

# Does life in unstable environments favour facultative selfing? A case study in the freshwater snail *Drepanotrema depressissimum* (Basommatophora: Planorbidae)

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Received: 19 May 2011 / Accepted: 22 August 2011 / Published online: 30 August 2011  
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**Abstract** One of the main advantages of self-fertilization is to provide reproductive assurance when pollen or mates are scarce. In plants, partial or facultative selfing limits the risk of pollination failure. In preferentially outcrossing species, this may result in mixed-mating. In hermaphroditic animals, recent studies suggest that mixed mating might be much rarer than in plants. However more studies are required to substantiate this claim, especially focusing on species whose lifestyle entails a high potential benefit of reproductive assurance via selfing. We studied a hermaphroditic snail, *Drepanotrema depressissimum*, which inhabits very unstable and fragmented freshwater habitats. Individuals often have to recolonize newly refilled ponds after long droughts, a situation of low population density and hence low mate availability in which selfing could be an advantage. We estimated selfing rates in natural populations from Guadeloupe (Lesser Antilles), and used laboratory experiments to characterize the reproductive behaviour and success of individuals with or without mates. We detected no sign of selfing in natural populations. Even when given no other option, isolated individuals were extremely reluctant to self. They produced either no or very small clutches, and in the latter case initiated egg-laying later than non-isolated individuals. Self-fertilized clutches suffered near-total (98%) inbreeding depression at the juvenile stage. The example of *D. depressissimum* therefore shows that a species can overcome periods of mate shortage and habitat instability without the potential to rely on facultative selfing. We hypothesize that metapopulation persistence in this landscape is probably related to a form of dormancy (aestivation in dry ground) rather than to recolonization by rare immigrants and reproductive assurance.

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**Electronic supplementary material** The online version of this article (doi:[10.1007/s10682-011-9520-8](https://doi.org/10.1007/s10682-011-9520-8)) contains supplementary material, which is available to authorized users.

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**Keywords** Life-history traits · Mating system · Waiting time · Selfing rate · Molluscs · Double-copulation

## Introduction

The evolution of the selfing rate in simultaneous hermaphrodites is a central issue in evolutionary biology. While the U-shaped distribution of selfing rates in plants has suggested that extreme selfing and extreme outcrossing were more evolutionary stable than mixed mating (Schemske and Lande 1985), the addition of new data has recently revealed a more substantial fraction of intermediate (0.3–0.7) selfing rates than previously thought (Vogler and Kalisz 2001; Goodwillie et al. 2005). Until recently, the distribution of selfing rate in animals was thought to be similar to that in plants, with an even higher fraction of mixed-maters (Jarne and Auld 2006). This idea has been challenged recently by Escobar et al. (2011) who argued that the selfing rates compiled by Jarne and Auld (2006) were partly compromised by methodological problems. Indeed, most of them rely on  $F_{IS}$  estimates, which are very sensitive to technical artefacts such as null alleles, and consequently overestimate selfing rates (Jarne and David 2008). A multilocus method insensitive to technical artefacts has been recently developed (David et al. 2007) but has not yet been widely used. In contrast to Jarne and Auld (2006), the data compiled by Escobar et al. (2011), based on this method, indicated a lack of mixed-maters (Escobar et al. 2011) in a set of hermaphroditic snails, the most represented group in the dataset of Jarne and Auld (2006). However, the sample size of Escobar et al. (2011) is too small (11 species), and we definitely need robust estimates in more species.

In contrast to animals, estimates of selfing rates in plants are not compromised by technical artefacts because they mostly rely on progeny-arrays, which are more robust than  $F_{IS}$ -based estimates (Jarne and David 2008). The significant frequency of mixed-mating in plants has mostly been discussed as a response to variation in environmental conditions, especially in metapopulations with frequent extinction-colonization cycles, where new populations must be founded by dispersing seeds (Pannell and Barrett 1998) and more generally to pollination uncertainty (Goodwillie et al. 2005; Cheptou and Massol 2009). By analogy, mixed-mating could be expected in animal species living in fragmented and fluctuating habitats with extinction-colonization dynamics or frequent population crashes. In such contexts, the possibility to self-fertilize eggs could provide reproductive assurance (Baker 1955), even in species where outcrossing is favoured under conditions of high mate/pollen availability. Thus, a variety of mating strategies can emerge between the two extreme strategies of obligate outcrossing and preferential selfing (Table 1). Among intermediate strategies, prior mixed-maters unconditionally self-fertilize a given fraction of their eggs, while occasional mixed-maters only rely on self-fertilization under specific conditions (mate or pollen scarcity) (Table 1). To place one species in this classification, we need not only good estimates of selfing rates in natural populations, but also information on how individuals react to changes in mate or pollen availability (Table 1).

In this study we focused on a hermaphroditic snail that lives in very unstable and fragmented habitats exposed to periodic population crashes (small tropical ponds that frequently dry out). Several strategies may facilitate species persistence in such conditions. Adult individuals can stay alive for some time in the dry ground (aestivation, Pointier and Combes 1976), which could be a way to avoid extinction and limit potential effects of mate shortage (as for seed dormancy in plants). However, self-fertilization could also be useful

**Table 1** A classification of mating systems in hermaphroditic animals (or plants, excluding the last line on copulation frequency) in relation to self-fertilization and expectations for several traits strongly associated to the evolution of the mating system (Selfing rate, reproductive output, inbreeding depression and copulation frequency)

	Type A	Type B	Type C	Type D
Mating system	Obligate outcrosser	Preferential outcrosser/ occasional mixed-mater	Prior mixed-mater	Preferential selfer
Definition	Practically no viable selfed offspring	Selfing occurs only under specific conditions (i.e., mate scarcity)	Some intermediate degree of unconditional selfing (prior selfing) usually occurs	Most eggs (80–100%) are selfed in all circumstances
Traits				
Selfing rates in random samples from natural populations	Always null	Usually null, sometimes positive	Always positive	Always close to 1
Reduction in reproductive output in the absence of mates	Very strong or total	Moderate	None or moderate	None
Inbreeding depression	High or total (= 1)	High	Intermediate	Low
Copulation frequency in the presence of mates	High	High	Intermediate	Low

when population density is very low (e.g., following recolonization by immigrants after local extinction when it occurs). We explored this possibility by investigating the mating strategy of this species. To this end, we sample natural populations and obtain robust molecular estimates of selfing rates. We then investigate the response of this species to situations of low mate availability. To this aim we quantify (1) how reluctant to self-fertilization individuals are in such situations; (2) how efficient self-fertilization is at producing viable offspring. Point (1) can be approached by studying experimentally how reproductive behaviour and reproductive output vary in response to mate availability while point (2) requires quantifying inbreeding depression.

Traits associated with different mating systems, and especially the negative correlation between inbreeding depression and selfing rate, are well-known in plants (Husband and Schemske 1996; Kalisz et al. 1999; Barrett et al. 2000; Stoeckel et al. 2008; Goodwillie et al. 2010) but less so in animals. However Escobar et al. (2011) have recently identified strong associations between the selfing rate, inbreeding depression, and the “waiting time” in animals. The waiting time is typical of predominant outcrossers, and refers to a tendency of individuals to delay their age at first reproduction when they have no mating opportunities (Tsitrone et al. 2003a; Escobar et al. 2009, 2011). Escobar et al. (2011) have compiled data on 22 pulmonate species (plus 3 other hermaphroditic animals) and concluded that most species fall into three “syndromes”: the first combined very high or total inbreeding depression, no selfing in natural populations, and very long waiting times (type A in Table 1); the second associated moderate inbreeding depression, rare selfing in the wild, and moderate waiting times (type B); and the third was characterized by very little inbreeding depression, high selfing rates and no waiting time (type D). Copulation frequency has also been suggested to correlate with the outcrossing rate (Doums et al. 1996, Tian-Bi et al. 2008). However very few studies simultaneously report behavioural traits (copulation frequency), life-history traits (waiting time), estimates of inbreeding depression and robust estimates of selfing rates. Our study illustrates how a combination of experimental, behavioural and molecular data can be used to place a species in the continuum of mating system strategies that exist (Table 1). Our expectation is that the lifestyle of the species studied here (*Drepanotrema depressissimum*) should encourage some degree of self-fertilization (types B, C or D; Table 1).

## Materials and methods

### Study species and sampling

*Drepanotrema depressissimum* is a hermaphroditic freshwater snail (Gastropoda: Basommatophora: Planorbidae) with a Neotropical distribution, mostly found in small tropical ponds with abundant vegetation (Pointier 2008). Our study was conducted in the Grande-Terre of Guadeloupe, Lesser Antilles where *D. depressissimum* is common. In this region the species inhabits a network of fragmented water bodies, and is mostly found in ponds that occasionally or regularly dry out (for periods of a few days to several months) during the dry season (December to June). Even when found in permanent water bodies, *D. depressissimum* is often more abundant on the ephemeral periphery of the habitat (puddles on pond or river margins temporarily flooded during the rainy season). Snail density show rapid fluctuation in time, with an alternation of long periods during which the species is not detected and fast development of dense populations (Pointier and Combes 1976; Pointier and David 2004; Pointier 2008). Individuals were collected at seven localities in 2007 or

**Table 2** Characteristics of seven populations of *Drepanotrema depressissimum* sampled in Guadeloupe

	Blonval	Poucet	Desbonnes	Geffrier	Pico	Grosse Roche	Sainte Rose	Mean
Coordinates	N 16°15' 41" W 61°19' 31"	N 16°13' 24" W 61°30' 34"	N 16°29' 12" W 61°27' 49"	N 16°19' 62" W 61°29' 92"	N 16°21' 11" W 61°27' 42"	N 16°14' 94" W 61°26' 27"	N 16°16' 74" W 61°20'93"	– –
Sampling year	2009	2009	2009	2009	2007	2007	2009	–
Waterbody	Temporary pond	Permanent canal	Ditch (to pond)	Permanent mangrove	Permanent pond	Temporary pond	Permanent pond	–
n	29	30	31	30	28	32	23	203
$N_a$	14.0	16.8	7.0	16.7	13.5	16.4	7.1	13.1
$H_o$	0.798	0.848	0.670	0.854	0.858	0.750	0.660	0.777
$H_e$	0.895	0.909	0.740	0.906	0.914	0.828	0.760	0.850
$f$	0.108	0.066	0.092	0.058	0.065	0.094	0.100	0.081
$s(f)$	0.195	0.124	0.168	0.109	0.122	0.172	0.182	0.153
$P(f = 0)$	0	0	0.04	0.01	0	0.02	0	–
$s(g_2)$	0	0	0	0.01	0.027	0.004	0.004	0
$P(g_2 = 0)$	0.543	0.555	0.879	0.173	0.117	0.372	0.415	–
$s'$ (ML)	0.036	0	0	0	0	0	0	0.000 <sup>a</sup>
CI95	0–0.103	0–0.040	0–0.063	0–0.034	0–0.049	0–0.074	0–0.077	0–0.014 <sup>a</sup>
$P(s'(ML) = 0)$	0.18	0.5	0.5	0.5	0.5	0.5	0.5	0.50 <sup>a</sup>

Geographic coordinates are in WGS 84.  $n$  is the sample size,  $N_a$  the mean number of alleles,  $H_e$  the gene diversity,  $H_o$  the observed heterozygosity, and  $f$  the inbreeding coefficient

We also provide the  $P$ -value for the null hypothesis  $f = 0$  based on 10,000 permutations. Estimates of the selfing rate ( $s'$ ) are based on the inbreeding coefficient ( $s(f)$ ), on  $g_2$  ( $s(g_2)$ ) and on a maximum-likelihood method ( $s'(ML)$ ); with CI95 the confidence interval at 95%. The  $P$ -values associated to the null hypotheses  $f = 0$  or  $s' = 0$  are also given

<sup>a</sup> Estimates using the ML method assuming that all populations have the same selfing rate

2009 (minimum sample size = 23 individuals) spanning a range of habitats (Table 2). Snails were killed in 70°C water for 1 min and preserved in 95% ethanol prior to genetic analysis.

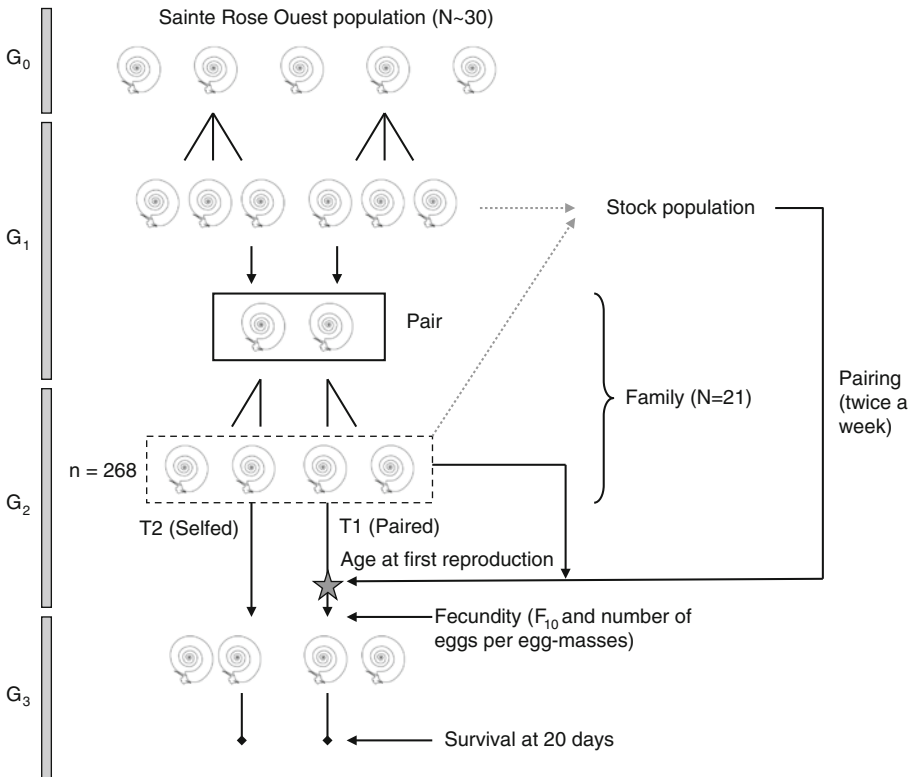
### Population genetic analysis

DNA was extracted using a Chelex<sup>®</sup> method (BIORAD). Genotypes were obtained at 12 polymorphic microsatellite loci (Supplementary Table S1; Nicot et al. 2009) based on three multiplex amplifications. PCRs were conducted in 10 µL final volume including 1 µL of primers (2 µM), 5 µL of QIAGEN multiplex PCR kit (QIAGEN, Inc.), 3 µL of water and 1 µL of genomic DNA (1/10 dilution). The PCR conditions were as in Nicot et al. (2009). Three micro litre of diluted amplicon from each multiplex amplification were pooled with 15 µL of de-ionized formamide and 0.2 µL GeneScan-500 LIZ Size Standard, and analysed on an ABI PRISM 3100 Genetic Analyser.

The number of polymorphic loci, number of alleles per locus ( $N_a$ ), observed heterozygosity ( $H_o$ ) and gene diversity ( $H_e$ , corrected for small sample size; Nei 1987) were computed for all populations and loci. Deviations of genotypic frequencies from Hardy–Weinberg equilibrium (HWE) at each locus and deviation from genotypic equilibrium between locus pairs were tested in each population using exact tests (Raymond and Rousset 1995a). Departures from HWE over all loci were tested using Fisher’s method for combining probabilities (Sokal and Rohlf 1995). Significant deviations from HWE were further investigated with specific tests for global heterozygote deficiency or excess. All calculations and tests were performed using Genepop version 4.0.9 (Raymond and Rousset 1995b) and Genetix 4.05.2 (Belkhir et al. 2000). Wright’s inbreeding coefficient  $F_{IS}$  was estimated using Weir and Cockerham (1984)’s estimator,  $f$ . Its significance was assessed using a permutation method (10,000 permutations) implemented in Genetix 4.05.2 (Belkhir et al. 2000). The selfing rate ( $\hat{s}$ ) was estimated using the two multilocus methods implemented in the software RMES (Robust Multilocus Estimates of Selfing, David et al. 2007). As these methods do not make use of heterozygote deficiencies and rely only on the correlations in heterozygosity among loci, they are not sensitive to null alleles and mis-scoring. Indeed scoring errors may reduce apparent heterozygosity at some loci, but only selfing produces individuals that tend to be homozygous at all loci at the same time. The first multilocus method is a point estimation based on  $g_2$ , the two-locus heterozygosity disequilibrium; the second is based on direct maximization of the likelihood of the multilocus heterozygosity distribution (David et al. 2007). We also provide estimates based on the relationship  $\hat{s}(f) = 2f/(1 + f)$  known to be sensitive to scoring errors (especially null alleles; see Jarne and David 2008) for comparison.

### Copulatory behaviour and response to mate availability in controlled conditions

We set up a quantitative genetic experiment so as to estimate three important parameters, namely (1) the frequency of copulations when mates are available (2) the changes in female reproductive investment when mates are lacking, quantified through the waiting time,  $\Delta_{\max}$  (see below) and differences in egg output between treatments, and (3) apparent inbreeding depression. We collected live individuals from one of the sampled populations (Sainte Rose Ouest). Thirty mature individuals ( $G_0$  snails) were brought back to the laboratory in Montpellier, France (Fig. 1). To minimize potential effects of the initial population state (environment, initial inbreeding) we used second-generation ( $G_2$ ) offspring obtained from paired  $G_1$  individuals.  $G_0$  adults were kept in groups in 150 mL plastic



**Fig. 1** Schematic diagram of the experimental protocol.  $G_i$  refers to generations. The waiting time was measured in  $G_2$  individuals as the difference in age at first reproduction between paired and selfed treatments. The copulatory behaviour was monitored in the paired treatment twice a week. The life-history traits measured were fecundity in  $G_2$  individuals and survival at 20 days in  $G_3$  individuals

boxes and allowed to lay eggs. We collected forty-four offspring ( $G_1$ ) and raised them in pairs of virgin individuals from different families in 75 mL boxes from which egg capsules were collected daily. The day on which egg-laying occurred was taken as age 0 for  $G_2$  individuals.  $G_2$  offspring of a given pair of  $G_1$  individuals will be referred to as a family. From each family we isolated approximately 10  $G_2$  offspring, half of which were attributed to experimental treatment 1 (i.e., paired) while the other was to treatment 2 (i.e., selfed, see below). The remaining offspring were grown collectively in two tanks, all members of a given family being maintained in the same tank, and marked with a dot of car paint. They will be referred to as stock populations. Throughout the experiments, snails were maintained at 25°C under a 12:12 photoperiod regime, and fed with finely-ground dried lettuce. Water collected from a subterranean spring (Lez, 10 km north of Montpellier) was changed twice a week.

Our study of the mating system in *D. depressissimum* included two experimental treatments (see Tsitrone et al. 2003b; Escobar et al. 2009, 2011 for similar protocols). In both treatments,  $G_2$  snails were isolated in 75 mL boxes. Snails from treatment 1 (paired) were paired for 3 h, twice a week, with a sexually mature mate (i.e., >6–7 mm in shell diameter) randomly drawn from the unrelated stock populations or from unrelated families in the same treatment. A different partner was used at each encounter. This treatment

provides enough contact time for mating to occur while limiting competition or other effects of social interactions due to pairing (see Doums et al. 1994). Snails from treatment 2 (selfed) never encountered mating partners and reproduced through selfing only. The experiment was initiated with 268 individuals from 22 families (1–16 individuals per family), but this reduced to 164 individuals and 21 families (1–8 individuals per family) because of mortality of  $G_2$  individuals.

Mating observation was initiated when individuals were 3–4 weeks old in the paired treatment that is before sexual maturity, in order to maximize our chance to record the first copulations. Preliminary observations (data not shown) indicated that copulation usually lasts for more than 30 min, and pairs were therefore checked every 20–30 min for the whole duration of the pairing period. A few pairs ( $N = 6$  individuals) had to be discarded from subsequent analysis of first copulations because copulations were not recorded. We obtained complete records of the sex role played by individuals and age at first copulation for 76 individuals (= 82 in the paired treatment minus the 6 with incomplete recordings). These individuals were paired for a few more sessions after first copulation until they had laid 10 eggs (see below).

The number of egg masses and eggs per egg mass laid by focal individuals was recorded twice a week in both treatments. Individuals from the paired treatment were discarded once they had laid more than 10 eggs. Individuals from the selfed treatment laid very few eggs, and fecundity was therefore monitored until death. Fecundity was compared between treatments based on the time required to lay the first 10 eggs—this will be referred to as  $F_{10}$ . A total of 532 and 300 egg masses (corresponding to 3,593 and 675 eggs) were counted in the paired and the selfed treatment, respectively. A subset of eggs (2,435 and 529 from treatments 1 and 2, respectively) was kept to estimate juvenile survival of  $G_3$  snails (number of living hatchlings 20 days after egg-laying).

The waiting time ( $WT$ ) is defined as the difference in age at first reproduction between the selfed treatment and the paired treatment. However, the waiting time does not appropriately describe the mating system in species extremely reluctant to self-fertilization since many isolated individuals die before ever starting to reproduce (Escobar et al. 2011). In such situations, the mating system is better described through  $\Delta_{\max}$ , which is the maximum difference, over all ages, between the cumulative proportions of individuals that have initiated egg production in the paired and the selfed treatment. Large significant values are expected in outcrossing species while values are close to 0 in selfing species. Positive waiting times and  $\Delta_{\max}$ , as well as reduced egg output under selfed treatment, are typical of preferentially outcrossing species (Tsitrone et al. 2003a; Escobar et al. 2011). Apparent inbreeding depression was estimated based on juvenile ( $G_3$ ) survival using the formula  $1 - W_2/W_1$  where  $W_2$  and  $W_1$  stand for juvenile survival in treatments 2 (selfed) and 1 (paired), respectively (Charlesworth and Charlesworth 1987). This is dubbed apparent inbreeding depression rather than inbreeding depression because we did not check that pairing resulted here in 100% outcrossing. If anything, apparent inbreeding depression therefore underestimates true inbreeding depression.

## Statistical analysis

The mating probability as male and as female was analysed as a function of age (polynomial fit) using an analysis of deviance with logit-link and Binomial error distribution. Age at first egg-laying in  $G_2$  individuals was analyzed using a mixed-model analysis of variance (ANOVA) with treatment as a fixed effect and family as a random effect. The additive genetic variance ( $V_A$ ) of age at first reproduction was estimated as twice the



among-family component of variance, and the narrow-sense heritability as the ratio of  $V_A$  to phenotypic variance ( $h^2$ ; Falconer and Mackay 1996). The variance among full-sib families, in addition to additive variance ( $1/2 V_A$ ), includes an unknown amount of dominance ( $1/4 V_D$ ) and maternal effects ( $V_M$ ). Therefore our estimates represent an upper limit to the true  $V_A$  and  $h^2$ . To test whether the observed  $\Delta_{\max}$  value exceeded the expectation under the null hypothesis of no effect of treatment on age at first egg-laying, we used the permutation procedure (1,000 permutations) described in Escobar et al. (2011).

The number of eggs per capsule was compared between treatments. Because the distributions were not normally distributed in both treatments, we tabulated the numbers of egg masses from paired and selfed treatments for each egg mass size, and tested whether the proportion of egg masses from the paired treatment increased or decreased with egg mass size using an analysis of deviance with logit-link and Binomial error distribution.  $F_{10}$  was similarly analysed using an inverse-link and Gamma error distribution.  $G_3$  survival was compared between treatments using an analysis of deviance by fitting a generalized linear model (GLM) with logit link and Binomial error distribution. All these analyses were computed using both R 2.11.0 (R-project, [www.r-project.org](http://www.r-project.org)) and JMP<sup>®</sup> 7.0 (2007 SAS Institute Inc.). To account for overdispersion, model comparison was conducted based on deviance ratios (approximately  $F$ -distributed; see Crawley 2005).

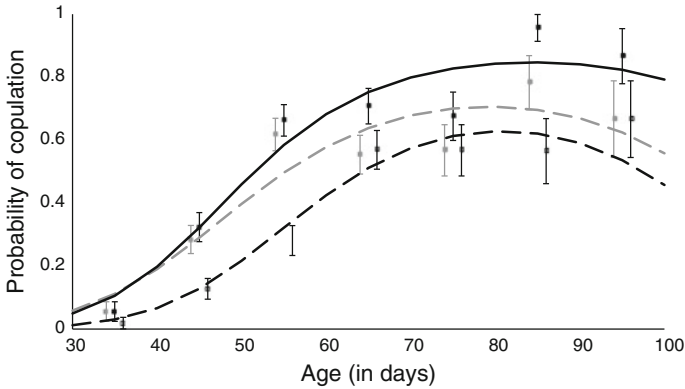
## Results

### Selfing rates and genetic diversity in the field

All loci were polymorphic in all populations (see Supplementary materials)—one of them, DDE14 failed to amplify in the Grosse Roche and Pico populations. The mean number of alleles per population ranged from 7.0 to 16.8 (Table 2). Gene diversity ranged from 0.74 to 0.91. Heterozygote deficiencies were detected in 19, out of 83, locus-population combinations. Three loci, DDE07, DDE08 and DDE23 accounted for 12 of these deviations (Supplementary Table S2). This resulted in significant departures from HWE (multilocus test,  $P < 0.0001$ ) in all populations. The inbreeding coefficient  $f$  value ranged from 0.058 to 0.108 (Table 2), producing estimates of the selfing rate,  $\hat{s}(f)$ , ranging from 0.109 to 0.195. In contrast, none of the multilocus estimates of selfing rate, whether based on  $\hat{s}(g_2)$  or on the ML method, significantly differed from 0 (Table 2). The ML method indicated no significant differences in selfing rates among populations, and the pooled estimate was 0.000, the upper bound of the 95% confidence interval being 0.014. This is much lower than the average  $\hat{s}$  derived from  $F_{IS}$  (0.153). 19 significant ( $P < 0.05$ ) genotypic disequilibria were detected out of 424 locus pairs-population combinations which is less than expected by chance at the 5% level.

### Copulatory behaviour

Copulations were recorded in 76 focal individuals from 21 families. Copulation usually started within the first 30 min of observation and lasted for more than 30 min. The frequency of copulation of focal individuals increased from 30 to 70 days of age then stabilized around 70% for both sex roles (Fig. 2). The first copulation occurred at 51.59 days ( $\pm 8.01SD$ ;  $N = 67$ ) and 55.12 ( $\pm 8.85SD$ ;  $N = 52$ ) for the male and female roles, respectively. Some snails ( $N = 27$ ) laid eggs without prior observation of copulation as female (Fig. 3). However the number of eggs of their first egg capsules ranged between

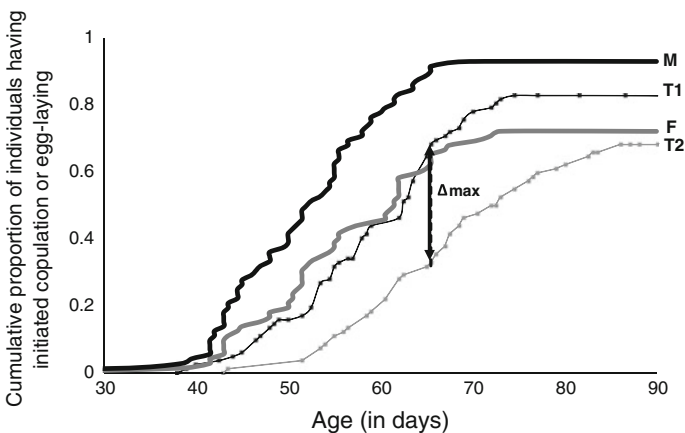


**Fig. 2** Mating probability (per 3-h pairing session) as a function of age. The observed means ( $\pm$  SD) are based on 52 and 67 data points for the female and male functions, respectively, and are indicated by squares (female: white; male: grey; both: black). Lines are fitted probabilities using a polynomial logit model with a quadratic effect (female: black dashed line; male: grey dashed line; both: black line)

five and eight, values which are large enough to suggest outcrossing (see below); this suggests that we failed to record their first copulation. We often observed two successive copulations with role-switching in the observation period; occasionally the second copulation sometimes started before the first one ended, with a transient situation of simultaneous reciprocal copulation (although we could not ascertain that simultaneous reciprocal sperm transfer actually occurred).

Life-history traits

Twenty-four selfed and eleven paired individuals (29 and 11%, respectively) never reproduced and were excluded from subsequent analyses. Selfed individuals initiated reproduction significantly later than paired individuals (6.67 days; Table 3; Fig. 3). The



**Fig. 3** Cumulative proportion of reproducers as a function of age in individuals from the paired treatment (black squares-T1) and the selfed treatment (grey squares-T2), and cumulative proportion of paired snails which copulated as male (dash black line-M) and female (grey dash line-F).  $\Delta_{max}$  is represented as a black arrow

**Table 3** Analysis of variance on the effect of treatment and family on the age at first reproduction in  $G_2$  individuals, and estimates of genetic variance ( $V_A$ ) and heritability ( $h^2$ )

Treatment	Factor (df)	var	$P$	$V_A$	$h^2$
Both treatments	Treatment (1)		$2 \times 10^{-4}$		
	Family (12)	20.57	0.008	41.13	0.30
	Interaction (12)	$2 \times 10^{-6}$	1.00		
	Error (82)	115.26			
Paired treatment	Family (12)	8.80	0.26	17.60	0.12
	Error (45)	135.13			
Selfed treatment	Family (12)	21.05	0.12	42.09	0.33
	Error (37)	104.80			

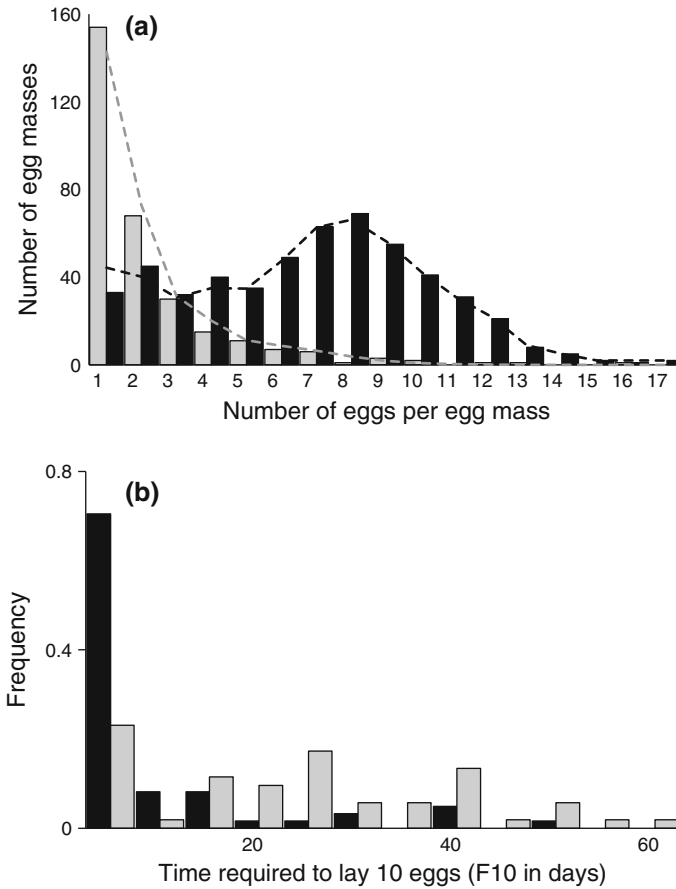
The analysis was conducted on both treatments (108 individuals, 13 families) and separately for the paired treatment (58 individuals, 13 families) and the selfed treatment (50 individuals, 13 families). Treatment has no estimate of variance because it is a fixed effect

maximum difference between selfed and paired curves ( $\Delta_{\max} = 0.35$ ; Fig. 3) occurred at 65.5 days and was highly significant ( $P < 0.001$ ). The ANOVA indicated no significant family-by-treatment interaction, but a significant family effect on the age at first reproduction (Table 3). The heritability estimate for age at first reproduction, combined over the two treatments, was 0.30. It was much higher in the selfed (0.33) than in the paired (0.12) treatment, though not significant in both cases due to limited statistical power. The family means of the age at first reproduction were positively correlated between treatments ( $r = 0.56$ ,  $P = 0.05$ ). The age at first egg-laying was also highly correlated with the age of first copulation as female ( $r = 0.62$ ,  $P < 0.0001$ ; Fig. 3). Individuals began laying eggs on average 9.15 days ( $\pm 9.94$ SD) after the first copulation as female.

The number of eggs per egg mass was much larger in the paired treatment than in the selfed treatment ( $6.75 \pm 3.32$ SD vs.  $2.25 \pm 2.10$ SD; Fig. 4a), and we significantly found more eggs in egg masses from the paired treatment than from the selfed treatment ( $F_{1,15} = 170.78$ ,  $P < 0.0001$ ). Egg masses from the selfed treatment were mainly composed of one or two eggs. In addition,  $F_{10}$ , the time required to lay 10 eggs, was significantly longer in the selfed treatment than in the paired treatment ( $21.9 \pm 15.3$ SD and  $7.7 \pm 10.1$ SD, respectively;  $F_{1,111} = 35.36$ ,  $P < 0.00001$ ; Fig. 4b). Offspring survival at 20 days (after egg-laying) was generally low, but paired snails survived much better than selfed ones ( $10.59\% \pm 0.46$ SE vs.  $0.19\% \pm 0.17$ SE,  $F_{1,132} = 14.34$ ,  $P = 0.0002$ ). Only one selfed juvenile indeed survived out of 529 eggs produced. The estimate of apparent inbreeding depression was  $0.98 \pm 0.02$ . The survival of juveniles produced by paired individuals that had not been seen copulating as female did not differ from the survival of juveniles produced by other paired individuals, suggesting again that they were outcrossed and that we missed the corresponding copulations.

## Discussion

Environmental instability is expected to result in frequent cycles of local extinction and colonization in the *D. depressissimum* metapopulation. In this context, it is usually assumed that selfing species, or species that can occasionally self-fertilize, are favoured because selfing provides reproductive assurance when mates are lacking. For example, in



**Fig. 4** **a** Distribution of the number of eggs per egg mass and **b** distribution of the time required to lay 10 eggs ( $F_{10}$ ) in the paired (black chart) and the selfed (grey chart) treatment. The lines in A correspond to fitted values from binomial models

plants, selfing is more frequent in early-succession annuals than in perennials (Barrett et al. 1996; Morgan et al. 1997). Contrary to this expectation none of the studied samples from natural populations of *D. depressissimum* showed any sign of self-fertilization, irrespective of their degree of polymorphism and environment type (river margins, ponds, mangroves, permanent or temporary habitats). It is important to note here that a different conclusion might have been reached if selfing rates had been computed from heterozygote deficiency (the  $F_{IS}$  method) rather than from the multilocus methods. Indeed all populations exhibited significant heterozygote deficiencies, and  $F_{IS}$ -based selfing rates ranged between 0.109 and 0.195 classifying *D. depressissimum* as a predominantly outbred species with some selfing. This reflects the fact that the  $F_{IS}$  method is highly sensitive to genotyping errors (e.g., null alleles or short-allele dominance), which tend to transform heterozygotes into apparent homozygotes (Jarne and David 2008). In agreement with this idea, heterozygote deficiencies were concentrated in a few loci (three), as expected if they were due to technical problems but not if they arise from inbreeding. Thus, the example of *D. depressissimum*

illustrates how purely outcrossing species can be mis-classified as occasional selfers using the  $F_{IS}$ -method.

In their large-scale analysis, Jarne and Auld (2006) obtained an almost flat distribution of selfing rates in hermaphroditic animals. However, these estimates were essentially derived from  $F_{IS}$ , and the authors cautioned against biased estimates in supposed mixed-maters. In contrast, the more recent analysis of Escobar et al. (2011) based on multilocus methods found a markedly U-shaped distribution in 11 snail species which were either pure outcrossers (selfing rate not significantly different from 0), or predominant selfers (selfing rate from 0.8 to 1). *Drepanotrema depressissimum* clearly ranks with the outcrossers. We are therefore still short of confirmed mixed-mating species in snails, and perhaps among hermaphroditic animals in general. The exceptions might be species exhibiting sexual polymorphisms such as the androdioecious freshwater shrimp *Eulimnadia texana* (Weeks et al. 2008). More studies are required to substantiate this claim. It also remains to be explained which forces might drive the selfing rate to extreme values in animals and less so in plants.

The molecular data clearly indicate that outcrossing is the dominant reproductive mode in natural populations of *D. depressissimum*. This shows that this species does not belong to preferential selfers nor to prior mixed-maters (i.e., strategies characterized by some degree of “prior” (unconditional selfing, Table 1). Yet we do not exclude the possibility that selfing occurs occasionally in the wild and has not been detected in our study. Assuming that selfing allows populations to persist and grow during episodes of very low density, such situations could be under-represented in our samples as collecting 20–30 individuals for genetic analysis then becomes too difficult. However, laboratory experiments allow us to measure how reluctant individuals were to self-fertilize when mate availability was reduced.

When opportunities to encounter mates were reduced to two 3-h periods per week (paired treatment), sexually mature individuals were eager to mate. Copulations in both roles take place in more than half of the occasions, mating probability ranging from  $0.67 \pm 0.08SD$  (for 70–80 days old snails) to  $0.96 \pm 0.04SD$  (for 80–90 days old snails), sometimes (21%) with bilateral copulation. In other predominantly outcrossing species such as *Physa acuta*, the same behaviour (swift mating after a few days of isolation) has been observed (e.g., Escobar et al. 2009). Individuals from predominantly selfing species are much more reluctant to copulate in similar conditions. For example, Tian-Bi et al. (2008) observed one copulation every (about) ten observation sessions in two populations of *Biomphalaria pfeifferi*. It therefore seems that monitoring the mating behaviour of sexually mature individuals, which have been isolated for a long enough time, is a simple and informative way to characterize the dominant mating system, at least in freshwater snails (Doums et al. 1996; Escobar et al. 2011).

Complete suppression of mating opportunities (selfed treatment) results in a drastic reduction in female reproductive output in *D. depressissimum*: many individuals (29%) never laid eggs, and egg-laying (when present) was delayed compared to paired individuals. The waiting time is approximately 6.7 days, or 11% of the age at first reproduction. This value is very significant, but might be deemed low in comparison to what has previously been measured in predominantly outcrossing animals, including snails (Escobar et al. 2011). Escobar et al. (2011) found positive waiting times in five Basommatophoran species: *Lymnaea stagnalis* (20.8 days), *Radix peregra* (12.6 days), *Physa acuta* (14.1 days), *Physa gyrina* (3.2 days) and *Biomphalaria glabrata* (3.5 days). The waiting time of *D. depressissimum* seems intermediate. Nevertheless, its mating system is better described through  $\Delta_{max}$  because many isolated individuals die before starting to reproduce,

and do not contribute to the estimation of the waiting time. We accordingly found a large and significant estimate of  $\Delta_{\max}$  (35%), which is consistent with the mean  $\Delta_{\max}$  estimated for outcrossed species in Escobar et al. (2011) of 41%. The age at first reproduction appears to be genetically variable, but the significant correlation in age at first reproduction between the two treatments, and the lack of significant interaction between treatment and family, suggests that the waiting time itself has little genetic variance. In addition to delayed reproduction, isolated individuals exhibited a much lower fecundity. Few egg masses were laid and usually contained a single egg, while the typical size in the paired treatment was 5–10 eggs. Additionally, we noted that many selfed egg masses differed in shape from outcrossed ones and sometimes included no egg at all (pers. Obs.). The decrease in reproductive output in isolated snails may be induced by the lack of either copulatory activity or other social interactions. However the positive correlation between age at first copulation (in the female role) and age at first egg-laying suggests a direct effect of copulation.

Finally, studying inbreeding depression allows us to evaluate the potential for the (few) selfed eggs to allow isolated individuals to found new populations and colonize available empty habitats. In our experiment the apparent inbreeding depression on early survival was extreme ( $0.98 \pm 0.02$ , at the juvenile stage). This estimation can only increase if we account for the fact that some of the individuals in the paired treatment might have self-fertilized some of their eggs. This implies that many lethal and sublethal recessive mutations are probably segregating in *D. depressissimum* populations (review in Jarne and Charlesworth 1993; Husband and Schemske 1996; Goodwillie et al. 2005). Juvenile survival was also low (even for outbred offspring), presumably because we have not been able to provide the optimal rearing conditions. However, it was clearly much lower in the selfed treatment with a single survivor at the juvenile stage (this individual was reared in isolation but died before reaching sexual maturity). Although field conditions might modify survival rates compared to laboratory conditions (Henry et al. 2005), it seems safe to assume that selfing can only exceptionally provide reproductive assurance to *Drepanotrema depressissimum*.

In summary, *D. depressissimum* presents an association of traits typical of a near-obligate outcrosser (type A in Table 1) and has very little opportunity to rely on self-fertilization to produce viable offspring in the absence of mates. Although the type A can be called a “near-obligate outcrosser” it is not comparable to a self-incompatible plant because most species retain the possibility to produce selfed eggs, although most of them are doomed to early mortality. In these conditions, the little selfing that might occur is probably not often a great help in demographic terms. Why the possibility to self-fertilize persists in these species is not clear. It might be complicated to eliminate any possibility of selfing in pulmonates where male and female gametes are produced in the same organ (Jarne et al. 2010); on the other hand when the production of selfed eggs is very low (as in *D. depressissimum*) the energetic cost of these few eggs is not very high and selection for a further reduction (or a higher delay before the onset of egg-laying) is probably not very strong. Heritable variation in waiting times also exists within species, and in *Physa acuta*, for example, it is correlated to inbreeding depression across populations (Escobar et al. 2009). It is therefore possible that populations of *D. depressissimum* that we have not sampled have lower inbreeding depression and less reluctance to self-fertilization than we observed in this study.

The characterization of *D. depressissimum* as a near-obligate outcrosser is contrary to our initial expectation and shows that a species can live in a frequently disturbed environment (sites that either dry out or substantially shrink during the dry season; Pointier and

Combes 1976; Pointier and David 2004) and form a viable metapopulation without relying on facultative self-fertilization to overcome episodes of low population density and associated Allee effects (i.e., mate shortage). In the metapopulation literature, there are two main categories of mechanisms, apart from selfing, that can play this role. The first is dispersal, provided that the number of immigrants is large enough to overcome Allee effects (Olivieri et al. 1995). The second is the constitution of seed banks (i.e., dormancy), or banks of resistant stages in animals, that can be characterized as “dispersal in time” (Venable and Brown 1988). Recent theoretical studies have indicated that dispersal and selfing can evolve as alternative strategies to persist in a metapopulation when mate or pollen shortage occurs occasionally within sites. This results in outcrossing-high dispersal versus selfing-low dispersal syndromes, because efficient dispersal suppresses the reproductive assurance advantage of selfing and vice versa (Cheptou and Massol 2009). A similar argument can probably be made for dormancy and selfing. Unfortunately we know little about dispersal in *D. depressissimum*. This species relies on passive terrestrial/aerial transport by birds or cattle to re-colonize sites because most of them are never connected to other sites through water, and although individual snails can survive some time in open air, they never actively move in this situation (pers. obs.). On the other hand, dormancy could play a role as this snail species can aestivate for several weeks in protected areas (e.g., under rocks or vegetation) of dried out sites (Pointier and Combes 1976). How many individuals can survive dry episode of several months (as commonly observed in natural habitats) is not known. However we found very high genetic diversity (overall gene diversity around 0.85) in our populations of *D. depressissimum* (indeed, the highest genetic diversities ever found in freshwater snails using any kind of molecular markers), which tends to suggest that bottleneck or founder effects are not as intense or common in this system as we previously thought.

More studies are required to confirm the role of aestivation in this species as well as in other species. Indeed, the ability to resist desiccation for some time is a capacity shared by many pulmonate snails, a group that dominates the dataset of Jarne and Auld (2006) on selfing rates in hermaphroditic animals and makes the totality of the dataset of Escobar et al. (2011). The absence of mixed-maters in the latter study could therefore reflect the fact that when inbreeding depression is high (as in predominantly outcrossing species), partial selfing is a more costly or infrequent evolutionary response to the constraints of living in unstable environments than aestivation or dispersal.

As mentioned in the introduction, mixed-mating seems frequent in plants, and is often perceived as a response to environmental instability. Many plant species, just like snails, are capable of efficient dispersion and seed dormancy, so why should mixed-mating be more frequent in plants than in snails? While population density, and mate encounter rates are probably the main determinant of outcrossing opportunities in mobile animals, plants are sessile and rely on the action of wind or insects for pollination. Then, pollination may fail even when the density of adult plants is high (Kalisz et al. 1999). In that case individual fitness cannot be restored through dormancy or dispersal, but only by autonomous pollination, especially in annual plants which cannot store energy for later reproduction episodes. This speculative idea has to be substantiated through more studies of mating systems in plant and animal species with different life styles.

**Acknowledgments** The authors thank V. Sarda for maintaining the mollusk room in working order, the SMGE at CEFÉ and the environmental genomics platform of the “Montpellier Environnement Biodiversité” Research Federation for help with genotyping. T. Lamy was supported by a fellowship from the French Ministry of Research. This work was funded by the CNRS, ANR JCJC-0202 to P. David; the Ecosystèmes

Tropicaux program (French ministry of environment) and a “Chercheurs d’avenir” Grant to P. David from the Région Languedoc-Roussillon.

**Conflict of interest** The authors declare no conflict of interest.

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