Journal of Insect Physiology 112 (2019) 39–47

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

LPS perception through taste-induced reflex in *Drosophila melanogaster*

Aya Yanagawa[a,*], Antoine Couto[b], Jean-Christophe Sandoz[b], Toshimitsu Hata[a], Aniruddha Mitra[b,c], Moutaz Ali Agha[d,e], Frédéric Marion-Poll[b,d,*]

[a] Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Japan
[b] UMR Evolution, Génomes, Comportement, Ecologie, CNRS, IRD, Univ Paris-Sud, Université Paris-Saclay, F-91198 Gif-sur-Yvette, France
[c] School of Biological & Environmental Sciences, Shoolini University, Solan, India
[d] AgroParisTech, Université Paris-Saclay, Paris, France
[e] Ÿnsect-Pôle Innovia, Damparis, France

ARTICLE INFO

Keywords:
Contact chemoreception
Wings
Drosophila
LPS
Gustation
Grooming reflex

ABSTRACT

In flies, grooming serves several purposes, including protection against pathogens and parasites. Previously, we found *Escherichia coli* or lipopolysaccharides (LPS) can induce grooming behavior via activation of contact chemoreceptors on *Drosophila* wing. This suggested that specific taste receptors may contribute to this detection. In this study, we examined the perception of commercially available LPS on *Drosophila* wing chemoreceptors in grooming reflex. Behavioral tests conducted with bitter, sweet and salty gustation such as caffeine, sucrose and salt, using flies carrying a defect in one of their taste receptors related to the detection of bitter molecules (*Gr66a, Gr33a*), sugars (*Gr5a, Gr64f*), or salt (*IR76b*). LPS and tastants of each category were applied to wing sensilla of these taste defectflies and to wild-type Canton Special (CS) flies. Our results indicate that the grooming reflex induced by LPS requires a wide range of gustatory genes, and the inactivation of any of tested genes expressing cells causes a significant reduction of the behavior. This suggests that, while the grooming reflex is strongly regulated by cues perceived as aversive, other sapid cues traditionally related to sweet and salty tastes are also contributing to this behavior.

1. Introduction

The induction and modification of behavior in *Drosophila* is dependent on chemical cues from the environment (Depetris-Chauvin et al., 2015). Grooming reflexes can be elicited in decapitated flies by contacting them with *Escherichia coli* or with commercially available lipopolysaccharides extracts (from Sigma: sLPS) (Yanagawa et al., 2014). Insects groom themselves for a number of purposes, including the maintenance of body integrity and the avoidance of noxious stimuli (Böröczky et al., 2013; Dethier, 1972; Newland, 1998; Page and Matheson, 2004). LPS is the principal component of the outer membrane of Gram-negative bacteria and it is considered as an endotoxin, which may elicit strong immune reactions in vertebrates (Rietschel and Brade, 1992) as well as in insects (Tanaka et al., 2009; Rao and Yu, 2010; Kazlauskas et al., 2016). In flies, the presence of peptidoglycans (PGN) but also of unknown impurities contained in sLPS extracts seem to be responsible for triggering grooming, as the response intensity to sLPS is higher than to PGN (Yanagawa et al., 2017). This behavior is quite efficient against microbes which require a physical contact to infect insects (Vega and Kaya, 2012). Hygienic behaviors such as grooming are efficient if they can also be triggered by volatiles or tastants which are a signature of the presence of such harmful microbes (Yanagawa and Shimizu, 2007; Yanagawa et al., 2009). In flies, specific olfactory receptors are devoted to the detection of chemicals from harmful microbes which modify the flies' orientation and oviposition abilities (Stensmyr et al., 2012), and specific contact chemicals induce grooming and feeding avoidance (Yanagawa et al., 2014). However, *Drosophila* habitats contain a great variety of microbes (Rolfs, 2005), which also trigger positive reactions such as feeding, oviposition, and courtship behavior (French et al., 2015; Hiroi et al., 2004; Joseph et al., 2009; Hu et al., 2015). Thus, although specific chemicals may trigger stereotyped aversive responses, microbial cues may elicit different responses depending on the context. In decapitated insects, the responses induced in these insects bypassed the downstream control normally exerted by higher-order nervous centers in intact animals. Using this approach allowed us to study the networks subtending grooming

Abbreviations: Conc, concentration; sLPS, lipopolysaccharide extracts from *Escherichia coli* obtained from Sigma Corp

* Corresponding authors at: RISH, Kyoto University, Uji city 611-0011, Kyoto, Japan (A. Yanagawa). UMR 9191 EGCE, CNRS, bat. 13, 1 rue de la Terrasse, 91198 Gif-sur-Yvette, France (F. Marion-Poll).

E-mail addresses: ayanagawa@rish.kyoto-u.ac.jp (A. Yanagawa), frederic.marion-poll@egce.cnrs-gif.fr (F. Marion-Poll).

https://doi.org/10.1016/j.jinsphys.2018.12.001
Received 13 September 2018; Received in revised form 7 November 2018; Accepted 2 December 2018
Available online 07 December 2018

behavior that involve local ganglia and not the brain. The observation of this simple neural network from input to output reflex is possible only with decapitated flies.

In our previous study (Yanagawa et al., 2014), only bitter and microbial substances seemed to be important for inducing hygienic behavior (i.e., the grooming reflex). This hypothesis was confirmed by Soldano et al. (2016), who reported that TRPA1 cation channels expressed in taste sensilla expressing *Gr66a* on labellum, regulate sLPS avoidance in *D. melanogaster*. Then, Raad et al. (2016) reported that *Drosophila* wings can detect sweet and bitter molecules with the corresponding *Gr* genes as on the proboscis. Here, to learn if microbial cues are attractive or aversive to *Drosophila melanogaster*, we assessed the role of gustatory receptors in eliciting grooming reflex either by using optogenetic stimulations of gustatory neurons or by monitoring flies' reactions to chemical stimuli in individuals subjected to genetic ablation of one category of taste neurons. In our optogenetic experiments, we investigated *Gr64f*, considered as a co-receptor for the detection of sugars (Jiao et al., 2008), and *Gr33a*, which is required for the detection of bitter substances (Weiss et al., 2011). We further examined the roles of *Gr5a*, *Gr66a*, and *IR76b* which are assumed to be involved in the detection of sugars, bitter substances and salt, respectively (Jiao et al., 2008; Weiss et al., 2011; Zhang et al., 2013). Grooming induction by the best agonists of these genes was also tested: this included caffeine (bitter) for *Gr66a*, sodium chloride (NaCl; salty) for *IR76b*, and sucrose (sweet) for *Gr5a* (Zhang et al., 2013). Our observations indicate that the expression of grooming reflexes not only requires functional bitter receptor genes and neurons but also gustatory genes and neurons related to the detection of sugars and salt. Lastly, to confirm the involvement of tested gene, *Gr* and *Ir* expressions in wing chemoreceptor cells were observed with a confocal microscope using GAL4 constructs to drive the expression of a fluorescent protein. Together with the confocal observation, the chemical composition of wing chemosensilla was investigated using Raman spectroscopy to see if internal structure of the chemosensillum support its functionability. It is said that Raman information of chemical compositions such as the C═O stretching of amidIindicates the presence a lymph meniscus, whose structure is important to deliver chemical cues to chemoreceptor cells (Valmalette et al., 2015).

2. Materials and methods

2.1. Flies

D. melanogaster flies were maintained on the standard cornmeal agar medium at 20 °C and 80% humidity. Most experiments were conducted on Canton Special (CS) flies. We also used mutants and UAS-Gal4 constructs listed in a separate table (Table S1).

2.2. Chemicals

sLPS (L2630, Lipopolysaccharides from *Escherichia coli* 0111:B4, Sigma), sucrose, caffeine, NaCl, and potassium chloride originated from Sigma-Aldrich (France and Japan) and were dissolved in distilled water. Five concentrations were prepared for all chemicals, LPS: 0, 0.1, 1, 5, 10 mg/ml, caffeine: 0, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} M, NaCl: 0, 10^{-3}, 10^{-2}, 10^{-1}, 1 M and sucrose: 0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M. The solutions with no chemical were used as control.

2.3. Optogenetic stimulation

For optogenetic experiments, we expressed a channel rhodopsin into *Drosophila* taste neurons in order to activate them with blue light. To accomplish this, we used the ubiquitous *UAS-Gal4* system (Brand and Perrimon, 1993) to express channelrhodopsin2 (ChR2) (Nagel et al., 2003; Hornstein et al., 2009) into cells expressing either *Gr33a*, which encodes for a receptor essential for aversive taste (Weiss et al., 2011), or *Gr64f*, which encodes for a receptor essential for appetitive taste (Dahanukar et al., 2007). Flies were beheaded by a single cut made at the neck with micro-scissors. Micro-scissors were washed and wiped with 70% ethanol before and after use. Beheaded flies were placed in the upright position on a clean paper sheet and the body was allowed to stand-up. Flies were then exposed during 3 min to blue light (> 25 W, 480 nm, COO-pE-100F-WH1-20, CoolLED, UK). During stimulation, headless flies were placed on filter paper in the dark. The blue light was the only light source in the dark room, therefore, the observation time was arranged as the same duration as the previous behavioral test (Yanagawa et al., 2014). Since the light stimulus was delivered for 3 min, the intensity of the grooming response was more variable than that induced by a quick brush with contact chemicals. Therefore, we scaled the grooming response from 0 to 5 (score 1: grooming occurrence (1–2 brushes); score 2: grooming persistence of more than 10 sec but less than 20 s; score 3: grooming persistence of more than 20 s but less than 1 min; score 4: grooming persistence of more than 1 min but less than 2 min; score 5: strong grooming which persisted for more than 2 min). Four-day old flies were used in all tests. Note that because pooled data suggested a sex difference in the grooming responses (grooming: $p < 0.001$, $\chi^2 = 23.4$, sex difference: $p = 0.007$, $\chi^2 = 7.36$ in *Gr33a-Gal4* × *UAS-ChR2* and grooming: $p < 0.001$, $\chi^2 = 56.7$, sex difference: $p = 0.27$, $\chi^2 = 1.22$ in *Gr64f-Gal4* × *UAS-ChR2*, logistic regression), the data from females and males were analyzed separately. Siblings, which did not carry the full construct, were employed as controls.

Flies carrying a *Gr-Gal4* construct were crossed with flies carrying UAS-channel rhodopsin *(UAS-CHR2)* (Bloomington Drosophila Stock Center, stock no. 28995). The genotype was Gr33a-Gal4[1]/CyO;Dr/TM3, Sb, Ser for the *Gr 33a-Gal4* construct, *Gr64f-Gal4 – 5/Cyo;MKRS/TM2(UBX)* for the *Gr 33a-Gal4* construct, where siblings expressed a curly wing phenotype and progenies displayed normal wings and *UAS-H134R-CHR2* for UAS-channel rhodopsin. During development, larvae were fed on normal medium supplemented with 1 mM *trans*-retinal (Hornstein et al., 2009).

2.4. Role of chemoreceptor genes in the induction of grooming

In order to examine *Gr* gene involvement in the detection of LPS, we used flies deprived of the gustatory cells expressing the tested gene (i.e. using a promoter of that gene driving the expression of GAL4). *Gr33-* and *Gr66a-GAL4* were chosen to test bitter perception, *Gr64f-* and *Gr5a-GAL4* were chosen to test sweet perception, and *IR76b-GAL4* was chosen to test salt detection. The Gal4 lines were crossed with UAS-diphtheria toxin *(UAS-DTI)* flies (Wang et al., 2004). Thus, in the progeny, all cells expressing GAL4 express the toxin, causing the death of the cell. We could thus select in the progeny individuals which expressed the construct (called progeny) and others (siblings) which did not express the phenotype. For *Gr33a*, the construct was *Gr33a-Gal4[1]/CyO*; *Dr/TM3, Sb, Ser* × *UAS-DTI/TM6b, Tb*, where the siblings expressed the tubby and the curly wing phenotype and the progenies had a normal body size and straight wings. For *Gr66a*, the construct was *w*; Gr66a-Gal4 (II)* × *UAS-DTI/TM6b, Tb*, where the siblings expressed the tubby phenotype and the progenies had a normal body size. For *Gr64f*, the construct was *Gr64f-Gal4 – 5/Cyo*; *MKRS/TM2(UBX)* × *UAS-DTI/TM6b, Tb*, where the siblings expressed the tubby and the curly wing phenotype and the progenies had a normal body size. For *Gr5a*, the construct was *p:Gr5a-Gal4/CyO*; *TM2/TM6B* × *UAS-DTI/TM6b, Tb*, where the siblings expressed the tubby and the curly wing phenotype and the progenies had a normal body size. For *IR76b*, the construct was *SP/CyO*; *IR76b-Gal4/TM3, Sb* × *UAS-DTI/TM6b, Tb*, where the siblings expressed the tubby and the ebony phenotypes and the progenies had a normal body size and body color. As an additional control, no involvement of water perception on grooming reflex was confirmed. In order to generate flies deprived of water cells, we crossed *ppk28-Gal4* flies with UAS-DTI flies (Wang et al., 2004). *ppk28* mediates the cellular

and behavioral response to water (Cameron et al., 2010). Since the balancer chromosome of this construction was TM6b, Tb, we could examine the progeny and select individuals which expressed the construction (called progeny) and others (siblings) which did not express the phenotype. For *ppk28*, the construction was *CyO/BI; ppk28-Gal4 × UAS-DTI / TM6b, Tb*, where the siblings expressed the tubby phenotype and the progenies had a normal body size.

2.5. Grooming in response to a chemical stimulation

Grooming induction was assayed using the method in Yanagawa et al. (2014, 2017), which reported together with movie files. Briefly, decapitated flies were placed on a filter paper at room temperature (22°−25 °C). We used a sharpened tooth pick previously soaked into the test solution, to gently touch their wings, margin (MR) III/IV. Their grooming activity was monitored by eyes and scored up to 3 min after the stimulation. 4-day old CS flies were tested with LPS, sucrose, caffeine and NaCl diluted in water. The concentrations of all compounds were physiological (Moon et al., 2006; Yanagawa et al., 2014) and the highest was set as that starts inducing the significantly strong reflex by the pilot test. Controls were performed by stimulating flies with distilled water. Each chemical was tested on 20 female and 20 male flies. Since the duration of single grooming differs by its intensity, a scoring system was employed to estimate the behavior: score 1: grooming occurrence (1 to 2 brush: < 10 s), score 2: grooming persistence more than 10 s but less than 20 s, score 3: grooming persistence more than 20 s but less than 1 min. The scoring had been simpler since the stimulus was applied with single contact, while the scoring in the blue light has higher level as the blue light stimulus was given continuously for 3 min.

2.6. Raman spectra of Drosophila chemosensory wing hair

Valmalette et al. (2015) reported that the Raman spectra can indicate the presence a lymph meniscus. Therefore the composition of wing chemosensory sensilla were examined with those spectra at 1350 cm^{-1}, 1540 cm^{-1} and 1610 cm^{-1}.

Sensilla at three locations on the wing margin were chosen to observe Raman spectra, and these values were then compared to that of small taste bristles on the leg (Sensillum S1, Fig. 6A), whose architecture is already known (Stocker, 1994). The yellow triangle on the linear wing vein pictured in Fig. 6BC indicates the three assessed sensilla. Sensillum S2 is on the wing margin 2 region, which is five sensilla away from sensillum S3-1. Sensillum S3-1 is located at the right of the image (Fig. 6C). Sensillum S3-2 is again five sensilla away from sensillum S3-1, toward the direction of wing margin 3. Approximate locations are illustrated in Fig. 6C. Although wing specimens were placed in a uniform manner, the way the laser beam captured the sensillum was highly variable owing to large divergence in wing curvature. As this factor seemed to largely affect Raman intensity, only spectra modes were examined. *Drosophila* hemolymph was collected from the thorax. It was smeared on a glass slide and dried for 1 day before observation. It is reported that the 1350 cm^{-1} band fits with the position of C−N stretching and N−H bending of aromatic amino acids, which is consistent with the expected presence of tryptophan, phenylalanine and tyrosine. 1540 cm^{-1} band corresponds to the position of the amide II bands resulting from the combination of N−H bending and C−N stretching, and a mode at 1610 cm^{-1} could be assigned to the C=O stretching of amide I, and these bands were deduced as the bands indicating a lymph meniscus (Valmalette et al., 2015).

A Renishaw's inVia Raman microscope and its Windows-based Raman Environment (WiRE2.0) software were used to obtain Raman spectra from wing taste sensilla. Raman spectral analysis used a wavelength of 514.5 nm (UV ready). Raman spectra were acquired using the method reported by Valmalette et al. (2015). Briefly, laser power was set at 5%, the spectrum range was 720–2400 cm^{-1}, and acquisition time was 30 s. *Drosophila* wings from live specimen were removed at the wing/thorax muscle junction and immediately placed on a glass slide. Raman spectra were obtained from the apical and basal area of the wing sensillum.

2.7. Confocal microscopy

In order to confirm gustatory gene expression on wing taste sensillum, RFP expression in taste cells encoding each tested Gr gene were observed using a confocal microscope (LSM-700; Carl Zeiss, Jena, Germany). Gal4 lines of *Gr64f, Gr5a, Gr33a, Gr66a*, and *IR76b* were crossed with an *UAS-RFP* line. Gr gene expression on the proboscis was used as a positive control, and siblings and CS flies were observed as negative controls. The expression pattern of *ppk28* was also visualized to confirm *Grs*, as *ppk28* is known to co-assemble with chemoreceptor cells (Hiroi et al, 2004; Cameron et al., 2010; Chen et al., 2010). Fly wings were removed from bodies and carefully mounted onto glass slides with a drop of Vectashield (Vector Laboratories, USA) to stabilize the fluorescence. Samples were scanned within one or two days after mounting or stored at 4 °C for longer latency. Observations were made with a water immersion objective (20× plan apochromat; 1.0NA) and RFP was excited with a 555 nm solid-state laser. Images were acquired with a resolution of 1024 × 1024 pixels, with 1 μm interval (z) between each optical section.

In order to confirm each *Gr, IR* and *ppk* gene expression on wing taste sensillum, RFP expressions in taste cells was observed. The progeny individuals which expressed the construct were found by its RFP expression. The gene construct was as follow: *Gr33a-Gal4[1]/CyO*; *Dr/TM3, Sb, Ser × b y1w DbGFP(x)*; *UAS-RFP(2), w**; *Gr66a-Gal4 (II) × b y1w DbGFP(x)*; *UAS-RFP(2), wUAS-MCD8RFPLexAop-MCD8GFP*; *Gr64f-Gal4(1)/TM3.ser × b y1w DbGFP(x)*; *UAS-RFP(2), Gr5a-Gal4/CyO*; *TM2/TM6B × b y1w DbGFP(x)*; *UAS-RFP(2), IR76b-Gal4/Cyo*; *Dr/TM3 × Gyc-89D-GFP*; *UAS-RFP and CyO/BI*; *ppk28-Gal4 × b y1w DbGFP (x)*; *UAS-RFP(2)*.

2.8. Statistical analysis

To examine concentration-dependent increases in grooming behavior in headless flies with respect to sex, chemical, and fly strain, a multiple logistic regression (JMP 10.0 software, SAS) was applied using the least square method. The independent variable (y) was the grooming score (ranking scale), and dependent variables (x_i) were concentration (ranking scale) and strain (categorical scale). The slopes of the regressions were compared. Additionally, Dunnett's test (JMP 10.0 software, SAS) was conducted to examine behavioral induction at each concentration with controls. Grooming induction in response to optogenetic stimulation was analyzed using the Mann-Whitney test.

3. Results

3.1. Optogenetic activation of taste neurons and grooming

To determine if grooming can be elicited by perception of aversive or attractive stimuli, we used UAS-Gal4 constructs to drive the expression of channel rhodopsin receptors sensitive to blue light (*ChR2*) in neurons, which express either *Gr33a* or *Gr64f*. First we confirmed that blue light does not affect grooming behavior in decapitated CS flies (grooming in females: $p = 0.32$, $\chi^2 = 1.0$ and grooming in males: $p = 0.25$, $\chi^2 = 1.34$, Fig. 1A). As expected from previous data (Yanagawa et al., 2014), the activation of bitter-sensitive (*Gr33a*) neurons induced significant grooming (Fig. 1B, females: $p = 0.044$, $\chi^2 = 4.07$, Mann-Whitney test, males: $p < 0.001$, $\chi^2 = 24.4$, males: $p < 0.001$, $\chi^2 = 15.5$, Mann-Whitney test).

During our observations, we noted that decapitated flies could use their forelegs to brush other legs and the thorax, while they used their hind legs to clean their abdomen and wings. We also noted that use of

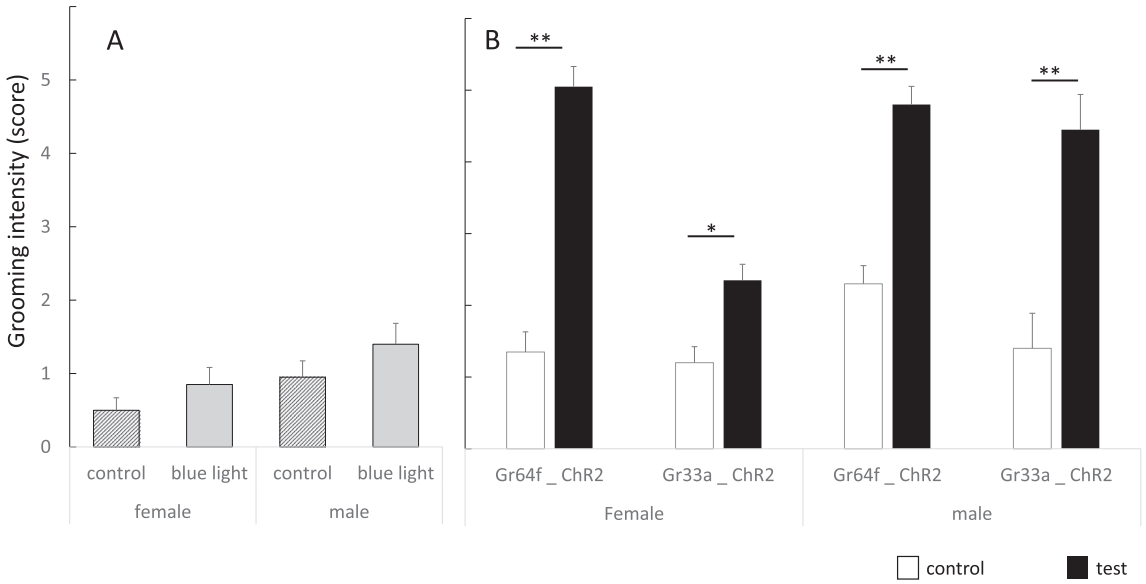


Fig. 1. Grooming responses in optogenetic experiments. A: 'control' is the grooming responses of CS flies under LED light, and 'test' is the grooming responses of CS flies under blue light. B: Black columns: Grooming responses in bitter taste-evoked flies, *Gr33a-Gal4* × *UAS-ChR*. White columns: Grooming responses in bitter taste-evoked flies, *Gr64f-Gal4* × *UAS-ChR*. 'control' presents the data from siblings and 'test' data from the progenies. Blue light was applied on the whole body. Headless flies were placed in the same position as control flies, however the experiment was conducted in a dark condition. 4-day old flies were used for all tests (n = 20 from each sex). Data represent mean ± SE. Asterisks show the significance in Mann-Whitney tests comparing the grooming response to the control (*p < 0.05, **p < 0.01).

the hind legs was the most common method of grooming (Seeds et al., 2014). Interestingly, females displayed intense cleaning of their abdomens following activation of *Gr64f* neurons (Fig. S1). Throughout these observations, males tended to groom their bodies and wings more than females in response to optogenetic activation (Fig. S1).

3.2. Responses to sLPS in taste-ablated flies

Since decapitated flies are capable of self-grooming following optogenetic or direct contact stimulation with specific chemicals (Yanagawa et al., 2014), we asked which taste neurons are necessary to induce grooming with sLPS. Water perception was confirmed not to be involved in sLPS inducing grooming reflex using *ppk28-Gal4* × *UAS-DTI* (Fig. 2). In order to address this, we expressed a diphtheria toxin (DTI) (Wang et al, 2004) into cells that express gustatory receptors by using specific GAL4 constructs: *Gr64f* and *Gr5a* (sweet), *Gr33a* and *Gr66a* (bitter), or *IR76b* (salt). When such flies are crossed with those carrying a UAS-DTI construct, in the progeny of these flies, all cells expressing *Grs/IR76b* express Gal4, which in turn drives the expression of DTI, thus killing these cells. In order to stimulate these flies, we gently brushed the margin of their wing with the tip of a toothpick that had been dipped in a sLPS solution at increasing concentrations, from 0.1 to 10 mg/mL. While sLPS induced a clear grooming response in control flies at high sLPS concentration, such response was absent in *Gr64f, Gr5a, Gr33a, Gr66a*, and *IR76b*-ablated flies (Fig. 3, Table S1).

3.3. Responses to sLPS and other tastants in Canton S flies

The data presented above suggest that other Gr genes than those linked to bitter detection (Yanagawa et al., 2014) may also be involved in the expression of grooming reactions. In the next experiment, we thus evaluated the response of normal flies (Canton S: CS) to increasing concentrations of sucrose, caffeine and sodium chloride. The grooming induction pattern induced by each chemical was compared to that induced by the microbial surface compound, sLPS. With the exception of sucrose, all contact chemicals induced concentration-dependent grooming responses ($p \leq 0.01$ Table S2). Moreover, a slight sex difference appeared in the responses to sLPS, NaCl, and sucrose (Table S2

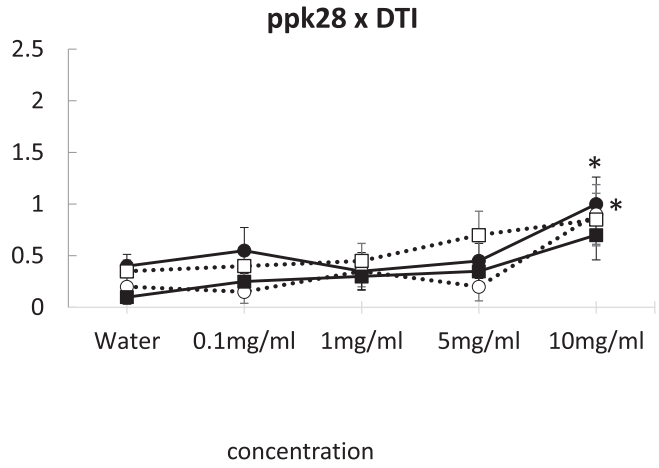


Fig. 2. Confirmation that water perception is not involved in grooming. In order to generate flies deprived of water cells, we crossed *ppk28-Gal4* flies with UAS-diphtheria toxin *(UAS-DTI)* flies. The grooming responses of decapitated flies were scored according to their intensity and duration. LPS was applied to the wings of 4 d old flies. Grooming responses induced in female progenies are illustrated by dark circles connected with a line, male progenies are illustrated by dark squares connected with a line, female siblings are illustrated by blank circles connected with a dotted line and male siblings are illustrated by blank squares connected with a dotted line. Data represents mean ± SE. Asterisks showed the significance in Dunnet test comparing grooming responses to water control (*p < 0.05, **p < 0.01).

and Fig. 4). The contact chemical which induced the clearest concentration-dependent grooming response was NaCl. Contrary to quinine tested previously (Yanagawa et al., 2014), which induces intense grooming in both females and males, grooming induction by caffeine was significant in females but not in males (females: $p = 0.003$, $\chi^2 = 4.24$, males: $p = 0.24$, $\chi^2 = 1.41$, logistic regression, Fig. 4). As for sucrose, a significant peak in grooming behavior appeared in males following application of 10^{-6} M sucrose ($p = 0.002$, Dunnet's test, Fig. 4B) but not at higher concentration.

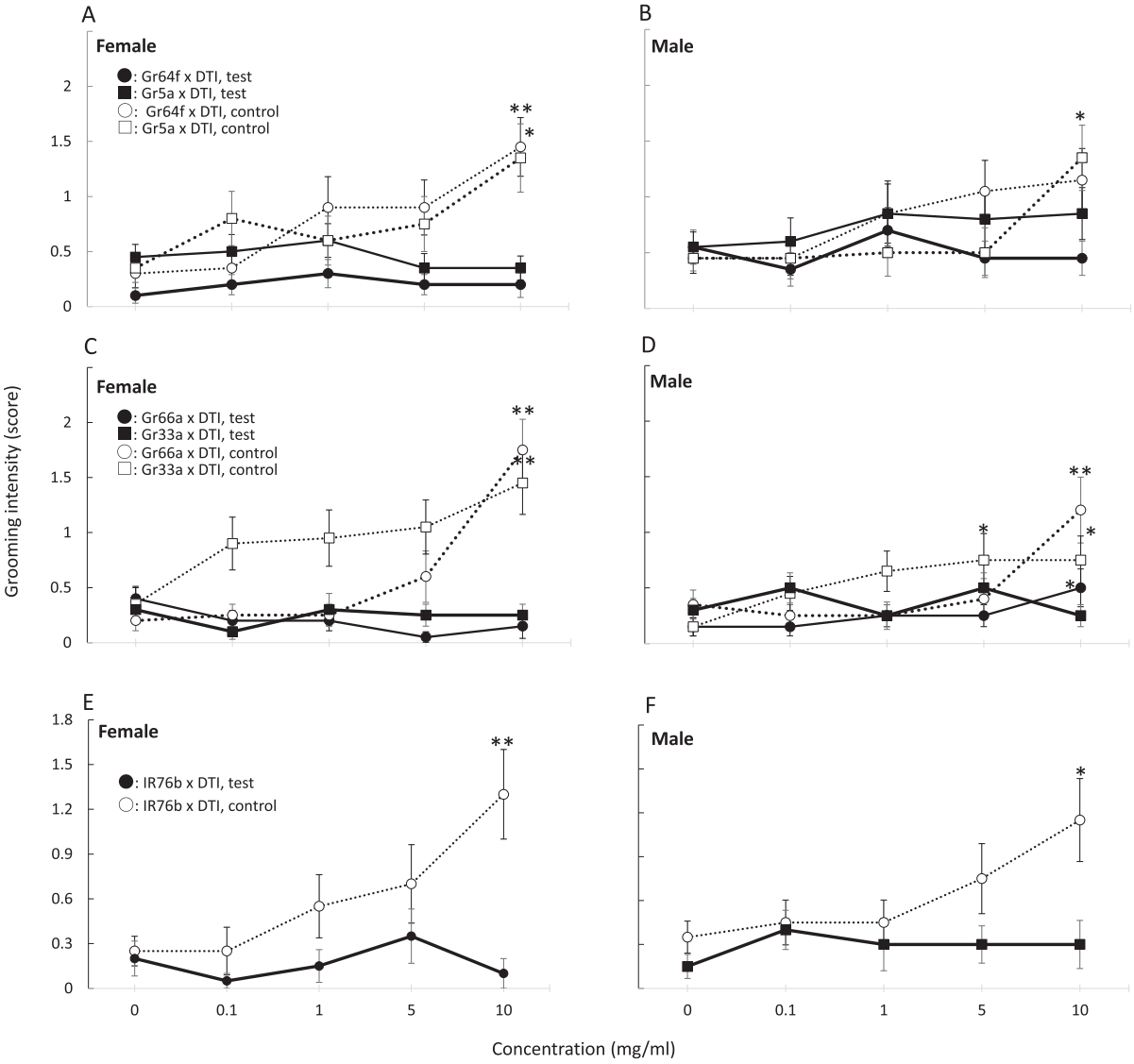


Fig. 3. Grooming responses in flies, whose gustatory receptor cells expressing particular Gr genes" were ablated. sLPS was applied to the wings of 4 d old flies (n = 20 for each sex). The grooming responses of decapitated flies were scored according to their intensity and duration. Data represent mean ± SE. Asterisks showed the significance in Dunnet tests to compare the grooming enhancement from control (*$p < 0.05$, **$p < 0.01$). A: grooming induction in females. B: grooming induction in males. Grooming responses induced in *Gr64f-Gal4* × *UAS-DTI* are illustrated by dark circles connected with a line, *Gr5a-Gal4* × *UAS-DTI* are illustrated by dark squares connected with a line, siblings of *Gr64f-Gal4* × *UAS-DTI,* control, are illustrated by blank circles connected with a dotted line and siblings of *Gr5a-Gal4* × *UAS-DTI,* control, are illustrated by blank squares with a dotted line. C: Grooming induction in females. D: grooming induction in males. Grooming responses induced in *Gr33a-Gal4* × *UAS-DTI* are illustrated by dark circles connected with a line, *Gr66a-Gal4* × *UAS-DTI* are illustrated by dark squares connected with a line, siblings of *Gr33a-Gal4* × *UAS-DTI,* control, are illustrated by blank circles connected with a dotted line and siblings of *Gr66a-Gal4* × *UAS-DTI,* control, are illustrated with blank squares connected with a dotted line. E: grooming induction in females. Grooming responses induced in *IR76b-Gal4* × *UAS-DTI* are illustrated by dark circles connected with a line, siblings of *IR76b-Gal4* × *UAS-DTI,* control, are illustrated by blank circles connected with a dotted line. F: grooming induction in males. Grooming responses induced in *IR76b-Gal4* × *UAS-DTI* illustrated by dark circles connected with a line, siblings of *IR76b-Gal4* × *UAS-DTI,* control, illustrated by blank circles connected with a dotted line.

3.4. Wing Gr gene expression

In order to see which of the gustatory genes studied above are expressed in wing sensilla, we drove the expression of a red fluorescent protein (RFP) in whole body using a UAS construct (Fig. 5). We found cells clearly marked along the wing margin only for *IR76b.* RFP expression patterns in *Grs* and its control: *ppk28* constructs were unspecific (Fig. S2). We checked the validity of all genetic constructs by monitoring RFP marking in the taste sensilla of the proboscis and confirmed that all coded genes were indeed expressed on the proboscis.

3.5. Raman spectra of Drosophila chemosensory wing hairs

We next addressed the structure of wing sensilla by measuring Raman spectra to complement the difficulties in RFP monitoring. According to Valmalette et al. (2015), Raman spectra can indicate the fuctionability of wing chemoreceptors together with its signature of internal structure. We successfully obtained the targeted molecular motifs of bands at 1350 cm^{-1}, 1540 cm^{-1}, and 1610 cm^{-1} (Fig. S3D–G). Leg sensillum was taken for a comparison with wing sensilla. The pattern of Raman spectrum varied depending on locations of sensilla. We classified sensilla into four groups according to the Raman spectra-determined modes (Fig. S3D–G), and found that the molecular

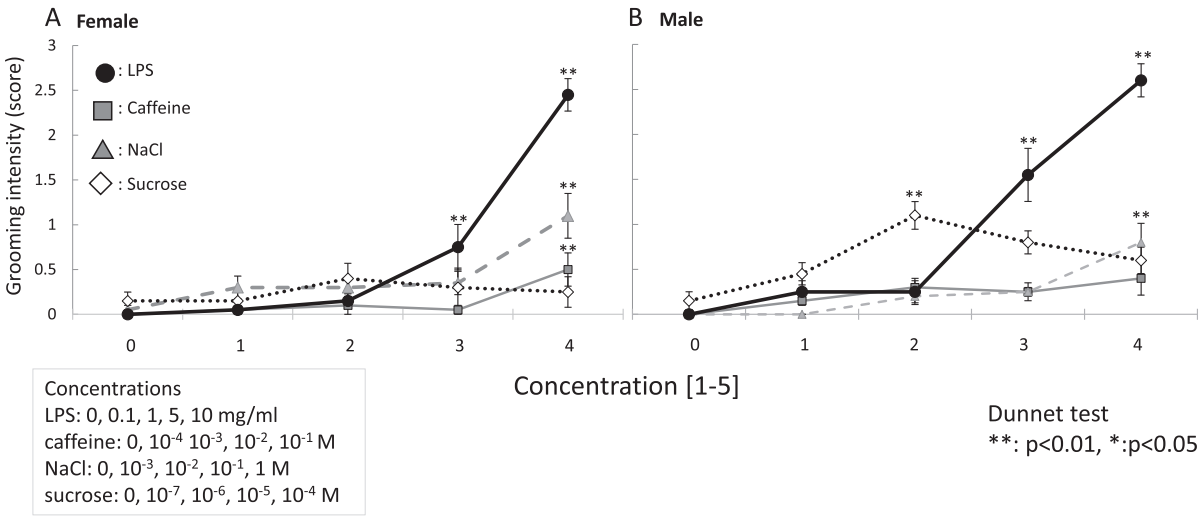


Fig. 4. Grooming responses induced by contact chemicals. A: Grooming induction in females. B: grooming induction in males. The grooming responses of decapitated flies were scored according to their intensity and duration. Each chemical was applied to the wings of 4 day old flies (n = 20 from each sex). Grooming responses to LPS are illustrated by dark circles connected with a line, responses to caffeine are illustrated by grey square connected with a line, responses to NaCl are illustrated by grey triangle connected with a dotted line and sucrose was illustrated with blank diamond with a dotted line. The lowest concentration '0' indicates control: water (no chemical). Concentration increase in LPS, 0: 0 mg/ml, 1: 0.1 mg/ml, 2: 1 mg/ml, 3: 5 mg/ml, 4: 10 mg/ml. Concentration increase in caffeine, 0: 0 M, 1: 10^{-4} M, 2: 10^{-3} M, 3: 10^{-2} M, 4: 10^{-1} M. Concentration increase in NaCl, 0: 0 M, 1: 10^{-3} M, 2: 10^{-2} M, 3: 10^{-1} M, 4: 1 M. Concentration increase in sucrose, 0: 0 M, 1: 10^{-7} M, 2: 10^{-6} M, 3: 10^{-5} M, 4: 10^{-4} M. Data represent mean ± SE. Asterisks show the significance in Dunnet tests comparing grooming responses to the water control (*p < 0.05, **p < 0.01).

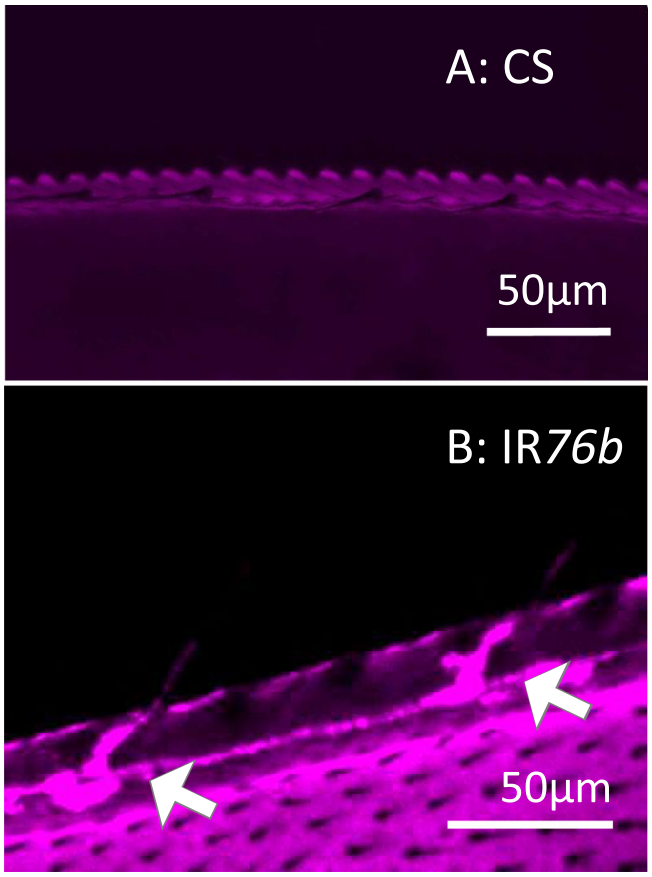


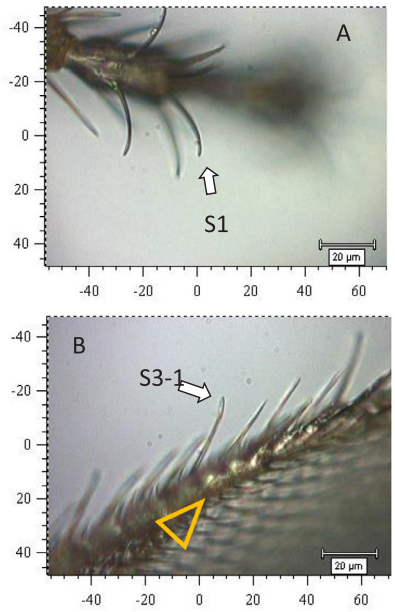
Fig. 5. Gustatory gene coded RFP expression in taste cells on wing sensillum. A: no expression on CS flies at margin 2 (control). B: IR76b expression (allow) on *Drosophila* wing at margin 2.

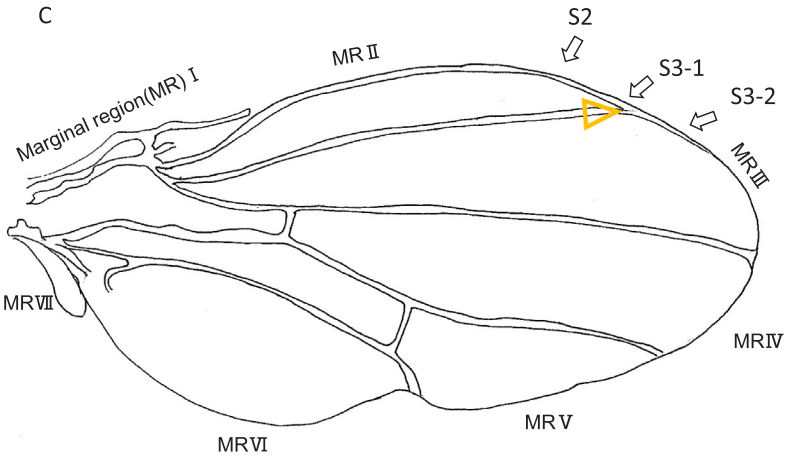
motifs of chemosensilla on the wing margin varied depending on the location (Fig. 6H). Clear bands consistently appeared in sensilla located on the legs (sensillum S1) and posterior wing margin (sensilla S3-1 and S3-2), but not on the anterior wing margin (sensillum S2). The data suggest that the chemical composition of the sensillum shaft of taste sensilla in marginal region III resemble that of taste sensilla on the leg, but differ from that of sensilla in region II.

4. Discussion

4.1. sLPS perception through gutatory genes

In this study, we attempted to address the question of how gustatory genes are involved in the grooming reflex induced by the taste perception at wing chemoreceptors. Our results suggest that the detection of sLPS involves cells expressing bitter and salt receptors. Additionally we found a dependency of grooming induction on cells expressing *Gr64f*, which are considered as sensitive to sugars (Jiao et al., 2008; Dahanukar et al., 2007; Slone et al., 2007; Jiao et al., 2007). We confirmed this by examining the grooming reactions induced by sLPS following the ablation of cells expressing different *Gr* genes. Simultaneous excitation of cells expressing *Gr64f*, *Gr33a* and *IR76b* could be important for eliciting the grooming reflex because if any of the cells expressing them is ablated, a reduction of grooming is observed. We also tested the responses to different tastants and to sLPS in control flies. The intensity of the grooming response induced by bitter substances was different between caffeine and quinine (Fig. 4). This may be because fruit flies would encounter less caffeine than quinine in their life. We found that chemoreceptors expressing *Gr64f* were important for expressing a grooming response to sLPS but not to sucrose (Figs. 3, 4). This is intriguing since *Gr64f* is expressed in most taste neurons responding to sugar on the proboscis (Jiao et al., 2008; Dahanukar et al., 2007). Taken together with our previous study, sLPS is indeed perceived by the receptor cells of aversive chemicals to flies but it might actually stimulate simultaneously variety of gustatory neurons expressing cells.





H

Raman spectrum pattern

Pattern	D	E	F	G
Basal area	+	-	+	-
Apical area	+	+	-	-
Possible Functionability	High	low	low	Non

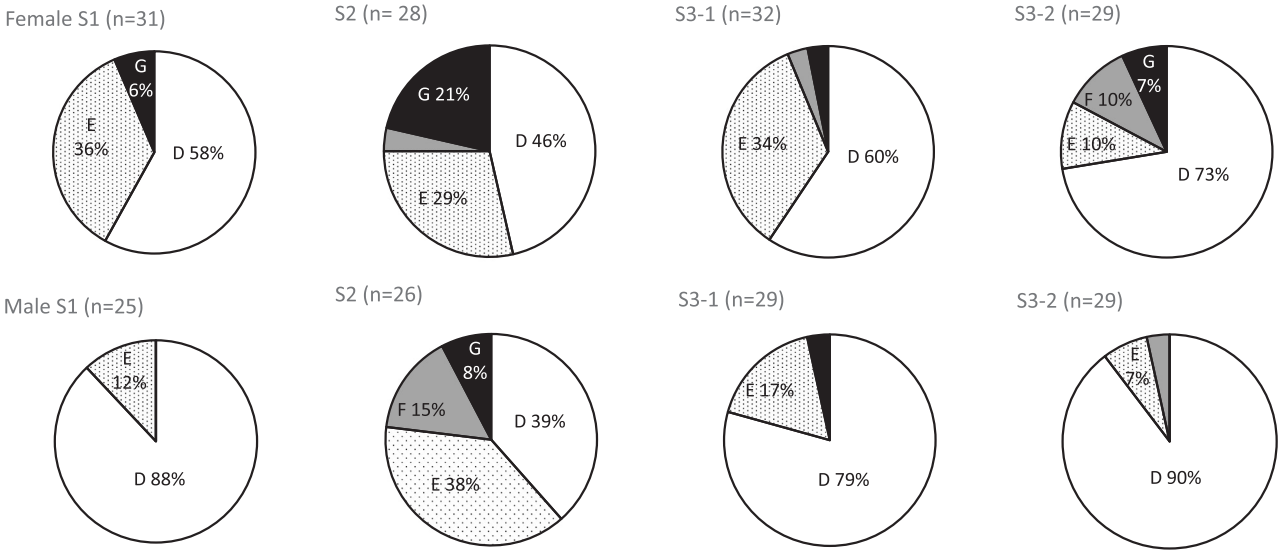


Fig. 6. Raman spectra of *Drosophila* chemosensory wing hair under 514.5 nm laser irradiation. A:Taste sensillum S1 on leg. B: sensillu, S3-1 on wing margin 2 region, which is at five sensilla away from sensillum S3-1. Sensillum S3-1 locates right at the landscape. Sensillum S3-2 is again at five sensilla away from sensillum S3-1 to the direction on wing margin 3 region.Yellow traingle on linear wing vein was a landscape for three sensillum as in B. D-G: Raman band pattern at apical/basal area of each sensillun S1 – S3-2. □D: White part indicates the sensilla, which had clear spectra both at apical/basal area. ■E: Dotted part indicate the sensilla, which had clear spectra only at aptical. ■F: Grey part indicates the sensilla, which had clear spectra only at basal area. ■G: Black parts indicates the sensilla, which had no spectra both at apical/basal area. H: The percentage of each sensilla that show each kind of spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. Responses to sLPS

Microbes contain various compounds on their surface including a mixture of proteins, nucleic acids, and, to a lesser extent, lipids and polysaccharides (Butt et al., 2016). In addition, insects have immune-receptors to microbial secondary metabolites (Salton and Kim, 1996). Chemical compounds indicating the presence of microbe can be also a cue for food source or for oviposition site. Therefore, understanding how insects perceive microbes requires a step-by-step approach. LPS consists of hydrophilic polysaccharide and lipophilic lipid moieties (Butt et al., 2016). The polysaccharide component comprises two distinct portions: a core oligosaccharide and a polysaccharide chain consisting of several repeating oligosaccharide units. The presence of phosphates, fatty acids, and acidic sugars in LPS makes it a unique anionic polyelectrolyte (Panda and Chakraborty, 1998). Electrodialyzed lipopolysaccharides could be rendered soluble by neutralizing them with alkali or with a basic amine (Galanos and Lüderitz, 1975). Though the water cells encoded by *ppk28* do not seem to play a role in the grooming reflex (Fig. 2) (Yanagawa et al., 2014), water molecules separate a large proportion of cations and basic amines from lipopolysaccharides where they neutralize negatively charged groups of the molecule. It is said that removal of these water molecules leads to acidic lipopolysaccharides, which can be converted to defined salt forms by neutralization with a given base (Galanos and Lüderitz, 1975). Our current findings—specifically that IR76 seemed important to perceive LPS on wing chemosensory sensilla—are in line with these aspects. *IR76b* has been reported not only to encode a receptor for NaCl (Zhang et al., 2013), but also a receptor detecting amino acids (Croset et al., 2016; Ganguly et al., 2017) and sourness in collaboration with IR25a (Chen and Amrein, 2017). Additionally, although the expression patterns of Grs on the wings were not clear, the expression was amenable with the RNA sequence data on *Drosophila* wing (Agnel et al., 2017). Supportively, we recorded similar Raman spectrum profiles from sensilla on the leg and on the wing marginal region III, suggesting that these sensilla are functional (Valmalette et al., 2015). On the other hand, we could not get the same spectrum from the *Drosophila* hemolymph. Though the Raman spectrum seems to indicate some internal structure of the chemoreceptor sensillum, to determine the molecular structure, which it can indicate, more investigations are required.

4.3. Role of sweet and salty testants in grooming reflex

In contrast to previous results, sugar cells turned out to be involved in eliciting grooming reflexes. Though the response induced by sucrose was low, the responses observed following optogenetic activation of cells expressing *Gr64f* and the decreased responses observed following the ablation of cells expressing *Gr64a/Gr5a* indicate the involvement of cells usually considered as allowing sweet perception. Moreover, supportively, we checked the involvement of sucrose perception in the grooming reflex by re-activating *Gr64a* cells from Δ*Gr64a* flies, and found that the re-activation of these cells recovered the response to LPS (Fig. S4). Nevertheless, it has been reported that taste sensilla on the proboscis and legs respond to sugars in the range of 1 mM to 1 M (Hiroi et al., 2002; Meunier et al., 2003). Our previous study implied that 100 mM sucrose already induced burst firing from wing sensilla (Yanagawa et al., 2014). In this study, male response at the surprisingly low concentration of sucrose implies that the grooming reflex induced by sLPS occurs in the consequence of the interactions from both bitter and sweet related cell activations. An alternative hypothesis could be that cells expressing *Gr64f* not only respond to sugars but that they could be activated by the lipophilic part of sLPS, in analogy with recent observations that indicate that *Gr64e* is involved in the detection of fatty acids (Masek and Keene, 2013; Tauber et al., 2017; Kim et al., 2018). As for *IR76b*, the expression pattern seemed quite similar to that of *IR52a* (Koh et al., 2014). Ionotropic receptor (IR) localization is known to be different from that of gustatory receptors (*Grs*) on the proboscis (Benton et al., 2009) and this also seems to be the case for the wing. The concentration, which starts inducing the significant grooming reflex was higher in bitter and unexpectedly lower in sugar from those that flies can sense with proboscis. The concentration that flies start to perceive the chemicals would be lower. Further work is needed to clarify the localization and function of chemoreceptor cells on the wing margin.

4.4. Gustatory receptor cells on wing, proboscis and legs

The variations of grooming reflex to sucrose and bitter or salty compounds remains unclear (Figs 4 and S2). Taste receptors on the legs seemed to perceive sLPS aversive as those on wings (Yanagawa et al., 2014). On the other hand, in our pilot test, flies drank the solution containing sLPS as much as they did water (*t* test, $p > 0.1$, Fig. S5). Together with the report that 1 mg LPS in 1 mL of 100 mM sucrose was negative in proboscis extension assay (Soldano et al., 2016), our behavioral assays suggest three possibilities: one is that insects regulate their reaction to microbes according to their concentration; the second is that sensilla on different body parts may play different roles in obtaining information from the surrounding world and the third is that LPS might inhibit sucrose perception. In conclusion, our study showed that aversive chemicals, and *Grs* related to the perception of aversive compounds are indeed crucial to inducing the grooming reflex; however, the behavioral response seemed to involve also taste cells and receptors implicated in the detection of tastants, such as salt and unexpectedly low concentration sugars. Further studies are necessary to better understand how flies detect and analyze the complex chemicals which are the signature of the many microbes which they encounter in their environment.

Author contributions

Aya Yanagawa: Conducting experiments, writing manuscript

Antoine Couto: helping confocal microscope observation, writing manuscript

Jean-Christophe Sandoz: supervising the confocal microscope observation, writing manuscript

Toshimutsu Hata: supervising the investigation with Raman spectroscopy

Aniruddha Mitra: helping the genetic experiments and writing manuscript

Moutaz Ali Agha: helping the genetic/behavioral experiments

Frédéric Marion-Poll: Supervising, validation of all data, writing manuscript

Competing interests

No competing interests.

Acknowledgments

We thank Dr K. Scott, Prof. J. Carlson, Dr L. Soustelle and Dr Y. Grau for sharing fly strains. This work was supported by Mishima Kaiun Memorial Foundation, the Research Institute for Sustainable Humanosphere, Kyoto University (grant no. 2016-5-1-1) and the Future Development Funding Program of Kyoto University Research Coordination Alliance. We thank Prof. C. Schal for thoughtful discussions and the support from OECD CRP fellowship 2015: Biological resource Management for Sustainable Agricultural system. Raman measurements were facilitated by a cooperative system of Research Institute for Sustainable Humanosphere (RISH), Kyoto University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

doi.org/10.1016/j.jinsphys.2018.12.001.

References

Agnel, S., Rocha, M., Robinchon, A., 2017. Transcriptome profiling of neurosensory perception genes in wing tissue of two evolutionary distant insect orders: Diptera (*Drosophila melanogaster*) and Hemiptera (*Acyrthosiphon pisum*). J. Mol. Evol. 85, 234–245.

Benton, R., Vannice, K.S., Gomez-Diaz, C., Vosshall, L.B., 2009. Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. Cell 136 (1), 149–162.

Böröczky, K., Wada-Katsumata, A., Batchelor, D., Zhukovskaya, M., Schal, C., 2013. Insects groom their antennae to enhance olfactory acuity. PNAS 110, 3615–3620. https://doi.org/10.1073/pnas.1212466110.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Butt, T.M., Coates, C.J., Dubovskiy, L.M., Ratcliffe, N.A., 2016. Chapter nine - entomopathogenic fungi: new insights into host-pathogen interactions. In: Advances in Genetics. Academic Press, pp. 307–364.

Cameron, P., Hiroi, M., Ngai, J., Scott, K., 2010. The molecular basis for water taste in *Drosophila*. Nature 465, 91–95. https://doi.org/10.1038/nature09011.

Chen, Y., Amrein, H., 2017. Ionotropic receptors mediate *Drosophila* oviposition preference through sour gustatory receptor neurons. Curr. Biol. 27, 2741–2750.

Chen, Z., Wang, Q., Wang, Z., 2010. The amiloride-sensitive epithelial Na+ channel PPK28 is essential for *Drosophila* gustatory water reception. J. Neurosci. 30, 6247–6252. https://doi.org/10.1523/jneurosci.0627-10.2010.

Croset, V., Schleyer, M., Arguello, J.R., Gerber, B., Benton, R., 2016. A molecular and neuronal basis for amino acid sensing in the *Drosophila* larva. Sci. Rep. 6, 34871. https://doi.org/10.1038/srep34871.

Dahanukar, A., Lei, Y.-T., Kwon, J.Y., Carlson, J.R., 2007. Two Gr genes underlie sugar reception in *Drosophila*. Neuron 56, 503–516.

Depetris-Chauvin, A., Galagovsky, D., Grosjean, Y., 2015. Chemicals and chemoreceptors: ecologically relevant signals driving behavior in *Drosophila*. Front. Ecol. Evol. 3. https://doi.org/10.3389/fevo.2015.00041.

Dethier, V.G., 1972. Sensitivity of the contact chemoreceptors of the blowfly to vapors. PNAS 69, 2189–2192.

French, A., Agha, M.A., Mitra, A., Yanagawa, A., Sellier, M.-J., Marion-Poll, F., 2015. *Drosophila* bitter taste(s). Front. Integr. Neurosci. 9. https://doi.org/10.3389/fnint.2015.00058.

Galanos, C., Lüderitz, O., 1975. Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. 54, 603–610.

Ganguly, A., Pang, L., Duong, V.K., Lee, A., Schoniger, H., Varady, E., et al., 2017. A molecular and cellular context-dependent role for Ir76b in detection of amino acid taste. Cell Rep. 18 (3), 737–750.

Hiroi, M., Marion-Poll, F., Tanimura, T., 2002. Differentiated response to sugars among Labellar Chemosensilla in *Drosophila*. Zool. Sci. 19 (9), 1009–1018.

Hiroi, M., Meunier, N., Marion-Poll, F., Tanimura, T., 2004. Two antagonistic gustatory receptor neurons responding to sweet-salty and bitter taste in *Drosophila*. J. Neurobiol. 61 (3), 333–342.

Hornstein, N.J., Pulver, S.R., Griffith, L.C., 2009. Channelrhodopsin2 mediated stimulation of synaptic potentials at *Drosophila* neuromuscular junctions. J. Viz. Exp. 16. https://doi.org/10.3791/1133.

Hu, Y., Han, Y., Shao, Y., Wang, X., Ma, Y., Ling, E., Xue, L., 2015. *Gr33a* modulates *Drosophila* male courtship preference. Sci. Rep. 5, 7777. https://doi.org/10.1038/srep07777.

Jiao, Y., Moon, S.J., Montell, C.A., 2007. *Drosophila* gustatory receptor required for the responses to sucrose, glucose, and maltose identified by mRNA tagging. Proc. Natl. Acad. Sci. U.S.A. 104, 14110–14115.

Jiao, Y., Moon, S.K., Wang, X., Ren, Q., Montell, Q., 2008. *Gr64f* is required in combination with other gustatory receptors for sugar detection in *Drosophila*. Curr. Biol. 18 (22), 1797–1801.

Joseph, R.M., Devineni, A.V., King, I.F., Heberlein, U., 2009. Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 106 (27), 11352–11357.

Kazlauskas, N., Klappenbach, M., Depino, A.M., Locatelli, F.F., 2016. Sickness behavior in honey bees. Front. Physiol. 7, 10.

Kim, H., Kim, H., Kwon, J.Y., Seo, J.T., Shin, D.M., Moon, S.J., 2018. *Drosophila Gr64e* mediates fatty acid sensing via the phospholipase C pathway. PLoS Genet. 14 (2), e1007229.

Koh, T.W., He, Z., Gorur-Shandilya, S., Menuz, K., Larter, N.K., Stewart, S., Carlson, J.R., 2014. The *Drosophila IR20a* clade of ionotropic receptors are candidate taste and pheromone receptors. Neuron 83 (4), 850–865.

Masek, P., Keene, A.C., 2013. *Drosophila* fatty acid taste signals through the PLC pathway in sugar-sensing neurons. PLoS Genet. 9, e1003710.

Meunier, N., Marion-Poll, F., Rospars, J.P., Tanimura, T., 2003. Peripheral coding of bitter taste in *Drosophila*. J. Neurobiol. 56, 139–152.

Moon, S.J., Kottgen, M., Jiao, Y.C., Xu, H., Montell, C., 2006. A taste receptor required for the caffeine response in vivo. Curr. Biol. 16, 1812–1817.

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., Bamberg, E., 2003. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. PNAS 100, 13940–13945. https://doi.org/10.1073/pnas.1936192100.

Newland, P.L., 1998. Avoidance reflexes mediated by contact chemoreceptors on the legs of locusts. J. Comp. Physiol. A 183, 313–324.

Page, K.L., Matheson, T., 2004. Wing hair sensilla underlying aimed hindleg scratching of the locust. J. Exp. Biol. 207, 2691–2703. https://doi.org/10.1242/jeb.01096.

Panda, A.K., Chakraborty, A.K., 1998. Interaction of mixed surfactants with bacterial *Lipopolysaccharide*. J Colloid Interface Sci. 203, 260–264.

Raad, H., Ferveur, J.F., Ledger, N., Capovilla, M., Robichon, A., 2016. Functional gustatory role of chemoreceptors in *Drosophila* Wings. Cell Rep. 15, 1442–1454.

Rao, X.J., Yu, X.Q., 2010. Lipoteichoic acid and lipopolysaccharide can activate antimicrobial peptide expression in the tobacco hornworm *Manduca sexta*. Dev. Comp. Immunol. 34, 1119–1128.

Rietschel, E.Th., Brade, H., 1992. Bacterial endotoxins. Sci. Am. 267, 54–61.

Rolfs, M., 2005. Clash of kingdoms or why *Drosophila* larvae positively respond to fungal competitors. Front. Zool. 2 (1).

Salton, M.R.J., Kim, K.S., 1996. Structure. In: Baron, S. (Ed.), Medical Microbiology, fourth ed. Galveston (TX): The University of Texas Medical Branch at Galveston.

Seeds, A.M., Ravbar, P., Chung, P., Hampel, S., Midgley, F.M., Mensh, B.D., Simpson, J.H., 2014. A suppression hierarchy among competing motor programs drives sequential grooming in *Drosophila*. eLife 3. https://doi.org/10.7554/eLife.02951.

Slone, J., Daniels, J., Amrein, H., 2007. Sugar receptors in *Drosophila*. Curr. Biol. 17, 1809–1816.

Soldano, A., Alpizar, Y.A., Boonen, B., Franco, L., López-Requena, A., Liu, G., Mora, N., Yaksi, E., Voets, T., Vennekens, R., Hassan, B.A., Talavera, K., 2016. Gustatory-mediated avoidance of bacterial lipopolysaccharides via TRPA1 activation in *Drosophila*. eLife 5, e13133.

Stensmyr, M.C., Dweck, H.K.M., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J., Grabe, V., Steck, K., Lavista-Llanos, S., Wicher, D., Sachse, S., Knaden, M., Becher, P.G., Seki, Y., Hansson, B.S., 2012. A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. Cell 151, 1345–1357. https://doi.org/10.1016/j.cell.2012.09.046.

Stocker, R.F., 1994. The organization of the chemosensory system in *Drosophila melanogaster* - a review. Cell Tissue Res. 275, 3–26.

Tanaka, H., Sagisaka, A., Fujita, K., Kaneko, Y., Imanishi, S., Yamakawa, M., 2009. Lipopolysaccharide elicits expression of immune-related genes in the silkworm *Bombyx mori*. Insect Mol. Biol. 18, 71–75.

Tauber, J.M., Brown, E.B., Li, Y.Y., Yurgel, M.E., Masek, P., Keene, A.C., 2017. A subset of sweet-sensing neurons identified by *IR56d* are necessary and sufficient for fatty acid taste. PLoS Genet. 13 (11), e1007059.

Valmalette, J.C., Raad, H., Qiu, N., Ohara, S., Capovilla, M., Robichon, A., 2015. Nano-architecture of gustatory chemosensory bristles and trachea in *Drosophila* wings. Sci. Rep. 5, 14198. https://doi.org/10.1038/srep14198.

Vega, F., Kaya, H., 2012. Insect Pathology, second ed. Academic press.

Wang, Z.R., Singhvi, A., Kong, P., Scott, K., 2004. Taste representations in the *Drosophila* brain. Cell 117, 981–991.

Weiss, L.A., Dahanukar, A., Kwon, J.Y., Banerjee, D., Carlson, J.R., 2011. The molecular and cellular basis of bitter taste in *Drosophila*. Neuron 69 (2), 258–272.

Yanagawa, A., Shimizu, S., 2007. Resistance of the termite, *Coptotermes formosanus* Shiraki to *Metarhizium anisopliae* due to grooming. BioControl 52 (1), 75–85.

Yanagawa, A., Yokohari, F., Shimizu, S., 2009. The role of antennae in removing entomopathogenic fungi from cuticle of the termite, *Coptotermes formosanus*. J. Insect Sci. 9.

Yanagawa, A., Guigue, A., Marion-Poll, F., 2014. Hygienic grooming is induced by contact chemicals in *Drosophila melanogaster*. Front. Behav. Neurosci. 8. https://doi.org/10.3389/fnbeh.2014.00254.

Yanagawa, A., Neyen, C., Lemaitre, B., Marion-Poll, F., 2017. The gram- negative sensing receptor PGRP-LC contributes to grooming induction in *Drosophila*. PLoS One 12 (11), e0185370. https://doi.org/10.1371/journal.pone.0185370.

Zhang, Y.V., Ni, J., Montell, C., 2013. The molecular basis for attractive salt-taste coding in *Drosophil*a. Science 340, 1334–1338.