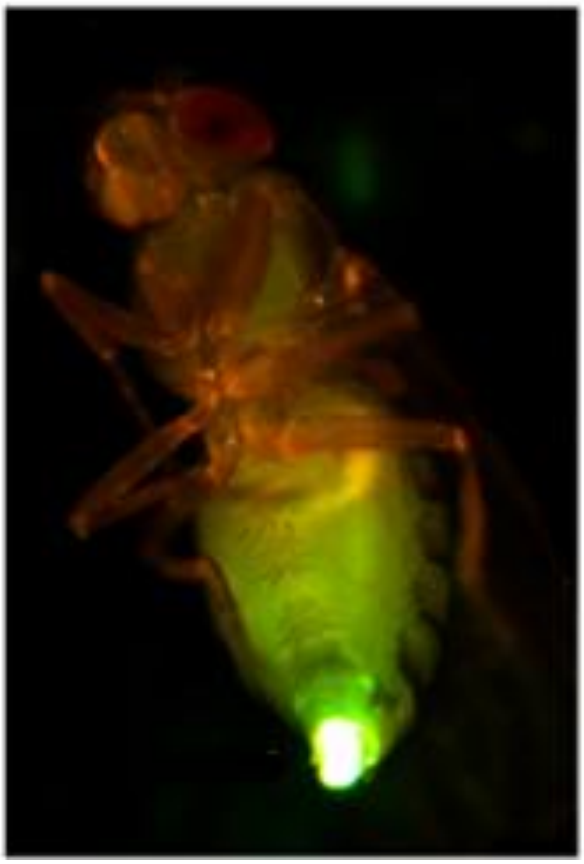
Pheromonal cues convey social information about favorable egg-laying site in Drosophila melanogaster



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2. Abstract

As far as egg-laying species are concerned, finding a suitable oviposition site is essential behavior for the survival of the species. But how do they learn this behavior? A model organism which is extensively used for studying behavior is the vinegar fly *Drosophila melanogaster.* Here we show that *Drosophila melanogaster* females use information from other flies when choosing a suitable oviposition site. Female *Drosophila melanogaster* leave a blend of pheromones behind in their territory that consists of the aggregation pheromone 11-cis Vaccenyl Acetate (cVA), which is obtained during copulation with males, and a specific combination of several Cuticular Hydrocarbon (CH) pheromones. This blend of pheromones affects other mated females behavior, because the food patches that were marked by mated females were preferred by mated females for oviposition. This signal that is left behind is highly specific, because neither male callers, nor virgin female callers with addition of ejected ejaculate were able to induce such a preference. In addition, females without CHs did not induce a preference for oviposition while an extract of a mated female fly did induce a preference. The mechanism by which the signal is perceived remains unclear, however, we did show that multiple olfactory sensory systems are needed. Our results show that *Drosophila melanogaster* female flies are able to learn from other mated female flies through a phenomenon called social learning.

3. Introduction

Oviposition is behavior many animals show, such as birds, fish, reptiles and insects. This behavior is important, because choosing a suitable egg-laying site directly affects the survival chance of offspring (Richmond & Gerking, 1979). Not only is this important for fitness at an individual level, but also in terms of the survival of species as a whole. By selecting a proper site, a female increases the chance of her offspring to survive and thereby increasing the chance to pass on a portion of her genes (Thorpe, 1945).

The oviposition behavior is considered very labile as it is influenced by many environmental factors (Richmond & Gerking, 1979). So how do females learn which sites are suitable for oviposition?
A possibility would be through learning by trial-and-error. This would mean that an egg-laying female would try out different sites, lay eggs on these different sites and determine by offspring survival rate whether a specific site is better than the previous or not. While learning through trial-and-error is a possibility, it is not a very efficient way, because producing eggs and finding a suitable site is costly in terms of energy. An alternative way and more efficient way would be through social learning, the process of learning by using information of others (Bandura, 1971). While this process in most cases requires less energy than learning by trial-and-error, there is an important side note that the information that is left behind by others, must be honest to the observers, otherwise the information cannot be trusted and usage of this information might not turn out to be more energy efficient (Laland, 2004).

An organism which is extensively used for studying behavior is the vinegar fly *Drosophila melanogaster*. *Drosophila* are considered social organisms and show social behavior such as aggression (Dow & von Schilcher, 1975; Baier et al., 2002), mating (McGraw et al., 2004; Dickson, 2008), aggregation (ref.) and communication (Billeter et al., 2009; Billeter & Levine, 2013). *Drosophila melanogaster* commonly live in groups. Group living elicits both benefits as well as disadvantages. Some of the disadvantages to living in groups are increased competition over food and mates, while on the other hand the benefits to group living are a decreased pressure of predation and an increased opportunity to find suitable mates. Next to living in groups, *Drosophila* are also known to communally lay eggs (Wertheim et al., 2002; Rohlfs & Hoffmeister, 2003.). This behavior is beneficial to larval offspring, because it has been shown that larvae cooperate by digestion of the food which in turn decreases the chance of detrimental fungal growth (Wertheim et al, 2002). Seemingly, the benefits outweigh the disadvantages because group living is prevalent. Typically, *Drosophila* mated females aggregate on food that contain yeast for oviposition (Hansson et al., 2012). Yeast contains important nutrients necessary for larval growth (Baumberger, 1917; Becher et al., 2012) as well as nutrients that are required for the production of eggs in females (Lebreton et al. 2012; Ribeiro and Dickson, 2010).

In addition to group living and communally egg laying, *Drosophila* have shown to be able to learn through social learning (Durisko & Dukas, 2013). This leads us to hypothesize that *Drosophila* might pass on information, or use information that is left behind by others, to determine the quality of a specific egg-laying site. If *Drosophila* do communicate about this, it is likely done by the use of pheromones, an important manner of communication in these flies. One of the pheromones that might play a role in this process is the aggregation pheromone 11-cis Vaccenyl Acetate (cVA) (Bartelt et al., 1985). This volatile compound is solely produced by males, females cannot produce it. However, certain quantities of this pheromone are transferred to females during mating and copulation (Wertheim et al., 2006; Lung and Wolf, 2001). The amount of cVA in an area holds information on the density of flies. This makes that the amount of cVA in a certain area can be considered to be an honest signal.(Wang & Anderson, 2009).

The pheromone cVA is not solely produced by *Drosophila*, other insects produce this aggregation pheromone as well (Ray et al., 2011). In addition to the pheromone cVA, *Drosophila* use other pheromones for communication and recognition of conspecifics, namely cuticular hydrocarbons (CH's). These pheromones are produced in the abdomen of the flies in specialized cells, called oenocytes. The flies cuticles are covered in these CH's and are released in the flies' surroundings

and the substrates they occupy. While there are multiple CH's, it's notable that only females produce CH dienes 7,11-HD, while males predominantly produce the monoenes CH 7-T (Billeter and Levine, 2013; Billeter et al., 2009).

All these findings, the living in groups and communally egg laying, need for yeast for larval survival growth and egg production, the ability to learn from others and communication through the use of pheromones, led to the hypothesis that females are able to learn to identify suitable egg-laying sites by communication through pheromones. This hypothesis led us to the following research questions: Do females leave information behind about suitable egg-laying sites? What is the composition of this signal and what is the mechanism underlying the perception of this signal?

4. Material & Methods

Fly stocks, food and rearing

For the experiments we used wild-type Oregon-R and the following mutant strains: Oenocyte-less (oe^-) (p{PromE(800)-gal4};p{TubP-gal80^{ts}}/p{UAS-hid}{UAS-nuclearGFP}) and as control (p{PromE(800)-gal4};p{Tub-gal80^{ts}}/p{UAS-nuclearGFP}). Ir8a$^-$ mutant flies (w^-;Ir8a^1) and Ir8a^1 rescue (w^-;Ir8a^1;p{Ir8a$^+$}) (Abuin et al., 2011), Orco$^-$ (w^-;Orco^1) and Orco$^-$ rescue (w^-; Orco1; pBac{Orco$^+$}) were gifts from R. Benton. (w^-;p{ProtamineB-eGFP}) (Jayamaiah Raja and Renkawitz-Pohl, 2005), Or65a$^-$ mutant flies (w^-;Or65a-gal4/p{UAS-kir2.1}) and (w^-;Or65a-gal4/p{UAS-TNT}) and as control to the Or65a$^-$ mutant flies (w^-;Or65a-gal4/w^{118}) Or67d$^-$ mutant flies (w^-;Or67d-gal4/p{UAS-kir2.1}) and (w^-;Or67d-gal4/p{UAS-TNT}) and as control to the Or67d$^-$ (w^-;Or67d-gal4/w^{118}), as control to the UAS-Kir2.1 and UAS-TNT we used the mutant flies (w^{118}/p{UAS-kir2.1}) and (w^{118}/p{UAS-TNT}), were obtained from the *Drosophila* Stock Center, Bloomington, IN, USA.

Flies were reared on "fly food" containing agar (10g/L), glucose (167mM), sucrose (44mM), yeast (35g/L), cornmeal (15g/L), wheat germ (10g/L), soya flour (10 g/L), molasses (30 g/L), propionic acid and Tegosept. Flies were raised in a 12:12 hour light/dark cycle (LD 12:12 at 25 °C). Oe$^-$ and Control flies were raised in the following conditions: The first 24 hours at 21 °C followed by 96 hours 12:12 at 25 °C. Virgins were collected using CO_2 anaesthesia and aged in plastic vials containing fly food, in same-sex groups of 20 and aged for 5-7 days prior to testing.

Oviposition assay

Mated females and males used as "signal-givers" or "markers", from here on named "caller", were mated in groups of 6 virgin females and 6 virgin males in a disposable Petri dish (55x8mm) containing a patch of fly food (25x1mm) at Zeitgeber Time (ZT) 0 at 21 °C for 2 hours. Virgin females and males used as callers were treated as mated female callers, except that no flies of the opposite sex were added to the disposable Petri dish. At ZT 2 caller flies were transferred to a 90x12mm Petri dish containing two food patches (25x1mm) placed 40 mm apart. Caller flies were either allowed to walk freely in the dish, able to visit both food patches or forced to occupy one of the food patches that were covered under the bottom of a 35x10mm Petri dish, for a period of 6 hours, after which flies and covers were removed. Dishes in which caller mated females had laid eggs during this period, were not used. Naive mated or virgin females and males, from here on named "responder", were transferred at ZT 9 to the 90x12mm dish previously housed with callers and were allowed to move freely in the dish to visit both food patches for 20 hours. The number of eggs laid on each food patch by responders was counted at the end of the experiment. Egg laying site preference was determined using the following equation: $\text{Egglaying preference} = \frac{(\#\text{eggs on marked food patch} - \#\text{eggs on unmarked food patch})}{\text{Total \#eggs}}$

Camera assay

To monitor the position and the time spent at certain locations of the dish of both caller and responder flies, a Logitech 910c webcam with Security Monitor Pro software (Deskshare Inc.) was used. Callers were monitored for 6 and responders were monitored for 12 hours, during which, at every 2 minutes, a photo of the dishes was taken. The position of each fly at the photo was determined by using the software ImageJ 1.48v (Wayne Rashband, USA). Positions in a radius of 27 or less mm around the marked food patches were considered as "near food" while positions in a radius of 27 or less mm around the unmarked food were considered as "away from food". Positions that were monitored that were not near one of the food patches were determined as a position without a choice and were excluded from the calculations. The food preference index was calculated using the following equation: $\text{Food preference} = \frac{(\#\text{positions near food} - \#\text{ positions away from food})}{\text{Total \# positions}}$

Sperm ejection observation

Sperm ejected by caller females was detected by using a MZ10F stereomicroscope equipped with filters for UV light and GFP detection (Leica Microsystems Ltd, Germany). Images were taken using a Leica DC250 camera connected to a computer with Leica Application Suite software.

Collection and analysis of chemicals

To determine the chemical compounds of male or female flies with different mating statuses, flies were anaesthetized by putting them in a fridge for a short period of time. After anaesthetized flies were placed in microvials containing 50µl hexane, standardised with 10ng/ml of octadecane (C18) and 10 ng/ml hexanosane (C26). Ejected sperm was collected using a clean metal pin and transferred to microvials containing the same internal standard solution as above. The microvials were vortexed for 2 minutes after which the flies, but not the sperm, were removed using a clean metal pin.

To determine the chemical compounds that callers leave behind in a dish, 4 Oregon-R mated or virgin females were housed in in a 40mmx10mm glass Petri dish. After 4 hours flies were removed and all sides of the dish were rinsed multiple times with 200µl of hexane, after which the hexane was collected in a 200µl microvial. The extract was reduced to 40µl under a flow of Nitrogen followed by an addition of 40µl of internal standard solution.

The extractions were analysed using an Agilent 7890 gas chromatograph with flame ionization detecor, an Agilent DB-1 column (Diameter: 0.180mm; Film 0.18µm) and a split-splitless injector set at 250°C with 40 ml/min splitless flow. Injector valve opened 1.5 minutes after injection in splitless mode with helium as carrier gas (flow 37.2 cm/sec). The oven temperature program begins at 50°C for 1.5 min, increasing with 10°C / minute to 150°C, after which the temperature increases at 4°C /minute to ultimately reach 280°C and hold this temperature for 5 minutes. ChemStation software (Agilent technologies was used to quantify the the compounds based on peak areas relative to internal standard C26.

Perfuming

For the perfuming experiment, extractions of mated and virgin females were made using the same method as above, except that they were put in a volume of 10µl of pure hexane. The extraction was then reduced to 2µl with a flow of Nitrogen to increase the concentration of the solvent. This solvent was deposited on a circle of 5mm cellulose chromatography paper (Ch1, Whatman) and air dried for 30 minutes to evaporate the hexane. The perfumed paper was then deposited onto one of the food patches, while as a control a same sized paper which was perfumed with hexane only, was put on the other food patch. Verification of the perfuming success was measured by extracting the papers using gas chromatography as described earlier.

Statistical analysis

Graphs and statistical analysis were performed using GraphPad Prism 5 (Graphpad Software Inc., USA) and R 3.2.0 (The R foundation for Statistical Computing). Because the data of all the preferences was not following a Gaussian distribution, exact two-tailed Wilcoxon Signed Rank tests were used to test if responders preferred one of the food patches. The null-hypothesis used in the test was that responders did not prefer one food patch over the other with no preference being valued as 0. The data was arranged as number of eggs (or positions) on the marked side and number of eggs (or positions) on the unmarked side.

Between groups comparisons were determined using a quasibinomial logistic regression on the proportion of eggs/positions on each food patch. The amount of eggs was counted in each experiment and statistically compared using the mean number of eggs. For multiple groups an ANOVA Wilk's lambda with Tukey Kramer two-tailed multiple comparison was used, while with two group comparison a Student's t-test was used.

5. Results

Mated, but not virgin females prefer high-level yeast food

Firstly, to determine if females are able to select an appropriate oviposition site, a camera experiment was done to investigate whether female flies prefer high-level yeast food (35 g/L) (High-quality) to low-level yeast food (0 g/L)(Low quality). Spending more time near one of the food patches would indicate a higher preference towards a certain food patch. Indeed, mated females, but not virgin females, spend more time near the high-level yeast food patch (Figure 1).

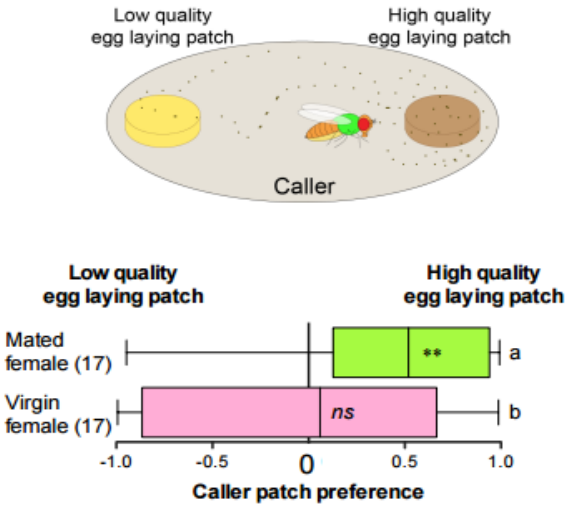

Figure 1. *Locations of caller flies near high- or low-quality food patches during 6 hours monitoring. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; **, p<0.01). Letters right to the box-plot indicate significant difference between groups by logistic regression.*

Mated females prefer a food patch previously visited by another mated female

A camera experiment was done to investigate whether mated females prefer a food patch previously visited by another female to a "fresh" food patch. Firstly, a caller female, mated or virgin, was allowed to freely move between the two different food patches. Next, the caller flies were removed and the low-level yeast food patch was replaced by an identical high-level yeast food patch. Consequently, a mated female responder, was allowed to visit the identical food patches, except for that one of the food patches that had been previously visited by another fly. More time spent near one of the food patches would indicate a preference. Responders showed preference towards food patches previously visited by mated, but not virgin females (Figure 2).

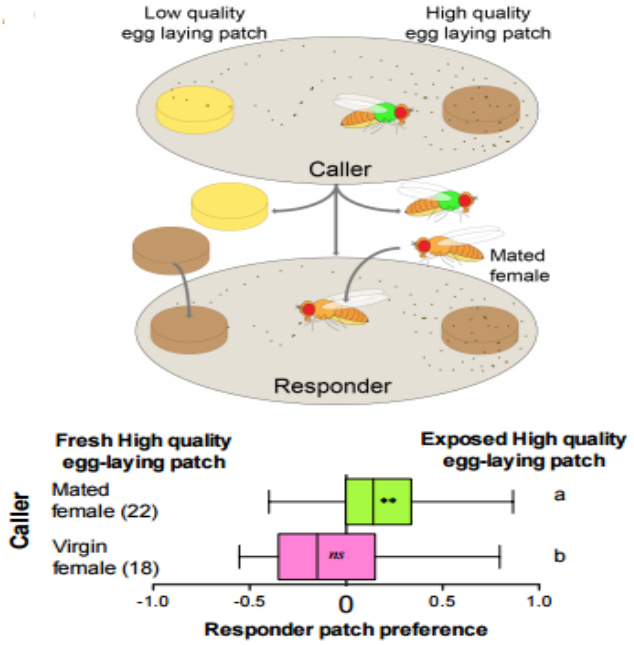

Figure 2. *Locations of responder flies near high-quality egg laying patch, "fresh" or "marked", during 12 hours of monitoring. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; **, p<0.01). Letters right to the box-plot indicate significant difference between groups by logistic regression.*

Mated females prefer to lay eggs on a food patch previously visited by another mated female

An oviposition assay was done to investigate whether responder flies prefer to oviposit on food patches previously visited by other females. Both virgin and mated female were used as caller. More eggs deposited on top of one of the food patches would indicate a preference towards that food patch. Responder mated females showed preference towards the food patch that previously had been visited by mated females. When the food patch previously had been visited by a virgin female, responders preferred the fresh food as oviposition site (Figure 3).

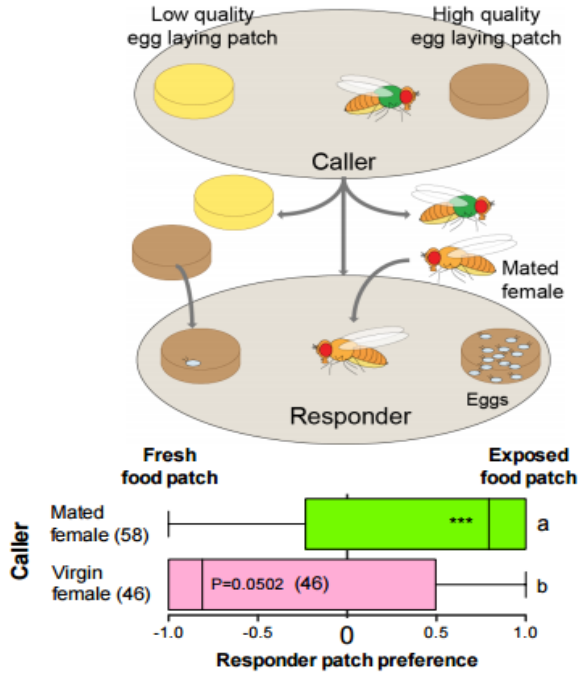

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Figure 3. *Egg-laying preference of a mated female between two high quality egg-laying patches, one of which was previously housed with either a mated or a virgin female free to visit both patches. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by logistic regression.*

Mated females eject excess sperm and mating plug near high-level yeast food

In the search for a possible signal that could be left behind by the mated female in the dish, dishes that had housed callers were observed through the microscope. With this microscope, equipped with UV-light and filter, residues of female ejections were found, i.a. containing excess sperm and mating plug. In a similar assay as that of the camera, the placement of female ejections were determined. Data showed that females prefer to deposit the ejaculate near or on top of high-level yeast food patches (Figure 4).

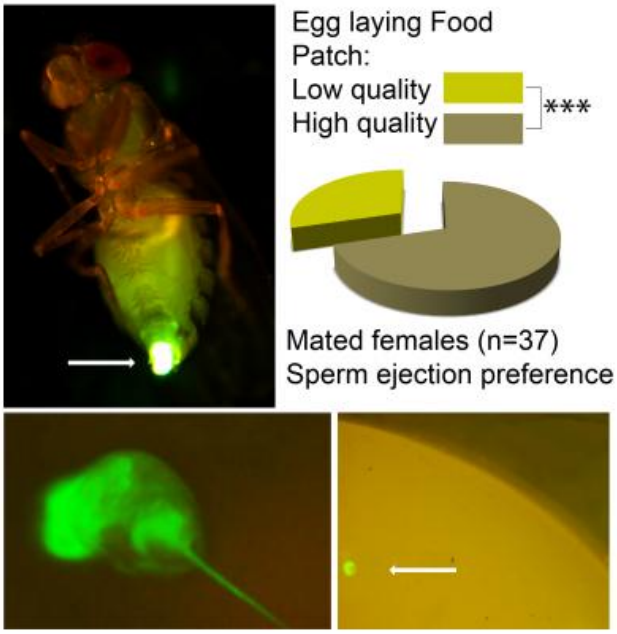

Figure 4. *Top left: Micrgraph of a mated female with green labelled sperm by ProtamineB:GFP, white arrowhead points to ejaculate that is being ejected. Bottom left: Micrograph of a GFP-fluorescing ejected. Top right: Proportion of females that ejected near or on top of low- or high-quality food patches. Bottom right: Micrograph of an ejected ejaculate deposited on a patch of food.*

Flies prefer food patches with ejected ejaculate

The ejected ejaculate and its placement indicated a possible signal. A camera assay was done to monitor if males, virgin and mated females were attracted to food patches that contained ejected ejaculate. A greater amount of positions near the “marked” food, compared with positions near “fresh” food, would indicate a preference towards that food patch. Results show that all groups preferred the “marked” food (Figure 5).

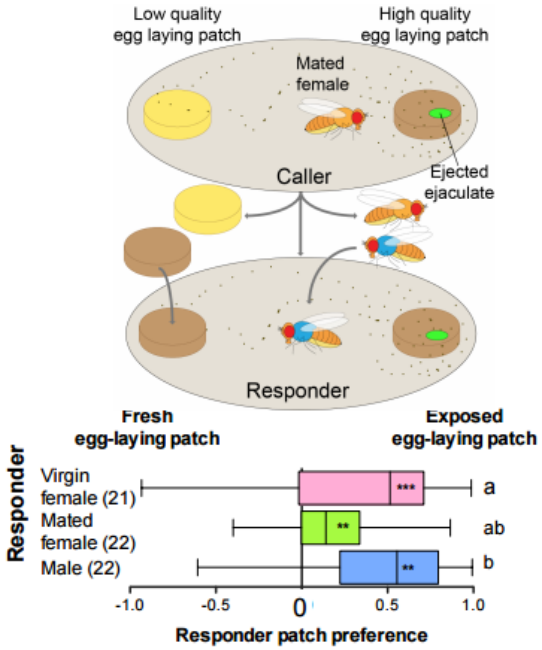

Figure 5. *Mated or virgin females and male responder preference between two food patches, one of which was previously exposed to a mated female. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; **, p<0.01; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

Mated females prefer to oviposit on food patches previously visited by a mated female, containing ejected ejaculate

An oviposition assay was done to found out if the candidate signal also contained possible information on the quality between the two food patches. In this assay the mated female caller was forced to occupy one of the identical food patches and eject, after which a mated female responder was free to oviposit on either food patches. Mated females showed that, even by forcing ejected ejaculates on a certain patch, they prefer to oviposit on food patches that previously had been occupied by mated females and contained ejected ejaculate (Figure 6).

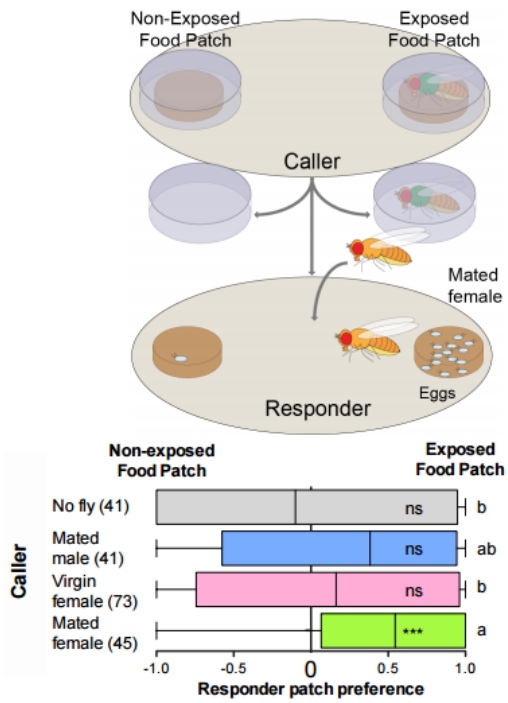

Figure 6. *Egg-laying preference of a responder female between two food patches of identical nutritional quality, one of which was previously forced-exposed to a fly of the indicated genotype and mating status. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

Females obtain and dispose of ejaculate, containing various pheromones, through copulation and ejection

Next, an analysis of the chemical composition of the candidate signal was done, to comprise possible compounds as a candidate. The analysis showed that the aggregation pheromone cVA was present, together with i.a. the cuticular hydrocarbons 7-T, 7,11-HD and 7-P (Figure 7).

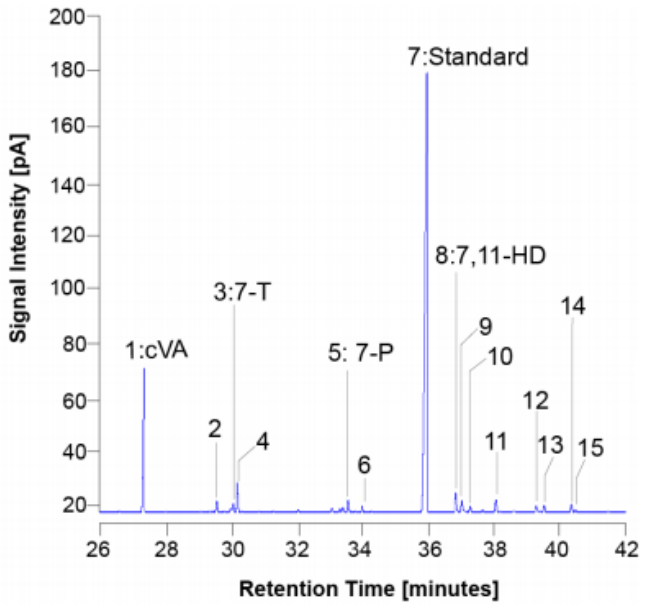

Figure 7. *Chromatogram of an ejected ejaculate. The area under the peak relates to the amount of compound plotted against the retention time. Numbers on the peaks refer to the identification and quantifications in Table S1.*

Another analysis was done to identify that females obtain the ejaculate through copulation and dispose of the ejaculate by ejecting it. Results show that the stage in which a female finds itself, affects the quantity of pheromones she possesses (Figure 8).

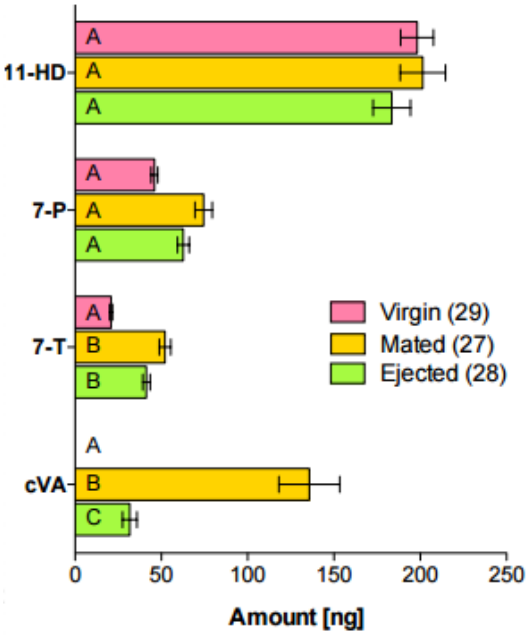

Figure 8. *Mean amount of CHs and cVA in a female with the specific mating status. Ejected: Mated female that has ejected the surplus sperm and mating plug; Mated: Mated female that has yet to eject; Virgin: unmated female. Error bars indicate SEM. Different letters indicate significant differences between groups, by Kruskal-Wallis ANOVA. Details in Table S2.*

Ejected ejaculate as a single factor is not sufficient to induce oviposition preference

To see if ejected ejaculate alone could induce a preference for oviposition towards one of the food patches, an oviposition assay was set up. Firstly, in half of the experimental dishes mated female callers were forced to eject near one of the food patches, after which the caller flies were removed as well as the eject. Then in all dishes ejects of females, that were forced to eject in a small Petri dish (35x10mm), were placed on top of one of the food patches. Preference of oviposition site towards food patches with eject would be indicated by a greater amount of eggs being laid on that particular food patch. Findings were, that ejaculate alone was not sufficient to induce a preference toward a particular site, the presence of a female was also a necessity (Figure 9).

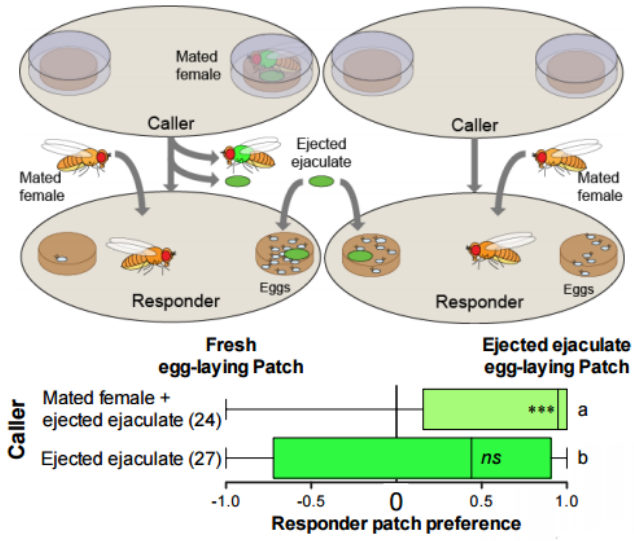

Figure 9. *Egg-laying preference of mated females on identical food patches, one of the patches has been previously visited by either a mated female and ejaculate of another fly or only an ejaculate. Possible ejaculates of female callers were removed to control for manipulation. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

Oviposition preference is influenced by the mating status of the female caller

An oviposition assay was done to disclose if the signal or marking consists of a combination of female pheromones and ejected ejaculate. For this assay, in half of the experimental dishes, we forced a mated

female caller to ejaculate, while in the other half we forced a virgin female caller for the same period of time. Then, we removed both callers and ejaculates. Other females were forced to ejaculate in a small Petri dish (35x10mm), after which the "fresh" ejaculates were transferred on top of the food patch that previously had been housed with either a mated or virgin female. Finally, mated female responders were allowed to move freely between both food patches and choose an oviposition site. Preference of oviposition site towards food patches with females and ejected ejaculate would be indicated by a greater amount of eggs being laid on that food patch. Results show that mated female responders did not prefer the food patch with ejaculate which had been housed with a virgin female to the fresh food patch. Food patches, visited by mated females with replaced ejected ejaculate, did show a preference towards the marked food patch (Figure 10).

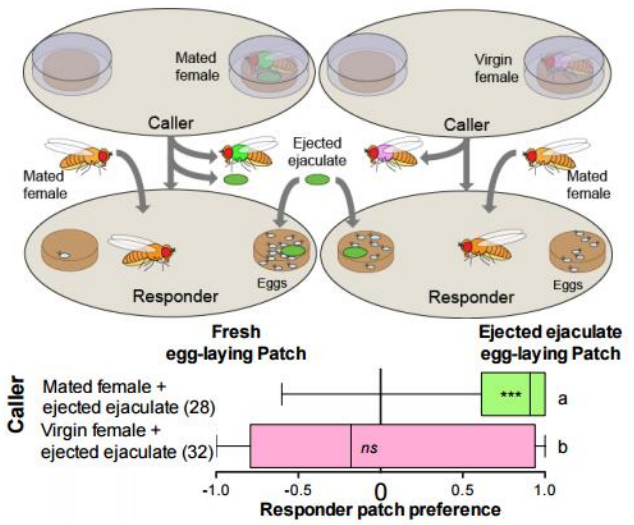

Figure 10. *Egg-laying preference of mated females on identical food patches, one of the patches has been previously visited by either a mated or virgin female. Possible ejaculates of female callers were removed and replaced by an ejaculated of another female to control for manipulation. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

Females leave different chemical compounds behind

The ejaculate contains the same compounds as mated females, however, ejaculate in itself cannot induce a preference to a certain food patch, nor can a virgin female accompanied by addition of an ejaculate. This leads to the assumption that mated females leave a different pheromonal pattern behind than virgin females. To identify the pheromonal patterns that were left behind, extractions of glass Petri dishes that had housed virgin or mated females were analysed. Chemical analyses showed that mated females leave the through male obtained pheromone cVA and increased levels of the CHs 7-T behind, in addition to the CH 7,11-HD that was also left behind by virgin females (Figure 11).

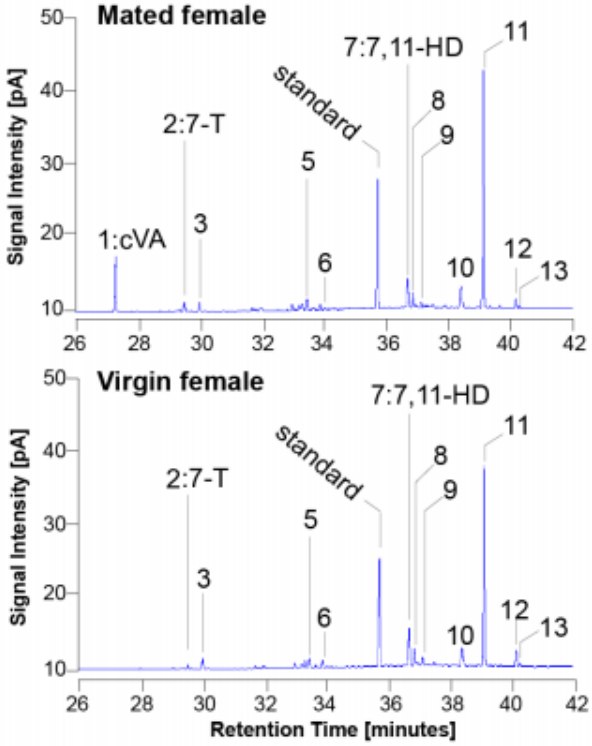

Figure 11. *Chromatogram of a glass dish which had been housed with either 4 mated female or 4 virgin females. The area under the peak relates to the amount of compound plotted against the retention time. Numbers on the peaks refer to the identification and quantifications in Table S3.*

Previous visits of males and virgin females do not influence oviposition preference

Another oviposition assay was set up, to exclude that virgin or mated males, that both have high levels of cVA and 7-T, are able to attract females to oviposit on a certain food patch. Male callers, both virgin and mated, were forced on one of the identical food patches for the same period of time as mated females callers. Female responders were then allowed to move freely and oviposit on both food patches and

preference would be indicated by a greater amount of eggs laid on the forced food patch. Neither virgin, nor mated males were able to induce a preference. As a control, both mated females callers (positive) as well as no caller flies (negative) were done at the same time (Figure 6).
To be absolutely certain that the food patches visited by mated females were more preferred, mated female callers were pitted against virgin female and virgin and mated male callers were forced to occupy one of the identical food patches. All setups resulted in responders preferring to oviposit on patches of mated female callers (Figure 12).

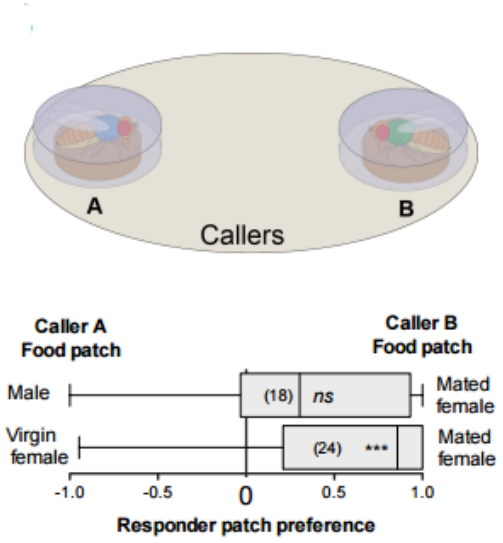

Figure 12. *Egg-laying preference of mated females on identical food patches, on one of the patches we forced a mated female while we forced either a male or virgin female on the other food patch. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

Cuticular hydrocarbons are a necessity for influencing oviposition preference

To verify that the preference towards food patches, which previously had been visited by mated females, was induced by the specific composition of CH's that females leave behind, we did an oviposition assay. In this assay we used various crosses of oe$^-$ flies as callers. Oe$^-$ flies lack oenocytes through genetic excision and therefore are unable to produce CH's. The crosses that were made, resulted in female callers that don't produce any CH's at all (♂oe$^-$ x ♀oe$^-$), don't produce female specific CH's (♂ctrl x ♀oe$^-$), don't produce male specific CH's (♂oe$^-$ x ♀ctrl) or females that do produce CH's (♂ctrl x ♀ctrl). Analyses of the chemical compounds of the crosses verified the success of the crosses (Table S2). These different mated female callers were forced on top of one of the food patches. Next, mated female responders were allowed to choose an oviposition site. Data shows that only ♂ctrl x ♀ctrl female callers induced a significant preference, while the other females did not induce a preference (Figure 13).

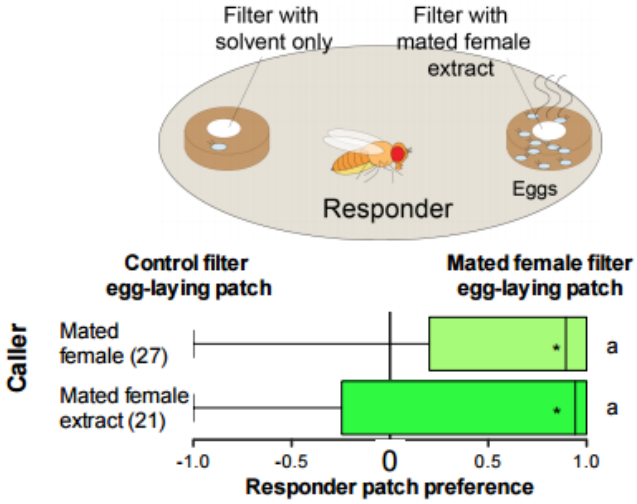

Figure 13 *Egg-laying preference of mated females on identical food patches, one of the food patches have previously been visited by a mated female or contains a paper with mated female extract. A paper with solely solvent has been placed on the other food patch for control.*

Mated females leave a specific blend of pheromones behind

A perfuming assay was done to directly show that the blend that mated female callers left behind, indeed is the factor that induced a preference for an oviposition site. Papers with extracts of mated females were pitted against papers with solvent only. Data shows that responder females preferred food patches with the extract, to the solvent only food patches (Figure 14).

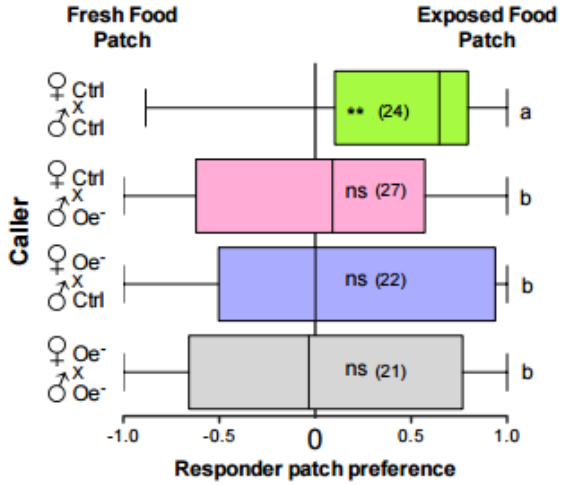

Figure 14. *Egg-laying preference of mated females on identical food patches, on one of the patches we forced a mated female from one of the groups on the left. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

Mated females use metabotropic odorant receptors to detect the pheromonal blend

We concluded from the previous steps that a specific pheromonal blend was left behind by mated females. The next step was to discover by which sensory circuits this pheromonal blend was perceived by other mated females. By an oviposition assay, we allowed mated female callers to leave a pheromonal blend on one of the food patches. As responders, we used mated females that were mutant to the Orco gene. This gene encodes for a co-receptor which is needed for the functioning of odorant receptors (Larsson et al., 2004.; van Naters & Carlson, 2007). Responders that were lacking the Orco gene (Orco$^-$) did not show preference for an oviposition site, while responders that had the Orco gene rescued, by getting a copy of a wild-type, did show preference (Figure 15).

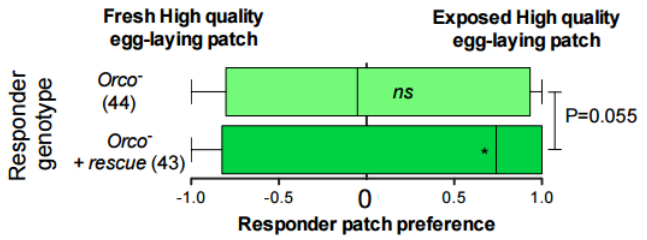

Figure 15. *Egg-laying preference of mated females on identical food patches either mutant to the Orco^1 gene or with a rescued gene, on one of the patches we forced a mated female caller before. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

Mated females use Ionotropic odorant receptors to select an oviposition site.

The indication that mated females need a functioning odorant co-receptor for detecting an appropriate site, leaded to the question if they also use other parts of the olfactory sensory system. Another part of this system does not require the above described functioning co-receptor, namely the Ionotropic receptors (Ir) (Rytz et al., 2013). In an oviposition assay the necessity of half of the ionotropic receptors was tested, by testing for a co-receptor of Irs (transcribed by the gene *Ir8a*) that allows flies to identify products of yeast fermentation. One of the mated female responders, either a mutant for Ir8a or a mutant which had been rescued by a wild-type copy, were allowed to choose between an unvisited food patch and fresh food patch. Results of the assay showed that responders that were mutant for the Ir8a gene did not show preference, while the rescued responders did (Figure 16).

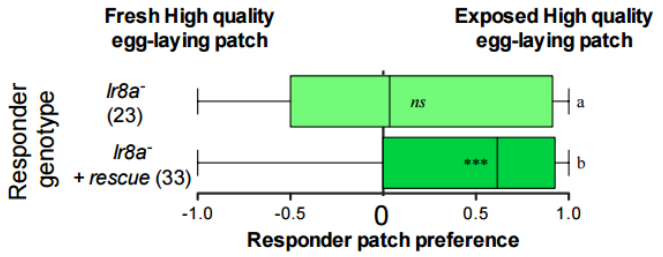

Figure 16. *Egg-laying preference of mated females on identical food patches either mutant to the Ir8a- gene or with a rescued gene, on one of the patches we forced a mated female caller before. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

What odorant receptors do mated female flies use for pheromonal blend identification?

The next step was to determine which odorant receptors specifically were needed by mated females to perceive the pheromonal blend. Odorant receptors that were selected as candidates were the receptors Or65a and Or67d. Both Or65a and Or67d receptors are known to be able to bind cVA and this binding induces behavioral responses such as aggregation and courtship in males (Liu et al., 2011.). In females the binding of cVA to Or56a affects remating (Lebreton et al., 2014.).

In an oviposition assay a variety of mated female responders were used with either a distorted or a properly working signaling function of the specific receptor neuron. Distortion of the function was accomplished by creating transgenic lines with a Gal4 gene under the promoter of the receptor neuron gene, to flies with UAS-Kir2.1, thereby creating flies that have the signaling of the receptor neuron blocked(w^{1118};Or65a-gal4/UAS-Kir2.1), or to flies with UAS-TNT, which results in ablation of the receptor neuron(w^{1118};Or65a-gal4/UAS-TNT). As a control to a possible effect of Gal4, transgenic lines of Gal4 flies with w^{1118} wild-type flies were set up. (w^{1118};Or65a-gal4/+ and w^{1118};Or67d-gal4/+). As a control to UAS-Kir2.1 or UAS-TNT a transgenic line of UAS-Kir2.1 or UAS-TNT and w^{1118} wild-type flies was set up (w^{1118};+/UAS-Kir and w^{1118};+/UAS-TNT). Results show that flies with distorted Or56a receptor neurons did not prefer either food patch, while the controls to Gal4 did show preference towards the previously visited food patch. The control to the UAS-Kir2.1 and UAS-TNT did not show preference.

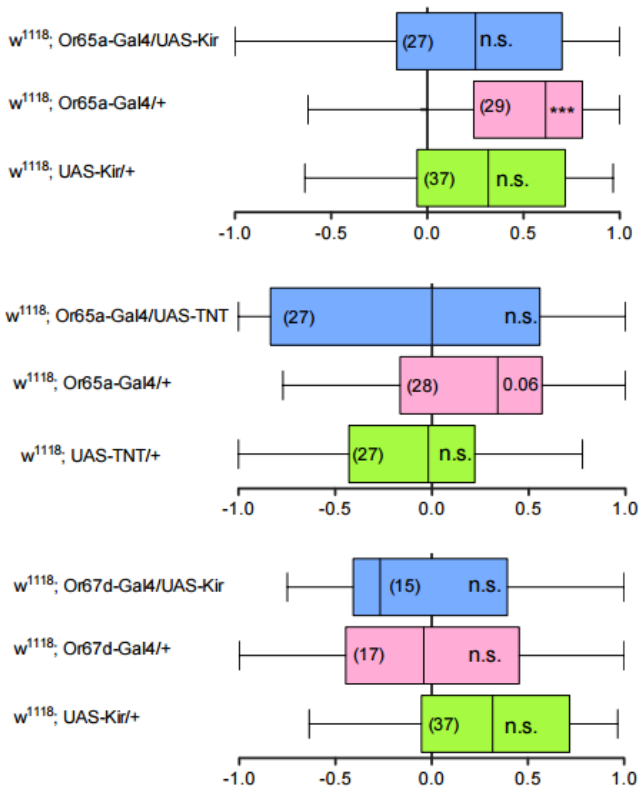

Figure 17. *Egg-laying preference of mated females on identical food patches , of which one previously had a mated female forced on. Left of the boxplot shows the genetic background of the particular groups. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

Are gustatory receptors involved for detecting the blend of pheromones by mated females?

In addition to perception through the olfactory sensory system, we searched for possible perception through the gustatory sensory system. The gustatory receptor Gr32a was of interest, because it is used to detect CHs in *D. melanogaster*, especially the CH 7-T (Moon et al., 2009). Transgenic lines were set up in the same way as for the above described Or67d receptor, resulting in lines with either blocked receptor neurons or lines as control to Gal4 and UAS-Kir2.1 with functioning receptor neurons. These transgenic flies were then used as mated female responders in an oviposition assay. The flies with

blocked Gr32a receptor neurons (w^{1118};Gr32a-Gal4/UAS-Kir2.1) did not show preference, while the control to the Gal4 (w^{1118};Gr32a-Gal4/+), did show preference. The control to UAS-kir2.1 flies did not show preference towards either fresh or previously visited food patch.

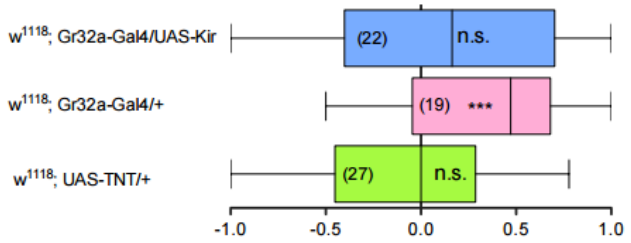

Figure 18. *Egg-laying preference of mated females on identical food patches, of which one previously had a mated female forced on. Left of the boxplot shows the genetic background of the particular groups. Two-tailed exact Wilcoxon signed rank test. (ns: non-significant; ***, p<0.001). Letters right to the box-plot indicate significant difference between groups by a logistic regression.*

6. Discussion

We conclude from our results that *Drosophila melanogaster* mated females prefer to spend time near food that is rich in yeast (Figure 1). These results are in accordance with other research that indicated that mated females require nutrients from yeast for the production of eggs. The fact that virgin females did not show a preference for food patches either high or low on yeast, in this respect, makes sense (Ribeiro and Dickson, 2010). Mated female responders also preferred to spend time and oviposit near food patches that had been previously visited by other mated females (Figure 2 & 3). These findings indicate that caller females indeed leave information behind and that responder females are able to sense these previous visitations. Remarkably, it seems that the response of mated females to food which had been previously exposed to virgin females, resulted in a preference to oviposit on the fresh food patch (Figure 3). While the result was not significant (p=0.0502), the trend that this result shows could be explained by the fact that the presence of virgin females, not only could attract male flies for mating, but also would increase competition over food while not contributing to the survival of the responder female offspring. Therefore, laying eggs on the unvisited patch would be a better choice in terms of fitness. Although, we can't clearly explain this trend, follow up experiments in which we forced virgin males and females on to one of the food patches, did not result in a preference towards the unvisited food patch (Figure 6).

While we also hypothesized that these preferences towards previously visited food patches might be due to excess sperm that caller mated females leave behind, results of the experiments of the sperm ejection contradicted this hypothesis(Figure 4 & 5). In addition to these findings, male or female flies with different mating status, with or without the ejection of excess sperm, did not induce such a preference towards oviposition site in the mated female responders' response (figure 9). Prior to our findings, we considered cVA as a candidate pheromone in the signaling of suitable oviposition sites, partly because of its use in different types of behavior and because it is known as an aggregation pheromone. However, we conclude from our results that cVA alone does not induce this response in responder mated females. This is mainly because cVA is effective over longer distances and our oviposition essay is done in small dishes. Because the essay is done in small compartments, they are completely saturated with cVA (Figure 11). The fact that only previous visitations of mated females or a mated female extract on a food patch induced a preference towards oviposition, tells us that the information that is left behind by caller females and the perception of this signal by responder females, is highly specific (Figure 10, 12, 13, 14).
Taken together, our results indicate that the information that is left behind by mated female callers can be considered to be an honest signal, as flies of the opposite sex and with different mating status were not able to induce such a response (Figure 6 & 12) . This is because the production and spreading of pheromones is energy consuming and that mated females have a highly specific pheromonal profile (figure 7 & 8), which is hard to imitate by other flies of different mating status. The consideration that it's an honest signal, together with findings from earlier research on social learning in *Drosophila melanogaster*, leads us to believe that mated females indeed use social information for determination of a suitable oviposition site. Thus, selection of a suitable oviposition site by *Drosophila melanogaster* mated females is mediated through the process of social learning.

To uncover the mechanism underlying the perception of suitable oviposition sites we set up experiments in which we tested the involvement of several receptors of the olfactory sensory system. A group of receptors, Ionotropic receptors, play a role in the identification of yeast fermentation. The results of the experiments in which mated females either lacked a copy of the Ir8a gene or that had the gene rescued, indicate that the ability to detect the fermentation products of yeast is important in the process of oviposition site selection (Figure 16). Mated females lacking the gene did not show a preference to either food patches. This result leads us to two hypotheses. Either females need a working copy of the Ir8a gene for the detection of yeast, which is an important factor in the determination of an oviposition site and without the ability to detect yeast, social information is not used for oviposition site selection. Or females are not able to perceive the signal of the mated caller females without a copy of the Ir8a gene, thus the Ir8a protein plays a role in the perception of the mix of pheromones. An experiment in which neither of the food patches contain yeast should determine which of the above hypotheses is true.

Next we tested the need of the ubiquitous co-receptor protein which is transcribed by the Orco gene. This co-receptor forms heteromeric complexes with odor receptor proteins and is important for proper functioning of the odorant receptors. As expected, in absence of the Orco gene, mated females did not show a significant preference towards either food patch, while mated females that were rescued with the Orco gene, did show a preference in oviposition towards the previously visited food patch (Figure 15). This result indicates that the co-receptor plays a role in the perception of the signal and implicates that working odorant receptors might play a role in the perception of the signal.

To further specify individual odorant and gustatory receptors and their contribution to oviposition behavior we selected candidate receptors. As shown in the results, we set up experiments that tested the role of the receptor neurons of Or65a, Or67d and Gr32a. Unfortunately, no conclusions can be drawn from the results of these experiments, as the control groups to both UAS-TNT and UAS-kir2.1 did not show a significant preference towards previously visited sites (figure 17 & 18). A possibility for these unexpected results could be that flies in the stock no longer contained flies with the indicated modification. Another possibility would be that there was a compromise during the setup of bottles of the UAS lines, while we consider this to be highly unlikely. Lastly, the modification itself, the insertion of the UAS element, could result in flies that show different behavior. While we don't know the exact reason, we assume that the unexpected results of the control group for UAS is due to a faulty stock bottle. To determine whether this assumption is true, flies of the stock itself have to be checked on their genome and the experiments with our candidate receptors have to be redone.

To further clarify the neuronal network that is involved in the decision making during selection of an oviposition site, more experiments have to be done. Unfortunately, due to the limited period of time that was available for this research, we cannot perform these additional experiments ourselves. If this research is to be continued by others, we recommend that methods in which the involvement of the odorant receptors is tested, is looked into more closely. The method that was used in this research is based on blocking the functioning, or ablation of the specific receptor neurons by a Gal4-UAS system. While this method is successfully used in many other researches, the blocking of the function of the neuron or ablation of the neuron itself could have an effect on other olfactory neurons in their proximity. In turn, the functioning and effect that these nearby odorant receptor neurons could alter and result in a difference in the behavior of the fly. Alternatively, experiments can be done with flies that do not produce the receptor protein by blocking the expression of the gene itself, leaving the specific odorant receptor neurons intact, as was done in the experiments in which we tested the need for a functioning odorant co-receptor.

Finally, we think it's noteworthy to describe an observation we did during a couple of our experiments. While we still hypothesized that the excess sperm and mating plug might be used as an indication of a suitable oviposition site towards other mated females, we observed remarkable behavior of mated female callers prior to ejection. With the excess sperm and mating plug still attached, mated female callers showed behavior that we would describe as a motion of the hind legs along the distal coronal side of the abdomen, towards the caudal end of the abdomen. After this motion, the flies rubbed both hind legs against each other and repeated these two motions multiple times. We did not observe this behavior in flies that already disposed of excess sperm and mating plug, but we don't exclude that these flies might have shown this particularly behavior. Other research has opted that this behavior is shown as part of grooming and removal of the excess sperm and mating plug, squeezing the plug slowly out (Lee et al., 2015). While this theory makes sense to us, we believe that this motion of the hind leg is not expressed to remove the plug and sperm, but a process in which female flies cover themselves and their surroundings in pheromones and CHs. This is mostly due to the fact that the area of the abdomen that is touched by the hind legs is also the area which holds the oenocytes. We also observed the exact moment at which a mated female fly disposed of the mating plug and excess sperm. This was done in a comparable way to the motion of egg-laying (Yang et al., 2008). We would describe the motion in which the flies disposed of the plug as pressing the end of the abdomen, and thereby part of the mating plug, on a surface while walking forward, smearing out the excess sperm and mating plug alongside. Further research has to be done to determine the nature of these particular behaviors and determine whether these behaviors play a role in the marking of an oviposition site.

All our results taken together, we conclude that *Drosophila melanogaster* mated females use social information left by other mated females for oviposition site selection, and therefore this can be seen as behavior that is learned through social learning. The implications of this research could be of importance at multiple levels. Our results are of importance for science, because it shows that Drosophila *melanogaster,* which is considered to be a model organism, use social information for oviposition site selection. This result could contribute to improvements in reproduction rate and thereby enhancing research that is done in this model organism in general. In addition, our findings confirm the earlier findings that *Drosophila* are able to learn through social learning. From an economical perspective, our findings can contribute to improvement in pest control. *Drosophila* is one of the major pests in food industry, costing this industry yearly billions of dollars. Through this new knowledge on oviposition site selection behavior, improvements to traps can be made to contend with *Drosophila* as a pest.

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8. Supplementary data

Number peak	Compound	Oregon-R (15)	Control (14)	Oenocyteless (14)
1	*c*VA	56,8 ± 11,0	65,90 ± 7,26	82,58 ± 11,3
2	7-Triscosene	3,4 ± 0,7	2,52 ± 0,4	0 ± 0
3	nC23	4,0 ± 0,7		
4	7-Pentacosene	3,9 ± 0,8		
5	7,11-HD	7,0 ± 1,2		0 ± 0
6	2-Me C26	4,9 ± 0,8	0.88 ± 0,4	0 ± 0
7	9-hepacosene	1,0 ± 0,3		
8	nC27	2,0 ± 0,8		
9	nC28	1,2 ± 0,6	1,38 ± 0,32	0 ± 0
10	7,11-ND	3,3 ± 0,7		
11	2-Me C28	0,4 ± 0,1		
12	2-Me C30	0,6 ± 0,3		

Table S1: Chemical content of single ejected ejaculate. *Mean (ng) ± SEM of compounds in the ejected ejaculate. Oregon-R: Oregon-R male mated with an Oregon-R female, Control: Control females mated with Control males, Oenocyteless: Oenocytless female mated with Oenocyteless female.*

	Compound	Mated (27)	Ejected (28)	Virgin (29)	*d.f.*	*Test*	*P value*
1	*c*VA	135.81 ± 17.65 [a]	31.73 ± 4.16 [b]		2	68.35	<0.001
2	7-Triscosene	52.14 ± 3.29 [a]	41.36 ± 2.19 [a]	20.91 ± 0.83 b	2	54.417	<0.001
3	nC23	78.83 ± 4.40	69.61 ± 3.76	69.99 ± 2.39	2	2.735	0.255
4	nC24	18.95 ± 1.47	17.43 ± 1.11	20.17 ± 1.08	2	1.898	0.387
5	2me C24	37.21 ± 2.49	34.82 ± 2.26	32.33 ± 1.31	2	1.418	0.468
6	9-Pentacosene	40.31 ± 2.31	36.35 ± 2.22	34.71 ± 1.39	2	4.203	0.122
7	7-Pentacosene	74.50 ± 5.00 [a]	62.77 ± 3.36 [a]	46.05 ± 1.93 b	2	32.555	<0.001
8	nC25	59.70 ± 4.08	53.59 ± 3.28	51.61 ± 1.84	2	1.284	0.526
9	7,11-HD	201.52 ± 13.13	183.60 ± 10.84	198.20 ± 9.55	2	0.558	0.757
10	2meC26	116.90 ± 7.51	105.30 ± 10.04	96.04 ± 5.38	2	3.871	0.144
11	nC27	21.07 ± 1.61	18.03 ± 1.38	18.52 ± 0.82	2	2.123	0.346
12	7,11-ND	81.72 ± 3.72	77.41 ± 5.13	81.26 ± 3.98	2	0.866	0.649
13	2-Me C28	23.45 ± 1.99	21.41 ± 1.68	21.41 ± 0.85	2	0.367	0.829

Table S2: Analyses by Gas Chromatography of Drosohopila melanogaster females. *Ejected: Mated female that has ejected the surplus sperm and mating plug; Mated: Mated female that has yet to eject; Virgin: unmated female. Mean ± SEM (ng). Different letters indicate significant differences between groups, by Kruskal-Wallis Anova. Number of replicates are shown next to the different groups*

Number Peak	Compound	Mated female	Virgin Female	*d.f.*	*Test*	*P value*
1	*c*VA	14.71 ± 2.47	-	-	-	-
2	7-T	3.92 ± 0.71	0.20 ± 0.20	1	13.322	0.003
3	nC23	4.29 ± 0.76	3.03 ± 0.77	1	1.354	0.248
4	9-Pentacosene	0.90 ± 0.45	0.57 ± 0.32	1	0.253	0.48
5	7-Pentacosene	5.93 ± 0.91	2.16 ± 0.74	1	7.229	0.219
6	nC25	1.29 ± 0.56	1.10 ± 0.51	1	0.07	0.52
7	7,11-HD	13.99 ± 1.31	14.79 ± 1.85	1	0.124 (A)	0.969
8	2meC26	8.11 ± 1.08	7.20 ± 0.93	1	0.414 (A)	0.414
9	nC27	3.79 ± 1.53	3.72 ± 1.48	1	0.061	0.955
10	7,11-ND	5.35 ± 0.72	5.95 ± 1.06	1	0.221 (A)	0.876

Table S3: Analyses by Gas Chromatography from glass dishes. *The data shows average amounts with SEM (ng). Kruskal Wallis with Dunns post hoc test values are shown except for (A) which are F values from an Wilk's lambda ANOVA with Tukey Kramer post hoc test.*