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Field and experimental evidence of preferential selfing in the freshwater mollusc *Lymnaea truncatula* (Gastropoda, Pulmonata)

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We have conducted a thorough study of the mating system of *Lymnaea truncatula*, the intermediate host of the liver fluke, using three approaches: (i) a population genetics study, (ii) controlled pairings in the laboratory and (iii) a progeny-array analysis. The population genetics study revealed high levels of inbreeding in the studied populations, with strong clues that the extensive heterozygote deficiencies observed are due to selfing. However, Wahlund effects may also arise due to recolonisations from different source populations after bottleneck events. A breeding experiment helped to disentangle the mating system and the Wahlund effects, and showed that high levels of selfing occurred in isolation and in controlled pairings. However, the progeny-array analysis performed after a high-density culturing of the snails suggests that substantial outcrossing may also occur.
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Introduction

In determining how genetic variation is inherited, mating systems are a key component of population structure and evolution. In contrast to gonochoric or strictly parthenogenetic species, hermaphroditic species can display a continuum of mating strategies, from complete selfing to outcrossing (Vogler and Kalisz, 2001). Furthermore, the mating system of such species has been proven to be highly variable, either spatially, between populations (eg Ellstrand *et al*, 1978; Coutellec-Vreto *et al*, 1997), or between families within populations (Ritland and Ganders, 1985), and/or temporally (eg Cheliak *et al*, 1985; Willis, 1993).

Geneticists and ecologists have proposed different hypotheses to account for the variability and evolution of mating systems in hermaphroditic species. From the genetic point of view, the higher efficiency of gene transmission through selfing compared to outcrossing (the so-called two-fold cost of outcrossing, see Fisher, 1941) is counterbalanced by inbreeding depression (Darwin, 1876). In selfing species, inbreeding depression is supposed to be lessened by the purging of deleterious alleles (Charlesworth and Charlesworth, 1987). This model leads to the prediction of fixation of either outcrossing or selfing strategies (Lande and Schemske, 1985). However, mixed-mating strategies are often observed (Vogler and Kalisz, 2001), and may result from the combination of both genetic and many environmental parameters exerting selection on mating systems (Waller, 1986; Uyenoyama *et al*, 1993). In particular, population density may be a key parameter determining mating systems: high density may increase outcrossing probabilities (Wright, 1946; Baker, 1967), and may enhance inbreeding depression (Schmitt and Ehrhardt, 1990). Population substructure may also affect mating systems through increasing mating among relatives (Ennos and Clegg, 1982; Uyenoyama, 1986; Ronfort and Couvet, 1995). Density may interact with population substructure, sometimes increasing biparental inbreeding (Ellstrand *et al*, 1978), although the reverse pattern may also be found: low density may also increase population structure and inbreeding (Williams, 1994; Gehring and Delph, 1999).

Mating system studies are common in the plant kingdom (for a review, see Schemske and Lande, 1985; Vogler and Kalisz, 2001). In contrast, such studies in animals are rather scarce (for a review, see Jarne and Charlesworth, 1993). They concern mainly freshwater snails, which are mostly hermaphroditic (review in Städler *et al*, 1995; Coutellec-Vreto *et al*, 1997; Städler and Jarne, 1997; Viard *et al* (1996, 1997), but see also Carlon, (1999) on corals). The hermaphroditic freshwater snail *Lymnaea truncatula* is the main intermediate host of the Trematode *Fasciola hepatica*, the liver fluke, and other Trematodes (Abrous *et al*, 1999; Abrous *et al*, 2000). *L. truncatula* populations frequently inhabit temporary ponds and ditches (eg Roberts, 1950; Morel-Vareille, 1973) and may be subjected to density variation associated with flooding and droughts, leading to bottlenecks and recolonisation events. As shown above,

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these density variations might be of particular relevance to mating system features.

Previous observations (Roberts, 1950; Kendall, 1953) reported the absence of copulation in *L. truncatula*. However, Morel-Vareille (1973) and Smith (1981) observed copulations in snails reared in the laboratory. Population genetics studies (Jabbour-Zahab *et al*, 1997; Meunier *et al*, 2001; Trouvé *et al*, 2003) suggest that this snail shows a predominantly selfing mode of reproduction, in contrast to other, closely related, lymnaeid snails, such as *L. peregra* (Bargues and Mas-Coma, 1997; Coutellec-Vreto *et al*, 1997). However, population genetics studies do not allow us to disentangle the effects of nonrandom mating and population substructure to account for high inbreeding values, or to take individual variation in the selfing rate into account.

Thus, to ascertain the mode of reproduction of *L. truncatula*, together with studying its variability within populations, we used three approaches: (i) inbreeding levels were estimated by a population genetic survey (ii) outcrossing rates were experimentally inferred using controlled pairings, and finally, (iii), parent–offspring comparisons allowed us to test for density and/or stress effects on the mating system.

Materials and methods

Study species

L. truncatula (or *Galba truncatula* Müller) is a pulmonate snail, living in wet pastures, ditch margins or any depression in the ground storing water, and more rarely on river banks (Morel-Vareille, 1973; Smith, 1981). Pulmonate snails have been shown to be able to store sperm for an average of 2 months (see Cain (1956) for *Lymnaea stagnalis*; Vianey-Liaud (1991) for *Biomphalaria glabrata*; and Wethington and Dillon (1991) for *Physa heterostropha*). Owing to sperm storage, the progeny of single isolated 'mothers' reflects past copulations and/or selfing.

Study sites

Adult snails, of more than 4 mm in size, were collected in two populations, hereafter referred to as Adriers and Migné, in Central France (departments of Vienne and Indre, respectively). The following coordinates of these two locations are given in the Lambert conic conformal projection system (zone II): $X = 484.9$ km, $Y = 2144.375$ km (Adriers) and $X = 525.15$ km, $Y = 2186.575$ km (Migné). The two locations are at a distance of ca. 70 km from each other. Both are side-road ditches, which frequently dry out in the summer (DR personal observation). In each population, sampling was made at three different dates: November 1998, April 2000 and November 2000. All individuals were sampled at a small scale (ca. 30 m along the ditch).

Microsatellite loci and analysis

DNA extraction methods, microsatellite loci and genotyping have already been described elsewhere (see Trouvé *et al*, 2000). The same three loci are used throughout all studies below (Genbank accession number AF226976, AF226980, AF226985). We chose these loci since they are the only variable ones in the populations studied. The Mendelian inheritance of the microsatellite loci was checked. The observed proportions of genotypes were compared to the expected Mendelian proportions, using a multinomial-law based test (M Raymond, personal communication).

The three approaches described below (population genetics, breeding and density experiments) are also summarised in Figure 1.

Population genetics study of the mating system

In each sample, Wright's inbreeding coefficient (F_{IS}) was computed, using FSTAT 2.9.3. (Goudet, 1995; Goudet *et al*, 1996), which provides Weir and Cockerham's (Weir and Cockerham, 1984) unbiased estimator f. The outcrossing rates (t) of each population were computed from the F_{IS} values according to the formula: $F_{IS} = 1 - t/1 + t$, which assumes inbreeding equilibrium (Crow and Kimura, 1970).

Breeding experiment

Snail pairing: We raised a laboratory generation of *L. truncatula* in order to obtain virgin snails and breed them. We chose to conduct within-population pairings to avoid biases due to a possible reproductive isolation between allopatric snails (see Städler *et al*, 1993; Trouvé *et al*, 1998),

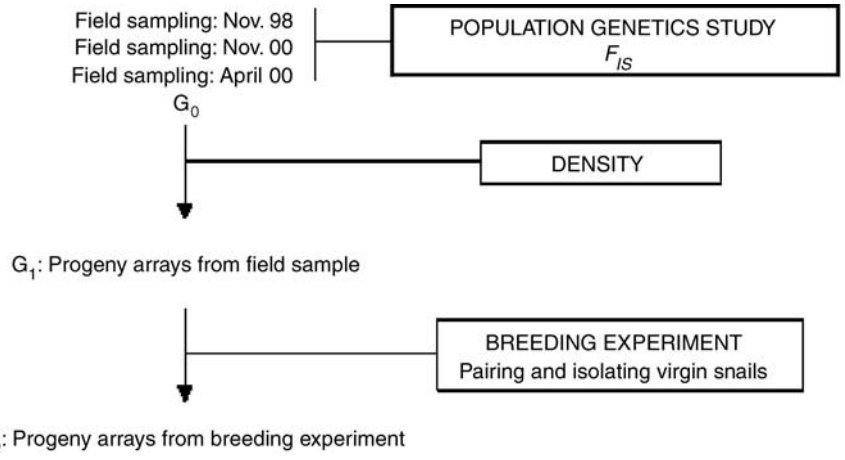

Figure 1 Experimental design.

even if this procedure increases genetic relatedness between parents. 'Mothers' (G_0 snails) from both populations were sampled in the field in April 2000 and allowed to lay eggs (G_1 snails) (Figure 1). All G_1 snails were isolated when their size reached 2 mm – sexual maturity has never been reported for snails smaller than 4 mm (Smith, 1981). Before pairing the G_1 snails, we genotyped the G_0 'mothers': this allowed us to partially control the crosses for genotype. We selected six G_0 founder snails in Adriers showing allelic variability. In Migné, the low genetic variability allowed us to select only four G_0 snails to found genotypically different G_1 families. From these G_0 snails, we obtained a number of virgin G_1 snails in each population. These snails were used to conduct six intrapopulation crosses in each population. The paired snails were stored in 70% ethanol, either after having laid at least a total of 20 eggs, or after 3 months of rearing. It was impossible to control the parental origin of egg clutches, as we kept the paired snails together throughout the experiment to increase mating probabilities. Parents (G_1) and a minimum of 20 hatchlings (G_2) were genotyped at the three microsatellite loci (Figure 1).

In the population of Adriers only, the existence of diagnostic loci (parents homozygous for different alleles) allowed a direct calculation of an outcrossing rate for each cross (counts of heterozygous progeny at the diagnostic loci).

Snail isolation: In order to test for the ability of *L. truncatula* to self-fertilise, we isolated at least one snail from each G_1 'family' used in the pair crosses, before sexual maturity (2 mm): 10 G_1 snails were isolated in Adriers and 13 G_1 snails in Migné. While isolated, the snails were allowed to grow and lay eggs during a period of 3 months (June–August 2000). Their progeny was removed just after hatching and stored in 70% ethanol. At the end of the experiment, one progeny was chosen at random from each population. A total of 20 hatchlings per progeny were genotyped and compared to their parental genotypes at the three microsatellite loci. Only one progeny was chosen to check for the validity of the chosen threshold size for isolation. If these progeny genotypes are in conformity with their parents' genotypes, the virginity of the isolated snail is confirmed.

Density experiment

In all, 50 mature snails per population were collected in April 2000. The snails from each population were kept together for 48 h in separated plastic boxes of 100 ml. Since densities have been estimated to reach 20 snails/m^2 in the field (Vareille-Morel *et al*, 1999), these experimental conditions represent higher densities than in the field. Then the snails were isolated in the laboratory in order to obtain individual egg clutches. No low-density control for this experiment was performed, as we consider that F_{IS} estimates should represent the average outcrossing rate in field density conditions (see also Figure 1).

After having laid at least 20 eggs, the mothers were stored in 70% ethanol before genotyping at the three microsatellite loci. Egg clutches were allowed to develop until hatching. The progeny snails of the different families were assayed at the same three microsatellite loci. Table 3 describes the number of families and progeny per family studied in the two populations.

Outcrossing rates were estimated using the multilocus mating system programme (MLTR, Ritland and Jain, 1981). This maximum likelihood-based programme estimates outcrossing rates from progeny-arrays assayed for markers, under the mixed mating model. We used the 'expectation-maximisation' (EM) method available in MLTR for the recursions because this method is more suited for highly inbred species. All variances of the estimates were calculated using 500 bootstraps. The MLTR programme allows the estimation of (i) population level outcrossing rates and (ii) family-level outcrossing rates.

(i) Multilocus outcrossing rates were computed in the population of Adriers. We estimated the paternal allele frequencies jointly with maternal ones, allowing them to differ. In the population of Migné, due to the absence of variability at two loci, we estimated a single-locus outcrossing rate. This probably leads to an underestimation of the outcrossing rate, because of biases due to biparental inbreeding (Ritland and Jain, 1981). In Migné, the outcrossing rate was also estimated with paternal allele frequencies constrained to maternal ones, because the lack of genetic variability may impede the joint estimate of many parameters. This is a conservative method as the outcrossing rate dropped down again when constraining paternal allele frequencies.

(ii) Family outcrossing rates were also estimated with the MLTR programme. The differences in outcrossing rates among families were tested using a G-log-likelihood ratio (see Sokal and Rohlf, 1995). The following parameters were computed:

- sum of the observed probabilities of outcrossing and selfing in one family (given by the family-level outcrossing estimate)
- and sum of their expected probabilities (given by the population-level outcrossing estimate).
- The likelihood of the departure from the expectations, L, is given by the ratio of these two sums, for one family. For all families, L is the product of all ratios. $G = 2 \ln L$ follows a χ^2distribution with (no. of family-1) d.f.

We computed a 99% confidence interval for the outcrossing rates obtained with the progeny-array analysis method, using the variance of the estimates computed with MLTR, and assuming that the outcrossing rate follows a Student-t distribution with (no. of family-1) d.f. We compared this confidence interval to the point estimates of outcrossing rates derived from the F_{IS} values.

Results

Mendelian transmission of the microsatellite loci

The Mendelian transmission of the three loci studied is confirmed (data available upon request), which allows us to analyse the following results.

Population genetic study of the mating system

Table 1 summarises the population genetics features of the snail populations. The number of alleles within populations is low: among the three loci analysed, the population of Migné has only one variable locus at two of the three sampling dates. The F_{IS} estimates and

Table 1 Population genetics study of the mating system of *L. truncatula*

Population *Date of sampling*	*Adriers* *November 1998*	*Adriers* *April 2000*	*Adriers* *November 2000*	*Migné* *November 1998*	*Migné* *April 2000*	*Migné* *November 2000*
Sample size	30	31	23	20	30	21
No. of variable loci	3	3	1	3	1	1
Mean no. of alleles per variable locus	2	2	2	2	2	2
F_{IS} all loci	0.928*	0.663*	1.000	0.933*	0.914*	1.000*
t_f	0.072	0.203	0	0.035	0.045	0

No. of variable loci: number of variable loci; mean no. of alleles per variable locus: mean number of alleles per variable locus; t_f: outcrossing rate computed from the F_{IS} values, $t_f = 1-s$, see Materials and methods. Significance of F_{IS} values: $^*P < 0.05$.

outcrossing rates for the two populations of snails at the three different dates are also given in Table 1. All F_{IS} estimates reach high values (0.66–1.00). As a consequence, the outcrossing rates derived from the F_{IS} estimates, that is averaged over different generations of snails and over all families of snails, are extremely low (range 0–0.203). In the population of Adriers, we note a lower F_{IS} value in April 00, whereas F_{IS} values in the population of Migné seem stable.

Breeding experiment

Snail isolation: Of the 13 snails isolated from Migné, 11 laid eggs, and three out of 10 for Adriers. The progeny from two isolated snails, one in each population, were analysed with the microsatellite markers. None of these isolated snails produced unexpected (ie outcrossed) offspring. We are therefore confident in the virginity of the snails used for self-fertility tests and for pairing.

Snail pairing: We report here the results for the three pairings that could be analysed, that is with parents of different genotypes (this was the case in Adriers only). Table 2 refers to the proportions of genotypes obtained in the progeny, given the parental genotypes. The progeny showed very low numbers of outcrossed descendants (see Table 2). From these results we conclude that these snails predominantly selfed when paired.

Density experiment

Population outcrossing rates: The estimates of the outcrossing rates are given in Table 3. The range of the outcrossing rates (0.232–0.389) indicates a mixed mating to a predominant selfing mode of reproduction for *L. truncatula*. For the population of Migné, the progeny-arrays outcrossing rates are unexpectedly high as compared to the outcrossing rates estimated from the F_{IS} values. The difference is significant at the 1% level. The point estimate of the outcrossing rate derived from the F_{IS} value in April 2000 ($t_f = 0.045$) lies outside the 99% confidence interval of the outcrossing estimates of the progeny-arrays (see Table 3). In Adriers, there is no significant difference between the outcrossing rates estimated in the two consecutive generations.

Family outcrossing rates: There are strong and significant differences in outcrossing rates among families: some families either completely self or completely outcross. However, complete outcrossing is rare (Table 4).

Table 2 Results of the breeding experiment

Cross name	*Locus*	*Parental genotypes*		*Offspring genotypes*	*No. offspring of given genotype*
		P#1	*P#2*		
Adriers Cross #1	1	114/116	114/114	114/116	10
				114/114	9
				116/116	1
	2	204/212	212/212	204/212	13
				212/212	4
				204/204	3
	3	117/117	122/122	117/122	1
				122/122	1
				117/117	18
t		0.05			
Adriers Cross #2	1	116/116	114/114	114/116	0
				114/114	4
				116/116	28
t		0			
Adriers Cross #3	1	114/116	114/114	114/116	20
				114/114	7
				116/116	5
	2	204/212	204/204	204/212	15
				204/204	11
				212/212	6
	3	117/117	122/122	117/122	0
				117/117	29
				122/122	3
t		0			

Genotypes given are allele sizes (in base pairs). Only loci are shown where parents have different genotypes. Offspring genotypes: expected genotypes in the progeny, given the parental ones, and given the parent's ability to either self or outcross. No. offspring of given genotype: observed number of offspring of a given expected genotype. *t*: outcrossing rate calculated from the comparison between offspring and parental genotypes (at diagnostic loci).

Discussion

A selfing syndrome in the field

The F_{IS} estimated in the field populations reached very high and significant values. These extensive heterozygote deficiencies are consistent with those previously found with more loci in the same locations (see Meunier *et al*, 2001; Meunier *et al*, unpublished data). Other studies conducted in Switzerland on the same species (Trouvé *et al*, 2003) have also shown high F_{IS} values for this snail (range 0.27–0.97). We are therefore confident

Table 3 Progeny-arrays analysis: population-level outcrossing rates

	Adriers	*Migné*
No. families	25	18
No.progeny	231	146
No.variable loci	3	1
t_m (SD)	0.232 (0.094)	NA
t_s (SD)	0.254 (0.114)	0.389 (0.166)
99% CI	[0.179;0.284]	[0.275;0.503]
t_f	0.203	0.045

No. families, no. progeny, no. variable loci: number of families, progeny assayed in both populations, and number of variable loci available for analysis. t_m: multilocus outcrossing rate inferred with the MLTR programme. t_s: single locus outcrossing rate. SD: standard deviation. 99% CI: 99% confidence interval for the outcrossing rates (see Materials and methods), computed for t_m (Adriers) and t_s (Migné). t_f: outcrossing rate estimated from the F_{IS} values.

Table 4 Progeny-arrays analysis: family-level outcrossing rates

	Adriers		*Migné*	
	Family	t_m	*Family*	t_s
	#1	1	#1	0.75
	#2	0.97	#2	0.68
	#3	0.52	#3	0.46
	#4	0.38	#4	0.38
	#5	0.36	#5	0.1
	#6	0.25	(13 families)	0
	#7	0.19		
	#8	0.13		
	#9	0.12		
	(16 families)	0		
G-test	$P<0.001$		$P<0.001$	

Only outcrossing families are detailed. t_m: multilocus outcrossing rate, and t_s: single-locus outcrossing rate, inferred with MLTR. *G*-test: *G*-log likelihood ratio test for the homogeneity of outcrossing rates among families.

that the small number of loci used in this study did not bring out any bias in the estimated F_{IS} values.

Such high heterozygote deficiencies are likely to arise through (i) high selfing rates in the populations and/or (ii) large Wahlund effects.

(i) Selfing directly reduces the heterozygosity in populations, and, as such, is the most obvious explanation for deviations from the Hardy–Weinberg equilibrium (HWE) in hermaphroditic species.

(ii) Heterozygote deficiencies may also arise because of Wahlund effects, if samples include two or more structured populations (Hartl and Clark, 1997). This may occur because of an inappropriate sampling scale. However, both populations sampled here were collected at a small scale (30 m along a ditch). If the high F_{IS} estimates we obtain were due to sampling biases, we should then conclude a very high substructuring of our snail populations, with very low rates of dispersal. Such Wahlund effects may also be caused by events of extinction/recolonisation of the snail populations. For example, if population substructure arises because of recolonizing snails coming from two unrelated populations, Wahlund effects may be only slowly reduced by subsequent matings, especially if selfing is common. Such events of extinction/recolonisation have been reported in surveys of *L. truncatula* population dynamics in the field (Roberts, 1950; Smith, 1981), and genetic evidence for a high intensity of drift and small N_e values in the snail populations also exist (Meunier *et al*, unpublished data).

Population dynamics and mating system may then both contribute to heterozygote deficiencies in our study populations. However, another study (Trouvé *et al*, 2003) surveyed more stable populations of *L. truncatula* (permanent habitats) and found very similar HWE deviations. Thus, it seems likely that selfing may contribute more to the high F_{IS} values obtained than does population substructure. The F_{IS} estimates themselves show a temporal variation in one of the two study populations (see Adriers, Table 1). Some populations may be far from an adaptive inbreeding equilibrium, as the amount of inbreeding seems to vary markedly over time. Disturbances to the environment, for example, those creating changes in density, might change the realised outcrossing rate.

Self-compatibility assessed in the laboratory

Self-compatibility has been assessed in the laboratory in isolating young snails before sexual maturity. These snails laid fertile eggs, which clearly proves the ability of *L. truncatula* to self. The snails that did not lay eggs while isolated may reflect (i) either cases of autosterility or, more probably, (ii) discrepancies in the delay to reach sexual maturity among snails, as the experiment was stopped after 3 months. In the genus *Physa*, sexual maturity has been proven to be delayed when snails are isolated (Wethington and Dillon, 1993; Tsitrone, 2003).

Our results showing self-fertilisation in *L. truncatula* are consistent with previous reports of self-compatibility in this snail reared in the laboratory (Roberts, 1950; Kendall, 1953). Furthermore, the breeding experiment seems to show a preference for selfing, as, at least in the population of Adriers, events of outcrossing are scarce, even if a sexually mature partner is available (see Table 2). However, in most experimental pairings, there was seemingly a trend for unequal progeny sizes of the parents (see Table 2). A bias due to differential maturation rate (and hence ability to reproduce) could have impeded copulation events between the snails, even if it was certain that both snails had reached sexual maturity at the end of the experiment (see Table 2). Therefore, we cannot rule out the possibility of having missed some outcrossing events, especially if the snails start selfing before outcrossing. However, similar breeding experiments in hermaphroditic snails have shown that outcrossed eggs are laid within 2 days when snails copulate, and that allosperm is used before autosperm (Paraense, 1955; Cain, 1956; Vianey-Liaud, 1991).

Thus, both the population genetics approach and the experimental analysis strongly suggest that *L. truncatula* is a preferential selfer.

Is there ability to outcross in special conditions?

Family-level estimated outcrossing rates: The progeny-array analysis showed an important and significant variance in the mating behaviour of different snails in the same population, giving thus additional information compared to F_{IS} estimates. This might indicate that the mating system may potentially evolve in the snail

populations, unless those differences in mating behaviour are entirely due to environmental effects.

Population-level estimated outcrossing rates: We submitted snails from the two localities to conditions of high density for a short time (48 h), allowing them to easily find sexual partners, and/or submitting them to stress. When analysing their progeny, we estimated an outcrossing rate consistent with the one derived from the F_{IS} estimate of the parents' sample, in the population of Adriers. However, in the population of Migné, we found more outcrossing after exposure of the snails to high densities. To explain this last result, we may hypothesise (i) that the difference observed is not caused by density exposure or conversely, (ii) that the experimental design did have an effect on the mating behaviour of the snails.

(i) *No density effect*. Progeny-array studies of mating systems usually show more inbreeding in the young progeny than in adults in the field and conclude the existence of inbreeding depression acting at a later stage (Godt and Hamrick, 1991; Morgante *et al*, 1991). Conversely, the young progeny seem here to be more outbred than the adult generation, suggesting outbreeding depression (ie selection against heterozygotes) occurring in the population of Migné. Evidence for outbreeding depression is usually found between distantly related populations, when crosses disrupt allelic coadaptations (Trouvé *et al*, 1998; Waser *et al*, 2000). The finding of outbreeding depression inside a single population seems thus unlikely, unless outcrosses are enforced in quasi-exclusive selfers showing very reduced gene dispersal and very high population substructure *in natura* (Parker, 1992; Quilichini *et al*, 2001). These lines of arguments seem to point to the alternative hypothesis of an effect of the density exposure on the snails.

(ii) *A density effect*? The experiment may have enhanced outcrosses in the Migné population. Negative effects of density on the apparent outcrossing rate have already been reported in plants (Ellstrand *et al*, 1978; Lu, 2000), which were interpreted as increased biparental inbreeding at high densities, resulting from reduced pollinator flight distances. Other empirical approaches have shown a positive effect of density on outcrossing rates (see Franceschinelli and Bawa (2000) in plants). In our study, density variations may be very likely to occur because of the instability of the habitat: differences in water availability through the seasons might induce successive increases and decreases in snail density. Our results suggest that the snail mixed-mating system may respond to density variations.

Other environmentally induced plasticity in mating strategies has already been shown to occur (see Trouvé *et al* (1999) in Trematodes; Waller (1980) in plants). Ecological parameters may then be of equal importance as genetics in shaping mating systems *in natura*. This leads to a very complex picture for mating system evolution: costs and benefits of mating strategies may vary widely according to environmental parameters, rendering predictions for the outcome of selection on the mating system more difficult.

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