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Ovipositor Extrusion Promotes the Transition from Courtship to Copulation and Signals Female Acceptance in *Drosophila melanogaster*

Highlights

- DNp13 activity induces full ovipositor extrusion
- Ovipositor extrusion is a response to the male courtship song
- Male licking an extruded ovipositor prompts copulation attempt
- Upon copulation attempt, receptive females retract the ovipositor to allow copulation

Authors

Cecilia Mezzera, Margarida Brotas, Miguel Gaspar, Hania J. Pavlou, Stephen F. Goodwin, Maria Luísa Vasconcelos

Correspondence

maria.vasconcelos@neuro.fchampalimaud.org

In Brief

Mezzera et al. uncover the significance of the communication between male and female that initiates the transition from courtship to copulation. A dual function of the fruit fly ovipositor is revealed: while its extrusion is necessary for initiating copulation by the male, its retraction signals female acceptance.

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Ovipositor Extrusion Promotes the Transition from Courtship to Copulation and Signals Female Acceptance in *Drosophila melanogaster*

Cecilia Mezzera,[1] Margarida Brotas,[1] Miguel Gaspar,[1] Hania J. Pavlou,[2] Stephen F. Goodwin,[2] and Maria Luísa Vasconcelos[1,3,*]

[1]Champalimaud Center for the Unknown, Lisbon 1400-038, Portugal
[2]Centre for Neural Circuits and Behaviour, University of Oxford, Oxford OX1 3SR, UK
[3]Lead Contact
*Correspondence: maria.vasconcelos@neuro.fchampalimaud.org
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SUMMARY

Communication between male and female fruit flies during courtship is essential for successful mating, but, as with many other species, it is the female who decides whether to mate. Here, we show a novel role for ovipositor extrusion in promoting male copulation attempts in virgin and mated females and signaling acceptance in virgins. We first show that ovipositor extrusion is only displayed by sexually mature females, exclusively during courtship and in response to the male song. We identified a pair of descending neurons that controls ovipositor extrusion in mated females. Genetic silencing of the descending neurons shows that ovipositor extrusion stimulates the male to attempt copulation. A detailed behavioral analysis revealed that during courtship, the male repeatedly licks the female genitalia, independently of ovipositor extrusion, and that licking an extruded ovipositor prompts a copulation attempt. However, if the ovipositor is not subsequently retracted, copulation is prevented, as it happens with mated females. In this study, we reveal a dual function of the ovipositor: while its extrusion is necessary for initiating copulation by the male, its retraction signals female acceptance. We thus uncover the significance of the communication between male and female that initiates the transition from courtship to copulation.

INTRODUCTION

Courtship behaviors allow animals to interact and assess their qualities before they commit to reproduction. In fruit flies, as with many animal species, the male decides whether to court, and the female decides whether or not to mate with the courting male [1]. Although this decision is crucial for reproduction, little is known on how the female communicates that she is receptive to copulation. Understanding the communication between males and females leading to copulation will shed light on this decision process. During courtship, the male performs a series of behaviors directed at the female consisting of tapping, following, vibrating a wing to generate the courtship song, quivering the abdomen, and licking [2–4]. At some point, the male attempts copulation. How the transition from courtship to copulation attempt is reached has not been explored. During the male display, the female exhibits behaviors that can be interpreted as mild rejections, possibly to increase sampling time [5, 6]. These behaviors are walking away or jumping, kicking, fending, wing fluttering, and curling [5–9]. In some *Drosophila* species, female behavioral displays associated with copulation have been identified. Such examples are observed in species in which, unlike *melanogaster*, the male courts in front of the female. Such signals include spreading the wings outward and upward or opening the labellum and "kissing" the male's extended labellum [10]. In *Drosophila melanogaster*, a few behaviors, such as partial ovipositor extrusion, abdominal preening, and droplet emission, have been associated with receptivity [11]. In addition, a progressive reduction in walking speed and increase in pausing have been observed before mating [12–14]. However, a conspicuous female acceptance signal has not been identified yet. The best candidate is ovipositor extrusion, since it is reported to occur before copulation [15]. However, ovipositor extrusion is also thought to block copulation and repel the male [2, 5, 11, 16]. During ovipositor extrusion, the female pushes her vaginal plates posteriorly, which results in their spread and the formation of a temporary tube-like structure protruding from the tip of the abdomen. It is thought that the extruded member is the ovipositor. Flies extrude the ovipositor to different extents. We refer to these as partial ovipositor extrusion (also called vaginal plate spreading) and full ovipositor extrusion, even though there is a continuum between the two as opposed to a sharp distinction. It is likely that the different extents of extrusion underlie the conflicting reports that ovipositor extrusion (1) predicts copulation, (2) blocks copulation, and (3) repels the male. Without the ability to manipulate the magnitude and dynamics of ovipositor extrusion, it has been difficult to establish its role in a conclusive manner.

Recently, a collection of GAL4 driver lines that provide genetic access to subsets of descending neurons, which have their cell bodies in the brain and project to the ventral nerve cord (VNC,

analog of the vertebrate spinal cord), has opened the possibility to address this issue [17]. In this collection, we identified a pair of descending neurons that command full ovipositor extrusion, allowing us to manipulate their activity and thus control the female's behavior. Here, we dissect the male-female interaction by manipulating the female's behavior. We show that ovipositor extrusion entices the male to attempt copulation, independently of whether it is a full or a partial extrusion. The crucial difference between ovipositor extrusion of virgin and mated females is that virgin females retract the ovipositor upon a copulation attempt, thus allowing copulation, whereas mated females do not, thus blocking copulation. Moreover, by removing female hearing or the male's ability to sing, we show that ovipositor extrusion is displayed in response to song perception. On the male side, we show that the male repeatedly probes the female genitalia with the proboscis (licking) independently of ovipositor extrusion display. We then show that it is the coincidence of ovipositor extrusion and licking that stimulates copulation attempt. Together, our findings identify a sequence of steps in the male-female communication during courtship, where courtship song leads to ovipositor extrusion and licking the extruded ovipositor prompts copulation attempt, which may, if the female is receptive, lead to copulation.

Our results shed light on the transition from courtship to copulation, which represents a dramatic behavioral switch from the appetitive to the consummatory stage of sexual behavior with consequences in the reproductive success of the animals.

RESULTS

Ovipositor Extrusion Requires Sexual Maturation and Varies with Mating Status

To understand the role of ovipositor extrusion, we first characterized when and how female flies display this behavior. For this, we paired naive males with females of different reproductive capacities: (1) sexually immature virgin, (2) adult virgin, and (3) adult 15–18 h after mating. As expected, most adult virgins engage in copulation shortly after courtship starts, while both immature virgins and mated females did not mate in the 20 min of the assay (Figure 1A). The absence of mating is not due to lack of courtship, but rather depends on the female's inner state, as the three female types are all courted, albeit with a small decrease of courtship index in the case of mated females (Figure 1B). Most naive males engage in courtship shortly after they encounter the female (Figure S1A); however, due to individual variability, some have very reduced courtship index. When analyzing the responses of the female to courtship, we annotated the behavior of the fly from the start of courtship up to 5 min or until copulation. We set a threshold of 20% for the courtship index for further behavioral analyses, since below this amount less than 1 min of courtship would be sampled and ovipositor extrusion happens at low frequency. We manually annotated the display of ovipositor extrusion during courtship to calculate the ovipositor extrusion index, defined as the time spent with the ovipositor extruded during courtship over the total time of courtship. We found that both virgin and mated females perform ovipositor extrusion while immature virgins never do it, indicating that sexual maturity is required for ovipositor extrusion display (Figure 1C), as had been reported previously [5, 12, 13, 18]. It has been previously reported that females can display either a complete or a partial extrusion; the latter also referred to as vaginal plate opening [11, 13, 16]. To elucidate whether virgin and mated females display different kinds of ovipositor extrusion, we annotated separately full and partial ovipositor extrusion depending on the length of the extruded ovipositor (Figure 1D). We found that, indeed, virgin and mated females perform ovipositor extrusion differently. Virgin females preferentially display partial ovipositor extrusion while mated females display partial and full ovipositor extrusion at similar levels (Figure 1E). Though in mated females we often observed that partial ovipositor extrusion happens in transition to full ovipositor extrusion. To test this possibility, we quantified the probability of partial ovipositor extrusion at the edges of full ovipositor extrusion bouts. We found that a large proportion of full ovipositor extrusion bouts are associated with partial bouts regardless of the mating status, but this probability is significantly higher in mated females than in virgin females (Figure 1F). In the converse quantification, we found that the probability of partial ovipositor extrusion bouts being associated with full ovipositor extrusion bouts is significantly higher in mated females than in virgin females (Figure 1G). These results indicate that mated females perform more frequently mixed partial and full ovipositor extrusions than virgin females. These quantifications need to be considered when interpreting partial and full ovipositor extrusion data. Quantification of ovipositor extrusion mean bout duration overall revealed that full extrusions have longer duration than partial ones (Figure S1B). We next asked whether this behavior is a response to courtship or a response to the simple presence of a male. For this, we paired a virgin female with a male that does not court using a mutant for *fruitless* [19]. Since courtship does not occur in this case (Figure 1H), we annotated ovipositor extrusion when the animals were less than 5.5 mm apart, which is a proxy for courtship [12]. The quantification shows that ovipositor extrusion is displayed exclusively toward a courting male (Figure 1I). Similarly, the quantification of ovipositor extrusion during courtship bouts and during the moments between courtship bouts shows that, in four out of thirty females, little amount of partial ovipositor extrusion is detected (Figure S1C). These correspond to lingering extrusion once the male stopped courting.

In summary, we found that ovipositor extrusion is only displayed by sexually mature females, exclusively during courtship, and that full and partial ovipositor extrusions are displayed differently by mature females according to their mating status. Based on our observations of this behavior, we hypothesize that ovipositor extrusion plays different roles in females at different internal states.

dsx-Positive DNp13 Neurons Control Ovipositor Extrusion

To infer the role for ovipositor extrusion in mating, we manipulated its display during courtship. To this end, we started by identifying which neurons in the female brain control ovipositor extrusion. We screened a collection of splitGAL4 lines that provide genetic access to small subsets of descending neurons, which have their cell bodies in the brain and project to the VNC [17]. We activated different sets of descending neurons using optogenetics while recording the fly's behavior (Figure 2A). We used a setup with red LEDs to activate the red-shifted channelrhodopsin, Chrimson [20], expressed in the target neurons, and infrared LEDs to record the fly's behavior with an infrared camera. We identified two descending neuron fly lines that induce full ovipositor extrusion

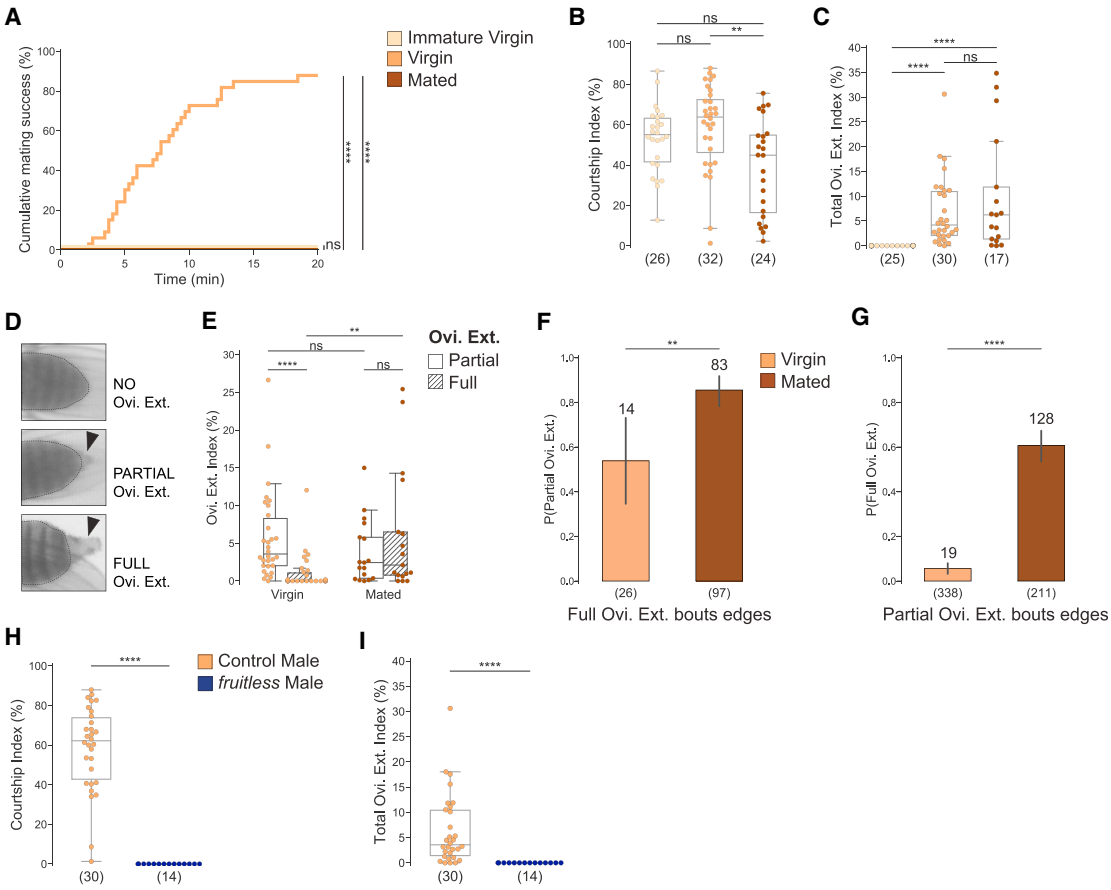

Figure 1. Female Ovipositor Extrusion Depends on Courtship and Varies with Sexual Maturity

(A) Cumulative copulation success of female flies of different stages: immature virgins, virgins, and mated females. Fisher's exact test.

(B) Male courtship index toward females of the indicated status. The courtship index is calculated as the amount of courtship happening since the start of courtship and for the next 5 min or until copulation. See also Figure S1A.

(C) Ovipositor extrusion index is shown as the ovipositor extrusion performed by the females during courtship.

(D) Images showing dorsal view of female abdomens either not extruding or performing partial ovipositor extrusion or full ovipositor extrusion. Dotted lines contour the edges of the abdomen. Black arrowheads indicate the ovipositor.

(E) Full and partial ovipositor extrusion indices of virgin and mated females are compared. See also Figure S1B.

(F) Probability of partial ovipositor extrusion at the edges of full ovipositor extrusion bouts for virgin and mated females.

(G) Probability of full ovipositor extrusion at the edges of partial ovipositor extrusion bouts for virgin and mated females.

(H) Courtship index of control and *fruitless* mutant males toward virgin females. Mann-Whitney test.

(I) Ovipositor extrusion performed by virgin females during courtship is compared to the ovipositor extrusion performed by virgin females that do not receive courtship (*fruitless* males). Mann-Whitney test. See also Figure S1C.

Boxplots: the outlines of the box represent the interquartile range and the whiskers indicate the variability outside the upper and lower quartiles. Each dot represents a fly. Unless otherwise specified, all statistical significance was acquired through Kruskall-Wallis tests, followed by pairwise comparisons using Mann-Whitney test with Bonferroni correction; ns = not significant, **$p < 0.01$, ****$p < 0.0001$. Sample size (number of flies or number of bouts) is shown in parentheses.

when activated (Figure 2B). One line labels DNp13 and DNp07 neurons (line SS01549), and the other line labels DNp13 and DNp30 neurons (line SS01581), suggesting that DNp13 neuron is responsible for full ovipositor extrusion. Moreover, activation of DNp07 neuron alone (line SS02276) does not elicit ovipositor extrusion; instead, DNp07 has been recently shown to be involved in landing behavior [21]. Altogether, the data suggest that DNp13 activation induces full ovipositor extrusion.

To describe the behavioral response of virgin female flies to the activation, we tested single flies upon a defined light stimulation protocol. We observed that about 60% of the tested flies did respond to the activation (Figure S2A). When responsive, flies display constant ovipositor extrusion bouts that match the length of the light stimulus (Figure S2B). In some cases, ovipositor extrusion persists after light-off. Most of the ovipositor extrusion generated is full; some partial ovipositor extrusion is observed at the edges of the ovipositor extrusion bouts, suggesting that it is a transition between null and full extrusion, similar to what we observed in unmanipulated mated females in response to courtship.

The anatomical characterization of line SS01549 revealed a sexually dimorphic projection within the abdominal ganglion,

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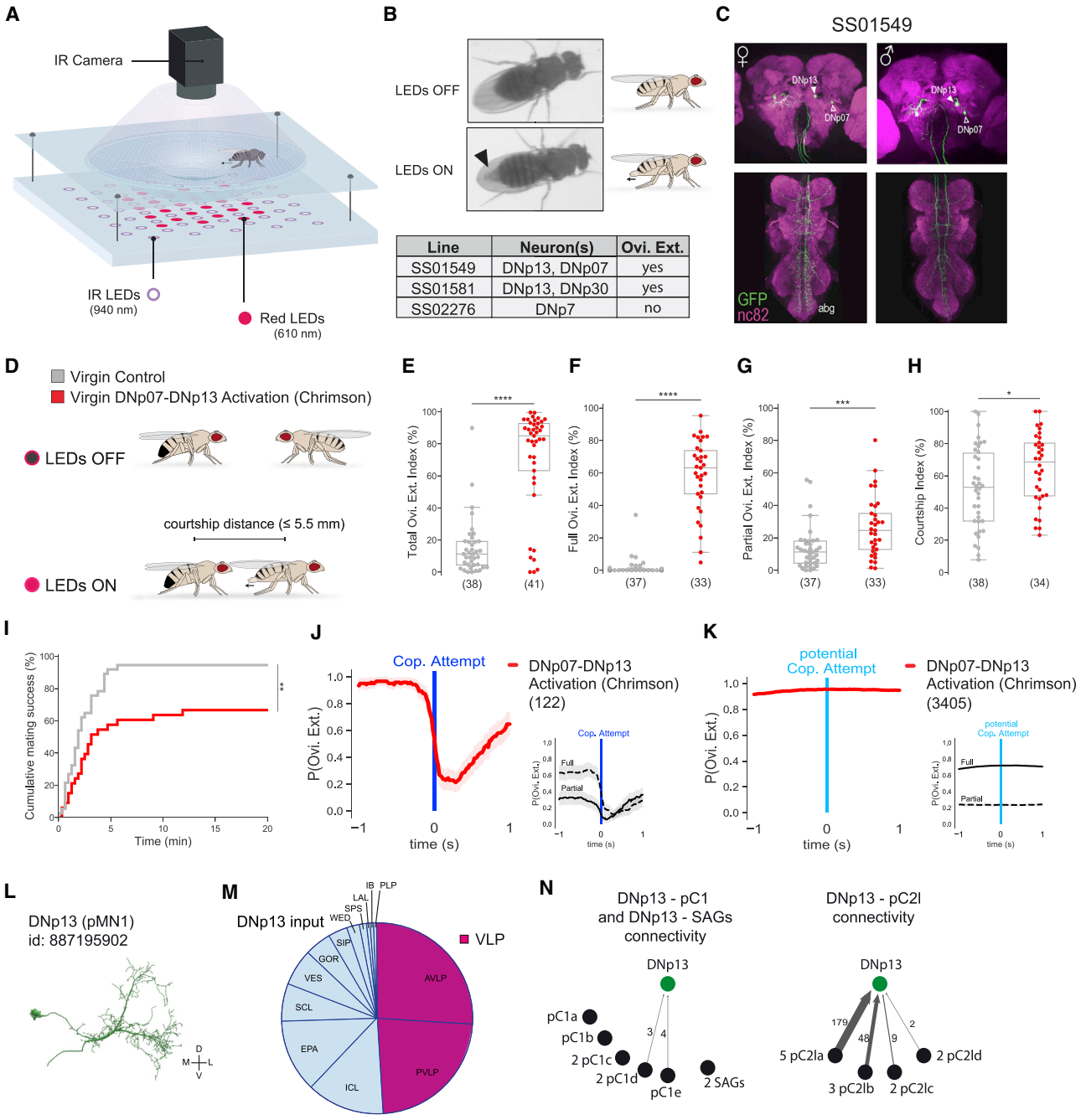

Figure 2. Inducing Ovipositor Extrusion Reduces Copulation

(A) Schematic of the behavioral setup used to screen the descending neuron collection of lines. A camera with an infrared (IR) filter recorded the females behavior upon optogenetic stimulation. A conical-shaped arena hosted one to five female virgins flies aged 4 to 8 days. Below the arena, strips of alternating IR and red LEDs are controlled through an electronic board by the software Bonsai. The IR LEDs were on for the whole duration of the video to allow recording; the red LEDs were switched on according to an arbitrary stimulation protocol.

(B) Top: images and schematic of virgin female flies showing the ovipositor extrusion behavior observed upon neuronal activation ("LEDs ON"). Black arrowhead indicates extruded ovipositor. Table: three of the tested lines and corresponding labeled neurons. In two of them, ovipositor extrusion was observed in response to the optogenetic stimulation. See also Figures S2A and S2B.

(C) Expression pattern of the line SS01549 labeling DNp13 and DNp07. The brain and ventral nerve cord (VNC) of male and female are shown from a posterior view. Empty and full arrowheads indicate DNp07 and DNp13 neurons, respectively. Neurons are labeled with anti-GFP and the tissue counterstained with nc82. abg, abdominal ganglion.

(D) Schematic of the closed-loop experiment performed in the same behavioral setup illustrated in (A). In this case, a male was paired with a female in the arena, and the red LEDs were switched on depending on the distance between the two animals, allowing stimulation of the female only when the male was at courtship distance.

(legend continued on next page)

which is the region within the VNC connected to the reproductive system. This innervation belongs to DNp13, since DNp07 does not innervate this area (Figure 2C) [21]. In brief, we found that activation of DNp13 is sufficient to induce full ovipositor extrusion, which allowed us to assess the role of ovipositor extrusion during courtship.

Inducing Full Ovipositor Extrusion May Impede Copulation

It has been proposed that full ovipositor extrusion has a repulsive role. It repels male courtship and impedes copulation [2, 5, 11, 16]. Having found DNp13, we could now investigate whether displaying full ovipositor extrusion is sufficient to change the behavior of the male and the outcome of courtship by inducing full ovipositor extrusion in virgin females. For this, we took advantage of the same setup built for the activation screen, now pairing a naive male with a female expressing *Chrimson* in DNp13 (together with DNp07). The test condition was fed all *trans*-retinal, which is necessary for Chrimson function, while the control condition was fed regular food. To restrict activation to moments of courtship, we adopted a closed-loop strategy [22], tracking in real-time the position of the two flies to turn on the light only when they were less than 5.5 mm apart (Figure 2D). By doing this, we induced ovipositor extrusion for most of the courtship bout (Figures 2E and S2C). Full ovipositor extrusion was prevalent, but partial ovipositor extrusion was also increased compared to controls (Figures 2F, 2G, and S2C). To address whether ovipositor extrusion impedes copulation, the females needed to have the ovipositor extruded for a large fraction of the courtship time. We observed that ovipositor extrusion index above 40% is distinguishable from control and that there is a discontinuity in the data points below 40% (Figure 2E). We therefore set a threshold of 40% for ovipositor extrusion index and selected pairs for further analysis according to this threshold. If full ovipositor extrusion is a rejection signal by itself, i.e., not carrying anti-aphrodisiac pheromones usually present in mated females [23–25], then male courtship would be reduced by increased amount of full ovipositor extrusion display. We observed, on the contrary, that males court retinal-fed females right after they encounter them, as they do with controls (Figure S2D), and that courtship index is slightly higher than control levels but still in the typical range for naive males (Figure 2H). The repulsive effect of full ovipositor extrusion described before [2, 11, 16] may require the association with anti-aphrodisiac pheromones that reduce courtship levels, suppress courtship initiation, and are present only in mated females [23–25]. Additionally, we cannot exclude that the increase in partial ovipositor extrusion, described to stimulate male courtship, compensates for the full ovipositor extrusion effect. However, inducing the female extrusion reduces the mating rate to 67.64%, significantly less than the 94.73% of the control (Figure 2I). This decrease might be due to either a reduced number of copulation attempts or to the fact that, when extruded, the ovipositor physically impedes copulation as postulated by Connolly and Cook [5]. We excluded the first possibility, as activated females elicit copulation attempt comparable to control ones (Figure S2E). If the ovipositor impedes copulation, we would expect a lower rate of copulation than that observed. By analyzing the probability of ovipositor extrusion before and after copulation attempt, we found that manipulated females, despite the activation, retract the ovipositor both from full and partial positions, while the male attempts to copulate (Figure 2J). We then compared the dynamics of the probability of ovipositor extrusion around copulation attempt with the probability of ovipositor extrusion when there is no copulation attempt but a potential for it (we called these moments "potential copulation attempts"). In order to define moments when copulation attempt could happen, we investigated which features characterize the second preceding copulation attempt. We found that 1 s before most copulation attempts, (1) the distance between the female and male body centers is within a range of 2–3.5 mm and (2) the flies are pausing (speed < 4 mm/s) (Figure S2F). We used distance and speed as parameters to define video frames in which the male is courting and there is potential for copulation attempt that is not fulfilled. We observed that the probability of ovipositor extrusion (both full and partial extrusion) is constant around a potential copulation attempt, in response to continuous light activation (Figure 2K). The fact that ovipositor extrusion is constant around potential copulation attempt indicates that the ovipositor retraction around copulation attempt is an active retraction that overrides the light activation. This finding suggests that the mating drive for virgin females overcomes the neuronal manipulation explaining why a significant fraction of activated females still allow copulation. Overall, our findings indicate that the ovipositor, when extruded persistently, impedes copulation. The extent to which females override the neuronal activation of DNp13, cease

(E) Quantification of the ovipositor extrusion performed by activated virgin females during courtship. Females not fed with retinal were used as controls. See also Figure S2C.
(F and G) Full (F) and partial (G) ovipositor extrusion performed by activated and control virgin females during courtship.
(H) Male courtship index toward activated and control females. See also Figure S2D.
(I) Cumulative copulation success of activated and control females. Fisher's exact test.
(J and K) Probability of activated females displaying ovipositor extrusion around copulation attempt (J) and around potential copulation attempt (K). Insets show the contributions of full and partial ovipositor extrusions to the probabilities. Shaded area represents the standard error of the mean. See also Figures S2E and S2F.
(L) Electron microscopy reconstruction of DNp13 neuron. Dorsoventral (DL) and mediolateral (ML) axes are indicated.
(M) Cake diagram showing the distribution of DNp13 inputs in the hemibrain, mostly located in the anterior and posterior ventrolateral protocerebrum (AVLP and PVLP), highlighted in magenta. ICL, inferior clamp; EPA, epaulette; SCL, superior clamp; VES, vest; GOR, gorget; SIP, superior intermediate protocerebrum; WED, wedge; SPS, superior posterior slope; LAL, lateral accessory lobe; IB, inferior bridge; PLP, posterior lateral protocerebrum.
(N) Connectivity graphs showing the connections of DNp13 neuron with pC1 and SAGs neurons (left) and with PC2l neurons (right). Synaptic connections are represented by arrows between the indicated neurons, and their strength is represented by the thickness of the line and indicated by the number of connections.
Boxplots: the outlines of the box represent the interquartile range and the whiskers indicate the variability outside the upper and lower quartiles. Each dot represents a fly. Unless otherwise specified, all statistical significance was acquired through Kruskall-Wallis tests, followed by pairwise comparisons using Mann-Whitney test with Bonferroni correction; ns = not significant, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Sample size (number of flies or copulation attempts/potential copulation attempts) is shown in parentheses.

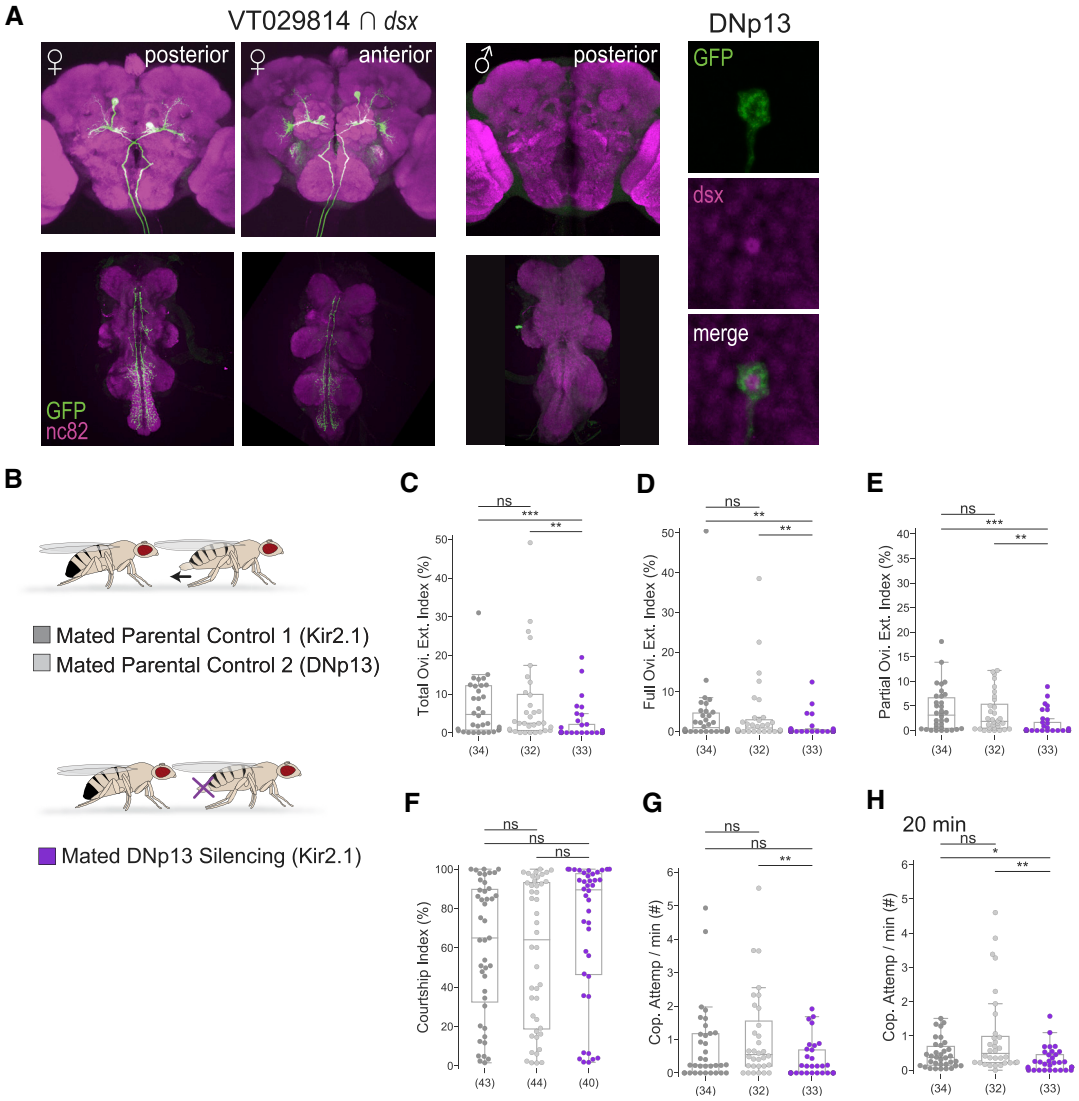

Figure 3. Ovipositor Extrusion Stimulates Male Copulation Attempt

(A) Left: brain and VNC of female (anterior and posterior views) and male (posterior view) showing the expression pattern of the line VT029814 intersected with *dsx*. Neurons are labeled with anti-GFP and the tissue counterstained with nc82. Right: DNp13 cell body from a female brain labeled with anti-GFP and anti-Dsx. See also Figure S3A.

(B) Schematic of the inhibition experiment: Kir2.1 was used to silence DNp13 in mated females, and the two parental lines were used as controls.

(C) Quantification of the ovipositor extrusion performed by silenced and control mated females during courtship.

(D and E) Full (D) and partial (E) ovipositor extrusion performed by silenced and control mated females during courtship.

(F) Male courtship index toward silenced and control mated females. See also Figures S3C and S3D.

(G) Number of copulation attempt events counted in the 5 min for silenced and control females.

(H) Number of total copulation attempt events counted during the whole video for silenced and control females.

Boxplots: the outlines of the box represent the interquartile range and the whiskers indicate the variability outside the upper and lower quartiles. Each dot represents a fly. All statistical significance was acquired through Kruskall-Wallis tests, followed by pairwise comparisons using Mann-Whitney test with Bonferroni correction; ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001. Sample size (number of flies) is shown in parentheses. See also Figure S3.

to fully extrude, and allow copulation may be dependent on the mating drive of the female.

Given that DNp13 is involved in a female courtship response, we wondered how it is connected to the known receptivity circuit. For this, we used newly released tools to identify synaptic partners based on electron microscopy data of the hemibrain of an adult female fly [26, 27]. We searched for the DNp13 neuron in the *neu*Print web interface and we visualized the neuron and the regions where it connects to other neurons in the brain (Figures 2L and 2M). We found that DNp13 has 2,554 inputs and 18 outputs in the brain. 45% of the input connections are located in the anterior and posterior ventrolateral protocerebrum (VLP) (Figure 2M), a region receiving higher-order auditory inputs [28–30]. We then investigated specific candidate DNp13

partners. We found that SAG neurons, carrying mating information from the VNC to the brain [31], do not directly connect to DNp13, while the pC1 cluster, involved in female receptivity and responsive to courtship song [32], was poorly connected with a few synaptic connections with pC1d and pC1e (Figure 2N, left). Whether these are the neurons within the cluster responding to the courtship song needs to be determined. In contrast, the pC2l cluster, involved in ovipositor extrusion [18] and responsive to song [33], has different subtypes highly connected to DNp13 (Figure 2N, right). This initial, though possibly incomplete, analysis of DNp13 connectivity indicates that the pC2l cluster is an entry point for future studies of ovipositor extrusion circuit.

Ovipositor Extrusion Is Associated with Male Copulation Attempt

Given that the DNp13 projections are dimorphic, we asked whether DNp13 co-expressed the sexual determination transcription factors *doublesex* (*dsx*) and *fruitless* (*fru*). We followed an intersectional approach using splitGAL4 with *dsx* promoter and FLP recombinase with *fru* promoter, which revealed that, in the female, DNp13 is *dsx*-positive and *fru*-negative (Figures 3A and S3A, respectively). Male DNp13 is not labeled with the *dsx* intersection, suggesting that it has a different genetic specification from the female neuron. We confirmed that DNp13 in the female is *dsx*-positive with Dsx immunostaining (Figure 3A, right panels). We reasoned that since DNp13 activation induces ovipositor extrusion, silencing DNp13 neurons may suppress ovipositor extrusion displays. To genetically access DNp13 specifically, we took advantage of the intersection with *dsx,* which labels only this pair of neurons. In DNp13 neurons, we expressed the inwardly-rectifier potassium channel *Kir2.1* that hyperpolarizes neurons, thus preventing action potentials [34]. We paired mated females with naive males and used parental lines as controls (Figure 3B). The neuronal manipulation was indeed able to suppress ovipositor extrusion with a reduction of both full and partial ovipositor extrusion, which is expected, since in mated flies, partial ovipositor extrusion is associated with full ovipositor extrusion bouts (Figures 3C–3E). The same manipulation did not affect the ovipositor extrusion performance in virgin flies (Figures S3B–S3E). We speculate that a different set of descending neurons controls ovipositor extrusion in virgins. Given that the ovipositor is extruded when females lay an egg, albeit in a very different posture from courtship ovipositor extrusion (Figure S3F), we investigated the requirement of DNp13 activity for egg laying. We showed that DNp13 silencing in mated females does not reduce the number of eggs laid (Figure S3G), indicating that ovipositor extrusion and oviposition are mediated by different mechanisms controlled by different command neurons, as previously reported [18]. We also examined, using *neu*Print, DNp13 connectivity with the recently identified egg-laying descending neurons (oviDNs) [35] and found no connections (data not shown).

Next, we silenced DNp13 to assess the effect of ovipositor extrusion suppression on copulation and courtship of mated females. Showing unwillingness to mate involves more than a single motor response. For instance, the females may walk or fly away, flutter the wings, or kick the male. Moreover, it is unlikely that removing a motor response changes the receptivity state of the female. If so, ovipositor extrusion suppression alone would not restore copulation of mated females. Indeed, we observed that mated females with suppressed ovipositor extrusion did not copulate in 20 min (Figure S3H). Courtship is also not affected by the ovipositor extrusion reduction, as courtship index and latency to courtship in silenced mated females do not differ from controls (Figures 3F and S3I), suggesting that, in our experimental conditions, full ovipositor extrusion does not repel the male. However, when ovipositor extrusion was suppressed, copulation attempt appeared reduced in the 5-min analysis, although significantly different only from one parental control (Figure 3G). To confirm this result and check whether this reduction persists over time, we annotated and analyzed the whole 20-min video duration. Indeed, we found that males attempt copulation significantly less with silenced mated females compared to controls (Figure 3H). This result indicates that female ovipositor extrusion prompts male copulation attempt in mated females. Whether this is also true for virgins will be addressed with a different approach in the following section.

Ovipositor Extrusion Is a Response to Male Courtship Song

To better understand ovipositor extrusion display during courtship, we investigated which male behavior elicits it. During manual annotation of the behavior, we noticed that ovipositor extrusion may be a response to male courtship song. To test this, we generated two conditions in which the courtship song component is eliminated. In the first, we removed the wings of the male (wingless), thus preventing song generation. In the second, we removed the aristae of the virgin female (aristaless), preventing song reception. The controls were intact flies of the same genotype (Figure 4A, first three panels). The courtship song is fundamental for copulation as either removal of male wings or removal of female aristae reduces copulation rate and increase its latency [13, 36–40]. We also observed that copulation is dramatically reduced (Figure S4A). We found that in the first 5 min, courtship index was very low for wingless condition (Figure S4B), and since a persistent courtship is a prerequisite to measure ovipositor extrusion, for this experiment we analyzed the parameters in the last 5 min before the end of the video or before copulation. In this case, males court at high levels, even more than in controls (Figure 4B), allowing the study of the effect of courtship song elimination on ovipositor extrusion. We found a significant reduction of ovipositor extrusion in the absence of courtship song or hearing (Figures 4C–4E), indicating that ovipositor extrusion (both full and partial) depends on courtship song perception. In mated females, the relationship between ovipositor extrusion and courtship song is less evident. In the last 5 min of the video, there is no significant difference in ovipositor extrusion index between wingless condition and control, being low in both conditions compared to the overall control measurements in this work (Figures S4C and S4D). This precludes a conclusion about the effect of song removal on ovipositor extrusion display. We analyzed the earliest time point when the male courtship is vigorous, from min 5 to 10. In this case, we observed regular levels of ovipositor extrusion in control females and a reduction in full ovipositor extrusion in females courted by wingless males (Figures S4E and S4F). These results suggest that song plays a role in stimulating ovipositor extrusion in mated females as well as in virgin females.

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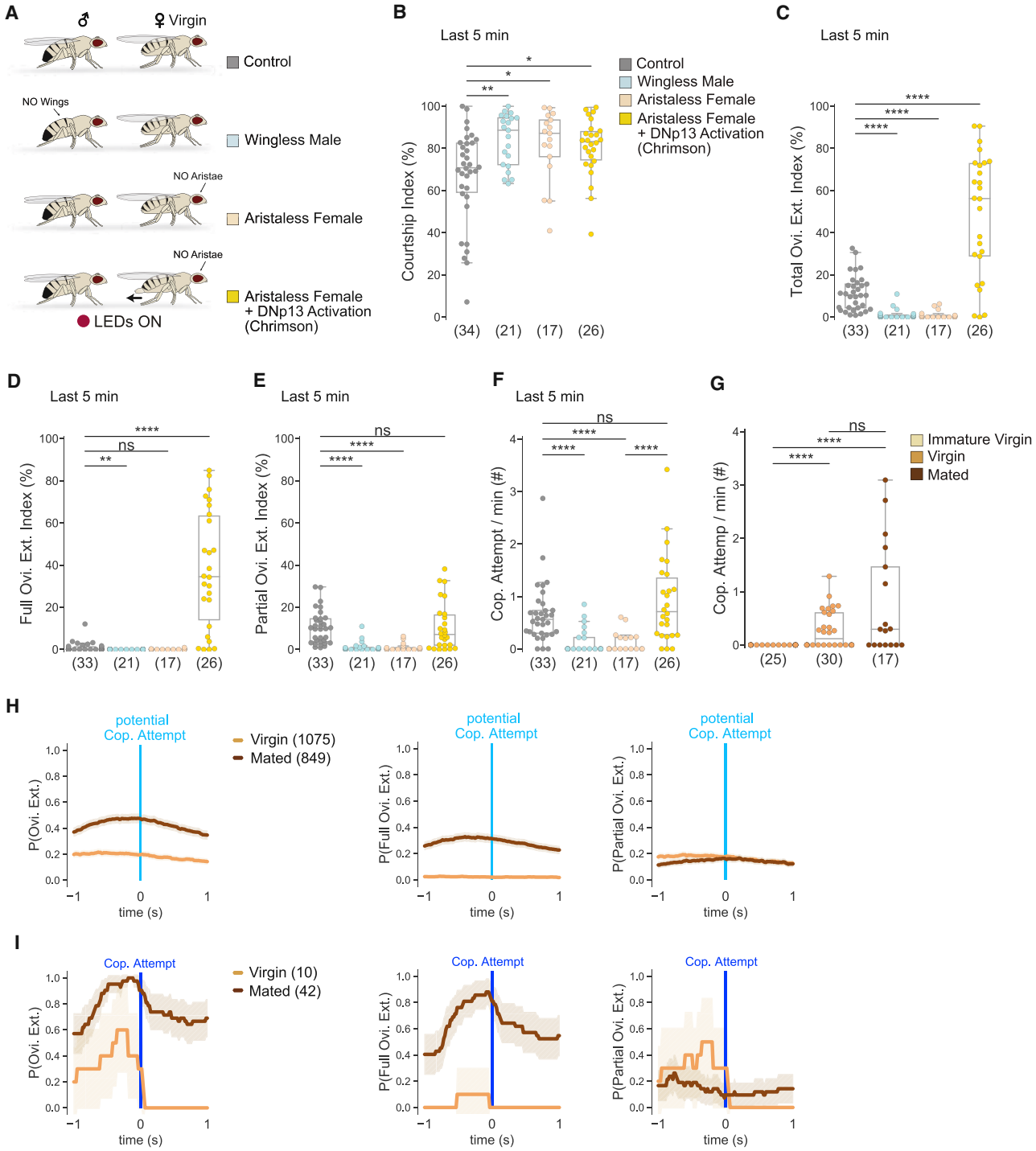

Figure 4. Ovipositor Extrusion Is a Response to Male Courtship Song and Precedes Copulation Attempt

(A) Schematic of the conditions used in the experiment: the male and female are indicated with the corresponding manipulation. Females are virgins in all conditions.

(B) Male courtship index in the last 5 min of the video in the control and different test conditions indicated in (A). See also Figure S4B.

(C) Quantification of total ovipositor extrusion performed by the females during courtship for all the conditions.

(D and E) Full (D) and partial (E) ovipositor extrusion performed by the females during courtship for all the conditions.

(F) Number of copulation attempt events per minute of courtship for all the conditions.

(G) Number of copulation attempt events per minute of courtship for females at different states.

(legend continued on next page)

Interestingly, when ovipositor extrusion was reduced, we found a significant reduction of male copulation attempt, both in virgin and mated females (Figures 4F and S4G), in line with our previous results with mated females upon silencing DNp13 (Figure 3H). This indicates that ovipositor extrusion stimulates copulation attempt also in virgin females. To further demonstrate copulation attempt-ovipositor extrusion dependency, we tested whether restoring ovipositor extrusion would restore male copulation attempt. We therefore optogenetically activated DNp13 neurons in aristaless virgins, which resulted in a very high ovipositor extrusion index, particularly full ovipositor extrusion. Partial ovipositor extrusion was similar to control levels (Figure 4A, bottom panel, and Figures 4C–4E). Indeed, artificially induced ovipositor extrusion can restore male copulation attempt to the copulation attempt levels of intact pairs (Figure 4F). Restoring copulation attempt did not restore copulation, indicating that reduced copulation is due to lack of female receptivity rather than the absence of attempts by the male (Figure S4A). These results show that not only mated but also virgin females use ovipositor extrusion to prompt copulation attempt. Earlier, we showed that immature virgins naturally do not perform ovipositor extrusion when courted (Figure 1C). We asked whether copulation attempt levels in immature virgins differ compared to mature females. We found that males do not attempt to copulate immature virgins, while copulation attempt is performed toward both virgin and mated (Figure 4G). Altogether, this set of experiments shows that ovipositor extrusion display depends on courtship song perception. Additionally, they reinforce and extend to virgin females the finding that ovipositor extrusion prompts copulation attempt.

Female Ovipositor Extrusion Precedes Male Copulation Attempt

Having established that copulation attempt depends on ovipositor extrusion, we set out to characterize the temporal dynamics of these two events. For this, we analyzed the probability of ovipositor extrusion around copulation attempt or potential copulation attempt. This analysis revealed that the probability of ovipositor extrusion usually is low when the male is courting close to the female, both for virgin and mated females (Figure 4H). In contrast, before copulation attempt, these probabilities are high (Figure 4I). For mated females, the probability of ovipositor extrusion drastically increases around 0.5 s before copulation attempt and remains high after copulation attempt. This result is consistent with an observation briefly mentioned in an early study that ovipositor extrusion is followed by copulation attempt in mated females [5]. We observe that full ovipositor extrusion is the main contribution to this probability. For virgins, the probability of ovipositor extrusion increases around 0.5 s before copulation attempt, but decreases immediately before copulation attempt. In this case, partial ovipositor extrusion is the main contribution to this probability. The exact timing of ovipositor retraction is uncertain since, as the male is about to attempt, he might reduce visibility of partial extrusion, and we only annotated what we clearly saw. As the attempt ends, visibility is restored. In the case of mated females, the ovipositor is usually visible when the male is attempting copulation because it is often fully extruded. These results show that ovipositor extrusion from both virgin and mated females precedes copulation attempt, but the outcomes are different. In mated females, ovipositor extrusion persists past the copulation attempt, which prevents copulation. In virgins, the ovipositor is retracted before the end of the attempt, eventually allowing copulation to happen.

Female Ovipositor Extrusion and Male Licking, Together, Prompt Copulation Attempt

If ovipositor extrusion alone was sufficient to trigger copulation attempt, we would expect to see that whenever ovipositor extrusion is increased upon neuronal activation, copulation attempt is increased to the same extent. However, this manipulation does not lead to increased copulation attempt past the control levels (Figures 4F and S2E). We hypothesized that ovipositor extrusion requires a second element to trigger copulation attempt. The male extends the proboscis to touch the female genitalia before the proper act of copulation attempt. We therefore annotated licking behavior defined as the male proboscis contacting female genitalia, as represented in Figure 5A. The analysis of licking behavior revealed that the probability of licking has a high peak immediately before copulation attempt (Figure 5B). In contrast, the probability of licking around potential copulation attempt is close to zero for both virgin and mated females (Figure 5C). This analysis shows that licking, as well as ovipositor extrusion, precedes copulation attempt. However, the temporal progression of licking and ovipositor extrusion differs. Licking is a short behavior that starts and ends immediately before copulation attempt. Ovipositor extrusion has a variable length and usually starts before licking (Figure 5D). This suggests that male licking is a response to female ovipositor extrusion. To investigate whether this is the case, we quantified licking toward females that display different levels of ovipositor extrusion: control ovipositor extrusion levels, low ovipositor extrusion levels in wingless and aristaless conditions, and high ovipositor extrusion levels in DNp13-activated aristaless females. We found that in all cases, licking levels were not altered (Figure 5E), showing that despite the temporal association between them, licking does not depend on ovipositor extrusion. Is there a significance to the fact that they are temporally associated just before copulation attempt? We hypothesize that male copulation attempt requires not the presence of ovipositor extrusion alone, but rather of both ovipositor extrusion and licking together. To address this question, we calculated the probability of an attempt, i.e., a copulation attempt or a successful attempt (copulation), when preceded by ovipositor extrusion alone, licking alone, or ovipositor extrusion and licking together in unmanipulated wild-type

(H and I) Probability of ovipositor extrusion around potential copulation attempt (H) and around copulation attempt (I) for virgin and mated females. Shaded areas represent the standard errors of the mean.

Boxplots: the outlines of the box represent the interquartile range and the whiskers indicate the variability outside the upper and lower quartiles. Each dot represents a fly. All statistical significance was acquired through Kruskall-Wallis tests, followed by pairwise comparisons using Mann-Whitney test with Bonferroni correction; ns = not significant, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Sample size (number of flies or copulation attempts/potential copulation attempts) is shown in parentheses. See also Figure S4.

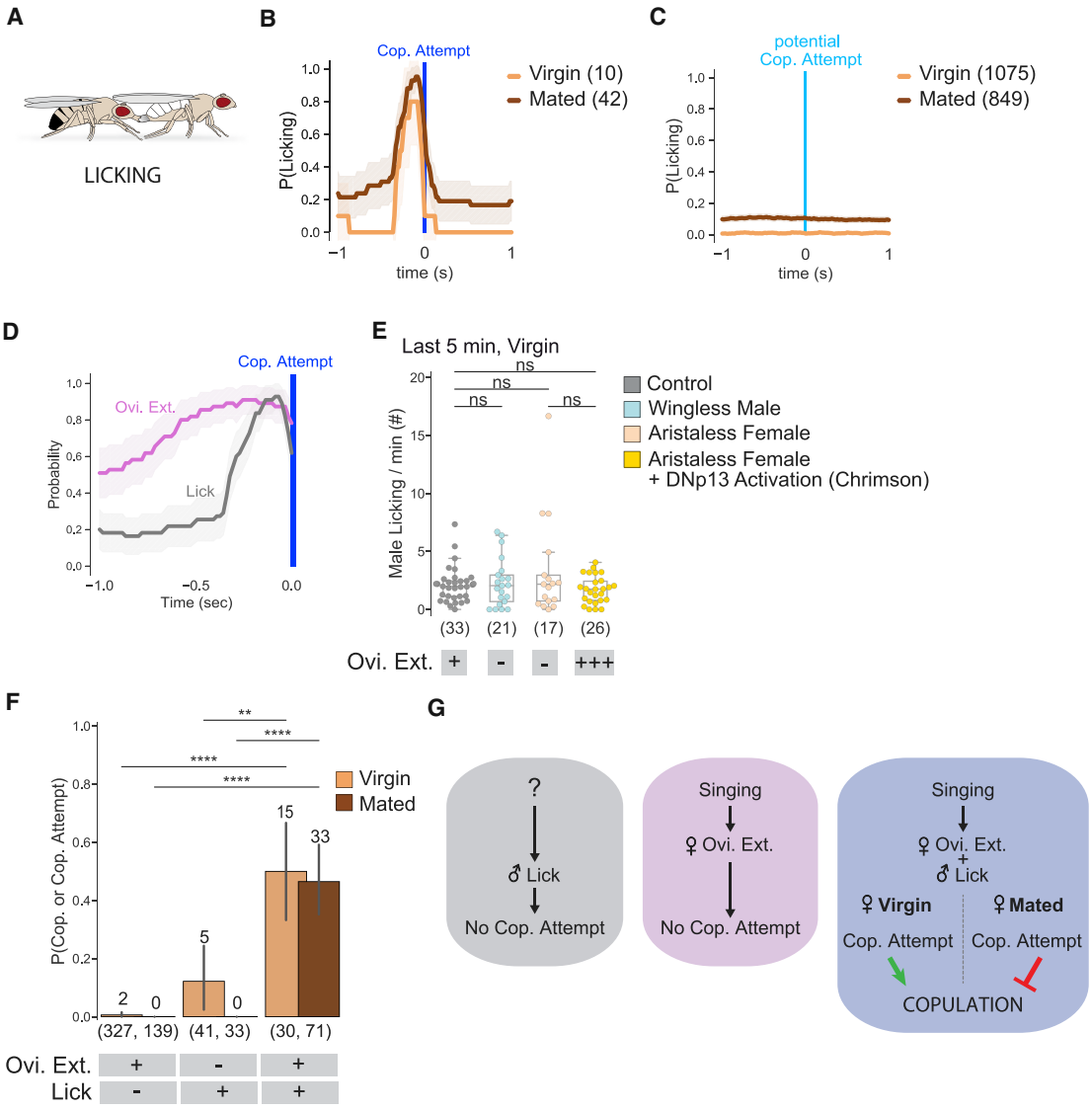

Figure 5. Licking and Ovipositor Extrusion Together Prompt Male Copulation Attempt

(A) Schematic drawing representing a male extending the proboscis and licking a female extruded ovipositor.

(B and C) Probability of licking around copulation attempt (B) and around potential copulation attempt (C) for virgin and mated females. Shaded areas represent the standard errors of the mean.

(D) Probabilities of ovipositor extrusion and licking before copulation attempt. Shaded areas represent the standard errors of the mean.

(E) Number of licking events toward virgin females displaying different ovipositor extrusion levels.

(F) Probability that copulation attempt or copulation is preceded by ovipositor extrusion alone, licking alone, or ovipositor extrusion and licking together for virgin and mated females. The number of total ovipositor extrusion alone, licking alone, and ovipositor extrusion together with licking events is indicated in parentheses below each condition. The number of events followed by copulation or copulation attempt is indicated on top of each condition. Number of copulations = 28 (virgin females); number of copulation attempts = 52 (10 for virgin females and 42 for mated females). Number of videos used for these calculations: n = 24 mated females and n = 32 virgin females. The number of total events is indicated in parentheses below each condition; the number of positive events is indicated on top of each condition. Number of copulations = 28; number of copulation attempts = 52 (10 for virgin females and 42 for mated females). Number of flies used for these calculations: n = 24 mated females and n = 32 virgin females. Error bars represent the bootstrapped SEM (n boot = 1,000).

(G) Schematic model representing the interactions between male and female involved in the transition from courtship to copulation.

Boxplots: the outlines of the box represent the interquartile range and the whiskers indicate the variability outside the upper and lower quartiles. Each dot represents a fly. All statistical significance was acquired through Kruskall-Wallis tests, followed by pairwise comparisons using Mann-Whitney test with Bonferroni correction; ns = not significant, **p < 0.01, ****p < 0.0001. Sample size (number of copulation attempts/potential copulation attempts, number of flies or number of events) is shown in parentheses.

females. This analysis revealed that ovipositor extrusion alone is not sufficient to induce copulation or copulation attempt. In the case of licking, we did observe a small probability of copulation attempt events for virgin females. However, when licking was observed along with ovipositor extrusion, these probabilities are considerably higher and significantly different from the single events alone for both virgin and mated (Figure 5F). These results indicate that licking an extruded ovipositor sways the male toward a copulation attempt.

DISCUSSION

The transition from courtship to copulation is a critical moment for the reproductive success of animals. The exact steps leading to this transition in any species remain largely uncharacterized [41]. In our work, we showed that in fruit flies, male licking and female ovipositor extrusion are involved in this transition (schema in Figure 5G). An important feature of this interaction is that the female mating status determines whether this transition is complete. We observed that virgin females retract the ovipositor upon a male's attempt, thus allowing copulation, whereas mated females do not, thus blocking copulation.

Virgin and mated females use a variation of the same behavior to stimulate and prevent copulation, respectively. This variation requires different descending neurons probably acting on common circuits. This could be a versatile and economic strategy to mediate opposite responses when the same individual uses one or the other variation depending on the circumstances.

Why would a mated female signal the male to attempt copulation while blocking intromission? In circumstances that we have not addressed in this study, mated females re-mate. For a few hours after mating, and given the appropriate context, mated females will eject the sperm and re-mate in an attempt to increase fecundity and offspring genetic diversity [25, 42]. In this case, prompting the male to attempt copulation makes sense, as it could lead to copulation. An additional role for full ovipositor extrusion in mated females, which we have not explored here, may be in announcing the female's current pheromonal composition. Ovipositor extrusion would be an efficient way of exposing the anti-aphrodisiac pheromones 3-O-acetyl-1,3-dihydroxyoctacosa-11,19-diene [24] present in the tip of the ovipositor and cis-vaccenyl acetate, mostly in the reproductive system [25, 43], which, together with 7-tricosene [23] present in the cuticle, indicate to an approaching male that the female has mated, and, depending on the combination and intensity of the chemical cues, the male may or may not initiate courtship.

In this work, we identified a pair of descending neurons that control full ovipositor extrusion. Full ovipositor extrusion can be induced in the virgin female by DNp13 activation, but activity in these neurons is not necessary for ovipositor extrusion in virgins. Although full and partial ovipositor extrusions do not have a sharp distinction, ovipositor extrusion is commanded by different neurons and controlled differently in virgin and mated flies. It remains to be elucidated which are the descending neurons controlling virgin ovipositor extrusion and how they interact with DNp13 to control similar behavior in females in different mating states.

Our work shows that, during the interaction between the sexes, the female responds to the male courtship song with ovipositor extrusion. However, it is apparent in the videos that song does not always lead to ovipositor extrusion. This response pattern suggests that ovipositor extrusion is not a reflexive reaction to courtship song, but rather arises from a temporal or multimodal integration. Further experiments are required to elucidate the nature of this association. How does the male verify that the song was heard? Our results indicate that the male is sampling the female genitalia with the proboscis throughout courtship. Presumably, licking is intended to probe the chemical landscape of the female genitalia, which is likely to change when the ovipositor is extruded; in this way, the male could sense when the female is responding to the song. We show that licking of the ovipositor elicits a copulation attempt. In line with early suggestions that compounds are released during ovipositor extrusion to stimulate the male [11], we speculate that a chemical compound is presented with the ovipositor by the female and sensed by taste neurons on the male proboscis. A gustatory signal, yet unidentified and common to virgin and mated females, would stimulate the male to attempt copulation. Having established that licking an extruded ovipositor is the starting point for the male to attempt copulation allows us to use the same starting point to study how the transition from courtship to copulation is processed in the male brain.

In conclusion, our work highlights how both sexes contribute to continuous communication during courtship that culminates in copulation attempt gated by the female ovipositor extrusion. Moreover, our findings open new avenues of study of the neuronal regulation of behaviors that lead to the transition from courtship to copulation and how this transition regulates neuronal activity.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.06.071.

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AUTHOR CONTRIBUTIONS

C.M. and M.L.V. conceived the project, designed the experiments, discussed the results, and wrote the manuscript. M.L.V. provided supervision. H.J.P. and S.F.G. generated dsx^{DBD}. C.M. performed the experiments and annotations with the support of M.B. C.M. analyzed the data with the support of M.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit anti-GFP	Molecular Probes	Cat# A11122; RRID:AB_221569
mouse anti-dsx	Developmental Studies Hybridoma Bank	Cat#AB_2617197; RRID:AB_2568832
mouse anti-bruchpilot (nc82)	Developmental Studies Hybridoma Bank	Cat#AB_2314866; RRID:AB_2314866
goat anti-rabbit AlexaFluor 488	Invitrogen	Cat# A11034; RRID:AB_2576217
goat anti-mouse AlexaFluor 594	Invitrogen	Cat# A11032; RRID:AB_2534091
Chemicals, Peptides, and Recombinant Proteins		
All trans-retinal	Sigma-Aldrich	Cat# R2500
VectaShield	Vector Laboratories	Cat# H1000; RRID:AB_2336789
Experimental Models: Organisms/Strains		
Canton-s (CS)	Lab stock	N/A
VT029814-p65ADZp(attP40); VT003280-ZpGDBD(attP2)	[17]	SS01549 (RRID:BDSC_75952)
VT008142-p65ADZp(attP40); VT046808-ZpGDBD(attP2)	[17]	SS01581 (RRID:BDSC_75904)
VT029814-p65ADZp(attP40); VT047755-ZpGDBD(attP2)	[17]	SS02276 (RRID:BDSC_75934)
+; +; UAS-Chrimson, mVenus	[20]	RRID:BDSC_55136
w*; UAS-Kir2.1; +	[34]	RRID:BDSC_6595
UAS-CD8-GFP; dsxDBD	[44]	N/A
FruLexA	[45]	N/A
LexAopFLP	[46]	N/A
UAS > STOP > CD8-GFP	[47]	N/A
fruGAL4	[19]	N/A
Software and Algorithms		
Caltech FlyTracker	[48]	N/A
JAABA	[49]	N/A
Bonsai	Bonsai website [22]	N/A
Python VideoAnnotator	Python VideoAnnotator website	N/A
Python	Python website	N/A
Fiji	Fiji website [50]	N/A

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Maria Luisa Vasconcelos (maria.vasconcelos@neuro.fchampalimaud.org).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Raw data supporting the current study have not been deposited in a public repository because of their large size but are available on request from the corresponding author.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fruit flies *D. melanogaster* were raised in standard cornmeal-agar medium, using Vienna food recipe (In 1 Liter of water: 80 g molasses-barley malt, 22 g beet syrup, 80 g corn flour, 18 g granulated yeast, 10 g soy flour, 8 g agar-agar, 8 mL proprionic acid, 12 mL 15% nipagin, 35 mL Bavistin), at 25°C and 70% relative humidity in a 12 h dark:12 h light cycle. Detailed information on fly

genotypes, housing and age for each experiment are indicated in the relevant Method Details. For strain details please see Key Resource Table.

METHOD DETAILS

Fly genotypes

The detailed genotypes per figure are as follows:

Males are always Canton-S (CS) except for 'fruitless', fru^{GAL4} in Figures 1D and 1E.

Figure 1:

w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}, *20xUAS-CsChrimson-mVenus(attp2)/+*

Figure 2:

w^-;VT029814-p65ADZp(attP40)/+;VT003280-ZpGDBD(attP2)/20xUAS-CsChrimson-mVenus(attp2)
Except for distance and speed plots which were calculated with flies from Figure 1

Figure 3:

Parental control 1: w^-;+;UAS-Kir2.1/+
Parental control 2: w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}/+
DNp13 silencing: w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}/ *UAS-Kir2.1*

Figure 4:

w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}, *20xUAS-CsChrimson-mVenus(attp2)/+*

Figure 5:

w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}, *20xUAS-CsChrimson-mVenus(attp2)/+*

Figure S1:

w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}, *20xUAS-CsChrimson-mVenus(attp2)/+*

Figure S2:

w^-;VT029814-p65ADZp(attP40)/+;VT003280-ZpGDBD(attP2)/20xUAS-CsChrimson-mVenus(attp2)
VT029814-p65ADZp(attP40)/UAS > STOP > CD8-GFP;VT003280-ZpGDBD(attP2)/Fru^{LexA}, *LexAopFLP*

Figure S3:

w^-;+;UAS-Kir2.1/+
w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}/+
w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}/ *UAS-Kir2.1*

Figure S4:

w^-;VT029814-p65ADZp(attP40) /+; dsx^{DBD}, *20xUAS-CsChrimson-mVenus(attp2)/+*

Immunostaining

Adult brains and VNCs were dissected in cold PBS and immediately transferred to cold PFA 4% in PBL (PBS with 0.12 M Lysine) and fixed for 30 min at RT, washed three times for 5 min in PBT (PBS with 0.5% Triton X-100) and blocked for 30 min at RT in NGS (Sigma) 10% in PBT. Primary antibodies were incubated for 72 h at 4°C. The following primary antibodies were used: rabbit anti-GFP 1:2000 (Molecular Probes, Cat#A11122), mouse anti-bruchpilot (nc82) 1:10 (Developmental Studies Hybridoma Bank, Cat#AB_2314866), anti-dsx (Developmental Studies Hybridoma Bank, Cat#AB_2617197). Samples were washed three times for 5 min in PBT and incubated in Alexa Fluor secondary antibodies 1:500 (Invitrogen) for 72 h at 4°C. Samples were washed three times for 5 min in PBT and mounted in VectaShield medium (Vector Laboratories, Cat#H1000). Images were captured on a Zeiss LSM 710 confocal microscope using a 25X immersion objective (Zeiss). After acquisition, color levels were adjusted using Fiji [50] for optimal display.

Connectome data analysis

DNp13 neuron was reconstructed from the hemibrain Electron Microscopy dataset of an adult female fly [27]. The *neu*Print framework, a database and web interface that allows to query specific neurons and their connections [26] was used to visualize DNp13

neuron in the right hemibrain and to identify the brain area distribution of its inputs. The connectivity graph was generated to display the existing synaptic connections between DNp13 and other neurons, listed in the following table.

Neuron	id	Alternative nomenclature
DNp13	887195902	DNp13(pMN1)_R
pC1a	5813046951	pC1a(PDM09)_R
pC1b	267214250	pC1b(PDM09)_R
pC1c	267551639	pC1c(PDM09)_R
pC1c	550319575	pC1c
pC1d	5813046951	pC1d(PDM09)_R
pC1d	5813063587	pC1d(PDM09)_R
pC1e	514850616	pC1e(PDM09)_R
pC2la	1135160387	PVL18a_pct(pC2la)(PVL18)_R
pC2la	1260655256	PVL18a_pct(pC2la)(PVL18)_R
pC2la	1167882816	PVL18a_pct(pC2la)(PVL18)_R
pC2la	1351674644	PVL18a_pct(pC2la)(PVL18)_R
pC2la	5813067886	PVL18a_pct
pC2lb	1075443614	PVL18a_pct(pC2lb)(PVL18)_R
pC2lb	1292022825	PVL18a_pct(pC2lb)(PVL18)_R
pC2lb	1351679002	PVL18a_pct(pC2lb)(PVL18)_R
pC2lc	642629827	PVL18a_pct(pC2lc)(PVL18)_R
pC2lc	1044071833	PVL18a_pct(pC2lc)(PVL18)_R
pC2ld	642629807	PVL18a_pct(pC2ld)(PVL18)_R
pC2ld	917553030	PVL18ola_pct
SAG	517587356	SAG(ADM09)
SAG	5812981862	SAG(ADM09)
oviDNa	550655668	-
oviDNb	519949044	-

Behavioral experiments

For all experiments, both males and females were collected soon after eclosion and kept in isolation at 25°C and 70% relative humidity until the experiment. Males were aged 4-8 days. Immature virgins were aged for 3 h, adult females were aged 4-8 days. For the closed loop experiment, single females were tested for ovipositor extrusion before each experiment. Flies that were not extruding the ovipositor in the first 20 s of activation were discarded. For mated female condition, females were removed from isolation and paired with 1-3 males 15-18 h before the experiment. If the vials where the mated females were kept had not progeny a few days later, the experiment was discarded. All experiments were performed at 25°C and 70% relative humidity, in dim light. For neuronal activation with optogenetics, virgin females were transferred to cornmeal-agar Vienna food containing 0.2 mM all trans-Retinal (Sigma, R2500) and reared in dim light until the experiment.

Activation screen

From the collection of lines labeling descending neurons [17] we selected one line for each descending neuron and tested around 60 lines in an optogenetic activation set-up.

Five females were gently aspirated and transferred to a custom-made circular arena with a conical-shaped bottom to avoid flies walking on the walls, as previously described [12, 51]. The arena is made of white Delrin with 11° sloped walls and 4 mm of height at the center. Flies are able to walk in a circle of ~3 cm diameter. The arena is topped with a lid made of plexiglass.

Movies were recorded at 60 frames per second with a camera mounted above the arena (PointGrey FL3-U3-32S2M-CS with a 5 mm fixed focal length lens (Edmund Optics)). Movies were acquired in dim light using a 940 nm LED strip (SOLAROX) and a Hoya 49 nm R72 infrared filter. Flies were recorded in grayscale (1021 × 1024 pixels, 60 frames per second). High-powered 610 nm LEDs were interspersed between the infrared LEDs on the backlight board. The arena was irradiated with a power in the range of 3.5-4 mV/cm^2. Bonsai [22] was used to acquire the movies and trigger the activation stimulus protocol. For each line three movies were recorded.

Egg laying

Single females were gently aspirated and transferred to 35 mm Petri dishes of 10 mm of height (Thermo Fisher Scientific) coated with apple agar (750 mL water, 250 mL apple juice, 19,5 g agar, 20 g sugar, 10 mL 10% nipagin) and incubated with a naive CS male for 2 h under constant observation. Plates where mating did not happen were discarded. After the incubation the male was removed and the female was kept in the plate for 24 h before eggs were counted.

Courtship assay

Single females were gently aspirated and transferred to the conical-shaped arena and paired with a naive male. For details about the experimental set-up, see Activation screen section. Flies were recorded for 20 min or until copulation. For the closed-loop experiment Bonsai was used to track the flies in real time and trigger the light switch according to the distance between them, with a threshold of 5.5 mm as we have previously shown that below this distance there is 95% likelihood of courtship [12].

Aristae and wings removal

For the aristae or wings removal to female and male flies respectively, individual flies were anesthetized with CO_2 approximately 15 h before the experiment. Either the aristae or the wings were bilaterally cut at their base with microscissors or microforceps (World Precision Instruments) under a scope. Flies were allowed to recover at 25°C until the experiment.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data processing

After movies were acquired, FlyTracker [48] was used to track the two flies and output information concerning their position, orientation, velocity, distance to the other fly, and wing angles, among others. Also, a Courtship Classifier developed in the lab using the machine learning-based system JAABA [49] was run to automatically identify courtship bouts. Subsequently, the in-house developed software PythonVideoAnnotator was used to manually annotate the time and duration of specific fly behaviors. Courtship events generated by JAABA were visualized in PythonVideoAnnotator to check and correct them if necessary. Annotations were done from the beginning of courtship and during five min or until copulation, except for aristaless and wingless experiment where the last five min of the video were used. The behaviors annotated were ovipositor extrusion, copulation attempts and licking. Copulation time was also annotated considering the whole duration of the video.

Quantification of behaviors

Data analysis was performed using custom Python scripts for all the experiments.

Courtship index was calculated as

$$\text{Courtship index} = \#\ \text{frames of courtship} / \#\ \text{total frames}$$

where the total number of frames is counted from the start of courtship and up to 5 min or until copulation.

Ovipositor extrusion index was calculated as

$$\text{Ovipositor extrusion index} = \#\text{frames of ovipositor extrusion} / \#\text{frames of courtship}$$

Copulation attempt number was calculated as the number of times the male attempts to copulate from the moment he initiates courtship and up to 5 min or until copulation. When copulation occurred within the analyzed 5 min, it was considered as an attempt (successful attempt).

For the experiment with *fruitless* mutant males, since courtship was absent, the ovipositor extrusion was annotated when the distance between the two animals was below 5.5 mm. This information was extracted from the FlyTracker output (see Data processing section).

To calculate the probability of either ovipositor extrusion or licking around copulation attempt we aligned all the copulation attempts events and we counted how many ovipositor extrusion or licking events were happening in each of the 60 frames preceding and following the onset of copulation attempt and normalized the counts over the number of copulation attempts. The potential copulation attempt events were calculated according to conditions of distance between the flies body centers within a range of 2-3.5 mm and speed of the female fly less than 4 mm/s, information was extracted from the FlyTracker output. To calculate the probability of either ovipositor extrusion or licking around potential copulation attempt we did the same calculations used for ovipositor extrusion and licking probabilities around copulation attempt this time aligning the potential copulation attempts events.

Statistical analysis

Prior to statistical testing, Levene's test was used to assess variance homogeneity and Shapiro-Wilk test was used to assess normality across all individual experiments. The data was not normally distributed in all the experiments. Fisher exact test and Mann Whitney test were used to perform pairwise comparisons. Kruskal-Wallis test was used to compare more than two groups and Bonferroni correction to p values was applied when multiple comparisons were performed. The sample size for each comparison is indicated in each plot. Some analyses were performed with specific thresholds, which will result in differences in the sample size for the same condition. The probability of copulation attempt or copulation given either ovipositor extrusion or licking was calculated as

the presence of copulation attempt or copulation at the offset of ovipositor extrusion or licking events, respectively, ± 10 frames of annotation error. The probability of copulation attempt or copulation given ovipositor extrusion and licking together was calculated as the presence of copulation attempt or copulation at the offset of corresponding licking events ± 10 frames of annotation error. The two events, licking and ovipositor extrusion, were considered happening together whenever any of their frames ± 10 frames of annotation error were overlapping.