

Reduced egg laying caused by a male accessory gland product opens the possibility for sexual conflict in a simultaneous hermaphrodite

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Abstract

Promiscuity, sperm storage and internal fertilization enhance sperm competition, which leads to sexual conflict whenever an advantageous trait for sperm donors is harmful to recipients. In separate-sex species, such conflicts can severely impact the evolution of reproductive characteristics, physiology and behaviours. For simultaneous hermaphrodites, the generality of this impact remains unclear and underlying mechanisms remain largely unexplored. In the hermaphrodite *Lymnaea stagnalis* several previous studies showed that investment in eggs differs depending on semen receipt, but these were inconsistent about the direction of change. We investigated whether the change in egg laying is caused by a seminal fluid component. By intravaginally injecting animals, we here reveal that a component of the seminal fluid inhibits egg laying, thus providing the first direct evidence for involvement of such components in competition for fertilization in hermaphrodites. We discuss the broad implications that this finding has on a number of previous studies performed in the same species.

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Keywords

Allohormone, insemination, Mollusca, Pulmonata, seminal fluid, sex peptide, snail

Introduction

In internally fertilizing animals sperm are usually accompanied by seminal fluid, which together compose the ejaculate. Within the ejaculate, natural selection favours substances that activate and nourish the sperm and that are thus essential for proper fertilization of oocytes (e.g. Mann and Lutwak-Mann, 1981). Additionally, post-copulatory sexual selection favours the evolution of seminal substances that further enhance fertilization chances by influencing female physiology (e.g. Arnqvist and Rowe, 2005), which thereby fall within the definition of allohormones (Koene and Ter Maat, 2001).

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Selection pressure on such substances is elevated because male reproductive success is usually dependent upon the number of matings obtained (Bateman, 1948). With the traditional division of the sexual roles, Bateman's principle thus results in males competing for fertilizations. However, although seminal fluid components can increase male reproductive success, they may at the same time reduce female reproductive success. If that is the case, the male and female objectives within a mating encounter are in conflict, and females may evolve resistance traits to counteract the effect of a specific seminal fluid component (persistence trait). This conflict can then drive the evolution of ejaculates towards complex mixtures of sperm and numerous seminal fluid components by antagonistic co-evolution (e.g. Gillott, 2002).

The best studied system, in which seminal peptides provide clear fitness advantages to males and cause clear fitness disadvantages to females, is the fruit fly *Drosophila melanogaster*. Around 20 accessory gland proteins (Acps) have been identified and in total 133 proteins have been shown to be transferred along with the sperm (Findlay et al., 2008). They have been found to affect females in several ways (Fowler and Partridge, 1989; Chapman et al., 1995). For example, Acp70A (also called sex peptide) decreases female receptivity and increases egg production (Wigby and Chapman, 2005). Rice (1996) showed, using an experimental evolution approach, that more competitive ejaculates can be detrimental to the survival of females. This reduced survival is most likely caused by Acp62F, which seems to protect sperm within the female tract but at the same time has a toxic effect on the female (Lung et al., 2002).

The above illustrates that such seminal peptides can enhance the male's reproductive success at the expense of the female's, causing a sexual conflict. There is, as yet, no clear evidence that any of these seminal substances is solely transferred to harm the female, and thereby inhibit e.g. remating. It rather seems that most of the negative effects are collateral damages due to toxicity of the substances that are targeting physiological processes in the female (Arnqvist and Rowe, 2005).

Seminal peptides thus have an essential role to play in species with separated sexes, i.e. gonochorists (Gillott, 2002; Arnqvist and Rowe, 2005). However, in species that possess both functional male and female reproductive organs (simultaneous hermaphrodites) the role of seminal fluid components has remained largely unexplored. Therefore, we here explore whether seminal products may also be involved in some of the conflicts found in simultaneous hermaphrodites. Clearly, being a simultaneous hermaphrodite adds a layer of complexity to sexual selection processes because each individual can perform both sexual roles and can gain male as well as female reproductive success through mating (Charnov, 1979; Michiels and Koene, 2006; Bedhomme et al., in press). Moreover, we know that most hermaphrodites store sperm from different donors and can digest excess sperm, which are conditions that enhance sperm competition (e.g. Koene et al., 2009).

Seminal fluids have been put forward to be responsible for changes in egg production observed in the simultaneously hermaphroditic model species, the great pond snail *Lymnaea stagnalis*. On the one hand, Van Duivenboden et al. (1985) showed that egg laying is suppressed after insemination and that this leads to reduced fecundity. On the other hand, Koene et al. (2006) found that controlled repeated mating seemed to

result in increased investment in egg production compared to once-mated individuals. Although these experiments differed in duration, i.e. weeks versus months, the results remain contradictory. Irrespective of the contradiction of change in egg production, both studies suggested that the change could be mediated by a component of the seminal fluid. Thus, there are a number of questions surrounding this issue that remain unanswered. In this paper, we therefore want to present some new evidence and, based on these new findings, re-evaluate some of the previous studies.

We will start by describing an experiment in which we tested the involvement of a seminal fluid component in changes in egg production in order to resolve the question about whether egg production is stimulated or suppressed by mating. Subsequently, based on this finding we will discuss its implications on the conclusions of previous findings, and then explore some of the possible mechanistic aspects underlying this change in egg laying. Finally, we will discuss whether this actually causes a sexual conflict and provide some fruitful directions for future research in simultaneous hermaphrodites.

Inhibition, not stimulation of egg laying after mating

Changes in egg production in *L. stagnalis* were initially mainly investigated in order to develop optimal breeding conditions. Several studies have looked at the effects of density on reproductive activity (defined as egg output, e.g. Mooij-Vogelaar et al., 1970). In controlled experiments where densities were increased from 2 to 10 individuals it was observed that egg laying decreased, while the opposite was true for cases where densities were decreased from 10 to 2 (Mooij-Vogelaar et al., 1970). If considered separately, the above results could be due to factors other than receiving sperm and seminal fluid. But, when mating opportunities change by changing densities from 1 to 12 and vice versa, the respective decrease and increase in egg laying were also observed (Van Duivenboden et al., 1985). Interestingly, more recent findings hinted at an increase in egg laying when more mating opportunities were given (Koene et al., 2006). Despite their opposite findings, the latter two studies both suggested, as one possibility, that the observed changes in egg laying could be due to a seminal fluid component transferred during mating.

In order to test the effect of the seminal fluid on egg laying we performed the following experiment. We obtained 50 adult specimens of *L. stagnalis* (L.) from our culture facility (shell lengths 30 ± 1 mm). During the experiments the animals were kept in individual perforated plastic jars, placed within a large tank under standard laboratory conditions, i.e. running low-copper water at 20°C with standard light conditions (light:dark=12:12h). Every day each snail was provided with an excess of lettuce discs each measuring 19.6 cm². These animals were randomly divided over two treatments (Control and Seminal fluid) in two replications (Per treatment: N=10 snails in replication 1 and N=15 snails in replication 2). For the statistical analysis of egg laying we nested the replications within treatment. The analyses were performed with JMP version 5.0.1 (SAS Institute Inc.). The data were tested for normality and nonparametric methods were used wherever necessary.

For the seminal fluid treatment, five adult donor snails were taken from the breeding facility and isolated for seven days to allow for prostate gland replenishment (De Boer et al., 1997, see below). During these days lettuce leaves were provided *ad libitum*. On day eight each donor snail (average shell length 31 mm \pm 8.7 SD) was anaesthetized by means of an injection of approximately 2 ml of 50 mM MgCl₂ through the foot. The prostate gland was then dissected out and its contents immediately expressed into a collection vial that was placed on ice. Subsequently, the seminal fluid was diluted with saline (see Loose and Koene, 2008) and prepared for immediate use. A control containing saline only was prepared at the same time (i.e. 0.00 dose). The seminal fluid dose, 0.25 prostate gland equivalent, approximates the estimated amount of seminal fluid transferred during one insemination (J.M. Koene, unpublished data).

For intravaginal injection, two 1 ml syringes were filled with the different solutions and each syringe was fitted with a blunted injection needle. A silicon tube with a diameter of 1 mm and a length of 10 cm, was slid over each injection needle. The randomly chosen experimental animals were inseminated intravaginally with either the control or the test solution in an alternating fashion. Prior to intravaginal injection, each snail was anaesthetized with 2 ml of 50 mM MgCl₂. This anaesthetic ensured that the animal was completely relaxed and maximally extended from its shell within seconds. The silicon tube was then carefully inserted into the female gonopore, now clearly visible as a white spot on the right side of the animal anterior to the right tentacle and male gonopore. A volume of 0.03 ml was then slowly injected after which the silicone tube was pulled out. Following this procedure, the shell length of each injected snail was measured and the snail was returned to its perforated plastic jar in a large tank, where it would recover over the next hours.

Following intravaginal injection, egg production was measured during one week. Egg masses were collected and the number of eggs per mass were counted. Egg counting was greatly facilitated by placing the egg masses in 70% alcohol, which turns the egg bodies white within 30 minutes. For Replication 1, to quantify daily consumption per snail its lettuce remains were collected and a picture was taken using a digital camera that was placed at a standardized distance. The surface of the remains of the lettuce discs was measured using ImageJ (NIH, Abramoff et al., 2004). Consumption was calculated by subtracting the leftover area from the total area of the lettuce provided (see De Visser et al. 1994). To verify the validity of this measure, we weighed different surfaces of lettuce, measuring between 0.19 to 19.60 cm², and found that the relation between surface area and weight in grams was highly significant (Linear regression: $R^2=0.97$, $N=40$, $P<0.001$). Thus, lettuce surface area can be used as a reliable measure for nutritional intake.

The results of this experiment indicate that there is a significant difference in the number of individuals laying eggs (Nested nominal logistic procedure: $\chi^2=6.02$, $df=2$, $P=0.036$). As shown in fig. 1, the presence of seminal fluid clearly suppressed egg laying in both replications of the experiment. Between egg laying individuals of both treatment no differences were found in number of eggs laid (Wilcoxon: Replication 1, $\chi^2=1.21$, $df=1$, $P=0.27$; Replication 2, $\chi^2=0.21$, $df=1$, $P=0.65$) or lettuce consumption (t test: Replication 1, $t_{16}=0.97$, $P=0.35$; not quantified for Replication 2).

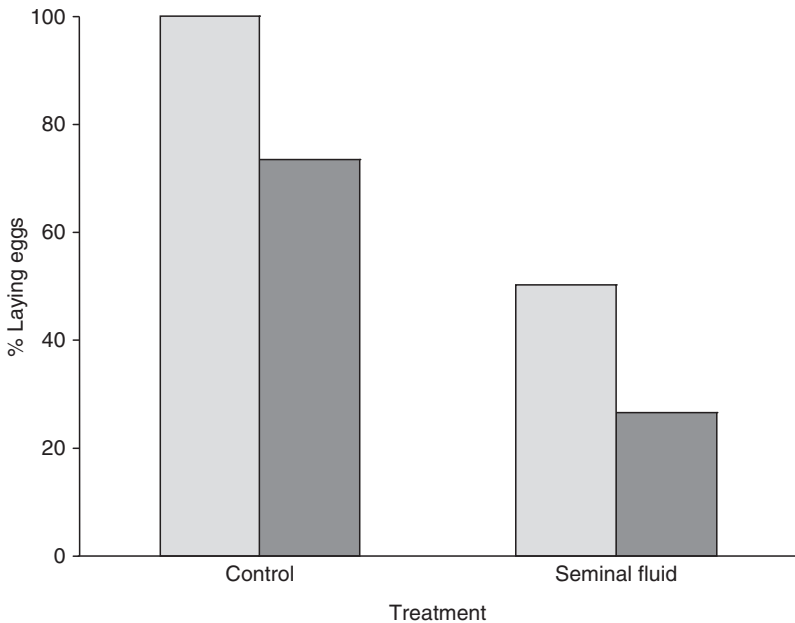


Figure 1. The occurrence of egg laying after intravaginal injection with a control substance or seminal fluid (0.25 gland equivalent) from the prostate gland. The percentage of animals laying eggs is shown for each treatment. The different shades of grey indicate the two replications.

From this experiment we can thus conclude that a seminal substance (probably a peptide) is indeed responsible for an inhibition of egg laying, not a stimulation. In the next section we now want to explore the implications of this finding on previous research investigating sex allocation. To do so, we will first provide a brief review of the regulation of male mating activity in this hermaphrodite.

Re-interpreting re-allocation of reproductive resources

The male behaviour consists of a fixed sequence of events that starts with shell mounting (reviewed in Jarne et al., 2009). The animal crawls to the tip of the shell in a counter clockwise fashion (circling). It then descends to the right side of the partner's shell where it positions itself on the edge. Circling and positioning can be repeated several times. When the correct position is found, the partially everted preputium becomes visible. Once the preputium, which carries the penis, is completely everted it probes to find the female opening. After one to several attempts, the penis is intromitted and semen is transferred (De Visser et al., 1994; De Boer et al., 1997).

Despite being a simultaneous hermaphrodite, *L. stagnalis* can only mate in one sexual role at the time. Hence, before mating the sexual roles need to be divided for that particular interaction. One determining factor seems to be sexual isolation, because after a period of several days of sexual isolation these animals are more eager to mate in the male role (e.g. Van Duivenboden and Ter Maat, 1985). During this isolation period

the animal's largest male gland, the prostate gland, also increases in size (De Boer et al., 1997). The prostate gland produces the seminal fluid that is added to the sperm prior to transfer to the partner. This increase in the size of this gland is detected by the central nervous system via a small branch of the penial nerve that runs along part of the vas deferens (De Boer et al., 1997). The nervous information is received in a brain area, called the anterior lobe, that controls male reproductive behaviour and seems to be evolutionarily conserved across the Gastropoda (Koene et al., 2000).

Koene and Ter Maat (2005) observed that sex role alternation, i.e. a second mating in which the individuals within a pair swap sexual roles, was mainly observed when both individuals were eager to mate. Thus, the swapping of sexual roles can be nicely explained by the motivation of both animals to mate in the male role. Moreover, the dependency of performing the male role on the available amount of seminal fluid (rather than sperm supply itself, De Boer et al., 1997) probably highlight the importance of substances in this fluid for successful fertilization. Several subsequent studies have further illustrated that the filling state of the prostate gland is not the only thing that counts in a mating encounter. For example, it was also shown that animals experience an increase in their male motivation when they encounter a new partner, even if they have just mated as a male (Koene and Ter Maat, 2007). Moreover, sperm donors seem also able to assess the mating history of their mating partner and donate sperm accordingly (Loose and Koene, 2008), but they do not seem to make a distinction between partners of different "quality" prior to mating (Koene et al., 2007, 2008).

The fact that male motivation largely originates from the amount of seminal fluid available in the prostate gland has also led to attempts to eliminate male behaviour. Indeed, when the nerve, running along part of the vas deferens, that innervates the prostate gland is cut, the animals essentially become females and no longer mate in the male role (De Boer et al., 1997). Thus, male mating behaviour and sperm transfer are eliminated, but the male reproductive organs remain otherwise intact and active (i.e. sperm and seminal fluid production continue, but reserves are not used). This observation has also prompted an attempt to quantify the investment in the male function. The details of the procedure are described in De Visser et al. (1994), but in brief, the set-up was as follows. In the experimental group male behaviour was eliminated by cutting the part of the vas deferens along which the nerve branch runs between the prostate gland and the central nervous system, they called these animals "non-copulants". The 2 control groups (together called "copulants") were untreated (copulants: control) and sham operated (copulants: sham). Animals were then kept in pairs within treatments and their consumption of the standardized amount of lettuce (39 cm²) was measured daily. Egg laying was also monitored daily whereas growth and dry weight were measured at the end.

After eliminating the male behaviour as described above, De Visser et al. (1994) recorded the change in investment in the female function as a result of this treatment compared to controls. They found that egg laying doubled in the animals in which the male behaviour had been eliminated. The interpretation of this finding was placed within hermaphrodite sex allocation theory (e.g. Charnov, 1979), which assumes a trade-off in investment between the male and female function. Hence the observed

difference in egg laying was interpreted as the shift of the allocation of energy that was no longer invested into the male function towards the female function.

Although the approach of the De Visser et al. (1994) experiment was very elegant (see also Koene, 2006), they seem not to have excluded a different explanation of their findings. What seems to have been previously overlooked, is that the animals in their experiment were all placed in pairs. They had two control groups, unoperated and sham-operated, which they used together as “copulants”. The experimental group, which were the “non-copulants”, showed increased egg laying. As a consequence, they are comparing two factors that they manipulated at the same time: 1. Elimination of sperm donation (i.e. male motivation) and 2. Elimination of sperm receipt. Hence, they cannot conclude as firmly as they do, that the observed change in egg production represents the amount of resources that are no longer invested into the male function and thus get reallocated towards the female function. The alternative explanation, that the change in egg laying is due to an absence of receipt of seminal fluid (including an egg laying suppression component) cannot be ruled out in their study.

Fortunately, a study that was done several years earlier can help to distinguish between the two alternative hypotheses. Van Duivenboden et al. (1985) performed an experiment that was very similar to the one done by De Visser et al. (1994). The crucial difference was that Van Duivenboden et al. (1985) did include the factor copulation separately (albeit sperm donation and receipt combined). Figure 2 depicts her findings, originally published in a table, in graphical form. For clear comparison, we have depicted them in the same way as the representation of the De Visser et al. (1994) data in Koene (2006). The first three groups on the left side essentially represent and confirm the findings of De Visser et al. (1994). Most interestingly, the right three groups show the same treatments but in this case copulation in either sexual role is excluded (i.e. individuals are isolated). This illustrates convincingly that the observed change in egg production is not due to a removal of the male behaviour, but rather due to an absence of insemination.

We are therefore now confident that the lower egg laying in copulating snails, compared to non-copulating snails, should be ascribed to an effect of receiving seminal fluid (containing the male accessory gland product that suppresses egg laying). Evidently, it is still unclear how the component of the seminal fluid inhibits the egg laying process in the recipient. To explore how this might work, we first need to have a brief look at the underlying mechanism regulating female reproduction in *L. stagnalis*.

How is the egg laying process affected in the recipient?

Egg laying is controlled by a bilateral group of neurons in the cerebral ganglia, the Caudo-Dorsal Cells (CDCs; Ter Maat et al., 1983). The importance of the CDCs was first revealed by cauterizing these cells, which completely stopped egg laying (Geraerts and Bohlken, 1976). These cells project into the neurohaemal area of the cerebral commissure, are electrically coupled, and send axons through the cerebral commissure to the contralateral cell cluster (Joose, 1964; Wendelaar-Bonga, 1971; Roubos, 1976;

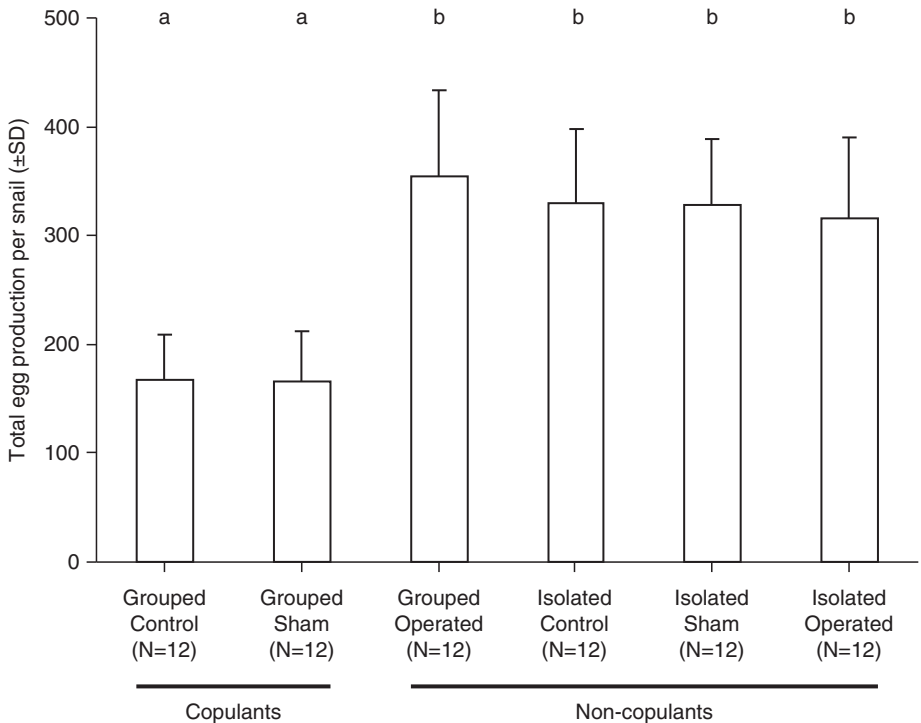


Figure 2. Total egg production per snail against the different operations and copulation opportunities. N indicates the number of individuals per treatment. The terms used in the text are also indicated on the x-axis. The overall ANOVA was significant (see Van Duivenboden et al., 1985). The post-hoc results of comparisons between the groups are indicated by different letters ($P=0.05$). However, note that the grouped animals were housed together and thus do not represent independent data points (i.e. pseudoreplicated).

De Vlieger et al., 1980). As a consequence, these cells show a synchronous, long-lasting discharge (De Vlieger et al., 1980; Ter Maat et al., 1986). That these CDCs are neurosecretory and that their products induce egg laying was first shown by injecting extracts from the cerebral commissure into the blood (e.g. Geraerts and Bohlken, 1976). The confirmation that the CDCs release hormones during their bursting activity comes from a very convincing *in vitro* experiment by Ter Maat et al. (1988). In that experiment the CDCs in one CNS preparation were induced to discharge. The reaction in a second preparation, that was placed adjacent in the same saline bath, was recorded and revealed that these CDCs also started a long lasting discharge. This unequivocally demonstrated that the initiation of this activity is chemically mediated.

It turns out that several peptides are involved and released into the blood (e.g. Geraerts et al., 1983, 1985; Geraerts and Hogenes, 1985). By now, 13 different peptides have been identified (Li et al., 1994; Jimenez et al., 2004) of which 11 are all encoded on the same gene, the CDCH-gene (e.g. Geraerts et al., 1983, 1985; Vreugdenhil et al., 1985, 1988). The key peptide for egg laying seems to be CDC-hormone (CDCH), because the injection of this peptide alone triggers egg laying (Ter Maat et al., 1987).

Egg laying behaviour of *L. stagnalis* consists of a fixed sequence of behavioural events at the end of which the egg mass is fixed to the substrate (reviewed in Jarne et al., 2009). Egg masses contain on average somewhere between 50 and 150 eggs, depending on the individual's body size (Koene et al., 2007). Animals stop laying eggs when the water gets too dirty. As a consequence, egg laying can be triggered by a transfer from dirty to clean water. Although this is known as the clean water stimulus (CWS), the effect is actually caused by a combination of clean water, clean surface and higher oxygen level (Ter Maat et al., 1983).

Understanding the regulation of egg laying and the CWS response is important for understanding the following experiment, in which the CWS was tested on snails that had just mated (Van Duivenboden et al., 1985). The details of the experiment can be found in the original paper, but in essence the following was done. Animals were kept individually in pots without changing the water for six days. The experimental snails were then placed in pairs and allowed to mate in one role while the control snails were not allowed to mate. All remained in dirty water. After mating each snail was transferred to a clean jar with clean water (i.e. given a CWS), as were the control animals. Egg laying was then observed for 4 hours. With this experiment Van Duivenboden et al. (1985) were able to show that immediately following a mating in the female role, the CWS does not elicit egg laying. For control animals and animals that mated in the male role they found that the CWS worked normally (Likelihood ratio $\chi^2=14.17$; $N=34$, $df=2$, $P<0.0001$). Their results, based on the published table, are graphically depicted in fig. 3.

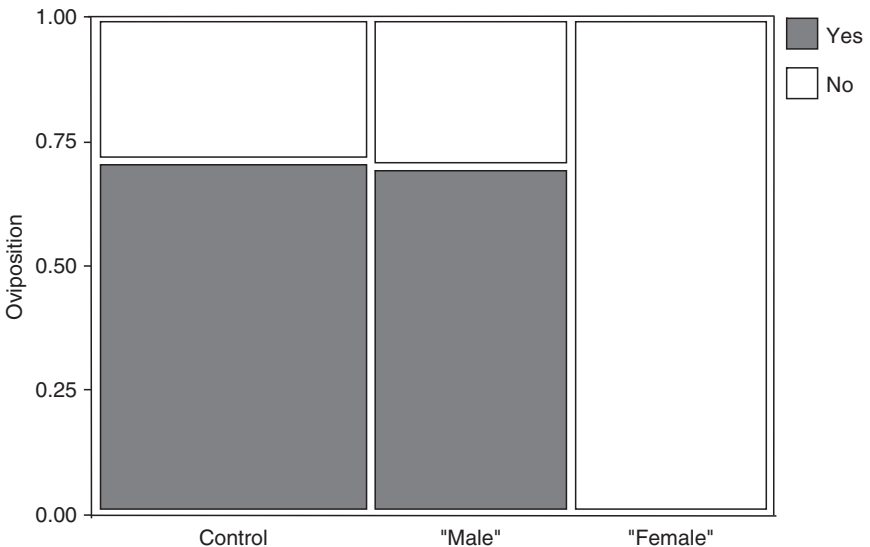


Figure 3. Egg laying (oviposition) response after a clean water stimulus (CWS). There were three experimental groups, animals that had not mated (Control, $N=14$), animals that had only donated semen by performing the male role only ("Male", $N=10$), and animals that only received semen by performing the female role only ("Female", $N=10$). Egg laying was scored at either occurring (yes) or not (no) and the proportion of animals ovipositing in each group is depicted. See Van Duivenboden et al. 1985 for original data.

The above clearly shows that already immediately following mating, egg laying is indeed inhibited in sperm recipients. This suggests that in these sperm recipients the CWS does not trigger the CDCs to initiate their long-lasting discharge (to release the egg laying peptides), as they normally would in response to an egg laying trigger. This makes it very tempting to speculate about a possible link between the seminal substance and CDC activity. Evidently, a very fruitful avenue for future research will be to investigate the mechanism underlying the inhibition of egg laying caused by the seminal substance.

Does suppression of egg laying reflect a conflict?

The foregoing has revealed that mating inhibits egg laying. The inhibition of egg laying in a mating partner seems counterintuitive, hence the question about why this occurs is very legitimate. One clear direction for future research should therefore focus on investigating the advantage(s) for the sperm donor as well as the possible resistance (counter-adaptations) to this by the sperm recipient.

As reviewed in Arnqvist and Rowe (2005), seminal substances can cause direct or indirect effects in the recipient. If the effect we observe in these pond snails represents a general inhibition of the female function, then the willingness to remate may also be reduced and that would provide a direct benefit for the sperm donor. However, no remating inhibition seems to occur in *L. stagnalis* because sperm recipients can be easily inseminated by a second donor within 24 hours or even immediately after insemination (respectively, Koene et al., 2009; Koene and Ter Maat, 2007). Alternatively, inhibited egg laying could be an indirect effect, i.e. a collateral effect brought about by a substance that is targeting a different physiological process in the female. In this context, one obvious principal, direct effect could aim at a higher paternity, for example via increasing storage of the donated sperm and/or sperm displacement of already-stored rival sperm. Another aim could be, by postponing egg laying, to commit the partner to invest more resources per egg, thus increasing egg quality (rather than quantity). Either of these different hypotheses could explain why egg laying is suppressed by seminal fluid and could be tested by using the intravaginal injection approach reported above.

In any case, the fact that the sperm donor can influence processes in the sperm recipient via the semen illustrates an important point. Although there has been a recent surge in sexual conflict research in hermaphrodites, most of these studies have focused on bizarre behaviours. Examples include dart shooting in land snails (Koene and Schulenburg, 2005), penis fencing in flatworms (Michiels and Newman, 1998), body piercing in earthworms (Koene et al., 2005) and stylet piercing in sea slugs (Anthes and Michiels, 2007). These extremes have highlighted the important role that sexual selection and sexual conflict play in the evolution of hermaphrodites. Our findings confirm that also in a relatively normal hermaphrodite, which simply transfers seminal substances along with the sperm during insemination rather than by using a stabbing device, sexual selection and conflict can be of equal importance.

Concluding remarks

Although many effects of seminal fluids are already known from animals with separate sexes, we have here presented the first clear example for the influence of seminal fluid on egg production in a simultaneous hermaphrodite. That the seminal fluid component, originating from the prostate gland, suppresses egg laying is of great consequence for the conclusions of several previous studies. Those previous studies could not distinguish between whether the observed changes in egg laying were caused by i) an active shift in resource allocation by the recipient in response to changed circumstances or ii) a seminal fluid component influencing egg production. This means, as argued above, that the observed changes in egg laying after removal of the male behaviour (De Visser et al., 1994) do not reflect a reallocation of freed-up male resources towards the female function, but rather an absence of the inhibiting effect of the seminal fluids received during inseminations (as is essentially confirmed by Van Duivenboden et al., 1985).

Our findings thus support the suggested inhibition of egg laying (Van Duivenboden et al., 1985), rather than the proposed stimulation (Koene et al., 2006), and show that this is indeed mediated by a component in the seminal fluid of *L. stagnalis*. Why then did these previous studies reach opposite conclusions? The explanation seems to lie in the different experimental procedures used. The most important difference lies in that the snails in the earlier study had continuous access to mates (Van Duivenboden et al., 1985), while in the later study restricted access was used (one day per week, Koene et al., 2006). Assuming that the decreased egg laying observed in the first study was caused by repeated receipt of seminal fluid, this may indicate that the second study underestimated the natural mating rates in groups. Evidently, this now needs to be tested, but recent work already indicates that mating rates may indeed be much higher (Koene and Ter Maat, 2007).

Our findings have another vital implication for research on hermaphrodites. Generally, hermaphrodites are flexible in optimizing their investment in growth, male and female reproduction depending on circumstances (Schärer and Ladurner, 2003). This is usually seen as beneficial, for example because a sperm recipient can invest resources based on mate availability and/or quality. However, as our data show, this flexibility may come at a cost, because it also gives sperm donors the opportunity to reroute resources in their partners (probably with the goal to assure optimal use of the donated sperm). If this goes at the expense of the sperm recipients, i.e. causes a conflict, this would clearly mean that flexible resource allocation is a mixed blessing for hermaphrodites (Bedhomme et al., in press).

To conclude in terms of future perspectives, revealing the exact biochemical identity of the seminal fluid component that is produced in the prostate gland, as well as its nucleotide sequence, is now imperative. Furthermore, the reason for and mechanism of suppression of egg laying warrant closer investigation. On a much more general note, the fact that we found a manipulative substance that is transferred along with the sperm (rather than via a stabbing device), implies that such substances may be very common in internally-fertilizing hermaphrodites.

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References

- Abramoff, M.D., Magelhaes, P.J. & Ram, S.J. (2004) Image Processing with ImageJ. *Biophotonics Intl.*, 11, 36–42.
- Anthes, N. & Michiels, N.K. (2007) Precopulatory stabbing, hypodermic injections and unilateral copulations in a hermaphroditic sea slug. *Biol. Lett.*, 3, 121–124.
- Arnqvist, G. & Rowe, L. (2005) *Sexual Conflict*. Princeton University Press
- Bateman, A.J. (1948) Intra-sexual selection in *Drosophila*. *Heredity*, 2, 349–368.
- Bedhomme, S., Arathi, S., Lankinen, Å., Bernasconi, G., Koene, J.M., Anthes, N. How does breeding system variation modulate sexual antagonism? *Biol. Lett.*, in press.
- Chapman, T., Liddle, L.F., Kalb, J.M., Wolfner, M.F. & Partridge, L. (1995) Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, 373, 241–244.
- Charnov, E.L. (1979) Simultaneous hermaphroditism and sexual selection. *Proc. Natl. Acad. Sci. USA*, 76, 2480–2484.
- De Boer, P.A.C.M., Jansen, R.F., Koene, J.M. & Ter Maat, A. (1997) Nervous control of male sexual drive in the hermaphroditic snail *Lymnaea stagnalis*. *J. Exp. Biol.*, 200, 941–951.
- De Visser, J.A.G.M., Ter Maat, A. & Zonneveld, C. (1994) Energy budgets and reproductive allocation in the simultaneous hermaphrodite pond snail, *Lymnaea stagnalis* (L.): a trade-off between male and female function. *Am. Nat.* 144, 861–867.
- De Vlioger, T.A., Kits, K.S., Ter Maat, A. & Lodder, J.C. (1980) Morphology and electrophysiology of the ovulation hormone producing neuro-endocrine cells of the freshwater snail *Lymnaea stagnalis* (L.). *J. Exp. Biol.*, 84, 239–271.
- Findlay, G.D., Yi, X., MacCoss, M.J. & Swanson, W.J. (2008) Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating. *PLoS Biol.*, 6, e178.
- Fowler, K. & Partridge, L. (1989) A cost of mating for female fruitflies. *Nature*, 338, 760–761.
- Geraerts, W.P.M. & Bohlken, S. (1976) The control of ovulation in the hermaphrodite freshwater snail *Lymnaea stagnalis* by the neurohormone of the caudo-dorsal cells. *Gen. Comp. Endocr.*, 28, 350–357.
- Geraerts, W.P.M. & Hogenes, T.M. (1985) Heterogeneity of peptides released by electrically active neuroendocrine caudodorsal cells of *Lymnaea stagnalis*. *Brain Res.*, 331, 51–61.
- Geraerts, W.P.M., Tensen, C. & Hogenes, Th.M. (1983) Multiple release of peptides by electrically active neurosecretory caudo-dorsal cells of *Lymnaea stagnalis*. *Neurosci. Lett.*, 41, 151–155.
- Geraerts, W.P.M., Vreugdenhil, E., Ebberink, R.H.M. & Hogenes, Th.M. (1985) Synthesis of multiple peptides from a larger precursor in the neuroendocrine caudo-dorsal cells of *Lymnaea stagnalis*. *Neurosci. Letters*, 56, 241–246.
- Gillott, C. (2002) Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Annu. Rev. Entomol.*, 48, 163–184.
- Jarne, P., Pointier, J.-P., David, P. & Koene, J.M. (2009) Basommatophoran Gastropods. In: A. Córdoba-Aguilar & J.L. Leonard (Eds.) *The Evolution of primary sexual characters in animals*, Oxford University Press, in press.

- Jiménez, C.R., Ter Maat, A., Pieneman, A., Burlingame, A.L., Smit, A.B., Li, K.W. (2004) Spatio-temporal dynamics of the egg-laying-inducing peptides during an egg-laying cycle: a semi-quantitative matrix-assisted laser desorption/ionization mass spectrometry approach. *J. Neurochem.*, 89, 865-875.
- Joosse, J. (1964) Dorsal bodies and dorsal neurosecretory cells of the cerebral ganglia of *Lymnaea stagnalis*. *Arch. Néerl. Zool.*, 16, 1–103.
- Koene, J.M. & Schulenburg, H. (2005) Shooting darts: co-evolution and counter-adaptation in hermaphroditic snails. *BMC Evol. Biol.*, 5, 25.
- Koene, J.M. & Ter Maat, A. (2001) “Allohormones”: A class of bioactive substances favoured by sexual selection. *J. Comp. Physiol. A*, 187, 323-326.
- Koene, J.M. & Ter Maat, A. (2005) Sex role alternation in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis* is determined by the availability of seminal fluid. *Anim. Behav.*, 69, 845-850.
- Koene, J.M. & Ter Maat, A. (2007) Coolidge effect in pond snails: Male motivation in a simultaneous hermaphrodite. *BMC Evol. Biol.*, 7, 212.
- Koene, J.M., Jansen, R.F., Ter Maat, A. & Chase, R. (2000) A conserved location for the central nervous system control of mating behaviour in gastropod molluscs: Evidence from a terrestrial snail. *J. Exp Biol.*, 203, 1071-1080.
- Koene, J.M., Loose, M.J. & Wolters, L. (2008) Mate choice is not affected by mating history in the simultaneously hermaphroditic snail *Lymnaea stagnalis*. *J. Mollus. Stud.*, 74, 331-335.
- Koene, J.M., Montagne-Wajer, K. & Ter Maat, A. (2006) Effects of frequent mating on sex allocation in the simultaneously hermaphroditic great pond snail. *Behav. Ecol. Sociobiol.*, 60, 332-338.
- Koene, J.M., Montagne-Wajer, K. & Ter Maat, A. (2007) Aspects of body size and mate choice in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. *Anim. Biol.*, 57, 247-259.
- Koene, J.M., Montagne-Wajer, K., Roelofs, D. & Ter Maat, A. (2009) The fate of received sperm in the reproductive tract of a hermaphroditic snail and its implications for fertilisation. *Evol. Ecol.*, 23, 533-543.
- Koene, J.M., Pfortner, T. & Michiels, N.K. (2005) Piercing the partner's skin influences sperm uptake in the earthworm *Lumbricus terrestris*. *Behav. Ecol. Sociobiol.*, 59, 243–249.
- Li, K.W., Jiménez, C.R., Van Veelen, P. & Geraerts, W.P.M. (1994) Processing and targeting of a molluscan egg-laying peptide prohormone as revealed by mass spectrometric peptide fingerprinting and peptide sequencing. *Endocrinology*, 134, 1812-1819.
- Loose, M.J. & Koene, J.M. (2008) The effect of body weight, insemination duration and rearing condition on sperm transfer in a simultaneous hermaphrodite. *Invert. Biol.*, 127, 162-167.
- Lung, O., Tram, U., Finnerty, C.M., Eipper-Mains, M.A., Kalb, J.M. & Wolfner, M.F. (2002) The *Drosophila melanogaster* seminal fluid protein Acp62F is a protease inhibitor that is toxic upon ectopic expression. *Genetics*, 160, 211-224.
- Mann, T. & Lutwak-Mann, C. (1981) *Male reproductive function and semen*. Springer-Verlag.
- Michiels, N.K. & Koene, J.M. (2006) Sexual selection favors harmful mating in hermaphrodites more than in gonochorists. *Integr. Comp. Biol.*, 46, 473-480.
- Michiels, N.K. & Newman, L.J. (1998) Sex and violence in hermaphrodites. *Nature*, 391, 647.
- Mooy-Vogelaar, J.W., Jager, J.C. & Van der Steen, W.J. (1970) The effect of density changes on the reproduction of the pond snail *Lymnaea stagnalis* (L.). *Neth. J. Zool.*, 20, 279-288.
- Rice, W.R. (1996) Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, 381, 232-234.
- Roubos, E.W. (1976) Neuronal and non-neuronal control of the neurosecretory Caudo-Dorsal Cells of the freshwater snail *Lymnaea stagnalis* (L.). *Cell Tiss. Res.*, 168, 11–31.
- Schärer, L. & Ladurner, P. (2003) Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. *Proc. R. Soc. London B*, 270, 935-941.
- Ter Maat, A., Dijkstra, F.A. & Bos, N.P.A. (1986) *In vivo* recording of neuroendocrine cells (caudo-dorsal cells) in the pond snail. *J. Comp. Physiol. A*, 158, 853-859.
- Ter Maat, A., Geraerts, W.P.M., Jansen, R.F. & Bos, N.P.A. (1988) Chemically mediated positive feedback generates long-lasting discharge in the molluscan neuroendocrine system. *Brain Res.*, 438, 77-82.

- Ter Maat, A., Lodder, J.C. & Wilbrink, M. (1983) Induction of egg-laying in the pond snail *Lymnaea stagnalis* by environmental stimulation of the release of ovulation hormone from the caudo-dorsal cells. *Int. J. Invert. Reprod.*, 6, 239-247.
- Ter Maat, A., Van Duivenboden, Y.A. & Jansen, R.F. (1987) Copulation and egg-laying behavior in the pond snail. In: H.H. Boer, W.P.M. Geraerts and J. Joosse (Eds.), *Neurobiology: Molluscan Models*, pp. 255-261. North-Holland Publishing Company Amsterdam, Oxford, New York.
- Van Duivenboden, Y. A. & Ter Maat, A. (1985) Masculinity and receptivity in the hermaphrodite pond snail, *Lymnaea stagnalis*. *Anim. Behav.*, 33, 885–891.
- Van Duivenboden, Y. A., Pieneman, A.W. & Ter Maat, A. (1985) Multiple mating suppresses fecundity in the hermaphrodite freshwater snail *Lymnaea stagnalis*: a laboratory study. *Anim. Behav.*, 33, 1184-1191.
- Vreugdenhil, E., Geraerts, W. M. P., Jackson, J. F. and Joosse, J. (1985) The molecular basis of the neuro-endocrine control of egg-laying behaviour in *Lymnaea*. *Peptides*, 6, 465-470.
- Vreugdenhil, E., Jackson, J.F., Bouwmeester, T., Smit, A.B., Van Minnen, J., Van Heerikhuizen, H., Klootwijk, J. & Joosse, J. (1988) Isolation, characterization, and evolutionary aspects of a cDNA clone encoding multiple neuropeptides involved in the stereotyped egg-laying behavior of the fresh-water snail *Lymnaea stagnalis*. *J. Neurosci.*, 8, 4184-4191.
- Wendelaar-Bonga, S.E. (1971) Formation, storage, and release of neurosecretory material studied by quantitative electron microscopy in the fresh water snail *Lymnaea stagnalis* (L.). *Z. Zellforsch.*, 113, 490-517.
- Wigby, S. & Chapman, T. (2005) Sex peptide causes mating costs in female *Drosophila melanogaster*. *Curr. Biol.*, 15, 316-321.