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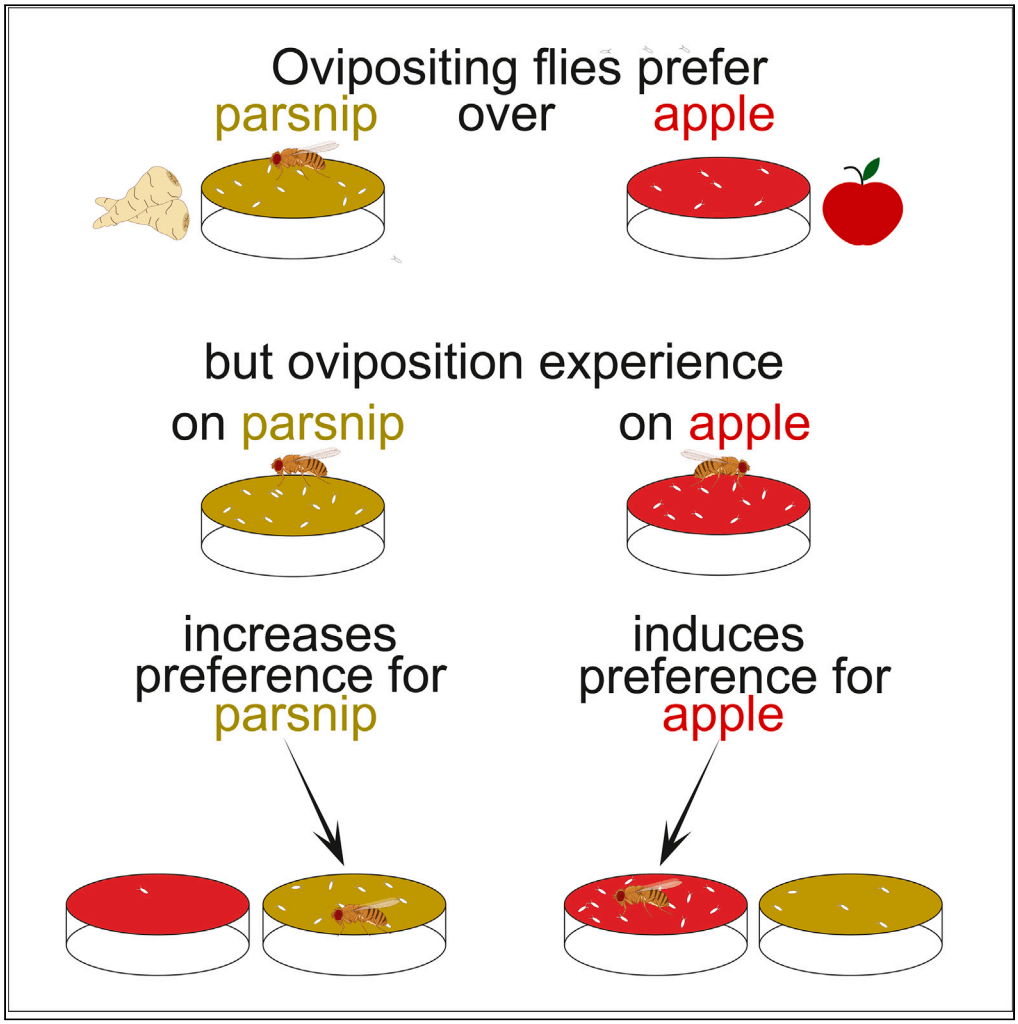

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Highlights

In *Drosophila*, oviposition on a substrate can change a fly's oviposition preference

Just sensing the substrate is not sufficient to change oviposition preference

Oviposition experience can form a long-term memory

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Oviposition experience affects oviposition preference in *Drosophila melanogaster*

Julio Otárola-Jiménez,[1,2] Nandita Nataraj,[1] Sonja Bisch-Knaden,[1] Bill S. Hansson,[1,3] and Markus Knaden[1,3,4,*]

SUMMARY

Learning, memorizing, and recalling of potential ovipositing sites can influence oviposition preference. Classical conditioning experiments have shown that vinegar flies can learn the association of olfactory, gustatory, or visual stimuli with either positive or negative unconditioned stimuli. However, less is known about whether similar associations are formed in an ecologically more relevant context like during oviposition. Our experiments reveal that *Drosophila melanogaster* females increase their preference for substrates they have already experienced. However, this change of preference requires that the flies not only smelled or touched the substrates but also oviposited on them. We furthermore show that such an experience results in long-term memory lasting for at least 4 days, i.e., a duration that so far was shown only for aversive conditioning. Our study thus reveals a different form of associative learning in *D. melanogaster* that might be highly relevant for settling novel ecological niches.

INTRODUCTION

Most insects do not provide direct care for their offspring. Therefore, it is crucial that female insects lay their eggs at places that provide good conditions for their offspring's survival. However, oviposition is an adaptive process,[1] as the decision and choice of an oviposition site can change depending on the situation of the female insect. The vinegar fly, *Drosophila melanogaster*, is a generalist ovipositing on different kinds of fermenting fruits or vegetables. Apart from substrate-related olfactory, gustatory, and visual cues[2–5] (that also can be driven by the presence of microorganisms,[3,6] of *Drosophila* larvae,[7–9] predators[10] or parasitoids[11]), other factors have been identified that can govern the female's decision (e.g., age,[12] ambient temperature,[13,14] or mating status[15]).

It is well known that vinegar flies can learn the association of olfactory,[16,17] gustatory,[18,19] or visual[20–22] cues with either positive unconditioned stimuli such as sugar rewards or negative ones such as electric shocks. However, it is not well known whether similar associations are formed in an ecologically more relevant context, i.e., when a fly approaches an oviposition source, evaluates it, and finally decides to lay eggs.

Associative learning can be formed by appetitive or aversive conditioning, which differ not only in the reinforcement used[23] but also in the neuronal circuits and neurotransmitters involved.[24–26] Therefore, memory is expected to be affected by the type of associative learning. However, in humans, aversive learning shows stronger acquisition than appetitive learning, and there seem to be no differences regarding the extinction of both memories.[27] In insects, a variety of paradigms with different ecological contexts or conditioned stimuli have been used for appetitive and aversive learning, which makes a general conclusion regarding the duration of memory after aversive and appetitive learning difficult.[28]

Oviposition learning in insects has been studied for several years, with a main focus on *Lepidoptera*.[29–36] However, it was also shown that the apple maggot fly (*Rhagoletis pomonella*) changes its oviposition preference due to oviposition-related learning.[37] Here, we show that oviposition preference of an individual vinegar fly is affected after experiencing an oviposition substrate. We furthermore investigate how, e.g., experience duration, the number of eggs laid, and the sensory input during the experience affect the change of oviposition preference and how long the formed memory lasts. Having established oviposition learning in the model insect *Drosophila*, with all its genetic tools, will facilitate future investigations regarding the neuronal circuits involved in this ecologically relevant insect behavior.

We find that

- Flies that have laid several eggs on either apple or parsnip substrate turn their preference toward this substrate.
- Just smelling the substrate or feeding from it is not sufficient to change the oviposition preference. Instead, female flies need to get access to the substrate and lay eggs on it to alter their subsequent oviposition preference.
- Associative learning is involved in the change of oviposition preference, with the olfactory cues acting as putative conditioning stimuli while the unconditioned stimuli during the oviposition process remain open.
- Egg-laying experience can result in long-term memory that lasts for at least 4 days.

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RESULTS AND DISCUSSION

Individual female flies rarely switch between oviposition substrates

Female *D. melanogaster* lay their eggs on various substrates.[38–41] We first asked whether gravid females (*Canton-S* wild-type flies) have an innate preference for agarose substrate containing either apple or parsnip puree when given a choice between the two. We offered these two substrates based on pilot experiments that revealed acceptance of both as oviposition substrates in *D. melanogaster*. Six-day-old virgin female flies were mated with males of the same age and were afterward kept individually. The flies were provided with a sucrose solution on a foam *Drosophila* plug, from which they could feed, but that did not elicit egg-laying behavior. After 24 h, individual female flies were transferred to a cage containing two small Petri dishes, one filled with apple puree and one filled with parsnip puree (Figure 1A). After 24 h, the number of eggs laid on each substrate was counted. Interestingly, only 17% of the flies tested (5 out of 30) laid eggs on both substrates, while the majority chose to lay eggs on only one of the substrates (n = 19 on parsnip, n = 6 on apple), resulting in an almost bimodal distribution of oviposition indices with a significant preference for parsnip (Figure 1B; this innate preference for parsnip was observed in all control experiments that we conducted along with the study). Therefore, we categorized the oviposition choice as eggs laid only on parsnip, only on apple, or on both substrates (Figure 1B, donut chart) (see Figure S4 to check the oviposition preference index of all the experiments). Interestingly, flies that oviposited on both substrates laid significantly more eggs in total than those flies that laid on a single substrate (Figure S3A). Furthermore, those flies that switched between substrates did not exhibit an oviposition preference neither for apple nor for parsnip (Figure S3B). The results show that maybe the choice of grouping eggs in one substrate over dividing them into two different places could be based on the number of eggs that the fly lays, which is basically explained by the extensive genetic variability among females.[42,43] In conclusion, most females avoided switching between oviposition substrates and had an innate preference for parsnip over apple.

The observed innate preference for parsnip over apple may be due to the high abundance of terpenes in the headspace of parsnip puree (Figure S1; Table S1), some of which have been shown to drive oviposition preference in *D. melanogaster*.[44] However, alcohols and esters present in the headspace of apple puree can also stimulate oviposition.[4,45] Apart from volatile cues, female flies are known to evaluate other factors when searching for a suitable oviposition site, preferring soft substrates[46,47] and the presence of nutritious microbes in fermenting fruit.[1,3,6,48] However, our substrates differed neither in softness nor in their content of living microbes as they were pasteurized. Another factor that could potentially influence the choice of an oviposition site is the increased preference of mated flies for a high-protein diet to support egg production.[49,50] This effect might have driven the innate preference for the, according to the manufactureŕs information, protein-rich parsnip over the sugar-rich apple in our study (although also sugar content can affect oviposition choice[3,51–55]).

The finding that most flies laid all their eggs on either parsnip or apple while only a few flies laid eggs on both substrates (Figure 1B) might be due to an idiosyncratic oviposition preference, with a minor subgroup of flies strongly preferring apple and the majority preferring parsnip. Studies on idiosyncratic behavior point out that genetic differences, even among flies of the same strain, can trigger significant differences in innate behavior.[56–58] Such individuality has recently been shown for learning capacity in *D. melanogaster*.[58] However, the observed binary choice might also be affected by learning. Flies that, for whatever reason, lay their first egg on one of the given substrates could increase their preference for this substrate and end up laying all their eggs on it.[36]

Oviposition experience can alter oviposition preference

Oviposition experience has been shown to influence the oviposition preference of the tobacco hawkmoth *Manduca sexta*[36] and the silk moth *Bombyx mori*.[29] This changed preference potentially can result in so-called oviposition constancy, where a female insect, despite its general acceptance of several hosts, finally ends up laying most of its eggs on only one host species (corresponding to the well-described flower constancy in bees).[35] We asked whether individual female flies would also change their oviposition preference after laying eggs on a given substrate. Therefore, 5-day-old virgin females were mated and afterward kept on sucrose solution for 24 h. These flies, however, were next allowed to oviposit on either apple or parsnip puree for 6 or 24 h ("training") before their preference was tested in a choice assay as described earlier. Interestingly, already 6 h (resulting on average in 5–6 eggs laid; Table S2) of experience on apple was enough to increase the flies' preference for oviposition on apple (Figure 1C). Although also the number of flies ovipositing on parsnip increased after 6 h of training on parsnip, the overall difference with and without training with parsnip was not significant (Figure S2A). Training for 24 h on either apple or parsnip (resulting on average in 20–25 eggs laid; Table S2) increased attraction to both of the corresponding substrates (Figures 1C and S2A). Taking into account that repetitive experience spread over time, i.e., so-called spaced training, affects the type of memory that is formed[59] and that female flies do not lay all eggs at once, but rather evaluate the oviposition substrate every time an egg is laid,[60] every single oviposition event potentially can be regarded as a single training. Therefore, the 6- or 24-h experiences resulting in several oviposition events in our assay would be comparable to spaced training. It has been shown that insects are capable of single-trial learning.[28] *Manduca sexta*, for example, increases its preference for a given host plant already after it has laid only a single egg.[36] We, therefore, also let flies oviposit only one egg on either apple or parsnip and tested their preference afterward. In neither case, however, did we observe increased attraction toward the substrate they oviposited on before. For a reason that we do not understand, flies after laying one egg on parsnip even turned their preference toward apple (Figure S2C). Apparently, only repeated oviposition experience can result in oviposition constancy, where flies prefer to lay eggs on substrates they have already experienced. In classical associative learning, sensory cues (conditioned stimuli [CS]) are associated with rewards or punishments (unconditioned stimuli [US]).[30,61] In our experiments, flies could experience either the sensation of food nutrients or stimuli related to oviposition itself as a reward (US). In order to disentangle these two hypotheses, we designed three experimental paradigms. First, we exposed mated flies to the headspace of either apple or parsnip but did not provide an oviposition substrate (Figures 1D and S2A). Therefore, the experience was restricted to olfactory cues. In a second experiment we provided virgin flies with full

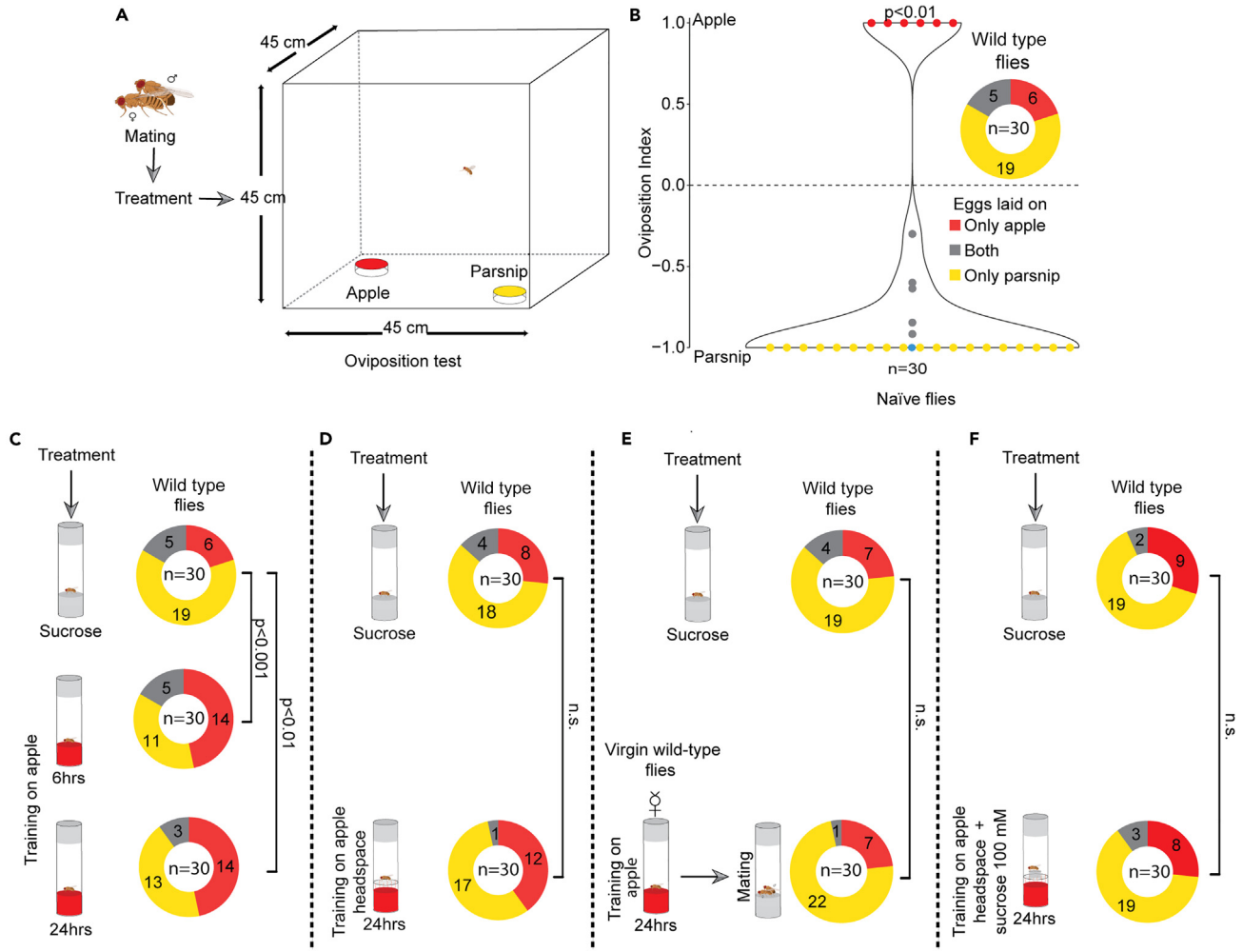

Figure 1. Oviposition experience affects innate preference

(A) Schematic of the paradigm to test oviposition preference of individual *D. melanogaster* females.

(B) Innate preference of naive female flies that were kept for 24 h on sucrose solution before choice tests. Violin plot, index of innate oviposition preference of individual flies (dots) calculated as (#eggs on apple-#eggs on parsnip)/total #eggs; light-blue dot, median of $n = 30$ flies; significant difference of median against zero ($p \leq 0.01$, Wilcoxon signed-rank test); donut chart, number of flies that laid eggs only on apple (red), only on parsnip (yellow), or on both substrates (gray).

(C) Oviposition preference of flies after training for 6 or 24 h on apple or on sucrose as control (same data as in B).

(D) Oviposition preference of mated flies after exposure to apple headspace (a mesh between fly and apple puree prevented direct contact). The few flies that laid eggs during exposure to headspace were excluded from former analysis.

(E) Oviposition preference of flies that experienced apple for 24 h as virgins and immediately afterward mated on sugar solution.

(F) Oviposition preference of mated flies that experienced apple headspace for 24 h with access to sucrose 100 mM in agarose 3% (w/v).

(C–F) Chi-squared goodness-of-fit test was used to compare oviposition preference of trained flies with that of control flies. In (C) Bonferroni-Holm correction for multiple tests was used. n within donut charts: number of flies tested; number in each section of donut: number of flies that laid eggs on each substrate. For total number of eggs laid per fly see Table S2.

access to apple, i.e., the substrate where we before had observed the strongest learning effect. Although the flies had direct contact to the substrate, they did not oviposit during the training phase due to their virgin state. Only afterward (before the test phase) we allowed them to mate. (Figure 1E). In a third experiment mated females were exposed to apple headspace and could access sugar as feeding cue (Figure 1F). Hence, the flies were exposed to apple headspace while they could feed from sucrose 100 mM in agarose 3% (w/v) but, despite being mated, did not oviposit during the training due to the hardness of the agarose (Figure 1F). After being exposed to the three different experiences, the oviposition preference was tested. We found that neither smelling the substrate as a mated fly, nor experiencing full contact with the substrate as a virgin fly, nor experiencing headspace during feeding as a mated fly was sufficient to change the fly's oviposition preference (Figures 1D–1F). These results thus suggest that contact to *and* oviposition on the substrate during training are necessary to establish oviposition constancy.

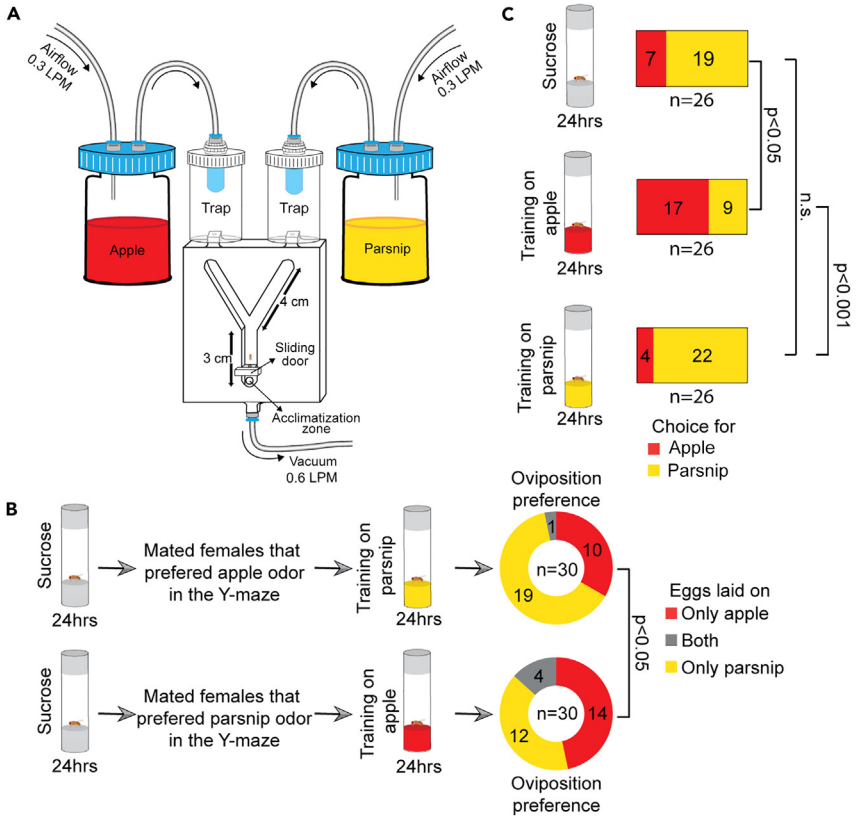

Figure 2. Odor preference is changed by oviposition experience

(A) Schematic of the Y-maze used to test olfactory attraction of individual females before and after oviposition experience. Flies were released into the acclimatization zone of the entrance arm; after 1 min, the opening of a wind-permeable sliding door enabled flies to reach the bifurcation of the Y-maze, where they could choose between the headspace from both substrates. Entering one of the traps was regarded as decision. Each fly had 5 min to decide.

(B) Females that innately preferred apple or parsnip odor were trained on the other food substrate for 24 h, and their oviposition preference was tested afterward. Chi-squared goodness-of-fit test was used to test the preferences of apple-trained vs. those of parsnip-trained flies. n in donut chart: number of tested flies; number in each section of donut: number of flies that laid eggs on each substrate.

(C) Olfactory preference of naive flies and flies that oviposited for 24 h either on apple or parsnip before. n below each bar graph: number of tested flies; number in each section of bar graph: number of flies that chose each odor. Two-sided Fischer's exact test with Bonferroni-Holm correction for multiple tests was used.

We next asked whether innate preferences for the headspace of either apple or parsnip would affect oviposition preference and learning. We, hence, used a Y-maze connected to headspace of apple in one arm and to parsnip in the other arm (Figure 2A) to select flies for their innate olfactory preference. Those flies that preferred apple odor were then trained on parsnip substrate for 24 h, whereas flies that preferred parsnip odor were trained on apple substrate. Afterward, the oviposition preference of the trained flies was tested (Figure 2B). In both groups, flies neglected their innate olfactory preference and instead preferred to oviposit on the substrates they had oviposited on before.

In all our experiments, it is likely that associative learning is involved. Therefore, the question arises, which CS and US are required.[62] In our paradigm, the conditioned stimuli could be the odor of the substrate. To test this, we used the Y-maze (Figure 2A), where the preference of female flies was tested after 24 h of oviposition experience on either apple or parsnip. The odor preference for the odor of apple or parsnip indeed changed depending on the training (Figure 2C), indicating that the odor preference is conditioned by oviposition experience. It should be mentioned here that apple and parsnip puree differ not only regarding their headspace but also slightly regarding their color. It might, hence, be possible that the flies in addition also used visual stimuli as a conditioned stimulus. However, the nature of the unconditioned stimulus, i.e., the reward, remains unclear. Nutrients such as sugars have been widely used as rewards for appetitive memory.[63,64] However, sensing those nutrients was insufficient to induce learning, as neither flies that experienced apple puree as virgins nor mated females that experienced apple headspace paired with sucrose (while they, however, could not oviposit) changed their oviposition preference. Therefore, it is likely that the unconditioned stimulus is directly linked to oviposition behavior. Future studies might reveal whether the act of oviposition itself is rewarding for female flies, as has been shown for ejaculation in male flies,[65] or whether cues detected, e.g., by sensory hairs on the female's ovipositor,[2,66] act as the rewarding, unconditioned stimulus in this context.

Complex associations learned by female insects during oviposition can influence subsequent oviposition decisions in moths,[29,31,36] beetles,[67] and apple maggot flies.[37] *D. melanogaster* has previously been shown to preferentially oviposit on substrates on which they were

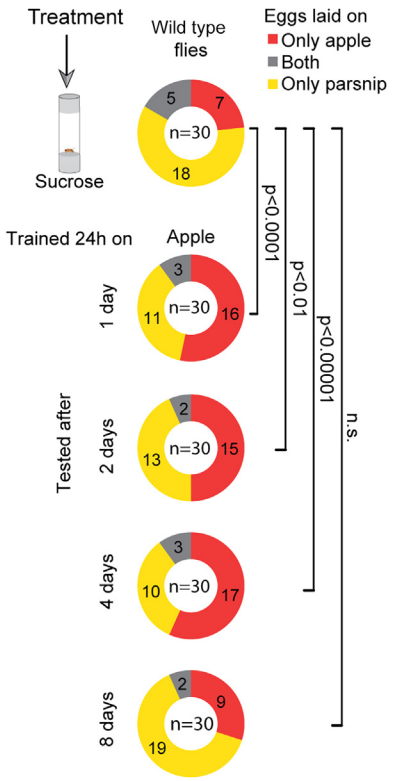

Figure 3. Oviposition experience results in long-term memory

Oviposition preference of individual flies trained for 24 h on apple puree and tested 1, 2, 4, or 8 days after training. Chi-squared goodness-of-fit test was used to test trained flies versus control flies, with a Bonferroni-Holm correction for multiple comparisons, also considering the use of the same control group in Figure S2B. n within donut charts: total number of flies tested; number in each section of donut: number of flies that lay eggs on each substrate. For total number of eggs laid per fly see Table S2.

reared as adults.[38,39] However, we found a change in oviposition preference only in those flies that were mated and had already oviposited on the substrate when they experienced it in the tests (Figures 1C and S2A), whereas experience gained before mating did not influence oviposition preference (Figure 1E). The mushroom bodies are involved in associative learning in flies,[68] and the contribution of different substructures of this brain region is quite well understood.[69,70] However, previous studies have used classical conditioning, in which a conditioned stimulus, e.g., an odor, was coupled with a well-defined unconditioned stimulus (either sugar or electric shock). Hence, future studies might reveal whether the same neural substrate is responsible for associative learning in the context of oviposition.

Oviposition preference remains altered for days following experience

Apple maggot flies[37] and tobacco hawkmoths[36] also prefer substrates on which they have already laid eggs, and both insects can remember those substrates for several days. Therefore, we next asked how long *D. melanogaster* would remember an oviposition experience. To address this question, we let flies lay eggs for 24 h on either apple or parsnip. Instead of testing their oviposition preference directly afterward, we isolated the flies from potential oviposition substrates for 1, 2, 4, or 8 days and then tested their preference for apple or parsnip (Figures 3 and S2B). Until day 4 after training on apple, the flies still exhibited an increased preference for apple. However, after 8 days the flies showed again their innate preference for parsnip, suggesting that the "apple-memory" lasted for at least 4 days after training but was gone after 8 days (Figure 3). To our knowledge, such long-term memory in *Drosophila* so far was observed in aversive learning only.[25,71–73] Interestingly, when flies were trained on parsnip, the increased preference for this substrate that was observed directly after training vanished already after one single day (Figure S2B). During training on parsnip, flies laid similar numbers of eggs like those that were trained on apple (Table S2). Therefore, different numbers of eggs laid cannot be the reason for the difference in LTM (long term memory) formed during apple and parsnip training. However, the apple food used has almost 4 times more sugar (mainly sucrose and fructose[74]) than the parsnip food. It is known that the nutritional value of sugars is evaluated and learned by *Drosophila*.[75] So, we next asked whether the low sugar content of parsnip might have inhibited the formation of an LTM. Sucrose or fructose was, therefore, added to parsnip food to mimic the sugar concentration of apple food. The now sugar-enriched parsnip was used during training and oviposition test. However, we still did not observe any increased preference for parsnip after 24 h (Figure S2B), suggesting that other factors are involved in the differential formation of LTM after training on apple

and parsnip. These findings suggest that female *D. melanogaster* can retain the memory of an oviposition site for at least 4 days. However, the duration of this memory seems to depend on the type of substrate.

In conclusion, we show that innate oviposition preference of *D. melanogaster* females can be changed by learning, with flies preferring substrates on which they have already laid eggs. Hence, our study reveals a novel form of associative learning in *D. melanogaster* that might be relevant for the colonization of new ecological niches.

Limitations of the study

As mentioned in the text, the experiments conducted in this study did not identify the unconditioned stimulus of the proposed new type of associative learning. As feeding experience without oviposition did not yield in any change of oviposition preference, oviposition itself might work as the reinforcing unconditioned stimulus. However, whether this is the case and how this stimulus would become detected remains elusive.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110472.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.O.J. and M.K.; methodology, J.O.J., N.N., and M.K.; investigation, J.O.J.; resources, M.K. and B.S.H.; analysis, J.O.J, S.B.-K., and M.K.; writing—original draft, J.O.J.; writing—review & editing, S.B.-K., M.K., and B.S.H.; visualization, J.O.J. and M.K.; supervision, M.K. and B.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
D(+)-sucrose (>99.5% p.a.)	Carl Roth GmbH	Cat#4621
D(-)-fructose (>99.5% p.a.)	Carl Roth GmbH	Cat#4981
Experimental models: Organisms/strains		
D. melanogaster (Dmel)	Hansson Lab Strain	N/A
Software and algorithms		
R 4.2.2	https://www.r-project.org/	RRID:SCR_001905
RStudio 2023.09.1	https://www.r-project.org/	RRID:SCR_000432
GraphPad Prism 9.4.0	https://www.graphpad.com/	RRID:SCR_002798

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Markus Knaden (mknaden@ice.mpg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Drosophila melanogaster

Flies were reared on yeast-cornmeal-agar medium (standard food), kept at 25°C with 70% humidity. A 12h:12h light:dark cycle was established. Flies were collected and sexed during the first 3 h after eclosion, using ice as an anesthetic. Groups of 5 female and 5 male flies were kept separately in small vials with standard food until the start of the experiments. Flies between 5-7 days old were used during experiments, except method indicates other age. Flies were treated under the same conditions for all experiments. The *D. melanogaster* (wild type) stock used in this study and associated references is listed in the key resources table.

For 1L of the standard food, the following ingredients were used: beet syrup (118 g), brewer's yeast (11g), agar powder (4.1 g), hot water (540 mL), corn grits (95 g), propionic acid (2.4 mL), Nipagin 30% (3.3 mL), and cold water (378 mL).

METHOD DETAILS

Oviposition substrates

Apple and parsnip purees (Hipp, Germany) were used as oviposition substrates. For each experiment, a freshly prepared substrate was used, in which the puree was combined with agarose to reach similar softness of both substrates. To prepare 100 g of substrate, 0.4 g of agarose (Agar-Agar, Kobe I Art.Nr 5210.2 Carl Roth GmbH) and 27 mL of distilled water were boiled. When this mixture reached ~60°C, 80 g of the puree was added and mixed. Then, 4.5 g of this mixture were transferred into narrow *Drosophila* tubes (28.5x95 mm, Dominique Dutscher) or small Petri dishes (35x10 mm, Ref.No. 734-2314, VMR). Small Petri dishes were stored in the refrigerator at 10°C until being used in oviposition assays. According to nutritional information from the manufacturer, parsnip puree has 2.7 g of sugars and 0.6 g of protein per 100 g, and apple puree has 10.7 g of sugars and 0.2 g of protein per 100 g. Both substrates differ slightly of color.

D(+)-sucrose (>99.5% p.a., Carl Roth GmbH) and D(-) fructose (>99.5% p.a., Carl Roth GmbH) were used to see their effect of the sugar-contect on long-term memory after training on parsnip (Figure S2B). Per 100 g of freshly prepared parsnip substrate, 6 g of sucrose or fructose were added to reach the same amount of sugars described for the apple substrate.

Collection of volatile chemical compounds (SPME)

The headspaces of both substrates were collected using solid-phase microextraction (SPME). A fiber coated with 50 μm Divinylbenzene layer and 30 μm Carboxene/Polydimethylsiloxane layer (gray fiber, 57328-U/Supelco) was used and conditioned at 280°C for 30 min before every extraction. 4.5 g of either apple or parsnip in small Petri dishes (35x10 mm, Ref. Nr. 734-2314, VMR) were kept in a closed GLS 80® Baffled bottle of 250 mL (222x101 mm, GLS 80 Duran) 1 hour before sampling the headspace with the fiber. Each headspace was sampled for 20 min and immediately analyzed by GC-MS (Figure S1).

Gas chromatography conditions

Volatile organic compounds (VOCs) were analyzed with an Agilent 6890N chromatograph coupled with an Agilent 5975B mass selective detector, equipped with an HP-5MS (5% phenyl-polymethylsiloxane, 30m x 0.25 mm, 0.25 μm film thickness; Agilent 190191S-433) capillary column. A constant column flow of 1.2 mL/min 99.999% pure helium was used as the carrier gas. The injector and detector temperatures were 250°C and 260°C, respectively. The oven temperature program was 40°C for 3 min, afterwards increased at 5°C/min to 260°C, and was finally kept for 5 min. The ionization energy of the mass spectra was recorded at 70eV. The quadrupole mass detector and ion source temperatures were 150°C and 230°C, respectively. The transfer line temperature was 280°C, and the mass spectra were scanned in the *m/z* range 29-350 amu at intervals of 1s. Identification of volatile compounds was performed by comparing the mass spectra with those from the database (NIST 17), and retention index (RI) values of the detected compounds were determined by comparison of the retention times with those for a series *n*-alkanes saturated standard, which was analyzed in a separate analysis under the same conditions (Figures S1B and S1C; Table S1).

Innate egg-laying assay of individual fly

A virgin female and a virgin male, 6 days old (wild-type), were placed together in a small vial with standard food. Once the female was mated, it was transferred to a small vial with a plug soaked in a 5% w/v sucrose solution and kept there for 24 h ("preparation time"). After this time, the mated female fly was placed in a cage (made of a synthetic mesh net, 45x45x45 cm) with two 3 cm Petri dishes containing 4.5 g of the oviposition substrates (apple or parsnip). The substrate position was always randomized. The innate test for oviposition preference was set at ZT8-ZT9 and was carried out for 24 h at 23°C, 70% relative humidity, and a 12:12 h light:dark cycle. Afterwards the number of eggs laid on both substrates was counted, and the oviposition index was calculated using the formula: (# eggs on apple - # eggs on parsnip)/total # of eggs (Figure 1B).

Training assay for 24 h

5-day-old individual virgin female and male flies (wild-type), were placed together in a small vial with standard food. After mating and 24 h of preparation time (see above), individual mated female flies were placed in another small vial containing 5 g of the oviposition substrate (either apple or parsnip) for 24 h ("training time"). Immediately after training, the number of eggs laid was counted, and the now experienced females were individually placed in a cage (made synthetic mesh net, 45x45x45 cm) with two 3 cm Petri dishes containing 4,5 g of the oviposition substrates (apple and parsnip, with randomized position), and their oviposition preference was tested as described for test of innate preference above (Figures 1C and S2A).

Training assay for 6 h

In order to test whether 6 hours of experience are enough to change oviposition preference, in a similar experiment 6-day-old virgin females and males were mated and the females were afterwards individually placed in a small vial with 5% w/v sucrose solution for 18 h of preparation time. Afterwards the female flies could experience either apple or parsnip substrate for 6 h. Immediately after this training, oviposition preference was tested as described for test of innate preference above (Figures 1C and S2A).

Training assay until the first egg laid

In order to test whether oviposition preference can be affected by a single egg laid, the flies were treated as before (training for 6 hours), but the training was interrupted after the flies had laid the first egg (which usually happened withing the first 2 hours. Every 20 min, the small vials with the single female flies were checked on a Leica MZ16 FA stereomicroscope, equipped with a mercury lamp Leica 106 z, using a Planachromatic 1x. Once the first egg was observed, the fly was immediately placed inside a cage and its oviposition preference was assessed as described above (Figure S2C).

Training assay restricted to substrate headspace

The following modifications followed the same steps as the training assay for 24 h. This time, however, a plastic mesh containing 0.5% m/v agarose was placed 2 cm above the oviposition substrate in the small vial used during training. Flies were exposed under the same conditions as in previous training but without access to the substrates for 24 h. Afterward, the flies' oviposition preference was tested (Figures 1D and S2A).

Training assay with virgin flies

In order to test whether oviposition behavior is needed to change oviposition preference, we allowed female flies to access the substrate only before they were mated, i.e. their experience included full access but no oviposition. For that, individual 5-day-old virgin females were placed in a small vial with a plug soaked in a 5% w/v sucrose solution and kept there for 24 h ("preparation time"). The females were afterwards transferred to another small vial containing 5 g of the apple oviposition substrate for 24 h ("training time"). After training, the virgin females were placed in a small vial with a plug soaked in a 5% w/v sucrose solution, where they were allowed to mate with a virgin male of the same age. Immediately after mating, their oviposition preference was assessed as mentioned before (Figure 1E).

Training assay with substrate headspace and sucrose 100 mM

In order to test whether flies change their oviposition preference, when they experience the substrate headspace while being allowed to feed from sucrose, 5-day-old mated females were placed in a small vial with apple substrate and a mesh that separated the substrate from the female. A small cup with 100mM sucrose dissolved in 3% agarose (w/v) was placed over the mesh, where the females could feed on but, due to the hardness of the agarose did not lay any eggs. Females were kept in this vial for 24 hours and then transferred into the cage to assess the oviposition preference as mentioned before (Figure 1F).

Y-maze assays

In order to test whether oviposition experience affects the preference for the headspace of a given substrate, we tested flies before and after experience with the substrate in Y-maze assay. The dimensions of the Y-maze can be found in Figure 2A, which has a metallic structure of Aluminum covered with two transparent plastic lids. Two 300 ml/min airflows were used and bubbled through a deionized water reservoir to create a moist airflow. One moist airflow was connected to a bottle with 120 g of apple puree. Another airflow was connected to a bottle with 120 g of parsnip puree. Afterward, both airflows passed through plastic traps. Finally, the airflows reached both arms of the Y-maze. The odor preference of individual flies was measured based on which trap the flies entered within 5 min. For each replicate, a single fly was put into the Y maze with an aspirator and allowed to acclimatize for 1 min. Experiments were conducted between ZT5 and ZT9. Only flies that reached the odor-trap within 5 min of test were used for the analysis. After five tested flies the odorants were switched between trials to control for a potential side bias.

Long-term memory assays

In order to test for long-term memory, we provided mated females with 24h experience on a given substrate as described above, but after the training period, the individual flies were placed in a small vial with a plug soaked in a 5% w/v sucrose solution and kept there for one, two, four or eight days ("waiting time"). Immediately after the waiting time, the flies' oviposition preference were tested as described (Figures 3 and S2B). Flies that laid eggs during the waiting period were discarded from the analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis tests, sample sizes, and corrections for multiple comparisons are given in the text and figure legends. Statistical tests and data visualization were performed with R (R version 4.2.2 (2022-10-31 ucrt)) and GraphPad Prism (version 9.4.0 (2022-06-07). For the control groups (i.e. naive flies), only flies that laid more than 2 eggs in the oviposition test were used. For the experiments with oviposition experience (training for 24 hours), only flies that lay more than 10 eggs during training were used.