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RESEARCH ARTICLE

Softness sensing and learning in *Drosophila* larvae

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ABSTRACT

Mechanosensation provides animals with important sensory information in addition to olfaction and gustation during feeding behavior. Here, we used *Drosophila melanogaster* larvae to investigate the role of softness sensing in behavior and learning. In the natural environment, larvae need to dig into soft foods for feeding. Finding foods that are soft enough to dig into is likely to be essential for their survival. We report that larvae can discriminate between different agar concentrations and prefer softer agar. Interestingly, we show that larvae on a harder surface search for a softer surface using memory associated with an odor, and that they evaluate foods by balancing softness and sweetness. These findings suggest that larvae integrate mechanosensory information with chemosensory input while foraging. Moreover, we found that the larval preference for softness is affected by genetic background.

KEY WORDS: Mechanoreception, Chemoreception, Sensory integration

INTRODUCTION

All organisms have to make appropriate food choices. When we find something to eat, we use vision, taste, olfaction and touch to evaluate whether it is edible (Drewnowski, 1997; Rolls, 2005; Verhagen and Engelen, 2006; Spence et al., 2016). Food texture is an important property of foods because we have to eat food that we can chew and swallow (Brown and Braxton, 2000; Jeltema et al., 2015). Thus, the integration of multisensory information is important (Rolls, 2005; Verhagen and Engelen, 2006). Studies in different animals have shown that food texture affects their food preference (in termites: Kasseney et al., 2011; in mice: Morris et al., 2012; in birds: Johansen et al., 2014). Until recently, however, little attention has been paid to the mechanical properties of foods.

Drosophila utilizes multisensory inputs to make decisions in feeding behavior (Hoffmann and Parsons, 1984; Dahanukar et al., 2007; Jeong et al., 2016). Recent studies have shown that adult fruit flies can discriminate the hardness of foods using two types of labellar mechanosensory neurons (Jeong et al., 2016; Zhang et al., 2016; Sánchez-Alcañiz et al., 2017) and that texture affects their preference for food (Jeong et al., 2016). Texture is an important food property for larvae as well as adults. In nature, larvae grow in fermenting and softened fruits or vegetables, which allows the larvae to dig into them and thus avoid predators and dehydration (Hwang et al., 2007; Robertson et al., 2013; Aggarwal et al., 2013). In feeding behavior, texture seems to be an especially critical factor for ingesting a sufficient quantity of food and increasing body size from the first to the third instar. Although a couple of recent studies have focused on how food texture affects larval behavior (Apostolopoulou et al., 2014; Kim et al., 2017), much remains unknown.

Another interesting issue is how larvae integrate chemosensory information with mechanosensory inputs. Larvae have three external sensory organs: the terminal (TO), ventral (VO) and dorsal organ (DO). In addition, they have four internal sensory organs: the dorsal (DPS), ventral (VPS) and posteriorpharyngeal sensilla (PPS), and the dorsal pharyngeal organ (DPO) (Gerber et al., 2004; Kwon et al., 2011; Apostolopoulou et al., 2015). These organs contain the dendrites of sensory neurons functioning in gustation, olfaction, thermosensation and hygrosensation (Singh and Singh, 1984; Stocker, 1994; Vosshall and Stocker, 2007; Klein et al., 2015; Apostolopoulou et al., 2015). In gustation, it is generally known that one neuron expresses multiple receptors, which sense a specific taste (Kwon et al., 2011; Apostolopoulou et al., 2015; Mishra et al., 2013b). In contrast, a recent study has shown that one taste neuron senses multimodal stimuli (van Giesen et al., 2016). Among the sensory organs in the larval head, morphological studies have reported that there are potential mechanosensory neurons in the TO and VPS (Apostolopoulou et al., 2015; Green et al., 1983; Becher et al., 2012; Sokolowski et al., 1984; Chu-Wang and Axtell, 1972; Rist and Thum, 2017). However, the behavioral function of the mechanosensory neurons has not been ascertained.

Drosophila melanogaster larvae are established as an excellent model for the study of learning and memory (Scherer et al., 2003; Gerber and Stocker, 2007; Gerber et al., 2009; Saumweber et al., 2018). Previous larval olfactory learning experiments have shown that sugars, low salt and aspartic acid act as rewards (Niewalda et al., 2008; Schipanski et al., 2008; Schleyer et al., 2015), and bitter taste and high salt act as punishment (El-Keredy et al., 2012; Niewalda et al., 2008; Dudai et al., 1976). In contrast to the extensive studies focusing on gustatory odor learning, far less is known about mechanosensory odor learning. Texture–chemosensory integration seems to be important in searching and foraging for appropriately textured food. Although previous studies have shown that larvae can learn electric shock and buzz as a punishment associated with an odor (Aceves-Piña and Quinn, 1979; Pauls et al., 2010; Eschbach et al., 2011), there are few studies showing that larvae can learn softness as a reward associated with an odor (Apostolopoulou et al., 2014). Saumweber et al. (2018) identified input and output neurons in the larval mushroom body that integrates multiple sensory input and past experience (Davis, 2004; Menzel, 2014; Owald and Waddell, 2015), and analyzed the functions of these neurons and the GABAergic anterior paired lateral (APL) neuron. These approaches should also shed light on future studies to reveal the neural network involved in softness learning.

Here, we studied how *D. melanogaster* larvae sense softness and how food softness affects their feeding preference. We reveal that

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they prefer softer food and can learn to associate appropriate softness with odor. Moreover, they can evaluate food quality by balancing softness and sweetness. We also show that genetic background affects the preference for softness and the amount of intake. These findings expand our understanding of how larvae sense softness and make decisions during feeding.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster were maintained on cornmeal–yeast–glucose–agar medium (Fukuoka: water 1 liter, corn meal 50 g, glucose 100 g, dry yeast 40 g, wheat germ powder 32 g, agar 7.7 g, propionic acid 5 ml and methyl paraben 1.17 g; Nagoya: water 1 liter, corn meal 35.5 g, glucose 100 g, dry yeast 45 g, agar 8 g, propionic acid 4 ml and 10% methyl *p*-hydroxybenzoate 3 ml) under a 12 h:12 h light:dark cycle at 25°C. We obtained similar results for softness preference using larvae raised on two different food recipes. We used third-instar larvae before the wandering stage collected from vials 5 or 6 days after egg laying for all the experiments except a preference experiment on first-instar larvae (Fig. 1F). Canton-S (CS) was used as a typical wild-type strain. To investigate the effects of genetic background, we used other wild-type strains (Oregon-R, Oregon-RC, Hikone-A-S, Urbana-S, Zimbabwe-S29, Berlin-K, Lausanne-S and Amherst-3) obtained from the Bloomington Drosophila Stock Center.

Larvae collection

First-instar larvae

We first collected a number of virgin females and males, and they were entrained under a 12 h:12 h light:dark cycle: light from 01:00 to 13:00 h; dark from 13:00 to 01:00 h. Virgin females were crossed with males at 12:00 h in a ratio of approximately 2:1. We put two pieces of filter paper on a Petri dish and plastic mesh on the filter paper. Then we poured apple juice onto the filter paper. Females were allowed to lay eggs on the Petri dish from 13:00 to 17:00 h. We collected first-instar larvae from 24 to 27 h after the start time of egg laying with a paint brush. After collecting 15 first-instar larvae, these were washed in a drop of distilled water and put onto a test agar plate with a paint brush. The first-instar larvae were used only for the double circle assay (Fig. 1F).

Third-instar larvae

Just before the experiments, we poured 15% glucose (Nihon Shokuhin Kako Co. Ltd, Japan, CAS: 50-99-7) solution into vials containing larvae and within 20 s collected floating animals. These were rinsed with distilled water on a stainless steel sieve (wire diameter 35 µm; mesh aperture 420 µm; Iida Manufacturing Co., Osaka, Japan) and were collected in a small droplet of distilled water with a paint brush. As shown in Fig. 1B,C, we tested the softness preference of larvae washed with distilled water, this result shows there are no effects of water used for washing larvae on softness preference.

Preference experiments

To evaluate preferences for softness, we used the double circle assay (Kudow et al., 2017). Approximately 1 h before tests, we prepared Petri dishes (55 mm diameter for third-instar larvae, AS ONE Co., Osaka, Japan; 35 mm diameter for first-instar larvae, Thermo Fisher Scientific). These consisted of both an inner circle (23 mm diameter for third-instar larvae; 10 mm diameter for first-instar larvae) and an outer circle. In the agar concentration preference tests, the inner circle was filled with several different concentrations of agar, and the outer circle contained 1.0% agar (2.0% only in Fig. 1E and 0.5% agar only in Fig. S1C,D). Agar was obtained from Sigma-Aldrich (A5431, purified powder; CAS: 9002-18-0). To investigate whether there is an interaction between sweetness and softness, the outer circle contained 0.5% to 2.0% agar with 80 mmol l^{-1} fructose (127-02765, Wako Pure Chemical Industries, Ltd, Osaka, Japan, CAS: 57-48-7), and the inner circle contained a fixed level of 0.5% agar with 10 mmol l^{-1} fructose. We placed 15 larvae on the inner circle, allowed them to move freely and counted the number of larvae in the inner circle every 5 min for 30 min. Then we calculated the preference index (PI) according to the following formula:

$$\mathrm{PI} = N_{\mathrm{inner}}/N_{\mathrm{total}}, \quad (1)$$

where N_{inner} is the number of larvae in the inner circle and N_{total} is the total number of larvae. In our previous study (Kudow et al., 2017), we obtained PI values by subtracting the values of the negative control. Here, we omitted the subtractions because the control values were close to zero (Fig. 1B,C, Fig. S1C,D). A higher PI shows that the larvae prefer the inner circle compared with the outer circle. To test the effect of light, we also performed the double circle assay in the dark. Immediately after placing 15 larvae on the inner circle, we placed them into a dark box. After 30 min, we counted the number of larvae and calculated PI values as described above.

To confirm the results of the double circle assay, we also performed the softness preference test using third-instar larvae in the agar split-plate assay. We put a half-dish silicon mold on one side of the Petri dishes (55 mm diameter, AS ONE Co.) and poured 1.0% agar onto the Petri dish. After cooling down, the other side was filled with 1.0% agar or 0.5% agar and left for 1 h before the test. Just before the test, we collected third-instar larvae as described above. We placed 15 larvae onto the middle of the test plate, allowed them to move freely for 30 min, and counted the number of larvae on each side and in the middle at 30 min. Then, we calculated the PI according to the following formula:

$$\mathrm{PI} = (N_{\mathrm{sideA}} - N_{\mathrm{sideB}})/(N_{\mathrm{sideA}} + N_{\mathrm{sideB}}), \quad (2)$$

where N_{sideA} and N_{sideB} are the numbers of larvae on side A and side B, respectively. A higher PI shows that they prefer side A compared with side B.

Measurement of larval intake

We measured the amount of intake of third-instar larvae in a similar way to Apostolopoulou et al. (2014). We prepared 0.5% and 1.0% pure agar and 0.5–2.0% agar containing 80 mmol l^{-1} fructose colored with Food Blue No. 1 (3.125 mg ml^{-1}, F0147, Tokyo Chemical Industry Co., Tokyo, Japan), and 0.5% and 1.0% pure agar and 0.5–2.0% agar containing 10 mmol l^{-1} fructose colored with Food Red No. 106 (5.0 mg ml^{-1}, F0143, Tokyo Chemical Industry Co.). We first measured larval intake on a wholly filled Petri dish. Feeding-stage third-instar larvae collected as described above were placed in, and allowed to feed in, a Petri dish (55 mm diameter for third-instar larvae, AS ONE Co.) filled with each solution. After 30 min in pure agar medium or after 10 min in fructose–agar medium, they were rinsed in distilled water, and 10 larvae were homogenized in 200 µl 1 mol l^{-1} ascorbic acid sodium salt (198-01251, Wako Pure Chemical Industries, CAS: 134-03-2) in a microcentrifuge tube with a pestle and centrifuged for 30 min at 16,000 ***g***. The absorbance of 2 µl of the supernatant at 630 nm (blue) and 565 nm (red) was measured with a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific) with software version 3.7.1. The absorbance of each single measurement was calculated

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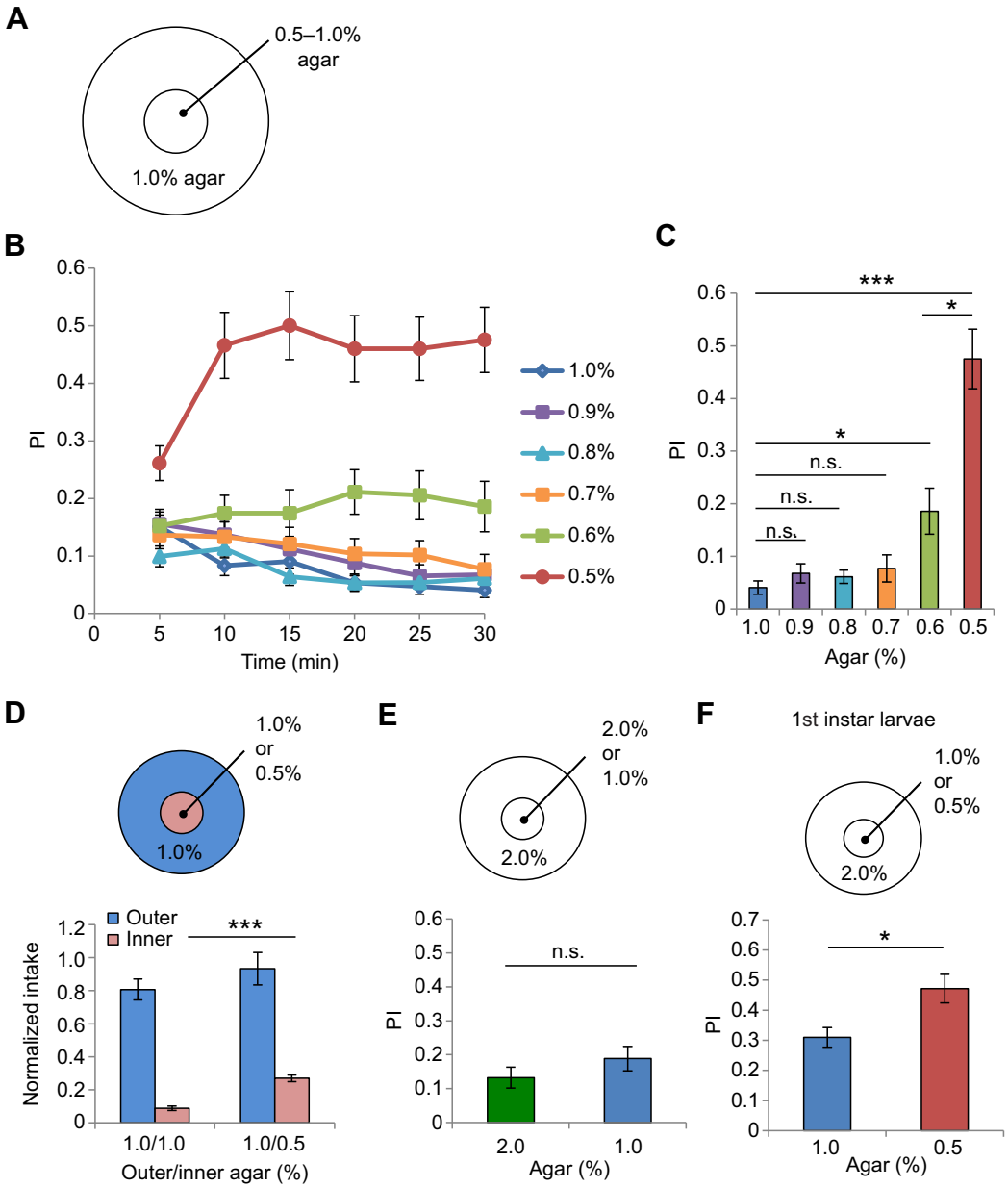

Fig. 1. Third-instar larvae prefer softer substrates. (A) The double circle assay was performed using feeding-stage third-instar larvae. (B) Time course of preference index (PI) values; the larvae preferred softer agar. (C) PI values at 30 min for each agar concentration in the inner circle; larvae preferred softer agar and PI values were highest when the inner circle was 0.5% agar [*N*=30 (1.0%), 30 (0.9%), 30 (0.8%), 15 (0.7%), 30 (0.6%), 10 (0.5%), respectively; Steel–Dwass tests: ***P<0.001; *P<0.05; n.s., not significant]. (D) Normalized intake of agar in the double circle assay. When the inner circle was 0.5% agar (right), the larvae ingested inner agar more than when the inner circle was 1.0% agar (left), indicating that they ingested pure agar and that their amount of intake corresponded to the preference for softness [*N*=9 (1.0%/1.0%), 12 (1.0%/0.5%); Mann–Whitney *U*-tests: ***P<0.001]. (E) PI values at 30 min when the outer circle was 2.0% agar and the inner circle was 2.0% (left) or 1.0% agar (right); larvae did not show a significant preference [*N*=20, 20 for 2.0% and 1.0% agar, respectively; Mann–Whitney *U*-tests: n.s., not significant], suggesting that they prefer absolute, not relative, softness. (F) Using a smaller Petri dish, the double circle assay was also performed on first-instar larvae. PI values at 30 min indicate that the larvae significantly prefer 0.5% agar to 1.0% agar (control) (*N*=20, 20 for 1.0% and 0.5% agar, respectively; Mann–Whitney *U*-tests: *P<0.05).

by subtracting the absorbance of the blank control (non-fed larvae) from that of fed larvae. To calculate the relative absorbances, we measured the absorbances of 3.125 mg ml^{-1} blue and 5.0 mg ml^{-1} red solutions and obtained the ratio (blue:red=1.96:1). The relative absorbances were calculated.

To measure intake in the double circle assay, we prepared a Petri dish (55 mm diameter for third-instar larvae, AS ONE Co.) that consisted of a red-colored inner circle (23 mm diameter) and a blue-colored outer circle, as described in the Preference experiments section, and left them for 1 h before the test. We measured the absorbance and calculated the relative absorbances of blue and red as described above. To calculate the normalized absorbance, the relative absorbance of the larvae fed on a specific solution was divided by the relative absorbance of the larvae fed on the wholly filled Petri dish of the same solution.

Video tracking of locomotion

We first prepared a Petri dish (90 mm diameter; Ina Optica Co. Ltd, Japan) filled with 2% agar and collected approximately 20 larvae by the above method. The video recording was performed on a light

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panel (Hakuba light viewer 5700; Hakuba Photo Industry Co. Ltd, Japan). We allowed one larva to crawl on the Petri dish and recorded its movement with a webcam (Logicool HD Webcam C615; Logitech, Japan) for approximately 2 min at 30 frames s^{-1}. Using AviUtl (http://spring-fragrance.mints.ne.jp/aviutl/aviutl99m.zip), we then segmented the movie into 30-s sections, starting from the point when the larva first moved, and saved them as AVI files. The movies were analyzed with Ctrax (Branson et al., 2009), and the crawling distance of the larval locomotion in 1/30 s was obtained by tracking the larval head position. We then calculated the total distance covered in 30 s by summing the distances covered in 1/30 s.

Olfactory learning experiment

The olfactory learning experiment was performed as described in Mishra et al. (2013a). The flies were maintained on Magdeburg standard food medium (water 1 liter, polenta 173.5 g, malt 86.7 g, molasses 54.2 g, soy flour 12.0 g, yeast 22.3 g, agar 9.0 g and methyl paraben 3.0 g) under a 12 h:12 h light:dark cycle at 25°C. Immediately before each experiment, we removed the food medium from a vial and collected the necessary number of feeding-stage third-instar larvae in tap water. For the learning experiments, we used Petri dishes of 90 mm diameter (Sarstedt, Nümbrecht, Germany) filled either with 0.5% agarose (Roth, Karlsruhe, Germany) or 1.0% agarose. We confirmed that agarose gel results in softness preferences similar to those of agar gel (Fig. S3). As the odor, we used 10 µl *n*-amyl acetate (AM; CAS: 628-63-7; Merck, Darmstadt, Germany) diluted 1:20 in paraffin oil. This odor was filled into Teflon containers that allowed the odor to evaporate, and these were placed on the Petri dish. The first group of 30 larvae was put on 0.5% agarose paired with AM (AM+;+indicates 0.5% agarose gel) and allowed to crawl freely. After 2.5 min, they were transferred to 1.0% agarose gel paired with no odor (empty; EM) and allowed to crawl for 2.5 min. The second group was trained reciprocally, i.e. AM/EM+. To investigate the effects of the training sequence, we also trained them with EM+/AM and EM/AM+. After repeating these trainings three times, the larvae were transferred to a test plate and given a choice of odor between AM and EM. As test plates, we used both 0.5% and 1.0% agarose gel. After 3 min, we counted the number of animals on the AM side, on the EM side and in the neutral zone, and calculated a preference index for *n*-amyl acetate according to the following formulas:

$$\text{Pref AM+/EM} = (N_{\text{AM}} - N_{\text{EM}})/N_{\text{total}}, \quad (3)$$

$$\text{Pref AM/EM+} = (N_{\text{AM}} - N_{\text{EM}})/N_{\text{total}}. \quad (4)$$

Thus, positive preference values represent a preference of the larvae for AM.

From these two reciprocal groups, we calculated the PI according to the following formula:

$$\text{PI} = (\text{Pref AM+/EM} - \text{Pref AM/EM+})/2. \quad (5)$$

Thus, positive PIs indicate appetitive associative memory.

Measurement of agar-gel hardness with an atomic force microscope (AFM)

We prepared 1.0% agar gel and 1.0% agar gel containing 80 mmol l^{-1} fructose (127-02765, Wako Pure Chemical Industries, CAS: 57-48-7) and cut the preparations into approximately 7×7 mm^2 fragments. All measurements were performed on the same day after preparing the agar gel. The Young's modulus of the gels was measured by the nanoindentation method with an MFP-3D system (Asylum Research, Oxford Instruments) at room temperature. We used an SD-Sphere-NCH-S-10 (NANOSENSOR, sphere diameter: 800 nm) cantilever for these measurements. More than 120 random points over the gel surface were tested, and force curve measurements were conducted. Young's modulus was calculated with the Asylum research software. The deflection sensitivity of the cantilever was calibrated by the spring constant value measured by the thermal noise method. The indentation depth is described in the legend to Fig. S4.

Statistical analysis

In the free locomotion experiments and the choice experiments, the data were compared using non-parametric statistics. When we compared across all groups in combination, we used the Steel–Dwass test. The Mann–Whitney *U*-test was used when we compared between two groups.

In olfactory learning experiments, the Mann–Whitney *U*-test was employed to compare two groups. Significant differences from zero were determined using one-sample sign tests. The data are presented as box plots, where the middle line represents the median, and the boundaries and whiskers show the 25th/75th and 10th/90th percentiles, respectively.

RESULTS

Larvae prefer softer places

To ascertain whether feeding-stage third-instar larvae show a preference for softness, we performed the double circle assay under light. The outer circle contained 1.0% agar, and the inner circle contained various concentrations of agar (Fig. 1A). We first put 15 larvae on the inner circle and counted the number of larvae in the inner circle every 5 min for 30 min. The PI values were close to zero when the inner circle contained 1.0–0.7% agar. However, when the inner circle contained 0.6% agar or 0.5% agar, the PI values were significantly higher compared with the PI values for the 1.0% control (Fig. 1B,C). These results indicate that feeding-stage larvae prefer softer places. Previous preference tests using larvae were mainly performed using split plates, and we asked whether the preference for softness we have found can be reproduced in split plates. The result shown in Fig. S1A demonstrates that larvae prefer softer places in this situation.

In the double circle assay, the larvae first went outside the inner circle during an approximately 30 s period (Fig. S1B), and then came back inside if the inner circle was softer than the outer circle (see Movie 1). If the agar concentration of the inner and outer circle was the same, the larvae did not choose the inner circle and there was no difference in PI values between 1.0% and 0.5% agar (Fig. S1C,D). Moreover, the larvae also showed low PI values when the outer circle was 0.5% agar and the inner circle was 1.0% agar (Fig. S1C,D). Third-instar larvae tend to disperse, and as a result they preferably accumulated near the edge of the test plate. These data suggest that they chose a softer place by preference, not by chance, in the double circle assay.

To investigate whether and how much pure agar the larvae ingest in the double circle assay, we measured the amount of larval intake in the double circle assay with colored foods. When the agar concentration was the same in the inner and outer circles (control), the normalized intake in the inner circle was low because they did not choose the inner circle and went to the outside one (Fig. 1D). However, when the inner circle was softer, the normalized intake in the inner circle was significantly higher than in the control (Fig. 1D). These results suggest that the preference for softness corresponds to their intake. Nevertheless, in a wholly filled plate, there was no significant difference by agar concentration (Fig. S2A). Our results

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do not coincide with the results given by Apostolopoulou et al. (2014), where the larvae ingested 1.0% agar more than 0.5% agar for 30 min. However, for 10 min, our results show a similar difference (Fig. S2B). We assume that 30 min might be too long to observe the differences in the amount of intake on agar concentrations shown in the previous study.

In terms of softness sensing, we wondered whether larvae prefer relative softness or absolute softness. To address this question, we performed the softness preference test at higher agar concentrations. Larvae did not show significantly different preferences in the choice between 1.0% and 2.0% agar (Fig. 1E), suggesting that larvae prefer absolutely softer agar, though we cannot exclude the possibility that the softness sensation by flies is saturated at agar concentrations higher that 1.0%. According to Kim et al. (2017), larvae on 2.0% agarose remain at the surface for ∼80% of the trial time, whereas larvae on 0.1–0.6% agarose dig into the substrate for almost the whole time. Thus, larvae appear to prefer softer places to dig into.

Larvae might prefer a softer place owing to the ease of crawling. We showed that locomotor speed does not differ on the different agar concentrations used in this assay (Fig. S1E), suggesting that it was not because of the ease of crawling that they chose softer agar. Indeed, Apostolopoulou et al. (2014) showed that larval crawling speed did not differ on 0.5%, 1.0% and 2.5% agarose, whereas higher concentrations affected it. Elements other than softness might also affect larval preference, for example, light intensity, the amount of moisture or osmotic pressure. Fig. S1F displays the result of the double circle assay in dark conditions. It shows that they also chose a softer place in dark conditions, suggesting that light intensity does not affect their preference. However, the PI values on 0.5% agar were lower (Fig. S1F) than in light conditions (Fig. 1C). Dombrovski et al. (2017) showed that larvae accumulate and form a larval cluster during digging behavior and that the cluster initiation needs visual input. From this finding we infer that when the double circle assay is carried out in the dark, larvae accumulate during digging less often than in the light, resulting in lower PI values. Unfortunately, our method cannot distinguish between the amount of moisture and osmotic pressure.

We performed the preference test for softness on first-instar larvae to establish whether they show preferences similar to those of third-instar larvae. In this assay, we used 1.0% or 0.5% agar for the inner circle. First-instar larvae preferred a lower concentration of agar, suggesting that first-instar larvae also prefer softer places (Fig. 1F). At the same time, we can see that the PI value on 1.0% agar was higher than the PI value in third-instar larvae (Fig. 1C). We believe that this inconsistency could be explained by locomotor differences between first- and third-instar larvae. According to Almeida-Carvalho et al. (2017), the turn rate of first-instar larvae is much higher than that of third-instar larvae, causing a reduced exploration range in first-instar larvae. For this reason, first-instar larvae might leave the inner circle less often than third-instar larvae in the double circle assay.

Larvae search for softer places using their memory of an odor

We investigated whether larvae can learn softness as a reward associated with an odor and search for appropriate softness using their memory of the odor. We trained larvae in a one-odor learning paradigm (Mishra et al., 2013a), tested their preference for odor, and calculated a PI value as described in the Materials and Methods (Fig. 2A). We used 0.5% and 1.0% agarose plates in the training procedure. Using agarose, we obtained the same result as for agar (Fig. S3A,B). Larvae were placed on the agarose plates for 2.5 min. Either a 0.5% or a 1.0% agarose plate was reciprocally paired with an odor. The training was repeated three times, and the odor test was performed on either a 0.5% or a 1.0% agarose plate. When we used 0.5% agarose as the test plate, the preference for odor was zero regardless of the agarose concentration paired with odor at training, the PI value being close to 0 (Fig. 2B). In contrast, when we used 1.0% agarose as the test plate, the larvae showed a positive preference for the odor paired with 0.5% agarose in training (Fig. 2C). In brief, when on a harder place, the larvae seek a softer place using their odor memory (Fig. 2D), implying that they can learn softness as a reward associated with an odor cue and search for an appropriate softness using their memory.

Larvae evaluate food by balancing softness and sweetness

Larvae feed on rotten and softened fruits in nature (Mccoy, 1962; Jaenike, 1983) and need to find a place that is both soft and sweet (Kim et al., 2017). Indeed, the hardness of the banana fruit *Drosophila* generally ingests is roughly the same as 0.5% agarose (Sánchez-Alcañiz et al., 2017). Thus, we wondered whether larvae can balance modalities between softness and sweetness. We first checked whether larvae can detect and are attracted by 10 mmol l^{-1} fructose and then whether they can discriminate between fructose concentrations of 10 and 80 mmol l^{-1} (Fig. 3A,B). Our results show that larvae prefer 10 mmol l^{-1} fructose in the center circle to 0 mmol l^{-1} in the outer circle (Fig. 3A). When the inner circle contains 80 mmol l^{-1} fructose and the outer circle contains 10 mmol l^{-1} fructose, a significantly higher PI is obtained (Fig. 3B), indicating that larvae can distinguish the difference in fructose concentration.

Next, we tested the interaction between sweetness and softness. The inner circle contained 10 mmol l^{-1} fructose, and the outer circle contained 80 mmol l^{-1} fructose. Adding fructose had no effect on the hardness of the agar gel (Fig. S4), and larvae did not gather in the inner circle when the inner and outer concentrations of fructose were the same (Fig. S5A). As shown in Fig. 1B, the larvae gradually started to gather in a softer place over the 30 min period. They started to sense fructose within 5 min and then the preference for 80 mmol l^{-1} fructose declined (Fig. S5B). If we compare preferences at 30 min, the PI values mainly reflect the preference for softness. Thus, we compared preferences at 10 min to determine the interaction of sweetness and softness, because the larvae seem to integrate these two sensory cues at 10 min (Fig. S5B). As a control, we first changed the agar concentrations of both the inner and outer circle, keeping the agar concentration of the inner circle the same as that of the outer circle (see legend to Fig. 3). The PI values at 10 min were almost zero regardless of the agar concentration (Fig. 3C). By contrast, when we fixed the agar concentration of the inner circle and changed the agar concentration of the outer circle, the larvae chose the softer place at 10 min even though the inner circle was less sweet than the outer circle (Fig. 3C). This result is similar to what was shown in a previous study using adult flies (Jeong et al., 2016). These findings suggest that larvae choose what to ingest by balancing sweetness and softness.

We also measured the amount of intake in the sweet–soft interaction test (Fig. 3C). We first measured relative intake on a wholly filled plate to normalize the intake on a double circle assay plate (Fig. S2B) (see Materials and Methods). When the outer circle was 1.0% agar, the amount of intake in the inner circle tended to increase if the inner circle was 0.5% agar in spite of the lower sweetness (Fig. 3D). When the outer circle was 2.0% agar, there was a significant difference between whether the inner circle was 2.0% or 0.5% (Fig. 3D). These results indicate that their intake correlates with preference in the double circle assay shown in Fig. 3C.

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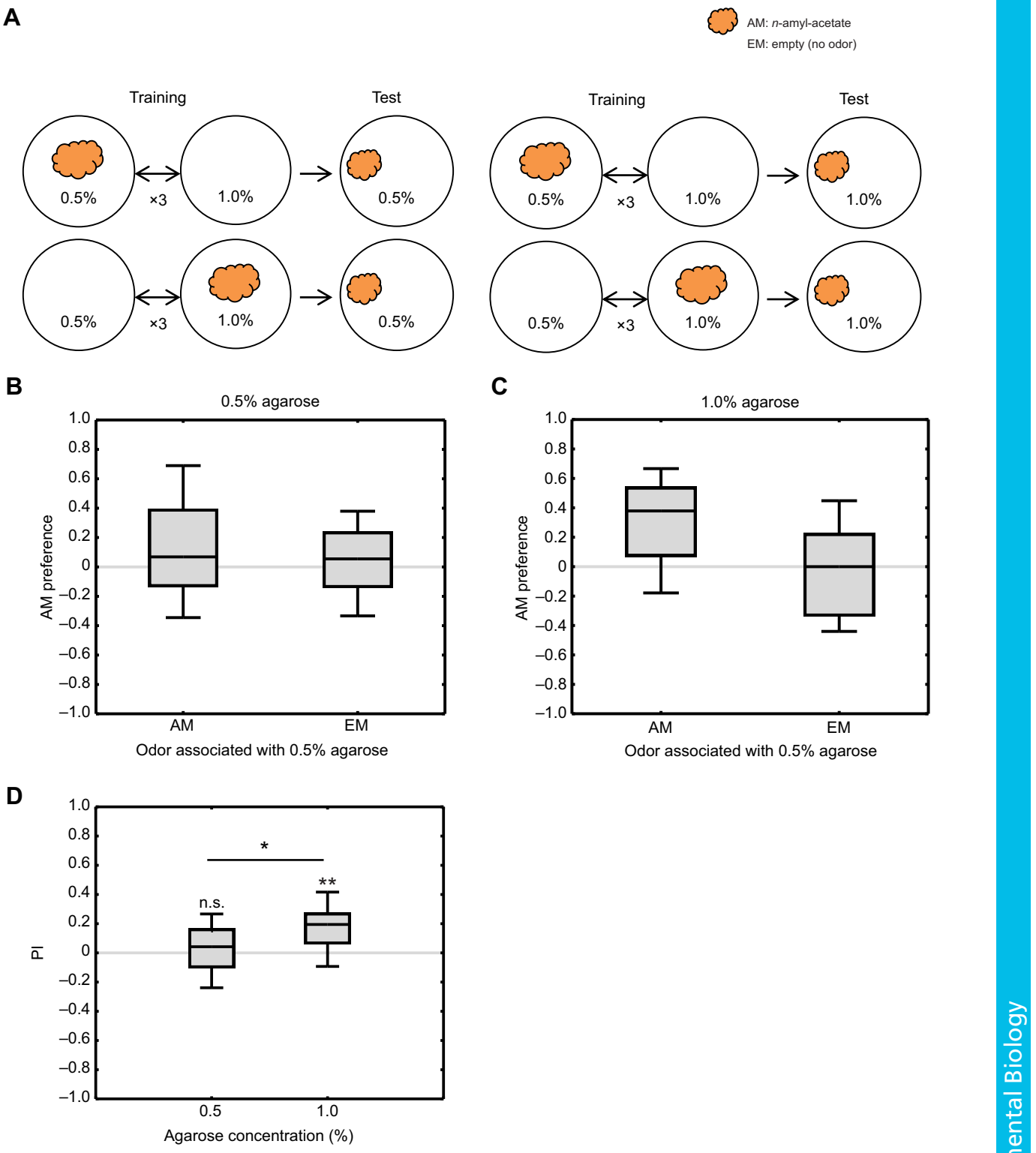

Fig. 2. Third-instar larvae can learn to associate 0.5% agarose with an odor. (A) Experimental design. (B,C) The preference index for AM. (B) Larvae did not show a preference for AM with 0.5% agarose as the test plate (*N*=24). If they were seeking an attractive place using olfactory memory, they should show a preference for odor. This result indicates that they were not searching for a softer place because the 0.5% agarose test plate was sufficiently soft for them. (C) In contrast, they showed a significant preference for odor with 1.0% agarose as the test plate (*N*=24). This result indicates that third-instar larvae placed on 1.0% agarose had learned to associate softness with odor and were searching for a softer substrate using their memory. (D) PI values at test. When we used 0.5% agarose as the test plate, they did not show a significant PI. However, they did show a significant PI when we used 1.0% agarose as the test plate. There is a significant difference between 0.5% agarose and 1.0% agarose. This result clearly indicates that they can learn to associate softness with odor (*N*=24, 24 for 0.5% and 1.0% agarose, respectively; one-sample sign test: ***P*<0.01; n.s., not significant; Mann–Whitney *U*-test: **P*<0.05).

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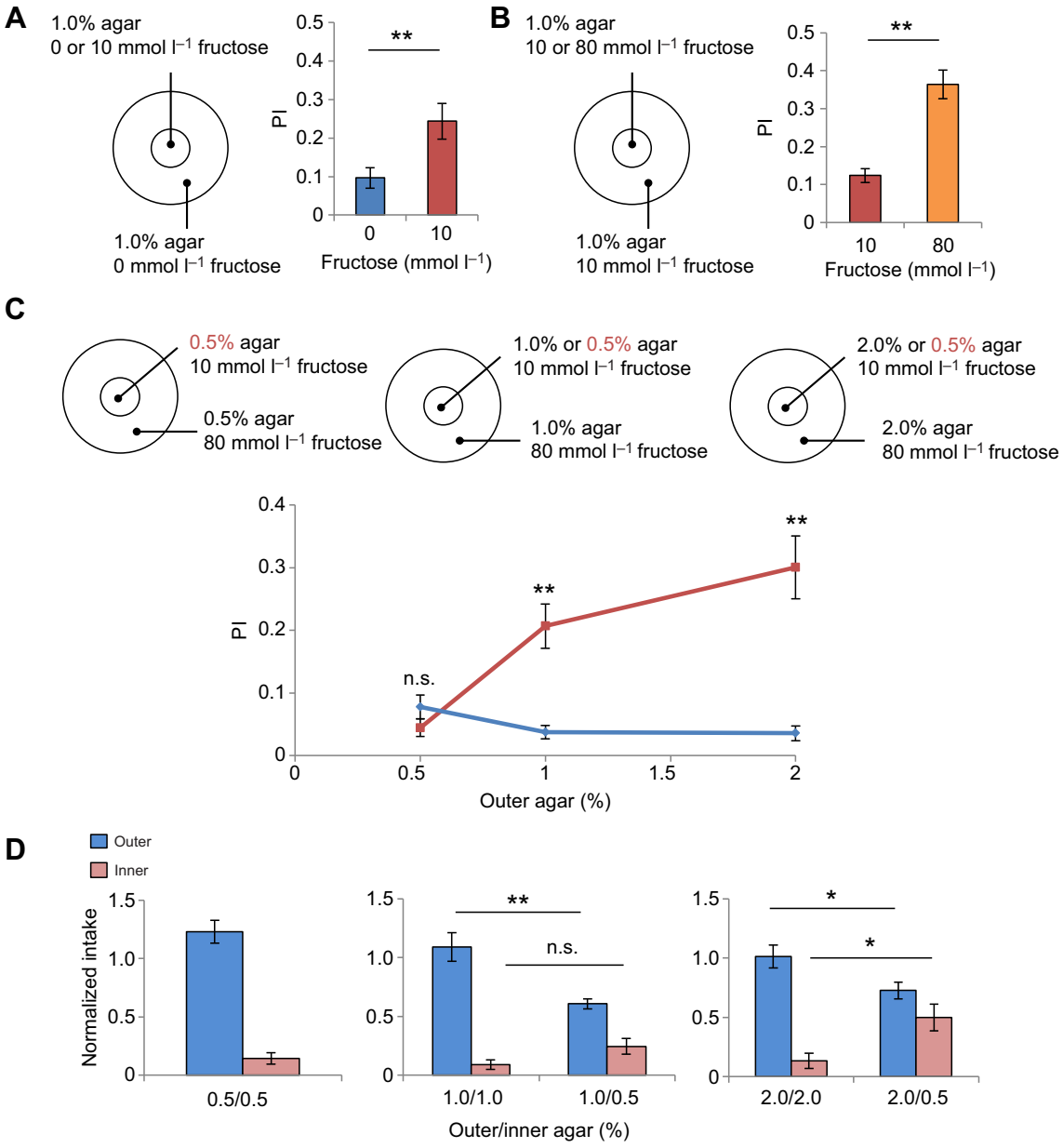

Fig. 3. Larvae evaluate food by balancing sweetness and softness. (A,B) PI values for 0, 10 and 80 mmol l^{-1} fructose. On the basis of the time course data (Fig. S5B), we used the PI at 10 min to draw a comparison between softness and sweetness. (A) PI values for 0 and 10 mmol l^{-1} fructose in the inner circle versus 0 mmol l^{-1} fructose in the outer circle; larvae can sense 10 mmol l^{-1} fructose as sweetness at 10 min (N=20, 20 for 0 mmol l^{-1} and 10 mmol l^{-1} fructose, respectively; Mann–Whitney U-tests: **P<0.01). (B) PI values for 10 and 80 mmol l^{-1} fructose in the inner circle versus 10 mmol l^{-1} fructose in the outer circle; they significantly prefer 80 mmol l^{-1} fructose compared with 10 mmol l^{-1} fructose at 10 min (N=20, 20 for 10 mmol l^{-1} and 80 mmol l^{-1} fructose, respectively; Mann–Whitney U-tests: **P<0.01). (C) We compared the PI value at 10 min in two different situations [N=20 (0.5%), 20 (1.0%) and 20 (2.0%) for blue and 20 (0.5%), 20(1.0%) and 20 (2.0%) for red, respectively; Mann–Whitney U-tests: **P<0.01; n.s., not significant]. The x-axis indicates the agar concentrations of the outer circle. The blue line shows the PI when the agar concentrations were the same in the inner and outer circles (control). The red line shows the PI when the inner circle was fixed at 0.5% agar, and the outer circle was changed from 0.5% to 2.0% agar. In both situations, the inner circle contained 10 mmol l^{-1} fructose, and the outer circle contained 80 mmol l^{-1} fructose. The harder the outer circle, the higher the PI value. Larvae prefer a softer substrate in spite of the lower concentration of fructose. (D) Normalized intake in the double circle assay during 10 min; agar concentrations correspond to the agar concentrations in the outer circle in B [N=12 (0.5%/0.5%), 13 (1.0%/1.0%), 13 (1.0%/0.5%),11 (2.0%/2.0%) and 11 (2.0%/0.5%), respectively; Mann–Whitney U-tests, *P<0.05, **P<0.01, n.s.: not significant]. When the outer circle was 1.0% agar, the absorbance of the inner circle showed no significant difference, but there was a tendency to increase if the inner circle was 0.5% agar in spite of the lower sweetness. When the outer circle was 2.0% agar, there was a significant difference in intake depending on whether the inner circle was 2.0% or 0.5% agar. These results indicate that the intake corresponds to preference in the double circle assay in C.

Genetic background affects the results of the double circle assay

We next asked whether mechanosensitive channels are involved in softness sensing and tested several mechanoreceptor mutant lines and their UAS-RNAi lines to see whether these flies fail to discriminate softness. In the course of these studies, we found that even control lines sometimes show a failure of softness discrimination. This prevented us from seeking the responsible mechanoreceptor, raising the possibility that genetic background might severely affect softness sensing. Thus, we tested nine

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available wild-type strains for softness sensing. We found that there is variation in the preference for softness among wild-type strains, indicating that genetic background might influence phenotypes in this assay (Fig. 4A). Three of the nine strains, Oregon-RC, Amherst-3 and Hikone-A-S, did not significantly prefer softer agar. Canton-S and Zimbabwe-S29 showed a similarly strong preference for softer agar, whereas the PI values for 0.5% agar among Lausanne-S, Urbana-S, Berlin-K and Oregon-R were lower than those of these two strains.

To investigate whether the preference for softness is related to ingestion behavior, we measured the intake of 1.0% and 0.5% agar in the wholly filled plate. There was variation in the amount of intake among wild-type strains (Fig. 4B). In six of the nine strains that preferred 0.5% to 1.0% agar in the double circle assay (Fig. 4A), there was no significant difference between 0.5% and 1.0% agar. However, the three strains that did not prefer 0.5% agar in the double circle assay (Fig. 4A), Oregon-RC, Amherst-3 and Hikone-A-S, ingested 0.5% agar significantly less than they ingested 1.0% agar. Unfortunately, we cannot conclude that the ingestion behavior is directly related to the preference for softness. Ingestion behavior might be affected by multiple genetic factors.

DISCUSSION

Drosophila melanogaster larvae prefer soft agar. Our associative learning experiments indicate that larvae can learn softness as a reward associated with odor and search for a softer substrate using their memory of the odor. Our results confirmed most of the results of Apostolopoulou et al. (2014). We also found that larvae evaluate food by balancing softness and sweetness and that genetic background affects the larval preference for softness.

Recent studies have reported that larvae show collective social behavior (Wu et al., 2003; Xu et al., 2008; Durisko et al., 2014; Dombrovski et al., 2017). In particular, larvae collectively dig into food at one place and form a larval cluster (Dombrovski et al., 2017). Our behavioral results might be affected by this collective effect, but the effect is likely to be limited because not all larvae were engaged in such digging behavior during the short observation period in our measurements.

To ascertain whether ingestion behavior is linked to the preference for softness, we measured the amount of intake in third-instar larvae. In double circle assay, when the inner agar was softer, larvae ingested more inner agar compared with the control during 30 min (Fig. 1D). The tendency was seen during 10 min in

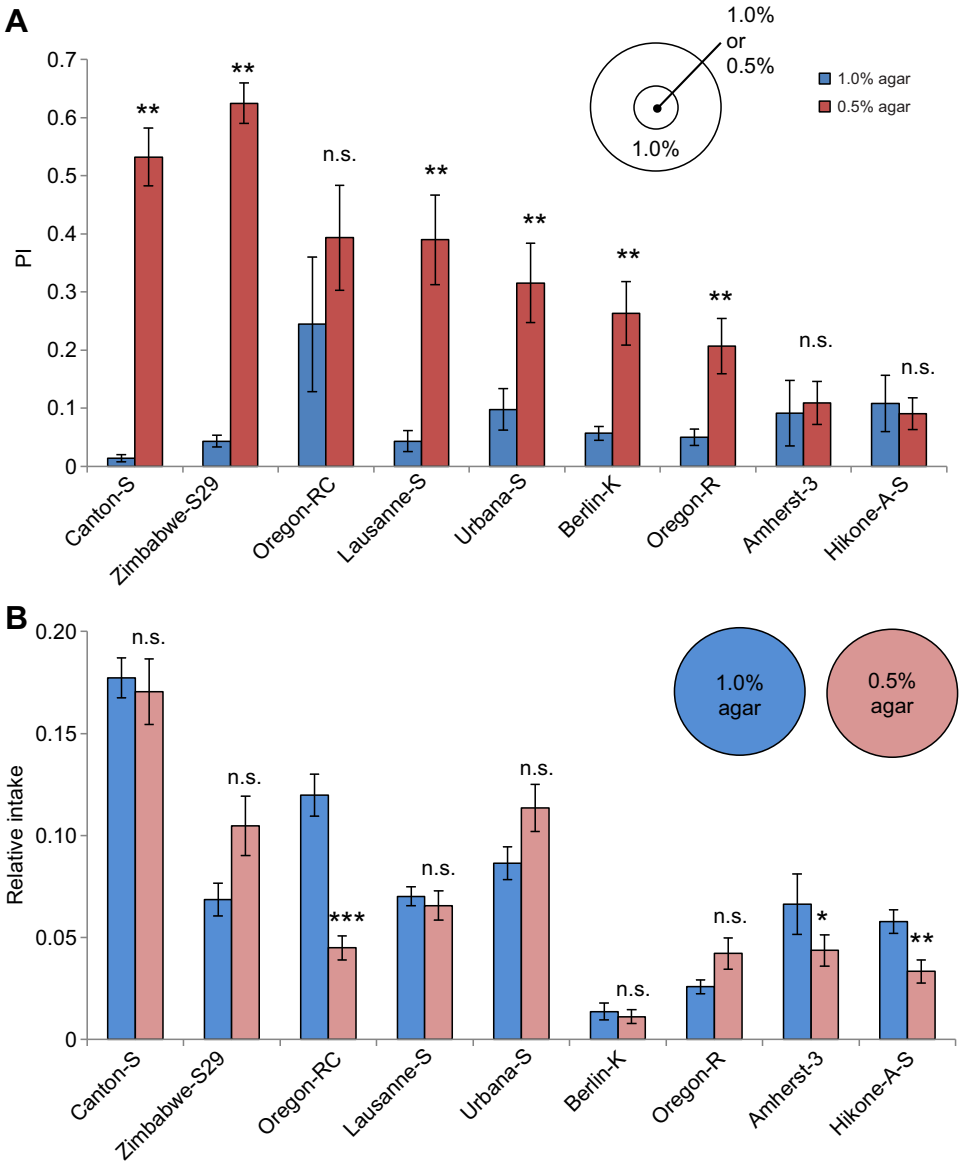

Fig. 4. Effect of genetic background. Softness preferences were tested in nine wild-type strains: Canton-S, Zimbabwe-S29, Oregon-RC, Lausanne-S, Urbana-S, Berlin-K, Oregon-R, Amherst-3 and Hikone-A-S (*N*=20, 20, 9, 20, 15, 20, 20, 8 and 8, respectively; Mann–Whitney *U*-tests: ***P*<0.01; n.s., not significant). The double circle assay was performed using 1.0% (outer) and 0.5% (inner) agar. (B) Relative intake during 30 min; to investigate whether the preference for softness corresponds to the amount of intake in wild-type strains, we measured and compared their intake of 1.0% and 0.5% agar colored with blue and red, respectively (*N*=12, 7, 9, 13, 8, 7, 8, 11 and 8 for blue and 14, 8, 9, 9, 10, 6, 11, 8 and 8 for red; Mann–Whitney *U*-tests: **P*<0.05; ***P*<0.01; ****P*<0.001; n.s., not significant).

the presence of fructose (Fig. 3D). In the wholly filled plate of pure agar, however, larvae ingested 0.5% agar as much as 1.0% agar for 30 min (Fig. S2A). Apostolopoulou et al. (2014) obtained a different result using a wholly filled plate of pure agar for 30 min, on which larvae ingested 0.5% agar less than 1.0% agar. We also measured the intake on the wholly filled plate adding fructose for 10 min, and larvae ingested 0.5% agar less than 1.0% agar (Fig. S2B). We suspect that the intakes on wholly filled plates of pure agar were saturated in our assay. Combining these results, however, we could not determine why larvae prefer softer substrates. There are two possible reasons. One is that this may enable them to escape predators or avoid dehydration. Another is the advantage for feeding conferred by the substrate condition that they choose, whether softer or harder.

We showed that larvae prefer softer substrates. We also demonstrated that larvae can learn softness as a reward associated with odor. These results support the previous report published by Apostolopoulou et al. (2014), which showed that larvae can discriminate differences in agarose concentration and are able to learn the softness of 0.5% agarose as a reward. We additionally showed that, when on a harder substrate, larvae search for a softer substrate using the memory associated with an odor. One should consider the possibility that 1.0% agarose acts as a punishment, but we assume that this is not likely. If it were so, larvae would show a negative preference for AM in the EM case. However, they showed a neutral preference for AM (Fig. 2C, right). Moreover, we found that if sweeter food is comparatively hard, larvae choose softer food even though it is less sweet. A similar interaction between softness and sweetness has recently been reported in adult feeding behavior (Jeong et al., 2016). Additionally, Apostolopoulou et al. (2014) showed that the larval behavioral response to bitter compounds is affected by agarose concentrations. In all of these cases, softness affects the preference for food.

Larvae show various behavioral patterns: crawling, shoveling and digging. It is important to establish by which behavioral process the larvae sense softness. The preference for softer agar is evident after 5 min of testing (Fig. 1B). At this time the larvae do not show digging behavior. In the olfactory learning experiment, 3 min is enough for the larvae to learn softness and to show their preference (Fig. 2D). It is possible that larvae can sense softness while they are crawling on the agar surface. However, we cannot exclude the possibility that they perform rapid shoveling behavior while they are crawling. Thus, we should identify the responsible mechanosensory neurons for softness sensing.

It is reasonable to postulate that a mechanosensitive channel is involved in softness sensation. Two TRP channels, NANCHUNG and NOMPC, have been found as receptors for texture sensing in adults (Jeong et al., 2016; Sánchez-Alcañiz et al., 2017, 2018). In larvae, these two TRP channels are known to function in sensing sound (Zhang et al., 2013), and NOMPC is required for gentle touch sensation (Tsubouchi et al., 2012; Yan et al., 2013). Thus, these are plausible candidates for the sensing of food texture in larvae. A next question is to identify the sensory neurons involved in texture sensing in larvae. There are three candidates. The first candidate is the chordotonal neurons. Recent studies in adults have shown that *nanchung* and *nompC*, which are expressed in mechanosensory neurons in gustatory sensilla, are required for sensing texture (Jeong et al., 2016; Sánchez-Alcañiz et al., 2017). In larvae, NANCHUNG and NOMPC are expressed in chordotonal neurons (Zhang et al., 2013; Gong et al., 2004; Lee et al., 2010), and these neurons are interspersed at regular intervals over their body wall (Makhijani et al., 2011; Grueber et al., 2002; von Hilchen et al., 2013). The second candidate is the multidendritic neurons tiling the body wall. These neurons are morphologically classified according to four classes, I–IV (Grueber et al., 2002). In particular, class III neurons sense mild mechanical stimuli (Tsubouchi et al., 2012; Yan et al., 2013), whereas class IV neurons sense nociceptive stimuli (Hwang et al., 2007). Moreover, transmembrane channel-like protein (TMC), which is expressed in multidendritic neurons in the labellum, is involved in sensing texture in adult flies (Zhang et al., 2016). Larval TMC is expressed in class I and II multidendritic neurons and is involved in sensory feedback for locomotion (Guo et al., 2016). If all or any of these body wall mechanosensory neurons are involved, larvae might discriminate softness by sensing differences in deflection of their body wall. The third candidate is the mechanosensory neurons in their head organs. As mentioned in the Introduction, larvae have three external and four internal sensory organs: the external ones are TO, VO and DO; the internal ones are DPS, VPS, PPS and DPO (Gerber et al., 2004; Kwon et al., 2011). According to previous morphological studies, there are potential mechanosensory neurons in TO and VPS (Apostolopoulou et al., 2015; Green et al., 1983; Becher et al., 2012; Sokolowski et al., 1984; Chu-Wang and Axtell, 1972; Rist and Thum, 2017). In the recent anatomical analysis of TO by Rist and Thum (2017), however, there is no UAS-GFP signal with any *Trp*-Gal4 lines in TO (i.e. nan-Gal4, nompC-Gal4 or iav-Gal4 and so on). Therefore, if larval head organs are involved in sensing texture, VPS is a more likely candidate than TO. We showed that larvae can integrate mechanical with chemosensory information (Fig. 3C), implying that mechanical information is important in feeding. Furthermore, Jeong et al. (2016) found that mechanosensory neurons in gustatory sensilla form an inhibitory synaptic output to chemosensory neurons that sense sweet taste in adult flies. In the light of these findings, mechanosensory neurons that sense food softness might be present in chemosensory organs.

Our initial attempt to identify the corresponding neuron and the receptor involved in softness sensing failed because of the presence of genetic variations between several wild-type strains, as shown in the Results (Fig. 4A). It would be interesting to determine why some strains do not prefer a softer substrate. Measurements of the amount of intake in the wholly filled plate also showed variation depending on the genetic background (Fig. 4B). Moreover, three strains that did not choose the softer agar were found to ingest 0.5% agar less than 1.0% agar, whereas other strains ingested 0.5% agar as much as 1.0% agar (Fig. 4B). One possible explanation is that they could not discriminate agar differences if they did not ingest 0.5% agar as much as 1.0% agar. Nevertheless, Canton-S flies prefer softer substrates for 10 min in spite of ingesting 0.5% agar less than 1.0% agar (Fig. 3C, Fig. S2B). Accordingly, we favor the hypothesis that the variation in the preference for softness was caused by a variation in feeding habitat. Kim et al. (2017) showed that *D. suzukii* larvae on 2.0% agarose spent more time digging than *D. melanogaster* larvae did, which seems to be caused by the difference in the flies' feeding habitat: *D. suzukii*, unlike *D. melanogaster*, grows on fresh (hard) fruits. Considered from this point of view, there is a possibility that genetic background affects feeding habits to generate the variation in the preference for softness and the amount of intake (Fig. 4A,B). If we can overcome the difficulties posed by the differences in genetic background, we will be able to identify the neural mechanisms of softness sensing.

Searching for softer food is adaptive for larvae in nature. Takamura (1984) showed that the hardness preferred by female flies to oviposit varies on the species of *Drosophila*, and that *D. melanogaster* females prefer 1.0% agar for their oviposition site.

Larvae constantly have to forage for an appropriate softness to dig into to feed, hide from predators or avoid dehydration. If they hatch on a harder substrate, they will have to search for a softer substrate. Indeed, in our olfactory learning experiments, the larvae searched for a softer substrate when they were on a harder substrate. It seems clear, therefore, that sensing texture is important for larvae in nature.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: T.T.; Methodology: T.T.; Formal analysis: N.K.; Investigation: N.K., T.T.; Data curation: N.K.; Writing - original draft: N.K.; Writing - review & editing: N.K., A.K., T.T.; Visualization: N.K.; Supervision: A.K., T.T.; Project administration: T.T.; Funding acquisition: A.K., T.T.

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Supplementary information
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