Evidence for age-dependent mating strategies in the simultaneous hermaphrodite snail, *Lymnaea stagnalis* (L.)

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SUMMARY

In many mating systems female reproductive capacity is a limiting resource over which males will compete. As a consequence, males and females have usually different fitness optimization strategies which may give rise to sexual conflict. Since simultaneous hermaphrodites have, in theory, the option to mate as male or as female at any time, conflict will occur if partners insist in taking the same role. Several lines of evidence exists that body size influences gender choice. However, growth in many invertebrates is indeterminate and therefore age is generally a covariant of size. We therefore investigated the effect of age on mating choices in the simultaneous hermaphrodite *Lymnaea stagnalis*. Using fully sexually mature animals sampled from three different age groups we show that copulation frequency declines with age. Specifically, in age-matched couples the frequency of primary and reciprocal copulations declines with age. Furthermore, the younger partner tends to mate as male with greater probability in couples of unequal age. Size was never a factor in the sex role preference of *Lymnaea*. Thus, young *Lymnaea* always attempt to copulate as male independent of the age of their partner, whereas senior snails act primarily as female. The sex role choices of middle-aged snails appear to depend on their partner's age. In addition, we demonstrate that the likelihood that an animal will copulate as male is not correlated with prostate gland size but correlates with the level of afferent electrical activity recorded in the nerve originating in the prostate gland. Together, our results indicate the existence of an age- and not size-dependent mating system in *Lymnaea*.

Key words: reciprocal copulation, mollusc, fitness, male sexual drive, sperm trading, size-assortative mating.

INTRODUCTION

Sexually reproducing organisms are under evolutionary selection pressure to maximize their Darwinian fitness through optimizing their sexual interactions with conspecifics. Although the process of sexual reproduction is symmetrical in the sense that (usually) two partners of the opposite sex contribute about equally to the genome of their offspring, it is often highly asymmetric in terms of gamete resource investment. In general terms, it has been argued that reproductive success of a female is much more closely tied to the number of oocytes she can produce and her ability to support the developing embryos, and therefore usually associated with a higher cost to produce offspring than the male quest for fitness, which is primarily limited by access to oocytes (Bateman, 1948). This sexual asymmetry thought to be rooted in the asymmetry in size of the male and female gamete, puts different evolutionary constraints on male and female reproductive systems. Consequently, the sexes of many species have different and sometimes conflicting fitness optimization strategies involving sexual conflicts over parameters such as male mating rate, female (re)mating, sperm digestion and competition (Chapman et al., 2003; Chapman, 2006). For example, a frequent phenomenon, particularly in species that do not engage in parental care, is that optimal mating rates for males exceed that for females (Bateman, 1948; Arnold, 1994). In the current context, nonselfing simultaneous hermaphrodites present a particularly interesting case. These organisms, at least in theory, have a choice in the matter of their sex roles anytime they encounter a potential mate. Hence, these animals are prone to develop sexual conflicts

over mating (Charnov, 1979). Clearly, a gender conflict will arise when two partners attempt to adopt the same sexual role (Leonard, 1991; Leonard, 2005; Leonard, 2006; Anthes et al., 2006). Thus, simultaneous hermaphrodites may face a dilemma in that to avoid a reproductive stalemate one of the partners has to compromise by assuming a less favourable sexual role.

The issue of sexual conflict in simultaneous hermaphrodites has attracted a lot of attention in recent decades and many theories have been advanced with regards to the nature and principles underlying the resolution of these conflicts (e.g. Leonard, 2005; Leonard, 2006; Anthes et al., 2006). One theory often put forward and observed in several molluscan species is that simultaneous hermaphrodites deal with this issue by means of 'sperm trading' through either sequential or simultaneous reciprocal copulations (Leonard and Lukowiak, 1984; Leonard, 1990; Leonard, 1991; Leonard, 2005; Anthes et al., 2005; Dall and Wedell, 2005; Koene and ter Maat, 2005; Jordaens et al., 2007). Another frequently proposed theory is that simultaneous hermaphrodites evolved size-assortative mating systems to abrogate mating conflicts (Angeloni et al., 2002; Angeloni, 2003). This theory builds on the presumption that larger individuals have more resources to invest in offspring and will therefore be better prepared to support the less economical female sex function. Various lines of evidence support this view (reviewed by Jordaens et al., 2007). For example, in many hermaphrodites, female fecundity, but not male fecundity, correlates significantly with body size (DeWitt, 1954; Ghiselin, 1969; Angeloni et al., 2002; Angeloni, 2003; Michiels et al., 2003; Gianguzzi et al., 2005; Jordaens et al., 2007).

Table 1. Survival characteristics of *Lymnaea* populations used in the present study

Population	a*	c*	s$^{\dagger}\times 10^{-2}$	Population survival (%) at sampling	Expected maximal age (months) within population
A	520	4.1	−0.27	99–95	28
B	545	4.1	−0.26	99–95	29
C	480	4.0	−0.29	80–90	27
D	542	4.3	−0.27	65–75	28

*a and c are Weibull parameters (a at 50% survival).
†s is the slope of the tangent of the survival curve at median age.

Other studies provide evidence for the existence of size-assortative mating strategies in various simultaneous hermaphrodites (Switzer-Dunlap et al., 1984; Baur, 1992; DeWitt, 1996; Yusa, 1996; Angeloni et al., 2002; Ohbayashi-Hodoki et al., 2004; Chaine and Angeloni, 2005). However, although most invertebrates are indeterminate growers, same-aged snails often differ greatly in size (Forbes and Crampton, 1942; Lam and Calow, 1989a; Lam and Calow, 1989b; Ward et al., 1997). It is therefore conceivable that whereas it is known that hermaphrodites change their reproductive strategies as they grow (Cadet et al., 2004), interpretation of these data could be confounded by the fact that for many of them age is a covariant of size.

In the present study we addressed the question of whether age instead of body size might be a parameter in the resolution of sexual conflicts over mating in simultaneous hermaphrodites. We investigated the significance of age in sex role preferences of the simultaneous hermaphrodite *Lymnaea stagnalis* (L.). In captivity, *Lymnaea* lives for about 1.5 to 3 years and matures sexually at an age of 2.5–3.5 months depending on the ambient conditions and feeding regimen (Hermann et al., 2007). Body growth is indeterminate in *Lymnaea* although it slows down substantial after about 9–11 months of age in *ad libitum* fed, captive populations (Janse et al., 1989). In consequence, young sexually mature animals are usually smaller than their more senior conspecifics although body size can differ greatly even within age cohorts (Lam and Calow, 1989a; Lam and Calow, 1989b; Ward et al., 1997) (and own unpublished observations). Although evidence exist that egg-laying slows down and may come to a halt in aged animals (Janse et al., 1989), very few if any studies have systematically investigated male and female copulation incidence across the species' life cycle. The data available suggest that, except for a brief period of ~2 weeks after maturation during which young sexually mature animals act exclusively as male, *Lymnaea* mates and reproduce as male as well as female for most of their adult life span (van Duivenboden and ter Maat, 1988). *Lymnaea* mates either unilaterally (i.e. each mating partner mates as either male or female) or in a sequential reciprocal fashion in which the partners reverse sex roles after completing their first copulation cycle (Van Duivenboden and ter Maat, 1985; Koene and ter Maat, 2005).

MATERIALS AND METHODS
Animal and culture conditions
The animals used in this study were raised 'in house' under strictly controlled ambient conditions (light:dark 12h:12h, ambient temperature 18–19°C, pH 7.8–7.9). Age-synchronized populations of up to 900 animals at the onset were divided over six 100-l glass tanks equipped with an external multi-stage powerfilter (Fluval 204; Rolf C. Hagen, Montreal Canada; i.e. maximal density of 1.5 snails l^{-1}). Water was treated by reverse osmosis and reconditioned to a conductivity of ~450 Ω.cm through the addition of Instant Ocean salts at 1 g per US gallon, ~0.25 g l^{-1} (i.e. artificial pond water; Aquarium Systems, Mentor, OH, USA). Calcium concentration was kept at saturation level (>50 mg l^{-1} as CaCO$_3$) through the addition of calcium carbonate (light powder; EMD analytics, Gibbstown, NJ, USA) to the tanks. In addition, animals had continuous access to sterilized cuttlefish (*Sepia officinalis*) bone (two to three per tank). The animals were fed *ad libitum* with lettuce and Aquamax-carnivorous Grower 600 trout pellets (Purina Mills LLC, St Louis, MO, USA).

Experimental animals were sampled from four different, age-synchronized populations. Survival characteristics of these populations were continuously monitored and evaluated using a Weibull failure model (Table 1, Fig. 1) (Slob and Janse, 1988). Model parameters of the populations included in the current study fell within range of those previously established for healthy, aging populations (Table 1) (Slob and Janse, 1988). The snails sampled from these populations contributed to three age cohorts with age ranges of, respectively, 150–170 days (~5 months), 220–255 days (~8 months) and 350–375 days (~12 months; Table 1, Fig. 1). For the sake of convenience we refer to these three cohorts as young, middle-aged and senior snails in the remainder of the text.

Behavioural observations
As per previously established protocols, male sexual drive was induced by socially isolating individuals in perforated separation chambers jars that were positioned in a large tray for 8 days (de Boer, 1998). Prior to social isolation, each snail was individually marked, and its shell length was determined. After the isolation period, couples were formed based on their shell length, that is, shell length differences between partners were kept at a minimum (i.e. size-matched) and each couple was placed in a 250 ml glass beaker filled with clean 200 ml artificial pond water.
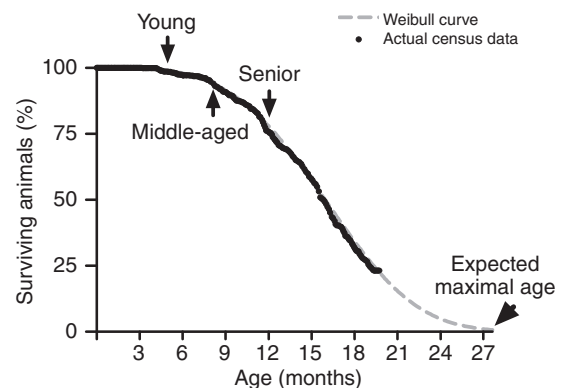

Fig. 1. Survival curve of a healthy laboratory population of *Lymnaea*. Example of a survival curve of a population used in the present study. Indicated are the approximate ages and survival percentages of young, middle-aged and senior snails used in the present study. The grey dotted line indicates the Weibull function fitting the survival data. Note that the expected maximal age of this population is 27 months.

In the present study, copulation was defined as the full male copulation behaviour repertoire including a complete and finalized intromission. The following behavioural acts were used to identify male copulation behaviour: mounting, circling, positioning, sham copulation and intromission (van Duivenboden and ter Maat, 1988). An intromission was considered finalized when the male removed its penis from the females receptacle and both snails disengage. In situations of a role reversal, that is after completion of the initial copulation, the sperm receiving snail reciprocates by initiating male copulation behaviour (mounting its partner), the first snail acting as male is referred to as the 'primary' male whereas it's partner acting as male after the reversal is referred to as the 'secondary' male. Behavioural observations were made for at least 5 h regardless of whether a copulation behaviour was initiated or not. Furthermore, in case of a primary copulation, observations continued for maximally 2.5 h or until completion of a role reversal (i.e. a full intromission of the secondary male).

After behavioural observations were finished, snails were re-grouped for 4 days in a community tank and subsequently isolated for another 8 days. After the second social isolation period animals were either re-tested on copulation behaviour or sacrificed for prostate gland removal.

Electrophysiology

The branch of the penis nerve connecting the CNS to the prostate gland, i.e. the penis nerve 1 (NP1), is thought to serve as a nervous pathway for male sexual drive (de Boer et al., 1997). To determine output from the prostate gland, extracellular recordings were made from NP1 (see also Hermann et al., 2007). To this end, snails were anesthetized, by submerging them for 5 min in a 20% Listerine solution, and subsequently de-shelled. After securing the snail in a small recording dish, the central nervous system (CNS) was exposed by means of a small incision made in the dorsal midline of the head region. NP1 was cut as close as possible to its branch point with the penis nerve proper and drawn into a glass microelectrode with a diameter just large enough to accommodate the nerve. Note that apart from the skin incision and transection of NP1, the preparation remained intact. The preparations were maintained in standard Hepes-buffered saline, composed of (in mmol l^{-1}): 51.3 NaCl, 1.7 KCl, 4.1 CaCl$_2$, 1.5 MgCl$_2$, and 10 Hepes (pH 7.9). Extracellular signals were amplified using a DAM-80 differential amplifier (World Precision Instruments, Sarasota, FL, USA), band-pass filtered at 10–1000 Hz, and digitized at 5 kHz sampling rate using a Digidata 1322A AD/DA converter (Axon Instruments, Union City, CA, USA). The digitized recordings were analyzed off-line with a spike train analysis program, i.e. software that uses waveform detection algorithms to capture and sort extracellular events (Spike 2, v.4.02a) and the total frequency of all waveforms was determined.

Data analysis and statistics

All experiments were performed at least in triplicate. Before statistical analysis, data were tested for normality (Kolmogorov–Smirnov one-sample test). When required, the data was logarithmically transformed. The correlation between shell length and shell width was calculated by means of a two-tailed Pearson followed by a linear regression. The effects of age on shell length, prostate gland mass, prostate gland-body wet mass ratio, and electrical activity of NP1 were tested by means of an unpaired Student's t-test. Associations were tested by means of χ^2 RxC tests. Unless stated otherwise, data average and dispersion are given as arithmetic means and standard error of the mean (s.e.m.) throughout the text. Percentages of snails performing intromission and primary

males performing intromission are presented with their 95% confidence intervals (CI$_{95\%}$) of ratios as calculated from the F distribution according to a modified Wald method (Agresti and Coull, 1998). A two-sided critical value of statistical significance of $P<0.05$ was adopted throughout the paper.

RESULTS
Female fecundity changes with age

Previous studies have shown that female reproduction (egg laying activity) changes with age in *Lymnaea* (Janse et al., 1989). Therefore, we first examined whether female reproductive activity changes in the cohorts used in the present study. To this end, 40 young, 40 middle-aged and 40 senior snails, were each placed in individual isolation chambers for 8 consecutive days and egg mass production was recorded. On average, all age groups produced the same number of egg masses per day (Fig. 2A; $F_{2,178}=1.276$, $P=0.28$). However, the egg masses produced by the young and senior snails were significantly smaller, i.e. they contained fewer egg capsules compared with those produced by the middle-aged snails (Fig. 2B; $F_{2,143}=30.18$ $P<0.0001$; Dunnett's multiple comparison test; young *versus* middle-aged $q=7.654$, $P<0.01$; middle-aged *versus* senior $q=4.473$, $P<0.01$). Thus, we conclude that fecundity changes with age in our populations.

Linear correlation between shell length, shell width and animal mass

Shell size is characterized by both shell length and shell width. To examine whether there is a linear relationship between both parameters, we measured *in situ* shell length and width of 167 snails randomly taken from young, middle-aged and senior *Lymnaea*. In addition, the mass of each snail was determined. Fig. 3 shows that there is a very strong linear correlation between *Lymnaea*'s shell length and shell width in the three age groups used (Pearson
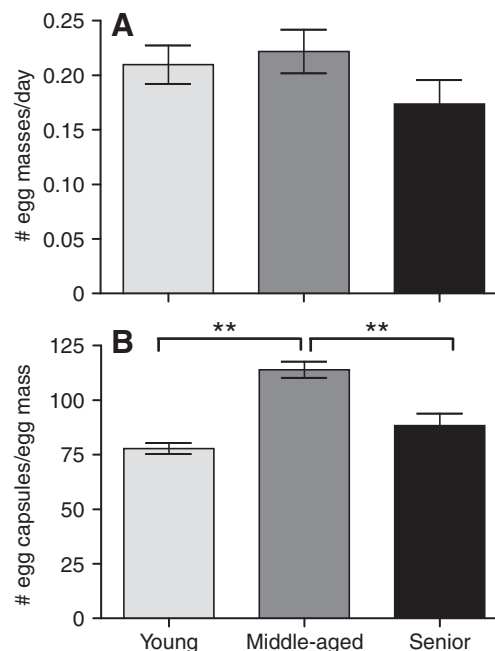
Fig. 2. Age-related fecundity in *Lymnaea*. (A) Young, middle-aged and senior snails deposit, on average, the same number of egg masses per day. (B) The number of egg capsules in each egg mass is significantly lower in young and senior snails compared with middle-aged snails. **$P<0.01$.
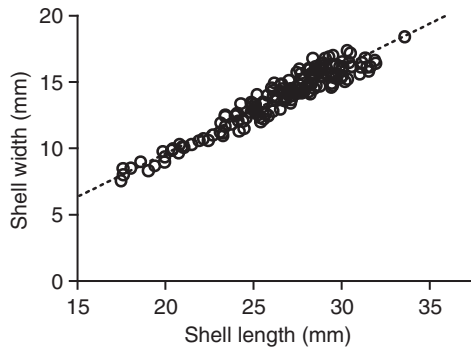
Fig. 3. Relationship between shell length and shell width in *Lymnaea*. Shell length and shell width are significantly correlated and can be described by a simple linear regression model. Pearson r=0.9571; goodness of fit R^2=0.9161; slope of linear regression 0.65±0.02.

r=0.9571; R^2=0.9161). Likewise there is a very strong correlation between shell-length and snail mass Pearson r=0.9351; R^2=0.8741) and shell width and snail mass (Pearson r=0.9436; R^2=0.8905). Thus shell length in *Lymnaea* is a reliable parameter to determine the size of an individual. It is noteworthy that there is a significant overlap between the shell lengths and widths measured in young snails (range of length 17.5–29.3 mm and width 7.6–16.2 mm) and those measured in middle-aged (range of length 23.4–31.9 mm and width 12.2–16.9 mm) and senior snails (range of length 24.6–33.6 mm and width 12.3–18.4 mm). This reiterates the fact that shell size can differ greatly even within age cohorts and that shell size is not a reliable parameter to determine age.

Copulation behaviour in encounters between animals of the same and different ages

To assess whether age is a variable in sexual preferences of *Lymnaea*, we examined the sexual interactions within pairs of animals sampled from young (Y), middle-aged (M) and senior (S) populations. Successful copulation was defined as a copulation cycle concluded by a full intromission by one of the partners.

Sexual interactions between age-matched individuals

First we tested copulation behaviour of same age peers. To this end, 40 young, 40 middle-aged and 20 senior snails were socially isolated for 8 days and subsequently paired to form, respectively, 20 young × young (Y×Y), 20 middle-aged × middle-aged (M×M) and 10 senior × senior (S×S) couples. The animals used in these tests were size matched (mean shell length ± s.d., 25.7±3.2 mm, 31.0±1.7 and 30.5±1.8 mm for young, middle-aged and seniors, respectively; the mean difference in shell length (± s.d.) between partners (smaller minus larger shell length) was –1.8±1.0 mm, –1.7±0.9 mm and –1.3±1.0 mm for the Y×Y, M×M and S×S pairs, respectively).

Copulation incidence (i.e. the occurrence of at least a unilateral copulation) differed substantially between the age groups, and were the highest in the Y×Y and the lowest in the S×S pairs (Fig. 4; $\chi^2_{(2)}$=7.428, P=0.027). Gender role was not determined by size. Thus, the number of times the smaller animal in a pair acted as primary male was not significantly different from the number of times the larger snail acted as primary male (P=0.23, P=0.12 and P=0.5 for Y×Y, M×M and S×S, respectively). Moreover, in all three age groups, there was no significant difference in shell length of animals that copulated as primary male, female or that did not copulate. Specifically, in the Y×Y pairs, the average shell length of primary males was 26.0±0.50 mm, of the females 25.8±0.50 mm
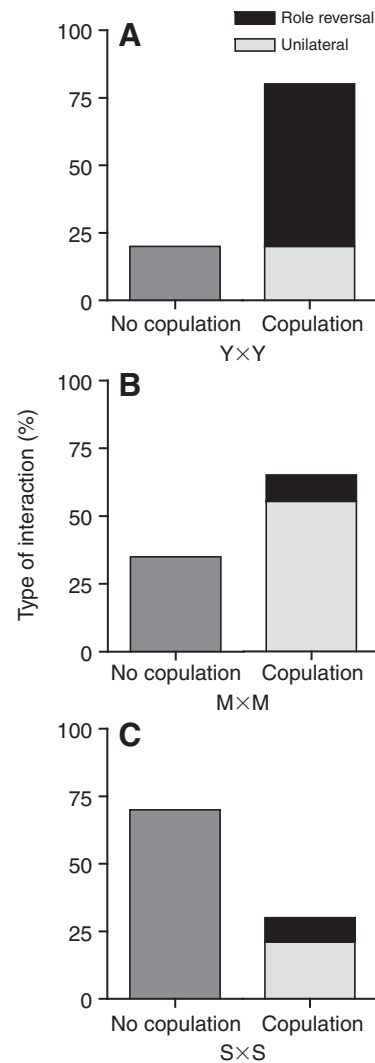

Fig. 4. Sexual interactions between same-aged *Lymnaea*. (A) Most of the young–young (Y×Y) couples show copulatory behaviour and most of these interactions are reciprocal, i.e. snails reverse roles after finishing the first copulation. (B) Most middle-aged–middle-aged (M×M) couples display copulatory behaviour. These interactions are, in general, unilateral. (C) Of the senior–senior (S×S) snail couples, only a minority show copulation behaviour and these interactions are mainly unilateral.

or that did not copulate 25.2±0.61 mm ($F_{2,39}$=0.5008, P=0.61). Similarly, the average shell length of the snails in the M×M pairs that acted as male was 31.1±0.32 mm, as female 30.9±0.42 mm or that did not copulate 30.9±0.49 mm ($F_{2,39}$=0.1267, P=0.88). The average shell length of the snails that acted as male in the S×S pairs was 30.0±0.09 mm, that acted as female 30.9±0.58 mm or that did not copulate 30.7±0.31 mm ($F_{2,19}$=0.7505, P=0.49).

Detailed behavioural comparison between the young, middle-aged and senior primary males indicated that the copulation behaviour was similar for most behavioural elements between the three age groups. That is, the total time spent on 'mounting', 'positioning', 'sham copulation' and 'intromission' was not different between the three age groups (mounting $F_{2,31}$=1.338, P=0.28; positioning $F_{2,132}$=0.6713, P=0.51; sham copulation $F_{2,67}$=1.441, P=0.24; intromission $F_{2,31}$=0.5999, P=0.56). The only behavioural difference occurred in the time spent circling ($F_{2,68}$=11.70, P<0.0001). That is, senior primary males circled significantly longer than young (P<0.001) and middle-aged males (P<0.05).

Intriguingly, besides differences in copulation incidence, the occurrence of role reversal differed between the age groups (Fig. 4, Fig. 7B; $\chi^2_{(2)}$=10.48, P=0.005). Specifically, whereas 75% of the young couples reversed roles the same happened in only 30% or fewer of the M×M and S×S couples. In all occasions that a role reversal was initiated it was finished with a full intromission of the secondary male. Taken together these data show that copulation frequency among same-aged partners declines with age (Fig. 7).

Sexual interactions between individuals of unequal age: middle-aged and seniors

The data presented above indicate that sexual proclivity of age-matched *Lymnaea* couples declines with age. In theory, this could be both due to a decline in the willingness and/or ability to copulate as male as well as female of either of the partners. To start addressing this question, we took a new set of middle-aged and senior animals from the same populations as the individuals used in the tests described above, and combined them into 20 middle-aged × senior (M×S) couples. As before, the animals were size matched [mean shell length ± s.d., 30.0±2.2 mm for middle-aged and 30.8±1.8 mm for senior snails; t=1.214 d.f.=38, P=0.23; mean length difference ± s.d., between partners (shell length of middle-aged minus shell length of senior) was –0.3±1.7 mm] and socially isolated before behavioural tests were performed.

Copulation was observed in 14 out of these 20 M×S couples and role reversal occurred in half of these 14 couples (Fig. 5A). On the occasions when a role-reversal was initiated it was always finished with a full intromission of the secondary male.

As observed in same-aged couples, the number of times a smaller animal acted as primary male was not significantly different from the number of times a larger snail acted as primary male (P=0.21). Moreover, there was no significant difference in shell length of animals that copulated as primary male (31±0.04 mm), as female (31±0.05 mm) or that did not copulate (30±0.07 mm; $F_{2,39}$=0.3180, P=0.73). Again, circling was the only element in the behavioural sequence that was different between the two age groups. That is, senior primary males increased their time spending on circling (t=2.181 d.f.=31, P=0.04) compared with the middle-aged primary males. All other behavioural elements were similar between the two age groups, i.e. there was no difference in the total time spent on mounting (t=1.131 d.f.=12, P=0.8), positioning (t=0.6495 d.f.=68, P=0.52), sham copulating (t=0.1422 d.f.=35, P=0.89) and intromission (t=0.3860 d.f.=12, P=0.71).

With 70% of the M×S couples copulating at least unilaterally, copulation frequency of M×S couples was very similar to that observed in age-matched M×M couples (cf. Fig. 4B, Fig. 5A). Hence, copulation frequency in couples with at least one middle-aged partner, was significantly higher than observed in age-matched S×S couples ($\chi^2_{(1)}$=4.6394, P=0.03). Interestingly, although the likelihood of middle-aged and senior animals acting as primary male was about equal (Fig. 5B), the odds of role reversal were highly dependent on age of the primary male. That is, whereas only one of the eight senior animals that acted as female reciprocated as male, all of the six middle-aged animals acting as female in the first round reciprocated as males (Fig. 7B). As a result, the proportion of middle-aged animals acting as either primary or secondary male significantly exceeded that of their older partners ($\chi^2_{(1)}$=4.912, P=0.03; Fig. 7A).

Both the relatively low copulation incidence in senior age-matched (S×S) couples (Fig. 4) and the virtual nonexistence of reciprocating senior partners in M×S couples (Fig. 5) seem to suggest that a growing proportion of older animals are predisposed to act as female, either by choice or necessity. Along this vein, we
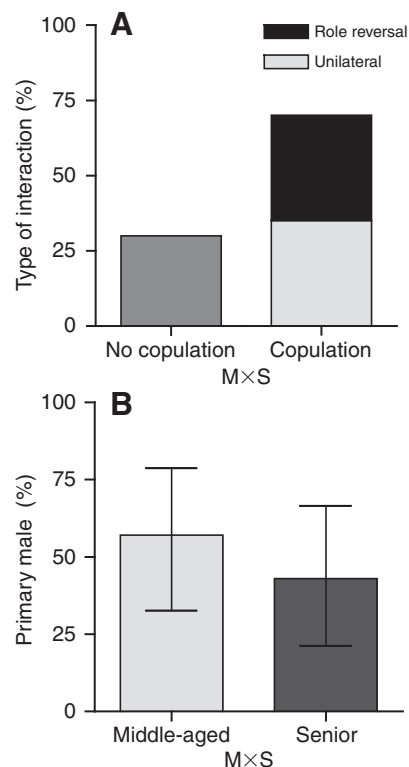
Fig. 5. Sexual interactions between middle-aged and senior *Lymnaea*. (A) Most couples of a middle-aged and a senior snail (M×S) display copulation behaviour. In half of these interactions the snails reversed roles after finishing the first copulation. (B) There is no difference in the percentage of middle-aged or senior animals acting as primary male.

tested whether individual senior snails tend to assume the female role during successive sexual encounters with novel partners. To this end, the animals used in the experiments described above were resocialized for 4 days in one common tank before being isolated again for 8 days. To avoid possible suppression of male copulation activity due to recognition of previous sexual partners [the 'Coolidge effect' (Koene and ter Maat, 2007)], each of the animals was presented with a novel partner thus creating 20 novel M×S and 10 new S×S couples (note: all partners were size-matched as before). The results obtained in this second round of trials were virtually identical to those of the first round. That is, S×S couples engaged with very low frequency in unilateral copulation (2 of 10) and even lower frequency in role reversal (1 of 10), whereas both occurred much more frequently in M×S couples (17 out of 20 copulations, 8 out of 17 performed role reversal). In the latter couples older partners never reciprocated as secondary male. Notably, however, there was no association between the primary gender role assumed by senior individuals in their first and second trial in the S×S couples or in the M×S couples ($\chi^2_{(4)}$=0.9524, P=0.92 for M×S couples and $\chi^2_{(4)}$=1.825, P=0.77 for S×S couples). Thus, these results do not support the conclusion that individual older snails lose their ability to enact both sex roles.

Role reversal is conditional upon age of the partner

The results presented above reveal an intriguing pattern with regards to the occurrence of a role reversal, i.e. it appears that the likelihood that an animal acting as female will reciprocate as male depends on the age of the partner. That is, most M×M sexual

interactions were unilateral. In other words, in general the animals acting as female did not reciprocate when presented with a partner of the same age. Yet, when presented with an older individual (i.e. M×S assays), all of the middle-aged animals acting as female reciprocated as males (c.f. M×S and M×M in Fig. 5A and Fig. 4B). This observation raises the question of how middle-aged animals respond when presented with a younger sexual partner. To address this question, we performed behavioural assays on 17 young ε middle-aged (Y×M) couples formed from animals that were socially isolated as described before. Thirteen out of these 17 couples copulated at least unilaterally, with the younger partners preferentially acting as primary male (Fig. 6; $\chi^2_{(1)}$=12.46, P<0.001). Importantly, there is no significant difference in shell length of animals that copulated as primary male, as female or that did not copulate (Fig. 6C; $F_{2,33}$=1.532, P=0.23) and detailed analysis of the behaviour indicated that there was no differences in any of the male copulation behaviour elements. Thus, the total time spend on mounting (t=1.562, d.f.=11, P=0.15), circling (t=0.7363, d.f.=27, P=0.47), positioning (t=1.375, d.f.=53, P=0.17), sham copulating (t=0.6070, d.f.=27, P=0.55) and intromission (t=1.739, d.f.=11, P=0.11) was similar between the two age groups.

In the two cases in which the middle-aged animals acted as primary male both young partners reciprocated. Remarkably however, role reversal occurred in only a small minority (18%) of the middle-aged animals copulating with junior primary males (Fig. 7B). These results stand in stark contrast with the behaviour of middle-aged animals in M×S couples in which all of them reciprocated (Fig. 5, Fig. 7B). As observed in all previous trials, if a role-reversal was initiated by the secondary male, it was always finalized with a full intromission.

Age difference is a factor in sex allocation of partners

When one brings all of the above results together, a pattern of changing sexual preferences over the life cycle of *Lymnaea* seems to appear. First, animals in the early phase of the species' life cycle (i.e. >95% population survival; see Fig. 1) tend to assure that they act as male (either primary or secondary), independent of the age of their partner (Fig. 7A). Senior animals (i.e. animals progressed in a later stage of the life cycle; see Fig. 1), by contrast, tend to act preferentially as females. The sexual behaviour of middle-aged animals depends on the age of their partner. Combined with similarly aged partners they either act as male or female. Combined with a younger partner they tend to act as female only. Combined with an older partner, they tend to execute both gender roles and will act as males with a similar probability as younger animals (Fig. 7A).

Male sexual drive and prostate gland

The data presented above suggests that relative motivation to copulate as male declines with age. According to de Boer et al. (de Boer et al., 1997), male sexual motivation in *Lymnaea* is strongly associated with increase prostate gland loading. This in turn, is reflected in an increase in afferent nervous activity originating in the prostate. In view of these data, we examined whether similar factors were involved and could explain the apparent decline in male sexual motivation in our aging animals. To this end, young, middle-aged and senior snails were isolated for 8 days and their prostate glands were subsequently dissected and weighted. In absolute terms, the prostate gland of socially isolated young animals were significantly smaller than that of both middle-aged and senior animals, but that no difference existed between the latter two groups (Fig. 8A; $F_{2,92}$=8.999, P<0.001; Y *vs* M Tukey HSD q=5.990,
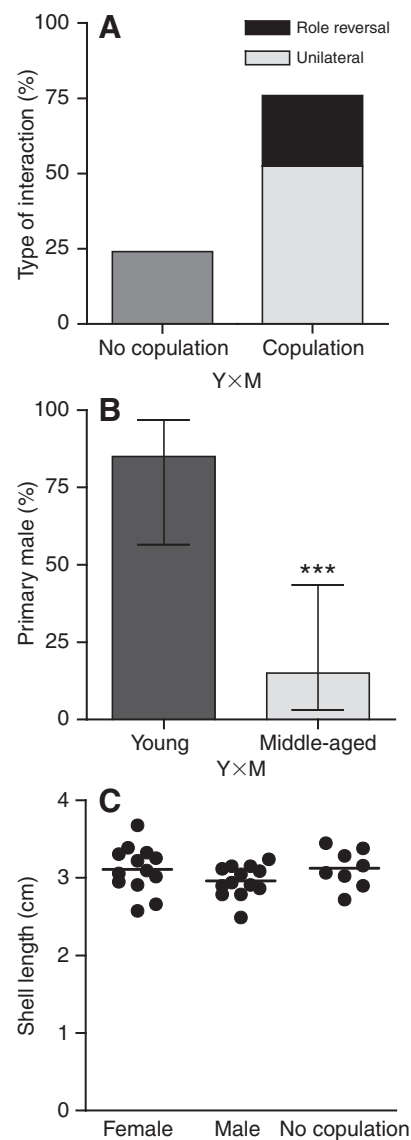

Fig. 6. Sexual interactions between young and middle-aged *Lymnaea*. (A) Most couples of a young and a middle-aged snail (Y×M) performed a copulation. The majority of these interactions were unilateral, i.e. the snails do not reverse roles after finishing the first copulation. Note that in the cases in which the middle-aged animals acted as primary male all young partners reciprocated. By contrast, role reversal occurred in only a small minority (18%) in the couples in which the middle-aged animals acted as female. (B) Younger snails act significantly more often as primary male than their middle-aged partner. (C) Shell length of snails that copulated as female did not differ of those that copulated as primary male or did not copulate at all. ***P<0.001.

P<0.001; Y *vs* S Tukey HSD q=3.606, P<0.05; M *vs* S Tukey HSD q=2.691, P>0.05). However, in relative terms (i.e. prostate gland mass relative to mass of the deshelled body) showed that, the prostate of young animals was slightly larger than that of senior animals (Fig. 8B; $F_{2,92}$=3.761, P=0.027; Tukey HSD q=3.707, P<0.05), but did not differ from that of middle-aged animals (Fig. 8B; Tukey HSD q=1.595, P>0.05). Relative size of prostate glands of middle-aged and senior animals did not differ (Fig. 8B; Tukey HSD q=2.671, P>0.05). Thus, these data suggest that mere prostate gland mass is not a probable factor in the more pronounced male sexual proclivity in younger snails.
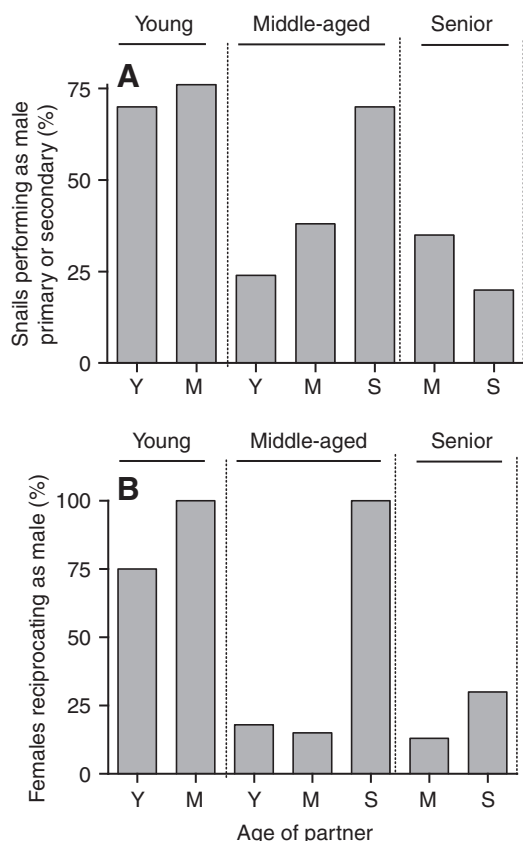
Fig. 7. Sexual behaviour in *Lymnaea* changes with age. (A) Young *Lymnaea* act most of the time as male (either primary or secondary), independent of the age of their partner. Middle-aged snails, however, increase their male performance (either as primary or secondary male) with increasing age of their partner. Senior snails rarely act as male. (B) Young *Lymnaea* readily reverse gender roles in the majority of copulations independent of the age of their partner. Middle-aged female snails, however, only reverse roles when their partner is older. Senior snails acting as female rarely reversed roles.
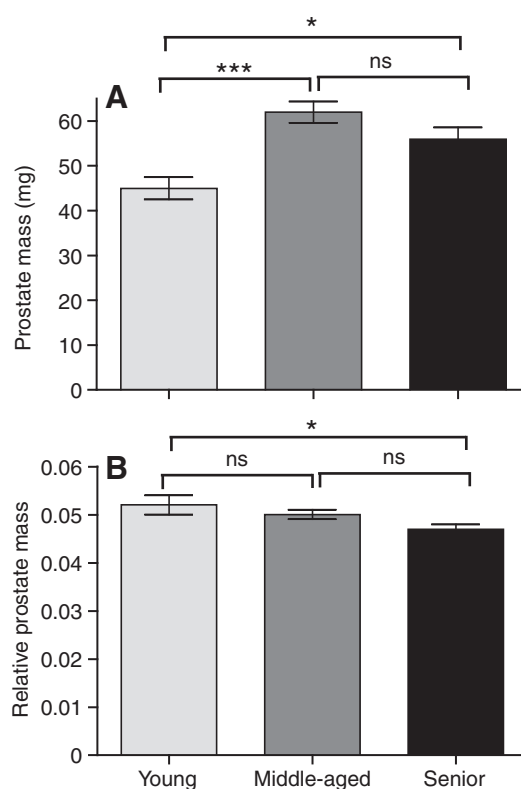

Fig. 8. Prostate gland mass of socially isolated *Lymnaea*. (A) The prostate glands of socially isolated young animals were significantly smaller than those of middle-aged and senior snails. The prostate gland mass of middle-aged and senior snails are not different. (B) The relative mass of the prostate gland (i.e. relative to the de-shelled body mass) of young animals is slightly but significantly larger compared with those of senior snails but are not different from those of middle-aged snails. The relative mass of the prostate gland between middle-aged and senior snails are not different; *$P<0.05$, ***$P<0.001$; ns, not significant.

De Boer et al. (De Boer et al., 1997) also showed that inflation of the prostate gland increases afferent electrical activity in the penis nerve 1 (NP1), the branch of the penis nerve that connects the prostate gland to CNS. They hypothesized that this mechanosensory response signals male sexual 'readiness' state to the CNS. Thus a change in mechanosensory output might cause a decline in male sexual behaviour. Based on this hypothesis, we recorded electrical activity in NP1 of young ($N=5$) and middle-aged ($N=7$) animals after 8 days of social isolation. Electrical activity in NP1 differed dramatically in these two groups (Fig. 9). Spontaneous activity recorded from NP1 in young animals was characterized by regularly occurring volleys of large-amplitude, high-frequency bursts (Fig. 9Ai). By contrast, NP1 recordings of middle-aged animals lacked such prominent bursts of extracellular spikes (Fig. 9Aii). Moreover, as summarized in Fig. 9B overall electrical activity in NP1 in middle-aged snails was significantly lower than that in young animals ($t=2.537$, d.f.=10, $P<0.05$). Although the evidence for a relationship between sensory information originating in the prostate gland and male sexual drive is thus far predominantly correlative, the above electrophysiological data are consistent with the idea that a decline in the afferent output from the prostate gland may be one of the factors in the decline in male sexual proclivity in older animals.

DISCUSSION

In this study we examined whether age is a factor in sex role preferences of the pond snail *Lymnaea stagnalis*. Since *Lymnaea* is a simultaneous hermaphrodite, individual snails have in theory the choice to mate either as male, female or both any time it encounters a mating partner. Its choice probably depends on numerous factors including those articulated by Bateman's principle but also by life cycle-related traits such as age, body size, mate size and quality, and copulation history. Thus, the optimal mating strategy of an individual may change over the course of its life and may depend on characteristics (size, age, quality) of its prospective mating partner, prompting us to investigate mating behaviour of age-matched as well as unequally aged pairs of *Lymnaea* taken from three phases of the organism's adult life.

Our key findings are summarized as follows. (1) Copulation frequency declines with age; (2) the incidence of reciprocal copulation declines with age in age-matched couples, but younger partners always reciprocate after mating as female with an older partner; (3) in couples of unequal age, the younger partner tends to mate as male with greater probability; (4) the likelihood that an animal will copulate as male is not correlated with prostate gland size but is correlated with the level of afferent electrical activity recorded in the nerve originating in the prostate gland.
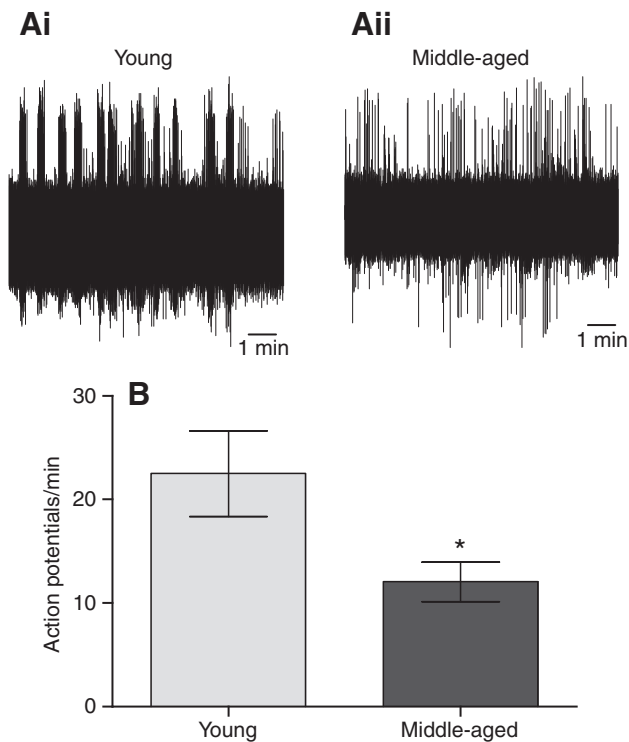
Ai Young

Aii Middle-aged

B

Fig. 9. Afferent output from prostate gland changes with age. (A) Example of spontaneous electrical activity recorded from NP1 in a young (Ai) and a middle-aged (Aii) animal. Note the prominent bursting activity in the young snail that is absent in the middle-aged snail. (B) Overall electrical activity in NP1 is, on average, significantly reduced in middle-aged snails; *$P<0.05$.

Are changes in mechanosensory output from the prostate gland a possible cause of the divergence of male sexual drive of younger and older animals?

Our results support the conclusion that the urge to copulate as male after a period of social isolation is the highest in younger animals. Prompted by De Boer et al. (De Boer et al., 1997), who proposed prostate gland loading as a major determinant of male sexual drive, we examined whether this phenomenon correlated with prostate gland size. Our results indicate that prostate gland size alone is not a likely explanation for the apparently higher male sexual drive of the younger partners. After all, the data shows that in absolute terms the youngest snails in our study tended to have the lightest prostate glands whereas there was no difference in prostate size of the middle-aged and senior snails, despite the fact that the former tended to act as male significantly more often than the latter in sexual encounters between them. Even in relative terms, the differences in prostate gland size (i.e. as a ratio of body mass) between the age groups are either nonexistent or too small to account for the predominantly male sex role adopted by the younger partners in couples of unequal age. This sentiment reiterates comments by Koene and ter Maat (Koene and ter Maat, 2007) who concluded that that *Lymnaea* can and is motivated to inseminate a new partner even though their prostate gland is partially depleted. However, the observation that afferent neural activity from the prostate gland is substantially higher in young animals than in older animals does suggest that perhaps not mass but some other (neuro-)physiological state of the gland, is a key factor in sex role determination in *Lymnaea*. This idea is certainly in keeping with findings by de Boer et al. (de Boer et al., 1997) who showed that inflation of the prostate gland resulted in

increased afferent electrical activity in the NP1 nerve from stretch activated mechanosensory neurons. These authors hypothesized that this mechanosensory response signals male sexual 'readiness' state to the CNS. From this perspective, our observation that afferent electrical activity of NP1 is substantially more intense in socially isolated young animals than in similarly treated middle-aged counterparts is very suggestive because it could be taken as an indication that decreasing male sexual drive in older animals involves a decline in stretch-activated mechanosensitive sensory output from the prostate gland and thus a reduced signal to the CNS of male 'readiness'.

Age- *versus* size-dependent mating

Various sex-allocation theories emphasize the importance of body size as a key feature influencing reproductive behaviours and sex allocation strategies of hermaphrodites in both the plant and animal kingdoms (e.g. Charnov, 1982; Klinkhamer et al., 1997; Angeloni et al., 2002; Angeloni, 2003; Cadet et al., 2004). For instance, the size-advantage model predicts, usually quite successful, the optimal body size (not age) at which sequential hermaphrodites should reverse sex (Ghiselin, 1969; Charnov, 1982). In simultaneously hermaphroditic plants, larger individuals have usually more resources to invest in reproduction [budget effect (Klinkhamer et al., 1997; Cadet et al., 2004)] and usually allocate more to their female function, resulting in a gradual shift in sex allocation with increasing size [i.e. growth (Cadet et al., 2004)]. In sperm-storing simultaneous hermaphroditic animals like *Lymnaea*, an individual's sex allocation strategy is thought to depend on its own body size relative to the size of its partner. It is therefore predicted that large animals with more reproductive resources invest a greater proportion of resources in female function than small animals (Anthes et al., 2006). Thus, when mating with a larger animal, more resources should be diverted to sperm transfer than when mating with a small animal (Angeloni et al., 2002; Jordaens et al., 2007). Empirical studies provide ample evidence for the existence of size-dependent mating (DeWitt, 1996; Yusa, 1996; Angeloni et al., 2002; Ohbayashi-Hodoki et al., 2004; Chaine and Angeloni, 2005; Pal et al., 2006; Jordaens et al., 2007) but also non size-dependent mating strategies in hermaphrodite gastropods species (Switzer-Dunlap et al., 1984; Baur, 1992; Koene et al., 2007). It is conceivable, however, that interpretation of some these studies is confounded by the fact that, for many of the species used, size is a covariant of age. In general, larger animals are older, however, the correlation between size and age is far from perfect. In fact, same-aged snails, including *Lymnaea* often differ greatly in size and same sized animals might be of different age [present study (Lam and Calow, 1989a; Lam and Calow, 1989b; Ward et al., 1997)]. Thus, size is an unreliable estimate of age and age could be a confounding effect when dealing with size-dependent mating strategies, i.e. some of the observed size-dependent mating strategies in simultaneous hermaphrodites might be (partly) due to age- and not size differences.

To our knowledge the present study, using simultaneous hermaphrodites, is the first in which the age of all animals is precisely known, and gender choice was investigated in fully sexually simultaneous hermaphrodites, i.e. all animals are active in both male (copulation) and female (egg laying) reproductive activities. As far as we are aware, the only two other studies dealing with age and gender choice either estimated age based on body mass and/or the youngest animals were not active in female reproductive roles (Switzer-Dunlap et al., 1984; Tomiyama, 1996). We show, that when combined with an older partner, younger animals act disproportionally more often as male (i.e. as reflected in a much

larger proportion of young animals copulating unilaterally as male in Y×M mating pairs compared with Y×Y mating pairs). Since copulation incidence in M×M mating pairs was only slightly (but not significantly) lower than that in Y×Y pairs, it seems unlikely that the uneven sex role distribution in Y×M matings is due to the older animals' inability to copulate as males. Rather it suggests the existence of an age-related shift in sex role preference from male to female. We are further strengthened in this opinion by the observation that Y×Y mating pairs usually engage in reciprocal copulation whereas M×M mating pairs usually copulate unilaterally. Importantly, size was never a factor in the sex role preference of *Lymnaea*. Although female fecundity in *Lymnaea* has been linked to body size (DeWitt, 1954) other studies have found no evidence to support the existence of size-assortative mating mechanisms in the species (Koene et al., 2007), corroborating our observations. Together, this clearly suggests that mating strategies in *Lymnaea* change with age not with size.

Reciprocal copulations

One of our most intriguing but also most puzzling observations is that the drive to reciprocate by a sperm receiving snail seems to depend on the age of the sperm donor. Previous studies have shown that role alternation in *Lymnaea* only takes place when both partners are motivated to mate in the male role, usually when both individuals have been socially isolated for several days. (Koene and ter Maat, 2005). This corresponds with our observation when dealing with young and middle-aged animals. That is, independent of the age of the sperm donor, animals in this age group, (nearly) always reciprocated after receiving sperm. By contrast, however, despite the fact that animals were isolated for 8 days and supposedly highly motivated to act as male, middle-aged and old snails are not willing to (or capable of) reciprocate after receiving sperm from a same-aged or younger partner. Even introducing a novel mating partner with the initially sperm receiving middle-aged animal, did not entice the latter animal to act as male. Intriguingly, middle-aged animals always reciprocated when coupled with an older aged animal. Thus, the chance *Lymnaea* reciprocates is inversely related with age.

Sperm trading through reciprocal copulation is thought to be one of the solutions to solve gender conflicts (Leonard and Lukowiak, 1984; Leonard, 1990; Leonard, 1991). To be able to work this way, sperm exchange should be conditional. Thus, it is thought that a key characteristic of sperm trading is that partners only donate sperm when there is a high likelihood of receiving sperm (Anthes et al., 2005; Anthes et al., 2006), although recent studies have shown that reciprocity does not prove sperm trading (Anthes and Michiels, 2005; Anthes et al., 2006). Whereas, the present observations with young middle-aged *Lymnaea* might suggest conditionality, our data with middle-aged and old snails indicate that copulation in *Lymnaea* is not always rewarded with reciprocation, supporting a similar observation made by Koene and ter Maat (Koene and ter Maat, 2005). In this context, a recent study by Webster et al. (Webster et al., 2003) is quite interesting. These authors showed that mate choice in the simultaneous hermaphrodite *Biomphalaria glabrata* is not under control of one individual, but rather depends upon both mating partners and that females can actively reject a (potential) partner (Webster et al., 2003). In the present study, we observed that in all situations the secondary male mounted its partner, thus initiated the start of a role reversal, it always ended with a full intromission of the secondary male. This suggests that in *Lymnaea,* the decision to reciprocate (or not) is made by the secondary male and not the partner. More detailed observations of the behaviour and their interaction of both sexes are, however, necessary to determine the potential active role of females in the initiation or rejection of a role reversal in this species.

Lymnaea can produce offspring *via* self-fertilization and cross-fertilization but once allosperm has been received outcrossing is favoured (Cain, 1956; Knott et al., 2003; Koene et al., 2008). Once sperm has been received most of the ejaculate is digested and only a small proportion of the sperm is stored (Geraerts and Joosse, 1984; Koene et al., 2008). De Visser et al. (De Visser et al., 1994) showed in young middle-aged *Lymnaea,* that half of the reproductive resources that are available are invested in the male function (copulation). In other words, reduced investment in male function (copulation) results in an increase in female reproduction (egg laying). Since the costs of copulating as male are high, animals have to be selective on their partner choice and optimize their male investment. Therefore, in order to maximize its chances of paternity, it is conceivable that the sperm donor manipulates the recipient such that resources will be allocated towards female (egg laying) rather than male reproduction. Although the existence of transfer of an allohormone to increase egg laying in the sperm donor has been suggested before (Koene et al., 2006), research is still ongoing to verify the release of such a factor and its physiological actions on the recipient.

In summary, this is the first evidence that simultaneous hermaphrodites display flexibility in individual *ad hoc* gender preferences and resource allocations based on its own and partner's age. Hence, mating strategies of simultaneous hermaphrodites within a population are not static but dynamic.

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