An uncoupling of male and sexual egg production leads to reduced inbreeding in the cyclical parthenogen *Daphnia*

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Cyclical parthenogenesis involves an alternation of parthenogenetic and sexual reproduction. In cyclical parthenogens with a short generation time, seasonal succession of clones switching to sexual reproduction at different periods of the growing season entails the risk of severe inbreeding with associated fitness costs. We show, however, that differences in genotype frequencies between males and sexual females result in a substantial reduction in the probability of selfing in natural *Daphnia* populations. This suggests that responses to male- and sexual egg-inducing stimuli may be uncoupled at the level of individual clones as a mechanism to avoid severe inbreeding.

Keywords: seasonality; inbreeding; mating patterns; cyclical parthenogenesis; clones; *Daphnia*

1. INTRODUCTION

Daphnia (Crustacea: Anomopoda) are medium-sized, freshwater, planktonic animals which are the major herbivores in many lakes and ponds. Most *Daphnia* species are cyclical parthenogens which reproduce by amictic parthenogenesis as long as the environmental conditions are favourable, resulting in the formation of all-female clones (Hebert 1987). When conditions deteriorate, the animals switch to sexual reproduction and produce males and haploid eggs which need to be fertilized (Hebert 1987). Fertilized sexual (ephippial) eggs are resting stages which can survive harsh conditions and hatch when conditions become favourable again. As the generation time of *Daphnia* is short, an average growing season of a population consists of multiple overlapping parthenogenetic generations. Clonal selection may result in pronounced shifts in genotype frequencies during the course of the growing season (Hebert 1974; Carvalho & Crisp 1987; De Meester 1996). Such temporal changes in clonal frequencies may lead to assortative mating if different clonal groups switch to sexual reproduction at different times in the growing season (figure 1). This assortative mating arises by random mating among clones differing in frequency, rather than from active choice of sexual individuals. Several studies have indeed reported pronounced differences in genotype frequencies between parthenogenetic and sexual females in natural *Daphnia* populations (Hebert 1974; Young 1979; Lynch 1983; Korpelainen 1986; Carvalho 1988).

Inbreeding depression, either resulting from the expression of (partially) recessive deleterious alleles or from the superiority of heterozygotes over homozygotes at specific loci, is an important selective force in many organisms

(Charlesworth & Charlesworth 1987). Several mechanisms have been reported to reduce selfing or mating with close kin in natural populations (Blouin & Blouin 1988; Jarne & Charlesworth 1993; Pusey & Wolf 1996). In *Daphnia*, males are produced parthenogenetically and are genetically identical to their mothers (Hebert 1987). Mating of a male with a female of the same clone is therefore genetically equivalent to selfing. The mean mortality rate among selfed offspring in *Daphnia* is twice as high as among outbred offspring under laboratory conditions (Innes 1989; De Meester 1993; Innes & Dunbrack 1993; Lynch & Deng 1994). In addition, Deng (1997) and Deng & Lynch (1997) documented that inbreeding results in a reduced developmental stability and an overall inferior fitness. This severe inbreeding depression indicates that clonal selfing does not occur frequently in natural populations (no purging of recessive deleterious mutations has occurred; Jarne & Charlesworth 1993).

The production of males and sexual eggs in *Daphnia* is triggered by similar environmental stimuli such as changes in photoperiod, food quantity and quality and infochemicals associated with crowding or predators (e.g. Stross & Hill 1965; Kleiven *et al.* 1992; Deng 1996). However, several laboratory studies have reported induction of males without subsequent production of sexual eggs (e.g. Ferrari & Hebert 1982; Hobaek & Larsson 1990). This may result in an important fitness cost, as the production of males is wasted energy when there are no sexual eggs to fertilize (Hebert 1987). We hypothesize that *Daphnia* clones have evolved a partial uncoupling of the production of males and sexual eggs as a strategy to reduce the even more important cost associated with inbreeding depression. The hypothesis that the uncoupling of male and sexual egg production has evolved as a mechanism to reduce the rate of selfing is difficult to test directly. However, we assess here whether such an uncoupling is observed in natural *Daphnia*

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Figure 1. A hypothetical scheme of temporal succession of different clonal groups (A–C) in a cyclical parthenogenetic *Daphnia* population. There is a seasonal succession of clonal groups associated with the differences in relative fitness ((a) clones A–C), with different clonal groups producing sexual eggs at different periods of the year (b). If the production of males in a given clone were tightly linked to the production of sexual eggs, this would lead to strong assortative mating. However, if males are produced in response to less genotype-specific stimuli than sexual eggs, assortative mating and the frequency of selfing are expected to be reduced (c). Whereas the patterns depicted for (a) parthenogenetic and (b) ephippial females have been reported in the literature, only scattered data are available on the genotype frequencies of males (c) in natural *Daphnia* populations.

populations under field conditions and whether it results in a lower probability of severe inbreeding. We studied the multilocus genotype (MLG) frequencies of males and parthenogenetic and sexual females in natural populations of *Daphnia magna* Straus and estimated the probability of ‘selfing’ for the observed genotype frequencies as well as for the hypothetical case of a perfect coupling of the production of males and sexual eggs. In addition to our own data, we included one unpublished (L. Y. Yampolsky, personal communication) and three published data sets (Hebert 1974; Lynch 1984; Ruvinsky *et al.* 1986) on the MLG frequencies of males and sexual and parthenogenetic females in natural *Daphnia* populations to verify whether they are in agreement with our hypothesis.

2. METHODS

(a) Sampling and electrophoretic analysis

We sampled eight *D. magna* populations. The *D. magna* population of Citadelpark, Small Pond (CSP, 0.03 ha) was repeatedly sampled during the period of sexual reproduction in autumn and winter 1992 (table 1). The populations of Oude Meren Pond 1 (OM1, 1.2 ha) and Driehoeksvijver (DV, 0.7 ha) were sampled at regular intervals during 1996 and 1997. In DV, the genotype frequencies of parthenogenetic and ephippial females and males were analysed for three dates in 1996 on which sexual females and males were abundant, whereas in OM1 one date in 1996 and three dates in 1997 were studied. The remaining five *D. magna* populations (Citadelpark, Big Pond (CBP, 0.15 ha), Fish Pond, Blankaart (FPB, 0.55 ha), Oude Meren Ponds 2 and 3 (OM2 and OM3, both *ca.* 1 ha) and Lebrader Teich, LT (*ca.* 75 ha)) were sampled once during a period of sexual reproduction (table 1). Sampling was done with a dip-net (mesh size

250 µm) taking care to sample the whole water column. The animals were transported alive to the laboratory and sorted within 1 h of collection. We randomly picked out *ca.* 50 adult parthenogenetic and sexual females and adult males from each sample. Although we took large samples, it was not always possible to collect 50 animals of each reproductive class (the sample size was >40 in 44 cases out of 60 and <30 in only six). Using cellulose acetate allozyme electrophoresis (Hebert & Beaton 1989), we screened for variation at four polymorphic enzyme loci: GPI (E.C.5.3.1.9), AAT (E.C.2.6.1.1), MDH (E.C.1.1.1.37) and MPI (E.C.5.3.1.8). MLGs were constructed from the genotype at each of these enzyme loci (all loci were polymorphic in all populations). We calculated Euclidean distances between the parthenogenetic female, ephippial female and male subpopulations for each date and population using the MLG frequencies as input data.

(b) Calculation of the probability of ‘multilocus genotype selfing’

As a measure of inbreeding, we calculated the probability of selfing at the MLG level for all populations and sampling dates. The probability of selfing (%) was determined as

$$\left(\sum_{i=1}^n m_i e_i \right) \times 100, \quad (1)$$

where m_i is the observed relative frequency (fraction of 1) of MLG $_i$ in the male subpopulation, e_i is the relative frequency of MLG $_i$ in the ephippial female subpopulation and n is the total number of MLGs in the sample. This formula only estimates the probability of selfing at the MLG level correctly under the assumption that there is no active mate choice, such that the mating probabilities are determined solely by the relative frequencies of mating

Table 1. *Multilocus selfing rates (%) calculated for each sample from all populations sampled in the present study using observed genotype frequencies of males ($M \times E$) or assuming that male and sexual egg production are tightly coupled ($E \times E$) or that all genotypes have the same male inducing responses ($P \times E$)*

(Population, population studied (for abbreviations, see §2(a)); number of MLGs (P) observed, the number of MLGs encountered in the sample of parthenogenetic females; number of MLGs expected, the number of MLGs expected in a sample of equal size as the sample of parthenogenetic females with equal number of allele copies as the real sample but assuming no clonal selection. Significance levels of difference between the observed and expected number of MLGs: * $p < 0.05$ and *** $p < 0.001$. P, parthenogenetic females; M, males; E, ephippial females.)

population	sampling date	number of MLGs (P) observed	number of MLGs expected ($\pm$ s.e.)	selfing rate (MLG)		
				$E \times E$	$M \times E$	$P \times E$
CSP	20 October 1992	3	22.6 $\pm$ 1.5***	75.8	11.9	13.1
	3 November 1992	4	32.0 $\pm$ 2.1***	73.5	32.9	34.2
	16 November 1992	3	35.6 $\pm$ 2.3***	51.6	42.8	50.3
	24 November 1992	4	38.2 $\pm$ 2.4***	82.1	37.0	54.0
	8 December 1992	3	11.9 $\pm$ 1.1***	100.0	22.2	32.7
	14 December 1992	3	9.4 $\pm$ 1.2***	92.3	44.5	12.5
	23 December 1992	3	12.8 $\pm$ 1.2***	92.3	24.2	7.6
	mean CSP	—	—	81.1	30.8	29.2 ^a
CBP	6 July 1993	6	9.7 $\pm$ 0.9***	51.1	6.3	62.7 ^a
DV	29 April 1996	21	30.6 $\pm$ 2.0***	11.2	6.1	4.8
	27 May 1996	23	29.9 $\pm$ 2.0***	9.7	5.3	4.9
	mean DV spring	—	—	10.5	5.7	4.9 ^a
	28 October 1996	25	29.5 $\pm$ 1.9*	6.9	6.6	5.4 ^a
FPB	3 August 1992	14	31.9 $\pm$ 2.2***	21.0	34.3	16.0 ^a
LT	20 August 1994	10	13.8 $\pm$ 1.2*	15.6	15.6	12.2 ^a
OM1	4 July 1996	12	21.3 $\pm$ 1.6***	81.4	4.3	11.6 ^a
	6 June 1997	12	28.8 $\pm$ 2.1***	43.1	37.6	22.5
	30 June 1997	17	35.1 $\pm$ 2.3***	16.1	16.2	10.4
	mean OM1 summer 1997	—	—	29.6	26.9	16.5 ^a
OM2	12 September 1997	5	27.6 $\pm$ 1.9***	100.0	92.5	58.8 ^a
	4 July 1996	25	40.4 $\pm$ 2.5***	24.7	5.7	8.3 ^a
OM3	22 May 1997	20	34.7 $\pm$ 2.2***	19.1	7.8	11.0 ^a
	18 June 1997	26	39.9 $\pm$ 3.3***	6.5	3.0	2.7 ^a
overall average ($n = 12$)		—	—	37.3	20.0	19.9

^a The value for the given date or population has been considered as an independent estimate (the data were treated as independent when obtained from a different population or growing season).

partners. As far as we know, there are no literature data indicating active mate choice or mate rejection in *Daphnia*.

The probability of selfing was also estimated for two hypothetical situations: (i) when the MLG frequencies of males are identical to those of ephippial females ($m_i = e_i$; this pattern will result when there is perfect coupling of the production of males and sexual eggs) and (ii) when the MLG frequencies of males are equal to those of parthenogenetic females ($m_i = p_i$, where p_i is the relative frequency of MLG i in the parthenogenetic subpopulation; this pattern will result when all genotypes in a given population have identical responses to male-inducing stimuli).

MLGs, as determined by four polymorphic loci, may contain several clones and should therefore be considered as clonal groups. Although our values for the rates of selfing are likely to overestimate the rates of true clonal selfing in most of the populations, this does not interfere with the test of our hypothesis, since it is very unlikely that the differences in the MLG frequencies among the reproductive classes observed by us will be cancelled by a different pattern occurring at the within-MLG level. It is much more likely that the patterns observed by us will be reinforced by genetic differences occurring at the within-MLG level, making our interpretation conservative.

The effect of an uncoupling of the responses to male- and sexual egg-inducing stimuli is expected to be strongest in

populations in which the probability of inbreeding is relatively high, i.e. in populations in which only a few clones switch to the production of sexual eggs at the same time. The strongest test of our hypothesis will thus be provided by populations in which clonal diversity is markedly reduced by clonal selection or random processes during the parthenogenetic growing season. Using Monte Carlo simulations, we estimated the number of MLGs for each population that would be expected in a sample with the same size as our sample of parthenogenetic females of that population and with the same number of copies of each allele (Levene 1949), but which would conform to Hardy–Weinberg expectations. Using these Monte Carlo simulations, we performed exact tests (J. Vanoverbeke, unpublished data) to determine whether the observed number of MLGs was significantly lower than under Hardy–Weinberg expectations. Deviations from Hardy–Weinberg equilibrium indicate the occurrence of clonal selection in the ongoing growing season or in previous growing seasons (gametic phase disequilibrium).

(c) Literature data

We know of four studies which have simultaneously determined the MLG frequencies of parthenogenetic females, sexual females and males of natural *Daphnia* populations (see table 2): Hebert (1974) studied *D. magna* in Harlton Pond (HP), UK (four

Table 2. *Multilocus selfing rates (%) calculated for data taken from the literature using the observed genotype frequencies of males ($M \times E$) or assuming that male and sexual egg production are tightly coupled ($E \times E$) or that all genotypes have the same male inducing responses ($P \times E$)*

(Population, population studied (for abbreviations, see the text); number of MLGs (P) observed, the number of MLGs encountered in the sample of parthenogenetic females. The average number of MLGs found in the parthenogenetic subpopulation are tabulated and whether the observed number of MLGs is in most cases lower than the expected one is indicated. Significance levels of difference between the observed and expected number of MLG: * $p < 0.05$ and *** $p < 0.001$. P, parthenogenetic females; M, males; E, ephippial females.)

population (reference)	species	number of samples	number of MLG (P) observed (mean)	selfing rate (MLG)		
				$E \times E$	$M \times E$	$P \times E$
HP (Hebert 1974)	<i>D. magna</i>	4	3.0*	78.0	54.5	58.9
BP (Lynch 1984)	<i>D. pulex</i>	9	7.6 ^a	33.6	32.3	30.3
Z (Ruvinsky <i>et al.</i> 1986)	<i>D. pulex</i>	1	5.0***	41.1	32.0	34.4
MZ (L. Y. Yampolsky, personal observation)	<i>D. magna</i>	1	27.0*	1.9	0.6	1.4

^a No significance.

samples in 1971 with one locus, MDH), Lynch (1984) studied *Daphnia pulex* in Busey Pond (BP), Illinois, USA (data on the three reproductive classes for a total of nine sampling dates in 1980–1982 with two loci, PGM and Pep2), Ruvinsky *et al.* (1986) studied *D. pulex* in pond Z (Z), Russia (one sample in 1981 with three Est loci) and L. Y. Yampolsky (personal communication) studied *D. magna* in a small pond in Moscow Zoo (MZ), Russia (one sample in 1988 with three loci, GPI, PGM and Est) (see also Yampolsky & Kalabushkin 1992). The data from these studies were used to calculate the probabilities of selfing as outlined above.

3. RESULTS

Tables 1 and 2 show the observed and expected numbers of MLGs in the parthenogenetic female subpopulation for all 12 populations studied. The observed number of MLGs was significantly lower than expected in all but one population. In particular, the difference is striking in CSP and OMI, indicating that a substantial genetic erosion had taken place during the preceding growing seasons in these populations. Figure 2 shows that the Euclidean distances, as determined by the MLG frequencies between males and parthenogenetic females, were lower than those between parthenogenetic and ephippial females in several populations, but the difference was not significant by the Wilcoxon matched-pairs test ($p = 0.48$). However, when considering the different samples of CSP separately, the Euclidean distances between males and parthenogenetic females were marginally significantly lower than between parthenogenetic and ephippial females (Wilcoxon matched-pairs test, pairing values according to date, d.f. = 6 and $p = 0.063$; see also figure 3).

Figure 4 plots the probability of MLG selfing for the observed genotype frequencies of males ($M \times E$) against the probability of selfing under the hypothesis of a perfect coupling of male and sexual egg production ($E \times E$). In most populations, the probability of selfing is lower for the observed MLG frequencies of males than under $E \times E$. The only exception to the pattern is population FPB, for which the probability of selfing under $M \times E$

Figure 2. The ratio of the Euclidean distance between the parthenogenetic female and male subpopulations (PM) to the Euclidean distance between the parthenogenetic female and ephippial female subpopulations (PE) in the different populations sampled during the present study, as calculated from the relative frequencies of MLGs (there are 12 independent data points, see table 1).

(34.3%) was higher than that under $E \times E$ (21.0%). For the CSP population, the average probability of selfing was reduced from 81.1 to 30.8% by the differences in the MLG frequencies between males and sexual females. The most striking reduction in the probability of selfing was observed in the OMI population sampled in July 1996 (from 81.4 to 4.3%). Under $E \times E$, the risk of selfing is particularly high in populations in which there are only a low number of MLGs (e.g. CSP and OMI). Although there is indeed a highly significant negative exponential relationship between the probability of selfing under $E \times E$ and the number of MLGs producing sexual eggs ($n = 12$, $\log Y = -0.87X + 1.98$, $r^2 = 0.73$ and $p = 0.00027$), this relationship is much less pronounced when the observed MLG frequencies of males are taken into account ($M \times E$; $n = 12$, $\log Y = -0.47X + 1.41$, $r^2 = 0.15$

Figure 3. MLG frequencies in the different reproductive categories (parth., parthenogenetic females; eph., ephippial females; males) on different sampling dates in the CSP population: (a) 20 October, (b) 3 November, (c) 8 December, (d) 14 December and (e) 23 December. (The data collected on 16 and 24 November are not shown because the number of males sampled on those dates was low; $n=8$ or 10.) Different hatching patterns indicate different MLGs, with identical hatching patterns representing the same MLGs across sampling dates.

Figure 4. Scatter plot of the probabilities of MLG selfing in 16 cyclical parthenogenetic *Daphnia* populations for the observed genotype frequencies of males and sexual females ($M \times E$) against the probability of clonal selfing for the hypothetical case that there will be perfect coupling of male and sexual egg induction responses in individual genotypes ($E \times E$). The bisecting line connects points for which the probability of selfing under $M \times E$ is identical to that under $E \times E$. For calculation of the probability of MLG selfing (%), see the text. Closed symbols are derived from the populations sampled by us (table 1), whereas the four open symbols are based on literature data (table 2).

and $p=0.12$). Overall, the probability of selfing differed strongly between populations, but was on average only approximately half as high for the observed genotype frequencies of males (20.0%) than under $E \times E$ (37.3%). This difference is significant by a Wilcoxon matched-pairs test pairing data according to the population ($n=12$ and $p=0.021$; including the literature data $n=16$ and $p=0.0045$). Tables 1 and 2 also show the probabilities of selfing under the hypothesis of a complete uncoupling of males and sexual egg production in a given genotype ($P \times E$). The probability of selfing under $M \times E$ (20%) is no higher than that under $P \times E$ (19.9%; Wilcoxon matched-pairs test $n=12$ and $p=0.53$; including the literature data $n=16$ and $p=0.92$).

4. DISCUSSION

In cyclical parthenogens in which the switch from parthenogenetic to sexual reproduction is triggered by environmental stimuli, it is expected that the induction of males and sexual eggs will be tightly coupled as a mechanism to increase the likelihood of fertilization. However, our data on *Daphnia* show that the MLG frequencies of ephippial females and males may differ substantially. Our results also suggest that the probability of MLG selfing is approximately halved by a non-perfect coupling compared to a perfect coupling of male and sexual egg production in natural *Daphnia* populations. In two out of the 12 populations for which data were analysed, the probability of selfing was found to be reduced from more than 50% to less than 10%. Given the more than two-fold higher mortality rate of selfed offspring (Innes 1989;

De Meester 1993; Innes & Dunbrack 1993; Lynch & Deng 1994), a reduction of the selfing rate may confer a substantial advantage on *Daphnia* genotypes which produce males in response to stimuli that are somewhat different from those which induce the production of sexual eggs.

MLGs, as determined by only a few polymorphic loci, may harbour several clones. As such, our estimates of the rates of 'multilocus selfing' no doubt overestimate the rates of true clonal selfing in most of the populations. However, the differences in genotype frequencies must result from differences in investment in male and female sexual eggs at the clonal level and no differences in the MLG frequencies among reproductive classes will be observed when the production of males and sexual eggs is tightly coupled in all clones. The limited resolution of having only four markers is much more likely to have obscured the reduction in inbreeding under $M \times E$ compared to $E \times E$ than to have revealed patterns that are not real, making our test of the uncoupling hypothesis conservative. Moreover, our markers may have been sufficient to detect almost all clones present in some of the populations. In CSP, for instance, screening of 904 individuals revealed only six genotypes, whereas more than 20 clones would be expected in each sample given the observed allele frequencies (table 1). In this population, our estimates of 'MLG selfing' may approximate the true rates of clonal selfing.

Our data clearly indicate that there is an uncoupling of investment in males and sexual eggs at the level of individual clones, resulting in a lowering of the probability of clonal selfing in natural *Daphnia* populations. An obvious mechanism by which a more random mating can be realized is by the evolution of less clone-specific responses to male-inducing than to sexual egg-inducing stimuli (see figure 1). This mechanism would explain why it is relatively easy to induce males in the laboratory without subsequent production of ephippial eggs (e.g. Ferrari & Hebert 1982; Hobaek & Larsson 1990). Our data on the Euclidean distances based on the MLG frequencies indicate that this particular mechanism may have operated in at least three of the 12 populations studied, including CSP (cf. the fact that the Euclidean distances between the parthenogenetic and male subpopulations are lower than the distances between the parthenogenetic and the ephippial subpopulations).

Although the avoidance of selfing can be a strong selective force, some assortative mating within seasonal clonal groups may be important in the inheritance of coadapted gene complexes which may be associated with seasonally specialized clones (see Pace *et al.* 1984; Carvalho 1987). Our data indicate that the genetic isolation between seasonal clonal groups is less pronounced than would be predicted from the genotype frequencies of sexual females alone, but do not indicate that mating is random. Indeed, we observed significant differences in the MLG frequencies between males and parthenogenetic females in all populations studied by us (with χ^2 -tests with sequential Bonferroni correction on the relative frequencies of MLGs representing > 5% of the population; no differences were observed on two of the sampling dates in the CSP population; see also the data provided by Yampolsky (1992) and Spaak (1995)).

Two other mechanisms which may reduce the probability of selfing in cladocerans have been previously described: the occurrence of non-male-producing clones (Innes & Dunbrack 1993) and the occurrence of a typical male photobehaviour which reduces spatial segregation between genotypes (De Meester 1992). Innes & Dunbrack (1993) reported the co-occurrence of non-male-producing females and females which produce both males and sexual eggs in natural *D. pulex* populations and interpreted the phenomenon as a mechanism for avoiding inbreeding depression. The absence of any production of males in some clones can be considered an extreme case of the uncoupling of the responses to male- and sexual egg-inducing stimuli. Obviously, the two mechanisms can operate together. De Meester (1992) observed that males of *D. magna* clones of which adult females differed strongly in phototactic behaviour (vertical distribution) all showed a similar phototactic behaviour which was intermediate to that of the females. These results were interpreted as a random mating promoting mechanism. Brewer (1998) also reported that males have a different depth selection behaviour than females.

It seems that different groups of cyclical parthenogenetic organisms may have evolved different mechanisms for avoiding strong inbreeding. In cyclically parthenogenetic monogonont rotifers, it has been shown that contact chemoreception involving glycoproteins is important in mate recognition (Snell & Hawkinson 1983; Gomez & Snell 1996) and there is evidence for recognition of clone mates in the genus *Asplanchna* (Gilbert 1976). Kin recognition during copulation may be important in rotifers, as it has been shown that *Asplanchna* strains suffer severe inbreeding depression (Birky 1967), while the production of males and sexual females in monogonont rotifers is intrinsically coupled, since unfertilized haploid eggs develop into males. In aphids, the production of males and sexual females also seems to be coupled. However, gynoparae and males are often winged and dispersal to other host plants is expected to result in very low probabilities of inbreeding (Newton & Dixon 1987).

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