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Sperm storage, sperm translocation and genitalia formation in females of the terrestrial isopod *Armadillidium vulgare* (Crustacea, Peracarida, Isopoda)

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ABSTRACT

We investigated sperm storage, sperm transfer from the oviduct to the seminal receptacle, and formation of the cuticular genitalia in female *Armadillidium vulgare* using light and electron microscopy. Apolysis of the genitalia within the oviduct forms a circum-genital lumen. During insemination this space is filled with immobile spermatozoa. Sperm transfer into the seminal receptacle takes place before oviposition. Within a peculiar proximal neck region of the oviduct spermatozoa are bundled and enveloped by a folded epicuticular layer. The envelope tightly surrounds the spermatozoa probably forming a seal against the main part of the circum-genital lumen. We propose that hydrostatic pressure produced by the muscle cells surrounding the oviduct leads to sperm transfer into the seminal receptacle. Within the seminal receptacle the sperm bundle forms a ring just around the orifice to the oviduct. At one side sheath-like extensions of epithelial cells surround the ring of spermatozoa holding it in place. At the other side oocytes would have access to the sperm during oviposition, probably allowing for fertilisation when they pass right through the ring of spermatozoa. After oviposition the new genitalia are formed from epicuticular folds, and cuticle secreted by the epithelial cells.

1. Introduction

The reproductive system of female *Armadillidium vulgare* comprises a pair of tubular ovaries and flat shaped oviducts, which are about 1 mm in length connecting the middle part of each ovary with the gonopores located near the bases of the fifth walking legs (Suzuki, 2001, 2002). The oviducts contain cuticular genitalia. These comprise a short distal segment that is in confluence with the cuticle of the integument and a cuticular tube that lines the oviduct from the distal segment to the short proximal region of the oviduct that is devoid of a cuticular lining (Suzuki and Ziegler, 2005). The cuticular tube has flat sheath-like lateral projections that stabilize the flat shape of the oviduct during intermoult. Depending on the reproductive condition of females the genitalia occur in two distinct states. After a parturial moult females develop genitalia in the reproductive state. In these, the lumen contains numerous branched cuticular folds, and the distal segment is funnel shaped. However, how this type of genitalia is formed, and the exact timing of their formation is still unknown. At a non-parturial moult, females form genitalia of the non-reproductive state which lumen is devoid of cuticular folds and which distal segment has a narrow lumen (Suzuki and Ziegler, 2005).

In *A. vulgare* females are receptive for copulation for about one week from the late stage C (Mead, 1976) to stage D (Moreau and Rigaud, 2002) of each parturial moult cycle. Insemination occurs through the lumen of the cuticular tube that is accessible to the male's genitalia. A large mass of spermatozoa apparently leaves the cuticular tube at its proximal pore causing the oviducts to swell (Suzuki, 2001, 2005). During swelling, the cross section of the oviduct changes from its flat to a circular shape. In a previous study we proposed that, before the oviduct can swell a circum-genital lumen has to form to provide enough space for storage of the sperm (Suzuki and Ziegler, 2005). For this the epithelium would have to separate from the cuticular tube and its sheath-like lateral projections. However, direct evidence for this hypothesis was lacking, and it was not yet clear if formation of the circum-genital lumen occurs before or during insemination.

After copulation a part of the spermatozoa move from the oviduct into the seminal receptacle located at the confluence of the oviduct to the ovary. This is of particular interest since Peracarida have immotile sperms that consist of a filamentous nucleus and a very long rod-shaped tail (Cotelli et al., 1976; Itaya, 1979). The

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mechanism of how these spermatozoa are moved is still unknown, as the exact timing of sperm transfer. Within two weeks after copulation, but before oviposition, females go through a parturial moult, which leads to the formation of a marsupium by the development of large leave shaped endopodids, the oostegites, at the first five walking legs. During moult the animals first shed the posterior half of their cuticle along with the distal segments of the genitalia (Suzuki, 2002). One day later the animals shed the anterior half of the cuticle. 5 h after the anterior moult, just before oviposition, the cuticular tubes of the genitalia are cast off from the oviducts and pushed into the marsupium together with part of the large mass of spermatozoa. This widens the gonopore to an oopore that has a size of about 500 μm (Suzuki, 2002, 2005).

During oviposition the mature oocytes pass right through the seminal receptacle. It is of particular interest that after a single insemination the seminal receptacle stores enough spermatozoa to fertilize several following broods for more than one year (Lueken, 1963; Suzuki, 2001). How these spermatozoa are retained within the seminal receptacle, although about 40 oocytes pass by within 2 h during each oviposition, is unknown. After oviposition females form a new pair of genitalia. How the oviduct forms the numerous branched cuticular folds within the cuticular tube is unknown.

In an attempt to further investigate the events occurring in the oviduct and seminal receptacle during the period of sperm storage, oviposition, and formation of the genitalia we analysed the structure of the oviduct in *A. vulgare* at various stages of the parturial moult cycle using light and electron microscopy. Our results confirm our previous hypothesis of sperm storage within the circum-genital lumen. We found that a specific neck region secretes a peculiar envelope structure around the spermatozoa, that may play a role in sperm displacement into the seminal receptacle. Furthermore we show that bundles of spermatozoa form a ring held in place by epithelial cells of the seminal receptacle. Oocytes pass right through the ring causing distorsions of the ring-structure that is restored after oviposition. Epicuticular folds within the new cuticular tube are formed from epicuticle during contraction of the oviduct.

2. Materials and methods

2.1. Animals

We analysed female *A. vulgare* (Latreille, 1804) at various stages of the parturial moult cycle. Adult females with 10–12 mm body length were collected during the hibernating period in mid-February in Kanagawa Prefecture (Suzuki, 2002). Almost all of these females had spermatozoa in the seminal receptacle and thus were non-virgin females. To obtain virgin *A. vulgare* young females were collected after their fifth moult and reared without males in the laboratory for one year (Suzuki, 2000). Virgin females were studied at a sexually mature size of 10–12 mm. Using light and electron microscopy we confirmed that virgin females carry no sperms within the seminal receptacles or the oviducts (not shown). From mid-February both virgin and non-virgin females were kept at 4 °C without males. After two months all females were in stage C of the moult cycle. They had immature ovaries, no marsupia, and oviducts containing genitalia in the non-reproductive state (Suzuki and Ziegler, 2005). In late March, the annual breeding season for *A. vulgare* in Kanagawa, the temperature of the culture was raised to 25 ± 2 °C at ambient light conditions (Suzuki, 2001). After one month almost all animals were in the late stage C or early stage D of the parturial moult cycle. Then, each female was kept individually at 25 ± 2 °C either together with two males for insemination or without males to avoid mating. Those with males were checked every 2 h in order to observe mating. After the first insemination, females were kept without males to prevent further copulations. From the time of the raise of the temperature to 25 °C, females were examined daily for the formation of calcium carbonate deposits on the first four sternites (Suzuki, 2002). Formation of the deposits indicates stage D of the moult cycle and an incomplete shape of the deposits indicates that the next moult will be a parturial moult (Steel, 1982; Moreau and Rigaud, 2002). In addition, we used females in defined time intervals before and after moult to analyse the formation of the new reproductive type genitalia. Table 1 gives the number, stage, and mating condition of females used for embedding and sectioning. In addition, we used 95 non-virgin and 48 females reared as virgins for light microscopic analysis of oocyte maturation and storage of spermatozoa.

2.2. Transmission electron microscopy

Animals were injected with about 10 μl of 12.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.3) into their hemocoelic space (Ziegler, 1997). Oviducts together with part of the ovaries were dissected in 2,5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol L^{-1} Na-cacodylate buffer (pH 7.3). Specimens were then fixed for 1 h in the same fixative and postfixed in 1% OsO_4 and 0.8% $K_3Fe(CN)_6$ in bidistilled water for another hour. Then the specimens were dehydrated in a series of isopropanoles and two times in propylene oxide, and embedded in Epon resin. Ultrathin sections were cut using a diamond knife (Diatome) on a Leica Ultracut microtome. Sections were transferred to carbon coated formvar films on EM grids, stained with 2% uranyl acetate and 0.3% lead citrate and viewed with a Zeiss EM10 or a Philips 400 electron microscope equipped with a F 114T TVIPS Fastscan digital camera. Photoshop was used to adjust contrast and brightness of the images.

2.3. Light microscopy

For light and polarised-light microscopic observations animals were dissected in crustacean physiological saline as described previously (Suzuki, 2001). Micrographs were obtained using an Olympus CX40 microscope equipped with a DP20-5 digital camera (Olympus). For observations on 0.5 μm thick sections of Epon resin-embedded material, sections were stained with an aqueous solution of 0.1% methylene blue and 1% borax. A Zeiss Axiophot microscope equipped with a Spot CCD camera (Visitron) was used to obtain digital images.

2.4. Analysis of oocyte and sperm ring diameters

Diameters were determined using light micrographs of whole ovaries and oviducts. The average of the two sperm rings and the

Table 1
Number of females used for light and electron microscopic observations of Epon-resinembedded material. The table provides the stage, time in hours to (−) and from (+) the anterior ecdysis and the number of females that have mated during the experiment before dissection. The asterisks indicate that one of these females was raised as a virgin and had mated before dissection.

Stage	Time	Total	Mated
C	192–240 (−)	2*	2
D	144–192 (−)	2	2
D	96–120 (−)	9*	5
E	20 (−)	3	2
A	2–5 and 5–7 (+)	9	4
A	10 and 20 (+)	7	0
B	40 (+)	2	0
C	80 and 120–150 (+)	6	0