High Genetic Load in the Pacific Oyster *Crassostrea gigas*

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ABSTRACT

The causes of inbreeding depression and the converse phenomenon of heterosis or hybrid vigor remain poorly understood despite their scientific and agricultural importance. In bivalve molluscs, related phenomena, marker-associated heterosis and distortion of marker segregation ratios, have been widely reported over the past 25 years. A large load of deleterious recessive mutations could explain both phenomena, according to the dominance hypothesis of heterosis. Using inbred lines derived from a natural population of Pacific oysters and classical crossbreeding experiments, we compare the segregation ratios of microsatellite DNA markers at 6 hr and 2–3 months postfertilization in F_2 or F_3 hybrid families. We find evidence for strong and widespread selection against identical-by-descent marker homozygotes. The marker segregation data, when fit to models of selection against linked deleterious recessive mutations and extrapolated to the whole genome, suggest that the wild founders of inbred lines carried a minimum of 8–14 highly deleterious recessive mutations. This evidence for a high genetic load strongly supports the dominance theory of heterosis and inbreeding depression and establishes the oyster as an animal model for understanding the genetic and physiological causes of these economically important phenomena.

WITH his "elm-oyster model," G. C. Williams (1975) drew attention to a similarity in the evolutionary biology of highly fecund plants and bivalve molluscs, namely the advantage of sexual reproduction in the face of intense natural selection on early life stages. Since then, other biological parallels between these disparate organisms have emerged. Both plants and bivalves, for example, tolerate extra or incomplete sets of chromosomes (polyploidy or aneuploidy; Beaumont and Fairbrother 1991; Guo and Allen 1994; Wang *et al.* 1999). Perhaps the most striking plant-bivalve parallel, and the feature motivating this study, is the expression of heterosis (hybrid vigor) for fitness traits like growth and survival. The fundamental genetic and physiological causes of heterosis remain poorly understood in plants after nearly a century of exploitation in crop improvement (Griffing 1990; Crow 1998; Lynch and Walsh 1998). An animal model of this important phenomenon could open up new opportunities for investigating genetic and physiological causes.

Heterosis in bivalves was first suggested in 1978 (Singh and Zouros 1978) by positive correlation between allozyme heterozygosity and fitness-related traits in individuals from natural populations. The causes of this marker-associated heterosis were vigorously debated by opposing camps essentially espousing the two classic explanations of heterosis, overdominance *vs.* dominance (or associative overdominance), while wrestling over the selective neutrality of allozymes (see Zouros and Pogson 1994; Britten 1996; David 1998). Under the overdominance hypothesis, selection acts directly on allozyme genotypes and heterosis is the result of functional superiority of allozyme heterozygotes. Under the associative overdominance hypothesis, allozymes are merely neutral indicators of selection against deleterious recessive mutations at linked loci having fitness effects.

Correlations between allozyme heterozygosity and fitness have been determined almost exclusively in samples from natural populations, generally using a flawed *ex post facto* protocol, in which individual heterozygosity was assayed after performance of communally held animals had been determined. Only more recently have hypotheses about heterosis in bivalves been addressed through experimental crosses of inbred lines (McGoldrick and Hedgecock 1997; Bierne *et al.* 1998). Experimental inbreeding and crossbreeding of the Pacific oyster *Crassostrea gigas* confirmed that F_1 hybrids are nearly always superior in yield to the best of the inbred parents (Hedgecock *et al.* 1995, 1996), which is the classical definition of yield heterosis (Griffing 1990). Controlled crossbreeding experiments have allowed analyses of the physiological components of growth heterosis (Hedgecock *et al.* 1996; Bayne *et al.* 1999). A first attempt to map the genetic basis of heterosis in oysters, using quantitative trait loci (QTL)-mapping methods, was stymied, however, by distortions of Mendelian segregation ratios (McGoldrick 1997). Similar distortions had been reported as early as 1975 (Wada 1975) for a variety of clams, mussels, and oysters (Wilkins 1976; Beaumont *et al.* 1983; Gaffney and Scott

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1984; Foltz 1986; Thiriot-Quievreux *et al.* 1992; Hu *et al.* 1993; Hu and Foltz 1996; McGoldrick and Hedgecock 1997; Launey 1998; McGoldrick *et al.* 2000).

Several characteristics of distorted segregation ratios in bivalves suggest the hypothesis that selection against recessive deleterious mutations at closely linked genes is responsible for non-Mendelian inheritance of markers. First, distortions are observed for both expressed (Foltz 1986) and unexpressed markers (Hu and Foltz 1996). Second, departures from classical Mendelian ratios occur in crosses of individuals collected from the wild but are exacerbated in families known to be inbred (McGoldrick and Hedgecock 1997; Bierne *et al.* 1998) or in samples taken from small hatchery stocks (Wilkins 1976). Finally, distortions of Mendelian segregation ratios grow more severe as progeny age (Gaffney and Scott 1984) and occur unpredictably among markers and families (McGoldrick and Hedgecock 1997). Under this hypothesis, we expect minimum distortion of segregation ratios at the earliest developmental stages, before defective genes are turned on and expressed.

Here, we report segregation ratios for 19 microsatellite loci in one F_3 and seven F_2 *C. gigas* families. We test whether segregation ratios are Mendelian in very early larvae, before expression of many genes. We then test whether segregation ratios become distorted by the juvenile stage in a manner consistent with differential mortality of individuals homozygous for recessive deleterious alleles. We fit significantly distorted segregation ratios at the juvenile stage to a two-locus selection model to estimate selection coefficients and recombination distances between markers and recessive mutations. Taking into account the proportion of the genome marked, we estimate a minimum genetic load for the wild founders of four inbred lines. Evidence for a high genetic load confirms a prediction of Williams' (1975) elm-oyster model and provides a coherent explanation for several phenomena reported for bivalve molluscs over the past 25 years—distortion of Mendelian segregation ratios in crosses, correlation of individual fitness with allozyme heterozygosity, correlation of growth with degree of somatic cell aneuploidy, and high levels of heterozygosity in gynogenetic diploids produced by blocking the second meiotic division.

MATERIALS AND METHODS

Biological material: Inbred lines of the Pacific oyster were derived from a naturalized population in Dabob Bay, Washington (Hedgecock 1994), either by self-fertilizing simultaneous hermaphrodites or mating full-sibs from pedigreed families (Hedgecock *et al.* 1995). Six to 9 years of controlled crosses yielded the one F_3 and seven F_2 hybrid families used in this study (Figure 1). Inbred lines 93-2 and 89-5 are each represented in five of the eight hybrid families examined; line 93-3 is represented in two families, and lines 89-6, 89-7, 93-7, and 93-9 are each represented in one family. Each family was derived from a single biparental cross, using standard methods of artificial fertilization and larval rearing (see Hedgecock *et al.* 1995).

Collection and treatment of samples: Samples were genotyped both at the first-swimming larval stage (6 hr postfertilization, n = 34–141 individuals per locus per family; mean, 80.7) and the juvenile stage (2–3 months old, n = 39–94; mean, 77.4). Six hours after fertilization, a sample of swimming trochophore larvae was siphoned off the beaker, transferred into 15-ml centrifuge tubes, and gently spun down. After removal of seawater, the larvae were washed once in 95% ethanol and then stored in ethanol at 4°. Prior to DNA extraction, larvae were individually collected under a dissecting scope and stored in 30 μl of 95% ethanol in 96-well PCR trays.

The remaining trochophore larvae were transferred to 100-liter fiberglass tanks and reared at 25° until metamorphosis (2–3 weeks). Cultures with a high percentage of pediveliger eyed larvae were provided with setting substrate of aged oyster shell (cultch), broken and sieved to ~1-cm^2 pieces. Cultch was inspected daily and refreshed in the larval tank when judged to have approximately one to three newly metamorphosed individuals (spat) per piece; spat on cultch were held and fed in the hatchery for about a week, then set out to grow in Tomales Bay, California. For families 2 × 5a, 5 × 2a, and 7 × 6, juveniles were sampled at 3 months of age (average shell height, 13 mm). A piece of mantle was taken from each specimen for DNA extraction and the body was frozen (−80°). For families 2 × 5b, 5 × 2b, 3 × 2, and 7 × 9, juveniles were sampled at 2 months of age; whole animals were frozen (−80°) and later the whole body was used for DNA extraction.

For pedigree information, gill samples were taken from the parents of each cross after fertilization and also from the frozen bodies of grandparents and other relatives. Alleles and genotypes can then be traced back to their respective parental inbred lines, except for families 7 × 6 and 7 × 9, for which tissue samples of great-grandparents and grandparents, respectively, were not available.

DNA extraction, PCR procedures, and electrophoresis: Because of the small amounts of tissue that were available, the Chelex extraction method (modified from Estoup *et al.* 1996) was used for the larvae. Alcohol was evaporated from larval samples at 37°. Then, 60 μl of 5% chelating resin (Chelex; Bio-Rad, Richmond, CA), 6 μl of TE (0.01 M Tris, 1 mM EDTA), and 3 μl of proteinase K (10 mg/ml) were added to each sample. The mixture was heated at 55° for 2 hr and boiled (100°) for 10 min. This reaction was performed using the Tetrad thermocycler from M. J. Research (Waltham, MA). The tray was then centrifuged at 3000 R/min for 5 min, and the supernatant was stored at −20° until used as template for PCR. DNA was extracted from juveniles following the same protocol but using 100 μl Chelex, 10 μl TE, and 5 μl proteinase K. DNA was extracted from adults using a standard phenol/chloroform protocol (Sambrook *et al.* 1989).

Nineteen microsatellite loci were individually amplified in this study (Table 1). Fourteen of them (*ucdCgi1*, *ucdCgi2*, *ucdCgi3*, *ucdCgi4*, *ucdCgi6*, *ucdCgi8*, *ucdCgi9*, *ucdCgi10*, *ucdCgi14*, *ucdCgi18*, *ucdCgi21*, *ucdCgi22*, *ucdCgi24*, *ucdCgi28*) were cloned at the Bodega Marine Laboratory (McGoldrick 1997; McGoldrick *et al.* 2000). The remaining 5 loci were taken from the literature (*CG49* and *CG108*, Magoulas *et al.* 1998; *L10*, *L16*, and *L48*, Huvet *et al.* 2000). PCR was performed in 10-μl reactions, containing 2 μl of the template DNA, 10 pmol of each primer (one fluorescently labeled), 1–2 mM $MgCl_2$, 80 μM of each dNTP, and 0.3 units of *Taq* DNA polymerase (Promega, Madison, WI). Thirty PCR cycles (30 sec at 94°, 1 min at T_m, 1 min at 72°, followed by a 5-min elongation step at 72°) were used to amplify the markers. T_m (the optimum annealing temperature) and the $MgCl_2$ varied depending on the locus (Table 1). PCR products were electrophoresed on 8% PAGE gels (acrylamide:bisacrylamide 29:1, 7 M urea), using 1× TBE (Tris

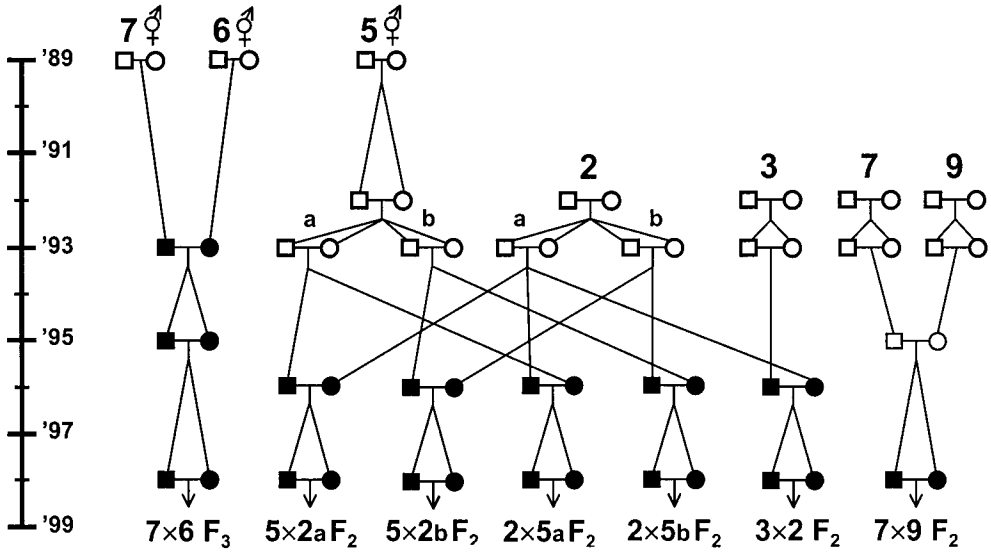

FIGURE 1.—Crosses of Pacific oysters from a natural population in Dabob Bay, Washington, which produced seven F_2 and F_3 hybrid families for segregation analysis (pedigree of the 5×3 F_2, which did not survive to the juvenile stage, is not shown). The earliest parents of each line were taken from the wild. Three crosses in 1989 were self-fertilizations of hermaphrodites; the remaining crosses in 1992 were fertilizations between wild males and females. Solid symbols indicate parents from which tissues and DNA were available for study.

borate EDTA) buffer. Results were visualized by scanning the gel using the FMBIO II fluorescence scanner (Hitachi Genetic Systems, Alameda, CA).

Segregation distortions: Different types of segregation were observed, depending on whether a pair of parents had two alleles (cross type $AA \times AB$ or $AB \times AB$), three alleles ($AB \times AC$), or four alleles ($AB \times CD$). Conformity to Mendelian phenotypic proportions was determined by chi-square test, with the level of significance adjusted for simultaneous multiple tests within each type of segregation (Bonferroni correction, RICE 1989). However, this correction may increase the type II error of not detecting segregation distortion, when selection is acting to distort some markers but not others (*i.e.*, the alternative hypothesis explaining segregation distortion is true some of the time).

Estimating selection and linkage in a two-locus selection model: When an F_2 individual is made homozygous identical by descent (IBD) for a particular allele at a marker locus, a region of chromosome around this marker is also made homozygous IBD. To explain deficiencies of marker IBD homozygotes, we use a linked selection model, in which the marked chromosomal region contains a single fitness gene, which is rendered homozygous IBD for a recessive deleterious allele. We adapt to F_1 crosses a maximum-likelihood model developed by HEDRICK and MUONA (1990) to estimate simultaneously the selection coefficient (s) of this deleterious gene and the map distance (c) between it and the marker, which would account for the observed departures from Mendelian segregation. We assume a marker locus with two alleles, A_1 and A_2, linked to a locus affecting viability with two alleles, l, the deleterious variant, and +, the wild-type allele. The parameters of the model are s, the selection coefficient for the deleterious allele, h, the dominance level of that allele, and c, the distance in map units between the marker and viability loci. Assuming that F_1 individuals are heterozygous A_1l/A_2+, then the frequencies in the F_2 progeny of the genotypes A_1A_1, A_2A_2, and A_1A_2 are

$$P_{11} = \frac{c^2s(2h-1) - 2cs(h-1) + (1-s)}{4 - s(2h+1)},$$

$$P_{12} = \frac{2(-c^2s(2h-1) + cs(2h-1) - hs + 1)}{4 - s(2h+1)},$$

$$P_{22} = \frac{c^2s(2h-1) - 2csh + 1}{4 - s(2h+1)}.$$

Because the model has only 2 d.f., it is not possible to estimate all three unknown parameters (h, s, and c). As a first approach, we assume h to be zero. We then jointly estimate s and c by a maximum-likelihood approach, through the calculation of the ratio of the probability of a given family array with c recombination to that with no linkage ($c = 0.5$). A general formula for that LOD score is $z = \Sigma N_i \log_{10}(P_i/P_i^*)$, where N_i, P_i, and P_i^* are the number observed, the proportion expected with recombination c, and the proportion expected with no linkage, respectively, for the ith genotypic class. The same approach is used for a three-allele cross $A_1A_2 \times A_1A_3$, where A_1 is linked with allele l. The expected frequencies in the F_2 progeny are P_{11} as above,

$$P_{12} = P_{13} = \frac{(-c^2s(2h-1) + cs(2h-1) - hs + 1)}{4 - s(2h+1)},$$

and P_{23} as P_{22} above.

Expected frequencies of the different genotypes are then recalculated given estimates for s and c. For three cases, in which deficiency of marker heterozygotes suggests partial dominance of the linked fitness mutation, there is a significant difference between observed from expected genotypic frequencies, assuming h is 0. In these cases, we increase h by 0.1, obtain new estimates for s and c, and again compare expected and observed genotypic proportions. The process is repeated until an h producing no significant difference between expected and observed genotypic frequencies is obtained.

Estimation of the total number of lethal genes: Standard calculation of the number of lethal equivalents (LYNCH and WALSH 1998) is not possible for oysters because of the difficulty of precisely measuring early survival (HEDGECOCK *et al.* 1995, 1996). The numbers of highly deleterious recessive ("lethal") mutations in wild founders of inbred lines 2, 3, and 5, and the F_1 hybrid grandparents of family 7×6, are estimated here by tracing each marked homozygous IBD chromosome segment back to its grandparental origin. We tally, for each lineage, the minimum number of highly deleterious recessive mutations implied by deficiencies of IBD homozygotes across families and linkage groups (see below). This tally is converted to an estimate of the lethal load in each of the diploid founders, by taking into account the proportion of the genome in linkage disequilibrium with markers segregating in F_2 descendants. Using a maximum-likelihood approach, we determine the maximum map distance from which a recessive lethal mutation ($s = 1$) could exert the least significant distortion

TABLE 1

Primer sequences and PCR conditions for 19 *C. gigas* microsatelite loci

Locus	GenBank accession no.	Primer sequences, 5′ to 3′ (forward/reverse)	Temp.	[$MgCl_2$] (mM)
ucdCgi1[a]		GATCGTTACTGTCAGTTGCTC TGCGGTGCTATTATGAACCA	55°	1.25
ucdCgi2[b]		TTGCAGGAAGCAAGAGATGA CTTGTTAACTGCCGGTGAGG	55°	2
ucdCgi3[a]		TTGAATGAACATCGTTTG GATCATAGAATAAACAGAAC	50°	2
ucdCgi4[a]	AF051170	ATAATAATTAAAGGGGTTAAGGGG GTGGTAGCAATTGTGTCCTATG	57°	1
ucdCgi6[b]	AF051172	AAGCAACTATCAGTTTTTGGTAGC AATGAGCTGACAGTTCATAGGC	55°	2
ucdCgi8[a]	AF0F1175	AAGAAGTTTGCATTATTAGTAGC AACTTGTGTAAAGCATCTG	50°	1.25
ucdCgi9[b]		TTAAACTTGTGTAAAGCATTTGG CGTTCATCGATTTTCGCAAT	53°	2
ucdCgi10[b]		TGCACCAATTTGAGATGTGA ACTGAGTTTGAAAATGTCACCG	50°	1.5
ucdCgi14[a]	AF051174	ACTCGGCGAAACTTCTAATAC TTAGCTGCCGCTCAAGTTTT	50°	1.25
ucdCgi18[b]		TCCATGTTTACTGCTACTTTTGG AAATGCTGTGCAGAGAAGCC	50°	1.5
ucdCgi21[b]		GCCCTCTAAATTAAAATCTCTCT CCGCCATAGGTTTGAAAATT	50°	1
ucdCgi22[a]		GGAAGAGGAATAGTCTACTTATGC GTCAGACGTTCCTAACTCTTC	43°	1.75
ucdCgi24[a]		CAGAGAGCCGGACTATTTC GCTCTTTGACACTATGCCGA	45°	1
ucdCgi28[b]	AF051178	TGTTTAATGATGTGTACCGCG ATCAAATTGGCTGTATTTACAGTG	53°	1.25
CG49[c]	Y12086	CATCAGGGGTAAATTAAAGTAAGC CCACAGACGATTTCATATATCCTG	55°	2
CG108[c]	Y12087	ATATGTAATGATTACGAAACTC GTATGAGATTTGGTTCCACC	55°	1.5
L10[d]	AF170850	GGTCAATTCAAAGTCAATTTCCC CATGTTTTCCCTTGACTGATCC	55°	1
L16[d]	AF170851	CGGACGAATAAGATATTTGGTC TGGATCTGCGCATCATCTCG	57°	1
L48[d]	AF170852	TCAAACCATCTGCTCGTCTACG TCCGAAAATCCAGGAATACCGG	60°	1.5

[a] Primers modified from those in McGoldrick (1997) and McGoldrick *et al.* (2000).
[b] Primers taken directly from McGoldrick (1997) and McGoldrick *et al.* (2000).
[c] Magoulas *et al.* (1998).
[d] Huvet *et al.* (2000).

of a marker segregation ratio. With selection against an IBD homozygote, the least significant deviation ($\alpha = 0.05$) from an expected 1:1:1:1 ratio that can be expressed in whole numbers for a sample size close to the mean ($n = 77.4$) is 24:24:24:9. Assuming that this least significant distortion is caused by a linked, lethal, recessive mutation, the model yields a maximum-likelihood solution for c of 18 cM; a mutation of smaller effect would have to be closer to cause the same distortion. Hence, we assume that a meiotic segregation, producing a microsatellite IBD homozygote, assays a 36-cM chromosomal segment for linked highly deleterious recessive mutations. To detect pairs of microsatellites close enough to have had their segregation ratios distorted by the same recessive lethal mutation, we performed linkage analysis on the 6-hr larval segregation data, using the LOD-score approach (Morton 1955). Distances between loci were calculated according to Haldane (1919), using the MAPMAKER software package (Lander *et al.* 1987). *C. gigas* has 10 pairs of metacentric or submetacentric chromosomes, with one chiasma per chromosome arm (Longwell *et al.* 1967; X. Guo, personal communication), so the total map length can be estimated as ~1000 cM.

The tally of lethal load, together with the sum of map units covered per founder genome, is carried out as follows, for different situations:

1. A single marker, yielding an IBD homozygote in one or more descendant F_2 or F_3 families, has a segregation ratio that conforms to Mendelian expectations; no highly deleterious recessive mutation is tallied for the 36-cM region of chromosome.

2. A single marker, with no linkage to other markers, shows a distorted segregation ratio in a single family: one highly deleterious recessive mutation is tallied for the appropriate grandparental lineage in a 36-cM region surrounding the marker.
3. A single marker (no linkage to other markers) is affected in several families because of descent from a shared grandparent: one lethal gene is tallied for the appropriate grandparental lineage over a 36-cM chromosomal segment.
4. Two linked markers <36 cM apart are affected in one family (or several related families): one lethal gene (not two) is tallied for the appropriate grandparent, over a chromosomal region of 18 cM + intermarker distance + 18 cM. When neither or only one of two markers is affected, no or one lethal is recorded, but genomic coverage is reduced, as above, for overlap.

The total number of highly deleterious lethal mutations detected by distorted segregation ratios is divided by the total centimorgans of coverage afforded by segregations producing IBD homozygotes and then multiplied by 1000 cM to produce an estimate of the number of lethal mutations per diploid genome. A 95% confidence interval is constructed around this estimate, using the binomial distribution to describe the probability, p, that IBD homozygotes are deficient in k of n informative segregations. The lower confidence limit is based on the smallest p_l, yielding k or fewer deficiencies of IBD homozygotes 2.5% of the time, while the upper confidence limit is based on the largest p_u, yielding k or more deficiencies of IBD homozygotes 2.5% of the time. The p_l and p_u are multiplied by n and divided by the genomic coverage to estimate the confidence limits. A web-based binomial probability calculator facilitated calculation of appropriate cumulative probabilities (http://faculty.vassar.edu/~lowry/VassarStats.html).

RESULTS

PCR results and validation of pedigree: DNA was extracted from a total of 1096 6-hr larvae. Seventy-six of these samples (6.9%) did not yield any PCR product. Similarly, DNA was extracted from 656 2- and 3-month-old spat with a yield of 98%. The DNA yield from a single 6-hr-old individual was enough to perform at least 25 PCR amplifications.

A few individuals (one to four in the combined larval and juvenile samples of five of the eight families) had multilocus genotypes inconsistent with parents and grandparents. Attributable to the contamination commonly observed among bivalve larval cultures (*e.g.*, Hedgecock *et al.* 1995; McGoldrick and Hedgecock 1997), these individuals were eliminated from further analysis.

Null alleles: Ninety-four segregation ratios were studied. Aside from the few individuals attributable to cross-contamination, no alleles were observed in progeny that were not seen in parents. However, in 15 cases, for which one parent appeared to be homozygous (*e.g.*, *AA*) and the other, heterozygous (*e.g.*, *BC*), some of the offspring unexpectedly appeared to be homozygous *BB* or *CC*. The most likely explanation is that the *AA* parent is actually a heterozygote for a null allele (*AØ*). Such a hypothesis could account for all the unexpected phenotypes observed. Inheritance models were modified accordingly before testing segregation ratios.

Segregation analyses: Results are sorted by life stage, 6-hr larvae *vs.* 2- to 3-month-old juveniles, to test the prediction that segregation is Mendelian in early larvae but becomes distorted in juveniles because of selection acting on deleterious recessive mutations linked to some markers.

Six-hour larvae: Taking into account the possibility of null alleles in the 15 cases mentioned above, no significant departures from Mendelian expectations are observed in 91 of 94 cases at the 5% significance level corrected for multiple tests. As the proportion of failures, 3/94, is within the conventional threshold of significance, we confirm the first part of our prediction that segregation ratios would conform to Mendelian expectations at an early stage.

A linkage analysis could be performed at this stage. Five linkage groups were found: {*Cgi3*-22 cM-*Cgi18*-8 cM-*Cgi8*-15 cM-*Cgi9*}, {*Cgi14*-18 cM-*Cgi21*}, {*Cgi28*-24 cM-*CG108*}, {*Cgi1*-28 cM-*L48*}, and {*Cgi22*-38 cM-*L16*}. The other loci appeared unlinked, although weak linkage of *Cgi2* and *CG49* (mean of 55 cM) was observed in six families.

Two- or 3-month-old spat: One family (5 × 3) did not survive to the juvenile stage, leaving only 86 segregations available for analysis at this stage. Thirty-one (36%) significant departures from Mendelian expectations are observed at the nominal significance level ($\alpha = 0.05$; Table 2, Figure 2). After correction for multiple testing, 18 segregation ratios (20.9%) remain significantly distorted. Thus, we confirm the second part of our prediction, that segregation ratios become distorted by the juvenile stage.

Analysis of the cases of significant segregation distortions: Significant distortions of segregation ratios in juveniles are broadly distributed over loci (17 of 19 loci are affected) and are associated with alleles coming from all lineages (10 from each of lines 93-2 and 89-5, 5 from 7 × 9, 4 from 7 × 6, and 2 from 93-3). The vast majority of significant segregation distortions, 24 of 31, result from deficiency of an expected homozygous genotype. In one extreme case (D1, Figure 2), a homozygote expected in 1 out of 4 offspring is absent from a sample of 87 individuals. In almost all cases, except for family 7 × 9 (grandparents unknown), both alleles of the homozygous genotype come from the same grandparent and are therefore IBD (cases indicated by arrows in Figure 2). In one case (B1, Figure 2), the homozygous genotype (*AA*) cannot be distinguished from a null heterozygote (*AØ*) but the combined A phenotype is at a lower than expected frequency.

These distortions result from homozygote disadvantage rather than heterozygote advantage. Deficiencies of IBD homozygotes, not excesses of heterozygotes, account for segregation distortion in almost all *AB* × *AB* crosses, in which the relative frequencies of *AB* heterozygotes can be compared to those of *AA* and *BB* homozy-

TABLE 2

Testing Mendelian segregation of 19 microsatellites in eight hybrid populations

	6-hr-old larvae		2- to 3-mo-old juveniles	
Segregation type	No. of tests[a]	Significant deviations from Mendelian ratios[b]	No. of tests[a]	Significant deviations from Mendelian ratios[b]
Two alleles				
AØ × *AB*	12	1 (1)	11	1 (1)
AA × *AB*	7	0	6	2 (1)
AB × *AB*	23	2 (1)	21	12 (5)
Three alleles				
AØ × *BC*	3	0	3	0
AA × *BC*	1	0	1	0
AB × *AC*	36	1 (1)	33	12 (9)
Four alleles				
AB × *CD*	12	0	11	4 (2)
Total	94	4 (3)	86	31 (18)

[a] Total number of segregations tested.

[b] Number of chi-square tests significant at the nominal level of significance ($\alpha = 0.05$) and (in parentheses) remaining significant after correction for multiple tests.

gotes (Figure 2C). The *AA* homozygote is deficient in 11 cases in this category (*AB* is deficient in case C12, Figure 2); however, in 7 of these cases, the ratio of *AB* to *BB* does not differ significantly from the expected 2:1. In 3 of 4 cases, for which the ratio of *AB* to *BB* is not 2:1 (C4, C6, and C7, Figure 2), the heterozygote is deficient, suggesting partial dominance of a deleterious mutation linked to the *A* allele (Table 3). The only case in which there is an excess of heterozygotes (C9, Figure 2) is equally well explained by two different recessive deleterious alleles, one linked to *A*, the other to *B*.

In 7 of 31 cases (Figure 2, A2, C12, D12, E1–4), significant departures from Mendelian segregation ratios appear not to conform to a simple model of linked deleterious recessive alleles. In cases E1–4, for example, every expected genotype in the progeny is heterozygous. However, in E1, E2, and E4, grandparental genotypes are deficient, suggesting the possibility that some F_2 progeny, though heterozygous at the marker, might still have been homozygous IBD for a linked, deleterious, recessive allele. This interpretation is supported by data from related families. For example, a heterozygous *Cgi8* genotype that is inherited from the line 93-2 grandparent is deficient in two cases, E2 and D12 (labeled *BD* and *BC*, respectively, in Figure 2). Moreover, one of the line 2-derived *Cgi8* alleles (*D* in E2 and *C* in D12) is highly deleterious when made homozygous IBD (*AA* in C2). Likewise, a line 5 grandparental allele in the deficient *Cgi8 AC* genotype of E2 is deficient as an IBD homozygote in case C11. A similar relationship exists between the deficiency of the line 2 grandparental *CG49 BD* heterozygote in case E4 and the IBD homozygote deficiency in case D5. In another three cases, homozygous genotypes are present at their expected frequencies while heterozygous genotypes are deficient (A2, C12, D12, Figure 2). Case D12 is explained above, and case A2 lacks the critical grandparental genotypes to determine cause. Only for cases C12 and E3, in which heterozygotes for independently derived alleles are deficient, is a more complex form of selection, probably involving epistatic interactions among genes, necessary to account for distortions of Mendelian segregation ratios. On the whole, the great majority, 28/30 or 93% of the significant distortions, can be attributed to selection against recessive deleterious mutations at linked fitness genes.

Estimation of the effect and the number of lethal genes: A linked selection model is applied to cases in segregation categories C and D (Table 3), to estimate selection coefficients and distances between markers and selected loci. We exclude cases C12 and D12, which had heterozygote deficiencies (see above), and C9 and D8, which appear to have had more than one lethal gene associated with them. At C9, there are deficiencies of both homozygotes, one IBD and the other not, while at D8 there are deficiencies of an IBD homozygote from line 2 and the line 5 grandparental heterozygote (Figure 2). In the remaining 20 cases (Table 3), segregation ratios can be explained by a recessive lethal or nearly lethal allele ($s = 0.9$–1) closely linked to the marker allele made homozygous IBD. Recombination rate, c, ranges from 0.01 to 22.7 cM, with means of 11.6 ± 7.3 cM for all cases significant at the nominal 5% level and 8.0 ± 6.2 cM for cases significant after Bonferroni correction of significance level.

For four inbred lines, we estimate the average number of lethal genes per wild founder, taking into account (i) the number of highly deleterious recessive alleles implied by deficiencies of IBD homozygotes in the F_2 or F_3, (ii) the proportion of the genome assayed by

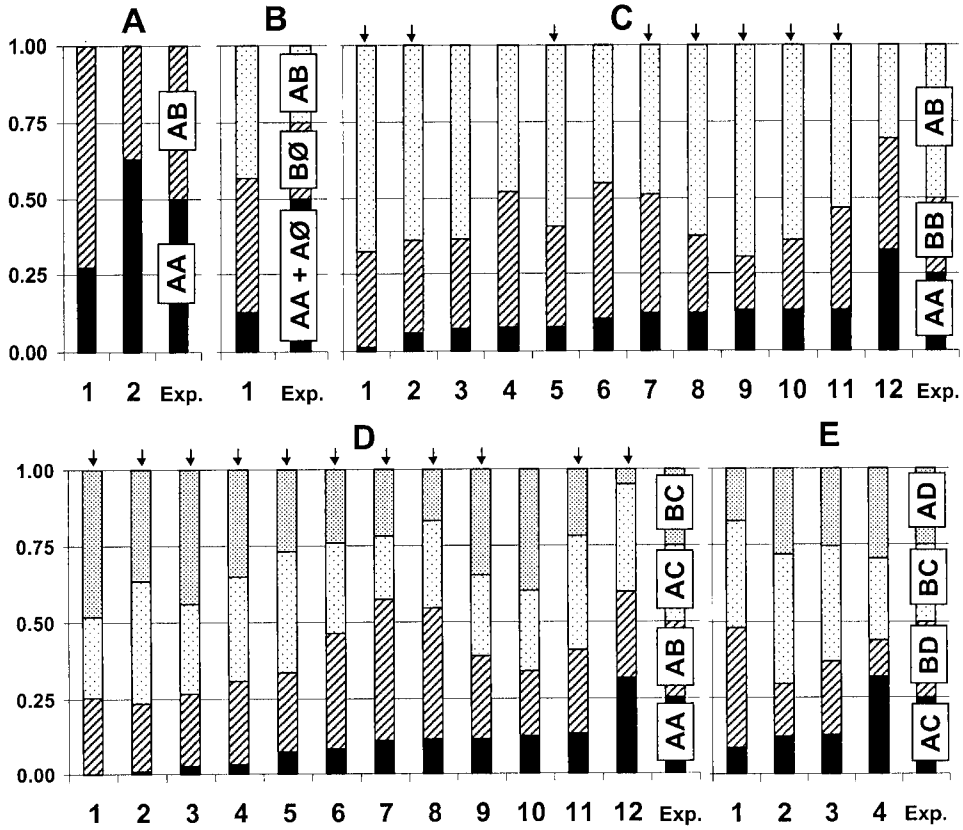

FIGURE 2.—Relative proportions of microsatellite DNA marker phenotypes in 31 cases, for which there is a significant departure ($\alpha = 0.05$) from classical Mendelian segregation ratios. Cases are grouped into A–E, according to type of segregation, as indicated in each by the far-right bar labeled "Exp.," which shows expected phenotypes and their Mendelian proportions. Arrows denote cases in which *AA* is homozygous identical by descent from a grandparental allele. Locus, family, grandparental genotypes (if known), and progeny sample size (in parentheses) by case are as follows: A1, *Cgi3*, 7 × 6, *AB* × *AB* (69); A2, *CG108*, 7 × 9 (73); B1, *Cgi18*, 2 × 5a, *A*− × *BØ* (78); C1, *CG108*, 2 × 5b *AC* × *B*− (86); C2, *Cgi8*, 5 × 2b, *BC* × *AD* (50); C3, *Cgi1*, 7 × 9 (55); C4, *Cgi28*, 7 × 9 (79); C5, *Cgi28*, 3 × 2, *BB* × *AC* (91); C6, *Cgi10*, 7 × 9 (75); C7, *L10*, 3 × 2, *AC* × *BD* (82); C8, *Cgi4*, 2 × 5b, *BB* × *AB* (72); C9, *Cgi28*, 7 × 6, *BB* × *AB* (79); C10, *Cgi6*, 2 × 5b, *BB* × *AC* (60); C11, *Cgi8*, 2 × 5b, *BC* × *AD* (75); C12, *L48*, 5 × 2b, *BC* × *AD* (82); D1, *Cgi1*, 7 × 6, *BC* × *AE* (87); D2, *L10*, 7 × 6, *AB* × *BC* (90); D3, *L10*, 5 × 2b, *AD* × *BC* (68); D4, *Cgi2*, 5 × 2a, *BC* × *AD* (93); D5, *CG49*, 5 × 2a, *BC* × *AD* (93); D6, *Cgi21*, 3 × 2, *BC* × *AD* (86); D7, *Cgi1*, 2 × 5b, *AD* × *BC* (82); D8, *L48*, 2 × 5b, *AD* × *BC* (77); D9, *Cgi24*, 2 × 5a, *BC* × *AA* (77); D10, *L16*, 7 × 9 (80); D11, *CG108*, 5 × 2a, *AB* × *BC* (91); D12, *Cgi8*, 3 × 2, *AD* × *BC* (82); E1, *Cgi1*, 3 × 2, *AC* × *BD* (81); E2, *Cgi8*, 2 × 5a, *BD* × *AC* (75); E3, *Cgi28*, 2 × 5a, *AD* × BC (78); E4, *CG49*, 3 × 2, *AC* × *BD* (84).

independent informative segregations for each founder, and (iii) the linkage relationships among markers (see MATERIALS AND METHODS; Table 4). We estimate that the founders (or the first generation hybrids of family 7 × 6) carried from 8 to 14 lethal genes, with 95% confidence limits for the better-sampled lines, 2, 3, and 7 × 6, ranging from 4 or 5 to 22 or 23 (Table 4). The average number of lethal genes per founder is 11.6, based only on segregations yielding IBD homozygotes. Counting two additional deleterious mutations inferred from significant deficiencies of grandparental heterozygotes (one each in lines 3 and 5), the average number of lethals per founder increases to 12.7.

DISCUSSION

Our results confirm the prediction that segregation of marker alleles in the Pacific oyster *C. gigas* is Mendelian in early developmental stages but becomes distorted as the progeny age. Analysis of segregation ratios confirms, moreover, that these distortions are largely attributable to selection against recessive deleterious mutations at fitness genes closely linked to the markers. There is no evidence for overdominance and only minor evidence for underdominance, possibly caused by epistasis.

Estimation of the genetic load: According to the linked selection model, deficiencies in IBD homozygote genotypes can be explained by the existence of closely linked highly deleterious alleles (Table 3). Because relative survival of genotypes is evaluated at 2–3 months rather than at sexual maturity, the severity of the segregation-ratio distortion may be underestimated and distances to linked markers overestimated. (This potential effect is illustrated by the increasing *c* with case number in Table 3, in which cases are ranked by severity of *AA* deficiency.) However, inspection of the cultch at the time of juvenile sampling did not reveal large numbers of dead individuals, nor were the spat overgrowing each other or crowding each other off the cultch. This suggests that most of the mortality occurred during the late larval stages or around the time of metamorphosis. The pattern of early mortality associated with IBD marker homozygotes supports the hypothesis of HUSBAND and SCHEMSKE (1996) that inbreeding depression in the early life stages of plants is caused mainly by lethal rather than mildly deleterious mutations.

The mean, over four inbred lines, of ~12 highly dele-

TABLE 3

Maximum-likelihood estimates of the selection coefficient (*s*) and recombination fraction (*c*) for a two-locus model explaining 20 cases of significantly distorted segregation ratios

Marker	Family	Case[a]	h^b	*s*	*c*
CG108	2 × 5b	C1	0.0	1.0	1.7
Cgi8	5 × 2b	C2	0.0	0.9	4.6
Cgi1	7 × 9	C3	0.0	0.9	6.8
Cgi28	7 × 9	C4	0.2	1.0	13.0
Cgi28	3 × 2	C5	0.0	1.0	12.3
Cgi1	7 × 9	C6	0.1	1.0	22.3
L10	3 × 2	C7	0.0	1.0	21
Cgi4	2 × 5b	C8	0.0	0.9	16.4
Cgi6	2 × 5b	C10	0.0	0.9	17.2
Cgi8	2 × 5b	C11	0.0	1.0	22.7
Cgi1	7 × 6	D1	0.3	1.0	0.01
L10	7 × 6	D2	0.0	1.0	1.8
L10	6 × 2b	D3	0.0	1.0	5.3
Cgi2	5 × 2a	D4	0.0	1.0	5.2
CG49	5 × 2a	D5	0.0	0.9	7.9
Cgi21	3 × 2	D6	0.0	0.9	7.6
Cgi1	2 × 5b	D7	0.0	0.9	12.6
Cgi24	2 × 5a	D9	0.0	1.0	19.8
L16	7 × 9	D10	0.0	0.9	16.6
CG108	5 × 2a	D11	0.0	0.9	17.4

[a] As referenced in Figure 2.

[b] Dominance of the allele under selection (*h*) is first assumed to be 0.0 to estimate *s* and *c*. In three cases, the observed genotype frequencies differ significantly from those expected; *h* is then increased by 0.1, *s* and *c* are recalculated, and the fit of observed to expected is checked, until agreement is reached.

terious recessive mutations per wild founder (range 8–14) may be compared to Bierne *et al.*'s (1998) estimate, from more limited data (four loci in two inbred families, grandparents not genotyped), of 15–38 genes causing inbreeding depression in the flat oyster *Ostrea edulis*. Likely, ours is a gross underestimate of the genetic load because of the following: (i) some lethal alleles would have been lost upon inbreeding of wild stocks; (ii) only 25% of lethal alleles carried by a grandparent become homozygous IBD in the F_2 generation; (iii) genomic coverage may be overestimated; and (iv) deleterious loci with little or mild effect could have been missed.

These minimum marker-based estimates of genetic load in oysters are larger, nevertheless, than the number of lethal equivalents estimated from inbreeding depression for other animals though comparable to the numbers for conifers. Data on effective number of lethals per gamete presented by Lynch and Walsh (1998; Tables 10.4–10.6), for example, imply means (and ranges) of lethal loads per individual of 2.8 (0.4–8.3) for mammals, 4.3 (1.9–5.8) for birds, 2.8 (2.1–3.7) for Drosophila, and 8.1 (3.4–10.8) for conifers. Marker-based mapping methods may provide slightly higher, though more accessible and accurate, estimates of genetic load for highly fecund species, for which early viability and inbreeding depression are difficult to quantify. Remington and O'Malley (2000), for example, estimate from amplified fragment length polymorphism markers that loblolly pine harbors >13 lethal equivalents compared to the 8.5 estimated from seed survival data.

Large genetic load is expected in highly fecund organisms (Williams 1975) and may be caused partly by the greater number of cell divisions, hence increased opportunities for mutation, necessary to produce each of the tens of millions of eggs or billions of sperm spawned each year. Still, the question remains whether mutational load alone explains distortion, typically, in 10% of marker segregation ratios examined in crosses of wild-caught bivalve molluscs (Wada 1975; Beaumont *et al.* 1983; Foltz 1986; Hu *et al.* 1993; McGoldrick *et al.* 2000). Segregation distortion in bivalves could be caused by a high mutation rate or by association of a large number of fitness genes with a handful of markers in a small genome (the haploid number of chromosomes is 10 in all cupped oysters, for example; Leitão *et al.* 1999). Thus, a lethal mutation rate greater than what is typical in other animals would be sufficient, but may not be necessary, to explain segregation distortion in bivalves.

Observation of numerous microsatellite null alleles might be consistent with a high mutation rate in the oyster, since a main cause of null or nonamplifying alleles at microsatellite loci is mutation in the priming sequence (Callen *et al.* 1993; Jones *et al.* 1998). Nonamplifying alleles were observed in each family at 1 or more loci. Twelve independent PCR-null alleles, derived from all lineages (4 from 93-2, 3 from 93-3, 2 each from 89-5 and 7 × 6, and 1 from 7 × 9), were segregating at 9 of the 19 loci. Such PCR-null alleles appear to be far more common in bivalves than in other organisms (McGoldrick *et al.* 2000), although a thorough quantitative review of the literature would be needed to confirm or refute this impression.

Implications for the cause of heterozygosity-fitness correlation: Our results appear to be consistent with the dominance (or associative overdominance) explanation of marker-associated heterosis in natural bivalve populations (Zouros and Pogson 1994; Britten 1996; David 1998) and of yield heterosis in crosses of inbred lines (Hedgecock *et al.* 1995, 1996). The large load of highly deleterious recessive mutations implies an even larger number of sublethal, subvital, or mildly deleterious mutations affecting survival and growth, which are positively correlated fitness traits for bivalves in the wild (David and Jarne 1997) and in artificial cultivation (Hedgecock *et al.* 1995, 1996). Under this hypothesis, heterosis results from the *general effect* (David *et al.* 1995) of selection against numerous deleterious recessive alleles, coupled with inbreeding or small effective population size, which promotes nonrandom associations

TABLE 4

Counting "lethal equivalents" in inbred lines of the Pacific oyster

Line	Tally of highly deleterious mutations[a]	No. of independent segregations[b]	Map length covered (cM)[c]	Estimated no. of lethal genes per founder genome[d]	Confidence limits (95%) on estimate[e]
Line 2	6	16	478	12.6	5.1–21.6
Line 3	1 (2)	5 (6)	132 (168)	7.6 (11.9)	0.2–21.3
Line 5	6 (7)	21 (23)	447 (511)	13.4 (13.7)	4.6–21.4
7×6 F_1	5	16	398	12.6	4.4–23.6

[a] Tally of highly deleterious recessive mutations ($s \approx 1.0$) implied by deficiencies of microsatellite IBD homozygotes. Numbers in parentheses for lines 3 and 5 include mutations implied by significant deficiencies of grandparental heterozygotes in F_2 or F_3 families.

[b] The number, over all descendant families and markers, of independent segregations producing an IBD homozygote for a grandparental allele. Numbers in parentheses include segregations producing deficiencies of grandparental heterozygotes.

[c] The sum of coverage provided by independent informative segregations, assuming that a recessive lethal mutation with 18 cM of a marker would produce a significant distortion of segregation ratio (see MATERIALS AND METHODS); coverage is adjusted for linkage of markers.

[d] The per genome estimate of "lethal equivalents" multiplies the number of lethal genes per map unit by 1000 cM, an estimate of the total map length of the oyster genome.

[e] Confidence limits, calculated from the exact binominal cumulative probability distribution (see MATERIALS AND METHODS), given n independent informative segregations (column 3) and k highly deleterious recessive mutations detected as deficiencies of IBD homozygotes (column 2).

(gametic phase disequilibria) between fitness genes and markers.

Although general effects have generally been dismissed for marine populations, which seem large and randomly mating, DAVID *et al.* (1997b) suggest that even small levels of inbreeding, ~1%, could maintain sufficient disequilibria among markers and fitness genes to produce the observed marker-associated heterosis. Moreover, the ratio of effective to actual population size in marine populations may be greatly reduced by high variance in reproductive success (HEDGECOCK 1994; LI and HEDGECOCK 1998), which could also generate temporary gametic phase disequilibria and marker-associated heterosis. BIERNE *et al.* (2000) show analytically and by simulations that nonequilibrium situations, such as sustained bottlenecks, can generate large associative overdominance. They also show that the association arises mostly from individual variation in inbreeding coefficient. Small population sizes, as well as recurrent inbreeding in large populations, would enhance inbreeding variance relative to that in large randomly mating populations. Species that have both a transient population fragmentation due to small reproductive groups (HEDGECOCK 1994; DAVID *et al.* 1997a,b) and high genetic load, such as shown in this study of the Pacific oyster, should show significant associative overdominance within local samples. Indeed, FUJIO (1982) showed negative correlation of meat weight with the inbreeding coefficient, F, and positive correlation of weight with number of heterozygous loci per individual from natural populations of the Pacific oyster in Japan. Fujio attributed the significant F (mean 0.152, range from 0.0 to 0.415 over 20 populations) to the tendency for oysters to form an inbreeding population structure.

At the same time, our results are inconsistent with overdominance as the principal explanation of marker-associated heterosis in natural bivalve populations. Overdominance at closely linked fitness genes should consistently favor marker heterozygotes, yet we see deficiencies of heterozygotes relative to homozygotes or no difference in the relative survival of *AB* and *BB* genotypes, when *AA* IBD homozygotes are deficient in $AB \times AB$ crosses (Figure 2C). Finally, instead of consistent superiority in the relative survival of heterozygotes across genetic backgrounds, we see consistent deficiencies of grandparental heterozygotes, again suggesting selection against deleterious recessive alleles. These interpretations are often supported by data from related families, showing deficiencies of IBD homozygotes for grandparental alleles.

Genetic load may explain other bivalve phenomena: A large load of deleterious recessive mutations also explains two other phenomena in bivalves. The first is negative correlation of growth rate with degree of somatic cell aneuploidy within and among families of the Pacific oyster (THIRIOT-QUIEVREUX *et al.* 1992; ZOUROS *et al.* 1996; LEITÃO *et al.* 2001). Given a high genetic load, aneuploid somatic cells would very likely be hemizygous for recessive deleterious mutations, resulting in the observed negative correlation of growth with degree of somatic cell aneuploidy. Thus, whether there is inbreeding or not in natural populations, bivalves may suffer substantially from the combined effects of a high load of recessive mutations and somatic cell aneuploidy.

The second phenomenon, reported for both the coot clam *Mulinia lateralis* and the Pacific oyster, is high or even complete retention of maternal allozyme heterozygosity in gynogenetic progeny produced by inhibition

of the second meiotic division (Guo and Gaffney 1993; Guo and Allen 1996). This observation has been explained as interference of a second crossover following formation of the first chiasma between centromere and marker. However, high heterozygosity in gynogenetic progeny could also be caused by mortality of non- or double recombinants, which would be homozygous for any lethal gene located on the same chromosome arm or distal to the second crossover, respectively.

An animal model for future study of heterosis: This study supports the dominance explanation of heterosis, a phenomenon likely to be as significant for the production of cultured oysters as it has been for crops (Crow 1998). Ironically, the oyster may provide a useful animal model for understanding the general phenomenon of heterosis. Analyses of the physiological basis of growth rate differences between inbred and hybrid oysters already suggest that heterosis is achieved by metabolic and feeding efficiencies that are not easily measured in plants (Hedgecock *et al.* 1996; Bayne *et al.* 1999). A genetic linkage map is being constructed by typing microsatellite markers in larvae, which show little distortion of segregation ratios. Subsequent mapping of QTL for heterosis and determination of their mode of action (additive, dominant, overdominant, or epistatic), together with physiological and functional genomic analyses of gene expression profiles in inbreds and hybrids, should shed light on the causes of this biologically and economically important phenomenon.

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