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Title: Olfactory receptors tuned to volatile mustard oils in drosophilid flies

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Abstract (248):

Plant toxins are effective defenses because they are aversive to most insects. The same molecules, however, are co-opted as host-finding cues by specialist herbivores. Although such behavioral shifts are central to our understanding of herbivorous insect diversification, it is not well understood how these behaviors evolve. We addressed this in *Scaptomyza flava,* a herbivorous drosophilid fly within a lineage that shifted to feeding on toxic mustard plants (Brassicales) less than 10 million years ago. *S flava* lost the ancestral attraction to yeast volatiles and the attendant chemoreceptors used to detect these odors. Here we report that *S. flava*, but not its close microbe-feeding relatives *Drosophila melanogaster* and *S. pallida*, is attracted to mustard host-plant odors, including volatile mustard oils (isothiocyanates or ITCs). Our genomic analysis uncovered three *S. flava* paralogs of an olfactory receptor gene (*Or67b*) that likely experienced recent positive selection. We then tested whether these chemoreceptors could underlie the observed attraction to volatile ITCs. Our *in vivo* recordings revealed that two of the *S. flava* Or67b proteins (Or67b1 and Or67b3) – but not the homologous Ors from microbe-feeding relatives – responded selectively and sensitively to volatile ITCs. These Ors are the first ITC chemoreceptors other than TRP channel family (*e.g.*, the TrpA1 'wasabi' receptor) known from any animal. Remarkably, *S. flava Or67b3* was sufficient to drive olfactory attraction toward butyl ITC when expressed in an attractive olfactory circuit. Our study illuminates that ancestrally aversive chemicals can be co-opted as attractants through gene duplication, leading to the origin of hedonic valence shifts in herbivorous insects.

43 **Significance Statement (120)**

44 Plant toxins trigger aversive olfactory (volatile-mediated) and gustatory (contact-mediated)
45 responses in animals. Paradoxically, toxic plants are often colonized by specialist insects that co-
46 opt these toxins as host-plant finding cues. The mechanisms underlying these behavioral shifts,
47 from indifference or repulsion to plant chemicals, to attraction, remain unclear. To address this,
48 we used a fly lineage, *Scaptomyza*, that switched from yeast-feeding to feeding on mustard plants
49 less than 10 million years ago. We found that *S. flava* is attracted to mustard-plant odors and
50 volatile mustard oils (isothiocyanates or ITCs) such as 'wasabi', a behavior enabled by the
51 evolution of genes encoding odorant receptors highly sensitive to ITCs. Our study illuminates
52 how insects colonize toxic host plants through duplication and ecological repurposing of genes
53 encoding pre-existing chemoreceptors.

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55

56

57 **INTRODUCTION**

58 Many plant compounds used in food, agriculture and medicine originally evolved as
59 toxins that deter and repel enemies (1). Among the most well-known compounds are those
60 reactive electrophiles that induce the sensation of pain, such as diallyl disulfide and thiosulfinates
61 produced by Alliaceae plants (*e.g.*, onions and relatives), α,β-unsaturated aldehydes found in
62 Lauraceae (*e.g.*, cinnamon and olives), and isothiocyanates (ITCs) from mustards and their
63 relatives in the Brassicales. Paradoxically, herbivores specializing on Brassicales evolved
64 electrophile diversion or resistance mechanisms and use these plants as a food source and habitat
65 (2, 3). Mustard specialists can be trapped with ITC baits, indicating that these insects use
66 "aversive" electrophiles for host finding (4). However, the mechanisms mediating the detection
67 of species-specific electrophiles and underlying shifts in hedonic valence, are unknown.

68 In animals, the only known and broadly conserved electrophile detecting sensors are
69 members of the Transient Receptor Potential (TRP) nociceptive channel family (5–7), and the
70 most studied among them is the Transient Receptor Potential Ankyrin-1 (TrpA1) (8–10).
71 Reactive electrophiles form covalent adducts with cysteine and lysine residues in TrpA1,
72 activating this non-selective cation channel and resulting in the perception of pain (9). A
73 reasonable hypothesis explaining the attraction of specialist mustard-plant insects to host-plant-
74 derived electrophiles is that the TrpA1 circuit has shifted from an aversive to an attractive one.
75 However, TrpA1 is a gustatory, non-selective electrophile sensor activated by myriad
76 electrophiles produced by plants in different orders, including diallyl disulfide, cinnamaldehyde,
77 and ITCs, rendering this chemoreceptor ineffective in signaling host-plant identity (7).
78 Alternatively, chemoreceptor proteins (*e.g.*, olfactory receptors -Ors- and/or ionotropic receptors
79 -Irs-) that commonly mediate responses to volatile compounds in insects (11, 12) are better

80 suited to evoke responses that signal volatile, host-specific electrophiles. Specificity for
81 ecologically relevant volatiles repeatedly arose through evolution of these olfactory
82 chemoreceptors (13, 14). In *D. sechellia*, a specialist of the toxic "noni fruit", Ir75b evolved high
83 sensitivity to a signature noni volatile through a single amino acid residue substitution (15).
84 Similarly, in other drosophilid species, Or22a (and Or22b) evolved to be narrowly tuned to host-
85 plant volatile esters (16, 17), facilitating host-plant specialization.

86 Here, we investigate the evolutionary and functional mechanisms underlying a major
87 shift in host plant use: olfactory specialization on mustard plants by *S. flava*. *S. flava* is a relative
88 of *D. melanogaster* nested phylogenetically in the paraphyletic *Drosophila* subgenus and is a
89 specialist herbivore of ITCs-producing Brassicales plants (18, 19). In behavioral olfactory
90 assays, we found that *S. flava*, but not its microbe-feeding relatives *S. pallida* or *D.*
91 *melanogaster*, is attracted to volatile ITCs and odors from Brassicales plants. We scanned the
92 genome sequence of *S. flava* to identify rapidly evolving chemoreceptors as candidates
93 underlying this behavioral shift, and identified a lineage-specific expansion of three paralogous
94 copies of the olfactory receptor gene *Or67b* that exhibited signatures of positive selection in the
95 *S. flava* lineage. This *Or*, in contrast, is present as a single copy and is under strong evolutionary
96 constraint in *S. pallida* and *D. melanogaster*. *In vivo* functional testing of Or67b copies from
97 these species shows that two *S. flava* Or67b receptors, but not the conserved Or67b proteins from
98 its microbe-feeding relatives, responded sensitively and selectively to volatile ITCs.
99 Furthermore, *D. melanogaster* flies expressing *S. flava Or67b3* were attracted to butyl ITC in
100 olfactory assays, indicating that this receptor is sufficient for attraction to volatile ITCs in this
101 context. We also found that ITCs are not detected through covalent modification of Or67bs, as
102 opposed to TrpA1 (*e.g.* (7)). Or67b *S. flava* proteins selectively shifted their odor-receptive range

103 to ITCs, an entirely new chemical class, from an ancestral olfactome of ketones, alcohols, and
104 aldehydes. In summary, we discovered an evolutionary mechanism by which specialist
105 herbivorous insects can shift hedonic valence from aversion to attraction towards host plant-
106 specific electrophiles. A novel ITC-sensitive olfactory receptor system supplements the
107 generalist TRP sensory system of a mustard specialist, an adaptation that evolved through gene
108 duplication and neofunctionalization.

109

110 **RESULTS**

111 ***S. flava* is attracted to mustard plant odors and volatile ITCs**

112 Because *S. flava* is a Brassicales specialist, we first determined whether this species is attracted
113 to mustard plant volatiles using a dual-choice olfactory assay (based on (20). We found that both
114 adult male and female *S. flava* are attracted to arugula *(Eruca vesicaria)* leaf odors (two-tailed
115 Binomial tests corrected for multiple testing using false discover rate (FDR), $p<0.05$; Fig. 1). In
116 contrast, *D. melanogaster* was neither attracted nor deterred by arugula odors but instead was
117 strongly attracted to apple cider vinegar volatiles (FDR, $p=0.0001$), in agreement with previous
118 results (*e.g.* (21)). In tests with apple cider vinegar, male *S. flava* preferred the odorless arm of
119 the maze (FDR, $p<0.05$) and females showed a similar tendency (FDR, $p=0.0541$). *S. pallida*
120 exhibited an intermediate behavior, distributing equally between the vinegar-laden and the
121 odorless arms of the maze. Overall, these results demonstrate that *S. flava*, but not *D.*
122 *melanogaster* or *S. pallida*, is attracted to mustard plant volatiles, and avoids odor sources that
123 strongly attract *D. melanogaster*, characterized by the presence of acetic acid and a variety of
124 ester, carbonyl, and hydroxyl-containing compounds (22, 23).

125 Brassicales plants produce ITCs upon tissue damage, which are highly toxic to non-
126 adapted insects (24). Yet, volatile ITCs are effective in capturing a variety of distantly related
127 specialist insects that have evolved mechanisms for coping with or preventing the formation of
128 ITCs during feeding (4). We investigated whether the sole presence of ITCs can mediate
129 olfactory attraction in *S. flava*. Adult female *S. flava* were attracted to both butyl ITC and sec-
130 butyl ITC (Fig. 1). Males, in contrast, were only attracted to butyl ITC, which suggests that at
131 particular ITCs might be involved in mediating sex-specific behaviors. Interestingly, *S. pallida*
132 oriented against butyl ITC (FDR adjusted p-value<0.05), which may be a strategy to avoid ITCs
133 as this species sometimes feed on decomposing – but not intact – mustard plant leaves. In
134 contrast, *D. melanogaster* flies distributed at random between the arms of the maze regardless of
135 the ITC compound tested (p>0.5), indicating that ITCs do not elicit olfactory attraction in this
136 species. In summary, these experiments demonstrate that *S. flava*, but not its microbe-feeding
137 relatives, is attracted to mustard plant volatiles and ITCs.
138

139 **Phylogenetic analysis identified *Or67b* paralogs as candidates mediating the detection of**
140 **ecologically relevant odorants**

141 To identify potential *S. flava* chemosensory receptors involved in the attraction of
142 mustard volatiles, we conducted a phylogenetic analysis of the Or protein sequences of *S. flava*
143 and four other *Drosophila* species to identify *Scaptomyza*-specific losses and gains (Fig. 2*A*).
144 The Or topology was largely congruent with previous *Drosophila* Or trees (25) except for some
145 deeper nodes, which had weak to moderate bootstrap support. In order to identify *Ors* evolving
146 under positive selection in *S. flava*, we inferred d*N*/d*S*, the ratio of nonsynonymous to
147 synonymous substitution rates, using a foreground/background branch test as implemented in

148 PAML (26). Of 75 *S. flava* branches tested, seven branch models (for paralogs of *Or63a, Or67b*
149 and *Or98a*, and a homolog of *Or67d*) indicated an *S. flava* foreground rate >1, consistent with
150 positive selection (Table S1). Of these receptors, homologs of *Or67d* detect the *Drosophila*
151 pheromone 11-cis-vaccenyl acetate (27) and likely function in intraspecific communication in
152 *Scaptomyza*, *Or63a* is only expressed in larvae (28), and the *Or98a*-like genes found in
153 *Scaptomyza* have no *D. melanogaster* homologs and are functionally uncharacterized. In
154 contrast, *Or67b* is expressed in adults and modulates oviposition behavior (29), making it a good
155 candidate. In a previous study, branch-site tests were also consistent with episodic positive
156 selection acting on *Or67b* paralogs from *S. flava* (19). After expanding the representation of
157 *Or67b* homologs in a phylogenetic analysis, and conducting branch tests in PAML on all
158 branches in the tree, we found support for elevated d*N*/d*S* exclusively in *S. flava* and *D.*
159 *mojavensis* branches (Fig. 2*B*; TableS1). Thus, we focused on *Or67b* paralogs for functional
160 studies.

161

162 ***S. flava* Or67b proteins respond specifically to mustard plant volatiles**

163 We wondered whether any of the three *S. flava* Or67b proteins could mediate the odor-
164 specific attraction of *S. flava* to mustard plant volatiles. First, we confirmed that all three
165 paralogs are expressed in adult *S. flava* (Fig. S2*B*). To study the odor-response profile of Or67b
166 across species, we used a *D. melanogaster* mutant that lacks its endogenous olfactory receptor
167 (Or22a) in the ab3A olfactory receptor neuron (known as the "empty neuron system" (30, 31).
168 We conducted single sensillum recordings from the ab3 sensilla in transgenic *D. melanogaster*
169 flies expressing each of the *S. flava* (*Sfla*) *Or67b* paralogs, as well as the *Or67b* orthologs from
170 *S. pallida* (*Spal*) and *D. melanogaster* (*Dmel*) (Fig. 3*A*). *Or22a* deletion in ab3A was confirmed

171 by the lack of responses in ab3A to ethyl hexanoate, a strong Or22a activator (Fig. S4*A*). We
172 found that ab3A neurons expressing any of the three *Sfla Or67b* paralogs responded to all
173 mustard plant volatiles tested (Fig. 3*B*; Mann-Whitney U tests adjusted for multiple comparisons
174 using FDR, $p<0.05$ in all cases). In contrast, neurons expressing *Dmel Or67b* responded strongly
175 to apple cider vinegar (FDR, $p<0.05$), but not to any of the mustard plant volatiles tested (FDR,
176 $p>0.1$). Neurons expressing *Spal Or67b* also showed strong responses when stimulated with
177 apple cider vinegar (FDR, $p<0.05$), but displayed only modest responses to arugula volatiles
178 (FDR, $p<0.05$). Interestingly, stimulation with apple cider vinegar decreased spiking activity of
179 neurons expressing *Sfla Or67b1* (FDR, $p<0.05$).
180 Although neurons expressing any of the *Sfla Or67b* paralogs responded to mustard plant
181 volatiles, they differed in their responsiveness. Stimulation with arugula volatiles, for instance,
182 elicited stronger responses in neurons expressing *Sfla Or67b1* (64 ± 7.8 spikes/sec; average ±
183 SE) than in neurons expressing *Sfla Or67b2* (24 ± 8.7 spikes/sec) or *Sfla Or67b3* (22.3 ± 2.3
184 spikes/sec) (Fig. 3*B*; Kruskal Wallis ANOVA, $p<0.005$, followed by post-hoc Tukey tests,
185 $p<0.05$; n=6 in each group). In contrast, stimulation with horseradish volatiles produced stronger
186 responses in neurons expressing *Sfla Or67b3* (32 ± 4.4 spikes/sec) than in *Sfla Or67b1* (8.75 ±
187 1.3 spikes/sec) or *Sfla Or67b2* (12.8 ± 2.3 spikes/sec) (Fig. 3*B*; Kruskal Wallis ANOVA,
188 $p<0.005$, followed by post-hoc Dunn's tests, $p<0.05$; n=6-9 in each group). Taken together, these
189 results demonstrate that *Dmel Or67b* and *Spal Or67b* are functionally similar and may represent
190 the ancestral phenotype, while *Sfla Or67b* paralogs are divergent and tuned to mustard plant
191 volatiles.
192

193 ***S. flava Or67b* paralogs respond selectively to ITCs and have different molecular receptive**
194 **ranges**

195 Upon herbivore damage, mustard plants release ITCs and nitriles in addition to green leaf
196 volatiles (GLVs) (32). To elucidate which odorants mediate activation of *Sfla* Or67b proteins, we
197 tested a panel of 44 odorants which include ITCs, nitriles, GLVs, *Dmel* Or67b activators bearing
198 various functional groups (33), and four ecologically relevant odorants which do not fall within
199 these categories. Because many compounds were tested, for statistical comparisons we selected
200 the odorants that produced the strongest average response across all Ors within each of the six
201 chemical categories tested (those odorants were butyl ITC, mandelonitrile, cis-3-hexenyl
202 butyrate, trans-2-pentenal, and geranyl acetate (Fig. 3*A* and *C*). Neurons expressing any of the
203 three *S. flava* paralogs responded to stimulation with butyl ITC (FDR, $p<0.05$), but not to
204 stimulation with acetophenone (FDR, $p>0.1$). Conversely, acetophenone evoked the strongest
205 excitatory responses from neurons expressing *Dmel Or67b* and *Spal Or67b* (respectively 175.7 ±
206 17.6 and 78 ± 15.2 spikes/second; average ± SE), while butyl ITC evoked no responses from
207 neurons expressing *Dmel Or67b* (FDR, $p>0.5$), and only produced a slight increase in spiking
208 activity (15 ± 4.2 spikes/second; average ± SE; FDR, $p<0.05$) from neurons expressing *Spal*
209 *Or67b*.

210

211 ***S. flava* Or67b1 and Or67b3 differ in their odor selectivity**

212 Although all *S. flava* paralogs are narrowly tuned to ITCs, they differ in their degree of
213 selectivity and sensitivity. Butyl ITC evoked strong responses in neurons expressing *Sfla Or67b1*
214 and *Or67b3* (196.3 ± 15.2 and 170.6 ± 11.2 spikes/second; average ± SE) that were not different
215 from each other (Kruskal-Wallis ANOVA followed by Dunn's tests; $p>0.05$), while stimulation

216 with this odorant produced only a modest response from neurons expressing *Sfla Or67b2* (26.7 ±
217 2.8 spikes/second; Dunn's tests, in both cases $p<0.05$). None of the 44 odorants tested evoked
218 strong responses from neurons expressing *Sfla Or67b2*, but it is possible that other compounds
219 not tested in this study, and in particular mustard plant volatiles (Fig. 3*C*), activate this receptor.
220 Next, because ITCs are highly diverse in structure (34), we hypothesized that a particular
221 subset of ITCs differentially activate *Sfla* Or67b1 and *Sfla* Or67b3. In order to test this, we
222 stimulated neurons expressing *Sfla Or67b1* and *Or67b3* with various concentrations of six ITCs
223 (3-methyl-thio-propyl ITC, butyl ITC, isobutyl ITC, sec-butyl ITC, benzyl ITC, and phenethyl
224 ITC) that evoked very strong responses (>123 spikes/second) from neurons expressing *Or67b3*
225 at 1:100 vol/vol (Fig. S4). At that concentration, only half of these odorants (3-methyl-thio-
226 propyl ITC, butyl ITC, isobutyl ITC, and benzyl ITC) produced moderate to strong responses
227 (41-147 spikes/second, depending on the odorant) from neurons expressing *Sfla Or67b1*. At
228 1:1,000 vol/vol, the responses of *Sfla* Or67b3 to stimulation with phenethyl ITC, sec-butyl ITC
229 and butyl ITC were different from those of *Sfla* Or67b1 (Mann-Whitney U tests adjusted for
230 multiple comparisons using FDR, $p <0.05$ in all cases). Thus, although both *Sfla* Or67b1 and
231 Or67b3 are highly selective and sensitive to ITCs, they have distinct odorant receptive ranges:
232 *Sfla* Or67b3 is broadly tuned and strongly responds to all ITCs tested, while *Sfla* Or67b1 has a
233 narrower receptive range and responds strongly only to a smaller subset of ITCs.
234

235 **Or67b response relatedness, specificities, and functional evolution**

236 We next compared the responses of all the Or67b proteins to all 44 odorants tested in a
237 multi-dimensional space using principal component analysis (Fig. 4*A*). Each dot in Figure 4A
238 shows the vectors quantifying the control-subtracted odor responses of each Or67b copy plotted

239 along the dimensions of the first three principal components, which captured 63.1%, 26.0%, and
240 9.0 % of the variance of the original high-dimensional data set. This analysis shows that: 1) most
241 of the ITCs cluster separately from the other odorants tested, contributing to most of the variance
242 (PC1) of the data set; 2) some *Dmel* activators and GLVs such as acetophenone and cis-3-
243 hexenyl-butyrate also produced distinct responses, contributing largely to the variance of PC2;
244 and 3) ITCs that activate *Sfla* Or67b1, such as sec-butyl ITC (but not butyl ITC) contribute to the
245 variance of PC3. We also constructed tuning curves for each Or67b protein (Fig. 4*B*). The
246 strongest responses (center of the distribution) from *Dmel* Or67b and *Spal* Or67b were evoked
247 by *Dmel* activators and GLVs, while the strongest responses from all *Sfla* Or67b paralogs were
248 evoked by ITCs. *Sfla* Or67b1 is particularly narrowly tuned to a few ITCs, as revealed by the
249 high kurtosis value of the distribution (Fig. 4*B*). Then we conducted a neighbor joining cluster
250 analysis based on the Euclidean distances between the odor-evoked responses from all the Ors.
251 Among the *S. flava* paralogs, Or67b1 and Or67b3 have the most similar (shorter Euclidean
252 distance) odorant responses (Fig. 4*C*).

253

254 **Activation of *S. flava* Or67b1 and Or67b3 requires the presence of the ITC functional**
255 **group**

256 The finding that *Sfla* Or67b1 and *Sfla* Or67b3 respond to ITCs suggest that both the
257 presence of the ITC functional group (–N=C=S) and the size of the molecule determine binding
258 specificity. These two factors are indeed important to evoke responses from TrpA1, a non-
259 selective electrophile chemoreceptor that relies on ligand-directed covalent modification (10).
260 We addressed whether this is also the case for *Sfla* Or67b1 and *Sfla* Or67b3, by testing their
261 responses to eleven electrophiles (non-ITCs) that activate TrpA1 through covalent linkages with

262 sulfhydryl side chains. Interestingly, none of the non-ITC electrophiles activated neurons
263 expressing *Sfla Or67b1*, and among these, only trans-2-pentenal produced a very small increase
264 in spike activity (4 ± 1.3 spikes/second) from neurons expressing *Sfla Or67b3* (Fig. 3*C*).
265 Additionally, we found that neurons expressing *Sfla Or67b* can respond to odor pulses that are
266 seconds apart (Fig. S5). Because recovery from ligand-induced covalent modifications takes at
267 least several minutes (and often hours) (7), these results suggest that activation of *Sfla* Or67b1
268 and Or67b3 by ITCs does not rely on the TrpA1-like covalent modification strategy.
269 We next investigated whether the presence of the ITC functional group (–N=C=S) is
270 necessary for determining the responses of *Sfla* Or67b1 and *Sfla* Or67b3. In order to test this, we
271 compared the responses of these Ors to benzyl ITC (BITC) and benzyl thiocyanate (-S≡C-N:
272 BTC) (Fig.4*B*), linkage isomers of the same molecular weight. We found that BITC produced
273 robust activity in neurons expressing *Sfla Or67b1* or *Sfla Or67b3* (Mann-Whitney U tests, in
274 both cases $p<0.002$), while BTC had little or no effect (Fig. 3*C* and 4*B*). This differential
275 activation pattern is likely due to the differences in the functional group because BITC and BTC
276 are not only isomers, but also have similar volatilities. These findings are consistent with the
277 hypothesis that *Sfla* Or67b1 and *Sfla* Or67b3 detect ITCs by interacting with the ITC (–N=C=S)
278 functional group selectively.
279

280 ***S. flava Or67b3* is sufficient to induce olfactory attraction to ITCs**

281 Because *Sfla* Or67b paralogs, but not the orthologs from *Spal* and *Dmel*, specifically
282 respond to ITC compounds, we asked whether these receptors can mediate olfactory attraction to
283 these odor compounds. We focused on *Sfla* Or67b3, because this Or has a broader sensitivity to
284 ITCs than *Sfla* Or67b1 (Fig. 4*B*). We investigated this by testing the behavioral responses of *D.*

285 *melanogaster* "empty neuron" mutants that expressed *Sfla Or67b3* or *Dmel Or67b* in the ab3A
286 neuron. As before, we used a dual-choice "Y-shaped" olfactometer offering an odorant stimulus
287 (butyl ITC 1:100 or 1:1,000 vol/vol) in one of the arms, and the solvent control in the other arm.
288 We found that at the 1:100 vol/vol concentration of butyl ITC the odor responses of flies
289 expressing *Sfla Or67b3* or *Dmel Or67b3* were different from the 50% random expectation (two-
290 tailed Binomial tests, $p<0.005$ and $p<0.01$ respectively), with flies orienting towards the odor in
291 ca. 70-71% of tests (Fig. 5). In contrast, at the lowest odor concentration (1:1,000 vol/vol), the
292 odor responses of flies expressing *Sfla Or67b3* – but not that of those expressing *Dmel Or67b* –
293 were different from the 50% random expectation (two-tailed Binomial tests, $p<0.00005$), with
294 flies choosing the odorous side of the maze in 83% of tests. Moreover, at the lower
295 concentration, the odor-responses of flies expressing the *S. flava* transgene were different from
296 those of flies expressing the *Dmel* transgene (Fisher Exact test, $p<0.05$). None of the three
297 genetic control *Dmel* lines showed a preference for either side of the maze (Binomial tests, $p>0.1$
298 in all cases), even at the 1:100 vol/vol concentration. These results demonstrate that *Sfla Or67b3*
299 can confer odor sensitivity to ITCs that persists at low (1:1,000 vol/vol) concentrations in a fly
300 that is otherwise not attracted to these odor compounds.
301

302 **Discussion**

303 Plants have evolved a myriad of toxic compounds to deter enemies which are generally
304 perceived as noxious by animals (1). In spite of this, the vast majority of herbivorous insect
305 species are specialized on one or a few host-plant lineages that contain aversive compounds such
306 as ITCs – the chemicals that give wasabi its characteristic pungent taste (35, 36). Through the
307 course of evolution, specialist insects co-opted these chemicals as cues for host-finding, feeding,

308 and egg laying (37). Here, we investigated how attraction to these ancestrally aversive chemical
309 cues evolved, using *S. flava*, a recently-derived herbivorous fly of mustard plants and their
310 relatives (Brassicales). In behavioral experiments we found that *S. flava*, but neither of its
311 microbe-feeding close relatives *S. pallida* or *D. melanogaster*, is attracted to mustard-plant
312 volatiles, including volatile ITCs (Fig. 1). We next searched for changes in chemoreceptor
313 proteins that could underlie this host plant-specific olfactory attraction. We found a candidate
314 olfactory receptor gene (*Or67b*), which was triplicated in a recent ancestor of *S. flava*, resulting
315 in three divergent and paralogous odorant receptor genes (*Sfla Or67b1-b3*; Fig. 2). These *Sfla*
316 Or67b paralogs evolved with rapid rates of amino-acid changing mutations, consistent with
317 episodic positive selection (Fig. 2*B*; TableS1). Next, using the "empty neuron" system of *D.*
318 *melanogaster*, we investigated the odor-response profiles of these receptors. Consistent with our
319 behavioral results, we found that two of the *Sfla* Or67b paralogs (*Sfla* Or67b1 and *Sfla* Or67b3)
320 specifically respond to stimulation with mustard-plant odors and volatile ITCs (Fig. 3 and Fig
321 S5). In contrast, *S. pallida* and *D. melanogaster* paralogs responded strongly to stimulation with
322 apple cider vinegar and a broad range of aldehydes, alcohols and ketones (Fig. 3 and Fig. 4),
323 consistent with their microbe-feeding habits. We also found that the presence of the ITC
324 functional group is necessary for activation of *Sfla* Or67b and furthermore, that activation of the
325 receptor likely does not involve a TrpA1-like covalent modification (Fig. 3, Fig. 4 and Fig. S5
326 (7)). Remarkably, when *Sfla Or67b3* was expressed in ab3A "empty neuron" system of *D.*
327 *melanogaster*, adult female flies were attracted to butyl ITC (Fig. 5). This suggests that *Sfla*
328 *Or67b3* may be sufficient for the behavioral valence shift observed in the evolution of mustard-
329 feeding *Scaptomyza*, when expressed in novel neural circuit that ancestrally mediates attraction.
330 Our results reveal the first Ors characterized from any animal that are tuned to mustard oils, and

331 illustrate an evolutionary mechanism by which mustard specialists can evolve attraction towards
332 otherwise aversive chemical compounds.

333 The gene triplication event that occurred in the *S. flava* lineage raises several interesting
334 molecular evolutionary questions. Gene duplication plays an important role in evolutionary
335 innovation (38) and several outcomes have been proposed regarding to how gene duplication
336 contributes to acquisition of novel gene functions (38, 39): (A) neofunctionalization occurs when
337 one of the two duplicated genes (paralogs) acquires a new function after accumulating *de novo*
338 mutations, while the other copy retains its ancestral function; (B) subfunctionalization is largely
339 considered a degenerative process whereby mutations accumulate in both copies, leading to
340 partitioning of the ancestral function between the two gene copies; (C) specialization occurs
341 when subfunctionalization and neofunctionalization evolve simultaneously, yielding gene copies
342 that are "functionally distinct from each other and from the ancestral gene" (40, 41). The
343 specialization model is supported by our data, as neither of the two outgroup Or67b proteins
344 (*Spal* Or67b and *Dmel* Or67b) responded to ITCs (neofunctionalization), coupled with the fact
345 that *Sfla* Or67b1 has a narrower breadth of tuning to ITCs than *Sfla* Or67b3
346 (subfunctionalization). Our conceptual model for this evolutionary hypothesis (Fig. 5) is that the
347 ancestral Or67b ("1") was broadly tuned to acetophenone, alcohols and ketones, and diverged
348 into a mustard oil-specific Or67b ("2") that lost sensitivity to these compounds and gained
349 responsiveness to ITCs in the *S. flava* lineage. This *Or* triplicated during the course of evolution,
350 ultimately giving rise to three Ors with different odor selectivity ("3"): (a) *Sfla* Or67b3, which
351 retained broad sensitivity to ITCs; (b) *Sfla* Or67b1, which become narrowly tuned to a subset of
352 *Sfla* Or67b3 activators; and (c) *Sfla* Or67b2, which lost sensitivity to a broad range of ITCs (at

353 least all the nine ITC compounds tested here) and likely retained or gained sensitivity to untested
354 compounds.

355 Across *Drosophila*, orthologous chemoreceptors respond in a species-specific manner to
356 ecologically relevant ligands. The best-studied of these is the *Or22a* gene, which has been
357 evolutionarily deleted in *S. flava* but not in *S. pallida (19)*. In several drosophilids, Or22a is
358 tuned to different ecologically relevant esters: ethyl-hexanoate in *D. melanogaster* (16), methyl-
359 hexanoate in *D. sechellia* (42), 3-methyl-2-butenyl acetate in *D. erecta* (17), and isobutyl acetate
360 in *D. suzukii* (43). Similarly, Ir75b is tuned to butyric acid in *D. melanogaster* and to hexanoic
361 acid in *D. sechellia* (15). The fact that the chemical classes are still conserved for each
362 chemoreceptor (i.e. esters in the case of Or22a and acids in the case of Ir75b), suggests that the
363 chemical niche space and underlying sensory mechanisms are relatively conserved across
364 species. In contrast, while *Spal* Or67b and *Dmel* Or67b respond to alcohols, aldehydes and
365 ketones, *Sfla* Or67b1 and *Sfla* Or67b3 respond selectively to volatile ITCs, thus acquiring a
366 previously undescribed sensitivity to an entirely different chemical compound class (Fig. 6*b*).
367 We hypothesize that the striking difference between Or67b-activating ligands among these
368 species resulted from the dramatic shift from feeding on microbes to feeding on live mustard
369 plant tissue that occurred in the evolution of herbivorous *Scaptomyza*.

370 TrpA1, which mediates taste-aversion to ITCs, is one of the most well-studied contact
371 chemoreceptors (7, 8, 10). However, volatile ITCs are widely used to trap agricultural pests of
372 Brassicales (4), and in agreement with this, the antennae of some of these insects respond to
373 volatile ITCs (44). Our study further advances the understanding of how volatile ITCs may be
374 detected by these insects. Furthermore, we uncovered a potential evolutionary mechanism, gene
375 duplication followed by neofunctionalization and subfunctionalization, by which ITC-responsive

376 Ors evolved in Brassicales specialists. More generally, our results suggest that major host shifts
377 and specialization events in herbivorous insects can result from simple genetic changes of large
378 effect in the peripheral nervous system, similar to the apple maggot fly *Rhagoletis pomonella*
379 (45). This in turn may explain why such events are so common across herbivorous insect
380 lineages, resulting in rapid rates of diversification and the most diverse guild of macroscopic life
381 to have evolved (46, 47).
382
383
384 **Material and Methods:**

385 Material and methods are summarized below. For complete details, see Supplemental Methods.
386 **Fly lines**
387 M2-MD refers to a CRISPR/Cas9-mediated deletion of *Or22a/b* and a knock-in of *Gal4*
388 and *DsRed* by homologous repair. *Gal4* is not functional but *DsRed* is expressed in the eye. The
389 functional absence of *Or22a* and *Or22b* genes in M2-MD flies was confirmed by
390 electrophysiological analysis on *Or22a/b* expressing neurons in wild-type flies (Fig. S3). The
391 four *UAS-Or67b* lines created in this study, the *UAS-Dmel Or67b* line, and the *UAS-dTrpA1*
392 (stock no. 26264) line was each crossed into the M2-MD line. The progeny were then used for
393 SSR recordings and behavioral experiments.
394
395 **Behavioral tests of olfactory attraction**
396 The olfactory responses of mated, non-starved *D. melanogaster* (Canton-S or transgenic),
397 *S. pallida*, and *S. flava* were tested using a dual-choice "Y-shaped" olfactometer based on one
398 previously published (20). Insects could walk upwind towards the "decision point" (intersection

399 of the short and long arms of the "Y") and turn towards either the odor-laden or the odorless arm
400 of the maze. Four insects were released at once (to increase experimental efficacy), but only the
401 first choice was considered.

402 The odorants (20 µl of 1:100 vol/vol mineral oil solution) used in experiments were butyl
403 ITC (Sigma-Aldrich, CAS # 592-82-5, USA) and sec-butyl ITC (Sigma-Aldrich, CAS # 15585-
404 98-5, USA). For tests with transgenic flies, responses were tested at 1:100 vol/vol and 1:1,000
405 vol/vol of butyl ITC. We also used apple cider vinegar (40 µl, O Organics, USA; 40 µl of
406 distilled water was as control stimulus in these tests). For tests of host orientation, two-four
407 leaves from young arugula plants grown in an insect and insecticide/pesticide free chamber or
408 greenhouse were excised just before tests.

409 For each odor/odorant, species, sex and genotype, the number of tests with insects
410 orienting towards the odorous arm and the odorless arm of the Y-maze was used to conduct two-
411 tailed Binomial tests (48). When appropriate, p-values were adjusted for multiple
412 testing/comparisons using the false discovery rate (FDR) method of Benjamin-Hochberg (49);
413 results were considered statistically significant if the Benjamini-Hochberg adjusted p-value was
414 <0.05. For all tests, we verified that the power was >0.8.

415

416 **Molecular phylogeny of drosophilid odorant receptors:**

417 Translations of *Ors* from *D. grimshawi*, *D. mojavensis, D. virilis* and *D. melanogaster*
418 (builds dgri r1.3, dmoj r1.3, dvir r1.07 and dmel r6.28, respectively) were downloaded from
419 Flybase (www.flybase.org, (50)) and *S. flava* sequences were obtained from ref. (19). Three
420 hundred and nine sequences were aligned in MAFFT v7.017 (51). Models were fitted to the
421 alignment using IQ-Tree and tested using the AIC criterion (52). A maximum likelihood (ML)

422 phylogeny was generated using the Or protein alignment in RAxML v8.2.10 (53). Orco
423 sequences were designated as the outgroup.

424

425 **Molecular phylogeny of drosophilid *Or67b* genes:**

426 *Or67b* CDS from *D. grimshawi*, *D. mojavensis, D. virilis D. sechellia, D. simulans*, *D. erecta, D.*
427 *yakuba, D. pseudoobscura, D. persimilis, D. ananassae, D. willistoni* and *D. melanogaster*
428 (builds dgri r1.3, dmoj r1.3, dvir r1.07, dsec r1.3, dsim r1.4, dere r1.3, dyak r1.3, dpse r3.2, dper
429 r1.3, dana r1.3, dwil r1.3 and dmel r6.28, respectively) were downloaded from Flybase
430 (www.flybase.org, (50)). The *S. pallida* DNA sequence was obtained through PCR. Sequences
431 were aligned, models fitted and chosen according to AIC (GTR+I+G) in IQ-Tree (52). Trees
432 were inferred using RAxML (v8.2.10) with the GTRCATI model and 1000 rapid bootstraps, and
433 MrBayes (v3.2.6) (54).

434

435 **Analysis of molecular evolution:**

436 CDS of homologs of every *Or* gene in *S. flava* found in the 12 *Drosophila* genome builds were
437 aligned to *S. flava Or* CDS. Homology was assessed according to inclusion in well supported
438 clades in the *Or* translation phylogeny above. Sequences were aligned in MAFFT (v7.017) (51).
439 Phylogenies were generated for every alignment using PhyML (55). If these trees showed >70%
440 bootstrap support for a topology contrary to the known species topology, or if the Or homology
441 group contained duplicates, these trees were used in PAML analyses instead of the species tree.
442 Branch models of sequence evolution were fit using PAML 4.9h (26). A foreground/background
443 branch model was fit for every *S. flava* tip branch and every ancestral branch in a *Scaptomyza*-

444 specific *Or* gene duplication clade, and compared in a likelihood ratio test to a null model with
445 one d*N*/d*S* rate for every unique phylogeny (total: 75 tests). After focusing on *Or67b*, patterns of
446 molecular evolution among the drosophilid *Or67b* homologs were explored using the expanded
447 *Or67b* CDS phylogeny above. Foreground/background branch models were fit for every branch
448 in the *Or67b* phylogeny with identical likely ratio tests performed as above (29 tests total Fig.
449 2*B*; Table S1). P-values were adjusted for multiple testing using the FDR method (49).

450

451 **Distance tree based on SSR recordings from neurons expressing *Or67b* transgenes**

452 A matrix of average responses from five *Or67b* transgene receptors to 44 odorant compounds
453 (1:100 vol/vol concentration) was produced (Table S3). Net responses were obtained by
454 subtracting the response to mineral oil or dimethyl sulfoxide solvents. A Euclidean distance
455 matrix was generated using the *dist* function from the R stats package (56). Receptor responses
456 were clustered by using the neighbor joining (NJ) algorithm on this distance matrix. Support for
457 clusters was assessed using 1,000 bootstraps of the original response matrix by generating
458 distance matrices and NJ trees on the pseudo-datasets in ape (v5.3) (57).

459

460 **Single sensillum recordings (SSR)**

461 Adult female flies were fed and then prepared for SSR as previously described (58).
462 Extracellular activity was recorded by inserting a tungsten electrode into the base of the ab3
463 sensillum. The following odor sources (all purchased in Berkeley, California, US unless
464 otherwise mentioned; 20 µl of material loaded on filter paper) were used: apple cider vinegar (O
465 Organics, USA), grated roots of *Wasabia japonica* (wasabi), organic roots of *Armoracia*
466 *rusticana* (horseradish), *Brassica rapa* (turnip), *Raphanus sativus* (daikon), and *Beta vulgaris*

467 (beet). *Eruca vesicaria* (arugula) was grown from seeds in the lab, and leaves of 3-8 weeks old
468 plants were used for odor stimulation. Roots or leaves were grated and homogenized using a
469 vegetable grater to a volume equivalent to ~500 µl. The "net number of spikes" were obtained by
470 counting the number of spikes during a 1-second window 0.2 seconds after the onset of
471 stimulation, and subtracting from this number the background spiking activity (number of spikes
472 during 1-second previous to the onset of odor stimulation). Data were analyzed using Mann-
473 Whitney Rank sum tests (for comparisons involving two means) and p-values were adjusted for
474 multiple comparisons using the FDR method, or by Kruskal-Wallis ANOVAs followed by
475 Dunn's or Dunnet's tests (for comparisons involving more than two means).

476

477 **Data Analysis and figure generation:**

478 All images and drawings are originals prepared by the authors. Figures were prepared via a
479 combination of WinEDR (v3.9.1), R Studio (v1.2.1335), Microsoft Excel (2016), Adobe
480 Illustrator (2019), Python, and Geneious (10.0.9).

481

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517 **Figure Legends:**

518

519 **Figure 1: Olfactory behavioral responses of *S. flava* and its microbe-feeding relatives *S.***
520 ***pallida* and *D. melanogaster* to mustard oil volatiles and ITCs.** (A) Schematic representation
521 of the dual-choice maze used to test the olfactory responses of flies. One arm of the maze was
522 connected to a 1-ml syringe containing a piece of filter paper loaded with an odorant (20 µl of a
523 1:100 vol/vol solution diluted in mineral oil) or apple cider vinegar (40 µl), while the other arm
524 was connected to a syringe containing a piece of filter paper loaded with the control stimulus (20
525 µl of mineral oil or 40 µl of water). In the case of arugula, 2-4 leaves were excised from a plant
526 just before tests and placed in a 5-ml syringe (the control syringe contained clean tissue paper).
527 In each test a group of non-starved flies (n=4) was anesthetized in ice during 50-60 seconds and
528 allowed to choose between the odor-laden or the odorless arm of the maze. Each test (maximum
529 duration=5 min) ended when the first insect (out of the 4 released) made a choice for one or the
530 other arm of the maze. (B) Olfactory behavioral responses of *D. melanogaster*, *S. pallida* and *S.*
531 *flava* to the odors and odorants indicated to the right. Data represent the proportion of tests in
532 which insects choose the odorous arm of the maze, and the error bars indicate the 95%
533 confidence interval of the proportion; the dotted reference line at 0.5 indicate no preference for
534 the odorous or odorless arm of the maze. Numbers between parentheses indicate the number of
535 tests with choices, either for the odorless or the odorous arm of the maze (on average, insects
536 made a choice in 82% of the tests). For each fly species and odor, data was analyzed using two-
537 tailed Binomial tests adjusted for multiple testing using FDR (adjusted p-values: * $p<0.05$, **
538 $p<0.01$, *** $p<0.005$, **** $p<0.001$). *S. flava* was attracted to mustard plant volatiles (arugula)
539 and ITCs and avoided apple cider vinegar volatiles, while *S. pallida* avoided butyl ITC. *D.*
540 *melanogaster* was attracted to vinegar but not to arugula volatiles or ITCs.

541 **Figure 2: Maximum likelihood (ML) phylogeny of *Or67b*s in Drosophilidae.** (A)
542 ML phylogeny reconstructed from protein translations of the *Ors* found in *S. flava*, *D.*
543 *melanogaster*, *D. grimshawi*, *D. virilis* and *D. mojavensis* genomes. Line width of branches are
544 proportional to bootstrap support. Green branches indicate *Scaptomyza Ors*. Gene names with
545 bold fonts include branches with estimated d*N*/d*S* >1 from branch tests conducted in PAML,
546 consistent with episodic positive selection. (B) ML phylogeny reconstructed from coding
547 sequence of *Or67b* orthologs of twelve *Drosophila* species, *S. pallida* and *S. flava*. All bootstrap
548 supports for the nodes are >80% and all posterior probabilities were >0.95 for the MrBayes tree.
549 Branches with significant support (FDR p-value< 0.05) for d*N*/d*S* values different from the
550 background rate are indicated with colored branch labels (blue where the foreground rate is less
551 than the background, and red where d*N*/d*S* is greater than the background). Only *S. flava* and *D.*
552 *mojavensis* branches have significantly elevated d*N*/d*S* according to branch model tests. *S.*
553 *flava*, *S. pallida* and *D. melanogaster* branches are indicated by fly icons colored as in Figure
554 1. Scale bars are in units of substitutions per site.
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564 **Figure 3: Odor responses of orthologous and paralogous Or67b from *D. melanogaster*, *S.***
565 ***pallida* and S. *flava* to mustard-plant volatiles.**
566 (A) *Or67b* paralogs and orthologs were expressed in a *D. melanogaster* mutant that lacks its
567 endogenous *Or* (*Or22a*) in the ab3A olfactory receptor neuron (30). Shown is a representative
568 trace of a single sensillum recording obtained from a *D. melanogaster* fly with *Dmel Or67b*
569 expressed in the ab3A neuron (*Or22a*$^{-/-}$ *Or22b*$^{-/-}$; *Or22a-Gal4/UAS-Dmel Or67b*) (top left).
570 Recordings from the ab3 sensilla produced spike trains from the two olfactory receptor cells
571 housed within this sensilla (ab3A, which expresses *Or67b*, and ab3B, which expresses the native
572 *Or85b*) that had distinct spike amplitudes. The ab3A neuron (labeled A) produced spikes of
573 larger amplitude than the ab3B neuron (labeled B). Scale bar: 10 mV. Bottom: representative
574 electrophysiological recordings obtained from the ab3 sensilla of flies expressing *Dmel Or67b*,
575 *Spal Or67b*, *Sfla Or67b1*, *Sfla Or67b2* or *Or67b3* in the ab3A neuron in response to stimulation
576 with acetophenone, butyl ITC, sec-butyl ITC and mineral oil (solvent control). The bars below
577 records indicate the onset and duration (1 sec) of the odor stimulation. Calibration bars: 10 mV.
578 (B) Responses (net number of spikes/second; average ± SE, n= 6-8 sensilla obtained from 2-4
579 females) evoked by stimulation with apple cider vinegar, mustard plant volatiles and non-
580 mustard plant volatiles (beet, control). Asterisks indicate significant differences between the
581 responses to stimulation with an odor and the control stimulus (beet) (Mann-Whitney U tests
582 corrected for multiple comparisons using FDR; adjusted p-values: * $p<0.05$; ** $p<0.01$; ***
583 $p<0.005$). Note that neurons expressing *Dmel Or67b* and *Spal Or67b*, but not those expressing
584 any of the *S. flava Or67b* paralogs, respond to apple cider vinegar. Conversely, only neurons
585 expressing *S. flava Or67b* paralogs respond to mustard plant volatiles; neurons expressing *Sfla*
586 Or67b3 responded to all mustard plant volatiles tested. (C) Responses (net number of spikes/sec;

587 average ± SE, n= 6-20 for each odorant, n=6-15 for mineral oil, obtained from 2-7 females)
588 evoked by stimulation with single odorants categorized as follows: *Dmel* Or67b activators
589 (Database of Odor Responses, (33); orange), ITCs (green), nitriles (blue), green leaf volatiles
590 (GLVs, purple), electrophiles/*TrpA1* activators (pink), other odorants not belonging to any of the
591 previous categories (gray), and the two solvent controls (black). The responses of the strongest
592 activator within each category (acetophenone, butyl ITC, mandelonitrile, cis-3-hexenyl butyrate,
593 trans-2-pentenal, and geranyl acetate) were compared against the mineral oil control. Asterisks
594 indicate significant differences between the responses to stimulation with each of these odorants
595 and the control stimulus (statistics as described in B). Note that ITCs strongly activate neurons
596 expressing *Sfla Or67b1* or *Sfla Or67b3*, and that for the most part these Ors do not respond to
597 any of the other odorants tested. Some ITCs, such as sec-butyl ITC and phenethyl ITC, only
598 activated neurons expressing *Sfla Or67b3*. *Spal* Or67b has an odorant response profile similar to
599 that of *Dmel* Or67b, responding mostly to stimulation with *D. melanogaster* activators and
600 GLVs.
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609 **Figure 4: Or67b orthologs and paralogs have distinct and non-overlapping odor-response**
610 **profiles, tuning breadths, and functional relatedness.** (A) Vectors quantifying the odor-
611 evoked responses (control-subtracted) were projected onto a two-dimensional subspace
612 determined by the first three principal components of the data-set (left: PC1 vs PC2; right: PC1
613 vs PC3). PC1-3 captured each 63.1%, 26.0%, and 9.0% of the variances of the original five-
614 dimensional data. Each dot represents one odorant, and the different colors and symbols indicate
615 different chemical categories (as in Figure 3C and in the inset, right graph). (B) Tuning curves of
616 each Or67b copy, showing the distribution of mean responses (n= 6-20) to the 44 odorants tested
617 (color-coded as in A). The odorants are displayed along the horizontal axis according to the
618 responses (net spikes/sec) they elicit from each Or. The odorants eliciting the strongest responses
619 for each Or are located at the center of the distribution, while weaker activators are distributed
620 along the edges of the distribution. Thus, the order of the odorants is different for the five Ors.
621 Note that the strongest responses (center of the distribution) from *Dmel* Or67b and *Spal* Or67b
622 are evoked by *D. melanogaster* activators and GLVs, while the strongest responses from all *Sfla*
623 Or67b paralogs are evoked by ITCs. The tuning breadth of each Or is quantified by the kurtosis
624 value (k) of the distribution with higher values indicating narrower odor-response profiles. The
625 chemical structure of the top six *Sfla* Or67b3 activators and benzyl thiocyanate (numbers in
626 green) are indicated to the right. (C) Neighbor joining cluster analysis for the five Or67b
627 orthologs based on the Euclidean distances between the odor-evoked responses of the receptors.
628 *Dmel* Or67b and *Spal* Or67b are consistently separated from the *S. flava* odor response profiles,
629 and *Sfla* Or67b1 and *Sfla* Or67b3 are consistently joined, having similar response profiles.
630
631

632 **Figure 5: *S. flava Or67b3* confers olfactory sensitivity to ITCs**

633 The responses of *D. melanogaster* flies expressing *Dmel Or67b* or *Sfla Or67b3* under the control
634 of *Gal4* in the "empty" neuron (ab3A neuron) and three control parental lines were conducted as
635 described in the caption to Figure 1. Genotypes are indicated at the bottom. One arm of the maze
636 was connected to a 1-ml syringe containing a piece of filter paper loaded with 20 µl of 1:100
637 vol/vol (gray circles) or 1:1,000 vol/vol (black rectangles) of butyl ITC, while the other arm was
638 connected to a syringe containing a piece of filter paper loaded with 20 µl of mineral oil. Data
639 represent the proportion of tests in which insects choose the odorous arm of the maze; the
640 deviation bars indicate the 95% confidence interval of the proportion. The dotted reference line
641 at 0.5 indicate no preference for either arm of the maze; the numbers between parenthesis
642 indicate the number of tests with choices for one or the other arm. Control flies (first three
643 columns) showed no preference for either arm of the maze, even when tested with the highest
644 concentration of butyl ITC (two-tailed Binomial tests, in all cases $p>0.1$). Note that flies carrying
645 the *UAS-Sfla Or67b3* transgene under the control of *Gal4* in the ab3A neuron were attracted to
646 both concentrations of butyl ITC (two-tailed Binomial tests, *** $p<0.005$; **** $p<0.001$). In
647 contrast, flies carrying the *UAS-Dmel Or67b* transgene under the control of *Gal4* were attracted
648 only to the highest concentration of the odorant (two-tailed Binomial tests, **$p<0.01$). At the
649 lowest concentration, the response of flies carrying the *S. flava* transgene was different from the
650 response of flies carrying the *D. melanogaster* transgene (Fisher Exact test, $p<0.05$). These
651 results indicate that in these flies, *Sfla Or67b3* is sufficient to confer attraction to butyl ITC, a
652 behavior that persists at low concentrations.

653

654

655 **Figure 6: A model for the evolution of *Or67b* and comparison of tuning curves of**
656 **orthologous Or22a, Ir75b and Or67b** (A) The evolution of *Scaptomyza Or67b* begins with a
657 shift in the tuning breadth and ligand specificity of an ancestral Or67b, from *Dmel* activators and
658 GLVs to ITCs (neofunctionalization). Subsequent gene triplication of *Sfla Or67b* gave rise to
659 three paralogous *Or67b* genes (*Sfla Or67b1, Sfla Or67b2,* and *Sfla Or67b3*). *Sfla* Or67b1
660 evolved to become more narrowly tuned, specializing in the detection of a few ITCs
661 (subfunctionalization), while *Sfla* Or67b3 kept its ancestral, more broadly tuned state, responding
662 to a broad range of ITCs. The ligands and tuning breadth of *Sfla* Or67b2 remain unresolved in
663 this model. (B) The ligand specificities of orthologous Or22a, Ir75b, and Or67b. The Or22a
664 orthologs from *D. melanogaster* (*Dmel* Or22a), *D. sechellia* (*Dsec* Or22a), *D. erecta* (*Der*
665 Or22a), and *D. suzukii* (*Dsuz* Or22a) are all strongly activated by the host-derived esters
666 indicated (top left; ref. 16, 17, 42, 43). The Ir75b orthologs from *D. melanogaster* (*Dmel* Ir75b),
667 and *D. sechellia* (*Dsec* Ir75b) are strongly activated by the two acids indicated (top right; ref.
668 15). *Dmel* Or67b and *Spal* Or67b are strongly activated by acetophenone, while *Sfla* Or67b
669 copies are activated by ITCs only (bottom). Note that Or22a and Ir75b orthologs are both
670 divergent but activated by ligands belonging to a single chemical class (esters and acids,
671 respectively). In contrast, the ligands of orthologous Or67b from *Dmel* and *Spal* are responsive
672 to a variety of chemical classes which include alcohols, aldehydes and ketones, whereas *Sfla*
673 Or67b orthologs are responsive to an entirely different chemical class (ITCs).
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831 **Supplemental Figure Legends:**

832

833 **Supplemental Figure 1: Protein alignment of *D. melanogaster*, *S. pallida* and *S. flava***
834 **Or67bs.**

835 Note the high number of identical amino acids across orthologs/paralogs (black squares). Also
836 shown are each of the seven predicted transmembrane domains (TM1-7). Darker colors illustrate
837 higher degrees of sequence similarity, and lighter colors denote residues with high variability in
838 sequence across paralogs and orthologs.

839

840 **Supplemental Figure 2: *Or67bs* are expressed in *Scaptomyza* spp.**

841 (A) Amplification of *Marf* from cDNA generated from whole body extracts of *S. flava* and *S.*
842 *pallida* adults (+RT). As expected, *Marf* does not amplify in templates treated with DNaseI
843 without reverse transcriptase (– RT). (B) Amplification of *Or67b* genes from *S. pallida* and *S.*
844 *flava* whole adult cDNA, indicative of *in vivo* transcription of *Or67b* genes in adult *Scaptomyza*.
845 Ladder is Gene Ruler™ 1 kb plus (ThermoFisher, USA).

846

847 **Supplemental Figure 3:**

848 (A) Lack of *Or22a* expression in the ab3A neuron in the M2-MD fly line ("empty neuron"
849 mutant) was verified by lack of electrophysiological responses to ethyl hexanoate (left). In
850 contrast, in wild-type flies (Canton-S), ab3A neurons respond strongly to simulation with this
851 odor compound (right). Data indicates the net number of spikes/sec (average ± SE, n= 6-7 for

852 each odor and control stimulation, obtained from 3 females). (B) Single odorants used for
853 statistical analysis in Fig. 3 were tested on the mutant ab3A neuron lacking its endogenous
854 olfactory receptor. Stimulation with any of the odorants failed to evoke spiking activity. (C)
855 *dTrpA1* was expressed in the ab3A neuron in the M2-MD fly line. None of the volatile ITCs or
856 electrophiles elicited spiking activity. For a list of chemical categories see Fig. 3.
857

858 **Supplemental Figure 4: Odorant dose–responses of *Sfla* Or67b1 and *Sfla* Or67b3**

859 Responses (net spikes/sec, average +/- SE, n=6, obtained from 2 animals) of *Sfla* Or67b1 and
860 *Sfla* Or67b3 to stimulation with increasing concentrations (vol/vol) of six different ITCs. The
861 responses of *Sfla* Or67b3 steadily increased with increasing the concentration of all ITCs tested.
862 *Sfla* Or67b1 and *Sfla* Or67b3 showed similar dose-response relationships in response to
863 stimulation with 3-methyl-thio-propyl-ITC, butyl ITC, and benzyl ITC. Stimulation with sec-
864 butyl ITC and phenethyl ITC produced only a very slight increase in spike activity (<10
865 spikes/second) from *Sfla* Or67b1 at any of the concentrations tested. The asterisks indicate
866 significant differences between the responses to the 1:10,000 vol/vol concentration and the
867 responses to the mineral oil control (Mann-Whitney U tests corrected for multiple testing using
868 FDR; adjusted p-values: * $p<0.05$).
869

870 **Supplemental Figure 5: *Sfla* Or67b3 has fast recovery.**

871 Representative electrophysiological recording obtained from the ab3 sensilla of flies expressing
872 *Sfla Or67b3* in response to stimulation with iso-butyl ITC following sec-butyl ITC with one
873 second interval between chemical administration. The horizontal bar denotes one second and the

874 vertical bar denotes 10 mV. *Sfla* Or67b3 sensitivity is recovered within seconds after stimulation
875 with ITCs.
876
877 **Supplemental Material and Methods:**
878 **Fly husbandry and lines**
879 *D. melanogaster* (Canton-S) were reared using standard cornmeal media, yeast, and agar
880 medium. Isofemale lines of *S. pallida* (collected in Berkeley, California, US) were maintained on
881 Nutri-Fly medium (Genesee Scientific). *S. flava* (collected in New Hampshire, US) were
882 maintained on fresh *Arabidopsis thaliana* and 10% honey water solution. All flies were cultured
883 at 23°C and 60% relative humidity under a 12-h light/12-h dark cycle. *S. flava* and *S. pallida*
884 were about 7-10 days old at the time of experiments; *D. melanogaster* (wild-type or transgenic)
885 were about 3-10 days old at the time of the experiments.

886 M2-MD refers to a CRISPR/Cas9 deletion of *Or22a/b* and a knock-in of *Gal4* and *DsRed*
887 by homologous repair. *Gal4* is not functional but *DsRed* is expressed in the eye. The functional
888 absence of *Or22a* and *Or22b* genes in M2-MD flies was confirmed by electrophysiological
889 analysis on *Or22a/b* expressing neurons in wild-type flies (Fig. S3). The M2-MD line was used
890 to generate flies expressing *Dmel Or67b*, *Spal Or67b* and *Sfla Or67b1-3* under the control of
891 *Gal4* in the ab3A "empty neuron". The *UAS-SflaOr67b1, UAS-Sfla Or67b2, UAS-Sfla Or67b3,*
892 and *UAS-Spal Or67b* strains were generated during this study.
893

894 ***Scaptomyza Or67b* gene cloning, UAS line generation, and verification of *S. flava Or67b***
895 **transcription**

896 The *UAS-Or67b* transgene lines were constructed as follows: RNA was extracted from
897 20–25 male and female, laboratory-reared, adult flies of *S. pallida* (collected from the White
898 Mountains, New Mexico) and *S. flava* (collected from near Portsmouth, New Hampshire). RNA
899 was extracted using Trizol (Thermo-Fisher, Waltham, MA) and precipitated with isopropanol.
900 Extracted RNA was treated with DNaseI; cDNA was then generated using qScript cDNA
901 Supermix (Quantabio, Beverly, MA). Absence of gDNA in cDNA preparations was verified by
902 attempting to amplify fragments of the *Marf* gene from reactions lacking reverse transcriptase
903 (Fig. S2; PCR conditions and primers detailed in (59)). CDS plus 7–9 bp of untranslated
904 sequence were amplified using High Fidelity Phusion Taq (New England BioLabs, NEB), 3%
905 DMSO v/v, and the PCR primers (Table S2) with the following program: initial denaturing at
906 98°C during 30 sec; 35 cycles at 98°C for 10 sec, 58°C for 30 sec, 72°C for 45 sec, and
907 extension at 72°C during 7 min. PCR fragments of the expected size were purified using
908 Qiaquick Gel purification kit protocol (Qiagen). An overhang was added to purified *Or67b*
909 amplicons with Taq polymerase (Fermentas) and cloned using the pGEM-T Easy cloning kit
910 protocol (Promega). Plasmids were extracted and purified using the GenElute plasmid miniprep
911 kit (Sigma-Aldrich, St. Louis, MO). EcoRI and KpnI cut sites were introduced using restriction
912 enzyme cut-site primers (Table S2) with 10 ng/µL diluted plasmids (as template) with 3%
913 DMSO vol/vol and the following program: initial denaturing at 98°C for 30 sec; 35 repetitions of
914 98°C for 10 sec, 55°C for 50 sec; 72°C for 45 sec; and final extension at 72°C for 7 min. The
915 pUAST attB plasmid (60) and the four *S. pallida* and *S. flava Or67b* PCR amplicons with RE
916 flanking sites were individually double-digested with KpnI and EcoRI high-fidelity enzyme in

917 cut smart buffer for 3 hours, according to the manufacturer's protocol (NEB). Cut fragments
918 were gel-purified using the Qiaquick Gel Cleanup Kit (Qiagen) and ligated in a 1:3 vector:insert
919 molar ratio using T4 ligase (Promega). Ligations were transformed into JM109 cells. Some cells
920 were preserved as glycerol stocks and a portion were sent for injection into the y^1 w^{67c23};
921 P{CaryP}attP2 fly line (BestGene Inc., Houston, Texas, USA). Transformants were selected
922 from individually injected flies with eye color rescue phenotypes. The four *UAS-Or67b* lines
923 created in this study, the *UAS-Dmel Or67b* line, and the *UAS-dTrpA1* (stock no. 26264) line was
924 each crossed into the M2-MD line. The progeny were then used for SSR recordings and
925 behavioral experiments.

926

927 **Behavioral tests of olfactory attraction**

928 The olfactory responses of mated, non-starved *D. melanogaster* (Canton-S or transgenic),
929 *S. pallida*, and *S. flava* were tested using a dual-choice "Y-shaped" olfactometer based on one
930 previously published (20). The "Y piece" of the olfactometer was a propylene connector (43 mm
931 long x 60 mm); the open ends of the arms of the "Y" were each connected to a 1-ml syringe
932 containing a piece of filter paper (600 x 50 mm) loaded with the odor or control stimuli.
933 Charcoal-filtered air was delivered to each of the two syringes using silicon tubing at 250
934 ml/min; thus, at the base of the maze air flow was approximately 500 ml/min. Two hours (in the
935 case of *D. melanogaster* and *S. pallida*) or 20 hours before tests (in the case of *S. flava*) insects
936 were gently anesthetized under CO_2 and placed in groups of four in open-top and mesh-bottom
937 cylindrical release containers (20 mm long x 10 mm diameter) constructed using silicon tube.
938 The open top of the containers was capped with a piece of cotton soaked in distilled water (in the
939 case of *D. melanogaster* and *S. pallida*) or with a piece of cotton soaked in 10% vol/vol aqueous

940 honey solution (in the case of *S. flava*). Before tests, each release tube was placed in ice during
941 45-60 seconds to slow down insect activity, the cotton cap was removed, and the open-top of the
942 tube was carefully slid into the open end of the Y maze. Thus, upon being released, insects could
943 walk upwind towards the "decision point" (intersection of the short and long arms of the "Y")
944 and turn towards either the odor-laden or the odorless arm of the maze. Although four insects
945 were released at once (to increase experimental efficacy), only the first choice (and the time of
946 the choice) was recorded; a choice was considered as such only if the insect walked past at least
947 10 mm into one of the arms, orienting upwind. The test was discarded if two or more insects
948 choose the two arms of the maze within a 3-second window of each other. Each test lasted a
949 maximum of five minutes, and each group of insects was used only once. As much as possible,
950 insects from the same cohort were tested in the same day with different odors/odorants; insects
951 from the three different species were also, as much as possible, tested in the same day with a
952 given odor/odorant/different fly genotypes. Tests with each species/stimulus were conducted in
953 at least five different days to compensate for possible day-to-day variations. Results from an
954 individual test session with a given odor/odorant were discarded if insects did not make a choice
955 is more than 50% of tests (this happened in less than 5% of experimental sessions). The position
956 of the odor and odorless arms was switched every 1-2 tests to control for positional asymmetries;
957 the mazes and odor sources were changed and replaced for clean/new ones every four tests or 10
958 minutes, whichever comes first.

959 The odor/odorant was loaded in a piece of filter paper and inserted into the 1-ml syringe
960 right before tests; control syringes had a piece of filter paper (3 x 40 mm) loaded with the
961 mineral oil solvent (for odorant solutions) or water (for odor mixtures). Experimental and control
962 filter papers were replaced by fresh ones every 4 tests or 10 minutes, whichever came first. The

963 odorants (20 µl of 1:100 vol/vol mineral oil solution) used in experiments were butyl ITC
964 (Sigma-Aldrich, CAS # 592-82-5, USA) and sec-butyl ITC (Sigma-Aldrich, CAS # 15585-98-5,
965 USA). For tests with transgenic flies, responses were tested at 1:100 vol/vol and 1:1,000 vol/vol
966 of butyl ITC. We also used apple cider vinegar (40 µl, O Organics, USA; 40 µl of distilled water
967 was as control stimulus in these tests). For tests of host-orientation, two-four leaves from young
968 arugula plants grown in an insect and insecticide/pesticide free chamber or greenhouse were
969 excised just before tests, and placed in 5-ml syringes connected to the Y-maze; control syringes
970 had two pieces of tissue paper. In all cases the Y-mazes, tubing and syringes were cleaned with
971 70% ethanol and allowed to air-dry before reusing. Experiments were conducted during the 2nd-
972 5th hour of the insects' photophase at 24 ºC under white light (Feit electric, 100 Watts; in the case
973 of *S. pallida* and *D. melanogaster*) or green light (Sunlite green, 100 Watts; in the case of
974 experiments with *S. flava*). A total of 1,061 (669 with wild-type flies of *D. melanogaster*, *S.*
975 *pallida* and *S. flava*; 392 with transgenic *D. melanogaster*) tests were and insects made a choice
976 in 84% (888) of tests.
977 For each odor/odorant, species, sex and genotype, the number of tests with the first insect
978 orienting towards the odorous arm and the odorless arm of the Y-maze were tested against a 50%
979 expected random distribution using two-tailed Binomial tests (48). P-values were adjusted for
980 multiple comparisons using the false discovery rate (FDR) method of Benjamini-Hochberg (49);
981 results were considered significant if the Benjamini-Hochberg adjusted p-value was <0.05. For
982 all tests, we verified that the power was >0.8.
983

984 **Molecular phylogeny of drosophilid Odorant Receptors (*Or*):**

985 Translations of *Ors* from *D. grimshawi*, *D. mojavensis, D. virilis* and *D. melanogaster* (builds
986 dgri r1.3, dmoj r1.3, dvir r1.07 and dmel r6.28, respectively) were downloaded from Flybase
987 (www.flybase.org, (50)). *S. flava* sequences were previously published (19). Three hundred and
988 nine sequences were aligned in MAFFT v7.017 with the E-INS-I algorithm and then manually
989 adjusted (51). Models were fitted to the alignment using IQ-Tree and tested using the AIC
990 criterion (52). A maximum likelihood (ML) phylogeny was generated using the Or protein
991 alignment in RAxML v8.2.10 with the CAT model of rate heterogeneity with seven distinct
992 categories, the JTT substitution matrix, empirical amino acid frequencies, and 1,000 rapid
993 bootstraps (53). Orco sequences were designated as the outgroup.

994

995 **Molecular phylogeny of drosophilid *Or67b* genes:**

996 *Or67b* CDS from *D. grimshawi*, *D. mojavensis, D. virilis D. sechellia, D. simulans*, *D. erecta, D.*
997 *yakuba, D. pseudoobscura, D. persimilis, D. ananassae, D. willistoni* and *D. melanogaster*
998 (builds dgri r1.3, dmoj r1.3, dvir r1.07, dsec r1.3, dsim r1.4, dere r1.3, dyak r1.3, dpse r3.2, dper
999 r1.3, dana r1.3, dwil r1.3 and dmel r6.28, respectively) were downloaded from Flybase
1000 (www.flybase.org, (50)). The *S. pallida* DNA sequence was obtained through PCR as described
1001 above; *S. flava* DNA sequences were previously published (19). Sequences were aligned, models
1002 fitted and chosen according to AIC (GTR+I+G) in IQ-Tree (52). Trees were inferred using
1003 RAxML (v8.2.10) with the GTRCATI model and 1000 rapid bootstraps, and MrBayes (v3.2.6)
1004 setting Nst to 6, nucmodel to 4by4, rates to Invgamma, number of generations to 125,000, burnin
1005 equal to 20% of generations, heating to 0.2, number of chains to 4, runs to 2 and priors set to
1006 default setting (54).

1007 **Analysis of molecular evolution:**

1008 CDS of homologs of every *Or* gene in *S. flava* found in the 12 *Drosophila* genome builds were
1009 aligned to *S. flava Or* CDS. Homology was assessed according to inclusion in well supported
1010 clades in the *Or* translation phylogeny above; *S. flava* sequences were previously published (19).
1011 Sequences were aligned in MAFFT (v7.017) (51) and adjusted manually to preserve codon
1012 alignments. *Or98a*-like genes found in subgenus *Drosophila* species were split into three
1013 separate clades, as were a group of *Or83c* paralogs not found in *D. melanogaster*, and a group of
1014 *Or85a*-like genes. All sequences examined of *Or46a* contain two alternatively spliced exons, so
1015 this gene was analyzed with all gene exon sequences in a single alignment as single taxa. *Or69a*,
1016 however, contains alternatively spliced exons only among *Sophophora* species. These alternative
1017 splice forms were analyzed as separate taxa. Phylogenies were generated for every alignment
1018 using PhyML (55) with the GTR+G substitution models. If these trees showed >70% bootstrap
1019 support for a topology contrary to the known species topology, or if the Or homology group
1020 contained duplicates, these trees were used in PAML analyses instead of the species tree. Branch
1021 models of sequence evolution were fit using PAML 4.9h (26). A foreground/background branch
1022 model was fit for every *S. flava* tip branch and every ancestral branch in a *Scaptomyza*-specific
1023 *Or* gene duplication clade, and compared in a likelihood ratio test to a null model with one d*N*/d*S*
1024 rate for every unique phylogeny (75 tests total). After focusing on *Or67b*, patterns of molecular
1025 evolution among the drosophilid *Or67b* homologs were explored using the expanded *Or67b*
1026 CDS phylogeny above. Foreground/background branch models were fit for every branch in the
1027 *Or67b* phylogeny with identical likely ratio tests performed as above (29 tests total Fig. 2*B*;
1028 Table S1). P-values were adjusted for multiple-testing using the FDR method (49).

1029 **Distance tree obtained from responses of ab3A neurons expressing *Or67b* transgenes**

1030 A matrix of average response of five *Or67b* transgene receptors to 44 odorant compounds (1:100
1031 vol/vol concentration) was produced (Table S3). Net responses were calculated by subtracting
1032 the response to mineral oil or dimethyl sulfoxide solvent from the odor-evoked responses. A
1033 Euclidean distance matrix was generated using the dist function of the R stats package(56).
1034 Receptor responses were clustered by using the neighbor joining (NJ) algorithm on this distance
1035 matrix. Support for clusters was assessed using 1000 bootstraps of the original response matrix
1036 by generating distance matrices and NJ trees on the pseudo-datasets in ape (v5.3) (57).

1037

1038 **Single sensillum recordings (SSR)**

1039 Female fed flies were prepared for SSR as previously described (58). We identified sensilla of
1040 targeted olfactory receptor cells using an Olympus BX51WI upright microscope with 10x and
1041 50x objectives (Olympus, UPlanFL N 10x, UPlanFL N 50x). Extracellular activity was recorded
1042 by inserting a tungsten electrode into the base of the ab3 sensillum. Signals were amplified
1043 10,000 x (A-M systems, Differential AC Amplifier model 1700), digitized using a 16-bit analog-
1044 digital converter, filtered (low cut-off: 300 Hz, high cut off: 3k Hz), and analyzed off-line with
1045 WinEDR (v3.9.1; University of Strathclyde, Glasgow). A tube delivering a constant flow of
1046 charcoal-filtered air was placed near the fly's head, and the tip of the stimulation pipette (50 ml)
1047 was inserted into the constant air stream. The stimulation pipette contained a piece of filter paper
1048 loaded with 20 µl of odorant solution or the solvent control. One second pulse of clean air was
1049 delivered to the stimulus pipette using a Stimulus Controller CS 55 (Syntech, Germany). We
1050 used three standard odors for identification of ab3 sensilla (all from Sigma-Aldrich, US, purity >
1051 1%): ethyl hexanoate (CAS # 123-66-0), ethyl acetate (CAS # 141-78-6) and 2-heptanone (CAS

1052 # 110-43-0). The following odor sources (all purchased in Berkeley, California, USA unless
1053 otherwise mentioned; 20 µl of material loaded on filter paper) were used: apple cider vinegar (40
1054 µl, O Organics, USA), grated roots of *Wasabia japonica* (wasabi), organic roots of *Armoracia*
1055 *rusticana* (horseradish), *Brassica rapa* (turnip), *Raphanus sativus* (daikon), and *Beta vulgaris*
1056 (beet). *Eruca vesicaria* (arugula) was grown from seeds at 23°C and 60% relative humidity in a
1057 12-hours light: 12-hours dark cycle, and leaves from 3-8 weeks old plants were used for odor
1058 stimulation. Roots and leaves were grated and homogenized using a vegetable grater to a volume
1059 equivalent to ~500 µl. The following odorants (all from Sigma-Aldrich, US) were diluted 1:100
1060 vol/vol in dimethyl sulfoxide: iodoacetamide (CAS # 144-48-9), N-methylmaleimide (CAS #
1061 930-88-1), N-hydroxysuccinimide (CAS # 6066-82-6), and 3-indol-carbinole (CAS # 700-06-1)
1062 and benzyl thiocyanate (CAS # 3012-37-1). All the other chemicals were diluted in mineral oil at
1063 1:100, 1:1,000, and 10,000 vol/vol. The "net number of spikes" were obtained by counting the
1064 number of spikes during a 1-second window 0.2 seconds after the onset of stimulation, and
1065 subtracting from this number the background spiking activity (obtained by counting the number
1066 of spikes in a 1-second window prior to the onset of the odor stimulation). Data was analyzed
1067 using Mann-Whitney Rank sum tests (for comparisons involving two means) and p-values were
1068 adjusted for multiple comparisons using the FDR method, or by Kruskal-Wallis ANOVAs
1069 followed by Dunn's tests (for comparisons involving more than two means).

1070

1071 **Principal Component Analysis (PCA).** For visualization, Principal component analysis (PCA)
1072 was done using Python.

1073

1074 **Data Analysis and figure generation:**

1075 All images and drawings are originals prepared by the authors. Figures were prepared via a

1076 combination of WinEDR (v3.9.1), R Studio (v1.2.1335), Microsoft Excel (2016), Adobe

1077 Illustrator (2019), Python, and Geneious (10.0.9).

Table S1: Parameters from Odorant Receptor molecular evolution analyses

Test*	Branch†	background dN/dS	foreground dN/dS	dN – dS	FDR adjusted p-value
OR	Or2a	0.10948	0.08368	-1.1523	0.4031933
OR	Or10a	0.16074	0.08826	-0.5837	**0.03599091**
OR	Or13a1	0.13815	0.95538	-0.0014	**8.36204E-06**
OR	Or13a2	0.14485	0.29386	-0.0114	0.5374215
OR	Or13a ancestor	0.1405	0.22696	-0.2232	0.1062494
OR	Or19a	0.27184	0.256	-0.3699	0.8337806
OR	Or22c	0.15572	0.25996	-0.1496	0.1475187
OR	Or23a	0.16331	0.2829	-0.2438	0.0934421
OR	Or24a	0.08324	0.09358	-0.5121	0.7036877
OR	Or30a	0.07027	0.10685	-0.4864	0.1396827
OR	Or33c	0.20981	0.15322	-0.402	0.2748754
OR	Or35a	0.10058	0.80581	-0.2963	**0.00245945**
OR	Or42a1	0.10806	0.11791	-0.336	0.7716366
OR	Or42a2	0.10578	0.80581	-0.012	**0.000970903**
OR	Or42a3	0.10571	0.80581	-0.0045	**0.000643469**
OR	Or42a2 and 3 ancestor	0.11046	0.08409	-0.5505	0.3637872
OR	Or43a	0.14775	0.08518	-0.3217	0.1557934
OR	Or45b	0.10067	0.10449	-0.3992	0.8803498
OR	Or46a	0.14313	0.20832	-0.2632	0.1151226
OR	Or47b	0.16957	0.15781	-0.3375	0.8025064
OR	Or49a	0.19774	0.17914	-0.5377	0.7036877
OR	Or49b	0.10784	0.05836	-0.3747	0.09592573
OR	Or56a	0.12598	0.05652	-0.5602	**0.004298517**
OR	Or59a	0.1085	0.31529	-0.2475	**0.000415309**
OR	Or59b	0.09936	0.23227	-0.4421	**0.000415309**
OR	Or63a1	0.12346	∞	**0.0172**	**3.51892E-05**
OR	Or63a2	0.12136	∞	**0.0274**	**1.35815E-09**
OR	Or63a ancestor	0.10903	0.56602	-0.1212	**5.0862E-13**
OR	Or67a1	0.20346	0.13116	-0.528	0.0934421
OR	Or67a2	0.2067	**0.06369**	-0.0693	**0.0004729**
OR	Or67b1	0.11719	0.87005	-0.0059	**2.5914E-07**
OR	Or67b2	0.11479	0.69462	-0.0253	**1.96849E-09**
OR	Or67b3	0.10844	**1.95161**	**0.0414**	**9.78045E-20**
OR	Or67b1 and 2 ancestor	0.11932	∞	**0.0282**	**3.90702E-08**
OR	Or67b1, 2 and 3 ancestor	0.11679	0.28275	-0.2591	**0.000585544**
OR	Or67c	0.05491	0.07255	-0.4886	0.4029281

OR	Or67d	0.12277	**7.22359**	**0.0948**	**2.56153E-05**
OR	Or69a	0.21665	0.12519	-0.4274	0.05279388
OR	Or71a	0.15846	0.25285	-0.3464	0.1151226
OR	Or74a1	0.13897	0.16825	-0.3766	0.5728869
OR	Or74a2	0.13723	0.18825	-0.4375	0.2932306
OR	Or82a	0.12569	0.18201	-0.2805	0.1926929
OR	Or83a	0.0967	0.13983	-0.4518	0.148133
OR	Or83c1	0.16267	0.21756	-0.4381	0.3145556
OR	Or83c2	0.22615	0.41682	-0.1561	0.0934421
OR	Or83c3	0.22488	0.39035	-0.2043	0.0934421
OR	Or83c2 and 3 ancestor	0.25561	0.17609	-0.2679	0.5410598
OR	Or85aLike	0.22488	0.39035	-1.8882	**0.001826812**
OR	Or85bc	0.19387	**0.07261**	-0.32	0.2783313
OR	Or85e	0.15034	0.20391	-0.3561	0.6592839
OR	Or85f	0.11206	0.13076	-0.4139	0.08611496
OR	Or88a	0.18031	0.09816	-0.2848	**0.003678095**
OR	Or92a	0.14147	0.32458	-0.3894	**3.51892E-05**
OR	Or94a	0.044	0.13816	-0.4616	0.1933
OR	Or94b	0.09284	0.14353	-0.5473	0.09592573
OR	Or98a1	0.09893	0.17168	**0.0013**	0.1796849
OR	Or98a3	0.18051	1.69406	-0.0093	0.6770641
OR	Or98a7	0.18219	0.28105	**0.0258**	**5.47517E-05**
OR	Or98a1 and 3 ancestor	0.14909	**2.74217**	-0.0113	**0.00014207**
OR	Or98a1, 3 and 7 ancestor	0.15018	0.81658	-0.6623	0.7716366
OR	Or98a2	0.18749	0.17369	-0.024	0.7716366
OR	Or98a6	0.24649	0.30314	-0.044	0.6592839
OR	Or98a2 and 6 ancestor	0.24491	0.30931	-0.33	0.0934421
OR	Or98a4	0.2739	0.14173	**0.0063**	**0.000676304**
OR	Or98a5	0.22865	**1.23866**	**0.01**	0.05279388
OR	Or98a4 and 5 ancestor	0.237	4.11669	-0.4823	**0.01935304**
OR	Or98b1	0.28857	**0.13007**	-0.8492	0.7613693
OR	Or98bLike	0.18605	0.20625	-0.8656	0.5728869
OR	OrN1	0.19137	0.15525	-0.3194	0.1960608
OR	OrN2 copy 1	0.02988	0.07861	-0.0006	0.1278416
OR	OrN2 copy 4	0.14809	0.21155	**0.0008**	0.05293525
OR	OrN2 copy 5	0.2495	0.94941	-0.6183	0.08611496
OR	OrN2 copy 6	0.24826	1.05198	-0.8924	0.1151226
OR	OrN2 copy 1 and 4 ancestor	0.26274	0.16626	-0.4861	0.5728869
OR	Orco	0.26137	0.17749	-0.2037	**0.02049901**

OR67b	*D. grimshawi*	0.24934	0.29184	-0.3355	0.7873258
OR67b	*S. flava* copy 3	0.1041	**1.803**	**0.0375**	**1.44394E-21**
OR67b	*S. flava* copy 2	0.10935	**0.7393**	-0.0208	**1.05769E-10**
OR67b	*S. flava* copy 1	0.11251	**0.62856**	-0.022	**1.5606E-06**
OR67b	*S. pallida*	0.1236	0.0506	-0.3076	**0.01604471**
OR67b	*D. mojavensis*	0.1137	**0.2053**	-0.8484	**0.009320281**
OR67b	*D. virilis*	0.119	0.1185	-1.4528	0.9933245
OR67b	*D. melanogaster*	0.11971	0.04714	-0.0423	0.4999046
OR67b	*D. sechellia*	0.1193	0.0796	-0.0368	0.6982622
OR67b	*D. simulans*	0.1202	0.0197	-0.0523	**0.04609565**
OR67b	*D. yakuba*	0.1198	0.0914	-0.1247	0.614736
OR67b	*D. erecta*	0.1193	0.1051	-0.1126	0.7873258
OR67b	*D. ananassae*	0.1329	0.0303	-1.7359	**5.2654E-10**
OR67b	*D. persimilis*	0.1188	0.1803	-0.0103	0.7873258
OR67b	*D. pseudoobscura*	0.119	0.1149	-0.0078	0.9933245
OR67b	*D. willistoni*	0.1288	0.0427	-1.4866	**1.97605E-05**
OR67b	Subgenus *Drosophila* Ancestor	0.1236	0.0564	-0.9778	**0.01967709**
OR67b	*Sophophora* minus D. *willistoni*	0.1212	0.0711	-0.5393	0.3034497
OR67b	virilis-repleta radiation	0.1196	0.0905	-0.1587	0.7873258
OR67b	Hawaiian radiation ancestor	0.1198	0.0979	-0.2595	0.7817405
OR67b	*Scaptomyza* genus ancestor	0.1221	0.0435	-0.2512	**0.04498955**
OR67b	*S. flava* copy 1, 2 and 3 ancestor	0.1094	**0.4081**	-0.1282	**4.06804E-07**
OR67b	*S. flava* copy 1 and 2 ancestor	0.1135	∞	**0.0276**	**4.5389E-09**
OR67b	*D. simulans* and *D. sechelia* ancestor	0.1205	0.0001	-0.0564	**0.01202915**
OR67b	*D. yakuba* and *D. erecta* ancestor	0.1221	0.0196	-0.1444	**0.00128517**
OR67b	melanogaster subgroup minus Dmel	0.1201	0.048	-0.0727	0.2412147
OR67b	melanogaster subgroup ancestor	0.125	0.0446	-0.5921	**0.003348616**
OR67b	melanogaster group ancestor	0.1207	0.0634	-0.2523	0.4817083
OR67b	pseudoobscura subgroup ancestor	0.1291	0.0342	-0.9693	**2.39766E-06**

Table S1: Parameters for background *versus* foreground branch tests in PAML. * OR = Branch tests for differences in dN/dS rates among *Scaptomyza flava* copies of odorant receptor genes. OR67b = Branch tests for differences in dN/dS rates among all individual branches in a phylogeny of *Or67b* homologs in *Scaptomyza* and *Drosophila* spp. † Terminal branches are indicated with gene names. Ancestral branches are indicated with the term “ancestor” and a list of the terminal branches or the clade subtended.

TableS2 PCR Primers

Primer Name	Sequence	Primer role
67b12Fb	cATGAAGGACTTATTGGATCTGGAGCTAG	CDS amplification
67b1Rb	agcgcaTTATTTGTCTCTCATATTGCTC	CDS amplification
67b2Rb	agcgcaTTATTCATCTCTCATGTTACGC	CDS amplification
67b3Fb	cATGAACTTATTGGATATGGAGCTAG	CDS amplification
67b3Rb	agcgcaTTATTTGTTAGATAAATTACGC	CDS amplification
Sp67bFb	cATGAAAAACCTATTGGACCGGAAG	CDS amplification
Sp67bRb	aaagtacaTCATTTGTTATTCATATTACGCAAAAG	CDS amplification
b1EcoRIF	GACgaattcATGAAGGACTTATTGG	RE cut site addition
b1KpnIR	GACggtaccTTATTTGTCTCTCATATTG	RE cut site addition
b2KpnIR	GACggtaccTTATTCATCTCTCATG	RE cut site addition
b3KpnIR	GACggtaccTTATTTGTTAGATAAATTACG	RE cut site addition
PalEcoRIF	GACgaattcATGAAAAACCTATTGGACC	RE cut site addition
PalKpnIR	GACggtaccTCATTTGTTATTCATATTACG	RE cut site addition

Tables S2: Nucleotides in lower case are either in untranslated sequence (CDS amplification) or are restriction enzyme cut sites (RE cut site addition). CDS amplification primers were used to amplify full *Or67b* CDS sequence from cDNA. Primers labeled “RE cut site addition” were used to engineer restriction enzyme cut-sites via PCR mutagenesis in order to ligate *Or67b* CDS sequences into the pUASTattB plasmid. All sequences are listed in a 5’ to 3’ orientation.

Table S3: Net number of spikes/second; average ± SE

	"Empty neuron"		WT Or22a	
Odors	**spikes**	**n**	**spikes**	**n**
ethyl hexanoate	0	7	118±7.7	7
mineral oil	0	7	6.33±1.7	6
acetophenone	0	6	NA	NA
butyl ITC	0	6	NA	NA
mandelonitrile	0	6	NA	NA
cis-3-hexenyl butyrate	0	6	NA	NA
trans-2-pentenal	0	6	NA	NA
geranyl acetate	0	6	NA	NA
mineral oil	0	7	NA	NA

	Dmel67b		Spal67b		Sfla67b1		Sfla67b2		Sfla67b3	
Odors	**spikes**	**n**	**spikes**	**n**	**spikes**	**n**	**spikes**	**n**	**spikes**	**n**
apple cider vinegar	96.3 ± 8.0	6	62 ± 10.4	7	-4.33 ± 2.0	6	6.67 ± 4.6	6	15 ± 2.1	6
arugula	19.7 ± 3.0	6	14 ± 3.4	7	64 ± 7.8	6	24 ± 8.7	6	22.3 ± 2.3	6
wasabi	2.67 ± 2.0	6	2.67 ± 1.11	6	15.3 ± 1.9	8	16.2 ± 2.5	8	13.7 ± 4.4	6
horseradish	3.67 ± 1.8	6	-0.33 ± 1.0	6	8.75 ± 1.3	8	12.9 ± 2.0	8	31.7 ± 4.4	6
turnip	1 ± 1.8	6	0.67 ± 0.7	6	11.8 ± 1.6	8	22 ± 2.7	8	34.3 ± 1.4	6
daikon	2 ± 3.2	6	-0.67 ± 0.8	6	14 ± 2.4	8	17.1 ± 3.4	8	19 ± 3.4	6
beet	8 ± 3.8	6	-0.33 ± 1.1	6	2 ± 1.30	6	-0.33 ± 1.7	6	4.33 ± 2.2	6
Dmel67b activators										
acetophenone	176 ± 18	7	78 ± 2.4	20	3.27 ± 1.2	11	4.5 ± 2.0	8	1.57 ± 1.3	14
cis-3-hexen-1-ol	56.9 ± 20	7	22 ± 4.1	7	8.67 ± 2.0	6	6.75 ± 1.3	8	2.67 ± 2.3	6
4-methylcyclohexenol	80.7 ± 8.7	6	33 ± 4.4	13	8.89 ± 4.3	9	1.56 ± 1.4	9	2.67 ± 1.2	6
phenethyl alcohol	140 ± 19	8	26.3 ± 4.5	6	2.67 ± 1.2	6	1 ± 1.2	6	3.67 ± 1.5	6
1-hexanol	118 ± 13	7	19.14286	7	13.7 ± 3.8	6	10.4 ± 4.6	9	8 ± 1.9	6
ethyl (S)-(+)-3-hydroxybutanoate	15.7 ± 3.3	7	12 ± 2.8	7	4.33 ± 4.4	6	10.4 ± 2.9	9	8 ± 2	6
2-hexanol	12.3 ± 5.2	8	11.7 ± 2.8	7	7.33 ± 2.1	6	9.5 ± 1.8	8	7 ± 3.1	6
benzyl alcohol	48.6 ± 2.9	6	14.3 ± 3.7	7	-1.33 ± 0.8	6	6.75 ± 1.8	8	5.67 ± 1.4	6
2-hexanone	40 ± 16	7	7.14 ± 2.0	7	9.33 ± 4.8	6	7.5 ± 2.0	8	7.33 ± 3.5	6
hexyl-formate	39.3 ± 8.7	6	16.3 ± 5.6	7	7 ± 2.0	6	8.89 ± 4.5	9	3 ± 0.9	6
phenethyl aldehyde	51.4 ± 10	7	29.4 ± 3.8	7	2 ± 2.3	6	2.86 ± 3.0	7	1 ± 0.7	6
2-heptanone	28 ± 4.1	7	13.1 ± 2.3	7	6.33 ± 2.4	6	15.3 ± 2.5	9	6 ± 1.9	7
ITCs										
butyl ITC	4.57 ± 1.6	7	15 ± 4.2	6	196 ± 15	7	22.7 ± 2.8	6	171 ± 11	7
ethyl ITC	0.33 ± 1.5	6	2.33 ± 0.95	6	24 ± 2.7	6	3.33 ± 3.9	6	13 ± 1.7	6
isobutyl ITC	1.67 ± 2.4	6	5.33 ± 2.6	6	74 ± 12	7	4.33 ± 1.8	6	139 ± 17	7
sec-butyl ITC	4.29 ± 1.0	7	5 ± 1.8	6	9.14 ± 2.7	7	5.33 ± 2.3	6	121 ± 17	8
allyl ITC	3.43 ± 0.8	7	9.75 ± 4.2	8	28.9 ± 5.3	7	6 ± 2.4	7	4.75 ± 3.7	8
3-methylthio propyl ITC	2 ± 1.8	6	6.33 ± 2.2	6	179 ± 9.2	6	33 ± 6.0	6	137 ± 17	8
benzyl ITC	2.29 ± 1.4	7	3.75 ± 1.9	8	81.7 ± 14.4	7	3.14 ± 1.3	7	134 ± 14	8
phenethyl ITC	6.57 ± 2.2	7	5.25 ± 2.8	8	4.8 ± 1.3	10	1.14 ± 0.86	7	158 ± 15	8
3-methoxybenzyl ITC	-0.67 ± 1.1	6	7 ± 3.3	6	4.57 ± 2.2	7	1.67 ± 0.80	6	3.14 ± 2.1	7
Nitriles										
mandelonitrile	45.3 ± 11	6	1 ± 1	6	1.33 ± 1.8	6	-0.29 ± 0.7	7	4 ± 3.0	6
adiponitrile	2.67 ± 2.5	6	0.33 ± 0.33	6	0.33 ± 0.3	6	0.67 ± 2.2	6	4.33 ± 1.8	6
propionitrile	5.33 ± 1.6	6	1.33 ± 0.4	6	2.3 ± 2.0	6	1.33 ± 1.1	6	4.33 ± 2.5	6
GLVs										
cis-3-hexenyl butyrate	91 ± 21	7	49.3 ± 8.0	6	5 ± 2.6	8	2 ± 1.8	6	11.8 ± 2.5	8
trans-2-hexen-1-ol	33 ± 6.0	6	51 ± 11	6	23.5 ± 5.6	8	3 ± 2.5	6	6.29 ± 2.2	7
trans-2-hexenyl acetate	55.7 ± 19	7	34 ± 6.8	6	2 ± 2.1	6	17.3 ± 2.5	6	10.3 ± 1.9	8
trans-2-hexenyl butyrate	29.7 ± 6.7	6	35.6 ± 8.2	6	1.71 ± 1.0	7	4 ± 1.5	6	12 ± 4.3	7
cis-3-hexenyl	43.3 ± 9.3	6	13.7 ± 2.6	6	1.67 ± 1.7	6	16 ± 5.3	8	11 ± 5.8	6

acetate	45.3 ± 9.3	6	13.7 ± 2.6	6	1.67 ± 1.7	6	10 ± 9.3	6	11 ± 3.8	6
TrpA1 activators/ electrophiles										
trans-2-pentenal	27.1 ± 20	7	6.33 ± 0.8	6	4.33 ± 2.9	6	4 ± 1.4	6	4 ± 1.3	6
citronellal	1.43 ± 1.6	7	7 ± 2.3	6	4.57 ± 1.6	7	9.14 ± 3.5	7	12 ± 2.8	11
cinnamaldehyde	12.3 ± 2.8	7	9 ± 3.8	6	9.25 ± 2.7	8	3 ± 1	6	6 ± 1.7	12
acrorelin	2.67 ± 2.6	6	6.33 ± 2.2	6	6.67 ± 1.9	6	7 ± 4.3	6	3.33 ± 1.3	6
iodoacetamide	1.43 ± 1.4	7	6 ± 1.9	6	7.33 ± 1.6	6	6 ± 2.4	6	4 ± 1.7	6
formaldehyde	3.14 ± 2.0	7	5.33 ± 1.8	6	6 ± 2.9	6	3 ± 1.4	6	2 ± 1.0	6
N-methylmaleimide	3.71 ± 1.0	7	8.67 ± 2.6	6	7.33 ± 1.8	6	10 ± 1.9	6	3 ± 1.3	6
N-hydroxysuccinimide	1.71 ± 1.3	7	4.67 ± 2.5	6	4.67 ± 2.6	6	8.67 ± 2.3	6	2 ± 0.7	6
methyl salicylate	-3 ± 1.8	7	0 ± 0.5	6	3 ± 3	6	-0.57 ± 1.8	7	5.33 ± 4.2	6
S-allyl 2-propene-1-sulfinothioate	5.66 ± 1.0	7	-0.33 ± 0.8	6	3.67 ± 1.5	6	0.57 ± 1.1	7	3 ± 1.5	6
diallyl disulfide	3.33 ± 3.3	7	0.67 ± 0.8	6	1.33 ± 0.7	6	1.14 ± 0.6	7	3.67 ± 2.1	6
Others										
geranyl acetate	5.67 ± 1.0	6	26 ± 6.5	6	8.5 ± 2.4	8	4.67 ± 2.0	6	5.25 ± 1.4	8
limonene	2.29 ± 2.4	7	5 ± 4.1	6	4.33 ± 1.6	6	1 ± 0.7	6	2.71 ± 1.5	14
indol-3-carbinol	4 ± 1.5	6	3.33 ± 2.6	6	5.67 ± 1.6	6	8.67 ± 1.1	6	3.33 ± 0.8	6
benzyl thyocyanate	2.57 ± 1.4	7	7.67 ± 1.4	6	8.67 ± 2.4	6	5 ± 1.3	6	8.67 ± 2.8	6
Solvent control										
DMSO	2.86 ± 1.6	7	10.7 ± 3.2	6	3.33 ± 1.0	6	7.67 ± 1.4	6	4.33 ± 2.9	6
mineral oil	3.73 ± 1.2	15	0.91 ± 0.87	11	1 ± 1.4	6	1 ± 1.8	6	0 ± 0.38	11

	Sfla67b1		Sfla67b3	
odorants	**spikes**	**n**	**spikes**	**n**
3-methylthio propyl ITC				
1/100	114±18	8	127±13	6
1/1,000	64±11	8	27.7±7.1	6
1/10,000	27.9±4.6	8	11.7±5.3	6
1/100,000	12±3.3	9	10±4.0	6
butyl ITC				
1/100	147±19	10	161±16	6
1/1,000	46.8±6.6	9	50.7±8.1	6
1/10,000	25.6±7.3	8	19.7±6.2	6
1/100,000	14.25±5	9	7.33±3.4	6
isobutyl ITC				
1/100	40.6±9.0	8	131±14	6
1/1,000	6.3±2.3	8	31.7±5.6	6
1/10,000	5.63±2.7	8	9±3.8	6
1/100,000	8.25±1.8	9	6±5.2	6
sec-butyl ITC				
1/100	5±1.7	9	124±13	6
1/1,000	4.5±1.6	9	26.3±4.2	6
1/10,000	5.63±1.8	8	13.7±4.6	6
1/100,000	10.5±3.5	8	9±4.5	6
benzyl ITC				
1/100	94.5±14.1	10	123±13	6
1/1,000	16.5±5.1	9	27.7±9.4	6
1/10,000	8.625±3.6	8	8±1.4	6
1/100,000	10.5±3.2	9	13±5.0	6
phenethyl ITC				
1/100	5.25±2.8	9	147±17	6
1/1,000	8.5±3.0	8	74±18	6
1/10,000	8.71±4.1	7	26.3±7.4	6
1/100,000	8±2.9	7	9.33±5.2	6
mineral oil	1±1.4	6	0±0.4	11

Tables S3: Responses (net number of spikes/second; average ± SE) of the ab3 sensilla of flies expressing either no Or22a ("Empty neuron"), wild-type *Or22a* (Canton-S), *Dmel Or67b, Spal Or67b, Sfla Or67b1, Sfla Or67b2 or Or67b3* to stimulation with the odors and number of recordings (n) are denoted.

Figure 1

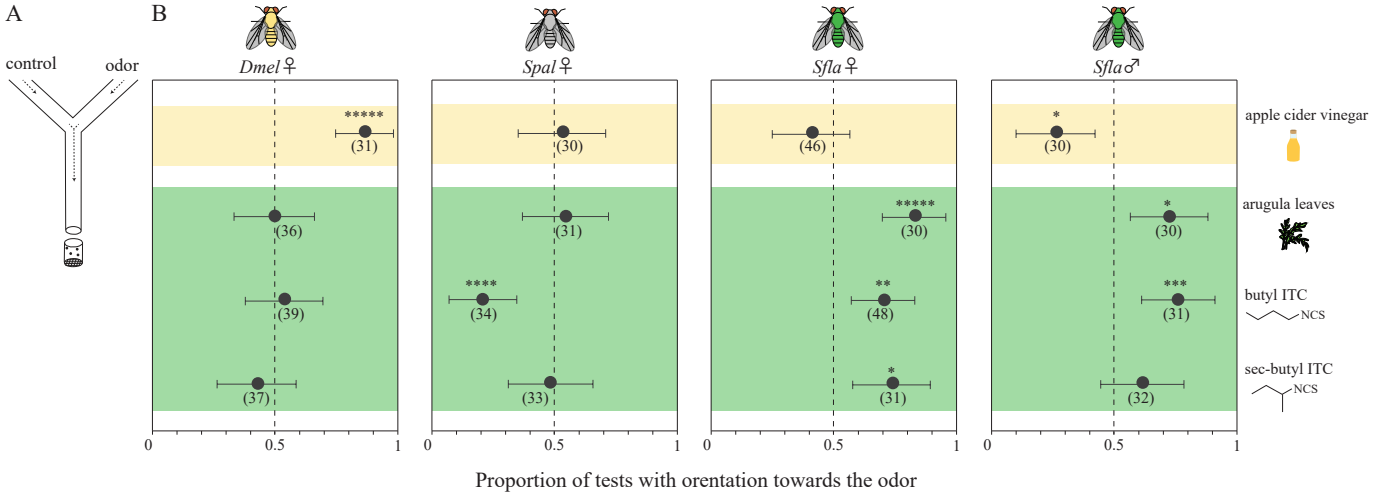
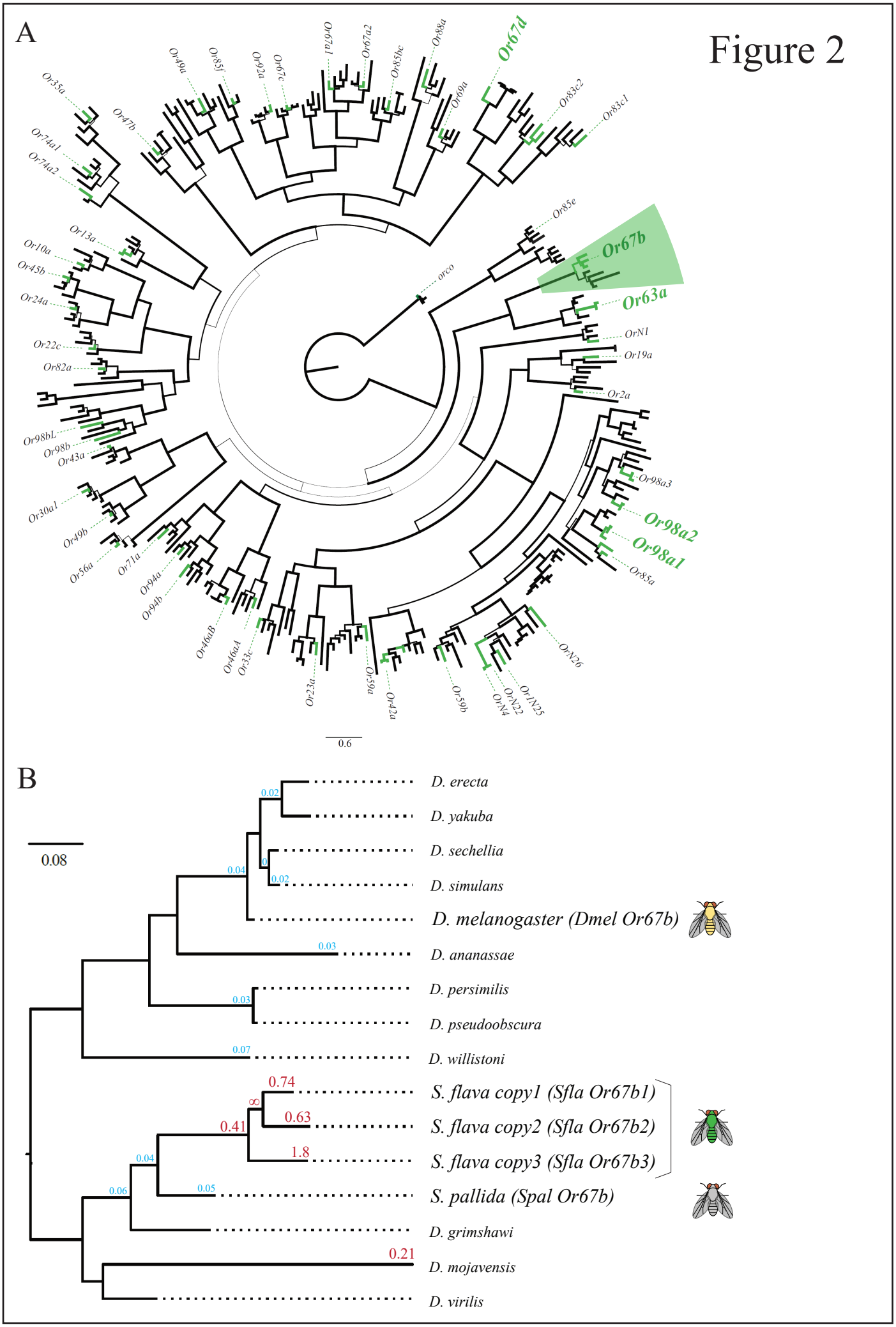

A
control
odor
B
Dmel ♀
Spal ♀
Sfla ♀
Sfla ♂
*****
(31)
(36)
(39)
(37)
(30)
(31)
****
(34)
(33)
(46)
*****
(30)
**
(48)
*
(31)
*
(30)
*
(30)
***
(31)
(32)
apple cider vinegar
arugula leaves
butyl ITC
NCS
sec-butyl ITC
NCS
0
0.5
1
Proportion of tests with orentation towards the odor

Figure 2

A


Or67d
Or67b
Or63a
Or98a2
Or98a1
Or35a
Or47b
Or74a1
Or74a2
Or49a
Or85f
Or92a
Or67c
Or67d1
Or67a2
Or85bc
Or88a
Or69a
Or83c2
Or83c1
Or85e
Or13a
Or10a
Or45b
Or24a
Or22c
Or82a
Or98bL
Or98b
Or43a
Or30a1
Or49b
Or56a
Or71a
Or94a
Or94b
Or46aB
Or46aA
Or33c
Or23a
Or59a
Or42a
Or59b
OrN4
OrN22
OrN25
OrN26
Or85a
Or98a3
Or2a
Or19a
OrN1
orco
0.6

B

0.08
D. erecta
D. yakuba
D. sechellia
D. simulans
D. melanogaster (Dmel Or67b)
D. ananassae
D. persimilis
D. pseudoobscura
D. willistoni
S. flava copy1 (Sfla Or67b1)
S. flava copy2 (Sfla Or67b2)
S. flava copy3 (Sfla Or67b3)
S. pallida (Spal Or67b)
D. grimshawi
D. mojavensis
D. virilis
0.02
0.04
0
0.02
0.03
0.03
0.07
0.74
∞
0.63
0.41
1.8
0.04
0.06
0.05
0.21

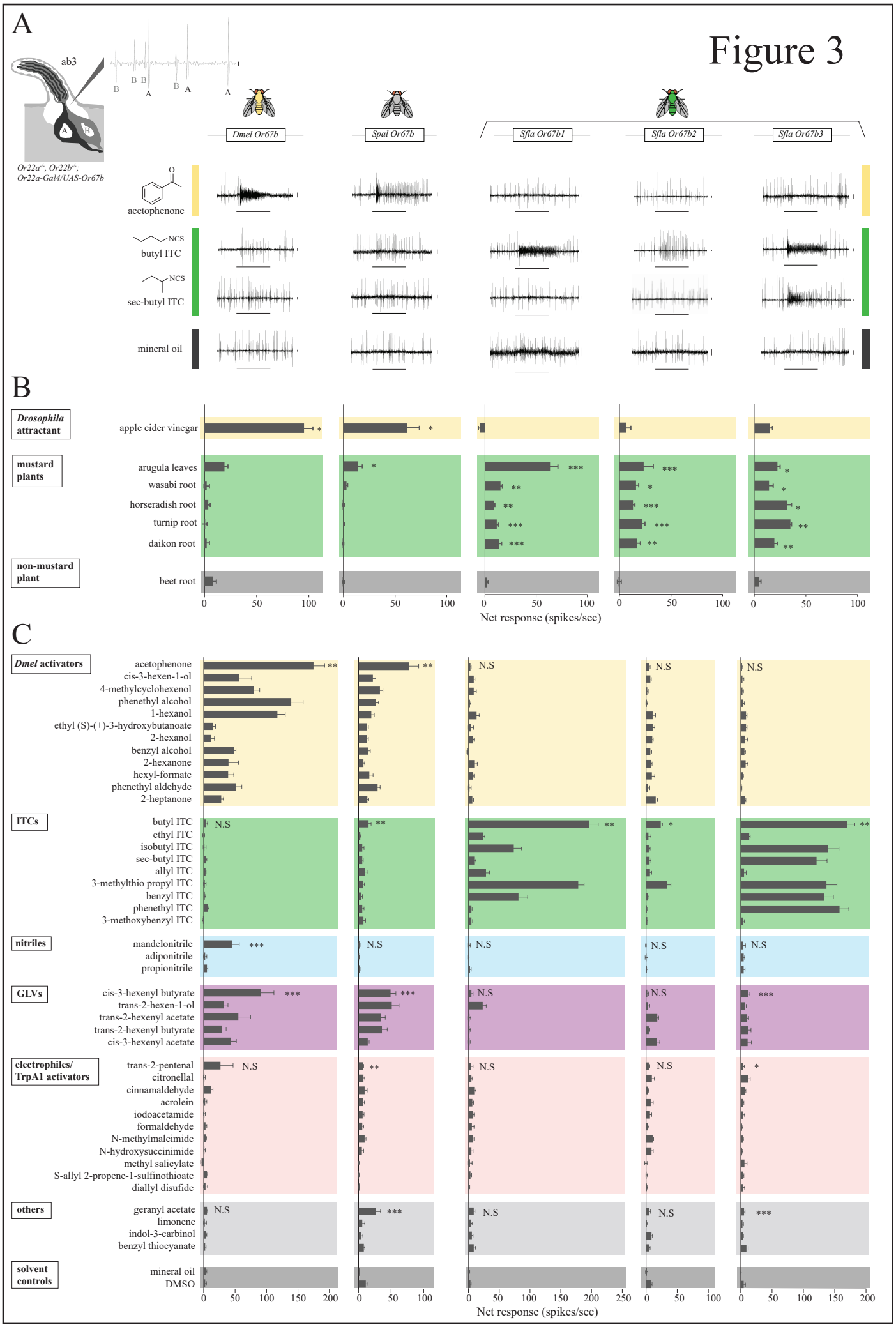
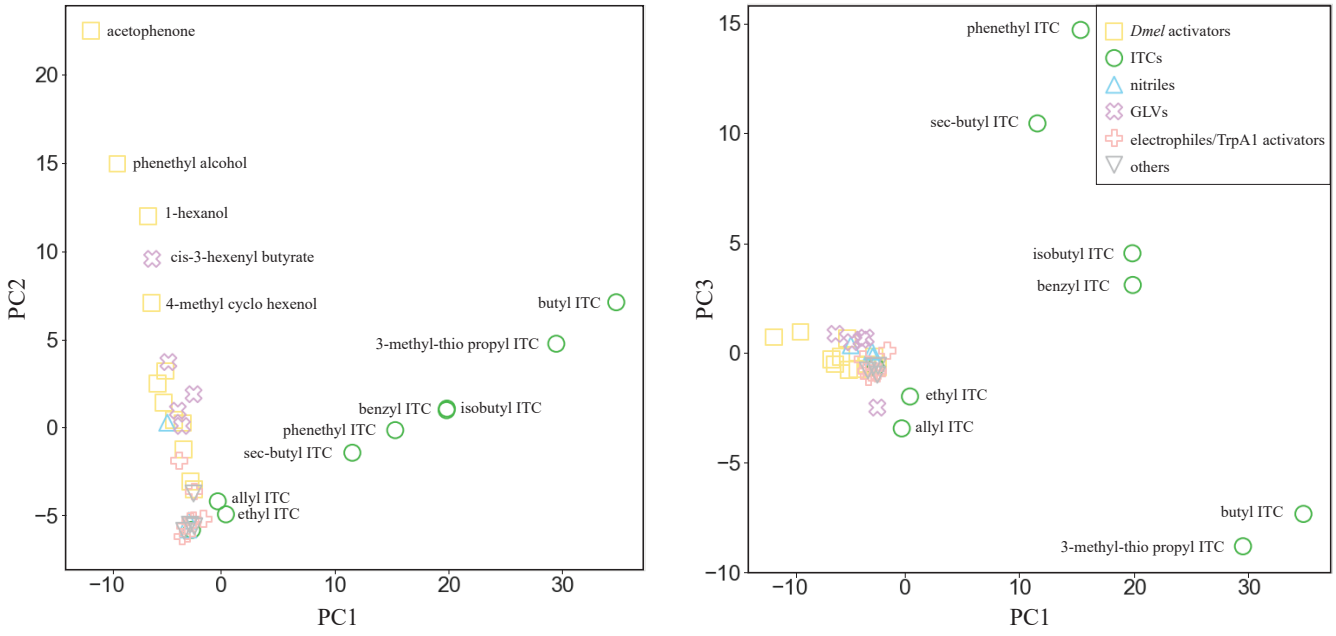


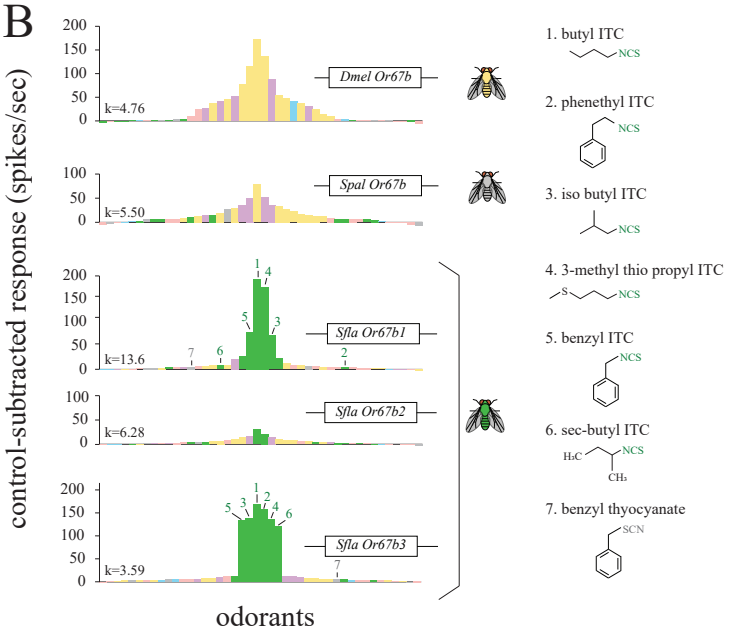
Figure 3
A
ab3
B
B B
A
B A
A
Or22a-/-, Or22b-/-;
Or22a-Gal4/UAS-Or67b
Dmel Or67b
Spal Or67b
Sfla Or67b1
Sfla Or67b2
Sfla Or67b3
acetophenone
butyl ITC
sec-butyl ITC
mineral oil
NCS
B
Drosophila attractant
apple cider vinegar
mustard plants
arugula leaves
wasabi root
horseradish root
turnip root
daikon root
non-mustard plant
beet root
Net response (spikes/sec)
C
Dmel activators
acetophenone
cis-3-hexen-1-ol
4-methylcyclohexenol
phenethyl alcohol
1-hexanol
ethyl (S)-(+)-3-hydroxybutanoate
2-hexanol
benzyl alcohol
2-hexanone
hexyl-formate
phenethyl aldehyde
2-heptanone
ITCs
butyl ITC
ethyl ITC
isobutyl ITC
sec-butyl ITC
allyl ITC
3-methylthio propyl ITC
benzyl ITC
phenethyl ITC
3-methoxybenzyl ITC
nitriles
mandelonitrile
adiponitrile
propionitrile
GLVs
cis-3-hexenyl butyrate
trans-2-hexen-1-ol
trans-2-hexenyl acetate
trans-2-hexenyl butyrate
cis-3-hexenyl acetate
electrophiles/ TrpA1 activators
trans-2-pentenal
citronellal
cinnamaldehyde
acrolein
iodoacetamide
formaldehyde
N-methylmaleimide
N-hydroxysuccinimide
methyl salicylate
S-allyl 2-propene-1-sulfinothioate
diallyl disufide
others
geranyl acetate
limonene
indol-3-carbinol
benzyl thiocyanate
solvent controls
mineral oil
DMSO
N.S
Net response (spikes/sec)

Figure 4

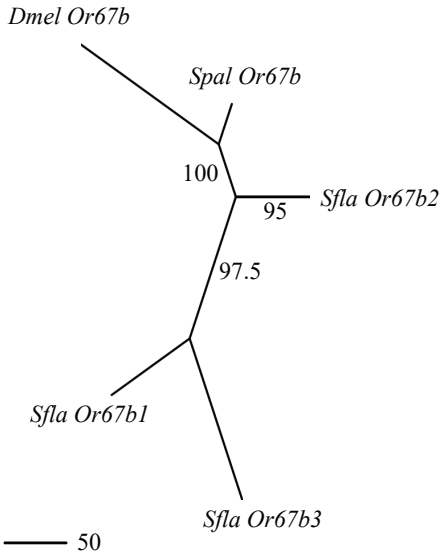
A


acetophenone
phenethyl alcohol
1-hexanol
cis-3-hexenyl butyrate
4-methyl cyclo hexenol
butyl ITC
3-methyl-thio propyl ITC
benzyl ITC
isobutyl ITC
phenethyl ITC
sec-butyl ITC
allyl ITC
ethyl ITC
PC1
PC2
PC3
Dmel activators
ITCs
nitriles
GLVs
electrophiles/TrpA1 activators
others

B


control-subtracted response (spikes/sec)
Dmel Or67b
k=4.76
Spal Or67b
k=5.50
Sfla Or67b1
k=13.6
Sfla Or67b2
k=6.28
Sfla Or67b3
k=3.59
odorants
1. butyl ITC
2. phenethyl ITC
3. iso butyl ITC
4. 3-methyl thio propyl ITC
5. benzyl ITC
6. sec-butyl ITC
7. benzyl thyocyanate

C


Dmel Or67b
Spal Or67b
Sfla Or67b2
Sfla Or67b1
Sfla Or67b3
100
95
97.5
50

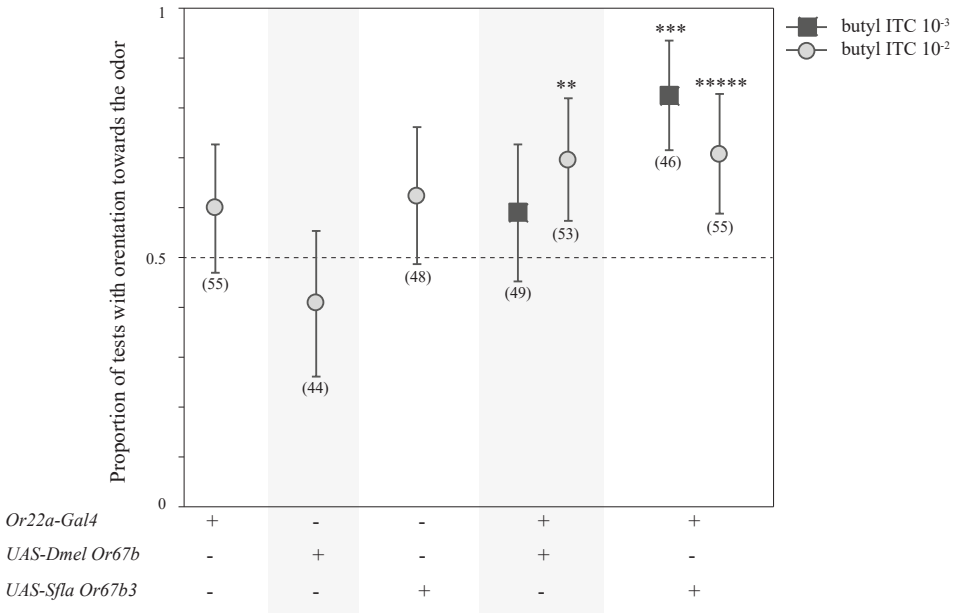
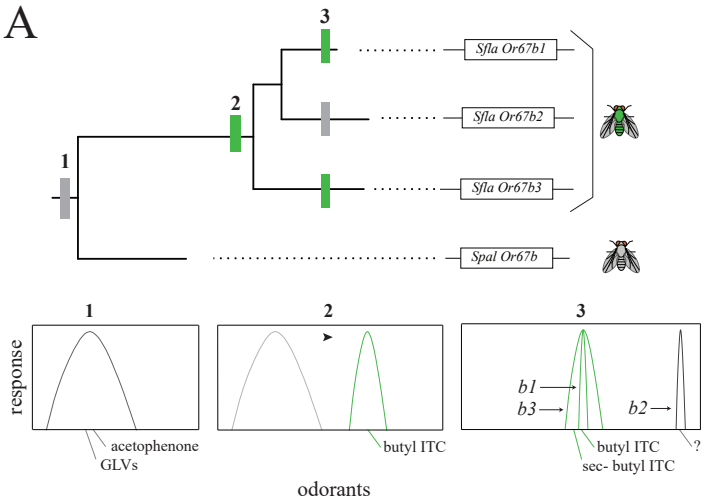

Proportion of tests with orientation towards the odor
1
0.5
0
butyl ITC 10-3
butyl ITC 10-2
***
**
*****
(55)
(44)
(48)
(49)
(53)
(46)
(55)
Or22a-Gal4 + - - + +
UAS-Dmel Or67b - + - + -
UAS-Sfla Or67b3 - - + - +

Figure 5

Figure 6

A


3
2
1
Sfla Or67b1
Sfla Or67b2
Sfla Or67b3
Spal Or67b
1
2
3
response
acetophenone
GLVs
butyl ITC
b1
b3
b2
butyl ITC
sec- butyl ITC
?
odorants

B

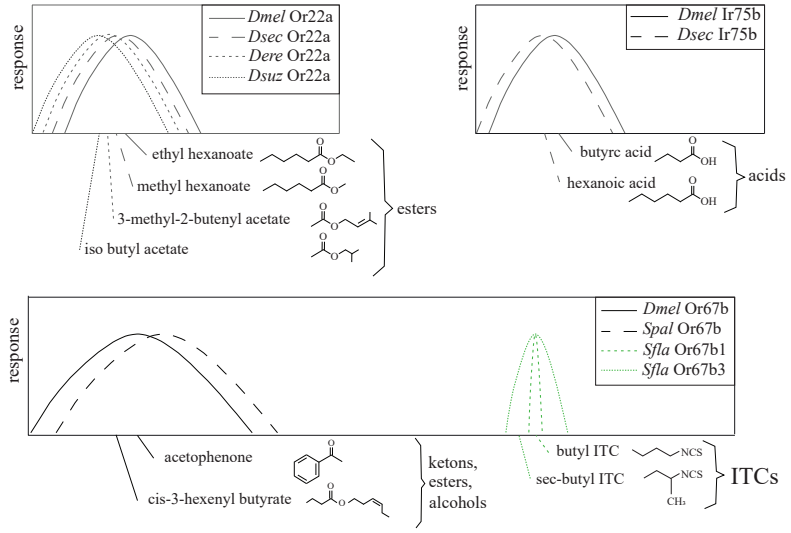

response
Dmel Or22a
Dsec Or22a
Dere Or22a
Dsuz Or22a
ethyl hexanoate
methyl hexanoate
3-methyl-2-butenyl acetate
iso butyl acetate
esters
response
Dmel Ir75b
Dsec Ir75b
butyrc acid
hexanoic acid
acids
response
Dmel Or67b
Spal Or67b
Sfla Or67b1
Sfla Or67b3
acetophenone
cis-3-hexenyl butyrate
ketons,
esters,
alcohols
butyl ITC
sec-butyl ITC
NCS
CH3
ITCs

Figure S1

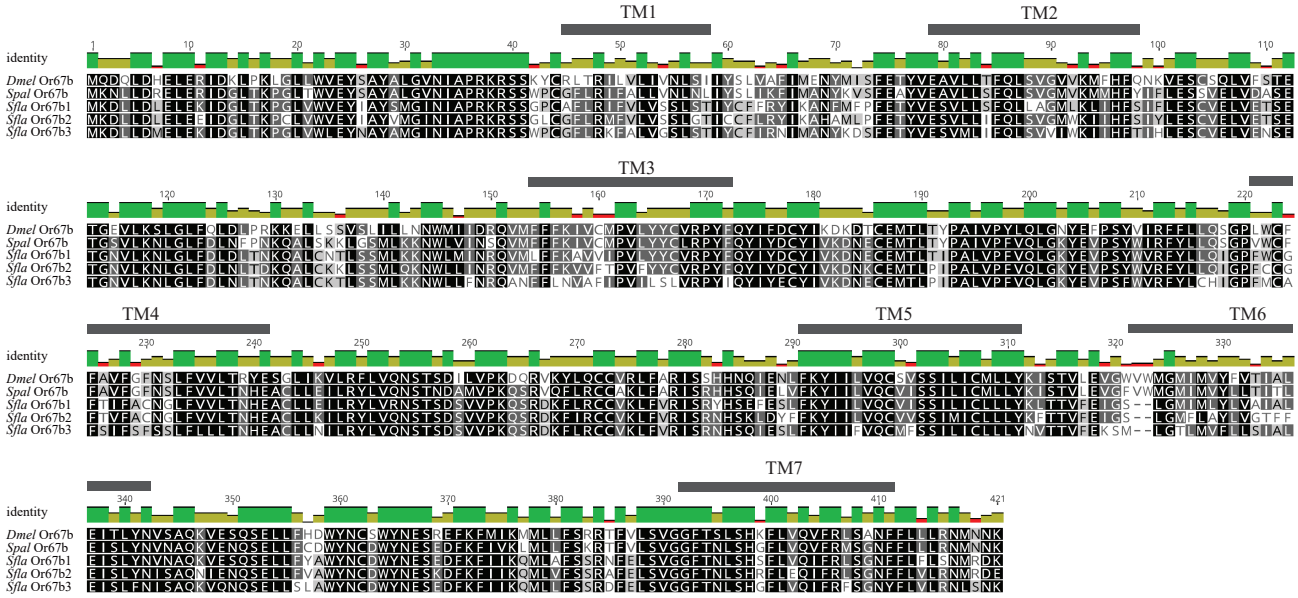

TM1
TM2
TM3
TM4
TM5
TM6
TM7
identity
Dmel Or67b
Spal Or67b
Sfla Or67b1
Sfla Or67b2
Sfla Or67b3

Figure S2

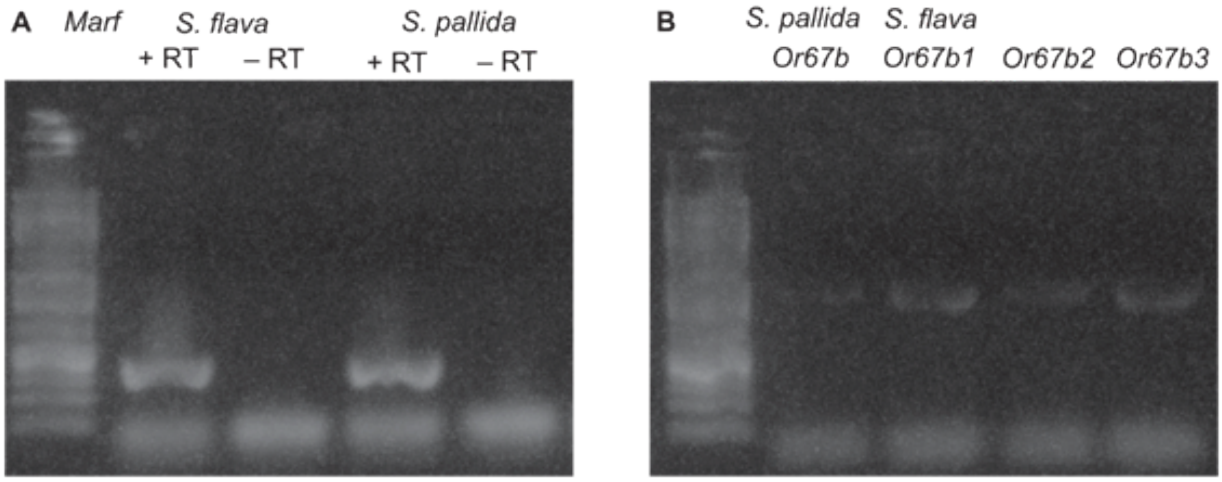
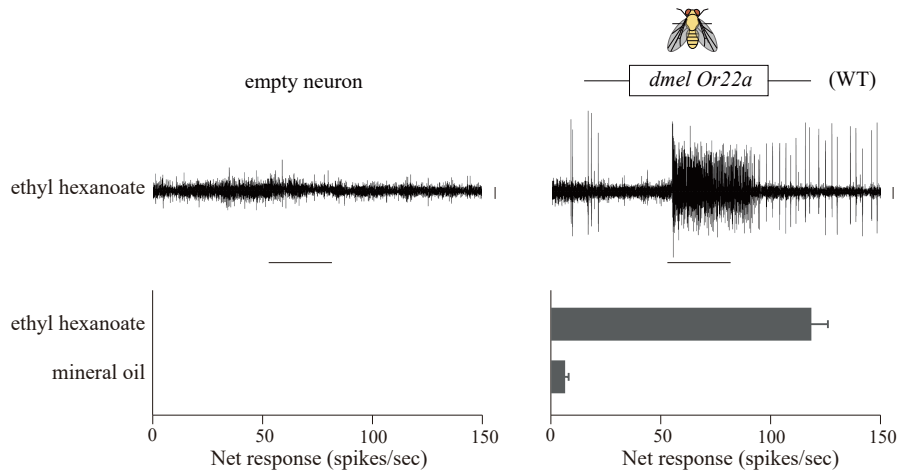
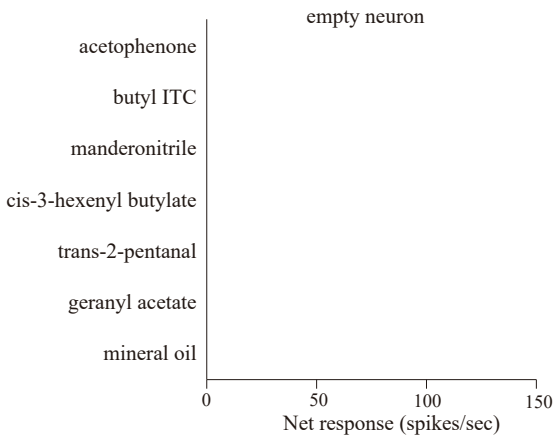

A
Marf
S. flava
+ RT
– RT
S. pallida
+ RT
– RT
B
S. pallida
Or67b
S. flava
Or67b1
Or67b2
Or67b3

Figure S3

A



B



C

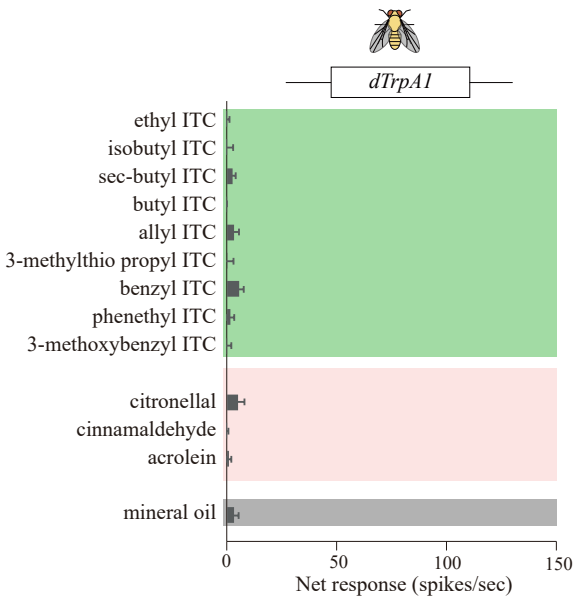
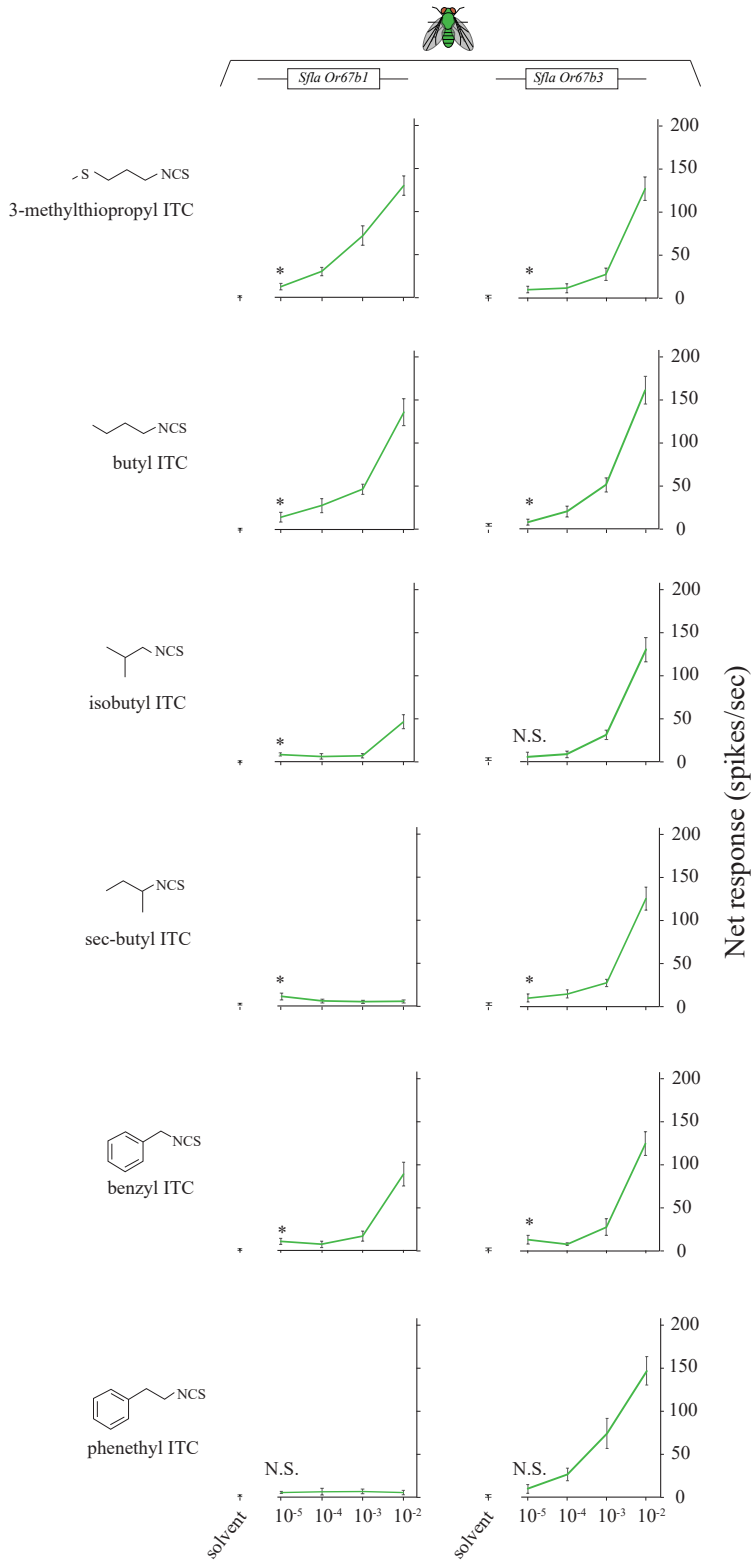


Figure S4


Sfla Or67b1
Sfla Or67b3
3-methylthiopropyl ITC
butyl ITC
isobutyl ITC
sec-butyl ITC
benzyl ITC
phenethyl ITC
NCS
S
N.S.
Net response (spikes/sec)
0
50
100
150
200
solvent
10^{-5}
10^{-4}
10^{-3}
10^{-2}

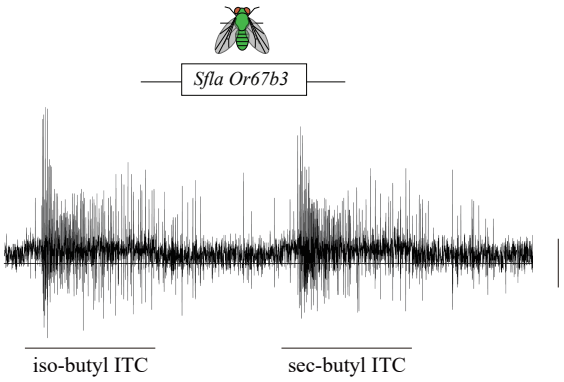



Figure S5