Genetics Research
June 2010 ; Volume 92 (3) : Pages 175-187
http://dx.doi.org/10.1017/S0016672310000248

Variance in the reproductive success of flat oyster *Ostrea edulis* L. assessed by parentage analyses in natural and experimental conditions

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Abstract:

In order to document further the phenomena of variance in reproductive success in natural populations of the European flat oyster *Ostrea edulis*, two complementary studies based on natural and experimental populations were conducted. The first part of this work was focused on paternity analyses using a set of four microsatellite markers for larvae collected from 13 brooding females sampled in Quiberon Bay (Brittany, France). The number of individuals contributing as the male parent to each progeny assay was highly variable, ranging from 2 to more than 40. Moreover, paternal contributions showed a much skewed distribution, with some males contributing to 50–100% of the progeny assay. The second part of this work consisted of the analysis of six successive cohorts experimentally produced from an acclimated broodstock (62 wild oysters sampled in the Quiberon Bay). Allelic richness was significantly higher in the adult population than in the temporal cohorts collected. Genetic differentiation (F_{st} estimates) was computed for each pair of samples and all significant values ranged from 0·7 to 11·9%. A limited effective number of breeders (generally below 25) was estimated in the six temporal cohorts. The study gives first indications of the high variance in reproductive success as well as a reduced effective size, not only under experimental conditions but also in the wild. Surprisingly, the pool of the successive cohorts, based on the low number of loci used, appeared to depict a random and representative set of alleles of the progenitor population, indicating that the detection of patterns of temporal genetic differentiation at a local scale most likely depends on the sampling window.

Keywords: *Ostrea edulis*, microsatellite, reproductive success, temporal cohort, brooding females

1. INTRODUCTION

The mating system can greatly influence the genetic structure of populations. Crosses between relatives and selfing reduce multilocus heterozygosity and increase gametic disequilibria in the resulting progenies (Hedrick 2000). At the population level, they also lead to a reduction of effective size and an increase of inter-population differentiation. Moreover, demographic fluctuations (caused by variable ecological conditions) may result in transient bottlenecks that are expected to have the same effect on population's diversity and differentiation (Cornuet & Luikart 1996). Marine species with high fecundity and high early mortality such as oysters (Elm-oyster model; Williams 1975), are particularly prone to display large variance in reproductive success, because of gametic (gamete quality, sperm-egg interaction) and zygotic (zygotic competition, differential viability of genotypes) effects (Boudry *et al*. 2002), contributing to a reduction of their effective population size. Hence, many marine species have a combination of high fecundity and narrow conditions for spawning success that may lead to wide individual variation in realised reproductive success, such that an annual cohort is the result of only a few spawning events or individuals (Hedgecock 1994).

The flat oyster, *Ostrea edulis,* an endemic European species, naturally occurs from Norway to Morocco in the North-Eastern Atlantic and in the whole Mediterranean Sea. It has been harvested for at least 6000 years (Goulletquer & Héral 1997). However, overharvesting and, more recently, the successive occurrence during the 1960's of two protozoan diseases caused by *Marteilia refringens* and *Bonamia ostreae* drastically decreased its production. For example, the French production was reduced from 20,000 tons in the 1950's to 1,900 tons nowadays (FAO 2007). Hence, the native European flat oyster is listed in the OSPAR (Oslo-Paris) Convention for the Protection of the Marine Environment of the North-East Atlantic (species and habitat protection). In the context of potential restoration along European coasts (Laing *et al*. 2005), it is important to assess the potential impact of hatchery-propagated stocks on the genetic variability and the effective population size of wild populations (Gaffney 2006). Therefore, information is needed about the genetic variability of hatchery-propagated stocks (Lallias *et al*. 2010) and the structure and dynamics of wild populations to ensure proper management of populations and aquaculture production.

The genetic structure of wild *O. edulis* populations has been analysed with microsatellite DNA (Launey *et al*. 2002) and mitochondrial DNA (12S) markers (Diaz-Almela *et al*. 2004). Genetic differentiation based on mitochondrial data was 10-fold greater

(F_{st} = 0.224; Diaz-Almela *et al*. 2004) than the one established on microsatellite data (F_{st} = 0.019; Launey *et al*. 2002). This quantitative difference of a factor of ten observed between the nuclear and mitochondrial F_{st} was proposed to be attributable to a reduced female effective population size. This could be explained by several factors: i) a biased effective sex-ratio towards males owing to the protandry of the species and the higher energy cost in oogenesis (Ledantec & Marteil 1976), leading to a lower probability of becoming female. This is aggravated by the *B. ostreae*-caused disease (Culloty & Mulcahy 1996) which induces high mortalities within 2-3 year-old adults, ii) a higher variance in female than male reproductive success (Boudry *et al*. 2002; Taris *et al*. 2009). Other explanations are: (1) N_{ef} is one-quarter of N_e and (2) F_{st} is proportional to (H_S-H_T) (H_S being the average subpopulation Hardy-Weinberg heterozygosity, H_T being the total population heterozygosity) and H_S approached 1.0 in the microsatellites used (Hedrick 2005a).

Heterozygote deficiencies with regard to Hardy-Weinberg equilibrium expectations are common in marine bivalve populations (Zouros & Foltz 1984; Huvet *et al*. 2000; Hare *et al*. 2006) and were reported in *O. edulis* for allozymes (Wilkins & Mathers 1973; Saavedra *et al*. 1987; Alvarez *et al*. 1989) and microsatellites (Launey *et al*. 2002). Microsatellite markers are particularly prone to PCR artifacts such as the presence of null alleles and upper allele drop-out, which are responsible for the commonly observed heterozygote deficiencies. Moreover, a positive correlation between multi-locus heterozygosity (MLH) and life history traits such as growth or survival was reported in *O. edulis* based on allozymes (Alvarez *et al*. 1989; Launey 1998) and microsatellite markers (Bierne *et al*. 1998). Two kinds of arguments were mentioned to explain heterozygote deficiencies and correlations heterozygosity-growth. The first hypothesis, overdominance, implies that selection acts directly on allozymic genotypes, questioning allozymes neutrality. This hypothesis was refuted by the evidence of the same phenomenon occurring with reputedly neutral markers like microsatellites (Bierne *et al*. 1998; Launey & Hedgecock 2001). The second hypothesis, associative overdominance, stipulates that marker polymorphism is neutral but reflects indirectly variation in loci linked to fitness by genetic correlations. Genetic markers, be they allozymes or microsatellites, can therefore either represent neutral loci in gametic disequilibrium with physically close loci under selection (local effect) or represent neutral markers of the overall genomic heterozygosity (general effect, David *et al*. 1995). Whether local or general, the associative overdominance hypothesis takes root in the characteristics of reproductive biology and dynamics of these species. Indeed, according to Bierne *et al*. (1998), an instantaneous reduced effective population size can induce gametic disequilibrium between genetic markers and loci

linked to fitness (local effect); whereas partial inbreeding can generate a variation in the global genomic heterozygosity between individuals (general effect). Li & Hedgecock (1998) in *Crassostrea gigas* and Hedgecock *et al*. (2007) in *O. edulis* highlighted the fact that, under local circumstances, the effective population size can be drastically reduced by a high variance in reproductive success, which could in turn generate a temporary gametic phase disequilibrium (reinforcing the associative overdominance hypothesis).

Variance in individual reproductive success among parents has also been documented under experimental conditions using controlled crossing (e.g. Hedgecock & Sly 1990; Hedgecock *et al.* 1992 and references therein; Petersen *et al.* 2008). The most direct evidence comes from studies of the Pacific oyster, *Crassostrea gigas*, in which changes in family representation in progenies resulting from factorial crosses were analysed using microsatellites markers for parentage analyses (Boudry *et al.* 2002; Taris *et al.* 2006). Their results showed large variance in parental contributions at several developmental stages, leading to a strong reduction of experiment-wide effective population size that could be attributed to four main factors: gamete quality, sperm-egg interaction, sperm competition and differential survival among families.

In order to document further the phenomena of variance in reproductive success both in natural and hatchery-produced populations of *O. edulis*, we performed two complementary studies to answer two questions: (1) Is it possible to detect a variance in reproductive success which could result in a reduced effective population size? (2) How is this variance temporally expressed? To answer these questions, brooding females were firstly sampled in the wild and the number of males fertilising each female estimated on the basis of microsatellite allele frequencies. Then, to get rid of drawbacks inherent to working with large natural populations and multiple environmental factors, parentage analyses were conducted under experimental conditions: successive cohorts were collected from a population of potential progenitors kept in hatchery, whose genotypes were known, in order to infer *a posteriori* the relative contribution of each. The results of these two studies are discussed in the light of previous studies of wild or hatchery-bred flat oysters.

2. MATERIALS AND METHODS

(i) Sampling and experimental design

First experiment - During summer 2001, 13 flat oysters, *O. edulis*, and the larvae present in their mantle cavity (i.e. brooding females) were collected when sampling individuals in Quiberon Bay (Brittany, France). This area represents a natural recruitment zone for this species. The sampling period extended from June to August:

- 26/06/2001: females F1 and F2
- 10/07/2001: females F4, F5, F6, F7 and F8
- 17/07/2001: females F9 and F10
- 08/08/2001: female F21
- 14/08/2001: females F22, F23 and F24

Second experiment - In November 2002, 62 adult oysters were sampled from a natural population in the same bay and transferred in raceways in the Ifremer experimental hatchery of La Tremblade (France). They were first anaesthetised with $MgCl_2$ (Culloty & Mulcahy 1992) to get biopsies of the gills for microsatellite genotyping. They were then conditioned for spawning, by increasing water temperature and food supply. Additional food consisted of three species of phytoplankton: *Isochrysis galbana*, *Chaetoceros calcitrans* and *Tetraselmis suecica*. Sieves were placed under the outflow pipe in order to collect larvae during the reproductive period (water flow: 150 l/h). The term "cohort" refers to larvae that were collected, just after their release, on these sieves. Sieves were checked daily to collect the larvae that were then kept in 70% ethanol for further genetic analysis. It is known that stocks of adult flat oysters produce larvae over an extended period, contrary to the cupped oysters which are mass spawners (Helm *et al*. 2004).

(ii) Genotyping

DNA extraction for adult oysters (gill tissue) was performed by a classical phenol/chloroform method (Sambrook *et al*. 1989). Eighty larvae per brooding female or per cohort were separated under binocular lens in a Dolfuss tank, and individuals were put in a 0.2 ml Eppendorf tube with 4 µl of 70% ethanol. Larval DNA extraction was performed by evaporating ethanol and adding 50 µl of extraction buffer (1.5 ml of 10X PCR Buffer, 75 µl of Tween 20, distilled water up to 15 ml) and 5 µl of Proteinase K (10 $mg.ml^{-1}$) (Taris *et al*. 2005). Larvae were incubated one hour at 55°C and 20 minutes at 100°C. Genomic DNA was kept at –20°C.

Four microsatellite loci were used: *Oedu*J12, *Oedu*U2, *Oedu*H15 and *Oedu*T5 described in Launey *et al*. (2002). Polymerase chain reactions (PCR) were performed in a 10

193 µl reaction mix containing 5 µl template DNA, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.25 µM of
194 each primer, 1 unit of Goldstar Licensed Polymerase (Eurogentec) and 1X polymerase buffer
195 (supplied by the manufacturer). The primers were synthesised by MWG Biotech with each
196 forward primer labelled with IRD-700 (*Oedu*J12 and *Oedu*U2) or IRD-800 (*Oedu*H15 and
197 *Oedu*T5). Amplifications were processed as follows: pre-denaturation (95°C, 5 min) followed
198 by 30 cycles of denaturation /annealing of primers / polymerisation (95°C, 20 s; T_a, 20 s;
199 72°C, 20 s) and a final elongation step (72°C, 30 min). The annealing temperature T_a of the
200 primer pair was respectively 50°C for *Oedu*J12, *Oedu*H15, and *Oedu*U2 and 53°C for
201 *Oedu*T5. Variation in fragment size was visualised by 6.5% polyacrylamide denaturing gels
202 run at 1500 V, 40 W, 40 mA, at 50°C on a LICOR® DNA sequencer. Genotypes were scored
203 with reference to individuals, whose alleles were of known size, and resulting data were
204 analysed with the Gene Profiler 4.0 software.
205
206 (iii) Genetic analysis
207
208 Microsatellite genetic polymorphism within the adult population and within each
209 temporal cohort was measured as the mean number of alleles per locus, the observed (H_o) and
210 expected unbiased (H_{nb}) heterozygosity (Nei 1978). Estimate of allelic richness (A) that uses
211 rarefaction to correct for unequal sizes (El Mousadik & Petit 1996) was also performed per
212 locus and sample with the program FSTAT version 2.9.3 (Goudet 1995). A Friedman test was
213 applied to detect differences in allelic richness among samples (Minitab 14.0): the adults and
214 progeny cohorts were the treatments and the loci were the blocks. F-statistics described by
215 Wright (1931) were computed according to Weir and Cockerham's estimators, using Genetix
216 4.1 software (Belkhir *et al*. 1996-2001). Deviations from Hardy-Weinberg equilibrium (F_{is})
217 were computed in the adult population and in each cohort. Moreover, genetic differentiations
218 between adult population and cohorts were estimated using Wright's fixation index F_{st},
219 estimated by θ (Weir & Cockerham 1984). The significance of departures from zero of F_{is} and
220 F_{st} was assessed by 1000 permutations of the appropriate data (alleles within individuals for
221 F_{is}, individuals among populations for F_{st}).
222 We used three different methods for estimating the effective number of breeders (N_b):
223 1) temporal moments method of Waples (1989), based on the changes of allelic frequencies
224 between the adult population and each of the cohorts (NeEstimator 1.3 software; Peel *et al*.
225 2004; http://www.dpi.qld.gov.au/28_6908.htm), 2) excess heterozygosity method
226 (NeEstimator 1.3 software) and 3) linkage disequilibrium (LD) method (LDNe program;

Waples & Do 2008). For the LD method, the P_{crit} value is the minimum frequency for alleles to be included in the analysis. We performed the analyses using a P_{crit} value of 0.05 or 0.01. There is a tradeoff between bias and precision: generally, the lower the P_{crit} value, the more precise but also the more biased the N_b estimates will be (Waples & Do 2009).

(iv) Paternal analysis of larvae collected in brooding females

For the larvae collected in the mantle cavity of 13 wild brooding females, only mothers' genotypes and adult population allelic frequencies were available. Because of the size of the studied population, it was indeed impossible to sample all its individuals in order to obtain genotypes of all possible fertilizing males. To determine the number of males that contributed to the progeny of each female, two parental reconstruction software were used, one based on Bayesian statistics, the other on a combinatory approach. Both used multilocus genotypes of the known parent and offspring to reconstruct the genotypes of unknown fathers contributing to the progeny array.

The mean numbers of males having fertilised each of the 13 brooding females analysed, as well as the standard error over the 1000 iterations, were first estimated using PARENTAGE 1.0, a software based on Bayesian statistics developed by Ian Wilson (Emery *et al*. 2001; http://www.mas.ncl.ac.uk/~nijw). In the input file, several priors concerning distributions of offspring among males were stated:

- an equivalent contribution (each male contributes equally to the offspring)
- a number of fathers between 1 and 60

The mutation rate, accounting for assignation failures, was stated as equal to 0.02.

We also used GERUD 2.0 (Jones 2005), based on a combinatory approach, which does not rely on the choice of priors. First, paternal alleles were established by subtraction. Then, an exhaustive search was performed, which tried every possible combination of paternal genotypes. The program provided all possible combinations of the minimum number of fathers. When several combinations of paternal genotypes were consistent with the progeny array, the solutions were ranked by likelihood, based on the segregation of paternal alleles in the general population according to Mendelian expectations. As this approach is computationally intensive, it is restricted to progeny arrays with less than six fathers (Jones & Ardren 2003). Therefore, it was computed only for females whose progeny presented a low number of alleles.

(v) Parentage analysis of temporal cohorts collected in the hatchery population

For the temporal cohorts collected in the hatchery, genotypes of all potential progenitors are known, but not their sex as flat oysters are alternative hermaphrodites and can change sex during the same reproductive season (personal observations). First of all, exclusion probabilities, which correspond to the probability that a parent taken at random in a population can be excluded, were computed. It is of prime importance to compute exclusion probability prior to any parentage analysis, to ensure that the set of molecular markers used is powerful enough to achieve successfully parentage analysis. Exclusion probabilities were computed for each locus separately (P_{El}) and for all loci progressively combined (P_{CE}) according to Chakraborty *et al*. 1988:

$$P_{El} = 1 - 4\sum_{i=1}^{n} p_i^2 + 2\left(\sum_{i=1}^{n} p_i^2\right)^2 + 4\sum_{i=1}^{n} p_i^3 - 3\sum_{i=1}^{n} p_i^4$$

where n is the number of alleles at locus l and p_i is the frequency of the ith allele.

And for L loci:

$$P_{CE} = 1 - \prod_{l=1}^{L} (1 - P_{El})$$

Exclusion probabilities computed for the pool of 62 potential progenitors were 73.7%, 94.5% and 98.3% for *Oedu*J12, *Oedu*U2 and *Oedu*T5 respectively. The combined exclusion probability obtained with the three loci was 99.9%.

For parentage assignment, the "Parental pair (sexes unknown)" option of CERVUS 3.0 (Marshall *et al*. 1998, Kalinowski *et al.* 2007) was used. It is a parental pair allocation program, based on a maximum likelihood approach. The statistic Delta is defined as the difference in LOD scores between the most likely candidate and the second most likely candidate. In the simulation of parental analysis, the proportion of loci typed was 0.93, the simulated genotyping error was set at 0.01, the number of candidate parents was 62 and the proportion of candidate parents sampled was set at 100%. Critical values of Delta were determined for 80% and 95% confidence levels based on simulations of 10 000 offspring.

3. RESULTS

(i) Genetic and paternity analyses of the brooding females collected in a natural population

Genotypes at 3 to 4 microsatellite loci were determined for 80 larvae collected in each brooding female. Beforehand, compatibility of maternal alleles was checked in each offspring; five females, respectively F4, F6, F8, F23 and F24 showed some larvae whose

genotypes were not compatible at locus *Oedu*H15. In these cases, the five females were apparently homozygous; mismatching arose from the presence of homozygous larvae for an allele different from the one of the corresponding female. Null alleles were suspected; females were most likely heterozygous for a null allele thus making the larvae heterozygous exhibiting a paternal allele and the suspected maternal null allele. This has already been reported for this locus (Launey *et al*. 2002). Consequently, genotypes at *Oedu*H15 were recoded to take into account the segregation of a null allele, before performing the paternity analyses. The number of alleles per locus was assessed in each progeny array for each female. Locus *Oedu*H15 presented a lower number of alleles, always below 12. Mean number of alleles per locus was highly variable, from 4.3 for F7 to 18.5 for F10 (Table 1).

Mean numbers of male parents as determined with PARENTAGE 1.0 was highly variable among females, from 2 to more than 40 (Table 1). Software GERUD 2.0 was used for the five progeny arrays showing the lowest number of alleles: F5, F7, F8, F21 and F24. Minimum numbers of fertilizing males were obtained (Table 1), as well as the genotypes of males contributing to each array. Each paternal genotype was associated with the number of larvae compatible with this genotype. Paternal contributions showed a very skewed distribution, with some males contributing to 50-100% of the progeny assay (Figure 1).

(ii) Genetic diversity, differentiation and effective number of breeders of temporal cohorts collected in hatchery

Six temporal cohorts were collected from the batch of adult oysters kept in the hatchery during a short period of time between the 14th of March 2003 and the 30th of March 2003, although the experiment was pursued until the end of June. These cohorts were named by the date of collection: 14, 17, 20, 22, 28 and 30/03/2003. The six cohorts were also pooled into a "Total cohort", for analysis. Multilocus genotypes (at *Oedu*J12, *Oedu*U2 and *Oedu*T5) were determined for the adult population and for 80 larvae from each cohort. The population of potential progenitors consisted of 62 adults. Linkage disequilibrium was computed for each pair of loci for the adults kept in hatchery with the option 2 of the web-based version Genepop software (genepop@wbiomed.curtin.edu.au). No significant linkage disequilibrium ($p > 0.63$ for each combination) was observed in the population of progenitors: the three loci studied segregate independently.

The values of allelic richness varied from 23.00 to 27.00 for the adult population depending on the locus (Table 2). For the six temporal cohorts collected, the values of allelic

richness varied from 11.52 to 20.33 depending on the locus and the cohort. For the Total cohort (6 pooled cohorts), allelic richness was 18.70 for *Oedu*J12, 21.07 for *Oedu*U2 and 18.86 for *Oedu*T5 (Table 2). Regarding the allelic richness across loci in the adult sample and the six cohorts, there were significant differences observed (Friedman test statistic S = 13.30, df = 6, p = 0.04). Values of observed and expected heterozygosity were high, above 0.9 (Table 2). Deviations from Hardy Weinberg equilibrium (F_{is}) were computed for the adults and the cohorts (Table 2). The global heterozygote deficiency was not significant for the population of progenitors. None of the heterozygote excesses observed in the cohorts was significant. The significant heterozygote deficiency observed for the cohort of 28/03/03 ($p < 0.05$) was attributable to locus *Oedu*J12 ($p < 0.001$ for this locus after Bonferroni correction).

Genetic differentiations (F_{st} values) were computed for each pair of samples (adult/cohort; cohort/cohort). All values were highly significant ($p < 0.001$ or $p < 0.01$). Genetic differentiation ranged from 0.7% (between cohorts 20/03/2003 and 22/03/2003) to 11.9% (between cohorts 14/03/2003 and 17/03/2003) (Table 3a). Genetic differentiations were also computed between the population of progenitors and the cohorts progressively pooled (Table 3b). With pooling, genetic differentiation became blurred, but was non significant only when all 6 pooled cohorts were compared to the progenitors.

The effective number of breeders (N_b) was computed for each temporal cohort, using three different methods. The N_b estimates varied according to the method used, but were generally below 25. The excess heterozygosity method and the linkage disequilibrium method (with a P_{crit} value of 0.05) gave consistently lower N_b estimates (Table 4). The temporal method and the linkage disequilibrium method (using a P_{crit} value of 0.01) gave very similar estimates. The cohort 17/03/2003 had the lowest N_b. For the Total cohort, the heterozygote excess and linkage disequilibrium methods gave N_b estimates ranging between 15 and 34, whereas N_b estimate was 96 based on the temporal method (Table 4).

(iii) Parentage analysis of temporal cohorts collected in hatchery

CERVUS 3.0 software was used to assign the most likely parental pair to each offspring of a progeny array. For each of six temporal cohorts collected in hatchery, the percentage of larvae that were assigned a parental pair ranged from 49% to 65% with a 95% statistical confidence, and from 68% to 88% with a 80% statistical confidence (Table 5). Out of 62 potential progenitors, 10 did not contribute, 15 contributed to only one cohort, 11 to two cohorts, 10 to three cohorts, 5 to four cohorts, 5 to five cohorts and 6 contributed to all 6

cohorts. Depending on the cohort, the number of contributing progenitors ranged from 19 (17/03/2003) to 28 (14/03/2003 and 28/03/2003) (Table 4).

It is apparent from Figure 2 that the total contribution of each progenitor, in terms of number of offspring, was very variable. For example, 10 progenitors contributed each to a single offspring (e.g. P007, P009, P018) whereas 4 progenitors contributed each to more than 40 offspring (P006, P026, P048 and P094). Also, it can be noticed that some parents contributed to successive cohorts (e.g. P014, P075 contributed to 28/03/2003 and 30/03/2003) while others contributed to cohorts spaced in time. For example, P083 contributed to two cohorts spaced by two weeks: 14/03/2003 and 28/03/2003. The contribution of this individual to these two cohorts was confirmed by the segregation of rare alleles (exhibited by only this individual): hence P083 exhibited a rare allele at locus *Oedu*U2, which was found in some larvae of these cohorts. Segregation of such rare alleles was used to check qualitatively the succession of some individuals along the time found with CERVUS. Results were consistent: rare allele analysis revealed contribution of P014 in 28/03/2003 and 30/03/2003; of P028 to 5 cohorts (from 17/03/2003 to 30/03/2003); of P045 in 14/03/2003; and of P061 in 20/03/2003 and 22/03/2003.

Finally, there was a succession in time of major contributing progenitors (Figure 3). The main progenitor of 14/03/2003 was P083, contributing to more than 20% of the progeny. P026 contributed to almost 50% of the 17/03/2003 cohort, whereas P006 contributed to almost 30% of the 20/03/2003 cohort. For the last three cohorts, no progenitor had a contribution over 20%.

4. DISCUSSION

The advent of molecular tools and methods for parentage analysis (reviewed in Jones *et al*. 2010) has greatly facilitated genetic investigation of mating systems and the evaluation of patterns and determinants of reproductive success in aquatic organisms. Genetic methods have recently added much insight into the reproductive and parental care behaviour of several fish species by analysing genetic parentage of broods collected in nature (Sefc *et al*. 2008, Tatarenkov *et al*. 2008, Byrne & Avise 2009). Moreover, genetic parentage analyses have been employed to gain a better understanding of the spawning behaviour and reproductive dynamics of captive fish broodstock held in commercial breeding tanks (Jeong *et al*. 2007, Herlin *et al*. 2008, Blonk *et al*. 2009). Finally, high variance in reproductive success has previously been reported in bivalves, both in natural populations (Li & Hedgecock 1998,

Hegdgecock *et al*. 2007, Arnaud-Haond *et al*. 2008) and in experimental conditions (Boudry *et al*. 2002, Petersen *et al*. 2008). To our knowledge, only a few studies combine experimental studies with observations in natural populations.

(i) Comparison of natural population and experimental hatchery conditions

The first part of our study allowed the estimation of the effective number of individuals contributing as fertilizing males to the progeny of brooding females *in natura*. Paternity analyses revealed that this number was highly variable (from 2 to more than 40, Table 1). Our results also revealed a high variance of the relative contribution of each male within a female (Figure 1). In the studied population, the number and spatial distribution of individuals was unknown, as well as effective sex ratio or local environmental conditions. Therefore, no hypothesis could be put forward to explain why a particular female was (or was not) fertilised by a particular male, or to explain the variance in the relative contribution of the males; this highlighted the need to work under experimental conditions in a controlled environment to mimic what happens at the population level. The experimental part of the present study was therefore performed to describe mating among individuals in more detail. In this second part of our study, as individuals of similar size and physiological condition were kept under common environmental conditions (temperature, food input), we could expect that all oysters would become mature around the same time. Moreover, progenitors were moved daily inside the tank aiming to avoid spatial effect on fertilisation caused by the direction of the water flow in the raceway. Thus variance in reproductive success was expected to be low. Consequently, the variance in relative contributions observed within each cohort truly represented intrinsic capacities (physiology, genetics) of individuals to reproduce. A similar approach was successfully used in the lion-paw scallop (Petersen *et al*. 2008). Furthermore, a comparable experimental design was successfully used to study the hypothesis that reproductive success is randomly distributed within spawning aggregations of Atlantic cod (Rowe *et al*. 2008). This indicates that experimental design might be of particular interest to understand better the behaviour of wild populations.

(ii) Variance in reproductive success and effective population size: implications

There is a relationship between reproductive biology (variance in reproductive success implying a low N_e) and temporary disequilibrium (or markers-based heterosis). To explain

heterozygote deficiencies and heterozygosity-growth correlations, the associative overdominance hypothesis postulates that selectively neutral markers are affected by selection operating on linked loci with effects on fitness. Genetic markers, be they allozymes or microsatellites, can therefore either represent neutral loci in gametic disequilibrium with physically close loci under selection (local effect) or represent neutral markers of the overall genomic heterozygosity (general effect, David *et al*. 1995). Analysis of distorted segregation ratios in *C. gigas* confirms that these distortions are mainly attributable to selection against recessive deleterious mutations of fitness genes linked to these distorted markers (Launey & Hedegecock 2001). David *et al*. (1997) suggest that even small levels of inbreeding can be sufficient to maintain disequilibrium between markers and fitness genes that causes the observed markers-associated heterosis. Moreover, the ratio N_e/N can be drastically reduced by a high variance in reproductive success (Hedgecock 1994, Launey & Hedegecock 2001, Hedrick 2005b, Hedgecock *et al.* 2007) that could generate temporary gametic disequilibrium and markers-associated heterosis.

This study highlighted the existence of variance in reproductive success as well as a locally reduced effective size in experimental (controlled) conditions. The two combined phenomena are compatible with the possibility of temporary gametic disequilibria, which favour the local effects hypothesis of associative overdominance. Moreover, variance in reproductive success highlighted in this study could be accentuated by variations in environmental conditions in the wild. Such a variance in reproductive success has been assessed previously in *C. gigas* by PCR-SSCP (Li & Hedgecock 1998) and by microsatellites (Boudry *et al*. 2002). This variance can be explained by asynchrony of maturation, as already observed some years in Brittany (France) with three successive spawns separated by about 2-3 weeks (Martin *et al*. 1995).

Effective number of breeders (N_b) is a fundamental parameter for the management of genetic resources and conservation biology because it influences the magnitude of genetic drift in the closed population under scrutiny. It determines the rate of inbreeding ($\Delta F=1/2N_b$) and hence the rate of genetic variability loss in a population. In species with overlapping generations however, the effective number of breeders per year (N_b) can differ from the population's long term effective size N_e. This study demonstrated a limited effective number of breeders in the six temporal cohorts, generally below 25 (Table 4). However, N_b (based on the temporal method) was computed between two successive generations therefore equilibrium was not achieved. Moreover, it is important to notice that effective sex ratio in the experimental population is unknown. Some features of life history of oysters tend to limit the

effective population size: a biased sex ratio and the fact that fertilisation takes place into the mantle cavity which pleads in favour of fertilisation by nearest neighbours (Saavedra *et al*. 1987). Moreover, analysis of genetic variability of a cohort collected early in the reproductive season in Sète in 1993 (Hedgecock *et al*. 2007) demonstrated that spat collected was issued from a small number of progenitors (probably less than 20). This is in agreement with the fact that sexual maturation is not synchronous in this species (Le Dantec & Marteil 1976) and that reproductive success can be highly variable in time, at least at the beginning of the reproductive season when only a few individuals are mature.

The occurrence of such gametic disequilibria is temporary therefore it is not always observed. These patterns of temporal genetic differentiation at the local scale were described as "chaotic genetic patchiness" (Johnson & Black 1984; David *et al*. 1997) because they are transient and do not represent a permanent structure. The ability of detecting them depends on the sampling window and time. Indeed, in the Hedgecock *et al*. (2007) study reported above, few individuals contributed to the recruited cohort probably because of the scarcity of oysters already mature at that time of the year. However, another similar study (Taris *et al*. 2009) collected successive 15-days cohorts later in the season and showed neither genetic differentiation between adults and cohorts nor temporal structuring of the genetic diversity observed with nuclear markers. This suggests that several differentiated cohorts were integrated into a wide 15-days cohort, erasing genetic differentiation: the sampling window (15 days) was perhaps too wide. This previous result obtained in the wild is supported by the study of our experimental population where F_{st} was computed between progenitors and successive cohorts, as well as pooled cohorts. A high genetic differentiation was found between the population of potential progenitors and the first cohort (F_{st} 3%, $p < 0.001$): this could be explained by a few individuals contributing the cohort. As soon as successive cohorts were pooled, more and more progenitors contributed to these cohorts and hence genetic differentiation faded to cancel finally when all the cohorts were pooled (Table 3b). Therefore the pool of successive cohorts appears to represent a random and representative set of alleles of the progenitor population. However, such a result is driven in a very large part by the limited power of the dataset, as F_{st} is estimated on only 3 loci. From Table 2, we can see that the allelic richness is still quite a bit lower in the pooled cohorts than in the parents. The pooled cohorts certainly seems to constitute a more representative set than any of the individual cohorts, but it would probably still be found to be differentiated from the parents if more loci had been used. The detection of this phenomenon depends probably on the sampling window: for Hedgecock et al.'s (2007) cohort, this window was 15 days in early

spring. In this study, we showed the existence of a genetic differentiation at a smaller stepping time: between two successive cohorts spaced of 2-3 days, different individuals contributed to the cohorts explaining the genetic differentiation observed.

Acknowledgements

We thank the technical team of Ifremer La Tremblade for their technical assistance, in particular Serge Heurtebise and Florence Cornette. Special thanks to Ian Wilson for his help with the PARENTAGE software. We would like to thank two anonymous referees for helpful comments on this manuscript.

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688

689 Table 1. Allelic polymorphism and paternity analysis of 13 brooding females sampled in a
690 natural population (Brittany, France). Numbers of alleles (N_a) per locus and mean number of
691 alleles are shown for 80 offspring of each female. n_{loci}: number of loci used for paternity
692 analysis. Number of fathers (N_f) contributing to each offspring has been determined by two
693 software, PARENTAGE 1.0 (Bayesian method) and GERUD2.0 (parental reconstruction).
694 Equivalent prior refers to the prior stating an equal contribution of males to the progeny. na:
695 not available (number of alleles too high).
696

Female	N_a					n_{loci}	Mean N_f (Parentage)	Minimum N_f
	J12	U2	H15	T5	Mean		(Equivalent prior)	(Gerud)
F1	18	18	10	-	15.3	3	35.5 (4.3)	na
F2	19	15	9	-	14.3	3	27.3 (2.9)	na
F4	11	15	10	17	13.3	4	24.0 (2.9)	na
F5	7	9	5	-	7	3	7.7 (0.7)	4
F6	14	16	6	12	12	4	14.2 (1.1)	na
F7	5	4	4	-	4.3	3	2.1 (0.3)	3
F8	8	9	7	6	7.5	4	6.9 (0.6)	4
F9	11	13	11	14	12.3	4	8.4 (1.5)	na
F10	21	23	11	19	18.5	4	44.5 (3.5)	na
F21	4	5	4	4	4.3	4	1.7 (0.9)	2
F22	17	25	10	18	17.5	4	34.2 (1.9)	na
F23	20	23	10	17	17.5	4	40.0 (4.1)	na
F24	7	8	6	9	7.5	4	6.3 (0.7)	5

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698

699 Table 2. Genetic diversity, test for Hardy-Weinberg equilibrium for a population of 62
700 potential progenitors and 6 cohorts obtained in an experimental hatchery. Number of samples
701 analysed (*N*), allelic richness (*A*), expected (H_{nb}) and observed (H_o) heterozygosity and F_{is}
702 estimates according to Weir and Cockerham (1984). Total cohort corresponds to the pooling
703 of the 6 temporal cohorts. Significance of F_{is} tested on 1000 permutations; NS corresponds to
704 non significant values of p, * of $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

705

		Adults	14/03/2003	17/03/2003	20/03/2003	22/03/2003	28/03/2003	30/03/2003	Total cohort
N		62	80	80	80	80	80	80	480
Scoring	J12	98.9%	95%	91.25%	97.5%	88.75%	93.75%	90%	92.92%
success	U2	100%	96.25%	92.5%	93.75%	90%	87.5%	87.5%	91.46%
	T5	98.9%	96.25%	95%	92.5%	96.25%	85%	82.5%	91.25%
A	J12	23.00	17.68	11.52	17.21	14.85	14.82	14.84	18.70
	U2	27.00	17.67	15.62	20.20	18.56	20.33	19.82	21.07
	T5	23.00	18.14	14.36	14.32	14.30	16.55	15.87	18.86
H_o	J12	0.895	1	0.836	0.859	0.930	0.733	0.944	0.883
	U2	0.906	0.922	0.919	0.987	0.912	0.944	0.971	0.943
	T5	0.916	0.883	0.829	0.878	0.935	0.868	0.909	0.884
H_{nb}	J12	0.928	0.889	0.768	0.835	0.900	0.880	0.897	0.910
	U2	0.947	0.883	0.849	0.887	0.911	0.915	0.932	0.931
	T5	0.914	0.852	0.774	0.819	0.855	0.895	0.878	0.890
F_{is} total		0.026NS	-0.069NS	-0.081NS	-0.072NS	-0.043NS	0.054**	-0.044NS	0.008NS

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707

Table 3. (a) Genetic differentiation between and within the population of potential progenitors and 6 cohorts obtained in an experimental hatchery. (b) Genetic differentiation between the population of potential progenitors and the 6 cohorts progressively pooled. F_{st} values per population pair (Weir & Cockerham 1984) are expressed in percentage and their significance tested by 1000 permutations: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS non significant.

(a)

	14/03/2003	17/03/2003	20/03/2003	22/03/2003	28/03/2003	30/03/2003
Adults	3^{***}	5.5^{***}	3.1^{***}	1.5^{***}	1.3^{***}	1.2^{***}
14/03/2003	-	11.9^{***}	7.1^{***}	5.9^{***}	5^{***}	5.1^{***}
17/03/2003	-	-	9.8^{***}	6.8^{***}	7.9^{***}	7.8^{***}
20/03/2003	-	-	-	0.7^{**}	4.2^{***}	4.5^{***}
22/03/2003	-	-	-	-	3^{***}	3^{***}
28/03/2003	-	-	-	-	-	1.8^{***}

(b)

	14	14+17	14+17+20	14+17+20+22	14+17+20+22+28	14+17+20+22+28+30
Adult	3^{***}	1.1^{***}	0.6^{**}	0.6^{**}	0.3^{*}	0.2^{NS}

Table 4. Estimated effective number of breeders N_b for each cohort by temporal and heterozygote (*H*) excess methods (using NeEstimator 1.3 software) and linkage disequilibrium (LD) method (using LDNe program). Variance intervals are given in brackets. $LD_{0.05}$: with lowest allele frequency used (P_{crit} value) of 0.05; $LD_{0.01}$: with P_{crit} value of 0.01. N_g (Real) is the number of progenitors having contributed to each cohort, determined by parentage analysis with CERVUS 3.0 software (80% statistical confidence). Total cohort corresponds to the pooling of the 6 temporal cohorts.

	N_b (Temporal)	N_b (H excess)	N_b ($LD_{0.05}$)	N_b ($LD_{0.01}$)	N_g (Real)
14/03/2003	21.0 [12.4-36.2]	10.9	3.5 [2.2-8.0]	21.8 [17.5-27.4]	28
17/03/2003	12.5 [7.8-19.6]	8.0	9.6 [3.8-17.7]	13.2 [9.6-17.8]	19
20/03/2003	21.0 [12.3-36.5]	9.0	5.7 [2.8-11.6]	18.2 [14.2-23.2]	21
22/03/2003	22.3 [12.9-39.6]	9.9	9.5 [4.1-15.5]	20.7 [15.8-27.4]	27
28/03/2003	33.2 [17.8-70.1]	27.0	14.3 [8.4-23.5]	20.9 [15.8-27.9]	28
30/03/2003	29.6 [16.3-58.9]	5.4	12.5 [7.4-20.2]	23.2 [17.5-31.2]	25
Total cohort	95.9 [43.7-347.4]	15.0	19.2 [13.2-26.5]	33.6 [29.8-37.9]	48

727 Table 5. Number of parentage assignments for 6 temporal cohorts collected in hatchery, using
728 CERVUS 3.0 software. N_{total}: number of larvae included in the analysis (genotyped for at least
729 2 loci). The critical Delta scores and expected number of parentage assignments were
730 determined by simulation of parentage analysis (see text).
731

Cohort	N_{total}	Confidence level of assignment	Critical Delta	Observed assignments	Expected assignments
14/03/2003	79	95%	1.38	40 (51%)	43 (54%)
		80%	0.00	64 (81%)	63 (80%)
		Unassigned		15 (19%)	16 (20%)
17/03/2003	75	95%	2.18	49 (65%)	26 (34%)
		80%	0.69	53 (71%)	42 (57%)
		Unassigned		22 (29%)	33 (43%)
20/03/2003	80	95%	1.41	48 (60%)	36 (45%)
		80%	0.69	57 (71%)	52 (65%)
		Unassigned		23 (29%)	28 (35%)
22/03/2003	77	95%	2.25	44 (57%)	34 (44%)
		80%	0.09	68 (88%)	57 (73%)
		Unassigned		9 (12%)	20 (27%)
28/03/2003	75	95%	2.02	37 (49%)	31 (42%)
		80%	0.32	56 (75%)	52 (70%)
		Unassigned		19 (25%)	22 (30%)
30/03/2003	75	95%	2.67	37 (49%)	27 (36%)
		80%	0.69	51 (68%)	49 (65%)
		Unassigned		24 (32%)	26 (35%)
Total cohort	461	95%	2.97	252 (55%)	143 (31%)
		80%	0.69	325 (70%)	289 (63%)
		Unassigned		136 (30%)	172 (37%)

732
733

734 Figure 1. Variance of reproductive success between males, determined with a parental
735 reconstruction software, GERUD2.0 (Jones 2005), for brooding females showing few alleles
736 in their offspring. First male refers to the male with the highest contribution to the offspring,
737 second male is the male with the second highest contribution. For each female, first to fifth
738 males refer to different males.
739
740 Figure 2. Total contribution of progenitors (in terms of number of offspring) to each of six
741 cohorts collected in an experimental hatchery. Parentage analysis was performed using a
742 parental pair categorical allocation software, CERVUS 3.0 (Marshall *et al*. 1998, Kalinowski
743 *et al*. 2007), with an 80% statistical confidence.
744
745 Figure 3. Percentage of contribution of each potential progenitor to each temporal cohort,
746 visualising the succession of major contributors over time. Parentage analysis was performed
747 using a parental pair allocation software, CERVUS 3.0 (Marshall *et al*. 1998, Kalinowski *et*
748 *al*. 2007), with an 80% statistical confidence.

Figure 1

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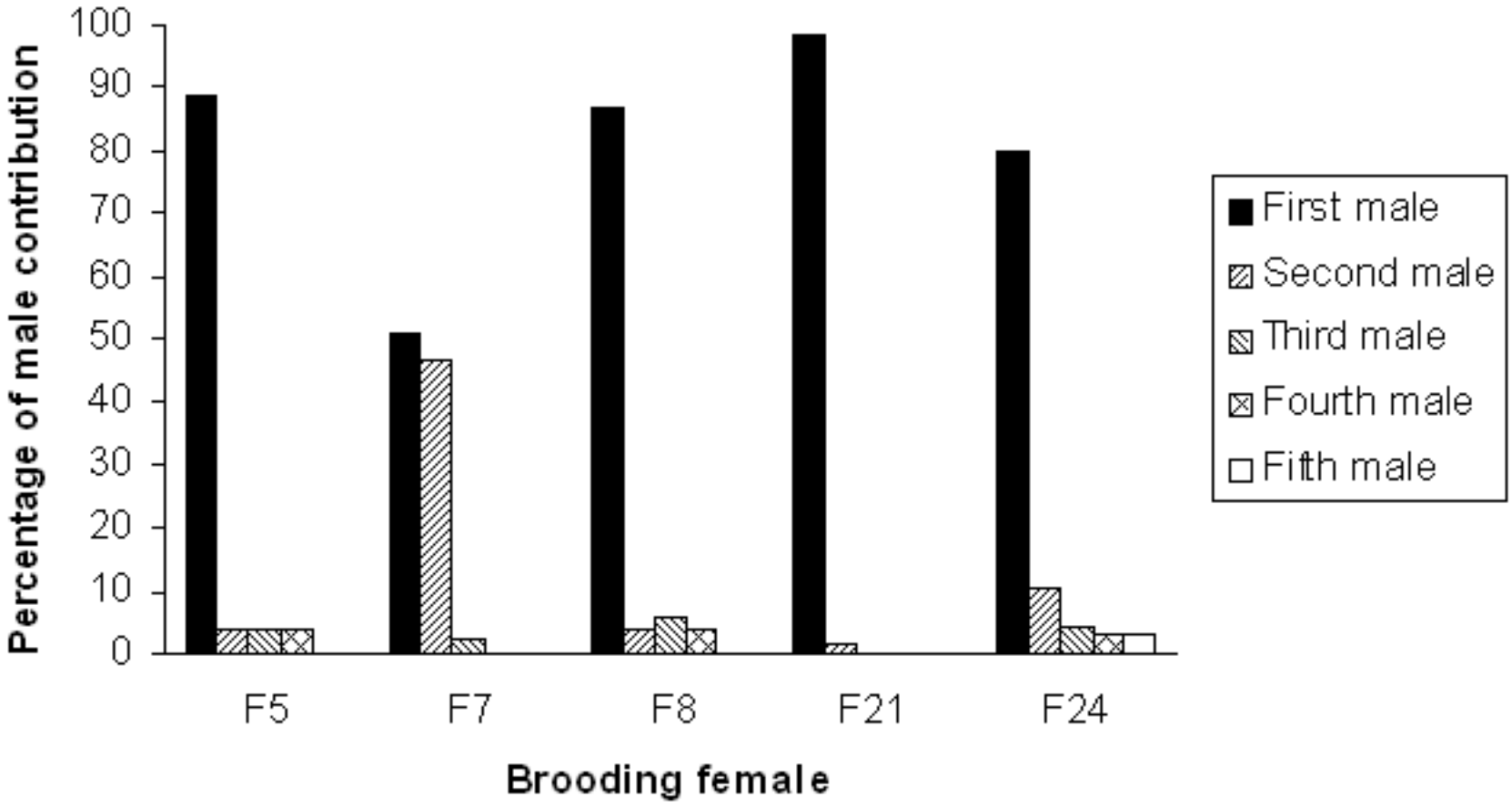

Figure 2

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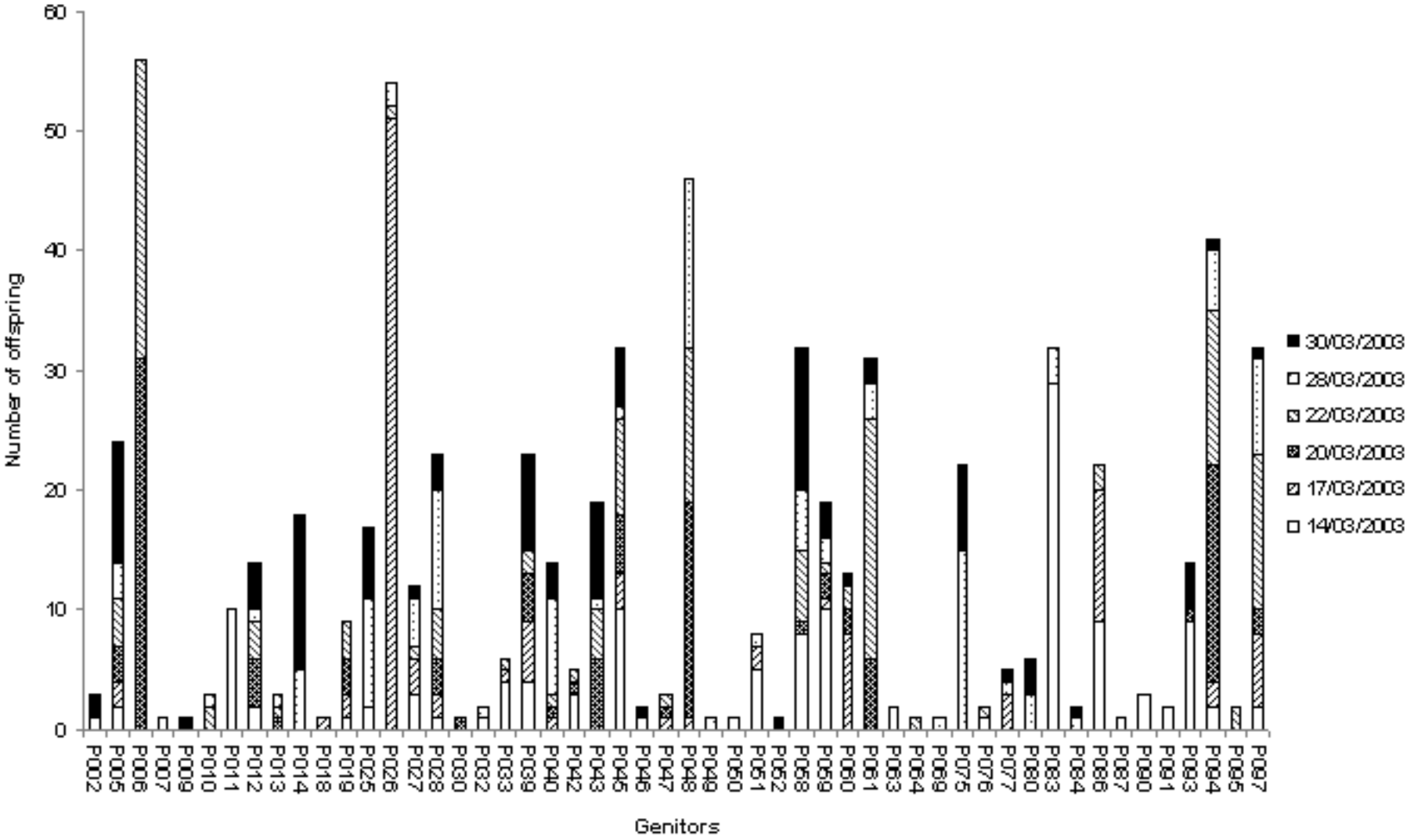

Figure 3

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