Loss of olfaction reduces caterpillar performance and increases susceptibility to a natural enemy

Qi Wang [1], Yufei Jia [1], Hans M. Smid [1], Berhane T. Weldegergis [1], Liana O. Greenberg [2], Maarten Jongsma [3], Marcel Dicke [1], Alexander Haverkamp [1,4]*

[1] Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands

[2] Biosystematics Group, Wageningen University & Research, Wageningen, the Netherlands

[3] Business Unit Bioscience, Wageningen University & Research, Wageningen, the Netherlands

[4] Lead contact

* Correspondence: alexander.haverkamp@wur.nl (A.H.)

Abstract

Insect herbivores such as caterpillars, are under strong selection pressure from natural enemies, especially parasitoid wasps. Although the role of olfaction in host-plant seeking has been investigated in great detail in parasitoids and adult lepidopterans, the caterpillar olfactory system and its significance in tri-trophic interactions remain poorly understood. In this study, we investigated the olfactory system of *Pieris brassicae* caterpillars and the importance of olfactory information in the interactions among this herbivore, its host plant *Brassica oleracea* and its primary natural enemy *Cotesia glomerata*. To examine the role of olfaction, we utilized CRISPR/Cas9 to knockout the odorant receptor co-receptor (*Orco*). This knockout (KO) impaired olfactory detection and primary processing in the brain. *Orco* KO caterpillars exhibited reduced weight and lost preference for their optimal food plants. Interestingly, the KO caterpillars also experienced reduced weight when challenged by the parasitoid *C. glomerata* whose ovipositor had been removed, and the mortality of the KO caterpillars under the attack of unmanipulated parasitoids increased. We then investigated the behavior of *P. brassicae* caterpillars in response to volatiles from plants attacked by conspecific caterpillars and volatiles from plants on which the caterpillars were themselves attacked by *C. glomerata*. After analyzing the volatile compounds involved in these interactions, we concluded that olfactory information enables caterpillars to locate suitable food sources more efficiently as well as to select enemy-free spaces. Our results reveal the crucial role of olfaction in caterpillar feeding and natural-enemy avoidance, highlighting the significance of chemoreceptor genes in shaping ecological interactions.

Keywords: insect larvae, natural enemy, olfaction, multitrophic interactions, Orco, host-plant selection

Introduction

Chemoreception plays a critical role in the insect life cycle. For instance, insects exploit chemical cues to locate food plants, to find and select mates, to evaluate oviposition sites, and to avoid natural enemies (Dicke & Grostal, 2001). Olfaction, a pivotal component of chemoreception, involves multiple processes in insects and has been extensively investigated in many species (Haverkamp et al., 2018), including its role in the interactions with conspecifics and natural enemies (Kannan et al., 2022). Thus far, the study of olfaction in insects has largely centered upon imagos, the final life stage of insects. However, chemical information likely also plays an essential role in host plant choice and natural-enemy avoidance during the immature life stage, which are especially pronounced in holometabolous insects. Nonetheless, the olfactory system in larval herbivores remains largely unexplored.

Tri-trophic interactions among plants, herbivores and natural enemies have been widely investigated in the last few decades, and chemoreception is considered to play an important role in these systems (Vet & Dicke, 1992; Turlings & Erb, 2018). In particular, abundant research has demonstrated that olfactory information, such as plant volatiles, is crucial in the coevolution of herbivores and natural enemies (Turlings & Erb, 2018; Dicke & Baldwin, 2010; Haverkamp & Smid, 2020). In addition to plant volatiles, chemicals emitted by the insects themselves also play an important role (Venugopal et al., 2020; Ebrahim et al., 2015). Under strong selection pressure from natural enemies, herbivores have developed several integrated avoidance strategies based on the detection of chemical cues. *Drosophila melanogaster* fruit flies, for example, prefer to lay eggs on citrus fruits because the volatiles emitted by citrus are repellent to the endoparasitoid *Leptopilina boulardi*, a natural enemy of fruit fly larvae (Dweck et al., 2013). Similarly, the brown planthopper *Nilaparvata lugens* deposits eggs on rice plants infested by the striped stem borer *Chilo suppressalis*, which is repellent to the egg parasitoid *Anagrus nilaparvatae* (Hu et al., 2020).

Insect olfaction is dominated by two classes of olfactory receptor genes, odorant receptors (ORs) and ionotropic receptors (IRs). ORs detect the majority of volatiles in the insect habitat. In all ORs the odorant receptor coreceptor (Orco) is required to form a functional ion channel in the membrane of the odorant receptor neurons (ORNs) (Sato et al., 2008; Wicher et al., 2008; Jones et al., 2005). Previous research indicates that silencing the *Orco* gene can lead to a severe olfaction deficiency, causing a loss of social behavior, impaired mating and significantly reduced foraging behavior (Trible et al., 2017; Yan et al., 2017; Fandino et al., 2019). Previous reports have also clearly shown that adult insect behavior

and neuronal development were greatly affected when *Orco* is knocked out (Trible et al., 2017; Yan et al., 2017). Yet, potential changes in olfaction-deficient larvae remain largely unexplored and little is known about how olfaction contributes to the co-adaptation of larvae and their natural enemies, especially in the larvae of non-model insects such as lepidopteran caterpillars.

Pieris brassicae is a very common butterfly species that causes substantial agricultural production loss across Europe and the Indian sub-continent (Feltwell, 1982; Hasan & Ansari, 2011; Hasan & Ansari, 2010). The butterflies utilize chemoreception to evaluate oviposition sites and host plants (van Loon et al., 1992a; van Loon et al., 1992b; Wang et al., 2023). The caterpillars also have a well-developed olfactory system (Wang et al., 2024) and chemical communication has been demonstrated to be involved in the interaction between herbivores and their natural enemies (Stam et al., 2014; Dicke & Baldwin, 2010). Natural enemies such as parasitoid wasps are highly attracted to volatiles from cabbage plants infested by *P. brassicae* caterpillars (Mattiacci et al., 1994). *P. brassicae* caterpillars are thus under strong selection pressure from natural enemies, though caterpillars can defend themselves by spitting at natural enemies while being attacked (Müller et al., 2003).

To gain comprehensive insight into the role of olfaction in *P. brassicae*, and to understand the role of olfaction in their coevolution with natural enemies, we employed a well-established research system utilizing *Brassica oleracea* as host plants, *P. brassicae* caterpillars as herbivores and *C. glomerata* parasitoid wasps as natural enemies. In this study, we knocked out *Orco* by CRISPR/Cas9 in *P. brassicae* to investigate how olfaction guides the host plant choice and natural-enemy avoidance of this insect herbivore. The knockout was verified in caterpillars by staining Orco and glomeruli in larval antennae and the larval antennal center (LAC) respectively and was further confirmed in butterflies by electrophysiological investigations. We also examined the egg-laying behavior of KO butterflies, which is the basis of establishing a homozygous KO insect colony for our subsequent studies. The development of caterpillars on cabbage plants was evaluated, and the performance of caterpillars under the threat of natural enemies was studied to investigate the ecological significance of larval olfaction in interacting with their natural enemies. The larval host-plant seeking behaviors were further evaluated to determine the importance of olfaction in locating food sources. Volatiles emitted by caterpillars and parasitoids were analyzed to identify candidate chemicals that might play a role in this interaction. Our study provides further knowledge on the olfactory system in insect herbivores and might help to clarify the ecological significance of olfaction in the interaction with their natural enemies.

Results

Orco knockout and neuronal deficiency

To test for potential off-target regions of our sgRNAs, we searched these sequences against the *Pieris brassicae* genome to identify any such regions. Neither did we find any off-target sites with a less-than-5nt alignment mismatch from Exonerate searching results, nor did we find any off-target sites with a less-than-4-nt alignment mismatch from CHOPCHOP searching results. Furthermore we did not find any developmental differences between wildtype (WT) animals and knockouts (KO) when caterpillars were raised on leaf discs in Petri dishes (Fig S1) or in the hatching rate of fertilized WT and KO eggs (Fig S2). Taken together these results indicate that no off-target sites were available for our sgRNA. Through screening all butterflies that were reared from injected eggs using PCR and sequencing, we identified an *Orco* mutant line in *P. brassicae*. This mutant line exhibited an 11-bp insertion and a four-bp deletion in the second exon, resulting in a shifted reading frame (Fig 1A). The Orco amino acid sequence showed a significant change mediated by an early stop codon (Fig 1B). These results indicate that the KO of the *Orco* gene was successful.

To further validate the KO in *P. brassicae* and investigate the neuronal mechanism underlying olfactory deficiency in KO caterpillars, we performed staining of Orco in third-instar larvae (L3) using a lepidopteran specific Orco antibody, which has been validated previously (Fandino et al., 2019; Wang et al., 2024; Nolte et al., 2016). In L3 WT caterpillar antennae, a total of 34 ORNs were successfully identified (Wang et al., 2024) (Fig 1C). However, no Orco-positive ORNs were found in the antennae of KO caterpillars (Fig 1D). Similarly, we detected a low number of Orco-positive neurons in the maxillary palps of L3 WT caterpillars (Fig 1E), but no Orco-positive signal was detected in the maxillary palps of KO caterpillars (Fig 1F). By tracing the ORN axons to their glomeruli in the brain, we observed some differences between WT and *Orco* KO caterpillars. In the larval antennal center (LAC), glomeruli displayed well-defined shapes with clear boundaries in the WT LAC (Fig 1G). However, in the KO LAC, the glomeruli were less clearly separated (Fig 1H). Only 7-12 glomeruli were identified in KO LAC, which was a significant reduction ($P = 0.0066$, Wilcoxon rank-sum test) compared to WT LAC (Fig 1I).

Butterfly electrophysiological response to chemical compounds and oviposition

We then conducted additional tests comparing the antennal electrophysiological responses of male and female butterflies from both genotypes using electroantennography (EAG), to further confirm the KO of *Orco* in butterflies. *Orco* KO butterflies of both sexes displayed a complete loss of responses to the tested chemicals with the exception of isovaleric acid, n-caproic acid, hexanal and 2,3-butanedione, where no significant difference was detected between KO and WT butterflies. Reduced responses were recorded in KO butterflies to 1-nonanal, (*Z*)-2-pentenal, (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate. Besides, WT male (n = 18) and female (n = 15) butterflies responded similarly to most of the volatile compounds with a few exceptions where WT females showed significantly different responses to 2-methylbutanal (P = 0.0183, Kruskal-Wallis test), 3-methylbutanal (P = 0.0030, Kruskal-Wallis test) and 3-pentanone (P = 0.0263, Student's t-test) compared to KO females (n = 17), and WT males (n = 18) responded significantly stronger to acetic acid than KO males (P = 0.0217, Student's t-test). Moreover, both WT males and females showed no response to benzoic acid, methyl ITC and 3-butenenitrile (P > 0.05, Student's t-test) (Fig 1J and 1K).

To investigate the role of olfaction in butterfly mating and oviposition, and evaluate the feasibility of establishing homozygous *Orco* KO colony for subsequent larval studies, pairs of newly emerged butterflies were collected and reared in a cage and allowed to mate freely. Upon dissection, it was found that KO butterflies (n = 14) mated fewer times than WT butterflies (P = 0.0199, n = 11; Wilcoxon rank-sum test), since the majority of the KO butterflies (9 out of 14) did not mate during the experiment (Fig S3). Out of the five KO butterflies that did mate, all but one mated singly. Of the WT butterflies, eight out of 11 mated, with most mating twice and one individual even thrice. Female KO butterflies also deposited fewer eggs than WT butterflies during the experiment (P < 0.0001, GLM negative binominal) (Fig S4). To determine the hatching rate, the number of newly hatched caterpillars of both genotypes were counted. It was found that virgin butterflies of both genotypes did not deposit any fertilized eggs, while mated butterflies exhibited similar fecundity with comparable hatching rates between the two genotypes without any significant difference (Fig S2).

Caterpillar performance on cabbage plants

151 To assess the ecological significance of knocking out *Orco* in *P. brassicae* caterpillars, we first
152 conducted a comparison of caterpillar growth between the two genotypes on their optimal host plant. The
153 results show that in the absence of parasitoids, WT caterpillars exhibited better performance, gaining
154 more weight compared to KO caterpillars (P = 0.0268, n = 17 for both genotypes; Student's t-test) (Fig
155 2A). However, we did not find significant survival difference between the two genotypes while collecting
156 caterpillars after the experiment (WT mortality = 8.8 %, n = 17; KO mortality = 2. 9 %; P = 0.0880, n =
157 17; Wilcoxon test). Nor was there a developmental difference when caterpillars were reared in a Petri
158 dish supplied with leaf discs (P=0.97, n = 10 for both genotypes; Wilcoxon test) (Fig S1). Subsequently,
159 to determine if the differences in feeding and survival are dependent on food-plant location, a two-choice
160 assay was conducted with cabbage leaf discs, tomato leaf discs and green paper discs in a glass Petri dish
161 to investigate the role of olfaction in host-plant selection (Fig 2B). Both genotypes of caterpillars chose
162 the cabbage leaf disc significantly more often than the green paper discs (P = 0.0016 for KO, χ^2 = 9.92;
163 P < 0.0001 for WT, χ^2 = 21.41; Chi-square test), and no difference was detected between the two
164 genotypes (P = 0.1460, χ^2 = 2.11; Chi-square test). Similarly, when the cabbage leaf disc was replaced
165 with a tomato leaf disc, both genotypes preferred the tomato leaf disc over the paper disc (P = 0.0143 for
166 KO, χ^2 = 6.00; P = 0.0009 for WT, χ^2 = 10.97; Chi-square test) and no difference was found between the
167 two genotypes (P = 0.5477, χ^2 = 0.36; Chi-square test). However, when caterpillars were provided with
168 both a cabbage leaf disc and a tomato leaf disc, a contrasting preference was observed between the two
169 genotypes (P = 0.0004, χ^2 = 12.68; Chi-square test). KO caterpillars significantly preferred the tomato
170 leaf disc (P = 0.0466, χ^2 = 3.96; Chi-square test), while WT caterpillars preferred the cabbage leaf disc
171 (P = 0.0028, χ^2 = 8.97; Chi-square test).

172 We then aimed to determine whether caterpillars would orient towards healthy plants, plants infested
173 with conspecifics and plants on which conspecifics are attacked by parasitoid wasps, using a Y-tube
174 olfactometer. We first compared the behaviors of both WT and *Orco* KO caterpillars in response to clean
175 air, volatiles from a healthy plant and volatiles from a caterpillar-infested plant (Fig 2C). Both WT and
176 *Orco* KO caterpillars tended to prefer a healthy cabbage plant to a clean air control, however WT
177 caterpillars were significantly attracted by volatiles from a healthy plant (P = 0.0164, χ^2 = 5.76; Chi-
178 square test) while the preference for *Orco* KO caterpillars was not significant (P = 0.2207, χ^2 = 1.50; Chi-
179 square test). Subsequently, we challenged the caterpillars with volatiles from a infested plant and a clean
180 air control and found that WT caterpillars prefer volatiles from the infested plant to the control (P =

181 0.0495, χ^2 = 3.86; Chi-square test), while *Orco* KO caterpillars did not show any preference between the
182 two provided options (P = 0.7055, χ^2 = 0.14; Chi-square test). We further compared larval host-plant
183 seeking behaviors with the presence of natural enemies on caterpillar-infested plants (Fig 2D). WT
184 caterpillars showed a significant preference for infested plants without parasitoid wasps *C. glomerata* (P
185 = 0.0067, χ^2 = 7.35; Chi-square test), while *Orco* KO caterpillars did not show any preference for either
186 odor source (P = 0.7055, χ^2 = 0.14; Chi-square test).

187 To understand the ecological significance of the odor-guided behavior for the natural enemy
188 avoidance in *P. brassicae* caterpillars, we then evaluated caterpillar performance under the potential
189 threat of natural enemies by exposing the caterpillar to parasitoids after removing the ovipositor of the
190 female wasps (Fig 2E). The results show a significantly higher weight in WT caterpillars than in KO
191 caterpillars (P = 0.0088, n = 8 for both genotypes; Student's t-test). To further explore the effect of
192 knocking out *Orco* on the interaction of the caterpillars with their natural enemies, we compared the
193 survival rates of caterpillars when exposed to two mated unmanipulated female *C. glomerata* wasps. The
194 findings show that KO caterpillars had significantly lower survival rates compared to WT caterpillars (P
195 = 0.0120, n = 7 for both genotypes; GLM beta-binomial) (Fig 2F).

196 Volatile headspace composition

197 Furthermore, we investigated if caterpillar- and parasitoid-derived chemicals are involved in the
198 behavioral responses. We identified a total of 45 chemical compounds among the five treatments
199 belonging to seven classes, including alcohols, aldehydes, aromatics, ketones, nitrogen and/or sulfur-
200 containing chemicals, terpenoids and others (Table S3). The contribution of identified chemicals towards
201 separating different treatment groups was analyzed using principal component analysis (PCA). This
202 analysis showed a significant separation of most groups with PC 1 carrying 65.4 % of the variance (P =
203 0.001, PERMANOVA). *Pieris brassicae* caterpillars (Pb, n = 10), *P. brassicae* caterpillar spit (Pb-S, n =
204 12), *C. glomerata* female wasps (Cg, n = 12) and caterpillar frass (Pb-Fr, n = 10) treatment groups were
205 clearly separated (Pb vs. Cg, Pb vs. Pb-S, Pb vs. Pb-Fr, Pb-S vs. Cg and Pb-Fr vs. Cg, P= 0.0013; Pb-S
206 vs. Pb-Fr, P= 0.0022 pairwise PERMANOVA, Fig 3A), whereas the identified and measured volatile
207 blend from Pb was similar to that of the *P. brassicae* caterpillars-*C. glomerata* parasitoids combination
208 (Pb-Cg, n = 14) (P = 0.0330, pairwise PERMANOVA). The amount of volatiles among the five
209 treatments was also compared. Most of the identified compounds were found in significantly higher

amounts in Pb-S and/or Pb-Fr compared to the other treatments (Fig 3B, Table S3). We also found that some chemicals are strongly correlated. The identified chemicals are classified into several subgroups in the hierarchical clustering heatmap according to the abundance among treatments. For instance, the seven chemicals (*Z*)-3-hexen-1-ol, 1-penten-3-ol, dimethyl disulfide, methyl (methylthio) methyl sulfide, dimethyl trisulfide, 2-methylbutanal and 3-methylbutanal formed a clade. Similarly, the six chemicals, 3-methylbutanoic acid, 1-pentanol, benzyl cyanide, 3-(methylthio) propanal, (*Z*)-2-hexen-1-ol and phenylacetaldehyde formed another clade. The clustering analysis demonstrated that chemicals within the same clade are highly correlated. The multivariate data analysis (MVDA) also showed similar clustering when comparing the different treatments (Fig S5-S9).

Based on the results of our chemical analysis, we selected 11 candidate chemicals (1-hexanol, 1-methoxy-2-propanol, 1-penten-3-ol, 2,3-butanedione, 3-methylbutanal, benzyl cyanide, dimethyl disulfide, pentanal, (*E*)-2-hexen-1-ol, (*Z*)-2-penten-1-ol and (*Z*)-3-hexen-1-ol) with high variable importance in the projection (VIP) scores (> 1.0) (Table S3-S8), and another four common plant volatiles (linalool, (*Z*)-3-hexenyl acetate, hexanal and (*E*)-2-hexenal). These odorants were chosen to test the behavioral response of L3 caterpillars. Using a custom-made multiple channel setup (Fig 3C), we conducted behavioral experiments with the caterpillars. The results showed that WT caterpillars displayed a preference for two plant chemicals, namely (*Z*)-3-hexen-1-ol and (*E*)-2-hexenal over paraffin oil ($P = 0.0482$ and $P = 0.0321$) (Fig 3D). In contrast, KO caterpillars did not exhibit any significant attraction or avoidance behavior towards the tested chemicals (Fig 3E). Additionally, we found that WT caterpillars were more active in the arena, moving around (Fig S10), while KO caterpillars were less active in the arena, often remaining at the same position within the channels (Fig S11).

Discussion

Chemical communication plays a significant role in the interaction between plants, herbivores and their natural enemies. Infested host plants emit herbivore-induced plant volatiles (HIPVs) and thereby attract natural enemies such as parasitoid wasps (Mattiacci et al., 1994). While the role of adult herbivores' olfaction in mediating these tri-trophic interactions has been investigated, the significance of caterpillar olfaction in tri-trophic interactions of plants, herbivores and their natural enemies has remained largely underexplored. In this study, we severely impaired the olfaction of *P. brassicae* by knocking out *Orco* using CRISPR/Cas9 to investigate the ecological importance of olfaction in host-plant choice and enemy

239 avoidance by *Pieris brassicae* larvae. The knockout of olfaction was successful, as we did not find Orco-
240 proteins in the antennae of *Orco* KO caterpillars and showed an altered brain structure in the *Orco* KO
241 caterpillars in comparison to WT caterpillars. Furthermore, the near complete lack of response to odors
242 by *Orco* KO butterfly antennae compared to WT confirms that olfaction was largely lost. Intriguingly,
243 although mating and oviposition were significantly reduced, KO butterflies still produce some
244 homozygous offspring. After this characterization of our mutant line, we aimed to test the role of larval
245 olfaction in a multitrophic framework. We found that the loss of olfaction had a significant effect on the
246 foraging behavior of the caterpillars, which exhibited a reduced growth rate, and a greater vulnerability
247 to the parasitoid wasp *C. glomerata.*

248 The perception of chemical cues is mediated by the chemosensory signal transduction in insect
249 antennae and further processing of the information in the brain. In *P. brassicae* KO caterpillar antennae,
250 no Orco protein was detected, and chemical signals can therefore not be converted into electrical signals
251 (Sato et al., 2008; Wicher et al., 2008). Interestingly, the non-functional Orco decreased the number of
252 glomeruli in the LAC, indicating that Orco is likely to play a role in the glomerular organization of the
253 LAC (Fig 1C-I). Similar changes have been reported for adult insects of a few hymenopteran species:
254 the ants *Harpegnathos saltator* and *Ooceraea biroi* and the honeybee *Apis mellifera*, where the number
255 and total size of glomeruli decreased while the volume of individual glomeruli increased in *Orco* KO
256 mutants (Trible et al., 2017; Yan et al., 2017; Chen et al., 2021). In contrast, the antennal lobe morphology
257 of *D. melanogaster* was not significantly changed when *Orco* was knocked out (Larsson et al., 2004). In
258 Lepidoptera, the size and number of glomeruli in *Helicoverpa armigera* were found to be comparable
259 between WT and KO adult moths without any significant difference (Fan et al., 2022). However,
260 silencing the pheromone receptor, *SlitOR5* led to a decreased size of a glomerulus in the adult moth
261 *Spodoptera littoralis* and knocking out *Orco* also led to a reduced size of OR-related glomeruli in adult
262 *Manduca sexta* (Koutroumpa et al., 2022; Fandino et al., 2019). The loss of olfactory glomeruli in the
263 caterpillar might, therefore, either indicate that the formation of glomeruli in the embryo is mediated by
264 a different mechanism than during pupation (Oland & Tolbert, 2011), or that the loss of neuronal activity
265 reduces axonal innervations and thereby the size of the glomeruli to an extent that they could no longer
266 be visualized by our method. However, the mechanism of glomerular formation and the roles that
267 olfactory genes play in this process needs to be further investigated in both immature and adult insects
268 (Williams et al., 2022).

269 The role of olfaction in the behavioral ecology of adult insects has already been studied in quite
270 some detail, and silencing *Orco* has been an important tool for unravelling the importance of chemical
271 communication in adults of various insect species. For instance, in *Orco* KO adults of several insect
272 species, there was a significant reduction in the electrophysiological responses to volatile chemicals such
273 as alcohols and esters (Fandino et al., 2019; Sun et al., 2020). Consequently, *Orco* mutants exhibited
274 notable changes in host-plant location and severely reduced fecundity due to impaired pheromone
275 perception and mating, which also resulted in significantly reduced egg-laying behavior (Fan et al., 2022;
276 Fandino et al., 2019; Liu et al., 2023). Similar to other species, *Orco* KO *P. brassicae* butterflies exhibited
277 a loss of electrophysiological responses to most plant volatiles we tested (Fan et al., 2022; Sun et al.,
278 2020) (Fig 1J and 1K), and the mating behavior of *P. brassicae Orco* KO butterflies was also disrupted
279 (Fig S3). However, we observed some differences: *P. brassicae Orco* KO butterflies were still able to
280 mate to a limited degree (Fig S2). Whereas *Orco* KO adults in some other insect species did not exhibit
281 any mating behavior (Fandino et al., 2019; Fan et al., 2022). This finding suggests that additional sensory
282 systems beyond olfaction, such as vision, may be involved in the mating processes of this day-active
283 butterfly (Carpenter & Sparks, 1982; Obara et al., 2008). Interestingly, while many *P. brassicae*
284 butterflies with impaired olfaction failed to mate, several KO butterflies still mate and deposit fertilized
285 eggs when the oviposition substrate was supplied in our laboratory setting. The mated *Orco* KO
286 butterflies laid a comparable number of fertilized eggs on cabbage plants to WT butterflies which is in
287 line with the results from different moth species (Fandino et al., 2019; Fan et al., 2022; Sun et al., 2023),
288 suggesting oviposition decisions are mostly guided by non-volatile compounds, when no host-plant
289 location is required (van Loon et al., 1992a). Furthermore, the hatching rate of eggs laid by mated *Orco*
290 KO butterflies was similar to that of WT butterflies (Fig S2), indicating that the reduced fecundity in
291 these insects is primarily due to impaired mating behavior and reduced mating frequency, which may
292 result from a lack of pheromonal communication.

293 When directly placed on their host plant, we found that WT caterpillars gained more weight than
294 *Orco* KO caterpillars (Fig 2A). We speculate that olfaction is needed to evaluate plant tissues and to
295 select different leaves or leaf parts in order to feed efficiently, even when the caterpillar is already on the
296 plant. Secondary metabolites are often unevenly distributed even within a single leaf, and these
297 concentration differences have been shown to influence the performance of different caterpillars (Kester
298 et al., 2002; Yuan et al., 2022). It is conceivable that these localized differences in plant secondary

299 metabolites are also detectable by the herbivore through its olfactory system (Hanson & Dethier, 1972).
300 The difference between WT and KO caterpillars was amplified in the presence of disarmed parasitoids,
301 which were able to attack but not injure the caterpillars, indicating that KO caterpillars were more
302 susceptible to harassment by their natural enemies (Fig 2E). We hypothesize that this further reduction
303 in caterpillar weight was due to the less efficient foraging behavior of the KO caterpillars, which resulted
304 in an extended time period in which the caterpillars were vulnerable to the harassment by the disarmed
305 parasitoids. This harassment would then further reduce the foraging efficiency of the KO caterpillars and
306 thereby exacerbate the effect seen in Fig 2A.

307 *P. brassicae* caterpillars are most vulnerable to *C. glomerata* during the first two larval instars. Due
308 to their reduced growth rate, KO caterpillars spent a longer time in these vulnerable stages and, therefore,
309 in a natural setting would likely have suffered more strongly from parasitoid attack than WT caterpillars
310 (Brodeur et al., 1996; Haverkamp & Smid, 2020). In addition, caterpillars might have been avoiding
311 plant parts with natural enemies similar to choosing infested plants without parasitoids over infested
312 plants with parasitoids present (Fig 2D). This suggests that caterpillars might be able to use their olfactory
313 system to find enemy-free spaces based on volatiles emitted by the plant or the frass and spit of
314 conspecifics. Similarly, when the caterpillars were exposed to unmanipulated parasitoids, the KO
315 caterpillars were more susceptible and exhibited a higher mortality rate, demonstrating again that
316 olfaction is crucial in larvae to survive under the pressure of natural enemies (Fig 2F). Subsequently, we
317 inferred that the differences in weight gain and survival rate between WT and KO caterpillars are due to
318 different sensitivity to plant volatiles. WT caterpillars can sooner reach a body size in which they are no
319 longer vulnerable to attack by *C. glomerata* by locating an available food source efficiently (Brodeur et
320 al., 1996) . The higher mortality rate of KO caterpillars might, therefore be partly due to a reduced
321 foraging efficiency caused by the lack of olfactory information, which in turn led to an extended period
322 in which the caterpillars were vulnerable to attack by their natural enemies. In addition, the WT
323 caterpillars might have been better able to use olfactory cues to avoid plant parts where conspecifics are
324 under the attack by parasitoids again reducing their mortality in comparison to KO caterpillars (Fig 2D).

325 Searching for host-plants suitable for feeding and free of natural enemies is largely accomplished
326 via the olfactory system of herbivorous insects (Carrasco et al., 2015; Hu et al., 2020). Without functional
327 ORs, *P. brassicae* caterpillars could still discriminate plant tissues from paper discs; however, *Orco* KO
328 caterpillars preferred the non-host tomato plant over their natural food plant (Fig 2B). We surmise that

329 OR-mediated olfaction is required in caterpillars to locate and identify their host plants, and losing this
330 part of their olfactory receptor repertoire would make it challenging to distinguish host from non-host-
331 plants (Liu et al., 2023). Nonetheless, *Orco* KO caterpillars still retain ORNs expressing ionotropic
332 receptors, which commonly detect acids and amines that have been found to elicit aversion in insect
333 herbivores (Zhang et al., 2019). Fatty acids are present at the edge of both artificially damaged and
334 caterpillar-infested cabbage leaves (Horikoshi et al., 1997), which, in the absence of ORs detecting
335 attractive plant compounds, may have caused avoidance behavior in the KO caterpillar to their natural
336 host-plant. Interestingly, the parasitoid *C. glomerata* also uses the fatty acids produced by the plant to
337 locate *Pieris* caterpillars, and it has been argued that herbivores might avoid these compounds to escape
338 from their natural enemies (Horikoshi et al., 1997; Zhang et al., 2019).

339 We found that WT caterpillars prefer caterpillar-infested plants free of wasps over caterpillar-
340 infested plants with wasps, which suggests that caterpillars are able to detect certain volatiles that are
341 derived from the interaction with caterpillars and wasps. By analyzing headspace samples of caterpillars,
342 parasitoids as well as the interaction of caterpillars and parasitoids, we successfully identified some
343 chemical compounds which were already known plant volatiles, such as alcohols and aldehydes.
344 However, we did not find any compounds that were specifically abundant in Pb-Cg or Cg. This suggests
345 that a volatile signal serving as a "danger signal" directly emitted by the wasps or caterpillars might not
346 exist, or it might not be detectable with our methods (Fig 3B). Alternatively, indirect cues such as the
347 volatiles emitted from the caterpillar spit during their defensive behavior might have triggered the
348 avoidance behavior of the WT caterpillars to infested plants with natural enemies. When we tested the
349 most abundant compounds in a two-choice behavioral assay against a solvent control, we found that *P.*
350 *brassicae* WT larvae showed a preference to the plant volatiles (*Z*)-3-hexen-1-ol and (*E*)-2-hexenal (Fig
351 3D), which are known HIPVs. Previous studies on other species have demonstrated that caterpillars are
352 capable of detecting different HIPVs (Di et al., 2017; de Fouchier et al., 2018). For caterpillars, HIPVs
353 can serve as indicators for the location of potential food plants and the presence of conspecifics (Di et
354 al., 2017; Zhang et al., 2019), but they also attract natural enemies and enhance the risk of being attacked
355 (Bernays, 1997; Ngumbi & Fadamiro, 2012; Yang et al., 2016). Most HIPVs identified in this study were
356 found abundantly in caterpillar saliva that caterpillars spit onto parasitoids to defend against attack. In
357 our study, caterpillars were attracted by HIPVs emitted by plants and by individual HIPVs (Fig 3D). We
358 speculate that caterpillars use low to intermediate concentrations of HIPVs to find suitable host-plants

359 and high concentrations to avoid sites where other caterpillars are under attack by their natural enemies.
360 Therefore, perceiving plant leaf volatiles and approaching the food plants accordingly will increase the
361 herbivores' feeding efficiency and enhance survival chances. Interestingly, isothiocyanates (ITCs) which
362 are hydrolyzed from glucosinolates after leave damage in crucifer plants, did not elicit strong responses
363 from the butterfly antenna (Fig 1 J and 1K). Because all receptors expressed in the caterpillar are also
364 present in the butterfly and because of the limited number of ORs and ORNs in larvae and low expression
365 levels of ORs (Wang et al., 2024), we found it less likely that ITCs are used by *P. brassicae* caterpillars
366 as foraging cues, even though these compounds are recognized by *Plutella xylostella* ORs (Liu et al.,
367 2020). In our study, volatile cues of wasps were only found in very low quantities and these compounds
368 hardly elicited any behavioral response in the caterpillars; however these chemicals might still play a
369 role in the interaction between the caterpillars and wasps at a close range (Ebrahim et al., 2015).
370 The way that larval insects react to the potential threat from their natural enemies is still largely
371 unknown for most insects. Our study that exploited CRISPR/Cas9 shows that olfaction is of high
372 significance to caterpillars for locating food sources and likely affects their survival when under the
373 selection pressure from natural enemies. We provided novel insights into the tri-trophic interactions of
374 plants, herbivores and their natural enemies from the perspective of a caterpillar and highlighted the role
375 of olfaction for both foraging and escaping natural enemies.

376

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381

382 Author contributions

383 Conceptualization, Q.W., M.D. and A.H.; Methodology, Q.W., M.J., A.H.; Investigation, Q.W., Y.J.,
384 H.S., B.T.W., L.O.G.; Writing - Original Draf, Q.W., A.H.; Writing - Review & Editing, Q.W., L.O.G.,

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Declaration of interests

The authors declare they have no competing interests.

Materials and methods

Plant and insect rearing

Cabbage plants (*Brassica oleracea* var. gemmifera cv. Cobelius; Brussels sprouts) and tomato plants (*Solanum lycopersicum* cv. moneymaker) were planted in individual pots and grown for four weeks after germination before being used in the experiments or to feed the caterpillars. The cabbage plants were reared under glasshouse conditions at 22 ± 3 °C, RH 50-80 % and 16 h light: 8 h dark cycles. *Pieris brassicae* caterpillars and butterflies as well as *C. glomerata* wasps were collected from laboratory colonies maintained at the Laboratory of Entomology, Wageningen University & Research, the Netherlands. The caterpillars were reared on Brussels sprouts plants at 22 ± 3 °C, RH 25-35 % with 14 h light: 10 h dark cycles. The adults were supplied with 10 % sugar water as nutrient under the same environmental conditions. After successful gene editing, KO insects were transferred and reared in a glass house compartment under conditions at 25 ± 3 °C, RH 25-35 %. Several KO caterpillars and butterflies from each generation were randomly collected for genotype screening to ensure that homozygosity remains. *Cotesia glomerata* adults were fed with organic honey, *P. brassicae* second-instar larvae (L2) were supplied to the parasitoid wasps for parasitization to maintain the colony. The wasp cocoons were collected in Petri dishes and incubated at 22 °C, RH 50-70 % with the same photoperiod as that of caterpillars. All the subsequent comparisons between WT and KO in this study were conducted in the glass house compartment under the rearing condition of KO insects unless specified.

Orco knockout

The second exon of *Orco* was targeted to knockout the gene. The sgRNA was designed with Geneious Prime 2021 (Geneious, New Zealand) by searching N20 target + NGG PAM sequences in the

409 second exon, sgRNA primers were selected according to activity scorings (Doench et al., 2016). The
410 designated sgRNA off-target regions were evaluated by searching against the genome by est2genome
411 function with a loose 50-score cut-off by using Exonerate 2.0 (Slater & Birney, 2005) and CHOPCHOP
412 v3 (Labun et al., 2019). The sgRNAs that had no off-target sites in coding areas were selected. sgRNA
413 templates were prepared by mixtures of 50 μL Q5 high-fidelity 2 × master mix (NEB, USA), 5 μL of the
414 following forward primers:
415 sgRNA1: ATTTAGGTGACACTATACATGTCAACTCTACTTAGGAGTTTTAGAGCTAGAAATA
416 GCAAG; sgRNA2: ATTTAGGTGACACTATACGATGAAGTAAACGAACTCAGTTTTAGAGCTA
417 GAAATAGCAAG; 5 μL constant reverse primer: AAAAGCACCGACTCGGTGCCACTTTTTCA
418 AGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC and 40 μL H_2O. The
419 mixture was incubated at 98 °C for 2 min; followed by 38 cycles of 98 °C for 20 s, 65 °C for 10 s and
420 72 °C for 10 s; finalized at 72 °C for 5 min. The PCR products were checked by 1.0 % gel electrophoresis
421 and purified by QIAGEN PCR purification kit (QIAGEN, Germany) according to the manufacturer's
422 instructions. sgRNA was synthesized by a reaction system of 2 μL 10 × SP6 enzyme mix (Invitrogen,
423 USA), 2 μL 10 × SP6 reaction buffer, 2 μL each of ATP, UTP, GTP and CTP, 200 ug sgRNA template
424 and add RNase-free H_2O up to 20 μL. The mixture was incubated at 37 °C for 4 hours, followed by
425 adding 1 μL Turbo DNase and incubating at 37 °C for 15 min. The reaction products were purified by
426 Monarch RNA cleanup kit (NEB, USA). The concentration of sgRNA was determined by DeNovix
427 (DeNovix, USA). sgRNA was mixed with Cas9 protein (NEB, USA) and incubated at 25 °C for 10 min.
428 Newly laid eggs, not older than half an hour, were collected from our laboratory colony and were
429 fixed on glass slides. The sgRNA/Cas9 mixture was colored with 1 μL food dye to stain the eggs as an
430 indicator that injection with a Femto-jet (Eppendorf, Germany) was successful. The injected eggs were
431 incubated at 25 °C for 4 to 5 days until hatching. The caterpillars were reared until the emergence of the
432 butterflies, a leg of each butterfly was cut for genotyping. gDNA of the leg samples was extracted with
433 MyTaq Extract-PCR Kit (Bioline, Germany). Mutant screening was performed by PCR with 15 μL 2 ×
434 MyTaq HS Red mix, 2 μL forward primer (TCTGGCTTCGGTATTACATTTC), 2 μL reverse primer
435 (CTTTTATGGCGTGTTTTATTTG), 2 μL gDNA template and 9 μL H_2O. The PCR products were
436 checked by gel electrophoresis and sequenced with the same primers by Eurofins (Eurofins, the
437 Netherlands).

ORN staining and axon tracing

L3 caterpillars (n = 6 for WT and n = 5 for KO) were anesthetized on ice and decapitated. Heads were cut along the midline with micro-scissors to facilitate penetration of the fixative and antibodies. The collected heads were immersed in freshly prepared 4 % formaldehyde in 0.1 M phosphate buffer at pH 7.3 and fixed overnight at 4 °C. The fixed samples were rinsed in 70 % ethanol, followed by incubation in a mixture of 96 % ethanol: 30 % hydrogen peroxide at 1:1 ratio for 7 days at 4 °C. The incubated samples were then dehydrated in a graded series of ethanol, degreased in xylene for two mins, rehydrated and incubated in 0.1 M phosphate buffered saline (Oxoid, Dulbecco A) with 0.5% triton X-100 (PBS-T) followed by four washes in PBS-T for 15 min each. Preincubation of head samples was performed in 10 % normal goat serum (Bio-connect services, The Netherlands) in PBS-T (PBS-T-NGS) for 1 hour, and then incubated in rabbit anti-Orco antibody which specificity was tested previously (Nolte et al., 2016) diluted in 1: 500 in PBS-T-NGS for three days at 4 °C, followed by 6 washes in PBS-T for 30 min each. Samples were subsequently incubated in goat anti-rabbit conjugated to Alexa fluor 488 (Thermo Fisher Scientific, USA) diluted 1:100 and TO-PRO-3 iodide (Thermo Fisher Scientific, USA) diluted 1:1000 in PBS-T-NGS for two days at 4 °C. Heads were washed two times in PBS-T for 30 mins and once overnight at 4 °C, following with two 1-hour PBS washes at room temperature. The samples were then dehydrated with a graded series of ethanol, cleared in xylene and mounted in DPX. Confocal microscopy was performed with a Leica Stellaris 5- DIM 8 confocal microscope (Leica, Germany), using a 63× oil-immersion plan APO objective NA1.4. and a spectrally flexible white light laser for excitation of the two fluorophores using the pre-sets for Alexa fluor 488 and To-Pro-3 iodide.

L4 caterpillars of similar head size were anesthetized on ice and immobilized in clay. The tip of each antenna was peeled, and a layer of petroleum jelly (Vaseline) was applied around the antenna. A 0.5 µL drop of 2.5 % biotin-dextran solution was added to the cut and covered with petroleum jelly, then incubated at 4 °C overnight. Brains were dissected in PBS, fixed in 4 % formaldehyde in PBS at 4 °C overnight, dehydrated in xylene and rehydrated in PBS-T, followed by three washes in PBS-T for 2 hours each. Brains were then incubated in 10 % NGS in PBS-T for 1 hour, followed by incubation in 1:200 streptavidin 488 and 1:1000 To-Pro iodide in PBS-T at 4 °C for three days. After further rinsing in PBS-T overnight, brains were mounted in DPX. Whole-mount samples were scanned using confocal microscopy with settings similar to those used for ORN staining.

467 # Electroantennography (EAG)

468 A panel of 54 chemicals (Table S1) including esters, alcohols, aldehydes, alkenes, isothiocyanates
469 (ITCs), heterocyclics and nitriles were selected from the literature (van Loon et al., 1992b; Liu et al.,
470 2020; Zhu et al., 2015; Bourne et al., 2023) and our current volatile identification data, to investigate the
471 EAG responses of both KO and WT butterfly antennae (n =15-18) of both sexes. Filter papers loaded
472 with 10 µL of selected chemicals, diluted in paraffin oil (10^{-2} v/v), were inserted into Pasteur pipettes,
473 clean filter paper loaded with 10 µL paraffin oil was employed as negative control. The left antennae of
474 butterflies were excised at the basal end and the distal tip was also removed for better conductivity.
475 Antennae were placed into glass capillaries filled with EAG Ringer solution. Glass capillaries were
476 placed on Ag-AgCl wires connected to a ground electrode and a 10× high-impedance DC amplifier
477 (Ockenfels Syntech, Germany). The electrical signals were transformed using an IDAC-232 (Ockenfels
478 Syntech, Germany) analog-digital converter connected to a personal computer. Signals were finally
479 recorded using the software EAG Pro (Ockenfels Syntech, Germany). The first puff of each chemical
480 was abandoned before the first round of testing. Antennae were exposed to the chemicals in random order
481 and were given intervals of at least 20 s for recovering until the baseline stabilized.

482 # Female oviposition behavior

483 A mating pair of newly emerged butterflies (n = 11 for WT and n = 14 for KO) were put in a cage
484 and supplied with a four-week-old Brussels sprouts cabbage plant as oviposition substrate and sugar
485 water as nutrients. The numbers of eggs were counted on a daily basis for seven days. The female
486 butterflies were then collected and dissected to compare mating frequency between WT and KO females.
487 Mating frequency was determined by the number of spermatophores dissected from the females'
488 abdomens. The eggs remained on the plants for another four to five days to allow hatching. The number
489 of caterpillars hatched enabled us to compare the number of fertilized eggs and hatching rate between
490 the two butterfly genotypes.

491 # Caterpillar performance on plants

492 Caterpillar performance on cabbage plants was evaluated by three different treatments:

493 (1) For each genotype, a group of ten L1 caterpillars (n = 17) was placed on one leaf of a four-week-
494 old cabbage plant. Caterpillars were reared for ten days, and more plants were provided when needed.
495 The caterpillars were then collected for weighing to compare the growth between the two genotypes.
496 (2) The growth of both genotypes of caterpillars (n = 8) was further compared by rearing in the
497 presence of *C. glomerata* parasitoid wasps from which the ovipositor had been removed (hereafter:
498 "disarmed"). Groups of ten L1 caterpillars were placed on individual four-week-old cabbage plants as
499 described above. The parasitoid wasps were anesthetized on a CO_2 plate and the ovipositor was removed
500 with fine tweezers to ensure that the parasitoid wasps can interact with the caterpillars but cannot
501 parasitize them. The activity of disarmed parasitoids was observed for several minutes to ensure that they
502 would behave similarly to unmanipulated parasitoids. Two disarmed parasitoid wasps were released in
503 each rearing cage 24 hours after the caterpillars had been placed on the plants. The disarmed parasitoid
504 wasps were replaced by newly disarmed parasitoids every day, the caterpillars were reared on the plants
505 for ten days and then collected for weighing.
506 (3) To compare the survival rate of caterpillars (n = 7 groups of ten caterpillars) on the plants under
507 the threat of *C. glomerata* parasitoid wasps, a group of ten L1 caterpillars was placed on a leaf of a four-
508 week-old cabbage plant two days prior to placing two female parasitoid wasps. The parasitoid wasps
509 were replaced every day to ensure that caterpillars were always exposed to natural enemies. The
510 caterpillars were collected after ten days to count the number that survived; the collected caterpillars
511 were further reared until they pupated or *C. glomerata* larvae emerged.

512 Larval host-plant seeking behavior

513 Caterpillar (n = 54-73) food-plant seeking behavior was evaluated in a greenhouse under the same
514 conditions in which the caterpillars were reared. Plant leaf discs or green paper discs (Trophée 1224
515 "Forest Green", Clairefontaine, France), with 12 mm diameter were placed at opposite positions along
516 the edge of a 188 mm-diameter Petri dish. A single L3 caterpillar was then placed at the center of the
517 arena. Each caterpillar was observed for 10 min, the first choice that the caterpillars made and the time
518 spent were recorded. The caterpillars were provided with a choice between either (i) a cabbage leaf disc
519 and a paper disc, (ii) a tomato leaf disc and a paper disc, or (iii) a cabbage leaf disc and a tomato leaf
520 disc. A choice was recorded when caterpillars began feeding on the cabbage leaf disc or contacted the
521 tomato leaf / green paper discs. New discs were used for every caterpillar and the positions of discs were

522 exchanged after every ten tests.

523 To further test the host-plant locating behavior of L3 caterpillars, we investigated the behaviors of
524 caterpillars in a Y-tube olfactometer (diameter 35 mm) with a built-in black Y-shaped wire, allowing
525 caterpillars to crawl along the wire and make a choice smoothly in the laboratory. Each arm was provided
526 with a 1.1 L/min inlet airflow which had been purified by charcoal and humidified by water before
527 entering the treatment jars. Healthy plants were untreated, infested plants were infested with 30 early L3
528 caterpillars for 24 hours and with caterpillars on plants while testing. Differently treated plants were
529 placed in jars to test caterpillar behaviors. The caterpillars had been starved for approximately 5 hours
530 before testing. Each caterpillar was tested and observed for 5 min, caterpillar behavior was monitored by
531 a camera. The position of inlet airflow was changed every five tests to avoid position bias.

532 Volatile analysis

533 Volatiles were collected from L3 *P. brassicae* caterpillars, three-day-old female *C. glomerata* wasps,
534 *P. brassicae* caterpillars and *C. glomerata* wasps together, caterpillar frass collected from L3 caterpillars
535 and caterpillar saliva also collected from L3 caterpillars using glass capillaries following Mattiacci et al.
536 (Mattiacci et al., 1994). Fresh frass and saliva samples were stored in a freezer at -20 °C and thawed
537 overnight at room temperature before volatile collection. Headspace collection of volatiles was
538 performed from five treatments: 20 female wasps (Cg) in a 10 mL glass vial (n =12); 20 L3 caterpillars
539 (Pb) in a 10 mL glass vial (n =10); 20 L3 caterpillars and 2 female wasps (Pb-Cg) in a 10 mL glass vial
540 (n =14); 100 mg caterpillar frass (Pb-Fr) in a 1.5 mL glass vial (n =10); and 50 μL caterpillar saliva (Pb-
541 S) in a 1.5 mL glass vial (n =12). Clean empty glass vials (10 mL and 1.5 mL) were used as a negative
542 control. Volatiles were collected through dynamic headspace sampling using Tenax TA adsorbent
543 material (20/35 mesh; Camsco, USA). Synthetic air (Air Synthetic 4.0 Monitoring from Linde Gas, the
544 Netherlands) at 110 mL min^{-1} to the samples in 10 mL vials and 55 mL min^{-1} to the samples in 1.5 mL
545 vials were constantly supplied as a carrier for the volatiles, while simultaneously the volatiles were
546 trapped by drawing air at 100 and 50 mL min^{-1}, respectively, through a stainless-steel tube filled with
547 200 mg Tenax TA for 2 h.

548 The collected volatiles were thermally released from the Tenax TA adsorbent using an Ultra 50:50
549 thermal desorption unit (Markes, UK) at 250 °C for 10 min under a helium flow of 20 mL min^{-1}, while
550 simultaneously re-collecting the volatiles in a thermally cooled universal solvent trap: Unity (Markes,

551 UK) at 0 °C. Once the desorption process was completed, the volatile compounds were released from
552 the cold trap by ballistic heating at 40°C s^{-1} to 280 °C, which was then kept for 10 min, while all the
553 volatiles were transferred to a ZB-5MS analytical column (30 mL × 0.25 mm ID × 1 mm F.T. with 10 m
554 built-in guard column (Phenomenex, USA), placed inside the oven of a Thermo Trace GC Ultra (Thermo
555 Fisher Scientific, USA), for separation of volatiles. The GC oven temperature was initially held at 40 °C
556 for 2 min and was immediately raised at 6 °C min^{-1} to a final temperature of 280°C, where it was kept
557 for 4 min under a constant helium flow of 1 mL min^{-1}. For the detection of volatiles, a Thermo Trace
558 DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, USA) coupled to the GC was operated
559 in an electron impact ionization (EI) mode at 70 eV in a fullscan mode with a mass range of 35 - 400 amu
560 at 4.70 scans s^{-1}. The MS transfer line and ion source were set at 275 and 250 °C, respectively.

561 Automated baseline correction, peak selection (S/N > 3) and alignments of all extracted mass signals
562 of the raw data were processed following an untargeted metabolomic workflow using MetAlign software,
563 producing detailed information on the relative abundance of mass signals representing the available
564 metabolites (Lommen, 2009). This was followed by the reconstruction of the extracted mass features into
565 potential compounds using the MSClust software through data reduction by means of unsupervised
566 clustering and extraction of putative metabolite mass spectra (Tikunov et al., 2012). Tentative
567 identification of volatile metabolites was based on a comparison of the reconstructed mass spectra with
568 those in the NIST 2008 and Wageningen Mass Spectral Database of Natural Products MS libraries, as
569 well as experimentally obtained linear retention indices (LRIs).

570 Caterpillar response to individual chemical compounds

571 L3 caterpillars (n = 31-50 for each compound, each genotype) were tested in a custom-made set-up
572 consisting of 25 separate arenas in the laboratory (Fig 3C). Each arena allowed caterpillars to move freely
573 along two channels of 90 mm located in opposite directions of a central opening, which permitted the
574 caterpillar to crawl into the arena. At the end of the arenas, airflows are introduced to deliver volatile
575 chemicals. The airflow was sucked out through the channel below the central opening simultaneously.
576 Single caterpillars were placed in the central well of the individual arenas before the test. Caterpillars
577 were given a choice between a selected volatile chemical of interest (Table S2) and paraffin oil. Input
578 odors were delivered at 1.1 L min^{-1} for both chemicals and solvent controls, the odors were sucked out
579 at 2.2 L min^{-1} at the same time from the center. The arena was cleaned by ethanol and dried by high-

pressure air. The position of chemicals was exchanged after each test. The behavior of caterpillars was recorded for 10 min at 100 frames per minute using a Canon TV lens JF16mm (Canon, Japan). Behavioral data was extracted by EthoVision XT 11.5 software (Noldus, The Netherlands). Data were removed from the dataset when the caterpillar stayed in the arena for less than 10 seconds. All tests were performed at room temperature.

Statistical analysis

Unless stated otherwise, statistical tests were performed in R (version 4.4.0; R Core Team, 2016) in combination with RStudio (Posit, USA). Data sets were tested for normal distribution using a Shapiro–Wilk test and for equal variances using Levene's test. The difference of glomerular number between WT and *Orco* KO caterpillars was tested with a Wilcoxon rank-sum test. Electroantennographic responses between the two genotypes were tested statistically by using the Student's t-test when the values were normally distributed and had homogenous variance or tested by using the Kruskal-Wallis rank-sum test when the requirements for the t-test were not met. The difference in butterfly mating frequency was analyzed by using the Wilcoxon rank-sum test; differences in oviposition dynamics were tested with a generalized linear model (GLM) with a negative binominal distribution. The development differences between WT and KO caterpillars on full plants and under the thread of disarmed parasitoids were tested using a Student's t-test, and survival rate difference between the two genotypes was tested using a GLM with a beta-binomial distribution. Caterpillar host-plant selection behaviors in Petri dishes and in the Y-tube were analyzed with a Chi-square test. Differences of the relative time spent by the caterpillars between the control side and the odor side of the multichannel arenas were compared by a non-parametric Wilcoxon rank-sum test. Principal component analysis (PCA) of volatile blends was achieved by MetaboAnalyst (Pang et al., 2024). Data were normalized by logarithmic transformation and scaled in auto mode. Ward's method and Euclidean distance were employed in the hierarchical clustering heatmap by default settings without clustering samples. In addition, the abundance of volatiles as peak heights was imported into SIMCA-P 17 statistical software (Umetrics, Sweden), the data was analyzed by multivariate data analysis (MVDA). Supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) was employed to compare and correlate treatment groups.

References

Bernays, E.A. (1997) Feeding by lepidopteran larvae is dangerous. *Ecological Entomology,* **22**, 121-123.

Bourne, M.E., Gloder, G., Weldegergis, B.T., Slingerland, M., Ceribelli, A., Crauwels, S., Lievens, B., Jacquemyn, H., Dicke, M. and Poelman, E.H. (2023) Parasitism causes changes in caterpillar odours and associated bacterial communities with consequences for host-location by a hyperparasitoid. *PLoS Pathogens,* **19**, e1011262.

Brodeur, J., Geervliet, J.B.F. and Vet, L.E.M. (1996) The role of host species, age and defensive behaviour on ovipositional decisions in a solitary specialist and gregarious generalist parasitoid (*Cotesia* species). *Entomologia Experimentalis et Applicata,* **81**, 125-132.

Carpenter, J.E. and Sparks, A.N. (1982) Effects of vision on mating behavior of the male corn earworm. *Journal of Economic Entomology,* **75**, 248-250.

Carrasco, D., Larsson, M.C. and Anderson, P. (2015) Insect host plant selection in complex environments. *Current Opinion in Insect Science,* **8**, 1-7.

Chen, Z., Traniello, I.M., Rana, S., Cash-Ahmed, A.C., Sankey, A.L., Yang, C. and Robinson, G.E. (2021) Neurodevelopmental and transcriptomic effects of CRISPR/Cas9-induced somatic *orco* mutation in honey bees. *Journal of Neurogenetics,* **35**, 320-332.

De Fouchier, A., Sun, X., Caballero-Vidal, G., Travaillard, S., Jacquin-Joly, E. and Montagne, N. (2018) Behavioral effect of plant volatiles binding to *Spodoptera littoralis* larval odorant receptors. *Frontiers in Behavioral Neuroscience,* **12**, 264.

Di, C., Ning, C., Huang, L.Q. and Wang, C.Z. (2017) Design of larval chemical attractants based on odorant response spectra of odorant receptors in the cotton bollworm. *Insect Biochemistry and Molecular Biology,* **84**, 48-62.

Dicke, M. and Baldwin, I.T. (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends in Plant Science,* **15**, 167-175.

Dicke, M. and Grostal, P. (2001) Chemical detection of natural enemies by arthropods: an ecological perspective. *Annual Review of Ecology and Systematics,* **32**, 1-23.

Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., Virgin, H.W., Listgarten, J. and Root, D.E. (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nature Biotechnology,* **34**, 184-191.

Dweck, H.K., Ebrahim, S.A., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., Hansson, B.S. and Stensmyr, M.C. (2013) Olfactory preference for egg laying on citrus substrates in *Drosophila*. *Current Biology,* **23**, 2472-2480.

Ebrahim, S.A., Dweck, H.K., Stokl, J., Hofferberth, J.E., Trona, F., Weniger, K., Rybak, J., Seki, Y., Stensmyr, M.C., Sachse, S., Hansson, B.S. and Knaden, M. (2015) *Drosophila* avoids parasitoids by sensing their semiochemicals via a dedicated olfactory circuit. *PLoS Biology,* **13**, e1002318.

Fan, X.B., Mo, B.T., Li, G.C., Huang, L.Q., Guo, H., Gong, X.L. and Wang, C.Z. (2022) Mutagenesis of the odorant receptor co-receptor (*Orco*) reveals severe olfactory defects in the crop pest moth *Helicoverpa armigera*. *BMC Biology,* **20**, 214.

Fandino, R.A., Haverkamp, A., Bisch-Knaden, S., Zhang, J., Bucks, S., Nguyen, T.a.T., Schroder, K., Werckenthin, A., Rybak, J., Stengl, M., Knaden, M., Hansson, B.S. and Große-Wilde, E. (2019) Mutagenesis of odorant coreceptor *Orco* fully disrupts foraging but not oviposition behaviors in the hawkmoth *Manduca sexta*. *Proceedings of the National Academy of Sciences of the United States of America,* **116**, 15677-15685.

Feltwell, J. (1982) *Large white butterfly: the biology, biochemistry and physiology of Pieris brassicae (Linnaeus),* Dr W. Junk Publishers, The Hague, The Netherlands.

Hanson, F.E. and Dethier, V.G. (1972) Role of gustation and olfaction in food plant discrimination in the tobacco hornworm, *Manduca sexta*. *Journal of Insect Physiology,* **19**, 1019-1031.

Hasan, F. and Ansari, M.S. (2010) Effect of different cole crops on the biological parameters of *Pieris brassicae* (L.) (Lepidoptera: Pieridae) under laboratory conditions. *Journal of Crop Science and Biotechnology,* **13**, 195-202.

Hasan, F. and Ansari, M.S. (2011) Population growth of *Pieris brassicae* (L.) (Lepidoptera: Pieridae) on different cole crops under laboratory conditions. *Journal of Pest Science,* **84**, 179-186.

Haverkamp, A., Hansson, B.S. and Knaden, M. (2018) Combinatorial codes and labeled lines: how insects use olfactory cues to find and judge food, mates, and oviposition sites in complex environments. *Frontiers in Physiology,* **9**, 49.

Haverkamp, A. and Smid, H.M. (2020) A neuronal arms race: the role of learning in parasitoid-host interactions. *Current Opinion in Insect Science,* **42**, 47-54.

Horikoshi, M., Takabayashi, J., Yano, S., Yamaoka, R., Ohsaki, N. and Sato, Y. (1997) *Cotesia glomerata* female wasps use fatty acids from plant–herbivore complex in host searching. *Journal of Chemical Ecology,* **23**, 1505-1515.

Hu, X., Su, S., Liu, Q., Jiao, Y., Peng, Y., Li, Y. and Turlings, T.C. (2020) Caterpillar-induced rice volatiles provide enemy-free space for the offspring of the brown planthopper. *eLife,* **9**, e55421.

Jones, W.D., Nguyen, T.A., Kloss, B., Lee, K.J. and Vosshall, L.B. (2005) Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Current Biology,* **15**, R119-121.

Kannan, K., Galizia, C.G. and Nouvian, M. (2022) Olfactory strategies in the defensive behaviour of insects. *Insects,* **13**, 470.

Kester, K.M., Peterson, S.C., Hanson, F., Jackson, D.M. and Severson, R.F. (2002) The roles of nicotine and natural enemies in determining larval feeding site distributions of *Manduca sexta* L. and *Manduca quinquemaculata* (Haworth) on tobacco. *Chemoecology,* **12**, 1-10.

Koutroumpa, F., Monsempes, C., Anton, S., Francois, M.C., Montagne, N. and Jacquin-Joly, E. (2022) Pheromone receptor knock-out affects pheromone detection and brain structure in a moth. *Biomolecules,* **12**, 341.

Labun, K., Montague, T.G., Krause, M., Torres Cleuren, Y.N., Tjeldnes, H. and Valen, E. (2019) CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research,* **47**, W171-W174.

Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H. and Vosshall, L.B. (2004) *Or83b* encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron,* **43**, 703-714.

Liu, X.L., Zhang, J., Yan, Q., Miao, C.L., Han, W.K., Hou, W., Yang, K., Hansson, B.S., Peng, Y.C., Guo, J.M., Xu, H., Wang, C.Z., Dong, S.L. and Knaden, M. (2020) The molecular basis of host selection in a crucifer-specialized moth. *Current Biology,* **30**, 4476-4482 e5.

Liu, X.Y., Yan, R., Chen, S.J., Zhang, J.L. and Xu, H.J. (2023) Orco mutagenesis causes deficiencies in olfactory sensitivity and fertility in the migratory brown planthopper, *Nilaparvata lugens*. *Pest Management Science,* **79**, 1030-1039.

Lommen, A. (2009) MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Analytical Chemistry,* **81**, 3079-3086.

Mattiacci, L., Dicke, M. and Posthumus, M.A. (1994) Induction of parasitoid attracting synomone in brussels sprouts plants by feeding of *Pieris brassicae* larvae: role of mechanical damage and herbivore elicitor. *Journal of Chemical Ecology,* **20**, 2229-2247.

Müller, C., Agerbirk, N. and Olsen, C.E. (2003) Lack of sequestration of host plant glucosinolates in *Pieris rapae* and *P. grarricae*. *Chemoecology,* **13**, 47–54.

Ngumbi, E. and Fadamiro, H. (2012) Species and sexual differences in behavioural responses of a specialist and generalist parasitoid species to host-related volatiles. *Bulletin of Entomological Research,* **102**, 710-718.

Nolte, A., Gawalek, P., Koerte, S., Wei, H., Schumann, R., Werckenthin, A., Krieger, J. and Stengl, M. (2016) No evidence for ionotropic pheromone transduction in the hawkmoth *Manduca sexta*. *PLoS One,* **11**, e0166060.

Obara, Y., Koshitaka, H. and Arikawa, K. (2008) Better mate in the shade: enhancement of male mating behaviour in the cabbage butterfly, *Pieris rapae crucivora*, in a UV-rich environment. *The Journal of Experimental Biology,* **211**, 3698-3702.

Oland, L.A. and Tolbert, L.P. (2011) Roles of glial cells in neural circuit formation: insights from research in insects. *Glia,* **59**, 1273-1295.

Pang, Z., Lu, Y., Zhou, G., Hui, F., Xu, L., Viau, C., Spigelman, A.F., Macdonald, P.E., Wishart, D.S., Li, S. and Xia, J. (2024) MetaboAnalyst 6.0: towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Research,* **52**, W398-W406.

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B. and Touhara, K. (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature,* **452**, 1002-1006.

Slater, G.S. and Birney, E. (2005) Automated generation of heuristics for biological sequence comparison. *BMC Bioinformatics,* **6**, 31.

Stam, J.M., Kroes, A., Li, Y., Gols, R., Van Loon, J.J.A., Poelman, E.H. and Dicke, M. (2014) Plant interactions with multiple insect herbivores: from community to genes. *Annual Review of Plant Biology,* **65**, 689-713.

Sun, H., Bu, L.A., Su, S.C., Guo, D., Gao, C.F. and Wu, S.F. (2023) Knockout of the odorant receptor co-receptor, orco, impairs feeding, mating and egg-laying behavior in the fall armyworm *Spodoptera frugiperda*. *Insect Biochemistry and Molecular Biology,* **152**, 103889.

Sun, H., Liu, F., Ye, Z., Baker, A. and Zwiebel, L.J. (2020) Mutagenesis of the orco odorant receptor co-receptor impairs olfactory function in the malaria vector *Anopheles coluzzii*. *Insect Biochemistry and Molecular Biology,* **127**, 103497.

Tikunov, Y.M., Laptenok, S., Hall, R.D., Bovy, A. and De Vos, R.C. (2012) MSClust: a tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. *Metabolomics,* **8**, 714-718.

Trible, W., Olivos-Cisneros, L., Mckenzie, S.K., Saragosti, J., Chang, N.C., Matthews, B.J., Oxley, P.R. and Kronauer, D.J.C. (2017) *Orco* mutagenesis causes loss of antennal lobe glomeruli and impaired social behavior in ants. *Cell,* **170**, 727-735 e10.

Turlings, T.C.J. and Erb, M. (2018) Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. *Annual Review of Entomology,* **63**, 433-452.

Van Loon, J.J.A., Blaakmeer, A., Griepink, F.C., Van Beek, T.A., Schoonhoven, L.M. and De Groot, A. (1992a) Leaf surface compound from *Brassica oleracea* (Cruciferae) induces oviposition by *Pieris brassicae* (Lepidoptera: Pieridae). *Chemoecology,* **3**, 39-44.

Van Loon, J.J.A., Frentz, W.H. and Van Eeuwijk, F.A. (1992b) Electroantennogram responses to plant volatiles in two species of *Pieris* butterflies. *Entomologia Experimentalis et Applicata,* **62**, 253-260.

Venugopal, U., Jayanthi, P.D.K., Kumar, P., Jagadeesh, K.S. and Mohan, K.M. (2020) Behavioural response of specific larval endoparasitoid, *Apanteles machaeralis* (Wilkinson) to volatile cues from its host insect, *Diaphania indica* (Saunders) and the host plant (*Cucumis sativus* L.). *Journal of Biological Control,* **34**, 132-139.

Vet, L.E. and Dicke, M. (1992) Ecology of infochemical use by natural enemies in a tritrophic context. *Annual Review of Entomology,* **37**, 141-172.

Wang, Q., Dicke, M. and Haverkamp, A. (2023) Sympatric *Pieris* butterfly species exhibit a high conservation of chemoreceptors. *Frontiers in Cellular Neuroscience,* **17**, 1155405.

Wang, Q., Smid, H.M., Dicke, M. and Haverkamp, A. (2024) The olfactory system of *Pieris brassicae* caterpillars: from receptors to glomeruli. *Insect Science,* **31**, 469-488.

Wicher, D., Schafer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H. and Hansson, B.S. (2008) *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature,* **452**, 1007-1011.

Williams, A.T., Verhulst, E.C. and Haverkamp, A. (2022) A unique sense of smell: development and evolution of a sexually dimorphic antennal lobe – a review. *Entomologia Experimentalis et Applicata,* **170**, 303-318.

Yan, H., Opachaloemphan, C., Mancini, G., Yang, H., Gallitto, M., Mlejnek, J., Leibholz, A., Haight, K., Ghaninia, M., Huo, L., Perry, M., Slone, J., Zhou, X., Traficante, M., Penick, C.A., Dolezal, K., Gokhale, K., Stevens, K., Fetter-Pruneda, I., Bonasio, R., Zwiebel, L.J., Berger, S.L., Liebig, J., Reinberg, D. and Desplan, C. (2017) An engineered *orco* mutation produces aberrant social behavior and defective neural development in ants. *Cell,* **170**, 736-747 e9.

Yang, G., Zhang, Y.N., Gurr, G.M., Vasseur, L. and You, M.S. (2016) Electroantennogram and behavioral responses of *Cotesia plutellae* to plant volatiles. *Insect Science,* **23**, 245-252.

Yuan, G.G., Zhao, L.C., Du, Y.W., Yu, H., Shi, X.B., Chen, W.C. and Chen, G. (2022) Repellence or attraction: secondary metabolites in pepper mediate attraction and defense against Spodoptera litura. *Pest Management Science,* **78**, 4859-4870.

Zhang, J., Bisch-Knaden, S., Fandino, R.A., Yan, S., Obiero, G.F., Große-Wilde, E., Hansson, B.S. and Knaden, M. (2019) The olfactory coreceptor IR8a governs larval feces-mediated competition avoidance in a hawkmoth. *Proceedings of the National Academy of Sciences of the United States of America,* **116**, 21828-21833.

Zhu, F., Broekgaarden, C., Weldegergis, B.T., Harvey, J.A., Vosman, B., Dicke, M. and Poelman, E.H. (2015) Parasitism overrides herbivore identity allowing hyperparasitoids to locate their parasitoid host using herbivore-induced plant volatiles. *Molecular Ecology,* **24**, 2886-2899.

Figure legends

Fig 1. *Orco* knockout by CRISPR/Cas9 and verification in *Pieris brassicae*. (A), gene structure of *Orco*. Yellow blocks indicate exons and blue block indicates target area in the second exon. Black solid line indicates intron. Black arrows indicate the designated cleavage site of the Cas9 protein. The target knockout area is magnified to show the sequence. Blue letters indicate base pairs in the second exon segment, purple letters indicate mutation sites. Protospacer adjacent motif (PAM) sequences are in blue bold. (B), predicted transmembrane structure of Orco. The left and right panels represent wildtype (WT) Orco and mutated Orco transmembrane domains, respectively. Orange blocks indicate ORN membrane, extracellular (Extra) and intracellular (Intra) are shown. White circles indicate amino acids of Orco, blue circles on the left panel indicate the target mutation area. Cyan circles and the brown circle on the right panel indicate the mutated area and the early stop codon, respectively. Orco-positive ORNs and larval antennal center. *P. brassicae* larval antennae and larval antennal center (LAC) staining. (C), Odorant receptor neurons (ORNs) were stained (green cells, several are indicated by white arrows) in WT larval antennae. (D), ORNs were not stained in *Orco* knockout (KO) larval antennae. (E), An ORN was stained (green cell, indicated by white arrow) in WT larval palps. (F), ORNs were not stained in KO larval palps. (G), glomeruli in the LAC of a WT brain. (H), glomeruli in the LAC of a KO brain. Glomeruli are indicated by white dashed circles in (G). In (H), the white dashed lines indicate the approximate position of missing glomeruli (not directly corresponding to panel (G)). (I), number of glomeruli counted in WT caterpillar brain (orange) and *Orco* KO caterpillar (blue) brain. A significant difference was detected by Wilcoxon rank-sum test. (J), electroantennogram (EAG) response of male butterflies (n = 18 for both WT and KO). (K), EAG response of female butterflies (n = 15 for WT and n = 17 for KO). Left panels represent wildtype (WT) butterfly EAG responses, and right panels represent are knockout (KO) butterfly EAG responses. Significant differences between WT and KO butterflies were identified using Student's t-test when the data was normally distributed and the variances were equal or using a Kruskal-Wallis rank-sum test when these criteria did not apply. Significance levels are indicated by asterisks, ns ($P > 0.05$), * ($0.01 < P < 0.05$), ** ($0.001 < P < 0.01$), *** ($P < 0.001$), - (no response recorded from WT butterfly antennae). Butterfly antennal responses (mV) to the tested chemicals are indicated by a color scale from navy (0 mV) via yellow to red (1.2 mV).

Fig 2. *Pieris brassicae* caterpillar growth and foraging behavior. (A), *P. brassicae* caterpillar growth

808 on cabbage plants, y axis shows weight (g) of WT and KO caterpillars after 10 days of feeding , n = 17.
809 (B), caterpillar behavioral choices in a Petri dish. The numbers of caterpillars that chose one of the two
810 discs are shown in the respective bars (n = 54 -73). Schematic drawing shows behavioral setup. Petri dish
811 diameter was 188 mm, disc diameters were 13 mm. Discs in the Petri dish represent cabbage, paper and
812 tomato leaf discs. Grey bars indicate paper disc choices, light green indicates cabbage leaf disc choices
813 and orange indicates choices for tomato leaf discs. (C), caterpillar behavioral choices in Y-tube
814 olfactometer without parasitoid wasps. Schematic drawing shows the Y-tube olfactometer. The dashed
815 line indicates a black metal Y wire in the center of glass Y-tube olfactometer. The main arm of the Y-
816 olfactometer is 200 mm length, the lateral arms are 275 mm length, the angle between the lateral arms is
817 80 °. Light green, dark green and grey bars represent choices for healthy plant, infested plant and no plant
818 respectively. Healthy plant, plants were not treated; no plant, an empty jar without any insect or plant;
819 infested plant, plants were infested by early L3 caterpillars. (D), caterpillar behavioral choices in Y-tube
820 olfactometer with parasitoid wasps. Different treatments are in different colors. Dark green, infested plant
821 - wasps; magenta, infested plant + wasps. Significant differences were tested between wildtype (WT)
822 and knockout (KO) caterpillars or between two discs by Chi-square test in panel B-D, P-values are shown
823 on the right side of each bar. (E), *P. brassicae* caterpillar growth when exposed to disarmed *C. glomerata*
824 female parasitoids, y axis shows weight (g) of caterpillars after 10 days of feeding, n = 8. Schematic
825 drawing shows disarmed female *C. glomerata* (ovipositor removed). (F), *P. brassicae* caterpillar survival
826 rate when exposed to healthy *C. glomerata* female wasps, n = 7. Significant differences in development
827 and survival rate were assessed using a one-tailed Student's t-test, P values are indicated above boxplots.
828 Schematic drawing shows a healthy female *C. glomerata* (unmanipulated). In both panels orange
829 boxplots indicate WT caterpillars, blue boxplots indicate *Orco* KO caterpillars.

830 **Fig 3. Overview of caterpillar- (*Pieris brassicae*) and parasitoid wasp- (*Cotesia glomerata*)**
831 **associated volatile compounds.** (A), PCA (Principal Component Analysis) two-dimensional score plot
832 of five treatment groups: Cg, *Cotesia glomerata* female parasitoid wasps (n = 12); Pb, *Pieris brassicae*
833 caterpillars (n = 10); Pb-Cg, *P. brassicae* caterpillars in the presence of *C. glomerata* female parasitoid
834 wasps (n = 14); Pb-Fr, *P. brassicae* caterpillar frass (n = 10); and Pb-S, *P. brassicae* spit (n = 12), based
835 on their volatile blend composition. (B), hierarchical clustering heatmap showing the abundance of each
836 identified volatile compound of each treatment. Clustering chemicals in the heatmap indicate higher
837 correlation. (C), schematic drawing of the custom-designed multi-channel arena. (D) wildtype (WT)

838 caterpillar behavioral preference to the tested 15 odorants. (E), *Orco* knockout (KO) caterpillar
839 behavioral preference to the tested 15 chemicals. Unfilled bars represent tests that exhibit no difference
840 between paraffin oil and the odorant of interest. Blue bars indicate the cumulative duration ratio that
841 caterpillars stayed in the paraffin oil zone, dark orange bars indicate the cumulative duration ratio that
842 caterpillars stayed in the odorant zone. Relative time spent in odorant zone = Cumulative duration in a
843 specific zone / Cumulative duration in the arena. Error bars indicate standard errors. In (D) and (E),
844 differences were tested between the two zones using a Wilcoxon rank-sum test, P values are presented
845 above bars (n = 31-50). For each comparison, left bar represents paraffin oil and right bar represents
846 chemical compound.
847

848 # Supporting information

849 **Fig S1. *Pieris brassicae* caterpillar development in a Petri dish environment.** X axis indicates the
850 date since caterpillars have been deposited in the Petri dish. Y axis indicates the weight of caterpillars
851 (Ln Weight). Caterpillar weight (n = 10 for both genotypes) in this figure is presented by the mean of a
852 group of caterpillars weight in each Petri dish on day 6.
853

854 **Fig S2. Egg-hatching rates by *wildtype* (n=11), *Orco*$^{-/-}$_fert (n=5) and *Orco*$^{-/-}$_unfert (n=9) butterflies.**
855 *Orco*$^{-/-}$_fert represents mated Orco female butterflies, *Orco*$^{-/-}$_unfert represents unmated female
856 butterflies. Differences were tested by using Student's t-test.
857

858 **Fig S3. Mating frequency of *Pieris brassicae* butterflies.** The boxplots indicate the number of
859 spermatophores in WT female butterflies (n = 11) and in KO butterflies (Orco-/-) (n = 14), the difference
860 was tested by using Wilcoxon rank-sum test. WT and Orco KO butterfly spermatophores are indicated
861 in orange and blue respectively.
862

863 **Fig S4. *Pieris brassicae* wildtype and *Orco* knockout (*Orco* $^{-/-}$) butterfly oviposition dynamics.** The
864 orange line indicates the number of eggs per female (mean ± SE) laid by wildtype (WT) butterflies (n =
865 11), the blue line indicates the number of eggs laid by Orco knockout (KO) butterflies (n = 14).
866 Differences were analyzed using a GLM with negative binominal distribution, difference test results are
867 shown in the line chart.
868

869 **Fig S5. Overview of volatile blends of *Pieris brassicae* caterpillars, *Cotesia glomerata* parasitoid**

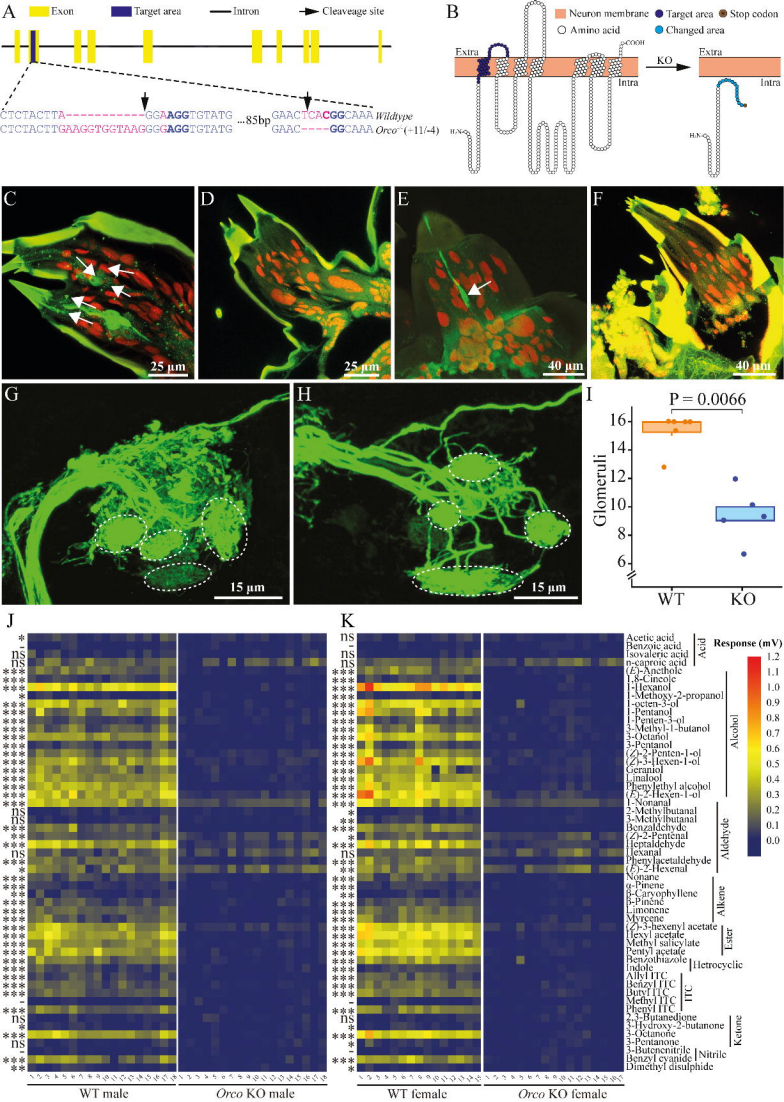
870 **wasps and caterpillars-parasitoid in interaction.** (A), OPLS-DA (Orthogonal Projection to Latent
871 Structures Discriminant Analysis) two-dimensional score plot of treatment groups: Cg, *Cotesia*
872 *glomerata* female parasitoid wasps (n = 12), based on their volatile content; Pb, *Pieris brassicae*
873 caterpillars (n = 10); Pb-Cg, *P. brassicae* caterpillars in the presence of *C. glomerata* female parasitoid
874 wasps (n = 14. (B), Loading plot showing the contribution of each identified volatile compound to the
875 separation of the different treatments. Volatiles closer to the treatment in the plot indicate higher
876 correlation. Numbers in the loading plot refer to the volatile compounds listed in Supporting information
877 Table S3.
878
879 **Fig S6. Overview of volatile blends of *Pieris brassicae* caterpillars and caterpillars-parasitoid**
880 **(*Cotesia glomerata*) in interaction.** (A), OPLS-DA (Orthogonal Projection to Latent Structures
881 Discriminant Analysis) two-dimensional score plot of treatment groups: Pb, *Pieris brassicae* caterpillars
882 (n = 10); Pb-Cg, *P. brassicae* caterpillars in the presence of *C. glomerata* female parasitoid wasps (n =
883 14), based on their volatile content. (B), Loading plot showing the contribution of each identified volatile
884 compound to the separation of the different treatments. Volatiles closer to the treatment in the plot
885 indicate higher correlation. Numbers in the loading plot refer to the volatile compounds listed in
886 Supporting information Table S3.
887
888 **Fig S7. Overview of volatile blends of *Pieris brassicae* caterpillar spit and caterpillars-parasitoid**
889 **wasp (*Cotesia glomerata*) in interaction.** (A), OPLS-DA (Orthogonal Projection to Latent Structures
890 Discriminant Analysis) two-dimensional score plot of treatment groups: P. brassicae spit (n = 12), Pb-Cg,
891 *P. brassicae* caterpillars in the presence of *C. glomerata* female parasitoid wasps (n = 14), based on their
892 volatile content. (B), Loading plot showing the contribution of each identified volatile compound to the
893 separation of the different treatments. Volatiles closer to the treatment in the plot indicate higher
894 correlation. Numbers in the loading plot refer to the volatile compounds listed in Supporting information
895 Table S3.
896
897 **Fig S8. Overview of volatile blends of *Pieris brassicae* caterpillar spit and *Pieris brassicae***
898 **caterpillar frass.** (A), OPLS-DA (Orthogonal Projection to Latent Structures Discriminant Analysis)
899 two-dimensional score plot of treatment groups: Pb-Fr, *P. brassicae* caterpillar frass (n = 10); and Pb-S,

900 *P. brassicae* spit (n = 12), based on their volatile content. (B), Loading plot showing the contribution of
901 each identified volatile compound to the separation of the different treatments. Volatiles closer to the
902 treatment in the plot indicate higher correlation. Numbers in the loading plot refer to the volatile
903 compounds listed in Supporting information Table S3.
904
905 **Fig S9. Overview of volatile blends of *Pieris brassicae* caterpillars, *Pieris brassicae* caterpillar spit**
906 **and *Pieris brassicae* caterpillar frass.** (A), OPLS-DA (Orthogonal Projection to Latent Structures
907 Discriminant Analysis) two-dimensional score plot of treatment groups: Pb, *Pieris brassicae* caterpillars
908 (n = 10); Pb-Fr, *P. brassicae* caterpillar frass (n = 10); and Pb-S, *P. brassicae* spit (n = 12), based on their
909 volatile content. (B), Loading plot showing the contribution of each identified volatile compound to the
910 separation of the different treatments. Volatiles closer to the treatment in the plot indicate higher
911 correlation. Numbers in the loading plot refer to the volatile compounds listed in Supporting information
912 Table S3.
913
914 **Fig S10. Heatmaps of WT caterpillar movement in response to the tested chemicals.** The chemical
915 compounds are indicated in the figures and color legend indicates the time (s) that each caterpillar stayed
916 at certain locations.
917
918 **Fig S11. Heatmaps of Orco KO caterpillar movement in response to the tested chemicals.** The
919 chemical compounds are indicated in the figures and color legend indicates the time (s) that each
920 caterpillar stayed at certain locations.
921
922
923 **Table S1. Chemical compounds that were used for the electroantennographical test.**
924
925 **Table S2. Chemical compounds that were used for the behavioral test in multi-channel arena.**
926
927 **Table S3. Volatile compounds detected in the headspace of the different treatment samples.** Cg,
928 *Cotesia glomerata* female parasitoid wasps (n = 12); Pb, *Pieris brassicae* caterpillars (n = 10); Pb-Cg, *P.*
929 *brassicae* caterpillars in the presence of *C. glomerata* female parasitoid wasps (n = 14); Pb-Fr, *P.*

930 *brassicae* caterpillar frass (n = 10); and Pb-S, *P. brassicae* spit (n = 12), are listed. Relative amounts of
931 volatiles are presented as average peak height (SE)/10^4. The volatiles are listed according to their elution
932 order in a chromatographic window. Volatiles with Variable Importance in the Projection (VIP) scores
933 equal to or higher than 1.0 (presented in bold), are considered important in separating the different
934 treatment groups of the given analysis. Significant differences among the five treatments detected by
935 Kruskal-Wallis test with Dunn's post-hoc test for multiple comparisons are indicated by letters in the
936 table. Different letters indicate significant difference ($P < 0.05$). NF, not found.
937
938 **Table S4. Volatiles used in separating *Cotesia glomerata* female wasps (Cg, n = 12), *Pieris brassicae***
939 **caterpillars (Pb, n = 10) and the interaction of *P. brassicae* caterpillars with *C. glomerata* female**
940 **wasps (Pb-Cg, n = 14) sample treatments (Fig S5).** Volatiles are listed according to ranking order of
941 their Variable Importance in the Projection (VIP) scores values, where those with VIP scores of equal to
942 or higher than 1.0, are considered important in separating the treatment groups of the given analysis.
943
944 **Table S5. Volatiles used in separating *Pieris brassicae* caterpillars (Pb, n = 10) and the interaction**
945 **of *P. brassicae* caterpillars with *Cotesia glomerata* female wasps (Pb-Cg, n = 14) sample treatments**
946 **(Fig S6).** Volatiles are listed according to ranking order of their Variable Importance in the Projection
947 (VIP) scores values, where those with VIP scores of equal to or higher than 1.0, are considered important
948 in separating the treatment groups of the given analysis.
949
950 **Table S6. Volatiles used in separating the interaction of *Pieris brassicae* caterpillars with *Cotesia***
951 ***glomerata* female wasps (Pb-Cg, n = 14) and *P. brassicae* caterpillar spit (Pb-S, n = 12), sample**
952 **treatments (Fig S7).** Volatiles are listed according to ranking order of their Variable Importance in the
953 Projection (VIP) scores values, where those with VIP scores of equal to or higher than 1.0, are considered
954 important in separating the treatment groups of the given analysis.
955
956 **Table S7. Volatiles used in separating the *Pieris brassicae* caterpillar spit (Pb-S, n = 12), and *P.***
957 ***brassicae* caterpillar frass (Pb-Fr, n = 10) sample treatments (Fig S8).** Volatiles are listed according
958 to ranking order of their Variable Importance in the Projection (VIP) scores values, where those with VIP
959 scores of equal to or higher than 1.0, are considered important in separating the treatment groups of the

given analysis.

Table S8. Volatiles used in separating the *Pieris brassicae* caterpillars (Pb, n = 10), the interaction of *P. brassicae* caterpillars with *Cotesia glomerata* female wasps (Pb-Cg, n = 14), and *P. brassicae* caterpillar frass (Pb-Fr, n = 10) sample treatments (Fig S9). Volatiles are listed according to ranking order of their Variable Importance in the Projection (VIP) scores values, where those with VIP scores of equal to or higher than 1.0, are considered important in separating the treatment groups of the given analysis.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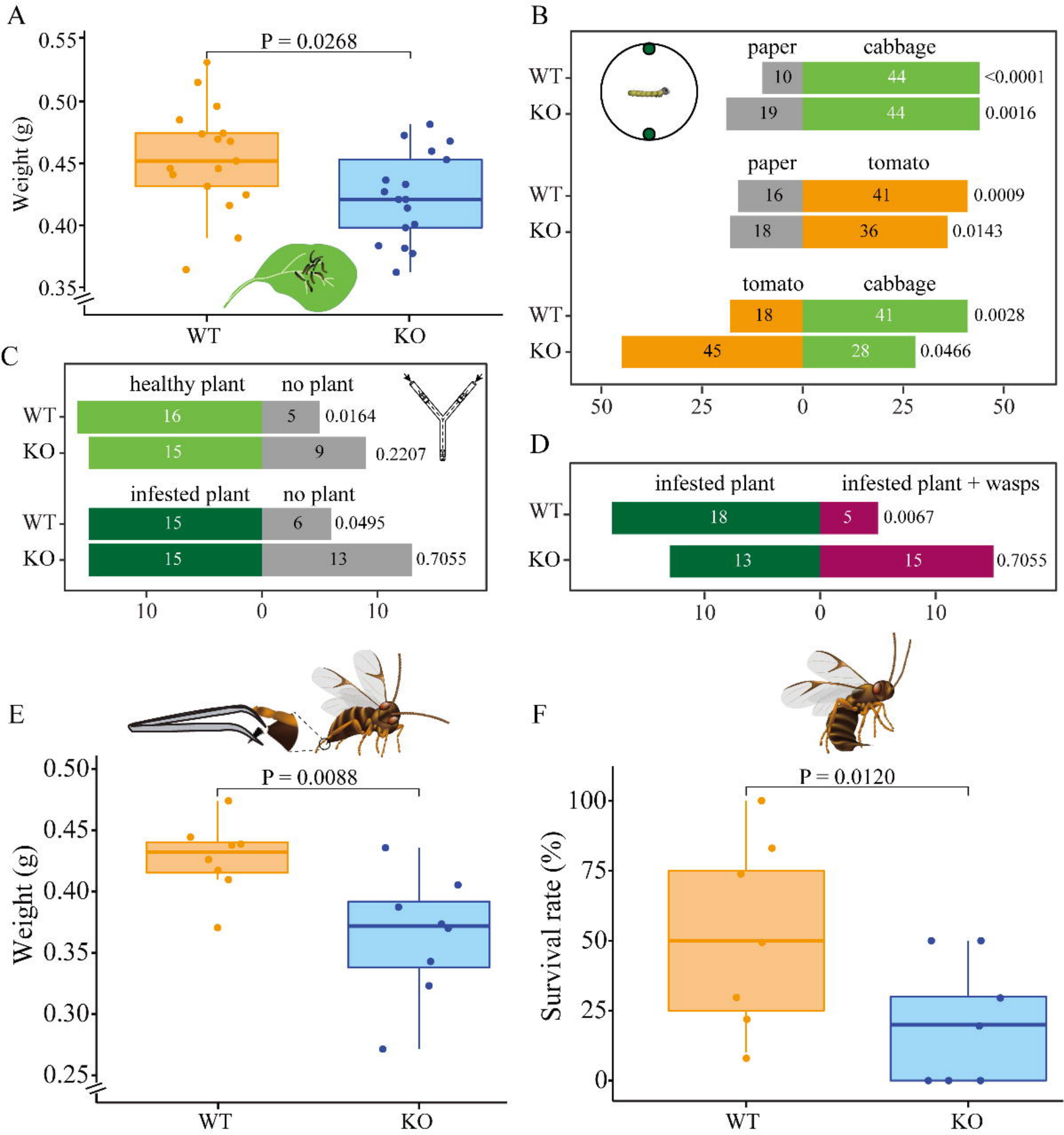
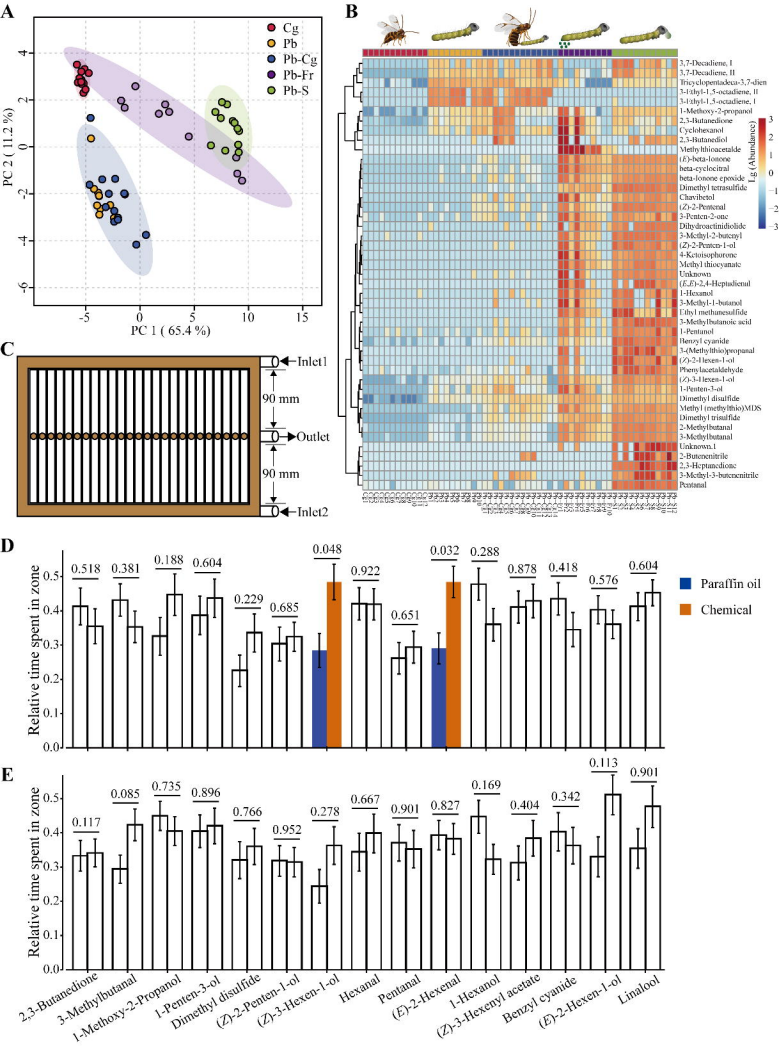
Target area
Stop codon
Amino acid
Changed area
Extra
Intra
COOH
KO
H2N
C
D
E
F
25 μm
25 μm
40 μm
40 μm
G
H
15 μm
15 μm
I
P = 0.0066
Glomeruli
16
14
12
10
8
6
WT
KO
J
K
WT male
Orco KO male
WT female
Orco KO female
Acetic acid
Benzoic acid
Isovaleric acid
n-caproic acid
(E)-Anethole
1,8-Cineole
1-Hexanol
1-Methoxy-2-propanol
1-octen-3-ol
1-Pentanol
1-Penten-3-ol
3-Methyl-1-butanol
3-Octanol
3-Pentanol
(Z)-2-Penten-1-ol
(Z)-3-Hexen-1-ol
Geraniol
Linalool
Phenylethyl alcohol
(E)-2-Hexen-1-ol
1-Nonanal
2-Methylbutanal
3-Methylbutanal
Benzaldehyde
(Z)-2-Pentenal
Heptaldehyde
Hexanal
Phenylacetaldehyde
(E)-2-Hexenal
Nonane
α-Pinene
β-Caryophyllene
β-Pinene
Limonene
Myrcene
(Z)-3-hexenyl acetate
Hexyl acetate
Methyl salicylate
Pentyl acetate
Benzothiazole
Indole
Allyl ITC
Benzyl ITC
Butyl ITC
Methyl ITC
Phenyl ITC
2,3-Butanedione
3-Hydroxy-2-butanone
3-Octanone
3-Pentanone
3-Butenenitrile
Benzyl cyanide
Dimethyl disulphide
Acid
Alcohol
Aldehyde
Alkene
Ester
Heterocyclic
ITC
Ketone
Nitrile
Response (mV)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0


A
P = 0.0268
Weight (g)
0.55
0.50
0.45
0.40
0.35
WT
KO
B
paper
cabbage
WT
KO
10
44
<0.0001
19
44
0.0016
paper
tomato
WT
KO
16
41
0.0009
18
36
0.0143
tomato
cabbage
WT
KO
18
41
0.0028
45
28
0.0466
50
25
0
25
50
C
healthy plant
no plant
WT
KO
16
5
0.0164
15
9
0.2207
infested plant
no plant
WT
KO
15
6
0.0495
15
13
0.7055
10
0
10
D
infested plant
infested plant + wasps
WT
KO
18
5
0.0067
13
15
0.7055
10
0
10
E
P = 0.0088
Weight (g)
0.50
0.45
0.40
0.35
0.30
0.25
WT
KO
F
P = 0.0120
Survival rate (%)
100
75
50
25
0
WT
KO


A
Cg
Pb
Pb-Cg
Pb-Fr
Pb-S
PC 2 (11.2 %)
PC 1 (65.4 %)
B
3,7-Decadiene, I
3,7-Decadiene, II
Tricyclopentadecu-3,7-dien
3-Ethyl-1,5-octadiene, II
3-Ethyl-1,5-octadiene, I
1-Methoxy-2-propanol
2,3-Butanedione
Cyclohexanol
2,3-Butanediol
Methylthioacetalde
(E)-beta-Ionone
beta-cyclocitral
beta-Ionone epoxide
Dimethyl tetrasulfide
Charvibetol
(Z)-2-Pentenal
3-Penten-2-one
Dihydroactinidiolide
3-Methyl-2-butenyl
(Z)-2-Penten-1-ol
4-Ketoisophorone
Methyl thiocyanate
Unknown
(E,E)-2,4-Heptadienal
1-Hexanol
3-Methyl-1-butanol
Ethyl methanesulfide
3-Methylbutanoic acid
1-Pentanol
Benzyl cyanide
3-(Methylthio)propanal
(Z)-2-Hexen-1-ol
Phenylacetaldehyde
(Z)-3-Hexen-1-ol
1-Penten-3-ol
Dimethyl disulfide
Methyl 1 (methylthio)MDS
Dimethyl trisulfide
2-Methylbutanal
3-Methylbutanal
Unknown,1
2-Butenenitrile
2,3-Heptanedione
3-Methyl-3-butenenitrile
Pentanal
Lg (Abundance)
C
Inlet1
90 mm
Outlet
90 mm
Inlet2
D
Relative time spent in zone
0.518
0.381
0.188
0.604
0.229
0.685
0.048
0.922
0.651
0.032
0.288
0.878
0.418
0.576
0.604
Paraffin oil
Chemical
E
Relative time spent in zone
0.117
0.085
0.735
0.896
0.766
0.952
0.278
0.667
0.901
0.827
0.169
0.404
0.342
0.113
0.901
2,3-Butanedione
3-Methylbutanal
1-Methoxy-2-Propanol
1-Penten-3-ol
Dimethyl disulfide
(Z)-2-Penten-1-ol
(Z)-3-Hexen-1-ol
Hexanal
Pentanal
(E)-2-Hexenal
1-Hexanol
(Z)-3-Hexenyl acetate
Benzyl cyanide
(E)-2-Hexen-1-ol
Linalool