Journal of Insect Physiology 99 (2017) 67–77

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

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

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ARTICLE INFO

Keywords:
Female remating
Female sexual receptivity
Accessory gland proteins
Sex peptide
yakuba complex
Drosophila

ABSTRACT

In sexual species, mating success depends on the male's 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 discovered 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.

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 (Birkhead 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

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http://dx.doi.org/10.1016/j.jinsphys.2017.03.008
Received 1 December 2016; Received in revised form 17 March 2017; Accepted 18 March 2017
Available online 22 March 2017

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 deployed, 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 test the conserved role of SP orthologs or Acps through a cross-experimental study. We show 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* reveals 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 5–8-day old virgin females were exposed to single virgin males of the same age for 3 h 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 h after the first mating, females were individually exposed to two new virgin males (6–8 days old) for 2 h. After each trial, unmated females were transferred into a new corn-meal food vial, until the next mating assay 24 h later. Such an assay was repeated every 24 h 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 alignments generated by CLUSTAL W software to identify suitable sequences (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-GAL4 (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 h 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 2.5 µL of Ringer's buffer. Excreted soluble proteins present in the AGs were recovered by vortexing and 10 min of centrifugation at 4 °C and 14 000 g. 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 1× 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 0.25×, 0.5×, 1×, 2×, 4× and 10× were used. For Acps, concentrations of 0.05×, 0.1×, 0.25×, 0.5×, 1.5× and 2× 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 h 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 (5–8 days old), into a standard corn-meal food vial and observed for mating receptivity for 3 h at 20–22 °C. These assays were carried out 4 h 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.

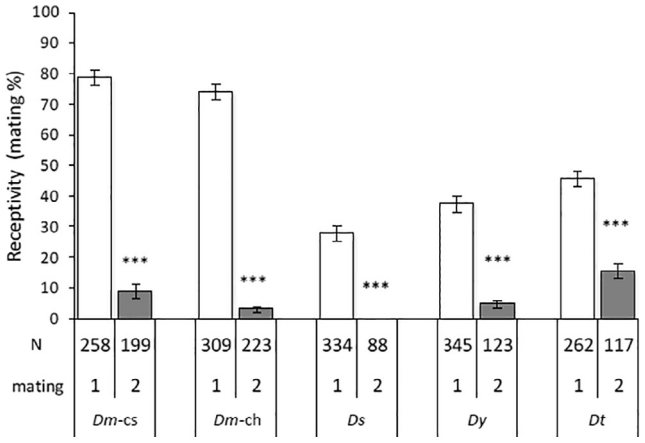

Fig. 1. Female receptivity during the first mating (white bars) and 24 h later (dark bars) in *D. melanogaster* (*Dm*-cs and *Dm*-ch), *D. santomea* (Ds), *D. yakuba* (Dy) and *D. teissieri* (Dt). Each bar represents the proportion ± standard error (SE). N is the number of tested females. Mating is the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

3. Results

3.1. Female sexual receptivity after mating

The proportion of sexually receptive females 24 h after mating decreased from 66% to 100% for all species and strains (Chi-square tests, df = 1, $P < 0.0001$ for the 5 conditions, Fig. 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.

To better characterize the mating system for each species and strain, the cumulative rate of female remating was plotted every 24 h after the first mating for four consecutive days (Fig. 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 (Fig. 2A). Interestingly, remating rates between sexual partners from the two *D. melanogaster* strains (*Dm*-cs females × *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 (Fig. 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.

The comparison of mating parameters (copulation latency and copulation duration) between the first and second matings is shown in Fig. 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 (Fig. 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 (Fig. 3B). *D. teissieri* showed the longest copulation duration (Tukey-Kramer tests, $P < 0.0001$).

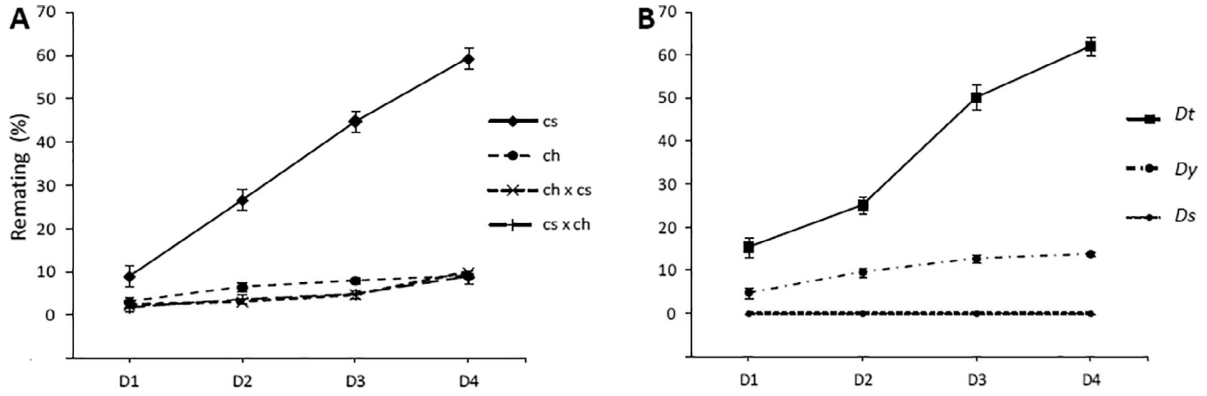

Fig. 2. Cumulative percentages (± SE) of female remating for four days after the first mating. Standard-errors are indicated on all curves, but some are too small to be visible. A-Results for the two different strains of *D. melanogaster* (cs and ch), with the heterogamic tests between cs females x ch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

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 (Fig. 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).

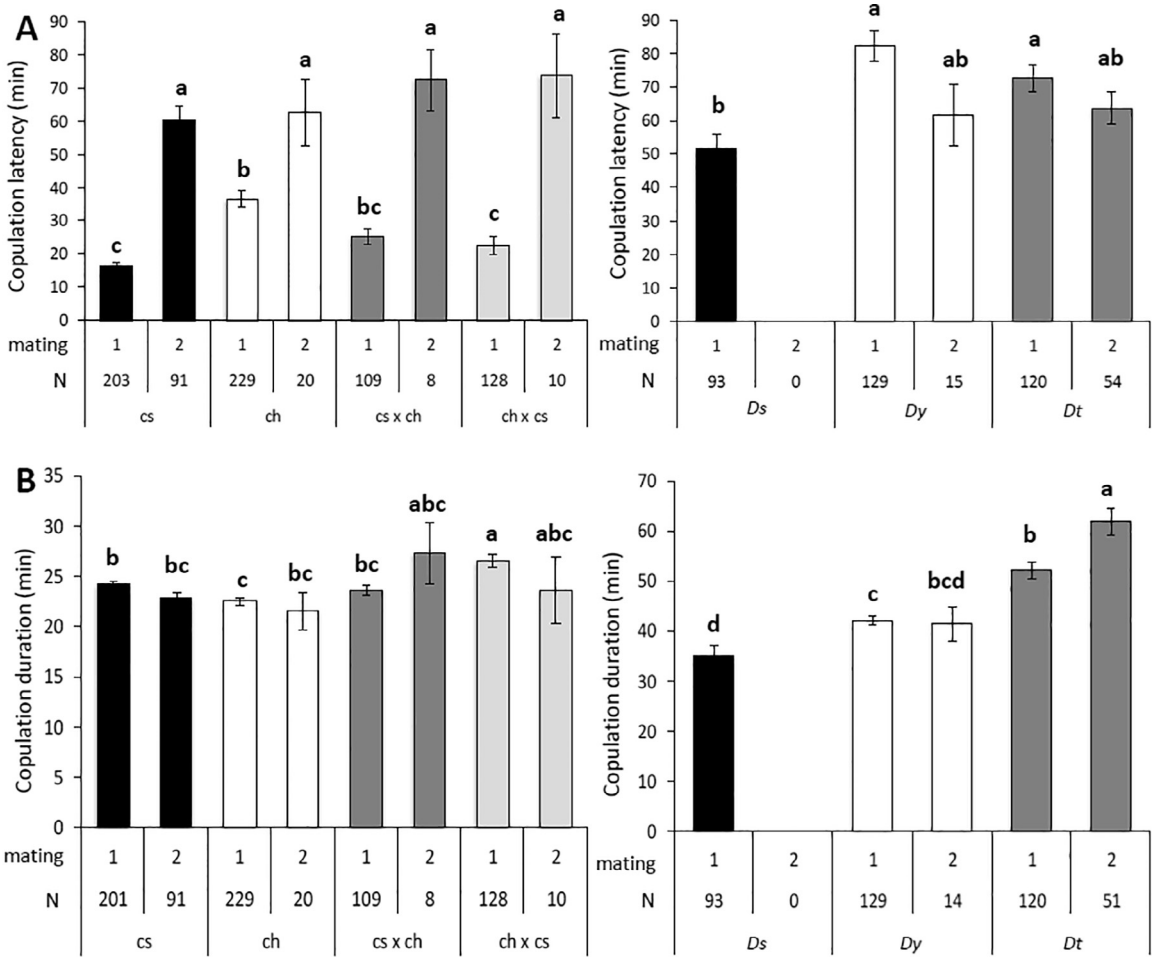

Fig. 3. Copulation latency (A) and copulation duration (B) between first and second mating. Bar charts on the left show results for the *D. melanogaster* strains: conspecific pairs (cs and ch) and heterospecific pairs (females from one strain crossed to males from the other strain, and *vice versa*). 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 ($P < 0.05$).

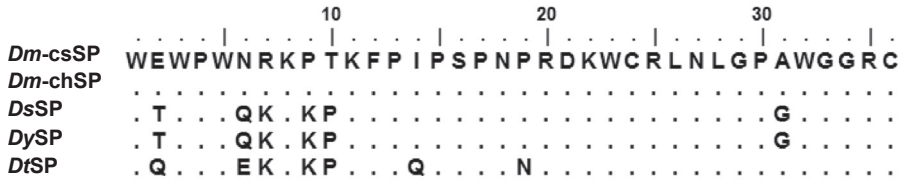

Fig. 4. Amino-acid sequences of the secreted SP of *D. melanogaster* Canton S (*Dm*SP-cs) compared to those of the other strains or species tested. Note that the two strains cs and ch have identical SP peptides, as do *D. santomea* and *D. yakuba*. *Dm*-cs*: D. melanogaster Canton S*; *Dm*-ch*: D. melanogaster* 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) (Fig. 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, $P < 1.3 \cdot 10^{-7}$ 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.

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, $P = 4.3 \cdot 10^{-6}$, and $P = 2.8 \cdot 10^{-6}$ for Canton S and Chavroches, respectively, Fig. 6). Interestingly, the SP ortholog of the *yakuba* complex species (*Ds/Dy*SP and *Dt*SP, Fig. 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).

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, $P = 0.061$, Fig. 7).

Interestingly, injection of Acps from Chavroches males lead to a stronger receptivity decrease than *Dm*SP in Canton S females (GLM, $P = 1.2 \cdot 10^{-11}$ and $P = 4.3 \cdot 10^{-6}$, respectively), while not in Chavroches females (GLM, $P = 0.009$, and $P = 2.949 \cdot 10^{-6}$, Figs. 6

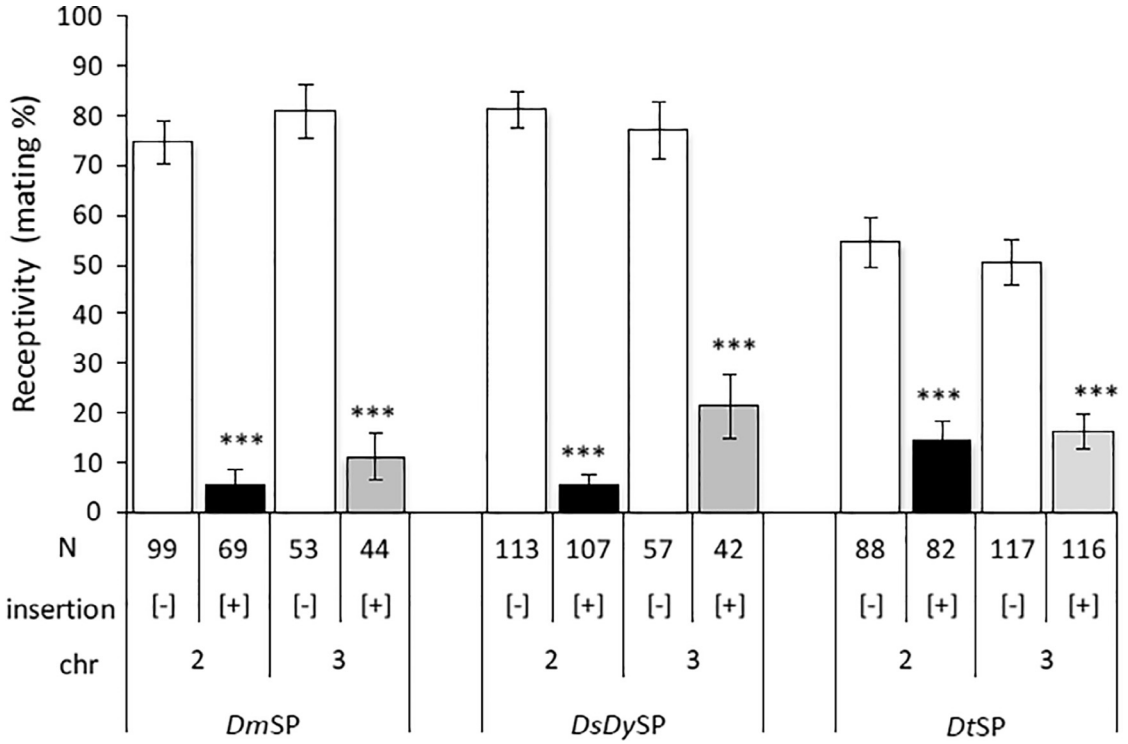

Fig. 5. Effect of transgenic insertion of the SP genes [+] on chromosomes 2 (dark bars) and 3 (grey bars) of *D. melanogaster* (*Dm*SP), *D. santomea/D. yakuba* (*DsDy*SP, the sequences being similar), and *D. teissieri* (*Dt*SP) in different *D. melanogaster* w^{118} females. The controls (white bars) 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. $^{***}P < 0.001$.

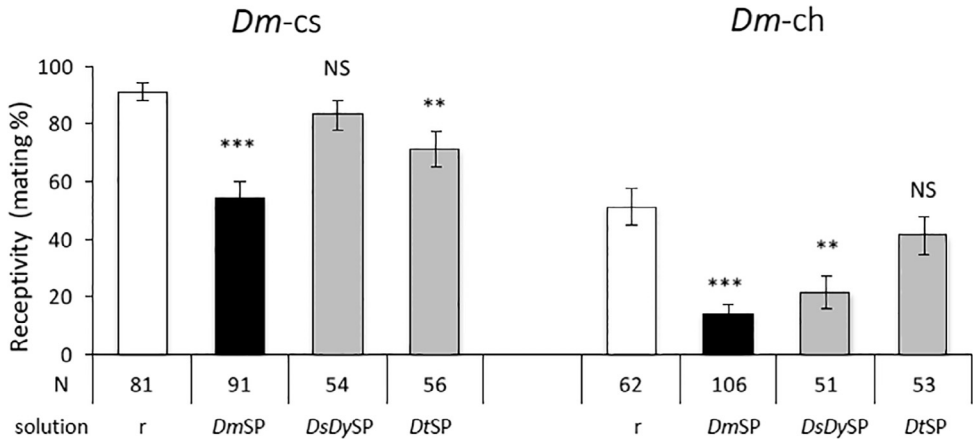

Fig. 6. Receptivity of *D. melanogaster* Canton S (*Dm*-cs) and Chavroches (*Dm*-ch) females 4 h after injection of synthetic SP solutions from *D. melanogaster* (*Dm*SP), *D. santomea*/*D. yakuba* (*DsDy*SP) and *D. teissieri* (*Dt*SP). Each bar represents percentage ± SE. Black bars indicate intra-specific 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 $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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, Fig. 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).

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, Fig. 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, $P \geq 0.055$).

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 (1×, GLM, $P = 4.3 \cdot 10^{-6}$ and $P = 3.7 \cdot 10^{-6}$, respectively, Fig. 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 (Fig. 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 2× and 4×, 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 1× and 10× concentrations, respectively). *D. teissieri* resembles *D. melanogaster* Canton S, with a strong reduction of female receptivity from the lowest concentration tested (1×, GLM, $P \leq 0.001$)

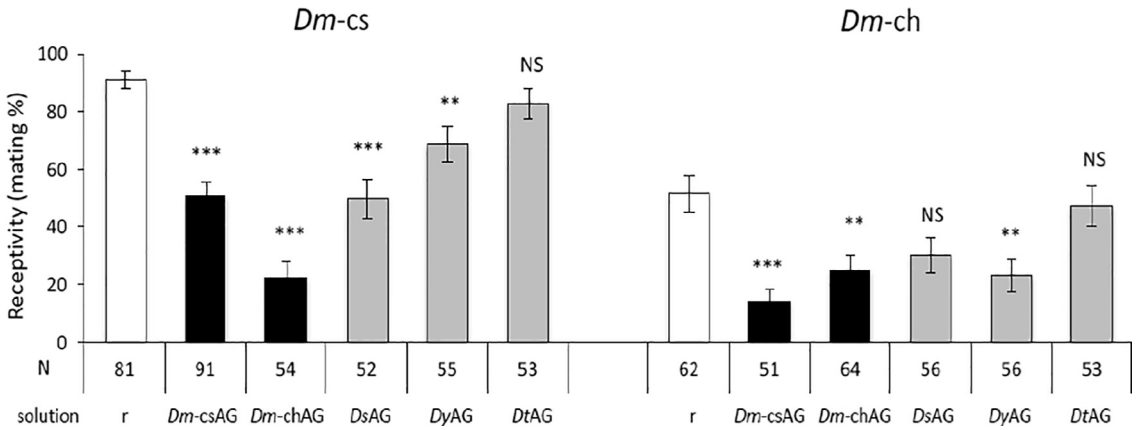

Fig. 7. Receptivity of *D. melanogaster* Canton S (*Dm*-cs) and Chavroches (*Dm*-ch) females 4 h after injection of accessory gland proteins from *D. melanogaster* Canton S (*Dm*-csAG) and Chavroches (*Dm*-chAG), *D. santomea* (*Ds*AG), *D. yakuba* (*Dy*AG) and *D. teissieri* (*Dt*AG), Each bar represents 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 $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

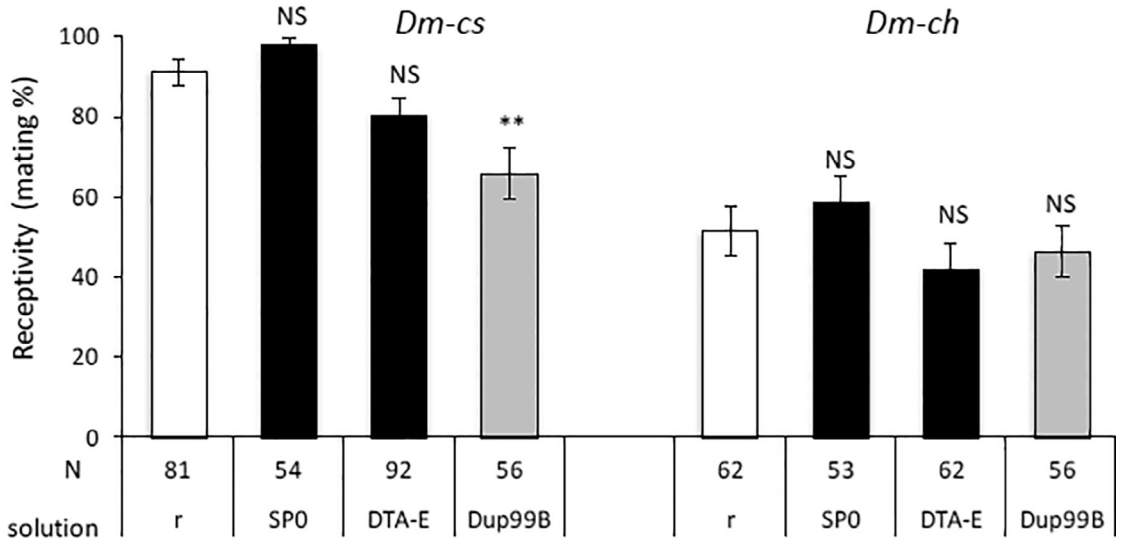

Fig. 8. Receptivity of *D. melanogaster* Canton S (*Dm*-cs) and Chavroches (*Dm*-cha) females 4 h after injection of accessory gland proteins from SP0 males or DTA-E males, or of Dup99B synthetic peptides. Each bar represents percentage ± SE. Black bars indicate injections of Acps; grey bars indicate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

but a decrease in female sexual receptivity with increasing *Dt*SP concentrations that is gradual but not significant from 1× to 10× (ANOVA, $F_{1,3} = 8.084$, $R^2 = 0.639$, df = 4, $P = 0.0655$).

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 0.5× in Canton S, while 1× was required in Chavroches (GLM, $P = 0.0006$ and $P = 0.014$ respectively, Fig. 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 0.05× to 1.5×). 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 $P > 0.371$, Fig. 11). This contrasts with what was observed in *D. teissieri* (GLM, P = 0.0002 for 1× and P = 0.0001 for 2×).

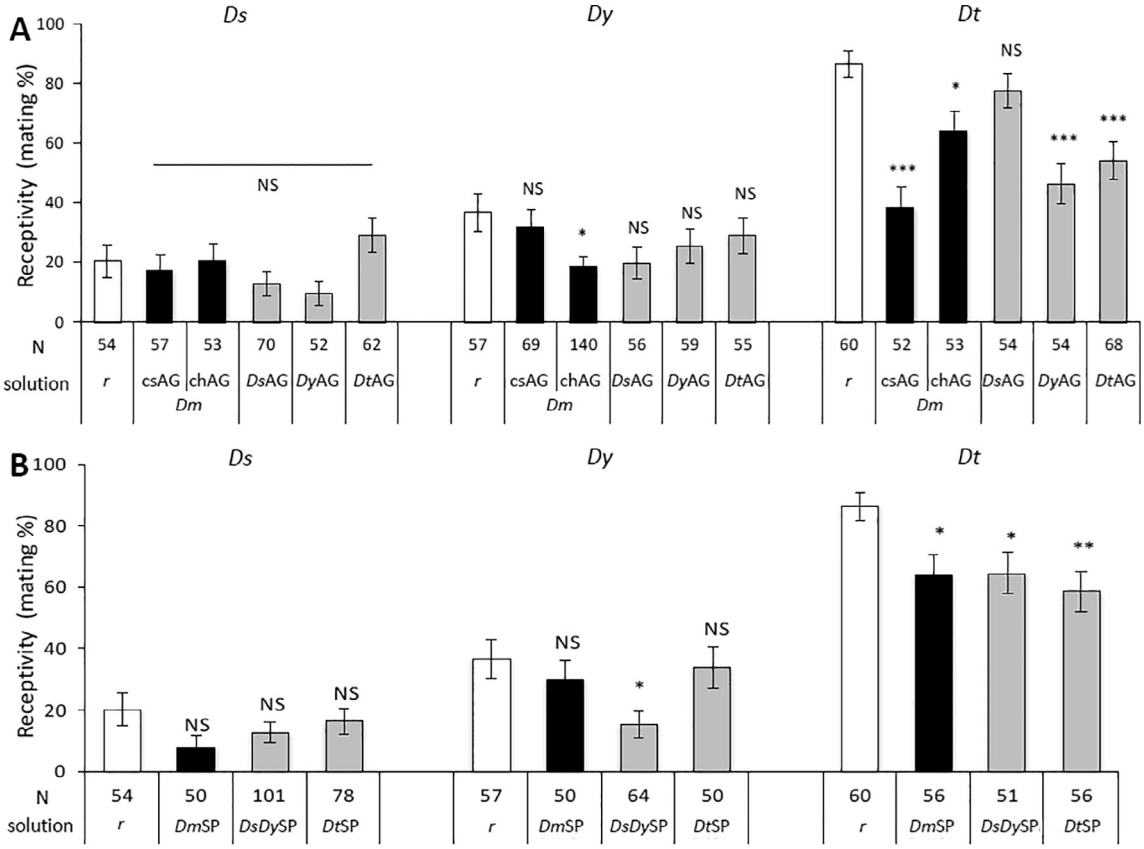

Fig. 9. Female receptivity 4 h after injection of Acps from accessory glands (A) of all species/strains from *D. melanogaster* Canton S (*Dm*-csAG), *D. melanogaster* Chavroches (*Dm*-chAG); *D. santomea* (*Ds*AG), *D. yakuba* (*Dy*AG) and *D. teissieri* (*Dt*AG). Injections of SP (B) from *D. melanogaster* (*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 $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

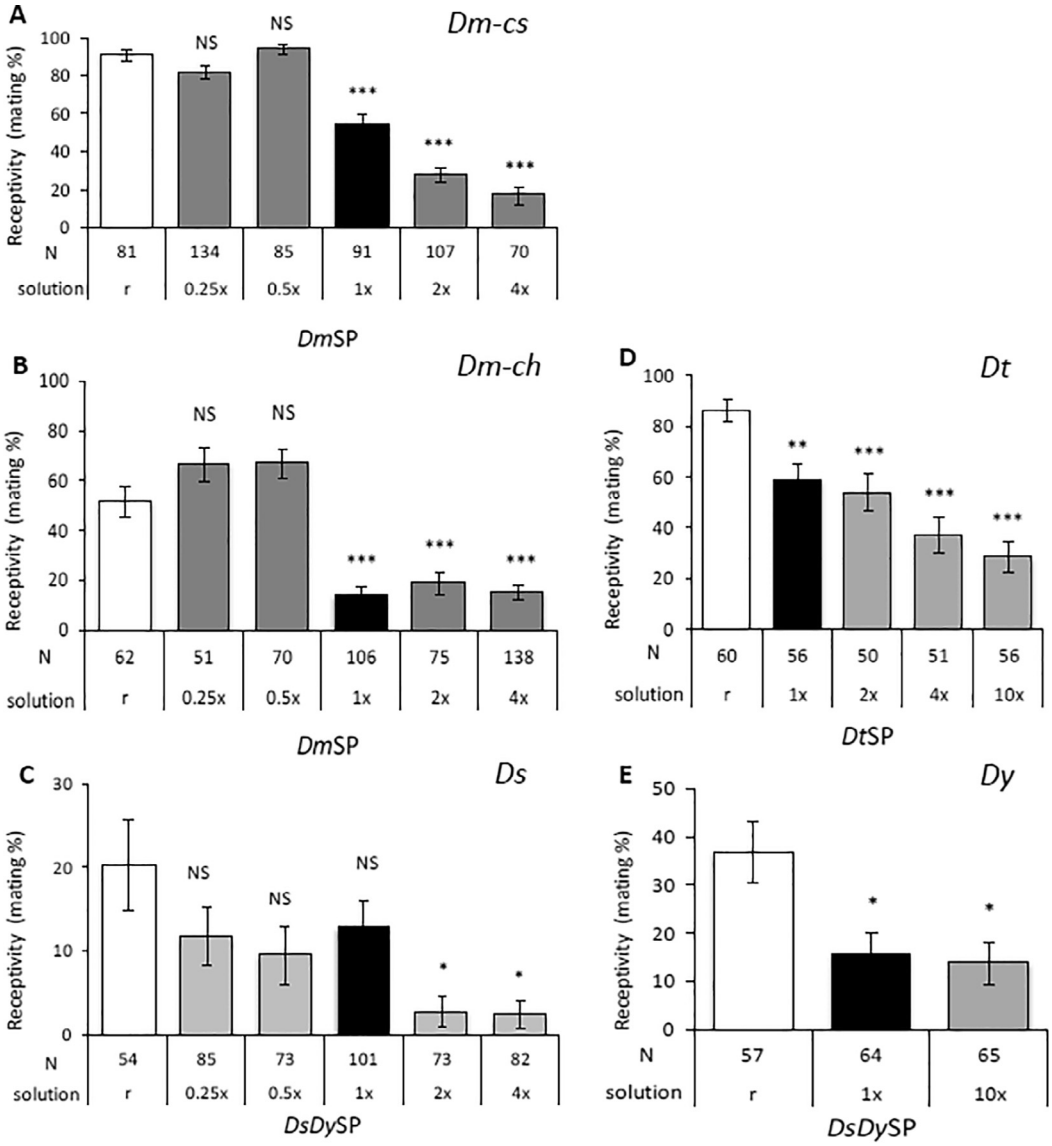

Fig. 10. Dose-dependent responses of SP orthologs on female receptivity 4 h after injection in *D. melanogaster* Canton S (A, *Dm*-cs) and Chavroches (B, *Dm*-ch), *D. santomea* (C, *Ds*), *D. teissieri* (D, *Dt*) and *D. yakuba* (E, *Dy*). Note that for the last two species an additional dose-dependent response (×10) 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 $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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 difference may respectively reflect a monoandrous *versus* a polyandrous remating pattern. 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

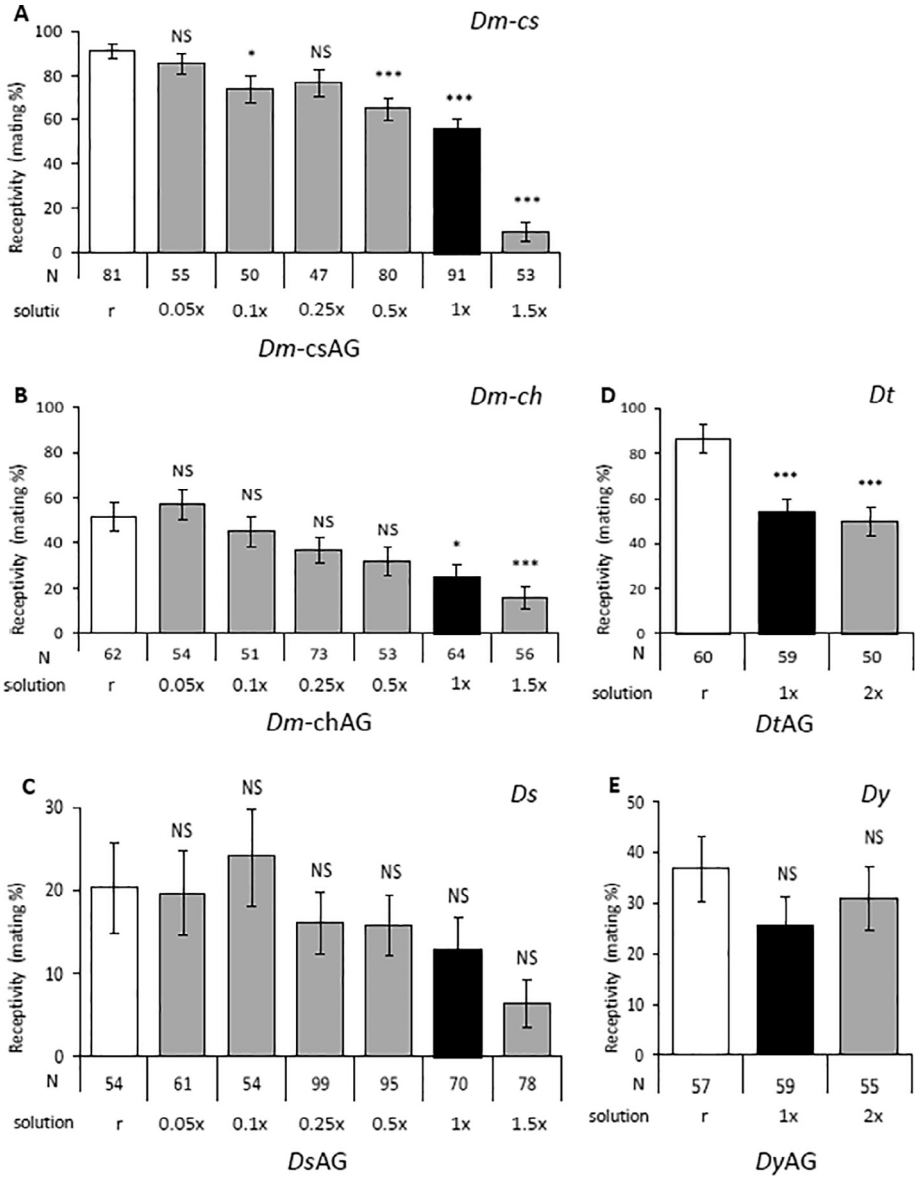

Fig. 11. Dose-dependent responses of Acps (AG) from the different species on female receptivity 4 h after injection in *D. melanogaster* Canton S (A) and Chavroches (B), *D. santomea* (C), *D. teissieri* (D) and *D. yakuba* (E). Each bar represents percentage ± SE. Black bars represent standard concentrations 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 $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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 the male-female postcopulatory interplay.

Four days after the first mating, females of the wild-type *D. melanogaster* strain (Chavroches) were found to remate at a dramatically lower frequency than females of the Canton S strain of the same species (9% and 63%, respectively) (Fig. 2A). The same variation in female sexual receptivity was found among species of the *yakuba* complex (Fig. 2B). As for Canton S females, *D. teissieri* females gradually recover their sexual receptivity from less than 10% up to about 60% four days after mating. This mating pattern may then be considered as polyandrous. In contrast, *D. santomea* and *D. yakuba* females strongly resemble those of the Chavroches strain of *D. melanogaster*, with only 10% of females recovering their sexual receptivity for remating after four days. We can consider that these species/strains are characterized by a monoandrous mating pattern. However, neither the copulation latency nor the copulation duration reliably reflect the different remating patterns since copulation latency after the second mating is significantly longer than after the first in both *D. melanogaster* strains. In contrast, copulation latency is shorter for the second mating in *D. yakuba* and *D. teissieri*, while these two species exhibit different remating patterns. Copulation duration is rather similar for both the first and second mating in almost all species, suggesting that males do not perceive sexual competition in these experimental conditions (Bretman et al., 2013). Hence, behavioral patterns may not be used as a proxy of the remating patterns seen here.

4.2. Evolutionary dynamics of postcopulatory interplay between males and females

The results of SP ortholog and Acp injections confirm data already published for *D. melanogaster*, and validate our protocol for the *yakuba* complex species. Therefore, our experiments on the effects of intraspecific Acp injections clearly attest that Acps alone were not responsible for female monogamy in the *yakuba* complex species.

In both strains of *D. melanogaster*, Acp injections drastically reduce

female sexual receptivity for at least 4 h after mating. However, the gradual increase in the remating rate evidenced in Canton S but not in Chavroches suggests a differential effect of Acps or male-female interplay. Indeed, when injected, Acps from Chavroches males drastically reduced sexual receptivity in the females of their own strain, but also in Canton S females. Several hypotheses may be raised to explain the long persistence of the decreasing receptivity in Chavroches females: (i) a prolonged effect of Acps compared to those from Canton S, (ii) a lower rate of sperm released from the female storage organs which delayed the recovery of her receptivity (Avila et al., 2011), (iii) other factors that may take over the 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 (Fig. 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.

Acknowledgments

We are very grateful to C. Vincent, C. Chevelie and Y. Ben Chehida for their input during the course of the experiments as well as to M. Bonneau and S. Nortier for their help in insect media preparation. We are very grateful to M. Wolfner for providing the SP0 and DTA-E lines. We thank P. Millares and J.-P. Le Caer for fruitful discussions, the reviewers for helpful comments, K. Kean and M. Eden for English editing. This work was supported by the Fondation pour la Recherche sur la Biodiversité Grant AAP-IN-2009-027 (DJ), the Agence Nationale de la Recherche Grant ANR 2010-BLAN-1705-01 (DJ) and the CNRS through the UMR 9191.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2017.03.008.

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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