The taste of vitamin C in *Drosophila*

Bhanu Shrestha [iD], Binod Aryal [iD] & Youngseok Lee[*] [iD]

Abstract

Vitamins are essential micronutrients, but the mechanisms of vitamin chemoreception in animals are poorly understood. Here, we provide evidence that vitamin C doubles starvation resistance and induces egg laying in *Drosophila melanogaster*. Our behavioral analyses of genetically engineered and anatomically ablated flies show that fruit flies sense vitamin C via sweet-sensing gustatory receptor neurons (GRNs) in the labellum. Using a behavioral screen and *in vivo* electrophysiological analyses of ionotropic receptors (IRs) and sweet-sensing gustatory receptors (GRs), we find that two broadly tuned IRs (i.e., IR25a and IR76b) and five GRs (i.e., GR5a, GR61a, GR64b, GR64c, and GR64e) are essential for vitamin C detection. Thus, vitamin C is directly detected by the fly labellum and requires at least two distinct receptor types. Next, we expand our electrophysiological study to test attractive tastants such as sugars, carboxylic acids, and glycerol. Our analysis elucidates the molecular basis of chemoreception in sweet-sensing GRNs.

Introduction

Vitamin C (ascorbic acid), the dietary antioxidant, is abundant in citrus fruits and vegetables. It plays a role in supporting daily activities and good health. It has a direct effect on energy levels, brain function, and cell metabolism (Poljsak & Ionescu, 2009). In addition, it has wound healing effect and the shortage of it causes the deadly disease, scurvy. Vitamin C cannot be stored in the body, so it needs to be supplied with diet. It is assumed that human adults require at least 40 mg of vitamin C every day. Several studies have suggested that it can influence insecticide resistance and oxidative stress in *Drosophila melanogaster* (Huang *et al*, 2006; González *et al*, 2018; Man Anh *et al*, 2019). Although the advantageous properties of the postingestive mechanism have been highlighted before, how the animal detects vitamins directly has been enigmatic.

Sense of taste plays a key factor in determining nourishing foods and avoiding dangerous toxins. Animals' ability to taste is crucial for recognizing beneficial substances in the environment (Shrestha & Lee, 2023). The taste of various macronutrients and micronutrients is required for the regulation of the feeding behavior of the animal (Freeman & Dahanukar, 2015; Delompré *et al*, 2019). *Drosophila melanogaster* offers an excellent choice for the researcher to perform conceivable research for the behavioral and neurophysiological analysis of taste (Moon *et al*, 2006; Aryal *et al*, 2022b). The distribution of gustatory organs throughout the body including the labellum, forelegs, pharynx, wings, and female genitalia makes the fruit fly interesting model animals (Vosshall & Stocker, 2007; Rimal & Lee, 2018; Chen & Dahanukar, 2020). The labellum has a special role in contact chemosensation as it contains special types of hair-like structure called taste sensilla. Each half of the labellum has 31 hair sensilla, which are further classified into the L (long), I (intermediate), and S (short) morphological subtypes according to the length of hair. The taste sensilla are made up of 2–4 chemosensory, 1 mechanosensory, and 3 supporting cells (Liman *et al*, 2014).

Also, most animals rely on chemosensory mechanisms to evaluate the beneficial and harmful value of their nutrition. Flies can taste macronutrients, such as carbohydrates, lipids, proteins, and micronutrients, such as minerals, in their peripheral sensory systems (Dahanukar *et al*, 2007; Ahn *et al*, 2017; Kim *et al*, 2018; Wu *et al*, 2021; Aryal *et al*, 2022a). However, it is unclear how vitamins, which are generally known as micronutrients, are sensed in the chemosensory organs.

Results and Discussion

Beneficial effect of vitamin C

Vitamin C is beneficial and gives pleasant taste to humans (Schiffman & Dackis, 1975; NTP, 1992). We aimed to decipher whether vitamin C has beneficial and attractive effects on fruit flies. On performing a starvation resistance assay, starved wild-type male and female flies lived significantly longer after feeding on micronutrient vitamin C (0.1–50 mM). This concentration protects cells from free radical damage (Fig 1A and B). We found that 1% agar (without any nutrients) caused lethality in 50% of the flies after 46 h (LT_{50}) in both sexes. However, the LT_{50} increased to 53.6 ± 5.14 and 56.6 ± 3.78 h in males and females, respectively, in the presence of 0.1 mM vitamin C. This effect increased in a dose-dependent manner. For example, the LT_{50} of males in the presence of 30 and 50 mM vitamin C were 99.3 ± 2.55 and 120.9 ± 2.31 h, respectively (Appendix Table S1). This indicates that feeding 50 mM vitamin C can extend the starvation resistance by 2.6 and

Department of Bio & Fermentation Convergence Technology, Kookmin University, Seoul, Korea
*Corresponding author. Tel: +82-2-910-5734; E-mail: ylee@kookmin.ac.kr

2.5 under starved conditions in males and females, respectively. Moreover, we wondered whether this starvation resistance can be regulated by another potent antioxidant, curcumin (Suckow & Suckow, 2006). Previously vitamin C and curcumin have extended the lifespan in a standard food condition (Suckow & Suckow, 2006; Suh et al, 2017). However, the LT_{50} decreased to 39.3 ± 3.88 and 46.0 ± 5.51 h in males and females, respectively, in the presence of 0.1% curcumin (Fig EV1A and B, and Appendix Table S2). Furthermore, 0.5% curcumin was very toxic without sugars (Fig EV1A and B, and Appendix Table S2). Therefore, the increased starvation resistance may be caused not by a simple antioxidant effect but by others. Vitamin C is a weak sugar acid, related to galactose (Vinson et al, 1992). When 50 mM galactose was fed, the LT_{50} increased, though not as much as vitamin C (Fig EV1C and D, and Appendix Table S3). This indicates that vitamin C enhances the starvation resistance properties by acting as a possible nutrient source rather than an antioxidant agent to the flies.

Next, we sought to understand whether flies are attracted to vitamin C. To test the palatability for vitamin C, we allowed females to lay eggs on 1 mM vitamin C + 2 mM sucrose versus higher concentrations of vitamin C + 2 mM sucrose for 18 h using binary oviposition choice assays (Fig 1C). Flies laid more eggs on the side plated with $\geq$ 5 mM of vitamin C. This indicates that flies can distinguish a higher concentration of vitamin C and are attracted to concentrations higher than 5 mM vitamin C. Females often lay eggs on the high nutritional food source. Therefore, we tested whether females lay more eggs in a dose-dependent manner (Fig 1D). Food containing 50 mM vitamin C induced a 4-fold higher number of eggs when compared with 1 mM vitamin C. These results indicate that vitamin C is beneficial for animal fecundity.

Vitamin C is considered a micronutrient to humans, but we found that flies can use it as a beneficial energy source like galactose. Therefore, flies increase survival rates and conduct egg-laying in a dose-dependent manner. Furthermore, flies prefer a higher concentration of vitamin C when finding better places for their progenies. The oviposition experiments can be mediated by short-term effects, such as attractive taste, and long-term effects, such as internal physiological changes.

Neuronal responses to vitamin C

Vitamin C is water-soluble and has a pleasant taste. To analyze its mode of action in the fly gustatory system, we tested all the sensilla in the labellum in the presence of vitamin C. Vitamin C induced strong neuronal activity from several L-type sensilla (L4 and L6 induced highest action potentials, with L5 and L7 inducing relatively milder potentials) but not from S- and I-type sensilla (Fig 1E and F). Moreover, there was a dose-dependent increase in action potentials on L6 sensilla (Fig EV2A). D. melanogaster has a strong preference for a vitamin-containing diet including folic acid (vitamin B9) and riboflavin (vitamin B2) (Wu et al, 2021). Thus, we compared the attractive properties of vitamin B and C via electrophysiology. However, neuronal responses were not significantly detected following stimulations with folic acid and riboflavin on L4, L6, and S6 sensilla (Fig EV2B).

Next, we tested flies in which neuronal inactivation was selectively induced in specific types of neurons via the expression of UAS-Kir2.1 under the control of GAL4s (Paradis et al, 2001). Flies

have two or four gustatory receptor neurons (GRNs) in each sensillum. Each type of GRN can be categorized based on functional GAL4 expressions (Rimal & Lee, 2018). Gr64f-GAL4 and Gr66a-GAL4 are expressed in sweet-sensing and bitter-sensing GRNs, respectively (Thorne et al, 2004; Dahanukar et al, 2007). Ir94e-GAL4 is expressed in one cell per L-type sensillum without overlapping with other GRNs (Jaeger et al, 2018). Furthermore, ppk23-GAL4 and ppk28-GAL4 are expressed in Ca^{2+}-sensing and water-sensing GRNs, respectively (Cameron et al, 2010; Lee et al, 2018). The induction of neuronal inhibition under the Gr64f-GAL4/+ background significantly suppressed the action potentials of L4 and L6 sensilla but not ppk23-GAL4, ppk28-GAL4, Gr66a-GAL4, and Ir94e-GAL4 (Fig 1G and H). This indicates that vitamin C activates sweet-sensing GRNs and may induce attractive signals to the brain.

Subsequently, we assessed whether the attraction to vitamin C was mediated via the labellum and legs because flies have multiple sensory organs responsible for sensing nonvolatile chemicals. To verify our findings, we used proboscis extension response (PER) assays and applied the stimulus to the labellum or forelegs. The test solution contained 2% sucrose or 50 mM vitamin C. We found a significant difference in PER between the test solutions when the stimulus was applied to the forelegs but not the labellum (Fig EV2C). Further, we confirmed that flies prefer a higher concentrations of vitamin C using the binary food choice assay (Fig EV2D). The preference for vitamin C was not affected by surgical removals of the forelegs, maxillary palps, or antennae, as well as of both maxillary palps and antennae (Fig EV2E). However, the preference was affected by the genetic ablation of sweet-sensing GRNs (Fig EV2F). These results indicate that vitamin C is tasted by the sweet-sensing GRNs in the labellum but not the legs or olfactory organs.

Molecular sensors of vitamin C

Vitamin C is also known as ascorbic acid. The ability to taste attractive carboxylic acids requires ionotropic receptors (IRs) and sweet-sensing GRs (Stanley et al, 2021; Shrestha & Lee, 2021a). We have previously reported that flies are highly attracted to glycolic, citric, and lactic acid and avoid tartaric and propionic acid (Rimal et al, 2019). Intriguingly, the activation of sweet-sensing GRNs by lactic acid has two distinct phases, as verified by calcium imaging in the brain (Stanley et al, 2021). IR25a mediates the onset response, while sweet GRs mediate the removal phases, verified as elevation of calcium responses upon removal of lactic acid, which cannot be quantified with tip recordings. Therefore, we tested a repertoire of putative taste receptors (31 candidate Ir and nine candidate Gr loss-of-function mutants) via electrophysiology on L4 and L6 sensilla to elucidate the vitamin C receptors (Fig 2A and B). This included the broadly required Ir25a and Ir76b (Zhang et al, 2013; Chen & Amrein, 2017; Ganguly et al, 2017; Lee et al, 2018; Dhakal et al, 2021; Shrestha & Lee, 2021a; Xiao et al, 2022; Aryal et al, 2022a). Ir7a and Ir62a are required for sensing acetic acid and Ca^{2+}, respectively (Lee et al, 2018; Rimal et al, 2019). Ir56d is essential for sensing hexanoic acid and carbonation buffer (Sánchez-Alcañiz et al, 2018; Dweck et al, 2022). Ir56b is essential for detecting attractive Na^+ (Dweck et al, 2022). We detected reduced responses from IR25a and IR76b alone (Fig 2A). The other 29 Ir mutants elicited normal responses to vitamin C. However, we cannot exclude other IRs that have not been tested. Next, we tested 9
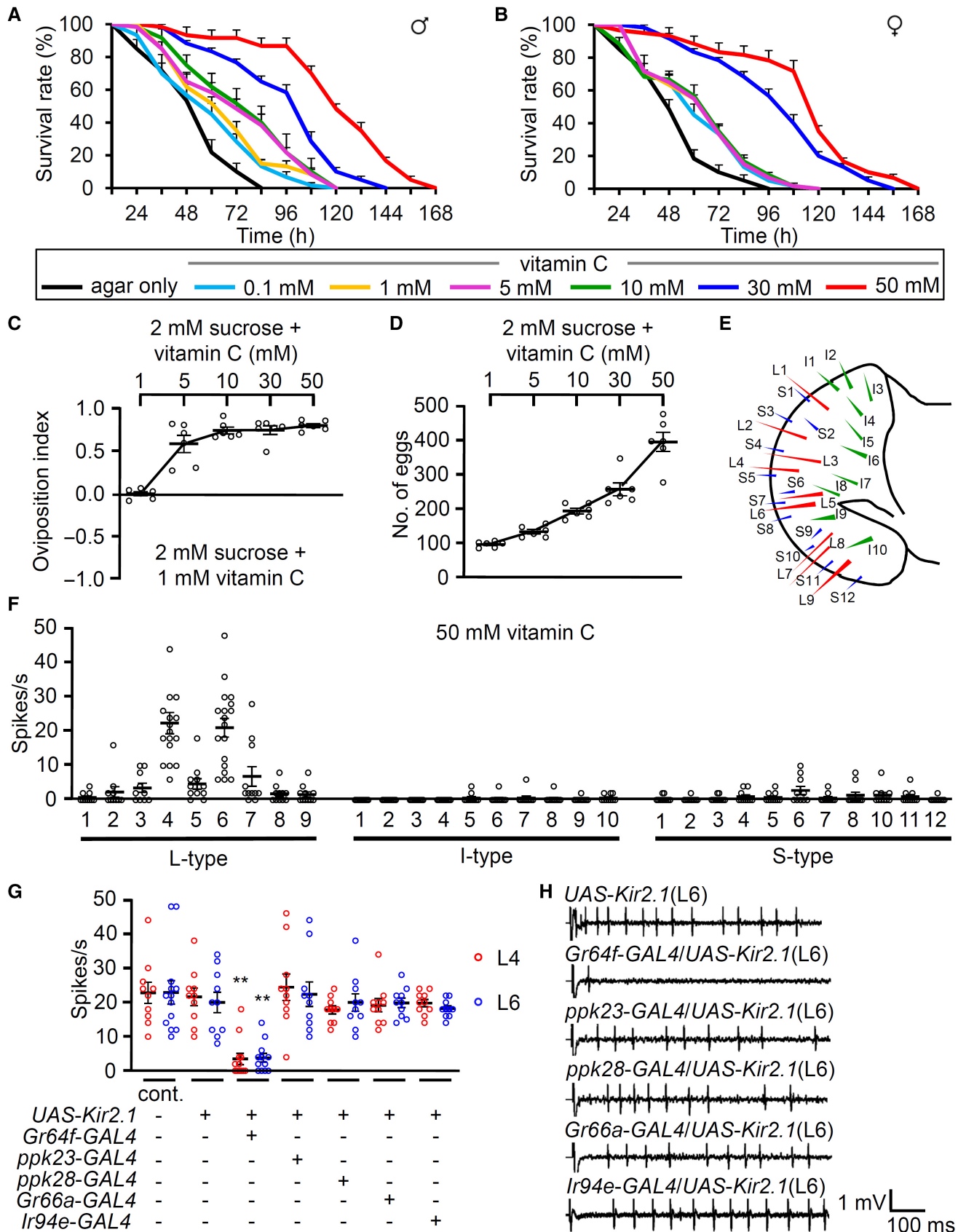
Figure 1.

Figure 1. Beneficial effect of vitamin C and neuronal response of sweet-sensing GRNs to vitamin C.

A, B Time-dependent effects of ingesting 0, 0.1, 1, 5, 10, 30, and 50 mM vitamin C without any further nutrition on the survival of wild-type control flies (w^{1118}) (A) males (B) females, n = 6. Kaplan–Meier survival analyses were performed to compare the survival rates of agar only and fed conditions at different concentrations of vitamin C (Appendix Table S1).

C Dose-dependent oviposition choice at indicated concentrations of vitamin C versus 1 mM vitamin C. Sucrose (2 mM) was included on both sides, n = 6.

D Number of eggs laid at the indicated concentrations of vitamin C with 2 mM sucrose, n = 6.

E Schematic illustration of taste sensilla on the fly labellum.

F Electrophysiology mapping of 31 sensilla with control to 50 mM vitamin C, n = 10–18.

G Tip recordings from the L4 and L6 sensilla of specific GRN-ablated flies with 50 mM vitamin C. +/− indicates the presence or absence of the transgene, respectively, n = 10–13.

H Representative sample traces of (G).

Data information: All error bars represent SEMs. Single-factor ANOVA coupled with the Scheffe's *post hoc* test was conducted to compare multiple datasets. Asterisks indicate statistical significance compared with the control. **P < 0.01.

Source data are available online for this figure.

Gr mutants (Fig 2B). From these putative sweet-sensing GRs, we identified that *Gr5a*, *Gr61a*, *Gr64b*, *Gr64c*, and *Gr64e* mutants had significantly reduced spike activities to vitamin C (Fig 2B). Then, we performed recovery experiments using its own *GAL4* drivers and sweet-sensing GRNs-expressing *Gr64f-GAL4* (Fig 2C). When each wild-type cDNA was expressed in the mutant background, the *Ir25a* and *Ir76b* mutants were fully rescued (Fig 2C). This indicates that IR25a and IR76b act in *Gr64f*-positive neurons. We also genetically recovered each *Gr* mutant by expressing each wild-type cDNA with its specific *Gr5a-GAL4* and *Gr61a-GAL4* or the broadly expressed sweet-sensing *Gr64f-GAL4* driver for each *Gr64* cluster mutant (Fig 2D). These data support the evidence that two coreceptor IRs, IR25a and IR76b, and the five sweet GRs are essential for detecting vitamin C in sweet-sensing GRNs. As previously reported (Stanley *et al*, 2021), it would be interesting to test whether two coreceptor IRs are required for the onset response by vitamin C stimulation and whether the other GRs are essential for the removal phase by calcium imaging. *Ir25a*, *Ir76b*, and the other *Gr*s are known to be expressed not only in labellum but also in the internal taste organ, pharynx (Chen & Dahanukar, 2017; Sánchez-Alcañiz *et al*, 2018). Furthermore, vitamin B preference is mediated by external and internal taste organs (Wu *et al*, 2021). It would be interesting to test the vitamin C response in the pharynx as well.

GR5a, GR61a, GR64b, GR64c, and GR64e are required in combination with IR25a and IR76b to detect vitamin C

To address how taste receptor activities are translated into behavioral responses, we adopted two different behavioral paradigms. First, we used the well-established binary food choice assay (Aryal *et al*, 2022b). Vitamin C physiologically activated sweet-sensing GRNs (Fig 1G and H); therefore, no sucrose was employed in the assays. This was verified by a preference of 50 mM vitamin C versus 1 mM in wild-type flies, while *Gr64f-GAL4/UAS-Kir2.1* flies had no preference (Fig EV2F). We screened the 31 *Ir* mutants with the same paradigm and found that only *Ir25a* and *Ir76b* were required for vitamin C attraction (Fig 3A). Next, we tested the nine sweet *Gr* mutants. Consistent with the electrophysiology results, *Gr5a*, *Gr61a*, *Gr64b*, *Gr64c*, and *Gr64e* mutant flies, but not *Gr43a*, *Gr64a*, *Gr64d*, and *Gr64f* mutants, were deficient in their attraction to vitamin C (Fig 3B). Rescue experiments revealed the expression of each wild-type cDNA under the control of its *GAL4* or the *Gr64f* promoter increased the preference index to the level of wild-type flies (Fig 3C

and D). This indicates that IR25a and IR76b function in the sweet-sensing GRNs with GR5a, GR61a, GR64b, GR64c, and GR64e when being attracted to vitamin C.

We employed a second behavioral paradigm to further investigate the role of the seven receptors (IR25a, IR76b, GR5a, GR61a, GR64b, GR64c, and GR64e) in the behavioral response to vitamin C. We used the oviposition assay, in which the flies were allowed to freely fly and walk in the arena. It is a complex process to determine how organisms integrate signals and are involved in decision-like processes and balances conflicting behavioral activities (Joseph *et al*, 2009). We used this paradigm to replicate the behavior of flies in a natural environment. First, 2 mM sucrose was included in the agarose on both sides to induce more egg-laying. Second, 1 mM versus 50 mM vitamin C was mixed on each side without any visible dyes. We counted the number of eggs over an 18 h period after the flies were adapted to the arena for 5–6 h. We found that sweet-GRNs-inhibited flies (*Gr64f-GAL4/UAS-Kir2.1*), but not others, had defects selecting the egg deposition site (Fig EV3A and B). Furthermore, the seven receptor mutants showed comparable defects to the sweet-GRNs-inhibited flies; these defects were completely recovered in the rescue experiments (Fig EV3C–E). Overall, the results of binary food choice feeding assays and oviposition assays were consistent. Flies preferred vitamin C, which is mediated by two broadly expressed IRs and five sweet GRs.

The oviposition behavior for attractive acids is mainly driven by tarsal taste neurons and requires IR25a and IR76b (Chen & Amrein, 2017). However, vitamin C, one of the attractive acids, functioned in the labellum when PER assays were performed (Fig EV2C). Therefore, we tested organ-ablated flies in oviposition (Fig EV4A–C). First, we found that oviposition preference to carboxylic acids (1 and 10% lactic acid, citric acid, and glycolic acid) is mediated by both forelegs and the labellum but not the antennae (Fig EV4A and B). Second, the flies that were ablated both organs (forelegs and labellum) have an additive effect on average but not significantly different from flies with only one of the organs ablated. Third, these results were consistent when vitamin C was tested (Fig EV4C). Therefore, we conclude that two distinct types of sweet-sensing GRNs in the labellum and forelegs drive egg-laying preference behavior.

We raised a question as to whether the increase in the number of eggs laid on the vitamin C in a dose-dependent manner (Fig 1D) can be seen in the mutants. Regulating the number of eggs laid is generally controlled by the physiological effect of the ingested

nutrients. Unlike most cases, the seven mutants, as well as sweet-GRNs-inhibited flies, laid a significantly lower number of eggs (Fig EV4D). Instead of attributing the reduction of egg numbers to the sensing of vitamin C, however, we expect that vitamin-insensitive mutants feed on less amount of vitamin C, which results in suppressed egg-laying.

Though the attraction to vitamin C was significant, flies showed only a slight attraction or stayed neutral to vitamin B2 and B9 in our binary food choice and oviposition assays (Fig EV5A and B). Taken together with behavioral assays and tip recordings with vitamin C, B2, and B9, we conclude that vitamin C can attract flies more than vitamin B2 and B9.
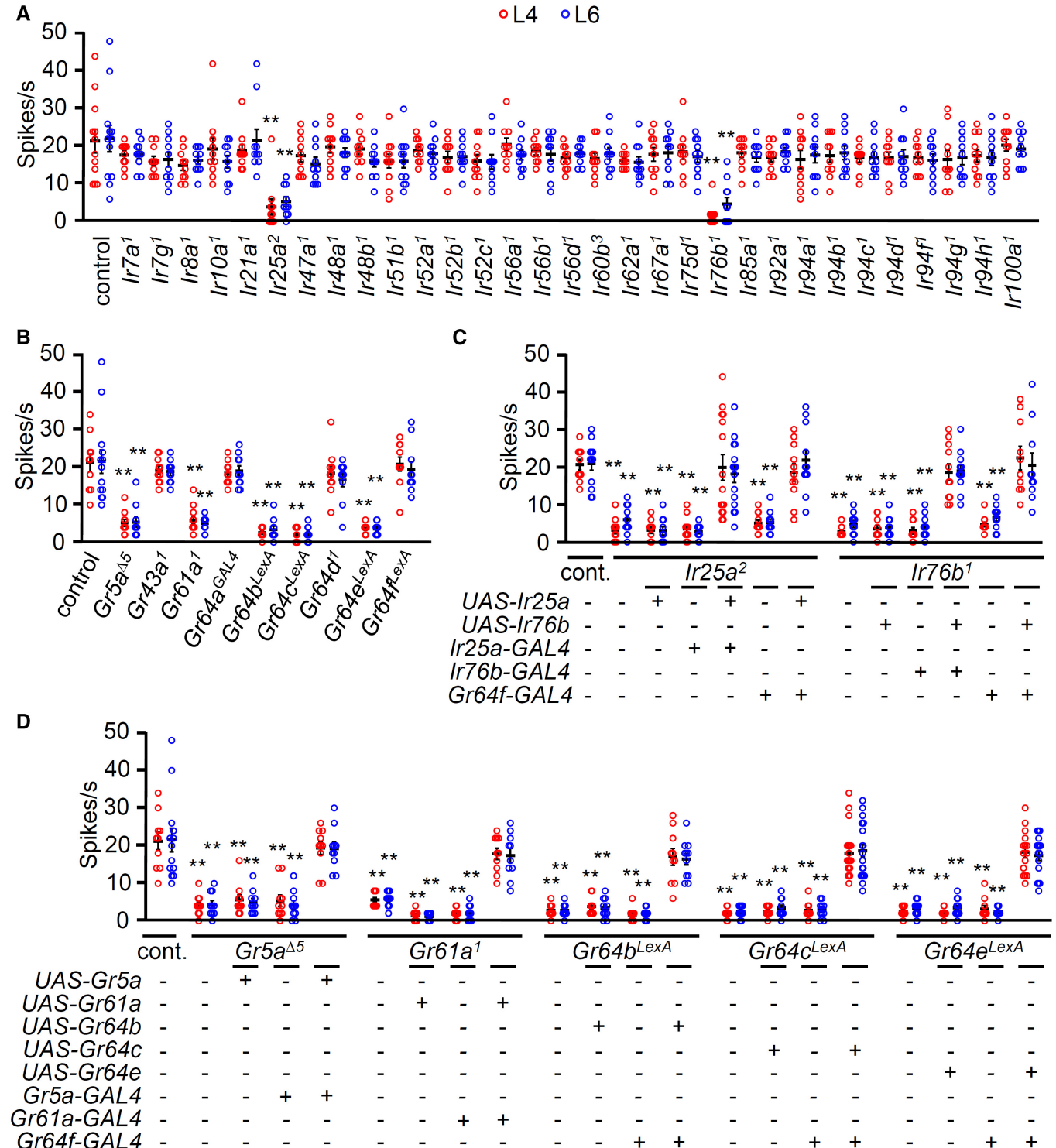

Figure 2.

Electrophysiological response profiles for candidate taste receptors to attractive tastants

Electrophysiological phenotyping of candidate *Ir* and *Gr* mutants, in combination with the use of its genetic rescues, have been conclusive in the identification of ligands and their receptors. Previous studies have sought to characterize these sweet taste receptor candidates by the combinational expression of two *Grs* in the *Gr64* cluster mutants or ectopic expression of one *Gr* in the empty olfactory neurons using the *GAL4/UAS* system (Jiao *et al*, 2007, 2008; Freeman *et al*, 2014; Kim *et al*, 2018). The recent discovery of six individual mutants of the *Gr64* cluster in addition to the other mutants allowed us to verify attractive tastant responses. Several studies have uncovered the functions of each sweet *Gr* gene (Jiao *et al*, 2007, 2008; Freeman *et al*, 2014; Fujii *et al*, 2015; Kim *et al*, 2018; Aryal *et al*, 2022a). However, some functions are controversial because they have used different mutants that may have affected other cluster genes. Fujii *et al* (2015) have generated many reagents to delete each of the six *Gr64* cluster genes to study the expression and behavioral analysis of each gene. However, these mutants were not evaluated at the level of single sensillum recordings. Therefore, we evaluated each *Gr64* mutant ($Gr64a^{GAL4}$, $Gr64b^{LexA}$, $Gr64c^{LexA}$, $Gr64d^1$, $Gr64e^{LexA}$, $Gr64f^{LexA}$) and other potential candidates, such as $Gr5a^{A5}$, $Gr61a^1$, $Ir25a^2$, and $Ir76b^1$, with the newly generated $Gr43a^1$ to delete *Gr43a* using homologous recombination via electrophysiology (Fig 4A and Appendix Fig S1). First, we tested four sugars. Using electrophysiological recordings from the L4 sensilla, we found a significant reduction in spike activity to sucrose in all six *Gr64* mutants. The *Gr64* gene cluster has a complex transcription unit that has a polycistronic transcription (Dahanukar *et al*, 2007; Jiao *et al*, 2007; Slone *et al*, 2007). Therefore, we also recovered all defects using rescue flies, using wild-type cDNA (red asterisks). The significance of this study is the following. Firstly, the functions of *Gr64b, Gr64c, Gr64d,* and *Gr64e* to sense sucrose were first identified in this study (Fig 4A and Appendix Fig S2A). The role of *Gr64a* and *Gr64f* to sense sucrose has been previously identified (Jiao

et al, 2007, 2008; Freeman *et al*, 2014). We also find conflicting evidence regarding the function of *Gr5a* in sucrose sensation. In previous research, *Gr64b* and *Gr5a* have been presented as sucrose sensors via PER assays (Fujii *et al*, 2015). However, we could not find any defect in *Gr5a* mutant testing sucrose. Second, we found a normal maltose response using $Gr64a^{GAL4}$ and $Gr64b^{LexA}$. However, the other four mutants from *Gr64c* to *Gr64f* had a significant reduction in neuronal activity (Fig 4A and Appendix Fig S2B). The mutant defects were fully rescued, except *Gr64e*. The rescue of *Gr64e* was significantly increased when compared with the mutant, but it did not reach the level of the control (black asterisk indicates a significant difference versus control). The roles of *Gr64c* and *Gr64d* in maltose detection were first identified here. However, we could not observe any reduced action potential from *Gr5a* and *Gr64a* mutants as compared to their previous role as maltose sensors (Jiao *et al*, 2007; Freeman *et al*, 2014; Fujii *et al*, 2015). Third, *Gr5a* with most *Gr64* cluster mutants except $Gr64c^{LexA}$, showed a significant reduction in glucose responses (Fig 4A and Appendix Fig S2C). This aligns with previous findings that *Gr5a* with *Gr64a* and *Gr64f* are glucose sensors (Jiao *et al*, 2007, 2008). Interestingly, $Gr61a^1$ was normal in this study, contrary to the previous finding that *Gr61a* has defects in its electrophysiology (Freeman *et al*, 2014). There was only a partial rescue of each mutant, including *Gr64a, Gr64b, Gr64d,* and *Gr64e* (Fig 4A, black asterisks). Nevertheless, we propose that *Gr64b, Gr64d,* and *Gr64e* are required for sensing glucose. Fourth, the internal fructose sensor, *Gr43a*, was not required for sensing fructose in the labellum. The fructose response profile was quite similar to glucose, except *Gr5a*. We confirmed the requirement of *Gr64a* to detect fructose (Freeman *et al*, 2014). We further identified that *Gr64b, Gr64d, Gr64e,* and *Gr64f* were essential for fructose-induced neuronal spikes (Fig 4A and Appendix Fig S2D). Therefore, we propose that *Gr64d, Gr64e,* and *Gr64f* are more commonly required for detecting sugars (sucrose, maltose, glucose, and fructose), whereas *Gr64a* and *Gr64b* are relatively specific to sucrose, glucose, and fructose (Fig 4B). Furthermore, *Gr64c* is specific to sucrose and maltose, whereas *Gr5a* is only specific to glucose

A

B

C

	cont.		*Ir25a²*					*Ir76b¹*				
UAS-Ir25a	-	-	+	-	+	-	+	-	-	-	-	-
UAS-Ir76b	-	-	-	-	-	-	-	-	+	-	+	-	+
Ir25a-GAL4	-	-	-	+	+	-	-	-	-	-	-	-
Ir76b-GAL4	-	-	-	-	-	-	-	-	+	+	-	-
Gr64f-GAL4	-	-	-	-	-	+	+	-	-	-	+	+

D

	cont.		*Gr5aΔ5*			*Gr61a¹*			*Gr64bLexA*			*Gr64cLexA*			*Gr64eLexA*		
UAS-Gr5a	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
UAS-Gr61a	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
UAS-Gr64b	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
UAS-Gr64c	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
UAS-Gr64e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
Gr5a-GAL4	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Gr61a-GAL4	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Gr64f-GAL4	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	+	+

Figure 3.

A

B

C

Figure 4.

◀

(Fig 4B). Interestingly, *Gr43a, Gr61a, Ir25a,* and *Ir76b* were dispensable to detect four sugars. For sugar detection, sweet GR members, but not IRs, are solely required.

Next, we tested the carboxylic acid responses (Stanley *et al*, 2021; Shrestha & Lee, 2021a). First, we confirmed that *Ir25a, Ir76b, Gr5a,* and *Gr61a* were similarly required for sensing lactic, citric, and glycolic acid (Fig 4A, and Appendix Fig S2E–G). The deficits were completely recovered by the wild-type cDNA expression of each gene. Although the role of *Gr64* cluster deletion mutant was identified (Stanley *et al*, 2021; Shrestha & Lee, 2021a), each *Gr64* cluster mutant has not been tested. Therefore, we further identified that *Gr64c, Gr64d,* and *Gr64e* were essential for the neuronal responses to lactic acid and citric acid (Fig 4A, and Appendix Fig S2E and F). However, *Gr64e* was dispensable in detecting glycolic acid (Fig 4A and Appendix Fig S2G). *Gr64a, Gr64b,* and *Gr64f* were not required for sensing any carboxylic acids. These results support the previous findings that both GRs and IRs are essential for detecting carboxylic acids as common sensors (Fig 4B). Although we did not reconfirm the separate roles of IRs and sweet GRs as stimulus onset (ON response) and removal phase (OFF response) sensors when the signals were conveyed to the brain (Stanley *et al*, 2021), we expanded the findings on carboxylic acids by identifying the individual GR64 cluster genes. Therefore, it will be interesting to test that newly identified each GR mutant is responsible for the OFF response by the way of brain calcium imaging. Overall, our results show that carboxylic acids differentially affect ligand-receptor interactions. *Gr5a, Gr61a, Gr64c, Gr64d, Ir25a,* and *Ir76b* were common sensors for the tested carboxylic acids, while *Gr64e* was specifically required for detecting lactic acid and citric acid (Fig 4B).

Glycerol is the backbone of triglyceride and nutritious. *Gr64e* is a glycerol sensor (Freeman *et al*, 2014; Kim *et al*, 2018). Additionally, *Gr64a, Gr64c,* and *Gr64d* are also identified as glycerol sensors in ectopic experiments (Freeman *et al*, 2014). *Gr64a, Gr64b,* and *Gr64c* with *Gr5a* have behavioral defects that attract glycerol (Fujii *et al*, 2015). Our electrophysiology revealed that all six *Gr64* mutants had significantly reduced spikes (Fig 4A and Appendix Fig S2H). The role of *Gr64e* and *Gr64f* were first identified in this study. Furthermore, *Gr43a,* but not *Gr5a,* was indispensable in detecting glycerol in the L7 sensilla. The rescued flies of *Gr64c* and *Gr64d* only partially recovered their physiological defects (Fig 4A and Appendix Fig S2H). The roles of *Ir25a* and *Ir76b* were not observed in the sensation of glycerol. This indicated that only sweet GRs except *Gr5a* and *Gr61a,* but not IRs, functioned in glycerol sensation. On comparing the neuronal responses to glycerol and vitamin C, the common sensors were *Gr64b, Gr64c,* and *Gr64e* (Fig 4B).

Gr5a, Gr61a, Ir25a, and *Ir76b* were specifically required for sensing vitamin C, while *Gr43a, Gr64a, Gr64d,* and *Gr64f* were specific to glycerol.

Overall, we propose that GR64c was a common receptor for detecting carboxylic acid, glycerol, and vitamin C (Fig 4C). GR64e was common for detecting sugars, glycerol, and vitamin C, while GR64d was a sole common receptor for sensing sugars and carboxylic acids (Fig 4C). There are multiple complex arrangements for forming heteromultimeric ion channels; therefore, we proposed a model in which distinct common GRs have category-dependent contributions to attractive taste depending on different categories (Fig 4C and Appendix Fig S2E, F, G and I). Furthermore, the requirement of IR25a and IR76b in combination with GRs to sense carboxylic acids and vitamin C should be further studied because it remains unclear how this complex physically works or how the intracellular signaling mechanisms of IRs and GRs converge. It would be interesting to study whether the gating or conformation of GRs would change with supplements of acidic compounds like carboxylic acids and vitamin C leading to additional ion flux. These can be enthralling mechanisms to be studied to link the relationship of GRs and IRs in future. Nevertheless, in this study, we showed how flies taste vitamin C.

Materials and Methods

Drosophila strains

In this investigation, the strain w^{1118} was employed as a control strain. All flies were kept at a constant temperature of 25°C and 50–60% humidified incubator with a 12-h light/12-h dark cycle. Male and female flies were utilized in the studies at random basis. The following lines were obtained from the Bloomington *Drosophila* Stock Center (https://bdsc.indiana.edu): *Ir7g¹* (BL42420), *Ir8a¹* (BL41744), *Ir10a¹* (BL23842), *Ir21a¹* (BL10975), *Ir48a¹* (BL26453), *Ir48b¹* (BL23473), *Ir51b¹* (BL10046), *Ir52b¹* (BL25212), *Ir52c¹* (BL24580), *Ir56b¹* (BL27818), *Ir56d¹* (BL81249), *Ir62a¹* (BL32713), *Ir67a¹* (BL56583), *Ir75d¹* (BL24205), *Ir85a¹* (BL24590), *Ir92a¹* (BL23638), *Ir94b¹* (BL23424), *Ir94d¹* (BL33132), *Ir94f¹* (BL33095), *Ir94g¹* (BL25551), *Ir100a¹* (BL31853), *Ir94e-GAL4* (BL81246), and *UAS-Kir2.1* (BL6596). The source of following lines was described earlier: *Ir7a¹, Ir47a¹, Ir52a¹, Ir56a¹, Ir60b³, Ir94a¹, Ir94c¹, Ir94h¹* (Rimal *et al*, 2019), *UAS-Ir25a* (Lee *et al*, 2018). C. Montell kindly provided strains *Ir76b¹, Ir76b-GAL4, UAS-Ir76b* (Zhang *et al*, 2013), *UAS-Gr64a, UAS-Gr64b* (Jiao *et al*, 2007), *UAS-Gr64c, UAS-Gr64d, UAS-Gr64f* (Jiao *et al*, 2008), and *UAS-Gr64e* (N/A). Further, J.

Carlson generously provided strains $Gr5a^{\Delta 5}$, $Gr5a^{\Delta 5}/FM7$;UAS-$Gr5a/$
CyO, $Gr5a^{\Delta 5}/FM7$;$Gr5a$-$GAL4/CyO$, $Gr61a^1$, $Gr61a$-$GAL4$, and
$Gr61a^1$;UAS-$Gr61a$ (Dahanukar *et al*, 2007). K. Scott provided
strains *ppk23-GAL4* (Thistle *et al*, 2012) and *ppk28-GAL4* (Cameron
et al, 2010). *Gr66a-GAL4*, *Gr5a-GAL4*, *UAS-Gr43a* (Thorne
et al, 2004), $Gr64a^{GAL4}$, $Gr64b^{LEXA}$, $Gr64c^{LEXA}$, $Gr64e^{LEXA}$, $Gr64f^{LEXA}$
(Fujii *et al*, 2015), *UAS-Gr64b*, *UAS-Gr64c*, and *UAS-Gr64e* were pro-
vided by H. Amrein. $Gr64d^1$ (Uchizono *et al*, 2017) was provided by
Dr. S.J. Moon. *Gr64f-GAL4* (Dahanukar *et al*, 2007) were obtained
from A. Dahanukar and L. Vosshall provided *Ir25a-GAL4* and $Ir25a^2$
(Benton *et al*, 2009) strains. *Gr43a-GAL4* (KDRC2785) was obtained
from Korea *Drosophila* Resource Center.

Chemical reagents

Vitamin C (L-ascorbic acid; CAS No 50-81-7), riboflavin (CAS No
83-88-5), folic acid (CAS No 59-30-3), tricholine citrate (CAS No
546-63-4), sucrose (CAS No 57-50-1), fructose (CAS No 57-48-7),
glucose (CAS No 50-99-7), maltose (CAS No 6363-53-7), glycerol
(CAS No 56-81-5), curcumin (CAS No 458-37-7), sulforhodamine B
(CAS No 3520-42-1), glycolic acid (CAS No 79-14-1), citric acid
(CAS No 77-92-9), and lactic acid (CAS No 50-21-5) were purchased
from Sigma-Aldrich Co. D(+)-Galactose (CAS No 59-23-4) was pur-
chased from Daejung chemicals & metals Ltd (Korea). Brilliant blue
FCF (CAS No 3844-45-9, Cat No 027-12842) was purchased from
Wako Pure Chemical Industry Ltd (Japan).

Generation of $Gr43a^1$ mutant lines

The $Gr43a^1$ mutation was generated by ends-out homologous
recombination (Gong & Golic, 2003). To create the construct for
injections, we amplified two 3-kb genomic fragments by PCR (NotI
and BglII were used) and subcloned the DNA into the pw35 vector.
Using the "A" of the "ATG" start codon as +1, the deleted region
was +711 to +1,666 (955 bp). The construct was injected into w^{1118}
embryos by BestGene Inc. We outcrossed the mutant with w^{1118} for
six generations.

Binary food choice assay

As in a prior study, binary food choice assays were performed
(Aryal *et al*, 2022b). In a humidified chamber, 50–70 flies (3–6 days
old; mixed sexes) were starved for 18 h. Next, two distinct food
sources containing 1% agarose were prepared. The first food source
had 1 mM vitamin C, while the second had higher concentrations of
vitamin C, as indicated in the Figures. These foods were combined
with either blue (brilliant blue FCF, 0.125 mg/ml) or red (sulforho-
damine B, 0.1 mg/ml) food coloring. In a 72-well microtiter dish
(Thermo Fisher Scientific, cat. no. 438733), the two-food mixtures
were placed in alternate wells. Within 30 min of food preparation,
approximately 50–70 starved flies were transferred to the plate. The
flies were allowed to feed for 90 min at room temperature by incu-
bating the dishes in a dark, humidified container. The tested flies
were frozen at −20°C. A stereomicroscope was used to examine the
color of their abdomens. Blue (N_B), red (N_R), and purple (N_P) flies
were tabulated based on ocular assessment. Based on the dye/
tastant combinations, the preference index (PI) was determined
using the following formula: $(N_B - N_R)/(N_R + N_B + N_P)$ or

$(N_R - N_B)/(N_R + N_B + N_P)$. PI values of 1.0 or −1.0 indicated that
the flies had a strong preference for one of the two food options. A
PI of 0.0 suggested that the flies had no bias.

Tip recording assay

Electrophysiology (i.e., tip recording assay) was performed as previ-
ously described (Lee *et al*, 2009). Flies (4–7-day-old males and
females) were anesthetized on ice. In the thorax of the flies, a refer-
ence glass electrode filled with Ringer's solution was introduced.
The electrode was then gradually extended towards the fly's probos-
cis. Adults that were 5–6 days old were prepared per cycle to avoid
any experimental biases. The same method was performed for
numerous rounds on different days. The sensilla were stimulated for
5 s in recording pipettes (10–20 mm tip diameter) attached to a pre-
amplifier with a mixture of chemical stimulants in a 30 mM tricho-
line citrate (TCC) solution (i.e., electrolyte solution). Using a
Syntech signal connection interface box and a 100–3,000 Hz band-
pass filter, the recorded signals were collected and amplified by 10×.
Action potential recordings were made at a sampling rate of 12 kHz
and analyzed with Autospike 3.1 (Syntech). All recordings were
performed at 1-min intervals to ensure appropriate signals. The
number of insects tested is shown by the dots in each figure.

Oviposition preference assay

We performed oviposition preference experiments as described ear-
lier (Shrestha & Lee, 2021b). Thirty to 40 flies (mixed sexes) aged 5–
7 days were acclimated in 1% agarose for 5–6 h in an egg-laying
chamber (Code No. FEC-50200, Hansol Tech, Korea). Following the
adaption process, the food supply was shifted to a 35 mm diameter
petri dish separated into two parts: one with 2 mM sucrose and
1 mM vitamin C, while the other side contained 2 mM sucrose with
the indicated concentrations of vitamin C (1–50 mM). This allowed
female flies to choose where they want to lay their eggs. The num-
bers of eggs laid on each portion were counted after 18-h incuba-
tion. Only the assays in which flies laid more than 50 eggs were
considered when calculating the oviposition index. The oviposition
index was determined as [{(number of eggs on choice A
side) − (number of eggs on choice B side)} ÷ (total number of eggs
laid)].

Egg count assay

For this assay, 10 females were permitted to lay eggs on media in an
egg-laying chamber overnight. After 18 h, the flies were transferred
to new vials and the number of eggs was counted immediately. The
indicated concentrations of vitamin C (1, 5, 10, 30, and 50 mM) in
1% agarose and 2 mM sucrose were tested. The numbers of eggs
are presented as dots in Fig 1D. The experiment was repeated six
times.

Proboscis extension response assay

The proboscis extension response (PER) experiment was performed
as described previously (Poudel *et al*, 2015). Twenty to 25 flies (3–
7-day-old males and females) were starved for 18–20 h in vials
containing 1% agarose. After anesthetizing briefly on ice, the flies

were trapped in a 20–200 μl pipette tip. The pipette tip's edge was chopped using a paper cutter blade to reveal the head. The head and proboscis were protruded outside the pipette tip to provide the stimuli to the proboscis. To stimulate the fly's tarsi, the head/proboscis and forelegs were pushed outside of the pipette tip without injuring any body parts. Water was first fed to remove any kind of thirstiness-based biases. Two percent sucrose alone was used for both the positive control and initial stimulation. The tastant stimuli were presented using Kimwipe paper as the medium. The tarsi or proboscis were exposed to wet wicks containing either 2% sucrose or 50 mM vitamin C. The flies were not included in the experiment if they did not react to the sucrose during the first exposure. The same circumstances as the initial exposures were used for the second exposure. For each test round, > 10 flies were used. The experiment was repeated six times.

Starvation resistance assay

Fly starvation resistance assays were conducted as previously described (Lee *et al*, 2018). The assays were performed with different concentrations of vitamin C only food (0, 0.1, 1, 5, 10, 30, and 50 mM), curcumin only food (0.1 and 0.5%), and galactose only food (10, 30, and 50 mM) without any further nutrition. Control food was 1% agar alone. Briefly, 3–5-day-old 10 male and 10 female flies were allowed to feed on the above-described food sources. The flies were observed every 12 h and then transferred to new vials containing the same food source. The survivability of flies was assessed up to 156 h, then the LT_{50} was calculated.

Quantification and statistical analyses

Data were analyzed using GraphPad Prism version 8.0 (RRID: SCR 002798). All trials were repeated on separate days. The number of trials for each experiment is represented by dots in the graphs. The standard error of the mean (SEM) is represented by error bars. Using single-factor ANOVA and Scheffe's *post hoc* analysis, multiple datasets were compared. Origin (Origin Lab Corporation, RRID: SCR 002815) was used for all statistical analyses. In the figures, asterisks denote statistical significance (*$P < 0.05$, **$P < 0.01$).

Data availability

The datasets produced in this study are available in the following database: Source data—BioStudies S-BSST991 (https://www.ebi.ac.uk/biostudies/studies/S-BSST991). Any additional information required to analyze the data reported in this paper is available from the lead contact upon request.

Expanded View for this article is available online.

Acknowledgements

Author contributions
Bhanu Shrestha: Conceptualization; formal analysis; validation; investigation; visualization; methodology; writing – original draft. **Binod Aryal:** Conceptualization; investigation; methodology. **Youngseok Lee:** Conceptualization; supervision; funding acquisition; writing – original draft; project administration; writing – review and editing.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

References

Ahn J-E, Chen Y, Amrein H (2017) Molecular basis of fatty acid taste in *Drosophila*. *Elife* 6: e30115

Aryal B, Dhakal S, Shrestha B, Lee Y (2022a) Molecular and neuronal mechanisms for amino acid taste perception in the *Drosophila* labellum. *Curr Biol* 32: 1376–1386

Aryal B, Dhakal S, Shrestha B, Sang J, Pradhan RN, Lee Y (2022b) Protocol for binary food choice assays using *Drosophila melanogaster*. *STAR protoc* 3: 101410

Benton R, Vannice KS, Gomez-Diaz C, Vosshall LB (2009) Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* 136: 149–162

Cameron P, Hiroi M, Ngai J, Scott K (2010) The molecular basis for water taste in *Drosophila*. *Nature* 465: 91–95

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

Chen Y-CD, Dahanukar A (2017) Molecular and cellular organization of taste neurons in adult *Drosophila* pharynx. *Cell Rep* 21: 2978–2991

Chen Y-CD, Dahanukar A (2020) Recent advances in the genetic basis of taste detection in *Drosophila*. *Cell Mol Life Sci* 77: 1087–1101

Dahanukar A, Lei Y-T, Kwon JY, Carlson JR (2007) Two gr genes underlie sugar reception in *Drosophila*. *Neuron* 56: 503–516

Delompré T, Guichard E, Briand L, Salles C (2019) Taste perception of nutrients found in nutritional supplements: a review. *Nutrients* 11: 2050

Dhakal S, Sang J, Aryal B, Lee Y (2021) Ionotropic receptors mediate nitrogenous waste avoidance in *Drosophila melanogaster*. *Commun Biol* 4: 1–10

Dweck HK, Talross GJ, Luo Y, Ebrahim SA, Carlson JR (2022) Ir56b is an atypical ionotropic receptor that underlies appetitive salt response in *Drosophila*. *Curr Biol* 32: 1776–1787

Freeman EG, Dahanukar A (2015) Molecular neurobiology of *Drosophila* taste. *Curr Opin Neurobiol* 34: 140–148

Freeman EG, Wisotsky Z, Dahanukar A (2014) Detection of sweet tastants by a conserved group of insect gustatory receptors. *Proc Natl Acad Sci USA* 111: 1598–1603

Fujii S, Yavuz A, Slone J, Jagge C, Song X, Amrein H (2015) *Drosophila* sugar receptors in sweet taste perception, olfaction, and internal nutrient sensing. *Curr Biol* 25: 621–627

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

Gong WJ, Golic KG (2003) Ends-out, or replacement, gene targeting in *Drosophila*. *Proc Natl Acad Sci USA* 100: 2556–2561

González E, Cruces MP, Pimentel E, Sánchez P (2018) Evidence that the radioprotector effect of ascorbic acid depends on the radiation dose rate. *Environ Toxicol Pharmacol* 62: 210–214

Huang AL, Chen X, Hoon MA, Chandrashekar J, Guo W, Tränkner D, Ryba NJ, Zuker CS (2006) The cells and logic for mammalian sour taste detection. *Nature* 442: 934–938

Jaeger AH, Stanley M, Weiss ZF, Musso P-Y, Chan RC, Zhang H, Feldman-Kiss D, Gordon MD (2018) A complex peripheral code for salt taste in *Drosophila. Elife* 7: e37167

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

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

Joseph RM, Devineni AV, King IF, Heberlein U (2009) Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in *Drosophila. Proc Natl Acad Sci USA* 106: 11352–11357

Kim H, Kim H, Kwon JY, Seo JT, Shin DM, Moon SJ (2018) *Drosophila* Gr64e mediates fatty acid sensing via the phospholipase C pathway. *PLoS Genet* 14: e1007229

Lee Y, Moon SJ, Montell C (2009) Multiple gustatory receptors required for the caffeine response in *Drosophila. Proc Natl Acad Sci USA* 106: 4495–4500

Lee Y, Poudel S, Kim Y, Thakur D, Montell C (2018) Calcium taste avoidance in *Drosophila. Neuron* 97: 67–74

Liman ER, Zhang YV, Montell C (2014) Peripheral coding of taste. *Neuron* 81: 984–1000

Man Anh H, Linh DM, My Dung V, Thi Phuong Thao D (2019) Evaluating dose-and time-dependent effects of vitamin c treatment on a parkinson's disease fly model. *Parkinsons Dis* 2019: 9720546

Moon SJ, Köttgen M, Jiao Y, Xu H, Montell C (2006) A taste receptor required for the caffeine response in vivo. *Curr Biol* 16: 1812–1817

National Toxicology Program, Institute of Environmental Health Sciences, National Institutes of Health (NTP) (1992) *National toxicology program chemical repository database.* Research Triangle Park, NC: U.S. Department of Health and Human Services

Paradis S, Sweeney ST, Davis GW (2001) Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. *Neuron* 30: 737–749

Poljsak B, Ionescu JG (2009) Pro-oxidant vs. antioxidant effects of vitamin C. In *Handbook of Vitamin C Research: Daily Requirements, Dietary Sources and Adverse Effects*, Kucharski H, Zajac J (eds), pp 153–183. New York, NY: Nova Science Publishers, Inc

Poudel S, Kim Y, Kim YT, Lee Y (2015) Gustatory receptors required for sensing umbelliferone in *Drosophila melanogaster. Insect Biochem Mol Biol* 66: 110–118

Rimal S, Lee Y (2018) The multidimensional ionotropic receptors of *Drosophila melanogaster. Insect Mol Biol* 27: 1–7

Rimal S, Sang J, Poudel S, Thakur D, Montell C, Lee Y (2019) Mechanism of acetic acid gustatory repulsion in *Drosophila. Cell Rep* 26: 1432–1442

Sánchez-Alcañiz JA, Silbering AF, Croset V, Zappia G, Sivasubramaniam AK, Abuin L, Sahai SY, Münch D, Steck K, Auer TO *et al* (2018) An expression atlas of variant ionotropic glutamate receptors identifies a molecular basis of carbonation sensing. *Nat Commun* 9: 1–14

Schiffman SS, Dackis C (1975) Taste of nutrients: amino acids, vitamins, and fatty acids. *Percept Psychophys* 17: 140–146

Shrestha B, Lee Y (2021a) Mechanisms of carboxylic acid attraction in *Drosophila melanogaster. Mol Cells* 44: 900–910

Shrestha B, Lee Y (2021b) Mechanisms of DEET gustation in *Drosophila. Insect Biochem Mol Biol* 131: 103550

Shrestha B, Lee Y (2023) Molecular sensors in the taste system of *Drosophila. Genes Genomics* 1–15 https://doi.org/10.1007/s13258-023-01370-0

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

Stanley M, Ghosh B, Weiss ZF, Christiaanse J, Gordon MD (2021) Mechanisms of lactic acid gustatory attraction in *Drosophila. Curr Biol* 31: 3525–3537

Suckow BK, Suckow MA (2006) Lifespan extension by the antioxidant curcumin in *Drosophila melanogaster. Int J Biomed Sci* 2: 402–405

Suh HJ, Shin B, Han S-H, Woo MJ, Hong K-B (2017) Behavioral changes and survival in *Drosophila melanogaster*: effects of ascorbic acid, taurine, and caffeine. *Biol Pharm Bull* 40: 1873–1882

Thistle R, Cameron P, Ghorayshi A, Dennison L, Scott K (2012) Contact chemoreceptors mediate male-male repulsion and male-female attraction during *Drosophila* courtship. *Cell* 149: 1140–1151

Thorne N, Chromey C, Bray S, Amrein H (2004) Taste perception and coding in *Drosophila. Curr Biol* 14: 1065–1079

Uchizono S, Itoh TQ, Kim H, Hamada N, Kwon JY, Tanimura T (2017) Deciphering the genes for taste receptors for fructose in *Drosophila. Mol Cells* 40: 731–736

Vinson JA, Courey JM, Maro NP (1992) Comparison of two forms of vitamin C on galactose cataracts. *Nutr Res* 12: 915–922

Vosshall LB, Stocker RF (2007) Molecular architecture of smell and taste in *Drosophila. Annu Rev Neurosci* 30: 505–533

Wu Q, Park SJ, Yang M, William WJ (2021) Vitamin preference in *Drosophila. Curr Biol* 31: R946–R947

Xiao S, Baik LS, Shang X, Carlson JR (2022) Meeting a threat of the Anthropocene: taste avoidance of metal ions by *Drosophila. Proc Natl Acad Sci USA* 119: e2204238119

Zhang YV, Ni J, Montell C (2013) The molecular basis for attractive salt-taste coding in *Drosophila. Science* 340: 1334–1338

Expanded View Figures
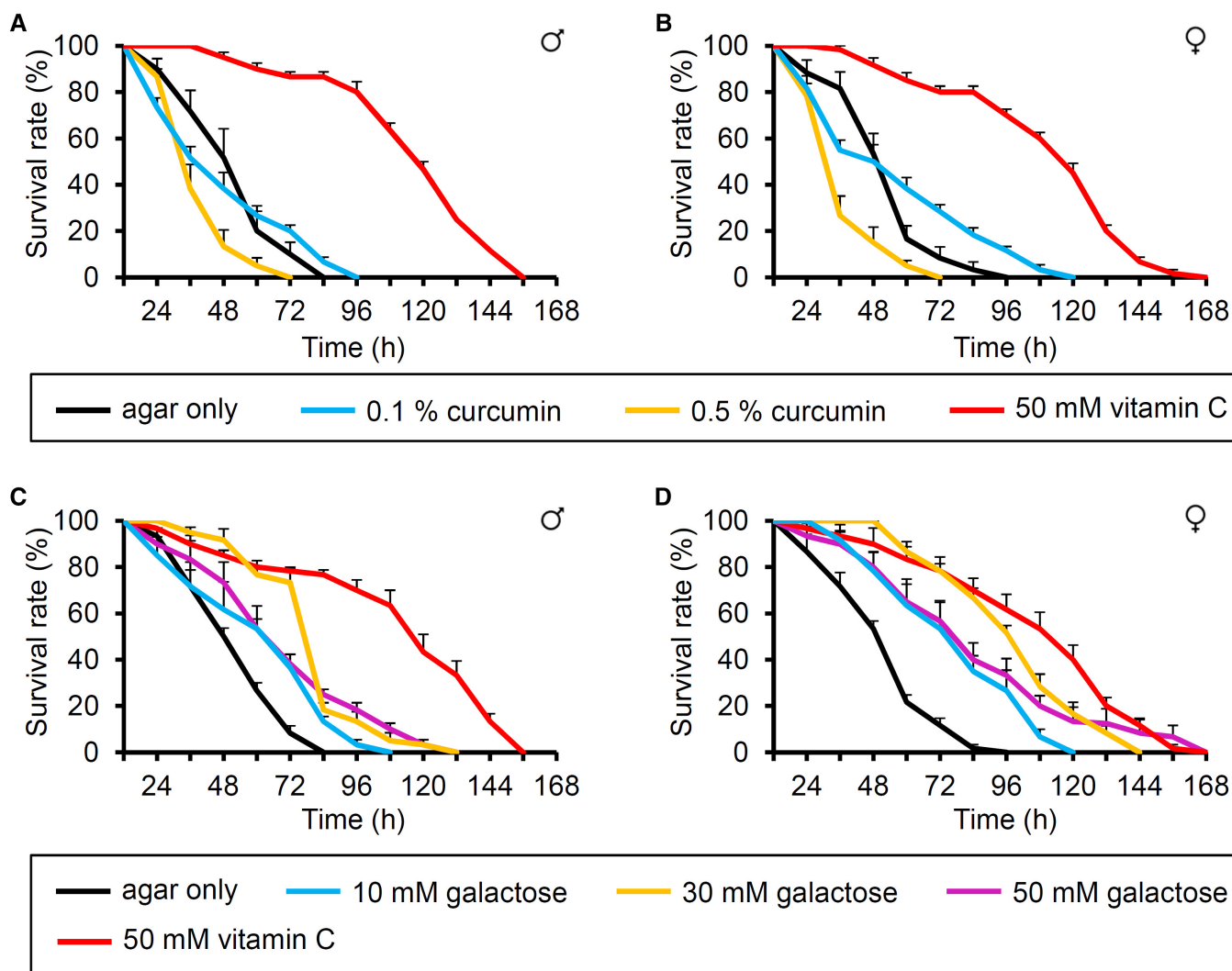
Figure EV1. Effect of curcumin and galactose food on starved flies.

A, B Time-dependent starvation resistance properties of flies to 0.1% (~3 mM), 0.5% curcumin, and 50 mM vitamin C food without the supplement of other nutrition in wild-type control flies (*w*[1118]) (A) males (B) females, *n* = 6.

C, D Time-dependent starvation resistance properties of flies to 10 mM galactose, 30 mM galactose, 50 mM galactose, and 50 mM vitamin C food without the supplement of other nutrition in wild-type control flies (*w*[1118]) (C) males (D) females, *n* = 6.

Data information: Error bars represent SEMs. Kaplan–Meier survival analyses were performed to compare the survival rates of agar only and fed conditions with the indicated concentrations of curcumin and galactose (Appendix Tables S2 and S3).

Figure EV2. Behavioral and electrophysiological analyses of Vitamin C.

A Tip recording of w^{1118} (control) flies at the indicated concentrations of vitamin C from L6 and S6 sensilla, $n = 10-12$.

B Neuronal firings from L4, L6, and S6 sensilla to folic acid and riboflavin at the indicated concentrations, $n = 10-18$.

C PER assays were given stimuli to the indicated organs: forelegs or labellum, $n = 6-8$.

D Dose-dependent binary food choice assays at different concentrations of vitamin C (1, 5, 10, 30, and 50 mM) versus 1 mM, $n = 6-7$.

E Binary food choice assays with different organ-ablated flies in the presence of 50 mM vitamin C versus 1 mM, $n = 6$.

F Binary food choice assays of specific GRN-ablated flies and control. +/− indicate the presence or absence of each transgene, respectively, $n = 6$.

Data information: All error bars represent the SEM. Multiple sets of data were compared using single-factor ANOVA coupled with the Scheffe's *post hoc* test. Asterisks indicate statistical significance compared with the control. $**P < 0.01$.
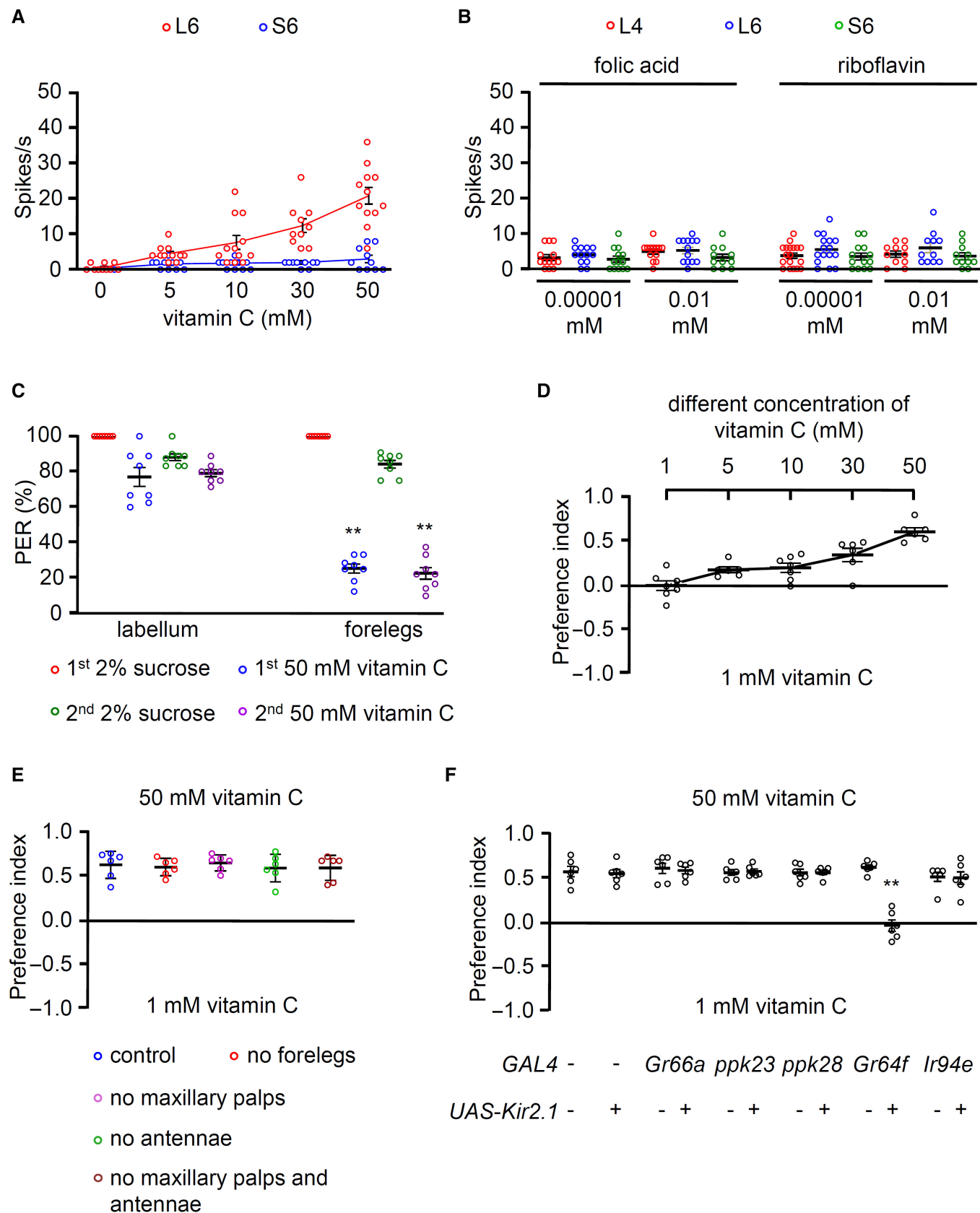
Figure EV2.

Figure EV3. Oviposition preference assays on vitamin C.

A Photograph of egg-laying plate to show the differentiating behavior of females in the presence of 50 mM vitamin C. Higher number of eggs were seen on the side of 50 mM vitamin C than 1 mM.

B Oviposition preference assays of specific GRN-ablated flies and control with the indicated concentrations of vitamin C, $n = 6$.

C Oviposition preference assays with $Ir25a^2$, $Ir76b^1$, $Gr5a^{\Delta 5}$, $Gr61a^1$, $Gr64b^{LexA}$, $Gr64c^{LexA}$, and $Gr64e^{LexA}$, $n = 6$–7.

D Oviposition preference assays to rescue $Ir25a^2$ and $Ir76b^1$ defects by expression of wild-type cDNA, $Ir25a^+$ and $Ir76b^+$, driven by its own $GAL4s$ and $Gr64f$-$GAL4$, $n = 6$.

E Rescue of the oviposition defects of $Gr5a^{\Delta 5}$, $Gr61a^1$, $Gr64b^{LexA}$, $Gr64c^{LexA}$, and $Gr64e^{LexA}$ using $GAL4/UAS$ system with the indicated $GAL4$s, $n = 6$. +/− indicate the presence or absence of the transgene, respectively.

Data information: All error bars represent the SEM. Multiple sets of data were compared using single-factor ANOVA coupled with the Scheffe's *post hoc* test. Asterisks indicate statistical significance compared with the control. $**P < 0.01$.
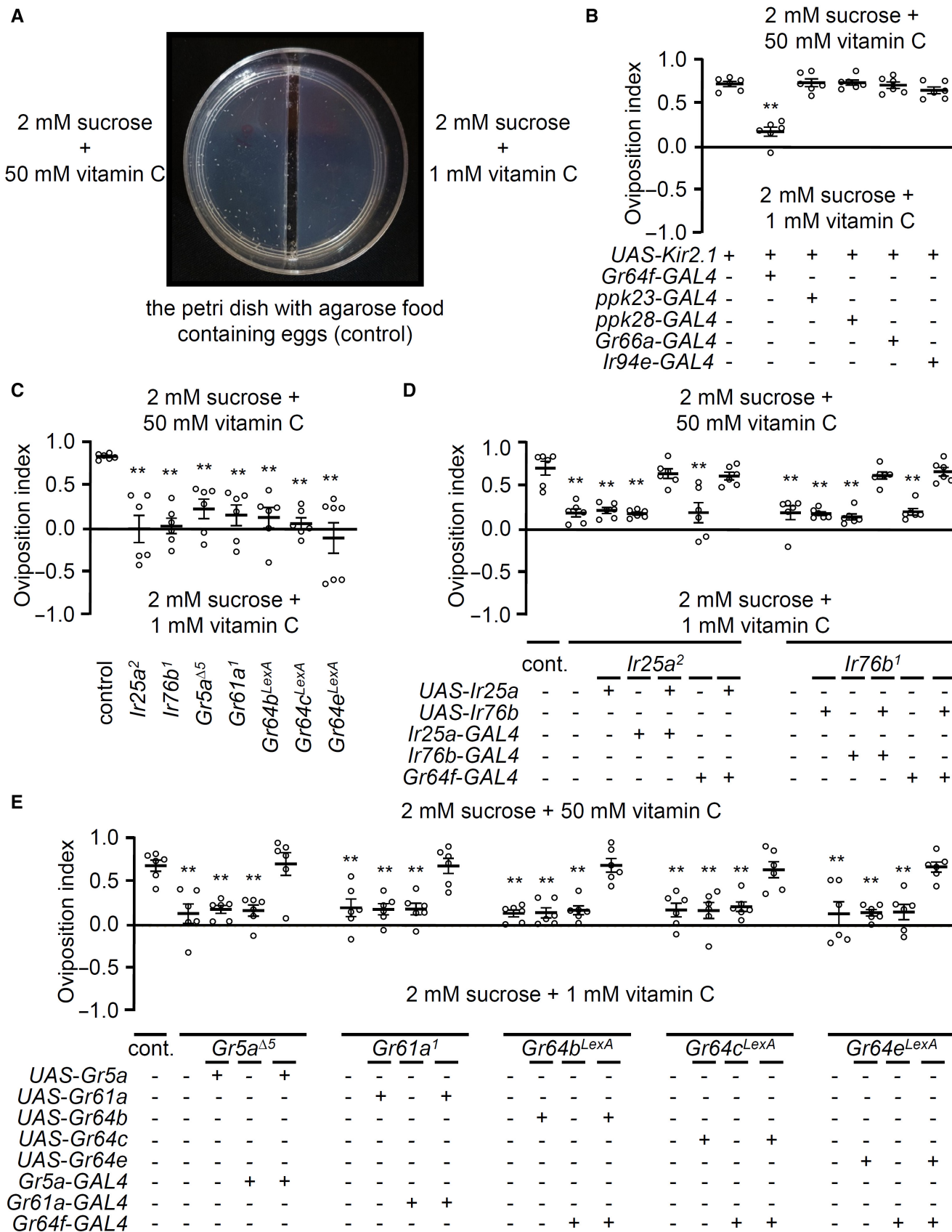
A

2 mM sucrose
+
50 mM vitamin C

2 mM sucrose
+
1 mM vitamin C

the petri dish with agarose food
containing eggs (control)

B

2 mM sucrose +
50 mM vitamin C

Oviposition index

2 mM sucrose +
1 mM vitamin C

UAS-Kir2.1 +	+	+	+	+	+
Gr64f-GAL4 -	+	-	-	-	-
ppk23-GAL4 -	-	+	-	-	-
ppk28-GAL4 -	-	-	+	-	-
Gr66a-GAL4 -	-	-	-	+	-
Ir94e-GAL4 -	-	-	-	-	+

C

2 mM sucrose +
50 mM vitamin C

Oviposition index

2 mM sucrose +
1 mM vitamin C

control, $Ir25a^2$, $Ir76b^1$, $Gr5a^{\Delta 5}$, $Gr61a^1$, $Gr64b^{LexA}$, $Gr64c^{LexA}$, $Gr64e^{LexA}$

D

2 mM sucrose +
50 mM vitamin C

Oviposition index

2 mM sucrose +
1 mM vitamin C

	cont.		$Ir25a^2$					$Ir76b^1$			
UAS-Ir25a	-	-	+	-	+	-	+	-	-	-	-	-
UAS-Ir76b	-	-	-	-	-	-	-	-	+	-	+	-	+
Ir25a-GAL4	-	-	-	+	+	-	-	-	-	-	-	-
Ir76b-GAL4	-	-	-	-	-	-	-	-	-	+	+	-	-
Gr64f-GAL4	-	-	-	-	-	+	+	-	-	-	-	+	+

E

2 mM sucrose + 50 mM vitamin C

Oviposition index

2 mM sucrose + 1 mM vitamin C

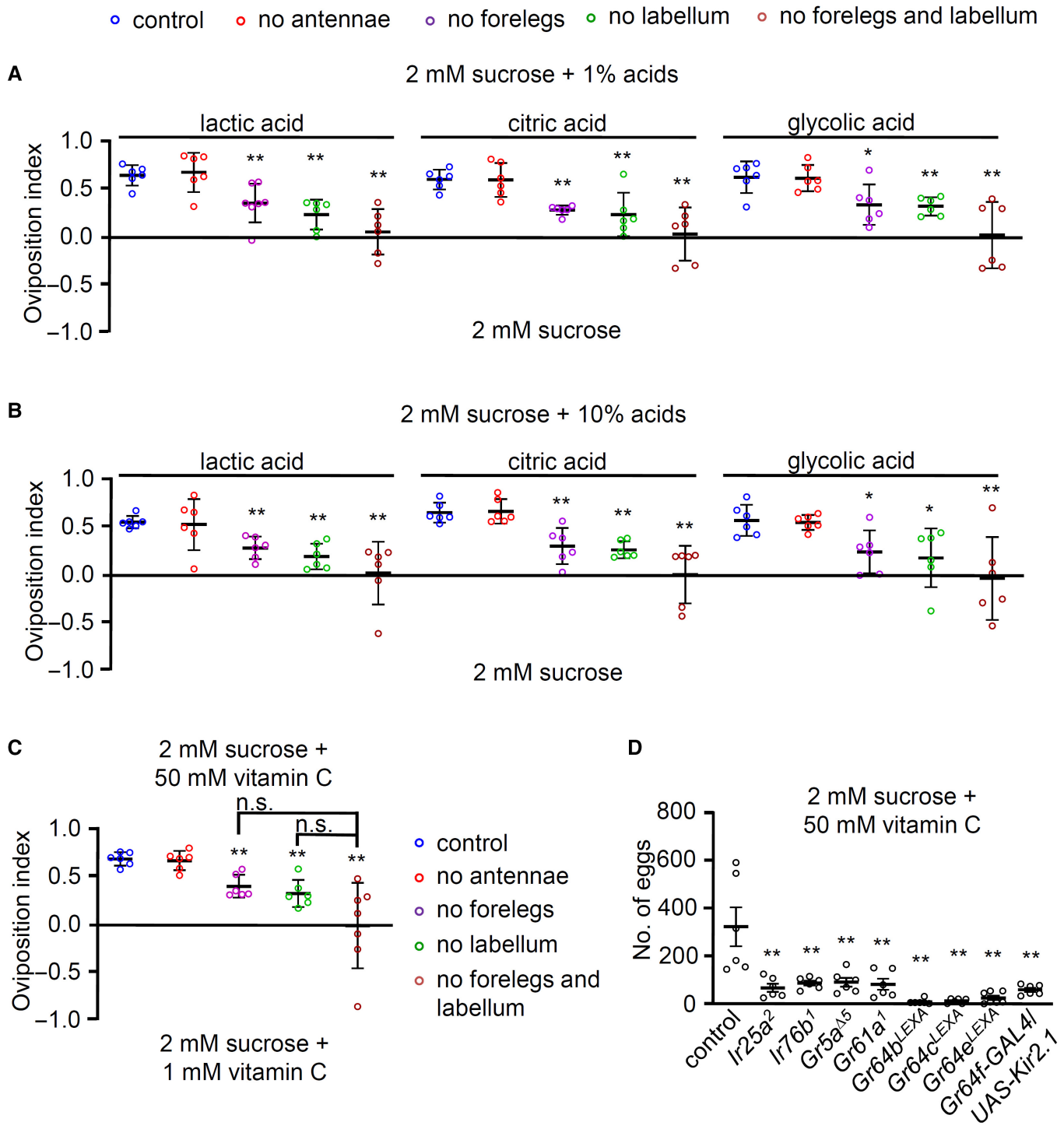|	cont.		$Gr5a^{\Delta 5}$			$Gr61a^1$			$Gr64b^{LexA}$			$Gr64c^{LexA}$			$Gr64e^{LexA}$		
UAS-Gr5a	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
UAS-Gr61a	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
UAS-Gr64b	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-
UAS-Gr64c	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
UAS-Gr64e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
Gr5a-GAL4	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Gr61a-GAL4	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Gr64f-GAL4	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	+	+

Figure EV3.

Figure EV4. Oviposition preference assays on acids.

A Oviposition assays of indicated organ-ablated flies and control at 1% carboxylic acids (lactic acid, citric acid, and glycolic acid), $n = 6$.
B Oviposition assays of indicated organ-ablated flies and control at 10% carboxylic acids (lactic acid, citric acid, and glycolic acid), $n = 6$.
C Oviposition assays of specific organ-ablated flies and control at 50 mM vitamin C versus 1 mM, $n = 6$.
D Number of eggs laid at 50 mM vitamin C mixed with 2 mM sucrose by $Ir25a^2$, $Ir76b^1$, $Gr5a^{\Delta 5}$, $Gr61a^1$, $Gr64b^{LexA}$, $Gr64c^{LexA}$, $Gr64e^{LexA}$, and vitamin C insensitive flies ($Gr64f$-GAL4/UAS-Kir2.1), $n = 6$.

Data information: All error bars represent the SEM. Multiple sets of data were compared using single-factor ANOVA coupled with the Scheffe's *post hoc* test. Asterisks indicate statistical significance compared with the control. *$P < 0.05$, **$P < 0.01$.

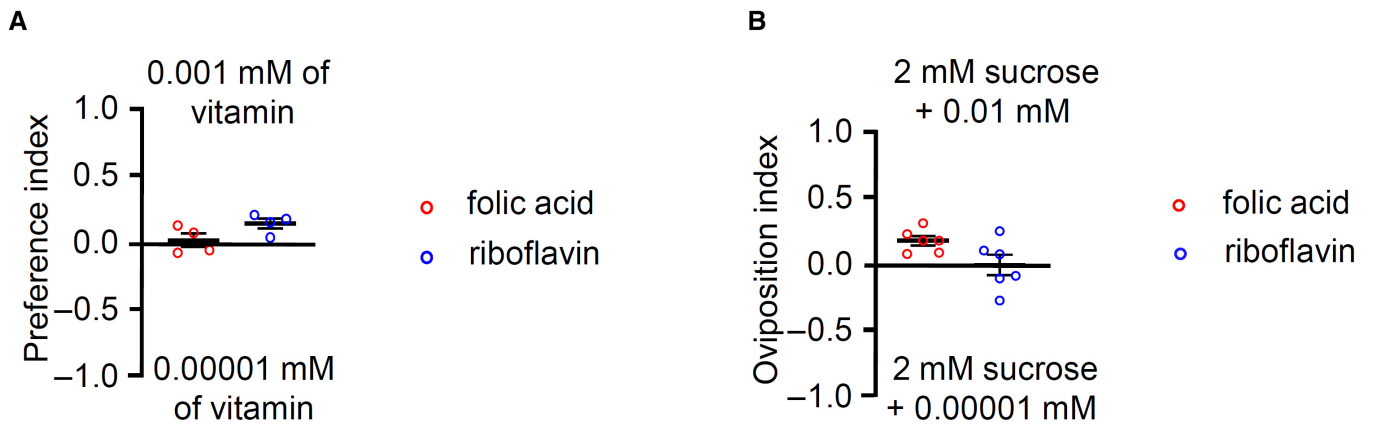**A**

B

Figure EV5. Binary food choice assay and oviposition preference assay with vitamin B2 and B9.

A   Binary food choice assays performed with wild-type flies in the presence of 0.001 mM versus 0.00001 mM riboflavin (vitamin B2) and folic acid (vitamin B9), *n* = 4.
B   Oviposition assays performed with wild-type flies, *n* = 6.

Data information: All error bars represent the SEM.

The Taste of Vitamin C in *Drosophila melanogaster*

Bhanu Shrestha[1], Binod Aryal[1], and Youngseok Lee[1,2,*]

Appendix-Table of contents

Appendix Table S1. Median lifespan (LT_{50}) and log-rank calculation of (A) male and (B) female related to Figure 1A and 1B. Kaplan-Meier survival analyses were performed to compare the performance of vitamin C unfed and fed conditions at different doses.

(A)

Line	Male						
conc. of vitamin C	0 mM	0.1 mM	1 mM	5 mM	10 mM	30 mM	50 mM
LT_{50} (hrs)	46.1 ± 4.27	53.6 ± 5.14	58.3 ± 5.32	71.0 ± 5.97	72.3 ± 9.17	99.3 ± 2.55	120.9 ± 2.31
Log-rank		$<10^{-2}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$

(B)

Line	Female						
conc. of vitamin C	0 mM	0.1 mM	1 mM	5 mM	10 mM	30 mM	50 mM
LT_{50} (hrs)	45.6 ± 3.17	56.6 ± 3.78	60.9 ± 5.61	61.5 ± 3.26	62.8 ± 2.97	102.0 ± 3.09	114.6 ± 1.60
Log-rank		$<10^{-2}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$

Appendix Table S2. Median lifespan (LT_{50}) and log-rank calculation of (A) male and (B) female related to Expanded View Figure 1A and 1B. Kaplan-Meier survival analyses were performed to compare the performance of (a) 0.1% curcumin, 0.5% curcumin, and 50 mM vitamin C fed conditions with unfed condition, (b) 0.1% curcumin and 0.5% curcumin fed conditions with 50 mM vitamin C fed conditions.

(A)

Line	Male			
conditions	Agar only	0.1% curcumin	0.5% curcumin	50 mM vitamin C
LT_{50} (hrs)	46.1 ± 4.12	39.3 ± 3.88	34.7 ± 2.63	117.2 ± 2.04
Log-rank (a)		0.9488	$<10^{-3}$	$<10^{-3}$
Log-rank (b)		$<10^{-3}$	$<10^{-3}$	

(B)

Line	Female			
conditions	Agar only	0.1% curcumin	0.5% curcumin	50 mM vitamin C
LT_{50} (hrs)	48.5 ± 2.47	46.0 ± 5.51	29.6 ± 0.95	116.3 ± 2.44
Log-rank (a)		0.0862	$<10^{-3}$	$<10^{-3}$
Log-rank (b)		$<10^{-3}$	$<10^{-3}$	

Appendix Table S3. Median lifespan (LT_{50}) and log-rank calculation of (A) male and (B) female related to Expanded View Figure 1C and 1D. Kaplan-Meier survival analyses were performed to compare the performance of (a) 10 mM galactose, 30 mM galactose, 50 mM galactose, and 50 mM vitamin C fed conditions with unfed condition, (b) 10 mM galactose, 30 mM galactose, and 50 mM galactose fed conditions with 50 mM vitamin C fed conditions.

(A)

Line	Male				
conditions	Agar only	10 mM galactose	30 mM galactose	50 mM galactose	50 mM vitamin C
LT_{50} (hrs)	46.6 ± 2.71	53.3 ± 7.95	76.7 ± 0.78	58.6 ± 4.91	117.0 ± 5.74
Log-rank (a)		0.0008	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
Log-rank (b)		$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	

(B)

| Line	Female				
conditions	Agar only	10 mM galactose	30 mM galactose	50 mM galactose	50 mM vitamin C
LT_{50} (hrs)	47.3 ± 2.52	73.0 ± 7.56	95.1 ± 2.34	75.6 ± 6.89	112.0 ± 5.93
Log-rank (a)		$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
Log-rank (b)		$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	

Appendix Figure S1
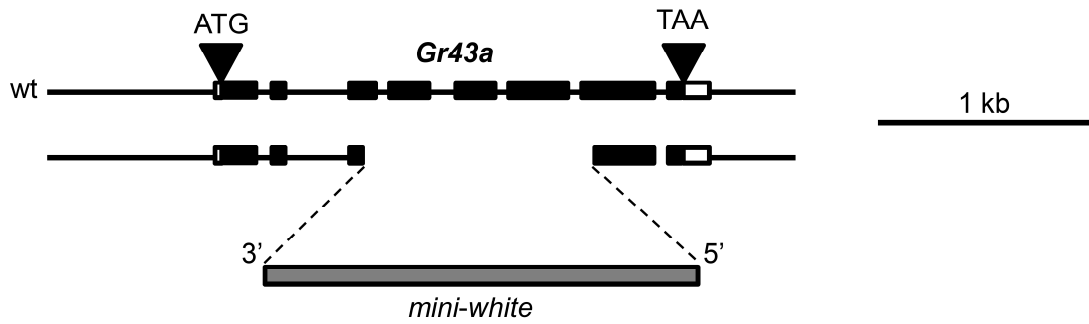
Appendix Figure S1. Gene structure of *Gr43a* and Generation of *Gr43a¹*.
Gr43a is composed of 8 exons. The mutant was generated by ends-out homologous recombination deleting 955 base pair. The deleted region ranged from +711 to +1666 using the "A" of the "ATG" start codon as a +1.
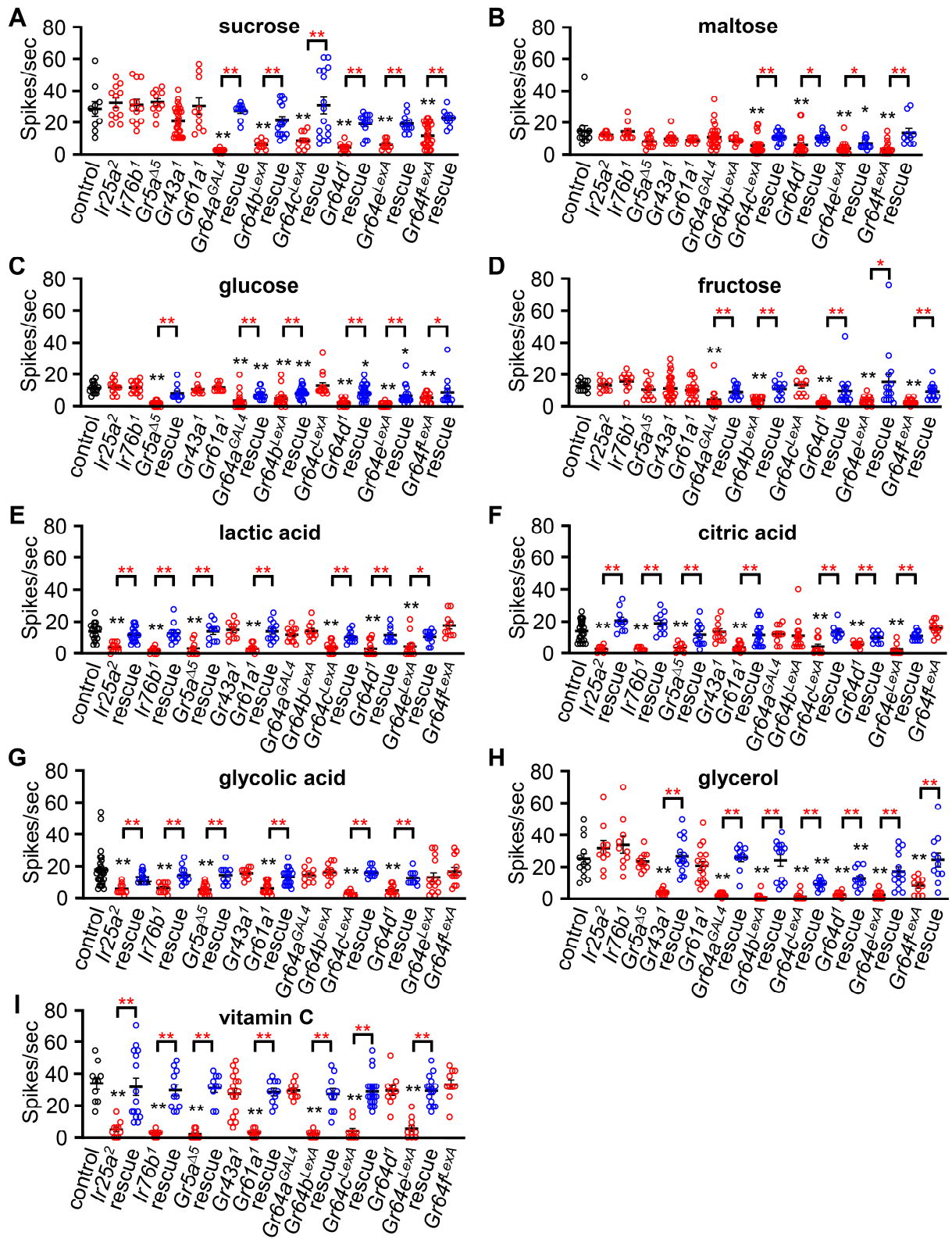
Appendix Figure S2. Tip recording analysis of sugars, carboxylic acids, glycerol, and vitamin C with 11 potential candidate mutants (related to Figure 4).
A-I Electrophysiology with the indicated mutants and control (w^{1118}) in the presence of 50 mM sugars (**(A)** sucrose, **(B)** maltose, **(C)** glucose, and **(D)** fructose), 1% carboxylic acids (**(E)** lactic acid, **(F)** citric acid, and **(G)** glycolic acid), **(H)** 10% glycerol and (**I**) 50 mM vitamin C. All the recordings were performed from L4 sensilla except for the glycerol where L7 sensilla was used. The defects of *Ir25a*, *Ir76b*, *Gr5a*, *Gr61a*, *Gr64a*, and *Gr64f* mutants were rescued by their own cDNA expression driven with their own *GAL4s*, while the rescue of *Gr64b*, *Gr64c*, *Gr64d*, and *Gr64e* were driven by *Gr64f-GAL4*, *n* = 10–29.
All error bars represent the SEM. Multiple sets of data were compared using single-factor ANOVA coupled with Scheffe's post hoc test. Black asterisks indicate statistical significance compared with the control and the red asterisks indicate statistical significance compared between the respective mutant and rescue lines. *p < 0.05, **p < 0.01.