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Molecular logic and evolution of bitter taste in *Drosophila*

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Summary

Taste systems detect a vast diversity of toxins, which are perceived as bitter. When a species adapts to a new environment, its taste system must adapt to detect new death threats. We deleted each of six commonly expressed bitter *Gustatory receptors (Grs)* from *Drosophila melanogaster*. Systematic analysis revealed that requirements for these *Grs* differed for the same tastant in different neurons, and for different tastants in the same neuron. Responses to some tastants in some neurons required four *Grs*, including Gr39a.a. Deletions also produced increased or novel responses, supporting a model of Gr-Gr inhibitory interactions. Co-expression of four *Grs* conferred several bitter responses to a sugar neuron. We then examined bitter coding in three other *Drosophila* species. We found major evolutionary shifts. One shift depended on the concerted activity of seven *Grs*. This work shows how the complex logic of bitter coding provides the capacity to detect innumerable hazards and the flexibility to adapt to new ones.

eTOC Blurb

Dweck and Carlson show that responses of some neurons to bitter tastants require four Gustatory receptors (*Grs*), and that coexpression of four *Grs* confers several bitter responses to sugar neurons. Loss of some *Grs* produces increased or novel responses, suggesting Gr-Gr inhibition. Two classes of bitter neurons arise in *D. melanogaster* via 7 *Grs*.

Keywords

Taste; *Drosophila*; taste receptor, bitter coding; evolution; *D. simulans*; *D. sechellia*; *D. erecta*

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AUTHOR CONTRIBUTIONS

H.K.M.D. designed and conducted the experiments. H.K.M.D. and J.R.C. wrote the paper.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

Introduction

The natural world abounds in toxins. Plants, which cannot flee their predators, synthesize toxic chemicals that deter them [1]. Animals, in turn, have evolved sensitive systems to detect toxins [2–4]. Taste systems allow recognition of many toxic chemicals as bitter. Such toxins are detected and encoded by bitter-sensing taste cells through mechanisms that are poorly understood. As a species evolves to inhabit a new niche, its taste system must evolve to detect the chemical threats in its new environment. Little is known about the logic of bitter coding, and even less is known about how this coding evolves.

Drosophila detects and avoids a wide variety of compounds that taste bitter to humans [5]. The main taste organ of the *Drosophila* head is the labellum, which contains 31 taste sensilla (Figure 1A) [6]. These sensilla fall into three morphological classes: Large (L), Intermediate (I) and Small (S) [7, 8]. Each sensillum contains a pore at the distal end. When the sensillum contacts a potential food source, compounds enter via the pore and may activate taste neurons within.

An electrophysiological analysis of the 31 sensilla with a diverse panel of bitter tastants identified four functional classes of bitter-sensitive sensilla, I-a, I-b, S-a, and S-b, each with a distinguishable response profile [5]. These I and S sensilla each have a neuron that responds to bitter compounds and that expresses members of the Gustatory receptor (Gr) family of taste receptors [9].

A collection of *Gr-GAL4* drivers representing the entire family of 68 Gr receptors was used to map Grs to these bitter-sensing neurons [5]. The molecular map identified four classes of neurons, in good agreement with the physiological map (Figure 1B). Five *Gr-GAL4* drivers were expressed in all bitter-sensing classes of the labellum, and 24 other drivers were expressed in some but not all classes. The five ubiquitous *Gr-GAL4* drivers identified “Commonly Expressed Receptors” (CERs): Gr32a, Gr33a, Gr39a.a, Gr66a, and Gr89a (Figure 1B). A sixth receptor, Gr93a, was detected in all bitter-sensing classes by immunohistochemistry [10]. We therefore now classify it as a CER.

A variety of studies have shown that some individual receptors are required for the response to particular bitter compounds in certain sensilla [10–16]. Most of these studies have examined one or a small number of tastants in one or a small number of sensilla. These studies have demonstrated that three of the CERs, Gr32a, Gr33a, and Gr66a, are required for responses to various bitter compounds.

We have analyzed the requirements for all six CERs. We generated new deletions of five *CER* genes using CRISPR-Cas9 genome editing. These five deletions, and an existing *Gr93a* deletion [10], were each crossed into a standard genetic background and tested against a panel of 21 diverse bitter compounds in all classes of sensilla.

The results reveal that within an individual sensillum, different *CERs* underlie the response to different tastants. The response to an individual tastant requires different *CERs* in different sensilla. Responses to some tastants in some sensilla require four *CERs*, including *Gr39a*, which had not previously been implicated in bitter taste. The removal of each *CER*

produces not only decreased responses, but also increased or novel responses to various tastants in various sensilla, supporting a model of extensive Gr-Gr inhibitory interactions. Analysis of bitter coding in three other *Drosophila* species revealed major evolutionary shifts in neuronal response specificities and in functional organization. One unique evolutionary feature of *D. melanogaster* arises through the concerted activities of seven *Gr* genes.

Results

We used CRISPR-Cas9 genome editing to create mutant alleles of *Gr32a*, *Gr33a*, *Gr39a.a*, *Gr66a*, and *Gr89a*. Each of these new mutant alleles, and an existing deletion of *Gr93a* [10], were backcrossed to our control genetic background for five generations to minimize genetic background effects.

We then tested the six mutants with a panel of 21 bitter compounds [5, 17, 18] in all four sensillum types (I-a, I-b, S-a, and S-b). Several kinds of phenotypes were observed. In 24% of the 504 mutation-tastant-sensillum combinations, the response was reduced compared to the response observed in our control genetic background (Figure 1C). The reduction was partial in approximately half of these cases and complete in the others.

By contrast, in some cases the response was greater in the mutant than in the control. We distinguished between a phenotype in which the control genotype shows no response but the mutant exhibits a response (“novel response”, Figure 1D, 4% of combinations), and a phenotype in which the control genotype shows a response but the mutant shows a greater response (“increased response”, Figure 1E, 4%).

Requirements for CERs differ among tastants and sensilla

Control I-a sensilla responded strongly to quinine (QUI), sparteine (SPS), escin (ESC), denatonium (DEN), berberine (BER), and lobeline (LOB), in agreement with previous findings (Figure 2 and Table S2; for ease of visualization, the tastants are listed in a different order for each sensillum type) [5, 19, 20]. The responses of I-a to all six of these tastants depend on three receptors, *Gr32a*, *Gr33a*, and *Gr66a*; mutations of any of these receptors result in a complete or near-complete loss of response. Responses to these six tastants were robust in mutants of the other three CERs. The reduced responses to these six tastants, represented in brown letters, are indicated in Figure 3.

I-b sensilla respond robustly to seven tastants of the panel: escin (ESC), theobromine (THE), saponin (SAP), coumarin (COU), theophylline (TPH), umbelliferone (UMB) and caffeine (CAF). Unlike I-a, in I-b the responses to different tastants required different subsets of CERs (Figures 2 and 3). Responses to five tastants (THE, COU, TPH, UMB, and CAF) required four receptors, *Gr33a*, *Gr39a*, *Gr66a*, and *Gr93a*. Response to ESC required three receptors: *Gr32a*, *Gr33a*, and *Gr66a*. Response to SAP required only two receptors, *Gr33a* and *Gr66a*.

S-a sensilla respond to a broad spectrum of tastants (Figure 2), which may reflect the broad array of receptors that they express (Figure 1B). As in I-b, responses to different tastants depended on different subsets of CERs. Responses to CAF and TPH were again reduced by

removal of any of four *CERs*, but most responses were reduced by removal of any of three, either *Gr32a*, *Gr33a*, or *Gr66a* in most cases, or *Gr33a*, *Gr39a*, or *Gr66a* in one case. Each of three other tastants required two *CERs*, but a different pair in each case.

S-b sensilla also express many *Grs* and responded broadly to many tastants. Some responses required four *CERs*, some three, some two, and some only one.

In summary, there is no *CER* that is required for response to all tastants in all sensilla. Rather, the requirements for *CERs* differ among tastants and among sensilla. Within an individual sensillum, responses to different tastants depend on different *CERs*, and the response to an individual tastant depends on different *CERs* in various sensilla. Deletion of one *CER* gene, *Gr89a*, did not reduce the response to any tastant in any sensillum. Responses to one tastant, aristolochic acid (ARI), were not reduced by removal of any *CERs*, a finding consistent with the discovery that responses to ARI depend on TRPA1 [21].

Deletion of *CERs* produced novel responses

Deletion of *CERs* produced 10 novel responses, in I-a, I-b or S-b (Figures 2 and 3; red in Figure 3). The novel responses were to a variety of compounds: seven compounds in I-a, two in I-b, and one in S-b. No novel responses were observed in S-a. Novel responses were observed following removal of some, but not all *CERs*.

In I-a, the deletion of either *Gr32a* or *Gr89a* produced novel responses to four compounds. A novel response to three other tastants, including ARI, could be produced by deletion of *Gr32a* but not by deletion of *Gr89a*. By contrast, in I-b a novel response to ARI could be produced by either *Gr32a* or *Gr89a*. All 10 of the novel responses were observed at lower concentrations (Figure S2).

Deletion of *CERs* produced increased responses

In each of the four sensillum types we found examples in which deletion of a *CER* led to an increase in the magnitude of a response observed in control sensilla (Figures 2 and 3). Most of these increases were observed in mutants of *Gr39a*, but deletions of *Gr32a*, *Gr33a*, and *Gr89a* also produced increased responses.

Removal of some receptors had dual effects. For example, while the *Gr39a* deletion increased responses to seven tastants in S-b, it decreased responses to three other tastants in S-a.

Gr39a.a is required for normal feeding and oviposition behavior

The *Gr39a* locus had previously been found to be required for normal sexual behavior [22]. Given our finding that it is required for physiological response to the bitter compounds CAF, COU, THE, TPH, and UMB in I-b, and for responses to some of these compounds in S-a and S-b (Figures 2 and 3), we wondered if it played a role in feeding and oviposition preferences.

We tested the *Gr39a* deletion mutant in the FLIC (Fly Liquid-Food Interaction Counter) assay (Figure 4A) [23, 24]. We gave the fly a choice between two wells, one containing

sucrose alone and the other containing sucrose and varying concentrations of a bitter compound. Whereas the control showed strong avoidance of the food containing bitter compounds, this avoidance was abolished or severely reduced by the *Gr39a* deletion in the case of all five bitter compounds (Figure 4B). As a control, we also tested DEN: the strong physiological responses to DEN were not reduced by deletion of *Gr39a* (Figure 2), and behavioral responses were not reduced either (Figure 4B).

In an oviposition paradigm, female flies were given a choice between a substrate containing sucrose alone or a mixture of sucrose and a bitter compound (Figure 4C). Consistent with previous results, control flies found low concentrations of some bitter compounds attractive, whereas high concentrations of all bitter compounds were aversive [25–27]. Deletion of *Gr39a* reduced the magnitude of preferences at one or more concentrations of all five bitter compounds (Figure 4D). As a control, oviposition preference was unchanged when DEN was tested.

Gr39a is one of a small number of *Gr* genes that is alternatively spliced [9, 28]. Our *Gr39a* deletion removes not only *Gr39a.a*, but also three other *Gr39a* splice forms. We asked whether the phenotypes could be rescued by the *Gr39a.a* splice form, which was the only splice form expressed in all bitter-sensitive sensilla in a *Gr-GAL4* analysis [5]. Expression of *Gr39a.a* completely restored the loss of electrophysiological response to I-b, S-a, and S-b (Figures 4E and 4F). The feeding and oviposition phenotypes with all five tastants were also completely rescued by expression of *Gr39a.a* (Figures 4G and 4H).

We constructed a 32-dimensional physicochemical space in which each dimension represents a molecular descriptor, *e.g.* carbon chain length [29]. The five bitter compounds that depend on *Gr39a.a*, CAF, COU, THE, TPH, and UMB, cluster together in this space (Figure S3A). The mean Euclidean distance among these five tastants is shorter than the mean distance among all 21 tastants (0.4 ± 0.04 vs. 2.4 ± 0.06 ; $p < 0.0001$, Mann-Whitney). The five clustered tastants are composed of exactly two fused heterocyclic rings (Figure S3B). In summary, the compounds that depend on a particular CER have a particular structure.

Gr39a.a operates in conjunction with three other Grs

Gr39a.a is one of several receptors that are required for response to CAF, COU, THE, TPH, and UMB. Besides *Gr39a.a*, *Gr33a*, *Gr66a*, and *Gr93a* are also required for responses to all five of these compounds in I-b sensilla (Figures 2 and 3). One possible interpretation of this surprisingly complex requirement is that these four Grs interact cooperatively to detect these tastants. An earlier study had misexpressed *Gr33a*, *Gr66a*, and *Gr93a* in a sweet-sensing neuron and had not detected a response to caffeine [14]. We asked whether *Gr39a.a* together with this trio would confer a response to bitter compounds.

We coexpressed all four Grs in a sugar-sensing neuron, using a *Gr5a-GAL4* driver. Although parental control strains responded only to sucrose, expression of *Gr33a*, *Gr39a.a*, *Gr66a*, and *Gr93a* conferred response to all five bitter compounds (Figures 5A and 5B). We confirmed these results by testing responses at lower concentrations of all five tastants (Figure 5C). We also found that *Gr93a*, *Gr33a*, and *Gr66a* were essential components (Figure S4).

Evolution of taste coding by the *Drosophila* labellum

The two classes of I sensilla are morphologically similar, but functionally distinct. CAF, COU, THE, TPH, and UMB elicit responses from I-b but not I-a; LOB, BER, and DEN elicit responses from I-a but not I-b. We wondered how such striking differences in response profiles evolve.

The sensitivities of taste sensilla presumably evolve to meet the needs of the organism to detect toxins in its environment. As species adapt to new environments, new threats must be detected. Little is known, however, about the mechanisms underlying the evolution of taste coding.

We extended our analysis to include the complete repertoire of taste sensilla on the labella of *D. simulans*, *D. sechellia*, and *D. erecta*, which diverged from *D. melanogaster* 2-3 million years ago (MYA), 3-5 MYA, and 10-12 MYA, respectively [30–33]. The numbers and positions of most sensilla are similar among the four species, suggesting that the developmental logic of patterning is broadly conserved (Figure S5A). In all species, none of the L sensilla respond to any of the bitter tastants (Figure S5B and Table S3). All species contain two S sensilla that showed no bitter responses. Hierarchical cluster analysis revealed that in each species, the sensilla divide into several functional classes (Figure 6). In each species, all L sensilla cluster together with two S sensilla to form a class that responds to none of the tested bitter compounds. All other functional classes are composed uniformly of sensilla of the same morphology, *i.e.* either small or intermediate. In each species, two functional classes consist exclusively of S sensilla, which we call S-a and S-b.

I sensilla have undergone major evolutionary shifts in their functional organization (Figure 6). Only in *D. melanogaster* do the I sensilla fall into two functional classes whose response profiles are starkly different, *i.e.* complementary with respect to the test panel. In *D. simulans*, there are again two classes, but their profiles are strikingly similar; they are distinguishable primarily by their different magnitudes of response to a single tested tastant, CAF ($p < 0.0001$ for differences in CAF response, Mann-Whitney; $p < 0.01$ for differences in entire profiles, $R = 0.75$; ANOSIM based on Bray-Curtis similarity; $n=10$). In *D. sechellia* and *D. erecta*, all I sensilla fall into a single class.

Other species show interesting similarities between the profiles of certain S and I classes. For example, in *D. simulans*, the profile of S-b is reminiscent of both I-a and I-b profiles (Figure 6). In *D. sechellia*, the profiles of S-a, S-b, and I-a are similar.

Major evolutionary shifts in sensitivity to particular compounds have occurred. For example, all bitter-sensing labellar sensilla in *D. simulans* respond to TPH and CAF; none do in *D. sechellia* (Figure S5B). THE elicits robust responses from *D. melanogaster*, but little or no response from sensilla in *D. simulans* or *D. sechellia*. *D. simulans* has evolved exceptionally strong responses to BER, stronger than any responses in *D. melanogaster* (Figure S5B). These differences between species may reflect differences in the prevalence of toxins that threaten these species in their natural environments. Alternatively, an individual species might be more or less sensitive to the lethality of a specific toxin, thereby increasing or decreasing the need to detect it with high sensitivity and reliability.

Molecular basis of the dichotomy in I sensilla of *D. melanogaster*

In the other three species all I sensilla were identical or very similar in their response profiles. How, then, did the two distinct response profiles of I sensilla evolve in *D. melanogaster*? Why do I-b, but not I-a sensilla, respond to CAF, COU, THE, TPH, and UMB?

We earlier carried out experiments with a Gr, Gr59c, which is not a CER, but rather is expressed in I-a but not I-b [19]. We found that misexpression of Gr59c in I-b induced a change in its response profile to one that was similar to I-a (Figure 7A). This finding suggested that in I-a, Gr59c acts in the suppression of responses to CAF and the other tastants that elicit responses from I-b. Consistent with this hypothesis, in a 17kb deletion mutant lacking *Gr59c* and several other genes, I-a sensilla underwent a phenotypic switch and adopted a profile like that of I-b, responding to CAF, COU, THE, TPH, and UMB (Figure 7A).

To investigate the molecular determinants of the I-a and I-b taste profiles, we first confirmed the finding that ectopic expression of Gr59c in I-b sensilla suppressed response to these tastants (Figure 7B); it also suppressed responses to most of these tastants in S sensilla (Figure S6A). We then used CRISPR-Cas9 genome editing to construct a *Gr59c* deletion whose endpoints lie within *Gr59c*. This more precise mutation also induced a phenotypic switch from I-a to I-b (Figure 7C, Figure S6B). These results support a model in which Gr59c acts in I-a to suppress the response to CAF, COU, THE, TPH, and UMB. Our analysis of *CER* deletions showed that Gr32a and Gr89a also suppress responses to CAF, TPH, THE, and COU in I-a (Figures 2 and 3). These results suggested that Gr59c, Gr32a, and Gr89a cooperate to suppress these responses in I-a; they cannot cooperate in I-b, which lacks Gr59c. In support of this notion of cooperation, we found that expression of Gr59c in I-b did not suppress response to the tested bitter compounds in *Gr32a* or *Gr89a* mutants (Figure S6C).

Next we asked whether the phenotypic switch from I-a to I-b depended on any other CERs. Since we had found that four Grs, Gr66a, Gr93a, Gr33a, and Gr39a, are necessary for the response of CAF, COU, THE, TPH, and UMB in I-b sensilla (Figure 2), and together they confer response to these tastants in sugar-sensing neurons (Figure 5), we asked whether they are required for the phenotypic switch from I-a to I-b that is observed in mutants of *Gr59c* and *Gr89a*.

To address this question we constructed a series of double mutants containing each of the four *Gr* mutations, *Gr66a*, *Gr93a*, *Gr33a*, and *Gr39a*, together with either the *Gr59c* or *Gr89a* deletion. All four genes, *Gr66a*, *Gr93a*, *Gr33a*, and *Gr39a*, are required for the phenotypic switch from I-a to I-b; in none of the double mutants did I-a respond to CAF, COU, THE, TPH, or UMB (Figure 7C).

Thus the generation of the two distinct I sensillum types in *D. melanogaster* require the deployment and activity of seven *Gr* genes. Taken together, the results support a model in which Gr39a.a, Gr33a, Gr66a, and Gr93a cooperate to induce the response to CAF, COU,

THE, TPH, or UMB in I-b, but that Gr32a, Gr59c, and Gr89a together suppress this induction in I-a (Figure 7D).

Discussion

The logic of bitter coding in *D. melanogaster*

One basic principle illustrated by our study is that the logic of bitter signaling is much more complex than that of olfactory signaling. Most olfactory receptor neurons express a single *Odor receptor (Or)* gene, together with a single co-receptor gene, *Orco* [34, 35]. The tuning profile of the neuron depends on the Or [36, 37]; trafficking of the Or to the membrane and its signaling depend on Orco [35, 38]. By contrast, requirements for signaling within bitter neurons are highly variable, depending on both the tastant and the neuron. For most bitter compounds several CERs are required to ensure a normal response, in addition to whichever Grs may be required from among those that are expressed in subsets of bitter-sensing neurons.

Within an individual neuron type, the responses to different tastants rely on different CERs. For example, in S-b sensilla, CAF depended on Gr33a, Gr39a, Gr66a, and Gr93a, whereas AZA depended on Gr32a and Gr66a. For an individual tastant, responses rely on different CERs in different neuron types. For example, SPS depended on Gr32a in S-a but not in S-b.

The number of CERs that are required for response was highly variable (Figures 2 and 3). The responses to several tastants in I-b sensilla depend on four CERs, Gr39a.a, Gr33a, Gr66a, and Gr93a. At the other extreme, SAP and THE depend on none of the CERs in S-a and S-b, and ARI depends on none of the tested CERs in any sensillum.

Not only does the response of I-b sensilla to several bitter compounds (CAF, COU, THE, TPH, and UMB) require Gr39a.a, Gr33a, Gr66a, and Gr93a, but the requirement is absolute: mutation of any of these four Grs virtually eliminates response. This finding argues against a model in which the four Grs act in two parallel, independent mechanisms that each confers a partial response within an I-b neuron. Rather, the finding supports a model in which the four Grs are obligate components of a single mechanism.

We know of no previous case in which four Grs, or four distinct members of any insect chemoreceptor family, have been shown to be required for a chemosensory response. Expression of the four Grs in a sugar-sensing cell also conferred response to these tastants. The four Grs could form a single heteromeric receptor, or perhaps two heteromeric complexes of which one acts as a receptor and the other a co-receptor that functions in its trafficking or signalling. A recent cryo-EM study revealed a tetrameric structure for Orco, which is a member of the superfamily that includes Grs [39]. It will be interesting to determine whether Grs also form tetramers.

Our finding that some neurons require different subsets of Grs for response to different tastants supports a model in which multiple heteromeric receptors form within the neuron. For example, in S-b, different heteromeric receptors might mediate responses to CAF and AZA. According to this model, the coexpression of many Grs within a neuron would allow

the formation of diverse multimeric receptors. This kind of intraneuronal combinatorial assembly of receptors could contribute to the ability of the taste system to detect a vast diversity of bitter compounds.

Consistent with this concept, mutations of *CERs* in some cases reduced but did not abolish response. Modest reductions in response occurred primarily in S sensilla, which express the greatest number of Grs [5]. Perhaps the proliferation of Grs in these sensilla provides multiple receptors through which a tastant can signal.

The simplest model of Gr function would predict that loss of any Gr would produce either a decrease in response, or no change in response if the Gr had a redundant function. However, we observed many cases in which the deletion of a *Gr* gene led to an increase in an existing response or a novel response.

This diversity of phenotypes has not been reported in the olfactory system. It supports a model of extensive Gr-Gr interactions within bitter neurons. A Gr may inhibit another Gr by binding to it, activate another Gr by binding to it, or compete with another Gr for binding to a required signaling factor. This model was elaborated largely to explain the results of a gain-of-function study in which a series of Grs were systematically misexpressed in bitter neurons [19].

The present loss-of-function study supports the model in two ways. First, the increased responses we observed when a CER was mutated may be interpreted in terms of inhibition. Such a Gr may partially inhibit the activity of another Gr by binding to it: when the former Gr is eliminated, the latter Gr then signals at a higher level. Second, the novel responses we observed when a CER was mutated may also be explained by inhibition. Such a Gr may completely inhibit the activity of another Gr by binding to it. Thus when the former Gr is mutated, the latter Gr provides a signal that is not observed in wild type.

Deletion of most *CERs* produced at least some decreases in response in at least some neurons. Loss of Gr89a, however, did not reduce the response to any tastant in any bitter neuron. Gr89a may be required for response to a bitter compound that we did not test, or its role may be largely redundant with another Gr, at least in the labellum. Loss of Gr89a was, however, accompanied by a novel response to TPH and some other tastants in I-a sensilla, suggesting that it interacts with and inhibits other Grs in these sensilla.

When we began this study we were attracted by a model in which CERs were largely distinct from other Grs in function, analogous to the distinction between Orco and Ors. Our finding that expression of four CERs in sugar neurons confers a response to bitter compounds does not support this model in its simplest form.

The evolution of bitter coding in *Drosophila*

We analyzed the coding of bitter tastants on the labella of three additional *Drosophila* species. Several conclusions emerged.

The functional organization of the sensilla was conserved in some respects. The numbers and positions of sensilla, although not identical, were largely conserved across species. L

sensilla did not respond to bitter compounds in any species; S and I sensilla responded in all species. In each species bitter-sensitive sensilla fell into several functional classes, each class composed solely of either S or I sensilla.

Other features showed evolutionary plasticity. Each class of S and I sensilla was distinct from all other classes in all other species ($p < 0.0001$; $R = 0.95-0.96$ for S-a, S-b, and I sensilla; ANOSIM based on Bray-Curtis similarity). Whereas in *D. melanogaster* the S sensilla are broadly tuned and the I sensilla are more narrowly tuned, in *D. simulans* and *D. sechellia* S-b sensilla are more narrowly tuned and have response profiles similar to those of their I sensilla counterparts. Thus tuning breadth can expand or contract over evolutionary time.

Sensitivity to individual tastants has also waxed or waned during the evolution of these species, e.g. the conspicuous absence or near absence of responses to CAF, TPH and THE in *D. sechellia*. *D. sechellia* is highly specialized in that it reproduces on the fruit of *Morinda citrifolia*, a tree of the coffee family [40]. Perhaps the loss of CAF (caffeine) response in *D. sechellia* represents an evolutionary mechanism for preventing high-level activation of aversive taste circuits by compounds in this fruit.

The evolutionary plasticity of bitter responses across *Drosophila* species is in contrast to the relatively conserved olfactory response profiles among antennal sensilla [41]. Most of the odor stimuli to which flies respond have a positive valence and signify the presence of a food source [42, 43]. The bitter stimuli tested here have a negative valence and signify the presence of a potentially lethal toxin. Perhaps a system that detects death threats is under particularly strong selective pressure to adapt rapidly to new environments.

The ability of the bitter detection system to adapt rapidly may be especially important if their host plants are evolving new toxins rapidly. Many plants are in an evolutionary arms race with insect herbivores and are under selective pressure to produce new toxins to defend themselves [44–46]. The rapid evolution of bitter coding in *Drosophila* may reflect the need to recognize toxins that plants evolve to defend themselves against any of a variety of other herbivores.

Gr39a has evolved quickly and has been implicated in sexual behavior [22]. We found that *Gr39a* is essential for the physiological response to bitter tastants and for feeding and oviposition behaviors. *Gr39a* is like *Gr32a* in being required for both sexual behavior and labellar bitter response [11, 14, 47]. However, unlike *Gr32a*, *Gr39a* is alternatively spliced, and a large exon that has been especially dynamic over evolutionary time encodes most of the *Gr39a.a* splice form [9, 22, 28, 48]. This splice form completely rescued the taste phenotypes. Perhaps another *Gr39a* splice form rescues the sexual behavior phenotype. We note that a *Gr39a.a-GAL4* driver is expressed not only in the labellum, but also in the legs [5, 49, 50].

Strikingly, most of the increased responses that we observed in our genetic analysis were observed in the *Gr39a* mutant (Figure 3). *Gr39a.a* could play a central role in the regulation of taste responses by inhibiting other Grs. Changes in the structure of *Gr39a.a* could

translate into changes in the levels or activity of several Gr complexes, thereby providing a flexible and efficient regulatory mechanism useful in adapting to new environments.

In a larger sense, interactions among Grs could provide a general mechanistic explanation for the relatively rapid evolution of bitter coding. The model of a network of Grs capable of both positive and negative interactions provides a means of adaptation that is not available to olfactory neurons that express only a single Or and Orco [34, 35].

We identified a mechanism through which two classes of I sensilla with different response profiles originate in *D. melanogaster* via the concerted action of seven *Gr* genes. No comparable mechanism has been identified in the olfactory system of *Drosophila* species. Rather, evolution of the olfactory system has been shown to proceed via changes in receptor sequences [51–53], expansion of the number of ORNs expressing a particular receptor [41, 54, 55], or changes in central circuitry [56].

Taken together our analysis supports a model of taste coding that contains many degrees of freedom. There are multiple bitter-sensing neurons with distinct response profiles, multiple Grs in each bitter-sensing neuron, and multiple splice forms for some Grs. At least seven Grs are required to specify the identities of the two I sensillum types in *D. melanogaster*, and our results support a model in which interactions among Grs underlie bitter coding. Together these degrees of freedom endow the taste system with a rich capacity to evolve and to facilitate adaptation to new chemical environments.

STAR METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, John R. Carlson (john.carlson@yale.edu). All unique and stable reagents generated in this study are available from the Lead Contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

***Drosophila* stocks**—Flies were reared on corn syrup and soy flour culture medium (Archon Scientific) at 25°C and 60% relative humidity in a 12: 12-hour light–dark cycle. *Gr93a*³ (LB27592) was obtained from the Bloomington *Drosophila* Stock Center. *UAS-myc-Gr66a* was obtained from Dr. Craig Montell. *D. simulans* (14021-0251.001), *D. sechellia* (14021-0248.27) and *D. erecta* (14021-0224.01) were obtained from the *Drosophila* Species Stock Center.

METHOD DETAILS

Transgenic flies—*Gr-GAL4* drivers (*Gr5a-Gal4*, *Gr66a-Gal4* and *Gr89a-Gal4*) are described in Weiss et al. (2011). *UAS-Gr* lines were generated by amplification of *Gr* coding regions from Canton-S cDNA. *UAS-Gr33a*, *UAS-Gr59c* and *UAS-Gr93a* were cloned into the pUAST expression vector and inserted into the genome using random transposon integration. *UAS-Gr39a.a* was cloned from gDNA into the pBI-UASCG expression vector and inserted into the genome with the phiC31 site-specific integration system (site #8622).

Gr Deletions—*Gr* deletions were generated using CRISPR/Cas9 homologous recombination. Guide RNAs (gRNAs) were designed using the flyCRISPR Optimal Target Finder. The gRNA plasmid was generated using pCFD4 (Addgene 49411), following the protocol described in (Port et al., 2014). Gibson Assembly was performed using Gibson Assembly Master Mix (New England BioLabs, E2611S). gRNA plasmid and a donor plasmid (pDsRed-attP, Addgene 51019) with homologous arms and a DsRed marker were injected into embryos by Bestgene, Inc. (Chino Hills, CA). DsRed positive alleles were then backcrossed to our control *w¹¹¹⁸* Canton-S line for five generations. Oligonucleotides used to generate gRNA and donor vectors, and to confirm *Gr* deletions, are shown in Table S1.

Bitter Tastants—Bitter tastants were obtained at the highest available purity from Sigma-Aldrich. The compounds are structurally diverse, including naturally occurring alkaloids, terpenoids, and phenolic compounds, and some synthetic compounds such as the insect repellent DEET (N, N-Diethyl-meta-toluamide). All tastants were tested at high concentrations, near the limit of their solubility. All tastants were dissolved in 30 mM tricholine citrate (TCC), an electrolyte that inhibits the water neuron [57]. All tastants were prepared fresh and used for no more than one day. For each set of experiments, all fly lines were tested in the same day by a single tastant. For electrophysiological recordings, tastants were tested at the following concentrations unless otherwise indicated: aristolochic acid (ARI), 1 mM; azadirachtin (AZA), 1 mM; berberine chloride (BER), 1 mM; caffeine (CAF), 10 mM; coumarin (COU), 10 mM; cucurbitacin I hydrate (CUC), 1 mM; N, N-Diethyl-m-toluamide (DEET), 10 mM; denatonium benzoate (DEN), 10 mM; escin (ESC), 1 mM; gossypol from cotton seeds (GOS), 1 mM; (–)-lobeline hydrochloride (LOB), 1 mM; myricetin (MYR), 1 mM; quinine (QUI), 1 mM; rotenone (ROT), 1 mM; saponin from quillaja bark (SAP), 0.1%; D-(+)-sucrose octaacetate (SOA), 1 mM; sparteine sulfate salt (SPS), 10 mM; strychnine nitrate salt (STR), 10 mM; theobromine (THE), 10 mM; theophylline (TPH), 10 mM; umbelliferone (UMB), 10 mM. All compounds were stirred for 24 hours. THE and UMB were additionally heated to increase their solubility, then cooled and tested while in solution.

Electrophysiology—Electrophysiological recordings were performed with the tip-recording method [58], with some modifications. 5-7 days old mated female flies were used. Flies were immobilized in pipette tips, and the labellum was placed in a stable position on a glass coverslip. A reference tungsten electrode was inserted into the eye of the fly. The recording electrode consists of a fine glass pipette (10–15 μm tip diameter) and connects to an amplifier with a silver wire. This pipette performs the dual function of recording electrode and container for the stimulus. Recording started the moment the glass capillary electrode was brought into contact with the tip of the sensillum. Signals were amplified (10x; Syntech Universal AC/DC Probe; www.syntech.nl), sampled (10,667 samples/s), and filtered (100–3000 Hz with 50/60-Hz suppression) via a USB-IDAC connection to a computer (Syntech). Action potentials were extracted using Syntech Auto Spike 32 software. Responses were quantified by counting the number of spikes generated over a 500 ms period after contact. Response to the TCC diluent was not subtracted in any case; TCC responses were small and are shown in Table S2. When recording from sensilla of a

particular class, *e.g.* I-a, all sensilla of that class, *i.e.* I₀-I₆, were tested. Sensilla on both halves of the labellum were tested.

Two-Choice Feeding Assay—Two-choice feeding experiments were performed using the FLIC (Fly Liquid-Food Interaction Counter) *Drosophila* Behavior System. In this paradigm, liquid food is contained in a well that is surrounded by a metal ring. To reach the food, a fly needs to stand on the ring; when the fly feeds, it closes an electrical circuit between the metal ring, the liquid food, and another metal sheet at the bottom of the well. An electrical signal is generated when the fly feeds, and such electrical signals serve as a good measure of ingestion [23]. This system was from Sable Systems International (North Las Vegas, NV) and was described by Ro et al. (2014). The FLIC Monitor Software (version 2.1, downloaded from wikiflic.com) was used to collect raw data from the *Drosophila* Feeding Monitor (DFM). Before experiments, 5–7 days old mated flies were transferred to a vial containing moistened Kimwipes and starved at room temperature for 18–21 hours. Each of the two channels of the DFM was loaded with either 100 mM sucrose alone or a mixture of 100 mM sucrose and the indicated concentration of bitter compound. An individual fly was introduced in an arena through a hole in an acrylic ceiling using an aspirator and allowed to choose between sucrose alone and the mixture of sucrose and bitter compound. The two-choice feeding assay was performed for 3 hours. To automate the analysis of raw FLIC data, we wrote custom software in the R programming language (Tam, 2017). Source code for the software can be obtained at <https://github.com/edrictam/FLIC-analysis> (with a copy archived at <https://github.com/elifesciences-publications/FLIC-Analysis>). Feeding Preference indices were calculated as (Total number of sips from sucrose alone-Total number of sips from sucrose and bitter compound)/Total number of sips. For each set of experiments, all fly lines were tested at the same time. Tastants were prepared fresh in water on the same day of the experiment.

Two-Choice Oviposition Assay—The two-choice oviposition assay was modified from Joseph et al. (2009), except that corn meal food was replaced with 1% agar containing 100 mM sucrose. Oviposition plates consisted of plastic Petri dishes (60 X 15 mm, Falcon), which were divided into two halves; each half contained either sugar or sugar mixed with a bitter compound. Twenty-five newly eclosed flies (five males, twenty females) were transferred to fly food vials and kept at 25°C and 60% relative humidity in a 12: 12-hour light-dark cycle. Flies, when 5-7d old, were placed into an oviposition cage (Genesee Scientific) without anesthesia through a small funnel that fits in the lid of the cage, and left for 24 hours in the dark. Eggs were counted from each substrate. An oviposition preference index was calculated as follows: (number of eggs on sucrose substrate – number of eggs on sucrose-bitter substrate)/(total number of eggs on both substrates).

QUANTIFICATION AND STATISTICAL ANALYSIS

Hierarchical cluster analyses were performed using Ward's method with PAST (paleontological statistics software package for education and data analysis; Hammer et al., 2001). This technique organizes the data into clusters based on the response profiles of each sensillum to the panel of tastants. Euclidean distances were calculated according to Ward's classification method for the hierarchical cluster analysis. Other statistical tests were

performed in GraphPad Prism (version 6.01). All error bars are SEM. Molecular descriptors were calculated by Dragon (<http://www.taletе.mi.it>). Descriptors were z-scores normalized for principal component analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DATA AND CODE AVAILABILITY

This study did not generate any datasets or new code. All raw data is available upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Highlights

- Responses of some neurons to some bitter tastants require 4 Grs, including Gr39a.a
- Co-expression of 4 Grs confers several bitter responses to a sugar neuron
- Loss of some Grs produces larger or new responses, suggesting Gr-Gr inhibition
- Evolutionary shifts in taste sensilla have arisen in *D. melanogaster* via 7 Grs

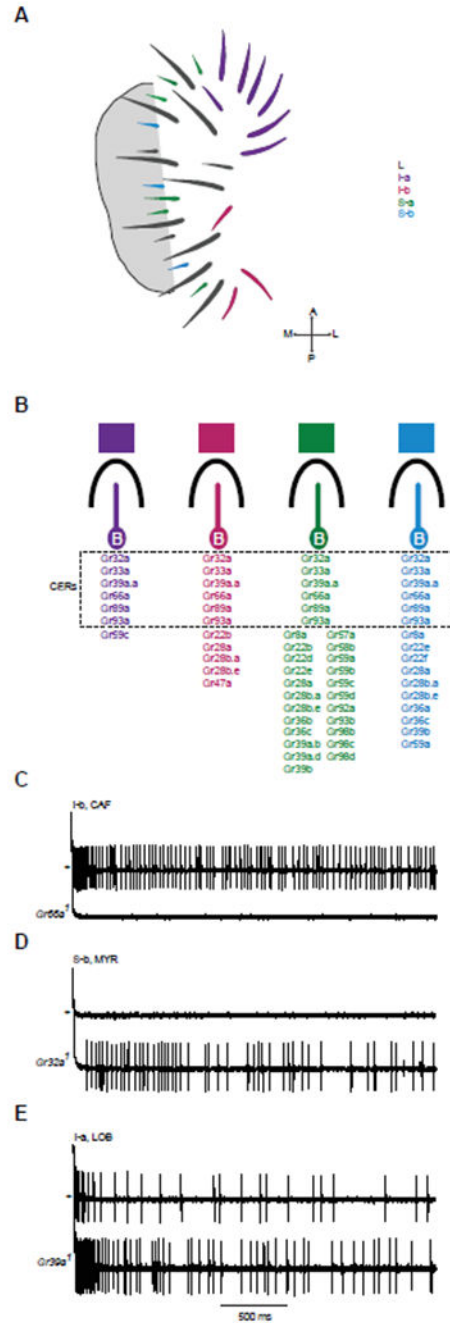


Figure 1. Labellar sensilla and sample phenotypes.

(A) Map of taste sensilla on the labellum. (B) The four classes of sensilla that contain a bitter-sensitive neuron, indicated by “B”. These sensilla all contain other neurons that are not shown. The bitter-sensitive neuron of each sensillum class expresses a different subset of *Gr-GAL4* drivers, as indicated. Five of these drivers are expressed ubiquitously in all four classes and identify receptors that are called Commonly Expressed Receptors (CERs). Other drivers are expressed in subsets of sensillum types. Although one of these other drivers, *Gr93a-GAL4*, is expressed in a subset of sensilla, immunohistochemical analysis indicates

that the Gr93a receptor is expressed in all bitter neurons; it is therefore classified as a CER and indicated as expressed in each sensillum type [10]. (C) Example of a physiological response that is lost in a *CER* mutant. CAF elicits a strong response from I-b in control flies, but not in *Gr66a* flies. (D) A novel response. MYR elicits no response from S-b in control flies, but elicits a response from *Gr32a* flies. (E) An increased response. LOB elicits a response from control flies, and a stronger response from *Gr39a* flies. Panels A and B are adapted from [19]. See also Table S1.

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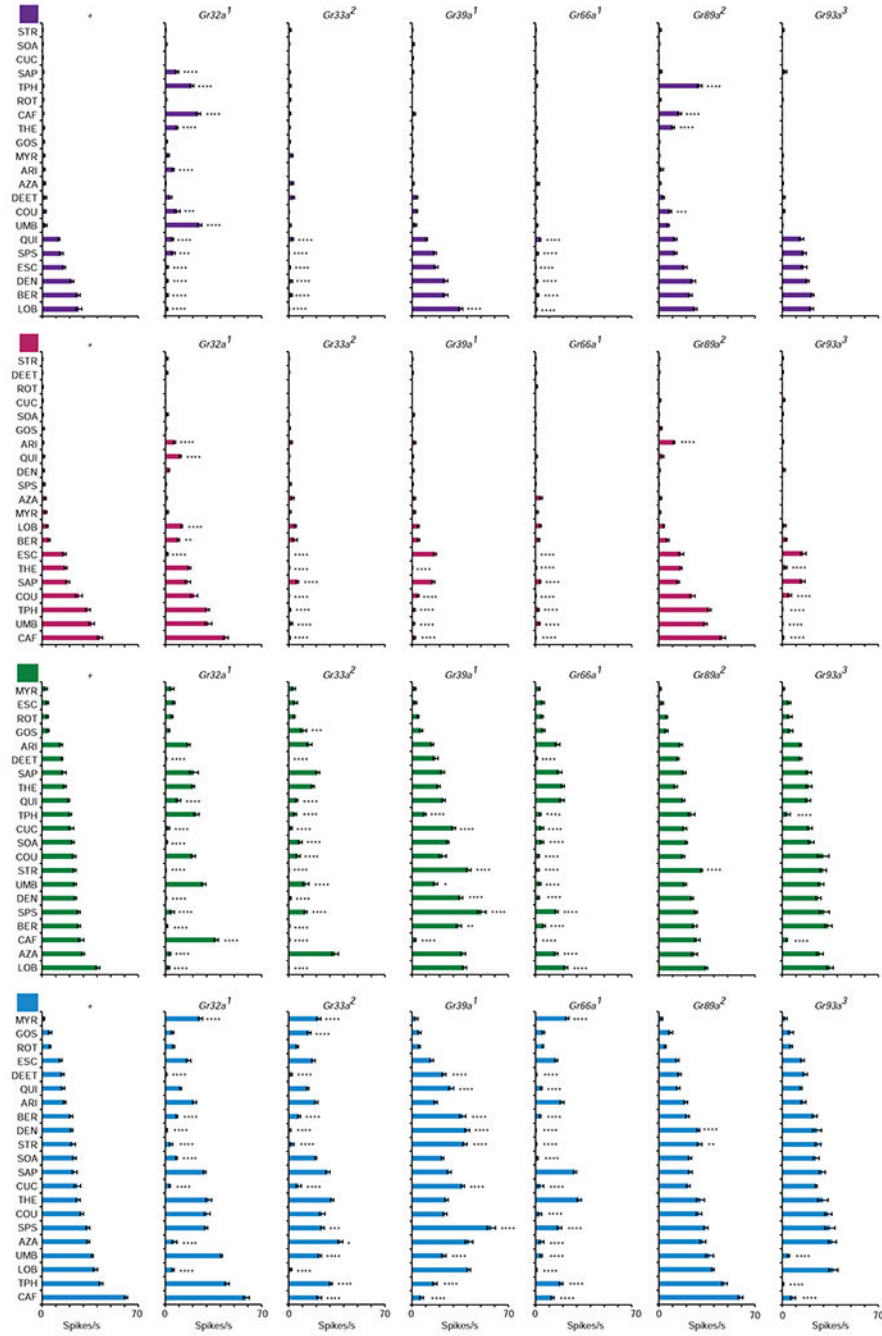


Figure 2. Responses to tastants of bitter-sensing sensilla in CER mutants. Each class of sensilla is tested with each of the 21 indicated tastants in each of the indicated 7 genotypes. For ease of visualization, the order of tastants is listed differently for each sensillum class. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; One-way ANOVA followed by Dunnett’s multiple comparison test, n = 10 for 98% of the 587 genotype-tastant-sensillum class combinations; n<10 for the remaining 2%. See also Figure S1 and Table S2.

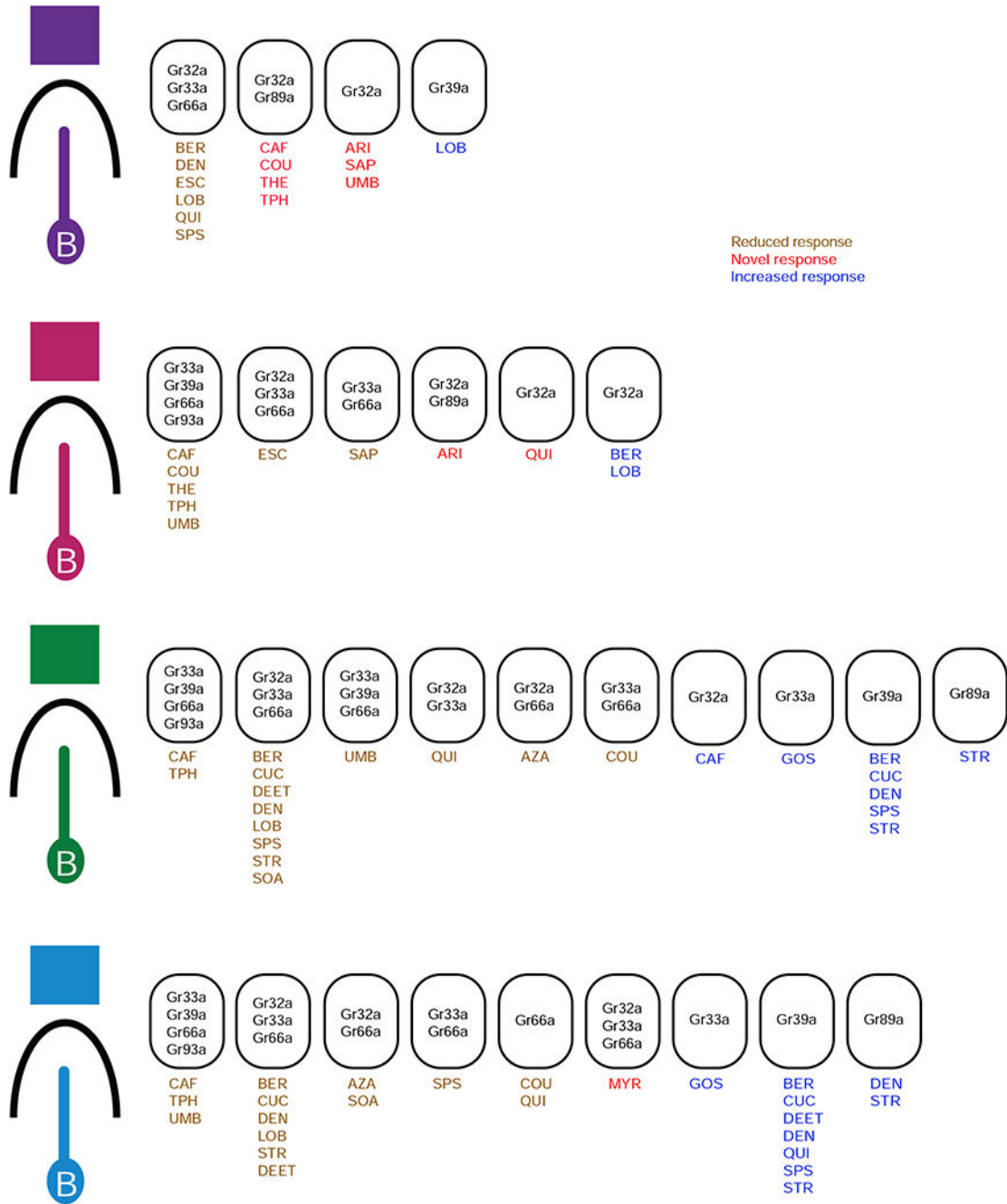


Figure 3. Summary of phenotypes produced by mutations of different *CERs* in different sensilla. In each sensillum type, mutations of the receptors within the same oval all produce the same kind of phenotype, *i.e.* reduced, novel, or increased responses, to the indicated tastants. Thus in I-a sensilla, deletions of either *Gr32a*, *Gr33a*, or *Gr66a* produce a reduced response to BER, DEN, ESC, LOB, QUI, and SPS; loss of *Gr32a* or *Gr89a* produce a novel response to CAF, COU, THE, and TPH; loss of *Gr32a* but not *Gr89a* produces novel responses to ARI, SAP, and UMB. See also Figure S2.

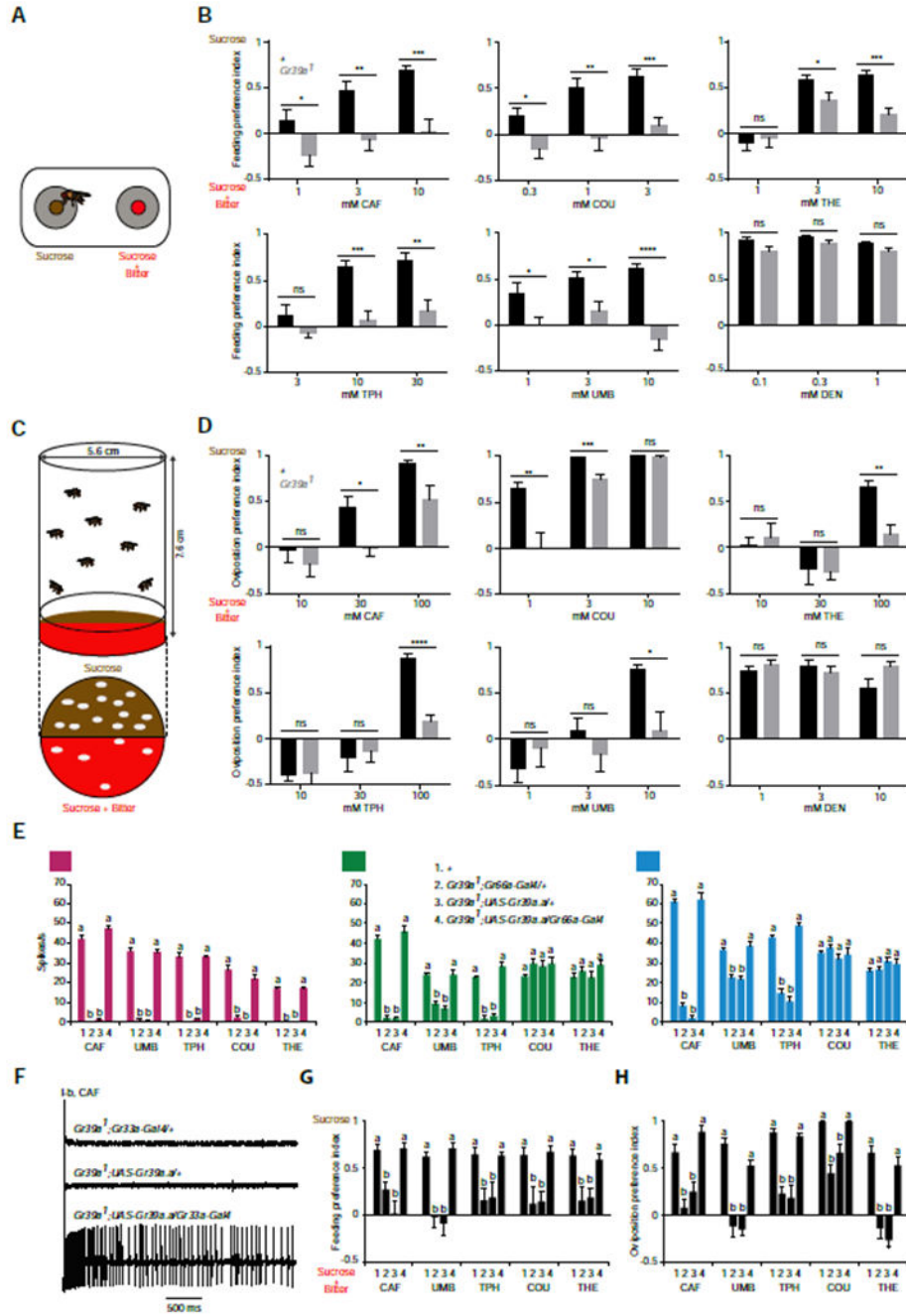


Figure 4. *Gr39a.a* is required for normal feeding and oviposition behavior.

(A) Measuring feeding preference with the FLIC assay. Flies are given a choice between a solution containing sucrose alone or sucrose mixed with a bitter compound. (B) *Gr39a* mutants have a reduced preference for all bitter compounds tested except DEN. Mann-Whitney test, n=9-12. (C) Oviposition preference paradigm. The oviposition preference is based on the number of eggs laid on each half of the plate. (D) *Gr39a* mutants have a reduced preference for all bitter compounds tested except DEN. Mann-Whitney test, n=9-10. (E) The physiological defect of *Gr39a* can be fully rescued by expression of the *Gr39a.a*

splice form. One-way ANOVA followed by Tukey's multiple comparison test, n=6-12. (F) Sample traces from the rescue experiment shown in E. (G) The feeding preference is rescued fully by a *Gr39a.a* transgene. Each tastant is tested at 10 mM except that COU was tested at 3 mM. One-way ANOVA followed by Tukey's multiple comparison test, n=10-18 (H) The oviposition preference is rescued fully by a *Gr39a.a* transgene. CAF, THE, and TPH were tested at 100 mM; UMB and COU were tested at 10 mM and 3 mM respectively. One-way ANOVA followed by Tukey's multiple comparison test, n = 10. In (E-H), values indicated with different letters are significantly different. The key shown for (E) applies to (G,H). See also Figure S3.

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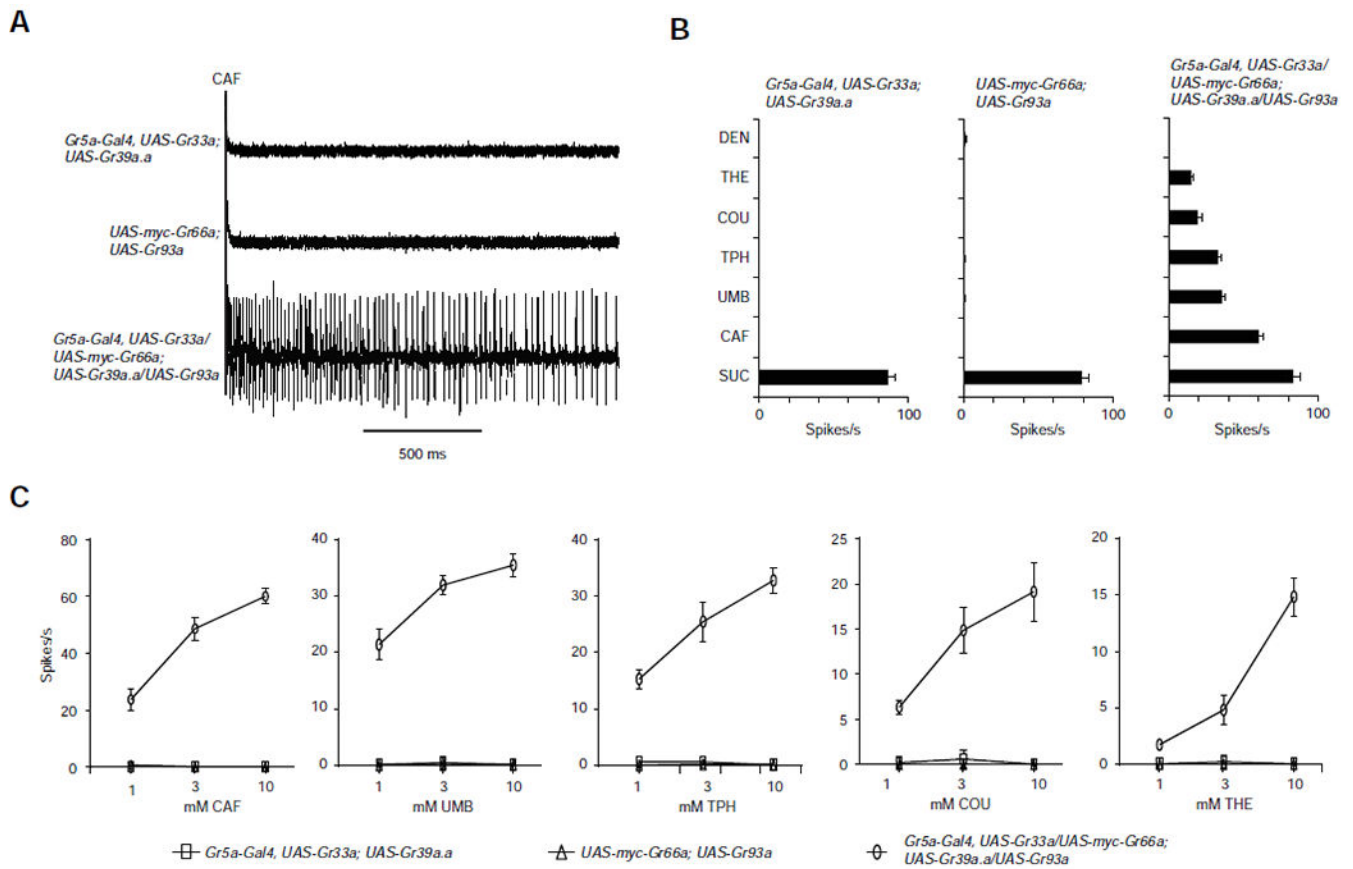


Figure 5. Coexpression of four Grs confers bitter response to sugar neurons.

(A) Coexpression of four Grs in a sugar-sensitive neuron confers response to CAF. (B)

Coexpression of four Grs in a sugar-sensitive neuron confers response to five bitter

compounds. N=65-10. (C) Coexpression confers response across several concentrations of

five bitter compounds. Recordings were from L sensilla. n=10. See also Figure S4.

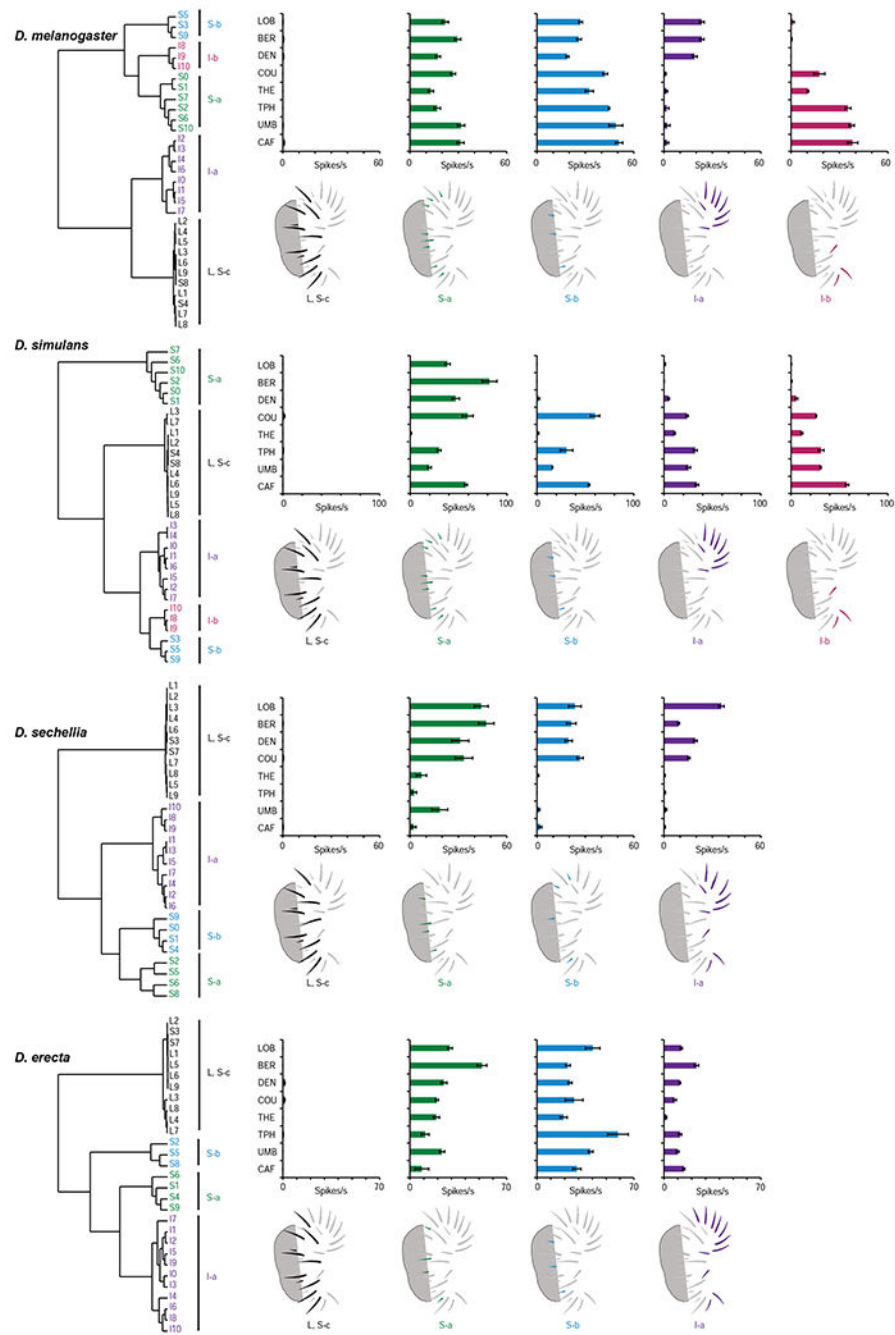


Figure 6. Clustering of labellar sensilla into functional classes.

The graphs show the mean response to each tastant for all sensilla of a functional class. The maps show the distribution of the sensilla of each class. Data are from Figure 6. n=3-10. See also Figure S5 and Table S3.

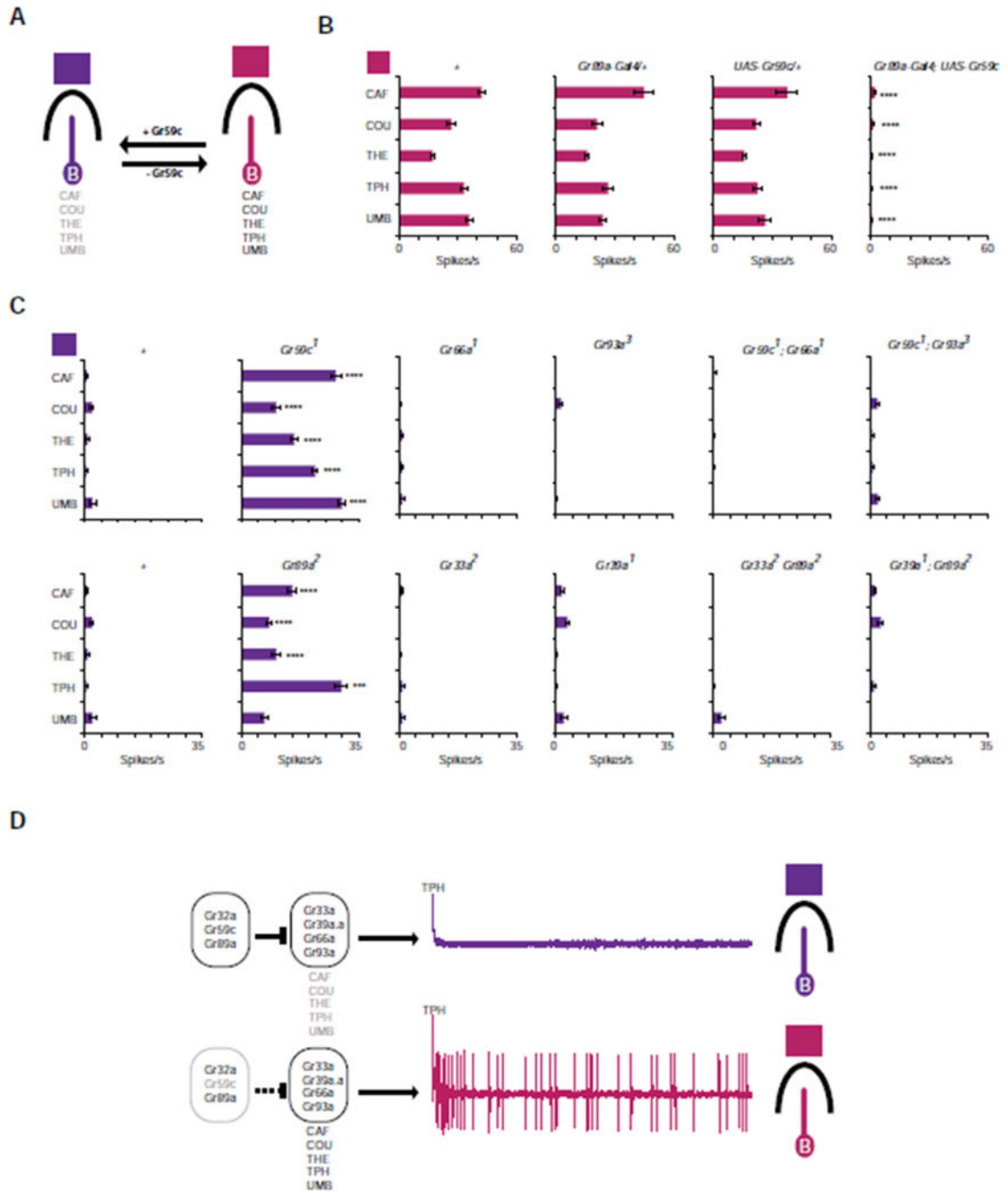


Figure 7. Regulation of I-a identity by seven Grs.

(A) Summary of previous data showing that misexpression of *Gr59c* in I-b induced a change in response profile to one resembling I-a, and that loss of *Gr59c* from I-a induced a change in profile to one resembling I-b [19]. (B) Confirmation that misexpression of *Gr59c* in I-b suppresses response to bitter compounds. The values for the control genotype “+” were from Figure 2. One-way ANOVA followed by Dunnett’s multiple comparison test, n=6-10. (C) New mutations confirm that loss of *Gr59c* or *Gr89a* from I-a leads to novel responses to TPH, CAF, THE, and COU; these responses depend on *Gr66a*, *Gr93a*, *Gr33a*, and *Gr39a* as

shown. One-way ANOVA followed by Dunnett's multiple comparison test, n=10-23. (D) Model for regulation of bitter response in I sensilla. Expression of four receptors, Gr33a, Gr39a.a, Gr66a, and Gr93a, together confer response to CAF, COU, THE, TPH, and UMB. In I-a, Gr32a, Gr59c, and Gr89a are all required to block the four receptors from conferring responses to these tastants. See also Figure S6.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chemicals, Peptides, and Recombinant Proteins		
Aristolochic acid (ARI)	Sigma-Aldrich	Cat# A5512
Azadirachtin (AZA)	Sigma-Aldrich	Cat# A7430
Berberine chloride (BER)	Sigma-Aldrich	Cat# Y0001149
Caffeine (CAF)	Sigma-Aldrich	Cat# C1778
Coumarin (COU)	Sigma-Aldrich	Cat# C4261
Cucurbitacin I hydrate (CUC)	Sigma-Aldrich	Cat# C4493
N,N-Diethyl-m-toluamide (DEET)	Sigma-Aldrich	Cat# 36542
Denatonium benzoate (DEN)	Sigma-Aldrich	Cat# D5765
Escin (ESC)	Sigma-Aldrich	Cat# E1378
(±)-Gossypol from cotton seeds (GOS)	Sigma-Aldrich	Cat# G8761
(-)-Lobeline hydrochloride (LOB)	Sigma-Aldrich	Cat# 141879
Myricetin (MYR)	Sigma-Aldrich	Cat# 72576
Quinine (QUI)	Sigma-Aldrich	Cat# 22620
Rotenone (ROT)	Sigma-Aldrich	Cat# 45656
Saponin (SAP)	Sigma-Aldrich	Cat# 47036
D-(+)-sucrose octaacetate (SOA)	Sigma-Aldrich	Cat# W303801
Sparteine sulfate salt (SPS)	Sigma-Aldrich	Cat# 234664
Strychnine nitrate salt (STR)	Sigma-Aldrich	Cat# S2880
Theobromine (THE)	Sigma-Aldrich	Cat# T4500
Theophylline (TPH)	Sigma-Aldrich	Cat# T1633
Umbelliferone (UMB)	Sigma-Aldrich	Cat# H24003
Experimental Models: Organisms/Strains		
D. melanogaster: <i>wCS; Gr32a¹</i>	Figure 2	N/A
D. melanogaster: <i>wCS; Gr33a²</i>	Figure 2	N/A
D. melanogaster: <i>wCS; Gr39a¹</i>	Figure 2	N/A
D. melanogaster: <i>wCS; Gr66a¹</i>	Figure 2	N/A
D. melanogaster: <i>wCS; Gr89a²</i>	Figure 2	N/A
D. melanogaster: <i>wCS; Gr93a³</i>	Bloomington Drosophila Stock Center	Stock# 27592
D. melanogaster: <i>wCS; Gr66a-Gal4</i>	Dr. John Carlson's lab	[5]
D. melanogaster: <i>wCS; UAS-Gr39a.a</i>	Dr. John Carlson's lab	N/A
D. melanogaster: <i>w; UAS-Gr33a</i>	Dr. John Carlson's lab	N/A
D. melanogaster: <i>w; UAS-Gr39a.a</i>	Dr. John Carlson's lab	N/A
D. melanogaster: <i>w; UAS-Gr66a</i>	Dr. Craig Montell's Lab	[13]
D. melanogaster: <i>w; UAS-Gr93a</i>	Dr. John Carlson's lab	N/A
D. melanogaster: <i>w; Gr5a-Gal4</i>	Dr. John Carlson's lab	[5]

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>D. melanogaster</i> : <i>wCS; Gr59c^l</i>	Figure 7	N/A
<i>D. melanogaster</i> : <i>wCS; Gr89a-Gal4</i>	Dr. John Carlson's lab	[5]
<i>D. melanogaster</i> : <i>wCS; UAS-Gr59c</i>	Dr. John Carlson's lab	[5]
<i>D. simulans</i>	Drosophila Species Stock Center	Stock# 14021-0251.001
<i>D. sechellia</i>	Drosophila Species Stock Center	Stock# 14021-0248.27
<i>D. erecta</i>	Drosophila Species Stock Center	Stock# 14021-0224.01

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