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Small effective number of parents (N_b) inferred for a naturally spawned cohort of juvenile European flat oysters *Ostrea edulis*

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Abstract:

The great fecundity and very high larval mortality of most marine invertebrates and fish make possible substantial variance in the number of offspring contributed by adults to subsequent generations. The reproductive success of such organisms may thus resemble a sweepstakes lottery, in which a minority of progenitors succeeds in replacing an entire population, while the majority fails to procreate. One specific prediction of this hypothesis, that genetic diversity of newly settled cohorts should be less than that of the adult population, is tested in the present study. Microsatellite DNA markers were examined in naturally spawned juvenile European flat oysters *Ostrea edulis* (L.), collected over a 12-day period in 1993 from the western Mediterranean Sea, near Sète, France (43°32′N, 3°56′E) and grown out for a period of up to 10 months. Variation in these juveniles was compared to that in a pooled sample of adults collected in 1994 from two locations (Thau Lagoon and Port St. Louis) that had statistically homogeneous allelic frequencies. Though nearly twice as large as the pooled adult sample, the juvenile sample had only 60% of the adult allelic diversity. Analyses of linkage disequilibrium and kinship, as well as estimation of the effective number of parents, suggested that 10–20 adults produced this juvenile cohort. This observation supports the hypothesis of sweepstakes reproductive success and suggests that partial inbreeding may occur even in species with large populations and dispersing planktonic larvae.

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Introduction

The abundance and wide distribution of many marine fish and invertebrates, having dispersing planktotrophic larval forms, leads to a natural presumption that such populations are very large and well mixed. Many genetic studies of marine populations have focused on the "well mixed" part of this presumption, describing spatial variation among adult populations and its inverse correlation with larval duration or dispersal (Avise 1994; Palumbi 1996). The actual extent of larval dispersal and its demographic and evolutionary consequences, however, continue to be much debated (Bonhomme and Planes 2000; Hellberg et al. 2002; Taylor and Hellberg 2003), particularly with respect to the design of marine reserves (Palumbi 2003). Less attention has been paid to the first part of the presumption that large numbers of adults routinely contribute to larval pools, to cohorts that subsequently recruit back into adult habitats, in sum, to the genetic and demographic continuity of these abundant marine species. By revealing chaotic genetic patchiness on small spatial and temporal scales, a few studies have suggested that dynamic processes underlie the typical genetic similarity of broadly distributed marine populations, (Johnson and Black 1984; Pudovkin and Balakirev 1985; Hedgecock 1986, 1994a,b; Watts et al. 1990; Edmands et al. 1996; David et al. 1997a; Li and Hedgecock 1998; Moberg and Burton 2000).

One hypothesis to explain these observations says that the presumption of large population size may be incorrect. Instead, high fecundity and early larval mortality create the potential for large variance in reproductive success (i.e. the number of offspring that an individual contributes to the next generation), which can reduce the effective size of marine invertebrate and fish populations. Reproduction of these marine organisms may be analogous to a sweepstakes lottery, in which there are relatively few big winners and many losers (Hedgecock 1986, 1994a; Waples 2002; Hedrick 2005). Sweepstakes reproductive success could generate chaotic patchiness in the genetic composition of recruits, as a result of the sampling variance associated with a small number of successful progenitors. Indeed, genetic variance among recruits to a local population has been shown to exceed genetic variance among adult populations on broad geographic scales (Watts et al. 1990; Edmands et al. 1996; Hedgecock et al. 1992; Hedgecock 1994b). The implication of sweepstakes reproductive success is that effective population sizes may be much smaller, perhaps orders of magnitude smaller than census sizes. As a result, genetic drift, hitherto regarded as negligible in abundant marine organisms, could play a substantial role in their evolution. Temporal genetic change has been confirmed in several studies of marine fish and invertebrates (Hedgecock 1994a; Hauser et al. 2002; Turner et al. 2002; Árnason 2004; Hoarau et al. 2005). The sweepstakes hypothesis further predicts less diversity within and more heterogeneity among cohorts of larvae and new recruits than exist in and among adult spawning populations on regional spatial or oceanographic basin scales. These predictions have been supported by several studies of marine fish and invertebrates (Ruzzante et al. 1996, 1999; David et al. 1997a; Li and Hedgecock 1998; Moberg and Burton 2000; Planes and Lenfant 2002). On the other hand, haplotype diversity was not significantly less in recruits relative to adults of the red sea urchin *Strongylocentrotus franciscanus* (Flowers et al. 2002).

Here, we provide a counterexample to that of Flowers et al. (2002), by analyzing polymorphism of four microsatellite DNA markers in newly recruited juveniles of the European flat oyster *Ostrea edulis* and comparing this to variability previously reported at the same markers in nearby adult populations (Launey et al. 2002). The flat oyster is an appropriate species with which to test the prediction that, under sweepstakes reproductive success, there should be lower genetic diversity in a cohort of new recruits. Although decimated by diseases, it is still abundant enough to be collected relatively easily in appropriate habitats. The flat oyster is a highly fecund species, with females spawning as many as 1-2 million eggs per brood (Cole 1941; Walne 1964). Though hermaphroditic and oviparous, with internal fertilization and egg brooding, the flat oyster alternates between releasing sperm and eggs and does not normally self-fertilize (Cole 1942; O'Foighil and Taylor 2000). Larvae are released, during summer months, after about 10 days of development and then spend another 12-15 days in the plankton before metamorphosis and settlement (Pouvreau 1977), a period that should be sufficient for dispersal and population mixing. Studies of genetic variability over the whole geographic range (from Norway to the Black Sea) do show significant amounts of interpopulation variation for allozymes, microsatellites, and mitochondrial DNA, with a suggestion of isolation by distance (Saavedra et al. 1995, Launey et al. 2002; Diaz-Almela et al. 2004). Obviously, to understand fully the species-wide balance between the unifying force of larval dispersal and the diversifying forces of genetic drift and selection, it is necessary to evaluate the effective sizes of local

populations. Towards this end, we compared the genetic diversity of a particular larval cohort to that of the adult population in the same region, which was previously studied by Launey et al. (2002).

Material and methods

Biological material

We collected juvenile European flat oysters (*Ostrea edulis* L.) from a wild population near Sète (specifically Palavas-les-Flôts, France, Mediterranean Sea, 43° 32' N, 3° 56' E). Collectors of fluted plastic tubes, ~1cm diam, were placed in the open sea in front of the IFREMER (Institut français de recherche pour l'exploitation de la mer) station at Palavas, at the end of summer 1993. The exact dates and depth of deployment of these collectors, which was carried out by IFREMER personnel, are unknown. After 12 d, the collectors were retrieved and placed in an experimental oyster rearing facility at the IFREMER station, under standard hatchery conditions (filtered sea water, unicellular algae fed ad libitum). On Feb 21, 1994, approximately 2500 juveniles were removed from the collectors; 102 were weighed and frozen for subsequent genotyping. The remaining oysters were put into plastic mesh bags and grown for another 17 wk in the nearby Thau Lagoon, at 1.5 m depth, on a commercial oyster farm near Bouzigues, France. On June 30, 1994, when they had reached an average live weight of 8.72 ± 0.55g, another 83 were weighed and stored for genotyping, bringing the total sample size to 185 individuals. Samples of the 1993 cohort were pooled for subsequent analysis, as described and justified in an online appendix. Comparison of these juveniles with local adult populations was afforded by the study of Launey et al. (2002), which reported microsatellite DNA marker data for two adult samples taken in 1994 from the nearby coast at Port Saint Louis (n=50) and from the Thau Lagoon, near Sète (n=49).

Molecular methods

DNA was extracted from gill samples of individual juveniles and adults using a classical phenol-chloroform method (Sambrook et al. 1989). Polymorphism has been analyzed for four of the five microsatellite loci described by Launey et al. (2002), *Oedu*H15, *Oedu*J12, *Oedu*O9 and *Oedu*T5. Briefly, PCR reactions were performed in a 10-µl reaction mix containing 2 µl template DNA, 1.5 µM $MgCl_2$, 75 µM each dNTP, 0.25 µM γ^{33}P-labelled forward primer, 0.4 µM reverse primer, 0.35 units of Goldstar Licensed Polymerase (Eurogentec) and 1× polymerase buffer (supplied by the manufacturer). Amplifications were made as follow: pre-denaturation (94°C-2 minutes), followed by 30 cycles of denaturation, annealing, and polymerization (94°C-1 min, T_a-1 min, 72°C-1 min 15 s) and a final elongation step (72°C-5 min). T_a is the optimal annealing temperature for each pair of primers (Launey et al. 2002). Amplification products were analysed on 7 M urea, 6% polyacrylamide gels, using individuals of known genotype as size markers.

Genetic variation within and between samples

Standard population genetic statistics were used to describe genetic variability within and among samples: the observed number of alleles per locus, the observed and expected heterozygosity per locus and averaged over loci, Weir and Cockerham's (1974) estimators of Wright's F-statistics, and Cockerham and Weir's (1977) digenic correlation coefficient, r, and its square, r^2, as an estimate of gametic phase disequilibrium. We also calculated $adj\ r^2 = r^2 - (1/S)$ to adjust the squared correlation for the number of individuals, S, typed for the two markers in question. Significance levels for statistics were assessed by appropriate permutation of alleles or genotypes within or between populations, using the Genetix 4.01 software package (Belkhir et al. 1996-2004).

The two adult samples were initially tested for homogeneity of allelic frequencies by assessing the significance of the standardized allele-frequency variance between them, F_{ST}. No significant difference was detected (F_{ST} = 0.001, P = 0.314), so the two samples were pooled for comparison of adults and juveniles.

Analysis of family structure

We conducted a likelihood analysis of the kinship between each pair of individuals in the adult (n=4753) and juvenile samples (n=16,110). Using the program Kinship (Goodnight and Queller 1999), we calculated the odds that each pair of individuals within a sample represents full-sibs or half-sibs rather

than a pair of unrelated individuals drawn from the adult population. The significance threshold for the log of the odds ratio (or LOD score) was taken from simulation results (10,000 simulations). Allelic frequencies of the pooled adult sample were used to calculate genotypic likelihood under the different kinship hypotheses (full-sib vs. unrelated, half-sib vs. unrelated).

Estimation of the effective number of parents (N_b)

We used three published methods for estimating the N_b of juveniles from genetic data (Waples 1991), based on (i) temporal change in allelic frequencies between adults and juveniles, (ii) average gametic phase disequilibrium for pairs of loci in the juvenile cohort, and (iii) excess heterozygosity of juveniles (Pudovkin et al. 1996). For the temporal method, we used the Nei and Tajima (1981) estimator of temporal variance of allele frequencies, F_c, and Pollak's (1983) estimator of N_k (equivalent to N_b for this adult vs. offspring comparison). For the gametic phase disequilibrium method, we used the formula of Waples (1991), based on the original suggestion of Hill (1981): $N_b = 1/[3(\overline{r^2} - 1/S)]$, where $\overline{r^2}$ is the mean squared Cockerham and Weir (1977) digenic correlation coefficient and S is the harmonic mean of the sample size per locus, 173.7. For the excess heterozygosity method, we calculated the average deviation, d, of observed juvenile heterozygosity from its Hardy-Weinberg (H-W) expectation over all alleles and estimated $N_b = 1/(2d) + 1/(2[d + 1])$, according to Pudovkin et al. (1996).

We also estimated N_b from rarefaction of alleles in juveniles with respect to adults, by simulating the generation of progeny cohorts from a limited number of adults drawn from a very large H-W equilibrium population (N = 10 million), having allele frequencies equal to those observed in the pooled adult sample. We note that this approach ignores the presence of additional rare alleles, which would be present in the larger base population, but preliminary simulations suggest that such rare alleles cause overestimation of N_b by the algorithm used here. At each step in the simulation, we generated 10,000 progeny cohorts of the same size as the juvenile sample from a certain number of parents, N_b; the simulation started with $N_b = 2$ and increased this number in subsequent steps, with actual N_b being calculated at each step, for each cohort, from the mean ($\overline{k}$, equal to 2 with constant N) and variance (V_k) in offspring numbers over all N individuals, according to $N_b = (4N - 4) / (V_k + \overline{k})$. We recorded at each step the median, 2.5%, and 97.5% percentile differences between the numbers of alleles in the 10,000 simulated progeny cohorts and the number of alleles in the observed adult sample. As an estimate of N_b compatible with our data, we took the mean of the modeled values that produced a median difference in allele number equal to the observed difference between parents and progeny, at each locus and over all loci. The lower (LCL) and upper confidence limits (UCL) for N_b corresponded to the model N_b values at steps, for which the 97.5% and 2.5% percentiles of the difference distribution, respectively, equaled the observed difference in allele numbers. When the difference in numbers of alleles skipped the observed difference between adjacent steps, we interpolated between the modeled N_b values. An example of simulation results is given in an online appendix to illustrate how the estimate and its confidence limits are obtained.

Results

Genetic variability within and between adult and juvenile samples

Average observed heterozygosity was 0.857 for both adult and juvenile samples (Table 1). The unbiased expected average heterozygosity values for juvenile and adult samples were 0.871 and 0.913, respectively, also not significantly different. The juvenile sample, despite its greater size, averaged significantly fewer alleles per locus than the adult sample, 13.75 vs. 23, respectively. Of the 95 alleles detected in this study, 52 were present in both the adult and juvenile samples, while another 40 were present only in the adult sample; three alleles – 225 at locus *Oedu*H15 and 142 and 184 at locus *Oedu*O9 – were restricted to the juvenile sample. Alleles present in the adult sample but absent in the juvenile sample were significantly less frequent than alleles that were present in both samples (mean 0.020 vs. 0.062, respectively, $P<0.003$), though they ranged in frequency as high as 0.073.

Departures from Hardy-Weinberg equilibria were analysed through the significance of the permutation test for the null hypothesis, $F_{IS} = 0$. Both adult and juvenile samples showed a large and highly significant heterozygote deficiency for locus *Oedu*H15 ($F_{IS} \approx 0.2$; $P < 0.0001$ for both samples, Table 1). This deficiency was likely explained by the presence of a non-amplifying, PCR-null allele at this locus. A null allele was observed for this locus in a controlled cross (S. Launey, unpublished), and heterozygote deficiency has been observed in 13 natural populations of adult flat oysters (Launey et al. 2002). The adult sample also showed a slight but significant deficiency of heterozygotes at the *Oedu*J12 locus. On the other hand, the juvenile population showed an excess of heterozygotes at *Oedu*J12, *Oedu*T5, and *Oedu*O9, the last being highly significant; averaged over these three loci, observed and expected heterozygosities were 0.917 and 0.881, respectively, and average $F_{IS} = -0.041$ was significantly < 0 (P=0.0016 in 10,000 permutations). Whereas gametic phase disequilibrium was significant in only one of six digenic combinations for the adult sample, it was highly significant in all six digenic combinations for the juvenile sample (Table 2). The adjusted mean squared digenic correlation ($adj\ \overline{r^2}$) for the juveniles, 0.0121, was significantly greater than that for the adults, 0.0004 (paired test $t = 15.25$, $P << 0.001$).

Finally, there was significant genetic divergence between the juvenile and adult samples. The $F_{ST} = 0.041$ (P<0.0001) between the two samples contrasted with the genetic homogeneity of the two adult samples ($F_{ST} = 0.001$, $P = 0.314$), which were pooled to make the juvenile-adult comparison.

Family structure

Kinship analysis revealed that 28% of juvenile pairs had a significantly greater likelihood of being full sibs than of being non-related; approximately equal proportions of pairs were significant at the 5%, 1%, and 0.1% levels of significance. By contrast, only 5% of adult pairs, most in the $0.01 < P \leq 0.05$ significance interval, were more likely to be full-sibs than to be unrelated (Table 3). Likewise, 26% of juvenile pairs had a greater likelihood of being half sibs than of being non-related, whereas only 2.8% of adult pairs were likely to be half sibs.

Estimation of the number of parents, N_b

We estimated N_b from the change in allelic frequencies between adult and juvenile samples, from gametic phase disequilibrium in the juvenile sample, and from excess heterozygosity in the juvenile sample (Table 4). The one-generation temporal variance in allele frequencies, $F_c = 0.052$, was larger than $F_{ST} = 0.041$, owing to differences in the way that these quantities are calculated; like F_{ST}, F_c was significantly larger than sampling variance. All three methods gave estimates of $N_b < 30$, with finite upper 95% confidence limits. The estimate and confidence interval from the temporal variance method was contained within the confidence interval for the heterozygote-excess estimate, which had to be based on only three loci, owing to a deficiency of heterozygotes attributable to null alleles at *Oedu*H15, and which therefore had the largest confidence interval. The gametic phase disequilibrium method, which was based on 1124 allelic pairs, yielded an estimate compatible with that from heterozygote excess but larger than the upper 95% confidence limit for the temporal variance estimate.

Estimates of the effective number of parents that contributed to the juvenile cohort, based on rarefaction of allelic diversity in juvenile compared to adult samples, were all quite small and consistent (Table 5). Across all four loci, the estimate of N_b was 13.7 with a narrow confidence interval, indicating

that more than 10 but fewer than 18 parents effectively contributed to this cohort of recruits. This estimate of N_b, together with its narrow confidence interval, was consistent with the temporal and heterozygote-excess estimates.

Discussion and conclusions

Genetic divergence observed between the geographically proximal samples of juvenile and adult *Ostrea edulis* from the western Mediterranean Sea was quite striking and unexpected, since marine fish and invertebrates with planktonic larvae are often assumed to comprise large, well-mixed populations. Indeed, this juvenile-adult difference contrasted sharply with the genetic homogeneity of adult samples from the Thau Lagoon and Port Saint Louis, France, which were pooled here for comparison with the juvenile sample. These same adult samples were part of a larger survey of microsatellite DNA markers (Launey et al. 2002; samples MWb, MWc), which reported genetic homogeneity over regional scales, such as the western Mediterranean, in substantial agreement with an earlier allozyme survey (Saavedra et al. 1995). Specifically, the level of juvenile-adult differentiation, F_{ST} = 0.041, was exceeded by only seven of 105 F_{ST} values reported by Launey et al. (2002) for pair-wise comparisons among 15 population samples stretching from Norway to the Black Sea. Five of the high F_{ST} values were found in comparisons between samples from Norway and the Mediterranean, one between Norway and Ria Formosa, Portugal, and one between Vigo, Spain, and Dubrovnik, Croatia. Thus, the juvenile-adult divergence reported here was as great as that between adult populations separated by thousands of kilometers and was thus consistent with previous reports of chaotic genetic patchiness in marine invertebrate populations cited in the Introduction.

Our finding of a divergent juvenile population is similar to that of Moberg and Burton's (2000) observation of genetic divergence between sea urchins recruits and adult spawning populations. Genetic divergence between adult and juvenile samples can be attributed to spatial variation among spawning populations (i.e. to source-sink dispersal) only by assuming the existence of a divergent, un-sampled, source population. Such an assumption, however, is supported neither by the large body of evidence for genetic similarity of marine populations with planktotrophic larvae nor by the particular evidence for weak population structure of the European flat oyster (Saavedra et al. 1995; Launey et al. 2002). In this case, moreover, a hidden source population would not only have to have divergent allelic frequencies but would also have to have significantly less allelic diversity than other Mediterranean populations.

Other causes, such as pre- or post-settlement selection, for example, could be invoked to explain differentiation of juveniles from adults. Selection during the larval phase would have had to have been very strong, however, to have caused such marked changes in allele frequencies and allelic diversity, and it would have had to have affected the four unlinked markers to a similar extent, which is unlikely (Lewontin and Krakauer 1973; Baer 1999). Selection after settlement is a more viable explanation, particularly since the cohort was reared for about 6 mo in a land-based aquaculture facility and then for a little more than 4 mo in the Thau Lagoon. Samples taken before and after the final grow-out period were genetically homogeneous, however, and mortality during the initial holding phase, though not documented, would have had to have been massive and noteworthy to account for the divergence (see online appendix for additional information on this point). Again, selection is unlikely to have reduced allelic diversity at all four unlinked markers simultaneously. Thus, neither spatial variation among adult spawning populations or selection, either before or after settlement, can adequately explain the peculiar genetic composition of the juvenile cohort we have studied.

Another potential explanation for the genetic divergence of adults and juveniles is sweepstakes reproductive success. A cohort of marine planktonic larvae or newly recruited juveniles is widely assumed to represent a very large number of parents. With sweepstakes reproductive success, however, cohorts of newly recruiting flat oyster juveniles could comprise individuals from relatively few full-sib or half-sib families. Reduced allelic diversity, excess heterozygosity, gametic-phase disequilibria, and substantial proportions of full- and half-sib relationships in this juvenile cohort are inconsistent with the presumption that this particular cohort was produced by a large number of adults but are consistent with the hypothesis of sweepstakes reproductive success.

Departures from Hardy-Weinberg equilibria observed in the juvenile sample were uncharacteristic of adult flat oyster populations, which generally conformed to random mating expectations (Launey et al.

2002). For example, after omitting *Oedu*H15, a locus known to have PCR null alleles, we found a significant excess of heterozygotes in the juvenile sample. In the Launey et al. (2002) study, F_{IS} for four loci, not including *Oedu*H15, was not significantly different from zero in 11 of 15 adult populations (including the two pooled here for the juvenile-adult comparison) and was significantly positive (deficiency of heterozygotes) rather than negative in the other four populations. On the other hand, excess heterozygosity with respect to Hardy-Weinberg expectations is characteristic of cohorts of progeny formed by a finite number of parents (Robertson 1965; Rasmussen 1979; Pudovkin et al. 1996; Cornuet and Luikart 1996; Balloux 2004).

Likewise, gametic-phase disequilibrium was highly significant for all pairs of loci in the juvenile sample, but was significant for only one pair of loci in the adult sample. The weighted mean squared digenic correlation, adjusted for sample size, was significantly greater for juveniles than for adults. Gametic-phase disequilibrium is unlikely to be maintained in large randomly mating populations, but can be generated by genetic drift in small populations (Hill 1981). In the case of a cohort of offspring from a randomly mating population, significant gametic-phase disequilibrium can be safely attributed to kinship among some proportion of the juvenile population.

Recent advances in the statistical power of detecting kinship in natural populations, made possible by PCR and highly polymorphic microsatellite DNA markers (Avise 1994, 2001), enables an explicit test of the sweepstakes-reproductive-success prediction that a cohort of juveniles might comprise full- and half-sib families. Full- and half-sib relatedness between individuals was significantly more likely in the juvenile sample of flat oysters (28% and 26%, respectively) than in the adult sample (5% and 2.8%, respectively). Relatedness in the juvenile sample is much greater than that detected by Herbinger et al. (1997) among larvae from a natural cohort of Atlantic cod (*Gadus morhua*), although the non-equilibrium genetic structure of this cohort was also interpreted as consistent with sweepstakes reproductive success (Ruzzante et al. 1996, 1999). Our finding of significant kinship in this particular cohort of flat oyster juveniles suggests a small effective number of parents at its origin.

Four estimates of N_b, particularly the temporal and allelic rarefaction estimates, agreed remarkably well for the small number of loci sampled. Differences among the estimates reflected differences in the statistical power of the four methods and in how well their assumptions were met in flat oyster populations. The allele-rarefaction method and temporal variance estimates had similarly narrow 95% confidence intervals, which were contained within the broader confidence interval for the excess heterozygosity method based on three loci. The estimator based on gametic phase disequilibrium had the narrowest 95% confidence interval, 24 to 31, which was surprising given Waples' (1991) suggestion that 10 or more independent loci may be required for precision. Precision in this case was increased by the high polymorphism of microsatellite DNA loci, which afforded correlation among 1124 pairs of alleles (965 degrees of freedom) and yielded a 95% confidence interval that did not include the point estimates from the other methods. This method is much more likely to be influenced by rare alleles than are the drift and allele-rarefaction methods, however. Also, unlike the previous estimates, which depend only on differences between the adult population and the observed cohort of juveniles, the estimate of N_b based on linkage disequilibrium reflects part of whatever genetic drift may have occurred in the grandparental generation (Waples 1991).

Estimates of N_b from genetic data support a prediction of the hypothesis of sweepstakes reproductive success that juvenile cohorts might have less genetic diversity than adult populations and falsify the null hypothesis that this naturally spawned cohort of flat oyster juveniles was produced by a very large number of parents. Small N_b provides a sufficient explanation for the non-equilibrium genetic structure of the juvenile sample, which is unexpected in light of the known equilibrium genetic structure of natural flat oyster populations. The question remains how fortuitous was this observation, i.e. how often and in what locations do marine fish or invertebrates with planktonic larvae produce such small-N_b cohorts. Future research should focus on genetic comparisons of adults with larval and early juvenile stages, in which the consequences of sweepstakes reproductive success may be most evident. Indeed, a cohort such as the one we studied could be recognized by its distinctive genetic profile at all stages of planktonic development, facilitating detailed oceanographic study. However, estimating the effective number of parents for larval or juvenile cohorts has two limitations that must be surmounted. First, statistical power in estimating N_b from genetic data is relatively weak. The typical study of mtDNA or a handful of microsatellite markers in samples of tens to hundreds of individuals may detect a true N_b possibly as large as a few hundred to a few thousand at most. However, an N_b of several thousand to several ten thousand would not be detectable but would still represent a substantial reduction, by several

orders of magnitude, of the effective number of breeders from the census numbers of most abundant marine fish and invertebrates. High-throughput genotyping is likely to be necessary to increase the statistical power of indirect genetic estimates of N_b for larval cohorts. A second limitation of this strategy is generally poor understanding of the environmental components of reproductive success in marine ecosystems. The number of different larval cohorts that recruit to a given place over a spawning season and the variation in this number among different species must be taken into account. Future studies will thus have to be of sufficiently large scale and careful design to account for the relevant physical and endogenous physiological and genetic factors that dictate the recruitment success of meroplanktonic marine life.

One of the main implications of our observation of a natural sweepstakes reproductive event is that related individuals issued from a limited number of progenitors settled close to each other. Conditions were thus set, in the vicinity sampled, for subsequent spawning between full- or half-sibs growing in close proximity to each another. The result could well have been production of some inbred families in the subsequent generation of larvae, perhaps enough to generate heterozygosity-fitness correlation through associative overdominance (Ohta 1971; Zouros and Pogson 1994; David et al. 1997b, David 1998; Bierne et al. 2000; Launey and Hedgecock 2001). The associative overdominance hypothesis is now amply supported by evidence for large mutational load and inbreeding depression in marine bivalves (Bierne et al. 1998; Naciri-Graven et al. 2000; Launey and Hedgecock 2001; Evans et al. 2003). Even small levels of consanguineous mating in such populations are likely to have measurable consequences on average fitness and more importantly on variance in fitness.

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Table 1. *Ostrea edulis*. Genetic variability in juvenile and adult samples of flat oyster. n_a is the number of alleles, and H_o and H_e are the observed and unbiased expected proportion of heterozygotes. F_{IS} estimates the deviation from HW equilibrium (* P<0.05, ** P <0.01, *** P <0.001). Data for adults from Launey et al. (2002).

Locus	Parameter	Juvenile	Adults
*Oedu*H15	Sample size	171	94
	n_a	11	16
	H_o	0.678	0.702
	H_e	0.841	0.893
	F_{IS}	0.194***	0.215***
*Oedu*J12	Sample size	183	96
	n_a	16	25
	H_o	0.918	0.885
	H_e	0.895	0.929
	F_{IS}	-0.026	0.047*
*Oedu*O9	Sample size	179	95
	n_a	16	24
	H_o	0.961	0.916
	H_e	0.906	0.895
	F_{IS}	-0.062**	-0.023
*Oedu*T5	Sample size	181	93
	n_a	12	27
	H_o	0.873	0.925
	H_e	0.843	0.933
	F_{IS}	-0.036	-0.009

Table 2. *Ostrea edulis*. Adjusted gametic phase disequilibrium, $adj\ r^2 = r^2 - (1/S)$, where S is the number of individuals typed for pairs of microsatellite DNA markers in adult (above diagonal) and juvenile (below diagonal) samples of flat oyster. Negative values, which result when $r^2 < 1/S$, are interpreted as zero. The null hypothesis of no gametic phase disequilibrium is tested by $\chi^2 = adj\ r^2 \times S \times df$, where $df = (m-1)(n-1)$ and m and n are the numbers of alleles at the two loci being considered; *** = P <<0.001, after Bonferroni adjustment for multiple testing.

	*Oedu*H15	*Oedu*J12	*Oedu*O9	*Oedu*T5
*Oedu*H15	-	-0.0008	0.0003	0.0036***
*Oedu*J12	0.0079***	-	-0.0008	-0.0006
*Oedu*O9	0.0127***	0.0133***	-	0.0012
*Oedu*T5	0.0161***	0.0117***	0.0117***	-

Table 3. *Ostrea edulis*. Likelihood ratio tests of full-sib (FS) and half-sib (HS) vs. unrelated hypotheses, for pairs of individuals in juvenile and adult samples. The body of the table shows the threshold LOD score for significance, the simulated type II error, and the percentage of pairs falling into three intervals of significance. The total percentage of significant tests is at the 5% level of significance. Total number of pair-wise tests is 16,110 for juveniles and 4753 for the pooled adults.

		Juveniles			Adults		
Test	*P* interval	LOD	Type II error	% pairs	LOD	Type II error	% pairs
FS	$0.01 < P \leq 0.05$	0.1755	0.112	9.92	0.1038	0.095	4.48
	$0.001 < P \leq 0.01$	1.0191	0.254	8.85	1.1258	0.250	0.44
	$P \leq 0.001$	1.8733	0.459	10.09	2.0187	0.476	0.15
	$P \leq 0.05$			28.26			5.07
HS	$0.01 < P \leq 0.05$	0.5460	0.467	10.19	0.5922	0.512	2.55
	$0.001 < P \leq 0.01$	1.0542	0.694	14.89	1.3518	0.791	0.23
	$P \leq 0.001$	2.3385	0.959	4.90	2.1855	0.942	0.04
	$P \leq 0.05$			25.98			2.81

Table 4. *Ostrea edulis*. Estimated effective number of breeders by temporal, linkage disequilibrium, and heterozygote excess methods.

Estimation Method	No. of loci	Parameter	N_b	95% CI
Temporal	4	F_c: 0.052	11.4	8.0 – 15.8
LD	4	r^2: 0.018	27.5	24.1 – 31.3
H excess	3	*d*: 0.026	19.7	10.3 – 368.3

Table 5. *Ostrea edulis*. Estimated effective numbers of parents for the juvenile sample, based on simulation of the difference in number of alleles between a sample from a large adult population (data from Launey et al. 2002) and a cohort of juveniles produced by a smaller number of breeders.

	Number of alleles			Estimate of the number of parents		
Locus	Adults	Juveniles	Difference	LCL	Median	UCL
*Oedu*H15	16	11	5	5.2	11.2	23.9
*Oedu*J12	25	16	9	8.7	17.6	34.5
*Oedu*O9	24	16	8	9.6	19.5	36.2
*Oedu*T5	27	12	15	4.1	7.9	14.4
All loci	92	55	37	9.6	13.7	18.0

Appendix

Small effective number of parents (N_b) inferred for a naturally spawned cohort of juvenile European flat oysters *Ostrea edulis*

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Comments on the cohort sampling

The cohort of naturally spawned juvenile flat oysters *Ostrea edulis* was sampled for genotyping at two time points, when the young juveniles were first removed from the collectors (February 21, 1994), before the grow-out period in the Thau Lagoon, and again at the end of the grow-out period (June 30, 1994). The original intent of the study was to look for correlation between growth and heterozygosity. In order to maximize association of genotype and growth, size-selective sampling was done, first to standardize the experimental group to a common size, by focusing on mean-sized juveniles (2-3 g live weight) and then, after growing these same-age and similar-size seed oysters, to contrast the largest (fastest growing) and smallest
(slowest growing) juveniles of this cohort. No correlation between microsatellite heterozygosity and growth was observed, but positive correlation of allozyme heterozygosity and growth was found (Launey 1998).

In this study, we focused on the comparison of allelic and genotypic diversity of the juvenile cohort with the diversity of nearby adult populations. For this purpose, we pooled all juvenile samples as representative of the cohort at settlement. We must consider, however, the extent to which selection among individuals after settlement and prior to sampling, rather than a small effective number of parents at the origin of the cohort, might have contributed to the observed difference in diversity between juveniles and adults. Observations on mortality, size, and genetic composition of the juvenile subsamples are relevant to this concern.

At the first sampling, juvenile oysters were removed from the collectors. While no data on mortality of oysters between the time of collection and first sampling were recorded, those present (YN, FB) do not recall large numbers of dead oysters on the collectors. The sample was divided roughly into three size fractions – small (n=927), intermediate (~2-3 g live weight, n=729), and large (n=892) seed oysters – and stocked into mesh bags for further growth. Forty-six and 56 oysters were sampled from the small and large groups, respectively, weighed individually, and frozen for subsequent genotyping; these samples are designated here as BS (before, small) and BB (before, big). Mean live weight of oysters in the BS sample was 1.27±0.05 g, while mean live weight of oysters from in the BB sample was 6.17±0.18 g (Fig. 1). The intermediate-sized group was planted at normal commercial densities of ~100 oysters per bag, but the small and large groups were stocked at higher densities, in three and four bags, respectively, for grow-out.

At the second sampling, the mortality of oysters in the small- and large-size seed groups was recorded (Table 1). Mortality of oysters in the small-size seed group was 60%, more than 1.5 times as large as mortality in the large-size seed group, 38%, though unequal densities in the bags confound these estimates. Mortality in bags containing the intermediate-size seed oysters was not recorded but was likely lower, owing to lower densities; mortality is generally positively correlated with stocking density in oyster cultivation (e.g. Bishop and Hooper 2005). Higher mortality in oysters reared from small-size seed could reflect selection against oysters homozygous for deleterious recessive alleles (Bierne et al. 1998; Launey and Hedgecock 2001), yet such selection, if present, did not produce a significant correlation of growth and heterozygosity at the microsatellite markers (Launey 1998).

From the bags stocked with intermediate-size seed oysters, 43 of the smallest oysters and 40 of the largest oysters were weighed and frozen for subsequent genotyping; these samples are designated AS (after, small) and AB (after, big). Mean live weight of oysters in the AS sample was 4.15±0.14 g, while mean live weight of oysters in the AB sample was 13.64±0.29 g (Fig. 1). The mean (±SE) live weight of the combined AS and AB samples was 8.72 ± 0.55 g (n=83).

The critical question is to what extent our inference of small N_b is potentially confounded by reductions in diversity resulting from selection after settlement and before sampling or by the non-random

sampling itself. Two pieces of evidence suggest that neither post-settlement selection nor non-random sampling is a sufficient explanation for the reduction in diversity observed in the juvenile cohort.

First, genetic differentiation among the four subsamples of the cohort was minimal, especially with respect to their differences from the adult sample. Numbers of alleles per locus and average expected heterozygosity were the same among the four subsamples (Table 2). Two comparisons of subsample BB, with BS and AS, had significant F_{ST}, after adjusting the threshold of significance for multiple tests ($\alpha = 0.05/10 = 0.005$; Table 3). The F_{ST} values (0.012 and 0.014), however, are much smaller than distances between any of the cohort samples and the adult sample. The non-significant allele-frequency variance between the small and big oysters sampled at the later time point (i.e. AS vs. AB) suggests that non-random sampling of individuals on the basis of size was, nevertheless, random with respect to allele frequencies at the microsatellite loci. Absence of differences in genetic diversity or allele-frequency variance between the February and June samples further suggests an absence of differential mortality of microsatellite marker genotypes over the 17 wk grow-out period in the Thau Lagoon.

Second, the proportion of pairs that showed significant full-sib relationship, using the Kinship program, as described in the main article, is large, whether the two individuals came from different groups or from the same group (Table 4). These proportions varied significantly among the paired-group comparisons (Table 5), with excesses of full sib pairs in the BB-BB, AS-AS, and interestingly AS-BB categories and deficiencies of full sib pairs in the AB-BS and BS-BS categories. Nevertheless, high proportions of full-sibs in small-big dyads strongly suggest that the same families were present in the large and small samples at each of the time points, though possibly at different frequencies, which could explain the differentiation of the BB group. That full sibs were shared across size extremes makes it difficult to imagine a large number of families that were restricted to the middle part of the size-frequency distribution and remained un-sampled. Non-random sampling on the basis of size is unlikely to have resulted in under-sampling of families in the juvenile cohort and an under-estimation of genetic diversity.

Thus, we find no support for the hypothesis that post-settlement selection reduced diversity in the juvenile cohort. Likewise, we find no support for the hypothesis that non-random sampling of oysters on the basis of size (growth rate) reduced diversity in the juvenile cohort by selecting against families that had intermediate rates of growth and were absent from the small and large extremes of the population sampled. In the absence of evidence for these two mechanisms, pooling of the subsamples of the juvenile cohort seems not only justified but also necessary to encompass the full diversity of the juvenile cohort for a comparison to the adult population.

Illustration of how the mean and confidence limits of N_b are obtained by simulation

As explained in the Materials and Methods of the article, at each step in the simulation, we generated 10,000 progeny cohorts of the same size as the juvenile sample from a certain number of parents, N_b, starting with $N_b = 2$ in the first step and increasing this number in subsequent steps. At each step, we recorded the median, 2.5%, and 97.5% percentile differences between the numbers of alleles in the 10,000 simulated progeny cohorts and the number of alleles in the observed adult sample. As an estimate of N_b compatible with our data, we took the mean of the modeled values that produced a median difference in allele number over all four microsatellite markers equal to the observed difference between parents and progeny, 37. The lower (LCL) and upper confidence limits (UCL) for N_b corresponded to the model N_b values at steps, for which the 97.5% and 2.5% percentiles of the difference distribution, respectively, equaled the observed difference in allele numbers (Table 6). When the difference in numbers of alleles skipped 37 between adjacent steps, *e.g.* from 38 at step 11 to 36 at step 12 (for LCL, Table 5), we interpolated between the modeled N_b values (to get, in this case, 9.65 as the mid-point between 9.21 and 10.09).

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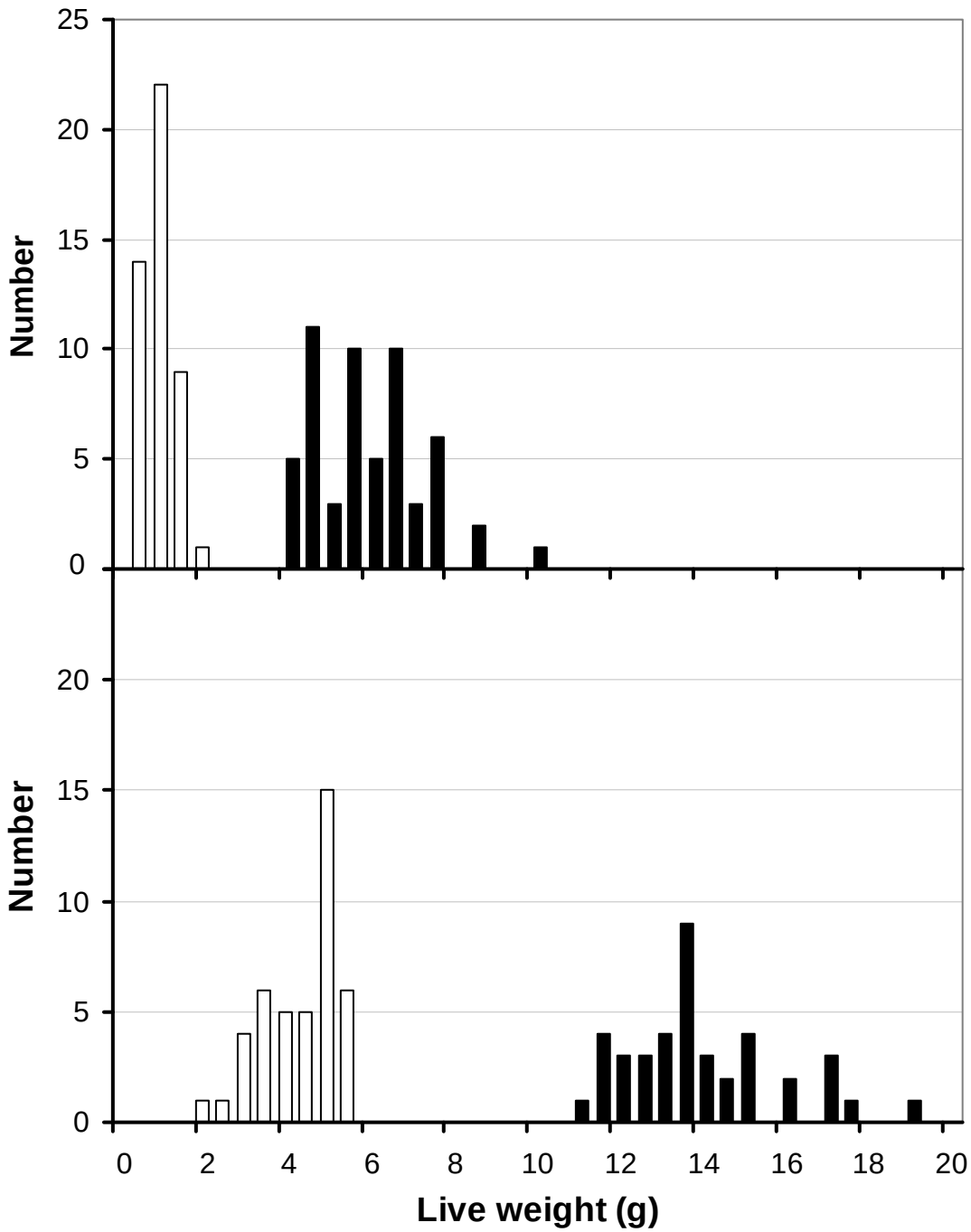

Fig. 1. *Ostrea edulis*. Size frequency distributions for four subsamples taken from the cohort of naturally spawned juveniles collected in 1993. A. Samples of small- and large-size seed oysters taken Feb. 21, 1994, when oysters were removed from the collectors. B. Samples of small- and large-size oysters selected from bags stocked with intermediate-size seed oysters and harvested June 30, 1994, after 17 wk of growth in the Thau Lagoon.

Table 1. *Ostrea edulis.* Mortality of oysters in small- and large-sized seed oysters reared in the Thau Lagoon for 17 wk

Group / bag	Starting no.	No. alive	No. dead	Mortality (%)
Small				
1	338	132	206	61
2	258	114	144	56
3	331	129	202	61
Sum	927	375	552	60
Large				
1	144	92	52	36
2	278	178	100	36
3	225	133	92	41
4	245	147	98	40
Sum	892	550	342	38

Table 2. *Ostrea edulis.* Numbers of alleles observed per locus and average unbiased expected heterozygosity in four juvenile cohort samples.

	*Oedu*H15	*Oedu*J12	*Oedu*O9	*Oedu*T5	H_e
BS	9	13	16	11	0.888
BB	10	15	15	12	0.844
AS	10	14	16	8	0.859
AB	9	11	14	11	0.876

Table 3. *Ostrea edulis.* F_{ST} in pair-wise comparisons between four juvenile cohort samples and the adult sample, above diagonal. Proportion of 10,000 permuted values producing an F_{ST} as large as the one observed, below diagonal; values in bold significant at the 5% level, adjusted for 10 tests.

	BS	BB	AS	AB	Adults
BS		0.0117	0.0100	0.0030	0.0272
BB	**0.0042**		0.0142	0.0114	0.0584
AS	0.0164	**0.0020**		-0.0010	0.0477
AB	0.2121	0.0133	0.5390		0.0352
Adults	**0.0000**	**0.0000**	**0.0000**	**0.0000**	

Table 4. *Ostrea edulis.* Proportion of intra- and inter- group dyads with a significant probability of being full siblings.

	Ind. 2			
Ind. 1	BS	BB	AS	AB
BS	0.227			
BB	0.268	0.364		
AS	0.260	0.333	0.357	
AB	0.216	0.282	0.291	0.239

Table 5. *Ostrea edulis.* Contingency χ^2 test of the proportion of dyads having significant full-sib (FS) vs. unrelated (UNR) relationships across 10 categories of comparisons between four spat samples. $\chi^2 = 161.58$, df = 9, $P < 0.001$.

Groups paired	Dyads		Expected counts		Contributions to χ^2		Sums
	FS	UNR					
BS-BS	235	800	294.6	740.4	12.07	4.80	16.87
BB-BS	691	1885	733.3	1842.7	2.44	0.97	3.41
BB-BB	561	979	438.4	1101.6	34.29	13.65	47.94
AS-BS	490	1396	536.9	1349.1	4.09	1.63	5.72
AS-BB	765	1531	653.6	1642.4	18.99	7.56	26.54
AS-AS	293	527	233.4	586.6	15.20	6.05	21.25
AB-BS	367	1335	484.5	1217.5	28.50	11.34	39.84
AB-BB	584	1488	589.8	1482.2	0.06	0.02	0.08
AB-AS	441	1076	431.8	1085.2	0.19	0.08	0.27
AB-AB	159	507	189.6	476.4	4.94	1.96	6.90

Table 6. *Ostrea edulis.* Estimation of N_b by simulation of progeny cohorts, using combined data for four microsatellite markers. The steps in the simulation at which the difference in number of alleles is equal to that observed between the adult and juvenile samples, 37, are marked in bold with asterisks. LCL is the lower 95% confidence limit on estimated N_b; UCL is the upper 95% confidence limit on estimated N_b.

		Adult-juvenile allele number difference		
Step number	Average N_b	LCL	Median	UCL
0	2.00	76	78	80
1	2.94	76	78	81
2	3.97	54	61	67
3	4.30	54	59	67
4	4.78	49	58	64
5	5.12	51	57	64
6	5.69	48	56	62
7	6.14	48	53	61
8	6.81	45	51	56
9	7.53	43	49	55
10	8.32	38	47	53
11	9.21	**38***	44	51
12	10.09	**36***	42	49
13	11.14	35	40	50
14	12.24	33	38	46
15	13.67	30	**37***	43
16	15.00	27	33	40
17	16.85	26	31	**38***
18	18.50	22	30	**35***