Lipstick on the collar

A possible female preference through pheromonal marking

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Abstract

Decision making requires information to lower uncertainty, especially in fitness affecting decisions such as mate choice. In situations with low certainty, mate-choice copying provides a low cost strategy using public information. Mate-choice copying has been tested in Drosophila melanogaster using visual cues. However, female pheromones transferred onto males during mating. This 'mark' might act source of public information, producing a female preference towards males with sexual experience. These experiments tested for the existence of a preference, as well as the necessity and the sufficiency of these pheromones to produce a preference. Although no significant results were found in during testing, there are indications that these pheromones are able to affect the mate-choice decision. When present, the female pheromones induced a slight preference for experienced males. However, the absence of pheromones induced a similar but opposite preference. The lack of a 'mark' steered the female choice away from experienced males and towards naïve males. As the results were not significant, the role of transferred female pheromones remains unclear. More research is needed to establish if his pheromonal mark is capable of inducing a preference on its own or it is just a small source of information in a complex decision-making process.

Introduction

Decision making

Animals are faced with choices throughout their lifetime. These can range from trivial to decisions able to impact an animal's life greatly. Depending on the choice, the outcome could either benefit an animal or put it at increased risk. Especially with fitness-affecting decisions, a wrong choice could spell disaster. As decisions are made in order to reach goals, the decision-maker must select a course of action best fitted to reach those goals. Sometimes a choice can be simple; having only a single goal to reach with a clear option to use. But when multiple goals and options are available, making a decisions becomes more difficult[1].

In order to identify the optimal decision, knowledge of the available options and their consequences is required. Decision-making, the process leading towards a choice of action, is gathering information until a decision can be made[2]. There will however remain a certain amount of uncertainty in the process, as there will be factors out of the decision maker's knowledge or control[3]. Gathering information allows an animal to better determine the probable gains and consequences of a choice [4].

Information

Information is anything that reduces uncertainty and can be acquired through direct interactions with the environment (*Personal Information*), or it can be gathered from others (*Social Information*)[5] (see Figure 1).

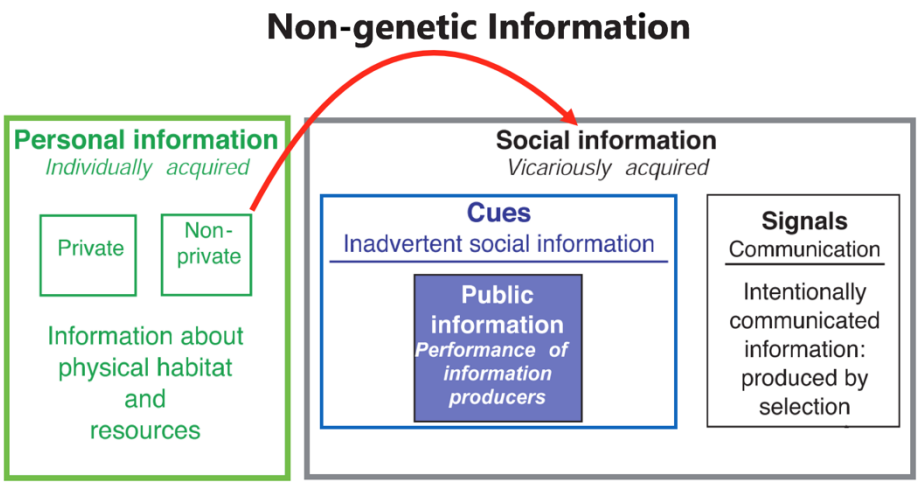

Figure 1 - Non-genetic information can be either personal information or social information. Personal information is acquired through direct interactions by an individual with its environment. When the acquired information is non-private, *social information* can be produced. This can be an intentional transfer of information (Signals) or inadvertent (Cues). Of the inadvertent social information, public information (PI) contains the information about the producer's performance (Adapted from Danchin et al. [5])

Personal information is the most reliable form of information, but is also costly to obtain. It requires the allocation of time and energy; recourses that could otherwise be spent on tasks such as foraging or reproduction. Socially acquired information is easier to acquire, but will be less reliable. *Social information* allows the decision-maker to obtain valuable information without costly trial-and-error interactions. The information however is more liable to be irrelevant, out-of-date or even deceitful[3,6]. *Personal information* would therefore be preferred when making a decision, as it allows for the largest reduction of uncertainty. But when *personal information* is too difficult or too costly

to acquire, animals rely more on *social learning* to come to a decision[4,7,8]. A common social learning strategy for decisions with high uncertainty is to copy the choice of others. *Public information* allows naïve individuals to learn successful strategies by observing others. The observer can then decide whether to copy the observed strategy or to try a different tactic[5,8].

Mate-choice

During an animal's life, mate–choice is one of the most important decisions. As sexual reproduction requires a male and female to pair in order to produce offspring, choosing a good mate can affect fitness greatly. In most species, the mate-choice decision lies with the females. As sperm has a lower energetic cost compared to eggs, it can be produced in greater quantities. This allows males to maximize reproductive success by mating with different females. A female's reproductive success on the other hand, does not depend solely on successful fertilization of the eggs. Due to the higher energetic cost and the limited amount of eggs produced, females are more '*invested'* in the eggs. To increase the quality of her offspring, a female must consider the quality of potential mates[9]. As a result, males will display courtship behaviour to show females they possess desirable qualities. The females then chooses a mate by either accepting or rejecting the male's advances [9,10]

As the male performs his courtship, the female must determine his quality. Social status, parental input and access to resources are qualities that provide the offspring with a *direct benefit* to survive or reproduce effectively. *Indirect benefits* are provided by the possession of high quality genes[9] and are harder to determine. The male's secondary sexual attributes were once thought to be honest indicators of these high quality or 'good' genes[11]. However, the process of sexual selection has allowed males to mimic certain desirable traits to improve their mating succes[12–14]. Females must now determine whether these traits are honest and representative of male quality. This sexual conflict between the male and female reproductive strategies increases a female's uncertainty during the mate-choice decision and the need for valuable information.

Mate-choice copying

Females need to decrease this uncertainty and observing the mate-choice others can provide useful information about performance. When a male is chosen by another female, he has been deemed to possess desirable traits. This *public information* elevates the quality of that male over others. Copying the choice can prove to be a beneficial strategy when there is limited time or it is too costly to acquire personal information about prospective mates[8,11]. Although mate-choice copying does not always provide a benefit[7,8,15], it has been found to occur in several different species such as fish, birds, flies and even humans[5,16,17]. Mate-choice copying also occurs in Drosophila melanogaster; a long time model-organism for genetics and sexual behaviour research[18–21]. Previously, research into D. melanogaster mate-choice has been focussed on males as male courtship-behaviour consists of several clearly distinguishable stages [18,21,22]. However, research into female sexual behaviour has progressed beyond the once thought simple acceptation of rejection behaviour [22].

Pre-copulatory behaviour in D. melanogaster can last up to several minutes and during this time females are able to gather information about prospective mates through several different sensory signals and cues[18,19,23]. This includes public information and D. melanogaster females have been shown to copy mate-choices in situations of high uncertainty[15,16]. Mery et al. tested the use of visual cues as a source of public information in the mate-choice decision of D. melanogaster females. Observing mating success of a certain coloured male provided females with positive information, whereas rejection provided negative information. Females preferred males of the colour associated

with positive information over males of the 'negative' colour, showing D. melanogaster females use visual cues to copy mate-choices[16].

Although observing a successful mating can provide positive information, observing a male mating multiple times can provide negative information. After multiple copulations, males are at risk of becoming semen limited. As the act of mating itself is costly for females[24], mating with these temporarily infertile males does not provide the fitness benefit of producing offspring to compensate for the costs of mating[15].

Research into mate-choice copying in D. melanogaster courtship has however focused on visual cues. Although visual cues are dominant in the male's decision to approach a female, chemosensory cues become more dominant during courtship[25]. In mate-choice copying, a difference between males was observed as one was chosen over another. This provides positive information about the chosen male's quality. Without visual cues, differences between suitors might be indicated through pheromones. Pheromones fulfil a variety of functions in D. melanogaster, including species recognition[23] and enhancing or decreasing attractiveness[26–28]. During mating, pheromones are exchanged between males and females [27,29,30] and as a result, males are 'marked' with female cuticular hydrocarbons[30]. As the involved cuticular hydrocarbons are volatile and dissipate over time[26], this 'mark' does not only indicate mating success, but that a male has mated recently.

The reliance on pheromones during mating paired with the knowledge mating provides a 'mark of approval', results into the hypothesis that females are able to use public information from female pheromones on males to determine male experience and copy mate-choice in situations of high uncertainty. To determine if D. melanogaster females do indeed copy mate-choices as a result of chemosensory cues, the research has been divided into three sets of experiments.

The first set of experiments aims to determine if virgin females show a preference for either males that mated (*experienced)* or males that have not mated (*naïve*). The second set investigates the effect of removing the female pheromones on males through the use of transgenic flies unable to produce cuticular hydrocarbons [31]. Males mated with these *oenocyteless* females will not receive female pheromones during mating and determines if the presence of female pheromones is necessary in order to create a preference. The last experiment tests if the presence of female pheromones on is sufficient to create a presence. Here the female is given a choice between two naïve males of which one has been perfumed with female pheromones.

During all experiments, only virgin females were used in order to eliminate female experience from the decision-making process. During these experiments females were not given a demonstration before the choice as they have in other experiments[15,16,18].

Materials and methods

Fly strains and rearing

All Drosophila stocks used during the experiments were reared on a medium of agar, sucrose, glucose, cornmeal, wheat germ, soy flour molasses, yeast, propionic acid and tegosept [30]. The same food is used in collection tubes and isolation vials. Flies were kept on a 12:12 hour light/dark cycle at 25°C. Virgin flies were collected using CO_2 anaesthesia and placed in vials of same-sex groups up to 20 flies per vial. Tests were conducted between 5 and 7 days after collection. Both the *Canton S* and *Oregon* R wildtype strains were used in this experiment, as well as two transgenic strains:

Oenocytless and control. *Oregon R* was only used to compare preferences between the two wildtype strains. *Canton S* acted as the wildtype for subsequent testing.

Creation of Oenocytless (Oe^-) and Control (Ctrl) females was done by crossing '1: PromE(800)-Gal4, tubP-Gal80ts;1' with either '1: UAS-StingerII, UAS-hid/CyO;1'(for Oe^-) or '1: UAS-StingerII;1' (Ctrl) according to Billeter et all[31]. After the cross, these flies were kept at 17°C instead of 25°C. Two days after collection Oe^- and Ctrl females were subjected to a heat shock at 29°C for 24hrs. Afterwards a visual check was conducted using a fluorescent microscope. As oenocyte cells are tagged, a lack of fluorescence in Oe^- flies indicates a successful cross.

Colouring flies

A different coloured fluorescent powder was applied to both males 24 hours before the test. The resulting pair of one pink and one green coloured male are easily distinguishable (Figure 2A). Applying the powder 24 hours before the test, allows the flies to groom themselves (Figure 2B), leaving only a small amount of powder on the back between the wings. Although not clearly visible with the naked eye, this amount is enough to identify the male using a UV flashlight.

Figure 2 - D. melanogaster flies after colouring. (A) Directly after colouring, flies are completely covered in brightly coloured powder. This will prompt the flies to start grooming. (B) Allowing flies to groom themselves clears off most visible powder, leaving small amounts visible under UV light. These powder traces allow for identification between the two males, whilst not influencing normal behaviour.

Male flies of the same age were split evenly into groups of 15 to 20 flies and transferred into a colouring cup using a mouth pipet. The colouring cup consists of a 5ml Eppendorf cup containing approximately 1mg of fluorescent powder. The colouring cup is then placed on a vortex three times for the duration of five seconds (with a five second pause in between) in order to cover the flies evenly with powder. The now coloured flies were then transferred to a fresh vial containing rearing medium and kept overnight at 25°C until the test.

Perfuming

Female hydrocarbons were extracted using hexane. 28 virgin *Canton S* females were placed into a clean 2ml glass vial and 1200µl hexane is added. After vortexing, the hexane is transferred to a new glass vial and the allowed to evaporate underneath a nitrogen flow. As a result, the vial is coated with female hydrocarbons. By placing 7 males into the coated vial and vortexing it, males were perfumed with one third of the female hydrocarbons. This is equivalent to the amount of hydrocarbons transferred during mating. As a control, *sham* treated males were created by vortexing 7 males in a clean glass vial.

Preference assays

All experiments use a basic preference assay consisting of three stages*: creating different males, isolation* and the *mate-choice assay.* During the *creation of different males,* two different coloured males of the same age were paired and treated separately before the test. Treatment for the *Preference*, *Time Delay* and *Necessity* experiments, consist of mating one of the virgin males to create an *Experienced* male. Both males are placed in separate mating dishes using a mouth pipet. Mating dishes consist of a 10x 8mm petri dish with a small opening to allow the transfer of flies. One male receives a virgin female to mate with, becoming *experienced*. The other male does not receive a female, remaining *naïve*. For the *Sufficiency* experiment, the males were either perfumed with female extract of given a sham treatment to create *perfumed* and *sham* males. As mated males are less inclined to directly mate again, both males were transferred to glass vials for *isolation*. Unless otherwise noted, males were isolated six hours before the mate-choice assay. The last stage consists of the mate-choice assay. Both males of a pair were removed from their isolation vials and placed into the same mating dish. The test starts when a virgin female is added and ends at the start of copulation. The colour of the chosen male is identified using an UV flashlight and the result registered. During the test, mating dishes were placed underneath a Logitech C920 webcam. The elapsed time from the female entering the dish until copulation was determined from the video using Vegas Pro 14 video editing software.

Creation of graphs and statistical analysis was performed using the Graphpad Prism 6 program. As the mate-choice consisted of two options, a two-tailed binomial test was used to determine the existence of a preference. The null-hypothesis was an absence of a preference, indicated by a ratio of 50/50 used in the binomial tests. Lastly, a two-tailed unpaired t-test was used to test the differences in mating duration.

Results

Preference

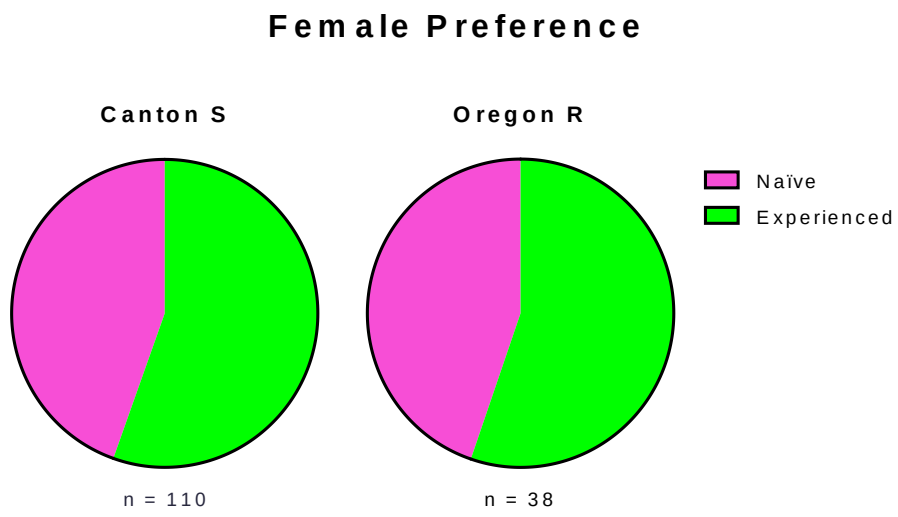

Figure 3 – Preference experiment 1: Preference in wildtype strains. Females were given a choice between an experienced and a naïve male. Of the 110 choices made by Canton S females, 61 resulted in the choosing of the experienced male and 49 choices for the naïve male. This translates to a ratio of 55.45/45.55 (p=0.2942) for experienced and naïve respectively. Of the 38 choices made by Oregon R females, 21 were experienced males and 17 were naïve males. This resulted in a 55.74/44.74 ratio (p=0.6271) in favour of the experienced males.

The first experiment tested for a preference between *experienced* and *naïve* males in the two wildtype strains. A pair of coloured virgin males was placed into separate mating dishes. One receives a virgin female to create the *experienced* male and the other remains alone during this time to create the *Naïve* male. After an isolation period of six hours to recover, both males were placed in a mating dish with a virgin female. The male with which she copulated was deemed to be chosen.

At first glance virgin females seem to have a slight preference towards the *experienced* males (See Figure 3). Around 55 percent of the choices made resulted in the female copulating with the experienced males. This small preference towards the experienced males was observed in both the Canton S and Oregon R strains. Although both strains show a ratio of around 55 to 45 percent in favour of the experienced males, the observed ratios did not differ significantly from the expected ratio of 50/50 (p= 0.2942 for Canton S and p= 0.6271 for Oregon R).

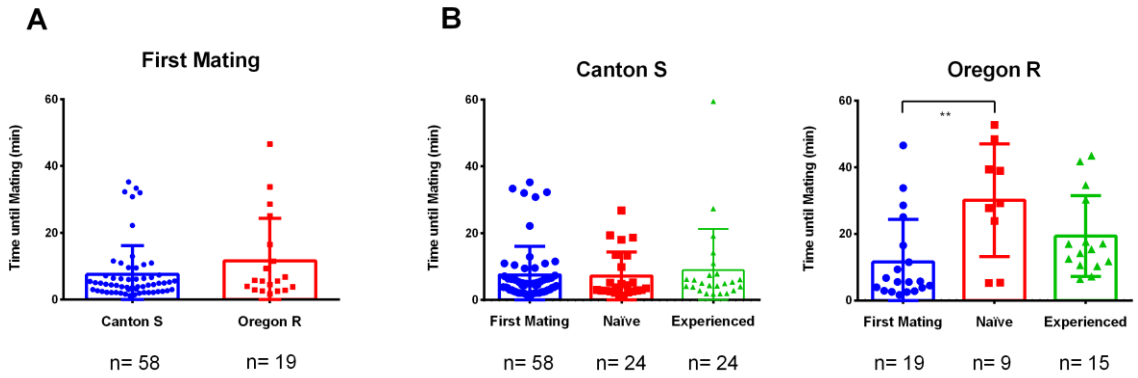

Figure 4 - Decision latency of experiment 1: (A) During the creation of experienced males no significant differences were observed between Canton S and Oregon R (p=0.1227). (B) The latency of choice was split for both possible outcomes and compared to the first mating. For Canton S, latency of both results did not differ significantly from the first mating. Oregon R showed a longer latency for both outcomes compared to the first mating. Although choices for the experience male did not take significantly longer (p=0.0813) they were on average 7 minutes slower. With an average of 18 minutes, the latency of choice in favour of naïve males was significantly longer compared to the first mating (p= 0.0033).

The creation of experienced males (first mating) and the preference tests were recorded using a webcam. The time spent from the moment the female entered the chamber until the start of successful copulation was extrapolated from the video (Figure 4). As decision-making takes time and a longer latency of the decision could indicate a "harder" decision[2]. Oregon R females are generally thought of as choosy compared to Canton S and should show a longer decision time.

During the creation of the experienced males, decision latency for Canton S and Oregon R did not differ significant from one another. As females were not given a choice between males as they are in the preference test, these times act as a baseline. The decision latency of Canton S females did not change compared to the first mating, nor was there any difference in decision time between choosing a naïve male and an experienced male. Oregon R females did show a significant difference in decision time (p=0.0033) when choosing naïve males compared to the first mating (Figure 4). It took females on average 30 minutes to decide when choosing the naïve male as compared to the 11 minutes it took during the first mating. Decisions resulting in the experienced male being successful also took longer on average (18 minutes), however this was not a significant difference (p=0.08). Seemingly, when given multiple options, Oregon R does seem the choosier strain of the two.

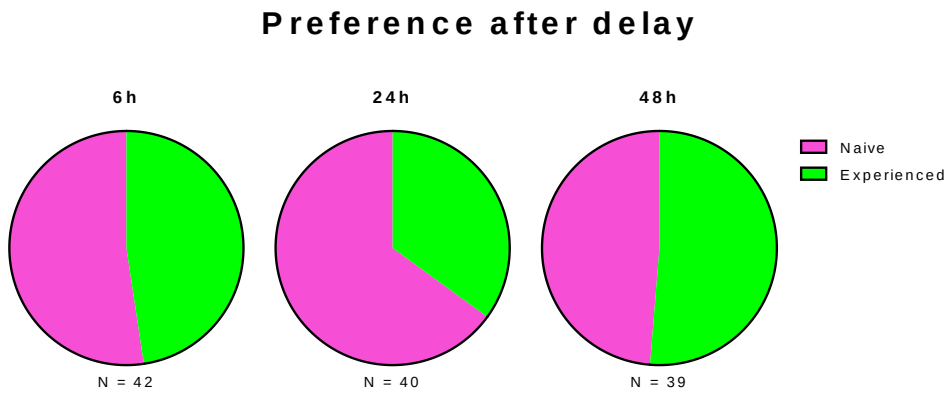

Figure 5 – Preference experiment 2: Preference after delay. Experienced males were created either 48, 24 of 6 hours before the preference test. Females did not show a preference in the 6-hour delay group (p=0.8776) nor in the 48-hour group (p=0.999). Females showed a slight preference towards the naïve males in the 24-hour group, although not significantly (p=0.0807).

During the second experiment, the times between the first mating and the preference test were varied. This was achieved by creating the experienced males either 6, 24 or 48 hours before the start of the preference test. As the female pheromones dissipate over time[26], the effect of the pheromones should diminish when time between matings is increased. Therefor the expectation was that a preference towards experienced males should be the strongest in the 6-hour group and the weakest or non-excitant in the 48-hour group.

Of the three groups, only the 48-hour group met expectations as no preference was found (figure 5). However, the 6-hour group and especially the 24-hour group showed curious results. The 6-hour group, which was expected to show the strongest preference, did not show a preference at all (Figure 5). The 24-hour group did show a preference, but this was in favour of the naïve males. Varying the time between matings did influence the mate choice.

Necessity

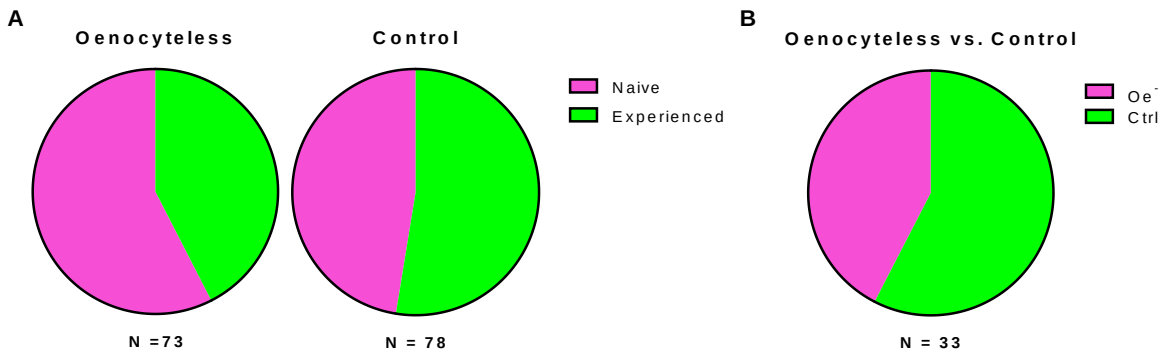

Figure 6 – Necessity tests. (A) Preference test between experienced and naïve males. In the oenocyteless group females show a small preference towards naïve males, choosing 42 out of 74 times in favour of the naïve males. This small preference was however not significant (p=0.2416). In the control group, no significant preference was found (p=0.7343). (B) When given a choice between two experienced males, females showed a slight preference towards the Ctrl mated males over Oe⁻ mated males. The preference however did not differ significantly from a 50/50 ratio (p=0.4869).

The third experiment was set up to test the necessity of the female pheromones to produce a preference. In the first of two tests, females were given a choice between a naïve male and an experienced male that was mated with either a oenocyteless (Oe^-) or a control (Ctrl) female. The difference between these experienced males lies in the transfer of female pheromones to the males

during mating. As Oe$^-$ females do not posess oenocyte cells, no pheromones are produced to transfer onto the males during mating. The resulting males are now experienced without the pheromone 'mark' to indicate they are indeed experienced. Ctrl females are still able to produce pheromones and males mated with the Ctrl females still receive pheromones during mating and will act as a control group.

Neither group showed a significant preference towards either male (figure 6A). However, the Oe$^-$ group did show a slight preference towards the naïve males. As the presence or absence of the pheromonal mark was expected to determine the preference, the control group was expected to produce a preference towards the experienced male and the Oe$^-$ group was not expected to show a preference towards either males. Although not expected, the lack of a preference in the control group is consistent with results from the preference experiments. The Oe$^-$ group, on the other hand, showed a slight preference towards the naïve males.

This result was supported by the second test of the experiment. During this test, females where given a choice between two experienced males. As one had mated with a Oe$^-$ female and the other with a Ctrl female, the only difference between the males should be the acquired pheromones. Although this test did show the males that received pheromones where preferred (figure 6B), receiving these pheromones might not be the source of the preference. As the presence of the pheromones did not produce a visible preference (figure 6A) and the absence produced a slight preference for naïve males (although not significant at p=0.2416), it might be the absence of pheromones that produces a preference against Oe$^-$ mated males.

Sufficiency

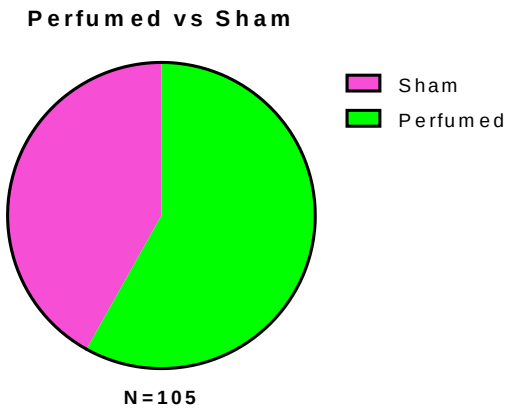

Figure 7 - Sufficiency test: Females where given a choice between two virgin males: one being perfumed and the other sham treated. Females showed a small preference in favour of the perfumed males over the sham treated males, choosing perfumed males 61 out of 105 times (58%). However, the observed preference was not significantly different from a 50/50 ratio (P=0.11).

The final experiment was set up to test the *sufficiency* of the pheromone to create a preference. Females were given a choice between two *virgin* males. By using extracted female cutticular hydrocarbons to *perfume* one of the two males, the equivalents of an experienced and a naïve male are created. The resulting males only differ in the presence of the female pheromones. During the other experiments experienced males could possibly benefit from experience besides the acquired pheromones. By using two virgin males, no male experience plays part in the decision making process. The perfumed males receive the same amount of female pheromones as would have been transferred during mating[30]. The other males receive a *sham* treatment to let the presence of the

pheromones be the only difference between the males. After the treatments both males were place in isolation for 6 hours before being placed in a mating dish with a virgin female.

As was expected, females showed a slight preference towards the perfumed males. However, like precious experiments the observed preference was not statistically significant (P=0.11). This was however the strongest observed preference with almost 60% of the results going in favour of the perfumed male. Although not significant, this result does suggest female pheromones are involved in the mate-choice process.

Discussion

The expectation was that the transfer of female pheromones to males is involved in creating a female preference for experienced males. The experiments were set up to test the existence of the *preference*, the *necessity* of female pheromones to observe the preference and lastly the *sufficiency* of the pheromones to create a preference.

Overall, no significant differences were found during these tests, as the ratio between choices seem to hover around the 50/50 mark. However, a case can be made that males who received female pheromones during the various tests (*Experienced, Ctrl mated* and *Perfumed)* have a slight edge in the decision process. In both preference tests with *Canton S* and *Oregon R*, a small preference was visible in favour of the *experienced* males. At 55% of the choices going towards the experienced males, the preference was not significant compared to the expected 50/50 ratio (p=0.29 for CS). As the preference was only slight, it could be explained by experience of the mated male. However, the same ratio persisted in the results from the *necessity* test. When were pitted against Oe^{-}-mated males, *Ctrl*-mated males seemed to be slightly more preferred (58% for Ctrl, p=0,48). In this test both males had mating experience and only differ in the presence of absence of female hydrocarbons. So the observed preference persisted without difference in male experience. This is further supported by the *sufficiency* test, where *Perfumed* males where preferred 58% of the time (p=0.11). A similar ratio was found in the precious tests. In this case however, experience was not a factor as both males were virgin.

Although the difference was not significant, results suggested the existence of a small preference towards males that received female pheromones. It further showed that this effect was independent of male experience as it was visible in all three tests. There do however remain question marks to preference's existence. During the time delay test the 6-hour group did not show the expected preference, but remained at a ratio of 50/50. The same was found in the *necessity* test where Ctrl-mated males did not stand out to naïve males. Both tests did show curious results when the pheromones where absent.

Where the presence of pheromones seemed to induce a small preference, the absence of the pheromone also results in a differing preference. During the *Necessity* test no preference inducing pheromones would have been transferred and therefore no preference towards either the naïve or the Oe^{-} mated males should occur. However, the naïve males actually seemed more preferable over Oe^{-}-mated males (58%; p=0.24). As both males lacked the female pheromones, the observed preference cannot be caused by them. However, changes in the male cuticular hydrocarbons could provide a possible explanation. Male pheromones are also transferred over to the female during mating, resulting in a lower amount of male pheromones [30]. Without a female 'mark' to indicate these lowered pheromone levels is due to previous mating, this cue could be interpreted as the male not being able to produce more pheromones. This would indicate to the female that the male is of a lower quality compared to the naïve males.

Similarly, this balance of male and female pheromones might explain the results of the *time delay* test. During the test, both the 6-hour and 48-hour groups showed a ratio around 50%, but the 24-hour group showed naïve males were preferred 65% of the time (p=0,08). As the female hydrocarbons are volatile, they dissipate over time. After 48-hours the pheromones should have dissipated and the two males would no longer differ in pheromones. The pheromones would not have dissipated entirely at the 24-hour mark, leaving a small trace on the males. During this time the male pheromones might not have been able to replenish, leaving the male with a lower amount of male pheromones and not enough female pheromones to offset the deficit. This again could result in a lower perceived male quality, and a preference towards the naïve males.

Pheromones are part of multiple male-female interactions that direct the mate-choice decision and the presence or absence of female pheromones on males seems to influence female choice. The observed preference was only small and no significant results were seen, leaving the existence of a preference uncertain.

One possible cause of this uncertainty could be the use of virgin males. By using virgin females over experienced females the effect of experience was removed from the mate-choice decision. Although this evened the playing field for both females, the first mating a female has might be the least important. D. melanogaster are not monogamous and females will mate multiply before laying eggs[9,10,32]. Generally, the last male she mates with sires the most of her offspring [30,33] This allows females to reduce fitness pressure, by mating and using secondary or tertiary matings to "Trade up" and improve the quality of her offspring [32].

Another possible cause could lie in the use of *Canton S* (CS) as the wildtype strain during most tests. Although CS is widely used because of its ease, it is generally thought of as being less *choosy* compared to strains like Oregon R (OR). During the *preference* tests, OR female took longer to make a decision compared to CS female (See Figure 4) indicating a more "thought out" decision[2]. As there was a small preference visible using CS, this could be stronger using OR. As such, using OR might provide clearer evidence of a preference. Although tests with OR where attempted, large scale testing failed with this experimental setup. In most cases, females refused to mate within the given timeframe. In this experiment, tests were performed under basic TL lights and not in temperature controlled rooms. Both are factors that could influence the mating behaviour of the flies. Controlling the test-environment would allow for conditions in which OR will be more inclined to mate. It might also be a good idea to include experienced females in order to evaluate if there is a difference in preference between virgin and experienced females. Lastly the male courtship between the two competing males should be scored. If a male has a higher success rate at a lower or similar amount of courtship could be an indicator of a preference. Differences in courtship behaviour could influence might also reveal if experience has an effect on courtship and courtship success.

These improvements should provide more insights into female decision-making and information gathering of female D. melanogaster, leading to improved understanding of the interactions and cues of the mate-choice-decision. As is stands, no significant results in favour of a preference created by female pheromones where observed in this experiment. There are indications however that these pheromones do in fact influence the decision-making process, although in what capacity and with what goal remains unclear. In the end, the female pheromone mark might just be a small cog in the clockwork of the mate-choice decision.

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