Gradual Release of Sperm Bound Sex-Peptide Controls Female Postmating Behavior in *Drosophila*

Jing Peng, Shanjun Chen, Susann Büsser, Huanfa Liu, Thomas Honegger, and Eric Kubli*
Zoological Institute
University of Zürich
Winterturinerstrasse 190
CH-8057 Zürich
Switzerland

Summary

Background: In many female insects, peptides transferred in the seminal fluid induce postmating responses (PMR), such as a drastic increase in egg laying and reduction of receptivity (readiness to mate). In *Drosophila melanogaster*, sex-peptide (SP) elicits short- and long-term PMR, but only the latter in the presence of stored sperm (sperm effect).

Results: Here, we elucidate the interaction between SP and sperm by immunofluorescence microscopy. Transgenic males were used to study the effects of SP modification on the PMR of females in vivo. We report that SP binds to sperm with its N-terminal end. In females, the C-terminal part of SP known to be essential to induce the PMR is gradually released from stored sperm by cleavage at a trypsin cleavage site, thus prolonging the PMR. These findings are confirmed by analyzing the PMR elicited by males containing transgenes encoding modified SPs. SP lacking the N-terminal end cannot bind, and SP without the trypsin cleavage site binds permanently to sperm.

Conclusion: By binding to sperm tails, SP prolongs the PMR. Thus, besides a carrier for genetic information, sperm is also the carrier for SP. Binding to sperm may protect the peptide from degradation by proteases in the hemolymph and, thus, prolong its half-life. Longer sperm tails may transfer more SP and thus increase the reproductive fitness of the male. We suggest that this could explain the excessive length of sperm tails in some *Drosophila* species.

Introduction

Despite their amazing species diversity, some reproductive traits are common to most insects [1–3]. For example, in many higher insects, mating elicits a drastic increase in egg laying and a reduction of receptivity (readiness to copulate). Thus, the postmating responses (PMR) are either evolutionarily old, or they evolve fast. They are induced by components of the seminal fluid transferred into the female during copulation and stored in the female genital tract [1, 2]. Due to its well-known genetics, combined with its amenability for biochemical, physiological, and genomic analysis, the model organism *Drosophila melanogaster* has become the best-studied species with respect to seminal peptides influencing postmating behavior in insects [4].

In *D. melanogaster*, the PMR are elicited by three male peptides [5–7]. Sex-peptide (SP) and Ovulin are products of the accessory glands, and the Ductus ejaculatorius peptide (DUP) is synthesized in the ejaculatory duct. They are transferred together with sperm into the female during mating [7]. SP and DUP induce both PMR. Ovulin elicits ovulation and oviposition on the first day after mating, but does not affect receptivity [8]. Mating with wild-type males induces the PMR for about 1 week; however, mating with males that do not transfer sperm elicits PMR only for one day (long-term and short-term PMR, respectively). (This phenomenon has been described as “sperm effect” by Manning, [9, 10]). Males lacking functional SP (SP0 males) elicit only weak PMR lasting about one day [11, 12]. Hence, in vivo SP is the major agent eliciting both the short- and the long-term PMR; Ovulin and DUP play only minor roles during the first day after copulation. Because SP0 males transfer and store sperm [12], sperm alone cannot induce the PMR, but stored sperm are essential for their persistence [9, 10]. Without sperm transfer, or injection of physiological amounts of the peptide, SP elicits only short-term PMR [7, 10, 13]. How do SP and sperm interact to support the long-term PMR? A possible mechanism could be binding of SP to sperm [14]. Here, we show that SP binds to sperm with the N-terminal end. During storage, SP is cleaved off from the tail, and the released C-terminal part elicits the long-term PMR.

**Results**

**Sex-Peptide Binds to Sperm Stored in the Genital Tract of the Female**

We used immunofluorescence to visualize putative binding of peptides to sperm. Sperm were isolated from females at different time intervals after mating and were then incubated with antibodies specific for SP, DUP, or Ovulin, respectively (Figure 1). DUP binds only to the head and only in the first few hours (data not shown), whereas Ovulin does not bind to sperm at all (Figure 1A). Furthermore, sperm is not autofluorescent at the wavelength used for this study (Figure 1A). Sperm isolated 5 hr after mating show binding of SP along the entire length of sperm (Figure 1B, and data not shown). (The antibody used for these experiments is specific for the fragment SPs20.) During storage in the female genitalia, SP is lost from the tail. Two days after mating, the signal intensity along the entire length of the tail, in comparison with the head, is weaker and spottier than immediately after mating (Figure 1C). Five days after mating, SP is almost absent from the tail (Figure 1D). However, the signal is still strong on the head in all stages. A quantitative analysis reveals that SP is gradually lost from the sperm tail (Figure 1E). Thus, SP indeed binds to sperm during several days, but it is lost from the tail while sperm are stored in the female genital tract.

*Correspondence: ekubli@zool.unizh.ch*
Figure 1. Sex-Peptide Binds to Sperm and Is Gradually Released from the Sperm Tail upon Storage in the Female Genital Tract
Panels in the horizontal rows show parts of the same sperm preparation: left, DIC; middle, antibody staining; right, staining of the nucleus with propidium iodide. Virgin females were used for all matings with wild-type males. Sperm was isolated from the female genital tracts at the time points indicated in the left panels. The scale bars represent 10 μm.

(A) Ovulin does not bind to sperm, nor is sperm autofluorescent. Antibody specific for Ovulin.
(B–D) SP binding to sperm isolated 5 hr, 2 days, and 5 days after mating, respectively. Antibody specific for amino acids SP6–20.
(E) Gradual loss of SP from the sperm tail. Relative intensity of tail labeling of sperm isolated at different time points after mating. Sperm head was taken as 100%. Standard deviation is indicated. For details, see Experimental Procedures.

In contrast, DUP binds to the sperm head only for a few hours (data not shown), and Ovulin does not bind at all (Figure 1A). This is consistent with the finding that DUP and Ovulin play only minor roles in the short-term PMR [8, 12]. Thus, we shall focus on the fate of SP bound to sperm.

A set of synthetic SP fragments was used to determine the part of SP interacting with sperm. The fragment SP1–11 competes for binding with full length SP1–36 (Figure 2A). Thus, SP binds to sperm with the N-terminal end. This part of SP stimulates juvenile hormone synthesis in vitro [15], but the C-terminal part is essential to elicit the PMR [13]. Thus, binding to sperm does not block the part of SP that is necessary to elicit the PMR. But, as shown below, full size SP bound to sperm is not able to elicit the PMR. How then is this achieved?

The C-Terminal Part of Sex-Peptide Is Gradually Cleaved off the Sperm Tail
Sex-peptide is eventually lost from the tail (Figures 1B–1E). Hence, reincubation of sperm isolated 5 days after mating with SP should yield labeled sperm tails. However, this is not the case; only the head is labeled (Figure 2B). “Sex-peptide-null” sperm isolated from females 5 days after mating with SP0 males does not produce any signal, as expected (Figure 2C), but incubation of such SP0 sperm with SP in vitro results in fully labeled sperm (Figure 2D). Thus, once sperm has been in contact with SP, binding of additional SP is not possible anymore (Figure 2B), but the mere storage of sperm in the genital tract in the absence of SP is not responsible for this effect (Figure 2D). The antibody used in the experiments reported thus far is specific for the fragment SP6–20 (AB SP6–20); hence it does not detect other parts of SP. If, for example, SP would be cleaved between the N-terminal part (site of SP attachment to sperm) and the part of SP recognized by AB SP6–20, the N-terminal part of SP remaining on sperm could not be detected by this antibody. Indeed, a putative trypsin cleavage site is localized near the N terminus of SP (R7-K8). An antibody specific for the fragment SP1–7 (AB SP1–7) reveals that this fragment is still bound to the sperm tail even 5 days after mating (Figure 2E). These findings show that the N-terminal end of SP remains bound to sperm (thus blocking full-length SP1–36 binding upon reincubation). The C-terminal part, known to be essential to elicit the PMR [13], is cleaved off and released.

The signal on the sperm head remains strong even after storage for 5 d in the female genital tract (Figure
Gradual Release of Sex-Peptide from Stored Sperm

Figure 2. Sex-Peptide Binds with its N-Terminal End to the Sperm Tail, the C-Terminal Part Is Cleaved off during Storage in the Female Genital Tract

Panels in the horizontal rows show parts of the same sperm preparation: left, DIC; middle, antibody staining; right, staining of the nucleus with propidium iodide. The scale bars represent 10 μm.

(A) Competition experiment. Incubation of sperm isolated from male seminal vesicles with SP_{1–36} (full-size SP) and a 200-fold excess of peptide fragment SP_{1–11}. Antibody: AB SP6–20. No full-size SP_{1–36} is bound, i.e., SP binds with the N terminus.


(B) Reincubation of sperm isolated 5 days after mating with wild-type male with synthetic SP. No SP_{1–36} is bound to the tail.

(C) Sperm isolated 5 days after mating with SP_{0} males and treated with AB SP6–20. No signal was detected.

(D) Sperm isolated 5 days after mating with wild-type males incubated with synthetic SP. SP binds to head and tail of sperm.

(E) Incubation of sperm 5 days after mating with wild-type males incubated with AB specific for the N terminus of SP (AB SP1–7). Head and tail of sperm are labeled. For details, see text.

1D). The label cannot be due to another molecule that, for example, shares sequence identity with SP. The only compound missing in the seminal fluid of SP_{0} males is SP, but incubation of sperm isolated from a SP_{0} male with the antibody AB SP_{6–20} does not yield any signal (Figure 2C). Hence, the labeling is due to SP.

Transgenic Males that Synthesize Modified Sex-Peptides that Cannot Be Cleaved from, or Cannot Bind to Sperm, Elicit Only Short-Term Postmating Responses

To confirm the above findings, three transgenes were introduced into flies with a SP_{0} background [12]: (1) the wild-type SP gene (control, transgenic wildtype = TG_{WT} males); (2) a modified SP gene coding for a SP mutated at a putative trypsin cleavage site (R_{7–Q_{7}}, K_{8–Q_{8}}) near the N terminus of SP (TG_{22–7} males) (Q maintains the polarity of the replaced amino acids); (3) a truncated SP gene with a deletion comprising the codons of the amino acids E_{2–R_{7}} (TG_{22–7} males; to allow appropriate processing of the signal peptide, W_{1} was not removed.). The DNA fragment –210 to +1 of the SP promoter that was used to drive the expression of the constructs induces expression of a lacZ reporter exclusively in the accessory glands of the male (Figures 3A and 3B). The transgenic males used for the experiments reported below contain one copy of the respective transgene (Figure 4A) in a SP_{0} background. The amount of SP produced in their accessory glands is about the same (Figure 3C).

Females mated with TG_{WT} males show normal PMR (Figures 4B and 4C). Thus, the amount of SP produced by one copy of the wild-type rescue construct is sufficient to support the PMR, as observed after mating with an Ore R wild-type male (Figures 4B and 4C). Because the accessory glands of all transgenic males produce the same amount of SP (Figure 3C), the results presented below are due to the modification of the SPs and not to varying amounts of SP production in the transgenic males. Females mated with TG_{90} or with TG_{22–7} males show only short-term PMR (Figures 4B and 4C). After 2 days, the PMR are lacking, as was also observed after matings with a SP_{0} male (compare green, blue, and red columns in Figures 4B and 4C). These short-term responses are due to the transfer of free functional SP not bound to sperm. In a mating with a wild-type male, about 3 pm SP are transferred per copulation [14]; only part of it is bound to sperm, the rest is free SP, which elicits a short-term PMR. Only the wild-type rescue males, TG_{WT}, elicit PMR indistinguishable from the PMR elicited from an Ore R wild-type male (yellow and black columns in Figures 4B and 4C). However, all males transfer and store sperm, and sperm of all transgenic males fertilize eggs. Sperm isolated from females 5 hr after mating with TG_{WT} (Figure 5A) or TG_{90} males show labeling of the tail, but no labeling is observed after mating
sequence [17]. These findings suggest that binding in the nervous system is responsible for eliciting the PMR, whereas binding in the genital tract may reflect the presence of a peptide transporter [7]. This interpretation is supported by the fact that the free (nonbound) modified SP\textsubscript{R7Q8} of TG\textsuperscript{QQ} males elicits the short-term responses, but the same SP\textsubscript{R7Q8} bound irreversibly to the sperm tail (Figure 5D) cannot elicit the long-term responses (Figures 4B and 4C). Hence, replacement of R\textsubscript{7} by Q\textsubscript{7} and K\textsubscript{8} by Q\textsubscript{8} does not affect SP function, but mutant SP\textsubscript{R7Q8} permanently bound to the tail cannot elicit the PM responses via the SP binding proteins present in the genital tract, i.e., these SP binding proteins are probably not receptors involved in the PMR. Furthermore, our results strongly suggest that the released C-terminal part of SP enters the hemolymph to reach its targets in the nervous system. Apparently the sperm tail is the only source of SP to sustain the long-term PMR. Our results are also in accord with the findings that the brain is the site of action of SP [18] and that ectopic expression of SP in the fat body of virgin females or injection of SP into the hemolymph does elicit the PMR [13, 19].

Evolutionary Considerations

Cooperative reproductive behavior of the two sexes promotes the evolutionary success of a species, but males and females also compete to control the number and genetic diversity of their offspring [1–4]. Because females are polyandrous [20], it is in the interest of the female to eliminate surplus SP, at the latest when sperm has been used up. In contrast, it is in the male’s interest to keep his mating partner monogamous. Thus, sexual conflict arises. Binding of SP to the sperm tail substantially increases the functional half-life of SP from 1 day to about 1 week, probably by hiding it from the hemolymph proteases. It is also in the male’s interest to transfer as much SP as possible. Because sperm serves as a carrier to transport and stabilize SP, selection may have favored long sperm tails binding more SP and thereby increasing the reproductive fitness of the male. This could explain the excessive length of sperm tails in some Drosophila species (D. melanogaster males produce sperm of 1.8 mm, and D. bifurca males produce sperm of 58 mm! [21]). Association of components of the male seminal fluid with sperm has also been reported for other insects, mammals, and birds ([22, 23], for review, see Neubaum and Wolfrner [24]). Binding of proteins that enhance male fitness to sperm may be a novel mechanism of general importance in insects and beyond. Selection experiments involving males either lacking functional SP completely [11, 12] or producing modified SPs (this work) should enable testing of the putative influence of SP on sperm tail length in D. melanogaster. Such experiments may lead to an understanding of male-male and male-female competition at a molecular level.

Conclusions

In sum, the PMR of D. melanogaster females can be divided into two phases: the short-term PMR and the long-term PMR, respectively. The short-term PMR
Figure 4. Transgenic Males Containing Modified Sex-Peptide Transgenes Either Lacking a Putative Trypsin Cleavage Site Or a Deletion in the N-Terminal Region of SP in a SP0 Background Elicit Only Short-Term Postmating Responses
(A) Amino acid sequences encoded by the transgenes. TGWT: wild-type SP gene (rescue construct, control); TGQQ: the putative trypsin cleavage site R7K8 (black arrow) has been replaced by Q7Q8; TGΔ2-7: N-terminal amino acids E2–R7 deleted. Red: Amino acids at the putative trypsin cleavage site.
(B) Oviposition of females mated with males of different genotypes. Standard deviations are indicated.
(C) Receptivity of females mated with males of different genotypes. Standard deviations are indicated. Males used for the crossings: Ore R, wild-type Oregon R stock; SP0, males lacking functional SP; transgenic males, TG, as above. Gray areas in (B) and (C) indicate duration of short-term postmating responses.

induced immediately after mating mainly by free SP, the long-term PMR, lasting about one week, by the C-terminal SP fragment cleaved from SP bound to the sperm tail. Both responses likely elicit the PMR by binding of SP to specific sites in the central and peripheral nervous systems [16, 17]. In addition, immediately after mating, the free SP probably also stimulates juvenile hormone synthesis because it contains the N terminus known to be essential for the stimulation of the corpus allatum in vitro [15]. The elucidation of the molecular mechanism supporting the persistence of the PMR in *D. melanogaster* may shed light on a fundamental aspect of insect reproduction in general.

**Experimental Procedures**

**Transgenic Flies, Fly Stocks, and Oviposition and Receptivity Tests**
SP wild-type rescue construct (TGWT): A 2 kb fragment of the SP gene was amplified with PCR (-870 bp: 5'-AGGACAGCACTGGTGTGTC; +1020 bp: 5'-TGGACGTGCTGATGCAG). This amplified fragment was cloned into the pGEM-T Easy vector and then ligated into the Smal and SpeI cutting sites of the PBS vector. Mutant constructs were made by site-selected mutagenesis (Mutagenesis kit from Stratagene) based on the wild-type rescue construct. Primers N-terminal deletion construct (TGΔ2-7): 5'-GGCAATTCGCTCCAGTCTCGGAAGCTCTAAGATTCCC and 5'-GAACACCTGTTAGCCCTTCA CGAATAGCCAAAGCC. Primers R7K8–Q7Q8 replacement construct (TGQQ): 5'-GAATGGCCGTGGAATTACGAGCCAGCAGCCTACAAAGTTTCC and 5'-GGAAACTTTGTAGGCTTCCACTGCTGATTCCACGGCCATTC. The fragments with the modified SP genes were cloned into the EcoRI and XbaI cutting sites of pW6 and subsequently transformed into w1118 flies by standard methodology. After crossing with SP0 flies [12], these constructs were expressed in a SP0 background. Transcription was driven by the SP promoter [25, 26].

For the oogenesis and receptivity tests, virgin females were collected from a VC-line (virginizer cross, [27]). Flies were reared on standard cornmeal-agar food in a 12 hr/12 hr light/dark photoperiod at 25°C. Oviposition and receptivity tests were performed as described by Chen et al. [28]. All experiments were repeated three times. Eighteen to twenty-four females were observed per experiment.

**Staining of Whole Hole Mount Preparations for β-Galactosidase; Western Blots**

Male genital tracts were dissected out from 5-day-old males. β-galactosidase staining was performed according to Bertram et al. [29]. Western blotting was performed as described by Sambrook and Russell [30]. A primary antibody specific for the fragment SPc20 was used. The secondary antibody was a goat anti-rabbit IgG peroxidase conjugate (Sigma A0545). The detection was performed with ECL Western-blotting detection reagents (RPN 2109, Amersham Pharmacia Biotech).

**Staining with Immunofluorescent Antibodies**
Sperm were dissected from uteri or seminal receptacles of females at different time points after copulation, fixed with 2% paraformaldehyde in PBS for 15 min at room temperature, washed, blocked with 0.2 M glycine in PBS for 30 min, and then washed three times. Sperm were blocked with 1% milk powder in PBS for 30 min, incubated with primary antibody (antibodies specific for fragments SPc20 or SPc7, respectively) in 0.1% milk powder-PBS for 1 hr at room temperature, and then washed three times in PBS. Incubation with secondary
Figure 5. Sex-Peptide Lacking the N-Terminal End Cannot Bind to Sperm.

SP containing a modified trypsin cleavage site binds to sperm permanently and cannot be cleaved. Females were mated to TGWT, TGΔ2−7, or TGQQ males, respectively. Horizontal rows show parts of the same sperm preparation: left, DIC; middle, staining with antibodies; right, staining of the nucleus with propidium iodide. The same antibody, AB SP6–20, specific for SP6–20, was used for all experiments. Genotype of the transgenic male is indicated in the left panels.

(A) Unmodified SP binds to sperm head and tail 5 hr after mating.
(B) SP lacking the N-terminal end cannot bind to sperm. Sperm isolated 5 hr after mating.
(C) Unmodified SP binds only to the sperm head 5 days after mating.
(D) SP containing a modified trypsin cleavage site binds to sperm head and tail even 5 days after mating. The scale bars represent 10 μm.

antibody: FITC-labeled anti-rabbit IgG (Bio-Rad) in PBS for 45 min at room temperature, washed, and incubated with propidium iodide (10 g/ml) for 20 min each at room temperature, washed three times, and mounted on glass slides in 90% glycerol, 10% PBS.

Incubation experiments with SP were done at a concentration of 200 pmol synthetic SP1–36/l. For the competition experiments with SP fragments, sperm was isolated from 3–5-day-old males. A 200-fold excess of fragment SP1–11 was added to the 200 pmol synthetic SP1–36/l.

Photography and Processing

The Reichert–Jung Polyvar microscope was equipped with Nomarski differential interference optics (DIC) and a set of fluorescent filters. For FITC: excitation filter 475–495 nm, emission filter BP 520–560 nm. For propidium iodide: excitation filter 510–560 nm, emission filter BP 590 nm.

Signals were detected with a CCD video camera (Hamamatsu C5404) linked to the microscope. Images were obtained with the Argus-20 imaging acquisition software (Hamamatsu Photonics) and processed with either National Institutes of Health (NIH) image-analysis software (Version 1.60) or Adobe Photoshop (Version 5.5) on a Macintosh computer online to the camera system.

Signal Intensity Measurements of SP Binding to Sperm

After collecting images, NIH image-analysis software (Version 1.60) was used to measure the intensity of signal in the sperm head and in the sperm tail, respectively. The signal intensity of the sperm head remains about the same over time, i.e., it is independent of the day of sperm isolation. In comparison with the head, the signal of the sperm tail diminishes over time. Because the intensity of the label is fading over time, we defined the signal intensity of the head as 100% for each preparation, and the signal intensity of the tail was calculated as relative intensity. For every time point, three batches of ten females each were processed. For one preparation, sperm were isolated from the ten mated females, pooled, and about 20 sperm were used for measurement.

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