




## RESEARCH PAPER

# Divergence of seminal fluid gene expression and function among natural snail populations

Yumi Nakadera<sup>1,2</sup>  | Alice Thornton Smith<sup>2</sup> | Léa Daupagne<sup>2</sup> |  
Marie-Agnès Coutellec<sup>3</sup> | Joris M. Koene<sup>2</sup>  | Steven A. Ramm<sup>1</sup> 

<sup>1</sup>Evolutionary Biology, Bielefeld University, Bielefeld, Germany

<sup>2</sup>Ecological Science, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

<sup>3</sup>ESE, Ecology and Ecosystem Health, INRAE, Rennes, France

## Correspondence

Yumi Nakadera, Ecological Science, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands.  
Emails: y.nakadera@vu.nl; yumi.nakadera@gmail.com

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## Abstract

Seminal fluid proteins (SFPs) can trigger drastic changes in mating partners, mediating post-mating sexual selection and associated sexual conflict. Also, cross-species comparisons have demonstrated that SFPs evolve rapidly and hint that post-mating sexual selection drives their rapid evolution. In principle, this pattern should be detectable within species as rapid among-population divergence in SFP expression and function. However, given the multiple other factors that could vary among populations, isolating divergence in SFP-mediated effects is not straightforward. Here, we attempted to address this gap by combining the power of a common garden design with functional assays involving artificial injection of SFPs in the simultaneously hermaphroditic freshwater snail, *Lymnaea stagnalis*. We detected among-population divergence in SFP gene expression, suggesting that seminal fluid composition differs among four populations collected in Western Europe. Furthermore, by artificially injecting seminal fluid extracted from these field-derived snails into standardized mating partners, we also detected among-population divergence in the strength of post-mating effects induced by seminal fluid. Both egg production and subsequent sperm transfer of partners differed depending on the population origin of seminal fluid, with the response in egg production seemingly closely corresponding to among-population divergence in SFP gene expression. Our results thus lend strong intraspecific support to the notion that SFP expression and function evolve rapidly, and confirm *L. stagnalis* as an amenable system for studying processes driving SFP evolution.

## KEYWORDS

accessory gland proteins, among-population divergence, functional assay, sexual conflict, sexual selection, simultaneous hermaphrodite

## 1 | INTRODUCTION

Seminal fluid proteins (SFPs, also referred to as accessory gland proteins or ACPs) contained in the ejaculate are transferred together

with sperm to mating partners. Laboratory studies have examined within-population variation of SFPs at sequence, expression and functional levels, and established that these proteins exhibit a wide range of functions, such as inducing ovulation or defending mating

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partners against sexually transmitted diseases (Gioti et al., 2012; McGraw, Clark, & Wolfner, 2008; Peng, Grassl, Millar, & Baer, 2016, review: Avila, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011; Chapman, 2001; Morrow & Innocenti, 2012; Poiani, 2006; Sirot, Wong, Chapman, & Wolfner, 2015). In addition to their immediate effects, SFPs may also be responsible for seminal fluid-mediated transgenerational effects (Bromfield et al., 2014; Crean, Kopps, & Bonduriansky, 2014; Priest, Roach, & Galloway, 2008; Simmons & Lovegrove, 2019). Moreover, although some effects of SFPs are essential and beneficial for both parents, other functions appear sexually antagonistic (Chapman, Liddle, Kalb, Wolfner, & Partridge, 1995; Patlar, Weber, Temizyürek, & Ramm, 2020, review: Sirot et al., 2015). For example, sex peptide, the best studied SFP in *Drosophila melanogaster*, makes females reluctant to re-mate (Chen et al., 1988). This effect is likely beneficial, in terms of reproductive success, for the SFP-transferring male, but not necessarily for the SFP-receiving female (Fricke, Wigby, Hobbs, & Chapman, 2009; Wigby & Chapman, 2005). Their sexually harmonious and antagonistic functions underlie the notion that SFPs are key mediators of sexual selection and sexual conflict (Sirot et al., 2015; Swanson & Vacquier, 2002a, b).

Another striking feature of SFPs is their rapid evolution, although their driving forces remain elusive. Based on interspecific comparisons, especially to date in mammals and insects, SFP genes often exhibit signatures of positive selection (Dorus, Evans, Wyckoff, Choi, & Lahn, 2004; Ramm, Oliver, Ponting, Stockley, & Emes, 2008; Rowe et al., 2020; Walters & Harrison, 2011) and overall evolve at higher rates than most other tissue-specific genes (Civetta & Singh, 1998; Good et al., 2013; Haerty et al., 2007). Proteomic investigations further confirm and expand this general trend of accelerated evolution in SFPs (Dean et al., 2009; Findlay, MacCoss, & Swanson, 2009; Ramm, McDonald, Hurst, Beynon, & Stockley, 2009). Thus, in the light of their known functions and evolutionary dynamics, it has frequently been proposed that SFP evolution is driven by sexual selection and associated sexual conflict (Sirot et al., 2015; Swanson & Vacquier, 2002a, b). Several cross-species studies have sought molecular evolutionary evidence in support of this hypothesis. For example, Dorus et al. (2004) found a positive correlation between the evolutionary rate of an SFP gene and the inferred level of sperm competition in primates. However, others did not observe such association (Hurle, Swanson, Comparative Sequencing Program, & Green, 2007 [but see Ramm et al., 2008]; Walters & Harrison, 2011; Good et al., 2013). Nevertheless, cross-species studies can only partially answer whether SFP evolution is driven by sexual selection, since macro-evolutionary trajectories of SFPs are obscured by uncertainty over ancestral states and other confounding factors, for example ecological factors (Perry, Garroway, & Rowe, 2017; Perry & Rowe, 2012, 2018). Moreover, it is also challenging to verify any association established between evolutionary changes and the functional consequences of SFPs. Although plausible, it therefore remains a largely unanswered question to what extent sexual selection provides a general explanation for rapid SFP evolution.

In this study, we therefore focused on an alternative means to study the driving force of SFP evolution, based on intraspecific variation in the expression of SFP genes and their biological functions. If sexual selection indeed drives the rapid evolution of SFPs, diversification of SFPs is expected to be detectable also at a within-species, among-population level. It is necessary to empirically test this hypothesis at different levels, as other studies have shown that a plausible explanation at one scale does not always apply to a different scale (Blanckenhorn, Stillwell, Young, Fox, & Ashton, 2006; Johnson & Wade, 2010; Perry & Rowe, 2012). For instance, male body size evolutionarily diversified more than female body size among species (Rensch, 1950), but this is not the general rule at a population level (Blanckenhorn et al., 2006). More importantly, intraspecific variation would more readily allow us to conduct experiments to test whether functional divergence in the strength of post-mating responses of mating partners induced by SFPs is correspondingly divergent, in order to examine the link between evolutionary and functional changes in SFPs.

Although fewer in number, intraspecific studies of SFP variation have already provided some important insights. In the early phase of SFP research, the detection of intraspecific variation indicated that sequence polymorphism of SFP genes is often maintained despite the fact that these proteins are expected to be under positive selection (Aguadé, 1998, 1999; Tsaur & Wu, 1997; Coulthart & Singh, 1988, but see Palopoli et al., 2008). Many subsequent studies extensively documented within-population variation of SFPs using inbred families from a single population, particularly focusing on the influence on sperm competition (e.g. sequence: Clark, Aguadé, Prout, Harshman, & Langley, 1995; Fiumera, Dumont, & Clark, 2005; Fiumera, Dumont, & Clark, 2007; Zhang, Clark, & Fiumera, 2013, gene expression: Patlar, Weber, & Ramm, 2019; Patlar & Ramm, 2020, protein abundance: Brandon, Heusnes, Caudle, & Fayrer-Hosken, 1999). More direct attempts to compare SFP divergence between populations have recently begun, providing empirical support for diversified SFPs, likely associating with differential effects (protein abundance: Baer, Zareie, Paynter, Poland, & Millar, 2012; Goenaga, Yamane, Rönn, & Arnqvist, 2015; Mangels et al., 2015). More importantly, Goenaga et al. (2015) have demonstrated that differences in SFP abundance were indeed associated with male performance in sperm competition and fecundity (also see Brandon et al., 1999).

Given the findings above, we aim here to expand the scope of studying intraspecific variation in SFPs, using the simultaneously hermaphroditic great pond snail, *Lymnaea stagnalis*, by utilizing the following advantages, especially the known functions of their SFPs and an established bioassay. First, this species is abundant, widespread, and populations are genetically well-structured (Bouétard, Côte, Besnard, Collinet, & Coutellec, 2014; Kopp, Wolff, & Jokela, 2012; Nakadera, Mariën, Van Straalen, & Koene, 2017; Puurtinen, Hytönen, et al., 2004; Puurtinen, Knott, Suonpää, Ooik, & Kaitala, 2004). Thus, we can readily sample from populations with distinct evolutionary histories. Second, it has been documented that SFPs in *L. stagnalis* have important functions in reproduction, affecting both egg production and subsequent sperm transfer of

mating partners: receiving the SFP LyAcp10 is known to delay egg laying (Koene et al., 2010), whereas two other SFPs, LyAcp5 and LyAcp8b, mediate a reduction in sperm transfer by recipient snails in their subsequent mating in the male role (Nakadera et al., 2014). Receiving seminal fluid is also suggested to increase egg size (Swart et al., 2020). Thirdly, we have an established SFP bioassay based on intravaginally injecting seminal fluid in *L. stagnalis* (Koene, Hoffer, & Brouwer, 2009; Koene et al., 2010; Nakadera et al., 2014). This species therefore provides an excellent opportunity to examine the functional consequences of intraspecific variation in SFP expression in controlled laboratory experiments. That is, we can specifically isolate SFP-mediated effects from potentially confounding influences including mate choice and sperm numbers.

We collected snails from four natural populations to establish iso-mother families in a 'common garden' laboratory environment, keeping snails at two different group sizes to control for social conditions that are a prominent source of variation in SFP expression (Hopkins et al., 2019; Ramm et al., 2015; Simmons & Lovegrove, 2017; Sloan, Lovegrove, & Simmons, 2018). Through a series of gene expression and follow-up functional assays of SFPs, we first tested whether seminal fluid expression indeed differs consistently among populations and then whether this leads to corresponding variation in partner responses to SFP receipt.

## 2 | MATERIALS AND METHODS

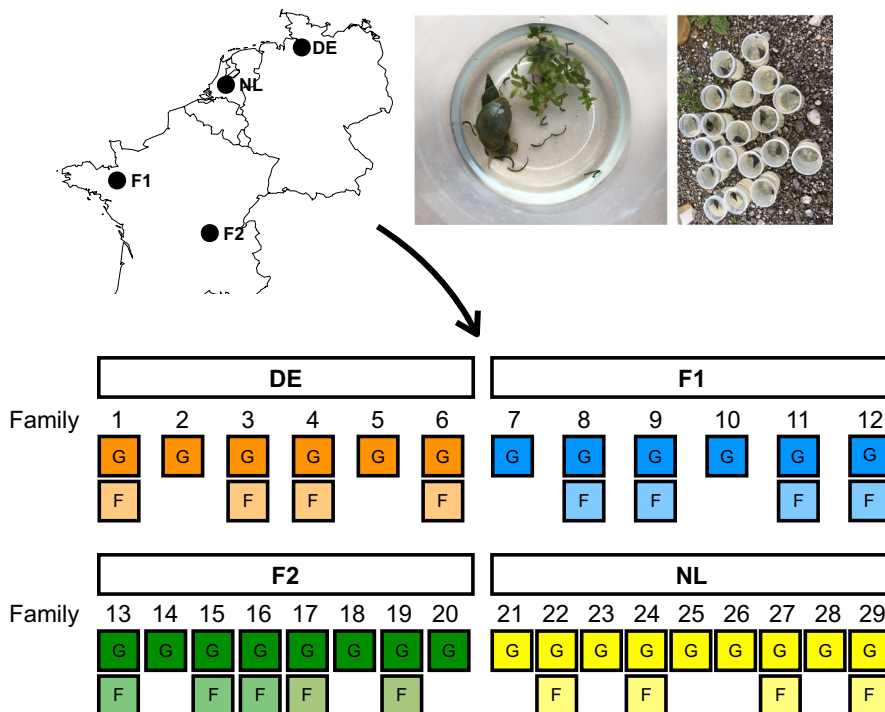
### 2.1 | Field collection

We collected *L. stagnalis* from four locations: Utrecht in the Netherlands (NL), Rennes and Lyon in France (F1 and F2, respectively)

and Hamburg in Germany (DE), from July to August 2017 (Figure 1, Table S1). Upon collection, we kept each snail in a small container to avoid copulation between collected individuals. Then, we transported the snails to the breeding facility at Bielefeld University to rear the next generation. To do so, we collected an egg mass from each snail laid in the laboratory to establish iso-mother families for each population (number of families: DE = 6, F1 = 6, F2 = 8, NL = 9; the egg collection dates after sampling ranged from 1 to 14 days, mostly within a week). Since this species shows multiply paternity (Nakadera et al., 2017), the offspring within such a family may not be full-sibs but are at least half-sibs. After on average 16 days of incubation (Figure S1), hatchlings came out of the egg mass. Two weeks after hatching, we randomly selected 20 individuals per family and transferred them into new larger containers and reared them for a further three weeks. This step was to standardize the density and social condition of juveniles across families and populations. During these rearing steps, we fed the snails with broadleaf lettuce and *Sepia* shell ad libitum and kept them in filtered water at 20°C under L:D = 12:12-hr conditions.

### 2.2 | Common garden design

We reared the field-derived snails under standardized laboratory conditions, in order to measure variation in SFP gene expression and their functions (Figure 1). We randomly assigned the five-week-old snails of each family to either a paired or a grouped treatment, as social conditions are potential factors to alter SFP gene expression (Nakadera, Giannakara, & Ramm, 2019; Ramm et al., 2015). To do so, we put together, respectively, either two or five snails from the same family into a container (150 × 90 × 80 mm) to rear



**FIGURE 1** Schematic overview of the experimental design. We collected field snails from four localities in 2017, and immediately after collection, we isolated the snails to avoid copulation between collected individuals. We established at least six iso-mother families for each population in the laboratory (see Methods). All the families were then used to quantify SFP gene expression, represented by squares labelled 'G'. A subset of at least four families from each population was then used for the SFP bioassay, represented by squares labelled 'F'. Note that, in each family, different sibs were used for the SFP gene expression and bioassays

them together until they became fully matured. We had two replicates for each family and group size combination. We provided a standardized amount of water (200 ml) and food per capita (broadleaf lettuce, ca. 19.6 cm<sup>2</sup> per day when they matured, Zonneveld & Kooijman, 1989). We also added *Sepia* shell into each container as a calcium source. We provided a new container with freshwater and checked the presence of egg masses in the container every week. We also measured the shell length of all the individuals every two weeks, using a Vernier calliper.

### 2.3 | SFP gene expression

When the field-derived snails had matured, we measured the level of SFP gene expression in the prostate gland, using quantitative reverse transcription PCR (qPCR). One week after all the replicates of a family laid egg masses, indicating their maturation, we randomly selected one snail from each container. Since the growth rate and timing of sexual maturation differed substantially across populations (Figures S2 and S3), we thus aimed to sample prostate glands in a similar developmental stage (= one week after their first egg laying), rather than at a fixed age. Also, we did not assign focal snails in advance, since all the snails in a container experienced the same social situation until the point of sampling. To randomly select a snail from each container, we detached the snails from the surface and gently swirled the water to blindly pick one individual. Then, we injected ca. 2 ml of 50 mM MgCl<sub>2</sub> into the foot for anesthetization. After removing the shell, we carefully dissected out the prostate gland. Given that the prostate gland consists of different cell types distributed along its anteroposterior axis (Koene et al., 2010), we cut it from top to bottom using a razor blade and stored half of the total prostate gland in 500 µl of RNAlater® (Ambion™) at -20°C until further processing.

For running qPCR, we followed the protocol published in Nakadera et al. (2019). In brief, we extracted total RNA from prostate glands, using TRI reagent (Sigma) and RQ1 DNase (Promega). Next, we synthesized cDNA from RNA extracts using the GoScript™ Reverse Transcription System (Promega). Then, we ran qPCRs to measure the expression of all six SFP genes known to date in *L. stagnalis* (*LyAcp5*, *LyAcp7a*, *LyAcp7b*, *LyAcp8a*, *LyAcp8b* and *LyAcp10*; Koene et al., 2010; Nakadera et al., 2019; Swart et al., 2019), using a CFX Connect Real-Time PCR Detection System (Bio-Rad). In a 96-well plate, we measured the gene expression of all six SFP genes, plus three housekeeping genes as reference [histone 2a (*Lhis*), ubiquitin-conjugating enzyme E2 (*Lubi*) and 14-3-3 protein zeta (*Lyh*); Davison et al., 2016]. We had three technical replicates for each gene on the same plate. In addition to negative controls, we prepared the plate controls to standardize the difference between plates using amplification efficiency (cDNA from a single individual, three dilution factors, two technical replicates, *LyAcp10*). The resulting raw cycle threshold (Ct) values from the software CFX Connect (ver. 3, Bio-Rad) were then used to calculate the relative expression level of SFP genes (see 'Statistical analysis' below).

### 2.4 | SFP bioassay

We next conducted a bioassay to characterize variation in SFP functions among field populations. In brief, we intravaginally injected seminal fluids of selected families into standardized focal snails, and then assessed the reproductive performance of focals, in terms of both egg production and sperm transfer in subsequent matings, as reported in previous studies (Koene et al., 2010; Nakadera et al., 2014; Swart et al., 2020).

For the SFP bioassay, due to logistic limitations we blindly selected approximately half of the families from each population (Figure 1, *N* = 18 families in total, including the laboratory strain). In order to obtain the material for this bioassay, we set aside 20 individuals of each family two weeks after hatching. We kept these snails in a large container and provided broadleaf lettuce and *Sepia* shell ad libitum. When these snails were fully matured, we transported them to the breeding facility of Vrije University Amsterdam, the Netherlands. We kept the snails in a perforated container placed in a large flow-through tank with low-copper water at 20 ± 1°C. The light-dark cycle is L:D = 12:12 hr. Again due to logistic limitations, we divided the whole experiment described below into seven runs. That is, we examined three randomly selected families and one control group in each run, and repeated this seven times. We conducted all of these bioassays in a single 3.5-week block. Note that, we chose to standardize the condition of focal snails from the laboratory strain and other unperceived factors (e.g. transportation) by carrying out this assay in a single time block. It, in turn, hindered us to use age-synchronized field-derived snails (range: 31 days in egg-laying date). That is because, for example, field-caught snails took varying amounts of time to acclimate and lay eggs in the laboratory.

We artificially injected the prostate gland extract of selected field-derived families into the standardized individuals from the laboratory strain (hereafter called focal snails). We followed the protocol of artificial injection published elsewhere (van Iersel, Swart, Nakadera, van Straalen, & Koene, 2014; Koene et al., 2009; Nakadera et al., 2014). Specifically for this experiment, we isolated the focal snails by keeping them in perforated containers for seven days, to standardize their mating history and increase their male mating motivation (Van Duivenboden & Ter Maat, 1985). After letting the field-derived snails acclimate to their new environment for at least one week, we isolated these snails for one week, in order to let them replenish the contents of their prostate glands (De Boer, Jansen, Koene, & Ter Maat, 1997; Van Duivenboden & Ter Maat, 1985). Next, we dissected prostate glands out from representatives of each target family. Then, we pooled and suspended the contents of prostate glands using *Lymnaea* saline solution, according to the estimated expenditure of an ejaculate in this species (equivalent to one third of the prostate gland secretion, Koene et al., 2009; Koene et al., 2010). Using 1-ml syringes and thin tubes (Silastic® Laboratory Tubing), we intravaginally injected prostate gland extract of field-derived snails into focal snails. In controls, we injected the same amount of saline solution into focals, and we aimed to have 10 replicates for each family.

The next day, we observed the focal snails' copulations and measured their sperm transfer. We placed a focal snail in a container with 400 ml of water and a standardized mating partner. The mating partners were from the laboratory strain, and we attached a bee tag on the shell of a partner for identification during the observation. Since focal snails had been sexually isolated for one week, they were more likely to act in the male role, compared with their partners that had been isolated for four days (De Boer et al., 1997; Van Duivenboden & Ter Maat, 1985). We carried out mating observations for six hours (9:00–15:00); we recorded the mating behaviour of 40 pairs every 15 min (Nakadera et al., 2015). When the partners started showing male behaviour (extending preputium towards female gonopore of a focal), we gently pulled the snails apart to avoid a focal snail receiving ejaculate from the partner. Due to logistical constraints, we aimed to measure sperm transfer of the first five focal snails that copulated in each family, following the established protocol (Loose & Koene, 2008; Nakadera et al., 2014). For those trials, immediately after a focal snail finished inseminating, we dissected out the vagina of partners, which is swollen with received ejaculate. Then, we placed the ejaculate containing duct into a tube with 200  $\mu$ l of saline solution and tore the duct using fine forceps to release sperm. After vigorous vortexing and transferring the duct into new solutions, we obtained 600  $\mu$ l of suspended sperm solution. Based on the counts from 5  $\mu$ l subsamples taken four times per replicate on an Improved Neubauer counting chamber, we estimated the number of sperm in the original solution. To quantify egg production, we digitally scanned egg masses of focal snails deposited two and four days after artificial injection (Van Iersel et al., 2014; Swart et al., 2020). Briefly, we scanned the egg masses using a flatbed scanner (CanoScan LiDE 700F Scanner), and based on these images, we manually counted the number of eggs and measured egg sizes using ImageJ. To estimate egg size, we randomly selected and measured the area of five eggs in an egg mass and calculated the average area as the proxy of egg size.

In total, 233 focal snails received prostate gland extracts via artificial injection, but we removed six samples from the following analyses due to handling errors during mating observation; 11 samples due to the shortage of partners (i.e. they did not encounter a mate); and 10 samples due to background mortality during the monitoring period of four days. From the remaining 206 samples, we observed that 151 focal snails showed courtship behaviour and 122 inseminations (overall mating rate: 59.0%).

## 2.5 | Statistical analysis

Life-history traits under a common garden design. We measured body size and the timing of maturation of field-derived snails, to test whether their life-history traits differ between populations. We compared the week when the snail started laying egg masses, using Kruskal–Wallis test, since it is a discrete variable. We used a nested ANOVA (Population and Family as fixed factor and Family is nested by Population) to test whether body size at the prostate gland sampling differed across populations. As expected, sampled populations

indeed differed in these life-history traits (body size at maturation:  $N = 419$ , nested ANOVA, Population:  $F_{3,384} = 67.31$ ,  $p < .001$ , Population/Family:  $F_{31,381} = 1.72$ ,  $p = .011$ , the timing of maturation: Kruskal–Wallis test,  $\chi^2_3 = 78.88$ ,  $p < .001$ : Figures S2–S3). These differences would support that these populations are genetically distinct, though we did not directly examine this. These life-history differences also justified our approach of sampling seminal fluid according to maturation stage rather than at a fixed age.

SFP gene expression. We ran qPCR for 92 individuals to partition variation in the relative expression of SFP genes between populations, families and treatments. We excluded the paired snails with extreme size difference ( $>7$  mm in shell length). Since it would be physically difficult for larger snails to inseminate the smaller partner, the mating frequency of these is expected to differ from pairs with similar body sizes. We included the replicates of the grouped treatment with four snails, instead of five, for the analyses as grouped treatment ( $N = 7$  of 92), since they were still under actual sperm competition, even if one snail had died. As a quality control of qPCR runs, we screened and removed the technical replicates that showed more than 1.0 difference in Ct value from the average of three replicates. In those cases, we re-calculated the average using the remaining two ( $N = 153$  of 2,484 runs). In a few cases, where all three replicates differed substantially from one another, all three replicates for the particular gene were excluded ( $N$  replicates with missing data = 22 of 828, 7 replicates of SFP genes, 15 replicates of reference genes). After we confirmed that the expression level of housekeeping genes could be used as reference by running nested ANOVAs (Treatment, Population and Family as fixed factor, Family is nested by Population, Figure S4), we used the average Ct value of three reference genes to calculate relative expression of SFP genes, using the formula  $2^{Ct(\text{Reference}) - Ct(\text{Target})}$  (Livak & Schmittgen, 2001). We then log-transformed the relative expression of SFP genes. To test whether SFP gene expression differs across populations and treatments, we used nested ANOVAs for each gene (Treatment, Population and Family as fixed factor, Family nested by Population). Moreover, we computed marginal and conditional  $R^2$  to evaluate the goodness of fit of our model (Nakagawa, Johnson, & Schielzeth, 2017; Nakagawa & Schielzeth, 2013). To do so, we fitted linear mixed models using Population as a fixed factor and Family as a random factor. In order to further visualize the data set of SFP gene expression, we ran a principal component analysis (PCA).

SFP bioassay. We focused on measuring the egg production (number of eggs, egg size) and sperm transfer of standardized focal snails that had each been artificially injected with prostate gland extracts from one of the field-derived families. In order to standardize the difference between experimental runs ( $N = 7$ ), we used the average value of a given trait of control snails as reference of each run. That is, we calculated the relative values of egg production and sperm transfer by subtracting the average of these traits in controls, which had saline solution injected ( $N$  without controls = 112 for egg production). To compare egg production, we had 219 snails that survived throughout our five-day monitoring period, and 155 of 219 snails laid at least one egg mass (1–3 egg masses laid, overall egg-laying rate 69.4%). We used



their total number of eggs laid and their egg sizes. During the mating observation, we counted the number of sperm transferred in 76 of 122 focal snails. To compare these three reproductive traits across populations and families, we used nested ANOVAs (Treatment, Population and Family as fixed factor, Family nested by Population). We ran all the statistical analyses in R (ver. 3.3.3, R Core Team 2018).

### 3 | RESULTS

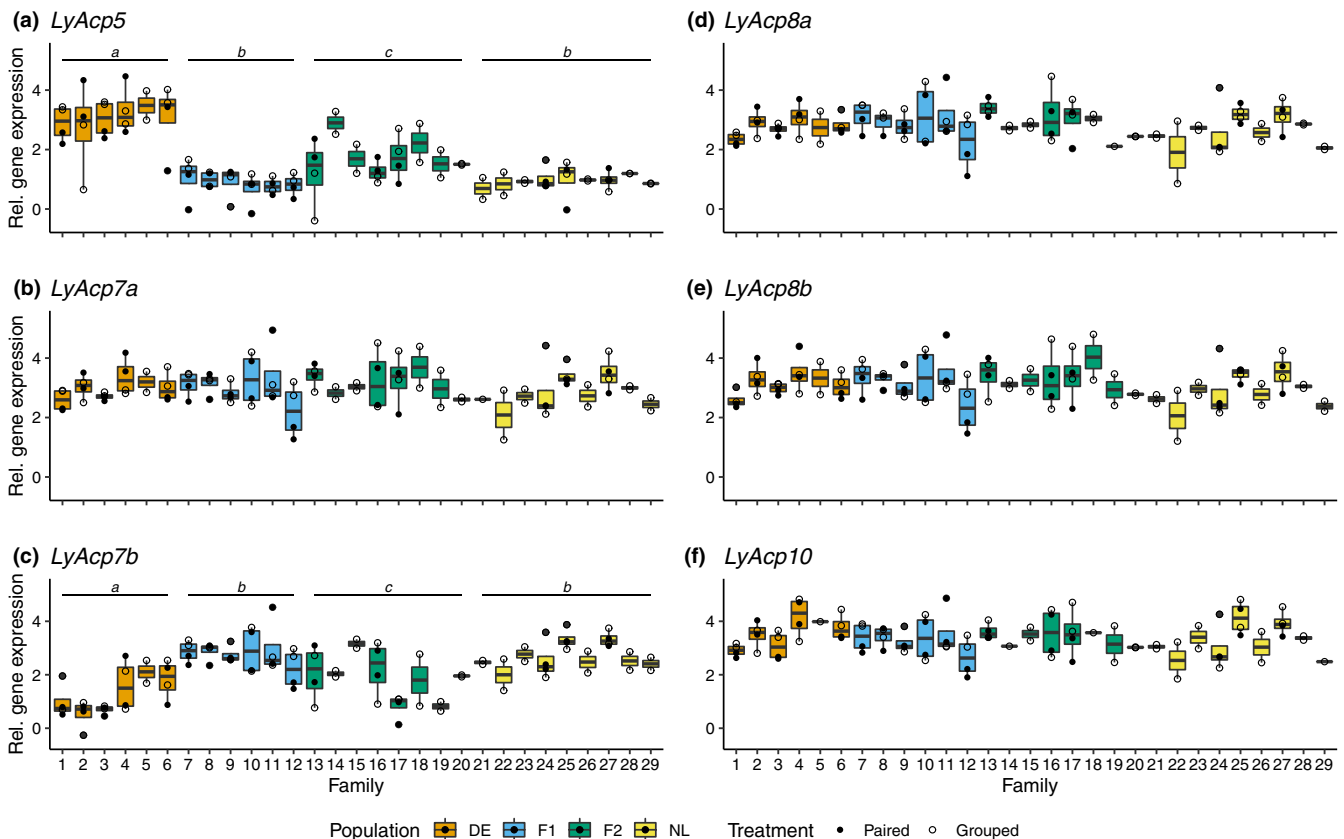
#### 3.1 | SFP gene expression differs among populations

Two of the six SFP genes varied in their expression by population. Specifically, the expression of *LyAcp5* was highest in DE and was higher in F2 than F1 and NL (Figure 2a, Table 1). In contrast, the expression of *LyAcp7b* was lowest in DE and was lower in F2 than F1 and NL (Figure 2c, Table 1). Since adding the factor of treatment did not change the outcome (i.e. paired and grouped snails did not differ in SFP gene expression, Table S2), we did not include the effect of treatment in subsequent analyses. The calculation of marginal and conditional  $R^2$  values suggested that, when the expression differs between populations, most variance is explained by population and much less so by

family (Table 1). Nevertheless, in the case of *LyAcp7b*, we detected a significant difference among families (Table 1). However, even here the marginal  $R^2$  showed that Population explained 39.3% of variance, and the combined effect of Population and Family was only 55.1% (Figure 2, Table 1). In order to further confirm the among-population divergence in SFP expression, we calculated principal components. PC1 explained 67.0% of overall variance, and PC2, 23.3% (Table 2). Based on the loadings for the different genes, PC1 appeared to represent overall seminal fluid expression level, except the expression of *LyAcp5*, a pattern which is consistent from previous experiments (Nakadera et al., 2019). In turn, PC2 captured well the among-population variation observed in the initial analyses, where we examined the expression variation of each SFP gene separately (Figure S5a). That is, the loadings for PC2 indicated that this axis is due to the differential expression of *LyAcp5* and *LyAcp7b* (Figure S5b). Overall, we detected significant variation in SFP gene expression between populations, but not so distinctively across families.

#### 3.2 | SFP gene expression changes are accompanied by functional divergence

When standardized focal snails received seminal fluid from the different field populations, they showed differential responses in egg



**FIGURE 2** Individual-, family- and population-level variation in SFP gene expression in *L. stagnalis*. Each data point represents a single replicate (individual) from a given family within a given population in one of the two treatments (paired—filled circles; grouped—open circles). Relative gene expression stands for  $\text{Log}_2(\Delta C_t)$ , and  $\Delta C_t$  is the difference in expression between reference genes and a target gene (see Methods). The characters above the plot of *LyAcp5* and *LyAcp7b* indicate the outcome of post hoc tests using Tukey's multiple comparison tests (populations labelled with different letters differ significantly from one another)

**TABLE 1** Variation of SFP gene expression and their functions between populations

Trait (N)	Nested ANOVA	Num. <i>df</i>	Denom. <i>df</i>	<i>F</i>	<i>p</i>	$R^2_m$	$R^2_c$
SFP gene expression							
<i>LyAcp5</i> (92)	Population	3	63	45.11	<b>&lt;.001</b>	0.630	0.630
	Population/Family	25	63	0.55	.948		
<i>LyAcp7a</i> (91)	Population	3	62	0.61	.609	0.020	0.053
	Population/Family	25	62	1.02	.454		
<i>LyAcp7b</i> (92)	Population	3	63	28.84	<b>&lt;.001</b>	0.393	0.551
	Population/Family	25	63	2.10	<b>.009</b>		
<i>LyAcp8a</i> (90)	Population	3	61	0.91	.442	0.029	0.056
	Population/Family	25	61	1.04	.438		
<i>LyAcp8b</i> (92)	Population	3	63	1.06	.371	0.035	0.085
	Population/Family	25	63	1.13	.340		
<i>LyAcp10</i> (88)	Population	3	59	0.58	.629	0.020	0.159
	Population/Family	25	59	1.36	.164		
SFP bioassay							
Egg number (112)	Population	4	94	6.32	<b>&lt;.001</b>	0.181	0.197
	Population/Family	13	94	1.11	.358		
Egg size (112)	Population	4	94	4.62	<b>.002</b>	0.114	0.231
	Population/Family	13	94	1.87	<b>.043</b>		
Sperm transfer (76)	Population	4	58	2.64	<b>.043</b>	0.087	0.406
	Population/Family	13	58	3.22	<b>.001</b>		

Note: We ran nested ANOVAs to test whether SFP gene expression and function differ between populations. In order to calculate marginal and conditional  $R^2$  ( $R^2_m$ ,  $R^2_c$ ), we fitted linear mixed models, using Population as a fixed factor, and Family as a random factor. *p* values shown in bold are less than .05.

	PC1	PC2	PC3	PC4	PC5	PC6
SD	2.005	1.182	0.606	0.375	0.204	0.186
Prop. Var.	0.670	0.233	0.061	0.023	0.007	0.006
Cum. Prop.	0.670	0.903	0.964	0.987	0.994	1.000
<i>LyAcp5</i>	0.080	-0.775	0.597	0.175	-0.074	-0.022
<i>LyAcp7a</i>	0.489	-0.022	-0.132	0.191	0.371	-0.754
<i>LyAcp7b</i>	0.258	0.616	0.738	0.080	-0.038	0.023
<i>LyAcp8a</i>	0.484	-0.001	-0.240	0.261	-0.800	0.028
<i>LyAcp8b</i>	0.486	-0.058	-0.151	0.312	0.465	0.652
<i>LyAcp10</i>	0.465	-0.124	0.033	-0.873	-0.010	0.074

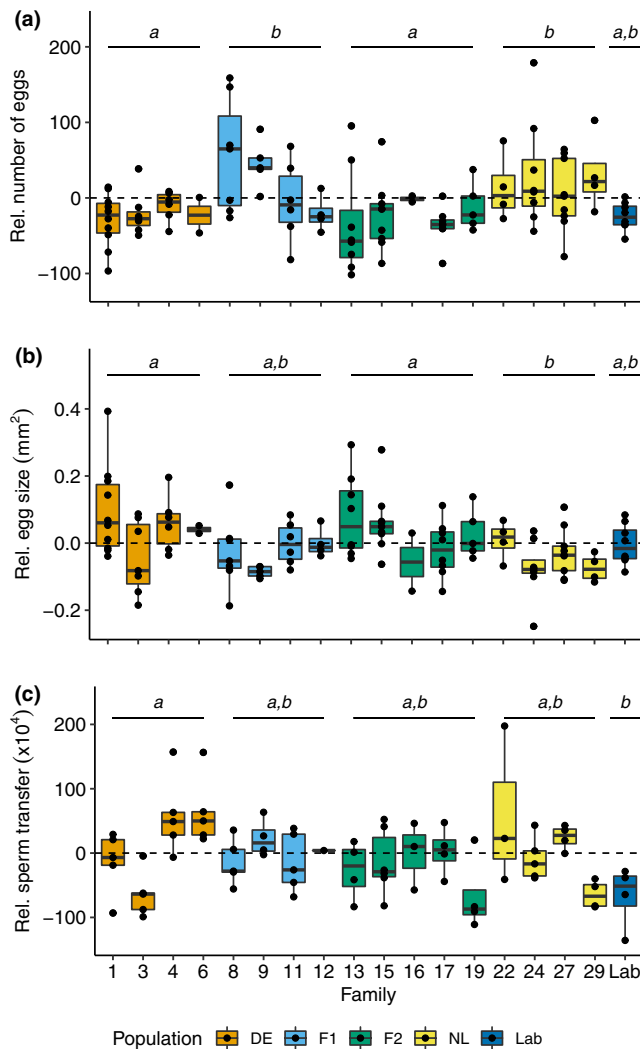
Note: The top three rows show the explained variance of each PC, and the rest shows the loadings of each SFP gene (*N* = 85). Visual presentation of PC1 and PC2 is in Figure S5.

**TABLE 2** Principal components of variation in SFP gene expression

production and sperm transfer (Figure 3). As reported previously, the snails injected with seminal fluid from the laboratory strain reduced egg production (Figure 3a, Koene et al., 2009, 2010). Interestingly, the focal snails injected with seminal fluid from DE and F2 produced significantly fewer eggs than the snails injected with seminal fluid from the other two populations (Figure 3a; Table 1). Furthermore, the snails injected with seminal fluid from DE and F2 laid smaller eggs (Table 1, Figure 3b). In this assay, we did not detect an increase in egg size of the focal snails injected with seminal fluid from the laboratory strain, although this

was expected based on indirect evidence from a previous study (Figure 3b, Swart et al., 2020). The  $R^2$  value indicated that the fixed factor Population explained most variance in egg number, and also explained an approximately equal amount of variance in egg size, as did the random factor, Family (Table 1).

For sperm transfer, the response to receiving SFPs was somewhat different. Although receiving SFPs from the laboratory strain indeed reduced the number of sperm transferred as previously reported (Figure 3c, Nakadera et al., 2014), seminal fluid from other populations did not induce such a response. In fact, seminal



**FIGURE 3** Individual-, family- and population-level variation in SFP functions in *L. stagnalis*. Each point indicates one standardized focal snail which had been injected with seminal fluid from a given family within a given population. For each trait, we calculated the average of control snails, which had been injected with saline solution. Based on this average (shown as dashed line), we calculated the relative value of each individual (see *Methods*). The characters above each population indicate the outcome of post hoc tests using Tukey's multiple comparison tests (populations labelled with different letters differ significantly from one another). Abb.: Rel. = relative. The colour codes for populations are the same as in Figure 2

fluid from DE actually increased the number of sperm transferred (Table 1, Figure 3c). Also,  $R^2$  indicated that the family structure accounted for most of the explained variance in sperm transfer, contrasting with the results for SFP gene expression and egg production (Table 1).

## 4 | DISCUSSION

We found clear evidence that SFP gene expression differs among natural populations of *L. stagnalis* and demonstrate using an artificial

injection that this likely has functional consequences for egg production and subsequent sperm transfer by SFP recipients. Specifically, the expression of *LyAcp5* and *LyAcp7b* was significantly different between the DE and F2 compared with the F1 and NL populations. Snails receiving seminal fluid from the DE and F2 populations produced fewer but larger eggs, and the snails receiving seminal fluid from the DE population transferred more sperm to subsequent mating partners.

Since the combination of a common garden experiment followed by artificial injection of standardized mating partners excludes various confounding factors, the detected SFP divergence in gene expression and functions implies that it is the variation in SFP expression that we have now documented that likely causes the variation in egg production and sperm transfer of mating partners. Previous studies showed that several factors alter SFP gene expression (e.g. developmental stage: Simmons et al. 2014, social condition: Hopkins et al., 2019, ecological conditions: Wigby & Chapman, 2005). Adopting a common garden design let us standardize these factors in SFP gene expression of field-derived snails. Also, we did not let field-derived snails copulate with laboratory snails, but artificially injected their seminal fluid. So, we can see the effects of injected seminal fluid on post-copulatory responses, without interferences of mating preference or mate choice of laboratory snails. However, one important proviso in this study is that we measured variation in only a subset of SFP genes. Thus, among-population variation of SFP expression might actually be greater than currently documented. This further implies that the functional effects we have observed are not necessarily mediated by these specific SFP genes (*LyAcp5*, *LyAcp7b*) that were observed to differ in expression. This discrepancy between SFP gene expression and functions applies particularly to sperm transfer, since the pattern with which this differed among populations did not obviously correspond to the pattern of gene expression (Nakadera et al., 2014). For egg production, the close correspondence between gene expression and functional responses would speak more in favour of the direct involvement of *LyAcp5* and/or *LyAcp7b*, but again we emphasize that this is a correlational pattern. Nevertheless, it is now clear from our study both that differential SFP composition is likely among populations and that this drives functional variation.

Our study also illustrates how insights from the laboratory are sometimes consistent in the field, and sometimes not. Firstly, we did not detect significant differences in SFP gene expression between paired and grouped snails, as recently observed also in the laboratory strain of *L. stagnalis* (Nakadera et al., 2019). This apparently confirms that individuals of this species do not change SFP production when they are exposed to ongoing sperm competition. On the other hand, SFP functions in *L. stagnalis* revealed in the laboratory are somewhat inconsistent with the SFP-mediated effects from field populations. For example, receiving *LyAcp5* has been reported to reduce the number of sperm transferred in a subsequent mating of recipient snails (Nakadera et al., 2014). Although we detected the differential expression of *LyAcp5* across populations, we did not observe differential reduction in sperm transfer in standardized snails.



In fact, snails from the DE population showed the highest expression of *LyAcp5* (Figure 2a), but their seminal fluid rather increased the number of sperm transferred subsequently by SFP recipients (Figure 3c). Given the low sample size at the population and family level of this study, we could not confidently conclude that the snails up-regulating *LyAcp5* increase the sperm transfer of mating partners in future. However, this seemingly contradictory outcome between SFP gene expression and its function might have various explanations. First, it is likely that SFP gene expression is not strictly equal to SFP production or its functional consequence, or there is high differentiation of SFPs themselves or their receptors between field and laboratory populations. It could also be that an unknown trait co-varied with the expression of *LyAcp5* and has an overriding effect on sperm transfer (Fay and Wittkopp 2008). Finally, it cannot be ruled out that different doses of *LyAcp5* affect sperm transfer of the laboratory strain snails very differently. All these scenarios are plausible, given the complex network of SFPs (Ayroles, Laflamme, Stone, Wolfner, & Mackay, 2011; Patlar et al., 2019) and intricate interactions between mating partners and rivals revealed in other SFP research (Clark, Begun, & Prout, 1999; Zhang et al., 2013). Nonetheless, our study emphasizes that the investigation of field populations is vital to validate and generalize the knowledge so far obtained largely from laboratory studies and often using only a small number of genotypes (Gasch, Payseur, & Pool, 2016).

A remaining question concerns whether the variation in SFP gene expression we have documented is also accompanied by sequence divergence in these SFPs themselves. We estimated the gene expression levels of SFP genes in prostate glands, but to date, we have not examined the sequences of SFP genes for polymorphism and divergence in this species. Since sequence polymorphism and divergence have been documented in several studies (e.g. *D. melanogaster*: Tsauro & Wu, 1997; Aguadé, 1998; *C. elegans*: Palopoli et al., 2008), investigating sequence variation of SFPs in *L. stagnalis* is an obvious priority, and would allow us to test whether such polymorphism is also detectable in a simultaneously hermaphroditic species, and gain a fuller appreciation of all aspects of seminal fluid divergence and their relative importance. Based on our current findings, we can now proceed to a more comprehensive investigation of SFP variation in the field. For instance, it would be highly pertinent to comprehensively identify all the SFPs of *L. stagnalis* using -omics methods since model species of SFP research usually express from 50 to several hundreds of SFPs (Siro et al., 2015), while to date we have full or partial sequence information on only eight in *L. stagnalis* (Koene et al., 2010). Moreover, future studies would certainly benefit from a wider and deeper sampling of populations, allowing us to examine the association between SFP expression and its functional consequences with greater statistical power. Also, it will be essential to validate the functions of SFPs, particularly *LyAcp5* and *LyAcp7b*, using molecular and transgenic approaches. Nevertheless, the current study provides vital groundwork to investigate the full spectrum of SFP variation in natural populations of *L. stagnalis*.

In sum, we found marked divergence of SFP gene expression among field populations in *L. stagnalis*. Our bioassay further revealed

functional divergence of seminal fluid between populations. Although further study is required, these two types of divergence hint that SFPs have diverged under selection, with gene expression changes driving functional divergence. Our study thus confirms *L. stagnalis* as a tractable model for quantifying intraspecific variation in SFPs and performing functional assays, helping to broaden our general understanding of SFP evolution and its driving force across animals and sexual systems.

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## AUTHOR CONTRIBUTIONS

YN, JMK and SAR conceived and designed the study. YN and MAC collected field snails. YN, ATS and LD conducted experiments and collected the data. YN analysed the data. YN and SAR wrote the manuscript with input from MAC, LA and JMK.

## PEER REVIEW

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## DATA AVAILABILITY STATEMENT

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## ORCID

Yumi Nakadera  <https://orcid.org/0000-0002-6342-4586>

Joris M. Koene  <https://orcid.org/0000-0001-8188-3439>

Steven A. Ramm  <https://orcid.org/0000-0001-7786-7364>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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