



## Effect of mating history on gender preference in the hermaphroditic snail *Physa acuta*

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Several internally fertilizing hermaphroditic animals can only perform one sexual role at a time. In such species, two individuals that engage in a copulation may have different interests in acting as male or female. A gender choice must be made which, if both individuals have the same preference, may give rise to a severe sexual conflict. Here we tested the hypothesis that gender choice could be influenced by mating history, using the freshwater snail, *Physa acuta*. We recorded the copulatory behaviour of 240 pairs composed of a focal individual and a partner, each either short- or long-isolated. We found that the time to the first copulation was unaffected by isolation status, suggesting that first contacts in this species are random processes. In contrast, the duration of copulations and the frequency of rejection behaviours suggested that individual gender preference switches from male biased to female biased as isolation increases. In addition, snails rejected copulations more frequently when presented to a partner with the same isolation status. Reciprocity, measured as the rate of gender swapping between the first and second copulations, was high irrespective of gender status. We suggest possible evolutionary causes for this gender preference switch and discuss its potential importance in natural population as well as its consequences for the maintenance of hermaphroditism.

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Hermaphroditic animals have long been considered as unaffected by sexual selection (Anderson 1994). Initially, these organisms were thought to have too low perceptual and cognitive abilities to show proper sexual preferences (Darwin 1871). More recently, it has been argued that the occurrence of both sexes within each individual may limit sexual selection for traits involved in mate choice (Greeff & Michiels 1999). However, hermaphroditic animals with internal fertilization show an impressive variety of sexual traits and behaviours such as penis chewing in *Ariolimax* (Leonard et al. 2002) or dart shooting in some

terrestrial snails (Pomiankowski & Reguera 2001; Schilthuisen 2005). These numerous and diverse reproductive features suggest that sexual selection does play a key role in the evolution of hermaphroditic mating systems (Schilthuisen 2005; Leonard 2006).

In species with separate sexes, sexual selection is driven by the conflicting interests of males and females. Gender role being fixed, it is not a source of conflict in these species. In contrast, when two hermaphroditic animals engage in a copulation, each of them may have an interest in acting as a male (donate autosperm), as a female (receive allosperm) or both. A gender choice must thus be made (Wethington & Dillon 1996). Models of sexual conflicts in hermaphrodites predict that there should be a preferred gender role (Bateman 1948; Charnov 1979; Michiels 1998). Because the male function (producing sperm) is thought to be energetically cheaper than the female one (producing ovules) (Locher & Baur 2000), the preferred gender should be male (Bateman's principle, Bateman 1948; Charnov 1979). In a theoretical paper,

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however, Greeff & Michiels (1999) have recently emphasized that acting as a female is not necessarily more costly than acting as a male notably in species with multiple mating and sperm digestion. Moreover, because of sperm competition and cryptic sperm selection, the variance in reproductive success is lower in females than in males. The preferred gender role may thus be female in a number of species (Gillespie's principle, Leonard & Lukowiak 1991; Leonard 1999). Although reaching opposite conclusions, these theories agree that in a given hermaphroditic species, one gender role should consistently be preferred over the other, thereby providing frequent opportunities for sexual conflict.

Recent empirical studies, however, suggest that there is not a consistently preferred gender role. Gender preference may even change within an individual during the course of a mating session as shown in *Navanax inermis* (Michiels et al. 2003). Various factors may affect the preferred gender role. First is body size (DeWitt 1991; Gianguzza et al. 2004; Ohbayashi-Hodoki et al. 2004), where bigger individuals usually prefer being female (Petersen & Fischer 1996; Angeloni & Bradbury 1999; Schärer & Wedekind 2001). Partner availability has also been shown to generate biases in gender allocation (Trouvé et al. 1999; Tan et al. 2004; Schärer et al. 2005; Schleicherova et al. 2006). There is also growing evidence that the time between two copulations (hereafter isolation time) may influence gender preference (Van Duivenboden & Ter Maat 1985; Wethington & Dillon 1996). It is commonly argued that the probability of initiating a copulation as a male increases with isolation time presumably because of the subsequent increase in stored autosperm (Van Duivenboden & Ter Maat 1985; Wethington & Dillon 1996; De Boer et al. 1997; Locher & Baur 1999; Michiels et al. 2003). Because females may pay minimal energetic costs during copulations and may benefit from sperm and seminal fluid digestion (Greeff & Michiels 1999), they are thought to be always receptive (Van Duivenboden & Ter Maat 1985; Wethington & Dillon 1996). These results were, however, obtained over short-isolation periods (a few days). It is questionable whether autosperm levels continue to increase with time over longer periods (Wethington & Dillon 1996). Moreover, in species where inbreeding depression is strong (and thus self-fertilization should be avoided), female receptivity is expected to be negatively correlated with isolation time (Wethington & Dillon 1996; Tan et al. 2004). Individuals isolated for long enough to lack allosperm should thus prefer the female role, until their allosperm store is replenished.

In the present paper, we study the hypothesis that isolation time influences gender preferences over long periods using the hermaphroditic snail, *Physa acuta*. We experimentally test the two predictions of this 'isolation-time' hypothesis. (1) As suggested by previous studies, individuals isolated for a few days should prefer the male role since their store of allosperm is not yet depleted and their store of autosperm should have been replenished. In contrast, individuals isolated for longer, should lack allosperm and thus prefer the female role. (2) If gender preference correlates with isolation time, then two individuals

that experienced the same isolation time should share the same gender preference. Conflicts should therefore be more intense between two individuals that have experienced the same isolation time than between two individuals with different isolation times.

## METHODS

### Species Studied

*Physa acuta* is a simultaneous and internally fertilizing hermaphroditic freshwater snail (Basommatophora) with a sperm storage organ (Paraense & Pointier 2003). Although self-fertilization is possible, it prefers outcrossing with selfing rates generally lower than 0.1 in natural populations (Jarne et al. 2000; Bousset et al. 2004). Large self-fertilization depression was detected in laboratory conditions (up to 0.9 over a full life cycle, Jarne et al. 2000). As in all Basommatophoran pulmonates, only one gender role can be performed at a time (Wethington & Dillon 1996). Gender roles can easily be distinguished. As in other closely related species (Van Duivenboden & Ter Maat 1985; DeWitt 1991), a typical copulation involves (1) approach and climbing onto the shell of an intended female by a male role snail, (2) crawling to the 'female' gonophore, (3) preputium eversion for intro-mission (copulations may last up to 30 min), and (4) dismounting (Wethington & Dillon 1996; Ohbayashi-Hodoki et al. 2004; Facon et al. 2006). Moreover, when acting as females, individuals can display rejection behaviours such as shell swinging and phallus biting (Wethington & Dillon 1996; Ohbayashi-Hodoki et al. 2004; Facon et al. 2006).

### Rearing Protocol

Twenty mature individuals ( $G_0$ ) were sampled in an irrigation canal in Ecublens (46°31'N, 6°32'E; Switzerland), 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), we collected clutches only during the first 2 days in the lab to obtain  $G_1$  offspring similar with respect to age and size. Two weeks later, 24  $G_1$  offspring per  $G_0$  individuals (20 families) were each isolated in 75-ml plastic boxes for 5 more weeks. After this period, individuals were allowed to mate freely as males and females, so as to avoid any bias due to reproductive history. To this purpose,  $G_1$  juveniles from different families were mixed in boxes of 20 individuals (one individual per family) during 2 weeks, after being marked with gouache paint previously shown to be harmless (Henry et al. 2003). *Physa acuta* reaches female maturity around 5 weeks (Tsitrone et al. 2003) after passing a short male stage ('slight protandry'; Wethington & Dillon 1993). Since individuals in this experiment are 9 weeks old, they should have received allosperm from one or more partners. The 24  $G_1$  juveniles of each family were then randomly assigned to one of the two treatments (Fig. 1): (1) 30 days of complete isolation (LI for long-isolation; 12 individuals/family), and (2) 24 days during

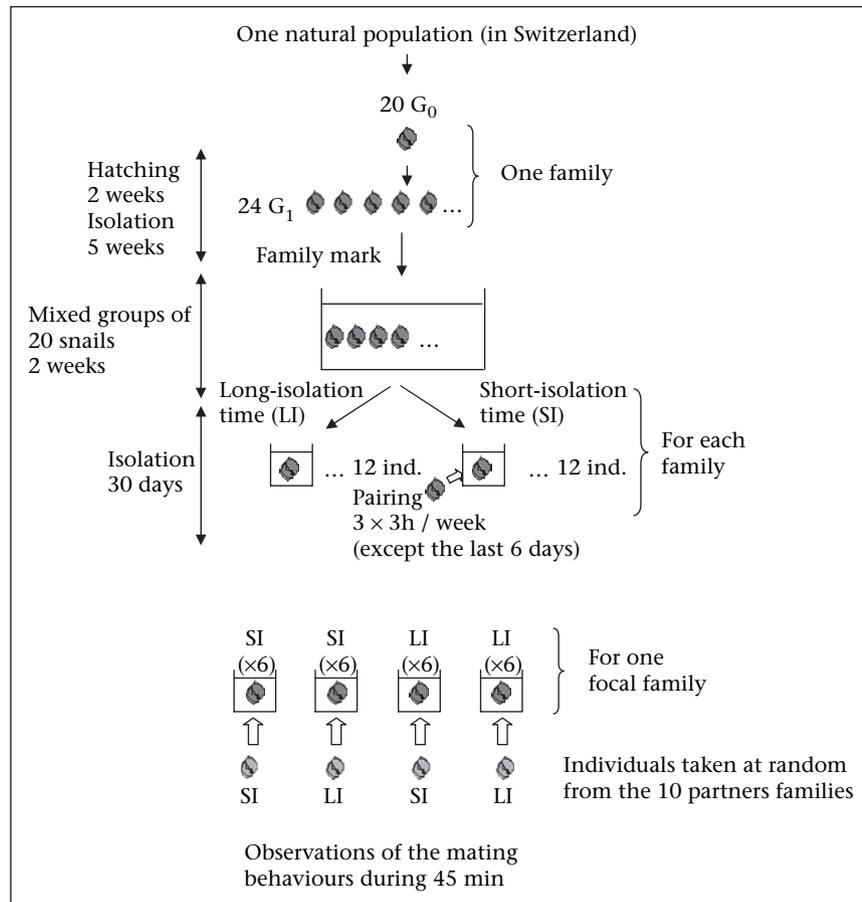


Figure 1. Schematic representation of the rearing protocol.

which they were presented a partner for 3 h every 2 days to permit copulations (see Tsitrone et al. 2003), followed by 6 days of complete isolation (SI for short-isolation; 12 individuals/family). Noticeably, an isolation of 6 days should allow snails to rebuild their store of autospERM (Wethington & Dillon 1996). All snails regularly laid eggs during these 30 days with no noticeable difference between isolation treatments in the rate of egg laying. Throughout the experiment, snails were maintained at 24°C under a 12:12-h light:dark photoperiod and fed ad libitum with boiled lettuce.

### Pair Tests

At the end of the rearing protocol, each of the 20 G<sub>0</sub> families was composed of 12 G<sub>1</sub> snails isolated for a long period (LI) and 12 snails isolated for a short period (SI). Ten families were randomly assigned to serve as sources of focal individuals and 10 as sources of partners (Fig. 1). Within each family, six LI focal snails and six SI focal snails were paired with a randomly assigned LI partner (denoted LI/LI and SI/LI, respectively), and six LI focal snails and six SI focal snails were presented a randomly assigned SI partner (denoted LI/SI and SI/SI, respectively). Each pair was observed during 45 min. This is generally sufficient for several copulations to take place (Facon et al. 2006). We recorded the time to each contact, copulation and

dismounting as well as the focal individual's gender role and the occurrence of rejection behaviours such as shell swinging and phallus biting being displayed by the individual playing the female role during copulations.

### Statistical Analyses

Statistical analyses were conducted using the software R (R Development Core Team 2004). Four variables were retained to describe mating sequences in *P. acuta*: the time to the first copulation of the focal snail as a female, the total duration of copulations of the focal snail as a female (the duration of male copulations being the complement of this measure), the proportion of all copulations of the focal snail as a female where rejection behaviours were observed (rejection behaviours are only visible from snails acting as females), and whether individuals in a pair exchanged their roles between the first and the second copulation. We first controlled for size (measured as the maximum length of the shell in mm) and family effects. As data did not conform to linear models assumptions, this test was achieved using randomization, permuting individuals among body size classes and families in the data set (10 000 random permutations). Significance was assessed through mean square testing, and a test was deemed significant at the 5% level when less than 5% of the randomized data sets gave a mean square larger than

**Table 1.** Predicted effects of mating history on the reproductive behaviours of *Physa acuta*

Variables	Comparisons		
	SI/SI vs. SI/LI	SI/LI vs. LI/SI	LI/SI vs. LI/LI
Time to first copulation as a female of the focal snail	<	>	<
Duration of copulation as a female for the focal snail	>	<	>
Proportion of copulations with focal snail rejection behaviours	= or >	>	= or <
Gender exchange between the first and second copulations	>	=	<

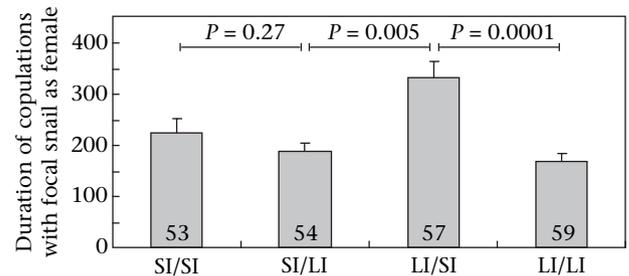
These expected effects are presented for pairs of snails composed of a focal snail and a partner each either short- or long-isolated assuming that (1) short-isolation snails (SI) prefer being male while long-isolation snails (LI) prefer being female and (2) conflicts are more intense in pairs of snails with identical isolation status than in pairs of snails with different isolation status. Pair types are denoted XX/YY where XX holds the isolation status of the focal snail and YY that of its partner.

the observed (e.g. Manly 1997). Neither shell size nor family effects were statistically significant, so they were not included in the subsequent analyses. We then tested whether pair type had an effect on the variables used to describe the mating behaviour of *P. acuta*. Treatment effect on gender exchange between the first and second copulation was tested using a generalized linear model with a quasi-binomial distribution to account for overdispersion. For the other three behavioural variables, data did not conform to analysis of variance assumptions, so we used randomizations (as for size and family effects), permuting individuals among treatments in the data set. When a significant treatment effect was found, the following pairwise comparisons were carried out using a sequential Bonferroni correction: SI/LI versus LI/SI, SI/LI versus SI/SI and LI/SI versus LI/LI (where in each pair, the first code holds the isolation status of the focal snail and the second one that of its partner). Thus, for the three tests, the lowest *P* value was compared with the corrected level  $\alpha/3$ , the second lowest *P* value to the corrected level  $\alpha/2$ , and the last *P* value to the level  $\alpha$ . The expectations for each test and variable are given in Table 1.

## RESULTS

Within the 45-min observation period, 94.8% of the pairs copulated at least once. The number of copulations per pair observed in 45 min ranged from 0 to 4, with a mean number of copulations of 1.8 per pair and an average total copulation time of 418 s per pair.

The time to first copulation as a female of the focal snail was not affected by the treatment ( $1200.1 \pm 39.8$  s,  $N = 189$ ,  $P = 0.44$ ). However, the duration of copulation as a female differed among treatments ( $P = 0.0001$ ; Fig. 2). For shortly isolated focal snails, the time spent copulating as a female did not differ significantly according to partner isolation status (SI/SI versus SI/LI,  $P = 0.27$ ). In



**Figure 2.** Effect of mating history on the total duration of copulation as female. The total duration of copulation as female (in seconds with standard errors) was measured on a focal snail either short- (SI/XX) or long-isolated (LI/XX) and confronted to a partner either short- (XX/SI) or long-isolated (XX/LI). Comparisons between pair types were made using 10 000 random permutations of individuals between pair types. *P* values of comparisons between pair types are given above the bars. Sample sizes are given on each bar.

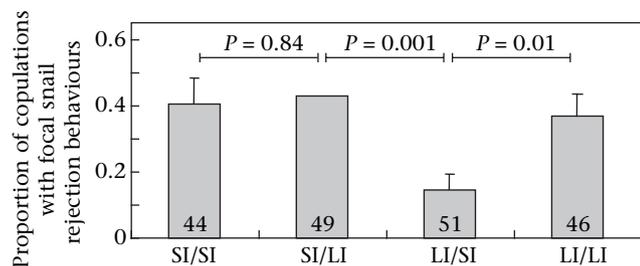
contrast, LI focal snails paired with a SI partner spent more time copulating as females than focal individuals from SI/LI pairs ( $P = 0.005$ ) or from LI/LI pairs ( $P = 0.0001$ ). This may be because snails spent more time copulating irrespective of their gender or because they indeed preferred female status and allocated a greater proportion of time to this gender. We therefore estimated the preference of focal snails for the female status by computing the relative difference between the times spent copulating as a female and as a male. The treatment affected this female bias ( $N = 223$ ,  $P = 0.003$ ). The gender preference of short-isolation focal individuals did not differ according to partner isolation status (SI/SI,  $0.14 \pm 0.10$ ,  $N = 54$  versus SI/LI,  $-0.05 \pm 0.08$ ,  $N = 52$ ,  $P = 0.19$ ). When presented to a partner of opposite isolation status, short-isolation focal individuals (SI/LI) appeared slightly male biased, while long-isolation focal individuals (LI/SI,  $0.36 \pm 0.08$ ,  $N = 57$ ) were female biased ( $P = 0.0009$ ). Long-isolation focal individuals were more female biased when presented to a short-isolation partner (LI/SI) than when presented to a long-isolation partner (LI/LI,  $-0.05 \pm 0.09$ ,  $N = 59$ ,  $P = 0.002$ ).

The proportion of copulations during which focal snails rejected their partner was also affected by the treatment ( $P = 0.007$ ; Fig. 3). When presented to a partner of opposite isolation status, long-isolation focal individuals (LI/SI) had a significantly lower rejection rate than short-isolation focal individuals (SI/LI) ( $P = 0.001$ ). Short-isolation focal snails had a high rate of rejection whatever the isolation status of their partner (SI/LI versus SI/SI,  $P = 0.84$ ). Long-isolation focal snails had a higher rejection rate when confronted to a long-isolation partner than with a short-isolation partner (LI/LI versus LI/SI,  $P = 0.01$ ).

Finally, the proportion of focal snails that changed their gender between the first and second copulations was not affected by pair types (74.7%,  $N = 150$ ,  $P = 0.84$ ).

## DISCUSSION

In this study, we showed that mating history could influence some components of gender preference in *P. acuta*



**Figure 3.** Effect of mating history on the frequency of rejections behaviours. The frequency of rejection behaviours (with standard errors) was measured as the proportion of copulations where the focal snail played the female role and rejected its partner through shell swinging and phallus biting. The focal snail either short- (SI/XX) or long-isolated (LI/XX) was confronted with a partner either short- (XX/SI) or long-isolated (XX/LI). Comparisons between pair types were made using 10 000 random permutations of individuals between pair types. *P* values of comparisons between pair types are given above the bars. Sample sizes are given on each bar.

using comparisons of the reproductive behaviours of short- and long-isolation snails when presented to partners either short- or long-isolated. Our first prediction was that snails isolated for short periods should prefer acting as males, whereas long-isolation snails should prefer acting as females. In our experiment, comparisons of short- and long-isolation snails presented to a partner of compatible (i.e. different) isolation status (second column of Tables 1 and 2) are partly congruent with this first prediction. When presented to a partner of a different isolation status, long-isolation snails copulated longer as females than short-isolation individuals and rejected copulations less frequently than short-isolation individuals. In contrast, the time to the first copulation as a female was not affected by the isolation status of either partner. This finding is not in line with the first prediction. Previous studies, however, have shown that the time of the first copulation was also unaffected by either partner size (Wethington & Dillon 1996) or partner relatedness (Facon et al. 2006) in the studied species. Overall, these results suggest that encounters

**Table 2.** Observed effects of mating history on the reproductive behaviours of *Physa acuta*

Variables	Comparisons		
	SI/SI vs. SI/LI	SI/LI vs. LI/SI	LI/SI vs. LI/LI
Time to first copulation as a female of the focal snail	=	=	=
Duration of copulation as a female for the focal snail	=	<	>
Proportion of copulations with focal snail rejection behaviours	=	>	<
Gender exchange between the first and second copulations	=	=	=

Pairs composed of a focal snail and a partner each either short-isolated (SI) or long-isolated (LI) were observed during 45 min and their mating behaviours recorded. Pair types are denoted XX/YY where XX holds the isolation status of the focal snail and YY that of its partner.

and first attempts at copulation are random or at least do not rely on chemical cues in *P. acuta*. Mating choices pertaining to partner identity and gender role may therefore involve early interruptions of copulations, notably through visible rejection behaviours from female acting snails rather than the avoidance of encounters with undesired individuals. That the copulation duration should determine the quantity of sperm transferred, however, needs to be confirmed by using more appropriate experiments.

In a previous study on another species of hermaphroditic snails, *Lymnea stagnalis*, it was found that individuals were equally receptive as females at all times (Van Duivenboden & Ter Maat 1985). In *P. acuta*, it seems that the probability to accept a copulation as a female increases with the time since last copulation (i.e. focal snails engaged in SI/LI pairs inflict more rejections and spend less time copulating as females than the ones in LI/SI pairs). This finding is also supported by the observation that virgin individuals (i.e. of infinite isolation time) prefer being female on their first copulation (Wethington & Dillon 1996).

Our second prediction based on the isolation-time hypothesis was that pairs of individuals with the same isolation status should show more frequent rejection behaviours and more reciprocity than pairs of snails with different isolation status. In the experiment (columns 1 and 3 of Tables 1 and 2), the proportion of copulations with the focal snail rejecting its partner was equally high among pair types except for LI/SI pairs. This again is not in line with our prediction. The explanation may be that rejection behaviours actually have two determinants: (1) short-isolation individuals prefer the male role and will thus frequently reject copulations regardless of the isolation status of their partners (SI/SI versus SI/LI) and (2) copulations where individuals have the same gender preference witness frequent rejection behaviours (LI/SI versus LI/LI). LI/SI pairs would therefore be the only situation where the focal snail often accepts copulations as a female and is with a partner of a compatible isolation status. In addition, the duration of copulation as a female for the focal snail is higher in LI/SI pairs than in any other type of pair. The gender alternation, measured as the rate of gender switching between the first and second copulation, was found to be equally high in all treatment pairs. Gender preference may therefore be more affected by the last copulation than by the earlier history of mating.

A potential mechanism to account for this effect of isolation time on reproductive behaviours is that individuals isolated for a long period should run out of allosperm and thus prefer the female role. In contrast, individuals isolated for a shorter period should still have large allosperm stores and should have had the time to replenish their store of autosperm since their last copulation (Wethington & Dillon 1996). Short-isolated individuals should thus increase their fitness by copulating as males. However, doubt was recently cast on this mechanism by the observation of a snail inseminated once that subsequently produced outcrossed offspring during several weeks (Dillon et al. 2005). If the allosperm obtained from a single copulation only is sufficient for this, why should individuals prefer the female role after 4 weeks of isolation? First, McCarthy (2004) suggested that female

motivation might be influenced by the quantity or maturity of their eggs. Here, all individuals regularly laid eggs during the rearing protocol. The rate of egg laying did not differ between short- and long-isolation snails. Wethington & Dillon (1996) also suggested that the rate of egg laying was constant in this species. Hence, the quantity and age of eggs should not differ between short and long-isolation snails. Only the store of allosperm should be lower in long-isolation snails than in short-isolation snails. Second, in a species where gonophores allow for multiple sperm stocks to compete for fertilization, the stimulus for female preference may be more related to allosperm quality than allosperm quantity. And sperm quality might decrease over time more rapidly than its quantity. Third, gender preference might not be controlled by the effective quantities of autosperm and allosperm but by an internal clock that itself evolved in response to sperm stores as for instance the time since last copulation. Evaluating whether the stores of auto- and allosperm are crucial determinants of gender preference would now require experiments based on the direct estimation of the relative quantities of auto- and allosperm, which may be a challenging task in some species.

*Physa acuta* dwells in large stable lakes and rivers but also in temporary and unstable habitats such as small ponds or irrigation canals (as the studied population). Its natural densities may thus show huge spatial and temporal variation, from less than one to several hundred individuals per m<sup>2</sup> (Henry et al. 2005; B.F. personal observation). Moreover, in Europe *P. acuta* is an invasive species. During the colonization of new habitats, it probably went through drastic founder effects (Jarne et al. 2000). Small densities (and isolation) are thus likely to be common in nature for this species. Whether allosperm is limiting in natural populations still remains to be shown. If it is, low-density populations could select for a switch from male to female-biased gender preferences with time since the last encounter. In contrast, in dense populations with no history of population fluctuations, individuals may lose their preference for the female role after long-isolation periods. Documenting such among-populations variation by extending the present study to several contrasted populations would help understanding whether this switch in gender preferences is adaptive or not.

As pointed out by several authors (for review see Arnqvist & Rowe 2005), a consistent preference for one gender is hardly compatible with the maintenance of simultaneous hermaphroditism. Gamete trading was suggested as a possible mechanism that would allow for the maintenance of simultaneous hermaphroditism (Dall & Wedell 2005; Leonard 2005). The present study, among others, suggests another possibility. Because hermaphroditic animals with internal fertilization are able to store sperm, they may gain fitness through sequential switches between male and female. The sequence of switches for a hermaphroditic species such as *P. acuta* could be as follows. Individuals are first mature as males (Wethington & Dillon 1993). When their female gonads are mature, they need to obtain allosperm (i.e. female-biased behaviour). Once enough allosperm is available for high-quality egg production, fitness is increased by adopting the male role. After copulating as

a male, autosperm store may be depleted and the female role will be preferred until the autosperm store is rebuilt (Wethington & Dillon 1996). If no partner is encountered for long enough that the allosperm store becomes depleted or decreases in quality, it becomes important to get new allosperm and the female role is preferred. Reciprocity would therefore be a consequence of each individual achieving its own strategy (Leonard 2006).

A growing body of experiments clearly establishes the complexity of mating behaviours in *P. acuta*. In the course of a reproduction, several decisions are to be made. If isolated, the question is when to self-fertilize. If conspecifics are present, the decision is over the choice of a partner to mate with. Size and relatedness were shown to influence this choice (Ohbayashi-Hodoki et al. 2004; Facon et al. 2006). Once a partner is chosen, both partners must agree on genders. Each of these choices has consequences on fitness and may be adaptive. To further understand how these choices evolve, we now need to gain insights into internal mechanisms of gamete production and postcopulatory phenomena in this species. In particular, it is crucial to determine the rate of sperm production and the dynamics of allosperm viability in the reproductive tract and to investigate if sperm digestion and sperm competition occur and if individuals are able to control the identity of the successful sperm (cryptic sperm selection).

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