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Among- and within-population variation in outcrossing rate of a mixed-mating freshwater snail

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Mixed-mating animals self-fertilize a proportion of their offspring. Outcrossing rate may covary with the ecological and historical factors affecting the population. Theory predicts that outcrossing is favored when inbreeding depression is high and when individual heterozygosity is important. Self-fertilization is predicted to be favored when costs of male function, or mate finding are high, for example, when empty patches are colonized by few individuals. In this study, we assessed primary (after hatching) and secondary (after juvenile mortality) outcrossing rates of two mixed-mating snail populations. Our purpose was to assess the variation in mating-system parameters and estimate significance of inbreeding depression for secondary outcrossing rate (the realized outcrossing rate of parents that produce the next generation). Secondary outcrossing rate was higher than the primary outcrossing rate in one of the two populations, suggesting considerable inbreeding depression. In the other study population, secondary outcrossing rates were found to increase when initially low, or decrease when initially high, depending on the family. Moderate outcrossing rates were found to be more stable. Parental inbreeding coefficients were close to zero in both populations. Outcrossing rate was much more variable among families in the population with the lower average outcrossing rate, suggesting that individuals differed considerably in their mating system. Our results add to recent studies suggesting that populations of mixed-mating animals may differ in their mating system parameters and expression of inbreeding depression.
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Introduction

Understanding the evolution of mixed-mating systems presents a considerable challenge for evolutionary theory (Lloyd, 1979; Holsinger, 1991, 1992; Jarne and Charlesworth, 1993; Latta and Ritland, 1994a). Self-fertilization increases genomic homozygosity and often leads to inbreeding depression, while outcrossing requires availability of mating partners and higher investment in male function (Lloyd, 1979; Charlesworth and Charlesworth, 1987; Jarne and Charlesworth, 1993). Theory predicts that mixed-mating systems are evolutionary stable under some specific assumptions (Lively and Lloyd, 1990; Charlesworth *et al*, 1991; Holsinger, 1991; Uyenoyama and Waller, 1991a, b; Jarne and Charlesworth, 1993; Latta and Ritland, 1993, 1994b). Many of the theoretical predictions rely on the level of inbreeding depression, its distribution among the inbreeding individuals, and on the possibility of purging the mutation load (Charlesworth and Charlesworth, 1987; Charlesworth *et al*, 1990; Uyenoyama and Waller, 1991a, b; Jarne and Charlesworth, 1993). For example, mixed-mating systems are predicted to be stable if fitness declines monotonically with consecutive rounds of self-fertilization (Maynard Smith, 1977, 1978; Latta and Ritland, 1994a), magnitude of inbreeding depression varies over time (Cheptou and Mathias, 2001), or the population is under specific kinds of density dependence (Cheptou and Dieckmann, 2002). Empirical studies have revealed that inbreeding depression may be severe in natural populations (Haag *et al*, 2002), and the corresponding selection may lead to rapid changes in the average inbreeding coefficients of the populations (recent reviews include Charlesworth and Charlesworth, 1987; Byers and Waller, 1999; Charlesworth and Charlesworth, 1999; Crnokrak and Roff, 1999).

In the analyses of mixed-mating systems, animals have received much less attention than plants (Jarne and Charlesworth, 1993, 1996). Recent studies with plants have emphasized the importance of within-population variation in the expression of inbreeding depression and evolution of mixed-mating systems (Dudash *et al*, 1997; Koelewijn, 1998; Mutikainen and Delph, 1998). Much less is known of the mixed-mating systems of animals. Two recent studies with freshwater snails, that addressed the magnitude of within-population variation in inbreeding depression, report large and significant variation in inbreeding depression among experimentally generated selfing and outcrossing lineages of *Lymnaea peregra* and *Physa acuta* (Coutellec-Vreto *et al*, 1998; Jarne *et al*, 2000). These studies also report significant within-population variation in selfing rates, as do studies by Viard *et al* (1997), Henry *et al* (2005), and our earlier study with *Lymnaea ovata* (Wiehn *et al*, 2002). All four studies suggest that the inbreeding depression and outcrossing rate of

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individuals vary within populations of mixed-mating freshwater snails.

In this study, we focused on variation in outcrossing rate before and after juvenile mortality. We designed an experiment to assess inbreeding depression and mating-system parameters among naturally fertilized progeny of two populations of hermaphroditic freshwater snails. We predicted that if inbreeding depression is important, the outcrossing rate of the offspring should increase with juvenile mortality (Ritland, 1990). We found evidence for inbreeding depression among the mixed-mating families of one study population, while in the other population outcrossing rate was high and less variable among families.

Materials and methods

Study system

Snails of the genus *Radix* (former *Lymnaea*) are mixed mating, simultaneously hermaphroditic freshwater animals (Jarne and Delay, 1990a, b; Coutellec-Vreto *et al*, 1998; Wiehn *et al*, 2002). The systematics of this group was recently revised using ribosomal DNA sequences (ITS-1 gene) by Bargues *et al* (2001). In their revision, the morphological species of *L. ovata*, which we used in this study, was assigned to either species *Radix peregra, R. ampla*, or *R. balthica* depending on the area where the sample originated. The haplotype of the snails used in this study identifies them with *R. balthica* (Mas Coma *et al*, unpublished). In this paper, we choose to call them *L. ovata* based on their morphology. *L. ovata* is a semelparous annual across its range in Central Europe (Wullschleger and Jokela, 2002), and prefers the shallow littoral zone of large lakes, where it may be found in variable densities in habitat patches of boulders and rocks (Wullschleger and Jokela, 1999). In Lake Zürich *L. ovata* lays eggs in the spring (April–May). Each snail may lay hundreds of eggs during the egg-laying period. The resulting cohort develops to maturity during the summer and overwinters as adults. In natural populations copulation takes place in spring, before and during the egg-laying period.

Snail collection and rearing methods

We collected egg-laying individuals from Kilchberg and Uerikon populations of Lake Zürich (distance 18.5 km) in late April 2000 using snorkeling equipment. Collection took place at the peak egg-laying period. Individuals were isolated after collection and transported alive to the laboratory at ETH-Zürich, where they were reared in isolation. Based on earlier experience, we knew that after isolation field-collected snails readily lay eggs that are fertilized using allosperm from copulations that took place earlier in the field (Jokela, unpublished). As allosperm can be stored for months (Boycott *et al*, 1930), field-copulated snails are able to choose it for fertilization in the laboratory. We collected eggs from each individual for a period of 14 days, snap froze two tissue samples of the parent in liquid nitrogen, and stored the samples in -80°C for later electrophoresis.

Experimental design

The purpose of this experiment was to study how the proportion of outcrossed offspring in each family changes due to natural mortality taking place as the cohort ages. Eggs of each parent ($N=60$ and $N=48$ parents for Uerikon and Kilchberg populations, respectively) were counted using a dissection microscope, and kept in 200-ml plastic cups till hatching. As the eggs were laid over a short period of time (14 days), hatching took place over 10 days in early June. Offspring of each family differed in age by less than 10 days and within family variation in length of hatching period was less than that. We counted the offspring and snap froze a random sample of the recently hatched offspring ($N=15$ offspring per family) for later electrophoresis. These samples were used to assess the outcrossing rate of each family at hatching. We refer to this sample as 'the first sample'.

At hatching, we chose 30 families from each population for further culturing (see below). The 30 families were chosen at random among those families that contained roughly 100 or more eggs ($\bar{x}=125$, SD $=30$, range 81–215 eggs). A hundred eggs equals to one or few clutches per female (Wiehn *et al*, 2002). The purpose of this lower limit to egg number was to guarantee that there would be enough offspring to sample later, after mortality had taken place. We cultured these snails, in family groups, in 8 l plastic containers that were continuously aerated. The volume in the containers was large enough to keep the water quality good during the experiment. Snails were fed frozen lettuce *ad libitum*, and the water in the containers was changed every 2 weeks. Containers were provided with a supply of chalk, which is important for shell growth.

We counted the number of snails in each family group roughly once a month (on 5th of June, 29th of June, 29th of July, and 4th of September). This allowed us to keep track of the mortality in each container. Our goal was to snap freeze a second sample ($N=20$ offspring per family) of each progeny when more than 50% of the hatched offspring had died. This allowed sufficiently large sample of the survivors to estimate the outcrossing rate, but presents significant mortality to estimate the difference in relative mortality of selfed and outcrossed offspring. The average level of mortality at the second sample was 57% (SD $=14.2$, range 26–90%). Note that the date of the second sample varied across families depending on the level of mortality. On the 4th of September, we terminated the experiment, and froze samples of those families that had not yet experienced 50% mortality (six families had experienced less than 40% mortality).

Estimation of outcrossing rate

We used cellulose acetate gel electrophoresis (CAGE) (Hebert and Beaton, 1989) to determine the genotype of each individual in the progeny array. Four polymorphic loci were available for Kilchberg (GPI, MPI, LAP, PGM), and three for the Uerikon (GPI, MPI, LAP) populations. Enzyme activity in LAP and PGM was very low for individuals in the first sample; therefore the data on many individuals in the first sample consists only of GPI and MPI results.

We used MLTR software (version 1.1, http://genetics.forestry.ubc.ca/ritland/) to derive family estimates of the outcrossing rate. As family estimation is prone to statistical problems, maximum likelihood estimates may

be biased upwards. These problems are more prevalent when the sample size is small and the level of polymorphism reduced (these details are documented in the latest version of the program manual, available from the above URL). Therefore, we examined our data carefully to exclude all families for which the software returned unreliable values for the outcrossing rate. We excluded all families for which the multilocus outcrossing rate was consistently too high ($t_m > 1$), either for the first, or for the second sample. Here, we took benefit of the Newton–Raphson algorithm to estimate the outcrossing rates; this algorithm returns high estimates for those families that lack sufficient resolution (see MLTR software manual). For the first sample this rule reduced the sample size from 48 and 60 families per population to 17 and 36 families for Kilchberg and Uerikon, respectively. These families were not among those cultured till the end of the experiment, therefore, for the second sample, we were left with eight and 14 families for the Kilchberg and Uerikon populations, respectively. Examination of the genotype data suggested that most of the excluded families had either a multiple heterozygote parent, or many offspring with a missing genotype in one of the two key loci (GPI or MPI). Although we lost many families using this rule, removing the problematic families is conservative as the true outcrossing rate of these families is not clear.

Estimation of inbreeding and outbreeding depression

Inbreeding (and outbreeding) depression can be derived from the magnitude of change in the outcrossing rate of a progeny array (Ritland, 1990). When outcrossing rate of the second sample (t_2) is higher than the outcrossing rate of the first sample (t_1), the difference in outcrossing rates is due to inbreeding depression, that is, poorer relative survival of the selfed offspring (Ritland, 1990). Outcrossing rate after mortality is therefore a function of inbreeding depression.

$$t_2 = \frac{N_O}{N_O + (1 - ID)N_S}, \quad (1)$$

where ID = inbreeding depression, N_O = number of outcrossed individuals at hatching, and N_S = number of selfed individuals at hatching. Numbers of outcrossed and selfed individuals may be written as a function of outcrossing rate at the first sample and initial progeny size (N).

$$N_O = t_1 N \quad (2)$$

$$N_S = (1 - t_1)N \quad (3)$$

Using these equations, inbreeding depression can be written as

$$ID = \frac{t_2 - t_1}{t_2 - t_1 t_2} \quad (4)$$

Note that this equation is the same as 1-Eq(8) in Ritland (1990). When outcrossing rate of the second sample is lower than that of the first sample ($t_2 < t_1$), the difference in outcrossing rate is due to outbreeding depression, that is, relative survival of the outcrossed offspring is lower than that of the selfed offspring. Then, the outcrossing rate of the second sample is a function of outbreeding depression (OD),

$$t_2 = \frac{(1 - OD)N_O}{(1 - OD)N_O + N_S} \quad (5)$$

Writing the numbers of outcrossed and selfed offspring using outcrossing rate of the first sample and total number of offspring (as above, equations (2) and (3)), the outbreeding depression is

$$OD = \frac{t_1 - t_2}{t_1 - t_1 t_2} \quad (6)$$

We calculated inbreeding/outbreeding depression for those families for which we had two reliable estimates of outcrossing rate using equations (4) and (6). We used these estimates to illustrate among-family variation in inbreeding and outbreeding depression in the two study populations. Outbreeding depression is often expressed as negative values that vary between 0 and -1, while inbreeding depression is expressed so that it varies between 0 and 1. We followed this practice in the analysis of the results. Note that the measure of inbreeding and outbreeding depression is based on relative survival of inbred and outbred individuals within a family. This measure is not sensitive to initial outcrossing rate, number of offspring in the family, or absolute level of mortality.

Statistical analyses

We first examined population genetic structure and heterozygote deficiency of the parents in the experiment using FSTAT 2.9.3.1 (http://www.unil.ch/izea/softwares/fstat.html) (Goudet, 1995). Then, we contrasted progeny traits (number of eggs, proportion of eggs hatching, and mortality rate of progeny after hatching) between completely selfed (determined by progeny-array analysis, see below) and mixed-mated progenies using a two-way ANOVA where population of origin (Kilchberg, Uerikon) and mating type (selfed, mixed mated) were used as fixed factors. Alternative analysis, using mating type as a covariate, violated the assumptions of ANCOVA (note that half of the families were selfing, see results), therefore, we used ANOVA with mating type as a fixed factor. We also calculated correlation coefficients between progeny traits and outcrossing rate of the progeny at hatching. Mortality rate after hatching was estimated as a daily rate (percent dead individuals/day) between 5th June and 29th June, the period during which the highest mortality was observed. The purpose of these analyses was to find out if the families with the highest fitness (many offspring, low mortality rate) also had the highest outcrossing rate.

We continued by comparing the estimated outcrossing rate at hatching (first sample) to the outcrossing rate observed after 50% mortality (second sample) using repeated measures analysis of variance. We were especially interested in the observed change in the outcrossing rate, as this would indicate that mortality of the offspring was biased towards either selfed or outcrossed offspring (within-effect 'SAMPLE'). Furthermore, the analysis allowed us to compare the mean outcrossing rate of the families of the two populations (between-effect 'POPULATION'), and compare the difference between the populations in the magnitude of

change in the outcrossing rate between the samples (within-effect 'SAMPLE × POPULATION').

For all analyses of variance described above the assumptions of normality of residuals and homogeneity of variances were fulfilled.

Results

Population genetic analysis of the parents used in this experiment revealed statistically significant population structure (test based on randomization of multilocus genotypes, available in FSTAT; $P<0.001$, 1000 randomizations, $\theta=0.074$). Largest differences in the allele frequencies between the two populations were found in GPI and LAP (Table 1). Snails of the two populations also differed by their average heterozygosity. While snails of Kilchberg showed only slight deficiency of heterozygotes (multilocus $F_{is}=0.037$), high heterozygote deficiency of Uerikon ($F_{is}=0.342$) suggests that self-fertilization has been common in the past.

High proportion of eggs in the experiment hatched (0.80 ± 0.029 (mean $\pm$ SE) and 0.78 ± 0.027, for Kilchberg and Uerikon, respectively; $t=0.521$, df $=103$, $P=0.604$). Peak offspring mortality took place during the first 4 weeks after hatching (Figure 1). Progeny array analysis suggested that half of the families were completely selfed (nine out of 17 families, and 18 out of 36 families for Kilchberg and Uerikon, respectively) (Figure 2). Neither the mean number of eggs, proportion of hatched offspring, nor mortality rate of the completely selfed families differed from those of the mixed-mated families (Table 2). Hence, we found no indication that selfing parents would have suffered from reduced fecundity, or that the progeny success would be lower than average in the completely selfed families.

Family outcrossing rate at hatching did not correlate either with the number of eggs laid by the parent ($r_s=-0.279$, $P=0.278$, $N=17$, and $r_s=-0.216$, $P=0.213$, $N=35$, for Kilchberg and Uerikon, respectively; Figure 2a and b), with the proportion of hatched offspring ($r_s=-0.429$, $P=0.085$, $N=17$, and $r_s=-0.012$, $P=0.947$, $N=35$, for Kilchberg and Uerikon, respectively; Figure 2c and d), or with the daily mortality of the offspring after hatching ($r_s=$ -0.340, $P=0.371$, $N=9$, and $r_s=0.281$, $P=0.310$, $N=15$, for Kilchberg and Uerikon, respectively; Figure 2e and f). Note that the lack of correlation was not due to lack of variation in the outcrossing rate (Figure 2). The result remained qualitatively identical when the analysis was conducted omitting the completely selfed families. These results indicate that outcrossing rate at hatching was a poor predictor of progeny quality at the family level.

Repeated measures ANOVA revealed that origin of the families affected the relative mortality of the outcrossed and selfed offspring (Table 3, Figure 3). More specifically, among the five families from Kilchberg that had been mixed mated, outcrossing rate increased consistently from hatching (first sample, t_{m1}) to 50% mortality (second sample, t_{m2}) (Figure 3), indicating that proportionally more selfed offspring died as the Kilchberg progeny developed during the summer. Note that only the mixed-mated families were included in this analysis because the outcrossing rate cannot respond to mortality in the completely selfed families. Among the more numerous mixed-mated Uerikon families, no consistent change in the outcrossing rate was observed (Figure 3). In families with low outcrossing rate, the proportion of outcrossed offspring increased with mortality, while in families with the highest outcrossing rate the proportion of outcrossed offspring tended to decrease with mortality (Figure 3). Families of different populations did not differ by their average outcrossing rate (Table 3). Three completely selfed Uerikon families were excluded from this analysis.

Above analysis indicates that some Uerikon progeny expressed outbreeding depression, while all Kilchberg progeny expressed inbreeding depression (Table 3, Figure 3). Among the five Kilchberg families, inbreeding

Table 1 Allele frequencies of the parent generation used in the experiment

Locus	*N*	*Allele 1*	*Allele 2*	*Allele 3*	F_{is}	P
GPI						
Kilchberg	51	0.382	0.618		−0.194	0.156
Uerikon	75	0.553	0.447		0.116	0.231
MPI						
Kilchberg	51	0.098	0.833	0.069	0.000	0.625
Uerikon	63	0.024	0.960	0.016	−0.023	0.894
LAP						
Kilchberg	43	0.465	0.535		−0.110	0.350
Uerikon	47	0.160	0.840		0.138	0.930
PGM						
Kilchberg	34	0.324	0.676		0.474	0.020
Uerikon	42	0.247	0.726		0.824	0.006

Samples also include individuals that did not lay eggs during culturing in the laboratory, and represent random samples from the wild. Multilocus F_{is} values were 0.037 ($P=0.331$) and 0.342 ($P=0.006$), for Kilchberg and Uerikon, respectively. Single- and multilocus tests for deviation from HW (F_{is}) were computed with FSTAT (randomization tests).

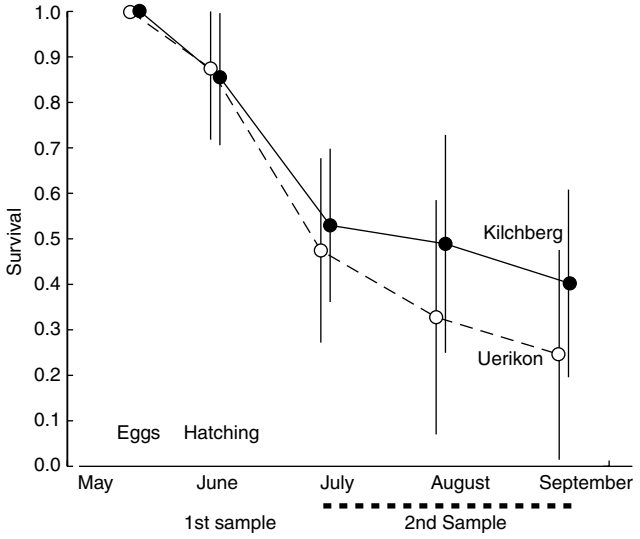

Figure 1 Survival curves of the snails during the experiment. Values show population mean $\pm$ SE of those Kilchberg and Uerikon families for which data were available for the whole time period presented in the figure. Therefore, the values for hatching success are higher than those given in the text.

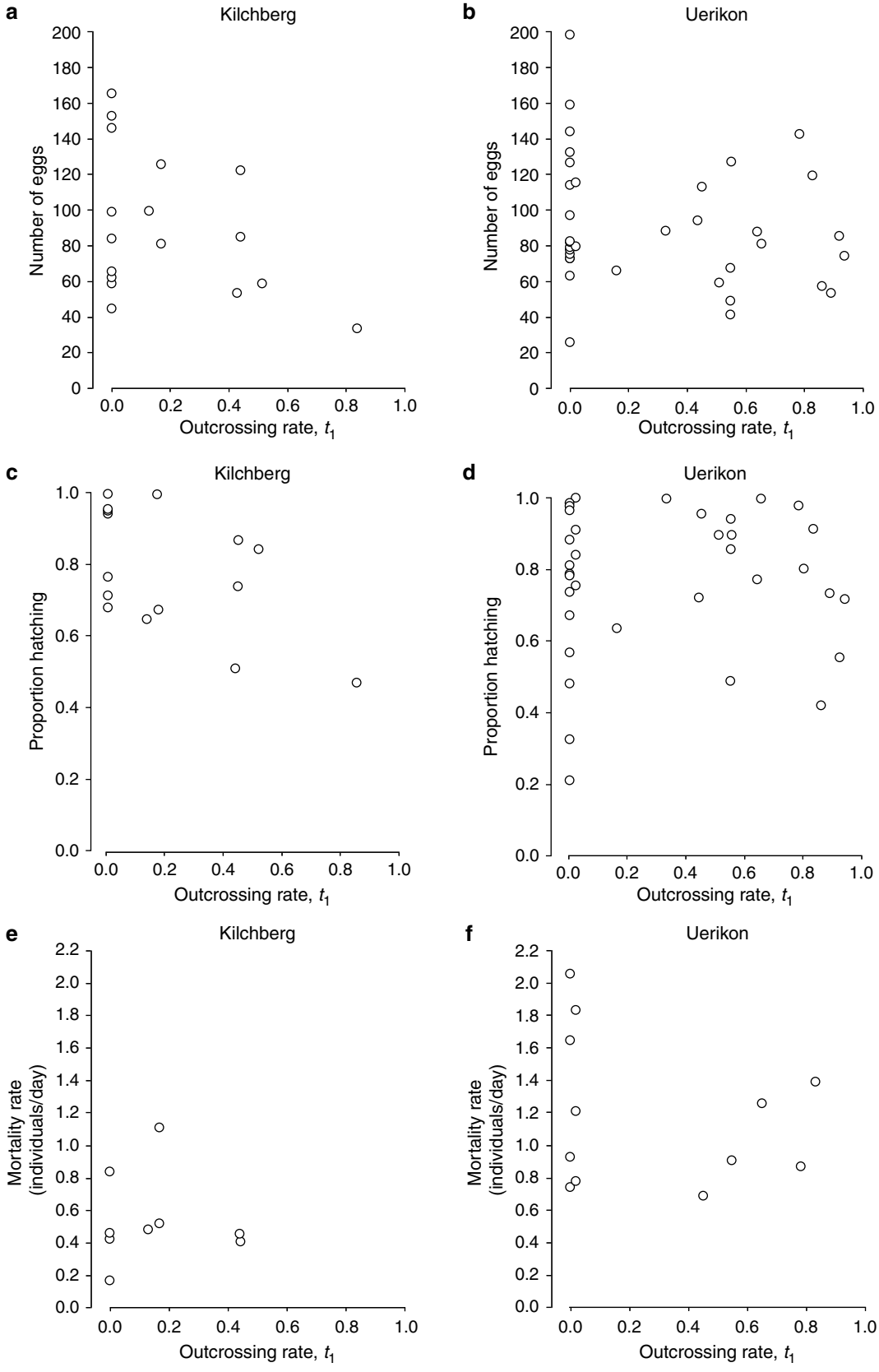

Figure 2 Relationship between family multilocus outcrossing rate at hatching (t_1) and (**a**, **b**) number of eggs, (**c**, **d**) proportion hatching, and (**e**, **f**) juvenile mortality rate of the offspring for the two study populations.

Table 2 Two-way analysis of variance of number of eggs, proportion hatching, and mortality rate between selfed and mixed-mated families (MTYPE) of Kilchberg and Uerikon populations (POPU)

Effect	*Number of eggs*				*Proportion hatching*				*Mortality rate*			
	df	*MS*	*F*	P	*df*	*MS*	*F*	P	*df*	*MS*	*F*	P
POPU	1	293	0.22	0.639	1	0.041	0.35	0.559	1	2.238	3.27	0.086
MTYPE	1	3218	2.45	0.124	1	0.043	1.06	0.308	1	0.081	0.12	0.735
POPU × MTYPE	1	33	0.03	0.873	1	0.150	3.38	0.061	1	0.613	0.90	0.355
Error	48	1316			48	0.041			20	0.684		

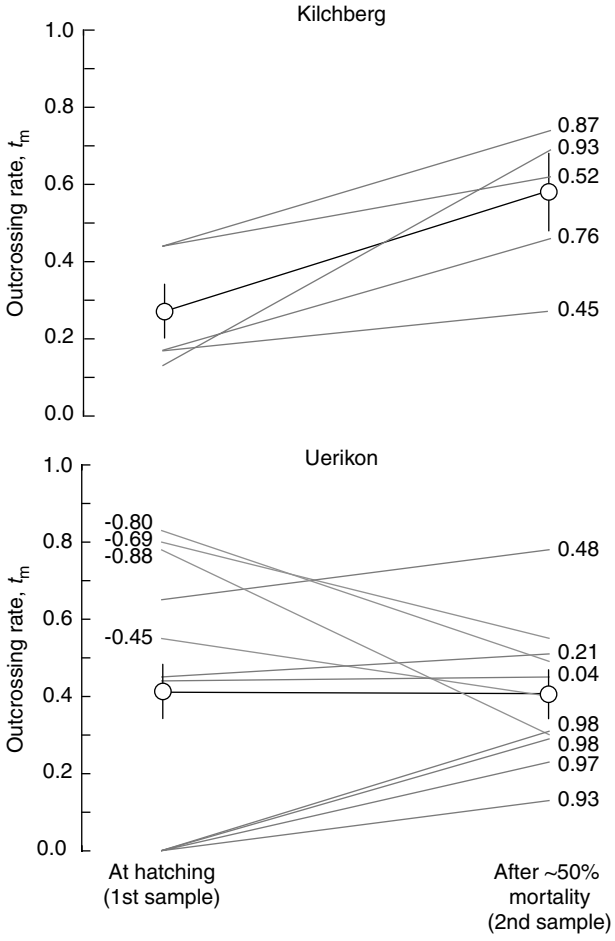

Figure 3 Multilocus outcrossing rate of each mixed-mated family at hatching and after ~50% mortality. Thin lines connect the values for same progeny. Thick black line connects mean values (±SE) for each population. Note that a positive slope indicates inbreeding depression and a negative slope indicates outbreeding depression. The inbreeding and outbreeding depression values for each family that were calculated with equations (4) and (6), respectively, are depicted on the right (inbreeding) and left (outbreeding) side of the panels. The outcrossing rate estimates that appear zero in the graph were 0.01 for each family.

depression appeared to be independent of the outcrossing rate at hatching (Figure 3, positive slopes of relatively same magnitude). Interestingly, among the Uerikon progenies inbreeding depression was found to be highest among those that had the lowest outcrossing rate at hatching (positive slopes in Figure 3), while the progeny with the highest outcrossing rate at hatching

Table 3 Results of repeated measures ANOVA to contrast the outcrossing rate at hatching to that after 50% mortality (within-subjects factor SAMPLE) in families of the two study populations (between-subjects factor POPU)

Effect	*df*	*MS*	*F*	P
Within-family				
SAMPLE	1	0.159	5.331	0.037
SAMPLE × POPU	1	0.171	5.720	0.031
Error	14	0.030		
Between-family				
POPU	1	0.002	0.024	0.880
Error	14	0.101		

expressed outbreeding depression (negative slopes in Figure 3). In general, relative mortality of selfed and outcrossed offspring differed the least in progeny that were produced by intermediate outcrossing (ie slope close to zero in Figure 3).

Discussion

Our results indicate that the studied *L. ovata* of Lake Zürich have a highly variable mixed-mating system, which is in line with our earlier studies on other populations of the same species (Wiehn *et al*, 2002), and with studies on closely related species (Coutellec-Vreto *et al*, 1997). Here, we also estimated among-family variation in fecundity, hatching rate and offspring survival. Our results revealed large variation in these traits, even among the completely selfed families. In fact, trait expression of the completely selfed families covered the full range of expression for all the measured traits (Figure 2). Furthermore, outcrossing rate at hatching turned out to be a poor predictor of progeny success (Figure 2). These results suggest that factors other than the outcrossing rate were important for variation in fitness – a finding that is in accord with some recent studies of mixed-mating plants (Dudash *et al*, 1997; Koelewijn, 1998; Mutikainen and Delph, 1998) and animals (Coutellec-Vreto *et al*, 1997, 1998; Jarne *et al*, 2000; Henry *et al*, 2005).

Our study populations differed in the degree of heterozygosity, suggesting that the Uerikon population had gone through higher degree of selfing in the past. Past inbreeding should have an effect on the expression of inbreeding depression, if purging of mutation load has occurred (Barrett and Charlesworth, 1991). We did not find any obvious difference in the performance of selfed and outcrossed families between the two study

populations; variation in most of the measured traits was as larger among the selfed families of each population than among the outcrossed families (Figure 2, Table 2). However, we found that in the Kilchberg population all of the mixed-mating families expressed inbreeding depression, while in the Uerikon population mixed-mating was associated with outbreeding depression when the outcrossing rate was high, and with inbreeding depression when the outcrossing rate was low (Figure 3). Unfortunately, it is difficult to draw far-reaching conclusions about the role of past inbreeding for the observed difference between the populations, because the range of outcrossing rates in the mixed-mating families of Kilchberg was rather narrow compared to Uerikon, and because only five mixed-mated families of Kilchberg were available for the analysis.

When examining Figure 3 in detail it appears that the relative mortality of selfed offspring (inbreeding depression) was highest when selfing rate was high, and lowest when outcrossing rate was high. This result was unexpected for us, and not anticipated by the theory. While we need to conduct further studies to verify this result over more general conditions, we discuss some implications of the result. Taken at its face value, our result suggests that intermediate families (initial outcrossing rate close to 0.5) are the ones where outcrossed and selfed offspring do not differ in their mortality rate. The result further suggests that the class of progeny (selfed or outcrossed) that is in the minority benefits as the progeny develop (a rare-type advantage).

We can think of two mechanisms that might have led to the effect we observed. First, maternal provisioning of eggs may be biased in favor of the rare type. Unfortunately, it is not known for how long juvenile snails rely on resources that are present in the egg. Given that the peak mortality took place in the few weeks following hatching (Figure 1), maternal provisioning may have been important for juvenile survival. Maternal provisioning might have differed between the early and late-laid egg clutches during the 14 days we allowed the snails to lay eggs. Provisioning of eggs may depend on the laying order, because the snails use their reserve energy to lay eggs, and die soon after the egg-laying period. However, it is not clear to us why the selfed (or outcrossed) eggs would be provisioned differently, depending on their frequency in the progeny. In many organisms egg size reflects maternal provisioning, therefore it would be helpful to look at variation in egg size and laying order with respect to selfing/outcrossing in future studies.

Second, we raised the progeny in family groups. Although we provided food *ad libitum*, it is not inconceivable that some interference-type competition might have been a factor affecting the observed mortality of the offspring. From classical competition theory we know that when the intraclass (eg among the selfed offspring) competition is harsher than the interclass competition (eg between selfed and outcrossed), negative effects of competition are stronger in the group that is more common (ie has higher density) (May, 1974). Such density-dependent effect would readily create a rare-type advantage. Indeed, mortality rate correlated positively with the initial number of offspring ($r_s = 0.50$, $df = 24$, $P = 0.014$), indicating that mortality rate was density dependent in our study families. However, inbreeding/outbreeding depression did not correlate with the initial density ($r_s = 0.17$, $df = 16$, $P = 0.534$) suggesting that density-dependent effects do not explain the observed inbreeding/outbreeding pattern.

To conclude, individual variation in costs and benefits of outcrossing suggest that simple explanation for the evolution of mixed-mating system in these snails may be difficult to find. We did not observe reduced fecundity of selfing individuals, which strikes a contrast to the results of earlier studies conducted with laboratory-reared snails (Coutellec-Vreto *et al*, 1998). In fact, some of the selfed families were large, had good hatching success, and low juvenile mortality (Figure 2). These selfing lineages should be competitive against the mixed-mated progeny. In the light of our results, stability of the mixed-mating system may not be a relevant issue to discuss (cf. Holsinger, 1988, 1991, 1992) before we have reliable estimates of the heritability of outcrossing rate in these snails. However, intermediate levels of outcrossing would be promoted by the fact that inbreeding depression was highest in highly selfed mixed-mated progeny and outbreeding depression was highest in the highly outcrossed mixed-mated offspring. Further studies are required to assess the generality of this result.

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