Behavior neurogenetics of gustation in Drosophila melanogaster

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Behavior neurogenetics of gustation in *Drosophila melanogaster*

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I. General introduction

Animals have to ingest essential nutrients from diets. To this end, various sensory systems are utilized to find and discriminate nutritious food in the outside world. First, animals seek and approach to candidate food sources by means of visual and olfactory cues. Subsequently, they contact to the unknown substance and ascertain whether it is beneficial or harmful for them relying on gustatory cues. In vertebrates, tastants are detected by taste cells located in taste buds on the surface of the tongue and palate epithelium, and basically recognized as five taste modalities (sweet, bitter, salty, sour, and umami) (Chandrashekar et al., 2006). Sweet taste is mostly induced by sugars, which are an essential energy source, and thus predicts potential food sources. Umami and salty tastes recognize amino acids and salt, both of which are also critical nutrients to maintain internal homeostasis. On the other hand, bitter taste elicits rejection against potentially poisonous chemicals and sour taste warns the existence of acidic and noxious chemicals.

These fundamental taste sensings are widely conserved between vertebrates and invertebrates, whereas mechanisms of taste perception are evolutionarily divergent (Liman et al., 2014). In vertebrates, the single heterodimer T1R2/T1R3 serves as a taste receptor for a wide range of sweet substances and the T1R1/T1R3 heterodimer functions as the umami receptor (Damak et al. 2003; Li et al. 2002; Nelson et al. 2001; Zhao et al. 2003). In contrast, insect gustatory receptor (GR) families, which were successfully identified through genome projects over the last two decades, are highly divergent (Clyne et al., 2000; Hill et al., 2002; The Honeybee Genome Sequencing Consortium, 2006; Wanner and Robertson, 2008). Moreover, while the mammalian sweet, umami, and bitter receptors are classified as G protein-coupled receptors, insect GRs are supposed to form ligand-gated ion channels as insect olfactory receptors (ORs), which are evolutionally related to GRs (Sato et al., 2008;
Animals occupy a wide range of ecological niches and therefore their feeding habits are also diverse among species. In several mammalian species, genetic variations in taste receptor genes have been revealed, which underlie the diversity of feeding habits. Domestic and wild cats, which are obligate carnivores, do not show preference for sweet compounds due to their insensitivity to sugars as a consequence of the pseudogenization of \textit{Tas1r2} gene (Li \textit{et al.} 2005). On the other hand, primarily herbivorous giant panda harbors pseudogenized \textit{T1r1} gene (Li \textit{et al.} 2010), which presumably induces loss of umami taste. These studies provide molecular explanations for alterations in feeding habit during the evolution of vertebrates. However, we do not yet know much about genetic variation in \textit{Gr} genes in insects. In chapter II, I examine the genetic variation in sugar taste sensitivity in a natural population of \textit{Drosophila melanogaster} as a model insect. Furthermore, I address to identify the \textit{Gr} gene responsible for variation in taste sensitivity to fructose in subsequent chapter III.

Information from the gustatory system allows animals to evaluate the quality of food. However, whether the food source is useful to an animal or not depends on their current physiological condition, and thus animals have to adjust the feeding behavior based on their internal state. To this end, animals show specific appetites for particular nutrients thorough both internal need- and state-dependent manners (Schulkin, 1991; Trumper and Simpson, 1993; Walker \textit{et al.}, 2015). Moreover, feeding behavior is regulated by circadian clocks as well as a wide range of behavior and physiology from mammals to insects (Allada and Chung, 2010; Green \textit{et al.}, 2008; Sarov-Blat \textit{et al.}, 2000; Xu \textit{et al.}, 2008), while the detailed mechanism is yet unclear. In chapter IV, I show that time-regulated feeding behavior for amino acids in mated females of \textit{Drosophila}.
II. Genetic variation in taste sensitivity to sugars in *Drosophila melanogaster*

1. Introduction

Gustation is an important sensory system regulating animal feeding behavior, and hence influences the selection and uptake of nutrients essential for survival and reproduction. Genetic variation in natural populations is a driving force in the process of evolution, but determining the evolutionary processes underlying taste sensitivities is challenging. In vertebrates, the T1R2/T1R3 dimer is the sole taste receptor for sweet substances, and recent genomic studies have elucidated the evolutionary lineage of the two genes in different mammalian species (Jiang et al., 2012; Max et al., 2001). In insects, little is known about genetic and physiological variations in taste sensitivities and their influence upon feeding preference. Recent research has suggested that mutational events influencing taste were key drivers of adaptation in *Drosophila* and in the cockroach (Wada-Katsumata et al., 2013; Wisotsky et al., 2011). *Drosophila melanogaster* is a useful model organism for the investigation of taste and feeding behavior (Gerber et al., 2009). Sugars are an essential energy source for flies and several gustatory receptor (Gr) family proteins function as sugar taste receptors (Fujii et al., 2015; Miyamoto et al., 2012; Montell, 2009). To date, most of the *Drosophila melanogaster* taste studies have used a few typical wild-type lines, such as Canton-S or Oregon-R; however, the diversity of genetic variation in taste sensitivity in the natural population remains uninvestigated. The *Drosophila melanogaster* Genetic Reference Panel (DGRP), which consists of inbred lines established from a natural population, enabled us to study genetic variations in a natural population (Huang et al., 2014; Mackay et al., 2012). The DGRP lines are fully sequenced, and SNP analyses have revealed gene networks associated with several quantitative traits, such as starvation resistance and olfactory behavior.
(Brown et al., 2013; Mackay et al., 2012; Swarup et al., 2013). I therefore studied sensitivities to sugars in the DGRP lines, with the aim of determining the extent of genetic variability in taste sensitivity in the natural population and further understanding the genes involved in sugar reception.

I show here that there are large strain differences in taste sensitivity to sugars among the DGRP lines. In particular, two-choice preference tests indicated that the preference for four kinds of sugar varies between lines. I then selected two lines showing opposing preferences for glucose and fructose, and compared their responses to glucose and fructose. The results indicated that sensitivity to fructose is responsible for the opposing preferences. Genetic analysis showed that high sensitivity to fructose is autosomal dominant over low sensitivity and that multiple loci control fructose sensitivity. Subsequently, I found the involvement of the Gr64a–Gr64f gene family in fructose sensitivity.
2. Materials and Methods

Fly stocks
Flies were reared on a cornmeal-agar-yeast-wheatgerm-glucose medium at 25°C under a 12 h light/dark cycle. The DGRP consists of inbred lines established by 20 generations of full-sib mating of the progeny of single female flies derived from natural populations in Raleigh, North Carolina, USA (Mackay et al., 2012). Seventy-six DGRP lines (Table 1) were obtained from the Bloomington Drosophila stock center (Indiana, USA). The strains were raised for several generations in our laboratory prior to experimentation. Chromosome exchanges were performed using two balancer strains, \( w^{+}; Kr^{H1}/CyO; D^{1}/TM3, Ser^{1} \) and \( w^{+}; CyO/In(2LR)bw^{Y1}, ds^{33k}dp^{ov1}b^{1}bw^{V1}; CxD/TM6B, Tb^{1} \). The DrosDel isogenic deficiency strains (Ryder et al., 2004; 2007) \( Df(2R)ED1715, Df(2R)ED2311, Df(3L)ED202, \) and \( Df(3L)ED4341 \) were obtained from the KYOTO Stock Center (Kyoto, Japan). \( Df(2R)ED1715, Df(3L)ED202, \) and \( Df(3L)ED4341 \) have breakpoints covering the \( Gr43a, Gr61a, \) and \( Gr64a–Gr64f \) genes, respectively. \( Df(2R)ED2311 \) was randomly selected as a control strain. These deficiency strains were homozygous lethal, so heterozygous (\( Df/+ \)) flies were tested for taste sensitivity. \( Gr43a^{GAL4}, Gr43a^{GAL4}; UAS-Gr43a, \) and \( Gr43a^{GAL4}; Cha^{7.4kb-GAL80/TM6b} \) lines were kindly provided by Dr. Hubert Amrein.

Chemicals
D-glucose was obtained from Sigma-Aldrich Corp. (St. Louis, USA); D-fructose, sucrose (highly purified) and D-sorbitol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); \( \alpha-D \)-trehalose was obtained from H+B Life Science Co., Ltd. (Tokyo, Japan); and Food Blue No. 1 and Food Red No. 106 were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).
Two-choice preference test

The two-choice preference test was performed as previously described (Hiroi et al., 2004). Put briefly, two pieces of filter paper were arranged in a diagonal arrangement on a Petri dish and wetted with 150 µl distilled water. Two further pieces were each soaked with 150 µl of one of two types of sugar solution, and individually colored with a blue (125 mg l⁻¹) or a red (250 mg l⁻¹) food dye. Glucose solution (32.5 mM) was consistently colored blue, and other tastants were colored red. The food dyes used in this study do not influence preference (Tanimura et al., 1982). Approximately 50–60 flies were starved for 20 h and were supplied only with water (Evian™). The flies were subsequently aspirated into the Petri dish and left in darkness for 1 h. Preference tests were performed during the 1 pm to 6 pm time window. Each line was tested on different days for three combinations of sugars. After freezing the flies, the abdomen coloration was observed using a compound stereomicroscope. The preference index (PI) was calculated using the following formulae: \( \frac{N_B + N_M^2}{2} / (N_B + N_M + N_R) \) (PI for the blue side) and \( \frac{N_R + N_M^2}{2} / (N_B + N_M + N_R) \) (PI for the red side), where \( N_B, N_R \) and \( N_M \) represent the number of flies colored blue, red and purple, respectively. The feeding ratio was calculated by \( \frac{N_B + N_M + N_R}{N_B + N_M + N_R + N_O} \), where \( N_O \) represents the number of uncolored flies. To determine the concentration of sugars, I first performed pilot experiments using Canton-S for three combinations of sugars. I changed the sugar concentrations, while fixing the glucose concentration at 32.5 mM, and determined the sugar concentrations so that Canton-S flies showed intermediate PI values. Then I tested several DGRP strains and confirmed that the PI values of the DGRP strains (76 strains for glucose vs. fructose, or 37 strains for glucose vs. sucrose and glucose vs. trehalose) were evenly distributed.

The PI values were assessed by the two-way analysis of variance (ANOVA) model \( Y = \mu + S + L + S \times L + \epsilon \), where \( \mu \) is the mean of the PI values, \( S \) is the fixed effect of sex, \( L \) is the random effect of DGRP line, \( S \times L \) is the sex-by-line interaction term, and \( \epsilon \) is the
environmental variance. For each individual sex, the ANOVA model \( Y = \mu + L + \varepsilon \) was used. Broad-sense heritabilities (\( H^2 \)), coefficients of genetic (\( CV_G \)) and environmental (\( CV_E \)) variance, and the cross-sex genetic correlations (\( r_{m\|f} \)) were estimated from the variance components as described previously (Mackay et al., 2012).

**Proboscis extension reflex test**

The proboscis extension reflex (PER) test was performed as previously described (Kimura et al., 1986). Put briefly, flies were food-deprived for 22 h and supplied with water only. Male flies were fixed on a plastic plate with myristyl alcohol (Nacalai Tesque, Inc., Kyoto, Japan) and left for 2 h in a moist chamber. The fixed flies were satiated with water prior to testing. Each fly was tested by stimulating the tarsal chemosensilla of one prothoracic leg with a small drop of sugar solution for 2 s, and the presence or absence of PER was recorded. The stimulations were performed in order from lower to higher concentrations of glucose and fructose solutions in turn, and the flies were tested with water between sugar stimulations. Exceptional flies that showed frequent positive responses to water were not included in the data set.

**Electrophysiology**

Electrophysiological recordings were performed on labellar chemosensilla using the tip-recording method, as previously described (Hiroi et al., 2002). Put briefly, a glass capillary (ERMA INC., Tokyo, Japan) filled with adult hemolymph-like saline (Hiroi et al., 2013) was inserted from the abdomen through to the labellum and connected to the ground. Labellar l-type chemosensilla of male flies were stimulated for 2 s with a 10–15 \( \mu \)m diameter glass capillary electrode filled with stimulus solution. Stimulations were performed in order from lower to higher concentrations of glucose and fructose solutions in turn (each also
containing 1 mM KCl as an electrolyte), and were started and finished with 1 mM KCl solution. The electric signals were amplified by a TastePROBE (Syntech, Kirchzarten, Germany) (Marion-Poll and van der Pers, 1996), and further amplified and filtered by a differential amplifier (Warner Instrument Corp., Connecticut, USA). The signals were digitized by a 16-bit A/D conversion card DT9804 USB A/D (Data Translation, Inc., Massachusetts, USA), and stored on computer. The recording data were analyzed using dbWave custom software provided by F. Marion-Poll (Marion-Poll, 1996).
3. Results

**Genetic variation in preference for sugars between DGRP lines**

To quantify how sugar preference varied between DGRP lines, I performed two-choice preference tests with two kinds of sugar with 76 DGRP strains. Previous studies predict that there are at least three separate sugar-receptor sites, for pyranose, furanose and trehalose, in *Drosophila* sugar-responsive neurons (Ishimoto and Tanimura, 2004). On the basis of this hypothesis, I chose three sugar combinations for the two-choice tests: glucose (pyranose) vs. fructose (furanose), glucose vs. sucrose (pyranose), and glucose vs. trehalose.

Figure 1 shows the preference for glucose and fructose in the 76 DGRP lines. The Preference Index (PI) values obtained for each line were distributed widely in the possible 0–1 range, indicating that there is an extensive and continuous difference in preference between the two sugars in the strains tested (Table 1). The broad-sense heritability of $H^2 = 0.662$ underscores substantial genetic variations in taste sensitivity to these sugars among the DGRP lines (Table 2 and 3). Similar preference results were observed between glucose and sucrose among the 37 DGRP lines, with a broad-sense heritability of $H^2 = 0.629$ (Figure 2A and Table 1–3). It is curious that extensive preference differences were observed between these two pyranose sugars. In the two-choice test between glucose and trehalose, I predicted that a dimorphic preference distribution would be observed due to the presence of a genetic dimorphism in taste sensitivity to trehalose (Tanimura et al., 1982). Contrary to expectation, however, the PI values again showed an extensive and continuous distribution in sugar preference, with a broad-sense heritability of $H^2 = 0.797$ (Figure 2B and Table 1–3). These data imply genetic variation in the sensitivity to glucose as well as trehalose. The extensive and continuous distributions observed in all three of the pairwise tests cannot be explained by genetic variation in sensitivity to single sugars alone. Therefore, our results indicate that taste sensitivities to all these sugars are polygenic in the tested population.
The analyses of variance (ANOVAs) indicate that the preference for sugars is sexually dimorphic (sex terms in Table 2). Moreover, there were significant sex-by-line interactions except for glucose vs. fructose ($P = 0.05$), indicating genetic variation in the magnitude of sexual dimorphism in the preference for sugars among DGRP lines (sex-by-line interaction terms in Table 2). However, considering the high cross-sex genetic correlations ($r_{MF} = 0.949$ for glucose vs. fructose, $r_{MF} = 0.840$ for glucose vs. sucrose, $r_{MF} = 0.914$ for glucose vs. trehalose; Table 3), the sex-specific effects on the variation in preference for sugars appear to be relatively small.

Since food intake is genetically correlated with starvation resistance in the DGRP lines (Garlapow et al., 2015), I tested the correlation between the preference for sugars and starvation resistance (Mackay et al., 2012) among the lines. The preference for sugars was shown to be largely independent of starvation resistance, although the variation in male PI values in glucose vs. trehalose showed a weak negative correlation with starvation resistance ($r = -0.334$, $P = 0.0436$; Table 4). By contrast, the feeding ratio of sugars was negatively correlated with starvation resistance in both sexes across all three combinations of sugars, as previously observed for food intake.

I further determined whether the PI values were correlated between the pairwise tests. Moderate positive correlations were observed in the distributions of PI values between glucose vs. fructose and glucose vs. sucrose in both male and female flies (Figure 3A). This correlation demonstrates that the perception mechanisms for fructose and sucrose have some functional overlap. The PI distributions for glucose vs. trehalose were not correlated with any other PI distributions (Figure 3B and C).

**Behavioral and nerve responses to glucose and fructose**

I found that the DGRP strains were variably sensitive to the sugars used in the two-choice
test; however, the pairwise test cannot determine whether the taste sensitivity to either sugar is affected. I therefore chose to further investigate the disparity between glucose and fructose sensitivity, and selected two lines, DGRP_301 (as a representative line preferring glucose to fructose) and DGRP_712 (as a representative line preferring fructose to glucose), for additional experimentation.

First, to determine whether the DGRP_301 and DGRP_712 flies exhibit different physiological responses to glucose and fructose, I performed PER tests by stimulating the tarsal chemosensilla with a range of glucose and fructose concentrations. There were no significant differences in the response to glucose between the two lines at any concentration (Figure 4A); by contrast, the response to fructose was significantly higher in DGRP_712, which preferred fructose in the two-choice test, than in DGRP_301 (Figure 4B). Specifically, the PER ratio in DGRP_301 did not exceed 0.7, even when flies were stimulated with the highest concentration of fructose (1M). These results demonstrate that the difference in the PI values between the two lines is attributable to fructose sensitivity, and thus I henceforth designate DGRP_712 as a fructose high-sensitivity line (HF), and DGRP_301 as a fructose low-sensitivity line (LF).

Next, to test whether the responses to glucose and fructose differ between the two lines at the gustatory receptor neuron level, I recorded nerve responses to these sugars from the l-type labellar chemosensilla by using the tip-recording method. Stimulation with sugar solution activates both the water-responsive and sugar-responsive receptor neurons, but the water response is inhibited by high osmolarity (Cameron et al., 2010; Evans and Mellon, 1962; Inoshita and Tanimura, 2006). To precisely count the spikes originating from the sugar-responsive receptor neuron, I first determined the water-response spikes by stimulating sensilla with different concentrations of sorbitol, which does not stimulate the sugar-responsive taste neuron (Fujita and Tanimura, 2011). Figure 5 indicates that the water
response is similarly inhibited by sorbitol in the two strains. I then subtracted the number of water spikes at the appropriate sorbitol concentration from the total number of spikes elicited by glucose and fructose. No significant difference in the number of spikes was observed between the two strains at any glucose concentration (Figure 6A and B). By contrast, DGRP_301 (LF) demonstrated a significantly lower response to fructose than DGRP_712 (HF) (Figure 6A and C), which led us to wonder whether the minimal response of LF at high fructose concentrations resulted from habituation caused by sequential stimulation. I therefore tested the responses in LF with 1 M fructose alone and found that the number of spikes did not increase (Figure 7). This indicates that the low activity was not caused by habituation and that the l-type labellar chemosensilla in LF is insensitive to fructose rather than merely exhibiting a low responsiveness. In summary, these data indicate that there is no difference between HF and LF in the response to glucose, and that the responsiveness to fructose in LF is strikingly lower than in HF both at the behavioral level and the gustatory receptor neuron level.

**Genetic analyses of fructose sensitivity**

To compare the fructose sensitivity between HF and LF in more detail, I performed two-choice tests using 3–4 different concentrations of fructose against 32.5 mM glucose. Fructose sensitivity was determined as relative to glucose sensitivity. Most HF flies preferred fructose at 20 mM, while most LF flies only preferred fructose at concentrations of 160 mM or above (Figure 8A); thus distinct, separate fructose sensitivity curves were observed for the two strains. I then reciprocally crossed the two lines and obtained sensitivity curves for the F<sub>1</sub> offspring to determine genetic dominance. The curves of the F<sub>1</sub> populations were similar to the HF curve, regardless of parental sex combination, suggesting that high sensitivity to fructose is autosomal dominant over low sensitivity (Figure 8A). I also recorded the nerve
responses of heterozygous flies obtained by reciprocal crossing of HF and LF (Figure 9). The results indicated that high sensitivity to fructose is autosomal dominant over low sensitivity, supporting the conclusion obtained by the behavioral results.

I then sought to discover whether autosome 2 or 3 is involved in the variability in fructose sensitivity. To this end, I used balancer chromosomes to establish two lines and tested their fructose sensitivity. In the first line, chromosomes 2 were HF-derived and chromosomes 3 were LF-derived (712; 301); the converse chromosome arrangement was present in the second constructed strain (301; 712). To exclude the possibility of using flies produced by rare recombination, I independently established five lines and selected one line per genotype by confirming that the sensitivity curves were similar between the lines (data not shown). The 301; 712 sensitivity curve was similar to the HF and F\textsubscript{1} curves (Figure 8B), while the 712; 301 curve was intermediate between the HF and LF curves. These data suggest that multiple loci from both autosomes are associated with the difference in fructose sensitivity between HF and LF, but that the major contributory locus is likely to be on chromosome 3.

Eight gustatory receptor (\textit{Gr}) genes on chromosome 2 or 3, \textit{Gr}43\textit{a}, \textit{Gr}61\textit{a}, and \textit{Gr}64\textit{a}–\textit{Gr}64\textit{f}, have been reported to be involved in sugar responses (Fujii \textit{et al.}, 2015; Miyamoto \textit{et al.}, 2012; 2013). Especially, GR43A functions as an internal fructose sensor in the brain (Miyamoto \textit{et al.}, 2012). I therefore asked whether these \textit{Gr} genes are associated with the difference in fructose sensitivity between HF and LF. In order to perform a genetic complementation test for fructose sensitivity, I chose the DrosDel isogenic deficiency strains \textit{Df}(2R)\textit{ED1715} (\textit{\DeltaGr}43\textit{a}), \textit{Df}(3L)\textit{ED202} (\textit{\DeltaGr}61\textit{a}), and \textit{Df}(3L)\textit{ED4341} (\textit{\DeltaGr}64). In addition, \textit{Df}(2R)\textit{ED2311}, whose breakpoint contains no \textit{Gr} gene, was used as a control strain. The heterozygotes of these deficient strains with HF (\textit{\DeltaGr}/HF) showed similar sensitivity curves to each other and to the HF curve (Figure 10A). On the other hand, the sensitivity curve of
heterozygous flies from LF with Df(3L)ED4341 (∆Gr64/LF) was apparently different from that of the other heterozygotes between LF and deficient strains (Df(2R)ED2311/LF, ∆Gr43a/LF, and ∆Gr61a/LF) and similar to the LF curve (Figure 10B). These results suggest that the Gr64a–Gr64f gene region might contribute to the difference in fructose sensitivity. It is intriguing that it is not the fructose receptor gene Gr43a but the Gr64a–Gr64f genes that are likely to be associated with variation in fructose sensitivity. I also tested fructose sensitivity in homozygous Gr43aGAL4 flies. The sensitivity curve of the Gr43aGAL4 flies was similar to the LF curve, although these flies showed a concentration-dependent preference for fructose, demonstrating the existence of another fructose receptor gene apart from Gr43a (Figure 11). Nevertheless, the rescue of the Gr43a gene (Gr43aGAL4, UAS-Gr43a) led to increased fructose sensitivity, comparable to that of HF. I further asked if the increase in fructose sensitivity is due to the rescue of Gr43a in the brain. Flies with restricted Gr43a expression in the brain (Gr43aGAL4/Gr43aGAL4; UAS-Gr43a/Cha7.4kb-GAL80) failed to rescue the phenotype, indicating that Gr43a expression in the peripheral organs notably affects fructose sensitivity. Taken together, peripheral GR43A is indeed involved in fructose sensitivity, although an additional fructose receptor protein is likely to exist.
4. Discussion

*Drosophila melanogaster* is an excellent experimental model for the study of evolution. Several studies have shown that differential behavioral traits can be identified in flies collected in natural populations, indicating the often polygenic nature of behavioral traits (Ehrman and Parsons, 1981). Previous research revealed the presence of genetic dimorphism with respect to taste sensitivity to trehalose in several *D. melanogaster* laboratory strains (Tanimura et al., 1982). However, the extent of gustatory genetic variation in natural populations is unknown. Recent molecular studies have revealed that several *Gr* family genes are implicated in sugar taste sensitivity (Freeman and Dahanukar, 2015; Fujii et al., 2015), and understanding the evolutionary processes underlying *Gr* gene diversification will provide valuable insights into diet, speciation, and colonization (Wisotsky et al., 2011).

The DGRP comprises a valuable resource for the elucidation of complex relationships between behavioral and physiological traits and genotypes, relationships that were not previously accessible through mutant analysis alone. In this study, I used the two-choice preference test with DGRP flies to show that there are genetic variations in sugar sensitivity in the wild-derived inbred *Drosophila* population. I performed two-choice preference tests between glucose and fructose, glucose and sucrose, and glucose and trehalose in the DGRP lines. PI values among the lines were evenly and continuously distributed for all three pairwise sugar comparisons, indicating that taste sensitivity to sugars is a polygenic trait. In the two-choice test, the flies were allowed to choose between two kinds of sugar. Flies are assumed to preferentially drink sugar that is more stimulative, and the choice of behavior therefore depends on the sensitivities of flies to the presented sugar types (Tanimura et al., 1982). Thus, the observed phenotypic variation might mostly be due to the difference in taste sensitivity to sugars. Our two-choice protocol lets flies choose sugars for one hour, so physiological and post-ingestive effects are unlikely to influence the preference for sugars.
Previous electrophysiological studies demonstrated that there are at least three separate sugar-receptor sites (for pyranose, furanose and trehalose) in the sugar-responsive neurons of larger flies and fruit flies, and that glucose and sucrose are co-detected by the pyranose site (Ishimoto and Tanimura, 2004). On the other hand, recent studies have implied that functional sugar receptors might serve as heterodimers or heteromultimers and that the constituent GR proteins appear to be partly redundant between receptors for sugars (Dahanukar et al., 2007; Fujii et al., 2015; Jiao et al., 2007; 2008; Slone et al., 2007). Variable preferences for the paired sugars in the DGRP population might be due to the variations in these Gr genes, which lead to the differences in ligand affinities and/or the kinetics of coupling the functional sugar-receptor proteins to transduction mechanisms.

The fructose sensitivities of the DGRP_712 (HF) and DGRP_301 (LF) strains were remarkably different, as determined through analyses at the behavioral level and the gustatory receptor neuron level. Surprisingly, l-type labellar chemosensilla in LF exhibited a minimal response to fructose, even at high concentrations. However, I did observe PER when stimulating the tarsus of a foreleg in LF with fructose, although the response was lower than that in HF. Similarly, in the two-choice preference test, LF flies preferred fructose to glucose only at high fructose concentrations. Hence, although the labellar nerve responses to fructose in LF are notably low, the flies retain some ability to detect fructose. Given that a previously identified fructose receptor gene, Gr43a, is expressed in tarsal taste sensilla but not in the labellum (Miyamoto et al., 2012; Fujii et al., 2015), an additional receptor for fructose should be involved in the fructose response of labellar sensilla, as our behavioral assay suggested. Moreover, I suggest that the Gr64a–Gr64f genes are involved in fructose sensitivity. This is consistent with previous observations that l-type labellar chemosensilla in flies partly deficient in the Gr64 region show no response to fructose (Dahanukar et al., 2007; Freeman et al., 2014).
Our studies revealed that there are genetic variations governing sensitivities to sugars in a natural population of *Drosophila*. It is a fascinating and challenging problem to understand why these genetic variations are present. It is interesting that extensive genetic variations were observed in taste sensitivity in the DGRP lines, because the lines are established from flies collected in the ‘Raleigh Farmers Market’ (Mackay *et al*., 2012). Determining how polymorphisms in sensitivity to sugars arise will contribute to understanding the mechanisms of changing taste sensitivity during incipient speciation and colonization and will also contribute to pest control (Wada-Katsumata *et al*., 2013).

The results of our genetic analyses imply that several genes participate in variation in sugar taste sensitivity, although I suggest that the sugar receptor genes *Gr64a–Gr64f* contribute to differences in fructose sensitivity between the two DGRP lines. The results obtained in this study provide a platform for genome-wide association studies by adding more phenotypic data of the DGRP lines, which will allow us to know the involvement of such genes in variations in sugar sensitivity. I also checked the SNPs of coding and regulatory regions of *Gr43a* and *Gr64a–Gr64f* in the DGRP lines by UCSC Genome Browser track at http://dgrp2.gnets.ncsu.edu/, and I found several nonsynonymous SNPs in coding regions of these genes, but so far I could not find plausible SNPs that might be associated with the fructose sensitivity dimorphism between DGRP_301 and DGRP_712. I also could not identify regulatory elements that might affect expression of these *Gr* genes. Further work should be carried out to reveal which of the *Gr64a–Gr64f* genes is involved in fructose sensitivity and to identify the genetic sequence variations associated with fructose sensitivity in the DGRP lines. Our current analyses suggest that such analyses are still painstaking because of our lack of knowledge regarding the genetic regulation of the *Gr64a–Gr64f* gene complex. Nonetheless, identifying genes associated with variation in taste sensitivity would enable us to explore the evolution of *Gr* genes in flies living in different locations and niches.
Table 1. Mean of Preference Index (PI) values and feeding ratios in the DGRP lines

<table>
<thead>
<tr>
<th>DGRP line</th>
<th>Preference Index (male)</th>
<th>Preference Index (female)</th>
<th>Glucose vs. Sucrose (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGRP_820</td>
<td>0.448</td>
<td>0.062</td>
<td>Var 0.0342 (0.0339, 0.0374, 0.0417)</td>
</tr>
<tr>
<td>DGRP_732</td>
<td>0.519</td>
<td>0.989</td>
<td>Var 0.0738 (0.0712, 0.0764, 0.0768)</td>
</tr>
<tr>
<td>DGRP_714</td>
<td>0.316</td>
<td>0.783</td>
<td>Var 0.0911 (0.0893, 0.0929, 0.0933)</td>
</tr>
<tr>
<td>DGRP_712</td>
<td>0.723</td>
<td>0.600</td>
<td>Var 0.0911 (0.0893, 0.0929, 0.0933)</td>
</tr>
<tr>
<td>DGRP_639</td>
<td>0.175</td>
<td>0.860</td>
<td>Var 0.0911 (0.0893, 0.0929, 0.0933)</td>
</tr>
<tr>
<td>DGRP_379</td>
<td>0.737</td>
<td>0.977</td>
<td>Var 0.0911 (0.0893, 0.0929, 0.0933)</td>
</tr>
<tr>
<td>DGRP_357</td>
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<td>DGRP_235</td>
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Mean and Variance of Preference Index (PI) values and feeding ratios in the DGRP lines.
Table 2. Analyses of variance of PI values of sugars

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<td>0.0246</td>
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</table>

df: degrees of freedom; MS: Type III mean squares; $\sigma^2$: variance component.
Table 3. Quantitative genetic analyses of preferences for sugars

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>glucose vs. fructose</th>
<th>glucose vs. sucrose</th>
<th>glucose vs. trehalose</th>
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<td>Mean</td>
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<td>0.495</td>
<td>0.392</td>
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<td>0.035</td>
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<td>Genetic standard deviation</td>
<td>$\sigma_G$</td>
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<td>Environmental variance</td>
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<td>Environmental standard deviation</td>
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<td>Phenotypic variance</td>
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<td>Coefficient of genetic variation</td>
<td>$CV_G$</td>
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<td>47.463</td>
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<tr>
<td>Coefficient of environmental variation</td>
<td>$CV_E$</td>
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<td>36.466</td>
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<td>Cross-sex genetic correlation</td>
<td>$r_{MF}$</td>
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Table 4. Phenotypic correlations between starvation resistance and feeding behaviors in the DGRP lines

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<th>Male</th>
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<td>$r$</td>
<td>$P$-value</td>
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<tr>
<td>Preference Index (glucose vs. fructose)</td>
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<tr>
<td>Preference Index (glucose vs. sucrose)</td>
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</tr>
<tr>
<td>Preference Index (glucose vs. trehalose)</td>
<td>-0.0985</td>
<td>0.5617</td>
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</table>

<table>
<thead>
<tr>
<th>starvation resistance vs.</th>
<th>Female</th>
<th>Male</th>
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<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$P$-value</td>
</tr>
<tr>
<td>feeding ratio of sugars (glucose vs. fructose)</td>
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<td>0.0038 **</td>
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<tr>
<td>feeding ratio of sugars (glucose vs. sucrose)</td>
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<td>0.0029 **</td>
</tr>
<tr>
<td>feeding ratio of sugars (glucose vs. trehalose)</td>
<td>-0.476</td>
<td>0.0029 **</td>
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</table>


**$P<0.01$; *$P<0.05$
6. Figures

Figure 1. Variation in taste preference for glucose and fructose among 76 DGRP lines.

Two-choice preference tests were performed upon 76 DGRP lines between 32.5 mM glucose and 20 mM fructose (n=5). Preference index (PI) values for glucose (mean ± SEM) in males (closed circle) and females (open triangle) are shown. PI values for each line are shown in rank order with respect to males.
Figure 2. Variation in taste preference between glucose and sucrose and between glucose and trehalose among 37 DGRP lines.

Two-choice preference tests were performed upon 37 DGRP lines between 32.5 mM glucose and (A) 8 mM sucrose (n=3), and (B) 80 mM trehalose (n=3). Preference index (PI) values for glucose (mean ± SEM) in males (closed circle) and females (open triangle) are shown. PI values for each line are shown in rank order with respect to males.
Figure 3. Correlation plots of sugar preference in the two-choice tests.

Correlation plots are shown based on the Preference Index (PI) values obtained from the two-choice preference tests (shown in Table 1). (A) Correlation between glucose vs. fructose and glucose vs. sucrose. (B) Correlation between glucose vs. fructose and glucose vs. trehalose. (C) Correlation between glucose vs. sucrose and glucose vs. trehalose. Significant positive correlations are observed between glucose vs. fructose and glucose vs. sucrose in both males and females (Pearson’s correlation coefficient test, **$P < 0.01$; female, $r = 0.39$; male, $r = 0.52$).
Figure 4. Behavioral responses of tarsal chemosensilla to glucose and fructose.

Proboscis extension reflex (PER) tests were performed with glucose (A) and fructose (B) in two lines that showed opposing preferences for glucose and fructose. At least 12 male flies were tested per strain, and the PER ratio was calculated (n=6). Error bars represent SEMs. Stimulations were performed with 3–1,000 mM glucose and fructose in DGRP_301 (prefers glucose to fructose, closed circle), and with 3–300 mM glucose and fructose in DGRP_712 (prefers fructose to glucose, open square). The two lines were significantly different in their responses to fructose (Mann–Whitney U test, *$P < 0.05$, **$P < 0.01$), but not to glucose.
Figure 5. Water spikes are inhibited by high osmolarity.

Water spikes were recorded from l-type labellar chemosensilla of DGRP_301 and DGRP_712 on stimulation with sorbitol solution. Each data point shows the number of spikes elicited by stimulation with 0, 10, 30, 100, 300 and 1,000 mM sorbitol solutions. Error bars indicate SEMs. There were no significant differences between the two lines at any concentration of sorbitol (One-way ANOVA; DGRP_301, n=9; DGRP_712, n=8).
Figure 6. Nerve responses of labellar chemosensilla to glucose and fructose.

Nerve responses were recorded from l-type labellar chemosensilla of DGRP_301 (closed circle) and DGRP_712 (open square). (A) Typical recordings obtained with 1 M glucose and 1 M fructose for 500 ms after the onset of stimulation. Arrows indicate the onset of stimulation. (B) and (C) Dose-response curves to 10–1,000 mM glucose and fructose, respectively. Dose-response curves were calculated by subtracting water spikes elicited by sorbitol solutions from the total spikes elicited by glucose and fructose solutions. Error bars indicate SEMs of the total number of sugar-induced spikes. Significant differences between the two lines were observed in the responses to fructose (One-way ANOVA, *P < 0.05, **P < 0.01; DGRP_301, n=13; DGRP_712, n=10), but not to glucose, at all concentrations.
Figure 7. Low fructose response in DGRP_301 is not due to stimulation habituation.

The number of spikes elicited by 1 M fructose in DGRP_301 as in Fig. 6C is shown here as ‘sequential stimulation’. Spikes were recorded from DGRP_301 1-type labellar chemosensilla on stimulation with only 1 M fructose (shown as ‘1 M fructose alone’). Error bars indicate SEMs. No significant difference was observed between the number of spikes elicited by 1 M fructose alone and the number of spikes elicited by sequential stimulation (One-way ANOVA, n.s. = not significant, n=10).
Figure 8. Genetic contributions of autosomes 2 and 3 to fructose sensitivity.

Sensitivities to fructose were determined using two-choice preference tests between 3–4 different fructose concentrations and 32.5 mM glucose. Sensitivity curves of DGRP_301 (low fructose sensitivity, closed circle) and DGRP_712 (high fructose sensitivity, open square) are indicated with grey dashed lines. (A) Sensitivity curves of F₁ offspring from DGRP_301 and DGRP_712 crossings. Two curves of F₁ offspring (black solid lines) are shown to account for reciprocal crossings (female DGRP_301 × male DGRP_712, closed triangle; female DGRP_712 × male DGRP_301, open triangle). (B) Sensitivity curves of the strains with autosomes from both DGRP_301 and DGRP_712. Strain 301;712 (closed triangle, black solid line) had fructose-low-sensitivity-line (LF)-derived chromosomes 2 and fructose-high-sensitivity-line (HF)-derived chromosomes 3, and strain 712;301 (open diamond, black solid line) had HF-derived chromosomes 2 and LF-derived chromosomes 3. The fructose concentrations used were 5, 10, 20, 40, 80 and 160 mM (n=5). Error bars indicate SEMs.
Figure 9. Nerve responses to sugars in F_{1} progeny of high and low fructose-sensitivity lines.

Nerve responses were recorded from l-type labellar chemosensilla of F_{1} offspring from DGRP_301 and DGRP_712 crossings. (A) and (B) Dose-response curves to 10–1,000 mM glucose and fructose, respectively. The vertical axis shows the total number of spikes including water spikes. Two black solid lines show the spikes of F_{1} offspring (female DGRP_301 × male DGRP_712, closed triangle; female DGRP_712 × male DGRP_301, open triangle). DGRP_301 and DGRP_712 curves are indicated with grey dashed lines. Error bars indicate SEMs. Significant differences were observed between DGRP_301 and the other three lines in the responses to 100–1000 mM fructose (One-way ANOVA with Scheffé post hoc tests, **P < 0.01; DGRP_301, n=13; DGRP_712, n=10; female DGRP_301 × male DGRP_712, n=10; female DGRP_712 × male DGRP_301, n=11), but not to glucose at any concentration. In addition, the numbers of spikes in response to 30 mM fructose were significantly different between DGRP_301 and DGRP_712 (**P < 0.01), between DGRP_301 and female DGRP_712 × male DGRP_301 (**P < 0.01) and between DGRP_712 and female DGRP_301 × male DGRP_712 (*P < 0.05).
Figure 10. The *Gr64* gene family is a candidate for the difference in fructose sensitivity between high and low fructose-sensitivity lines.

Genetic complementation of fructose sensitivity was examined between *Gr* gene-deficient strains and (A) DGRP_712 (HF) or (B) DGRP_301 (LF). The DrosDel isogenic deficiency strains *Df(2R)ED2311, Df(2R)ED1715, Df(2R)ED2311, Df(3L)ED202*, and *Df(3L)ED4341* are denoted as *ED2311*, *ΔGr43a*, *ΔGr61a*, and *ΔGr64*, respectively. Fructose sensitivity was determined as in Fig. 8. Sensitivity curves of the heterozygotes are indicated with black solid lines; *ED2311/HF* or LF (closed triangle); *ΔGr43a/HF* or LF (open triangle); *ΔGr61a/HF* or LF (closed diamond); and *ΔGr64/HF* or LF (open diamond). The HF (open square) and LF (closed circle) curves are indicated with grey dashed lines. The fructose concentrations used were 5, 10, 20, 40, 80, 160 and 320 mM (n=3–5). Error bars indicate SEMs.
Figure 11. Gr43a mutant flies can distinguish differences in fructose concentration.

Sensitivity curves of $Gr43a^{GAL4}/Gr43a^{GAL4}$ (closed triangle), $Gr43a^{GAL4}/Gr43a^{GAL4}$; $UAS-Gr43a/UAS-Gr43a$ (closed diamond), $Gr43a^{GAL4}/Gr43a^{GAL4}$; $UAS-Gr43a/Cha^{7.4kb}\cdot GAL80$ (open diamond) are indicated with black solid lines. The DGRP_301 (closed circle) and DGRP_712 (open square) curves are indicated with grey dashed lines. Fructose sensitivity was determined as in Fig. 8. The fructose concentrations used were 5, 10, 20, 40, 80, 120 and 160 mM ($n=4–5$). Error bars indicate SEMs.
III. Deciphering the genes for taste receptors for fructose in *Drosophila*

1. Introduction

Sweet taste is an essential chemosensory modality enabling animals to detect sugars, a critical energy source for survival, and facilitate consumption of energy-rich foods. In mammals, a wide range of sugars are all recognized by a single heterodimeric taste receptor T1R2/T1R3 expressed on the surface of taste cells in the tongue (Damak *et al*., 2003; Li *et al*., 2002; Nelson *et al*., 2001; Zhao *et al*., 2003). In *Drosophila melanogaster*, sugars are detected by sugar receptors expressed in sugar-responsive gustatory receptor neurons (GRNs), which are housed in chemosensilla present on the various taste organs; labellum, legs, and pharyngeal sense organs (Montell, 2009; Stocker, 1994). Studies over the past decade have suggested that nine of the 68 gustatory receptors (GRs) serve as sweet taste receptors (Dahanukar *et al*., 2007; Fujii *et al*., 2015; Jiao *et al*., 2007; 2008; Miyamoto *et al*., 2012; Slone *et al*., 2007). For example, Gr5a is required for trehalose sensing and also broadly mediates responses to several other sugars (e.g. glucose, maltose, and sucrose) along with Gr64f (Dahanukar *et al*., 2007; Jiao *et al*., 2008). In contrast, Gr43a is narrowly tuned to sense fructose and sucrose (Miyamoto *et al*., 2012). However, we do not yet clearly understand how these GRs for sugars function as sugar receptors.

Earlier studies on natural variation in the taste sensitivity to trehalose contributed to finding the *Tre* locus, which led to the identification of the trehalose receptor gene Gr5a (Dahanukar *et al*., 2001; Tanimura *et al*., 1982; Ueno *et al*., 2001). Recently, over 200 sequenced inbred lines, the *Drosophila melanogaster* Genetic Reference Panel (DGRP), have been established from a natural population, which enables the dissection of various natural phenotypic variations (Huang *et al*., 2014; Mackay *et al*., 2012). Using the DGRP lines, a previous study of ours revealed that taste sensitivities to glucose, fructose, and sucrose as well
as trehalose are polygenic among the DGRP lines (Uchizono and Tanimura, in press). Moreover, behavioral tests using two DGRP lines that have a different sensitivity to fructose have shown that the \textit{Gr64a–Gr64f} locus is involved in fructose sensitivity as well as \textit{Gr43a}, the narrowly tuned fructose receptor gene.

Here we examine the association of individual \textit{Gr64a–Gr64f} genes with fructose sensitivity. Expression levels of \textit{Gr64d} and \textit{Gr64e} mRNA in labella differ between the two DGRP lines, which show high and low sensitivities to fructose, respectively. Furthermore, electrophysiological recordings of GRNs show that deletion of individual \textit{Gr64a–Gr64f} genes gives rise to distinct labellar responses to fructose and glucose. Similarly, behavioral responses to fructose and glucose are different among \textit{Gr64a–Gr64f} mutant flies. These analyses verify the contribution of the \textit{Gr64a–Gr64f} gene locus to taste sensitivity to fructose, as well as the several other previously suggested sugars.
2. Materials and Methods

Fly stocks

Two wild-derived, inbred DGRP lines, DGRP_301 and DGRP_712, were obtained from the Bloomington Drosophila stock center (Indiana, USA) (Mackay et al., 2012). $Gr64a^{GAL4}$, $Gr64b^{LEXA}$, $Gr64c^{LEXA}$, $Gr64e^{LEXA}$, and $Gr64f^{LEXA}$ flies were donated by H. Amrein (Texas A&M Health Science Center, USA) (Fujii et al., 2015). $Gr64d^{T14A322}$ flies were established and provided by H. Kim and J. Y. Kwon (Sungkyunkwan University, Korea). Flies were reared on a cornmeal-agar-yeast-wheatgerm-glucose medium at 25°C under a 12 h light/dark cycle.

Chemicals

D-glucose was obtained from Sigma-Aldrich Corp. (St. Louis, USA); D-fructose and D-sorbitol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and Food Blue No. 1 and Food Red No. 106 were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Quantitative PCR (qPCR) assay

Total RNA was extracted using TRIzol reagent (Invitrogen) from 200 labella of 4-5-day-old flies and purified with RNeasy micro kit (QIAGEN) according to the protocol provided by the manufacturers. cDNA was synthesized from the total RNA using Superscript III (Invitrogen) following the protocol provided. The qPCR was carried out using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) with an Mx3000P qPCR system (Agilent Technologies). 1 μL of synthesized cDNA in a 20 μL volume was amplified with 0.5 μM primers as follows: 10 min at 95°C, then 40 cycles of 15 s at 95°C, 30 s at 60°C, and 1 min at 72°C. A house-keeping gene $Gapdh2$ was used as the internal control to normalize mRNA levels. The data finally obtained were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and
We confirmed that all primer sets we used did not yield any non-specific amplification by a melting curve analysis using the products of qPCR. Triplicate reactions for each of three biological replicates were performed for each sample.

qPCR primer pairs for *Gr64a* were: GTGTGCTACCAACTGCTAAATGTC (forward) and ACCTCGTTTGAGACTCTCATTG (reverse), for *Gr64b*: CTATCGGTTCACGGCGAGTAC (forward) and ACTGGGTGCGCTCCATATTG (reverse), for *Gr64c*: CTCAGTAGTTGCCCCCTTG (forward) and ATCCGCATAGTTGACCGAACACCTTTGC (reverse), for *Gr64d*: TGCTTCCGAATGAAACCTTTGC (forward) and CTTGCAATTGCGGAACAGC (reverse), for *Gr64e*: ACCTTCGCCCTGGAACCTTTAACG (forward) and CCTGCACTGCAATTAATAGTCC (reverse), for *Gr64f*: CCGCAGTACAAGACACAGTGC (forward) and TCCGCAAAGACAGCATCATCC (reverse), and for *Gapdh2*: CTACCTGTTCAA GTTCGATTGCAC (forward) and AGTGGACTC CACGATGTATTG (reverse).

**Tip recordings**

Action potentials were recorded from l-type labellar chemosensilla using the tip-recording method, essentially as described in Uchizono and Tanimura (in press). Recordings were performed on L3, L5, and L7 sensilla with 100 mM fructose, 100 mM glucose, and 100 mM sorbitol solution. To precisely count the spikes originating from the sugar-responsive receptor neuron, the number of water spikes elicited by 100 mM sorbitol concentration was subtracted from the total number of spikes elicited by 100 mM fructose or 100 mM glucose in each sensillum.

**Two-choice preference test**
The two-choice preference test was performed as previously described (Hiroi et al., 2004). The flies were starved (supplied only with water) on the basis of the time taken for 10% of the flies to die in each strain, and then given choices between 32.5 mM glucose colored with blue food dye and different concentrations of fructose (2–320 mM) colored with red food dye for 1 h in darkness. Fructose sensitivity was thus determined as relative to glucose sensitivity, as carried out previously (Uchizono and Tanimura, in press). The preference index (PI) for fructose was calculated using the following formulae: 

\[ \frac{N^R + N^M/2}{N^B + N^M + N^R} \] 

where \( N^B \), \( N^R \) and \( N^M \) represent the number of flies colored blue, red and purple, respectively.
3. Results and Discussion

Expression levels of \textit{Gr64d} and \textit{Gr64e} genes in labella differ between strains showing high- and low-sensitivity to fructose

The \textit{Gr64a--Gr64f} gene locus has been implicated in the difference in fructose sensitivity between two wild-derived inbred lines, DGRP\_301 and DGRP\_712 (Uchizono and Tanimura, in press). Sensitivity to fructose differs between the two lines in taste organs, labellum and tarsi; the labellar nerve response to fructose in DGRP\_301 (fructose low-sensitivity line, to which we refer as LF) is notably lower than the response in DGRP\_712 (fructose high-sensitivity line, to which we refer as HF). To determine which gene of the \textit{Gr64} family is involved in fructose sensitivity, we first quantified the expression levels of the \textit{Gr64a--Gr64f} genes in the labella of LF and HF flies by qPCR. Unexpectedly, the expression levels of two genes, \textit{Gr64d} and \textit{Gr64e}, were significantly higher in LF than HF (Figure 1). Consistent with these results, DGRP\_712 (HF) has a deletion of 38 bp (3L: 4,032,908–4,032,945, relative to the reference allele in the DGRP lines) in exon 3 of the \textit{Gr64d} gene (DGRP Freeze 2 genome browser, http://dgrp2.gnets.ncsu.edu). These results raise the possibility that down-regulation of these genes results in higher sensitivity to fructose, even though these mRNA levels might not necessarily reflect the translation levels. In addition, the expression pattern of these \textit{Gr64a--Gr64f} genes recalls previous 5’- and 3’-RACE experiments, which suggested the bicistronic transcription of \textit{Gr64bc} and \textit{Gr64de} in addition to single mRNAs of \textit{Gr64a}, \textit{Gr64e}, and \textit{Gr64f} (Dahanukar \textit{et al.}, 2007). Transcriptions of \textit{Gr64d} and \textit{Gr64e} may be partly down-regulated together in the labellum of HF flies. Furthermore, the expression level of \textit{Gr64a} in labella was quite low in both lines, which is consistent with a previous expression analysis of \textit{Gr64a}\textsuperscript{GAL4} using a UAS-RFP reporter (Fujii \textit{et al.}, 2015). \textit{Gr64f} also showed surprisingly low expression levels in both lines. Given that \textit{Gr64f} has been reported to be widely expressed in sugar-responsive GRNs in the labellum
(Dahanukar et al., 2007; Fujii et al., 2015; Weiss et al., 2011), the translational level of Gr64f might be distinct from the mRNA level.

**Labellar and behavioral responses to fructose are altered by deletion of Gr64a–Gr64f genes**

In the light of the qPCR results, we wondered whether deletion of Gr64d or Gr64e gene affects labellar response to fructose. We then recorded labellar nerve responses to fructose in mutant flies of each Gr64a–Gr64f gene; Gr64a\textsuperscript{GAL4}, Gr64b\textsuperscript{LEXA}, Gr64c\textsuperscript{LEXA}, Gr64d\textsuperscript{T14A322}, Gr64e\textsuperscript{LEXA}, and Gr64f\textsuperscript{LEXA}. Interestingly, Gr64d\textsuperscript{T14A322} and Gr64f\textsuperscript{LEXA} showed higher responses to 100 mM fructose than the other mutant flies, implying that loss of Gr64d and Gr64f genes may induce enhanced fructose sensitivity in the labellum (Figure 2A). The higher response to fructose in Gr64d\textsuperscript{T14A322} is likely to be consistent with the lower expression of Gr64d in the labellum of HF flies. Given that sugar receptors function as multimeric complexes composed of two or more subunits (Dahanukar et al., 2007; Jiao et al., 2007; 2008; Slone et al., 2007), one of the plausible interpretations of these results is that GR64D and GR64F competitively interact with a member of a functional fructose receptor to form another receptor, as is the case for GR64E, which was suggested to be shared by GR64A and GR64B to form distinct sugar receptors (Yavuz et al., 2014). However, we were not able to find which gene of the Gr64 family is the member of the functional fructose receptor since these mutant lines, except for Gr64d\textsuperscript{T14A322} and Gr64f\textsuperscript{LEXA}, showed considerably lower responses to fructose, similar to the LF flies; especially in Gr64a\textsuperscript{GAL4} and Gr64b\textsuperscript{LEXA} the numbers of spikes elicited by fructose were close to zero.

We then further examined the behavioral responses to fructose in these mutant lines. Sensitivity curves for fructose, determined by two-choice preference tests using different concentrations of fructose against a constant concentration of glucose, successfully
distinguished the fructose sensitivities in HF and LF lines (Uchizono and Tanimura, in press). Thus, sensitivity curves were employed to compare the behavioral responses to fructose in mutant flies of each $Gr64a$–$Gr64f$ gene. To this end, we first tested glucose sensitivity in each mutant line. Labellar responses to glucose were found to be diminished in $Gr64c^{LEXA}$ and $Gr64f^{LEXA}$ (Figure 2B). The impaired glucose response in $Gr64f^{LEXA}$ is compatible with previous studies showing that $Gr64f$ is required for glucose sensing (Jiao et al., 2008; Fujii et al., 2015), whereas there seems to be no mention of the involvement of $Gr64c$ in glucose response so far. The $Gr64c$ gene locus deleted in $Gr64c^{LEXA}$ may be required for glucose response in the labellum, but this will need to be validated by further work.

Figure 3 shows the sensitivity curves of each of the $Gr64a$–$Gr64f$ mutant flies. Interestingly, these six curves can be classified into three groups; (i) $Gr64a^{GAL4}$ and $Gr64e^{LEXA}$, (ii) $Gr64b^{LEXA}$, $Gr64c^{LEXA}$, and $Gr64d^{T14A322}$, (iii) $Gr64f^{LEXA}$. In contrast to the notably low fructose responses in their labellum, $Gr64b^{LEXA}$ and $Gr64c^{LEXA}$ flies preferred higher concentration of fructose. The discrepancy might be explained by fructose responses in the other taste organs and also by impaired glucose sensitivity in the case of $Gr64c^{LEXA}$. In addition, $Gr64f^{LEXA}$ flies preferred 2 mM fructose to 32.5 mM glucose, presumably due to the higher response to fructose and lower response to glucose. While these results suggested that $Gr64a$–$Gr64f$ gene loci are indeed associated with fructose sensitivity, nevertheless we were not able to identify the fructose receptor gene from the $Gr64a$–$Gr64f$ genes. Our current analyses obviously suggest the limitations of a study focusing on single genes from $Gr64a$–$Gr64f$. Further rescue experiments of these genes should thus be performed in a combinatorial manner as undertaken by Yavuz et al. (2014) to ascertain the organization of the functional fructose receptor. Our observations would also be helpful for this further study.
4. Figures

![Image of a bar graph]

**Figure 1. Comparisons of the expression levels of Gr64a–Gr64f genes in the labella of strains showing high- and low-sensitivity to fructose**

RNA expression levels of the Gr64a–Gr64f genes in labella were compared between DGRP_301 (LF) and DGRP_712 (HF) by qPCR assays. The dark and light gray bars represent the relative mRNA levels of each Gr64 gene normalized by the level of Gapdh2 gene in DGRP_301 and DGRP_712, respectively (Gr64b, Gr64d, and Gr64f, n=3 in triplicate; Gr64a, Gr64c, and Gr64e, n=5 in triplicate). Error bars represent SEMs. The relative expression levels of each Gr64 gene were compared between the two lines by Student's *t*-test or Welch's *t*-test (**P < 0.01).
Figure 2. Gustatory nerve responses of labellar chemosensilla to fructose and glucose in
*Gr64a–Gr64f* mutant flies

Responses of l-type sensilla to 100 mM fructose (A) and 100 mM glucose (B) were recorded
in *Gr64a*<sup>GAL4</sup> (n=9), *Gr64b*<sup>LEXA</sup> (n=6), *Gr64c*<sup>LEXA</sup> (n=6), *Gr64d*<sup>T14A322</sup> (n=9), *Gr64e*<sup>LEXA</sup> (n=9),
and *Gr64f*<sup>LEXA</sup> (n=6) flies. The dark gray bars represent the number of sugar spikes per second,
which was calculated by subtracting water spikes elicited by 100 mM sorbitol solutions from
the total spikes elicited by 100 mM fructose or 100 mM glucose solutions in each sensillum.
Bars denoted by the same letter do not differ significantly (*P* > 0.05, one-way ANOVA with
Tukey–Kramer post hoc tests). For comparison, the numbers of spikes elicited in DGRP_301
and DGRP_712 (Uchizono and Tanimura, in press) are also shown by light gray bars. Error
bars indicate SEMs.
Figure 3. Behavioral responses to fructose and glucose in Gr64a–Gr64f mutant flies

Relative sensitivity to fructose against glucose sensitivity was determined by two-choice preference tests between 32.5 mM glucose and different concentrations of fructose. PI values for fructose are shown at the following concentrations of fructose: 5, 20, 80, and 320 mM in Gr64a\textsuperscript{GAL4} (closed circle, n=5); 2, 5, 10, and 20 mM in Gr64b\textsuperscript{LEXA} (open circle, n=5), Gr64c\textsuperscript{LEXA} (closed square, n=5), and Gr64d\textsuperscript{T14A322} (open square, n=5); 2, 5, and 10 mM in Gr64e\textsuperscript{LEXA} (closed triangle, n=5) and Gr64d\textsuperscript{T14A322} (open triangle, n=4). Error bars indicate SEMs.
IV. Mated *Drosophila melanogaster* females consume more amino acids during the dark phase after amino acid deprivation

1. Introduction

In a wide range of organisms, many biological events in physiology, behavior, and metabolism are restricted to a particular time of day. These rhythmic oscillations are gated not only by environmental cues but also by the internal circadian clock (Andreani *et al*., 2015). The circadian clock is composed of the transcriptional-translational feedback loop (TTFL), which has been identified as the core mechanism inducing specific circadian behaviors (Andreani *et al*., 2015; Tomioka and Matsumoto, 2010). In *Drosophila melanogaster*, CLOCK (CLK)/CYCLE (CYC) heterodimers (CLK/CYC) bind to E-box sequences and induce transcription of several key transcription factors, including *period* (*per*), which represses the activation of CLK/CYC to ensure circadian oscillation of the TTFL. It is well known that flies carrying a mutated version of *period* (*per^0*) show arrhythmicity in many aspects of behavior and physiology regulated by the circadian clock (Andreani *et al*., 2015).

Feeding behavior is one of the activities regulated by the circadian clock. Mutations in circadian genes alter the feeding rhythm in both flies and mice (Green *et al*., 2008). In *Drosophila*, for example, *takeout* (*to*), induced by *Pdp1ε*, which is one of the feedback loop components, modulates feeding behavior by conveying temporal information about the internal nutritional state (Benito *et al*., 2010; Sarov-Blat *et al*., 2000); moreover, the fat body, an important tissue for energy storage that is known to express clock genes, might also control the feeding behavior of flies (Xu *et al*., 2008). Although feeding behavior is controlled by the circadian clock, the detailed mechanism through which the clock induces feeding at a particular time of day has not yet been elucidated.
Animals need to ingest appropriate nutrients depending on their internal state to maintain nutritional homeostasis, and thus their feeding behavior is dependent on their developmental, reproductive, or internal physiological state (Cripps and Williams, 1975; Raubenheimer and Simpson, 1997; Walker et al., 2015; Woodring et al., 1979). Animals increase their feeding preference for a particular nutrient when they are deficient in that nutrient (Schulkin, 1991; Tordoff, 2001; Trumper and Simpson, 1993). Amino acids are important nutrients for development and reproduction, and a specific hunger for proteins or amino acids has been reported in several species (Dethier, 1976; Hawkins et al., 1994; Simpson et al., 1991). In Drosophila, removal of amino acids from the food source prevents larval development, and the lack of only one essential amino acid prevents female flies from laying eggs (Sang and King, 1961). It was reported recently that Drosophila deprived of amino acids show an enhanced preference for them (Toshima and Tanimura, 2012).

In many insects, including Drosophila, mating causes dramatic changes in female physiology and/or behavior (Haussmann et al., 2013). Female flies alter their feeding behavior towards yeast-containing food (Ribeiro and Dickson, 2010) as well as salt (Walker et al., 2015) after mating. The post-mating switch in female behavior is triggered by Sex Peptide (SP), a seminal protein transferred to the female during copulation (Chen et al., 1988; Peng et al., 2005). SP activates a specific receptor, the Sex Peptide Receptor (SPR), which is broadly expressed in the female reproductive tract and nervous system (Yapici et al., 2008). Though the period of locomotor activity rhythm does not differ between males, virgin females, and mated females, the sleep status during the light phase is dramatically changed in mated females compared with virgins, and this change results from the involvement of the post-mating SP/SPR signal (Issac et al., 2010; Oh et al., 2014). Although feeding behavior and metabolic status are both circadian-regulated, we do not yet understand the relationship between a specific hunger for a particular nutrient and the circadian rhythm. I focused in this
study on the feeding behavior after amino acid deprivation and asked whether flies consumed more amino acids at a certain time of the day, despite urgently requiring amino acids. Only mated females showed a higher level of feeding on amino acids in the dark phase than in the light phase; wild-type (CS) males, virgin CS females, and per<sup>0</sup> flies did not. This suggested that only mated females had a specific reason for changing their amino acid consumption over a day. I then investigated whether the post-mating responses (PMRs) of females were related to the time-dependent consumption of amino acids. Time dependency in amino acid consumption was still observed in egg production mutants, indicating that egg-laying behavior itself is not important in increasing amino acid consumption during the dark phase of the 12 h light: 12 h dark (LD) cycle. The flies lacking the SP/SPR signal showed partly diminished amino acid consumption during the dark phase, compared with CS mated females. This suggests that the post-mating SP/SPR signal promotes amino acid consumption during the dark phase by interacting with the circadian clock.
2. Materials and Methods

Fly stocks

Canton-Special (CS) was used as a wild-type control strain in all experiments. *pep*° (Konopka and Benzer, 1971) was used as a representative clock gene mutant strain. *SP°*/\(\Delta^{130}\) flies were generated by crossing *SP°*/TM3,Sb to \(\Delta^{30}/TM3,Sb\) stocks, and *SP* null mutant males (Liu and Kubli, 2003) were used in experiments. A *SPR* mutant strain, *Df(1)Exel6234*, was donated by Y. J. Kim (Gwangju Institute of Science and Technology, Gwangju, Korea). *ovoD1*/CS sterile females were generated by crossing *ovoD1* males to CS females; the *ovoD1* strain is maintained with a compound-X chromosome and was obtained from the Bloomington *Drosophila* Stock Center. All fly stocks were raised on a standard cornmeal-agar-yeast-glucose medium (SM) under LD cycles at 25 °C.

Amino acid deprivation

Adult flies were collected within 24 h of eclosion and raised on an amino acid-deficient glucose [aa(-)] medium containing 90.08 g glucose, 1 g sodium hydrogen carbonate, 0.7 g potassium dihydrogen phosphate, 3.9 g di-potassium hydrogen phosphate, 0.2 g magnesium sulfate, 0.1 g phosphatidylcholine (dissolved in 1 ml ethanol), 2 ml propionic acid, and 9 g agar in 1L water [modified from Toshima *et al.* (2014)]. Female and male flies were raised together on the medium, and flies were transferred to fresh aa(-) medium every other day.

Chemicals

Chemicals used in the amino acid-deficient glucose [aa(-)] medium were obtained from the following sources: D-glucose was obtained from Sigma-Aldrich (St. Louis, USA); sodium hydrogen carbonate and magnesium sulfate were obtained from Wako Pure Chemical Industries (Osaka, Japan); potassium dihydrogen phosphate, di-potassium hydrogen
phosphate, L-α-phosphatidylcholine, propionic acid, and agar (purified powder) were all obtained from Nacalai Tesque (Kyoto, Japan).

The stock amino acid mixture, based on a previous study (Toshima et al., 2014) and used in all experiments, was made up 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. The stock amino acid mixture was used at 1/10 dilution in all experiments. Special grade amino acids were obtained from Nacalai Tesque, Wako Pure Chemical Industries, or Sigma-Aldrich. Food dyes (Food Blue No. 1 and Food Red No. 106) were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan).

Two-choice preference test

The two-choice preference test was performed as previously described (Toshima and Tanimura, 2012). A piece of chromatography paper was soaked with 150 µl 10 mM glucose solution colored with red food dye, and another piece of chromatography paper was soaked with 150 µl amino acid mixture colored with blue food dye. The food dyes do not influence taste preference at the concentration used in this assay (Tanimura et al., 1982; Toshima and Tanimura, 2012). Approximately 50–60 flies were raised on aa(-) medium for 2 days, and then tests were performed at four Zeitgeber times (ZTs) under LD cycles or at four circadian times (CTs) in constant darkness (DD) or constant light (LL) (Fig. S1A). The flies were placed in a Petri dish containing chromatography papers bearing glucose and amino acids and tested for 2 h. After the test period, flies were frozen and their abdominal colorings were observed under a compound stereomicroscope. Flies were classified into red (R), blue (B), purple (M), and uncolored (O) groups, and feeding ratios (FRs) of glucose and amino acids were calculated using the following formulae:
FR of glucose: \((N^R + N^M/2) / (N^R + N^B + N^M + N^O)\)

FR of amino acids: \((N^B + N^M/2) / (N^R + N^B + N^M + N^O)\)

where \(N^R\), \(N^B\), \(N^M\), and \(N^O\) were the number of flies with red, blue, purple, and uncolored abdomens, respectively.

**CAFE assay**

The capillary feeder (CAFE) assay was modified from the previously reported method (Fujita and Tanimura, 2011; Ja et al., 2007) as follows: four microcapillary tubes were inserted in a Buzz Plug, which was placed into an experimental vial with a piece of wet Kimwipe on the bottom. In a two-choice assay, two of the capillary tubes were filled with 10 mM glucose solution colored with 250 mg/L red food dye, and the other two tubes were filled with amino acid mixture colored with 125 mg/L blue food dye. In a no-choice assay, all capillaries were filled with either glucose solution or the amino acid mixture.

Male and female flies, which eclosed within a 24 h period, were kept together on aa(-) medium for 2 (Figure 1) or 3 days (Figure 5) under LD cycles. Separate groups of 10 male or 10 female flies were then transferred to fresh aa(-) medium and kept for another 2 (Figure 1) or 1 day(s) (Figure 5). In the experiment shown in Figure 5, virgin females eclosing within a 12 h period were kept alone on aa(-) medium for 4 days. After 4 days on aa(-) medium, flies without anesthesia were introduced into experimental vials, and the intake of glucose and amino acids during the light and dark phases was measured. Three control vials without flies were included in each experiment to measure the amount of evaporation; the mean amount of evaporation was subtracted from the decrease in each tested tube.

**Measurement of circadian rhythms and sleep**

Flies were kept on SM under LD cycles at 25 °C for 2–5 days post-eclosion. Virgin and
mated females were then loaded individually into 5×65 mm glass capillary tubes containing agar gel with 100 mg/ml glucose (Itoh et al., 2011). A Drosophila Activity Monitor system (Trikinetics; Waltham, MA, US) was used to record locomotor activity for 4 days under LD cycles, followed by 7 days in DD. The periods were calculated by chi-square periodogram analysis with the significance level set to \( \alpha = 0.05 \) (Sokolove and Bushell, 1978), programmed by the Matlab R2007b software (MathWorks Inc.). Flies with a chi-square statistic \( \geq 10 \) over the significance line were scored as rhythmic (Lim et al., 2007). The 4 days of data recorded under LD cycles were used to assess waking, sleep, and walking activity. The data were analyzed using a custom-written Excel macro (Pfeiffenberger et al., 2010). Sleep was defined as \( \geq 5 \) min of inactivity (zero infrared beam crossings). Walking activity was defined as the number of times the infrared beam was crossed.

**Oviposition assay**

Female flies eclosing within 24 h were collected and placed, together with male flies, on SM or aa(-) medium for 4 days under LD cycles at 25 °C. Groups of 3–5 mated females, together with 1–2 males, which had been kept on SM or aa(-) medium, were then placed in a Petri dish (55 mm diameter) containing SM or aa(-) medium. Dyes (62.5 mg/L blue food dye and 125 mg/L red food dye) were mixed with SM to enable easy recognition of eggs on the medium. The number of eggs laid during light and dark phases was counted under a compound stereomicroscope. To calculate the number of eggs laid by individual females, the total number of eggs on the plate was divided by the number of surviving females at the end of the assay.
3. Results

**Females ingested more amino acids in the dark phase than in the light phase**

Deprivation of amino acids induces a specific appetite, and thus flies increase amino acid consumption to meet their internal needs (Toshima and Tanimura, 2012). The two-choice CAFE assay (between glucose and amino acids) was used to quantify consumption of glucose and amino acids, and thus determine whether amino acid-deprived flies ingested more amino acids at a constant rate across the day. CS flies were kept on aa(-) medium under LD cycles for 4 days before the assays (Figure 1A).

No difference across a day was observed in the consumption of either glucose or amino acids in CS males (Figure 1B); CS females, however, significantly increased their amino acid intake during the dark phase, relative to the light phase, although their glucose intake was constant between the light and dark phases of the LD cycle (Figure 1B). This suggests that females alter their intake of amino acids between the light and dark phases.

We wondered whether clock genes were involved in the differences in consumption of amino acids between the light and dark phases, and thus measured glucose and amino acid intakes in a strain carrying a mutant clock gene (per°). Glucose and amino acids intakes in per° flies were constant between the light and dark phases in both sexes, suggesting that consumption of amino acids in females is under control of the circadian clock.

Since the assay gave flies a choice between glucose and amino acids, I wondered whether the presence of glucose was affecting the level of amino acid intake. I therefore performed a no-choice CAFE assay of glucose and amino acid consumption. The glucose intake of CS flies was unchanged between the light and dark phases, but the amino acid intake of females increased during the dark phase relative to the light phase (Figure 1C), which is the same result as that obtained in the two-choice CAFE assay. Likewise, there were no significant differences between the light and dark phases in glucose and amino acid intakes in...
per$^b$ flies. I concluded, therefore, that glucose intake was independent of the change between the light and dark phases in amino acid intake. Notably, the total amount of feeding throughout the day was greater in per$^b$ than in CS, which is consistent with a recent report (Allen et al., 2016). Taken together, these results show that female flies ingested greater quantities of amino acids during the dark phase, and that amino acid consumption was controlled by the circadian clock.

**Feeding preference for glucose and amino acids does not show a circadian pattern**

Several previous studies have demonstrated that deprivation of yeast or amino acids increases the feeding preference for yeast or amino acids, as well as increased consumption (Ribeiro and Dickson, 2010; Toshima and Tanimura, 2012). To determine whether the feeding preference for amino acids also changed across a day in females deprived of amino acids, I examined the feeding preference for amino acids at four different time points (ZT 0–2, 6–8, 12–14, and 18–20) across a 24 h day using the two-choice preference test (Figure 2A). Both male and female CS flies kept on aa(-) medium under LD cycles preferred amino acids to glucose (Figure 2B and D), as previously reported (Toshima and Tanimura, 2012). Surprisingly, unlike the level of intake, the FR of amino acids (percentage of flies that preferred amino acids to glucose), as well as that of glucose, in both male and female CS flies remained unchanged across the four different time points (Figure 2B; one-way ANOVA, $p > 0.05$).

To test if there was a masking effect of light on the feeding preference during the light phase, I repeated the two-choice tests in DD at four CTs (0–2, 6–8, 12–14, and 18–20). Again, neither males nor females showed any significant differences in the FRs of glucose and amino acids across the four CTs in DD (Figure 2B; one-way ANOVA, $p > 0.05$), suggesting that there was no masking effect of light on the feeding preference. In addition, to determine
whether there was a masking effect of dark on the feeding preference during the dark phase, the two-choice tests were repeated in constant light (LL). In LL, flies of both sexes showed constant FRs of glucose and amino acids at the four CTs (Figure 2B; one-way ANOVA, \( p > 0.05 \)), demonstrating no masking effect of dark on the feeding preference for glucose and amino acids.

To explore whether clock genes were involved in keeping the feeding preference constant across a day, I performed pairwise tests with \( \text{per}^0 \) flies (Figure 2C and D). As with CS flies, the FRs of glucose and amino acids remained constant in \( \text{per}^0 \) flies across four different time points in LD, DD, and LL (Figure 2C; one-way ANOVA, \( p > 0.05 \)). For comparisons between levels of intake (Figure 1) and FRs, I integrated the FRs at ZT 0–2 and 6–8 as the light (L) phase, and at ZT 12–14 and 18–20 as the dark (D) phase (Figure 2D). No significant differences were observed between light and dark phases in either CS or \( \text{per}^0 \) flies. The feeding preferences for glucose and amino acids thus appeared to be independent of light and dark signals, and even of the circadian clock.

**Mating induces an increased consumption of amino acids during the dark phase**

As only CS females showed a difference between the light and dark phases in the level of amino acid intake, it is possible that a specific behavior controlled by the circadian clock underlies amino acid intake in these flies. I first focused on levels of sleep and locomotor activity in CS and \( \text{per}^0 \) females. In both CS and \( \text{per}^0 \) females, the total amount of time spent awake was higher in the light phase than in the dark phase, while the total amount of sleep in the light phase was lower than in the dark phase (Figure 3A and B). CS females had significantly higher activity levels during the dark phase than the light phase; by contrast, the activity levels of \( \text{per}^0 \) flies were significantly higher during the light phase than the dark phase (Figure 3C). Nevertheless, both CS and \( \text{per}^0 \) females show different patterns in the levels of
sleep and locomotor activity between the light and dark phases, which is not consistent with the observation that only CS females show difference in the level of amino acid intake between the light and dark phases. These results imply the levels of sleep and locomotor activity might not be the specific behavior controlled by the circadian clock underling amino acid intake.

Next, I focused on egg-laying behavior of CS and per\textsuperscript{0} females. I counted the numbers of eggs laid by females kept on aa(-) medium for 4 days (Figure 4A; see materials and methods). As the mean number of eggs laid per female under these conditions was less than one, consistent with an earlier report (Sang and King, 1961), I repeated the assay using CS and per\textsuperscript{0} females kept on SM for 4 days (Figure 4B). The mean number of eggs laid per female in the test using SM was greater than 10. The number of eggs laid by CS females in the light phase was significantly higher than in the dark phase, whereas there was no difference in the mean number of eggs laid by per\textsuperscript{0} females in the light and dark phases. These results imply that the difference in amino acid intake between light and dark phases might be related to egg-laying behavior.

As mating elicits increased egg laying (Liu and Kubli, 2003) and amino acid consumption is important for egg laying by mated females (Figure 4B), I hypothesized that mating facilitated amino acid consumption during the dark phase by interacting with the clock that caused eggs to be laid in a circadian manner. I first examined whether virgin CS females showed differences in amino acid consumption between the light and dark phases of the LD cycle using the no-choice CAFE assay. To examine the effect of mating more precisely, mated CS females were also tested again (Figure 5A) by placing females with males for 1 day longer than in the previous no-choice CAFE assay (Figure 1A). As I expected, the consumption of amino acids by virgin CS females during the dark phase was dramatically
lower than that of mated CS females; moreover, virgin CS females did not show differences in amino acid consumption between the light and dark phases (\(t\)-test, \(p = 0.275\)).

To determine whether the post-mating switch regulating amino acid consumption involved the SP and SPR pathway, amino acid consumption by CS females mated with \(SP\) mutant males and by \(SPR\) mutant females mated with CS males was measured using the no-choice CAFE assay (Figure 5B). Both groups of females still showed significant differences in amino acid consumption between the light and dark phases, although \(p\) values were greater than 0.01 (CS females mated with \(SP\) mutant males: \(t\)-test, \(p = 0.015\); \(ΔSPR\) females mated with CS males: \(t\)-test, \(p = 0.042\)). The levels of amino acid intake of CS females mated with \(SP\) mutant males and mated \(SPR\) mutant females were significantly lower than that of mated CS females only in the dark phase. In addition, virgin \(SPR\) mutant females did not show differences in amino acid consumption between the light and dark phases (\(p = 0.309\) with \(t\)-test), as previously observed in CS virgin females. These results indicated that mating must be the trigger to increase amino acid consumption during the dark phase of the LD cycle.

To determine whether the increase in amino acid consumption by mated females during the dark phase was due to amino acid deprivation resulting from egg laying, I examined consumption of amino acids by females carrying the dominant \(ovo^{D1}\) mutation (Figure 5C); such females lack the ability to produce eggs due to an arrest in egg development. Significant differences in amino acid consumption between the light and dark phases were still observed in mated \(ovo^{D1}\) females, although the level of intake of \(ovo^{D1}\) females was much greater than that of mated CS females. Elevated food consumption by \(ovo^{D1}\) females has been previously reported to result from an increase in the volume of food consumed per proboscis extension (Wong et al., 2009). I also tested virgin \(ovo^{D1}\) females and found no significant differences in intake between the light and dark phases (\(t\)-test, \(p = 0.086\), similar to the result.
obtained from virgin CS females. These results suggest that egg-laying behavior is not itself necessary to produce the difference in amino acid consumption between the light and dark phases.

To rule out the possibility that the absence of differences in amino acid consumption by virgin CS and ovo^{D1} females between the light and dark phases resulted from behavioral arrhythmicity (as in per^{0} females), I examined their locomotor activity rhythms (Table 1). While all the per^{0} females tested showed arrhythmic locomotor activity, the rates of rhythmicity in CS and ovo^{D1} virgin females were greater than 90% and the mean period of the locomotor activity rhythm was within 0.5 h of 24 h, which is considered the wild-type locomotor phenotype of *Drosophila*. The locomotor activity rhythms of SP and SPR mutants also had normal periods (Table 1). These results strongly indicate that an increase in amino acid consumption during the dark phase is triggered by mating, and that signaling through the SP/SPR pathway promotes amino acid consumption during the dark phase by modulation of the circadian clock.
4. Discussion

The feeding preference of *Drosophila* for proteins or amino acids increases in response to deprivation of these nutrients (Ribeiro and Dickson, 2010; Toshima and Tanimura, 2012; Vargas *et al*., 2010). Given that there is a daily rhythm in a variety of metabolic, physiological, and behavioral processes, I wondered whether flies showed diurnal changes in feeding behavior for particular nutrients, such as amino acids, required for those processes. I found that mated females showed such a diurnal change, increasing consumption of amino acids during the dark phase when deprived of amino acids throughout the day.

Our two-choice and no-choice CAFE assays showed that the consumption of amino acids was elevated during the dark phase only in mated females. Thus, amino acid consumption appears to be regulated in mated females during the light and dark phases. On the other hand, both male and female flies in CS and *per*^0^ did not change their feeding preference for either glucose or amino acids across a 24 h day in our two-choice preference tests. Since there was a disparity between constant feeding preference and fluctuating consumption of amino acids across a day, the circadian clock may modulate feeding behavior independently of pathways associated with appetite.

Interestingly, a previous study using the CAFE assay reported that *w^1118* flies showed a peak of sucrose consumption in the early daytime (Xu *et al*., 2008), whereas I did not observe any change in glucose consumption between the light and dark phases. I compared food consumption over 12 h for each of the light and dark phases (i.e., both phases include the dawn, which is around the peak time of sucrose consumption). Even if there were an underlying diurnal rhythm in glucose intake, it is possible that total glucose consumption might show no difference between the two phases due to the long measurement interval in our tests. Nevertheless, mated females exhibit the difference in the consumption of amino acids
between the two phases, implying glucose and amino acid consumption are independently regulated by the circadian clock.

Mating drastically changes the physiological status of females and modifies feeding behavior to meet the internal demands for nutrients (Haussmann et al., 2013; Ribeiro and Dickson, 2010; Vargas et al., 2010; Walker et al., 2015). Food consumption is up-regulated after mating, depending on egg production (Barnes et al., 2008; Carvalho et al., 2006). Mating and egg-laying behavior have been shown to be rhythmic (Howlader and Sharma, 2006), and I also observed a difference between the light and dark phases in the average number of eggs laid per female over 12 h. I furthermore found that there was no difference between the light and dark phases in the average number of eggs laid by per^0 females. McCabe and Birley, however, reported that per^0 females still showed an egg-laying rhythm (McCabe and Birley, 1998), which appears to contradict our results. As periodicity of the egg-laying rhythm was disrupted in LN_c-ablated flies (Howlader et al., 2006) and per^0 females kept under DD showed inconsistent periods of egg laying (Xu et al., 2011), it is possible that the periodicity of the egg-laying rhythm of per^0 females is altered or has a greater variance, even under LD conditions. Thus, if the peak and trough times of the egg-laying rhythm of per^0 females fall at the lights-on and -off times, respectively, the average number of eggs laid per female over 12 h (our experimental condition) is likely to be equal in the light and dark phases. Alternatively, although I measured numbers of eggs laid over 24 h, these data might not include the peak if the period in per^0 females is longer than 24 h. Nevertheless, our observation that per^0 females showed no differences in either the level of amino acid intake or egg laying between light and dark phases implies that the link between amino acid intake and egg-laying behavior is via the clock gene, per.

Given the correlated rhythmicity of amino acid intake and egg laying, there are three possibilities: (i) the egg-laying rhythm controlled by the circadian clock drives the rhythmic
consumption of amino acids; (ii) the circadian clock regulates the level of amino acid intake to render egg laying rhythmic; or (iii) the clock synchronizes the two behaviors in parallel. Our observation of increased amino acid consumption during the dark phase by $ovo^{D1}$ females disproves the first hypothesis. In addition, the mean number of eggs laid by $per^0$ females was lower than that by CS females regardless of light and dark phases. A previous report that “wrong time” feeding could reduce a fly’s reproductive capability (Xu et al., 2011) supports hypothesis (ii), namely, that an arrhythmic intake of amino acids results in the reduction in the number of eggs laid by $per^0$ flies; however, it is not possible yet to reach a definite conclusion on whether hypothesis (ii) or (iii) is correct. Additionally, the increased intake of yeast following mating was recently reported to result from the action of SP on SPR-expressing SP sensory neurons (SPSNs) in the reproductive tract, which is also necessary for the modulation of egg laying (Walker et al., 2015). The circadian clock may interact with the mating signal downstream of these SPSNs to induce rhythmic feeding on amino acids and egg laying. Further study is necessary to identify which clock-regulated neuron interacts with the mating signal to modulate amino acid intake and egg-laying behavior.
5. Table

Table 1. Free-running periods of the flies used in the CAFE assay

<table>
<thead>
<tr>
<th>Lines</th>
<th>Period</th>
<th>SEM</th>
<th>N</th>
<th>R%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>per</em>^0^ female (mated with <em>per</em>^0^ male)</td>
<td>-</td>
<td>-</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>CS virgin female</td>
<td>24.23</td>
<td>0.07</td>
<td>32</td>
<td>93.75</td>
</tr>
<tr>
<td>CS female (mated with CS male)</td>
<td>23.87</td>
<td>0.06</td>
<td>30</td>
<td>86.67</td>
</tr>
<tr>
<td>CS female (mated with <em>SP</em>/Δ^130^ male)</td>
<td>24.22</td>
<td>0.05</td>
<td>33</td>
<td>96.97</td>
</tr>
<tr>
<td><em>Df(1)Exel6234</em> (Δ<em>SPR</em>) virgin female</td>
<td>24.06</td>
<td>0.05</td>
<td>31</td>
<td>83.87</td>
</tr>
<tr>
<td><em>Df(1)Exel6234</em> (Δ<em>SPR</em>) female (mated with CS male)</td>
<td>24.12</td>
<td>0.07</td>
<td>33</td>
<td>100</td>
</tr>
<tr>
<td><em>ovo</em>^Dp^/CS virgin female</td>
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<td>0.04</td>
<td>34</td>
<td>97.06</td>
</tr>
<tr>
<td><em>ovo</em>^Dp^/CS female (mated with CS male)</td>
<td>24.47</td>
<td>0.07</td>
<td>33</td>
<td>96.07</td>
</tr>
</tbody>
</table>

N indicates number of flies analyzed.

R% indicates percent flies with detectable rhythmicity.
6. Figures

A

mix females and males

separate females and males

eclosion

aa(-) medium

CAFE assay

eclosion

aa(-) medium

CAFE assay

Day 1  Day 2  Day 3  Day 4  Day 5

B

two-choice CAFE

<table>
<thead>
<tr>
<th></th>
<th>glucose</th>
<th>amino acids</th>
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<tr>
<td></td>
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<td>CS L D</td>
</tr>
<tr>
<td></td>
<td>per^0</td>
<td>per^0</td>
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</table>

C

no-choice CAFE

<table>
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<th>glucose</th>
<th>amino acids</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CS L D</td>
<td>CS L D</td>
</tr>
<tr>
<td></td>
<td>per^0</td>
<td>per^0</td>
</tr>
</tbody>
</table>
Figure 1. Females ingest an increased quantity of amino acids during the dark phase.

The experimental scheme is indicated in (A). Each 12 h light (L) phase is shown by a white box and each 12 h dark (D) phase by a gray box. Intake of glucose and amino acids during L and D phases was quantified using the capillary feeder (CAFE) assay. Assays were performed in the two-choice situation (B) between glucose and amino acids (n = 3 or 4 trials) and in the no-choice situation (C) (n = 3 or 4 trials). The quantity of intake per single fly is shown. Intake during L and D phases is indicated by orange and blue bars, respectively; filled bars represent CS flies; hatched bars represent per⁰ flies. Error bars indicate SEM. *p < 0.05 for comparisons between L and D phases using the Student’s t-test.
Figure 2. Taste preference for amino acids does not show daily rhythmicity.

(A) Diagram of two-choice preference tests. The flies were kept on aa(-) medium for 2 days, and then offered a choice between glucose or amino acid mixture for 2 h (indicated as T) at each time point under LD cycles, DD, or LL (n = 3 or 4 in quintuple). Each light (L) phase is shown by a white box and each dark (D) phase by a gray box. (B and C) Feeding ratios (FRs) between glucose (red lines) and amino acids (blue lines) of CS (B) and per\(^0\) (C) flies are shown at four Zeitgeber (ZT) or circadian (CT) time points. \(p > 0.05\) for all comparisons between feeding ratios across the four time points under LD, DD, and LL using one-way ANOVA. (D) Feeding ratios at ZT 0–2 and 6–8 in (B) and (C) were integrated as the feeding ratio in the L phase (orange bars). Feeding ratios at ZT 12–14 and 18–20 in (B) and (C) were integrated as a feeding ratio in the D phase (blue bars); filled bars represent CS flies; hatched bars represent \(per^0\) flies. \(p > 0.05\) for all comparisons made between L and D phases using Student’s \(t\)-test. Error bars indicate SEM.
Figure 3. Females show differences in sleep status and locomotor activity between the light and dark phases.

Behaviors of CS and per$^o$ flies were recorded under LD cycles for 4 days at 25 °C. L and D represent the results obtained during light (orange bars) and dark (blue bars) phases, respectively; filled bars represent CS flies; hatched bars represent per$^o$ flies. (A) the total time spent awake (min) over 4 days, (B) the total amount of sleep time (min) over 4 days, (C) the total walking activity over 4 days (times) in CS (n = 32) and per$^o$ (n = 31) females. Error bars indicate SEM. *** $p < 0.001$ for comparisons between L and D phases using the Student’s $t$-test.
Figure 4. Number of eggs laid in the light and dark phases differs for CS flies.

The oviposition assay was performed for 12 h starting at either ZT 0 (for the light phase) or ZT 12 (for the dark phase). L and D represent results obtained during the light (orange bars) and dark (blue bars) phases, respectively; filled bars represent CS flies; hatched bars represent per⁰ flies. (A) The mean number of eggs laid during L and D phases on aa(−) medium by an amino acid-deprived female (n = 20). (B) The mean number of eggs laid during L and D phases on a standard cornmeal-agar-yeast-glucose medium (SM) by a female raised on SM (n = 20). Error bars indicate SEM. **p < 0.01 and ***p < 0.001 for comparisons between L and D phases using the Student’s t-test.
Figure 5. A post-mating signal elevates amino acid consumption during the dark phase.

(A) The experimental scheme for the capillary feeder (CAFE) assays. Each L phase is shown by a white box and each D phase by a gray box. (B and C) Amino acid consumption during L (orange bars) and D (blue bars) phases was quantified using no-choice CAFE assays with the following strains: virgin CS females and CS females mated with CS or SP$^{9}$/A$^{130}$ males (B; n = 3 or 4 trials); virgin Df(1)Exel6234 (shown as ΔSPR) females and Df(1)Exel6234 females mated with CS males (B; n = 4 trials); and virgin ovo$^{D1}$/CS females and ovo$^{D1}$/CS females mated with CS males (C; n = 4 trials). Intake per single fly is shown. Error bars indicate SEM. *p < 0.05 and **p < 0.01 for comparisons between L and D phases for each type of female in (B) and (C) using the Student’s t-test. p > 0.05 for all comparisons during L phase among females in (B) using one-way ANOVA. *p < 0.05 and **p < 0.01 for all comparisons during D phase among females in (B) using one-way ANOVA followed by post hoc Bonferroni/Dunn test. *p < 0.05 and **p < 0.01 for comparisons between ovo$^{D1}$/CS virgin and mated females in (C) using the Student’s t-test.
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