ORIGINAL ARTICLE

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Effects of frequent mating on sex allocation in the simultaneously hermaphroditic great pond snail (*Lymnaea stagnalis*)

Abstract Simultaneous hermaphrodites have the opportunity to control the allocation of resources to the male and female function depending on the circumstances. Such flexibility also provides the possibility to influence sex allocation in the mating partner. To investigate this idea, we measured egg production (female investment) and sperm production as well as prostate gland size (both are part of male investment) under different mating regimes in the great pond snail *Lymnaea stagnalis*. We find no evidence for the prediction from sex allocation theory that sperm production increases with mating frequency. However, we do find that animals with more mating opporunities develop smaller prostate glands, in which seminal fluid is produced. Moreover, repeated mating increases egg production, thus shifting allocation towards the female function, and probably decreases growth. So, our data hint at a three-way trade-off between part of the male function (prostate gland), female function, and growth. Interestingly, sex allocation seems to be shifted in the opposite direction from the one predicted by theory. We discuss how this feminization is suggestive of a direct manipulation by the sperm donor, probably to stimulate immediate sperm use.

Keywords Copulation · Hermaphrodite · Reproduction · Sperm competition · Sexual conflict · Allohormone · Pulmonata

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Introduction

Hermaphrodites need to divide the resources allocated to reproduction between their male and female functions and can do this in a phenotypically plastic way (López and Domínguez 2003; Schärer and Ladurner 2003; Tan et al. 2004; Schärer et al. 2005; Lorenzi et al. 2005). This makes them ideally suited for situations in which mate availability is unpredictable (Ghiselin 1969). Hence, short-term adjustments in sex allocation allow individuals to adapt to environmental factors affecting size and composition of the mating group. Sex allocation theory predicts that male allocation should increase with the number of mating opportunities (e.g., Hamilton 1967; Charnov 1982).

For simultaneous hermaphrodites, sex allocation theory assumes that a fixed portion of the energy budget is allotted to male and female reproduction (Charnov 1979; Kooijman 1993). This implies that there has to be a trade-off between the male and female function within the hermaphrodite. However, recent studies suggest that resources can also be reallocated to reproduction from the energy stores used for growth and maintenance (i.e., resource allocation; Yund et al. 1997; Koene and Ter Maat 2004). This idea is further supported by research in which sexually reproducing individuals grew less (*Phys*a: Tsitrone et al. 2003; *Celleporella*: Hughes et al. 2002; *Pseudosuccinea*: Gutiérrez et al. 2002; *Helobdella*: Tan et al. 2004; *Ophryotrocha*: Sella and Lorenzi 2003) and also developed smaller seminal-fluid-producing prostate glands (*Lymnaea*: Koene and Ter Maat 2004).

A trade-off between growth and reproduction suggests that animals have to decide when to reduce investment in growth and start producing offspring. In the spermcast mating hermaphrodite *Celleporella* this transition is determined by the receipt of spermatozoa (Hughes et al. 2002). In the internally fertilizing hermaphroditic freshwater snails, the receipt of semen initiates the female function (*Lymnaea*: Van Duivenboden 1983; *Biomphalaria*: Paraense 1993; *Physa*: Tsitrone et al. 2003). Accelerating the onset of the partner's female function increases the male reproductive success of the sperm donor because the donated sperm will be used for fertilizing the first eggs. Such a manipulation is

favored by sexual selection and similar mechanisms have also been reported in species with separate sexes (for reviews see Gillott 2002; Koene and Ter Maat 2001, 2002).

The above illustrates that sperm donors can gain a fitness advantage by rerouting resources in their partners to the female function to assure optimal use of their sperm, i.e., feminization (Michiels 1998). Feminization can be accomplished by inducing egg laying or by preventing partners from remating, leaving the sperm recipient no other option than laying more eggs. However, this feminization is not necessarily beneficial for the recipient because its resources may be shifted away from optimal allocation. Hence, mates may not agree about the way in which resources are allocated in their partners, which could lead to sexual conflict over sex allocation or even resource allocation.

Lymnaea stagnalis is a simultaneous hermaphrodite that can mate in the male and female role, but within a copulation one sexual role is performed (Van Duivenboden and Ter Maat 1988) after which role alternation is possible (Koene and Ter Maat 2005). Although self-fertilization is an option, outcrossing is preferred and allosperm stores are sufficiently large to fertilize eggs during approximately 3 months (Cain 1956). When the male function is eliminated experimentally, the female reproductive output roughly doubles, which shows that reallocation of energy between male and female function does occur (De Visser et al. 1994; Koene, in press). The earlier onset of egg laying after the reception of sperm (van Duivenboden 1983) is attended by reduced body growth as well as reduced prostate gland development (Koene and Ter Maat 2004), suggesting a trade-off between growth on the one hand and sexual functions on the other. In addition, it is in agreement with the idea that feminization, i.e., early onset of egg laying, results from manipulation of the sperm receiver by the sperm donor. In this study, we have experimentally manipulated the opportunities to copulate to investigate whether this feminization only concerns the onset of egg laying, or whether longer-lasting feminizing effects are associated with multiple copulations.

Materials and methods

Forty immature specimens of the pond snail *L. stagnalis* of the same age and with a shell length of 15 mm were obtained from our laboratory culture (Van Der Steen et al. 1969). They were housed individually in perforated polyethene jars (460 ml) in the same experimental tank with continuous water exchange. The water was kept at 20°C and the light/dark cycle was 12:12 h. During the experiment, the snails were provided with one circular disc of lettuce with a surface area of 19.6 cm^2 each day. Because this was slightly below their maximum food intake, and thus completely consumed, the total energy intake of individuals was equal without causing nutritional stress (Locher and Baur 2002). To maintain equal food intake, animals from both treatments were not fed on grouping days (see below), because mating animals do not feed during these 24-h mating period (unpublished data).

After three weeks, when the animals had reached adulthood and were 24±1 mm, they were individually marked with nail polish and randomly divided in four equal groups, resulting in two replicates for each treatment. Each group was transferred to a perforated polyethene box (5,600 ml) and the snails were allowed to copulate for 24 h. All the snails were then returned to their original jar. For two groups, this was the only mating opportunity and these groups will be referred to as the once-grouped treatment. For the other two groups, the grouping procedure was repeated once every week for another 7 weeks. These two groups will be referred to as the repeatedly-grouped treatment. We know
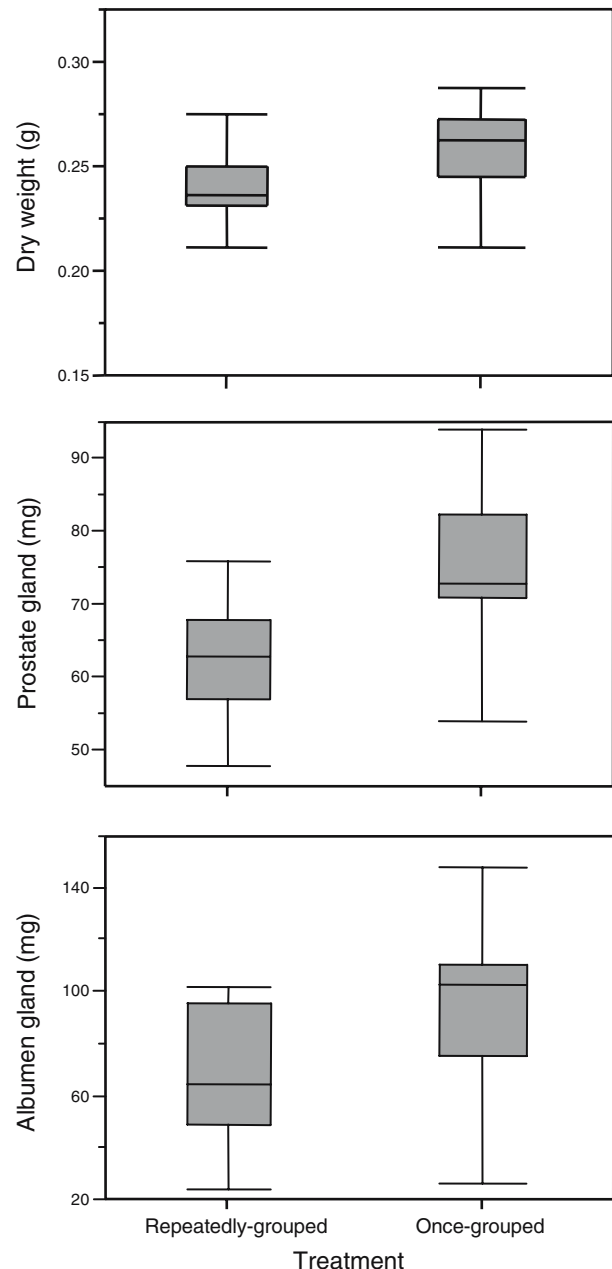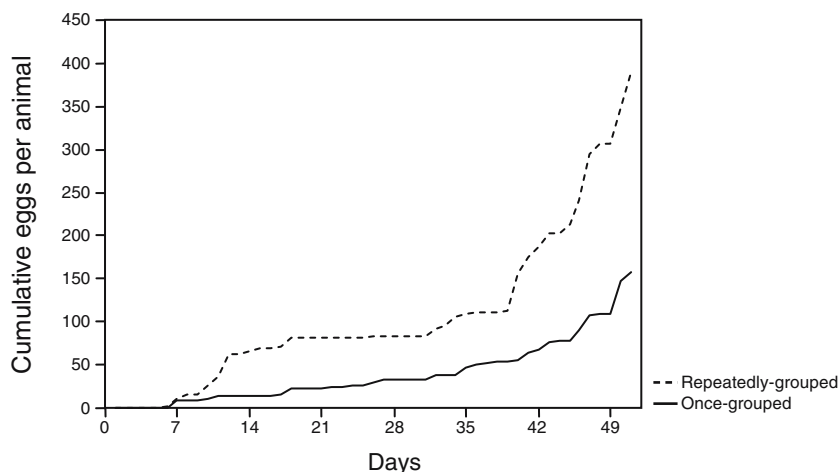

Fig. 1 Dry weights and gland weights of the animals in the once- and repeatedly-grouped treatment. The *box plots* indicate median, 25th and 75th quartile, and range

Fig. 2 Cumulative plot showing the total number of eggs per animal for the once- and repeatedly-grouped treatment


from previous work that within a 24-h mating period, animals donate and receive sperm at least once (Van Duivenboden and Ter Maat 1988; De Boer et al. 1997; Koene and Ter Maat, unpublished data). We are therefore confident that all animals donated and received sperm at least once during each grouping. Because these snails can store enough sperm to fertilize eggs for approximately 3 months (Cain 1956), the once-grouped snails did not suffer from allosperm depletion during the 8 weeks of the experiment.

Throughout the experiment, egg laying was recorded daily for each individual. One repeatedly-grouped animal died during the course of the experiment. At the end of the experiment, 7 days after the last grouping (to allow for prostate gland replenishment, see De Boer et al. 1997), body weight and shell length were measured. The animals were then anaesthetized with 50 mM $MgCl_2$ after which the shell was removed and weighed. The albumen gland and prostate gland were dissected out and their wet weights were determined. Finally, the wet and dry weights (after freeze-drying) of the remaining soft body parts were measured. All weights were determined using a semimicro-analytical balance (type R160D, Sartorius, Göttingen, Germany). Five randomly chosen animals of each treatment were

fixed in Bouin, after removal of the glands, for histological processing. After fixation, the gonadal tissue was embedded in paraffin. Serial sections of 7 μm were stained using the HE method (haematoxylin and eosin). A naïve observer took digital photographs of sections where both sperm and eggs were present in the acini of the ovotestis. Subsequently, the number of oocytes, and the area of oocytes, aggregations of sperm heads, and Sertoli cells were measured using ImageJ (NIH, USA). All area measurements were corrected for the total area of the acinus.

For the statistical comparisons of the experimental treatments, we report the averages and standard deviations as well as the F-values with degrees of freedom and p-values from the nested analysis of variance (ANOVA). Because during the period where the animals were allowed to copulate, four groups were formed (two in each treatment) these groups were nested within the treatment factor. For all correlations we indicate the Spearman correlation coefficient (r), sample size (n), and p-value. For the area measurements, we report the averages and standard deviations as well as the F-values with degrees of freedom and p-values from a nested ANOVA, with individuals nested within treatment.

Fig. 3 Size of albumen gland vs the total number of eggs produced by each individual. The *line* shows the reciprocal (hyperbolic) fit (see "Results") and the accompanying *dashed lines* the 95% confidence intervals. The *dotted lines* indicate the estimated minimal and maximal size of the albumen gland
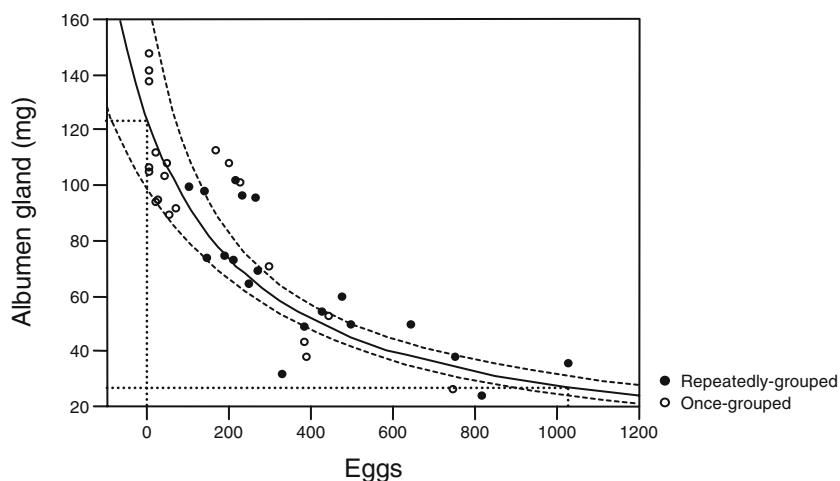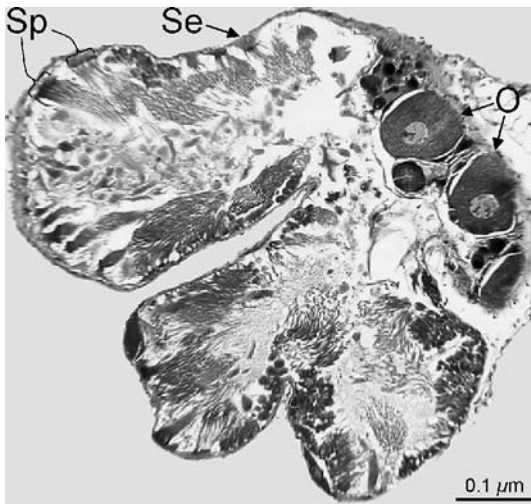
Fig. 4 A section through an acinus of the ovotestis. The oocytes (O), sperm (Sp), and Sertoli cells (Se) are labelled

Results

Repeatedly-grouped and once-grouped animals did not differ in wet body weight (1.59±0.12 and 1.66±0.11 g, respectively; $F_{1,25}=1.79$; $p=0.192$) or shell height (3.65± 0.13 and 3.60±0.13 cm, respectively; $F_{1,32}=1.33$; $p=0.257$). The dry weights between the two treatments show a larger difference, but this is also not significant (0.24±0.02 and 0.26±0.03 g, respectively; $F_{1,25}=3.73$; $p=0.0648$). Shell weight was significantly lower in repeatedly-grouped than in once-grouped animals (0.52± 0.06 and 0.56±0.06 g; $F_{1,35}=4.52$; $p=0.0405$). Figure 1 shows the dry weights for the two treatments. The figure also shows the differences in gland weights. The wet weights of prostate glands (62.5±7.7 and 75.3±10.9 mg; $F_{1,35}=18.26$; $p=0.0001$) and albumen glands (65.4±24.8 and 94.5±33.3 mg; $F_{1,35}=9.50$; $p=0.004$) were significantly larger in once-grouped than in repeatedly-grouped individuals.

In both treatments, egg laying started before the second grouping day of the repeatedly-grouped treatment. The total number of egg layers in the two groups was not different (Fisher's exact test: $n=40$; $p=0.11$) neither was the onset of egg laying, i.e., the day that the first egg mass was laid (Wilcoxon: $X^2=0.055$, $p=0.82$). Repeatedly-grouped animals laid more than twice as many eggs in total as once-grouped animals (382.9±256.9 and 153.7±202.0 eggs, respectively; $F_{1,35}=9.82$; $p=0.0035$; Fig. 2), while the total number of produced egg masses was not significantly different (5.9± 3.1 and 3.9±4.1 masses, respectively; $F_{1,35}=2.88$; $p=0.0984$). Thus, the difference in fecundity is due to the number of eggs per egg mass (69.4±32.5 and 33.2± 17.0 eggs/mass; $F_{1,30}=14.41$; $p=0.0007$). The weight of the albumen gland shows a negative correlation with the total number of eggs laid ($r=-0.831$, $n=39$, $p<0.0001$). Rather than showing this linear correlation, Fig. 3 shows the regression fit after a reciprocal transformation of the data on the y-axis, i.e., $1/y$ ($R^2=0.720$; $F_{1,37}=95.2$; $p<0.0001$). This regression fitted the data best and explains 72% of the
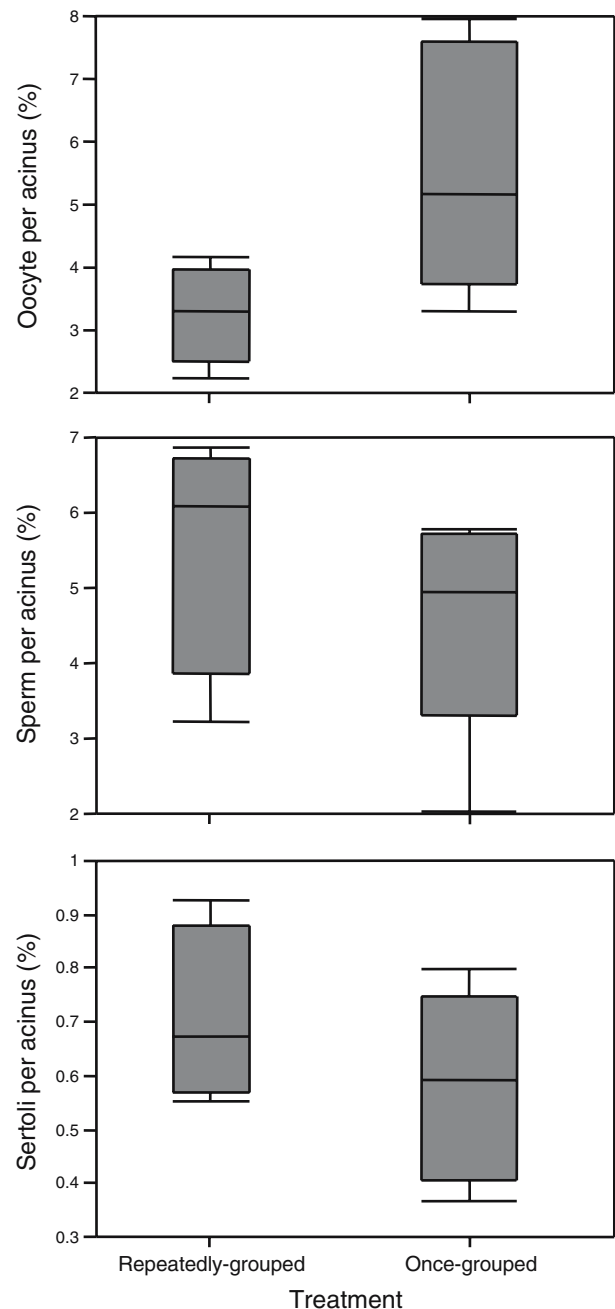

Fig. 5 The area measurements of the ovotestis in the once- and repeatedly-grouped snails. The area measurements of oocytes, sperm, and Sertoli cells are shown as a percentage of the total area of the acinus. The *box plots* indicate median, 25th and 75th quartile, and range

variation, rather that the 69% explained by the linear correlation. Additionally, the weight of the albumen gland was positively correlated with the number of days since the last egg mass was produced ($r=0.607$, $n=39$, $p<0.0001$). Thus, when more eggs are produced, the albumen gland is smaller, which is consistent with previous findings that this gland is empty after egg laying (De Jong-Brink et al. 1982; Koene and Ter Maat 2004). Moreover, the fitted curve of Fig. 3 can be used to estimate the average size of

the albumen gland when no eggs are laid by looking at the *y*-value when eggs = 0, which is 125 mg. Based on his curve, the size of the albumen gland at the maximum observed egg laying (1,023 eggs) is estimated at 27 mg.

Figure 4 shows a section through an acinus in which oocytes, sperm and Sertoli cells were measured. Area measures are expressed as a percentage of total acinus area. The number of oocytes per acinus area as well as the size of the oocytes were not significantly different between treatments (respectively, 0.0070±0.0041 vs 0.0093±0.0066 oocytes/acinus area; $F_{1,7}$=0.64; p=0.450 and 3.06±2.14 vs 5.20±4.37% of acinus area; $F_{1,7}$=3.425; p=0.107). Also, comparisons of the sperm area (respectively, 5.79±2.94 and 5.33±2.70% of acinus area; $F_{1,7}$=1.003; p=0.350) and the Sertoli cell area (0.73±0.50 and 0.50±0.34% of acinus area; $F_{1,7}$=2.199; p=0.182) did not reveal a significant difference between the two treatments. Figure 5 shows the percentage of the total area of the acinus occupied by oocytes, sperm, and Sertoli cells.

Discussion

Sex allocation theory in simultaneous hermaphrodites predicts that individuals with more mating opportunities should increase investment in male reproduction (Greeff and Michiels 1999; Charnov 1980, 1982). And, under the general assumption that hermaphrodites invest a fixed amount of resources in reproduction (e.g., Charnov 1979), this increase should be traded off with a decreased investment in female reproduction. This prediction from sex allocation theory is supported by experimental work on animals (Raimondi and Martin 1991; Trouvé et al. 1999; Locher and Baur 2000; Schärer and Ladurner 2003; Tan et al. 2004; Schärer et al. 2005; Lorenzi et al. 2005) as well as plants (López and Domínguez 2003). We find no evidence that frequently mating pond snails (*L. stagnalis*) increase their sperm production because the measured parameters are not higher. But contrary to this prediction, we find that these snails develop smaller prostate glands, where seminal fluid is produced. Moreover, female investment increases when these snails mate more often, while growth may be reduced.

Effect on the male function

Sertoli cells, which are the "nurse" cells that coordinate spermatogenesis, have previously been used as a measure for the area occupied by sperm heads (França and Russel 1998; Vileda et al. 2003). Nonetheless, although the trend goes in the predicted direction, we found no difference in this measure between treatments. Neither did we find any difference in the measured sperm areas. Because the latter areas are relatively small, they may not be a reliable measure for sperm production. Evidently, the lack of difference may also be due to the small sample sizes. But irrespective of changes in male investment in gamete production, we find clear evidence for inhibited development of the prostate gland in the animals that had more

mating opportunities. In the following, we will explain how we arrive at this conclusion.

We know from many previous studies that after 7 days of sexual isolation, animals are eager to mate in the male role (e.g., Van Duivenboden and Ter Maat 1988; De Boer et al. 1997; Koene and Ter Maat 2005). Additionally, the number of days to the onset of egg laying is not different between the treatments, indicating that matings were successful in the once-grouped snails. If no semen had been received, this latter treatment would have delayed its onset of egg laying by several weeks (Van Duivenboden 1983). We can therefore safely assume that in each 24-h grouping all the animals mated at least once as male and female (see Van Duivenboden and Ter Maat 1988; De Boer et al. 1997; Koene and Ter Maat, unpublished data). Hence, the repeatedly-grouped animals evidently spent more time and energy on copulation, and had a higher seminal fluid and sperm turnover. From De Boer et al. (1997; reviewed in Koene, in press), we also know that this sexual isolation period allows enough time for the prostate gland to be maximally filled with seminal fluid. In our experiment, we waited 7 days after the last 24-h grouping before determining gland weights to ensure that prostate glands were maximally filled in both treatments. We can therefore infer that the observed difference in prostate gland size between the two treatments is due to slower development of this gland in the repeatedly-grouped snails.

Effects on the female function

As reported in this study, increased mating opportunities result in an increased investment in egg laying. In insects, such an increase in female reproductive output can often be explained by the donation of nuptial gifts by males (e.g., Arnqvist and Nilson 2000). Similarly, in internally fertilizing hermaphrodites, the specialized organs for digestion of sperm and seminal fluid could provide a direct source of energy (Greeff and Michiels 1999). However, the observed effect in *L. stagnalis* cannot be attributed (exclusively) to energy gained through digestion of semen because then the reduction in development of the prostate gland (i.e., seminal fluid production) should not have been observed. Moreover, because *L. stagnalis* and simultaneous hermaphrodites in general usually mate an equal number of times in both sexual roles, the net gain will be zero (or even less than zero because nuptial gift production will be more costly than the energy extracted by gift digestion). Because the increase in egg laying occurs after the initial onset of egg laying, our data suggest that this is not only a mechanism to initiate the female function (e.g., Gillott 2002) or to delay selfing and avoid inbreeding (Tsitrone et al. 2003). The latter is in agreement with the lack of inbreeding depression in natural populations of *L. stagnalis* from Finland (M. Puurtinen, personal communication).

Previous research has shown that egg masses of animals that are hormonally induced to lay (via an injection of the egg laying hormone CDCH) contain more eggs when their

previous egg mass was laid longer (Ter Maat et al. 1983). Hence, the size of an egg mass could be determined by the number of ripe oocytes that are available. However, the clear difference in egg laying in the present study is not reflected in either the number or size of the oocytes in the ovotestis, but is reflected in the size of the albumen gland. This organ, which is the major gland that provisions the eggs, is smaller in the repeatedly-grouped treatment because it is continuously used, while its products accumulate in the once-grouped animals. This finding suggests that egg mass size depends mainly on the size of the albumen gland. This is consistent with the previous finding that albumen glands are heavier when eggs were laid longer (Koene and Ter Maat 2004, see also De Jong-Brink et al. 1982). For the garden snail *Cantareus aspersus* (previously *Helix aspersa*) it has recently been shown that the number of ripe oocytes in the ovotestis provides a permissive signal for the occurrence of egg laying (Antkowiak and Chase 2003). Given our findings, we predict that egg laying behavior of *L. stagnalis* depends mainly on the availability of albumen secretion.

Contrary to our results, Van Duivenboden et al. (1985) reported that a suppression of egg laying occurs when *L. stagnalis* is allowed to mate often. However, that study only looked at the effect of frequent mating by grouping adult animals during 1 week, while we allowed initially juvenile animals to mate for 24-h periods and followed individual animals over a much longer time span. Differences between the two studies may therefore result from different experimental set-ups. For example, by only allowing the animals to interact for 24-h, rather than permanently grouping them for a week, our experimental set-up excludes other interfering effects of living in groups. Additionally, we made sure that the energy intake of all individuals was equal, which was not done in the other study. Finally, the size and mating history of the animals used in both studies was different (adults of 30–32 mm in Van Duivenboden et al. 1985; virgins of 24 mm in this study) and represents an age difference of approximately 1 month. The discrepancy between the two studies may therefore also reflect a difference in strategies at different times in the life cycle, although we think that this latter explanation is rather unlikely.

Effects on growth?

Throughout our experiment we kept the energy intake equal for all individuals without causing nutritional stress. Using the same approach, a previous study showed that the earlier onset of egg laying by the semen decreases allocation towards both growth and prostate gland development (Koene and Ter Maat 2004, note that those data should all be graphed in grams). Such a growth difference is in agreement with the observation that dry weight of pond snails decreases with increased egg laying (Zonneveld and Kooijman 1989). Our current data suggest that growth may also be affected after this initial onset of egg laying, although much less pronounced. We find a significant difference in shell weight but no significant difference in dry weight (only a trend in the predicted direction) between the treatments.

If growth is indeed affected by the number of mating opportunities, this would imply that a three-way trade-off exists between growth, male and female reproduction. Several recent studies suggest that such a three-way trade-off may exist (Yund et al. 1997; Koene and Ter Maat 2004). If true, this would mean that resources can be reallocated to reproduction from the growth and maintenance budget. As a consequence, the basic theoretical assumption that hermaphrodites have a fixed amount of resources that they can allocate to the male or female function (Charnov 1979; Pen and Weissing 1999), may need to be reconsidered. Clearly this warrants further investigation in future studies.

In conclusion, our data suggest that in pond snails that mate more often, sperm production is probably not increased, while prostate gland development is clearly inhibited. Besides, we find an increase in female reproduction and indications for a decrease in growth. These results not only indicate a trade-off between male (seminal fluid) and female (egg) investment, but are suggestive of a three-way trade-off with growth. Interestingly, contrary to the prediction of sex allocation theory that increased mating opportunities should shift allocation towards the male function, we find evidence for feminization. Such feminization may be the result of the individuals optimizing the genetic diversity of their offspring, which predicts that repeated matings with the same partner will not cause the effects we observed in this study. Alternatively, an allohormone in the semen may be transferred to increase egg laying in the mating partner.

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