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Mating behavior and ultrastructural aspects of copulation in the rotifer Asplanchna brightwelli

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Mating

Males and females were crossed simply by placing 1-3 virgin females with 6-10 males in a small amount of culture fluid. The virginity of the females required for genetic studies (Birky, 1965, 1967b) or examination of sperm-egg interaction (present investigation) was assured by staggering the mictic cycles a day apart for 3-4 consecutive days (Birky, 1967b). In this manner, when males were observed in the most advanced culture, the second culture begun one day later contained young, virgin, mictic females with haploid oocytes, capable of being fertilized. However, not every female in the culture was affected by tocopherol (Birky, 1968) and in the second culture both mictic and amictic females were being born consecutively. Since no morphological difference between mictic and amictic females was discernable (Bentfeld, 1971a,b), the presence of haploid or diploid oocytes was not known at the time of the matings and numerous repetitions were required (see Birky, 1964). Specific techniques for observing copulation and spermogenesis stages of development of the rotifiers can be found in Aloia & Moretti (1973a,b).

Literature Cited


MATING BEHAVIOR AND ULTRASTRUCTURAL ASPECTS OF COPULATION IN THE ROTIFER ASPLANCHNA BRIGHTWELLI

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ALOIA, R. C. & MORETTI, R. L. 1973. Mating behavior and the ultrastructure of copulation in the rotifer Asplanchna brightwelli. Trans. Amer. Micros. Soc., 92: 371-380. Copulation in the Asplanchna rotifiers appears to occur by hydropic semenation in which the male copulatory organ is pressed tightly against the female integument with the subsequent transfer of spermatocytes through the integument and into the pseudocoel. The mating behavior transcends four successive stages of (1) normal contact of the male, (2) body arching of the male, (3) copulatory organ attachment, and (4) copulation. The process requires about 60 sec, during the latter part of which the male is pulled behind the female solely by the copulatory organ attachment. Electron microscopic examination of this attachment reveals an extraneous substance between the tightly juxtaposed surfaces which may function in some manner in formation of the firm bond between the female integument and the male copulatory organ.

The genus Asplanchna may reproduce sexually or asexually by female parthenogenesis. The sexual or mictic cycles occur at various times of the year in nature (Birky & Gilbert, 1971) or in response to the addition of vitamin E to the culture medium (Aloia & Moretti, 1972a; Birky, 1967; Gilbert & Thompson, 1969). The first author wishes to thank the Department of Biology for providing teaching assistantships and NIH training grant ES 00084-04 during the course of this research; also Dr. W. W. Thomson for his excellent mentorship in electron microscopy. Both authors extend their thanks to Dr. C. W. Birky, Jr. and M. J. Bentfeld for valuable commentary and guidance during the investigation. We also thank Dr. E. T. Fenselayer and I. W. Sherman for their critical reading of the original manuscript and Kaylyn Gary for her patience and expert assistance in typing this manuscript.
placed over the drop and in contact with it. Culture medium in the center of the plastic petri dish. Six or eight males and two females were placed in a drop of light source and exposing for with a light meter simply by placing red, green, and blue filters over a bright light source and exposing for 1/50, 1/125, and 1/250 seconds. A simulated compression chamber for photography was made with coverslips and the lid of a plastic petri dish. Six or eight males and two females were placed in a drop of culture medium in the center of the plastic petri dish lid. Two coverslips were added to each side of the drop of culture medium to support a third coverslip placed over the drop and in contact with it.

**MATERIALS AND METHODS**

The stock culture of the rotifer *Asplanchna brightwelli* was kindly provided by C. W. Birky. Culture and maintenance techniques of the rotifers, as well as the induction of males into the female cultures, are modified procedures of Birky (1964) and have been previously outlined (Aloia & Moretti, 1973a).

**Light Microscopy**

Swimming activity and mating of *A. brightwelli* were determined by visual observation and photography. A Nikon microflex Dark Box mounted to an American Optical binocular dissection microscope was used to photograph mating rotifers with Agfa Isopan Record 35 mm, 800 ASA, black and white film. Photography was performed without a light meter simply by placing red, green, and blue filters over a bright light source and exposing for 1/50, 1/125, and 1/250 seconds. A simulated compression chamber for photography was made with coverslips and the lid of a plastic petri dish. Six or eight males and two females were placed in a drop of culture medium in the center of the plastic petri dish lid. Two coverslips were added to each side of the drop of culture medium to support a third coverslip placed over the drop and in contact with it.

**Electron Microscopy**

To fix rotifers in the process of copulation, one or two females were placed with 8 or 10 males in a drop of culture medium and observed at a magnification of 10 to 20X until copulation was underway. Ten to 20 sec after the final attachment of the male copulatory organ to the female cuticle (see Results) the fixative was added. This was accomplished by placing several drops of fixative adjacent to the medium of copulating rotifers and pushing the two solutions together. This was the only method proving gentle enough not to rupture the copulating pair. However, even this technique disrupted 30 out of 36 pairs (83%), and of the six pairs successfully fixed only three were successfully processed for electron microscopy.

The primary fixative used was 2.5 or 4% glutaraldehyde (EM grade from Polysciences) buffered with 0.067 M or 0.1 M cacodylate buffer to pH 7.2, with sucrose added to a final concentration of 0.5 or 1%. Fixation was carried out at room temperature for 90-120 min followed by a rinse of 60-120 min at room temperature in the cacodylate buffer. This was followed by 90 min in 1% cacodylate buffer or 2% unbuffered OsO4. The animals were then rinsed again in the cacodylate buffer for 60-120 min before dehydration in a graded series of ethanol and embedded in Spurr’s epoxy (Spurr, 1969). The rotifers were infiltrated in Spur’s (hard) medium for 2-4 hr at 4°C and oven cured at 70°C for 8-12 hr.

Large size Irwin loops (Sargeant-Welch & Co.) were used to transfer the pairs of copulating rotifers through the 25 mm x 9 mm Syracuse watch glasses containing the various processing fluids.

After embedding, silver-gold sections were cut with an LKB ultramicrotome, expanded with xylene vapors, and picked up on uncoated 100, 150, and 200 mesh copper grids. The sections were stained with Reynolds lead citrate only and examined in a Philips 300 electron microscope at 60-80 kv.

**RESULTS**

**Swimming Activity**

The females of *A. brightwelli* swim in a smooth, gliding pattern along a straight course until another animal or the wall of the culture dish is encountered. At this time they simply glance or graze off the surface of the encountered object, sometimes turning 180 degrees in the process, and resume swimming along another straight course.

This almost-rhythmic forward motion of the female is strikingly contrasted to that of the male rotifer, which is frequently interrupted by a spasmodic, jerking activity often accompanied by a change in direction. The males usually swim with a slight propensity toward a circular pattern, with their ventral surface facing the center of the arc.

**Courtship**

The complete mating process of *A. brightwelli* requires about 45-60 sec and can be divided into four successive stages: (1) coronal contact, (2) body arching,
The mating process appears to be initiated following a random encounter of a male and female. The first step after encounter involves placement of the male corona in close apposition to the cuticular surface of the female (Fig. 2A). The female continues her smooth swimming motion, only occasionally contracting the body wall musculature causing a brief interruption in her forward progress. The male follows along, with his coronal region remaining closely associated with the female's integument. From 16 mm cinematographic observations, this association sometimes appears to be more than a simple anatomical juxtaposition. On some occasions it appears to be a firm attachment which causes the cuticle of the female to become visibly taut in the region associated with the corona of the male as the female twists and turns during swimming. This initial phase of attachment usually lasts 3–5 sec.

After forming the association of the male corona and female integument, the male begins to hunch or arch his body and move the copulatory organ closer to the body of the female (Fig. 2B). The male may remain in this position for a few seconds or he may continue the arching process (Fig. 2B'), bringing the copulatory organ in close contact with the cuticular surface of the female. During this hunching phase (Figs. 2B and 2B') the male begins to move across the surface of the female, usually around the short axis of her body. This migration occurs in such a manner that the corona of the male remains contiguous with the female integument at all times.

During these first two stages, requiring 10–15, sometimes 20 sec, both male and female may discontinue the courtship activity. The female may rotate around the long axis of her body or completely contract her body musculature causing the male to lose contact. The male may halt the courtship behavior simply by straightening his body and swimming away.

If courtship is continued, the third stage of the process ensues; the copulatory organ of the male is attached to the female integument. This attachment has been observed to occur at almost every possible locus around the surface of the female body; near the coronal area or at the posterior end of the female at the level of the gastric glands, stomach, and vitellarium. During this 10–20 sec attachment phase the male remains in a hunched position with both the corona area and the tip of the copulatory organ in contact with the female integument (Fig. 2C).

After attachment, the corona of the male is withdrawn from the body of the female and the male assumes his normal straight or erect position (Fig. 2D). This phase of actual copulation usually lasts 20–45 sec, although one unusually prolonged contact of 65 sec was recorded on 16 mm film. The final attachment of the copulatory organ to the female integumental surface apparently is a strong bond, since the female, being ca. 19–3 times the size of the male, can continue to swim and drag the male along solely by the copulatory organ. However, this bond between the copulatory organ and the female is of such a nature that it also allows the female to rotate 360° around the point of attachment without rupturing the union.

Figs. 1A, 1B. Light photomicrograph of side view and top view of male rotifer. Note the two testes and urethral opening (UR, 1A). × 100.

Figs. 2A–2D. Light photomicrographs of mating sequence of Asplanchna brightwelli. (Figure 2A = coronal contact; Figures 2B and 2B' = body arching; Figure 2C = copulatory organ attachment; Figures 2D and 2D' = copulation.) Arrowhead points to the tip of the copulatory organ in each successive stage. × 100.
After completing the copulatory process, the male simply swims away in his usually salutary fashion. The female, however, begins a series of body wall contractions, interrupting her forward motion for approximately 10–20 sec prior to resuming her normal smooth swimming behavior.

Anomalous Mating

The males of A. brightwelli have been observed to mate with other males, taken from artificial mictic cultures containing vitamin E. They have also mated with young females of similar size taken from artificial mictic cultures without vitamin E. Normally, these matings have occurred between two individuals. However, occasionally, relationships such as two or three males simultaneously attached to one female, or one male copulating with one female while another male copulates with her have been observed.

Electron Microscopy of Mating

Light microscopic observations indicate that the tip of the copulatory organ is tightly pressed against the female integument during copulation. These observations appear confirmed by examination of copulating rotifers with the electron microscope. Figure 3 illustrates a section through the periphery of the copulatory organ and the female integument. A closer examination of the juxtaposed cuticles indicates that an additional substance is present. It can be seen as a thin, wispy fibrous layer running perpendicular to the outer cuticular fibers, described by Koehler (1965b), and parallel to the surface of the cuticle (Fig. 4). Figure 6 shows that the more tightly pressed cuticular surfaces separate at their outer boundaries, this extra fibrous material adheres closely to the surface of the female integument. In this region the fibrous substance is arranged in two distinct layers parallel to the female integument (EF, Fig. 8). A similar extra fibrous substance can also be seen when examining the urethra of a male rotifer fixed while copulating. The fibrous material is found between the walls of the urethra arranged perpendicular to the cuticular fibers (Fig. 7). An extra fibrous substance such as this is never present in the urethra of non-copulating male rotifers. In this region between the extreme tip of the urethral canal and the juxtaposed female cuticle of copulating rotifers, an amorphous, fuliginous material can be observed (Fig. 3). This substance is found in the central planes through the longitudinal axis of the copulatory organ and completely fills the triangular-shaped cavity formed by the tip of the male urethra and the female cuticle. Additionally, Figures 4, 5, and 7 illustrate some ultrastructural differences between male and female integuments (see Koehler, 1965b, 1968). Throughout the entire investigation, the rigid rods synthesized by the non-functional spermatozoa were never observed more anterior than the sperm duct region of the testis. They were not observed at any point along the male urethra nor within the female pseudocoel of copulating females.

Discussion

Mating Behavior

The mating behavior of the closely related pleomate rotifer Brachionus has been accurately described by Gilbert (1963). The male of this genus must come into normal head-on contact with the corona of the female before the characteristic mating reaction ensues. This reaction begins by the male arching his body and moving around the surface of the female's body keeping one or both extremities of his body in contact with hers. This movement around the female continues until the male copulates or swims away; the female apparently has a passive role. Copulation usually occurs in the coronal region of the female with the male penis piercing the body wall to inject spermatozoa. After insemination, the male is often dragged behind the female until he is able to break loose and swim away.

There are many similarities in the mating behavior of Brachionus and Asplanchna. For example, the mating behavior of the Asplanchna male begins with the male hunching his body and migrating over the surface of the female. He then copulates and is dragged along behind the female for variable distances prior to swimming away. However, there are several interesting differences in the copulation behavior of the two genera. Asplanchna males may initiate the mating response by contacting any other rotifer (male, mictic female, or amictic female) at any point on the body surface; whereas, Brachionus males will only initiate the mating response if they contact the coronal area of the female, which apparently emits an aromatic chemical stimulus. If this aromatic compound is not emitted from the coronal region of the females, or if the chemical stimulus is too weak to be detected, males will not copulate with them (Gilbert, 1963). This implies that the pre-isolating mechanism for the conservation of genetic material which exists in Brachionus species does not function in the Asplanchna rotifers. It appears to be only by chance that asplanchnoid male gametes will reach receptive haploid eggs of mictic females. Secondly, the Asplanchna females are not as passive as the Brachionus females, since they may discontinue the courtship process during the first three stages of the mating activity. Thirdly, copulation occurs at any point along the body of the female in Asplanchna and not primarily on the softer, larger coronal area, as with Brachionus females. Lastly, the copulatory organ of Asplanchna does not appear to be inserted through the female integument as with Brachionus. Rather, copulation occurs by attachment of the copulatory organ to any point along the female body with subsequent insemination through the integument. This observation also indicates that the mechanism of copulation of Asplanchna rotifers described by Whitney (1913) and Hyman (1951) appears to be incorrect. Copulation does not appear to occur by insertion of the male copulatory organ through the female integument (Whitney, 1913), nor does it occur by insertion directly into the cloaca of the female (Wesenberg-Lund, 1925).

Electron Microscopy of Mating

Cursory examination of the juxtaposed integuments of copulating rotifers (Figs. 4, 5, 6) reveals a slight difference in thickness. The Dense Lamellar Band (Koehler, 1965b, 1968) and the hypodermal region appear wider in the female integument than in the integument of the tip of the male copulatory organ. Koehler (1965b, 1966) has illustrated several strands of rough endoplasmic reticulum in the female hypodermis lying parallel to the cuticular surface (Figs. 4, 5). Additionally, between the reticular strands profiles of hypodermal bulbs (investigations of the outer cuticular membrane through the dense lamellar layer and into the hypodermis) are observed (Figs. 3, 4, 5). These bulbs have been found in all rotifer integument examined to date (Koehler, 1968) and have been shown to function in the deposition of fibrinous cellular material on the external portion of the cuticle during embryonic development (Brodie, 1970).

Contrasted to this integumental morphology, that of the male rotifer appears to be less well developed in the region of the copulatory organ. Koehler (1968) demonstrated that the hypodermal bulbs of males were half the size of female bulbs, and as seen in Figures 4, 5, and 6 they are also fewer in number. Also the male hypodermis does not appear to be packed with ribosomes and rough endoplasmic reticulum.
The electron microscopical examination of the copulatory organ also reveals no specialized cuticular or muscular structure to which the mechanism of attachment to the female integument can be attributed per se (see Hyman, 1951, for description of stylets on the copulatory organ of other rotifers). Both light and electron microscopical examination of copulation indicate that the copulatory organ is simply attached to the outside of the female integument. Electron microscopy reveals that there is an extra fibrous substance between the surface of the juxtaposed male and female integuments and within the narrow urethral channel of copulating males. Since this additional fibrous substance is never observed on the cuticular surface of non-copulating rotifers, it seems credible to assume that it is in some fashion associated with the formation of the bond between the male copulatory organ and female integument. In addition to the flocculent substance within the cavity formed by the tip of the male urethra and the female integument (Fig. 5), this extra fibrous substance may represent a secretory product of the prostate glands.

In a subsequent report it will be shown that there are seven prostate gland cells in the male copulatory organ extending posteriorly from the urethral-vesiculae junction to the anteriormost tip of the testis (Alolia & Moretti, 1973b). During and only during copulation these gland cells have been observed to secrete their amorphous, powdery content into the basal region of the urethra. It seems plausible that the extra fibrous and flocculent material seen between copulating rotifers may represent the prostate gland secretion which assumes various morphological appearances depending upon its location. In other words, the prostate secretion may assume a fibrous nature between the tightly apposed walls of the male urethra and between the tightly pressed copulatory organ and female integment, while assuming a more expanded or flocculent form in the open cavity formed by the tip of the urethra and the female integment. Depending on the chemical nature of this prostate secretion it may indeed serve as a glue-like substance establishing the firm bond which allows the male to be pulled behind the female solely by the copulatory organ attachment.

Examination of the copulating rotifers indicates that the rigid rods were never observed more anterior than the sperm duct region of the testis. This evidence does not appear to confirm the hypothesized function of the rigid rods as projectiles to rupture the female integument during copulation, but neither does it automatically refute their participation in hypodermic penetration. The observations of this investigation indicate that the copulating animals were
chemically fixed prior to the time of rigid rod ejection. After complete attachment of the copulatory organ to the female, 10-20 sec elapsed prior to the addition of fructose to allow for the formation of a firm bond. It appears that insemination probably occurs at a later time. Perhaps insemination occurs immediately prior to the separation of the two animals at the end of copulatory attachment.

**Literature Cited**


**DIRECT DEPOSITION AT HIGH RESOLUTION OF SPECIFIC METALS FROM SOLUTIONS AT RADIOACTIVE SITES IN TISSUE SECTIONS**

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NORMANDIN, D. K. 1973. Direct deposition at high resolution of specific metals from solutions at radioactive sites in tissue sections. *Trans. Amer. Micr. Soc.*, 92: 381-398. A new direct method of marking sites labeled with beta emitting isotopes in electron microscope sections has been found. This method does not require the use of a photographic emulsion. It has the following advantages. (1) The time of exposure is shortened to seconds. (2) The size of the marked area may be reduced to approximately twenty-five angstrom units. (3) The size of the marked area may be varied according to experimental design. (4) No special techniques in terms of light temperature or special atmospheres are necessary. (5) Localization may be found throughout the depth of the section. (6) A double fixation procedure with aminium may be used to increase contrast and yet not disturb or mask the deposition of the marker metal. (7) More than one beta emitting isotope may be used simultaneously, each labeling a different material, with the identities of the labeled material substances distinguishable according to microcrystalline size.

The methods presently used to demonstrate radioactive labels within electron microscope sections are essentially similar to the methods used for light microscopy.

Much careful research has been done (Budd & Salpeter, 1969; Caro, 1962; 1969; Caro & Van Tubergeren, 1962; Hampton & Quastler, 1961; Koehler, Mocke, & Frey-Wyssling, 1963; Salpeter, 1969; Salpeter & Bachmann, 1964; Salpeter, Bachmann & Salpeter, 1969) to determine, and to reduce, the size of the developed silver particles within the emulsion; but the average minimal size obtainable is still approximately 1,000 Å, which agrees with the data supplied by Kodak on N.T.E. emulsion and with the physical limitations discussed by Spence (1966). This size is much too large for certain critical determinations, such as those involving the specific localization of a particle relative to an intracellular membrane or small organelle.

It occurred to the author that the sizes of the deposited particles would be greatly reduced, as in physical development (Haynes & Shockley, 1935; Mees, 1969), if solutions of silver or other metallic halide salts were used in direct contact with the labeled sections, instead of applying photographic emulsions containing rather large halide grains spaced in a relatively rigid dispersion pattern to record the radioactivity.

By the method developed, the size of the final particle can be reduced tenfold or more and the time of exposure reduced from days or months to 20 sec.

**Materials and Methods**

Three NIH albino mice, two days old, were used. One mouse was injected intraperitoneally with 12 microcuries of 14C labeled thymidine, another with 12

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