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Genotypic selection in *Daphnia* populations consisting of inbred sibships

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Abstract

The genetic basis of fitness reduction associated with inbreeding is still poorly understood. Here we use associations between allozyme genotypes and fitness to investigate the genetic basis of inbreeding depression in experimental outdoor populations of the water flea, *Daphnia magna*. In *Daphnia*, a phase of clonal reproduction follows hatching from sexually produced resting eggs, and changes in genotype frequencies during the clonal phase can be used to estimate fitness. Our experiment resembles natural colonization of ponds in that single clones colonize an empty pool, expand asexually and produce sexual offspring by selfing (sisters mate with their clonal brothers). These offspring diapause and form populations consisting of selfed sibships in the following spring. In 12 of 13 experimental populations, genotypes of selfed hatchlings after diapause conformed to Mendelian expectations. During the subsequent ca. 10 asexual generations, however, genotype frequencies changed significantly at 19 of 27 single loci studied within populations, mostly in favour of heterozygotes, with heterozygosity at multiple loci affecting the change in genotype frequency multiplicatively. Because variance in heterozygosity among siblings at a given marker reflects only heterozygosity in the chromosomal region around this marker, our results suggest that selection at fitness-associated loci in the chromosomal regions near the markers were responsible for these changes. The genotype frequency changes were more consistent with selection acting on linked loci than on the allozymes themselves. Taken together, the evidence for abundant selection in the chromosomal regions of the markers and the fact that changes in genotype frequencies became apparent only after several generations of clonal selection, point to a genetic load consisting of many alleles of small or intermediate effects, which is consistent with the strong genetic differentiation and repeated genetic bottlenecks in the metapopulation from which the animals for this study were obtained.

Introduction

Inbreeding depression is the reduction in fitness as a result of increased homozygosity of offspring from consanguineous matings (Charlesworth & Charlesworth, 1987). Its widespread occurrence in the wild (Keller & Waller, 2002) indicates that many natural populations are polymorphic at loci affecting fitness ('fitness loci'). Indeed, genetic variation for fitness and inbreeding depression are closely connected, and studying the genetic basis of inbreeding depression may allow conclusions about the genetic basis of fitness variation, that is about the number of fitness loci and their distribution

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across the genome, as well as about selection and dominance coefficients and possible epistatic interactions of alleles at these loci (Charlesworth & Charlesworth, 1999). These parameters are important for a range of evolutionary questions, such as the maintenance of sexual reproduction, the evolution of diploidy, and the evolution of breeding systems in plants (Charlesworth & Charlesworth, 1998).

One way to study the genetic basis of inbreeding depression is to investigate the association of genotypes at neutral markers with fitness in sibling offspring from crosses between closely related parents (Hedrick & Muona, 1990; Fu & Ritland, 1994, 1996; see also Carr & Dudash, 2003). This approach is related to genetic mapping of fitness loci (Stuber *et al.*, 1992) and is referred to as marker-assisted inference of inbreeding depression (Charlesworth & Charlesworth, 1999). Inbred siblings have the same expected inbreeding coefficient because they share the same pedigree, but they may vary in realized inbreeding levels because of the finite number of independently segregating chromosomal regions (Franklin, 1977; Strauss, 1986; Bierne *et al.*, 2000). Under close inbreeding, this variation may be substantial and can cause substantial fitness variation (Wang *et al.*, 1999). However, because of independent segregation of different chromosomal regions, the genotype at a particular marker will be correlated only with the genotypes at loci in its own local chromosomal region (the 'marker region') and carries no information about genotypes at unlinked loci. Hence, whereas variation in fitness among siblings may be due to selected loci anywhere in the genome, only the part that is due to fitness loci in the marker region should cause differences in the performance of marker genotypes. Differences in the performance of marker genotypes can thus be used to infer selection in the marker regions.

Several studies have used this approach to investigate the genetic basis of inbreeding depression (Strauss, 1986; Leary *et al.*, 1987; Hedrick & Muona, 1990; Fu & Ritland, 1994, 1996; Mitchell-Olds, 1995; Bierne *et al.*, 1998; Kärkkäinen *et al.*, 1999; Remington & O'Malley, 2000; Launey & Hedgecock, 2001). Most of these studies have focused on the degree of dominance and/or overdominance (e.g. Fu & Ritland, 1994; Mitchell-Olds, 1995; Kärkkäinen *et al.*, 1999). However, with this method it can be difficult to distinguish between linkage of markers with truly overdominant loci and apparent overdominance caused by linkage with two or more recessive deleterious alleles in repulsion on different homologous chromosomes (Frydenberg, 1963; Ohta, 1971). Nonetheless, the design is very useful for studying the genetic basis of fitness variation. The abundance and distribution of fitness loci throughout the genome can be estimated from the proportion of markers that show differences in performance among genotypes, because each marked region that shows such differences must contain at least one locus with an effect on fitness (e.g. Strauss, 1986; Bierne *et al.*, 1998). Furthermore, epistatic interactions among fitness loci can be investigated by assessing the linearity of the relationship between fitness and the proportion of markers with a given genotype (Fu & Ritland, 1996). This method also works if potentially non-neutral markers, such as allozymes are used (e.g. Fu & Ritland, 1994; Kärkkäinen *et al.*, 1999). In this case, fitness differences among genotypes may be due to the marker loci themselves or due to other loci in the marker region.

In the present study, we investigated the relationship between allozyme genotypes and fitness in a system in which the experimental conditions outlined above occur in nature, because natural populations often consist entirely of inbred sibships. Our study species, *Daphnia magna*, is a small, diploid freshwater crustacean that reproduces by cyclical parthenogenesis. It occurs in southern Finland in systems of small ponds, which form a large metapopulation with frequent extinction and recolonization (Pajunen, 1986; Pajunen & Pajunen, 2003). Recolonization often occurs by the arrival of just a single female (Haag *et al.*, 2005), which expands parthenogenetically to produce a large population of genetically identical individuals. To survive the subsequent winter, resting eggs must be produced sexually, which leads, under these circumstances, to mating between males and females of the same clone (genetically equivalent to selfing). Consequently, the individuals that hatch in the next spring represent a selfed sibship, and, at each locus that was heterozygous in the parent clone, hatchling genotypes are expected to conform to Mendelian ratios (assuming no selection during the resting egg stage).

The aim of this study was to experimentally investigate genotypic selection in the season following colonization by single individuals, and thereby to gain insights into the genetic basis of inbreeding depression in *D. magna*. We set up 13 experimental populations, each founded by a single individual, in large outdoor containers. The sexually produced resting eggs over-wintered, and, in the following season, we estimated deviations from expected Mendelian frequencies at different sampling times and changes in allozyme genotype frequencies between them. The founder individuals were obtained from the natural metapopulation. Hence we studied only effects due to loci segregating within natural populations. Because of parthenogenetic reproduction, the offspring genotypes were preserved during the entire season. Investigating changes in genotype frequencies across a large part of the season allowed even small potential fitness differences among genotypes to become visible through several generations of clonal selection (ca. 10 generations in our experiment). Furthermore, the accumulated deviations from Mendelian genotype frequencies at the end of the experiment provided a compound fitness estimate, including survival and hatching of resting eggs, asexual reproduction, and survival during

the parthenogenetic phase. Obtaining a compound fitness estimate may be especially important because the genetic basis of compound fitness is likely to differ from that of individual fitness components. Finally, because fitness differences may be accentuated by competitive conditions in natural populations, the experiment was conducted outdoors, under ambient conditions and without the addition of food.

Methods

Parental generation

Females sampled from natural populations in May–July 2001 were placed individually into jars in the laboratory, where they reproduced parthenogenetically, each female founding a clone. The clones were screened by cellulose acetate electrophoresis for heterozygosity at five allozyme loci, *Aat* (enzyme commission number 2.6.1.1), *Fum* (4.2.1.2), *Mpi* (5.3.1.8), *Pgi* (5.3.1.9), and *Pgm* (5.4.2.2, Hebert & Beaton, 1993). On 17 August 2001, 100 individuals of each of 13 clones, heterozygous at 1–4 marker loci, were released separately into outdoor containers, filled with 30 L of 20 μm-filtered natural pond water. The 13 clones originated from 11 different ponds. During the rest of the summer (for ca. 2.5 months), these genetically uniform (clonal) populations reproduced under ambient temperatures and photoperiod. No food was added during the entire experiment, so the populations fed entirely on the local community of phytoplanktonic algae and other microorganisms. Resting eggs were then produced sexually by matings within the clones (females can produce sons and daughters clonally), generating an expected inbreeding coefficient $F = 0.5$ with respect to the parents.

Offspring generation

During the winter all containers froze solid (volumes were reduced to ca. 7 L at the beginning of November to prevent bursting of containers), ensuring that only resting eggs survived to the next spring. These resting eggs initiated the offspring generation, each offspring being an independent, genetically unique realization of the selfing process. For each locus, at which the parent clone was heterozygous, the expected genotype frequencies in the offspring generation are the Mendelian frequencies, 1 : 2 : 1 (because each population was founded by just one clone, each polymorphic locus had exactly two alleles). Significant deviations from these expectations at any sampling date during the clonal phase of the offspring generation indicate the occurrence of selection or genetic drift prior to sampling.

In early May 2002, the containers were refilled to 30 L and all hatchlings were counted and removed to a replicate bucket (6 L), free of resting eggs. Hatching started on 5 May and slowed down around 20 May. The average number of hatchlings per population was 532.9 (Table 1). Once hatching ended, the individuals were transferred back to their original 30-L containers, and, for populations with > 300 hatchlings, a first random sample was genotyped (all sample sizes and sampling dates are given in Table 1). For populations with < 300 hatchlings, the first sampling took place only on 16 June, which allowed these populations to increase in number (by asexual reproduction) reducing the effect of sampling on genotype frequencies. To assess changes in genotype frequency during clonal selection, additional random samples from each population, taken in August and October were genotyped. Sexually produced resting eggs that appeared were unlikely to hatch during the experiment because they require a hatching stimulus (e.g. freezing or drying).

Data analysis

For each sampling date, the genotype frequencies at each locus and in each population were compared with expected Mendelian frequencies by χ^2-tests. To examine whether deviations from Mendelian expectations were predominantly in favour of certain genotypes or alleles, we first tested for heterozygote excess by analysing changes in mean heterozygosity between sampling dates (using polymorphic loci only) and by comparing observed heterozygote frequencies with the expected 50%. Population mean values and standard errors were calculated by averaging heterozygosity across individuals, whereas mean values and standard errors for single loci were calculated by averaging across populations.

Secondly, heterozygosity was expected to increase if the performance of individuals heterozygous for the marker region ('marker region heterozygotes') exceeded the average performance of marker region homozygotes. To analyse whether the performance of marker region

Table 1 Number of hatchlings, sample sizes and sampling dates for each experimental population.

Population	Hatchlings (*N*)	First sample (Date)	First sample (*N*)	23 Aug 2002 (*N*)	10 Oct 2002 (*N*)
1	243	16 Jun 2002	72	70	72
2	1102	24 May 2002	66	144	71
3	262	16 Jun 2002	72	48	72
4	685	30 May 2002	60	48	72
5	160	16 Jun 2002	72	144	72
6	722	24 May 2002	72	48	72
7	1325	24 May 2002	72	72	72
8	904	24 May 2002	72	72	72
9	375	24 May 2002	53	70	38
10	123	16 Jun 2002	72	72	72
11	64	16 Jun 2002	72	144	72
12	702	30 May 2002	56	72	72
13	261	16 Jun 2002	72	48	72
All	6928		883	1052	901

heterozygotes exceeded that of both homozygotes (and not just the average of the homozygotes), we used the graphical method by Fu & Ritland (1994), which classes genotype frequencies into different categories, consistent with different modes of selection (overdominant, partly recessive, partly dominant, underdominant) at fitness loci in the marker region. Genotype frequencies consistent with overdominance (either true overdominance or apparent overdominance caused by linkage to several loci with deleterious alleles in repulsion) are evidence for a superior performance of heterozygotes compared with both homozygotes in that region.

Thirdly, to test whether deviations from Mendelian expectations were due to superior performance of specific alleles, we tested for a change and deviation from expected 50% frequency in allele frequencies, averaged across populations. Significant deviations in favour of certain alleles would suggest selection on the allozyme itself, or significant linkage disequilibrium between allozymes and selected loci across the whole metapopulation.

Under the multiplicative fitness model, the relationship between the natural logarithm of fitness and the number of heterozygous loci is expected to be linear (Fu & Ritland, 1996). To test for epistatic interactions between different chromosome regions, we therefore estimated the fitness of pooled classes of multi-locus genotypes with different numbers of heterozygote regions. An estimate of relative fitness w was obtained by calculating the difference between the logit-transformed observed (p_{obs}) and expected (p_{exp}) frequencies of multi-locus genotypes with a given number of heterozygous markers: $\ln w = \ln [p_{obs}/(1 - p_{obs})] - \ln [p_{exp}/(1 - p_{exp})]$. Expected frequencies were calculated according to Mendelian expectations. This estimates how strongly the frequency of a given genotype diverges from its expected frequency relative to all other genotypes in the population (Hartl & Clark, 1997).

To assess the strength of clonal selection, we estimated a modified version of the relative Shannon–Wiener diversity J'. This diversity index varies from zero to 1, attaining one if all observed classes (multi-locus genotypes) are equally frequent. It is estimated by $J' = [-\Sigma(p_i \log p_i)]/\log k$, where p_i is the frequency of class i and k is the number of classes (Zar, 1999). Because in our experiment not all multi-locus genotypes were expected to be equally frequent under neutrality, we divided the observed numbers by their Mendelian probabilities before calculating the 'corrected' frequency of each multi-locus genotype. Thus, $J' = 1$ if all genotypes occurred exactly at their expected frequencies. The number of multi-locus genotypes k was determined for each actual sample separately as the mean number of multi-locus genotypes observed in 10 000 samples of the same size from a 'virtual population' in which genotype frequencies conformed to the neutral expectations.

Finally we assessed whether there were changes in linkage disequilibrium and allele frequencies during the season. Expected allele frequencies before selection were 50% with two alleles at each locus, and linkage disequilibrium between a pair of loci before selection would suggest physical linkage. These tests were carried out with the program FSTAT (Goudet, 2003).

Results

Genotype frequencies after hatching

In the first sample in May/June 2002, single-locus genotype frequencies closely fitted Mendelian expectations (Figs 1 and 2). Only two of the 27 single-locus estimates within populations differed significantly (population 8, *Mpi*, $\chi^2_2 = 6.6$, $P < 0.05$, and population 11, *Fum*, $\chi^2_2 = 24.3$, $P < 0.001$). Population 11 had the smallest number of hatchlings, only 64 (Table 1) and was also the only population in which heterozygosity deviated significantly from 50% (Fig. 3, Table 2). Average heterozygosity across populations in the first sample was 0.48 (SE = 0.017, Fig. 3, Table 2). There was weak linkage disequilibrium between *Fum* and *Pgi* in the two populations where both loci were polymorphic, suggesting that these two markers are located on the same chromosome. Based on the genotype frequencies in the first sample (May/June 2002), the estimated map distance (Hedrick, 2000) between *Fum* and *Pgi* in the two populations was 36 and 39 cM respectively (46 and 51 cM after Kosambi correction, $P < 0.05$ and $P = 0.08$, Table 3). All other pairs of markers appeared to be unlinked ($P > 0.17$) in the first sample.

Genotype frequencies in August and October

In the two subsequent samples in August and October, many of the genotype frequency estimates had diverged from the Mendelian expectations (Figs 1 and 2). In August, 16 of 27 single-locus estimates within populations differed significantly ($P < 0.05$) from Mendelian ratios (12 after sequential Bonferroni correction), and 19 of 27 estimates in October (10 after sequential Bonferroni correction). Heterozygosity increased during the season in all populations except one (Fig. 3, Table 2). Across populations, the increase in heterozygosity was significant from May to August, but not from August to October (Wilcoxon signed-rank test, $N = 13$, $P = 0.014$ and $P = 0.8$ respectively). In August, mean heterozygosity across populations was 0.55 (SE = 0.032), in October 0.57 (SE = 0.035); the October sample was significantly different from 50% (Wilcoxon signed-rank test, $N = 13$, $P = 0.021$, Table 2). Nineteen of 27 single-locus heterozygosity estimates within populations were > 0.5 in August and 24 of 27 estimates in October (Table 2). This represents a general test of heterozygote excess across the whole data because under neutrality, 50% of the estimates are expected to be larger than 0.5. The binomial probabilities associated with the observed values are 0.052 for August and 5×10^{-5} for October

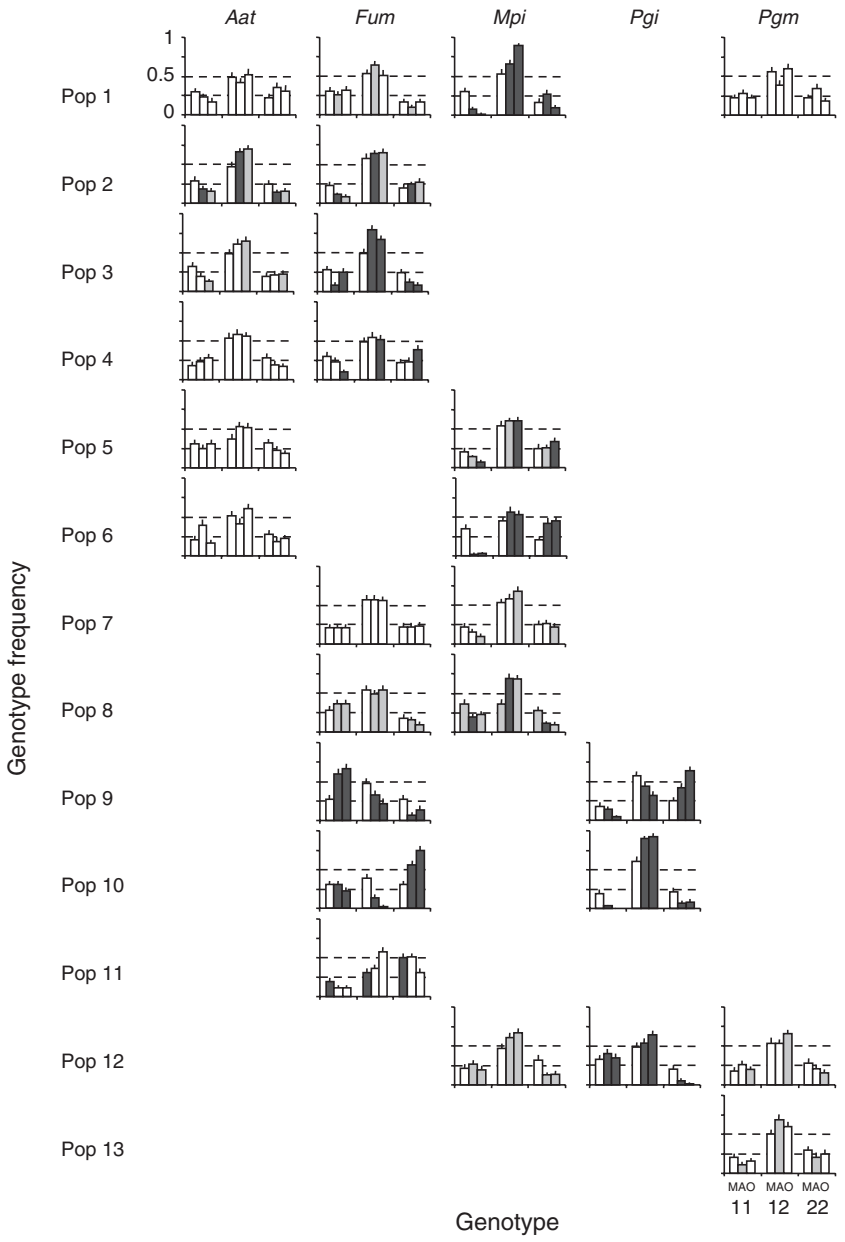

Fig. 1 Genotype frequencies at each locus and in each population. The left hand triplet within each small graph shows the frequencies of homozygotes for allele 1 (see Table 4 for allele identities), the middle one shows heterozygote frequencies, and the right hand triplet shows homozygote frequencies for homozygotes with allele 2. For each genotype, the first bar is its frequency in May (M), the second in August (A), and the third in October (O). Grey bars indicate samples in which genotype frequencies differed significantly ($P < 0.05$) from Mendelian expectations, black bars samples, in which these deviations were significant also after sequential Bonferroni correction.

(two-tailed tests). Each of the five loci showed significant deviations from Mendelian expectations and significant heterozygote excess in at least some populations (Fig. 1, Table 2), suggesting that significant changes in genotype frequencies were not confined to specific regions.

Mode of selection at fitness loci in the marker regions

Of the 19 single loci that showed significant deviations from Mendelian genotype frequencies, the graphical method of Fu & Ritland (1994) identified 10 that showed heterozygote excess consistent with overdominance (or apparent overdominance) in the marker region, six showed heterozygote excess consistent with (partial) recessivity of a deleterious allele in the marker region, and three showed heterozygote deficits, consistent with (partial) dominance of a deleterious allele or underdominance in the marker region (Fig. 2). In August, the respective numbers of loci suggesting overdominance, partial recessivity and heterozygote deficit were nine, two and five of 16 significant deviations from Mendelian expectations (Fig. 2). Direct inference of dominance at fitness loci from these data relies on the strong assumption that only one locus causes deviations from Mendelian ratios in any given marker region. Furthermore, the exact classification of regions consistent with different

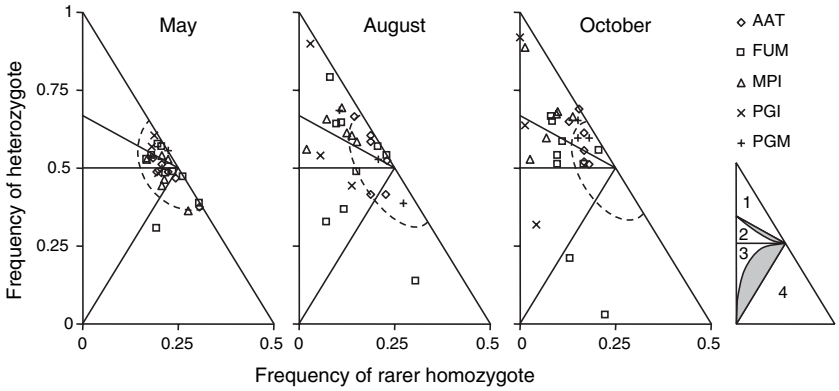

Fig. 2 Single-locus genotype frequencies within populations plotted according to Fu & Ritland's (1994) method. The small triangle to the right indicates the inferences for points in the different regions: overdominance (1), partial recessivity of a deleterious allele (2), partial dominance of a deleterious allele (3), and underdominance (4), assuming a single selected locus linked to the marker. The grey areas are areas of overlap between (1)/(2) and (3)/(4), respectively, where the form of selection depends on the degree of linkage between the selected locus and the marker locus. The regions in the large triangles are for complete linkage, and the three dividing lines indicate full recessivity (between 1 and 2), additivity (between 2 and 3), and full dominance (between 3 and 4). For more details, see Fu & Ritland (1994).

modes of selection depends on linkage between the marker and the fitness loci; the above figures are for complete linkage (for more details see Fig. 2 and Fu & Ritland, 1994). However, the data indicate that in the vast majority of the significant cases, heterozygosity increased in a manner consistent with either partial recessivity of a deleterious allele in the marker region or (in more than half of the significant cases) with true or apparent overdominance.

At the end of our experiment, 10 of 27 single loci showed significantly ($P < 0.05$) unequal allele frequencies within populations (Table 4). However, the alleles that became more frequent differed among populations (Table 4), and no allele was significantly more common than expected across populations (t-tests, all $P > 0.2$).

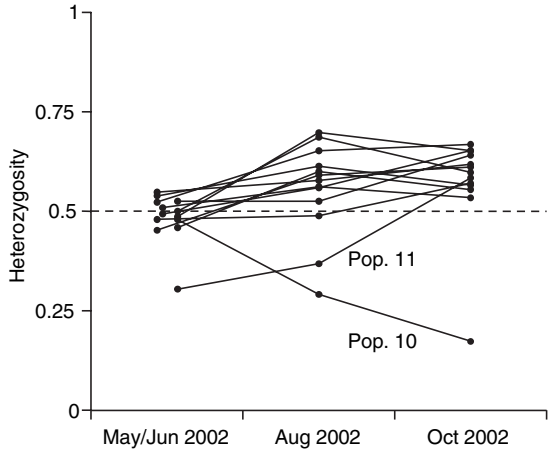

Fig. 3 Changes in heterozygosity in each of the 13 experimental populations during the course of the season. Populations 10 and 11 are labelled separately. There are three different sampling dates in May/June 2002 (see Table 1).

Epistasis

The natural logarithm of our fitness estimate increased in most populations linearly with the proportion of heterozygous markers available (Fig. 4). Across populations, a quadratic regression did not reduce the residual variance compared with a linear regression (both $r^2 = 0.135$), and the average of the quadratic coefficients within each population was not significantly different from zero (mean = −0.16, SE = 0.31, $t_{10} = -0.52$, n.s.). If homozygotes rather than heterozygotes increased in frequency the assessment of epistasis between heterozygous regions is problematic. This was the case in populations 9 and 10. However, excluding them from the analysis did not change the results.

Clonal diversity

Clonal diversity was high in May, consistent with the good agreement with Mendelian genotype frequencies (Fig. 5). However, it was positively correlated with the number of hatchlings ($r = 0.62$, $N = 13$, $P < 0.05$), suggesting that in populations with few hatchlings some selection or genetic drift may have already occurred prior to the first sampling date. During the season, clonal diversity decreased (mean ± SE, May: 0.95 ± 0.01, October: 0.80 ± 0.05, Wilcoxon signed-rank test, $N = 13$, $P < 0.0001$, Fig. 5), but in most populations it remained moderately high, suggesting that clonal selection did not lead to an extreme reduction in the number of clones present. The exceptions were two populations, in which clonal diversity decreased strongly (Fig. 5). It is noteworthy that these were the only two populations that had a significant homozygote excess at one or both loci in the end of the season. This is consistent with the idea that the populations became dominated by a few clones and

Table 2 Heterozygosity for each population and studied locus at each of the sampling dates.

	May/June 2002						August 2002						October 2002					
Population	*Aat*	*Fum*	*Mpi*	*Pgi*	*Pgm*	All loci	*Aat*	*Fum*	*Mpi*	*Pgi*	*Pgm*	All loci	*Aat*	*Fum*	*Mpi*	*Pgi*	*Pgm*	All loci
1	0.49	0.53	0.53		0.56	0.52	0.41	0.64*	0.66*		0.39	0.53	0.52	0.51	**0.89****		0.6	**0.64****
2	0.47	0.58				0.52	**0.67****	**0.64*****				**0.65****	**0.69****	0.65*				**0.67****
3	0.49	0.49				0.49	0.6	**0.79****				**0.7*****	0.65*	0.67**				**0.65*****
4	0.53	0.48				0.51	0.58	0.54				0.56	0.56	0.51				0.53
5	0.38*		0.54			0.46	0.52		0.6*			0.60*	0.51		0.6			0.56
6	0.51		0.44			0.48	0.42		0.56			0.49	0.61		0.53			0.57
7		0.57	0.53			0.55		0.57	0.58			0.58		0.56	0.68**			**0.62****
8		0.54	0.36*			0.45		0.49	**0.69****			0.59*		0.54	0.68**			0.61**
9		0.47		0.6		0.54		**0.33****		**0.9*****		0.61**		**0.21*****		**0.92*****		0.57
10		0.39		0.57		0.48		**0.14*****		0.44		**0.29*****		**0.03*****		0.32**		**0.17*****
11		**0.31****				**0.31****		**0.37****				**0.37****		0.58				0.58
12			0.46	0.48	0.54	0.49			0.61	0.54	0.53	0.56			0.67**	0.64*	0.65*	**0.65*****
13					0.5	0.5					0.69*	0.69*					0.6	0.6
All	0.48	0.48	0.48	0.55	0.53	0.48	0.53	0.50	**0.62****	0.63	0.53	0.55	0.59*	0.47	0.67*	0.63	0.62*	**0.57***

Bold face values were significant after sequential Bonferroni correction (single loci within populations: 27 tests, population averages: 13 tests, locus averages: 5 tests, overall average: 1 test). Underlined values indicate that heterozygotes had a higher estimated fitness than the fittest of the two homozygotes (i.e. region 1 in Fig. 2). Single-locus estimates within populations were evaluated using two-tailed binomial tests. 'All' indicates averaging across populations, evaluated by one-sample *t*-tests (single-locus estimates) or one-sample Wilcoxon signed-rank tests (overall average).
Significance levels are abbreviated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

that random hitchhiking of marker genotypes with dominant clones may have occurred in these two populations. Interestingly, for both populations we had used *Fum* and *Pgi* as marker loci, the two markers, which showed some degree of linkage.

Discussion

Evidence for genotypic selection among selfed siblings

We found marked changes in allozyme genotype frequencies during the phase of clonal reproduction in *D. magna* populations consisting of selfed sibships. We believe that these changes reflect selection at genes in the regions of our markers, rather than neutral processes. First, the average number of hatchlings was > 500 and genotype frequencies in the first sample were in good agreement with Mendelian expectations. This suggests that initial clonal diversities were high enough to limit strong sampling effects. Secondly, clonal diversities declined only moderately during the course of the experiment, suggesting limited scope for hitchhiking through random associations of marker genotypes with particularly fit clones (see below). Thirdly, changes in genotype frequencies were nonrandom with respect to genotype: heterozygotes became more frequent in almost all populations. Changes in genotype frequencies because of genetic drift are expected to increase heterozygosity only in 50% of all cases. Taken together, our observations thus strongly suggest that selection caused genotype frequencies changes in these populations. Furthermore, because the populations consisted of selfed sibships, only selection at genes in the marker regions (potentially including the markers themselves) that were heterozygous in the founding individuals could contribute to the observed changes in genotype frequencies. Hence these loci are segregating in the natural metapopulation from which our founding individuals were collected. As this metapopulation is characterized by frequent single-genotype bottlenecks following natural colonization of empty ponds (Haag *et al.*, 2005), genotypic selection among selfed siblings is expected to occur also in the natural metapopulation.

Predominant increase in heterozygosity

The increase in heterozygosity could partly be explained by linkage to a locus segregating for a (partly) recessive deleterious allele, but in several cases we found evidence that this increase was because of true or apparent overdominance (Fig. 2). True overdominance is caused by at least one locus with heterozygote advantage in the marker region, whereas apparent overdominance is caused by two or more loci in the marker region with partially recessive deleterious alleles in repulsion. We cannot distinguish between these two mechanisms, although there is little evidence that true overdominance is a common phenomenon (Charlesworth & Charlesworth, 1999). However, regardless of the exact mechanism, the increase in heterozygosity in many of the marker regions suggests that selfing in these *D. magna* populations leads to strong inbreeding depression (Haag

Table 3 Linkage disequilibrium between loci *Fum* and *Pgi* in populations 9 and 10 in the sample in May/June 2002, shortly after hatching.

		N individuals	
Genotype *Fum*	Genotype *Pgi*	Population 9	Population 10
MM	MM	5	1
SM	MM	3	3
SS	MM	2	9
MM	MF	9	15
SM	MF	14	15
SS	MF	9	11
MM	FF	0	6
SM	FF	8	10
SS	FF	3	2
		Population 9	Population 10
Inferred parental genotype*		M-M/S-F	S-M/M-F
P-value for association (two-tailed)†		0.08	0.012
Adjusted recombination frequency‡		0.39	0.36
Kosambi map distance (cM)		51	46

**Fum-Pgi*; the alleles connected by a horizontal line are inferred to have been present on the same homologous chromosome in the parent because they co-occurred at increased frequency in offspring homozygotes.
†Calculated using FSTAT (Goudet, 2003).
‡Calculated using method described in Hedrick (2000).

Table 4 Allele frequencies within populations in October.

Population	Allele 1	Allele 2	Frequency of allele 1
1	*Aat-a*	*Aat-c*	0.43
2	*Aat-a*	*Aat-b*	0.5
3	*Aat-b*	*Aat-c*	0.45
4	*Aat-a*	*Aat-c*	0.56
5	*Aat-b*	*Aat-c*	0.56
6	*Aat-b*	*Aat-c*	0.47
1	*Fum-a*	*Fum-c*	0.58
2	*Fum-a*	*Fum-b*	0.41*
3	*Fum-a*	*Fum-b*	0.58
4	*Fum-a*	*Fum-b*	0.35***
7	*Fum-a*	*Fum-b*	0.49
8	*Fum-a*	*Fum-b*	0.63**
9	*Fum-a*	*Fum-b*	0.76****
10	*Fum-a*	*Fum-b*	0.24****
11	*Fum-a*	*Fum-b*	0.40*
1	*Mpi-a*	*Mpi-b*	0.46
5	*Mpi-a*	*Mpi-b*	0.37**
6	*Mpi-a*	*Mpi-b*	0.29****
7	*Mpi-a*	*Mpi-b*	0.44
8	*Mpi-a*	*Mpi-b*	0.56
12	*Mpi-a*	*Mpi-b*	0.53
9	*Pgi-a*	*Pgi-b*	0.46
10	*Pgi-a*	*Pgi-b*	0.20****
12	*Pgi-a*	*Pgi-b*	0.67****
1	*Pgm-a*	*Pgm-b*	0.52
12	*Pgm-a*	*Pgm-b*	0.52
13	*Pgm-a*	*Pgm-b*	0.45

Two-sided significance levels are given for a binomial test of equal frequency (*$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

et al., 2002), as the effect of all regions must be stronger than for just our marked regions. Furthermore, our observations suggest that many loci contribute to inbreeding depression (see below for a tentative estimation of the number of loci).

Selection at allozyme loci vs. selection at linked loci

We cannot entirely exclude the possibility that genotypic selection was because of selection at the allozymes themselves. Indeed, allozyme variation has sometimes been suggested to be overdominant (reviewed in David, 1998). However, in our experiment, single-locus heterozygosity increased in some populations, but not in others (Fig. 1), and the frequency of specific alleles increased in some populations but decreased in others (Table 4). If selection acting on allozyme markers would explain our results, we would therefore need to invoke qualitatively different selection pressures on given allozyme loci in different populations, which appears not very plausible. Furthermore, genotype–environment interactions, which could, in theory, explain such different selection pressures, are unlikely in our experiment because all populations had the same starting conditions and were placed close to each other at the field site. Hence, our results suggest selection at linked loci, not on the allozyme markers.

Number of fitness-associated loci

The mean size M of a marker region after one generation of selfing can be estimated by $M = L[(1/Y) - (e^{-Y}/Y)]$, where L is the total length of the average chromosome (in cM) and Y is the mean number of crossovers per bivalent (Strauss, 1986). The haploid chromosome number in *D. magna* is 10. So the total genome size is 10 L, and F_{SC}, the proportion of the genome marked by a single marker, is $F_{SC} = [(1/Y) - (e^{-Y}/Y)]/10$ (Bierne *et al.*, 1998). Assuming that the average number of crossovers per bivalent Y is between one and three ($Y = 2$ in *D. pulex*, a related species, Cristescu *et al.*, 2006), then F_{SC} is between 3.2% and 6.3% of the total genome, and hence we estimate that the genome of *D. magna* contains between 16 and 32 independent regions after one generation of selfing.

By the end of our experiment, 70% (19/27) of the single-locus genotype frequencies within populations showed significant deviations from Mendelian expectations. Hence, we estimate that, in an average maternal individual used in our experiment, 70% of all independent regions, that is 11–22 regions, contain at least one heterozygous fitness locus. As an estimated 30% of

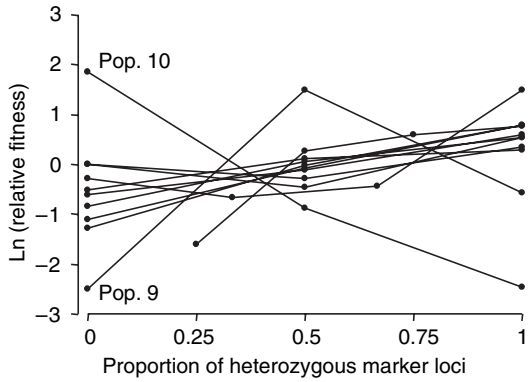

Fig. 4 Average of the natural logarithm of relative fitness of multilocus genotypes with different fractions of heterozygotes among the polymorphic markers, based on the sample of October 2002. Only populations with > 1 polymorphic loci were considered. The two populations with evidence for homozygote excess (at least at one locus) are labelled separately. For one population, the relative fitness of genotypes with zero heterozygous loci could not be estimated because no such genotypes were observed.

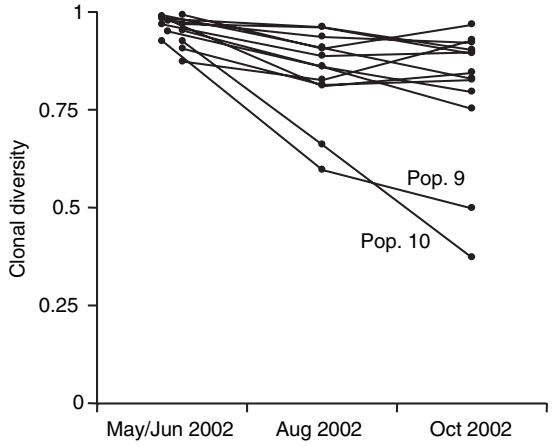

Fig. 5 Changes in clonal diversity in each of the 13 experimental populations during the course of the season. The two populations with strongly reduced clonal diversity are labelled separately. Clonal diversity was estimated by a modified version of the relative Shannon–Wiener index (see Methods).

regions contained no detectable fitness locus, assuming that the number of fitness loci per region follows a Poisson distribution leads to an estimated average number of fitness loci per marked interval of $\mu = 1.2$. Then a tentative estimate of the total number of heterozygous fitness loci detectable in our experiment in an average individual is 19–38. The same Poisson assumption also suggests that an estimated 34% of regions have > 1 fitness locus, which may have contributed to our observation of apparent or true overdominance. Our estimate of the number of fitness loci should be treated with caution because it is obtained from only five distinct marker loci whose distribution in the genome is unknown so that the local recombination rates are also unknown. It nevertheless suggests that, even in highly subdivided populations subject to repeated genetic bottlenecks, individuals may be heterozygous at many loci affecting compound fitness.

Genetic load in the study population

At first sight our estimate of the number of fitness-associated loci is similar to estimates obtained by other studies using the same method (e.g. Strauss, 1986; Bierne *et al.*, 1998; Remington & O'Malley, 2000; Launey & Hedgecock, 2001) or different methods (e.g. Charlesworth & Charlesworth, 1998; Latter, 1998). These studies all suggest a high number (10 to several tens) of loci contributing to inbreeding depression. Furthermore, Deng & Lynch (1997) estimated the genome-wide deleterious mutation rate U as well as the mean selection coefficient s and the mean dominance coefficient h in two *Daphnia* species. They found for clutch size $U = 0.99$, $h = 0.36$, $s = 0.21$ and $U = 0.69$, $h = 0.23$, $s = 0.07$ in *D. pulicaria* and *D. arenata* respectively. Assuming mutation-selection balance and no overdominant loci, the average number of deleterious mutations n per individual can be estimated from these data as $n = U/hs$ (Haldane, 1927), thus $n = 13$ and 43 for the two species. If overdominant loci are present, this is likely to be an overestimate.

Most previous estimates of the number of fitness-associated loci are for specific fitness components, such as clutch size (Deng & Lynch, 1997) or early survival (e.g. Remington & O'Malley, 2000; Launey & Hedgecock, 2001). In contrast, in our experiment, differential survival of resting eggs, hatching of resting eggs, asexual reproduction, and survival during the parthenogenetic phase may have contributed to deviations from Mendelian expectations. The accumulation of genotype frequency changes during ca. 10 generations of clonal selection increased our power to detect such changes (e.g. Pujol *et al.*, 2005), allowing us to detect even small changes. Furthermore, we did not observe clear deviations from Mendelian expectations early in our experiment. This contrasts with observations by Innes (1989), who found that genotype frequencies of selfed *D. obtusa* already significantly deviated from Mendelian expectations shortly after hatching. Similarly, in constructing a genetic map of *D. pulex*, several chromosome regions had significant deviations from Mendelian segregation (Cristescu *et al.*, 2006), presumably caused by differences in early survival among genotypes. One important difference between these studies and ours is that they mostly used individuals from large, outbreeding populations, whereas we used individuals from a metapopulation that is characterized by strong genetic differentiation and strong genetic bottlenecks during colonization (Haag *et al.*, 2005, 2006). Our results thus suggest that in this metapopulation the genetic load for traits related to diapause is low and that the total genetic

load (Ebert *et al.*, 2002; Haag *et al.*, 2002) consists of a large number of loci with alleles of mostly weak or intermediate effects rather than lethal alleles. This is consistent with the idea that lethals may be purged owing to strong genetic population structure and repeated founder events (Nei, 1968; Glémin, 2003). Moreover, because of strong population structure, a relatively large part of the genetic load may be fixed locally rather than segregate within local populations (Haag *et al.*, 2005, 2006). While this part of the genetic load contributes to strong fitness differences between inbred and outcrossed (between-population hybrid) genotypes (Ebert *et al.*, 2002), only loci segregating within populations may have contributed to the effects observed here. Together, the consequences of metapopulation structure may explain why the fitness effects in our study became apparent only after several generations of clonal selection even though we used a more general measure of fitness than earlier studies.

No evidence for epistasis

The logarithm of relative fitness increased roughly linearly with the number of heterozygous markers, supporting a multiplicative fitness model. Although this does not exclude epistatic interactions among particular fitness loci, it seems to contrast with earlier evidence for a nonlinear decrease of the natural logarithm of fitness with the degree of inbreeding in *D. magna* from the same metapopulation (Salathé & Ebert, 2003). The reason for this difference is not clear, but one major difference from the present study is that Salathé & Ebert (2003) used crosses between populations. Overall, there is little empirical support for epistasis averaged across loci (e.g. Fu & Ritland, 1996; Elena & Lenski, 1997; Peters & Keightley, 2000). Our study is not conclusive in this respect, but the possible difference between interactions among loci within populations vs. in crosses between populations may warrant further attention.

Clonal selection

Clonal selection in the offspring generation was important in our experiment because it increased the power to detect genotypic selection. On the other hand, clonal selection can cause random associations of marker genotypes with particularly fit clones (e.g. Lynch, 1984). If populations become dominated by a few superior clones, changes in genotype frequencies could therefore reflect hitch-hiking of certain genotypes in superior clones rather than selection. However, the number of hatchling clones in our experiment was high and in most populations, clonal diversity decreased only moderately during the study. Furthermore, chance associations with superior clones are expected to occur equally often for homozygous as for heterozygous marker genotypes. Therefore, our results are clearly inconsistent with the hypothesis that evolution was dominated by random association between marker genotype and high fitness clones.

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