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AUTHOR(S):

Yanagawa, Aya; Chabaud, Marie-Ange; Imai, Tomoya; Marion-Poll, Frederic

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1 Olfactory cues play a significant role in removing fungus from the body surface of
2 *Drosophila melanogaster*

3 Aya Yanagawa¹, Marie-Ange Chabaud², Tomoya Imai¹, Frederic Marion Poll^{3, 4}

4

5 1 RISH, Kyoto University, Uji city, Japan 611-0011, Japan

6 2 UMR Physiologie de l'Insecte : Signalisation et Communication, INRA Centre de
7 Versailles, F-78026 Versailles Cedex, France

8 3 UMR Evolution, Génomes, Comportement, Ecologie, CNRS, IRD, Univ Paris-Sud,
9 Université Paris-Saclay, F-91198 Gif-sur-Yvette, France

10 4 AgroParisTech, F-75005 Paris, France

11

12 **Abstract**

13 Many insects and Dipterans in particular are known to spend considerable time
14 grooming, but whether these behaviors actually are able to remove pathogenic fungal
15 conidia is less clear. In this study, we examined whether grooming serves to protect
16 flies by reducing the risk of fungal infection in *Drosophila melanogaster*. First, we
17 confirmed that fungi were removed by grooming. Entomopathogenic, opportunistic, and
18 plant pathogenic fungi were applied on the body surface of the flies. To estimate
19 grooming efficiency, the number of removal conidia through grooming was quantified
20 and we successfully demonstrated that flies remove fungal conidia from their body
21 surfaces via grooming behavior. Second, the roles of gustatory and olfactory signals in
22 fungus removal were examined. The wildtype fly Canton-S, the taste deficiency mutant
23 *poxn 70*, and the olfactory deficiency mutant *orco1* were used in the tests. Comparisons
24 between Canton-S and *poxn 70* flies indicated that gustatory signals do not have a
25 significant role in fungal removal via grooming behavior in *D. melanogaster*. In
26 contrast, the efficiency of conidia removal in *orco1* flies was drastically decreased.
27 Consequently, this study indicated that flies rely on mechanical stimulus for the
28 induction of grooming and olfaction for more detailed removal.

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32 **Keywords**

33 *Drosophila melanogaster*, grooming behavior, fungus, insect pathogen

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37 **1 Introduction**

38 Dipterans spend a significant amount of time grooming (Dawkins and Dawkins,
39 1976). Grooming behaviors involve brushing the body and the wings with the legs and
40 cleaning the legs and the antenna with the mouthparts. It is reported that grooming may
41 help clean external chemosensory receptors (Böröczky et al., 2013) and contributes to
42 removal of dust particles (Phillis et al., 1993). However, there are very limited data to
43 support the hypothesis that grooming behavior plays a role in the resistance against
44 microbial infection. Most dipterans live in highly humid habitats containing microbes
45 (Rohlf, 2008) and frequently perform spontaneous grooming (Szebenyi, 1969). It is
46 reported that flies decrease spontaneous exploratory activity when they perceive the
47 presence of other individuals on food resources (Kamyshev et al., 2002). Instead, flies
48 increase individual behaviors, such as preening (when the legs are rubbed together),
49 which are interpreted as signaling movements that maintain flies at a certain minimum
50 distance apart from each other (Connolly, 1968; Kamyshev et al., 2002). Grooming
51 systematically occurs after egg laying (Rieger et al., 2007; Yang et al., 2008). Considering
52 that many microbes can eventually invade insects through their cuticles, self-grooming
53 in Diptera may help to prevent infections from microorganisms living in their habitats.

54 In insects, hygiene behavior is realized as an integral part of the strategy to cope
55 with pathogens (Vega and Kaya, 2011). If the purpose of grooming is directly linked to
56 the need for cleaning the body from potential ectoparasites, then this behavior may be
57 triggered by signals emanating from microorganisms. Several recent observations
58 performed on social insects indicate that grooming is involved in the resistance against
59 pathogen infection (Zhukovskaya et al., 2013). Spores of entomopathogenic fungi first
60 adhere to the cuticle and then penetrate the surface of the insect by sending hyphae
61 through the epidermis (Yanagawa et al., 2008). Mutual contacts like allogrooming in
62 several species of termites makes them less prone to infection by pathogens (Boucias et
63 al., 1996; Shimizu and Yamaji, 2002; Traniello et al., 2002; Yanagawa and Shimizu,
64 2007). In honeybees, allogrooming is used to remove debris and parasitic mites (Peng et
65 al., 1987; Bozic and Valentincic, 1995; Rath, 1999). It is also known that ants use
66 grooming to protect themselves from ectoparasites (Tranter and Hughes, 2015; Westhus
67 et al., 2014; Okuno et al., 2012). *Drosophila* performs self-grooming, although no reports
68 demonstrated the effects of self-grooming on the removal of parasites in *Drosophila* by
69 using bioassays. Self-grooming is often triggered by touch (Page and Matheson, 2004) or
70 by noxious chemicals (Newland, 1998; Elwood, 2011) detected with nociceptive receptors,
71 which respond to damage or by taste sensilla. The stimulated part of the body or
72 appendage is moved away from the stimulus, and upon increasing stimulation, a

73 brushing movement is generated in either of the legs and directed to the site of
74 stimulation (Dürr and Matheson, 2003). Considering these reports, the central nervous
75 system has an important role in generating self-grooming behaviors (Yellman et al.,
76 1997).

77 We investigated whether self-grooming contributes to preventing infection from fungi
78 in fruit flies, *D. melanogaster*. First, the susceptibility of the wildtype *D. melanogaster*
79 strain “Canton-S” to three fungal species and isolates: The entomopathogen *Beuveria*
80 *bassiana* F1286, the opportunist *Aspergillus niger* ASN5131, and the plant pathogen
81 *Fusarium oxisporum* 544H had been tested. Then conidia removal from the *Drosophila*
82 body surface of all three fungal species of three *D. melanogaster* strains: The wildtype
83 “Canton-S”. The taste mutant strain “*poxn 70*”. The olfactory deficiency mutant strain
84 “*orco1*”. In this study, we confirmed that flies remove fungal conidia by comparing three
85 strains of fungi with different virulence levels. We then examined the roles of taste and
86 olfactory signals.

87

88

89 **2 Materials and methods**

90 **2.1 Insects**

91 *Drosophila melanogaster* were maintained on a standard cornmeal agar diet and at
92 20°C and 80% RH. The wildtype strain Canton-S was used for all experiments. The *poxn*
93 *70* (Yanagawa et al., 2014) and *orco1* strains (Bloomington stock # 23129) were used in
94 the behavioral assays with *Beuveria bassiana*. In order to establish if these responses
95 were mediated by taste sensilla, we performed the same experiments on flies deprived
96 of their external taste chemoreceptors by means of a *poxn 70* mutation, which deters
97 development of external chemoreceptors (Nottebohm et al., 1994). To investigate the
98 importance of olfactory perception on fungal removal, we used *orco1* mutant flies. *Or83b*
99 is abolished in *orco1* mutant flies. This protein is essential for *Drosophila* olfaction
100 (Lausson et al., 2004). Four-day-old flies were used in all experiments. All experiments
101 were conducted in a room without window and under normal room light. All rooms were
102 maintained at 23-26 °C. Flies were placed in the experiment room for about one hour
103 before use to get use to the new environment so that the light in the test room was not
104 affecting the behavior of the insect.

105

106 **2.2 Fungi and preparation of conidial suspension**

107 Three different fungi were used in our experiments: *Beuveria bassiana*, *Aspergillus*
108 *niger*, and *Fusarium oxysporum*. *B. bassiana* is an entomopathogenic fungus, which is

109 known to infect *Drosophila* (Clarkson and Charnley, 1996; Lemaitre et al., 1997). *A. niger*
 110 is an opportunistic microbe (Klainer and Beisel, 1969) and *F. oxysporum* is a plant
 111 pathogen (Snyder and Hansen, 1940).

112 Laboratory maintained isolates were used for the experiments. *B. bassiana* F1286 was
 113 maintained on L-broth agar (1% polypeptone, 0.3% yeast extract, 2.0% sucrose, 0.5%
 114 NaCl, and 2.0% agar) at 25°C. *A. niger* ASN5131 and *F. oxysporum* 544H were
 115 maintained on potato dextrose agar (PDA) (0.4% potato extract, 2.0% glucose, and 1.5%
 116 agar) at 25°C. Conidia were harvested from 10-day-old to 15-day-old cultures using a
 117 brush and were suspended in various solutions as follows. The conidial suspensions (A
 118 series) of all fungal strains were prepared in a 0.025% aqueous solution of Tween 20 to
 119 evaluate virulence. These solutions were diluted 10¹, 10², 10³, and 10⁴ times. On each
 120 PDA petri dish, 0.1 ml of the diluted suspension was pipetted and then spread using a
 121 sterilized glass spreader. The Petri dishes were incubated at 25°C for 3 days. The
 122 numbers of colony-forming units per milliliter (CFU/ml) were determined on the basis of
 123 the numbers of colonies on these PDA plates. To detect the conidia on the cuticles, the
 124 conidia were surface-labeled with 0.01% fluorescein isothiocyanate solution (FITC,
 125 Sigma Chemical) according to the protocols outlined by Hung and Boucias (1992). The
 126 FITC-labeled conidia in a 0.025% aqueous solution of Tween 20 were counted using a
 127 Thoma hemocytometer (Erma Inc, Japan) and adjusted to a concentration of 1.0 × 10⁷
 128 conidia/ml (B series). Over 95% of viability was confirmed on both series of conidia
 129 suspension.

130

131 2.3 Fly susceptibility to fungal infection

132 We first tested the susceptibility of the flies to each fungal strain. For inoculation,
 133 Canton-S flies were collected and placed on ice for 3–5 minutes to induce light anesthesia.
 134 The flies were then placed in microcentrifuge tubes containing the conidial suspensions
 135 (A series) (*A. niger*, 1.63 × 10⁷ CFUs/ml; *F. oxysporum*, 1.30 × 10⁷ CFUs/ml; and *B.*
 136 *bassiana*, 6.25 × 10⁸ CFUs/ml). The flies were submerged in conidial suspensions with
 137 gentle swirling for 5 seconds and allowed to dry on a Whatman No. 1 filter paper. When
 138 they recovered from anesthesia and started to move, a group of 10 flies (5 male and 5
 139 female) were transferred on a filter paper disc to 90 × 15 mm Petri dishes and fly medium
 140 in a cup (10 × 5 mm). Flies treated only with a 0.025% aqueous solution of Tween 20 were
 141 reared as controls. They were incubated at 25°C and 60% RH in the dark room. Mortality
 142 and median lethal dose (LD₅₀) values were calculated seven days after inoculation.

143

144 2.4 Removal of conidia from the fly cuticle

145 Flies were inoculated with the FITC-labeled conidial suspensions (B series), as
146 described above. After treatment with FITC-labeled conidia, the flies were incubated at
147 25°C. At intervals of 0, 3, 24, 48, and 72 hours, 10 flies were removed and stored at -
148 20°C. Flies were carefully mounted in a drop of Vectashield (Vector Laboratories, USA)
149 to stabilize the fluorescence and were examined using an epifluorescence microscope
150 (Axioplan, Carl Zeiss, Germany) at 200×magnification through a common UV filtering
151 cubes FT510. Photos were taken with a charge-coupled device camera (DP74, Olympus,
152 Japan). Four defined sites (head, thorax, wing, and abdomen) were examined on each fly
153 for attachment of conidia, which was calculated in relation to the whole body. To compare
154 the attachment and persistence of the three different fungi (*A. niger*, *F. oxysporum*, and
155 *B. bassiana*), the number of conidia on the insect body surface was counted. We then
156 examined the abilities of the three different *Drosophila* strains (wild-type fly Canton-S,
157 taste deficient mutant *poxn70*, and olfactory deficient mutant *orco1*) to remove conidia.
158 The *B. bassiana* suspension (B series) was used to compare fungus removal ability in
159 flies, as it had the best initial attachment. Removal efficiency for the initial attachment
160 was compared using the removal index (RI) (number of conidia attached to the insect
161 body surface at each time interval)/(number of conidia initially attached to the insect
162 body surface).

163

164 2.5 Taste signals and the induction of grooming

165 Grooming induction was assayed in decapitated four-day-old Canton-S flies using the
166 method described by Yanagawa et al. (2014). Olfaction is perceived by antennae and
167 maxillary palps, and gustation is perceived by the proboscis, legs, wings, and genitalia
168 (Vosshall and Stocker, 2007). Since decapitated flies were employed in this test, the
169 influence of olfaction was ruled out and only taste signals were examined. Decapitated
170 flies are capable of self-grooming movements either spontaneously or following specific
171 stimulation, such as touching. These movements mostly involve the meta-thoracic legs,
172 which are raised and moved independently in a succession of strokes. The legs brush the
173 wings, abdomen, and dorsum, or are extended under the abdomen and touch each other
174 in a series of reciprocal sliding movements. Flies were placed on ice for 3–5 minutes to
175 induce light anesthesia. They were then placed under a stereoscope. Ten flies were then
176 decapitated using a single cut at the neck made by micro-scissors. The decapitated flies
177 then awoke over the next 2–3 minutes. They were placed in an upright position and
178 allowed to recover. In order to stimulate the flies, the wings, forelegs, or hindlegs were
179 gently touched using a sharpened toothpick previously soaked in a test solution. The test
180 solution consisted of a series conidial suspensions *A. niger* ASN5131, *F. oxysporum* 544H

181 and *B. bassiana* F1286 (A series). They were counted using a Thoma hemocytometer and
182 adjusted to a concentration of 1.0×10^7 conidia/ml. These solutions were diluted 10^1 , 10^2 ,
183 10^3 , and 10^4 folds to examine the concentration-dependence of the reaction. The
184 bioassays were performed at room temperature on standing flies placed on a piece of
185 paper. The room temperature kept at about 20°C. Grooming behavior after touching by
186 the toothpick was observed and quantified by a scale. A score of 0 indicates no behavioral
187 induction, a score of 1 indicates 1-2 grooming behaviors (or less than 10 seconds), a score
188 of 2 indicates 3-6 grooming behaviors (or less than 20 seconds), and a score of 3 indicates
189 a strong grooming induction (more than 20 seconds). Twenty female and 20 male flies
190 were tested for each fungus.

191

192

193 2.6 Olfactory signals and fungal removal

194 First, grooming performance was tested in *orco1* flies, since they failed to remove
195 fungal conidia from their body surface. To confirm this, we treated flies with chalk
196 powder and examined whether they could clean the dust. Visual comparisons were made
197 with Canton-S flies 6 hours after treatment.

198 We then determined whether fungal odor enhances or induces the hygiene behavior
199 in both Canton-S flies and *orco1* flies. Since *B. bassiana* is the representative
200 entomopathogenic fungus to *D. melanogaster* and its conidia attachment and removal
201 efficiency are the largest in three tested fungi as well, we have used *B. bassiana* for this
202 test. The GC profile of the fungal odor is also available only on *B. bassiana* from previous
203 study (Yanagawa et al., 2011). Three to five intact *Drosophila* were placed in a 20-ml vial
204 and exposed to control air for at least 10 minutes. Stimulus air containing *B. bassiana*
205 odor was then provided for 3 minutes. Spontaneous grooming was observed for 3 minutes
206 prior to the onset of the stimulus. The time intervals that a sample fly devoted to
207 grooming behavior were added to obtain a numeral conversion for grooming.

208 Airflow was controlled using a three-way cock. Two sides of the cock were connected
209 to a bottle (30 ml) that contained an odor source. One side of the cock was connected to
210 air from 1 ml of 1.0×10^7 /ml *B. bassiana* conidial suspension, and the other side was
211 connected to 1 ml of 0.025% Tween 20 solution as a control. Stimulus and control air both
212 flowed into the three-way cock and the air offered to the flies was regulated by the cock.
213 Fresh air was pumped into the system using a diaphragm pump (AP-115 Iwaki air pump;
214 Iwaki Co., Ltd., Japan) and cleaned through serially connected bottles containing silica
215 gel, molecular sieves 3A and 5A, and active carbon. The cleaned airflow was divided into
216 two channels using a Y-shaped connector. Each air channel was connected to a bottle (30

217 ml) that contained one of the odors being tested. This bottle was then connected to one
 218 side of the three-way cock. The flow in each channel was regulated to 400 ml/minute
 219 using an inline flowmeter. Twenty flies were examined per experiment. These
 220 experiments were carried out in the laboratory, and test arena was maintained at 20°C
 221 and about 69% RH under room light conditions.

222

223 2.7 Fungal avoidance

224 Since avoidance is another major hygiene behavior aimed at preventing infection, we
 225 determined whether chemical signals induce any reactions in different behavioral
 226 paradigms involving the same fungi. To assess avoidance due to chemical signals, the
 227 visitation test, as described by Marella et al. (2006), was used, with modifications
 228 (Supplementary Fig. S1). Canton-S flies were starved for 22 hours using a wet filter
 229 paper disc and were transferred to cylindrical containers (height, 7 cm; diameter, 3 cm,
 230 polystyrene). The tube bottom was separated into two parts and each part was filled with
 231 1 ml of 1% agarose containing 100 mM sucrose. The surface of one side was treated with
 232 20 µl of the tested solutions (1.0×10^7 conidia/ml of *A. niger*, *F. oxysporum*, and *B.*
 233 *bassiana*), and the other side was treated with 20 µl of a 0.025% aqueous solution of
 234 Tween 20. The solutions were spread onto the filter paper using a spreader. In the control
 235 set, both sides were treated with a 0.025% aqueous solution of Tween 20. For the negative
 236 control set, one side was treated with 20 µl of 10^{-1} M quinine, and the other side was
 237 treated with 20 µl of 0.025% aqueous solution of Tween 20. Approximately 40 flies were
 238 placed in a bottle and allowed to explore the agarose for 30 minutes. The visitation rate
 239 was estimated by providing flies access to agar on the bottom of a test tube. One-half of
 240 the agar was treated with a chemical and the other was not. By sampling the number of
 241 flies in each area at regular intervals (every 30 seconds over 30 minutes), we can compute
 242 a mean preference index ($PI = (n1-n2)/(n1+n2)$) and monitor the number of flies visiting
 243 both substrates ($n1+n2$). The number of flies on each side was recorded every 30 seconds
 244 using digital photographs, which were then manually counted. Data were obtained from
 245 10 replicates for each substance.

246

247 2.8 Statistical analysis

248 We used multiple logistic regression analysis to examine conidia removal from the
 249 insect surface and concentration-dependent increases in grooming behavior in
 250 decapitated flies with respect to sex, chemicals, and fly strains. Dunnett's tests were used
 251 to compare RI values used to determine conidia removal efficiency from the initial
 252 attachment, and PI values used to compare preferences in in the visiting test. To

253 determine the odor-induced increase in grooming, Kruskal-Wallis tests were used to
 254 compare the time that flies dedicate to each behavior. JMP 10.0 software (SAS) was used
 255 for all analyses.

256

257 3 Results

258 3.1 Fly susceptibility

259 Mortality at one week rearing was as follows. *B. bassiana*: 67%, *A. niger*: 25%, *F.*
 260 *oxysporum*: 0%, and controls: 0%. The LD₅₀ values of the fungi in *D. melanogaster* were
 261 *B. bassiana* F1286: $\geq 4.16 \times 10^6$ CFU/ml, *A. niger* ASN5131: $> 1.63 \times 10^7$ CFU/ml, and *F.*
 262 *oxysporum* 544H: $> 1.295 \times 10^7$ CFU/ml. *Drosophila* were more susceptible to *B. bassiana*
 263 than *A. niger* and *F. oxysporum*. The LD₅₀ values are provided in Supplementary Table
 264 S1.

265

266 3.2 Attachment and removal of fungal conidia on the *Drosophila* cuticle

267 The binding of the FITC-labeled conidia to the defined sites on the surfaces of the flies
 268 was quantified using an epifluorescence microscope. Attachment and persistence of FITC-
 269 labeled conidia on the fly cuticle are illustrated in Fig. 1 according to fungal strain, time,
 270 and sites of attachment. There was a significant reduction in the number of attached
 271 conidia on the insect surface (*B. bassiana* on Canton-S flies: $p < 0.01$, $F = 60.32$; *A. niger*
 272 on Canton-S flies: $p < 0.01$, $F = 18.10$; and *F. oxysporum* on Canton-S flies: $p < 0.01$, $F =$
 273 44.22 ; logistic regression). There was no sex difference in conidium removal efficiency (p
 274 > 0.1 in all strains on the entire body surface). The number of attached conidia at the
 275 initial stage clearly reflected fungal virulence. *B. bassiana* conidia has higher
 276 attachment than the other strains (Figs. 1 and 2). Both Canton-S flies and *poxn* 70 flies
 277 removed the conidia to a similar extent (*B. bassiana* on *poxn* flies: $p < 0.01$, $F = 61.74$)
 278 (Fig. 3). In contrast, *orco1* flies failed to remove the conidia (*B. bassiana* on *orco1* flies: p
 279 > 0.01 , $F = 61.74$) (Fig. 3). Sex differences were observed only in Canton-S flies at the
 280 wing site (Supplementary Fig. S2). More conidia stayed on the wings in female flies. This
 281 indicates that female flies rely more on both gustatory and olfactory signals to remove
 282 fungi from the wings when compared to the male flies (Student T test: $p < 0.05$ at all
 283 time intervals) (Supplementary Fig. S2). This difference was not observed in *poxn* 70 or
 284 *orco1* flies (Student T test: $p > 0.1$ at all time intervals).

285

286 3.3 Taste signals in the induction of grooming

287 We scored grooming responses following contact with the tip of a small wood stick
 288 dipped into a solution of water mixed with different solutions. The stimulus was brought

289 into contact with the margins of the wings, the front legs, or the hind legs. We first tested
 290 the different fungal suspensions (F1286, ASN5131, and 544H). None of the fungal
 291 suspensions induced grooming in the flies (*B. bassiana* F1286: foreleg, concentration, χ^2
 292 = 0.399, $p = 0.983$; sex, $\chi^2 = 0.540$, $p = 0.970$; hind leg, concentration, $\chi^2 = 4.658$, $p =$
 293 0.324 ; sex, $\chi^2 = 7.040$, $p = 0.134$; wing, concentration, $\chi^2 = 7.886$, $p = 0.096$; sex, $\chi^2 = 3.529$,
 294 $p = 0.474$; *A. niger* ASN5131: foreleg, concentration, $\chi^2 = 1.936$, $p = 0.748$; sex, $\chi^2 = 0.235$,
 295 $p = 0.994$; hind leg, concentration, $\chi^2 = 1.819$, $p = 0.769$; sex, $\chi^2 = 2.109$, $p = 0.716$; wing,
 296 concentration, $\chi^2 = 0.627$, $p = 0.959$; sex, $\chi^2 = 2.144$, $p = 0.709$; *F. oxysporum* 544H: foreleg,
 297 concentration, $\chi^2 = 6.566$, $p = 0.161$; sex, $\chi^2 = 7.687$, $p = 0.104$; hind leg, concentration,
 298 $\chi^2 = 2.335$, $p = 0.674$; sex, $\chi^2 = 2.464$, $p = 0.651$; wing, concentration, $\chi^2 = 4.045$, $p = 0.400$;
 299 sex $\chi^2 = 6.876$, $p = 0.143$; logistic regression).

300

301 3.4 Olfactory signals in fungal removal

302 Flies successfully cleaned the chalk dust from their bodies. There was no visible
 303 difference in the cleaning of the dust between Canton-S flies and *orco1* flies. This
 304 indicates that olfaction does not influence dust removal.

305 Grooming induced by fungal odor was examined using the odor exposure test. Behaviors
 306 of *Drosophila* during the air exposure experiments are illustrated in Fig. 4. In addition
 307 to grooming, two new conditions were observed; 1) 'stay' which means no moving
 308 (standing still) and 2) 'activity', which encompasses all other movements except from
 309 grooming. Mostly, flies walked or ran in 'activity' status. Since there was significant
 310 difference in grooming behavior between females and males (grooming, $\chi^2 = 10.641$, $p =$
 311 0.001 ; stay, $\chi^2 = 5.367$, $p = 0.023$; activity, $\chi^2 = 5.367$, $p = 0.023$; Kruskal-Wallis test),
 312 behaviors were analyzed by females and males independently. We observed more
 313 running behavior in female Canton-S flies ($\chi^2 = 8.526$, $p = 0.004$, Kruskal-Wallis test),
 314 however, no other significant behavior effect was observed during exposure to the
 315 harmful fungus air (Canton-S flies_female: grooming, $\chi^2 = 0.047$, $p = 0.829$; stay, $\chi^2 =$
 316 0.6812 , $p = 0.409$; activity, $\chi^2 = 1.294$, $p = 0.255$; Canton-S flies_male: grooming, $\chi^2 =$
 317 0.019 , $p = 0.892$; stay, $\chi^2 = 1.657$, $p = 0.198$; activity, $\chi^2 = 8.526$, $p = 0.004$; *orco1*
 318 flies_female: grooming, $\chi^2 = 0.001$, $p = 0.978$; stay, $\chi^2 = 0.106$, $p = 0.745$; activity, $\chi^2 =$
 319 1.058 , $p = 0.304$; *orco1* flies_male: grooming, $\chi^2 = 0.105$, $p = 0.745$; stay, $\chi^2 = 0.009$, $p =$
 320 0.925 ; activity, $\chi^2 = 0.000$, $p = 1.000$; Kruskal-Wallis test).

321

322 3.5 Fungal avoidance

323 No sex differences were found in the PI indexes ($p = 0.45$, analysis of variance, Fig. 5).
 324 The PI measured during the control treatment was 0.04 ± 0.04 . The flies visited both

325 sides of the non-treated agar equally and exhibited a strong aversion to quinine in the
 326 negative control test (PI = -0.71 ± 0.06 , $p < 0.001$, Dunnett's test). The flies did not typical
 327 preference or avoidance behaviors in response to any of the fungal suspensions (*B.*
 328 *bassiana*: PI = 0.10 ± 0.07 , $p = 0.14$; *A. niger*: PI = -0.11 ± 0.04 , $p = 0.92$; and *F. oxysporum*:
 329 PI = -0.06 ± 0.04 , $p = 1$ in Canton-S flies; *B. bassiana*: PI = 0.01 ± 0.06 , $p = 1$ in *poxn* flies;
 330 Dunnett's test).

331

332 4 Discussion

333 Grooming behavior seems to have diverse roles. Indeed, many factors involved in this
 334 behavior are still unknown. In this study, we examined the roles of gustatory and
 335 olfactory signals on fungus removal. First, we successfully demonstrated that flies
 336 remove fungal conidia from their body surfaces via grooming behavior. Comparisons
 337 between wildtype Canton-S flies and the chemical mutants *poxn 70* and *orco1* indicated
 338 that gustatory signals do not have a significant role in fungal removal via grooming
 339 behavior in *D. melanogaster*, although olfactory signals are involved in this behavior. It
 340 seems that flies rely on mechanical stimulation for the induction of grooming and on
 341 olfaction for more detailed removal.

342 *D. melanogaster* remove microbes, such as ectoparasites, from their surfaces via
 343 grooming behavior (Fig. 1). The flies removed conidia from all fungal strains.
 344 Differences in the initial attachment numbers for each strain, which reflect the
 345 virulence levels of the different fungi, support our previous findings that attachment
 346 ability is important in estimating fungal virulence (Yanagawa et al., 2008). FITC-
 347 labelled fungal conidia enabled us to visualize fungal ectoparasites and monitor their
 348 behavior on the host surface. The design of the bioassay was another key for the
 349 quantitative observation of conidial removal. Spraying has usually been used to apply
 350 fungi onto flying insects. However, this method requires large amounts of conidial
 351 solution, which are difficult to produce at the laboratory level (Ingris et al., 2012).
 352 Moreover, the *Drosophila* rearing conditions used (vial with a medium-covered bottom)
 353 (Greenspan, 2004) prevented us from using other methods, such as immersion or
 354 droplet application, which are usually used for beetles. These methods created
 355 humidity levels that are too high for flies to survive. Indirect applications, such as
 356 embrocation using a soft brush, which is usually used for worms, are also problematic,
 357 as they may lead to damage to the wings of the flies. We avoided all of the above
 358 problems by using a flat arena (Supplementary Fig. S3). After the flies were immersed
 359 in the conidial suspension, they were able to dry themselves on the filter paper and
 360 came into contact with wet food after they were fully dried.

361 Grooming seems to be triggered by mechanoreceptors (Page and Matheson, 2004) or
362 taste sensilla (Newland, 1998) in most other insects. However, many recent studies
363 have reported that odors from bacteria and yeast modulate fly behavior. These odors
364 are detected by *D. melanogaster* using specialized olfactory receptor proteins (Becher et
365 al., 2012; Stensmyr et al., 2012; Kapsetaki et al., 2014; Dweck et al., 2015; Falchi et al.,
366 2015). Comparisons of conidia removal in Canton-S flies and *orco1* flies indicate that
367 olfactory signals play a significant role in the removal of *B. bassiana* conidia from the
368 *Drosophila* body surface. The fact that *orco1* mutants were able to clear up chalk
369 powder indicates that there may be a unique role for olfactory cues in fungus removal.
370 Experiments using *poxn* flies indicate that taste signals are not important in removing
371 fungal conidia from the body surfaces of *D. melanogaster*, as *poxn70* flies display
372 almost the same conidia removal efficiency as Canton-S flies. Moreover, there was no
373 grooming induction by fungus-related taste stimuli. We have demonstrated that
374 gustatory stimuli from bacteria are involved in grooming reflexes (Yanagawa et al.,
375 2014). The results of the grooming induction test in this study therefore indicate that
376 *Drosophila* use microbial signals from *E. coli* and fungi differently in the induction of
377 grooming behavior. This is because gustatory signals from suspensions of *E. coli* induce
378 grooming while the same is not true of suspensions of fungi. Phillis et al. (1993) have
379 reported detailed grooming induced by mechanical stimuli in *D. melanogaster*. Conidia
380 were attached everywhere on the surface of the flies, and some *B. bassiana* conidia
381 were attached directly to sensory hairs. This observation supports the role of
382 mechanoreceptors in fungal grooming. In addition, considering the success of the *orco*
383 flies in removing chalk powder, it seems that removal of foreign objects via grooming
384 mainly relies on mechanical stimulation. Conidial attachment most likely leads to
385 mechanical stimulation, which then induces the removal of all foreign organisms on the
386 insect's surface. In Canton-S flies, however, the more highly virulent strain, *B.*
387 *bassiana*, was more carefully removed, as the conidia reduction was significant at all-
388 time intervals. The higher level of initial attachment was persistent (Fig. 1). Although
389 the numbers of conidia decrease substantially over time, a marked reduction was
390 observed in the numbers of FITC-labeled conidia associated with virulence. Notably,
391 significant differences were observed in conidium removal from the wings between the
392 two sexes in Canton-S flies, but not in *poxn70* or *orco1* flies. This supports the idea that
393 both taste and olfactory signals are used for fungal cleaning in intact flies, especially in
394 female flies.

395 Flies usually do not move in the direction of harmful microbial odors (Stensmyr et al.
396 2012). Although we do not yet know whether flies possess specialized olfactory receptor

397 proteins to the volatile compounds of *B. bassiana*, in our previous study, we detected 1-
398 octen-3ol in odors from *B. bassiana* (Yanagawa et al., 2011). This compound is a well-
399 known aversive odorant to flies (Silbering et al., 2011). This may explain the higher
400 levels of running/walking activity in female Canton-S flies after exposure to the musty
401 odor. It is reported that termites generally avoid odors from entomopathogenic fungi,
402 but move toward these odors to remove it when they sense the presence of pathogens
403 nearby (Yanagawa et al., 2015). The odor from the pathogenic mite fungus *Neozygites*
404 *floridana* is known to be an attractive signal for males upon their mating and
405 facilitates the transmission of the fungus to healthy individuals (Trandem et al., 2015).
406 This suggests that fungal signals have differing significance to host insects when they
407 are mixed with other odors based on the insect's condition/situation. It is possible that
408 fungi have also developed the ability of using insect perception during their evolution
409 and produce or potentially modify their odors. Fungal odors are known to attract
410 *Drosophila* larvae when the fungal colony is still young (Rohlf, 2005). Since they have
411 more interactions with general contaminating fungi, the insects may rely on fungal
412 odors to find food. Nevertheless, the manner by which insects perceive microbes is still
413 ambiguous. Insect behavioral reactions to microbial signals may be regulated by the
414 delicate balance between neural regulatory pathways that perceive odors as beneficial
415 signals denoting a food source, oviposition site, or mating individual, and those
416 perceiving odors as harmful signals denoting microbial infection.

417 Insects often groom themselves spontaneously. This grooming behavior is increased
418 following the introduction of environmental changes, such as those caused by changes
419 in odor, taste, air, light, or physical contact (Zhukovskaya et al., 2013). The factors
420 involved in this behavior are varied. It was interesting that *D. melanogaster* were
421 found to possess different neural cascades used to trigger grooming by different types
422 of microbe. More research on how insects use signals from microbes will lead to a
423 broader understanding of ecological interactions in nature.

424

425

426

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432

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565

566 **Figure legends**

- 567 Fig. 1. Attachment and persistence of FITC-labeled conidia on the cuticle of the fruit fly
568 *D. melanogaster* wildtype strain Canton-S.
569 Verticals bars represent standard errors. The results of the Tukey-Kramer honest
570 significant difference test are indicated by letters ($p < 0.05$).
- 571
- 572 Fig. 2. Initial attachment of FITC-labeled conidia from *B. bassiana*, *A. niger*, and *F.*
573 *oxysporum* on the wings of Canton-S flies

574 Scale bars indicate 300 μ m.

575

576 Fig. 3. Attachment and persistence of FITC-labeled conidia on the cuticle of the fruit fly
577 *D. melanogaster*

578 Black square shapes indicate Canton-S flies treated with *B. bassiana*, white triangles
579 indicate *poxn 70* flies treated with *B. bassiana*, and white circle indicate *orco1* flies
580 treated with *B. bassiana*. Removal efficiency is assessed using the removal index (***: p
581 < 0.01 , **: $p < 0.05$, *: $p < 0.1$, Dunnett's test). Verticals bars represent standard errors.

582

583 Fig. 4. Grooming behavior of *D. melanogaster* strain Canton-S and *orco1* induced by
584 olfactory chemicals from the fungus *B. bassiana*. Behavior increase/decrease following
585 fungal odor exposure. The grooming behavior was estimated using the time devoted to
586 grooming during a 3-minute observation period. $n = 40$ (20 female and 20 male flies).
587 (***: $p < 0.01$, **: $p < 0.05$, *: $p < 0.1$, Kruskal-Wallis test).

588

589 Fig. 5. Fungal avoidance by the fruit fly *D. melanogaster* wild type strain Canton-S.
590 Visiting preference/aversive responses were examined using the preference index (PI)
591 (***: $p < 0.01$, **: $p < 0.05$, *: $p < 0.1$, Dunnett's test). If PI is low (left), that indicates
592 avoidance and if high (right), that indicates attraction. Horizontal bars represent
593 standard errors.

594

595 Supplementary Fig. S1. Visitation test model arena.

596 About 40 flies were introduce to the polystyrene container from the hole at top. The taste
597 preference index (PI) was calculated as (number flies on test substance side – number
598 flies on water side)/(total number of flies). Data were obtained from 10 replicates for each
599 substance.

600

601

602 Supplementary Fig. S2. Attachment and persistence of FITC-labeled conidia from *B.*
603 *bassiana* on wings of Canton-S, *poxn70*, and *orco1* flies

604 The conidia removal efficiency was described by the removal index. Verticals bars
605 represent standard errors. The results of Dunnett's tests are indicated by asterisks (*: p
606 < 0.05 , **: $p < 0.01$). $n = 20$ from each sex.

607

608 Supplementary Fig. S3. Design for the rearing of conidia-treated flies used in the
609 bioassays

610 (a) Assay kits before use. (b) Assays using conidia-treated flies.

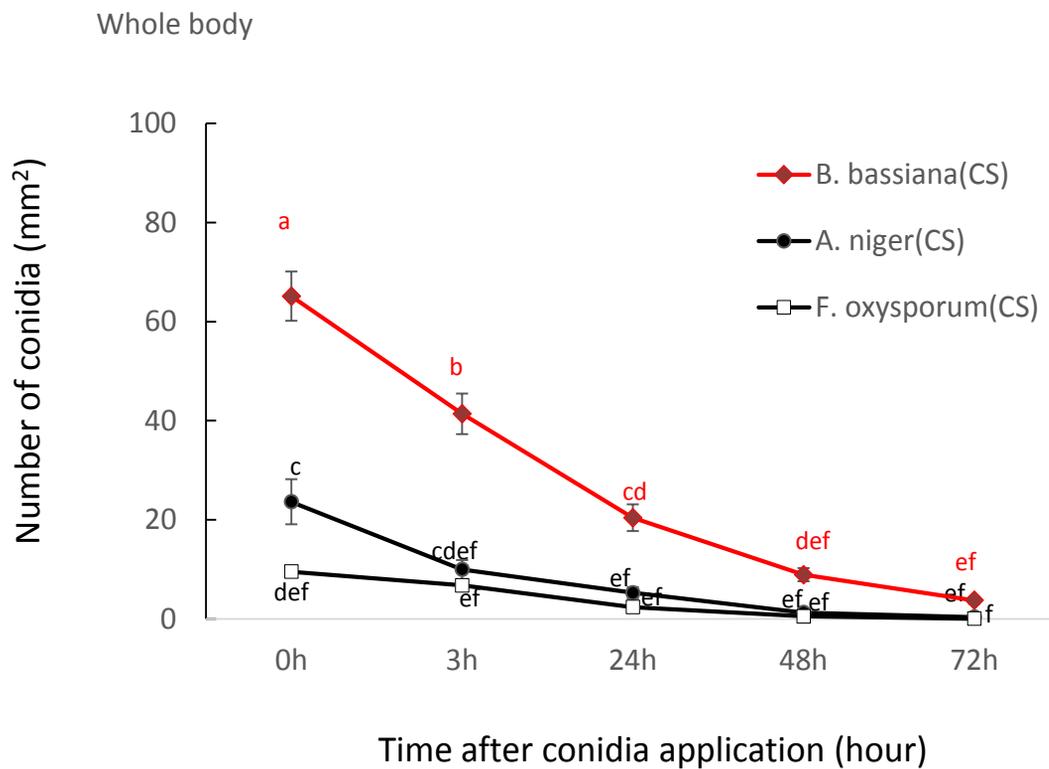


Fig. 1. Attachment and persistence of FITC-labeled conidia on the cuticle of the fruit fly *D. melanogaster* wildtype strain Canton-S.

Vertical bars represent standard errors. The results of the Tukey-Kramer honest significant difference test are indicated by letters ($p < 0.05$)

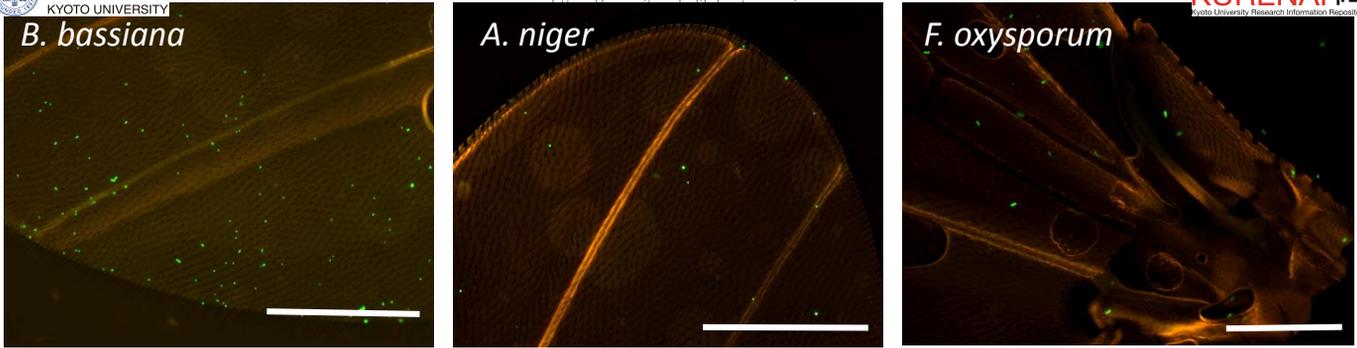


Fig. 2. Initial attachment of FITC-labeled conidia from *B. bassiana*, *A. niger*, and *F. oxysporum* on the wings of Canton-S flies
Scale bars indicate 300 μ m.

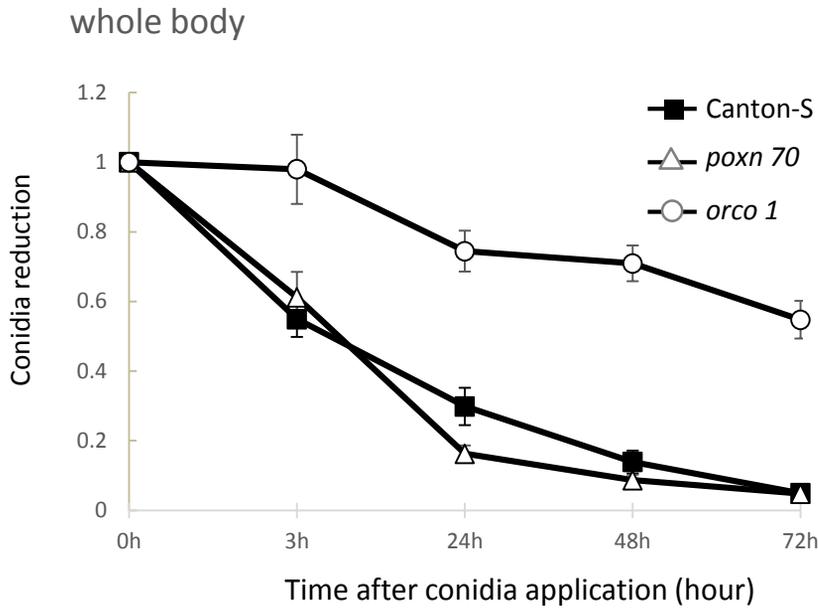


Fig. 3. Attachment and persistence of FITC-labeled conidia on the cuticle of the fruit fly *D. melanogaster*

Black square shapes indicate Canton-S flies treated with *B. bassiana*, white triangles indicate *poxn 70* flies treated with *B. bassiana*, and white circle indicate *orco1* flies treated with *B. bassiana*. Removal efficiency is assessed using the removal index (***: $p < 0.01$, **: $p < 0.05$, *: $p < 0.1$, Dunnett's test). Vertical bars represent standard errors.

Fig. 4

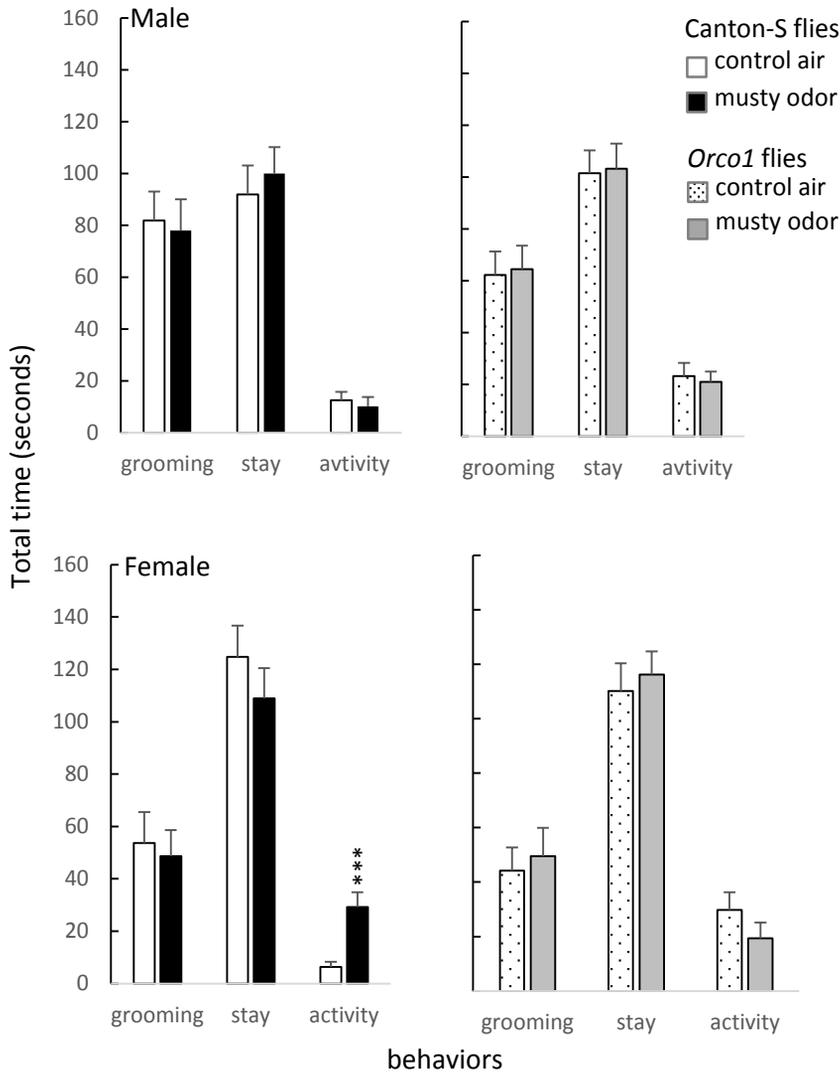


Fig. 4. Grooming behavior of *D. melanogaster* strain Canton-S and *orco1* induced by olfactory chemicals from the fungus *B. bassiana*. Behavior increase/decrease following fungal odor exposure. The grooming behavior was estimated using the time devoted to grooming during a 3-minute observation period. n = 40 (20 female and 20 male flies). (***: p < 0.01, **: p < 0.05, *: p < 0.1, Kruskal-Wallis test).

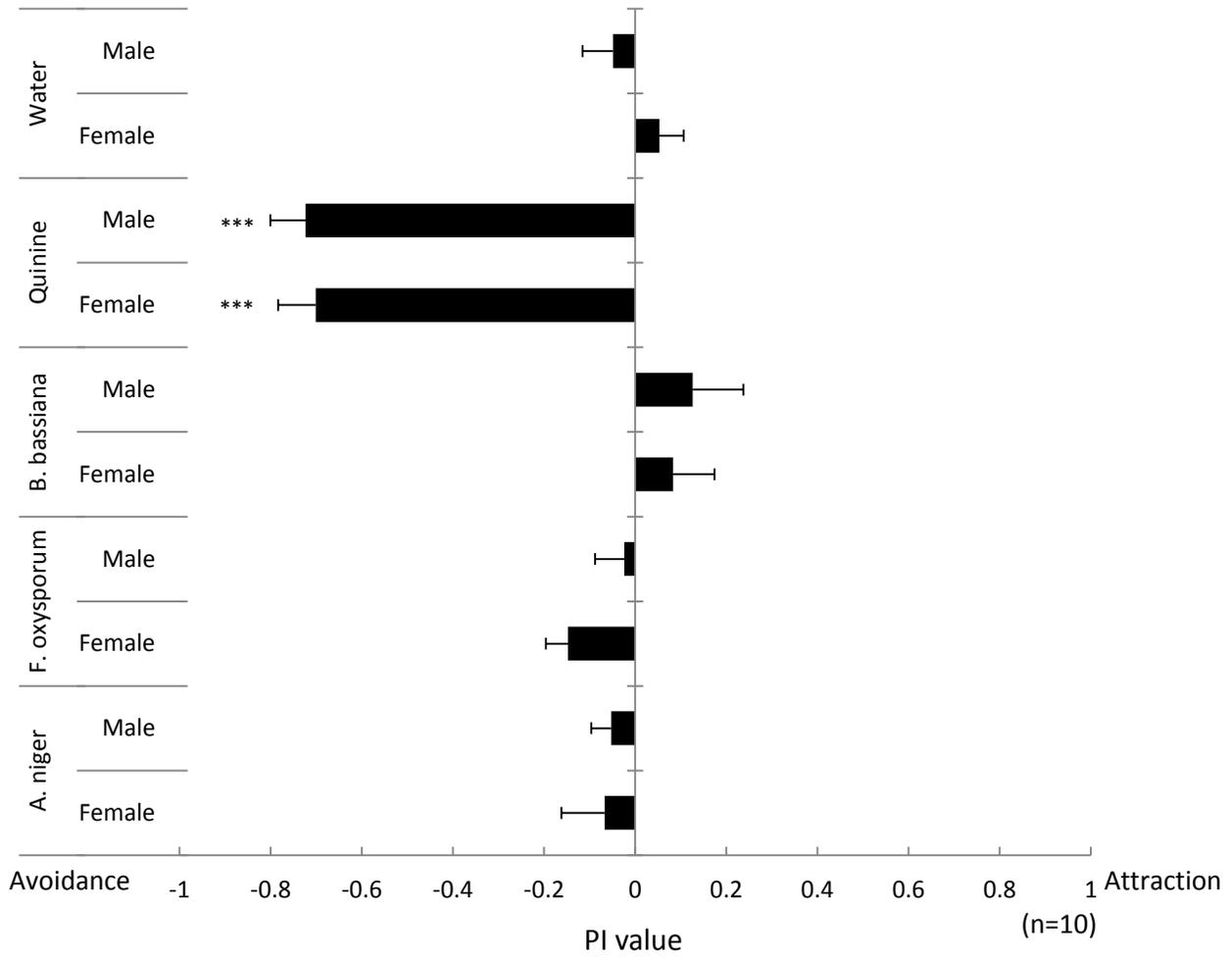


Fig. 5. Fungal avoidance by the fruit fly *D. melanogaster* wild type strain Canton-S. Visiting preference/aversive responses were examined using the preference index (PI) (***: $p < 0.01$, **: $p < 0.05$, *: $p < 0.1$, Dunnett's test). If PI is low (left), that indicates avoidance and if high (right), that indicates attraction. Horizontal bars represent standard errors.

Supplemental table 1 LD50 of *D. melanogaster* to each fungal strain after 1 week rearing

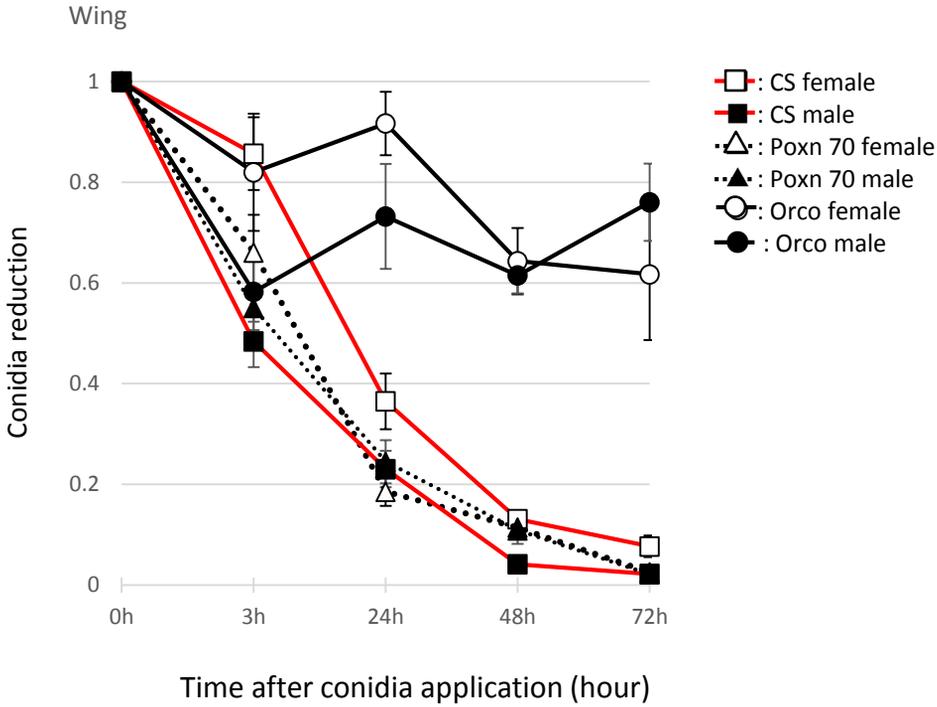
<i>B. Bassiana sensu stricto</i> Origin: <i>Bombyx mori</i> (Japan)	Laboratory maintain strain F1286 Last retrieve with <i>Drosophila melanogaster</i> in 2016
Female + Male	$\geq 4.163 \times 10^6$
Female	$= 6.250 \times 10^6$
Male	$\geq 2.901 \times 10^6$
<i>A. niger</i> Origin: NBRC#105649 (U.S.A)	Laboratory maintain strain 5131 Since 1990
Female + Male	$> 1.633 \times 10^7$
Female	$> 1.633 \times 10^7$
Male	$> 1.633 \times 10^7$
<i>F. oxysporum</i> Origin: <i>Palmier datier</i> (France)	Laboratory maintained strain 544H Since 1988
Female + Male	$> 1.295 \times 10^7$
Female	$> 1.295 \times 10^7$
Male	$> 1.295 \times 10^7$



Fig. S1

Supplementary Fig. S1. Visitation test model arena.

About 40 flies were introduced to the polystyrene container from the hole at top. The taste preference index (PI) was calculated as (number flies on test substance side – number flies on water side)/(total number of flies). Data were obtained from 10 replicates for each substance.



Supplementary Fig. S2. Attachment and persistence of FITC-labeled conidia from *B. bassiana* on wings of Canton-*S*, *poxn70*, and *orco1* flies
The conidia removal efficiency was described by the removal index. Verticals bars represent standard errors. The results of Dunnett's tests are indicated by asterisks (*: $p < 0.05$, **: $p < 0.01$). $N = 20$ from each sex.

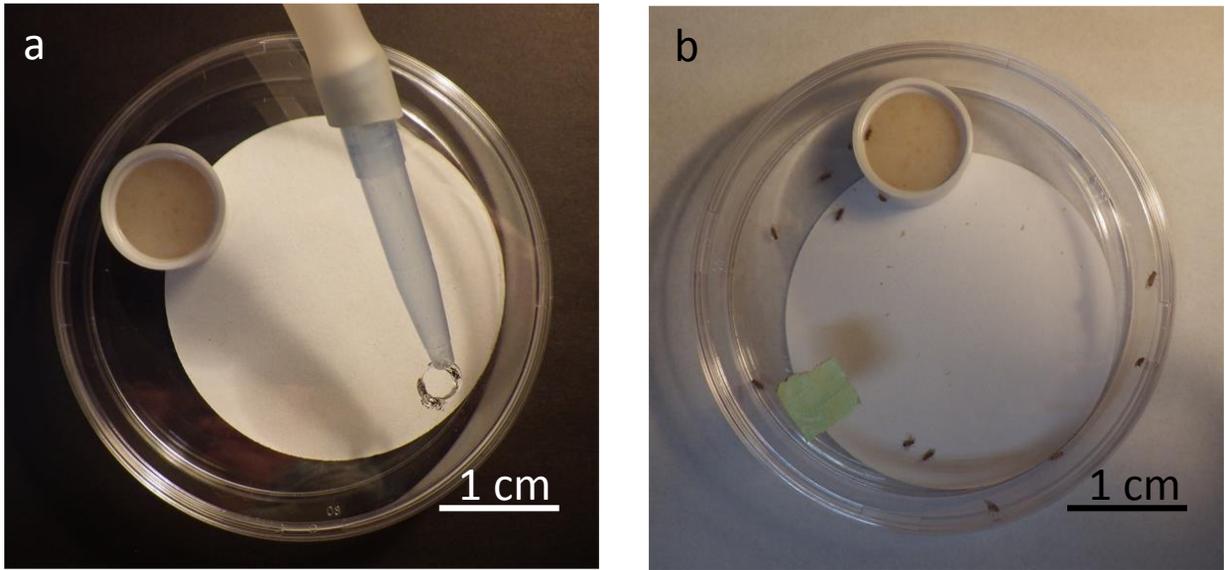


Fig. S3. Supplementary Fig. S3. Design for the rearing of conidia-treated flies used in the bioassays
Assay kits before use. (b) Assays using conidia-treated flies.