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Mate Copying chez la drosophile : importance évolutive et bases mécanistiques

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Mate-copying in drosophila: evolutionary importance and mechanistic bases

« C'est la science qui m'a conduit à la conclusion que le monde est bien plus compliqué que ce qui peut être expliqué par la science. »

Allan Sandage

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M. Monier, S. Nöbel, A.-C. Dagaeff, L. Polizzi, G. Isabel and E. Danchin. The Hexagon - A New Tool for Multiple Simultaneous Demonstrations in Mate-Copying. **Measuring Behaviour 2018** (5-8 juin 2018, Manchester, UK). Présentation orale.
https://www.measuringbehavior.org/files/2018/MB2018%20Proceedings.pdf

M. Monier, S. Nöbel, G. Isabel and E. Danchin. Mate-Copying : mechanistics bases of a social learning in *Drosophila melanogaster*. **CRCA doc-post-doc symposium** (26 septembre 2018, Toulouse). Présentation orale.
http://rainbiodoc.wixsite.com/crca2018/program

M. Monier, S. Nöbel, E. Danchin and G. Isabel. Dopamine and serotonin are both required for mate-copying in *Drosophila melanogaster*. **Ecology & Behavior 2019** (20-24 mai 2019, Toulouse). Poster.

Enseignements en Licence Biologie Cellulaire et Physiologie :

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Vulgarisation scientifique :

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Encadrement de stagiaires :

2018 : Romain Di Stasi, M1 ; Tristan Lafont, L3.
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Résumé

La copie de partenaire, ou « *mate-copying* » est un comportement bien documenté chez de nombreuses espèces, parmi lesquelles des animaux en apparence aussi rudimentaires que *Drosophila melanogaster*. Chez cette espèce d'insecte, lorsqu'une femelle observe une autre femelle s'accoupler avec un mâle d'un certain phénotype, sa préférence pour les mâles de ce phénotype augmente. Autrement dit, elle copie la préférence de partenaire de la femelle démonstratrice. Ce comportement constitue un modèle d'apprentissage social observationnel que l'on peut exploiter tant au niveau des mécanismes proximaux (par exemple comportementaux et neurobiologiques) que distaux (par exemple pour son influence sur l'évolution). Dans ce travail, ces deux aspects du mate-copying sont abordés. Le premier chapitre de ma thèse étudie la stabilité de cette stratégie de choix de partenaire en fonction de conditions environnementales sociales, particulièrement sur la disponibilité apparente des mâles, et sa stabilité dans le temps (mémoire à long terme). J'ai montré que les femelles adaptent leur sélectivité en fonction de la disponibilité apparente des mâles, mais sans impact sur leur capacité à copier le choix de la femelle démonstratrice. J'ai aussi contribué à montrer que les femelles peuvent former une mémoire sociale à long terme (24h) impliquant la synthèse protéique. Les deuxième et troisième chapitres abordent les mécanismes cognitifs du mate-copying. Ainsi, j'ai montré que les neurotransmetteurs dopamine et sérotonine sont impliqués dans cet apprentissage ; j'ai montré également que le récepteur dopaminergique DAMB (*DopAmine Mushroom Bodies*) est requis pour cette mémoire sociale à long terme, mais pas à court terme, suggérant l'implication d'un autre récepteur dopaminergique que DAMB dans cet apprentissage social. J'ai enfin élaboré un nouveau protocole de démonstrations basé sur des photographies, qui contribuera à la caractérisation plus efficace des signaux visuels nécessaires, et à moyen terme, des mécanismes neurobiologiques. Enfin, j'ai montré que le mate-copying est un apprentissage basé sur le trait du mâle impliqué dans l'acceptation et non le rejet par la femelle démonstratrice, et impliquant des réseaux neuronaux dopaminergiques en jeu dans l'apprentissage aversif olfactif.

Abstract

Mate-copying has been reported in many Vertebrate and Invertebrate species, including animals as simple in appearance as *Drosophila melanogaster*. In this species, when a female observes another female mating with a male of a given phenotype, his attraction to other males of this phenotype increases. In other words, she copies the mate preference of the demonstrator female. This behavior constitutes a powerful model of social observational learning in animals, both for proximate mechanisms (for instance behavioral and neurobiological) as well as ultimate mechanisms (notably, as it takes part to sexual evolution). The present work studied these two aspects of mate-copying. The first chapter tested the stability of mate-copying across environmental social conditions, more specifically, apparent availability of males, and across time (long-term memory). I showed that, while sex-ratio affects female choosiness positively, *Drosophila* females seem to have evolved a mate-copying ability independently of sex-ratio. I also participated in showing that females can form a social long-term memory (24h) involving protein synthesis. Chapters 2 and 3 deal with cognitive mechanisms in mate-copying. I showed that it involves the neurotransmitters dopamine and serotonine, while the dopaminergic receptor DAMB (DopAmine Mushroom Bodies) is required for this social long-term memory, but not for short-term memory, which suggests that another dopaminergic receptor is also involved in this social learning. I designed and tested a new protocol of demonstrations based on photographs, which will ease the study of the visual cues necessary for this behavior, and later the study of the neurobiological mechanisms. Finally, I showed that mate-copying is a learning based on on the trait of the male accepted by the demonstrator female, and not on the rejected one, and I found that, counter-intuitively, dopaminergic networks involved are those for aversive, not appetitive, olfactory learning.

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Introduction

Sexual selection

Natural selection continuously selects for the individuals that have the higher chance of survival or reproduction in a given context. Thus, individuals that are less adapted to their environment, for instance because they are weak, sick or disabled, have lower chances of survival and will be counter-selected. However, in some species, traits that can appear as a disadvantage persist or strengthen over generations. It is the case for instance in several birds with long ornamented tails like in the peacock. Such ornaments can be viewed as handicaps with respect to escaping predators. The same holds for the bright colors of many birds species that prevent them from easily hiding. The solution of this apparent paradox is another evolutionary mechanism: sexual selection. This concept was first proposed by Darwin in *The Origin of Species* (Darwin, 1859), and later developed in his book (Darwin, 1871) as he felt that natural selection alone was unable to account for certain types of non-survival adaptations.

Sexual selection occurs when members of one sex select members of the other sex to mate with (inter-sexual selection), or when members of the same sex (usually males) compete with each other for access to the other sex (intra-sexual selection). In this paradigm, exaggerated traits (ornaments, colors...) can be an advantage to successfully find a mate and reproduce (Figure 1). Sexual selection was later developed by Fisher (Fisher, 1930), who proposed several hypotheses to explain and describe it. Notably, the Fisher runaway process suggests that male ornaments and female preference for these ornaments are both heritable, with a co-evolution of both, which can lead to a positive feedback, selecting for the most extreme ornaments in males together with the highest preference for these traits in females. This mechanism is a possible explanation for the highly diverse and often astonishing ornaments of animals and plants. In plants, selection is actually performed by another agent, which is the pollinator, but the result is still that exaggerated traits like colorful flowers that we find very beautiful are in fact a byproduct of sexual selection.

Figure 1: Examples of sexual selection. A. male peacock. ©Tuo Yang. B. male paradise bird. ©Tim Laman.

In 1948, Bateman published an experimental study of Drosophila's reproduction in which he demonstrated sexual selection (Bateman, 1948). He reported that in that species the reproductive success depends on the number of successful matings in males, but not in females, for which one mating is usually sufficient to maximize their reproductive success. Moreover, he observed that the reproductive success is highly variable in males, depending on male-male competition intensity. In other words, females are the choosy sex in *Drosophila* as this is the case in most species. Apart from these few studies, sexual selection has been largely overlooked for more than a century, with a revival starting in the 1980s, in particular with the work from Lande (Lande, 1981) and Zahavi (Zahavi, 1975, 1977). Since that decade, sexual selection has become one of the most prominent subject studies in behavioral ecology (reviewed in Danchin and Cézilly, 2008).

Mate choice, Mate-copying

Choosing a mate is a decision with major fitness consequences, particularly for individuals that have few partners in their lifetime, because the quality of the mate affects the fitness of their progeny. In *Drosophila*, a study conducted on wild flies found that females mate four to six times in their whole life (Imhof et al., 1998), so it is of no surprise that they built strategies to maximize their chances of choosing a suitable partner. During male courtship, for instance, females can discriminate courtship songs from two closely related species (Kyriacou and Hall, 1982), and they show much higher preference for the courtship songs of males of their own species.

Apart from personal assessment of male quality, females also developed an economical mate-choice strategy: mate-copying. After witnessing the mate-choice of another female between two males of different phenotypes, females build a clear preference for the male phenotype they saw being chosen over the one that was rejected during the demonstration. This behavior was described in several taxa. First descriptions of mate-choice copying came from field studies of lekking birds and mammals (reviewed in Gibson and Höglund, 1992). It was then reported in fish, in the guppy *Poecilia reticulata* (Dugatkin and Godin, 1993), in birds with the Japanese quail, *Coturnix coturnix japonica,* (White and Galef Jr, 1999), in mammals: humans (Waynforth, 2007) and the Nordway Rat *Rattus Norvegicus* (Galef et al., 2008), and finally, Frederic Mery and collaborators demonstrated mate-copying in an invertebrate for the first time using *Drosophila melanogaster* in 2009 (Mery et al., 2009). In this species, females are able to memorize and copy the mate-choice decision of a demonstrator female after watching her freely choosing between two artificially dusted green and pink males (Dagaeff et al., 2016, Figure 2).

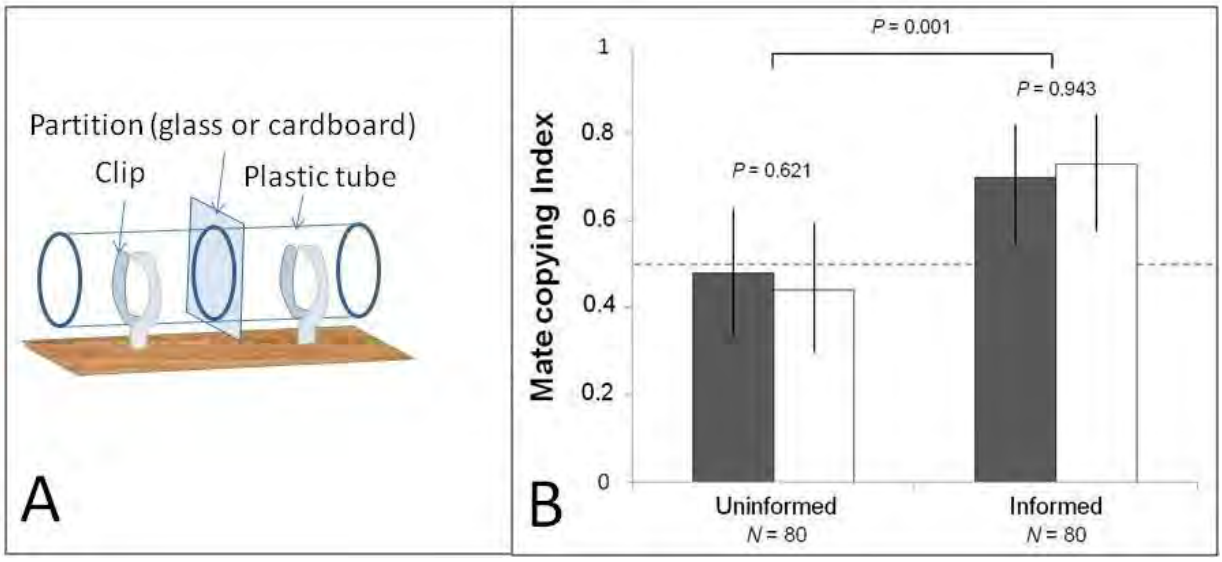

Figure 2: Mate-copying in *D. melanogaster* using artificial colors. A. Experimental device used in Dagaeff et al. 2016. **B.** Mate-copying index of drosophila females after different treatments. Informed flies: females that saw a demonstration in which the pink (grey bars) or the green (white bars) male was preferred, while the other male color was rejected. Uninformed flies: the partition between observer and demonstrators was opaque. P values: pairwise comparisons. Vertical bars: 95% Agresti-Coull confidence intervals; horizontal dashed line: expected value if females chose randomly. B is excerpted from Dagaeff et al. 2016, Figure 4.

In the past decade, Danchin and Isabel and their collaborators studied mate-copying from an evolutionary point of view (Loyau et al., 2012; Germain et al., 2016; Danchin et al., 2018; Nöbel et al., 2018b, 2018a), gathering increasing knowledge on this social behavior and its evolutionary consequences. At the time I started my PhD, mate-copying in *Drosophila* had constituted a promising model to study the cognitive mechanisms of social learning in general (Dagaeff, 2015), although this field was really emerging.

Evolutionary importance: selection, arbitrary traditions

Mate-copying can be individual-based, when the observer female develops a preference for the very same male she saw being successful with another female. This form of mate-copying, without generalization, cannot persist in time, and can have drawbacks for the copier female, in particular disease transmission, and in some species in which males are sperm-limited (for instance, in drosophila, see Demerec and Kaufman, 1941; Loyau et al., 2012), the female will have less offspring with a single mating when her suitor already mated with another female just before. As a matter of fact, female fruit flies tend to avoid mating with a male they saw being chosen just before (Loyau et al., 2012).

Another form of mate-copying is trait-based copying (Bowers et al., 2012), in which the female builds a preference for any male bearing the same trait as the successful male. For

instance, in *D. melanogaster*, observer females witnessing a choice between a pink and a green wild-type males later copy the preference for the chosen color when given the choice between a green and a pink curly-winged males, or between a pink and a green white-eyed males (Danchin et al., 2018). Thus, drosophila females do develop a preference for a trait.

The important point is that only the trait-based copying can be transmitted among interacting individuals within a population, potentially leading to the emergence of local cultural traditions for an arbitrary trait (i.e. a trait not necessarily revealing the fitness of the males). As a matter of fact although interesting in itself, learning to prefer the very specific male that was chosen during the demonstration cannot be transmitted over generations because the potential transmission chain generated by such a social learning would end with the death of that male.

Persistent local traditions then constitute a form of selection that can impact the evolution of male traits in the population, as females select some male traits (the preferred traits) against others. In other words, there would be a form of sexual selection that would not be genetically based, but would rather result from social learning.

Social learning

Building tools, learning a language, choosing a mate, all involve some learning, and some innate capacities. Learning from the other's experience is probably the main learning method in *Homo sapiens*, who evolved a brain well-fitted for this purpose. Cecilia Heyes, in her book "cognitive gadgets, the cultural evolution of thinking" (Heyes, 2018), proposes that "*the minds of human babies are only subtly different from the minds of newborn chimpanzees. We are friendlier, our attention is drawn to different things, and we have a capacity to learn and remember that outstrips the abilities of newborn chimpanzees. Yet when these subtle differences are exposed to culture-soaked human environments, they have enormous effects. They enable us to upload distinctively human ways of thinking from the social world around us".* In other words, our high capacity to socially learn is a major trait of our species, and we use social learning extensively to adapt to our environment. This use of social learning has the potential to lead to the emergence of cultural processes, that then become part of inheritance (that is parent-offspring resemblance), which may then interact with genetic evolution in affecting the evolutionary fate of populations.

As a consequence, illnesses that affect social skills (e.g. autism spectrum disorders) or learning capacities in general usually cause strong disabilities. It thus appears of major interest to disentangle the cognitive processes underlying social learning in humans. This can be studied by cognitive sciences, psychology, as well as behavior biology. The last discipline takes advantage of inter-species similarities in the brain structures, genomes, and protein interaction networks to study complex processes using an easier-to-study species.

Many animal species have been shown to be capable of social learning (Galef, 1985; Brown and Laland, 2003; Galef and Laland, 2005; Leadbeater and Chittka, 2007; Battesti et al., 2015). For instance, Nordway Rat pups have been shown to learn avoidance of a poisoned food by observing and copying their parent's diet (Galef and Clark, 1971). In social insects, in

particular in honeybees, social learning abilities have been observed since ancient Greece: Aristotle himself, in his descriptions of animal species, praised the "extraordinary intelligence" of honeybees. In these species, information given by the relatives allows learning new foraging areas and synchronization of the nest activities (Leadbeater and Chittka, 2007).

More recently, social learning was demonstrated in non-social insects, for instance in the Wood cricket *Nemobius sylvestris* (Coolen et al., 2005), and in fruit flies. In the latter insect, Sarin and Dukas (Sarin and Dukas, 2009), and later Battesti et al. (Battesti et al., 2015) observed that oviposition site choice is heavily influenced by previous social interactions.

In this context, fruit flies constitute a particularly suitable animal model as they can be used to study social learning mechanisms at the molecular, cellular and behavioral levels (Leadbeater, 2009). In the next section I illustrate the major importance of the fruit fly as a model animal in the past and present for biology.

Drosophila as a model organism

D. melanogaster entered in the history of scientific research at the beginning of the twentieth century, when Thomas Hunt Morgan used it in his "flyroom" (Figure 3).

Figure 3: Morgan's fly room, around 1920. Courtesy of American Philosophical Society. CC BY 4.0.

The reasons of the success of this little dipter are many. First of all, it is cheap and easy to breed, needing only a small tube with corn flour-yeast medium, where it can reproduce quickly and in large proportions. It has a short generation time: at 25°C, the eggs laid by a female (up to 100 per day) will develop into a larva, a pupa, and finally a sexually mature adult after only eleven days (Figure 4).

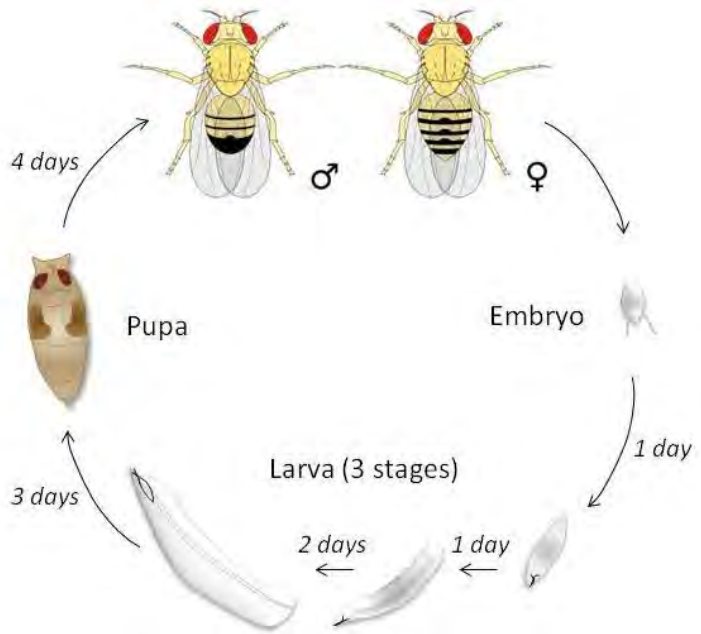

Figure 4: Drosophila life cycle. The eggs laid by a female undergo several larval stages after hatching, then enter the pupal stage during which they undergo metamorphosis, leading to the imago (i.e. adult). Newly emerged adults become sexually mature a few hours after emergence. Approximate durations of each stage are indicated for standard rearing conditions at 25°C.

The sexual behavior of both male and female drosophila is accurately described in the literature (Villella and Hall, 2008), and relatively easy to measure in the lab. Briefly, fruit flies acquire sexual maturity several hours after emergence (Manning, 1967). Before sexual maturity, females reject all males for copulation. Sexually mature, young virgin females are highly attractive to males (Tompkins and Hall, 1981), which courtship is stereotyped (Villella and Hall, 2008). The first easily observable behavior of the courtship sequence is the "singing", when the male extends a wing to emit the courtship song. In all behavioral experiments I used this singing behavior as a measure of courtship initiation. Then the male chases the female, contacts its genital parts and tries to mount the female by bending its abdomen. Copulation acceptance in *D. melanogaster* is under female control (Connolly and Cook, 1973; Kimura et al., 2015), that is, there is no forced copulation in the wild.

Historically, *D. melanogaster* was first used in genetics studies, but is now a broadly used model organism in many kinds of studies. Its genome was sequenced in 2000 (Adams et al., 2000), it has 170 Mbp (per haploid genome) which is rather small compared to a mammal's. For instance, the mouse's genome is 2.5 Gbp big (Church et al., 2009), and contains about 14,000 genes, while human genome has about 20,000 (Salzberg, 2018). A very detailed annotation of *D. melanogaster* genome is now available (flybase.org), and reveals that not less than half the genes has an ortholog in the human genome, making the fruit flies an excellent model to study many human diseases (Yamaguchi, 2018) like Parkinson, Alzheimer, cancer (Enomoto et al., 2018), immune system diseases, among others (Jeibmann and Paulus, 2009; Apidianakis and Rahme, 2011). On a structural point of view, the *Drosophila* genome is composed of four pairs of chromosomes, it is easy to observe in salivary gland cells of the larva, as they contain polytene chromosomes (massive duplication

of each strand without cytoplasmic division) and this last property eases the establishment of precise genomic cartography.

Because of these properties, researchers developed a large diversity of genetic tools to modify the fly's genome, drive the expression of a gene (ectopic or not), or modify the activity of a given structure or cell. Drosophilists, all around the world, constituted banks of strains, developmental and genomic data in which researchers can pick according to their needs. Thus, it is now relatively easy to build a custom-made drosophila strain that fits exactly someone's needs. During my PhD, I used some of these genetic tools, in particular revolving around the UAS-Gal4 system.

The UAS-Gal4 tool is a yeast genetic expression regulatory system (Figure 5): Gal4 codes for a transcription factor that specifically recognizes an enhancer sequence called UAS (Upstream Activating Sequence) localized upstream a gene which expression will be activated when GAL4 binds to the enhancer. This system was used to express genes in animal cells (Kakidani and Ptashne, 1988; Webster et al., 1988) and has been used in *Drosophila* since that time (Fischer et al., 1988). Briefly, a UAS sequence (or several UAS sequences, to increase expression level) is placed upstream an interest gene "geneA" (from drosophila, or ectopic like the jellyfish green fluorescent protein gene) and introduced in the genome of the fly by genetic engineering. The fly strain will not express it in absence of Gal4 (theoretically, because in some cases a slight "leak" of gene expression can be observed). In parallel, the gene Gal4 can be introduced in the genome of a fly, in a random place: if the gene is downstream a promoter, it will be expressed with the spatio-temporal pattern determined by this promoter. For instance, if Gal4 is localized downstream of the gene of Tryptophan Hydroxylase, it will be specifically expressed in every cell expressing this gene, which is, for the adult stage, in the serotoninergic system in theory. When the Gal4 line is crossed with the line containing UAS-geneA, geneA will then be expressed with the spatio-temporal pattern determined by the position of the Gal4 (Figure 5). By doing so, it is possible to express a gene of interest with the desired spatio-temporal pattern, thanks to huge banks of Gal4 lines (VDRC for instance).

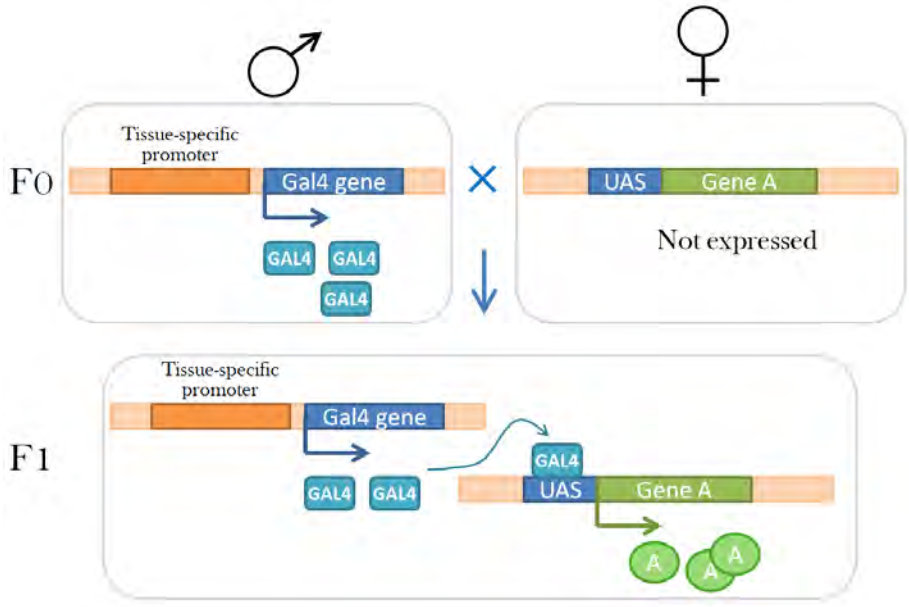

Figure 5: UAS/Gal4 genetic expression system. One parental line contains the UAS-GeneA while the

other contains the Gal4 under the control of a specific promoter. The progeny will thus inherit both transgenes which will result in the expression of geneA in a specific tissue at a specific stage, determined by the orange promoter.

Thanks to the genetic tools developed in drosophila and made easily available, the fruit fly has become a major model organism for the study of a large diversity of complex physiological processes, and the neurobiological processes governing learning and memory is not the least.

Drosophila in associative learning

Associative learning occurs when an individual experiences directly or observes a pairing between a conditional stimulus (CS) that is initially neutral, e.g. a blue circle, and a unconditional stimulus (US), either appetitive or aversive, e.g. sugar or electric shocks. The pairing will result in building a memory, appetitive or aversive depending on the valence of the US: later when the animal experiences the CS alone, it will display an approach or an avoidance behavior because it has associated the CS to a rewarding state or a punishment state, respectively. The most famous historical description of such a behavior is certainly that of Pavlov (Pavlov, 1927), who trained a dog to salivate at the sound of a bell, because this cue predicted the arrival of food in the training phase.

D. melanogaster has been broadly used in associative memory research (the basis of which started with Quinn et al., 1974): in olfactory learning and memory for example (Zars et al., 2000; Isabel et al., 2004; Scheunemann et al., 2012; Cognigni et al., 2018) as well as in visual learning for instance (Brembs and Heisenberg, 2000; Liu et al., 2006; Vogt et al., 2014, 2016). In olfactory learning in particular, the protocols to study associative olfactory learning were established decades ago (Quinn et al., 1974) and are still in use today. The ease of training and testing a large amount of flies altogether relatively quickly lead to outstanding progress in discovering the mechanisms underlying this form of learning. In particular, the different temporal phases of appetitive and aversive learning (Isabel et al., 2004; Trannoy et al., 2011), the neural structures and circuits involved are now well-described (Cognigni et al., 2018), and constitute a good basis for any study about other forms of learning in *Drosophila*, or olfactory learning in other species.

Several authors suggested that social learning should be studied as a form of associative learning. In particular, in *Drosophila* mate-copying, it was suggested (Avarguès-Weber et al., 2015) that male color and observation of the demonstrator trio could mediate a conditioned and an unconditioned stimulus, respectively. This hypothesis is interesting because it can help designing several experiments that will elucidate the nature of the cues needed to elicit mate-copying, and how they are conveyed and processed from the sensory organs to the high-order integration systems of the drosophila. These are some of the questions I tackled during my PhD. Building a parallel between what is known from olfactory learning and our social learning paradigm, we can make several assumptions that I summarize in the above figure (Figure 6). Briefly, the CS could be mediated by visual system neural networks, while the unconditioned stimulus should require dopamine, the neurotransmitter signaling the

valence of the US in many olfactory and visual learning processes (Riemensperger et al., 2005, 2011; Aso et al., 2012; Burke et al., 2012; Liu et al., 2012; Vogt et al., 2014).

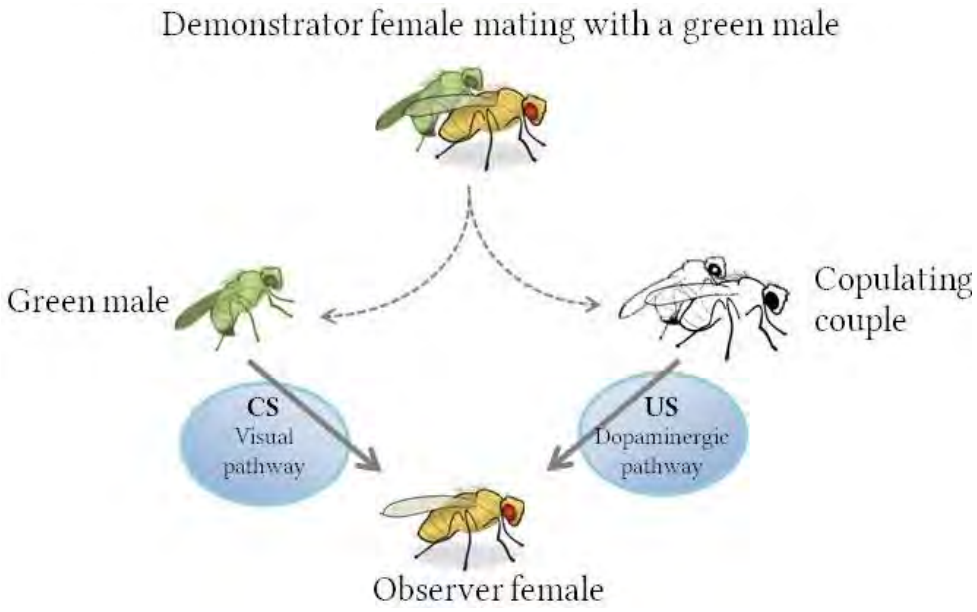

Figure 6: Mate-choice demonstration described in the CS/US paradigm. The observation of the copulating pair by the observer female can be divided into two components: the color of the successful male is the CS, while the observation of a couple successfully mating is an US (appetitive). US and CS converge on a coincidence detector in the female brain, Rutabaga (Nöbel et al, in prep).

The fly brain

Generalities

The brain of adult drosophila (Figure 7) is composed of 100,000 neurons and has been recently mapped with precision (Zheng et al., 2018). Although flies have a rather small number of brain neurons, they are capable of highly diverse and sophisticated behaviors, like courtship dance, olfactory and visual learning, fighting, or copying. This modest size and the capacity to display complex behaviors make them very well fitted for the study of cognitive processes.

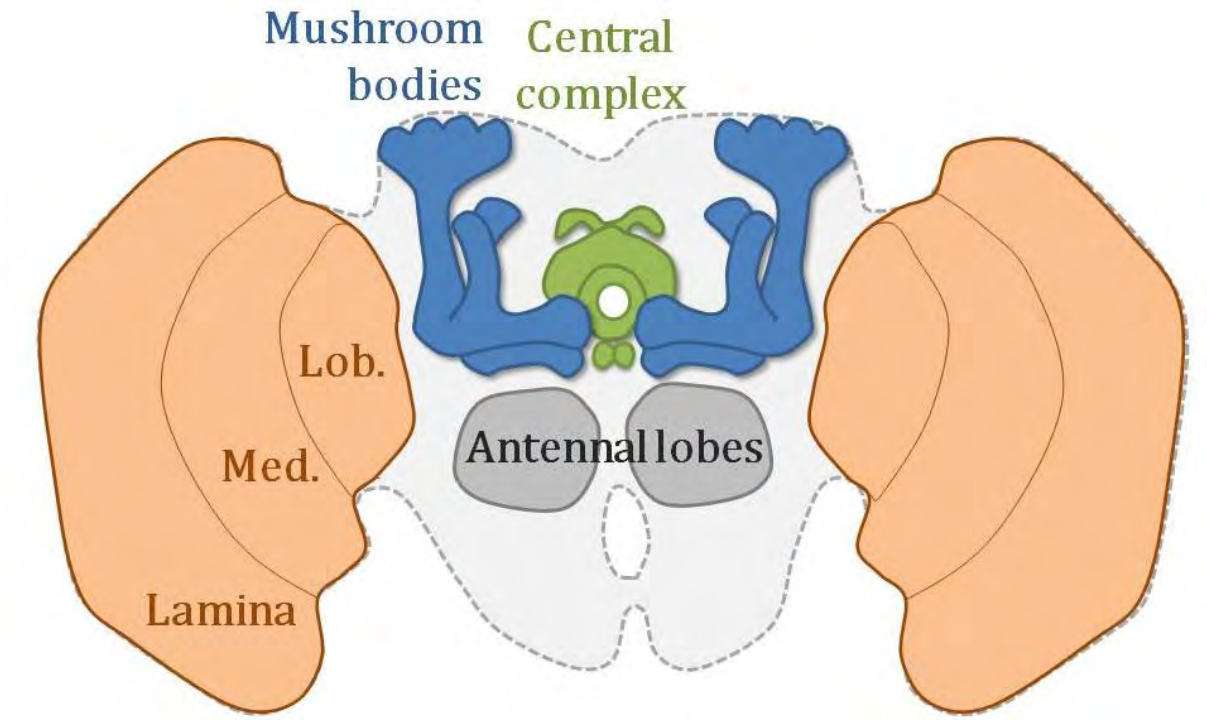

Figure 7: Drosophila adult brain. Lamina, medulla (Med.) and lobula (Lob.) are devoted to visual information primary processing. Antennal lobes process olfactory information; central complex and mushroom body are higher-order integration structures. Other brain structures are not depicted.

In particular, the brain of adult Drosophila comprises two structures involved in higher-order integration of sensory stimuli from the fly's environment, and thus responsible for learning and memory from different sensory modalities. These two structures are the mushroom bodies and the neuropils of the central complex.

The Mushroom bodies

The mushroom body (MB, Figure 8) is a higher processing center in the insect brain, it is functionally equivalent to the hippocampus of mammals (Davis and Han, 1996; Barnstedt et al., 2016). It is composed of Kenyon cells (in blue on Figure 8), about 2,200 per hemisphere (Kahsai and Zars, 2011), which mainly receive inputs from the antennal lobe's projection neurons (Lin et al., 2007). The wiring between projection neurons of the antennal lobe and Kenyon cells of the MB is largely random, which may contribute to maximize the memory capacity of this mini brain (Caron et al., 2013). Some Kenyon cells (for instance the dorsal accessory Kenyon cells) are not contacted by projection neurons of the antennal lobe but receive inputs from other sensory modalities. MB lobes are the main output sites of this structure, but they also receive inputs from neurons of other brain structures. Kenyon cells form cholinergic synapses (Barnstedt et al., 2016) with 21 types of mushroom body output neurons (MBONs), organized into a highly-complex, multi-layered network (Aso et al., 2014a, 2014b). MBONs project to several neuropils of the fly brain, and three MBON types also constitute a feedforward loop by contacting the MB lobes. Synapses between KCs and

MBONs are modulated by 20 different types of dopaminergic neurons (Aso et al., 2014b). Kenyon cells activity is also modulated by GABAergic neurons from the Anterior Paired Lateral neuron (APL). This regulation can suppress learning (Liu and Davis, 2009) and can sustain labile memory and/or anesthesia-resistant memory (Pitman et al., 2011; Wu et al., 2013). The Dorsal Paired Median (DPM) neuron is required to consolidate mid-term memory via serotonin (Lee et al., 2011). Interestingly, APL and DPM are functionally connected with gap junctions, and they are critical for memory (Wu et al., 2011).

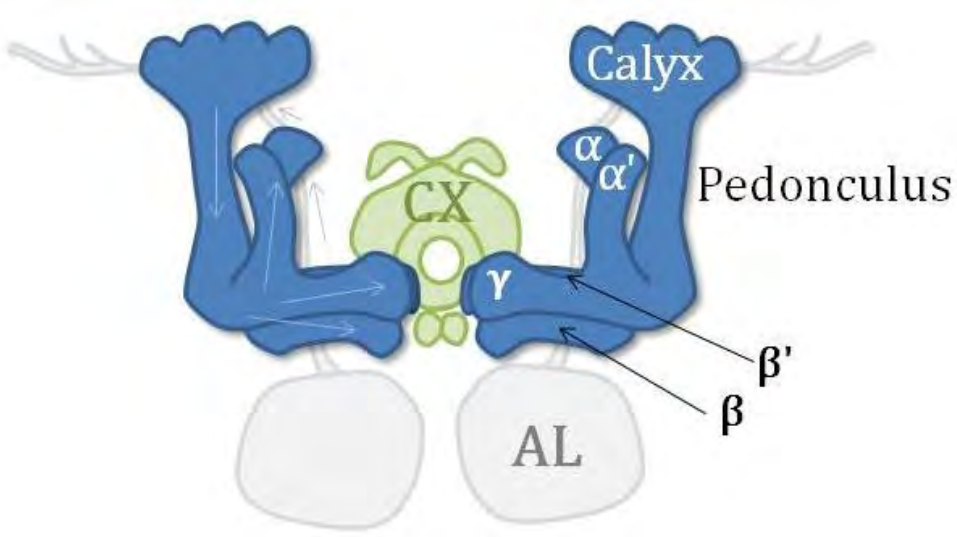

Figure 8: drosophila mushroom body. The mushroom bodies of the adult fly brain are composed of three lobes: alpha/alpha', beta/beta', and gamma, composed of Kenyon cells axons, soma being located on top of the calyx (not represented). Arrows on the left part indicate information flow in the neurites: Projection neurons from the antennal lobe send axons to the calyx (grey arrows) and to medial structures of the fly brain. Information circulates from the dendrites located in the calyx, to the lobes (blue arrows) in which Kenyon cells axons contact dendrites of mushroom body output neurons (MBONs). AL: antennal lobe, CX: central complex.

This brain structure is the center of formation and storage of associative memory (Heisenberg et al., 1985; Belle and Heisenberg, 1994; Dubnau et al., 2001; McGuire et al., 2001; Cognigni et al., 2018). Notably, dopamine receptors and Rutabaga Adelylate Cyclase are specifically required in the mushroom body for olfactory memory formation and stability (Zars et al., 2000; McGuire et al., 2003; Isabel et al., 2004; Aso et al., 2012; Scheunemann et al., 2012; Waddell, 2013). MB cells play a key role in formation and storage of olfactory short-term memory, in courtship conditioning memory, and in regulating the transition from walking to rest (reviewed in Zars, 2000; Riemensperger et al., 2011; Cognigni et al., 2018). Moreover, MBs are known to be involved in visual memory: they are required for visual context generalization (Liu et al., 1999), and they allow stabilization of visual memories in changing contexts (Brembs and Wiener, 2006). Finally, γ neurons of the mushroom bodies mediate memorization of simple associations between color stimuli and an expected outcome (sugar reward or electric shock punishment, Vogt et al., 2014).

The central complex

The central complex (CX) is a medial structure of the fly brain composed of several thousands of neurons organized into four neuropils: protocerebral bridge, fan-shaped body, ellipsoid body, and the two noduli (Figure 9). The different structures are inter-connected into a complex and multilayered network called a connectome, and display strong inter-hemispheric connections through chiasmata (Pfeiffer and Homberg, 2014). Central complex noduli receive inputs from visual processing structures (lamina, lobula and medulla) connected to compound eyes.

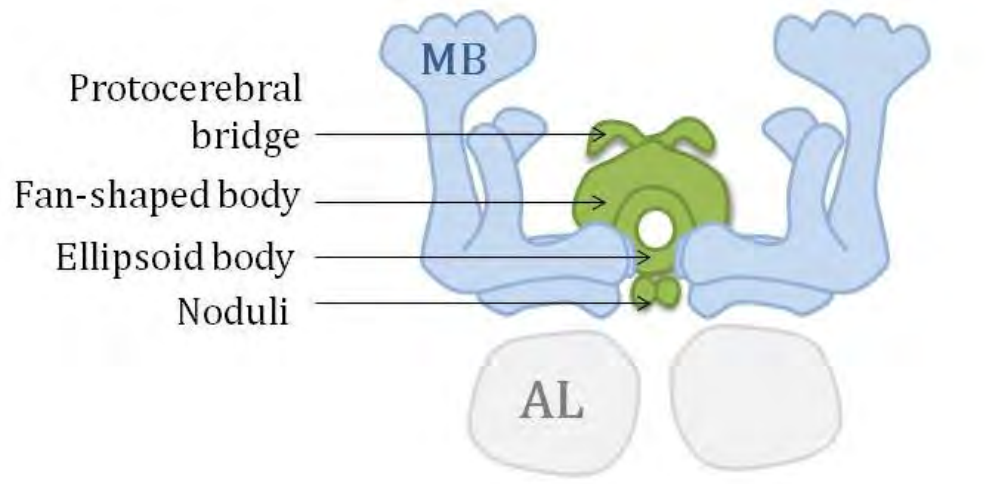

Figure 9: Anatomy of the central complex of the adult fly brain. Neuropils of the CX are represented in green. MB: mushroom bodies, AL: antennal lobes, are represented for the purpose of orientation.

The central complex has been shown to be involved in complex behaviors, notably during flight. It allows spatial navigation in insects (Webb and Wystrach, 2016). It is involved in landmark detection, angular position detection, and perception of body position, but also in visual pattern memory (Liu et al., 2006; Pan et al., 2009). In particular, the ellipsoid body is involved in visual place learning and short-term orientation memory (Neuser et al., 2008; Seelig and Jayaraman, 2013; Pfeiffer and Homberg, 2014), as well as in NMDA-receptor dependent long-term memory consolidation in olfactory learning (Wu et al., 2007). Thus visual learning and memory are achieved through dynamic interactions between the ellipsoid-body and the fan-shaped body.

Mushroom bodies and the central complex are the two key structures in learning and memory. Their differential implication depends on the temporality and the sensory modality of the learning experience, but visual learning often requires both structures. Locomotion state (flying or walking) may also play a role in selecting the neural pathway involved in visual information memorization (Kottler and van Swinderen, 2014). To wrap everything up, we can hypothesize that in mate-copying, observational learning is allowed by the MB and/or the CX.

Questions and hypotheses tackled in my PhD

During my PhD, I tried to address several questions linked to the evolutionary importance of mate-copying on one hand, and to the mechanisms underlying this social learning on the other hand.

I first investigated the stability of this mate-choice strategy: stability in time and robustness to male availability. The aim was to evaluate the robustness of this behavior, in order to determine the evolutionary relevance of the behavior, and use mate-copying as a model for the study of social learning mechanisms.

In the second part I investigated a part of neuronal mechanisms involved in the short term memory and the long term memory of social learning. I first focused on the roles of dopamine and serotonin in short/mid-term memory of mate-copying, then on the dopaminergic receptor DAMB, that is specifically required for long-term memory formation in appetitive and aversive olfactory learning (Musso et al., 2015; Plaçais et al., 2017).

In the third part, I tried to find what are the necessary cues for mate-copying. Based on the strong assumption that only vision is needed (as glass partitions do not allow olfactory cues), I proposed a new protocol based on virtual demonstrations using pictures, in order to see whether fruit flies can mate-copy out of a picture. My hypothesis was that it is the case. I also disentangled positive from negative information (that is, female acceptance for a male trait, from female rejection for a male trait) during the demonstration of a mate-copying experiment to determine which from the positive or the negative part was necessary to elicit learning and copying in observer females.

Chapter I. Evolutionary importance of mate-copying

I first studied the evolutionary importance of mate-copying, and more precisely its stability across environment and across time. The aim of this first chapter is to bring pieces of evidence that mate-copying is a general, well-established strategy of mate-choice, and is thus a good model to study the mechanisms of a social learning and the potential outcomes of such a strategy on a long term.

In the wild, fruit flies do not live alone: they aggregate on food patches (Rodrigues et al., 2015; Keesey et al., 2016) where they meet individuals of their own species and of other species. In these groups, inter-sex encounters can lead to copulations and same-sex encounters can give rise to competition, for instance for access to mates. Here, I first studied the effect of female competition on mate-copying scores and choosiness of female observers. In the conditions studied in the first experiment (part A-1), the sex-ratio or number of competitor females had no effect on mate-copying scores. This may result from several contradictory effects cancelling each other out, but all in all the pattern I mesured is an indication that mate-copying is a stable mate-choice strategy, that has a certain robustness to the social context. This work was published in *Current Zoology* in January 2018. In a second experiment (part A-2), I investigated whether the pattern I observed could be due to the sex-ratio itself, by changing the sex-ratio during the demonstration while the number of observer females remained constant on one hand, and on the other hand this experiment tested the effect of male phenotypic rarity during the demonstration on mate-copying scores. These two experiments gave measures of the stability of mate-copying across different environmental conditions.

In a second step (part B), I participated to studying whether female fruit flies can form a long-term memory of a mate preference after watching several mate-choice demonstrations. This experiment thus measured the stability of mate-copying across time.

A. Stability in environment: study in a context of competition for access to males

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Article

Article

Effects of a sex ratio gradient on female mate-copying and choosiness in *Drosophila melanogaster*

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Abstract

In many sexually reproducing species, individuals can gather information about potential mates by observing their mating success. This behavioral pattern, that we call mate-copying, was reported in the fruit fly *Drosophila melanogaster* where females choosing between 2 males of contrasting phenotypes can build a preference for males of the phenotype they previously saw being chosen by a demonstrator female. As sex ratio is known to affect mate choice, our goal was to test whether mate-copying is also affected by encountered sex ratios. Thus, we created a gradient of sex ratio during demonstrations of mate-copying experiments by changing the number of females observing from a central arena 6 simultaneous demonstrations unfolding in 6 peripheral compartments of a hexagonal device. We also tested whether the sex ratio experienced by females during demonstrations affected their choosiness (male courtship duration and double courtship rate) in subsequent mate-choice tests. Experimental male:female sex ratio during demonstrations did not affect mate-copying indices, but positively affected the proportion of both males courting the female during mate-choice tests, as well as male courtship duration, the latter potentially explaining the former relationship. As expected, the sex ratio affected female choosiness positively, and *Drosophila* females seem to have evolved a mate-copying ability independently of sex ratio, and a capacity to adapt their choosiness to male availability. This suggests that, as in many animal species, individuals, especially females, can adapt their mate choice depending on the current sex ratio.

Key words: competition, *Drosophila melanogaster*, experimental protocol, mate-copying, social learning, sex ratio.

Choosing a mate is a major fitness-affecting decision in any sexually reproducing organism. In species where females invest more than males in the production of a single offspring, females are selected to become the choosy sex (Johnstone et al. 1996; Trivers 1972). Furthermore, in males of such species, natural and sexual selection shape traits that are related to male quality, which in turn can be used by females to assess male quality because these traits reveal that they are better fathers providing better or more resources to the female or the offspring (Candolin 2003). Male mating success is thus affected by various parameters revealing their intrinsic quality

(Weatherhead and Boag 1995), such as their size, ornaments, bright colors, or songs as this was documented in many animal taxa including vertebrates and invertebrates (Partridge and Farquhar 1983; Searcy 1992; Madsen et al. 1993; Andersson 1994; Aspi and Hoikkala 1995; Bateman et al. 2001; Dreher and Pröhl 2014; reviewed in Danchin and Cézilly 2008).

Alternatively but not exclusively, females can get further information about potential mates by observing their mating success (Danchin et al. 2004). We call such observational learning mate-copying. It leads females to either mate preferentially with the specific male they saw being chosen by another female (individual-based copying, Pruett-Jones 1992; Bowers et al. 2012), or with a male showing similar characteristics as the male they saw being chosen by another female (trait-based copying, Bowers et al. 2012). Trait-based mate-copying is particularly interesting because learning to prefer males of a given phenotype rather than a specific male (Witte et al. 2015) may potentially lead to the establishment of persistent local traditions in mate choice (Danchin et al., submitted for publication), which in turn may strongly affect sexual selection differentially across populations, thus setting the stage for speciation.

Mate-copying has been reported in many social and non-social species, including fish (Dugatkin and Godin 1993), birds (White and Galef 1999), humans (Waynforth 2007), and other mammals (Galef et al. 2008), as well as in 1 insect, *Drosophila melanogaster* (Mery et al. 2009; Loyau et al. 2012; Dagaeff et al. 2016; Germain et al. 2016; Danchin et al., submitted for publication; Nöbel et al., submitted for publication). In particular, *D. melanogaster* females can perform trait-based mate-copying after watching only a single live demonstration of 1 female copulating with a male of a given phenotype and 1 male of another phenotype being rejected (Dagaeff et al. 2016; Danchin et al., submitted for publication; Nöbel et al., submitted for publication). More generally, it is now accepted that many animal species from a vast array of taxa can learn from others (i.e., socially learn), particularly in the context of mate choice (Avital and Jablonka 2000; Danchin et al. 2004; Galef and Laland 2005).

Sex ratio is known to affect male–male competition in a mate-choice context in various species (Lawrence 1986; Jirotkul 1999; Weir et al. 2011), probably because it affects the availability of potential partners. For instance, in *D. melanogaster*, male sexual behavior is influenced by the number of rivals (Bretman et al. 2009), and male sperm depletion starts after just one copulation, so that the number of emerging offspring is divided by more than 3 after 4 consecutive copulations (Demerec and Kaufmann 1941; Lefevre and Jonsson 1962; Loyau et al. 2012). Thus, females are also expected to adapt their sexual behavior to the sex ratio (i.e., to the number of competitor females), for instance by accepting mates more readily when the male-to-female ratio is low, that is, when the number of potential female competitors is high.

Females can use 2 different sources of information to select a male partner: females might rely on their personal assessment of males' courtship during the mate-choice test, or only rely on the social information provided by demonstrator females during the demonstration. The latter option is probably more economical in a context of high level of female competition, as mate choice is costly to females (reviewed in Reynolds and Gross 1990; Andersson 1994; Vakirtzis 2011). For instance, females invest time and energy to assess male quality, and male courtship may sometimes be harmful to them (Andersson 1994). Moreover, the risk of losing potential mates to competitors increases with the time spent in assessing males. Under high competition scenario, one could expect females to use the more easily gathered social information only, thus minimizing risks of losing potential mates to competitors. In contrast, under low female competition (i.e., in male-biased sex ratios), this risk is minor, allowing them to spend time in male assessment.

So far, no study has investigated the effect of sex ratio on mate-copying or social learning in general. As in the more general context of mate choice, we can expect females to show contrasting choosiness when being within groups of varying sex ratios (Berglund 1994; Jirotkul 1999; Passos et al. 2014). For example, being under strong competition to access males, females learning within a mixed-sex group mainly composed of females might be much more prone to accept the very first male that courts them, and thus, ignore social information. Contrastingly, females learning within a mixed-sex group essentially composed of males can be expected to be much choosier and to take the time to gather more information about the various potential males before selecting one of them.

Here, we studied mate-copying along a gradient of group sex ratio during demonstrations. All mate-copying designs in *Drosophila* involve a demonstration phase (or simply demonstration) followed by a mate-choice test. We manipulated the population sex ratio during demonstrations by varying the number of observer females (and thus group size) in the central arena of a hexagonal experimental device (see "Materials and Methods" section). By assuming that observer females can assess and remember the group size and sex ratio they experienced during the demonstration, we predicted that along our gradient of increasing sex ratios during demonstrations, observer females would become more and more choosy during the subsequent mate-choice test, leading them to 1) accept copulation slower (i.e., a longer delay between first courtship and copulation initiation), and thus 2) increasing the rate of replicates in which both males courted the female before copulation initiation ("double courtship rate"), a parameter with methodological implications for future mate-copying experiments.

Concerning mate-copying, we could predict at least 3 possible outcomes, according to how we envisage the group size and sex ratio effects. The group size effect can be either positive or negative: first, females might learn better in a group than alone, thus predicting a positive mate-copying to group size relationship in a form of "social social-learning." Inversely, in large groups of females (i.e., at female-biased sex ratios) group members may start to disturb and stress each other, thus hampering proper learning and leading to a negative mate-copying to group size relationship in our system. On the other hand, sex ratio could have a negative effect: we could expect that under female-biased sex ratios during demonstration (i.e., in large groups) observer females will adopt the less costly strategy and thus favor social information use over the time-consuming assessment of both males, thus leading to a negative mate-copying to sex-ratio relationship. These potential and contradictory effects of group size and sex ratio on mate-copying, being non-exclusive, might also cancel out each other, leading to no detectable pattern in mate-copying with group size and/or sex ratio. They may also lead to an optimal group size at which mate-copying is more efficient. The mate-copying to group size and sex ratio relationship should thus depend on the relative importance of these various potential effects, so that we did not have clear predictions.

Materials and Methods

Fly maintenance

Wild-type Canton-S flies were raised in 30 ml vials containing 10 ml of a standard corn flour—agar—yeast medium. They were maintained at 25 ± 1°C, 59 ± 5% humidity in a 12 h/12 h light/dark cycle.

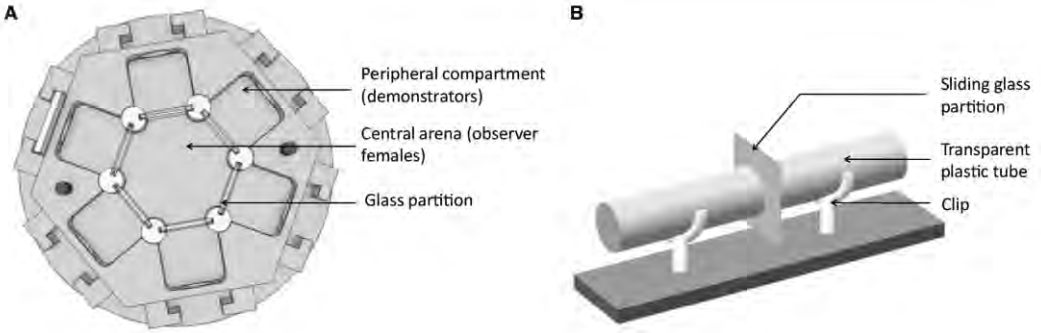

Figure 1. Experimental set-up used for demonstrations and mate-choice tests. (**A**) Demonstrations took place in a hexagon device (Danchin et al., submitted for publication). Observer females were placed in the central arena and were able to observe 6 demonstrator trios of 1 female copulating with a male of 1 color plus an apparently non-preferred male of the other color placed in the 6 peripheral compartments. In all 6 demonstrations the female copulated with the male of the same color so that the social information provided was consistently favoring males of that color. The device "Hexagon" can be purchased from Toulouse Tech Transfer and Paul Sabatier University through a Material Transfer Agreement. (**B**) Mate-choice tests unfolded in double plastic tubes separated by a microscopy cover slide. The observer female was placed on one side, and 2 virgin males, 1 green and 1 pink, on the other side. The test began by lifting the partition, allowing males and females to meet.

Virgin flies were collected daily within 7 h after emergence, sexed without anesthesia, and kept in same-sex groups of 7 in a vial with medium. Experiments were conducted on 3–5 days old flies and fly manipulation was performed by gentle aspiration, using a glass pipette, tubing, and gauze.

Experimental protocol

All experiments were conducted under similar conditions as fly maintenance. Air pressure at the airport Toulouse-Blagnac weather station was in the range of 1,004–1,034 hPa and was previously shown to constitute a highly reliable proxi of atmospheric pressure in the experimental room (Dagaeff et al. 2016). Contrasting male phenotypes were created by randomly dusting them with green or pink powders (Mery et al. 2009) 20–30 min prior to use them as demonstrators or potential mates, so that they could clean the excess of dust. Demonstrations were run according to the "speed learning" design (Dagaeff et al. 2016) within a hexagon device (Figure 1A) composed of 1 central arena devoted to female observers (2.7 cm × 1.5 cm, volume = 8.6 cm^3) and 6 peripheral compartments devoted to demonstrators (1.5 cm × 1.5 cm × 1.5 cm each) separated from the central arena by a glass partition (0.8 mm thick). The 4 experimental groups differed in the number of observer females: 1, 6, 12, and 24 observer females in the central arena, being able to witness 6 simultaneous demonstrations each ongoing in 1 of the 6 peripheral compartments. Each demonstration involved 1 female apparently choosing the same male color as the other demonstrator females and rejecting the male of the other color. Thus, with a total of 6 demonstrator females and 12 demonstrator males, with 1, 6, 12, and 24 observer females in the central arena, the sex ratio within a hexagon ranged from 1.7 to 1, 0.67, and 0.4 males per female, respectively. Mate-choice tests were run using a device made of a double plastic tube (0.8 cm × 3 cm each) separated by a microscopy cover slide (1.6 cm × 1.6 cm, Figure 1B).

Before the beginning of the demonstration, observer females were placed in the central arena. Demonstrator females were placed individually in small plastic tubes with 2 males of the color chosen for the demonstration of that hexagon. As soon as copulation started, the couple and a male of the opposite color were transferred carefully into a peripheral compartment of the hexagon in which the copulation continued. Demonstrations started when the first demonstrator trio was transferred, and ended when all 6 couples were broken, or as soon as new courtship occurred in 1 peripheral compartment after the end of copulation, despite the fact that some of the other copulations might still be ongoing. Thus, demonstration length varied from 16 to 24 min. Then, all observer females were removed by gentle aspiration and placed all together in a food vial until the mate-choice test. For the condition with 24 observer flies, only 12 randomly chosen observer females were kept together in a vial for the test. Results were obtained from 57, 16, 13, and 14 demonstration blocks (i.e., hexagons) for conditions with 1, 6, 12, and 24 observer flies, respectively.

The mate-choice tests started 45–60 min after the end of the demonstration (for practical reasons, half of the flies were tested after 45 min and the other half after 60 min). Each observer fly was placed in one side of a double plastic tube device, and a pair of males, one of each color, in the other side. The males used in the test phase came from a different vial than those used in the demonstration, and were powdered 20–30 min before the beginning of the test. After 2 min resting, the partition was removed, thus beginning the test. The first wing vibration of a male was recorded as courtship initiation, as well as, the color of the courting male, the time of the beginning of copulation, and the color of the chosen male. In trials in which both males courted the female before she chose, the median time of double courtship was 68 s, and the minimum time of double courtship was 1 s.

After the end of the experiment, flies were transferred into a vial and euthanized in a freezer. As in previous studies (Dagaeff et al. 2016; Danchin et al., submitted for publication; Nöbel et al., submitted for publication) mate-choice tests were successful if they led to a copulation and if both males courted the observer female before the initiation of the copulation, as this was the only situation when observer females were visibly in a situation of choice.

Mate-copying index

Replicates in which the observer female copulated with the male of the phenotype preferred during the demonstration (copied) were attributed a mate-copying score of 1, and 0 in the opposite case. The mate-copying index (MCI) was calculated as the mean mate-copying score for each treatment, which reveals female preference in the corresponding experimental group. MCIs significantly higher than 0.5 (random choice) reveal mate-copying.

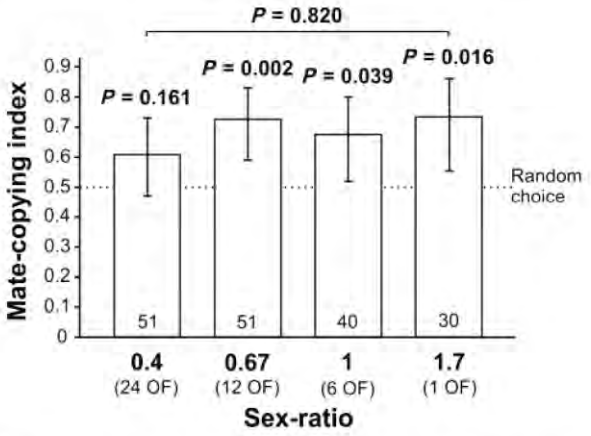

Figure 2. Mate-copying indices along a sex-ratio gradient. Mate-copying indices above 0.5 indicate a preference for the male of the color chosen during the preceding demonstration. OF, observer females; vertical bars: Agresti–Coull intervals. Apart from binomial tests provided above bars, statistical analyses detected a significant negative effect of first-courting male and an almost significant positive effect of air pressure (see text). Sample sizes are provided at the bottom of each bar.

Statistical analysis

Analyses were conducted with the R software 3.4.0 (R Core Team 2017). For each treatment, the departure from random choice was tested with a binomial test. Mate-copying scores were then analyzed in a generalized linear mixed model (GLMM) with binary logistic regression (package lme4, Bates et al. 2015). We analyzed all successful replicates, that is, replicates in which both males courted the female before she mated (172 trials out of 455). We tested the effect of potential confounding parameters in univariate tests and found no significant effect (time of the demonstration, color of the male chosen by the demonstrator female, and delay between demonstration and test: GLMM, Wald χ^2-test, $N=172$, $\chi^2=1.516$, 0.020, and 0.205, $P=0.218$, 0.889, and 0.651, respectively). Starting models included sex ratio, normalized air pressure (measured air pressure minus the average air pressure in our data-set, which was 1,022 hPa), and air pressure change within 6 h before the experiment as fixed effects. Air pressure and its variations were introduced into the model because they were shown to influence mate-copying in *D. melanogaster* (Dagaeff et al. 2016). We also included "first-courting male" as a fixed effect: this parameter takes the value 1 if the color of the first courting male was the "preferred" color in the preceding demonstration, and 0 in the opposite case. For instance, if demonstrator females in the hexagon mated with green males, then during the mate-choice test, if the new green male was the first one to court the observer female, the value was 1. We first introduced a random block (i.e., hexagon) effect into the model in order to account for the non-independence of observer flies from the same hexagon. However, as models including this effect always had higher Akaike Information Criteria (AIC, Akaike 1969) and because this effect could potentially capture part of the main effect as treatments differed from one block to the next one we only report on models that did not include the hexagon as a random effect. Results were not qualitatively affected by the exclusion of that random effect. The significance of fixed effects was tested using Wald χ^2 tests implemented in the ANOVA function of the car package (Fox and Weisberg 2011). All starting models included interactions between fixed effects. We applied a backward selection method using *P*-values, by dropping out non-significant effects, starting with the highest order interaction. We used AIC to determine the final model(s).

To test experimental effects on the rate of double courtship, we analyzed the number of males courting the female in a GLMM with binary logistic regression (1 vs. 2 males courted before copulation initiation). We analyzed all trials in which the female copulated after at least 1 male courted her (441 trials out of 455). The dependent variable "number of males courting" was set to 1 when both males courted, and 0 if only one of the males courted. All potential confounding parameters (time of the test, test chamber ID in the set of 6 tube designs, delay between demonstration and test) were found non-significant in univariate tests (GLMM, Wald χ^2-test, $N=441$, $\chi^2=1.299$, 3.390, and 1.738, $P=0.255$, 0.640, and 0.187, respectively). Thus, the starting model included sex ratio, normalized air pressure, time when the first courtship began, and first-courting male as well as their interactions as fixed effects.

The log-transformed courtship duration was analyzed in a LMM with logistic regression. We analyzed all trials with detailed times of courtship and copulation initiation (432 trials out of 455). Log-transformation (natural log) was used to achieve a Gaussian distribution of that variable. All potential confounding parameters (time of the test, test chamber ID in the set of 6 tube designs, delay between demonstration and test) were non-significant in univariate tests (LMM, $N=432$, $F=1.039$, 0.997, and 0.228, $P=0.309$, 0.419, and 0.633, respectively). The starting model thus included sex ratio, log-transformed time of first courtship initiation, and first-courting male as fixed effects.

Results

Mate-copying along a gradient of sex ratio

We analyzed mate-copying indices in a GLMM with binary logistic regression. The starting model included the sex ratio as a continuous variable, first-courting male, and normalized air pressure, as well as air pressure changes within 6 h before the experiment as fixed effects. None of the interactions were significant ($P>0.14$ in all cases). Sex ratio had no effect on mate-copying (GLMM, Wald χ^2-test, $N=172$, $\chi^2=0.052$, $P=0.820$; Figure 2), nor did air pressure changes within 6 h before the experiment (GLMM, Wald χ^2 test, $N=172$, $\chi^2=0.25$, $P=0.616$). The selected model included normalized air pressure (GLMM, Wald χ^2 test, $N=172$, $\chi^2=3.81$, $P=0.051$, positive effect) and first courting male (GLMM, Wald χ^2 test, $N=172$, $\chi^2=4.13$, $P=0.042$, negative effect). The MCI was higher when the first male courting in the test was the one of the color that was rejected by the demonstrator females.

Thus, we did not find any significant relationship of mate-copying with sex ratio (Figure 2). When analyzing mate-copying indices in each group using binomial tests, we found a significant departure from random choice for the groups with sex ratios of 1.7, 1, and 0.67 males per female, but not in the group with a sex ratio of 0.4 (Figure 2), although the trend was in the same direction with a tendency to choose the male of the color selected during the demonstration.

Rate of double courtship along a sex ratio gradient

We measured the rates of both males courting the female ("double courtship rate"), that is, the proportion of trials in which both males courted the female before she initiated mating with one of them. The starting binary logistic regression model included the sex ratio as a continuous variable, first-courting male, and normalized air

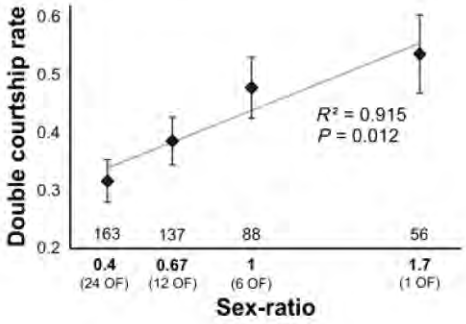

Figure 3. Female choosiness measured as double courtship rate. Rates are expressed as the number of trials in which both males courted the female on the total number of trials. OF, observer females. Error bars represent SEM. Sample sizes are provided above the *X*-axis.

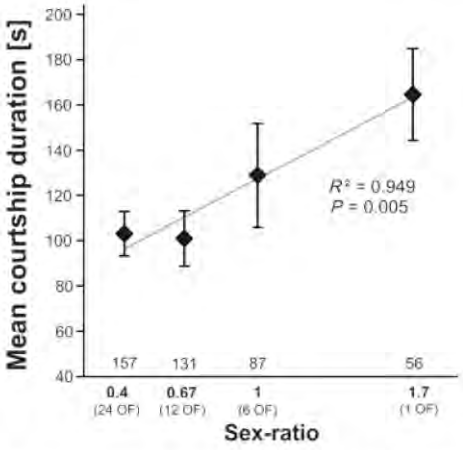

Figure 4. Courtship duration measured as mean latency between time of first courtship initiation and copulation initiation along the sex-ratio gradient. OF, observer females. Error bars represent SEM. Sample sizes are provided above the *X*-axis.

pressure, as well as the log-transformed time of first courtship initiation as fixed effects. None of the interactions were significant ($P > 0.69$ in all cases). First-courting male had no effect on the double courtship rate (GLMM, Wald χ^2 test, $N = 441$, $\chi^2 = 1.15$, $P = 0.283$). The selected model included sex ratio (GLMM, Wald χ^2 test, $N = 441$, $\chi^2 = 6.333$, $P = 0.012$, positive effect, Figure 3), normalized air pressure, and log-transformed time of first courtship initiation (GLMM, Wald χ^2 test, $N = 441$, $\chi^2 = 5.818$ and 8.978, $P = 0.016$ and 0.003, positive and negative effects, respectively). As expected, we found that the double courtship rate increased along the sex-ratio gradient (Figure 3). Females thus appeared to be able to assess the sex ratio during demonstrations, remember it, and adapt their behavior during the subsequent mate-choice test accordingly.

Courtship duration along the sex ratio gradient

Because copulation initiation in *D. melanogaster* is mainly under female control (Connolly and Cook 1973; Kimura et al. 2015), we tested whether the decrease in the double courtship rate was due to faster acceptance of copulation in groups with lower sex ratios during the demonstration. In a preliminary model analyzing number of males courting as a fixed effect depending on log-transformed courtship duration, we first found that these 2 variables were highly correlated (LMM, $N = 432$, $F = 124.3$, $P < 0.001$, positive effect), which supports our hypothesis: in trials in which both males courted, the latency between first courtship and copulation initiation was the highest. We then analyzed the delay between first courtship initiation and copulation initiation along the sex ratio gradient (Figure 4). In a LMM with logistic regression in which the response variable was the log-transformed courtship duration, including the sex ratio as a continuous variable, first-courting male, and the log of the time of first courtship initiation, none of the interactions were significant ($P > 0.32$ in all cases). The first-courting male index was not associated with courtship duration (LMM, $N = 432$, $F = 0.048$, $P = 0.827$), while sex ratio (Figure 4) and time of first courtship initiation were (LMM, $N = 432$, $F = 7.828$ and 14.19, $P = 0.005$ and <0.001, positive and negative effect, respectively). Thus, as expected, copulation occurred faster in low sex ratio conditions, suggesting that observer females were much quicker in accepting copulation in such situations.

Discussion

We investigated sex-ratio and group size effects during mate-choice demonstrations on the observer females' tendency to copy the mate choice of demonstrator females for a specific male phenotype as well as their choosiness in the subsequent mate-choice tests. We expected that along a gradient of increasing sex ratio during demonstrations, observer females would accept copulation slower, thus increasing the double courtship rate. We had at least 3 contradictory predictions concerning the mate-copying to sex ratio and group size relationship, so that we had no specific expectation about the direction of the effect. The relationship of mate-copying to group size could be either (i) positive, because of some social facilitation in mate-copying or (ii) negative, as a result of decreasing disturbance by other females with decreasing group size, and the relationship of mate-copying to sex ratio could be (iii) negative, as a result of decreasing female competition for males when sex ratio increases. Finally, a combination of these effects could also produce an optimal group size and sex ratio at which social learning is maximized.

As expected, we found that the frequency of both males courting the observer female and the delay between first courtship initiation and copulation initiation increased along the increasing experimental sex-ratio gradient (and decreasing group size). This supports our hypothesis that female choosiness decreases when sex ratio gets more female biased. Interestingly, females copied on average the observed mate-choice decisions for a certain male phenotype regardless the experienced sex ratio. We can thus conclude that mate-copying seems to be similarly efficient under all tested sex ratio conditions. In accordance, none of our initial hypotheses was supported.

No detectable effect of sex ratio on mate-copying

Although non-significant the MCI of the largest group (24 observer females, sex ratio of 0.4 males per female) did not differ from those of the other treatments, suggesting that females did not learn better in a group. There seemed to be no "social social-learning" in this observational learning paradigm, which is in accordance with the fact that in a form of olfactory learning, flies tested in groups show increased memory retrieval compared with flies tested individually, but training condition (single vs. group) does not affect memory formation (Chabaud et al. 2009). Similarly, in large groups of observer females (i.e., with female-biased sex ratios), we did not detect any evidence for increased disturbance among flies that would have

hampered proper learning, as mate-copying appeared relatively unchanged with the size of the group of observer females. Finally, we found no evidence that females learning in female biased groups favor social information use over the personal assessment of male's quality. This might be explained by a lack of quality differences among presented males that led females to rely preferably on social information gathered during demonstrations. Another explanation is that several contradictory effects were ongoing simultaneously and cancelled out each other. Unfortunately, our experimental design did not allow us to disentangle group size from sex ratio effects as they co-varied in our design.

The ability to copy may be an adaptation to the naturally crowded conditions existing on rotten pieces of fruit to which females are attracted both as sources of food and egg laying site (Rodrigues et al. 2015; Keesey et al. 2016). In these aggregations, copulations are common (Danchin E, personal observation) and surrounding females have the opportunity to watch the mate choice of other females, thus setting the stage for mate-copying in natural situations.

As for methodological implications, our results suggest that it is possible to increase the number of observer females in the central arena of the hexagon during demonstrations at least up to 12 without affecting mate-copying efficiency. This could help in designing future mate-copying experiments, for instance by increasing the number of males in the peripheral compartments, and thus, increasing the sex ratio in order to increase double courtship rates without affecting mate-copying scores. This would allow us to extend the proportion of replicates in which females are visibly in a situation of choice during the mate-choice test.

All in all, our results suggest that mate-copying is quite robust to sex ratio conditions as well as group size differences in general. While this might be caused by contradictory effects of varying sex ratios and group sizes cancelling each other out, the high observed robustness in mate-copying may suggest a real importance of social information use in mate choice (see also Danchin et al. 2004; Galef and Laland 2005). In general, selection might have favored the evolution of mate-copying under varying environmental conditions. In particular, the Fisher runaway process (Fisher 1930) predicts that females should conform to the local preference because male descendants of females mating with the locally non-preferred males would inherit of the non-preferred trait, thus counter-selecting them for as long as the local preference persists (Danchin et al., submitted for publication). There is thus strong selection for conforming to the majority. In our experiments, females saw all 6 demonstrator females having apparently chosen to copulate with the same male color morph and rejecting the other, revealing a very strong local preference. In such conditions, the Fisher runaway process predicts that they should build a preference for that type of males independently of the other local conditions.

Female choosiness increased with sex ratio

From a female's point of view, a female-biased local sex ratio is concomitant with high competition for mates, and thus, females should accept any encountered mate more readily and more quickly. Following this logic, we found that females were quicker in initiating copulations under female-biased sex ratios. Similarly, double courtship rates, which constitute a positive proxy of female choosiness in *Drosophila*, decreased when the sex ratio shifted from male-to-female bias. However, the rate of double courtship likely depends on both sexes as males can be more or less interested in courting the female (Eastwood and Burnet 1977; Clowney et al. 2015), and females can exhibit varying choosiness (Maklakov and Arnqvist 2009). Nonetheless, because all males used during mate-choice tests were new and naïve males, they had no information on the previous demonstration and thus could not have generated the observed pattern. They all saw a young virgin female, which should be attractive *per se* (Tompkins and Hall 1981). Contrastingly, observer females having been under varying sex ratios during the demonstration are more likely to have driven the observed pattern. All observer females of all experimental groups were raised in unisex groups for 3–5 days from hatching to experiment, and thus all of them experienced the same situation of acute lack of potential mates for a long period before experiments. As a consequence, the difference in choosiness observed between these groups should only come from the difference in the sex ratio and group size experienced during the ~20 min of the demonstration. In effect, demonstrations constituted the first time since sexual maturity in which observer females were in the presence of males, and vice versa, which might have made them highly sensitive to the presence of mates, potentially explaining the fact that the short demonstration period was sufficient to elicit differential behavior in females according to the sex ratio and group size they experienced just before.

Results on double courtship rates and on courtship to copulation latency were probably not independent because the shorter the courtship, the shorter the time for the second male to court the female. However, globally our results support our hypothesis that varying levels of competition along the sex-ratio gradient affected female choosiness in *Drosophila*. Similarly, it was shown in several species (Berglund 1994; Passos et al. 2014; Pompilio et al. 2016), as well as in a theoretical study (Bleu et al. 2012), that females tend to maximize the chances of mating with a high-quality male in male-biased sex ratios, while minimizing the risk of remaining unmated in female-biased sex ratios. For similar reasons, the positive relationship between sex ratios and rates of both males courting the observer female during the mate-choice test appears adaptive.

Mechanisms of sex ratio detection

Concerning the mechanisms by which females detected the local sex ratio, the characteristics of our hexagon device implies that observer females could only perceive sex ratio visually, which is consistent with the fact that flies can recognize visual patterns and sense motion and color (Behnia and Desplan 2015; Liu et al. 2006; reviewed in Guo et al. 2017), and may even be able to visually recognize individual males and behave differently relative to them according to these males' past experience (Loyau et al. 2012). Our results thus support the idea that *Drosophila* probably use vision in much more diverse and subtle contexts than previously thought (Loyau et al. 2012). More generally, insect cognitive capacities are being discovered as surprisingly sophisticated. For instance, honeybees *Apis mellifera* have been shown to be able to count visually (Chittka and Geiger 1995; Gross et al. 2009; reviewed in Dacke and Srinivasan 2008). The question of the existence of such skills in *Drosophila* remains open. It would be interesting to study how and for how long such specific behavior can last after the demonstration, in order to assess the dynamics of *Drosophila* mating behavior determinants.

Effect of first-courting male on MCI

We found the fact that the first courting male was or was not of the same color that was selected during the demonstration significantly affected mate-copying. Among the group of flies that chose after a double courtship, mate-copying indices were higher when the first

courting male had the color that was rejected during the demonstration. A female has 2 options when the first male courted: accept copulation with the courting male or wait for the second male to start courting. A copier observer female would be less likely to wait for her "non-preferred" male to court her if the first male to court her is of the color that she learnt to prefer during the previous demonstration: a situation that most often would lead to a single courtship before copulation. Contrastingly, a non-copier female or a copier female first courted by the male of her non-preferred color would both be more likely to wait for the second male to court her. Because in our analyses of mate-copying indices, it was necessary to discard mate-choice tests in which only one male courted the female before the onset of the copulation to only keep situations in which females were in a real situation of choice between the 2 males (see "Materials and Methods" section), we could thus expect a lower proportion of copiers in the group "First-courting male = 1" (i.e., females that were first courted by their preferred male), than in the group "First-courting male = 0" (i.e., females that either had no preference or that were first courted by the male of their non-preferred phenotype), as we found. Thus, our measured MCI is conservative.

Effect of atmospheric pressure on *Drosophila* sexual behavior

As in a previous study (Dagaeff et al. 2016), we found a significant positive effect of air pressure on the rate of double courtship, and a slight positive effect on mate-copying. Mating behavior was previously found to be correlated with atmospheric pressure in *D. pseudoobscura* (Ankney 1984) and in *D. melanogaster* (Austin et al. 2014). Bad weather can mean death for small insects, and it seems adaptive for them to be able to anticipate weather variations, like air pressure changes, to find a shelter and then save energy in bad weather. Interestingly, flies from the same population were found to differ in response to air pressure: under low pressure some individual flies reduced their mating activity, while others increased it (Austin et al. 2014). Such a polymorphism might reveal phenotypic variation in relation to dispersal. Similarly, mate-copying was found to be reduced under low atmospheric pressure (Dagaeff et al. 2016). Here, we further found that *D. melanogaster* females seem to become much less choosy under bad weather forecast (revealed by lower double courtship rates), probably because they act as quick as possible in such cases.

In conclusion, we provide evidence that sex ratio as a proxy of female–female competition may affect female choosiness in *D. melanogaster*. We did not find any relationship between sex ratio and mate-copying efficiency, suggesting that mate-copying is fairly robust to variation in this environmental condition. We speculate that this may be partly because our experimental design did not allow us to separate the contradictory effects of sex ratio on the propensity to mate-copy, or because selection favors copying in general independently from sex ratio conditions. In terms of evolution, our findings suggest that females may have acquired the ability to mate-copy independently of group size and sex ratio, without hampering their capacity to adapt their choosiness to the current population sex ratio, which determines the relative availability of male partners.

Author Contributions

M.M. carried out the experiments, performed the analysis, and drafted the manuscript; S.N. contributed in the analysis and writing of the manuscript; E.D. and G.I. designed the experiment and jointly supervised all steps in the process. All authors gave final approval for the publication.

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2- Effect of sex-ratio and phenotype commonness on mate-copying scores and choosiness

Introduction

In the experiment just above, I showed that sex-ratio impacts female selectivity while mate-copying scores do not differ significantly across a sex-ratio gradient. In order to manipulate the sex-ratio during the demonstration, I changed the number of females in the central arena of the hexagon during the demonstration. Thus the effects I observed could either be due to the sex-ratio, or more directly, to the number of competitor observer females. In this second experiment, I thus tried to disentangle sex-ratio effect from the effect of the number of competitors. To do so, I kept the latter parameter constant (12 observer females in the central arena, so 18 females in total with the 6 demonstrators), while the number of males in the peripheral compartments varied. My hypothesis was that female selectivity would be higher when the sex-ratio (males/females) is higher, i.e. when the intensity of the competition to access males is lower. This should be seen in higher courtship duration and higher rate of double courtship, in the group with the highest sex-ratio.

Because the proportions of pink and green males in the hexagon were not equal in one of the treatments, I could also analyze the effect of phenotypic rarity (or phenotype commonness) on the mate-copying scores. My hypothesis was that the least common the chosen phenotype, the stronger the strength of social information, and the higher the mate-copying scores.

Methods

Behavioral experiment

I used the hexagon, with 12 observer females and 6 demonstrator females, and a varying number of demonstrator males: 12 (control treatment corresponding to the treatment with 12 observer females in the previous experiment); 18 males with 9 of each color; 18 males with 6 of the preferred color and 12 of the rejected color (Figure 1). After the end of all copulations in the peripheral compartments (or as soon as a male starts fighting or courting after the end of the copulation), the observer females are removed, and the mate-preference test takes place in the classical tubes set-up, after 50 or 65 min (6 females tested at 50 min, 6 tested at 65 min). During the resting time, observer females are placed altogether in a food vial. The treatment in which proportions of pink and green males in the demonstration are different from 50/50 tested for the very first time in our model the effect of phenotype commonness.

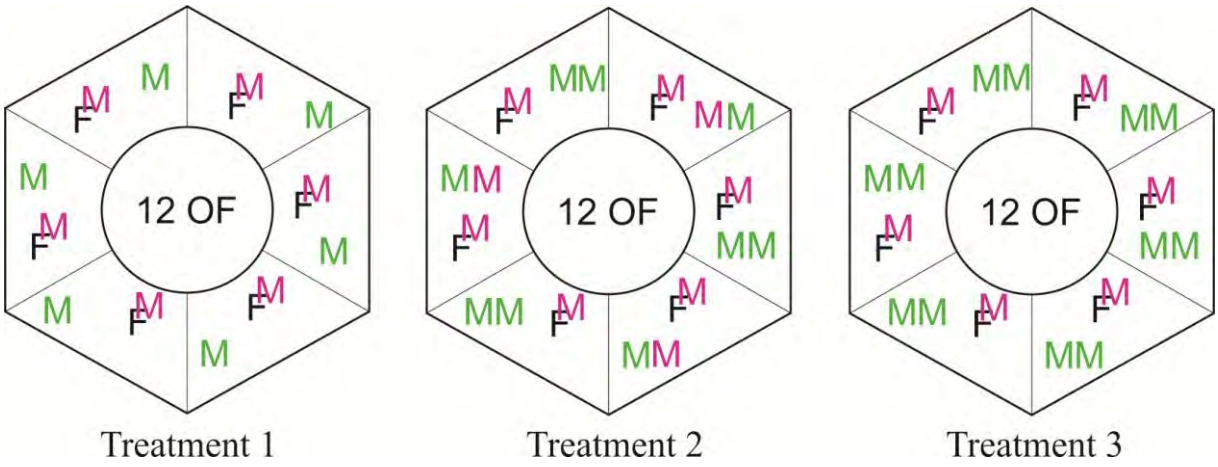

Figure 1: Three different treatments were made by changing the composition of peripheral chambers during the demonstration. 12 OF: 12 observer females in the central arena. M: male, F: female. Example with pink males preferred. In treatment 1, 12 observer females observe 6 demonstrator females mating with 6 pink males while 6 green males are rejected. The sex-ratio is thus 0.67 male per female and pink and green males are equally common. Treatment 2 is composed of 6 demonstrator females mating with 6 pink males, while 9 green and 3 pink males are rejected. Thus, sex-ratio is 1 and pink and green males are equally common. In treatment 3, 6 pink males are mating and 12 green males are rejected: demonstrator females prefer the rare phenotype, sex-ratio is 1.

Statistical analyses

Analyses were conducted with R version 3.4.0. As in all my experiments, mate-copying scores were analyzed in females that chose after both males courted; the other trials (one male courting and/or no copulation) were excluded from the analyses. For each group, the departure of the mate-copying index from random choice was measured using a binomial test. A GLMM test between the three groups measured the effect of treatment on MCI: the starting model included treatment, normalized air pressure, normalized air pressure changes in the 6 hours before demonstration, log-transformed time when demonstration started, first-courting male (see part I-A-1, Methods, subsection Statistical analysis), and all interactions with potential biological sense, as well as a random block effect (6 females par block, tested in the same time). The selected model (backward selection approach using the AIC) included normalized air pressure changes, treatment and log-transformed time when demonstration started. Another GLMM tested the effect of phenotypic rarity on MCI: the starting model included phenotypic rarity (a parameter set to 1 if both male phenotypes are equally common in the demonstration, else 0), normalized air pressure, normalized air pressure changes, log-transformed time when demonstration started, interaction between air pressure and air pressure changes, and interactions between phenotype rarity and each of the previous parameters, as well as a random block effect. The selected model included phenotypic rarity, normalized air pressure changes and log-transformed time when demonstration began. A GLMM model tested the effect of sex-ratio on the double courtship rate. The response variable was a binomial variable taking the value 1 if both males courted before copulation, and 0 if only one male courted the female. Trials in which the female did not mate were excluded from the analysis. Fixed effects of the GLMM model were sex-ratio, normalized air pressure, chamber of the test box, and interactions between sex-ratio and the other variables, as well as a random block effect. The selected model included sex-ratio and

chamber and the random block effect. Finally, a LMM model tested the effect of sex-ratio, log-transformed time when courtship starts and interaction between them on courtship duration, with a random block effect. The selected model contained the variables without interaction.

Results and discussion

Mate-copying index

Mate-copying indices significantly above 0.5 for control (sex-ratio = 0.67) and treatment 2 (sex-ratio = 1; equal proportion of pink and green males in the hexagon) reveal preference for the phenotype chosen by the demonstrator females (Figure 2), while treatment 3 (sex-ratio = 1; 6/18 males of the preferred phenotype) did not reveal mate-copying. A GLMM comparing the three groups did not reveal a significant difference between the three treatments (GLMM, Wald χ^2 test, N = 174, χ^2 = 4.01, P = 0.134), but when testing for the effect of phenotype commonness, this parameter has a significant effect on mate-copying scores (GLMM, Wald χ^2 test, N = 174, χ^2 = 4.44, P = 0.035).

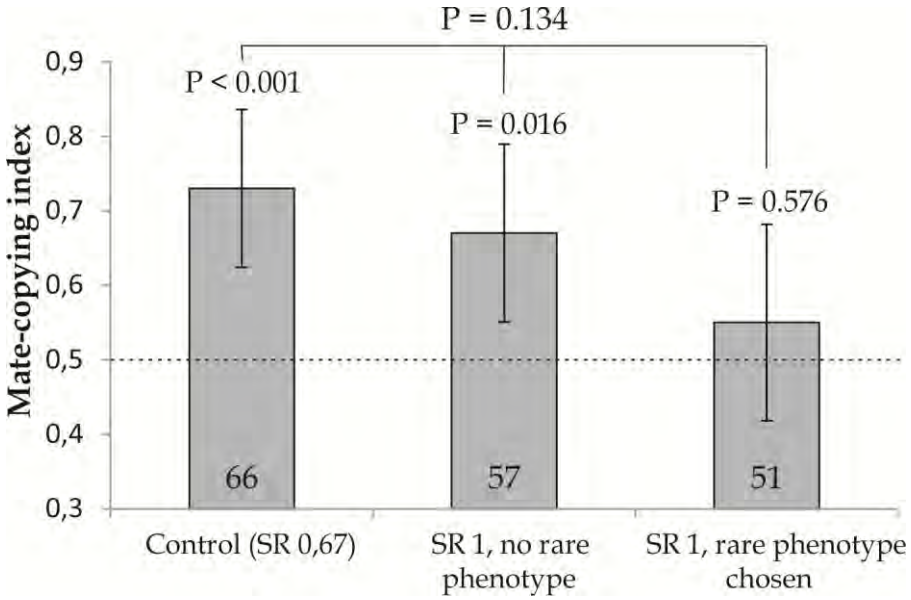

Figure 2: Mate-copying indices for the 3 treatments. Statistics: binomial tests (above each bar); GLMM of the effect of the treatment on MCI. Error bars: Agresti-Coull 95% confidence intervals. Inside each bar: sample size. Dashed line indicates random choice.

Moreover, the GLMM model showed that atmospheric pressure and its variations within 6 hours before the experiment had no effect on the scores (P = 0.367). Contrastingly, I found a possibly strong, although slightly effect of the time when demonstration starts (GLMM, Wald χ^2 test, N = 174, χ^2 = 3.66, P = 0.056), with comparatively better scores in the afternoon compared to the morning (MCI 0.7 for the afternoon compared to 0.6 in the morning, Figure 3). Given that the different treatments were distributed evenly enough

during each experimental day, this might be due to the experimenter being more relaxed or more accurate in the afternoon, or related to the circadian rhythm of the flies that might learn better in the afternoon compared to the morning.

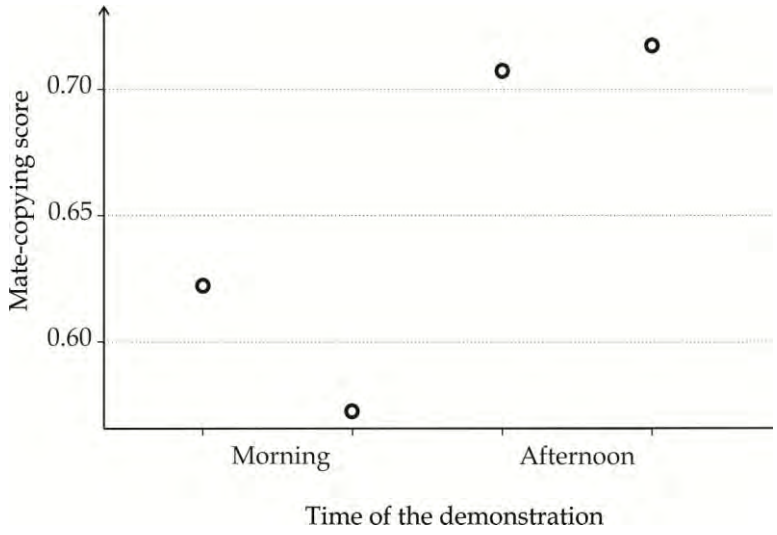

Figure 3: Mate-copying scores depending on the time when demonstration started. From left to right, early morning (09:00) to late afternoon (15:30). All treatments are pooled together. The scores were better in the afternoon compared to the morning. Sample sizes of each group (left > right): 45, 42, 41, 46.

Double courtship rate and courtship duration

I measured the proportion of samples in which both males courted the females before she accepted to mate, and I found a non-significant tendency to a lower rate of double courtship in the control group (treatment 1, sex-ratio = 0.67) compared to the two other groups (with sex-ratios of 1), in a GLMM testing the effect of sex-ratio on double courtship rate (GLMM, Wald χ^2 test; N = 414, χ^2 = 1.19, P = 0.276, Figure 4). The effect of chamber was almost significant (P = 0.055), with chambers A and B having comparatively lower double courtship rates than the four other chambers, which is difficult to explain. Prior to the test, males are introduced in the second tube of each device, starting with the one in chamber A, then in chamber B, etc. Thus, females in the first chambers (A and B) can observe males longer than those in the last chambers before the beginning of the test, so one might suggest that females in the first chambers had a higher chance to preselect their partner just by observing them through the glass partition before the test started, which could have lead to this lower frequency of double courtships.

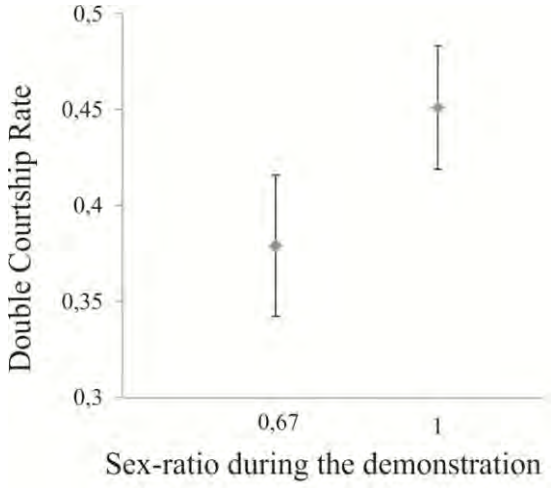

Figure 4: Sex-ratio effect on the double courtship rate. Conditions 2 and 3 are pulled together as they have the same sex-ratio. The values are similar to those of the previous experiment. Sample sizes: 174, 240.

When testing the effect of sex-ratio on courtship duration in a LMM, I found a slight albeit non-significant effect (LMM, N = 414, F = 1.68, P = 0.195, Figure 5), with longer courtships in the groups with a higher sex-ratio. Time when courtship starts had a strong effect (LMM, N = 414, F = 4.08, P = 0.043): the later the courtship started, the shorter it lasted. Also, rates of double courtship and courtship durations are coherent with what I could measure in the previous experiment (see part I-A-1).

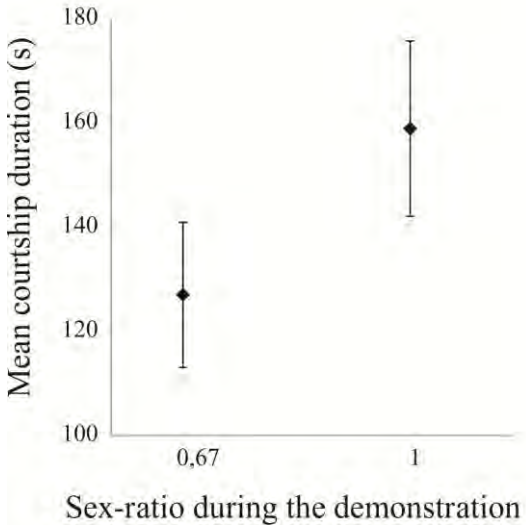

Figure 5 : Mean courtship duration depending on the sex-ratio. Conditions 2 and 3 are pulled together as they have the same sex-ratio. The values are similar to those of the previous experiment. Sample sizes: 174, 240.

Conclusions

The first aim of this experiment was to disentangle the effect of sex-ratio from the effect of the number of competitors on female selectivity. My results are going in the expected direction, with values close to what I measured in the previous experiment. Unfortunately, the treatments I tested were not sufficiently different in terms of sex-ratio to show any significant effect of this parameter without a very large sample size. The difficulty was also that I could not reach extreme values without creating additional issues, like, too many flies in the peripheral compartments that would dilute the information, or too few flies in the peripheral compartments, that would have not allowed giving the same amount of information to the observer females. Thus, although this experiment is not absolutely conclusive, it strongly suggests that the sex-ratio in itself is influencing female selectivity, with females being less selective under female-biased sex-ratio. Concerning the previous experiment, we can thus say that the effect I observed is due to the sex-ratio, alone or in addition to the number of competitors itself.

Concerning the effect of phenotype commonness on mate-copying scores, the results I measured were unexpected: my hypothesis was that mate-copying index would be higher than in control groups, and finally I could not even detect a preference for the phenotype chosen by the demonstrators: observer females of this group did not copy. Thus, the fact that the preferred phenotype is rare does not increase the strength of social information provided in the demonstration. Contrarily, my results suggest that females tend to prefer the most common phenotypes. Treatment 3 would be a limit situation, with opposite effects of mate-copying and phenotype commonness cancelling each other out in the particular situation I studied. This tendency to prefer common phenotypes could somehow be a consequence of conformity, as the most common phenotypes may result from a preference for this phenotype in the previous generation, or from a higher fecundity of the fathers bearing the common trait value. Anyways, this question needs to be further studied; as such a behavior would imply that the settlement and invasion of new phenotypes in this species would be disadvantaged. In other words, this preference for common phenotypes would favor common phenotypes and disfavor rare phenotypes. It would be interesting to confirm my results with a larger experiment with various proportions of males from the preferred phenotype, to see if there is a positive correlation between mate-copying scores and commonness of the phenotype preferred by the demonstrator females.

B. Stability across time: long-term memory and emergence of stable traditions

Temporal stability can be seen at two different scales: (1) how long an observer female can remember and copy the information provided in the demonstration, and (2) how long this arbitrary tradition can be transmitted from teachers to pupils. Both questions have been answered in a paper in *Science* (Danchin et al., 2018), to which I participated. Here, I report

the part of this article that I took part in (criterion 3 of durable social learning), and provide an answer to the first question.

Introduction

In the wild, fruit flies often live in dense population on food sources like rotting fruits. In these habitat patches, copulations are common and it is thus very likely that virgin females witness several mate-choice demonstrations before they have the opportunity to mate. In a previous experiment conducted by Anne-Cécile Dagaeff, 24-h memory was not obviously detectable after only one presentation of the mate-choice demonstration (Dagaeff, 2015, Doctoral thesis). In olfactory aversive learning, a repeated presentation of the same pairing CS-US can lead to persistent memory in fruit flies, provided that the training sessions are separated temporally with resting intervals of sufficient duration (Tully et al., 1994; Beck et al., 2000; Pagani et al., 2009). We thus tried to apply the same kind of protocol in mate-copying, presenting five times a mate-choice demonstration to an observer female, with 15 min resting intervals between each presentation. Our hypothesis was that females would be able to remember the information 24h later, if it was presented repeatedly with resting intervals, as in olfactory learning. Moreover, long-term memory in olfactory learning is dependent on *de novo* protein synthesis (Tully et al., 1994). In order to see if a form of social long-term memory had this characteristic, we also trained and tested females that were fed with an inhibitor of protein synthesis prior to the demonstrations, and compared their scores with those of untreated flies.

Methods

Behavioral experiment and treatments

We used adult Canton-S flies at 3-5 days after emergence. In order to inhibit protein synthesis, females were fed cycloheximide overnight before the experiment (sucrose 5 %, cycloheximide 35 mM in mineral Evian® water), while control females received vehicle solution alone, both were given on a Whatman paper soaked with 125 µL of solution. To elicit long-term memory, females were allowed witnessing five successive demonstrations of a female mating with a male of one color, while a male of the other color was apparently rejected. Demonstrations occurred in tube devices but instead of introducing three demonstrators and allowing the female to choose one of the males, we introduced a couple as soon as they started mating, plus a male of the opposite color. Demonstrator flies were then removed as soon as the copulation finished. Two demonstration steps were spaced by 15-30 min resting intervals ("spaced training"). One "uninformed" group received the vehicle solution and had an opaque partition separating the observer and the demonstrator, thus providing no information about mate preference. Two other groups, one receiving vehicle and the other receiving cycloheximide, could watch the demonstration through a transparent partition and were thus informed about the mate preference. Finally, a fifth group received a cycloheximide treatment but could see only one demonstration and was tested immediately after, in order to verify that cycloheximide did not impair social learning.

Analysis

Mate-copying scores were analyzed using the R software version 3.3.2 (2016). Departure from random choice was tested for each group in a binomial test. We then did a GLMM model testing the effect of treatment on mate-copying scores. The starting model comprised treatment, experimenter-ID and air pressure as well as a random block effect, and the selected model contained only treatment and a random block effect.

Results

Females receiving the sucrose treatment ("informed") copied when tested after 24h (Figure1), while uninformed flies did not. Flies that could not do protein synthesis during the demonstration because they received cycloheximide were not able to build a long-term memory, while their capacity to learn from the classical protocol remained unchanged, showing that their short-term memory was not impaired. Thus, flies can build a long-term memory of a mate preference from a spaced training, and this memory is dependent on protein synthesis.

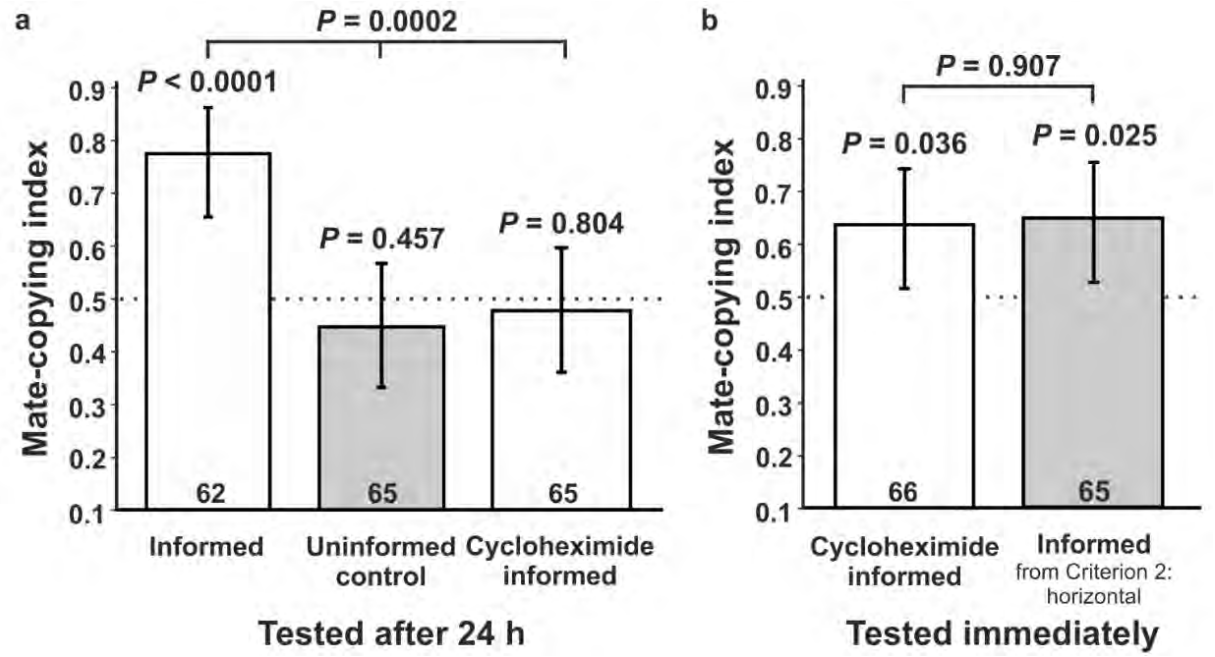

Figure 1: Mate-copying index of flies 24 h after a spaced training. a. Flies tested after 24h; **b.** Flies tested immediately, to control that the effect of cycloheximide was specifically on long-term memory. Statistics: binomial tests (above each bar), GLMM. Dashed line indicates random choice, sample sizes are indicated inside the bars. Uninformed control is a group of flies that received a sucrose treatment (control treatment) but had the demonstrations occurring behind an opaque partition, preventing them to see. Figure excerpted from Danchin et al., 2018, figure 3.

Discussion

This experiment showed that females are able to build a long-term memory of a mate preference, and this memory, like in olfactory learning, depends on *de novo* protein synthesis. Moreover, the discovery that flies have all cognitive capacities to transmit mate-preferences culturally on the long-term (Danchin et al., 2018) makes this behavior quite stable at the individual level (at least for 24h) as well as at the population level. In olfactory learning it was shown that flies can remember for several days (Tully et al., 1994), it could be interesting to know how long flies can remember in our paradigm.

Conclusion

In this first chapter, I investigated the environmental and temporal stability of mate-copying. I first showed that this strategy is stable across a gradient of number of observer females during the demonstration, and in different sex-ratio conditions. My second experiment also showed that, contrary to female competition, male phenotypic rarity during the demonstration impacted mate-copying scores and could abolish the effect of social information on female mate-choice. Mate-copying is thus a mate-choice strategy that has some robustness, but is sensitive to at least one environmental condition: male phenotypic rarity. In these two experiments, all treatments I applied only impacted the demonstration, while the test remained unchanged. I thus studied the impact of different parameters on the acquisition of the social learning, not on the retrieval. Finally, these experiments revealed that mate-copying is a promising model for the study of a social learning, as it is in the same time, a robust strategy, but also dependent on some environmental conditions.

The demonstration that flies can form a long-term memory of a mate preference opens great perspectives, as it shows the potential evolutionary impact of this social learning, and in the same time, constitutes a new field of exploration on the mechanisms of long-term memory in social learning, as it is likely that the mechanisms of long-term memory differ from those of short-term memory, like in olfactory learning (Isabel et al., 2004).

Chapter II: Neuronal mechanisms of mate-copying

Fruit flies possess several neurotransmitters that altogether ensure and modulate the great diversity of the behaviors and physiological functions in the fly brain. Some of these molecules also exist in Vertebrates, like glutamate, acetylcholine, GABA, dopamine and serotonin, among others. Dopamine and serotonin are known to be involved in olfactory and visual learning in drosophila. In this second chapter, I first studied whether dopamine and serotonin are involved in mate-copying, using a pharmacological approach, and then I focused on the role of one dopaminergic receptor expressed in MBs, DAMB (DopAmine Mushroom Bodies).

A. Roles of dopamine and serotonin in observational social learning: a pharmacological study

Context and overview

In this article, I used a pharmacological approach to test the role of serotonin and dopamine in mate-copying. I reduced dopamine or serotonin synthesis in adult virgin females by feeding 3-iodotyrosine (3-IY) and DL-para-chloro-phenylalanine (PCPA), respectively, and then tested their mate-copying performance with the classical experimental design (speed learning). I found that drug-treated females with reduced dopamine or serotonin did not mate-copy, indicating that both are required for social learning. These results give a first insight into the mechanistic pathway underlying social learning in *D. melanogaster*. This work was published in *Frontiers in Behavioral Neuroscience* in January 2019.

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BRIEF RESEARCH REPORT
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Dopamine and Serotonin Are Both Required for Mate-Copying in *Drosophila melanogaster*

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Mate-copying is a form of social learning in which the mate-choice decision of an individual (often a female) is influenced by the mate-choice of conspecifics. *Drosophila melanogaster* females are known to perform such social learning, and in particular, to mate-copy after a single observation of one conspecific female mating with a male of one phenotype, while the other male phenotype is rejected. Here, we show that this form of social learning is dependent on serotonin and dopamine. Using a pharmacological approach, we reduced dopamine or serotonin synthesis in adult virgin females with 3-iodotyrosine (3-IY) and DL-para-chlorophenylalanine (PCPA), respectively, and then tested their mate-copying performance. We found that, while control females without drug treatment copied the choice of the demonstrator, drug-treated females with reduced dopamine or serotonin chose randomly. To ensure the specificity of the drugs, the direct precursors of the neurotransmitters, either the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) or the serotonin precursor 5-L-hydroxytryptophan (5-HTP) were given together with the drug, (respectively 3-IY and PCPA) resulting in a full rescue of the mate-copying defects. This indicates that dopamine and serotonin are both required for mate-copying. These results give a first insight into the mechanistic pathway underlying this form of social learning in *D. melanogaster*.

Keywords: fruit fly, mate choice, social learning, social memory, 3-iodotyrosine (3-IY), DL-para-chlorophenylalanine (PCPA), L-3,4-dihydroxyphenylalanine (L-DOPA), 5-L-hydroxytryptophan (5-HTP)

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INTRODUCTION

Many animal species from a vast array of taxa can learn from others (i.e., social learning), particularly in the context of mate-choice (Avital and Jablonka, 2000; Danchin et al., 2004; Galef and Laland, 2005). Such observational learning can lead to mate-copying (Pruett-Jones, 1992), when females mate preferentially with a male showing similar characteristics as the male they saw being chosen by another female (trait-based copying, Bowers et al., 2012).

In *Drosophila melanogaster*, females are able to perform mate-copying (Mery et al., 2009) after watching only a single live demonstration of one female copulating with a male of a given phenotype and one male of another phenotype being rejected (Dagaeff et al., 2016; Danchin et al., 2018; Nöbel et al., 2018).

Despite some promising studies, research about the mechanisms of social learning in general and observational social learning in particular are still at the beginning (Burke et al., 2010; Debiec and Olsson, 2017; Kavaliers et al., 2017; Allsop et al., 2018). While social learning mechanisms are poorly known in any organism, *D. melanogaster* with its mini yet highly

structured brain (100,000 cells) is one of the most favorable model species to dissect the neuronal processes of learning. Mostly, studies focused on simple kinds of learning tasks, where flies can learn from their own experience (non-social learning task), that are easier to standardize and historically well studied, like olfactory or visual associative learning (Quinn et al., 1974; Vogt et al., 2014, 2016; Cognigni et al., 2018). Thus, while the mechanisms of non-social learning in *Drosophila* are now well-described, the neurotransmitters and neural structures involved in observational social learning in *Drosophila* are unknown.

The formation of non-social associative memory requires dopamine in *D. melanogaster*: during the olfactory or visual learning process, it mediates aversive or appetitive unconditional stimuli (Riemensperger et al., 2005, 2011; Aso et al., 2012; Burke et al., 2012; Liu et al., 2012; Vogt et al., 2014), while serotonin is required for aversive place memory (Sitaraman et al., 2008), and for olfactory learning and memory (Johnson et al., 2011; Lee et al., 2011). Based on the fact that visual and olfactory learning share common neurotransmitters and neural structures (Vogt et al., 2014), we hypothesized that our model of observational social learning, mate-copying, involves the same two neurotransmitters. To address this, we used a pharmacological approach to reduce dopamine or serotonin synthesis with specific inhibitors of the limiting-step-enzyme of the synthetic pathway: 3-iodotyrosine (3-IY) inhibits tyrosine hydroxylase that catalyzes L-DOPA formation from tyrosine, and DL-para-chlorophenylalanine (PCPA) inhibits tryptophan hydroxylase that catalyzes 5-HTP formation from tryptophan, respectively. Young sexually mature virgin females were fed one of these drugs and their mate-copy ability was tested after a single demonstration. To ensure specificity of the drugs, we also had two rescue treatments in which the female received the drug (3-IY or PCPA) together with the immediate precursor of the neurotransmitter (L-DOPA or 5-HTP, respectively), so that the level of dopamine or serotonin was less reduced than with 3-IY or PCPA alone.

MATERIALS AND METHODS

Fly Maintenance

Wild-type Canton-S flies were raised in 30 ml food vials containing standard corn flour-yeast-agar medium. The room was maintained at 25 ± 0.8°C, 60 ± 3% humidity, with a 12 h:12 h light:dark cycle. Virgin flies were collected daily for the experiments and sexed without anesthesia, by gentle aspiration using a glass pipette, tubing and gauze. Flies were then kept in single-sex groups in food vials until the experiment started. As *D. melanogaster* females are reluctant to re-mate (Chapman et al., 2003), each female was used only once as demonstrator or observer.

Drug Treatment

The solutions were freshly prepared every week in vehicle (sucrose 5% in mineral water Vittel®) and 200 ml were poured on a Kimwipe paper (1.5 cm × 3.5 cm) deposited in a 15 ml Falcon tube. Nine 1-day-old virgin females were introduced in the tube for the length of the treatment, at 18°C, 12 h:12 h light:dark cycle. To explore dopamine effect, flies were fed with 3-IY (10 mg/ml, Sigma I8250) and/or L-DOPA (1 mg/ml, Sigma D9628) for 36–40 h (Bainton et al., 2000; Seugnet et al., 2008). To explore serotonin effect, flies were fed with PCPA (10 mg/ml, Sigma C6506) and/or 5-HTP (16 mg/ml, Sigma H9772) for 3 days, with papers being changed once during the 3 days period (Dierick and Greenspan, 2007; Plaçais et al., 2012). We used a high 5-HTP concentration (30% more than in Dierick and Greenspan, 2007) to ensure rescued serotonin levels in PCPA-treated flies in our conditions. This concentration did not affect mate-copying in flies fed with 5-HTP (**Figure 2**). The treatment "vehicle" consisted of vehicle solution given during 36–40 h or 3 days.

Mate-Copying Experiment

Flies were tested 3–4 days after eclosion. Experiments were conducted in the same conditions as fly maintenance. We used the same tubes set-up (double plastic tube (0.8 × 3 cm each) separated by a thin glass partition) and the speed-learning protocol as described in Dagaeff et al. (2016) except that mate-choice tests were run either immediately to test learning, or 3 h 20 ± 15 min after the demonstration, a time when associative memory in drosophila is composed of consolidated and labile memories (Folkers et al., 1993), two memories with independent pathways (Isabel et al., 2004; Scheunemann et al., 2012) so that we could detect a learning and/or memory defect. Artificial male phenotypes were obtained by randomly dusting them in green or pink (neutral trait) using colored powders (green: Shannon Luminous Materials, Inc., #B-731; red: BioQuip Products, Inc., #1162R). Demonstrations in tube set-ups showed a demonstrator female choosing between the two male phenotypes while the treated female could observe through a transparent partition. After the end of the copulation of demonstrator flies, each observer female was either directly tested or placed individually in a food vial until the test. The mate-choice test then involved two new virgin green and pink males placed in a tube with the observer female. Time when courtship began (first wing vibration) and color of the male, as well as time when copulation started and color of the chosen male were recorded.

Mate-Copying Index

Observer females that chose the same male color as the demonstrator for copulation (copied) were given a mate-copying score of 1, and females that chose the opposite color were given a score of 0. A mate-copying index (MCI) was calculated as the mean mate-copying score per treatment. Samples in which only one male courted the female before she initiated mating were not used for the analysis of the mate-copying performance because only when both males showed their interest the female was unambiguously in a position of choice. Samples in which no copulation occurred after 30 min were excluded from the analyses.

Ethics Statement

Behavioral observations of *D. melanogaster* required no ethical approval and complied with French laws regarding animal welfare. We kept the number of flies used in this study as small as

possible. We handled flies by gentle aspiration without anesthesia to minimize damage and discomfort. After the experiments, individuals were euthanized in a freezer.

Analyses

Mate-copying scores were analyzed with the R software 3.4.0 (R Core Team, 2017). For each treatment, the difference from random choice was tested with a binomial test. For global comparisons, mate-copying scores were analyzed in a generalized linear mixed model (GLMM) with binary logistic regression (package lme4, Bates et al., 2014). A random block effect introduced into the models accounted for the non-independence of observer flies from the same block of 6 demonstrations and tests. The significance of fixed effects was tested using Wald chi-square tests implemented in the ANOVA function of the car package (Fox and Weisberg, 2011). Starting models included treatment, air pressure at the time of the test, and its variation within the 6 preceding hours, and interactions between these effects. We used a backward selection approach using *P*-values, removing the highest order interaction as soon as it was non-significant. The final model was always chosen as the one with the lowest Akaike Information Criteria (AIC, Akaike, 1969). Two-by-two comparisons between treatments were done using *post hoc* X^2 tests.

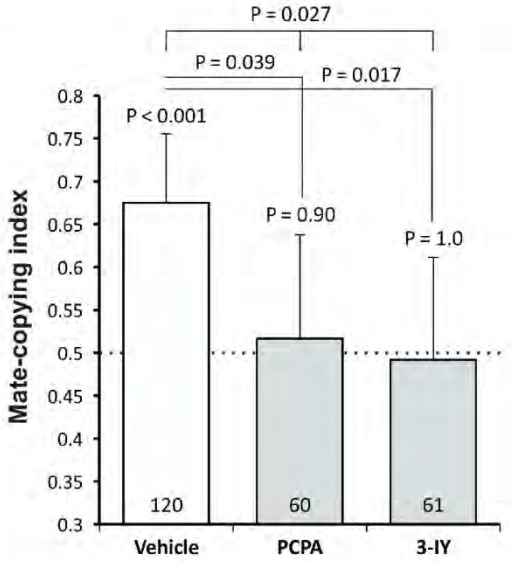

FIGURE 1 | Mate-copying index measured 3 h after the demonstration. Numbers inside the bars represent the sample size. Dashed line indicates random choice. Error bars: Agresti-Coull intervals. Just above bars: binomial tests. Two-by-two comparisons: *post hoc* chi-square tests. Test between all groups: GLMM.

RESULTS

PCPA and 3-IY Impair Learning or Memory in Mate-Copying

We first tested whether females' mate-copying performance was affected after a PCPA or a 3-IY treatment. We analyzed the mate-copying scores (**Figure 1**) and found that females fed with the vehicle mate-copied, while females lacking serotonin or dopamine did not. We then compared the three groups and found a significant difference (GLMM, $N = 241$, $X^2 = 7.26$, $P = 0.027$), which we also found when comparing PCPA- or 3-IY-treated flies to the vehicle (**Figure 1** and **Supplementary Table S1**). Thus, PCPA and 3-IY impaired mate-copying in these conditions. We also measured courtship duration in each group and found no statistical difference between them (**Supplementary Figure S1**).

Dopamine and Serotonin Are Both Required for Learning in a Mate-Copying Context

We then tested female mate-copying immediately after the demonstration, in order to study learning capacities only, and not memory retention. To ensure that the mate-copying defects observed in **Figure 1** depend on lacking dopamine or serotonin, and not to a side-effect of the drug, we added four more treatments: PCPA with 5-HTP, 5-HTP, 3-IY with L-DOPA and L-DOPA. We measured mate-copying scores in all groups (**Figure 2**) and found that all groups copied except PCPA and 3-IY treated females. We compared mate-copying scores in the five groups that copied and found no statistical difference (GLMM, $N = 345$, $X^2 = 1.72$, $P = 0.79$), indicating that 5-HTP and L-DOPA given alone did not alter mate-copying ability, and could rescue mate-copying in females treated with the inhibitor. Additionally, we found a significant difference between inhibitor-treated flies and flies fed with the vehicle or rescued flies (**Figure 2** and **Supplementary Table S2**). Thus, flies lacking dopamine

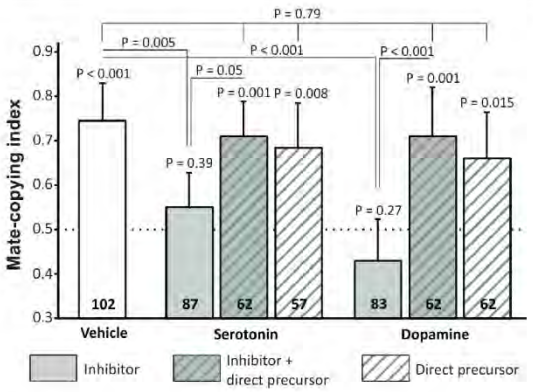

FIGURE 2 | Mate-copying index measured immediately after the demonstration. Flies received the following treatment (from left to right): vehicle, PCPA, PCPA + 5-HTP, 5-HTP, 3-IY, 3-IY + L-DOPA, L-DOPA. Numbers inside the bars represent the sample size. Dashed line indicates random choice. Error bars: Agresti-Coull intervals. Just above bars: binomial tests. Two-by-two comparisons: *post hoc* chi-square tests. Test between all groups that copied: GLMM.

or serotonin are not able to learn a mate preference from the demonstration.

DISCUSSION

We found that dopamine and serotonin are both required in learning during mate-copying. Observer females lacking these neurotransmitters were unable to learn the successful male phenotype in the demonstration while control females receiving the vehicle solution, females treated with 5-HTP or L-DOPA, and females treated with the precursor together with the inhibitor copied the choice of the demonstrator immediately after the demonstration. This is in accordance with other studies showing that dopamine and serotonin are required for learning. In an olfactory learning task, dopamine is required to mediate the unconditional stimulus after a single training phase (Riemensperger et al., 2011; Aso et al., 2012; Burke et al., 2012; Liu et al., 2012; Lin et al., 2014; Vogt et al., 2014). Alterations in behavioral tracking were reported in flies lacking dopamine (Andretic et al., 2005), but dopamine-deficient flies were shown to have no alteration in visual perception and display a normal electroretinogram (Riemensperger et al., 2011). Thus, the defects we observed are not due to deficient vision, although we cannot exclude attention deficiency in dopamine-depleted flies. Serotonin is necessary to form place memory (Sitaraman et al., 2008) and associative olfactory learning memory (Johnson et al., 2011; Lee et al., 2011). Mate-copying can be compared to associative learning with pairing between a conditional and an unconditional stimulus (Avarguès-Weber et al., 2015): the conditional stimulus would be the color of the male copulating with the demonstrator female while the unconditional reinforcing stimulus could be the observation of the copulation. Under these circumstances, dopamine would mediate the reinforcing stimulus. Our results provide one more indication that the pathways underlying memory formation are comparable for visual social information and for olfactory information, and it was shown that both share mushroom body circuits for memory consolidation (Vogt et al., 2014). Mate-copying was also described in many vertebrates (Dugatkin and Godin, 1993; White and Galef, 1999; Waynforth, 2007; Galef et al., 2008), so the mechanistic results discovered in *Drosophila* could be a starting point for such studies in vertebrates, as many vertebrate pathways and genes have homologs in *Drosophila*.

We showed that dopamine and serotonin are both required in mate-copying. This result paves the way for further studies of the neural pathways underlying social observational learning in *D. melanogaster*. The next step is now to dig into the role of each of these neurotransmitters, by assessing the neural structures and the receptors involved in this social learning task.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MM carried out the experiments, performed the analyses, and drafted the manuscript. SN contributed in the writing of the manuscript. ED and GI designed the experiments and jointly supervised all steps in the process. All authors gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00334/full#supplementary-material

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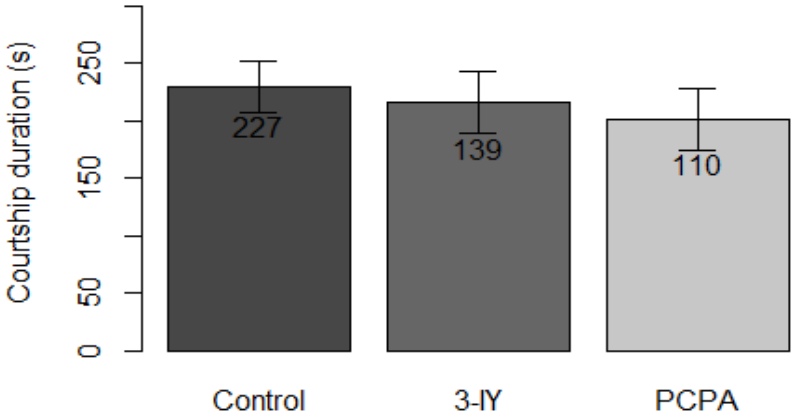
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary information

Supplementary table 1: post-hoc χ^2 tests comparing groups of flies from figure 1

Groups compared	N	χ^2	P-value
PCPA to vehicle	180	4.27	0.039
3-IY to vehicle	181	5.72	0.017



Supplementary figure 1: Mean courtship duration for each treatment. Numbers inside bars represent the sample size. Log-transformed courtship duration was analyzed in a linear mixed model (LMM) with logistic regression. All trials with detailed times of courtship and copulation initiation were analyzed. Log-transformation (natural log) was used to achieve a Gaussian distribution of that variable. The starting model included treatment and log-transformed time when first courtship began. The selected model included this last parameter alone. Treatment effect was found non-significant (LMM, N = 476, χ^2 = 4.73, P = 0.094), while time when first courtship began had a significant effect (P < 0.001, the later the courtship began the shorter it was).

Supplementary table 2: Post-hoc χ^2 tests comparing groups of flies from figure 2.

Groups compared	N	χ^2	P-value
PCPA to vehicle	189	7.78	0.005
PCPA to PCPA + 5-HTP	149	3.82	0.05
3-IY to vehicle	185	18.6	<0.001
3-IY to 3-IY + L-DOPA	145	10.9	<0.001

B. Role of DAMB

Introduction

In the previous experiment, I showed that dopamine is involved in mate-copying. In drosophila, this neurotransmitter can target four receptors: dDA1 (also known as DopR), and DAMB (also known as Dop1R2 or DopR2) are members of the D1-like family (a subclass that comprises dopamine receptors coupled to a stimulatory Gs or Gq protein), while D2R is coupled to inhibitory Gi/Go. A fourth receptor, DopECR, is activated by both dopamine and ecdysteroids (Srivastava et al., 2005). Dopamine, and thus dopaminergic receptors, is known to regulate a wide diversity of functions, like courtship and receptivity, locomotion, sleep, learning and memory (reviewed in Riemensperger et al., 2011; Waddell, 2013; Yamamoto and Seto, 2014; Ichinose et al., 2017). In olfactory learning, DdA1 is required to mediate the unconditional stimulus in both appetitive and aversive learning (Kim et al., 2007; Qin et al., 2012), while DAMB is specifically involved in both appetitive and aversive long-term memory formations (Musso et al., 2015; Plaçais et al., 2017). To go further into the role of dopamine in mate-copying, I chose to study the role of DAMB (DopAmine Mushroom Bodies), a dopaminergic receptor expressed in mushroom bodies (Kondo et al., 2020), the center of higher cognitive processes in insects. First, DAMB is involved in long-term memory in olfactory learning, and long-term memory is of particular interest in our paradigm as it is essential to allow the emergence of stable traditions on the populational, multigenerational level. Moreover, *damb* flies display normal short and mid-term memory, which provides a control for the experiments. In effect, up to now, we were not able to find a proper control for color vision, thus, it appears difficult to draw strong conclusions from experiments showing an absence of mate-copying in a mutant fly, without the proof that this mutant has no color vision impairment; especially because dopamine is known to be involved in visual processes, notably attention (Riemensperger et al., 2011). For these two reasons, I chose to start studying the roles of dopaminergic receptors with DAMB.

DAMB is expressed in mushroom body neurons at the adult stage and in the third instar larva (Han et al., 1996), more precisely in the α'β' lobes and in γ neurons (Kondo et al., 2020), and is also expressed in part of the central complex: in the noduli and a part of the fan-shaped body (Kondo et al., 2020, Figure S5). This dopaminergic receptor is a "D1-like" GPCR (G-protein coupled receptor) first thought to be coupled to Gs that stimulates adelylate cyclase activity (Han et al., 1996), however, it was shown that it activates Gq much more efficiently (Himmelreich et al., 2017) and thus leads to [Ca^{2+}] intracellular increase (Figure 1).

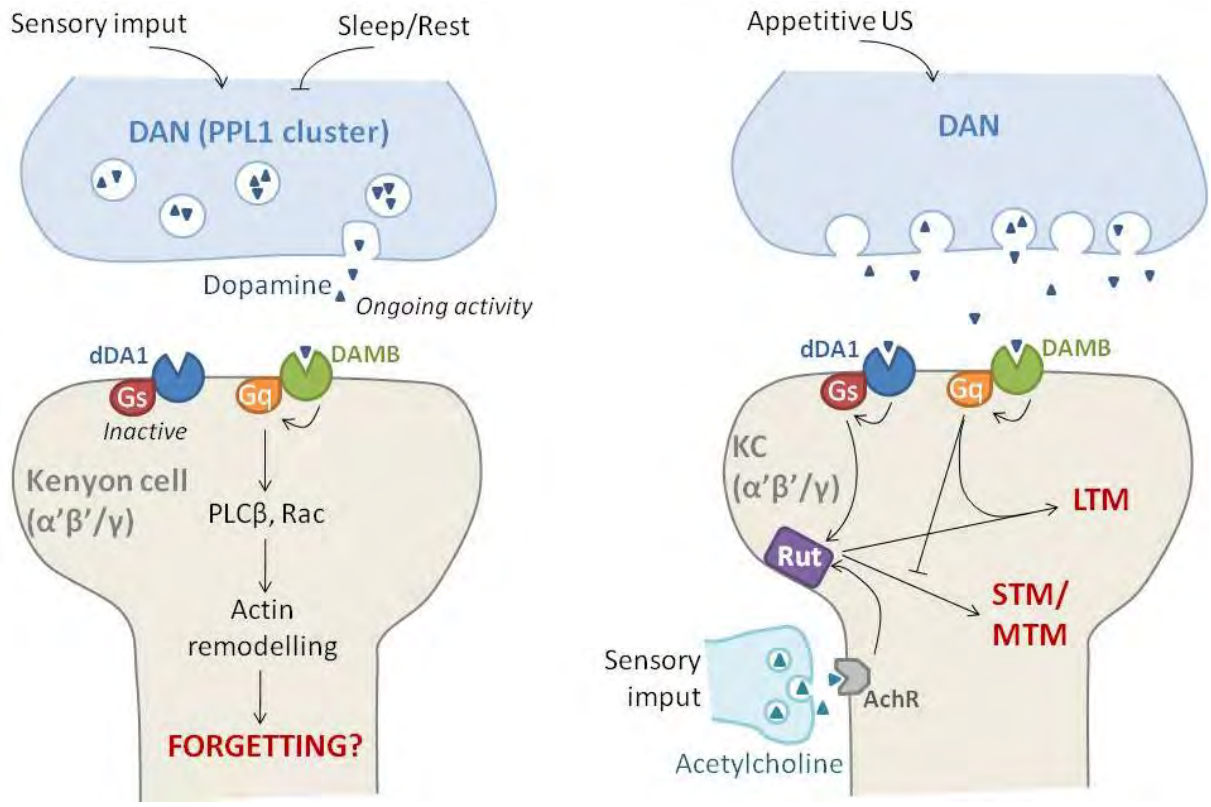


Figure 1: Localization of DAMB and its activity in drosophila memory. DAMB has a role in memorisation, and a possible role in forgetting (Berry et al., 2012).

Methods

Flies

I used Canton-S flies from the wild-type strain and *damb* mutants (Knock-Out). In the second experiment I used the strain *w+;;UAS-DAMB-RNAi* (110947/KK from Vienna Drosophila Ressource Center) that expresses RNA interference (RNAi) anti-DAMB transcript under the control of a Gal4-activated promoter (Plaçais et al., 2017), and I crossed it with *w-;;VT30559-Gal4* (Gal 4 expressed in the whole mushroom body) to target the mushroom body neurons, and with wild-type flies as a control. I also crossed the Gal4 line with the wild-type strain as a control, I thus had four lines to test (Table 1).

Table 1: Name and genotype of the observer females tested in the LTM experiment. Four different genotypes were tested to investigate whether DAMB is required in mushroom bodies for long-term memory in mate-copying. Note that all flies have at least one wild-type copy of the *white* gene required for a proper vision.

Name	Genotype	Description
MB/+	*w-/w+;;VT30559/+*	Control for the Gal4 driver VT30559
MB/RNAi	*w-/w+;;VT30559/UAS-DAMB-RNAi*	Reduced DAMB expression in MB
WT	*w+;;*	Wild-type control
RNAi/+	*w+;;UAS-DAMB-RNAi/+*	Control for the UAS line

Behavioral test

I used the protocol described in I-A, Methods for the test of long-term memory: females had five demonstrations of already formed couples, separated by resting intervals of 10-15 min. They were tested 21 to 24 h after the end of the demonstration. For the speed learning experiment, I used the design described in II-A, Methods: control and *damb* flies received a sucrose treatment for 36-40 h prior to the experiment, then had one demonstration in the classical set-up, and were placed individually in food vials between the end of the demonstration and the beginning of the test 3 h after. The reason why I used this protocol is that I tested *damb* flies together with the drug-treated flies described in II-A, and used the same control flies (WT flies that received a sucrose treatment).

Analyses

Data were analyzed as in II-A, Methods, with the following GLMM models, all including block as a random effect: for comparison between *damb* and control flies in speed learning (Figure 2, left), the starting model comprised genotype and normalized air pressure in Toulouse Airport weather station, and interactions between these two parameters. The selected model comprised genotype alone. For comparison between *damb* and control flies in LTM (Figure 2, right), the starting model included genotype, normalized air pressure in the room and normalized total duration of the five demonstration steps, and all interactions between these parameters. The selected model comprised genotype, and normalized demonstration duration. For comparison between the four different genotypes in experiment 2 (Figure 4), the starting and selected models comprised genotype.

Results

In short-term memory, *damb* flies are able to learn as well as control flies (Figure 2). I found no statistical difference between the two groups in a GLMM model (N = 102, $\chi^2_1 = 0.005$, P = 0.944). Contrastingly, in long-term memory, while wild-type flies show a strong tendency to copy, *damb* flies choose randomly, revealing the absence of long-term memory. The difference between the two groups is significant: GLMM with Wald χ^2 test: N = 62, $\chi^2 1 = 4.22$, P = 0.040. In the selected model, normalized demonstration duration had a slight non-significant effect on mate-copying scores: N = 62, $\chi^2 1 = 2.77$, P = 0.096 (Figure 3).

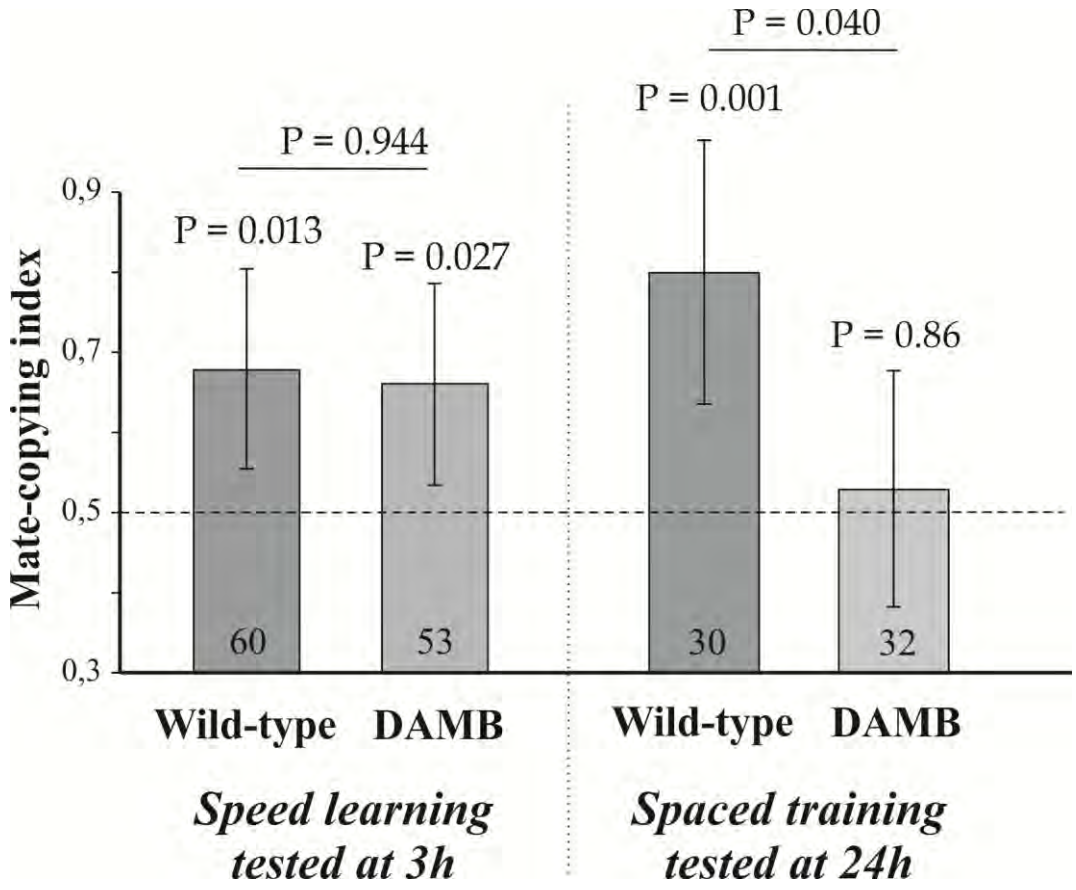


Figure 2: Mate-copying index of Wild-type and *damb* flies. Left: tested 3 h after one demonstration. Right: tested 24 h after a spaced training (5 demonstrations). Numbers inside the bar indicate the sample size. Error bars represent Agresti-Coull 95 % confidence intervals. Statistics: binomial tests just above the bars, and GLMM tests comparing the two treatments. Dashed line indicates expected results under random choice.

I tested the effect of demonstration total duration on mate-copying scores, and I found a positive correlation (Figure 3): the longer the demonstration, the higher the scores, but the effect is not significant in the selected model (comprising genotype and normalized demonstration duration, with block as a random effect), probably because the sample size is low.

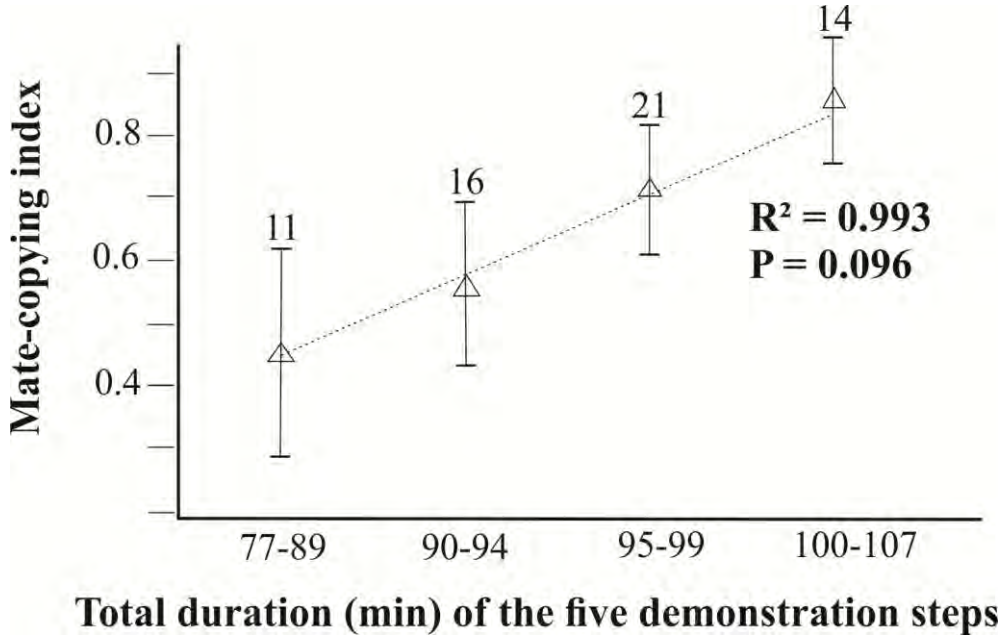


Figure 3: Mate-copying scores of control and *damb* flies (pulled together) depending on demonstration duration. Flies that copulated after a double courtship are divided in four groups of equivalent sizes depending on the duration of the demonstration they had. Mate-copying scores correlate with total demonstration duration, although the effect is not significant. Error bars represent standard error of the mean, sample sizes are indicated above each bar.

In a second step, I tried to locate the neural structure in which DAMB is required for long-term memory in mate-copying. To do so, I used the UAS-Gal4 system with RNAi anti-DAMB, to reduce the expression of the receptor selectively in the mushroom bodies. I then measured the mate-copying index of these flies 24h after a spaced training (Figure 4), but unfortunately I did not manage to finish the experiment because of technical issues. The wild-type control as well as the Gal4 and UAS controls display normal learning scores, while we do not have evidence that flies expressing RNAi in the whole mushroom bodies learn (Binomial test, N = 30, P = 0.36).

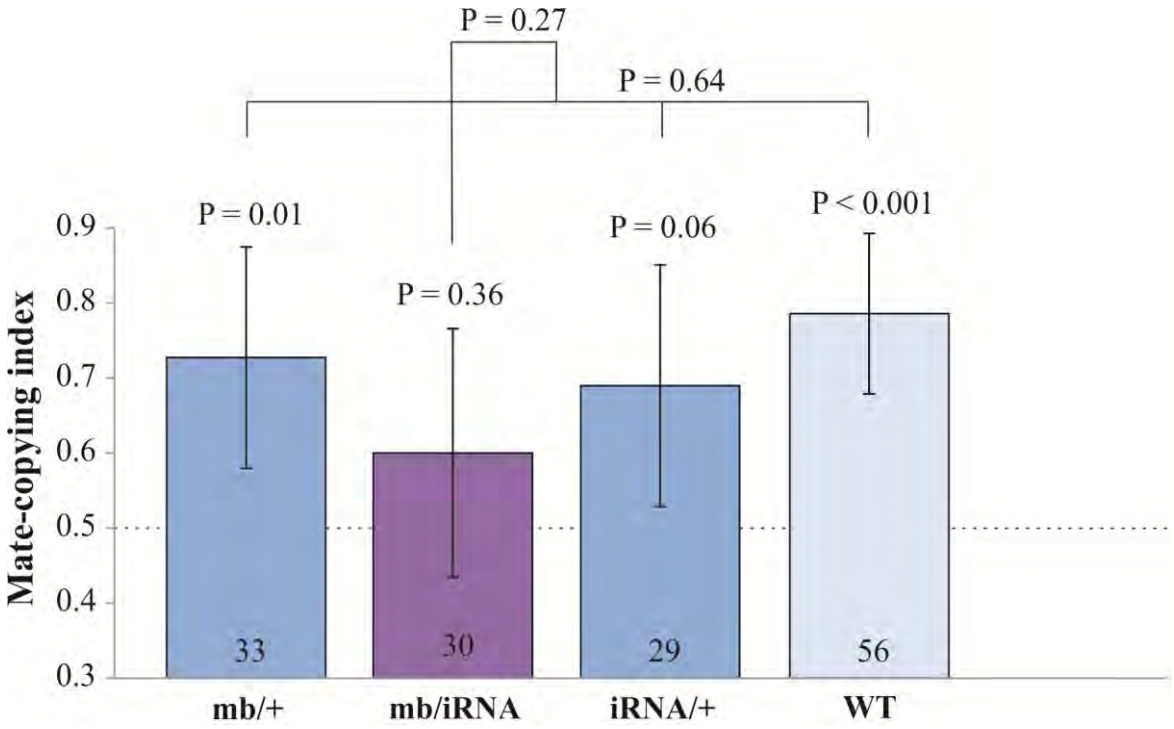


Figure 4: Mate-copying index of observer females of different genotypes, tested 24h after 5 spaced demonstrations. For the exact genotypes, please refer to table 1 in the methods. Light blue = wilt-type flies. Blue = control flies (normal DAMB expression), Purple = females with reduced DAMB expression in the MB. Error bars represent Agresti-Coull intervals. Statistics: binomial tests (just above each bar), GLMM with Wald χ^2 test.

There is no significant difference between control groups and flies with reduced DAMB expression in the MB (GLMM, Wald χ^2 test, N = 148, $\chi^2_1 = 1.22$, P = 0.27), however, the trend is in the expected direction as females expressing RNAi in the MBs tend to have a lower score than the controls. I cannot conclude whether DAMB is required in the mushroom bodies for long-term memory in mate-copying, but the partial results tend to support this hypothesis.

Discussion

DAMB is involved in long-term memory formation, as in olfactory memory. The tendency that longer demonstrations are correlated to higher mate-copying scores might mean that longer demonstrations lead flies to form a more robust memory of the mate preference. In this view, one can assume that four demonstrations only would have led to undetectable mate-copying. However, another possible explanation is that external factors like air pressure conditions influence both demonstration length and learning capacities of observer females. In effect, the length of the demonstrations depends on flies behavioral variables like stress level for instance, and we can assume that both demonstrator and observer flies are submitted to the same external factors that influence these variables in a positive or a negative manner. It would be interesting to find other measures of the quality of a demonstration, and to study correlations between these variables and mate-copying scores.

The precise region of the fruit fly brain where the receptor is needed for the behavior is still to be discovered. I tried to test flies with reduced expression of DAMB in the MB via anti-DAMB RNAi, but despite several attempts to perform the experiment, I could not obtain conclusive results. In particular, some control lines (not shown here) displayed very low mate-copying scores, which raises the question of whether these lines have some unknown genetic mutations. Moreover, it is possible, in view of my preliminary results (Figure 4), that the reduction in DAMB expression is not sufficient to abolish long-term memory in our paradigm. It thus seems better to use the opposite strategy, that is, testing *damb* flies expressing DAMB only in the mushroom bodies (thanks to the line *;UAS-DAMB;damb*). Last, at the time I performed the experiments (2018), there were no precise information on the precise expression pattern of DAMB in MB and CX. I thus tested several Gal4 drivers (for MB, but also for CX, not shown). In view of the results of Kondo et al. (2020), the driver for MBs was relevant as VT30559 labels all MBs' lobes (Plaçais et al., 2017).

Finally, it would be highly useful to develop a lighter protocol for the study of LTM in mate-copying, because the protocol I used is very long and delicate, and thus is not adapted to answer precise genetic questions that require testing many different genotypes.

Conclusion

In this second chapter, I showed that the neural processes underlying mate-copying require dopamine and serotonin. I found that the dopaminergic receptor DAMB, known to be involved in olfactory learning for long-term memory and not for short-term memory, is required in the same way for mate-copying. This brings a new piece of indication that different types of learning can share the same neural networks. Finally, my attempt to localize the brain region in which DAMB is required was not very successful, raising the need for a lighter protocol that would allow crossing the bridge to a wide exploration of the neural mechanisms of this observational social learning.

Chapter III. Relevant cues in mate-copying

I studied the cues used by the observer females to form a memory of a mate preference. First, I disentangled positive from negative information in the demonstration, to see which one (or whether both) is required in mate-copying. Then, using virtual demonstrations, I tried to refine what are the minimal visual and temporal characteristics of the demonstration allowing females to mate-copy.

A. Disentangling positive and negative information in mate-copying

While the neurobiological mechanisms underlying learning coming from an animal's own experience are largely investigated, neurobiology of social learning is more scarcely addressed, especially in invertebrates. In this part, I provide evidence that mate-copying occurs through learning based on acceptance cue. Using a new protocol for the mate preference demonstration, I disentangled positive from negative information in the demonstration (original idea from Arnaud Pocheville), while they are classically provided simultaneously, and I found that females copy the acceptance, but not the rejection, of a male.

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In the second part, I went further in the mechanisms, exploring the roles of populations of dopaminergic neurons known to be involved in appetitive and in aversive olfactory learning.

Female fruit flies copy the acceptance, not the rejection, of a mate

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Abstract

Preferences and avoidances can be socially transmitted, in particular in the case of mating preferences. *Drosophila melanogaster* females that witness another female's mate choice can memorize and copy her preference. However, in mate-copying in *Drosophila*, it is not known whether information lies in the acceptance of the chosen phenotype, the avoidance of the rejected one, or both; as classical mate-copying designs provide both types of information to observer females in the demonstration. To disentangle the respective roles of positive and negative information in mate-copying, we performed experiments in which demonstrations provided only one type of information at a time. We showed that positive information is sufficient to trigger mate-copying: observer females prefered males of phenotype A after watching a female mating with a male of phenotype A in the absence of any other male. Conversely, giving negative information only (by showing a demonstrator female actively rejecting a male of phenotype A) did not affect observer female's mating preference. This suggests that in mate-copying experiments in *Drosophila*, the informative part of demonstrations lies in the copulation with a given male, which in turns suggests that the underlying mechanisms may be shared with those involved in appetitive memory in non-social associative learning.

Keywords

Drosophila melanogaster, mate-copying, social learning, appetitive learning, aversive memory, indirect learning.

Introduction

Preferences as well as avoidances can be transmitted through social learning. Social learning allows an individual to learn about its environment at a lower cost than with a trial-and-error tactic, potentially affecting fitness positively (Boyd and Richerson, 1995). In mammals, Norway rat pups were shown to avoid poisoned food after observing and copying their parent's diet (Galef and Clark, 1971). Such kind of learning can be observed especially in animals with prolonged maternal care (Mirza and Provenza, 1990), or in social insects, where social information is used in finding new foraging areas and synchronizing nest activities

(Leadbeater and Chittka, 2007). Social information is also used in non-social insects like Drosophila (Mery et al., 2009; Sarin and Dukas, 2009; Lone and Sharma, 2011), notably in mate-choice. Mate-choice constituting a major fitness impacting decision, it is thus no surprise that animals often use multiple information sources for mate-choice (Danchin et al., 2004).

The learning processes of *Drosophila melanogaster* have been extensively studied for the last decades in several forms and sensory modalities in direct associative learning (Quinn et al., 1974; Tempel et al., 1983; Wolf and Heisenberg, 1991; Tully et al., 1994; Schwaerzel et al., 2003; Isabel et al., 2004; Aso et al., 2010; Vogt et al., 2014, 2016; Cognigni et al., 2018). Direct associative learning occurs when the animal experiences by itself the association between conditional and unconditional stimuli (with or without being active). On the contrary, indirect associative learning involves a demonstration and no direct experience of the stimuli association. Typically, social learning is an indirect form of learning (Olsson et al., 2007) in which, a focal individual observes a demonstrator or teacher experiencing the association between a cue and a reward. The mechanisms of social learning in general and social learning in insects in particular are now under investigation (Burke et al., 2010; Debiec and Olsson, 2017; Kavaliers et al., 2017; Allsop et al., 2018), but we are still far from understanding them thoroughly. In particular, the question of the extent of the overlap between pathways of social learning and the better studied direct associative learning remains poorly explored (Heyes, 1994; Heyes and Pearce, 2015; Leadbeater and Dawson, 2017).

Here, we focused on a form of observational social learning called mate-copying. Described in many vertebrate and invertebrate species (reviewed in Varela et al., 2018), mate-copying occurs when after observing the mate-choice of demonstrator individuals the preference of the observer individuals is biased towards either the specific male chosen during the demonstration (individual-based mate-copying) or towards males of similar phenotypes (trait-based mate-copying; Bowers, Place, Todd, Penke, & Asendorpf, 2012). The latter can strongly affect evolution (Agrawal, 2001; Witte et al., 2015) as it can considerably amplify sexual selection on male traits. Trait-based mate-copying has been described and studied in *Drosophila* for a decade (Mery et al., 2009; Dagaeff et al., 2016; Nöbel, Allain, et al., 2018; E. Danchin et al., 2018; Nöbel, Danchin, et al., 2018; Monier et al., 2018, 2019), and constitutes a powerful model to dissect the mechanisms of observational social learning (Monier et al., 2019). A first question concerns the stimuli that elicit mate-copying, to refine experiments on both behavioural and neurobiological mechanisms. In the mate-copying design in *Drosophila*, the demonstration involves a female choosing between two males of contrasting phenotypes (randomly and artificially dusted in pink or green) in front of a naïve observer female, which thus gathers positive information for the successful male A and negative information for the rejected male B. Here, we provided only one kind of information (positive or negative) at a time, and then measured a preference bias in the observer female immediately after the demonstration, offering her the choice between a new green and a new pink male. To do so, we had two types of demonstrations plus a control with usual demonstrations. In the first type of demonstration, the demonstrator female copulated with a male of a given colour ("acceptance" treatment providing positive information), while in the second type of demonstration the female actively rejected the male of a given colour ("rejection" treatment providing negative information). In view of previous results, we hypothesized that flies receiving only positive information would copy the choice of the demonstrator, whereas flies receiving only negative information would not. This is because

the real information in choice seems to be in the copulation itself: this was suggested by the fact that Dagaeff et al. (Dagaeff et al., 2016) found no difference in mate-copying scores between trials in which observer females could watch the courtship plus the copulation during the demonstration and trials in which the observer female only saw the copulation). Rejection, on the other hand, does not necessarily carries information about male quality as non-receptive females reject all males independently from their quality.

Methods

Fly maintenance

Wild-type Canton-S *Drosophila melanogaster* were raised in 30 ml vials on standard corn flour- agar-yeast-medium at 25 ± 1 °C and 56 ± 4 % relative humidity, in an artificial 12 h – 12 h light/dark cycle. Newly emerged, virgin flies (male and female) were collected daily and sexed without anaesthesia, by gentle aspiration using a glass pipette, tubing and gauze. They were kept in unisex groups of 7 females and 15 males and used for the behavioural experiments when 3-5 days old. For the experiments, males were dusted with artificial green (Shannon Luminous Materials, Inc. #B-731) and pink (BioQuip Products, Inc. #1162R) powders, and let in a food vial for 20-30 min to allow them cleaning the excess of dust before being transferred to the experimental set-up. All males were randomly assigned to one colour. After the experiments, observers and demonstrators were euthanized in a freezer (12h at -20 °C).

Animal welfare

Animals used in this study were neither harmed, food or drink deprived, nor anesthetized. We kept their number as small as possible and they were gently handled with a mouth aspirator.

Behavioural assay

Experiments were conducted in the double plastic tube devices (see Dagaeff et al., 2016). We applied three different treatments: a control treatment, an acceptance treatment, and a rejection treatment (figure 1). For each treatment, the demonstration comprised two successive 30 min phases (1 and 2, figure 1) which order was reversed from one trial to the next. In the acceptance and the rejection treatments, phase 1 demonstration consisted of a 30 min presentation of a single male, pink or green (alternating from one trial to the next for each treatment). This ruled out a potential novelty effect (i.e. the discovery of one male colour during the test), which could occur if the observer female has only seen one male colour before the mate-choice test. As that male was alone, this did not provide any social information about its attractiveness. In phase 2, a male of the opposite colour was presented together with a demonstrator female. The demonstrator female was either virgin (acceptance

treatment) or recently mated (rejection treatment). Recently mated D. melanogaster females actively reject courting males (Kimura et al., 2015), so the observer female in rejection treatment could witness rejection of one male, providing negative information for this male colour. Contrastingly, observer females in the acceptance treatment could see the demonstrator mating with the male, which provided positive information for this male colour. The few trials in which the virgin female constantly rejected the male were included in the rejection group. Similarly, trials in which the mated female copulated with the male were included in the acceptance treatment group as they in fact conveyed positive information. To ensure that the female really had access to negative information in the rejection treatment, we checked that the male courted the female and was rejected. Trials in which no courtship happened were discarded. In the control treatment, the observer female was alone during phase 1, and during phase 2 we introduced in the opposite compartment a virgin demonstrator female, a pink and a green males. The observer female could thus witness the courtship of the two males and the choice of the demonstrator female. Trials in which the female did not mate within the 30 min of the demonstration were discarded. After the end of copulation of the demonstrator female, or after 30 min of rejection of the male, demonstrator flies were removed and two new virgin males, one of each colour, were placed in the tube. After 5 min, the partition separating males and female was removed, beginning the mate-choice test. During the test, we recorded the time of the first wing extension ("singing") of a male as the beginning of courtship of this male, and its colour, as well as the time when copulation began and the colour of the chosen male. As in previous studies (Dagaeff et al., 2016; Danchin et al., 2018; Monier et al., 2018, 2019; Nöbel et al., 2018b), trials in which only one male courted the female before the onset of the copulation were discarded because only when both males showed interest towards the female she was unambiguously in a position to choose.

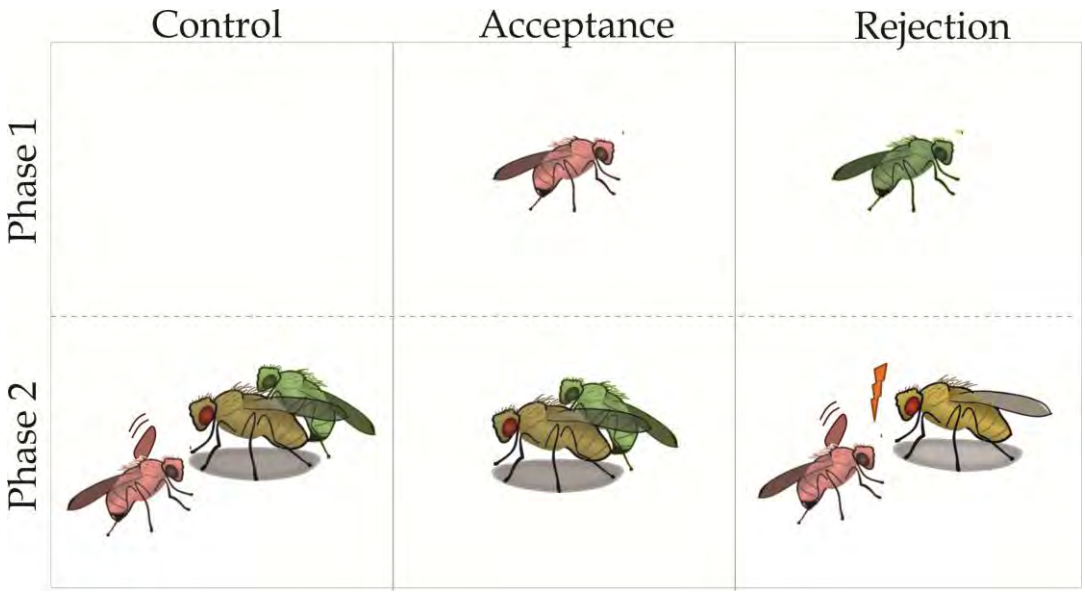

Figure 1: Demonstrations presented to observer female in each of the three treatments.

Each phase lasts 30 min. Order of phases 1 and 2 was reversed from one trial to the next, and we also did the same demonstrations with reversed colours.

Mate-copying index

For each trial, we computed a mate-copying score as a binomial variable taking the value 1 when the observer female mated with a male of the colour preferred (or not rejected) by the demonstrator female, and 0 in the opposite case. For instance, if the demonstration showed a female rejecting a pink male (rejection treatment), the mate-copying index was 1 if the observer female mated with a green male in the test, and 0 if she mated with a pink male. We then calculated the mate-copying index for each group as the mean of mate-copying scores. For the analyses, we took all trials in which a copulation occurred after both males courted the female during the test (192 trials), because only when both males showed interest towards the female she was unambiguously in a position to choose. Mate-copying indices significantly above 0.5 indicate that observer females were biased in their mate choice towards the colour preferred or not rejected by the demonstrator, and thus reveal mate-copying.

Statistical analyses

Raw data of the behavioural experiment has been uploaded as supplementary material. We analyzed the data using the version 3.5.1 of the R software (R Core Team, 2018). For each treatment, we measured the departure from random choice with a binomial test. We then ran GLMM (generalized linear mixed models) with binary logistic regression (package lme4; Bates, Mächler, Bolker, & Walker, 2014) between the three groups in order to see if treatment and normalized air pressure (air pressure at the beginning of the trial minus mean air pressure in the whole data set) have an effect on mate-copying scores. We included a random block effect to account for the non-independence of the set of six trials trained and tested in parallel in the same observation box. We used Wald chi-square tests implemented in the ANOVA function of the car package (Fox and Weisberg, 2011) to test the significance of fixed effects. The starting model included two fixed effects (treatment, normalized air pressure) and interaction between them, and the final models were obtained through a backward selection approach, removing the interaction as it was non-significant. We then selected a model with the lowest Akaike Information Criterion (AIC, Akaike, 1969). Finally, we did two-by-two comparisons between groups using Pearson's Chi-squared test with Yates' continuity correction.

Results

We measured mate-copying scores after a demonstration showing either a female accepting a male, a female rejecting a male, or a female accepting a male while rejecting the other. Observer females that received positive information for one phenotype and negative information for the other one during the demonstration (control treatment) copied the choice

of the demonstrator (binomial test, N = 63, P = 0.043, figure 2). Females that received only positive information, by watching a demonstrator female accepting copulation with a male, also copied the demonstrators apparent preference (binomial test, N = 65, P < 0.001; right bar of figure 2). Contrastingly, females that only saw a male rejected by a female (negative information only) did not show a preference for the opposite phenotype, or in other words, they did not avoid mating with the male of the phenotype that was rejected by the demonstrator (binomial test, N = 64, P = 0.532, figure 2). We compared the mate-copying scores of the three groups in a GLMM including treatment, normalized air pressure and interactions between them, as well as a block random effect. Air pressure was added to the model because it was found that mate-copying scores are sensitive to this weather variable (Dagaeff et al., 2016). In the selected model, that comprises treatment plus normalized air pressure and the random block effect, treatment effect on mate-copying scores was significant (GLMM, Wald χ^2 test, N = 192, $\chi^2 2 = 9.26$, P = 0.010) while normalized air pressure was not (GLMM, Wald χ^2 test, N = 192, $\chi^2 1 = 0.64$, P = 0.423). Finally, we did two-by-two comparisons between groups in post-hoc χ^2 tests, and found a significant difference between acceptance and rejection treatment groups (N = 129, $\chi^2 1 = 8.63$, P = 0.003), but neither between control and acceptance (N = 128, $\chi^2 1 = 0.77$, P = 0.373) nor between control and rejection (N = 127, $\chi^2 1 = 3.53$, P = 0.060). Thus, positive information for a certain phenotype appeared sufficient to elicit mate-copying, but not negative information in our experimental conditions.

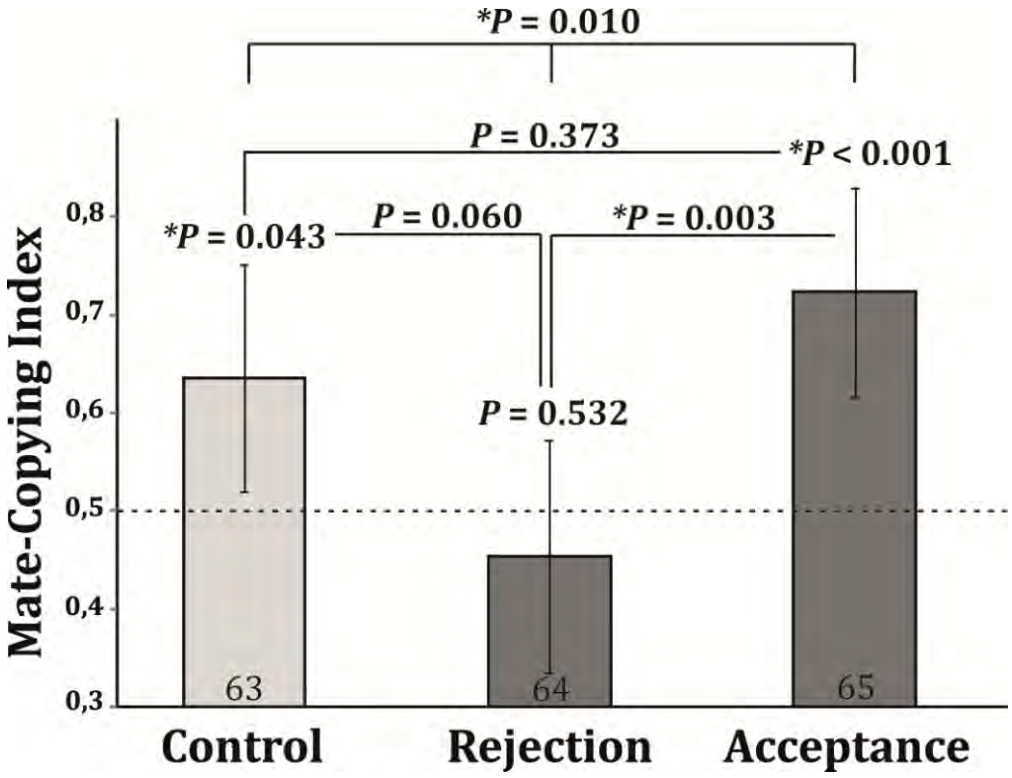

Figure 2: Mate-copying index after a single demonstration.

Observer females received the following treatments: positive and negative information (Control), negative information only (Rejection), and positive information only (Acceptance). Dashed line indicates expectations under random choice. Sample sizes are indicated inside the bars. Error bars

represent Agresti-Coull intervals. Above bars are the P-values of binomial tests for each group, of post-hoc χ^2 tests between two groups, and of GLMM test between the three groups. P-values under significance threshold (< 0.05) are highlighted by a star.

Discussion

Our experiment aimed at disentangling positive from negative information during observation of mate-choice decisions in *D. melanogaster*. We found that control females learned and copied the choice of the demonstrator females, as in previous studies (Dagaeff et al., 2016; Danchin et al., 2018; Nöbel et al., 2018b; Monier et al., 2019), and so did females receiving positive information. In contrast, females receiving only negative information did not significantly avoid the colour they saw being rejected. Thus, positive information is sufficient to elicit mate-copying after one demonstration in fruit flies.

Our negative result in the rejection treatment suggests that one rejection demonstration was not a strong-enough cue to elicit avoidance behaviour in the observer female, probably because a female can reject a male for reasons that are independent from male quality, like, a non-receptive status (Connolly and Cook, 1973; Neckameyer, 1998), which is actually the case in our study. Recent research on aversive olfactory memory in *Drosophila* showed that spaced training with sequences of conditioned stimuli (CS) reinforced with an aversive cue (CS+) followed by another CS without reinforcement (CS-) leads to an approach for the CS-, a "safety memory" (Jacob and Waddell, 2019), when the fly is later tested with a combination CS-/novel odour. Thus, a sequence of several rejection demonstrations (showing first the rejected male and then the single one, repeated several times) might elicit aversive learning and/or approach of the other male phenotype. In sailfin mollies (*Poecillia latipina*), females copied the rejection of a male (Witte and Ueding, 2003), but the set-up used was quite different from ours, in particular as the rejection demonstration consisted of a sequence of 12-min video of four different females escaping from a courting male, we can thus think that the rejection cue is stronger than in the present study, as several model female consistently reject the male. Similarly, a study in humans found that women, but not men, decrease their interest for a relationship to a model after watching a speed-dating video in which this model and a potential partner show mutual lack of interest (Place et al., 2010). This can indicate that above the experimental conditions, different species use different social cues in mate-copying. Finally, our results show that in the classical mate-copying experiment in *Drosophila*, the rejected male shown in the demonstration does not seem to be the relevant cue that biases the preference of the observer female. Moreover, one could wonder if the presentation of a male of the opposite colour together with the copulating pair in the classical demonstration could constitute a distractive stimulus rather than only a neutral additional cue. This could explain the non-significant tendency to display higher scores for the acceptance treatment compared to the control (figure 2): the observer female might have, to a lesser extent, associated the single male to the positive unconditional stimulus (US) provided by the copulating pair, in the presence of a rejected demonstrator male.

Our finding that acceptance of a male by the demonstrator is sufficient to elicit a preference for this phenotype in the observer female suggests that mate-copying is achieved

through acceptance learning, likely involving networks of appetitive learning. Several authors suggested that social learning can have an associative explanation (Avarguès-Weber et al., 2015; Heyes and Pearce, 2015; Leadbeater and Dawson, 2017), but it still has to be demonstrated. In asocial learning, like olfactory, associative, direct learning, pairing between a conditioned stimulus (CS; for instance, odour A) and an appetitive US (sucrose) lead flies to prefer odour A over B even in absence of any reward (Tempel et al., 1983), because they associate odour A to a rewarding state (Schultz et al., 1997). In our social learning paradigm, we can speculate that the relevant cues eliciting learning are the colour of copulating males and the observation of a couple of flies successfully mating. In this view, the copulating pair would mediate the appetitive US, while male colour would be the CS (Avarguès-Weber et al., 2015). Under this hypothesis, it could be interesting to study whether mate-copying mechanisms resemble those of visual, appetitive, associative learning, given that its neural bases are now well-studied (Vogt et al., 2014, 2016).

More generally, understanding how social learning works can only help sharpening our view on the evolution of the different types of learning: this would allow building accurate theories about the evolution of behaviour, cognition and culture in invertebrates.

Acknowledgements and author contributions

We would like to thank Nathalie Partuisot for help in flycare, Audrey Dussutour for providing access to her food production equipment. GL, LF and MM carried out the experiments. GL and MM performed the analyses, MM drafted the manuscript and supervised GL. SN supervised LF. SN, ED and GI designed the experiment, ED and GI jointly supervised all steps in the process. All authors gave final approval for publication.

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Investigation of the dopamine neurons required in speed learning

This experiment was started as a part of Guillaume Lespagnol's master project that I supervised. GL set the protocol for the demonstration and collected most of the data. I continued data collection with the help of Sabine Noëbel, and I performed statistical analyses.

Context

My previous experiment shows that mate-copying is achieved through learning based on acceptance cue, and not on rejection cue. This result gives indication on how flies learn, and can orient the exploration of the underlying neural mechanisms, as we can make the assumption that mate-copying is an appetitive learning.

In the second chapter I showed that dopamine is required for mate-copying in a speed learning design. In olfactory learning, dopa decarboxylase DDC-gal4 neurons (that is 118 dopaminergic neurons from the Paired Anterior Medial –PAM– cluster innervating almost all of the MB horizontal lobes) are responsible for appetitive learning (Liu et al., 2012; Shyu et al., 2017, reviewed in Vogt et al., 2014). On the contrary, aversive olfactory learning and aversive taste learning are under the control of TH-Gal4 labeled neurons, and more precisely those from the Paired Posterior Lateral (PPL1) cluster (Riemensperger et al., 2005; Kirkhart and Scott, 2015).

I thus made the assumption that in mate-copying, DDC-Gal4 dopaminergic neurons, but not TH-Gal4 neurons, would be required for correct learning, similarly to what is known in olfactory learning. I blocked TH-Gal4 or DDC-Gal4 dopaminergic neurons in observer females during the mate-choice demonstration, and measured effects on mate-copying scores. To prevent developmental effects that could result from a lifetime impairment of some dopaminergic neurons activity, I used a conditional inactivation system: the Shibire thermosensitive protein (Kitamoto, 2001) was expressed either in DDC-Gal4, or in TH-Gal4 cells, which resulted in a blockade of synaptic transmission from these cells when flies are placed at restrictive temperature (33°C).

Methods

Fly strains

I crossed *w+;;UAS-Shi^{ts}* flies with *w-;;TH-Gal4* and *w-,DDC-Gal4;;* lines. I then tested the female progeny of each crossing, that is, flies expressing one copy of each transgene, and having one wild-type copy of the *white* gene required for proper vision. The genotypes of the

tested females are: *w+/w-;;TH-Gal4/UAS-Shi^{ts}*, and *w+/w-,DDC-Gal4;;+/UAS-Shi^{ts}*, hereinafter referred to as DDC>Shi(ts) and TH>Shi(ts), respectively. TH-Gal4 and DDC-Gal4 are expressed in distinct but overlapping groups of dopaminergic neurons projecting to the mushroom bodies (Liu et al., 2012).

Behavioral test

As the thermosensitive Shibire blocks neuronal transmission at restrictive temperature only (Kitamoto, 2001), i.e. over 29°C, flies are assumed to have normal behavior in the classical rearing conditions at 25°C. This allows a precise temporal control of the activity of specific sets of neurons. To activate the neuronal blockade, observer females were put at a restrictive temperature (33°C) 30 min prior to the experiment, and were maintained at this temperature during the demonstration thanks to a heating mat under the observer's tubes. Demonstration occurred in classical devices, and observer females were then removed and placed individually into food vials at 25°C for 3-4 hours to ensure that the neuronal blockade had stopped before the time of the test

Statistics

Data were analyzed as in II-A, Methods. The starting GLMM model, including block as a random effect, comprised genotype, normalized air pressure changes in the six preceding hours, normalized air pressure at the time when the demonstration began, and all interaction between them, plus experimenter-ID (3 different experimenters did this experiment). The selected model comprised genotype only.

Results

Females in which TH neuronal activity was blocked during the demonstration (TH>Shi(ts), Figure 2, left bar) exhibited no mate copying (binomial test, N = 49, P = 0.57), whereas flies in which DDC neurons were blocked during the demonstration (DDC>Shi(ts), Figure 2, right bar) copied the choice of the demonstrator (binomial test, N = 39, P = 0.024, Figure 2). I compared the scores from these two groups in a GLMM model and found a significant difference (GLMM with Wald χ^2 test, N = 88, $\chi^2_1 = 4.14$, P = 0.042, Figure 2). Thus, blocking TH-Gal4, but not blocking DDC-Gal4 dopaminergic neurons during the mate choice demonstration impairs proper learning in a speed learning design.

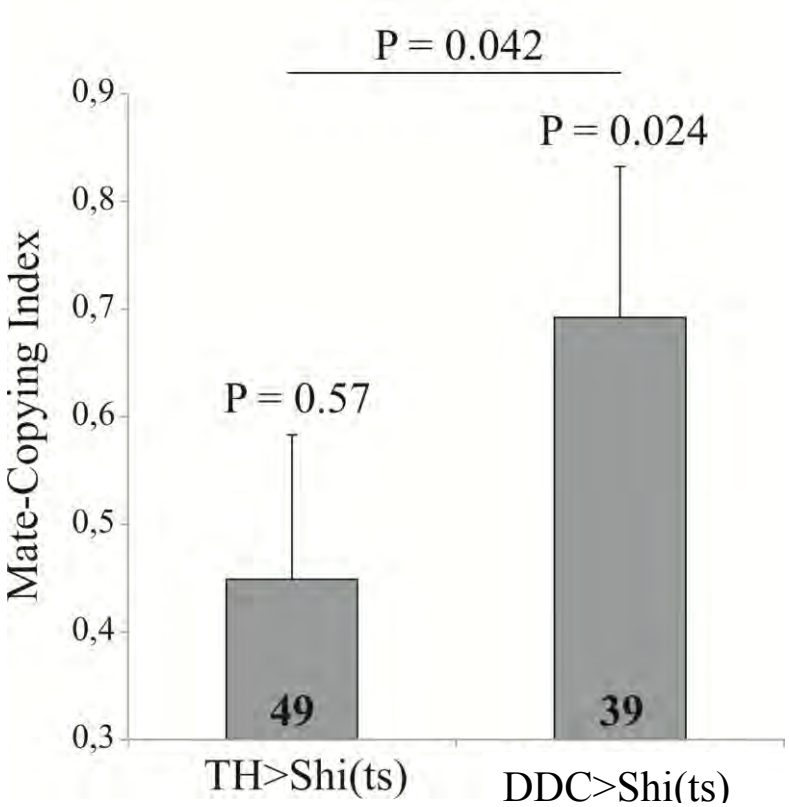

Figure 2: Mate-copying indices of flies trained with neuronal blockade. Flies were tested after a single demonstration when they were warmed at 32°C to activate Shibire. **TH>Shi(ts):** females in which TH neuronal activity is blocked during the demonstration (genotype: *w+/w-;;TH-Gal4/UAS-Shi^{ts}*). **DDC>Shi(ts):** females in which DDC neuronal activity was blocked during the demonstration (genotype: *w+/w-,DDC-Gal4;;+/UAS-Shi^{ts}*). Inside bars: sample size. Statistics indicate the P-values of binomial tests and of a GLMM comparing the effect of treatment in both groups.

Discussion

Dopamine is involved in mate-copying in a speed learning design (Monier et al., 2019). Here, I tested the involvement of two different groups of dopaminergic neurons known to be involved in olfactory learning, and I found that blocking TH-Gal4 neurons impaired learning, while blocking DDC-Gal4 neurons did not affect mate-copying scores. Thus, my hypothesis is invalidated, mate-copying in *D. melanogaster* has not the same mechanism as an appetitive olfactory learning, at least concerning the dopaminergic neurons involved. However, to conclude that TH-Gal4 neurons are the dopaminergic cluster involved in social learning in mate-copying, it is necessary to do additional tests: observer females should be tested after a demonstration at permissive temperature to validate the absence of any impairment when TH-Gal4 neurons are not blocked. Moreover, fruitflies can detect temperature changes (Bang et al., 2011; Tomchik, 2013; Barbagallo and Garrity, 2015) and display genetically controlled temperature preference behaviors, with an optimum at 24°C for wild-type flies. Thus, doing the demonstration at 33°C could have impaired proper learning because of the aversive valence of the temperature stimulus. The fact that DDC>Shi(ts) females have normal mate-copying scores is thus an important control that the experimental conditions of the behavioral test can allow an observer female to learn and copy.

When Shibire is expressed in TH-Gal4 neurons, submitting flies to a restrictive temperature for 40 min or more just after learning is known to greatly reduce forgetting (Berry et al., 2012; Berry and Davis, 2014) in olfactory aversive learning, because DAMB is expressed in the target neurons of TH-Gal4 and this dopaminergic receptor promotes forgetting. TH-Gal4 is also a neuronal cluster involved in cold detection (Tomchik, 2013). Taken together, these results indicate that TH-Gal4 neurons are involved in many functions and it could be good to confirm these results by an experiment that does not involve temperature shifts: for instance, by expressing Kir2.1 into TH-Gal4 neurons at the adult stage, which would silence them (Baines et al., 2001; Hodge, 2009).

Finally, this experiment strikingly suggests that the mechanisms underlying observational social learning may be distinct from those involved in appetitive memory in non-social associative learning, although they share some common characteristics. This exciting fact invites to a deeper study of the neuronal processes. Detailed research of structures and networks may involve testing many different treatments, and it is crucial to first try making the experiment as simple, fast and standardized as possible.

B. Development of a protocol of demonstrations using virtual stimuli

Introduction

Virtual stimuli are now used in a wide variety of behavioral experiments (reviewed in Chouinard-Thuly et al., 2017). These methods can offer many advantages when they are used properly; notably, they can allow studying new questions that are not possible to study otherwise, and they can offer new ways of studying behavioral questions.

I tried to elicit mate-copying in observer females by presenting them a picture of a demonstration (copulating couple and a rejected male) instead of live flies. The aim of this experiment was primarily to show that pictures can be used instead of live animals during the demonstration, with similar mate-copying scores. Such a discovery would then open the door for a lighter, more efficient, more homogenous, and simpler method for the study of mate-copying in *Drosophila*.

Methods

Fly maintenance

Canton-S wild-type flies were reared as in previous experiments. Virgin flies were collected daily and sexed without anesthesia, and kept in unisex groups until use at 2-5 days.

Pictures

Flies were semi-constrained in a square, transparent plastic box 1.8 x 1.8 cm^2, closed with a white foam plug, so that flies could have a volume of about 1 x 1 x 0.4 cm^3 in which they could walk and interact for several minutes.

Pictures were taken with a camera Panasonic DMC FZ300 (25-600mm equivalent lens), under white light, at 3-5 cm of the flies. Pictures were then re-treated with Corel Photopaint to intensify the green and pink dusting of the males (green painting #00FF00, pink paintings #FF00FF and #FF0066), lighten the background (first protocol) or remove it (second protocol). On each painting in the final form, two couples of the same color and two rejected males of the opposite color were present together, in different positions (one topview and one frontview), for each protocol (Figure 1). The size of the flies on the printed picture (printed on glossy photopaper in the photographer studio ABCD Pictures, Castanet-Tolosan) was about 2.5 mm.

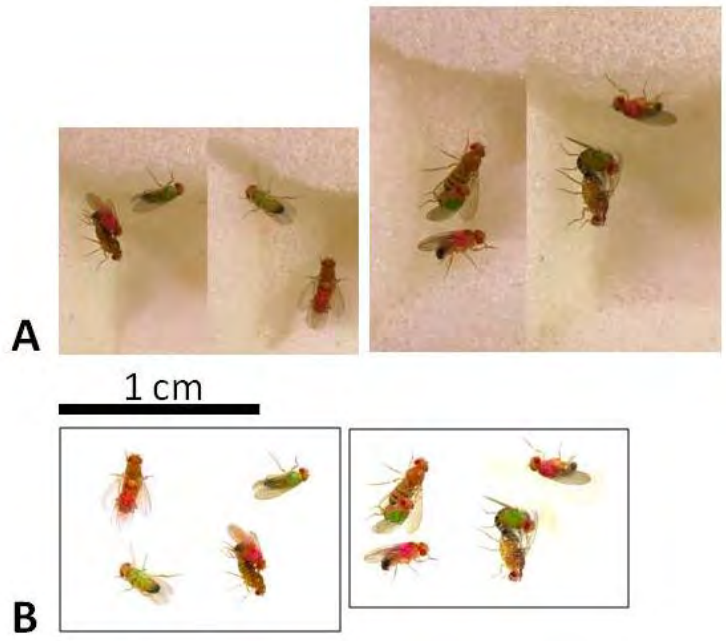

Figure 1: Pictures presented to the observer flies as a demonstration. A: in the first protocol, B: in the second protocol. Note that the same raw pictures were used for both protocols (two different green couples, two different pink couples, and two different rejected males of each color).

Behavioral test

In both protocols, pictures were presented at 0.9 to 1.2 cm to the glass partition (which was fixed to the plastic tube). In each block of six trials, three pictures showed green males copulating, while the three other showed pink males. The attribution of a picture to the observer fly was random and kept as blind as possible: each picture was paired to a device and only the number of the device was noted at the demonstration step. The color of the preferred male in the demonstration was noted after the end of the experiment. In the first protocol, observer females were first offered to observe two live, green and pink males, presented in the opposite compartment, in a classical tube device. After 5 min of "pre-demonstration", females were transferred to another device with a unique compartment facing the picture (Figure 2). Picture presentation lasted for 25 min, then females were transferred back to the classical device for the test. In the second protocol, I used devices with 2 tubes and 2 glass partitions (Figure 2), the observer female was placed in the tube and the central partition was put as soon as the female was in the second compartment. The demonstration consisted in 30 min presentation of the picture, then the picture was hidden behind a white cardboard and two virgin males were introduced for the test. Thus, the second protocol had no pre-demonstration and observer females were not transferred from a device to another during the experiment.

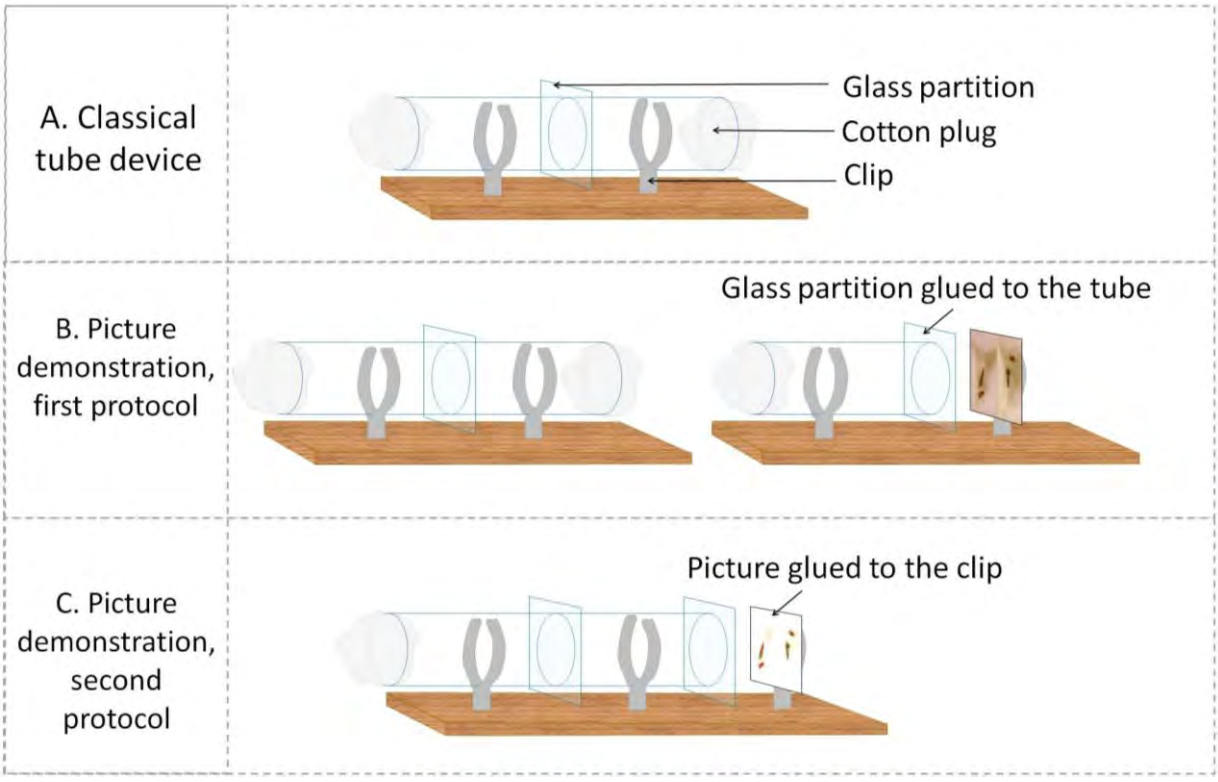

Figure 2: Devices used in the picture demonstration. A. Classical tube device used in the experiments with live demonstrations. B. Devices used in the first protocol of this experiment: pre-demonstration and test take place in the left device (classical device), while the demonstration with a picture takes place in the right device. This protocol thus requires two transfers of the observer female. C. Device used in the second protocol: the observer female is placed in the central compartment, and after the end of the demonstration the picture is hidden behind a white cardboard and males are introduced in the left compartment for the test.

Analyses

Data are analyzed with the R software version 3.4.0 (R Core Team, 2018). For each condition, the departure from random choice was analyzed with a binomial test. Mate-copying scores were then analyzed in generalized linear mixed models (GLMM) with binary logistic regression (package lme4, Bates et al., 2014). A random block effect was introduced into the models to account for the non-independence of observer flies from the same block. The significance of fixed effects was tested using Wald chi-square tests implemented in the ANOVA function of the car package (Fox and Weisberg, 2011). Starting models included treatment (protocol A or protocol B), normalized air pressure at the time of the test, and its normalized variation within the 6 preceding hours, and color of the successful male in the demonstration, as well as interactions between these effects. I used a backward selection approach using P-values, removing the highest order interaction as soon as it was non-significant. The final model was chosen as one with the lowest Akaike Information Criterium (AIC, Akaike, 1969).

Results

In both protocols, observer females copied the choice of the virtual demonstrator presented on the picture (binomial tests, N = 64 and 72, P = 0.033 and 0.003 respectively, Figure 3). Thus, females can recognize and use social information presented on a picture. Both protocols produce positive results, the difference between them is not significant: GLMM with Wald χ^2 test, N = 136, χ^2_{10} = 2.12, P = 0.15, the selected model comprises protocol, color of the male chosen in the demonstration, normalized air pressure, air pressure variations, and all interactions between the three last parameters, as well as a random block effect.

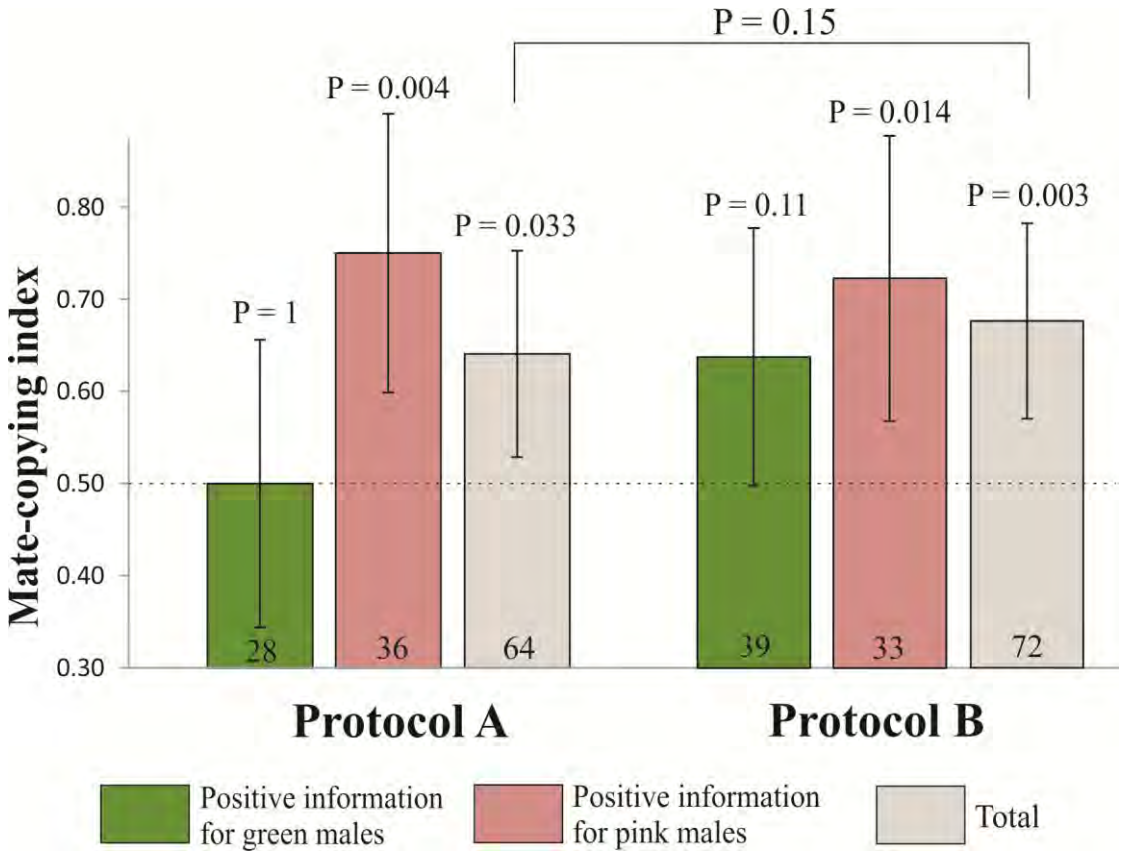

Figure 3: Mate-copying index of females that had a picture demonstration. Left bars: protocol A, right bars: protocol B. Grey bars represent the total dataset. As results are different depending on the picture shown, results for each picture are also represented: pink bars represent the MCI for females that could observe a picture on which two pink males are successful while two green males are apparently rejected; green bars represent the MCI of flies that had the opposite demonstration, i.e., positive information for green males and negative information for pink males. Error bars represent Agresti-Coull intervals. P-values are the results of binomial tests for each group, and of a GLMM (see Analyses in the Methods section).

I looked into more details into the results of each experiment and I recognized that the color of the males copulating on the picture presented was affecting the scores, particularly in protocol A (Figure 2). I thus did GLMM models with Wald χ^2 test on data from each protocol, in order to see if the color of the preferred male in the demonstration affected mate-copying scores.

For protocol A, the selected model comprised air pressure, air pressure changes, color of the male as well as all interactions between the three parameters and a random block effect. The interaction between the three fixed effects had a significant effect on mate-copying scores: $N = 64$, $\chi^2_9 = 4.37$, $P = 0.037$. To examine further the effect of male color, I thus ran a second analysis after removing data with the lowest air pressure values (N = 7 data points discarded), the starting model integrated color of the male, air pressure variations, interaction between them as fixed effects, and the selected model included color of the male only, this parameter having a significant effect on MCI: $N = 57$, $\chi^2_3 = 4.88$, $P = 0.027$. I chose

to remove data from the analysis instead of splitting my dataset into two subsets and running two parallel analyses because the dataset is already rather small.

For protocol B, the selected model comprised air pressure changes, color of the males and interaction between them, as well as a random block effect. The interaction had a significant effect on MCI: N = 72, $\chi^2_5 = 5.59$, P = 0.018: green demonstrations elicited mate-copying when air pressure was decreasing or stable, while pink demonstrations elicited mate-copying when air pressure was stable or increasing.

In a nutshell, the color of the male receiving positive information in the demonstration impacted mate-copying scores in both protocols. As I used only one picture for each color, it is plausible that this effect is driven by the picture itself (position of the flies for instance), and not by the color of the male.

Discussion

Drosophila females are able to perform mate-copying after observing a picture of copulating and rejected flies for 25-30 min only. This astonishing discovery could mean that they recognize the picture as male and female flies, and that they can detect that the female and one male are copulating while the male of the opposite color is single.

This ability can surprise in such a simple and small organism, as we can imagine that it requires complex cognitive phenomena to associate a pictured fly with a living congener providing social cues. However, things might have to be considered in a much simpler way: fly brains might be "tuned" to recognize anything that has roughly the size and shape of a fly as a fly, and everything that has roughly the size and shape of a copulating pair as a copulating pair, even when these objects are not moving. A study showed that male flies initiate courtships towards magnets as if they were female flies, provided that those magnets have roughly the size of a female fly and that they move at the speed of a fly (Agrawal et al., 2014). Our human brain is also tuned to quickly recognize human faces in our environment (Hadjikhani et al., 2009), for instance a "surprised face" in the Moon, a phenomenon called pareidolia. This ability of *Drosophila* females to mate-copy based on fly pictures might actually reveal a "pareidolic-like" behavior.

One can suppose that this ability helps to quickly grasp social information from the environment, and could help flies to locate members of their own species and aggregate on food patches and oviposition sites for instance. Moreover, the pictures presented in this experiment are high quality pictures in which the flies are, to a human eye at least, very resembling. As copulating flies usually stay immobile for roughly the entire duration of the mating (personal observation) when they are not disturbed, and as flies are tiny and have a "depth" of about one millimeter, one can think that a picture of a couple is somehow not that different in appearance from a real couple.

Finally, my experiment shows that virtual stimuli can be used in *Drosophila* in complex social learning situations. This replacement of live flies with pictures was initially mainly motivated by technical considerations that are simplifying, accelerating and standardizing the whole experiment. This aim has been reached and the perspectives of the experiment are

much larger than a simple improvement in techniques. I thus decided to push further my investigations, which I describe in the next part.

Acknowledgements

I would like to thank Antoine Wystrach who made the first pictures of the flies, that I used for protocols A and B. I also thank David Villa who took many pictures and helped a lot with polishing the raw pictures, and Guillaume Isabel and Arnaud Pocheville who suggested using pictures instead of movies. Finally, I would like to thank the "video project" team: Brice Ronsin, Christian Rouvière, and Patrick Arrufat.

C. How far can we simplify the stimulus without losing its ability to elicit mate-copying?

Introduction

My previous experiment showed that *Drosophila* females can copy with a picture demonstration. The results I obtained in the second experiment (protocol B; Figure 3, right bars) are comparable to what we usually see in similar conditions (one demonstration in tube, test 0-4 h after) with a live demonstration. Moreover, Sabine Nöbel showed that females that sequentially observed five different pictures of a couple mating plus a rejected male, always with the same color associations, learned and copied after 24h (Nöbel et al., in prep.), as we showed for five live demonstrations (chapter I, B), under similar conditions (5 x 20 min demonstrations spaced by 15 min resting intervals, according to the "LTM protocol" described in chapter I, B). Thus, it is possible to elicit mate-copying by presenting a picture of a demonstration. This result opens a wide window on several fields of exploration: the study of mate-copying mechanisms on a much larger scale, due to the standardization and simplification of the demonstration that considerably lightens the whole experimental process, and also the dissection of the stimulus, that can now be controlled and artificially modified.

In this third part, I studied which characteristics of the demonstration are necessary and sufficient to elicit mate-copying in a speed learning design. To do so, after a first step aiming at determining the minimal demonstration duration required for a proper learning, I gradually simplified the picture used in the demonstration step in an attempt to determine the minimal cue required to elicit mate-copying.

Methods

Rearing of the flies was conducted as in the previous experiments, and experimental conditions were the same as in protocol B (III-B, Figure 2, bottom panel).

In the first experiment, testing the effect of demonstration duration, I used the same pictures as in protocol B of the previous experiment (III-B, Figure 1-B), i.e. two couples and two rejected males per picture, with a white background.

In the second experiment, I modified the pictures, creating three different conditions (with two different pictures per color per condition, which makes 12 different pictures in total). Starting pictures were taken with Antoine Wystrach (CRCA) or by David Villa (Sciencimage). Two pictures of a demonstration were selected for each color (two pictures showing a copulation with a green male, two showing a copulation with a pink male, all with a rejected male of the opposite color). The treatment described in III-B, protocol B was applied, that is, a white background, and colors intensified with the pencil tool of the software. This first set of pictures was used as a control treatment (Figure 1). The same four pictures were also modified to create simpler stimuli: legs were erased and the whole fly except the wings was covered by even colors. In the treatment "dots" (Figure 1), the colors were brown #B7702C for the fly bodies, pink #FA2F35, and green #76B018 for the colored dot on the male back, and dark red #B41912 for the fly eyes. Colors were chosen visually to resemble as much as possible to those on real colored drosophila, but more intense for the pink and green. Finally, in the treatment "painted" (Figure 1), the brown color was replaced with either green or pink, so that the whole couple was colored like the chosen male.

Figure 1: Two examples of a picture transformation for the three treatments. From left to right: control (picture treated as described in III-B-protocol B: white background, intensified colors), "dots"

(pink or green dot on the back of the male), and “painted” (whole male and whole couple colored in pink or green).

Analyses were conducted as described previously, with binomial tests and GLMM with Wald χ^2 tests including a random block effect. The starting model for the comparison of the different demonstration durations included demonstration time as a continuous variable, and normalized air pressure in the experimental room, and interaction between them, as fixed effects. The selected model comprised the two parameters without interaction.

Results

In a first experiment, I compared the mate-copying results for three different durations of the demonstration: 5 min presentation, 15 min presentation or 30 min presentation (control condition). Females that could watch the demonstration for 15 min, as well as control females, learned and copied the choice of the virtual demonstrator (Binomial test, N = 67 and 64, P = 0.014 and 0.004, respectively; Figure 2).

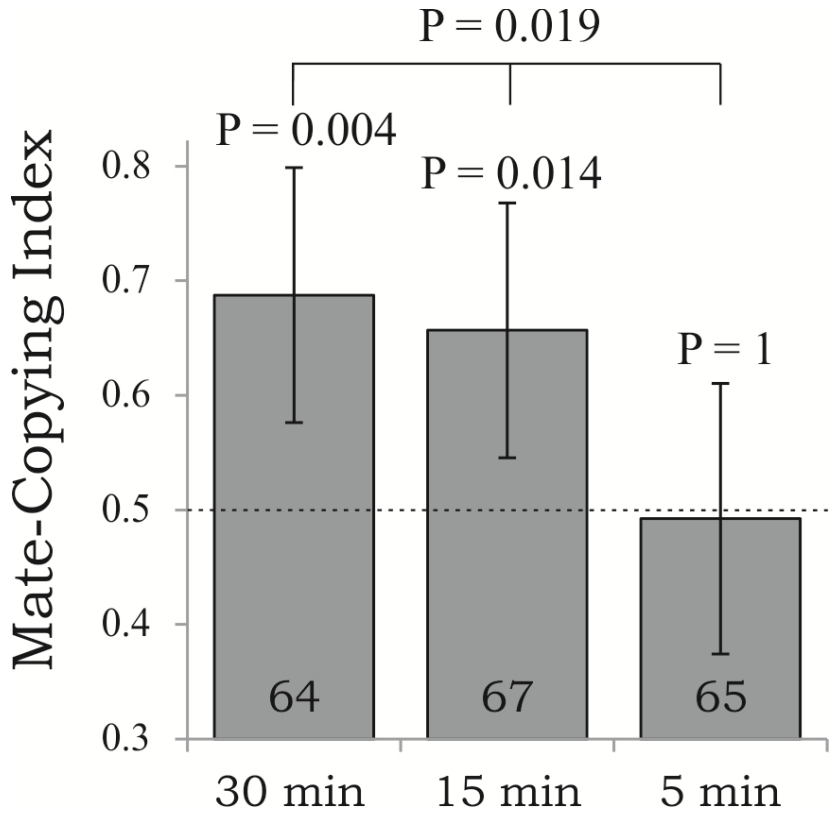

Figure 2: Effect of demonstration duration on mate-copying scores. Mate-copying scores of females that observed the picture demonstration for 30 min, 15 min or 5 min before the mate-choice test. Inside bars: sample size. Statistics indicate the P-values of binomial tests and of a GLMM comparing the effect of treatment in the three groups. Error bars represent Agresti-Coull 95% confidence intervals, and the dashed line indicates expected results under random choice.

In the second experiment, I measured the mate-copying scores of females that could watch either a picture (control), or a simplified picture ("dots" and "painted" treatments, Figure 3) for 20 min. However, COVID-19 outbreak interrupted the experiment and the data collected is not sufficient to draw any conclusion.

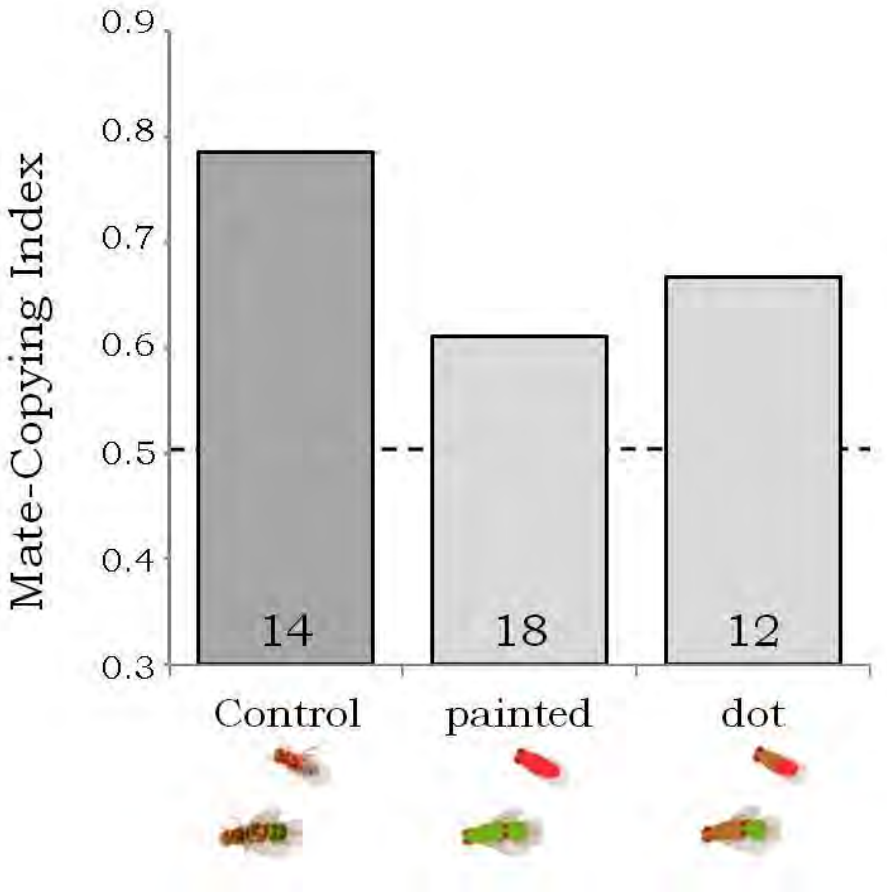

Figure 3: Effect of a picture simplification on mate-copying scores. Inside bars: sample size. The dashed line indicates expected results under random choice. No statistical test was applied because of the very low sample size.

Discussion

I showed that females are able to learn and copy from a picture, and in this last part I modified the visual cue in order to find which characteristics allow flies to do mate-copying. I found that 15 min of demonstration are sufficient to elicit mate-copying, but 5 min are not. Then I simplified the picture and measured the scores, but the amount of data collected does not allow concluding. We can however imagine different scenarii.

If the conditions "painted" and "dot" both elicit mate-copying like the control, this would mean that either flies still recognize the drawings as congeners, or another learning mechanism is occurring, like a sort of imprinting (Lorentz, 1941).

If females watching the control picture can learn and both other conditions give non-significant results, this would mean that the modification of the picture removed the salience

of the stimulus. A technical explanation can simply be that the colors chosen were not recognized as the powder dusts applied on males used for the test. A mechanistic explanation would be that flies notice that modified pictures do not present actual congeners, which would mean that *Drosophila* vision is good enough to detect details on a picture, like legs or abdominal stripes, and that the preference for an artificially colored phenotype can only be elicited by the presentation of realistic flies. In other words, flies would be able to visually recognize individuals from their own species, using visual cues present on the control picture and absent from the modified pictures. Several studies found that species recognition in drosophila can be mediated by pheromonal cues (Antony and Jallon, 1982; Keesey et al., 2016), or by the courtship song of males (Schilcher, 1976; Talyn and Dowse, 2004), but visual cues involved in this function have not been explored so far.

Finally, a third possible result could be that only the condition "painted" does not lead to mate-copying: my hypothesis would then be that the very colorful drawings of this condition do not reflect the colors on the males used in the test, and that an "enhanced" stimulus (brighter colors than in a live demonstration) does not increase the scores. To test this hypothesis, one could replace the bright green and pink colors by an averaging of all the colors present on the couple and on the male in the control picture.

All in all, the experiments I conducted in this part bring a proof of concept that picture demonstrations and picture modification can be used not only to study mate-copying, but also to study species visual recognition and to explore many different types of social and non-social learning.

Conclusion

In this third chapter, I showed that females copy the acceptance, not the rejection of a mate, and that TH-Gal4, but not Ddc-gal4 dopaminergic neurons, are required for proper learning in a speed learning design. This result completes my finding that dopamine is involved in mate-copying, but a lot still has to be done in the exploration of the neuronal networks involved in mate-copying in short- and middle-term memory. I also found that a picture of a copulating couple plus a rejected male could elicit mate-copying in observer females, which will help efficiently in further investigation of the neuronal mechanisms, in particular for the study of long-term memory that requires several demonstrations. Finally, I started to use the pictures as a mean to explore the characteristics of the visual cue that leads to mate-copying. I found that the demonstration duration can be reduced to 15 min, which shortens the experiment, and I brought first pieces of evidence that the manipulation of the picture can be a great tool to explore species visual recognition.

General discussion

Overview

This work brings several new elements in the study of mate-copying, and in its use as a model of social observational learning.

First of all, it establishes that this social behavior is rather robust to demonstration conditions, and gives rise to a long-term memory of a mate preference when demonstrations are sequential, with resting intervals. These characteristics support our assumption that this model can be of great interest in the study of social learning in *Drosophila* and of its potential consequences in terms of cultural heredity.

In the second chapter, I showed that neural mechanisms of mate-copying present some similarities with those of pavlovian, non-social, visual or olfactory learning, which suggests that several types of learning share common mechanisms and pathways. From a technical point of view, this second chapter, however, also shows the technical limits of the classical experimental design in the study of genetically modified flies, in particular in long-term memory, because experiments are very heavy and delicate, which hampers our ability to deeply investigate neural mechanisms by testing a diversity of genotypes.

In the third chapter, I provided some responses to the problems raised in the second chapter: first of all, the elaboration of a protocol of virtual demonstrations can allow a great gain in time, homogeneity, and simplicity, by standardizing the protocol. This increases our capacities in terms of testing many demonstration conditions in parallel or sequentially, thus opening new avenues of exploration. Moreover, the replacement of live demonstrators with pictures of flies allows us to investigate several questions: first, the relevance of classifying different learning types dependent on the nature of the stimulus (social versus non-social) could be questioned by experiments of stimulus simplification. In particular, if mate-copying is a visual associative learning, what is the unconditional stimulus on the picture? If females learn to prefer a color after watching a demonstration in which pictures are modified, is it because they still recognize a couple and this has an appetitive value, or is another phenomenon occurring? Second, modifying pictures can also be a way of exploring species visual recognition in *Drosophila*. To explore the first and the second point, it would be necessary to compare the neuronal pathways involved in learning with live versus highly simplified demonstrations that nonetheless still trigger mate-copying.

Mate-copying in the population

From the lab to the wild

Female fruit flies can learn and copy whatever the number of co-observer females during the demonstration, and in a range of 0.7 to 1.7 male:female sex-ratios in experimental conditions. In natural conditions, *D. melanogaster* often live in dense populations on food patches, together with other species (Markow, 2015), and they can interact with each other (Kacsoh et al., 2018). Copulations often occur while females are young, sexually mature adults, as

randomly collected flies in the wild all produce progeny (Markow, 2011), and as it seems that non-mating is costly to wild females (Markow, 2011). Hence, female fruitflies probably observe copulations commonly, in various sex-ratio and density conditions. Having acquired the ability to quickly grasp and use social information provided by their conspecific's mate choice even in crowded conditions gives an evolutionary advantage when competition for access to mates and short lifespan do not allow too much indecision.

However, my results obtained with Canton-S strain in laboratory conditions should be repeated using a wild population before one can claim that natural populations of flies do behave like the laboratory Canton-S strain on this particular ability. For instance, in mate-copying, the demonstration duration is 30 min in the speed learning design, and up to 3 hours in the protocol for long-term memory. However, it seems unlikely that a fruit fly would stay for 30 min in front of a demonstration. What probably happens in the wild is that fruitflies travel frequently from one food patch to another one, and they probably observe a high number of copulating couples, each for a brief lap of time. Somehow, this makes their ability to detect the preference of the majority highly relevant ecologically, as the observation of a single couple in the wild may not be enough to elicit a mate preference. About this point, a project currently conducted in our group aims at estimating how many demonstrations a female can observe simultaneously.

Finally, there is a lack of information on how drosophila behave in the wild, notably in terms of distances travelled. Subsequently, it would be interesting to investigate further the ecology of drosophila in the wild, while very little is known for the moment (Markow, 2015). As a matter of fact, some behavioral traits primarily demonstrated on laboratory strains, under artificial conditions, can be quite different to what *D. melanogaster* actually does in the wild. For instance, several studies reported a cost of multiple mating in *D. melanogaster* females (Bateman, 1948; Wigby and Chapman, 2005) that decreases their lifespan, due to effects of ejaculate components on female physiology, while an experimental study on wild female fruitflies found that mated females live longer than virgin females (Markow, 2011), and another study on lab strains measured no difference in lifespan between monogamous and polyandrous females (Castrezana et al., 2017), except when polyandrous females mated with virgin males only, which decreased their lifespan. To wrap everything up, experimental conditions can greatly affect behavioral and physiological variables.

Again, about the duration of the demonstration, circumstances in the wild are different from laboratory conditions. Maybe, in the wild, when fruit flies are not stressed at all, a short demonstration of a few minutes (that is likely to be observable by freely moving flies) can be sufficient to elicit memory. The need for a 20-min long demonstration might be a consequence of the manipulation stress of observer females. We observed an experimenter effect on learning scores: naïve experimenters undergo a "training period" before they master the experiment and obtain significant mate-copying scores with the control treatments. Training duration varies a lot among experimenters, from a few days up to 6+ weeks. The relationship between stress and learning and memory is complex (Gewirtz and Radke, 2010) and poorly explored in insects. Recently, stress pathways were studied in honeybees (Even et al., 2012), and anxiety pathways in *Drosophila* (Mohammad et al., 2016), but not in relationship with learning. The study in *Drosophila* revealed striking behavioral resemblance with mice, and the effects of anxiety and stress on learning in rodents were depicted in several studies: stress decreases the response of serotoninergic neurons signaling reward and cue (Zhong et al., 2017), potentially contributing to an anhedonia state. Injecting

corticosterone (the hormone of stress in mammals) to mice 1-3 hours after appetitive learning has a positive or neutral effect (depending on the learning task) on 24 hours memory (Micheau et al., 1984). Stress hormones in rodents and humans modulate learning and memory, positively or negatively depending on the context (McGaugh and Roozendaal, 2002). Finally, the effect of stress on learning and memory depends both on the type of stress (notably, chronic stress/anxiety or acute stress) and the type or learning task. In our case, we have strong indication that the type of stress induced by manipulation negatively affects mate-copying as inexperienced manipulators often measure lower mate-copying scores than experienced ones.

Under certain conditions, copying can be costly for the female (Witte et al., 2015). In *D. melanogaster*, Sabine Nöbel showed that it was possible to modify the preference for curly males that produce lower-fitness offspring (Nöbel et al., 2018b). Thus, we can manipulate the system so as to lead mate-copying to have a negative effect on offspring viability and fitness. Yet, my results on the environmental stability, together with Nöbel et al. results, suggest that mate-copying as a mate-choice strategy is robust to several environmental conditions. Finally, my finding that female choosiness can vary depending on the female competition context shows that *D. melanogaster* females can display strategies that allow a compensation of the possible costs associated with this social learning strategy.

A striking fact that has to be taken into account is that all the results I presented in this manuscript exclude females that selected a male to mate with before the second male started courting. I considered, as in previous studies (Dagaeff et al., 2016; Danchin et al., 2018; Nöbel et al., 2018b) that only when both males show their sexual interest the female is really in a position to choose. Nevertheless, this selection leads to the exclusion of 50-75 % of the data collected on Canton-S flies, depending on the experiments and experimenters. Actually, without doing this selection, no bias in mate preference was detectable in the group of informed observer females, except in the first data set I collected (for the experiment published in *Current Zoology*) in which a significant proportion of about 55 % of the females chose the color that was preferred during the demonstration. This has major consequences: if our Canton-S population is representative of a wild drosophila population (in particular in terms of proportion of females quickly mating with the first male courting), and if the experimental conditions somehow reflect natural conditions, there is no chance that a tradition lasts longer than the very first transmission step –whatever the weather.

Under such circumstances, building hypotheses and models of long-term transmission of an arbitrary trait in a wild drosophila population risks being like building castles in the air. Alternatively, one can argue that in the wild, drosophila females are choosier and that the naturally crowded conditions they experience in the wild make it unlikely that they are not in a position to choose between several potential suitors. This assumption is supported by the fact that in the first study of mate-copying in *Drosophila,* Frederic Mery and his collaborators (Mery et al., 2009) used a different strain (the Chavroche strain, caught in the wild a few years before) and observed strong mate-copying without selecting data based on the number of males courting the observer female. Thus, laboratory strains differ in behavioral traits like female choosiness, and it is thus delicate to extrapolate our findings to wild fruit fly populations. The Canton-S strain has been reared in laboratory for more than 75 years (Stern

and Schaeffer, 1943), which is more than two thousand generations in controlled conditions. This undeniably impacted behavioral traits that often evolve quickly with environmental changes (reviewed in Wong and Candolin, 2015). It is thus not unlikely that lab-reared Canton-S females evolved to a lower choosiness as selective pressure on progeny health is much lower than in the wild. The Canton-S strain is not the best one to study the ecology of the species, and it would be very informative to test mate-copying in one or several wild-caught strains of drosophila, as their choosiness (and their sexual behavior in general) might slightly differ from that of Canton-S, with major evolutionary consequences. On the other hand, as it is the most broadly used *D. melanogaster* strain, Canton-S is much easier to work with when it comes to the use of genetic constructs, because the genetic background is more similar between the two parental lines, which decreases the risk of side effects. Moreover, it can make the experiments more easily reproducible by another researcher.

In a nutshell, it could be greatly interesting to test mate-copying in wild-caught *D. melanogaster* from two or three different places, in a naturalistic protocol, and compare the results with Canton-S, as this would finally inform us about the capacity of *D. melanogaster* to transmit mate preferences culturally in the wild. It would also cast light on the evolution of Canton-S in the lab.

Influence of phenotype commonness

Contrary to the sex-ratio, phenotype commonness can influence mate-copying scores. Somehow, this could be related to the experiment with picture demonstration: seeing more of one color elicits a preference for this color compared to the other one. In the experiment testing the effect of sex-ratio and phenotype commonness on mate-copying scores, "more" of one color means that the proportion of each colored phenotype in the male population during the demonstration is not fifty-fifty, while in the picture demonstration experiment, seeing more of one color means that the surface of the couple, bearing the color of the chosen male, is greater than the surface of the single male. This is of course an interpretation that should be tested.

Anyway, my experiment needs a complementary treatment in which demonstrator females prefer the most common phenotype: it would be very interesting to check that in this condition female build a strong preference for the phenotype that was both preferred and more common during the demonstration. My expectation is that mate-copying score in this condition would be a bit higher than in the control condition, but not significantly so: a gradient of four or more different conditions of phenotype commonness during the demonstration would probably reveal a significant effect of the proportions of pink and green males on the mate-copying scores, but it is difficult to predict the type of the relationship (linear or not), as the preference of demonstrator females also strongly influences the preference of observer females. One can imagine an additional experiment in which females would observe different proportions of pink and green males, without copulation, and see if this demonstration affects mate preference.

Finally, my experiment was a pilot study that opens new questions to further explore, and that could help better understanding the relationship and relative strength of different social factors influencing a female's mate choice in *D. melanogaster*.

From a technical point of view, I calculated the sex-ratio as total number of males over total number of females in the hexagon during the demonstration. However, the operational sex-ratio (OSR) usually takes into account the receptive status of females as it is calculated as the number of males ready to mate divided by the number of males and females ready to mate (Kvarnemo and Ahnesjo, 1996). However, in my study, it is difficult to estimate what should be taken into account for the calculation of an OSR: at the time of the demonstration, both males and females that are involved in a copulation are not "ready to mate", one may thus calculate the sex-ratio as the number of non-mating males over the number of non-mating flies in the hexagon. Alternatively, as males can theoretically re-mate quickly after a first sexual encounter (Demerec and Kaufman, 1941), they might be considered as ready to mate while their female partner might not, which ends up in a third different estimate of sex-ratio. As these alternative ways of measuring the sex-ratio seemed equally challengeable to me, I chose the simplest one.

Mate-copying across time

Fruit flies are able to learn mate preferences from a single demonstration, and can remember this information for at least 24 hours, in a process involving protein synthesis. The fact that protein synthesis is involved is similar to the long-term memory in olfactory learning (Tully et al., 1994). In all organisms, long-term memory formation requires protein synthesis after training (mouse: Barondes and Cohen, 1967; rat: Daniels, 1971; praying matis: Jaffé, 1980; chicken: Rose and Jork, 1987).

In drosophila, depending on the type of learning, memory retention time can differ: typically, in appetitive olfactory learning, a single conditioning trial can elicit long-term memory that is still present after several days (Krashes and Waddell, 2008), while in aversive olfactory learning, a single conditioning trial leads to short and mid-term memory, but no long-term memory (Tully et al., 1994). Nonetheless, in aversive learning, anesthesia-resistant memory independent of protein-synthesis can persist for several days after repetitive training (Tully et al., 1994). Similarly, in honeybees, 24-h memory in appetitive olfactory learning can be independent of protein synthesis (Wittstock et al., 1993; Wüstenberg et al., 1998). Depending on the protocol and the insect model, the duration of each type of memory can thus vary. In mate-copying, Anne-Cécile Dagaeff showed that one demonstration could be sufficient to elicit a preference in the observer female 6 h after the demonstration, but the memory does not last up to 24 hours (Dagaeff, 2015 and Sabine Nöbel, unpublished results). When using spaced training, with five sequential demonstrations separated by resting intervals, observer females memorize and copy immediately after and 24 hours after. It would be interesting to test flies at different times after the end of the demonstration, in

order to measure the kinetics of memory decay. Moreover, in olfactory learning, when several training sessions are presented without resting intervals (massed conditioning), flies do not form long-term memory (Tully et al., 1994). In mate-copying, we never tried to do massed conditioning, while this kind of protocol might be as naturalistic or more naturalistic as speed learning or spaced training. It might thus lead to the formation of a persistent memory like the spaced demonstrations we presented: in effect, contrary to olfactory conditioning, the demonstrations in our social learning paradigm are long and females are never forced to observe it, it is thus likely that they are not submitted to the stimuli all the time of the demonstration.

It is noticeable that however appetitive, socially learning to prefer a mate is apparently not that striking a piece of information that it can be memorized on the long term after only one demonstration. A possible explanation can come from the fact that fruit flies are conformist in their mating preferences: they copy the majority (Danchin et al., 2018). One can think that having such an ability to grasp and memorize the preference of the majority supposes that a single demonstration will not reach a threshold leading to long-term memorization. Moreover, in appetitive olfactory learning, *D. melanogaster* has good 24 h memory after a single, 2-min long training session, only if individuals are starving at the time of the test (Krashes and Waddell, 2008). In the case of mate-choice, there is no such thing as starvation, as choosing one male among others is generally not a life-or-death decision.

In a transmission chain, each observer female becomes a potential demonstrator when it then chooses a mate, creating one more transmission step. But during the night, there can be no observation, so no demonstration, and presumably very few mating as fruit flies are crepuscular animals that sleep during the night (Hendricks et al., 2000). The possibility of long-term memory in mate-copying is therefore crucial in allowing a possible persistence of mating traditions. Moreover, even during the day, environmental conditions are not always favorable to mating, in particular, if the weather is bad, courting and choosing a mate may not be a priority (Austin et al., 2014). Regarding this point, although many (if not all) experiments about mate-copying in *D. melanogaster* found a correlation between atmospheric pressure (considered as a proxy for weather) and mate-copying scores, it was never verified that the perception of unstable, decreasing or low air pressure by fruit flies was the only weather-related cause of low mate-copying scores. Testing this would be doable with the use of mutant flies (deaf flies) that do not sense pressure variations.

Memory duration is an important factor to evaluate the ecological importance of this social behavior. Further studies should specify these points, and provide a better understanding of how environmental and experimental conditions influence the strength and duration of memory in mate-copying.

Social cognition

Like many animals, fruit flies are able to behave socially, that is, to adapt their behavior to the social context. This kind of behavior involves cognitive capacities, like social information acquisition, processing, storage and retrieval. Social competence is a trait of behavioral performance that quantifies how well an individual performs a complex social task, like

choosing a mate for instance. It comprises cognitive traits as well as other traits related to any function involved in the social task (Varela et al., 2020).

Depending on the context, evolution can favor social competence or on the contrary, non-social competence, and since both are under selective pressure, it is possible that animals developed behavioral and cognitive traits that are specifically adapted to social or non-social competence. With this in mind, one can suggest that there can be specific cognitive mechanisms for social tasks that are different from those for non-social tasks (Rosati, 2017). Social and non-social cognition could involve specialized cognitive modules devoted to a particular type of task, or rather general processes that are adapted to both types of tasks – an hypothesis that has sometimes been named "associationist explanation" (Reader, 2016). The debate between these two apparently contradictory views will probably wait for the discovery of neural networks and brain structures involved in each type of learning to be closed. However, findings in one species may not be transferrable to a general knowledge of how cognitive networks are organized in other animal species.

About this debate, Cecilia Heyes (Heyes, 1994, 2012) proposed that social learning can be social at two levels: it can simply be that the learned information is provided by another individual, or it can require specialized cognitive abilities devoted to that social situation. It is likely that the first type of social learning involves general-purpose cognitive mechanisms while the second one requires specialized networks. Thus, depending on the species and on the type of social learning task, the underlying processes could be fundamentally different.

Concerning mate-copying, can we still call it "social learning" when flies copy out of a picture, or even more, out of a drawing? Finally, many questions remain unanswered without a deep jump into the neural mechanisms underlying mate-copying, and more specifically, each kind of situation in which flies are able to copy a mate preference (short or long-term memory, from live flies, photos or drawings). The true strength of *D. melanogaster* in this domain is that the mechanisms of several kinds of learning have already been precisely explored, which provides a very interesting set of genetic and technical tools, apparatuses and hypotheses to begin with.

Several species demonstrated a particular ability to learn socially: in social corvids (Templeton et al., 1999) individuals learn faster socially than individually, which is not the case in a non-social corvid species. This is also the case of chimpanzees, but not of dogs (Wobber and Hare, 2009). This shows that some species that have a high level of sociality co-evolved cognitive abilities particularly well-fitted for social learning specifically. On the contrary, in some other taxa, a social learning task is simply associative learning (Dawson et al., 2013), and a recent study modeling social learning as associative learning found that this theory could explain the emergence of most kinds of social learning (Lind et al., 2019).

Between these two cases, there are many examples of animals in which social learning is probably often more than simple association, as they can modulate their propensity to copy depending on their social relationship with the demonstrator. For instance, chimpanzees modulate their level of copying depending on the level of assumed knowledge of the demonstrator (Kendal et al., 2015). Similarly, in mice, social learning about a biting fly is modulated by kinship and by social status (Kavaliers et al., 2005): observers from the same family have higher learning scores, and individuals learn better from a dominant than from a subordinate. On the contrary, bumblebees seem to lack this capacity, which leads them to make suboptimal choices by indiscriminate copying (Avarguès-Weber et al., 2018). In fruit

flies, Anne-Cécile Dagaeff studied the effect of a genetic variation of the *foraging* gene in the demonstrator and in the observer in mate-copying scores (Dagaeff, 2015), but the results were inconclusive. In our mate-copying experiments, the observer and the demonstrator come from two different tubes, and it would thus be interesting to test if mate-copying scores are different between a situation in which observer and demonstrator are siblings, kept together from emergence, and a situation in which the two females do not know each other. As there is indication that *D. melanogaster* can recognize each other (Loyau et al., 2012), it is possible that the level of familiarity impacts the strength of mate-copying.

Finally, is it really relevant to oppose social learning and associative learning? Any learning type requires an association between several stimuli (internal or external), so even the more complex social learning imaginable involves stimuli association. The question is more about the way stimuli are processed in the brain of the animal: is there a specific network activated when the stimuli have a social component? In humans, the same two structures take part in social as well as pavlovian fear learning, but these two forms of learning differentially activate the network (Lindström et al., 2018). How is the use of this specialized network selected? And what is the advantage of having distinct networks for social and asocial learning?

Neuronal mechanisms of a social learning

Social learning has long been considered as a trait specific to "complex" animals like primates and other mammals, and eusocial insects. It has remained underexplored in all other animals for the last decades. Moreover, experiments that study social learning are often more complex to design than experiments on non-social, olfactory or visual learning. In the last decades, the number and diversity of taxa in which at least one form of social learning was found has dramatically increased. On the other hand, the term of "social learning" is really vast and gathers forms of learning involving contrasted neural mechanisms into a given species. It thus appears difficult to speak about mechanisms of social learning, one might better speak about mechanisms of *a* social learning.

In the last decades, many researchers investigated the mechanisms of different forms of social learning and social transmission in animals (reviewed in Olsson et al., 2020). Understanding social learning mechanisms is a key point in better understanding the dynamics of transmission (Reader, 2016), as the type of learning mechanisms will greatly impact the type of transmission dynamics, and in better knowing what are the required capacities to learn socially, which would broaden our view of what species can learn socially (Reader, 2016).

Concerning cues responsible for social learning, in rodents, social transmission from mother to pups of a fear response that can be memorized for days involves olfactory cues (Debiec and Sullivan, 2014), and social fear conditioning can be elicited by distress vocalizations alone (Kim et al., 2010). These cues that indicate the demonstrator's fear or

distress provoke a strong, information-specific activation of the amygdala (the center of fear in mammals) which leads to changes in exploratory behavior (Knapska et al., 2006). This emotional contagion is supposed to have evolutionary functions (Dezecache et al., 2015), as emission and reception of emotional states are costly. In the case of mate-copying, one can wonder if there is a social transmission of a positive "emotional state" between the copulating demonstrator female and the observer female. A study found that *Drosophila* is able to transmit and receive visual information about the presence of a threat (a parasitoïd wasp), even between two closely relative species (Kacsoh et al., 2018, 2019). This communication is mainly visual, and if it can work for transmitting fear signals, one can imagine that it could also be the case for transmitting pleasure signals, as choosing a good mate, like protecting its progeny from parasitoid wasps, is highly fitness-relevant. The communication of the presence of a threat involves the visual system (notably, L2 and L4 neurons from the lamina, that take part to motion detection), and region 5 of the fan-shaped body (Kacsoh et al., 2019). The method they use (selective inactivation of brain regions using the thermosensitive *Shibire* under the control of a spatially restricted Gal4 promoter) is easily transposable to our model. Interestingly, Balint Kaksoh and his collaborators also found that an artificial activation of the brain regions involved (with TrpA1) could accelerate learning. It would be interesting to elaborate similar experiments in mate-copying, by silencing or activating the same brain regions during the mate choice demonstration, and measure effects on mate-copying scores.

Brain structures involved in social fear transmission were described in rodents (Olsson et al., 2007; Twining et al., 2017; Allsop et al., 2018) and primates (Burgos-Robles et al., 2019). In humans, a study revealed that individuals with autism spectrum disorder and normal IQ had a different pattern of neural activation compared to neurotypical controls in a social learning task, although their performance in solving the task was similar (Schipul et al., 2012). In drosophila, some sensory signals are conveyed to higher brain centers by a different, overlapping circuit depending on stimulus intensity (Lin et al., 2013). On the other hand, memories acquired through different sensory modalities can share neural circuits (Vogt et al., 2014). The correspondence between sensory modalities / type of memory and brain structures or neural circuits involved is thus highly complex, especially in mini brains like those of Insects that evolved an economical design of brain circuits.

Social learning –as any form of learning– can trigger neurogenetic changes in the brain structures involved in the learning task (Cui et al., 2017). It would be interesting to carry transcriptomic analyses on different regions of the observer fly brain after the mate-copying demonstration (particularly after the five spaced demonstrations of the long-term memory protocol, as stable memories require changes in gene expression). This could reveal two pieces of information: which structures are involved in this social long-term memory, and which genes have modulated expression.

In appetitive learning, social transmission of food preference in rats requires muscarinic transmission in the basolateral amygdala (Carballo-Márquez et al., 2009). We can make a parallel between this social learning and mate-copying, as in both cases, a preference is elicited in the observer by cues about the demonstrator's choice. There is no known equivalent to the mammal's amygdala in fruit flies, but they do have muscarinic neurotransmission: it is notably involved in olfactory learning (Bielopolski et al., 2019), and modulation of muscarinic reception in mushroom bodies can enhance or suppress olfactory

learning (Gai et al., 2016). It would be interesting to explore the function of cholinergic transmission in drosophila mate-copying.

Mate-copying as a form of associative learning

Mate-copying is an observational learning in which the visualization of a female copulating with a male of a given phenotype elicits a preference for this particular male phenotype. In the demonstration, the two important elements are the copulating female, and the phenotype of the male. As written in the introduction, the male phenotype could be a conditioned stimulus mediated by the visual pathway, while the copulating female would constitute the unconditional, appetitive stimulus, and would involve dopaminergic pathway and visual pathway.

At first sight, one could propose that mate-copying is an appetitive associative learning, however my results of the neuronal blockade experiment showing that TH neurons, but not Ddc neurons, are required in this learning apparently go against this hypothesis. In this experiment, observer flies are submitted to a temperature shift during the demonstration. In olfactory aversive learning, the aversive cue can be a temperature of 34°C (Galili et al., 2014) so in the neuronal blockade experiment the demonstration could be considered as mediating an aversive cue because of the temperature shift. This makes the fact that Ddc>Shi(ts) females learn a bit surprising, as one could assume that presenting the demonstration together with an aversive cue would not elicit a preference for a given phenotype, or could even elicit an aversion. But maybe the appetitive valence of observing copulation overcomes the aversive value of the heat stimulus. Indeed, submitting TH-Gal4>UAS-Shi^{ts} flies to 34°C did not affect appetitive memory in an experiment studying the roles of TH neurons in appetitive and aversive olfactory learning (Schwaerzel et al., 2003). This result indicates that temperature is not a sufficiently aversive cue to prevent appetitive learning.

It would be interesting to submit observer females to appetitive or aversive stimuli during the demonstration and measure effects on mate-copying scores. For instance, we can imagine that an electric shock could impair (or reverse) the preference, depending on its intensity, while an appetitive stimulus like sugar could increase the scores or increase memory duration. This would allow studying how different learning modalities can interact with each other.

Maybe, from a flie's point of view, mate-copying from pictures is not exactly the same process as mate-copying from live demonstrators. It is possible that both share many common characteristics but present tiny differences linked to the fact that live flies offer a social situation that pictures do not. Exploring these differences could teach us a lot on the specificities of social learning.

Future directions

This thesis opens many new perspectives of research. Concerning the cognitive mechanisms of mate-copying, I brought first elements and raised intriguing points, in particular, with the discovery that the dopaminergic neurons involved in mate-copying are not those required for appetitive olfactory learning. Thus, it would be interesting to first test TH>Shi(ts) females at 25°C to make sure that they can learn and rule out a problem with the strain. This step being fulfilled, the use of more precise Gal4 drivers would allow refining the group of dopaminergic neurons required for mate-copying in a speed learning design. A final and very elegant experiment would then be to activate these neurons with optogenetics while presenting a male of a given color, and then test the preference of the female. Optogenetic tools have been developed in *D. melanogaster* in the past years (Dawydow et al., 2014) and allow evoking neuronal activity using a light beam. This neuronal activity can be restricted to the desired region using the UAS-Gal4 system to drive the expression of "ChR2-XXL" transgene, which is a mutant form of channelrhodopsin-2 providing very good results in living drosophila (Dawydow et al., 2014). With this tool, it would be possible to activate the neuronal activity of the specific dopaminergic neurons identified as necessary for short / mid-term memory in mate-copying, to provide the unconditional stimulus while presenting a male of a given color.

The localization of DAMB receptor required for long-term memory formation in mate-copying still has to be discovered. The use of picture demonstrations, and of the *damb* mutant with re-expression only in precise regions of the mushroom bodies or the central complex, should greatly help in this exploration, but it would be useful to verify at the end of the experiment that the results are the same when demonstrations involve real flies, by testing in a protocol using live demonstrations the genotype that re-expresses DAMB in the region involved in social LTM with pictures.

Concerning pictures, their use could be strength not only in the discovery of neural mechanisms, but also as stated above, in the exploration of the cues necessary in species recognition. One can also imagine using the same kind of stimuli in other contexts, like preference for egg laying sites: if females observe pictures of flies around or on a substrate, will this increase the chance that they select it for egg laying compared to another one? The comparative exploration of the neural processes involved in a type of learning using pictures versus using live flies might also reveal interesting differences. We can then imagine replacing pictures with videos to observe the effect of motion in the artificial stimuli.

Another interesting avenue that I opened is the effect of phenotype commonness, as this is indeed a crucial parameter for tradition in a population. Experiments in the hexagon could be a good way to start exploring this effect, first by observing the responses to a gradient of phenotype commonness for a given color preference. The use of pictures in this kind of experiments could also help.

Concerning the occurrence of mate-copying in wild populations, exploring this field could help better understanding the evolution of the species. We could test several species of *Drosophila* for existence of mate-copying (particularly long-term memory in mate-copying), and do a comparative genomic and/or transcriptomic analysis of the species in which this behavior is present versus absent. This would give exciting insight into the evolution of this behavior in an insect species.

More generally, in this work I tried to consider mate-copying on two different but complementary aspects: ecology and evolution on the one hand, and molecular and cellular processes on the other hand. These two aspects, macroscopic and microscopic have historically been too often considered separately, while it is much more enriching to consider them altogether, as they are in close link. Recent and current works are progressing in this direction.

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