Sperm Depletion in Singly Mated Females of the Mexican Fruit Fly (Diptera: Tephritidae)[1]

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Abstract Female Mexican fruit flies, *Anastrepha ludens* (Loew), have the capacity to produce more than 1,000 eggs over their lifetime, but fertility of the eggs will depend on the female's capacity to store semen or to replenish semen through remating or both. The two parameters are interrelated in that sexual receptivity depends to a large degree on insemination status. In this controlled study, we measured sperm depletion in singly mated females with continuous access to oviposition substrates by using a squash technique. The spermathecae were sequentially emptied of sperm over the 3 weeks following copulation. Under laboratory conditions, sperm was always found in the ventral receptacle up to 4 weeks following copulation, but was empty in almost all females by the fifth week. These results mirrored previous measurements of egg fertility in singly mated females, which declines at 26–40 d postcopulation.

Key Words sterile insect technique, sperm storage, fecundity, fertility

In the United States, outbreaks of the Mexican fruit fly (mexfly), *Anastrepha ludens* (Loew), are suppressed by the sterile insect technique (SIT). Population suppression with SIT is based on the concept that matings between wild females and sterile males are barren and the resulting reduction in reproductive rate will eventually drive the outbreak to extinction. Naturally, only matings between sterile males and wild females advance this outcome. Matings between fertile pairs and sterile pairs will delay or deter the outcome. One of the more important parameters in this dynamic is the fecundity of fertile mated females. Under laboratory conditions, McPhail and Bliss (1933) recorded a female laying eggs up to an age of 95 d. Under the conditions of the experiment, it was not known how frequently she had oviposited or if she had remated over that span. Berrigan et al. (1988) stated that the reproductive capacity was "over 1000 eggs over a female's lifespan." Under experimental conditions, Mangan (1997) recorded a maximum lifetime production of 1,522 eggs with a 74% fertility rate.

Although these individual records are of interest, the typical or average values may be of greater relevance from a programmatic standpoint. Mangan (1997) found

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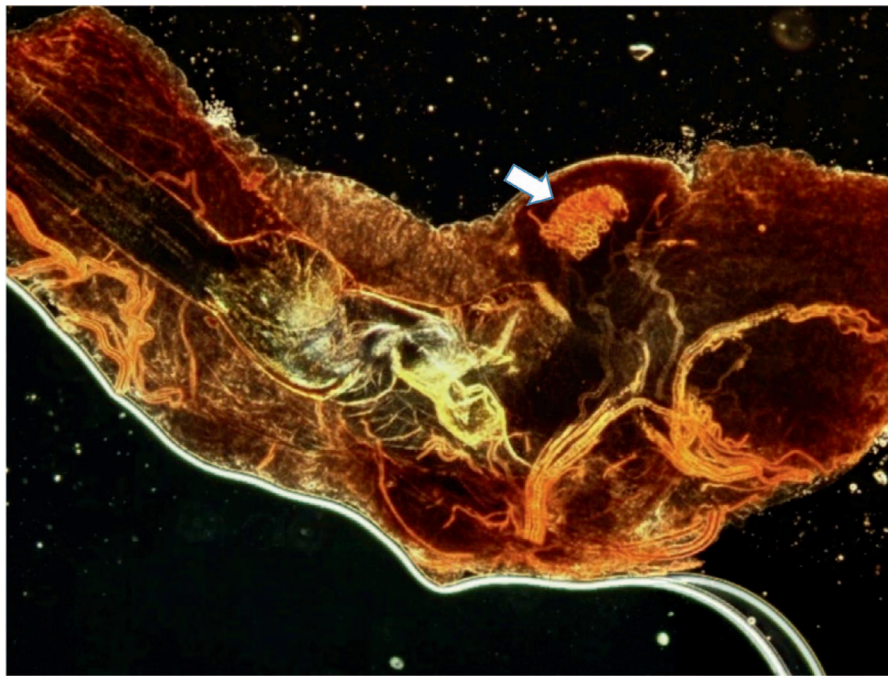

Fig. 1. Dark field photomicrograph (400×) of female reproductive tract showing the ventral receptacle that consists of the stalked cluster of alveoli (arrow) within the fertilization chamber.

that the age to which mexflies could lay fertile eggs was quite variable among laboratory strains with means from 26 to 40 d for singly mated females and maximums from 42 to 84 d. In that regard, females will continue to lay eggs, fertile or not, as long as oviposition substrate is available. Infertility could result from depletion of sperm or from sperm inviability due to age of the semen. Thus, sperm storage capacity could be an important factor limiting lifetime fecundity depending on receptivity to remating. Aluja et al. (2009) found that wild female mexflies were sexually recalcitrant for a period of 12–19 d following copulation, with 30% of females remating thereafter. In a closely controlled set of experiments, Abraham et al. (2016) found that female *A. ludens* were significantly more likely to remate if not inseminated at the first copulation. These studies suggest that females have the ability to detect a need to replenish the stores of sperm needed for fertility.

As in other species of *Anastrepha*, *A. ludens* has four sperm storage organs: three spermathecae and a seminal receptacle consisting of a cluster of alveoli (Fig. 1) within the ventral receptacle (Martinez and Hernandez-Ortiz 1997). The ventral receptacle is the fertilization chamber and because of that function it is the most important storage organ. The spermathecae function as long-term storage vessels. Thomas et al. (2014) found that the ventral receptacle was the first organ to receive semen during copulation with the spermathecae receiving and storing the overflow. Studying the medfly, Twig and Yuval (2005) found that the amount of semen in the

ventral receptacle remained constant for 18 d postcopulation, whereas the amount of semen in the spermathecae gradually decreased. Our experiments were designed to test if a similar "recharging" process occurs in the mexfly and to measure the rate of sperm depletion in singly mated females with continual access to oviposition substrate.

Materials and Methods

Test insects. The flies for these tests were from a breeding colony maintained by United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) at its Moore Air Base Facility near Edinburg, TX. The colony is an isofemale line originating with an outbreak of wild flies captured in Willacy County TX, in April 2008. The colony is maintained in a room with constant 25°C and 55 ± 5% relative humidity with a combination of fluorescent and window lighting. The larvae were reared on a meridic diet (Spishakoff and Hernandez-Davila 1968) with adults fed a diet designed to maximize adult longevity (Martinez et al. 1987).

Experimental design. Prior to the tests, puparia of the same approximate age were held in emergence grids. Newly emerged flies were separated by gender into male or female only cages. Under standard laboratory conditions, females become sexually mature beginning at 9 d postemergence (Servin-Villegas and Jimenez-Jimenez 1995). Therefore, testing began when the flies were 14 d old. In nature, Mexican fruit flies mate at dusk (Robacker et al. 1991). Therefore, on the day of the test, 120 naive males were introduced into one of two caged mating arenas containing 60 virgin females 1/2 h before sunset. An observer at each arena captured copulating pairs in a petri dish as they initiated coitus, recording the time from when copulation began for each pair. Only mated females that were in coitus for at least 15 min were used for the sperm depletion test. This procedure was repeated on two replicate occasions with 50 copulating pairs isolated on each occasion. The next morning, the mated females were placed into a Plexiglas observation cage containing food and water. Two citrus fruits (oranges or grapefruit or both) were placed into the cage to provide the females with an oviposition substrate. The fruits were replaced at 3–4 d intervals. At weekly intervals, five females were sacrificed by placement into saline solution for dissection (in some cases fewer, depending on survival).

Dissection and scoring for sperm presence. The spermathecae and ventral receptacle were separately dissected from each female by using a Vannas-style spring scissors and microforceps in sterile saline. Each organ was separately mounted on a microscope slide with a drop of aceto–orcein stain in the case of the spermathecae (no stain for ventral receptacle) and topped with a coverslip. Gentle pressure was then applied to the coverslip with a pencil eraser observed with the aid of a Nikon SMZ dissecting stereomicroscope at a magnification of 40× until spermatozoa were expressed or rupture of the organ was achieved or both. The stain is necessary because the spermathecae contain a filamentous secretion that can be mistaken for sperm. For visualizing and scoring for the presence or absence of spermatozoa, the slide was moved to a Nikon Eclipse 80i compound microscope with oil immersion objective at 1,000× (Fig. 2). All specimens were photographed

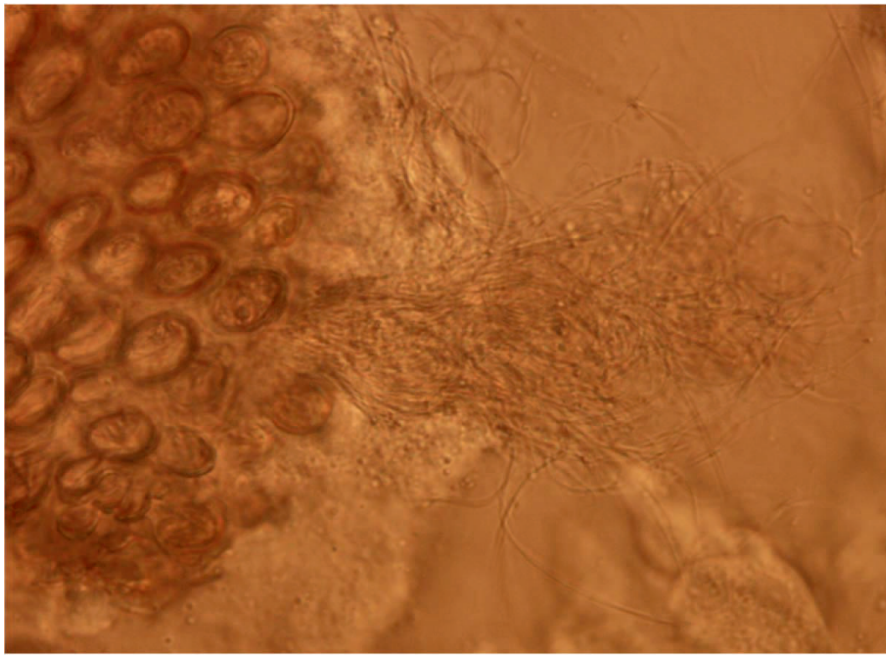

Fig. 2. Bright-field photomicrograph (1,000×) of spermatozoa exuding from a squash of the ventral receptacle.

with a Nikon DS-Fi1 camera attachment with digital sight monitor system and video software.

Statistical analysis. Differences among means were analyzed by pairwise *t*-tests. Correlation values were calculated by least squared regression (Sokal and Rohlf 1973). Probabilities were calculated using National Council for Social Studies (NCSS) Statistical Software (Silver Springs, MD).

Results and Discussion

In both replicates, all females were positive for sperm in the ventral receptacle until the fifth week postcopulation, at which time all females in both replicates were negative with the exception of one female that had only a small number of sperm remaining. In contrast, all females in both replicates were negative for sperm in the spermathecae by the fourth week postcopulation, whereas all females had sperm in at least one spermatheca at 3 weeks postcopulation (Table 1). There was a statistically significant depletion of sperm stored in the spermathecae over time postcopulation. That is, the percentage of spermathecae positive for spermatozoa was significantly reduced each week ($t = 12.9$, $P = 0.003$ in pair-wise comparison of the first to the second week, and $t = 8.2$, $P = 0.007$ comparing the second to the third week). This result suggests that the spermathecae were depleted sequentially rather than simultaneously. Also, whereas the reduction in fertility was abrupt and

Table 1. Sperm-positive females by ventral receptacle (VR+) and by spermathecae (St+) and mean percent of positive spermathecae by time postcopulation.

Weeks postcopulation	VR+ Females (*n*)	St+ Females (*n*)	St+ Organs (*n*)	Mean ± SE*
1	10 (10)	10 (10)	19 (30)	63.3 ± 10.0 a
2	10 (10)	7 (10)	9 (30)	30.0 ± 3.3 b
3	8 (8)	8 (8)	11 (24)	45.8 ± 4.1 c
4	9 (9)	0 (6)	0 (18)	0 ± 0 d
5	1 (6)	0 (6)	0 (18)	0 ± 0 d

*Means followed by same letter are not significantly different.

non-linear ($r^2 = 0.199$), the reduction in the percentage of spermathecae containing sperm was correlated with time postcopulation ($r^2 = 0.694$).

The results show that not only is the ventral receptacle the first storage organ to receive sperm, it is also the last storage organ that contains sperm as that commodity is depleted. Given that the ventral receptacle is the organ where fertilization of the egg is accomplished, this sequence is logical. Indeed, studies on related species such as *Anastrepha suspensa* (Loew) (Fritz 2004) and *Anastrepha obliqua* (Macquart) (Perez-Staples and Aluja 2006) similarly recorded the relative importance of the ventral receptacle for sperm storage. One might expect that there would be a feedback system regulating the flow of sperm from the spermathecae to the ventral receptacle assuring that the latter is recharged as fast as it is depleted, and that appears to be the case. Although the database in this study is not exhaustive, the results of the two replicates were consistent between one another and previously published studies. Specifically, our results showing depletion of sperm at 28–35 d postcopulation in both replicates closely mirrors those in the study by Mangan (1997) in which females produced fertile eggs for means of 26–40 d when oviposition substrate was continuously available. The data thus support the hypothesis that infertility resulted from sperm depletion rather than sperm inviability.

Although the functional relationship between the sperm storage organs, wherein the spermathecae recharge the depleted ventral receptacle, seems straightforward in *A. ludens,* there is evidence that other considerations, including sperm competition, may have an influence. Perez-Staples and Aluja (2006) found that *A. obliqua* females hold proportionately larger amounts of sperm in the ventral receptacle compared to other species of *Anastrepha* and hypothesize that this is an adaptation to the phenology of its host plant, resulting in a narrower window of oviposition opportunities. In that regard, we note that the number of alveoli in the ventral receptacle differs among the species of *Anastrepha* according to Martinez and Hernandez-Ortiz (1997), and it is likely that such differences are related to the ecology of the ovipositional host. In our preparations, it appears that *A. ludens* has approximately 200 alveoli, about the same as reported by Fritz and Turner (2002) for *A. suspensa*. Those authors found that each alveolus contained from 0–4 coiled

(mean 2.5) spermatozoa, with a capacity of 440–800 total sperm. And, although those authors described the stalked alveoli as "a cluster of grapes," a more appropriate analogy might be to a "blackberry," as the alveoli are arranged around a central space or lumen. The same authors reported finding up to 300 spermatozoa present in the receptacle lumen of *A. suspensa*. In that regard, our prior-mated status technique (Thomas et al. 2014) relied on staining of the ventral receptacle in order to visualize sperm bundles as inclusions within the alveoli. The refinement used in this study, a simple squash technique to visualize the presence of sperm in the ventral receptacle, was found to be reliable without staining. Given the statement by Fritz (2004) that the ventral receptacle does not lend itself to the squash technique, it may be that the sperm visualized by us were those situated in the lumen of the seminal receptacle, as the alveoli themselves do not appear ruptured in our preparations. It is unclear how sperm are released from the alveoli at the time of fertilization, although Fritz and Turner (2002) hypothesize that they may be expressed by muscular contraction of the receptacle.

From a practical perspective, managers of SIT programs need to maintain high overflooding ratios not just to minimize fertile matings, but to mitigate against remating and prolonged fecundity as well. Also, because the mated status of female flies is used to gauge the efficacy of sterile males, our present and previous studies affirm the importance of including the ventral receptacle in that measurement and not just the spermathecae.

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