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2

3 Title:

4 **Acute ozone exposure impairs detection of floral odor, learning, and**
5 **memory of honey bees, through olfactory generalization.**

6

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17

18 **Abstract (max 300 words) (word count: 299)**

19

20 Air pollution stemming from human activities affects the environment in which plant and
21 animal species live and interact. Similar to primary air pollutants which are emitted, secondary
22 air pollutants, such as tropospheric ozone (O₃) formed from nitrogen oxides, are also harmful
23 to human health and plant physiology. Yet, few reports studied the effects of O₃ on pollinators'
24 physiology, despite that this pollutant, with its high oxidative potential, likely affects pollinators
25 behaviors, especially the perception of signals they rely on to navigate their environment.
26 Volatile Organic Compounds (VOCs) released by plants are used as signals by different
27 animals. For pollination services, VOCs attract different insects to the flowers and strengthen
28 these interactions. Here, we used the honey bee *Apis mellifera* as a model to characterize the
29 effects of acute exposure to different realistic mixing ratios of O₃ (80-, 120-, and 200-ppb) on
30 two crucial aspects: first, how exposed honey bees detect VOCs; and second, how O₃ affects
31 these pollinators' learning and memory processes. With electroantennogram (EAG) recordings,
32 we showed that increasing O₃ mixing ratios had a biphasic effect: an initial 25% decrease of
33 the antennal activity when bees were tested directly after exposure (O₃ direct effect), followed
34 by a 25% increase in activity and response when bees were allowed a two-hour rest after
35 exposure (O₃ delayed effect). In parallel, during olfactory conditioning, increasing O₃ mixing
36 ratios in both exposure protocols scarcely affected olfactory learning, followed by a decrease
37 in recall of learned odors and an increase of response to new odors, leading to a higher
38 generalization rate (*i.e.*, discrimination impairment). These results suggest a link between O₃-
39 related oxidative stress and olfactory coding disturbance in the honey bee brain. If ozone affects
40 the pollinators' olfaction, foraging behaviors may be modified, in addition with a possible long-
41 term harmful effect on pollination services.

42

43 **Keywords.** Air pollution; volatile organic compounds; olfaction; generalization; *Apis*
44 *mellifera*; plant-pollinator interactions.

45

46 **Abbreviations.** CS: Conditioned Stimulus; EAG: electroantennography; NO: new odor; PER:
47 Proboscis Extension Reflex; O₃: ozone; US: Unconditioned Stimulus; VOCs: volatile organic
48 compounds.

49

50

51 **Highlights** (max 85 characters including spaces per bullet point)

52

- 53 • Ozone (O₃) at 80 ppb decreases the recall of learned odor association.
- 54 • O₃ increases the response to new odor suggesting higher generalization.
- 55 • Directly after O₃ exposure at 80 & 200 ppb, antennal activity decreases.
- 56 • But after a two-hour rest, O₃ increases antennal activity at 120 & 200 ppb.
- 57 • O₃ affects olfactory detection and perception of VOCs in honey bees.

58

59 **1. Introduction.**

60

61 Plants release many Volatile Organic Compounds (VOCs) to attract pollinators and seed
62 dispersers, in order to insure their reproduction (Raguso 2008, Muhlemann et al. 2014, Burkle
63 & Runyon 2019). These compounds spread with a concentration gradient, the higher the
64 concentration the closer to the plant; this enables for instance the pollinators to locate the plant
65 accurately, using floral VOCs as olfactory cues from both long and short distances (Cardé &
66 Willis 2008, Riffell et al. 2008, Dötterl & Vereecken 2010). Thanks to the combination of the
67 VOCs released, insect pollinators are able to extract information such as identifying the plant
68 species, assessing the nectar availability and the quality of the resource (Howell & Alarcón
69 2007, Wright et al. 2009). However, since the beginning of the industrial era, this chemical
70 communication between plants and pollinators has been challenged by several factors (Yuan et
71 al. 2009, Jürgens & Bischoff 2017), affecting recognition of host plants by the insects, which
72 in turn may affect the crucial pollination services provided by them.

73 Air pollution is one of the most impacting environmental hazards arising from human activities
74 (W.H.O. Occupational & Environmental Health 2006): urbanization and industrialization lead
75 to several environmental issues with negative impacts on climate and air quality (Baklanov et
76 al. 2016). Among the different pollutants in the atmosphere, the rates of tropospheric ozone
77 (O_3) started to increase since the pre-industrial era and will continue to do so for the next
78 decades (Vingarzan 2004). Tropospheric O_3 , through its high oxidative potential and the
79 increased production of reactive oxygen species, can affect the balance between oxidative stress
80 and anti-oxidant defenses; O_3 has a direct influence on plant physiology, causing oxidative
81 damages (Iriti & Faoro 2007, Pinto et al. 2007). Further emission of VOCs by plants can be
82 affected by O_3 exposure (Peñuelas et al. 1999, Holopainen & Gerchenzon 2010), including
83 floral compounds (Saunier & Blande 2019). Additionally, O_3 can directly react with VOCs

84 released by these plants in the atmosphere (Holopainen & Blande 2013, Blande et al. 2014,
85 Dubuisson et al. 2022). Incidentally, O₃ and other air pollutants will impact plant-pollinator
86 communications (Girling et al 2013, Lusebrink et al. 2015, Farré-Armengol et al. 2016, Fuentes
87 et al. 2016, Ryalls et al. 2022). While few studies reported the direct effects of air pollution on
88 insect pollinators through carbon oxides, nitrous oxides, and diesel exhausts (Leonard et al.
89 2019, Reitmayer et al. 2019), especially on their learning abilities, the effects of O₃ are still less
90 known and described.

91 One of the most studied pollinators, the honey bee *Apis mellifera*, is a polylectic pollinator,
92 meaning the foragers do not have one specialized interaction with one plant, but instead visit
93 different flowers and plant species in order to collect nectar and pollen (Robertson 1925; also,
94 see definitions in the appendix of Müller & Kuhlmann 2008). Honey bees are able to navigate
95 their environment and successfully recognize and remember patches of resource-bearing
96 flowers *via* visual and olfactory memory associations (Chittka & Raine 2006, Dötterl &
97 Vereecken 2010). In order to recognize them, bees need first to detect the signals released by
98 plants; for olfactory signals, they can achieve that through VOCs detection and signaling: first,
99 the VOCs are detected by the antennae, through olfactory receptor neurons (Kaissling 1971,
100 Jung et al. 2014), then the olfactory message is mediated towards the honey bee brain; initially
101 processed through the antennal lobes, then carried up to the mushroom bodies *via* projection
102 neurons (Strausfeld 2002, Paoli & Galizia 2021). The mushroom bodies, which receive
103 information from all the sensory modalities, are the higher structures of the honey bee brain,
104 and where the memory processes take place (Dujardin 1850, Menzel & Giurfa 2001). This
105 whole progression describes the olfactory pathway, which is classically stimulated for the
106 proboscis extension reflex (PER) conditioning through appetitive reward presentation (Takeda
107 1961, Bitterman et al. 1983). The association between olfaction and PER ultimately represents

108 the basis for olfactory learning and memory in honey bees (Menzel et al. 1993, Menzel & Giurfa
109 2001).

110 The effect of oxidative stress on pollinators, and honey bees specifically, has been mostly
111 studied under the scope of pesticide poisoning and subsequent detoxification processes
112 (Kodrick et al. 2015, Chaitanya et al. 2016). While the effects of insecticides are largely
113 reported on many aspects of learning and memory, very few looked at the effects of exogenous
114 oxidative stress. Farooqui (2008) reported the effects of injections of ferrous ammonium citrate,
115 source of oxidative stress, in the antennal lobes, and looked at the olfactory learning and recall.
116 Bees subjected to iron-induced oxidative stress had a consistent effect on recall of the learned
117 association, and the acquisition was also affected (Farooqui 2008). In the same way, Leonard
118 and colleagues (2019) measured the effects of carbon oxides, nitrous oxides and particle matter
119 altogether, on olfactory learning and memory of honey bees. Pollution treatments were based
120 on peak concentrations measured at city street levels. After a short exposure from low- to high-
121 concentration treatments, bees exerted a lower learning rate than the control group and their
122 olfactory memory was impaired (Leonard et al. 2019). Moreover, Dötterl and colleagues (2016)
123 reported the effect of O₃ fumigation on the detection of floral VOCs by cut antenna of honey
124 bee using electroantennography (EAG) recordings: O₃ induced a decrease of the antennal
125 activity when stimulated by VOCs (Dötterl et al. 2016). But the O₃ mixing ratio used in this
126 report (1000 ppb) was highly exceeding naturally-occurring mixing ratios (Vautard et al. 2005).
127 More recently, Vanderplanck et al. (2021) also reported the direct effects of O₃ exposure, using
128 field-relevant mixing ratios (from 80 to 200 ppb), on antennal activity and behavior of two
129 insect pollinators: the fig wasp *Blastophaga psenes* and the bumble bee *Bombus terrestris*. For
130 the latter, increasing mixing ratios of O₃ decreased the antennal activity and suppressed the
131 innate attraction to natural VOCs for both pollinators (Vanderplanck et al. 2021). To date, there

132 are no reports on the effects of O₃ exposure on olfactory learning and memory in honeybees, or
133 any other insect.

134 In the present study, we tested the effect of exposing honeybees to naturally occurring mixing
135 ratios of O₃ (Vautard et al. 2005) on both their ability to detect, learn, and recall floral VOCs.
136 To do so, in control conditions, honeybees were exposed to four different O₃ mixing ratios
137 during one hour; directly after exposure, learning and detection abilities were tested with two
138 different synthetic VOCs mimicking floral scent, using respectively PER conditioning and
139 EAG recordings. Based on the aforementioned literature results, we hypothesize that exposure
140 to increasing mixing ratios of O₃ will (1) decrease the learning rate of exposed bees and affect
141 their memory, and (2) decrease the EAG activity of exposed antennas. We also tested the effect
142 of a two-hour rest to assess O₃ delayed effect: we investigated how honey bees may cope with
143 the O₃ exposure, and determined its effect on perception and detection as well. The effect of a
144 potential recovery after an acute stress on insects has been mostly reported for cold/chill
145 tolerance experiments, with various effects on metabolic rate and neuromuscular functions
146 (Lalouette et al. 2011, MacMillan et al. 2014, Overgaard & MacMillan 2017). But we did not
147 find any evidence for a recovery after acute O₃ stress. Hence, we can only hypothesize that the
148 two-hour rest in absence of O₃ exposure should reduce the decrease in olfactory learning and
149 antennal activity, so the parameters measured should be similar to control groups.

150

151 **2. Material and Methods.**

152

153 *2.1. Animal samplings*

154 Honey bees (*Apis mellifera*) were collected from three different hives at the apiary located at
155 the Terrain d'Expérience on the CNRS campus (43°38'19 N, 3°51'49 E), from the LabEx
156 CeMEB platform (Centre Méditerranéen pour l'Environnement et la Biodiversité). We
157 temporarily blocked the hives' entrance, and identified returning foragers thanks to their loaded
158 pollen baskets. Adult bees were collected in small clear plastic tubes and placed shortly under
159 ice (< 5 minutes, no direct contact). While asleep, bees were individually placed and attached
160 to 3-cm high Teflon holders. Once awake, they were given sugar water (50% w/w sucrose) to
161 recover from cold anesthesia, and placed to rest overnight in an incubator at 33.5°C ± 0.2°C /
162 RH>65%. Experiments were conducted from April to September 2021.

163

164 *2.2. Chemical compounds and preparations*

165 We used synthetic versions of two extremely common floral scents ([Knudsen et al 2006](#)), *i.e.*
166 R-linalool (97 % pure; CAS: 78-70-6), and eucalyptol (99 % pure; CAS: 470-82-6), used as
167 VOCs for electrophysiological recordings (EAG) and associative conditionings (PER), at a
168 unique concentration of 100 µg/µL. Depending on the experiment, different solvents were used:
169 paraffin oil for EAG procedure, and 100% ethanol for PER procedure. For each procedure, a
170 volume of 10 µL of VOC preparation was deposited on a single 20 × 10 mm Whatman no.1
171 piece of paper, placed in a Pasteur pipette for odor delivery. All compounds were purchased
172 from Sigma Aldrich (St-Louis, MO, USA).

173

174 2.3. Ozone mixing ratios & exposures

175 For each procedure, O₃ exposure was performed as previously described ([Vanderplanck et al.](#)
176 [2021](#)). In short, bees fixed in holders were placed in a 500-mL glass bottle acting as a fumigation
177 chamber, connected to an O₃ analyzer-generator (Model 49i, Thermo Fischer, Franklin, MA,
178 USA), with a pump pushing the O₃ through Teflon tubes at a 1.5 L/min flow rate. Ozone was
179 produced through oxygen photolysis subjected to UV radiation at 185-nm wavelength. By
180 adjusting the solenoid valves opening level, we can produce fixed mixing ratios of O₃: for both
181 procedures, we tested four different mixing ratios: 0 ppb (control group), 80 ppb, 120 ppb, and
182 200 ppb, similar to those previously tested and reported ([Vanderplanck et al. 2021](#)). These
183 mixing ratios correspond to values commonly reported in the South of France: the mean value
184 of O₃ measured during a summer day (80 ppb), the peak value reached during this same summer
185 day (120 ppb), and the highest O₃ mixing ratio recorded in the South of France during the last
186 20 years (*i.e.* the heatwave in 2003) ([Vautard et al. 2005](#)). To maintain the O₃ at desired ratio
187 and flow rate, air was extracted at the same 1.5 L/min flow rate, from the fumigation chamber
188 toward the O₃ analyzer. To keep the honey bees in a humid environment during exposure, a
189 piece of Whatman paper was imbibed with distilled water and placed in the fumigation
190 chamber.

191 Each treated group was exposed for one hour; from here, we either tested the exposed bees right
192 away, to assess **ozone direct effects** (a), or placed them back in the incubator for a 2-hr rest
193 before testing them, to assess **ozone delayed effects** (b). This paradigm serves a proxy to
194 foraging behaviors where bees can be exposed to O₃ pollution outside (a) and return to the
195 colony to recover from possible oxidative stress (b).

196

197 2.4. *Olfactory conditioning (PER)*

198 Effects of O₃ exposure on honey bees' learning ability were assessed through a classical
199 olfactory conditioning using the proboscis extension reflex (PER; [Bitterman et al. 1983](#)). Honey
200 bees underwent an acquisition phase where they learned to associate a neutral odor (later acting
201 as the conditioned stimulus, CS) with an appetitive reward eliciting the PER (acting as the
202 unconditioned stimulus, US). One trial of training session consists of the CS presentation for 7
203 seconds, accompanied by the US presentation (a small drop of sucrose solution) for 5 seconds;
204 both stimuli overlapped for 3 seconds. This trial was repeated five times, with a 1-minute
205 interval (minimum) between trials. This acquisition phase results in long-term memory
206 formation ([Gerber et al. 1998](#), [Démares et al. 2014](#)). This memory can be tested afterwards with
207 recall tests: it consists of the presentation of CS alone, to check if the bee remembers and recalls
208 the reinforced association by exerting the PER. In parallel, we presented a new odor (NO) to
209 check for generalization, and to test the specificity of the CS response. Similar to the training
210 sessions, stimuli were randomly presented and separated by 1-minute interval, one hour after
211 the last trial of the acquisition phase.

212 Ozone exposures, training sessions and memory tests were performed between late morning
213 and mid-afternoon (from 09.00AM to 04.00PM). Bees were fed briefly in the early morning
214 and late-afternoon (08.00AM and 06.00PM): this allowed to check for normal PER to sucrose
215 feeding. Bees not responding to sucrose were discarded from the experiments. Bees responding
216 to the CS odor at the first acquisition trial were also discarded from analysis. The odors used as
217 CS were the aforementioned VOCs, R-linalool and eucalyptol, equally randomized within
218 treated groups. When one VOC was used as CS, the other was used as NO. In total, between all
219 protocols, O₃ mixing ratios, and VOC tested as CS (N=16 groups), we used 308 adult honey
220 bees for this section (ranging from 18 to 22 bees per group).

221

222 2.5. *Electroantennography (EAG)*

223 EAG recordings allow assessment of VOCs detection from individualized antenna.
224 Specifically, it measures the amplitude of depolarization of all active olfactory sensory neurons
225 in response to an olfactory stimulus (Roelofs 1984). Here, we measured how O₃ exposure might
226 affect such amplitude in response to either R-linalool or eucalyptol stimulation. After O₃
227 exposures, the right antenna of individual exposed honey bees was cut and mounted between
228 glass capillaries, filled with Ringer's solution (composition, in mM: NaCl, 131; KCl, 5; CaCl₂,
229 2; NaHCO₃, 29; pH 7). Those were connected to silver electrodes of an EAG probe. A
230 continuous humidified air flow (450 mL/min), purified by activated carbon, was blown at the
231 antenna through a circulating tube to keep it from drying. Single VOCs were applied to a
232 Whatman no.1 paper placed in a Pasteur pipette; the tip of this pipette was inserted in a hole in
233 the circulating airflow tube. A short pulse of purified air (0.5 second, 900 mL/min) through the
234 Pasteur pipette released the VOC in the airflow leading to the antenna. The continuous air flow
235 and the pulsed air stimulation are both set and delivered by the stimulus controller (CS-55),
236 recorded by an acquisition controller (IDAC-2), and analyzed with the GcEad 1.2.5 software
237 (all EAG equipment purchased from Ockenfels SYNTECH Buchenbach, Germany). The
238 maximum amplitude of depolarization was recorded for each stimulus. Intervals between two
239 stimulations were at least 45 seconds (up to one minute), in order for the antennal activity to
240 return to a stable baseline. Every antenna went through a balanced randomized sequence of
241 VOC stimulations, with paraffin controls always coming first and last of this sequence. The
242 response amplitude of each VOC was adjusted with subtracted mean paraffin-response from
243 their own sequence. In total, with all the exposure protocols and O₃ mixing ratios (N= 8 groups),
244 we tested 208 antennae, each tested for both VOCs (n=26 for each group).

245

246 2.6. *Statistical analyses*

247 To assess honey bees' performance in learning, we performed generalized linear mixed models
248 (GLMM) with a binomial distribution on the PER response of honey bees (binary data) with
249 'O₃ mixing ratio' and 'acquisition trials' as fixed factors, along with their interactions. Bee
250 individual was implemented as a random factor in the model. As reported in Tables 1&2, each
251 GLMM resulted in F-values as statistical indicators related to degrees of freedom, which
252 enabled us to calculate a p-value for each factor and interaction. Between the two VOCs used
253 as CS and the two exposure protocols, we performed four GLMMs for each combination of CS
254 & exposure protocol. Memory recall tests were also analyzed using GLMM with 'O₃ mixing
255 ratio' and 'odor tested' (CS vs NO) as fixed factors, and 'bee individual' as random factor. In
256 GLMM pairwise comparisons between all groups were conducted using contrasts, and p-values
257 were adjusted with False Discovery Rate (FDR) corrections. In addition to memory recall test
258 analyses, we assessed the distribution of response categories at one-hour time point. Responses
259 to memory tests fell into four distinct categories: PER to CS only ('CS⁺'), response to new odor
260 only ('CS⁻/NO⁺'), response to both CS and NO ('All Odors'), and response to none ('no PER').
261 For each condition, all bees were categorized this way and the overall frequency distribution of
262 the four categories was compared with the response distribution of the control group using
263 Pearson's chi-square tests (as described in [Urlacher et al. 2017](#)). For post-*hoc* comparisons
264 against control group, subsequent chi-square tests were done and adjusted with the FDR
265 method.

266 For EAG recordings, we focused on the effect of O₃ mixing ratio on EAG response amplitude,
267 depending on the VOC tested for stimulation (eucalyptol & R-linalol) and on the exposure
268 protocol (*i.e.* direct & delayed effect): we performed one-way ANOVAs with 'O₃ mixing ratio'
269 as factors, followed by post-*hoc* contrasts corrected with the FDR method. All statistical
270 analyses were conducted using IBM SPSS 23 and GraphPad Prism 7. All data used for each

271 figure are reported in Supplementary Table 1. All statistical analysis results with complete
272 numbers are reported in Supplementary Table 2.

273

274 **3. Results.**

275 *3.1. Ozone alters VOC perception*

276 3.1.1. Effect on the responses to reinforced VOC

277 3.1.1.1. *Effect on learning*

278 For each O₃ exposure protocol and each VOC used as conditioned stimulus (CS) for the
279 association with sugar reward, acquisition is achieved (Figs 1 & 2): the olfactory learning rate
280 is significantly increasing with each trial increment, for both eucalyptol and R-linalool (GLMM
281 ‘acquisition trial’ factor; Table 1, O₃ direct effect, $p < 0.001$, see also Fig. 1A; O₃ delayed effect,
282 $p < 0.001$, see also Fig.1D; Table 2, O₃ direct effect, $p < 0.001$, see also Fig.2A; O₃ delayed
283 effect, $p < 0.001$, see also Fig.2D). Also, O₃ mixing ratios affect the level of PER response rate
284 in each condition (Table 1, GLMM ‘O₃ mixing ratio’ factor; Eucalyptol, O₃ direct effect, $p =$
285 0.001 ; O₃ delayed effect, $p = 0.038$; Table 2, R-linalool, O₃ delayed effect; $p = 0.023$). In all
286 these conditions, bees exposed to 80 ppb of O₃ have a significantly lower acquisition rate than
287 control bees, especially when eucalyptol is used as CS (see Figs 1A&D and Fig. 2D; for
288 complete data, see Supplementary Table 1). There is only one condition where the O₃ does not
289 significantly affect the acquisition rate, when R-linalool is used as CS and bees are tested right
290 away after exposure (Table 2, GLMM ‘O₃ mixing ratio’ factor, O₃ direct effect, $p = 0.121$; also
291 see Fig. 2A), although the tendency follows the same pattern, *i.e.*, a lower learning rate for 80-
292 ppb O₃-exposed bees compared to control bees. Interestingly, in almost every condition, the
293 learning rate of honey bees exposed to 200 ppb of O₃ does not significantly differ from the one
294 of control bees nor bees exposed to 80 ppb and 120 ppb, independent on VOC or exposure
295 protocol (except for eucalyptol used as CS under O₃ direct effect; see Supplementary Table

296 2A). In summary, O₃ does not affect the ability to acquire the association, but limits the PER
297 rate of exposed bees: olfactory learning is less efficient after O₃ exposure.

298

299 3.1.1.2. *Effect on recall*

300 One hour after the last acquisition trial, recall trials are performed; the CS presented alone is
301 tested along with a new odor (NO) to test for olfactory memory specificity. For each O₃
302 exposure protocol and each VOC used as CS, independently of O₃ mixing ratios, the global
303 PER response rate to CS is always significantly higher than the response rate to the NO (GLMM
304 ‘odor tested’ factor; Table 1, O₃ direct effect, $p < 0.001$, see also Fig 1B; O₃ delayed effect, $p =$
305 0.003 , see also Fig. 1E; Table 2, O₃ direct effect, $p = 0.002$, see also Fig 2B; O₃ delayed effect,
306 $p = 0.009$, see also Fig 2E;). The O₃ mixing ratios only impact significantly the CS recall of
307 bees trained when eucalyptol is used as CS, right after O₃ exposure (GLMM ‘O₃ mixing ratio’
308 factor; Table 1, O₃ direct effect, $p = 0.006$, see also Fig. 1B). Contrast comparisons show that
309 the group exposed to 80 ppb of O₃ has a significantly lower PER rate to CS compared to the
310 control group, 15.8% vs 78.9% respectively (GLMM *post-hoc* CS recall comparisons; 80 ppb
311 vs control, $p < 0.001$). In short, depending on the nature of the VOC, O₃ affects the CS recall
312 or not (eucalyptol vs R-linalool, see Figs 1B/1E & 2B/2E, respectively).

313

314 3.1.2. *Effect on new odors presentation*

315 While the specific response to CS is altered, we also observed an increased response rate to the
316 new odor presentation. More specifically, under O₃ direct effect, with eucalyptol used as CS,
317 bees exposed to 200 ppb of O₃ have a significantly higher response rate than bees exposed to
318 control bees (GLMM *post-hoc* NO response comparisons; 200 ppb vs control, $p = 0.006$). For
319 this specific condition, the interaction ‘O₃ mixing ratio * odor tested’ was close to significance

320 (Table 1, $p = 0.095$), meaning the effect of O₃ can depend on the type of test performed (here,
321 CS vs NO). But it was the only instance where it was this close, all the other interactions being
322 not significant for the other conditions (Tables 1&2).

323 Moreover, as all the ‘odor tested’ factors were statistically significant in the memory analyses,
324 this meant that the overall responses to CS were higher than the overall responses to NO. As a
325 follow-up, we performed post-*hoc* contrasts to test which specific groups showed significant
326 difference between CS and NO responses. As a result, only the control groups showed
327 statistically higher CS response compared to NO responses (Fig. 1B, O₃ direct effect, CS =
328 eucalyptol, CS vs NO rate, $p < 0.001$; Fig. 1E, O₃ delayed effect, CS = eucalyptol, CS vs NO
329 rate, $p = 0.001$; Fig. 2B, O₃ direct effect, CS = R-linalool, CS vs NO rate, $p = 0.002$; Fig. 2E,
330 O₃ delayed effect, CS = R-linalool, CS vs NO rate, $p < 0.001$). All the O₃-exposed groups did
331 not have statistically different response rates between CS and NO (see Supplementary Table
332 2B). Also, the difference between the PER rates to CS and NO for the control groups is around
333 55% in average (ranging from 44.4 % to 78.9 % difference), while for the O₃-exposed groups
334 the CS-NO difference is around 17.5% (ranging from 5.3% to 31.6% difference). Given all
335 these points, this shows that O₃ decreases the difference between learned odor and new odor
336 response rates.

337 These observations were corroborated with the frequency analysis of the distribution of PER
338 response categories. In two conditions, we observe significant global differences in honey bee
339 response’s distributions. The first condition is when eucalyptol is used as CS, under O₃ direct
340 effect exposure protocol (Fig. 1C; Pearson χ^2 , $p < 0.001$). Pairwise comparisons against control
341 group reveal different observations: each O₃-exposed group significantly differs in response
342 distribution compared to control (Fig. 1C). Distribution of PER responses in the control group
343 was 78.9% of response to the learned odor (CS⁺ category) and 21.1% of no response to any
344 odor (‘no PER’ category). For the O₃-exposed groups, there are two types of difference from

345 control distribution: for the 80-ppb group, a decrease of responses to CS alone (15.8% CS
346 response rate) with an increase of no responses (84.2% ‘no PER’ response rate) (χ^2 , $p < 0.001$)
347 resulting in an opposite shift in distribution compared to control; while for the 120-ppb and
348 200-ppb groups, again a decrease of responses to CS alone (25.0% and 31.6% respectively) but
349 here associated with an increase of all responses (20.0% and 36.8% respectively), not observed
350 in control group, when eucalyptol is used as CS (120-ppb: χ^2 , $p = 0.003$; 200-ppb: χ^2 , $p = 0.004$).

351 The second condition is when R-linalool is used as CS, under O₃ delayed effect exposure
352 protocol (Fig. 2F; Pearson χ^2 , $p = 0.041$). Distribution of PER responses in the control group
353 was, in order, 50.0% of response to CS alone, 13.6% to both CS and NO (‘All odors’ category),
354 and 36.4% of no response to any odor (‘no PER’ category). Here, pairwise comparisons against
355 control group reveal only one O₃-exposed group differing significantly from control group in
356 terms of response distribution: the 80-ppb group has fewer response to CS alone (5.3%) and an
357 increase of responses to all odors (63.2%) (Fig. 2F; 80-ppb: χ^2 , $p = 0.020$). For the groups
358 exposed to higher mixing ratios of O₃, although a few bees appear to respond to the NO only
359 (CS⁻/NO⁺ category), which is never observed in the control group, their overall response
360 distributions are not statistically different from the control group (120-ppb: χ^2 , $p = 0.137$; 200-
361 ppb: χ^2 , $p = 0.249$). For the other two conditions, the same pattern ‘decreased response to CS
362 alone with increased response to all odors’ was consistent with O₃ mixing ratios but the
363 response distributions were not statistically different from their respective control groups
364 (Pearson χ^2 ; CS: eucalyptol, O₃ delayed effect, $p = 0.198$; CS: R-linalool, O₃ direct effect, $p =$
365 0.505 ; see Supplementary Table 2C).

366

367 *3.2. Ozone alters VOC detection*

368 The electrophysiological recordings of antennal activity reveal significant effects of O₃
369 exposure in three conditions over four, either decreasing or increasing compared to control,
370 depending on the exposure protocol (Table 3; VOC: eucalyptol, O₃ direct effect, $p < 0.001$;
371 VOC: R-linalool, O₃ direct effect, $p < 0.001$; VOC: R-linalool, O₃ delayed effect, $p = 0.049$).
372 When testing the effect of O₃ treatment directly after exposure, and stimulated with eucalyptol,
373 we measured a significant decrease of EAG activity for 80-ppb and 200-ppb treated bees (0.72
374 mV and 0.73 mV, respectively) compared to control bees (0.98 mV amplitude) (Fig. 3A; VOC:
375 eucalyptol; control vs 80 ppb, $p = 0.002$; control vs 200 ppb, $p = 0.002$). We measured the same
376 significant decrease with R-linalool stimulation, where EAG activity for 80-ppb and 200-ppb
377 treated bees (1.97 mV and 2.39 mV, respectively) was lower than the one of control bees (2.85
378 mV) (Fig. 3C; VOC: R-linalool; control vs 80 ppb, $p < 0.001$; control vs 200 ppb, $p = 0.022$).
379 In contrast, the delayed effect of O₃ increases the EAG activity of treated bees compared to
380 control, but only significantly for antennas tested with R-linalool: the higher the O₃ mixing
381 ratio, the higher the amplitude of the recorded activity. In this case, the bees treated with 120
382 ppb of O₃ show a tendency of increased activity (3.65 mV), while bees treated with 200 ppb of
383 O₃ display a significant increase in amplitude (3.75 mV) compared to control (2.93 mV) (Fig.
384 3D; control vs 120 ppb, $p = 0.071$; control vs 200 ppb, $p = 0.039$). Finally, the only condition
385 where O₃ does not statistically affect the EAG response amplitude of the treated bees is when
386 we stimulated the antenna with eucalyptol after the two-hour rest (Table 3; O₃ delayed effect,
387 $p = 0.430$; see Fig.3 and Supplementary Table 2D). While R-linalool and eucalyptol display the
388 same pattern of response within the same exposure protocol, the biological effect of the
389 increased EAG activity with the increase in O₃ mixing ratios is only statistically significant with
390 R-linalool (Figs 3B & 3D). We hypothesize that this is due to the difference in absolute
391 amplitude between R-linalool stimulations and eucalyptol stimulations, around 3 mV and 1 mV,

392 respectively. Overall, these EAG findings highlight the fact that the effects of O₃ on antennae
393 depend more on physiological dynamics (*i.e.* direct vs delayed) rather than VOC nature.

394

395 **4. Discussion / Conclusion.**

396

397 In this study, under controlled conditions, we reported for the first time the effects of O₃
398 exposures on honey bees' olfactory learning and memory, along with its impacts on antennal
399 activity and responses. We tested the olfactory modality by using common VOCs found in
400 flowers, R-linalool and eucalyptol (Knudsen et al 2006). We found that O₃ affects both
401 detection at the antenna level (Fig.3) and perception at a more global level (Figs 1&2).
402 Respectively, these effects of O₃ strongly depend on the time elapsed since O₃ exposure on one
403 hand, and the VOCs tested on the other hand.

404 *Ozone affects olfactory conditioning and memory recall of VOCs*

405 Ozone exposure had a significant effect on olfactory conditioning, during acquisition (olfactory
406 learning) and recall (olfactory memory), which is in line with previous results: a decrease in
407 olfactory learning and CS recall due to iron-induced oxidative stress (Farooqui 2008). Right
408 after exposure, for O₃ direct effect, the mixing ratio of 80 ppb of O₃ significantly decreased the
409 acquisition rate and the recall rate of eucalyptol used as CS (60 % difference compared to
410 control, Figs 1A & 1B). For the delayed effect, this decrease after 80 ppb of O₃ was still
411 observable on both acquisition and recall of eucalyptol as CS (respectively 50 % and 30%
412 difference compared to control, Figs 1D & 1E). For olfactory conditioning using R-linalool as
413 CS, the acquisition rate right after O₃ exposure was not significantly impaired, but slightly
414 decreased for the O₃-exposed groups (around 25% difference compared to control for 80-ppb
415 and 120-ppb groups, Fig.2A), and the recall was not significantly affected (less than 20%

416 difference with control group response rate, Fig.2B). For the O₃ delayed effect, the acquisition
417 rate of R-linalool used as CS was significantly decreased for groups exposed to 80 ppb & 120
418 ppb, compared to control group (30% difference, Fig.2D). The recall rate was decreased for
419 these two groups as well, compared to control, but not significantly (25% difference, Fig.2E).
420 Overall, the CS acquisition rate and recall rate were mostly impacted by the lower mixing ratio
421 tested (80 ppb), and less by the other mixing ratios, independent of the exposure protocols used.
422 On one hand, the specific response to the learned VOC during recall was decreased; on the
423 other hand, the response to a new odor presented during recall was increasing with the increase
424 of O₃ mixing ratios (Figs 1&2). There was a significantly higher response to the new odor when
425 eucalyptol was used as CS, testing O₃ direct effect, in parallel with a statistically different
426 distribution of PER response types between control bees and 200-ppb exposed bees (Fig.1C).
427 These results strongly indicate a generalization of the new odor response to the learned odor
428 (CS response). This is also underlined by the limited difference between CS-NO response rates
429 for O₃-exposed groups (17.5% average difference) compared to control (55% average
430 difference). All in all, this means that the PER responses are less specific to the only reinforced
431 VOC. While responses to new odor always increase with increasing mixing ratios of O₃, it is
432 only statistically different with eucalyptol as CS, testing O₃ direct effect, suggesting the
433 generalization might be dependent on VOCs used as CS and on exposure protocols.

434 The decrease of CS recall in parallel with the increased responses to new odor is reported for
435 the first time for the effect of O₃ on the honey bee's olfactory memory. But a similar effect has
436 been reported when measuring the impacts of certain pesticides on honey bee behavior, *e.g.* the
437 effect of the phenylpyrazole fipronil (El Hassani et al. 2009), but also when inhibiting the
438 glutamate chloride (GluCl) neurotransmission (Boumghar et al 2012, Démares et al. 2014).
439 Fipronil targets the GABA chloride channels and the GluCl channels (Cole et al 1993, Barbara
440 et al. 2005, Janssen et al. 2007, Wolstenholme 2012), which are expressed in insect interneurons

441 in all areas of the honey bee brain, and are involved in inhibitory neurotransmission (Bicker et
442 al. 1987, Bicker 1999, Démares et al. 2013). At sublethal doses, the oxidative damages of
443 fipronil have been observed at the enzymatic level on different species including honey bees:
444 more specifically, fipronil modulates the activity of catalase, alkaline phosphatases and
445 carboxylesterases (Carvalho et al. 2013; see Wang et al. 2016 for review) thus we hypothesize
446 a possible correlation between oxidative stress, interneurons functions, and olfactory memory.
447 This was not the aim of that study, but future studies will enable us to provide a basis for this
448 claim; for instance, by studying the effects of antioxidant treatments on olfactory recall of honey
449 bees undergoing oxidative stress. Interneurons are also present in the optic lobes and other brain
450 areas of the honey bee (Strausfeld 2002, Démares et al. 2013). Since O₃ exposure may affect
451 the neurotransmission in interneurons, we can suspect effects on the visual system as well. Here
452 we report O₃ effects at a global physiological level, encompassing learning, memory, olfaction,
453 and potential metabolic effects due to oxidative stress: we cannot dissociate them *only* through
454 a behavioral basis, even though odor generalization potentially suggests an effect at the central
455 level. In order to begin to disentangle each effect, we measured and reported effects at the
456 peripheral level on the antennae.

457 *Ozone affects olfactory detection of VOCs at the antennal level*

458 Ozone exposure had a biphasic effect on the antennal responses to VOCs stimulation,
459 depending on the exposure protocol. With the first protocol, the direct effect of O₃ decreased
460 the amplitude of VOC-evoked antennal activity, for both eucalyptol and R-linalool
461 (Fig.3A&3C). Surprisingly, the antennal responses were statistically lower than control with
462 the 80-ppb and 200-ppb O₃ mixing ratios, but not with 120 ppb, which was not different from
463 any other group. While the global effect is in line with previous results reported on bumble bees
464 (Vanderplanck et al. 2021), we can only hypothesize that this effect of slight rebound with 120-
465 ppb O₃ direct exposure is either species-specific or protocol-dependent. When tested with the

466 second protocol, the O₃ delayed effect increased the amplitude of antennal responses with
467 increasing mixing ratios for both eucalyptol and R-linalool, the latter being statistically different
468 from the control group responses (Fig.3D). We hypothesize that this is due to the difference in
469 absolute amplitude between R-linalool stimulations and eucalyptol stimulations, around 3 mV
470 and 1 mV, respectively. Overall, these EAG findings highlight the fact that the effects of O₃ on
471 antennae depend more on physiological dynamics (*i.e.* direct vs delayed) rather than VOC
472 nature. This delayed effect operated a reversal from decreased response to increased response.
473 We hypothesize that this biphasic effect could be due to two elements: a direct mechanistic
474 effect of O₃ on the antenna decreasing its activity, and a delayed activation threshold acting
475 more globally on the whole bee physiology and counteracting O₃-induced damages, eventually
476 leading to increased antennal activity. It is possible that the activation threshold might be related
477 to anti-oxidant pathways, which we will test in future works. Additionally, since we observed
478 O₃ direct effects starting at 80 ppb on perception and detection, we will also need to test lower
479 mixing ratios to test the sensitivity thresholds from which the honey bees show behavioral and
480 electrophysiological changes, from ‘no observable adverse effect’ to ‘lowest observable
481 adverse effect level’.

482 *Ozone potentially disrupts the link between detection and perception*

483 The observable effects between exposure protocols, O₃ direct effect vs. delayed effect,
484 independent of VOCs used CS, are: (1) a decrease of PER acquisition rate; (2) a decrease of
485 response to CS and a slight increase of response to all odors, on the PER response distribution;
486 and not least (3), an increase of the antennal activity, with the response amplitudes increasing
487 with increasing O₃ mixing ratios (Fig. 3). While the direct effect of O₃ decreases the antennal
488 activity and can affect the learning rate of eucalyptol as CS, the delayed effect of O₃ seems to
489 correlate with both increased antennal activity and increased generalization response: indeed,
490 when exposed to O₃ and conditioned right away with associative learning, exposed bees are

491 tested *one hour after* for recall, which can relate to a transition from direct to delayed O₃ effect
492 (Fig. 3). While the delayed effect of O₃ correlates with an increased generalization, we are
493 aware that we cannot properly conclude about discrimination between CS and NO responses: a
494 discriminative CS⁺/CS⁻ associative learning would have been more appropriate (Sandoz et al.
495 2001). Nonetheless, this simple conditioning already offers a good basis for future studies on
496 the effects of O₃ on learning and memory.

497 This report shows a clear effect of O₃ on detection and perception of VOCs by honey bees. The
498 surprising aspect of the O₃ direct effect is the asymmetrical effect between eucalyptol and R-
499 linalool. This can be interpreted as a subtle effect on olfactory coding, at the antennal lobe level
500 (Sachse et al. 1999, Paoli & Galizia et al. 2021). Every VOC detected at the antennal level
501 activates a different set of olfactory receptor neurons, which in turn will activate specific sets
502 of glomeruli (Deisig et al. 2006): each VOC has its own activation pattern at the antennal lobe
503 level. The biphasic effect of O₃ (direct and delayed) on the antennal activity can be transferred
504 onto the glomeruli activity. The generalization of odors is asymmetrical, meaning that
505 responding to odor A while being conditioned to odor B is not equal to responding to odor B
506 while being conditioned to odor A (Sandoz et al. 2001, Paoli & Galizia 2021). Hence, we can
507 underline an asymmetrical effect of generalization between eucalyptol and R-linalool, which
508 could be related to an O₃-altered glomerular pattern activation during acquisition. Exposed
509 honey bees can learn, even an altered odor, but the least resilient pattern (the most sensitive to
510 O₃ alteration) will be generalized during memory recall.

511 The reported generalization effect of O₃ on olfaction, coupled with a possible effect on vision
512 of the forager honey bee, can impact foraging activity and success, as this has been reported
513 with the effects of pesticides (Weick & Thorn 2002, Decourtye et al. 2004, Mustard et al. 2020).
514 Since foragers are the honey bees that will be the most likely exposed to tropospheric O₃, two
515 directions can stem from this: (1) we need to characterize how forager honey bees are resilient

516 to oxidative stress and air pollution; and (2) other castes of the honey bee colonies, living inside
517 hives, may be much less exposed to ozone. Interestingly, while 500-ppm O₃, up to 1000 ppm,
518 was suggested as a viable inside-hive treatment to control pests and decrease pesticides residues
519 (James 2011), the naturally-occurring mixing ratio of O₃ inside hives has never been tested nor
520 reported yet. Forager bees will rely on multiple signals and cues, not only olfactory ones such
521 as VOCs. While the effects of O₃ on plants and VOCs can lead to disturbed emissions and alter
522 the integrity of the chemical signal (Peñuelas & Staudt 2010, Jürgens & Bischoff 2017,
523 Dubuisson et al. 2022), bees may still be able to forage relying on different salient cues such as
524 shapes and colors for navigation, flower approach and recognition (Chittka & Raine 2006,
525 Raine & Chittka 2007, Dötterl & Vereecken 2010, Blande 2021). The association between floral
526 bouquet and nectar/pollen reward can be affected as the O₃ may level all olfactory cues. From
527 the forager standpoint, the olfactory landscape under tropospheric O₃ exposure can appear
528 “flatten”, that is to say, less salient and less heterogenous (Jürgens & Bischoff 2017, Conchou
529 et al 2019). To put it another way, every odor will smell the same for the foragers affected by
530 O₃. Although this situation should *not* prevent honey bees from foraging, they might do so less
531 efficiently.

532 In this changing environment, the resilience to oxidative stress from air pollution may play a
533 key role in the adaptation of pollinators' behaviors. While different sensory modalities may be
534 affected, such as olfaction –as reported in this study– and perhaps vision as well, pollinators
535 will either rely on different cues, reinforcing the prevalence of one unaffected modality, or will
536 adapt to an equally changing environment (Leonard et al. 2019, Blande 2021). The pollination
537 services provided by pollinators are already affected by different factors (Goulson et al. 2015,
538 Potts et al. 2016). Now, the follow-up questions are: how fast does the pollution arising from
539 human activities affect the environment? And from that, how resilient the pollinators can be, to
540 continue providing such services? Ryalls and colleagues (2022) recently provided insights for

541 the latter, reporting a striking decrease of in-field pollinator counts and flower visits linked to
542 air pollution from ozone and diesel exhaust. This might reflect the effects of air pollution on
543 pollinators' olfaction and vision, or more broadly the ability to navigate their environment. Air
544 pollution affects the in-field presence of the main groups of pollinators (Ryalls et al. 2022),
545 potentially leading to harmful effects to pollination services.

546

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548

549 Contributions: FD: Investigation, Methodology, Supervision, Formal analysis, Data curation,
550 Validation, Visualization, Writing (original draft, review and editing). LG: Investigation,
551 Methodology, Formal analysis, Data curation, Writing (review & editing). PC: Investigation,
552 Methodology, Writing (review & editing). BL: Methodology, Supervision, Validation, Writing
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568

569 **6. Bibliography/References.**

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792

793 **7. Legends of figures and tables**

794

795 **Figure 1. Olfactory learning and recall tests of O₃-exposed bees, with eucalyptol used as**
796 **conditioned stimulus (CS⁺).** After one hour of O₃ exposure, bees were trained either right after
797 exposure (*O₃ direct effect: A-C*) or after a 2-hr rest (*O₃ delayed effect: D-F*). Acquisition phase with five
798 trials (T1 to T5) of O₃-exposed bees (**A & D**); black line, control group; blue line, 80-ppb exposure; yellow
799 line, 120-ppb exposure; and orange line, 200-ppb exposure. The same color code is used for the next
800 panels and figures; numbers between parentheses indicate group size. (**B & E**) Recall test and response
801 to a new odor (NO, here R-linalool tested) one hour after the last acquisition trial T5. (**C & F**) Distribution
802 of PER response categories for each treated group. Four categories total for each combination of
803 response, either CS alone (CS⁺) or NO alone (CS⁻/NO⁺), or both ('All Odors'), or none ('no PER'). Stars
804 indicate statistical differences against control groups while dots indicate tendencies (FDR adj.p-values:
805 * < 0.05; ** < 0.01; *** < 0.005; ● < 0.1).

806

807 **Figure 2. Olfactory learning and recall tests of O₃-exposed bees, with R-linalool used as**
808 **conditioned stimulus (CS⁺).** After one hour of O₃ exposure, bees were trained either directly after
809 exposure (*O₃ direct effect: A-C*) or after a 2-hr rest (*O₃ delayed effect: D-F*). Acquisition phase with five
810 trials (T1 to T5) of O₃-exposed bees (**A & D**). This figure uses the same color code as Figure 1, numbers
811 between parentheses indicate group size. (**B & E**) Recall test and response to a new odor (NO, here
812 eucalyptol tested) one hour after the last acquisition trial T5. (**C & F**) Distribution of PER response
813 categories for each treated group. Four categories total for each combination of response, either CS
814 alone (CS⁺) or NO alone (CS⁻/NO⁺), or both ('All Odors'), or none ('no PER'). Stars indicate statistical
815 differences against control groups while dots indicate tendencies (FDR adj.p-values: * < 0.05; *** < 0.005;
816 ● < 0.1).

817

818 **Figure 3. Electroantennographic recordings of O₃-exposed bees, with eucalyptol stimulations (A-**
819 **B) and R-linalool stimulations (C-D).** Antennae of exposed honey bees (n=26 for each bar) were
820 collected and mounted either directly after O₃ exposure (*O₃ direct effect: A & C*) or after a 2-hr rest (*O₃*
821 *delayed effect: B & D*). This figure uses the same color code as previous figures. For each panel, stars
822 indicate statistical differences from control (* FDR adj. *p*-value < 0.05), while a dot indicates a tendency
823 but not statistically different from control (• FDR adj. *p*-value < 0.10).

824

825 **Table 1. Results of the GLMMs with binomial distribution, performed on acquisition and recall**
826 **phases of PER conditioning with Eucalyptol used as CS, and separated by exposure protocols**
827 **(direct vs delayed effect).** For the acquisition phase, 'O₃ mixing ratio' and 'Acquisition trial' were used
828 as GLMM factors, along with their interaction 'Mix ratio * Trial'. For the recall phase, 'O₃ mixing ratio' and
829 'Odor tested' were used as GLMM factors, along with their interaction 'Mix ratio * Odor'. F-values
830 correspond to statistical indicators related to inter- and intra-group degrees of freedom (df), giving a
831 calculated *p*-value for each factor and interaction. *P*-values lower than the 0.05 alpha-level are
832 statistically significant and indicated by the following symbols (* ≤ 0.050, ** < 0.010, *** < 0.005, ns >
833 0.050), while values higher than 0.05 are not statistically significant (ns > 0.050). This table is related to
834 results reported in Figure 1.

835

836 **Table 2. Results of the GLMMs with binomial distribution, performed on acquisition and recall**
837 **phases of PER conditioning with R-linalool used as CS, and separated by exposure protocols**
838 **(direct vs delayed effect).** For the acquisition phase, 'O₃ mixing ratio' and 'Acquisition trial' were used
839 as GLMM factors, along with their interaction 'Mix ratio * Trial'. For the recall phase, 'O₃ mixing ratio' and
840 'Odor tested' were used as GLMM factors, along with their interaction 'Mix ratio * Odor'. F-values
841 correspond to statistical indicators related to inter- and intra-group degrees of freedom (df), giving a
842 calculated *p*-value for each factor and interaction. *P*-values lower than the 0.05 alpha-level are
843 statistically significant and indicated by the following symbols (* ≤ 0.050, ** < 0.010, *** < 0.005), while
844 values higher than 0.05 are not statistically significant (ns > 0.050). This table is related to results
845 reported in Figure 2.

846

847 **Table 3. Results of ANOVAs performed on EAG antennal activity responses following eucalyptol**
848 **or R-linalool stimulations.** For each VOC tested, the 'O₃ mixing ratio' factor was tested. Each ANOVA
849 returned a statistical indicator (F-value) related to inter- and intra-group degrees of freedom (df), giving
850 a calculated p-value. P-values lower than the 0.05 alpha-level are statistically significant and indicated
851 by the following symbols (* \leq 0.050, *** $<$ 0.005), while values higher than 0.05 are not statistically
852 significant (ns $>$ 0.050). This table is related to results reported in Figure 3.

853

854 **8. Supplementary Information**

855

856 *8.1. Supplementary Table 1*

857 Complete data used for Figures 1 & 2 on PER responses (sheet A), and Figure 3 on EAG
858 activity (sheet B).

859 *8.2. Supplementary Table 2*

860 Complete statistical numbers for GLMM on acquisition (sheet A) and on recall (sheet B),
861 for PER response distribution (sheet C), and for EAG analyses (sheet D).

862

Figure 1.

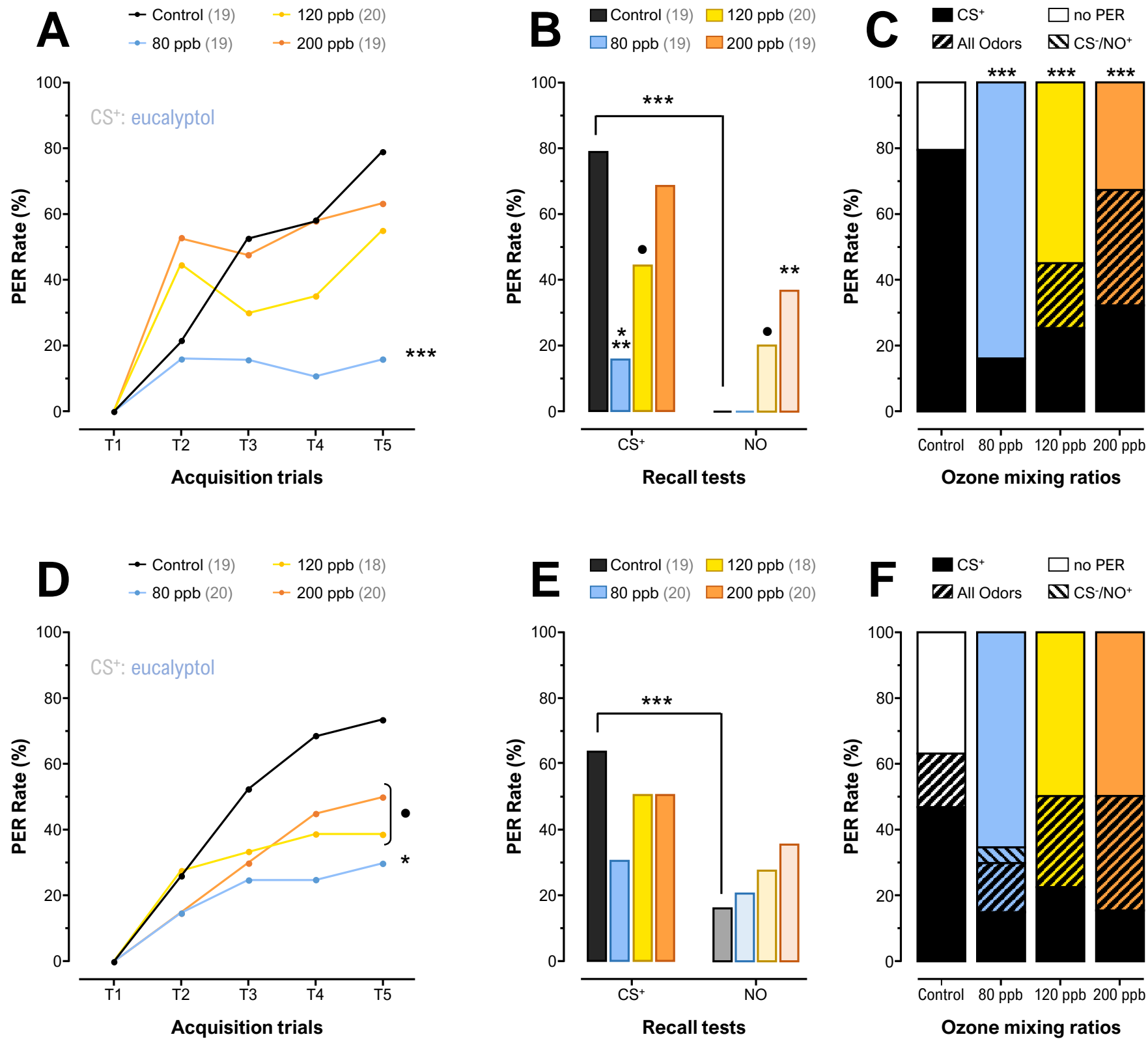


Figure 2.

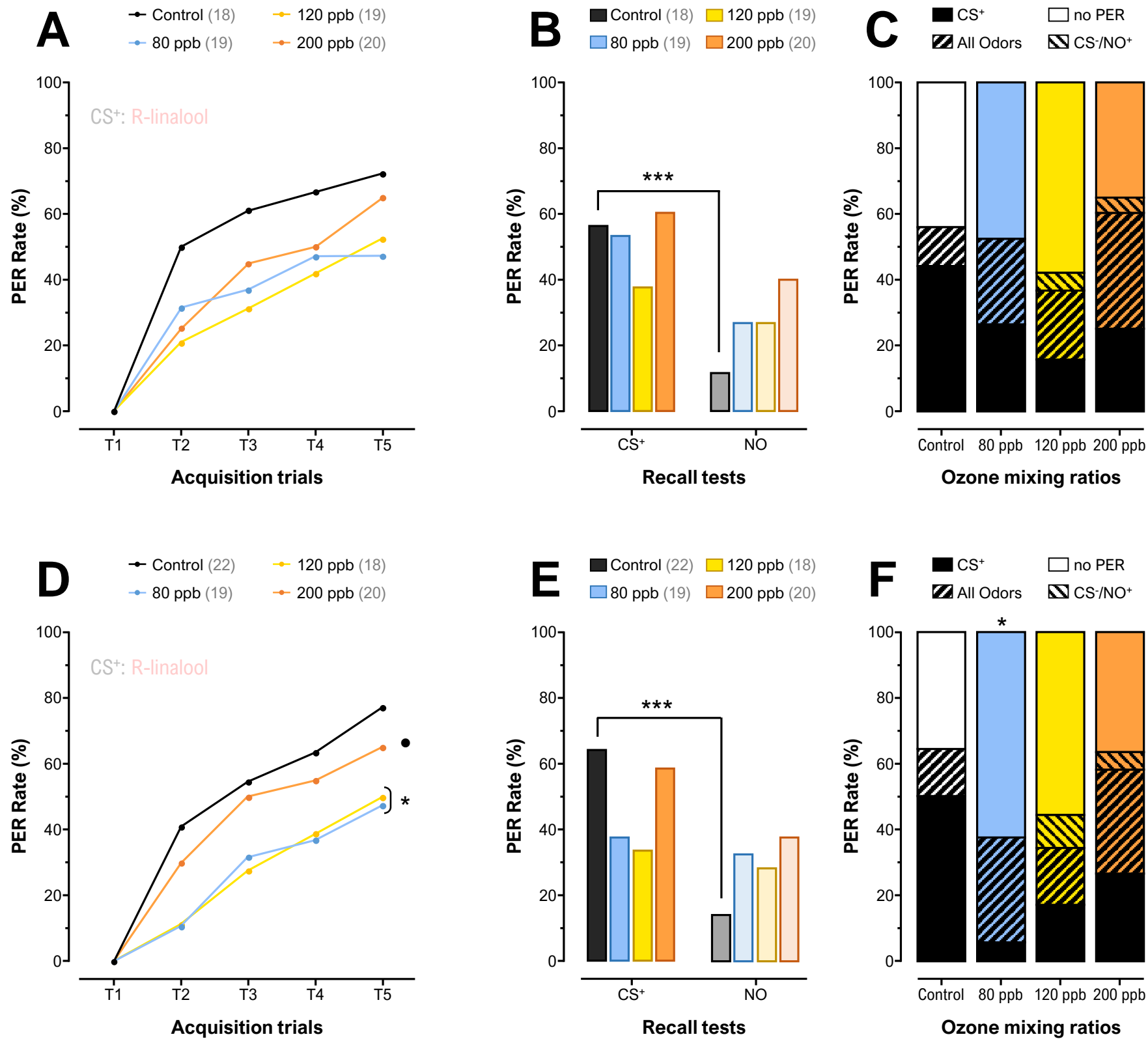


Figure 3.

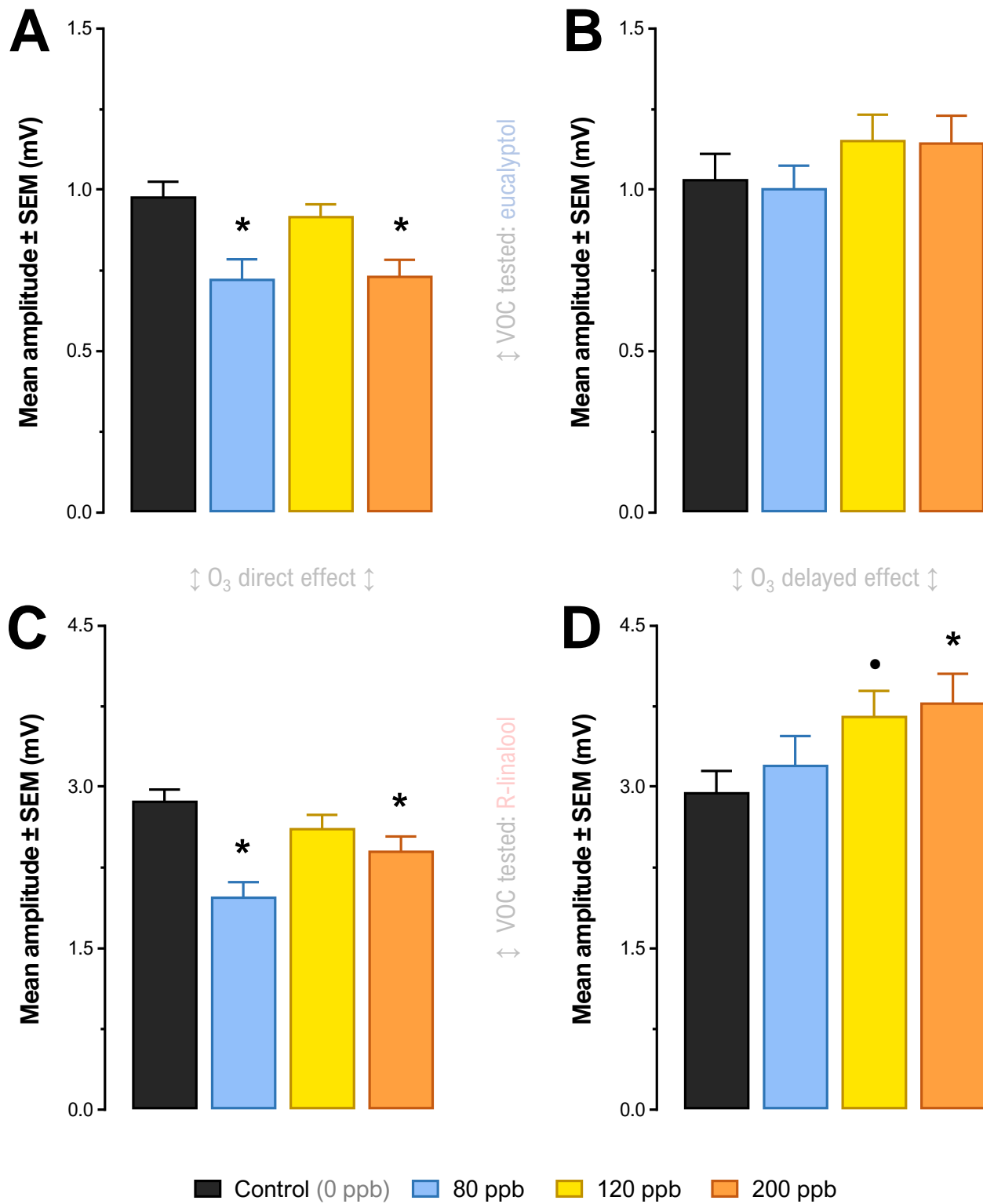


Table 1. Results of the GLMMs with binomial distribution, performed on acquisition and recall phases of PER conditioning with Eucalyptol used as CS, and separated by exposure protocols (direct vs delayed effect). For the acquisition phase, 'O₃ mixing ratio' and 'Acquisition trial' were used as GLMM factors, along with their interaction 'Mix ratio * Trial'. For the recall phase, 'O₃ mixing ratio' and 'Odor tested' were used as GLMM factors, along with their interaction 'Mix ratio * Odor'. F-values correspond to statistical indicators related to inter- and intra-group degrees of freedom (df), giving a calculated p-value for each factor and interaction. P-values lower than the 0.05 alpha-level are statistically significant and indicated by the following symbols (* ≤ 0.050, ** < 0.010, *** < 0.005, ns > 0.050), while values higher than 0.05 are not statistically significant (ns > 0.050). This table is related to results reported in Figure 1.

Eucalyptol as CS		Acquisition (Learning Phase)			Recall (Memory Phase)			
		O ₃ Exposure	GLMM factors	F value	df	p-value	GLMM factors	F value
One hour (direct effect)	O ₃ Mixing Ratio	5.385	3,365	0.001 ***	O ₃ Mixing Ratio	4.323	3,146	0.006 **
	Acquisition Trial	6.857	4,365	< 0.001 ***	Odor tested	14.909	1,146	< 0.001 ***
	Mix Ratio * Trial	0.965	12,365	0.482 ns	Mix Ratio * Odor	2.159	3,146	0.095 ns
One hour + 2h rest (delayed effect)	O ₃ Mixing Ratio	2.842	3,365	0.038 *	O ₃ Mixing Ratio	0.954	3,146	0.416 ns
	Acquisition Trial	8.437	4,365	< 0.001 ***	Odor tested	8.932	1,146	0.003 ***
	Mix Ratio * Trial	0.394	12,365	0.965 ns	Mix Ratio * Odor	1.032	3,146	0.380 ns

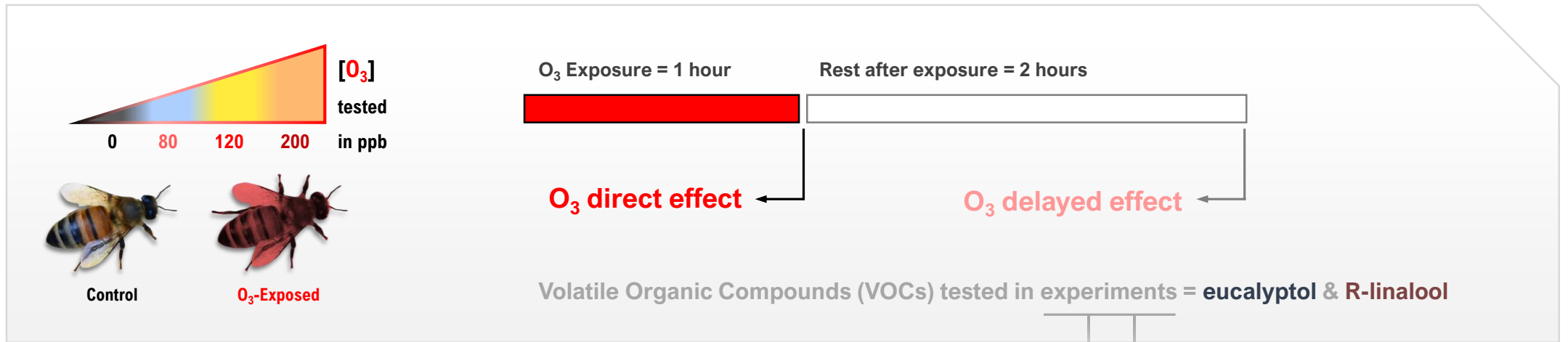
Table 2. Results of the GLMMs with binomial distribution, performed on acquisition and recall phases of PER conditioning with R-linalool used as CS, and separated by exposure protocols (direct vs delayed effect). For the acquisition phase, 'O₃ mixing ratio' and 'Acquisition trial' were used as GLMM factors, along with their interaction 'Mix ratio * Trial'. For the recall phase, 'O₃ mixing ratio' and 'Odor tested' were used as GLMM factors, along with their interaction 'Mix ratio * Odor'. F-values correspond to statistical indicators related to inter- and intra-group degrees of freedom (df), giving a calculated p-value for each factor and interaction. P-values lower than the 0.05 alpha-level are statistically significant and indicated by the following symbols (* ≤ 0.050, ** < 0.010, *** < 0.005), while values higher than 0.05 are not statistically significant (ns > 0.050). This table is related to results reported in Figure 2.

R-Linalool as CS		<u>Acquisition (Learning Phase)</u>				<u>Recall (Memory Phase)</u>				
		O₃ Exposure	GLMM factors	F value	df	p-value	GLMM factors	F value	df	p-value
One hour (direct effect)	O ₃ Mixing Ratio	1.951	3,360	0.121	ns	O ₃ Mixing Ratio	1,323	3,144	0.269	ns
	Acquisition Trial	10.038	4,360	< 0.001	***	Odor tested	10,282	1,144	0.002	***
	Mix Ratio * Trial	0.174	12,360	0.999	ns	Mix Ratio * Odor	0,924	3,144	0.431	ns
One hour + 2h rest (delayed effect)	O ₃ Mixing Ratio	3.225	3,370	0.023	*	O ₃ Mixing Ratio	0,869	3,148	0.459	ns
	Acquisition Trial	11.670	4,370	< 0.001	***	Odor tested	6,997	1,148	0.009	**
	Mix Ratio * Trial	0.213	12,370	0.998	ns	Mix Ratio * Odor	1,870	3,148	0.137	ns

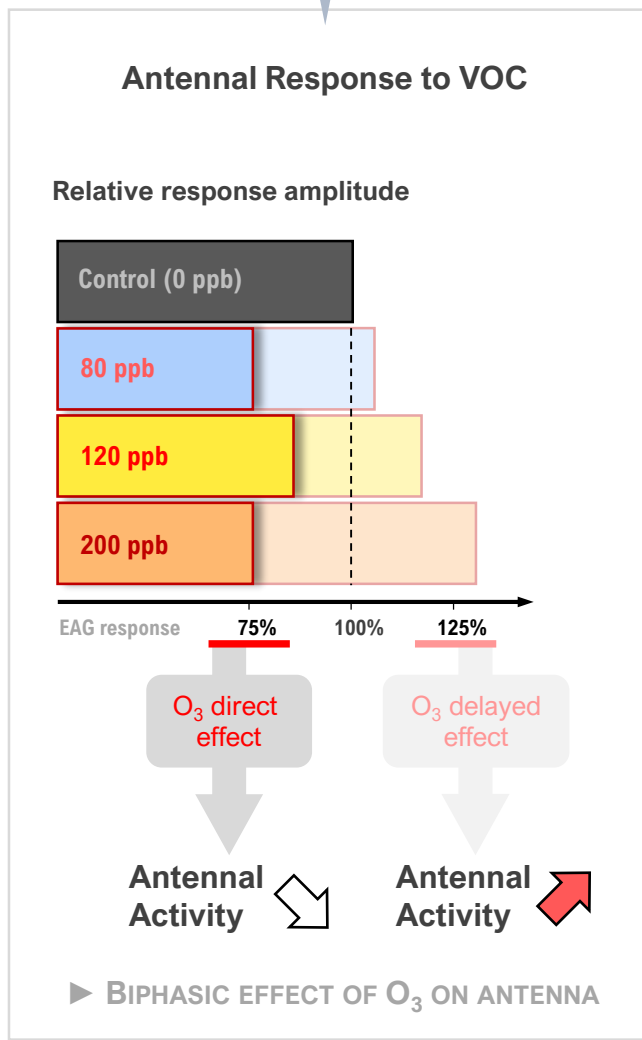
Table 3. Results of ANOVAs performed on EAG antennal activity responses following eucalyptol or R-linalool stimulations. For each VOC tested, the 'O₃ mixing ratio' factor was tested. Each ANOVA returned a statistical indicator (F-value) related to inter- and intra-group degrees of freedom (df), giving a calculated p-value. P-values lower than the 0.05 alpha-level are statistically significant and indicated by the following symbols (* ≤ 0.050, *** < 0.005), while values higher than 0.05 are not statistically significant (ns > 0.050). This table is related to results reported in Figure 3.

VOC tested	ANOVA	Ozone exposure: 1 hour			Ozone exposure: 1 hour + 2-hour rest			
		F value	df	p-value	ANOVA	F value	df	p-value
Eucalyptol	O ₃ Mixing Ratio	6.230	3,100	< 0.001 ***	O ₃ Mixing Ratio	0.928	3,100	0.430 ns
R-Linalool	O ₃ Mixing Ratio	8.080	3,100	< 0.001 ***	O ₃ Mixing Ratio	2.710	3,100	0.049 *

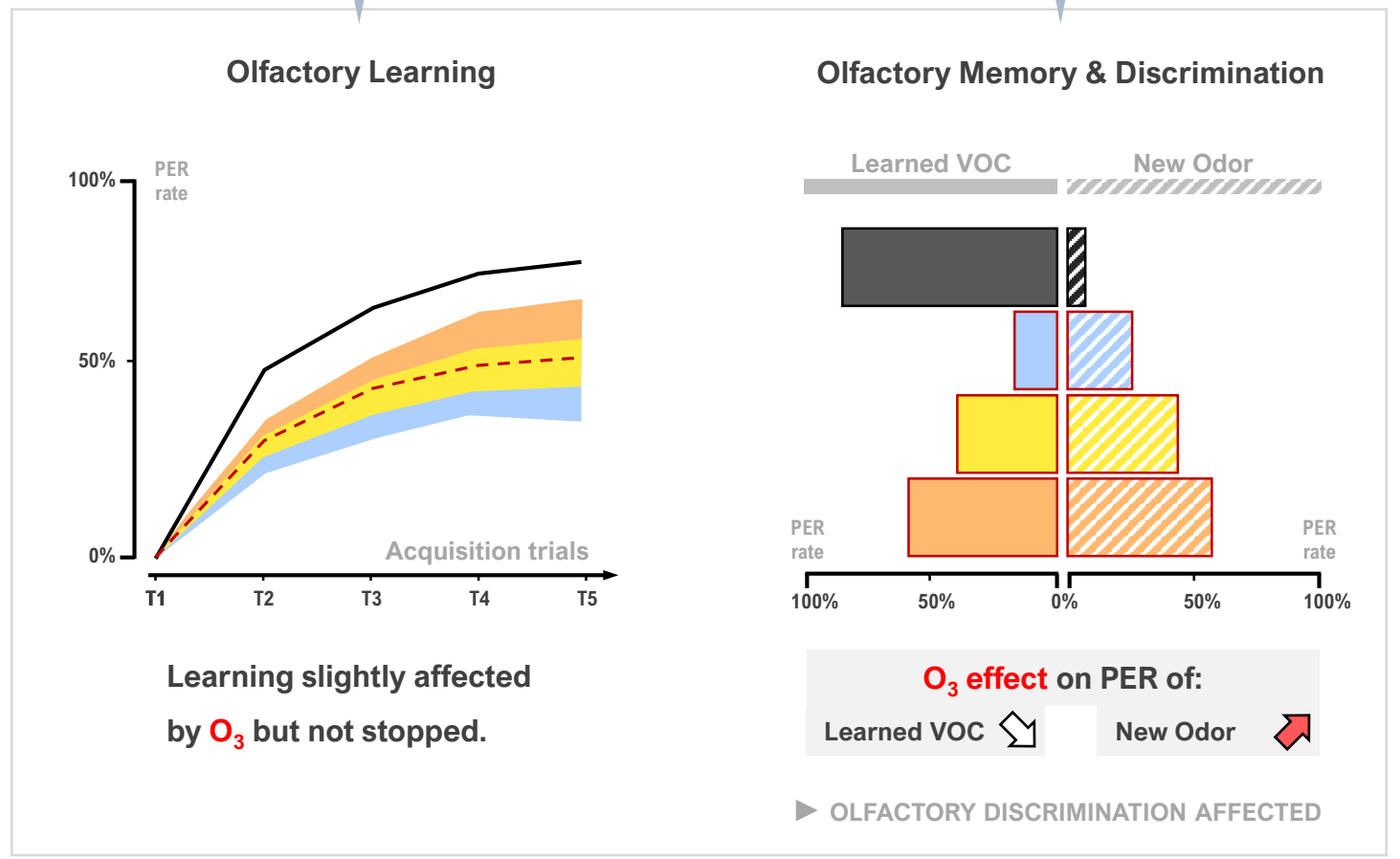
IMPACTS OF OZONE (O_3) ON OLFACTORY DETECTION AND PERCEPTION OF HONEY BEE *Apis mellifera*



EAG RECORDINGS



PER CONDITIONING (NO DIFF. BETWEEN DIRECT AND DELAYED EFFECTS)



► EFFECT OF O_3 ON HONEY BEE OLFACTORY GENERALIZATION