Unprecedented dynamics of seminal fluid replenishment after mating

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Abstract

Seminal fluid proteins (SFPs) play vital roles for optimizing reproductive success in diverse animals. Underlining their significance, SFP production and transfer are highly plastic, e.g., depending on the presence of rivals or mating status of partners. However, surprisingly little is known about replenishing SFPs after mating. It is especially relevant in multiple mating species, as they would continuously produce and use SPFs throughout their reproductive life. Here we examined the expression pattern of SFP genes after mating in the great pond snail, Lymnaea stagnalis. Our results show that only two of the six SFP genes investigated here were up-regulated after mating, indicating that L. stagnalis replenishes seminal fluid in a protein-specific manner. In addition, we suggest that SFP replenishment is plastic depending on the mating history of female-acting snails. Our results shed light on unexplored aspects of SFP replenishment, thereby expanding the understanding of reproductive strategies in animals.

Introduction

Seminal fluid proteins (SFPs, also referred to as accessory gland proteins or ACPs) are part of the non-sperm component of an ejaculate, and consist of up to several hundreds of proteins (Sirot et al. 2015). Although SFPs were initially considered as merely assisting the functioning of sperm, it has since become clear that they also mediate other important and diverse processes in reproduction (McGraw et al. 2016). For example, SFPs facilitate the initiation of healthy pregnancy in humans (Bromfield 2014) and induce oviposition after mating in many insects (Avila et al. 2011). Underlining these significant functions, males have been shown to adjust SFP production as well as SFP transfer depending on the presence of rivals (e.g., Ramm et al. 2015; Nakadera et al. 2019) or mating status of partners (Sirot et al. 2011). Thus, this is often explained as males 'tailoring' SFP composition of their ejaculate for each mating to optimize their reproductive success.

However, although SFP production is well known to be plastic, their replenishment has received surprisingly little attention. This is a non-trivial knowledge-gap in multiple mating species, as refilling seminal fluid is expected to be dynamic depending on their past and future copulations. For instance, male *Drosophila melanogaster*adjust the amount of specific SFPs to transfer, depending on whether the female is virgin or not (Sirot et al. 2011). Such protein-specific adjustment would affect the subsequent SFP replenishment in the male's accessory gland organ(s). Also, males often alter SFP production depending on prevailing sperm competition risk (e.g., Fedorka et al. 2011; Ramm et al. 2015; Bartlett et al. 2017; Mohorianu et al. 2017; Simmons and Lovegrove 2017; Hopkins et al. 2019) as well as depending on on-going sperm competition (e.g., Sloan et al. 2018; Ramm et al. 2019; Nakadera et al. 2019). This plastic SFP production and transfer implies that males predict and prepare for future mating opportunities. Thus, it is likely that refilling seminal fluid after mating is highly plastic, although empirical data for such patterns over time are largely missing up to now.

To the best of our knowledge, SFP replenishment within the accessory gland has been investigated only in *D. melanogaster* and our model species, the great pond snail *Lymnaea stagnalis* (see below). In*D. melanogaster* , transferring ejaculate indeed triggers the replenishment of Acp95EF (Herndon et al. 1997). Furthermore, it takes at least three days to fully replenish two SFPs, sex peptide and ovulin (Sirot et al. 2009). Also, when enlarging our scope to general protein replenishment, this yields very few studies. One example comes from snake venom, also a complex mixture of proteins, for which it was reported that the production of the different classes of protein occur in parallel when the venom gland is refilled (Currier et al. 2012). Therefore, we consider that filling this knowledge gap of SFP replenishment would not only expand the knowledge of SFP expression and male reproductive strategies, but also stimulate studying the replenishment of other proteins in various biological contexts.

In this study, we examined the dynamics of SFP replenishment after mating in the great pond snail *Lymnaea stagnalis* . To do so, we let the snails copulate, then examined SFP gene expression at 3, 24, 48 and 192 h after mating. A previous study showed that this species increases the production of LyAcp10 one day after mating (Swart et al. 2019). However, such an increase was not always observed in another study (Nakadera et al. 2019). In this experiment, we included all SFP genes identified in this species ($N = 6$, Koene et al. 2010; Nakadera et al. 2019), to monitor if all SFPs replenish in parallel after mating. It has also been shown that virgin snails express SFP genes lower than snails with mating opportunities (Nakadera et al. 2019; 2020). This expression pattern led us to predict that SFP production would be low after a long absence of mating. In sum, we predicted that, in this species, (1) insemination triggers SFP production, and (2) the expression of all SFP genes decreases when they are fully replenished in the seminal fluid producing prostate gland. Furthermore, we examined whether SFP replenishment occurs in parallel across all SFP genes.

Material and Methods

We used the lab culture of *L. stagnalis* maintained at Vrije Universiteit Amsterdam. All the snails are kept in a flow-through tank with low copper water maintained at 20 ± 1 °C under dark:light cycle of 12:12h. In this experiment, we used adult snails (4-month-old). As mentioned, this species is a simultaneous hermaphrodite, but individuals copulate unilaterally. That is, one individual acts in the male role, and the other in the female role. Afterwards, they can swap their sex roles and copulate again (Koene and Ter Maat, 2005). In addition, this species is promiscuous as exemplified by the fact that they can inseminate more than once within 24 hours (Koene and Ter Maat, 2007).

To estimate the expression level of SFP genes at several time points after mating, we let the snails copulate under observation. First, to increase their male mating motivation, we isolated the snails for eight days, by keeping one individual per 460-ml perforated container placed in a flow-through tank (Van Duivenboden and Ter Maat 1985; De Boer et al. 1997). During isolation, we fed ca. 19.6 cm^2 of broad leaf lettuce per day per capita, which is slightly less than their maximum food intake (Zonneveld and Kooijman 1989). Next, we placed two individuals together in a container to let them mate. We size-matched pairs of snails to reduce the effect of body size on sex role decision (Nakadera et al. 2015), marked snails on their shell with waterproof marker for identification during observations. During the mating observation, we recorded their mating behavior every 15 min (No contact, mounting, probing, intromission: see Jarne et al. 2010). After insemination finished, we immediately separated the pair to prevent a second copulation, and isolated the male-acting snails (hereafter called donor) until their designated sampling time. We ran this experiment twice (total N : 3 h = 4, 24 h = 4, 48 h = 6, 192 h = 5).

To estimate the expression level of SFP genes, we collected prostate glands of donors in four different time intervals, which were 3, 24, 48, 192 h after mating in the male role. First, we injected ca. 2 ml of 50 mM $MgCl_2$ into foot for anesthetization. Then, we quickly dissected out a prostate gland, placed the tissue into an 1.5 ml Eppendorf tube, and immediately after the collection, we snap froze the collected samples using liquid nitrogen. The samples were stored at -80 °C until further processing.

Next, we isolated total RNA using trizol-chloroform, following the classic protocol. In brief, we homogenized the tissue with trizol, added chloroform for phase separation, and precipitated RNA pellet using 2-propanol.

After washing the pellet using 75 % ethanol, we applied DNAse treatment. After the quality control of extracted total RNA using Nanodrop and electrophoresis, we synthesized cDNA using the MML-V Reverse transcriptase kit (Promega). Then, we conducted quantitative PCR (qPCR) to estimate the relative expression levels of SFP genes, using NO-ROX SYBR® Green mix (BioLine) and thermal cycler (CFX-96, Bio-Rad). We examined all the known SFP genes ($N = 6$) with two technical replicates, and used two house-keeping genes as reference (Beta-tubulin, Ubiquitin, Davison et al. 2016; Young et al. 2019; Table S1). For primer designing, we applied the following thresholds: annealing temperature 59-60 °C, GC contents = 40-45 %, amplicon melting temperature = 80-85 °C. To calculate the relative, normalized gene expression ($2^{-[?][?]Ct}$, Livak and Schmittgen 2001), we used the software CFX Manager v3.1. We confirmed that the expression of reference genes was not significantly different across treatments (Fig. S1).

To examine the temporal expression changes of each SFP gene after mating, we used two-way ANOVAs. We used Hours after mating and experimental block (Exp) as fixed factors. When there was a significant difference between time after mating, we used Tukey's Honest Significant Differences test (HSD). To visualize the overall change in SFP gene expression over time, we reduced the dimensions of expression data using principal component analysis (PCA), and tested the expression pattern over time using two-way ANOVAs again. We performed all the analyses with R (ver. 4.0.3, R Core Team).

Results

We detected that mating triggers SFP replenishment in two SFP genes. *LyAcp5* 's expression level significantly increased 48 hours after mating, and reduced 192 hours after mating (ANOVA, $F_{3,11} = 5.61$, $P = 0.014$, Fig. 1, Table 1). Also, *LyAcp8b* showed a significant change in expression level over time (ANOVA, $F_{3,11} = 4.18$, $P = 0.033$, Fig. 1, Table 1), which is likely due to the increased expression 48 hours after mating (Tukey HSD test, 48 h - 3 h: adjusted $P = 0.054$, 48 h – 24 h: adjusted $P = 0.057$). In contrast, the four remaining SFP genes did not show a significant change in expression level throughout our monitoring, suggesting that the production of these SFPs did not increase after mating (Fig. 1, Table 1).

In order to visualize the overall change in SFP gene expression, we conducted a PCA and the patterns showed that half of expression variation is explained by the factor Hour after mating (PC1, 50.0 % explained, Table 2), although we did not detect a significant difference between treatments for this component (ANOVA, $F_{3,11} = 2.73$, $P = 0.095$, Fig. S2, Table S2). In contrast, PC2 seemingly explained the difference between SFP genes (PC2: 26.5 % explained, Fig. S2, Table 2). Although it is only close to statistical significance, this component tended to differ across Hours after mating (ANOVA, $F_{3,11} = 3.50$, $P = 0.053$, Table S2).

Discussion

Our data reveal a much more dynamic and complex pattern of replenishment of SFP than we predicted for this snail species. We found that *L. stagnalis* increases the transcription of two SFP genes 48 h after mating, supporting that transferring ejaculate indeed initiates SFP replenishment. However, four out of six SFP genes did not change their expression level after mating, implying that SFP replenishment occurs in a protein-specific manner. Lastly, even though seminal fluid reserves in the prostate gland are fully replenished after one week, the transcription of SFP genes seem high, contrasting with the low SFP expression of virgin snails previously reported (Nakadera et al. 2019). Below, we discuss the implications of these findings.

We found that the expression of the genes coding LyAcp5 and LyAcp8b increased 48 h after mating in the male role, supporting the importance of the functions of these proteins that are known to reduce sperm transfer of recipients in their subsequent mating as sperm donor (Nakadera et al. 2014). The first study that identified these SFPs showed that this species uses approximately one third of the amount of seminal fluid stored in the prostate gland for one insemination (Koene et al. 2010). For promiscuous species, it is very intuitive that they refill their seminal fluid after using up (part of) their supply, as shown in *D. melanogaster* (Herndon et al. 1997). Nevertheless, we did not detect signs of increased production after mating in the other four SFP genes studied here (Fig. 1). This may imply that SFP replenishment occurs in a protein-specific manner. Furthermore, this species might replenish specific SFPs depending on its own mating history as well as that of its partner(s). In this species, mating history indeed affects sperm transfer and SFP transcription

(Loose and Koene 2008; Nakadera et al. 2019). These studies suggest that *L. stagnalis* allocates specific SFPs differently to an ejaculate, depending on the mating history of donors and recipients, which leads to protein specific SFP replenishment. Also, it has been shown that receiving LyAcp5 and LyAcp8b reduces sperm transfer in a subsequent mating (Nakadera et al. 2014). Thus, increased production of LyAcp5 and LyAcp8b may hint at the intention of donors to reduce sperm transfer of their mating partners and, overall, supports the flexible and complex nature of SFP replenishment.

Comparing our results to a previous study also points out that SFP replenishment in *L. stagnalis* is affected by the status of female-mating snails (hereafter, recipient). Given the data of Swart et al. (2019), we predicted that the expression of *LyAcp10* increases 24 h after mating, but we did not observe such a pattern (see also Nakadera et al. 2019, Fig. 1). The cause of this deviation probably lies in that we decided to standardize the mating history of donors and recipients by isolating them for eight days, while Swart et al. (2019) used eight-day isolated individuals as donors, and non-isolated individuals as recipients. Such a difference in mating history in recipients could cause differential allocation of SFPs due to the following details of mating behaviours in *L. stagnalis* . When two isolated, male-mating motivated, snails meet, the recipient snails in the first mating tends to twist their body and grab the shell of the donor, so that the recipient can act as male immediately after the first mating (Koene and Ter Maat 2005). It is conceivable that this position squeezes the preputium of the donor and might thereby reduce efficient seminal fluid transfer. Also, this species transfers sperm at the very end of the insemination phase (Weggelaar et al. 2019), but it is still possible that donors transfer non-sperm components, including seminal fluid, prior to sperm during the preceding insemination duration. Given this reasoning, we examined whether the gene expression of SFPs 48 h after mating correlated with insemination duration from our behavioural observation, but did not observe any association (data not shown). Nonetheless, these details could explain why we did not see the expected increase of *LyAcp10* expression 24 h after mating as Swart et al. (2019), suggesting that this species alters SFP transfer and replenishment depending on the mating history of recipients.

We originally predicted that SFP expression would be reduced 192 h after mating, but this was not fully supported. 192 h is sufficient for these snails to become fully motivated to copulate as male (Van Duivenboden and Ter Maat 1985), based on the completed filling state of their prostate glands (De Boer et al. 1997). Moreover, previous studies showed that virgin snails show reduced SFP production (Nakadera et al. 2019, 2020). Therefore, we predicted that SFP production would be very low one week after mating in this species. However, our data do not fully reflect that (Fig. 1). This pattern either suggests that one week was too short for this species to down-regulate SFP production, or past mating experience had changed their reproductive physiology to produce SFPs permanently (White et al. 2021).

Our study also provides several cautionary pointers for predicting and interpreting gene regulation patterns of SFPs. First, we estimated the abundance of mRNA, which indicates the degree to which the protein production machinery is at work, but does not strictly reflect the amount of protein produced and/or present in the gland; a standard caveat when using qPCR (Futcher et al. 1999). For example, post-transcriptional regulation, translation efficiencies and turnover rate of each protein could disturb the direct relationship between the amount of mRNA and protein products (Futcher et al. 1999; Pratt et al. 2002). Second, SFP expression can be highly flexible and as we explained above, a slight change of experimental design can already have unexpectedly strong impact on the transcriptome. In our case, a slight deviation of protocol using snails directly from our mass culture as recipient did reveal the potential high plasticity on SFP expression depending on the mating history of recipients (Swart et al. 2019). Lastly, timing is essential to capture the expected up- and down-regulation of target genes. Based on our previous study (Swart et al. 2019), we expected that most expression changes would occur one day after mating. However, it turned out that this rather occurs between 24-48 h after mating, or not at all. Therefore, it is vital to carefully plan and conduct pilot experiments before investigating SFPs using extensive and expensive approaches, such as RNAseq.

In sum, we investigated SFP replenishment of *L. stagnalis* to contribute to filling the knowledge gap in SFP research. Our investigation indeed supported that insemination triggers up-regulation of SFP genes, but

the result also suggested that it proceeds in a SFP-specific manner. Furthermore, our results showed that SFP replenishment is plastic depending the mating history of recipient snails. Lastly, we found that not all SFP genes are down-regulated 192 h after mating, although the seminal fluid producing prostate gland is fully replenished by then. Given these outcomes, we believe our study expands the understanding of SFP dynamics and reproductive strategies in animals and suggests that this might also be the case for other glandular systems involving protein replenishment.

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Author contribution

JMK conceived and designed the study. YK and JM conducted experiments and processed the samples. YN and JMK analysed the data and wrote the manuscript with input from JM and YK.

Data accessibility

All data of this research will be deposited in an open-access and permanent data depository (e.g., Dryad), upon the acceptance of publication.

Competing interests.

None.

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Figures and tables

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Fig. 1. Temporal variation of SFP gene expression. Symbols indicate experimental blocks. The small letters above the bars indicate the outcome of post-hoc test (Tukey HSD, $P > 0.05$). Note that we detected significant difference in expression of *LyAcp8b*across hours after mating, but it was only close to significance when applying the post-hoc test.

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Fig. 2. PC loadings in SFP gene expression after mating. PC1 seems associating with Hours after mating, and PC2 with different SFP genes.

Table 1. The expression difference of each SFP gene. ANOVA ($N = 19$)

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Table 2. PC variance.

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Supplementary information

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Figure S1. Expression of reference gene. We confirmed that our reference genes are expressed consistently across the treatments (ANOVA, Tub: Hours after mating, $F_{3,11} = 0.33$, $P = 0.806$, Exp: $F_{1,11} = 0.98$, $P = 0.344$, Hours after mating × Exp, $F_{3,11} = 0.39$, $P = 0.760$, UbiE: Hours after mating, $F_{3,11} = 0.62$,$P = 0.618$, Exp: $F_{1,11} = 0.47$, $P = 0.509$, Hours after mating × Exp, $F_{3,11} = 0.22$,$P = 0.880$).

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Fig. S2. SFP gene expression along principal components.

Table S1. Primer data.

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Table S2. SFP gene expression along principal components.

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