

## Topic Introduction

# Measurement of *Drosophila* Reproductive Behaviors

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Courtship behaviors in *Drosophila melanogaster* are innate and contain highly stereotyped but also experience- and state-dependent elements. They have been the subject of intense study for more than 100 years. The power of *Drosophila* as a genetic experimental system has allowed the dissection of reproductive behaviors at a molecular, cellular, and physiological level. As a result, we know a great deal about how flies perceive sensory cues from potential mates, how this information is integrated in higher brain centers to execute reproductive decisions, and how state and social contexts modulate these responses. The simplicity of the assay has allowed for its broad application. Here, we introduce methods for studying male and female innate reproductive behaviors as well as their plastic responses.

## REPRODUCTIVE BEHAVIORS

### The Fly Courtship Ritual: A Behavioral Duet

Choosing a mating partner is crucial for reproduction and has profound consequences in evolution. Courtship rituals provide opportunities for animals to assess the suitability of a prospective mate and increase their chances of reproductive success. In *Drosophila*, courtship involves a series of progressive and interactive innate behaviors that culminate in copulation (Sturtevant 1915; Bastock and Manning 1955; Bennet-Clark and Ewing 1967; Spieth 1974; Ferveur 2010; Yamamoto and Koganezawa 2013). Although the courtship sequence is genetically determined and “hardwired” in the nervous system, it is plastic and results from the complex interaction between both mating partners (Auer and Benton 2016; Ellenderson and von Philipsborn 2017; Aranha and Vasconcelos 2018; Rings and Goodwin 2019). Prospective mates exchange visual, chemical, tactile, and auditory cues (Krstic et al. 2009; Agrawal et al. 2014; Yew and Chung 2017). Such multimodal sensory integration allows flies to assess sex, species, and mating status, and to decide whether to progress with courtship and engage in copulation.

The courtship ritual begins with the male orientating toward the female and circling around her (Fig. 1A; Cook 1979; Markow 1987). Male flies identify and approach a potential mate using visual, olfactory, and auditory cues (Ejima and Griffith 2008; Kimura et al. 2015; Ribeiro et al. 2018; Nojima et al. 2021). She initially escapes by running away, but he responds by chasing her (“following”) (Fig. 1A). As courtship progresses, the male touches the female body with his forelegs (“tapping”)

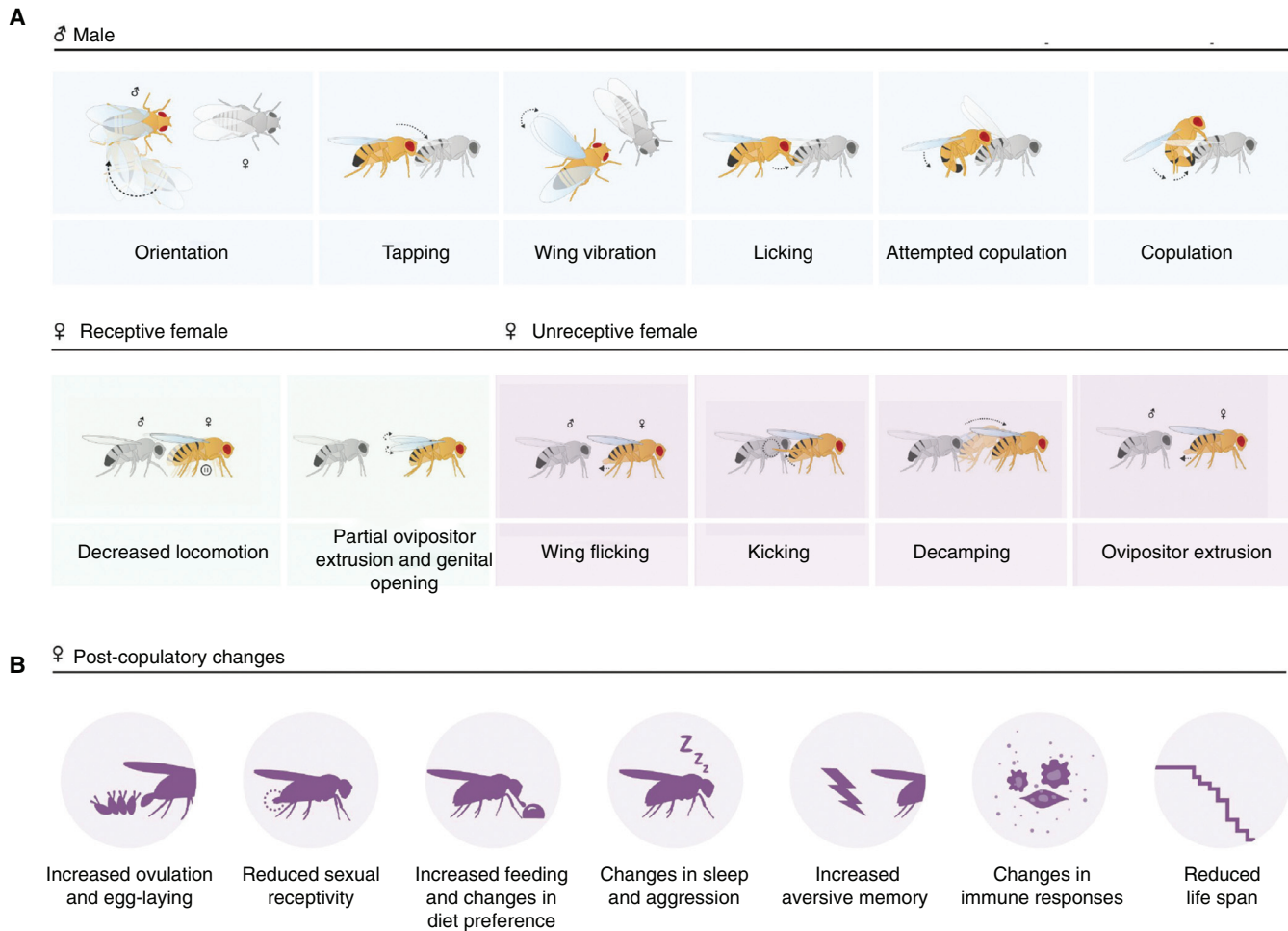
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From the *Drosophila* Neurobiology collection, edited by Bing Zhang, Ellie Heckscher, Alex C. Keene, and Scott Waddell.

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Advanced Online Article. Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top107866



**FIGURE 1.** *Drosophila* reproductive behaviors. (A) *Drosophila* male and female behaviors during courtship. (B) *Drosophila* female postmating responses.

(Fig. 1A), which allows him to sample gustatory pheromones on her cuticle (Kohatsu et al. 2011; Clowney et al. 2015). Female-specific pheromones, such as 7,11-heptacosadiene, act as aphrodisiac cues, stimulating male courtship. Male–male courtship is prevented by male-specific gustatory and olfactory pheromones, including 7-tricosene and 11-*cis*-vaccenyl-acetate (cVA). Similarly, the male sexually stimulates the female, increasing the chances of copulation. One of the main aphrodisiac stimuli produced by males is courtship song, generated by the sustained vibration of one wing (“singing”) (Fig. 1A). The male song pattern is formed of recurrent “sine” and “pulse” components. The time between consecutive pulses, defined as the “interpulse interval,” is a species-specific signal (Bennet-Clark and Ewing 1969; Swain and von Philipsborn 2021). Whereas the male courtship song induces females to slow down, facilitating copulation, it stimulates other males to court (Crossley et al. 1995; Zhou et al. 2015). Males can further increase female receptivity through the generation of substrate-borne vibrations by abdomen quivering (Fabre et al. 2012). The female responds to the courtship display, providing sensory feedback. This allows the male to adjust the courtship sequence and increase the probability of success. For instance, based on visual cues, males update the amplitude and type of courtship song with female distance (Coen et al. 2016). If the female is receptive, the male licks the female genitalia (“licking”) (Fig. 1A) and bends his abdomen to attempt copulation. Courtship persistence depends on female-derived multimodal stimuli, and the male may repeat the steps several times. Upon successfully mounting the female, he ejaculates sperm and seminal fluid. In copula, the female produces wing vibrations, which may influence male inseminate

transfer and enhance the male's reproductive success (Kerwin et al. 2020). Protocol: **Single-Pair Courtship and Competition Assays in *Drosophila*** (von Philipsborn et al. 2023a) will introduce approaches for quantifying different components of the courtship ritual.

## Female Responses during Courtship

The courtship ritual provides females time to evaluate a variety of sensory cues during courtship before accepting a male for copulation (Bastock and Manning 1955). The courtship song is one of the most important cues used by females to assess the species and fitness of a male and represents a key stimulator of receptivity (Swain and von Philipsborn 2021). Volatile and nonvolatile pheromones (like cVA and 7-tricosene, respectively) also act as aphrodisiac cues for females (Brieger and Butterworth 1970; Laturney and Billeter 2014). The developmental stage and mating status of the female also influence whether she will copulate. Young females, which are sexually immature, are unlikely to copulate (Fig. 1A). They reject courting males by running away, decamping, leg kicking, and wing flicking. In contrast, older females that are sexually mature tend to quickly accept a suitable male if they have not previously mated. These virgin females signal acceptance by slowing down, partially extruding their ovipositor, and opening their vaginal plates (Bussell et al. 2014; Mezzera et al. 2020; Wang et al. 2021).

Although *Drosophila* virgin females accept a partner to copulate with low selectivity, females become choosier in further copulation events. This is driven by reduced olfactory sensitivity to a male pheromone and allows females to initially prioritize sperm acquisition and then secure high-quality offspring (Kohlmeier et al. 2021). Several studies have provided evidence for mate-choice behavior in female flies: Females prefer to mate with good-condition males over poor-condition males and prefer to mate with partners that live or were raised on a similar diet. The latter phenomenon depends on pheromone profiles that are linked to the gut microbiome (Battesti et al. 2012). Protocol: **Single-Pair Courtship and Competition Assays in *Drosophila*** (von Philipsborn et al. 2023a) will introduce approaches for quantifying female sexual receptivity during courtship.

## Postmating Female Behaviors

Once mated, females undergo physiological and behavioral changes (postmating responses) (see Protocol: **Female Fly Postmating Behaviors** [von Philipsborn et al. 2023b]) that are triggered by the sex peptide and other male seminal fluid proteins and are mediated by the female nervous system (Chen et al. 1988; Chapman et al. 2003; Kubli 2003; Häsemeyer et al. 2009; Avila et al. 2011; Rezaval et al. 2012, 2014, 2016). For instance, females become less receptive to copulation, actively rejecting males. A mating plug forms in the female reproductive tract from secretions of the male ejaculatory bulb and aids sperm storage. A few hours after mating, females eject the mating plug along with unstored sperm and male-repelling cVA, a process that is under neuronal control and increases remating (Manier et al. 2010; Lee et al. 2015; Laturney and Billeter 2016). Mated females reject courting males by kicking their legs toward the suitor, curving their abdomen, and extruding the ovipositor (Fig. 1A). This rejection behavior is used by researchers to train male flies (see Protocol: **Courtship Conditioning/Suppression Assays in *Drosophila*** [von Philipsborn et al. 2023c]). The decrease in sexual receptivity in mated females lasts for ~8–10 d and is largely mediated by seminal fluid proteins (Kubli and Bopp 2012). Females remate more when they are in social groups or in the presence of food (Gorter et al. 2016).

Mated females produce and lay up to 80 eggs per day (Fig. 1B). They probe different substrates and select an optimal site for laying their eggs, which increases the chances of offspring survival (Joseph et al. 2009; Stensmyr et al. 2012; Dweck et al. 2013; Lin et al. 2015). Females tend to deposit their eggs in acetic acid-containing food and avoid substrates comprising toxic hexanoic acids, octanoic acids, or harmful microbes. Several assays have been developed to measure female postmating behaviors, including rejection behaviors during courtship, remating rate, oviposition, and egg-laying substrate choice (see Protocol: **Female Fly Postmating Behaviors** [von Philipsborn et al. 2023b]).

A series of postmating changes support the metabolic and nutrient demands associated with increased egg laying. These include an increase in food intake and preference for certain nutrients such as yeast, salt, and polyamines (Ribeiro and Dickson 2010; Walker et al. 2015; Hussain et al. 2016). In addition, mated females show increased aggression (Bath et al. 2017), decreased siesta sleep (Isaac et al. 2010), enhanced immune responses (Peng et al. 2005; Kapelnikov et al. 2008), and increased memory performance (Scheunemann et al. 2019). However, reproduction comes with a cost; excess mating events and receipt of seminal fluid proteins decrease female life span and reproductive success (Chapman et al. 1995; Wigby and Chapman 2005).

## Modulation of Male Courtship Behaviors by Physiological State, External Context, and Experience

Mating drive in male flies is homeostatically regulated (Zhang et al. 2016; Liu et al. 2019), such that repeated events of successful mating that are accompanied by reduction of available inseminate reduce the motivation to court virgin females. Although male courtship actions are considered innate responses to the presence of female flies, certain aspects of this complex behavior are flexible and modulated by prior experience, current internal state, and the social context in which it occurs. Such behavioral flexibility is advantageous in a dynamic and unpredictable environment such as the social domain, particularly in a polyandrous species such as *Drosophila*, in which females mate with multiple males within a single fertility period. To cope with this challenge and maximize their paternity rates, male flies need to identify the optimal mating partner and adjust their investment in mating according to their mating drive, experience, and perceived level of competition.

Naive males court both virgin and mated females but learn through experience to discriminate between them, thus minimizing futile investments in nonreceptive female flies. Early studies by Siegel and Hall (1979) showed that male flies that experience failure to mate upon interaction with nonreceptive previously mated females show decreased motivation to court (courtship suppression) (see Protocol: **Courtship Conditioning/Suppression Assays in *Drosophila*** [von Philipsborn et al. 2023c]). Courtship suppression is an important experimental paradigm for studying genes and neuronal circuits that mediate short- and long-term memory (Siegel and Hall 1979; Tompkins et al. 1983; Ejima et al. 2007; Keleman et al. 2012; Zhou et al. 2012). The anti-aphrodisiac male-specific pheromone cVA plays a key role in this conditioned response, as male flies learn to associate its presence on mated females with the failure to mate.

Successful sexual experience, on the other hand, can enhance male mating success in a competitive mating assay (see Protocol: **Single-Pair Courtship and Competition Assays in *Drosophila*** [von Philipsborn et al. 2023a]). When tested against a naive rival, experienced males display longer song bouts and more copulation attempts and eventually win the race and copulate (Saleem et al. 2014). Although some find that older male flies have a longer latency to court and invest less time in courtship (Churchill et al. 2019), others find that older male flies (7-d-old) have a copulation advantage over young male flies (2-d-old) in competition assays. This advantage of older male flies is explained by increased mating drive due to enhanced sensitivity to the pheromone palmitoleic acid (Lin et al. 2016). Last, the perceived level of competition also regulates certain aspects of male courtship. Males raised in complete isolation exhibit more singing than socially raised flies (Inagaki et al. 2014).

Taken together, these findings highlight the importance of carefully controlling mating history, age, and housing conditions of experimental flies when performing any of the assays described in the accompanying protocols.

Perceiving the presence of rival male flies affects male investment in copulation and regulates production and allocation of sperm and seminal fluid proteins (Wigby et al. 2020). Exposure to rival males modulates copulation duration (Bretman et al. 2009; Kim et al. 2013). The presence of rivals before mating also influences the production and transfer of sperm (Garbaczewska et al. 2013) and seminal proteins that enhance postmating response such as sex peptide and ovulin (Wigby et al. 2009).

The outcome of aggressive encounters between male flies impacts mating behavior in males. Loser flies exhibit less mating than winner flies (Teseo et al. 2016). Winners have higher reproductive

success than losers, but losers compensate for this disadvantage by enhancing the duration of their copulation events (Filice and Dukas 2019). Sexually deprived male flies that repeatedly experience rejection by mated female flies cope with this challenge by extending their copulation duration and increasing the expression of seminal proteins such as sex peptide to facilitate stronger postmating responses in female flies (Omesi et al. 2021).

## Balancing Courtship Motivation with Other Drives

Feeding, mating, and fighting for resources are essential for survival and reproduction, but fulfilling these needs simultaneously is not always possible. How do animals choose to prioritize one behavior over others? How decision-making in different situations occurs in the brain is still highly elusive; however, it is known that behavioral choices are affected by the internal state of the fly, previous experience, and external context.

Several studies have focused on how flies balance the needs of sleeping and courtship (Beckwith and French 2019). In certain contexts, courtship is prioritized over sleep (Fuji et al. 2007; Beckwith et al. 2017; Chen et al. 2017; Machado et al. 2017). For instance, when fed, males sacrifice nighttime sleep to engage in courtship. However, when deprived of yeast, males favor sleep over courtship (Duhart et al. 2020).

Another conflict arises when flies are deprived of both food and sex and prioritize searching for either resource. Male flies socially isolated posteclosion and sex- and food-deprived for 24 h prioritize feeding on sucrose over courtship (Cheriyamkunnel et al. 2021). However, this behavioral choice is plastic and can be fine-tuned by the fly's nutritional state and sex drive (e.g., whether the male has previously mated), as well as the quality of the available food source (e.g., nutritious vs. noncaloric sugar). A gut-derived hormone released after food consumption mediates a rapid switch to courtship (Lin et al. 2022). Sugar feeding, however, can also suppress courtship, a phenomenon called “post-prandial inhibition” (Zhang et al. 2022).

Mounting an immune response is energetically “costly”; therefore, infections are typically thought to reduce the amount of energy available for other activities such as mating. Surprisingly, both male and female fruit flies infected with several bacterial pathogens, including deadly pathogens, show normal levels of courtship and mating success (Rose et al. 2022). This suggests that reproductive behaviors are preserved in flies infected with bacteria.

Finally, during an agonistic interaction, males choose whether to engage in fighting (Hoopfer et al. 2015; Koganezawa et al. 2016). Recent fighting and mating encounters, as well as social experience, play an important role in this choice (Kim et al. 2018). Although defeat or group rearing reduces aggression (Wang et al. 2008; Penn et al. 2010), recent mating experience increases aggression (Yuan et al. 2014).

## METHODS FOR MEASURING REPRODUCTIVE BEHAVIORS

The study of *Drosophila* reproductive behaviors has a long history. In 1915, Sturtevant observed male courtship and copulation in single-pair and competitive assays, trying to assess the importance of different sensory modalities for mating. The procedures for these simple behavioral experiments (see Protocol: **Single-Pair Courtship and Competition Assays in *Drosophila*** [von Philipsborn et al. 2023a]) have not changed much, although today's use of video recordings greatly facilitates semimanual scoring and allows the use of automated tracking algorithms (ctrax [Kabra et al. 2013; Robie et al. 2017], together with FixTRAX [Bentzur et al. 2021], or FlyTracker [Eyjolfsson et al. 2014]) and machine learning algorithms such as JAABA (Kabra et al. 2013) for automated and precise analysis of fly interactions.

Sturtevant (1915), rather lapidary, concluded “A female that is ready to mate will accept any male, and a male that is ready to mate will do so with the first female that will allow him to.” Decades later, Bastock and Manning (1955), who were trained by Niko Tinbergen, revived the interest in studying reproductive behaviors, introducing ethograms, more detailed analysis of behavioral sequences, tran-

sitions, and the interdependence of behavioral steps. In Hall's laboratory (Siegel and Hall 1979), it was first shown that *Drosophila* courtship, formerly often viewed as a "fixed action pattern," is modified by experience and can be suppressed in conditioning assays (see Protocol: **Courtship Conditioning/Suppression Assays in *Drosophila*** [von Philipsborn et al. 2023c]). These assays are suitable to test for learning ability and memory performance.

One hallmark behavioral step of male courtship, unilateral wing vibration, was shown in the 1960s to constitute an acoustic stimulus (Bennet-Clark and Ewing 1967). In Protocol: **Probing Acoustic Communication during Fly Reproductive Behaviors** (von Philipsborn et al. 2023d), we describe how this "love song" is recorded, quantified, and played back during behavioral assays.

Female reproductive behaviors might be not as conspicuous as male courtship but have a large impact on fecundity and sexual selection. They include postcopulatory behaviors such as egg laying and mating plug ejection (see Protocol: **Female Fly Postmating Behaviors** [von Philipsborn et al. 2023b]) that the female executes in the absence of males, although they are still influenced by the inseminate of her mate.

Basic assays for measuring reproductive behaviors are easy to perform and do not require expensive and sensitive equipment. Without directly contradicting Sturtevant (1915), we can emphasize that there are still many things to discover about which genes, neurons, circuits, external and internal stimuli, and overall mechanisms make a female or a male "ready to mate." New behavioral details can be addressed and uncovered by various modifications and refinements. For example, all of the accompanying assays can be combined with optogenetic manipulation of defined neurons at defined times of the behavioral sequence by adding an appropriate illumination setup and closed-loop control systems. Pioneered by the group of D. Yamamoto (Kohatsu et al. 2011; Kohatsu and Yamamoto 2015), several laboratories have tested aspects of reproductive behavior in tethered flies walking on air-supported balls (O'Sullivan et al. 2018; Ribeiro et al. 2018; Jung et al. 2020; Hindmarsh Sten et al. 2021; Loesche and Reiser 2021). Male flies in such a setup respond to female cues and can be induced to sing. The setup can be combined with virtual reality stimulations and/or calcium imaging of brain neurons or flight muscles.



## CONCLUDING REMARKS

Similar patterns of social behaviors are seen in very different animals. These shared properties suggest that certain social behaviors originated early in evolution, and that similar ancient biological principles and genes are involved in these processes. As such, reproductive behaviors in *Drosophila* are an excellent system to explore many questions in contemporary neuroscience including sensory integration, mechanisms underlying dimorphic behaviors, decision-making, processing of motivation and reward, learning via experience, and the control of motor actions. During the last decade, brain research has undergone a technology-driven revolution with the emergence of high-resolution behavior analysis, multiphoton imaging, optogenetics, live neurotransmitter-release measurement, connectomics, circuit tracing, single-cell sequencing, and expansion sequencing. With these tools, we can use courtship behavior in *Drosophila* to go all the way from molecules to cells and to circuits and uncover shared mechanisms and principles that are conserved across species.

## ACKNOWLEDGMENTS

We thank Assa Bentzur and Julia Ryvkin for insightful comments on this text. Work in our laboratory was supported by the Lundbeckfonden under grant number DANDRITE R248-2016-2518 to A.C.v.P., the Israel Science Foundation grant 174/19 to G.S.-O., and BBSRC (BB/S009299/1), Wellcome Trust (214062/Z/18/Z), and Royal Society Research (RGS\R2\180272) grants to C.R.

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*Cold Spring Harb Protoc*; doi: 10.1101/pdb.top107866; published online February 13, 2023

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