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Hybrid vigor in Pacific oysters: an experimental approach using crosses among inbred lines

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Abstract

Two competing genetic hypotheses for heterosis, dominance and overdominance, have been championed to explain positive correlations between allozyme heterozygosity and fitness-related traits for bivalve molluscs. To begin to test these hypotheses, we made controlled crosses among inbred lines of the Pacific oyster *Crassostrea gigas*. In such mating experiments, heterosis (h_p) can be defined and quantified through ANOVA as $Q/L > 1.0$ or < -1.0, where L is the trait-difference between the two parental inbred lines and Q is twice the deviation of the hybrid from the mid-parent value (Griffing, 1990).

Inbred lines of the Pacific oyster were made by selfing hermaphrodites; brood stock pedigrees and a grand mean fixation index of $F=0.5$ were confirmed by allozymes. In two separate experiments pairs of inbred lines were crossed in 2×2 fashion to produce two inbred and two hybrid progeny genotypes. Each genotype was replicated by pairwise matings and replicates were grown in multiple containers (8 l plastic bags), from which data on larval mortality (proportional decrement in population number per day) and size (shell-height) were obtained. In the first experiment, the two inbred genotypes differed significantly from each other in daily larval mortality (-0.115 vs. -0.881) and both hybrids had lower mortalities than inbred genotypes (-0.034 and -0.078); mean h_p, 1.15, was significantly greater than 1.0. By Day 14, larvae of one hybrid group were significantly smaller than those of the inbred genotypes (241.0 μm vs. 253.5 and 259.2 μm, respectively); only a very small part of this difference could be attributed to negative correlation between larval density and growth. Negative heterosis for larval size was highly significant ($h_p = -5.36$, $P<0.001$) and persisted to the juvenile stage (mean $h_p = -1.34$, ns, for shell-height at Day 154 and $h_p = -7.77$, $P<0.001$, for live-weight at Day 163).

In the second experiment, inbred genotypes again differed significantly in daily larval mortality (-0.341 vs. -0.654). One hybrid group had significantly less daily mortality than either parent (-0.110), but the other was similar to the best parent (-0.347), so that for one hybrid, $h_p=2.47$, $P<0.01$, and for the other, $h_p=0.96$, ns). Variance among genotypes for larval shell-length was highly significant on Days 2, 7, and 14, with heterosis evident on Days 2 and 7 ($h_p=1.04$ and $h_p=3.84$, respectively). At 11 months of age, hybrid shell-height averaged 150% of the shell-height

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for the best inbred parent and $h_p \approx 7.7$. These measurements of heterosis, both positive and negative, implicate a third explanation of heterosis, epistasis. Intercrosses of F_1 hybrids can be used to discriminate among dominance, overdominance and epistasis hypotheses for quantitative trait loci (QTL) causing hybrid vigor.

Keywords: Heterosis; Growth; Survival; *Crassostrea gigas*; Inbred lines; Fixation

1. Introduction

The genetic and physiological bases of hybrid vigor or heterosis are poorly understood even for major agricultural crops (Griffing, 1990). Three alternative genetic explanations exist for the phenomenon, dominance, overdominance, and epistasis (reviewed by Wright, 1977). Debate over the first two hypotheses was renewed in the 1980s by reports that individual heterozygosity at allozyme-coding loci is positively correlated with fitness-related traits, primarily growth rate and metabolism in cohorts of juvenile bivalves (reviewed by Zouros and Foltz, 1987; Pogson and Zouros, 1994). Paradoxically, deficiencies of allozyme heterozygotes with respect to random mating proportions have also been commonly observed in bivalves, and the magnitude of heterozygote deficiency at a locus has been correlated with its contribution to the heterozygosity-fitness correlation (Gaffney et al., 1990). The causes of these correlations have been difficult to resolve without experiments.

The debate over the causes of heterosis is of more than academic interest, as the outcome could have substantial implications for developing genetic improvement programs for cultured bivalve molluscs. Additive genetic variance for production-related traits has been measured in a number of bivalves (reviewed by Gjedrem, 1983; Lannan, 1980; Mallet et al., 1986; Rawson and Hilbish, 1990) so that well designed individual or family selection programs are likely to result in improvement. Nevertheless, were a large fraction of genetic variance in production-related traits non-additive, especially overdominant, crossbreeding among inbred lines might be preferable to linebreeding as a means of improving bivalves. Substantial non-additive, though not necessarily overdominant components of genetic variance have been observed for larval survival (Lannan, 1980) and body size at harvest for the Pacific oyster *Crassostrea gigas* (Hedgecock et al., 1991).

To try both to resolve the causes of heterosis and to decide whether crossbreeding should be considered as a component of breeding programs for the Pacific oyster, we have adopted a classical experimental approach, controlled crosses among inbred lines. Cupped oysters like *Crassostrea gigas* can be inbred more rapidly than farm or genetic-model animals because hermaphroditism, occasionally simultaneous but more typically alternating, permits self-fertilization (Lannan, 1971). In factorial crosses between two inbred lines, heterosis (or potence, h_p) for one or more traits can be defined as $Q/L > 1.0$ or < -1.0, where Q and L are the quadratic and linear contrasts estimated from analysis of variance (ANOVA), corresponding to twice the deviation of the hybrid from the mid-parent value and the difference between the mean trait-values of the two parental inbred lines, respectively (Griffing, 1990).

2. Materials and methods

2.1. Inbred lines

Inbred lines were initiated in 1989 by Ken Cooper (Coast Oyster, now Coast Seafoods Co., Bellevue, Washington), who combined sperm and eggs from simultaneous hermaphrodites collected from the naturalized population of *Crassostrea gigas* in Dabob Bay, Washington. First generation progeny (G_1) from these self-fertilizations were grown on long lines in Quilcene Bay, Washington, and Humboldt Bay, CA; subsamples of the lines were transferred in 1991 to Hog Island Oyster Co., Tomales Bay, CA, near the Bodega Marine Laboratory. Lines are named hereafter by number, i.e. Line 89-5 is Line 5 or simply 5.

2.2. Experimental Design

Two experiments were made in this study, both full factorial matings between males and females from two inbred lines. The four genotypes produced by these factorial mating experiments are numbered sequentially, starting with the inbred cross of the first line and listing the male parent first; for example, in the first experiment, the four genotypes are: (1) 4×4, inbred genotype for Line 4; (2) 4×5, hybrid genotype with male parent 4; (3) 5×4, reciprocal hybrid genotype with male parent 5; (4) 5×5, inbred genotype for Line 5. Each genotype was replicated by independent, pairwise matings; these replicates are denoted by letters after the genotype symbol, i.e. 4×4A is the full-sib mating of male A and female A from Line 4, 4×5A is the hybrid cross of male A from Line 4 with female A from Line 5, etc.

Hatchery methods were substantially those described by Breese and Malouf (1975). Prior to spawning, oysters were sexed by microscopic examination of biopsied gonadal tissue-smears. Prospective parents were segregated by sex to different rooms to prevent accidental fertilizations. Eggs were gently stripped into seawater, washed, counted, resuspended at 1000 eggs ml^{-1}, allowed to stand with gentle aeration for 30–45 min. in order to develop resistance to polyspermy (Stephano and Gould, 1988), and divided into two batches for inbred and outbred crosses. A small aliquot of unfertilized eggs was reserved and subsequently examined for evidence of accidental fertilization. None was ever observed. Sperm was extracted from males by Pasteur pipette, suspended in small volumes of seawater, and examined microscopically for motility. The bodies of all parents were put in labelled plastic bags and frozen at −70°C for subsequent allozyme electrophoresis.

Eggs were fertilized by adding several millilitres of dense sperm suspension per liter of eggs; samples of the sperm–egg suspension were quickly examined microscopically, and if necessary, more sperm was added to achieve a bound-sperm:egg ratio of about ten. Sperm was allowed 2–5 min to fertilize eggs at 1000 eggs ml^{-1}; fertilized eggs were then stocked into 8 l plastic bags (Borgeson et al., 1989) at five zygotes ml^{-1}. Samples of the fertilized eggs were examined microscopically after several hours to determine percent fertilized. Larval cultures were kept at 20–24°C, fed *Isochrysis galbana* (Caicos Islands strain) according to a standard schedule, and screened and resuspended in fresh seawater every 4 days. At Day 18, a plastic-mesh pouch, containing oyster-shell broken and screened to a

size of about 0.5 cm^2, was placed in each larval culture bag. Settlement was allowed over 15–17 days. Spat on cultch were transferred to seed bags and placed in off-bottom cages in Tomales Bay for growout.

2.3. Experiment 1: 89-4 × 89-5

The first cross of Lines 4 and 5 was made on 14 May 1993. Three males and three females were available for 89-4. Two surviving 89-5 G_1 individuals were both female; three G_2 males for this line were therefore obtained from the second generation of 89-5 spawned in 1992. Thus, initially, genotypes 4 × 4 and 5 × 4 were each represented by three replicates, while genoytpes 4 × 5 and 5 × 5 were each represented by two replicates. Each of the ten replicate crosses was initially stocked into four 8 l bags.

The numbers of larvae per bag on Days 2, 6, and 14 were estimated from counts made by screening larvae, concentrating them in an appropriate smaller volume (usually in 1 l at Day 2, 80–100 ml, thereafter), and taking ten, 0.25 ml samples from this concentrate while plunging to keep it well mixed. The total numbers of larvae counted on Days 2, 6, and 14 were 1363, 7968, and 5473, respectively. Larvae remaining in the concentrate were immediately returned to a fresh 8 l bag. Sampled larvae were counted under a dissecting scope, then, unless too few in number, were placed in tissue-culture well-plates and preserved with buffered formalin for subsequent measurements of shell-length. Because of low rates of fertilization or poor survival or both, replicate larval cultures were either lost or pooled; as early as Day 2, for example, replicate 5 × 5B was lost entirely and the four bags within each of replicates 5 × 4A, 5 × 4B, 5 × 4C, and 5 × 5A were pooled into one bag. Larval counts for all 3 days are thus available for only seven replicates, 4 × 4A, B, C; 4 × 5A, B; 5 × 4C; 5 × 5A.

Preserved larvae ($n = 722$) from the Dy 14 sample were recorded on video tape, using an inverted microscope at 100×. The video tape was projected on a monitor, and shell-lengths were measured by vernier calipers; measurements were calibrated by recording the stage micrometer at the same magnification. Because of low numbers in replicates 4 × 4A and 5 × 4C, larval size data are available on only five of the seven surviving replicates, comprising three of the four genotypes.

Larvae began to settle after 14 days and were provided with cultch from Day 18 to Day 33. All spat for each of the seven surviving replicates were combined into a single plastic cage; the seven cages were tagged and transferred to Tomales Bay on 24 June, 41 days after fertilization. Shell-lengths and live-weights for juveniles from all seven replicates were measured with vernier calipers and top loading balance, respectively, on 16 October 1993 ($n = 337$); individuals ($n = 238$) from replicates 4 × 4B, 4 × 5B, and 5 × 5A were re-weighed on 1 February 1994.

2.4. Experiment 2: 89-6 × 89-7

A cross of inbred Lines 6 and 7 was made on 29 June 1993. Three female parents but only one male parent were available from each of these two lines. Allozyme analysis on 30 June revealed that female parent 7C was not from inbred Line 7, so all crosses involving this parent were destroyed. Thus, initially, genotypes 6 × 6 and 7 × 6 were each represented

by three replicates, while genotypes 6 × 7 and 7 × 7 were each represented by two replicates. Each of the ten replicate crosses was initially stocked into three, 8 l bags.

Larvae from each bag-culture were counted on Days 2 (n = 20,532), 7 (n = 6487), and 14 (n = 6136), as described for the previous experiment except that, to increase the precision of population estimates, 24, 0.25 ml samples were taken at concentrations designed to yield a total of about 400 larvae per bag per count. Numbers of larvae measured for shell-length on Days 2, 7, and 14 were 1335, 1268, and 1427, respectively. Survival of bags and replicates was much better in this experiment, so that all replicates were present through Day 7; by Day 14, both replicates of 7 × 7 were reduced to numbers too low to sample.

Larvae were provided with cultch from Day 18 to Day 35, when spat for each of the ten replicates were placed in tagged plastic cages. The ten cages were transferred to Tomales Bay on 4 August, 36 days after fertilization. Shell-lengths for juveniles from eight surviving replicates (all but one individual of genotype 7 × 7 were lost) were measured by vernier calipers on 4 June 1994 (n = 454).

2.5. *Allozyme typing*

To confirm pedigree, parents from Lines 4, 5, 6, and 7 were typed at 24 allozyme markers by methods described elsewhere (Hedgecock and Sly, 1990; Hedgecock, 1994). Having originated from self-fertilization, no inbred line should be segregating for more than two alleles at any locus. Phenotypes for each oyster were compared to the allozyme profile previously determined for their line (McGoldrick and Hedgecock, unpublished data, 1995). Also, average heterozygosity in the first inbred generation (H_{G1}) should be half that of the line's founder (H_P), so that the mean fixation index, $F = 1 - (H_{G1}/H_P)$, should be 0.5. Mean fixation indices were calculated from observed H_{G1} and an H_P inferred from first generation segregation data (not shown); an exact two-tailed probability for the number of heterozygous genotypes observed in each family was obtained from the appropriate cumulative binomial distribution, using a spreadsheet (Quattro Pro, v. 6.0, Novell, Inc.).

2.6. *Statistical Analysis*

Traits analyzed were: (1) daily mortality ($M_{di,df} = \log[N_f/N_i]/d_{f-i}$), where N_f and N_i are final and initial population sizes (as estimated from counts adjusted for destructive sampling) and d_{f-i} is the interval in days between counts; (2) shell-length (μm) for larvae; (3) shell-height (cm) for juveniles; (4) natural log of live-weight (g) for juveniles.

ANOVA or ANCOVA of experimental results was performed, following Griffing (1990), using the general linear model (GLM) procedure of the Statistical Analysis System (SAS) for the personal computer (SAS Institute Inc., 1988). Genotype and replicate matings within genotypes were random effects. The general ANOVA table for testing the significance of genotype and of replicates-within-genotype, when available, and for estimating the linear and quadratic contrasts L and Q is given in Table 1. Significance testing of main effects employed type III sums of squares and expected mean squares. For three of four analyses of Experiment 1, replicates-within-genotype had to be used as the mean square error and could not itself be tested by variation among observations within replicates. Owing to unbalanced designs, variance components for the orthogonal contrasts did not sum to

Table 1
Analysis of variance (ANOVA) for a full-factorial cross of two inbred lines of Pacific oysters, producing four genotypes, with three, replicate pair-matings nested within genotype, and observations on four larval cultures per replicate, a total of 48 larval cultures. Degrees of freedom (d.f.) correspond to an analysis of daily mortality in which there is only one observation per larval culture; d.f. for sampling error would be larger for observations of individual shell-lengths within cultures. MS is the mean square symbol for observational components of variance; E(MS) is the expected mean square allowing calculation of causal components of variance. Partitioning of genotype effects into linear and quadratic orthogonal contrasts is also shown (see text); Q2 and Q3 refer to the two reciprocal hybrid genotypes

Source	d.f.	MS	E(MS)
Genotypes (G)	3	MS_G	$\sigma^2_e + 4\sigma^2_{R(G)} + 12\sigma^2_G$
Linear [G(L)]	1	$MS_{G(L)}$	$\sigma^2_e + 4\sigma^2_{R(G)} + 12\sigma^2_{G(L)}$
Quadratic [G(Q2)]	1	$MS_{G(Q2)}$	$\sigma^2_e + 4\sigma^2_{R(G)} + 12\sigma^2_{G(Q2)}$
Quadratic [G(Q3)]	1	$MS_{G(Q3)}$	$\sigma^2_e + 4\sigma^2_{R(G)} + 12\sigma^2_{G(Q3)}$
Replicates [R(G)]	8	$MS_{R(G)}$	$\sigma^2_e + 4\sigma^2_{R(G)}$
Sampling Error	36	MS_e	σ^2_e
Total	47		

that for genotype and are thus considered approximate. Significance of these contrasts was tested with the same error term as that used to test mean square for genotype; the components themselves were approximated by their relative contribution to the sum of their mean squares. Potence values ($h_p = Q/L$) for the two reciprocal hybrids were obtained as the ratio of SAS estimates for the contrasts.

To test whether variation in larval density among bag-cultures might have confounded differences between genotypes, we performed regression analyses on shell-length means and numbers of larvae per bag on Day 14 for Experiment 1 ($n = 14$) and on Days 2, 7, and 14 for Experiment 2 ($n = 22$, 23, and 20, respectively).

3. Results

3.1. Experiment 1: 89-4×89-5

Genotypes at 24 allozyme loci for the six 89-4 brood stock used for this experiment are each consistent with the multilocus genotype inferred for the hermaphroditic founder of this line. The average heterozygosity for these G_1 individuals, 0.14, compared to that inferred for the founder, 10/24 allozyme loci or 0.42, yields a mean fixation index, $F = 1 - (0.14/0.42) = 0.67$. Likewise, the five brood stock from Line 5 each have allozyme genotypes consistent with that inferred for the founder, and $F = 1 - (0.18/0.5) = 0.63$. Mean F for Line 4 is significantly greater than the expected 0.5 ($P = 0.0135$), but mean F for Line 5 is not ($P = 0.067$).

Larval density on Day 2 averaged 2.84 larvae ml^{-1} and ranged from 0 to 4.65 larvae ml^{-1}. By Day 6, mean density was 1.67 larvae ml^{-1} with a range from 0.01 to 3.65, and by Day 14 mean density was 1.13 larvae ml^{-1} with a range from 0 to 1.84. The ANOVA model for daily larval mortality over the Day 2–6 interval of this experiment is not significant (not shown). For the Day 6–14 interval, however, genotypic variance (σ^2_G) is highly

Table 2
ANOVA results for Experiment 1, a full-factorial cross of inbred Pacific oyster lines 89-4 and 89-5. Traits are $M_{6,14}$, daily mortality between Days 6 and 14 of larval culture; Sl_{14}, larval shell-length (μm) on Day 14; Sh_{154}, juvenile shell-height (cm) on Day 154; and Lwt_{263}, juvenile live-weight (g; log-transformed for analysis) on Day 263. Results are given as A, percent contribution of variance components (see Table 1) to total variation, and B, significance of each component

Variance component	Traits							
	$M_{6,14}$		Sl_{14}		Sh_{154}		Lwt_{263}	
	A	B	A	B	A	B	A	B
σ^2_G	95.2	**	2.5	**	8.0	ns	6.4	**
$(\sigma^2_{G(L)})$	(50.4)	**	(0.1)	ns	(5.1)	ns	(0.2)	ns
$(\sigma^2_{G(Q2)})$	(29.6)	**	(2.4)	***	(0.4)	ns	(6.2)	***
$(\sigma^2_{G(Q3)})$	(15.1)	*	–	–	(2.4)	ns	–	–
$\sigma^2_{R(G)}$	–	–	–	–	24.9	***	–	–
σ^2_e	4.8		97.5		67.1		93.6	

ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3
Trait values or means for genotypes and replicates in Experiment 1, a full-factorial cross of inbred Pacific oyster Lines 89-4 and 89-5. Traits as in Table 2. Daily mortalities are single observations per replicate based on total larval counts across all bag cultures on Days 6 and 14

Trait	Genotype	Replicates			LS Means[a]
		A	B	C	
$M_{6,14}$	(1) 4×4	−0.168	−0.159	−0.016	−0.115[a]
	(2) 4×5	−0.0[b]	−0.069	–	−0.034[a]
	(3) 5×4	–	–	−0.078	−0.078[a]
	(4) 5×5	−0.881	–	–	−0.881[b]
Sl_{14}	(1) 4×4	–	246.0	255.5	253.5[a]
	(2) 4×5	258.9	217.2	–	241.0[b]
	(3) 5×4	–	–	–	–
	(4) 5×5	259.2[c]	–	–	259.2[a]
Sh_{154}	(1) 4×4	2.43	3.35	2.18	2.65[b]
	(2) 4×5	2.86	3.00	–	2.93[b]
	(3) 5×4	–	–	2.13	2.13[c]
	(4) 5×5	3.63	–	–	3.63[a]
Lwt_{263}	(1) 4×4	–	2.28	–	2.28[a]
	(2) 4×5	–	1.47	–	1.47[b]
	(3) 5×4	–	–	–	–
	(4) 5×5	2.60	–	–	2.60[a]

[a] Least square means sharing superscripts are not significantly different by Student-Newman-Keuls' multiple range test.[b] Day 14 population estimate greater than Day 6, post-count estimate; Day 14 estimate thus set equal to Day 6 estimate.[c] Mean of observations on pooled-replicate culture.

significant in the ANOVA model and accounts for 95.2% of variance in daily mortality (Table 2). Both hybrid genotypes have lower mean daily mortalities than the two inbred genotypes (Table 3), although neither has significantly lower mortality than 4×4. The contrast between mean daily mortalities for the two inbred genotypes ($\sigma^2_{G(L)}$) is highly significant and accounts for half of total variance. The quadratic contrasts are both significant, although that for 4×5 ($\sigma^2_{G(Q2)}$) is highly significant and accounts for about twice as much variation as that for 5×4 ($\sigma^2_{G(Q3)}$; Table 2). Potence values for the reciprocal hybrids and for the hybrid mean are all greater than one (Table 6A).

The genotype effect in the ANOVA model for larval shell-length on Day 14 (Sl_{14}) is also highly significant, although σ^2_G accounts for only 2.5% of total variance in this trait (Table 2). Owing to low numbers of 5×4 larvae on Day 14, no data are available for this genotype. For this trait, the two inbred genotypes are not significantly different from each other, but both are significantly larger than the 4×5 larvae. Nearly all of the genotypic variance is attributable to the single quadratic contrast $\sigma^2_{G(Q2)}$, which is highly significant (Table 2). The potence value in this trait is −5.36 (Table 6A). Regression of mean Sl_{14} on the number of larvae per bag on Day 14 was not significant ($P=0.159$).

The ANOVA model for juvenile shell-height on Day 154 (Sh_{154}) is highly significant, but replicate-within-genotype is the only significant source of variance (Table 2), owing primarily to variation among replicates for 4×4 (Table 3). Hybrid genotype 5×4 is smaller than both inbred genotypes, while the reciprocal hybrid, 4×5, is somewhat larger than the smaller of the two inbred genotypes, 4×4. By Day 263, however, differences among mean live-weights for replicates 4×4B, 4×5B, and 5×5A, Table 3) are highly significant, accounting for 6.4% of total variance (Table 2). Almost all of this variance is attributable to the highly significant negative deviation of the 4×5 hybrid from the average inbred value, which yields a potence value of −7.77 (Table 6A).

3.2. 2: 89-6×89-7

Genotypes at 20 allozyme loci for the four 89-6 brood stock used for this experiment are each consistent with the multilocus genotype inferred for the Line 6 founder. However, average heterozygosity for the G_1, 0.20, is identical to that of the founder, which yields a mean fixation index, $F=0.0$, highly significantly less than the expected 0.5 ($P=0.000025$). Of four brood stock from Line 7 examined, one female has an allozyme genotype that is inconsistent with that inferred for the founder, at six of 20 loci; offspring from this female are not included in subsequent analyses. Genotypes for the remaining three brood stock are consistent with the founder, and $F=1-(0.12/0.29)=0.59$, slightly but not significantly greater than the expected 0.5 ($P=0.629$).

Larval density on Day 2 averaged 2.27 larvae ml^{-1} and ranged from 0.07 to 5.36 larvae ml^{-1}. By Day 7, mean density was 0.82 larvae ml^{-1} with a range from 0.0 to 2.72, and by Day 14 mean density was 0.66 larvae ml^{-1} with a range from 0.01 to 2.76. Variance among genotypes makes up 65.8% of total variance in daily larval mortality between Days 2 and 7. Nearly all of genotypic variance appears to be attributable to the quadratic contrast for the 7×6 hybrid ($\sigma^2_{G(Q3)}$, Table 4), which has the lowest mean daily mortality of any genotype (Table 5). Variance among replicates-within-genotype is non-significant. The

Table 4
ANOVA results for Experiment 2, a full-factorial cross of inbred Pacific oyster Lines 89-6 and 89-7. Traits are $M_{2,7}$, daily mortality between Days 2 and 7 of larval culture; Sl_2, Sl_7, and Sl_{14}, larval shell-lengths (μm) on days 2, 7, and 14; Sh_{340}, juvenile shell-height (cm) on Day 340. Results are given as A, percent contribution of variance components (see Table 1) to total variation, and B, significance of each component

Variance component	Traits									
	$M_{2,7}$		Sl_2		Sl_7		Sl_{14}		Sh_{340}	
	A	B	A	B	A	B	A	B	A	B
σ^2_G	65.8	**	2.2	**	33.7	**	32.3	**	19.0	***
$(\sigma^2_{G(L)})$	(8.0)	ns	(1.0)	**	(1.1)	ns	–		(0.2)	ns
$(\sigma^2_{G(Q2)})$	(6.2)	ns	(0.7)	*	(6.8)	ns	–		(11.0)	**
$(\sigma^2_{G(Q3)})$	(51.5)	**	(0.5)	*	(25.2)	**	–		(7.8)	*
$\sigma^2_{R(G)}$	0.0	ns	0.0	ns	7.6	***	1.9	***	0.4	ns
σ^2_e	34.2		97.8		59.3		65.8		80.6	

ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Table 5
Trait means for genotypes and replicates in Experiment 2, a full-factorial cross of inbred Pacific oyster Lines 89-6 and 89-7. Traits as in Table 4

Trait	Genotype	Replicates			LS Means[a]
		A	B	C	
$M_{2,7}$	(1) 6×6	−0.252	−0.313	−0.458	−0.341[a]
	(2) 6×7	−0.384	−0.309	–	−0.347[a]
	(3) 7×6	−0.064	−0.114	−0.153	−0.110[a]
	(4) 7×7	−0.521	−0.787	–	−0.654[b]
Sl_2	(1) 6×6	72.4	72.1	71.5	72.0[b]
	(2) 6×7	74.3	73.8	–	74.0[a]
	(3) 7×6	74.1	73.4	74.0	73.8[a]
	(4) 7×7	74.6	73.2	–	73.9[a]
Sl_7	(1) 6×6	100.1	101.9	107.5	103.2[c]
	(2) 6×7	107.0	119.3	–	113.1[b]
	(3) 7×6	132.1	123.2	131.4	128.9[a]
	(4) 7×7	98.7	82.5	–	90.6[d]
Sl_{14}	(1) 6×6	207.9	205.5	205.0	206.1[b]
	(2) 6×7	221.0	231.2	–	226.1[b]
	(3) 7×6	268.0	261.7	281.3	270.3[a]
	(4) 7×7	–	–	–	–
Sh_{340}	(1) 6×6	4.04	3.92	4.14	4.03[a,b]
	(2) 6×7	6.68	6.42	–	6.55[a]
	(3) 7×6	6.48	5.82	6.00	6.10[a]
	(4) 7×7	–	3.35[b]	–	3.35[b]

[a] Least square means sharing superscripts are not significantly different by Student-Newman-Keuls' multiple range test.[b] Observation for sole surviving individual.

Table 6
Potence values ($h_p = Q/L$) for mortality and shell-size traits in inbred and hybrid genotypes produced by factorial crosses between inbred lines of Pacific oysters. Potence values for the reciprocal hybrids are symbolized as $_2h_p$ and $_3h_p$, while that for the hybrid mean is h_p

A. Experiment 1: 89-4×89-5.	Traits			
	$M_{6,14}$	Sl_{14}	Sh_{154}	Lwt_{263}
$_2h_p$	1.21	−5.36	−0.96	−7.77
$_3h_p$	1.10	–	−1.72	–
h_p	1.15	–	−1.34	–
B. Experiment 2: 89-6×89-7.	Traits			
	$M_{2,7}$	Sl_2	Sl_7	Sh_{340}
$_2h_p$	0.97	1.14	2.59	8.40
$_3h_p$	2.47	0.94	5.10	7.09
h_p	1.72	1.04	3.84	7.74

potence value for 6×7 is just slightly less than 1.0, but potence values for the reciprocal hybrid, 7×6, and the hybrid mean are both much larger than 1.0 (Table 6B).

Genotypic variance is a highly significant component of variation in larval shell-lengths on Days 2, 7, and 14 and constitutes nearly a third of total variance for Sl_7 and Sl_{14} (Table 4). Contrasts are possible only in the Day 2 and Day 7 ANOVA because 7×7 was not sampled on Day 14. The linear contrast and the quadratic contrast for 6×7 are significant only for Sl_2, while the quadratic contrast for 7×6 is significant for both Day 2 and Day 7 shell-length measurements. Although highly significant, $\sigma^2_{R(G)}$ constitutes only a small fraction of total variance in Sl_7 and Sl_{14}. Potence for 6×7 hybrid shell-length is greater than 1.0 for Days 2 and 7 (Table 6B). Potence for 7×6 hybrid shell-length is 0.97 on Day 2 but 5.1 on Day 7. Potence for the hybrid shell-length mean is greater than 1.0 on both days. The regression of mean shell-length on number of larvae per bag for Day 2 is significant ($P = 0.034$) but with a very small coefficient (-0.000); the Day 7 and 14 regressions are not significant ($P = 0.65$ and 0.79, respectively).

Variation among genotypes makes up a highly significant 19% of total variation in shell-height for the 11 month old juveniles from this cross (Table 4). Unfortunately, only a single 7×7 individual survived to this age. The shell-height of this single individual is included in the ANOVA reported in Table 4 and Table 5 so that L and Q can be estimated. Results and least squares means for ANOVA with and without this individual (the latter not shown) are practically identical for the three remaining genotypes. The linear contrast is not significant, but both quadratic contrasts are significant with $\sigma^2_{G(Q2)}$ being perhaps slightly larger than $\sigma^2_{G(Q3)}$ (Table 4). Mean shell-heights for the hybrids are 150% of the mean shell-height for the 6×6 inbred genotype (Table 5). Potence values for hybrid genotypes range from 7.1 to 8.4 (Table 6).

4. Discussion

In hybridizing inbred lines of the Pacific oyster *Crassostrea gigas*, we found allozyme markers essential for verifying the pedigrees of parents. While the majority of the inbred

brood stock had alleles consistent with the inferred genotype of their line's hermaphroditic founder, contaminants were detected. Indeed, a third 2×2 experiment conducted in 1993 had to be destroyed for this reason, and in the 6×7 cross, one of the female 89-7 parents was found to be a contaminant and her progeny were destroyed.

A grand mean fixation index calculated for all confirmed inbred brood stock, over all allozyme markers and all four inbred lines, is 0.47, not statistically different from the expected 0.5. There is, nevertheless, significant heterogeneity in fixation among lines; two Lines (5 and 7) do not deviate significantly from 0.5, but one (Line 4) is significantly overfixed and another (Line 6) is very significantly underfixed. These two results, as well as significant heterogeneity in fixation among allozyme markers, also obtain in a larger study involving these and three other inbred lines (McGoldrick and Hedgecock, unpublished data, 1995).

Replication is also important in studying the quantitative traits of aquatic animals. Variation among different larval bag-cultures of the same progeny group was the sampling error in our experiment. However, we also attempted to estimate the experimental error associated with the use of different parents from incompletely inbred lines. Unfortunately, loss of replicate larval cultures in the first experiment forced us to use variation among replicate crosses within genotype as the sampling error in three of four subsequent analyses. Only for juvenile shell-height were we able to estimate a variance component for cross replication; $\sigma^2_{R(G)}$ was highly significant in this case, owing primarily to variation among replicates for the 4×4 inbred genotype (Table 3) and accounting for nearly 25% of the total variation (Table 2). In the better balanced second experiment, however, experimental error was estimable for all five traits. Here, $\sigma^2_{R(G)}$ was non-significant for three traits and highly significant for two others. Even when significant, $\sigma^2_{R(G)}$ accounted for only a small percentage of total variation (Table 4). The rank orders of genotypic means for larval shell-length on Days 7 and 14 and for juvenile shell-height on Day 340 (i.e. $7\times6>6\times7>6\times6>7\times7$) are remarkably consistent across replicates (Table 5), perhaps because only a single male parent from each line was used and the cross replicates were thus paternal half-sibs. Interestingly, the hybrids reverse rank size-order by 11 months of age.

Despite some variability among replicates, the rank orders of inbred and hybrid genotypes in both experiments are maintained from the larval to the juvenile and adult stages. This observation is relevant to observations of widespread deficiencies in heterozygous allozyme genotypes in bivalve populations, particularly at settlement. Heterozygote deficiency is puzzling in light of evidence that individual heterozygosity and fitness-related traits are positively correlated in juvenile molluscs. One explanation of this paradox is that the relative fitnesses of allozyme heterozygotes and homozygotes reverse between the larval and juvenile stages (Singh and Green, 1984). Our results are inconsistent with this hypothesis.

Our experiments demonstrate significant non-additive genetic components of variance in larval mortality, larval size, and juvenile size. Deviations of hybrid genotypes from the mean of the inbred genotypes (mid-parent value) were significantly greater than 0.0 for all traits in both crosses, which allows us to reject additive gene action as an explanation for the relative performance of hybrid genotypes. These findings are consistent with those from other studies that have detected significant non-additive components of variance for similar traits in Pacific oysters (Lannan, 1980; Hedgecock et al., 1991).

The nature of the non-additive genetic variance in these traits is more problematic. An operational definition of heterosis that may be applied to the results of 2×2 crosses among inbred lines is $h_p = Q/L > 1.0$ or < -1.0, where Q is twice the deviation of the hybrid from the mid-parent value and L is the difference in the mean trait values for the two parental inbred genotypes (Griffing, 1990). Q and L are estimated as linear and quadratic contrasts from ANOVA. For alternative genotypes at a single locus, $h_p > 1.0$ could only be explained by overdominance. For quantitative-trait differences among inbred lines and their hybrid offspring, however, h_p (or potence) greater than 1.0 may be explained by any of three hypotheses for heterosis, dominance, overdominance, or epistasis. Nevertheless, potence at least provides an appropriate experimental measure of the phenomenon of interest.

The great majority of potence values for traits measured in our experiments are greater than 1.0 (or less than -1.0), suggesting pervasive heterosis. Of the total of 14 h_p values calculated for hybrid genotypes, 11 (79%) are greater than 1.0 (or less than -1.0); all six h_p values calculated for hybrid means are greater than 1.0 (or less than -1.0; Table 6). These are the first direct measurements of heterosis that have been made for quantitative traits in bivalve molluscs. The three potence values that are less than 1.0 are only slightly so, suggesting complete dominance for these traits. These results indicate that heterosis (or negative heterosis) for fitness- and production-related traits can be readily observed and quantified in crosses among inbred lines of oysters. Although density varied widely among larval cultures, regression analyses of mean size on larval number per bag-culture indicate that larval density has little or no effect on larval growth and could not have confounded our findings concerning heterosis.

Physiological analyses of these same experimental progenies, which will be reported elsewhere, fully support the results presented here for growth rate, at both the larval and adult stages. Routine rates of larval metabolism measured as mass-specific rates of oxygen consumption are inversely proportional to the growth rates of inbred and hybrid genotypes (N. Appelmans and D. Manahan, unpublished data), as expected from previous studies on genotype-dependent scope for growth (Koehn and Shumway, 1982; Garton et al., 1984; Hawkins et al., 1986; Hawkins et al., 1989). Larval 4×5 hybrids, for example, consume 35% more oxygen than the 4×4 inbreds, indicating a lower scope for growth, as reflected in the negative growth heterosis reported here. Negative heterosis for resting metabolic rate ($h_p = -1.16$) has also recently been measured for the adult progeny from this first cross (B. Bayne, unpublished data). However, the larval 6×6 inbreds consume 42% more oxygen than hybrid genotypes, which supports the positive growth heterosis observed in the second experiment. Rates of protein accretion differ significantly between inbred and hybrid larvae and are attributable to differences in rates of protein degradation or turnover (Vavra and Manahan, unpublished data, 1995). Again, protein turnover is higher in the 4×5 hybrids than in the 4×4 inbreds, while the reverse is observed in the case of the 6×7 hybrids, in accord with the negative and positive growth heterosis measured in these experiments. Adult hybrids from the second experiment are more than twice as efficient in absorbing ingested organic material than their inbred counterparts (B. Bayne, unpublished data), suggesting that heterosis for growth may be achieved not only by greater metabolic efficiency but also by greater nutrient absorption on the part of hybrids.

Our observation of negative heterosis for larval and juvenile size in the first experiment (Table 6A) is novel and unexpected. Under neither of the two most commonly championed

hypotheses of heterosis, dominance or overdominance, is a hybrid expected to be worse than both of its inbred parents. This negative heterosis cannot be attributed to outbreeding depression, as our inbred lines were extracted from the same natural population, not from genetically distant sources. Epistasis is the only hypothesis that can explain the expression of both positive and negative heterosis in crosses between inbred lines of common origin.

The genetic basis of the heterosis that we have observed, both the positive and negative, can now be further explored by means of quantitative-trait loci (QTL) mapping (Edwards et al., 1987; Paterson et al., 1988; Stuber et al., 1992; Jacob et al., 1991; Andersson et al., 1994). Intercrosses among the F_1 hybrids from the 4×5 and 6×7 crosses will permit observations of statistical associations between quantitative traits and molecular markers as these segregate and assort by linkage group in the F_2 generation. The additive, dominant, and epistatic effects of chromosome segments can be also estimated in such experiments. Twenty-five polymorphic allozyme loci are already available for QTL-mapping, and we are in the process of developing additional microsatellite nuclear DNA markers with the immediate goal of having at least two markers for each of the ten linkage groups in the oyster.

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