

# Post-copulatory behaviour in the female fruit fly *Drosophila melanogaster*

Meghan Laturney

## KEY WORDS

Diptera, neurons, pheromones, reproduction, sexual conflict, sperm

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Sexual reproduction is a combination of cooperation and conflict. Superficially, progeny production is the ultimate collaboration between the sexes, but below the surface males and females employ strategies to maximize their own reproductive success at a cost to the other. To gain a better understanding of how and why the interests of the sexes diverge during reproduction, I investigated post-copulatory sexual conflict in the fruit fly, *Drosophila melanogaster*. During copulation, males of this species apply chemicals to their female mates, diminishing female attractiveness. This tactic, known as chemical mate-guarding, is an adaptation evolved to reduce the probability of female remating and increase male reproductive output. After mating, however, I found that females employ a counter-adaptation, known as sperm ejection, which allows females to remove antiaphrodisiac pheromones, regain their attractiveness and increase polyandrous behaviour. Furthermore, I determined that the timing of sperm ejection is not only influenced by external cues of social context but is also actively controlled by the females' internal central nervous system. By regulating the timing of ejection, I found that females can adjust their remating rate, resulting in altered patterns of paternity, increased brood genetic diversity, and ultimately elevated female reproductive success. Therefore, female control over sperm ejection represents a female post-copulatory mechanism of sexual conflict resolution.

## Reproduction: Cooperation and conflict

Sexual reproduction is the most widely employed form of progeny propagation across taxa. That means for most species, males and females must mate in order to produce offspring. Initially, mating is beneficial to both males and females as it allows the pair to pass on their genes to future generations resulting in a mutual gain in reproductive success. This collaboration is, however, prone to exploitation.

Conflict between the sexes can arise when one partner pursues personal reproductive success at a cost to their previous mate. For example, one approach to increase male or female reproductive success is to escalate sexual activity by mating more often (Jennions & Petrie 2000). Since selection favours the individual who maximizes their own fitness, regardless of the consequences to others, behaviours like remating that increase an individual's personal offspring production will be selected for. Female remating, or otherwise known as polyandry, is no different. In general, polyandry is beneficial to females as it usually results in an increase in offspring quality and/or quantity. However, it is costly to her male mates as each copulation event introduces new sperm that must compete for access to a finite number of eggs, reducing the reproductive output of each male. Although polyandry is beneficial for females, it drastically reduces the siring capabilities of males, creating conflict between the sexes.

## Male-derived mechanisms of sexual conflict

Sexual conflict theory (Chapman *et al.* 2003) predicts that males should develop, over evolutionary time, adaptations to reduce polyandry and thereby maximizing the reproductive gain with each mating. Indeed, various mate-guarding strategies are observed in many different species. As insects relay heavily of chemical communication to advertise species, age, sex, and even experience, it is not surprising that chemical mate-guarding is a common method within this class (reviewed by Malouines 2017). This tactic involves males producing and transferring chemicals, known as antiaphrodisiac pheromones (AAPs), to their mates during copulation. For example, male fruit flies (*Drosophila melanogaster*) produce large quantities of two compounds: cis-vaccenyl acetate (cVA) and 7-tricosene (7-T). Although these chemicals are nearly absent on virgin females, they are transferred to females during mating (Jallon 1984, figure 1). As these chemicals act as AAPs, marked females elicit less courtship/copulation attempts from potential male suitors, resulting in a reduced polyandry and a gain in reproductive success of her initial mate (Scott 1986, Zawistowski & Richmond 1986).

## Identifying the female response

Male-derived adaptations like chemical mate-guarding that shift the tendency of polyandry towards a male-preferred

## NEV-Dissertatieprijs 2017

Tijdens de 29e Nederlandse Entomologendag (Ede, 15 december 2017) is de tiende NEV-Dissertatieprijs uitgereikt aan Dr. Meghan Laturney, voor haar proefschrift 'The second sex. Functions and mechanisms of sperm manipulation in female *Drosophila melanogaster*', op 8 december 2016 verdedigd aan de Rijksuniversiteit Groningen. De prijs bestaat uit een geldbedrag plus een oorkonde en wordt jaarlijks toegekend voor het beste proefschrift op het gebied van de entomologie, verdedigd aan een Nederlandse universiteit in het voorgaande academische jaar (1 september – 31 augustus). De jury was unaniem in haar keuze voor het proefschrift van Meghan Laturney. Dit proefschrift levert een belangrijke en vernieuwende bijdrage aan het verontwikkelde veld van seksuele selectie door een actieve rol van vrouwtjes bloot te leggen in seksuele conflicten. Het juryrapport vermeldt verder dat het proefschrift zeer helder is geschreven met veel diepgang in de inleiding en discussie, dat het onderzoek sterk vernieuwend en integratief is, en het een brede combinatie van fascinerende experimenten

bevat. Deze indrukwekkende combinatie van experimenten varieert van gedrag tot neurologisch tot genetisch onderzoek, waarbij zeer goed gebruik is gemaakt van de breedte van technieken die beschikbaar zijn in het modelorganisme *Drosophila*.

During the 29th Annual Dutch Entomologists Meeting (Ede, 15 December 2017), the tenth Netherlands Entomological Society (NEV) Dissertation Award was presented to Dr. Meghan Laturney, for her thesis 'The second sex. Functions and mechanisms of sperm manipulation in female *Drosophila melanogaster*', on December 8th, 2016 at the University of Groningen. This prize comprises a sum of money and a certificate of appreciation, and is awarded for the best doctoral thesis in the field of entomology, defended at a Dutch university in the preceding academic year (1 September – 31 August). The committee was unanimous in selecting Meghan Laturney's thesis as the best. This thesis makes a significant and important contribution to the far-developed field of sexual selection, by revealing an



active role of females in sexual conflict. The committee especially praises the clear writing, with a comprehensive introduction and discussion, the novelty of the integrative research, and the broad combination of fascinating experiments. This impressive combination of experiments varies from behaviour to neurological to genetic research, taking advantage of the wide range of techniques that are available in the model organism *Drosophila*.

optimal threaten female reproductive success. As polyandry offers various benefits to females (reviewed by Parker & Birkhead 2013), sexual conflict theory predicts that females should develop counter-adaptations to restore their polyandrous behaviour.

Although theoretically anticipated, examples of female mechanisms that reduce the influence of male mate-guarding tactics like AAPs are lacking – and it is not that surprising. Generally speaking, examination of the impact females have over their own reproduction has been mainly limited to pre-copulatory mate choice. In contrast, the influence males have over their paternal reproductive success is arguably the most studied system and includes investigations for both pre- and post-copulatory traits. Indeed, the male-focussed research has demonstrated how powerful *Drosophila* can be in identifying mechanisms that underlie a complex mating system. However, the deficiency in the literature regarding the female regulation of reproduction, specifically the response to male-derived restrictions, limits our understanding of the co-evolution that likely exists between the sexes.

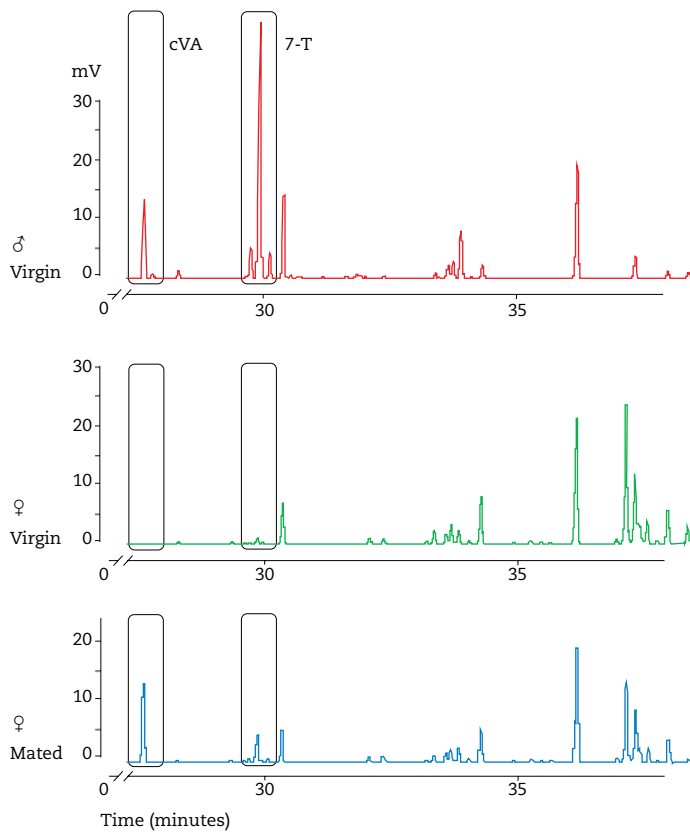
To identify mechanisms that females use to control their own reproduction including those that females may have evolved to neutralize or offset male-derived attempts of control, the best strategy was to utilize and supplement our current understanding on mechanisms of male reproduction. Since much of the male-centered research identifying the mechanisms that control reproductive behaviours has employed *Drosophila*, making use of this same model organism to initiate an investigation on the female complement to this control was an obvious starting point.

Despite the constraints of chemical mate-guarding, *D. melano-*

*gaster* Meigen females are polyandrous. When collected from wild populations, females typically have sperm from multiple males within their sperm storage organs (reviewed by Gowaty 2012); and females from inbred lab strains also accept multiple partners in a single day (Billeter *et al.* 2012, Gorter *et al.* 2016). In order to identify how females achieve rematings, I first examined what females do after copulation. *D. melanogaster* females have a variety of post-mating behaviours including the ejection of unstored sperm a few hours after mating (reviewed by Laturney & Billeter 2014), which has been linked to the end of the sperm storage process (Manier *et al.* 2010) and marking of egg-laying sites (Duménil *et al.* 2016). Interestingly, the timing of sperm ejection has also been linked to the outcome of sperm competition in a twice-mated female where longer intervals between remating and ejection after remating were associated with inflated degree of last male sperm precedence (LMSP) (Lüpold *et al.* 2013). Together, these results suggest that some female post-mating behaviour, namely sperm ejection and remating, likely have large impacts on the paternity outcomes of a polyandrous female. For this reason, I first explored the impact of female sperm ejection on the potency of chemical mate-guarding.

### Sperm ejection and chemical mate-guarding

As reviewed above, females acquire both cVA and 7-T during copulation. cVA is a male-specific pheromone produced in the male reproductive tract (Brieger & Butterworth 1970). 7-T, on the other hand, is a cuticular hydrocarbon produced in subepidermal abdominal cells just under the cuticle or skin of the fly.

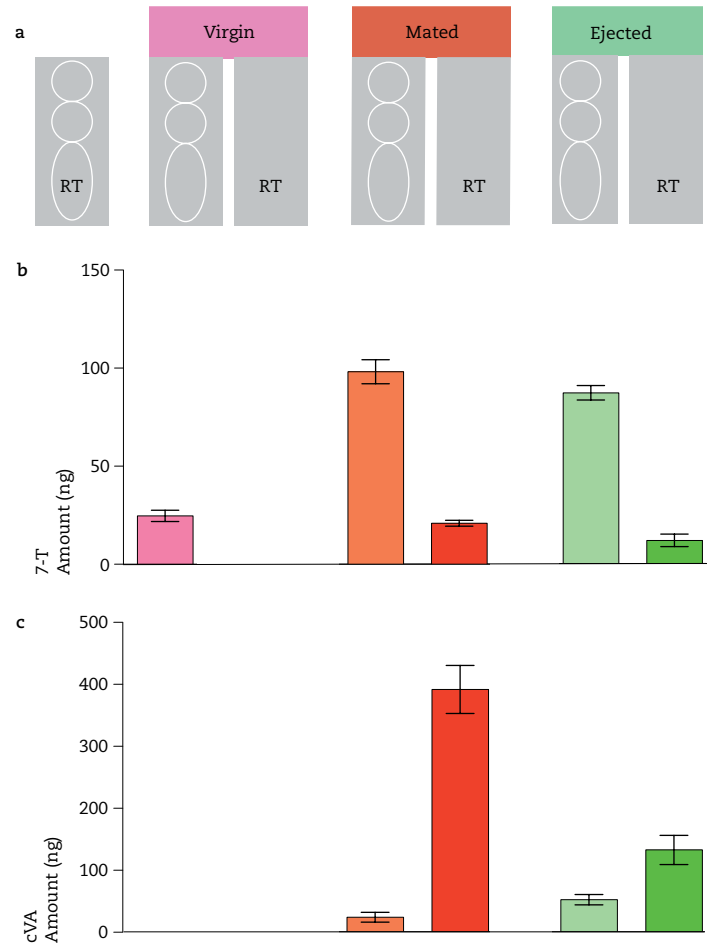


**1. Males transfer chemicals to females during copulation. Chromatograph of a virgin male (top), virgin female (middle), and mated female (bottom). Black boxes highlight cis-vaccenyl acetate (cVA) and 7-Tricosene (7-T). Gas chromatography separates chemicals by size (time in minutes on the x-axis), allowing for the identification of pheromones and their relative amounts. cVA and 7-T are found in relatively high amounts on males and absent on virgin females. Thus, the presence of cVA and 7-T on mated females is due to transfer during copulation.**

**1. Mannetjes dragen chemische stoffen over op vrouwtjes tijdens een paring. Chromatogram van een maagdelijk mannetje (boven), maagdelijk vrouwtje (midden) en een gepaard vrouwtje (onder). Cis-vaccenyl acetaat (cVA) en 7-Tricosen (7-T) worden weergegeven met zwarte boxen. Gaschromatografie deelt een mengsel op in losse chemische stoffen op basis van grootte (tijd in minuten op de x-as) wat de mogelijkheid biedt om feromonen te identificeren en te kwantificeren. cVA en 7-T komen in relatief grote hoeveelheden voor bij mannetjes en zijn afwezig bij maagdelijke vrouwtjes. De aanwezigheid van cVA en 7-T bij gepaarde vrouwtjes komt daarom door overdracht tijdens een paring.**

In other words, cVA is produced and stored in the interior of the male and 7-T on the exterior. Given the different spatial origins of these chemicals, I explored where on female these chemicals accumulate during mating and if these chemicals can be removed. To do so, we analysed the chemical profiles of virgin, mated, and ejected females using gas chromatographic analysis (see figure 2 for details). To determine the location of the chemicals we analysed the reproductive tracts separate from the rest of the animal. As expected, we found that 7-T was transferred to the exterior of the females and cVA to the interior of the reproductive tract. Although mated females were not able to remove the male-derived 7-T from their cuticle, we found that over 80% of the cVA was eliminated from the reproductive tract after sperm ejection (Laturney & Billeter 2016, figure 2). Thus, sperm ejection removes some AAPs from females – but is it enough to influence female attractiveness?

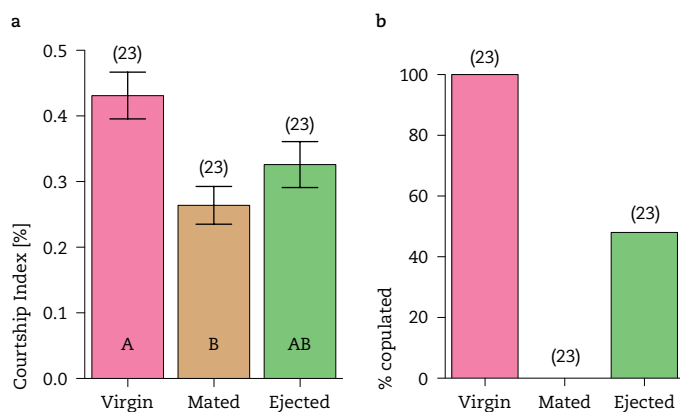
To determine if ejection influenced attractiveness, we placed a single virgin, mated, or ejected female into a small chamber with a single male, observed the pair for ten minutes,



**2. Mated females remove cVA from their reproductive tract via sperm ejection. (a) To explore the fate of cVA and 7-T, we examined the chemicals found on/in virgin females (pink), mated non-ejected females denoted as 'mated' (orange); and mated-ejected females denoted as 'ejected' (green). We removed the reproductive tracts of females and analysed the chemical profiles of the body minus the reproductive tract (indicated with three circles to represent the three body segments) or the isolated reproductive tract ('RT'). (b) Females gain 7-T during mating. The chemical is transferred to the outside of the female and is unaffected by sperm ejection. (c) Females gain cVA during mating and this chemical is largely contained within the reproductive tract of a mated female. Due to its location, females remove the majority of cVA via sperm ejection.**

**2. Gepaarde vrouwtjes verwijderen cVA uit hun reproductieve organen door sperma te dumpen. (a) Om te onderzoeken wat er met cVA en 7-T gebeurt hebben we de chemische stoffen geanalyseerd bij maagdelijke vrouwtjes (roze), gepaarde vrouwtjes die geen sperma gedumpt hebben genoteerd als 'mated' (oranje) en gepaarde vrouwtjes die wel sperma gedumpt hebben genoteerd als 'ejected' (groen). We hebben de reproductieve organen van de vrouwtjes verwijderd en het chemische profiel geanalyseerd van de rest van het lichaam (weergegeven met drie cirkels die het driedelige lichaam representeren) of het geïsoleerde reproductieve orgaan ('RT'). (b) Vrouwtjes verkrijgen 7-T tijdens paren. Deze chemische stof wordt overgebracht op de buitenkant van het vrouwtje en wordt niet beïnvloed door het dumpen van sperma. (c) Vrouwtjes krijgen cVA tijdens het paren en deze chemische stof bevindt zich hoofdzakelijk in het reproductieve orgaan van gepaarde vrouwtjes. Door deze lokalisering kunnen vrouwtjes het overgrote deel verwijderen doormiddel van het dumpen van sperma.**

and calculated the percentage of time the male spent courting. We found that virgin females are courted the most, mated females courted the least, and ejected females courted at an intermediate rate, suggesting that mated females become more attractive after they eject (figure 3a). Additionally, we also observed that although half of the ejected females remated, none of the mated non-ejected females accepted a new male partner,



**3. Removal of cVA via sperm ejection is associated with increased courtship and remating.** (a) Mean courtship index of one naïve male with one female of indicated mating status. Differences between groups were determined by One-way ANOVA followed by Tukey's post-hoc tests. Error bars indicate s.e.m. (b) Proportion of females that copulated within a 30-minute observation period when placed with a naïve male ( $X^2$  test,  $X^2=46.0387$ ,  $p<0.00001$ ).

**3.** Verwijdering van cVA via het dumpen van sperma is geassocieerd met een verhoogde kans op opnieuw paren. (a) Gemiddelde mannelijke aandacht (ook wel 'courtship') van één naïef mannetje met één vrouwtje met de aangegeven paringsstatus. Verschillen tussen de groepen is vastgesteld met een eenzijdige ANOVA opgevolgd door Tukey's post-hoc-testen. Foutbalken geven de standaardfout (s.e.m.) weer. (b) Proportie van vrouwtjes die binnen een 30-minuten observatie interval gepaard hebben met een naïef mannetje ( $X^2$ -test,  $X^2=46.0387$ ,  $p<0.00001$ ).

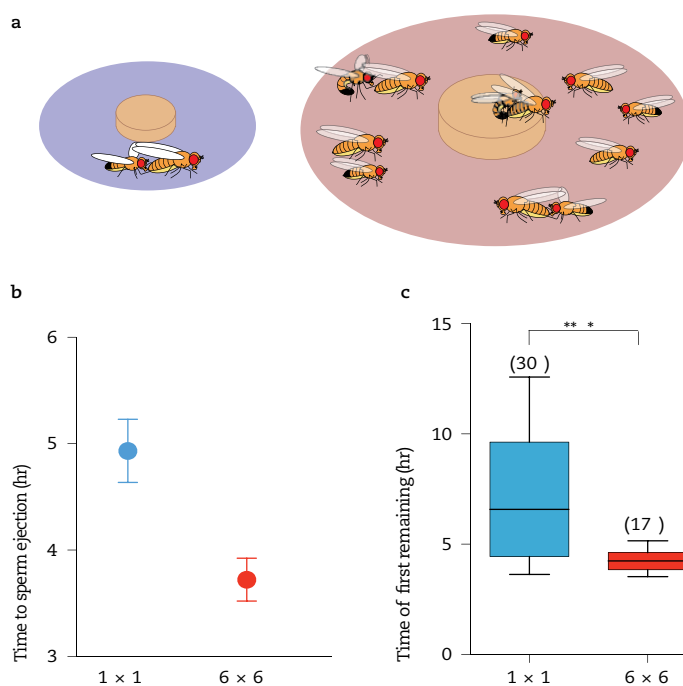
suggesting a relationship between ejection, attractiveness and polyandry (figure 3b). In support of this, we found that both the timing of ejection (time between mating and ejection) and timing of remating are both reduced when females are in a larger social context (figure 4).

Together, these results reveal that female post-mating behaviour can counter-act male mate-guarding techniques. Once a female ejects, she elicits more courtship from potential male suitors – often leading to polyandrous behaviour. As the timing of both sperm ejection and remating are advanced when more males are present, it begs the question: do females have active control over the timing of these post-copulatory behaviours?

### Timing of ejection under neuronal control

The movement of the ejaculate within the female reproductive tract is a highly controlled process. Investigations into the fate of sperm have revealed a collaboration between male-derived seminal compounds and female cellular substrates including the involvement of the central nervous system (Arthur *et al.* 1998, Ravi Ram & Wolfner 2007, Schnakenberg *et al.* 2011). However, the mechanisms controlling the expulsion of the unstored sperm from the female reproductive tract following mating, known as sperm ejection, are not fully understood.

To identify neurons that control sperm ejection and/or modulate the timing of ejection, we made use of a well-established technique in *Drosophila*, the binary Gal4-UAS system used for targeted gene expression (Brand and Perrimon 1993). We independently gained genetic access to a number of small populations of neurons and forced the expression of a temperature sensitive cation channel, dTrpA1 (Hamada *et al.* 2008), that can activate neuronal firing; or a temperature sensitive allele of dynamin, Shibire<sup>ts1</sup> (Kitamoto 2001) that can block synaptic transmission to silence neuronal activity. At a permissive tem-



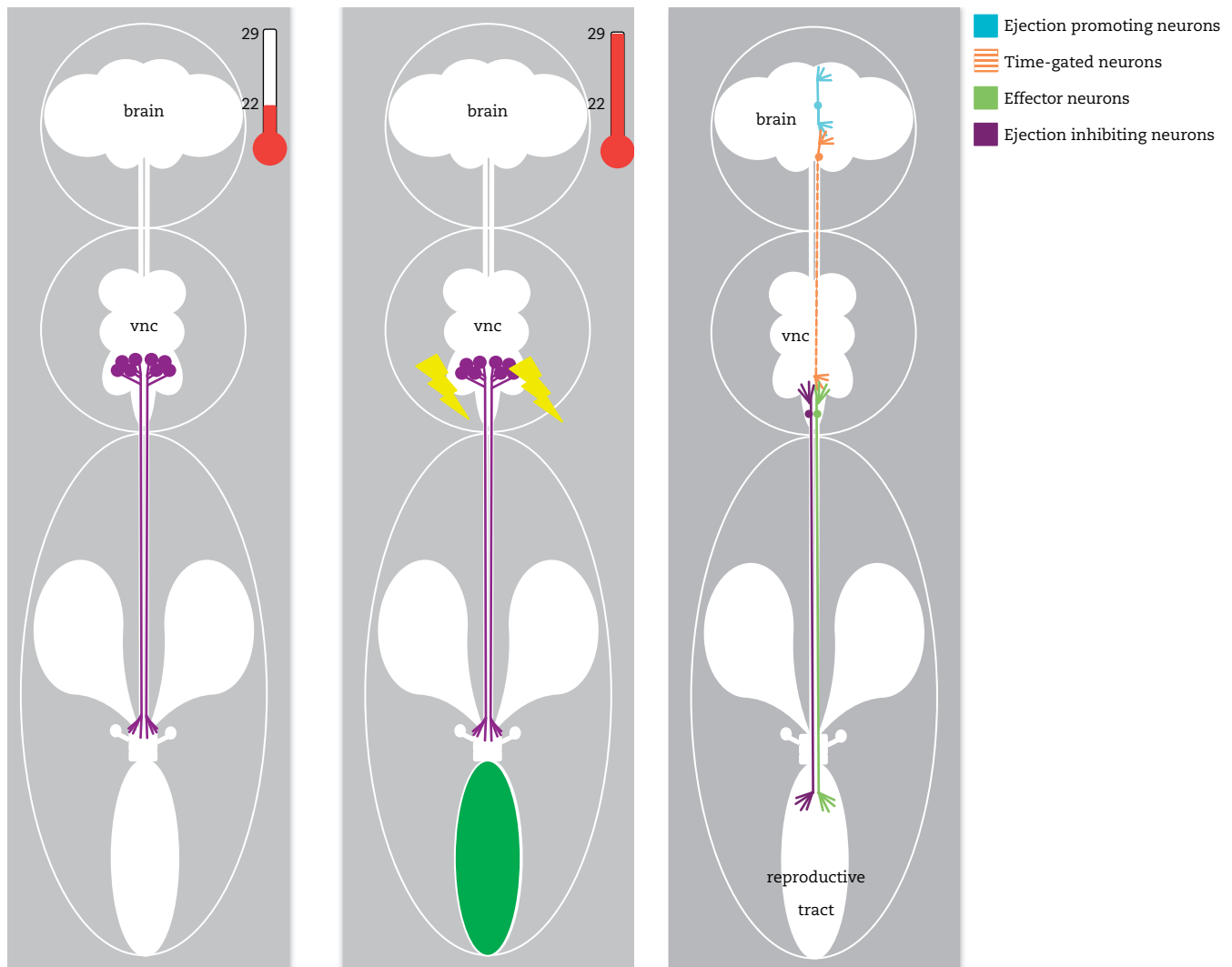
**4. Social context reduces the latency of sperm ejection and remating.** (a) Flies were either housed in pairs (1 female and 1 male) or in groups (6 females and 6 males) in the presence of food (brown disk). (b) Mean (+/- s.e.m.) time from start of mating to female sperm ejection when mated in either pairs (blue) or in groups (red). General linear model of the data revealed a significant effect of social context on the timing of ejection (GLM:  $p=0.0055$ ,  $**p<0.01$ ). (c) Time between virginal mating and second mating (remating) for flies either held in pairs (blue) or in groups (red). Bootstrap analysis revealed that remating latency is significantly advanced in groups relative to pairs ( $***p<0.001$ ).

**4.** Sociale context verkort de tijd tot het dumpen van sperma en opnieuw paren. (a) Vliegen zijn getest in koppels (1 vrouwtje en 1 mannetje) of in groepen (6 vrouwtjes en 6 mannetjes), in de aanwezigheid van voedsel (bruine schijf). (b) Gemiddelde (+/- s.e.m.) tijd van spermadumpen, van het begin van paren tot het vrouwtje het sperma dumpt, voor vrouwtjes in koppels (blauw) of groepen (rood). Algemeen lineair model van de data onthult een significant effect van sociale context op de timing van het dumpen van sperma (GLM:  $p=0.0055$ ,  $**p<0.01$ ). (c) Tijd tussen maagdelijke paring en tweede paring voor vliegen getest in koppels (blauw) of in groepen (rood). Bootstrap-analyse laat zien dat het interval tot opnieuw paren significant verkort is groepen vergeleken met koppels ( $***p<0.001$ ).

perature (under 25 °C), neurons expressing this machinery display normal activity. However, if the fly is placed above this threshold, neurons that express this machinery are forced to either fire or be silenced, respectively. If the specific population of neurons are involved in sperm ejection, then manipulating their activity should result in aberrant ejection behaviour.

We identified a small population of neurons that innervate the female reproductive tract that inhibit ejection upon activation (figure 5). This suggests that directly after mating, this neuron is active, allowing females to keep the ejaculate in the reproductive tract while she is storing sperm. A few hours later this neuron likely becomes inhibited and the female is free to discard the ejaculate including the AAPs. Moreover, it is likely that the activity of this neuron is dependent on social cues, allowing for the social modulation of the timing of ejection that we observed (figure 4). Additionally, we found three populations of neurons that promote sperm ejection (figure 6). These neurons likely behave in an inverse fashion with regard to the previous neuron: inhibited directly following mating and become active when the female ejects.





**5. Activation of *dsx*-expressing neurons inhibit sperm ejection.** Females were first mated to transgenic males that express green fluorescent labeled sperm. Once mated, the sperm (green) can be visualized in the uterus of the female. Using the Gal4-UAS system, we expressed temperature sensitive tools specifically in these neurons (purple), allowing us to modulate the activity of these neurons in an otherwise normally behaving fly. The head of the fly contains the brain, the thorax contains the ventral nerve cord (vnc), and the abdomen contains the reproductive tract. Females either remained at 22 °C or were placed at 29 °C (temperature indicated in the upper right corner).

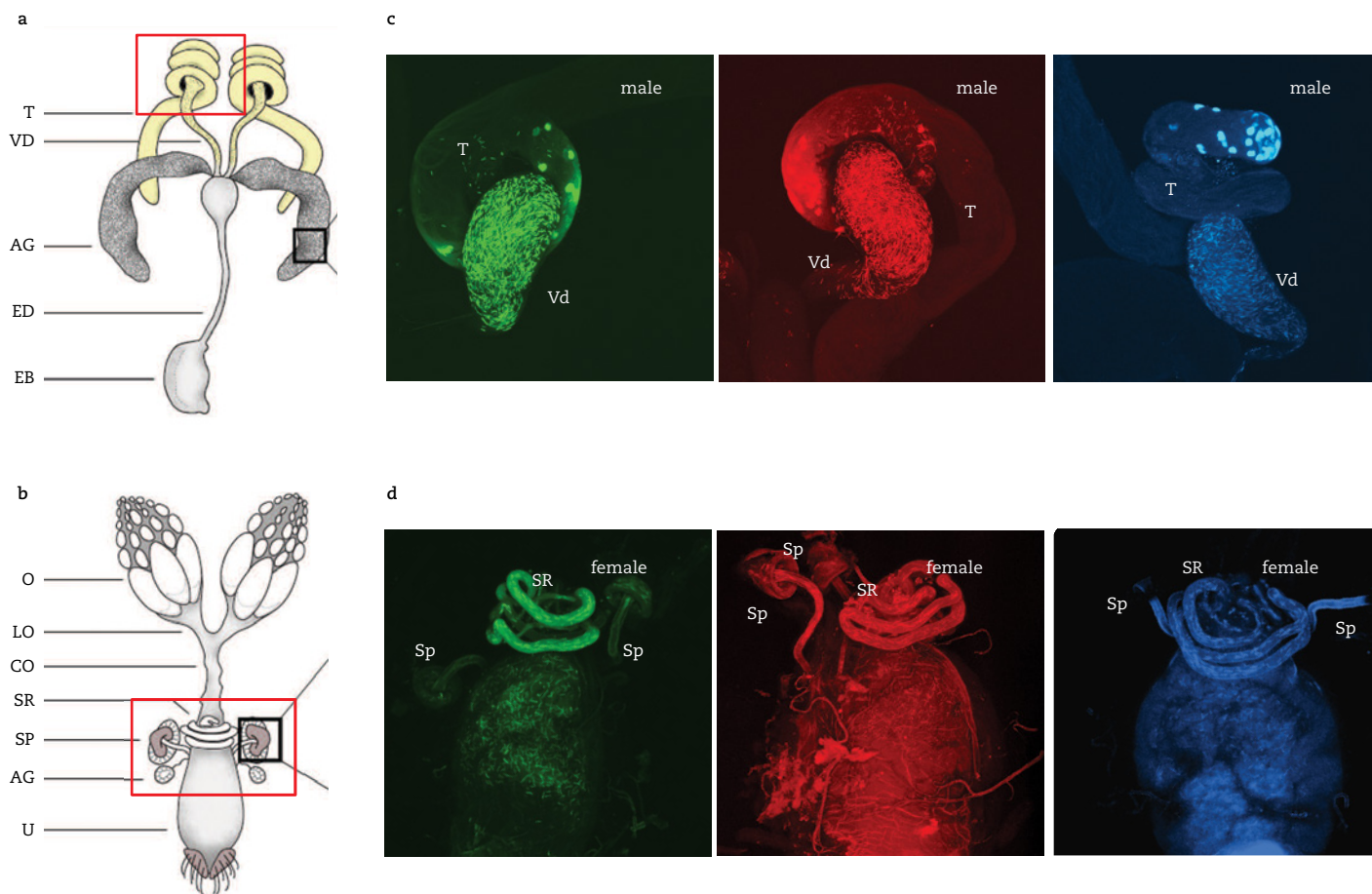
**5.** Activatie van neuronen die *dsx* aanmaken onderdrukt het dumpen van sperma. Eerst zijn de vrouwtjes gepaard met transgene mannetjes die groen fluorescerend gelabeld sperma produceren. Na paring kan het sperma (groen) gevisualiseerd worden in de baarmoeder van de vrouwtjes. Met het Gal4-UAS-systeem hebben we temperatuurgevoelige tools specifiek in deze neuronen (paars) tot uiting gebracht. Dit stelt ons in staat om de activiteit van deze neuronen te manipuleren in een vlieg die zich verder normaal gedraagt. In het hoofd van de vlieg bevindt zich het brein, in de borstkas zit het centrale zenuwstelsel (vnc) en in de onderbuik zitten de reproductieve organen. Vrouwtjes verbleven bij 22 °C of werden overgebracht naar 29 °C (temperatuur staat rechts bovenin de hoek aangegeven).

To summarize, we identified neurons in the female central nervous system that control sperm ejection. We artificially activated or silenced small populations of neurons in a recently mated female and observed altered ejection behaviour. From these results, we were able to identify different populations of neurons that either inhibit or promote this post-copulatory behaviour, allowing us to propose a model of the neuronal circuitry underlying sperm ejection (see figure 6 for details). As we observed that the timing of both ejection and remating

**6. Proposed neuronal circuitry that supports sperm ejection.** The three body segments of the fly are represented by three white circles. Neurons are unilaterally presented in this diagram for clarity. The *dsx*-expressing neurons (purple), when active, inhibit sperm ejection, likely reducing activity within a few hours after mating, allowing the female to eject. The effector neuron (green), when active, promotes spontaneous ejection any time after mating. This population of neurons is, however, regulated by the time-gated neuron (orange), protecting against premature ejection, allowing the female to store sperm. The time-gated neuron likely receives input from higher-order ejection promoting neurons (blue) in the brain to cause the female to eject a few hours after mating.

**6.** Voorgesteld neuronaal netwerk dat het dumpen van sperma ondersteunt. De drie lichaamsdelen van de vlieg zijn weergegeven met drie witte cirkels. Neuronen en hun geassocieerde functie zijn weergegeven. Neuronen zijn eenzijdig weergegeven voor de helderheid. Wanneer de neuronen die *dsx* aanmaken (paars) actief zijn, remmen ze het dumpen van sperma. De activiteit van deze neuronen is waarschijnlijk verlaagt binnen een paar uur na paring wat het vrouwtje in staat stelt om te dumpen. Wanneer de effectorneuronen (groen) actief zijn, bevordert dit spontane dumping op elk willekeurig moment na paring. Deze populatie neuronen wordt op zijn beurt gereguleerd door het tijdgevoelige neuron (oranje) wat een vrouwtje beschermt tegen te vroeg dumpen en in staat stelt om sperma op te slaan. Het tijdgevoelige neuron ontvangt waarschijnlijk signalen van hogere orde neuronen in het brein die dumpen bevorderen (blauw), met als resultaat dat het vrouwtje een paar uur na paring het sperma dumpst.

are variable and that the circuitry supporting sperm ejection is poised for plasticity, we next asked: what is the function of this flexibility? What aspect (if any) does the timing of remating influence?



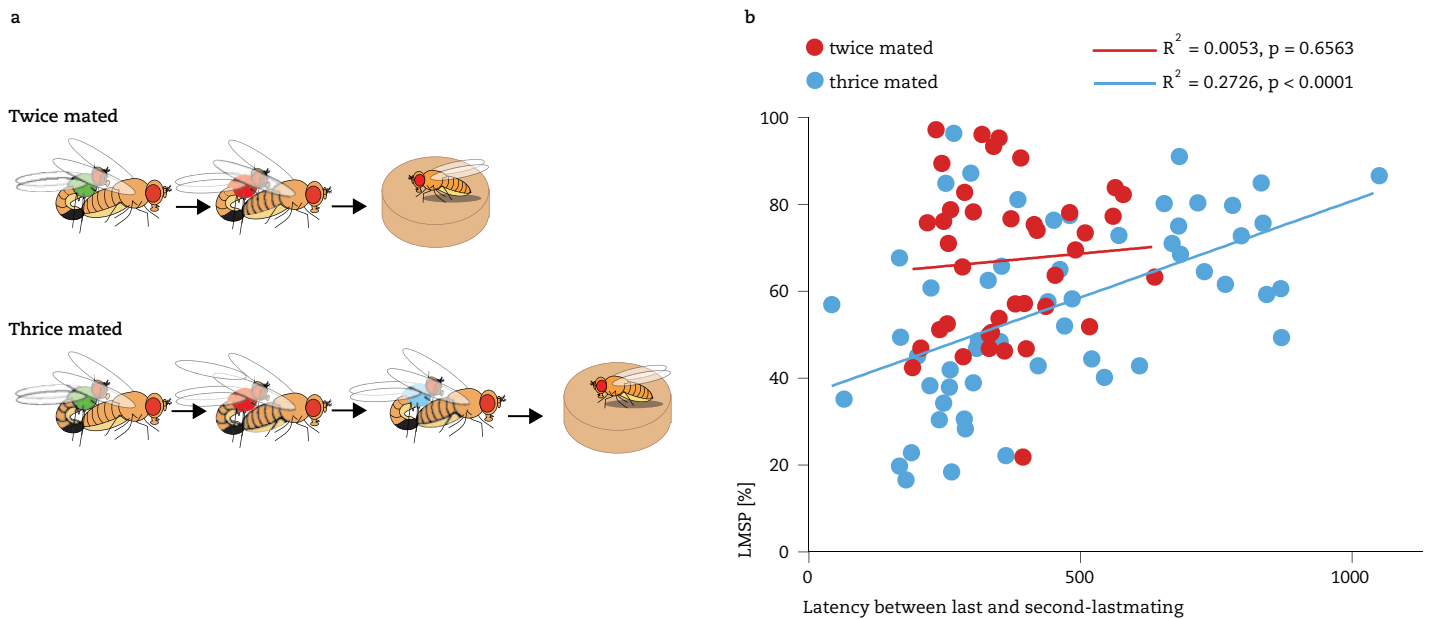
**7.** Transgenic males with fluorescently tagged sperm allow for the visualization of sperm within male and female reproductive tract. (a-b) Diagram of the male and female reproductive tract. Modified from Schnakenberg et al. 2012. The reproductive systems are shown in ventral view, with anterior to the top. (a) The male reproductive tract contains a pair of testes (T), which connect through vasa deferentia (VD) to the anterior ejaculatory duct. A pair of lobed accessory glands (AG) is surrounded by a sheath of muscle that presumably squeezes the secretions of the cells into the ejaculatory duct (ED) and bulb (EB) to mix with sperm and other SFPs (seminal fluid proteins). (b) The female reproductive tract contains a pair of ovaries (O), from which mature eggs pass to the lateral oviducts (LO), which join to form the common oviduct (CO). Eggs are activated in the common oviduct before passing to the uterus (U), where fertilization takes place. The entrance to the egg, or micropyle, is adjacent to the openings of the ducts to the spermathecae (SP) and seminal receptacle (SR). Small accessory glands (AG) also connect through ducts to the anterior-dorsal uterus. (c-d) Sperm visualized in the male and female reproductive tract. Images are maximum projections of confocal stacks. (c) Micrographs of male testes expressing fluorescent protein labeled sperm heads: green fluorescent protein (GFP), red fluorescent protein (RFP), and blue fluorescent protein (BFP), respectively. Location of the testes (T) and vas deferens (Vd), are indicated. (d) Micrographs of female sperm storage organs and anterior uterus of wild-type females mated to a transgenic male expressing fluorescent protein labeled sperm heads: GFP, RFP, and BFP, respectively. Location of the seminal receptacle (SR), paired spermathecae (Sp), and uterus/bursa (Bursa), are indicated.

**7.** Transgene mannetjes met fluorescerend gelabeld sperma maken het mogelijk om sperma te visualiseren in mannelijke en vrouwelijke reproductieve organen. (a-b) Diagram van mannelijke en vrouwelijke reproductieve organen. Aangepast vanuit Schnakenberg et al. 2012. De reproductieve systemen zijn weergegeven vanuit ventraal perspectief, waarbij anterior boven is. (a) De mannelijke reproductieve organen bestaan uit de testikels (T) die via de vas deferens (VD) in verbinding staan met de voorste zaadleider. Een set van gelobde bijklieren 'accessory glands' (AG) is omgeven door een schede van spieren die de afscheidingen van de cellen in de zaadleider (ED) en 'ejaculatory bulb' (EB) persen om ze met sperma en andere SFPs (seminal fluid proteins) te mixen. (b) De vrouwelijke reproductieve organen bestaan uit de eileiders (O) die rijpe eitjes doorgeven aan de laterale eileiders (LO) welke samenkomen tot een gemeenschappelijke eileider (CO). Eitjes raken geactiveerd in de gemeenschappelijke eileider voordat ze in de baarmoeder (U) aankomen, waar vervolgens de bevruchting plaatsvindt. De ingang naar de eitjes, ook wel 'micropyle', ligt naast de doorgang van de leiders naar de opslagorganen 'spermathecae' (SP) en 'seminal receptacle' (SR). Kleine bijklieren 'accessory glands' (AG) staan ook via leiders in verbinding met de anterieure-dorsale baarmoeder. (c-d) Sperma gevisualiseerd in mannelijke en vrouwelijke geslachtsorganen. De afbeelding is een samenvoeging van confociaal dwarsdoorsnede opnames. (c) Micrograaf van mannelijke testikels met fluorescerend gelabelde spermakoppen: respectievelijk groen fluorescerend eiwit (GFP), rood fluorescerend eiwit (RFP) en blauw fluorescerend eiwit (BFP). De locatie van de testikels (T) en vas deferens (Vd) zijn weergegeven. (d) Micrograaf van vrouwelijke sperma opslagorganen en anterieure baarmoeder van wild-type vrouwtjes gepaard met transgene mannetjes met fluorescerend gelabelde spermakoppen: respectievelijk GFP, RFP en BFP. De locatie van de 'seminal receptacle' (SR), 'spermathecae' (SP) en baarmoeder (Bursa) zijn weergegeven.

### Timing of ejection/remating influences patterns of paternity

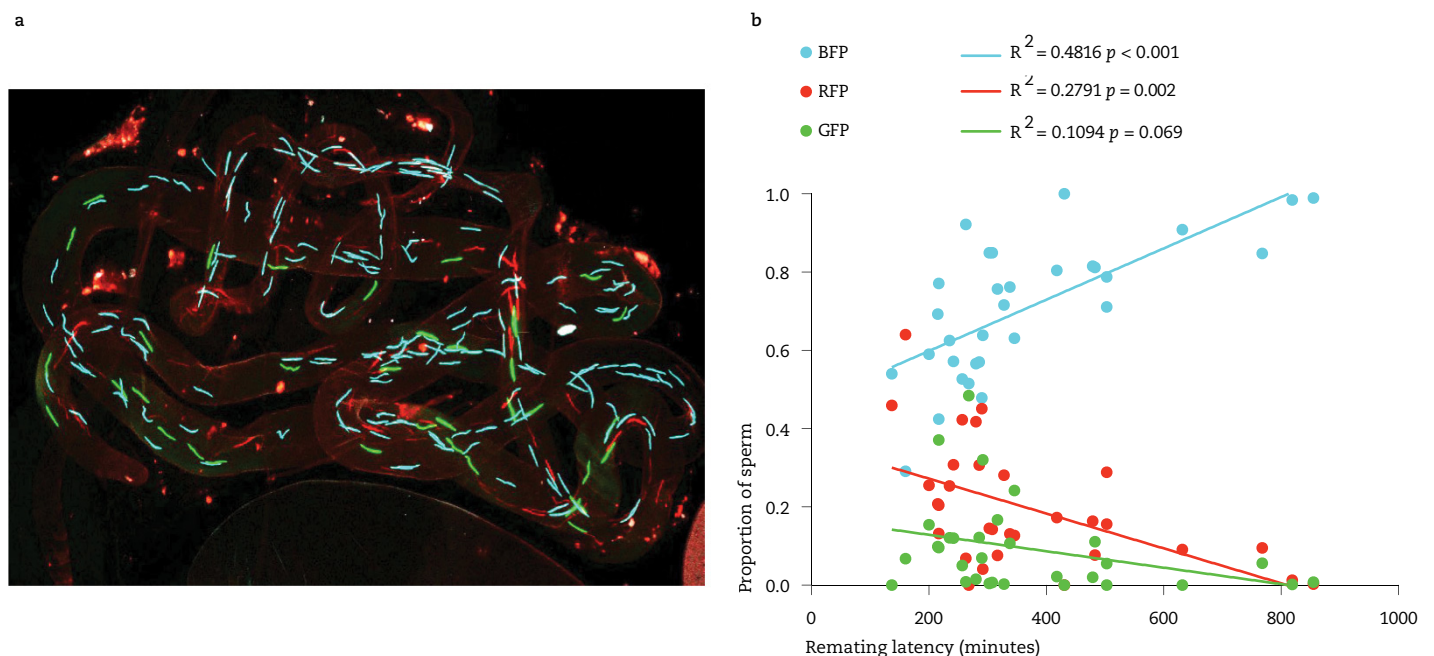
When females remate, sperm from multiple males interact within the female reproductive tract and compete for a chance at fertilization. The typical outcome of this interaction is LMSP: unequal distribution of paternity in favour of the last mate. As one of the proposed benefits of polyandry is the production of a genetically diverse brood, females should develop mechanisms to modulate paternity to maximize reproductive success.

Most of what is known about LMSP in *Drosophila* comes from laboratory studies that make use of a very specific paradigm. Researchers first mate a female, isolate her for one to five, then mate her with a phenotypically distinct male. However, in other species, LMSP is influenced by the amount of time between copulation and the number of male partners a female has, indicating that mating rate may contribute to a female's ability to modulate paternity patterns. Indeed, numerous findings suggest that *Drosophila* females can temper the outcome of sperm



**8.** Manipulating neuronal activity causes spontaneous ejection in a time dependent manner. (a) Females were sequentially exposed to two or three different males, expressing either green, red, or blue transgenic fluorescent sperm. Time interval between mating was recorded. After mating, females were either placed in a food vial to lay eggs and paternity of offspring was determined or were flash frozen, their reproductive tracts removed, and stored sperm was genotyped and counted. (b) Correlation between LMSP and remating latency between last and penultimate mating (RFP males for twice-mated females represented in red; BFP for thrice-mated females represented in blue). Strength of the relationship was assessed with a Pearson's correlation test.

**8.** Manipulatie van neuronale activiteit veroorzaakt spontane tijdsafhankelijke dumping van sperma. (a) Vrouwjes zijn opeenvolgend blootgesteld aan twee of drie verschillende mannetjes met groen, rood of blauw fluorescerend sperma. Het tijdsinterval tussen paringen werd vastgesteld. Na paring zijn de vrouwjes óf teruggeplaatst in voedsel buisjes om eieren te leggen waarna het vaderschap van de nakomelingen bepaald is óf ze zijn ingevroren waarna hun reproductieve organen zijn verwijderd om het opgeslagen sperma te genotypen en tellen. (b) Correlatie tussen LMSP (*last male sperm precedence*) en tijd tot opnieuw paren tussen laatste en een-na-laatste paring (RFP-mannetjes voor tweemaal gepaarde vrouwjes weergegeven in rood; BFP voor driemaal gepaarde vrouwjes weergegeven in blauw). Mate en statistische significantie van de correlatie is vastgesteld met een Pearson's correlatietest.



**9.** Increased remating rate reduced storing of third mate in thrice-mated females. (a) A confocal micrograph of the seminal receptacle of a thrice-mated female holding green, red, and blue fluorescently labeled sperm. (b) Correlation between proportion of green, red, or blue sperm in storage in the seminal receptacle and remating latency between penultimate and last mating in thrice-mated females. Strength and statistical significance of the relationship was assessed with a Pearson's correlation.

**9.** Verhoogde mate van opnieuw paren verlaagd de opslag van het derde mannetje in driemaal gepaarde vrouwjes. (a) Een confocaal micrograaf van het sperma opslag orgaan 'seminal receptacle' van driemaal gepaarde vrouwjes met groen, rood en blauw fluorescent gelabeld sperma. (b) Correlatie tussen proporties groen, rood of blauwe sperma opgeslagen in de 'seminal receptacle' en tijd tot opnieuw paren tussen een-na-laatste en laatste paring in driemaal gepaarde vrouwjes. Mate en statistische significantie van de correlatie is vastgesteld met een Pearson's correlatietest.



competition (for example Billeter *et al.* 2012, Chow *et al.* 2010, 2012). Nevertheless, because the current paradigm holds female mating rate steady, the relationship between mating rate and paternity outcomes in this species has not been fully explored. If females can modulate the genetic diversity of their offspring by modifying remating behaviour, then this classic paradigm would fail to capture this.

We explored the effect of two variables (number of mates and length of remating latency) on LMSP by modifying the paradigm in which it is tested and show that female mating rate can indeed lead to a breakdown in LMSP. We generated three types of transgenic males that produce fluorescent sperm (green, red, or blue), allowing us to assess the paternity of the offspring and the male-specific sperm fate inside the female reproductive tract (figure 7). Instead of using the typical mating paradigm, we accommodated varying remating latency and varied the number of mating events (figure 8a). We found that LMSP was significantly reduced in thrice-mated females with short remating intervals (figure 8b); coinciding with reduced last-male sperm storage (figure 9). This means that females who were faster to remate produced more even ratio of sons from green, red, and blue fathers compared to females who were slower to remate. These results provide evidence that by modulating remating rate, females can skew the outcome of sperm competition (Laturney *et al.* 2018). As LMSP threatens to reduce brood genetic diversity, females may increase remating rate to influence the relative ratio of male-specific sperm within her storage organs, ultimately producing a more balanced brood with respect to paternity.

## Conclusion

These experiments, reviewed above, represent a response to the lack of understanding of the role that females play in reproduction in general and in sexual conflict specifically. After mating, females are able to actively modulate the timing of sperm ejection and by extension the removal of male-derived AAPs, resulting in a gain in attractiveness and an increased probability of polyandrous behaviour. This control of remating behaviour provides females with the ability to influence the patterns of paternity of her resulting brood and therefore maximizing her reproductive success. This research is a small step to not just identify the influence that females have in remating, biasing patterns of paternity, or manipulating sperm within her reproductive tract, but ultimately to integrate that knowledge with previous findings on the male contribution and gain a full understanding of the complex interaction between the two sexes. As reproduction is the ultimate collaboration so too should be the research that investigates it and the literature that reports it.

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## Samenvatting

### Post-copulatiegedrag in vrouwtjes van de fruitvlieg *Drosophila melanogaster*

In diersoorten die zich seksueel voortplanten moeten een mannetje en vrouwtje paren om nakomelingen te produceren. Ondanks deze samenwerking kan er ook conflict ontstaan wanneer een van de seksen het reproductieve succes maximaliseert ten koste van de andere sekse. Wanneer een vrouwtje bijvoorbeeld meermaals paart, verhoogt dit haar hoeveelheid nakomelingen, maar vermindert meestal het aantal nakomelingen van het eerste mannetje. Polyandrie is daarom een conflict tussen de seksen. Seksueel-conflicttheorie suggereert dat gedurende evolutie, mannetjes tactieken ontwikkelen om meermaals paren van het vrouwtje tegen te gaan. Vrouwtjes daarentegen moeten manieren ontwikkelen om dit te voorkomen en zodoende meermaals te paren. Hoewel er meerdere voorbeelden bestaan van hoe mannetjes de vrouwelijke reproductie manipuleren, blijft onze kennis van vrouwelijke mechanismen die de reproductie na een eerste paring beïnvloeden achter. Om te begrijpen waarom en hoe vrouwtjes hun gedrag na paren aanpassen, heb ik de vrouwelijke invloed op een welbekende mannelijke tactiek in de fruitvlieg *Drosophila melanogaster* onderzocht. Tijdens een paring deponeert een mannetje naast zaadcellen ook chemische stoffen die de aantrekkelijkheid verminderen in de reproductieve organen van het vrouwtje. Met een chemische analyse van maagdelijke en gepaarde vrouwtjes heb ik gevonden dat *D. melanogaster*-vrouwtjes een deel van deze stoffen kunnen verwijderen door het mannelijke ejaculaat te dumpen. Na deze verwijdering ontvangt een vrouwtje meer mannelijke aandacht en is de kans groter dat ze opnieuw paart; haar aantrekkelijkheid is verhoogd. Daarnaast heb ik gevonden dat het interval tot opnieuw paren een grote invloed heeft op het aandeel in vaderschap: hoe eerder een vrouwtje opnieuw paart, hoe evenrediger de verdeling van vaderschap over de verschillende mannetjes. Als laatste heb ik neuronpopulaties in het vrouwelijk centrale zenuwstelsel geïdentificeerd die betrokken zijn bij het manipuleren van het ejaculaat in de vrouwelijke reproductieve organen en daarbij waarschijnlijk het interval tot dumpen beïnvloeden. Deze resultaten illustreren de invloed die vrouwtjes op reproductie hebben en dragen bij aan ons begrip van de complexe interactie tussen de twee seksen.



Meghan Laturney

University of Groningen

Groningen Institute for Evolutionary Life Sciences

Evolutionary Genetics, Development & Behaviour group

The Netherlands

Current address:

University of California, Berkeley

Department of Molecular and Cell Biology

United States

meghan.laturney@gmail.com