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 SFPs 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 three out 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). Moreover, SFPs play crucial roles in sperm competition, e.g., by reducing remating rate of females or changing sperm velocity (e.g., Fiumera et al. 2007; Bartlett et al. 2017). Underlining the significance of SFP functions in sperm competition, a few studies in insects reported that consecutive mating make males deplete SFPs more quickly than they do sperm (*Drosophila melanogaster* : Lefevre and Johnson 1962, bedbug *Cimex lectularius* : Reinhardt et al. 2011, south American fruit fly*Anastrepha fraterculus* : Abraham et al. 2020). Furthermore, previous studies observed that males adjust SFP production as well as SFP transfer depending on the presence of rivals (e.g., *D. melanogaster* : Fedroka et al. 2011; Mohorianu et al. 2017; Hopkins et al. 2019, field cricket *Teleogryllus oceanicus* : Simmons and Lovegrove 2017; Sloan et al. 2018, chinook salmon *Oncorhynchus tshawytscha* : Bartlett et al. 2017, house mouse *Mus musculus domesticus* : Ramm et al. 2015, flatworm *Macrostomum lignano* : Ramm et al. 2019, pond snail *Lymnaea stagnalis* : Nakadera et al. 2019) or mating status of partners (*D. melanogaster* : Sirot et al. 2011, red junglefowl *Gallus gallus* : Alvarez-Fernandez et al. 2019). This observed plasticity is often explained as males 'tailoring' SFP composition of their ejaculate for each mating to optimize their reproductive success under varying levels of expected sperm competition.

However, although SFP production and its transfer are well known to be plastic in some taxa, 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 *D. melanogaster* adjusts the amount of specific SFPs to transfer, depending on whether

the female is virgin or not (Sirot et al. 2011). Such protein-specific adjustment of SFP transfer would affect the subsequent SFP replenishment in the male's accessory gland organ(s). That is, the most recent usage of SFPs would affect which SFPs would be more replenished than other SFPs. Also, males often alter SFP production depending on prevailing sperm competition risk (e.g., Ramm et al. 2015; Hopkins et al. 2019) as well as depending on on-going sperm competition (e.g., Sloan et al. 2018; 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 in only a few Diptera species and our model species, the great pond snail *Lymnaea stagnalis* (see below). In*Drosophila* , it has been well established that mating triggers up-regulation of transcription and translation in male accessory glands, likely to replenish SFPs (Bauman 1974; Schmidt et al. 1985; DiBenedetto et al. 1990; Monsma et al. 1990; Bertram et al. 1992; Yamamoto et al. 1998; Herndon et al. 1997; Redhai et al. 2016; Leiblich et al. 2012, 2019). Several studies monitored the size of male accessory glands after mating to see the time window of SFP replenishment (*D. melanogaster* : Hopkins et al. 2019, Queensland fruit fly*Bactrocera tryoni* : Radharkrishnan and Taylor 2008, stalk-eyed fly*Cyrtodiopsis dalmanni* : Rogers et al. 2005, but see*Bactrocera dorsalis* : Wei et al. 2015), based on the correlation between the size of male accessory glands and amount of secretion in*Drosophila* (Ravi Ram and Ramesh 2002). To date, only two studies in *D. melanogaster* measured how long it takes to refill SFPs at protein level (Coleman et al. 1995; Sirot et al. 2009). Sirot et al. (2009) showed that full replenishment of two SFPs, sex peptide and ovulin, was complete within three days (Sirot et al. 2009, see also Hopkins et al. 2019). 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). Given above, we consider that the knowledge of protein-specific replenishment of SFPs would expand the understanding 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 *L. stagnalis* . To do so, we let the snails copulate, then examined SFP gene expression at 3, 24, 48 and 192 h after mating. The rationale of finishing our monitoring after one week is that previous studies show that these snails get highly motivated to copulate as male after eight days of social isolation (Van Duivenboden and Ter Maat 1985), and this male mating motivation is driven by the fullness of the prostate gland (De Boer et al. 1997). Moreover, it has been shown that this species increases the production of LyAcp10 one day after mating (Swart et al. 2019). However, such an increase at 24 h after mating was not 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 how these SFPs get replenished 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). Although this species is a simultaneous hermaphrodite, 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 relatively 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), and 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, and the sample size is not fully balanced due to a few handling errors (total N : 3 h = 4, 24 h = 4, 48 h = 6, 192 h = 5).

To estimate the expression level of SFP genes, we sacrificed the donor snails to collect their prostate glands 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 a generalized linear model (GLM) with gamma distribution, due to the expression data being skewed. We used expression levels as the dependent variable, and Hours after mating and experimental block (Exp, N = 2) as fixed, categorical factors. Subsequently, we corrected the p values using false discovery rate (FDR) correction. When there was a significant difference between Hours after mating, we used Tukey's Honest Significant Differences (Tukey HSD) test. To visualize the overall change in SFP gene expression over time, we reduced the dimensions of expression data using principal component analysis (PCA). In addition, we tested the created PC scores using GLM with gaussian distribution with the same model above and FDR correction. We performed all the analyses with R (ver. 4.0.3, R Core Team).

Results

First, we examined the expression of each separate SFP gene, and detected that the expression of *LyAcp8b* significantly increased 48h after mating (Fig. 1, Table 1). We also found that *LyAcp5* and*LyAcp8a* expression altered significantly after mating, although post-hoc testing did not show any significant difference between specific time points (Fig. 1, Table 1). For *LyAcp5* , this seems due to the difference in expression between 48 h and 196 h, and for*LyAcp8a* (very similar to LyAcp8b) expression increased 48 h after mating. Two samples showed consistently high expression in*LyAcp5* , *LyAcp8a* and *LyAcp8b* (#92, #110), but not in the other genes and we could not find any technical or biological features explaining this pattern (e.g., RNA extraction

date, body size). In LyAcp8a and *LyAcp8b* , we detected significant differences between experimental blocks, but not interaction with Hours after mating. In contrast, the three remaining SFP genes did not show any significant change in expression level throughout our monitoring, suggesting that the production of these SFPs did not increase after mating (Fig. 1, Table 1). We also like to note that the expression at 196 h after mating is not always low, compared to 3 h after mating (Fig. 1), while by that time the prostate gland is expected to be fully replenished.

Next, we inspected the overall change in expression across all SFP genes. To do so, we conducted a PCA to create representative variables for overall SFP gene expression. PC1 explained 50.0% of the total variance, and this variable seems to correspond with Hours after mating, although this is not the case after FDR correction (Fig. S2, Table 2, Table S2). In contrast, PC2 explained 26.5% of the total variance, and seemingly explained the difference between SFP genes, again after FDR correction statistical significance disappeared (Fig. S2, Table 2, S2). The important, additional insight from PCA is that the expression of SFP genes after mating differed between separate SFP genes, which is visualized by the directions of PC loadings (Fig. 2, Table 2).

Discussion

Our data reveal a much more dynamic and complex pattern of replenishment of SFPs than we predicted for this snail species. We found that *L. stagnalis* increases the transcription of a SFP gene 48 h after mating, supporting that transferring ejaculate indeed initiates SFP replenishment. However, three 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 (Van Duivenboden and Ter Maat 1985; De Boer et al. 1997), 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, LyAcp8a 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). 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. Also, we did not detect signs of increased production after mating in the other three SFP genes studied here (Fig. 1). This may imply that SFP replenishment occurs in a protein-specific manner. In this species it has been established that, mating history indeed affects sperm transfer and SFP transcription (Loose and Koene 2008; Nakadera et al. 2019). Collectively, 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.

The timing of elevated SFP gene expression was rather unexpected, and currently we do not have a fully-satisfactory explanation for why this is the case. Single insemination should be sufficient to see the signal of SFP replenishment, because this species uses approximately one third of the amount of seminal fluid stored in the prostate gland for one insemination (Koene et al. 2010). Thus, we expected that this promiscuous species would refill its seminal fluid immediately after using up (part of) its supply, as shown in *D. melanogaster* (e.g., Monsma et al. 1990). Although, statistically speaking, we did detect the elevated expression of two SFP genes (*LyAcp5* , *LyAcp8a*), the up-regulation of *LyAcp8b* was observed 48 h after mating, which is much later than expected. Based on our knowledge about the biology of this species, we consider it unlikely that the up-regulation of SFP genes happened earlier than 3 h after mating in *L. stagnalis* , although in *Drosophila* , it occurs within 1 h after mating. The reproductive nature of *L. stagnalis* is slightly more promiscuous and much slower than *D. melanogaster* . For example, the courtship and insemination of *L. stagnalis* usually take several hours, and they can inseminate twice per day (Koene and Ter Maat 2007). Moreover, even if they elevated SFP gene expression immediately after mating, it would not cease within 3 h after mating. However, for the time being we do not have a suitable explanation nor reference to argue why they up-regulate SFP genes so late.

The discrepancy between the results from a previous study and ours suggests that SFP replenishment in *L. stagnalis* is affected by the mating history of female-mating snails (hereafter, recipient). Swart et al. (2019) examined the expression of one SFP gene, *LyAcp10* , after mating. To do so, they let eight-day isolated donors inseminate non-isolated recipients. Then, they found that the expression of*LyAcp10* significantly increased 24 h after mating. In our experiment, however, we used both isolated donors and recipients, and we did not detect any change of *LyAcp10* expression throughout our monitoring (Fig. 1, also see Nakadera et al. 2019). The comparison of the experimental setups and outcomes between these two studies implies that the mating history of recipients has strong impacts on SFP replenishment of donors. Although this hypothesis may be surprising, it is also supported from the perspective of their mating behaviour. When two isolated, male-mating motivated, snails meet, the recipient snails in the first mating tends to twist their body and grab the shells of their donors, so that the recipient can act as male immediately after the first mating (see photos in Koene and Ter Maat 2005). It is conceivable that this position of recipient snails squeezes the preputium of donors and might thereby reduce efficient seminal fluid transfer. The effect of squeezing is likely more relevant to SFP transfer than sperm transfer, since this species spends most of insemination duration for transferring non-sperm components (Weggelaar et al. 2019). 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 insights from other studies 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. Our prediction stemmed from previous study in *D. melanogaster* (Sirot et al. 2009), as well as following reproductive biology of this species. 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 did 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. The latter is not such a far-fetched hypothesis, since mated females often experience drastic changes triggered by SFPs (e.g., White et al. 2021). Also, we like to emphasize that our study species is simultaneously hermaphroditic, and we cannot rule out long-lasting effects of receiving SFPs, next to the known short-term effects (Nakadera et al. 2014). Therefore, in future studies we will also need to consider that mating experience might mediate long-lasting effects on SFP expression. Moreover, we want to point out that there is a lack of study focusing on this feature of SFP expression, although relatively high expression of SFP genes long after mating was reported in a previous study in mice showing that that SFPs undergo considerable turnover even without copulation or presence of rivals (Claydon et al. 2012).

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 measured SFP gene expression after mating in *L. stagnalis* to expand the knowledge of protein-specific SFP replenishment. 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 on 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 protein-specific replenishment might also be the case in other glandular systems involving protein replenishment.

Appendix

For qPCR, we used the sets of primers listed in Table S1. In order to validate the chosen housekeeping genes as reference, we confirmed that these genes were indeed 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 x 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 x Exp, $F_{3,11} = 0.22$, P= 0.880: Fig. S1). To see the overall pattern of SFP expression after mating, we conducted PCA and found that PC1 is mostly corresponding to Hour after mating, and PC2 is for SFP genes (Fig. S2, Table S2)

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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 (N = 2). 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*LyAcp5* and *LyAcp8a* across hours after mating, but post hoc comparisons between individual time points were all only close to significance, indicated by *a* ' (*LyAcp5* , 48 h vs. 196 h:$P = 0.082$, *LyAcp8a* , 24 h vs. 48 h: $P = 0.069$).

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Fig. 2. PC loadings in SFP gene expression after mating. Based on PC loadings shown as black arrows and Fig. S2 in appendix, PC1 seems associated with Hours after mating, and PC2 with different SFP genes.

Table 1. The expression difference of each SFP gene after mating. We used GLM with gamma distribution to see if SFP gene expression altered ($N = 19$). Then, we adjusted P values using FDR. Significance after FDR correction is indicated by asterisks.

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Table 2. PC variance and loadings.

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Appendix

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Figure S1. Expression (threshold cycle, Ct) of reference gene.

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Fig. S2. SFP gene expression along principal components.

Table S1. Primer data.

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Table S2. The expression pattern of all SFP genes combined in principal components. We carried out GLM with Gaussian distribution for PC score to test if overall SFP expression alters after mating. After FDR correction, we did not detect any significant difference.

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