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RESEARCH ARTICLE

## Experimental evidence of inbreeding avoidance in the hermaphroditic snail *Physa acuta*

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**Abstract** Inbreeding depression should select for inbreeding avoidance behaviours. Here we test this hypothesis in two populations of the simultaneous hermaphroditic freshwater snail *Physa acuta*. We recorded the copulatory behaviour of 288 pairs of sib-mates, non-kin mates from the same population, or non-kin mates from two different populations. We find that kin discriminatory behaviours exist in this species, exclusively expressed by individuals playing the female role. We discuss the relevance of our finding in the context of the evolution of recognition systems and the consequences of such a behaviour in natural populations.

**Keywords** Mating behavior · Inbreeding avoidance · Mate choice · Freshwater snail · Hermaphroditic · Intersexual conflict

### Introduction

Spatial and temporal landscape heterogeneity may cause populations to undergo small effective size or punctual bottlenecks. By restricting opportunities for mating, small populations foster inbreeding via mating among relatives. Such crosses often result in depressed fitness progeny, a phenomenon referred to as inbreeding depression (Keller and Waller 2002; Glémin et al. 2003). In species suffering from inbreeding depression, selection should theoretically favour the emergence of mechanisms to avoid crosses between closely related

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individuals (Pusey and Wolf 1996; Meagher et al. 2000; Kruuk et al. 2002; Tregenza and Wedell 2002). In animals, these include sex-specific dispersal (Perrin and Mazalov 1999; Lehmann and Perrin 2003) and kin-discriminatory mate choice (Pusey and Wolf 1996; Reinhold 2002).

Mate choice entails either pre-copulatory partner choice behaviours (Blouin and Blouin 1988; Simmons 1991), or post-copulatory mechanisms. The latter may for instance rely on a female mating multiply to avoid having all its eggs fertilised by a poor sperm donor (i.e. dilution effect; Pizzari et al. 2004) as well as female cryptic sperm selection (Bishop 1996; Birkhead and Pizzari 2002). Furthermore as males are thought to have a higher reproductive potential than females (Bateman 1948; Trivers 1972), inter-sexual conflicts may exist over inbreeding avoidance behaviours (Smith 1979; Parker 1983; Perrin and Mazalov 2000), females being expected to be choosier than males. Congruently, in some species where males have a high investment in sperm production (e.g. nuptial gift), males should be as choosy as females (e.g. Lewis et al. 2004).

Both inbreeding avoidance and sex-specific inbreeding avoidance have been reported from numerous species with separate sexes (e.g. Pizzari et al. 2004, see Pusey and Wolf 1996 for a review). Inbreeding avoidance is also known from hermaphroditic plants in the form of incompatibility loci (e.g. Charlesworth and Awadalla 1998; Glémin et al. 2001). In simultaneous hermaphroditic animals, where sex-biased dispersal is impossible, inbreeding avoidance can only rely on mate choice.

Reproductive structures of hermaphroditic animals often show amazing complexity, and mating behaviour can be elaborate (Michiels 1998; Schilthuisen 2005). Prolonged courtship gives ample opportunity for partner assessment, which may set the stage for mate choice. Several studies have been carried out in hermaphroditic animals to test mate choice with respect to different parameters such as body size (e.g. Vreys and Michiels 1997; Ohbayashi-Hodoki et al. 2004; Jordaens et al. 2005) or geographic origin (Baur and Baur 1992). However, studies investigating the consequences of relatedness on sex-specific mate choice in hermaphroditic animals are still scarce (but see Peters and Michiels 1996; Baur and Baur 1997). One possible reason is that many hermaphroditic species have simultaneous bilateral sperm transmission. In such cases, both individuals play symmetrical roles, and distinguishing between male-specific and female specific mate choice behaviours is difficult.

*Physa acuta* is a good candidate for investigating sex-specific inbreeding avoidance behaviours, as in this simultaneous hermaphroditic freshwater snail, individuals may be either male or female at each copulation (but not the two simultaneously). Sex-roles can thus easily be distinguished. Although self-fertilisation is possible, *P. acuta* is a preferential outcrosser with selfing rates generally lower than 0.1 in natural populations (Jarne et al. 2000; Bousset et al. 2004). Large self-fertilisation depression was detected both in laboratory conditions (up to 0.9 over a full life-cycle, Jarne et al. 2000) and in the field (Henry et al. 2003). The ecology of the species also implies that it is likely to undergo inbreeding through extremely small population sizes. It is an invasive species and European populations were recently founded presumably from a North American source (Dillon et al. 2002) through demographic and genetic bottlenecks. Moreover, the species often dwells in temporary habitats and thus goes through recurrent bottlenecks and/or extinctions (Städler and Jarne 1997). Even large populations may show drastic variations in effective size. Critical ingredients are thus gathered for *Physa acuta* to exhibit kin discrimination in its mate choices.

Here we test whether *P. acuta* exhibits inbreeding avoidance behaviours both at the scale of one population (kin vs. sympatric) and at the scale of two populations (kin vs. allopatric). We find that inbreeding avoidance behaviours exist in this species and are mainly due to kin rejection expressed when playing the female role.

## Material and methods

### Populations studied

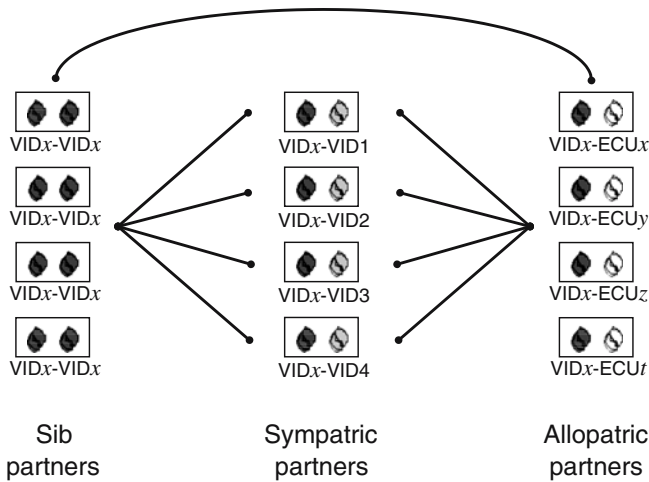
Two populations were studied and, respectively sampled at Vidy (VID; 46° 31' N, 6° 36' E) and Ecublens (ECU; 46° 31' N, 6° 32' E) near Lausanne (Switzerland). These populations differ with respect to environmental conditions. Standing in a harbor of lake Léman, VID exhibits a permanent water regime and temperatures are relatively stable. On the contrary, ECU is a small irrigation canal often dry in summer and very cold in winter. Although densities can drastically vary in both populations (B. Facon, pers. obs.), the carrying capacity and effective size are doubtlessly higher in VID than in ECU. The dynamics of inbreeding depression, the intensity and efficiency of selection may thus differ between these two populations. They will therefore be treated separately in the analysis.

### Rearing protocol

Throughout the experiment, snails were maintained at 24°C under a 12L:12D photoperiod and fed *ad libitum* with boiled lettuce. Mature individuals ( $G_0$ ) were sampled in each population (in December 2004), brought back to the laboratory, and isolated in 75 ml plastic boxes filled with water. As body size and age may influence the mating behaviour of *P. acuta* (Ohbayashi-Hodoki et al. 2004),  $G_1$  offspring similar with respect to size and age were obtained by collecting clutches only during the first 2 days in the lab. This restricted the experiment to 15 families of 16  $G_1$  offspring for ECU and 16 families of 16  $G_1$  offspring for VID. If reared in isolation, these snails take longer to reach sexual maturity (Tsitrone et al. 2003) and are craving so much for allosperm than they are not expected to show any discrimination concerning their partner's identity on the first encounter (Facon pers. obs.).  $G_1$  juveniles from different families were thus mixed in boxes of 20 individuals, as soon as they were large enough (>2 mm) to be marked with gouache paint previously shown to be harmless (Henry et al. 2003). Groups were carefully composed so as to avoid any bias due to relatedness in their breeding environment. This treatment lasted until all snails were sexually mature. It thus allowed individuals to mate freely (around 50 days after clutches were produced; see Monsutti-Grecescu 1998). Individuals were then isolated during 10 days. This ensures that all tested individuals will seek for new copulations during the pair tests (Wethington and Dillon 1996).

### Pair tests

We recorded the mating behaviour of individuals from 12 families in each population when they were presented to partners of different levels of relatedness. Figure 1 shows the experimental design used for the population VID, the same design was used for ECU. Twelve focal  $G_1$  individuals per family were tested. Four were presented to a partner sampled among the 12 families of the other population. Pairs were carefully chosen to avoid any association between families from the two populations. Four were presented to a non-kin partner of the same population sampled among the three remaining families not serving as tested families for ECU and the four remaining families for VID. The last four  $G_1$  individuals of each family were presented to a sib. Once formed, each pair was observed during one full hour. All observations were obtained within a 3-days period.



**Fig. 1** Pairs tested for their reproductive behaviour in each family. The design is given for a VID family but is similar for an ECU. Twelve individuals of the tested family VID<sub>x</sub> are divided into three groups of four. Individuals of the first group receive a sib partner. Individuals of the second group are confronted to a partner of the same population (one of the four VID families used as sources of such partners). Individuals of the third group receive a partner randomly drawn in a family of the other population ECU. Lines show the pair comparisons used for the statistical analysis, which results are shown in Tables 1 and 2

A typical copulation involves (i) approach and climbing onto the shell of an intended female by a male role snail, (ii) crawling to the female gonophore, (iii) preputium eversion for intromission (copulations may last up to 15 min), and (iv) dismounting (Ohbayashi-Hodoki et al. 2004). Male rejection behaviours, if they exist (as suggested by McCarthy 2004) would take the form of mounting avoidance and early dismounting. Female rejection behaviours may express through shell swinging and phallus biting. During the 1 h observation, we recorded the time to each contact, copulation and dismounting. For each copulation, we also recorded individual gender role and occurrence of rejection behaviour. The following variables were analysed: time to first copulation ( $T_{1st\ cop}$ ), total duration of all observed copulations (phase iii) during the observation period ( $D_{cop}$ ) and proportion of copulations where rejection occurred ( $P_{rej}$ ).

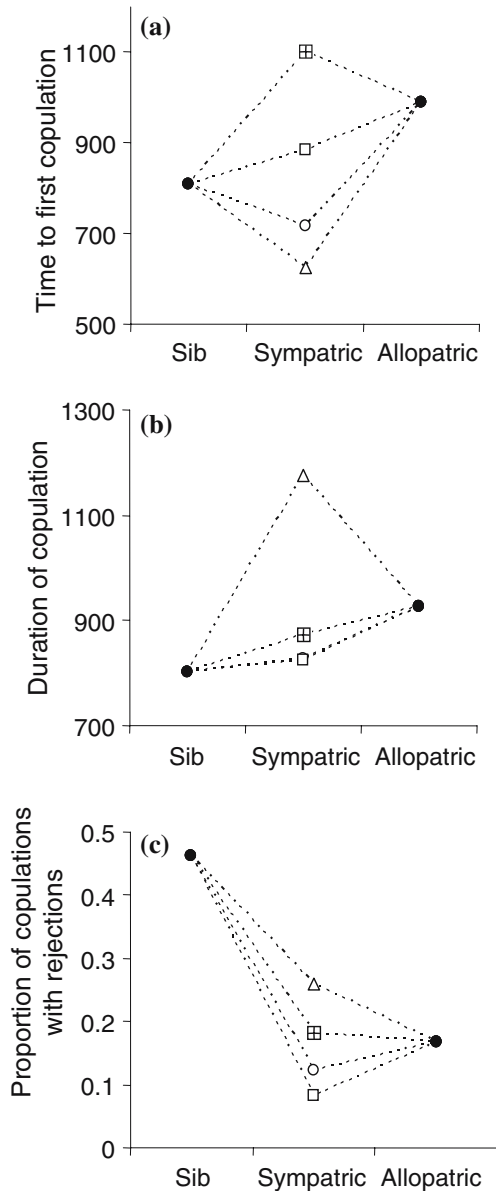
### Statistical analyses

Statistical analyses were conducted using the software R (R development Core team 2004). For the three variables, averages of the four replicates per family were calculated for each mating type. Differences between kin and non-kin mating (Sib vs. Sympatric and Sib vs. Allopatric) were tested using a unilateral matched-pairs Wilcoxon test, with the expectation that non-kin partners copulate earlier ( $T_{1st\ cop}$ ), have a longer copulation period ( $D_{cop}$ ) and a lower rejection propensity ( $P_{rej}$ ) than kin partners. Differences between sympatric and allopatric mating trials were tested using a bilateral matched-pairs Wilcoxon test, as we had no *a priori* expectations for these. In order to avoid pseudo-replication caused by the availability of only three ECU and four VID families of sympatric partners, differences involving sympatric pairs were tested separately for each sympatric family (Fig. 1). Differences between the two populations were checked using a bilateral Wilcoxon test on the sib and sympatric mating only, as allopatric crosses of each population are not independent.

**Results**

Within the 1 h observation period, 94.7% of the pairs copulated at least once. The number of copulations observed in 1 h ranged from 0 to 7 for a mean number of copulations of 2.58 and a mean copulation time of 308.0 s. Figures 2 and 3 show the mean family reaction norms for VID and ECU, respectively. Mean values of the traits over all families as well as *P*-values are given in Tables 1 and 2.

**Fig. 2** Effect of relatedness on mating behaviour in the population VID. **(a)** Time of first copulation ( $T_{1stcop}$ ). **(b)** Total duration of copulation ( $D_{cop}$ ). **(c)** Proportion of copulations with rejections ( $P_{rej}$ ). For the sympatric treatment, mean behaviours were shown separately for each partner family (square: VID1; circle: VID2; triangle: VID3; cross: VID4). Standard errors and statistical significance of the paired tests are given in Table 1. The mean number of copulations was 2.60 for sib pairs, 2.79, 2.82, 4.09, 2.33, respectively, for sympatric pairs involving VID1, VID 2, VID3 and VID4, and 2.85 for allopatric pairs

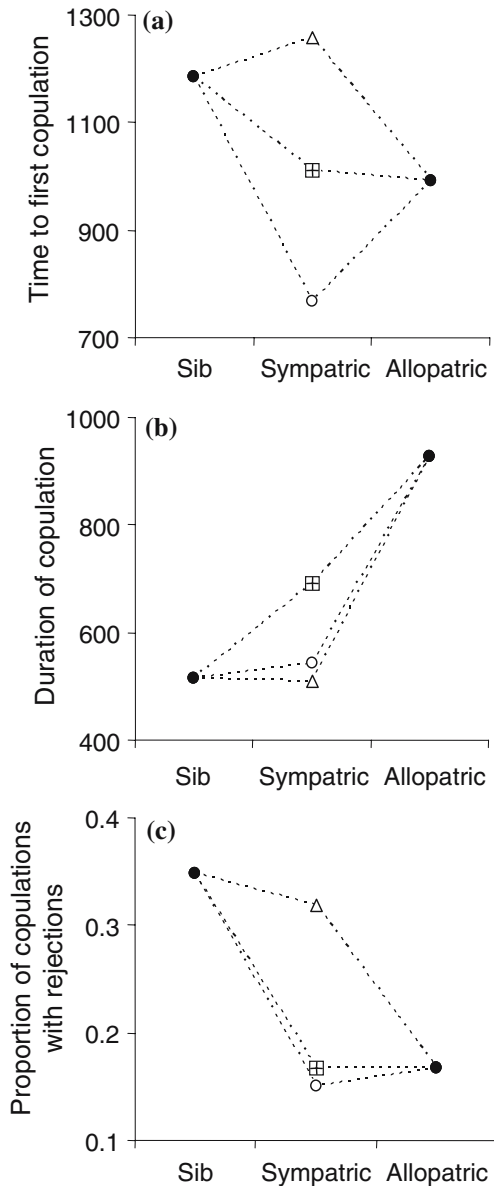


VID population

The time to first copulation was not significantly different between the three treatments except between allopatric mating and the sympatric line VID3 (Table 1 and Fig. 2a).

Kin partners tended to mate for shorter time than non-kin but this trend was significant only for allopatric partners and for the sympatric family VID3 (Table 1 and Fig. 2b).

**Fig. 3** Effect of relatedness on mating behaviour in the population ECU. **(a)** Time of first copulation ( $T_{1stcop}$ ). **(b)** Total duration of copulation ( $D_{cop}$ ). **(c)** Proportion of copulations with rejections ( $P_{rej}$ ). For the sympatric treatment, mean behaviours were shown separately for each partner family (cross: ECU1; circle: ECU2; triangle: ECU3). Standard errors and statistical significance of the paired tests are given in Table 2. The mean number of copulations was 1.96 for sib pairs, 2.37, 2.17, 2.07, respectively, for sympatric pairs involving ECU1, ECU2 and ECU3 and 2.85 for allopatric pairs



**Table 1** Mean values of the three traits for each kind of cross and results of the Wilcoxon tests for the VID population

	Kin	<i>P</i> -value	VID1 VID2 VID3 VID4	<i>P</i> -value	Allopatric
T <sub>1stcop</sub>	808.37±100.97	0.58	885.5±149.70	0.38	990.24±128.07 <i>P</i> = 0.95
		0.16	715.56±170.33	<b>0.10</b>	
		0.14	624.75±99.10	<b>0.01</b>	
		0.90	1100.0±212.34	0.52	
D <sub>cop</sub>	801.88±72.38	0.71	824.09±215.72	0.70	927.25±73.60 <b><i>P</i> = 0.05</b>
		0.62	828.25±199.16	0.56	
		<b>0.05</b>	1176.0±159.45	0.19	
		0.48	871.82±207.30	0.32	
P <sub>rej</sub>	0.46±0.10	<b>0.02</b>	0.08±0.04	0.32	0.17±0.03 <b><i>P</i> = 0.02</b>
		<b>0.05</b>	0.12±0.08	0.33	
		<b>0.08</b>	0.26±0.07	0.56	
		<b>0.01</b>	0.18±0.08	1	

The left (right) “*P*-value” column contains *P*-values of Wilcoxon tests between sib (allopatric) mating and sympatric mating with each sympatric family. The *P*-Value in the “Allopatric” column corresponds to differences between sib and allopatric mating

**Table 2** Mean values of the three traits for each kind of cross and results of the Wilcoxon tests for the ECU population

	Kin	<i>P</i> -value	ECU1 ECU2 ECU3	<i>P</i> -value	Allopatric
T <sub>1stcop</sub>	1185.83±132.71	0.19	1010.25±175.17	0.85	990.24±128.07 <i>P</i> =0.17
		0.62	1257.05±180.65	0.37	
		<b>0.03</b>	767.0±121.64	0.19	
D <sub>cop</sub>	514.15±63.30	0.16	692.27±187.06	0.24	927.25±78.02 <b><i>P</i>=0.001</b>
		0.55	507.95±87.27	<b>0.01</b>	
		0.54	543.25±71.86	<b>0.02</b>	
P <sub>rej</sub>	0.35±0.09	<b>0.06</b>	0.17±0.07	0.92	0.17±0.04 <b><i>P</i>=0.05</b>
		0.27	0.32±0.10	0.19	
		<b>0.03</b>	0.15±0.04	0.72	

The left (right) “*P*-value” column contains *P*-values of Wilcoxon tests between sib (allopatric) mating and sympatric mating with each sympatric family. The *P*-value in the “Allopatric” column corresponds to differences between sib and allopatric mating

Rejections were more frequent between kin partners than between allopatric partners and between sympatric partners (Table 1 and Fig. 2c). There were no significant differences in the proportions of rejections between sympatric and allopatric partners.

ECU population

The time to first copulation did not significantly differ between the three types of mating except between sib mating and the sympatric family ECU3 (Table 2 and Fig. 3a).

Kin partners tended to mate for a shorter time than non-kin but this trend was significant only for allopatric partners. Allopatric partners also mated significantly longer than sympatric partners for two sympatric families (Table 2 and Fig. 3b).

The type of mating strongly affected the proportion of copulations with rejections inflicted by female-role snails (Table 2 and Fig. 3c). Rejections were more frequent between kin partners than between allopatric partners and between sympatric partners for two sympatric families. There were no significant differences of proportion of rejections between sympatric and allopatric partners.

### Comparisons between populations

There was no difference between the two populations with regards to rejection propensities ( $P_{\text{rej}}$ ). When given a kin partner, VID copulated earlier ( $T_{1\text{stcop}}$ ,  $P=0.05$ ) and had a longer copulation period ( $D_{\text{cop}}$ ,  $P=0.005$ ) than ECU. When given a sympatric partner, VID also copulated earlier ( $T_{1\text{stcop}}$ ,  $P=0.09$ ) and had a longer copulation period ( $D_{\text{cop}}$ ,  $P=0.02$ ) than ECU. Noticeably in both populations, the consequences of relatedness on mating behaviour differed according to the family considered.

## Discussion

### Evidence of inbreeding avoidance behaviours

Our results reveal the existence of kin discriminatory mate choice behaviours in *Physa acuta*. The higher the partners relatedness, the more frequent the rejection attempts. These rejections take the form of shell swinging and phallus biting inflicted by female role snails to their partner. It has been suggested that rejections by male acting snails also exist, for instance through mounting avoidance and/or interrupted copulations (McCarthy 2004). When two individuals separate without obvious female rejection, it is difficult to attribute the separation to a male rejection with certainty. The effect of male rejection may, however, be measured through an increased time to first contact, an increased time to first copulation and shortened copulations. We found no evidence of a difference in the time to first contact (result not shown) or the time to first copulation between kin and non-kin pairs, suggesting that male acting snails do not avoid copulation with kin partners. Copulation duration tended to be shorter for kin than non-kin pairs, the trend being only clearly significant in population ECU. But this pattern could stem from a higher female rejections rate. Therefore discrimination as male if it exists seems not as strong as discrimination as female. This corroborates the hypothesis that male function is cheaper than the female in this species.

This finding raises the question of how recognition is achieved. Freshwater snails can perceive chemical signals in the water and on the ground. In particular, growth (Kawata and Ishigami 1992), fecundity (Levy et al. 1973) and motion (Karowe et al. 1993) differ in presence of conspecific signals as compared to signals from other species. In the snail *Biomphalaria glabrata*, mate choice based on partner infection status was suggested to involve the recognition of chemical compounds in the snail's mucus or shell composition, comparable to the vertebrate MHC, which may be detected during the mutual allo-grazing periods that precede copulation (Webster et al. 2003). Here we find that neither the time to first contact nor the time to first copulation are affected by partner relatedness. As snails are relatively mobile, this suggests that recognition is not mediated by chemical compounds dissolved in the water but rather occurs at penis intromission or later trough contact signals (e.g. as in rotifers, Snell and Morris 1993).



In our experiment, greater rejection rates were associated with shorter copulations as expected. More surprising is that this trend was only clearly significant for comparisons with allopatric partners. One can then wonder whether rejections are an efficient mean to end up an unwilling copulation in the wild. Beyond a potential lack of statistical power for comparisons implying sympatric partners, one can imagine that the “no choice” design gives an advantage to males in the inter-sexual conflict over mate choice. In this experimental design, only one male is available and the female is confronted several times to the same partner. The efficiency of rejections is thus reduced. In nature on the contrary, individuals might have fewer opportunities to meet again after an aborted copulation. That a female successfully rejects a male gives her access to other males with different relatedness. Rejections could thus prove more efficient at shortening unwilling copulations in nature than suggested by our experiment.

Genetic load has been shown to vary among families (Jarne et al. 2000; Henry et al. 2003). If fully adaptive, inbreeding avoidance should depend on the number of deleterious mutations carried in a family, i.e. the family genetic load. Avoiding mating with kin is only adaptive in families with a high genetic load. Then mating choices should differ between families. Although the present design does not allow testing for such differences, inbreeding avoidance behaviours show some variation among families. For instance, some families seem to reject kin partners less than others. Family differences could be due to either genetic or early environmental processes. Checking whether inbreeding avoidance is conditional on family genetic load requires further investigation. In particular, a simultaneous assessment of family mate choice behaviour and inbreeding depression is clearly needed.

#### Implications for natural populations

Whether the pre-copulatory behaviours of inbreeding avoidance highlighted here are a major determinant of mating patterns in natural populations needs to be further investigated. Wethington and Dillon (1993) have detected incompatibility between gametes of the same individual and to a lesser extent between gametes of different individuals. This suggests that the mechanistic basis for female cryptic sperm selection exist, although conclusive evidence is still lacking. Post-copulatory mate choice may thus reinforce inbreeding avoidance in this species. If cryptic sperm selection is possible, conflicts should be less intense when several partners are available as compared to a one-to-one situation. When several partners are available, low quality copulations may have little consequences on fecundity and offspring performance. In one-to-one interactions, conflicts should intensify, both individuals willing to be a male in presence of a sib (Angeloni et al. 2002). The relative importance of pre- and post-copulatory mate choice could thus well differ according to partner availability in the wild.

The existence of kin discriminatory mate choice was expected in this species. First it suffers from high levels of inbreeding depression (Jarne et al. 2000), possibly linked to the recurrent bottlenecks that may have accompanied its recent colonisation of Europe (Dillon et al. 2002). Second, *P. acuta* exhibits delayed selfing, that can be seen as a form of inbreeding avoidance (Tsitroni et al. 2003). Inbreeding depression is thus high enough in this species for some inbreeding avoidance to evolve. Restricting inbreeding avoidance to self-fertilisation avoidance would only be adaptive in well-mixed and large populations where the probability to mate with a kin is low. Congruently with this idea, it seems that ECU, the small population, exhibits greater choosiness than VID, the large lake population.

More surprising is the existence of behaviours favouring mating with allopatric over sympatric partners. In population ECU, snails also mate longer and make less rejection attempts when interacting with an allopatric partner than with a sympatric partner. These behaviours may simply exist as a by-product of the evolution of a kin-discrimination system selected at a local scale. Alternatively they may be selected directly. That ECU exhibits greater preference for VID individuals than the reverse is consistent with the selection hypothesis: small populations are more subject to drift and random fixation of deleterious mutations. The benefit of heterosis through allopatric mating might thus be greater for ECU than VID.

For the two populations considered here, no evidence of outbreeding avoidance was found at the scale considered here. This differs from a previous experiment where greater avoidance rates were found in allopatric pairs as compared to sympatric ones using two American populations of *Physa acuta* (called *P. gyrina* in the original paper, McCarthy 2004). *P. acuta* has a world-wide distribution and occupies a great variety of habitats (temporary or permanent ponds, lakes, rivers...). In Europe, population differentiation at neutral loci has been documented at several scales (Bousset et al. 2004), and the same is likely true for loci and traits under selection. If local adaptation occurs, outbreeding depression and thus outbreeding avoidance should be found at some scale. Since inter-fertility between *P. acuta* and two other *Physa* species has recently been documented (Dillon et al. 2002), the scale at which outbreeding depression could occur is huge. Fitness estimates of crosses of increasing partner distance, ranging from self-fertilisation to inter-specific cross, could allow determining the optimal distance of crosses in this species. This large scale makes *P. acuta* an ideal animal model for investigating the relative importance of genetic load and local adaptation in natural populations.

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