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## Differential Electrophysiological Responses to Odorant Isotopologues In *Drosophilid* Antennae

Odorant isotopologues evoke differential EAGs

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1 ***DIFFERENTIAL ELECTROPHYSIOLOGICAL RESPONSES TO ODORANT***  
2 ***ISOTOPOLOGUES IN DROSOPHILID ANTENNAE***

3  
4 Abbreviated title: Odorant isotopologues evoke differential EAGs

5  
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69 **ABSTRACT**

70

71 Olfaction presents the ultimate challenge to molecular recognition as thousands of  
72 molecules have to be recognized by far fewer olfactory receptors. We have presented  
73 evidence that *Drosophila* readily distinguish odorants based on their molecular  
74 vibrations using a battery of behavioral assays suggesting engagement of a molecular  
75 vibration-sensing component. Here we interrogate electrophysiologically the antennae  
76 of four *Drosophilids* and demonstrate conserved differential response amplitudes to  
77 aldehydes, alcohols, ketones, nitriles and their deuterated isotopologues. Certain  
78 deuterated odorants evoked larger electroantennogram (EAG) amplitudes, while the  
79 response to the normal odorant was elevated in others. Significantly, benzonitrile  
80 isotopologues were not distinguishable as predicted. This suggests that isotopologue-  
81 specific EAG amplitudes result from differential activation of specific olfactory  
82 receptors. In support of this, odorants with as few as two deuteria evoke distinct EAG  
83 amplitudes from their normal isotopologues and this is independent of the size of the  
84 deuterated molecule. Importantly, we find no evidence that these isotopologue-  
85 specific amplitudes depend on perireceptor mechanisms or other pertinent physical  
86 property of the deuterated odorants. Rather, our results strongly suggest that  
87 *Drosophilid* olfactory receptors are activated by molecular vibrations differentiating  
88 similarly sized and shaped odorants *in vivo*, yielding sufficient differential  
89 information to drive behavioral choices.

90

91 **SIGNIFICANCE STATEMENT**

92

93 Insects can behaviorally discriminate odorants from their deuterated isotopologues. It  
94 remains unclear whether discrimination occurs because olfactory receptors detect  
95 their distinct molecular vibrations, or because isotopologues differ sufficiently in  
96 other properties to be perceived differentially. We report electrophysiological  
97 recordings, taken from the antennae of several *Drosophilid* species exposed to a range  
98 of odorant isotopologues. We find that in almost all cases of odorant and species  
99 tested, electroantennogram amplitude, but not kinetics, differs in response to  
100 hydrogen-only and deuterated odorants. Inhibition of enzymes that prevalently  
101 catabolize odorants has no effect on the isotopologue-specific response. Hence, we  
102 conclude that *in vivo*, isotopologue differentiation is not a perireceptor event, but  
103 likely occurs at the receptor itself.

104 **INTRODUCTION**

105

106           Thousands of odorants are discriminated with exquisite specificity by a far  
107 smaller number of olfactory receptors (ORs). Multiple receptors are activated by  
108 particular odorants, fewer by others, and diverse odorants can activate the same OR  
109 (Buck, 2004). Therefore, ORs likely recognize multiple, potentially distinct,  
110 molecular features and functional groups of odorant molecules, including their  
111 molecular vibrations as previously suggested (Turin, 1996; Franco et al., 2011).  
112 Odorants containing non-exchangeable deuterium in place of hydrogen (isotopically  
113 substituted odorants, or isotopologues) retain the shape, physical properties and  
114 ground-state conformations of their normal counterparts (Wade, 1999). However,  
115 they are vibrationally distinct, because the C-H stretch for example, vibrates with a  
116 frequency of 2950-3000  $\text{cm}^{-1}$ , whereas the C-D at 2150  $\text{cm}^{-1}$  due to the additional  
117 neutron in deuterium (Turin, 1996; Wade, 1999; Haffenden et al., 2001). Hence,  
118 discrimination of deuterated from non-deuterated (normal) isotopologues by ORs is  
119 consistent with detection of odorant molecular vibrations. Recognition of odorant  
120 vibrational modes could provide additional discriminatory cues, which together with  
121 their size and functional groups, could contribute to the OR recognition repertoire,  
122 hence to olfactory selectivity. Behavioural experiments strongly suggest that the  
123 insect olfactory system detects molecular vibrations and uses them to drive  
124 behavioural choices (Franco et al., 2011; Bittner et al., 2012; Gronenberg et al., 2014).  
125 Importantly, *Drosophila* trained to avoid a deuterated odorant exhibit learned aversion  
126 for the chemically unrelated nitrile functional group, which however shares  
127 vibrational frequency with the C-D stretch at 2150  $\text{cm}^{-1}$  (Franco et al., 2011).

128           Alternative explanations to vibration-based isotopologue discrimination have  
129 been suggested because deuterated compounds are slightly heavier due to the extra  
130 neutrons. This could result in differential isotopologue diffusion through the sensillar  
131 lymph to the ORs, or differential isotopologue affinity for ORs, or for odorant binding  
132 proteins (OBPs). OBPs are small proteins in the lymph surrounding the receptor  
133 thought to be involved in odorant transport (Heydel et al., 2013; Leal, 2013). In  
134 addition, enzymatic processing in the OR-surrounding lymph has been suggested as a  
135 contributor to isotopologue differentiation (Block et al., 2015), because of potential  
136 differences in their chemistry. Such activities are largely mediated by P450  
137 cytochromes (CYPs) in *Drosophila* (Wang et al., 1999; Younus et al., 2014) and

138 vertebrate olfactory organs (Schilling et al., 2010). These considerations prompted us  
139 to address the potential contribution of enzymatic and kinetic effects of odorant size,  
140 functional groups and other parameters to differential isotopologue detection.  
141 Furthermore, because of these considerations we addressed these questions *in situ*,  
142 rather than in heterologous expression systems, where lack of peri-receptor enzymes  
143 and the OBP milieu (Heydel et al., 2013; Leal, 2013), could compromise  
144 isotopologue discrimination.

145         Given the mammalian complexity, with large numbers of ORs and millions of  
146 olfactory sensory neurons (OSNs) (Godfrey et al., 2004; Malnic et al., 2004; Patel et  
147 al., 2014), we employed *Drosophila* because it is well suited for an *in vivo* approach,  
148 can differentiate isotopologues (Franco et al., 2011; Bittner et al., 2012) and follows  
149 the same organizational principles as vertebrate olfactory systems (Leal, 2013; Martin  
150 et al., 2013). *Drosophila melanogaster* expresses 62 heterodimeric ORs residing  
151 within broadly stereotypically distributed sensilla in the fly antenna (Hallem et al.,  
152 2004; Tunstall et al., 2012). *Drosophila* ORs contain an odorant engaging variable  
153 subunit and a common co-receptor encoded by the *Orco* gene (Larsson et al., 2004;  
154 Sato et al., 2008; Wicher et al., 2008) and this is a major difference with the  
155 monomeric mammalian receptors. In addition, although fly ORs also contain seven  
156 trans-membrane domains, they are distinct from the mammalian receptors, which  
157 belong to the typical G-protein coupled receptor (GPCR) family (Buck, 2004). At  
158 least 14 additional receptors related to ionotropic glutamate receptors (IRs) are also  
159 present in the antenna and often co-reside with particular ORs within specific OSNs  
160 (Abuin et al., 2011; Rytz et al., 2013).

161         Although *Drosophila* ORs differ structurally from their mammalian  
162 counterparts, they offer the major advantage of being readily amenable to direct  
163 activity measurements of single OSNs, or populations of OSNs *in vivo*. Electro-  
164 antennograms (EAGs) probe the sum of receptor activities in the antenna, or at least  
165 in the broader vicinity of the electrode probe (Ayer et al., 1991; Ayer et al., 1992). We  
166 assessed the physiological response of *Drosophila* antennae to multiple odorant  
167 isotopologues in live animals and generalized our findings to other species within the  
168 genus *Drosophila* covering the 40 million years that separate *Drosophila*  
169 *melanogaster* from *Drosophila virilis* (Ashburner et al., 1981; Markow et al., 2006)  
170 using both electrophysiological (EAGs) and behavioral approaches.

171

172 **MATERIALS AND METHODS**

173

174 **EAG measurements.** Recordings were obtained from immobilized 3-10 day old  
175 females that were maintained at 20C and 40-60% relative humidity. Each fly was  
176 immobilized by insertion into the narrow end of a truncated plastic yellow pipette tip,  
177 with the head facing upward protruding from the open end and was then placed under  
178 a dissecting microscope (Olympus SZX16). The antenna was lifted and fixed on a  
179 coverslip with the aid of a glass micropipette tip. Recording and reference glass  
180 microelectrodes filled with 0.17M NaCl (Venard et al., 1984) were placed in the third  
181 antennal segment and the eye (ground) respectively. The recording electrode was  
182 placed in the middle of the dorso-ventral axis of the inner-facing (away from the eyes)  
183 lateral side of the 3rd antennal segment near the area that the antennal nerve exits.  
184 The signal was amplified through a patch-clamp amplifier (MultiClamp 700B,  
185 Molecular Devices), fed into a computer via a 16-bit Analogue-to-Digital converter  
186 (Digidata 1440A, Molecular Devices) with a sampling rate of 4 kHz and analyzed  
187 with pCLAMP 10.3 software (Molecular Devices).

188 The stimulus was applied using a controller (Stimulus Controller CS-55,  
189 Syntech) generating a continuous, humidified, charcoal-filtered air flow of 1.0 L/min  
190 to which the odorant stimulus was added at a flow rate of 0.5 L/min. The stimulus  
191 was carried in a continuous airstream and was interchanged with plain air  
192 automatically to avoid mechanical stimulation of the antennae. The airstream was  
193 directed at the fly through a plastic tube (1 cm diameter), which was fixed in position  
194 by a manipulator such that its output was ~1 cm away from the head of the fly.

195 All odorants were tested for purity by gas chromatography (Tables 2, 3 and 5)  
196 after the completion of each experimental set employing them to ascertain lack of  
197 possible degradation during the experimental protocol. Each odorant was diluted in  
198 isopropyl myristate-IPM (Sigma Aldrich), and 20  $\mu$ L of the stimulus solution were  
199 applied on a strip of filter paper (0.3 cm x 5 cm, Whatman, GE Healthcare), which  
200 was then inserted into a Pasteur pipette. This was then attached to the stimulus  
201 carrying tubing of the CS-55. Each fly was stimulated twice with a single  
202 isotopologue pair (normal and deuterated). The duration of the stimulus was 0.5 sec  
203 and the order the isotopologues were presented was random.

204 To eliminate variation in the amount of odorant present in the first stimulus,  
205 the EAG amplitude was measured from the pre-stimulation baseline to the maximal

206 odorant-induced polarization only for the second stimulus with each isotopologue.  
207 The peak amplitude difference was calculated for each pair of odorants given to a fly  
208 and reported as the means of absolute differences with values above zero representing  
209 cases where the response of the h-odorant was higher than that of its isotopologue and  
210 less than zero if *vice versa*. Differences in response to the two isotopologues for each  
211 fly were evaluated by paired sample t-tests (Excel).

212 Rise time values were calculated as the time required to achieve two-thirds of  
213 the maximal amplitude, whereas fall time values were measured as the time necessary  
214 to recover to one-third of the maximal amplitude after stimulation as described  
215 previously (Alcorta, 1991). Differences were evaluated for each fly by paired sample  
216 t-tests (Excel).

217 The following odorants were used: benzaldehyde (Sigma-Aldrich and Fluka  
218 Analytical), 2-hexanone (Aldrich), 1-hexanol (Fluka analytical and Lluch Essence  
219 SL), benzonitrile (Sigma-Aldrich), ethanol (Fisher), 1-pentanol (Ernesto Ventos), 1-  
220 octanol (Sigma-Aldrich), acetophenone (Fluka analytical and Puriss grade), benzoic  
221 acid (Sigma). All deuterated odorants (benzaldehyde-d6, 2-hexanone-1,1,1,3,3-d5, n-  
222 hexyl-1,1-d2 alcohol, n-hexyl-5,5,6,6,6-d5 alcohol, n-hexyl-d13 alcohol, benzonitrile-  
223 d5, ethyl alcohol-d6, 1-pentyl-d11 alcohol, 1-octyl-d17 alcohol, aceto-d3-phenone,  
224 acetophenone-2',3',4',5',6'-d5, acetophenone-d8 and d5-benzoic acid) were from  
225 CDN Isotopes.

226

227 **Enzyme inhibition assays.** *Drosophila* P450 CYPs (Wang et al., 1999; Younus et al.,  
228 2014), were inhibited with piperonyl butoxide (PBO) based on the protocol of Wang  
229 *et al* and the involvement of these enzymes in methanol detoxification (Wang et al.,  
230 2013).

231 **Lethality assays.** Ten 3-5 day old adult flies were placed in glass vials (75x26 mm)  
232 containing 3 mL of minimal food (1% agar and 2% sucrose), the indicated amount of  
233 pure methanol (%v/v) and 30 $\mu$ L of a 1:3 PBO:acetone solution or acetone vehicle  
234 alone as indicated, applied on a 0.5x5 mm piece of Whatman paper. The number of  
235 dead flies was scored after 18 h at 25C and the experiment was repeated eight  
236 independent times.

237 **Physiology assays.** After 18 h of exposure to PBO, flies were taken from glass vials  
238 and were mounted for EAGs as described above. For recovery experiments, flies  
239 exposed to PBO for 18 h were transferred without anesthesia to vials containing

240 minimal food without drug and were assayed for EAG responses 18 h later. Control  
 241 flies were treated with vehicle alone (acetone) on minimal media for the same time as  
 242 experimental animals.

243

244 **Behavioral assays.** All *Drosophila* species were reared and maintained on standard  
 245 fly food (Acevedo et al., 2007) at 25C, except for *D. pseudoobscura*, which were  
 246 reared and maintained at 22C to account for their Alpine habitat (Kuntz et al., 2014).  
 247 Handling before and during the behavioral experiments was as described previously  
 248 (Franco et al., 2011). Conditioning for *D. pseudoobscura* was performed at 24C and  
 249 70% humidity. The glass vials containing the odorants were sealed with a rubber plug  
 250 that allowed an air stream of 500 mL/min to pass through. In order for a constant  
 251 surface area to be maintained, all odorants were diluted to a total of 1 mL IPM. The  
 252 amounts of odorants used were determined empirically such that as to evoke a similar  
 253 naïve responses (Franco et al., 2011) and are shown in Table 1. The amounts below  
 254 refer to those used for training and testing with each isotopologue such that 5  $\mu\text{L}/\text{mL}$   
 255 were used for training *D. melanogaster* with h-HEL and 5  $\mu\text{L}/\text{mL}$  of each for testing.  
 256 Conversely, 1  $\mu\text{L}/\text{mL}$  were used for training *D. melanogaster* with d13-HEL and  
 257 1  $\mu\text{L}/\text{mL}$  of each isotopologue for testing.

258

	h-hexanol+IPM	d13-hexanol+IPM
<i>D. melanogaster</i> (w1118)	5 $\mu\text{L}/\text{mL}$	1 $\mu\text{L}/\text{mL}$
<i>D. simulans</i>	2 $\mu\text{L}/\text{mL}$	5 $\mu\text{L}/\text{mL}$
<i>D. pseudoobscura</i>	2 $\mu\text{L}/\text{mL}$	2 $\mu\text{L}/\text{mL}$
<i>D. virilis</i>	20 $\mu\text{L}/\text{mL}$	20 $\mu\text{L}/\text{mL}$
<i>D. melanogaster</i> (Canton S)	2 $\mu\text{L}/\text{mL}$	2 $\mu\text{L}/\text{mL}$

259 Table 1. Concentrations of hexanol isotopologues used for conditioning experiments.

260

261 **Olfactory conditioning.** Groups of 40-60 flies were placed into the training arm of a  
 262 standard olfactory conditioning maze (Franco et al., 2011) and were presented for 1  
 263 minute with the odorant while receiving 12 electric foot-shocks of 90V DC each  
 264 lasting 1.2 seconds. The training odor was then cleared from the training tube with 15  
 265 seconds of room air. Subsequently the flies were transferred to the choice point of the  
 266 maze where an air stream carrying an odorant met with another that passed over IPM

267 only. The flies then had 90 seconds to move away or towards the test odorant, which  
268 was either the same as the training odorant or its isotopologue. The assay was  
269 executed in groups of three trials starting with naïve avoidance of an odorant *versus*  
270 the solvent (IPM)-scented air. Another group of flies was tested with a test  
271 isotopologue identical with the one used for training (odor + shock). Finally, for the  
272 third group of flies, the training odorant was the other isotopologue of the test odorant.  
273 After every trial, the flies were collected and counted and a distribution index was  
274 calculated by subtracting the number of flies that did not avoid the test odorant from  
275 those that did, divided over the total counted from each trial. Results were analyzed  
276 parametrically using planned comparisons (Least Squares Method) and the statistical  
277 program JMP (SAS).

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287 **RESULTS.**

288

289 *Differential EAG responses to isotopologues.*

290 Receptors may be activated or inhibited by odorants (Hallem et al., 2006;  
291 Carey et al., 2011), but EAGs do not differentiate between such events (Alcorta,  
292 1991). Rather, the EAG amplitude reflects the sum of receptor activities as voltage  
293 changes upon odorant exposure. If engagement of odorant isotopologues results in  
294 differential patterns of OR activation and inhibition, it will yield differential voltage  
295 changes reflected by distinct EAG properties (Alcorta, 1991; Ayer et al., 1992).

296 We used this approach to interrogate the overall electrophysiological response  
297 of *Drosophila* antennae to previously described (Franco et al., 2011) and new odorant  
298 isotopologue pairs in three concentrations (0.01%, 0.1% and 1% v/v). Initially, we  
299 used isotopologue pairs of common functional groups representing simple alcohols  
300 (1-hexanol-HEL), aromatics and aldehydes (benzaldehyde-BNZ), simple ketones (2-  
301 hexanone-HEN) and nitriles (benzonitrile-BNL). The purity of all odorants was  
302 ascertained by gas chromatography (Table 2, 3 and 5). The recording electrode was  
303 placed approximately in the same location of the midpoint along the dorso-ventral  
304 axis of the inner lateral side of the antenna in all animals tested. Individual flies were  
305 challenged with isotopologue pairs delivered in random order (see Materials and  
306 Methods). We avoided using acids (ie acetic acid) because these activate IRs  
307 preferentially (Benton et al., 2009; Rytz et al., 2013). Therefore with the odorants  
308 used we are assaying primarily the response of the OR class of receptors.

309 Average EAG traces from four isotopologue pairs at three different  
310 concentrations are shown in Figure 1 A, F, K and P. It is evident that the overall  
311 structure and details of the traces are distinct for each odorant as expected, but except  
312 for the EAG amplitude, they were similar for all odorant isotopologue pairs. Maximal  
313 amplitudes were consistently different between isotopologues at the lower dilutions  
314 and we concentrated on that aspect for this study. Interestingly, h-HEL always evoked  
315 larger EAG amplitudes than d-HEL (Fig 1 C-D) and the same for HEN isotopologues  
316 (Fig 1 M-N) at the  $10^{-2}$  and  $10^{-3}$  dilutions. In contrast, BNZ isotopologues yielded the  
317 opposite result with d6-BNZ evoking a much larger response than the normal odorant  
318 in the same dilutions (Fig 1 H-I). Differences in response amplitude were not apparent  
319 at the lowest odorant concentration ( $10^{-4}$ ), for all odorants tested, potentially reflecting  
320 a loss of isotopologue selectivity at the higher dilutions.

321 To quantify these isotopologue-specific EAG differences and to normalize the  
322 obvious inter-animal variability, we subtracted the absolute maximal amplitude  
323 elicited by the deuterated odorant from that of its normal counterpart per animal. We  
324 then estimated the means of those differences per isotopologue pair ( $\Delta$  amplitude),  
325 which along with their standard errors (SEM) are shown in Figure 1 E, J, O, T.  
326 Positive  $\Delta$  amplitude values arise when the EAG for the normal odorant is larger than  
327 that of its deuterated isotopologue (Fig 1 E, O), whereas the converse yields negative  
328 values (Fig 1 J). If the response to the two isotopologues is similar or identical, their  $\Delta$   
329 amplitude would be zero. Because isotopologues are not expected to yield differences  
330 (Keller et al., 2004; Block et al., 2015), we used this as a null hypothesis in testing for  
331 significance. Similar results for hexanol and benzaldehyde isotopologues were  
332 obtained with the Canton-S strain of *D. melanogaster* (not shown), indicating that  
333 these responses are typical of the species and not strain-specific.

334 The C $\equiv$ N and C-D bonds shares the  $\approx 2150\text{ cm}^{-1}$  vibrational frequency (Franco  
335 et al., 2011). Consequently, deuterated BNL contains both components that share the  
336  $2150\text{ cm}^{-1}$  vibration, while the normal odorant contains only that of the C $\equiv$ N bond. If  
337 the differential amplitudes depended on molecular vibrations and not the mere  
338 presence of deuterium, then the BNL isotopologues would be expected to yield  
339 similar EAGs. In fact, average traces were nearly indistinguishable at all dilutions  
340 tested (Fig 1 P) and the peak amplitudes were not significantly different at all  
341 dilutions (Fig 1 Q-S). This is further illustrated by all of the  $\Delta$ -amplitudes (Fig 1 T),  
342 which were not different from zero. Collectively then and in accord with predictions  
343 based on calculations, three of the four isotopologue pairs yielded differences in EAG  
344 responses, while the fourth did not, as expected. This is not consistent with the null  
345 hypothesis expecting identical responses from structurally identical molecules and  
346 agrees with prior reports (Franco et al., 2011; Bittner et al., 2012; Gane et al., 2013;  
347 Gronenberg et al., 2014) and with the hypothesis that molecular vibrations play a role  
348 in differentiating isotopologues.

349 Impurities are unlikely to account for these differences as hexanol and  
350 hexanone isotopologues were of very high purity (Table 2). Furthermore, deuterated  
351 benzaldehyde, which elicits higher amplitudes, is more pure than its normal  
352 isotopologue (Table 2). This is not consistent with the notion that higher EAG  
353 amplitudes may result from contaminants activating additional ORs. In fact, the main  
354 benzaldehyde contaminant was benzoic acid as mass spectroscopy identified (not

355 shown). Although counterintuitive given the higher EAG amplitudes from d6-BNZ,  
356 we investigated whether the presence of benzoic acid could account for the larger  
357 amplitude. We obtained pure benzoic acid isotopologues (Table 2), which yielded  
358 characteristically low signals (mean EAG amplitude for normal benzoic acid= $0.122 \pm$   
359  $0.221$  mV), even at a much higher concentration ( $10^{-3}$ ) than present in BNZ samples  
360 (estimated from the gas chromatographs at  $<10^{-4}$ ). EAGs evoked by d5-benzoic acid  
361 were slightly higher than those from the normal compound (mean EAG amplitude=  
362  $0.096 \pm 0.174$  mV), with  $\Delta$ amplitude not significantly different from 0 ( $\Delta$  amplitude=  
363  $0.026 \pm 0.273$  mV;  $p=0.927$ ). Therefore, the EAG amplitude differences of BNZ  
364 isotopologues are not attributable to the main contaminants in the preparations. In  
365 support of this, normal 2-hexanone yielded higher EAG amplitudes (Fig 1 M, N),  
366 although the deuterated isotopologues had an additional impurity expected to yield  
367 larger EAG amplitudes if contaminants were determinants of the differential  
368 isotopologue response. Importantly, the fact that responses are higher at least for d6-  
369 benzaldehyde (Fig 1 H, I, J), indicates that evaporation rates, potentially reduced for  
370 the slightly heavier deuterated odorants are unlikely to be important for the observed  
371 differential effects.

372 To further ascertain that contaminants were not the source of the amplitude  
373 differences, we obtained normal odorants from different sources and different lots of  
374 their deuterated isotopologues. For emphasis, the purity profiles of the new odorants  
375 are presented separately on Table 3 and were in fact different than the original set  
376 (Table 2). For example, h-1-HEL in table 3 contains a lot more trace impurities than  
377 that from Fluka (Table 2). In contrast, lot X421P8 of d13-1-HEL (Table 3) contains  
378 fewer impurities than the original (lot X241P13, Table 2), although from the same  
379 source (CDN). Similar purity profile differences characterized the different lots and  
380 sources of benzaldehyde (Table 2 *versus* Table 3) and acetophenone isotopologues  
381 (Table 3 *versus* Table 5). Results from the new independent odorant set are  
382 summarized on Table 4. Despite the differences in trace impurities from the  
383 previously used odorants, similar isotopologue-specific differences in amplitude,  
384 albeit somewhat different in absolute value were observed with the different lots of  
385 HEL and BNZ (Table 4). Normal HEL contains fewer impurities than d13-HEL  
386 (Table 2) in the lots surveyed in Fig 1. However, an independent lot of h-HEL  
387 containing more impurities (Table 3) still yielded amplitudes larger than its purer  
388 deuterated isotopologue (Table 4). A similar effect was uncovered for ACP

389 isotopologues (Table 4 versus Fig 2N). For BNZ where the deuterated isotopologue  
390 elicits larger EAG amplitude, increased impurities in the d6-BNZ isotopologue (Table  
391 3) still resulted in significantly different negative  $\Delta\text{amp}$  (-0.628 on table 4 *versus* -  
392 0.752 on Fig 1) for the  $10^{-2}$  dilution. Therefore, the amplitude differences between  
393 isotopologues remain significant irrespective of isotopologue source and lot. These  
394 results are discordant with the notion that impurities underlie the isotopologue-  
395 specific differential amplitudes. Hence, contaminating trace impurities cannot account  
396 for the differential response of the *Drosophila melanogaster* antenna to odorant  
397 isotopologues.

398         Significantly, the main measurable electrophysiological difference at the level  
399 of total antennal activity in response to odorant isotopologues is the amplitude of the  
400 EAG responses. As expected, different odorants yielded EAG traces with different  
401 overall shapes, which however were similar if not identical for isotopologues of the  
402 same odorant (Fig 1A, 1 F and 1 K). In fact, EAGs in response to BNZ+HEN  
403 mixtures can be differentiated from those of either odorant alone in our preparation  
404 (not shown), in accord with our contention. It follows then that despite the limited  
405 resolution, if distinct ORs were activated by one isotopologue of a pair, the shapes of  
406 the resultant EAG curves would be predicted to diverge in a manner akin for curves  
407 produced by different odorants. Instead, our results are consistent with differential  
408 activation of the same ORs, or engagement of perhaps overlapping, yet distinct OR  
409 sets by each isotopologue.

410

411 ***The degree of deuteration does not affect the isotopologue-specific EAG amplitude.***

412         A previous publication (Gane et al., 2013), suggested that the number of  
413 deuteriums and vibrational modes seems to be important in humans, since subjects  
414 discriminated cyclopentadecanone (28 C-H/C-Ds) isotopologues, but not the much  
415 smaller acetophenone (8 C-H/C-Ds). Therefore, we investigated whether the degree  
416 of deuteration may correlate with the differential isotopologue-specific EAG  
417 amplitudes in *Drosophila*. This is of particular interest because of reports suggesting  
418 that deuteration increases polarity in proportion with the number of deuteria and  
419 number of heteroatoms (Wade, 1999). This decrease in hydrophobicity is apparent in  
420 the slight, but consistently faster GC elution time of deuterated isotopologues (Table 2,  
421 3 and 5). Therefore, we reasoned that if the number of deuteriums *per se*, or the  
422 changes in polarity due to their presence were important for differentiation, then

423 partially deuterated odorants should evoke distinct responses from perdeuterated ones.  
424 Thus, d2-hexanol would not be readily differentiated from its normal counterpart, or  
425 at least not as well as the perdeuterated (d13-hexanol) isotopologue. In addition to  
426 hexanol, we also used isotopologues of the aromatic ketone acetophenone (ACP),  
427 chemically distinct from hexanol, which in addition to the aromatic ring contains a  
428 polar ketone functional group. The purities of ACP isotopologues are reported on  
429 Table 5.

430 As apparent by the representative traces in Fig 2 A, normal hexanol yielded  
431 significantly higher EAG amplitudes from those elicited by all of its deuterated  
432 isotopologues and this is reflected in the data from partially deuterated *versus* normal  
433 isotopologue pairs in Fig 2 B, C, and D. This surprising result was independently  
434 verified by challenging individual flies with the di-deuterated *versus* perdeuterated  
435 hexanol, which yielded strikingly similar traces illustrated in Fig 2 E and detailed in  
436 Fig 2 F, underscoring the lack of significant EAG differences. Calculation of  $\Delta$ -  
437 amplitude for each deuterated and normal isotopologue revealed that it was not  
438 significantly different across the range of hexanol deuteration tested (Fig 2 G).  
439 Similar responses were obtained over a range of dilutions of d2 and d13-HEL (Table  
440 6). The mean amplitudes were proportional to the dilutions of the odorants as  
441 expected. Importantly, the response amplitudes generated by each of the  
442 isotopologues at a given dilution were not significantly different from each other as  
443 the p values and the near zero  $\Delta$  amplitude values indicate (Table 6). It should be  
444 noted that the results from the  $10^{-2}$  HEL isotopologue dilution are independent of and  
445 confirm those reported in Fig 2F and G.

446 Similar results were obtained with acetophenone isotopologues (Fig 2 H-N),  
447 where again the normal ACP yielded significantly larger EAG amplitudes than any of  
448 the deuterated odorants (Fig 2 I, J, K). Importantly, the  $\Delta$ -amplitude between normal  
449 and either tri-, penta- or the perdeuterated odorants were statistically indistinguishable  
450 (Fig 2 N), a conclusion confirmed independently by the identical EAGs elicited when  
451 individual animals were challenged with d3- and d8-acetophenone (Fig 2L, M). Again,  
452 the lack of difference in amplitudes yielded by the d3 and d8 isotopologues held over  
453 a range of dilutions (Table 6) and confirmed the results mentioned above.

454 Therefore, the lack of EAG amplitude differences between perdeuterated and  
455 partially deuterated isotopologues of HEL and ACP is not a consequence of saturating  
456 odorant at the  $10^{-2}$  dilution. It follows then that incorporation of as few as two

457 deuteria is sufficient to evoke measurable differential EAG amplitudes from their  
458 normal isotopologues. Moreover, the effect of multiple C-D bonds in a molecule does  
459 not appear to be linear.

460         Reduced volatility of the deuterated odorants does not explain the EAG  
461 amplitude differences because if it were responsible, isotopologues with fewer  
462 deuteria (ie d2-hexanol and d3-acetophenone), would be expected to elicit similar or  
463 identical EAG amplitudes to their normal counterparts. Furthermore, differences  
464 cannot be attributed to impurities, as the hexanol isotopologues are highly pure (Table  
465 2, 3) and impurities vary in the preparations in a discordant fashion with the  
466 amplitude differences reported here. In agreement, independent lots of ACP  
467 isotopologues at the  $10^{-2}$  dilution (Table 3), yielded similar results (Table 4) as those  
468 in Fig 2K.

469         In the case of hexanol and acetophenone isotopologues, the number of  
470 deuteria varied, but the size of the molecule that carried them was constant. We aimed  
471 to independently verify these surprising results by asking whether the size of the  
472 deuterated molecule is important for the differential EAG amplitudes. To keep the  
473 functional group unaltered and stereochemistry as similar as possible, we selected  
474 four alcohols including 1-hexanol to ask whether size may be a relevant  
475 differentiating parameter and tested normal and deuterated pairs at the  $10^{-2}$  dilution.  
476 With the exception of 1-pentanol where the difference was marginal (Fig 3 B),  
477 ethanol and octanol isotopologues evoked highly significant EAG differences (Fig 3  
478 A, D). However, unlike the response to hexanol isotopologues (Fig 3 C), where the  
479 normal odorant elicited larger EAG amplitudes, deuterated ethanol, pentanol and  
480 octanol evoked larger responses (Fig 3E). This difference does not appear to depend  
481 on the size of the molecule since hexanol is in the middle of the size range, or the  
482 number of deuteria it carries (13), which is also in the middle of the range (6-17)  
483 examined.

484         Because EAGs report total OR activity, the response summarizes the  
485 activation and inhibition of ORs responsive to a particular isotopologue. Maximally  
486 activated ORs are likely to contribute the majority of the activity reported in EAG  
487 amplitudes. It follows then that the isotopologue-specific EAG amplitudes reflect  
488 differences in the subsets of maximally activated ORs. The direction of the  $\Delta$ -  
489 amplitude difference between hexanol and the other alcohols may be consequent of  
490 the number or ORs activated or inhibited by each odorant isotopologue. Hence, within

491 the subset of hexanol activated ORs, the majority yield maximal activities with the  
492 normal odorant and fewer with d13-HEL. The converse could be the case for ORs  
493 activated by ethanol pentanol and octanol as suggested by their  $\Delta$  amplitudes (Fig 3  
494 E). Alternatively, particular ORs respond only to one of the two isotopologues and if  
495 so, more appear responsive to normal than d13-HEL with the converse for ethanol  
496 pentanol and octanol.

497 Interestingly, the  $\Delta$  amplitude ranges are similar for ethanol with 6 deuteria  
498 and octanol with 17 (Fig 3 E). Therefore, as suggested above, increasing the number  
499 of deuteria does not proportionally increase the EAG difference from that elicited by  
500 the normal odorant. Moreover, inasmuch as odorant size determines volatility, it does  
501 not predict which isotopologue will evoke the larger EAG, as exemplified by the  
502 opposite  $\Delta$  amplitude effects for pentanol and hexanol. Therefore, the differential  
503 responses to isotopologues appear to depend primarily on the presence of deuterium  
504 and to be independent of its actual numbers per molecule. Collectively, the results do  
505 not support the hypothesis that deuteration-mediated polarity changes are responsible,  
506 or contribute to the differential EAG amplitudes evoked by deuterated vs normal  
507 odorants. In contrast, the results are consistent with the notion that ORs are sensitive  
508 to the characteristic vibrational frequency of at least two, perhaps a single C-D bond  
509 to differentiate deuterated odorants from their normal isotopologues in *Drosophila*.  
510

511 ***Perireceptor effects are not major contributors to isotopologue differentiation.***

512 Perireceptor mechanisms that potentially interplay or interfere with direct  
513 odorant engagement by the ORs have recently been reported (Heydel et al., 2013) and  
514 could in principle affect differential isotopologue perception. The OBPs bind odorants  
515 with high affinity, probably based on their overall shape or functional groups and act  
516 as carriers to the ORs (Swarup et al., 2011; Leal, 2013). Although isotopologues do  
517 not differ in shape, EAG amplitude differences may reflect differential diffusion or  
518 transport through the sensillar lymph of the slightly heavier and more polar deuterated  
519 isotopologues to the ORs.

520 To investigate whether isotopologues are transported differentially to the  
521 respective ORs, we estimated the time required to reach two-thirds of the maximal  
522 EAG amplitude (rise time) upon odorant exposure (Alcorta, 1991). The results  
523 presented in Fig 4 A-H did not reveal significant differences (paired t-tests) in rise  
524 time for all isotopologues tested except for ethanol, where rise times in response to

525 the deuterated odorant were found significantly slower in 7 of the 9 animals tested  
526 (Fig 4 F). Because the deuterated isotopologue evoked a slower response, it may  
527 reflect less efficient OBP engagement or transport to the cognate OR because of its  
528 increased polarity. However, this is unlikely to be generalized and underlie the higher  
529 EAG amplitudes evoked by deuterated pentanol and octanol (Fig 3 E), because their  
530 rise times were not significantly different from those elicited by their normal  
531 counterparts (Fig 4 G, H). Moreover, rise time differences were not seen for hexanol,  
532 where the normal isotopologue evokes higher EAG amplitudes (Fig 3 E). Similar data  
533 were obtained with higher isotopologue dilutions (not shown). Collectively,  
534 isotopologue-specific rise time differences were not observed in 7 of the 8 odorant  
535 pairs tested and ethanol may be an exception because of its size. Therefore, our data  
536 do not support the notion that the isotopologue-specific EAG amplitude differences  
537 we uncovered (Fig 1, Fig 2 and Fig 3) reflect differential diffusion or OBP-dependent  
538 transport (Heydel et al., 2013; Leal, 2013) to the ORs.

539 Furthermore, we determined the time required for the response to decay to one  
540 third of the maximum (fall time), as a measure of OR activity after stimulus removal.  
541 We reasoned that the slightly heavier deuterated isotopologues may require more time  
542 to be cleared from the respective ORs, evoking longer OR activities, perhaps  
543 underlying the differential EAG amplitude. Overall the data (Fig 4 I-P), do not reveal  
544 isotopologue-specific significant differences in fall time for all odorants tested except  
545 for hexanol (Fig 4 I), which is also illustrated by the difference in the recovery (right  
546 side) portion of the average traces at  $10^{-2}$  and  $10^{-3}$  in Fig 1A. This was also observed  
547 with the alternative HEL isotopologues on Table 6 (not shown). However, the longer  
548 time required to return to baseline upon d13-HEL exposure does not correlate with the  
549 differential amplitudes, as it is the normal isotopologue which evokes the higher EAG  
550 (Fig 1 A-E). Collectively the data indicate that in general, OR engagement by the  
551 slightly heavier deuterated odorants and residual OR activity are not differentially  
552 affected by isotopologues as to underlie the observed EAG amplitude differences.

553 Biotransformation and detoxification enzymes contribute to odorant  
554 inactivation in the perireceptor space (Martin et al., 2013). The main such enzymes  
555 are of the cytochrome P450 family in the *Drosophila* antenna (Wang et al., 1999;  
556 Brandt et al., 2002). If biotransformation is required before OR engagement for  
557 odorants used in this study, deuterated odorants could be processed at a different rate  
558 from their normal isotopologues as described previously (Swiderek et al., 2013).

559 Differences in processing rates could result in submaximal OR activation, which  
560 could account for the observed amplitude effects. Alternatively, P450 activity may be  
561 necessary to clear the odorant from the perireceptor space and deuterated odorants  
562 may in fact be cleared at a lower rate yielding differential EAG properties and  
563 amplitudes. Therefore we sought to inhibit the P450 family and determine the effects  
564 on the EAGs of selected isotopologue pairs.

565 CYPs were inhibited with piperonyl butoxide (PBO), using the protocol of  
566 Wang *et al* (2013). Initially, we verified that PBO exposure enhanced methanol  
567 toxicity as reported (Wang *et al.*, 2013). Then the lowest effective PBO concentration  
568 that enhances methanol toxicity without being excessively toxic itself was determined  
569 as 0.25% (v/v). If P450s were essential for isotopologue differentiation, their  
570 inhibition should eliminate the isotopologue-specific amplitude differences. Control  
571 EAG traces upon stimulation with hexanol isotopologues yielded differences  
572 comparable with those described and quantified in Fig 1 (Fig 5 B green vs dark blue  
573 trace). Flies exposed to PBO for 18 h responded to hexanol isotopologues  
574 differentially (Fig 5 B light blue vs dark grey trace), albeit with reduced EAG  
575 amplitudes (Fig 5 C). This suggests that PBO inhibits enzymes necessary for  
576 activation of all ORs contributing to the differential maximal EAG amplitudes, or that  
577 the reduced response is due to compound toxicity on the ORs. However, although the  
578 EAG amplitudes were reduced, the isotopologue-specific differential effect remained  
579 similar to that in untreated animals (Fig 5 D). Moreover, PBO treatment did not  
580 differentially affect the rise and fall times in response to hexanol isotopologues (not  
581 shown), suggesting that odorant transport and OR engagement were not affected.

582 To determine whether the reduced amplitude was caused by OR depression, or  
583 attrition due to PBO toxicity, we allowed the flies to recover for 18 h following PBO  
584 treatment. Surprisingly, recovery resulted in EAG amplitudes virtually  
585 indistinguishable from controls (Fig 5 B, yellow vs green and dark blue vs magenta  
586 traces and Fig. 5 C). Again the isotopologue-specific amplitude differences in all  
587 three groups were not significantly different (Fig 5 D). Rise and fall times in flies  
588 recovered from PBO treatment were also similar to those from controls (not shown).  
589 Therefore, PBO treatment is not irreversibly toxic to ORs and P450 family enzymes  
590 do contribute to maximal hexanol detection, but their activity does not affect  
591 isotopologue differentiation. Similar results were obtained with the unrelated aromatic  
592 ACP isotopologues (Fig 5 E, F), indicative of the generality of the above conclusions.

593 We also tested 1-octanol, where the deuterated odorant elicited larger EAGs. In this  
594 situation, PBO treatment did not abolish (Fig 5 G), but reduced the isotopologue  
595 differences such that they became marginally significant (Fig 5 H). This appears to  
596 result from reduced PBO-mediated inhibition of the amplitude elicited by h-1-OCT  
597 relative to that from its deuterated isotopologue as suggested by their  $\Delta$  amplitude.  
598 Therefore, P450s could be important for perireceptor processing or clearance of  
599 normal 1-octanol, but this is a likely exception and alone does not account for the  
600 specific EAG differences of other isotopologue pairs.

601 Collectively our data does not lend support to the notion that in general,  
602 isotopologues are engaged or processed differentially enough by the *Drosophila*  
603 antenna to correlate with the observed significant differences in EAG amplitudes.  
604 This agrees with kinetic analyses indicating that P450s do not degrade deuterated  
605 substrates faster than their normal counterparts (Guengerich, 2013).

606

607 ***Evolutionary conservation of the differential isotopologue response.***

608 Isotopologue discrimination salient to behavioral choices has been described  
609 recently for *Drosophila* and bees (Franco et al., 2011; Bittner et al., 2012; Gronenberg  
610 et al., 2014). Isotopologue discrimination limited to a single or a few odorants has  
611 also been reported for the flour beetle *Tribolium castaneum*, the American cockroach  
612 *Periplaneta americana* (Meloan et al., 1988) and more recently in humans for a  
613 macrocyclic musk (Gane et al., 2013), indicating broad evolutionary conservation.  
614 Because *Drosophila* ORs are highly diverse and fast-evolving (de Bruyne et al., 2009),  
615 we wondered whether the specific responses to isotopologues described herein for  
616 *Drosophila melanogaster* would be conserved within the genus. Hence, we used  
617 EAGs to ask whether representative species within the genus respond similarly to  
618 isotopologues. Towards that end we used hexanol, benzaldehyde, hexanone, and  
619 benzonitrile isotopologues to obtain EAGs from the equivalent location as that for *D.*  
620 *melanogaster* antennae in the sibling species *Drosophila simulans*, the more distant  
621 *Drosophila pseudoobscura* and *Drosophila virilis* which is separated from *D.*  
622 *melanogaster* by over 40 million years (de Bruyne et al., 2009).

623 All species responded differentially to hexanol isotopologues (Fig 6A-C) and  
624 importantly, as for *D. melanogaster* (Fig 1 D), the normal odorant evoked  
625 significantly higher amplitudes than d13-HEL (Fig 6 D). Furthermore, like for *D.*  
626 *melanogaster* (Fig 1I), d6-benzaldehyde evoked significantly higher amplitudes than

627 its normal counterpart in *D. simulans* and *D. pseudoobscura* (Fig 6 E, F, H). However,  
628 d6-BNZ evoked marginally larger amplitudes in the distant *D. virilis* (Fig 6 G, H). A  
629 similar pattern was uncovered with 2-hexanone isotopologues, where *D.*  
630 *melanogaster* (Fig 1N), *D. simulans* and *D. pseudoobscura* responded with  
631 significantly larger EAG amplitudes to the normal than the deuterated odorant (Fig 6 J,  
632 K), but again the difference was marginal for *D. virilis* (Fig 6 K). Importantly, *D.*  
633 *simulans*, *D. pseudoobscura* and *D. virilis* presented highly variable and not  
634 significantly different responses to BNL isotopologues (Fig 6 M-O). Therefore,  
635 similar to *D. melanogaster* these species spanning the breadth of the genus, do not  
636 differentiate benzonitrile isotopologues, indicating that the mechanism of  
637 isotopologue differentiation is conserved and involves detection of molecular  
638 vibrations.

639         In summary, although to different degrees in accord for species occupying  
640 diverse niches and separated by significant evolutionary distance, members of the  
641 genus *Drosophila* presented conserved differential EAG amplitudes to odorant  
642 isotopologues. Significant differences in the magnitude of the differential response  
643 among the species were detected for benzaldehyde (Fig 6 B) and hexanone (Fig 6 C)  
644 and this is not totally unexpected considering the evolutionary distance and the high  
645 variability of ORs. Importantly however, the isotopologue from each pair eliciting the  
646 higher EAG response was conserved and so was the inability to distinguish the  
647 benzonitrile isotopologues. This likely reflects isotopologue-specific activities of OR  
648 subsets for sibling and distant species and suggests that as for *D. melanogaster*  
649 (Franco et al., 2011; Bittner et al., 2012), isotopologues may elicit differential  
650 behavioral responses.

651         We selected hexanol isotopologues to address this question, because they  
652 elicited equivalent responses in terms of  $\Delta$ amplitude (Fig 6 D). We simplified the  
653 Pavlovian conditioning assay used previously (Franco et al., 2011), reasoning that the  
654 isotopologue in whose presence animals are punished should be selectively avoided  
655 even in the absence of an unpunished odorant. In contrast, if not differentiated, both  
656 isotopologues should elicit identical responses. Comparative pilot experiments  
657 indicated that this modified assay affords better resolution than Pavlovian  
658 conditioning (not shown). Odorant concentrations (Table 1 in Materials and Methods),  
659 were adjusted in control experiments as before (Franco et al., 2011), to elicit as  
660 balanced a response from naïve animals as possible.

661 Naïve *D. melanogaster* strain  $w^{1118}$  flies showed mild avoidance for h-hexanol  
662 (naïve-Fig 7 A), which was significantly enhanced upon prior punishment in its  
663 presence (h-HEL-trained Fig 7 A). However, h-hexanol avoidance after punishment  
664 in the presence of d13-HEL was not enhanced and remained significantly different  
665 from the performance of animals trained with normal hexanol (d13-HEL-trained Fig 7  
666 A). In the converse experiment, avoidance of d-hexanol by naïve animals (which was  
667 similar to that of the h- isotopologue), was significantly enhanced by prior  
668 punishment in its presence (d13-HEL-trained Fig 7 B). However, punishment in the  
669 presence of the normal odorant did not result in enhanced d13-HEL avoidance (h-  
670 HEL-trained Fig 7 B). Identical results were obtained with the Canton-S strain of *D.*  
671 *melanogaster* (Fig. 7 C, D), demonstrating the stability and reproducibility of the  
672 assay. Therefore, in accord with prior results with other odorants (Franco et al., 2011),  
673 *D. melanogaster* readily differentiate hexanol isotopologues at the behavioral level  
674 and respond accordingly.

675 Importantly, *D. simulans* (Fig. 7 E, F), *D. pseudoobscura* (Fig. 7 G, H) and *D.*  
676 *virilis* (Fig. 7 I, J) also readily differentiated between hexanol isotopologues, albeit  
677 with lower performance indices for *D. virilis*. Therefore, flies within the genus  
678 *Drosophila* present conserved selective avoidance of isotopologues linked to electric  
679 footshocks. This indicates that these species differentiate isotopologues not only  
680 electrophysiologically at the level of the receptors, but also as salient stimuli to drive  
681 behavioral choices in agreement with previous publications (Franco et al., 2011;  
682 Bittner et al., 2012; Gronenberg et al., 2014).

683 **DISCUSSION**

684 We demonstrate that a range of odorant isotopologues elicited distinct  
685 electrical changes in *Drosophilid* antenna, yielding isotopologue-specific EAG  
686 amplitudes. As described previously (Franco et al., 2011; Bittner et al., 2012) and  
687 herein, such differential responses are behaviorally salient, suggesting that  
688 isotopologues are evaluated and identified as distinct odors in higher brain centers  
689 such as the lateral horn and the mushroom bodies (Galizia, 2014). The contribution of  
690 such higher brain centers to isotopologue identification driving quantifiable  
691 behavioral choices is illustrated by the reported graded behavioral responses to  
692 partially and perdeuterated ACP isotopologues (Franco et al., 2011; Bittner et al.,  
693 2012), which was not observed at the level of OR activities (Fig 2 A-G).

694 Our results indicate strongly that ORs differentiate odorant isotopologues  
695 based on the presence of C-D bonds. Because odorants do not bind ORs covalently,  
696 the known differential strengths of the C-H and C-D bonds (Wade, 1999; Swiderek et  
697 al., 2013) are not possible mediators of isotopologue differentiation. Furthermore, the  
698 effects of deuteration on non-covalent interactions such as hydrogen bonding, ionic  
699 and van der Waals interactions are negligible (Wade, 1999; Swiderek et al., 2013),  
700 indicating that these physical properties also do not contribute to isotopologue  
701 differentiation. Conversely, deuteration is reported to increase polarity (Wade, 1999),  
702 a modification of potentially significant consequences to the hydrophobic odorant  
703 molecules. Reduced hydrophobicity could decrease the time deuterated odorants  
704 transit the aqueous sensillar lymph reflected as an isotopologue-specific reduction in  
705 rise time, which was not observed (Fig 4). OBPs are the likely escort of the  
706 hydrophobic odorants through the aqueous lymph to the ORs. OBPs appear to bind  
707 specific odorants, suggesting selectivity based on structural determinants (Heydel et  
708 al., 2013; Martin et al., 2013). If so and because isotopologues are of identical  
709 structure, it is unlikely that differential OBP binding mediates isotopologue  
710 differentiation. Because deuterated odorants are slightly heavier, OBPs could escort  
711 isotopologues to ORs at different rates potentially reflected in the rise times. However  
712 with the exception of ethanol, isotopologue-specific differences in rise times were not  
713 observed (Fig 4), suggesting that generally, OBPs are not major contributors to  
714 isotopologue differentiation.

715 Furthermore, the isotopologue-specific EAG amplitude differences do not  
716 depend on antennal enzymes of the cytochrome P450 family (Fig 5). Additional

717 enzymes of the glutathione S-transferase, monooxygenase and UDP-  
718 glucuronosyltransferase families have been reported in the sensillar lymph (Wang et  
719 al., 1999; Martin et al., 2013), but as they mainly function in rapid odorant  
720 inactivation they are unlikely to contribute to isotopologue differentiation. These  
721 enzymes may influence signal duration, but not its onset or amplitude as reported for  
722 another *Drosophila* sensillar enzyme, the EST-6 esterase (Chertemps et al., 2015).  
723 Therefore, EAG amplitude-dependent differentiation is unlikely the consequence of  
724 enzymatic activities in the sensillar lymph, strongly suggesting that perireceptor  
725 events do not contribute significantly to isotopologue differentiation.

726         Therefore, our collective results in agreement with previous evidence (Franco  
727 et al., 2011; Bittner et al., 2012), strongly suggest that *Drosophilid* ORs are sensitive  
728 to the vibrations of odorant molecules since this is the main physical property relevant  
729 to olfaction that differentiates the isotopologues.

730         It is not unreasonable to expect that partially deuterated isotopologues would  
731 yield responses similar to those from normal odorants. However, d2- *versus* d13-HEL  
732 and d3- *versus* d8-ACP yielded practically superimposable traces (Fig 2 E, L) over a  
733 range of concentrations (Table 6). Moreover, rise times were nearly identical ( $0.078 \pm$   
734  $0.002$  sec for normal,  $0.072 \pm 0.003$  sec for d2 and  $0.071 \pm 0.002$  sec for d13-HEL;  
735  $0.052 \pm 0.003$  sec for normal,  $0.059 \pm 0.001$  sec for d3 and  $0.055 \pm 0.001$  for d8-  
736 ACP) at the  $10^{-2}$  dilution. The notion that a few or a single C-D bond suffices for  
737 isotopologue differentiation is also in accord with published evidence that the C-D  
738 vibration at  $2150\text{ cm}^{-1}$  is perceived (Franco et al., 2011) as that of the  $\text{C}\equiv\text{N}$  functional  
739 group with which it shares vibrational frequency. This is independently supported at  
740 the OR level where the vibrational frequency of  $\text{C}\equiv\text{N}$  in benzonitrile overlaps that of  
741 the C-D bond in deuterated BNL. Hence, in a manner similar to partially deuterated  
742 and perdeuterated compounds, the two BNL isotopologues yield identical EAG traces  
743 (Fig 1 P-T).

744         Therefore, it appears that *Drosophila* ORs differentiate isotopologues on the  
745 basis of few bonds or vibrations with frequencies distinct from that of the typical C-H  
746 vibration *circa*  $3000\text{ cm}^{-1}$ . However, typical odorants do not contain deuterium, but  
747 rather functional groups that present distinct vibrational modes and frequencies,  
748 which have been proposed to mediate odorant character (Turin et al., 2003). Thus, our  
749 observation that as few as a couple of C-D bonds suffice to differentiate isotopologues  
750 indicates that ORs are likely differentially activated by the characteristic vibrational

751 frequencies of functional groups on odorants they engage. Single odorants activate  
752 more than one OR albeit to different extents [(Hallem et al., 2004; Yao et al., 2005;  
753 Hallem et al., 2006; Galizia, 2014) and DoOR data base ([http://neuro.uni-  
754 konstanz.de/DoOR/default.html](http://neuro.uni-konstanz.de/DoOR/default.html))]. In light of our data, this suggests that odorant size,  
755 shape or hydrophobicity allows it to engage a number of distinct ORs, but activation  
756 of a particular one, or a subset of receptors depends on the vibrational frequencies of  
757 odorant functional groups or structures, as previously suggested (Turin, 2002; Turin et  
758 al., 2003; Solov'yov et al., 2012).

759         Enantiomers have the same size, hydrophobicity and functional groups, but  
760 different shape, so when insects discriminate them (Ulland et al., 2006; Sato et al.,  
761 2015) it may be based on the later, although the majority of enantiomeric pairs smell  
762 identical (Turin et al., 2003). Because ORs must be chiral, then enantiomers may  
763 engage different receptors based on their shape differences but still activate them via  
764 the vibration sensing mechanism previously proposed (Turin, 1996; Turin et al., 2003;  
765 Solov'yov et al., 2012).

766         Isotopologues do not differ in shape and functional groups but rather in their  
767 vibrational modes. So, do they engage and activate the same ORs, or a subset of the  
768 receptors that the normal odorant engages? The similarity of EAG shapes elicited by  
769 isotopologues (Fig 1. Fig 2), suggests they do not engage completely different ORs,  
770 which would likely yield EAGs of distinct shapes. Rather isotopologues likely engage  
771 the same set of ORs probably based on their shape, size or hydrophobicity, but do not  
772 activate them equally and this is reflected in the EAG amplitude differences. Because  
773 EAGs report total OR activity, traces summarize the activation and inhibition of ORs  
774 responsive to a particular odorant, with maximally activated ORs likely to contribute  
775 differentially to the EAG amplitude. Hence, each HEL isotopologue for example,  
776 activates maximally a subset of ORs that typically engage hexanol and the activities  
777 of these differentially activated ORs is reflected in the distinct amplitudes.

778         Alternatively, both isotopologues activate the same exact ORs, but each to a different  
779 extent. Therefore, we propose that the isotopologue-specific EAG amplitudes reflect  
780 differences in the subsets of maximally activated ORs, or the degree of activation of  
781 the same ORs.

782         This model makes specific experimental predictions, currently under  
783 investigation. Imaging of OR activity yields both a specific pattern of receptors  
784 activated to different extents (Korsching, 2002; Silbering et al., 2012). The first

785 possibility suggests that isotopologues will share the overall pattern of OR activation,  
786 but differ in the degree of activation of particular receptors. Alternatively, whereas  
787 both isotopologues will activate common receptors, each will also activate additional  
788 unique ORs. While this manuscript was in revision, independent experiments on bee  
789 antennal lobe glomeruli imaged while the animals were exposed to deuterated  
790 isotopologues of different odorants were reported (Paoli et al., 2016). Specific  
791 glomeruli in the same animal were found preferentially activated by one of the  
792 isotopologues, others exhibited the same response to both and other glomeruli were  
793 inhibited by one of the pair. The responses of these specific glomeruli were conserved  
794 in all individual animals tested, strongly indicating that as in *Drosophila*, bee ORs  
795 discriminate isotopologues. Since, isotopologues generate distinct activation maps  
796 that include differential activation, inhibition or equal activation of specific bee ORs,  
797 it is likely that a similar situation occurs in *Drosophila*. This suggests that both types  
798 of responses presented as alternatives above are likely to be uncovered in *Drosophila*  
799 as well. In summary, the experiments detailed herein and the complementary imaging  
800 experiments in bees demonstrate that isotopologue discrimination underlies a general  
801 property of their olfactory systems, strongly indicating in turn that molecular  
802 vibrations contribute to smell in insects.

803           Whether molecular vibrations are a component of vertebrate and human  
804 olfaction is contested at the moment (Block et al., 2015; Turin et al., 2015; Vosshall,  
805 2015). Behavioral experiments on humans indicated that subjects were able to  
806 discriminate cyclopentadecanone isotopologues (Gane et al., 2013), but not those of  
807 the much smaller ACP (Keller et al., 2004; Gane et al., 2013). In contrast, differential  
808 activation by isotopologues was not reported when mouse ORs were expressed in  
809 cultured human kidney cells (Block et al., 2015). Although perireceptor effects are not  
810 important in fly isotopologue discrimination, they may in fact be required for humans  
811 (Schilling et al., 2010) and clearly such mechanisms are lacking in tissue culture.  
812 Given that vertebrate ORs are GPCRs, while insect ORs are not, direct measurements  
813 of OR activity *in situ* akin to these described herein and in bees (Paoli et al., 2016) are  
814 essential to address the issue definitively. A mouse or other vertebrate model is likely  
815 to serve that purpose and conversely fly dimeric ORs expressed in a heterologous  
816 system will complement these experiments.

817           The notion that like hearing and color vision, smell is at least in part, a spectral  
818 sense is indeed attractive, but additional experiments are needed to establish it fully

819 and explore and define its parameters with precision. We have shown in *Drosophila*  
820 and is reported for bees (Paoli et al., 2016), that at least for some odorants one  
821 component of OR activation is conferred by molecular vibrations. Molecular size,  
822 shape or hydrophobicity of the odorant molecules are also likely involved, in  
823 combination with molecular vibrations or alone, for selective OR activation. Odour  
824 character has been proposed to be determined largely by molecular vibrations, while  
825 odour intensity by its shape (Turin et al., 2003). To address this working hypothesis  
826 requires electrophysiological recordings from single *Drosophila* ORs. However, given  
827 our data and those from bees (Paoli et al., 2016), utilization of molecular vibrations to  
828 maximally activate specific receptors from those engaged by odorants based on the  
829 shape or size alone would provide additional selectivity and specificity. We do not  
830 know at the moment whether molecular vibrations are essential for activation of all  
831 ORs in *Drosophila* and bees, but clearly they are for some. It would appear then that  
832 olfaction in *Drosophila* combines both chemical (functional groups, shape) and  
833 spectral (vibrations) components and may be evolutionarily intermediate between the  
834 purely spectral senses (vision and hearing) and a purely chemical one (taste).

835         The exact mechanism that selectively activates ORs based on the molecular  
836 vibrations of odorant molecules is unclear at the moment. Because electron transfer in  
837 biological systems is not uncommon, theoretical models suggesting an electron  
838 tunneling mechanism for vibrational sensing (Turin, 1996; Brookes et al., 2007;  
839 Bittner et al., 2012; Solov'yov et al., 2012) are likely. However, such models are still  
840 incomplete because the structure of insect and vertebrate ORs is currently unknown  
841 and therefore it is difficult to estimate the reorganization energy of the putative  
842 odorant-interacting sites that would be permissive to electron tunneling. Very recently  
843 however, Reese et al (Reese et al., 2016) suggested that reorganization of molecular  
844 vibrations at the OR binding site are minimal and hence cannot suppress the proposed  
845 electron transfer mechanism (Brookes et al., 2007) of the odorant vibrational  
846 frequencies as has been suggested before (Block et al., 2015). Additional theoretical  
847 and experimental considerations on the vibration sensing mechanism can be found in  
848 several recent publications (Vosshall, 2015; Paoli et al., 2016; Reese et al., 2016).

849         Although our results do not address these models directly, identification of  
850 *Drosophila* ORs differentially responsive to one isotopologue as in bees (Paoli et al.,  
851 2016) may offer an experimental approach. If the mechanism of vibrational detection  
852 requires electron movement, then amino acids acting as donors and acceptors may

853 characterize ORs responding differentially to isotopologues of a particular odorant.  
854 Our evidence that isotopologue detection is broadly conserved within the genus will  
855 likely help define such amino acids, essential for future functional physiological,  
856 imaging and behavioral experiments on single receptors *in vivo*.  
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1078 **FIGURE LEGENDS.**

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1080 **Table 2. Purity of normal and deuterated isotopologues of the standard odorant**  
1081 **set.**

1082 Results from gas chromatograms of normal, partially deuterated and perdeuterated  
1083 odorants used in the standard odorant set and relevant related odorants. The percent  
1084 (Area %) of the total that constitutes the main species within each preparation is  
1085 shown in magenta as are those of all other species.

1086 For the hexanol isotopologues, purity of all preparations was greater than 99.6%.

1087 Contaminants varied with minimal overlap among the isotopologues and with the  
1088 most abundant contaminant at 0.1-0.2%, but most others at least 10-fold lower.

1089 Normal benzaldehyde purity was 98.7%, but the most abundant contaminant was  
1090 benzoic acid (peak #7), while the contribution of others was negligible. Similarly for  
1091 d6-BNZ with 99.4% purity the most abundant contaminant was d5-benzoic acid (peak  
1092 #7) as indicated by mass spectroscopy (not shown). Solid normal and deuterated

1093 benzoic acid was dissolved in ethanol to generate a 1% solution, which did not show  
1094 additional contaminants (benzoic acid isotopologues at 19.730 and 19.658 Ret times).

1095 Both hexanone isotopologues exhibited over 99.8% purity, while the contribution of  
1096 contaminants was negligible.

1097 Normal benzonitrile was totally pure, while the deuterated isotopologue was 99.8%  
1098 pure with negligible contribution from contaminants.

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1113 **Figure 1. Isotopologue-specific EAG responses.**

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1115 Average EAG traces elicited by three concentrations of the normal (dark blue) or  
1116 deuterated (magenta) isotopologues of four representative odorants are shown in A, F,  
1117 K and P. The shaded area indicates the timing and duration of odorant stimulation,  
1118 while the scale is shown in the bottom right of each trace.

1119 The actual amplitude values elicited by the normal or deuterated isotopologues of 1-  
1120 hexanol-HEL (B, C, D), benzaldehyde-BNZ (G,H, I), 2-hexanone-HEN (L,M, N) and  
1121 benzonitrile-BNL (Q, R,S) are represented by pairs of blue dots and magenta squares  
1122 respectively shown for each individual, with the number of animals tested indicated in  
1123 the abscissa as “fly number”. The ordinate scales have been adjusted to allow  
1124 maximal resolution. The significance of isotopologue-specific amplitude differences  
1125 were evaluated per fly using paired sample t-tests and is indicated on the top left of  
1126 each panel.

1127 Quantification of the collective differential response per odorant dilution was  
1128 achieved by subtracting the absolute values of the h-odorant amplitude from that of  
1129 the d-odorant for each fly tested and the mean ( $\Delta$  amplitude) and  $\pm$  SEM are shown in  
1130 (E, J, O and T). Therefore, the probabilities that  $\Delta$  amplitude per odorant dilution is  
1131 different than 0 are the same as shown on the respective panels. The actual  $\Delta$   
1132 amplitude values and their SEMs are summarized below.

Dilution	Mean h-	SEM	Mean d-	SEM
HEL $10^{-2}$	13.7872	0.5066	11.8815	0.4079
HEL $10^{-3}$	7.4245	0.2400	6.3346	0.2194
HEL $10^{-4}$	4.5079	0.1636	4.3553	0.1808
BNZ $10^{-2}$	8.5526	0.3489	9.3040	0.4044
BNZ $10^{-3}$	4.2038	0.1121	4.4785	0.1531
BNZ $10^{-4}$	1.8785	0.1916	1.9165	0.1940
HEN $10^{-2}$	11.4822	0.4242	9.6779	0.1681
HEN $10^{-3}$	4.2479	0.2185	3.4943	0.1723
HEN $10^{-4}$	2.7123	0.2251	2.6894	0.2495
BNL $10^{-2}$	4.4556	0.4427	4.5258	0.4009
BNL $10^{-3}$	2.7186	0.1895	2.7848	0.1660
BNL $10^{-4}$	2.1769	0.1641	2.1159	0.1596

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1154 **Table 3. Purity of independent lots and sources of normal and deuterated**  
1155 **isotopologues.**

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1157 Results from gas chromatograms of normal and perdeuterated odorants from distinct  
1158 lots and sources as indicated from those on Table 2 and Fig 1. The percent (Area %) of the total that constitutes the main species within each preparation is shown in magenta. The batch numbers of isotopologues distinct from those on Table 1 are indicated. Distinct trace impurities were present in the 1-hexanol preparation from Lluçh Essences compared to that from Fluka (Table 2). The deuterated 1-hexanol contained less contaminants, which however constituted a slightly larger fraction of the sample, since pure d13-1-HEL exhibited 99.5% purity in this sample compared to 99.7% in the one on Table 2. Similarly, h-BNZ contained less contaminants than that on table 2, but it constituted less percent of the sample (94.4% vs 98.7% on table 2), because a larger proportion was benzoic acid (Ret Time 19.153). d6-BNZ also contained additional contaminants from that on table 2 (Ret Times 8.105, 8.937) and contained a larger percent (5.03%) deuterated benzoic acid than the batch on table 2. Nevertheless the EAG responses of the two sets of batches were similar. In contrast, the normal ACP sample was more pure than the original one (table 4) and the same was true for the d8-ACP sample. Based on retention times there are no common contaminants in the two isotopologue preparations.

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1176 **Table 4. Amplitude differences of odorant isotopologues from distinct batches**  
1177 **and sources.**

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1179 Mean amplitudes  $\pm$  SEM of 1-hexanol (HEL), benzaldehyde (BNZ) and acetophenone

1180 (ACP) isotopologue pairs distinct from those in Fig 1 and Fig 2 derived from the

1181 indicated (n) animals. Isotopologue pairs were used at the  $10^{-2}$  dilution. The

1182 probability that the mean amplitudes evoked by the two isotopologues are

1183 significantly different is shown per odorant.

1184 **Table 5. Purity of normal and deuterated isotopologues of additional odorants.**  
1185 Results from gas chromatograms of normal, partially deuterated and perdeuterated  
1186 additional odorants used herein. The percent (Area %) of the total that constitutes the  
1187 main species within each preparation is shown in magenta.  
1188 For acetophenone isotopologues, purity of all preparations was greater than 99.7%.  
1189 The contribution of all contaminants was negligible as it ranged below 0.1%.  
1190 Both ethanol isotopologues are 100% pure. For pentanol, the normal odorant was  
1191 nearly pure (99.9%), while the deuterated isotopologue was highly pure at 99.5%,  
1192 with two main albeit low-level contaminants, peak #1 at 0.16% and peak #2 at 0.33%.  
1193 For octanol, the normal isotopologue was highly pure (99.4%), while the deuterated  
1194 isotopologue is equally pure (99.5%). Although more in number, the contribution of  
1195 contaminants in the deuterated isotopologue is minor, except for the peak at 5.692  
1196 contributing 0.26% to the total. This peak is shared with the normal isotopologue at  
1197 5.607 and contributes 11% of the total while the major contaminating peak at 8.821  
1198 contributes 0.36%.  
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1202 **Figure 2. The degree of deuteration does not affect the isotopologue-specific**  
1203 **differential response.**

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1205 Average EAG traces of normal (dark blue) *versus* deuterated (magenta) odorants are  
1206 shown in A and H. When two deuterated isotopologues are compared (E, L), the blue  
1207 trace corresponds to the least deuterated species. The gray area on the left side of the  
1208 traces indicates the timing and duration of odorant stimulation, while the scale is  
1209 shown in the bottom right of each trace.

1210 Raw amplitudes elicited in response to normal and d2-hexanol (B), d5-hexanol  
1211 (C) and d13-hexanol (D). The ordinate scales have been adjusted to allow maximal  
1212 resolution. The number of flies tested with each isotopologue pair is shown in the  
1213 abscissas, with each pair of blue dots and magenta squares representing the responses  
1214 from single flies. Similarly, raw amplitudes in response to the di-deuterated hexanol  
1215 (dark blue diamonds) *versus* the perdeuterated odorant (magenta squares) are shown  
1216 in F. The significance of isotopologue-specific amplitude differences were evaluated  
1217 per fly using paired sample t-tests and is indicated on the top right of each panel.  
1218 These differential responses are quantitatively represented in G and ANOVA  
1219 indicated significant differences [ $F_{(3,32)} = 17.739$ ,  $p < 0.0001$ ], which were revealed by  
1220 LSM contrast analysis to be due to the difference of the d2 vs d13  $\Delta$  amplitude (open  
1221 bar) from the other three ( $p=0.0006$ ,  $<0.0001$  and  $<0.0001$  respectively in order of  
1222 increasing deuteration). In contrast, comparing the  $\Delta$  amplitudes of each partially  
1223 deuterated odorant over the normal isotopologue with each other, did not reveal  
1224 significant differences (h/d2 vs h/d13  $p=0.087$ , h/d2 vs h/d5  $p=0.110$  and h/d5 vs  
1225 h/d13  $p=0.940$ ).

1226 Similarly, raw amplitudes elicited in response to normal and d3-acetophenone  
1227 (I), d5-acetophenone (J) and d8-acetophenone (K). The ordinate scales have been  
1228 adjusted to allow maximal resolution, while the number of flies tested with each  
1229 isotopologue pair is shown in the abscissas. Each pair of blue dots and magenta  
1230 squares representing the responses from single flies. Raw amplitudes in response to  
1231 the d3-acetophenone (dark blue diamonds) *versus* the perdeuterated odorant (magenta  
1232 squares) are shown in M. The significance of isotopologue specific amplitude  
1233 differences were evaluated per fly using paired sample t-tests and is indicated on each  
1234 panel. These differential responses are quantitatively represented in N and ANOVA  
1235 indicated significant differences [ $F_{(3,32)} = 6.331$ ,  $p=0.002$ ], which were revealed by  
1236 LSM contrast analysis to be due to the difference of the d3 vs d8  $\Delta$  amplitude (open

1237 bar) from the other three ( $p=0.0075$ ,  $0.0017$  and  $0.0005$  respectively in order of  
1238 increasing deuteration). In contrast, comparing the  $\Delta$  amplitudes of each partially  
1239 deuterated odorant over the normal isotopologue with each other did not reveal  
1240 significant differences (h/d3 vs h/d5  $p= 0.438$ , h/d3 vs h/d8  $p= 0.245$  and h/d5 vs h/d8  
1241  $p= 0.7287$ ).

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1250 **Table 6. Lack of differences in the amplitudes of partially and perdeuterated**  
1251 **odorant pairs over a range of dilutions.**

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1253 The dilutions and the resultant mean amplitudes  $\pm$  SEM of perdeuterated and  
1254 minimally deuterated 1-hexanol (HEL) and acetophenone (ACP) isotopologue pairs  
1255 collected from the indicated (n) number of animals are shown. The resultant mean  
1256  $\Delta$ amplitude  $\pm$  SEMs are also shown, as well as the probabilities from paired t-tests (p  
1257 t-test) that the mean responses to each pair of isotopologues at each dilution are  
1258 significantly different. Significant differences were not uncovered.

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1260 **Figure 3. Isotopologue-specific EAG amplitudes of normal and deuterated**  
 1261 **alcohols.**

1262  
 1263 (A, B, C, D). The maximal amplitudes for each fly tested with the normal (dark blue  
 1264 dots) or deuterated (magenta squares) isotopologue of the 2-carbon ethanol and d6-  
 1265 ethanol (ETL), the 5-carbon pentanol and d11-pentanol (PNL), the 6-carbon hexanol  
 1266 and d13-hexanol (HEL) and the 8-carbon octanol and d17-octanol (OCT) at the  $10^{-2}$   
 1267 dilution. The number of flies tested with each isotopologue is shown in the abscissas  
 1268 with each pair of blue and magenta dots representing the responses per single fly,  
 1269 while the ordinate scales have been adjusted for maximal resolution. The significance  
 1270 of the uncovered differences after paired t-tests is shown on the graphs. The data in C  
 1271 are the same as in Fig 1D.

1272 (E) Quantification of the collective differential response per odorant isotopologue  
 1273 shown as  $\Delta$  amplitude  $\pm$  SEM. The data for the hexanol isotopologues are the same  
 1274 as in Fig 1. The actual  $\Delta$  amplitude values and their SEMs are summarized below.

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1276	Odorant	Mean h-	SEM	Mean d-	SEM
1277	ETL	3.0746	0.1671	4.1313	0.1733
1278	PNL	10.6673	0.3975	11.1639	0.4391
1279	HEL	13.7872	0.5066	11.8815	0.4079
1280	OCT	6.4527	0.2811	7.0699	0.2752

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1304 **Figure 4. Isotopologues elicit similar EAG activation and decay rates.**

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1306 The times required to achieve two-thirds of the maximal amplitude (time to rise, A-  
1307 H) and times to recover to 1/3 of the maximal amplitude (time to decay, I-P) are  
1308 shown for isotopologues of all odorants used in this study. Dark blue and magenta  
1309 triangles are used for the rise times due to normal and deuterated isotopologues  
1310 respectively and conversely dark blue and magenta diamonds are used for decay times.  
1311 The number of flies tested with each isotopologue is shown in the abscissas and the  
1312 ordinate scales have been adjusted for maximal resolution. The probability that paired  
1313 t-tests uncovered isotopologue specific differences in rise and decay times is shown  
1314 with significant differences in bold and boxed. HEL: 1-hexanol, BNZ:benzaldehyde,  
1315 HEN: 2-hexanone, BNL: benzonitrile, ACP: acetophenone, ETL: ethanol, PNL: 1-  
1316 pentanol and OCT: 1- octanol.

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1326 **Figure 5. P450 inhibition does not alter the isotopologue-specific differential**  
1327 **responses.**

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1329 (A) Flies were exposed to piperonyl butoxide (PBO) dissolved in acetone. The mean  
1330 lethality  $\pm$  SEM due to exposure to 0.25 % (v/v) PBO in acetone alone (PBO), 5 and  
1331 7% methanol (MTL) alone (v/v in minimal food) and combinations thereof is shown  
1332 (n=8 for each). PBO significantly (t-test derived probabilities shown above the bars  
1333 compared as indicated) augmented the lethality precipitated either by 5% or 7%  
1334 methanol demonstrating that under the conditions of the experiment it actually  
1335 inhibits *Drosophila* P450s.

1336 (B) Flies were exposed to PBO, or the acetone vehicle (-PBO) for 18 h at 25C.  
1337 Another group of animals were exposed to PBO for 18 h and then allowed to recover  
1338 on minimal food for another 18 h (REC). Average EAG traces from treated (+PBO),  
1339 control (-PBO) and recovered (REC) flies exposed to normal (h-HEL) and d13-  
1340 hexanol (d-HEL) isotopologues. The gray area indicates the timing and duration of  
1341 stimulation.

1342 (C) The mean EAG amplitudes  $\pm$  SEM of treated, untreated and recovered animals  
1343 demonstrated that the differential responses to HEL isotopologues remain significant  
1344 (probabilities from paired t-tests shown above the relevant bars) despite the PBO  
1345 treatment.

1346 (D)  $\Delta$  amplitudes calculated from the data in C. ANOVA [ $F_{(2,23)} = 0.819$ ] did not  
1347 indicate significant differences ( $p=0.453$ ) among groups indicating that the  
1348 isotopologue-specific differences remain despite the PBO treatment.

1349 (E) Mean EAG amplitudes  $\pm$  SEM of PBO and vehicle treated (-PBO) animals  
1350 exposed to normal and d8-acetophenone (ACP) show that the isotopologue-specific  
1351 responses remain significant (paired t-test probabilities above the respective bars)  
1352 despite PBO treatment.

1353 (F)  $\Delta$  amplitudes calculated from the data in E. The isotopologue-specific differences  
1354 remain despite PBO treatment as ANOVA [ $F_{(1,14)} = 0.087$ ] did not indicate significant  
1355 differences ( $p=0.773$ ) among groups.

1356 (G) Mean EAG amplitudes  $\pm$  SEM of PBO and vehicle treated (-PBO) animals  
1357 exposed to normal and d17-octanol (OCT) indicate a marginal ( $p=0.0152$ , paired t-  
1358 tests) isotopologue-specific response after PBO treatment.

1359 (H)  $\Delta$  amplitudes calculated from the data in G. As indicated in G, the isotopologue-  
1360 specific differences are decreased upon PBO treatment as ANOVA [ $F_{(1,16)} = 7.727$ ]  
1361 indicated a significant difference ( $p=0.013$ ) among groups.  
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1365 **Figure 6. The differential response to isotopologues is conserved within the**  
1366 **genus *Drosophila*.**

1367  
1368 Raw EAG amplitudes from the antennae of *D. simulans* (*D.sim*), *D. pseudoobscura*  
1369 (*D.pse*) and *D. virilis* (*D.vir*) in response to hexanol (A-C), benzaldehyde (E-G),  
1370 hexanone (I-K) and benzonitrile (M-O) isotopologues (dark blue dots for the normal  
1371 and magenta squares for the deuterated odorant). The ordinate scales have been  
1372 adjusted to allow maximal resolution. The probability (paired t-tests) that  
1373 isotopologue-specific differences are uncovered is indicated on the panels.  
1374  $\Delta$  amplitudes calculated from the data in the previous panels are shown in D for  
1375 hexanol, H for benzaldehyde, L for hexanone and P for benzonitrile. The relevant  $\Delta$   
1376 amplitudes for *D. melanogaster* from Fig 1 are added for comparison. ANOVA did  
1377 not indicate ( $F_{3,34}=0.992$ ,  $p=0.409$ ) significant differences in  $\Delta$  amplitudes for HEL  
1378 (D), or BNL ( $F_{3,28}=1.518$ ,  $p=0.232$ ) (P). However ANOVA indicated significant  
1379 differences in  $\Delta$  amplitudes for BNZ isotopologues ( $F_{3,31}=9.672$ ,  $p < 3.25 \times 10^{-5}$ ), which  
1380 subsequent Tukey HSD indicated were due to differences in the  $\Delta$  amplitude from *D.*  
1381 *simulans* and *D. pseudoobscura* compared to that from *D. virilis* ( $\alpha=0.05$ ). Similarly,  
1382 ANOVA indicated differences ( $F_{3,31}=10.802$ ,  $p=0.232$ ) in  $\Delta$  amplitudes elicited by  
1383 HEN exposure (L). Tukey HSD revealed that the  $\Delta$  amplitudes of *D. melanogaster*  
1384 and *D. simulans* were significantly different from those of *D. virilis* ( $\alpha=0.05$ ).

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1390 **Figure 7. Behavioral discrimination of 1-hexanol isotopologues within the genus**  
1391 **Drosophila.**

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1393 The mean 1-hexanol isotopologue avoidance  $\pm$  SEM of complementary experiments  
1394 is shown. The Drosophila strains and species tested are indicated on the right of each  
1395 pair of experiments. The graphs are shown horizontally to reflect the actual  
1396 distribution of the flies in the left and the right arms of the T-maze. Room air is  
1397 shown delivered on the right arm, whereas the odorant on the left, although in  
1398 actuality the side of air and odorant delivery were alternated semi-randomly. Open  
1399 bars indicate the naïve response to the indicated isotopologue *vs* room air. In A, C, E,  
1400 G, I, flies were exposed to 12-90 Volt electric footshocks (thunderbolts) in the  
1401 presence of either normal (h-HEL-gray bars) or perdeuterated (d13-HEL-black bars)  
1402 1-hexanol and then tested for avoidance of the normal isotopologue *versus* air. The  
1403 complementary experiments are shown in B, D, F, H, J, with flies exposed to electric  
1404 footshocks (thunderbolts) in the presence of either normal (h-HEL-gray bars) or per-  
1405 deuterated (d-HEL-black bars) 1-hexanol and then tested for avoidance of the per-  
1406 deuterated odorant *versus* air.

1407 Differences in the performance of each group were investigated by an initial ANOVA  
1408 followed by LSM contrast analysis. The group trained to avoid the same isotopologue  
1409 as used for testing was denoted as the control group and the probabilities that it  
1410 performed differently than naïve or animals trained to the other isotopologue are  
1411 shown above each relevant bar.  $n \geq 8$  for all groups.

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h-1-Hexanol (Fluka Analytical)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	4.314	BB	0.0273	2.69500	1.63299	0.04475
2	5.653	BB	0.0369	7.43677e-1	3.22039e-1	0.01235
3	6.214	BB	0.0578	6012.02588	1353.56238	99.82841
4	14.426	BB	0.1929	6.89491	4.49524e-1	0.11449

d5-1-Hexanol						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.904	BB	0.0257	1.00243	6.49066e-1	0.01407
2	4.796	BB	0.0339	6.67262	3.27978	0.09365
3	5.215	BB	0.0326	2.84580	1.35165	0.03994
4	6.232	BV	0.0739	7097.00391	1337.32166	99.60153
5	6.688	VB	0.0422	5.86693	2.26736	0.08234
6	7.574	BB	0.0327	8.28919	3.91570	0.11633
7	8.113	BB	0.0335	9.32212e-1	5.06874e-1	0.01308
8	10.646	BB	0.0317	2.78344	1.65248	0.03906

h-Benzaldehyde (Sigma)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.083	BB	0.0214	2.73390e-1	2.07438e-1	0.00248
2	2.445	BB	0.0212	8.67375e-1	6.65289e-1	0.00788
3	5.213	BB	0.0273	3.35924e-1	2.02958e-1	0.00305
4	8.686	BB	0.0668	1.08576e4	2400.08643	98.68858
5	9.365	BB	0.0290	3.97409	2.20585	0.3612
6	10.762	BB	0.0309	2.21929	1.13301	0.02017
7	19.732	BBA	0.0395	136.61024	50.65681	1.24170

h-Benzoic acid						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	19.730	BBA	0.0439	85.00277	29.25834	1.00e2

h-2-hexanone						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.122	VV	0.0172	1.22825	1.17401	0.01777
2	3.393	BV	0.0410	6898.30908	2597.11719	99.81266
3	7.690	BB	0.0329	3.76977	1.76718	0.05455
4	10.191	BB	0.0277	7.94950	4.47342	0.11502

h-Benzonitrile						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	10.234	BB	0.0557	9508.75098	2276.43311	1.00e2

d2-1-Hexanol						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	4.622	BB	0.0301	4.95090e-1	2.61661e-1	0.00676
2	4.847	BB	0.0326	2.15994	1.02467	0.02950
3	5.638	BV	0.0310	1.22029	6.18148e-1	0.01667
4	6.277	BB	0.0710	7312.0483	1446.4130	99.88262
5	6.953	BB	0.0404	1.71584	5.82760e-1	0.02344
6	9.090	BB	0.0251	8.65162e-1	5.27801e-1	0.01182
7	11.268	BB	0.0280	1.23281	7.18195e-1	0.01684
8	11.420	BB	0.0258	3.41531e-1	2.24189e-1	0.00467
9	13.871	BB	0.0324	5.62412e-1	2.92345e-1	0.00768

d13-1-Hexanol (lot X241P13)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	3.645	BB	0.0328	4.77799e-1	2.67615e-1	0.00709
2	4.734	VB	0.0309	12.45923	6.33606	0.18490
3	5.429	BB	0.0496	3.09593	8.68689e-1	0.04594
4	5.695	BB	0.0364	8.49879e-1	3.48745e-1	0.01261
5	6.127	BB	0.0810	6720.69971	1285.26160	99.73692
6	7.432	BB	0.0368	8.44513e-1	3.97840e-1	0.01253

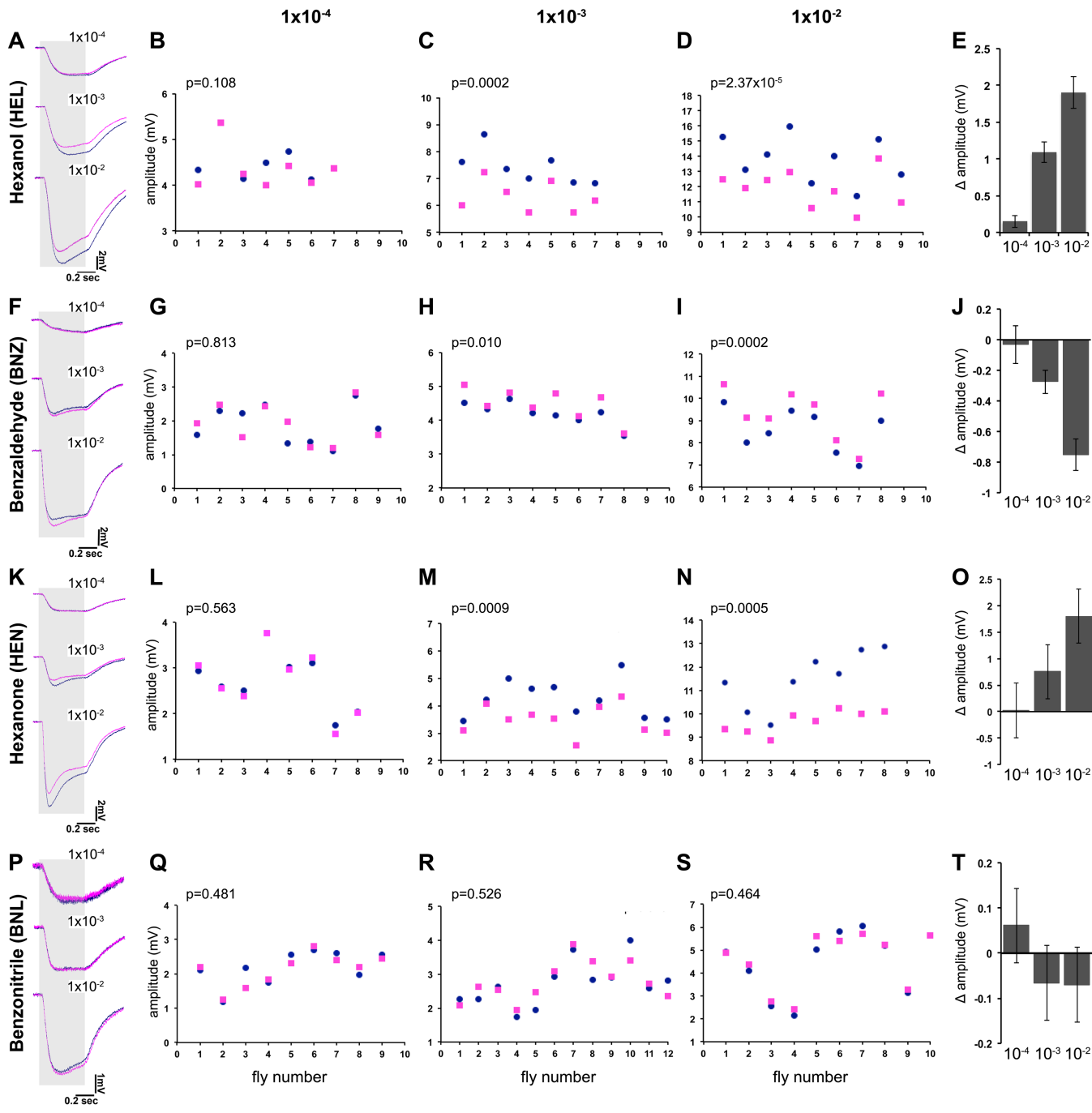
d6-Benzaldehyde (lot H468P23)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.702	BB	0.0403	4.31283	1.78300	0.00508
2	8.669	BB	0.0718	8558.87891	1929.26196	99.39384
3	10.612	BB	0.0438	1.06592	4.46972e-1	0.01238
4	12.554	BB	0.0410	9.16474	5.10110	0.10643
5	13.386	BB	0.0584	5.53546	1.52484	0.06428
6	15.041	BB	0.0461	12.58218	4.87559	0.14612
7	19.918	BB	0.0484	19.53590	7.04030	0.22687

d5-Benzoic acid						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	19.658	BBA	0.0395	284.06458	105.29233	1.00e2

d5-2-hexanone						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.567	BB	0.0252	3.98876	3.51804	0.05061
2	2.792	BV	0.0310	4.36951e-1	2.68910e-1	0.00554
3	3.342	VB	0.0505	7864.65332	2381.03467	99.79115
4	3.766	BB	0.0427	11.39421	4.32301	0.14458
5	4.857	BB	0.0323	6.40037e-1	3.06994e-1	0.00812

d5-Benzonitrile						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	4.644	BB	0.0344	2.18002	1.13441	0.01571
2	5.958	BB	0.0351	4.69176e-1	2.36558e-1	0.00338
3	6.316	BB	0.0311	18.41002	9.86647	0.13265
4	7.883	BB	0.0400	2.80853	1.02908	0.02024
5	10.188	BB	0.0808	1.38512e4	2657.15698	99.80494
6	15.243	BB	0.0389	6.83049e-1	2.96831e-1	0.00492
7	19.561	BB	0.0578	2.51985	7.02758e-1	0.01816

TABLE 2



h-1-Hexanol (Lluch Ess)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.196	BB	0.0369	5.72620	2.58805	0.10832
2	2.569	BV	0.0214	5.22487	4.06881	0.09884
3	2.726	VV	0.0331	4.29227	2.37241	0.08120
4	2.856	VV	0.0308	2.58679	1.32312	0.04893
5	3.148	VV	0.0475	3.80892	1.12580	0.07205
6	3.408	VB	0.0350	3.19802	1.62208	0.06050
7	4.004	BB	0.0818	2.04721	3.23609e-1	0.03873
8	5.228	BB	0.0435	3.19114	1.18053	0.06037
9	5.738	BB	0.0815	5256.20166	1146.01575	99.43107

d13-1-Hexanol (X421P8)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	3.327	BB	0.0345	9.75216	6.15056	0.15369
2	4.297	BB	0.0433	15.99059	8.01434	0.25201
3	5.572	BB	0.0964	6313.49365	1076.56616	99.49862
4	6.808	BB	0.0544	3.50448	1.18921	0.05523
5	12.926	BB	0.0764	2.56706	5.30815e-1	0.04046

h-Benzaldehyde (Fluka Analytical)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.375	BB	0.0224	6.39770e-1	4.53907e-1	0.00680
2	8.249	BB	0.0506	8977.63086	2349.54297	95.44216
3	8.921	BB	0.0426	9.60250	3.23823	0.10209
4	10.373	BB	0.0419	8.93681e-1	3.07697e-1	0.00950
5	12.829	BB	0.0340	6.93815e-1	3.11654e-1	0.00738
6	19.153	BB	0.0427	416.89743	139.98267	4.43208

d6-Benzaldehyde (X261P20)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.377	BB	0.0267	5.28992e-1	3.69073e-1	0.00567
2	8.105	BV	0.0544	1.22637	3.22554e-1	0.01314
3	8.307	VB	0.0731	8838.80566	2099.49609	94.72651
4	8.937	BB	0.0342	14.52237	7.00919	0.15564
5	10.126	BB	0.0512	1.07901	3.21227e-1	0.01156
6	11.935	BB	0.0376	1.19985	5.46938e-1	0.01286
7	12.754	BB	0.0581	2.19288	6.07426e-1	0.02350
8	14.382	BB	0.0350	1.63216	7.62619e-1	0.01749
9	19.128	BB	0.0530	469.68039	133.57043	5.03362

h-Acetophenone (Fluka Puriss)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	9.530	BB	0.0452	1.14808	3.80043e-1	0.01397
2	9.819	BB	0.0802	1.13668	2.06503e-1	0.01383
3	10.160	BB	0.0545	8210.81641	1976.72363	99.89956
4	11.114	BV	0.0329	7.13131e-1	3.34774e-1	0.00868
5	11.176	VB	0.0833	3.74978	6.13817e-1	0.04562
6	13.617	BB	0.0512	1.50747	4.48821e-1	0.01834

d8-Acetophenone (G466P32)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	7.637	BB	0.0381	1.46471	6.08103e-1	0.01922
2	8.188	BB	0.0365	1.16085	4.74491e-1	0.01523
3	10.084	BB	0.0605	7616.92383	1759.73987	99.93954
4	11.941	BB	0.0893	1.98253	3.24316e-1	0.02601

TABLE 3

<b>Odorant (from Table 3)</b>	<b>Mean Amplitude</b>	<b>n</b>	<b><math>\Delta</math>(Amp)</b>	<b>p (t-test)</b>
h-1-HEL (Lluch Ess)	11.9367 $\pm$ 0.6336	7	<b>0.5841 <math>\pm</math> 0.094</b>	<b>&lt;8x10<sup>-4</sup></b>
d13-1-HEL (X421P8)	11.3525 $\pm$ 0.6546			
h-BNZ (Fluka Analytical)	5.6187 $\pm$ 0.1380	8	<b>-0.6275 <math>\pm</math> 0.08</b>	<b>&lt;1x10<sup>-4</sup></b>
d6-BNZ (X261P20)	6.2462 $\pm$ 0.1642			
h-ACP (Fluka Puriss)	6.0601 $\pm$ 0.4386	10	<b>0.3103 <math>\pm</math> 0.062</b>	<b>&lt;7.1x10<sup>-4</sup></b>
d8-ACP (G466P32)	5.7498 $\pm$ 0.4073			

TABLE 4

h-Acetophenone (Fluka analytical)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	8.634	VV	0.0532	1.75445	5.22483e-1	0.02044
2	9.091	VB	0.0379	1.11003	4.33768e-1	0.01293
3	9.499	BB	0.0409	1.46442	5.19445e-1	0.01706
4	10.206	VB	0.0451	9.35422e-1	3.11201e-1	0.01090
5	10.909	VV	0.0605	8552.97363	1899.87683	99.66466
6	11.145	VV	0.0351	2.05978	8.85598e-1	0.02400
7	11.272	VV	0.0302	2.00964	1.05744	0.02342
8	11.337	VV	0.0255	1.28859	7.71482e-1	0.01502
9	11.391	VB	0.0300	1.06645	5.65312e-1	0.01243
10	11.826	BB	0.0296	7.87661	4.26206	0.09178
11	12.824	BB	0.0267	3.46171	1.94807	0.04034
12	12.967	BV	0.0318	7.98368e-1	3.91961e-1	0.00930
13	13.008	VB	0.0279	6.91700e-1	3.68989e-1	0.00806
14	14.378	BB	0.0265	4.26080	2.42068	0.04965

d3-Acetophenone						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	9.212	BB	0.0459	6.59789e-1	2.27296e-1	0.00659
2	10.060	BB	0.0432	9.67617	3.62314	0.09659
3	10.384	BB	0.0443	9.54238	3.44315	0.09526
4	10.844	BV	0.0763	9988.40723	1933.71594	99.71190
5	11.217	VB	0.0400	7.89751e-1	3.28895e-1	0.00788
6	11.785	BB	0.0682	1.58633	5.11016e-1	0.01584
7	12.807	BB	0.0334	5.71362	3.11383	0.05704
8	13.684	BB	0.0369	8.92039e-1	4.17685e-1	0.00891

d5-Acetophenone						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	10.866	BB	0.0933	9092.35840	1730.31287	99.98919
2	11.796	BB	0.0357	9.82952e-1	4.83287e-1	0.01081

d8-Acetophenone (lot G466P29)						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	8.259	BB	0.0367	1.77587	8.40201e-1	0.01898
2	8.850	BB	0.0328	1.34282	6.81408e-1	0.01435
3	10.820	BB	0.0933	9347.32031	1778.28320	99.90145
4	11.766	BB	0.0379	1.43155	6.45780e-1	0.01530
5	12.484	BB	0.0379	2.74861	1.23874	0.02938
6	13.712	BB	0.0391	1.48917	6.42145e-1	0.01592
7	16.677	BB	0.0313	4.32479e-1	2.30103e-1	0.00462

h-Ethanol						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.418	BB S	0.0205	4076.57593	3284.19873	1.000e2

d6-Ethanol						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.387	BB	0.0360	4830.66064	2824.42505	1.000e2

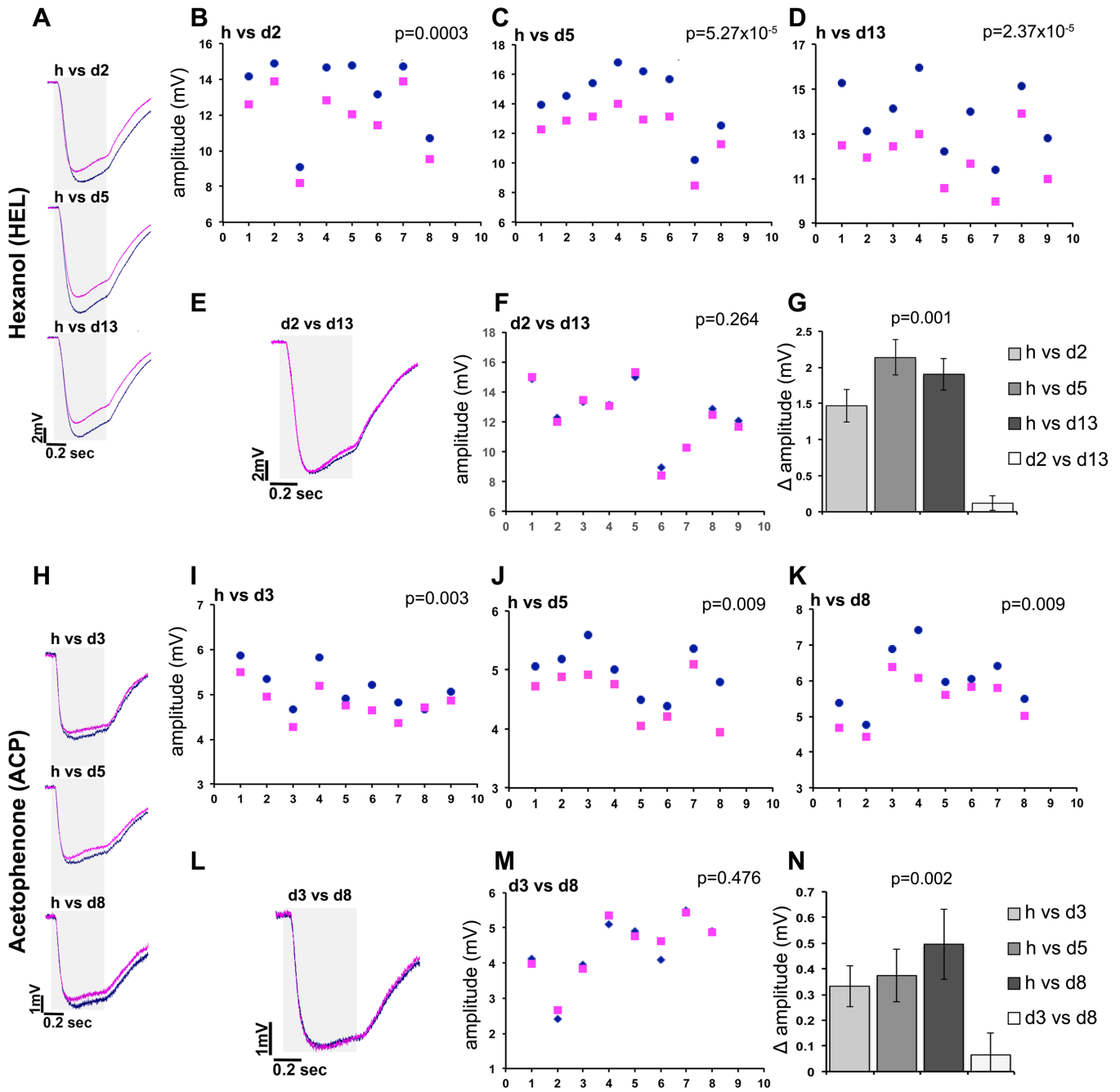
h-1-Pentanol						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	2.711	BB	0.0231	6.06239e-1	4.13028e-1	0.00853
2	4.421	BB	0.0363	1.78695	7.93818e-1	0.02515
3	5.023	BB	0.0618	7100.83350	1662.52856	99.94445
4	5.750	BB	0.0231	1.55354	1.05958	0.02187

d11-1-Pentanol						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	3.443	BB	0.0421	11.47077	6.04865	0.15594
2	3.984	BB	0.0381	24.50063	12.94281	0.33307
3	4.528	BB	0.0792	7319.98291	1443.77832	99.51099

h-1-Octanol						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	5.607	BB	0.0376	8.33811	4.49661	0.10979
2	6.696	BB	0.0325	2.99909	1.70881	0.03949
3	8.018	BV	0.0456	1.06406	4.85292e-1	0.01401
4	8.165	VB	0.0400	2.65737	1.29295	0.03499
5	8.821	BB	0.0560	27.26840	7.94571	0.35904
6	9.302	BB	0.1036	7552.50998	1054.55908	99.44269

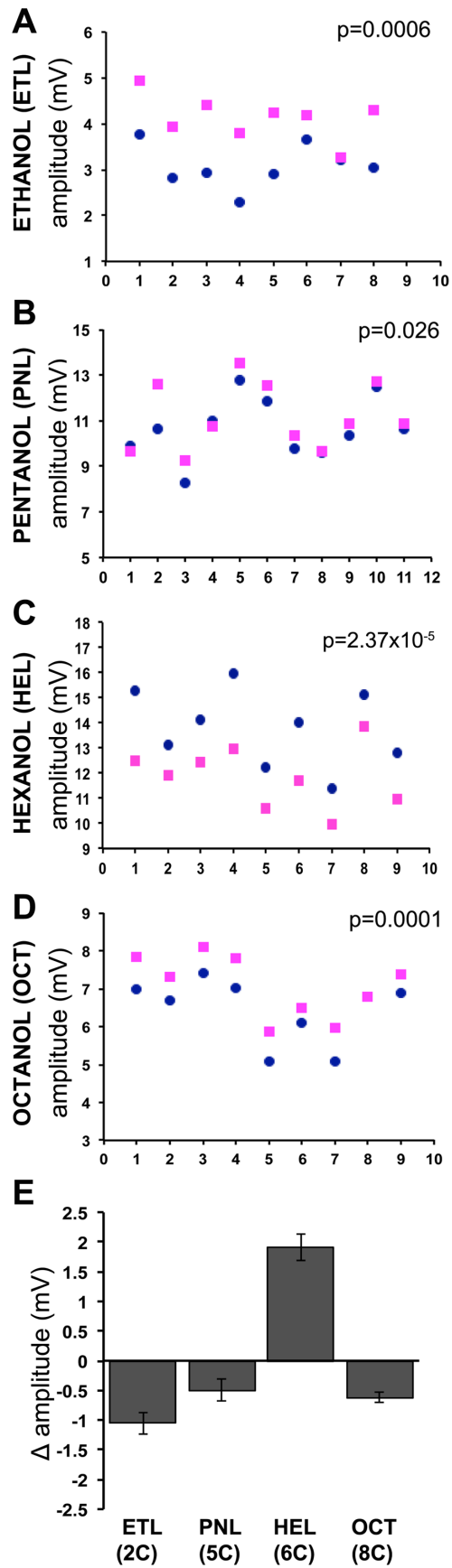
d17-1-octanol						
Peak #	RetTime (min)	Type	Width (min)	Area (pA*s)	Height (pA)	Area %
1	5.692	BB	0.0379	23.70400	12.84659	0.25730
2	7.063	BB	0.0385	11.61009	6.02909	0.12603
3	8.644	BB	0.1126	9162.00488	1206.64636	99.45199
4	9.924	BB	0.0483	1.24629	5.13031e-1	0.01353
5	10.233	BB	0.0438	4.07517	1.99705	0.04424
6	11.328	BB	0.0524	4.05706	1.45943	0.04404
7	13.140	BB	0.0522	2.07261	7.51371e-1	0.02250
8	13.614	BB	0.0537	1.37058	4.24300e-2	0.01488
9	14.565	BB	0.0490	2.34988	9.43809e-1	0.02551

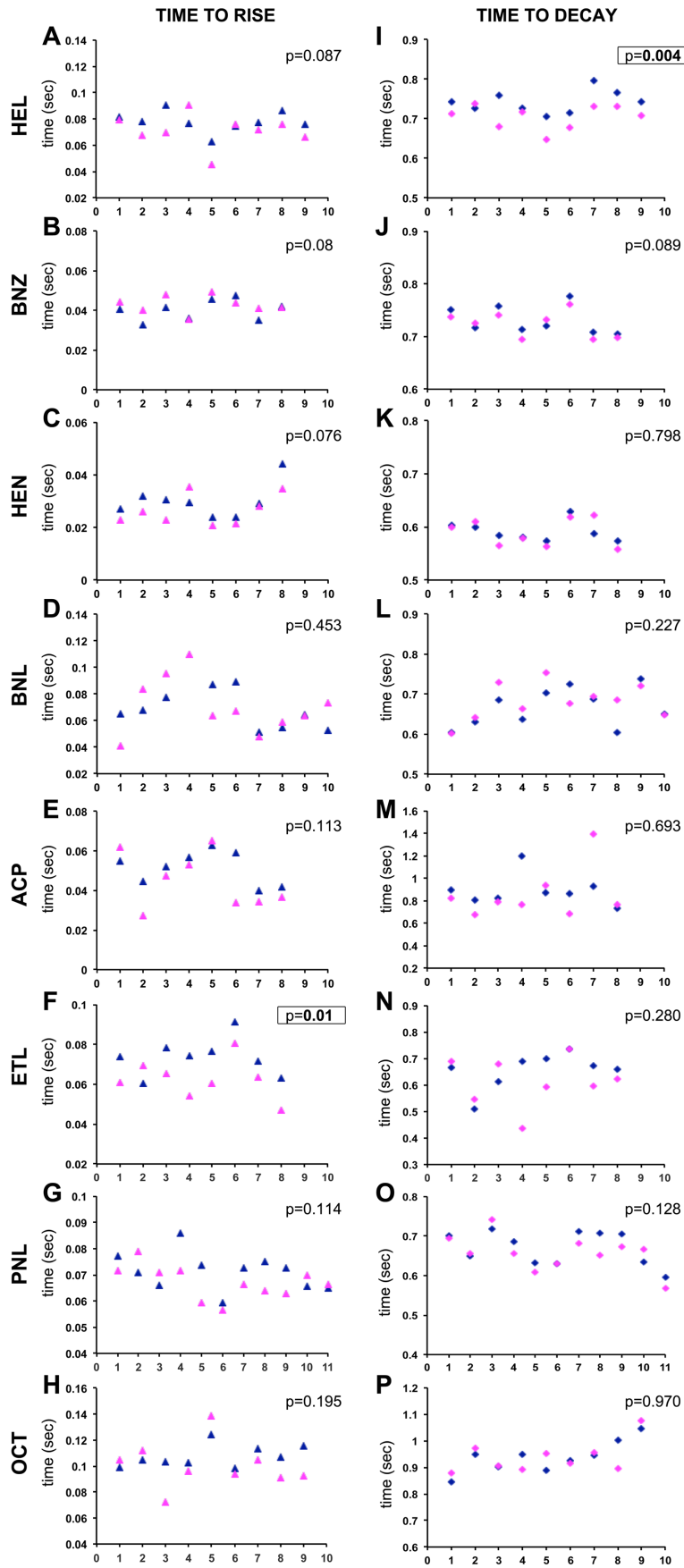
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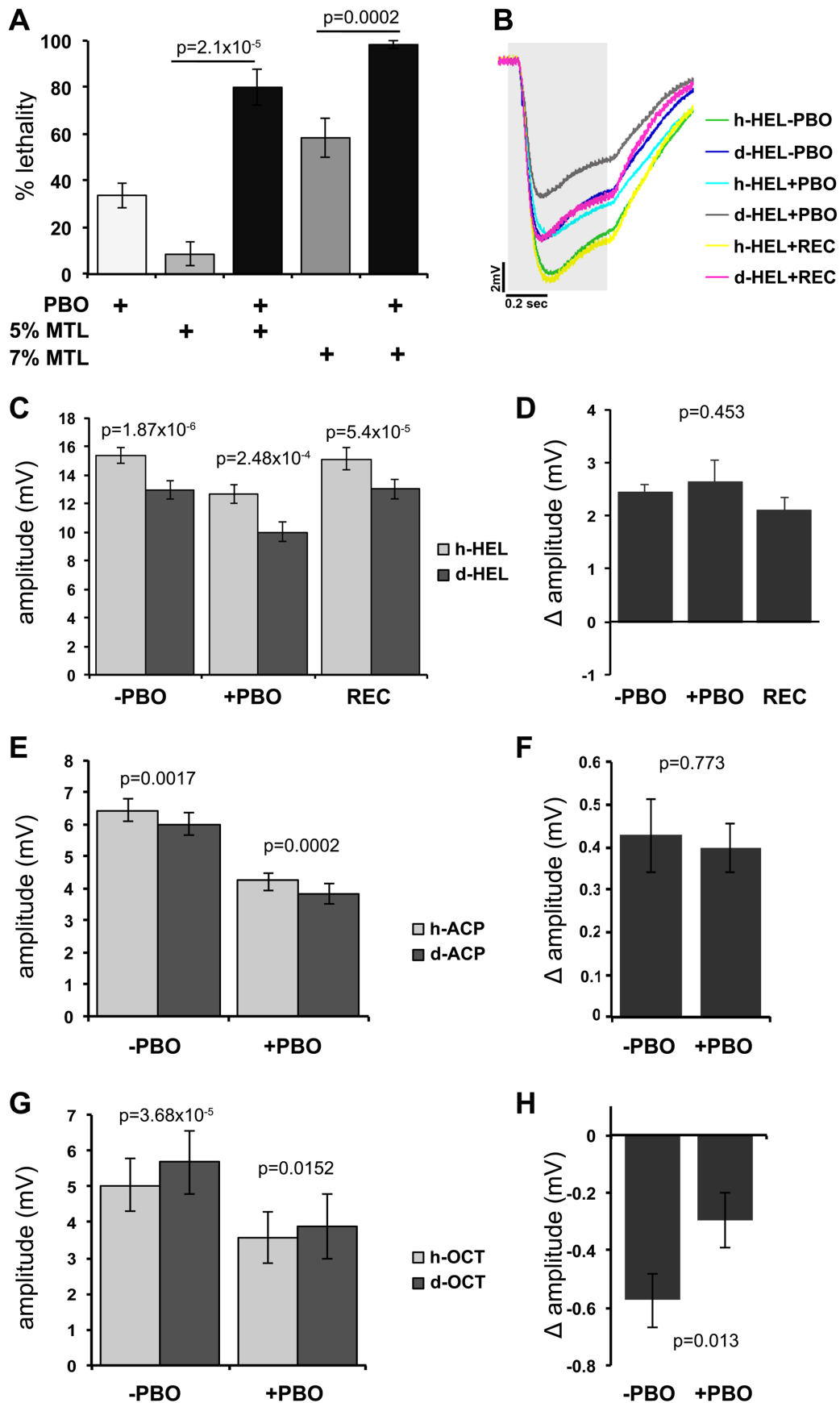


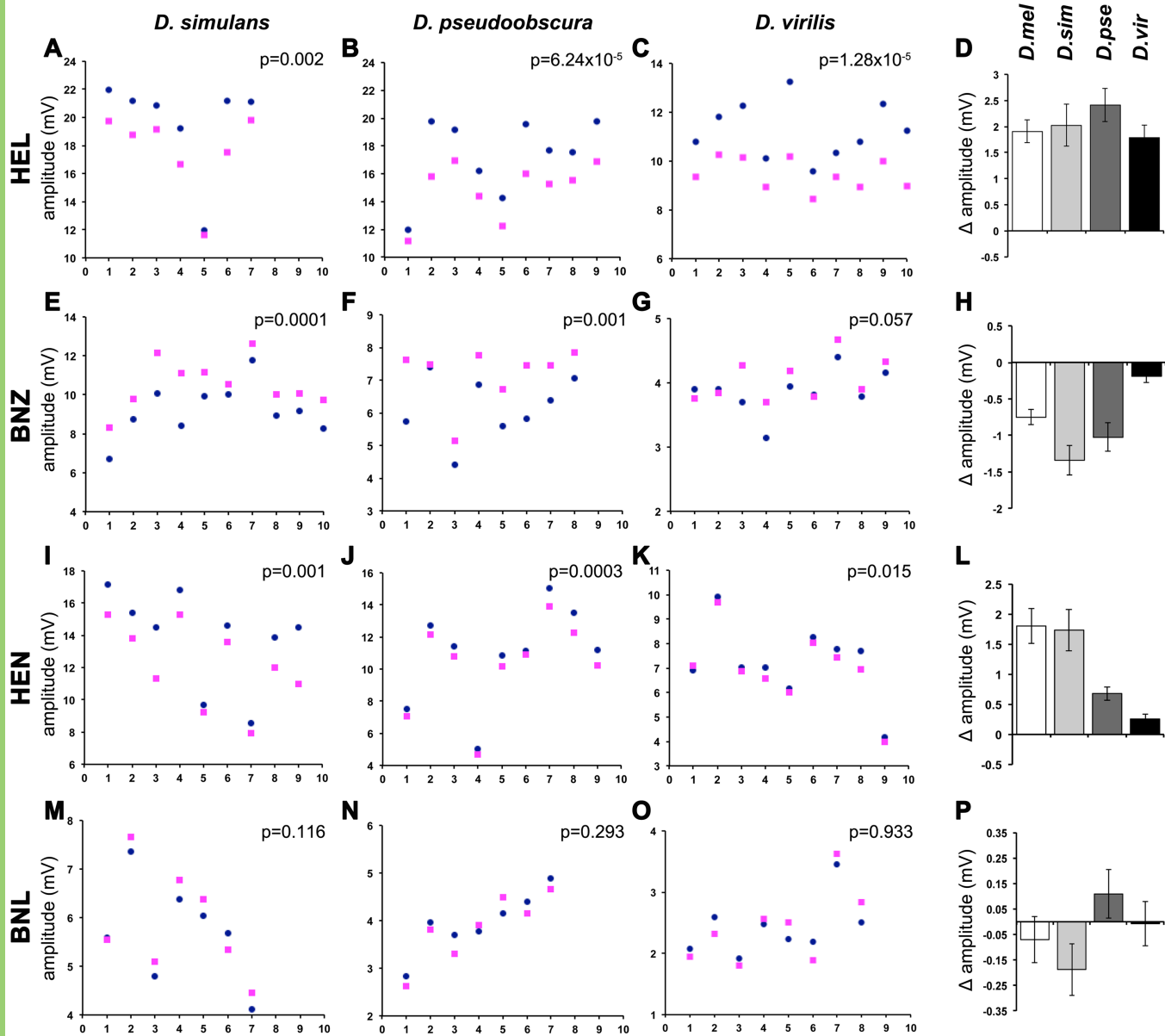
Odorant	Mean Amplitude	n	Mean $\Delta$ (Amp)	p (t-test)
d2-1-HEL $1 \times 10^{-4}$ d13-1-HEL $1 \times 10^{-4}$	4.0675 $\pm$ 0.1789 4.1068 $\pm$ 0.1697	7	<b>-0.0392 <math>\pm</math> 0.009</b>	<b>=0.3213</b>
d2-1-HEL $1 \times 10^{-3}$ d13-1-HEL $1 \times 10^{-3}$	6.4915 $\pm$ 0.1603 6.3477 $\pm$ 0.1641	7	<b>0.1438 <math>\pm</math> 0.1292</b>	<b>=0.3085</b>
d2-1-HEL $1 \times 10^{-2}$ d13-1-HEL $1 \times 10^{-2}$	12.2325 $\pm$ 0.4389 12.3246 $\pm$ 0.3804	7	<b>-0.092 <math>\pm</math> 0.195</b>	<b>=0.653</b>
d3-ACP $1 \times 10^{-4}$ d8-ACP $1 \times 10^{-4}$	3.4181 $\pm$ 0.2490 3.3743 $\pm$ 0.2554	7	<b>0.0437 <math>\pm</math> 0.054</b>	<b>=0.4490</b>
d3-ACP $1 \times 10^{-3}$ d8-ACP $1 \times 10^{-3}$	4.6298 $\pm$ 0.2093 4.7302 $\pm$ 0.2235	10	<b>-0.1004 <math>\pm</math> 0.064</b>	<b>=0.1542</b>
d3-ACP $1 \times 10^{-2}$ d8-ACP $1 \times 10^{-2}$	6.2129 $\pm$ 0.6041 6.1682 $\pm$ 0.5080	7	<b>0.0447 <math>\pm</math> 0.133</b>	<b>=0.7478</b>

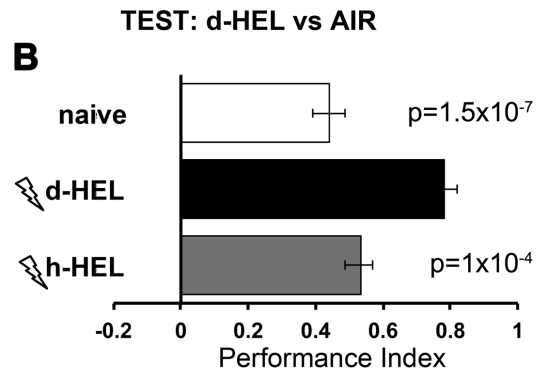
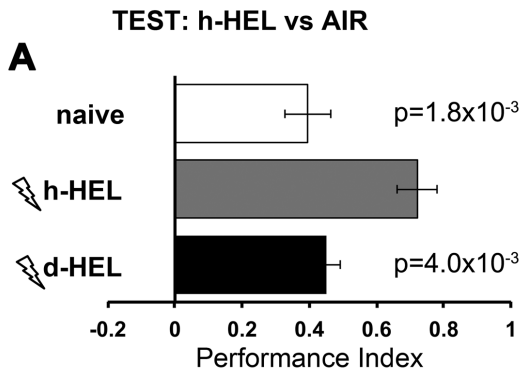
TABLE 6



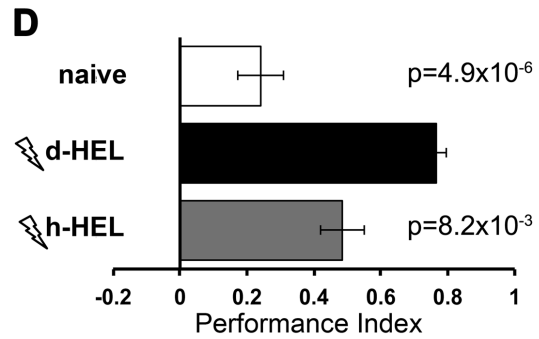
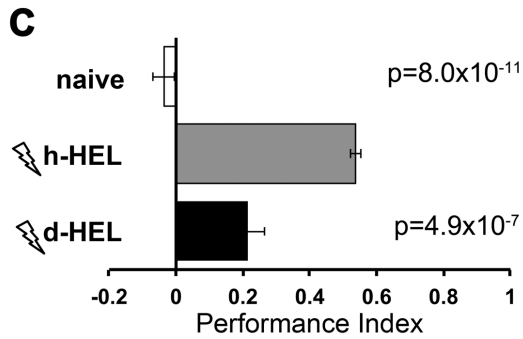




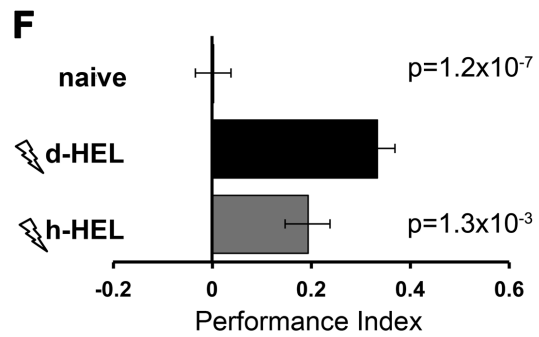
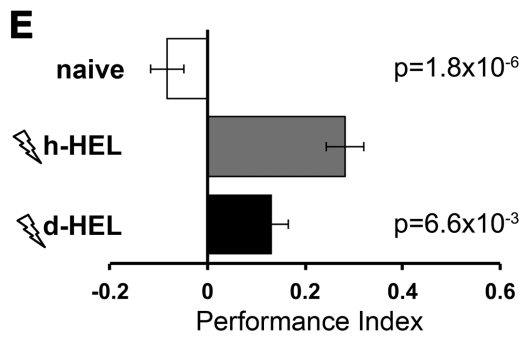




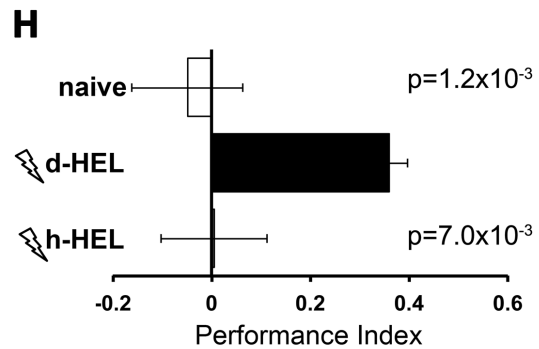
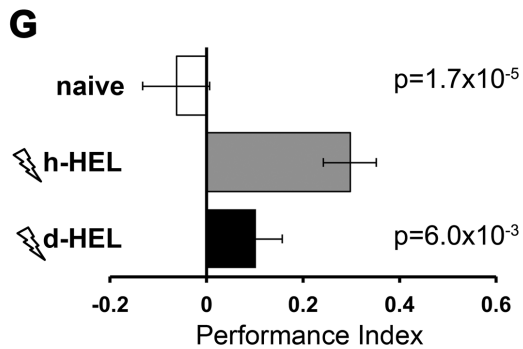
*D. melanogaster*  
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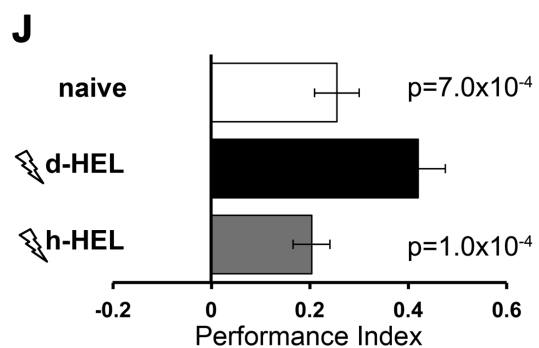
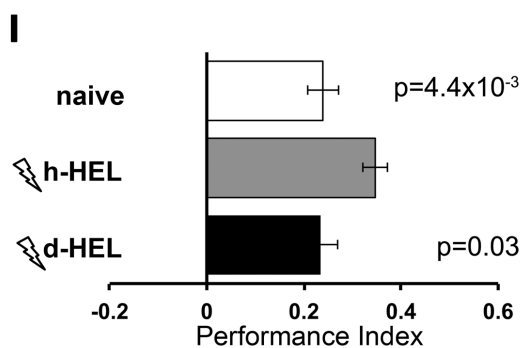
*D. melanogaster*  
CS



*D. simulans*



*D. pseudoobscura*



*D. virilis*