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Dissertação

Mestrado em Biologia Evolutiva e do Desenvolvimento

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Abstract

Non-genetic information has been classically overlooked in the light of evolutionary processes. It was shown, however, that non-genetic information can also be transmitted inter generationally, thus being a potential source of evolutionary change. Social information, a form of non-genetic information, can lead, through social learning, to cultural inheritance. Culture can be defined as the socially inherited fraction of the phenotypic variance. Four criteria were proposed to test cultural inheritance of a trait: social learning, inter-generational transmission, durability and generalization. The first and fourth criteria were already verified for mate-choice copying in *Drosophila melanogaster.* Mate-choice copying occurs when observer females change their initial mating preferences for those of other females. The main goal of this work was to test the fulfillment of the third criterion in this context: socially acquired information must last long enough to be transmitted to other individuals. It was thus attempted to verify if female copied preferences are maintained for at least 5 hours. Furthermore, an alternative to classic testing of female mate-choice copying was investigated. The aim was to find a positive correlation between affiliative and mating behaviors, thus allowing for the multiple testing of the same females, this correlation was not found so the analyses of the females' copying behavior depended on their mating decisions. The main experiment was not conclusive relatively to the occurrence of mate choice copying, although this behavior has been already verified in the same line of flies. This discrepancy is probably due to small sample size. It was found however that the proportion of correct choices did not vary between test times, a good indicator that social information might last at least 5 hours. Further experiments are needed to draw more conclusive interpretations, but results here obtained do not reject the hypothesis, providing preliminary evidence that the third criterion can be verified for mate-choice copying in *D. melanogaster.*

Key words - Social information, Mate-choice copying, Cultural evolution, *Drosophila melanogaster*, Social influence durability.

Resumo

A Vida é um fenómeno de transferência de Informação entre níveis de organização não-redundantes. Esta transferência de informação pode ocorrer tanto de uma forma horizontal (intrageracional) como vertical (intergeracional). A transferência de informação não-genética, tem sido classicamente considerada como pouco relevante para os processos evolutivos, por oposição à informação genética. No entanto muitos autores têm reconhecido que a informação não-genética pode ser também transferida entre gerações (e.g. Dawkins 1976). Entre esta pode referir-se a informação social - informação adquirida através de outros indivíduos, que, por aprendizagem social pode conduzir à hereditariedade cultural (ou social). Foi proposta uma reformulação da genética quantitativa clássica de forma a incluir formas de informação não-genética no cálculo de heritabilidade. Em substituição da divisão clássica da variância fenotípica numa componente genética e outra não-genética (e a interacção entre as duas), a variância fenotípica passa então a ser dividida numa componente transmitida e uma componente não transmitida, sendo que a primeira inclui tanto informação genética como não-genética. A cultura é definida neste esquema como a parte da variância fenotípica transmitida que é herdada através de aprendizagem social. Para identificar as características que são sujeitas a evolução cultural foram propostos quatro critérios: (1) deve ocorrer aprendizagem social; (2) transferência de informação deve ocorrer também de forma intergeracional; (3) a influência social deve durar tempo suficiente para os seus efeitos serem observados por outros; (4) a influência social deve ser generalizável.

A escolha de parceiro reprodutivo é um fenómeno no qual a influência social foi já demonstrada em diversas espécies de peixes, aves, mamíferos (incluindo humanos), embora o cumprimento simultâneo dos quatro critérios nunca tenha sido testado. Mery & Varela *et al.* (2009) descreveram pela primeira vez num invertebrado, *Drosophila melanogaster, a* existência de cópia da escolha de parceiro reprodutivo, bem como o cumprimento do primeiro e quarto critérios de evolução cultural.

O principal objectivo deste estudo foi então testar o cumprimento do terceiro critério de evolução cultural, no âmbito das preferências sexuais de *Drosophila melanogaster.* Este é um dos dois critérios ainda por verificar e está directamente implicado no cumprimento do segundo critério - transmissão intergeracional. Para tal o trabalho de Mery & Varela *et al.* (2009) foi adaptado de forma a permitir testar a durabilidade da informação adquirida socialmente e tentar verificar se a informação social é retida o tempo suficiente de forma a poder ser transmitida a outros indivíduos.

O protocolo utilizado por estes autores consiste em apresentar a fêmeas virgens as escolhas de parceiro reprodutor por outras fêmeas. Nestas demonstrações são utilizados machos de fenótipos artificiais distintos, corados com pós de cores diferentes. As fêmeas observadoras obtêm informação positiva sobre a atractividade dos machos que vêem acasalar e informação negativa sobre os machos que vêem ser consistentemente rejeitados. Para as demonstrações positivas, é colocado num

compartimento adjacente ao da fêmea observadora um macho com uma fêmea virgem, que está disposta a acasalar. Nas demonstrações negativas, um macho de fenótipo diferente é colocado com uma fêmea que acasalou previamente, uma vez que as fêmeas de *Drosophila melanogaster* exibem um período refractário após o acasalamento, durante o qual rejeitam outras tentativas de acasalamento, providenciando assim informação negativa sobre esse macho. Três conjuntos de cada tipo de demonstração são apresentados de forma alternada e no final a fêmea observadora é colocada juntamente com uma díade de machos de fenótipos diferentes, sendo registado com qual destes ela acasala. Os autores observaram que ocorre cópia da escolha de parceiro reprodutivo (*mate choice copying*) em *Drosophila melanogaster*: as fêmeas observadoras exibem uma preferência por machos do fenótipo que viram a acasalar, em detrimento de machos do fenótipo rejeitado.

Numa primeira fase deste estudo, enquanto experiência preliminar, procurou-se encontrar uma alternativa ao teste de preferência utilizado previamente, contabilizando se o tempo passado perto de cada macho poderia estar correlacionado com a preferência da fêmea. O objectivo era poder testar repetidamente a mesma fêmea observadora, sem o constrangimento do período refractário, para averiguar se as preferências se mantêm ao longo do tempo. Por outro lado, esta alternativa permitiria também evitar factores de confusão relacionados com a competição entre os machos que ocorrerá durante a fase final do teste. Para tal colocou-se uma fêmea num pequeno tubo e, em tubos adjacentes separados por uma partição transparente, dois machos de fenótipos diferentes. Este sistema era depois filmado durante 10 minutos e a localização da fêmea registada (do lado esquerdo, direito ou no centro do compartimento). Após esse período as partições eram retiradas e observava-se com qual dos machos a fêmea acasalava, correlacionando esta escolha com o tempo passado junto de cada macho. Enquanto controlo, foram realizados ensaios em que os machos foram substituídos por fêmeas e ainda outros em que a fêmea estava sozinha no sistema, tendo sido registado de igual forma o tempo passado de cada lado do compartimento. Os resultados obtidos não revelaram a existência de uma correlação entre o tempo dispendido pela fêmea junto de cada macho e a sua escolha.

As experiências de durabilidade foram então conduzidas utilizando o protocolo original, uma vez que não foi possível desenhar outro protocolo a partir dos resultados obtidos na primeira experiência. No entanto, o protocolo original foi modificado de forma a existirem dois momentos de teste distintos, logo após as demonstrações ou apenas 5 horas mais tarde. As fêmeas eram distribuídas aleatoriamente em dois grupos a serem testados num dos dois momentos. Por outro lado, procurou-se diminuir o tempo das demonstrações e aumentar o rendimento dos ensaios. Assim, em vez de uma hora, as demonstrações passaram a durar apenas 20 minutos e, para as demonstrações positivas, passou a transferido para o sistema um casal já em cópula. Desta forma foi também possível reduzir o tempo que as fêmeas permanecem no sistema experimental, sem acesso a alimento. Esta segunda experiência não obteve

resultados conclusivos relativamente à existência de cópia da escolha de parceiro reprodutivo, embora este fenómeno tenha sido já observado neste sistema, utilizando a mesma linha de moscas. A ausência de resultados conclusivos pode dever-se por um lado ao menor número de dados obtidos, relativamente à amostra utilizada pelos outros autores, ou ainda às alterações que foram efectuadas no protocolo original (diminuição da duração das demonstrações). Relativamente à influência do tempo na cópia das fêmeas, não se encontraram diferenças significativas entre os dois momentos de teste, o que é uma boa indicação de que a informação adquirida socialmente poderá ser retida durante pelo menos 5 horas.

No geral, os resultados obtidos não rejeitam a hipótese de que o terceiro critério é cumprido no contexto da cópia da escolha de parceiro reprodutivo em *D. melanogaster.* Assim, embora sem resultados conclusivos, este estudo põe em evidência a necessidade de efectuar mais experiências nesta linha de investigação. Por um lado é necessário recolher um maior número de dados de forma a confirmar as tendências reveladas neste trabalho e, caso seja confirmada a retenção da informação social durante as 5 horas, testar intervalos de tempo maiores. Por outro lado, é também pertinente testar alternativas ao protocolo utilizado, no seguimento da experiência preliminar. A confirmação do terceiro critério de evolução cultural é uma condição para que o segundo critério - transmissão de informação social entre gerações – também se cumpra. Assim, torna-se relevante explorar mais pormenorizadamente a durabilidade de informação social de forma a poder desenhar um protocolo para o teste do segundo critério e observar o cumprimento de todos os critérios, que revelaria a existência de evolução cultural no contexto da cópia da escolha de parceiro reprodutivo em *Drosophila melanogaster*.

Palavras chave - Informação social, Cópia da escolha de parceiro reprodutivo, Evolução cultural, *Drosophila melanogaster*, Durabilidade de influência social.

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Introduction

Life is a phenomenon of information transfer across non-redundant organization levels (Laborit 1995). This information transference can occur both horizontally, (intra generationally), and vertically, from parents to offspring (inter generationally) (Dudai 1989, Avital and Jablonka 2000, Laland *et al.* 2000, Galef and Giraldeau 2001, Danchin *et al.* 2011). Different types of biological information exist, the main division line occurring between the information that is transmitted through the nucleic acids (genetic), and every other kinds of information that do not involve nucleic acids (non-genetic) (Danchin and Wagner 2010). Genetic information has been typically regarded as the only form of relevant information for evolution (e.g. Andersson 1994; Andersson & Simmons 2006). However, various forms of non-genetic information have also been recognized as being inherited across generations, hence potentially effecting evolutionary processes (Coleman 1977, Danchin *et al.* 2004, Mameli 2004).

In changing environments, genetic information is frequently not sufficient to perform adjustments and living organisms are thought to use other forms of information in fitness affecting decisions (Calow 1976). Indeed, other individuals' interactions with the environment – classically known as public information (Valone & Templeton 2002; Danchin *et al.* 2004), but more recently referred to as social information (Danchin *et al.* 2008, Wagner & Danchin 2010) – is one type of non-genetic information that is broadly used by animals during foraging, habitat and mate-choice decisions (see reviews in Galef & Giraldeau 2001, Valone & Templeton 2002, Danchin *et al.* 2004, Danchin *et al.* 2008).

In particular, the use of social information is related to the cognitive process of social learning. Although there is considerable semantic debate about the term social learning, it is used here in its broadest sense, that is, when «learning is influenced by observation of, or interaction with, another organism (typically conspecifics) or its products» (Leadbeater & Chittka 2007). In this broad meaning, social learning includes a series of cognitive processes such as imitation, copying, imprinting and teaching, among others. Social learning is now thought to be a major evolutionary process as it has the potential to lead to another form of non-genetic inheritance, often called cultural (or social) inheritance (Danchin *et al.* 2004, 2011). Social inheritance comprises all the situations in which information is somehow exchanged among individuals by social learning (Dawkins 1976; Danchin & Wagner 2010).

How can social learning lead to a form of non-genetic inheritance?

The study of the importance of non-genetic components of inheritance to evolution requires the development of an evolutionary framework that would include both genetic and non-genetic components of inheritance (Dawkins 1976, Danchin *et al.* 2004, Mameli 2004, Danchin & Wagner 2010, Danchin *et al.* 2011). In quantitative genetics, however, variation is classically studied by splitting phenotypic variance into different components, in order to filter the genetic component of the variation among individuals. The idea is to extract the part of the phenotypic variance that is transmitted across generations, as this is classically considered the only part that can lead to evolutionary change.

Phenotypic variance (VP) is, thus, classically partitioned into its genetic and environmental components (VG and VE), plus their interaction (VG*E), (Eq. 1). Genetic variance (VG) is further partitioned into three components: the variance due to dominance effects (VD), epistatic interactions among *loci* (VI) and additive genetic variance (VA). VD and VI result from gene associations that are irrelevant for reproduction because they are not transmitted as such to the progeny. Only the additive component of genetic variance, the sum of the small co-adapted effects of all the genes acting on a trait, is transmitted as such to the offspring. Geneticists thus define narrow sense heritability (h^2) as the ratio VA/VP, which is the part of phenotypic variance that is genetically transmitted. This definition assumes that the contribution of VE can be reduced to 0, and more importantly, assumes that only genetic inheritance matters in evolution (Falconer & Mackay 1996).

$$V_P = V_G + V_E + V_{G*E} \qquad \textbf{(1)}$$

$$h^2 = \frac{V_A}{V_P} \qquad \textbf{(2)}$$

However, this assumption may be viewed as a reductionist approach, because, as Darwin and Wallace stressed more than 150 years ago, the evolutionary potential of a trait depends on the part of the phenotypic variance that is transmitted. This statement is true independently of the mechanism of transmission. It can be genetic and non-genetic, as other mechanisms may also be implicated in parent-offspring resemblance. One of these mechanisms is most probably social learning, since it may lead offspring to behave as their parents. Hence, it was recently proposed to decompose phenotypic variance in a different way. Instead of the classical distinction between genetic VG and environmental variance VE (Eq. 1), VP should be partitioned into transmitted VT and non-transmitted variance VNT (Eq.3). VT includes not only genetic, but also any form of non-genetic information that is inherited (Fig. 1). The idea is that to estimate the full evolutionary potential of a trait, all components of phenotypic variance that can be inherited across generations should be considered. The term Inclusive heritability (IH^2) was then proposed in order to encompass the effect of all forms of inheritance that may affect evolution (Eq. 4) (Danchin and Wagner 2010, Danchin *et al.* 2011).

$$V_P = V_T + V_{NT} \tag{3}$$

$$IH^2 = \frac{V_T}{V_P} \tag{4}$$

VT thus comprises different inheritance systems: epigenetic, parental effects, habitat inheritance and social inheritance. Each of these systems has different properties and must be studied in order to distinguish their direct and interacting effects in evolutionary changes.

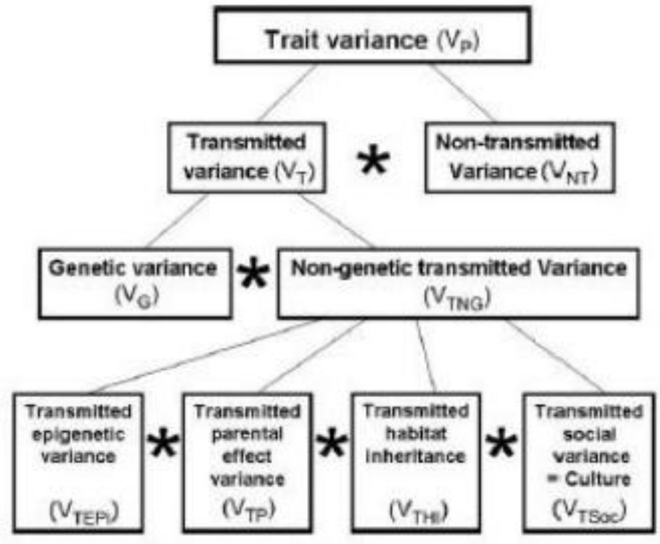


Fig. 1 Alternative decomposition of phenotypic variance (extracted from Danchin & Wagner 2010)

What is culture for an evolutionary biologist?

This inclusive view of heritability leads to a basic definition of animal culture, which could be defined as the part of the phenotypic variance that is inherited through social learning (Dawkins 1976; Danchin & Wagner 2010). One of the most original characteristic of culture, in opposition to genetic variance, is that cultural transmission can occur not only vertically (from parents to offspring), but also horizontally (among individuals of the same generation) and obliquely (from older, non-parental, to younger individuals). Oblique and horizontal transmissions, which are supposed to be inexistent in the genetic inheritance of eukaryotes, imply that cultural innovations can spread much faster than genetic innovations (i.e. mutations) (Dawkins 1976, Danchin *et al.* 2004).

Although here referred in an evolutionary perspective, the study of cultural inheritance has been dominated by human sciences, and, as such, the bulk of the works published so far on non-human cultural evolution consists of theoretical models

(Boyd & Richerson 1983, Feldman & Cavalli-Sforza 1984, Laland & Brown 2006). There is a lack of uniformity in experimental research that is related to the lack of operational tools to study this subject in non-human animals. In order to overcome this flaw, a set of four testable criteria was proposed to identify behavioral traits potentially culturally inherited (Danchin & Wagner 2010).

For a trait to be considered at least partially culturally inherited, the following conditions must be fulfilled: (1) the trait must be socially learned, (2) it must be transmitted across generations, preferably from older to younger individuals. The latter condition implies that (3) the modifications in the phenotype induced by social learning must last long enough to allow it to be observed and learnt by other individuals, and (4) inherited social information must be generalized and used in different contexts. The first two criteria are the most important (Danchin & Wagner 2010).

As stated above, the first criterion, social learning, has been consistently reported in several contexts, such as foraging, habitat and mate-choice (e.g. Laland & Williams 1997, Doligez *et al.* 2002, Dugatkin *et al.* 2003, Witte & Noltemeier 2002).

Although first thought to be restricted to vertebrates, social learning can also occur in invertebrates (Coolen *et al.* 2005, Leadbeater & Chittka 2007, Mery & Varela *et al.* 2009). There are some behavioral traits which are, most likely, subjected to cultural evolution, such as the dialects of songbirds and cetaceans and the tool manufacturing and use in primates and birds (Rendell & Whitehead 2001, Van Schaik *et al.* 2003, and Hunt & Gray 2003). However, even for those traits, the simultaneous fulfillment of the four criteria has not been demonstrated yet. It is thus necessary to start the study of animal cultural evolution recurring to the recently presented conceptual tools, paving the way to future work on the impact of cultural transmission on species ecology and evolutive trajectories (Danchin *et al.* 2011).

Mate choice copying: a matter of culture?

In the present work, mate-choice copying in *Drosophila melanogaster* was studied in the context of cultural evolution. Mate-choice copying is a type of social information use that occurs when observing individuals learn the mating choices of other (model) individuals and change their mate-choice behavior accordingly. The use of social information for mate choice appears to be more common in females - though it has been also reported in males (in fish: Schlupp & Ryan 1997, Widemo 2006, Plath *et al.* 2009). Mate-choice copying has thus been classically defined as a situation where the probability of choice of a given male by a given female is either greater or less than the absolute probability of choice depending on whether the male mated previously or was avoided, respectively (Pruett-Jones 1992, Galef & White 2000).

Since the first and fourth criteria of culture have been tested already in mate-choice copying experiments with fruit flies (Mery & Varela *et al.* 2009; see more details below), the goal here was to test whether female copying of mating preferences in this species verifies the third criterion, which is about the durability of the social influence. In the future, the verification of all four criteria in *D. melanogaster* would imply that cultural transmission is not limited to vertebrates but can also affect the evolutionary pathway of a vast array of animal taxa (Griffin 1976, Danchin *et al.* 2011).

The choice of reproductive partners – i.e. mate choice – exists probably in most sexually reproducing animals, and should have a considerable impact on fitness (Janetos 1980). Three sources of information have been proposed to affect the choice between mate alternatives: (1) individual assessment through sensorial systems (genetic information); (2) former individual experience (personal information) and (3) social information derived from other individuals' choices (social information) (Wagner & Danchin 2010). The use of social information in the context of mate choice is considered as a complementary strategy, rather than an alternative one, to the use of personal or genetic information (Sirot 2001). Social information should have more influence on mating decisions with the decreasing quality of the other types of information. In this way, copying is thus to be more likely when the alternative mates don't show marked differences (Godin & Dugatkin 1996, Dugatkin & Godin 1992). Additionally, the importance of social information is more considerable when reproductive experience is smaller. Young individuals are more likely to copy others, choosing to copy old, rather than young conspecifics and older individuals in general don't copy the choices of younger ones (Dugatkin & Godin, 1993, Castellano *et al.* 2012).

Two different explanations have been proposed for the evolution of copying, in the context of mate choice. The first is cost avoidance, as the assessment of the quality of potential mates involves costs such as time, energy, increased risk of predation and exposure to sexually transmitted parasites. This explanation has been empirically explored in a few studies, but so far neither its confirmation nor its exclusion has been achieved (Briggs *et al.* 1996, Dugatkin & Godin 1998). The second hypothesis focus on the uncertainty and error component of the mate assessment process and considers copying as an adaptation to facilitate discrimination. As individuals estimate mate quality via various physical, behavioral and social cues, these estimates are subjected to errors that could be diminished by the observation of other individuals' choices. This is particularly relevant for sexually inexperienced individuals, whose evaluations of mate quality have usually a higher error component, and also when alternative mates are phenotypically resemblant (Gibson & Höglund 1992). Even if poor decisions can be sometimes copied, copying is usually a better alternative than random choice in these situations (Wade & Pruett-Jones 1990, see review in Varkitzis 2011).

Mate-choice copying has already been found in a diverse array of organisms, such as: fishes (Dugatkin 2003, Witte & Noltemeier 2002), birds (Galef & White 2000), fruit flies (Mery & Varela *et al.* 2009) and humans (Waynforth 2007). Mery & Varela *et al.* (2009) were the first to describe mate-choice copying in an invertebrate: the fruit fly, (*Drosophila melanogaster*). In their work, the fulfillment of the first and fourth criteria of cultural evolution is also demonstrated. These criteria correspond, respectively, to the social learning ability and the capacity to generalize social information in different contexts. In one of the experiments, virgin females were presented with two artificial male phenotypes (colored with pink and green powders) and could observe one type of males successfully copulating, while males from the contrasting phenotype were consistently rejected. After the demonstrations, the females were allowed to choose between males of each phenotype. The results demonstrate that observer females relied on social information and thus showed a preference for the "successful" males that were accepted by other females. The fulfillment of the fourth criterion was also demonstrated, given that females displayed a preference for a phenotype and not the particular individuals. In a second experiment, social learning was demonstrated as well by showing that females, after having observed poor condition males being preferred for copulations by demonstrator females, changed their initial mating preferences for the good condition males to those in poor condition. This shows how the genetic information of an individual can be overridden by social information when there is inconsistency between the two types of information (Mery & Varela *et al.* 2009, Danchin *et al.* 2010).

Learning and memory in *D. melanogaster* – durability of social influences

The assessment of the durability of social information is very important as the modifications in the phenotype induced by social learning must last long enough to allow it to be observed and learnt by other individuals.

The study of learning and memory in Drosophila has been using mostly olfactory classical and operant conditioning protocols. Within this line of research, four main categories of memory have been described, with decay rates ranging from 1h to a week or more. They are the short-term memory that decays over 1 hour, mid-term memory that decays over 4-5 hours, anesthesia-resistant memory, lasting up to 4 days, and long-term memory that can last a week or even longer. These different types of memory are associated with different pathways that can involve, or not, protein synthesis (Dubnau & Tully 1998, Yurkovic *et al.* 2006). Also, for visual learning, three categories of memory have been reported, analogous to the ones described for olfactory conditioning (Xia *et al.* 1997, Siwicki & Ladewski 2003).

Even if these results are not relative to social learning, nor to the copying behavior, in particular, the fact that flies can retain information learnt in conditioning protocols from 1 hour to a week can be used as an indicator in studies related with the durability of social information in Drosophila mate-choice copying behavior, even if learning in social contexts is more complex. It possibly involves multiple sensory modalities and elements of both classical and operant conditioning (Yurkovic *et al.* 2006). The goal of the present project was to put in place this type of experiment, by testing female flies' mid-term mate-choice copying memory. According to the mate-choice copying hypothesis and to the previous study of this phenomenon in *D. melanogaster* (Mery & Varela *et al.* 2009), it was expected to find evidence of copying – and, by consequence, of social learning – in the studied population. Complementarily, according to the durability criterion of the cultural evolution hypothesis, it was predicted that mate-choice copying could be found even after five hours (mid-term memory) from the transfer of social information.

Methods

Culture stocks

All experiments were conducted using a population of wild type *D. melanogaster*, derived from flies caught in Chavroche (France), in 2007. Eggs, larvae and adult flies were maintained at 18-22^{o}C, at approximately 60% of humidity and a 12:12-h day/night cycle – the experiments being conducted in the same conditions. The stock population and the individuals used in the experiments were maintained in 8mL vials containing sugar-agar-yeast medium. All flies were manipulated by aspiration, without recurring to any anesthesia type, as it has been shown to affect both memory and normal mating behavior of the flies (Xia *et al.* 1997, Barron 2000). Virgin males and females were collected on the day of eclosion and kept in separate vials, with approximately 10 individuals each, for 5 days.

Climatic variables

Throughout all experimental work, several climatic variables (temperature, humidity and atmospheric pressure) were registered, two times a day (morning and afternoon), with the aid of a mobile meteorological station. Although the room where the fly stocks were maintained and the experiments conducted had controlled temperature and humidity (only the atmospheric pressure could not be controlled), there was always some fluctuation of these variables. The data obtained was used in order to control for the effect of any of those variables in the results obtained, as fruit flies are small-bodied poikilotherms and thus extremely sensitive to climatic variables, such as temperature and humidity (Sayeed & Benzer 1996). In *Drosophila subobscura* it was found that atmospheric pressure might also have a major influence in mating behavior (30% of the variation in number of copulations was attributable to atmospheric pressure) (Ankney 1984). The inclusion of climatic variables in behavioral studies is thus pertinent, even when experiments are conducted in relatively controlled environments. Atmospheric pressure is not usually controlled but it should also be monitored, as it is related to wind velocities and thus small flying organisms, such as fruit flies, can be expected to be also sensible to this variable.

Experimental set-up and the original protocol

The experimental set-up consisted of three transparent plastic vials of approximately 3 cm long and 1 cm of diameter that provided three compartments, separated by two removable transparent microscope cover glasses (Fig. 2).

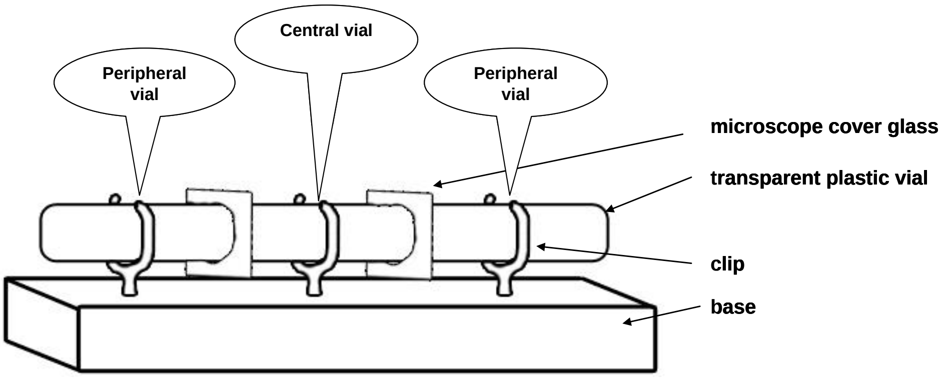


Fig 2. Experimental set-up

As already referred in the introduction, the protocol used in Experiment 1 in Mery & Varela *et al.* (2009) was adapted for this project. This protocol will be described here and the included changes presented afterwards.

In this protocol a virgin observer female, placed in a small plastic tube (the central vial in Figure 2), was given the opportunity to observe the mating choices of other females. The demonstrator couples were placed in a second tube (on one of the peripheral tubes in Figure 2), adjacent to first one and separated from it by a microscope cover glass. Two distinct male phenotypes were created by artificially dusting them with pink or green powders. These males were then used alternately in two types of demonstrations: positive and negative. For positive demonstrations, a male of one phenotype was placed, for one hour, with a virgin female that was receptive for copulation. The occurrence of copulation during this period provided positive social information about the male attractiveness to the observer female. In a second demonstration step, the pair of demonstrator flies was replaced, for another hour, by a male from the contrasting phenotype and a recently mated female. Recently mated females are obtained by placing a virgin female in a vial with two males, prior to the experiment, for at least one hour. Because female Drosophila refuse copulations after recent matings (Barnes *et al.* 2008), these demonstrations provide negative social information about that male. This two hour sequence is repeated two more times, for a total of three positive and three negative demonstrations, performed in an alternate fashion. In the seventh hour, mate-choice copying is tested. In this test phase, two males, one from each phenotype, are placed in the peripheral vials, one on the left side and the other on the right side. The glass partitions are then removed and the mating choice of the observer female is recorded.

Experiment 1: Alternative protocol to assess female preference

When accounting for the durability of the social information in female mate choice behavior, two types of preference tests can be performed: (1) by repeatedly testing the mate-choice copying behavior of each observer female at various time points after the demonstration steps; or (2) by testing each female only once, with groups of females being randomly tested at different time points. The second alternative is more resource and time consuming, since two samples of observer females have to be tested simultaneously. For the first alternative, only one sample is needed, but the existence of a refractory period in recently mated females renders it impossible to test them repeatedly, as preference is obtained by registering which male copulates with the female. Furthermore, in the original protocol, the copulation of the female with one of the males during the final test phase is viewed as a sign of her preference. However, as both males are able to interact with the female and with each other as well, male competition and male choice can also be affecting the outcome of the test. To avoid the confounding effects that these phenomena may have, but to allow, at the same time, for the multiple testing of individual females, it was necessary to search for another behavior that would equally positively correlate with female choice. In organisms like fish and birds (Dugatkin *et al.* 2003, Cummings & Mollaghan 2006, Hoi & Griggio 2011) it was already verified that the affiliation time (the time spent next to each male, but without contact and consequently without copulation) can be used as a preference measure, thus acting as a proxy for mate choice without the need for actual mating.

In this first experiment, the objective was, therefore, to test if the same was true for *D. melanogaster*, that is, if the amount of time spent by the female next to each male before copulation correlates positively with its mating preference. In this experiment, 5-days-old virgin female flies were placed in the central compartment of the experimental apparatus and they were taped for 10 minutes, using a digital video camera. The central vial of the experimental set-up (Figure 2) was previously divided in three sections (each 1 cm long) using a permanent marker. In the peripheral compartments were placed virgin males previously colored with pink and green powders. The videos obtained were divided in approximately 300 frames each and in each frame it was recorded the position of the female in the compartment (left, right or middle sections). At the end of the 10 minutes, the glass partitions were removed and the female was left to interact with the males for as long as 1 hour. The color of the male with which the female mated was also registered. The objective was thus to test if the proportion of time spent by the female in the section next to each male correlated with the color of the chosen male.

Two controlling experiments were also conducted, placing females instead of males in the peripheral compartments, or leaving them empty, and the observer female was similarly recorded for 10 minutes. These trials had the objective to analyze differences of behavior according to the occupation of the peripheral compartments: it was expected that females would spend more time in the right and left sections if peripheral compartments were occupied by conspecifics, rather than empty, and that there should be an increase in this proportion if these were males.

Experiment 2: Testing the third criterion of cultural evolution

To test the fulfillment of the third criterion of cultural evolution, it is necessary to determine for how long the social influence induced in female mate-choice can last. According to the cultural evolution theory, a behavioral trait can be further transmitted to other individuals only if its influence lasts for a considerable fraction of an animal's life. In the present work, the original protocol used by Mery and Varela *et al.* (2009) described above was modified to test the durability of the social influences in D. melanogaster. Also, according to the results from experiment one – no correlation was found between the females' affiliation and mating behaviors (see the details in the Results' section) –, the test phase was conducted according to the second type of preference test (described above): the observer females were divided in two groups, those from one group being tested ready after the demonstration phase, and the others being transferred to separate vials with nutritional medium and tested only 5 hours after (mid-term memory).

Further modifications to the original protocol were conducted in order to reduce the time spent by the observer females in the experimental set-up, as the original protocol required the females to spend 6 hours watching the demonstration sequence, without nutritional medium, before the test phase: both positive and negative demonstrations were thus shortened to 20 minutes, as the duration of copulation in *D. melanogaster* is commonly between 15 an 25 minutes (MacBean & Parsons 1967). To respect the 20 minutes interval, positive demonstrations were controlled by introducing in the peripheral vial already copulating couples, which were then watched by the observer female for at least 10 minutes. The couples were removed as soon as copulation ended to prevent the observer females from watching the rejections of further copulation attempts by the males. Therefore, positive demonstrations had a variable duration of 10 to 20 minutes and negative demonstrations an invariable duration of 20 minutes.

Statistical Analysis

All statistical analyses were performed using Microsoft Office Excel 2007 and StatsoftStatistica 8.0.

In all statistical tests the alpha-level for significance is 0.05.

Experiment 1: Alternative protocol to assess female preference

For the analysis of the first set of experiments, two distinct dependent variables were used: the proportion of frames spent in the right side of the compartment relative to the total number of frames (R/Total) and the proportion of time spent in the right side, considering only the time spent in the right and left side of the compartment, excluding thus the time spent in the middle(R/R+L). Both variables are continuous and have a normal distribution (R/Total: Shapiro-Wilk W=0.99, p=0.41; R/R+L: Shapiro-Wilk W=0.98, p=0.26).

Data were analyzed using the General Linear Model procedure (GLM), including categorical (the Design and Male Colour variables) and continuous predictors (the climatic variables). A Tukey honest significant difference (HSD) test was performed for the pair-wise comparisons of group means between designs (factor levels) (McNeil *et al.* 1996).

The Akaike Information Criterion was used as a model selection criterion. This criterion is useful when models with different number of parameters (simple versus more complex models testing for interactions between predictor variables) yield different results at the p-value level. The model with the smallest AIC value is suggested to be the one best fitting the data, the one that minimizes the distance between the model and the truth. As the number of parameters is considered, it reduces the probability of overfitting (excessive introduction of parameters). It is calculated as AIC= 2lmax - 2k, where lmax is the maximum log-likelihood and k the number of parameters. It is also important to consider the difference between AIC values (Δ*i*). If the models have a small difference (<2) relatively to the model with the smallest value, they are all considered to have a substantial empirical support. In these situations where more than one model is equally good in explaining the data, AIC weights can be obtained. The relative likelihood of the models given the data (exp(-0.5*Δ*i*)) is used to calculate the Akaike weights (*wi*) for each model, (equation 5). Akaike weights can be considered as the weight of evidence, or the probability, that the model *i* is the best approximating model, given the data and the set of candidate models (Burnham & Anderson 2002). Furthermore, the ratio between the Akaike weight of the model with lowest AIC value and the weight of other model gives a measure of how many times the first model is more likely to represent the best explanation for the data obtained, when compared with the other.

$$w_i = \frac{exp(-0.5 * \Delta_i)}{\sum_{r=1}^{R} exp(-0.5 * \Delta_r)} \qquad \textbf{(5)}$$

Experiment 2: Testing the third criterion of cultural evolution

Three dependent variables were used in this analysis: the color of the male that successfully mated with the female in the test phase (the Chosen Male), the choice of females as a function of the phenotype used in the positive demonstrations (Correct Choice) and the number of successful essays (essays where females actually mated, relative to those where no mating occurred, Rate of Success). All these variables are binary and were, therefore, analyzed using a Generalized Linear Model procedure (GZLM): the logistic regression, which is the canonical link function for binary distributions (Dunteman & Monn-Ho 2006).

Results

Experiment 1: Alternative protocol to assess female preference

1.1 Does the time spent by females affiliating with the males relates with which male is chosen for copulation afterwards?

In order to assess if time spent next to a male could be used as a predictor of female mate choice, both the proportion of frames spent in the right side of the central vial of the experimental set-up (Figure 2), relative to the total number of frames (R/Total) and relative to the sum of frames spent on the right and left sides (R/R+L) were used as dependent variables, and it was investigated if this proportion was related with the female choice afterwards (mating with the male on the right or left sides). Contrary to other studies on fish and birds (Dugatkin *et al.* 2003, Cummings & Mollaghan 2006, Hoi & Griggio 2011), no statistically significant relationship was found between R/total or R/R+L and the males chosen by the observer females. (See table 1)

Table 1 GLM for the relationship between R/Total and R/R+L and the males chosen by females

		N	DF	MS	F	p
R/Total	**Chosen Male**	29	1	0.000302	0.121	0.730493
R/R+L	**Chosen Male**	29	1	0.000724	0.245	0.624909

1.2 Is there a significant effect of the demonstrator flies in the affiliation behavior of the observer females?

The design (observer females with demonstrator males, F+M; observer females with demonstrator females, F+F; or observer female alone, F alone) has a significant effect in the time spent by the observer females on the right side of the compartment relative to the total time of the experiment (R/Total, p=0.0077). For R/R+L, the value obtained is also very close to significance (p=0,0503). (See details in table 2).

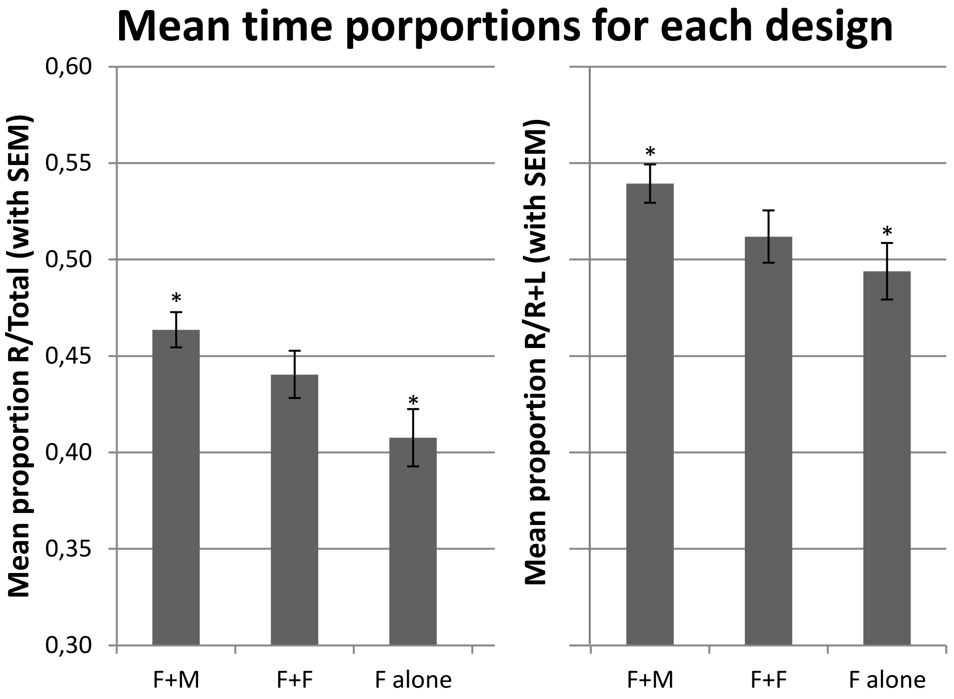
A post-hoc pair wise comparison revealed that there is a significant difference between the first and third designs (F+M and F alone): when there are males on the extremity tubes, the time spent on the right side is significantly different from the design where extremity tubes are empty. (Tukey HSD test, p= 0.005648). (See full comparison in table 3). The main result and the examination of the graphic show that there is a gradient and that there is an attraction to conspecifics that is more pronounced towards males, as expected (Graphic 1).

Table 2 GLM for the design effect on R/Total and R/R+L

		N	DF	MS	F	p
R/Total	**Design**	89	2	0.02338	5.156	**0.007676**
R/R+L	**Design**	89	2	0.01541	3.096	0.050309

Table 3 Pair wise comparisons Tukey HSD test. Variable R/total: between MSE = 0.00453, df = 86 ; variable R /R+L: between MSE = 0.00498, df = 86

		F+M	F+F	F alone
R/Total	**F+M**		0.388245	**0.005648**
	F+F	0.388245		0.148345
	F alone	**0.005648**	0.148345	
R/R+L	**F+M**		0.298594	**0.040410**
	F+F	0.298594		0.587298
	F alone	**0.040410**	0.587298	



Graphic 1 Proportion (with standard error of the men) of time spent on the right side of the compartment in the three designs, for both R/Total and R/R+L.

1.3 Effect of climatic variables in females' behavior.

As controls to the behavior of the observer females, several predictor variables were analyzed, such as male color, room temperature, humidity and atmospheric pressure. As a whole, these variables didn't show a significant effect in the flies' behavior. (See detailed analysis for male color, humidity and atmospheric pressure in Appendix I tables S1 to S7). Nonetheless, for room temperature, when included in a simple model as the main effect, this variable seems to have an influence in the time spent by females in the right side of the compartment for the R/R+L variable (p=0.033) – the same is not verified for R/total (p= 0.067). (See table 4). However, when including the effect of design and the interaction between design and temperature, the effect of temperature is no longer present. (See tables 4-6).

An Akaike's Information Criterion was used to select between the models. Although the simple model with temperature has the lowest AIC value (-216.925), the difference between AIC values revealed that the model including only the design variable have a substantial empirical support as well (ΔDesign<2). The model including the interaction has considerably less support than the other two (ΔTemperature*Design>2). Furthermore, the evidence ratio for temperature versus design (wTemperature/ wDesign) is only 1.271, thus the model "temperature" is only 1,27 times more likely to be the best explanation for the data than the model "Design". It can be concluded that the model "Temperature" has a relatively weak support as the best model, given that the weights of the two models are very close. (For AIC detailed analysis see tables S8 and S9 in Appendix I).

Table 4 GLM for the effect of temperature on R/total and R/R+L

		N	DF	MS	F	p
R/Total	**Temperature**	89	1	0.016627	3.44297	0.066908
R/R+L	**Temperature**	89	1	0.023270	4.64815	**0.033847**

Table 5 GLM for the effect of temperature and design on R/total and R/R+L

		N	DF	MS	F	p
R/Total	**Temperature**	89	1	0.001266	0.276786	0.600186
	Design	89	2	0.015702	3.433354	**0.036820**
R/R+L	**Temperature**	89	1	0.001024	0.203766	0.652848
	Design	89	2	0.004285	0.852956	0.429768

Table 6 GLM for the effect of temperature, design and the interaction between both on R/total and R/R+L

		N	DF	MS	F	P
R/Total	**Design**	89	2	0.009230	2.060663	0.133837
	temperature	89	1	0.007102	1.585631	0.211481
	Design*temperature	89	2	0.008491	1.895695	0.156661
R/R+L	**Design**	89	2	0.002376	0.466680	0.628716
	temperature	89	1	0.000010	0.002032	0.964155
	Design*temperature	89	2	0.002168	0.425756	0.654695

Experiment 2: Testing the third criterion of cultural evolution

2.1. Controlling for the females preference for the males pink or green artificial phenotypes

2.1.1. Is there an effect of male color on female mate choice behavior?

The effect of male color in female choice was investigated to discard a bias towards one of the artificially created male phenotypes. No significant effect was found, as expected: females did not mate preferentially with males of one color. (see table 7)

Table 7 Logistic Regression for the effect of male color on mating choice by females

	N	DF	Wald-Stat	p
Mating	92	1	1.556270	0.212212

2.1.2. Is the proportion of copying also dependent on male color?

The color of the males used in the positive demonstrations did not have a significant effect on the number of correct choices. Correct choices (the behavior of mate-choice copying by females) seem not to depend on male color, as expected. (See table 8).

Table 8 Logistic Regression for the effect of positive demonstration male color (Color +) on proportion of correct choices.

	N	DF	Wald-Stat	p
Color +	92	1	1.451314	0.228317

2.2. Did females mate-choice copy?

There is no evidence for the occurrence of mate choice copying: contrary to our predictions the proportion of correct choices (copying behaviour) is not significantly higher than the proportion of incorrect ones (non-copying behavior). (Mean proportion of correct choices: 0,565217). (See table 9).

Table 9 Logistic Regression for proportion of correct choices.

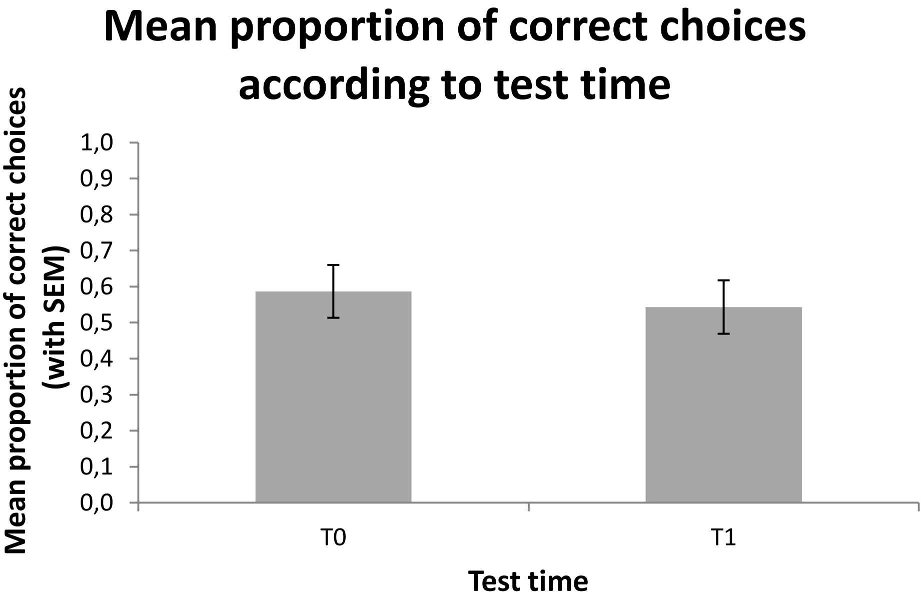
	N	DF	Wald-Stat	p
Correct choice	29	1	1.556270	0.212212

2.3 Did the females memorize the information for a mid-term period of time?

In order to test for the durability of the socially acquired information in the females copying behavior, the proportion of correct choices was compared between test times. The proportion of correct choices does not differ significantly between test times as expected, suggesting that the information provided by the males is retained during the 5 hours hiatus between test times, which corresponds to the flies' mid-term memory capacity. (See table 10, Graphic 2).

Table 10 Logistic Regression for the test time effect on the number of correct choices .

	N	DF	Wald-Stat	p
Test Time	92	1	0.176802	0.674136



Graphic 2 Proportion (with standard error of the mean) of correct choices in both test times: immediately after demonstrations (T0) and after 5 hours (T1).

2.4. Did the duration of the positive demonstrations have any influence on the females' correct choices?

In this experiment, positive demonstrations had variable durations (from 10 to 20 minutes). The effect of the total amount of time covered by the 3 positive demonstrations on the proportion of correct choices was investigated, however, no significant effect was found. (See tables 11 and 12).

Table 11 Logistic Regression model for the duration effect of the positive demonstrations, test time and the interaction on the proportion of correct choices

	N	DF	Wald- Stat.	p
Test time	92	1	0.187841	0.664719
Sum duration+	92	1	0.692970	0.405156
Test time* Sum duration+	92	1	0.163863	0.685625

Table12 Logistic Regression model for the duration effect of the positive demonstrations and test time on the proportion of correct choice

	N	DF	Wald- Stat.	p
Test time	92	1	0.072542	0.787671
Sum duration+	92	1	0.888244	0.345954

2.5. Did temperature, humidity and atmospheric pressure have any effect on the proportion of correct choices?

Since fruit flies' behavior is known to be affected by climatic variables, it was tested (controlled) if temperature, humidity and atmospheric pressure had a significant effect on the proportion of correct choices. No significant effect of these climatic variables was found. (See table 13).

Table 13 Logistic Regression model for the effect of temperature, humidity and atmospheric pressure on correct choice proportion

	N	DF	Wald – Stat.	P
Temperature	92	1	0.098662	0.753441
Humidity	92	1	0.163880	0.685609
Atmospheric Pressure	92	1	0.664380	0.415018

2.6. Was the proportion of successful essays affected by climatic variables?

Given that the proportion of successful essays (those where copulations occurred in the test phase) is only 55%, a supplementary analysis was conducted in order to investigate whether there was some effect of the meteorological variables in the success rate.

Neither temperature, humidity, atmospheric pressure nor difference of atmospheric pressure had a significant effect on this proportion. For atmospheric pressure difference (morning pressure on day 2 - afternoon pressure day 1) N was severely reduced as data from essays conducted on Mondays could not be used: Sunday atmospheric pressure values were not available. (See table 14).

Table 14 Logistic Regression model for effect of Temperature, humidity, atmospheric pressure and atmospheric pressure difference in the number of successful essays.

	N	DF	Wald - Stat.	p
Temperature	167	1	1.745654	0.265193
Humidity	167	1	2.603439	0.186424
Atmospheric Pressure	167	1	0.937598	0.106632
Pressure difference	80	1	0.620722	0.430779

Discussion

Alternative protocol to assess female preference

As already referred, in several species the time spent next to each male can be used as a proxy for mate choice without the need for actual mating. For *D. melanogaster* however, the same result was not confirmed in the present work. The first experiment conducted showed that, in *D. melanogaster,* it is not possible to use the time spent by females next to each male as a proxy of mating preference. Having an alternative behavior to test mate choice would be useful, as it would allow the multiple testing of females, and also to overcome the confounding factors associated with male competition during the test phase of experiment 2.

Although not wielding results relatively to the utilization of this method to assess female preference, results showed that female fruit flies modify their behavior according to the presence or absence of conspecifics. Moreover, they discriminate between male and female conspecifics, spending more time next to males than females, which can be related to the assessment of prospective mates (Graphic 1). It could be valuable to repeat this experiment increasing the time of assessment. On the other hand, since drosophila are known to depend on cuticular hydrocarbons both for mate choice within species and species recognition (Howard *et al.* 2003), it would be also interesting to use mesh partitions instead of glass ones, allowing observer females to sense male cuticular hydrocarbons.

In this first experiment, temperature seemed to have some effect on the time spent on the right side of the compartment relatively to the time spent on the right and left sides (R/R+L). However, it was found, through further investigation, that this effect was diluted in more complex models, that included also the effect of design. After applying the Akaike's Information Criterion, it was seen that although the model with only the temperature effect is the one with the lowest AIC value, the model with design as the main effect still has substantial support (ΔAIC<2). Furthermore, the analysis of the weight of each model reveals that the selected model (temperature) is not convincingly the best as the evidence ratio relatively to the model Design is only (1.271): the model "Temperature" is 1,27 times more likely to be the best explanation when compared with the model "Design".

Although temperature has an effect on R/R+L, which must be more closely controlled in future experiments, it seems to be a rather marginal effect when compared to other models. Additionally, the fact that for the variable R/Total there is no significant effect of temperature also leads to the conclusion that the design effect (significant for both variables) should be considered the principal effect. The difference in results can be due to the fact that R/Total includes all the behavioral components, choice (time spent in the right and left sides of the compartment) and no choice (time spent in the middle of the compartment), and that R/R+L only includes choice. R/R+L can be considered to be less adequate to evaluate the differences between designs, as it excludes a behavioral component that varies plainly with the design: when with other females or when alone, females diminish the time spent in the extremities of the compartment.

Testing the third criterion of cultural evolution

The durability of social information is directly related to the third criterion of cultural evolution: social information has to last long enough to be observed and transmitted to other individuals. The question of how long social information must be retained to be observed and transmitted to others has to be approached taking into account the organisms life histories. For *Drosophila melanogaster*, lifespan is relatively short (it can go up to a couple of months), and they are ready to reproduce shortly after eclosion. In the light of fruit flies' life history, it could be thus expected that even short lengths of time (as 5 hours) might be enough to allow the spread of social information. In relation to the memory types already described, the 5 hours range corresponds directly to mid-term memory, as it has been defined in the literature (Dubnau & Tully 1998, Yurkovic *et al.* 2006), but does not exclude the possibility that anesthesia-resistant memory and long-term memory could also be implied. The testing of other time lengths would allow to verify, in more detail, which type of memory is more commonly associated with social learning.

In the present work there are no consistent results relatively to the existence of mate choice copying - it wasn't possible to replicate the results obtained by the previous authors. This can be due to a lack of data, as in Mery & Varela *et al.* (2009) the data amount was nearly twice the one obtained in this work. Also the fact that the demonstrations period was shortened from 1 hour to 10 to 20 minutes could be preventing the effective transmission of social information about the males performance with the demonstrator females. The fact that the total duration of the demonstrations did not show any effect on the proportion of correct choices, leads to suspect that the use of shorter demonstrating periods might not be influencing the results. Nonetheless, the maximum duration 20 minutes for the demonstrations is still quite different from 1 hour periods. More data is thus needed in order to disentangle the effects of the lack of data and of protocol modifications, as well as to confirm the occurrence of mate choice copying. Moreover, in Nature, females are not expected to watch the courtship displays and mating behaviors of other couples for as long as 1 hour, which leads to the suspicion that, if mate choice copying is indeed occurring and affecting natural populations, shorter demonstration periods should be enough for the acquisition of social information. It should also be interesting to test for this hypothesis in the future.

Assuming the occurrence of mate choice copying (which was already verified in this context and with the same line of fruit flies), and considering only the data where it actually occurred, since there was no significant difference between the number of correct choices at the first and second test times (T0 and T1), it can be inferred that mate choice copying occurrence is not different between test times and thus that social information is kept for as long as 5 hours.

Although the refractory period of female fruit flies poses a problem for the multiple testing of the same females, repeated mating is a common behavior in Nature (Milkman & Zeitler 1974). As the remating average interval is of 5 days in fruit flies (Pitnick 1991), it would be interesting to test the same females 5 days after the demonstration phase and verify if the acquired preferences are maintained at the second mating event. This could be done including both situations where demonstrations are repeated between the first and second mating events and when only the first set of demonstrations is done.

The fulfillment of the second criterion of cultural evolution (inter-generational transmission of information) is dependent on the verification of the third one (the durability of the information). Due to fruit flies' short lifetime and relatively reduced number of reproductive events of females, parents are not expected to transmit mate preferences to their offspring. However, cultural transmission can happen also in oblique fashion and thus a protocol involving an information transmission chain, with first and second order observers, can be developed: females that observe the demonstrations can subsequently be used as demonstrators for new observer females. In order to design such a protocol, it is necessary to know the span of social information. In a recent study, the spread of social information within *Drosophila melanogaster* groups was studied relatively to egg-laying site preference. In this study, a transmission chain was designed, using first and second order observers (Batestti *et al.* 2012). The results obtained by these authors suggest that social information maintenance over time within groups is possible, although depending on the group dynamics. In the context of oviposition site preference, the personal assessment is relatively easy and thus previous social information was found to be overridden by personal information after some time. These results shed some light on the possibility that social information in the context of mate choice copying might also persist in groups and thus be transmitted in an oblique fashion between generations.

In general, the results here presented don't reject the hypothesis that the third criterion can be verified for mate choice copying in *D. melanogaster*. However, further experiments confirming these results and exploring also the existence of intergenerational transference of social information in the context of mate choice are needed to draw more clear interpretations regarding the possible existence of cultural evolution in sexual selection.

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Appendix I

Table S1 GLM for the effect of male colour on R/Total and R/R+L

		N	DF	MS	F	p
R/Total	**male colour**	29	1	0,000119	0,048	0,828914
R/R+L	**male colour**	29	1	0,000386	0,129821	0,721421

Table S2 GLM for the effect of humidity, design and the interaction between both on R/total and R/R+L

		N	DF	MS	F	p
R/Total	**Design**	89	2	0,001470	0,31857	0,728073
	Humidity	89	1	0,002024	0,43853	0,509670
	Design*Humidity	89	2	0,000200	0,04335	0,957600
R/R+L	**Design**	89	2	0,000455	0,08831	0,915567
	Humidity	89	1	0,000086	0,01660	0,897794
	Design*Humidity	89	2	0,000004	0,00071	0,999285

Table S3 GLM for the effect of humidity and design on R/Total and R/R+L

		N	DF	MS	F	p
R/Total	**Humidity**	89	1	0,006561	1,4544	0,231167
	Design	89	2	0,025288	5,6058	0,005166
R/R+L	**Humidity**	89	**1**	0,000242	0,0480	0,827029
	Design	89	2	0,015475	3,0751	0,051356

Table S4 GLM for the effect of humidity on R/Total and R/R+L

		N	DF	MS	F	p
R/Total	**Humidity**	89	1	0,002750	0,5512	0,459826
R/R+L	**Humidity**	89	1	0,000106	0,0202	0,887369

Table S5 GLM for the effects of atmospheric pressure, design and the interaction between both on R/total and R/R+L

		N	DF	MS	F	p
R/Total	**Design**	89	2	0,006129	1,346952	0,265656
	Atm pressure	89	1	0,009699	2,131572	0,148067
	Design * Atm pressure	89	2	0,006144	1,350333	0,264787
R/R+L	**Design**	89	2	0,002405	0,471627	0,625648
	Atm pressure	89	1	0,000115	0,022460	0,881235
	Design * Atm pressure	89	2	0,002381	0,466881	0,628591

Table S6 GLM for the effect of atmospheric pressure and design on R/Total and R/R+L

		N	DF	MS	F	p
R/Total	**Atmospheric pressure**	89	1	0,000041	0,008957	0,924823
	Design	89	2	0,016798	3,661528	0,029828
R/R+L	**Atmospheric pressure**	89	1	0,000005	0,001031	0,974458
	Design	89	2	0,011071	2,198761	0,117212

Table S7 GLM for the atmospheric pressure effect on R/Total and R/R+L

		N	DF	MS	F	p
R/Total	**Atmospheric pressure**	89	1	0,013209	2,713264	0,103125
R/R+L	**Atmospheric pressure**	89	1	0,008678	1,677274	0,198713

Table S8 Akaike's Information Criterion model comparison. Variable: R/R+L; T- Temperature; D- Design, T*D- Temperature * Design. Smallest AIC value presented in bold. Δi – Akaike differences

Model	DF	AIC	L.Ratio - Chi2	p	**Δ*i* (AIC*i*-AIC*T*)**
T	1	**-216.925**	4.667086	0.030746	-216.925+ 216.925 =0
D	2	-216.446	6.187734	0.045326	-216.446 + 216.925 = 0.479
T*D	3	-214.689	6.430405	0.092448	-214.689 + 216.925 = 2.236

Table S9 Akaike's Information Criterion model comparison. Variable: R/R+L. T- Temperature; D- Design, T*D- Temperature * Design. Δi – Akaike differences; RL- relative likelihood; *w*i - Akaike weight; ΣRL- sum of relative likelihoods; wT- Akaike weight for temperature model.

Model	AIC	Δ*i*	**RL exp(-0.5*Δi)**	***wi* (RL*i*/ΣRL)**	***wi ratio* *(wT/wi)***
T	-216.925	0	1	0,473047	1
D	-216.446	0.479	0,787021	0,372298	1,270614
T*D	-214.689	2.236	0,415406	0,154655	3,058731
			ΣRL= 2,113954		