Published in final edited form as:

Methods Mol Biol. 2012 ; 845: 455–468. doi:10.1007/978-1-61779-539-8_32.

***Drosophila melanogaster* as a model organism for invasive aspergillosis**

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Abstract

Mammalian hosts have traditionally been considered the “gold standard” models for studying pathogenesis and antifungal drug activity in invasive aspergillosis (IA). Nevertheless, logistical, economical and ethical constraints make these host systems difficult to use for high-throughput screening of putative *Aspergillus* virulence factors and novel antifungal compounds. Here, we present *Drosophila melanogaster*, a heterologous non-vertebrate host with conserved innate immunity and genetic tractability, as an alternative, easy-to-use, and inexpensive pathosystem for studying *Aspergillus* pathogenesis and antifungal activity. We describe three different infection protocols (i.e., injection, rolling, ingestion) that introduce *Aspergillus* conidia at different anatomical sites of *Toll*-deficient *Drosophila* flies. These reproducible assays can be used to (1) determine the virulence of various *Aspergillus* strains, and to (2) assess the anti-*Aspergillus* activity of orally absorbed antifungal agents *in vivo*. These methods can also be adapted to study pathogenesis and antifungal drug activity against other medically important human fungal pathogens.

Keywords

Drosophila; fruit fly; invertebrate mini-host model; *Aspergillus*; aspergillosis; virulence; pathogenesis; antifungal efficacy

1. INTRODUCTION

IA is the leading cause of infectious death in patients with leukemia and recipients of allogeneic hematopoietic stem cell transplantation (1, 2). Although significant advances have occurred over the past decade in antifungal treatment, patients who develop IA still have unfavorable prognosis, reflecting their significant net state of immunosuppression and the suboptimal *in vivo* efficacy of modern antifungals (1, 2). Thus, new antifungal drug

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⁶It takes ~ 5–10 min to inject 10 flies.

development and introduction of novel therapeutic strategies are important directions in *Aspergillus* research (3).

Since the early 2000s, new antifungal drugs with promising *in vitro* anti-*Aspergillus* activity have been added to our armamentarium against IA: the new-generation broad-spectrum triazoles and the echinocandins. Because *in vitro* susceptibility testing of antifungals alone or in different combinations does not reliably correlate with *in vivo* clinical efficacy (4, 5), evaluation of the activity of antifungals relies on studies in animal models of IA, typically using immunocompetent or immunosuppressed mammals such as rodents, rabbits and guinea pigs (6–9). Use of these conventional host systems is costly, time-consuming and poses ethical controversies, especially when it comes to testing various antifungal combinations that requires large number of animals. Not surprisingly, use of these animal models is typically limited to testing only one *Aspergillus* isolate in a small number of animals.

The recent completion of the *Aspergillus fumigatus* genome sequencing project (10), along with significant strides in fungal genetics has led to a surge of genetic information pertaining to the contribution of individual genes to *Aspergillus* virulence. For instance, *Aspergillus* strains with defects in siderophore biosynthesis (*sidA*, *sidC*, *sidD*, *sidF*) (11, 12), melanin (*alb1*) (13) or gliotoxin production (*gliP*) (14), PABA metabolism (H515) (15), thermotolerance (*cgrA*) (16), ras signaling (*rhbA*) (17) or starvation stress response (*cpcA*) (18) have been shown to be hypovirulent in mammalian models of IA. Several other molecular factors that may be required for an *Aspergillus* strain to be an effective pathogen are likely to be discovered in the near future. This explosion in functional genomics creates the need for high-throughput screening strategies capable of determining the role of individual *Aspergillus* genes in virulence. Because studying the pathogenesis of IA in conventional mammalian models is labor-intensive, expensive and has logistical limitations, these hosts present a significant ‘bottleneck’ in large-scale screening of putative *Aspergillus* mutants.

Because of these limitations, several studies of pathogenesis in *Aspergillus fumigatus* and a variety of other fungal and non-fungal human pathogens have been recently reported in non-vertebrate mini-hosts, including the fruit fly *Drosophila melanogaster* (19–23), the roundworm *Caenorhabditis elegans* (24), the greater wax moth *Galleria mellonella* (25), and the amoebas *Acanthamoeba castellanii* and *Dictyostelium discoideum* (26, 27). Besides their genetic tractability, the availability of robust research tools (i.e., full-genome microarrays and RNA interference libraries) (28) and the fact that they are easy-to-use, inexpensive and less time consuming than mammalian host systems, critical components of their innate immunity are evolutionarily conserved through mammalian hosts, making these invertebrate pathosystems appealing for studying microbial pathogenesis (29, 30).

Drosophila in particular has two distinct, highly conserved signaling pathways that are critical for defending against invading pathogens: *Imd* against Gram-negative bacteria, and *Toll* against fungi and Gram-positive bacteria (31). The *Toll* signaling pathway, which was initially discovered as a key regulator of embryonic dorsoventral patterning in *Drosophila* (19), is a protease cascade homologous to complement activation by the lectin pathway in

mammals (31). Upon fungal challenge, *Toll* activation leads to downstream production of potent fungicidal peptides that protect flies against fungi (19, 20). On the other hand, *Drosophila* mutants lacking different components of the *Toll* cascade are highly susceptible to an array of fungal microorganisms including *Aspergillus fumigatus*, *Aspergillus terreus*, *Cunninghamella bertholletiae*, *Rhizopus oryzae*, *Mucor circinelloides*, *Scedosporium prolificans*, *Scedosporium apiospermum*, *Fusarium moniliforme*, *Candida albicans* and *Cryptococcus neoformans* (19, 23, 32–37).

Because flies can be grown, manipulated, and analyzed in large numbers in a time efficient manner and with significantly less labor and cost than conventional animal models, *Drosophila* can be used as an *in vivo* model system for large-scale screening of *Aspergillus* virulence factors and of drugs for anti-*Aspergillus* activity (32, 38). Herein, we describe three infection assays that introduce *Aspergillus* conidia (1) directly into the fly hemolymph (injection assay), (2) at the gastrointestinal mucosa (ingestion assay), or (3) on the skin surface (rolling assay) (32). All three assays are easy to perform and result in reproducibly high mortality in *Toll*-deficient flies after *Aspergillus* challenge as opposed to WT *Drosophila*, which are resistant to IA (32). A comparative analysis of hypovirulent *Aspergillus* strains between mice and *Drosophila* reveals high-level concordance between these host systems for testing *Aspergillus* virulence factors (39). Moreover, treatment of *Aspergillus*-infected *Toll*-deficient flies with voriconazole results in significant improvement in survival and reduction in tissue fungal burden (32). Finally, treatment of *Aspergillus*-infected *Toll*-deficient flies with the combination of voriconazole and terbinafine was found to be synergistic (32), in agreement with the *in vitro* synergism of these agents against *Aspergillus fumigatus* (40). Hence, *Drosophila melanogaster* fruit flies with impaired *Toll* pathway can be successfully used for studying the pathogenesis of IA and the efficacy of antifungal drugs (used alone or in combination) against *Aspergillus*.

2. MATERIALS

2.1 *Aspergillus* inoculum preparation

1. *Aspergillus fumigatus* clinical isolate AF293 wild-type (WT) strain and *gliP*-deleted *Aspergillus fumigatus* strain derived from AF293 (14). Of note, AF293 is the strain used in the *Aspergillus* genome sequencing project (http://www.sanger.ac.uk/Projects/A_fumigatus/) (10). Other hypovirulent *Aspergillus fumigatus* strains along with their isogenic WT strains can also be used (e.g., *alb1*-deleted *Aspergillus fumigatus* strain B-5233/RGD12-8 and its isogenic WT *Aspergillus* strain, B-5233) (13).
2. YAG agar plates: 15 g agar, 10 g glucose, 5 g yeast extract, 10 mL of 1 M MgSO₄, 2 mL vitamin mix (1 g p-aminobenzoic acid, 1 g niacin, 1 g pyridoxine HCl, 1 g riboflavin, 1 g thiamine HCl, 1 g cholin HCl, 2 mg d-biotin in 1 L distilled water; store at 4°C in the dark after autoclaving), and 1 mL trace elements (100 mL 0.25 M EDTA pH 8.0, 1 g FeSO₄·7H₂O, 8.8 g ZnSO₄·7H₂O, 0.4 g CuSO₄·4H₂O, 0.15 g MnSO₄·4H₂O, 0.1 g Na₂B₄O₇·10H₂O, and 0.1 g NaMoO₄·2H₂O in 1 L distilled water; store at room temperature) in 1 L distilled water. Pour autoclaved

medium into sterile petri dishes (~20–25 mL per dish) and allow to solidify overnight at room temperature. Store at 4°C for up to 3 month

3. Sterile disposable petri dishes 100 × 15 mm (BD Biosciences).
4. Glass spreaders.
5. Glycerol.

2.2 *Drosophila* infection assays

1. Adult fly lines (*see* Note 1): Oregon^R WT flies have a functional *Toll* pathway and are inherently resistant to *Aspergillus* challenge; *Tl^{r632}/Tl^{r-XA}* *Toll*-deficient flies have a null (*Tl^{r-XA}*) and a thermosensitive loss-of-function (*Tl^{r632}*) allele and lack a functional *Toll* pathway making them susceptible to *Aspergillus* and other fungal infections when maintained at 29°C. At the end of the experiment, infected flies should be killed by freezing at –20 °C and disposed of as a biohazard material.
2. Fly food: 4.4% cornmeal, 3% yeast, 1% agar, 0.6% sucrose, 0.36% propionic acid, and 0.11% Tegosept (Genesee Scientific Corporation). Aliquot 10–15 mL per fly food vial.
3. Stereoscopic microscope equipped with a controllable CO₂-flow fly pad (Fig. 1).
4. Fly incubators with high humidity capacity (60– 75%), adjustable temperature, and a 12-hour light/12-hour dark cycle.
5. Size 0 paintbrush (See Note 2).
6. Tungsten stainless steel needle (tip diameter, 0.01 mm), held in a pin vise (Ernest F. Fullam).
7. Bunsen burner.
8. Fly food vials containing 15 mL YAG medium. Pour the autoclaved medium in empty fly vials and let overnight at room temperature to solidify. Store at 4°C for up to 3 months until use.
9. Fly food vials (Genesee Scientific Corporation).
10. Sterile disposable petri dishes (100 × 15 mm) (BD Biosciences).

2.3 Fly tissue fungal burden quantification by qPCR

1. 0.85% NaCl.
2. Bead-beater homogenizer.
3. DNeasy Kit (Qiagen).

¹For breeding flies, incubation at the optimal humidity and temperature is required. It is necessary to add distilled water to the vials every 3–5 days to prevent them drying out. Also, flies should be maintained at 25°C as this temperature increases the yield of emerging adults. Under these optimal conditions, it takes ~8–12 days from the time the female flies lay their eggs in the fly food until the adults emerge.

²To avoid injuring flies, use a paintbrush to handle them.

4. Oligonucleotide primers and FAM-TAMRA probe. The sequences of *Aspergillus fumigatus* 18S rRNA (GenBank accession no. AB008401) gene-specific primers and dual-labeled fluorescent hybridization probe are as follows: forward primer, 5'-GGCCCTTAAATAGCCCGGT-3'; reverse primer, 5'-TGAGCCGATAGTCCCCCTAA-3'; and probe, 5'-FAMAGCCAGCGGCCCGCAAATG-TAMRA-3' (Applied Biosystems).
5. PCR components: TaqMan Universal PCR Master Mix (Applied Biosystems), MicroAmp optical 96-well reaction plates (Applied Biosystems), MicroAmp optical caps (Applied Biosystems), and ABI PRISM 7900HT sequence detection system (Applied Biosystems).

2.4 Fly antifungal treatment

1. Antifungal agent(s), e.g., voriconazole (Sigma), or other orally absorbable drugs, e.g., terbinafine (Novartis). Prepare stock solutions of 40 mg/mL. Inactive dry yeast granules (Genesee Scientific Corporation).
2. Spatula.

3. METHODS

3.1 *Aspergillus* inoculum preparation

1. Streak frozen glycerol stock of *Aspergillus fumigatus* AF293 (or any other *Aspergillus* strain of interest) onto YAG agar plates and incubate at 37°C for 24 hours.
2. Inoculate a single colony using a sterile loop onto a YAG agar plate and incubate at 37°C for 72 hours.
3. A uniform lawn of *Aspergillus* conidia forms on the agar surface. Collect conidia from the surface of the agar plate by adding 0.5 ml of autoclaved water and using a glass spreader.
4. Count the conidia by using a hemocytometer.
5. Prepare working solutions of *Aspergillus* conidia with the desired concentration (range: 10^7 – 10^{10} conidia/ml).

3.2 *Drosophila* infection assays

3.2.1 Injection assay

1. Anesthetize flies by placing them on the CO₂-flow fly pad (Fig. 1; *see* Note 3).
2. Sterilize a tungsten needle with a flame, and after the needle cools off dip it into the *Aspergillus* conidial suspension (*see* Note 4).
3. Insert the needle midway into the dorsolateral aspect of the fly thorax (Fig. 2a).

³To anesthetize flies, remove the fly food vial cover and rapidly reverse the vial and attach it onto the fly pad. By doing that, flies will fall on the surface of the pad and will fall asleep within 5–10 s.

⁴Vortex the *Aspergillus* suspension in between fly inoculations to ensure that all flies have been infected with a similar inoculum.

4. Inject 30–50 female flies per group of interest (age, 2–4 days; *see* Notes 5 and ⁷) (19, 32).
5. Return the injected flies to the fly food vial (*see* Note 8).
6. For controls, inject 30–50 female flies (age, 2–4 days) with a sterile needle (septic injury control).
7. Observe the flies closely over the first 3 hours post-injection. Flies that die within this 3-hour period post-infection (typically < 5%) have not died from aspergillosis but because of injection injury. Exclude these flies from the survival analysis.
8. Maintain infected flies at 29°C, the temperature at which susceptibility to infection is maximal due to the temperature-sensitive loss of function (*Tlr632*) allele.
9. Transfer the flies to new food vials every 2 days and monitor mortality (See Note 9).

3.2.2 Ingestion assay

1. Grow a fresh lawn of *Aspergillus* conidia in the YAG-containing fly vials by adding 100 µl of a 10⁸ conidia/ml solution to the surface of the agar and incubating at 37°C for 72 h.
2. Place 30–50 female flies (age, 2–4 days) into the vials and let them feed on *Aspergillus* conidia for 6–8 h (Fig. 2b; also *see* Note 10).
3. As starvation controls, place 30–50 female flies (age, 2–4 days) into vials that contain YAG medium only for 6–8 h (starvation control).
4. Observe the flies closely over the first 3 hours post-ingestion. Flies that die within this 3-hour period post-infection (typically < 1%) have not died from aspergillosis but because of starvation and/or stress related to the procedure. Exclude these flies from the survival analysis.
5. Maintain infected flies at 29°C, the temperature at which susceptibility to infection is maximal due to the temperature-sensitive loss of function (*Tlr632*) allele.
6. Transfer infected flies to fresh food vials every 2 days and monitor mortality every 3–6 h (*see* Note 11).

⁵Age plays a critical role in fly survival following aspergillosis (i.e., 10–15-day-old flies are more susceptible to *Aspergillus* infection compared to 2–4-day-old flies). Therefore, it is important to use 2–4-day-old flies in all experiments (42). Also, we recommend the use of female flies as they are larger, easier to handle, and relatively more resistant to injection injury compared to male flies.

⁷One of the advantages of using flies instead of rodents is that flies can be grown and analyzed in large numbers in a time efficient and economical manner. As such, it is feasible to obtain enough flies to use ~30–50 flies per group for virulence and antifungal efficacy studies, and this provides sufficient power for statistical analyses.

⁸Anesthetized infected flies should be returned to food vials placed on their side until the flies recover from anesthesia. This usually only takes a few minutes and prevents flies from sticking to the food and dying.

⁹Typically, using this inoculum level, *Toll*-deficient flies infected with WT *Aspergillus* begin dying ~48 h postinjection and universally succumb to aspergillosis within 6–8 days postinfection (32). In contrast, OregonR WT flies start dying ~96 h postinjection, with ~25% mortality by day 8 postinfection (32).

¹⁰Do not allow flies to feed on *Aspergillus* for more than 6–8 h because they will die of dehydration/starvation as YAG medium and conidia are not optimal nutrition (32). For example, 24 h of feeding will result in ~50% fly mortality.

¹¹Typically, *Toll*-deficient flies infected by ingestion with WT *Aspergillus* begin dying ~48 h postinfection, with 80–90% mortality occurring within 6–8 days postinfection (32). In contrast, OregonR WT flies start dying ~96 h postinfection and only develop ~10% mortality by day 8 postinfection (32).

3.2.3 Rolling assay

1. Anesthetize female flies (age, 2–4 days) by placing them on the CO₂-flow fly pad for 3–4 min (*see* Notes 3 and ¹²).
2. Transfer anesthetized flies to the surface of a YAG petri dish containing a pre-grown fresh carpet of *Aspergillus* (Sunheading 3.1).
3. Roll flies on the conidial lawn for 2 min (Fig. 2c) to coat the entire fly surface in *Aspergillus* conidia (Fig. 2d).
4. After rolling, transfer flies into temporary vials for 1–2 hours. During these 1–2 h. During this time, flies will wake up and move around with a substantial amount of *Aspergillus* conidia falling off (Fig. 2d) onto the food surface (*see* Note 13).
5. After this 1–2 h recovery period, transfer the flies to fresh vials.
6. As rolling-associated injury controls, roll 30–50 female flies (age, 2–4 days) on empty petri dishes that do not contain agar for 2 min.
7. Observe the flies closely over the first 3 h post-rolling. Flies that die within this 3-hour period post-infection (typically < 1%) have not died from aspergillosis but because of rolling injury. Exclude these flies from the survival analysis.
8. Maintain infected flies at 29°C, the temperature at which susceptibility to infection is maximal due to the temperature-sensitive loss of function (*Tlr632*) allele.
9. Transfer infected flies to fresh food vials every 2 days and monitor mortality every 3–6 h (*see* Note 14).

3.3 Fly tissue fungal burden quantification by qPCR

1. Store groups of 20 infected or control uninfected flies at –80°C.
2. When ready to proceed with DNA extraction, wash flies twice with 0.85% NaCl to remove conidia from their exterior.
3. Homogenize flies in 1 mL PBS.
4. To create samples for a 7-point standard curve, spike groups of 20 uninfected flies with 101–107 AF293 conidia and process in the same way as sampled flies.
5. Extract DNA by using the DNeasy tissue kit following the manufacturer's instructions.

¹²Anesthetized flies should be allowed to rest for 3–4 min on the CO₂-flow fly pad before rolling, instead of the few seconds that would otherwise be sufficient to anesthetize them. By allowing a longer period, flies remain anesthetized during rolling, allowing for uniform exposure to *Aspergillus*. If flies do wake up during rolling, they move around the petri dish and are not exposed to *Aspergillus* in a uniform manner.

¹³The intermediate 1–2-h step is required, as without it the surface of the food becomes covered with *Aspergillus* conidia that will have fallen from the fly surface/wings (Fig. 2d). The fallen conidia prevent the flies from feeding and also continually expose the flies to *Aspergillus* and would lead to the death of a substantial proportion of flies within the initial 24-h period (~50%).

¹⁴*Toll*-deficient flies infected with WT *Aspergillus* by rolling start dying ~48 h postinfection and develop ~75% mortality within 6–8 days postinfection (32). In contrast, OregonR WT flies start dying ~96 h post-ingestion and only develop ~10% mortality by day 8 postinfection (32).

6. Prepare a 25- μ L PCR reaction using 12.5 μ L of PCR 2 $\times$ master mix, 6.1 μ L of water, 5 μ L of DNA sample, 0.5 μ L of the probe (200 nM), and 0.45 μ L each of the forward and reverse primers (900 nM). Run a PCR reaction as follows: 2 min at 50°C, followed by 10 min at 95°C, and then, 15 s at 95°C, followed by 1 min at 65°C; the latter two steps are repeated for 40 cycles.
7. Interpolate the threshold cycle (CT) for each sample from the 7-point standard curve.
8. Report qPCR results as conidial *A. fumigatus* DNA equivalents (see Note 15).

3.4 Fly antifungal treatment

3.4.1 Preparation of voriconazole-containing fly-food vials

1. Flame-sterilize a spatula and use it to make superficial abrasions on the fly food surface.
2. Add voriconazole or the antifungal drug of choice on the surface of the fly food (see Notes 16 and ¹⁷).
3. Fill a 1-ml pipette tip with dry inactive yeast particles and slowly drop them on the damp food surface (Fig. 3a; see Notes 18 and ¹⁹).
4. Allow the vials to sit for 24–48 h at room temperature to dry prior to use, otherwise flies will stick to the damp surface and will die. Vials are now ready to use for the antifungal protection experiments.

3.4.2 Treatment of *Drosophila* with antifungal drugs

1. Place 30–50 female flies (age, 2–4 days) in empty vials for 6–8 h to starve (Fig. 3b). (see Note 20).

¹⁵The measurement unit “conidial equivalents” is used to quantify *Aspergillus* fungal burden and infers that only one nucleus is present per fungal cell, as in the conidial developmental program of growth of *Aspergillus*. To calculate tissue fungal burden as conidial equivalents, a standard curve is first prepared by homogenizing 20 uninfected flies in 1 mL of PBS containing 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10², or 10¹ conidia. After DNA extraction, PCR is run and the threshold cycle (CT) value corresponding to each conidial inoculum is obtained. Then, the samples from infected flies are processed, DNA is extracted, PCR is run, and the CT values from infected flies are obtained. Then, the PCR software will calculate the fungal burden as conidial equivalents based on the CT value of the sample and the CT values of the standard curve (41).

¹⁶An optimal volume of diluted drug to add to the surface of fly food is 200 μ L. If more is added, it will not be absorbed by the fly food and yeast granules; flies will get stuck in the food and die. Insufficient volume will not fully soak the added yeast granules and exposure to the drug will be suboptimal.

¹⁷Antifungal drugs should be prepared as high-concentration stock solutions, e.g., 40 mg/mL. This allows testing of very high antifungal drug concentrations, or combination of drugs, in the fly food without having to exceed the optimal 200- μ L volume.

¹⁸The dried yeast granules should not be dispensed all at once. Add a small amount initially, allow them soak up the drug, and then add some more. Continue until all of the yeast granules are soaked by the drug volume you have added. This process should take ~5 min. Ensure that you add only the amount of yeast granules necessary to saturate with the volume of the drug (Fig. 3a). Excessive yeast granules will expose the flies to yeast particles that are not soaked with the antifungal drug. This will lead to suboptimal exposure of the flies to the drug tested. On the other hand, insufficient yeast particles results in flies getting stuck in the food since the yeast granules will not be sufficient to soak the added volume of drug and the food surface will be sticky.

¹⁹Yeast granules are essential for antifungal drug ingestion by the flies. If the antifungal drug is added directly to the fly food surface without yeast particles, two problems can occur. First, absorption of the drug will be erratic because flies will not eat as much of the drug-containing fly food, and second, the granules help absorb the liquid drug preventing the flies from sticking in the food and dying. Vials should be prepared in small batches and used within 5–10 days. This will prevent the yeast granules from drying out.

²⁰Starvation for 6–8 h encourages better ingestion of the antifungal containing food. However, flies should not be starved for longer periods as many flies will die, e.g., starvation for 24 h will result in the deaths of 50–75% of flies.

2. After this starvation period, transfer flies into the antifungal-containing vial and allow feeding for 24 h before infecting with *Aspergillus*. As an alternative to prophylaxis, the therapeutic effect of the drug can be determined by starting treatment immediately after infection.
3. Maintain infected flies at 29°C, the temperature at which susceptibility to infection is maximal due to the temperature-sensitive loss of function (*Tlr632*) allele.
4. For controls, infect 30–50 female flies (age, 2–4 days) with *Aspergillus* and place them in vials with fly food that does not contain the antifungal agent.
5. Transfer infected flies to fresh antifungal-containing food vials every 24 h and monitor mortality every 3–6 h (see Note 21).

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²¹Typically, *Toll*-deficient *Drosophila* infected with *Aspergillus* and exposed to voriconazole have significantly better survival compared to untreated flies. At day 8 postinfection, survival of voriconazole-treated flies is ~50–60%, ~40–50%, and ~50–60% following injection, ingestion, and rolling infection, respectively (as compared to survival of <5%, ~20–25%, and ~10–20% in untreated flies). Finally, survival of flies treated with the combination of voriconazole and terbinafine at day 8 postinfection by rolling is ~80%, suggesting synergy between these two antifungal drugs, as survival of flies treated with either drug was much lower (32).

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Fig. 1.
A CO₂-flow fly pad used to anesthetize *Drosophila* flies.

Fig. 2.

Drosophila infection routes. (a) Injection assay. A CO₂-anesthetized fly is pricked at its dorsolateral thorax with a needle previously dipped in a concentrated *Aspergillus* solution. (b) Ingestion assay. A group of flies feeds on the surface of a fresh lawn of *Aspergillus* conidia pre-grown inside a YAG-containing fly vial. (c) Rolling assay. A group of anesthetized flies were rolled for 2 min on a petri dish covered by a fresh carpet of conidia. (d) After rolling, *Aspergillus* uniformly covers the surface of the flies.

Fig. 3. Antifungal therapy. (a) Dry yeast granules are dispensed onto antifungal drug previously added onto the fly food surface. Note how the yeast particles are entirely soaked by the antifungal agent. (b) Prior to exposure to antifungal drug-containing food vials, flies are starved for 6–8 h in empty vials.