168/160	0.256		0.270			
Oedu.B0	*098/098*	0.198		0.154			
	098/101	0.244	G = 1.671	0.192	G = 7.859	Gh = 1.584	Gp = 7.946
	096/098	0.267	NS	0.321	P = 0.049	NS	P = 0.0471
	096/101	0.291		0.333			
Oedu.T5	*124/124*	0.198		0.128			
	124/128	0.139	G = 11.013	0.308	G = 7.316	Gh = 7.35	Gp = 10.979
	106/124	0.372	P = 0.0117	0.295	NS	NS	***P*** = **0.0118**
	106/128	0.291		0.269			
Oedu.J12	*224/224*	0.198		0.218			
	230/230	0.244	G = 1.587	0.115	G = 11.337	Gh = 4.707	Gp = 8.217
	224/230	0.558	NS	0.667	***P*** = **0.0034**	**NS**	P = 0.0164

B. Analysis of allelic proportions for type 2 crosses

Loci	Parent P1: Day 10 (n = 86)	Parent P1: Day 70 (n = 78)	Parent P1: Temporal heterogeneity	Parent P1: Pooled test	Parent P2: Day 10 (n = 86)	Parent P2: Day 70 (n = 78)	Parent P2: Temporal heterogeneity	Parent P2: Pooled test
	158/168				*158/160*			
Oedu.09	SD = 0.16	SD = 0.26			SD = 0.05	SD = 0.05		
	G = 2.289	G = 5.186	Gh = 0.375	Gp = 7.1	G = 0.186	G = 0.205	Gh = 0.391	Gp = 0
	NS	P = 0.0228	NS	***P*** = **0.0077**	NS	NS	NS	NS
	096/098				*098/101*			
Oedu.B0	SD = 0.12	SD = 0.31			SD = 0.07	SD = 0.05		
	G = 1.165	G = 7.506	Gh = 1.571	Gp = 7.1	G = 0.419	G = 0.205	Gh = 0.624	Gp = 0.610
	NS	***P*** = **0.0062**	NS	***P*** = **0.0077**	NS	NS	NS	NS
	106/124				*124/128*			
Oedu.T5	SD = 0.33	SD = 0.13			SD = 0.14	SD = 0.15		
	G = 9.285	G = 1.289	Gh = 1.689	Gp = 8.885	G = 1.68	G = 1.854	Gh = 3.534	Gp = 0
	P = **0.0023**	NS	NS	***P*** = **0.0029**	NS	NS	NS	NS

n, number of individuals analyzed; P, probability to accept the null hypothesis (type I error); NS, not significant at $\alpha = 5\%$; probabilities in bold are significant at the experimentwise level (A: $\alpha = 0.00625$ for genotype frequencies at days 10 and 70, $\alpha = 0.0125$ for global analyses, B: $\alpha = 0.0083$ for allelic frequencies at days 10 and 70, and $\alpha = 0.0167$ for global analyses); SD, segregation deviation [(allele observed frequency − 0.5)/0.5].

TABLE 2

Segregation statistics for cross C2

A. Analysis of genotypic proportions

Loci	Progeny genotypes	Day 1 (n = 78) Genotype frequencies	Day 1 (n = 78) G test	Day 10 (n = 80) Genotype frequencies	Day 10 (n = 80) G test	Day 70 (n = 80) Genotype frequencies	Day 70 (n = 80) G test	Global analyses Temporal heterogeneity	Global analyses Pooled test
Oedu.09	*168/168*	0.192		0.112		0.125			
	168/158	0.295	G = 2.149	0.325	G = 10.427	0.275	G = 9.173	Gh = 2.946	Gp = 18.803
	150/168	0.282	NS	0.313	P = 0.0153	0.350	P = 0.0271	NS	$\mathbf{P = 0.0003}$
	150/158	0.231		0.250		0.250			
Oedu.B0	*098/098*	0.295		0.187		0.312			
	101/101	0.282	G = 1.876	0.163	G = 7.445	0.063	G = 19.609	Gh = 19.445	––
	098/101	0.423	NS	0.65	P = 0.0241	0.625	$\mathbf{P = 5.5 \times 10^{-5}}$	$\mathbf{P = 0.0006}$	
Oedu.T5	*124/124*	0.192		0.250		0.262			
	124/128	0.282	G = 2.149	0.313	G = 2.067	0.188	G = 2.627	Gh = 6.388	G = 0.455
	106/124	0.231	NS	0.237	NS	0.313	NS	NS	NS
	106/128	0.295		0.200		0.237			
Oedu.J12	*224/230*			0.350		0.275			
	224/246	—	—	0.225	G = 5.898	0.212	G = 2.685	Gh = 2.283	G = 3.3
	232/230			0.262	NS	0.200	NS	NS	NS
	232/246			0.163		0.313			

B. Analyses of allelic proportions for type 2 crosses

Loci	Parent P1 Day 1 (n = 78)	Parent P1 Day 10 (n = 80)	Parent P1 Day 70 (n = 80)	Parent P1 Temporal heterogeneity	Parent P1 Pooled test	Parent P2 Day 1 (n = 78)	Parent P2 Day 10 (n = 80)	Parent P2 Day 70 (n = 80)	Parent P2 Temporal heterogeneity	Parent P2 Pooled test
		168/150					*168/158*			
Oedu.09	SD = 0.02	SD = 0.12	SD = 0.2			SD = 0.05	SD = 0.15	SD = 0.05		
	G = 0.051	G = 1.253	G = 3.222	Gh = 1.224	Gp = 3.302	G = 0.205	G = 1.807	G = 0.2	Gh = 0.529	Gp = 1.683
	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		124/106					*124/128*			
Oedu.T5	SD = 0.05	SD = 0.12	SD = 0.1			SD = 0.15	SD = 0.02	SD = 0.15		
	G = 0.205	G = 1.253	G = 0.801	Gh = 2.242	Gp = 0.017	G = 1.854	G = 0.05	G = 1.807	Gh = 3.694	Gp = 0.017
	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		224/232					*230/246*			
Oedu.J12		SD = 0.15	SD = 0.02				SD = 0.23	SD = 0.05		
	—	G = 1.807	G = 0.05	Gh = 1.232	Gp = 0.625	—	G = 4.085	G = 0.2	Gh = 3.058	Gp = 1.227
		NS	NS	NS	NS		P = 0.0433	NS	NS	NS

n, number of individuals analyzed; P, probability to accept the null hypothesis (type I error); NS, not significant at α = 5%; probabilities in bold are significant at the experimentwise level (A:α = 0.00045 for genotype frequencies at days 1, 10, and 70, α = 0.0125 for heterogeneity tests, and α = 0.0167 for global tests, B: α = 0.00625 for allelic frequencies at days 1, 10, and 70, and α = 0.0167 for global tests); SD, segregation deviation [(allele observed frequency − 0.5)/0.5]; —, nonavailable data; ––, test not possible.

TABLE 3

Homozygotes vs. heterozygotes: analysis of single-locus heterozygote proportions

	Cross C1				Cross C2			
Loci	Day 10 (n = 86)	Day 70 (n = 78)	Temporal heterogeneity	Pooled test	Day 1 (n = 78)	Day 10 (n = 80)	Day 70 (n = 80)	Temporal heterogeneity	Pooled test
Oedu.09	D = 0.07	D = 0.179			D = 0.077	D = 0.183	D = 0.167		
	G = 1.322	G = 8.864	Gh = 2.112	Gp = 8.074	G = 1.467	G = 9.530	G = 7.718	Gh = 2.288	Gp = 16.427
	NS	**P = 0.0029**	NS	**P = 0.0045**	NS	**P = 0.002**	**P = 0.0055**	**NS**	**$P = 5.1 \times 10^{-5}$**
Oedu.B0	D = 0.07	D = 0.128			D = −0.154	D = 0.3	D = 0.25		
	G = 1.322	G = 4.271	Gh = 0.543	Gp = 5.05	G = 1.854	G = 7.312	G = 5.053	Gh = 9.903	
	NS	P = 0.0388	NS	P = 0.0246	NS	P = 0.0068	P = 0.0246	**P = 0.0071**	— —
Oedu.T5	D = 0.07	D = 0.162			D = 0.077	D = 0	D = −0.017		
	G = 1.322	G = 7.109	Gh = 1.452	Gp = 6.979	G = 1.4667	G = 0	G = 0.066	Gh = 1.255	Gp = 0.278
	NS	P = 0.0077	NS	**P = 0.0082**	NS	NS	NS	NS	NS
Oedu.J12	D = 0.116	D = 0.333							
	G = 1.165	G = 8.835	Gh = 2.033	Gp = 7.967	—	—	—	— —	
	NS	**P = 0.003**	NS	**P = 0.0048**					

n, number of individuals analyzed; D, [(number of observed heterozygotes/number of expected heterozygotes) − 1]; P, probability to accept the null hypothesis (type I error); NS, not significant at α = 5%; probabilities in bold are significant at the experimentwise level (A:α = 0.00625 for heterozygote frequencies at days 10 and 70, α = 0.0125 for global tests, B:α = 0.00556 for heterozygotes frequencies at days 1, 10, and 70, and α = 0.0167 for global tests; —, nonavailable data; — —, test not possible.

d.f. = 2, P = 0.012) but not between days 10 and 70 (Gh = 0.718, d.f. = 2, P = 0.6983).

Heterozygosity–growth correlation: Figure 2 presents the MLH–size relationship. For each cross and at each stage, individuals ranking in higher size quartiles show higher MLH. Product—moment correlations are all significant and consistent over time in each cross (cross 1 day 10: r = 0.51, d.f. = 82, $P < 0.001$; day 70: r = 0.52, d.f. = 74, $P < 0.001$; cross 2 day 1: r = 0.46, d.f. = 74, $P < 0.001$; day 10: r = 0.37, d.f. = 78, $P < 0.001$; day 70: r = 0.39, d.f. = 78, $P < 0.001$). Results on single-locus comparisons between heterozygote and homozygote growth (Tables 4) are more puzzling because they lack consistency. Nevertheless, d, which corresponds to the mean standardized growth of heterozygotes minus the mean standardized growth of homozygotes, is always positive and significantly different from zero in four out of eight cases in cross C1, whereas it is positive, except for *Oedu*.T5 at day 10, and significantly different from zero in six out of nine cases in cross C2 (Table 4). In both crosses and from day 1 to day 70, Fisher's procedure showed that over all loci, heterozygotes were significantly larger than homozygotes (cross 1 day 10: χ^2 = 31.5, d.f. = 8, P = 0.0001; day 70: χ^2 = 26.9, d.f. = 8, P = 0.0007; cross 2 day 1: χ^2 = 16.1, d.f. = 6, P = 0.0132; day 10: χ^2 = 19.6, d.f. = 6, P = 0.0033; day 70: χ^2 = 24.8, d.f. = 6, P = 0.0004).

At the genotypic level, it is impossible to find any evidence for significant differences in growth between the various heterozygotes for type 2 segregations (three heterozygous and one homozygous genotypes in the progeny). For type 1 segregations (*Oedu*.J12 in cross C1 and *Oedu*.B0 in cross C2), it is interesting to compare the two homozygous genotypes (Figure 3). Homozygotes are always smaller than heterozygotes, and one of the two homozygotes is smaller than the other. One-tailed t tests were performed and pooled between stages using Fisher's method for combining independent test results, showing for cross C1 that the only significant difference in size is found for *224/230* and *230/230* genotypes (*224/230 vs. 230/230*: χ^2 = 24.02, $P = 7.9 \times 10^{-5}$; *224/224 vs. 224/230*: χ^2 = 8.89, P = 0.064; *224/224 vs. 230/230*: χ^2 = 8.51, P = 0.074). For cross C2, all the differences in size are found significant (*098/098 vs. 098/101*: χ^2 = 10.06, P = 0.04; *098/101 vs. 101/101*: χ^2 = 15.33, P = 0.004; *098/098 vs. 101/101*: χ^2 = 12.39, P = 0.014).

Allele-specific biases: Homozygotes for two different alleles were found for one locus in each cross (*Oedu*.J12 for cross C1 and *Oedu*.B0 for cross C2; Tables 1 and 2). Allele frequencies for these two loci in the natural population are illustrated in Figure 4 (S. Launey, unpublished data). A bias against both types of homozygotes (*224/224* and *230/230* for *Oedu*.J12, *099/099* and *102/102* for *Oedu*.B0) was observed in both cases (see Tables 1A and 2A for viability and Figure 3 for growth).

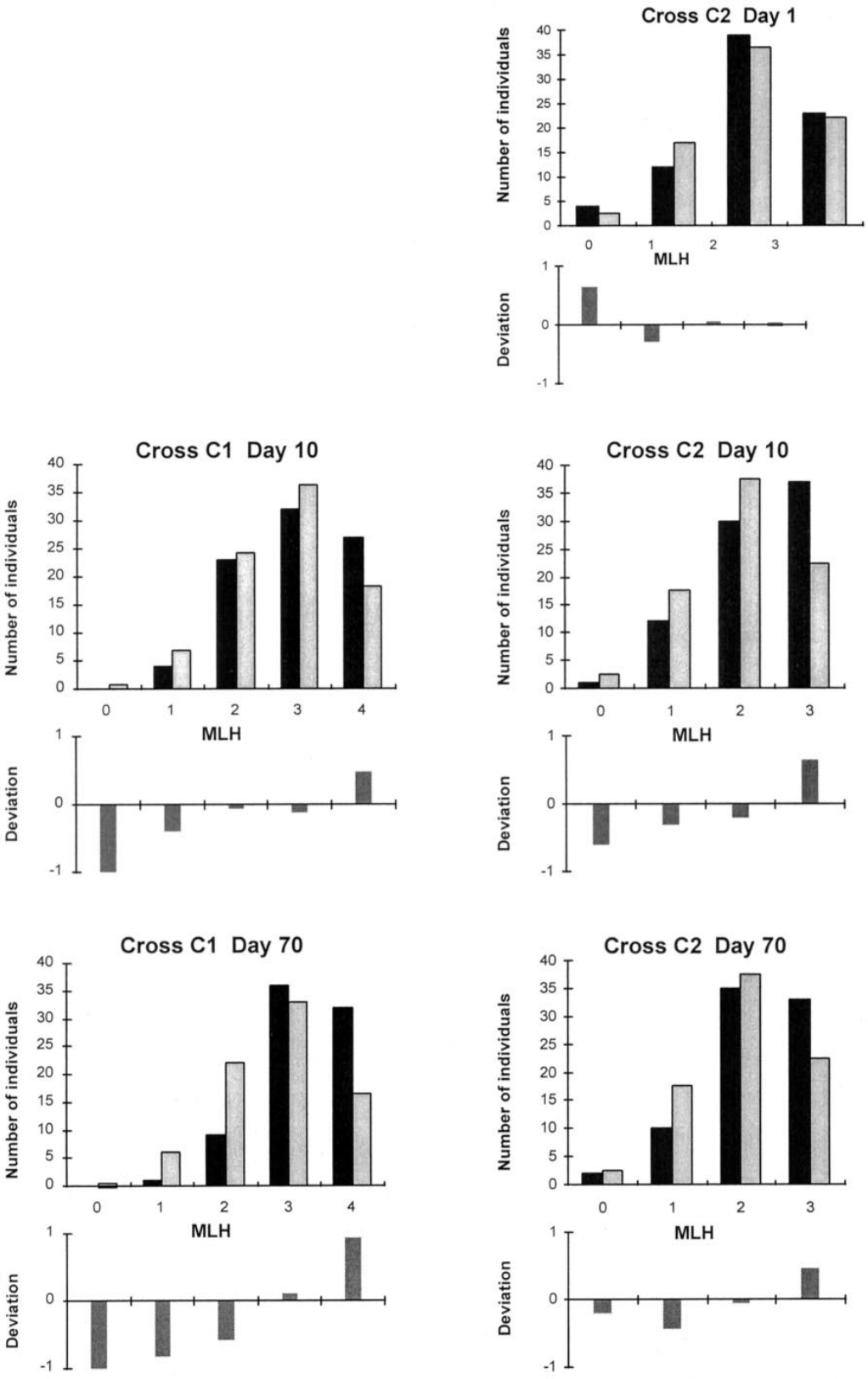

Figure 1.—*O. edulis.* Observed (in dark) and expected (in light) multilocus heterozygosity distributions (MLH, number of heterozygous loci per individual) for crosses C1 and C2 at days 1, 10, and 70 after spawning. Deviations, corresponding to [(observed heterozygosity − expected heterozygosity)/expected heterozygosity], are presented under each distribution.

The bias was stronger and significant for the less common allele (*230*) at locus *Oedu*.J12, but on the contrary, it was stronger for the more common allele (*099*) at locus *Oedu*.B0. For *Oedu*.T5 and *Oedu*.O9 loci, there was no observed tendency towards a bias for the allele of the homozygote genotype to be rare in the natural population.

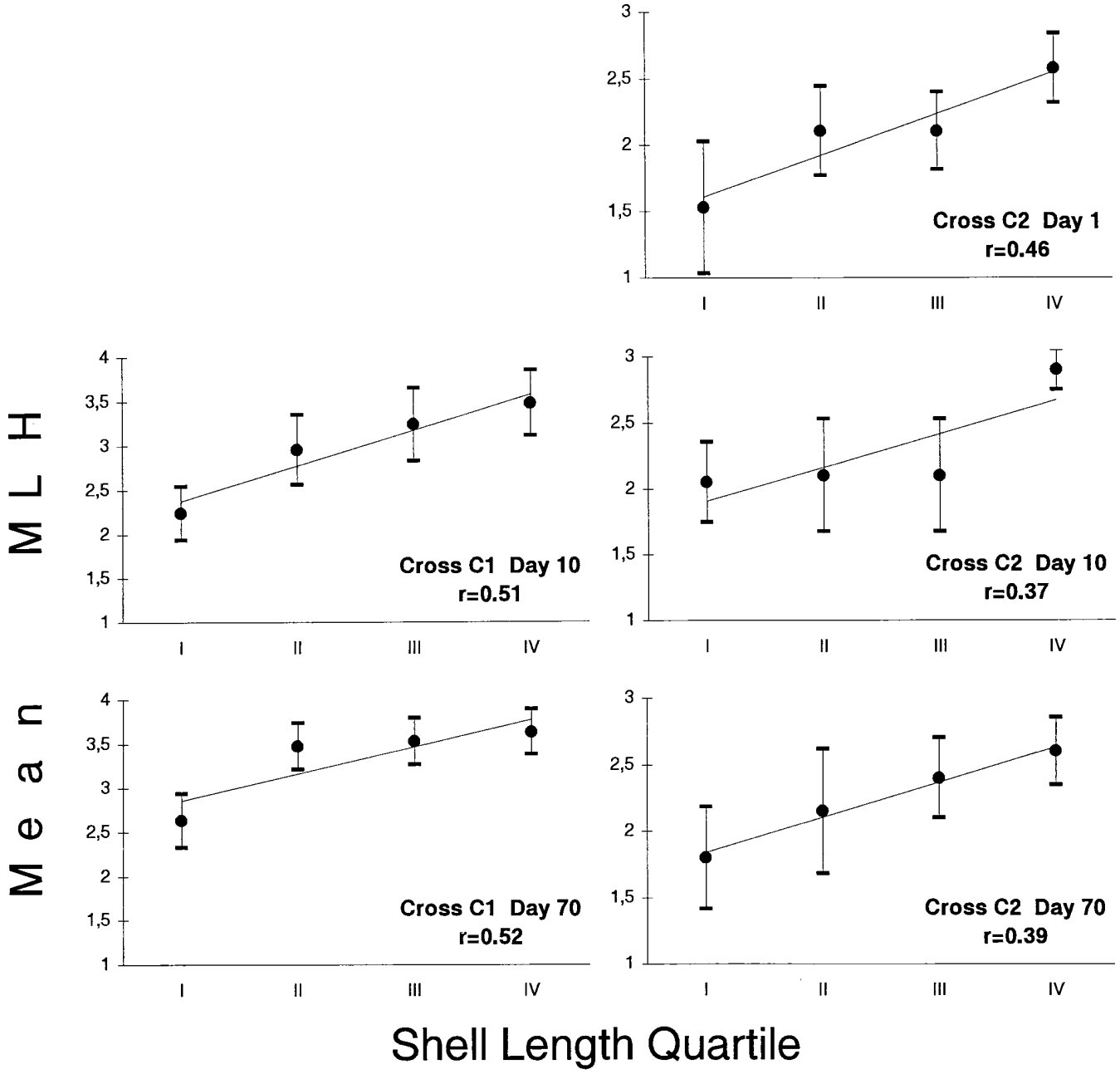

Figure 2.—*O. edulis*. Mean heterozygosity (number of heterozygous loci, 95% C.I.), in size quartiles for crosses C1 and C2 at days 1, 10, and 70 after spawning. Linear tendency curves are plotted, and *r* values are moment–product correlations. NS $P > 0.05$, * $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, and *** $P < 0.001$.

DISCUSSION

Our work is one of the first multilocus analyses performed on single bivalve larvae. Hu *et al.* (1992) were the first to have genotyped single *C. virginica* larvae using an allozymic microelectrophoretic method. Only one polymorphic locus (*Pgi*), however, has been routinely scored in their study. More recently, Hu and Foltz (1996) have tested the Mendelian inheritance of a set of polymorphic scnDNA markers in the same species, and Foltz and Hu (1996) have subsequently used them to genotype natural populations of larvae and postlarvae. ScnDNA polymorphisms are anonymous nuclear sequences amplified by PCR (followed by restriction enzyme digestion) that are supposed to be neutral so that they share some characteristics in common with microsatellites. The method we used in this study is straightforward and gives the possibility of amplifying up to 20 loci per larva as small as 160 μm. This allowed us to follow genotypic frequencies at different stages of the early life cycle of *O. edulis* and to determine at which stage Mendelian expectations are observed, and hence, to detect when potential selection effects take place.

Absence of early heterozygote deficiencies: For cross C1, a Mendelian segregation was observed at the end

TABLE 4

Growth comparison between single-locus heterozygotes and homozygotes

Loci	Day 1	Day 10	Day 70
Cross 1	—	$n = 86$	$n = 78$
Oedu.09	—	$d = 0.090^{NS}$	$d = 0.358^{**}$
Oedu.B0	—	$d = 0.022^{NS}$	$d = 0.017^{NS}$
Oedu.T5	—	$d = 0.034^{**}$	$d = 0.309^{**}$
Oedu.J12	—	$d = 0.036^{***}$	$d = 0.143^{NS}$
Cross 2	$n = 78$	$n = 80$	$n = 80$
Oedu.09	$d = 0.027^{*}$	$d = 0.099^{**}$	$d = 0.028^{NS}$
Oedu.B0	$d = 0.006^{NS}$	$d = 0.07^{**}$	$d = 0.129^{*}$
Oedu.T5	$d = 0.026^{*}$	$d = -0.003^{NS}$	$d = 0.186^{***}$

n, number of individuals analyzed; *d*, mean standardized growth of heterozygotes − mean standardized growth of homozygotes.

Significance of Fisher satistics of analysis of variance: ^{NS}not significant at $\alpha = 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

of the larval stage (day 10). Heterozygote excesses were, however, detected subsequently (day 70), especially for *Oedu*.J12. Temporal heterogeneity was not detected at the single-locus level, but was detected at the multilocus level (Figure 1). Hence, selection occurred between these two stages and also possibly before, *i.e.*, during settlement (metamorphosis and very early juvenile stage). For cross C2, departure from Mendelian expectations was already present at the end of the larval stage (day 10), when a single test level of significance was considered, but not at day 1. Thus, selective effects certainly occurred earlier than in cross C1, during the free larval stage (see results with locus *Oedu*.B0 and the two-locus MLH distribution analysis of heterogeneity). A strong selection was thus detected during the early stage of the life cycle, during the larval stage, and through settlement, *i.e.*, before juveniles reach the size at which allozymes can be used, with a minimal mortality differential, expressed as the percentage of homozygotes dying for genetic reasons, on the order of 25–50%.

Foltz and Hu (1996) analyzed 400 wild-caught larvae

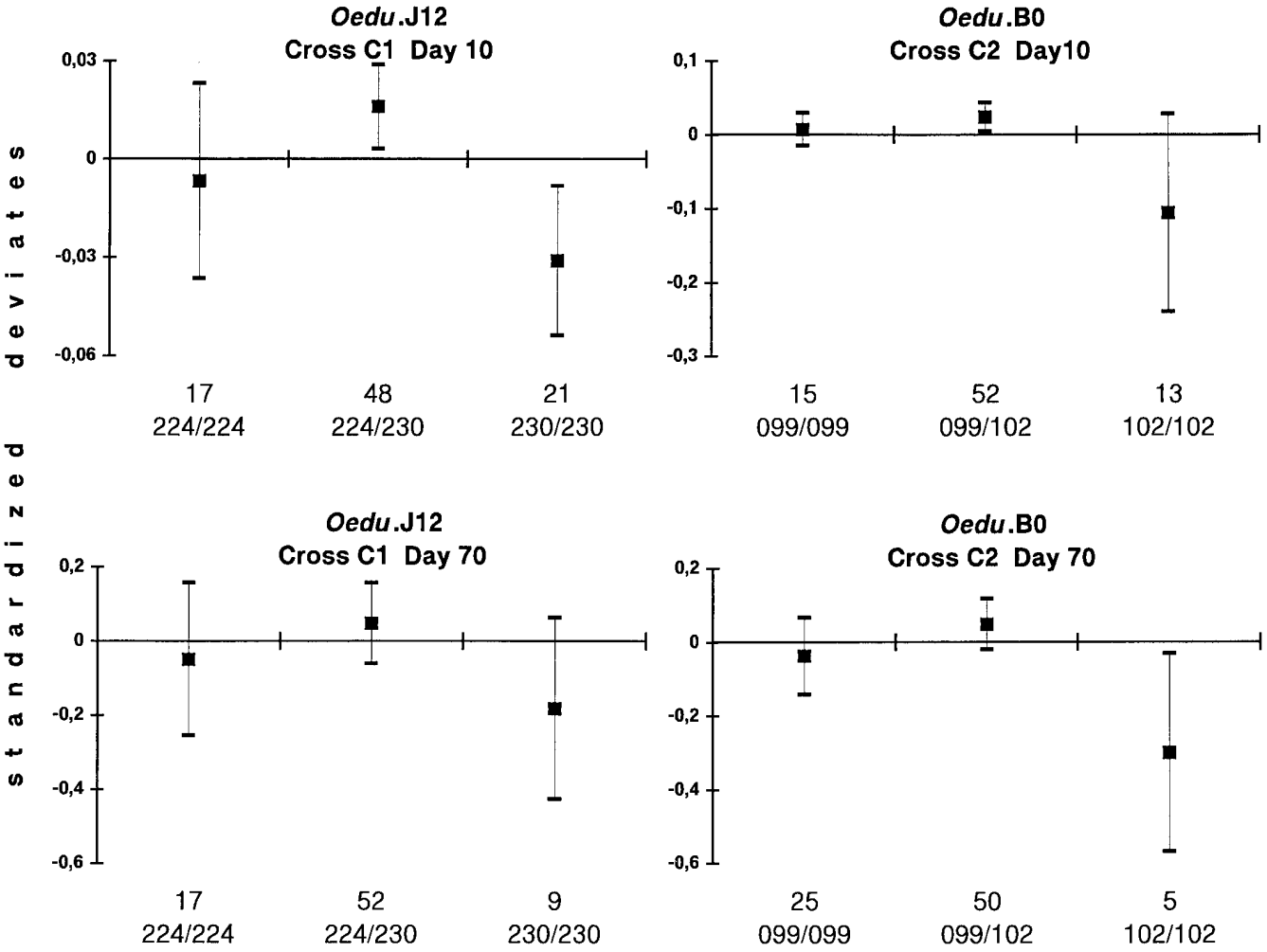

Figure 3.—*O. edulis*. Standardized deviates (95% C.I.) for type 1 segregation (one heterozygous and two homozygous genotypes in the progeny) for crosses C1 and C2 at days 10 and 70, using *Oedu*.B0 or *Oedu*.J12 microsatellite markers. The genotypes and the number of individuals per genotype are indicated at the bottom of each graph.

and postlarvae for five scnDNA markers and observed a tendency toward heterozygote deficiencies already detected at the premetamorphic stage, but no up- or downward trend for the deficiencies between the two stages. In our case, however, we did not find heterozygote deficiencies; on the contrary; our sequential results show that heterozygotes may have an advantage in larval life. This observation, that of Hu and Foltz (1996) on crosses between unrelated individuals, and that of Hedgecock et al. (1996), who observed heterosis in hybrid larvae, rule out the "biphasic selection" at nonenzyme loci, a family of hypotheses that envision a selection in favor of homozygotes during the larval phase, followed by selection in favor of heterozygous juveniles.

Correlations with fitness-associated genes: All loci exhibited significant heterozygote excesses on pooled data in C1 (three at the experimentwise level and one above the single-test level, Table 3), and so did two out of three loci in C2 as early as day 10. Under the hypothesis that microsatellite loci are neutral, these results suggest that these markers cosegregate with fitness-associated genes (FAGs), whether the latter are true overdominant loci or deleterious recessives. No such phenomenon has been recorded in pair crosses between unrelated animals in allozyme studies (Beaumont *et al.* 1983; Beaumont 1991; Gaffney and Scott 1984; Adamkewicz *et al.* 1984; Foltz and Chatry 1986; Mallet *et al.* 1986; Alvarez *et al.* 1989). In sib crosses, however, the probability of homozygosity around a marker which is itself in homozygous condition is increased. Strauss (1986) estimated the fraction of the genome marked for identity by descent under selfing in *Pinus attenuata*. He demonstrated that M, the mean chromosome length

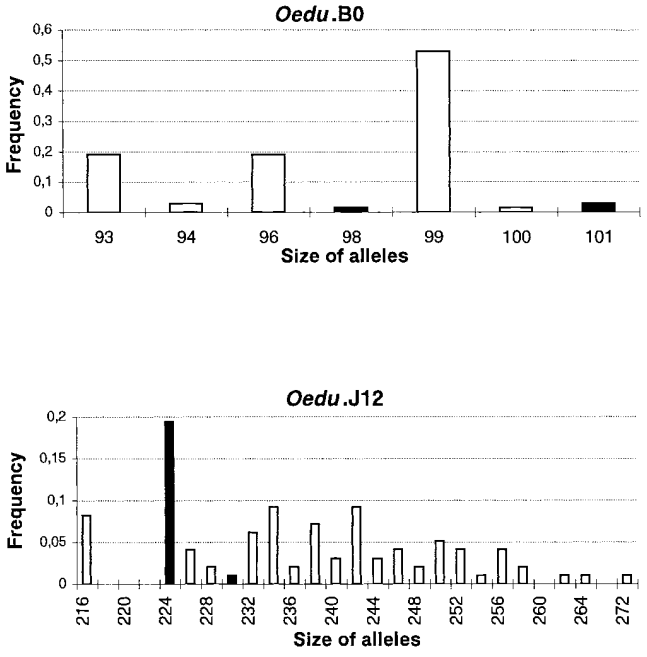

Figure 4.—Allele frequencies for *Oedu*.B0 and *Oedu*.J12 in a natural population of *O. edulis* from Quiberon Bay.

associated with a marker, is a function of Y, the mean number of chiasmas per bivalent. $M = L[(1/Y) - (e^{-Y}/Y)]$, where L is the total length (in map unit) of the average chromosome. In sibcrosses, pieces of chromosomes showing identity by descent come from grandparents, and two generations have to be taken into account. This doubles the mean number of chiasmas so that $M = L[(1/2Y) - (e^{-2Y}/2Y)]$. Knowing that the haploid chromosome number is 10 in *O. edulis*, the total genome size is 10 L, and then F_{sc}, the fraction of the genome marked by each locus in a sibcross, is $F_{sc} = [(1/2Y) - (e^{-2Y}/2Y)]/10$. Usually, $1 < Y < 2.5$ and then $2\% < F_{sc} < 4.3\%$ of the total genome.

In cross C1, whose offspring were analyzed at four microsatellite loci, at least three and probably four of them showed a higher viability of heterozygotes, suggesting that between three and four survival-associated genes lie within 8–17% of the genome, which is a total number of such genes comprised between 17 and 38. In cross C2, where two out of three markers showed the same phenomenon, the estimates drop to 15–33 FAGs, which is still very high. Even though overdominance effects cannot be distinguished from deleterious recessive effects at this stage, the above figures point to a potentially high genetic load in *O. edulis*. Alternatively, we may have underestimated the percent of total genome marked by each microsatellite locus. This could be the case if the number of recombinationally effective crossovers per chromosome were smaller than suspected, or if crossovers were primarily concentrated in recombinational hot spots. Intriguing results that support the latter hypothesis have been published recently for the bivalve *Mulinia lateralis* (Guo and Allen 1996), where it appears that a single recombinational hot spot is present in the proximal region of each chromosome arm.

To further discriminate between the hypothesis of the high number of independently segregating FAGs and that of segregation of mostly very large chromosomal fragments, we can ask whether or not the observed variance in fitness fits the assumed number of FAGs. This should be weak if many FAGs were implied and stronger if the genome of the grandparents were inherited in large blocks. The intensity of the MLH–fitness correlation is a good estimate of this variance. We do find such a correlation for survival. The better viability of heterozygotes is also confirmed by the MLH distribution biased toward high heterozygosities (Figure 1). We cannot, however, reason further with survival because we are missing the genotypes of larvae that died. Nevertheless, this is not the case of the correlation of MLH with growth, another fitness-associated trait that we consider next.

Heterozygosity–growth correlation: The single-locus analysis of growth is somewhat complex. The effects of a single locus on growth are sometimes not consistent from one cross to the other. Locus *Oedu*.B0, for instance, does not exhibit significant differences in size between

heterozygous and homozygous genotypes in cross C1, while it does in cross C2 at days 10 and 70. Moreover, when heterozygotes are significantly bigger at one stage, the same effect should be observed at older stages. This happened only for *Oedu*.O9 and *Oedu*.T5 in cross C1 and for locus *Oedu*.B0 in cross C2. Two hypotheses can be suggested to explain that significant differences do not always persist: (1) As variance in size increases with age, it is more and more difficult to get a significant difference, especially since the number of homozygotes in the successive samples has decreased because of genotypic selection against them. This could explain the results obtained at locus *Oedu*.J12 in cross C1, which is not far from significant at day 70. (2) Smaller individuals were also those who died between two consecutive stages. This could explain that a smaller *d* was found at day 70 than at day 10 at locus *Oedu*.O9 in cross C2. The results at locus *Oedu*.T5 for cross C2, however, remain very puzzling because analysis fails to show a significant *d* at day 10, while it does at days 1 and 70.

Another possibility is that selection occurs at the multilocus level as a result of pleiotropic effects of the above-mentioned selective mortality, with growth as only one component of survival. Indeed, a significant MLH–growth correlation was observed at each stage for each cross, confirming the advantage of multiheterozygous genotypes. Because multiheterozygotes were larger than multihomozygotes at a given stage, significant locus-specific effects may show up sporadically, with no reason why this should be concordant among stages.

Nevertheless, MLH at four loci in cross C1 explains on average 17% of the total growth variability, whereas MLH at three loci in the cross C2 explains on average 10% of the total growth. Assuming no linkage disequilibrium between *Oedu*.O9 and *Oedu*.J12, 8–17.2% of the genome is marked by our microsatellites in cross C1, and the three loci of cross C2 mark 6–13%. These very rough estimates are in agreement with each other if the recombinational size of the *O. edulis* genome stands on the low side of our confidence interval for the parameter *Y* (see above).

The rare-allele paradox: McGoldrick and Hedgecock (1997) analyzed the progeny of self-fertilized hermaphrodites of the Pacific oyster *C. gigas* with 14 allozyme loci. For most loci and families, they observed a bias against homozygotes for the allele of lesser frequency in natural populations. Only two loci showed a bias against homozygotes for the more common allele. This was considered as an argument against the associative overdominance hypothesis because this hypothesis does not explain why recessive fitness mutations should be preferentially in *cis* configuration with rarer allozyme alleles. In our study, contrasting results were obtained because a bias against homozygotes with the less common allele was observed for *Oedu*.J12, whereas an opposite trend was recorded for *Oedu*.B0. These results are not similar to that of McGoldrick and Hedgecock (1997) because the lower-ranked alleles were not necessarily rare in their study. Moreover, direct effects or regulatory epistasis (McGoldrick and Hedgecock 1997) cannot be invoked for microsatellites. In this study, fewer loci and families were analyzed, but the larger sample sizes (near 80 individuals) offer the opportunity to find significant effects easily, even with the more common alleles (see results with *Oedu*.J12). It can be suggested that the genetic environment of a frequent allele is more easily purged from deleterious recessives because the frequency of the corresponding homozygote genotype is high. In self-fertilization or in full sib crosses where linkage disequilibrium is high, one or several deleterious alleles may be linked with the neutral marker in much higher probability for rare alleles than for common alleles in a natural population. This would explain why it is easiest to find biases with less common alleles. In other words, when linkage disequilibrium increases as a result of inbreeding, the genetic background of the marked unit has to be taken into account (Charlesworth 1991).

The mutation rate for microsatellite loci is usually very high, so the effect of linked FAGs in the natural population should be more important than that of allozymes. Actually, each microsatellite allele is, on the average, both younger and in a lesser frequency than an allozymic electromorph that may correspond to a whole class of sequences. Allozymes are thus expected to show less linkage disequilibrium with their surroundings than microsatellites. If the associative overdominance hypothesis explains heterotic effects in the natural population of marine bivalves, it should, therefore, occur even with hypervariable neutral loci. This question, however, is not relevant to what happens in inbred crosses because homozygotes are actually true autozygotes, which always implies maximum linkage with the genetic background. The explanation for the difference between microsatellites and allozymes, particularly for the behavior of their rare alleles, should be sought elsewhere if confirmed.

CONCLUSION

For each locus where a homozygote–heterozygote comparison was possible, we detected an effect on viability and/or growth before the spat stage. This constitutes an experimental proof that microsatellites, considered neutral by themselves, can be linked with FAGs and can provide indicators of selection processes at linked loci (Slatkin 1995; Charlesworth 1991). The linkage disequilibrium observed in crosses between full sibs is certainly more important that what it would be for all other reasons (population structure, migration, looser inbreeding relationships, etc.), and this is a probable reason for such a strong signal. The present results suggest that there is a potentially high genetic load in *O. edulis*. This genetic load is most likely caused by FAGs, which

seem to be quite well spread out in the genome so that any microsatellite that is picked up randomly will have one or several of them in its vicinity. It can be hypothesized that the *O. edulis* genome has a small enough recombinational size so that big pieces of chromosomes are made homozygous in inbred crosses. This potential for inbreeding depression has already been shown by numerous studies of performance in sib families, and was observed at the larval stage (Longwell and Stiles 1973; Beaumont and Abdul-Matin 1994; Ibarra *et al.* 1995; Lannan 1980; Mallet and Haley 1983), at the spat stage (Mallet and Haley 1983) and the adult stage (Beattie *et al.* 1987). Unfortunately, studies of inbreeding depression are seldom assisted by marker analysis, except by measuring the fixation indices in the consecutive inbred generations. Classically, such studies conducted on naturally outbred species show a slower rate of decrease in heterozygosity than the one predicted under inbreeding theory (Rumball *et al.* 1994). Fu and Ritland (1996) introduced this approach to infer quantitative trait loci for inbreeding depression (what we call FAGs here) in self-fertile organisms. It is based upon selfing a parent that is heterozygous for several unlinked codominant markers and then analyzing the fitness of the progeny according to genotypes. Mapping FAGs and estimating their number in various organisms would be of great interest for understanding the basis of inbreeding depression and heterosis in natural populations. Using selfing is easiest because the 1:2:1 ratio is always expected and the two homozygotes are always autozygotes. Using full sibs is also possible, as demonstrated here, for species in which selfing is impossible. Genotyping grandparents and parents is then necessary to control that homozygotes in the progeny are real autozygotes, and obtaining multi-autozygous genotypes can sometimes be difficult.

This study also confirms that biphasic selection (Zouros and Foltz 1984; Mallet *et al.* 1985; Singh and Green 1984; Blanc and Bonhomme 1986; Hawkins *et al.* 1989; Gaffney 1990), may be ruled out as a causative explanation of early heterozygote deficiencies, and it shows that heterozygote advantage may appear very early, between days 1 and 10, as in Cross C2.

Moreover, our results show that a strong selection occurs during the larval stage or at settlement. Selection during settlement has already been recognized by Foltz and Hu (1996), but selection during the free larval stage was never proven experimentally. The evidence for larval selection that this study provides may help us to understand the origin of MLH–fitness correlation in natural populations.

Larval selection can first modify genotypic frequencies and complicate genetic analysis. The results observed for the linkage of *Oedu*.09 and *Oedu*.J12 could be an example because genotypic selection on epistatic interactions might be able to alter or to fake genetic linkage. Moreover, if inbreeding ever occurs in wild populations of marine bivalves, selection against homozygotes during the larval stage may conceal the homozygote excess to some extent and lead to underestimate the inbreeding coefficient when analyses are done at the spat stage. If at a locus all homozygotes caused by partial inbreeding die during the larval stage, the heterozygote deficiency and the apparent heterozygotes advantage can be lost.

The magnitude of heterozygote deficiencies in wild populations of marine bivalves make the inbreeding hypothesis *a priori* not very likely to account for. Other hypotheses, such as null alleles, seem good alternatives, but a small fraction of inbreeding is enough to explain the MLH–fitness correlation if a sufficient genetic load does exist, as suggested in this study. The inbreeding hypothesis, also termed general effect hypothesis (David *et al.* 1995), is usually based on a lesser fitness of inbred *vs.* noninbred individuals. Our study shows that different individual fitnesses may exist among individuals that have a homogeneous inbreeding coefficient. These results may complicate the general effect model and its expectations.

The authors are very indebted to D. Hedgecock and P. David for detailed discussions and criticisms while working on this paper, to J. Goudet and Ph. Borsa for their comments on the manuscript, and to anonymous referees for their constructive remarks. The authors also express their thanks to P. Phélipot and C. Ledu for the larval rearing in La Tremblade. This research was funded in part by IFREMER URM 16.

LITERATURE CITED

Adamkewicz, L. S., R. Taub and J. R. Wall, 1984 Genetics of the clam *Mercenaria mercenaria*. II. Size and genotype. Malacologia **25:** 525–533.

Alvarez, G., C. Zapata, R. Amaro and A. Guerra, 1989 Multilocus heterozygosity at protein loci and fitness in the European oyster, *Ostrea edulis* (L.). Heredity **63:** 395–372.

Avise, J. C., 1994 *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.

Beattie, J. H., J. Perdue, W. Hershberger and K. Chew, 1987 Effect of inbreeding on growth in the Pacific oyster *Crassostrea gigas*: J. Shell. Res. **6:** 25–28.

Beaumont, A.R., 1991 Genetic studies of laboratory reared mussels, *Mytilus edulis*: heterozygote deficiencies, heterozygosity and growth. Biol. J. Linn. Soc. **44:** 273–285.

Beaumont, A. R. and A. K. M. Abdul-Matin, 1994 Differences in morphology, survival and size between self and cross fertilized larvae of *Mytilus galloprovincialis*: J. Mar. Biol. Assoc. U.K. **74:** 445–448.

Beaumont, A. R., C. M. Beveridge and M. D. Budd, 1983 Selection and heterozygosity within single families of the mussel, *Mytilus edulis* (L.). Mar. Biol. **4:** 151–161.

Blanc, F., and F. Bonhomme, 1986 Polymorphisme génétique des populations naturelles de mollusques d'intérêt aquicole, pp. 59–78 in *Selection, Hybridization and Genetic Engineering in Aquaculture*, Vol. I. H. Heenemann GmbH & Co., Berlin.

Charlesworth, D., 1991 The apparent selection on neutral marker loci in partially inbreeding populations. Genet. Res. **57:** 159–175.

Côrte-Real, H. B. S. M., P. H. W. Holland and D. R. Dixon, 1994 Inheritance of a nuclear DNA polymorphism assayed in single bivalve larvae. Mar. Biol. **120:** 415–420.

David, P., B. Delay, P. Berthou and P. Jarne, 1995 Alternative models for allozyme-associated heterosis in the marine bivalve *Spisula ovalis*. Genetics **139:** 1719–1726.

David, P., P. Berthou, P. Noel and P. Jarne, 1997a Spacially structured recruitment and population dynamics in a marine bivalve: *Spisula ovalis*. Oecologia **111:** 331–340.

David, P., M.-A. Perdieu, A.-F. Pernod and P. Jarne, 1997b Fined grained spatial and temporal population genetic structure in the marine bivalve *Spisula ovalis*. Evolution **51:** 1318–1322.

Foltz, D. W., 1986 Null alleles as a possible cause of heterozygote deficiencies in the oyster *Crassostrea virginica* and other bivalves. Evolution **40:** 869–870.

Foltz, D. W., and M. Chatry, 1986 Genetic heterozygosity and growth rate in the Louisiana oysters (*Crassostrea virginica*). Aquaculture **33:** 157–165.

Foltz, D. W., and Y. P. Hu, 1996 Genetics of scnDNA polymorphism in juvenile oysters (*Crassostrea virginica*). Part II: Heterozygote deficiency and gametic desequilibrium in natural population samples. Mol. Mar. Biol. Biotech. **5:** 130–137.

Fu, Y. B., and K. Ritland, 1996 Marker-based inferences about epistasis for genes influencing inbreeding depression. Genetics **144:** 339–348.

Gaffney, P. M., 1990 Enzyme heterozygosity, growth rate and viability in *Mytilus edulis*: another look. Evolution **44:** 204–210.

Gaffney, P. M., 1994 Heterosis and heterozygote deficiencies in marine bivalves: more light? pp. 146–153 in *Genetics and Evolution of Aquatic Organisms*, edited by A. R. Beaumont. Chapman & Hall, London.

Gaffney, P. M., and T. M. Scott, 1984 Genetic heterozygosity and production traits in natural and hatchery populations of bivalves. Aquaculture **57:** 261–269.

Gaffney, P. M., T. M. Scott, R. K. Koehn and W. J. Diehl, 1990 Interrelationships of heterozygosity, growth rate and heterozygote deficiencies in the coot clam *Mulinia lateralis*. Genetics **124:** 687–699.

Guo, X., and S. K. Allen, Jr., 1996 Complete interference and nonrandom distribution of meiotic crossover in a mollusc, *Mulinia lateralis* (Say). Biol. Bull. **191:** 145–148.

Hare, M. P., S. A. Karl and J. C. Avise, 1996 Anonymous nuclear DNA markers in the American oyster and their implications for the heterozygote deficiency phenomenon in marine bivalves. Mol. Biol. Evol. **13:** 334–345.

Hawkins, A. J. S., B. L. Bayne, A. J. Day, J. Rusin and C. M. Worral, 1989 Genotype-dependent interrelations between energy metabolism, protein metabolism and fitness, pp. 283–292 in *Reproduction, Genetics and Distributions of Marine Organisms*, edited by J. S. Ryland and P. A. Tyler. Olsen & Olsen, Fredensborg, Denmark.

Hedgecock, D., 1994 Does variance in reproductive success limit effective population sizes of marine organisms? pp. 122–134 in *Genetics and Evolution of Aquatic Organisms*, edited by A. R. Beaumont. Chapman & Hall, London.

Hedgecock, D., D. J. McGoldrick, D. T. Anahan, J. Vavra, N. Appelmans *et al.*, 1996 Quantitative and molecular genetic analysis of heterosis in bivalve molluscs. J. Exp. Mar. Biol. Ecol. **203:** 49–59.

Hu, Y.-P., and D. W. Foltz, 1996 Genetics of scnDNA polymorphisms in juvenile oysters, *Crassostrea virginica*: characterizing the inheritance of polymorphisms in controlled crosses. Mol. Mar. Biol. Biotechnol. **5:** 123–129.

Hu, Y.-P., R. A. Lutz and R. C. Vrijenhoek, 1992 Electrophoretic identification and genetic analysis of bivalve larvae. Mar. Biol. **113:** 227–230.

Ibarra, A. M., P. Cruz and B. A. Romero, 1995 Effect of inbreeding on growth and survival of self fertilized catarina scallop larvae, *Argopecten circularis*. Aquaculture **134:** 37–47.

Katoh, M., and D. W. Foltz, 1988 Determination of null allele frequency at an allozyme locus in a natural oyster population. J. Shell. Res. **7:** 203.

Koehn, R. K., and P. M. Gaffney, 1984 Genetic heterozygosity and growth rate in *Mytilus edulis*. Mar. Biol. **82:** 1–7.

Koehn, R. K. and S. E. Shumway, 1982 A genetic/physiological explanation for differential growth rate among individuals of the American oyster *Crassostrea virginica* (Gmelin). Mar. Biol. Lett. **3:** 35–42

Koehn, R. K., W. J. Diehl and T. M. Scott, 1988 The differencial contribution by individual loci of glycolysis and protein metabolism to the relationship between heterozygosity and growth in the coot clam, *Mulinia lateralis*. Genetics **118:** 121–130.

Longwell, A. C., and S. S. Stiles, 1973 Gamete cross incompatibility and inbreeding in the commercial American oyster *Crassostrea virginica* Gmelin. Cytologia **38:** 521–533.

Lannan, J. E., 1980 Broodstock management of *Crassostrea gigas*. IV. Inbreeding and larval survival. Aquaculture **21:** 353–356.

Mallet, A. L., and L. E. Haley, 1983 Effect of inbreeding on larval and spat performance in the American oyster. Aquaculture **33:** 229–235.

Mallet, A. L., E. Zouros, K. E. Gartner-Kepkay, K. R. Freeman and L. M. Dickle, 1985 Larval viability and heterozygote deficiency in populations of marine bivalves: evidence from pair matings of mussels. Mar. Biol. **87:** 165–172.

Mallet, A. L., E. Zouros, K. E. Gartner-Kepkay and K. R. Freeman, 1986 Genetics of growth in blue mussels: family and enzyme-heterozygosity effects. Mar. Biol. **92:** 475–482.

McGoldrick, D. J., and D. Hedgecock, 1997 Fixation, segragation and linkage of allozyme loci in inbred families of the Pacific oyster *Crassostrea gigas* (Thunberg): implication for the causes of inbreeding depression. Genetics **146:** 321–334.

Mitton, J. B., 1993 Theory and data pertinent to the relationship between heterozygosity and fitness pp. 17–41 in *The Natural History of Inbreeding and Outbreeding—Theoritical and Empirical Perspectives*, edited by N. W. Thornhill. University of Chicago Press, Chicago.

Naciri, Y., Y. Vigouroux, J. Dallas, E. Desmarais, C. Delsert *et al.*, 1995 Identification and inheritance of $(GA/TC)_n$ and $(AC/GT)_n$ repeats in the European flat oyster *Ostrea edulis* (L.) Mol. Mar. Biol. Biotechnol. **4:** 83–89.

Ohta, T., 1971 Associative overdominance caused by linked detrimental mutations. Genet. Res. **18:** 277–286.

Peterman, R. M., and M. J. Bradford, 1987 Wind speed and mortality rate of a marine fish, the northern anchovy (*Engraulis mordax*). Science **235:** 354–356.

Pogson, G. H., and E. Zouros, 1994 Allozyme and RFLP heterozygosities as correlates of growth rate in the scallop *Placopecten magellanicus*: a test of the associative overdominance hypothesis. Genetics **137:** 221–231.

Roughgarden, J., S. Gaines and H. Possingham, 1988 Recruitment dynamics in complex life cycles. Science **241:** 1460–1466.

Rumball, W., I. R. Franklin, R. Frankham and B. L. Sheldon, 1994 Decline in heterozygosity under full-sib and double first-cousin inbreeding in *Drosophila melanogaster*. Genetics **136:** 1039–1049.

Singh, S. M., and R. H. Green, 1984 Excess of allozyme homozygosity in marine molluscs and its possible biological significance. Malacologia **25:** 569–581.

Sokal, R. R., and F. J. Rohlf, 1996 Biometry, Ed. 3. Freeman & Co., New York.

Slatkin, M., 1995 Hitchhiking and associative overdominance at a microsatellite locus. Mol. Biol. Evol. **12:** 473–480.

Strauss, S. H., 1986 Heterosis at allozyme loci under inbreeding and crossbreeding in *Pinus attenuata*. Genetics **113:** 115–134.

Strobeck, C., 1979 Partial selfing and linkage: the effect of a heterotic locus on a neutral locus. Genetics **92:** 305–315.

Walsh, P. S., D. A. Metzger and R. Higuchi, 1991 Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques **10:** 506–513.

Zouros, E., 1993 Associative overdominance: evaluating the effects of inbreeding and linkage desequilibrium. Genetica **89:** 35–46.

Zouros, E., and D. W. Foltz, 1984 Possible explanations of heterozygote deficiency in bivalve molluscs. Malacologia **25:** 583–591.

Zouros, E., and G. H. Pogson, 1994 Heterozygosity, heterosis and adaptation, pp. 135–146 in *Genetics and Evolution of Aquatic Organisms*, edited by A. R. Beaumont. Chapman & Hall, London.

Zouros, E., S. M. Singh and H. E. Miles, 1980 Growth rate in oysters: an overdominant phenotype and his possible explanations. Evolution **42:** 1332–1334.

Zouros, E., S. M. Singh, D. W. Foltz and A. L. Mallet, 1983 Post settlement viability in the American oyster (*Crassostrea virginica*): an overdominant phenotype. Genet. Res. **41:** 259–270.

Communicating editor: A. G. Clark