



OPEN **Optokinetic response in *D. melanogaster* reveals the nature of common repellent odorants**

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Animals' ability to orient and navigate relies on selecting an appropriate motor response based on the perception and integration of the environmental information. This is the case, for instance, of the optokinetic response (OKR) in *Drosophila melanogaster*, where optic flow visual stimulation modulates head movements. Despite a large body of literature on the OKR, there is still a limited understanding, in flies, of the impact on OKR of concomitant, and potentially conflicting, inputs. To evaluate the impact of this multimodal integration, we combined in *D. melanogaster*, while flying in a tethered condition, the optic flow stimulation leading to OKR with the simultaneous presentation of olfactory cues, based on repellent or masking compounds typically used against noxious insect species. First, this approach allowed us to directly quantify the effect of several substances and of their concentration on the dynamics of the flies' OKR in response to moving gratings by evaluating the number of saccades and the velocity of the slow phase. Subsequently, this analysis was capable of easily revealing the actual effect, i.e. masking vs. repellent, of the compound tested. In conclusion, we show that *D. melanogaster*, a cost-affordable species, represents a viable option for studying the effects of several compounds on the navigational abilities of insects.

Keywords *Drosophila melanogaster*, Repellents, Optokinetic response, Navigation

Abbreviations

C	Control group
E05	Group exposed to eugenol 0.5% solution
E1	Group exposed to eugenol 1% solution
HOKN	Head optokinetic nystagmus
I05	Group exposed to IR3535 0.5% solution
I1	Group exposed to IR3535 1% solution
L05	Group exposed to lemongrass 0.5% solution
L1	Group exposed to lemongrass 1% solution
IR	Infra-red
ISI	Inter-saccade interval
MOP	Mineral oil phase
OF	Optic flow
OKN	Optokinetic nystagmus
OKR	Optokinetic response
OP	Odorant phase
P05	Group exposed to picaridin 0.5% solution
P1	Group exposed to picaridin 1% solution

The navigational abilities of insects are remarkably close, if not equal, to the skills exhibited by animals commonly addressed, incorrectly, as “more evolved” or, better, as more complex. Meanwhile, the debate is still ongoing, asking whether these notable similarities may be identified as analogies due to the convergent evolution of independent nervous systems^{1–3}, rather than reflecting homologies originating from the last common ancestors

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between Invertebrates and Vertebrates^{4,5}. The study of insects' navigation flourished since the end of the 19th century⁶, resulting nowadays in extremely refined studies from the anatomical, physiological, engineering, and physics point of view. Nevertheless, at present, unravelling the components and processes which generate the repertoire of behaviours adopted by the insects when exploring and moving in the environment is becoming of even more importance for tackling the challenges placed by some species to agriculture and human health.

In Europe, the increasing presence of alien insect species, exacerbated by the occurring variations in climate, is posing a serious threat to cultivation, as is the case for the dipteran *D. suzukii* from Southeast Asia⁷, and to public health, which is endangered by several vector-transmitted diseases spread by insects like mosquitoes and ticks⁸. Moreover, in 2012, the approval of the EU Biocidal Product Regulation thinned the list of compounds available for companies and consumers to distribute and purchase⁹. Regarding the public health aspect, and particularly the threat posed by mosquitoes¹⁰, a deeper comprehension of the effects of the repellent compounds still publicly available for production and commercialization could increase the efficacy of repellent products and enhance the global protection against vector insects in the population. However, identifying a suitable approach to study these aspects is still challenging: the rearing of mosquitoes and other pest species requires special attention and carefulness (see the latest 2021–22 FAO/AIEA guidelines^{11,12}), which are not always easy to satisfy in public laboratories with limited spaces and resources. In turn, *D. melanogaster*, a well-known model organism, does not require extensive care, especially when dealing with wild-type strains, and it also shares with mosquitoes the same brain organization and the sensitivity to some known mosquitoes' repellents. Reports in mosquitoes and in the fruit fly (*D. melanogaster*) have confirmed this view^{13,14}.

Therefore, we considered whether *D. melanogaster* could be a suitable organism for investigating both the raw repellent effect of the substances of choice, and their possible influence on visually guided behaviours which may impact the navigational abilities of insects. This strategy would open the possibility to utilize the extremely sophisticated behavioural, neurophysiological, and genetic techniques available in this model organism, using it as a probe organism in screening for the effects of promising repellent compounds. However, the ecological differences between different insect species should always be accounted for, as one compound could be in theory repellent to one species but not to another or have opposite effects for larval and adult stages.

We tackled these questions by setting up a behavioural paradigm with adult *Drosophila* in fixed tethering conditions, where the tethered fly is not able to rotate around the vertical axis. We investigated the OKR of these flies presented with a moving grating visual stimulus^{6,15,16} during the concurrent administration of different compounds chosen among the ones already being tested on mosquitoes, namely: eugenol, lemongrass, picaridin, and IR3535¹³.

Our research extends the body of literature investigating the OKR in flies or the effects of odorants on their flight conducted in the past years^{17–19}, as well as studying the multisensory integration in flies' navigation²⁰, for instance, on vision and olfaction in presence of attractive odour cues²¹.

As readout, being the fly unable to turn we chose to track the dynamics of the orientation of the head, focusing on the movements elicited by the optokinetic stimulation. In flies, this stimulation elicits nystagmus-like sawtooth head oscillations, which closely resemble the typical optokinetic nystagmus (OKN), observed for eye movement in other animals, humans included. These nystagmus-like head movements, which herein we will refer to as “head optokinetic nystagmus” (HOKN) are characterized, as in the classical OKN, by two alternate phases, a slow and a fast phase, taking place when the visual system is properly stimulated. The slow phase, consensual to the movement of the visual panorama, encapsulates the reflexive response to the motion of the visual field (or visual stimulus) and is generated by the optokinetic neuronal circuits. This slow phase is immediately followed by the fast phase, identifiable as a rapid ‘reset’ movement (usually referred as saccades²²) of the head in the direction opposite to the moving panorama¹⁵. Said fast phase happens as the flies, while following a moving panorama, come closer to the torque limit of their heads (without necessarily reaching it) and reset their gaze to keep pursuing the shifting visual stimulus¹⁵. This resembles the eye dynamics found in humans and mammals, where the fast phase is generated by resetting circuits located in the pontine reticular formation for the horizontal OKN²³.

In our study, we looked for the differences in the HOKNs during the OKR of flies exposed to a plume flowing in the opposite direction of the visual stimulus movement and carrying a repellent compound. Our working hypothesis was that if the repulsive cue evoked by the odorant had been strong enough, this would have interfered with the dynamics of the elicited HOKNs. We found that aversive compounds can affect the OKR of flies, decreasing the number of HOKNs and increasing the delay in between each event, while leaving the intrinsic dynamics of the process (i.e. the angular velocity during the slow phase) unaltered.

We think our findings demonstrate that this kind of approach is feasible, being relatively simple to set up, and affordable. Moreover, it could be extended to other *Drosophila* species or, with adequate adjustments, other insects, being a *trait d'union* between studying their navigational abilities and the necessity of testing suitable components used in products for both agriculture and health care.

Results

Measuring HOKN in flies

To investigate the effect of multimodal sensory integration on the fly optokinetic response, we identified relevant kinetic parameters of the head movements associated with the presentation of a visual stimulation based on horizontally moving grating (Fig. 1a). We considered that the head movement represents a good proxy for the OKR, presenting an OKN-like pattern with a slow turning phase in the same direction of the grating motion, followed by a fast reset phase (saccadic like²²) in the opposite direction (Fig. 1c). We quantified these responses, using an automated behaviour analysis pipeline to extract the total number of HOKNs during the stimulation period, the characteristic inter-saccadic interval, and the corresponding angular velocity for the slow phase. For the considered grating movement speed (60 deg·s⁻¹), the acquisitions presented a consistent pattern across the

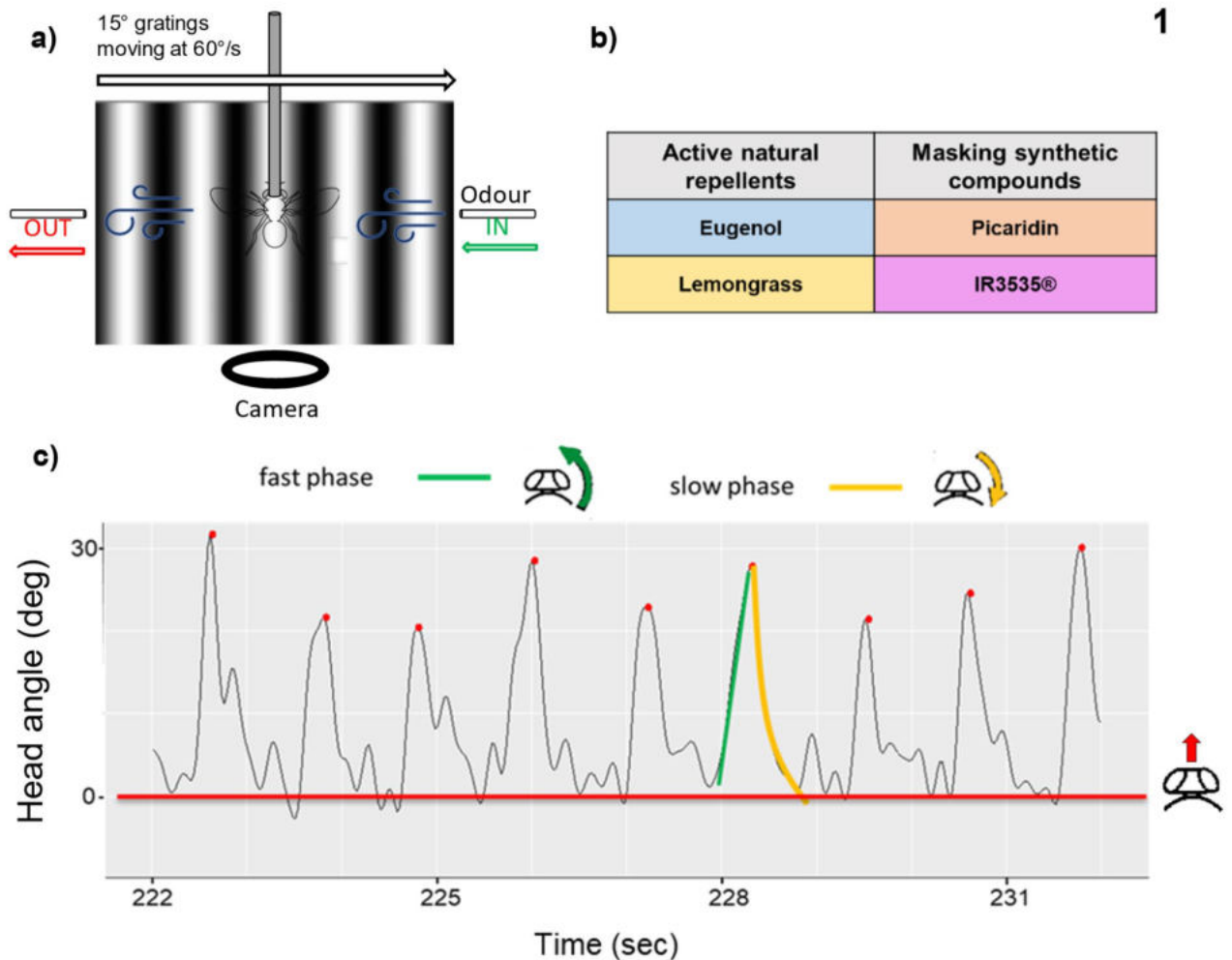


Fig. 1. Experimental paradigm. **(a)** Setup scheme: the whole apparatus is placed in a dark chamber and the illumination is achieved through infra-red LEDs. The fly is glued to a pin by the thorax and is placed on a fixed support (unable to rotate around the z-axis). A screen for projecting the visual stimuli (gratings pattern drifting to the right of the animal at $60 \text{ deg}\cdot\text{s}^{-1}$) is placed in front of the fly. The fly is suspended inside a continuous air flow, administered through one delivery and one recycle tube placed at 1 cm to the right and 2 cm to the left of the fly respectively; the air flow moves right to left, opposite to the visual stimulus (left to right). The camera is placed below the animal. **(b)** Tested compounds: we chose 4 substances classified by their nature and known mechanism of action¹³. Two compounds are natural repellents with an active repulsing effect on insects (eugenol and lemongrass). The other two are synthesized substances (picaridin and IR3535) with a masking action mechanism, binding and making less volatile the molecules they are mixed with. **(c)** HOKN identification: we identified the onset of HOKN events by the peaks corresponding to the end of the flies' reset head-saccades (fast phase²²). As the fly experiences and follows the optic flow (OF) generated by the frontal drifting visual stimulus, its head can reach the resting position (red line and arrow), or surpass it, getting closer to its angular limit. Then, a reset saccade occurs, allowing the fly to keep pursuing the OF. HOKNs are characterized by a slow phase (gold), a pursuit movement concordant with the OF, followed by a rapid reset saccade, the fast phase (green), in the opposite direction of the moving visual stimulus. As reported in the Methods, we identified the HOKNs by tagging the peaks in the raw tracks of the head position with the 'find peaks' function in MATLAB and extracted the velocities of the slow phase ((peak location + 18 frames)/time) and the fast phase ((peak location - 5 frames)/time, not analysed).

different flies tested (good vs. discarded), confirming that this kind of head movements can be considered a reliable readout of the OKR response.

Design of the multimodal sensory input protocol

Along with the visual stimulation, we designed a protocol to assess the impact of a multimodal sensory integration on the HOKN features, delivering an odorant flow directed opposite to the grating movement and with either neutral odour (plain mineral oil solution) or odorant compounds (Fig. 1a). The 'Control' group only experienced

the neutral mineral oil throughout all the trials (MOP phase, explained further below). The compounds were chosen considering two odorant types, a group of two natural repellents, namely eugenol and lemongrass oil, along with a group of synthetic compounds, picaridin, and IR3535 (Fig. 1b), classified according to reported test on mosquitoes; this classification considers the observed mechanisms of action for the two groups, as the natural substances have an active repelling effect, while the synthetic ones act as masking agents which reduce the volatility of molecules relevant to the insects' antennae¹³. All the compounds were diluted in mineral oil and tested with two concentrations (0.5% and 1% in volume). Groups of flies, all naive to the odorants, were tested for a single odorant at a single concentration. We designed a multimodal integration protocol with an initial time segment (40 s) where flies were exposed to visual stimulation with neutral airflow. This consisted of a stripe fixation phase (SF, 10 s) followed by drifting gratings (Mineral Oil Phase, MOP, 30 s). In the following segment visual stimulation was paired with the odorant stimulation (Odorant Phase, OP, 30 s) during the grating motion phase. This same trial was repeated six times separated by a 20 s (Pause) dark period (Fig. 2).

Repellents do not alter the HOKN's intrinsic kinetics

Even though we did not expect the olfactory processing channel to directly impact the neuronal components in charge of the HOKN motor execution, we analysed whether the presence of a repellent gradient opposed to the optic flow direction could impact the tracking process of the fly. Therefore, to check this first hypothesis, we initially focused on the velocity of the slow phase, when the head of the insect, following the optic flow direction, moves toward the odour source. Comparing this parameter between MOP and OP, in general, we did not find any significant difference (Suppl. Fig. 2 and Suppl. Table 1). The only exception from this generalized trend is represented by the condition with lemongrass at 1% ($N=14$), showing a significant reduction in the mean slow-phase velocity ($60.21 \pm 21.29 \text{ deg}\cdot\text{s}^{-1}$ (MOP) vs. $52.93 \pm 17.71 \text{ deg}\cdot\text{s}^{-1}$ (OP), Wilcoxon signed-rank test, p -value: 0.01172), although the contribution of one outlier during the L1 OP phase could be the culprit. Moreover, these values did not change over the trials (Suppl. Fig. 2).

Odorant presentation induces a decrease in the HOKN number

To further evaluate the possible effect of the odorants, we looked at the number of HOKNs and their relative change in numerosity from MOP to OP (Fig. 3), with respect to the corresponding values measured in the control group, exposed only to mineral oil across the whole protocol (fake OP). We observed a generalized trend for the natural repellents, with a lower number of OP HOKNs (normalised to the number of flies in each group) in all groups and concentrations when compared to MOP. Values are presented as mean \pm standard deviation (SD), and p -values were obtained through multiple 2-sample tests for equality of proportion with continuity correction: [Controls (C), $N=5$] MOP 169.40 ± 89.80 vs. OP 175.60 ± 50.71 HOKNs (103.65%); [Eugenol 0.5% (E05), $N=8$] 124.62 ± 62.77 vs. 91.50 ± 29.56 (73.42%), $p=3.326e^{-7}$; [Eugenol 1% (E1), $N=15$] 201.46 ± 83.02 vs. 160.06 ± 87.67 (79.45%), $p=1.149e^{-6}$; [Lemongrass 0.5% (L05), $N=8$] 136.87 ± 33.41 vs. 120.87 ± 40.53 (88.31%), $p=0.00862$; [Lemongrass 1% (L1), $N=14$] 188.78 ± 43.84 vs. 143.57 ± 51.26 (76.04%), $p=3.232e^{-8}$. The effect was significant at both the concentrations tested with no statistically significant evidence for a dose-dependent effect, except between L05 and L1 ($p=0.0056$). Interestingly, for the synthetic repellent, two different scenarios emerged, with Picaridin showing a decrease in the number of HOKNs: [Picaridin 0.5% (P05), $N=7$] MOP 160.57 ± 75.91 vs. OP 117.28 ± 32.42 HOKNs (73.04%), $p=1.027e^{-7}$; [Picaridin 1% (P1), $N=10$] 152.60 ± 65.49 vs. 119.50 ± 60.57 (78.30%) $p=3.918e^{-6}$, similarly to natural repellents, and IR3535 resulting the only odorant not affecting the number of OP HOKNs: [IR3535 0.5% (I05), $N=8$] 177.87 ± 108.99 vs. 175.75 ± 73.34 saccades (i.e. HOKN) (98.80%); [IR3535 1% (I1), $N=8$] 107.87 ± 72.95 vs. 105.50 ± 70.77 (97.79%).

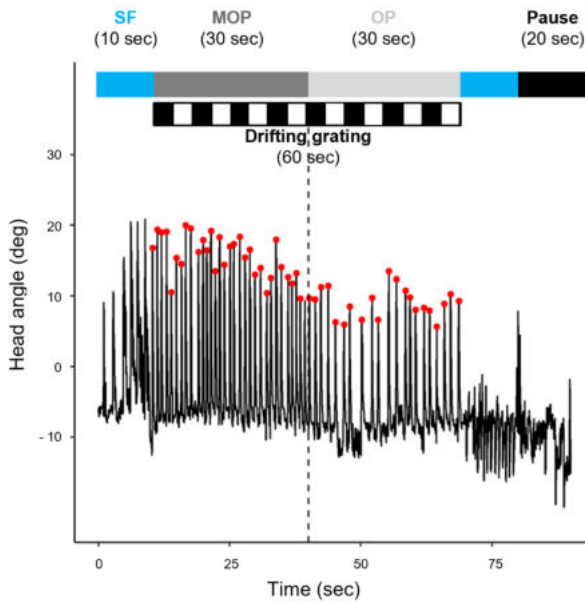
Inter-saccadic interval increases in the presence of the repellent

Focusing on the origin of the reduction in the number of HOKNs, we considered two scenarios. The first one, where the input from the olfactory channel, stimulated by the repellent, could with time overtake the HOKN mechanism, leading to a progressive decrease in the number of the HOKNs. The second one is where the visual and the olfactory channels co-exist, and the repellent interference does not suppress the normal HOKNs but rather modulates their frequency. We then looked at the Inter-Saccadic Interval (ISI), the time interval separating two consecutive HOKNs, comparing this parameter during MOP and OP (Fig. 4).

As expected, the control group, exposed only to mineral oil, did not show any appreciable difference in the ISI, showing a substantial overlap of the corresponding distribution in MOP and OP (2 W-ANOVA [$F=35.338$, num. d.f. = 17.0, denom. d.f. = 7451.4, p -value $< 2.2e^{-16}$] + post-hoc Games-Howell test). The results were different for the repellents, as visible in Fig. 4, where MOP and OP distributions of the ISI in both concentrations are shown for each compound against the C ($N=5$) ISI distribution (black line).

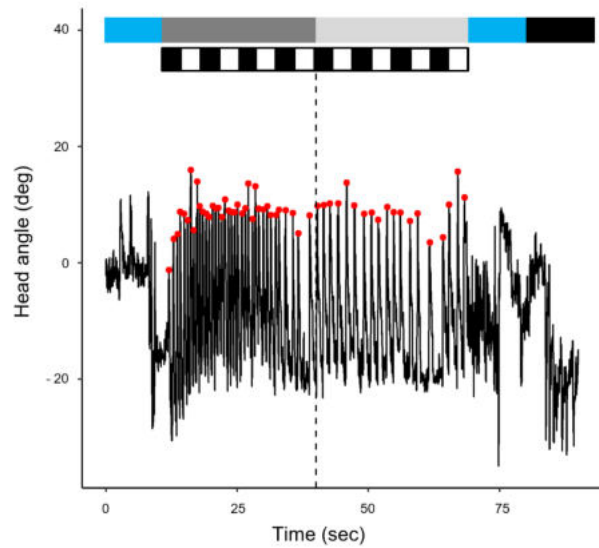
As for the natural repellents, eugenol and lemongrass, the distributions are characterized by a significant increase in the ISI for most of the concentrations (Fig. 4a, b): E05 ($N=8$), mean 1.18 ± 1.53 (SD) vs. 1.61 ± 1.63 s (+36%, $p=5.9712e^{-6}$); E1 ($N=15$), 0.76 ± 0.8 vs. 1.14 ± 0.92 s (+50%, $p=1.2536e^{-6}$); L05 ($N=8$), 1.13 ± 1.28 vs. 1.27 ± 1.42 s (+12.5% [ns]); L1 ($N=14$), 0.77 ± 0.84 vs. 1.00 ± 1.10 s (+30%, $p=6.7175e^{-9}$). Conversely, in the case of the synthetic compounds (Fig. 4c, d), the scenario reflects the data related to the number of peaks, with no significant differences in the ISI distribution for IR3535 at both tested concentrations: I05 ($N=8$), mean 0.89 ± 1.3 (SD) vs. 0.87 ± 1.00 s (-2.25% [ns]) and I1 ($N=8$), 1.20 ± 1.67 s vs. 1.22 ± 1.58 s (+1.6% [ns]). Picaridin, on the other hand, shows features more like those observed in natural repellents also in the ISI rather than more similar to the other synthetic masking agent, with a clear increment in the mean ISI at both concentrations: P05 ($N=7$), mean 0.87 ± 1.01 (SD) vs. 1.30 ± 1.92 s (+49%, $p=1.2860e^{-6}$); P1 ($N=10$), 0.94 ± 1.18 s vs. 1.18 ± 1.43 s (+25%, $p=0.0004$). It is also to be noted that MOP values from E05, E1, L05, L1, and I1 significantly differ

Eugenol

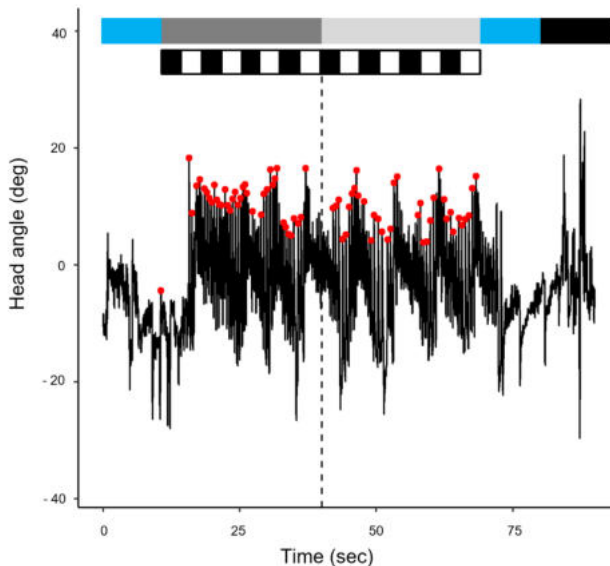


Lemongrass

2



Picaridin



IR3535

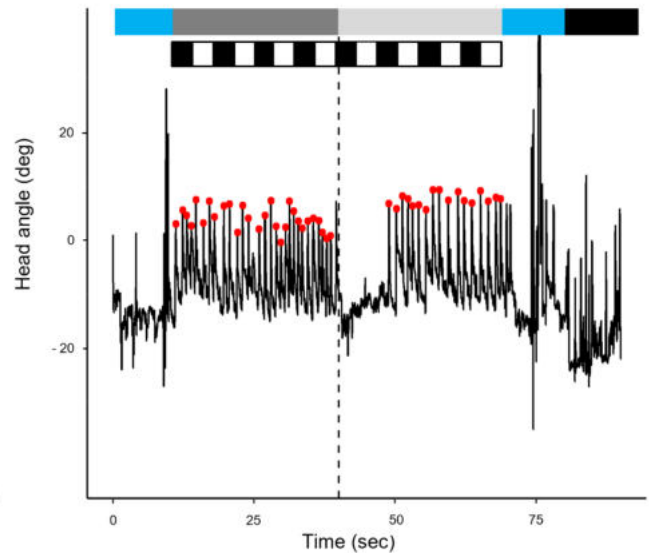


Fig. 2. Raw recordings. Zoom of the 4th trial for one raw track from each group: each track shows a visible change in the number and/or inter-saccadic intervals in between events, as reported further down the result section, during the Mineral Oil Phase (MOP), when no odour is present, or the Odorant Phase (OP), when repellents are delivered to the fly. The shown paradigm was repeated six times, for a total of 600 s in each experiment (one fly). The 0° angle corresponds to the fly's straight head position with respect to the body axis, while positive shifts indicate head left turns (away from the direction the gratings are moving) and negative ones correspond to right turns (concordant with the gratings movement). The corresponding full tracks are plotted in Supplementary Fig. 1.

from C MOP phase ($p = 0.0079, 0.0043, 0.047, 0.010, \text{ and } 0.012$ respectively), possibly due to a carry-over effect from previous trials.

Inter-saccadic interval decreases with the trials

Additionally, we noticed a trend while looking at ISI value variations across trials in the groups (see Supplementary Fig. 4). As shown in Fig. 5, there is a subtle trend towards the ISI reduction as the trials proceed (1st to 6th) and it was detectable in both the MOP and the OP. The mean values and SDs are shown below (Table 1). We

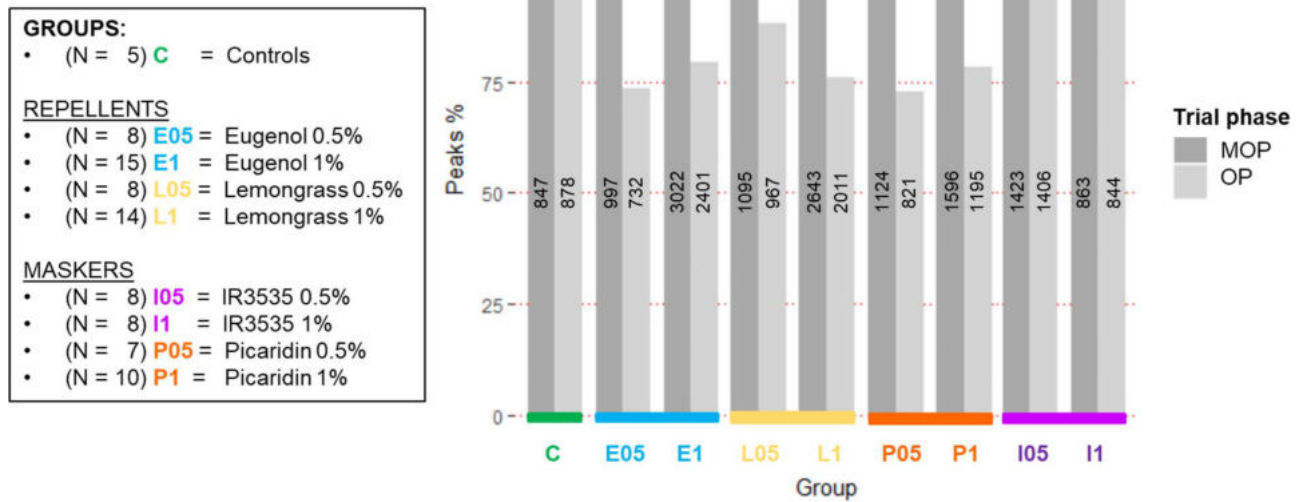


Fig. 3. HOKNs number and proportion. The bar plot shows the relative proportion of MOP and OP HOKNs normalised on the MOP to the flies' number in each group; values in bold within each bar refer to the total number of HOKN identified during each phase in each group. p-values result from multiple 2-sample tests for equality of proportion with continuity correction, to verify if the observed differences in proportions between MOP and OP differed significantly from the C group and between the two concentrations within each group. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$.

first evaluated this trend applying a simple linear model (LM). We found a slight but significant effect on the ISI with the increasing trial number, in all groups (C: $p = 2.00e^{-9}$; E05: $p = 2.84e^{-11}$; E1: $p = 5.50e^{-11}$; L05: $p = 0.035$; L1: $p = 9.21e^{-7}$; P05: $p = 1.03e^{-12}$; I05: $p = 0.00248$; I1: $p = 2e^{-16}$) except for P1, as well as a significant effect dependent on the (MOP and OP) phase in most groups (E05: $p = 5.75e^{-8}$; E1: $p = 3.94e^{-9}$; L05: $p = 0.013$; L1: $p = 2e^{-16}$; P05: $p = 6.69e^{-11}$; P1: $p = 1.64e^{-6}$) except C, I05 and I1. Then we tested the mean values of ISI in the 1st trials against the 6th and found a significant reduction of the ISI, both during MOP and OP (Wilcoxon signed-rank test, refer to Table 1 for all the means \pm SD and p-values), in C ($N = 5$, MOP: 2.21 ± 1.91 vs. 1.06 ± 0.71 s) and I1 ($N = 8$, MOP: 3.4 ± 2.56 vs. 1.64 ± 1.16 s; OP: 3.4 ± 3.5 vs. 0.96 ± 0.48 s), and also during the OP in the 1% concentrations of both E1 ($N = 15$, OP: 0.91 ± 0.29 s), and L1 ($N = 14$, OP: 1.7 ± 1.26 vs. 0.96 ± 0.22 s). Moreover, returning back to the underived data (the number of HOKN over time in each group, not shown), we normalized them for the number of flies in each group, checked if there was a meaningful difference in this variable through groups in our original dataset (Kruskal-Wallis test, chi-squared = 476.83, d.f. = 8, $p < 0.001$ and Dunnett test with Bonferroni correction for pairwise comparisons, see Supplementary Table 2), and we also checked through a permutation test of 10^4 iterations that the observed differences, and a better value for the test, could not be replicated by randomly re-assigning the observations within the groups ($p = 0.0001$). Then we looked for the best fitting from different generalized linear models on the collective data (not separated for MOP or OP, since again there was no significant difference in their distributions, nor a clear contribution from those phases in the LM) from C and I05, which did not show significant differences. We found the best fit to be a second-order polynomial regression, with the following R-squared values: C, 0.10596417; E05, 0.10085333; E1, 0.02766739; L05, 0.02097825; L1, 0.15916139; P05, 0.14127738; P1, 0.04623636; I05, 0.05301220; I1, 0.27136975.

We also calculated an "Effect Index (E.I.)", a qualitative index, describing the ISI differences observed over time matching the OP and MOP from their corresponding groups:

$$\left(E.I. = \frac{(ISI_{OP} \text{ 6th trial} / ISI_{OP} \text{ 1st trial})}{(ISI_{MOP} \text{ 6th trial} / ISI_{MOP} \text{ 1st trial})} \right)$$

This index describes ideally the possible strength each compound could have in perturbing this slight decline in ISI (Table 2). C, L05 and I05 groups show scores close to 1 suggesting that the diminution in the ISI could be similar between MOP and OP, and not much affected by the presentation of compounds. On the other side, most of the other groups have scores lower than 1, pointing to a major reduction happening during the MOP (i.e. the repellent effectively alters said reduction during the OP). Exception from this general trend is represented by the group P1, characterized by a score greater than 1, associated with a change in the ISI occurring mostly during the OP but showing a greater reduction in the ISI with respect to the control group.

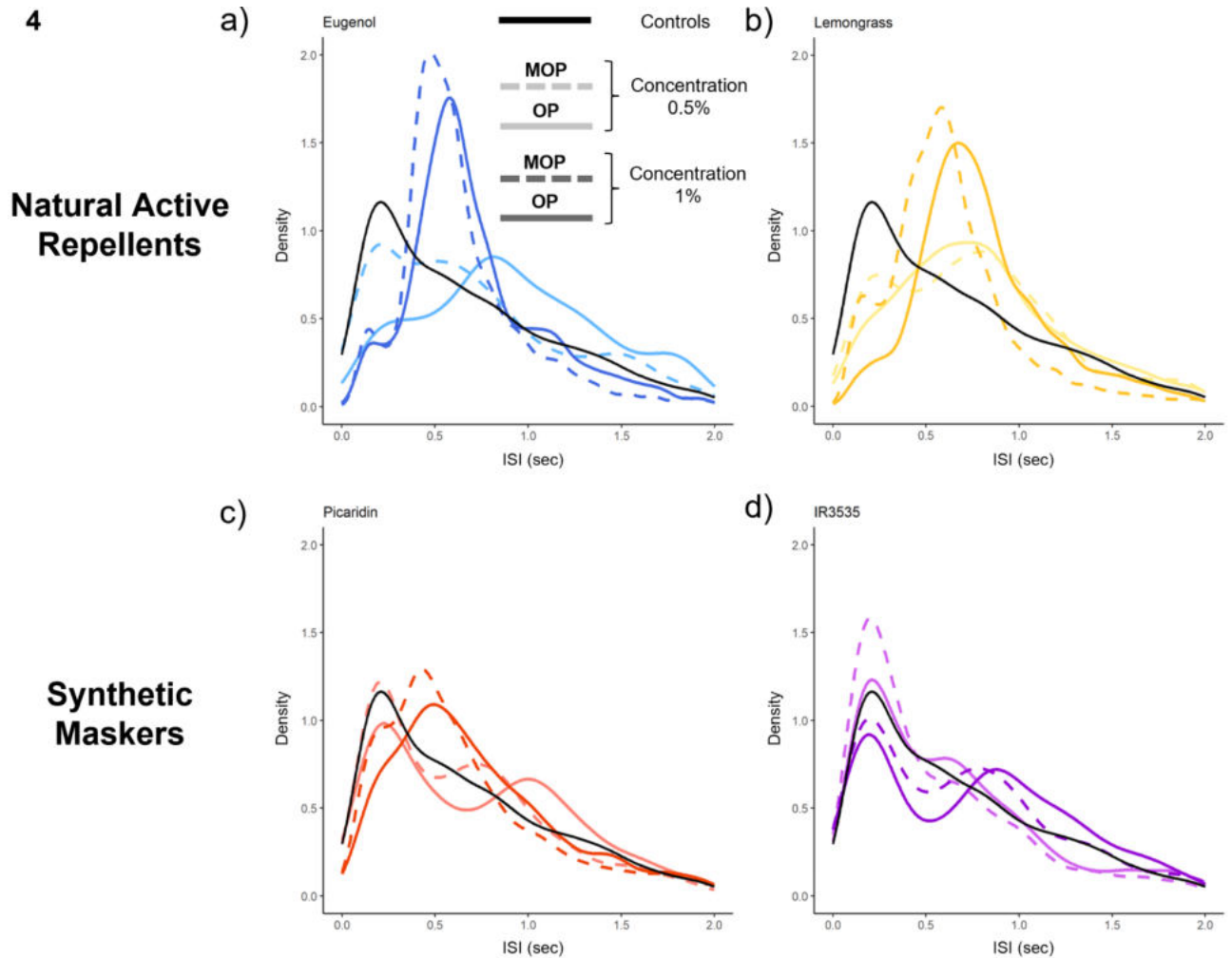


Fig. 4. ISI distribution. The plots show the distribution of the ISI in the four groups, following the same colour scheme from the above figures. The lesser concentrations are represented by the lighter colours, and the MOP and OP are shown as dashed or continuous lines, respectively. The continuous reference black lines represent the whole Control group's ISI, unsplit into MOP and OP since there was no statistically significant difference between the two. Statistical analysis was achieved through a 2 W-ANOVA followed by a post-hoc Games-Howell test (the relevant p-values are reported in the main text 'Inter-saccadic interval increases in the presence of the repellent' section). Density is normalised over the entire range of ISI values, so that the total probability under the curve equals 1: density values are not direct probabilities, but rather indicate the relative likelihood of the ISI values.

Discussion

Odor plumes are extremely important for insects' foraging and exploration, and successful navigation relies on the multisensory integration of environmental and internal sensory inputs^{24,25}. Attractive odours were reported for increasing the gain of gaze-stabilizing optomotor reflexes (body motor responses elicited by the movement of the visual field, which oppose the image drift on the retina²³), maintaining the animal aligned within an invisible plume and facilitating the localization of attractive odorant sources while navigating in the environment²⁶, and also for enhancing the final behavioural output through synergistical interaction with other sensory cues, like demonstrated for the visual, olfactory, and thermal receptors synergy in the landing response of *Anopheles coluzzii* mosquitoes²⁷. However, in the evolutionary war between plants and insects, the former have been producing a variety of odorous-repellent substances or compounds that mask the odorous cues. And, although numerous studies have analysed the integration of attractive odorous stimuli with other sensory modalities in insects, mainly vision, relatively few studies are dedicated to investigating the interaction between olfactory and visual stimuli competing for one against the other. This becomes every day more important, in light of recent EU regulations pushing the use of natural aversive substances in pest control in agriculture to replace currently⁹. In this context, we observed that when a competing aversive odour is presented to flies undergoing optic flow stimulation (Table 3):

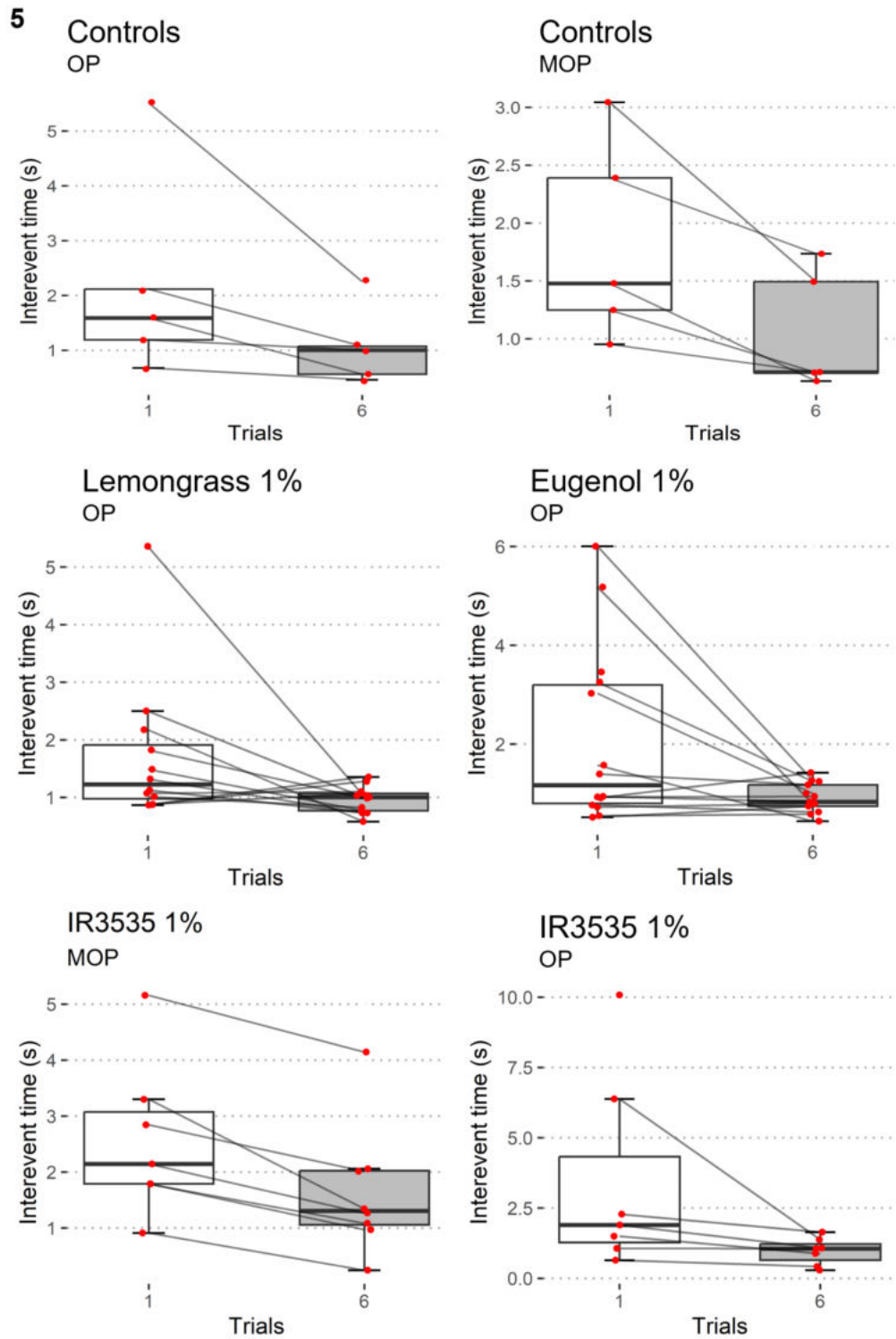


Fig. 5. Mean ISI values between the 1st and 6th trials. Standard boxplots representing the median ISI values divided by trial and mediated by subject (only statistically significant comparisons from Wilcoxon signed-rank tests are shown).

1. the number of HOKN reduces, and their temporal distribution becomes more dispersed, reflecting an increment of the time interval separating consecutive events (ISI).
2. the velocity of the slow phase, associated with the movement tracking process of the flies, does not change overall.

Group	ISI values at 1st and 6th trial (seconds)					
	1st trial	6th trial	p-value	1st trial	6th trial	p-value
	MOP			OP		
Controls	2.21 ± 1.91	1.06 ± 0.71	0.03	1.82 ± 0.86	1.05 ± 0.51	0.03
Eugenol 0.5%	1.70 ± 0.85	1.88 ± 2.24	ns	2.58 ± 1.37	1.56 ± 1.06	ns
Eugenol 1%	1.33 ± 1.11	0.78 ± 0.32	ns	2.08 ± 1.80	0.91 ± 0.29	0.04
Lemongrass 0.5%	1.47 ± 0.73	1.13 ± 0.62	ns	2.15 ± 1.41	1.34 ± 0.55	ns
Lemongrass 1%	1.24 ± 1.05	1.03 ± 0.69	ns	1.70 ± 1.26	0.96 ± 0.22	0.02
Picaridin 0.5%	2.44 ± 2.35	0.99 ± 0.57	ns	6.23 ± 9.77	1.15 ± 0.43	ns
Picaridin 1%	1.59 ± 1.20	1.39 ± 1.01	ns	1.55 ± 0.74	2.65 ± 2.96	ns
IR3535 ⁺ 0.5%	1.72 ± 1.58	1.16 ± 0.78	ns	1.61 ± 1.44	1.08 ± 0.54	ns
IR3535 ⁺ 1%	2.56 ± 1.38	1.64 ± 1.16	0.01	3.40 ± 3.50	0.96 ± 0.48	0.01

Table 1. ISI values at 1st and 6th trial. Mean values ± SD for both MOP and OP phases with the relative p-values obtained through a Wilcoxon signed-rank test.

Group	ISI 6th/ISI 1st		E.I.
	MOP	OP	
Controls	0.4796	0.5769	1.2028
Eugenol 0.5%	1.1058	0.6046	0.5476
Eugenol 1%	0.5864	0.4375	0.7459
Lemongrass 0.5%	0.7687	0.6232	0.8107
Lemongrass 1%	0.8306	0.5647	0.6798
Picaridin 0.5%	0.4057	0.1845	0.4549
Picaridin 1%	0.8742	1.7096	1.9556
IR3535 ⁺ 0.5%	0.6744	0.6708	0.9946
IR3535 ⁺ 1%	0.4823	0.2823	0.5853

Table 2. Effect Index. The E.I. (values rounded to 4th decimal, formula reported above) suggests when the increase in performance (reduced ISI) of flies was more prominent during the experiment. The reduction is similar between MOP and OP with the values close to 1. Lower E.I. values point a major reduction during the MOP (when repellents were not being delivered), while higher values (P1) are associated with the prevalence of ISI change during the OP, in the second half of the grating presentation. Compounds and concentrations exhibiting lower E.I. could be then considered more effective in impairing the stimulus habituation when present.

	Peaks %	Slow ph. velocity	ISI	E.I.
Eugenol	↓↓↓	- -	↑↑↑	↓↓↓
Lemongrass	↓↓↓	- ↓	- ↑	- ↓
Picaridin	↓↓↓	- -	↑↑↑	↓↓↓
IR3535 ⁺	- -	- -	- -	- ↓

Table 3. Visual scheme resuming the results. Controls did not show any significant difference and thus are not included.

Additionally, we observed a slight decrease in the ISI as the trials proceeded, paired, in some of the groups, with an increment in the number of recorded HOKNs as a function of time.

Along with this general trend, a peculiar result emerges from the comparison between the repellent and masker sub-groups. As mentioned above, the substances chosen are classified as natural repellents (eugenol and lemongrass) or masking agents (IR3535 and picaridin) for mosquitoes. In these insects, the two natural repellents strongly activate olfactory neurons, while the masking substances do not activate olfactory neurons substantially¹³. Regarding *D. melanogaster*, lemongrass has been observed to be repellent in addition to being lethal as well after 96 hours of exposure^{28,29}, and eugenol was shown to alter behaviour in adult animals and to also affect cardiac rhythm in larvae^{30,31}. Considering, on the other hand, the two masking substances, the repellent action of these was also demonstrated in *Drosophila* with contrasting results: both IR3535 and picaridin

have shown repellent action in a choice inhibition test¹⁴ but, in another experiment, the ‘masking’ effect of the former seemed more evident than a repellent action²⁹.

Our experiments with *Drosophila* revealed that picaridin induces HOKN responses closer to the natural repellents than to an expected masker as IR3535 which in turn appears to have no effects on all the analysed parameters of the optokinetic response (OKR), as expected (Table 3). Taken together, our results show that repellents have an opposite action on the OKR compared to what was observed using attractors³², however, the mechanism of action seems to differ. Attractors can increase the gain of the OKR, improving it and resulting in an enhanced ability to localize resources (e.g. food). In the presence of a repellent, on the other hand, a decrease in the gain of the OKR is not observed (except in the higher concentration of lemongrass and with limited effects), but rather we do note a reduction in it in terms of HOKN. In humans and mammals, a decrease in the gain of the optomotor response is associated with spatial disorientation: a reduction in nystagmic beats is observed, for instance, in conditions of inattention, when individuals do not pay attention at all or only partially to the visual stimulus³³. It can then be assumed that the decrease in the number of HOKNs observed in the presence of repellents in flies may reduce the attentive ability and the resources allocated for localizing the source of the odour while maintaining the ability of the optokinetic system to react promptly to perturbations. Moreover, a decreasing gain due to exposure to a repellent could underlie a possible, maybe dose-dependent, heavier effect if not toxicity of the compound (like the anaesthetic effect described in Weineck et al.³¹, which should then be avoided because of the fallout it could have on other sensitive insect species, like pollinators). Instead, synthetic maskers gave different results. IR3535 did not affect both HOKN’s number and HOKN’s slow phase velocity. On the other hand, picaridin significantly reduced the number of HOKNs (MOP vs. OP within the group), without altering the slow phase velocity. Therefore, in our study, IR3535 appears to be neutral to a stimulus eliciting the OKR. Indeed, Yoon and Taak²⁹ found a similar “masking” effect of IR3535 in their experiments; however, conclusions are still unclear, as it has been observed that this compound elicits pb1 ORN-A neurons response in *Drosophila*¹⁴, while calcium imaging does not reveal activation of antennal neurons in *Anopheles* after stimulation with the same compound, leaving doubts about how the substance exerts its physiological action. A different and more complex scenario is the outcome we observed after stimulation with picaridin. We found that picaridin significantly reduced the number of HOKNs. Similarly to IR3535, picaridin was shown to activate pb1 ORN-A neurons in *Drosophila*¹⁴, while also having a similar profile in *Anopheles*¹³. Nevertheless, calcium imaging of antennal neurons showed some response in these cells after stimulation with picaridin (Fig. S3 in Afify et al.¹³), therefore suggesting that for both species picaridin may not be, after all, a neutral compound. Our data, obtained using a straightforward behavioural assay, agrees with these reported observations.

The visual information collected by the *ommatidia* photoreceptors is processed in the optic lobe, which consists of the *lamina*, *medulla*, *lobula*, and *lobula* plate. Three distinct longitudinal channels of visual information processing (ON-OFF and colours) associated with horizontal analysis have been identified, which are able to distinguish the differences in movement and contrast between *ommatidia*³⁴. The visual information, processed at the level of the optic lobe, is then sent to three main nerve structures: the lateral cerebral ganglion, the central complex, and the mushroom bodies. The lateral cerebral ganglion (precisely in its PLP, PS, and PVLP components) is involved in the sensorimotor processing of decision-making processes, as it is the site of a multisensory convergence and the origin of numerous motor descending pathways³⁵. Afferents to these regions from the tangential cells of the optic lobe³⁶ are involved in the processing of optomotor signals. Descending projection neurons, in addition to reaching the motor neuropile that innervates the indirect flight muscles and the leg muscles, also reach the motor neuropile for the neck muscles^{35,37–40}. Thus, through this pathway involving specific Descending Neurons (DN9), motor signals originating from optic lobe tangential cells and processing visual optic flow will eventually reach motor neurons regulating head movements^{35,41}. Attractive odour plumes can increase the gain of optomotor response in *Drosophila*: this modulation of the visuomotor response is mediated by a specific *lobula* plate tangential interneuron, called Hx, which activity is enhanced whenever optic flow inputs are paired with odour inputs⁴². Octopaminergic Tdc2 neurons, activated by odour inputs, are responsible for the modulation of Hx and of T4/T5 columnar neurons which in turn are also activated by optic flow inputs above tangential cells^{32,42}. It was also suggested that T4/T5 neurons expressing octopaminergic receptors could be modulated by octopaminergic neurons and that this modulation can reverse the aversive effect of a visual object on fly behaviour³².

Our results show a modulatory effect on the HOKN by repellents (eugenol, lemongrass, and picaridin to some extent) and no effects by masking odour plumes (IR3535). Therefore, it is possible to hypothesize that an octopaminergic modulation onto T4/T5 neurons and/or specific multimodal tangential cells (Hx tangential neuron) could take place when aversive compounds are delivered to the fly. Moreover, since DNs receive extensive dendritic contacts in the *lobula* plate, the aforementioned modulation may be reflected on the final motor output, thus possibly being also accountable for the alterations we found in the number of HOKNs.

One last intriguing outcome of our experimental paradigm is the slight progressive increase in the number of HOKNs, and consequently reduced ISI duration, as the trials proceeded. To describe this aspect, we calculated the Effect Index (E.I.). Interestingly, the worst index values (i.e. most intrusive effect on the variable) resulted from the provenly effective repellents, but not for the Control and the IR3535 0.5% groups as expected. Notably, though, a quite low score of the index is indeed related to a greater concentration of IR3535, which we did not expect. Moreover, in the Lemongrass group, the score is lower the higher the concentration, while the trend appears inverted in Eugenol, and Picaridin (which exhibits both the most extreme scores in Table 2). This may suggest an effect of concentration, even in what we thought was an almost neutral compound from our other results (IR3535), suggesting a possible lower saturation point for Eugenol and Picaridin with a consequent diminishing effect. Altogether, these results suggest both the possibility that flies can learn and improve their responsiveness to a prolonged constant optokinetic stimulation, and that being exposed to the repellents we

tested could interfere, up to compound-specific saturation concentrations, with this process, effectively altering the flies' learning dynamics in repellents unsaturated conditions.

In conclusion, we have shown here the application of a behavioural assay based on multisensory stimuli competition that can catch subtle differences in the action of aversive or neutral odorant compounds. It is important to note how recent works have shown that the flies can move the retina independently⁴³ and discovered that head movements are dependent on the head angle during visual stimulation³⁷. Although studying these aspects was beyond the scope of this study, considering these factors in future studies could lead to a deeper and more comprehensive understanding of multisensory integration in insects.

We think this work represents an ideal preliminary workbench to instruct more in-detail analyses, at the cellular or circuit level, to dissect the underlying neuronal mechanisms and potentially the impact of the compounds on the brain function.

Materials and methods

Fly strains

Berlin-K adult female flies aged 3–6 days were tested within 6 h from the light onset. Females were separated from males and collected under CO₂ anaesthesia, then given at least 24 h to recover.

The rearing was conducted in vials containing 10 ml of standard cornmeal medium, with a 12 h:12 h dark-light cycle with controlled temperature (26 °C, at 45 ± 10% humidity).

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

The number of the analysed flies and total peaks, after pruning unsuccessful experiments and/or tracking, is reported in Fig. 3 ('Results' section). Each recording was manually checked before the tracking, and only the flies which flew continuously throughout the duration of the experiments were tracked. Likewise, bad tracking results, which could be caused by illumination issues and/or excessive leg movements from the fly's self-grooming interfering with the tagging of the antennae, were discarded altogether and are not part of the analysis shown in this paper.

Fly preparation

Flies were transferred from the rearing vial to an empty one which was then put in ice. After the cold anesthetization, single flies (one at a time) were transferred to the mounting block, which could be kept cold (down to +4 °C) via a Peltier platform laid on a fan heat sink, and carefully placed upright inside the dedicated groove of the mounting support. The Peltier temperature was set to 14 °C to minimize the cold experienced by the flies. The tip of a 34-gauge dispensing needle (BSTEAN, Shenzhen Hemasi E-Commerce Co., Ltd., PRC) was dipped in UV hard resin (DecorRom, Shenzhenshi Baishifuyou Trading Co., Ltd., PRC) removing the excess quantity (barely one drop remaining on the tip). The needle was then placed on one support angled at 60° with respect to the fly's horizontal body axis and, with the aid of a micromanipulator, lowered onto the fly, touching the centre of the thorax; the resin was then cured for 45–60 s with a UV torchlight to glue the animal to the pin. The whole tethering procedure took about 2 min. Flies were let to recover from the procedure for about 5–10 min; during this period, periodical small air puffs were delivered to verify the gluing had been performed correctly, as well as the willingness of the animal to fly (if it was not flying already). Badly glued or unwilling flies were discarded. Once the fly was glued and actively flying, the pin was transferred and mounted inside the experimental setup.

Experimental apparatus

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

A vertical support, ending with a syringe attachment, where the pin together with the glued fly can be secured, was suspended above the monochrome camera (MQ003MG-CM, Ximea GmbH, Germany) equipped with an infra-red (IR) bandpass filter; two IR (850 nm) LEDs (M850L3, Thorlabs Inc., US) provide the necessary illumination. The camera resolution was VGA 0.3 MP 648 × 488 pixels, with a pixel size of 7.4 μm and a maximum frame rate of 500 fps.

The other components of the apparatus include:

- the projector (Lightcrafter 4500, Texas Instruments Inc.), placed in front of the screen, with a refresh rate of 60 Hz, with a maximal output illuminance of 621.5 lx at the centre of the screen and 435 lx at 45° of azimuthal deflection;
- an adjustable, curved, hand-crafted screen (radius = 6.5 cm, height = 13 cm), made of parchment paper, placed in front of the animal (distance of 5 cm), with an illuminance attenuation factor of 10. The actual projected display had an azimuth of ± 90°, an elevation of ± 45°, and a resolution of 1280 × 800 pixels;
- the custom odour-*delivery* system made up of plastic tubing of various diameters, assorted luers (Ark-Plas Products Inc., US), glass capillaries (GB150F-10, Science Products GmbH, Germany), an Arduino (UNO REV3, Arduino, US) controlled solenoid valve (SIRAI Elettromeccanica S.r.l., Italy), two glass vials containing the solutions, and an air pump (Air Professional 150, PRO.D.AC INTERNATIONAL S.r.l., Italy) for the vaporizing;
- the custom odour-*recycling* system, consisting of plastic tubing, an externally alimented suction unit (VN-C4 vacuum pump, You Cheng Industrial Co., Ltd., Taiwan), plus the glass flask used to achieve negative pressure and the subsequent suction.

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

Due to computer specifications limitations, we had to use two different computers, one for running the protocol while the second took care of the recording. The two machines were made to communicate via a U3-LV Labjack (Labjack Corporation, US): the recording of the experiment, controlled by the first computer, would only start after receiving the signal sent from the second computer when starting the protocol through the dedicated script.

We wrote down custom scripts in MATLAB, to control the camera and the recording, and in Python, to control the presentation of the visual stimuli (designed through the open-source PsychoPy[®] toolbox, Open Science Tools Ltd., UK), the modulation of the solenoid valve, and also for synchronizing the start between the protocol script and the recording.

The actual framerate of the video acquisition was in the range 74–80 fps. Variations occurred between different recordings due to the required optimization and adjustments to the illumination and the exposure in different recordings.

Repellent compounds

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

Experimental paradigm

The paradigm was structured in 6 (six) repetitions of one trial (Fig. 2), where the flies faced visual stimulation (moving in the same direction throughout all the experiment) both in the absence and presence of the repellent odour plume. The trial was structured as follows:

- a. **SF**: one 15° vertical black bar on a white background, presented in the middle of the screen (duration: 10 s).
- b. **MOP + OP**: a mask of gratings made up of 15° alternate black and white vertical bars (spatial wavelength of 30°) moving clockwise at a fixed speed of 60 deg·s⁻¹ for a duration of 60 s.
- c. Same as (a.).
- d. **Pause** in darkness (duration: 20 s).

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

The air plume was continuous for the duration of the trials and coming from the right to the left of the animal, or in the direction opposite to the optokinetic stimulation (Fig. 1a). Every time the gratings phase (b.) reached its half (30 s), the solenoid valve was switched through the Arduino board, as the pre-defined switch state was properly set to 'ON' from within the Python script. The activation of the valve switched the air intake and output from the odourless vial (pure mineral oil, MOP phase) to the repellent one (compound in solution with mineral oil, OP phase), so that, in every trial, the flies experienced 30 s of visual stimulation within an odour neutral air flow followed by another 30 s of the same visual stimulus but in presence of the competing odorous compound. As reminder, the Controls group never experienced the OP, but two consecutive MOPs instead.

Data extraction

The tracking of the animal was conducted offline through the self-contained MATLAB program 'Flyalyzer'⁴⁴: after setting the body axis and the head node (neck) position, the ROI over the flies' antennae was manually adjusted for the positioning, the number of tracked pixels, the clustering method (k-means), and for the dark/light contrast threshold; only yaw turns were modelled. We wrote a custom MATLAB script to analyse the raw data (head position over time). Firstly, we applied a low-pass filter with a cut-off frequency at 8 Hz to reduce the high frequency noise components. Then, for each video, the timestamps were reshaped to the actual acquisition's framerate and realigned to those related to the timings of the visual stimuli, generated by the Python script during the experiments. We exploited a native MATLAB function ('*find peaks*'), manually adjusting the thresholds for the magnitude to account for a minimum height of the peak and the minimum distance between consecutive, to identify the peaks (location and timestamp) on the track corresponding to the HOKN events (Figs. 1c and 2). For calculating the slow phase velocities, we initially superimposed the tracks from each HOKN with a permissive frame window (30 frames post peak), then, we manually adjusted the thresholds related to the relevant number of pre- and post-peak frames (5 and 18 respectively), which we observed were accounting for most of the head position movement. We then computed the velocities (slow phase only) as the delta of the head position divided by the time corresponding to the 18 post-peak frames.

For further clarification: it is known that flies also perform a small fraction of co-directional saccades when presented with moving gratings¹⁵, therefore, the raw head tracks contained both positive and negative peaks. Since we were not interested in the co-directional saccades, we wrote the script to only tag the (positive, in our coordinates system) HOKNs peaks (end of the head-reset fast movements) and the related slow phases. Subsequent data elaboration, plotting, and statistical analysis were performed in RStudio through custom-made scripts.

Statistical analysis

The whole analysis was conducted with a significance level $\alpha = 0.05$.

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

The statistical relevance of the observed differences in the number of events (HOKN) was assessed through paired Z-tests with continuity correction, checking if the proportion of HOKNs identified during the MOP differed significantly with respect to the OP between the “Controls” group and the other compounds plus within same compounds at different concentrations.

ISI data were not normally distributed nor homogenous in variances. However, given the large amount of data, we analysed the ISI distribution opting for a 2-Way ANOVA (accounting for the presence/absence of the compound during the OKR task and the groups of repellent tested) corrected for the non-heteroscedasticity, followed by a post-hoc Games-Howell test corrected for non-normality of the data.

Confrontations between the 1st and 6th trials ISI (both MOP and OP), as well as the slow-phase velocity profiles, were performed for each group through the Wilcoxon signed-rank test, as data were not normally distributed.

Data availability

An R-markdown file retracing the content of the paper and the relevant produced dataset are available in the “HOKN-Flies” repository at Github.com (<https://github.com/enda92/hokn-flies.git>); in addition, the pre-processed data, along with a video sample, are hosted in the “Optokinetic response in *D. Melanogaster* reveals the nature of common repellent odorants” repository at Zenodo.com (DOI: 10.5281/zenodo.11183967). The Arduino and Python scripts, related to the experimental paradigm control, and the scripts written for the data analysis (mostly included in the markdown document), can be shared by the authors upon motivated request.

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Author contributions

GMM, PV, AD, and AM designed the experiment. GMM, MB, MAZ, and AM, set up the experimental apparatus and wrote the related scripts. GMM designed the final experimental paradigm and acquired the data. GMM, MAZ, and AM carried out the pre-processing and data analysis. GMM drafted the manuscript and prepared the figures and tables, AM and MDM drafted the Discussion section. All authors equally contributed to the interpretation of data and the revision of the manuscript.

Declarations

Competing interests

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Additional information

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