

1 ***orco* mutagenesis causes loss of antennal lobe glomeruli and impaired social behavior in**  
2 **ants**

3

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16

17 Life inside ant colonies is orchestrated with a diverse set of pheromones, but it is not clear how  
18 ants perceive these social cues. It has been proposed that pheromone perception in ants evolved  
19 via expansions in the numbers of odorant receptors (ORs) and antennal lobe glomeruli. Here we  
20 generate the first mutant lines in ants by disrupting *orco*, a gene required for the function of all  
21 ORs. We find that *orco* mutants exhibit severe deficiencies in social behavior and fitness,  
22 suggesting that they are unable to perceive pheromones. Surprisingly, unlike in *Drosophila*  
23 *melanogaster*, *orco* mutant ants also lack most of the approximately 500 antennal lobe glomeruli  
24 found in wild-types. These results illustrate that ORs are essential for ant social organization, and  
25 raise the possibility that, similar to mammals, receptor function is required for the development  
26 and/or maintenance of the highly complex olfactory processing areas in the ant brain.

27

28 Ants live in complex societies and display sophisticated social behavior, including reproductive  
29 division of labor between queens and workers, behavioral division of labor between nurses and  
30 foragers, the formation of adaptive foraging networks, nestmate vs. non-nestmate discrimination,  
31 and collective nest construction (David Morgan, 2009; Grüter & Keller, 2016; Richard & Hunt,  
32 2013; Leonhardt et al., 2016). All of these behaviors are largely mediated via chemical  
33 communication using a wide range of pheromones (David Morgan, 2009; Grüter & Keller, 2016;  
34 Richard & Hunt, 2013; Leonhardt et al., 2016). However, the receptor families involved in  
35 pheromone perception have not been functionally characterized, in part because the complex life  
36 cycle of ants has hindered the development of functional genetic tools (Schulte et al., 2014;  
37 Grüter & Keller, 2016; Kohno et al., 2016; Reid & O’Brochta, 2016). In *Drosophila*, pheromone  
38 receptors have been identified that belong to multiple insect chemosensory receptor families,  
39 including odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), and  
40 pickpocket channels (PPKs) (Kohl et al., 2015). Ants have numbers of GRs, IRs, and PPKs that  
41 are typical for insects, while their OR repertoire is highly expanded (Zube et al., 2008; C. D.  
42 Smith et al., 2011; C. R. Smith et al., 2011; Zhou et al., 2012; Oxley et al., 2014; McKenzie et  
43 al., 2016) (Figure 1A and Supplementary Table 1). This raises the possibility that the expansion  
44 of ORs specifically, rather than chemoreceptors in general, may underlie the evolution of  
45 complex chemical communication in ants. Ants also have exceedingly large numbers of  
46 glomeruli in their antennal lobes, which likely mirror their expanded OR gene repertoire  
47 (McKenzie et al., 2016) (Supplementary Table 1). Insect ORs function as chemosensory  
48 receptors by forming ligand-gated ion channels through the formation of dimers with the highly-  
49 conserved co-receptor protein Orco (Larsson et al., 2004; Jones et al., 2005; Sato et al., 2008).  
50 *orco* null mutants in fruit flies, locusts, mosquitoes, and moths therefore lose OR function and

51 show impaired responses to odorants such as food volatiles and sex pheromones (Asahina et al.,  
52 2008; DeGennaro et al., 2013; Koutroumpa et al., 2016; Li et al., 2016; Yang et al., 2016).

53

54 To study the role of ORs in ant biology, we developed a CRISPR/Cas9 protocol and created *orco*  
55 mutants in the clonal raider ant, *Ooceraea biroi* (formerly *Cerapachys biroi* (Borowiec, 2016)), a  
56 promising genetic model system (Oxley et al., 2014). We confirmed the identity of *orco* in the *O.*  
57 *biroi* genome (Supplementary Table 2, Supplementary Figure 1) and designed and synthesized a  
58 guide RNA (gRNA) to target *orco* (Figure 1B). We injected Cas9 and gRNA into 3,291 eggs less  
59 than 5h of age and produced 42 G0 adults, some of which displayed mutations in at least 97% of  
60 PCR amplicons of the *orco* target site (Figure 1C, Supplementary Text). G0 mutations in the  
61 germline can be inherited by G1s to produce stable modifications to the genome (Reid &  
62 O'Brochta, 2016). Given that *O. biroi* reproduces through parthenogenesis (Oxley et al., 2014),  
63 stable mutant lines can be clonally propagated from individual mutant G1s and subsequent  
64 generations without the need for crosses. *orco* loss-of-function mutant lines are thus derived  
65 from G1 eggs with independent frameshift mutations in both *orco* alleles. We recovered a  
66 diverse set of *orco* mutant lines, including two *orco*<sup>wt/-</sup> lines with one frameshift allele and five  
67 *orco*<sup>-/-</sup> lines with two frameshift alleles (Figure 1D). The phenotypes reported below were  
68 consistent across the two *orco*<sup>wt/-</sup> lines and across the five *orco*<sup>-/-</sup> lines, respectively.

69 Descriptions of the specific lines used in each experiment along with the associated phenotypes  
70 are given in Supplementary Table 3.

71

72 In *D. melanogaster*, all antennal lobe glomeruli that have been examined remain present in *orco*  
73 mutants, implying that OR function is not required for gross antennal lobe morphology (Larsson

74 et al., 2004; Chiang et al., 2009). These results contrast strongly with mice, where olfactory  
75 receptor function and neuronal activity are essential for the formation and maintenance of the  
76 analogous brain region, the olfactory bulb (Yu et al., 2004). However, *D. melanogaster* has only  
77 60 ORs and a similar number of glomeruli, while mice possess over one thousand olfactory  
78 receptors and glomeruli. This striking difference suggests that highly complex olfactory systems  
79 must rely on receptor function for their development and/or maintenance. Ants have highly  
80 expanded numbers of ORs and antennal lobe glomeruli (Zube et al., 2008; Oxley et al., 2014)  
81 (Supplementary Table 1), raising the possibility that the development and/or maintenance of ant  
82 antennal lobes may require additional mechanisms to exceed the complexity found in other  
83 insects. To address whether OR function might be required for the structure of the ant antennal  
84 lobe, we imaged brains using confocal microscopy, measured antennal lobe volumes and the  
85 number of glomeruli, and reconstructed antennal lobes in wild-type and *orco* mutant adults in *O.*  
86 *biroi* and *D. melanogaster*. We found that the antennal lobes of *orco*<sup>-/-</sup> ants measured only one  
87 third the volume of wild-type and *orco*<sup>wt/-</sup> antennal lobes, and approximately 82% of the  
88 glomeruli were lost (Figure 2A,B and Supplementary Video 1). However, all six glomeruli in the  
89 T7 cluster of the ant antennal lobe, which is believed to be innervated by olfactory sensory  
90 neurons that do not express ORs (Nakanishi et al., 2010; McKenzie et al., 2016), were still  
91 present in *orco*<sup>-/-</sup> individuals. No differences were observed in the volume of the protocerebrum,  
92 mushroom bodies, or central complex relative to wild-types ( $P = 0.45, 0.17, \text{ and } 0.20,$   
93 respectively; *t*-test). In contrast, we detected no significant difference in antennal lobe volumes  
94 and only minor potential differences in glomerulus numbers between wild-type and *orco*<sup>-/-</sup> flies  
95 (Figure 2C,D, Supplementary Video 1, and Supplementary Text). These results demonstrate that  
96 development and/or maintenance of ant antennal lobes are indeed dependent on *orco* function.

97 While further experiments are required to describe precisely how this striking phenotype arises,  
98 we hypothesize that ants, similar to mammals, may employ ORs in the development and/or  
99 maintenance of antennal lobe glomeruli, possibly allowing them to evolve larger and more  
100 complex antennal lobes than other insects (Supplementary Table 1).

101  
102 Based on the general observation that ants are often repelled by the smell of permanent markers,  
103 we developed a simple assay to test whether *orco* mutants have compromised chemosensory  
104 abilities. We found that wild-type and *orco*<sup>wt/-</sup> *O. biroi* are indeed strongly repelled by lines  
105 drawn with Sharpie™ permanent marker, and rarely contact or cross Sharpie lines (Figure 3A-  
106 C). However, we found that *orco*<sup>-/-</sup> *O. biroi* are significantly less repelled by Sharpie (Figure  
107 3A-C and Supplementary Video 2), implying that *orco* is required to perceive the odorants that  
108 cause Sharpie lines to be repulsive. These results suggest that *orco* mutant ants possess general  
109 chemosensory deficiencies, similar to *orco* mutants in other types of insects (Asahina et al.,  
110 2008; DeGennaro et al., 2013; Koutroumpa et al., 2016; Li et al., 2016; Yang et al., 2016).

111  
112 Pheromone trails are a major feature of chemical communication in many ants, and are important  
113 for coordinating collective behaviors such as foraging and nest relocation (Zube et al., 2008;  
114 David Morgan, 2009). To test whether *orco* influences the ability of *O. biroi* to follow  
115 pheromone trails, we set up 5 experimental colonies composed of 12-14 identically-reared G1s  
116 with wild-type, *orco*<sup>wt/-</sup>, and *orco*<sup>-/-</sup> genotypes, and individually tagged each ant with color dots  
117 (Figure 3D). We recorded videos of each colony and used a novel custom-built automated  
118 behavioral tracking system employing painted color tags (rather than paper barcodes (Mersch et  
119 al., 2013)) to individually identify the ants and quantify their behavior (Figure 3D,

120 Supplementary Figure 2, and Supplementary Videos 3,4). We disturbed each colony at the  
121 beginning of each video. During the ensuing period of high activity, we created a 2-D histogram,  
122 or density map, of movement for each ant, and measured the Pearson correlation coefficient of  
123 this density map with the density map of the other ants in the colony, reasoning that density maps  
124 would be more highly correlated when ants were following pheromone trails (Figure 3E,F). To  
125 provide a null expectation, we also compared the density maps of individual ants to a  
126 randomized density map of other ants in the colony (Figure 3E,F). We found that the density  
127 maps of individual ants had significantly higher correlations with the density maps of the rest of  
128 the ants in the colony than with the randomized density maps in wild-type, but not *orco*<sup>-/-</sup>, ants  
129 (Figure 3F; average correlation coefficients were 0.31 versus 0.04 in wild-type and 0.07 versus  
130 0.02 in *orco*<sup>-/-</sup>, respectively). These findings imply that trail following behavior is reduced or  
131 absent in *orco*<sup>-/-</sup> ants, likely because they are unable to perceive chemical pheromone trails.

132

133 Nesting behavior, and the formation of aggregations more generally, is a ubiquitous feature of  
134 social insect biology (Depickere et al., 2004). Immediately upon eclosion, we noticed that some  
135 G1s did not nest with other ants, but instead showed a wandering phenotype (Figure 3G). In a set  
136 of 16 G1 colonies, we used this wandering phenotype to identify colonies containing *orco*<sup>-/-</sup> ants  
137 with 100% accuracy ( $P < 0.001$ , Fisher exact test, see Methods). To more precisely measure  
138 nesting behavior in *orco* mutants, we recorded and analyzed 24 hr videos of each experimental  
139 colony. We found that wild-type and *orco*<sup>wt/-</sup> ants aggregated into tight clusters and exhibited  
140 little movement outside the cluster, while *orco*<sup>-/-</sup> ants frequently exited the cluster and wandered  
141 around the dish (Figure 3H, Supplementary Video 3). Overall, *orco*<sup>-/-</sup> ants spent a significantly  
142 larger fraction of time without contact with other ants when compared to wild-type and *orco*<sup>wt/-</sup>

143 ants (Figure 3I). These findings demonstrate that typical nesting behavior is compromised in  
144 *orco*<sup>-/-</sup> ants. This observation is consistent with the idea that *orco* mutants are unable to perceive  
145 odorants, such as aggregation pheromones (Bell et al., 1972; Depickere et al., 2004; Li et al.,  
146 2016), that might be involved in nesting behavior.

147

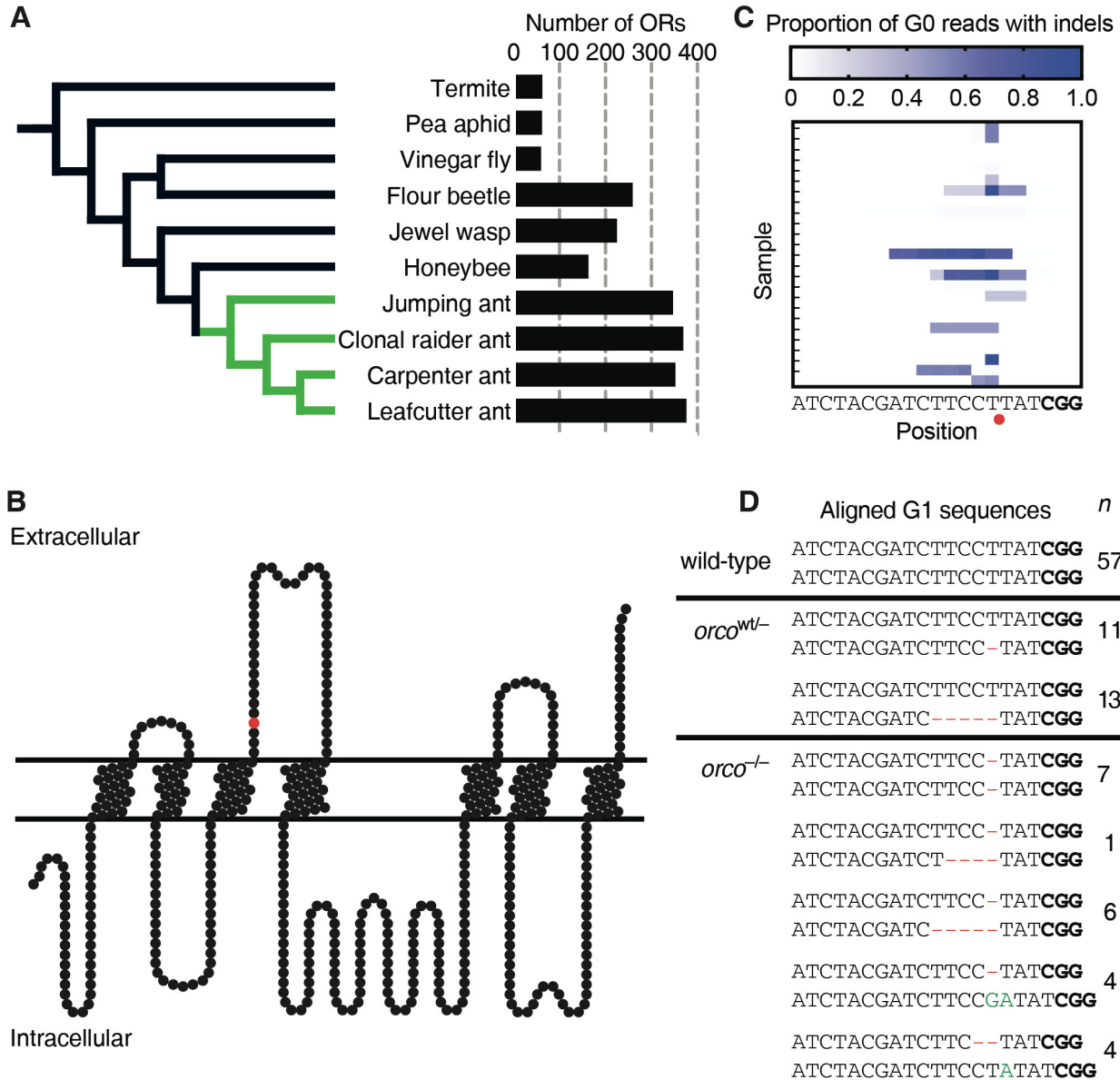
148 Finally, we investigated whether *orco* mutations have an effect on fitness by measuring egg-  
149 laying and survival rates of wild-type and *orco* mutant ants. We found that *orco*<sup>-/-</sup> ants laid  
150 significantly fewer eggs than wild-types and *orco*<sup>w<sup>t</sup>-/-</sup> ants over a two week period (Figure 4A),  
151 and *orco*<sup>-/-</sup> ants exhibited significantly higher mortality than wild-types over a 34 day period  
152 (Figure 4B). This suggests that the *orco* mutant phenotype has serious consequences for ant  
153 fitness (for a discussion of the potential role of off-target effects see Supplementary Text). It is  
154 possible that these fitness effects result because *orco*<sup>-/-</sup> ants are unable to integrate into the  
155 colony, as wandering behavior and reduced fitness are also seen in wild-type ants that are kept in  
156 social isolation (Koto et al., 2015). While we observed many striking deficiencies in *orco*<sup>-/-</sup> ants,  
157 it is important to note that these ants are viable, feed, lay eggs, and may still exhibit some typical  
158 social behaviors. For example, we have observed *orco*<sup>-/-</sup> ants groom eggs, touch other ants with  
159 their antennae, and elicit alarm responses (Supplementary Videos 3,4). Thus, these *orco* mutants  
160 will provide an important resource to study the role of ORs in ant biology in the future.

161

162 We have demonstrated that *orco* is crucial for many aspects of ant biology, including antennal  
163 lobe morphology, individual response to repulsive odorants and pheromones, and fitness. These  
164 results illustrate the functional significance of the striking expansion of ORs and antennal lobes  
165 in ants relative to other insects, and imply that the expansion of ORs may have been an important



166 component of the evolution of eusocial behavior in ants (Zube et al., 2008; C. D. Smith et al.,  
167 2011; C. R. Smith et al., 2011; Zhou et al., 2012; Oxley et al., 2014; McKenzie et al., 2016)  
168 (Figure 1A, Supplementary Table 1). Major transitions in evolution require the coordinated  
169 action of individuals to operate as a functional, higher-level unit (Maynard Smith & Szathmary,  
170 1997). During the transition from solitary to eusocial living in ants, this coordination was largely  
171 achieved via pheromones, and our study illustrates some of the dramatic consequences that are  
172 suffered by individuals deficient in pheromone perception. Importantly, the greatly reduced  
173 antennal lobes of *orco* mutant ants suggest that the mechanisms underlying the development  
174 and/or maintenance of ant olfactory systems differ from *D. melanogaster*, and could be  
175 dependent on receptor function as is the case in mammals. These findings also open the  
176 possibility that ants could serve as complementary models to better understand the evolution and  
177 development of complex chemosensory systems.



178

179 **Figure 1: Number of OR genes and *orco* mutagenesis.** (A) Phylogeny with numbers of ORs

180 for ants (green) and other insects (black), showing ant OR expansion (Supplementary Table 1).

181 (B) Position of predicted CRISPR/Cas9 cut site in Orco protein model (red circle). Frameshift

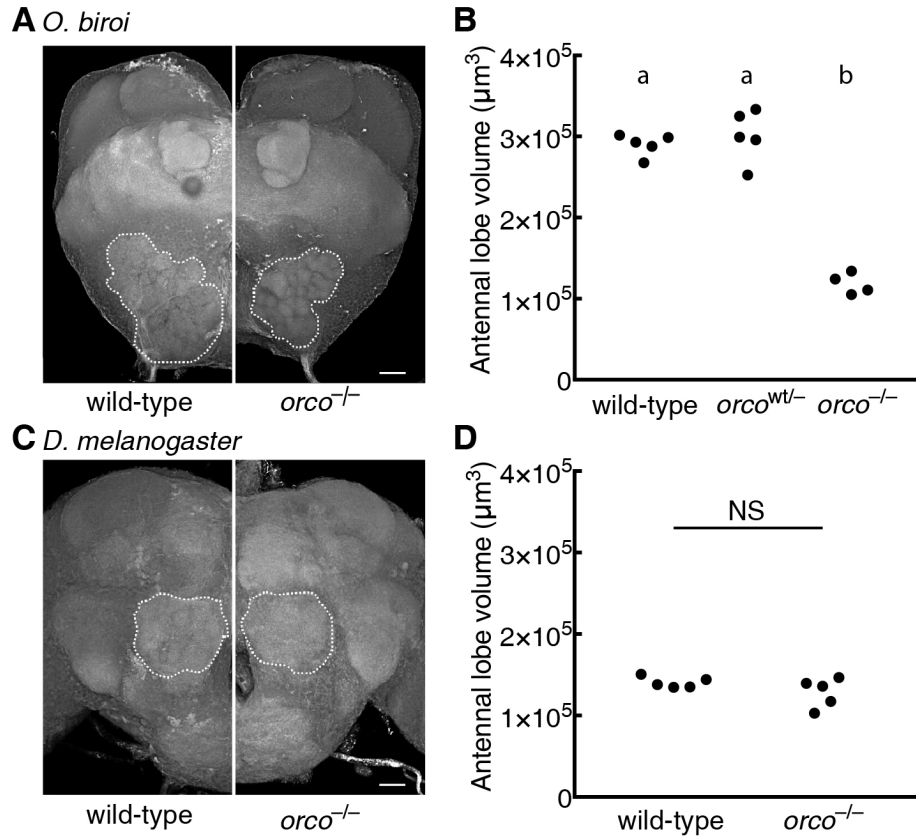
182 mutations at this position truncate the wild-type protein between the third and fourth

183 transmembrane domains, and the resultant mutant protein is unlikely to form functional ion

184 channels. (C) Proportion of Illumina sequencing reads of *orco* amplicons with insertions or

185 deletions (indels) relative to gRNA sequence in G0s, showing mutation rates of at least 97% in

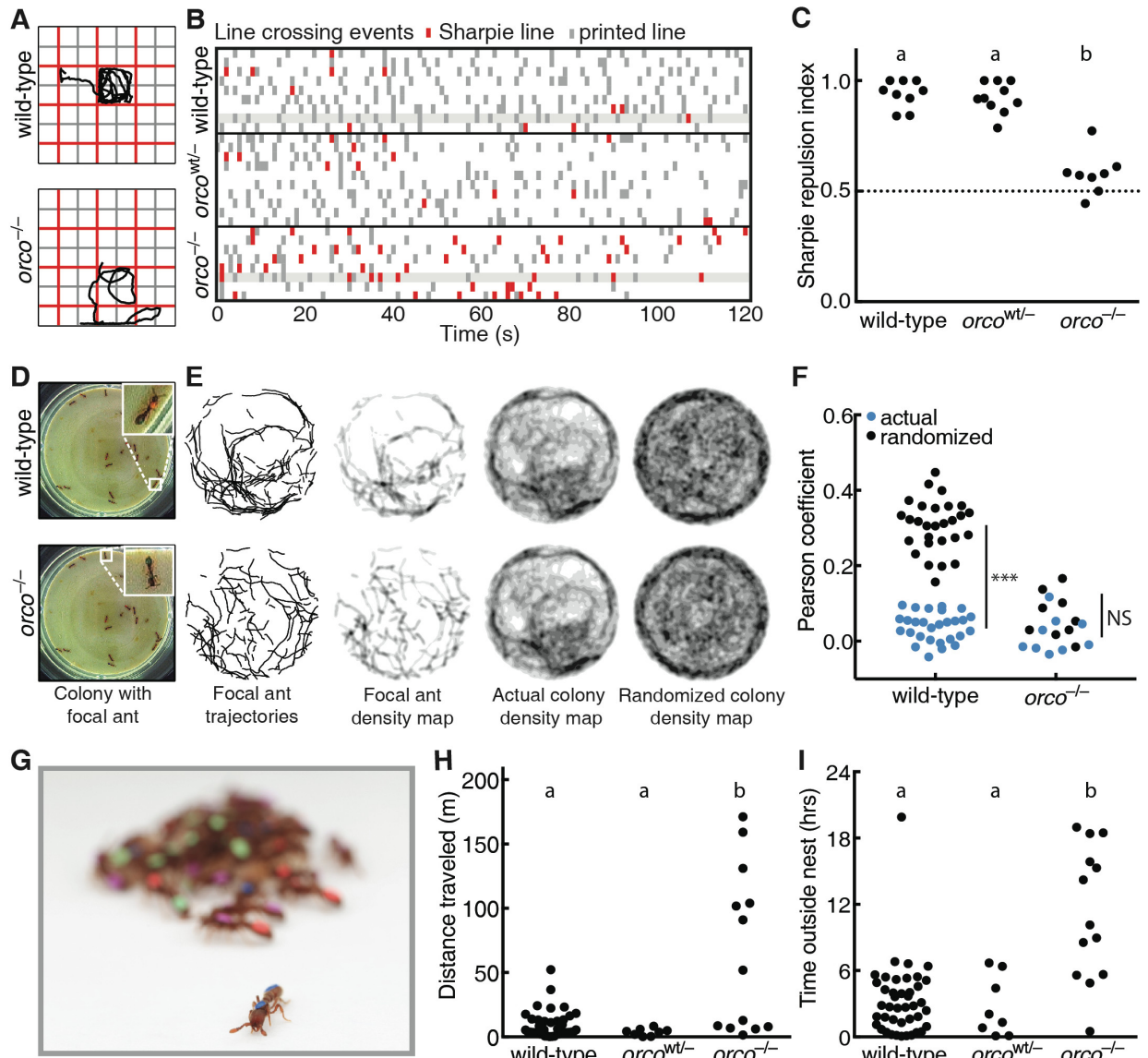
186 some individuals. Red circle indicates predicted CRISPR/Cas9 cut site. Protospacer adjacent  
187 motif (PAM) in bold. **(D)** Wild-type *orco* sequence compared to sequences for the two *orco*<sup>wt/-</sup>  
188 and the five *orco*<sup>-/-</sup> mutant lines based on G1 individuals. Deletions are shown in red and  
189 insertions in green. *orco*<sup>-/-</sup> ants have two frameshift alleles and are therefore expected to be  
190 complete loss-of-function *orco* mutants. *n* indicates the number of ants of each line used across  
191 all experiments in this study. PAM in bold.



192

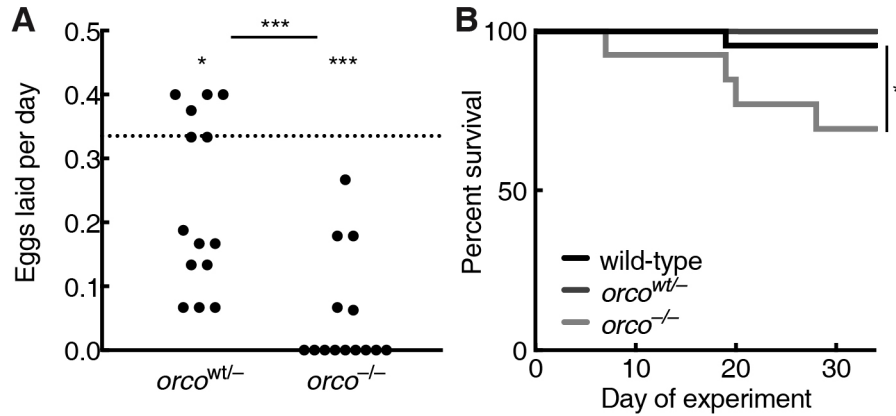
193 **Figure 2: Reduced antennal lobes in *orco*<sup>-/-</sup> ants.** *O. biroi*: (A) Dorsal (n-ventral) 3D  
194 projections of wild-type and *orco*<sup>-/-</sup> brains. Antennal lobes indicated by dashed lines. *orco*<sup>-/-</sup>  
195 antennal lobe is highly reduced relative to wild-type. Two *orco*<sup>-/-</sup> ants had 90 and 91 glomeruli  
196 relative to 493 and 509 glomeruli for two wild-type ants (one of the wild-type reconstructions  
197 has been published previously (McKenzie et al., 2016)); small differences between replicates  
198 within treatments might reflect reconstruction errors or actual biological variation. (B) Antennal  
199 lobe volumes for wild-type, *orco*<sup>wt/-</sup>, and *orco*<sup>-/-</sup> ants. *orco*<sup>-/-</sup> ants, but not *orco*<sup>wt/-</sup> ants, have  
200 significantly smaller antennal lobes than wild-type. *D. melanogaster*: (C) Anterior (n-ventral) 3D  
201 projections for wild-type and *orco*<sup>-/-</sup> brains. Antennal lobes indicated by dashed lines. *orco*<sup>-/-</sup>  
202 antennal lobe is similar to wild-type. Two *orco*<sup>-/-</sup> flies had 43 and 44 glomeruli, and two wild-  
203 type flies each had 46 glomeruli. These glomerulus numbers were higher than has been

204 previously reported, which is likely due to differences in sample preparation and imaging  
205 techniques. Slight differences in glomerulus numbers between replicates may be due to  
206 reconstruction errors, or may reflect modest antennal lobe phenotypes in *orco* mutant flies  
207 (Supplementary Text). **(D)** Antennal lobe volumes for wild-type and *orco*<sup>-/-</sup> flies. Volumes of  
208 wild-type and *orco*<sup>-/-</sup> antennal lobes are not significantly different ( $P = 0.20$ , *t*-test). Scale bars  
209 are 20  $\mu\text{m}$ . NS: not significant. Genotypic classes marked by different letters are significantly  
210 different ( $P < 0.05$ ) after ANOVA followed by Tukey's test (B).



211  
 212 **Figure 3: Deficient olfactory and social behavior in *orco*<sup>-/-</sup> ants.** (A) Example trajectories of  
 213 wild-type and *orco*<sup>-/-</sup> ants in Sharpie assay. (B) Line crossing events for wild-type, *orco*<sup>wt/+</sup>, and  
 214 *orco*<sup>-/-</sup> ants in Sharpie assays, with ants from Figure 3A highlighted. Wild-type and *orco*<sup>wt/+</sup> ants  
 215 cross Sharpie lines (red) less frequently than printed lines (grey), but *orco*<sup>-/-</sup> ants cross both lines  
 216 at approximately equal frequencies. (C) Repulsion indices for wild-type, *orco*<sup>wt/+</sup>, and *orco*<sup>-/-</sup>  
 217 ants in Sharpie assays. Repulsion index is calculated as proportion of printed line crosses. *orco*<sup>-/-</sup>  
 218 ants, but not *orco*<sup>wt/+</sup> ants, are significantly less repelled than wild-types. (D) Example colony  
 219 used for trail pheromone analysis. The same colony, containing a mixture of wild-type, *orco*<sup>wt/+</sup>,

220 and *orco*<sup>-/-</sup> ants, is shown twice, with a wild-type or *orco*<sup>-/-</sup> focal ant highlighted. **(E)** Example  
221 trail pheromone analysis. Trajectories, during which ants were moving and edges were excluded,  
222 were used to create 2-D histograms, or density maps, for each ant in the colony. These density  
223 maps were compared to the actual and randomized density maps for all other ants in the colony.  
224 The wild-type density map is more strongly correlated with the actual colony density map than  
225 with the randomized colony density map, while the *orco*<sup>-/-</sup> density map is poorly correlated with  
226 both colony density maps. **(F)** Pearson correlation coefficients for individual ant density maps  
227 with the actual or randomized density map of the other ants in the colony. Pearson correlation  
228 coefficients for wild-type ants, but not for *orco*<sup>-/-</sup> ants, were significantly higher in actual than  
229 randomized density maps (sample sizes were too small to test *orco*<sup>wt/-</sup>). **(G)** Example colony  
230 showing an individual outside of the nest. **(H)** Distances traveled in 24 hr videos by ants in  
231 experimental colonies. *orco*<sup>-/-</sup> ants, but not wild-type or *orco*<sup>wt/-</sup> ants, exhibit a wandering  
232 phenotype. **(I)** Time without contacting other ants in 24 hr videos. *orco*<sup>-/-</sup> ants spend more time  
233 without contact than wild-type or *orco*<sup>wt/-</sup> ants. \*\*\**P* < 0.001; NS: not significant. Genotypic  
234 classes marked by different letters are significantly different (*P* < 0.05) after ANOVA followed  
235 by Tukey's test (C), or from log-likelihood ratio tests on generalized linear mixed models  
236 followed by Tukey's tests with colony as a random variable and actual/randomized maps (F) or  
237 genotypic class (H,I) as fixed variables.



238

239 **Figure 4: Reduced fitness in *orco*<sup>-/-</sup> ants.** (A) Eggs laid per day over a two week period by  
240 *orco*<sup>wt/-</sup> and *orco*<sup>-/-</sup> ants relative to wild-type average (dotted line). *orco*<sup>-/-</sup> ants laid significantly  
241 fewer eggs than *orco*<sup>wt/-</sup> ants. Both *orco*<sup>wt/-</sup> and *orco*<sup>-/-</sup> ants laid significantly fewer eggs than the  
242 wild-type average of 0.34 eggs per day. Wild-type data are given as an average, rather than  
243 individual values, because most ants in each colony were wild-type and it was therefore not  
244 possible to obtain individual egg-laying rates for wild-type ants (see Methods). (B) Survival of  
245 identically-reared and age-matched wild-type ( $n = 42$ ), *orco*<sup>wt/-</sup> ( $n = 7$ ), and *orco*<sup>-/-</sup> ( $n = 13$ ) ants  
246 over a 34 day period. Survival of *orco*<sup>-/-</sup> ants was significantly lower than that of wild-type ants.  
247 Survival of *orco*<sup>wt/-</sup> ants was not statistically tested due to small sample size, but no trend toward  
248 reduced survival was observed. \* $P < 0.05$ ; \*\*\* $P < 0.001$ ; NS: not significant.  $P$  values from an  
249 unpaired two-way Wilcoxon test (comparison of *orco*<sup>wt/-</sup> and *orco*<sup>-/-</sup> egg-laying rates) and one-  
250 way Wilcoxon tests (comparisons of *orco*<sup>wt/-</sup> and *orco*<sup>-/-</sup> egg laying rates to wild-type) using the  
251 mean egg-laying rate of wild-type ants in this experiment (A) or from a Fisher exact test (B).

252



## 253 **Materials and Methods**

### 254 **Confirmation of the *O. biroi orco* gene identity**

255 Candidate *orco* orthologs for eight insect species were detected as reciprocal best hits  
256 using phmmer (Eddy, 1998) with *D. melanogaster orco* (flybase id: FBgn0037324) as the initial  
257 query sequence (Supplementary Table 2). To confirm orthology, homologs  $\pm 50\%$  the length of  
258 *orco* were aligned with MAFFT (Kato & Standley, 2013) using default parameters. This  
259 alignment was then used to construct a bootstrapped phylogeny with RAxML (Stamatakis,  
260 2006), providing unambiguous support for a single copy ortholog of *orco* in *O. biroi*  
261 (Supplementary Figure 1).

262

### 263 **Design of *orco* gRNA**

264 Identification of cut sites and assessment of off-target sites was performed using the  
265 script `cris.py`, part of the `genomepy` package (commit #94cc628), available at  
266 <https://github.com/oxpeter/genomepy>. The genomic sequence for *O. biroi orco* was searched on  
267 both strands for the CRISPR guide RNA (gRNA) recognition sequence 5' - N<sub>20</sub>NGG - 3' using  
268 BLASTN (Altschul et al., 1990) and checked for off-target hits using CRISPRseek (Zhu et al.,  
269 2014). We detected no off-target sites with 2 or less mismatches and only one site with 3  
270 mismatches for our *orco* gRNA, which is expected to lead to low or no off-target cutting (Fu et  
271 al., 2013) (see Supplementary Text).

272

### 273 **CRISPR reagent preparation**

274 Recombinant Cas9 protein was purchased from PNAbio, and gRNA was synthesized as  
275 described previously (Kistler et al., 2015). Activity of Cas9 and gRNA was validated using an in-  
276 vitro digestion assay from New England Biolabs. Immediately prior to injection, Cas9 and  
277 gRNA were mixed in water to produce a solution with 100 ng/ $\mu$ L Cas9 and 10 ng/ $\mu$ L gRNA.

278

### 279 **Colony maintenance and egg collection**

280 *O. biroi* colonies were maintained in circular Petri dishes (50 mm diameter, 9 mm height)  
281 with a plaster of Paris floor ca. 4 mm thick. Colonies were fed 3 times weekly with fire ant  
282 (*Solenopsis invicta*) brood and cleaned and watered at least once per week. Eggs for injections  
283 were collected from egg-laying units consisting of 70 *O. biroi* individuals without larvae or  
284 pupae. Eggs were collected and placed on double-sided tape with the ventral side up on a glass  
285 slide, and injected into the anterior end (Oxley et al., 2014). Slides were prepared with up to 80  
286 eggs for injection and ~25 control eggs to validate incubation conditions. For additional  
287 information about egg collection see Supplementary Text.

288

### 289 **Egg injection, incubation, and rearing**

290 Injection needles were prepared as in previous studies (Lobo et al., 2006). Injections were  
291 performed using an Eppendorf Femtojet with a Narishige micromanipulator. The Femtojet was  
292 typically set to Pi 1800 hPa and Pc 500 hPa. Needles were broken by gently touching the needle  
293 against a capillary submerged in halocarbon oil. Alternatively, sharper needles were generated by  
294 setting the Femtojet to maximum pressure (6000 hPa) and lightly touching the capillary against  
295 fibers on the tape. Data in this manuscript result from a combination of both methods.

296

297 Eggs were submerged in a drop of water immediately prior to injection, and dried after  
298 injection. Eggs were pierced with the needle and injected for 1-2 seconds. Eggs less than 5 hrs

299 old were injected, corresponding to a time when eggs are in a syncytial stage of development  
300 with <100 nuclei (Oxley et al., 2014). We injected 100-300 eggs per day. After injection, eggs  
301 were incubated in humid plastic boxes prepared with a plaster of Paris floor moistened with  
302 distilled water.

303

304 Two *O. biroi* clonal lines, which are genetically distinguishable at the mitochondrial  
305 *cytochrome oxidase subunit 1* (CO1) gene (Kronauer et al., 2012), were used in this study. All  
306 experimental ants belonged to Line B, while Line A ants were only used as chaperones to raise  
307 experimental Line B individuals. Experimental ants were reared by placing Line B larvae (G0s)  
308 or eggs (G1s and subsequent generations) in colonies of 20 Line A chaperones, and chaperones  
309 were removed once the callows had eclosed. This rearing method results in a small fraction of  
310 Line A offspring of chaperones in colonies with the G0s and subsequent generations. For this  
311 reason, all individuals were genotyped following experiments, and Line A individuals were  
312 removed from all analyses. All experimental colonies in this study had eggs removed twice  
313 weekly so that adults were maintained without larvae or pupae. All individuals, including those  
314 that died during experiments, were genotyped (see below) to determine their clonal line, and  
315 *orco* amplicons from Line B individuals were sequenced to determine *orco* genotype. For  
316 additional information about egg injection, incubation, and rearing see Supplementary Text.

317

### 318 **Tagging**

319 All ants in behavioral and fitness experiments (Figures 3,4) were tagged with two color  
320 dots, one on the thorax and one on the gaster, using uni-Paint markers (models PX-20 or PX-21)  
321 such that each individual could be identified within the colony (Figure 3D,G). For automated  
322 behavioral tracking, four colors were used (blue, green, orange and pink) for a total of 16 unique  
323 combinations. Ants were tagged with a randomly assigned color pair at least 10 days prior to any  
324 behavioral experiments. Tagged ants had a leg removed for genotyping and sequencing either  
325 before (Figure 2A,B; Figure 3A-C) or after (Figure 3D-I; Figure 4) experiments.

326

### 327 **Genotyping**

328 To distinguish Line A and Line B, eggs and adults were genotyped using PCR of  
329 mitochondrial *COI* with standard DNA barcode primers (Folmer et al., 1994) followed by a  
330 restriction digest with MwoI from New England Biolabs. This enzyme cuts the PCR product  
331 derived from Line B, but not from Line A.

332

### 333 **Sequencing**

334 To screen for *orco* mutations, we designed PCR primers that flanked the *orco* cut site and  
335 sequenced the resulting PCR products using Sanger and Illumina sequencing. Primer sequences  
336 were: F:

337 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCAACTTGCTGTAAATTTGGAT

338 R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCTTCTTGGTCGGCGGTA.

339

340 Illumina methods followed a previously described protocol (Kistler et al., 2015). Primers  
341 included tails at the 5' end (underlined) that were used as adapters to add indices to individual  
342 samples for Illumina sequencing (Kistler et al., 2015). Sequences were aligned to the *orco*  
343 genomic sequence and reads at each base pair that aligned with an insertion or deletion were  
344 counted with the script crispralign.py from the genomepy package, available at

345 <https://github.com/oxpeter/genomepy>. *orco* amplicons from 25 of 42 recovered G0s were  
346 subjected to Illumina sequencing (Figure 1C). Three of these individuals, all of which were  
347 found to have nearly 100% mutation rates, displayed a wandering phenotype similar to the  
348 wandering phenotype observed in G1s, indicating that somatic CRISPR in G0s may be useful for  
349 functional genetic studies even in the many social insect species where it is not logistically  
350 possible to generate or maintain stable mutant lines (Schulte et al., 2014).

351

### 352 **Identification of mutant sequences**

353 Mutant lines were identified via Sanger sequencing of eggs and adults of G1s and  
354 subsequent generations. All Sanger sequencing traces were scored manually. For the G1 dataset  
355 (below), manual identifications were verified by checking for misalignment against a reference  
356 sequence using MEGA (Kumar et al., 2016) and by using the program Mutation Surveyor  
357 (Softgenetics) for automated allele identification. Mutant lines were defined as groups of ants  
358 that possess identical *orco* genotypes, and *orco* amplicons from representatives of each mutant  
359 line were Illumina sequenced and individual reads were manually inspected to ensure both  
360 alleles were properly identified.

361

### 362 **Glomerulus counts and antennal lobe volumes**

363 One of the wild-type *O. biroi* antennal lobe reconstructions was based on published data  
364 (McKenzie et al., 2016). For the remaining data, *D. melanogaster* and *O. biroi* brains were  
365 dissected in PBS and immediately transferred to a fixative solution of either 1% glutaraldehyde  
366 or 2% PFA and 2.5% glutaraldehyde in PBS, and fixed at room temperature on a shaker for 1-30  
367 days. To dehydrate, brains were rinsed in PBS and then suspended for 5 minutes each in an  
368 ascending series of 50%, 70%, 90%, 95%, 100%, 100%, and 100% ethanol. Brains were cleared  
369 and mounted in methyl salicylate. Glutaraldehyde-enhanced autofluorescence was imaged using  
370 a confocal laser scanning microscope (Zeiss LSM 8800) with excitation by a 488 nm laser.  
371 Three-dimensional projections were created from confocal image stacks using Fluorender (Wan  
372 et al., 2012). Three-dimensional reconstructions of glomeruli and antennal lobes were produced  
373 by manually segmenting confocal image stacks using the Segmentation Editor plugin in the Fiji  
374 distribution of ImageJ (Schindelin et al., 2012). Antennal lobe volumes were calculated using the  
375 Object Counter3D ImageJ plugin (Bolte & Cordelieres, 2006), blindly with respect to genotype.

376

### 377 **Sharpie assay**

378 Sharpie assays were conducted with tagged ants (Figure 3A-C) on printer paper in a  
379 5.25x5.25 in arena bounded by a clear acrylic barrier. Six horizontal and 6 vertical black lines  
380 were printed on the paper using an HP LaserJet printer (Figure 3A,B). Immediately before each  
381 assay, 3 alternating horizontal and vertical black lines were traced with red Fine Point Sharpie  
382 Permanent Marker (item 30002). Then the ant was placed in the center of the grid. A 2 min video  
383 was recorded and the number of times the ant crossed black and Sharpie lines was manually  
384 counted (Supplementary Video 2). A Sharpie repulsion index was calculated as the ratio of black  
385 line crosses to total line crosses. Once the experiment had concluded, we determined that low  
386 numbers of line crosses caused the repulsion indices to be unreliable, and we therefore excluded  
387 four assays that had less than 10 line crosses total. As a positive control, a wild-type worker was  
388 assayed after each assay with a low repulsion index to ensure the Sharpie lines retained a  
389 repulsive effect. All positive controls had high repulsion indices and as a population were  
390 statistically indistinguishable from the other wild-type workers assayed ( $P = 0.42$ ,  $t$ -test).

391

## 392 **G1 preparation for behavior and fitness experiments**

393 G1 rearing resulted in a set of 34 colonies containing a mixture of G1 ants and Line A  
394 progeny of chaperones. These colonies were used to identify mutants for egg-laying, automated  
395 behavioral tracking, and survival experiments (Figure 3D-I, Figure 4, Supplementary Table 3).  
396 Once each colony started producing eggs, we collected all eggs 5 times over a 14-16 day period.  
397 *COI* amplicons from all eggs were genotyped to identify Line A and Line B eggs, and *orco*  
398 amplicons from Line B eggs were Sanger sequenced to identify *orco* mutants. We sequenced  
399 2,184 eggs from the 533 Line B ants in these colonies, corresponding to ca. 4 eggs per ant.  
400 During the period of egg collection and one week after egg collection had concluded, we  
401 subjectively determined whether any individuals in any given colony displayed a wandering  
402 phenotype. Colonies in which *orco* mutant eggs were detected or in which wandering phenotypes  
403 were observed were selected for the egg-laying dataset.

404

## 405 **Egg-laying dataset**

406 We included 16 colonies in the egg-laying dataset. A subset of ants in these colonies were  
407 later also used for behavioral and survival experiments (Supplementary Table 3, see below).  
408 After the experiments had been concluded, all ants had a leg removed, from which *orco*  
409 amplicons were sequenced. For each *orco*<sup>wt/-</sup> and *orco*<sup>-/-</sup> adult we identified, we counted the  
410 number of eggs of its genotype in its colony. If several adults of the same genotype were  
411 identified in a colony, for each individual we calculated the number of eggs of that genotype  
412 divided by the number of adults of that genotype. We used a two-tailed Wilcoxon test to test  
413 whether *orco*<sup>wt/-</sup> or *orco*<sup>-/-</sup> G1s produced different numbers of eggs than the average of wild-type  
414 G1s in this experiment (Figure 4A).

415

## 416 **Behavioral and survival dataset**

417 Before removing legs from ants for genotyping, workers from colonies that produced a  
418 high frequency of *orco* mutant eggs or contained individuals with wandering phenotypes were  
419 pooled to create 5 experimental colonies with a mixture of 12-14 G1 wild-type and *orco* mutant  
420 ants. Before pooling, all workers in these colonies were individually tagged with two color dots.  
421 These 5 colonies were recorded in 24 hr videos.

422

423 Experimental *O. biroi* colonies initially contained a total of 68 G1 ants, with 42 wild-  
424 type, 8 *orco*<sup>wt/-</sup>, and 14 *orco*<sup>-/-</sup> individuals (Figure 1D). These colonies also contained 4 *orco*  
425 mutant individuals with in-frame mutations, which were not included in the current analyses  
426 because sample sizes were too small. G1s in experimental colonies varied in *orco* genotype but  
427 were otherwise identical in rearing methods, genetic background, and did not differ  
428 systematically in age. Before the start of each 24 hr video, colonies were cleaned and the plaster  
429 was moistened. For four weeks after the video was recorded, we also recorded survival of all ants  
430 in the 5 experimental colonies.

431

## 432 **Video recording and automated behavioral tracking**

433 Automated behavioral tracking was performed in custom-made tracking setups under  
434 constant illumination. Temperature was maintained at 25°C. Videos were acquired using C910  
435 Logitech USB webcams controlled with custom MATLAB (version R2016a, The MathWorks,  
436 Inc.) software at 10 fps at 960x720 pixel resolution (13 pixels/mm).

437

438 Tracking was performed blind with respect to genotype. Videos were processed and  
439 analyzed using custom MATLAB software. In each frame, ants were segmented from the  
440 background of the dish using a fixed threshold. Resulting components, or blobs, were linked into  
441 trajectories using the optical flow computed between consecutive frames (Horn–Schunck method  
442 (Barron et al., 1994)). Trajectories ended and new ones were initiated whenever blobs split or  
443 merged between two consecutive frames. Trajectories stored the following data, collected from  
444 the respective blob in each frame: centroid (position of center of mass), orientation (angle  
445 between the major axis of the best-fitting ellipse and the horizontal axis) and area (in mm<sup>2</sup>).

446

447 We used a threshold size to select trajectories that corresponded to a single ant and lasted  
448 longer than two seconds. Each trajectory was then assigned a combination of color tags using a  
449 custom classification algorithm. For each experiment, at least 500 frames per ant were manually  
450 identified to create a training set, 70% of which was used to train, and 30% of which was used to  
451 validate an automated identity classifier.

452

453 For each trajectory, a naive Bayes color classifier (Fletcher et al., 2011) was used to  
454 compute the pixel color probabilities for each pixel in the blob of each frame for six color  
455 classes: the four tag colors, the ant cuticle color and the color of the plaster of Paris  
456 (Supplementary Figure 2). Predicted probabilities for all four tag colors were used to determine  
457 whether both tags were visible and, if so, the orientation of the ant in the frame was deduced  
458 from the relative position of the tags with respect with the cuticle color. If both procedures were  
459 successful, the pixel color probabilities were fed into another naive Bayes classifier to assign an  
460 identity to the ant.

461

462 For each trajectory, frames were tested in a random order until 20 frames were  
463 successfully identified or no more frames were available. If at least one frame was identified, the  
464 trajectory, and thus all its underlying positions, was assigned the most frequently predicted  
465 identity. The identity classifier had an empirical error rate of 20% on single frames. However,  
466 the error rate decreased with the number of frames tested within a trajectory. Overall, we  
467 estimate that less than 10% of the total identified positions were misclassified, a performance  
468 equivalent to that reported previously for a functionally similar tracking setup for ants (Mersch et  
469 al., 2013).

470

## 471 **Behavioral analysis**

472 Cleaning and watering the nests at the beginning of each experiment caused the ants to  
473 actively move around the dish. We used this initial period of high activity to measure trail  
474 following behavior, reasoning that trail pheromones may cause two or more ants to move along  
475 the same path within the dish. For each ant, we measured the correlation between its own  
476 movement and the movement of the remaining ants in the colony during the first hour of the  
477 tracking experiment. As *O. biroi* have a tendency to walk along the edges of the dish, we  
478 discarded segments of trajectories close to the edge of the dish and included only segments  
479 where the ants moved faster than 1 mm/s continuously for at least 1 second and without contact  
480 with any other ant. We then computed a 2-D histogram, or density map, for each ant by counting  
481 the number of times one of the remaining positions in the 970x720 pixel original image fell into  
482 each bin of a 120x90 bin grid. We then computed the Pearson correlation coefficient between the

483 density map of each ant and a density map constructed from the trajectories of all ants in the  
484 colony but the focal ant (Figure 3E). For each ant, the actual correlation coefficient provided an  
485 estimate of the correlation of movement of that ant with the actual movement of other ants in the  
486 colony, which presumably results from following pheromone trails.

487  
488 As a baseline comparison, the density map of each ant was correlated with a randomized  
489 density map constructed by rotating the trajectories of all ants but the focal ant in the colony by a  
490 random angle around the center of the dish (Figure 3E). This randomized correlation coefficient  
491 provided an estimate of the correlation of the movement of that ant with the randomized  
492 movement of other ants in the colony. This residual correlation reflects the portion of the  
493 correlation that is due to non-local effects such as turning frequencies, linear and angular  
494 velocity dynamics, and radial preference for certain regions of the petri dish. After examining the  
495 data, two experimental colonies were excluded from the trail following analysis because they did  
496 not form clear trails during the videos, resulting in Pearson correlation coefficients of  
497 approximately zero for all ants in the colony.

498  
499 To measure wandering phenotypes, in each experiment we calculated the total distance  
500 traveled by each ant over the 24 hr video by computing the distances between all pairs of  
501 successive positions in meters in all identified trajectories. Time without contact was calculated  
502 as the total time each ant was identified in each experiment (since ants were only identifiable  
503 when they were separated from other ants). This provides a minimum estimate of the time  
504 without contact for each ant, given that it was also possible for ants to be spatially separated from  
505 other ants, yet unidentifiable, for example if their posture did not allow the detection of both  
506 tags.

## 507 508 **Statistics**

509 Behavioral tracking and antennal lobe volume measurements were performed blindly  
510 with respect to genotype. Other analyses were not performed blindly with respect to genotype.  
511 Mixed model statistics were performed in R v 3.3.1 using the *lmer* function in the lme4 library as  
512 described previously (Ulrich & Schmid-Hempel, 2012; Ulrich & Schmid-Hempel, 2015). All  
513 other statistics were performed using GraphPad Prism 7. Normality was determined by  
514 D'Agostino-Pearson normality tests. Datasets used for ANOVA analyses had equal variance  
515 across treatments. Single groups were compared against a predicted mean using two-tailed  
516 Wilcoxon tests. Proportional data were compared between treatments using a Fisher exact test.  
517 Unpaired two-tailed Student's *t*-tests or paired Wilcoxon tests were used to compare two groups,  
518 when appropriate, and two-way ANOVAs followed by Tukey's multiple comparisons test were  
519 used to compare more than two groups.

## 520 521 **Data and code availability**

522 Raw data are available in Supplementary Table 3. gRNA design and sequencing analyses  
523 were performed using the script `crispralign.py` from the genomepy package, available at  
524 <https://github.com/oxpeter/genomepy>.

525

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- 684

## 685 **Supplementary Text**

### 686 ***D. melanogaster* glomerulus counts**

687 Our reconstructions of *D. melanogaster* antennal lobe glomeruli yielded different  
688 glomerulus numbers than what has been published previously (Supplementary Table 1). Our  
689 reconstructions also showed small differences in glomerulus numbers between wild-type and  
690 *orco*<sup>-/-</sup> flies (Figure 2). To address this possibility, we imaged and reconstructed two additional  
691 *D. melanogaster* antennal lobes, one from an *orco*<sup>-/-</sup> and one from a wild-type individual.  
692 These reconstructions were not performed strictly *de novo*, as the *O. biroi* and *D.*  
693 *melanogaster* reconstructions reported in the main text, but by referring to the published map of  
694 the *D. melanogaster* antennal lobe (Laissue et al., 1999; Laissue & Vosshall, 2008). Due to  
695 differences in sample preparation and imaging methods, it was not possible to unambiguously  
696 match each glomerulus to the published map. However, we identified structures in our newly  
697 reconstructed wild-type and *orco*<sup>-/-</sup> antennal lobes that corresponded to all published wild-type  
698 glomeruli. These results suggest that *orco*<sup>-/-</sup> flies have no systematic reduction in the number of  
699 antennal lobe glomeruli compared to wild-types, although it is possible that some neighboring  
700 glomeruli in *orco*<sup>-/-</sup> flies have divisions that appear less distinct or may even be fused relative to  
701 wild-types (Figure 2).  
702

### 703 **Off-target effects**

704 It has been shown that in some cases CRISPR/Cas9 injections can lead to off-target  
705 mutagenesis that in turn can give rise to non-specific phenotypes. However, we consider this to  
706 be highly unlikely in the current study due to the following reasons. First, we used a high-quality  
707 reference genome to design the gRNA in this study to have no additional target sites in the  
708 genome that are likely to lead to off-target cutting (Fu et al., 2013; Oxley et al., 2014) (see  
709 Methods). Second, mutations induced by Cas9 are stochastically generated (Fu et al., 2013), such  
710 that any off-target effects would likely be present in some G1 lines but not others. The  
711 phenotypes we report are consistent across five independently generated *orco*<sup>-/-</sup> lines, and we do  
712 not observe the same phenotypes across two independently generated *orco*<sup>wt/-</sup> lines  
713 (Supplementary Table 3). Third, the striking reduction of *orco*<sup>-/-</sup> antennal lobes relative to  
714 *orco*<sup>wt/-</sup> and wild-type antennal lobes is a phenotype specific to the ant chemosensory system that  
715 is unlikely to arise from random off-target effects. This phenotype provides a direct functional  
716 link between the *orco*<sup>-/-</sup> genotype and the chemosensory deficiencies described in this study.  
717 Importantly, the antennal lobe phenotype was entirely discrete: every *orco*<sup>-/-</sup> brain had  
718 substantially smaller antennal lobes than any *orco*<sup>wt/-</sup> or wild-type brain, even though this  
719 phenotype was measured across multiple independently derived *orco*<sup>-/-</sup> and *orco*<sup>wt/-</sup> lines (Figure  
720 2b, Supplementary Table 3). Therefore, while we cannot exclude the possibility that our  
721 injections gave rise to some level of off-target mutations, it is unlikely that the specific  
722 phenotypes reported in this study arise from off-target effects.  
723

### 724 **Additional egg collection, injection, and rearing methods**

725 We observed that the presence of eggs in *O. biroi* colonies inhibits the production of new  
726 eggs and employed this observation for efficient egg collection. Egg-laying units were left with  
727 eggs for 7 days to inhibit worker egg-laying. On day 0 eggs were removed to release inhibition,  
728 and on day 2 eggs were removed again to further prevent inhibition. This led workers to  
729 synchronously activate their ovaries, and on days 5, 6, and 7 eggs were collected for injection.  
730 Following day 7, eggs were not collected from these colonies for 7 days, causing workers to

731 become inhibited, and the protocol was then repeated.

732

733 Eggs were collected from colonies under a stereoscope using insect pinning needles. On a  
734 typical injection day, eggs were removed from colonies from 10-11 am, and those eggs were  
735 used as uninjected incubation controls or fostered into rearing units. Eggs were collected for  
736 injections from 2-3 pm and 6-7 pm and injected from 3-4 pm and 7-8 pm, respectively.

737 Therefore, all injected eggs were less than 5 hrs old, when *O. biroi* eggs are in a syncytial stage  
738 of development with <100 nuclei (Oxley et al., 2014). Typical egg-laying units produced 2-5  
739 eggs per day, and we collected from up to 60 egg-laying units, injecting 100-300 eggs per day.

740

741 The anterior end of *O. biroi* eggs is slightly wider than the posterior end, and the ventral  
742 surface is concave while the dorsal surface is convex. To inject, eggs were placed on double-  
743 sided 3M tape (Model S-10079 from Uline) on a glass slide, with the anterior end forward and  
744 the ventral side upward. Eggs were injected into the anterior end, where nuclei are located in  
745 early *O. biroi* embryos (Oxley et al., 2014). The ventral side was placed upward, so that larvae  
746 hatched with the mouth facing away from the tape, which facilitated successfully recovering  
747 larvae from the tape. To inject, eggs were individually submerged in 1-2  $\mu$ L drops of water. Eggs  
748 were gently pierced with the needle and injected for 1-2 seconds. During successful injections,  
749 little or no cytoplasm is discharged from the egg when the needle is removed. Preliminary trials  
750 showed that injection under liquid was necessary to remove the needle without rupturing the  
751 chorion, and that water led to higher survival than Ringer, PBS, or halocarbon oil. A video was  
752 recorded of every injection session, allowing us to verify that hatching larvae had been  
753 successfully injected.

754

755 Preliminary injections were conducted using multiple batches of reagents and variable  
756 Cas9 and gRNA concentrations. These trials suggested that hatch rates varied inversely with  
757 cutrates. Batch effects in hatch rates were observed across different days of injection within the  
758 same experimental treatment, requiring multiple injection days and large numbers of eggs (~400)  
759 to accurately estimate hatch rates of any given experimental treatment. In the final injection  
760 round, eggs were injected with either low (1800 hPa) or high (6000 hPa) pressure (see Methods),  
761 with sharper needles and lower injection volumes used in injections with high pressure. 46 of  
762 2535 eggs (1.8%) hatched after injections with low pressure, and 58 of 756 eggs (7.6%) hatched  
763 after injections with high pressure. 25 of the 42 G0s were Illumina sequenced, and we observed  
764 an average of at least 27% cutrates at the predicted cut site resulted from low pressure injections  
765 ( $n = 17$ ) relative to 22% from high pressure injections ( $n = 8$ ). *orco*<sup>w<sup>l</sup>-</sup> and *orco*<sup>-/-</sup> G1s were  
766 recovered from G0s injected with each method.

767

768 Following injections, slides with eggs were incubated in air-tight plastic boxes (0.9L  
769 SpaceCube boxes from ClickClack). Incubation boxes were prepared with a plaster of Paris floor  
770 (85 g plaster of Paris mixed with 50 mL distilled water). The plaster was dried completely after  
771 casting, and water was then added until the plaster became saturated with moisture to determine  
772 the saturation volume. The plaster was then dried completely once again, after which 20% of the  
773 saturation volume of distilled water was added. This procedure produced suitable incubation  
774 conditions for 2 weeks, after which the plaster was discarded. Incubating eggs were checked  
775 daily, and any water that had condensed on the eggs was removed with Kimwipes<sup>TM</sup> tissue.  
776 Fungus frequently grew on injection slides. Growth was controlled by spacing the eggs ~2 mm

777 apart and mechanically breaking up fungal hyphae and overgrown eggs in 100% ethanol using  
778 insect pinning needles. This egg-incubation protocol yielded ~60% hatch rates of uninjected  
779 control eggs, which is similar to hatch rates of eggs in laboratory colonies.

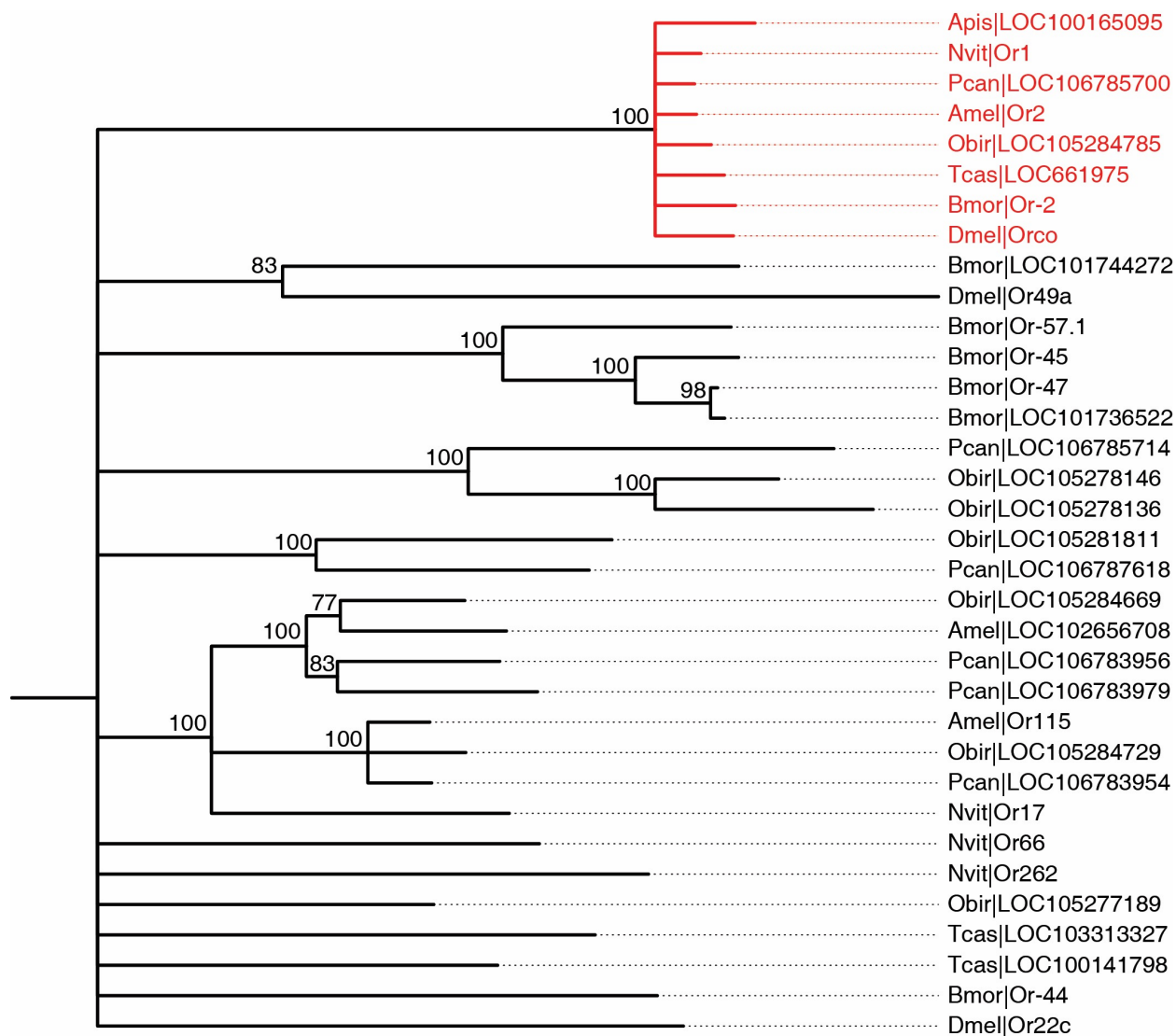
780

781 To synchronize hatching of larvae from injected eggs, eggs injected on days 5, 6, and 7  
782 were incubated at different temperatures. Preliminary trials showed that eggs incubated at 25 °C  
783 hatch after 9-10 days, while eggs incubated at 30 °C hatch after 7-8 days. We therefore incubated  
784 eggs injected on days 5 and 7 at 25 °C and 30 °C, respectively, while eggs injected on day 6  
785 were incubated at 25 °C for the first 5 days and then at 30 °C until hatching. This protocol  
786 resulted in most larvae hatching on days 14 and 15. Once hatching had commenced, larvae were  
787 manually removed from the egg membrane with an insect pinning needle, taking care to prevent  
788 them from becoming stuck to the double-sided tape. Eggs that were expected to hatch overnight  
789 were wrapped with a sheet of Parafilm® (stretched to be as thin as possible) to prevent larvae  
790 from falling onto the tape.

791

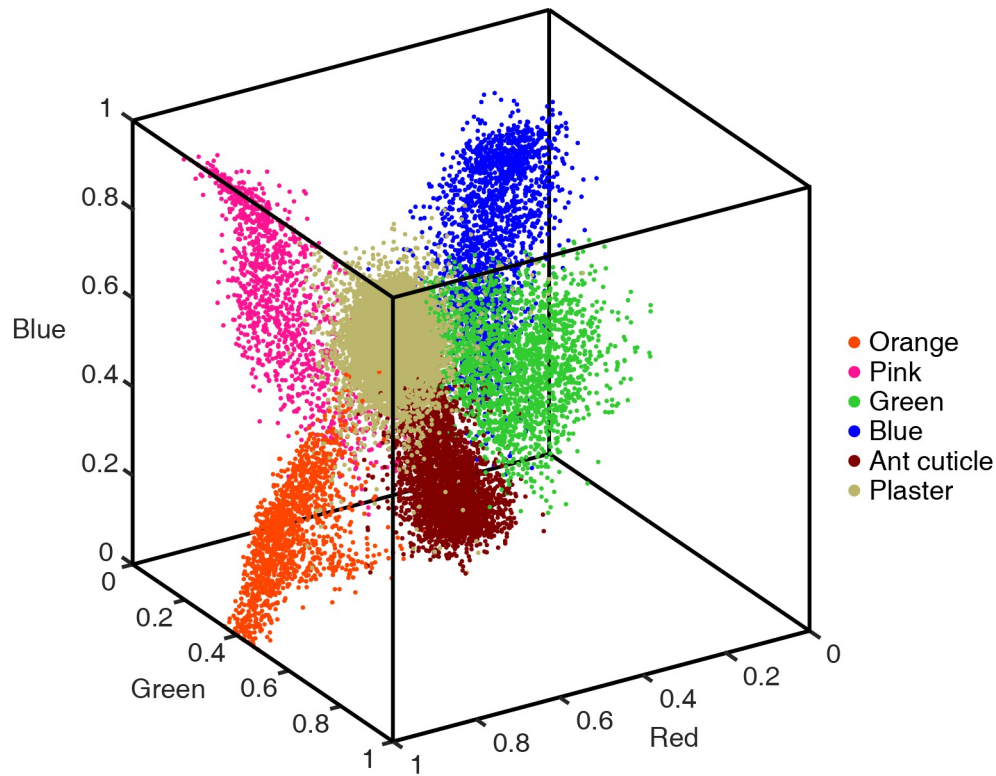
792 To rear G0 larvae, uninjected control eggs slightly older than the eggs injected on day 5  
793 were placed with ~20 adult Line A workers in a Petri dish with a plaster of Paris floor and  
794 maintained at 25 °C. These eggs hatched slightly earlier than the injected eggs, priming the  
795 workers to rear larvae derived from injected eggs. When the larvae hatched from injected eggs,  
796 control larvae were replaced with experimental (injected) larvae. Preliminary trials showed that  
797 higher survival was obtained by fostering a minimum of 7 larvae at a time, so control larvae were  
798 added to experimental larvae if insufficient experimental larvae were available. The G0 adults  
799 reported in this study therefore include an unknown fraction of adults derived from control  
800 larvae. Survival of larvae under these conditions was approximately 50%.

801



802  
803 **Supplementary Figure 1: Phylogeny of *orco* and other odorant receptor genes in insects.**  
804 *orco* orthologs in red. *O. biroi orco* is included in a clade with the *orco* genes from all other  
805 studied insect species (100% bootstrap support). All nodes with less than 75% bootstrap support  
806 have been collapsed for clarity. All other bootstrap values are shown at the respective node.  
807 Species are indicated by a four letter code: Apis – *Acyrtosiphon pisum*; Nvit – *Nasonia*  
808 *vitripennis*; Pcan – *Papilio canadensis*; Amel – *Apis mellifera*; Obir – *Ooceraea biroi*; Tcas –  
809 *Tribolium castaneum*; Bmor – *Bombyx mori*; Dmel – *Drosophila melanogaster*. NCBI gene  
810 symbols are shown on the right. The scale bar indicates an average of 0.3 substitutions per site.

811



812

813 **Supplementary Figure 2: Color definition for automated behavioral tracking in RGB**  
814 **space.** Measurements were collected manually from the four different tag colors (orange, pink,  
815 green and blue), the plaster (background) and the ant cuticle. These colors are clearly  
816 distinguishable in RGB space, allowing automated behavioral tracking based on identifying the  
817 color tags of each ant.

818 **Supplementary Table 1: OR and glomerulus numbers for ants and other insects.**

819 For social insects, glomerulus counts refer to workers. OR numbers refer to putatively functional  
820 genes.

Common name	Scientific name	OR number	Antennal lobe glomerulus number
Dampwood termite	<i>Zootermopsis nevadensis</i>	63 (Terrapon et al., 2014)	70-74 (Terrapon et al., 2014)
Pea aphid	<i>Acyrtosiphon pisum</i>	62 (Smadja et al., 2009)	25-40 (Kollmann et al., 2011)
Vinegar fly	<i>Drosophila melanogaster</i>	60 (Robertson et al., 2003)	42 (Laissue et al., 1999; Laissue & Vosshall, 2008)
Red flour beetle	<i>Tribolium castaneum</i>	259 (Engsontia et al., 2008)	~70 (Dreyer et al., 2010)
Jewel wasp	<i>Nasonia vitripennis</i>	225 (Robertson et al., 2010)	Not published
Honey bee	<i>Apis mellifera</i>	163 (Robertson et al., 2010)	156-166 (Flanagan & Mercer, 1989)
Jerdon's jumping ant	<i>Harpegnathos saltator</i>	347 (Zhou et al., 2012)	178* (Hoyer et al., 2005)
Clonal raider ant	<i>Ooceraea biroi</i>	369 (Oxley et al., 2014)	493-509 (McKenzie et al., 2016)
Florida carpenter ant	<i>Camponotus floridanus</i>	352 (Zhou et al., 2012)	434-464 (Zube et al., 2008)
Big headed leafcutter ant	<i>Atta cephalotes</i>	376 (Engsontia et al., 2015)	349 (Kelber et al., 2009)

821 \*Note that this estimate was generated using sections rather than whole mount brains and is  
822 therefore likely an under-estimate of the true number of glomeruli.



823 **Supplementary Table 2: HMMer alignment scores for best matches in eight insect genomes**  
 824 **to the *D. melanogaster* (*orco*) and *O. biroi* (*LOC105284785*) *orco* orthologs.**

825 The best three matches for *O. biroi* and *D. melanogaster* and the best match for each remaining  
 826 species are shown. aa: amino acids. Full names of species are: *Ooceraea biroi*, *Drosophila*  
 827 *melanogaster*, *Nasonia vitripennis*, *Papilio canadensis*, *Apis mellifera*, *Tribolium castaneum*,  
 828 *Acyrtosiphon pisum*, and *Bombyx mori*.

Gene	Species	Length (aa)	E-value <i>cf</i> <i>orco</i>	HMMer score <i>cf</i> <i>orco</i>	E-value <i>cf</i> <i>LOC1052</i> <i>84785</i>	HMMer score <i>cf</i> <i>LOC105284</i> <i>785</i>
<i>LOC105284785</i>	<i>O. biroi</i>	478	3.10e-188	625.9	0.00e-00	1094.6
<i>LOC105278136</i>	<i>O. biroi</i>	673	2.60e-22	78.9	1.10e-25	89.9
<i>LOC105284729</i>	<i>O. biroi</i>	818	7.00e-20	70.9	2.80e-20	72.1
<i>orco</i>	<i>D. melanogaster</i>	486	0.00e-00	1099.4	1.00e-187	624.4
<i>Or22c</i>	<i>D. melanogaster</i>	402	7.70e-14	51.2	1.80e-14	53.2
<i>Or49a</i>	<i>D. melanogaster</i>	396	1.60e-12	46.9	6.70e-14	51.3
<i>Or1</i>	<i>N. vitripennis</i>	475	2.70e-192	639.4	2.10e-256	850.7
<i>LOC106785700</i>	<i>P. canadensis</i>	494	2.60e-190	632.5	2.20e-260	863.4
<i>Or2</i>	<i>A. mellifera</i>	478	3.00e-197	655.4	1.40e-261	867.5
<i>LOC661975</i>	<i>T. castaneum</i>	475	3.00e-206	685.3	9.00e-202	670.5
<i>LOC100165095</i>	<i>A. pisum</i>	486	1.30e-159	532.0	8.20e-159	529.4
<i>Or-2</i>	<i>B. mori</i>	476	1.80e-201	669.7	1.00e-189	630.9

830 **Supplementary Table 3: Genotypes and raw data for all ants included in the present study.**  
831 Supplementary Table 3 is available in the Supplementary Materials. For an explanation of which  
832 ants were used in which experiments, see Methods. #-XX (e.g., 3-GO) refers to G1 ants in the  
833 datasets from the 5 experimental colonies. # gives the colony number, and XX gives the color  
834 tags of the individual ants. These colonies were used for all automated behavioral tracking and  
835 survival data (Figures 3D-H, and 4B). Some of these ants were also used for glomerulus counts,  
836 antennal lobe volume, and egg-laying data (Figures 2A, 2B, 4A). E-# (e.g., E-1) refers to  
837 additional G1 ants used for antennal lobe volume and egg-laying data (Figures 2B and 4A). A-#  
838 (e.g., A-1) refers to wild-type ants used for glomerulus counts and antennal lobe volume (Figure  
839 2A and 2B). S-# (e.g. S-1) refers to G2 ants used for Sharpie assays (Figure 3A-C).

840 **Supplementary Video 1: Antennal lobe reconstructions of wild-type and *orco*<sup>-/-</sup> adults in *O.***  
841 ***biroi* and *D. melanogaster*.**

842 Antennal lobes are reduced in *orco*<sup>-/-</sup> relative to wild-type in ants, but not in flies. T7 glomeruli  
843 in *O. biroi* are labeled in white. Size standards are 20 μm.

844

845 **Supplementary Video 2: Sharpie assay.**

846 Videos of exemplar wild-type and *orco*<sup>-/-</sup> ants in Sharpie assays (using the same individuals as in  
847 Figure 3A,B). The wild-type ant is strongly repelled by Sharpie lines (red) but the *orco*<sup>-/-</sup> ant is  
848 not. Videos are of 2 minute assays playing back at 4x speed.

849

850 **Supplementary Video 3: Wandering behavior.**

851 Automated behavioral tracking of an experimental colony, showing trajectories and  
852 identification of ants based on the following color tags: O: orange, P: pink, G: green and B: blue.  
853 Note that individual identities are lost when ants form clusters. This two minute video of an  
854 experimental colony shows the wandering behavior of *orco*<sup>-/-</sup> ants (PP and GG; the first letter  
855 refers to the thorax tag, the second letter to the gaster tag). OP, which never leaves the nest  
856 cluster and therefore is never identified, is *orco*<sup>wt/-</sup>; all other ants are wild-type. PP and GG are  
857 seen antennating each other at 0:57 s.

858

859 **Supplementary Video 4: Additional social behaviors.**

860 Ants are the same as in Supplementary Video 3. This video initially shows an *orco*<sup>-/-</sup> ant, PP,  
861 grooming a pile of eggs. At 0:31 s PP begins to move, seemingly eliciting an alarm response that  
862 causes the other ants in the colony to move toward PP. Note that GG, the second *orco*<sup>-/-</sup> ant in  
863 the colony, does not become active. PP then moves toward the edge of the dish, possibly  
864 depositing a chemical pheromone trail that is followed by some of her nestmates. Note that by  
865 the end of the video a clear pheromone trail has formed that many of the wild-type ants are  
866 following.