Modulation of feeding response to amino acids depending on nutritional state in Drosophila melanogaster

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Amino acids are important nutrients for all animals. How can animals detect and regulate amino acid intake? Not only vertebrates, but also insects, have a decision-making process that determines the intake of appropriate nutrients to maintain health. We found that the fruit fly *Drosophila melanogaster* has a specific hunger for amino acids. Flies showed increased amino acid feeding behaviour when they were reared on an amino acid-free diet. Adult *Drosophila* can survive without ingesting amino acids; however, amino acids are necessary for propagation. To identify the gustatory receptor neurons required for amino acid feeding responses, I performed two-choice preference tests using several mutants and transgenic flies. I found that *poxn* mutants, which had no external taste sensation, preferred amino acids over low-concentration sugar, indicating that external gustatory information is not essential for amino acid detection. Flies in which sugar-sensing neurons were genetically silenced showed an amino acid feeding response, suggesting that sugar-sensing neurons are not necessary for amino acid feeding behaviour. Additionally, neurons that express GR43a or IR76b might be involved in amino acid feeding. RNAi screening studies revealed that several neuropeptides may also contribute to amino acid feeding responses depending on amino acid deprivation. Larval *Drosophila* show a preference for amino acids. Our previous study showed that genetic variation affects amino acid feeding in adult flies. Therefore, I performed choice tests with amino acids using larvae of high-responsive and low-responsive strains with respect to amino acids. Low-responsive strains did not always show low responses to amino acids at the larval stage. Furthermore, larval learning experiments revealed that all 20 amino acids individually acted as appetitive rewards for larvae.
I. General introduction

Animals require appropriate nutrients to maintain health. In humans, deficiencies in essential nutrients cause sickness; however, excessive nutrients cause diseases, such as diabetes and obesity. Equipped with a variety of sensory systems, e.g., gustation, olfaction and vision, animals search for nutritive foods and avoid harmful substances. Based on taste information, animals have to decide to eat or not to eat. For hungry animals, a sweet or fatty taste sensation is a reward, and these sensations induce feeding. In mammals, the hypothalamic satiety centre controls the feeding amount (Debons et al., 1970). A decision-making process exists in a wide range of taxa, including mammals and invertebrates. Mice, flies and nematodes were used in recent studies to investigate the molecular and cellular mechanisms underlying decision-making (Yapici et al., 2014). Mammals and insects share many common physiological systems and have a degree of genetic homology. Although insects have a simple nervous system compared with mammals, they do not simply respond to environmental stimuli, but regulate their behaviour via a complicated decision-making process. Accordingly, insects are thought to be excellent models to investigate the neuronal and molecular mechanisms involved in feeding regulation.

The gustatory system of insects is different from that of mammals. In mammals, taste cells are housed in taste buds and gustatory neurons are synaptically connected with taste cells to send information to the brain. In insects, primary gustatory receptor neurons (GRNs) directly project to the taste centre in the brain. GRNs are distinct from olfactory neurons in terms of the expression pattern of receptors. In the olfactory system, each neuron expresses a single receptor, while GRNs express several types of receptors. The availability of various genetic tools makes the fruit fly Drosophila melanogaster an excellent model to study sensory systems and feeding regulation mechanisms. In silico analyses identified a family of 68 gustatory receptors (GRs), some of which are expressed in GRNs housed in hair-like structures called
sensilla (Montell, 2009). Taste sensilla are located on the labellum, pharynx, legs, wing margins and ovipositor. GRN dendrites extend to the pore at the sensillum tip. The axons of labellar and pharyngeal GRNs project to the suboesophageal ganglion (SEG) in the brain (Kwon et al., 2014). When flies are walking and detect a ‘tasty’ sensation via their legs, the information is conveyed to the SEG and is integrated, and motor neurons subsequently elicit proboscis extension to initiate feeding.

Labellar chemosensilla are classified into three types, l-, i- and s-type, depending on length. The l-type and s-type sensilla house four GRNs, i.e., W (water), S (sugar), L1 (low concentration of salt) and L2 (high concentration of salt) cells (Tanimura et al., 2009). In s-type sensilla, L2 cells sense bitter compounds and salt. The i-type sensillum harbours two cell types, one that responds to sugar and a low concentration of salt and another that responds to a high concentration of salt and bitter compounds. Together, W, S and L1 cells sense appetitive taste information, and L2 cells sense aversive information.

Flies need to make a decision regarding intake quantity depending on their hunger state. Neuropeptides and peptide hormones regulate physiology and behaviour in insects (Nässel & Winther, 2010). Insulin is broadly conserved among animal species as a metabolism regulator for carbohydrates and fats. In Drosophila, insulin-like peptides and downstream proteins are important in developmental and growth processes, carbohydrate and lipid metabolism, and the regulation of life span (Brogiolo et al., 2001). Neuropeptide F (NPF) is a member of the vertebrate neuropeptide Y (NPY) superfamily. Drosophila NPF functions in the regulation of foraging, feeding and appetitive memory performance (Krashes et al., 2009). A number of studies indicate that neuropeptides contribute to feeding regulation in Drosophila. Additionally, Gruber et al. (2013) demonstrated that an increase in haemolymph osmolality is a critical factor determining satiation. These findings clarify a fraction of insect feeding regulatory mechanisms; however, it is unknown whether divergent nutrients are regulated independently.
To dissect gene functions and the molecular and neuronal mechanisms of various behaviours, *Drosophila* genetics provide a variety of sophisticated tools. For example, the Gal4/UAS system enables precisely controlled targeted gene expression in specific cells (Brand & Perrimon, 1993). When a Gal4 line with a specific promoter is crossed with a UAS line that includes a gene that induces cell death, specific, targeted cells can be genetically ablated. A variety of UAS lines have been generated; for instance, UAS-Kir codes inwardly rectifying potassium channels and can silence neurons via the overexpression of potassium channels (Lu, 2004). UAS-GFP induces the expression of green fluorescent protein and is widely utilised as a cell marker.

The identification of genes and molecules that are involved in particular behaviours is an ultimate goal of animal behaviour studies. Genome-wide association (GWA) studies have become a powerful tool to investigate gene functions owing to recent developments in genome sequencing techniques. *Drosophila* have an advantage for GWA studies because their short generation time enables inbred lines to be easily established. A collection of 192 inbred strains, referred to as the *Drosophila melanogaster* Genetic Reference Panel (DGRP), was generated using single mated females from wild populations, followed by 20 generations of sib-mating (Mackay et al., 2012). The DGRP facilitates genotype–phenotype mapping using *Drosophila* genetics methods. Genome sequence data and gene expression data are available for these lines; therefore, a variety of phenotypes were studied to identify the underlying genes by GWA analyses (Ayroles et al., 2009; Swarup et al., 2013).
II. Exploring amino acid-sensing neurons and mechanisms of amino acid feeding modulation induced by amino acid deprivation in *Drosophila melanogaster*

1. Introduction

Amino acids are important nutrients for animals as they are protein components. Some amino acids are precursors of neurotransmitters or neurotransmitters themselves, e.g., serotonin is the product of tryptophan and glycine functions as a neurotransmitter (Wu, 2009). Animals cannot synthesise essential amino acids; therefore, they have to obtain them from ambient food sources. Essential amino acids in *Drosophila* include the essential amino acids in humans plus arginine (Sang & King, 1961). A deficiency in one of the essential amino acids prevents female flies from producing eggs.

Amino acids have an ‘umami’ taste. In 1908, the Japanese scientist Ikeda discovered sodium glutamate, which contributes to food palatability, and named umami the fifth basic taste in addition to sweet, bitter, salty and sour. Especially in Japanese cuisine, umami plays an important role in creating a unique taste. An umami taste may indicate the presence of amino acids and proteins in food. In mammals, the heterodimer T1R1/T1R3 functions as an amino acid receptor (Nelson et al., 2002). How do flies detect amino acids? Yeast is thought to be a main protein source for *Drosophila*, as they prefer to ingest matured fruits and tree sap, which are rich in yeast. Yeast deprivation causes enhanced yeast feeding, indicating that flies can modulate feeding behaviour depending on internal protein deficiency (Ribeiro et al., 2010). However, it is unknown whether flies sense the taste of proteins or not. Sweet and bitter tastes are comparatively well-studied (Fujii et al., 2015; Weiss et al., 2011); however, neither amino acid receptors nor amino acid-sensing neurons have been identified in *Drosophila*.

Gr5a, Gr43a and Gr64a–f are expressed in sweet-sensing neurons. Gr43a is a fructose receptor (Miyamoto et al., 2012). Gr32a, Gr33a, Gr66a and Gr93a are required for responses to caffeine and other bitter compounds (Lee et al., 2009). The ligands for
a number of GRs have not been identified. *Gr2a* is expressed in pharyngeal taste organs, but not in labella or tarsi (Wang et al., 2004). Larval gustatory organs also express *Gr2a* (Kwon et al., 2011). These facts raise the possibility that *Gr2a* functions in the detection of important tastants from the larval to adult stage.

In addition to GRs, ion channels and other receptors also contribute to gustatory sensation. An osmosensitive ion channel, *ppk28*, is expressed in water-sensing neurons (Cameron et al., 2010). Recently, an ionotropic receptor, *IR76b*, was identified as a salt receptor (Zhang et al., 2013). Benton et al. (2009) revealed that genes in the IR family code for an independent class of chemosensory receptors in insects. For example, several IRs function as olfactory pheromone receptors and others are expressed in taste neurons (Croset et al., 2010). IRs are related to vertebrate ionotropic glutamate receptors, making them excellent candidates for amino acid receptors.

Here, I found that adult *Drosophila* regulate the feeding preference for amino acids depending on internal nutritional state. To study the amino acid-sensing mechanism, I performed two-choice preference tests using two groups of flies, one that was nutritionally satiated and another that was amino acid-deficient. I tested several receptor mutant strains and flies in which particular GRNs were genetically silenced to explore defects in amino acid feeding responses. I also investigated whether neuropeptide receptor neurons are involved in amino acid feeding behaviour.
2. Materials and Methods

Fly strains

*Drosophila melanogaster* were reared on standard cornmeal-yeast-glucose-agar medium under a 12h:12h light:dark cycle (lights on at 06:00 and off at 18:00) at 25 ºC. Canton-Special (CS) and *w*°118 were used as wild-type strain. *poxn*70–23/CyO was crossed to a deficiency strain, *Df(2R)42WMG/CyO*, and *poxn*70–23/*Df(2R)WMG* flies were used as the *poxn* mutant strain (Awasaki & Kimura, 1997). *Gr64f-Gal4, Gr5a-Gal4, and Gr43a-Gal4* were provided by J. R. Carlson. *Gr2aGal4* and UAS-neuropeptide receptor dsRNA strains were kindly provided by SJ. Moon. *Gr43aGal4* was provided by H. Amrein. UAS-GFP, and nSyb-Gal4 were obtained from the Bloomington stock center (Indiana University, U.S.A.). IR-Gal4 strains and IR mutants were provided by R. Benton.

Amino acid deprivation

Flies eclosed within 12 h were placed in vials containing either standard medium (aa (+)) or glucose plus ions medium (aa (-)). aa (+) media contained 100 g glucose, 50 g cornmeal, 40 g dry yeast, 31.7 g wheat germ, 7.7 g agar, 5 ml propionic acid, and 11.7 ml 4-hydroxybenzoate (10% in 90% ethanol) in 1000 ml water. aa (-) media contained 90 g glucose, 1 g sodium hydrogen carbonate, 0.7 g potassium dihydrogen phosphate, 3.9 g di-potassium hydrogen phosphate, 0.2 g magnesium sulphate, 0.1 g phosphatidylcholine (10% in 99% ethanol) in 1000 ml water. Ion composition was based on the reference to the defined medium (Chakraborty et al., 2009). The vials were replaced with new vials every 2 days. Female and male flies were mixed and placed under these conditions for 5 or 6 days after eclosion until tests.

Chemicals

D-glucose was obtained from Sigma-Aldrich (St Louis, MO, U.S.A.). Sodium hydrogen carbonate, potassium dihydrogenphosphate, di-potassium hydrogen phosphate, and
magnesium sulphate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Agar (purified powder) and phosphatidylcholine were obtained from Nacalai Tesque (Kyoto, Japan). Amino acids of special grade were obtained from Nacalai Tesque, Wako Pure Chemical Industries, or Sigma-Aldrich. The composition of the amino acid mixture was as follows: 0.5 mM tyrosine, 2 mM arginine, 3.5 mM aspartic acid, 4 mM glutamic acid, 5 mM tryptophan, and 10 mM each of alanine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, and valine. This solution corresponds to a total of 165 mM amino acids and was used at 1/10 dilution. Food Blue No. 1 and Food Red No. 106 dyes were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan).

**Two-choice preference test**

A two-choice test on a Petri dish was performed as previously described (Hiroi et al., 2004). Four pieces of square filter paper (20x20 mm, Whatman 3MM CHR) were placed on the bottom of a Petri dish (90 mm diameter). Two pieces in diagonal position were wetted with 150 µl distilled water to humidify the dish and to allow the flies to drink water. Then, 150 µl of glucose solution (coloured with the red dye, 250 mg in 1000 ml water) was applied to one of the remaining pieces of paper, and amino acid solution (coloured with the blue dye, 125 mg in 1000 ml water) was applied to the other. After applying the solutions, the Petri dish was immediately overlaid. Approximately 40–50 flies were introduced into the Petri dish using a fly aspirator. Petri dishes were placed in a humidified Peltier incubator (CN-25C, Mitsubishi Electric Co.) in darkness for 2 h. Flies were killed by freezing. Colouring of the abdomen was observed under a compound stereomicroscope. The feeding ratio for the blue side was defined as \((B+M/2)/(B+R+M+O)\) and the feeding ratio for the red side was defined as \((R+M/2)/(B+R+M+O)\), where \(B\), \(R\), \(M\), and \(O\) represent the number of flies coloured blue, red, purple, and uncoloured, respectively. Colour was classified by comparing with standard solutions mixed at different ratios, and ‘purple’ was judged when one
colour was mixed at a ratio of more than 20% of the other colour. The experiments using IR-Gal4 and IR mutant strains were conducted in R. Benton’s lab, University of Lausanne, Switzerland.

**Gr2a expression profile**
Gr2a\textsuperscript{Gal4} females were crossed with UAS-GFP males, and F1 females were dissected. Dissected mouth parts were fixed in 4\% formaldehyde and mounted on slide glass with 70\% glycerol. The image was acquired with a Leica TCS SP8 STED laser confocal microscope (LSM).
3. Results

*Drosophila* have specific hunger for amino acids

I performed two-choice preference tests between a low concentration of glucose and amino acids (Fig. 1; Toshima & Tanimura, 2012). In most cases, feeding tests are conducted using starved animals to maximize the amount of intake, however, we cannot precisely discriminate between the hunger for sugar and amino acids. Therefore I used flies without food-deprivation. aa (+) flies were reared with standard medium for 5–6 days and satiated both with sugar and amino acids. On the contrary, aa (-) flies were reared with glucose medium and satiated with sugar but not with amino acids. Fig. 1A shows the result of two-choice preference test between glucose and 20 amino acid mixture. In all groups, approximately 20% of flies ingested glucose solution. On the other hand, the ratio of flies ingested amino acid mixture was significantly higher in aa (-) flies compared to aa (+) flies in both sexes, meaning that amino-acid-deficiency induced enhanced feeding response to amino acids. This result suggests that flies have specific hunger for amino acids and can modulate the feeding behaviour to amino acids depending on their internal nutritional state. When I tested with individual amino acids, aa (-) flies showed strong feeding response to phenylalanine and cysteine, while not to glycine (Fig. 1B–D).

External taste organ is not essential for amino acid detection

To know how flies can detect amino acids, two-choice preference tests were performed with *pox-neuro* (*poxn*) mutant, in which external chemosensilla are transformed into mechanosensilla. *poxn* flies do not have taste reception on the labellum and the tarsi. When amino acid mixture solution was presented, amino acid feeding was enhanced by amino-acid-deprivation (Fig 2A). Furthermore, *poxn* preferred amino acid mixture over glucose, indicating that *poxn* mutant can detect amino acids. *poxn* significantly preferred glycine, while they did not show preference for phenylalanine and cysteine (Fig. 2 B–D). *poxn* mutant may sense glycine, but might not be able to discriminate
between phenylalanine or cysteine and glucose. The pharyngeal taste organs of poxn are intact (LeDue et al., 2015). Therefore poxn might detect several amino acids including glycine by using pharyngeal taste sensation. Taken together with previous study (Toshima & Tanimura, 2012) phenylalanine and cysteine might be sensed by external labellar sensilla, while glycine might be sensed by pharyngeal taste organs.

**Sweet sensing neurons are not necessary for amino acid feeding behaviour**

Amino acids might be sensed by several different taste organs. Are amino acids sweet for flies? Using the Gal4/UAS system, I performed two-choice preference tests using flies in which particular GR-expressing neurons were genetically silenced. I used Gr5a-Gal4 and Gr64f-Gal4 as Gal4 drivers, because both genes are known to be expressed broadly in sweet taste-sensing neurons. Gr5a has been identified as trehalose receptor (Ueno et al., 2001). Gal4 lines were crossed with the UAS-TNT line. In the F1 flies, UAS-TNT gene produce tetanus toxin under the expression of Gal4 gene, therefore the neurons that are expressing Gr5a or Gr64f are silenced. I tested whether these sweet-taste-blind flies can show feeding response to amino acid mixture (Fig 3). Both in Gr5a-Gal4>UAS-TNT and Gr64f-Gal4>UAS-TNT, female flies exhibited significant increase of amino acid preference by amino-acid-deprivation. These results indicate that sweet sensing neurons are not essential for amino acid feeding behaviour.

I next tested the contribution of Gr43a, which is known to be a fructose receptor, to amino acid feeding. Gr43a\textsubscript{Gal4} is Gal4 knock-in strain produced by H. Amrein’s group (Miyamoto et al., 2012). Since the Gr43a gene is replaced with Gal4 gene, Gr43a\textsuperscript{Gal4} strain is mutant of the Gr43a gene. When I performed two-choice preference test between glucose and amino acid mixture with Gr43a\textsuperscript{Gal4}, aa (-) flies showed enhanced preference for amino acid mixture (Fig. 4A). On the contrary, Gr43a\textsuperscript{Gal4}>UAS-TNT and Gr43a-Gal4>UAS-TNT flies did not show significant increase of amino acid preference after amino-acid-deprivation (Fig 4B, C). These results suggest that GR43a itself is not an amino acid receptor, while Gr43a-expressing neurons might play an important role
for amino acid feeding regulation caused by amino-acid-deficiency.

**The role of pharyngeal taste neurons**

Gr43a is expressing in pharyngeal taste organs and brain neurons (Miyamoto et al., 2012). Miyamoto et al. reported that Gr43a-expressing brain neurons function as a sensor of fructose concentration in the haemolymph and promote feeding in hungry flies. Which neurons are required for amino acid feeding regulation? Gr64f is co-expressed with Gr43a in LSO, one of the pharyngeal taste organs (LeDue et al., 2015). In my two-choice preference tests, Gr64f-Gal4>UAS-TNT flies responded to amino acid (Fig. 3), meaning that at least Gr43a-expressing neuron in LSO are not required for amino acid feeding regulation.

Gr2a is not expressed in labellar GRNs, but is expressed in pharyngeal taste organs. To confirm the expression pattern of Gr2a, I observed the mouth part of the flies in which Gr2a expressing neurons were labeled by GFP using LSM (Fig. 5A). I obtained Gr2a\textsuperscript{Gal4} knock-in strains from S.J. Moon, therefore in Gr2a\textsuperscript{Gal4}>UAS-GFP flies, GFP expression should accurately demonstrate the Gr2a expression. Gr2a expression was observed in the neurons in LSO and VCSO. Next I tested two-choice preference tests with Gr2a mutant line and the flies in which Gr2a expressing neurons were silenced using the Gal4/UAS system (Fig. 5B–D). Gr2a\textsuperscript{Gal4} is a Gr2a mutant strain (Fig.5B). In Gr2a\textsuperscript{Gal4}>UAS-TNT and Gr2a-Gal4>UAS-TNT flies, Gr2a expressing neurons are silenced by tetanus toxin (Fig. 5C, D). In all flies, aa (-) flies showed significantly increased feeding to amino acid mixture, indicating that Gr2a and Gr2a-expressing neurons are not essential for amino acid feeding controlled by amino-acid-deprivation.

**Several neuropeptides might be involved in amino acid feeding regulation**

Several neuropeptides are known to be involved in feeding regulation, such as insulin-like peptides and NPF. I did screening test of two-choice preference tests using
flies in which gene expression of neuropeptide receptors were knocked down by dsRNA. UAS-neuropeptide receptor dsRNA strains were crossed to nSyb-Gal4 strain, therefore the gene expression of particular neuropeptide receptor was knocked down in all neurons in F1 flies. Fig. 6 demonstrates the distribution of deprivation-induced changes in amino acid preference among 28 lines. The graph shows the feeding ratio for amino acid of aa (-) flies minus the feeding ratio for amino acid of aa (+) flies. Strains are presented with the order of low-responding (left) to high-responding (right) in females’ value. When flies showed significant difference of the feeding ratio for amino acid mixture between aa (+) and aa (-), I considered that the strain showed wild-type phenotype of amino acid feeding behaviour. RNAi flies of allatostatin A receptor, allatostatin C receptor, CG4395, Oamb, and Dilp receptor did not showed significant increase of amino acid preference after amino-acid-deprivation in both males and females. These neuropeptide receptor neurons might be involved in amino acid feeding behaviour.

**IR25a and IR76b neurons are essential for amino acid feeding behaviour**

To investigate whether IRs function as amino acid receptors, I performed two-choice preference tests between glucose and amino acid mixture with IR mutants and the flies in which IR-expression neurons were silenced by the Gal4/UAS system. After a screening of two-choice preference tests between glucose and amino acid mixture with the flies in which particular IR-expressing neurons were silenced, I found that flies did not show the enhanced preference to amino acids after amino-acid-deprivation when IR25a-Gal4 and IR76b-Gal4 were used as Gal4 drivers.

*IR25a* is known to be broadly expressed in gustatory and olfactory neurons. The flies in which IR25a-expressing neurons were silenced did not show feeding response to amino acid mixture depending on amino-acid-deprivation, however, *IR25a* mutant flies showed significantly increased preference to amino acids (Fig. 7). These results indicate that IR25a itself is not necessary for amino acid feeding but
IR25a-expressing neurons are involved in amino acid feeding behaviour.  

*IR76b* is also expressing in a number of GRNs. When IR76b-expressing neurons were genetically silenced, flies did not show the enhanced feeding response to amino acid mixture (Fig. 8A). Furthermore, amino acid feeding behaviour was abolished in two *IR76b* mutants, suggesting that IR76b is essential for amino acid feeding depending on amino-acid-deprivation (Fig. 8B, C).
4. Discussion

The results of this study suggest that *D. melanogaster* modulate feeding preference for amino acids depending on the internal amino acid level. Flies that were replete with sugar showed selective feeding behaviour toward amino acids, suggesting that flies have a ‘specific hunger’ for amino acids. External taste organs are not essential for amino acid feeding as a *poxn* mutant showed amino acid feeding. The stimulation of labellar sensilla with amino acids was sufficient to induce a proboscis extension reflex. Taken together, amino acids are sensed at several different physical locations. The ablation of sugar-sensing neurons did not induce defects in the amino acid feeding response. This result is consistent with observations that flies can discriminate between glucose and amino acids in two-choice preference tests. However, neurons expressing *Gr43a*, a sugar receptor, were necessary for amino acid feeding responses. These neurons might be required to sense an amino acid deficiency. Additionally, *IR76b* mutants did not exhibit an amino acid feeding response, indicating that the expression of *IR76b* is essential for amino acid detection. Several neuropeptides are also involved in the signalling pathway for amino acid feeding.

In Canton-S *Drosophila* reared in aa (-) conditions, there was no significant enhancement in glycine feeding (Fig. 1). Genetic variation affects amino acid feeding regulation, as other wild-type strains show an enhanced preference for glycine in aa (-) conditions (Toshima et al., 2014). Therefore, it is not surprising that the *poxn* mutant showed a significant increase in the preference for glycine after amino acid deprivation (Fig. 2). CS flies did not show a proboscis extension reflex when labellar sensilla were stimulated with glycine, yet glycine was the most highly preferred amino acid in a previous two-choice preference test using aa (+) flies (Toshima & Tanimura, 2012). The observed preference for glycine in the *poxn* mutant supports the hypothesis that glycine is detected by pharyngeal taste organs.

How can flies modulate feeding behaviour based on internal nutritional state? After starvation, sugar neurons show an elevated firing frequency in response to glucose
stimulation (Meunier et al., 2007). Starved flies exhibit a significant elevation in taste responsiveness to low concentrations of sucrose and increased expression of a sugar receptor gene (Nishimura et al., 2012). These studies suggest that peripheral taste sensitivity is modulated by nutrient deprivation. An intriguing question is whether amino acid deprivation induces an increase in amino acid sensitivity in peripheral systems without changes in sensitivity to sugar. According to our results using poxn mutants, pharyngeal taste organs might be important for amino acid feeding modulation. Labellar taste sensitivity can be investigated by proboscis extension reflex tests or electrophysiological recordings; however, it is difficult to study the activity of pharyngeal taste organs using these methods. For future studies, we need to establish in vivo imaging techniques to monitor the activity of pharyngeal taste cells.

*Drosophila* can learn the nutritional value of non-sweet sugars (Fujita and Tanimura, 2011; Burke and Waddell, 2011). Furthermore, mutant flies that lack taste reception can detect nutritional sugars (Dus et al., 2011). These studies indicate that flies have internal nutritional sensors and can modulate their feeding behaviour. The target of rapamycin (TOR) signalling pathway is conserved in most eukaryotes and is a nutrient sensor that regulates cell growth (Chantranupong et al., 2015). This pathway may contribute to feeding modulation. Additionally, autophagy is controlled by insulin/TOR signalling. Autophagy in ovaries is induced by starvation (Barth et al., 2011). After 5 days of amino acid deprivation, autophagy might occur in female ovaries.

Recent studies revealed that mated females show an increased preference for yeast and that yeast deficiency increases the preference for yeast (Vargas et al., 2010; Ribeiro & Dickson, 2010). These studies clarified that the TOR/S6K signalling pathway is involved in the regulation of yeast feeding. My study demonstrated that *Drosophila* can sense amino acids and raised the possibility that flies modulate yeast feeding via the detection of free amino acids. Locust nymphs that are deficient in protein show an
increased response to an amino acid mixture in electrophysiological studies (Simpson et al., 1991); however, functional analyses have not been performed in locusts.

The results of a screening test with neuropeptide dsRNA transgenic flies indicated that several neuropeptides are involved in the amino acid feeding response (Fig. 6). In most cases, females showed a higher Δ feeding ratio than males. A similar tendency was observed in wild-derived strains of the DGRP (Toshima et al., 2014). The requirement for amino acids should be higher in females than males, as females have to produce eggs. Interestingly, females had a lower Δ feeding ratio than males in capability and hugin receptor RNAi flies. In these strains, males showed significant differences between aa (+) and aa (-) conditions, but females did not. These two neuropeptides were related to the pyrokinin-like peptide, which is involved in a variety of physiological and behavioural processes, including reproduction and feeding, in insect species (Nässel & Winther, 2010). Additionally, these neuropeptide receptors are expressed in the SEG, which is the centre of gustation in the brain. In particular, hugin signalling may regulate feeding motivation in Drosophila (Melcher et al., 2007). These neuropeptide receptors might be involved in amino acid feeding promotion in females.

Both male and female RNAi flies with disrupted allatostatin A receptor, allatostatin C receptor, CG4395, Oamb and Dilp receptor did not show amino acid feeding responses (Fig. 6). A previous study reported that allatostatin A regulates metabolism and feeding decisions in Drosophila (Hentze et al., 2015). Oamb is an octopamine receptor gene. Octopamine induces and modulates signal transduction pathways, similar to norepinephrine in vertebrates. Various insects employ octopaminergic modulation, such as feeding and dancing behaviours in honeybees and learning and memory in Drosophila (Farooqui, 2012). Dilp is a Drosophila insulin-like peptide and is involved in the regulation of metabolism (Brogiolo et al., 2001). These neuropeptides are related to feeding or metabolism regulation, and might also act as regulators of amino acid feeding. It is interesting that flies in which NPF receptor neurons were knocked down showed a comparatively higher feeding response to amino
acids (Fig. 6).

IR25a is conserved in molluscs, nematodes, crustaceans and insects (Croset et al., 2010). It might act as a co-receptor, similar to OR83b, for olfactory receptors. However, an IR25a mutant showed an amino acid feeding response that depended on amino acid deprivation, while IR25a-Gal4>UAS-TNT flies did not (Fig. 7). These results indicate that IR25a does not function as a co-receptor in amino acid sensing. By contrast, neither IR76b-Gal4>UAS-TNT flies nor IR76b mutant flies showed amino acid feeding responses (Fig. 8). IR76b might function as a co-receptor to detect several substances, including NaCl and amino acids. IR76b is broadly expressed in gustatory neurons; therefore, it might be necessary in all IR-expressing neurons. If IR76b functions as a co-receptor for amino acid sensing, which IR is the amino acid receptor? I performed two-choice preference tests using several fly strains in which IR-expressing neurons were genetically ablated, but could not identify IR-expressing neurons associated with defects in amino acid feeding behaviour (data not shown). To identify neurons, individual amino acids might need to be tested based on the present results, which suggest that amino acid mixtures exhibit multiple tastes. A two-choice preference test is appropriate for screening owing to its handiness; however, most strains do not show a strong preference for individual amino acids using this method. Accordingly, the establishment of calcium imaging techniques may be necessary to identify amino acid-sensing neurons and receptors.
5. Figures

Figure 1
Two-choice preference test between amino acids and glucose using wild-type, CS flies. Graphs are modified from Toshima & Tanimura (2012). Tests were conducted between 10 mM glucose and 1/10 amino acid mixture (16.5 mM in total) (A) and between 5 mM glucose and 10 mM phenylalanine (B), cysteine (C), and glycine (D). The height of each column represents the ratio of coloured flies. In each column, the blue portion indicates the ratio of flies that ingested amino acid, and the red portion indicates the ratio of flies that ingested glucose. Significant differences in the proportion of flies that preferred amino acids were observed in both sexes between the aa (+) and aa (-) groups, except for glycine (Mann-Whitney test, *P<0.05, **P<0.01, N=5). No significant differences in the proportion of flies that preferred glucose were observed in both sexes between the aa (+) and aa (-) groups for amino acid mixture, phenylalanine or cysteine (Mann-Whitney test, P>0.10, N=5).
Figure 2
Two-choice preference test using poxn flies. Column representation is the same as in Fig. 1. Significant differences in the proportion of flies that preferred amino acids were observed between the aa (+) and aa (-) groups for amino acid mixture and glycine (Mann-Whitney test, *$P<0.05$, **$P<0.01$, $N=8$).
Figure 3

Two-choice preference test between 10 mM glucose and 1/10 amino acid mixture using the flies in which sugar-sensing neurons were genetically silenced. Female flies showed significant difference in the proportion of flies that preferred amino acid mixture between the aa (+) and aa (-) condition when both Gr64f-Gal4 (A) and Gr5a-Gal4 (B) were used as Gal4 drivers (Mann-Whitney test, **$P<0.01$, $N=10$).
Figure 4

Two-choice preference test between 10 mM glucose and 1/10 amino acid mixture using Gr43a mutant (A) and the flies in which Gr43a-expressing neurons were genetically silenced (B, C). Gr43a mutant flies showed significant difference in the proportion of flies that preferred amino acid mixture between the aa (+) and aa (-) condition (A), but Gr43a-Gal4>UAS-TNT and Gr43a-Gal4>UAS-TNT did not (B, C). (Mann-Whitney test, **P<0.01, N=10).
Figure 5

(A) The image of mouth part of Gr2aGal4>UAS-GFP fly under LSM. Gr2a-expressing neurons are labeled by GFP. Gr2a is expressing in the LSO neurons (filled arrowhead) and in the VCSO neurons (open arrowhead).

(B–D) Two-choice preference test between 10 mM glucose and 1/10 amino acid mixture using Gr2a mutant (B) and the flies in which Gr2a-expressing neurons were genetically silenced (C, D). All flies showed significant difference in the proportion of flies that preferred amino acid mixture between the aa (+) and aa (-) condition. (Mann-Whitney test, *P<0.05, **P<0.01, N≥6).
Figure 6

Mean preference changes between aa (+) and aa (-) flies are shown by $\Delta$ feeding ratio values. $\Delta$ feeding ratio is calculated as the feeding ratio for amino acid mixture in aa (-) minus the feeding ratio for amino acid mixture in aa (+). Each column represents the fly strains used as UAS-dsRNA that were crossed with nSyb-Gal4 line. Asterisks indicate significant differences of the feeding ratio for amino acid mixture between aa (+) and aa (-) flies (Mann-Whitney test, *$P<0.05$, **$P<0.01$, N=6).
Figure 7

(A) Two-choice preference test between 10 mM glucose and 1/10 amino acid mixture using the flies in which IR25a-expressing neurons were genetically silenced. In IR25a-Gal4>UAS-TNT flies, IR25a-expressing neurons were silenced by tetanus toxin. IR25a-Gal4>UAS-IMPTNT is a control strain, as IMPTNT codes for an inactivated tetanus toxin.

(B) Two-choice preference test between 10 mM glucose and 1/10 amino acid mixture using IR25a mutant.

Control flies and IR25a mutant flies showed significant difference in the proportion of flies that preferred amino acid mixture between the aa (+) and aa (-) condition. (Mann-Whitney test, **P<0.01, N≥7).
Figure 8

(A) Two-choice preference test between 10 mM glucose and 1/10 amino acid mixture using the flies in which IR76b-expressing neurons were genetically silenced. In IR76b-Gal4>UAS-TNT flies, IR76b-expressing neurons were silenced by tetanus toxin. IR76b-Gal4>UAS-IMPTNT is a control strain, as IMPTNT codes for an inactivated tetanus toxin. Female flies of IR76b>UAS-TNT showed enhanced feeding to glucose after amino-acid-deprivation. In control flies, female showed enhanced feeding to amino acid mixture after amino-acid-deprivation. (Mann-Whitney test, *P<0.05, **P<0.01, N=5).

(B, C) Two-choice preference test between 10 mM glucose and 1/10 amino acid mixture using IR76b mutants. In both strains, flies did not show significant difference in the proportion of flies that preferred amino acid mixture between the aa (+) and aa (-) condition. (Mann-Whitney test, N≥7).
III. Amino acid preference and learning in *Drosophila* larvae

1. Introduction

It is intriguing to ask how learning and memory processes are involved in the regulation of feeding behaviour. Mice prefer a nutritive sweetener to a non-nutritive sweetener in a choice experiment, suggesting that mice can evaluate the post-ingestive reward (Domingos et al., 2011). *Drosophila* are also able to learn the nutritional value of non-sweet sugars (Fujita and Tanimura, 2011; Burke and Waddell, 2011). A learning process is involved in these phenomena. To investigate feeding motivation, associative learning, such as Pavlov’s classical conditioning experiments using dogs, is often employed. *Drosophila* can develop a robust association between an odour, the conditioned stimulus, and a food reward, the unconditioned stimulus, when both stimuli are presented together (Davis, 2011). When the unconditioned stimulus is appetitive taste, a reward memory is created. When the unconditioned stimulus is an aversive taste or electric shock, punishment learning occurs. Flies are attracted to or avoid an unconditioned stimulus associated with a reward or punishment, respectively.

Adult *Drosophila* can survive without obtaining amino acids. However, larval *Drosophila* need to ingest a protein source for growth. Adult flies showed a preference for amino acids in amino acid-deprived conditions; accordingly, larvae might show a stronger preference than adults. In fact, larvae prefer to stay on agar medium containing amino acids compared with pure agar without amino acid deprivation (Miura, 2014). However, preferred amino acids differ between larvae and adults; for example, adults prefer cysteine, glycine and phenylalanine, whereas larvae prefer aspartic acid, cysteine and glutamic acid (Toshima & Tanimura, 2012; Miura, 2014).

Larval chemosensory organs are located on the anterior tip of the head; they include the dorsal, terminal and ventral organs (Stocker, 2008). The terminal organ mainly functions as an olfactory organ. The dorsal and ventral organs have gustatory functions. The expression profiles of GR genes are different between larvae and adults.
Larval pharyngeal taste organs are the dorsal pharyngeal sense organs and ventral pharyngeal sense organs, which are located behind the mouth-hooks, and the posterior pharyngeal sense organs, which is located far more posteriorly. Surprisingly, most larval pharyngeal neurons persist throughout metamorphosis and are integrated into the adult nervous system (Gendre et al., 2004).

The larval brain consists of approximately 10,000 neurons, which is ten times fewer than the adult brain. Nevertheless, larvae are intelligent enough to exhibit associative learning of odours and tastes, i.e., after repetitive training for an association between an odour and a sweet taste reward, larvae are attracted to the learned odour (Gerber & Hendel, 2006). Larvae can learn an odour associated with aspartic acid in like wise odour-sugar association (Shleyer et al., 2015). This finding enabled a new approach to characterise the amino acid-sensing mechanism in peripheral systems.

Gustatory sensing differs between larvae and adults. Do larvae have a distinct amino acid feeding regulation system from adults? I utilised larvae of selected DGRP lines and performed a two-choice assay to measure the preference for amino acids. In adults, the RAL-427, RAL-732 and RAL-852 lines showed high responses to amino acids and the RAL-335, RAL-379, RAL-786 and RAL-705 showed low responses to amino acids in amino acid-deprived conditions (Toshima et al., 2014). I found that adults in low-responsive lines do not always exhibit low responses at the larval stage. Furthermore, I examined whether larvae learn all 20 amino acids, in addition to aspartic acid. Individually, the 20 amino acids acted as appetitive rewards for larvae.
2. Materials and methods

Fly strains

A collection of inbred lines of *Drosophila melanogaster* (DGRP) was established from female flies collected in Raleigh, NC, USA (Ayroles et al., 2009; Mackay et al., 2012). DGRP lines were obtained from the Bloomington Drosophila Stock Center, IN, USA. Amino acid high-responding lines (RAL-427, RAL-732, and RAL-852) and low-responding lines (RAL-335, RAL-379, RAL-786, and RAL-705) were selected and used based on amino acid preference tests of adults (Toshima et al., 2014). Flies were reared on standard cornmeal-yeast-glucose-agar medium under a 12h:12h light:dark cycle (lights on at 06:00 and off at 18:00) at 25 ºC. Canton-Special (CS) was used as a wild-type strain in the learning experiments.

Chemicals

D-glucose was obtained from Sigma-Aldrich (St Louis, MO, U.S.A.). Sodium hydrogen carbonate, potassium dihydrogenphosphate, di-potassium hydrogen phosphate, and magnesium sulphate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Amino acids of special grade were obtained from Nacalai Tesque, Wako Pure Chemical Industries, or Sigma-Aldrich. The composition of the amino acid mixture was as follows: 0.5 mM tyrosine, 2 mM arginine, 3.5 mM aspartic acid, 4 mM glutamic acid, 5 mM tryptophan, and 10 mM each of alanine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, and valine. This solution corresponds to a total of 165 mM amino acids and was used at 1/10 dilution. N-amyl acetate (AM) was obtained from Merck (Darmstadt, Germany) and diluted 1:50 in paraffin oil. 1-octanol (OCT) was obtained from Sigma-Aldrich (Seelze, Germany). Agarose and fructose were obtained from Roth (Karlsruhe, Germany).

Choice test
The method of larval choice test followed Miura’s double-circle style (Miura, 2014). Petri dishes were prepared by filling outside with 1 % agar, and inside with 1 % agar plus amino acids (in/out = agar/amino acids). Inside and outside circle was separated by using 50 mL centrifuge tube (WATSON CO. LTD., Japan; 225 mm diameter). 5 day-old third-instar larvae were collected just before the test or 4 hours before for amino-acid-deprivation. I poured 15 % glucose solution to the vials containing medium and larvae, and collected the floated larvae. Amino-acid-deprivation group was placed in the distilled water containing glucose and ions for 4 hours. Larvae were rinsed by distilled water on stainless sieve (IIDA MANUFACTURING CO. LTD., Osaka, Japan; 420 µm mesh), and then collected on Kimwipes paper. 10 larvae were placed at the centre of Petri dish by using small brush. I counted the number of larvae located within the central circle every 5 minutes until 30 minutes. Preference index (P.I.) of each time points was calculated as follows:

P.I. = #inside the central circle / 10 (#total)

**Learning experiment**

Larval learning experiment followed the method of B. Gerber’s group (Shleyer et al., 2015; Fig. 9). Approximately 30 larvae were trained by 3 cycles of paired presentation of AM with an amino acid and OCT with a tasteless agarose (AM+/OCT). Then larvae were transferred to an agarose test dish and given the choice between the two odours. After 3 minutes larvae on either side were counted and olfactory preference was calculated as follows:

Olfactory preference = (#AM - #OCT) / #total

The centre line (1 cm width) between AM side and OCT side was neutral zone, then the larvae within the neutral zone were included neither #AM nor #OCT. Positive preference values indicate larvae preferred AM. For each group of larvae trained the association of AM and an amino acid (AM+/OCT), a second group was trained the
association of OCT and the same amino acid (AM/OCT+). From two reciprocally trained groups of larvae, we calculated a learning index (L.I.) as follows:

$L.I. = \frac{\text{olfactory preference (AM+/OCT) – olfactory preference (AM/OCT+)}}{2}$

Positive values indicate appetitive and negative values indicate aversive associative memory.
3. Results

Amino-acid-deprivation did not induce preference enhancement for amino acids in larvae

Miura found that CS larvae prefer to stay on the agar containing amino acids and that aspartic acid is the most preferred amino acid (Miura, 2014). I tested whether amino acid high-responding strains in DGRP (Toshima et al., 2014) show stronger preference to amino acids after amino-acid-deprivation. I compared two groups of larvae; one was collected from medium and was immediately tested, the other was placed in the distilled water containing glucose and ions for 4 hours before the test. Two high-responding lines, RAL-427 and RAL-732 were tested by larval choice test of double-circle method established by Miura (Miura, 2014) (Fig. 10). In this experiment, the value of control is the base line as centre circle is filled with pure agar in the control tests. Both in RAL-427 and RAL-732, larvae significantly preferred amino acid mixture and aspartic acid as compared to agar control ($P<0.01$). When I compared no-deprivation and amino-acid-deprivation groups, RAL-427 showed significantly decreased preference for aspartic acid by amino-acid-deprivation (Fig. 10A). RAL-732 showed smaller preference index for aspartic acid than RAL-427, however, did not demonstrate preference changes between no-deprivation and amino-acid-deprivation groups (Fig. 10B).

The larvae of amino acid low-responding strains at adult stage are not always low-responsive to amino acids

By the screening of two-choice preference test using adult DGRP strains, we previously found RAL-427, RAL-732, and RAL-852 as amino acid high-responding lines, and RAL-335, RAL-379, RAL-705, and RAL-786 as amino acid low-responding lines (Toshima et al., 2014). I performed larval choice assay with these lines to know whether responsiveness to amino acids is conserved among different developmental stages. Two low-responding lines, RAL-335 and RAL-705, showed significantly low preference for
amino acid mixture as compared to other lines (Fig. 11). This result suggests that adult low-responding lines are not always low-responsive to amino acid mixture at larval stage.

**20 amino acids individually work as rewards in associative learning**

I next asked whether individual amino acids work as rewards in CS larvae. Schleyer et al. previously reported that CS larvae can develop associative memory of odour and aspartic acid (Schleyer et al., 2015). Therefore I tested all 20 amino acid individually. Third instar larvae of CS were trained with one amino acid in the presence of odour, and tested whether they are attracted to the learned odour or not. Positive learning index indicates appetitive and negative learning index indicates aversive associative memory. The learning indices of all 20 amino acids were significantly difference from pure agarose as the control (Fig. 12). However, there was no significant difference among 20 amino acids, indicating that all 20 amino acids were equally work as rewards for larvae.
4. Discussion

In the present study, the larval choice test revealed that amino acid deprivation does not increase the preference for an amino acid mixture in larvae. For one strain in which adults exhibit high responses to amino acids, larvae showed a decreased preference for aspartic acid after amino acid deprivation. Low-response strains with respect to adults did not always exhibit low responses at the larval stage.

In our previous study using DGRP flies, several high-response and low-response strains with respect to amino acids were identified (Toshima et al., 2014). In the current study, I detected two low-responding lines (out of four total lines), i.e., RAL-379 and RAL-786, that showed amino acid preferences during the larval stage that were as high as three high-responding lines (Fig. 11). These results suggest that the response level to amino acids depends on the developmental stage. In the adult stage, four low-responding strains showed equally low responses to amino acids; however, the mechanisms underlying the low preferences might differ between RAL-379 and RAL-786 and the other two strains. For example, RAL-335 and RAL-705 flies might not be able to detect amino acids as evidenced by the low response in both larval and adult stages, while RAL-379 and RAL-786 might have defects in the internal monitoring system to detect amino acid deficiencies.

Adult flies show strong feeding responses to amino acids after amino acid deprivation; therefore, I predicted that larvae would show a stronger response owing to their requirement for amino acids for growth. Surprisingly, larvae did not show an increase in amino acid preference after 4 hours of deprivation; rather, RAL-427 flies showed a decreased preference for aspartic acid (Fig. 10). Third instar larvae develop into pupae when the supply of food is stopped. Four hours of amino acid deprivation might induce a developmental switch and accordingly might affect amino acid feeding regulation. Feeding motivation might have an influence on this process because starvation stress resistance differs among DGRP strains (Ayroles et al., 2009). Larvae must obtain amino acids for development; thus, low-responding strains should have a
disadvantage with respect to fitness. However, considering that low-responding lines are present in natural populations, an ability to detect amino acids might not be essential in natural environments. *Drosophila* prefer fruits and oviposit on them; these fruits may supply sufficient amino acids. Interestingly, adult flies also tend to exhibit a reduced preference for aspartic acid after amino acid deprivation (Toshima & Tanimura, 2012).

Adult flies did not show a strong preference for amino acids without amino acid deprivation. In studies of amino acid feeding behaviour using adults, I consistently compared aa (+) with aa (-) groups; therefore, I need to consider both the influence of the peripheral sensing system and the homeostatic monitoring system. By contrast, larvae presented a detectable preference for amino acids without amino acid deprivation. A recent study also revealed that larvae avoid an amino acid-imbalanced diet via dopaminergic neurons in the brain (Bjodal et al., 2014). Larvae are valuable for future studies of the detailed mechanisms of amino acid sensing in peripheral systems.

Sugar and a low concentration of salt are reinforcers of appetitive memory (Gerber & Stocker, 2007; Niewalda et al., 2008). Here, I discovered that all 20 amino acids individually act as rewards for *Drosophila* larvae. Feeding satisfaction can be studied by assessing the motivation to approach a conditioned odour (Krashes et al., 2009; Gruber et al. 2013). Our findings that larvae develop an appetitive memory to an amino acid taste enables a new approach to study decision-making involved in amino acid feeding. It is interesting that all 20 amino acids were equal rewards in our learning experiment, despite previous observations based on choice tests that individual amino acids induce different preferences (Miura, 2014). Innate preference and reward effects might be controlled by distinct input neurons.

Single amino acids modulate feeding and associative learning in the honeybee (Simcock et al., 2014). Honeybees may require amino acid detection mechanisms since they have to nurse larvae, which require a continuous supply of amino acids for growth. Social insects have comparatively large brains and present complicated learning behaviours; therefore, honeybees are frequently used for learning and memory studies (Sandoz,
2011). We found that *Drosophila* larvae with 10,000 neurons in the brain have an ability to create appetitive memories for the 20 individual amino acids, and this fact should accelerate studies of the learning and memory mechanisms for amino acid rewards.
Approximately 30 larvae were introduced into the training dish filled with agarose or agarose plus an amino acid. Larvae were transferred to the dish of the other odour/taste combination after 5 minutes. After 3 cycles of training, larvae were transferred to the test dish filled with agarose. After 3 minutes larvae on either side were counted.

When odour A and B were AM and OCT, respectively, larvae learn the association of OCT and an amino acid (AM/OCT+). When odour A and B were OCT and AM, respectively, larvae learn the association of AM and an amino acid (AM+/OCT).
Figure 10
Larval choice tests between pure agarose and amino acids using RAL-427 (A) and RAL-732 (B). No-deprivation group was tested immediately after the collection from medium, and amino-acid-deprivation group was placed in the distilled water containing glucose and ions for 4 hours before the test. The number of larvae that were located within the centre circle was counted every 5 minutes for 30 minutes, and P.I. values were calculated. The graphs show the mean P.I. value of 6 time points. RAL-427 larvae showed significantly reduced preference for 10 mM aspartic acid after 4 hours of amino-acid-deprivation (Mann-Whitney test, *P<0.05, N≥10). In all groups, significantly higher preferences to amino acids were observed as compared with control.
Figure 11

(A) Larval choice tests between pure agarose and 1/10 amino acid mixture using seven DGRP strains. Three strains are amino acids high-responding strains in adults (High), and four strains are amino acids low-responding strain in adults (Low) according to our previous study. Time course of P.I. for 30 minutes are shown.

(B) To compare amino acid preference of each strain, mean P.I. of 6 time points are shown. RAL-335 and RAL-705 showed significantly lower preference for amino acid mixture than the other strains (Mann-Whitney test, \( P<0.01, N\geq10 \)).
Learning indices for 20 individual amino acids and pure agarose (PUR) are presented with box plots (middle line: median, 25% / 75%: box boundaries, 10% / 90%: whiskers). The concentration of amino acid was 10 mM, except for tyrosine (1 mM). There was no significant difference among amino acids (Kruskal-Wallis tests), however, L.I. for all 20 amino acids showed positive values (one-sample-sign-tests).
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