Drosophila female sexual behavior induced by sterile males showing copulation complementation

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Females of most animal species are usually inseminated by more than one male, which allows sperm from different males to compete for fertilization. To prevent invasion of sperm from other males, *Drosophila* males elicit a rejection behavior in their mates after copulation. Using *paired* mutant males that, for the lack of accessory glands, are sterile, we show that this rejection behavior is induced exclusively by the secreted accessory gland products transferred to the female during copulation. Moreover, the activities of sperm and accessory gland products are complementary and interdependent: both sperm fertility and rejection behavior depend on accessory gland products whose prolonged activities, in turn, require the presence of sperm. Fertility of sperm from *paired* males can be restored by accessory gland products of spermless males in ''copulation complementation'' experiments. Our observations may have important implications for the role of sexual behavior in evolution and for the treatment of male sexual dysfunction in humans.

Sperm competition is a common phenomenon among most animal species and is a powerful evolutionary force that influences both behavior and physiology (1, 2). It is encountered when a female copulates with more than one male during a single fertile period. In many animals, the last male to copulate with a female fathers most of her offspring (3–5). In *Drosophila*, this so-called second- or last-male sperm precedence (6, 7) is explained by two mechanisms: the sperm of earlier matings is either displaced by the fresh sperm or incapacitated by the seminal fluid produced by the accessory glands of the last male (8). However, earlier matings counteract this effect in females by drastically enhancing egg laying (oviposition) and repressing sexual receptivity, i.e., the acceptance of males for remating, and thus maximize the number of eggs fertilized by sperm before the next mating (9–11). Both behavioral responses can persist for 9–11 days after a single mating event (12, 13) and require, in addition to sperm, the transfer during copulation of components secreted by the male accessory glands (9–11, 14, 15). XO males, which produce accessory gland secretions (seminal fluid) but no sperm, induce in their mates a transient increase in oviposition and a decrease in receptivity for only 1 day (14). Similar responses were observed after transplantation of a whole accessory gland (16, 17) or injections of its extract (18, 19) into the abdomen of virgin females. Mated females that have received neither sperm nor seminal fluid show no response (15). Based on these results, it has been proposed that both responses are composed of two phases: a short-term phase lasting for 1 day and a long-term phase persisting for an additional 8–10 days (20). The short-term phase is thought to be activated only by seminal fluid (''seminal effect'') (14, 16–19) and the long-term phase, only by sperm (''sperm effect'') (13). According to this model, when males producing sperm, but no accessory gland secretions, are mated, the recipient females are expected to display both responses only with a long-term phase. However, because no *Drosophila* mutants with a specific loss of male accessory gland functions have been available, it had not been possible to test this prediction.

The *Drosophila paired* (*prd*) gene, initially identified as a member of the pair-rule gene family required for the establishment of positional information along the anteroposterior axis in the *Drosophila* embryo (21), encodes a transcription factor whose N-terminal moiety includes two DNA-binding domains, a paired-domain, and a *prd*-type homeodomain (22–24). Because all known *prd* mutant alleles are lethal during embryogenesis and display a pair-rule cuticular phenotype (25), the adult functions of *prd* remained unknown. Here, we show that *prd* is essential for accessory gland development, for *prd* mutants, rescued to adulthood by two differently modified *prd* transgenes, possess severely reduced or no accessory glands and are sterile. This enabled us to determine the independent contributions of seminal fluid and sperm to elevated oviposition and reduced receptivity after matings with (*i*) *tudor* (*tud*) males that produce seminal fluid but no sperm and (*ii*) *prd* males that generate sperm but no seminal fluid. We found that oviposition is stimulated by both seminal fluid and sperm, whereas receptivity is inhibited only by seminal fluid. In both cases, the activities of seminal fluid are transient, and the extent of their persistence strongly depends on the presence of sperm. Finally, we have demonstrated that fertility can be restored by ''copulation complementation'' experiments, in which seminal fluid from sterile *tud* males complements sperm from sterile *prd* males after consecutive matings.

Materials and Methods

Dissection of Accessory Glands. Accessory glands were dissected from 3-day-old males in PBST (PBS + 0.05% Tween-20), mounted, and photographed under Nomarski optics with a ×10 lens on a compound microscope. ''Wild-type'' males were from the w^{1118} stock. *Df(2L)Prl*/$prd^{2.45}$; *prd*-SN20/ry^{506} males were produced from a cross between $prd^{2.45}$/*SM1*; *prd*-SN20 females (26) and *Df(2L)Prl*/*SM1*; ry^{506} males. *Df(2L)Prl*/$prd^{2.45}$; *prd*-Gsb males were obtained from a cross between $prd^{2.45}$/*SM1*; *prd*-Gsb females and *Df(2L)Prl*/*SM1*; *prd*-Gsb males (27). w^{1118}; *Df(2L)Prl*/$prd^{2.45}$; *prd*Res males were generated from a cross between w^{1118}; $prd^{2.45}$/*SM1*; *prd*Res females and w^{1118}; *Df(2L)Prl*/*SM1*; *prd*Res males (28).

Oviposition and Receptivity Assay. The oviposition assay was performed as described previously (15). Determination of receptivity was carried out according to Chen *et al.* (19). Females were of the Oregon-R strain. ''Wild-type'' males were from the parental w^{1118} stock of *prd*Res transformants. Spermless *tud* males were generated by crossing tud^{1} *bw sp* females (29) with w^{1118} males. *prd* mutant males were obtained as w^{1118}; *Df(2L)Prl*/$prd^{2.45}$; *prd*Res males from the cross described above.

To determine the effect of the constitutively expressed sex peptide (SP) on oviposition, *ry* flies and YPhsSPg females were obtained as ry^{506} and ry^{+} progeny, respectively, from a cross between ry^{506} females and YPhsSPg/ry^{506} males (30).

Abbreviation: SP, sex peptide.

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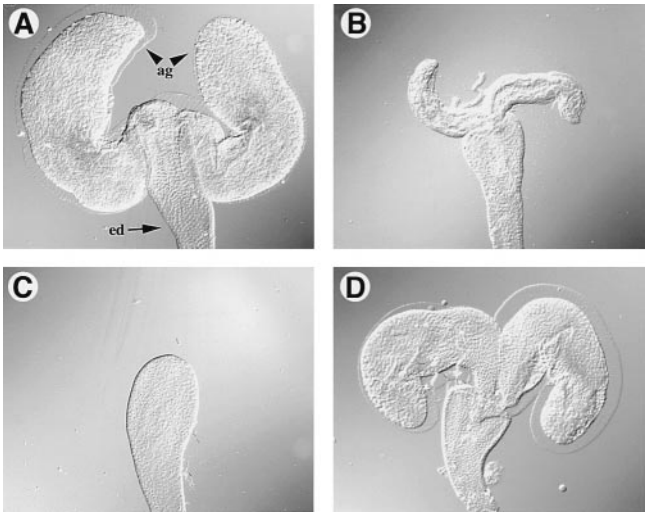

Fig. 1. *prd* is essential for accessory gland development. Accessory glands from a wild-type male (*A*) and from *Df(2L)Prl*/*prd*$^{2.45}$ mutant males rescued either by two copies of *prd*-Gsb (*B*) or *prd*Res (*C*) or by one copy of *prd*-SN20 (*D*) are shown. ag, accessory glands; ed, ejaculatory duct.

Copulation Complementation Assay. Virgin Oregon-R females were mated to the first male a single time and allowed to remate with the second male for 1 h at the intervals indicated in Table 1. Remated females were removed, stored individually, and scored for production of offspring.

Results and Discussion

***prd* Is Essential for Accessory Gland Development and Male Fertility.** A *prd* transgene consisting of the transcribed portion of *prd* as well as of neighboring upstream (10.0-kb) and downstream (5.9-kb) sequences, *prd*-SN20, rescues *prd* null mutants to fertile adults (26) and, hence, includes all enhancers of *prd* required for viability and fertility. Two additional *prd* transgenes, *prd*-Gsb, in which the coding region of *prd*-SN20 has been replaced by that of *gsb* (27), and *prd*Res, in which the distal 5 kb of the downstream region of *prd*-SN20 are missing (28), are also able to rescue *prd* mutants to viable adults (27, 28). However, in these cases all rescued males are sterile, whereas rescued females are fully fertile (ref. 28; data not shown), which implies that the wild-type *prd* gene includes, in addition to its embryonic functions, functions required for male fertility. Further studies showed that the 5 kb of *prd* downstream sequences of *prd*-SN20 that are missing in *prd*Res harbor all of the enhancers that are necessary and sufficient for this male fertility function of *prd* (unpublished results). Because the rescued, but sterile, *prd* males exhibit a normal copulation behavior, the observed male sterility apparently does not derive from any behavioral abnormalities. Dissection of the male sexual organs reveals no obvious morphological defects in the testes (data not shown), the ejaculatory duct (Fig. 1), and the ejaculatory bulb (data not shown) in the rescued *prd* mutant flies. In addition, a normal amount of sperm is produced in the testes of mutant males and is transferred to females during copulation (data not shown). However, the accessory glands are severely reduced in *prd* males rescued by *prd*-Gsb (*cf.* Fig. 1 *B* with *A*) and completely absent from *prd* males rescued by *prd*Res (ref. 28; Fig. 1*C*). It is probable that the impaired accessory glands render the *prd* mutant males sterile. Apparently, sperm is unable to fertilize eggs in the absence of accessory gland secretions. Consistent with this explanation, we find that *prd* males rescued by *prd*-SN20 have accessory glands of normal size (Fig. 1*D*) and are fertile. We conclude that *prd* is essential for normal accessory gland development and, hence, for male fertility.

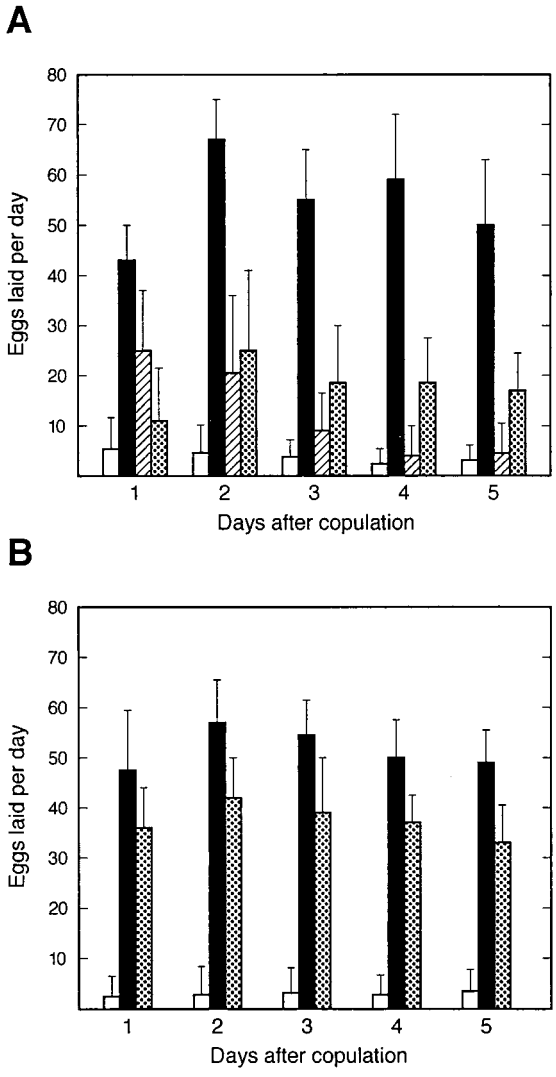

Fig. 2. Effects of seminal fluid and sperm on oviposition. (*A*) Time course of average number of eggs laid per day by virgin females (open bars) and females mated a single time to wild-type (solid bars), *tud* (hatched bars), or *prd* (stippled bars) males. (*B*) Time course of average number of eggs laid per day by virgin *ry* females (open bars), YPhsSPg females (stippled bars), and *ry* females mated a single time to *ry* males (solid bars).

Effects of Seminal Fluid and Sperm on Oviposition. To determine the individual contributions of the seminal fluid and sperm to female behavior in oviposition and sexual receptivity, three types of males were tested by matings with wild-type Oregon-R females. One set of males was sons of *tud*^{1} mothers (*tud* males), which produce and transfer normal amounts of seminal fluid (31), but lack sperm (15, 29). The second set of males was *prd* null mutants rescued by *prd*Res (*prd* males), which produce and transfer sperm, but have no accessory glands, whereas *w*^{1118} males, which produce both seminal fluid and sperm, served as "wild-type" control.

We first examined the effects of seminal fluid and sperm on oviposition. Whereas virgin females deposit only about 4 eggs per day, females lay an average of 43 eggs on the first day after a single mating with wild-type males (Fig. 2*A*). The oviposition of these females is enhanced further to 67 eggs on the second day

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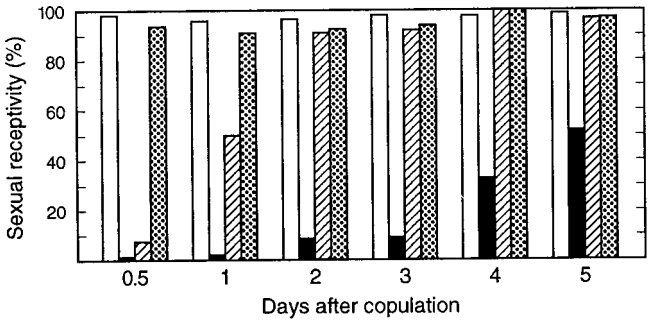

Fig. 3. Effects of seminal fluid and sperm on sexual receptivity. Time course of sexual receptivity of virgin females (open bars) and females mated a single time to wild-type (solid bars), *tud* (hatched bars), or *prd* males (stippled bars).

and remains elevated, albeit at slightly lower levels, for at least 5 days after mating (Fig. 2*A*). Females mated with *tud* males show a partial stimulation of oviposition during the first day after mating and lay an average of 25 eggs or about 60% of the clutch induced by mating with wild-type males (Fig. 2*A*). Oviposition induced by *tud* males declines to 30% of the full response during the second day and virtually reaches virgin levels after only 3 days (Fig. 2*A*). Mates of *prd* males lay an average of 11 eggs on the first day or about 25% of the response to wild-type males. This stimulation is enhanced slightly, but remains constant at 30–35% of the level of oviposition induced by wild-type males for at least 5 days (Fig. 2*A*). Thus, maximum stimulation of oviposition is induced only by the transfer of both sperm and seminal fluid, which contribute about 30–40% and 60–70%, respectively, as reflected by the initial stimulation induced by only sperm or seminal fluid (Fig. 2*A*). Moreover, contrary to the prevailing model (14, 16–19), sperm without accessory gland secretions is able to elicit only a third of the long-term response in wild-type oviposition. Therefore, the remaining two-thirds must be caused by accessory gland secretions. However, whereas elevated oviposition induced by sperm without accessory gland fluid can last for more than 5 days, oviposition induced by accessory gland secretions in the absence of sperm is rather transient and can persist longer only if sperm is also present (Fig. 2*A*). Most likely, in the absence of sperm, accessory gland products are subject to either rapid degradation or diffusion into the hemolymph and, hence, rapidly lose their inductive capacity. This capability is prolonged by sperm, which might stabilize accessory gland products or control their release into the hemolymph through yet-unknown mechanisms. Consistent with this explanation, a prolonged stimulation of oviposition corresponding to about 70% of the full wild-type male-induced response (Fig. 2*B*) is observed in virgin females carrying the YPhsSPg transgene, which, under the control of the *yp1* enhancer, constitutively expresses the SP (30), a key component of seminal fluid (19).

A contribution by sperm to the short-term stimulation of oviposition also was suggested by elegant experiments of Kalb *et al.* (15), who removed the main cells from the accessory glands by cell-specific expression of intracellular diphtheria toxin subunit A. Mates of such males exhibit a 50% stimulation of egg laying on the first day (15). However, a contribution by the remaining intact secondary cells of the accessory glands in these males (15) cannot be ruled out.

Effect of Seminal Fluid and Sperm on Female Receptivity. We also investigated the effect of seminal fluid and sperm on female receptivity. Mature virgin females mate readily, but reject subsequent copulation attempts during the first 3 days after mating until the original receptivity is restored gradually after another 7–8 days (ref. 12; Fig. 3). Mates of *prd* males accept further copulation at a rate indistinguishable from that of virgin females at all tested time points (Fig. 3), which suggests that sperm cannot elicit any rejection behavior in females in the absence of seminal fluid. Interestingly, females mated to *tud* males display nearly complete rejection 12 h after copulation (Fig. 3). Receptivity is recovered in about 50% of such females after 1 day and in almost all females after 2 days (Fig. 3). These results indicate that the rejection behavior is triggered exclusively by accessory gland secretions and that its persistence depends on the presence of sperm, which may stabilize the activity of accessory gland products. The essential component in the seminal fluid responsible for the induction of the female rejection behavior and dependent on the stabilizing effect of sperm is most likely the SP, since virgin females constitutively expressing SP in their fat bodies from a YPhsSPg transgene manifest a constitutive rejection behavior at a rate similar to that of mated females (30).

Mated females also become less attractive to males (32). Although the drop in female attractiveness is not triggered by the receipt of sperm or accessory gland products, its persistence depends on the presence of sperm (33). Hence, sperm may also stabilize the unknown factor(s) that diminish the female sexual attractiveness.

Fertility Can Be Restored by Copulation Complementation. Despite their success in courtship and mating to wild-type females (data not shown), neither *prd* nor *tud* males are fertile, which demonstrates that both seminal fluid and sperm are necessary for male fertility. In a normal mating event, accessory gland products and sperm are provided by the same male and transferred to its mate at the same time, about 5–10 min after mating has begun (34). To test whether seminal fluid from *tud* males and sperm from *prd* males can complement each other to restore male fertility, virgin females were mated with both males in a defined sequence and scored for the production of offspring. In the first series of experiments, females inseminated by *prd* males were mated with *tud* males after the intervals indicated in Table 1. Such females occasionally produce a few offspring (Table 1), which are derived from *prd* males (data not shown) as expected. It is not surprising that only few females were fertilized in this consecutive mating experiment (Table 1) because most of the sperm received from the first male is known to be either displaced from, or incapacitated in, the female sperm-storage organs during a second mating by the seminal fluid from spermless males (7, 8). Despite this reported ability to incapacitate sperm received during the first mating from a *prd* male, seminal fluid delivered during the second mating by *tud* males was able to confer fertility to and thus "capacitate" infertile sperm stored in females for 2 days after its reception from *prd*

Table 1. Copulation complementation: Fertility by sequential matings with infertile males

		Days between copulations				
First male	Second male	1	2	3	4	5
prd	*tud*	3/307	2/353	0/405	0/382	0/332
tud	*prd*	0/671	0/519	0/532	0/579	0/505

Indicated are the number of fertile females/number of females mated with both *prd* and *tud* males.

males, albeit at low efficiency (Table 1). Complementation was no longer observed after a 2-day interval (Table 1), consistent with the finding that sperm, after being stored in females for 2 days, is rendered more susceptible to incapacitation by the second male's seminal fluid (8). In the second series of experiments, females first mated to *tud* males were mated with *prd* males after the same intervals as before (Table 1). Although females impregnated with seminal fluid of *tud* males are reluctant to copulate again during the first day (Fig. 3), they do so in a crowded situation. Nevertheless, with more than 500 females scored for each time point, no offspring were produced (Table 1). A probable explanation is that in the absence of sperm, at least one accessory gland product that is crucial for male fertility is very unstable and subject to rapid inactivation after transfer to the female genital tract.

Conclusions

We have shown that the induction of a prolonged female rejection behavior, i.e., the efficiency of the first male to prevent subsequent matings and thus competition by a second male's sperm, requires both seminal fluid and sperm factors. Although SP and, perhaps, additional seminal fluid factors are sufficient only to induce the full rejection behavior for a half-day, their activity is prolonged for several days by unknown sperm factors. In addition, we have demonstrated that sperm fertility depends on fresh seminal fluid. The factors of the seminal fluid required for sperm fertility are short-lived, but their effect on sperm fertility, in turn, is prolonged by sperm factors because "copulation complementation," a technique applied here to restore fertility of two sterile males, is successful only if sperm deposited by *prd* males is capacitated by seminal fluid subsequently supplied by *tud* males, but not *vice versa*. Thus, the male's reproductive success requires both seminal fluid and sperm factors whose activities depend on each other. Because the activities of sperm and seminal fluid are expected to be under strong selection, their interdependence might have played a crucial role during evolution. Our observation that sperm capacitation by seminal fluid is essential for male fertility in *Drosophila*, as has been long known in humans, may imply further that its mechanism has been conserved from insects to man.

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