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The role of ovipositor extrusion during courtship in female
Drosophila melanogaster

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Abstract

Courtship is the set of behavioural interactions between sexes, with the objective of copulation, as well as the survival and fitness of the species. *Drosophila melanogaster* courtship includes a complex series of behaviours and it is well characterized at the circuit level. As in most species, *Drosophila melanogaster* males court possible partners, but the decision whether to mate or not is up to the females. Male courtship has been the focus of most courtship *Drosophila melanogaster* studies. Contrastingly, we still have a very poor insight on female behaviour, as well as the neuronal circuits underlying it. It is known that the female performs accepting and rejecting behaviours during courtship. Among these responses, Ovipositor Extrusion (OE) is a behaviour exclusive of courtship, which indicates its importance on this process. OE is characterized by the pushing of the vaginal plates posteriorly, projecting from the tip of the abdomen as a tube-like structure. It can be performed in two different forms: partially, when only the distal part of the ovipositor is extruded and fully, when the ovipositor is completely extruded. Classical studies consider OE a rejection behaviour, since it was observed that OE prevents copulation. Nevertheless, OE is a rare event and both receptive (virgin) and unreceptive (mated) females perform it, which complicates its interpretation. The main goal of our project is to unravel the role of female *Drosophila melanogaster* OE during courtship.

An activation screen of a collection of lines was previously performed in Luísa Vasconcelos's Lab. It revealed a line with two pairs of command neurons (P7 and P13) for ovipositor extrusion, which was called OE line. To further study the role of OE, we performed neuronal manipulation experiments on this line: for activating the neurons, we used Chrimson; for silencing the neurons, we used Kir2.1. We set out to record behavioural interactions between a pair of flies in a behavioural setup that, due to its high resolution, allows to dissect meticulously male courtship behaviours, as well as female responses. We annotated and analysed OE, copulation, courtship and attempted copulation behaviours. Additionally, we worked on the development of an automatic classifier for OE using JAABA software. In neuronal activation experiments, we used a closed-loop system where proximity of the male induced optogenetic activation on the female. To highlight potential advantages or disadvantages of OE, we recorded competition experiments between a virgin female that extrudes often her ovipositor and a virgin female that extrudes her ovipositor normally for a male. We also performed inactivation experiments with Kir2.1 in virgin and mated females.

We found that, of the two neurons, P13 is the only one responsible for OE. We also found that inhibition of OE leads to a marked decrease in the number of attempted copulation events, suggesting OE as enticing for this behaviour. Additionally, we show that overexpression of OE leads to a decrease in copulation, which seems to indicate that OE acts as a physical barrier for copulation. However, and despite continuous neuronal activation, the intermittency of OE allows females to mate. Lastly, we show that males prefer to court and copulate with a female that performs OE normally rather than a female that performs OE more often.

We believe that our findings contribute to the understanding of the role of OE, both in behavioural and neuronal terms. We found that P13 is the neuron responsible for this behaviour. We show that OE has a different meaning, depending on the sex: for females, full OE functions as a physical barrier for impeding male copulation; regarding the male, OE is enticing for attempted copulation events, whilst it has a rejecting connotation for copulation itself. Further behavioural and genetical studies will help to unveil the role of OE.

Keywords: *Drosophila melanogaster*, courtship, ovipositor extrusion, genetics.

Resumo

A corte é o conjunto de interações comportamentais que ocorrem entre os dois sexos, com o objectivo de cópula, assim como a manutenção da sobrevivência e fitness das espécies. A corte de *Drosophila melanogaster* inclui uma série de comportamentos complexos e encontra-se bem caracterizada ao nível de circuitos neurais. Os factores de transcrição *Fruitless (fru)* e *Doublesex (dsx)* especificam redes neurais específicas do sexo, que determinam comportamentos com dimorfismo sexual. À semelhança do que ocorre na maior parte das espécies, os machos da espécie *Drosophila melanogaster* cortejam possíveis parceiras, mas a decisão de acasalar ou não é tomada pelas fêmeas. A corte inclui uma série de comportamentos fixos efectuados pelo macho: primeiramente, este orienta-se em relação à fêmea; segue-a; toca-a no abdómen; produz uma canção de corte através da vibração de uma asa; lambe a sua genitália e, por fim, tenta copulá-la. A corte por parte dos machos tem sido o foco da maior parte dos estudos de corte de *Drosophila melanogaster*, visto que é composta por uma série de acções conspícuas facilmente quantificáveis. Em contraste, pouco se sabe sobre o comportamento da fêmea, assim como os circuitos neuronais responsáveis pelo mesmo. Sabe-se que a fêmea responde às acções do macho, efectuando comportamentos que variam em termos de frequência e tipo, dependendo da sua receptividade: as fêmeas maduras são altamente receptivas, copulando com os machos; por outro lado, as fêmeas que já acasalaram passam por várias alterações a nível fisiológico e comportamental. Estas não são receptivas sexualmente e colocam um número significativamente maior de ovos. Sabe-se também que as fêmeas executam comportamentos de aceitação e rejeição durante a corte. Entre estas respostas, a Extrusão do Ovipositor (EO) é um comportamento exclusivo da corte, o que indica a sua importância neste processo. A EO é caracterizada pelo empurrar posterior das placas vaginais, que projectam da ponta do abdómen como uma estrutura em forma de tubo. Pode ser efectuada de duas formas diferentes: parcialmente, quando apenas a parte distal do ovipositor é extruída, e completamente, quando o ovipositor é extruído na sua totalidade. Estudos clássicos consideram a EO como um comportamento de rejeição: Bastock e Manning encontraram uma tendência significativa para a corte do macho parar após a ocorrência da EO; Connolly e Cook sugeriram que a EO tem como função principal prevenir a cópula, visto que este comportamento é efectuado principalmente por fêmeas acasaladas. Contudo, Welbergen *et al.*, sugerem que a OE induz comportamentos de corte do macho, tais como tentativa de cópula e canção de corte. Labsleiz *et al.* demonstra que a extrusão parcial estimula a corte por parte do macho, enquanto a extrusão completa a inibe. Contudo, o facto de a EO ocorrer em baixa frequência e ser efectuada tanto por fêmeas (virgens), como não receptivas (acasaladas) dificulta a sua interpretação. A disponibilidade de tecnologias, tais como ferramentas de manipulação genética de um número restrito de células, assim como a observação de estruturas de pequenas dimensões recorrendo a gravação de vídeo de alta resolução, permitem estudar melhor a corte em *Drosophila melanogaster*. O objectivo principal deste projecto é desvendar o papel da EO da fêmea *Drosophila melanogaster* na corte.

Um screen de activação de uma colecção de linhas que marcam interneurónios descendentes foi efectuado anteriormente no laboratório de Luísa Vasconcelos. Este revelou uma linha com dois pares de interneurónios-comando (P7 e P13) para a EO, a qual chamámos OE line. Para estudar a EO, utilizámos esta linha para efectuar experiências de manipulação neural. Para activação neuronal, utilizámos uma ferramenta optogenética: Chrimson, um canal controlado pela incidência de luz. Esta ferramenta permite estimulação cerebral directa em *Drosophila melanogaster*. A fim de haver uma produção funcional de de Chrimson, é necessário fornecer t-Retinal como suplemento alimentar. Este facto permitiu criar o nosso grupo de controlo para experiências de activação neuronal, constituído por fêmeas sem t-Retinal como suplemento alimentar. Para inactivação neuronal, utilizámos o canal de

potássio de rectificação Kir2.1. Este causa sobreexpressão dos canais de potássio que se encontram abertos no potencial de repouso da membrana, levando à supressão da actividade eléctrica dos neurónios onde é expresso. Gravámos as interacções comportamentais entre um par de moscas num setup comportamental durante 20 minutos ou até à ocorrência de cópula. Este setup, devido à sua elevada resolução, permite compreender detalhadamente os comportamentos de corte do macho, assim como as respostas da fêmea. Posteriormente, procedemos à anotação manual de comportamentos de EO, cópula e tentativa de cópula. Em relação à anotação de corte, utilizámos um classificador automático previamente desenvolvido no laboratório, recorrendo ao software JAABA. Tendo em conta este software, trabalhamos no desenvolvimento de um classificador automático para a EO, a fim de reduzir o trabalho providenciado pelas anotações manuais. Em experiências de activação neuronal, utilizámos um sistema closed-loop, onde a proximidade do macho induzia activação optogenética na fêmea. Numa tentativa de sublinhar potenciais vantagens ou desvantagens da EO, filmámos experiências de competição entre uma fêmea virgem que extruía o seu ovipositor frequentemente e uma fêmea virgem que extruía o seu ovipositor normalmente por um macho. Efectuámos também experiências de inactivação neuronal em fêmeas virgens e acasaladas. Por último, realizamos ainda experiências de activação neuronal contínua a fêmeas virgens com t-Retinal pertencentes a diferentes linhas, em que a EO ocorre.

Descobrimos que, dos dois neurónios, apenas o P13 é o responsável pela EO e que este é positivo para o gene *dsx* e negativo para o gene *fru*. Verificámos que o silenciamento do neurónio P13 em fêmeas acasaladas levou a uma inibição total da EO, assim como a um decréscimo demarcado no número de tentativas de cópula, sugerindo que a EO é atractiva para este comportamento. Também verificámos que a activação neuronal da OE line levou a uma sobreexpressão da EO em fêmeas virgens, assim como a um decréscimo na cópula. Tal parece indicar a EO como uma barreira física para a ocorrência de cópula. Contudo, e apesar da activação neuronal contínua, a intermitência da EO permite que as fêmeas ainda consigam acasalar, o que parece demonstrar que a EO não é totalmente eficaz a bloquear a cópula. Demonstrámos também que os machos preferem cortejar e copular com uma fêmea que extrui normalmente o seu ovipositor do que uma fêmea que o extrui mais frequentemente, o que indica que a EO não é propriamente vantajosa para a fêmea. Descobrimos ainda que o silenciamento do neurónio P13 em fêmeas virgens não leva à inibição da EO. Tal pode ser explicado pelo facto de o neurónio P13 apenas actuar em eventos de EO completa e as fêmeas virgens apenas executarem EO parcial.

Acreditamos que estas descobertas contribuem para a compreensão do papel da EO, tanto em termos comportamentais, como em termos de redes neurais envolvidas neste comportamento. Descobrimos que o neurónio P13 é o responsável pela EO. Também descobrimos que a EO tem um significado diferente, dependendo do sexo: no caso das fêmeas, a extrusão completa funciona como uma barreira física, que impede a cópula; em relação ao macho, a EO é atractiva para tentativas de cópula, enquanto tem uma conotação de rejeição para a cópula em si. Estudos futuros a nível comportamental e genético ajudarão a compreender melhor o papel da EO. Nesse sentido, seria interessante estudar qual o factor que leva ao despoletar da EO na fêmea, assim como compreender um pouco melhor a componente química deste comportamento.

Palavras-chave: *Drosophila melanogaster*, corte, extrusão do ovipositor, genética.

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Chapter I

Introduction

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1.1. Courtship from an evolutionary point of view

Courtship is defined as the behavioural interactions and displays between males and females, with the evolutionary objective of copulation and, ultimately, perpetuation of the species. Courtship interactions are guided by several sensory modalities that need to be understood in conjunction, such as vision, olfaction, gustation, audition and mechanosensation¹. In addition, internal physiological states and individual experience also contribute to generate this diversity of behavioural outputs (reviewed in ²).

Courtship behaviours are thought to be important for demonstrating both the appropriateness (species, sex and mating status) and the fitness of the individual to potential partners³. Both sexes play necessarily different roles in the courtship process, within a given species, contributing differentially to its reproductive success⁴. Typically, the male recurs to multiple sensory modalities to search for females and determine if they are receptive and fit. If so, the male performs courtship displays. Males can also use the acquired information in this process to change the course of courtship. These mechanisms for identification of appropriate mating partners are essential for species propagation (reviewed in ⁵). Regarding the role of the female, she decides to accept or not a courting male. This process of sexual selection of the best mate has a great importance in the evolution of animal communication, as well as of a given species (reviewed in ⁶).

Courtship behaviour is elicited by the crucial role of the nervous system. This system is formed during development by networks of interacting genes, where similar networks assemble the physiological structures required for the generation of behaviour patterns (reviewed in ⁷). Neural circuits mediating courtship are developmentally programmed, as it is an innate behaviour that can be detected in naïve animals without previous learning or experience⁸. Nevertheless, these innate behaviours also exhibit flexibility, since they are modulated by the internal state, as well as social and environmental cues^{9,10}.

1.2. *Drosophila melanogaster* as a model organism

Drosophila melanogaster is a versatile model organism that presents several technical advantages: it is inexpensive and easy to culture in laboratory conditions, has a short life cycle and produces a large number of progeny. It has been used for over a century to study a wide scope of biological subjects, including behaviour at its molecular, cellular and evolutionary levels (reviewed in ¹¹).

Although this model organism is a simple animal, with a simple nervous system, only composed of ~100 000 neurons¹², its behavioural repertoire and its underlying brain circuits are complex enough to pose interesting questions. Additionally, *Drosophila melanogaster* genome is completely sequenced¹³ and can be genetically modified with several genetical tools. The development of binary expression systems, such as the GAL4/UAS system¹⁴, as well as the generation of fly strain collections enable the access and identification of neurons or populations of neurons that are involved in neural circuits underlying complex behaviours^{12,15}.

Courtship behaviour in *Drosophila melanogaster* is an example of a complex, multistep¹⁶ (further described in 1.2.2. section) and one of the best understood behaviours at the circuit level. The neural circuitry involved in this behaviour will be described in 1.2.1. Work on *Drosophila melanogaster* courtship can reveal essential mechanisms regarding communication between animals.

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1.2.1. Neural networks in *Drosophila melanogaster*

To choose their mates, *Drosophila melanogaster* males and females display sexually dimorphic courtship behaviours. This represents a suitable model for studying the neural circuitry of innate social sexually dimorphic behaviours (reviewed in ¹⁷). Genes of the sex-determination cascade provide a starting point for the identification of these neural circuit components. These genes mediate the development and differentiation of sex-specific tissues, establishing sex-specific neural circuitry and physiology¹⁸. *Fruitless (fru)* and *doublesex (dsx)* are two pivotal transcription factors of the sex-determination hierarchy that specify sex-specific behaviours^{17,19}. *fru* and *dsx* are expressed in sensory neurons, interneurons and motoneurons, which suggests their organization into circuit elements: they can receive, process and transfer information that controls courtship behaviours. Comparing males and females gives an insight into how distinct behaviour outputs may be wired via shared circuits that operate differently and/or sex-specific circuits that are the product of the combination of unique circuit elements (reviewed in ¹⁷).

1.2.2. Courtship behaviour in *Drosophila melanogaster*

Courtship in *Drosophila melanogaster* includes a complex series of behaviours^{16,20}. As in most species, the male courts possible partners and the female is the one deciding whether to mate or not¹⁹. Courtship comprises a series of stereotyped actions that were firstly described by Sturtevant²¹. When a pair of flies from opposite sexes comes close, they sense each other instantly¹⁶. Males detect suitable courtship objects by relying on vision and chemical signals, such as volatile pheromones detected by the olfactory system, as well as non-volatile hormones detected by the gustatory system²². If the male perceives that these signals are indicating mating success, he initiates courtship by orientating directly towards the female, almost simultaneously tapping her abdomen with his forelegs^{20,23}. The male then follows the female while she moves or circles her, if she is stationary¹⁶. He also extends one wing or the other at an angle of 45° to his main body axis. This extension of the wing is accompanied by its vibration, which is a species-specific acoustic signal, known as the courtship song^{16,24}. Afterwards, the male extends his proboscis and licks the female's genitalia, which presages his first copulation attempt, characterized by an abdominal bending towards the female^{16,20}. If the male fails to copulate, he might engage in courtship bouts before finally copulating^{16,25}. If the female is receptive, they ultimately copulate¹⁶ (Fig. 1.1.).

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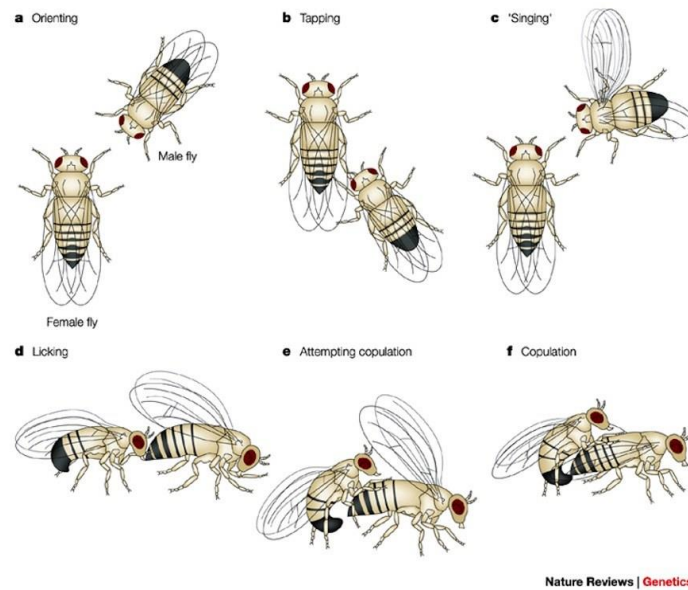


Figure 1.1.: Sequence of courtship behaviours performed by the male *Drosophila melanogaster* towards a female. a) The male orientates towards the female, following her afterwards; b) taps her abdomen; c) produces the “courtship song” by vibrating one wing or the other. d) Lastly, he licks the female’s genitalia and e) attempts to copulate with her. f) This can result in copulation (from Sokolowski, 2001, Nature Reviews Genetics).

1.3. Courtship behaviour under the female’s perspective

Male flies show a very conspicuous set of actions that are easy to quantify. For this reason, the male has been the focus of most courtship *Drosophila melanogaster* studies. His behaviour has been analysed with fine genetic tools, which have brought insight into physiological and cellular processes underlying sensory perception. Furthermore, these behavioural analyses have also provided knowledge regarding neural connectivity and processing of male sexual behaviour (reviewed in ⁶).

Contrastingly, very little is known about how the female brain receives and processes internal and external cues to respond to courtship: she presents a subtle, yet robust behaviour, seen as limited only to accept or reject the male (reviewed in ²⁶). This asymmetric level of information between sexes hampers the understanding of the interactive process underlying sexual communication and reproduction. Taking into consideration that different challenges are faced by the two sexes, understanding female behaviours provides an opportunity to acquire more knowledge on the underlying genetic basis of species-specific behaviour, as well as neural and molecular groundwork of conspecific interactions. It also enables to unravel evolutionary aspects related to species formation and isolation by sexual selection (reviewed in ⁶). Nevertheless, this scenario has been progressively changing: more insight into female specific behaviours is starting to be obtained: recent progress will be specified in section 1.5.

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1.4. Changes with life stage in females

It is known that the female responds to the male's actions by performing behaviours that vary in frequency and type, depending on her receptivity^{24,27}. Sexually immature females are unreceptive²⁷. The onset of their receptivity takes place only approximately 48 hours after emergence from the pupal case^{28,29}. Mature virgin females show a high level of receptivity and copulate when confronted with a courting male. These mated females undergo profound behaviour and physiological changes³⁰, due to the post-mating switch: for eight to ten days after mating, they become temporarily sexually unreceptive to further copulation, until the depletion of live sperm in the reproductive tract. They also exhibit longer latencies to mate^{29,31}. In addition, these post-mating changes in behavioural responses induce a significant increase in oviposition and changes in dietary preferences²⁹. This set of responses are mainly elicited by Sex Peptide (SP), a 36 amino-acid-long male peptide. SP is synthesized in the accessory glands of the male and transferred during copulation to the female³²⁻³⁴. It elicits these post-mating changes via a receptor expressed in *fru* and *dsx*-positive sensory neurons, as well as the proprioceptive neuronal marker *pickpocket* (*ppk*) located in the female uterus³⁵⁻³⁷. These neurons send their projections to the abdominal ganglia, connecting to the *dsx*-expressing SAG neurons, which have their axons projected to the dorsal proto-cerebrum³⁸.

Post-mating responses of *Drosophila melanogaster* are a clear example of the behavioural switch that occurs due to the need of responding differently to the same stimulus, which is allowed by the functional reconfiguration of existing neural circuits (reviewed in³⁹).

1.5. Female behavioural responses to male courtship behaviours

Although there is a poor understanding of the female courtship behaviours, some of them have been described in the literature. During courtship, there is a dynamic interaction that is established between sexes, which suggests that the female is not a passive agent throughout the course of this process²¹. Nevertheless, females often attempt to escape as a first reaction to the male's courtship, even when they are receptive virgins²⁷. They may perform escaping behaviours to gain more time to evaluate the male's courtship behaviour to measure directly and indirectly his fitness²⁵. Decamping, i.e., running or jumping, is one of the most frequent behaviours exhibited by the female^{27,40}. In addition, she might flick her wings^{20,27,40} and depress or elevate the tip of the abdomen, to prevent genital contact²⁷. If stationary, she often reacts by kicking with her hindlegs^{20,27,41}, extruding the ovipositor^{20,41} or fending²⁷. These behaviours have been generally assumed as "rejecting behaviours"^{20,27,40}, having the objective of minimising or preventing copulation (Fig. 1.2). Sturtevant mentioned repelling behaviour in his original description of courtship behaviour in *Drosophila melanogaster*: "Occasionally a female seems to frighten off a male by spreading her wings and moving quickly towards him"²¹. As rejection behaviours are performed by both receptive and unreceptive females, their interpretation is more difficult to understand^{20,24,27,42}.

If the female is receptive, she reduces her locomotor activity^{43,44} and performs abdominal grooming^{24,42}, actively allowing copulation. These are considered accepting behaviours (Fig. 1.2.).

It is important to mention that, most of times, these behaviours are not exclusively associated to the context of courtship.

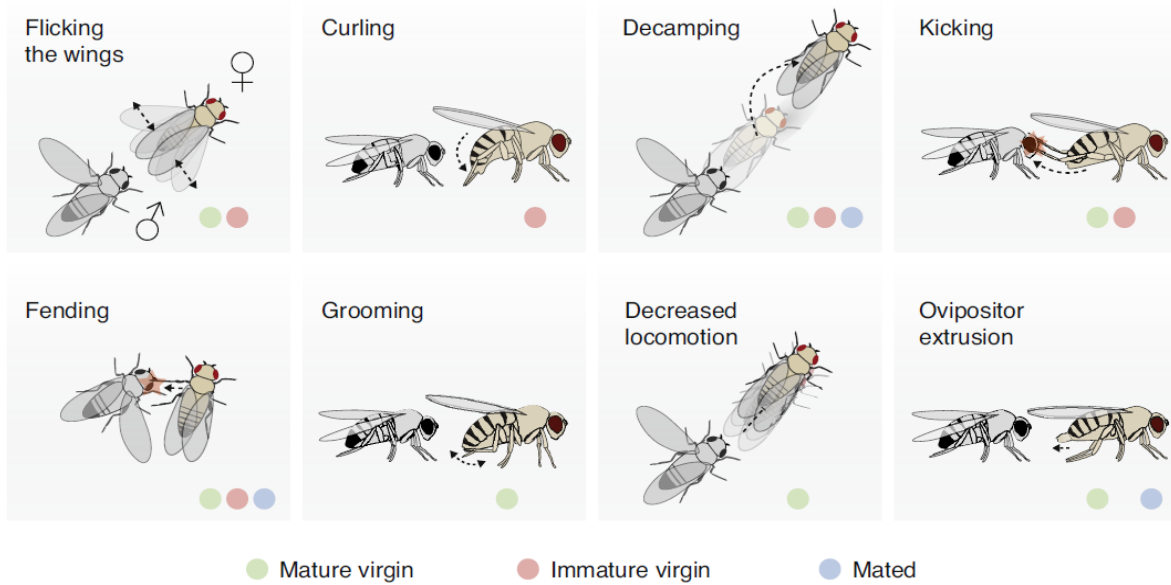


Figure 1.2.: Courtship behaviours in female *Drosophila melanogaster* at different receptivity states: immature virgin, mature virgin and mated females. Flicking the wings: Wings undertake one or more lateral flicks; Curling: The female depresses her abdomen; Decamping: the female runs, jumps, or flies away (basically, she escapes); Fending: to keep distance from the other flies, the female extends her leg laterally; Grooming: the female grooms her abdomen; Decreased locomotion: before copulation, the female reduces her velocity; Ovipositor extrusion: the female pushes the vaginal plates posteriorly and they project from the tip of the abdomen as a tube-like structure. (from Aranha and Vasconcelos, 2018, Current Opinion in Neurobiology).

1.6. Ovipositor Extrusion, a behaviour exclusive to *Drosophila melanogaster* courtship

Ovipositor extrusion (OE) is a behaviour exclusive to courtship⁴⁴, which indicates its importance for these behavioural interactions. This behaviour varies with mating status: immature virgins do not display OE^{43,44}; mature virgins perform it, even though it is not their most frequent courtship response²⁷, and it represents the main response of mated females to this context²⁷. It was firstly described by Rendel as the “pushing of the vaginal plates posteriorly so that they protrude from the tip of the abdomen as a tube-like structure”, in *Drosophila subobscura* and *Drosophila melanogaster* (reviewed in ²⁷). Current representations of *Drosophila melanogaster* reproductive system are very similar to the first description of this system and the ovipositor is few times mentioned in these studies^{45,46} (Fig. 1.3.). It is thought that this structure corresponds to the terminal part of the uterus, which is extruded from the vaginal aperture. Although it is known that the ovipositor has an egg-laying function, oviposition and OE display different motor output and are triggered by different neuronal circuits: whilst the former is induced by *dsx*-positive female-specific pMN2 neurons, the latter is regulated by sexually dimorphic pC2I interneurons in the brain⁴⁷

From classical studies, OE is thought to be a rejection behaviour. Rendel, in 1945, found it to be a typical reaction of the fertilised female to a courting male²⁷. Bastock and Manning, in 1955, found a significative tendency for the cessation of male’s courtship following female’s extrusion. Nevertheless, there were some cases when extrusion was preceded by attempted copulation²⁰.

Connolly and Cook, in 1973, studied the rejecting responses of female *Drosophila melanogaster*. They observed that old virgin females, 28 days old, and mated females, 4 days old, performed OE as a principal and exclusive to wing extension. They also observed that attempted copulation tended to occur after OE. They also suggested that the major effect of OE is preventing copulation. By observing mated females performing frequently OE, they raised the hypothesis that either the behaviour of the male is altered, or there is a mechanical prevention of copulation, or both²⁷.

Welbergen *et al.*, in 1987, observed mixed-sex pairs of *D. melanogaster* and *D. simulans*. It was suggested that OE, together with standing and preening, are important inducers of wing vibration, licking and attempted copulation in the male⁴⁸. Cobb and Ferveur, in 1995, also verified in *D. sechellia* that female extrusion is immediately preceded by courtship male licking²⁵. Both these studies indicate male arousal by this female behaviour.

A revision of *Drosophila melanogaster* courtship carried by Labsleiz, Ferveur and Everaerts, in 2006, found that two defined types of ovipositor induce antagonistic effects on male courtship: whilst extrusion of the distal part of the ovipositor entices male's courtship, the complete extrusion of this structure repels the male. It was hypothesized that partial extrusion, abdominal preening and droplet emission are related behaviours that disperse actively compounds present on the female's abdomen²⁴.

Despite the information provided by these studies, it is not clear how OE contributes to the progression of courtship, since it is performed both by receptive and unreceptive females, as well as it is a rare event²⁷.

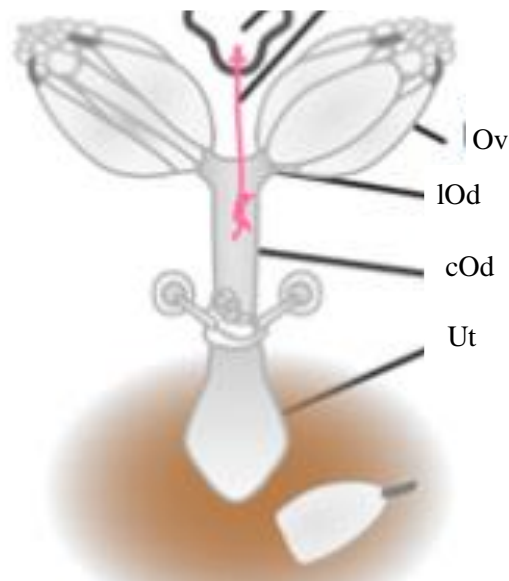


Figure 1.3.: Representation of the female *Drosophila melanogaster* reproductive tract: Ov: ovary; IOd: lateral oviduct; cOd: common oviduct; Ut: uterus. (adapted from Rubinstein and Wolfner, 2013, PNAS).

1.7. Tools for probing neuronal function

The availability of new technologies allows advances in *Drosophila melanogaster* courtship studies. These enable targeting gene manipulation to a restricted number of cells, as well as observing structures and objects of very small size, recurring to cell-activity imaging and high-resolution video-tracking of flies (reviewed in ²⁶).

One of the most powerful approaches for addressing gene function in vivo is temporal and spatial targeting of gene expression. The GAL4/UAS system is an example of this. In this system, the

responder, which is the gene of interest, is controlled by the presence of the UAS element, consisting of five tandemly arrayed and optimized GAL4 binding sites. Since the transcription of the responder requires GAL4, the absence of GAL4 in the responder lines keeps them in a transcriptionally silent state. For transcription activation, responder lines are mated to lines expressing GAL4 in a specific pattern, which constitutes the driver. Their progeny express the responder in a transcriptional pattern which reflects the GAL4 pattern of the driver¹⁴.

Taking advantage of the modularity of transcription factors first shown in GAL4, the Split GAL4 tool is an alternative approach for manipulation of more restricted neuronal activity. In this system, transcriptional factors often consist of functional domains for transcription-activation (AD) and site-specific DNA-binding (DBD) and can be independently targeted using two different promoters. It is only at the intersection of the expression pattern of these two promoters that the transgenic expression will be driven at appropriate promoter sites. Split GAL4 is compatible with the numerous UAS-effector lines available in *Drosophila melanogaster*⁴⁹.

Considering recent technological advances, a very relevant genetic tool to our work was developed recently: a collection of Split-Gal4 lines that label descending interneurons, which are neurons that carry information from the brain to the thoracic ganglia and that are present in multiple circuits in the brain⁵⁰. These are putative command neurons. This collection comprises cell-type specific lines with an expression limited to a single type of descending interneuron. These lines can be used to evaluate the consequences of manipulating the cells they label, which presents an insight into their possible roles in behavioural circuits⁵¹.

Optogenetic tools enable the targeting of specific neurons using light-sensitive proteins, such as ion channels, enzymes and ion pumps, as well as manipulating their physiological state through illumination⁵². Chrimson, an example of an optogenetic tool, is a light-gated channel. Its excitation spectrum is red-shifted by 45 nm more relatively to previous channelrhodopsins and enables experiments in which the use of red light is preferable, such as deep tissue targeting, or experiments in which blue light is visually distracting. It also allows direct brain stimulation through the cuticle of *Drosophila*, as well as reduction of visual system-triggered responses⁵³. The inwardly rectifier potassium channel Kir2.1 is also a tool that enables manipulation of neuronal activity. By overexpressing potassium channels that are open at resting membrane potential, electrical activity is suppressed. When this overexpression occurs, it leads to an increased potassium efflux and membrane hyperpolarization setting the resting membrane potential below the threshold required to fire action potentials⁵⁴.

1.8. An activation screen revealed a line that, by neuronal activation, leads to OE occurrence

Previously to my arrival to Luísa Vasconcelos's lab, an activation screen of Fly Descending Interneuron Split-GAL4 lines was performed to look for lines that display a female response to courtship. One of these lines, 1549-AD;1549-DBD, when activated with Chrimson, lead to the occurrence of OE. This line labels two pairs of command neurons for OE, P7 and P13. For simplification, this will be referred as the OE line.

To confirm which neurons involved in the OE line were responsible for OE behaviour, activation with Chrimson was performed in other lines that label either P7 or P13 neurons. Lines with the P13 neuron, when activated, led to the occurrence of OE. Contrastingly, OE did not occur with the activation of lines involving only the P7 neuron. It was found that the P13 is the neuron responsible for OE. This fact was confirmed by the activation of only this neuron, which led to the occurrence of OE (Fig. 1.4.)

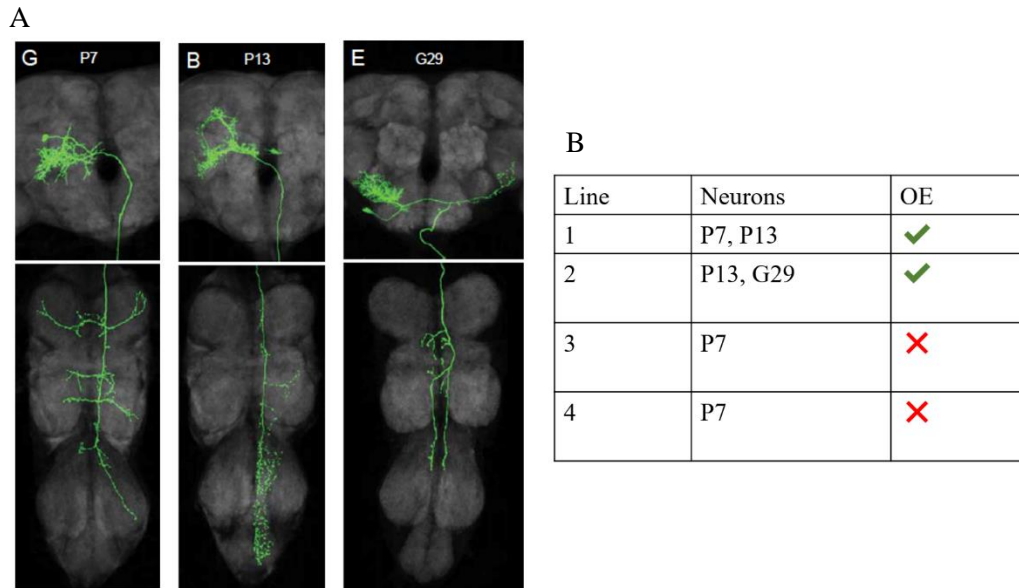


Figure 1.4.: P13 is the neuron responsible for OE. A) Immunostaining of P7, P13 and G29 neurons, which are labelled in some of the lines included in Janelia's Fly Descending Interneuron collection of lines (provided by Janelia Research Campus); B) Neuronal activation with Chrimson on the different lines. With this experiment, it was found that P13 is the neuron responsible for OE. Experiment done by Cecilia Mezzera, a postdoctoral researcher in Luísa Vasconcelos's lab.

1.9. Objectives

The main goal of this project is to unravel the role of the female *Drosophila melanogaster* OE during courtship. Particularly, we aim to study the meaning of the OE for the male when presented by virgin and mated females.

Since we know which neurons are involved in OE behaviour, we will take advantage of the tools that enable neuronal manipulation. More particularly, we will perform neuronal activation experiments with Chrimson, that determine sufficiency, as well as neuronal silencing with Kir2.1, that establish necessity. These manipulations and their effects in courtship will bring insight into the meaning of OE.

Chapter II

Methodologies

2.1. Fly stocks

Drosophila melanogaster flies were raised in standard cornmeal-agar medium at 25 °C, 60-70% relative humidity, in a 12 h:12 h dark:light cycle. The following strains were used:

- Wild-type *Canton-S* (CS)
- *w*; *1549-AD*; *1549-DBD*;
- *w*, *1549-AD*; *dsx-DBD*;
- *w*; *1549-AD*; *dsx-DBD*, *UAS-Chrimson*;
- +; +; *UAS-Chrimson-mVenus*;
- *w**; *UAS-Kir2.1*; +;
- *w*; +; +;

2.2. Behavioural Setup

The behavioural setup consists of a camera (Point Grey FL3-U3-32S2M-CS) with a 5 mm fixed focal length lens (Edmund Optics) mounted above a conical-shaped arena. The conical arena is made of white Delrin with 11° sloped walls and 4 mm of height at the centre. When gently aspirated into the arena, flies can walk in a circle of ~3 cm diameter. This chamber provides several advantages: it limits flies to a shallow volume of space, which leads to the occurrence of interactions within a monolayer of individuals; it decreases the frequency in which flies overlap or occlude each other and restricts variability in flies' appearance⁵⁵. The arena is topped with a lid made of plexiglass. Videos were acquired in the dark, using a 940 nm LED array (SOLAROX) placed below the arena. The infrared light illuminates the arena without interfering with the fly behaviour and is captured by the camera due to an infrared filter (Hoya 40 mm R72) (Fig. 2.1.)

Flies were recorded in grayscale (1024x1024 pixels, 60 frames per second).

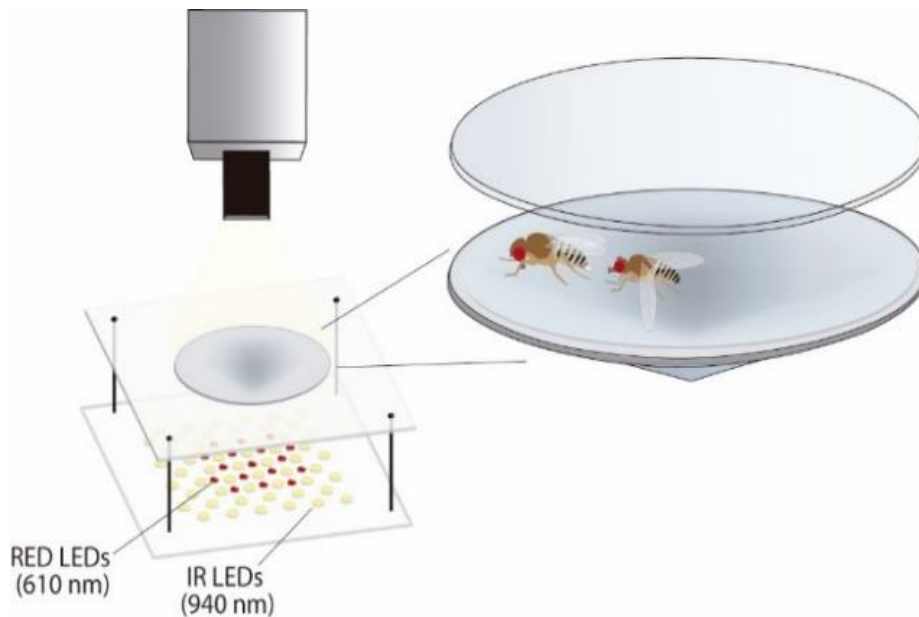


Figure 2.1.: Behavioural setup for neuronal activation experiments. It consists of a camera mounted above a conical arena. Below the arena, there is placed an infrared LED array (940 nm), as well as red LEDs (610 nm). Red LEDs are used for neuronal activation experiments, which will be detailed in section 2.3.1. (illustration is courtesy of Cecilia Mezzera).

2.3. Behavioural Experiments

The progeny of the crosses eclosed in ten days. Virgin females and males were collected after their eclosion, housed individually until behavioural experiments, and aged for 4-8 days at 25°C or for 8-14 days at 18°C.

15 hours prior to the experiments, fly food was placed on the arenas, and cleaned on the experiment day. Food is known to improve courtship behaviour⁵⁶. Also 15 hours before the experiments, flies must be kept at 25°C to acclimatize to the temperature of the behaviour room.

Circadian clocks drive courtship and mating behaviour. Male-female couples are highly active throughout the night and early morning and display a short duration rest phase around dusk⁵⁷. Regarding this fact, behavioural experiments were recorded between 09h30 and 13h00.

For all behavioural experiments involving pairs, we used naïve males, since previously mated males learn from their sexual experiences and adjust their behaviours accordingly to gain advantages in terms of mating⁵⁸.

Video tracking of behaviour, as well as closed-loop (control systems that use the output of a system to control or modify the input⁵⁹) control of stimulation require the combination of different hardware and software components. For these two tasks, Bonsai, a modular, open source visual programming framework that acquires and processes online data streams, was used⁶⁰ (Fig. 2.2.). All assays were performed at 25°C with 70% relative humidity.

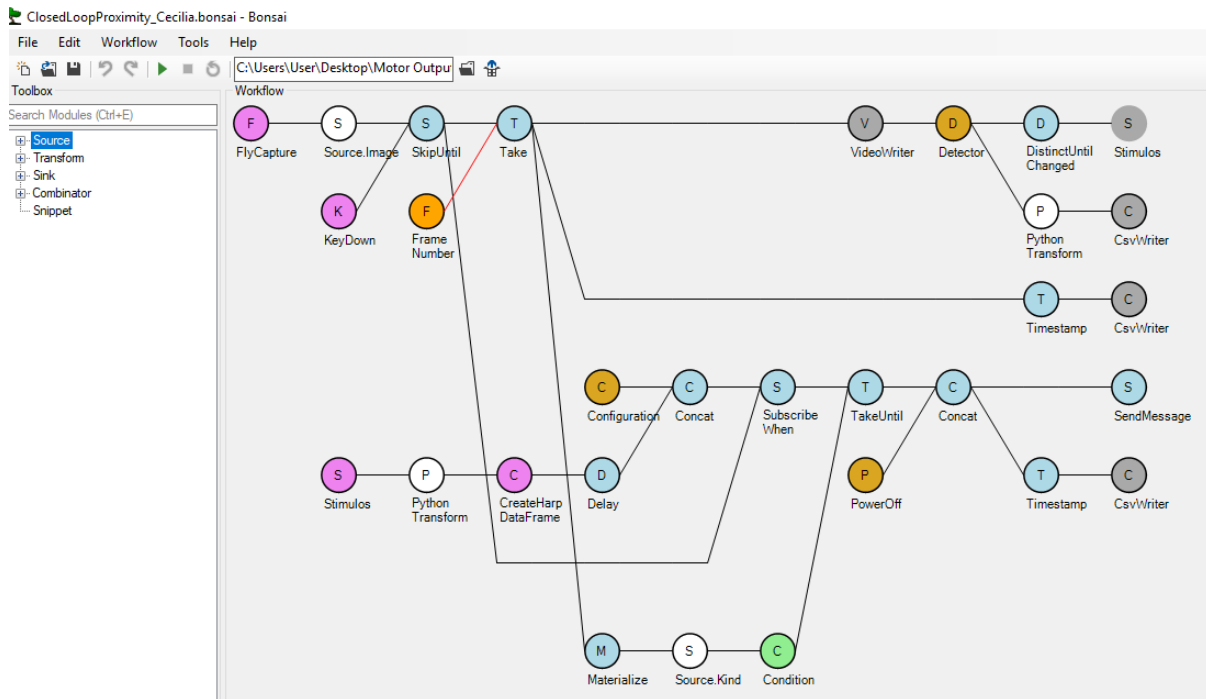


Figure 2.2: Bonsai file for closed-loop activation experiments. Bonsai has the objective of simplifying and accelerating the development of software for acquiring and processing the several heterogeneous data sources commonly used in neuroscience. It allows closed-loop control of stimulation, where the action of the animal directly modulates manipulation under the user's control.

2.3.1. Activation experiments

In *Drosophila melanogaster* system, it is necessary dietary supplementation of the all-trans-retinal (t-Retinal) co-factor to produce functional Chrimson. It is also critical to raise the flies fed with t-Retinal in the dark, since Chrimson can be inactivated by excessive light exposure, due to its photosensitivity⁶¹. For activation experiments, we supplemented t-Retinal to the food of manipulated females, which allows them to perform Chrimson-induced behaviours. These females were kept at least three days in the dark, which are necessary to achieve enough activity of Chrimson. Female flies raised on food without t-Retinal constitute the control group for these activation experiments.

For optogenetic stimulation, we used 610 nm red LEDs.

2.3.1.1. Neuronal activation experiments in virgin females

Behavioural interactions between a *w; 1549-AD/+; 1549-DBD/UAS-Chrimson*; virgin female and a CS naïve male were recorded for 20 minutes or until copulation. We decided to record for these durations, since flies copulate within 5-10 minutes⁶². Every time the recorded pair was at courtship distance (5,5 mm or less), the red LED would switch on in a closed-loop manner, causing the activation of the neurons and, consequently, the occurrence of OE.

2.3.1.2. Neuronal activation in competition experiments between females

Competition between a *w; 1549-AD/+; 1549-DBD/UAS-Chrimson*; virgin female raised on food supplemented with t-Retinal and a *w; 1549-AD/+; 1549-DBD/UAS-Chrimson*; virgin female without t-Retinal as a food supplement for a CS naïve male was recorded for 20 minutes or until the occurrence of mating. The flies were under constant illumination from the red LED.

2.3.1.3. 5-minute neuronal activation experiments in females from three different strains

w; 1549-AD/+; 1549-DBD/UAS-Chrimson; , *w; 1549-AD/+; dsx-DBD/UAS-Chrimson*; and *w; 1549-AD/+; dsx-DBD, UAS-Chrimson/UAS-Chrimson*; virgin females raised in t-Retinal food were recorded during 5 minutes under red light constant illumination.

2.3.2. Neuronal inactivation experiments

Neuronal inactivation experiments were both performed in virgin and mated females. Behavioural interactions between a *w; 1549-AD/ UAS-Kir2.1; dsx-DBD/+*; female and a CS naïve male were recorded for 20 minutes or until the occurrence of copulation. *w; 1549-AD/+; dsx-DBD/+*; and *w; UAS-Kir2.1/+; +*; females constituted the control groups for these experiments.

For experiments involving mated females, a virgin female was mated with a virgin male 15 hours before the experiments. For the behavioural experiment, a new naïve male was added to the experimental set up with the previously mated female.

2.4. Video processing and automatic classification of behaviour

The recorded videos of the behavioural experiments were processed in a modified version of the software Caltech FlyTracker, a system that tracks pairs of fruit flies⁶³. Before processing the videos in Caltech FlyTracker, it was necessary to perform two different steps: first, videos were run in Calibrator, a function that enables the user to define resolution (frame rate and diameter of the chamber) (Fig. 2.3. A), experimental setup parameters (definition of number, shape and flies per chamber) (Fig. 2.3. B) and parameter tuning (foreground and body thresholds of the flies) (Fig. 2.3. C).

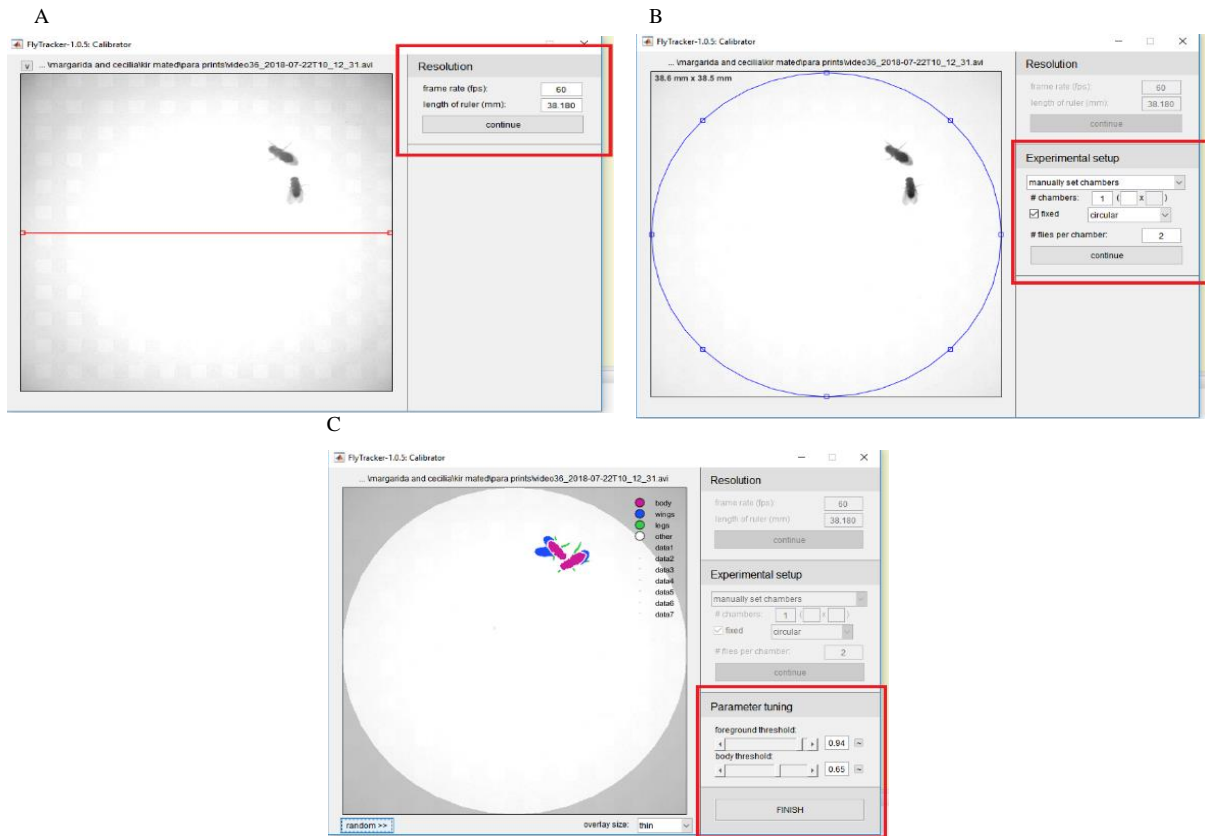


Figure 2.3.: Calibrator function. A) Definition of resolution: frame rate and diameter of the chamber; B) Definition of experimental setup parameters: definition of number, shape and flies per chamber; C) Definition of parameter tuning: foreground threshold and body threshold.

The second step involves the use of Visualizer, a function that displays the video image and tracks the pair of flies (ellipse, trail wings and legs). It allows to correct the flies' identity in terms of sexes when it is swapped throughout the video (Fig. 2.4.). Videos are then processed in Caltech FlyTracker, which returns as an output information regarding flies' distance, velocity and position.

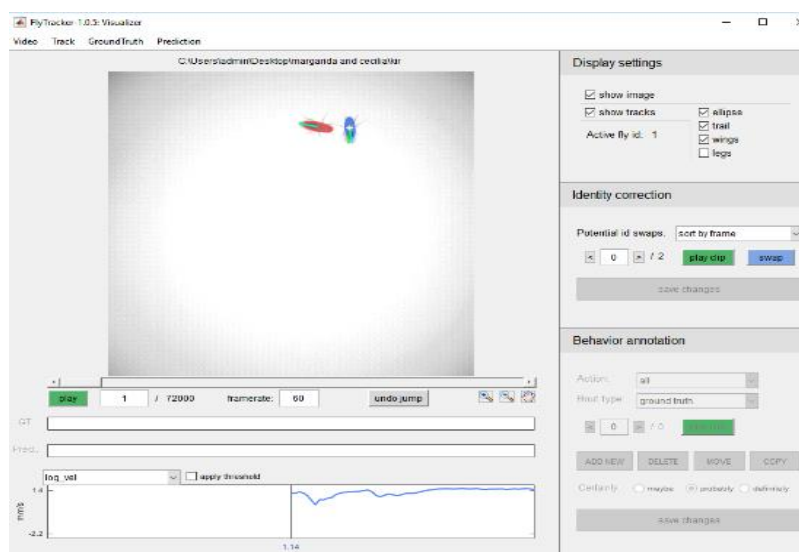


Figure 2.4.: Visualizer function. This function allows the correction of flies' identity in terms of sexes.

For the classification of courtship behaviours, a Janelia Automatic Animal Behaviour (JAABA) classifier was used (Fig. 2.5.). This classifier was previously developed in the lab and presents a high accuracy (approximately 90%). JAABA is a machine learning-based system that allows the creation of automatic behaviour classifiers⁶⁴. Post-processing tools were applied after the classifiers' predictions to avoid classifying bouts shorter or longer than normal behavioural bouts. For this, we just considered the occurrence of the behaviour when the number of frames including it was higher than 30. We also used JAABA software to develop an automatic classifier for OE. Its development will be detailed in section 3.6.

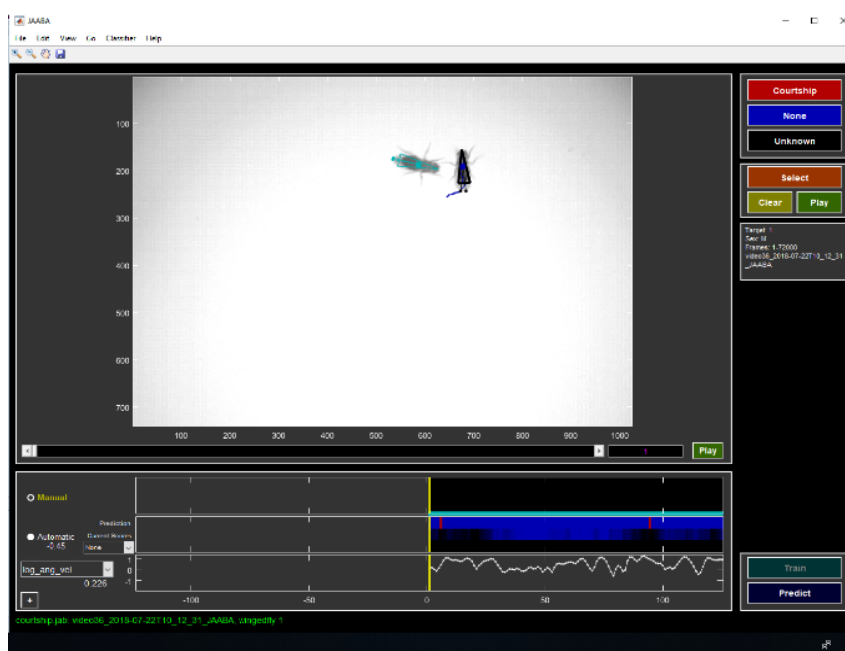


Figure 2.5.: JAABA automatic classifier for courtship. JAABA allows automatically computing interpretable, quantitative measures of animal behaviour.

2.5. Manual annotations of behaviour

Behaviours, such as full OE, partial OE, attempted copulation and copulation were manually annotated in PythonVideoAnnotator, an in-house developed software (Fig. 2.6.). It is a graphical application written in Python, whose main goal is to analyse videos and label events notes⁶⁵. Courtship annotations generated by the automatic JAABA classifier were converted from .jab format to .csv, with a script developed in the lab. This script allowed the annotations to be read in PythonVideoAnnotator, which enabled the user to check if the output from JAABA coincided with the actual occurrence of courtship. Manual annotations were done from the moment the male initiates courtship, after confirming the first annotation returned by the automatic courtship classifier, up to ten minutes or until copulation. We chose this amount of time to annotate, since flies start their courtship ritual within 5-60 seconds and copulate within 5-10 minutes⁶².

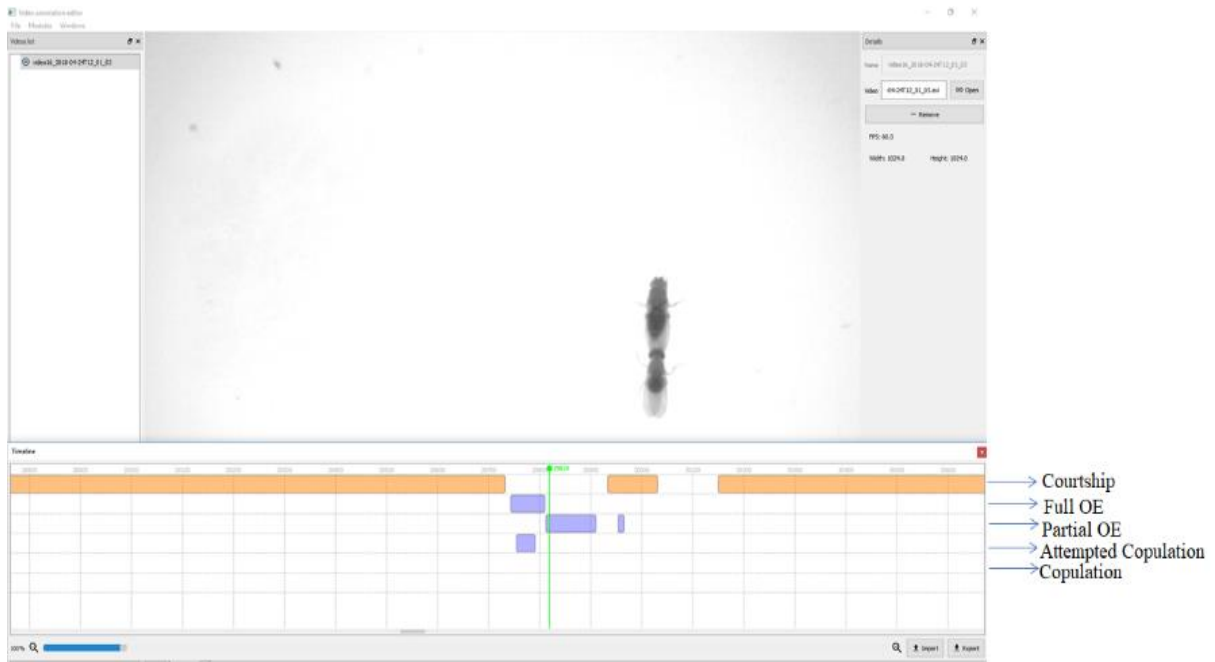


Figure 2.6.: PythonVideoAnnotator. PythonVideoAnnotator allows annotating events, while analysing videos. The order of the behavioural annotations is depicted on the right.

2.6. Graphical representations and statistical analysis

Annotations from PythonVideoAnnotator and JAABA software were read in in-house developed Python scripts. These use the information depicted in the annotations for the development of graphical representations. Afterwards, statistical tests were performed. To calculate the percentage of occurrence of copulation or courtship, Fisher's exact test was done. To compare courtship behaviours between control and manipulation groups, Mann-Whitney U test were applied. In the case of the experiment containing two controls and a manipulated group, Kruskal-Wallis was applied. For further pair comparison between these three conditions, Mann-Whitney U test was performed. Bonferroni correction was also used for p-value adjustment.

Chapter III

Results

This project was developed in close collaboration with Cecilia Mezzera, a postdoctoral fellow in Luísa Vasconcelos's lab.

OE is a female response exclusive of courtship⁴⁴, which indicates its important role in this set of behavioural interactions. We proposed to unravel the role of female *Drosophila melanogaster* OE during courtship. Since we knew which neurons are involved in OE behaviour, we performed neuronal activation experiments, as well as neuronal silencing experiments. These manipulations and their consequences in courtship will further clarify the meaning of OE.

3.1. OE line-Chrimson neuronal activation in virgin females

Depending on their receptivity state, female responses to male courtship vary in type and frequency^{24,27}. Virgin females are receptive, mating eventually with the courting male. However, they still perform rejection behaviours as a first reaction to male courtship²⁷. Firstly, we asked whether overexpression of OE in virgin females affected courtship. We know that OE is overexpressed by neuronal activation of OE line-Chrimson female flies. To answer our question, the behavioural interactions between an OE line-Chrimson virgin female fly and a wild-type virgin male fly were recorded for 20 minutes or until copulation. The expression of Chrimson was driven in the neurons of the OE line for neuronal activation. To be functional, Chrimson needs t-Retinal, which is not produced by fruit flies⁶¹. We supplemented it to the food, and used female flies raised in normal food as a control group. Every time the recorded pair was at courtship distance ($\leq 5,5$ mm), the 610 nm LED would switch on, leading to the activation of the neurons. Consequently, the manipulated female would extrude the ovipositor every time the male courted her. We then manually annotated OE events in the videos since the beginning of courtship to ten minutes or until copulation. Female flies perform two types of OE depending whether the ovipositor is extruded partially or completely²⁴. Considering this aspect, we annotated separately these two forms of behaviour, referring to them as 'partial OE' and 'full OE', respectively. We also annotated copulation events, as well as attempted copulation events since the first courtship bout up to 10 minutes or until copulation. Additionally, we ran the automatic courtship classifier (JAABA) previously developed in the lab, which gives as output annotations of courtship behaviour, allowing to measure several parameters of courtship. These behavioural annotations were depicted in a raster plot that provided a global overview of the interactions between the pairs of flies (Fig. 3.1.). Then, we proceeded to obtain quantifications of these behaviours.

We started by asking if the neuronal activation was inducing an increase in the amount of OE performed by the manipulated females. We calculated the percentage of total, full and partial OE relative to video duration. These measures correspond to the amount of time the female performs the sum of both types of OE, full OE and partial OE, respectively, since the beginning of the video up to 10 minutes or until copulation. We observed a dramatic increase in OE, namely for full OE (Fig. 3.2. A, B). Wild-type virgin females usually perform partial extrusion. This information was further verified in the control group of this experiment (Fig. 3.2. C).

Then, we looked at the consequences of OE overexpression in copulation. We calculated the percentage of copulation, defined by the percentage of flies that mated within the duration of the video. We found that overexpression of OE led to a significant reduction in copulation. Although the OE seems to function as a physical barrier, females are still able to mate (Fig. 3.2. D). The fact that OE does not effectively block copulation might have two explanations: either the female voluntarily retracts the ovipositor to mate or there is an intermittence in neuronal activation, leading to a discontinuous OE

occurrence. We also measured the time until copulation occurred (latency of copulation), in minutes, and we found no significant differences between groups (Fig. 3.2. E)

Since copulation decreases when OE is overexpressed, we asked whether courtship was also affected by this manipulation. We measured the percentage of videos where courtship occurs. No significant differences were observed between groups (Fig. 3.2. F). We determined the percentage of courtship per fly, defined as the fraction of time the male spends on courtship since the start of the video up to ten minutes or until the occurrence of mating, relative to the video duration. Also, no statistical differences were observed between control and manipulated flies (Fig 3.2. G). Lastly, we calculated the latency to court, which is the time, in minutes, until the male begins courtship. Likewise, there were no differences between groups (Fig. 3.2. H).

We did not observe any effects on courtship in our experiments. Nevertheless, we decided to see if attempted copulation, a specific courtship behaviour, was affected by OE overexpression. We then determined the percentage of attempted copulation duration per fly, which corresponds to the fraction of time the male spends on attempted copulation events since the start of the video up to ten minutes or until mating occurs, relative to the video duration. We also calculated the number of copulation attempts per minute. Both these statistics indicate that OE overexpression did not affect significantly attempted copulation events (Fig. 3.2. I, J).

These results show that neuronal activation of OE line-Chrimson t-retinal females leads to OE overexpression: females perform significantly more OE, namely full OE (Fig. 3.2. A, B). These results also show that OE leads to a marked decrease in copulation (Fig. 3.2. D).

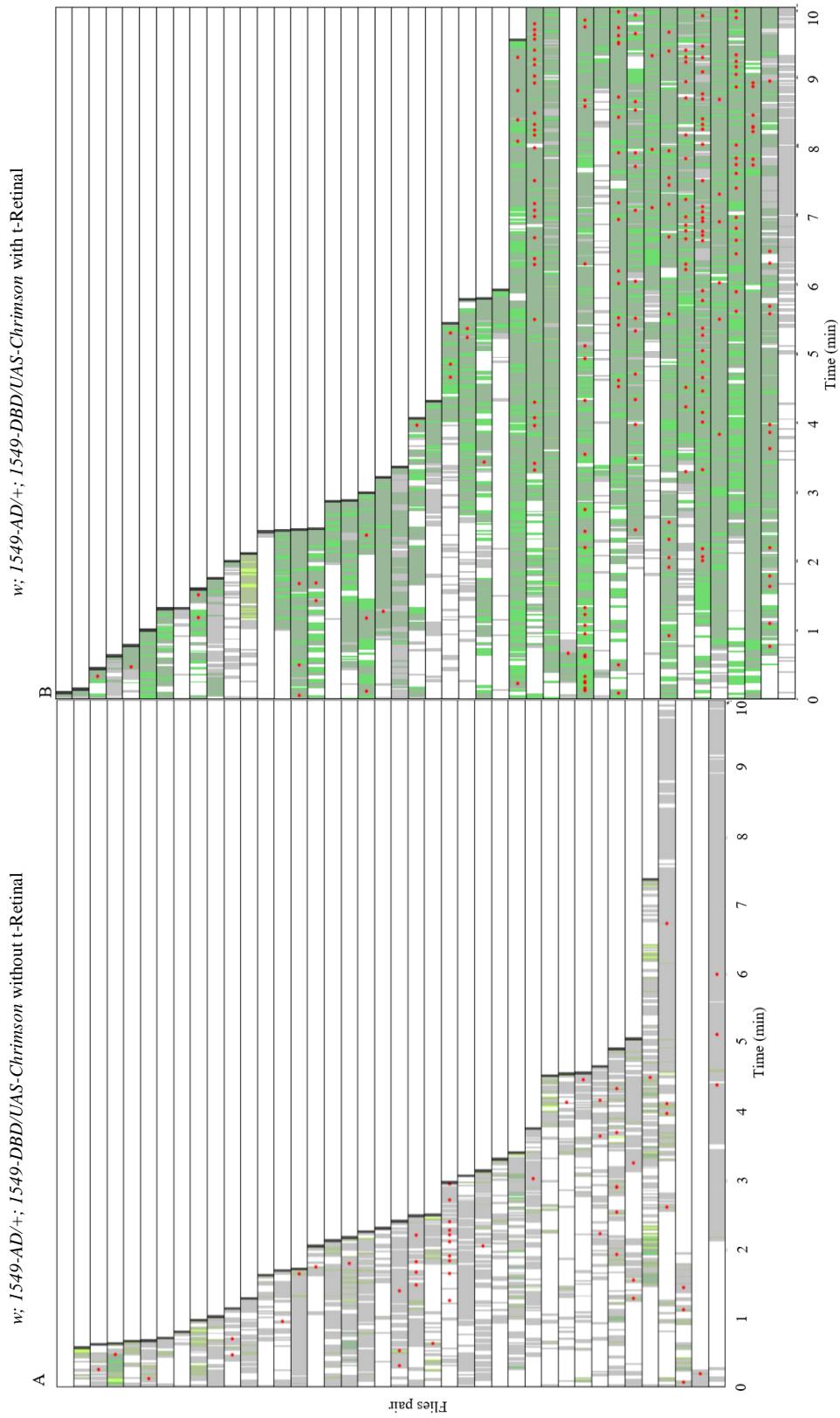


Figure 3.1.: Temporal dynamics of the behavioural interactions between a wild-type male and a virgin female in the first 10 minutes of the video. Each line is one recorded pair of flies. A) Virgin control (n = 40); B) Virgin with overexpression of OE (n = 44).

Chapter III - Results

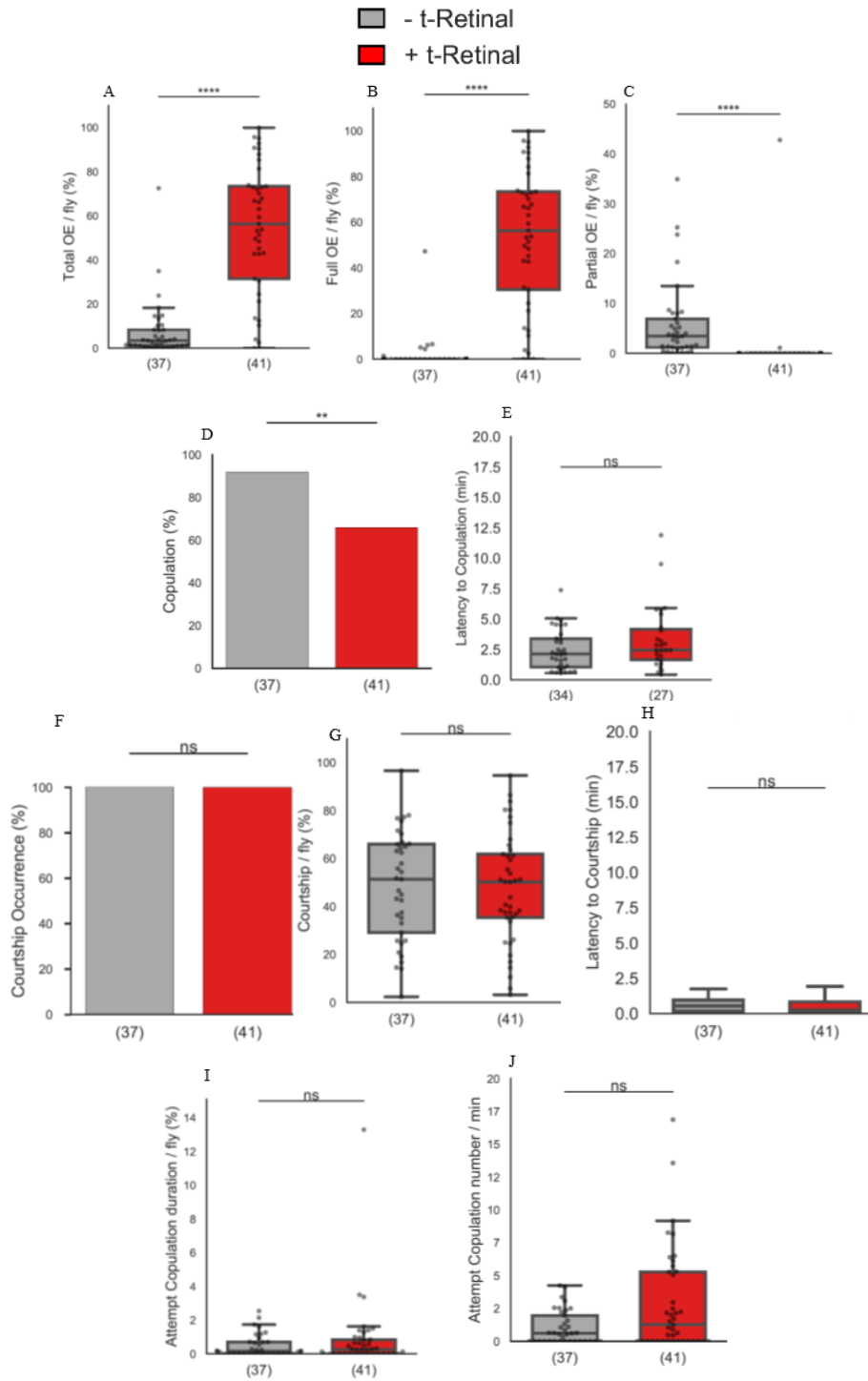


Figure 3.2.: OE overexpression in OE line-Chrimson virgin females leads to an increase in OE, as well as to a significant decrease in copulation. A) Total OE duration; B) Full OE duration; C) Partial OE duration; D) Quantification of mating; E) Time (in minutes) until the occurrence of copulation; F) Quantification of courtship; G) Male courtship index, during the first 10 min of courtship or until copulation; H) Time (in minutes) at which the male begins courtship; I) Quantification of attempted copulation events; J) Number of attempted copulation events per minute. Sample size is shown in parenthesis. ns, not significant, ** $p < 0.01$, **** $p < 0.0001$. Fisher's exact test for occurrence of mating and courtship and Mann-Whitney U test for the other statistical analysis.

3.2. OE line-Chrimson neuronal activation competition between females

To further dissect the role of OE, we wanted to infer if performing OE is advantageous or disadvantageous for the female. For this purpose, we tested whether the male would prefer a female that often extrudes her ovipositor or a female that performs OE normally. Two females, an OE line-Chrimson virgin female raised without t-Retinal and an OE line-Chrimson virgin female supplemented with t-Retinal, were placed in the arena along with a wild-type naïve male. Their behavioural interactions were recorded for 20 minutes or until mating. The flies were under constant 610 nm red LED illumination, which leads to the continuous overexpression of OE in OE line-Chrimson virgin females with t-Retinal.

Firstly, we asked whether the male would prefer to copulate with a female with overexpressed OE or a female that extrudes normally her ovipositor. We observed a higher percentage of copulation with control females (64,7%), in comparison to females that extruded the ovipositor constantly (29,4%) (Fig. 3.3. A). We also measured the latency to copulate and we did not find significant differences between the two females (Fig. 3.3. B).

Next, we asked whether the male showed any courtship preference. We calculated the percentage of courtship per female and noticed that control females were more courted than females with overexpression of OE (Fig. 3.3. C). Latency to court was also evaluated and there were no significant differences between females (Fig. 3.3. D).

We then calculated the time and number of attempted copulation events. No significant differences were observed between control and tested females (Fig. 3.3. E, F).

These results indicate that males prefer to copulate and court with females that perform normal OE rather than females that extrude more frequently the ovipositor.

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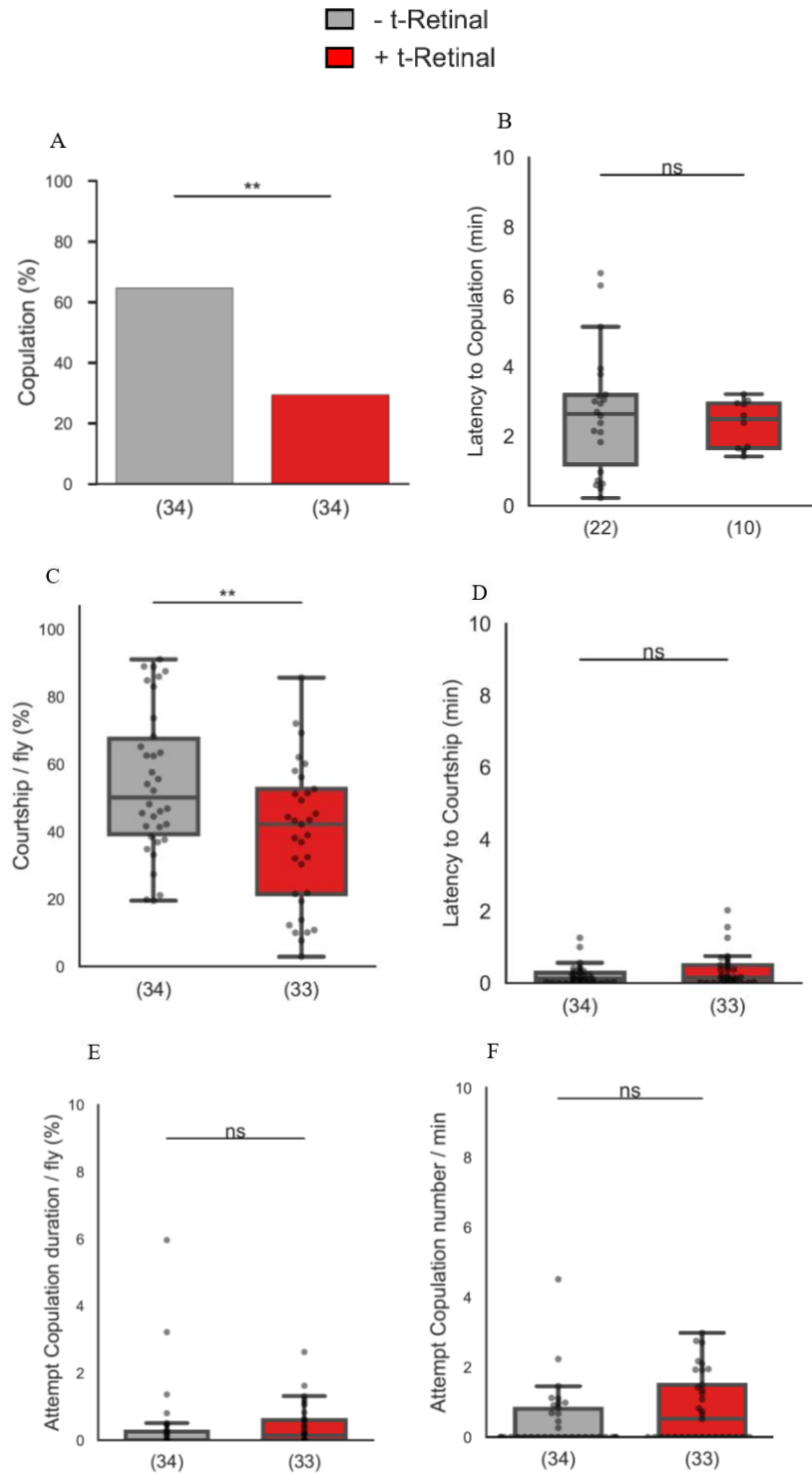


Figure 3.3.: Males prefer to copulate and court with females that extrude their ovipositor normally rather than females that extrude their ovipositor more frequently. A) Quantification of mating; B) Time (in minutes) until the occurrence of copulation; C) Male courtship index, during the first 10 min of courtship or until copulation; D) Time (in minutes) at which the male begins courtship; E) Quantification of attempted copulation events; F) Number of attempted copulation events per minute. Sample size is shown in parenthesis. ns, not significant, ** $p < 0.01$. Fisher's exact test for occurrence of mating and courtship and Mann-Whitney U test for the other parameters.

3.3. 5-minute OE activation

We performed OE neuronal activation experiments with OE line, which labelled P7 and P13 neurons, crossed with Chrimson (see section 3.1.). These experiments showed that OE overexpression led to a decrease in copulation but does not block completely this action (Fig. 3.2. D). We hypothesized that, despite continuous neuronal activation of OE line, it does not lead to a continuous activation of OE, which causes intermittences in the behaviour.

Cecilia Mezzera found that P13 is the neuron responsible for OE. Additionally, she also found that P13 is *dsx*-positive. Crossing the AD element of OE line with the *dsx* DBD element led to the creation of the P13-OE line.

Taking advantage of the availability of two lines that can manipulate OE, we wanted to validate our hypothesis regarding the intermittent character of OE. In case it verified, we wanted to check which line, when crossed with one copy of Chrimson, lead to a higher level of OE activation. For this purpose, OE-Chrimson and P13-OE-Chrimson lines female flies raised on food supplied with t-Retinal were presented to constant 610 nm LED light for 5 minutes. We also tested females from a line with two copies of Chrimson. This is a recombinant line, P13-OE-Chrimson, crossed with Chrimson.

We manually annotated OE events for the whole video duration. Since effective OE activation is characterized by full OE, we annotated separately ‘partial OE’ and ‘full OE’. Based on these annotations, raster plots were then generated. These depicted the temporal occurrence of the two different forms of OE for each manipulated female.

We found that OE-Chrimson line displayed a higher level of activation in comparison to the other two lines. During the 5 minutes activation, OE line female flies mainly displayed full OE, as well as they seemed to show a short time latency to extrude their ovipositor. Out of 20 tested flies, only 2 of them did not perform any type of extrusion (Fig. 3.4. A).

Opposingly, OE-P13-Chrimson line obtained the lower level of activation: the predominant form of extrusion is partial, having 5 out of 20 flies just performed partial OE during the 5 minute-activation. Only 4 of the flies extruded fully their ovipositor consistently through time. 3 of them did not extrude their ovipositor at any time. Regarding latency to OE, they seemed to take more time to perform extrusion in comparison to the OE line and, even when they perform it earlier, it was not a continuous event (Fig 3.4. B).

These results show that, of the three lines tested, OE-line crossed with one copy of Chrimson is the one that leads to a higher level of OE activation. Furthermore, they also seem to indicate that continuous neuronal activation does not result in continuous OE. The intermittency of OE allows females to mate.

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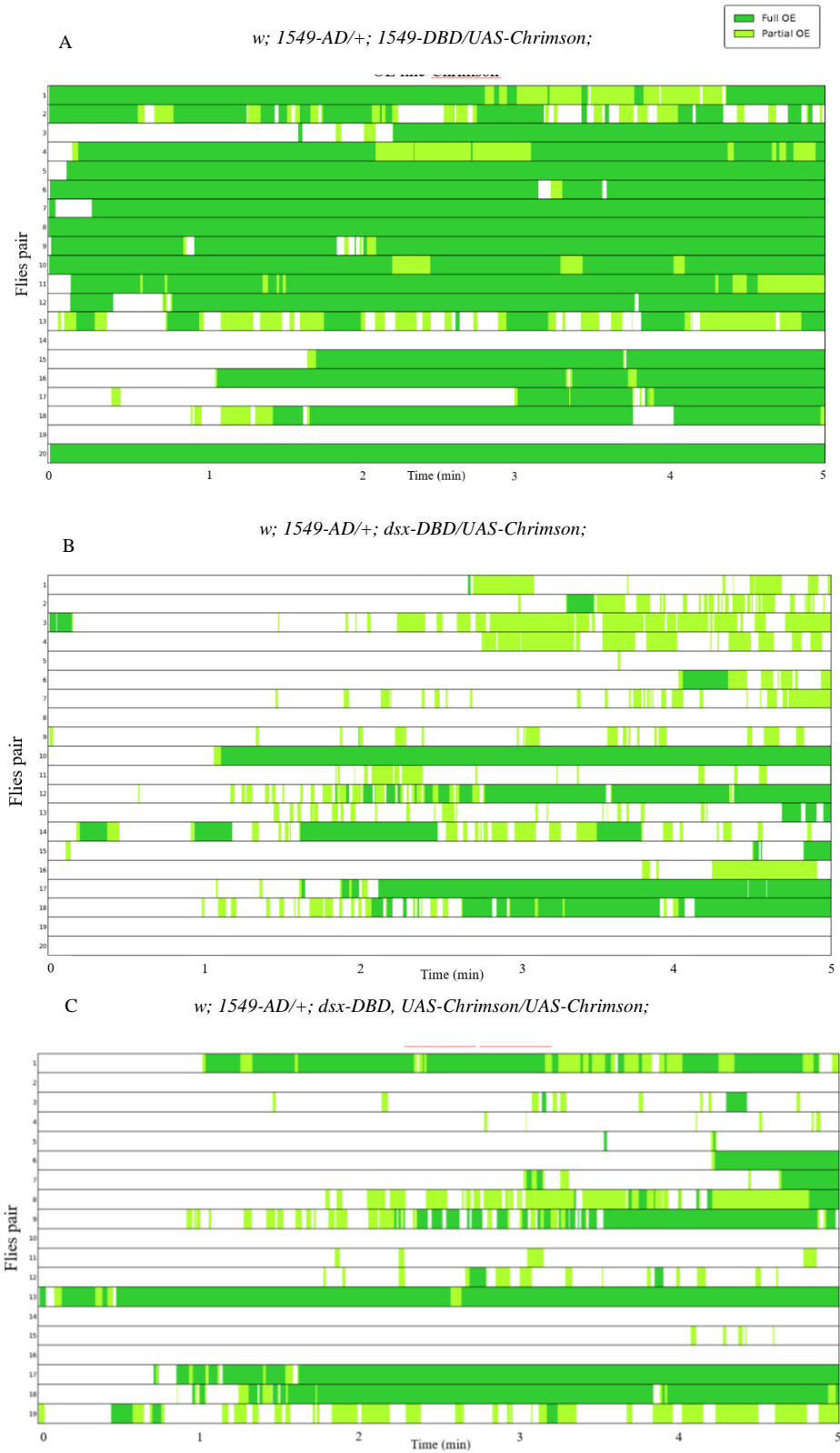


Figure 3.4.: Full and partial OE events during the five minutes of constant red LED illumination in t-Retinal virgin females. A) OE-Chrimson line (n = 20); B) P13-OE-Chrimson line (n = 20); C) P13-OE-Chrimson-Chrimson line (n =19). Each line represents one fly.

3.4. OE-P13-line silencing in virgin females

OE overexpression leads to a significant decrease in copulation in virgins (Fig 3.2. D). We next asked if, by inhibiting OE, we would observe an increase in copulation.

By the time we were posing these questions, we obtained the P13-OE line, which only labels the P13 neuron. When the expression of Chrimson was driven in the P13 neuron, OE overexpression would occur. We then used the P13-OE line in these experiments of OE inhibition. For this purpose, we silenced the P13 neuron with Kir2.1 and test the effect of this manipulation in courtship. As control lines, we used Kir2.1 crossed with the white mutant line and P13-OE line crossed with the white mutant line. We recorded the behavioural interactions between a virgin female and a naïve male for 20 minutes or until the occurrence of copulation. Then, we manually annotated full OE, partial OE, copulation and attempted copulation, as well as we ran the automatic courtship classifier. With these annotations, we obtained the temporal dynamics of behavioural responses between the male and the female (Fig. 3.5). We then quantified these behaviours.

We first checked whether silencing P13 neuron was inducing a decrease in the amount of OE. We found that P13 silencing in virgin females did not lead to a significant full nor partial OE inhibition (Fig. 3.6. A, B, C). We confirmed that Kir2.1. was functioning correctly. Regarding this result, we could not draw any conclusions about the effect of OE silencing on virgin females. Thus, we did not calculate measurements for the other behaviours.

These results show that silencing of the P13 neuron did not lead to OE inhibition. Yet, similarly to OE line-Chrimson neuronal activation in virgin females experiment (Fig. 3.2. C), results in control groups confirm that partial OE is the main form of extrusion performed by virgin females.

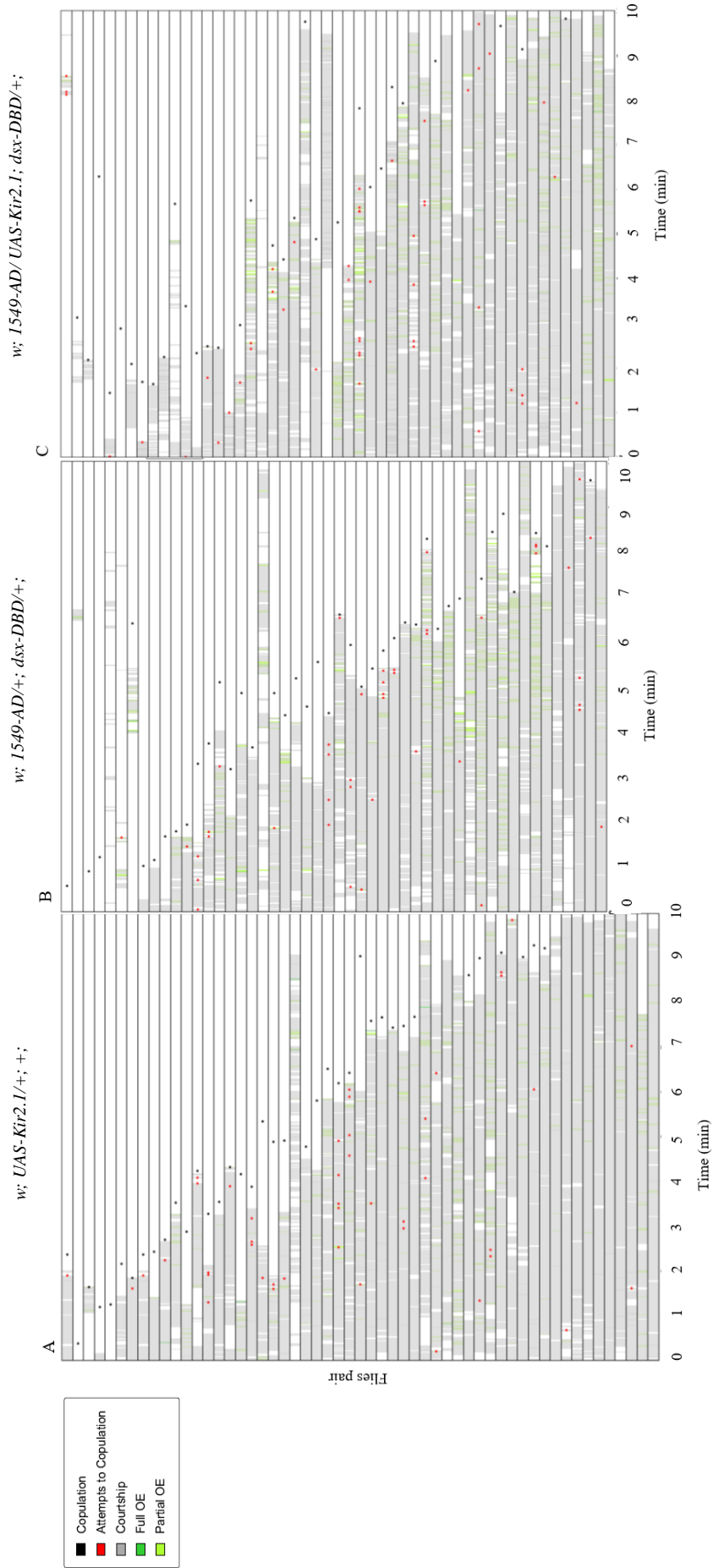


Figure 3. 5.: Temporal dynamics of the behavioural responses between a wild-type male and a virgin female in the first 10 minutes of the video. Each line is one recorded pair of flies. A) and B) Virgin controls (n = 55 and n = 50, respectively); C) Virgin with inhibition of OE (n = 51).

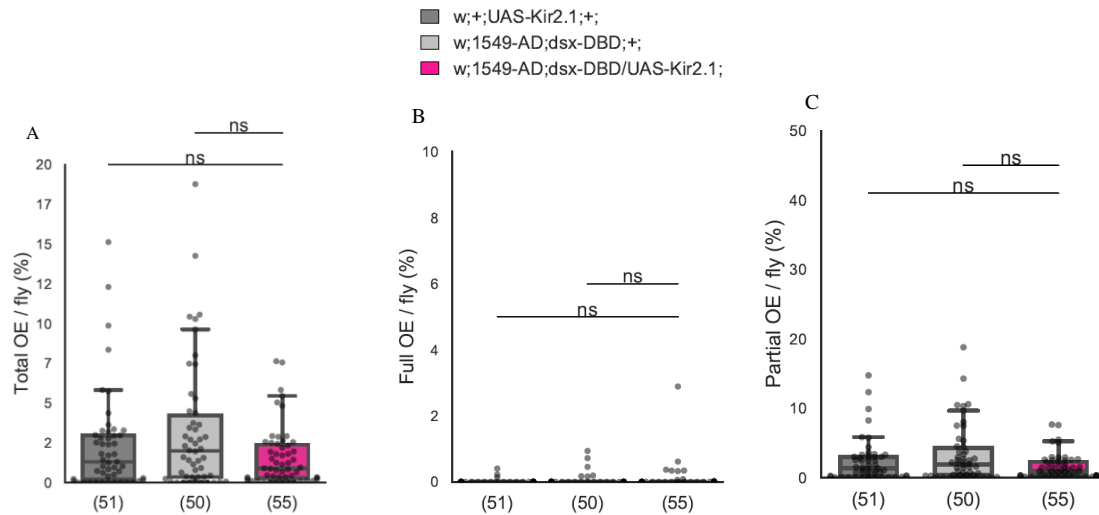


Figure 3.6.: Silencing the P13 neuron in virgin females does not lead to OE inhibition. A) Total OE duration; B) Full OE duration; C) Partial OE duration. Sample size is shown in parenthesis. ns, not significant. Kruskal Wallis with *post-hoc* Mann-Whitney U test with Bonferroni correction of the p-value for the other statistical analysis.

3.5. OE -P13-line silencing in mated females

Silencing the P13 neuron in female virgins did not lead to any significant effects (Fig. 3.6.). Mated females display OE much more often comparing to virgins, since it is their main response to courtship²⁷. We then further investigated the possibility that silencing the P13 neuron in mated females would lead to effective OE inhibition. To address this question, we carried the same protocol as presented in 3.4. with mated females. We obtained the global overview of the interactions between the pairs and proceeded to obtain behavioural quantifications (Fig. 3.7.).

Indeed, we found a reduction of both types of OE in manipulated flies, in comparison to control ones (Fig. 3.8. A, B, C). These results indicate that silencing P13 neuron with Kir2.1. in mated females leads to OE inhibition. Furthermore, control groups of this experiment confirm that mated females perform mainly full OE (Fig. 3.8. B).

Since OE overexpression led to a marked decrease in copulation, we asked whether silencing the P13 neuron would induce mated females, normally unreceptive, to mate again. This manipulation did not restore copulation in mated females (Fig. 3.8. D).

We then asked whether P13 silencing would lead to alterations in courtship. We looked at courtship occurrence, percentage of courtship per fly and latency to courtship. No significant differences between groups were observed in any of these measurements of courtship (Fig. 3.8. E, F, G). It is important to point out that all courtship indexes were higher than 60%. These are higher than the ones obtained in OE-Chrimson line neuronal activation experiments (3.2. G), which were approximately 50%. This seems to indicate that the LED is affecting the male's courtship performance.

However, we found that attempted copulation was influenced by OE inhibition. Both the time the male spent in attempted copulation and the number of attempts exhibited a dramatic decrease in the group of females with OE inhibited (Fig. 3.8. H, I). These results indicate that OE influences copulation attempts.

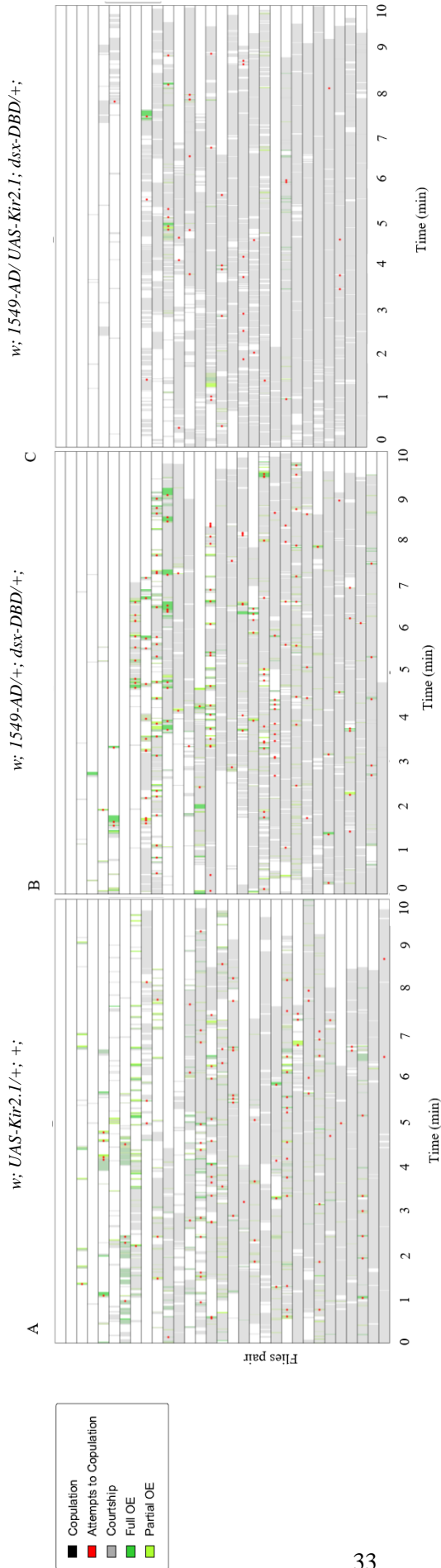


Figure 3.7.: Temporal dynamics of the behavioural interactions between a wild-type male and a mated female in the first 10 minutes of the video. Each line is one recorded pair of flies. A) and B) Virgin controls (n = 31 and n = 31, respectively); C) Virgin with inhibition of OE (n = 29).

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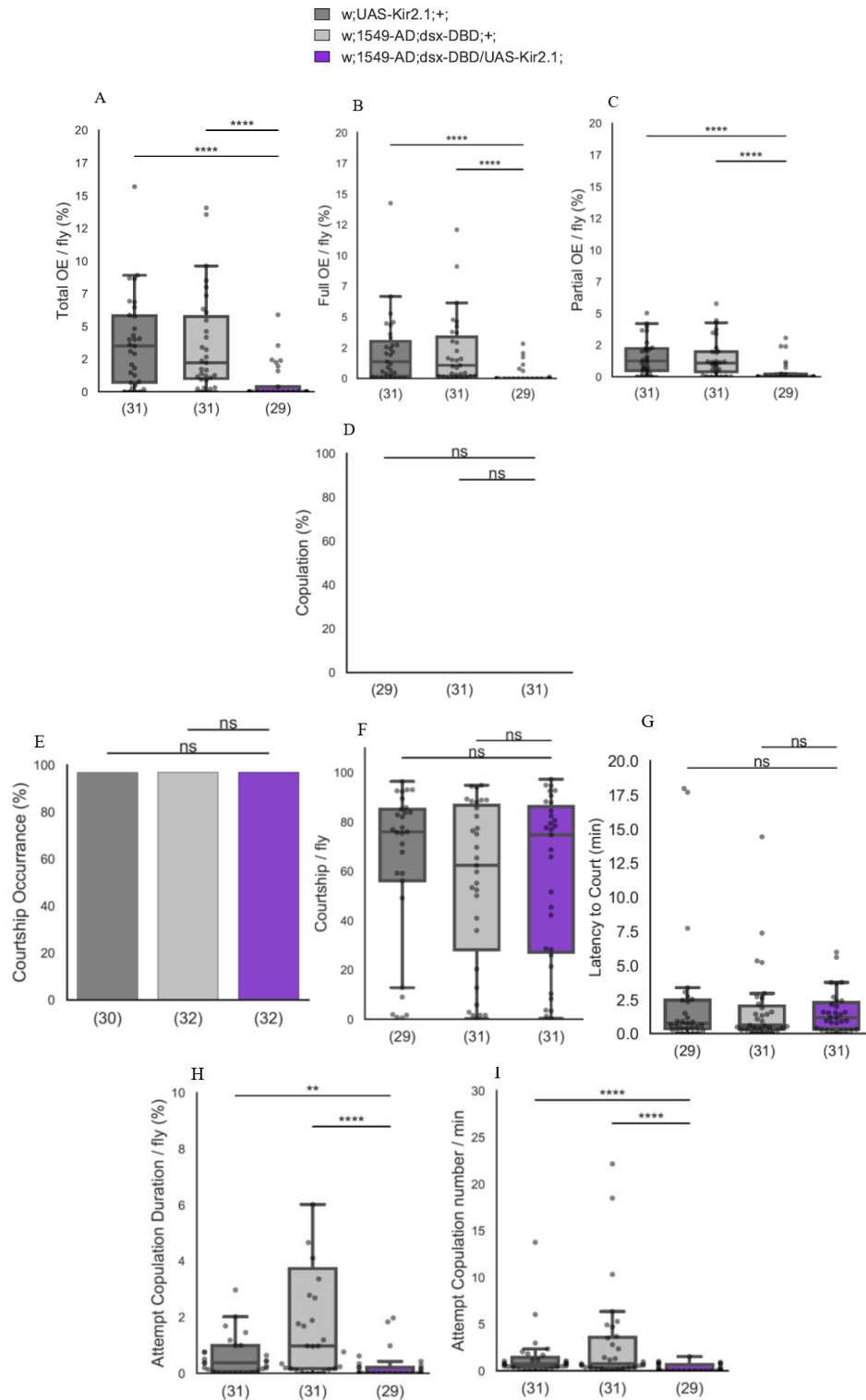


Figure 3.8.: Silencing the P13 neuron in mated females leads to total OE inhibition, as well as a marked decrease in attempted copulation events. A) Total OE duration; B) Full OE duration; C) Partial OE duration; D) Quantification of mating; E) Quantification of courtship; F) Male courtship index towards females of the indicated genotype, during the first 10 min of courtship; G) Time (in minutes) until the male begins courtship; H) Quantification of attempted copulation events; I) Number of attempted copulation events per minute. Sample size is shown in parenthesis. ns, not significant, **p < 0.01, ****p < 0.0001. Fisher's exact test for occurrence of mating and courtship and Kruskal Wallis test, with *post-hoc* Mann-Whitney U test with Bonferroni correction of the p-value for the other statistical analysis.

3.6. Development of an automatic OE classifier

Manual annotation of behaviour can be a time-consuming task. Automated, high-throughput detection of behavioural output can solve this issue, as well as provide more accurate and detailed quantification of behaviour. We then set out to develop an automatic OE classifier. For this purpose, we used JAABA software, which had been previously used to develop other behaviour classifiers in the lab. With this software, users label the behaviour in question of the animal in a small set of video frames, according to their intuition regarding the structure of the behaviour. Then, it uses machine learning techniques that convert the users' manual labels into behaviour detectors. These can be used to automatically classify the behaviours of animals^{64,66}.

The first step of developing a classifier in JAABA is the selection of per-frame features. Per-frame features are computed from the input trajectories and give information about the animal state in the current frame. We developed several different classifiers for OE, based on different per-frame features. The first classifier had all the per-frame features selected. Then, after labelling some frames with this first classifier, we looked at the histograms of the per-frame features over labelled data. We found differences between the histogram over OE labels and the another one over non-OE labels for position and distance per-frame features. We then developed another classifier based on these two per-frame features. We also developed other classifiers based on body axis, velocity or on both these per-frame features. We chose these two per-frame features, since the length of the female body axis becomes longer when OE occurs, as well as female velocity seems to decrease when she extrudes the ovipositor.

Next step was training the classifier. For this, we annotated whether the fly was displaying OE ([Behaviour]) or not ([None]). After labelling some bouts, we trained the classifier. We also took into consideration not labelling frames whose behavioural class was not clear, as well as having a balanced number of examples from both behavioural classes. For this last aspect, after training the classifier for the first time, we tried to label only examples with low confidence or that were incorrectly predicted.

Afterwards, we proceeded to perform ground-truthing. This step quantitatively measured the classifier's performance, considering its accuracy in predicting the occurrence of behaviour in frames not included on the training step. For this purpose, we annotated a random selection of frames, being blind to the classifier's predictions on these frames. These were used as the ground-truth against which the classifier's predictions were compared. It is important to use an unbiased method to choose frames to label. We used a balanced random selection of frames, which is suitable for behaviours that occur in low frequencies, which is the case for OE. In this selection, JAABA selects bouts to label randomly, but increases the weight of intervals that contain predicted positive frames. In ground-truthing mode, it is possible to label behaviour, depending on the user's confidence on the frames: frames that were clearly the behaviour or not the behaviour were labelled as Important [Behaviour] and Important [None], respectively; frames in which the behaviour or not-behaviour is somewhat present, but not clear for the user are labelled as [Behaviour] or [None]. Lastly, the classifier compared our labels with its predictions and returned a table with the accuracy on ground-truthing labels. The columns of this table correspond to the classifier's prediction, and the rows to the manual labels. Each element of the table corresponds to the number and percentage of frames with the given type of user labels that have the given prediction.

All the classifiers obtained accuracies under 80% for [Behaviour] Important frames, which means that, if applied, the classifiers would return several false negatives. Accuracies for [None] Important frames were always lower than the ones obtained for [Behaviour] Important frames, which indicates that these classifiers would consider several false positives.

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The low accuracy values indicate that the OE classifiers that we developed are not accurate enough in detecting the occurrence of OE, nor detecting the absence of the behaviour. Therefore, we decided not to use it and to obtain the behaviours annotations manually, using PythonVideoAnnotator software. Low resolution of the video, together with the ovipositor being a small structure with little contrast with the background can explain why it is difficult to train the software to automatically detect OE. Also, the features that JAABA relies on may not be the most precise to lead to the creation of a classifier for this behaviour. Other alternatives must be pursued in the future for the further development of an automatic OE classifier.

Chapter IV

Discussion

The aim of this project was to unveil the role of the female *Drosophila melanogaster* OE during courtship. Since we identified command neurons for OE, we went on performing neuronal activation, using Chrimson, and neuronal silencing experiments, using Kir2.1. We then looked at different aspects of courtship to see how they were affected with these manipulations.

Firstly, we found that OE inhibition in mated females led to less attempted copulation events performed by the male. This further indicates that OE seems to entice copulation attempts. Two hypotheses can provide an explanation for this finding: either the male is attempting to copulate with OE to try to lead to its retraction and, consequently, get the possibility of mating, or OE presents chemical cues that may be attractive for the male. Some previous studies have observed an association between OE and attempted copulation events. Connolly and Cook observed that attempted copulation tends to occur after OE²⁷. Welbergen *et al.* also indicated OE as one of the enticing behaviours for attempted copulation events in *Drosophila melanogaster* studies⁴⁸. Both these studies are in the same line as our findings. Our results argue that the OE and attempted copulation relationship is causal.

We also showed that OE overexpression in virgin females led to a marked decrease in copulation. Nevertheless, OE does not block completely copulation, since neuronal activation of OE, although continuous, caused an intermittent display of the behaviour. Connolly and Cook had already suggested that the major effect of extrusion is the prevention of copulation. However, they make this statement regarding mated females, raising the hypothesis that either the behaviour of the male is altered, or there is a physical prevention of copulation, or both²⁷. If we are establishing this link between OE overexpression and copulation, one could expect that, likewise, copulation would be restored when OE was inhibited in mated females. Surprisingly, we did not verify this scenario. Mated females, due to the post-mating switch, are not receptive²⁹, and this information can be carried by different sensory cues present in different sources and not only by the ovipositor itself.

Furthermore, we showed that when the male is presented with the choice of a female that extrudes more often the ovipositor or a female that extrudes normally the ovipositor, he chooses to copulate and court with the latter one. The expression of this preference by the male shows that OE is not necessarily advantageous for the female. The result regarding copulation preference provides further support to OE functioning as a physical barrier for mating, despite not being totally effective.

OE overexpression and inhibition did not lead to any alterations in courtship when single pairs were tested. Nevertheless, attempted copulation, which is an example of a male courtship behaviour, was affected by OE inhibition. We then conclude that courtship is a rough parameter to measure the consequences of manipulating OE: specific male courtship behaviours might be more informative for this purpose.

Previous studies carried by Labsleiz *et al.* suggested antagonistic roles for the two forms of OE: extruding the ovipositor distally is enticing for male courtship, whilst its complete extrusion inhibits courtship²⁴. Connolly and Cook also indicated that mated females performed OE as their main response²⁷. In this project, we could start to establish a link between OE type, female status and its meaning underlying courtship aspects. In control groups, we observed that virgin females perform mainly partial extrusion, as well as mated females perform mainly full OE. This may be related to the fact that virgins are receptive, so partial OE might have an enticing role for courtship, whilst mated females are unreceptive²⁷, which may explain why full OE seems to act as a physical barrier for copulation.

P13 silencing in virgin females did not lead to OE inhibition. One possible explanation is that P13 is only involved in full OE and virgin females only perform partial extrusion. OE overexpression

in virgin females leads to a marked increase in full OE. Likewise, P13-silenced mated females have all forms of OE inhibited.

Cecilia Mezzera also performed egg-laying experiments in P13 neuron-silenced mated females, to investigate if this neuron was also involved in oviposition behaviour. Our results indicated that these females do not have their egg laying affected, which indicates that P13 neuron is not involved in the ovipositor behaviour.

We also showed that OE-Chrimson line is the one that displays a higher level of neuronal activation. This result was not expected, since OE-P13-Chrimson line, as well as OE-P13-Chrimson-Chrimson line only label P13 neuron, which would lead to a finer activation of OE. An explanation for this result might be the lower expression levels of OE-P13 line.

The present study identifies P13 as the neuron responsible for OE. We show that, depending on the sex, OE has a different meaning. In the case of the female, full extrusion has a rejecting connotation, impeding copulation when it occurs. Considering the role of OE for the male, it seems to entice attempted copulation events, while it appears to have a rejecting connotation for copulation itself. These findings contribute to the unravelling of the role of the female during courtship, both in behavioural terms, as well as regarding its underlying neural networks. This ultimately yields understanding of sexual communication and reproduction interactions, as well as species evolution.

In this project, we studied the effects of OE overexpression in virgin females, but we did not perform neuronal activation experiments on mated females. We also found out that the red LED used in neuronal activation experiments might be affecting the male's performance, but we did not look for this in further detail. We also demonstrated the intermittency of OE, despite continuous neuronal activation. Although, since one of our questions regarding this experiment was which line a higher level of OE activation had, we just performed a qualitative analysis. A quantitative analysis would provide further detail on the intermittent character of OE. While annotating videos of P13-neuron silencing mated females, we observed that females performed a frequent behaviour, which consisted on a highly specific abdominal bending, but we did not study this in further detail. We believe that it is a compensatory behaviour that the female performs when she is not able to extrude the ovipositor. Also, with these studies, we show that OE affects attempted copulation events but not courtship in general. Yet, we do not know the impact of OE on other specific male courtship behaviours. Also, with these studies, we have an insight about the impacts of OE on male courtship, but we did not have evaluated the factors that lead to OE occurrence. We hypothesise that male courtship song triggers OE. In fact, Connolly and Cook obtained data that indicate wing extension as an important factor for the causation of OE²⁷. This project also brought knowledge in the physical role of OE. Yet, experiments regarding its role from a chemical point of view are lacking. We observed that, before performing attempted copulations, males first lick the female's OE. It is known that most of the chemical substances that act during courtship are thought to be detected by gustation in the acts of tapping and licking²². This might indicate that OE has enticing chemical cues for the male.

For future studies, it would be interesting to:

- Perform neuronal activation experiments in mated females. For these experiments, we would use the same set up, but lower the intensity of the red LED in a way that would not compromise the male's performance and neuronal activation in females;
- Regarding the 5-minute neuronal activation experiments, quantify the OE duration (total, full and partial) relative to the video duration;

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- Identify possible compensatory behaviours for OE in P13-silenced mated females. To study this, we need to watch the videos again and annotate for these behaviours;
- Study if other specific male courtship behaviours, such as the “courtship song”, are affected by OE overexpression and inhibition. For this purpose, it is necessary to watch the videos again and perform manual annotations for these behaviours;
- Observe if OE occurs when the courtship song is eliminated. To evaluate this, behavioural experiments between a wingless male and a wild-type female, as well as between an aristaless female and a wild-type male would be recorded;
- Study licking and attempted copulation male behaviours, considering the context of OE. For this purpose, we would put a small tip of wax in the male’s proboscis, to inhibit licking, as well as in the tip of the female’s abdomen, to impede copulation attempts.

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