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

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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 lepidopteran, the caterpillar olfactory system and its significance in tri-trophic interactions remains 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 knock out 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 plant. 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 selecting 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, many insects are holometabolous and spend a significant portion of their lives as larvae, during this life stage chemical information likely play an essential role in host-plant choice and natural-enemy avoidance. 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 (Turlings & Erb, 2018; Vet & Dicke, 1992; Wang, 2023). In particular, abundant research has demonstrated that olfactory information, such as plant volatiles, are crucial in the coevolution of herbivores and natural enemies (Dicke & Baldwin, 2010; Haverkamp & Smid, 2020; Turlings & Erb, 2018). In addition to plant volatiles, chemicals emitted by the insects themselves also play an important role (Ebrahim et al., 2015; Venugopal et al., 2020). Under the 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, brown planthoppers deposit their eggs on rice plants infested by the striped stem borer, 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) (Jones et al., 2005; Sato et al., 2008; Wicher et al., 2008). 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 (Fandino et al., 2019; Trible et al., 2017; Yan et al., 2017). Previous reports have also clearly shown that adult insect behavior

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

71 *Pieris brassicae* is a very common butterfly species that causes substantial agricultural production
72 loss across Europe and the Indian sub-continent (Feltwell, 1982; Hasan & Ansari, 2010; Hasan & Ansari,
73 2011). The butterflies utilize chemoreception to evaluate oviposition sites and host-plants (van Loon et
74 al., 1992a; van Loon et al., 1992b; Wang et al., 2023a). The caterpillars also have a well-developed
75 olfactory system (Wang et al., 2023b) and chemical communication has been demonstrated to be
76 involved in the interaction between herbivores and their natural enemies (Dicke & Baldwin, 2010; Stam
77 et al., 2014). Natural enemies such as parasitoid wasps are highly attracted to volatiles from cabbage
78 plants infested by *P. brassicae* caterpillars (Mattiacci et al., 1994). *P. brassicae* caterpillars are thus under
79 strong selection pressure from natural enemies, though caterpillars can defend themselves by spitting at
80 natural enemies while being attacked.

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

Results

Orco knockout and neuronal deficiency

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 knockout (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; Nolte et al., 2016; Wang et al., 2023b). In WT L3 caterpillar antennae, a total of 34 ORNs were successfully identified (Wang et al., 2023b) (Fig 1C). However, no Orco-positive ORNs were found in the antennae of KO caterpillars (Fig 1D). Similarly, we detected an Orco-positive neuron 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 compared to WT LAC ($P = 0.0066$, Wilcoxon rank-sum test) (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 acetic acid, n-caproic acid and hexanal, where no significant

difference was detected between KO and WT butterflies. Reduced responses were recorded in KO butterflies to 1-nonanal, (*Z*)-2-pentenal and (*E*)-2-hexenal. Besides, WT male and female butterflies responded similarly to most of the volatile compounds with a few exceptions where females showed slight responses to 1-methoxy-2-propanol oil ($P < 0.0001$, Kruskal-Wallis test) and methyl ITC compared to paraffin oil ($P < 0.0001$, Student's t-test) while males did not. Moreover, *Orco* KO males did not show significantly different responses to 3-methylbutanal and 3-pentanone compared to WT males. *Orco* KO females did not show significantly different responses to methyl ITC, 2,3-butanedione and dimethyl disulphide compared to WT females (Fig 2A and 2B).

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 mated fewer times than WT butterflies ($P = 0.0199$, Wilcoxon rank-sum test), since the majority of the KO butterflies (9 out of 14) did not mate during the experiment (Fig 2C). Out of the five KO butterflies that did mate, all but one mated singly. Of the wildtype butterflies, all but 3 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 2D). 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 (S1 Fig).

Caterpillar performance on cabbage plants

To assess the ecological significance of knocking out *Orco* in *P. brassicae* caterpillars, we first conducted a comparison of caterpillar growth between the two genotypes on their optimal host-plant. The results show that in the absence of parasitoids, WT caterpillars exhibited better performance, gaining more weight compared to KO caterpillars ($P = 0.0268$, Student's t-test) (Fig 3A). However, there is no developmental difference while being reared in a Petri dish in which a group of caterpillars were supplied with cut out leaf discs (S2 Fig). Subsequently, to determine if the differences in feeding and survival are dependent on food-plant location, a two-choice assay was conducted with cabbage leaf discs, tomato leaf discs and green paper discs in a glass Petri dish to investigate the role of olfaction in host-plant selection

151 (Fig 3B). Both caterpillar genotypes chose the cabbage leaf disc significantly more often than the green
152 paper discs (P = 0.0016 for KO; P < 0.0001 for WT, Chi-square test), and no difference was detected
153 between the two genotypes (P = 0.1460, Chi-square test). Similarly, when the cabbage leaf disc was
154 replaced with a tomato leaf disc, both genotypes preferred the tomato leaf disc over the paper disc (P =
155 0.0143 for KO; P = 0.0009 for WT, Chi-square test) and no difference was found between the two
156 genotypes (P = 0.5477, Chi-square test). However, when caterpillars were provided with both a cabbage
157 leaf disc and a tomato leaf disc, a contrasting preference was observed between the two genotypes (P =
158 0.0004, Chi-square test). KO caterpillars significantly preferred the tomato leaf disc (P = 0.0466, Chi-
159 square test), while WT caterpillars preferred the cabbage leaf disc (P = 0.0028, Chi-square test).

160 We then aimed to investigate if caterpillars could locate the optimal host-plant when supplied with
161 differently treated plants using a Y-tube olfactometer. We compared the behaviors of both WT and KO
162 caterpillars in response to clean air, a healthy plant and a caterpillar-infested plant (Fig 3C). Both WT
163 and KO caterpillars tended to prefer a healthy cabbage plant to a clean air control, however WT
164 caterpillars were significantly attracted by a healthy plant (P = 0.0164, Chi-square test) while the
165 preference for KO caterpillars was not significant (P = 0.2207, Chi-square test). Subsequently, we
166 challenged the caterpillars with a healthy plant and an infested plant and found that WT caterpillars prefer
167 a healthy plant to an infested plant (P = 0.0495, Chi-square test), while KO caterpillars did not show any
168 preference between the two provided plants (P = 0.7055, Chi-square test). We further compared larval
169 host-plant seeking behaviors with the presence of natural enemies on caterpillar-infested plants (Fig 3D).
170 WT caterpillars showed a significant preference for infested plants without parasitoid wasps *C.*
171 *glomerata* (P = 0.0067, Chi-square test), while KO caterpillars did not show any preference for either
172 odor source (P = 0.7055, Chi-square test).

173 Next we tested whether these differences in the foraging behavior also lead to differences in the
174 development and survival of *P. brassicae* caterpillars when exposed to their natural enemies. We first
175 evaluated caterpillar performance under the potential threat of natural enemies by exposing them to
176 female parasitoids from which the ovipositor had been removed, so that these parasitoids could attack,
177 but not kill the caterpillars (Fig 3E). The results show a significantly higher weight in WT caterpillars
178 than in KO caterpillars (P = 0.0088, Student's t-test). To further explore the effect of knocking out *Orco*
179 on the interaction of the caterpillars with their natural enemies, we compared the survival rates of
180 caterpillars when exposed to two mated unmanipulated female *C. glomerata* wasps. The findings show

181 that KO caterpillars had significantly lower survival rates compared to WT caterpillars (P = 0.0120, GLM
182 beta-binomial) (Fig 3F).

183 Volatile headspace composition

184 Finally, we investigated if caterpillar- and parasitoid-derived chemicals are involved in the
185 behavioral responses. We identified a total of 45 chemical compounds among the five treatments
186 belonging to seven classes, including alcohols, aldehydes, aromatics, ketones, nitrogen and/or sulfur-
187 containing chemicals, terpenoids and others (S3 Table). The contribution of identified chemicals towards
188 separating different treatment groups was analyzed using principal component analysis (PCA). This
189 analysis showed a significant separation of most groups with PC 1 carrying 65.4 % of the variance (P =
190 0.001, PERMANOVA). *Pieris brassicae* caterpillars (Pb), *P. brassicae* caterpillar spit (Pb-S), *C.*
191 *glomerata* female wasps (Cg) and caterpillar frass (Pb-Fr) treatment groups were clearly separated (Pb
192 vs. Cg, Pb vs. Pb-S, Pb vs. Pb-Fr, Pb-S vs. Cg and Pb-Fr vs. Cg, P= 0.0013; Pb-S vs. Pb-Fr, P= 0.0022
193 pairwise PERMANOVA, Fig 4A), whereas the identified and measured volatile blend from *P. brassicae*
194 caterpillars (Pb) was similar to that of the *P. brassicae* caterpillars-*C. glomerata* parasitoids combination
195 (Pb-Cg) (P = 0.0330, pairwise PERMANOVA). The amount of volatiles among the five treatments was
196 also compared. Most of the identified compounds were found in significantly higher amounts in Pb-S
197 and/or Pb-Fr compared to the other treatments (Fig 4B, S3 Table). We also found that some chemicals
198 are strongly correlated. The identified chemicals are classified into several subgroups in the hierarchical
199 clustering heatmap according to the abundance among treatments. For instance, the seven chemicals (*Z*)-
200 3-hexen-1-ol, 1-penten-3-ol, dimethyl disulfide, methyl (methylthio) methyl sulfide, dimethyl trisulfide,
201 2-methylbutanal and 3-methylbutanal formed a clade. Similarly, the six chemicals, 3-methylbutanoic
202 acid, 1-pentanol, benzyl cyanide, 3-(methylthio) propanal, (*Z*)-2-hexen-1-ol and phenylacetaldehyde
203 formed another clade. The clustering analysis demonstrated that chemicals within the same clade are
204 highly correlated. The multivariate data analysis (MVDA) also showed similar clustering when
205 comparing the different treatments (S3-S7 Fig).

206 Based on the results of our chemical analysis, we selected 11 candidate chemicals (1-hexanol, 1-
207 methoxy-2-propanol, 1-penten-3-ol, 2,3-butanedione, 3-methylbutanal, benzyl cyanide, dimethyl
208 disulfide, pentanal, (*E*)-2-hexen-1-ol, (*Z*)-2-penten-1-ol and (*Z*)-3-hexen-1-ol) with high variable
209 importance in the projection (VIP) scores (> 1.0) (S3-S8 Table), and another four common plant volatiles

210 (linalool, (*Z*)-3-hexenyl acetate, hexanal and (*E*)-2-hexenal). These odorants were chosen to test the
211 behavioral response of L3 caterpillars. Using a custom-made multiple channel setup (S7 Fig), we
212 conducted behavioral experiments with the caterpillars. The results showed that WT caterpillars
213 displayed a preference for two plant chemicals, namely (*Z*)-3-hexen-1-ol and (*E*)-2-hexenal over paraffin
214 oil (P = 0.0482 and P = 0.0321) (Fig 4D). In contrast, KO caterpillars did not exhibit any significant
215 attraction or avoidance behavior towards the tested chemicals (Fig 4E). Additionally, we found that WT
216 caterpillars were more active in the arena, moving around (S8 Fig), while KO caterpillars were less active
217 in the arena, often remaining at the same position within the channels (S9 Fig).

218 Discussion

219 Chemical communication plays a significant role in the interaction between plants, herbivores and
220 their natural enemies. Infested host-plants emit herbivore-induced plant volatiles (HIPVs) and thereby
221 attract natural enemies such as parasitoid wasps (Mattiacci et al., 1994). While the role of adult herbivores'
222 olfaction in mediating these tri-trophic interactions has been investigated, the significance of caterpillar
223 olfaction in tri-trophic interactions of plants, herbivorous and their natural enemies has remained largely
224 underexplored. In this study, we severely impaired the olfaction of *P. brassicae* by knocking out *Orco* by
225 CRISPR/Cas9 to investigated the ecological importance of olfaction in host-plant choice and enemy
226 avoidance by *Pieris brassicae* larvae. The knockout of olfaction was successful, as we did not find Orco-
227 proteins in the antennae of *Orco* KO caterpillars and showed an altered brain structure in the *Orco* KO
228 caterpillars in comparison to WT caterpillars. Furthermore, the near complete lack of response to odors
229 by *Orco* KO butterfly antennae compared to wildtype confirms that olfaction was largely lost.
230 Intriguingly, although mating and oviposition were significantly reduced, KO butterflies still reproduce
231 some homozygous offspring. After this characterization of our mutant line, we aimed to test the role of
232 larval olfaction in a multitrophic framework. We found that the loss of olfaction had a significant effect
233 on the foraging behavior of the caterpillars, which exhibited a reduced growth rate, a greater vulnerability
234 to the parasitoid wasp *C. glomerata.*

235 The perception of volatile chemicals is mediated by the olfactory signal transduction cascade in the
236 insect antennae and the further processing of information in the brain. In the *P. brassicae* KO caterpillar
237 antennae, no Orco protein was detected, and chemical signals can therefore not be converted into
238 electrical signals (Sato et al., 2008; Wicher et al., 2008). Interestingly, the malfunctional Orco decreased

239 the number of glomeruli in the LAC, indicating that Orco is likely to play a role in the glomerular
240 organization of the LAC (Fig 1C-I). Similar changes have been reported for adult insects of a few
241 hymenopteran species: the ants *Harpegnathos saltator* and *Ooceraea biroi* and the honeybee *Apis*
242 *mellifera*, where the number and total size of glomeruli decreased while the volume of individual
243 glomeruli increased in *Orco* KO mutants (Chen et al., 2021; Trible et al., 2017; Yan et al., 2017). In
244 contrast, the antennal lobe morphology of *D. melanogaster* was not significantly changed when *Orco*
245 was knocked out (Larsson et al., 2004). In Lepidoptera, the size and number of glomeruli in *Helicoverpa*
246 *armigera* were found to be comparable between WT and KO adult moths without any significant
247 difference (Fan et al., 2022). However, silencing the pheromone receptor, *SlitOR5* led to a decreased size
248 of a glomerulus in the adult moth *Spodoptera littoralis* and knocking out *Orco* also led to a reduced size
249 of OR-related glomeruli in adult *Manduca sexta* (Fandino et al., 2019; Koutroumpa et al., 2022). The
250 loss of olfactory glomeruli in the caterpillar might, therefore, either indicate that the formation of
251 glomeruli in the embryo is mediated by a different mechanism than during pupation (Oland & Tolbert,
252 2011), or that the loss of neuronal activity reduces axonal innervations and thereby the size of the
253 glomeruli to an extent that they could no longer be visualized by our method. However, the mechanism
254 of glomerular formation and the roles that olfactory genes play in this process needs to be further
255 investigated in both immature and adult insects (Williams et al., 2022).

256 The role of olfaction in the behavioral ecology of adult insects has already been studied in quite
257 some detail, and silencing *Orco* has been an important tool for unravelling the importance of chemical
258 communication in adults of various insect species. For instance, in *Orco* KO adults of several insect
259 species, there was a significant reduction in the electrophysiological responses to volatile chemicals such
260 as alcohols and esters (Fandino et al., 2019; Sun et al., 2020). Consequently, *Orco* mutants exhibited
261 notable changes in host-plant location and severely reduced fecundity due to impaired pheromone
262 perception and mating, which also resulted in significantly reduced egg-laying behavior (Fan et al., 2022;
263 Fandino et al., 2019; Liu et al., 2023). Similar to other species, *Orco* KO *P. brassicae* butterflies exhibited
264 a loss of electrophysiological responses to most plant volatiles we tested (Fan et al., 2022; Sun et al.,
265 2020) (Fig 2A and 2B), and the mating behavior of *P. brassicae Orco* KO butterflies was also disrupted
266 (Fig 2C). However, we observed some differences: *P. brassicae Orco* KO butterflies were still able to
267 mate to a limited degree (Fig 2D). Whereas *Orco* KO adults in some other insect species did not exhibit
268 any mating behavior (Fan et al., 2022; Fandino et al., 2019). This finding suggests that additional sensory

269 systems beyond olfaction, such as vision, may be involved in the mating processes of this day-active
270 butterfly (Carpenter & Sparks, 1982; Obara et al., 2008). Interestingly, while many *P. brassicae*
271 butterflies with impaired olfaction failed to mate, several KO butterflies still mate and deposit fertilized
272 eggs when the oviposition substrate was supplied in our laboratory setting. The mated *Orco* KO
273 butterflies laid a comparable number of fertilized eggs on cabbage plants to WT butterflies which is in
274 line with the results from different moth species (Fan et al., 2022; Fandino et al., 2019; Sun et al., 2023),
275 suggesting oviposition decisions are mostly guided by non-volatile compounds, when no host-plant
276 location is required (van Loon et al., 1992a). Furthermore, the hatching rate of eggs laid by mated *Orco*
277 KO butterflies was similar to that of WT butterflies (S1 Fig), indicating that the reduced fecundity in
278 these insects is primarily due to impaired mating behavior and reduced mating frequency, which may
279 result from a lack of pheromonal communication.

280 When directly placed on their host-plant, we found that WT caterpillars gained more weight than
281 *Orco* KO caterpillars (Fig 3A). We speculate that olfaction is needed to evaluate plant tissues and to
282 select different leaves or leaf parts in order to feed efficiently, even when the caterpillar is already on the
283 plant. Secondary metabolites are often unevenly distributed even within a single leaf, and these
284 concentration differences have been shown to influence the performance of different caterpillars (Kester
285 et al., 2002; Yuan et al., 2022). It is conceivable that these localized differences in plant secondary
286 metabolites are also detectable by the herbivore through its olfactory system (Hanson & Dethier, 1972).
287 The difference between WT and KO caterpillars was amplified in the presence of disarmed parasitoids,
288 which were able to attack but not injure the caterpillars, indicating that KO caterpillars were more
289 susceptible to harassment by their natural enemies (Fig 3E). *Pieris brassicae* caterpillars are most
290 vulnerable to *C. glomerata* during the first two larval instars. Due to their reduced growth rate, KO
291 caterpillars spent a longer time in these vulnerable stages and, therefore, in a natural setting would likely
292 have suffered more strongly from parasitoid attack than WT caterpillars (Brodeur et al., 1996;
293 Haverkamp & Smid, 2020). Similarly, when the caterpillars were exposed to unmanipulated parasitoids,
294 the KO caterpillars were more susceptible and exhibited a higher mortality rate, demonstrating again that
295 olfaction is crucial in larvae to survive under the pressure of natural enemies (Fig 3F). Subsequently, we
296 hypothesized that the differences in weight gain and survival rate between WT and KO caterpillars are
297 due to different sensitivity to plant volatiles. By locating an available food source efficiently, WT
298 caterpillars can sooner reach a body size in which they are no longer vulnerable to attack by *C. glomerata*

299 (Brodeur et al., 1996). The higher mortality rate of KO caterpillars is, therefore, at least partly, a result
300 of a reduced foraging efficiency caused by the lack of olfactory information, which in turn led to an
301 extended period in which the caterpillars were vulnerable to attack by their natural enemies.

302 Searching for host-plants suitable for feeding and free of natural enemies is largely accomplished
303 via the olfactory system of herbivorous insects (Carrasco et al., 2015; Hu et al., 2020). Without functional
304 ORs, *P. brassicae* caterpillars could still discriminate plant tissues from paper discs; however, *Orco* KO
305 caterpillars preferred the non-host tomato plant over their natural food plant (Fig 3B). We surmise that
306 OR-mediated olfaction is required in caterpillars to locate and identify their host-plants, and losing this
307 part of their olfactory receptor repertoire would make it challenging to distinguish host from non-host-
308 plants (Liu et al., 2023). Nonetheless, *Orco* KO caterpillars still retain ORNs expressing ionotropic
309 receptors, which commonly detect acids and amines that have been found to elicit aversion in insect
310 herbivores (Zhang et al., 2019). Fatty acids are present at the edge of both artificially damaged and
311 caterpillar-infested cabbage leaves (Horikoshi et al., 1997), which, in the absence of ORs detecting
312 attractive plant compounds, may have caused avoidance behavior in the KO caterpillar to their natural
313 host-plant. Interestingly, the parasitoid *C. glomerata* also uses the fatty acids produced by the plant to
314 locate *Pieris* caterpillars, and it has been argued that herbivores might avoid these compounds to escape
315 from their natural enemies (Horikoshi et al., 1997; Zhang et al., 2019).

316 We found that WT caterpillars prefer caterpillar-infested plants free of wasps over caterpillar-
317 infested plants with wasps, which suggests that caterpillars are able to detect certain volatiles that are
318 derived from the interaction with caterpillars and wasps. By analyzing headspace samples of caterpillars,
319 parasitoids as well as the interaction of caterpillars and parasitoids, we successfully identified some
320 chemical compounds which were already known plant volatiles, such as alcohols and aldehydes.
321 However, we did not find any compounds that were specifically abundant in Pb-Cg or Cg. This suggests
322 that a volatile signal serving as a "danger signal" directly emitted by the wasps or caterpillars might not
323 exist, or it might not be detectable with these methods (Fig 4B). Alternatively, indirect cues such as the
324 volatiles emitted from the caterpillar spit during their defensive behavior might have triggered the
325 avoidance behavior of the WT caterpillars to infested plants with natural enemies. When we tested the
326 most abundant compounds in a two-choice behavioral assay against a solvent control, we found that *P.*
327 *brassicae* WT larvae showed a preference to the plant volatiles (*Z*)-3-hexen-1-ol and (*E*)-2-hexenal (Fig
328 4D), which are known HIPVs. Previous studies on other species have demonstrated that caterpillars are

329 capable of detecting different HIPVs (de Fouchier et al., 2018; Di et al., 2017). For caterpillars, HIPVs
330 can serve as indicators for the location of potential food plants and the presence of conspecifics (Di et
331 al., 2017; Zhang et al., 2019), but they also attract natural enemies and enhance the risk of being attacked
332 (Bernays, 1997; Ngumbi & Fadamiro, 2012; Yang et al., 2016). Most HIPVs identified in this study were
333 found abundantly in caterpillar saliva that caterpillars spit onto parasitoids to defend against attack. In
334 our study, caterpillars were attracted by HIPVs emitted by plants and by individual HIPVs (Fig 4D). We
335 speculate that caterpillars use low to intermediate concentrations of HIPVs to find suitable host-plants
336 and high concentrations to avoid sites where other caterpillars are under attack by their natural enemies.
337 Therefore, perceiving plant leaf volatiles and approaching the food plants accordingly will increase the
338 herbivores' feeding efficiency and enhance survival chances. In our study volatile cues of wasps were
339 only found in very low quantities and these compounds hardly elicited any behavioral response in the
340 caterpillars, however these chemicals might still play a role in the interaction between the caterpillars
341 and wasps at a close range (Ebrahim et al., 2015).

342 Larval insects are under a strong selection pressure to the detect optimal food sources and enemy
343 free spaces. Our study use a CRISPR/Cas9 knockout mutant line to investigate the significance of
344 olfactory information for the survival and development of *P. brassicae* caterpillars. We provided novel
345 insights into the tri-trophic interactions of plants, herbivores and their natural enemies from the
346 perspective of a caterpillar and highlighted the role of olfaction for both foraging and escaping natural
347 enemies.

348

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352

353 Author contributions

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Declaration of interests

The authors declare they have no competing interests.

Data availability

All data sets and scripts related to this study are available at the 4TU.ResearchData repository:

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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 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 compartment under the rearing condition of KO insects unless specified.

Orco knockout

The second exon of *Orco* was targeted to knock out the gene. The sgRNA was designed with Geneious Prime 2021 (Geneious, New Zealand) by searching N20 target + NGG PAM sequences in the second exon, sgRNA primers were selected according to activity scorings (Doench et al., 2016). The designated sgRNA off-target regions were evaluated by searching against the genome by est2genome function with a loose 50-score cut-off of using Exonerate 2.0 (Slater & Birney, 2005) and CHOPCHOP v3 (Labun et al., 2019). The sgRNAs that had no off-target sites in coding areas were selected. sgRNA templates were prepared by mixtures of 50 µL Q5 high-fidelity 2 × master mix (NEB, USA), 5 µL of the

389 following forward primers:
390 sgRNA1: ATTTAGGTGACACTATACATGTCAACTCTACTTAGGAGTTTTAGAGCTAGAAATA
391 GCAAG; sgRNA2: ATTTAGGTGACACTATACGATGAAGTAAACGAACTCAGTTTTAGAGCTA
392 GAAATAGCAAG; 5 μL constant reverse primer: AAAAGCACCGACTCGGTGCCACTTTTTCA
393 AGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC and 40 μL H_2O. The
394 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
395 72 °C for 10 s; finalized at 72 °C for 5 min. The PCR products were checked by 1.0 % gel electrophoresis
396 and purified by QIAGEN PCR purification kit (QIAGEN, Germany) according to the manufacturer's
397 instructions. sgRNA was synthesized by a reaction system of 2 μL 10 × SP6 enzyme mix (Invitrogen,
398 USA), 2 μL 10 × SP6 reaction buffer, 2 μL each of ATP, UTP, GTP and CTP, 200 ug sgRNA template
399 and add RNase-free H_2O up to 20 μL. The mixture was incubated at 37 °C for 4 hours, followed by
400 adding 1 μL Turbo DNase and incubating at 37 °C for 15 min. The reaction products were purified by
401 Monarch RNA cleanup kit (NEB, USA). The concentration of sgRNA was determined by DeNovix
402 (DeNovix, USA). sgRNA was mixed with Cas9 protein (NEB, USA) and incubated at 25 °C for 10 min.
403 Newly laid eggs, not older than half an hour, were collected from our laboratory colony and were
404 fixed on glass slides. The sgRNA/Cas9 mixture was colored with 1 μL food dye to stain the eggs as an
405 indicator that injection with a Femo-jet (Eppendorf, Germany) was successful. The injected eggs were
406 incubated at 25 °C for 4 to 5 days until hatching. The caterpillars were reared until the emergence of the
407 butterflies, a leg of each butterfly was cut for genotyping. gDNA of the leg samples was extracted with
408 MyTaq Extract-PCR Kit (Bioline, Germany). Mutant screening was performed by PCR with 15 μL 2 ×
409 MyTaq HS Red mix, 2 μL forward primer (TCTGGCTTCGGTATTACATTTC), 2 μL reverse primer
410 (CTTTTATGGCGTGTTTTATTTG), 2 μL gDNA template and 9 μL H_2O. The PCR products were
411 checked by gel electrophoresis and sequenced with the same primers by Eurofins (Eurofins, the
412 Netherlands).

413 ORN staining and axon tracing

414 L3 caterpillars (n = 6 for WT and n = 5 for KO) were anesthetized on ice and decapitated. Heads
415 were cut along the midline with micro-scissors to facilitate penetration of the fixative and antibodies.
416 The collected heads were immersed in freshly prepared 4 % formaldehyde in 0.1 M phosphate buffer at
417 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.

Electroantennography (EAG)

A panel of 54 chemicals (S1 Table) including esters, alcohols, aldehydes, alkenes, isothiocyanates (ITCs), heterocyclics and nitriles were selected from the literature (Bourne et al., 2023; Liu et al., 2020; van Loon et al., 1992b; Zhu et al., 2015) and our current volatile identification data, to investigate the EAG responses of both KO and wildtype (WT) butterfly antennae (n =15-18) of both sexes. Filter papers

447 loaded with 10 μL of selected chemicals, diluted in paraffin oil (10^{-2} v/v), were inserted into Pasteur
448 pipettes, clean filter paper was employed as negative control. The left antennae of butterflies were excised
449 at the basal end and the distal tip was also removed for better conductivity. Antennae were placed into
450 glass capillaries filled with EAG Ringer solution. Glass capillaries were placed on Ag-AgCl wires
451 connected to a ground electrode and a 10× high-impedance DC amplifier (Ockenfels Syntech, Germany).
452 The electrical signals were transformed using an IDAC-232 (Ockenfels Syntech, Germany) analog-
453 digital converter connected to a personal computer. Signals were finally recorded using the software
454 EAG Pro (Ockenfels Syntech, Germany). The first puff of each chemical was abandoned before the first
455 round of testing. Antennae were exposed to the chemicals in random order and were given intervals of
456 at least 20 s for recovering until the baseline stabilized.

457 Female oviposition behavior

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

466 Caterpillar performance on plants

467 Caterpillar performance on cabbage plants was evaluated by three different treatments:
468 (1) For each genotype, a group of ten L1 caterpillars (n = 17) was placed on one leaf of a four-week-
469 old cabbage plant. Caterpillars were reared for ten days, and more plants were provided when needed.
470 The caterpillars were then collected for weighing to compare the growth between the two genotypes.
471 (2) The growth of both genotypes of caterpillars (n = 8) was further compared by rearing in the
472 presence of *C. glomerata* parasitoid wasps from which the ovipositor had been removed (hereafter:
473 "disarmed"). Groups of ten L1 caterpillars were placed on individual four-week-old cabbage plants as

474 described above. The parasitoid wasps were anesthetized on a CO_2 plate and the ovipositor was removed
475 with fine tweezers to ensure that the parasitoid wasps can interact with the caterpillars but cannot
476 parasitize them. Two disarmed parasitoid wasps were released in each rearing cage 24 hours after the
477 caterpillars had been placed on the plants. The disarmed parasitoid wasps were replaced by newly
478 disarmed parasitoids every day, the caterpillars were reared on the plants for ten days and then collected
479 for weighing.

480 (3) To compare the survival rate of caterpillars (n = 7 groups of ten caterpillars) on the plants under
481 the threat of *C. glomerata* parasitoid wasps, a group of ten L1 caterpillars was placed on a leaf of a four-
482 week-old cabbage plant two days prior to placing two female parasitoid wasps. The parasitoid wasps
483 were replaced every day to ensure that caterpillars were always exposed to natural enemies. The
484 caterpillars were collected after ten days to count the number that survived; the collected caterpillars
485 were further reared until they pupated or *C. glomerata* larvae emerged.

486 Larval host-plant seeking behavior

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

497 To further test the host-plant locating behavior of L3 caterpillars, we investigated the behaviors of
498 caterpillars in a Y-tube olfactometer (diameter 35 mm) with a built-in black Y-shaped wire, allowing
499 caterpillars to crawl along the wire and make a choice smoothly in the laboratory. Each arm was provided
500 with a 1.1 L/min inlet airflow which had been purified by charcoal and humidified by water before
501 entering the treatment jars. Healthy plants were untreated, infested plants were infested with 30 early L3
502 caterpillars for 24 hours and with caterpillars on plants while testing. Differently treated plants were

503 placed in jars to test caterpillar behaviors. The caterpillars had been starved for approximately 5 hours
504 before testing. Each caterpillar was tested and observed for 5 min, caterpillar behavior was monitored by
505 a camera. The position of inlet airflow was changed every five tests to avoid position bias.

506 Volatile analysis

507 Volatiles were collected from L3 *P. brassicae* caterpillars, three-day-old female *C. glomerata* wasps,
508 *P. brassicae* caterpillars and *C. glomerata* wasps together, caterpillar frass collected from L3 caterpillars
509 and caterpillar saliva also collected from L3 caterpillars using glass capillaries following Mattiacci et al.
510 (Mattiacci et al., 1994). Fresh frass and saliva samples were stored in a freezer at -20 °C and thawed
511 overnight at room temperature before volatile collection. Headspace collection of volatiles was
512 performed from five treatments: 20 female wasps (Cg) in a 10 mL glass vial (n =12); 20 L3 caterpillars
513 (Pb) in a 10 mL glass vial (n =10); 20 L3 caterpillars and 2 female wasps (Pb-Cg) in a 10 mL glass vial
514 (n =14); 100 mg caterpillar frass (Pb-Fr) in a 1.5 mL glass vial (n =10); and 50 µL caterpillar saliva (Pb-
515 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
516 control. Volatiles were collected through dynamic headspace sampling using Tenax TA adsorbent
517 material (20/35 mesh; Camsco, USA). Synthetic air (Air Synthetic 4.0 Monitoring from Linde Gas, the
518 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
519 vials were constantly supplied as a carrier for the volatiles, while simultaneously the volatiles were
520 trapped by drawing air at 100 and 50 mL min^{-1}, respectively, through a stainless-steel tube filled with
521 200 mg Tenax TA for 2 h.

522 The collected volatiles were thermally released from the Tenax TA adsorbent using an Ultra 50:50
523 thermal desorption unit (Markes, UK) at 250 °C for 10 min under a helium flow of 20 mL min^{-1}, while
524 simultaneously re-collecting the volatiles in a thermally cooled universal solvent trap: Unity (Markes,
525 UK) at 0 °C. Once the desorption process was completed, the volatile compounds were released from
526 the cold trap by ballistic heating at 40°C s^{-1} to 280 °C, which was then kept for 10 min, while all the
527 volatiles were transferred to a ZB-5MS analytical column (30 mL × 0.25 mm ID × 1 mm F.T. with 10 m
528 built-in guard column (Phenomenex, USA), placed inside the oven of a Thermo Trace GC Ultra (Thermo
529 Fisher Scientific, USA), for separation of volatiles. The GC oven temperature was initially held at 40 °C
530 for 2 min and was immediately raised at 6 °C min^{-1} to a final temperature of 280°C, where it was kept
531 for 4 min under a constant helium flow of 1 mL min^{-1}. For the detection of volatiles, a Thermo Trace

532 DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, USA) coupled to the GC was operated
533 in an electron impact ionization (EI) mode at 70 eV in a fullscan mode with a mass range of 35 - 400 amu
534 at 4.70 scans s^{-1}. The MS transfer line and ion source were set at 275 and 250 °C, respectively.

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

544 Caterpillar response to individual chemical compounds

545 L3 caterpillars (n = 31-50 for each compound, each genotype) were tested in a custom-made set-up
546 consisting of 25 separate arenas in the laboratory. Single caterpillars were placed in the central well of
547 the individual arenas before the test. Caterpillars were given a choice between a selected volatile
548 chemical of interest (S2 Table) and paraffin oil. Input odors were delivered at 1.1 L min^{-1} for both
549 chemicals and solvent controls, the odors were sucked out at 2.2 L min^{-1} at the same time from the center.
550 The arena was cleaned by ethanol and dried by high-pressure air. The position of chemicals was
551 exchanged after each test. The behavior of caterpillars was recorded for 10 min at 100 frames per minute
552 using a Canon TV lens JF16mm (Canon, Japan). Behavioral data was extracted by EthoVision XT 11.5
553 software (Noldus, The Netherlands). Data were removed from the dataset when the caterpillar stayed in
554 the arena for less than 10 seconds. All tests were performed at room temperature.

555 Statistical analysis

556 Unless stated otherwise, statistical tests were performed in R (version 4.4.0; R Core Team, 2016) in
557 combination with RStudio (Boston, USA). Data sets were tested for normal distribution using a Shapiro–
558 Wilk test and for equal variances using Levene's test. The difference of glomerular number between

559 wildtype and *Orco* knockout caterpillars was tested with a Wilcoxon rank-sum test.
560 Electroantennographic responses between the two genotypes were tested statistically by using the
561 Student's t-test when the values were normally distributed and had homogenous variance or tested by
562 using the Kruskal-Wallis rank-sum test when the requirements for the t-test were not met. The difference
563 in butterfly mating frequency was analyzed by using the Wilcoxon rank-sum test; differences in
564 oviposition dynamics were tested with a generalized linear model (GLM) with a negative binominal
565 distribution. The development differences between WT and KO caterpillars on full plants and under the
566 thread of disarmed parasitoids were tested using a Student's t-test, and survival rate difference between
567 the two genotypes was tested using a GLM with a beta-binomial distribution. Caterpillar host-plant
568 selection behaviors in Petri dishes and in the Y-tube were analyzed with a Chi-square test. Differences of
569 the relative time spent by the caterpillars between the control side and the odor side of the multichannel
570 arenas were compared by a non-parametric Wilcoxon rank-sum test. Principal component analysis (PCA)
571 of volatile blends was achieved by MetaboAnalyst (Pang et al., 2024). Data were normalized by
572 logarithmic transformation and scaled in auto mode. Ward's method and Euclidean distance were
573 employed in the hierarchical clustering heatmap by default settings without clustering samples. In
574 addition, the abundance of volatiles as peak heights was imported into SIMCA-P 17 statistical software
575 (Umetrics, Sweden), the data was analyzed by multivariate data analysis (MVDA). Supervised
576 orthogonal partial least-squares discriminant analysis (OPLS-DA) was employed to compare and
577 correlate treatment groups.

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Figures and figure legends

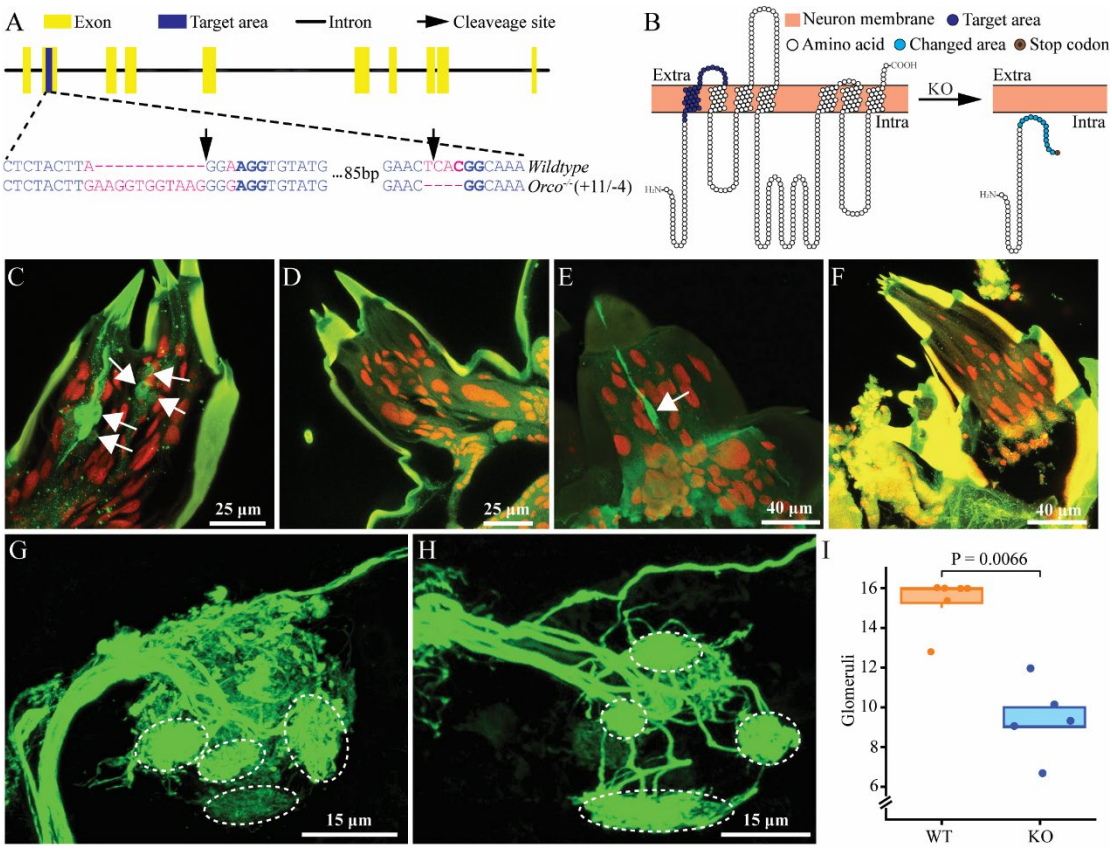

Fig 1. *Orco* knockout by CRISPR/Cas9 and verification in *Pieris brassicae*. (A), gene structure of *Orco*. Yellow blocks indicate exons and blue block indicates target area in the second exon. Black solid line indicates intron. Black arrows indicate the designated cleavage site of the Cas9 protein. 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 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 wildtype (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) and (H). (I), number of glomeruli counted in WT caterpillar brain (orange) and *Orco* KO caterpillar (blue) brain. Significant difference was detected by Wilcoxon rank-sum test.

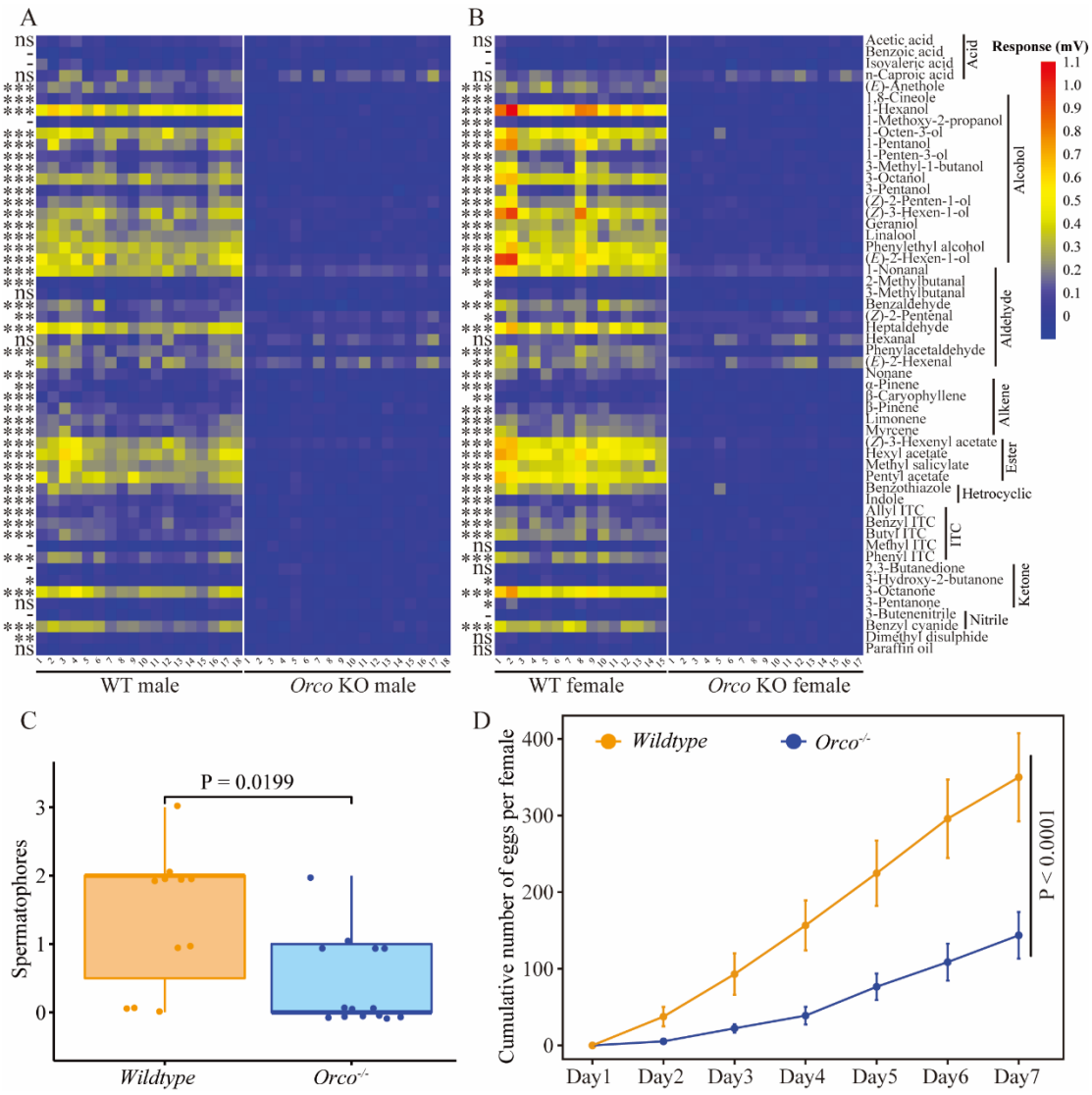

Fig 2. *Pieris brassicae* butterfly electrophysiological responses to chemical compounds and mating and oviposition dynamics. (A), electroantennogram (EAG) response of male butterflies (n = 18 for both WT and KO). (B), EAG response of female butterflies (n = 15 for WT and n = 17 for KO). Left panels 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. Significant difference 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 (1.1 mV). (C), mating frequency of *P. brassicae* butterflies. The boxplots indicate the number of spermatophores in WT female butterflies (n = 11) and in KO butterflies (*Orco*$^{-/-}$) (n = 14), the difference was tested by using Wilcoxon rank-sum test. (D), *P. brassicae wildtype* (WT) and *Orco*$^{-/-}$ knockout butterfly oviposition dynamics. The orange line indicates the number of eggs per female (mean ± SE) laid by wildtype butterflies (n = 11), the blue line indicates the number of eggs laid by *Orco* knockout butterflies (n = 14). Differences were analyzed using a GLM with negative binominal distribution, difference test results are shown in the line chart.

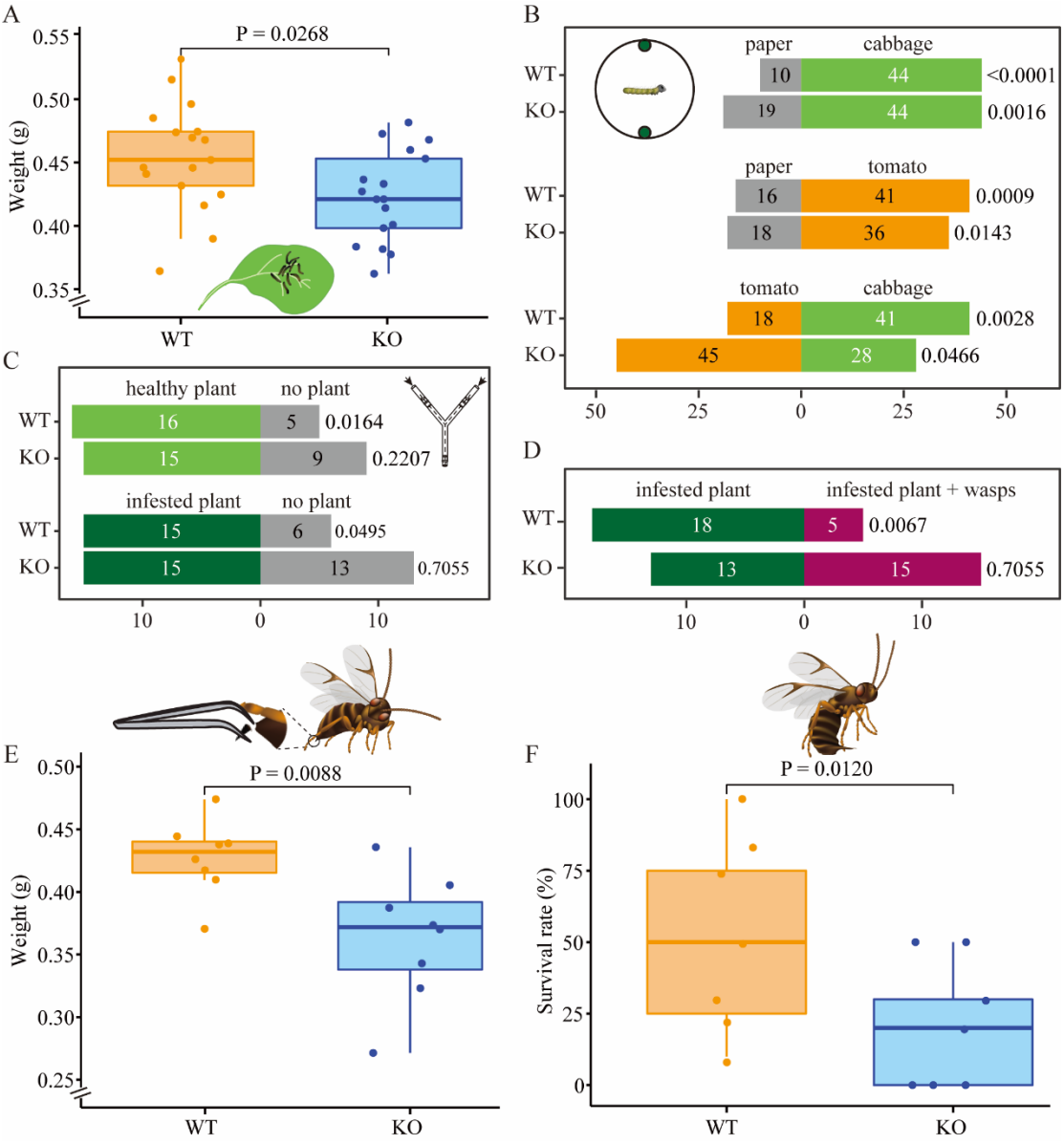

Fig 3. *Pieris brassicae* caterpillar growth and foraging behavior. (A), *P. brassicae* caterpillar growth on cabbage plants, y axis shows weight (g) of WT and KO caterpillars after 10 days of feeding , n = 17. (B), caterpillar behavioral choices in a Petri dish. The numbers of caterpillars that chose one of the two discs are shown in the respective bars (n = 54 -73). Schematic drawing shows behavioral setup. Petri dish diameter was 188 mm, disc diameters were 13 mm. Discs in the Petri dish represent cabbage, paper and tomato leaf discs. Grey bars indicate paper disc choices, light green indicates cabbage leaf disc choices and orange indicates choices for tomato leaf discs. (C), caterpillar behavioral choices in Y-tube olfactometer without parasitoid wasps. Schematic drawing shows the Y-tube olfactometer. The dashed line indicates a black metal Y wire in the center of glass Y-tube olfactometer. The main arm of the Y-olfactometer is 200 mm length, the lateral arms are 275 mm length, the angle between the lateral arms is 80 °. Light green, dark green and grey bars represent choices for healthy plant, infested plant and no plant respectively. Healthy plant, plants were not treated; no plant, an empty jar without any insect or plant; infested plant, plants were infested by early L3 caterpillars. (D), caterpillar behavioral choices in Y-tube olfactometer with parasitoid wasps. Different treatments are in different colors. Dark green, infested plant - wasps; magenta, infested plant + wasps. Significant differences were tested between wildtype (WT) and knockout (KO) caterpillars or between two discs by Chi-square test in panel B-D, P-values are shown on the right side of each bar. (E), *P. brassicae* caterpillar growth when exposed to disarmed *C. glomerata* female parasitoids, y axis shows weight (g) of caterpillars after 10 days of feeding, n = 8. Schematic drawing shows disarmed female *C. glomerata* (ovipositor removed). (F), *P. brassicae* caterpillar survival rate when exposed to healthy *C. glomerata* female wasps, n = 7. Significant differences in development and survival rate were assessed using a one-tailed Student's t-test, P values are indicated above boxplots. Schematic drawing shows a healthy female *C. glomerata* (unmanipulated). In both panels orange boxplots indicate wildtype caterpillars (WT), blue boxplots indicate *Orco* knockout caterpillars (KO).

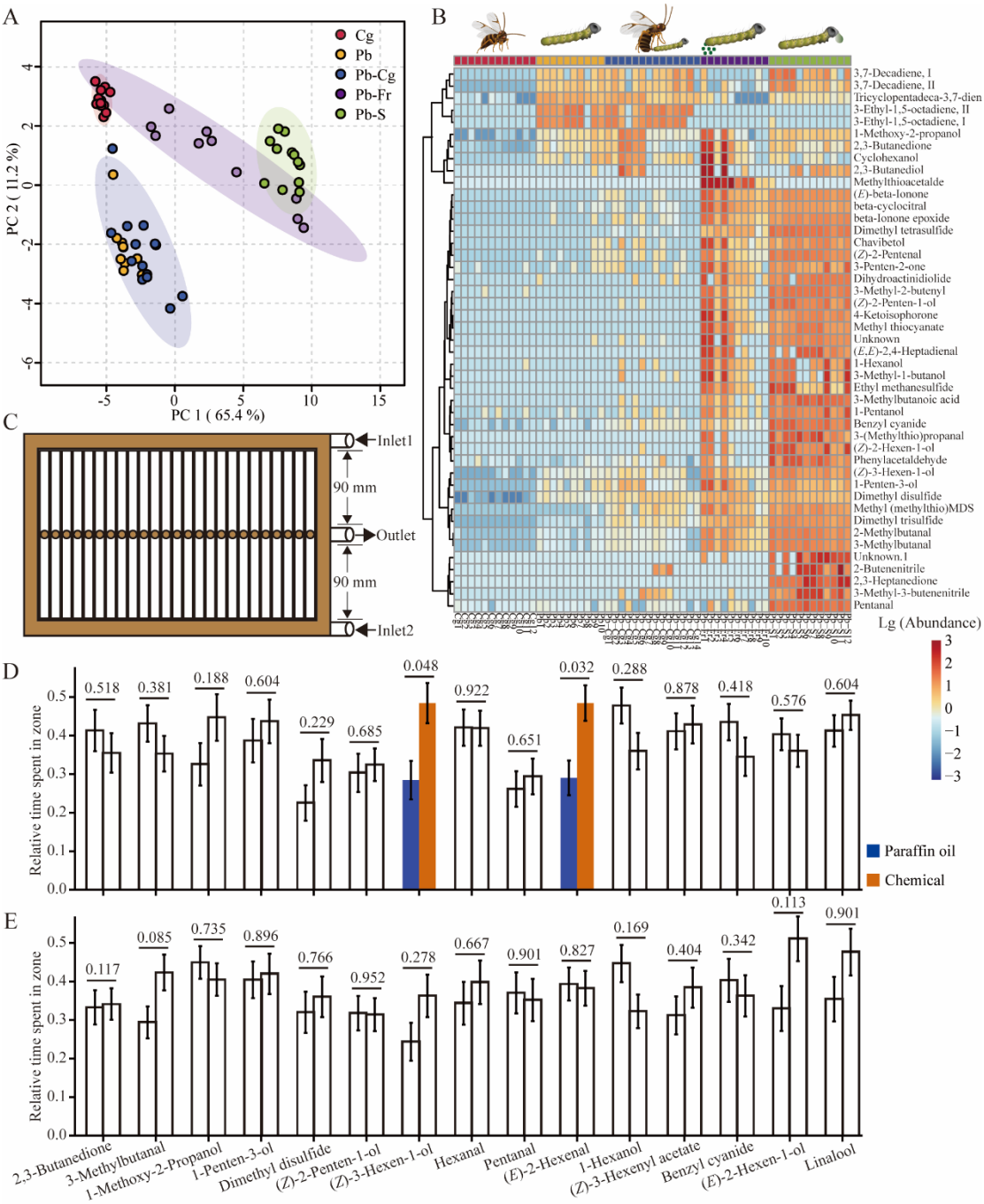

Fig 4. Overview of caterpillar- (*Pieris brassicae*) and parasitoid wasp- (*Cotesia glomerata*) associated volatile compounds. (A), PCA (Principal Component Analysis) two-dimensional score plot of five treatment groups: Cg, *Cotesia glomerata* female parasitoid wasps (n = 12); Pb, *Pieris brassicae* caterpillars (n = 10); Pb-Cg, *P. brassicae* caterpillars in the presence of *C. glomerata* female parasitoid wasps (n = 14); Pb-Fr, *P. brassicae* caterpillar frass (n = 10); and Pb-S, *P. brassicae* spit (n = 12), based on their volatile blend composition. (B), hierarchical clustering heatmap showing the abundance of each identified volatile compound of each treatment. Clustering chemicals in the heatmap indicate higher correlation. (C), schematic drawing of the custom-designed multi-channel arena. (D) wildtype (WT)

847 caterpillar behavioral preference to the tested 15 odorants. (E), *Orco* knockout (KO) caterpillar
848 behavioral preference to the tested 15 chemicals. Unfilled bars represent tests that exhibit no difference
849 between paraffin oil and the odorant of interest. Blue bars indicate the cumulative duration ratio that
850 caterpillars stayed in the paraffin oil zone, dark orange bars indicate the cumulative duration ratio that
851 caterpillars stayed in the odorant zone. Relative time spent in odorant zone = Cumulative duration in a
852 specific zone / Cumulative duration in the arena. Error bars indicate standard errors. In (D) and (E),
853 differences were tested between the two zones using a Wilcoxon rank-sum test, P values are presented
854 above bars (n = 31-50). For each comparison, left bar represents paraffin oil and right bar represents
855 chemical compound.
856