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Control of mating plug expelling and sperm storage in *Drosophila*: A gynandromorph- and mutation-based dissection

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Introduction: In this study, we analyzed gynandromorphs with female terminalia, to dissect mating-related female behaviors in *Drosophila*. *Materials and methods:* We used gynandromorphs, experimentally modified wild-type (Oregon-R) females, and mutant females that lacked different components of the female reproductive apparatus. *Results:* Many of the gynandromorphs mated but did not expel the mating plug (MP). Some of these – with thousands of sperm in the uterus – failed to take up sperm into the storage organs. There were gynandromorphs that stored plenty of sperm but failed to release them to fertilize eggs. Expelling the MP, sperm uptake into the storage organs, and the release of stored sperm along egg production are separate steps occurring during *Drosophila* female fertility. Cuticle landmarks of the gynandromorphs revealed that while the nerve foci that control MP expelling and also those that control sperm uptake reside in the abdominal, the sperm release foci derive from the thoracic region of the blastoderm. *Discussion and conclusion:* The gynandromorph study is confirmed by analyses of (a) mutations that cause female sterility: *Fs(3)Avar* (preventing egg deposition), $Tm2^{gs}$ (removing germline cells), and $iab\text{-}4^{DB}$ (eliminating gonad formation) and (b) by experimentally manipulated wild-type females: decapitated or cut through ventral nerve cord.

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INTRODUCTION

During mating – that lasts for 15–20 min in *Drosophila melanogaster* – about 3,000–4,000 sperm are transferred along with the seminal fluid into the uterus (Adams & Wolfner, 2007; Bloch Qazi et al., 2003). From about the 5th min of mating, some of the accessory gland proteins coagulate in the distal part of the uterus and form a gelatinous structure called the mating plug (MP; Adams & Wolfner, 2007; Avila et al., 2015; Lung & Wolfner, 2001; Mattei et al., 2015). The MP is not only a physical barrier that prevents sperm loss but may also facilitate sperm storage by confining the sperm to the anterior region of the uterus adjacent to the openings of the sperm storage organs: the seminal receptacle and the paired spermathecae (Adams & Wolfner, 2007; Bairati & Perotti, 1970; Lung & Wolfner, 2001; Polak et al., 1998). The MP may also prevent consecutive mating (Hosken et al., 2009).

Sperm storage begins already during copulation and is completed by about 1 hr following mating (Bloch Qazi & Wolfner, 2003). About 800–1,000 sperm are stored in the storage organs, about 80% in the seminal receptacle, and the rest in the spermathecae (Bloch Qazi et al., 2003; Bloch Qazi & Wolfner, 2006). Over 40% of the stored sperm fertilize the mature egg during the upcoming days (Neubaum & Wolfner, 1999). Fertilization happens when an egg – on its way from the ovaries into and through the oviduct – enters the uterus, shortly before egg deposition. As a rule, only one sperm is present per fertilized egg in *Drosophila* (Hildreth & Lucchesi, 1963).

In the different insect species, the MP may gradually dissolve, pushed out by the first egg to be deposited, or can also be expelled along with the non-stored sperm (Bertram et al., 1996; Bloch Qazi et al., 2003; Polak et al., 1998; Takami et al., 2008). In *D. melanogaster*, the MP leaves the uterus about 6 hr following mating at the time when the MP imposed prevention from remating taken over by another mechanism (Hasemeyer et al., 2009). The "second" mechanism is established and maintained by some of the accessory gland proteins of which sex peptide is of outstanding importance

in control of the so-called postmating responses (Kubli & Bopp, 2012; LaFlamme et al., 2012; Rezával et al., 2012; Yang et al., 2009). Following depletion of the stored sperm in 5–7 days, the females become receptive again, mate, and possess the postmating responses.

The different steps in the female reproductive behavior are coordinated by both chemical and neural signaling (Bussell et al., 2014; Feng et al., 2014; Garaulet et al., 2014; Hasemeyer et al., 2009; Heifetz et al., 2014; Kubli & Bopp, 2012; LaFlamme et al., 2012; Middleton et al., 2006; Rezával et al., 2012, 2014; Yang et al., 2009; Zhou et al., 2014). Genetic dissection using gynandromorphs revealed four groups of neurons – called foci – in the head of the *Drosophila* females that control (a) the receptivity to male courtship, (b) the detection of insemination, (c) the rate of egg production, and (d) the site of egg deposition. There were three foci identified in the thorax that control egg transfer (a) from the ovaries into the uterus, (b) the release of sperm from the storage organs, and (c) oviposition (Szabad & Fajszi, 1982; Tompkins & Hall, 1983). The focus for female sex appeal resides in the abdomen (Jallon & Hotta, 1979). However, a few steps in the female reproductive behaviors remained to be elucidated and stimulated this study.

By combining genetic and physiological techniques, in this study, we report novel features of MP expelling, sperm uptake from the uterus into the storage organs, and sperm utilization in *Drosophila*. Analysis of (a) gynandromorphs; (b) wild-type females that were decapitated, or their ventral nerve cord (VNC) was cut through; and (c) females from which the germline cells were genetically deleted clearly showed that MP expelling is a female-specific behavior. Fate-mapping – based on the sex of the cuticle landmarks in gynandromorphs – located the MP-expelling nerve focus in the abdomen. Analysis of $iab\text{-}4^{DB}$ homozygous females – that mate but fail to expel the MP – placed the primordium of the corresponding focus into the 9th parasegment (i.e., the 3rd–4th abdominal segment).

We describe that sperm uptake from the uterus into the storage organs is a female-specific behavior: some of the gynandromorphs mate and carry plenty of sperm in the uterus and none in the storage organs. Gynandromorph fate mapping located the sperm uptake focus into the abdomen. Since the $iab\text{-}4^{DB}$ homozygous females mate but fail to take up sperm into the storage organs, we concluded that the corresponding control focus derives from the 9th parasegment. It is different from the one that controls MP expelling. Using the above types of flies, we show that the stored sperm are depleted only when fertilized eggs are produced. We also discuss the mechanism of egg transfer from the ovaries into the uterus and verify that the focus for sperm release from the storage organs resides in the thorax.

MATERIALS AND METHODS

In this study, we used gynandromorphs, experimentally modified wild-type (Oregon-R) females and mutant females that lacked different components of the female reproductive apparatus. The gynandromorphs were generated by crossing *XX* (*y v f mal* homozygous) females with X/Y; $lds^{Hor\text{-}D}$/*TM3*, *Sb Ser* males (Szabad et al., 1995; for a description of the genetic symbols, visit the FlyBase at http://flystocks.bio.indiana.edu). The *X* chromosome carried the wild-type alleles of the *y*, *v*, *f* and *mal* recessive marker mutations and thus the *XX* females appear wild type. The $lds^{Hor\text{-}D}$ mutation renders the *X* chromosome unstable during spermatogenesis, such that it may be lost in the descending zygotes during early embryogenesis (Szabad et al., 1995; Szalontai et al., 2009). Loss of the *X* chromosome leads to the formation of *XX/X0*, female/male mosaics, gynandromorphs in which the male tissues show the phenotypes of the above marker mutations. Since the line that separates the female and the male tissues is random, the gynandromorphs possess a wide range of reproduction-related behaviors (Cook, 1978; Hall, 1978; Hotta & Benzer, 1972, 1976; Janning, 1978; Szabad & Fajszi, 1982; Szabad et al., 1995; Tompkins & Hall, 1983). Only those gynandromorphs were considered in this study that had female genitalia and could thus mate. They were collected as virgins, brought, and kept together with males for the first 2 days of their life. In the males, green fluorescent protein (GFP)-tagged Don Juan protein highlighted the sperm tail (Santel et al., 1997). Two groups of gynandromorph were studied. In the first group (89 gynandromorphs), where we focused on MP expelling and sperm uptake from the uterus into the storage organs, the gynandromorphs were separated from the males after 2 days, kept for one additional day before dissection and analysis of their internal reproductive organs. In the second group (115 gynandromorphs), we focused on sperm storage and sperm release from the storage organs. The mated gynandromorphs were separated from the males and were individually transferred into plastic tubes. Mating was inferred from the green fluorescent light emitted by the stored GFP-tagged sperm in the internal genitalia as observed in a dissecting microscope equipped with a GFP-detection setup. Nineteen such tubes have been glued together in a block, which rested on a Petri dish containing apple juice egg-laying medium. The Petri dish was changed in 24-hr intervals, such that a tiny drop of live yeast was placed onto the food to each of the gynandromorphs every day. The number of eggs and the hatching larvae were counted for single gynandromorphs throughout 7–9 days. Before dissection of the gynandromorphs, the genotypes (whether *XX* female, *X0* male or mosaic) of the following cuticle landmarks were determined: antenna, palpus, orbital bristles, wing, notum, legs, and the 2nd–6th tergites and sternites (Hotta & Benzer, 1972; Janning, 1978). Following dissection, the internal reproduction organs were analyzed: type and condition of the gonads, the oviduct, the sperm storage organs, and the presence/absence of the MP. While the GFP-highlighted sperm glow green, the MP was identified through its strong yellowish autofluorescence in a fluorescence microscope. Numbers of the stored sperm heads were counted in a phase contrast microscope.

Wild-type females were collected as virgins and aged for 3–4 days when they were brought together with males in which GFP highlighted the sperm. To elucidate the mechanism of MP expelling, sperm uptake from the uterus into the storage organs, and sperm release, one of the following operations was carried out in less than 1 min after mating: (a) decapitation, (b) the VNC was cut through with a fine

iridectomic scissor between the 3rd and the 4th sternites, (c) abdomens were isolated by constricting the abdomen at its anterior end with a *ca.* 20-µm-thick plastic thread loop before cutting away the head and the thorax, (d) egg deposition was prevented by sealing the vaginal opening with a tiny drop of Loctite instant adhesive. To make the VNC visible for severing, we generated *elav-Gal4; UAS-EGFP* females in which the VNC glowed green. The females were briefly narcotized with ether before operations or dissection.

The egg producing but non-ovipositing *Fs(3)Avar*/+ females carried one of the dominant female sterile mutations (Erdélyi & Szabad, 1989). Females without germline cells descended from $Tm2^{gs}$ homozygous mothers. $Tm2^{gs}$ is a grandchildless mutation that blocks germline cell formation without affecting function of the mesoderm-derived components of the gonads (Barth et al., 2011; Erdélyi et al., 1995). Females without ovaries were homozygous for $iab\text{-}4^{DB}$ (Busturia et al., 1989). The $iab\text{-}4^{DB}$ homozygous mutant flies lack gonads as a consequence of the failure of gonadal soma formation due to homeotic transformation (Boyle & DiNardo, 1995; Cumberledge et al., 1992; Karch et al., 1985; Tremml & Bienz, 1989).

Statistical significance between frequencies of the different types of gynandromorphs was analyzed using the Fisher's exact test. The *t*-test was used in comparing the mean values of the stored sperm.

RESULTS

Gynandromorphs and MP expelling

Random distribution of relatively large patches of female (*XX*) and male (*X0*) tissues is a characteristic feature of the *Drosophila* gynandromorphs. Therefore, sex – whether female, male, or mosaic – of their reproductive organs and the nerve foci that control sexual behavior show much variation and were of great use in both fate-mapping organ primordia and the dissection of reproductive behaviors (Hotta & Benzer, 1972, 1976; Janning, 1978; Szabad & Fajszi, 1982; Szabad & Nöthiger, 1992; Tomkins & Hall, 1983; Villella & Hall, 2008). As described here, an analysis of gynandromorphs led to the discovery of novel features of the *Drosophila* female reproductive behavior and rough localization of the corresponding control nerve foci.

There were 1,466 gynandromorphs collected in this study of which 460 had female genitalia and 256 of these mated. Eighty-nine of the 256 gynandromorphs were included in the study that focused on MP expelling and sperm uptake from the uterus into the storage organs. Another 115 gynandromorphs served to study sperm utilization and fertility. The internal genitalia were thus dissected and analyzed in 204 gynandromorphs.

Of the 204 gynandromorphs, 24 did not expel the MP (Fig. 1). They might have failed to carry out this female-specific function because the foci that control MP expelling were composed from male cells. To locate the MP expelling focus, we made use of the principles of blastoderm fate mapping: primordia of the control foci lean closest to those cuticle landmarks that contain most frequently male tissue in

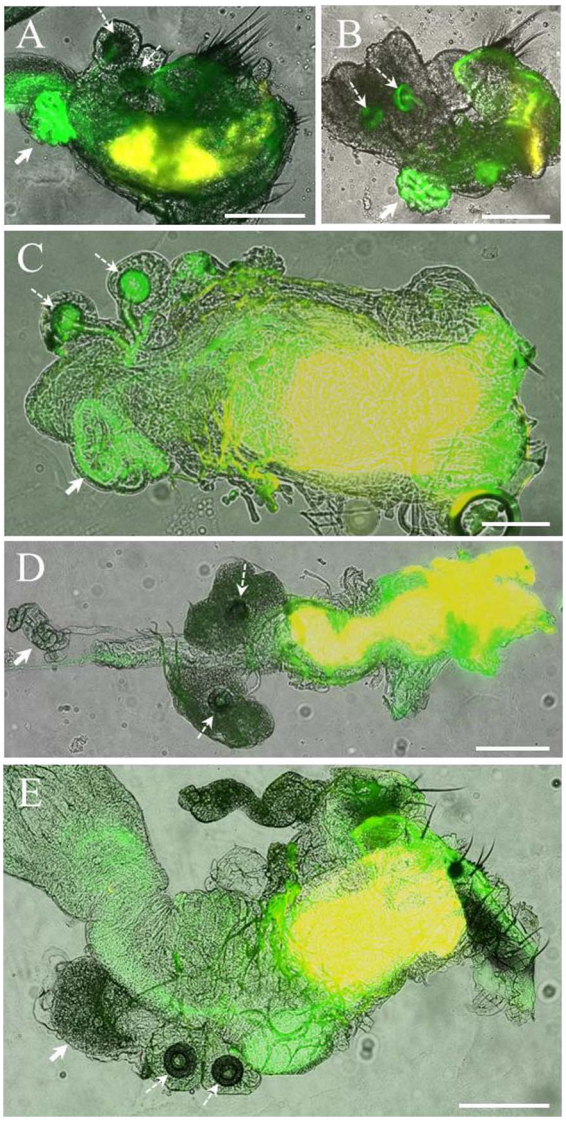

Fig. 1. Mating plug and sperm in females and gynandromorphs. Overlay of three photographs: bright field, green fluorescence emitted by GFP-highlighted sperm and mating plug with yellow autofluorescence. Arrows point to the sperm storage organs: the thick and the thin dashed arrows on the seminal receptacle and the spermathecae, respectively. (A) Wild type shortly after mating. (B) Wild type 1 day after mating. (C) Gynandromorph that did not expel the MP plug and stored sperm in the storage organs. (D) Gynandromorph that did not expel the MP and failed to take up sperm into the storage organs. (E) An $iab\text{-}4^{DB}$ homozygous female that did not expel the MP and failed to store sperm in the storage organs. Scale bars = 200 µm

the 24 gynandromorphs (Hotta & Benzer, 1972; Janning, 1978). It appears that the MP-expelling focus derives from the abdominal blastoderm region; (a) while the entire head and thoracic cuticle were female in 5 of the 24 non-expelling gynandromorphs, at least some of the tergites and/or sternites contained male cells in every case (Fig. 2). (b) While the frequencies of the head and thorax cuticle landmarks with male cells were not significantly different between the MP expelling and the non-expelling gynandromorphs, the male cells were significantly more frequent in the sternites and tergites in the non-expelling ones (Table 1).

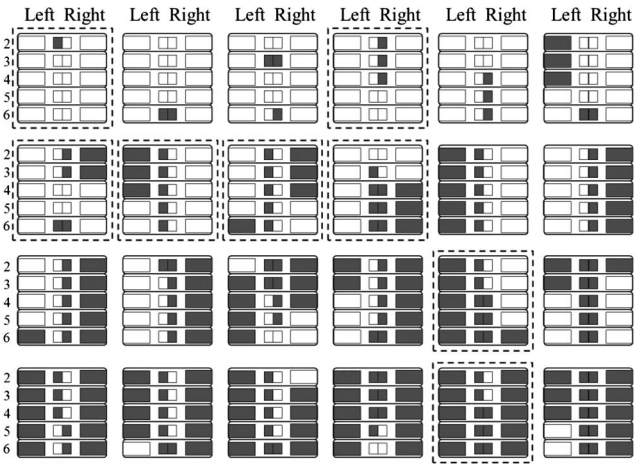

Fig. 2. Distribution of female (gray) and male cell containing (white) tergites and sternites in the 24 gynandromorphs that did not expel the mating plug. Numbers on the left delineate the 2nd–6th abdominal segments. The tergites are symbolized by the rectangles along the right and the left sides of the charts. The squares in the middle of the segments correspond to the sternites. Pictures framed in dashed line correspond to those gynandromorphs that did not take up sperm from the uterus into the storage organs

Nonetheless, the site of the focus could not be precisely determined, since the abdominal segments rather uniformly contained male cells.

The MP-expelling focus appears to be domineering in action (cf. Hotta & Benzer, 1972, 1976), i.e., male focus on either side of a gynandromorph is sufficient to prevent MP plug expelling: although all the sternites and the tergites were composed of female cells on one side of the abdomen in nine of the 24 gynandromorphs, yet they failed to expel the MP (Fig. 2) Such gynandromorphs also appear among those that expel the MP, although at a much higher frequency; 9/24 vs. 131/180, $p < .01$. There are two explanations for the formation of the unusual gynandromorphs, which, for example, can expel the MP, although their tergites and/or sternites contain male cells. (a) The relatively large distance between the progenitor cells of the neurogenic region from which the foci nerve cells derive and the dorsal ectoderm sites that give rise to the sternites and the tergites. The 84 Sturt width of the blastoderm fate map leaves plenty of room for the formation of such gynandromorphs (Janning, 1978; for an illustration, visit the http://www.sdbonline.org/fly/atlas/0607.htm website of Hartenstein, 1993). (b) It is also possible that the MP expelling focus is submissive in action, i.e., the combined absence of foci function on both sides leads to the failure of the process. A direct analysis of the abdominal ganglia of gynandromorphs – whether their nerve cells on the two sides are female, male, or both – may reveal the domineering or the submissive nature of the MP-expelling focus.

Gynandromorphs and sperm uptake for storage

Among the 24 gynandromorphs that mated but did not expel the MP and therefore carried plenty of sperm in the uterus, 8 failed to take up sperm from the uterus into the storage organs even though their internal genital apparatus appeared normal (Fig. 1). This observation clearly shows that sperm uptake from the uterus into the storage organs is a female-controlled process and sperm do not enter the storage organs on their own or are not squeezed in there.

Cells of the sperm uptake focus originate from the abdominal region of the blastoderm: the abdominal cuticle landmarks significantly more frequently contained male cells in the 8 gynandromorphs as compared to those 196 that stored sperm (Table 1; Fig. 2). However, and as in case of the MP-expelling focus, the cuticle patterns of the gynandromorphs do not allow precise localization of the site of the sperm uptake focus. Whether the sperm uptake foci are domineering or submissive in action cannot be determined based on the sex of the tergites and the sternites.

Gynandromorphs and fertility

Of the 256 gynandromorphs set aside for the analysis of sperm utilization and fertility, 115 lived long enough to be included in this study: their egg and progeny production were analyzed in the absence of males for 7–9 days before dissection and analysis of the reproductive organs. Of the 115 gynandromorphs, 40 mated, expelled the MP, stored sperm, and deposited eggs and larvae hatched from at least some of the eggs. Of the 40 gynandromorphs, 35 resembled the wild-type females: they deposited 42.9 ± 10.7 (mean $\pm$ *SD*) eggs per day and larvae hatched from almost all of their eggs during the first 4–5 days. After running out of the stored sperm, their egg production rate dropped to 1–3 eggs per day and larvae did not hatch from these eggs. Their sperm storage organs were practically empty when dissected. Although mated and stored sperm, 5 of the 40 gynandromorphs deposited only 1–9 eggs per day. Larvae hatched from all their eggs throughout the test period. There were still plenty of stored sperm in these gynandromorphs 9–11 days after mating. They might have not detected fertilization and/or were defective in increasing the egg production rate as discussed earlier (Szabad & Fajszi, 1982).

Forty-five of the 115 gynandromorphs mated, expelled the MP, and stored the sperm, although they deposited eggs larvae did not hatch from their eggs. Thirty-six of the 45 gynandromorphs deposited 39.2 ± 8.3 eggs per day throughout the test period at a frequency not different from the above 42.9 ± 10.7 ($t = 1.631$; $p = .11$). These gynandromorphs detected mating, set high egg production rate but failed to release sperm to fertilize the eggs. Nine of the 45 gynandromorphs deposited 1–9 eggs per day. They were probably defective in sensing mating and/or setting high egg production rate. The failure to release sperm is best indicated by the average of the 391 ± 97 sperm in the storage organs upon dissection (Table 2). This value is not significantly different from the number of the stored sperm (432 ± 84) in those 14 sibling gynandromorphs in which the eggs were not transferred from the ovaries into the uterus or from 402 ± 95 counted in those 16 in which eggs did not form (Table 2; $t = 0.910$; $p > .05$). In agreement with a previous report (Szabad & Fajszi, 1982), it appears that the primordial cells of the sperm release focus derive from the thoracic blastoderm region: the legs contain more frequently male cells in the 45 gynandromorphs that deposited unfertilized eggs as compared to those 40 that produced offspring

Table 1. Frequencies of cuticle landmarks with male cells in the different types of gynandromorphs

Type of the gynandromorph		Gynandromorph	Head			Thorax				Abdomen						
			Antenna	Palpus	Orbital bristles	Wing	Notum	Leg 1	Leg 2	Leg 3	Tergite 2	Tergite 3	Tergite 4	Tergite 5	Tergite 6	Sternite 2	Sternite 3	Sternite 4	Sternite 5	Sternite 6
Was the mating plug expelled?	Yes (control)	180	0.24	0.26	0.24	0.30	0.32	0.26	0.26	0.29	0.24	0.26	0.28	0.30	0.26	0.20	0.25	0.27	0.23	0.10
	No	24	0.33	0.33	0.32	0.35	0.40	0.35	0.40	0.44*	0.48**	0.46**	0.50**	0.60**	0.56**	0.50**	0.48**	0.48**	0.52**	0.35*
Were sperm taken up into the storage organs?	Yes (control)	196	0.26	0.28	0.25	0.34	0.33	0.30	0.28	0.32	0.27	0.29	0.30	0.32	0.29	0.24	0.27	0.30	0.26	0.12
	No	8	0.25	0.25	0.25	0.50	0.56	0.38	0.50	0.63*	0.63**	0.63**	0.63**	0.75**	0.63**	0.56**	0.56**	0.44	0.50*	0.38*
Were the eggs fertilized?	Yes (control)	40	0.18	0.21	0.16	0.30	0.29	0.23	0.23	0.24	0.25	0.29	0.34	0.31	0.29	0.21	0.18	0.20	0.24	0.20
	No	45	0.21	0.23	0.21	0.39	0.38	0.34	0.34	0.37	0.28	0.28	0.26	0.27	0.20	0.28	0.27	0.27	0.32	0.21

Note. * and **Significantly different from the control at $p < .05$ and $p < .01$, respectively; Fisher's exact test.

($p = .09$ vs. $p = .21$–1.00 for the other cuticle landmarks; Table 1).

Although mature eggs formed in 14 of the 115 gynandromorphs, those were not deposited (Table 2). The 14 gynandromorphs were of three types. (a) Not a single egg entered the oviduct (9 gynandromorphs. The ovaries were attached to the oviduct and the attachments appeared normal. (b) In a number of gynandromorphs, a single egg became stuck in one of the lateral oviducts (3 gynandromorphs). (c) An egg – with decomposed cytoplasm – was present in the uterus usually along with another egg in one of the lateral oviducts (2 gynandromorphs). These observations indicate that egg transfer is a three-step process: from the ovaries into the oviduct, through the oviduct into the uterus and egg deposition. The three types of the egg-non-depositing gynandromorphs suggest that different nerve foci control the three steps. The gynandromorph-based dissection of egg transfer is beyond the topic of the present paper.

In 16 of the 115 gynandromorphs eggs did not form either because the ovaries were missing or only the mesoderm-derived components of the ovaries were present without germline cells (Table 2; see also Szabad & Nöthiger, 1992). Although the above 30 gynandromorphs never laid eggs, they expelled the MP showing that this process is indeed a female-specific, nerve-controlled behavior, and is independent of egg deposition.

Experimentally altered wild-type females

Mating brings about remarkable changes (postmating responses) within hours in the behavior of the wild-type *Drosophila* females: they store sperm in the storage organs, get rid of the MP along with the excess sperm, turn non-receptive and start to produce eggs with high rate (Fig. 1). Practically, all the eggs are fertilized and larva hatch from every one of them (Table 3). The changes are largely related to the sex peptide, a small seminal protein (Kubli & Bopp, 2012). Upon depletion of the stored sperm in about a week, the females become receptive again, mate, and possess the postmating responses.

The decapitated females, whose head was removed within a minute after mating, expel the MP, take up sperm into the storage organs and store plenty of sperm (Table 3). Such females deposited only few eggs all of which were fertilized and larvae hatched from every one of them (Table 3). Importantly, the behavior of those females that were first decapitated and mated afterward (4 in 103) was identical to those in which the head was removed right after mating. The low egg deposition rate is the consequence of the lack of nutrient supply and/or the absence of the foci in the head that detect mating and establish high egg production and deposition rates (Szabad & Fajszi, 1982). As compared to the newly mated control females, the amount of the stored sperm did not change noticeably in 7 days in the decapitated females. The above observations show that foci controlling MP expelling, sperm uptake from the uterus into the storage organs, egg transfer from the ovaries into and through the oviduct, sperm release from the storage organs and egg deposition reside outside the head.

Table 2. Number of stored sperm in different types of gynandromorphs[a]

Features of the gynandromorphs	Sperm stored in the storage organs[b]	
	Mean ± *SD*	*N*
Mating plug not expelled and sperm taken up into the storage organs[a]	441 ± 140	16
Mating plug expelled, sperm stored, and eggs deposited but not fertilized	391 ± 97	36
Mating plug expelled, sperm stored, and eggs did not leave the ovaries	432 ± 84	14
Mating plug expelled, sperm stored, and ovaries did not form or did not contain germline cells	402 ± 95	16

Note. SD: standard deviation.
[a]Counted 7–9 days after separation from the males, on the 9th–11th day of their life. [b]Sperm in the uterus were not considered.

Obviously, nerve foci that coordinate reproduction exert activities through the musculature of the ovaries and the internal genital apparatus (Bussell et al., 2014; Feng et al., 2014; Garaulet et al., 2014; Hasemeyer et al., 2009; Heifetz et al., 2014; Middleton et al., 2006; Rezával et al., 2012, 2014; Zhou et al., 2014). After cutting through the VNC – within 1 min following mating – the MP was not expelled for at least 5 days and consequently sperm remained in the uterus (Table 3). Remarkably, despite severing the VNC, sperm were normally taken up into the storage organs. In the VNC-cut-through females, the eggs did not leave the ovaries to enter the lateral oviduct and sperm were not depleted from the storage organs. Identical effects were observed in the isolated abdomens that were separated from the rest of the body within 1 min following mating: the MP was not expelled and although a huge number of sperm remained in the uterus as many sperm were taken up into the storage organs as in the normal females. In the isolated abdomens, the mature egg did not leave the ovaries to enter the oviduct (Table 3). The lack of MP expelling and egg transfer from the ovaries into the oviduct clearly shows the involvement of the VNC in these processes. That sperm uptake into the storage organs is normal in the VNC-severed females and in the isolated abdomens is unexpected knowing that the extremely condensed small abdominal nerve center is located in the posterior thorax and may be expected to exert activities through the VNC (Middleton et al., 2006; Power, 1948). Perhaps, there is a VNC independent, not yet identified nerve route that controls sperm uptake. It is also possible that mating triggers sperm uptake within a minute and thus severing the VNC or the removal of the head and the thorax cannot prevent sperm uptake.

There appears to be a tight correlation between the deposition of fertilized eggs and sperm depletion from the storage organs. When only few or no eggs are deposited essentially as many sperm remain stored in the storage organs after 4–9 days as in the newly mated females. Females in which the genital opening was sealed within a minute following mating support this statement; they cannot expel the MP and store as many sperm in the storage organs after 7 days as seen in the newly mated females (Table 3).

Genetically altered females

It was suggested earlier that the *Drosophila* females get rid of the MP during deposition of the first egg in (Bloch Qazi et al., 2003; Neubaum & Wolfner, 1999; Polak et al., 1998). This assumption may appear to be correct as the *Fs(3)Avar*/+ females produce mature egg at a rate as the wild-type females; however, they never deposit a single egg. They do not expel the MP and the sperm remain in the uterus up to at least 9 days following mating (Table 3). They take up and store sperm in the storage organs as the wild-type females. Ovarian chimeras revealed that the unusual behavior of the *Fs(3)Avar*/+ females is the consequence of altered somatic function and is not ovary-related: while chimeras with normal soma and *Fs(3)Avar*/+ ovaries produce offspring from the *Fs(3)Avar*/+ ovaries, those with *Fs(3)Avar*/+ soma and normal ovaries do not deposit eggs (Erdélyi & Szabad, 1989).

The analysis of females in which the germline cells are missing and only the somatic components of the gonads form clearly showed that the ability to expel the MP does not require the deposition of mature egg (Table 3). Such females that descended from mothers homozygous for the grandchildless mutation $Tm2^{gs}$ (Erdélyi et al., 1995) expel the MP alike the wild-type females (Table 3). The soma functions normally in such females as was shown by the behavior of germline chimeras that developed following the transplantation of normal germline founder cells into $Tm2^{gs}/Tm2^{gs}$-derived host embryos; eggs developed and offspring descended from such germline chimeras (Barth et al., 2011). As expected, the grandchildless-derived females take up and store sperm as the wild-type females. However, the number of the stored sperm does not decrease noticeably in time and thus support the correlation between deposition of fertilized eggs and depletion of the stored sperm (Table 3).

While the ovaries are missing in the $iab\text{-}4^{DB}/iab\text{-}4^{DB}$ females, the external and the internal genitalia appear normal. In such flies, the absence of *iab-4* function leads to the failure of gonadal soma and therefore leads to gonad formation (Bender, 2008; Boyle & DiNardo, 1995; Cumberledge et al., 1992; Garaulet et al., 2014; Karch et al., 1985). Although the ovaries of the $iab\text{-}4^{DB}$ homozygous females are missing, they elicit courtship and mate. They do not expel the MP, fail to take up sperm from the uterus into the storage organs, and carry sperm in the uterus even 9 days after mating (Fig. 1; Table 3). The lack of MP expelling is most likely the result of homeotic transformation of the 9th parasegment to the 8th (Karch et al., 1985) that alters neural patterning and reproductive behavior (Garaulet et al., 2014). The finding that the $iab\text{-}4^{DB}$ homozygous females fail to take up sperm from the uterus into the storage organs confirms that the process is a female-controlled process.

Table 3. Mating plug expelling, sperm uptake and storage, egg transfer, and sperm storage depletion in different types of females

Specimen[a]		Features		Is mating plug expelled?	Sperm location	Egg transfer		Depletion of stored sperm
						From ovary into oviduct	Through oviduct into the uterus	
Females[a]	Wild type	Normal	Functional ovaries. Eggs produced, fertilized, deposited (about 70 per day), larvae hatch	Yes	SR and STs	Yes	Yes	Completed in about seven days
		Decapitated	Functional ovaries. Eggs produced, fertilized, deposited (less than one per day), larvae hatch	Yes	SR and STs	Yes	Yes	None (day 7)
		Ventral nerve cord cut through	Functional ovaries. Eggs produced, mature but do not enter the oviduct	No	SR and STs and uterus	No	No	None (day 5)
		Isolated abdomen	Functional ovaries. Eggs do not enter the oviduct	No	SR and STs and uterus	No	No	None (day 4)
		Sealed vaginal opening	Functional ovaries. Eggs produced, mature as in wild type but (except one) do not enter the oviduct	No	SR and STs and uterus	Yes	Yes	None (day 7)
	Mutant	*Fs(3)Avar*/+	Functional ovaries. Egg produced and mature. Only a single egg enters the oviduct but is not transferred into the uterus	No	SR and STs and uterus	Yes	No	None (day 9)
		Daughters of $Tm2^{gs}$ homozygous mothers	No germline cells in the ovaries. Only the mesoderm-derived components of the ovaries develop	Yes	SR and ST	–	–	None (day 9)
		$iab\text{-}4^{DB}/iab\text{-}4^{DB}$	Ovaries do not develop	No	Uterus	–	–	None (day 9)

Note. SR: seminal receptacle; ST: spermathecae.
[a]There were at least 21 specimen analyzed for each type.

DISCUSSION

Reproduction in the animal kingdom is a multicomponent process. It includes not only the origin, formation and function of the germline, and the reproductive organs, but also those groups of the nerve cells that coordinate organ functions as well as the sexual interplay between the mates. The dissection of sexual behavior in *Drosophila* commenced with the classical papers of Hotta and Benzer (1972, 1976). By making use of gynandromorphs, they not only revealed the sequential steps in courtship behavior but also determined the sites on the blastoderm fate map of the controlling nerve foci and their domineering or submissive mode of action. Their statement "*Additional foci must surely exist for other detailed steps*" encouraged further studies to dissect sexual behavior (Cook, 1978; Hall 1978; Szabad & Fajszi, 1982; Tomkins and Hall 1983). The present paper is a late continuation of the project. The combined use of gynandromorphs and also experimentally or and genetically modified females not only revealed the existence of new steps in the *Drosophila* female reproductive behavior but was also of use in localizing the primordia of the control foci on the fate map. We report here novel features of *Drosophila* female reproduction and deal mostly with the control of MP expelling, sperm uptake from the uterus into the storage organs, and to some extent with egg transfer through the oviduct and sperm utilization.

MP expelling

The finding that there are gynandromorphs and also females in which eggs do not form and yet they expel the MP clearly shows that the MP is not pushed out by the first deposited egg and that MP expelling is a female-specific behavior. This conclusion is supported by the existence of gynandromorphs that mate but do not expel the MP most likely because the control foci are composed from or contain at least a few male cells. The correlation between the lack of MP expelling and the regular presence of male cells in the tergites and the sternites in the MP-non-expelling gynandromorphs indicates that the MP-expelling focus derives from the abdominal blastoderm region. This conclusion is in agreement with the observations that while the decapitated females expel the MP, those with cut through VNC and also the isolated abdomens cannot do so. Since the abdominal ganglia reside in the posterior thorax in the adults (Power, 1948; Middleton et al., 2006), blocking their action (by severing the VNC) or their removal (isolated abdomens) is expected to prevent MP expelling. However, other than locating the MP-expelling focus into the abdomen, neither gynandromorph fate mapping nor the experimental modifications of wild-type females allowed precise localization of the focus. Analysis of *iab-4*^{DB} homozygous females – that mate but fail to expel the MP – indicates that the MP expelling focus derives from the 9th parasegment that includes the posterior compartment of the 3rd and the anterior of the 4th abdominal segments): in the *iab-4*$^{-}$ mutant flies the ectoderm of the 9th parasegment is transformed to the 8th (Karch et al., 1985). It appears that the transformation leads to the failure to form of the MP expelling focus. That this focus derives from the 9th parasegment is supported by a report of Cumberledge et al. (1992). To overcome the lack of gonadal soma formation in the *iab-4*$^{-}$ homozygous flies, they transplanted wild-type cleavage nuclei into cleavage *iab-4*$^{-}$ mutant embryos at the presumptive site for gonadal soma formation. Not only ovaries formed in two of the developing chimeras but they also deposited eggs. It appears that the cells that formed around the wild-type nuclei not only gave rise to the gonadal soma, but also to at least some of the neuroectoderm cells from which the MP-expelling focus derived. Existence of these two chimeras suggests that the MP-expelling focus derives from the 9th parasegment or from the nearby blastoderm cells.

Sperm uptake

The existence of gynandromorphs, which carry ample of sperm in the uterus and none in the storage organs, clearly shows that sperm uptake from the uterus into the storage organs is a female-specific behavior and is in accordance with a previous report by Arthur et al. (1998). The observation that the abdominal cuticle plates of the sperm-non-uptake gynandromorphs contain more frequently male cells than in those siblings that store sperm indicates that the sperm uptake focus derives from the abdominal blastoderm region. This conclusion is supported by the finding that the *iab-4*^{DB} homozygous females mate but do not take up sperm to store and suggests that primordial cells of the sperm uptake focus derive from the 9th parasegment of the blastoderm embryo. This finding is supported by a report that those few eggs did not hatch that were deposited by the very rare females from which the *iab-4*-encoded miRNAs had been removed (Bender, 2008). Although such females mated, their eggs might have not been fertilized possibly because of the lack of stored sperm. Unfortunately, Cumberledge et al. (1992) did not report whether any of the chimeras that developed following the transplantation of normal cleavage nuclei into *iab-4*$^{-}$ mutant embryos took up sperm for storage or their egg hatched and thus an experimental verification for the origin of the sperm uptake focus from the 9th parasegment is yet to come.

It is very unlikely that MP expelling and sperm uptake are controlled by the same focus. If the same focus controlled MP expelling and sperm uptake, only two types of behaviors would be expected: (a) expel the MP and store sperm and (b) do not expel the MP and do not store sperm. However, some of the gynandromorphs do not expel the MP but store sperm and a few were seen to mate and yet did not store sperm (regrettably, such gynandromorphs were not concerned in this study). A direct analysis of the nerve cells – whether they are *XX* or *X0* – in the abdominal ganglia of the gynandromorphs that possess different types of behavior could settle not only the above issues but also the domineering or submissive nature of the MP expelling and the sperm uptake foci.

Egg transfer from the ovaries into the oviduct

Although mature egg formed in 14 of the 115 gynandromorphs, none were deposited for one of the three reasons: (a) the eggs did not leave the ovaries to enter the lateral

oviducts; (b) an egg entered the lateral oviduct but was not transferred through the oviduct into the uterus; and (c) although an egg reached into the uterus, it was not deposited. Behavior of the 14 gynandromorphs indicates that the different steps of egg transfer are controlled by different foci. These foci are outside the head, as the decapitated females can deposit a number of eggs. Analyses of *iab-4*$^{-}$ mutant females also revealed the importance of the 9th parasegment in egg transfer control. Ovaries form and develop normally in females homozygous for weak *iab-4*$^{-}$ mutant alleles. However, the ovaries either do not become attached to the lateral oviducts or the attachments are abnormal and nonfunctional. In few cases, an egg enters the lateral oviduct but becomes stuck there and is never transferred into the uterus (Cumberledge et al., 1992; Garaulet et al., 2014). The importance of the interplay between the ovary and the lateral oviducts is elegantly illustrated by the *iab-4*$^{-}$-related ovarian chimeras. When *iab-4*$^{-}$ larval ovaries were transplanted into normal host larvae, although the ovaries grew normally and became attached to the lateral oviduct in each and every of the developing chimeras, the ovary–oviduct junction was deformed and eggs did not enter the oviduct (Cumberledge et al., 1992). This finding clearly shows that the initial step of egg transfer is ovary-dependent and needs a 9th parasegment-derived component, perhaps the pedicels at the base of the ovarioles and/or the contractions in the ovaries (Bloch Qazi et al., 2003; Middleton et al., 2006). When normal larval ovaries were transplanted into *iab-4*$^{-}$ mutant host larvae, the ovaries developed normally. Although the ovaries became attached to a lateral oviducts and an egg entered the lateral oviduct, it was not transferred into the uterus. This shows that egg transfer through the oviduct into the uterus needs contribution from the 9th parasegment (Cumberledge et al., 1992). In the absence of *iab-4* function, the excitatory motoneuron subset – that innervates the radial muscles of the oviduct and is located in the posterior VNC – is abnormal and accounts for the failure of egg transfer through the oviduct in the *iab-4*$^{-}$ mutant females (Garaulet et al., 2014). Involvement of the 9th parasegment-derived components is also supported by results of the somatic chimeras that originated following the transplantation of normal cleavage nuclei into *iab-4*$^{-}$ mutant embryos at the presumptive 9th parasegment (Cumberledge et al., 1992). Apparently, the cells that formed around the transplanted cleavage nuclei populated not only the presumptive gonadal soma but also at least part of the neuroectoderm that gives rise to the foci that control egg transfer through the oviduct. This result suggests that the egg transfer foci that control egg transfer from the ovaries into the lateral oviduct and through the oviduct derive from or nearby the 9th parasegment blastoderm region. The focus that controls egg deposition appears to be located in the thorax (Szabad & Fajszi, 1982).

Sperm release from the storage organs

It appears that there is a correlation between the deposition of fertilized eggs and the depletion of the stored sperm. The sperm storage is depleted in about a week in both the wild-type *Drosophila* females and in about 30% of the gynandromorphs that deposit dozens of fertilized eggs every day. In contrast to the offspring producers, the amount of the stored sperm does not appear to change in time in those that do not produce mature egg or fail to deposit those. The failure to release the stored sperm in those gynandromorphs that deposit unfertilized eggs shows that sperm release is also a female-specific behavior. A comparison of the cuticle of the sperm releasing and the non-releasing gynandromorphs suggest that the control focus is located in the thorax: the legs are composed from or contain male cells slightly more frequently in the non-releaser than in the release gynandromorphs. However, and possibly due to the large distance between the primordia of the legs and the sperm release foci on the blastoderm fate map, the gynandromorph-based mapping is not as precise as in case of the MP expelling and the sperm uptake foci. The assumption that the sperm release focus derives from the thoracic region is in agreement with the findings that while the decapitated females produce very few fertilized eggs, this never happens in the isolated abdomens and the females with cut-through VNC. Even though the analysis of gynandromorphs, experimentally or genetically modified females revealed novel features of the *Drosophila* female reproductive behavior, the wise statement of Hotta and Benzer is still on: "*Additional foci must surely exist for other detailed steps*."

CONCLUSION FOR FUTURE BIOLOGY

Reproduction may be considered as a puzzle with the pieces to identify and paste together in a logical way. The gynandromorph-based dissection of the reproduction puzzle in *Drosophila* began with the pioneering work of Hotta and Benzer (1972, 1976) and was followed by a number of contributors over the past decades. The present paper is a recent addition to the puzzle: it reveals MP expelling, sperm uptake and sperm release, as well as pieces of the puzzle. Hopefully, a detailed analysis of the corresponding neural foci will follow some day. The mutation-altered reproductive behaviors, the use of the ovarian, germline, and nuclei injection chimeras brought another tools in resolving the puzzle (Cumberledge et al., 1992; Garaulet et al., 2014). Most likely, combined efforts of the "old" and the new molecular techniques will create a high-resolution image of the reproduction puzzle over the upcoming decades.

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