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► **To cite this version:**

Fabien Démares, Laëtitia Gibert, Benoît Lapeyre, Pierre Creusot, D Renault, et al.. Ozone exposure induces metabolic stress and olfactory memory disturbance in honey bees. *Chemosphere*, 2024, 346, pp.140647. 10.1016/j.chemosphere.2023.140647 . hal-04338015

HAL Id: hal-04338015

<https://hal.science/hal-04338015>

Submitted on 29 Feb 2024

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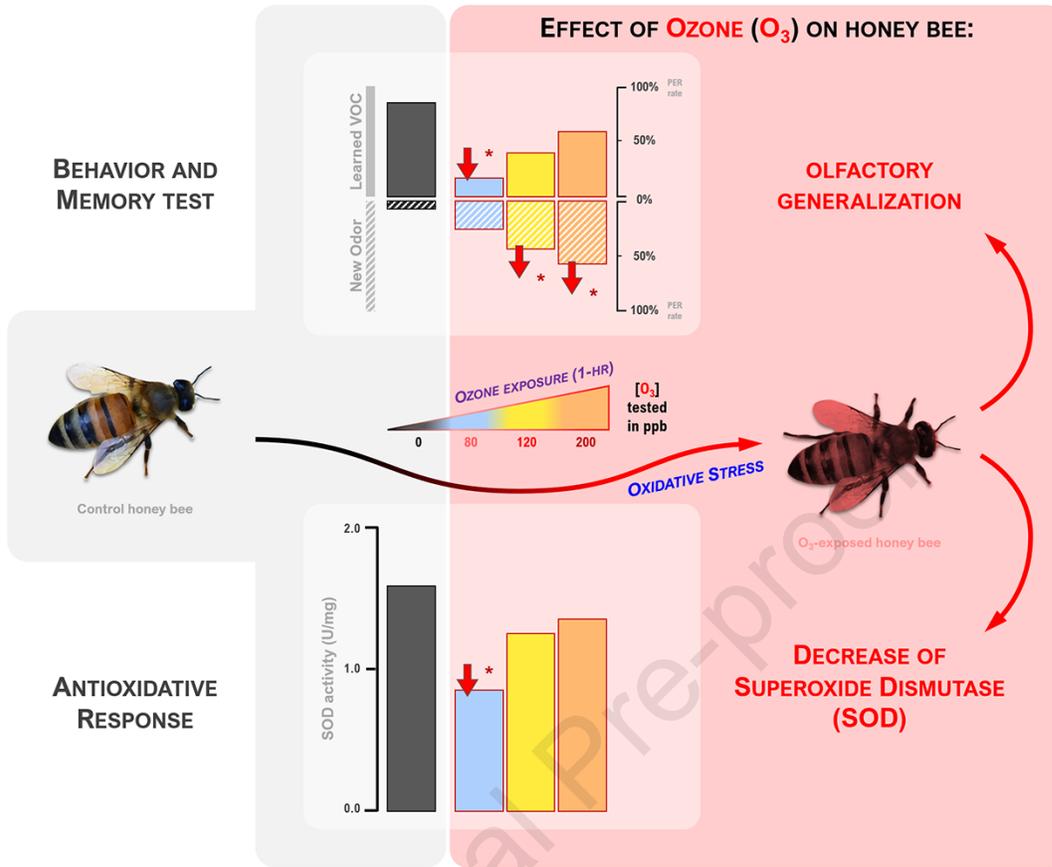
RESEARCH PAPER – CHEMOSPHERE (MS CHEM123733-R2)

Ozone exposure induces metabolic stress and olfactory memory disturbance in honey bees.

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Author Contributions Statement

Contributions: FD: Investigation, Methodology, Supervision, Formal analysis, Data curation, Validation, Visualization, Writing (original draft, review and editing). LG: Investigation, Methodology, Formal analysis, Data curation, Writing (review & editing). PC: Investigation, Methodology, Writing (review & editing). BL: Methodology, Supervision, Validation, Writing (review & editing). DR: Resources, Supervision, Formal analysis, Validation, Writing (original draft, review & editing). MP: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Formal analysis, Validation, Visualization, Writing (original draft, review and editing).



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2

3 **Ozone exposure induces metabolic stress and olfactory memory disturbance in**
4 **honey bees.**

5

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16

17 **Abbreviations.** AO; antioxidant; EAG: electroantennography; GC: Gas Chromatography;
18 GPX: Glutathione Peroxidase; O₃: ozone; ORN: Olfactory Receptor Neuron; PER: Proboscis
19 Extension Reflex; SOD: Superoxide Dismutase; TAC: Total Antioxidant Capacity; VOCs:
20 volatile organic compounds.

21

22 *Word count – revised R2: Abstract (297 words), Main body with figure legends (6908 words).*

23

24 **Abstract**

25 Human activities, urbanization, and industrialization contribute to pollution that affects climate
26 and air quality. A main atmospheric pollutant, the tropospheric ozone (O₃), can damage living
27 organisms by generating oxidative radicals, causing respiratory problems in humans and
28 reducing yields and growth in plants. Exposure to high concentrations of O₃ can result in
29 oxidative stress in plants and animals, eventually leading to substantial ecological
30 consequences. Plants produce volatile organic compounds (VOCs) emitted in the environment
31 and detected by pollinators (mainly by their antennae), foraging for nutritious resources. Several
32 pollinators, including honey bees, recognize and discriminate flowers through olfactory cues
33 and memory. Exposure to different concentrations of O₃ was shown to alter the emission of
34 floral VOCs by plants as well as their lifetime in the atmosphere, potentially impacting plant-
35 pollinator interactions. In this report, we assessed the impacts of exposure to field-realistic
36 concentrations of O₃ on honey bees' antennal response to floral VOCs, on their olfactory recall
37 and discriminative capacity and on their antioxidant responses. Antennal activity is altered
38 depending on VOCs structure and O₃ concentrations. During the behavioral tests, we first check
39 consistency between olfactory learning rates and memory scores after 15 minutes. Then bees
40 exposed to 120 and 200 ppb of ozone do not exert specific recall responses with rewarded VOCs
41 90 minutes after learning, compared to controls whose specific recall responses were consistent
42 between time points. We also report for the first time in honey bees how the superoxide
43 dismutase enzyme, an antioxidant defense against oxidative stress, saw its enzymatic activity
44 rate decreases after exposure to 80 ppb of ozone. This work tends to demonstrate how hurtful
45 can be the impact of air pollutants upon pollinators themselves and how this type of pollution
46 needs to be addressed in future studies aiming at characterizing plant-insect interactions more
47 accurately.

48

49 **Keywords.** Lavender; VOC; Olfactory generalization; Oxidative stress; Superoxide
50 Dismutase; Hormesis.

51 1. Introduction

52 Pollution arising from human activities, urbanization and industrialization, constitutes a
53 major environmental hazard affecting climate and air quality (Baklanov *et al.* 2016, W.H.O.
54 Occupational & Environmental Health, 2006). Among atmospheric pollutants, tropospheric
55 ozone (O₃) plays an important part in global warming issues, with its concentration steadily
56 increasing for the past four decades, and predicted to keep increasing over the next 30 years
57 (Cooper *et al.* 2014, Gaudel *et al.* 2018, Shen *et al.* 2016, Sicard *et al.* 2017, Yeung *et al.* 2019).
58 Ozone is a powerful oxidant, generating highly-reactive oxidative radicals, causing subcellular
59 damages (DNA, lipids or proteins); eventually, this creates an imbalance in antioxidant
60 mechanisms, resulting in oxidative stress (OS) in many organisms (Chaitanya *et al.* 2016). It
61 leads to respiratory problems for humans, lung capacity deficiency or increased inflammatory
62 response, leading to asthma (Nuvolone *et al.* 2018); it also decreases growth and yields in
63 plants, leading to increased susceptibility to environmental stressors (Brosset *et al.* 2020,
64 Emberson 2020, Jurán *et al.* 2021). Exposure to O₃ and the resulting OS may alter emission and
65 detection of all the signals involved in species recognition. This can negatively impact the
66 interactions between plants and animals, and can lead to major ecological consequences.

67 To interact with other organisms, plants produce complex blends of volatile organic
68 compounds (VOCs) (Ueda *et al.* 2012): one major interaction is the attraction of pollinators
69 through floral VOCs to ensure plant reproduction (Abbas *et al.* 2017). However, exposure to
70 elevated concentrations of ozone can alter the emission of floral VOCs by plants (Saunier &
71 Blande 2019, Otieno *et al.* 2023) as well as floral VOCs once they are in the atmosphere (Farré-
72 Armengol *et al.* 2016, Dubuisson *et al.* 2022, Cook *et al.* 2020, Langford *et al.* 2023). Since
73 pollinators rely on the proportion of VOCs in floral blends to have specific flower recognition
74 (Raguso 2008, Proffit *et al.* 2020), this can drastically impact pollinator attraction and
75 consequent plant-pollinator interactions (Farré-Armengol *et al.* 2016, Langford *et al.* 2023,

76 Saunier *et al.* 2023). Nonetheless, a pollinator can learn to associate an ozone-altered floral odor
77 with a reward which would theoretically mitigate the adverse effects that ozone can have on
78 plant-pollinator interactions (Cook *et al.* 2020). Yet, these considerations do not take into
79 account effects of ozone on perception of sensory cues, and on learning and memory abilities
80 of pollinators. But to which extent does ozone affect behavioral responses of insect pollinators?
81 Short exposure to elevated O₃ concentrations affects detection of floral VOCs by pollinators as
82 well as their innate attraction to this signal (Vanderplanck *et al.* 2021a). It was also
83 hypothesized that O₃ affected VOCs perception of honey bees, *Apis mellifera* (Dötterl *et al.*
84 2016). Other behavioral responses were more thoroughly studied and reported that O₃ exposure
85 affects the motility and the recover physiology of fig wasps *Blastophaga psenes* (Vanderplanck
86 *et al.* 2021b). Many reports observed effects of O₃ exposure on different behaviors, but only a
87 few are interested in physiological bases and structures affected by such exposure.

88 Coupled with their innate attraction to VOCs and their foraging activity, various pollinators
89 rely on learning and perception of sensory cues to find and track resources (Conchou *et al.*
90 2019, Liu *et al.* 2022 *et al.*). Amongst several generalist pollinators, honey bees were
91 extensively studied and known for their ability to navigate their environment and search for
92 floral resources (pollen and nectar) from many different plants (Robertson, 1925). They manage
93 to recognize and discriminate flowers through vision, olfaction, and memory (Chittka & Raine
94 2006, Dötterl & Vereecken 2010). Floral VOCs stimulate insects' olfactory pathway, starting
95 with antennal *detection* through olfactory receptor neurons (Kaissling 1971, Jung *et al.* 2014),
96 then to *perception* at higher levels in the brain, through antennal lobes and mushroom bodies
97 (Dujardin 1850, Strausfeld 2002). Associating the olfactory pathway activation by floral VOC
98 and presentation of sugar rewards leads to the proboscis extension reflex (PER), the cornerstone
99 of classical conditioning among bees (Takeda 1961, Bitterman *et al.* 1983). Associative
100 conditioning is the core of olfactory learning procedure, eventually establishing olfactory

101 memory (Menzel & Giurfa 2001). In a previous study, we reported that a short exposure of
102 honey bees to ozone could modify their antennal activity in response to very common floral
103 VOCs as well as their ability to learn these VOCs (Démarets *et al.* 2022). These common floral
104 compounds were linalool and 1-8 cineol (eucalyptol) and are largely found in several plant
105 families (Knudsen *et al.* 2006). Are these O₃ effects still observed using different VOCs, less
106 abundant in the environment but still relevant to pollinators? To test this, we used compounds
107 extracted from lavender, and we hypothesized that similar effects as reported previously (a
108 decreased amplitude of antennal response) should be observed upon *detection* depending on the
109 VOCs structure and O₃ concentrations. We also aim to evaluate whether exposure of bees to
110 ozone can alter a well-established olfactory memory of more specific floral compounds by
111 assessing *perception* through olfactory memory tests. We hypothesized that, under O₃
112 influence, olfactory recall rates would decrease and generalization (unspecific response to non-
113 associated odors) would increase. Moreover, describing further impacts of ozone on pollinators'
114 physiology means going deeper into molecular mechanisms behind O₃-induced OS; but
115 virtually no study reported such a step yet. Hence, we checked if OS, through O₃ exposure,
116 would change any antioxidant (AO) parameter, especially AO enzyme rates. Antioxidant
117 enzymes are molecules protecting cells from oxidative damage generated by reactive oxygen
118 species (ROS), expressed in several organisms, including insects (Felton & Summers 1995).
119 These enzymes are essential for maintaining cellular homeostasis and were measured in many
120 insects, under different OS modalities like cold tolerance, heavy metal soil-poisoning or low
121 level of oxygen (Lalouette *et al.* 2011, Lawniczak *et al.* 2013, Renault *et al.* 2016). So far, no
122 AO enzymatic measurements were reported on honey bee under ozone influence. Thus, we
123 present the first attempt to assess total AO capacity and two specific AO enzymes (glutathione
124 peroxidase and superoxide dismutase) of honey bees under OS. Here we can only hypothesize

125 that with higher O₃ concentrations, we would measure higher AO enzymatic levels to counteract
126 OS.

127

128 **2. Methodology**

129 *2.1. Animal samplings*

130 Honey bees (*Apis mellifera*) were collected at the experimental field's apiary (CNRS
131 campus, 43°38'19N, 3°51'49E), from LabEX CeMEB platform (Centre Méditerranéen pour
132 l'Environnement et la Biodiversité) from three different hives. Entrances were blocked
133 momentarily to identify returning foragers with their loaded pollen baskets. Foragers were
134 collected in clear plastic tubes and placed shortly under ice. While asleep, bees were
135 individually placed and attached to 3-cm high Teflon holders. Once awoken, they were briefly
136 given sugar water (50% w/w sucrose) to recover from cold anesthesia, and placed in an
137 incubator at 33.5°C ±0.2°C, RH>65% to rest overnight. Experiments were conducted from
138 April to November 2021.

139

140 *2.2. Chemical preparation and analyzes*

141 We used lavender, an emblematic plant of the Mediterranean region, as a model of a
142 generalist plant that is widely visited by bees. As the VOCs emitted by lavender and detected
143 by bees have never been described before, we used gas chromatography-mass spectrometry
144 (GC-MS) analyses and electroantennodetection (GC-EAD) recordings to characterize these
145 VOCs (Supplementary Figures S1 & S2, and methods).

146

147 *2.2.1. VOCs used*

148 For electrophysiological recordings (EAG section 2.4) and associative conditionings &
149 memory tests (PER section 2.5), synthetic standards of VOC identified in the previous
150 paragraph were used: camphor (CAS: 464-48-2), and linalyl acetate (CAS: 115-95-7). For each
151 experiment, 10 μ L of VOC preparation was put on a Whatman no.1 paper (20 \times 10mm), tested
152 afterwards on antennas or behaving individuals. The two VOCs, the solvents, and compounds
153 used for Ringer's solutions were all purchased from Sigma-Aldrich (St-Louis, MO, USA).

154

155 2.3. Ozone treatment and concentrations

156 For each procedure, O₃ exposure was performed as previously described (Vanderplanck *et*
157 *al.* 2021a, Démares *et al.* 2022). Bees fixed in holders were placed in a 500-mL glass bottle
158 acting as a fumigation chamber, connected to an ozone analyzer-generator (Model 49i, Thermo
159 Fischer, Franklin, MA, USA), with a pump pushing ozone through Teflon tubes at 1.5 L/min
160 flow rate. Ozone was produced through oxygen photolysis subjected to UV radiation at 185-
161 nm wavelength. For both procedures, we tested four different concentrations: 0 ppb (control
162 group), 80 ppb, 120 ppb, and 200 ppb, similar as previously reported (Vanderplanck *et al.*
163 2021a, Démares *et al.* 2022). These concentrations have field-realistic relevance and correspond
164 to: mean value of O₃ measured during a summer day (80 ppb), peak value reached during this
165 same summer day (120 ppb), and highest O₃ concentration recorded in the South of France
166 during the 2003 heatwave (Vautard *et al.* 2005). To maintain O₃ at a desired concentration and
167 flow rate, air was extracted at the same flow rate, from the fumigation chamber toward the
168 ozone analyzer. To keep the honey bees in a humid environment during exposure, a Whatman
169 paper was imbibed with water and placed in the fumigation chamber. Each treated group was
170 exposed for one hour and tested shortly after.

171

172 2.4. Antennal activity

173 Electroantennographic (EAG) recordings measure amplitudes of depolarization of all active
174 olfactory sensory neurons in response to olfactory stimuli (Roelofs 1984). We previously tested
175 how O₃ exposure affected the antennal response to R-linalool and eucalyptol stimulations
176 (Démarens *et al.* 2022), and we did the same here for camphor and linalyl acetate. After O₃
177 exposure, the right antenna of O₃-exposed individuals was cut and mounted between glass
178 capillaries, filled with Ringer's solution (composition, in mM: NaCl, 131; KCl, 5; CaCl₂, 2;
179 NaHCO₃, 29; pH 7). Capillaries were connected to silver electrodes of an EAG probe. The
180 antenna was placed in a continuous humidified air stream (450mL/min) to keep it from drying.
181 Single VOCs were applied to a Whatman no.1 paper placed in a Pasteur pipette; the tip of this
182 pipette was inserted in a hole in the circulating airflow tube. A short pulse of purified air
183 (500ms, 900mL/min) through the Pasteur pipette released the VOC in the airflow leading to the
184 antenna: this stimulation was recorded by an acquisition controller IDAC-2, and analyzed with
185 GcEad 1.2.5 software (all EAG equipment purchased from Syntech Kirchzarten, Germany).

186 In a preliminary experiment, we performed EAG on unexposed bees to determine the dose-
187 response relationship for each VOC using eight different concentrations (from 0.1 µg/µL to 300
188 µg/µL) of each VOC. Then, for exposed bees, we used a short range of three different
189 concentrations (3, 10 and 30 µg/µL in 100% paraffin solvent) corresponding to the linear
190 ascending phase of the dose-response curves for camphor and linalyl acetate (Supplementary
191 Figure S2 and supplementary methodology). The maximum amplitude of depolarization was
192 recorded for each stimulus. Intervals between two stimulations were at least 45 seconds (up to
193 one minute) for antennal activity to return to a stable baseline. Every antenna went through a
194 balanced randomized sequence of VOC stimulations, with paraffin (negative control) always
195 coming first and last of this sequence, and R-linalool (positive control) coming second and
196 penultimate of the same sequence (not shown in results). The response amplitude of each VOC

197 was adjusted with the subtracted mean paraffin-response from their own sequence. In total we
198 recorded 95 antennae, each of them tested for both VOCs (n=22 to 25 for each group of O₃
199 concentrations exposure).

200

201 2.5. Olfactory conditioning and recall

202 Consequences of O₃ exposure on honey bees' olfactory memory were assessed with
203 classical conditioning through proboscis extension reflex (PER; Bitterman *et al.* 1983). To test
204 the effects of ozone solely on recall phase, we exposed bees after the acquisition training session
205 (see Figure 2A). First, the acquisition phase consisted of learning to associate a neutral odor
206 (later acting as *conditioned stimulus*, CS) with an appetitive reward eliciting PER (acting as
207 *unconditioned stimulus*, US). One trial of training session goes as follows: it starts with CS
208 presentation for 7 seconds, then followed by US presentation (a small drop of sucrose solution)
209 for 5 seconds; both stimuli overlapped for 3 seconds. One training session consists of five trials,
210 with a 1-minute interval (minimum) between trials. This acquisition phase results in memory
211 formation (Gerber *et al.* 1998, Démares *et al.* 2014) which can be tested afterwards with recall
212 tests: CS is presented alone without any reward, to check if the bee recalls the reinforced
213 association by extending its proboscis. In parallel, a new odor (NO) is presented to check for
214 generalization, and to test the specificity of CS response. VOCs used as CS and NO were
215 camphor and linalyl acetate at 30 µg/µL (in 100% ethanol solvent). VOCs were applied on
216 individual Whatman papers before every morning conditioning sessions, left 10 minutes
217 minimum under a ventilation hood in order for the ethanol to evaporate, then placed in small
218 vials and linked to the odor delivery apparatus. When we tested one VOC as CS, the other one
219 was used as NO. For memory tests, VOC stimuli were randomly presented and separated by 1-
220 minute interval. To determine differences between before and after exposure, we tested the

221 olfactory memory after 15 minutes and 90 minutes after the last trial of acquisition phase, when
222 the one-hour O₃ exposure took place in-between these recall tests.

223 Ozone exposures, training sessions and memory tests were performed between late-morning
224 and mid-afternoon (from 11.00AM to 04.30PM) in ventilated boxes. VOCs puffed toward bees
225 were aspirated at the back of the ventilated boxes connected to an active vacuum system (with
226 integrated activated-charcoal filter where all odors were trapped). This was a precaution to
227 ensure that the experimental room remain as olfactory 'neutral' as possible and not saturated
228 with tested VOCs. Bees were fed briefly in the early-morning and late-afternoon (08.30AM and
229 06.30PM): this allowed to check for normal PER to sucrose feeding. Bees not responding to
230 sucrose were discarded from experiments. Bees responding to CS odor at the first acquisition
231 trial were also discarded from analysis. Between all O₃ concentrations exposure, and VOCs
232 tested as CS (N=8 groups), we used 165 adult honey bees for this section (n=19 to 24 bees per
233 group).

234

235 2.6. Determination of total AO capacity and AO enzyme activities

236 To measure the effects of O₃ exposure on honey bee physiology, we tested different
237 parameters linked to AO pathways. After exposing bees to O₃, using the same procedure
238 described above, we measured total protein content as well as total AO capacity (TAC) and the
239 activity of two different enzymes: Glutathione Peroxidase (GPX) and Superoxide Dismutase
240 (SOD). We tested 8 bees (two hives, four bees from each) for each O₃ concentration (0, 80, 120,
241 and 200 ppb) for one-hour exposure, alongside an additional control group referred as *Sham*:
242 bees were handled the same way as the exposed bees minus the fumigation phase. It was a
243 handling control to see if there was an effect of the exposure procedure on enzymatic parameters

244 whatsoever. A total of 40 bees were collected in May 2021, and used for these biochemical
245 tests.

246 Right after exposure, and just before we sacrificed them, we put each individual bee to sleep
247 in a 1.5-mL Eppendorf tube on ice, noted its fresh weight (in mg), and quickly place all tubes
248 at -80°C for at least 24 hours before sample preparation. In all tubes containing one bee each,
249 we added two tungsten beads and 1.5 mL of extraction buffer (HPLC-grade water, Tris 50mM,
250 pH 7.8) for sample grinding. Samples were vortexed for 2 minutes at 30 Hz, kept at 4°C . To
251 separate solid particles from supernatant, we centrifuged samples at 180 g for 10 minutes at
252 4°C . We assessed protein mass (in mg per 100 mg bee weight) through Bradford reaction
253 (Sigma-Aldrich, Ref.B9616500ML) reading absorbance at 595 nm. To determine TAC and the
254 AO enzymes activities, we used molecular kits from Cayman Chemical (Ann Arbor, MI, USA)
255 and followed the manufacturer's instructions for each kit, with minor modifications as reported
256 hereafter.

257

258 2.6.1. Total Antioxidant Capacity (TAC)

259 This test was a first approach to assess if damages resulting from ROS and O_3 exposure
260 were counterbalanced by AO systems, including enzymatic activities. The overall level of
261 counteractivity is named total AO capacity. The procedure is based on Trolox standard
262 oxidation rates by metmyoglobin (Cayman Chemical, Ref.709001). For each bee sample, we
263 took $5\ \mu\text{L}$ of supernatant, added $10\ \mu\text{L}$ metmyoglobin and $150\ \mu\text{L}$ of chromogen. Reaction was
264 initiated by hydrogen peroxide ($40\ \mu\text{L}$). Absorbance was read at 750 nm between 15-20 minutes
265 after reaction initiation. TAC measurements were assessed against the Trolox standard activity
266 (in mM).

267

268 2.6.2. Glutathione Peroxidase (GPX)

269 To test if O₃ exposure had any impact on AO enzymes, we tested the glutathione
270 peroxidase enzymatic level. The procedure is based on GPX standard rate (change in
271 absorbance through time) through coupled glutathione reductase activity and NADPH
272 oxidation reaction (Cayman Chemical, Ref.703102). For each bee sample, we added 20 µL of
273 supernatant to a well containing a [1:1:1] mix of reaction buffer, co-substrate and NADPH, 150
274 µL total (50 µL each). Reaction was quickly initiated by cumene hydroperoxide (20 µL) and
275 incubated at room temperature. Absorbance was read at 340nm, starting 2-3 minutes after
276 reaction initiation and every minute for 5-10 minutes. The result is a difference in absorbances
277 between two time points, divided by time interval. GPX sample measurements were assessed
278 against the GPX standard rate (in µM/min.mL).

279

280 2.6.3. Superoxide Dismutase (SOD)

281 To further examine specific O₃ effects, we also tested enzymatic levels of superoxide
282 dismutase. Here the procedure is based on SOD standard rate and its oxidation through xanthine
283 oxidase reaction (Cayman Chemical, Ref.706002). For each bee sample, we took 10 µL of
284 supernatant, added to a well containing 200 µL of radical detector. Reaction was initiated by
285 xanthine oxidase (20 µL) and incubated at room temperature. Absorbance was read at 440-
286 460nm, from 30 minutes after reaction initiation. SOD sample measurements were assessed
287 against the SOD standard activity (in U/mg).

288

289 2.7. Statistical analyzes

290 For EAG recordings, we performed linear mixed models (LMMs) with a normal distribution
291 on response amplitudes, ‘O₃ concentration’ and ‘VOC concentration’ as fixed factors with their
292 interaction, and ‘bee individual’ and ‘hive ID’ used as random factors. LMMs were separated
293 for each VOC. All LMMs resulted in F-values as statistical indicators and *p*-values for each
294 factor and interaction. Pairwise comparisons between all groups and within groups were
295 conducted using contrasts, and *p*-values were adjusted with False Discovery Rate (FDR)
296 corrections. To clarify, comparisons *between* groups correspond to groups of bees that have
297 been exposed to two different ozone concentrations, but with the same VOC concentration
298 (antennal responses from *different* bees). Comparisons *within* group correspond to responses to
299 different concentrations of the same VOC for *one* group exposed to *one* ozone concentration
300 (antennal responses from the *same* bees).

301 Performances in PER experiments, acquisition and recall phases, were assessed with
302 generalized linear mixed models (GLMMs) with binomial distribution for PER response
303 (binary data as presence/absence) with the following fixed factors: ‘O₃ concentration’ and ‘test
304 trial’ for acquisition phases, and ‘O₃ concentration’ and ‘Odor tested’ (CS.vs.NO) for recall
305 phases, all terms with their interactions. ‘Bee individual’ and ‘hive ID’ were used as random
306 factors. GLMMs were separated for each VOC used as CS. Again, GLMMs resulted in F-values
307 as statistical indicators and *p*-values for each factor and interaction. Pairwise comparisons
308 between all groups were also conducted using contrasts, and *p*-values were FDR-corrected. In
309 addition to memory recall test analyses, the distribution of response categories was assessed.
310 There are only 4 categories of responses to a recall test with two odors: PER responses to CS
311 only (‘CS⁺’), responses to NO only (‘CS⁻/NO⁺’), responses to both odors (‘All’), and no
312 response at all (‘no PER’). For each VOC used as CS, and for both recall tests at 15 and 90
313 minutes, bees were categorized and frequency distributions for each exposed group were
314 compared to the frequency distribution of control group through Pearson’s χ^2 tests (Urlacher *et*

315 *al.* 2017). For post-*hoc* comparisons against control groups, new pairwise χ^2 tests were done
316 and FDR-adjusted.

317 To test physiological parameters, we performed linear mixed models (LMMs) with a normal
318 distribution on physiological rates or measures, ‘O₃ concentrations’ as fixed factor, and ‘hive
319 ID’ as random factor. Pairwise comparisons between all groups were done using contrasts, and
320 *p*-values were FDR-adjusted. All statistical analyses were done using IBM SPSS 26. All data
321 used for every figures are reported in Supplementary Tables 1, while all statistical numbers for
322 every tests are reported in Supplementary Tables 2.

323

324 **3. Results**

325 *3.1. Ozone differentially affects honey bee’s antennal responses*

326 Antennal activities of bees depended on the level of O₃ exposure and on the VOC
327 concentration tested. Both linear models for overall responses were significant whether with
328 camphor stimulations ($F_{11,273}=16.72$; $p<0.001$) or with linalyl acetate ($F_{11,273}=24.53$; $p<0.001$).
329 For both VOCs tested, each fixed factor had a significant effect on the response amplitude, *i.e.*
330 the O₃ concentration (‘O₃ concentration’; VOC=camphor, $F_{3,273}=12.47$, $p<0.001$; VOC=linalyl
331 acetate, $F_{3,273}=22.77$, $p<0.001$), and the VOC concentration (‘VOC concentration’;
332 VOC=camphor, $F_{2,273}=69.33$, $p<0.001$; VOC=linalyl acetate, $F_{2,273}=91.85$, $p<0.001$). The
333 interaction between factors was only significant when linalyl acetate was tested (‘O₃
334 concentration*VOC concentration’; VOC=camphor, $F_{6,273}=1.09$, $p=0.370$; VOC=linalyl
335 acetate, $F_{6,273}=2.36$, $p=0.031$). For ‘VOC concentration’, there were statistical differences in
336 response between VOCs at 3 $\mu\text{g}/\mu\text{L}$, 10 $\mu\text{g}/\mu\text{L}$, and 30 $\mu\text{g}/\mu\text{L}$, from lowest-to-highest response
337 amplitudes (see pairwise comparisons in Supplementary Tables 2A). Furthermore, details of O₃
338 effects on responses to each VOC revealed compelling differences. First, at 3 $\mu\text{g}/\mu\text{L}$ for both

339 VOCs, no statistical difference calculated between O₃-exposed groups (Figure 1). But then,
340 with 10 and 30 µg/µL, O₃ had a differential effect depending on VOC tested.

341 With camphor, the main effect was a significantly lower amplitude response for the 80 ppb
342 O₃-exposed group, first compared to the 200-ppb group at VOC 10 µg/µL (0.320 mV vs 0.518
343 mV, pairwise comparison, $t=3.350$, $p=0.006$), then compared to all three groups –0, 120, and
344 200ppb– at VOC 30µg/µL (0.404 mV), corresponding to at least 35% lower response compared
345 to control and other exposed groups. Also, mean amplitude responses to camphor for control
346 group and 120 ppb exposed group were never statistically different (Figure 1A).

347 With linalyl acetate, the main effect is a significantly higher mean amplitude response for
348 the group exposed to 200 ppb of O₃, compared to control group and 80-ppb group at VOC
349 10µg/µL (control vs 200 ppb: 0.272 mV vs 0.400 mV, pairwise comparison, $t=3.404$, $p=0.002$;
350 80 ppb vs 200 ppb: 0.185mV vs 0.400 mV, pairwise comparison, $t=3.85$, $p=0.001$), and then
351 compared to all the groups at VOC 30µg/µL (0.740 mV), corresponding to, at least, 55%
352 increase in response compared to control and other exposed groups (Figure 1B). For the 80-ppb
353 O₃-exposed group, it had the lowest amplitude response (in similar fashion to the response to
354 camphor), always statistically different from the 120-ppb group, but never different from
355 control group.

356

357 *3.2. Ozone at 80 ppb decreases olfactory recall*

358 To test the effect of O₃ exposure solely on olfactory memory, we ensured that the groups of
359 bees were balanced with similar PER conditioning rates at the last acquisition trial and that their
360 first recall test 15 minutes after –and just before O₃ exposure– had similar results. From this
361 point on, anything that differed at the 90-minute recall test was solely because of the effect of
362 O₃ exposure (Figure 2A).

363 Bees trained to associate camphor as CS reached acquisition rates between 80% and 89.5%,
364 and showed similar performances at the 15-minutes recall tests, with high response rates to CS
365 between groups (81% to 90%), and low response rates to NO (0% to 5.3%) (see Supplementary
366 Results and Figure S3). After 1-hour O₃ exposure, we tested the olfactory recall 90 minutes
367 after acquisition. Groups of bees that were exposed to O₃ all had lower response rates to CS
368 (33.3% to 42.1%) compared to control group (72.7%) (control vs 80 ppb: $t=2.065$, $p=0.079$;
369 control vs 120 ppb: $t=2.803$, $p=0.032$; control vs 200 ppb: $t=2.250$, $p=0.075$), only statistically
370 significant at 120-ppb exposure, and a statistical tendency after FDR-correction for 80-ppb and
371 200-ppb O₃-exposed bees (Figure 2B).

372 Similarly, bees trained to associate linalyl acetate as CS reached acquisition rates between
373 87.5% and 95.2%; then at the 15-minutes recall test, they showed high response rates to CS
374 between groups (83.3% to 95.2%), and low response rates to NO (4.8% to 8.3%)
375 (Supplementary Results and Figure S3). Like camphor used as CS, after 1-hour O₃ exposure
376 when we tested the olfactory recall 90 minutes after acquisition and using linalyl acetate as CS,
377 there were lower responses to CS from all the O₃-exposed groups compared to control group
378 (75.0% vs 40.0%) (control vs 80 ppb: $t=3.214$, $p=0.009$; control vs 120 ppb: $t=2.480$, $p=0.041$;
379 control vs 200 ppb: $t=2.033$, $p=0.086$) statistically significant at 80-ppb and 120-ppb exposure,
380 and a statistical tendency after FDR-correction for 200-ppb O₃-exposed bees (Figure 2C).

381

382 *3.3. Higher ozone concentration increases olfactory generalization*

383 Ozone exposure also had an additional effect on the response to the new odor NO. After
384 exposure, for both VOCs used as CS, O₃-exposed groups had higher response rates to NO
385 compared to control group. Moreover, it was O₃-concentration dependent, the higher the

386 concentration, the higher the response to NO (Figures 2B & 2C). Once again, the effects varied
387 depending on tested VOCs.

388 When camphor was used as CS, responses to NO increased with O₃ concentrations, but
389 there was merely a tendency for the 200-ppb group compared to control (4.5% vs 35.0%)
390 (control vs 200 ppb: $t=2.498$, $p=0.078$ after FDR correction). When linalyl acetate was used as
391 CS, responses to NO were statistically higher for the 120-ppb and 200-ppb groups compared to
392 control (respectively 37.5% and 42.1% vs 4.8%) (control vs 120 ppb: $t=2.825$, $p=0.015$; control
393 vs 200 ppb: $t=2.903$, $p=0.024$).

394 Distributions of PER response categories were also significantly different from control after
395 O₃ exposure. With camphor as CS (Pearson $\chi^2=23.413$, $p=0.005$), almost all distributions from
396 O₃-exposed groups were significantly different from control responses' distribution (control vs
397 80 ppb: $\chi^2=6.003$, $p=0.111$; control vs 120 ppb: $\chi^2=12.984$, $p=0.005$; control vs 200 ppb:
398 $\chi^2=13.602$, $p=0.005$). Proportion of response to CS only (CS⁺) decreased with the increase in
399 O₃ concentration, while the proportion of response to 'All Odors' increased (Figure 2B). As
400 with linalyl acetate as CS (Pearson $\chi^2=24.866$, $p=0.003$), all distributions from O₃-exposed
401 groups were significantly different from control responses' distribution (control vs 80 ppb:
402 $\chi^2=12.245$, $p=0.007$; control vs 120 ppb: $\chi^2=14.626$, $p=0.003$; control vs 200 ppb: $\chi^2=15.085$,
403 $p=0.005$). With the increase in O₃ concentration, the proportion of response to CS only (CS⁺)
404 decreased very slightly, while the proportion of response to 'All Odors' increased; in addition,
405 the proportion of response to NO only (CS⁻/NO⁺) also appeared in all exposed groups, while
406 absent from control (Figure 2C).

407

408 3.4. Ozone and its effects on physiological parameters

409 3.4.1. Bee mass and amount of protein

410 We measured each bee individually after O₃ exposure and right before sacrificing them.
411 Although there was a 10-mg difference in fresh mass among experimental groups exposed to
412 80 and 120 ppb O₃ (above 100-mg average) and group exposed to 0 and 200 ppb O₃ and the
413 sham group (93-mg average) (Figure 3A), it was not statistically significant (LMM ‘ozone
414 concentration’, $F_{4,35}=1.340$, $p=0.275$). When assessing protein mass, the difference was about
415 0.7 mg over 7-mg average for all groups (Figure 3B), but again was not statistically significant
416 (LMM ‘ozone concentration’, $F_{4,35}=0.720$, $p=0.582$).

417

418 3.4.2. Total antioxidant capacity

419 Since O₃ exposure results in OS damages to the organism, we measured the total AO
420 capacity of O₃-exposed honey bees to check how the TAC might vary among the experimental
421 groups. The TAC activity ranged from 351 ± 58 mM up to 381 ± 30 mM (Figure 3C) and was not
422 statistically different among measured groups (LMM ‘ozone concentration’, $F_{4,35}=0.450$,
423 $p=0.772$), which was not expected.

424

425 3.4.3. AO enzyme activities

426 In parallel we proceeded to measure specific activities of two enzymes linked with AO
427 defense system in insects: GPX and SOD. While GPX rate for the 80-ppb O₃-exposed group
428 (1.98 ± 0.12 $\mu\text{M}/\text{min.mL}$) was slightly higher than the rest, ranging from 1.62 ± 0.12
429 $\mu\text{M}/\text{min.mL}$ to 1.73 ± 0.08 $\mu\text{M}/\text{min.mL}$ (Figure 3D), there was no statistical difference in GPX
430 rates among all measured groups (LMM ‘ozone concentration’, $F_{4,35}=1.670$, $p=0.179$).

431 However, SOD activity was significantly decreased for the 80-ppb O₃-exposed group (0.88
432 ± 0.16 U/mg) compared to all the other groups, ranging from 1.33 ± 0.11 U/mg to 1.56 ± 0.12

433 U/mg (Figure 3E) (LMM ‘ozone concentration’, $F_{4,35}=4.010$, $p=0.009$; also see all data in
434 Supplementary Table 1E, and all statistical pairwise comparisons in Supplementary Table 2E).
435 In addition, groups exposed to high concentration of O₃ had slightly lower SOD rates (120 ppb:
436 1.33 ± 0.11 U/mg; and 200 ppb: 1.38 ± 0.14 U/mg) compared to control groups (Sham: 1.51
437 ± 0.15 U/mg; and 200 ppb: 1.56 ± 0.12 U/mg). After O₃ exposure, effects on AO enzymes were
438 mixed, with only the activity of SOD being significantly impacted.

439

440 **4. Discussion / Conclusions**

441 We confirmed how ozone alters honey bees' ability to detect and perceive volatile
442 compounds. Antennal activity and olfactory recall were altered differentially depending on O₃
443 concentrations (80 ppb vs 120 ppb & 200 ppb) and on the nature of tested VOCs. Ozone
444 exposure also increased response rates to new chemical cues, reinforcing the hypothesis of
445 olfactory generalization issue (Démares *et al.* 2022). With altered olfactory detection and
446 perception, different physiological parameters could vary with ozone exposure and OS. Body
447 mass, protein mass and TAC of honey bees were similar between control and O₃-exposed
448 groups. But SOD activity significantly decreased in honey bees after a 1-hour O₃ exposure at
449 80 ppb, and it is the first time this is reported in the literature.

450 Most studies investigated atmospheric pollution impacts on pollinators' attraction through
451 changes in pollutant-altered floral blends (Farré-Armengol *et al.* 2016, Jürgens & Bischoff
452 2017, Cook *et al.* 2020, Murawska *et al.* 2021, Ryalls *et al.* 2022; Saunier *et al.* 2023). Only
453 few publications examined impacts of pollution exposure on insect behaviors (Leonard *et al.*
454 2019, Vanderplanck 2021a, Démares *et al.* 2022). By exposing bees to ozone after olfactory
455 conditioning and testing olfactory memory before and after O₃ exposure, we clearly
456 demonstrate how a reward-associated VOC does not induce specific responses in individuals
457 exposed to high concentrations compared to controls (Figures 2 and S3). We observed a clear

458 difference between the first recall test 15 minutes after conditioning and the second recall test
459 90 minutes after conditioning and when the 1-hour O₃ exposure was over; bees exposed to
460 ozone had lower recall rates to the rewarded VOC (CS) and higher recall rates to the unrewarded
461 VOC (NO), depending on O₃ concentration. Ozone affects honey bee olfactory memory in
462 similar fashion as other environmental pollution such as diesel exhaust (Leonard *et al.* 2019).
463 Additionally, the O₃ effect on olfaction and recall is not linear with its concentration but also
464 depends on the VOC used: at 80 ppb, antennal responses to camphor decrease while responses
465 to linalyl acetate increase at 200 ppb. The situation is similar with olfactory perception: for 80-
466 ppb ozone-exposed bees, there is a significant decrease of recall responses to linalyl acetate
467 used as CS but not significant for camphor used as CS, while at 120 ppb and 200 ppb there are
468 significant increased PER responses to camphor used as NO but just a tendency at 200 ppb for
469 linalyl acetate used as NO. There is an asymmetric O₃ effect dependent on VOCs, which was
470 also previously described with eucalyptol and R-linalool (Démare *et al.* 2022). Camphor and
471 eucalyptol both contain an aromatic ring (“closed” carbon chain), while R-linalool and linalyl
472 acetate have 7-carbon “open” chains: this structural difference underlines how these VOCs are
473 differentially coded within the honey bee’s olfactory system (Sachse *et al.* 1999, Deisig *et al.*
474 2006). Olfactory coding takes place at the brain level, inside antennal lobes, more specifically
475 stemming from patterns of glomerular activation (Paoli & Galizia 2021). A differential
476 olfactory response ‘recall/generalization’ was previously described with the effect of a
477 phenylpyrazole insecticide (El Hassani *et al.* 2009), underlying the role of inhibitory GABA
478 and glutamate-chloride neurotransmission in antennal lobes (Boumghar *et al.* 2012, Démare
479 *et al.* 2014). Oxidative stress through fipronil, a phenylpyrazole insecticide targeting GABA
480 and glutamate-chloride channels, was previously reported (Wang *et al.* 2016): in honey bees,
481 fipronil treatments altered several AO enzymes’ activities (catalases, phosphatases,
482 carboxylesterases)(Carvalho *et al.* 2013) but neither GPX nor SOD were assessed. It was

483 reported that OS through actions of different pesticides leads to subcellular damages, especially
484 to endoplasmic reticulum and mitochondria, and eventually lead to apoptosis in Asian honey
485 bees *Apis cerana* (Kumar *et al.* 2022). In this report, we did not assess tissue damages from
486 ozone exposure, and since TAC remained unchanged, GPX was slightly modified, and the only
487 significant effect was on SOD (decreased activity at 80 ppb), we can only infer damages were
488 minimal compared to those with pesticides. Other lines of thoughts to explain the singular effect
489 on SOD could be either a deterioration of the enzyme itself, or a decreased metabolic activity
490 that did not reach a sufficient threshold to ‘activate’ the whole AO defenses (which should be
491 observable through TAC level). The mitochondrial target is an interesting lead to look for in
492 future experiments: mitochondrial activity in neurons can be modified by OS and change
493 overall neuronal functions, whether at the antenna level (detection issue) or higher in the
494 olfactory pathway (coding issue and/or perception issue). Glial cells also provide damage repair
495 and protective support to neurons (Edenfeld *et al.* 2005, Giraldo *et al.* 2021), so this needs to
496 be assessed in parallel for the ‘OS/AO activity’ balance. Furthermore, we also observed a non-
497 linear biphasic effect of O₃ with a different exposure protocol (Démarets *et al.* 2022), which we
498 coined under the term of *stress response hormesis*: a well-known toxicological response
499 commonly described for insect/pest responses under pesticide influence (Archer *et al.* 2014,
500 Cutler & Rix 2015). What is interesting here is that we observe it with a very different stressor
501 which affects living organisms able to exhibit AO responses, such as honey bees (and perhaps,
502 more generally, insects) but also plants, which also display hormesis stress responses under
503 tropospheric O₃ exposure (Agathokleous *et al.* 2019, 2020). This underlines the fact that
504 hormesis responses to O₃ may be a general phenomenon across taxa (Agathokleous *et al.* 2019,
505 2020).

506 Trying to unravel the honey bee defense system, by exploring other molecular targets linked to
507 the OS/AO activity balance, would be interesting to look for in other honey bee castes. Queens

508 for instance, individuals living much longer than any worker bee, would require a strong AO
509 defense and effective damage repair system; or drones, whose olfactory system is more attuned
510 to pheromonal detection and works differently than those of regular foragers (Kropf *et al.* 2014,
511 Villar *et al.* 2015). Since O₃ exposure impacts foraging behaviors of other pollinator species
512 (*i.e.*, bumblebees in Saunier *et al.* 2023), it would be interesting to compare which parameters
513 (pollen load, flying activity, navigating) are impacted in similar fashions in honey bees. Also,
514 as tropospheric O₃ varies during the day, and seasonally during the year, another direction to
515 follow could be assessing the effects of longer acute exposures to lower O₃ concentrations on
516 one hand, or assessing impacts of chronic exposures to O₃ on the other hand; these setups might
517 be closer to realistic field conditions, and it would be interesting to check if the pollinator's
518 OS/AO activity balance is stable between seasons. Going through longer multiple periods of
519 O₃ exposures and other ROS stressors could lead to variations of TAC levels, potentially
520 leading to a gradual exhaustion of immune defenses (Kodríck *et al.* 2015, Chaitanya *et al.*
521 2016).

522 Impacts of O₃ on pollination were investigated by a handful of publication so far. From this
523 relatively-recent standpoint, O₃ has a three-way impact on pollination and plant-pollinator
524 interactions: by affecting plant physiology, by altering floral VOCs structure and lifetime in the
525 environment, and by directly impacting olfactory detection and perception of pollinators (Farré-
526 Armengol *et al.* 2016, Conchou *et al.* 2019, Vanderplanck 2021a, Dubuisson *et al.* 2022,
527 Démares *et al.* 2022, Saunier *et al.* 2023, Langford *et al.* 2023). Is there any strategy to help
528 decreasing the actions of ozone in the environment? A first strategy would prioritize the culture
529 of plants that produce stable VOCs under oxidative conditions. For instance, camphor is
530 relatively unaffected by ozone oxidation (Dubuisson *et al.* 2022). A second strategy in parallel
531 could focus on plants that enable pollinators' self-medication behaviors, *i.e.* pollinators may
532 choose to favor plants that will produce nutritious molecules that reduce OS by up-regulating

533 AO enzymatic rates, and eventually protect pollinators' health and physiology. There are a
534 couple of research projects documenting this behavior (Erler & Moritz 2016, Spivak *et al.* 2019,
535 Gekière *et al.* 2022) that will need to be assessed during in-field experiments. If all else fails,
536 will pollinators and plants be flexible enough to adapt to fluctuating environmental conditions
537 and global warming? (Gérard *et al.* 2020). Plants may favor the production and emission of
538 VOCs resistant (or tolerant) to increasing O₃ concentrations in the environment, along with
539 other atmospheric pollutants; and pollinators will either rely more on other cues rather than
540 VOCs (shapes and colors of plants, for instance), or become more resilient to environmental
541 oxidative damages, in order to successfully reach floral resources and further
542 perpetuate/maintain the important ecosystemic service that is pollination.

543 5. Contributions & Acknowledgments

544

545 Contributions: FD: Investigation, Methodology, Supervision, Formal analysis, Data curation,
546 Validation, Visualization, Writing (original draft, review and editing). LG: Investigation,
547 Methodology, Formal analysis, Data curation, Writing (review & editing). PC: Investigation,
548 Methodology, Writing (review & editing). BL: Methodology, Supervision, Validation, Writing
549 (review & editing). DR: Resources, Supervision, Formal analysis, Validation, Writing (original
550 draft, review & editing). MP: Conceptualization, Funding acquisition, Project administration,
551 Resources, Supervision, Formal analysis, Validation, Visualization, Writing (original draft,
552 review and editing).

553

554 The authors declare no known competing financial interests or personal relationships that could
555 have appeared to influence the work reported in this paper. We would like to acknowledge: the
556 technical team managing the *Terrain d'Expériences* (TE) platform [LabEx CeMEB, the French
557 National Research Agency (ANR) under the “Investissements d’Avenir”] at the CEFÉ/CNRS
558 campus, and once again Pierrick AURY and David DEGUELDRE for their skills and assistance
559 with creating equipment for the conditioning and the O₃ exposure; and all the people involved
560 in managing the apiary and providing hives for this project (through “Lune de Miel” foundation
561 and *Montpellier Université d'Excellence* (MUSE) under the program “Investissements
562 d’Avenir” reference ANR-16-IDEX-0006). We also would like to thanks the *Plateforme*
563 *d'Analyse Chimique en Ecologie* (PACE) (LabEx CeMEB) for hosting the GC-EAD and GC-
564 MS analysis. We also thank the two anonymous reviewers for their constructive comments and
565 suggestions. This work is a contribution to the French National Research Program for
566 Environmental and Occupational Health of ANSES (program 2018/1/138), and partly funded
567 by the International Research Project (IRP)-CNRS-MOST and MUSE.

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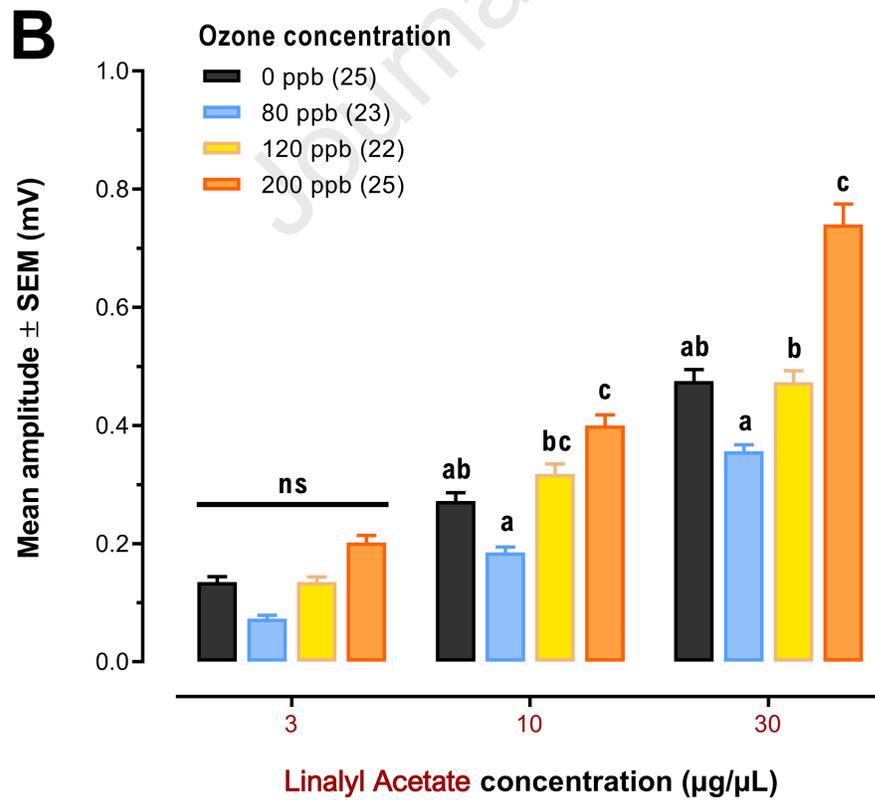
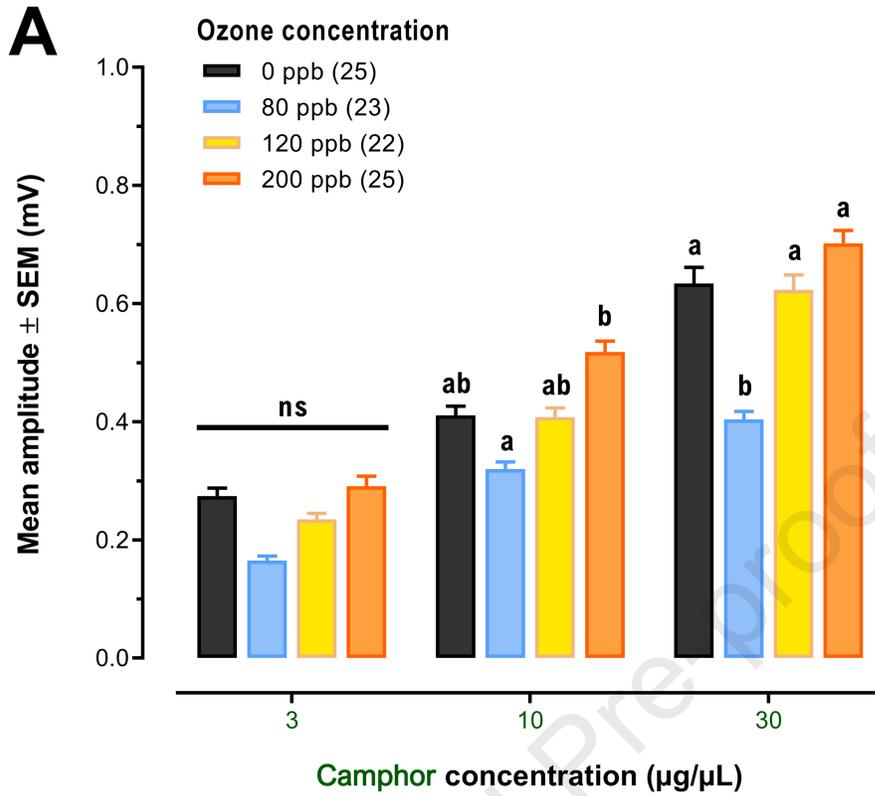
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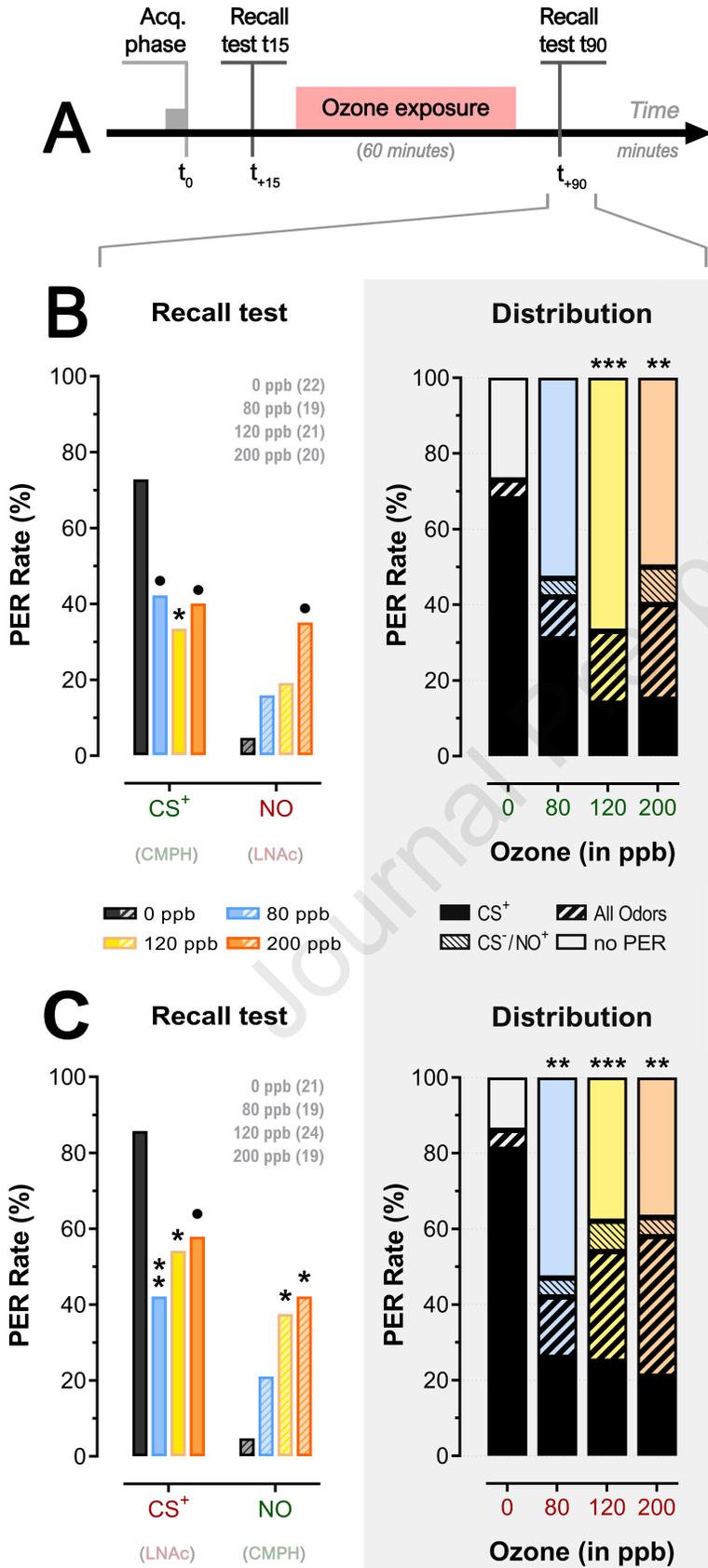
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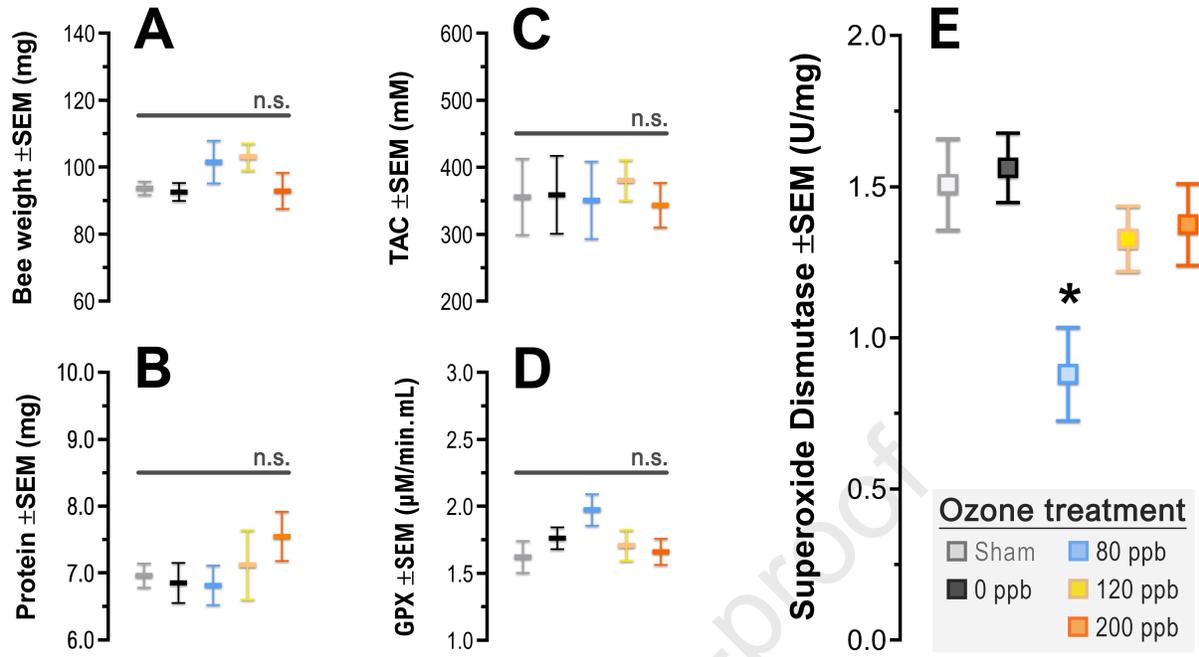
Figure 1. Electroantennogram (EAG) recordings of honey bee antenna activity evoked by camphor and linalyl acetate puff presentations. The mean amplitude response was recorded with camphor (**A**) or linalyl acetate (**B**) at three increasing concentrations (3, 10, and 30 $\mu\text{g}/\mu\text{L}$) from antennae of bees previously exposed to different concentrations of ozone: 0 ppb (control group in **black**), 80 ppb (**blue**), 120 ppb (**golden yellow**), and 200 ppb (**orange**). For each concentration, pairwise comparisons indicate which groups are statistically different from each other with different letters (p -value < 0.05). Groups with the same letter or a letter in common are not different from each other. All data can be found in Supplementary Table 1A, and corresponding stats in Supplementary Table 2A.

Figure 2. Olfactory recall tests of ozone-exposed bees, with either camphor (CMPH) or linalyl acetate (LNAc) used as the conditioned stimulus (CS*). (**A**) Schematic outline depicting the chronology of the ozone exposure protocol used in the present report. Before ozone exposure, bees were randomly attributed a group and trained through proboscis extension reflex (PER) classical conditioning. Results from the acquisition phase and the first recall test at 15 minutes are available in Supplementary Materials (Figure S3). (**B**) Results of recall tests (left panel) and its corresponding distribution of PER response categories (right panel, gray shading) 90 minutes after the last acquisition trial, with CMPH used as CS. In recall tests, bees were subjected to the learned odor and to a new odor (here, linalyl acetate as NO). (**C**) Results of recall tests (left panel) and its corresponding distribution of PER response categories (right panel, gray shading) 90 minutes after the last acquisition trial, with LNAc used as CS. In recall tests, bees were subjected to the learned odor and to a new odor (here, camphor as NO). For each odor response, pairwise comparisons indicate which groups are statistically different from the control group. For the distribution panels, four categories total for each combination of response, either CS alone (CS⁺) or NO alone (CS⁻/NO⁺), or both ('All Odors'), or none ('no PER'). In all panels, stars indicate statistical differences against control groups while dots indicate tendencies (FDR adj. p -values: ● < 0.10; * < 0.05; ** < 0.01; *** < 0.005).

Figure 3. Physiological parameters and enzymatic levels of ozone-exposed bees. To assess the impact of ozone-induced oxidative stress on antioxidant pathway, we measured different parameters such as bee weight (**A**) and their related protein mass (**B**), the total antioxidant capacity TAC (**C**), and the activity of two specific enzymes involved in the antioxidant pathway: the glutathione peroxidase GPX (**D**) and the superoxide dismutase SOD (**E**). This figure uses the same color code as the previous figures, with one additional group called 'sham' (in light gray) which corresponds to bees prepared the same way as the 0-ppb control group but sacrificed right before the ozone exposure protocol. Stars indicate statistical differences against all groups (FDR adj. p -values: $* < 0.05$). All data can be found in Supplementary Table 1C.







Ozone exposure induces metabolic stress and olfactory memory disturbance in honey bees.

Highlights (max 85 characters including spaces per bullet point)

- Ozone (O₃) at 80 ppb **decreases olfactory recall** of honey bees.
- O₃ at higher concentrations (200ppb) **increases olfactory generalization.**
- Antennal activity is differentially altered depending on O₃ level & VOC structure.
- After 1-hr O₃ exposure, only one of two tested antioxidant enzymes is impacted.
- Activity rate of **superoxide dismutase is decreased** after 80-ppb O₃ exposure.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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