Evolution, 52(2), 1998, pp. 394–402

GENETIC DETERMINANTS OF PROTANDRIC SEX IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* THUNBERG

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Abstract.—A unique feature of sex in *Crassostrea* oysters is the coexistence of protandric sex change, dioecy, and hermaphroditism. To determine whether such a system is genetically controlled, we analyzed sex ratios in 86 pair-mated families of the Pacific oyster, *Crassostrea gigas* Thunberg. The overall female ratios of one-, two-, and three-year-old oysters were 37%, 55%, and 75%, respectively, suggesting that a significant proportion of oysters matured first as males and changed to females in later years. Detailed analysis of sex ratios in factorial and nested crosses revealed significant paternal effects, which corresponded to two types of sires. No major maternal effects on sex were observed. Major genetic control of sex was further indicated by the distribution of family sex ratios in two to four apparently discreet groups. These and other data from the literature are compatible with a single-locus model of primary sex determination with a dominant male allele (*M*) and a protandric female allele (*F*), so that *MF* are true males and *FF* are protandric females that are capable of sex change. The rate of sex change of *FF* individuals may be influenced by secondary genes and/or environmental factors. Strong maternal and weak paternal effects on sexual maturation or time of spawning were also suggested.

Key words.—*Crassostrea*, dioecy, evolution, hermaphroditism, mollusc, oyster, protandry, sex, sex change, sex determination, sexual maturation.

Received January 2, 1997. Accepted January 28, 1998.

The evolution of sex continues to be a major problem in biology. Despite the prevalence of sex in the vast majority of higher organisms, it remains questionable whether the benefits of sexual reproduction outweigh the costs (Michod and Levin 1987; Maynard Smith 1989). Adding to the confusion is the fact that the mode of sexual reproduction often varies greatly among closely related species, and the same mode may evolve independently in many distant groups. While strictly genetic or chromosomal dioecy is widespread, other types of reproduction such as hermaphroditism, parthenogenesis, and biased sex ratio appear to be the rule in many species. Whether these variations are responses to natural selection or due to phylogenic mishaps remains a mystery (Morton 1991; Heller 1993). Understanding the origin and maintenance of these variant forms of sexual reproduction may provide important clues to the evolution of sex.

As a group, molluscs exhibit highly diverse modes of sexual reproduction, ranging from functional hermaphroditism, alternative sexuality, to strict dioecy and genetic determination (Coe 1943; Guo and Allen 1994a). Although dioecy appears to be the norm in molluscs, about 40% of the 5600 genera are either simultaneous or sequential hermaphrodites (Heller 1993). Some seemingly closely related species may exhibit very different modes of sexuality. For example, while most species of *Pecten* are hermaphrodites, *P. magellanicus* is dioecious; conversely, within the predominantly dioecious genus *Cardium*, a few species (e.g., *C. crassum* and *C. ciliatum*) are hermaphroditic (Purchon 1968). This diversity of sexual forms makes molluscs an interesting group for the study of the evolution of sex and sex determination.

The sexuality of *Crassostrea* oysters, which is generally referred to as protandric dioecy, is especially interesting. The two sexes are usually separate in these oysters. However, juveniles usually mature as males and change to females later in life (Coe 1943; Galtsoff 1964). In addition to protandric sex change and dioecy, functional hermaphroditism is also present at a low but persistent frequency. The coexistence of protandric sex change, dioecy, and functional hermaphroditism within the same population (or family) raises several interesting questions. Is such a system under genetic control? If so, how did it evolve and how is it maintained?

Although interest in the sexuality of oysters dates back over a century (Hoek 1883; Kellogg 1892), studies to date have been mostly limited to the documentation of sex change and sex ratio of wild oysters. For a long time, the inability to make controlled matings has prohibited experimental examination of the genetic mechanisms of sex determination in *Crassostrea* oysters. The prevailing hypothesis is that *Crassostrea* oysters have protandric alternative sexuality, and that sex is determined by environmental factors such as food supply and water temperature (Coe 1936; Galtsoff 1964; Quale 1988). Involvement of genetic factors was suspected, but without supporting evidence (Coe 1932, 1943; Needler 1942).

Haley (1977, 1979) was the first to provide some evidence for genetic control of sex in the American oyster, *C. virginica*. Among five families produced by controlled mating, two families sired by the same male had significantly different sex ratios at one and two years of age. Based on the sex ratio variation among those five families, Haley proposed a three-locus model for sex determination, each with two additive alleles, *m* for maleness and *f* for femaleness, with the *m*:*f*

ratio determining sex. Haley's model and conclusions have been largely ignored, probably because they were based on data from only five families.

In this study, we analyzed sex ratios in 86 pair-mated families of the Pacific oyster (*C. gigas* Thunberg), in an attempt to determine the extent of genetic influence on sex determination. Our analyses revealed strong evidence for major genetic control in a seemingly complex system of the protandric dioecy with extensive sex change. A single-locus model of sex determination is proposed for the Pacific oyster, based on family sex ratios from this study and other data from the literature.

Materials and Methods

The sex-ratio data presented in this report were obtained from a study conducted between 1988 and 1991, whose original goal was to estimate heritability for growth-related traits for the Pacific oyster. In 1988 and 1989, eight sets of families were made in the hatchery of Coast Seafood Company, Quilcene, Washington. These sets of families were produced from three base populations: (1) the hatchery stock at Coast Seafood Company (sets H1, H2, and H3); (2) the wild population from Dabob Bay, Washington (sets D1, D2, and D3); and (3) the wild population from Willapa Bay, Washington (sets W1 and W2). Among the eight sets, H1 and D1 were factorial crosses in which each of eight males (sires) was crossed to the same three females (dams). The other six sets were nested crosses in which each of eight males was crossed to three different females per male, thus a total of 24 females was used in these nested crosses. Not all families were available for this study.

All families were produced using artificial spawning techniques (Guo et al. 1993). Gametes were obtained by dissecting gonads. Eggs were passed through a 60-μ nytex screen to remove large tissue debris, and rinsed on a 25-μ screen. About 1.5 million eggs were used for each family. Fertilizations were conducted in 4-L beakers at 23–26°C. The salinity of the seawater was about 30 ppt. After fertilization, each family was cultured in a 150-L tank, and the tanks of families of the same set shared the same water bath during larval culture. Feeding and larval care were conducted according to Breese and Malouf (1975). Spat were collected on oyster shells and cultured on intertidal longlines. Shells carrying spat from each family were randomly assigned to blocks on longlines. Whenever possible, each set was cultured at multiple sites in Washington and California. Sets deployed at different sites were labeled as: D1E, D3E, H1Q, H2E, H3E, W1A, W1E, W2E, and W2Q. The first letter in the label refers to the base population where parents of the set were chosen: D = Dabob Bay, Washington; H = Hatchery (Coast Seafood Company); and W = Willapa Bay, Washington. The number designates the replicate for a set (8 × 3) of families produced from a given base population, each replicate from a different group of parents. The last letter refers to the sites where the set was cultured: A = Allyn, Washington; Q = Quilcene, Washington; and E = Eureka, California.

Oysters from the various sets of families were sampled and sexed at one, two, and three years of age. Since oysters have no secondary sexual characteristics, sex was determined by microscopic examination of gonad smears. Oysters with eggs were classified as females, and those with sperm were classified as males. Oysters with both sperm and eggs were classified as hermaphrodites, and oysters that contained neither were classified as "no-gametes."

Percentages of females were used for sex ratios. Hermaphrodites and no-gamete oysters, both rare, were excluded from sex-ratio calculations. For analysis of family sex ratio, only families that contained <10% of no-gamete oysters and ≥10 sexed oysters (excluding hermaphrodites) were used. The same criteria were applied to data reviewed from other studies. Most families used in this study had a sample size of 20–60 and no no-gamete individuals.

Sex-ratio differences among families and groups of families were tested by the likelihood-ratio chi-square (or *G*-statistics) analysis of independence. Unless otherwise indicated, the 95% confidence level was used for statistical comparisons. In nested crosses, maternal and paternal effects on sex were analyzed in a series of two-way (factor × sex) contingency tables (Sokal and Rohlf 1995). In factorial crosses, the three-way (male × female × sex) tables were analyzed through a loglinear procedure using SYSTAT 6.0 (Wilkinson 1996). The full loglinear model is defined as:

$$LnF_{ijk} = \text{mean} + S_i + D_j + X_k + SD_{ij} + SX_{ik} + DX_{jk} + SDX_{ijk}, \quad (1)$$

where F_{ijk} is the expected value for the cell frequency; S_i, D_j, and X_i are the effects due to sire (paternal), dam (maternal), and sex; SX_{ij}, DX_{jk}, and SD_{jk} are the two-way interactions; SDX_{ijk} is the three-way interaction. In the loglinear procedure, each term of the full model is removed one at a time, and the resulting short models are tested for fit with the observed frequency. The simplest model that does not significantly differ from the data is accepted.

Results

Overall Sex Ratio

At one year of age, 94 families belonging to four sets of families, H1Q, H3E, W1A, and W2E, were sampled and sexed. Other sets could not be sampled because of time and labor constraints. The overall sex ratio of the four sets was about the same, with female ratios ranging from 34.2% to 38.8% (Table 1). On average, the one-year-old oysters consisted of 37.1% females and 62.9% males, excluding hermaphrodites and no-gametes. Hermaphroditism was a rare condition among the oysters sampled, accounting for only 1.1% of the one-year-old oysters. The number of no-gamete oysters was small, except for H1Q families, where 17% of all oysters sampled had no gametes. H1Q families were sampled in late August, which was late in the spawning season. The oysters without gametes had probably spawned out before sampling.

At two years of age, sexes were determined for another 63 families from four sets, D1E, H2E, D3E, and W2Q. Among the four sets of families sampled, the average sex ratio was 55% females and 45% males (Table 1). Compared with the sex ratio at one year of age, there was a considerable increase in the percentage of females, suggesting that substantial sex

TABLE 1. Overall sex composition of Pacific oysters produced by factorial and nested crosses. Female and male percentages were calculated from pooled data, excluding hermaphrodites and no-gamete individuals.

Experiment	Cross type	No. of families	No. of oysters	Female (%)	Male (%)	Hermaphrodite (%)	No-gamete (%)
One Year Olds:							
H1Q	Factorial	22	599	34.2	65.8	2.2	17.2
H3E	Nested	24	602	38.8	61.2	1.3	4.4
W1A	Nested	24	272	37.5	62.5	0.7	0.4
W2E	Nested	24	1025	37.9	62.1	0.3	5.0
Average				37.1	62.9	1.1	6.8
Two Year Olds:							
D1E	Factorial	18	391	66.0	34.0	2.6	1.0
H2E	Nested	24	570	56.9	43.1	0.0	0.4
D3E	Nested	9	90	49.4	50.6	0.0	1.1
W2Q	Nested	12	111	47.2	52.8	4.5	0.0
Average				54.9	45.1	1.8	0.6
Three Year Olds:							
W1E	Nested	10	90	75.0	25.0	1.1	1.1

change had occurred from males to females. The frequencies of hermaphrodites and no-gamete oysters were 1.8% and 0.6%, respectively, similar to that at one year of age.

At three years of age, sex was determined for only two sets of families, H2Q and W1E. However, data from H2Q families were not used because of the small number of individuals that survived and the possibility that these families were contaminated with an overset of wild spat (some of the H2Q oysters were obviously small for three year olds). Of the 90 oysters representing W1E families, 75% were females. The same set of families, W1A, had a female ratio of 37.5% at one year of age.

Unfortunately, not a single set of families was sampled twice at the same grow-out site. Two nested sets of families, W2 and W1, were sampled twice, each time at a different location (E and Q, and A and E, respectively). These data were not analyzed also because only a small number of oysters (< 12) per family were sampled at the second sampling date.

Family Differences and Parental Effects

One Year Old.—Among the four sets of families sampled at one year of age, only two nested sets of families, W2E and H3E, were used for analysis of family differences and parental effects. The other two sets were disqualified because of high proportions of no-gamete oysters (H1Q, factorial) and small sample sizes (W1A, nested).

Although the overall sex ratio for the one year old oysters from different sets was about the same (Table 1), sex ratio varied greatly among families within each set. For the nested set W2E, some families had only 10–20% females, while others had 60–71% females (Table 2). As indicated by likelihood-ratio chi-square analysis of independence, the effects of family on sex were highly significant ($P = 0.000$) (Table 3). The family differences were primarily due to paternal effects ($P = 0.000$). Female effects (within males) were significant in only two of the eight sires, and the P-values were marginally significant (0.038 and 0.036). The total female effects (combined over all sires) were not significant ($P = 0.17$). The strong parental effects in W2E were primarily due to two types of sires: those with low female ratios (sire 1, 5–8) ranging between 16.3% and 34.2%, and those with high female ratios (sire 2–4) ranging between 50.8% and 60.4% (Table 2). Sires 2–4 were not significantly different from each other, but all had significantly higher female ratio than the

TABLE 2. Sex composition of nested families (W2E) of the Pacific oyster at one year of age. Bold type indicates the mean female-ratio for each sire.

Sire		Dam	Female	Male	Hermaphrodite	No-gamete	%Female
1	×	1	17	53	0	0	24.3
		2	10	48	0	5	17.2
		3	3	22	0	0	12.0
							17.8
2	×	4	22	9	0	1	71.0
		5	19	24	1	2	44.2
		6	27	14	0	0	65.9
							60.4
3	×	7	25	25	0	2	50.0
		8	27	15	0	5	64.3
		9	17	14	0	0	54.8
							56.4
4	×	10	27	18	2	8	60.0
		11	31	37	0	4	45.6
		12	29	33	0	4	46.8
							50.8
5	×	13	14	33	0	1	29.8
		14	9	18	0	0	33.3
		15	17	38	0	2	30.9
							31.3
6	×	16	13	18	0	2	41.9
		17	7	18	0	1	28.0
		18	2	18	0	0	10.0
							26.6
7	×	19	18	39	0	6	31.6
		20	7	17	0	0	29.2
		21	10	14	0	0	41.7
							34.2
8	×	22	6	30	0	5	16.7
		23	2	14	0	0	12.5
		24	9	34	0	3	19.6
							16.3

TABLE 3. Likelihood-ratio chi-square analysis of independence between factors and sex at year one in the nested family set W2E.

Factor		χ^2	df	P-value
Family (total)		117.8	23	0.000
Sire		96.2	7	0.000
Dam/within	Sire 1	2.2	2	0.339
	Sire 2	6.5	2	0.038
	Sire 3	1.9	2	0.380
	Sire 4	2.6	2	0.273
	Sire 5	0.1	2	0.951
	Sire 6	6.6	2	0.036
	Sire 7	1.0	2	0.608
	Sire 8	0.6	2	0.725
	Total dam	21.6	16	0.170

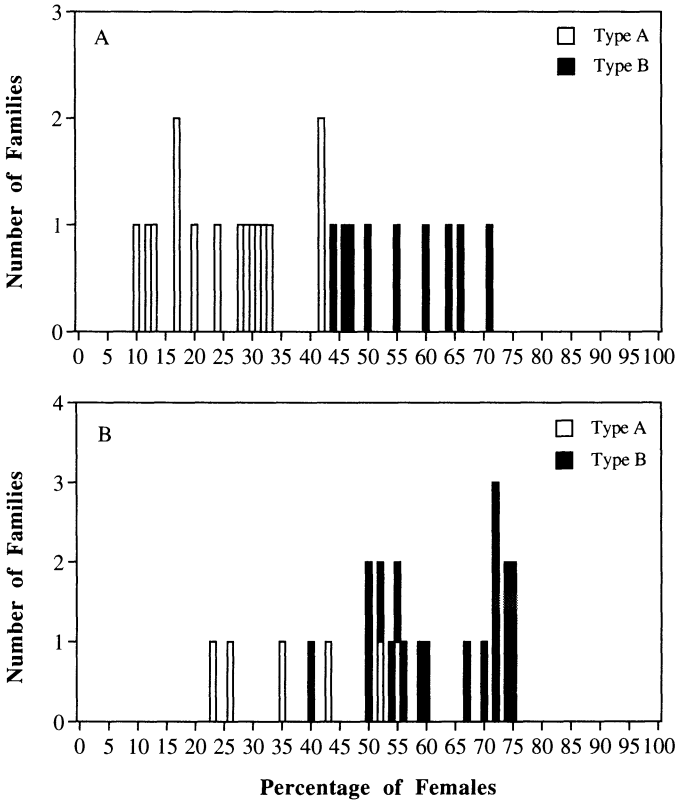

FIG. 1. Distribution of family sex ratio from two sets of 8 male × 3 female nested crosses of the Pacific oyster. (A) Set W2E, one year old. (B) Set H2E, two years old. Families were characterized according to the single-locus model: type A for *MF* × *FF* and type B for *FF* × *FF* (male × female) matings.

TABLE 4. Sex composition of nested families (H2E) of the Pacific oyster at two years of age. Bold type indicates the mean female ratio for each sire.

Sire		Dam	Female	Male	Hermaph-rodite	No-gamete	%Female
1	×	1	14	14	0	0	50.0
		2	13	11	0	0	54.2
		3	15	5	0	0	75.0
							59.7
2	×	4	19	13	0	0	59.4
		5	18	7	0	1	72.0
		6	6	3	0	0	66.7
							66.0
3	×	7	11	10	0	0	52.4
		8	6	17	0	0	26.1
		9	10	18	0	0	35.7
							38.1
4	×	10	21	7	0	0	75.0
		11	14	6	0	0	70.0
		12	18	7	0	0	72.0
							72.3
5	×	13	15	10	0	0	60.0
		14	14	5	0	0	73.7
		15	14	13	0	0	51.9
							61.9
6	×	16	13	13	0	0	50.0
		17	20	7	0	0	74.1
		18	8	12	0	0	40.0
							54.7
7	×	19	18	15	0	0	54.5
		20	5	17	0	0	22.7
		21	3	4	0	0	42.9
							40.0
8	×	22	14	11	0	0	56.0
		23	18	7	0	0	72.0
		24	16	13	0	0	55.2
							61.1

other sires (1, 5–8). With few exceptions, the female ratio of individual families could be assigned to two major groups according to sire types: one between 10% and 42% and one between 44% and 71% (Fig. 1A). Each of the two major groups might be tentatively divided into two subgroups, although these subgroups were not well separated. The two subgroups within the low female-ratio groups were largely due to sire difference, while the two subgroups within the high female-ratio group were due to dam differences. Families within each of the four subgroups were not significantly different in sex ratio as tested by chi-square analysis of independence ($P = 0.669$–0.912). When families within a subgroup were pooled, all four subgroups were significantly different from each other as indicated by both test of independence and Tukey comparison of arcsine-transformed female ratios ($P < 0.01$).

In the other one-year-old set (H3E, nested), likelihood-ratio chi-square analysis of 18 families (6 × 3) showed almost identical patterns. (Families from two sires were disqualified because of small sample size). Sex ratio differed significantly among individual families ($\chi^2 = 41.6$, df = 17, $P = 0.001$). Paternal effects were highly significant ($\chi^2 = 24.9$, df = 5, $P = 0.000$). Within sires, maternal effects were significant ($P = 0.04$) in only one of the six sires. The overall female effect was not significant ($\chi^2 = 16.7$, df = 12, $P = 0.174$).

Two Years Old.—Only two sets of families, H2E and D1E, were used for analysis, and the other two sets of families, D3E and W2E, contained only a few qualified families and could not be used.

For the two-year-old oysters, sex ratio also varied greatly among families within each set. In the nested set H2E, family female ratio ranged from 22.7% to 75.0% (Table 4). The likelihood-ratio chi-square test of independence showed the same pattern as in the one-year-old sets, that is, strong family effects ($\chi^2 = 51.1$, df = 23, $P = 0.001$), strong paternal effects ($\chi^2 = 27.1$, df = 7, $P = 0.000$), and weak overall maternal effects ($\chi^2 = 24.1$, df = 16, $P = 0.09$). Within sires, female effects were only significant in one of the eight sires with a marginal P-value of 0.045. Two sires (3 and 7) had significantly lower female ratios than the other sires (Table 4). Although the female effects were not statistically significant in most sires, there were some differences among females. For example, within a number of sires, some females produced about 50% females, while others produced about 75% females (Table 4). The distribution of family female ratio in H2E also appeared to be discontinuous (Fig. 1B). Although the two sire groups were not well separated, families within the high-female-ratio sires clearly formed two distinct subgroups due to differences among dams: one between 67% and 75% females and the other between 50% and

TABLE 5. Sex composition of factorial families (D1E) of the Pacific oyster at two years of age. Bold type indicates the mean female ratio for each sire.

Sire		Dam	Female	Male	Hermaphrodite	No-gamete	%Female
1	×	1	8	5	0	0	61.5
		2	8	4	0	0	66.7
		3	12	4	2	0	75.0
							67.7
2	×	1	4	4	0	0	50.0
		2	10	7	1	0	58.7
		3	14	4	2	1	77.8
							62.2
3	×	1	10	14	0	0	41.7
		2	4	14	0	0	22.2
		3	16	8	0	0	66.7
							43.5
4	×	1	19	6	0	1	76.0
		2	18	8	3	0	69.2
		3	22	7	0	0	75.9
							73.7
5	×	1	9	10	0	1	47.4
		2	24	10	2	0	70.6
		3	17	10	0	0	63.0
							60.3
6	×	1	18	4	0	1	81.8
		2	11	4	0	0	73.7
		3	25	5	0	0	83.3
							79.5

60% females. The two subgroups were significantly different ($P < 0.001$) from each other as indicated by both the chi-square test of independence and Tukey comparison of arcsine-transformed female ratios. The grouping of low-female ratio families was less certain because of the small number of families examined.

The two-year-old factorial set, D1E, provided stronger evidence for the differential parental effects. The raw sex-ratio data for D1E are presented in Table 5. The factorial data were analyzed with the loglinear procedure, where paternal and maternal effects were tested together with greater statistical power (Table 6). Expected frequencies from the full model with all three two-way interactions did not differ significantly from the observed frequencies (df = 10, $P = 0.473$). The removal of the sire (paternal) term and its higher level interactions, for example, would make the shorter model significantly ($P = 0.0003$) different from the observed data, suggesting that sire effects were important. On the other hand, the shorter model with the term dam (maternal) and its higher interactions removed still fitted the observed data ($P = 0.1755$). The P-value on the right column showed whether the model without a specific term was significantly different from the full model. The shorter model with dam and its interactions removed was not significantly different from the full model in fitting the observed data (df = 14, $P = 0.1110$). Results from the loglinear procedure clearly demonstrated that paternal factors had strong influence on sex, and maternal effects did not.

Three Years Old.—For the three-year-old oysters, all 10 families had a sample size of 12 or smaller. Only seven families had a sample size of more than nine. Individual families could not be compared because of the small sample size. After pooling dams (with > 9 oysters) within sires, three sires obtained a sample size of 20–24. Two of the sires had 83.3% females, and the other had 55% females, a difference that is marginally significant ($\chi^2 = 4.26$, df = 1, $P = 0.039$).

TABLE 6. Analysis of a factorial cross (D1E) by a loglinear procedure.[1]

Terms tested hierarchically	The model without the term			Removal of term from model		
	χ^2	df	P-value	χ^2	df	P-value
Sire	64.08	30	0.0003	54.45	20	0.0000
Dam	30.29	24	0.1755	20.65	14	0.1110
Sex	72.97	18	0.0000	63.34	8	0.0000
Sire × dam	20.17	20	0.4472	10.54	10	0.3948
Sire × sex	29.44	15	0.0141	19.80	5	0.0014
Dam × sex	14.96	12	0.2437	5.32	2	0.0699

[1] The observed data were explained by a full model that included the three terms and all two-way interactions ($\chi^2 = 9.64$, df = 10, $P = 0.4729$). P-values on the left indicate whether the full model without the term differs significantly from the observed data, and P-values on the right indicate if the removal of the term from the model causes significant decrease in fitting the data.

Genetic Influence on Maturation

One factorial set of families contained a large fraction of no-gamete oysters (17% on average) and permitted the examination of sexual development and maturation for genetic influence. These no-gamete oysters were apparently early spawners that, at the time of sampling, had already released their gametes. Therefore, a difference in the frequency of no-gamete oysters represents a difference in the time of spawning, and possibly the rate of sexual maturation. Careful examination revealed significant family differences in the frequency of no-gamete oysters. Some families had as high as 32–52% no-gamete oysters, and others had none (Table 7). The family difference in frequency of no-gamete oysters is highly significant ($\chi^2 = 83.8$, df = 17, $P = 0.000$). Families from female 2 had considerably lower numbers of no-gamete oysters than families from the other two females. Analysis by the loglinear procedure suggested that the ratio of no-gamete oysters was significantly affected by maternal factors, and maternal and paternal interaction (model $\chi^2 = 20.3$, df = 20, $P = 0.4418$). Paternal factors alone were not significant. If these differential effects are true, genes that affect

TABLE 7. Percentage of oysters without gametes in an 8 × 3 factorial cross (H1Q) at 17 month postfertilization. Sample sizes are given in parentheses.

Sire	Dam 1	Dam 2	Dam 3	Mean
1	51.9 (27)	0.0 (37)	19.4 (31)	23.8
2	35.7 (28)	7.7 (26)	32.3 (31)	25.2
3	36.0 (25)	15.4 (26)	22.2 (27)	24.5
4	13.8 (29)	0.0 (30)	7.4 (27)	7.1
5	7.1 (28)	0.0 (05)	—	3.6
6	18.5 (27)	6.3 (16)	27.6 (29)	17.5
7	10.0 (30)	6.3 (32)	—	8.2
8	5.7 (35)	3.8 (26)	44.4 (27)	18.0
Mean	22.3	4.9	25.6	

sexual maturation may be linked to female sex factors or regulated differently in the two sexes.

Discussion

Alternating sexuality, as well as rare but persistent simultaneous hermaphroditism, in oysters suggested to earlier workers that sex in these bivalve molluscs is largely environmentally determined (Coe 1932; Galtsoff 1964; Quayle 1988). The extent of genetic sex determination was a matter of speculation (Coe 1943) until controlled crosses were first employed with the American oyster, *C. virginica* (Haley 1977, 1979). With this study, we greatly extend (from 5 to 86) the number of oyster families from controlled crosses in which sex ratio has been studied, and we provide compelling evidence of genetic determination of sex in oysters.

For our entire study population of the Pacific oyster, *C. gigas*, we can confirm the features of hermaphroditism and protandric dioecy that have long drawn attention to the determination of sex in oysters. The frequency of simultaneous hermaphrodites (1.3%) in our study population is comparable to that reported previously from the American and Pacific oysters (ranging 0.4–1.0%; Amemiya 1929; Burkenroad 1931; Coe 1932, 1936; Needler 1932a,b), further substantiating simultaneous hermaphroditism as a rare but consistent feature of sex in *Crassostrea* oysters. Unfortunately, the low frequency of simultaneous hermaphroditism prevented an analysis of family differences. Next, the overall sex ratio of one, two, and three year olds in our study population clearly show protandric dioecy—adults mature first predominantly as males and change to females in subsequent years. In the American oyster, most individuals are male at the first breeding season, but the proportion of females increases thereafter, passing equality at two to three years and resulting in an excess of females in the oldest oysters (Coe 1932; Galtsoff 1964; Mackie 1984). Sex ratio in the Pacific oyster has not been as well documented. In one randomly mated group, 37.7% of one-year-old Pacific oysters was females (Allen 1987), a percentage almost identical to the overall percentage of females observed in this study. Analyses of sex ratios in families from controlled crosses, which provide evidence for genetic determination of sex, are considered next.

Differential Parental Effects

All datasets analyzed in this study clearly demonstrate that sex determination in the Pacific oyster is strongly influenced by paternal effects, while maternal effects are weak. It should be pointed out that the experimental design used in this study, which was originally intended for estimating heritability in growth traits, was not the best for comparing parental effects. The nested design was especially limiting, because females were nested under males. Sire effects were tested by pooling females within each sire, and random variation among dams within sires could potentially inflate the sire effects. There were only three females per male, and the chance of detecting female differences (within males) might be reduced. On the other hand, the total number of females (24) used in each nested cross was large and should be a fairly good representation of possible genotypic differences. The factorial families were more informative, but the unbalanced 6 × 3 (male × female) design might be biased against detecting female variation. Given the magnitude of the difference between paternal and maternal effects, however, a change in experimental design is unlikely to change the conclusion—paternal effects are the principal genetic determinant of sex ratio.

The discontinuous distribution of family female ratio supported the conclusion that sires (paternal effects) set the range for family sex ratios, and dams (maternal effects) only affect sex ratio within that range. This pattern was particularly clear in W2E, where the family female ratio was distributed in two major groups according to sire types. Within the ranges of each of the two sire-dictated groups, family female ratio might vary according to dam or sire differences and be further divided into two subgroups (Table 1, Fig. 1A). Although the maternal effects were not statistically significant, they could lead to considerable variation in family female ratio within the range set by sire. The distinctive subgroup formation within high-female-ratio sires in H2E, for example, was primarily attributable to maternal differences (Fig. 1B). The trend of the segmented distribution of family female ratio is clear, although statistically the groups are difficult to define because of the small number of families available. The fact that the two major groups (peaks) directly corresponded to the two types of sires in W2E (Fig. 1A), suggests that the segmented distribution is not totally artificial.

Genetic Determinants of Sex

The significant family differences and differential parental effects on sex ratio as revealed in this study strongly argue for genetic control over sex determination. Further, the finding of two sire types and the segmented distribution of family sex ratio in two to four groups or subgroups, suggests that family sex ratio is not controlled by a large number of genes. Polygenic traits are usually distributed randomly around a single population mean. If the two to four family (sub)groups truly reflect genotypic differences, sex in the Pacific oysters may be explained by a genetic mechanism involving a small number of loci.

We propose that the sex in the Pacific oyster is primarily determined by a single locus with a dominant maleness (*M*) allele and an allele for protandric femaleness (*F*). *MF* genotypes are true males that do not change sex, while *FF* are protandric females that mature as males at the juvenile stage and can change sex during later years. Thus, there are two genotypes for males: the true males (*MF*) and the protandric males (*FF*), but only one genotype for the female (*FF*), which fits well with the two types of sires and the relative weak maternal effects observed in this study. Additional loci may affect the rate of sex change in *FF* oysters.

The single-locus model is primarily based on the distinction between two types of males, the true males and protandric males. Although this designation is well supported by our data, it did not originate from this study and was actually first proposed by Coe (1932) based on his histological studies of gonadal development. "Since there is such a wide diversity in the abundance and size of the ovocytes in the sexually mature young males of *O. virginica*, it is pertinent to inquire whether there may not be two genetically distinct types of these males. . . . It is conceivable that those males with but

TABLE 8. Family genotypes and female ratios predicted by a single-locus model in the Pacific oyster, assuming *MF* are true males and *FF* are protandric females that have a 50% chance of changing from males to females each year.

Family type	Parental genotype			Offspring genotype		Family female ratio at		
	Male	×	Female	*MF*	*FF*	1 yr	2 yr	3 yr
A:	*MF*	×	*FF*	*MF*	*FF*	25 ± 10%	37.5 ± 10%	45 ± 0.5%
B:	*FF*	×	*FF*		2 × *FF*	50 ± 20%	75.0 ± 20%	90 ± 10%

few and very small ovocytes are genetically 'true males' while those with more numerous and larger ovocytes may represent the protandric males."

Coe (1932) did not further develop his concept of "true males" and protandric males owing to lack of corroborative evidence, (he wrote, "it would probably be unwise to speculate further in this connection until more complete evidence is available"). Our demonstration of significant paternal effects on sex and of two types of sires, provides evidence supporting Coe's conception of heterogametic males.

The existence of true males in a protandric system has been documented in other species. In the clam *Mercenaria* (*Venus*) *mercenaria*, for example, all juveniles are males, while adults have a strict 1:1 sex ratio (Loosanoff 1937). Obviously, half of the juvenile males in *M. mercenaria* are true males, and the other half are protandric males and have uniformly changed to females before reaching the adult stage. In the Pacific oyster, sex change is gradual and variable.

Under the single-locus model, mating between the two sexes can produce two types of families: one contains 50% true males and 50% protandric females (type A), and the other contains 100% protandric females (type B) (Table 8). The family sex ratio sampled at a given time is determined first by the family type (A or B), and then by the rate of sex change of protandric females, which may be regulated by secondary genes and environmental factors. The fact that the sex ratio varied among families of the same type (and that the discontinuous distribution of family female ratio was sometimes difficult to define), is indicative of the involvement of secondary genes and environmental factors in sex change.

It is interesting to note that, despite diverse genetic backgrounds and grow-out environments, the overall female ratio of all four sets of the one-year-old families seems to be conspicuously close to 37.5% (Table 1). In fact, an independent study of the Pacific oyster also found a very similar sex ratio, 37.7% females (Allen 1987). If the rate of sex change in the *FF* oysters is affected by secondary genes and environment factors, then why do these populations consistently produce a female ratio around 37.5%?

This question may be answered under certain assumptions. It could be due to the uniformity of hatchery environment and to selection for a population equilibrium so that *FF* oysters have a 50% chance of changing to females at the population level. If we assume that the two types of families in Table 8 are present at a 1:1 ratio in these populations, 50% sex change of the 75% *FF* oysters would give a 37.5% female ratio at the population level. Under these assumptions, the expected family female ratio (population mean) is about 25% for type A families and 50% for type B families at one year of age (Table 8). These numbers are not significantly different (P = 0.223 and 0.744) from the observed female ratio for the two sire groups in W2E (Table 2).

The single-locus model supplemented with some genetic and environmental influence on the rate of sex change fits most of our data. At this stage, we cannot prove this model or exclude others. In the American oyster, Haley (1977, 1979) proposed a three-locus model and further divided sex-changing oysters into two types: those changing from males to females, and those changing from females to males. In Haley's model, there are two additive alleles, *m* for maleness and *f* for femaleness, at a minimum of three loci, and sex is determined by the *m:f* ratio. Oysters with *m:f* ratios of 6:0, 5:1, 4:2, 3:3 are true males; 2:4 are males that change to females; 1:5 are females that change to males; and 0:6 are true females. Haley's three-locus model has some difficulties. First, at the present time, there is no indication that American oysters changing from males to females are genetically different from those changing in the reverse direction. On the contrary, the same oyster may go through multiple sex changes (Galtsoff 1964; Haley 1979). If there were a specific genotype for female-to-male sex change, mating of female-reversed males with true females would produce families with 90–100% females in the first year, which was not observed among the 86 one-year-old families in this study. Secondly, multiple genotypes for true males are unnecessary and unstable, and one or two loci may become fixed for the *m* allele, ending with a single-locus system. By invoking a three-locus model instead of one- or two-locus models, the attempt was to account for all the variation in the sex ratios which, we think, is not necessary because sex changes may be subject to influence by secondary genes and environmental factors.

Likewise, our simple genetic model of sex determination need not explain the rare but consistent simultaneous hermaphroditism in oyster populations. Simultaneous hermaphroditism may have two potential causes in *Crassostrea* oysters: one developmental and one genetic. Developmental abnormalities, especially at the time of sex change, may accidentally activate spermatogenesis and oogenesis simultaneously. Genetic abnormalities such as triploidy, aneuploidy, unequal crossover, or heterologous translocation involving major sex-determination genes may also cause hermaphroditism. A large proportion of induced triploids in the Pacific oyster (15–49%) were hermaphrodites (Allen 1987; Guo and Allen 1994b). Aneuploidies involving sex chromosomes are found in 0.2% of human newborns (Hecht and Hecht 1987), and may also occur in oysters. In the Pacific oyster, spontaneous triploidy occurs at an estimated frequency of 1.3% at embryonic stages (Guo et al. 1992), which may potentially create 0.2–0.5% hermaphrodites. It is also possible that, owing to sampling or classification errors, only a fraction of reported cases are "true" hermaphrodites.

Evolutionary Implications

Evolution of sexuality in molluscs has been the subject of much discussion but little agreement. The most controversial point is the sexuality of the ancestral molluscs. Because most primitive molluscs are dioecious, it has been generally assumed that dioecy is the ancestral sexuality in molluscs (Purchon 1968). On the other hand, Mackie (1984) argued that the protandric sex change was the primitive sexuality, and both dioecy and functional hermaphroditism evolved from protandric sex change. More broadly, however, there is little dispute that dioecy in most higher organisms evolved from hermaphroditism (Morell 1994). One indication is that the primary gonad of most dioecious species including humans is hermaphroditic, and sexual differentiation is achieved by selectively suppressing the development of the opposite sex (Haqq et al. 1994; Parkhurst and Meneely 1994; Pieau 1996). Because the primary gonad of *Crassostrea* oysters is bisexual (Coe 1932), we believe that functional hermaphroditism is also the ancestral sexuality for *Crassostrea* oysters.

If the single-locus model of sex determination is correct, *Crassostrea* oysters may actually exemplify an intermediate stage in the evolution from hermaphroditism to strict dioecy. The present sexuality of *Crassostrea* oysters may have evolved in two phases, starting from a functional hermaphrodite. The first phase was the elimination of self-fertilization through the evolution of protandric sex change or rhythmic (or alternate) sexuality, similar to that in the extant species of *Ostrea* oysters. *Ostrea* oysters mature as males at juvenile stage and function alternatively as females and males in subsequent seasons (Coe 1943). The second phase was the evolution of primary sex determination genes to achieve a more balanced sex ratio. If most individuals practice protandric sex change at the same time, natural selection would favor mutations that either change sex early (true females) or not at all (*MF* true males). Under the proposed model, the Pacific oyster has apparently gained a dominant *M* allele for not changing sex (in *MF* true males), but has still not obtained an allele that would make *FF* oysters true females. The emergence of true males may be advantageous for a protandric system, so that populations consisting of old individuals are guaranteed with the presence of males. The absence of strict dioecy in *Crassostrea* oysters could be due to a lack of mutations that cause instantaneous sex change in *FF* oysters, and not an adaptation to unique life history or environment as commonly believed. Similar conclusions were made from studies of molluscan hermaphroditism in a variety of environments (Heller 1993). Nevertheless, there have been suggestions that the unique features of sexual reproduction in molluscs are strategies adapted for unique environments (Morton 1991).

Acknowledgments

We thank P. M. Gaffney, E. Zouros, and anonymous reviewers for constructive suggestions and help with data analysis. The study was funded in part by a grant from the Western Regional Aquaculture Consortium. Hatchery and broodstock support were provided by the Coastal Seafood Company (Washington, USA). Mr. Thomas Bettinger assisted with data collection. This is publication no. 32100-98-1, NJAES/IMCS.

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