Mating rate influences female reproductive investment in a simultaneous hermaphrodite, *Lymnaea stagnalis*

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As a consequence of differential investment in gametes, males and females adopt different strategies to maximize their fitness. Females, by definition, produce large, costly gametes and fitness is limited mainly by resources that can be allocated to eggs and maternal care. In contrast, males often produce millions of sperm, which, in theory, could fertilize all the eggs in a population. Therefore, the fitness of males is limited by access to eggs carried by females and selection will favour males that can obtain, besides many copulations, the highest proportion of fertilizations from copulations (Bateman 1948; Anthes et al. 2010). As a result of male strategies aimed at increasing paternity, females often experience costs of mating multiply (Chapman et al. 2003). Such mating costs can include impaired immunity (Rolf & Siva-Jothy 2002), exposure to parasites, increased predation risk and physical injuries (Crudgington & Siva-Jothy 2000). In addition, males often transfer not only sperm during copulation but also seminal fluid compounds that can influence the recipients' behaviour and physiology, for instance by increasing current investment in offspring at the expense of future reproduction (Chapman et al. 1995). Such male seminal fluid compounds can prevent females from reaching their reproductive optima (Arnqvist & Rowe 2005). Despite these negative effects of mating, females generally seem to copulate more often than appears to be necessary to fertilize all eggs. Why then do females mate multiply? Evidence reveals that not only is multiple mating driven by male promiscuity, but it can also provide benefits to females that can counterbalance costs (Arnqvist & Nilsson 2000; Jennions & Petrie 2000; Simmons 2005). These benefits may be either direct or indirect. Direct benefits to females can be accrued through, for example, guarding behaviour by sires or nutritious nuptial gifts. In the field cricket *Gryllus lineatus*, for instance, females that mated multiply and thus received multiple spermatophores lived 32% longer and produced almost twice as many offspring as singly mated individuals (Wagner et al. 2001). A meta-analysis of the effects of polyandry in insects also found evidence for positive effects of mating with multiple males on female fitness, including increased egg production and higher percentages of hatched eggs (Arnqvist & Nilsson 2000). Indirect benefits may result from an improved genetic make-up or genetic diversity of offspring (Møller 1997).
The bulk of work on benefits and costs of polyandry has focused on separate-sex species, even though hermaphroditism is present in 20 of the 28 more speciose animal phyla (Michiels 1998; Anthes 2010) and is estimated to be present in 5% of animal species (Jarne & Auld 2006) and most plants. Although sexual selection was once thought to be absent from the lower animal classes (Darwin 1871), its presence is now well established in hermaphrodites (Anthes et al. 2010; Pelissié et al. 2012 and references therein) and considered an important evolutionary force (Charnov 1979; Michiels 1998; Arnqvist & Rowe 2005; Koene 2012). Many hermaphroditic species mate promiscuously even when damaging mating tactics are employed, for example stabbing mating partners with so-called ‘love-darts’ in land snails (Koene & Schulenburg 2005), piercing the body with copulatory setae in earthworms (Koene et al. 2005) and hypodermic insemination in marine flatworms (Michiels & Newman 1998). This is thought to occur because individuals can compensate for fitness losses in one sexual function via their other sexual function and it has therefore been suggested that sexual conflicts between mating partners can become more costly for hermaphrodites than for separate-sex species (Michiels & Koene 2006).

Similar to the situation in separate-sex species, in hermaphrodites the extent to which benefits can counterbalance mating costs depends on many variables, such as food and mate availability and whether or not individuals can phenotypically adapt to current conditions (Schärer 2009). For example, in the hermaphroditic marine slug Chelidonura sandrana investment per offspring is increased in polyandrous animals compared to animals that have repeatedly mated with the same individual, and maternal investment peaks at intermediate mating rates (Sprenger et al. 2008a, b). In this study, we focused on the simultaneously hermaphroditic pond snail Lymnaea stagnalis. This species mates unilaterally and has the option to reciprocate in the other sexual role (i.e. role alternation: Koene & Ter Maat 2005). It fertilizes internally, prefers outcrossing, and its egg production has been shown to be influenced by mating rate (Koene et al. 2009a, b, 2010; Jarne et al. 2010). Intermediate mating rates (animals grouped once a week) resulted in enhanced female reproductive output compared to snails that mated only once (Koene et al. 2006). However, even higher mating rates (continuous access to one or more partners) led to strongly depressed oviposition rates (Van Duivenboden et al. 1985; De Visser et al. 1994). The negative effect of copulation on the number of eggs laid by the female copulation partner (hereafter recipient) is due to repeated receipt of ejaculates, which contain bioactive male accessory gland products (Koene et al. 2009a, 2010), but also to male (hereafter donor) expenditure on expensive ejaculates (Hoffer et al. 2010). In addition, received sperm (allosperrm) can be stored and used for months in L. stagnalis; hence female fertility can be maintained at low mating rates (Cain 1956; Y. Nakadera, C. Blom & J.M. Koene, unpublished data).

The above-mentioned results suggest a sexual conflict in L. stagnalis, since mating rates exceed those optimal for recipients. However, it remains unclear whether decreased reproductive investment in terms of egg production is ameliorated by possible benefits accrued through multiple mating and polyandry. The main goal of the present study was therefore to integrate previous treatments into one comprehensive study to assess the effect of mating opportunity on fecundity and female reproductive investment. To that end, we subjected pond snails to treatments that provided either no mating (self-fertilization) or various mating opportunities that comprised one-time mating, and repeated and continuous access to either the same or different potential partners. Under these conditions, paired snails copulate in both sexual roles and mating rates are highest when continuously paired (Koene &

![Figure 1. Schematic overview of the experimental set-up and procedures. Only the first 2 weeks of a total of 10 weeks are shown, as later weeks are a repetition of days 8-14. Treatment labels are explained in the main text. Focal animals are shown in white. Grey-coloured snails indicate either the same partner (light grey shell) or a different partner (dark grey shell). Dashed squares indicate experimental jars and dashed diagonal lines indicate a physical separation in the jar. Focal snails are shown as copulating as a female, but in reality individuals mated in both sexual roles. Snail outlines were redrawn after De Boer et al. (1996).](image-url)
Figure 2. Growth and female reproductive investment of *Lymnaea stagnalis* during 10 weeks. Treatments are denoted above the graphs (‘Cont.’ equals ‘Continuous’). (a) Growth measured as shell length of snails over time. (b) The number of eggs produced weekly within each treatment. (c) Dry weight of egg masses produced per week. Letters in (b) and (c) above the graph indicate Tukey post hoc results on the total number of eggs produced per week and the mean dry weight of egg masses produced per week, respectively. (d) To indicate how the investment per offspring changed over time, this variable was calculated by dividing the weekly dry weight of egg masses by the weekly number of eggs. Letters in (d) denote post hoc t test results (see text). Treatments not connected by the same letter are significantly different. The dots and bars represent means ± 1 SEM. Note that in (b), (c) and (d) the scale on the X-axis is different from that in (a), because no egg masses were laid during the first experimental week.
By tracking growth, egg production and investment in eggs under controlled feeding conditions we tested the effect of mating opportunity and partner availability on female reproductive investment.

METHODS

Experimental Set-up

All specimens of *L. stagnalis* were obtained from our laboratory culture. In the breeding facility and experimental tank the low-copper water was kept at 20 °C and the light:dark cycle was 12:12 h. In total 154 juvenile animals with a shell size of 16 mm were individually raised to maturity in 2 weeks in perforated plastic jars (460 ml) in an experimental tank, which had continuous water exchange. These focal snails were each fed a circular disc (19.6 cm²) of lettuce per day, which was slightly below their maximum daily food intake and therefore completely consumed. An additional set of 88 snails of the same size and age, similarly treated as above, was used as partners for the focal snails. For these partner snails we did not collect any body size or egg-laying data. In between mating trials these animals were kept isolated to ensure eagerness to donate sperm (De Boer et al. 1997; Koene & Ter Maat 2005).

Upon maturity the focal animals were randomly divided over the following seven treatments (see also Fig. 1) and individually labelled using numbered tags (Het Bijenhuis, The Netherlands) that were fixed to the shell with a cyanoacrylate-based glue. The control treatment consisted of unmated virgin animals that produced eggs through selfing (No mating, Selfing). For the second treatment focal snails were allowed to be inseminated only once at the start of the experiment (Once Mated). The third treatment (Once + Presence) was only different from the previous one in that, after having been inseminated once, the same partner joined the focal snail every week but they were unable to copulate owing to a plastic mesh that divided the jar into two compartments (but allowed for water exchange between the compartments). The presence of a snail in this treatment was to check for possible effects other than those caused by copulation, and was done at the same time intervals as the following two repeated mating treatments. In the fourth and fifth treatment focal snails were allowed to copulate every 7 days with either the same (Repeated, Same) or a different (Repeated, Different) partner each time. During these mating trials the mating behaviour was observed. Animals were returned to their individual jars as soon as they had mated reciprocally or when the trial had lasted 8 h. For the sixth treatment focal snails were continuously paired to the same individual during the whole experimental period (Continuous, Same). Finally, for the seventh treatment focal snails were also continuously allowed to mate, but were provided with a different partner every week (Continuous, Different) on days when mating trials took place. Each of the first five treatments contained 22 focal snails. In the last two treatments, with continuous mating opportunities, it was impractical to score egg laying individually of both focal snails; therefore these data were averaged over the pairs (i.e. N = 11), thus also avoiding pseudoreplication. For the treatments with different partners we ensured that partners were never paired more than once with the same focal snail. For these treatments (i.e. Repeated, Different and Continuous, Different) we reused each partner snail by moving it over to a different focal snail within the same treatment, thus ensuring that these partners all had the same mating history and eagerness. Figure 1 provides an overview of the experimental schedule.

During the whole experimental period, which lasted 10 weeks, egg masses were collected once a week from the jars of the focal snails. Given that allosperm is stored for up to 3 months in *L. stagnalis* and used for outcrossing (Cain 1956), egg masses from all treatments except Selfing contained largely outcrossed offspring (thus, any changes measured will be caused by mating regime and not by a switch from outcrossing to selfing). Each egg mass was collected in a separate, preweighed Eppendorf tube filled with 70% ethanol. The ethanol turns the egg bodies white within 30 min, thus facilitating counting of the eggs. Since the lipid content of egg masses is insignificant compared to other constituents (less than 0.5%, Wijisman & Van Wijck-Batenburg 1987), we assumed egg mass weight loss from lipid extraction to be negligible. Following counting, the egg masses were lyophilized overnight in a freeze-dryer. For each individual, the dry weight of egg masses produced during 1 week was then determined on a closed microbalance (Sartorius, model 1712 MP8, Göttingen, Germany). Shell length was measured weekly with callipers to the nearest 0.5 mm. At the end of the experiment all animals were killed and separated from their shells and freeze-dried in punctured 2 ml Eppendorf tubes. After drying, each individual was weighed on a closed microbalance. As body size and shell length are closely correlated (Zonneveld & Kooijman 1989), measuring shell length allowed us to check whether possible treatment differences in fecundity were the result of differential investment in growth.

Statistical Analysis

All variables were checked for conformation to model assumptions of parametric tests. Final shell size was analysed with a generalized linear mixed model (GLMM). In addition, we inferred growth rates by fitting Von Bertalanffy growth curves to the weekly measurements of shell length with a fixed initial size of 16 mm for all individuals (e.g. Zonneveld & Kooijman 1989; Koene et al. 2008). The onset of egg laying was analysed using proportions of individuals producing egg masses in each week. When an experimental snail died, the lack of reproductive data was taken as a missing value with its predictable effect on the degrees of freedom in statistical analyses. The survival analysis itself was performed assuming a nonconstant hazard with a Weibull distribution. We tested the effect of treatment on total fecundity and total dry weight of egg masses by one-way variance analyses. The effect of time on measured variables was analysed with several GLMMs, with weekly number of eggs and egg mass dry weight as the dependent variables, treatment and time (in weeks) as fixed factors, and snail identity as a random factor. To compare differences between treatments we performed Tukey post hoc tests (α = 0.05) with Bonferroni corrections for multiple testing. Changes in investment per offspring may show patterns of interest. However, calculated new variables (by dividing the weekly dry weight of eggs masses by the number of eggs) can result in erroneous conclusions (Packard & Boardman 1999). Therefore, we constructed an ANCOVA in which number of eggs and time were taken into account as covariates. In this way, we controlled for effects of fecundity and time on the dry weight of egg masses, thus enabling us to test for differences in investment per offspring. In addition, possible differences in survival were approached with a survival analysis. All statistical analyses were performed in R version 2.8.1 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org).

RESULTS

Growth and Survival

We found no significant differences in shell growth (GLMM: $F_{5,144} = 3.47, P = 0.064$; Fig. 2a, Table 1) or final body weight between the animals in the different treatments (ANOVA: $F_{5,113} = 1.090, P = 0.373$). Fitted growth curves of the Von
Bertalanffy type on the weekly measured shell lengths provided us with estimates of final shell size as well as growth rate (Table 2). The 95% confidence intervals of these estimates all overlapped. On the one hand, this corroborates that final shell size did not differ between treatments and, on the other hand, shows that growth rate, the temporal investment in bodily growth, was the same for all treatments (Table 2). Survival analysis did reveal significant differences in survival between treatment groups ($\chi^2_G = 20.92, P = 0.002$). In the No mating, Selfing and the Once, Presence treatment no snails died. In the Once Mated treatment one snail died, while two died in the Repeated, Different treatment. Four snails died in the Repeated, Same and the Continuous, Same treatment, while six died in the Continuous, Different treatment.

**Egg Production**

The weekly number of eggs produced was clearly affected by treatment (GLMM: $F_{6,792} = 45.82, P < 0.001$; Fig. 2b, Table 1). Most notably, Tukey post hoc tests revealed that continuously paired individuals produced significantly fewer eggs in total (i.e. over the whole experimental period) than most of the treatments that had less frequent mating opportunities. Being virgin (No mating, Selfing) or the presence of an inaccessible mating partner (Once, Presence) had no effect on the total number of eggs produced; neither did the identity of the partner (Same versus Different partner treatments). For all but the continuously paired treatments, there was a significant difference in the proportion of individuals laying eggs during the second experimental week ($\chi^2_G = 10.05, P = 0.040$; during the first week no egg masses were produced). This difference was due to only seven of 22 virgins laying eggs (31.82%), while for the once and repeated treatments the average was 12.5 out of 22 (56.82%). In all subsequent weeks, the proportion of animals laying eggs was not significantly different between treatments.

**Investment in Eggs**

Like egg production, the weekly dry weight of egg masses was affected similarly by the different treatments (GLMM: $F_{6,792} = 32.54, P < 0.0001$; Fig. 2c, Table 1). These results demonstrate an increase in weekly investment in eggs over time up to mating rates of once per week. Continuously paired snails, however, differed from these treatments in that their slopes were significantly shallower (Fig. 2c). We tested, with an ANCOVA, for the effect of treatment on the dependent variable weekly dry weight of egg masses with time (weeks) and number of eggs as covariates. As expected, the main source of variation in dry weight of egg masses was the number of eggs present in these masses (covariate number of eggs: $F_{1,798} = 2405.53, P < 0.0001$). In addition to these covariate effects, the ANCOVA revealed a significant effect of the treatment ($F = 6.798, P = 0.001$). The latter finding means that, because this test took the two covariates (number of eggs and time) into account, the treatment affected the average investment per offspring. To illustrate this clearly, we depicted the investment per offspring (weekly dry weight of egg masses divided by number of eggs) in Fig. 2d. Post hoc test results derived from output of the ANCOVA described above show that repeatedly mated and continuously mated snails invested more per offspring than unmated and singly mated individuals (Fig. 2d).

**DISCUSSION**

Our study documented that mating rate alters female reproductive investment, but not growth, in the pond snail *L. stagnalis*. It is worth noting that earlier estimates relied solely on egg numbers, while we included dry weights. At mating rates above once a week (continuously paired treatments) overall female investment was strongly suppressed in comparison with the treatments with lower mating rates. This suppression probably results from receipt of male accessory gland products, transferred by the donor, that have been shown to increase the oviposition interval in recipients (Koene et al. 2009a, 2010). Earlier work (Van Duijenboden et al. 1985), where a recovery of inhibited egg laying was observed after 6 days, further strengthens this interpretation. The latter indicates that at short insemination intervals the inhibition of egg laying is maintained and allows no time for recovery to the higher fecundity level at lower mating frequencies. Based on previous work, such high mating rates are expected to be at least 1.5 times per week (a highly conservative estimate, see Koene & Ter Maat 2007).

When the snails were grouped only once a week, we found that egg production remained high. These treatments showed a higher investment per offspring (i.e. dry weight per egg) than treatments in which animals mated never or only once. Within the repeatedly mated individuals the treatment that received a different mating partner each week invested more per egg, which hints at a potential benefit in being polygamous. Such a polygamy effect was not detected in the continuously paired treatments, probably because of the continuous suppression of egg laying and higher mating costs. The latter interpretation is corroborated by the result that mortality rates were generally higher in our treatments with higher mating opportunity. At the other end of the spectrum (i.e. no mating at all), we found that selfing individuals invested less dry
weight per egg than the other treatments. This lower female reproductive investment by virgins might represent a way to avoid costs of self-fertilization (Escober et al. 2011), as current reproductive investment would trade off against future reproductive investment (e.g. Wedell et al. 2006).

Given the above, the current findings suggest that a mating frequency around once per week resulted in optimal investment in eggs in terms of both number and dry weight. This is further supported by the finding that the presence of an unreachable mating partner (treatment Once + Presence) did not affect these reproductive traits. In other words, the effect was not due to chemicals or pheromones that are released by a potential partner, but was caused by actual mating.

Since we kept food intake equal across treatments, we can conclude that growth was not affected by treatment, and we have no evidence that resources were invested elsewhere. However, in the course of the experiment growth (and ageing) did reveal a clear increase in investment per offspring over time that was independent of treatment. Hence, larger (and older) individuals had higher female reproductive output, as predicted by size advantage models (Ghiselin 1969; Charnov 1982). A number of empirical studies have shown this relationship in hermaphroditic animals (e.g. DeWitt 1996; Yusa 1996; Angeloni et al. 2002; Ohbayashi-Hodoki et al. 2004; Jordaeus et al. 2007) and in hermaphroditic plants (Klinkhamer et al. 1997; Cadet et al. 2004). While for L. stagnalis a mating preference based on size was not found previously (Knoune et al. 2007), more recent work does demonstrate that this species mates relatively more often as a female as it becomes older (Hermann et al. 2009). Thus, our results are consistent with age-dependent sex allocation, but targeted experiments are needed to exclude the possibility that older snails also transfer larger ejaculates per mating.

While we attribute the differences in female reproductive output mainly to the transfer of bioactive compounds from the male accessory gland (see above; Knoune et al. 2009a, 2010), there are two other options to consider. First, like all Basommatophoran gastropods, these pond snails have a specialized gametolytic organ that digests the bulk of received sperm (Knoune et al. 2009b; Jarne et al. 2010). Our results partly support the hypothesis of energy investment into the transfer of bioactive compounds from the male (treatment Once + Presence). Second, polyandrous snails could potentially optimize quality. We do report some evidence here that investment per offspring was increased in polyandrous snails compared to multiple inseminations by the same partner (in repeatedly mated treatments).

One of the few studies to report direct positive effects (larger egg capsules and offspring) of multiple mating and polyandry in hermaphrodites was performed on the opisthobranch Cheilodonura sandrana (Sprenger et al. 2008a). In that study, however, mating histories of the sperm donors in the different treatments were not equal. The ‘same’ partners may have been sperm and/or seminal fluid depleted, while the ‘different’ partners were not. In the present study, we circumvented this problem by standardizing the donors’ mating history (as was done in later C. sandrana studies, e.g. Sprenger et al. 2008b). In our set-up this also resulted in a standardized intermating interval, thus ensuring that, at maximum, each individual received and donated once during each observation session in once- and repeatedly mated individuals. Unfortunately, this standardization introduced a potential confounding factor in the different partner treatments, because the same partners were reused (for practical reasons) and offered to another focal snail within the treatment, thus potentially inflating effects of partners with particularly high fecundity or potent ejaculates.

Although intermediate mating rates seem to maximize female reproduction, copulations occur more frequently when L. stagnalis is given the opportunity, suggesting that mating rates are male driven (as predicted by Michiels & Koene 2006). This situation clearly generates potential for sexual conflict and the evolution of female resistance traits (Lessells 2006), as occurs for instance in water striders (Arnvist & Rowe 2002; Han & Jablonski 2009).

In hermaphroditic snails, resistance against insemination has been described for the pulmonate Physa acuta, which performs rejection behaviours such as shell swinging and penis biting (Wethington & Dillon 1996; Ohbayashi-Hodoki et al. 2004; Facon et al. 2006). Such behavioural and morphological mechanisms to avoid being inseminated remain largely undescribed in L. stagnalis (Van Duivenboden & Ter Maat 1988) but now seem very relevant given our findings.

Ultimately, hermaphrodites are selected to maximize their total reproductive success, that is, fitness through both reproductive functions (Anthes et al. 2010). Similar results to ours were previously interpreted as a shift in sex allocation towards the male reproductive function (De Visser et al. 1994), as predicted by theoretical models (Charnov 1980, 1982). However, it was recently shown that reallocation towards the male function and sexual conflict both contributed to reduced female investment (Hoffer et al. 2010). The latter, in combination with the current finding, illustrates that, to address sex allocation and the reallocation of reproductive resources properly, a major objective for future research should be to assess male and female reproductive success simultaneously. The present study now provides us with the relevant information about which parameters to measure as well as which treatments to use. In addition, we can evaluate whether increased maternal investment per offspring improves offspring quality and performance in this species, as suggested by theory (Jennions & Petrie 2000; Simmons 2005).

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