Genet. Res., Camb. (2008), **90**, *pp.* 61–72. © 2008 Cambridge University Press
doi:10.1017/S0016672307008919 Printed in the United Kingdom

Prior selfing and the selfing syndrome in animals: an experimental approach in the freshwater snail *Biomphalaria pfeifferi*

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(*Received 23 July 2007 and in revised form 5 October 2007*)

Summary

Inbreeding species of hermaphroditic animals practising copulation have been characterized by few copulations, no waiting time (the time that an isolated individual waits for a partner before initiating reproduction compared with paired individuals) and limited inbreeding (self-fertilization) depression. This syndrome, which has never been fully studied before in any species, is analysed here in the highly selfing freshwater snail *Biomphalaria pfeifferi*. We conducted an experiment under laboratory conditions over two generations (G_1 and G_2) using snails sampled from two populations (100 individuals per population). G_1 individuals were either isolated or paired once a week (potentially allowing for crosses), and monitored during 29 weeks for growth, fecundity and survival. Very few copulations were observed in paired snails, and there was a positive correlation in copulatory activity (e.g. number of copulations) between the male and female sexual roles. The waiting time was either null or negative, meaning that isolated individuals initiated reproduction before paired ones. G_2 offspring did not differ in hatching rate and survival (to 28 days) between treatments, but offspring from paired individuals grew faster than those from isolated individuals. On the whole, the self-fertilization depression was extremely low in both populations. Another important result is that paired G_1 individuals began laying (selfed) eggs several weeks prior to initiating copulation: this is the first characterization of prior selfing (selfing initiated prior to any outcrossing) in a hermaphroditic animal. A significant population effect was observed on most traits studied. Our results are discussed with regard to the maintenance of low outcrossing rates in highly inbreeding species.

1. Introduction

The evolution of self-fertilization versus cross-fertilization is a central issue in evolutionary biology, and has been the focus of a substantial amount of both empirical and theoretical work (reviewed in Jarne & Charlesworth, 1993; Husband & Schemske, 1996; Goodwillie *et al.*, 2005). The distribution of selfing rates among species is typically U-shaped, although this is more marked in plants than in animals (Goodwillie *et al.*, 2005; Jarne & Auld, 2006). Our focus here will be on the highly selfing side of this distribution. Theoretical genetic models balancing the automatic genetic advantage of selfing with inbreeding depression (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987) predict that high selfing rates should be associated with low inbreeding depression ($<0{\cdot}5$), a pattern that has indeed

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found substantial empirical ground (Husband & Schemske, 1996). These models also predict pure selfing as an equilibrium state. This, on the other hand, is rarely observed, and a few per cent outcrossing is often detected, especially in animals (Jarne & Auld, 2006). The evolution of high selfing rates is also associated with a suite of morphological, behavioural and life-history traits (Jain, 1976). For example, cleistogamous (i.e. closed) flowers have very reduced petals and produce little pollen (e.g. Schoen & Lloyd, 1984). The reproductive timing might also differ between selfing and outcrossing within single individuals. The set of traits associated with the evolution of high selfing rates can thus be described as a selfing syndrome (see Lloyd, 1979).

These topics have been studied far more in plants than in animals (Jarne & Auld, 2006), especially for highly selfing species. Despite the fact that the model species *Caenorhabditis elegans* is a self-fertilizing hermaphrodite, the most thorough information so far comes from work on freshwater snails (Jarne & Charlesworth, 1993). In these animals, the selfing syndrome has been defined as 'high selfing rate, low inbreeding depression and a limited propensity to copulate' (Doums *et al*., 1996). More recent work indicates that a limited waiting time (the time that an isolated individual waits for a partner before initiating reproduction compared with paired snails) is also expected in highly selfing species in which outcrossing requires copulation (Tsitrone *et al*., 2003*a*, *b*; Charbonnel *et al*., 2005). However, even in snails, the suite of traits 'inbreeding depression, mating activity and waiting time' has not been studied experimentally in any species. For example, the waiting time has not been estimated in selfers, and no quantitative analysis of the copulatory activity has been conducted.

The reproductive timing of selfing relative to outcrossing has also not been thoroughly evaluated in animals. In plants, selfing can result from competition between outcross and selfed pollen (competitive selfing), can occur prior to outcrossing (prior selfing), or can be delayed until after all opportunities for outcrossing have been expended (delayed selfing; Lloyd, 1979, 1992). There is ample evidence for these three selfing modes in plants. Far less is known in those hermaphroditic animal species in which outcrossing requires copulation (note that many hermaphroditic species release gametes in the external world, especially among marine species, and do not copulate). Delayed selfing has been characterized in snails and cestodes (Tsitrone *et al*., 2003*a*; Schjørring, 2004): isolated individuals reproduce later than individuals that have access to mates. This delay is the abovementioned waiting time, and is a function of, among others, inbreeding depression and the probability of finding a mate. It has been interpreted as a reproductive assurance mechanism (Tsitrone *et al*., 2003*a*). On the other hand, we are not aware of any report of prior selfing in animals. It is defined here by selfing occurring prior to outcrossing in the presence of sexually mature partners. Prior selfing might be selected when the probability of being outcrossed is low (e.g. pollination or partner limitation) and/or inbreeding depression is weak (Lloyd, 1992). A second goal of this work was to test for the occurrence of prior selfing in an animal species, and to propose some explanations for its evolution.

In order to characterize the selfing syndrome and the occurrence of prior selfing in an animal species, we conducted experiments in a hermaphroditic freshwater snail. Snails from this group (Basommatophora) have a single reproductive gland, the hermaphroditic ovotestis. Outcrossing requires copulation while selfing is strictly an internal process (review in Jarne *et al*., 1993). A recent synthesis showed that selfing rates range from 0 to 1 among basommatophoran species, with several species exhibiting high selfing rates (Jarne & Auld, 2006). We used the African species *Biomphalaria pfeifferi* as model species in this work. It is an interesting model for investigating the selfing syndrome and prior selfing. Previous work indeed reported selfing rates on the order of 0·9 in natural populations of *B. pfeifferi* from Madagascar (Charbonnel *et al*., 2002*b*, 2005). However, copulation does occur, and the copulatory sequence has already been detailed (Rupp & Woolhouse, 1999). Indirect evidence suggested that inbreeding depression is limited in this species (Charbonnel *et al*., 2005). However, we do not know of any formal investigation of the waiting time, and more generally of the selfing syndrome. Our work included individuals from two populations from Côte d'Ivoire. We analysed several life-history traits over two laboratory generations for estimating the waiting time and inbreeding depression. The copulatory activity was analysed in individuals from the first laboratory generation.

2. Materials and methods

(i) *The species studied*

The hermaphroditic freshwater snail *B. pfeifferi* (Planorbidae; Basommatophora) is the main intermediate host of the trematode *Schistosoma mansoni*, the agent of intestinal bilharziasis, in Africa and Madagascar (Brown, 1994). This species occupies a variety of more or less permanent waterbodies including streams, irrigation channels and dam lakes (Woolhouse, 1992; Brown, 1994; Charbonnel *et al*., 2002*a*, *b*). Populations may experience density variation associated with flooding and droughts, leading to bottlenecks and recolonization events (Jarne & Delay, 1991; Charbonnel *et al*., 2002*b*). A consequence is limited neutral variability within populations

and fairly large genetic differentiation among populations (Charbonnel *et al*., 2002*a–c*). Genetic analyses at both family and population levels indicated high selfing rates in *B. pfeifferi* (Charbonnel *et al*., 2002*b*, 2005).

Individuals begin laying eggs when around 2–3 months old (Loreau & Baluku, 1987) and egg capsules generally contain fewer than 10 eggs (Lévêque, 1980). Hatching occurs about 2 weeks after egg-laying (Loreau & Baluku, 1987). The mating behaviour shows little variability among basommatophorans. It is a unilateral process, one individual playing the male role while the other plays female, but role-switching can occur (Jarne *et al*., 1993; Facon *et al*., 2006). Rupp & Woolhouse (1999) describe the whole process in *B. pfeifferi*. The male behaviour comprises a fixed sequence of events, including a circular movement on the partner's shell and positioning on the left side of the aperture (shell mounting and positioning), extension of the penis towards the partner's gonopore (under the shell), penetration and sperm transfer into the partner's vagina. The male partner is sometimes prevented from mating by the female partner retreating into its shell. The sperm transferred during mating is stored and might be used for several weeks for ova fertilization, either in highly outcrossing (Vianey-Liaud, 1998; Wethington & Dillon, 1997) or highly selfing (De Larambergue, 1939) species, although the temporal dynamics of allosperm exhaustion has not been evaluated in *B. pfeifferi*.

(ii) *Populations studied and rearing conditions*

The individuals studied were sampled (mid-February 2005) in two urban sites – Doyagouiné I (DOY) and Quartier-Treize (QTT) – located in the city of Man (western Côte d'Ivoire) and separated by 1·7 km. DOY is an irrigated paddy field (temporary habitat) and QTT a permanent pond. Fifty-seven and 62 G_0 individuals were collected in DOY and QTT, respectively, and brought alive to the laboratory. Twenty individuals measuring 3–4 mm in shell diameter were chosen at random per population. These individuals were immature, since sexual maturity is reached at a shell size larger than 4 mm in this species (Loreau & Baluku, 1987; Baluku & Loreau, 1989). Individuals were isolated in 200 ml transparent plastic boxes filled with 125 ml of dechlorinated tap water. Throughout the experiment, snails were maintained at 21–24 °C (water temperature in boxes) under a 12L : 12D photoperiod. They were fed *ad libitum* with boiled lettuce. Food and water were changed twice per week.

(iii) *General outline of protocol*

The general outline of the protocol, spanning two generations (i.e. G_0, G_1 and G_2), is given in Fig. 1. The G_0 individuals began laying eggs 1 month after isolation (mid-March). The egg capsules were collected daily over 10 days (20–30 March). The G_1 offspring were assumed to be selfed products because their parents were immature when isolated. After hatching, juvenile G_1 were maintained with their parents for approximately 1 week. Twenty-five G_1 individuals were collected at random in each of the 20 G_0 boxes (1–15 April). The 500 G_1 individuals (1·5–2 mm in shell length) per population were randomly allocated to groups of five individuals for 6 weeks (15 April 15–28 May). One hundred individuals were chosen at random (i.e. 1 G_1 per group) in each population when they reached a 2·5–4 mm in size (that is before sexual maturity). In each population, 50 individuals were randomly assigned to one of the two treatments: T_1 individuals were kept isolated throughout the experiment and therefore self-fertilized; T_2 individuals were paired once a week for 10 hours (08:00–18:00 hours), and were otherwise isolated. This treatment allows copulation without the depressing effect of pairing on fitness (grouping effect in Doums *et al*., 1994). There was no significant difference in shell diameter among treatments (*t*-test, $P > 0{\cdot}78$ in both populations). The T_2 treatment began when individuals were 69 days old, on average. Several life-history traits were monitored, including survival, fecundity and growth, over about 6 months (29 weeks). The mating behaviour of T_2 individuals was observed over the same period. At week 18, egg capsules were collected in the two populations and treatments in order to estimate hatching rate of G_2 individuals and, their growth and survival over a month.

(iv) *Mating parameters*

The mating behaviour was analysed in T_2 snails. Their partners were drawn from a stock of adult G_1 individuals, born from G_0 individuals originating from the same sites as the experimental snails. Mates were marked with nail polish and reared in the conditions described above. They were isolated 2 weeks prior to pairing in order to stimulate copulation (see Vianey-Liaud & Dussart, 2002; Tsitrone *et al*., 2003*b*). Experimental snails were monitored for 10 hours once a week, and were not fed during this pairing period. Copulation was considered successful when the male-acting individual inserted its penis into its partner's gonopore. We recorded the number and duration of copulations per T_2 individual, as well as the sexual role assumed (male or female). Copulation duration was defined as the time from penis intromission to penis retraction of the male-acting individual provided. That penis retraction was actually followed by partners' separation. At the beginning of treatments, the mean sizes were respectively 7·53 mm and 2·66 mm for mates and experimental G_1 individuals in

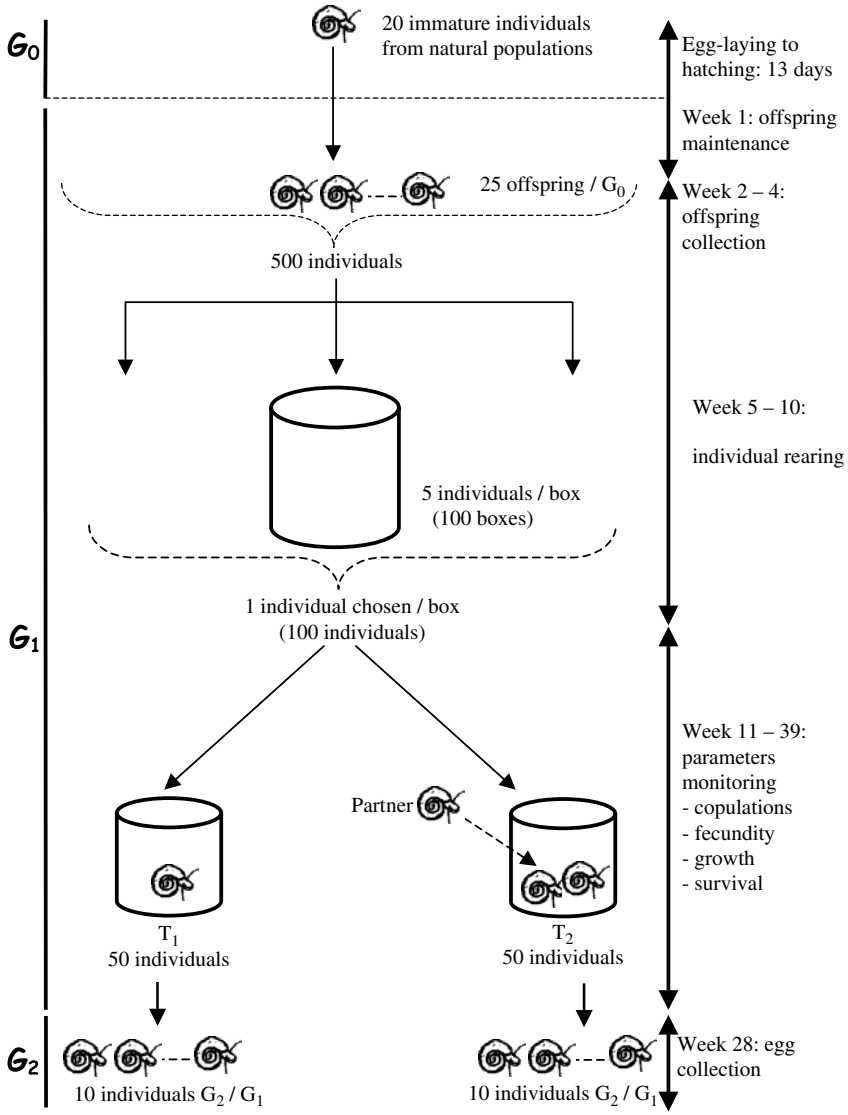

Fig. 1. Schematic diagram of the experimental protocol that was conducted in the two populations. G_i refers to generation. Twenty field-caught G_0 individuals were isolated, and 25 G_1 offspring were collected per G_0 individual (500 individuals overall). They were placed in groups of five individuals. One individual was sampled per box at week 10, and was attributed either to treatment 1 (T_1; isolation) or to treatment 2 (T_2; partner provided once a week). Individuals were monitored over 29 weeks for copulatory activity, fecundity, growth and survival. Ten offspring per individuals were collected for estimating survival and growth. For further details see text.

DOY, and 7·08 mm and 2·66 mm in QTT respectively, but the difference between mates and experimental snails decreased quickly over the course of the experiment. Choosing large partners ensured that they were sexually mature as both male and female.

(v) *Life-history traits*

Size (shell diameter) of G_1 snails was measured weekly to the nearest 0·01 mm using an electronic (Digimatic) calliper. This allowed estimating size at both first reproduction and death, as well as growth. Individual survival was monitored three times a week. Individuals were checked every 2 days for their first egg capsule, and fecundity (number of eggs, of egg capsules and of eggs per capsule) was subsequently monitored twice a week. Egg capsules laid during the pairing periods, including those of T_1 individuals, were discarded. The reason is that it was not possible to know which individuals of a given pair laid egg capsules during the pairing period of T_2 individuals. From the weekly distribution of eggs laid, we inferred the time to reach the peak of egg production per individual. Capsules from the eighteenth week from both T_1 and T_2 individuals were maintained in the boxes in which they were laid. At this point, 40 (80 %) T_2 individuals from DOY and 42 (84 %) from QTT had already copulated as female at least once (i.e. received allosperm from their partner). The hatching rate was recorded daily. Ten G_2 individuals (siblings)

https://doi.org/10.1017/S0016672307008919 Published online by Cambridge University Press

were chosen at random per G_1 individual from both treatments, and they were reared together. Survival and size were measured at 7 and 28 days. Size was estimated twice a week from two G_2 individuals haphazardly chosen in each box.

(vi) *Statistical analyses*

Most analyses were conducted using the generalized linear model (GLM) framework (Crawley, 2005). The effects of population and treatment, as well as their interaction, were tested on continuous variables (age and size at both first reproduction and death, time to reach the peak of egg production, growth) using a Normal error. The same effects were tested on the categorical variables (number of eggs, of egg capsules and of eggs per capsule) using a Poisson error (log link). The waiting time was estimated within populations as the mean age at first reproduction of T_1 individuals minus that of T_2 individuals. Phenotypic correlations were estimated between pairs of traits within both populations using Spearman rank-order correlation coefficients. The mortality of G_1 individuals was compared across treatments within and between populations using a *G*-test. A GLM was used to test for the effects of population, treatment and their interaction on hatching rate, survival at 7 and 28 days (Binomial error) and on size at 7 and 28 days (Normal error) of G_2 individuals.

A GLM was used to test the effects of population and sexual role on age and size at first copulation and duration of copulations (Normal error) in T_2 individuals from both populations. The number of copulations was analysed using a Poisson error. Individuals that did not copulate as both male and female during the experiment or that died before copulating (eight in DOY and one in QTT) were discarded from the analyses. An analysis of repeatability (Lessells & Boag, 1987) was used to compare the duration of, and the duration between, successive copulations at individual level. This amounts to partitioning the variance into intra- and inter-individual components. The interdependence between copulation parameters, as well as between life-history traits and copulation parameters, was assessed using Spearman rank-order correlation coefficients. The occurrence of prior selfing was evaluated by comparing the age at first reproduction to the age at first copulation as female using Wilcoxon signed-rank test. All analyses were performed using STATISTICA (version 6.0 for Windows), except for the *G*-test which was performed using STATA 9.

Differences in fitness between treatments might derive from a difference in either the fecundity of G_1 individuals, or hatching rate, survival and size (at both 7 and 28 days) of G_2 individuals. This has collectively been referred to as self-fertilization depression. Note though that G_2 fitness only might be due to inbreeding depression (Jarne *et al.*, 1991). We will denote δ_p the parental part (G_1) of self-fertilization depression and δ_o that in offspring. δ_p and δ_o were estimated as $1-(W_1/W_2)$, with W_i the values of the *i*th treatment. Note that δ_p was estimated only in those T_2 individuals that copulated as female throughout the experiment (40 in DOY and 45 in QTT), and δ_o only in the offspring of G_1 individuals that copulated as female before the eighteenth week, that is when egg capsules were collected (40 and 42 individuals in DOY and QTT, respectively).

3. Results

(i) *Life-history traits and pairing treatments in G_1 individuals*

Reproduction was initiated at 104·05 and 107·21 days of age in T_1-DOY and T_2-DOY respectively, and 88·52 and 95·66 days in T_1-QTT and T_2-QTT respectively. The age at first reproduction was significantly affected by both the population and treatment effects (Table 1). Isolated individuals (T_1) began laying eggs earlier than individuals that were offered a partner (T_2). The waiting time is therefore negative, and its absolute value is 3·1 days in DOY and 7·1 days in QTT. The size at first reproduction (5·03 mm in both treatment of DOY, and 5·17 and 5·24 mm in T_1 and T_2 of QTT, respectively) was influenced by the population factor only (Table 1). None of the three estimates of fecundity (number of eggs, number of capsules and number of eggs per capsule) was affected by the treatment or population factors (Table 1). This was also true for the time to reach the peak of egg production and age at death of individuals dying before the end of the experiment (Table 1). Growth was only influenced by the population factor, and was slightly faster in DOY than in QTT. The number of survivors at week 29 (end of the experiment) was 36 and 19 for T_1 and T_2, respectively, in DOY ($G=11{\cdot}9$, $P=0{\cdot}001$), and 21 and 6 in QTT ($G=15{\cdot}2$, $P<10^{-4}$). Mortality was therefore lower in T_1 than in T_2 individuals. Although no significant effect of treatments was detected on age at death, there was a marked difference in QTT, with T_1 individuals dying younger by 30·5 days than T_2 individuals. More generally, T_1 individuals dying during the experiment were smaller than T_2 individuals (Table 1).

We estimated correlations between life-history traits in each population and treatment (Web Table 1). Given the large number of estimated correlation coefficients, we discuss only general trends indicated by low probability values, providing a conservative point of view. The fecundity parameters were on the whole negatively correlated with age at first reproduction, but most significant correlations were found

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Table 1. *Mean values of estimates of several life-history traits for isolated* (T_1) *and paired* (T_2) G_1 *individuals from two populations* (*DOY and QTT*) *of* Biomphalaria pfeifferi

	DOY		QTT				
Parameters	T_1	T_2	T_1	T_2	P_P	P_T	$P_{P\times T}$
Age at first reproduction	104·0 (1·867; 44)	107·2 (1·746; 48)	88·5 (2·157; 44)	95·7 (1·770; 50)	$<10^{-4}$	0·011	0·330
Size at first reproduction	5·03 (0·038; 44)	5·03 (0·046; 48)	5·17 (0·046; 44)	5·24 (0·052; 50)	10^{-4}	0·412	0·492
No. of egg capsules per day	0·485 (0·026; 44)	0·518 (0·027; 48)	0·452 (0·030; 44)	0·482 (0·019; 50)	0·741	0·758	0·996
No. of eggs per day	2·21 (0·114; 44)	2·26 (0·117; 48)	1·96 (0·145; 44)	2·12 (0·091; 50)	0·356	0·614	0·782
No. of eggs per capsule	4·557 (0·091; 44)	4·363 (0·110; 48)	4·336 (0·17; 44)	4·398 (0·10; 50)	0·776	0·966	0·423
T peak eggs	124·9 (2·847; 44)	121·6 (2·847; 48)	137·5 (1·99; 44)	118·0 (0·53; 50)	0·498	0·088	0·226
Growth	2·85 (0·116; 50)	2·86 (0·133; 50)	2·64 (0·082; 50)	2·51 (0·060; 50)	0·003	0·494	0·423
Age at death	204·0 (14·31; 14)	203·2 (9·30; 31)	175·2 (12·47; 29)	205·7 (3·58; 44)	0·139	0·095	0·079
Size at death	6·27 (0·253; 14)	7·07 (0·145; 31)	6·98 (0·284; 29)	7·24 (0·101; 44)	0·038	0·012	0·200

The standard deviations and numbers of individuals analysed are given in parentheses. 'T peak eggs' refers to the time period required to reach the peak of egg production. Age and size at death are given for those individuals that died during the 29 weeks of the experiment. Age and time period are in days, and size is in millimetres. Growth estimates are given as the mean ratios of final size over initial size. P_P, P_T and $P_{P\times T}$ are the probabilities associated with the population, treatment and interaction effects respectively.

in T_2 individuals from QTT (Web Table 1). As expected, the fecundity parameters were in most cases positively correlated with growth in both populations and treatments (all $P<0{\cdot}05$), except for the number of eggs per capsule and the time period required to reach the peak of egg production, which were both negatively correlated with growth in T_2 individuals of QTT. Other significant correlations were found between pairs of life-history traits, but they were not consistent either across treatments or across populations (Web Table 1).

(ii) *Mating activity of paired individuals*

Individuals began copulating around 150–160 days (Table 2), and there was a significant effect of the population factor. Size at first copulation ranged from 6·08 to 6·27 mm, but neither the population, nor the sexual role (male or female) explained its variation. The mean number of copulations (male + female) ranged from 4·71 (DOY) to 7·51 (QTT), i.e. 0·16 to 0·26 copulations per snail and week over the whole experiment. Population was the only significant effect (Table 2). The experimental individuals spent more time copulating as female than as male, and no interaction with the population parameter was detected. Copulation duration showed significant repeatability for the male role in QTT.

We estimated a large number of phenotypic correlations between the mating parameters (size and age at first reproduction, number of copulations, etc.), and again we extract here only those that were significant at $P<10^{-3}$ in both populations, providing a conservative point of view (Web Table 2). There was a significant positive correlation between age at first copulation as male and as female, and age at first copulation in a given role was also positively correlated with size at first copulation in the same role. The lifetime duration of copulations (male + female) was positively related to the lifetime number of copulations. Individuals copulating a lot as male also copulated a lot as female in both time and number, but the trend is weaker in DOY. A less significant ($P=0{\cdot}02$), though interesting, result is the negative correlation between age at first copulation as male and the lifetime number of copulations.

Interestingly, all T_2 individuals began copulating long after initiating egg-laying, and the difference was 59 days in DOY and 39 days in QTT for copulation as female (compare the first row in Tables 1 and 2). This was highly significant whether the first male or the first female copulation was considered (Wilcoxon signed-rank test, $P<10^{-4}$ in all cases). An analysis of correlation between life-history traits on one side and mating parameters on the other suggested that both fecundity (number of egg capsules) and age/size at

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Table 2. *Mean values of estimates of copulation parameters for paired (T_2) G_1 individuals from two populations (DOY and QTT) of* Biomphalaria pfeifferi

	DOY			QTT					
Parameters	♂	♀	Total (♂+♀)	♂	♀	Total (♂+♀)	P_P	P_T	$P_{P\times T}$
Age at first copulation	154·2 (5·30; 40)	155·9 (3·94; 40)	–	135·6 (4·23; 44)	134·7 (2·63; 45)	–	10^{-4}	0·265	0·715
Size at first copulation	6·26 (0·108; 40)	6·27 (0·090; 40)	–	6·13 (0·084; 44)	6·08 (0·088; 45)	–	0·148	0·471	0·706
No. of copulations	2·31 (0·204; 42)	2·40 (0·212; 42)	4·71 (0·355; 42)	3·50 (0·336; 49)	4·06 (0·275; 49)	7·51 (0·613; 49)	$<10^{-4}$	0·111	0·204
Duration of copulations	110·1 (14·93; 42) {−0·007; 0·795}	132·9 (13·37; 42) {−0·012; 0·933}	243·0 (20·76; 42) {−0·008; 0·816}	195·0 (22·73; 49) {0·020; 0·023}	222·4 (21·49; 49) {0·015; 0·410}	417·4 (39·42; 49) {0·04; 10^{-4}}	$<10^{-4}$	0·032	0·679
Time between successive copulations	5·96 (0·552; 28)	4·64 (0·459; 31)	4·16 (0·375; 38)	4·42 (0·437; 37)	3·12 (0·322; 40)	2·45 (0·165; 44)	$<10^{-4}$	0·088	0·363

Age and time are in days, and size in millimetres. For the duration of copulations and the time between successive copulations, we also give the repeatability and its associated *P* value in parentheses.

death were positively associated with the lifetime number and duration of copulations (as male, female, or both) in QTT (all $P<0{\cdot}025$; Web Table 3), though not in DOY. No other tendency was detected in this analysis.

The influence of individual size on fecundity and mating behaviour was evaluated using regression analyses conducted on monthly values in the third and fifth months. The number of eggs increased with individual size in both treatments in QTT and in T_1 in DOY (all *P* values $<0{\cdot}05$), but no significant relationship was detected between the number and duration of copulations as either male or female and individual size.

(iii) *Life-history traits in G_2 individuals and self-fertilization depression*

The hatching rate ranged from 56% to 78% (Table 3), and was affected neither by the treatment nor by the population factor (and the interaction was not significant). Offspring from paired individuals from DOY had a lower hatching rate, though, when analysed separately ($F=11{\cdot}59$, $P=0{\cdot}001$). Survival was higher than 91% over both the first 7 days and the 7–28 day period, and did not differ between treatments and populations. There was a significant effect of the treatment factor on growth over both the first 7 days and the 7–28 day period, but no significant effect of population and no interaction (Table 3).

Consequently, no depression on either parental fecundity and offspring survival was detected (Table 3). The significant increase in hatching rate of offspring from isolated individuals compared with those from paired individuals in the DOY population is an indication of outcrossing rather than inbreeding depression. However, some inbreeding depression was detected on offspring growth during both periods considered, with estimates ranging between 0·135 and 0·173 depending on population and period (Table 3).

4. Discussion

(i) *The selfing syndrome*

(a) *Self-fertilization depression*

No difference in fecundity was detected between G_1 individuals of the two treatments. However juveniles (G_2) from the paired treatment grew faster than those from the isolated treatment, but their hatching rate was lower (in DOY only). On the whole, these results suggest that self-fertilization depression is non-existent to low. Any depression would indeed have resulted in lowered fitness components in T_1 individuals, even if the selfing rate is high (Jarne *et al.*,

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Table 3. *Mean estimates of parental* (G_1) *fecundity and of offspring* (G_2) *hatching rate, survival and growth for isolated* (T_1) *and paired* (T_2) *individuals from two populations* (*DOY and QTT*) *of* Biomphalaria pfeifferi

Parameters	Population	T_1	T_2	δ	P_P	P_T	$P_{P\times T}$
Fecundity	DOY (G_1)	2·21 (0·114; 44)	2·35 (0·123; 40)	0·061	0·275	0·499	0·943
	QTT (G_1)	1·96 (0·175; 44)	2·12 (0·121; 45)	0·068			
Hatching rate	DOY (G_2)	0·783 (0·039; 44)	0·560 (0·042; 40)	−0·398	0·995	0·100	0·100
	QTT (G_2)	0·760 (0·047; 37)	0·674 (0·041; 38)	−0·128			
Survival							
7 days	DOY (G_2)	0·982 (0·007; 38)	0·985 (0·017; 35)	0·003	0·950	0·978	0·952
	QTT (G_2)	0·968 (0·014; 36)	0·989 (0·006; 32)	0·050			
28 days	DOY (G_2)	0·936 (0·020; 38)	0·941 (0·017; 35)	0·005	0·896	0·999	0·878
	QTT (G_2)	0·912 (0·029; 36)	0·957 (0·011; 32)	0·047			
Growth							
0–7 days	DOY (G_2)	1·18 (0·022; 76)	1·37 (0·020; 70)	0·143	0·369	$<10^{-4}$	0·372
	QTT (G_2)	1·18 (0·032; 72)	1·43 (0·043; 64)	0·173			
7–28 days	DOY (G_2)	1·72 (0·038; 76)	2·09 (0·051; 70)	0·170	0·515	$<10^{-4}$	0·270
	QTT (G_2)	1·74 (0·040; 72)	2·01 (0·056; 64)	0·135			

The standard deviations and numbers of individuals analysed are given in parentheses. Fecundity is the number of eggs per day, and growth is the ratio of final size over initial size. δ is the associated self-fertilization (or inbreeding) depression. P_T, P_P and $P_{P\times T}$ are the significance values of the treatment factor, the population factor and their interactions in the generalized linear model associated with these parameters.

1993). Moreover, experimental conditions enforcing the presence of partners tend to decrease the selfing rate in selfing snail species, as shown in another highly selfing snail species, *Bulinus truncatus*, using molecular markers (P. David and P. Jarne, unpublished data). Low inbreeding depression is consistent with an average selfing rate of about 90% in *B. pfeifferi* (Charbonnel *et al*., 2005). Genetic models of the evolution of the selfing rate indeed predict limited inbreeding depression (<0·5) in such species (Charlesworth & Charlesworth, 1999; Goodwillie *et al*., 2005). The review of the empirical evidence in plants confirmed this prediction (Husband & Schemske, 1996), although exceptions can be found (e.g. see Ishida, 2006 for a case of high inbreeding depression in a selfer). A similar result has previously been found in *Bulinus truncatus* (Jarne *et al*., 1993). The timing of inbreeding depression over the life-cycle also differs between inbreeders and outbreeders, with less inbreeding depression in early stages in inbreeders (Husband & Schemske, 1996). This might explain the slight inbreeding depression on growth, as opposed to no or negative inbreeding depression on the hatching rate.

(b) *The waiting time*

Tsitrone *et al*. (2003*a*) suggested that isolated individuals, compared with paired individuals, should delay their age at first reproduction (self-fertilization) in order to wait for future outcrossing. Their model was built for outcrossing species in which selfers incur a strong cost of inbreeding depression. The model also involves external mortality sources, the probability of finding a partner and resource allocation as parameters. The waiting time has indeed been documented in the snail *Physa acuta* (Tsitrone *et al*., 2003*b*; Escobar *et al*., 2007) and the cestode *Schistocephalus solidus* (Schjørring, 2004). An intuitive extension of this model to preferentially selfing species is that no waiting time is expected. Note that 'waiting time' may not have the same meaning in outcrossing and in selfing species, even if it is evaluated experimentally using the same protocol. In

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self-fertile, preferentially outcrossing species, the waiting time refers to the difference between the age at first reproduction of outcrossing (paired) individuals and selfing (isolated) individuals. In selfing species, the waiting time is still the difference in age at first reproduction between paired and isolated individuals, but both types of individuals are at least highly, or perhaps purely, selfing. In the first experimental analysis in animal selfers, we show here that the waiting time is slightly negative (3 and 7 days in DOY and QTT, respectively). A possible explanation for these negative values is that paired individuals were provided a partner once a week for a period representing about 7% of their time. Previous work in *B. truncatus* showed that pairing has a negative effect on traits such as fecundity as a result of resource reallocation (the grouping effect; Doums *et al.*, 1994), and might have delayed the onset of reproduction in paired individuals from our experiment. Further work should aim at evaluating whether the (negative) waiting time is heritable, as found by Tsitrone *et al.* (2003*b*) and Escobar *et al.* (2007) in the outcrosser *P. acuta*. How it evolves when switching between low and high selfing rates is an open question.

(c) *Limited mating activity*

The mating activity has been analysed in several species of freshwater snails (see e.g. Rupp & Woolhouse, 1999; Facon *et al.*, 2006). These experimental studies provided detailed analyses of mating sequences, including courtship and copulation. However, the mating activity was followed from a few hours to a few days. Our goal here was rather to provide a quantitative estimate over a full life-cycle and to estimate the age at first copulation. We recorded few mating events per individual with, on average over 6 months, 0·16 (DOY) to 0·26 (QTT) copulations as both male and female per 10 hour pairing period. This is not much lower than the 0·39 to 0·75 copulations recorded over a similar (and single) period by Rupp & Woolhouse (1999). Many more copulations have been observed over similar periods in outcrossing species. For example, Rupp & Woolhouse (1999) recorded from 1·20 to 2·57 matings per night in *Biomphalaria glabrata*. In *P. acuta*, Facon *et al.* (2006) observed that copulation occurred within an hour in 95% of 288 pairs studied, and counted 2·58 copulations per hour. On the other hand, copulation duration was much longer in our experiment (about 50 minutes) than has been observed in the outcrosser *P. acuta* (13–14 minutes in Wethington & Dillon (1996) and 5–8 minutes in Facon *et al.* (2006)). Mating is preceded by a courtship phase during which the prospective male partner positions itself on the shell of the female partner, who may be more or less receptive, and show rejection behaviour. This courtship phase lasted for 1–2 hours in our experiment, which is also much longer than values reported in *P. acuta* (Wethington & Dillon, 1996; Facon *et al.*, 2006). We hypothesize that the whole mating process (courtship and mating) is much shorter in outcrossing than in selfing species. In other words, mating might be less costly in outcrossing than in selfing species. This implicitly means that there is a significant reward for single mating events in highly selfing species, especially since inbreeding depression is limited. However, the repeatability of copulation duration was significant only for the male role in QTT. Low repeatability was also found in an analysis of mating activity in the land snail *Arianta arbustorum* (Locher & Baur, 2000). This result suggests that the heritability of this trait is low (see Falconer & Mackay, 1996), and selection rather inefficient.

Our experiment also allows some insights into sex allocation in a hermaphroditic organism, a topic of major importance for the evolution of gender (Charnov, 1982; Anthes *et al.*, 2006). Equal investment in the male and female behaviour was our null hypothesis. We actually observed that the experimental individuals spent more time copulating as female than as male. An explanation might be that mates were larger than experimental snails, especially during the first weeks of the experiment. However, the expected preferred role of small individuals is male rather than female, assuming that the female function is more costly (see Charnov, 1982 for theory; Vianey-Liaud, 1998 for results in snails). We also did not observe a shift towards the female role when experimental individuals grew older (and larger). Moreover, we did not find any trade-off between the male and female mating parameters (age and size at first copulation, number and duration of copulations), rather observing significant positive correlations. This is considered in more detail below.

(d) *Conclusion on the selfing syndrome*

As expected, our work indicates limited self-fertilization depression, no (or even negative) waiting time and a limited number of copulations per time unit in the highly selfing *B. pfeifferi*. These are characteristics of the selfing syndrome in snails, as defined by Doums *et al.* (1996), paralleling the occurrence of characters promoting self-fertilization in highly selfing plants (Jain, 1976; Schoen & Lloyd, 1984). The definition of the selfing syndrome can be extended to all life-history traits that are associated with the evolution of high selfing rates. This especially includes developmental, morphological or physiological traits directly involved in reproduction, such as the amount of sperm and seminal fluids produced and transferred during mating. These traits deserved specific investigations. The evolution of high selfing rates also

affects the distribution of genetic variability, with for example less variability within populations than in outcrossers and a reduced strength of natural selection (Jarne, 1995; Charlesworth, 2003). Although not part of the selfing syndrome *per se*, the distribution of genetic variability should be considered when analysing the selfing syndrome, because it drives the evolution of life-history traits (Charlesworth & Charlesworth, 1995). *B. pfeifferi* indeed displays limited neutral variability, even at large geographic scales (Charbonnel *et al.*, 2002*a–c*). The current study suggests that the amount of phenotypic variation may substantially vary among populations, QTT exhibiting significantly more variation in most traits than DOY (results not shown). However, this issue should be addressed using appropriate experimental designs.

(ii) *Prior selfing with residual outcrossing*

Individuals from the paired treatment began laying eggs 40–50 days before initiating copulation with their partners, whether in the male or in the female role. As far as we know, this is the first characterization of prior selfing in animals. Prior selfing has otherwise been detected, and largely studied, in plants (Lloyd, 1992; Davis & Delph, 2005). Pulmonate snails produce both sperm and ovules in a single reproductive structure referred to as the ovotestis, and self-fertilization occurs within the hermaphroditic part of the reproductive tracts (see Jarne *et al.*, 1993), while cross-fertilization requires copulation. Evolving prior selfing therefore seems easy from a functional point of view. However, this does not tell us why prior selfing has evolved in *B. pfeifferi*. Using a general phenotypic model, Lloyd (1992) showed that prior selfing may evolve when inbreeding depression is not too high (i.e. $<0{\cdot}5$), all the more when discounting of female gametes is limited. Inbreeding depression in *B. pfeifferi* seems indeed low enough, but we have no information on gamete discounting and more generally in hermaphroditic animals.

Limited inbreeding depression and reproductive assurance, associated with recurrent demographic bottlenecks, should lead to extremely high selfing rates in *B. pfeifferi* (Charbonnel *et al.*, 2002*b*,*c*). However, the observed mean selfing rate per population is about 90% (Charbonnel *et al.*, 2005), as observed in other species of freshwater snails (Viard *et al.*, 1997; Trouvé *et al.*, 2004). An open question is what maintains this residual outcrossing rate.

(iii) *Correlations between life-history and behavioural traits*

We detected in our analysis a series of positive phenotypic correlations among mating parameters (e.g. age and size at first copulation), among life-history traits, and between mating parameters and life-history traits (e.g. number and duration of copulation vs number of eggs). Moreover, these correlations were more often detected in QTT than in DOY (see Web Appendixes 1 and 2). Similar results have been found in other studies of snails (Locher & Baur, 2000; Vianey-Liaud & Dussart, 2002). This is surprising since some negative correlations were expected in our study, as a result of re-allocation between fitness-associated traits (Reznick, 2000). However, when resource assimilation is genetically variable, genotypes with high assimilation rates have more resources to allocate to all aspects of their life history, and positive genetic correlations should arise (Van Noodwijk & de Jong, 1986). As far as fecundity is concerned, this is a plausible explanation in our experiment since the number of eggs increased with individual size in both populations. On the other hand, no correlation was found for mating activity.

We found no difference in fecundity between selfing and paired individuals, whether we considered the period prior to first copulation (in T_2 individuals) or after first copulation (results not shown). The partner presence therefore does not affect the fecundity of T_2 individuals, and no resource reallocation to reproduction occurs. We also failed to find a difference in growth between treatments prior to copulation of T_2 individuals. However, T_1 individuals grew faster than T_2 individuals after copulation was initiated (results not shown), suggesting a possible trade-off between mating activity and growth. Why this trade-off does not translate into a fecundity/mating activity trade-off remains unclear. Fecundity and size are indeed correlated in freshwater snails (Norton & Bronson, 2006).

(iv) *Population effect*

A population effect was detected for most traits studied (see Tables 1–3). For example, QTT individuals had their first copulation earlier, copulated more frequently and longer than individuals from DOY, and exhibited higher fecundity, growth and survival as well. More variance in traits was also found in QTT than in DOY, and phenotypic correlations between traits were more often significant in QTT than in DOY. This is indicative of more genetic variance in QTT than in DOY. The explanation might depend on the particular ecological conditions experienced by these two populations. QTT is a permanent pond, while DOY is a paddy field where water is not available all the time. There is ample evidence in freshwater snails that water permanence affects the amount of neutral genetic variability (e.g. Trouvé *et al.*, 2003; Bousset *et al.*, 2004), and this is also true in *B. pfeifferi* (Charbonnel *et al.*, 2002*a–c*). However,

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the amount of neutral genetic variability might not be representative of that at loci encoding life-history traits. For example, water permanence is not a good indicator of quantitative genetic variation in the selfing freshwater snail *Galba truncatula* (Chapuis *et al*., 2007). An alternative explanation is that DOY individuals are less well adapted than QTT ones to the laboratory conditions proposed here.

P.J. dedicates this manuscript to Deborah Charlesworth, and warmly thanks her for help and guidance over the last (almost) 20 years. We thank members of the non-governmental organization ODAFEM for help during snail sampling, Chitou Bassirou for statistical advice, and Gonédélé Bi Sery, Patrice David, Juan Escobar, Coulibaly Yahya and two reviewers for discussion and/or constructive comments on an earlier draft of this manuscript. This work received financial support from the Swiss Development Cooperation (SDC) allocated to the Centre Suisse de Recherches Scientifiques (CSRS) via a project entitled 'Contribution to the process of national reconciliation in Côte d'Ivoire' (AVS).

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