Cell Tissue Res (1994) 275:467–479

Cell & Tissue Research



# Ultrastructural examination of the insemination reaction in *Drosophila*

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Received: 4 May 1993 / Accepted: 1 July 1993

**Abstract.** The insemination reaction is a swelling of the female vagina caused by the male ejaculate. This postmating phenomenon is common among species in the genus *Drosophila*. It could act as a plug securing male paternity. It is not clear, however, what benefits it provides to the female. The structure formed in the female vagina is expelled in some species and disappears gradually in others suggesting different phenomena. Based on ultrastructural examination of the vaginal contents of five *Drosophila* species (*D. mettleri*, *D. nigrospiracula*, *D. melanogaster*, *D. mojavensis*, and *D. hexastigma*), we propose three terms to describe these vaginal structures: the sperm sac, the mating plug, and the true insemination reaction. Each term describes a distinct structure associated with a specific female postmating behavior. This study questions the concept of the insemination reaction as a single phenomenon and discusses its possible functions from an evolutionary perspective.

**Key words:** Vagina – Insemination reaction – Postmating behavior – Evolution – Sperm competition – Sexual selection – Paragonia – *Drosophila* (Insecta)

## Introduction

Sexual selection is a major selective force acting on both sexes in the context of reproductive fitness (Thornhill 1979). The reproductive success of males depends on the sperm that accomplish fertilization of the eggs (Parker 1984). Females of many species, especially insects, store sperm and remate several times; therefore, it is likely that ejaculates from several males will compete for the fertilization of the eggs (Parker 1970). Males have evolved behavioral, morphological, and physiological adaptations to secure their paternity against other males. Male mechanisms to ensure paternity can be beneficial or costly to females (Knowlton and Greenwell 1984). Cases where there is conflict between the interests of the sexes pose interesting evolutionary questions (Trivers 1972; Parker 1984).

The insemination reaction, which occurs in many species of *Drosophila*, is an enlargement of the vagina produced by the male ejaculate (Patterson 1946). In intraspecific matings the reaction appears to prevent the female from remating temporarily (Patterson 1947). Therefore, the insemination reaction secures the male paternity of offspring. It is not clear, however, whether this postmating plug provides any selective benefit to the female in which it evolved (Maynard Smith 1956). Parker (1970), discussing the insemination reaction in the context of sperm competition, suggested that the insemination reaction does not have to confer selective advantages to the female; it could have evolved through competition among the males. The insemination reaction could be a trait evolving in females due to the advantage conferred to her male offspring (Fisher 1958).

The insemination reaction influences the postmating behavior of the female. Patterson (1947) stated that most females will expel an excess of sperm together with the insemination reaction materials around 6 to 8 h after mating. This suggests that the female plays an active role in removing sperm from her vagina. However, Lee (1950) and Asada and Watanabe (1987) reported that the insemination reaction disappears gradually from the vagina. A gradual clearance of the reaction suggests the possibility of an ongoing physiological process rather than a female-controlled behavior. Because the two ways of bringing the reaction to its end – expulsion and gradual disappearance – suggest major differences in female control of the outcome of a mating, we decided to compare the structures of the reaction masses formed in different species of *Drosophila*.

In the present study, using ultrastructural and biochemical techniques, we compared the structures formed in the female vagina after mating in five *Drosophila* species: *D. mettleri*, *D. nigrospiracula*, *D. mojavensis*, *D.*

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*hexastigma*, and *D. melanogaster*. The first four species belong to the repleta group, a large group of mainly cactophilic *Drosophila*, which show great variation in reproductive characters. *Drosophila melanogaster* was chosen because it has a postmating behavior similar to some of the cactophilic flies although it is phylogenetically distant from the repleta group. These five species previouly have been classified in the three different categories of the insemination reaction first proposed by Wheeler (1947). *Drosophila melanogaster* is listed in class I, as there is no observable insemination reaction, but the female expels a droplet of sperm after mating (Wheeler 1947). *Drosophila mojavensis* has a very striking enlargement, class III, of the vagina that disappears gradually as first demonstrated by Patterson (1946). *Drosophila hexastigma* is also listed in class III, since it has a major enlargement of the vagina, but Wheeler (1947) found that *D. hexastigma* females also extrude a solid structure after mating. More recently *D. mettleri* has been classified as class II, with a mild reaction, and *D. nigrospiracula* as class I, with no observable reaction, by Markow and Ankey (1988). However, Heed (1990) found that both of these species discard a large mass of sperm after mating.

Based on the ultrastructural and biochemical results reported here and our ongoing studies of the postmating behavior of females, we believe that among these five species there are three distinctive postmating phenomena: the "sperm sac" present in *D. mettleri, D. nigrospiracula,* and *D. melanogaster*; the "true insemination reaction" present in *D. mojavensis;* and the "mating plug" present in *D. hexastigma*. To understand the delineations of the sperm sac, mating plug, and true insemination reaction inside the female vagina (see Lee 1950 for definition of this term), the structure of the vaginal wall of each species was also examined. Our new classification brings into question the role of the insemination reaction as a single entity and contributes to our understanding of the diversity of reproductive traits among *Drosophila* species.

## Methods and materials

### *Animals*

The strains of cactophilic flies used in this study were: *Drosophila mettleri* A855 and *D. nigrospiracula* A855, both collected in Cerro Colorado, 24 kms northeast of Puerto de la Libertad (Mexico) in February, 1984; *D. mojavensis* A730 from Tonichi (Mexico), collected in March, 1978; and *D. hexastigma* A842 from Zapotitlan Salinas (Mexico), collected in March, 1983. These cactophilic flies were reared in the laboratory in 8-dram shell vials containing standard food (yeast-agar-banana-malt-syrup). Virgin males and females were immobilized and separated on ice within 24 h after eclosion. About 25 flies per vial were stored at room temperature until used in the experiment between 11 and 14 days posteclosion. The *D. melanogaster* used were the Oregon R-C strain. In this species the males and females were also separated on ice within 8 h after eclosion and used in the experiment between 4 and 6 days posteclosion.

On the day of the experiment approximately 30 virgin males and 20 virgin females were placed in Petri dishes with pieces of food on the sides. After 8 h the flies were removed and the expelled structures were collected. The structures expelled by *D. hexastigma* were collected after a 24-h period.

### *Electron microscopy*

The vaginal structures expelled by the females and the reproductive organs of virgin and recently mated females were prepared for light- and electron-microscopic examination. *Drosophila mettleri, D. nigrospiracula, D. melanogaster,* and *D. hexastigma* females expel the structures formed in the vagina on the surface of the food media, where they can be found and collected. The expelled structures were collected from the Petri dishes and placed in fixative overnight. Females were dissected in saline solution and their reproductive organs were also placed in fixative overnight. The fixative solution contained 2.5% glutaraldehyde, 0.5% paraformaldehyde, 0.18 mM $CaCl_2$, 0.58 mM sucrose, and 0.1 M sodium cacodylate buffer, pH 7.4. After fixation the tissues were osmicated en bloc in 0.05%–1.0% $OsO_4$, and then dehydrated through a graded series of ethanols and embedded in Epon/Araldite. For light microscopy, sections were cut at 1-μm thickness, stained with toluidine blue, and mounted in Permount. For electron microscopy, thin sections were cut on a diamond knife, placed on clean grids, stained with lead citrate, and examined in a JEOL 1200EX electron microscope.

For light microscopy, we examined the vaginas of 8 *D. mettleri* females interrupted during the late stages of mating or immediately after mating and 3 discarded sperm-containing structures; 8 *D. nigrospiracula* vaginas of recently mated females and 2 discarded sperm-containing structures; 11 *D. mojavensis* vaginas 40 min after mating; 3 *D. hexastigma* vaginas between 1 to 4 h after mating and 5 extruded structures; and 2 *D. melanogaster* vaginas of recently mated females and 2 extruded sperm-containing structures.

For electron microscopy we examined 3 extruded sperm-containing structures of *D. mettleri* and 3 vaginas in the last stages of mating or right after mating; 2 *D. nigrospiracula* vaginas after mating and 2 discarded sperm-containing structures; 2 *D. mojavensis* vaginas 40 min after mating; 2 *D. hexastigma* vaginas from 1 to 4 h after mating and 2 extruded structures; and 2 *D. melanogaster* vaginas after mating and 2 expelled sperm-containing structures.

### *Electrophoresis*

We used one-dimensional electrophoresis to compare the chemical contents of the sperm sacs in *D. mettleri, D. nigrospiracula,* and the mating plug in *D. hexastigma*. Fifteen virgin males and 15 virgin females, 11–15 days old, were placed in Petri dishes with small pieces of food in the sides. After 24 h 10 sperm sacs or matings plugs of each species were collected from the Petri dishes and placed in an ice-cold homogenizer with 200 μl of 5% trichloric acid. The homogenate was transferred to a plastic tube and the homogenizer was rinsed with 200 μl of 5% trichloric acid that was also added to the plastic tube. Then, the homogenate was centrifuged for 5 min, at 12000 *g* and the supernatant was carefully removed and either used immediately or stored at −90° C.

The proteins in the supernatant were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) on 10% acrylamide gels containing 0.1% SDS. The gels were silver-stained as described by Merril et al. (1983). Sigma standards were used.

## Results

### *Drosophila mettleri*

In previous studies *D. mettleri* has been classified as having a mild insemination reaction (Markow and Ankey