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.

1. Introduction

Dipterans spend a significant amount of time grooming (Dawkins and Danks, 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 (Boroczky 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 (Kamyshov 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; Kamyshov 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; Tranielo et al., 2002; Yanagawa and Shimizu, 2007). In honeybees, allogrooming is used to remove debris and parasitic mites (Peng et al., 1987; Bozic and Valentine, 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 nociceptor 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. melanogaster* strain “Canton-S” to three fungal species and isolates: The entomopathogen *Beauveria bassiana* F1286, the opportunist *Aspergillus niger* ASN5131, and the plant pathogen *Fusarium oxysporum* were tested. Then conidia removal from the *Drosophila* body surface of all fungal species and three *D. melanogaster* strains: The wildtype “Canton-S”. The taste mutant strain “poxn 70”. The olfactory deficiency mutant strain “orco1”. In a 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 20 °C and 80% RH. The wildtype strain Canton-S was used for all experiments. The *poxn 70* (Yanagawa et al., 2014) and *orco1* strains (Bloomingstock # 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. OskB is abolished in *orco1* mutant flies. This protein is essential for *Drosophila* olfaction (Laussou 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 °C. 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; Lemaître 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 I-broth agar (1% polypeptone, 0.3% yeast extract, 2.0% sucrose, 0.5% NaCl, and 2.0% agar) at 25 °C. *A. niger* ASN5131 and *F. oxysporum* F44H 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 obtained by noxious chemicals (Newland, 1998; Elwood, 2011) detected with nociceptor 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).

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 min 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 × 10^6 CFUs/ml). The flies were submersed in conidial suspensions with gentle swirling for 5 s 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

Flies were inoculated with the FITC-labeled conidial suspensions (B series), as described above. After treatment with FITC-labeled conidia, the flies were incubated at 25 °C. At intervals of 0, 3, 24, 48, and 72 h, 10 flies were removed and stored at −20 °C. Flies were carefully mounted in a drop of Vectashield (Vector Laboratories, USA) to stabilize the fluorescence and were examined using an epifluorescence microscope (Axioplan, Carl Zeiss, Germany) at 200 × magnification through a common UV filtering cubes (FTS10). Photos were taken with a charge-coupled device camera (DP74, Olympus, Japan). Four defined sites (head, thorax, wing, and abdomen) were examined on each fly for attachment of conidia, which was calculated in relation to the whole body. To compare the attachment and persistence of the three different fungi (*A. niger*, *F. oxysporum*, and *B. bassiana*), the number of conidia on the insect body surface was counted. We then examined the abilities of the three different *Drosophila* strains (wild-type fly Canton-S, taste deficient mutant *poxn 70*, and olfactory deficient mutant *orco1*) to remove conidia. The *B. bassiana* suspension (B series) was used to compare fungus removal ability in flies, as it had the best initial attachment. Removal efficiency for the initial attachment was compared using the removal index (RI) (number of conidia attached to the insect body surface at each time interval)/(number of conidia initially attached to the insect body surface).

2.5. Taste signals and the induction of grooming

Grooming induction was assayed in decapitated four-day-old Canton-S flies using the method described by Yanagawa et al. (2014). Olfaction is perceived by antennae and maxillary palps, and gustation is perceived by the proboscis, legs, wings, and genitalia (Voshall and Stocker, 2007). Since decapitated flies were employed in this test, the influence of olfaction was ruled out and only taste signals were examined. Decapitated flies are capable of self-grooming movements either spontaneously or following specific stimulation, such as touching. These movements mostly involve the meta-thoracic legs, which are raised and moved independently in a succession of strokes. The legs brush the wings, abdomen, and dorsum, or are extended under the abdomen and touch each other in a series of reciprocal sliding movements. Flies were placed on ice for 3–5 min to induce light anesthesia. They were then placed under a stereoscope. Ten flies were then
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