# Manuscript Details

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| **Manuscript number** | IP_2016_339 |
| **Title** | Male accessory gland proteins affect differentially female sexual receptivity and remating in closely related Drosophila species |
| **Article type** | Research Paper |

**Abstract**

In sexual species, mating success depends on male capacity to find sexual partners and on female receptivity to mating. Mating is under evolutionary constraints to prevent interspecific mating and to maximize the reproductive success of both sexes. In Drosophila melanogaster, female receptivity to mating is mainly controlled by Sex peptide (SP, i.e. Acp70A) produced by the male accessory glands with other proteins (Acps). The transfer of SP during copulation dramatically reduces female receptivity to mating and prevents remating with other males. To date, female postmating responses are well-known in D. melanogaster but have been barely investigated in closely-related species or strains exhibiting different mating systems (monoandrous versus polyandrous). Here, we describe the diversity of mating systems in two strains of D. melanogaster and the three species of the yakuba complex. Remating delay and sexual receptivity were measured in cross-experiments following SP orthologs or Acp injections within females. Interestingly, we highlighted strong differences between the two strains of D. melanogaster as well as among the three species of the yakuba complex. These results suggest that reproductive behavior is under the control of complex sexual interactions between the sexes and evolves rapidly, even among closely-related species.

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| **Keywords** | female remating; female sexual receptivity; accessory gland proteins; Sex peptide; yakuba complex; Drosophila |
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| **Suggested reviewers** | Bashishth N. Sing, Christophe Bressac, Mariana Wolfner |

## Submission Files Included in this PDF

**File Name [File Type]**

Response to the reviewers.docx [Response to Reviewers]

Denis&al_JIP_revision.docx [Manuscript File]

To view all the submission files, including those not included in the PDF, click on the manuscript title on your EVISE Homepage, then click 'Download zip file'.

Dear Editor,

Thank you very much for your interest in our manuscript, previously titled “Female remating and differential effects of male accessory gland proteins on female receptivity in closely-related *Drosophila* species” and for this opportunity to revise it. We are grateful to the reviewers for their constructive comments, and have thoroughly revised the manuscript according to their suggestions. Here are our detailed responses to those comments, with the original comments in bold.

Various changes have been made in all the sections and the text has been reorganized, notably in the introduction section, to facilitate the reading. The title has also been changed to: “Male accessory gland proteins affect female sexual receptivity and remating in closely related *Drosophila* species”, which gives a more direct information about the subject of the work. A second native English proof-reader has carefully revised the paper.

We hope that you will find this revised version acceptable for publication in the Journal of Insect Physiology.

Sincerely yours,

**Reviewer(s)' Comments to Authors:**

**Reviewer 1:**

**Paper is well organized and writen, it's main weakness is its length that exceeds usual papers in this field; it renders the topic sometimes hard to follow.**
The paper has been shortened by about 20% in various parts (introduction section from 74 to 64 lines, discussion section from 165 to 118 lines, conclusion section from 13 to 10 lines, and reference section from 74 to 59 references. The concerns in the different sections are now more focused to facilitate reading, eliminating the detailed literature survey as well as extensive citations and discussion. The highlights have also been modified so as not to exceed the 85 characters available.

**Major comments:**
**1. in introduction and discussion, the 'female side' is less considered than male's, in terms of receptivity to sex peptides, saturation of receptors... Is it really not documented in the litterature?**
Indeed, Reviewer 1 is right and our work does not explore the perception of the SP signal at the level of the SPR receptor in females. In *Drosophila*, the location of the SPR receptor and its role in modifying post-reproductive behavior are well documented (Ottiger *et al*., 2000; Yapici *et al*., 2008) as well as its association to neurons expressing genes of sexual determinism as fru/ ppk and dsx (Kvitsiani & Dickson, 2006, Yang *et a*l., 2009, Rideout *et al*., 2010, Rezaval *et al*., 2012). However, these considerations go beyond the scope of the present paper and additional information on this theme would make the text too long. Following the previous remark, we decided to focus on the link between male seminal peptide and female receptivity.

Some works have also been dedicated to the study of SPR receptor in females. The affinity of SP ligand to SPR has been measured as well as the minimum concentration producing an effect, or the colocalisation of SP and SPR using various methods (Ding *et al :* 2003; Yapici *et al*., 2008; Yang *et al*., 2009 ; Tsuda *et al*., 2015). However, to our knowledge, no study has investigated the saturated SP dose for SPR, in correlation with its functionality. Such a study would be very interesting and would be the subject of another paper.

**2. are they other peptides tranferred by mates or other ways which change the behaviour of the receptive individual?**

Firstly, the SP network proteins (Ravi Ram and Wolfner, 2007; Findlay *et al*., 2014) have been added in the introduction section for more precision (from line 95 to line 102). Secondly, there are some other peptides than Acps that are also transferred within the seminal fluid during mating that change the sexual behavior, as Dup99B (mentioned in the paper section 2.5.2, 393, 399, 546), Esterase-6 (line 544) or PebII (line 544). These possibilities have been included in the discussion section and the corresponding references now added (Gilbert, 1981; Bretman *et al.,* 2010).

Reproductive success also depends on the persistence of courting males and on the emission of aphrodisiac pheromones, which promote recognition of sexual partners and thus mating. These pre-copulatory elements, although decisive, are not the subject of our work, which is centered on the post-copulatory interactions and more precisely on the decrease in female sexual receptivity. Another way for the male to ensure paternity and avoid sperm competition is to make the female unattractive to rival males (Wedell, 2005). So, the moth male *A. assectella* deposits pheromones on the female, which repels other males (Lecompte *et al*., 1998), while the butterfly male *H. erato* transfers an anti-aphrodisiac that alters the female's odor (Gilbert, 1976). In the butterfly, *P. napi*, decreased susceptibility is induced by the additional storage of non-fertilizing apyrene sperm (Cook and Wedell, 1999). Thus, many factors can change the behavior and the sexual receptivity of partners. However, those considerations go too far beyond our purpose as we have not studied this kind of pheromones and we also know that the *Drosophila* species studied here do not possess apyrene sperm (Joly *et al*., 1989). We therefore highlight this complexity in the discussion section (lines 549-551 and 560-561).

**3. introduction and discussion are focused on drosophila, could some examples being found in other insects?**

We apologize for not being clear enough about the fact that a number of references concern species other than *Drosophila*. We mainly focus on insect species, because the literature on seminal protein and postmating sexual interactions is very abundant on this taxonomical group, but not exclusively. However, the first two paragraphs of the introduction section cite references to a wide range of taxonomical species, from invertebrates to vertebrates, while the other paragraphs are more devoted to the *Drosophila* literature.

As suggested by Reviewer 1, we have now cited the species concerned in various places throughout the text.

**Minor comments:**

**1. lines are not numbered, it is hard to refer to typos. Some may be due to mac-windows conversions.**

We apologize for the typo conversion problems that did indeed occur, particularly in some figures during the pdf production. We have now numbered the lines, fixed all the typos errors and carefully checked the final file.

**2. in figs showing a cumulative percentage, how was calculated the SD? Usually such data are on the whole population, then without SD.**
It was indeed the SE and not the SD that was indicated in figure 2. This has been corrected correspondingly.

**3. in some figures (3 , 9 and others), "minutes" are "min" or "mn", correct it. The symbol " is reserved to angles minutes, it is inapriopriate for durations.**
The abbreviation of minutes was corrected in figure 3. In other figures, the symbol " was due to a conversion problem during the pdf production. This has been corrected in the revision file and all the figures within the pdf file carefully checked.

**4. In fig 5, are the chromosomes indicated by numbers (2 or 3) or by the bar colors, or both?**
In figure 5, the chromosomes are indicated both by color (which discriminates between inserted transgenes with SP gene – black or grey – or without SP gene – white bars) and numbers. A clarification has been added in the legend.

**5. At the beginning of discussion, a summary of results would be welcome.**
Following the Reviewer 1 suggestion, the first paragraph of the discussion section was deleted and the section now begins more directly with the following paragraph of the submitted file, which summarizes the results of the present study.

**6. The end of first paragraph of the discussion is an introduction.**
As indicated above, the first paragraph has been deleted.

**7. the conclusion is a summary of the paper, it does not give a take home message.**
The conclusion section has been changed and shortened to deliver a more direct take-home message, highlighting the intraspecific variation in *D. melanogaster* and the specificity of *D. teissieri*.

**Reviewer 2:**

**However, I am sorry to say that the quality of the presentation is lacking. The manuscript is littered with grammatical errors, awkward sentences, and cosmetic mishaps that could have been remedied by careful revisions. This is unfortunate given that the manuscript is authored by six individuals. It would have assisted the reviewer greatly if the manuscript included line-numbering so as to facilitate pointing out these errors. I point out a subset of these errors below, but these suggestions are by no means comprehensive, and I urge the authors to give careful consideration to each paragraph.**
We apologize for the numerous grammatical errors, awkward sentences, and cosmetic mishaps; however, a native English proof-reader did revise the submitted draft. For the revised draft, we have made numerous changes throughout the text according to both Reviewer's suggestions. We have also asked another native English proof-reader to carry out

a more rigorous revision. Moreover, the revised draft has been carefully checked for editing errors and cosmetic mishaps. The text is now line-numbered, and the pdf file has been carefully checked, including figures.

**Abstract:**

**1. Sex Peptide is the name of the protein, thus, no need to use "the" or "-". It is already a proper noun. Correct throughout.**

This has been corrected throughout the text.

**2. "Here, we describe.....*depending on time*". time of what? Needs clarification.**

This confusing part of the sentence has been deleted without changed the meaning.

**3. "conspecific and hetero-specific". no "-".**

This has been corrected.

**4. "Additionally, it highlights the role of Acps in female postmating responses". This is already known, and shouldn't be the concluding statement of the abstract.**

The Reviewer's statement is right and this part of the abstract has been deleted.

**Introduction:**

**p1. 2nd paragraph: That first sentence doesn't make sense. It reads as if RNAi and genomic methods cause a drop in female sexual receptivity.**

We apologize for this misinterpretation of the sentence. The sentence has been deleted to follow the new organization of the introduction section.

**p1. 2nd paragraph: Why be specific about "12 functional classes" but not mention even one of them?**

In response to the Reviewer's suggestion, some details about the functional classes have added, now focusing on the SP network instead of the number of functional classes; however, the major protein classes have been indicated in lines 95-98. Their conservation across the animal kingdom is paradoxical given their function, which can sometimes be extremely diverse, as indicated lines 101-103.

**Overall, give careful consideration to the introduction. As is it contains many erroneous statements that are difficult to understand.**

The text, including introduction section, has been carefully revised and changed in many parts. The statements with the references have been thoroughly controlled and rephrased when necessary.

**Materials and Methods:**

**section 2.2, p.6: "remated females were discarded"????? You mean "unmated"?**

The Reviewer is right. This part has been rephrased and placed before the next sentence to be clearer regarding the protocol used.

**The "cumulate/cumulated" is incorrect. Should be "cumulative".**

This has been corrected.

**section 2.3: "Specific primer pairs... were designed with CLUSTAL W software .. using specific primers". Surely this can't be right.**
The sentence has been corrected and the last part deleted.

**section 2.4: What does it mean for "inserted sequences" to be "controlled by PCR"? Is this to check the genotype of transformed flies? Not clear.**
Yes, the Reviewer is right: the transformed lines were controlled by PCR. We have changed the text accordingly.

**section 2.5.1: Should be "denoted", not "noted".**
The sentence has been rephrased to say that 4 AGs/ µL corresponds to 1x in the dose-dependent response experiment.

**section 2.5.2: Is Table S1 the correct reference for this?**
The Table S1 was already cited in sections 2.3 and 2.4 and does not need to be cited in the section 2.5.2. We have deleted the reference as suggested.

**section 2.5.3: dose-dependant?**
Yes, we have changed dose-response to dose-dependent response throughout the text.

**section 2.5.6: No need to specify "Mac OS X".**
Following the Reviewer's suggestion, the last part of the sentence has been deleted.

**Results:**
**It is generally best to insert the figure reference at the end of the sentence or before punctuation, not mid-sentence.**
The position of the references of figures has been checked throughout the text; some have been deleted, others moved elsewhere in the sentence.

**Figure 3: Fix "min" vs "mn". Is there a significance to the quotation marks in B-left?**
The abbreviations of minutes have been homogenized throughout the figures; the problem of pdf conversion of the text within the figures has been solved. We apologize for the inconvenience in the submitted draft. This was carefully checked in the revised draft.

**section 3.2: non-annotated = unannotated.**
This has been changed.

**Figure 9: What does the "#" mean? ALso note errors in axis text, e.g. solu<on"**
Again, we apologize for the pdf conversion problems. All the figures are now correctly transformed and the pdf file thoroughly verified.

# Male accessory gland proteins affect differentially female sexual receptivity and remating in closely related *Drosophila* species

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**both contributed equally to the work*

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**Running title:** Female receptivity in *Drosophila*

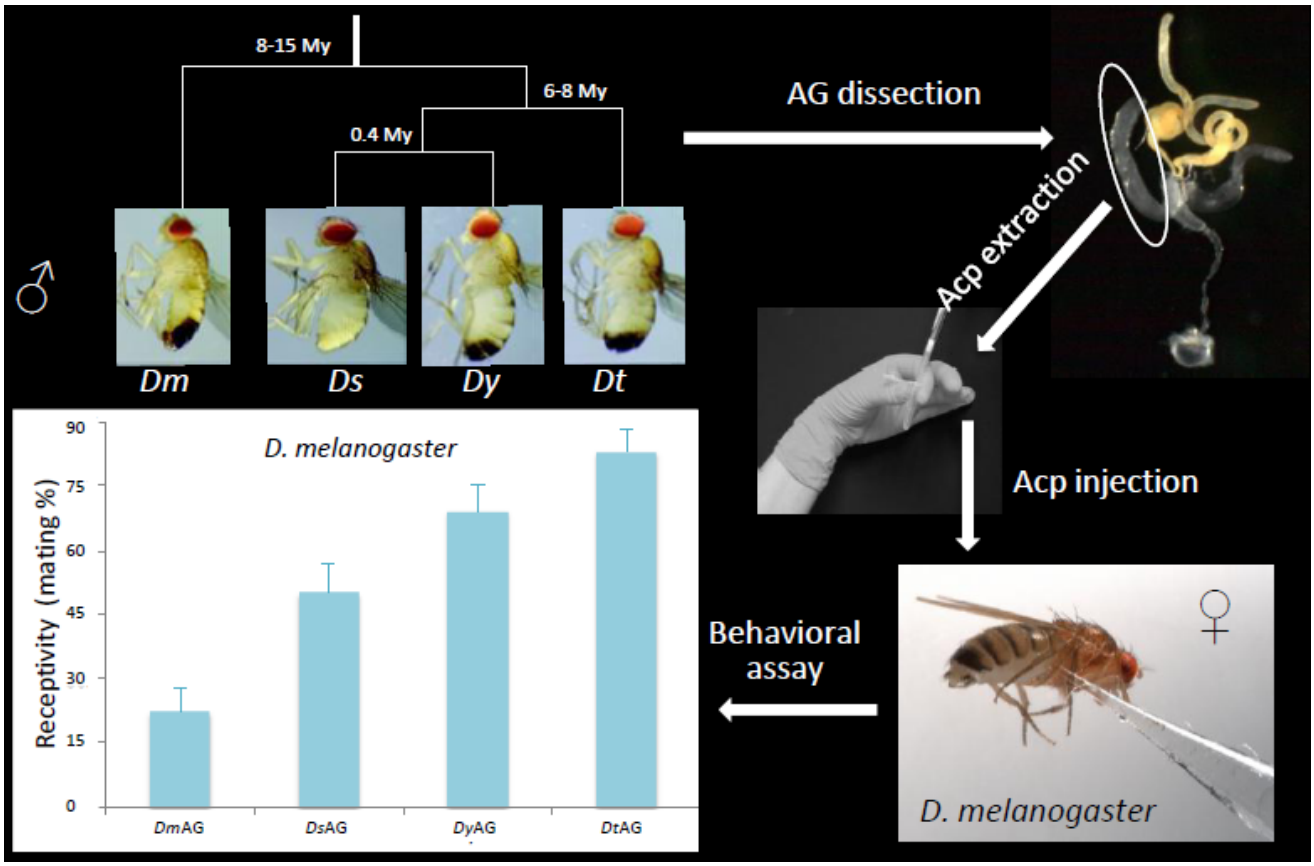
**Abstract**: In sexual species, mating success depends on male capacity to find sexual partners and on female receptivity to mating. Mating is under evolutionary constraints to prevent interspecific mating and to maximize the reproductive success of both sexes. In *Drosophila melanogaster*, female receptivity to mating is mainly controlled by Sex peptide (SP, *i.e*. Acp70A) produced by the male accessory glands with other proteins (Acps). The transfer of SP during copulation dramatically reduces female receptivity to mating and prevents remating with other males. To date, female postmating responses are well-known in *D. melanogaster* but have been barely investigated in closely-related species or strains exhibiting different mating systems (monoandrous *versus* polyandrous). Here, we describe the diversity of mating systems in two strains of *D. melanogaster* and the three species of the *yakuba* complex. Remating delay and sexual receptivity were measured in cross-experiments following SP orthologs or Acp injections within females. Interestingly, we highlighted strong differences between the two strains of *D. melanogaster* as well as among the three species of the *yakuba* complex. These results suggest that reproductive behavior is

under the control of complex sexual interactions between the sexes and evolves rapidly, even among closely-related species.

**Key words**: female remating; female sexual receptivity; accessory gland proteins; Sex peptide; *yakuba* complex; *Drosophila*

**Highlights**

- Drosophila Sex peptide (SP) and Acps regulate female remating behavior
- We tested for conserved role of SP orthologs and Acps in closely-related species
- Female receptivity was strongly reduced in species of the *yakuba* complex
- Female remating rates highly depended on the species origin of SPs and Acps
- Females of *D. santomea* and *D. yakuba* were more resistant to SP and Acp effects



## 1. Introduction

Female polyandry is a major component of mating systems, driving a series of processes implicated in postcopulatory sexual selection. Even if males deliver enough sperm to ensure fertilization of eggs during a single mating event, female remating has been widely reported in many taxonomical groups (Birkead and Møller, 1998). Considerable efforts to understand why females mate several times have highlighted a number of genetic and non-genetic benefits (Jennions and Petrie, 2000). However, female polyandry reduces male paternity assurance, and various coercive adaptations have evolved to force decreased female remating in response to such conflict. These include quality and persistence of male courtship and mate guarding (Thornhill and Alcock, 1983; Birkhead *et al.*, 1985; Edward *et al.*, 2014), coercive behaviors (Clutton- Brock and Parker, 1995), mating plug (Avila *et al.,* 2011) and a series of compatible signal-reception systems involving ejaculate components in males and receptors in females (Wolfner, 2009; Sirot *et al.*, 2009; Avila *et al.*, 2015). These male adaptations induce a refractory period of female sexual receptivity that can sometimes last until female death (blowfly: Gillott, 2003; mosquitos: Helinski *et al.*, 2012).

In insects, the decrease of female sexual receptivity after mating depends on seminal fluid proteins mostly produced by male accessory glands (Acps) and transferred during mating (Mediterranean and South-American fruit flies: Miyatake *et al.*, 1999 and Abraham *et al.,* 2016, respectively; Lepidoptera: Wedell, 2005; mosquitoes: Dottorini *et al.,* 2007; Drosophila: Sirot *et al.*, 2009). Acps functional classes are widely conserved across the animal kingdom, including vertebrates and mammals, the majority being proteases, protease inhibitors, lectins, prohormones, mediators of an immune response, and lipid metabolism categories (Chapman, 2008; Findlay *et al.*, 2008). In contrast, the primary sequences of Acps exhibit evolutionary patterns that are far more rapid than those of proteins not involved in reproduction (Swanson and Vacquier, 2002; Haerty *et al.,* 2007; Walters and Harrison, 2010). The diversity of Acp effects on female postmating responses has raised various questions with respect to the evolutionary conservation of their functions, their functional redundancy, and the female phenotype induced (Wolfner, 2002; Avila *et al.*, 2011).

Among Acps, Sex peptide (SP, Acp70A) is one of the major agents eliciting female decreased receptivity in *D. melanogaster* (Kubli, 1992). Experimental injections of synthetic SP into the abdomen of virgin females showed that the C-terminal part of SP is essential to reduce sexual receptivity (Schmidt *et al.*, 1993). Such an effect was also shown among the closely-related species *D. simulans, D. sechellia* and *D. yakuba*, but not outside the *melanogaster* group, since no inhibitory effect was detected on virgin females of the more distant species of the *obscura* or *willistoni* groups (Tsuda *et al.*, 2015). However, SP orthologs have been found in all species of the Sophophora subgenus studied to date (Kim

*et al.,* 2010), which raises questions about their functional roles with regards to between-species variations in female sexual receptivity inhibition (Singh *et al*., 2002). Interestingly, when injected into the *Helicoverpa armigera* moth, SP from *D. melanogaster* was found to significantly deplete the female pheromone production, reducing their attractiveness and receptivity (Fan *et al.,* 2000). In *D. melanogaster*, the N-terminal part of SP has been shown to reduce female pheromone production, possibly through juvenile hormone activation (Bontonou *et al*., 2015). Moreover, SP acts within a network of Acps including two C-type lectins (CG1652 and CG1656), a serine protease homolog (CG9997), a cysteine-rich secretory protein (CG17575) (Ravi Ram and Wolfner, 2007, 2009) and a serine protease, seminase (CG10586) (LaFlamme *et al*., 2012) that are required for SP binding to sperm, causing long-term inhibition of female sexual receptivity when sperm are stored (Peng *et al*., 2005). Three additional proteins (two serine proteases, Aquarius CG14061 and Intrepid CG12558, and a cysteine-rich secretory protein, Antares CG30488) were positioned upstream within this network (Findlay *et al.,* 2014).

The diversity of the Acp effects on female postmating physiology and behavior raises various questions with respect to the female phenotype induced. Indeed, female postmating response is known to vary widely across populations of a single species, but its measurement largely depends on the experimental protocol (Singh *et al*., 2002). *Drosophila* species are good candidates to address these questions thanks to the in-depth knowledge of their genomes, the numerous genetic tools that can be developed, and the diversity of the mating strategies observed within this species group (Joly *et al*., 1991; Chang, 2004; Markow and O'Grady, 2005).

In the present study, our goal is to characterize the effects of Acps and SP orthologs to measure, under a standard protocol, the duration and extent of the female sexual refractory period in species of the *yakuba* complex. We tested the conserved role of SP orthologs or Acps through a cross-experimental study. We showed significant differences in the duration of the inhibition of the female sexual receptivity, not only among species, but also between strains of a single species. Finally, the constitutive expression of SP orthologs in *D. melanogaster* revealed a significant effect on female behavior, which suggests divergent evolutionary interplay between sexes.

## 2. Material and methods

### *2.1. Fly stocks*

All flies were grown on standard corn-meal medium with live-yeast granules at 21°C under natural photoperiod. Virgin flies were collected at emergence, under $CO_2$ anesthesia, sexed and kept sex-separated in vials with food, until their use.

The *D. melanogaster* strains used were the laboratory wild-type Canton S (*Dm*-cs) strain and a wild-type strain collected in Chavroches (*Dm*-ch, 46° 21' 0" North, 3° 36' 0" East, France, Gif stock 2008); *D. santomea* originating from São Tomé Island (*Ds*, Gif stock 390-2, collected in the Obo Forest, 1998); *D. yakuba,* a Cameroun strain (*Dy*, Gif stock 115, collected in Kounder, 1967) and *D. teissieri,* a multi-female strain from Zimbabwe (*Dt*, Gif stock 308-1, collected in the Chirinda Forest, 1991).

To highlight the role of SP in the reduction of female sexual receptivity in both strains of *D. melanogaster (Dm-cs and Dm-ch)*, we used Acp extracts from two different types of male mutants: SP0 that do not produce SP (Liu and Kubli, 2003), and DTA-E (Kalb *et al.*, 1993), which are sperm-less and lack Acps produced from the main cells (96% of the accessory glands). DTAE-males do, however, secrete proteins from secondary cells of accessory glands, as well as the ejaculatory bulb and duct (Gligorow *et al.,* 2013).

### *2.2. Female remating rate assays*

Single 5 to 8-day old virgin females were exposed to single virgin males of the same age for 3 hours at 20-22°C. After copulation, the mated females were separated from the males and kept individually in corn-meal food vials until the next mating trial. Unmated females and those that did not produce larvae after the first mating were discarded. 24 hours after the first mating, females were individually exposed to two new virgin males (6 to 8 days old) for 2 hours. After each trial, unmated females were transferred into a new corn-meal food vial, until the next mating assay 24 hours afterwards. Such an assay was repeated every 24 hours for four consecutive days after the first mating. The cumulative percentage of remated females was calculated for each of the four days of the experiment. Between 250 and 300 females were tested at the starting point. Copulation latency (*i.e.* the time between the male being introduced into the female-containing vial and copulation) and copulation duration were recorded for first and second matings.

### *2.3. SP ortholog transcript sequencing*

Total RNA was extracted from the two strains of *D. melanogaster* and from the *D. santomea, D. yakuba* and *D. teissieri* species using TRIzol®Reagent from Life Technology according to the manufacturer's instructions. RNA preparations were reverse-transcribed using 1 µg of RNA (Invitrogen). RT-PCR experiments were performed on five

whole-body adult flies. Specific primer pairs, which annealed to the SP ortholog sequences, were designed using CLUSTAL W software (Thompson *et al.,* 1994, Table S1). Amplified PCR products were sequenced to check the identity of the ortholog sequences.

### *2.4. SP ortholog expression in* D. melanogaster *transgenic flies*

Effects of over-expression of SP genes (*Dm*SP from *D. melanogaster*, *Ds*SP from *D. santomea,* or *Dt*SP from *D. teissieri*) on *D. melanogaster* female receptivity were studied using the ubiquitous daughterless-gal (da-gal) driver. Additionally, the SP cDNA ortholog sequences (from ATG to STOP) were also cloned into the *p {UAST}* plasmid using the primers shown in Table S1. Transgenic flies were generated by *P*-mediated germline transformation in *D. melanogaster* $w^{118}$ embryos (Bestgene Inc). Two independent lines containing a single insert were obtained: one insert on the chromosome 2 was associated with the SM5 Balancer and one on the chromosome 3 with the TM3 Balancer. The transformed lines were controlled by PCR, followed by DNA sequencing to confirm the expected SP sequences. Similar transgenic lines without any SP gene insert were kept and used as a control.

To test sexual receptivity, three SP transgenic 5-day-old females (either *Dm*SP, *Ds*SP or *Dt*SP) were exposed to seven wild-type virgin *Dm*-cs males for 3 hours at 20-22°C. At least 42 females were tested for each condition.

### *2.5. Solutions for injections*

#### *2.5.1. Accessory gland proteins (Acps) extracts*

Males were briefly anesthetized on ice, and their accessory glands (AG) were carefully extracted from the abdomen and isolated from the internal reproductive tract. For each sample, ten AG (five males) were pooled in 2.5µL of Ringer's buffer. Excreted soluble proteins present in the AGs were recovered by vortexing and 10 minutes of centrifugation at 4°C and 14 000g. The supernatant was collected, adjusted to 2.5 µL using a SpeedVac® and kept at -20°C until use. This protocol allowed us to apply a standard concentration of 4 AGs/µL that corresponds to 1x in the dose-dependent response experiment.

#### *2.5.2. SP ortholog and Dup99B synthetic peptides*

Three SP orthologs were synthetized by the Proteogenix® society: *Dm*SP from *D. melanogaster*, *Ds*SP from *D. santomea* and *Dt*SP from *D. teissieri,* and one Dup99B peptide from the *D. melanogaster* sequence, all with at least 95% purity as measured by HPLC. Sharing the same SP protein sequences, synthetic *Dm*SP was used for both strains

of *D. melanogaster.* Similar peptide was also obtained for *D. santomea* and *D. yakuba species* (*i.e., Ds/Dy*SP). The synthetic peptides were dissolved in Ringer's buffer and injected into females at an efficient concentration of 1.32 pmol for SP and 1.16 pmol for Dup99B, which is some two-fold higher than the critical concentration determined previously (Schmidt *et al*., 1993).

*2.5.3. Acp extracts and synthetic SP ortholog dose-dependent responses*

To evaluate the dose-dependent responses of SP orthologs and Acps on female receptivity, we performed a series of injections with different concentrations of SP orthologs or Acps. For SP orthologs, concentrations of 0.25x, 0.5x, 1x, 2x, 4x and 10x were used. For Acps, concentrations of 0.05x, 0.1x, 0.25x, 0.5x, 1.5x and 2x were used. All preparations were kept at -20°C before use.

*2.5.4. Female injections with synthetic SP orthologs and Acp extracts*

Five day-old wild-type virgin females were anesthetized on ice and injected in the upper right part of the ventral face with 50 nL of SP or Acp solutions using a Nanoject II© (Drummond Scientific Company). As a control, some of the virgin females were injected with 50 nL of Ringer's buffer. Needles were made with a PC-10 needle puller (Narishige) producing a 0.3-0.5 µm diameter needle, which limited damage to the female's body. The needles were first filled with mineral oil (Oil 3S Prolabo Voltalef®, viscosity cPo 25°C 115) followed by the different solutions of interest. Injections were performed manually under a light microscope. Injected females were then left to recover in food vials for 4 hours before being tested for sexual receptivity.

*2.5.5. Receptivity assays after injections*

Three injected females (either with SP orthologs, Acps or Ringer's buffer) were introduced along with seven wild-type virgin males (5 to 8 days old), into a standard corn-meal food vial and observed for mating receptivity for 3 hours at 20-22°C. These assays were carried out 4 hours after injection. Female sexual receptivity was measured as the number of mated females. At least 50 females were tested for each condition.

*2.5.6. Statistical analyses*

Data analyses were performed with Excel (ver. 14.6.8), JMP (ver. 9, SAS Institute) and R software (ver. 2.12.1 with the help of "multicomp" and "faraway" packages). Chi-square tests (with Yates correction) were used to compare female receptivity between the first and the second mating, and also to compare female receptivity between strains containing [+] or not containing [-] the SP gene. Tukey-Kramer tests were used to compare

copulation latency and duration of copulation between first and second mating events in the female remating experiment.

A linear model was fitted to analyze female receptivity after injection of Ringer's buffer, while a generalized linear model GLM (binomial family) was used to test the fixed effects of treatments, injected solutions, species and origin (intraspecific or interspecific) for interspecific analysis. Each treatment was compared to the corresponding control (Ringer-injected females) as the reference (model intercept). A Bonferroni correction for multiple tests was applied in all cases. Finally, the dose-dependent response experiment in *D. teissieri* was analyzed by linear regression.

## 3. Results

### *3.1. Female sexual receptivity after mating*

The proportion of sexually receptive females 24 hours after mating decreased from 66% to 100% for all species and strains (Chi-square tests, df = 1, $P < 0.0001$ for the 5 conditions, Figure 1). A strong variation in female sexual receptivity was observed. *D. melanogaster* females had the highest receptivity values, with 79% and 74% of mated females in *Dm*-cs and *Dm*-ch, respectively. *D. yakuba* and *D. teissieri* displayed intermediate values of 37% and 46%, respectively and *D. santomea* had the lowest value with only 28% of females being receptive.

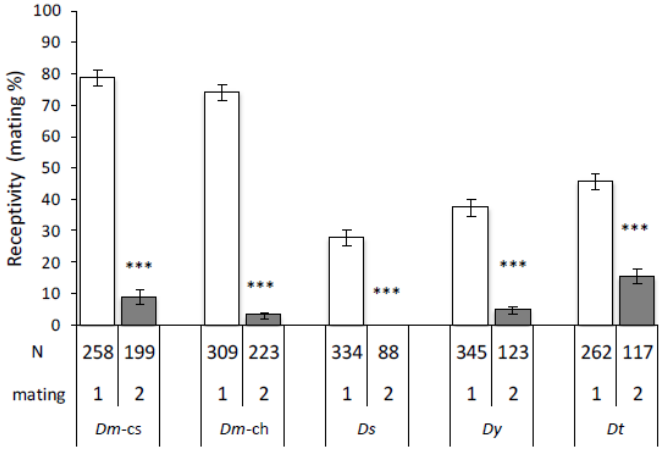



Fig. 1. Female receptivity during the first mating (white bars) and 24 hours later (dark bars) in *D. melanogaster* (*Dm*-cs and *Dm*-ch), *D. santomea* (Ds), *D. yakuba* (Dy) and *D. teissieri* (Dt). Each bar represents the proportion ± standard error (SE). N is the number of tested females. Mating is the mating rank. Note that for *D. santomea*, none of the 88 first-mated females was receptive. *** $P < 0.001$.

To better characterize the mating system for each species and strain, the cumulated rate of female remating was plotted every 24 hours after the first mating for four consecutive days (Figure 2). We observed a strong difference between the two strains of *D. melanogaster* (*Dm*-cs and *Dm*-ch) with less than 10% of *Dm*-ch females being receptive to remating after four days compared to 60% of *Dm*-cs females (Figure 2A). Interestingly, remating rates between sexual partners from the two *D. melanogaster* strains (*Dm*-cs females x *Dm*-ch males and *vice-versa*) were very similar to that of the *Dm*-ch strain. The intra-strain specific difference was as high as the difference between species of the *yakuba* complex (Figure 2B). Also, *D. teissieri* showed a remating slope similar to *Dm-cs* while *D. santomea* and *D. yakuba* were more similar to *Dm*-ch. In contrast, *D. santomea* females never remated during the course of the experiment.

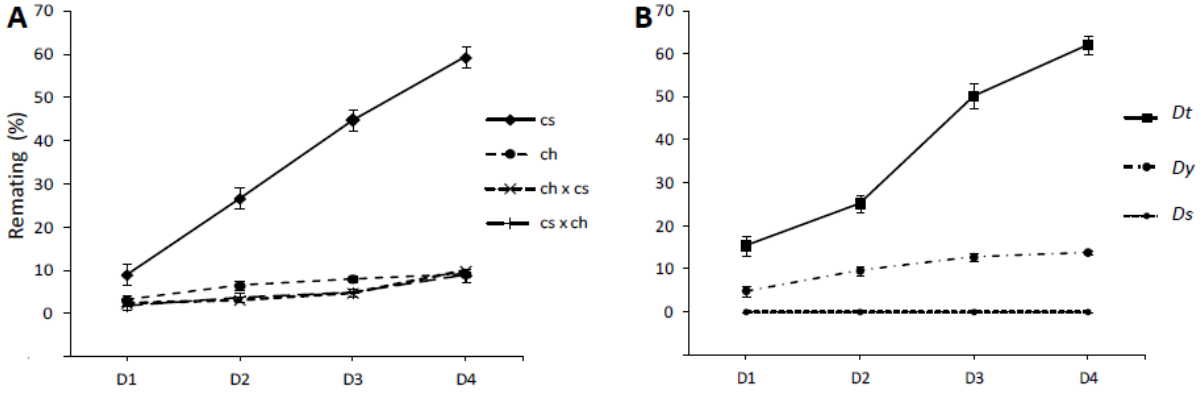



Fig. 2. Cumulative percentages (± SE) of female remating for four days after the first mating. Standard-errors are indicated on all curves, but some are too small to be visible. A- Results for the two different strains of *D. melanogaster* (cs and ch), with the heterogamic tests between cs females x ch males and ch females x cs males. B- Results for the species of the *yakuba* complex, *i.e*. *D. santomea* (*Ds*), *D. yakuba* (*Dy*) and *D. teissieri* (*Dt*).

The comparison of mating parameters (copulation latency and copulation duration) between the first and second matings is shown in Figure 3. Clearly, the copulation latencies of the *D. melanogaster* strains were shorter in the first mating than in the second, which was not the case for the species of the *yakuba* complex (Figure 3A). There was more variation among the former than among the latter (Tukey-Kramer tests, $P < 0.05$). The lack of data for the second mating in *D. santomea* was due to the fact that none of the 88 tested females remated. In contrast, the copulation duration was relatively homogeneous between strains of *D. melanogaster,* with the second mating being slightly shorter than the first one. However, there was more variation between species of the *yakuba* complex (Figure 3B). *D. teissieri* showed the longest copulation duration (Tukey-Kramer tests, $P < 0.0001$).

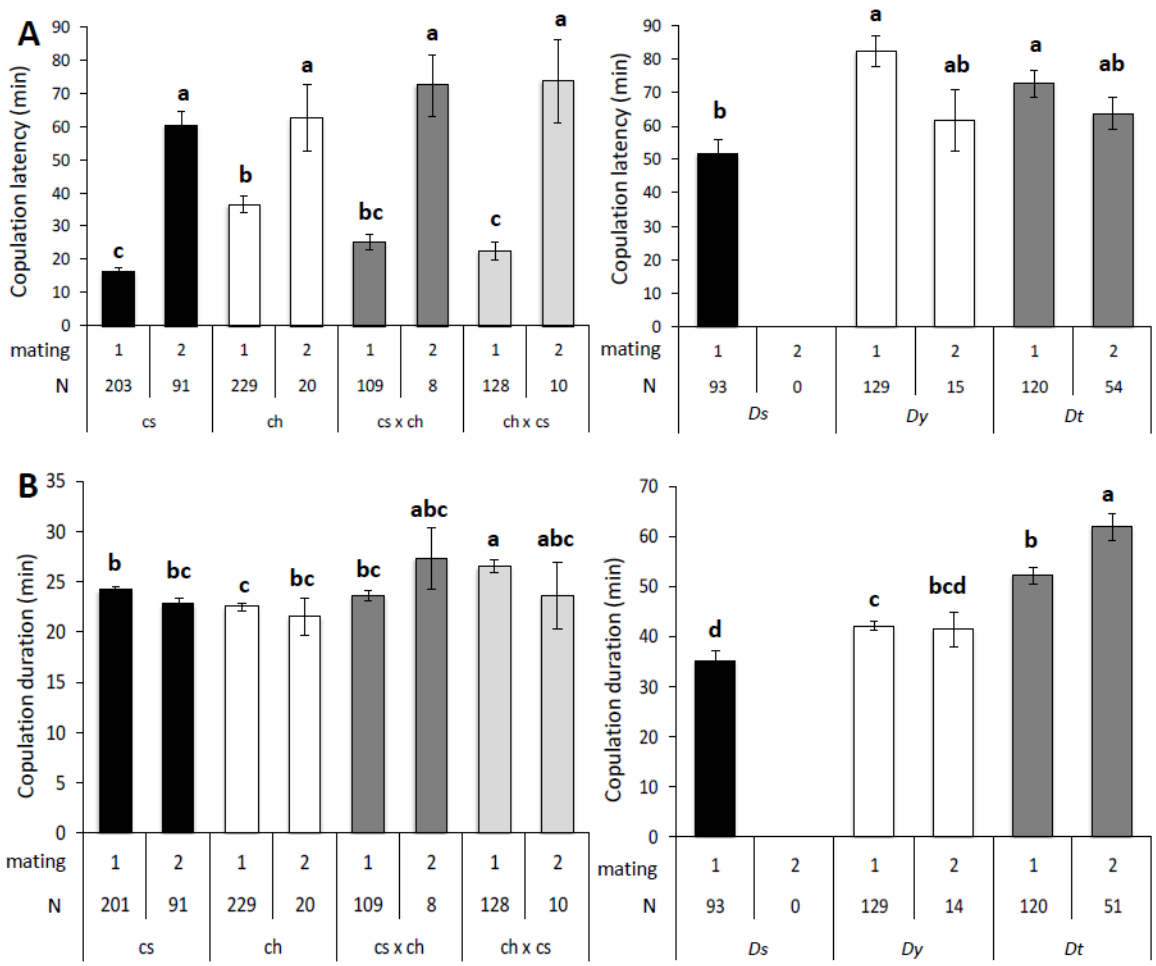



Fig. 3. Copulation latency (A) and copulation duration (B) between first and second mating. Bar charts on the left show results for the *D. melanogaster* strains: conspecific pairs (cs and ch) and heterospecific pairs (females from one strain crossed to males from the other strain, and *vice-versa*). Bar charts on the right are for the species of the *yakuba* complex (*D. santomea*: *Ds*, *D. yakuba*: *Dy*, and *D. teissieri*: *Dt*). Each bar represents mean ± SEM. Numbers of repetitions (N) are indicated below the bars. Different letters above the bars indicate significant differences between mean values based on multiple-comparison Tukey-Kramer tests ($P < 0.05$).

### *3.2. Comparison of SP ortholog sequences*

To date, SP ortholog sequences are only available for five species of the *melanogaster* complex (*i.e. D. melanogaster, D. simulans, D. sechellia, D. yakuba* and *D. erecta*) and only as unannotated sequences for *D. santomea* (http://genomics.princeton.edu/ AndolfattoLab/Dsantomea_genome.html). From these data,

we generated primers to determine the nucleotide sequences of SP orthologs in the species and strains studied here (GenBank, LT629262 and LT629263). As observed, the translated sequences of SP corresponding to mature peptides of both *D. melanogaster* strains were identical, as for the *D. santomea* and *D. yakuba* pair (Figure 4). The SP orthologs shared 83% aminoacyl identity. *Dt*SP was more divergent, sharing 80.5% identity with that of *Dm*SP. Moreover, the C-terminal parts of the peptide (17-36) responsible for the modification of female sexual behavior (Ottiger *et al*., 2000) were widely conserved (95% identity).

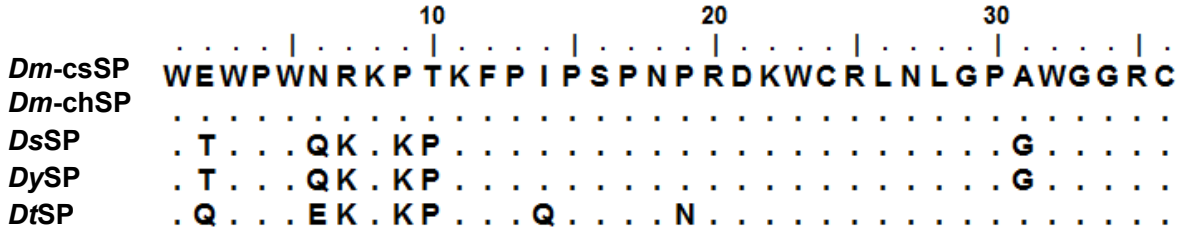



Fig. 4. Amino-acid sequences of the secreted SP of *D. melanogaster* Canton S (*Dm*SP-cs) compared to those of the other strains or species tested. Note that the two strains cs and ch have identical SP peptides, as do *D. santomea* and *D. yakuba*. *Dm*-cs*: D. melanogaster Canton S; Dm*-ch*: D. melanogaster* Chavroches; *Ds: D. santomea; Dy: D. yakuba*; *Dt: D. teissieri*.

Furthermore, the SP protein sequences from the strains we used were similar to those available in Flybase (http://flybase.org) for *D. melanogaster* (ID CG17673) and *D. yakuba* (ID GE21959), in spite of some differences in nucleic sequences. This high level of protein identity supports the functional conservation of the role of SP orthologs on female sexual receptivity.

### *3.3. Ectopic expression of SP orthologs in transgenic* D. melanogaster *flies*

To investigate the conserved role of the SP ortholog sequences on *D. melanogaster* females, we produced transgenic lines expressing SP from *D. melanogaster* SP (*Dm*SP), *D. santomea* (the same as *D. yakuba, Ds/Dy*SP) or *D. teissieri* (*Dt*SP) (Figure 5). Transgenic insertions were performed on chromosomes 2 and 3 to eliminate a role of positional insertion within the genome. As expected for *Dm*SP, female receptivity of the transgenic lines was significantly lower than the control lines without SP expression; similar results were obtained with *Ds/Dy*SP and *Dt*SP (GLM, $P < 1.3\ 10^{-7}$ for all conditions, *i.e.* species and chromosome insertion). Receptivity reduction ranged from 68% to 93% between control and transgenic

lines, which suggests a non-causal relationship between the decrease in female receptivity and the origin or location of the transgenes.

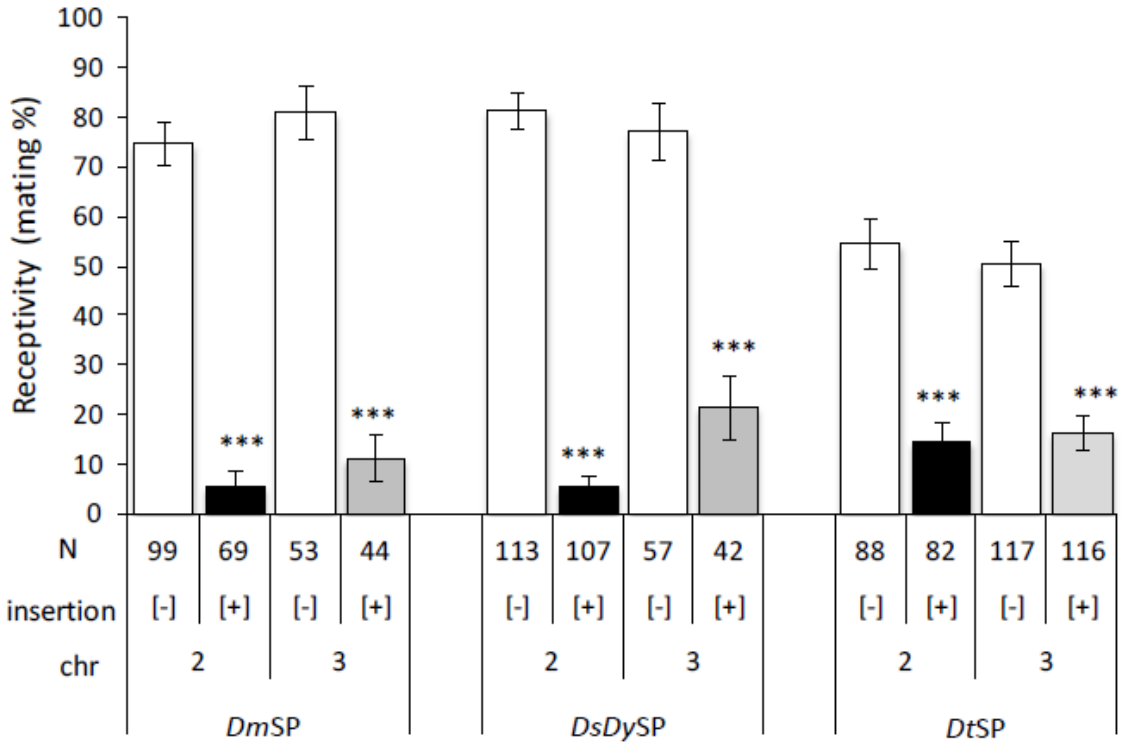



Fig. 5. Effect of transgenic insertion of the SP genes [+] on chromosomes 2 (dark bars) and 3 (grey bars) of *D. melanogaster* (*Dm*SP), *D. santomea*/*D. yakuba* (*DsDy*SP, the sequences being similar), and *D. teissieri* (*Dt*SP) in different *D. melanogaster* $w^{118}$ females. The controls (white bars) correspond to the insertion of the transgene without the SP gene [-] in all lines. Each bar represents percentage ± SE. N is the number of tested females. *** $P < 0.001$.

### *3.4. Female receptivity after SP ortholog or Acp injections into* D. melanogaster

Consistent with previous findings (Schmidt *et al.*, 1993; Ottiger *et al.*, 2000), conspecific injections of *Dm*SP significantly decreased female receptivity in both *D. melanogaster* Canton S and Chavroches strains, compared to Ringer's buffer injections (GLM, $P = 4.3\ 10^{-6}$, and $P = 2.8\ 10^{-6}$ for Canton S and Chavroches, respectively, Figure 6). Interestingly, the SP ortholog of the *yakuba* complex species (*Ds/Dy*SP and *Dt*SP, Figure 6) had weaker but still significant effects on female sexual receptivity in both *D. melanogaster* strains (GLM, $P = 0.164$ for san/ *Ds/Dy*SP and $P = 0.007$ for *Dt*SP in Canton S and $P = 0.004$ for san/ *Ds/Dy*SP and $P = 0.560$ for *Dt*SP in Chavroches).

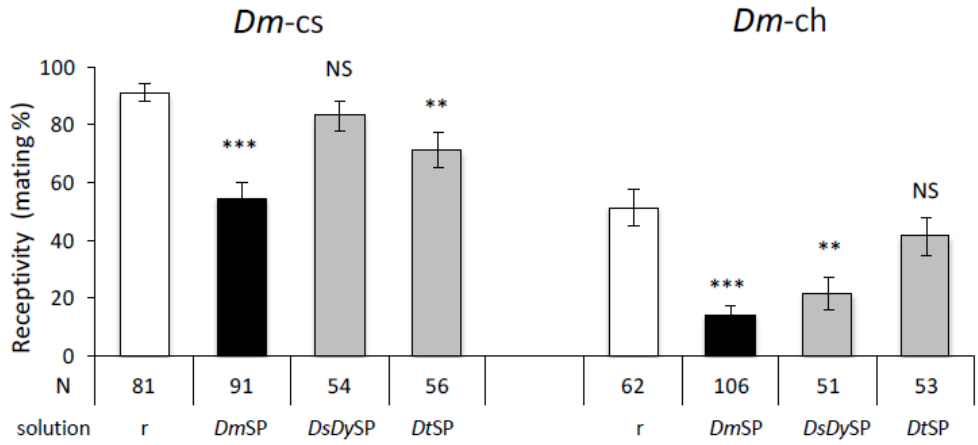



Fig. 6. Receptivity of *D. melanogaster* Canton S (*Dm*-cs) and Chavroches (*Dm*-ch) females 4 hours after injection of synthetic SP solutions from *D. melanogaster* (*Dm*SP), *D. santomea* / *D. yakuba* (*DsDy*SP) and *D. teissieri* (*Dt*SP). Each bar represents percentage ± SE. Black bars indicate intra-specific SP injections; grey bars indicate inter-specific SP ortholog injections. *P*-values are calculated against the Ringer treatment (r). N is the number of tested females. NS $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

In *D. melanogaster* (Canton S and Chavroches), the Acp extracts from different species and strains induced significant reductions of female sexual receptivity (GLM, $P < 0.003$) except the extracts from *D. teissieri* (GLM, $P = 0.152$ in Canton S and $P = 1$ in Chavroches) and *D. santomea* in Chavroches females (GLM, $P = 0.061$, Figure 7).

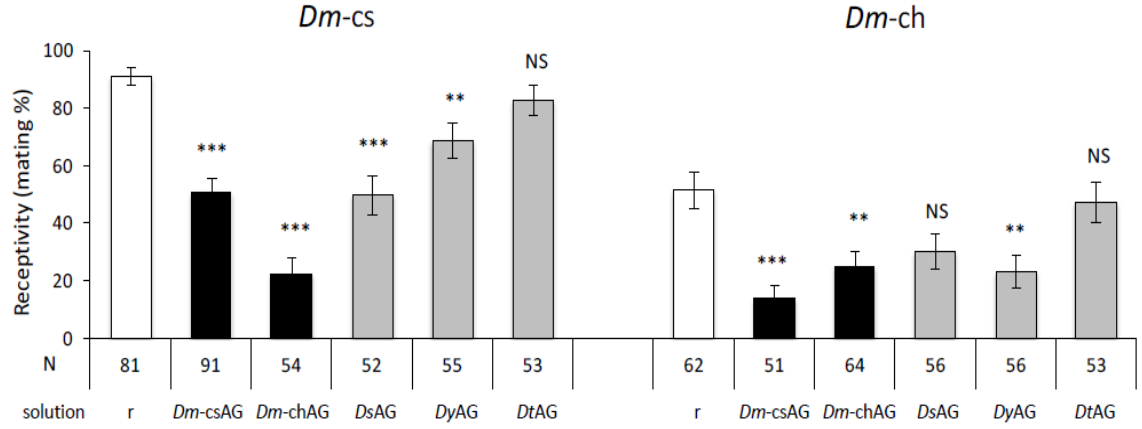



Fig. 7. Receptivity of *D. melanogaster* Canton S (*Dm*-cs) and Chavroches (*Dm*-ch) females 4 hours after injection of accessory gland proteins from *D. melanogaster* Canton S (*Dm*-csAG) and Chavroches (*Dm*-chAG), *D. santomea* (*Ds*AG), *D. yakuba* (*Dy*AG) and *D. teissieri* (*Dt*AG), Each bar represents percentage ± SE. Black bars indicate intra-specific SP injections; grey bars indicate inter-specific SP ortholog injections. The *P*-values were

calculated against the Ringer treatment (r). N is the number of tested females s. NS $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Interestingly, injection of Acps from Chavroches males lead to a stronger receptivity decrease than *Dm*SP in Canton S females (GLM, $P = 1.2\ 10^{-11}$ and $P = 4.3\ 10^{-6}$, respectively), while not in Chavroches females (GLM, $P = 0.009$, and $P = 2.949\ 10^{-6}$, Figures 6 and 7). *D. teissieri* was the only species whose SP ortholog (*Dt*SP) or Acps (*Dt*AG) induced very low or no significant effect on either Canton S or Chavroches females (GLM, $P = 0.007$ and $P = 0.152$ for Canton S females and $P = 0.560$ and $P = 1$ for Chavroches females).

The effect of *Dm*SP as a main actor reducing female receptivity in *D. melanogaster* was confirmed by injection of Acp extracts from the null mutant SP0 (which does not produce any SP) or from the DTA-E mutant (which lacks Acps, including SP). In both cases, these two similar control injections did not reduce the receptivity when compared to Ringer injections (GLM, $P \geq 0.173$ for both Canton S females and $P = 1$ for both Chavroches females, Figure 8). Moreover, as expected from data in the literature (Saudan *et al.*, 2002), our results show that another seminal peptide, Dup99B from the ejaculatory duct, also produced a significant decrease in female receptivity in Canton S females, but interestingly not in Chavroches females (GLM, $P = 0.008$ and $P = 1$, respectively).

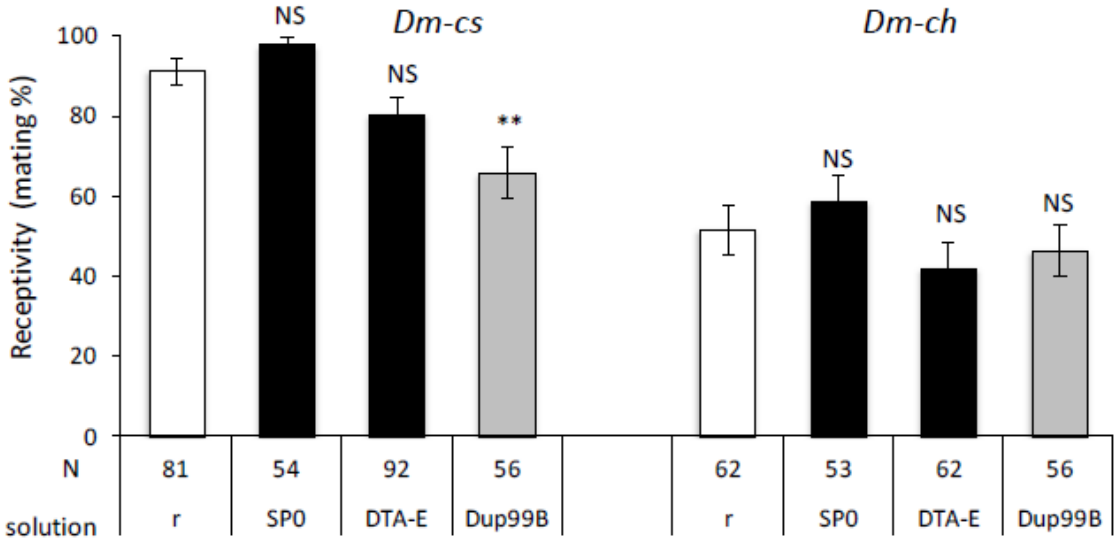



Fig. 8. Receptivity of *D. melanogaster* Canton S (*Dm*-cs) and Chavroches (*Dm*-cha) females 4 hours after injection of accessory gland proteins from SP0 males or DTA-E males, or of Dup99B synthetic peptides. Each bar represents percentage ± SE. Black bars indicate injections of Acps; grey bars indicate injection of synthetic peptide. The *P*-values were calculated against the Ringer treatment (r). N is the number of tested females. NS $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

### 3.5. Female receptivity after SP ortholog or Acp injections in species of the yakuba complex

Contrasting with results for *D. melanogaster* strains, injection of SPs or Acps whatever the species origin, had very limited effects on sexual receptivity of *D. santomea* and *D. yakuba* females (GLM, $P > 0.247$ for all conditions, except for *Ds/Dy*SP and *Dm*-chAG in *D. yakuba,* which showed 58% and 50% decreases of female receptivity, $P = 0.036$ and $P = 0.225$, respectively, Figure 9). Therefore, larger effects were found in *D. teissieri* females (GLM, $P < 0.013$ for all conditions except for Ds-AG, $P = 0.216$). In this species there was no significant difference in the decrease in female sexual receptivity between Acps and SPs whatever the species and strains of *D. melanogaster* (GLM, $P \geq 0.055$).

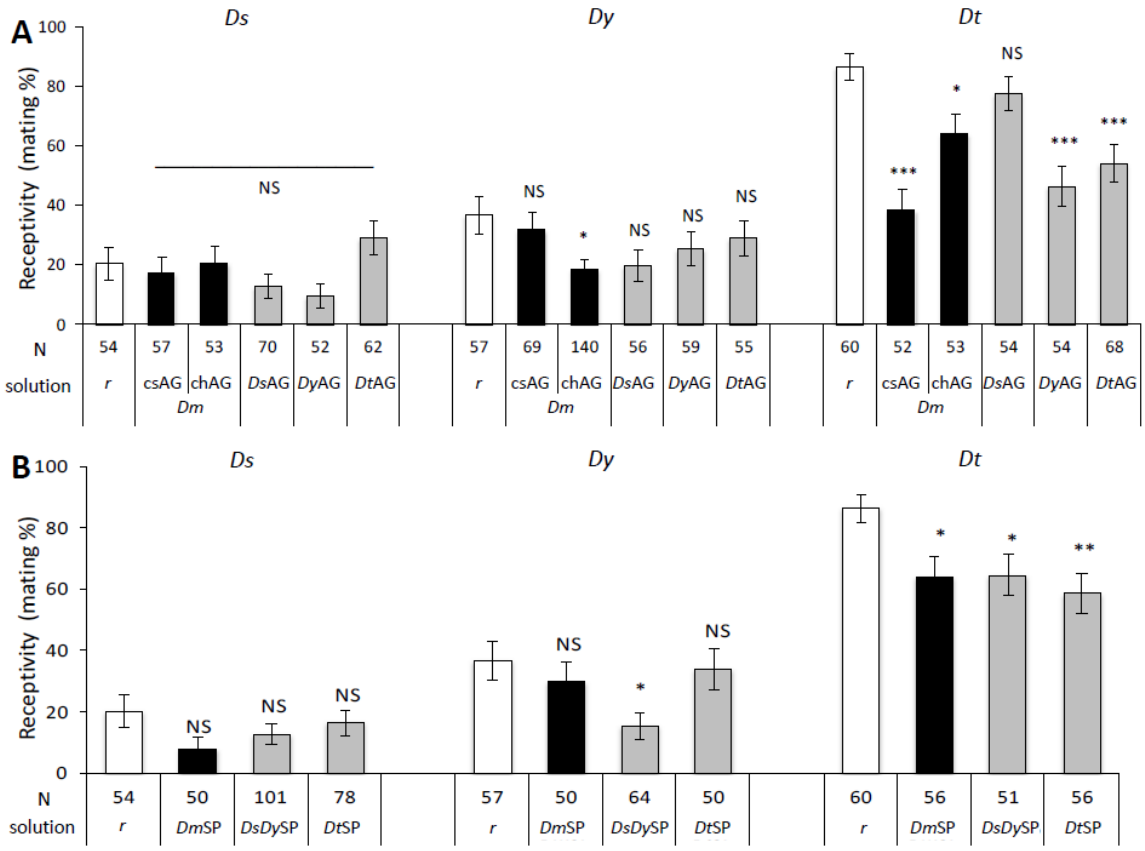



Fig. 9. Female receptivity 4 hours after injection of Acps from accessory glands (A) of all species/strains from *D. melanogaster* Canton S (*Dm*-csAG), *D. melanogaster* Chavroches (*Dm*-chAG); *D. santomea* (*Ds*AG), *D. yakuba* (*Dy*AG) and *D. teissieri* (*Dt*AG). Injections of SP (B) from *D. melanogaster* (*Dm*SP), *D. santomea* / *D. yakuba* (*DsDy*SP) and *D. teissieri* (*Dt*SP). Each bar represents percentage ± SE. Black bars indicate solutions from *D. melanogaster,* grey bars indicate solutions from species of the *yakuba* complex. *P*-values were calculated against the Ringer treatment (r). N is the number of tested females. NS $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

### *3.6. Dose-dependent responses of SP orthologs and Acps*

These experiments tested the possibility that females from the species of the *yakuba* complex were less sensitive than *D. melanogaster* at similar concentrations of SP orthologs and Acps, in particular at the standard dose used in the previous experiment. Synthetic *Dm*SP significantly decreased female receptivity in both *D. melanogaster* Canton S and Chavroches females at the standard dose (1x, GLM, $P = 4.3\ 10^{-6}$ and $P = 3.7\ 10^{-6}$, respectively, Figure 10). However, at higher concentrations, the reduction was gradual in Canton S females (ANOVA, $F_{1,4} = 19.974$, $R^2 = 0.791$, df = 5, $P = 0.011$) while values dropped drastically in Chavroches females (Figure 10). A 87% decrease was found in female receptivity in *D. santomea* at higher concentration of *DsDy*SP (GLM, $P = 0.021$ and $P = 0.016$ for the last two concentrations 2x and 4x, respectively); similarly a 58% and 62% decrease was found in female receptivity in *D. yakuba* (GLM, $P = 0.027$ and $P = 0.017$ for the 1x and 10x concentrations, respectively). *D. teissieri* resembles *D. melanogaster* Canton S, with a strong reduction of female receptivity from the lowest concentration tested (1x, GLM, $P \leq 0.001$) but a decrease in female sexual receptivity with increasing *Dt*SP concentrations that is gradual but not significant from 1x to 10x (ANOVA, $F_{1,3} = 8.084$, $R^2 = 0.639$, df = 4, $P = 0.0655$).

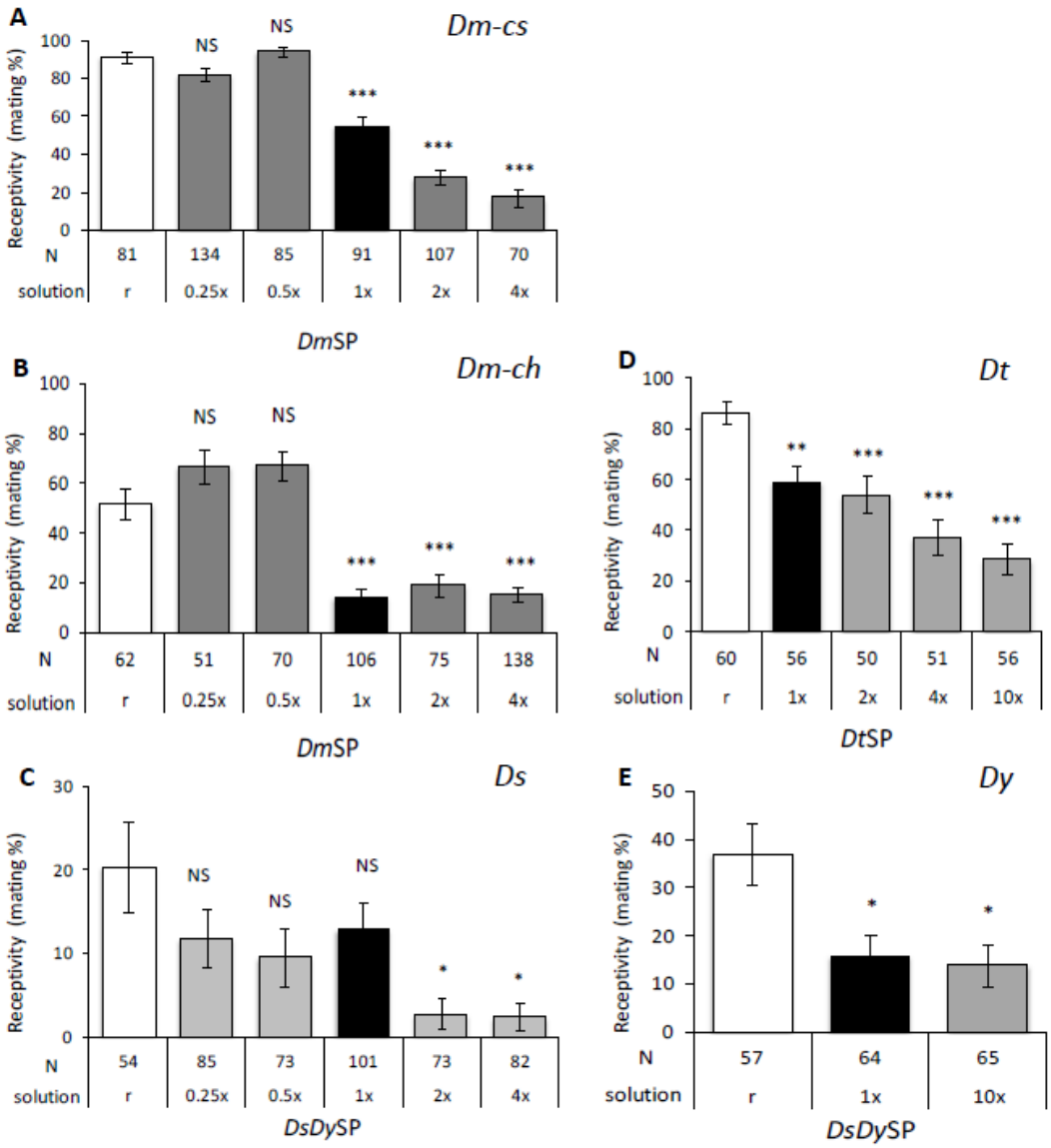



Fig. 10. Dose-dependent responses of SP orthologs on female receptivity 4 hours after injection in *D. melanogaster* Canton S (A, *Dm*-cs) and Chavroches (B, *Dm*-ch), *D. santomea* (C, *Ds*), *D. teissieri* (D, *Dt*) and *D. yakuba* (E, *Dy*). Note that for the last two species an additional dose-dependent response (x10) was performed to amplify the effect. Each bar represents percentage ± SE. Black bars represent standard concentrations of SP orthologs. Grey bars represent dilutions and concentrations of SP orthologs from the standard one. White bars represent injections of Ringer solution. *P*-values were calculated against the Ringer treatment (r). N is the number of tested females. NS $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The results were very similar for the Acp dose-dependent responses in all species/strains analyzed. A significant effect on *D. melanogaster* female receptivity was obtained at 0.5x in Canton S, while 1x was required in Chavroches (GLM, $P = 0.0006$ and $P$

= 0.014 respectively, Figure 11), with a gradual decrease in this latter strain (ANOVA, $F_{1,5}$ = 33.195, $R^2$ = 0.842, df = 6, *P* = 0.002 from 0.05x to 1.5x). Both in *D. santomea* and *D. yakuba* there was no significant effect in spite of a decrease in female receptivity that reached 68% and 31%, respectively (GLM, *P* > 0.128 and *P* > 0.371, Figure 11). This contrasts with what was observed in *D. teissieri* (GLM, P = 0.0002 for 1x and P = 0.0001 for 2x).

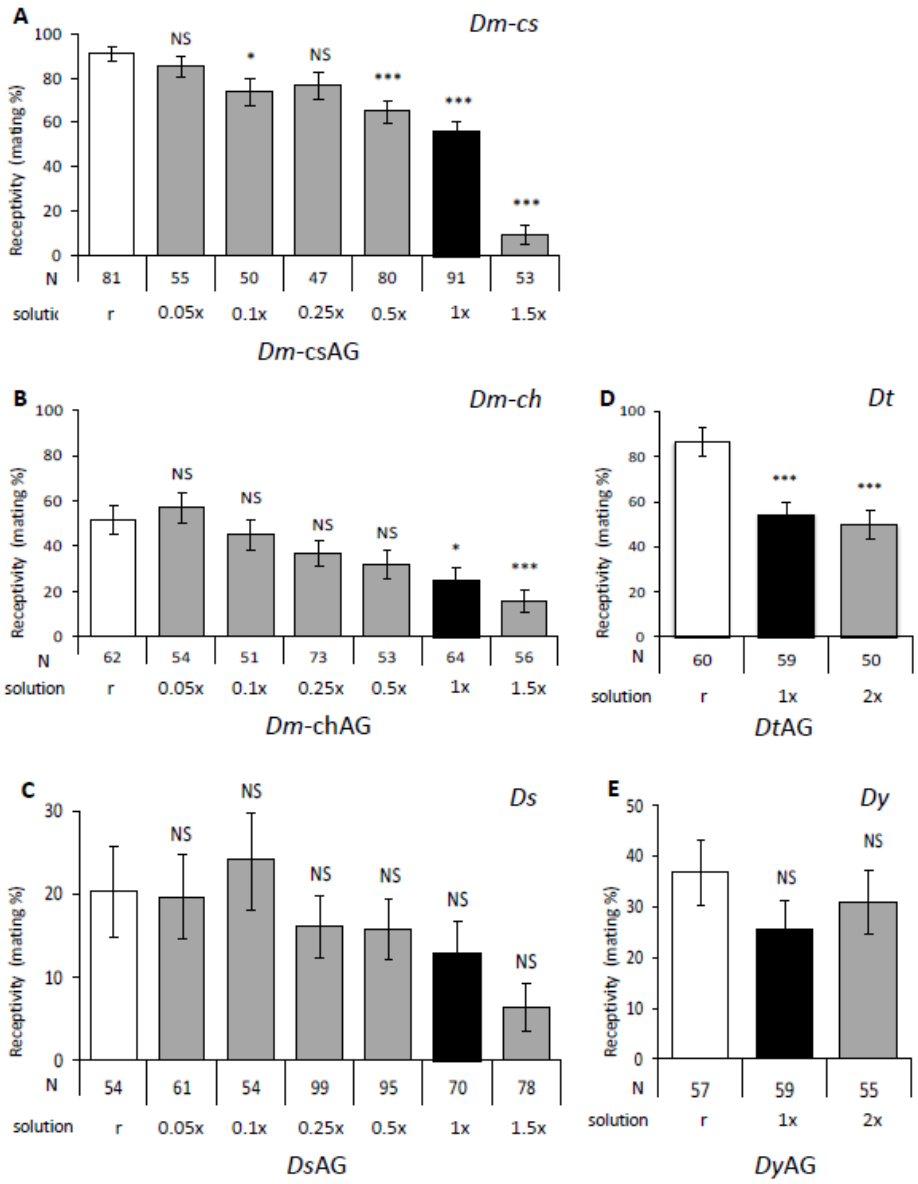



Fig. 11. Dose-dependent responses of Acps (AG) from the different species on female receptivity 4 hours after injection in *D. melanogaster* Canton S (A) and Chavroches (B), *D. santomea* (C), *D. teissieri* (D) and *D. yakuba* (E). Each bar represents percentage ± SE. Black bars represent standard concentrations of Acps. Grey bars represent dilutions and concentrations of Acps from the standard one. White bars represent injections of Ringer solution. *P*-values were calculated against the Ringer treatment (r). N is the number of tested females. NS *P* > 0.05; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

## 4. Discussion

Our experiment on the remating pattern showed a long- (*D. melanogaster* Chavroches, *D. santomea* and *D. yakuba*) *versus* a short-lasting (*D. melanogaster* Canton S, *D. teissieri*) decrease in female receptivity: this evidenced monoandrous *versus* polyandrous remating patterns, respectively. Indeed, depending on the species or strains, we highlighted different effects of male Acp extracts and SP orthologs on female sexual receptivity. Using transgenic *D. melanogaster* lines expressing the SP orthologs of the *yakuba* complex, we showed that all genes had similar biological activity to that of the *Dm*SP. However, effects of SP ortholog and Acp injections were context-dependent. Overall, the *D. melanogaster* and *D. teissieri* females were highly sensitive to conspecific or SP orthologs, as well as to conspecific or heterospecific Acps. In contrast, *D. santomea* and *D. yakuba* females were more resistant to both conspecific SP orthologs and Acps. This is confirmed following increasing concentrations of SP ortholog or Acp injections in conspecific conditions: we showed strong effects on female receptivity in both *D. melanogaster* and *D. teissieri*, while effects were weak (SP orthologs) or null (Acps) in *D. santomea* and *D. yakuba*. All these results suggest species-specific molecular dialogue between the sexes.

### *4.1. Intra and interspecific variation of the remating frequency*

Female remating appears to be under the control of many factors. Among these, the reproductive characteristics (ejaculate volume, female reproductive physiology, Kelly and Jennions, 2011) and environmental conditions (temperature, population size and density or distribution of resources) are crucial (Gromko *et al*., 1984; Aluja *et al*., 2009; Best *et al*., 2012). Singh *et al*. (2002) already reported a wide intraspecific variation of the female remating frequency in *D. melanogaster* from 15% up to 84%. Using the same experimental design, we showed that *D. melanogaster* females and *yakuba* complex species females, exhibit a strong decrease in sexual receptivity during the day after the first mating. However, our results indicate that the duration of the refractory period varied among the strains and species considered.

Using different strains of *D. melanogaster,* we (i) validated our protocol regarding previous published studies, as Canton S is one of the main strains used for most of the work on sexual behavior found in the literature, (ii) compared intra *versus* interspecific variations of female postmating sexual receptivity with closely related species of the *yakuba* complex, (iii) assessed the speed of evolution of the mating patterns in *Drosophila* and the fine-tuning of

507 the male-female postcopulatory interplay.

508 Four days after the first mating, females of the wild-type *D. melanogaster* strain
509 (Chavroches) were found to remate at a dramatically lower frequency than females of the
510 Canton S strain of the same species (9% and 63%, respectively) (Figure 2A). The same
511 variation in female sexual receptivity was found among species of the *yakuba* complex
512 (Figure 2B). As for Canton S females, *D. teissieri* females gradually recover their sexual
513 receptivity from less than 10% up to about 60% four days after mating. This mating pattern
514 may then be considered as polyandrous. In contrast, *D. santomea* and *D. yakuba* females
515 strongly resemble those of the Chavroches strain of *D. melanogaster,* with only 10% of
516 females recovering their sexual receptivity for remating after four days. We can consider that
517 these species/strains are characterized by a monoandrous mating pattern. However, neither
518 the copulation latency nor the copulation duration reliably reflect the different remating
519 patterns since copulation latency after the second mating is significantly longer than after the
520 first in both *D. melanogaster* strains. In contrast, copulation latency is shorter for the second
521 mating in *D. yakuba* and *D. teissieri,* while these two species exhibit different remating
522 patterns. Copulation duration is rather similar for both the first and second mating in almost
523 all species, suggesting that males do not perceive sexual competition in these experimental
524 conditions (Bretman *et al.,* 2013). Hence, behavioral patterns may not be used as a proxy of
525 the remating patterns seen here.
526
527
528 *4.2. Evolutionary dynamics of postcopulatory interplay between males and females*
529
530 The results of SP ortholog and Acp injections confirm data already published for
531 *D. melanogaster*, and validate our protocol for the *yakuba* complex species. Therefore, our
532 experiments on the effects of intraspecific Acp injections clearly attest that Acps alone were
533 not responsible for female monogamy in the *yakuba* complex species.

534 In both strains of *D. melanogaster,* Acp injections drastically reduce female sexual
535 receptivity for at least 4 hours after mating. However, the gradual increase in the remating
536 rate evidenced in Canton S but not in Chavroches suggests a differential effect of Acps or
537 male-female interplay. Indeed, when injected, Acps from Chavroches males drastically
538 reduced sexual receptivity in the females of their own strain, but also in Canton S females.
539 Several hypotheses may be raised to explain the long persistence of the decreasing
540 receptivity in Chavroches females: (i) a prolonged effect of Acps compared to those from
541 Canton S, (ii) a lower rate of sperm released from the female storage organs which delayed
542 the recovery of her receptivity (Avila *et al.,* 2010), (iii) other factors that may take over the
543 effect of Acps with time, either intrinsic (mechanical, physiological or biochemical, including

other seminal peptides but not Acps, as Esterase-6 (Gilbert, 1981) or PebII (Bretman *et al.,* 2010) or extrinsic, such as nutritional status, as was shown in Tephritidae (Aluja *et al.,* 2009; Abraham *et al.,* 2011). Interestingly, we showed that Dup99B, already identified as a player of female postmating responses in *D. melanogaster* (Saudan *et al.,* 2002; Kubli, 2003), had a significant effect on Canton S females but not on Chavroches females in our experimental conditions. Additionally, some Acps of the SP network (Ravi Ram and Wolfner, 2007; Findlay *et al.,* 2014) or some proteins of the seminal fluid could play a crucial role in Chavroches. These hypotheses are currently under consideration.

In the two species *D. santomea* and *D. yakuba,* SP ortholog and Acp injections, whatever their origin, had no effect. This lack of significant reduction of female receptivity was unexpected with regard to the corresponding female remating frequency and pattern of polyandry. For *D. yakuba*, this result also contrasts with data from Tsuda *et al*. (2015), who showed a reduced receptivity after *Dm*SP injection. The difference may be due to the concentration of injected SP orthologs, which was four times higher than in our study, highlighting once again a dose-dependent effect. In contrast, *D. teissieri* showed reduced female receptivity after SP ortholog or Acp injections, whatever their origin (Figure 9). Together, these results suggest that other factors may play a role to explain *D. santomea* and *D. yakuba* female monoandry. It was previously shown that long-lasting effects of male seminal fluid depend on the presence of sperm within the female storage organs in *D. melanogaster* (Schnakenberg *et al.*, 2012). It can be hypothesized that the amount of sperm released by *D. santomea* and *D. yakuba* females could be very low compared to other species, a question that deserves further investigation. Some differences in biological activities of SP orthologs and Acps are unlikely to be involved since they both triggered significant effects after ectopic expression or female injections, respectively, in *D. melanogaster*. This suggests that the molecular pathway known to be activated through the stimulation of the neuronal SP receptor (Yapici *et al.,* 2008; Kubli and Bopp, 2012) is conserved.

Moreover, the absence of SP ortholog effects, whatever their origin, in the *D. santomea and D. yakuba* species, may be interpreted by two main scenarios:

(1) Either the female receptor has evolved in such a way that it cannot recognize any SP orthologs, or some degradation process within the female reproductive tract may inactivate/degrade the peptide. However, such a hypothesis is not likely since the SP receptor was shown to be highly conserved among species of the *melanogaster* subgroup (Kim *et al.,* 2010).

(2) SP orthologs or Acps were injected in too small quantity/concentration compared to the amount transferred by males during mating. Our dose-dependent response experiments confirm this hypothesis since most of the strains/species exhibit a

significant reduction of female receptivity at higher concentrations. A similar dose-dependent response pattern was evidenced in mosquito females of *Aedes albopictus* and *A. aegypti* (Helinski *et al*., 2012).

Together, our results suggest that some strains/species may be more resistant to SP orthologs than others and that higher concentrations of the peptides may trigger significant biological activity. It was previously shown that genetic variation of male SP expression levels may vary with the refractory period duration in *D. melanogaster* females (Smith *et al.*, 2009, but see Chow *et al*., 2010). While no data are available regarding the quantity of SP found in females of the *yakuba* complex species, further experiments are necessary to investigate this question.

### *4.3. Conclusion*

The most intriguing result of this study is the strong difference observed between the two strains of *D. melanogaster* as well as the difference between *D. teissieri* and its sister species of the *yakuba* complex. *D. teissieri* exhibits the most divergent SP ortholog sequence, and also the most contrasting SP ortholog and Acp sensitivities, which confirms the more distant phylogenetic relationship within the *yakuba* complex (Lachaise *et al*., 2004). Previous data had suggested a geographical disruption of the reproductive system of *D. teissieri* with respect to male genitalia and sperm size from Southeast to Northwest in tropical Africa (Joly *et al.,* 2010). Our present work reinforces the reproductive system specificity of this species, but also the diversity among the species of the *melanogaster* subgroup of the molecular, physiological and behavioral interplay between the sexes.

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Table S1. List of the primers used to clone SP and its orthologs (the restriction sites are in bold).

| | |
|---|---|
| | *D. melanogaster* |
| *Dm*SP DIR EcoRI: | ACG **GAA TTC** ATG AAA ACT CTA GCT CTA TTC |
| *Dm*SP REV kpnI: | ACG **GGT ACC** TTA ACA TCT TCC ACC CCA GGC |
| | *D. santomea* |
| *Ds*SP DIR EcoRI: | ACG **GAA TTC** ATG AAC ACA GTA GCT CTC CTC |
| *Ds*SP REV kpnI: | ACG **GGT ACC** TTA GCA TCT TCC TCC CCA GCC |
| | *D. teissieri* |
| *Dt*SP DIR EcoRI: | ACG **GAA TTC** ATG AAA ACA GTA GCA CTC CTC |
| *Dt*SP REV kpnI: | ACG **GGT ACC** TTA GCA TCT TCC TCC CCA GGC |