

Video Article

Effect of Male Accessory Gland Products on Egg Laying in Gastropod Molluscs

Sander van Iersel¹, Elferra M. Swart¹, Yumi Nakadera¹, Nico M. van Straalen¹, Joris M. Koene¹¹Department of Ecological Science, Faculty of Earth and Life Sciences, VU UniversityCorrespondence to: Joris M. Koene at joris.koene@vu.nlURL: <http://www.jove.com/video/51698>DOI: [doi:10.3791/51698](https://doi.org/10.3791/51698)Keywords: Physiology, Issue 88, Allohormone, Fresh-water snail, Gastropod, *Lymnaea stagnalis*, Mollusc, Pond snail, Prostate, Semen, Seminal fluid Sexual selection, Sperm

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Abstract

In internally fertilizing animals, seminal fluid is usually added to the spermatozoa, together forming the semen or ejaculate. Besides nourishing and activating sperm, the components in the seminal fluid can also influence female physiology to augment fertilization success of the sperm donor. While many studies have reported such effects in species with separate sexes, few studies have addressed this in simultaneously hermaphroditic animals. This video protocol presents a method to study effects of seminal fluid in gastropods, using a simultaneously hermaphroditic freshwater snail, the great pond snail *Lymnaea stagnalis*, as model organism. While the procedure is shown using complete prostate gland extracts, individual components (*i.e.*, proteins, peptides, and other compounds) of the seminal fluid can be tested in the same way. Effects of the receipt of ejaculate components on egg laying can be quantified in terms of frequency of egg laying and more subtle estimates of female reproductive performance such as egg numbers within each egg masses. Results show that seminal fluid proteins affect female reproductive output in this simultaneous hermaphrodite, highlighting their importance for sexual selection.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51698/>

Introduction

Upon transfer, spermatozoa are usually accompanied by seminal fluid produced by male accessory glands. While some ejaculate components play roles in nourishing and activating sperm¹, others influence female physiology in order to increase fertilization success of the sperm donor²⁻⁴. Such effects are especially selected for, via sexual selection, when a species is highly promiscuous and has efficient sperm storage and sperm digestion. Under such conditions, sperm donors compete heavily for the fertilization of eggs⁵. While there is abundant correlational evidence for the importance of seminal fluid substances for male fertilization success, direct evidence is more rare and few studies have delved into the details of the physiological change and/or substances involved⁶.

In species with separate sexes (*i.e.*, males and females; gonochorists) there are a few well-investigated model species when it comes to seminal fluid composition and their effects. Examples include the fruit fly, *Drosophila melanogaster*⁶, the malaria mosquito, *Anopheles gambiae*⁷, and the red flour beetle, *Tribolium castaneum*⁸. Such seminal fluid studies are much rarer when it comes to species that are simultaneously hermaphroditic (*i.e.*, individuals are functionally male and female at the same time), even though this mode of reproduction is common throughout the animal kingdom⁹. The most notable hermaphroditic example of the transfer of an accessory gland substance is probably the shooting of so-called love-darts in land snails^{2,10}, where the substance is transferred by the sperm donor via hypodermic injection^{11,12}, *i.e.*, not along with the spermatozoa. But there are many simultaneous hermaphrodites that transfer male accessory gland products along with their sperm, such as the model species used here, the great pond snail *Lymnaea stagnalis*. Previous research has already demonstrated that the receipt of seminal fluid influences the female reproductive output of this species¹³⁻¹⁵, and several of the involved seminal fluid proteins have been identified^{16,17}.

The overall goal of this protocol, which is an extension of the methods reported in Koene *et al*¹⁶, is to test effects of seminal fluid proteins on various parameters of egg laying in gastropod molluscs. This is accomplished by dissection of the prostate gland (*i.e.*, the male accessory gland producing the seminal fluid) of sexually isolated donor snails from a standardized laboratory culture. Second, the donor's sperm is collected from the seminal vesicles and different treatment solutions are made. Subsequently, test snails are anaesthetized and intravaginally injected with a test solution (for example, prostate gland extract alone, prostate gland extract with sperm, sperm alone, carrier medium alone). These test snails are then monitored for the following days to weeks, under standard conditions, to record egg laying. Egg masses are collected, digitally scanned for counting and measuring various parameters, and then put in individual vials for hatching. After about two weeks, the hatchlings are counted and digitally scanned. All the scans, of both egg masses and hatchlings, are then analyzed using a freely-available image analysis software package.

For this protocol the laboratory strain of the great pond snail, *Lymnaea stagnalis*, is used, which is bred at VU University in Amsterdam. This species serves as a model system for investigating questions about sexual selection and reproduction in hermaphrodites. The animal's relatively large size makes it experimentally very accessible. In addition, because it is used as a model species in many biological disciplines we already know a lot about the basic biology of these animals. This method will help to study questions about processes of sexual selection that are mediated via seminal fluid proteins in general. In addition, because these are simultaneous hermaphrodites, it is possible to address whether such proteins act in similar and/or different ways as they do in species where the sexes are separated^{16,17}.

Protocol

1. Breeding Facility

1. Obtain adult specimens of *Lymnaea stagnalis* (L.) from a laboratory culture like the one at VU University.
2. Maintain the low-copper water at 20 °C in both the breeding facility (molluscarium) and experimental tanks, which have continuous water exchange.
3. Set the light/dark cycle at 12 hr:12 hr.
4. For breeding, feed the adult snails pesticide-free lettuce *ad libitum*, feed the young juveniles with TetraPhyll (fish food) flakes. Maintain snails in separate tanks according to their age, starting from egg masses which are laid within a 24 hr time frame.
5. Once snails are removed from the breeding tanks they are not returned, to avoid effects of having been kept under slightly different conditions as well as to prevent pseudoreplication.

2. Dissection

1. Isolate the snails for 1 week, and feed them *ad libitum*, to ensure that they have full sperm and seminal fluid stores.
2. Prepare a stereo microscope, a dissection plate with pins, small surgical scissors, coarse and fine forceps, a 10 ml syringe filled with a 50 mM MgCl₂ solution, injection needles (0.3 mm x 13 mm) and some paper towels.
3. Euthanize a snail by injecting MgCl₂ solution (generally 3 ml or more for adult snails with a shell length of 31.6 ± 2.1 mm and wet weight of 2.89 ± 0.55 g). Penetrate the foot with the injection needle at a 45° angle and inject by gently applying pressure continuously until the snail relaxes and remains extended (within seconds). Remove the injection needle and check whether the snail is euthanized (for example, by checking whether the tentacle withdrawal reflex is absent).
4. Carefully remove the shell following its curvature by using coarse forceps. Halfway, gently detach the columellar muscle from the shell by scraping with the forceps. Gently twist the snail out of its shell, following the winding direction of the shell.
5. Place the snail on the dissection plate and pin down the back end of the foot. Then, place a pin through the head and put some tension on the snail to facilitate dissection.
6. Localize the female gonopore, which will later on be a reference point for dissection.
7. Using the surgical scissors and fine forceps, make the first incision, just under the edge of the mantle and cut towards the female gonopore. When one of the scissors' blades is pushed under the body wall, lift it up slightly to ensure that only the body wall, and none of the internal organs, is cut. Continue cutting above the gonopore towards the head and cut in a straight line.
8. Localize the prostate gland (posterior) and seminal vesicles (anterior). Dissect these out carefully and keep them on ice. Use these to make the test solutions containing seminal fluid and/or sperm. Use physiological *Lymnaea* saline (5.83 mmol/L CaCl₂·2H₂O, 3.76 mmol/L MgCl₂·6H₂O, 42.69 mmol/L NaCl, 37.53 mmol/L KCl) as a carrier medium.
9. Collect the seminal fluid that is present in the lumen of the prostate gland as well as the sperm from the seminal vesicles. Subsequently, suspend both in saline in separate vials by gently expressing their contents and removing the organ tissue with a forceps. Keep on ice and use the same day. Later, mix these suspensions to create the different treatment solutions.

3. Intravaginal Injection

1. Before starting the intravaginal injection procedure, prepare a silicon shell-mold (made to fit the tip of the shell), 1 mm syringes, 10-12 cm silicon tubing with a diameter of 1 mm, blunted injection needles (0.3 mm x 13 mm), coarse forceps and the same 10 ml syringe filled with a 50 mM MgCl₂ solution fitted with a sharp injection needle.
2. Collect the sperm, needed to add to the seminal fluid (or individual seminal fluid proteins) in some treatments; a biologically relevant dose equals 1/3 of a prostate gland and seminal vesicle per injection.
NOTE: In the representative results section (**Figure 1**) the results of an experiment with three different treatments are shown: Control (carrier medium, *i.e.*, saline), Ovipostatin (one of the identified seminal fluid proteins) alone and Ovipostatin accompanied with sperm. In addition, to estimate the biologically relevant dose in other species, determine the difference in weight of the prostate gland of individuals that have recently mated and individuals that remained sexually-isolated¹⁶.
3. Prepare 1 ml syringes for each of the prepared test solutions. Fill each syringe with one solution and ensure that there are no air bubbles inside.
4. Fit a blunted injection needle on each syringe.
5. For injection use a 1 mm diameter silicon tube with a minimum length of 10 cm.
6. Carefully slide the silicon tube over the injection needle, making sure not to pierce the tube, and remove the air from the tube by gently applying the pressure on the syringe.
7. Anaesthetize a snail by following the same protocol as in point 2.3. This time make sure not to inject more than 2-3 ml of 50 mM MgCl₂.
8. Lay the snail in the shell-mold to keep it in a stable position.
9. Locate the female gonopore, which is clearly visible as a white spot on the right side of the animal, anterior to the right tentacle and male gonopore (this location also applies to other species of freshwater snails). Make sure to have unobstructed access.
10. Use a forceps to hold the end of the silicon tube and gently insert approximately 2-4 mm of the silicon tube into the female gonopore.

11. Carefully apply pressure to the syringe and inject 0.03 ml of the test solution. Wait half a minute to allow the pressure in the tube to spread into the female tract before removing the tube.
12. Return the treated snail to its isolation container, where it can be monitored, and allow it to recover from sedation in the experimental tank.

4. Bioassay

1. Label each snail for identification and measure its shell length. The latter is a good indicator of the size, which is relevant when quantifying reproductive output.
2. Keep each snail isolated in a 460 ml container that is perforated to allow for water exchange within the experimental tank. It is important to place all containers in the right orientation, with the perforations in the direction of the water flow, to allow for efficient water exchange.
3. During the bioassay, feed snails regularly with a standardized amount of lettuce. Use a round, metal puncher to cut lettuce. In this protocol lettuce discs of 19.6 cm² are used, which approximates the amount they maximally can eat in one day.
4. Collect an egg mass using the flat side of a spatula and scrape the egg mass from the container wall. Use the spoon side to collect the egg mass.
NOTE: Opaque egg masses that have just been laid cannot be used for scanning (they become transparent within a few hours after laying). For the model species in this protocol, *Lymnaea stagnalis*, check for egg masses every day, since they produce 2-3 egg masses per week. This can differ in other species.
5. Scan the collected egg masses digitally, using a (laptop) computer containing the scanner driver, a flat bed scanner, a standard millimeter scale, a spatula, transferring plates and glass tiles.
6. Place each egg mass on the transferring plates and repeat until the plates are full. Subsequently, dab the egg masses dry on paper towel and transfer them onto the glass tiles in the same order as on the transferring plates.
7. Turn each tile upside down (the egg masses will stick to the glass tile) and carefully place it on the flatbed scanner, just under the millimeter scale. Apply a bit of pressure to flatten the egg masses, which will distribute the eggs evenly so they are all visible within the same focus plane. Fit the tiles with a standardized amount of tape around two edges to assure that all eggs are flattened equally.
8. Adjust the scanner settings. It is important to adjust the resolution to its maximum, make a preview scan, crop the working area, and adjust the brightness and contrast. Scan the egg masses.
9. Place each egg mass in its own labelled 20 ml glass vial for further development and hatching.
10. Refresh the water in hatching vials every other day to keep it properly oxygenated. Do this according to hatchling numbers and size in the following order: decanting/pouring off (no hatchlings), overflowing (start of hatching), and extracting out the water with a pipette (many hatchlings).

5. Measuring and Counting Eggs

1. Install the freely-available image analysis software ImageJ (NIH, USA) and the cell counter plugin.
2. Open ImageJ as well as a spreadsheet program to transfer the data to. Then open a scanned picture of egg masses in ImageJ.
3. Set the scale by using the zoom tool to navigate to the millimeter scale. Zoom in and adjust the window for 1 mm to fill up the screen. Draw a line using the line tool between the two lines that indicate 1 mm. Navigate to Analyze, and Set scale. In the pop-up menu the measured length option becomes visible, adjust Known distance to 1 and unit of length to mm. Check the box Global, and close the window.
4. Set the preferred measurements (length, width, circumference) by navigating to Analyze, and Set Measurements by selecting Area and Feret's Diameter (length and width). Click OK to confirm and continue with the image analyses.
5. To measure the eggs, zoom in on an egg of the first egg mass that needs to be measured. A zoom of 800% is recommended. Then, using the elliptical tool draw an oval exactly around the egg, press ctrl+D to draw the circumference on the image (to prevent measuring the same egg twice). Then press ctrl+M to record the measure of the properties of the oval (length, width, circumference); a pop-up screen opens displaying the measurements.
NOTE: The use of the elliptical tool to measure eggs does not apply to all species. For species with non-uniformly shaped eggs, use the freehand selections instead.
6. Measure as many eggs as necessary and then save the measures as .xls worksheet. Optionally, copy the data directly into a worksheet.
7. Subsequently count the eggs within an egg mass, by using the counter window and put the counts manually into the worksheet.
8. Alternatively, count the individual eggs per egg mass with the help of the cell counter plug-in. For this, navigate to Plug-ins, then, Analyze and select Cell counter. The cell counter window will pop up, check the 'keep original' box and press 'initialize'. A new counter window appears, select the crosshair tool from the toolbar and select a counter type in the cell counter menu. Count the individual eggs by clicking on the individual eggs in the cell counter window. Observe the total count next to the counter type in the cell counter menu. Copy this number manually to a spreadsheet or obtain the results by pressing Results.
9. Count multiple egg masses within one counting event by adding counter types, if necessary.

Representative Results

Figure 1 shows the percentage of animals laying eggs after intravaginal injection of either the control substance (saline), Ovipostatin, or Ovipostatin accompanied with sperm. N = 15 for each treatment; see Koene *et al.*¹⁶ for details and statistics (Source: **Figure 3** from Koene *et al.* 2010, PLoS ONE).

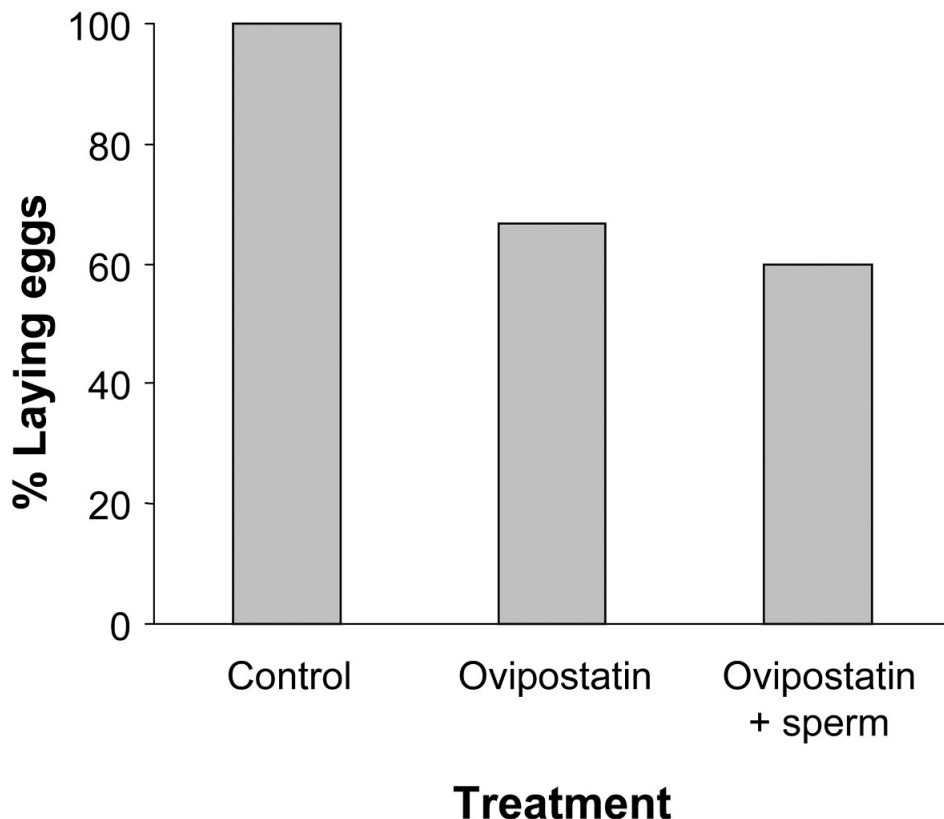


Figure 1. Effect of Ovipostatin on egg mass production of *Lymnaea stagnalis*. The graph shows the percentage of animals laying eggs after intravaginal injection of either the control substance (saline), Ovipostatin, or Ovipostatin accompanied with sperm. N=15 for each treatment; see Koene *et al.* 2010 details and statistics. Reprint with permission from Koene *et al.* 2010 PLoS ONE 5: e10117.

Discussion

The presented protocol shows how to collect seminal fluid and how to test this in a biologically meaningful way. Although this procedure here is shown using the prostate gland extract, the effect of a single purified seminal fluid protein can also be tested following the same procedure. Obviously, in such tests it is always crucial to include proper controls (sham treatments) to ensure that the procedure and/or the carrier medium do not affect the parameters in questions^{14,16}. Using this method, it has already been shown that there is a single seminal fluid protein, namely Ovipostatin, that mediates a reduction in egg laying^{14,16}. This protein, produced in the prostate gland and transferred during mating along with the spermatozoa, represents the first fully characterized seminal fluid component in a simultaneous hermaphrodite and does, so far, not show any resemblance to known proteins.

The finding that Ovipostatin reduces egg mass production in the recipient corroborates earlier work indicating that substances in the semen may influence egg laying¹³⁻¹⁵. By transferring biologically relevant amounts (see 3.2¹⁶), one can convincingly demonstrate that it is a seminal fluid protein, and not the presence of spermatozoa themselves, that mediates the observed reduction in egg laying (**Figure 1**). Given that repeated receipt of seminal fluid via natural matings increases the investment per egg¹⁵, it now becomes highly relevant to quantify other parameters of egg laying. The presented protocol now provides the necessary methods to do so. Although no effect was found of Ovipostatin on hatching success of the eggs laid by the recipient¹⁶, there may be other seminal fluid proteins that act in concert with this protein. Hence, parameters that need to be quantified in this context - next to laying frequency and egg numbers - include egg size, hatching time, hatchling development and hatchling size. In addition, it is possible to look at effects on other female processes than egg laying, such as sperm storage and usage, as well as the allocation of reproductive resources towards the female and male function^{14,17}. Clearly, the presented method is instrumental in addressing these followup issues, both in *Lymnaea stagnalis* and in other fresh water snails.

In sum, experiments using this protocol show that transferred seminal fluid proteins affect the female reproductive performance of the recipient in these simultaneous hermaphrodites. Given that few studies have addressed this, it is likely to be much more common in internally fertilizing hermaphrodites than is currently reflected in the scientific literature. Especially when one realizes that pre-copulatory mate choice and sperm competition have been shown to play an important role in these hermaphrodites¹⁴, which highlights the potential of such male accessory gland proteins to be sexually selected in this system. Finally, this research helps to support the developing notion that seminal fluid proteins are of equal importance for simultaneous hermaphrodites as they are for separate-sexed species.

Disclosures

The authors have nothing to disclose.

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