

1 **Repellent olfactory cues influence the optomotor response** 2 **modulation in *Drosophila melanogaster*.**

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18 **Keywords:** *Drosophila melanogaster*; Repellents; Optomotor response; Olfaction;
19 Spatial navigation.

20

21 **Abstract**

22 Animals need to precisely perceive and integrate the environmental cues to orient and
23 select the appropriate motor responses required for navigating. This is the case, for
24 instance, of the optokinetic reflex (OKR) and the optomotor response (OMR) in
25 *Drosophila melanogaster*, where optic flow stimulation modulates the head or the body
26 and legs motor activity respectively. Despite large bodies of literature on both the OKR
27 and the OMR, there is still a limited understanding, in flies, of the impact on these
28 responses of concomitant, and potentially conflicting, sensory inputs. To investigate
29 this aspect, we used fruit flies walking on a sphere, presented with optic flow

30 stimulation leading to the OMR together with the simultaneous exposure to olfactory
31 stimulation, either using established repellent or masking compounds. We analysed the
32 effect of different substances, and of their concentration, on the dynamics of the flies'
33 response to moving gratings, evaluating the fly walking path as well as average speed
34 and duration. This analysis revealed several alterations between the compounds tested,
35 in agreement with reported data on the simpler OKR. In conclusion, we show that
36 concomitant exposure to repellents and maskers may consistently affect fundamental
37 processes (the OKR and OMR) available to insects for informing themselves while
38 navigating through the environment.

39

40 **Introduction**

41 The navigational abilities of insects are remarkably close, if not equal, to the skills
42 exhibited by vertebrates. Besides, navigational skills are fundamental for everyday
43 activities, like searching for food, escaping from hazards, and social interactions.
44 Unravelling the components and processes which generate and influence the
45 repertoire of behaviours adopted by insects when exploring and moving in the
46 environment is becoming of even more importance for tackling the challenges placed
47 by some species to agriculture and human health.

48 In Europe, the increasing presence of alien insect species, exacerbated by the
49 important climate variations, is posing a serious threat to cultivations, as is the case for
50 the dipteran *D. suzukii* ^[1], which seriously threatens soft summer fruit cultivations.
51 Likewise, public health is endangered by several disease-transmitting insect vectors
52 such as mosquitoes and ticks which are expanding their geographical areas ^[2].
53 Moreover, the 2012 EU Biocidal Product Regulation, for the sake of the population's
54 safety and environment preservation, shortened the list of compounds available for
55 companies to distribute and consumers to purchase ^[3]. A deeper comprehension of the
56 effects of the repellent compounds publicly available for production and
57 commercialization would increase the efficacy of repellent products and enhance
58 global protection against pests. This is particularly important for the threat posed by

59 mosquitoes, which can become vectors of several relevant diseases [4]. Indeed, an
60 efficient approach to study the problem could rely on the use of a more readily
61 manageable and well-known model organism, such as the fruit fly *Drosophila*
62 *melanogaster*. *Drosophila* is more easily reared and managed in the laboratory
63 compared to mosquitoes, allows for sophisticated genetic manipulation, as well as deep
64 behavioural and neurophysiological analysis; moreover, it shares the same brain
65 organisation [5,6] and sensitivity to some known mosquito repellents [7,8]. In fact, reports
66 in these species confirm said olfactory sensitivity to a series of compounds, both
67 natural - eugenol (**E**), lemongrass (**L**) - and synthetic - picaridin (**P**), IR3535® (**I**) -
68 independently from their ecological differences [7,8].

69 Recently there's been renewed interest and application of new protocols for studying
70 OMR responses in mosquitoes [9,10], when, in the past, studies on the mosquito's visual
71 system were fewer and constrained on visually guided behaviours [11]. Given the similar
72 response to visual and olfactory stimuli between mosquitoes and fruit flies, we thus
73 opted for investigating the possible influence of repellents on visually guided
74 behaviours in *D. melanogaster* by evaluating the multimodal sensory integration of
75 vision and olfaction with respect to the OMR outcome.

76 To this end, we set up a behavioural paradigm similar to others described in literature
77 [12,13] where adult female *Drosophila* were tethered at their back and with their legs
78 walking on the surface of a sphere in response to moving grating visual stimuli [14-16]. The
79 paradigm envisages a temporal segment where visual stimulation is paired with the
80 administration of different odorous repellent compounds (Fig. 1). We evaluated ten
81 different conditions with both repellents (eugenol and lemongrass) and maskers
82 (picaridin and IR3535®) delivered at two volume concentrations of **0.5%** and **1%** in
83 mineral oil, which was used alone in the control condition.

84 Our research follows up on our previous work on the OKR and aligns with other studies
85 on the effects of odorants on *Drosophila* flight and walking conducted in the past years
86 [17-21]. We aimed with the current work at analysing these effects within a more complete
87 locomotion scenario - the OMR - which incorporates the proprioceptive motor

88 feedback from the flies' legs and needs to regulate their movement pace and patterns
89 [22]. We thus tracked the sphere's movement to reconstruct the flies' fictive walking
90 paths in order to extract the trajectories and other kinematic parameters in response
91 to the concomitant exposure of naive flies to optokinetic stimulation and to a gaseous
92 plume carrying one of the compounds. Since it is known that aversive compounds can
93 affect the OKR modulation in flies by decreasing the number of head-like optokinetic
94 *nystagmi*, while leaving the intrinsic dynamics unaltered [23], we first considered
95 whether this was also the case for the OMR. Our results show that, indeed, the
96 compounds tested do affect the output and dynamics of the OMR, both qualitatively
97 and quantitatively. Moreover, we found evidence that some of the synthetic masker
98 compounds, typically found as active ingredients in some commercially available
99 products, are not as neutral to insects as it is expected, rather they can interfere with
100 fundamental processes underlying navigation in these (and most likely other) species.

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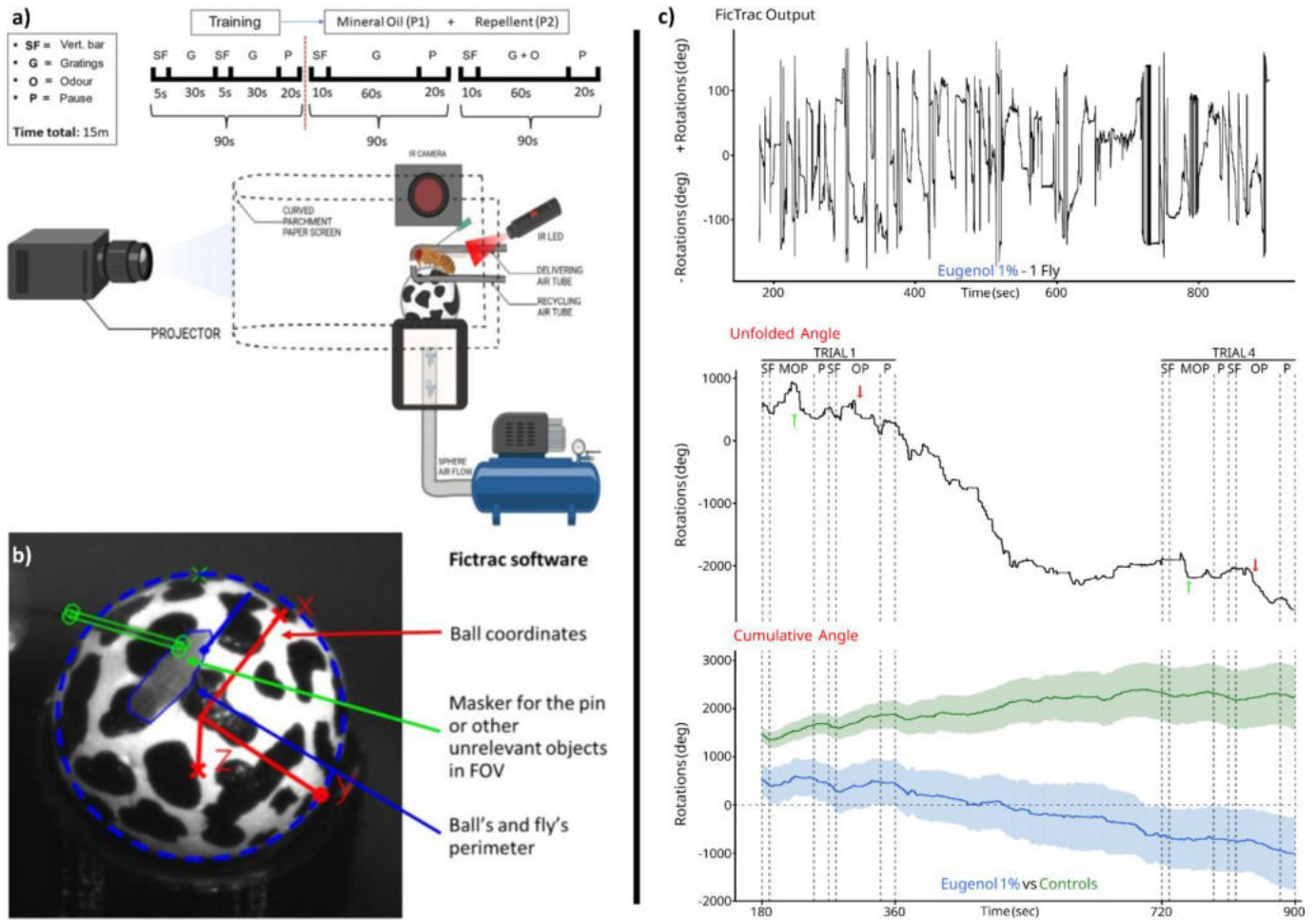


Figure 1. Methods and paradigm. (a) Experimental paradigm sequence and cartoon of the experimental apparatus. Created in BioRender. (b) FicTrac^[41] GUI before processing one of our recordings. Blue ROIs identify the limits of the sphere and of the fly. Green ROIs mask object irrelevant or interfering with the tracking. Red arrows set the X, Y, Z axis allowing for the correct extraction of the space coordinates. (c) In descending order: example of one FicTrac^[41] raw output, deconvoluted data, and one sample of the cumulated tracks from Controls (green) and Eugenol 1% (blue) groups (mean \pm s.d.). **SF**: stripe fixation. **MOP**: mineral oil phase (plain oil + gratings). **OP**: odorant phase (oil + odorant + gratings; Controls experienced 2 consecutive MOP instead). **P**: pause (darkness). Note that the training portion (the first part of the paradigm) was excluded from the analysis. Regarding the *original* versus *transformed* output from FicTrac^[41] calculations: when the limit of ± 3 radians are reached, the coordinate system is flipped to the opposite sign. Inversion points were identified as those points where the first derivative exceeded 2 times the standard deviation.

102 Pilot study's results

103 *Gratings' period, speed, and aversive odour influence the flies' response the optomotor*
 104 *stimulation*

105 First and foremost, we needed to test and tune our experimental apparatus; besides,
 106 we also wanted to set a suitable visual stimulus for the subsequent experiments. For
 107 achieving this, we conducted an initial set of experiments (Suppl. Fig. 1 and 2; Suppl. Fig
 108 1a shows the experimental paradigm sequence) by testing gratings optomotor

109 stimulation at different combinations of spatial period (λ) and velocity (v), adapting the
110 experimental paradigm found in Götz (1973) [24]. In this and other studies of his, Götz
111 analysed the visual control of locomotion on tethered *D. melanogaster* flies; in the 1973
112 study in particular, he had utilized the visual stimulation we replicated in order to
113 investigate the phenomenon on flies tethered to a magnetic sledge performing
114 stationary walking on a rubber sphere laid on servomechanisms. Two main findings of
115 his afore cited work in particular should be mentioned here: i) leg contribution to the
116 movement was observed to be equal for each pair of legs; ii) he identified the “limit of
117 resolvability”, which is the minimum λ value (around 9.0° in walking flies) before
118 observing an inversion of rotational movement (majority of movement is performed
119 opposing the actual stimulus direction).

120 In our pilot set of experiments (Suppl. Fig. 1 and 2), we first tested for different sets of
121 velocities for the gratings mask at, in our opinion, a low λ (12°) but nonetheless higher
122 than the inversion point identified by Götz [15,24]:

- 123 A. $15^\circ\cdot\text{s}^{-1}$
- 124 B. $30^\circ\cdot\text{s}^{-1}$
- 125 C. $60^\circ\cdot\text{s}^{-1}$

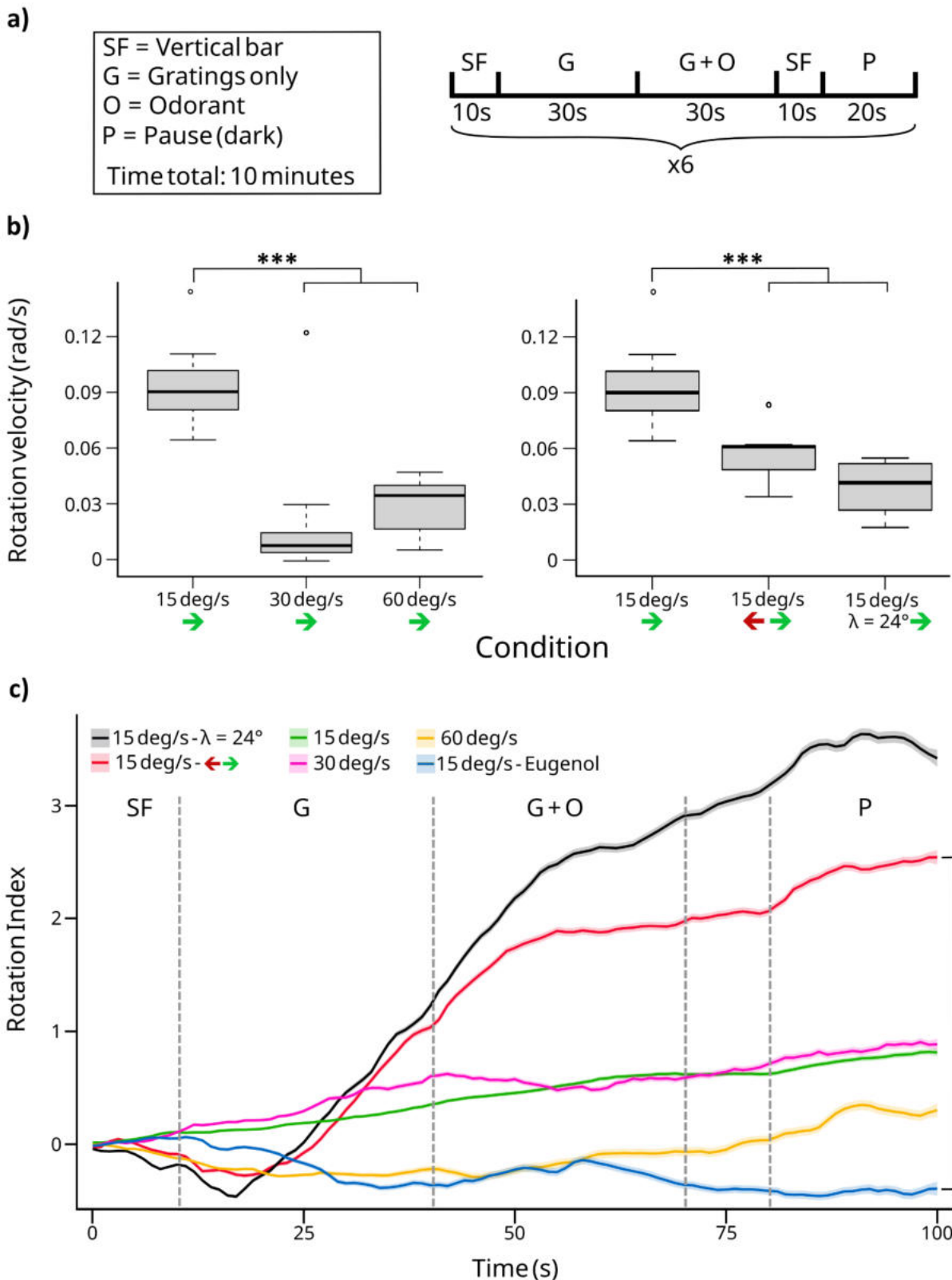
126 We observed that the slowest velocity (**A**) elicited the higher mean speed in our flies
127 (Suppl. Fig. 1b); therefore, we kept this V value while introducing other three sets of
128 conditions:

- 129 D. concurrent aerosol administration of a mineral oil solution containing eugenol
130 (1% in volume) in the opposite direction of the visual stimulation
- 131 E. alternating direction of movement for the gratings ($v = 15^\circ\cdot\text{s}^{-1}$, $\lambda = 12^\circ$)
- 132 F. doubled spatial period ($v = 15^\circ\cdot\text{s}^{-1}$, $\lambda = 24^\circ$)

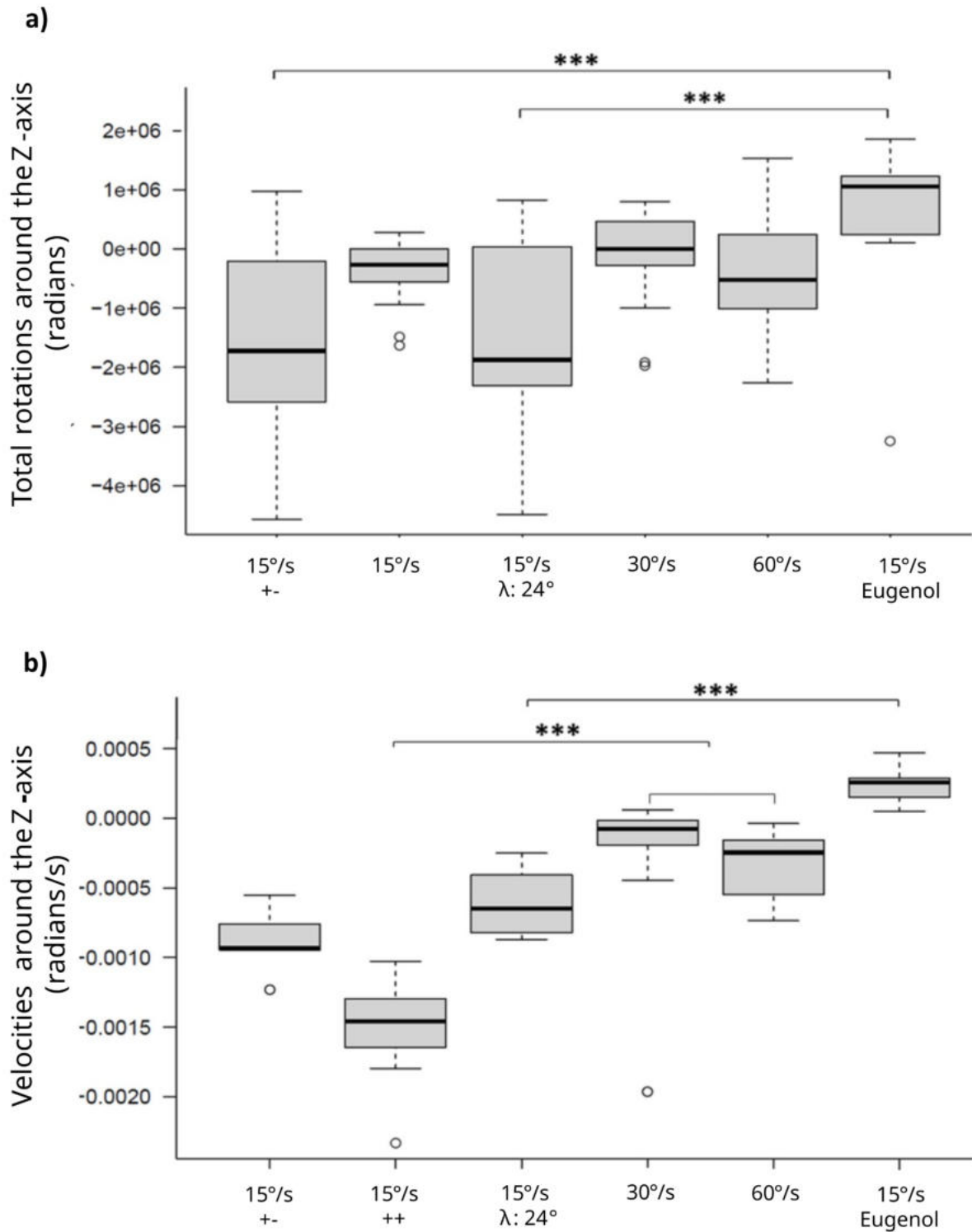
133 Although mean velocities decreased significantly in **E** and **F** (Suppl. Fig. 1b), the mean
134 quantity of movement resulted higher than in the baseline **A** (Suppl. Fig. 1c). We
135 measured this as ‘Rotation Index’ (**R.I.**), which was calculated as the measured amount
136 of rotational movement (around the z-axis, subjects’ mean) during a trial divided by the
137 expected amount of the same motion at set velocity in ideal conditions (*i.e.*, if the
138 animals were moving continuously at constant speed). Mean R.I. values higher than |1|

139 indicates that the flies turned more than expected and with a higher degree of
140 variability in speed and acceleration than an ideal subject perfectly matching the
141 stimulus speed and moving in a uniformly fashion. Both the **E** and the **F** condition
142 exhibited higher index value, the latter being higher. Notably, the **D** condition gave
143 preliminary insights on the efficacy of the substance, clearly moving mainly in the
144 opposite direction , with respect to the **A** baseline condition, as visible in the overall
145 rotations and velocity (Supp. Fig. 2, $p < 0.0005$), as well as in the mean R.I. (Suppl. Fig.
146 1c; $p < 0.0005$, see Methods).

147 Considering the results from the pilot experiment altogether, we selected for the next
148 experimental paradigm the conditions which promoted the highest velocity ($\nu = 15^\circ\text{s}^{-1}$)
149 and amount of rotation ($\nu = 15^\circ\text{s}^{-1}$, $\lambda = 24^\circ$).



Supplementary Figure 1. Pilot study paradigm and results. (a) Experimental paradigm: each trial is repeated 6 times over a span of 10 minutes and starts with a single black vertical bar (6°) on a white background for 10 seconds to centre the flies' attention towards the middle of the screen and to elicit movement. Subsequently a mask of gratings runs for 1 minute; in the eugenol group, after 30 seconds, the air current is switched to come from a vial containing the solution with the repellent. The trial ends with 20 seconds of darkness and then a new trial starts. All conditions had $\lambda = 12^\circ$, except for the one identified by $\lambda = 24^\circ$. (b) Standard boxplots of velocity profiles. P-values: *** = 0.0005 (ANOVA + Tukey HSD test). (c) Rotation Index for each experimental condition (mean value among trials \pm s.e.m.): the index was calculated as the mean measured amount of rotational (over the z-axis) movement during a trial divided by the theoretical amount of the same motion which would have been performed at set velocity if the animals were moving at constant speed. Values higher than $|1|$ indicates the flies turned more than expected and with a higher degree of variability in speed and acceleration than an ideal subject perfectly matching the stimulus speed and moving in a uniformly fashion. P-values were computed on the regression line slopes: *** = 0.0005 (Tukey adjustment).



Supplementary Figure 2. Summary of rotations and velocities. (a) Standard boxplots for the total amount of rotations around the vertical (Z) axis: in the “ $15^\circ \cdot s^{-1} +$ ” condition the gratings move at $15^\circ \cdot s^{-1}$ in the opposite direction to the “ $15^\circ \cdot s^{-1}$ ” one; in the “*eugenol*” condition the gratings speed is set at $15^\circ \cdot s^{-1}$ gratings and the repellent is presented throughout the 2nd half of the OK stimulation; in one condition λ was increased to 24° , instead of 12° . (b) Standard boxplots for the average velocity of the same conditions as in (a); p-values: ** = 0.005, *** = 0.0005 (ANOVA + Tukey HSD test).

152 Results

153 1. Odour presentation influences the fly walking trajectories and the OMR.

154 Initially, we aimed at assessing the relative effect of the odour presentation with
155 respect to the concomitant visual stimulation, that, depending on the specific condition
156 (compound and concentration), could potentially result either in a suppression or
157 mild/strong modulation of the OMR.

158 We found that several groups, such as those exposed to 1% eugenol (**E1**) and 1% IR3535
159 (**I1**), but also 0.5% lemongrass (**L05**) and 0.5% Picaridin (**P05**), showed altered
160 trajectories (mixed or opposite rotation direction) with respect to Controls (**C**). In
161 particular, the analysis of the cumulative trajectories over the whole experiment (Fig.
162 2) and the cumulative relative number of rotations (Fig. 3) performed in the direction
163 coherent (+) or non-coherent (-) with the grating's direction (only taking into account
164 the periods when the grating mask was active) revealed distinct trajectory patterns and
165 preferred + or - direction of movement correlated with the different odorants. Suppl.
166 Figure 3 shows the average number of (+) or (-) rotations per fly occurring in the
167 absence (**MOP**) and in the presence (**OP**) of repellent for each group of flies.

168 Considering the mean cumulative trajectories during the whole task (Fig. 2a), the
169 repellent **E1** (n = 10, p-value = 0.006) and the masker **I1** (n = 10, p-value = 0.037) showed
170 major differences from the **C** (n = 13) group, exhibiting the opposite rotation direction,
171 which is non-coherent with the experienced visual stimulation and moving away from
172 the odour source. In contrast, **E05** (n = 7), **L1** (n = 12), **P1** (n = 12), and **I05** (n = 13) are not
173 significantly different with respect to **C**. Moreover, **L05** (n = 10) and **P05** (n = 11),
174 approached statistically significant differences with respect to **C** (p-values = 0.07 and
175 0.13 respectively), showing an intermediate behaviour with respect to the controls and
176 the groups showing the most non-coherent rotations (*Kruskal-Wallis: chi-squared =*
177 *26.104, d.f. = 8, p-value = 0.001. Post-hoc: Dunnet test with Benjamini-Hochberg*
178 *correction*).

179 In particular:

180 • The repellent Eugenol and the masker IR3535 show a similar trend of
181 concentration-dependent impact: at low concentration it does not appear on the
182 walked trajectories any clear repellent effect, rather an opposite trend if any. However,
183 the repellent action becomes detectable and significant at higher concentrations.

184 • The repellent Lemongrass and the masker Picaridin show an opposite trend,
185 characterized by a significant repellent effect at lower concentrations, while showing a
186 diminished if not opposite effect at higher concentrations, possibly due to saturation.

187 These differences in the cumulative trajectories were confirmed by the cumulative
188 mean amount of movement observed in the direction coherent (+) or non-coherent (-)
189 with the visual stimulation (Fig. 3). Here, without considering the initial 180 seconds of
190 the training period, both **E1** and **I1** exhibited a reversed ratio for the preferred direction
191 of movement with respect to **C**. Furthermore, a similar, significant, difference was also
192 observed for **L05** and **P05** (see Table 1 for the mean values \pm standard deviations
193 alongside the Bonferroni adjusted p-values for the equality of proportions test).

194 When considering the mean values by subject (Suppl. Table 1), the trends observed for
195 the cumulative trajectories and relative quantity of (+/-) rotations were confirmed. No
196 significant differences were found between MOP and OP for any group: **C** flies moved
197 more in the direction coherent with the gratings, as expected, as well as **E05** and **I05**.
198 Moreover, while **L1** and **P1** had a slight preference towards (+) directions during the
199 MOP – which reversed during OP – **E1**, **L05**, **P1** and **I1** all preferred the (-) direction
200 (Kruskal-Wallis: $\chi^2 = 4.7795$, $d.f. = 17$, $p\text{-value} = 0.9983$), while significant
201 differences were present for the preferred direction of rotation (Kruskal-Wallis: $\chi^2 = 306.25$,
202 $d.f. = 35$, $p\text{-value} < 2.2e^{-16}$. Post-hoc: Dunnet test with Benjamini-
203 Hochberg correction).

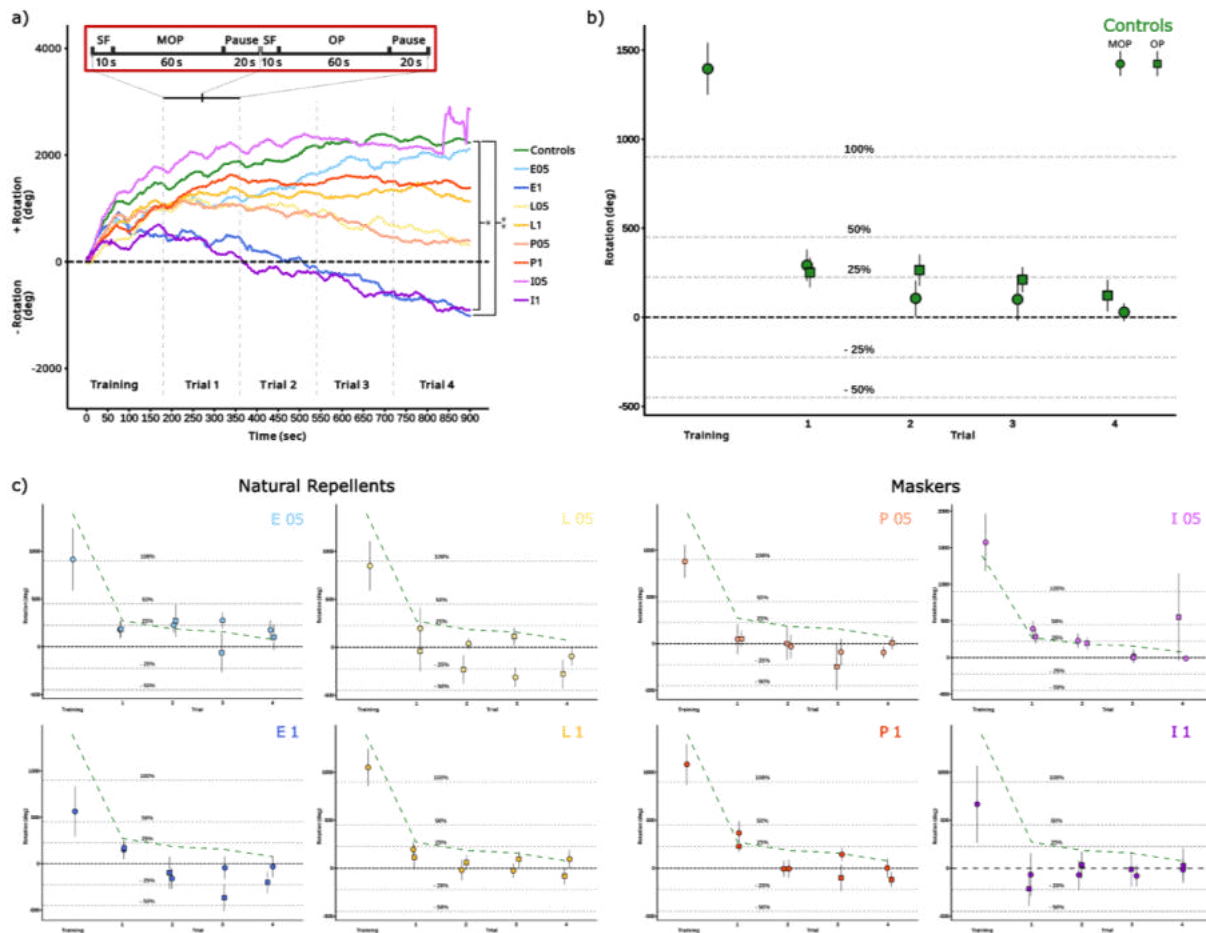
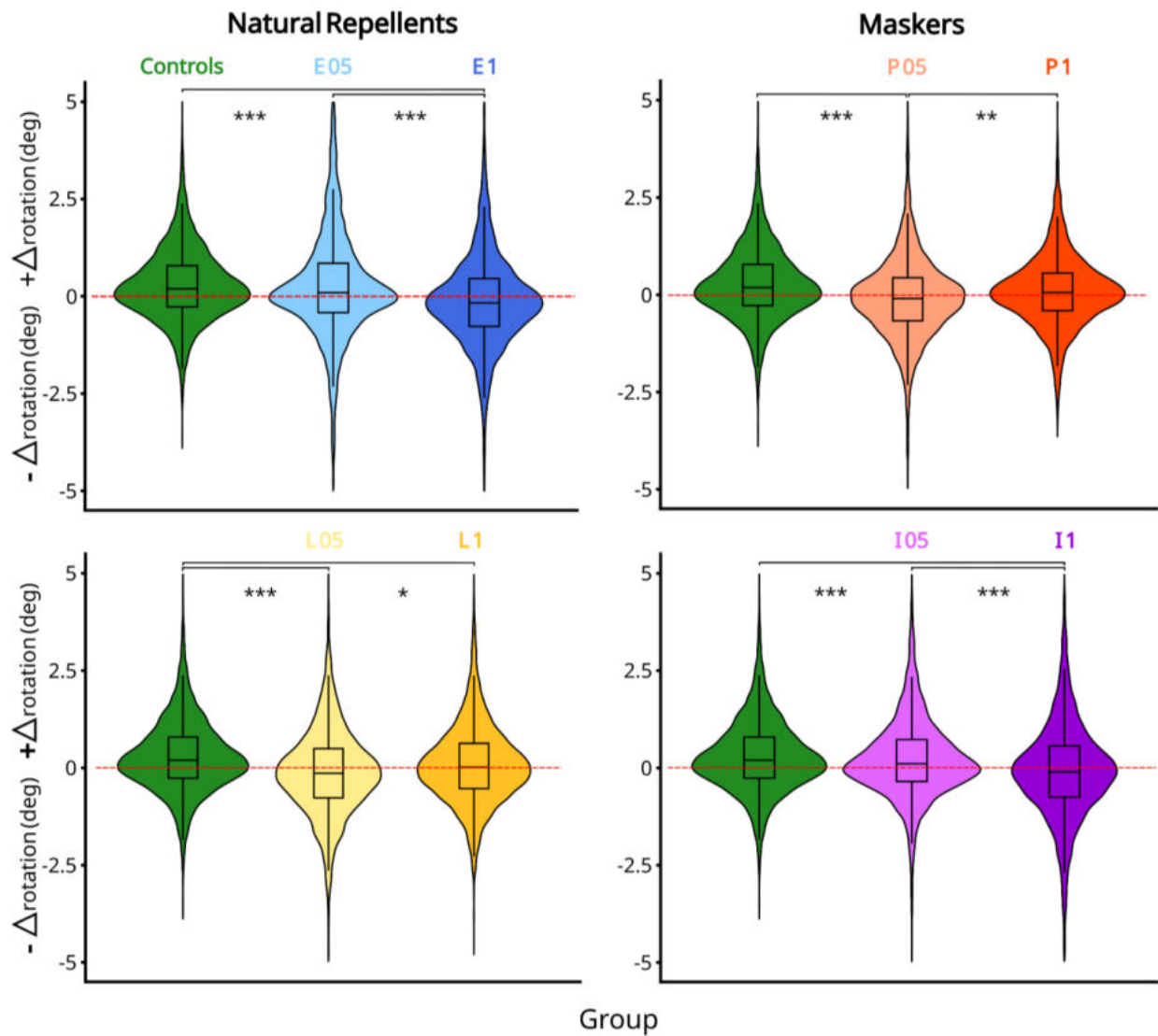
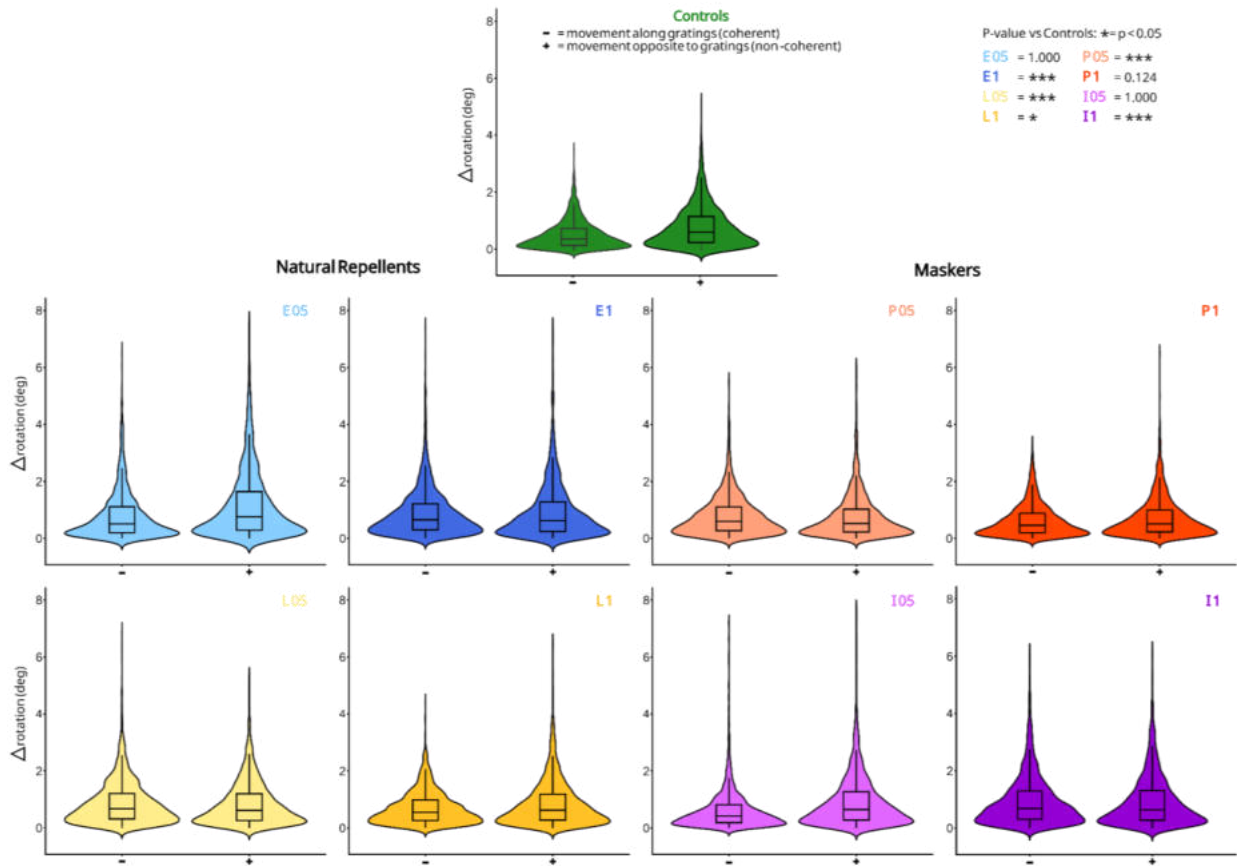


Figure 2. Cumulative trajectories and the average amount of rotation per trial (MOP and OP) for each compound. (a) Cumulative group trajectories: **L1** and **P1**, as well as **E05** and **I05** behave similarly to **C**; **P05** and **L05** have p-values close to significant (0.13 and 0.07 respectively, 0.04 and 0.02 without adjustment); **E1** and **I1** clearly tend to move in the opposite direction, away from the odour source (p-values 0.006 and 0.037 respectively). P-values were obtained from a *Kruskal-Wallis* and *post-hoc Dunnett* test with *Benjamini-Hochberg* adjustment on the subject mean differential position value each 100 milliseconds. **(b)** Average rotation per trial in the Control group. **(c)** Average rotation per trial in all the test groups; a green reference dashed line identifies the Control values shown in **(b)**. * < 0.05, ** < 0.005.



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Figure 3. Violin plots of the Δ rotation angle. C flies rotate more in the direction concordant with the gratings movement, as expected. The same is true for E05 and I05. L1 and P1: a slight preference towards *concordant* (+) directions can be observed (see Suppl. Fig. 3 for the MOP/OP division). E1, L05, P05 and I1 all show a tendency for the *opposite* (-) directions both during MOP and OP, although this is visible as *opposite* (-) overall trajectories only in E1 and I1 (Fig. 2a). *: $p < 0.05$, ***: $p < 0.0005$; see Table 1 for detailed number of rotations and p-values (pairwise comparison of proportion with Bonferroni p-value adjustment).



Supplementary Figure 3. Violin plots of the Δ rotation angle during MOP and OP phases. Same plot as in Figure 3, except MOP and OP are shown separately.

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| Group | Trajectory (mean rotations) | coherent movement (mean + rotations) | non-coherent movement (mean - rotations) | P-value vs Controls |
|--------------------------|--------------------------------|--|--|---------------------------|
| Controls (n = 13) | 6. ± 2.1e-3 | 6.71 ± 2 e-3 | 2.87 ± 1.4e-3 | - |
| Eugenol 0.5% (n = 7) | 5.72 ± 3.1e-3 | 8.56 ± 3.3e-3 | 4.80 ± 2.4e-3 | 1.000 |
| Eugenol 1% (n = 10) | -2.98 ± 2.8e-3 | 5.41 ± 2.6e-3 | 6.78 ± 2.3e-3 | < 2e ⁻¹⁶ |
| Lemongrass 0.5% (n = 10) | 0.72 ± 2.3e-3 | 4.89 ± 2.1e-3 | 6.59 ± 2.1e-3 | 6.3e ⁻¹⁰ |
| Lemongrass 1% (n = 12) | 2.98 ± 2.1e-3 | 5.75 ± 2.2e-3 | 4.57 ± 1.6e-3 | 0.008 |
| Picaridin 0.5% (n = 11) | 0.92 ± 2.2e-3 | 4.65 ± 2.1e-3 | 5.63 ± 1.9e-3 | 1.7e ⁻¹¹ |
| Picaridin 1% (n = 12) | 3.69 ± 1.9e-3 | 5.23 ± 1.9e-3 | 3.83 ± 1.6e-3 | 0.124 |
| IR3535 0.5% (n = 10) | 7.77 ± 6.1e-3 | 8.82 ± 6.9e-3 | 4.22 ± 3. 6e-3 | 1.000 |
| IR3535 1% (n = 13) | -2.69 ± 2.6e-3 | 5.64 ± 2.4e-3 | 6.78 ± 2.4e-3 | < 2e ⁻¹⁶ |

Table 1. Concordant vs discordant number of rotations. Values relative Fig. 3 panels. Cumulative values (mean ± standard deviation calculated on the variation in position every 100 milliseconds) per group: trajectories include the training period (first 180 seconds). Values for the mean amount of movement concordant (+) or discordant (-) with the gratings' direction only include the proper trials (1 ~ 4) during which the grating mask is projected on screen. P-values related to the relative +/- rotation proportions are obtained through pairwise comparison of proportions with Bonferroni correction.

| Group | MOP | | | OP | | |
|--------------------------|---------------------|---------------------|---------|---------------------|---------------------|---------|
| | Mean - rotations | Mean + rotations | P-value | Mean - rotations | Mean + rotations | P-value |
| Controls (n = 13) | 10.06 ± 1.81 | 12.71 ± 3.93 | 0.00140 | 10.64 ± 2.54 | 12.54 ± 3.96 | 0.00096 |
| Eugenol 0.5% (n = 7) | 12.85 ± 1.51 | 15.68 ± 4.23 | 0.00048 | 12.60 ± 2.33 | 15.56 ± 4.61 | 0.00019 |
| Eugenol 1% (n = 10) | 15.10 ± 4.73 | 12.05 ± 1.46 | 0.00007 | 14.51 ± 2.29 | 11.07 ± 1.53 | 0.00077 |
| Lemongrass 0.5% (n = 10) | 15.21 ± 4.14 | 14.06 ± 4.55 | 0.00045 | 15.28 ± 4.20 | 13.06 ± 2.97 | 0.00004 |
| Lemongrass 1% (n = 12) | 12.63 ± 4.28 | 13.63 ± 4.28 | 0.00006 | 12.76 ± 2.85 | 12.48 ± 2.70 | 0.00007 |
| Picaridin 0.5% (n = 11) | 13.69 ± 5.64 | 11.80 ± 2.50 | 0.00028 | 14.33 ± 5.46 | 12.55 ± 3.60 | 0.00009 |
| Picaridin 1% (n = 12) | 10.58 ± 3.32 | 11.89 ± 3.25 | 0.00250 | 10.88 ± 3.65 | 10.85 ± 2.09 | 0.00520 |
| IR3535 0.5% (n = 10) | 11.46 ± 2.00 | 12.74 ± 2.79 | 0.00009 | 14.27 ± 8.58 | 16.11 ± 15.11 | 0.00006 |
| IR3535 1% (n = 13) | 15.48 ± 8.43 | 12.28 ± 3.31 | 0.00015 | 15.28 ± 7.93 | 11.81 ± 3.41 | 0.00025 |

Supplementary Table 1. Average per fly concordant vs discordant number of rotations. Values shown as mean ± sd. In all groups MOP and OP do not present statistically significant differences (*Kruskal-Wallis: chi-squared = 4.7795, d.f. = 17, p-value = 0.9983*). Conversely, direction within MOP or OP is significant (*Kruskal-Wallis: chi-squared = 306.25, d.f. = 35, p-value < 2.2e-16 + Dunnet test with Benjamini-Hochberg correction*).

217 2. Mean rotation velocity increases as flies move against the direction of gratings.

218 Next, we evaluated whether the presence of repellents could influence the dynamics of
219 the rotational locomotor pattern induced by the visual stimulation and analysed the
220 velocity profiles for each group of flies. First, for each group, we computed the average
221 angular velocity ($\omega_{\text{avg}} = \frac{\theta_2 - \theta_1}{t_2 - t_1}$) with respect to the z-axis during the presentation of visual
222 stimulus (moving gratings on screen) across the 4 trials (Fig. 4 and Tables 2 – 3).
223 Decomposing the data according to the movement directionality, revealed that, for the
224 coherent (+) direction, with respect to the control condition, only **E05** group shows a
225 higher angular velocity. For the coherent (+) direction this effect was, however, not
226 detected when further segregating the data according to the phase (MOP or OP),
227 possibly because of the relatively smaller sample size. The velocity profiles of all the
228 other groups were similar (3W-Robust ANOVA: “group”, $F = 17.04$, $p\text{-value} = 0.061$;
229 “direction”, $F = 3969.60$, $p\text{-value} = 0.0001$; “group: direction”, $F = 34.93$, $p\text{-value} 0.0010$;
230 “stim (MOP/OP)” and related interactions not significant. Post-hoc: *mcppb20* test with
231 Bonferroni correction).

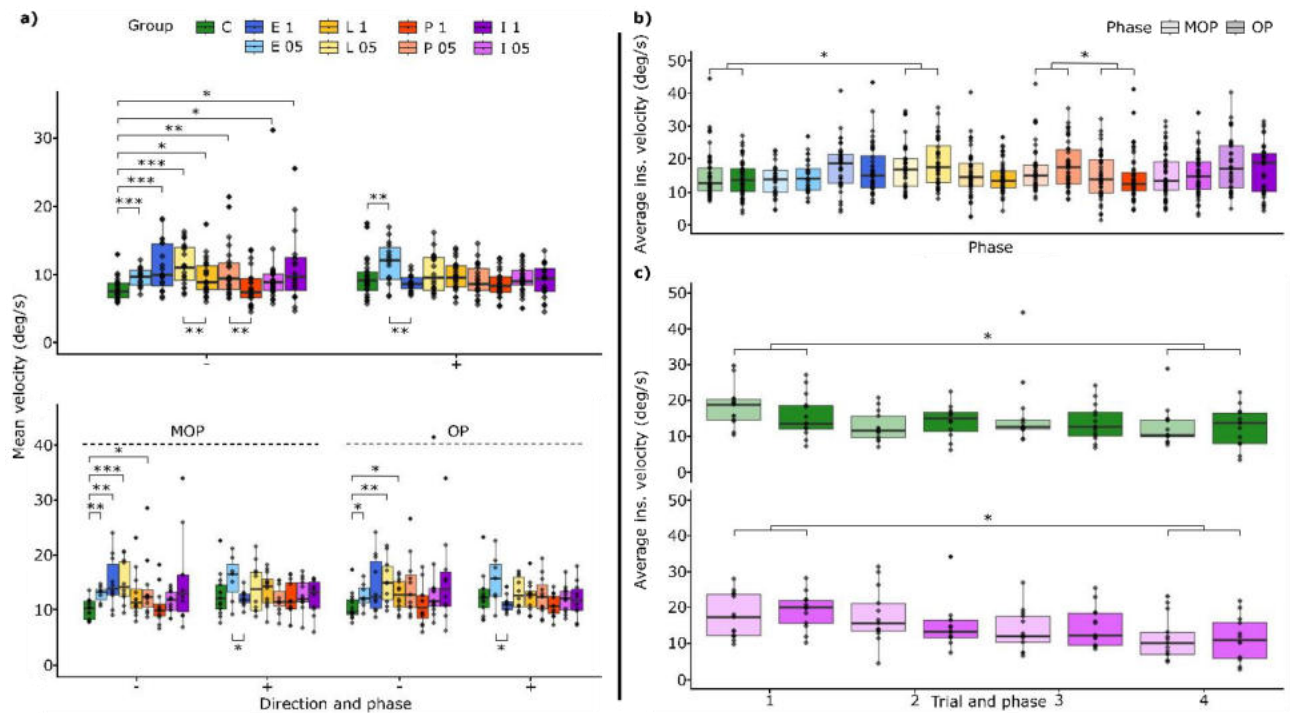
232 In contrast, for the non-coherent (-) direction, several differences could be appreciated:
233 apart from **P1**, all groups showed increased velocity of rotation with respect to controls.
234 Moreover, while **E05 – E1** and **I05 – I1** didn’t show a significant difference between
235 concentrations (**E1** and **I1** values were more spread), this was instead true for **L05 – L1**
236 and **P05 – P1**. When separating between MOP and OP, we found that **E05** and **L05**
237 exhibit increased velocity during both phases, while in the case of **P05** and **E1** the major
238 contribution came from the MOP. **L1**, conversely, moved faster only when the repellent
239 was actively delivered to flies during the OP.

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241 3. Overall instantaneous velocity profiles remain unchanged, with a trend towards
242 reduced values as trials proceed.

243 Next, we wondered whether during the protocol flies could show effects due to
244 muscular fatigue. We then calculated the instant angular velocities ($\omega_{\text{ins}} = \frac{d\theta}{dt}$) integrating
245 the information from all the three axes of rotation (Table 4). After testing, the overall

246 ω_{ins} profiles were similar across all groups, except for **L05** (p-value = 0.0077), which
247 showed higher speeds with respect to **C** (see Fig. 4b and Table 4). For thoroughness, on
248 inspection of the plot **P05** and **I1** also appeared to stand out, but the p-values were just
249 short of significance (0.0733 and 0.0749 respectively). Picaridin was the only repellent
250 showing a significant difference between the two tested concentrations, with **P05**, the
251 lower one, showing higher instantaneous velocity (p-value = 0.0476, 3W-ANOVA for
252 *Aligned Rank Transformed Data: “group”, F = 4.59, p-value = 1.75e⁻⁵; “trial”, F = 3.68, p-*
253 *value = 0.0118; “stim (MOP/OP)” and interactions not significant. Post-hoc: EMMs*
254 *pairwise comparison with Bonferroni correction*).
255 Additionally, as the statistical test showed a slightly positive dependence from the
256 “trial” variable, we analysed each group and found a positive correlation in the **C** and
257 **I05** group. For both (see Fig. 4c – 4d), the velocity values showed a slight progressive
258 reduction along the trial sequence, with a significant difference between the 1st and the
259 4th trial for both **C** (p-value = 0.0325) and **I05** (p-value = 0.0011) which also revealed a
260 positive response between the 1st and 3rd trial (p-value = 0.0407). No other group
261 exhibited a similar trend (2W-ANOVA for *Aligned Rank Transformed Data: “trial”, F =*
262 *3.49, p-value = 0.0185; “stim” (MOP/OP) and interaction not significant + EMMs pair-*
263 *wise comparison with Bonferroni correction*) and **C** group(2W-ANOVA for *Aligned Rank*
264 *Transformed Data: “trial”, F = 3.49, p-value = 0.0426; “stim” (MOP/OP) and interaction*
265 *not significant*).



266

Figure 4. Mean velocity and averaged instantaneous velocities. **Legend:** group colours are shown in the upper left panel with the same label in the main text; in panels **b, c, d**, degree of transparency relates to MOP (lighter) and OP (darker). **(a) Top left:** standard boxplots for the mean speed of flies during the optomotor stimulation in both directions, concordant (+) or discordant (-) with the gratings' movement. **Bottom left:** same plot separated between MOP and OP (test: 2W or 3W-Robust ANOVA + post-hoc *mcppb20* test with Bonferroni correction, see main text and **Tables 2 – 3**). **(b)** Standard boxplots for the averaged instantaneous velocity mediated by subject (see **Table 4**); **(c)** Average ins. velocity along trials of Controls and IR3535 0.5% respectively (test: 2W-ANOVA for Aligned Rank Transformed Data + EMMs pairwise with Bonferroni correction, see main text). P-values: * < 0.05, ** < 0.005, *** < 0.0005.

| Group | Mean rotation velocity (deg-sec-1) | | | | | |
|--------------------------|------------------------------------|--------------|---------|--------------|--------------|---------|
| | MOP | | | OP | | |
| | - | + | P-value | - | + | P-value |
| Controls (n = 13) | 7.55 ± 0.22 | 9.53 ± 0.47 | ns | 7.98 ± 0.30 | 9.41 ± 0.47 | ns |
| Eugenol 0.5% (n = 7) | 9.64 ± 0.18 | 11.76 ± 0.50 | ns | 9.45 ± 0.28 | 11.67 ± 0.55 | ns |
| Eugenol 1% (n = 10) | 11.33 ± 0.56 | 9.04 ± 0.17 | ns | 10.88 ± 0.63 | 8.30 ± 0.18 | 0.0296 |
| Lemongrass 0.5% (n = 10) | 11.40 ± 0.49 | 10.55 ± 0.54 | ns | 11.46 ± 0.50 | 9.79 ± 0.35 | ns |
| Lemongrass 1% (n = 12) | 9.47 ± 0.51 | 10.24 ± 0.37 | ns | 9.57 ± 0.34 | 9.36 ± 0.32 | ns |
| Picaridin 0.5% (n = 11) | 10.27 ± 0.67 | 8.85 ± 0.30 | ns | 10.75 ± 0.65 | 9.41 ± 0.43 | ns |
| Picaridin 1% (n = 12) | 7.94 ± 0.40 | 8.92 ± 0.39 | ns | 8.16 ± 0.43 | 8.14 ± 0.25 | ns |
| IR3535® 0.5% (n = 10) | 9.45 ± 0.24 | 9.56 ± 0.33 | ns | 10.70 ± 1.02 | 12.08 ± 1.80 | ns |
| IR3535® 1% (n = 13) | 11.61 ± 1.00 | 9.21 ± 0.39 | ns | 11.46 ± 0.95 | 8.86 ± 0.41 | ns |

267

Table 2. Average MOP and OP rotation velocities. Values relative to **Fig. 4a** shown as mean ± SD. P-value(s) obtained from within-group paired t-tests on MOP/OP data (Eugenol 1%: t = -2.58 - d.f. = 9).

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| | | | Mean rotation velocity (-) pairwise p-values | | | | | | | |
|-------------|---|----------|--|--------|---------|--------|--------|------|--------|--------|
| | | | E 0.5% | E 1% | L 0.5% | L 1% | P 0.5% | P 1% | I 0.5% | I 1% |
| A L L | - | Controls | <0.0001 | 0.0004 | <0.0001 | 0.0068 | 0.0020 | ns | 0.0108 | 0.0108 |
| | + | | 0.0160 | ns | ns | ns | ns | ns | ns | ns |
| M O P | - | | 0.0016 | 0.0016 | <0.0001 | ns | 0.0284 | ns | ns | ns |
| | + | | ns | ns | ns | ns | ns | ns | ns | ns |
| O P | - | | 0.0476 | ns | 0.0028 | 0.0400 | ns | ns | ns | ns |
| | + | | ns | ns | ns | ns | ns | ns | ns | ns |

Table 3. P-values from the Robust ANOVA and the post-hoc mcppb20 tests. Significance relative to **Fig. 4a**. To reduce the effect of outliers the tests (*Robust ANOVA*, see text and Methods) were run after trimming the outliers with values present in the bottom (<10%) and top (>90%) of the data. Post-hoc mcbbp20 tests p-values were adjusted with Bonferroni correction for multiple comparisons.

272

| Group | Average instantaneous angular velocity (deg.sec-1) | | |
|--------------------------|--|---------------|--------------------------|
| | MOP | OP | P-value (vs Controls) |
| Controls (n = 13) | 14.91 ± 6.78 | 14.96 ± 8.5 | / |
| Eugenol 0.5% (n = 7) | 13.54 ± 4.88 | 14.59 ± 5.02 | ns |
| Eugenol 1% (n = 10) | 17.74 ± 7.12 | 16.73 ± 7.76 | ns |
| Lemongrass 0.5% (n = 10) | 17.73 ± 7.27 | 18.91 ± 7.00 | 0.0077 |
| Lemongrass 1% (n = 12) | 15.60 ± 6.80 | 14.42 ± 4.78 | ns |
| Picaridin 0.5% (n = 11) | 16.84 ± 7.00 | 18.26 ± 7.08 | ns |
| Picaridin 1% (n = 12) | 14.84 ± 6.85 | 14.12 ± 7.21 | ns |
| IR3535® 0.5% (n = 10) | 15.14 ± 6.82 | 19.14 ± 30.56 | ns |
| IR3535® 1% (n = 13) | 18.19 ± 8.08 | 17.19 ± 7.61 | ns |

Table 4. Mean and standard deviation values for the instantaneous angular velocity. Values referring to **Fig. 4b**. No significant differences were found between **MOP** and **OP**. The only significant difference with respect to **C** is observed for **L05**; **P05** and **P1** are the only pair with a within group significant difference ($p = 0.0476$). P-values extracted from *Aligned Rank Transformation ANOVA + post-hoc Estimated Marginal Means with Pairwise Comparisons (EMMs) and Bonferroni correction*, see main text.

273 4. *Low concentrations of natural repellents reduce the time interval between bursts*
274 *of movement.*

275 Following the observations on the average velocity, we reasoned that the higher
276 velocity found during Eugenol and Lemongrass (all concentrations) stimulation might
277 be due to an increased sensory engagement causing livelier patterns of movement. We
278 thus checked whether repellent delivery was affecting how frequently flies alternated
279 pauses and bouts of locomotor activity, and how long these periods of
280 activity/inactivity lasted.

281 For this purpose, we calculated the angular acceleration values ($\alpha_{ins}(z) = \frac{d\omega}{dt}$) of the
282 trajectory around the z-axis to extract “go” and “stop” epochs based on the relative
283 acceleration values with respect to the time averaged value. We found that, across the
284 4 trials, the average number of “stop” and “go” did not change between groups (see Fig.
285 5b, further down); also, the average duration of the movement bouts (Fig. 5a, top) did
286 not show appreciable differences with respect to **C** (MOP: mean $369.37 \pm$ SD 56.20
287 milliseconds, OP: 370.40 ± 48.37 ms) except in the case of the **P05** group (MOP: 336.47
288 ± 40.67 ms, OP: 334.12 ± 49.86 ms), which exhibited slightly shorter bouts (p-value =
289 0.0003, 2W-ANOVA for Aligned Rank Transformed Data: “group”, $F = 6.89$, p-value =
290 $8.79e-9$; “stim (MOP/OP)” and interaction not significant+ EMMs pairwise comparison
291 with Bonferroni correction). On the other hand (Fig. 5a, bottom), the average duration
292 of the “stop” periods was reduced for low concentrations of the natural repellents **E05**
293 and **L05** (MOP: 171.15 ± 30.89 ms, OP: 174.80 ± 30.29 ms; p-value = 0.0042; MOP: 180.43
294 ± 55.94 ms, OP: 183.85 ± 42.51 ms; p-value = 0.0266) with respect to **C** (MOP: $199.17 \pm$
295 38.90 ms, OP: 196.51 ± 42.58 ms) but without appreciable difference between the MOP
296 and OP (2W-ANOVA for Aligned Rank Transformed Data: “group”, $F = 4.24$, p-value =
297 $5.41e-5$; “stim (MOP/OP)” and interaction not significant + EMMs pairwise comparison
298 with Bonferroni correction).

299

300 5. The direction of the acceleration at movement onset is random and not influenced
 301 by odour presentation

302 One last parameter we considered was the orientation of each first bout of movement
 303 performed by the flies after a “stop” while the visual stimulation was present (Fig. 5c).
 304 Our goal was to see whether initiation of movement could be directed and shaped by
 305 the interaction with the concomitant odour. In this respect our expectation was to see
 306 a decisive shift away from the odour, at least in those groups which were showing a
 307 decisively negative (**E1, I1**) or at least mixed (**L05, L1, P05, P1**) response with respect to
 308 the repellent.

309 In the end, we did not find any significant differences: in each group, the movement
 310 onset direction seemed to initiate randomly at the beginning of locomotion bouts.

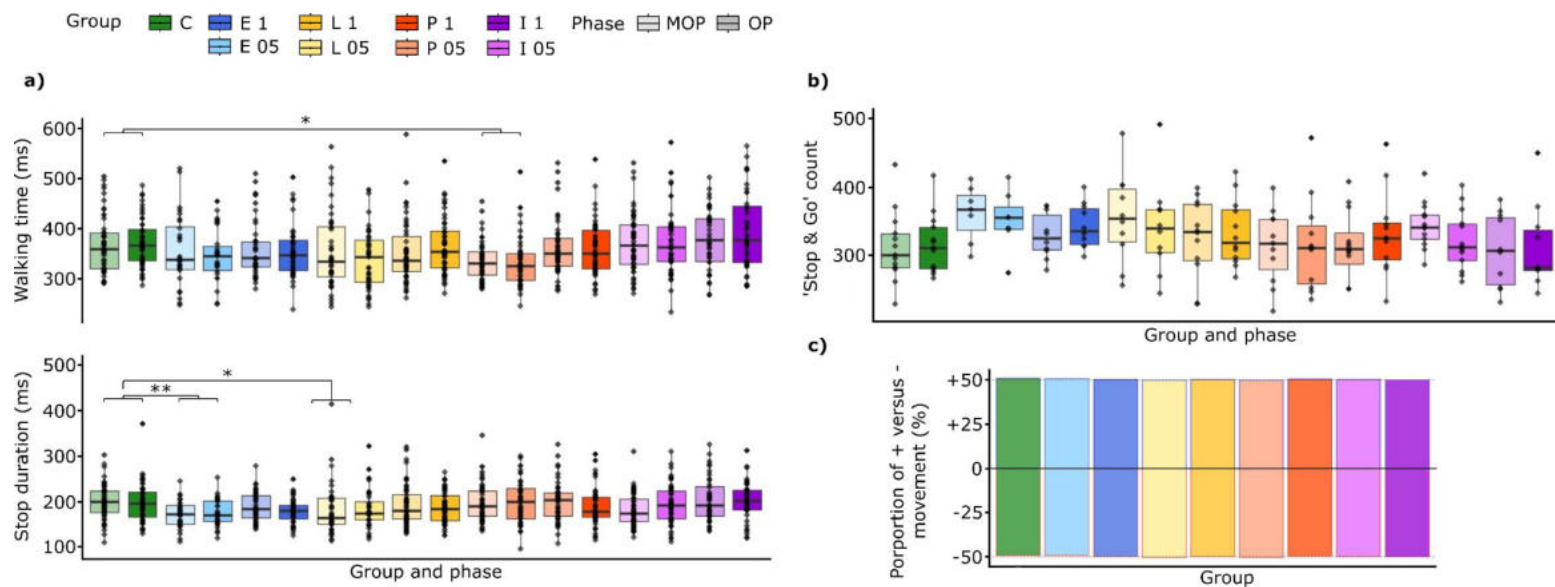


Figure 5. Mean velocity and averaged instantaneous velocities. Legend: group colours are shown in the upper left panel with the same label in the main text; in panels **a** and **b**, the degree of transparency relates to MOP (lighter) and OP (darker). **(a)** Standard boxplots for the mean duration (milliseconds) of walking (“Go”) and idle (“Stop”) periods (tests: 2W-ANOVA for Aligned Rank Transformed Data. **(a)**: “group”, $F = 6.89$, $p\text{-value} = 8.79e-9$; “stim (MOP/OP)” and interaction not significant + EMMs pairwise comparison with Bonferroni correction; **(b)** “group”, $F = 4.24$, $p\text{-value} = 5.41e-5$; “stim (MOP/OP)” and interaction not significant+ EMMs pairwise comparison with Bonferroni correction [see main text for significant post-hoc $p\text{-values}$]). **(b)** Standard boxplots for the “Go” and “Stop” count, mediated between subjects, in each group and phase (test: Pearson’s Chi-squared $X = 2.07 - p = 0.97$). **(c)** Proportion of the direction of the first movement at the onset of walking episodes concordant (+) or discordant (-) with the gratings’ direction of movement. (test: Pearson’s Chi-squared $X = 5.16 - p = 0.74$ respectively). P-values: * < 0.05, ** < 0.005.

311

312 Discussion

313 A resume of the results we observed in our experiment is shown in Table 5 below.

314

315

| | Trajectories | | Avg. speed (MOP -) | | Avg. speed (OP -) | | 'Stop' count | | 'Stop' length | | 'Go' length | |
|------------|--------------|----|--------------------|----|-------------------|----|--------------|----|---------------|----|-------------|----|
| | 0.5% | 1% | 0.5% | 1% | 0.5% | 1% | 0.5% | 1% | 0.5% | 1% | 0.5% | 1% |
| Eugenol | ++ | -- | ↑ | ↑ | ↑ | = | = | = | ↓ | = | = | = |
| Lemongrass | - | + | ↑ | = | ↑ | = | = | = | ↓ | = | = | = |
| Picaridin | - | + | ↑ | = | = | = | = | = | = | = | ↓ | = |
| IR3535 | ++ | -- | = | = | = | = | = | = | = | = | = | = |

Table 5. Recap of the results for the walking experiment. Red “-” indicates the direction discordant with the grating mask movement, while the green “+” is for concordant directions; “=” means no change is observed.

316 From the qualitative point of view, it stands out immediately that the presence of
 317 repellents alters the prevalent direction of movement of flies undergoing the OMR
 318 stimulation. As mentioned in the Methods section, we decided *a priori* to exclude from
 319 the analysis those animals which did not show a pattern of rotation consistent with the
 320 OMR during the training phase and, therefore, it is safe to assume that the observed
 321 magnitude of *contra*-gratings movement was indeed elicited by the aversive effect of
 322 the delivered odours.

323 Most of the differences are found within the natural repellent groups (Eugenol and
 324 Lemongrass), but at least some of the measured parameters are indicative of an
 325 unexpected behaviour in response to the so-called maskers. Regarding the natural
 326 repellents, flies exposed to highest (1%) Eugenol concentration show a marked
 327 alteration in their walked path, moving away from the repellent source (in the opposite
 328 direction of the OMR stimulus). A similar effect, although not significant is observed in
 329 the lowest (0.5%) Lemongrass concentration. As per the maskers, picaridin-exposed
 330 flies show mixed trajectories and suggest a possible perturbation in their action
 331 planning – the consequent motor programs – similarly to the flies experiencing the
 332 proper repellents. On the other hand, many of the analysed features do not appear as
 333 altered in flies exposed to IR3535: notably, while low concentrations of this masker do
 334 not alter trajectories with respect to the control group, the higher concentration (1%)

335 stands out showing a strongly opposite direction of rotation with respect to Controls,
336 just as in the case of the highest eugenol concentration.

337 Another noteworthy aspect is the difference shown by the two concentrations utilized
338 for all odorants in our experiments. When such concentration-related mixed responses
339 are detected, a trend appears for a stronger/relevant response at the lower
340 concentrations tested. Again, we tested compounds (Eugenol, Lemongrass, Picaridin,
341 and IR3535) and concentrations (0.5% and 1%), which proved to be functional for
342 studying the response to the OKN stimulation. In turn, the results on the OMR here
343 suggest that the modulation acting on the final behavioural output is possibly not
344 peripheral, but could be highly sensitive to low concentrations, thus resulting in the
345 rapid saturation of the system. However, another possible, and not necessarily
346 exclusive, explanation may be that what we are observing is not a “simple” variation in
347 the quantitative aspect of a single response, but rather that different outputs are
348 selected among a possible repertoire of behaviours, depending on the perceived
349 strength of the odorant. The conceivable uncertainty present in conditions of
350 attenuated olfactory stimulation – which would hypothetically involve a greater level of
351 integration and a consequently larger “computational” load in the selection of the most
352 suitable behaviour – would be lost at system saturation, promoting a more decisive and
353 unequivocal response, much as we observed in the comparison between the low and
354 high eugenol concentrations.

355 In fact, we did not find any difference between the MOP and OP phases within each
356 combination of odorant and concentration, as instead was the case in our previously
357 reported OKR-flight paradigm ^[23]. This is likely due to the greater complexity of the
358 control mechanisms of the OMR, which rather than being immediately impacted by the
359 administration of the odorants is possibly remodelled in a slower and perhaps more
360 constrained manner ^[25].

361 In our OMR task, flies can be seen as engaged in a response guided towards a preferred
362 direction by the elicited optic flow. Something similar, although not OMR related, was
363 described in Frighetto *et al.* (2019) ^[26]: the presentation of a (visual) distractor was
364 sufficient to cause a temporary disengagement from a Buridan paradigm. In our results

365 it may be that this is not happening during the OMR (as was instead previously observed
366 by us to occur in the case of the OKR ^[23]) in which case the response was not suppressed
367 but rather modulated. The odours act as distractors involving a different sensory
368 pathway, which may be possibly harder to integrate than a second visual distractor. The
369 more complex responses appreciated during the OMR could be therefore explained by
370 a remodulation or reshaping occurring within an already engaged motor program,
371 instead of a process involving the selection of a new action after disengaging from the
372 ongoing one.

373 Indeed, in these experiments, OMR is observed during grounded locomotion, which is
374 the complex result of the interaction between the local thoracic circuits, called CPGs,
375 generating spatio-temporally organised patterns of movements of the animal
376 appendages and their modulation by both the cerebral ganglia, the central complex
377 (CX), the mushroom bodies (MB) and the gnathal ganglia, as reviewed in Emanuel *et al.*
378 (2020) ^[27].

379 It is known that the cerebral ganglia, which are centres integrating both visual and
380 olfactory information, are involved in the regulation of probably mostly inhibitory
381 walking behaviours, as demonstrated in experiments where insects from different
382 species deprived of the circumesophageal connectives – which link the gnathal and
383 cerebral ganglia – have long and unoriented bouts of movement ^[27]. In fruit flies, the
384 inhibitory effect of the MB was demonstrated by Martin, Ernst and Heisenberg (1998)
385 ^[28], who observed highly increased walking activity in flies with ablated MB or defective
386 Kenyon cells; meanwhile, Serway *et al.* (2009) ^[29] and Mabuchi *et al.* (2016) ^[30]
387 demonstrated the MB role in enhancing activity when initiating light-evoked walking
388 and maintaining the physiological daily rhythmic activity ^[27,29,30].

389 Concurrently, it has been shown that mutant flies with alterations of the CX's subunits
390 present reduced walking activity in terms of unreliability of movement initiation and
391 reduced speed or increased lag between consecutive bouts ^[27,31–33]. Moreover, in
392 cockroaches, it has been demonstrated that a reduction in the CX's synaptic drive
393 diminishes the excitatory activity on the thoracic CPGs with decreased activity of the
394 octopaminergic neurons ^[34].

395 In fruit flies, at least two neuropeptides, tachykinin and the short neuropeptide F, are
396 expressed within the CX; their suppression leads, respectively, to increased bout
397 frequency and augmented speed and distances walked [27,35].

398 As in the OKR, where specific DNs vehicle the information down to the motor neurons
399 regulating head movements (DN9), the modulation on dopaminergic DNs – with
400 possible circuits to the ventral nerve cord for both the CX and MB [36,37]: CX → *lateral*
401 *accessory lobes (LAL)* → *posterior slope (PS)*; MB → *superior medial protocerebrum (SMP)*
402 → LAL → PS – reaching the thorax, could be a good candidate. However, when Tschida
403 and Bhandawat (2015) [38] studied a couple of such DNs, they found evidence of the
404 correlation between the activity of those DNs and specific patterns of leg movement
405 and walking speed, but they did not notice any difference when stimulating flies with
406 attractive odours eliciting strong responses in terms of leg movements. This
407 observation showed that, at least in these neurons, the major modulation happens
408 during leg movement and may not be influenced by the nature of the stimulus
409 provoking them. Interestingly, recent studies (Feng *et al.*, 2024; Rayshubskiy *et al.*, 2025)
410 [39,40] characterized several DNs types (DNa01, DNa02, DNa03) involved specifically in
411 turning control during walking, which nonetheless do have indirect connections with
412 wings' motor neurons and are engaged in stimulus-directed steering in walking (DNa2)
413 [40], or have been shown to also promote steering during flight (DNa3) [39].

414 The quantitative changes we illustrated here are indeed partly described by an
415 increased average velocity and a reduced interval between bouts (“stop” length),
416 compatible with reduced inhibitory regulation from the MB-CX, which in turn would
417 promote an increased motor activity in presence of the aversive odour. In just one
418 specific case, **E1**, a minor increase in velocity was also associated with a trajectory of
419 decisively opposite direction with respect to the control group. It is possible to assume
420 a more complicated scenario than during a rigidly tethered flight OKR paradigm, which
421 may involve more complex responses to arousal and novelty (see for example **E05** which
422 trajectories are akin to controls while presenting an enhanced motor activity despite
423 experiencing a known, to *D. melanogaster*, repellent stimulus) as well as decision-
424 making processes gated through the CX-MB.

425 One last point worth mentioning is that we did not observe, apparently, any evidence
426 for possible memorization and learning processes in the terms of an enhancement of
427 the response as a function of time, as no major differences in the variables we measured
428 did change over the trials. On the other hand, neither a “decline” in performance was
429 observed. The only exception to this trend was found in the average velocities of **C** and
430 **I05**, which showed a decline over time. Possible explanations may be fatigue or
431 habituation. However, we consider the case of fatigue less likely, as we would have
432 expected still a more widespread decrease in the average speed even in presence of
433 stronger aversive odours, which provoked a change in the direction of movement. It
434 seems therefore likely that perceived aversive odours could have helped keeping the
435 flies engaged during the task, as they actively tried to move away, but did not trigger a
436 – maybe unnecessary – learning process for achieving a “better” escape behaviour as
437 the aversive stimulation kept being around. However, resolving these last speculations
438 will require ad hoc experimental paradigms.

439 In conclusion, the results we obtained show that the substances commonly used in
440 many publicly available products effectively affect fundamental mechanisms, like the
441 OKR, or intervene in the modulation of more complex processes, as in the OMR, which
442 constitute the basis of a complex repertoire of behaviours concurring to the
443 navigational abilities of insects. As we still have limited knowledge on how and why the
444 final output (behaviour) we observe from this multimodal sensory integration is
445 achieved and selected among other possibilities, the fact that some compounds could
446 act on unpredicted components of behaviours in a plethora of species sharing the same
447 neural architecture and organisation should be worrisome: more study is necessary to
448 explore and better characterize the deeper effects of repellent substance in order to
449 fine tune their application while avoiding any abuse which could become harmful
450 against unwanted or protected targets, such as pollinator insects.

451

452 **Materials and methods**

453 *Fly strains*

454 The Berlin-K strain was kindly provided by Prof. Christian Wegener (University of

455 Würzburg).

456 Flies were reared in vials containing 10 ml of standard cornmeal medium, with a 12h:12h
457 dark-light cycle with controlled temperature (26 °C, at 45 ± 10% humidity). Female flies
458 were separated from males and collected under CO₂ anaesthesia within 24 h from pupal
459 eclosion, then given at least 24 hours to recover. Adult female flies aged between 3 and
460 6 days were tested within 6 hours from the light onset.

461 Every group of flies was evaluated for only one (1) repellent and concentration, meaning
462 that one group experienced, e.g., eugenol, concentrated at either 0.5% or 1%.

463 Each recording was manually checked before the tracking, and only the flies which
464 walked consistently throughout the duration of the experiments were tracked.
465 Likewise, bad tracking results, which could be caused by illumination issues and/or
466 excessive self-grooming by the flies, were discarded altogether and are not part of the
467 analysis shown in this paper.

468

469 *Fly preparation*

470 Flies were transferred from the rearing vial to an empty one which was then put on ice.
471 After the cold anesthetization, single flies (one at a time) were transferred to the
472 mounting block, which could be kept cold (down to +4° C) via a Peltier platform laid on
473 a fan heat-sink and carefully placed upright inside the dedicated groove of the
474 mounting support. The Peltier temperature was set to 14° C to minimize the cold
475 experienced by the flies. The tip of a 34-gauge dispensing needle (BSTEAN, Shenzhen
476 Hemasi E-Commerce Co., Ltd., PRC) was dipped in UV hard resin (DecorRom,
477 Shenzhenshi Baishifuyou Trading Co., Ltd., PRC) removing the excess quantity (barely
478 one drop remaining on the tip). The needle was then placed on a support angled at 120°
479 with respect to the fly's horizontal body axis (considering the frontal portion of the
480 thorax as the 0° angle) and, with the aid of a micromanipulator, lowered onto the fly,
481 touching the centre of the thorax; the resin was then cured for 45 - 60 seconds with a
482 UV torchlight to glue the animal to the pin. The whole tethering procedure took about
483 2 minutes. Flies were let to recover from the procedure for about 15 minutes while
484 resting on a piece of paper of appropriate dimensions. The flies were then transferred

485 and mounted inside the experimental setup, and we verified that they could easily move
486 while on the Styrofoam sphere. Badly glued or unwilling flies were discarded.

487

488 *Experimental apparatus*

489 The experiment was conducted in a home-built dark chamber (components bought
490 from Thorlabs Inc., US), with side access, containing all the hardware.

491 A support, mounted on a micromanipulator, ends with a syringe attachment where the
492 pin together with the glued fly can be secured, by the side of the monochrome camera
493 (MQ003MG-CM, Ximea GmbH, Germany). The camera is equipped with an infra-red
494 (IR) bandpass filter and two IR (850 nm) LEDs (M850L3, Thorlabs Inc., US) provide the
495 necessary illumination. The camera resolution was VGA 0.3 MP 648 × 488 pixels, with a
496 pixel size of 7.4 μm and a maximum frame rate of 500 frames per second.

497 The other components of the apparatus include:

- 498 ▪ the projector (Lightcrafter 4500, Texas Instruments Inc.), placed in front of the
499 screen, with a refresh rate of 60 Hz, with a maximal output illuminance of 621.5
500 lux at the centre of the screen and 435 lux at 45° of azimuthal deflection.
- 501 ▪ an adjustable, curved, hand-crafted screen (radius = 6.5 cm, height = 13 cm), made
502 of parchment paper, placed in front of the animal (distance of 5 cm), with an
503 illuminance attenuation factor of 10. The actual projected display had an azimuth
504 of ± 90°, an elevation and depression angle of 22.5°, and a resolution of 1280 x
505 800 pixels.
- 506 ▪ the custom walking machinery, composed of the 3D-printed, M-shaped, sphere
507 holder, a Styrofoam ball hand-painted with an irregular black spot pattern, and
508 the air jet pump (ADD HERE) to keep the sphere slightly floating.
- 509 ▪ the custom odour-delivery system made up of plastic tubing of various
510 diameters, assorted luers (Ark-Plas Products Inc., US), glass capillaries (GB150F-
511 10, Science Products GmbH, Germany), an Arduino (UNO REV3, Arduino, US)
512 controlled solenoid valve (SIRAI Elettromeccanica S.r.l., Italy), two glass vials
513 containing the solutions, and an air pump (Air Professional 150, PRO.D.AC
514 INTERNATIONAL S.r.l., Italy) for the vaporizing.

515 ▪ the custom odour-recycling system, consisting of plastic tubing, an externally
516 alimented suction unit (VN-C4 vacuum pump, You Cheng Industrial Co., Ltd.,
517 Taiwan), plus the glass flask used to achieve negative pressure and the
518 subsequent suction.

519 The tubing was placed perpendicularly to the fly, at around 1 cm and 2 cm for the
520 delivery (on the fly's right) and the recycling (on the fly's left) system, respectively.
521 Different (clean) tubing, glass capillaries, and vials were used for the different
522 compounds.

523 Differently from our previous work, we used a dedicated computer (Workstation HP
524 Pro Tower 400 G9 PCI: Win 10; Intel 12th Gen. 12500 6 cores 3 GHz processor; 16 GB
525 RAM) both for delivering the stimuli and the recording, which was achieved directly
526 through the Ximea Software GUI. A U3-LV Labjack (Labjack Corporation, US), in tandem
527 with an Arduino Uno (Arduino[®], US) system, controlled the synchronization between
528 the stimuli delivery and the recording timings of the camera, which analogically
529 received inputs from the Labjack-Arduino system.

530 We programmed the Arduino Uno to control the relevant camera triggers synchronized
531 to the Python script controlling the presentation of the visual stimuli (designed through
532 the open-source PsychoPy[®] toolbox, Open Science Tools Ltd., UK), and the modulation
533 of the solenoid valve. Sampling rate of the camera was locked at 200 frames per second.

534

535 *Repellent compounds*

536 The chosen repellent substances (eugenol, lemongrass oil, picaridin, and IR3535[®] *alias*
537 'Nb[n-N-butyl-N-acetyl] aminopropionic acid ethyl ester') were purchased at the
538 highest purity available (min. 95 %) from Biosynth[®] Ltd, UK.

539

540 *Pilot study experimental paradigm*

541 The paradigm is structured similarly as described in the further section (seen Suppl.
542 Fig. 1 for a graphical representation). This paradigm derives from a former version we
543 utilized in a previous study where the two MOP (Mineral oil only Phase) and OP
544 (Odorant Phase) phases run consecutively (30 sec. each) with no pause in between.

545 *Experimental paradigm*

546 The paradigm was structured in 2 (two) quick “training” phases to get flies acquainted
547 to their tethered condition, followed by 4 (four) repetitions of the proper trial, which
548 was subdivided in 2 portions (Fig. 1a). Within the training, as well as during the first part
549 of the trial, flies faced visual stimulation (moving in the same direction throughout all
550 the experiment; same as in the second part) without experiencing any odour, as they
551 were instead delivered a plume coming from a plain mineral oil solution (Mineral Oil
552 Phase, MOP). Within the second part of the same trial, the solenoid valve switched at
553 the set time, causing the air plume to come instead from the vial containing the
554 repellent-enriched mineral oil solution (Odorant Phase, OP). The training and trial were
555 structured as follows:

556 Training

- 557 a. **SF**: one 12° vertical black bar on a white background, presented in the middle of
558 the screen (duration: 5 seconds).
- 559 b. **MOP**: a mask of gratings made up of 12° alternate black and white vertical bars
560 (spatial wavelength of 24°) moving clockwise at a fixed speed of 15 deg·s⁻¹ for a
561 duration of 60 seconds.
- 562 c. Same as (a.).
- 563 d. Same as (b.).
- 564 e. **Pause** in darkness (duration: 20 seconds).

565 Trial

- 566 a. **SF**: one 12° vertical black bar on a white background, presented in the middle of
567 the screen (duration: 10 seconds).
- 568 b. **MOP + OP**: a mask of gratings made up of 12° alternate black and white vertical
569 bars (spatial wavelength of 24°) moving clockwise at a fixed speed of 15 deg·s⁻¹ for
570 a duration of 60 seconds.
- 571 c. **Pause** in darkness (duration: 20 seconds).

572 Stimuli were drawn at the maximum contrast available, resulting in a Michelson
573 contrast of 0.61.

574 For further clarification, the air plume was continuous, from the start of the training to

575 the end of the trials and was coming always from the same direction, from the right to
576 the left of the animal, which was the direction opposite to the optokinetic stimulation.
577 The Control group never experienced the OP, but two consecutive MOPs instead.

578

579 *Data extraction*

580 Recordings were manually checked and only flies which kept an active walking
581 behaviour (i.e., moving > 90% of the time during the optomotor task) were kept and
582 analysed.

583 The tracking of the animal was conducted offline through the self-contained FicTrac^[41]
584 program: we manually set from the GUI, for each recording, the sphere's ROI and the
585 masks for identifying the fly and the pins (Fig. 1b); further adjustments were made to
586 the settings file for the binarization and the thresholding of the input video, as
587 suggested by the FicTrac's author. The recording was checked live to assess the
588 correctness of the automated tracking procedure through the debugging FicTrac
589 feature.

590 The FicTrac output data (X, Y, Z coordinates, and the timestamps) were imported into
591 RStudio for the subsequent analysis (Fig. 1c).

592 X, Y, and Z data were smoothed applying a simple moving average (SMA) with 10 *sample*
593 *length* before further calculations of the related per-fly weighted means. Mean
594 velocities were computed as the total path length (sum of the position's differentials
595 absolute values in degrees) walked over 60 seconds (trial duration). Instant velocities
596 and accelerations were not directly imported from the FicTrac output, but rather
597 manually computed after smoothing the position data with a Savitzky-Golay low-pass
598 filter with a cut-off at 10 Hz to remove high frequency noise.

599 Regarding the analysis on the “stop” and “go” windows, we defined as “stops” the time
600 windows where $\alpha_{\text{ins}} < 10\%$ of the time-averaged α_{ins} . Equally, “go” were defined by values
601 higher than the set 10% threshold, plus we only considered periods as proper “go”
602 phases those lasting at least 100 milliseconds. Then, we tagged the average α_{ins} values
603 as coherent (+) or non-coherent (-) over the minimum span of *bona fide* “go” periods we
604 set (100 ms), when the ratio of the negative α_{ins} versus positive α_{ins} showed to be

605 unbalanced towards one of the two (-/+) α_{ins} with a minimum threshold of 60%.

606

607 *Statistical analysis*

608 The whole analysis was conducted in R-Studio (v4.4.1.) with a significance level $\alpha = 0.05$.

609 Data were checked for normality (Shapiro-Wilkins test) and heteroscedasticity
610 (Bartlett's test when the distributions were not normal) across groups.

611 In the pilot experiment, statistical significance regarding velocity and rotation amount
612 were assessed through ANOVA + Tukey HSD. The differences between trajectories were
613 analysed by comparing the slopes of the regression lines, obtained from the 'lm'
614 function, with the 'emmeans' and 'pairs' functions (EMMs, "emmeans" R package).

615 In regard to the main experiment, the statistical significance for trajectories and
616 quantity of movement (not all groups had normal distributions, and the variance was
617 no homogeneous) was assessed through Kruskal-Wallis plus post-hoc Dunnet test and
618 Pairwise Test for Equality of Proportions respectively, both adjusted with Benjamin-
619 Hochberg correction for multiple comparisons – which we preferred to the Bonferroni
620 correction (used later) due to the multiple levels in the test. We also run Wilcoxon
621 signed rank exact test comparison within each group to compare the result with the
622 multifactorial Kruskal Wallis + Dunnet test, but we did not find significant differences
623 between the "MOP/OP" phases.

624 In regarding to the analysis of the mean velocities, to reduce the effect of outliers, we
625 compared the results from the Kruskal-Wallis and post-hoc Dunnet test with robust
626 ANOVA for trimmed means followed by the related post-hoc mcppb20 test with
627 Bonferroni adjustment ("WSR2" R package), which we refer to in the results as it proved
628 more conservative.

629 Not all groups had their instantaneous velocities values normally distributed; therefore,
630 for a multi-factorial analysis of velocities and "Stop" and "Go" periods we opted for a
631 non-parametric ANOVA after aligned rank transformation test ("ARTool" R package) for
632 assessing the relevant effects and interactions to then build a linear model for
633 subsequent Estimated Marginal Means (EMMs, "emmeans" R package) computation and
634 pairwise comparisons with Bonferroni adjustment.

635 **Abbreviations**

636 **C:** control group; **CX:** central complex; **E05:** group exposed to eugenol 0.5% solution;
637 **E1:** group exposed to eugenol 1% solution; **HOKN:** head optokinetic nystagmus; **I05:**
638 group exposed to IR3535[®] 0.5% solution; **I1:** group exposed to IR3535[®] 1% solution; **L05:**
639 group exposed to lemongrass 0.5% solution; **L1:** group exposed to lemongrass 1%
640 solution; **IR:** infra-red; **MB:** mushroom bodies; **MOP:** mineral oil phase; **OF:** optic flow;
641 **OKR:** optokinetic response; **OMR:** optomotor response; **OP:** odorant phase; **P05:** group
642 exposed to picaridin 0.5% solution; **P1:** group exposed to picaridin 1% solution; **SF:**
643 stripe fixation.

644

645 **Data Availability**

646 An R-markdown file retracing the content of the paper is available in the “OMR-
647 Repellents” repository ([Github](#)), where a google-drive link is also provided to access the
648 original generated Dataframes. Additionally, the pre-processed data, along with a video
649 sample are hosted in the “*Repellent olfactory cues influence the optomotor response*
650 *modulation in Drosophila melanogaster - Data*” repository at Zenodo.com (DOI:
651 <https://doi.org/10.5281/zenodo.18416157>).

652

653 **Author contributions**

654 GMM, PV, AD, and AM designed the experiment. GMM, MB, and AM set up the
655 experimental apparatus and wrote the related scripts. GMM designed both the
656 experimental paradigms. GMM and SZ acquired the data. GMM carried out the pre-
657 processing (together with SZ) and the data analysis. GMM drafted the manuscript, with
658 contribution from MB, AM, and MDM, and prepared the figures and tables. All authors
659 contributed to the interpretation of data and the revision of the manuscript.

660

661 **Conflict of interest statement**

662 The authors have declared no conflict of interest.

663 Entostudio S.r.l. is not involved in the production nor the distribution of any of the
664 tested compounds and did not receive any funding related to the research presented
665 in this article.

666 The work presented in this paper was funded by the PON - DM 1061 PhD scholarship
667 from Italian Ministry for University and Research (MUR) assigned to Menti G. M., the
668 Università degli Studi di Padova's DOR funding to Megighian A and Dal Maschio M.

669

670 **Acknowledgements**

671 Authors thank Prof. Christian Wegener, from Biocenter - University of Würzburg (DE),
672 who kindly provided the Berlin-K *Drosophila* strain, and Dr. Paola Cisotto, from the
673 Biology Department - University of Padova (IT), who supported us with the caring and
674 nurturing of the flies' strains.

675

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