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

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Abstract

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

Keywords
Drosophila melanogaster, grooming behavior, fungus, insect pathogen

1 Introduction

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

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

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

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

2 Materials and methods

2.1 Insects

Drosophila melanogaster were maintained on a standard cornmeal agar diet and at 20ºC and 80% RH. The wildtype strain Canton-S was used for all experiments. The *poxn 70* (Yanagawa et al., 2014) and o*rco1* strains (Bloomington stock # 23129) were used in the behavioral assays with *Beauveria bassiana*. In order to establish if these responses were mediated by taste sensilla, we performed the same experiments on flies deprived of their external taste chemoreceptors by means of a *poxn 70* mutation, which deters development of external chemoreceptors (Nottebohm et al., 1994). To investigate the importance of olfactory perception on fungal removal, we used *orco1* mutant flies. *Or83b* is abolished in *orco1* mutant flies. This protein is essential for *Drosophila* olfaction (Lausson et al., 2004). Four-day-old flies were used in all experiments. All experiments were conducted in a room without window and under normal room light. All rooms were maintained at 23-26 ºC. Flies were placed in the experiment room for about one hour before use to get use to the new environment so that the light in the test room was not affecting the behavior of the insect.

2.2 Fungi and preparation of conidial suspension

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

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

Laboratory maintained isolates were used for the experiments. *B. bassiana* F1286 was maintained on L-broth agar (1% polypeptone, 0.3% yeast extract, 2.0% sucrose, 0.5% NaCl, and 2.0% agar) at 25ºC. *A. niger* ASN5131 and *F. oxysporum* 544H were maintained on potato dextrose agar (PDA) (0.4% potato extract, 2.0% glucose, and 1.5% agar) at 25°C. Conidia were harvested from 10-day-old to 15-day-old cultures using a brush and were suspended in various solutions as follows. The conidial suspensions (A series) of all fungal strains were prepared in a 0.025% aqueous solution of Tween 20 to evaluate virulence. These solutions were diluted 10^1, 10^2, 10^3, and 10^4 times. On each PDA petri dish, 0.1 ml of the diluted suspension was pipetted and then spread using a sterilized glass spreader. The Petri dishes were incubated at 25°C for 3 days. The numbers of colony-forming units per milliliter (CFU/ml) were determined on the basis of the numbers of colonies on these PDA plates. To detect the conidia on the cuticles, the conidia were surface-labeled with 0.01% fluorescein isothiocyanate solution (FITC, Sigma Chemical) according to the protocols outlined by Hung and Boucias (1992). The FITC-labeled conidia in a 0.025% aqueous solution of Tween 20 were counted using a Thoma hemocytometer (Erma Inc, Japan) and adjusted to a concentration of 1.0×10^7 conidia/ml (B series). Over 95% of viability was confirmed on both series of conidia suspension.

2.3 Fly susceptibility to fungal infection

We first tested the susceptibility of the flies to each fungal strain. For inoculation, Canton-S flies were collected and placed on ice for 3–5 minutes to induce light anesthesia. The flies were then placed in microcentrifuge tubes containing the conidial suspensions (A series) (*A. niger*, 1.63×10^7 CFUs/ml; *F. oxysporum*, 1.30×10^7 CFUs/ml; and *B. bassiana*, 6.25×10^8 CFUs/ml). The flies were submerged in conidial suspensions with gentle swirling for 5 seconds and allowed to dry on a Whatman No. 1 filter paper. When they recovered from anesthesia and started to move, a group of 10 flies (5 male and 5 female) were transferred on a filter paper disc to 90 × 15 mm Petri dishes and fly medium in a cup (10 × 5 mm). Flies treated only with a 0.025% aqueous solution of Tween 20 were reared as controls. They were incubated at 25°C and 60% RH in the dark room. Mortality and median lethal dose (LD_{50}) values were calculated seven days after inoculation.

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 epifluoresence 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

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

2.6 Olfactory signals and fungal removal

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

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

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

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

2.7 Fungal avoidance

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

2.8 Statistical analysis

We used multiple logistic regression analysis to examine conidia removal from the insect surface and concentration-dependent increases in grooming behavior in decapitated flies with respect to sex, chemicals, and fly strains. Dunnett's tests were used to compare RI values used to determine conidia removal efficiency from the initial 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 LD_{50} 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 LD_{50} 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 epifluoresence 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

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

3.4 Olfactory signals in fungal removal

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

Grooming induced by fungal odor was examined using the odor exposure test. Behaviors of *Drosophila* during the air exposure experiments are illustrated in Fig. 4. In addition to grooming, two new conditions were observed; 1) 'stay' which means no moving (standing still) and 2) 'activity', which encompasses all other movements except from grooming. Mostly, flies walked or ran in 'activity' status. Since there was significant difference in grooming behavior between females and males (grooming, $\chi^2 = 10.641$, $p = 0.001$; stay, $\chi^2 = 5.367$, $p = 0.023$; activity, $\chi^2 = 5.367$, $p = 0.023$; Kruskal-Wallis test), behaviors were analyzed by females and males independently. We observed more running behavior in female Canton-S flies ($\chi2 = 8.526$, $p = 0.004$, Kruskal-Wallis test), however, no other significant behavior effect was observed during exposure to the harmful fungus air (Canton-S flies_female: grooming, $\chi^2 = 0.047$, $p = 0.829$; stay, $\chi^2 = 0.6812$, $p = 0.409$; activity, $\chi^2 = 1.294$, $p = 0.255$; Canton-S flies_male: grooming, $\chi^2 = 0.019$, $p = 0.892$; stay, $\chi2 = 1.657$, $p = 0.198$; activity, $\chi^2 = 8.526$, $p = 0.004$; *orco1* flies_female: grooming, $\chi^2 = 0.001$, $p = 0.978$; stay, $\chi^2 = 0.106$, $p = 0.745$; activity, $\chi^2 = 1.058$, $p = 0.304$; *orco1* flies_male: grooming, $\chi^2 = 0.105$, $p = 0.745$; stay, $\chi^2 = 0.009$, $p = 0.925$; activity, $\chi^2 = 0.000$, $p = 1.000$; Kruskal-Wallis test).

3.5 Fungal avoidance

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

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

4 Discussion

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

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

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

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

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

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

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Figure legends

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

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). Verticals bars represent standard errors.

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).

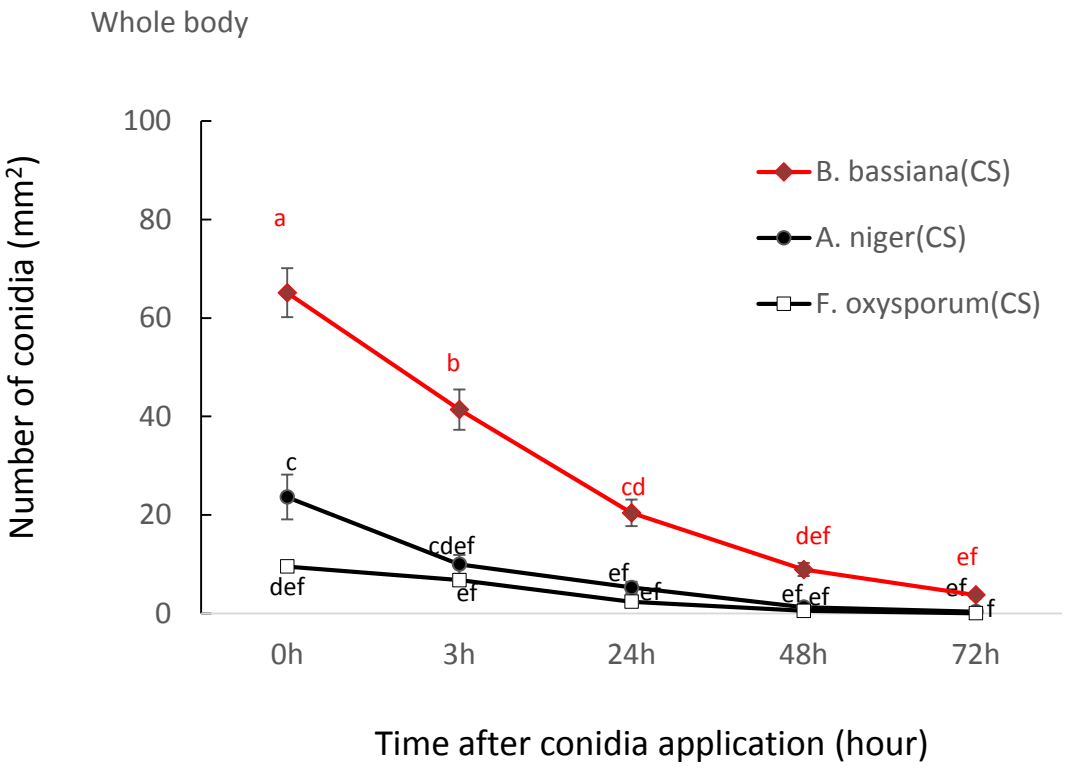
Fig. 5. Fungal avoidance by the fruit fly *D. melanogaster* wild type strain Canton-S.
Visiting preference/aversive responses were examined using the preference index (PI) (***: $p < 0.01$, **: $p < 0.05$, *: $p < 0.1$, Dunnett's test). If PI is low (left), that indicates avoidance and if high (right),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

Supplementary Fig. S1. Visitation test model arena.
About 40 flies were introduce 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.

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

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



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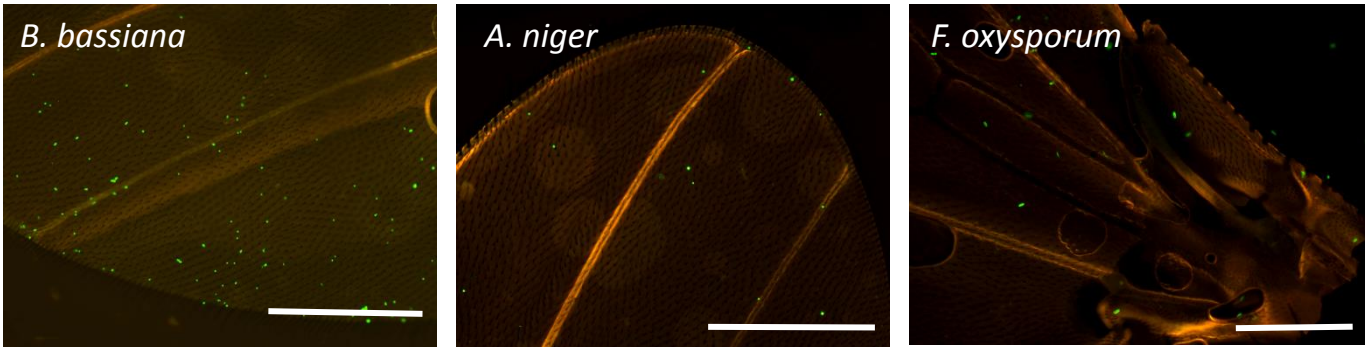

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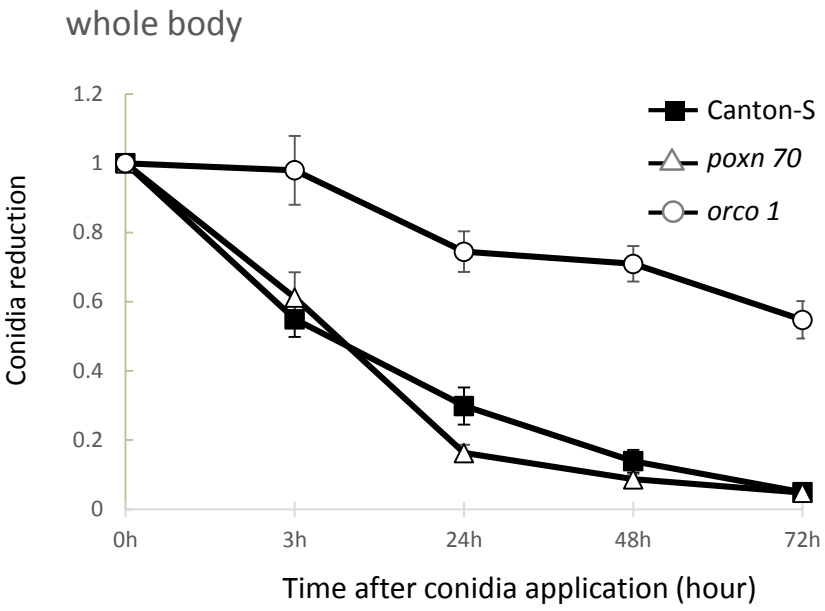

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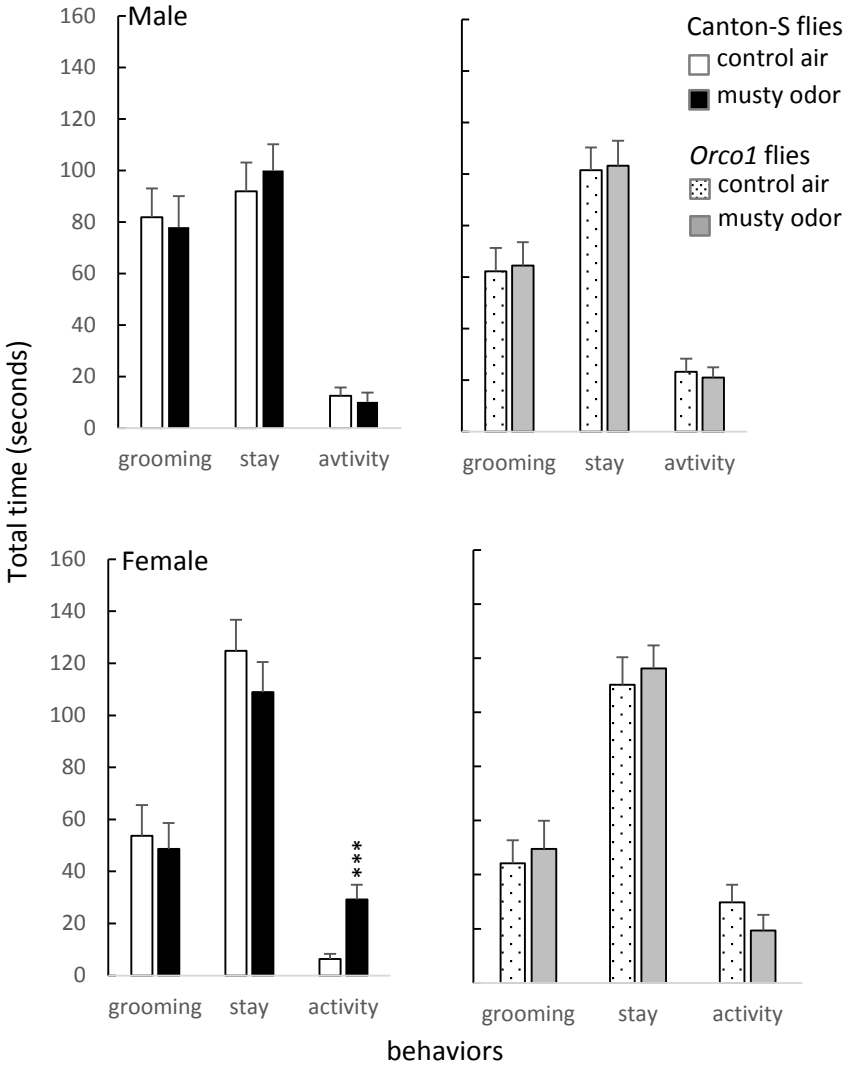

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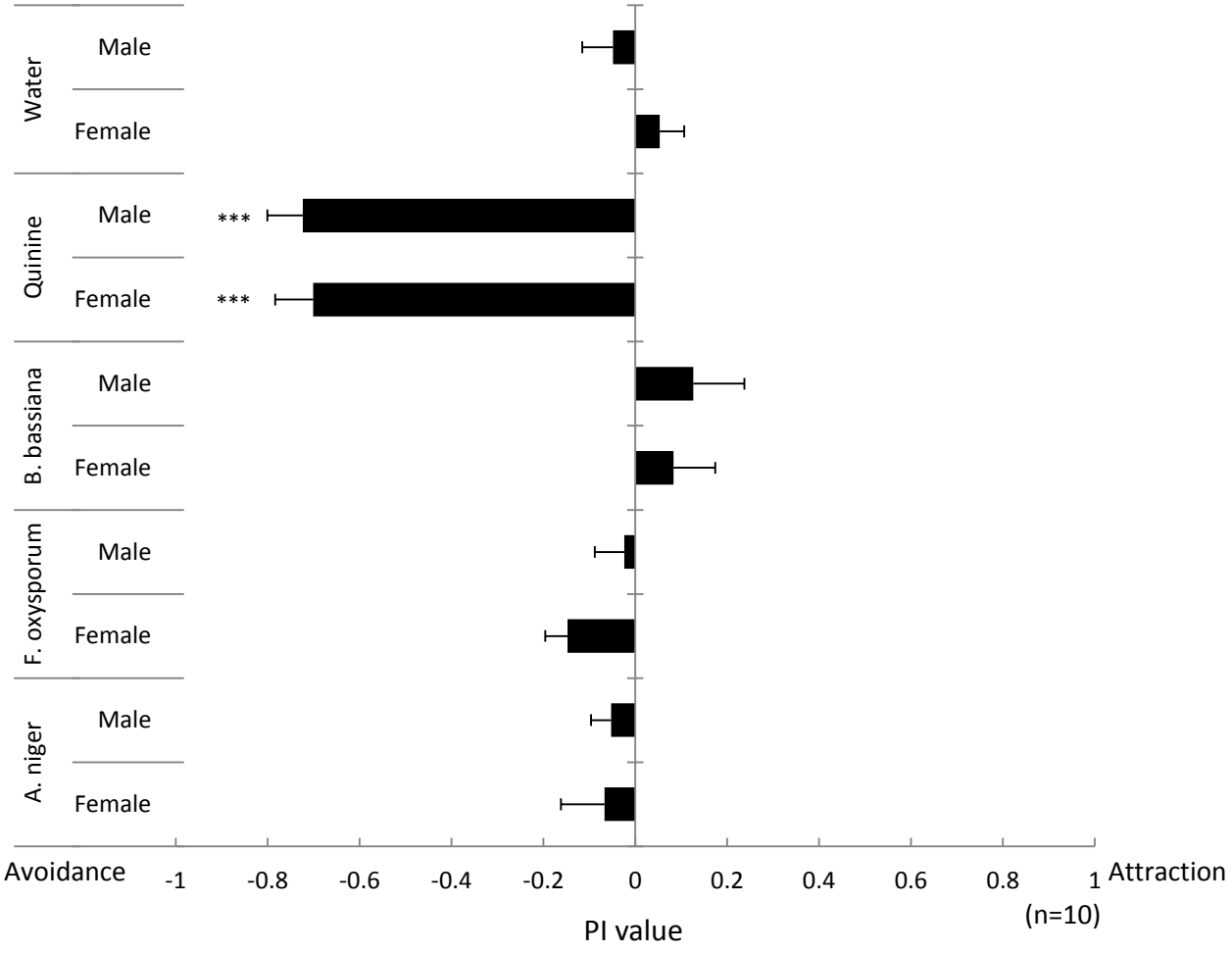

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Supplemental table 1 LD50 of *D . melanogaster* to each fungal strain after 1 week rearing

B. Bassiana sensu stricto Origin: *Bombyx mori* (Japan)	Laboratory maintain strain F1286 Last retrieve with *Drosophila melanogaster* in 2016
Female + Male	$\geq 4.163 \times 10^6$
Female	$= 6.250 \times 10^6$
Male	$\geq 2.901 \times 10^6$
A. niger 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	$> 1.633 \times 10^7$
F. oxysporum Origin: *Palmier datier* (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 introduce 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.

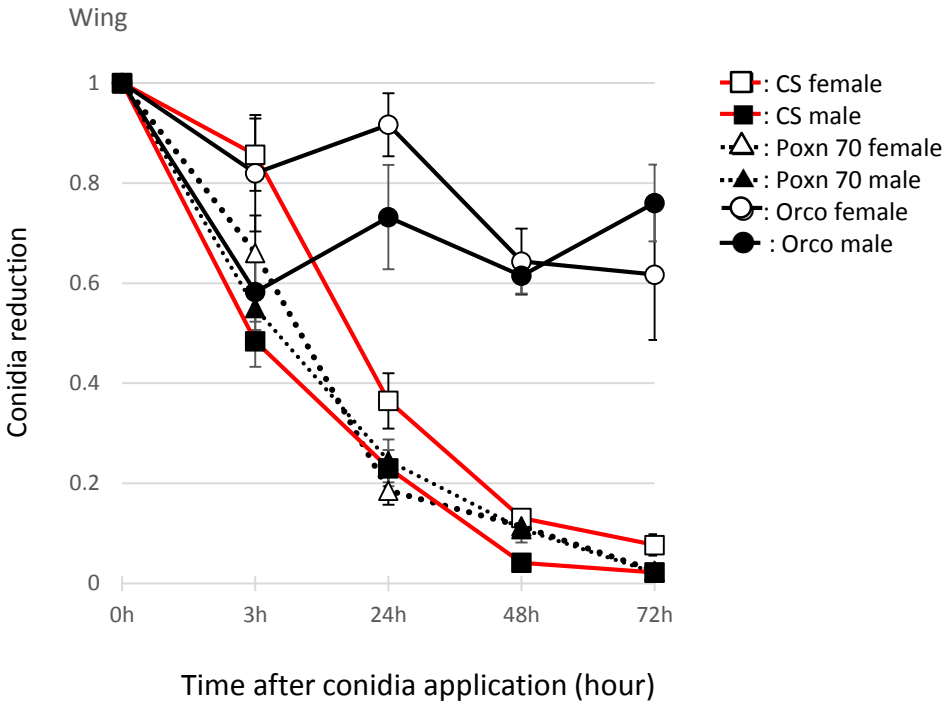

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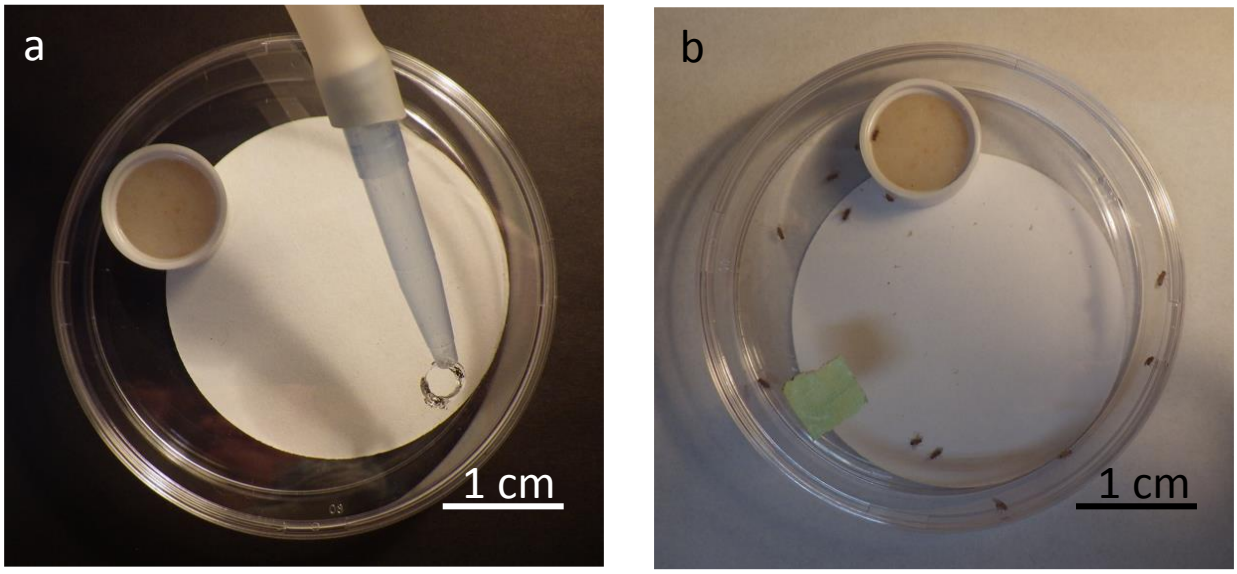
Fig. S2



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