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Dynamics of seminal fluid production 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. This is especially relevant in species that mate multiple times, as they 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 two out of the six SFP genes investigated here were upregulated 1 week after mating. Surprisingly, most SFP genes did not change their expression immediately after mating. Even after 1 week, when supposedly seminal fluid is fully replenished, the expression of SFP genes is rather high. In addition, the difference with previous studies hints at the possibility that SFP production after mating is plastic and depends on the mating history of female-acting snails. Our results shed light on unexplored aspects of SFP production, thereby expanding the understanding of reproductive strategies in animals.

KEYWORDS
accessory gland proteins, ACPs, gene expression, mating history, mollusc, reproduction

1 | INTRODUCTION

Seminal fluid proteins (SFPs, also referred to as accessory gland proteins or ACPs) are part of the nonsperm 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. For example, SFPs facilitate the initiation of a healthy pregnancy in humans (Bromfield, 2014; McGraw et al., 2016) and induce oviposition after mating in many insects (e.g., 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., Bartlett et al., 2017; Fiumera et al., 2007). Underlining the significance of SFP functions in sperm competition, a few studies in insects reported that consecutive mating make males deplete SFPs faster than sperm (*Drosophila melanogaster*: Lefevre & Jonsson, 1962; Linklater et al., 2007, 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*: Fedorka et al., 2011; Hopkins et al., 2019; Mohorianu et al., 2017, field cricket *Teleogryllus oceanicus*: Simmons & 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 transfer are well known to be plastic in some taxa, their replenishment has received surprisingly little attention. This is a nontrivial 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 need to be more replenished than other SFPs. Also, males often alter SFP production depending on prevailing sperm competition risk (e.g., Hopkins et al., 2019; Ramm et al., 2015) as well as depending on on-going sperm competition (e.g., Nakadera et al., 2019; Sloan et al., 2018). 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 upregulation of transcription and translation in male accessory glands, likely to replenish SFPs (Baumann, 1974; Bertram et al., 1992; DiBenedetto et al., 1990; Herndon et al., 1997; Leiblich et al., 2012, 2019; Monsma et al., 1990; Redhai et al., 2016; Schmidt et al., 1985; Yamamoto et al., 1988). 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*: Radhakrishnan & 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* (Ram & 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 3 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 the 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 production in the prostate gland after mating in the great pond snail *L. stagnalis*. To do so, we let the snails copulate, then measured SFP gene expression at 3, 24, 48, and 192 h after mating using reverse-transcription quantitative PCR (qPCR). Monitoring for 1 week after mating is considered reasonable since these snails become highly motivation to copulate as male after 8 days of social isolation (Van Duivenboden & Maat, 1985), and the fullness of the prostate gland is the main driver of this motivational state (De Boer et al., 1997). Moreover, it has been shown that this species increases the production of one of the known SFPs, LyAcp10, 1 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 examined the expression of six SFP genes identified in previous studies, to monitor how these SFPs get replenished after mating (*LyAcp5*, *LyAcp7a*, *LyAcp7b*, *LyAcp8a*, *LyAcp8b*, *LyAcp10*: Koene et al., 2010; Nakadera et al., 2019; Swart et al., 2019). Although many more SFPs are expected in this species (e.g., 174 well established SFPS in *D. melanogaster* [Hurtado et al., 2022; Wigby et al., 2020], 827 SFPs in house sparrows [Rowe et al., 2020], Reviewed in Sirot et al., 2015), these six SFPs of *L. stagnalis* are highly abundant and have known full or partial sequences. The functions of some of these SFPs are also revealed, as receiving LyAcp10 (also called ovipostatin) delays egg laying (Koene et al., 2010), and receiving LyAcp5 or LyAcp8b reduces sperm transfer in a subsequent mating (Nakadera et al., 2014). In addition, receiving SFPs increases the occurrence of female mating avoidance behavior (Daupagne & Koene, 2020). Since virgin snails express SFP genes less than snails with mating opportunities (Nakadera et al., 2019), it 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 or not.

2 | RESULTS

First, we examined the expression of each SFP gene separately. We excluded two outliers in the time point of 48 h after mating from further analysis (Figure S2). The expression of two out of six SFP genes (*LyAcp7a*, *LyAcp8a*) significantly increased 192 h after mating (Figure 1 and Table 1). The expression of *LyAcp8b* was altered significantly after mating, without showing any significant difference between specific time points in the post hoc test. For the expression of *LyAcp5*, we observed a high variation as shown in previous studies (Nakadera et al., 2019, 2020). In *LyAcp8a* and *LyAcp8b*, we detected significant differences between experimental blocks and the interaction with hours after mating (Table 1). In contrast, the three remaining SFP genes did not show any significant change in expression level throughout our monitoring (Figure 1 and Table 1), but we like to note that the expression at 196 h after mating was

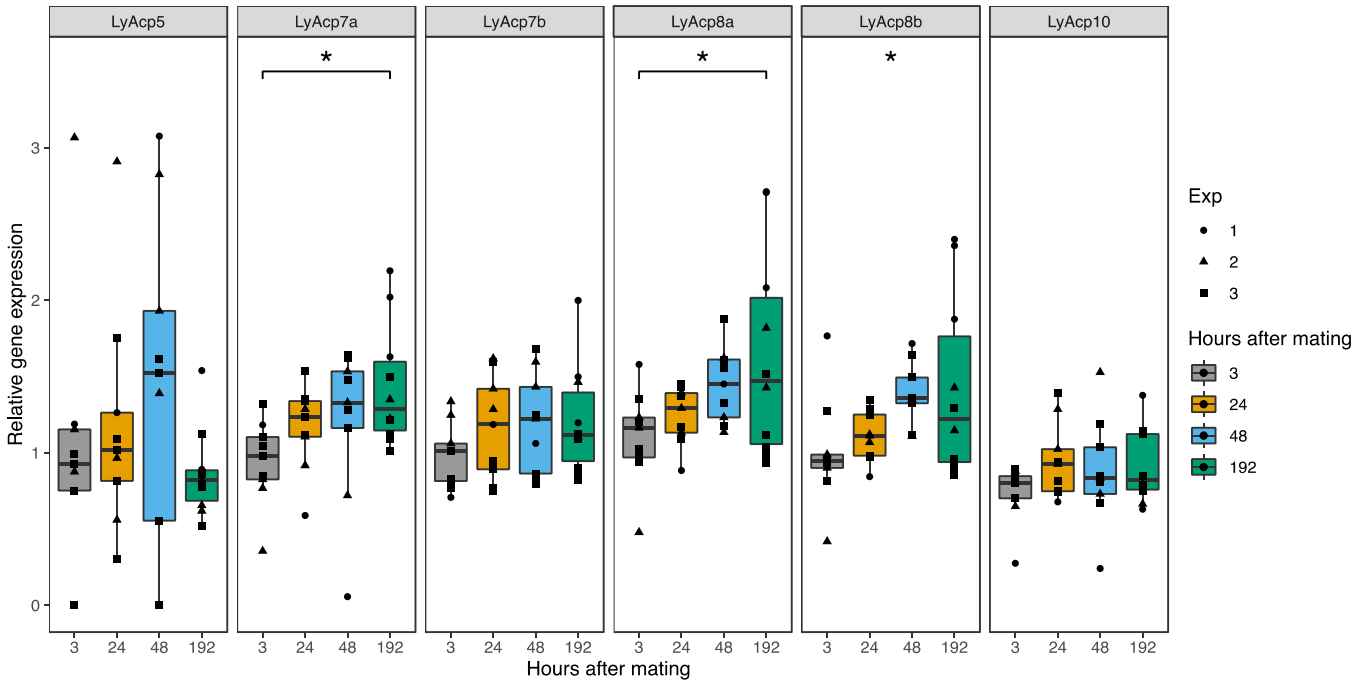

FIGURE 1 Temporal variation of SFP gene expression. Symbols indicate experimental blocks ($N = 3$). The asterisks above the bar plots indicate the outcome of GLM whether the gene expression differs across time points, and the bars immediately below show the outcome of post-hoc testing (Tukey HSD, $p > 0.05$). Note that we detected a significant difference in expression of *LyAcp8b* across hours after mating, but post hoc comparisons between individual time points were all only close to significance (3 h vs. 192 h: $p = 0.073$). Abbreviations: HSD, honest significant differences; SFP, seminal fluid protein.

rather elevated compared to 3 h after mating, while by that time the prostate gland is expected to be fully replenished.

Next, we examined the overall change in expression across all SFP genes (Figure 2). To do so, we conducted a principal component analysis (PCA) to create representative variables for overall SFP gene expression. PC1 explained 46.4% of the total variance, and this variable corresponds with the temporal variation of SFP gene expression as observed in the individual SFP genes (hours after mating: $p < 0.001$, Exp: $p = 0.003$, interaction: $p < 0.001$: Figure 2a–c). In contrast, PC2 explained 25.9% of the total variance, and seemingly explained the difference in expression between each SFP genes, based on the different directions of PC loadings (Figure 2a). As expected from previous analysis, the clustering of SFP gene expression showed that the expression of *LyAcp5* differed compared to other genes, and *LyAcp7b* and *LyAcp10* clustered together (Figure 2d). In sum, this outcome shows that ca. 25% of overall variance was explained by differential expression of SFP genes after mating.

3 | DISCUSSION

Our data revealed a much more complex pattern of SFP production after mating than we predicted in this snail species. We found that *L. stagnalis* increases the transcription of two SFP genes 192 h after mating, and three out of six SFP genes did not change their expression level after mating, suggesting that transferring ejaculate does not necessarily initiate upregulation of SFP genes. Lastly, even though seminal fluid reserves in the prostate gland are fully replenished after 1 week (De Boer et al., 1997; Van Duivenboden & Maat, 1985), the transcription of SFP genes was higher 192 h after mating than that of 3 h after mating. Last, we found that SFP production after mating occurs in a protein-specific matter, supported by the different directions of PC loadings of SFP genes. Below, we discuss the implications of these findings.

Unexpectedly, we did not find support for increased SFP production after mating in *L. stagnalis*. Our data showed that the expression of the genes coding for LyAcp7a and LyAcp8a increased 192 h after mating in the male role, which is too long after mating to conclude that ejaculate donation initiates SFP replenishment. Moreover, three SFP genes did not change their expression after mating. It is unlikely that the upregulation of SFP genes happened and finished earlier than 3 h after mating in *L. stagnalis* (Swart et al., 2019). Also, we expected that a single ejaculate donation is 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., Hopkins et al., 2019; Monsma et al., 1990; Sirot et al., 2009). Also, *L. stagnalis* is slightly more promiscuous. For example, the courtship and insemination of

TABLE 1 The expression difference of each SFP gene after mating.

	df	Deviance	*p*	Adj. *p*
LyAcp5				
Hours after mating	3	1.73	0.312	0.312
Exp	2	5.30	0.004	0.006**
Hours after mating × Exp	6	4.23	0.190	0.227
LyAcp7a				
Hours after mating	3	1.19	<0.001	<0.001***
Exp	2	0.26	0.079	0.094
Hours after mating × Exp	6	3.31	<0.001	0.000
LyAcp7b				
Hours after mating	3	0.31	0.212	0.254
Exp	2	1.07	<0.001	0.001***
Hours after mating × Exp	6	0.52	0.276	0.276
LyAcp8a				
Hours after mating	3	1.52	<0.001	<0.001***
Exp	2	1.56	<0.001	<0.001***
Hours after mating × Exp	6	2.52	<0.001	<0.001***
LyAcp8b				
Hours after mating	3	1.21	<0.001	<0.001***
Exp	2	2.16	<0.001	<0.001***
Hours after mating × Exp	6	1.65	<0.001	<0.001***
LyAcp10				
Hours after mating	3	0.27	0.192	0.254
Exp	2	0.22	0.139	0.139
Hours after mating × Exp	6	0.74	0.041	0.062

Note: We used GLMs with Gaussian distribution to see if SFP gene expression altered. Then, we adjusted *p* values using FDR. Significance after FDR correction is indicated by asterisks.

Abbreviations: EXP, experiment; FDR, false discovery rate; SFP, seminal fluid protein.

L. stagnalis usually take several hours, and they can inseminate twice per day (Koene & Ter Maat, 2007), and reproduce for many months (e.g., Nakadera et al., 2015). Last, we need to emphasize that our method estimated the abundance of messenger RNA (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). Nonetheless, we do not have a concrete explanation why transferring ejaculate does not trigger SFP replenishment at all in this study.

Another unexpected outcome was that SFP gene expression was high 1 week after mating, even though the seminal fluid reserve is full (De Boer et al., 1997; Van Duivenboden & Maat, 1985). Our recent transcriptomic investigation supports this pattern, as these SFP genes were observed to be upregulated constantly (Nakadera et al. in prep.). As reported in *D. melanogaster* (Monsma et al., 1990), Nakadera et al. (2019) observed the low SFP gene expression of virgin snails in *L. stagnalis*, compared to mated individuals. Collectively, it might imply that, regardless of the recent mating history, mated males or donors maintain high transcription of SFP genes.

In addition, our data supports that SFP production occurs in a protein-specific matter across the time span with a standardized mating history. We consider it is an important, but not unexpected feature of SFP expression, since seminal fluid is a complex mixture of proteins (e.g., Perry et al., 2013) and their expression forms complex networks (Ayroles et al., 2011; Mohorianu et al. 2018; Patlar et al., 2019). Furthermore, a previous study in mice showed that SFPs undergo considerable turnover even without copulation or presence of rivals (Claydon et al., 2012).

The discrepancy between the results from a previous study and ours hints at the significant role of mating history of female-acting snails (hereafter, recipient) on SFP replenishment in *L. stagnalis*. Swart et al. (2019) examined the expression of one SFP gene, *LyAcp10*, after mating. To do so, they let 8-day isolated donors inseminate nonisolated recipients. Then, they found that the expression of *LyAcp10* in sperm donors 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 (Figure 1, also see fig. S1 of Nakadera et al., 2019; Swart 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. This hypothesis is also supported from the perspective of their mating behavior. When two isolated, male-mating motivated, snails meet, the recipient snails in the first mating tend 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 fig. 3 in Koene & 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 seems to spend most of the insemination duration for transferring nonsperm 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 behavioral 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), implying that this species alters SFP transfer and replenishment depending on the mating history of recipients. Further investigation on SFP transfer, mating history and mating behavior would be necessary to examine this hypothesis in future.

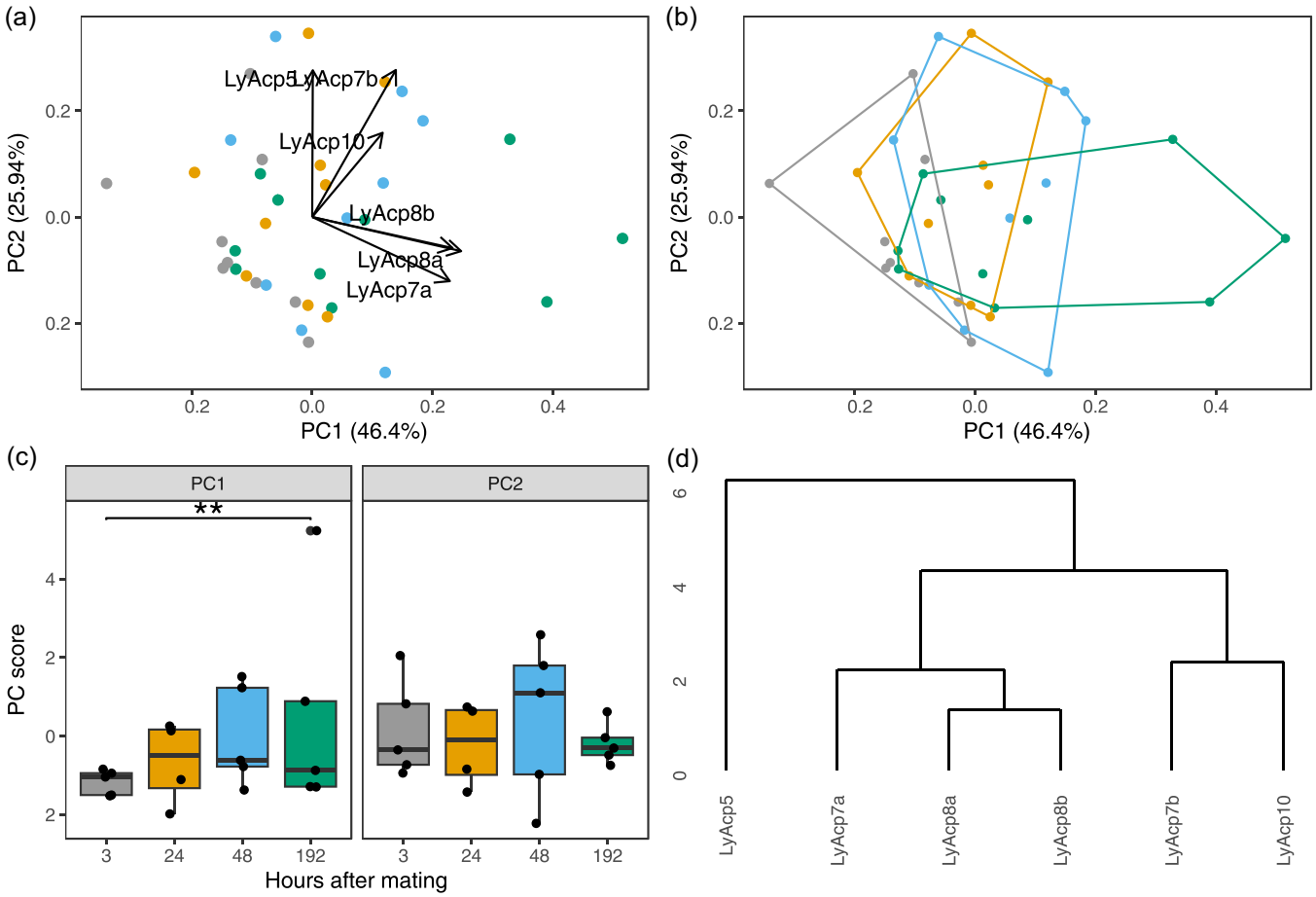

FIGURE 2 Variation of overall SFP gene expression after mating. The colors are corresponding to hours after mating. (a) PC loadings in PC spaces, shown as black arrows. (b) individual data points in PC space. (c) PC scores between hours after mating. The asterisks above the bar plots indicate the outcome of GLM whether the gene expression differs across time points, and the bars immediately below show the outcome of post-hoc test (Tukey HSD, $p > 0.05$). Based on these outcomes of the PCA, we consider that PC1 seems associated with hours after mating, and PC2 with different SFP genes. (d) The outcome of clustering between SFP genes. Abbreviations: HSD, honest significant differences; PC, principal component; PCA, principal component analysis; SFP, seminal fluid protein.

In sum, we measured SFP gene expression after mating in *L. stagnalis* to expand the knowledge of SFP replenishment. Our investigation did not support that insemination triggers upregulation of SFP genes, and found that SFP gene expression was elevated 1 week after mating, even though their seminal fluid is fully replenished by then. Our monitoring also revealed protein-specific expression of SFPs. Given these outcomes, our study emphasizes the need of further investigations of SFP replenishment in diverse model systems, as this aspect of SFP expression may contain unexpected implications to expand the understanding of SFP function and its evolution.

4 | MATERIALS 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 12:12 h. In this experiment, we used adult snails (4 months 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 & Ter Maat, 2005). In addition, this species is relatively promiscuous as exemplified by the fact that they can inseminate more than once within 24 h (Koene & Ter Maat, 2007).

To estimate the expression level of SFP genes at different time points after mating, we let the snails copulate under observation. First, to increase their male mating motivation, we isolated the snails for 8 days, by keeping one individual per 460-mL perforated container placed in a flow-through tank (De Boer et al., 1997; van Duivenboden & Maat, 1985). 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 & 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 three times (Exp 1 = 6, Exp 2 = 11, Exp 3 = 20, total *N*: 3 h = 9, 24 h = 9, 48 h = 9, 192 h = 10).

To estimate the expression level of SFP genes after mating, we euthanized the donor snails to collect their prostate glands at the designated time point after mating. We chose to monitor four different time intervals, which were 3, 24, 48, 192 h after mating in the male role. To collect their prostate glands, first, we injected ca. 2 mL of 50 mM $MgCl_2$ into foot for anesthetization. Then, we dissected out a prostate gland, placed the tissue into a 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 complementary DNA using the MML-V Reverse transcriptase kit (Promega). Then, we conducted 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; Johnson & Davison 2019; Young et al. 2019; Table S1). For primer designing, we applied the following thresholds: annealing temperature 59°C–60°C, GC contents = 40%–45%, amplicon melting temperature = 80°C–85°C. To calculate the relative, normalized gene expression ($2^{-\Delta\Delta Ct}$; Livak & Schmittgen, 2001), we used the software CFX Manager v3.1. We confirmed that the expression of reference genes was not significantly different across treatments (Figure S1).

To examine the temporal expression changes of SFP genes after mating, we used a generalized linear model (GLM) with Gaussian distribution. We used expression levels as the dependent variable, and hours after mating and experimental block (Exp, *N* = 3) 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 test. To visualize the overall change in SFP gene expression over time, we reduced the dimensions of expression data using PCA. In addition, we tested the created PC scores using GLM with Gaussian distribution with the same model above and FDR correction. Last, we run hierarchical clustering with complete linkage method to see the grouping of SFP gene expression. We performed all the analyses with R (ver. 4.0.3, R Core Team, 2018).

AUTHOR CONTRIBUTIONS

Yvonne Kortsmit: Investigation; data curation; writing - review & editing. **Janine Mariën**: Investigation; data curation; writing - review & editing. **Joris M. Koene**: Conceptualization; writing - review & editing; supervision; writing - original draft. **Yumi Nakadera**: Writing - original draft; visualization; formal analysis; writing - review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data of this research are openly avaialbe (doi:10.48338/VU0-YO96TU).

ETHICS STATEMENT

This study includes experimental work on an invertebrate, a common freshwater snail species. While formal ethical approval is not required, the research has been conducted in accordance with ASAB and institutional guidelines.

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