Sex peptide receptor is not required for refractoriness to remating or induction of egg laying in *Aedes aegypti*

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SPR not required for *A. aegypti* post-mating behaviors

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Abstract

Across diverse insect taxa, the behavior and physiology of females dramatically changes after mating – processes largely triggered by the transfer of seminal proteins from their mates. In the vinegar fly *Drosophila melanogaster*, the seminal protein sex peptide (SP) decreases the likelihood of female flies remating and causes additional behavioral and physiological changes that promote fertility including increasing egg production. Although SP is only found in the *Drosophila* genus, its receptor, sex peptide receptor (SPR), is the widely-conserved myoinhibitory peptide (MIP) receptor. To test the functional role of SPR in mediating post-mating responses in a non-*Drosophila* dipteran, we generated two independent *Spr*-knockout alleles in the yellow fever mosquito *Aedes aegypti*. Although SPR is needed for post-mating responses in *Drosophila* and the cotton bollworm *Helicoverpa armigera*, *Spr* mutant *Ae. aegypti* show completely normal post-mating decreases in remating propensity and increases in egg laying. In addition, injection of synthetic SP or accessory gland homogenate from *D. melanogaster* into virgin female mosquitoes did not elicit these post-mating responses. Our results indicate that *Spr* is not required for these canonical post-mating responses in *Ae. aegypti*, indicating that unknown signaling pathways are likely responsible for these behavioral switches in this disease vector.

Introduction

Female insects undergo major changes after mating that alter their behavior and physiology, ultimately contributing to successful reproduction. Much of our understanding of these changes comes from work in *Drosophila melanogaster*, where post-mating changes are primarily initiated by seminal fluid proteins derived from male reproductive organs and transferred to females within the ejaculate (reviewed in AVILA *ET AL.* 2011; WIGBY *ET AL.* 2020). Many post-mating changes in *D. melanogaster* are induced by a 36 amino acid seminal "sex peptide" (SP); its most emblematic effects are decreasing the female's receptivity to remating and increasing her egg production (CHEN *et al.* 1988; CHAPMAN *et al.* 2003; LIU AND KUBLI 2003). SP exerts many of its effects through a G protein-coupled receptor, SPR (the sex peptide receptor) that is expressed in neurons as well as in reproductive tract tissues (YAPICI *et al.* 2008; HASEMEYER *et al.* 2009; YANG *et al.* 2009).

Impairment or loss of SPR in *D. melanogaster* leads to a reduction in post-mating egg laying, and to refractoriness to remating (YAPICI *et al.* 2008), as well as impairing additional post-mating changes in sleep patterns (GARBE *et al.* 2016), sperm release (AVILA *et al.* 2015), long-term memory (SCHEUNEMANN *et al.* 2019), and gut growth (WHITE *et al.* 2021). These responses have been shown to be integrated largely through the Sex-Peptide sensory and abdominal ganglion neurons (SPSN-SAG network) (FENG *et al.* 2014; WANG *et al.* 2020; WANG *et al.* 2021; reviewed in OKAMOTO AND WATANABE 2022). Consistent with the rapid evolution seen for many reproductive proteins (e.g. CIVETTA AND SINGH 1998; SWANSON AND VACQUIER 2002; HAERTY *et al.* 2007), SP is only found in some species of *Drosophila* (TSUDA AND AIGAKI 2016). However, SPR is

conserved across several *Drosophila* species (KIM *et al.* 2010) and other insects (YAPICI *et al.* 2008). This widespread sequence conservation likely reflects SPR's role as a myoinhibitory peptide (MIP) receptor, which has been considered to be its ancestral function. It is suggested that SPR was hijacked or co-opted to a reproductive function in lineages where it acquired expression in the female reproductive tract (KIM *et al.* 2010; POELS *et al.* 2010; TSUDA AND AIGAKI 2016). Experiments aimed at investigating a reproductive role for MIPs suggest that they do not induce post-mating responses, as mating receptivity was not altered by either pan-neuronal knockdown of MIPs or injection of MIPs in *D. melanogaster* females (KIM *et al.* 2010).

SPR has also been found to be important for post-mating responses in some insects in addition to *Drosophila*. RNAi knockdown of SPR expression in olive fruit flies (*Bactrocera oleae*) and Oriental fruit flies (*Bactrocera dorsalis*) led to lower oviposition rates (ZHENG *et al.* 2015; GREGORIOU AND MATHIOPOULOS 2020). Additionally, mutation of the SPR gene in *B. dorsalis* resulted in a reduction in egg-laying ability, viability of eggs laid, and underdeveloped ovaries (CHEN *et al.* 2023). Injection of *D. melanogaster* SP into the cotton bollworm, *Helicoverpa armigera* (which has no obvious SP gene in its genome) suppressed sex pheromone production leading to decreased calling behaviors; these effects did not occur if SPR was knocked down simultaneous with SP injection, indicating SP function is through SPR (HANIN *et al.* 2012). Moreover, *H. armigera* females with little to no SPR laid significantly fewer eggs after mating and displayed altered pheromone calling behavior and remating rates after 24 and 48 h (HANIN *et al.* 2012; LIU *et al.* 2021). A reproductive role for SPR was also reported in the tobacco cutworm *(Spodoptera litura*): females with SPR knockdown laid fewer eggs

than controls after injection of male accessory gland lysate and remained receptive to mating (LI *et al.* 2014).

Collectively, these studies indicate that SPR plays important post-mating roles in several insect taxa. However, the role of SPR in medically important mosquitoes, such as *Aedes aegypti*, has not been determined. If SPR is involved in inducing post-mating responses it could potentially be a target for mosquito reproductive control efforts (CATOR *et al.* 2021). RNASeq analyses of *Ae. aegypti* have identified SPR transcripts in neuronal and reproductive tissues (ALFONSO-PARRA *et al.* 2016; MATTHEWS *et al.* 2016), consistent with its expression patterns in those *Drosophila* species where it exerts a reproductive role (TSUDA *et al.* 2015). Although the *Ae. aegypti* genome does not contain a recognizable SP gene (our unpublished genome searches), neither does the genome of *H. armigera*, where SPR is necessary for inducing post-mating responses (HANIN *et al.* 2012; LIU *et al.* 2021).

To test the functional role of SPR in mediating *Ae. aegypti* post-mating responses, we employed CRISPR/Cas9 genome editing to generate two independent mutant alleles in the SPR gene. We tested female mutants for fertility and survival, as well as for the most salient reproductive phenotypes: post-mating egg laying and decreased mating receptivity, both of which are induced by seminal fluid proteins (CRAIG 1967; LEAHY AND CRAIG 1965; HELINSKI *et al.* 2012; VILLARREAL *et al.* 2018). Our data indicate that despite the sequence conservation of SPR, it does not play a detectable role in these post-mating changes in *Ae. aegypti*. Consistent with this conclusion, our injection of synthetic *Drosophila* SP, which can bind to and activate *Ae. aegypti* SPR *in vitro* (YAPICI *et al.* 2008; KIM *et al.* 2010) or of *Drosophila* male accessory gland extracts

into virgin or gravid *Ae. aegypti* females gave no effect on these post-mating behaviors. The lack of effects of SPR knockout on post-mating egg-laying and receptivity in this dipteran, in contrast to its activity in the dipteran *D. melanogaster* and the lepidopteran species *H. armigera* and *S. litura*, suggests that SPR may have been repetitively co-opted for a reproductive function. Our results indicate that unknown signaling pathways are likely responsible for the post-mating switches governing long-term refractoriness to remating and induction of egg laying in *Ae. aegypti*.

Materials and methods

Our groups generated *Spr* knockout alleles and characterized them independently. These mutant alleles, which were unique and in different genetic backgrounds, yielded the same results where compared. We refer to these alleles, generated at Cornell and Johns Hopkins respectively, as $Spr^{\Delta 235}$ and Spr^{ECFP}.

Mosquito rearing

$Spr^{\Delta 235}$: The $Spr^{\Delta 235}$ NHEJ allele was generated in a Thai background (e.g. HELINSKI AND HARRINGTON 2011), and its controls and backcrosses were of that background. Our Thai colony of *Ae. aegypti* derived from field-collected mosquitoes (15°72'N, 101°75'E) maintained since 2009 with annual supplementation and a homozygous transgenic line with dsRed-labelled sperm (SMITH *et al.* 2007) were used. After vacuum-hatching eggs, larvae were reared under uniform conditions to ensure medium body size adults (HELINSKI AND HARRINGTON 2011). Mosquitoes were maintained at 28°C and 70% RH with a 10 h light:10 h dark cycle that included two hours of simulated dusk and dawn. Virgin males and females were obtained by separating pupae by sex prior to adult

eclosion. All mosquitoes were maintained on 10% w/v sucrose. Biological replicates were derived from independently hatched cohorts.

Spr^{ECFP}: The *Spr*^{ECFP} HDR allele was generated in the *LVPib12* strain (NENE *et al.* 2007), which also served as the genetic background for assays with this mutant allele. Mosquitoes were maintained with a 12 h light:dark photoperiod at 27°C and 80% relative humidity using a standardized rearing protocol (WOHL AND MCMENIMAN 2023a). Adult mosquitoes were provided constant access to a 10% w/v sucrose solution and virgins were isolated as pupae and sexed within 12 h of emergence. The *Exu-Cas9* strain (marked with Opie2-dsRed) expressing *Cas9* under the maternal germline promoter *exuperantia* (LI *et al.* 2017) was used for CRISPR/Cas9 mutagenesis. *Exu-Cas9* was backcrossed to *LVPib12* each generation for stock maintenance.

SPR mutant generation via CRISPR

Spr$^{\Delta 1235}$: We generated a mosquito line harboring a NHEJ-based deletion in exon 2 of *Spr* (AAEL019881), by CRISPR/Cas9-based editing according to the procedures in Kistler *et al.* (2015). Two sites for Cas9 cleavage targeting exon 2 were identified using CHOPCHOP (LABUN *et al.* 2016) and validated *in vivo*. Guide RNAs were synthesized *in vitro* using the MEGAScript kit (Thermo Fisher Scientific) from DNA generated from a template-free PCR consisting of a primer targeting the Cas9 cut site with a T7 promoter and a universal reverse primer (sequences listed in Table 1). After reaction purification with MEGAClear (Thermo Fisher Scientific) and size confirmation using a Bioanalyzer 2100 (Agilent Technologies), 40 ng/ul each of the two gRNAs together with 333 ng/ul

Cas9 (PNA Bio) was injected into Thai strain embryos by the University of Maryland Insect Transformation Facility (https://www.ibbr.umd.edu/facilities/itf). After G_0 females were backcrossed to Thai wild type males, blood fed and allowed to lay eggs, genomic DNA from the G_0 mosquitoes was extracted using Puregene reagents (Qiagen) and PCR was used to detect deletions in the *Spr* gene (Table S1, Fig S1A). Eggs from deletion-positive females were hatched and back-crossed for five generations. Heterozygous males and females were crossed, and progeny screened for genotype using genomic DNA extracted from a single leg (SMITH *et al.* 2018). Males and females homozygous for the *Spr* deletion were crossed together to generate a stable mutant line ($Spr^{\Delta 235/\Delta 235}$). A wild type control line ($Spr^{+/+}$) derived from a backcross of heterozygous $Spr^{\Delta 235/+}$ individuals was also maintained. $Spr^{\Delta 235}$ heterozygotes ($Spr^{\Delta 235/+}$), derived from a cross between $Spr^{\Delta 235}$ homozygotes and this wild type line, served as an additional control.

Spr^{ECFP}: We generated a disruptive insertion in exon 2 of *Spr* using CRISPR/Cas9-mediated homologous recombination. Guide RNAs were designed targeting exon 2 of *Spr* using CHOPCHOP (LABUN *et al.* 2016) and validated individually with *in vitro* cleavage assays using a PCR amplicon spanning the putative cut sites, each *in vitro* transcribed gRNA (MEGAscript, Invitrogen) and Cas9 protein (PNA bio). A single gRNA with validated cleavage activity was chosen for incorporation into a synthetic gBlock (IDT) that contained the *Ae*. *aegypti U6* (*AAEL017774*) promoter, a modified gRNA scaffold and a terminator (CHEN *et al.* 2021) for subsequent subcloning into the backbone of the following HDR donor plasmid. We first integrated homology arms into

the base donor plasmid pSL1180-HR-PUbECFP (Addgene #47917) (McMeniman *et al.* 2014). Homology arms 1425 bp (left) and 1797 bp (right) in length flanking the gRNA cut site were amplified with CloneAmp (Takara) from a consensus genomic DNA clone covering *Spr* exon 2 and flanking introns (primers listed in Table S2). We PCR-amplified the gBlock using primers with 5' InFusion adaptors (Table S2). Next, we integrated the gBlock fragment with the U6 expression cassette into the backbone of this donor plasmid at the NdeI restriction site using InFusion cloning. This yielded a final construct (pMW001) containing a U6 expression cassette and HDR cassette targeting *Spr* exon 2.

We microinjected 300 ng/ul of pMW001 (endotoxin-free) into the posterior pole of *Exu-Cas9* pre-blastoderm stage embryos (*LVPib12* strain*)* using an Eppendorf FemtoJet 4X. Transformed G_1 larvae with constitutive ECFP fluorescence were identified by screening for fluorescent bodies (PUb-ECFP) at the L3-L4 stage. Transgenic animals were then outcrossed to *LVPib12* for two generations before crossing the lines to generate a homozygous viable *Spr* mutant strain ($Spr^{ECFP/ECFP}$). Insertion of the disruptive donor cassette into *Spr* exon 2 was confirmed by PCR amplification of genomic DNA using a three primer genotyping strategy (Table 2, Fig S1B) where one forward primer was centered on the CRISPR cut site so that it would only anneal to the wild type allele, one forward primer was placed in the polyubiquitin (PUb) sequence in the integrated cassette, and one reverse primer was nested in the right homology arm. This yielded a 342 bp amplicon for the wild type allele with an intact gRNA site, and a 524 bp amplicon for the mutant allele.

Mating, fertility, and body size of the *Spr$^{\Delta 235}$* allele in Thai background

Mating was examined by mating *en masse* two-to-three-day-old homozygous (*Spr$^{\Delta 235/\Delta 235}$*) or heterozygous (*Spr$^{\Delta 235/+}$*) females to three-to-four-day-old heterozygous males. Mating was performed in an 8 L container for 24 h in the absence of 10% sucrose. Mated females were dissected to confirm mating status by scoring the presence of sperm in the spermathecae. Eggs from both homozygous and heterozygous females mated to heterozygous males had similar hatch rates. Two biological replicates were performed from independently hatched cohorts. To determine relative body sizes of the mosquitoes, we used wing-length measurements as a proxy (NASCI 1990).

Post-mating receptivity

Spr$^{\Delta 235}$ allele in Thai background: A single two-to-three-day-old homozygous (*Spr$^{\Delta 235/\Delta 235}$*) or heterozygous (*Spr$^{\Delta 235/+}$*) female was released into an 8 L container with 10 three-to-four-day-old heterozygous (*Spr$^{\Delta 235/+}$*) males and observed closely. Once the female mated (maintained a copula for >8 sec), the mating pair was immediately collected and the female was placed in a 0.5 L cup. The mated male was excluded from additional matings and the mating arena was replenished with an additional virgin male. Two three-to-four-day-old males with dsRed-marked sperm were introduced into each 0.5 L cup either immediately or 24 h after the first female mating and then held together for 24 h. The lower reproductive tract of each female was then dissected and the spermathecae and bursa examined under a fluorescence microscope for the presence of red sperm along with non-fluorescent sperm, indicative of remating. As a control,

individual virgin homozygous females or virgin heterozygous females with two dsRed-sperm males were placed together in 0.5 L cups for 24 h (n=10 for each genotype for each replicate) and the dissected reproductive tracts of females were examined to make sure the dsRed-sperm males could mate successfully with $Spr^{\Delta 235}$ mutant females. Two biological replicates from independently hatched cohorts were conducted. The genotype order of initial matings was reversed in replicate two to avoid bias.

Spr^{ECFP} in LVPib12 background: To similarly test if Spr^{ECFP} mutants were refractory to remating, a single four-to-five-day-old homozygous ($Spr^{ECFP/ECFP}$) or heterozygous ($Spr^{ECFP/+}$) female was released into a 355 ml insulated solo cup with 10 five-to-six-day-old wild type ($Spr^{+/+}$) *LVPib12* males and observed closely. Once the female mated (maintained a copula for >8 sec), the mating pair was immediately collected. The male was removed and discarded, and the female was placed in a separate solo cup with other newly mated females. This mating scheme was then replicated until the target number of copulated females per genotype was obtained. After 1-1.5 h following their first mating, these females were introduced into a 20.3 x 20.3 x 20.3 cm cage (Bioquip, 1450A) with five-to-six-day-old males with dsRed-marked sperm (SMITH *et al.* 2007). The female to male ratio was 1:2 and they were held together for 24 h at which point males were removed by aspiration. The lower reproductive tract of each female was then dissected and the spermathecae examined under a fluorescence microscope for the presence of red sperm, indicative of remating.

Control matings were performed between homozygous ($Spr^{ECFP/ECFP}$) females (n=32) and wild type ($Spr^{+/+}$) *LVPib12* males as described above to estimate insemination rates after the first mating (31/32 mated). As a supplemental control to validate mating compatibility in this post-mating receptivity assay, homozygous ($Spr^{ECFP/ECFP}$) females (n=20) were placed with two times the number dsRed-sperm males in a 20.3 x 20.3 x 20.3 cm cage (Bioquip, 1450A) for 24 h and the dissected reproductive tracts of females were examined for the presence of red sperm (20/20 mated). Finally, twenty virgin females of each genotype ($Spr^{ECFP/ECFP}$ and $Spr^{ECFP/+}$) were dissected to confirm virginity (20/20 for each genotype were unmated).

Spr^{ECFP} Oviposition Assays

For oviposition assays with virgin or mated wild type ($Spr^{+/+}$), heterozygous ($Spr^{ECFP/+}$) or homozygous ($Spr^{ECFP/ECFP}$) females, three-to-five day old females were blood fed to repletion using anesthetized Swiss-Webster mice (Johns Hopkins University Animal Care and Use Committee, Approval Number: MO21H373). For assays with mated females, all crosses were made between virgin females of the target genotype and two-day-old wild type virgin males. Crosses were established with a sex ratio of 1 female: 2 males in a 20.3 x 20.3 x 20.3 cm cage (Bioquip, 1450A) with constant access to 10% sucrose. These small group crosses were left to mate for 48h before blood feeding the females. 72 hours post blood feeding, females were placed in single oviposition vials, each containing a filter paper cone moistened with 4mL dH_2O (Wohl and McMeniman 2023b). Females were allowed to lay eggs on the filter paper for 48 hours at which point they were removed from vials and eggs were counted. Egg papers from females who

251 died before collection were not counted. Counts were analyzed statistically as described
252 below.
253
254 **Drosophila sex peptide injection**. Synthetic sex peptide (3 pmol; CanPeptide) in 69 nl
255 *Aedes* saline (HAYES 1953) was injected into the thorax of virgin two-to-five-day-old Thai
256 background *Ae. aegypti* (n=18) and Canton S *D. melanogaster* females (n=30) with a
257 Nanoject II (Drummond, Broomall, PA). Controls included injection with *Aedes* saline
258 and a non-injected group. Males were introduced (ratio 1:1) 12 h after injection. Mating
259 events were confirmed by immediate direct observation for *Drosophila*. The number of
260 eggs laid by females in the treatment groups was recorded. *Ae. aegypti* females were
261 examined for sperm presence or absence after being held with males for two days.
262
263 To independently test if *D. melanogaster* sex peptide (dSP) could induce oviposition
264 behavior in the *Ae. aegypti LVPib12* genetic background, we injected synthetic dSP
265 (Aapptec, sequence: WEWPWNRKOTKFOIOSONORDKWCRLNLGPAWGGRC with a
266 disulfide bridge between the two cysteines) (CHEN *et al.* 1988) dissolved in PBS (Gibco,
267 pH 7.2) into the thorax of virgin females. We injected a concentration series spanning the
268 dynamic range of dSP concentrations known to elicit oviposition responses in *D.*
269 *melanogaster*. Specifically, we performed 150 nl injections of 10 uM, 100 uM and 1 mM
270 dSP which equates to 1.5 pmol, 15 pmol and 150 pmol of dSP respectively. First, three-
271 to-five-day-old virgin females were blood fed using anesthetized Swiss-Webster mice,
272 and 48 h later, synthetic dSP or *D. melanogaster* male accessory gland homogenate
273 (dMAG), *Ae. aegypti* male abdominal tip (aeMAT) homogenate (preparation described in

next section) and PBS controls were injected into the thorax with a Nanoject II (Drummond). Oviposition was assayed as described above.

Accessory Gland Homogenate Injection Assays

We tested both the effects of injecting *Drosophila* and *Aedes* male accessory gland (MAG) homogenates on mating and egg laying:

To first evaluate the effect of *Drosophila* MAG (dMAG) on Thai *Ae. aegypti* mating behavior and oviposition, accessory glands were dissected from 38 six-day-old virgin *D. melanogaster* Canton S males in 38 µl *Aedes* saline (HAYES 1953). The tissues were ground, sonicated in a water bath for 15 sec, and then spun at 13,400 rpm for 15 minutes at 0ºC. The supernatant was removed and virgin Thai *Ae. aegypti* females (three-to-six days old) were injected in the thorax with 0.25 µl of *D. melanogaster* homogenate (equivalent to 0.25 of an accessory gland). Females injected with *Aedes* saline were used as controls as well as females injected with *Ae. aegypti* male accessory gland extract (aeMAG); and females that were mated without injection. Females were divided into two cohorts. One group was tested for refractoriness to mating by placing females in individual 0.5 L cups with males (aged seven-to-nine days) 3 days after injection for 2 days (n=10). Two days later, females were removed and their spermathecae dissected to determine insemination status. The other group was tested for oviposition behavior. Females were blood fed on a human arm (M.E.H.) 4 days after injection. Unfed females were removed and offered blood the following day. Three to four days after feeding, fed females were placed in individual cups (0.5 L) for oviposition. Four days later, females were removed

297 and the number of eggs for each female was counted. Live females with zero eggs were
298 dissected to determine insemination status (only when females had been exposed to
299 males) and for the presence of fully developed eggs in ovaries. Virgin females injected
300 with saline and non-injected females mated with males from the same cohort were used
301 as controls.
302
303 In the second independent assessment of the impact of *Drosophila* and *Aedes* accessory
304 gland extracts on egg laying, *Drosophila* MAGs were prepared by dissecting accessory
305 glands from 50 fly abdomens (Canton-S strain) into 50 ul of PBS (Gibco, pH 7.2) and
306 homogenized with a pellet pestle motor (Kimble). This mixture was centrifuged at 14,000
307 x g for 30 minutes at 4°C. The supernatant was applied to a 0.22 um filter column
308 (Millipore) and centrifuged at 14,000 x g for 10 minutes at 4°C. The liquid flow through
309 (dMAG) was kept at 4°C and injected within one week of preparation. *Aedes* accessory
310 gland homogenates were prepared using an identical protocol from three-to-five-day-old
311 *LVPib12* virgin males that had been anesthetized on ice with the following modifications:
312 The male abdominal tip (MAT) was dissected into a drop of PBS by grasping the genital
313 claspers with forceps and pulling the terminal abdominal segment away from the rest of
314 the abdomen. This isolated the last segment of the abdomen including the male
315 accessory glands and other reproductive organs. MAT from 200 males were dissected
316 into 200 uL PBS (Gibco, pH 7.2) on ice (HELINSKI *et al.* 2012). The liquid flow through
317 (aeMAT) was kept at 4°C and injected within two weeks of preparation.
318

To test if SPR is required for the post-mating reduction in mating receptivity resulting from injection of *Ae. aegypti* MAG (aeMAG) homogenate, Thai wild type and $Spr^{\Delta 1235}$ homozygous females were injected with aeMAG as previously described (AMARO *et al.* 2021). Modified PBS buffer alone (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 3 mM KH_2PO_4, 2 mM $CaCl_2$ pH 7.0) served as a control. Two days after injection, females were blood fed on a human host (S.P.) for 8 minutes, fully-engorged females were separated individually into 0.5 L cups and 2 wild type Thai males were introduced 5 days post-blood feeding. After 2 days female spermathecae were dissected and scored for the presence or absence of sperm.

Cell-based assays

Cell-based assays were performed as in Duvall *et al*. (2019). Briefly, HEK293T cells (Thermo Fisher Scientific) were maintained using standard protocols in a Thermo Scientific water jacketed carbon dioxide incubator. Cells were transiently transfected with 0.5 μg each of plasmid expressing GCaMP6s (CHEN *et al.* 2013), mouse Gqα15 (OFFERMANNS AND SIMON 1995), and SPR (AAEL019881) using Lipofectamine 2000 (Invitrogen). Transfected cells were seeded into 96 well plates (Greiner Bio-one) and incubated overnight in Fluorobrite DMEM media (Thermo Fisher Scientific) supplemented with Fetal Bovine Serum (Invitrogen) at 37°C and 5% carbon dioxide. Cells were directly imaged in 60μL Fluorobrite DMEM media (Thermo Fisher Scientific) using GFP-channel fluorescence of a BioTek Synergy Neo plate reader with liquid handling system and automated dispenser. Peptides were prepared at 3x concentration in reading buffer [Hank's Balanced Salt Solution (GIBCO), 20mM HEPES (Sigma-

Aldrich), pH 7.4]. Wells were imaged every 0.5 seconds for 3 minutes in kinetic mode. 30μL of peptide was added to each well after 15 seconds of baseline fluorescence recording. Normalized responses were calculated as $(\Delta F/F_0)_{experimental} - (\Delta F/F_0)_{no\ receptor\ control}$. AstAR (AAEL006076) response to Ast1 was used as a positive control in each plate. *Ae. aegypti* peptides previously detected in Predel *et al.* (2010) and tested *in vitro* in Duvall *et al.* (2019) were synthesized by Bachem and maintained as lyophilized powders or 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) concentrated stock solutions at -20°C. All peptides were tested at a 10 μM final concentration with < 1% DMSO final concentration. 11 – 14 replicates were performed/transfection and each experimental well included matched positive and negative (no receptor transfected) control wells in the same plate. Any well in which the matched positive control response was < 0 was excluded from analysis.

Data analysis. Power analysis using G*power (FAUL *et al.* 2007) was used to determine appropriate sample size prior to experiments based on preliminary data. Statistical analysis was performed using SPSS (IBM Statistics, version 25). A Levene's test was first performed to determine homoscedasticity of male and female body size data, then wing lengths were compared with Kruskal-Wallis and Mann-Whitney tests. Replicate effects were analyzed and replicates were combined when appropriate. Remating was analyzed using a binary generalized linear mixed model to compare differences by time points and genotype ($Spr^{\Delta 235/\Delta 235}$ or heterozygous ($Spr^{\Delta 235/+}$); to compare remating in the Spr^{ECFP} mutants vs. wild type, a two sample proportions test was performed. Egg counts from oviposition assays were analyzed with Graphpad

Prism 9.4.1 software with a one-way ANOVA followed by *post-hoc* t-tests between experimental groups. *P* values were adjusted for multiple comparisons with Dunnett's correction when the comparisons were all against a control group; and adjusted with Tukey's correction when comparisons were made between experimental groups. In cell-based assays fluorescence signal responses were analyzed with Graphpad Prism 9.3.1 software with one sample t and Wilcoxon Signed Rank test.

Results

Generation and characterization of *Aedes aegypti Spr* mutants

To test whether sex peptide receptor (SPR) is required for canonical post-mating receptivity and egg laying behaviors in *Ae. aegypti*, we generated two independent mutant alleles in the wild type *Spr* gene locus using CRISPR/Cas9 mutagenesis (Fig 1A).

We first generated a NHEJ allele ($Spr^{\Delta 235}$) in the Thai *Ae. aegypti* genetic background by injecting two gRNAs targeting exon 2 of *Spr* (the first coding exon of this gene) along with Cas9 protein into pre-blastoderm stage embryos (Fig 1B). Subsequently using PCR genotyping (Fig S1A), we successfully recovered the $Spr^{\Delta 235}$ mutant allele consisting of a 235 bp deletion (bp 50-285 in the open reading frame) with an insertion of 15 bp in this exon.

We next generated an independent HDR allele (Spr^{ECFP}) in the *LVPib12 Ae. aegypti* genetic background by injecting a donor construct with a gRNA expression cassette in

its backbone into pre-blastoderm stage embryos from the germline *Exu-Cas9 Ae. aegypti* strain (Fig 1C). In this allele, a 2617 bp cassette was inserted by homology-dependent repair into exon 2 of *Spr*. This disruptive insertion included a stop cassette and marks mutants visibly with enhanced cyan fluorescent protein (ECFP) (Fig 1C) and can be detected in the *Spr* locus by a custom genotyping assay using PCR (Fig S1B).

Predicted amino acid alignments indicate that relative to the wild type Spr^{+} allele, both $Spr^{\Delta 235}$ and Spr^{ECFP} mutant alleles are likely truncated before the first predicted transmembrane domain of SPR (Fig 1D). Specifically, the $Spr^{\Delta 235}$ allele has a frameshift mutation in the *Spr* coding sequence that yields a premature stop codon at amino acid 28, while precise insertion of a stop codon in Spr^{ECFP} directly after amino acid 47 is similarly predicted to prematurely terminate translation.

Spr knockout mutants are viable and fertile.

We determined that both of the *Spr* mutant alleles that we generated ($Spr^{\Delta 235}$ and Spr^{ECFP}) were homozygous viable. We have successfully maintained these homozygous mutant lines for more than six years and one year respectively since their establishment, using standard protocols.

Detailed morphometric and fitness characterization of the $Spr^{\Delta 235}$ allele, revealed that homozygous mutant males were smaller (average = 1.99 to 2.16 mm wing length) than wild type ($Spr^{+/+}$) or heterozygous ($Spr^{\Delta 235/+}$) males reared under same conditions (2.13 to 2.23 mm) across replicates and remating experiments; $Z = -3.79$, $P<0.00$ (wild type),

411 Z = -4.05, P<0.00 (heterozygous). Mutant females were smaller in body size compared
412 to wild type and $Spr^{\Delta 235/+}$ females in replicate 1: $Spr^{\Delta 235/\Delta 235}$ female = 2.53 ± 0.15 mm;
413 $Spr^{+/+}$ female = 2.78 ± 0.20 mm (Z = -4.039, df <0.01), but not significantly different in
414 Replicate 2: $Spr^{\Delta 235/+}$ female = 2.9 ± 0.06 mm; $Spr^{\Delta 235/\Delta 235}$ female = 2.88 ± 0.08; $Spr^{+/+}$
415 female = 2.91 ± 0.05. Mutant females mated successfully with males at the same
416 percentage as controls, as demonstrated by the presence of sperm in their
417 spermathecae after 24 h [96% $Spr^{\Delta 235/\Delta 235}$ (87/90) and 99% $Spr^{\Delta 235/+}$ (91/92) females
418 mated with $Spr^{\Delta 235/+}$ males].
419
420 **SPR *in vitro* response screen**
421 The smaller body size of our *Spr* mutants prompted us to investigate the nature of the
422 ligand of SPR. Work from Kim *et al.* (2010) suggests that *Aedes* SPR can be potently
423 activated by non-SP, non-MIP ligands found in whole mosquito extracts. We performed
424 *in vitro* assays to profile *Ae. aegypti* SPR responses to various classes of
425 neuropeptides. We expressed *Ae. aegypti* SPR cDNA in HEK293T cells co-transfected
426 with a promiscuous G protein (OFFERMANNS AND SIMON 1995) and the genetically
427 encoded calcium sensor GCaMP6s (CHEN *et al.* 2013). Activation of a given receptor is
428 read out by calcium-induced increase in fluorescence of GCaMP6s. SPR responses
429 were profiled to a 10μM dose of 50 known neuropeptides. Although modest responses
430 were noted to several neuropeptides (Ast3, AT, sNPF1, sNPF2+4, TKRP2, TKRP3),
431 these showed low efficacy compared to positive controls (AstAR activation by Ast1
432 peptide) (Fig S2). We did not observe activation by MIP peptides tested, nor by HP-I, a
433 peptide known to play a role in short-term receptivity suppression (DUVALL *et al.* 2017).

We did not identify additional high efficacy agonists of SPR among our neuropeptide panel.

Spr knockout females show normal changes in post-mating refractoriness.

Mated *Ae. aegypti* females quickly become refractory to subsequent mating (CRAIG 1967; HELINSKI *et al.* 2012; DEGNER AND HARRINGTON 2016). While initial changes are induced by a seminal peptide HP-1 (DUVALL *et al.* 2017), this effect is temporary, acting only within one hour of mating. The nature of the molecule(s) that induce(s) lifelong refractoriness in these females is unknown.

We initially observed that $Spr^{\Delta 235/\Delta 235}$ homozygous mutant females are as likely to mate with a second male as are $Spr^{\Delta 235/+}$ control females, regardless of the time after the first mating (Fig 2A; p = 0.88 and p = 0.18 for tests immediately, and 24 h, after mating, respectively). To confirm this result, we injected male accessory gland homogenate, which is known to induce mating refractoriness (HELINSKI *et al.* 2012), from wild type Thai males into both $Spr^{\Delta 235/\Delta 235}$ and wild type Thai females and found no difference in mating receptivity when males were presented 5 days after injection [$Spr^{\Delta 235/\Delta 235}$, 1.37% females remated (n = 74); wild type, 1.16% females remated (n = 86)].

Similarly, we observed in an independent post-mating receptivity assay, that $Spr^{ECFP/ECFP}$ homozygous mutant females are as likely to mate with a second male as are $Spr^{EFCP/+}$ control females (Fig 2B; Z = 0.88, p = 0.37). In this assay, females were

tested for their propensity to remate within 1-1.5 h after initial mating and we similarly detected no significant differences in post-mating receptivity.

Thus, *Spr* does not seem to be necessary for induction of post-mating refractoriness, including short, medium or long-term refractoriness, in *Ae. aegypti* females.

Spr is not required for egg laying in _Aedes aegypti_.

Mating stimulates oviposition in gravid *Aedes aegypti* females (LANG 1956; JUDSON 1967), and this effect is mediated by unknown protein/s transferred to females in male accessory gland fluid (LEAHY AND CRAIG 1965; HISS AND FUCHS 1972). To test whether SPR is required for post-mating oviposition behavior in *Ae. aegypti*, we next tested whether egg laying was impacted using the Spr^{ECFP} mutant allele.

We determined that gravid wild type $Spr^{+/+}$, heterozygous $Spr^{ECFP/+}$ and homozygous $Spr^{ECFP/ECFP}$ mutant females laid very few eggs if they were not mated: 6.5 ± 6.5, 8.8 ± 4.9 and 17.5 ± 8.8 eggs, respectively (mean ± S.E.M., Fig 2C). We hypothesized that if SPR was required for post-mating oviposition behavior, homozygous $Spr^{ECFP/ECFP}$ females would not lay eggs even after mating. However, the number of eggs laid by mated homozygous *Spr* mutant females (90.4 ± 10.0 eggs) was not significantly different from that laid by heterozygous (92.9 ± 3.1 eggs, comparison: p = 1.000) or wild type females (mean: 105.3 ± 3.5, comparison: p = 0.774) (mean eggs ± S.E.M., Fig 2C).

We conclude that SPR is not required for post-mating oviposition in *Ae. aegypti*.

Neither *Drosophila melanogaster* sex peptide nor male accessory gland homogenate induces mating refractoriness in *Aedes aegypti* females.

We next tested whether injection of synthetic *D. melanogaster* SP (dSP) could induce mating refractoriness in *Ae. aegypti,* as demonstrated for *D. melanogaster* and *H. armigera* (CHEN *et al.* 1988; SCHMIDT *et al.* 1993; FAN *et al.* 1999; FAN *et al.* 2000).

We first confirmed activity of synthetic dSP by injecting 3 pmol (or saline) into 30 virgin *D. melanogaster* and assessing activity. Mating refractoriness assays revealed that only 6.6% of SP-injected *D. melanogaster* female flies mated, compared with 63% of those injected with saline. Similarly in egg laying assays, 46.7% of SP-injected *D. melanogaster* virgin female flies laid eggs compared to 0% of *D. melanogaster* females injected with saline. These data are all consistent with previous reports of the effects of dSP on mating refractoriness and egg laying in *D. melanogaster* (CHEN *et al.* 1988; SCHMIDT *et al.* 1993; LIU AND KUBLI 2003).

We then examined the effect of injecting synthetic dSP (3 pmol) or saline vehicle on mating refractoriness in the Thai *Ae. aegypti* strain using virgin females. In these assays, at 12 h post-injection, injected females were allowed to mate for 48 h and afterwards, females were dissected and examined for sperm in their spermathecae. We observed that sperm were present in all dSP-injected and all saline-injected females,

indicating that dSP had no effect on *Ae. aegypti* mating receptivity in this context (Fig 3A).

Consistent with our results with synthetic SP, when 0.25 male *D. melanogaster* male accessory gland (dMAG) equivalents were injected into virgin Thai *Ae. aegypti* females (0.25 ul homogenate), we also found no effect of dMAG on mating receptivity (Fig 3B). We conclude that intrathoracic injection of synthetic dSP as well as homogenate from dMAG, the source of endogenous SP, is insufficient to induce mating refractoriness in *Ae. aegypti*.

Neither *Drosophila melanogaster* sex peptide nor male accessory gland homogenate induces egg laying in *Aedes aegypti* females.

Intrathoracic injection of *Ae. aegypti* male accessory gland homogenate has previously been shown to be sufficient to stimulate oviposition behavior in virgin gravid *Ae. aegypti* females (LEAHY AND CRAIG 1965; JUDSON 1967; HISS AND FUCHS 1972). Independently, our two groups next assessed whether intrathoracic injection of *D. melanogaster* homogenates putatively representing the complement of seminal fluid proteins from this species, inclusive of dSP, could similarly act to induce oviposition in virgin *Ae. aegypti* females.

In our first assay series in the Thai *Ae. aegypti* strain, we confirmed that injection of *Ae. aegypti* accessory gland homogenate (aeMAG) triggers oviposition from virgin gravid females (66.0 ± 8.0), approaching levels of egg laying observed from mated females

(62.1 ± 8.1) (mean eggs ± S.E.M, Fig 3C). In contrast, virgin gravid females injected with dMAG laid very few eggs (5.3 ± 4.2) not significantly different from saline injected controls (5.4 ± 5.0).

Our second assay series in the *LVPib12* strain similarly revealed a crude homogenate prepared from the terminal abdominal segment of virgin males (*Ae. aegypti* male abdominal tip: aeMAT) containing the accessory glands and all other male reproductive glands was sufficient to induce oviposition behavior when injected into the thorax of virgin gravid females. Females injected with only PBS solvent laid on average 4.2 ± 3.3 eggs whereas those injected with aeMAT laid on average 60.7 ± 13.0 eggs (mean eggs ± S.E.M. aeMAT vs PBS control, $p < 0.0001$, Fig 3D). In contrast, dMAG did not stimulate egg laying from virgin gravid *Ae. aegypti* females (9.2 ± 8.6 eggs), yielding a similar level of oviposition to those injected with the PBS solvent control (mean eggs ± S.E.M., dMAG vs PBS control: $p = 0.992$, Fig 3D).

Finally, to probe whether *Drosophila* sex peptide (dSP) was capable of stimulating oviposition behavior in *Ae. aegypti*, we injected synthetic *D. melanogaster* sex peptide (dSP) over a concentration series ranging from 1.5 pmol to 150 pmol (Fig 3D) into virgin gravid females from the *LVPib12* strain. We determined that dSP injection did not have an effect on egg-laying in this context. The number of eggs (mean eggs ± S.E.M.) laid by females injected with 1.5 pmol dSP (0.3 ± 0.2, vs PBS control: $p = 0.997$), 15 pmol dSP (15.3 ± 10.0, vs PBS control: $p = 0.787$) and 150 pmol dSP (11.0 ± 6.7, vs PBS control: $p = 0.961$) did not differ significantly relative to the PBS solvent control.

We conclude that intrathoracic injection of dMAG as well as synthetic dSP are insufficient to stimulate oviposition behavior in virgin gravid *Ae. aegypti* females. However, aeMAT and aeMAG is sufficient to stimulate oviposition behavior within this context.

Discussion

Elucidating the molecular pathways involved in establishing post-mating responses in *Ae. aegypti* from both males and females is important to our understanding of their reproductive biology and has implications for strategies for vector control. Here, we explored the role of SPR, a critical female receptor in some other insects, including the dipteran *D. melanogaster,* in mediating multiple post-mating responses. The primary *D. melanogaster* SPR ligand responsible for controlling reproductive outcomes is SP, a seminal peptide that is not found in insects outside *Drosophila* genus (TSUDA AND AIGAKI 2016; MCGEARY AND FINDLAY 2020). Interestingly, injecting *D. melanogaster* SP into unmated female *H. armigera* moths reduces egg laying, suppresses pheromone synthesis, and reduces calling behaviors (FAN *et al.* 1999; FAN *et al.* 2000; HANIN *et al.* 2012), thus mimicking post-mating responses analogous to those induced by SP in *Drosophila*. However, roles of SPR, and effects of SP were not consistent across insects. For example, injection of SP^{21-36} into the tarnished plant bug *Lygus herperus* had no effect on mating receptivity and although this region of SP binds *in vitro* to SPR from *D. melanogaster* and *H. armigera* it was unable to bind to SPR from *L. herperus* (HULL AND BRENT 2014). Although *Ae. aegypti*

oviposition was reported to be induced by surgical implantation of whole *D. melanogaster* accessory glands (LEAHY 1967) or intrathoracic injection of whole *Drosophila* male body lysates in the thorax of blood fed, virgin females (HISS AND FUCHS 1972), it is not known what male molecule(s) had this effect nor what receptor they bound to in the female. It should be noted that the stimulation of oviposition with intrathoracic injection of *Drosophila* male accessory gland homogenate into gravid virgin *Ae. aegypti* females was not observed when independently tested by both groups. In contrast, we found that *Ae. aegypti* accessory gland homogenate preparations were fully sufficient to evoke the post-mating receptivity and egg laying phenotypes that we characterized in this study consistent with previous reports (HISS AND FUCHS 1972; HELINSKI *et al.* 2012).

Genes corresponding to a SP homolog are not readily identified in insects outside *Drosophila*, but sequences homologous to SPR can be identified in various insect groups, including *Ae. aegypti*.; the mosquito SPR even binds Drosophila SP *in vitro*, albeit weakly (KIM *et al.* 2010; LEE *et al.* 2020). Given the presence of SPR in *Ae. aegypti*, the parallels between post-mating responses in *Ae. aegypti* and *D. melanogaster*, and the importance of SP in inducing post-mating response in *H. armigera,* we tested here whether SPR is required for post-mating responses in *Ae. aegypti*. We generated two independent SPR null mutations by CRISPR/Cas9 gene editing and compared mutant and control females for a variety of post-mating responses.

First, we tested whether SPR is needed for the post-mating drop in female receptivity. Female *Ae. aegypti* that have mated are refractory to subsequent mating

(CRAIG 1967; HELINSKI *et al.* 2012); ~25% do not remate immediately after mating, and all the mosquitoes eventually establish strong refractoriness 16-20 h after the initial mating (DEGNER AND HARRINGTON 2016). In both of the *Spr* mutant alleles ($Spr^{\Delta 235}$ and Spr^{ECFP}) that we characterized, functional knockout of SPR does not affect the establishment of remating refractoriness, regardless of the interval between mating opportunities.

Stimulation of egg development and oviposition is another post-mating response by *Ae. aegypti* females (LANG 1956; JUDSON 1967). Spr^{ECFP} knockout females oviposited a similar number of eggs as heterozygous females after mating and blood feeding, demonstrating that SPR is not required for this post-mating process. Knockdown or knockout of SPR in various insect systems have noted a reduced number of eggs laid (LI *et al.* 2014; ZHENG *et al.* 2015; GREGORIOU AND MATHIOPOULOS 2020; LIU *et al.* 2021), although the degree of reduction varies and it often is not a complete lack of egg laying. Consistent with the lack of effect of SPR knockout on mating receptivity and on oviposition, no phenotypes were observed when two commercial sources of synthetic dSP (at a biologically-effective dose for *D. melanogaster*) were injected independently into unmated *Ae. aegypti* females. The lack of any observable impact of intrathoracic microinjection of synthetic sex peptide on *Ae. aegypti* egg laying thus mirrored that of injecting dMAG homogenate which had the effect as described above.

SPR was reported to bind myoinhibitory peptides (MIPs), often at stronger affinities than SP, leading to the hypothesis that SPR is an ancestral MIP receptor (KIM *et al.* 2010; POELS *et al.* 2010). MIPs in insects are involved in control of hindgut and oviduct muscle contraction (SCHOOFS *et al.* 1991; BLACKBURN *et al.* 1995; BLACKBURN *et*

616 *al.* 2001; PALUZZI *et al.* 2015; LUBAWY *et al.* 2020), modulating nutritional preferences
617 (HUSSAIN *et al.* 2016; MIN *et al.* 2016), maintaining sleep states in *D. melanogaster* (OH
618 *et al.* 2014), as well as involvement in ecdysis (DAVIS *et al.* 2003; KIM *et al.* 2006a; KIM
619 *et al.* 2006b; SANTOS *et al.* 2007). MIPs have been shown to have allostatic activity by
620 inhibiting JH synthesis in the cricket *Gryllus bimaculatus* (LORENZ *et al.* 1995) and
621 Brown-winged green bug *Plautia stali* (MATSUMOTO *et al.* 2017). The *B. mori* peptide
622 PTSP inhibits ecdysone synthesis from the prothorasic gland (HUA *et al.* 1999). There
623 are five identified MIPs in *Ae. aegypti* (PREDEL *et al.* 2010; SIJU *et al.* 2014) with positive
624 staining in the CNS with an anti-MIP antibody (KIM *et al.* 2010), though intriguingly a
625 non-MIP ligand for *Ae. aegypti* SPR was partially purified (KIM *et al.* 2010). In our *in vitro*
626 assay we found that *Ae. aegypti* SPR showed modest responses to Ast3, AT, sNPF1,
627 sNPF 2+4, TKRP2, TKRP3 but we did not observe significant responses to HP-I, a
628 peptide known to be found in male accessory gland and transferred to females during
629 mating (NACCARATI *et al.* 2012; DUVALL *et al.* 2017) nor did we identify novel high
630 efficacy ligands for SPR among our cohort of 50 peptides. Unlike Kim *et al.* (2010), we
631 did not observe responses from MIP peptides tested in this assay (MIP 1, 4, and 5),
632 although we note that our assay differs in cell type and sensor, and that although a
633 10μM dose of peptide has been used successfully to identify ligand/receptor interactions
634 (DUVALL *et al.* 2019) we acknowledge that a single dose of ligand may not be sufficient
635 to identify all potential SPR agonists. Given no strong phenotype in the SPR knockout
636 mosquitoes characterized in the assays presented here, it is still possible that there
637 could be a subtle effect with other known functions of MIPs. It is also possible there are

638 pathways where ligands other than MIPs bind SPR that were not included in our peptide
639 panel or belong to a different class of ligands.

640 As highlighted above, SPR disruptions in different insects can have varying
641 effects on reproductive traits. Our contribution characterizing the effects of lack of SPR
642 in *Ae. aegypti* helps contribute to the evolutionary understanding of this key receptor.
643 The SPR is flexible to evolve binding to similar ligands for different functions, some of
644 which have been used in reproduction. Unknown signaling pathways operating during
645 transfer of *Ae. aegypti* seminal fluid thus likely underlie long-term refractoriness to
646 remating and induction of egg laying in this important disease vector.

647

648 **Data availability statement:** Strains and plasmids are available upon request. The
649 authors affirm that all data necessary for confirming the conclusions of the article are
650 present within the article, figures, and tables.

651

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Table S1. Primers for generation and verification of the *Spr*$^{\Delta 235}$ NHEJ allele

Underlined sequences in guide primers are complementary to the universal primer for use in template-free PCR to generate the DNA template for guide RNA synthesis. Bolded bases indicate the targeted *Spr* gene sequence.

	Sequence (5' – 3')
gRNA universal reverse; Kistler 2015	AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA GTT GAT AAC GGA CTA GCC TTA TTT TAA CTT GCT ATT TCT AGC TCT AAA AC
Guide 1	GAA ATT AAT ACG ACT CAC TAT AG**G TAG ATG CCA GGA TCC AGT G**GT TTT AGA GCT AGA AAT AGC
Guide 2	GAA ATT AAT ACG ACT CAC TAT A**GG CGA TAT CTC TAA TGT CCT** GTT TTA GAG CTA GAA ATA GC
SPR genotyping Forward	TCG TCT CAC AGC TAG AAT TGC
SPR genotyping Reverse	CAT CAG CAC GAA GTT GGT CG

673 **Table S2. Primers for generation and verification of the *Spr^{ECFP}* HDR allele**

674 Underlined sequences indicate adapters for InFusion cloning, bold sequence indicates
675 *LoxP* sites incorporated into the cassette to facilitate potential *Cre-LoxP* mediated
676 excision of the integrated marker cassette, and lowercase letters indicate a triple stop
677 cassette sequence.

	Sequence (5' – 3')
guideRNA for SPR exon 2	AAC AGC GGA GAC GCC TCA AG
Forward Left Homology Arm (5' SalI)	AAT GGC CAT GGG ACG TCG ACC GTC TTT CGG AGA CAT CGG CA
Reverse Left homology arm (5' SalI)	TTA CCT CAG GTC GAC **ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA T**tc agc cta cgt taC TTG AGG CGT CTC CGC TGT T
Forward Right homology arm (5' AgeI)	ATA GTT CTA GAG GTA CCG GT**A TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT** TGG TGG CGA ACG AAT GCT GTA
Reverse right homology arm (5' AgeI)	GGC TAA CGT TAA CAA CCG GTC AAA CTG TGG TTC ACT CGA ACC G
gBlock Forward	GGC CTA GGA TGC ATA GAA TGA AAT CGC CCA TCG
gBlock Reverse	CAG GCG GCC GCC ATA AAA AAA GCA CCG ACT CGG
SPR insertion genotyping forward; cut site	CGC CTC AAG TGG TGG C
SPR insertion genotyping forward; polyUb cassette	GCT TTC TTT CTT GTC TGG CTC

SPR insertion genotyping reverse; right homology arm	GTT TCA CAG GAC ACG TTC AG

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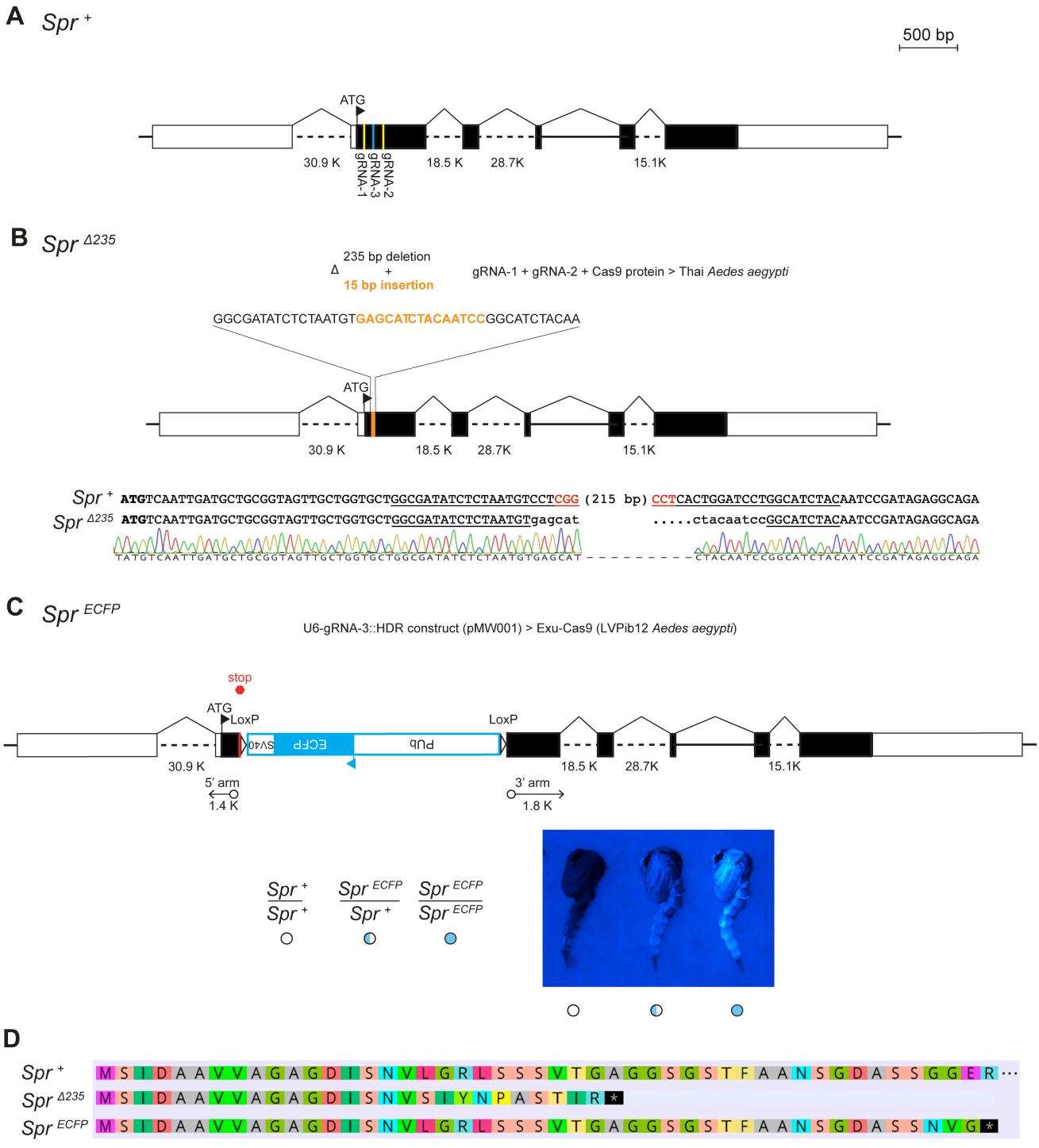

Figure 1: Generation of *Aedes aegypti* sex peptide receptor mutants.
(A) Diagram of the wild type *Ae. aegypti* sex peptide receptor (*Spr*$^{+}$) genomic locus. Boxes denote exons which are shaded with white for untranslated regions and black for the open reading frame. Guide RNA sites used for generating mutants shown as yellow and blue lines. (B) Diagram of the *Spr*$^{\Delta 235}$ mutant allele generated by deletion of 235 bp and insertion of 15 bp using gRNA1 and gRNA2. Sanger trace (below) validates the altered sequence of this allele relative to wild type. Translational start site is bold, gRNA target site underlined, and PAM sequence highlighted in red. (C) Diagram of *Spr*^{ECFP} mutant allele generated by insertion of a disruptive ECFP expression cassette via homology directed repair. Representative images of wild type (open circle), heterozygous (half cyan circle) and homozygous (full cyan circle) *Spr*^{ECFP} mutant pupae are shown below. (D) Predicted amino acid alignments of wild type and truncated *Spr*$^{\Delta 235}$ and *Spr*^{ECFP} mutant alleles. Premature stop codons are indicated by the black box with star.

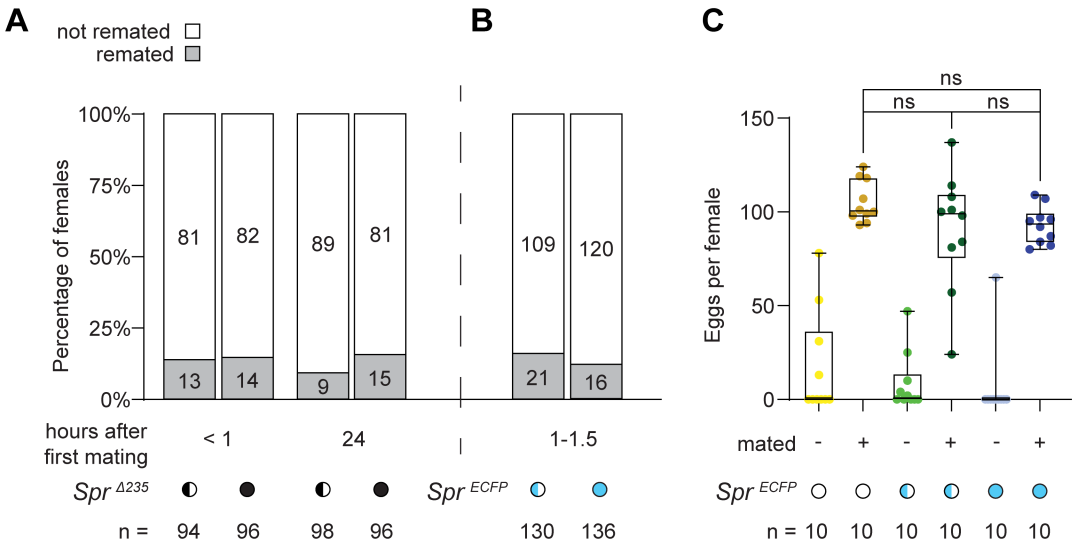

Figure 2: Sex peptide receptor is not required for refractoriness to remating over varied timescales or induction of egg laying in *Aedes aegypti*.

(A) Percent of *Spr*$^{\Delta 1235}$ heterozygous (half black circle) and homozygous mutant (full black circle) females that remated (gray shading) immediately (<1 hr) or 24 h after their first mating. Raw number of females in each remating category are denoted within each bar. Cumulative sample size (n) from two independent biological replicates indicated. (B) Percent of *Spr*^{ECFP} heterozygous (half cyan circle) and homozygous (full cyan circle) mutant females that remated (gray shading) 1 to 1.5 h after their first mating. Raw number of females in each remating category are denoted within each bar. Sample size indicated below genotypes. (C) Eggs laid per female for wild type (open circle), *Spr*^{ECFP} heterozygous (half cyan circle) and *Spr*^{ECFP} heterozygous (full cyan circle) mutant genotypes after a bloodmeal that were either virgin (-) or mated (+). n.s. = p > .05 (Dunnett's multiple comparisons test). Box plots show median, interquartile range, and maximum and minimum values.

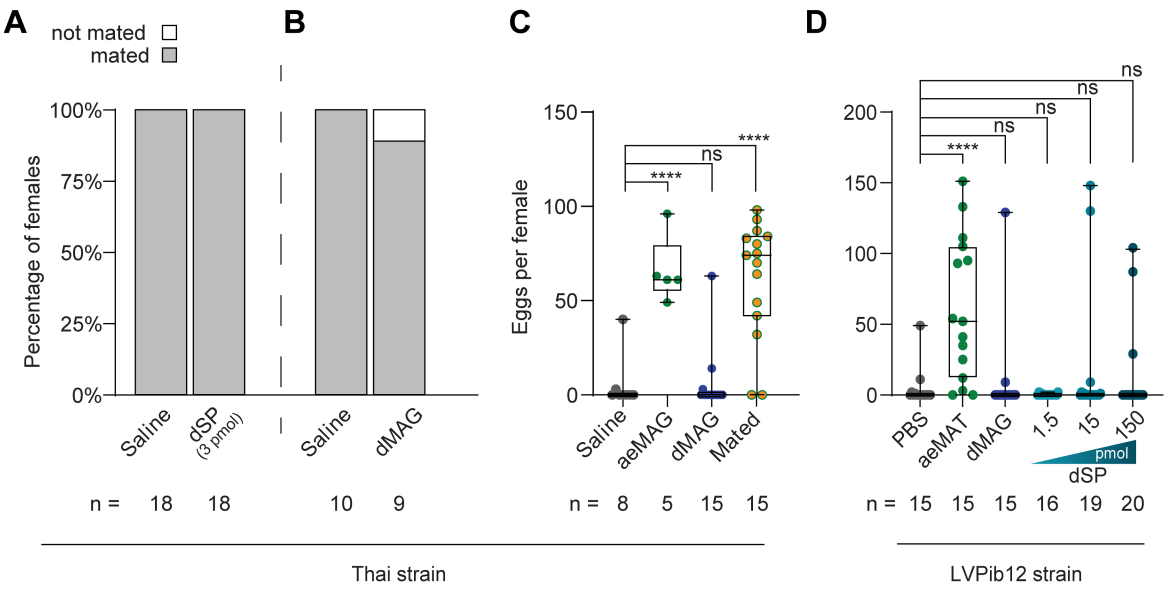

Figure 3: *Drosophila melanogaster* sex peptide and accessory gland extracts do not induce post mating behaviors in *Aedes aegypti* females.
(A) Percentage of virgin *Ae. aegypti* females injected with saline vehicle or synthetic *Drosophila* sex peptide (dSP) that mated (gray shading). (B) Percentage of virgin *Ae. aegypti* females injected with saline vehicle or *Drosophila* male accessory gland (dMAG) homogenate that mated (gray shading). (C) Eggs laid per virgin gravid *Ae. aegypti* female after injection with saline vehicle or male accessory gland homogenate from *Aedes aegypti* (aeMAG) or *Drosophila melanogaster* (dMAG), or normal mating. n.s. = p > .05, **** p < .0001 (Dunnett's multiple comparisons test). (D) Eggs laid per virgin gravid *Ae. aegypti* female after injection with saline vehicle (PBS), male abdominal tip homogenate from *Aedes aegypti* (aeMAT), dMAG, or different dosages of dSP. n.s. = p > .05, **** p < .0001 (Dunnett's multiple comparisons test). Box plots show median, interquartile range, and maximum and minimum values.

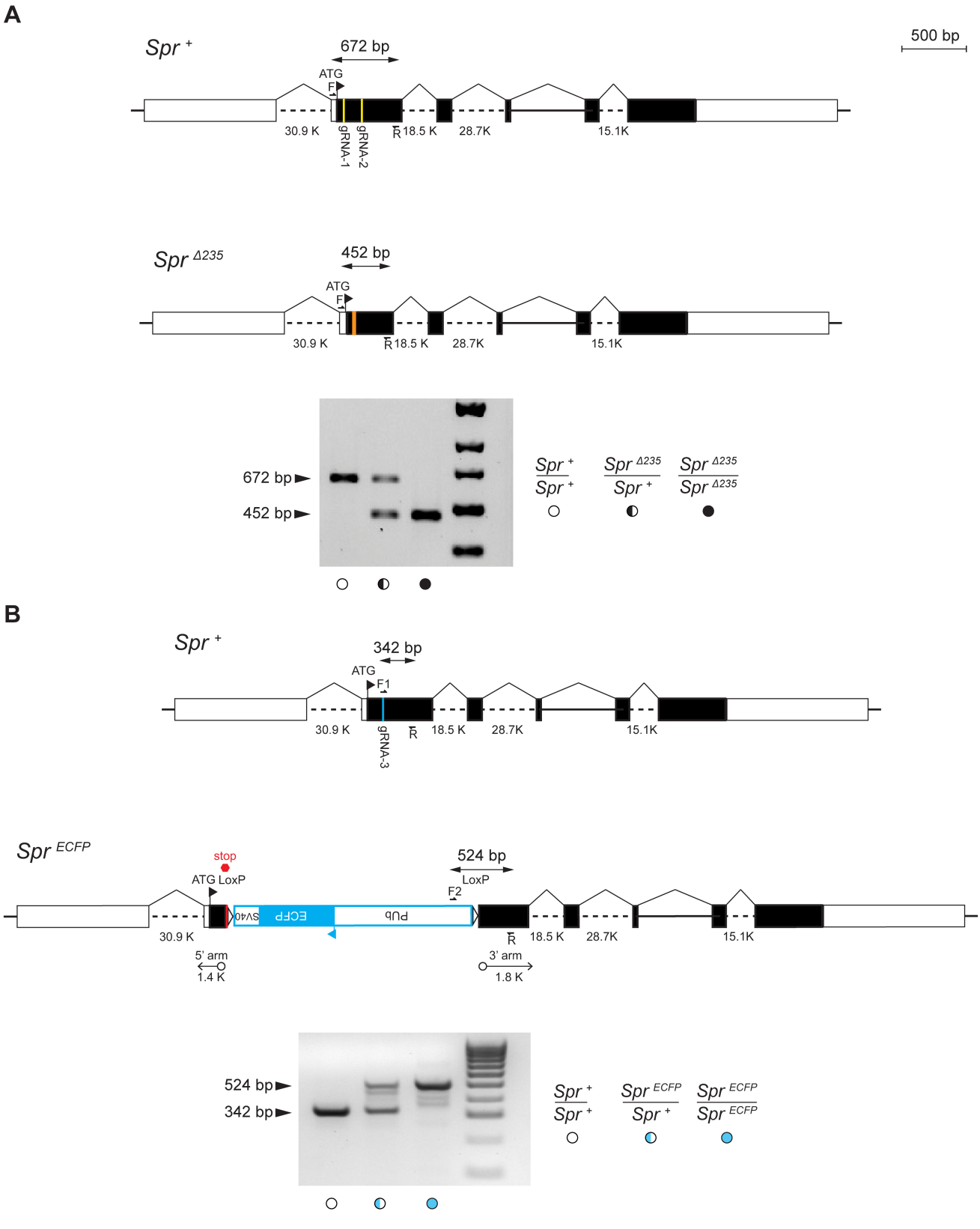

Figure S1: PCR genotyping assays for *Aedes aegypti* sex peptide receptor mutant alleles.
(A) Genotyping assay for the $Spr^{\Delta 235}$ NHEJ allele: a two primer PCR assay with forward (F) and reverse (R) primers anchored in Exon 2 yields a 672 bp amplicon for the wild type Spr^{+} allele and a 452 bp amplicon for the $Spr^{\Delta 235}$ mutant allele. (B) Genotyping assay for the Spr^{ECFP} HDR allele: a three primer PCR assay was used where one forward primer (F1) was centered on the CRISPR cut site in Exon 2 so that it would only anneal to the wild type allele, one forward primer (F2) was placed in the polyubiquitin (PUb) sequence in the integrated cassette; and one common reverse primer (R) was nested in the right homology arm in Exon 2. This yields a 342 bp amplicon for the wild type Spr^{+} allele with the intact gRNA site (amplified by F1/R), and a 524 bp amplicon for the Spr^{ECFP} mutant allele (amplified by F2/R).

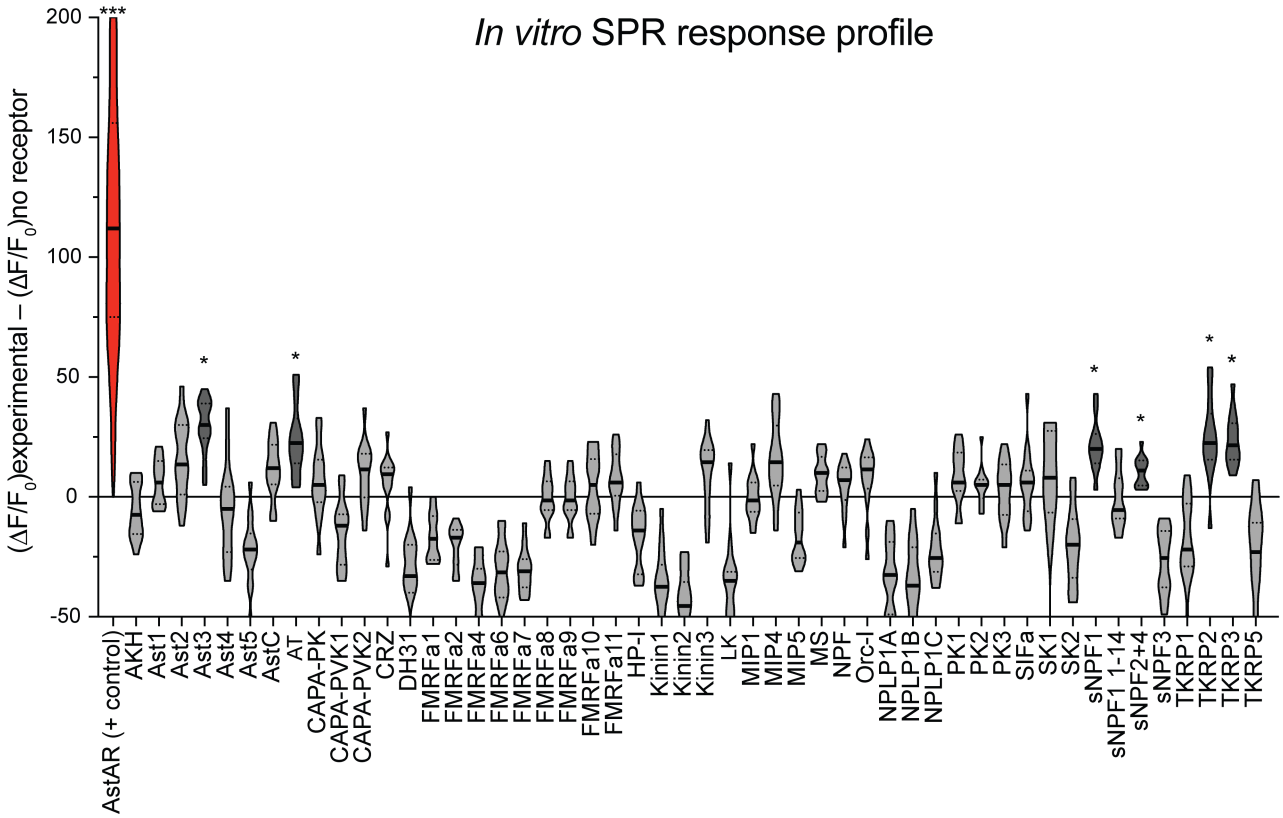

Figure S2: *In vitro Aedes aegypti* SPR responses to neuropeptide panel.
In vitro response profile of *Ae. aegypti* sex peptide receptor (AAEL019881) to a panel of 50 neuropeptides. Fluorescence signal was calculated as $(\Delta F/F_0)_{experimental} - (\Delta F/F_0)_{no\ receptor\ transfected\ control.}$ AstAR (AAEL006076) response to Ast1 was used as a positive control in each plate. (n = 11 – 14 replicates/transfection). * $p < 0.001$, *** $p < 0.0001$ (one sample t and Wilcoxon Signed Rank test, hypothetical value = 0).