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# Temporal expression profile of an accessory-gland protein that is transferred via the seminal fluid of the simultaneous hermaphrodite *Lymnaea stagnalis*

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## ABSTRACT

Male accessory-gland proteins are known to affect female physiology in multiple ways, maximizing a male's reproductive success—often at a cost to the female. Due to this inherent sexual conflict, accessory gland proteins (ACPs) are generally studied in separate-sex organisms. While ACPs have also been identified in simultaneous hermaphrodites as an important part of post-copulatory sexual selection processes, their study has lagged behind that of ACPs in organisms with separate sexes. In the great pond snail, *Lymnaea stagnalis*, an ACP affecting egg laying, ovipostatin, is produced in the prostate gland. Based on the published partial *Ovipostatin* gene sequence, we now provide the complete mRNA and gene sequences, and confirm that gene expression is prostate gland-specific. More importantly we observed a significant increase in *Ovipostatin* expression in sperm donors after ejaculation. *Ovipostatin* gene expression did not differ between donors giving their ejaculate first (primary donors) and those donating an ejaculate after having been inseminated (secondary donors). These observations support a role for ovipostatin in reproduction and highlight the importance of standardizing the time point when measuring expression levels of ACPs.

## INTRODUCTION

In species that reproduce by internal fertilization, males transfer complex ejaculates into females, delivering sperm in fluid containing a cocktail of proteins (Birkhead, Hosken & Pitnick, 2008). Accessory-gland proteins (ACPs; also referred to as seminal fluid proteins, SFPs) are produced in male accessory glands and can affect both male and female fitness. Upon receipt, ACPs have been shown to affect female physiology, behaviour, immunity and life history (Perry, Sirot & Wigby, 2013). Males achieve these changes either by inducing oviposition (Hirata, 1981; Chapman *et al.*, 1995), decreasing female sexual receptivity after copulation (i.e. latency to copulate, Wolfner, 1997), or inhibiting other males from accessing female gametes (i.e. mating plug formation, Wigby & Chapman, 2005; Avila *et al.*, 2010), thereby facilitating sperm storage (Tram and Wolfner, 1999; Wigby *et al.*, 2009; Zizzari, Smolders & Koene, 2014). Particularly in species where females mate with multiple partners, ACPs can play an important role in post-copulatory sexual selection, by affecting sperm storage, sperm digestion and sperm displacement, thereby reducing competition between rival males and increasing male reproductive success (Córdoba-Aguilar, Uhía & Cordero Rivera, 2003; Ramm, Parker & Stockley, 2005; Wigby *et al.*, 2009; Bussiere *et al.*, 2010).

The role of ACPs is well established in internally fertilizing gonochoristic species such as rats (Ramm *et al.*, 2005), bees (Baer *et al.*, 2009) and fruit flies (Ravi Ram & Wolfner, 2007; Kelleher *et al.*, 2009; Avila *et al.*, 2010). In the last of these, ACPs have been shown to be strategically allocated to females depending on the level of sperm competition (Wigby *et al.*, 2009). In organisms where both sexes occur within a single individual, such as in simultaneous hermaphrodites (hereafter referred to as hermaphrodites), the study of ACPs lags some way behind. Some studies have analysed the effect of ACPs transferred with love darts in land snails (e.g. Chase & Blanchard, 2006; Stewart *et al.*, 2016). It has been argued that ACPs are a key evolutionary driving force for hermaphrodites and form an important part of postcopulatory sexual-selection processes (Nakadera & Koene, 2013; Ramm, 2017).

In the hermaphroditic freshwater pulmonate, the great pond snail *Lymnaea stagnalis* (Linnaeus, 1758), the presence of ACPs was initially inferred from a decrease in oviposition in multiply mated individuals compared with isolated individuals (van Duivenboden, Pieneman & Ter Maat, 1985). Later, an analysis of the seminal fluid of this species was conducted by identifying some of the secretory products produced by its male accessory gland, the prostate gland (Koene *et al.*, 2010). Eight of these ACPs were individually tested to determine effects on oviposition, growth, lettuce consumption and hatching of the oviposited eggs. A protein was found to be responsible for decreasing oviposition and was named ovipostatin (or *Lymnaea* accessory gland protein 10 or LyAcp10;

Koene *et al.*, 2010; Nakadera *et al.*, 2014; van Iersel *et al.*, 2014). Using the bioactive HPLC fraction, a partial amino-acid sequence was obtained for ovipostatin (Koene *et al.*, 2010). This protein has so far only been found in the genome of one other species (the freshwater snail *Biomphalaria glabrata*, Adema *et al.*, 2017) and does not resemble other known proteins (Koene *et al.*, 2010). Other studies have since established that the decrease in egg-laying found in copulating individuals is accompanied by an increase in investment per egg (Hoffer *et al.*, 2012) and that this may be responsible for better egg development and hatching (Hoffer *et al.*, 2017). More recently, it has been discovered that two of the novel ACPs (LyAcp5 and LyAcp8b) possibly target the male function of a recipient: Nakadera *et al.* (2014) reported that, after receiving ACPs during insemination, the recipient transferred less than half the sperm in the male role in a subsequent mating than an uninseminated counterpart, with a significant decrease in paternity gain. ACPs in simultaneous hermaphrodites may therefore be targeting both the male and female function of a female receiving partner.

In this study, we first provide a complete sequence of the *Ovipostatin* gene. We subsequently determine the temporal expression profile of *Ovipostatin* in multiple tissues of the sperm donor after the prostate has been depleted (i.e. several hours after copulation, De Boer *et al.*, 1997). This was done to find out whether the expression of *Ovipostatin* is prostate-specific and whether the production of this ACP changes over time. In addition, we test whether *Ovipostatin* expression is influenced by the donor's mating history by determining if recently inseminated (secondary) donors show reduced expression compared with uninseminated (primary) donors. If this were the case, and if ACP expression is a proxy for replenishment of spent seminal fluid (Wigby *et al.*, 2009), this would be in line with the reduction in sperm transfer observed by Nakadera *et al.* (2014).

## MATERIAL AND METHODS

### *Study organism and experimental conditions*

*Lymnaea stagnalis* is widely distributed across the Northern Hemisphere (e.g. Pfleger & Chatfield, 1983). It can be reared successfully under laboratory conditions and is easy to handle, making it a suitable model organism (e.g. Koene & Ter Maat, 2005). The Vrije Universiteit Amsterdam maintains a continuous laboratory culture of this species, consisting of breeding tanks of various age-synchronized cohorts, ranging from eggs through to 4-month old snails. All the breeding and experimental laminar-flow basins are maintained under similar environmental conditions: oxygenated low-copper water at a constant temperature of 20 °C (±1 °C) and a light:dark regime of 12 h:12 h. The cultures are alternately fed broadleaf lettuce or fish flakes (TetraPhyll, Tetra GmbH, Melle, Germany) three times per week (e.g. Koene & Ter Maat, 2005).

### *Molecular characterization of the Ovipostatin gene*

*Ovipostatin genomic DNA.* Genomic DNA was isolated from foot tissue of Vrije Universiteit *L. stagnalis* stock animals using a modified protocol of the SV DNA isolation kit (Promega®): the tissue was homogenized in Nuclei Lysis Solution and Proteinase K; the homogenate was incubated for 15 min at 65 °C prior to further lysis with Promega DNA Lysis Buffer; after centrifugation, the DNA was isolated from the supernatant using the spin columns of Promega®.

We performed PCR with TaKaRa LA taq® polymerase (TaKaRa Bio Inc., Kusatsu, Japan) using primers Ls_ovi_OPS-F and Ls_ovi_OPS-R (see Supplementary Material Table S1 and Fig. S1 for all primer sequences and their positions). The cycling programme was: 1 min at 94 °C followed by 2 cycles of 1 min at 94 °C, 1 min at 64 °C and 8 min at 68 °C; 2 cycles of 1 min at 94 °C, 1 min at 62 °C and 8 min at 68 °C; 32 cycles of 1 min 94 °C, 1 min 60 °C and 8 min 68 °C; a final extension of 10 min at 68 °C. Amplicons were purified, ligated into pGEM-T vector, transformed in *E. coli* XL-1 Blue and sequenced as described above.

The gDNA fragments obtained were aligned with an unpublished gene prediction extracted from the genomic data to which we have access as part of the *L. stagnalis* genome consortium (Lsta_scaffold5:2642153–2646668; genome publication in prep.) along with the partial sequence previously obtained from Edman sequencing (GenBank acc. no. GQ906707, Koene *et al.*, 2010). With two additional primers (Ls_629438F and Ls_49805R), a final gap was bridged and a consensus sequence was completed. The complete gDNA sequence was submitted to GenBank (acc. no. KT378132).

We translated the coding sequence using ExPASy Translate (Swiss Institute of Bioinformatics, SIB) and used SignalP v. 4.1 (Petersen *et al.*, 2011) to identify the signal peptide. Subsequently, we determined the theoretical isoelectric point (pI; i.e. the pH at which the protein's net charge is zero) and molecular weight (Mw) using ExPASy Compute pI/Mw (SIB) and checked for known protein domains using SMART (Letunic & Bork, 2017).

*Ovipostatin messenger RNA.* Prostate tissue samples of *L. stagnalis* were obtained from 2 to 3 month old, sexually mature adult snails from the Vrije Universiteit Amsterdam breeding stock. To remove the prostate, the snails were sacrificed by injecting 2 ml 50 mM $MgCl_2$ through the foot, directly into the haemocoel. The prostate was quickly dissected out, snap-frozen in liquid nitrogen and stored at −80 °C.

The *Ovipostatin* fragment was amplified by PCR using cDNA derived from six individuals based on the *Ovipostatin* sequence GQ906707 (Koene *et al.*, 2010). RNA was isolated from the prostate tissue using the Promega® SV RNA isolation kit (Promega Corporation, Madison, WI) with minor modifications. For reverse transcription, we used the M-MLV reverse-transcription protocol of Promega® in a PTC-100 thermal cycler (BioRad Laboratories). PCR was performed with primers Ls_ovi_OPS-F and Ls_ovi_OPS-R at an annealing temperature of 55 °C for 30 cycles, using the Go-taq DNA polymerase kit (Promega®). Amplification products were purified, ligated into pGEM-T vector (Promega®) and cloned into *E. coli* XL-1 Blue (Stratagene Corporation, La Jolla, CA). Positive clones were cultured and plasmids were isolated using SV miniprep DNA isolation kit (Promega®). For each sample of prostate tissue, we sequenced four positive clones on an ABI 3100, using the Big Dye sequencing chemistry of Applied Biosystems (Thermo Fisher Scientific, Waltham, MA). Consensus sequences were generated using Vector NTI® software (Invitrogen, Thermo Fisher Scientific, Waltham, MA).

The full length cDNA of the *Ovipostatin* gene was obtained with 3′RACE and 5′RACE. For 3′RACE we applied the protocol of the Gene Racer™ kit (Invitrogen, Thermo Fisher Scientific). After transcription, we used the primer Ls_ovi_OPS-F or a nested primer Ls_ovi_168F in combination with the antisense Gene Racer™ 3′RACE primer in PCR. For the 5′RACE, we used the SMART™ RACE cDNA amplification kit from Clontech® (Clontech Laboratories, Mountain View, CA). After transcription, we performed PCR with the primer Ls_ovi_OPS-R or a nested antisense primer Ls_ovi_278R in combination with primer mix UPM from the Clontech kit.

PCR cycling was the same for both 3′RACE and 5′RACE. PCR conditions were as follows: 2 min at 95 °C followed by 5 cycles of 30 s at 94 °C, 30 s at 64 °C and 1 min at 72 °C; 5 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C; a final extension phase at 72 °C for 7 min.

Fragments were purified, ligated in pGEM-T vector, transformed into *E. coli* XL-1 Blue and sequenced as described above. The *Ovipostatin* full length mRNA sequence has been submitted to GenBank (acc. no. KT378132.1).

*Quantitative PCR assays*. We performed RNA isolation and cDNA transcription from experimental tissues as described above. For quantitative PCR (qPCR), we developed a primer set based on Exon 4 from the *Ovipostatin* mRNA. The RNA input was normalized with two reference genes, *Elongation Factor 1-alpha* (*EF*; DQ278441.1) and *Beta-tubulin* (*β-tubulin*; X15542.1) (e.g. Carter *et al.*, 2015). All primer sets were developed using Primer Express v. 1.5 (Applied Biosystems) with the following parameters: annealing temperature 60 °C, GC content 45–55%, amplicon length 80–120 bp and amplicon melting temperature 78–85 °C (Supplementary Material Table S1, Fig. S1).

Plate results were collected in a single 'gene study' and mean normalized expression values (MNEs) were calculated using the Comparative CT ($\Delta\Delta C_T$) method (Pfaffl, 2001) with the Bio-Rad CFX Manager software (v. 3.1, BioRad Laboratories). Basic settings were manually adjusted, including primer efficiencies and baseline threshold (set at $10^3$ relative fluorescence units, RFU). In order to determine PCR efficiency, standard curves were obtained for the primer sets with six four-fold dilutions of a standard batch *L. stagnalis* cDNA in duplicate. Primer PCR efficiencies were then set to 96.4% for *EF*, 94.4% for *β-tubulin* and 113.3% for *Ovipostatin*. Real-time qPCR was performed on the cDNA samples to yield cycle threshold values ($C_t$) in duplicate for target genes and reference genes.

### *Temporal Ovipostatin expression profiles across multiple tissues*

*Ovipostatin* expression was compared over time in six different tissues, to determine where and when *Ovipostatin* is expressed. Because Ovipostatin is a known component of an ejaculate, donors were dissected at set time points after having copulated in the male role. Twenty nonvirgin 3-month-old *L. stagnalis* were obtained from the breeding tank and isolated for 8 days in perforated plastic containers (460 ml). During the experiment, each snail was fed one broadleaf lettuce disc/day (*c.* 19.6 $cm^2$), which is slightly below their optimum (Zonneveld & Kooijman, 1989). Eight days in isolation is sufficient to determine if the slightly protandrous snails are fully hermaphroditic, which they should be when 3 months old (indicated by egg-laying), and to increase male mating motivation (van Duivenboden, 1983; van Duivenboden & Ter Maat, 1985; Koene & Ter Maat, 2005; Koene, Loose & Wolters, 2008). Subsequently, the isolated snails (donors) were size-matched (on the basis of shell length, SL) to 20 partners (recipients) selected directly from the breeding tank; this was done to remove body-size bias in sex-role decisions (Nakadera *et al.*, 2014) and gave a total of 20 pairs. The donors were marked with coloured nail varnish (Fenwick & Amin, 1983), to distinguish them from the recipients. *Lymnaea stagnalis* copulates unilateral, with one partner assuming the male role (primary donor) while the other

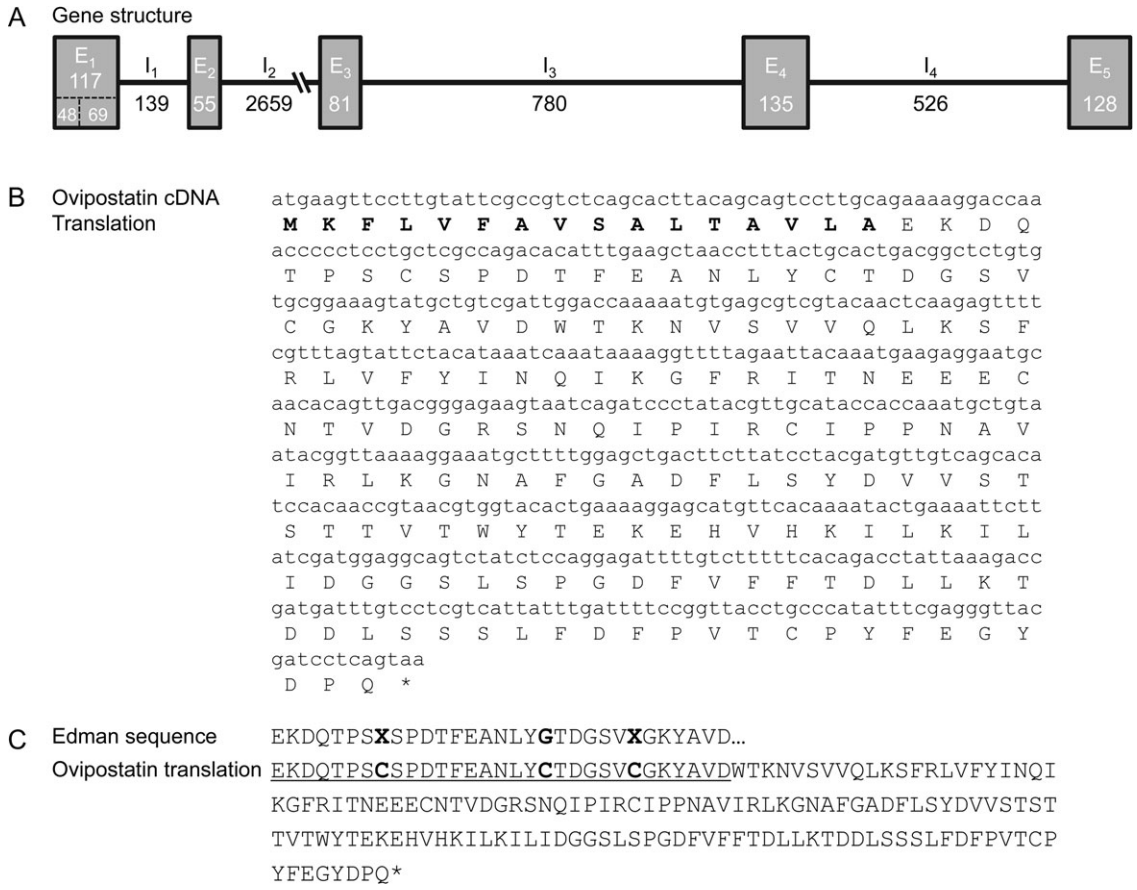


**Figure 1.** Gene, transcript and mature protein of *Ovipostatin* (LyACP10) in *Lymnaea stagnalis*. **A.** The gene consists of four introns ($I_{1-4}$) and five exons ($E_{1-5}$) of varying length (bp). Note the separation between the coding region for the signal peptide and the start of the mature protein (dotted lines) in the first exon. The second intron ($I_2$) is long, so has been abbreviated (dashed line) in the figure, but all the other parts are to scale. **B.** The cDNA sequence of the *Ovipostatin* gene accompanied by the translated precursor protein (i.e. including the signal peptide, shown in bold). Amino acids are centred under their respective codons and the asterisk indicates the stop codon. **C.** The original partial Edman sequence from Koene *et al.* (2010) aligned with the complete ovipostatin translation. The previously unidentified positions (X) and one variable position are indicated in bold.

assumes the female role (recipient). Thus, it is easy to identify the sexual role of copulating snails. During observation, the pairs were placed in plastic containers (460 ml) filled with water from the laminar-flow basins. All copulations were observed until the end (i.e. penis and preputium withdrawal). Following the end of copulation, donors were placed in isolation, while noncopulating individuals were removed from the experiment. The donors were then randomly allocated to a time point postcopulation (3, 24, 48 and 196 h) and dissected. After the donors were sacrificed for dissection, six tissues were snap-frozen in liquid nitrogen and stored at −80 °C. These tissues are classified as follows: two neutral tissues (from the buccal mass and foot, i.e. not involved in reproduction); two female tissues (oothecal and albumen gland) and two male tissues (preputium and prostate gland). We performed RNA isolation, cDNA transcription and qPCR as described above.

### *Expression profiles in prostate glands of primary and secondary donors*

One hundred and twenty nonvirgin 3-month-old *L. stagnalis* were obtained from the breeding tank and isolated for 8 days (see above). Subsequently, the snails were size-matched (based on SL), giving a total of 60 pairs, with both partners in each pair equally motivated to mate in the male role. The pairs were then allocated to a treatment, either a single mating, where we collected the primary donors, or a reciprocal mating, where we collected the secondary donors. Although mating is not obligately reciprocal, after sexual isolation the frequency of role switching is high (van Duivenboden & Ter Maat, 1985; De Boer *et al.* 1997), resulting in a second copulation (reciprocal mating by a secondary donor) (Koene & Ter Maat, 2005; Koene *et al.*, 2010). One of each pair was marked with coloured nail varnish (Fenwick & Amin, 1983), distinguishing the individuals for the purpose of observation. The pairs were placed in plastic containers (460 ml) filled with water from the laminar-flow basins. During 6 h of observation, we recorded three levels of interaction: (1) no contact; (2) mounting or probing (courtship behaviour) and (3) intromission (insertion of male copulatory organ into female gonopore), every 15 min or until the end of a single or reciprocal mating encounter. Of the 60 pairs of snails, two-thirds copulated, giving a total sample size of 41; all noncopulating pairs were removed from the experiment. The donors from these 41 pairs were allocated to a time point postcopulation (3, 18, 24, 48 and 196 h) and kept in isolation until the set time. Between two and five primary and secondary donors were dissected at each time point after copulation. The prostate glands were extracted, snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA isolation, cDNA synthesis and qPCR were performed as outlined above.

Data that were not normally distributed (Shapiro-Wilk test) were log-transformed. As multiple-significance tests were performed, *P* values were Bonferroni-adjusted by multiplying each nominal *P* value from the ANOVA test by the number of tests performed. All statistical analyses were performed using IMB SPSS v. 21 (IMB, Armonk, NY).

## RESULTS

### *Ovipostatin gDNA and cDNA*

The genomic consensus sequence (gDNA) for *Ovipostatin*, from the ATG start codon up to the TAA stop codon, has a total length of 4,515 bp (Fig. 1). The *Ovipostatin* gene is composed of five exons, respectively 117, 55, 81, 135 and 128 bp long, and four introns, respectively 139, 2,659, 780 and 526 bp long (Fig. 1A). All introns begin with GT and end with AG, and represent consensus sequences for splicing signals. The resulting full-size transcript (cDNA) sequence is 516 bp and encodes for a 183 amino acid (aa) long precursor protein with the first 16 aa forming the signal peptide (Fig. 1B). The mature 167 aa protein has a predicted theoretical pI of 4.74 and Mw of 18.78 kDa. No SMART domains were detected. A comparison between the translated *Ovipostatin* cDNA and the previously published partial amino acid sequence (Koene *et al.*, 2010) elucidated the unidentified positions (indicated as 'X' in Fig. 1C) and confirms those that were determined through Edman sequencing (except for position 19 in the mature protein, where G is replaced by C).

### *Temporal Ovipostatin expression profiles across multiple tissues*

To determine *Ovipostatin* expression in the prostate gland and other tissues, we analysed the relative normalized expression level in the time series of 3, 24, 48 and 192 h after copulation. We did this for three individuals/time frame for the two neutral tissues, two female organs and two male organs. A significant effect of time was found for *Ovipostatin* expression in the prostate gland ($F_{3,\ 9} = 7.539$, $P = 0.019$; Fig. 2). Post-hoc comparisons using a Bonferroni correction indicated a significant increase in mean transcription score between the expression level at 3 and 24 h ($P < 0.05$), while expression at the other two time points did not differ significantly from the 3 and 24 h treatments (Fig. 2). *Ovipostatin* mRNA activity was very low in the other tissues tested and no significant differences were found in expression over time in any of these tissues ($P > 0.05$; data not shown). The amplicon from the oothecal gland was largely absent.

### *Expression profiles in prostate glands of primary and secondary donors*

To determine if there is a difference in expression between primary and secondary donors, the expression of *Ovipostatin* in the prostate gland (normalized with respect to the reference genes) was investigated for both types of donors. Of the 41 pairs that copulated, 25 were primary donors and 16 secondary donors. The primary and secondary donors were split up into a time series of 3, 18, 24, 48, 96 and 192 h after donating an ejaculate during copulation, giving a minimum of two samples per time point (Supplementary Material Fig. S2). A two-way ANOVA was conducted to examine the effect of donor type and time on the expression profile of *Ovipostatin* in the prostate (log-transformed) and this revealed that the two donor types did not differ in their level of *Ovipostatin* expression ($F_{1,\ 41} = 2.175$, $P = 0.15$). There was a significant effect of time on the expression profile ($F_{5,\ 41} = 5.132$, $P = 0.002$), but no significant interaction was found between time and donor type ($F_{5,\ 41} = 1.70$, $P = 0.166$).

## DISCUSSION

In this study we extend and confirm the complete sequence of the *Ovipostatin* gene, showing that the full transcript of the mature protein LyACP10 consists of 167 aa, elucidate all previously unidentified aa positions and detect one variable position (this may be due to heterogeneity in the population; Koene *et al.*, 2010). In addition, we show that the expression of *Ovipostatin* increases after copulation in the male role and that it is tissue-specific, being expressed, as suggested by Koene *et al.* (2010), mainly in the prostate gland. Interestingly, we found no evidence for our expectation that expression would differ between the different donor types. In what follows we discuss these findings in greater detail and explore the implications of this work for future research.

The finding that there is a temporal change in expression of *Ovipostatin* after the prostate has been largely depleted (i.e. when an ejaculate has been donated, see De Boer *et al.*, 1997), strongly suggests that much of this ACP has been transferred to the partner and that its production is increased. In other words, transcription in this case seems to be a reasonable proxy for replenishment of spent seminal fluid. The timing of increased expression seems to

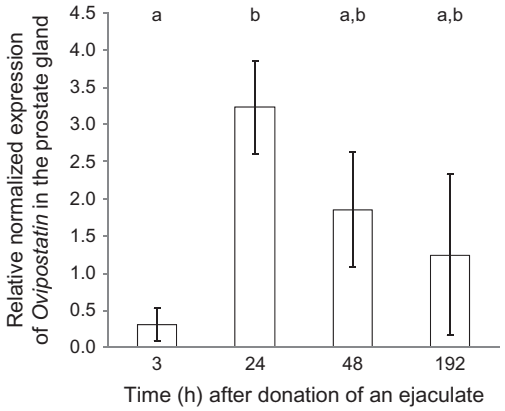


**Figure 2.** Change in expression over time of the target gene (*Ovipostatin*) in the prostate gland of sperm donors of *Lymnaea stagnalis*. Expression is shown relative to the internal reference genes (*Elongation factor 1α* and *β-tubulin*) and for the different time points (h) after the individual had copulated in the male role (i.e. had donated sperm from seminal vesicles and seminal fluid from prostate gland). Letters indicate statistical post-hoc significance at $P < 0.05$. Error bars indicate standard deviations.

correspond with what is known about the increase in the size of the prostate gland. After donation of an ejaculate the prostate is drastically reduced in size. Over the days following mating, the prostate gland increases in size, with the more enlarged states providing a permissive signal for male mating (van Duivenboden, 1983; van Duivenboden & Ter Maat, 1985; De Boer *et al.* 1997; Koene & Ter Maat, 2005; Koene *et al.*, 2008). In other words, the motivation for male mating increases with the duration of sexual isolation (i.e. prostate gland product is stored and not spent), reaching a plateau after 192 h (8 days). Nevertheless, individuals that have undergone such periods of isolation are known to be able to ejaculate again within 24 h (Koene & Ter Maat, 2007), which indicates that relatively rapid replenishment of the products of the prostate gland can occur and/or that smaller amounts of the products are transferred in subsequent copulations. Our results show that the highest levels of *Ovipostatin* mRNA were found within 24 h postcopulation, so this agrees with the physiological demands placed on this gland.

The comparison of *Ovipostatin* expression at various time points after copulation across different tissues indicates that transcription increased only in the prostate gland. This suggests that *Ovipostatin* has tissue-specific RNA expression and regulation. Tissue-specific expression of ACPs has also been found in a range on invertebrate taxa, including crickets (Simmons *et al.*, 2014), fruit flies (Wagstaff & Begun, 2005; Haerty *et al.*, 2007) and tiger beetles (Rodríguez-García, Machado & Galián, 2015). Such sex-specific proteins are generally thought to be under positive selection, exhibiting higher levels of polymorphism and changes in abundance with age, when compared with non-sex-related proteins. We also expected the level of expression to differ depending on the donor's male mating history (i.e. being a primary or secondary donor), similar changes in allocation of different ACPs having been reported in *Drosophila* (Sirot *et al.*, 2001). Nevertheless, we found no such difference between the different donor types, even though these are known to be affected by other ACPs that reduce the amount of sperm that they transfer (Nakadera *et al.*, 2014). This may indicate that the amount of ovipostatin transferred is not modulated depending on the donor's male-mating history. Evidence for this is provided by the experiments of Hoffer, Ellers, and Koene (2010), which showed that although more sperm was transferred to feminized recipients (equivalent to isolated recipients), prostate weight did not differ, suggesting that the amount of accessory-gland products transferred does not change. However, it should be noted that the Hoffer *et al.* (2010) study considered only the total weight of the prostate gland and not the amounts of individual ACPs. Our findings suggest that work on the latter would be an interesting line of inquiry for future research.

Given the expression patterns of *Ovipostatin* over time, and assuming that this reflects overall activity of the prostate gland, investment into the male function after a single ejaculation event can be hypothesized to change over time. In other words, a sexually isolated snail (represented in our study by treatment 192 h post-ejaculation) with a fully replenished prostate gland can be expected to invest minimal resources in that part of its male function. Except for a slight investment in maintenance and renewal of the ACP stores, the sexual resources that remain 192 h after ejaculation are allocated to the female function. However, after a copulation event in the male role, resources are necessary to replenish the prostate gland with accessory-gland products. Such a change in female investment, as a consequence of only donating ejaculates, has been previously reported, with a *c.* 50% reduction in egg laying in snails restricted to the male function (Hoffer *et al.*, 2010), indicating likely investment in both sperm and seminal fluid. Taken together, this supports the suggestion that allocation of resources to the male function is relatively high in this species and that changes in investment with respect to sexual function are rather flexible (Koene, 2017).

In summary, we show that *Ovipostatin* expression profiles change in the prostate after copulation, highlighting that this ACP is transferred during sexual encounters and has to be replenished afterwards. The expression does not seem to depend on the donor's mating histories, as investigated here. Our data do, however, highlight the importance of temporal standardization when such expression is measured. To determine whether *Ovipostatin* expression is a good proxy for prostate-gland replenishment, the expression of other ACPs in the prostate gland should now be examined. The role of these proteins in the reproductive process and their potentially different allocation should also be assessed. Work is needed on other snail species in which such ACPs have already been identified (e.g. *Biomphalaria glabrata*, Adema *et al.*, 2017) or remain to be discovered. Finally, our finding that ACP production in a simultaneous hermaphrodite seems to be regulated in a similar way to separate-sexed organisms, suggests that such proteins are at least as important in the post-copulatory processes of hermaphrodites as they are in organisms with separate sexes.

## SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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