Disentangling female postmating responses induced by semen transfer components in a simultaneous hermaphrodite

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Optimizing reproductive success is an essential part of evolution for both sexes. Females can optimize mating by avoiding superfluous mating advances and insemination, since both take time away from other activities and may incur costs related to sperm receipt. While many separate-sexed organisms are known to exhibit mate avoidance, much less is known about this for simultaneous hermaphrodites. We addressed this here, using a simultaneously hermaphroditic species that can choose to mate in either of the two sex roles during each mating interaction. Recently, avoidance behaviours in response to natural matings were observed in the hermaphroditic pond snail *Lymnaea stagnalis*, potentially deterring insemination. To disentangle whether such behaviours are mediated by the mechanical act of mating or the receipt of accessory gland proteins and/or sperm, we intravaginally injected individuals with control or test fluids. Our results show that the avoidance behaviours, crawl-out and biting, were more frequently expressed when individuals were inseminated with accessory gland proteins and/or sperm. These behavioural components of the recipient increased time in courtship prior to insemination, which is concordant with the hypothesis that the partner tries to discourage the potential sperm donor from inseminating. Understanding the mechanism underlying the effects that molluscan accessory gland proteins induce contributes to our understanding of the molecular basis of the recipient's (behavioural) responses as well as how such biochemical postcopulatory strategies evolve.
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Many organisms have evolved behavioural, morphological or physiological reproductive traits and strategies to optimize their reproductive success. That this applies to both separate-sexed and hermaphroditic species is now generally accepted. In fact, hermaphroditic organisms are now seen as essential models to fully comprehend the evolution of reproductive strategies and how sexual selection occurs without sexual dimorphism (e.g. Anthes et al., 2010; Pélissié, Jarne, & David, 2012). Initially, most ecologists and evolutionary biologists, including Darwin (1871), thought that sexual selection could not occur in simultaneously hermaphroditic animals and would therefore not impact their evolution.

Darwin was essentially trying to explain secondary sexual characteristics but considered that lower animals, including hermaphrodites, would not be capable of mate choice or experience competition for mates, primarily because of their 'too imperfect senses and much too low mental powers' (Darwin, 1871, page 321).

Since then, however, numerous studies have revealed that being hermaphroditic does not eliminate sexual selection and that hermaphroditic species display numerous reproductive strategies aimed at achieving higher reproductive success (e.g. Ghiselin, 1969; Charnov, 1979; Arnold, 1994; Morgan, 1994; Greeff & Michiels, 1999a, 1999b; Anthes, Putz, & Michiels, 2006, 2010; Schärer et al., 2012; for angiosperms/plants see e.g. Willson, 1979; 1994). Such strategies appear to be rather essential in simultaneous hermaphrodites because both male and female reproductive strategies are used at the same time and need to be optimized (Charnov, 1979; Morgan, 1994; Anthes et al., 2010; Schärer & Pen, 2013). This defining feature of simultaneous hermaphrodites generally implies flexible sex allocation towards their male or female functions depending on the circumstances (Charnov, 1982). While males and females differ in their optimal mating rates (as pointed out by Bateman, 1948), there is no a priori reason to assume that hermaphrodites should deviate from this principle (e.g. Anthes et al., 2010; Hoffer, Mariën, Ellers, & Koene, 2017). Although Bateman's (1948) experiment has received criticism based on experimental design, data collection and repeatability (e.g. Gowaty, Kim, & Anderson, 2012; Gowaty, Kim, & Anderson, 2013), the above-mentioned basics of the Darwin—Bateman paradigm generally do

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seem to hold (e.g. Janicke, Häderer, Lajeunesse, & Anthes, 2016). After all, sperm donors aim to maximize their reproductive success by increasing successful copulations, while sperm recipients favour lower mating rates due to inherantly higher investment in reproduction, i.e. anisogamy and the resulting Bateman's principle apply (Anthes et al., 2010; Hoffer et al., 2017). Differential importance of additional matings for reproductive success between sperm donor and recipients can fuel sexual conflict over mating rate (Parker, 1979; Bradbury & Andersson, 1987; West-Eberhard et al., 1987; Rice, 1996; Alexander et al., 1997; Hosken et al., 2001; Cordero & Andrés, 2002).

Obtaining more mating partners is not the only way male reproductive success can improve. It is now generally accepted that processes occurring both before and after insemination can affect sexual selection in hermaphrodites. That sexual selection often occurs at the postcopulatory level is not entirely unexpected, given that mate choice based on secondary sexual characteristics (pre-copulatory sexual selection) is absent by definition (but note that not all secondary traits need to be visual). Sperm donors, however, do employ various precopulatory tactics to find mates and to increase their chances of fertilizing females' eggs. After a successful sexual encounter, postcopulatory processes can take place in the form of sperm competition (Parker, 1970) and cryptic female choice (Eberhard, 1996). These processes may be even more important for the reproductive success of hermaphroditic species, particularly gastropods, since individuals not only mate with multiple partners but can also store sperm for months to years (Kupfernagel, Rusterholz, & Baur, 2010; Baur, 1998, 2007; Nakadera, Blom, & Koene, 2014).

For internally fertilizing organisms, the transfer of semen (i.e. sperm and accessory gland products; Zizzari, Smolders, & Koene, 2014) is an important postcopulatory strategy. Numerous studies have revealed that the receipt of semen influences female physiology and renders a female unwilling to remate (e.g. Koene & Ter Maat 2001; Liu & Kubli, 2003). This is generally caused by accessory gland products (ACPs), which are a broad range of biological substances consisting primarily of proteins and peptides secreted by male accessory glands that modulate female reproductive physiology and behaviour, and thereby their receptivity (Koene & Ter Maat, 2001; Perry, Sirot, & Wigby, 2013; Sirot, Wong, Chapman, & Wolfner, 2015). For example, physical barriers to block multiple inseminations (e.g. mating plugs) are induced by the receipt of seminal fluid (Gillot, 1988; Lung & Wolfner, 2001). In many insects, the transfer of spermatozoa and ACPs also induce behavioural changes in females that trigger avoidance of remating (e.g. Koene, 2012).

In *Drosophila melanogaster*, an ACP called sex peptide has been shown to control female reproductive processes, increasing egg laying and refractoriness to remating (Liu & Kubli, 2003; Peng et al., 2005). Likewise, for the simultaneously hermaphroditic land snail *Euhadra quaesita*, the accessory gland products that are transferred along with love darts during courtship appear to reduce the likelihood of remating (Kimura, Shibuya, & Chiba, 2013). In another simultaneous hermaphrodite, the great pond snail, *Lymnaea stagnalis*, behavioural observations suggest that individuals are more reluctant to mate in the female role after they have received an ejaculate (Moussaoui, Verdel, Benbellil-Tafoughalt, & Koene, 2018). In the latter study, recently inseminated snails appeared to discourage reinsemination by a novel partner by either crawling above the water line (crawl-out behaviour) or actively biting the partner. However, as in other examples from simultaneous hermaphrodites, it remains unknown whether such avoidance behaviours are induced by specific components of semen (ACPs and/ or sperm) or mating itself. Indeed, several studies on separate-sexed species have revealed the importance of sperm for

inducing specific female behaviours (Aranha & Vasconcelos, 2018). Based on the current evidence found in *L. stagnalis*, it is conceivable that sperm, ACPs, a combination thereof or the mechanical act of mating trigger specific female behavioural responses. That such responses may be expressed in order to avoid or discourage a mating partner is supported by the fact that specific ACPs have been reported to reduce egg laying (Koene et al., 2010) as well as sperm transfer by the recipient (Nakadera, Swart, Hoffer, Den Boon, Ellers, Koene, 2014), and seem to outweigh the potential benefit of better hatching eggs (Hoffer et al., 2017).

Using the well-studied *L. stagnalis* as a model, here we aimed to discover the trigger for female remating reluctance. Our goal was to reach a better understanding of the role that ACPs and/or sperm play in the expression of female behaviour (Note that to be able to expand on the work of Moussaoui et al. (2018), we adhere to the terms they used for female behaviours.). Rather than natural inseminations, we performed intravaginal injections, allowing us to determine the separate effects of the different components (i.e. ACPs and sperm) that are transferred during mating.

METHODS

Study Species

Lymnaea stagnalis is a large, air-breathing, aquatic snail highly suited for experimental laboratory studies; indeed, it can be bred in large densities under specific artificial conditions without being physiologically affected. As mentioned above, it is a simultaneous hermaphrodite that can copulate in either the male or female role, meaning that as one snail performs in the male role (i.e. donor) by inseminating its partner, the other performs in the female role (i.e. recipient). This distinctive feature allows us to visually discriminate the individual's sexual role during copulation, thus enabling us to separate male and female responses to being inseminated. The male courtship consists of a fixed set of behaviours: mounting, circling, positioning, partial/total eversion and probing (de Boer et al., 1997) followed by insemination (i.e. the transfer of semen). Each insemination lasts approximately 20—70 min. After insemination, the recipient either directly digests the received sperm (allosperm) in the bursa copulatrix or stores it for roughly 2 months (Nakadera, Blom et al., 2014).

Snail Maintenance

We used the laboratory strain of *L. stagnalis* from the snail-breeding facility of the Vrije Universiteit, Amsterdam (The Netherlands). This strain has been bred for approximately 50 years but originates from a wild population in a nature area near Eemnes (The Netherlands). Snails are mass reared in groups of the same age and fed copious amounts of lettuce. Each age group is separated into 220-litre tanks with 20—22 °C low-copper water with continuous laminar flow at 200 litres/h with a light:dark cycle of 12:12 h.

Snail Features and Isolation

Mature, 3-month-old individuals of *L. stagnalis* were used, with a shell length of 25—30 mm to avoid the confounding factors of age and size (Nakadera et al., 2015). They were randomly divided into two categories: one group was designated to mate in the male role (donor) and the other to mate in the female role (recipient). The focal sperm recipients were marked with a small dot of nail polish so we could distinguish them from their partner (i.e. donor). First, donors were isolated for 7 days in perforated polyethene jars (460 ml), i.e. each individual was placed in one jar within a laminar

flow-through tank. This sexual isolation period induces an enlargement of the prostate gland and ultimately increases the snails' motivation to mate in the male role (Van Duivenboden & Ter Maat, 1988; De Boer et al., 1997; Koene & Ter Maat, 2005; Koene & Ter Maat, 2007). Recipients were isolated for only 4 days, which is enough to remove the known physiological effects of previous inseminations (Koene, Montagne-Wajer, Roelofs, & TerMaat, 2009, 2010; Hoffer et al., 2010) without increasing their motivation to mate in the male role (Van Duivenboden and Ter Maat, 1985).

Role of ACPs and/or Sperm in Female Behaviour

We used the complete prostate gland extracts (i.e. seminal fluid-producing organ) to procure ACPs and the seminal vesicles (i.e. autosperm storage organ) to procure sperm. We used the equivalent of one-third of the content of one prostate gland extract and/or the content of one seminal vesicle (Koene et al., 2010) as the intravaginal injection dose per individual, dissolved in carrier medium (physiological *Lymnaea* saline: 5.83 mM $CaCl_2.2H_2O$, 3.76 mM $MgCl_2.6H_2O$, 42.69 mM NaCl and 37.53 mM KCl). Each 10 ml syringe containing a test solution was fitted with a blunt injection needle (0.3 mm × 13 mm). A 1 mm diameter silicon tube was carefully slid over the injection needle and any air was removed from the tube by gently applying pressure on the syringe. We then anaesthetized the recipient individuals with 2—3 ml of 50 mM $MgCl_2.6H_2O$ by penetrating the middle of the foot with a sharp injection needle at a 45° angle towards the head and gently applying pressure continuously until the snail relaxed and remained extended (within seconds; after which the needle can be removed). Immediately afterwards we intravaginally injected them with one of the three experimental ejaculates or a control solution (see Nakadera Swart et al., 2014; Van Iersel, Swart, Nakadera, Van Straalen, & Koene, 2014). The control consisted of an intravaginal injection of the carrier medium. The experimental ejaculates were either ACPs only, ACPs + sperm or sperm only. For each intravaginal injection, once the female gonopore of the anaesthetized snail was visible and reachable with a silicon-tube-fitted syringe, a forceps was used to gently insert approximately 2—4 mm of the tube into the pore. By carefully applying pressure to the syringe, 0.03 ml of the test solution was injected after which we allowed 30 s for the pressure in the tube to spread into the female tract before removing the tube and returning the treated snail to its isolation container.

Two days after the intravaginal inseminations, one focal recipient from each treatment was matched with a donor. We decided to wait 2 days after insemination to avoid potential behavioural effects of anaesthesia (Moussaoui et al., 2018). We continuously observed and noted the following female mating behaviours for 4—6 h: (1) locomotion, i.e. the individual moves while attached to the surface; (2) crawl-out, i.e. the individual tries to escape and positions its body and shell above the water line; (3) retraction, i.e. the individual uses the shell as a hiding place; (4) biting, i.e. the mounted individual uses its radula to bite the body or shell of its partner (5) floating, i.e. the individual moves while not attached to the surface. The cumulative number of minutes for which the focal snail expressed each behaviour were calculated. For the donors, we recorded the total duration of courtship (i.e. time until insemination); and the total duration of insemination.

Individuals that did not feed and/or lay eggs during the isolation period, which was rare, were excluded from observations. Pairs in which focal recipients mated in the male role first were excluded from the analyses, as we were only interested in female postmating responses of inseminated individuals (17 for ACP only, 20 for ACP + sperm, 18 for sperm only and 19 for saline control). In total, we observed 110 complete matings (29 for ACP only, 26 for ACP + sperm, 28 for sperm only and 27 for the saline control).

Statistical Analysis

Statistics were performed with R software (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Normality (Shapiro test) and homoscedasticity (Bartlett test) conditions were not met, so Kruskal—Wallis tests followed by pairwise Wilcoxon tests were used to compare (1) the total duration of each mating behaviour between treatments and (2) the courtship time/time until insemination/insemination time between treatments. Since the behavioural observations were divided over several consecutive days (each containing pairs of every treatment to avoid introducing a confounding variable), we used a Scheirer—Ray—Hare test which confirmed that the random factor 'day of observation' did not have any effect on the outcome.

Ethical Note

The research adheres to the ASAB/ABS Guidelines for the Use of Animals in Research, the legal requirements of the Netherlands and all institutional guidelines. The housing and breeding conditions have been optimized for this species and are fully standardized (Van Der Steen, Van Den Hoven, & Jager, 1969). The experimental procedures were designed to minimize adverse effects on the snails. We kept the number of individuals used and/or killed to a minimum while ensuring we had enough replicates for statistical testing.

RESULTS

Behaviour Duration

The total duration of crawl-out behaviour (Fig. 1a, Appendix Table A1) differed significantly between the treatments and control (Kruskal—Wallis test: $\chi^2 = 15.93$, $P = 0.001$). Recipients inseminated with ACPs only, ACPs + sperm or sperm only expressed crawl-out behaviour for significantly longer than the control group (post hoc pairwise Wilcoxon tests: $P < 0.05$). The total durations of crawl-out were not significantly different between the three treatment groups (post hoc pairwise Wilcoxon tests: $P > 0.05$). Similarly, the total duration of biting behaviour (Fig. 1b, Appendix Table A1) differed significantly between the treatments and control (Kruskal—Wallis test: $\chi^2 = 41.07$, $P < 0.001$; Appendix Table A1). Recipients inseminated with ACPs only, ACPs + sperm or sperm only showed significantly longer biting behaviour than the control group (post hoc pairwise Wilcoxon tests: $P < 0.05$). Moreover, snails inseminated with ACPs + sperm showed longer biting behaviour than snails from the other two treatments (post hoc pairwise Wilcoxon tests: $P < 0.05$). No differences were found for the duration of floating and retraction behaviours (Fig. 1c and d, Appendix Table A1; Kruskal—Wallis tests: $P > 0.05$).

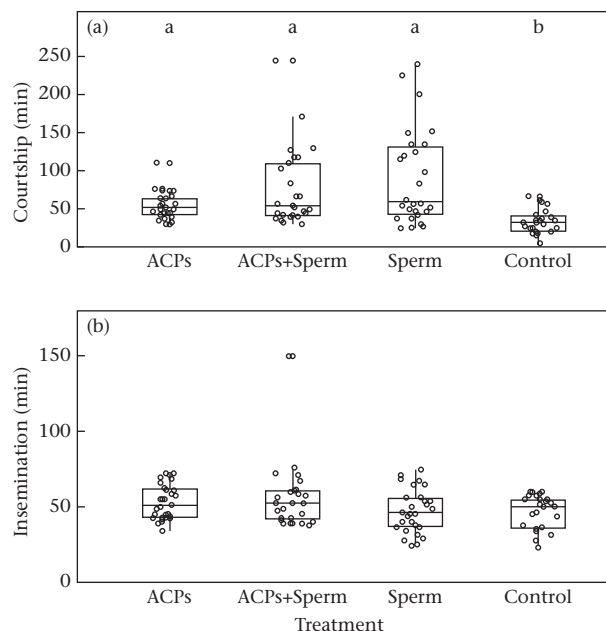### Courtship and Insemination Time

The courtship time (i.e. the time between the start of the experimental observation and the final mounting that led to insemination) was statistically different between the treatments and control (Fig. 2a, Appendix Table A1; Kruskal—Wallis test: $\chi^2 = 13.77$, $P = 0.003$). The courtship time was significantly shorter in the control group (post hoc pairwise Wilcoxon test: $P < 0.05$). However, insemination duration did not statistically differ between treatments and control (Fig. 2b, Appendix Table A1; Kruskal—Wallis test: $\chi^2 = 5.66$, $P = 0.13$).
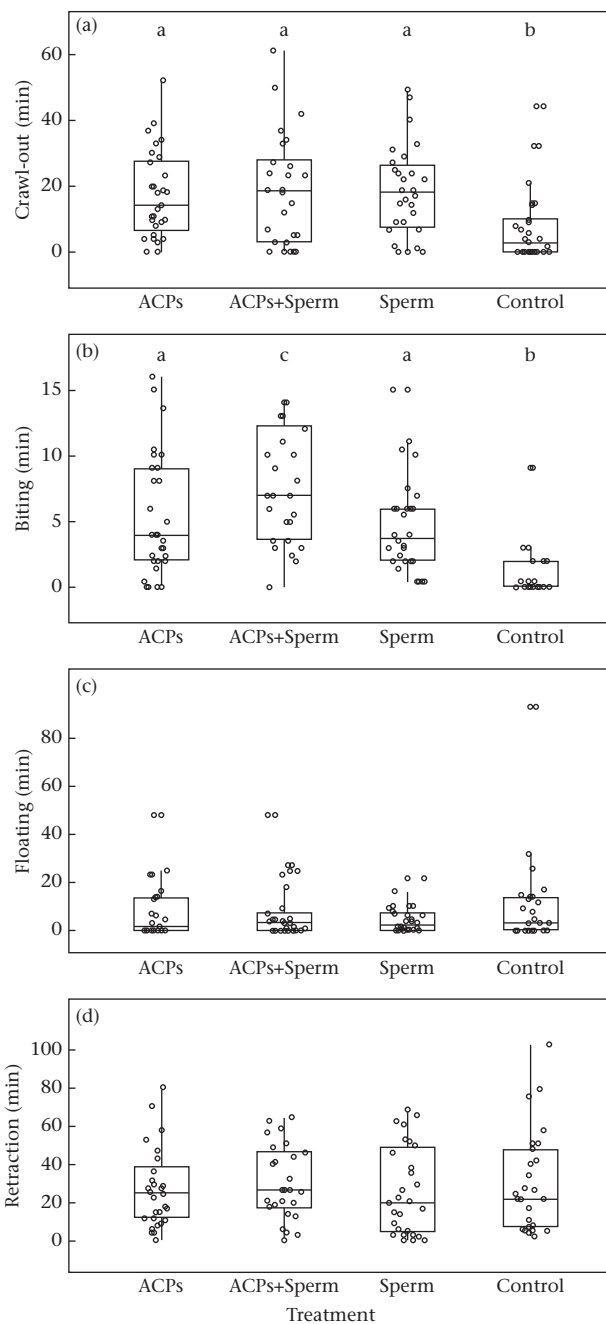
Figure 1. Total duration (min) of the recipient's behaviours during mating. (a) Crawl-out, (b) biting, (c) floating and (d) retraction, compared for the intravaginally inseminated experimental ejaculates (ACPs only, ACPs + sperm, Sperm only) and the saline control. In the box plots, the horizontal lines indicate the median (50th percentile) and quartiles (25th and 75th percentiles), the vertical whiskers indicate the range and the open circles the jittered data points. Significant post hoc differences between treatments are indicated with different letters (pairwise Wilcoxon test: *P* < 0.05).

Figure 2. (a) Courtship duration (min) and (b) insemination duration (min) for the intravaginally inseminated experimental ejaculates (ACPs, ACPs + sperm, Sperm) and the saline control. In the box plots, the horizontal lines indicate the median (50th percentile) and quartiles (25th and 75th percentiles), the vertical whiskers indicate the range and the open circles the jittered data points. Significant post hoc differences between treatments are indicated with different letters (pairwise Wilcoxon test: *P* < 0.05).

DISCUSSION

With our experiment we can confirm that the female behaviours observed in *L. stagnalis* in response to an additional insemination attempt were not induced by the mechanical act of mating. Our findings indicate that individuals that were artificially inseminated with ACPs only, ACPs + sperm and sperm only displayed more of the female behaviours suggested to function as avoidance of additional insemination (both crawl-out and biting behaviours)

compared to control individuals inseminated with saline. This is further supported by the fact that the time to reach insemination was delayed for the donors, meaning that courtship lasted longer. Hence, based on our findings, we can confirm that chemical cues from the ejaculate (i.e. the sperm and/or ACPs), rather than physical cues, induce behavioural changes in the sperm recipients.

Interestingly, our results do not show clear differences between the effect of ACPs only and sperm only, so both seem to induce such behaviours. Indeed, no differences in durations of behaviours or courtship were found between individuals inseminated with ACPs only and individuals inseminated with sperm only. This result is not in line with the vast majority of studies on separated-sex species (mainly insect species) where remating avoidance seems to be generally due to the receipt of ACPs and not the sperm itself. In *D. melanogaster*, for instance, the receipt of only ACPs during copulation induces refractoriness to remating: mated females actively reject courting males (Chapman et al., 2003; Hasemeyer, Yapici, Heberlein, & Dickson, 2009; Liu & Kubli, 2003; Ram & Wolfner, 2009; Yang et al., 2009; Yapici, Kim, Ribeiro, & Dickson, 2008). In addition, females of the Queensland fruit fly, *Bactrocera tryoni*, when mated to sterile males (i.e. irradiated males that store little or no sperm) display no difference in postmating receptivity compared with mates of nonsterile males, implying that ACPs and not sperm itself are responsible for such behaviours (Harmer, Radhakrishnan, & Taylor, 2006).

Although male ACPs generally induce refractoriness in females, some authors have proposed that sperm might also play an essential role in the long-term refractoriness of females (Manning, 1962; Gillot, 1988, 2003, Peng et al., 2005). In *D. melanogaster*, female refractoriness to remating persists for at least 5 days after a normal mating while this response only lasts 1 or 2 days after mating with males that transfer only ACPs and not sperm. The more rapid return of female receptivity after a spermless insemination reveals a 'sperm effect' (Gillot 2003). Peng et al. (2005) highlighted

that sperm is the carrier for the sex peptide, an ACP inducing short- and long-term female postmating responses. By binding to sperm, sex peptide prolongs the reduced female readiness to remate (Sirot et al., 2014). Such results are in line with what we observed in terms of biting behaviour: snails inseminated with ACPs + sperm showed more biting behaviour than snails inseminated with ACPs or sperm only, suggesting a cumulative effect. Possibly, an association between sperm and one or more of the ACPs could be responsible for the 'sperm effect' observed in *L. stagnalis*.

Notwithstanding the above, it remains surprising that artificial insemination with sperm induced refractoriness in females. As far as we know, no other studies have reported this effect. We therefore recommend future research to take a proteomic approach to ACP transfer during mating. Combined with genetic methods such as knockouts, CRISPR-Cas editing or RNAi (e.g. Wigby & Chapman, 2005; Weber, Giannakara, & Ramm, 2019), this would allow researchers to create sperm donors that lack one specific ACP in order to identify which protein decreases female receptivity. Our expectation is that this would be a different ACP from those already functionally identified in this species (Koene et al., 2010; Nakadera, Swart et al., 2014).

Given our findings, it would be worth investigating the details of how these ACPs achieve their effects since there are various possible scenarios. For example, an ACP can directly pass through the wall of the female reproductive tract and enter the recipient's haemolymph (thus reaching the central nervous system or another target organ directly). Alternatively, it can stimulate a specific receptor located within the reproductive tract itself that would subsequently initiate the synthesis or release of a hormonal substance that then affects the target organ. It is also possible that such a receptor is present on sensory neurons within the female tract that can relay the information via a nervous connection to the central nervous system, A final possibility is that the ACP can trigger a more general immune or stress response (as has been reported for mice, *Mus musculus*, and fruit flies: reviewed in McDonough, Whittington, Pitnick, & Dorus, 2016) that, as a side-effect, results in a behavioural change that makes the recipient more reluctant to remate.

The approaches and possible mechanisms suggested above, which are not exhaustive, will be instrumental for understanding how molluscan ACPs exert their effects, and whether these are specifically targeted or side-effects of a more general response (which is suggestive since sperm also induced a behavioural change in the recipient). Moreover, determining the mechanisms underlying the apparent function of ACPs will contribute to our understanding of the molecular basis of the recipient's (behavioural) responses. Furthermore, it informs about how such biochemical postcopulatory strategies evolve and adds to the understanding of postcopulatory sexual selection processes in simultaneous hermaphrodites.

Acknowledgments

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Appendix

Table A1
Summary statistics showing the mean and SD for each treatment shown in Figs. 1 and 2

Treatment	ACPs	ACPs + sperm	Control	Sperm
Crawl-out (min)	17.4 ± 13.2	18.7 ± 16.8	7.2 ± 10.8	18.6 ± 13.6
Biting (min)	5.3 ± 4.7	7.7 ± 4.3	1.0 ± 1.9	4.7 ± 3.6
Floating (min)	7.5 ± 11.3	7.5 ± 11.6	9.9 ± 18.7	4.5 ± 5.5
Retraction (min)	26.7 ± 20.4	30.2 ± 19.5	29.7 ± 26.6	25.9 ± 23.1
Courtship (min)	53.2 ± 18.5	75.6 ± 51.8	33.6 ± 16.6	88.3 ± 62.2
Insemination (min)	53.6 ± 11.5	56.3 ± 22.3	47.0 ± 11.6	47.8 ± 14.4
Sample size (*N*)	29	26	27	28