Ethanol-guided behavior in *Drosophila* larvae

Isabell Schumann¹, Michael Berger², Nadine Nowag¹, Yannick Schäfer², Juliane Saumweber³, Henrike Scholz² & Andreas S. Thum¹,³*

Chemosensory signals allow vertebrates and invertebrates not only to orient in its environment toward energy-rich food sources to maintain nutrition but also to avoid unpleasant or even poisonous substrates. Ethanol is a substance found in the natural environment of *Drosophila melanogaster*. Accordingly, *D. melanogaster* has evolved specific sensory systems, physiological adaptations, and associated behaviors at its larval and adult stage to perceive and process ethanol. To systematically analyze how *D. melanogaster* larvae respond to naturally occurring ethanol, we examined ethanol-induced behavior in great detail by reevaluating existing approaches and comparing them with new experiments. Using behavioral assays, we confirm that larvae are attracted to different concentrations of ethanol in their environment. This behavior is controlled by olfactory and other environmental cues. It is independent of previous exposure to ethanol in their food. Moreover, moderate, naturally occurring ethanol concentration of 4% results in increased larval fitness. On the contrary, higher concentrations of 10% and 20% ethanol, which rarely or never appear in nature, increase larval mortality. Finally, ethanol also serves as a positive teaching signal in learning and memory and updates valence associated with simultaneously processed odor information. Since information on how larvae perceive and process ethanol at the genetic and neuronal level is limited, the establishment of standardized assays described here is an important step towards their discovery.

Communication with the environment through chemical signals is an essential process for the survival of most if not all organisms. Specialized signal transduction pathways are used to detect chemical cues and convert information into neuronal activity that induces appropriate behavioral output¹. Important insights into principals of chemosensory perception and information processing are provided by genetically modifiable organisms such as the fruit fly *D. melanogaster*²–⁵. This includes also the larval central nervous system with its simpler structure consisting of only about 10,000 neurons. Larvae provide access to combinations of genetic tools, robust behavioral assays, the possibility of transgenic single-cell manipulation, and even connectome data of the central nervous system⁶–¹⁴. Various studies have identified chemosensory stimuli that larvae perceive from their environment. Most odorants, are attractive to larvae in a dose-dependent manner¹⁵–¹⁷. Likewise, larvae show dose-dependent responses to gustatory cues¹⁸–²². Even the characteristics of the substrate seem to influence larval chemosensory responses²³,²⁴. However, to understand how larvae orient in their complex chemosensory environment, further studies are needed on additional environmental occurring stimuli, such as ethanol.

Most *Drosophila* species are saprophagous and feed on decaying sweet substrates like rotting fruits, which contain ethanol produced by natural fermentation²⁵. Adult flies exhibit acute ethanol responses similar to those of mammals: as ethanol concentration increases, flies exhibit locomotor stimulation, loss of postural control, and eventually sedation²⁶. With repeated exposure, adults develop tolerance to the effects of ethanol²⁷. It is even assumed that *D. melanogaster* has altered its ecological niche to benefit from food sources characterized by a higher alcohol concentration²⁸. *D. melanogaster* has an unusually high alcohol dehydrogenase (Adh) activity within its genus, which allows to deal with higher ethanol levels to occupy microhabitats that are not accessible to other species such as *Drosophila simulans*, which have a lower Adh activity²⁹. In addition, larvae are even able to perceive and selectively consume food containing ethanol when they are infected by parasitic wasps³⁰. A higher ethanol in the larval hemocoel due to increased consumption of ethanol enriched food leads to enhanced death of the growing parasites and thus increases the larval survival rate. This means that *D. melanogaster* larvae can adjust their alcohol consumption depending on the particular situation, and use it as a kind of medical treatment. Accordingly, ethanol is an important ecological parameter, which has selected to the establishment of specific larval sensory systems, physiological adaptations and related behaviors.

¹Department of Genetics, Leipzig University, 04103 Leipzig, Germany. ²Department of Biology, University of Cologne, 50674 Cologne, Germany. ³Department of Genetics, Institute of Biology, Faculty of Life Sciences, Leipzig University, Talstraße 33, 04103 Leipzig, Germany. ⁴email: andreas.thum@uni-leipzig.de
Therefore, it is plausible that several studies have been able to show that *D. melanogaster* larvae preferentially migrate to substrates that contain ethanol. For instance, different wild-type strains derived from Australian populations revealed strong preferences for 6% ethanol31–33. Even 17% ethanol was attractive for two larval strains that were either homozygous for the AdhF or the AdhS allele15. AdhF and AdhS describe two functional allozymes that exist in natural populations of *Drosophila* species on several continents35,36. The polymorphism is maintained by natural selection; while AdhF is showing a higher enzymatic activity for ethanol, AdhS is more resistant to higher environmental temperatures37. However, there are also studies in which ethanol is not attractive to larvae38,39, which complicates the interpretation of the various results. Therefore, we analyzed ethanol guided behavior by using a set of simple and robust experimental designs.

In recent years, a set of well-defined larval behavioral assays was established to investigate substrate choice, feeding, survival, and associative learning and memory.14,18,19. It is therefore possible to analyze how larvae react to individual chemical components of its environment. Fructose, for example, provides nutrition and acts as a positive teaching signal during learning20,21. On the other hand, many chemicals that humans categorize as bitter are also repulsive to the larvae (for example among others caffeine, denatonium, or quinine) and can act as a negative teaching signal (caffeine, quinine)18,19,22,24. Sodium chloride even proved to be dichotomous, being attractive and a positive teaching signal at low concentrations and repellent and a negative teaching signal at high concentrations41,42. Using a set of standardized assays, we have now addressed the effect of ethanol on larval behavior. Our results support previous studies suggesting that the odorant ethanol is attractive to larvae in a dose dependent manner at moderate naturally occurring concentrations and has a positive effect on larval survival. The larval ethanol choice is based on smell and taste. Finally, ethanol can be used in associative olfactory learning paradigms to establish appetitive memories as it provides a positive teaching signal.

Results

*Drosophila melanogaster* larvae are attracted to ethanol. Several studies have repeatedly shown that *D. melanogaster* larvae are attracted to ethanol31–33. However, in almost all studies there are slight differences in the applied assays, used substrates and mode of ethanol presentation. This might influence the observed results. In some cases ethanol is attractive and in others not35,38,39. Therefore, we first reanalyzed the attractiveness of ethanol in a widely applied standardized assay. We analyzed the preference of wild-type Canton-S to ethanol by observing their approach behavior to a substrate that contains different concentrations of ethanol (Fig. 1). The ethanol concentration ranged from 1 to 50%. Ethanol concentrations above 50% prevent the agarose plates from solidifying and thus could not be analyzed. We found that Canton-S and w1118 larvae are attracted to ethanol in a dose-dependent manner (Fig. 1b). The attractiveness of ethanol peaked at around 4–10% ethanol for Canton-S. For 10% and 20% ethanol Canton-S-larvae showed higher preferences than w1118 larvae. Additionally, we observed the attractiveness to 8% ethanol of the Canton-S larvae for 120 min (Fig. 1c). The attraction to ethanol remained equally stable and no sedative effect on larval locomotion was seen during this prolonged ethanol exposure. However, larvae respond differently over time to 20% ethanol. The initial attractiveness turns into an aversion after about 15 min of ethanol exposure (Fig. 1c). To investigate the influence of ethanol pre-exposure on the substrate choice of larvae we compared how animals reared on standard food (containing 1% ethanol) versus animals reared for one or two generations on ethanol-free food respond to 8% ethanol (Fig. 1d). Canton-S larvae grown for one or two generations on ethanol-free food preferred the ethanol containing site similar to larvae raised on standard food. These results show that *D. melanogaster* larvae are attracted to ethanol in a concentration-dependent manner.

Ethanol attraction changes during post-embryonic development. The life cycle of *D. melanogaster* comprises three larval stages (L1, L2 and L3), clearly separated by two molting stages, which occur approximately 48 and 72 h after egg laying (AEL). To understand whether ethanol choice changes during larval development, we analyzed eight groups of Canton-S larvae from 36 – 120 h AEL every 12 h for their 8% ethanol attraction (Fig. 2). We chose this concentration because it was most preferred by the larvae in the first experiment (Fig. 1). Each of the tested groups showed an attraction to ethanol (Table 1). However, during the first and second molt, the preferences are reduced (Fig. 2, Table 1). Furthermore, in L3 larvae the preference decreases with age.

Ethanol has nutritional benefit for the larva. Ethanol can be used as energy source (reviewed in43). However, the nutrient gain for the larva appears to be lower, as survival rates on ethanol substrates are lower than those of adult *Drosophila*4,45 and also than those of larvae on sugar diets49. To complicate matters, ethanol is pharmacologically active and in higher concentrations toxic and needs to be neutralized46–48. These diametrical differences in the applied assays, used substrates and mode of ethanol presentation. This might influence the observed results. In some cases ethanol is attractive and in others not35,38,39. Therefore, we first reanalyzed the attractiveness of ethanol in a widely applied standardized assay. We analyzed the preference of wild-type Canton-S to ethanol by observing their approach behavior to a substrate that contains different concentrations of ethanol (Fig. 1). The ethanol concentration ranged from 1 to 50%. Ethanol concentrations above 50% prevent the agarose plates from solidifying and thus could not be analyzed. We found that Canton-S and w1118 larvae are attracted to ethanol in a dose-dependent manner (Fig. 1b). The attractiveness of ethanol peaked at around 4–10% ethanol for Canton-S. For 10% and 20% ethanol Canton-S-larvae showed higher preferences than w1118 larvae. Additionally, we observed the attractiveness to 8% ethanol of the Canton-S larvae for 120 min (Fig. 1c). The attraction to ethanol remained equally stable and no sedative effect on larval locomotion was seen during this prolonged ethanol exposure. However, larvae respond differently over time to 20% ethanol. The initial attractiveness turns into an aversion after about 15 min of ethanol exposure (Fig. 1c). To investigate the influence of ethanol pre-exposure on the substrate choice of larvae we compared how animals reared on standard food (containing 1% ethanol) versus animals reared for one or two generations on ethanol-free food respond to 8% ethanol (Fig. 1d). Canton-S larvae grown for one or two generations on ethanol-free food preferred the ethanol containing site similar to larvae raised on standard food. These results show that *D. melanogaster* larvae are attracted to ethanol in a concentration-dependent manner.

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Larvae perceive ethanol as an olfactory cue. Initially we performed substrate choice experiments that allowed larvae to get into direct contact with ethanol in addition to smelling the odorant source (Figs. 1, 2). To disentangle the olfactory from contact cues we refined our behavioral approach by presenting ethanol in custom-made Teflon containers with perforated lids. This eliminates a physical contact with the substrate to specifically address the olfactory response of larvae (Fig. 4). We compared the attraction of 8% ethanol to three well-known attractive odorants: 1-octanol (1-OCT), amyl acetate (AM), and benzaldehyde (BA)15–17.

*Canton-S* larvae showed olfactory preference for all four odorants (Fig. 4b). Thus, *Drosophila melanogaster* larvae perceive ethanol as an attractive olfactory stimulus. In the next step we analyzed whether the larva can detect other odorants in a homogeneous ethanol background, which is a basic requirement for further odorant-ethanol learning experi-

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**Figure 1.** Ethanol attraction of *Drosophila melanogaster* larvae. (a) Scheme of the experimental procedure. Larvae were allowed to choose within 5 min between control agarose and a substrate containing different concentrations of ethanol ranging from 1 to 50%. (b) Wild-type *Canton-S* (red) and mutant w^{1118} (white) larvae are attracted to ethanol following a Gaussian-shape like dose response curve. The highest behavioral response (preference index (Pref)) was seen at 8% ethanol for both strains (Pref*{Canton-S} = 0.62, ci = 0.53–0.68, Pref*{w^{1118}} = 0.60, ci = 0.51–0.74). All groups are significant different from zero, except for 1% w^{1118} (one-sample t test, p < 0.17). Multiple comparison indicates a significant difference between the two genotypes for 10% and 20% ethanol (Wilcoxon-Rank, p_{10%} < 0.001, p_{20%} < 0.001). Letters above each box-plot indicate differences between only *Canton-S* (red) and mutant w^{1118} (white) larvae, respectively. (c) Larval 8% and 20% ethanol substrate choice over a test period of 120 min. *Canton-S* larvae did not vary in their attraction within 120 min to 8% ethanol (Kruskal Wallis, p < 0.032). (Pref*{8%} = 0.55, ci = 0.47–0.6, Pref*{10min} = 0.65, ci = 0.53–0.69, Pref*{10min} = 0.63, ci = 0.6–0.73, Pref*{120min} = 0.53, ci = 0.5–0.67, Pref*{120min} = 0.5, ci = 0.45–0.61, Pref*{120min} = 0.57, ci = 0.39–0.59). However, the initial attraction for 20% ethanol turns into an avoidance after about 15 min of ethanol presentation (Pref*{5min} = 0.15, ci = 0.03–0.25, Pref*{10min} = 0.1, ci = 0.04 to 0.25, Pref*{15min} = 0.16, ci = 0.38 to 0.1, Pref*{30min} = 0.33, ci = 0.52 to 0.05, Pref*{60min} = 0.46, ci = 0.64 to 0.27, Pref*{120min} = 0.62, ci = 0.5 to 0.69). Indicated values show medians; error bars represent standard errors. (d) *Canton-S* larvae raised on standard food that contains 1% ethanol (ethanol +) shows a substrate choice behavior that was not different from larvae that were raised for one (ethanol—[1]) or two generations (ethanol—[2]) on ethanol free food (TukeyHSD, p_{standard-w/o} < 0.202, p_{standard-w/o2} < 0.639). Note a slight increase in ethanol substrate choice with increasing generations of ethanol food free raised flies (TukeyHSD, p < 0.03). Differences against zero are indicated in red and black at the bottom of each panel. Sample size for each box plot is n = 16. Significant differences between the two groups are given with letters. Preference scores and statistical tests underlying the different indices are documented in the Supplementary material.
ments (Fig. 4d). Since BA triggered the greatest behavioral response, we used this stimulus to test whether larvae can still perceive it on 5%, 8%, 10% and 20% ethanol containing agarose plates (Fig. 4d). For all four ethanol concentrations wild-type Canton-S larvae were similarly attracted to BA (Fig. 4d). Therefore, in the assay D. melanogaster larvae could distinguish BA from a week or high concentrated ethanol background.

Ethanol provides a teaching signal for larval olfactory learning. Larvae of D. melanogaster are able to associate odorants (conditioned stimulus) with cues of different sensory modalities (unconditioned stimulus) to establish appetitive or aversive olfactory memories. So far, tastants, temperature, vibration, electric shock, and light have been identified as teaching signals (reviewed in14). In contrast to adult flies, ethanol has not yet been tested as reinforcer. Therefore, we performed standardized learning experiments to validate the potential of ethanol in differential conditioning. In this assay larvae are trained three times with a given concentration of ethanol (2.5%, 8%, or 20% as a teaching signal) and then tested on an agarose plate for their odorant preference between the previously ethanol paired and the non-paired odorant (Fig. 5). Higher ethanol concentrations of 8% and 20%, in contrast to a lower concentration of 2.5%, provide an appetitive teaching signal for larval olfactory learning (Fig. 5b). For appetitive olfactory learning with fructose, it has been reported that larvae show no
Figure 3. Larval survival on diets containing different concentrations of ethanol. (a) Scheme of the experimental procedure. Independent groups of 12 second instar Canton-S larvae were put into food vials that contained an agarose substrate plus different concentration of ethanol. Surviving of larvae and pupae were counted every 24 h. Water was added every 24 h to avoid dehydration. Pupation was used as measure of survival. (b) Diagram shows Kaplan–Meier survival curves of larvae. Larvae were put either on 0% (green), 4% (light blue), 8% (blue), 12% (black), or 20% ethanol (dark blue) diet for eight days. 12% and 20% ethanol significantly reduced larval survival as most of the animals died within one day (survival rate\textsuperscript{12\% ethanol} = 21.40%, survival rate\textsuperscript{20\% ethanol} = 12.40%). Larvae reared on 0% and 8% ethanol showed a nearly similar survival (Log-Rank-test, p < 0.001, survival rate\textsuperscript{0\% ethanol} = 44.90%, survival rate\textsuperscript{8\% ethanol} = 60.90%). In contrast 4% ethanol diet significantly increased larval survival compared to the 0% ethanol control diet (Log-Rank-test, p < 0.001, survival rate\textsuperscript{0\% ethanol} = 74.90%). (c) Diagram shows Kaplan–Meier survival curves of the related pupation rates of the surviving animals shown above raised at 0% (green), 4% (light blue), 8% (blue), 12% (black), or 20% ethanol (dark blue) diet. 4% ethanol diet significantly increased the pupation rate compared to larvae reared at 0% ethanol diet (Log-Rank-test, p < 0.001, pupation rate\textsuperscript{0\% ethanol} = 35.8%, pupation rate\textsuperscript{4\% ethanol} = 73.80%). In contrast 8%, 12%, and 20% ethanol diet significantly reduced the pupation rate compared to larvae reared at 0% ethanol diet (Log-Rank-test, p\textsuperscript{0\%–8\% ethanol} < 0.001, p\textsuperscript{0\%–12\% ethanol} < 0.001, p\textsuperscript{0\%–20\% ethanol} < 0.001, pupation rate\textsuperscript{8\% ethanol} = 15.6%, pupation rate\textsuperscript{12\% ethanol} = 0.5%, pupation rate\textsuperscript{20\% ethanol} = 12.2%). Single scores and statistical tests underlying the different survival curves are documented in the Supplementary material. Sample size for each Kaplan-Meier survival curve is n = 16.
memory when tested in the presence of the unconditioned stimulus. This is probably because in the presence of food the larvae do not have to search for food41,49. This also seems to be the case for ethanol, since larvae trained with 8% ethanol only recall odor-ethanol memory on control agarose, but not on a test plate containing 8% ethanol (Fig. 5c). Ethanol memory also appears to be somehow similar to fructose memory, as even 2 M fructose added to the test plate prevents the recall of the odor-ethanol memory.

Ethanol does not alter appetitive and aversive olfactory memory. Treating larvae with 20% ethanol for 20 min impairs aversive odorant-heat shock memory after one-odor conditioning at 35 °C50. The effect of ethanol was absent, when the odorant was paired with a higher temperature heat-shock of 41 °C, which likely forms a stronger memory unsusceptible for ethanol treatment. Therefore, we similarly investigated whether the 20 min incubation with 20% ethanol interferes with larval olfactory memory formation when the odorant is paired with fructose or salt (Fig. 6). We found that both, aversive salt memory (1.5 M NaCl; Fig. 6a) and appetitive sugar memory (0.01 M fructose; Fig. 6b), showed no reduction after ethanol treatment in comparison to H2O treated larvae. Therefore, the olfactory memory reinforced by salt or fructose was not altered by ethanol treatment.
Figure 5. Larval olfactory learning reinforced by three different ethanol concentrations. (a) Scheme of the experimental procedure. Canton-S were trained three times with two odorants (AM and BA) and either 2.5%, 8%, or 20% ethanol as reinforcer. Olfactory memory is quantified by the performance index (PI). (b) Larvae trained with 8% and 20% ethanol concentration show a significant appetitive memory ($PI_{8\% \text{Ethanol}} = 0.20$, $CI = 0.11–0.26$, $PI_{20\% \text{Ethanol}} = 0.20$, $CI = 0.05–0.24$, one-sample t test, $p_{8\%} < 0.001$, $p_{20\%} < 0.005$). When 2.5% ethanol was used as a teaching signal no memory was detectable ($PI_{2.5\% \text{Ethanol}} = 0.83$, $CI = −0.07$ to 0.08, one-sample test, $p_{2.5\%} < 0.828$). Accordingly, larvae trained with 8% and 20% ethanol behaved differently from larvae trained with 2.5% ethanol (Dunn’s Multiple Comparison test, $p < 0.001$ and $p < 0.003$, respectively). (c) Larvae trained with 8% ethanol concentration as teaching signal and tested on control agarose showed a significant appetitive memory ($PI = 0.20$, $CI = 0.11–0.26$, one-sample t test, $p < 0.001$); if tested in the presence of the teaching signal of 8% ethanol or 2 M fructose (2 M FRU), appetitive associative memory is not expressed ($PI_{8\% \text{Ethanol}} = −0.03$, $CI = −0.17$ to 0.05, $PI_{2\times \text{MFRU}} = −0.05$, $CI = −0.11$ to 0.01) as it is not significantly different from zero (one-sample t test, $p_{8\% \text{Ethanol}} < 0.260$, $p_{2\times \text{MFRU}} < 0.080$) and not significantly different from each other (paired t-test, $p < 0.902$). Differences against zero are indicated in red at the bottom of each panel. Sample size for each box plot is $n = 15$. Significant differences between the groups are indicated with letters; Preference scores and statistical tests underlying the different indices are documented in the Supplementary material.
Due to the numerically relatively simple anatomy of the larva, it has an enticing analytical power based on the combination of genetic tractability, the availability of robust behavioral assays, the possibility for transgenic single cell manipulation, and an emerging synaptic connectome of the complete central nervous system. The focus of this work was on the behavioral description of ethanol driven larval behavior in order to provide a basis for subsequent genetic, molecular and cellular studies.

Ethanol is an important stimulus in the environment of adult and larval *D. melanogaster* (reviewed in51,52). The concentration of ethanol in the natural habitat of this species vary between 0.6% in ripe hanging fruits and up to 4.5% in rotting ones53. In some man-made environments, such as wine cellars, the ethanol concentration can reach more than 10%53–55. Given that adult *D. melanogaster* often carry yeast to the egg laying sites and inoculate ripening fruits, the induced fermentation and associated ethanol production is likely to result in higher concentrations at the larval stage56. Therefore, the naturally occurring range of ethanol concentrations experienced by a larva is in agreement with most of the concentrations tested in our behavioral analysis (Figs. 1, 3, and 5). Only the highest concentration tested (20%, 30%, and 50% ethanol) do not occur in the natural habitat of the larva. Of course, one has to keep in mind that our experiments were performed under laboratory conditions using a substrate of agarose mixed with ethanol. Due to the evaporation of ethanol, which is indeed volatile, especially higher ethanol concentrations could have been lower in reality. However, these effects are likely to be small, as studies have shown that within the first few hours, ethanol levels drop only slightly in a 13% ethanol containing Petri dish55. This corresponds approximately to the period of preparation of the plates and the conduction of the ethanol attraction and learning experiments. Similarly, evaporation seems to be of little relevance in the survival experiments, where the experimental vials were changed every day. It was shown that the ethanol concentration in vials closed with polyurethane bungs remains very stable over several days (in bottles with 6% ethanol in the medium the fall is to 3.5% after six days)55.

The ethanol attraction is based on various sensory modalities. We show that *D. melanogaster* larvae preferentially migrate to substrates containing ethanol, regardless of whether they have been previously exposed to ethanol or not. In contrast to adult *Drosophila* 57, exposure over several days does not increase the attractiveness of ethanol to larvae (Fig. 1d). Larval attraction appears to follow a Gaussian-shape like dose response curve reaching a plateau between 4 and 10% (Figs.1 and 2). Our results are in agreement with several published findings that have shown larvae prefer 1%58, 2.5%59, 5%59, 6%51–53, 10%50,69, 17%54, 20%59 and even pure ethanol58. Thus, our standardized experimental design provides a robust behavioral assay that allows to identify the neural and molecular basis of larval ethanol substrate choice. However, it must be mentioned that there are some studies on larval ethanol substrate choice, where the tested animals show no or only weak attraction to ethanol concentrations from 2 to 6%32,34,39,60 and even aversion at higher concentrations39,60. However, it is not clear whether, in addition to methodological matters such as a low volume of applied ethanol38, other factors such as the enzymatic activity of Adh, the surrounding temperature, specific pre-treatments and the precise experimental procedure are responsible for this32–34,39,59. Likewise, there are changes across larval development. During first and second molting, the attractiveness of ethanol is reduced (Fig. 2). During these

![Figure 6](https://www.nature.com/scientificreports/)
developmental stages, larval locomotion is generally reduced as they renew their exoskeleton on which all external sensory organs are also located. Our results also suggest that certain mutations may alter the attraction to ethanol, as w1118 mutants showed a reduced attraction to higher ethanol concentrations compared to the wild-type (Fig. 1). The white gene encodes for an ABC transporter a key player of the eye pigmentation pathway. In addition, white mutant flies also possess abnormally low levels of the biogenic amines serotonin, dopamine, and histamine. Whether this plays a role in the lower attractiveness of these mutants towards 10% and 20% ethanol, however, remains to be investigated.

Substrate attraction tests (Figs. 1 and 2) allow larvae to come into direct contact with ethanol. Therefore, larvae can potentially be guided by different inputs: the sense of smell or taste, but also by a caloric gain or a pharmacological effect. Examples for such cases are reported for adult D. melanogaster. In this work, we have separated gustatory and olfactory inputs from each other. (Please note that the pharmacological effect can only be analyzed by behavioral changes and measurement of endogeneous ethanol concentration in the larvae, which were not done in our study). To investigate whether larvae specifically perceive and behaviorally respond to ethanol as an odorant, we have filled the stimulus in containers that allow ethanol to evaporate, but prevent the larvae from directly contacting it and thus excludes feeding or ingesting it (Fig. 4). This has not been studied so far. Larval ethanol attraction for the odorant stimulation was clearly evident (Fig. 4), but reduced compared to the substrate attraction of ethanol (Fig. 1). The behavioral response is almost halved from a median of 0.61 (Fig. 1) to 0.33 (Fig. 4). This means that (1) larvae indeed do perceive ethanol via the olfactory signaling pathway and (2) larvae can perceive ethanol not only as an odor.

Ethanol provides a positive teaching signal. To our knowledge it has not yet been tested whether larvae can utilize ethanol as a positive teaching signal (unconditioned stimulus) in learning and memory assays. Our results suggest that this is the case for concentrations of 8% and 20% ethanol (Fig. 5). Therefore, not only adult flies can use ethanol as a teaching signal, but also larvae. The initial memory of adult flies, however, is averse and only changes after 24 h into a positive long-term memory that lasts several days. However, these results are based on multiple training stimuli that are separated in time (spaced training). Adult training protocols more similar to the larval procedure used here are also able to induce appetitive memories. Thus, the adult behavioral response is much more similar to the larval one than suspected at first glance. In addition, we
gain increasing insight to understand what stimulation the larva classifies as rewarding. Chemosensory stimuli include ethanol, low salt concentrations, amino acids, ribonucleosides and various sugars (e.g., sucrose, glucose, maltodextrin, sorbitol, ribose), even though some cannot be metabolized (arabinose, xylitol). Without a direct comparison, it is of course difficult to assess how rewarding ethanol is for the larva. However, a comparison of published values suggests that fructose and a low salt concentration, which are used in most studies, lead to reward memories that are about twice as strong.

We also do not yet have any insights into the neuronal and molecular organization of larval ethanol learning and memory. However, the assay described here allows their analysis. It is worth noting that ethanol memory cannot be retrieved on a fructose test plate (Fig. 5). According to Schleyer and colleagues *D. melanogaster* larvae search for food after the conditioning phase based on their acquired experience. Thus, the odorant paired with ethanol predicts a certain gain. At the moment of testing, the animals expect such a gain and compare it with their current environmental input. It seems that the larvae do not only expect a positive gain in this situation but also its specific quality. This was shown as sugar memory could be recalled on a positive aspartic acid containing test plate and vice versa. Following the same logic, the absence of ethanol memory recall on a fructose plate thus means that larvae do not distinguish between ethanol and fructose quality. Therefore, it is possible that fructose and ethanol processing circuits overlap in the larval brain. In adult flies food reward and ethanol circuits overlap in context of regulating ethanol preference. Thus is tempting to speculate that this is the case in larvae as well. However, to exclude that sugar presence just distracts larvae from odorant attraction in our experiments further work has to be done.

**Outlook.** Which cells and molecules might be involved in the perception of ethanol in larvae? Based on studies on olfactory ethanol sensing in adult flies, the octopaminergic system and different olfactory receptors can be considered as an analysis entry point. Using the Flywalk or a two odor vial assay to measure naive adult olfactory output, it was shown that the olfactory co-receptor Orco plays an essential role in both assays when ethanol was applied. For the Flywalk analysis the effect could even be refined to the olfactory receptor genes Or42b and Or95a. At the larval stage, only Or42b is expressed in a single ORN of the dorsal organ, the main larval olfactory sense organ. Or42b responds to ethyl acetate, ethyl butyrate, propyl acetate and pentyl acetate; chemicals that are all highly attractive to larvae. It is tempting to expect that Or42b serves a similar function at the larval stage. However, ethanol has not yet been tested, and we lack any molecular and neuronal information on its perception in the larva. With respect to learning and memory, we have recently obtained a cellular understanding of the involved neuronal pathways of the larval brain. Four dopaminergic neurons of the primary protocerebral anterior medial (pPAM) cluster encode fructose dependent teaching signals and these neurons are directly connected to the mushroom body, the larval memory center. Hence, it is now possible to test whether aspects of the ethanol reward learning and memory are also processed by the same neuronal mushroom body network. This would also reveal a conservation of the functional patterns throughout development, since adult appetitive ethanol memory also requires dopaminergic cells and the mushroom body.

**Methods**

**Fly strains.** Fly strains were kept on standardized cornmeal medium containing 1% ethanol at 25 °C and 65% humidity under a 14:10 h light:dark cycle. Adult flies were transferred to new food vials every 72 h. Larvae were taken from food vials and briefly washed in tap water to remove food residues. Larvae were then collected in a 12 h cycle starting 36 h after egg laying (AEL).

**Substrate choice.** For gustatory preference tests, Petri dishes (85 mm diameter; Greiner) were filled with 2.5% agarose substrate (VWR Life Science; type number: 97062-250). Agarose and ddH2O were mixed and boiled in a microwave oven. A thin layer of the hot mixture was filled into Petri dishes. After cooling, agarose was removed from half of the plate and re-filled with 2.5% agarose substrate containing different concentration of 99.8% ethanol (1%, 2%, 4%, 8%, 10%, 20%, 30%, or 50% ethanol; CHEMSOLUTE; type number: 2273.1000). In all experiment the final volume was kept the same. No obvious change of the texture of the plates was seen with increasing ethanol concentrations up to 50%. Therefore, we currently have no evidence that our results are affected by texture effects, although some are described in the literature. To keep an evaporation effect as low as possible, groups of 30 larvae of different ages were placed immediately after plate preparation (in the order of maximum 45 min) in the middle of the Petri dish and allowed to crawl for 5 min (or up to 120 min) at room temperature (RT). Then, larvae were counted on each side of the Petri dish. A preference index (Pref) was calculated by subtracting the number of larvae on the control agarose side (#nS) from the number of larvae on the side with a stimulus (#S), divided by the total number of larvae (#total): Pref= (#S−#nS)/#total. A positive Pref indicates attraction and a negative aversion to the presented stimuli.

**Olfactory attraction.** To test for olfactory attraction, Petri dishes (85 mm diameter; Greiner) were filled 2.5% agarose substrate or with 2.5% agarose substrate with different concentration of ethanol (5%, 8%, 10% and 20%). Agarose and ddH2O were mixed and boiled in a microwave oven. A thin layer of the hot mixture was filled into Petri dishes. Closed Petri dishes were kept at room temperature and used on the same day. To test the olfactory stimuli of ethanol, either 8% ethanol, benzaldehyde (BA; Sigma-Aldrich, type number: 102213897; undiluted), amyl acetate (AM; Sigma-Aldrich, type number: 102172386); diluted 1:250 in paraffin oil, 1-octanol...
(1-Oct; Sigma-Aldrich, type number: 101858766 undiluted) or distilled water was filled into custom-made Teflon containers (4.5 mm diameter) with perforated lids and placed on each side of the plate. Immediately after plate preparation, groups of 30 larvae were placed in the middle of the Petri dish and allowed to crawl for 5 min at RT. Then, larvae were counted on each side of the Petri dish. A preference index (Pref) was calculated by subtracting the number of larvae on the side without an odor (#nO) from the number of larvae on the side with an odor (#O), divided by the total number of larvae (#total): Pref = (#O - #nO)/#total. A positive Pref indicates an attractiveness; a negative Pref represents an avoidance.

Olfactory ethanol learning and memory. All experiments were performed on Petri dishes filled with either 2.5% control agarose or 2.5% agarose containing different concentrations of ethanol (8% or 20%), 0.01 M fructose (Carl Roth®; type number: 4981.2) or 1.5 M sodium chloride (Carl Roth®; type number: 3957.2). Agarose and ddH2O were mixed and boiled in a microwave oven. A thin layer of the hot mixture was filled into Petri dishes. Closed Petri dishes were kept at room temperature and used on the same day. As olfactory stimuli, we filled 10 µl AM, and 10 µl BA, in custom-made Teflon containers (4.5 mm diameter) with perforated lids. Immediately after plate and container preparation, groups of 30 larvae were placed in the middle of the Petri dish containing a control agarose substrate. All experiments were conducted at RT. Larvae were exposed to AM and allowed to crawl for 5 min. Then, larvae were transferred with a small brush to a new Petri dish containing a positive (ethanol, fructose) or negative (NaCl) reinforcer and exposed to BA for 5 min. Each transfer usually takes about one minute. After three training cycles, larvae were placed on a new Petri dish containing control agarose substrate or agarose substrate containing 8% ethanol, 0.01 M fructose, or 1.5 M sodium chloride and exposed to AM and BA on opposite sides for 5 min. Then, larvae were counted on each side of the Petri dish. A second group of larvae was trained via a reciprocal training regime. For each group an independent olfactory preference index was calculated as described above. A performance index (PI) was calculated by adding the Pref of the first training group (Pref1) to the Pref of the second training group (Pref2) and dividing them by the number of experimental groups (#2): PI = (Pref1 + Pref2)/#2. A positive PI indicates an attractiveness; a negative PI represents an avoidance.

Larval survival on ethanol. To investigate larval survival in the presence of ethanol, 12 wild-type L2 stage larvae were placed in vials containing either 1% control agarose substrate or 1% agarose substrate plus different concentrations of ethanol of 4%, 8% or 20%. Three drops of tap water were daily added to prevent larvae from dehydrating. Larvae that were alive and later the pupal stage were counted from day 1 to day 9. The data are shown as Kaplan–Meier survival curves. The percentage of survival and pupation were calculated as follows: Percentage = (number of living larvae or pupae / total number of larvae or pupae) × 100. For each condition 16 independent experimental groups were analyzed (n = 16).

Data analysis and visualization. Groups that did not violate the assumption of normal distribution (Shapiro–Wilk test) and homogeneity of variance (Bartlett’s test) were analyzed with parametric statistics: paired t-test (comparison between two groups) or One-way ANOVA followed by planned pairwise comparisons between the relevant groups with a Tukey honestly significant difference (HSD) post hoc test (comparisons between groups larger than two). Experiments with data that were significantly different from the assumptions above were analyzed with non-parametric tests, such as Wilcoxon signed rank test (comparison between two groups) or Kruskal–Wallis test followed by Dunn’s multiple pairwise comparison (comparisons between groups larger than two). To compare single genotypes against chance level, we used one-sample t test. All statistical analyses and data visualization were done with R (V 3.5.1) in RStudio by using stats, dunn.test and ggplot2 package8. Figure panels were edited with Adobe Illustrator CS5 (San Jose, CA, USA). The significance level of statistical tests was set to 0.05; the shown confidence interval (CI) is the 95% confidence interval of the data plots. Data are displayed as box plots with the median as the middle line, the box boundaries as 25% and 75% quantiles and the whiskers as 1.5 times the interquartile range. Outliers are shown as dots directly above or below the box plots. Larval and pupal survival rates are shown as Kaplan–Meier curves. Further details are documented in the Supplemental Data files.

Received: 7 February 2021; Accepted: 27 May 2021
Published online: 10 June 2021

References
1. Kandel, E. R., Schwartz, J. H., Jessell, T. M., Siegelbaum, S. A. & Hudspeth, A. J. Principles of Neural Science Vol. 5 (McGraw-Hill Education Ltd, 2012).
2. Gerber, B., Stocker, R. F., Tanimura, T. & Thum, A. S. Smelling, tasting, learning: Drosophila as a study case. Annu. Rev. Neurosci. 28, 205–239. https://doi.org/10.1146/annurev.neuro.28.060604.093910 (2005).
3. Vosshall, L. B. & Stocker, R. F. Molecular architecture of smell and taste in Drosophila. Annu. Rev. Neurosci. 30, 505–533. https://doi.org/10.1146/annurev.neuro.30.051606.094306 (2007).
4. Liman, E. R., Zhang, Y. V. & Montell, C. Peripheral coding of taste. Neuron 81, 984–1000. https://doi.org/10.1016/j.neuron.2014.02.022 (2014).
5. Dahanukar, A., Hallem, E. A. & Carlson, J. R. Insect chemoreception. Curr. Opin. Neurobiol. 15, 423–430. https://doi.org/10.1016/j.conb.2005.06.001 (2005).
6. Li, H. H. et al. A GAL4 driver resource for developmental and behavioral studies on the larval CNS of Drosophila. Cell. Rep. 8, 897–908. https://doi.org/10.1016/j.celrep.2014.06.065 (2014).
54. McKenzie, J. A. & McKechnie, S. W. A comparative study of resource utilization in natural populations of Drosophila melanogaster and D. simulans. Oecologia 40, 299–309. https://doi.org/10.1007/BF00358500 (1979).

55. Gibson, J. B., May, T. W. & Wilks, A. V. Genetic variation at the alcohol dehydrogenase locus in Drosophila melanogaster. FASEB J. 9, 225–233. https://doi.org/10.1096/fj.9.3.1401880210 (1994).

56. Stamps, J. A., Yang, L. H., Morales, V. M. & Boundy-Mills, K. L. Drosophila regulate yeast density and increase yeast community similarity in a natural substrate. PLoS ONE 7, e42238. https://doi.org/10.1371/journal.pone.0042238 (2012).

57. Devineni, A. V. & Heberlein, U. Preferential ethanol consumption in Drosophila models features of addiction. Curr. Biol. 19, 2126–2132. https://doi.org/10.1016/j.cub.2009.10.070 (2009).

58. Rodrigues, V. Olfactory behavior of Drosophila melanogaster. Basic Life Sci. 136, 361–371. https://doi.org/10.1007/978-1-4684-7968-3_26 (1980).

59. Depiereux, E. et al. Serotonin is necessary for place memory in Drosophila. Proc. Natl. Acad. Sci. U S A 105, 5579–5584. https://doi.org/10.1073/pnas.0710168105 (2008).

60. Das, G., Lin, S. & Waddell, S. Remembering components of food in Drosophila. Front. Integr. Neurosci. 10, 4. https://doi.org/10.3389/fintNeuro.2016.00004 (2016).

61. Geer, B. W., Dybas, L. K. & Shanner, L. J. Alcoholic dehydrogenase and ethanol tolerance at the cellular level in Drosophila melanogaster. J. Exp. Zool. 250, 22–39. https://doi.org/10.1002/jez.1402500106 (1989).

62. McKenzie, J. A. & Parsons, P. A. Alcohol tolerance: An ecological parameter in the relative success of Drosophila melanogaster and Drosophila simulans. Oecologia 10, 373–388. https://doi.org/10.1007/BF00345738 (1972).

63. Geer, B. W., Langevin, M. L. & McKechnie, S. W. Dietary ethanol and lipid synthesis in Drosophila melanogaster. Biochem. Genet. 33, 607–622 (1995).

64. McClure, K. D., French, R. L. & Heberlein, U. A Drosophila model for fetal alcohol syndrome disorders: Role for the insulin pathway. Dis. Model. Mech. 4, 335–346. https://doi.org/10.1242/dmm.006411 (2011).

65. Kaun, K. R., Azanchi, R., Maung, Z., Hirsh, J. & Heberlein, U. A Drosophila model for alcohol reward. Nat. Neurosci. 14, 612–619. https://doi.org/10.1038/nn.2805 (2011).

66. Nunez, K. M., Azanchi, R. & Kaun, K. R. Cue-induced ethanol seeking in Drosophila melanogaster is dose-dependent. Front. Physiol. 9, 438. https://doi.org/10.3389/fphys.2018.00438 (2018).

67. Mishra, D., Thorne, N., Miyamoto, C., Jagge, C. & Amrein, H. The taste of ribonucleosides: Novel macronutrients essential for larval growth are sensed by Drosophila gustatory receptor proteins. PLoS Biol. 16, e2005570. https://doi.org/10.1371/journal.pbio.2005570 (2018).

68. Yoshimura, N., Hidaka, H., Kominami, E. & Doguchi, J. Alcohol-seeking behavior is enhanced by dietary ethanol in Drosophila. Chem. Senses 26, 563–570. https://doi.org/10.1093/chemse/bjae051 (2005).

69. Shinohara, K., Azanchi, R. & Heberlein, U. Olfactory attraction to ethanol is mediated by a G protein-coupled receptor and signal transduction pathway in Drosophila. J. Biol. Chem. 278, 3204–3209. https://doi.org/10.1074/jbc.M303292200 (2003).

70. Toshima, N., Kantar Weigelt, M., Weiglein, A., Boetzl, F. A. & Gerber, B. An amino-acid mixture can be both rewarding and aversive to larval Drosophila melanogaster. Oecologia 176, 191–198 (2014).

71. Nunez, K. M., Azanchi, R. & Kaun, K. R. Cue-induced ethanol seeking in Drosophila melanogaster is dose-dependent. Front. Physiol. 9, 438. https://doi.org/10.3389/fphys.2018.00438 (2018).

72. Steck, K. et al. A high-throughput behavioral paradigm for Drosophila olfaction: The Flywalk. Sci. Rep. 2, 361. https://doi.org/10.1038/srep00361 (2012).

73. Ogueta, M., Cibik, O., Eltop, R., Schneider, A. & Scholz, H. The influence of Adh function on ethanol preference and tolerance in adult Drosophila melanogaster. Chem. Senses 35, 813–822. https://doi.org/10.1093/chemse/bjq084 (2010).

74. Schleyer, M., Miura, D., Tanimura, T. & Gerber, B. Learning the specific quality of taste reinforcement in larval Drosophila. Elife 4, e10754. https://doi.org/10.7554/eLife.04711 (2015).

75. Schneider, A. et al. Neuronal basis of innate olfactory attraction to ethanol in Drosophila. PLoS ONE 7, e52007. https://doi.org/10.1371/journal.pone.0052007 (2012).

76. Keesey, I. W. et al. Alcohol boosts pheromone production in male flies and makes them sexier. bioRxiv https://doi.org/10.1101/2020.08.09.242784 (2020).

77. Steck, K. et al. A high-throughput behavioral paradigm for Drosophila olfaction: The Flywalk. Sci. Rep. 2, 361. https://doi.org/10.1038/srep00361 (2012).

78. Ogueta, M., Cibik, O., Eltop, R., Schneider, A. & Scholz, H. The influence of Adh function on ethanol preference and tolerance in adult Drosophila melanogaster. Chem. Senses 35, 813–822. https://doi.org/10.1093/chemse/bjq084 (2010).

79. Schleyer, M., Miura, D., Han, K. A., Stocker, R. F. & Thum, A. S. The role of dopamine in Drosophila larval classical olfactory conditioning. PLoS ONE 4, e5897. https://doi.org/10.1371/journal.pone.0005897 (2009).

80. Rohwedder, A. et al. Four individually identified paired dopamine neurons signal reward in larval Drosophila. Curr. Biol. 26, 661–669. https://doi.org/10.1016/j.cub.2016.01.012 (2016).

81. Saumweber, T. et al. Functional architecture of reward learning in mushroom body extrinsic neurons of larval Drosophila. Nat. Commun. 9, 1104. https://doi.org/10.1038/s41467-018-03130-9 (2018).

82. Lytovka, R. et al. Reward signaling in a recurrent circuit of dopaminergic neurons and peptide-containing Kenyon cells. Nat. Commun. 10, 3097. https://doi.org/10.1038/s41467-019-11092-1 (2019).

83. Wickham, H. ggplot2: Elegant Graphics for Data Analysis (Springer-Verlag, 2016).

Acknowledgements
The authors thank Wolf Huetteroth, Tilman Triphan, Bert Klagges and Astrid Rohwedder for fruitful discussions and comments on the paper. This work was supported by grants from the German Research Foundation (DFG) to AST (TH1584/3-1, TH1584/6-1 and TH1584/7-1) and HS (HS675/10-1).
Author contributions
I.S. and A.S.T. outlined the manuscript. I.S., M.B., H.S. and A.S.T. developed the design of the methodology; I.S., M.B., N.N., Y.S. and J.S. conducted the experiments; I.S. prepared and created the analysis and visualization of the data; I.S., M.B., H.S. and A.S.T. wrote and finalized the manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-91677-3.

Correspondence and requests for materials should be addressed to A.S.T.

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