A novel method for infecting Drosophila adult flies with insect pathogenic nematodes

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Drosophila has been established as an excellent genetic and genomic model to investigate host-pathogen interactions and innate immune defense mechanisms. To date, most information on the Drosophila immune response derives from studies that involve bacterial, fungal or viral pathogens. However, immune reactions to insect parasitic nematodes are still not well characterized. The nematodes Heterorhabditis bacteriophora live in symbiosis with the entomopathogenic bacteria Photorhabdus luminescens, and they are able to invade and kill insects. Interestingly, Heterorhabditis nematodes are viable in the absence of Photorhabdus. Techniques for infecting Drosophila larvae with these nematodes have been previously reported. Here, we have developed a method for infecting Drosophila adult flies with Heterorhabditis nematodes carrying (symbiotic worms) or lacking (axenic worms) their associated bacteria. The protocol we present can be readily adapted for studying parasitic strategies of other insect nematodes using Drosophila as the host infection model.

Introduction

The use of insect models to investigate pathogenic infection processes and host innate immune mechanisms offers certain advantages over the use of mammalian models. Insects are easy to maintain in large quantities and their small size facilitates artificial infections and extractions of tissues. Insects also share many physiological processes with mammalian hosts, and they can be used as efficient models to discover toxins and virulence factors some of which are also required to subvert mammalian defenses.1-6

Drosophila melanogaster has been established as an ideal model for studying host-pathogen interactions, as it benefits from the development of a wide range of molecular and cellular tools, impressive advances in the application of high throughput forward and reverse genetic/genomic screens and the body of knowledge accumulated by thousands of investigators.7 In addition, innate immunity in Drosophila can be studied as an integrated system at the level of the whole organism.8 In recent years, genetic studies in Drosophila have resulted in the discovery of distinct immune signaling pathways in response to microbial infections.9 These advances have enjoyed a high profile because they have produced the major spin-off of rekindling interest in the innate immune system of mammals. Largely thanks to work done first on Drosophila, it is now recognized that the nuclear localization of nuclear factor kappa B (NFkB) transcription factors is a universal feature of innate immune reactions,10,11 and that Toll-like receptors are of outstanding importance in transducing innate immune responses in vertebrates.12-15

Drosophila has a multilayered immune response consisting of humoral and cellular mechanisms.16 The hallmark of the Drosophila host defense is the definition of two main signaling pathways, Toll and immune deficiency (Imd), which lead to the activation of distinct members of the NFkB family of transcription factors, and result in the expression of hundreds of
target genes, including those encoding antimicrobial peptides (AMPs). Activation of these pathways depends on recognition of certain microbial elicitors such as bacterial peptidoglycans and fungal glucans. Two other signaling pathways, the c-Jun N-terminal kinase (JNK) and Janus kinase-signal transducer and activator of transcription (JAK-STAT), also participate in regulating immunity effector genes in Drosophila. The body cavity of Drosophila, like that of all arthropods, is filled with a circulating hemolymph that contains both free-floating and sessile blood cells (hemocytes). These are responsible for a number of cellular defenses, while they can take part in humoral reactions. In addition, Drosophila can activate complex proteolytic cascades that regulate coagulation and melanization of hemolymph, defenses associated with the production of reactive oxygen and nitrogen species, and epithelial responses in the gut that also play important roles in fighting microbial infections.

Drosophila has previously been used as a model host to investigate immune responses to diverse pathogenic organisms, including bacteria, fungi, viruses and parasitoid wasps. Recent work has also begun to use the power of Drosophila to dissect the molecular basis of the insect immune response to the combined insult of insect parasitic nematodes and their mutualistic bacterial pathogens. Unlike many animals associated with bacterial symbions, entomopathogenic nematodes are viable in the absence of their mutualistic bacteria. Consequently, each partner of the mutualistic relationship can be separated and studied in isolation or in combination, thus enabling pathogenesis to be studied individually or together.

In addition, to being highly virulent parasites of insects, Heterorhabditis bacteriophora nematodes maintain a mutualistic relationship with the entomopathogenic bacteria Photorhabdus luminescens. In particular, the bacteria are found in the gut of infective juvenile (IJ) worms that are able to attack and invade insects. Once inside the insect, the IJ regurgitates Photorhabdus into the hemolymph where the bacteria begin to divide exponentially producing a wide range of toxins and hydrolytic enzymes that result in insect death. At the same time, the IJ exits diapause and develops into an adult hermaphrodite nematode in a process called IJ recovery. The adult hermaphrodite lays eggs that hatch and develop through four juvenile stages into adult nematodes. Remarkably a single IJ entering an individual insect will result in the production of >100,000 IJs over a timescale of 2–3 weeks. This extremely efficient relationship provides a fascinating model system for studying bacterial pathogen, nematode-vector and insect host interactions.

Protocols for infecting Drosophila larvae with Heterorhabditis nematodes have recently been published; however, it is not currently known how Drosophila adults respond to nematode infection. Interestingly, differences have been found between the adult fly and larval immune systems that are probably due to physiological differences between larvae and adults, and differences in their lifestyles. Here we report the development of a new assay for infecting adult flies with axenic and symbiotic worms (Fig. 1). We have used this method to monitor fly survival and nematode load and show that Heterorhabditis nematodes are able to develop in Drosophila adults. In future studies, we will employ this infection protocol to assess immune gene expression in nematode infected wild-type (WT) and mutant adult flies in order to elucidate how parasitic infections are sensed, and how their presence is communicated both within and among cells and tissues of the host.

Figure 1. Outline of the method for infecting Drosophila adult flies with Heterorhabditis nematodes. We use 4–6 d old adult flies in the infection experiments. Nematode infective juveniles (IJ) are kept in sterile water in tissue culture flasks. Filter paper discs are transferred to the bottom of small plastic cups. Approximately 1,000 nematodes are pipetted onto the filter papers and 10 flies are transferred to each test cup. A second identical plastic cup is inserted into the test cup to restrict movement of flies between the filter papers and the bottom of the inserted cup.
Experimental Design

Fly strain. We used 4–6 d old adult flies of the Drosophila strain Oregon². Both male and female flies were used in the assays. Flies were reared at relatively low densities (20 individuals per vial) to prevent overcrowding conditions. All experiments were performed using flies from the same batch, whenever possible, to reduce variation.

Bacterial/nematode strains. Heterorhabditis bacteriophora nematodes and Photorhabdus luminescens subsp. laumondii strain TT01 bacteria were used in all experiments. We also used 2–4 weeks old Heterorhabditis Ij, as newly hatched and old nematodes show reduced infectivity and host seeking behavior (unpublished data). To generate axenic nematodes, we used bacteria of the Photorhabdus temperata mutant strain RET16 that support the growth of Heterorhabditis without colonizing the nematodes.31 Nematodes were cultured on lipid agar plates supplemented with carbencillin and gentamicin. This resulted in selection of Photorhabdus luminescens cells that were obtained by the worms. Consequently, nematodes fed on RET16 bacteria turn to axenic (Photorhabdus-free) once they complete their life cycle.

Materials

Organisms. Insects. Oregon² adult flies (kindly provided by Prof. J.M. Reichhart, University of Strasbourg and CNRS, Strasbourg, France); Galleria mellonella sixth instar larvae (PetCo).

Nematode species. Heterorhabditis bacteriophora strain TT01.

Bacterial strains. (a) Photorhabdus luminescens subsp. laumondii strain TT01 (kindly provided by Dr David Clarke, University College Cork, Cork, Ireland)

(b) RET-16 mutant, a GFP-labeled variant of Photorhabdus temperata strain NC1 (gentamicin resistant) (kindly provided by Dr Todd Ciche, Michigan State University)

(c) Escherichia coli (streptomycin resistant) (ATCC #25254), kindly provided by Prof. Michael R. Strand, University of Georgia).

Reagents. Luria-Bertani (LB) broth (Miller) (1% tryptone, 0.5% yeast extract, 1% NaCl) (Amresco, J106) Bacteriological agar (Amresco, J637) Corn Syrup (MP Biochemicals, 101413) Cod liver oil (MP Biochemicals, 901405) 0.5M magnesium chloride solution (Acros organics, 7791-18-6) Gentamicin (stock solution 10 mg/mL) (VWR, 97061-370) Carbenicillin (stock solution 100 mg/mL) (VWR, 97063-146) Phosphate-buffered saline (PBS) (Fisher Scientific, BP399-500) Sterile water: autoclaved distilled water 1% Bleach solution for nematode sterilization 10% Bleach solution for cleaning surfaces Ethanol (Absolute), 70% solution for surface sterilization (Decon-Labs, 2716) Active dry yeast (Fisher Scientific, S802452)

Instant Drosophila media, Equation 4-24 (Carolina Biol. Supply Co., 173202) 0.85% Sodium chloride solution (Fisher Scientific, BP358-1) Glycerol (Fisher Scientific, 633-500) Mineral oil (Alpha Aesar, 8020-83-5) Equipment. Fly incubators (25°C temperature, 60–75% humidity, 12-h light/12-h dark photoperiod. Paintbrush (size 0) (Fisher Scientific) General-purpose microbiological incubator (VWR)

Petri dishes (15 × 15 mm) (VWR, 25384-236) Petri dishes (100 × 15 mm) (VWR, 25384-342) Compartmentalized Petri dishes (Bi-plates (100 × 15 mm, 2 sections) (Fisher Scientific, 0875150) Whatman filter paper, Grade 1 (90 mm diameter) (Whatman, 1001090) Whatman filter paper, Grade 1 (150 mm diameter) (Whatman, 1001150) Graduated disposable pipettes (25 mL) (Corning)

One liter bottles (Corning) 96 well plates (Corning) Microscopy glass slides (Fisher Scientific, 12-550-15) Glass coverslips (60 × 24 mm) (Fisher Scientific, 12-545-M) Cell culture flasks, 80 cm² culture area (Thermo, 153732) Cell culture flasks, 175 cm² culture area (Thermo, 156502) Plastic cups 0.5 Oz (Solo Cup Co., P050-0100) 15 mL conical polypropylene tubes (VWR, 21008-103) 50 mL conical polypropylene tubes (Falcon, 352098)

Kimbirips (4 × 8 in) (Fisher Scientific, S47299)

Orbital shaker (New Brunswick) Needle puller P-1000 (Sutter Instruments)

Borosilicate capillary needles, Sutter Instruments (OD: 1.0 mm, ID: 0.50 mm, B100-50-10)

Centrifuge with interchangeable rotors for 1.5 mL microcentrifuge tubes and 15, 50 mL conical tubes (Eppendorf)

Steremicroscope (Tri-Tech)

Nanoject II (Drummond) 10 mL disposable syringe (BD Medical, 14-823-2A) 25 G syringe needle (Fisher Scientific, BDC-5124) 0.22 μm syringe filter sterile (Fisher Scientific, 09-719C)

Color tape (Fisher Scientific, 15-901-15A) 1.5 mL microcentrifuge tubes (US Scientific, 1615-5510)

Nanodrop spectrophotometer (Thermo Scientific)

Disposable spectrophotometer cuvettes (VWR, Cat. No. 89005-758) Micropipettes (10, 100, 200, 1000 μL) (Rainin)

Drosophila vials (VWR, 734-2255) Disposable bacterial spreaders (VWR, 89042-018) Drummond #5 Forceps (Fine science tools, 11252-40)

Pneumatic pump for pipettes (VWR, 53502-211) Water bath, Isotemp 215 (Fisher Scientific)

Graduated 100 mL cylinder (Fisher Scientific)

Carbon dioxide (CO₂) anesthetizing system for Drosophila (Genesee Scientific)

Rubbermaid container, 6.5 qt. (Rubbermaid, 3Q31)

UV Transilluminator, Spectrolinker XL-1000 (Spectronics corporation)

Setup of reagents. Drosophila food preparation. Prepare Drosophila instant media according to the manufacturer’s instructions. Briefly, take one small scoop of media and add it to a Drosophila vial. Add 10 mL of sterile water and let the mix

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sit for 2 min; add 5–10 yeast granules and cover the tubes with cotton balls. Label accordingly. Always use fresh vials when preparing fly cultures.

**Preparation of antibiotic stocks.** Prepare stock solutions of gentamicin (10 mg/mL) and carbenicillin (100 mg/mL) by diluting the antibiotics in sterile water and filtering the solution through a 0.2 µm syringe filter. Prepare rifampicin stock solution (75 mg/mL) by dissolving the antibiotic in DMSO and vigorous vortexing (no sterilization is required). Keep antibiotic stocks at -20°C in the dark.

**Preparation of lipid agar plates.** They are used for growing axenic nematodes. Add 25 g of LB broth, 17 g of bacteriological agar, 10 mL of corn syrup in 1 L of double distilled water and autoclave the solution. After autoclaving, keep the solution at 60°C in a water bath. Add 5 mL of cod liver oil (autoclaved), 1 mL of 0.5 M MgCl₂, and carbenicillin, gentamicin antibiotics (final concentration in water: 100 µg/mL and 0.75 µg/mL, respectively). Mix well and pour into one side of a compartmentalized-plate. The plates are wrapped in aluminum foil and stored at 4°C until needed.

**Preparation of LB agar plates.** These are used for culturing bacteria. Plain LB agar plates are prepared by mixing 25 g of LB, 17 g of agar in 1 L of double distilled water and the solution is autoclaved. A separate batch of LB agar plates supplemented with rifampicin (75 µg/mL) is prepared to test for the presence of Photorhabdus bacteria in axenic worms. Rifampicin plates should always be filter sterilized.

**Culturing symbiotic nematodes: Timing 14–20 d.** To grow and amplify Heterorhabditis symbiotic nematodes, we use caterpillars of the greater wax moth Galleria mellonella. The larvae are infected with nematodes carrying *P. luminescens* strain TT01 bacteria.

1. **Plate preparation: 1 h.** Cover the bottom of a large Petri dish (150 mm diameter) with a single filter paper, add 1 mL of sterile water and transfer 6–10 Galleria larvae. Pipette a drop of water containing 50–100 IJ on each insect.

**Procedure**

1. **Procedure 1.** Incubation: 11 d. Galleria larvae succumb to nematodes 2 d after the infection. Heterorhabditis/Photorhabdus infected Galleria larvae exhibit a characteristic “red brick” color (Fig. 2A). Insects turning black or gray are contaminated by other pathogens and they should be immediately discarded. Monitor the survival of larvae daily and add a few drops of water, if needed, to keep the insect cadavers moist but not soaked.

2. **Procedure 2.** Water trap preparation: 1 h. On day 11 after infection, transfer the Galleria larvae to a water trap. This consists of a large Petri dish (150 × 20 mm), inside of which a smaller standard size Petri dish (100 × 15 mm) is placed upside down. We place a piece of filter paper (cut as shown on Fig. 2B) on top of the small Petri dish and fill the large Petri dish with water until the edges of the filter paper are submerged.

3. **Procedure 3.** Insect cadavers as prey for nematodes: 10 min. Using a pair of plastic forceps, gently transfer and arrange the infected Galleria larvae on the filter paper of the water trap. Place the traps in a Rubbermaid container to prevent contamination of the

4. **Procedure 4.** Water trap preparation: 1 h. On day 11 after infection, transfer the Galleria larvae to a water trap. This consists of a large Petri dish (150 × 20 mm), inside of which a smaller standard size Petri dish (100 × 15 mm) is placed upside down. We place a piece of filter paper (cut as shown on Fig. 2B) on top of the small Petri dish and fill the large Petri dish with water until the edges of the filter paper are submerged.

5. **Procedure 5.** Insect cadavers as prey for nematodes: 10 min. Using a pair of plastic forceps, gently transfer and arrange the infected Galleria larvae on the filter paper of the water trap. Place the traps in a Rubbermaid container to prevent contamination of the
Nematodes usually emerge from the dead cadavers by other pathogens. Store in the dark until the JJ emerge from the insect cadavers.

(5) Nematode harvesting: 30 min. Nematodes usually emerge from the dead insects 14–20 d after infection. Emerging nematodes directly fall into the water allowing for easy collection. They can be readily collected from the water trap using a 100 mL graduated cylinder. Fill the cylinder to the top (100 mL mark) with water containing nematodes and let the worms settle at the bottom (this takes approximately 30 min). Remove 85 mL of water (down to the 15 mL mark) using a volumetric pipette. Fill back up with fresh water to the 100 mL mark, let the nematodes settle, and remove the excess water as indicated above. Repeat three times until the water in the cylinder is clear. Transfer 20 mL aliquots in small culture flasks, and store in the dark. Nematodes can be collected daily for at least 2 weeks or longer depending on the number of worms required for experiments.

NOTE: It is important to achieve optimum numbers of nematodes in each flask: high density will result in nematode aggregations and eventually nematode death; low density will not produce the appropriate number of worms required for the experiments. We keep around 1,500–2,000 JJ/mL of water. Shake flasks regularly to aid aeration. Nematodes can be stored at room temperature (RT) (~2°C) for months. We always use less than 1-mo-old nematodes in our experiments.

Culturing axenic nematodes: timing 14–20 d. Axenic nematodes (nematodes lacking Photorhabdus bacteria) are cultured in bi-plates on a bacterial lawn produced by P. temperata mutant strain RET16.

(6) Bacterial inoculation and plating: 18 h. Grow P. temperata RET16 bacteria in LB broth at 30°C with vigorous agitation. Nematodes can be cultured until the appropriate number of worms required for the experiments. We use a nanoinjector and glass capillary needles for delivering precise doses of bacteria into flies.

(7) Incubation of nematodes mixed with bacterial lawn: 30 min–12 d. Transfer 500–1,000 surface-sterilized JJ to the center of the bacterial lawn; gently agitate plates to distribute the worms evenly and incubate at 30°C for 12 d.

NOTE: A container filled with sterile water is placed in the incubator to prevent desiccation. Sterile water (approximately 400 μL) is also added to the plates after 1 week (or whenever necessary), to moisten the agar.

(8) Water trap setup: 10 min. After 12 d of incubation, fill the other half of the bi-plate with sterile water and incubate at RT to allow emerging JJ to fall into the water trap as they migrate from the agar to the water.

(9) Nematode collection: 1 h. After 14–20 d, JJ nematodes are collected from the water traps. Aspirate the water using a 10 mL disposable pipette and replace with fresh water. Transfer the water containing nematodes to a culture flask. Wash nematodes several times with sterile water and store flasks in the dark. Nematodes should always be surface sterilized before use, as described above.

(10) Testing axenicity: 2–3 d. To determine whether axenic Heterorhabditis contain Photorhabdus cells, pellet a sample of newly generated axenic nematodes (200 μL of a nematode suspension in a 1.5 mL tube) for 5 min at 8,000 rpm. Remove the supernatant and homogenize the worms using a plastic pestle. Add 100 μL of LB broth, mix and plate out the solution on LB agar plates supplemented with rifampicin (see above); incubate for 2 d at 30°C. The absence of bacterial colonies indicates that no P. luminescens cells are present in the worms.

Surface sterilization of nematodes: timing 1 h. (11) Pelleting nematodes: 10–15 min. Centrifuge 20 mL of a nematode suspension in a 50 mL conical tube at 1,500 rpm (standard speed used in all steps) for 5 min; discard the supernatant.

(12) Bleach treatment: 10–15 min. Resuspend the nematode pellet in 20 mL of 0.1% bleach solution in water, gently invert the tube several times, incubate for 7 min at RT and centrifuge for 5 min at 1,500 rpm. Remove the bleach solution leaving the pellet in approximately 1 mL of solution left, add fresh water (40 mL), mix gently and repeat centrifugation step. Repeat four times. Nematodes are resistant to low concentrations of bleach. We did not observe any negative effects of bleach on the fitness or survival of the nematodes.
suspension corresponding to an OD of 0.1 (approximately 400–700 CFUs for *P. luminescens* and 1,000–4,000 CFUs for *E. coli*). Transfer infected flies to fresh vials and monitor their survival daily and up to 7 d.

Nematode infection assays: 5–7 d. (19) Preparation of infection units: 45 min–1 h. Cover the bottom of the cups with 4–5 pieces of filter paper; use the bottom of the cup as measurement.

(20) Mixing nematodes and flies: 15 min. Surface sterilized nematodes in solution (100 IJ/fly) are pipetted to each infection unit. Flatten the filter paper at the bottom of the cup using a micropipette tip. Add more water to the filter paper as needed (usually in aliquots of 100 µL) until it is attached to the bottom of the cup; this step is important because extra water keeps the nematode parasites active.

Tip: For four layers of filter paper, the maximum total volume of added water is approximately 600 µL; for five filter papers it is about 800 µL. Once nematodes and flies are added to the cup, a second empty cup is placed on top and secured using tape (Fig. 1). Keep the infection units at RT in a humid box (e.g., a pipette tip box lined with wet filter paper).

NOTE: It is important to prevent desiccation. The addition of extra water to the cups depends on nematode density: the higher the density, the more water needs to be added, and vice versa. Excess water will result in fly drowning; lack of water will result in desiccation and nematode death. It is also important to flatten the filter papers at the bottom of the cup to avoid trapping of flies in between the filter papers. Cups should be prepared 15–30 min before use.

Survival experiments: 7 d. (21) Set up: 30 min. Infect 4–6 d old Oregon* R* adult flies with 100 IJ/fly as described above.

(22) Monitoring survival: 7 d. Count and record the number of dead flies daily.

Dose-response survival experiments: 7 d. (23) Set up: 30–45 min. Infect 4–6 d old Oregon* R* adult flies with 10, 25, 50 and 100 nematode IJ/fly. Estimate nematode numbers as described above.

(24) Monitoring survival: 7 d. Incubate infected and uninfected control flies for 7 d at RT and count the number of dead flies daily.

Persistence experiments: 2 d. (25) Set up: 30 min. Infect 4–6 d old Oregon* R* adult flies with 100 IJ/fly as described above. Incubate flies at RT.

(26) Nematode counting: 2 d. At 24 and 48 h post-infection, wash infected (dead or alive) flies in a drop of 1x PBS to remove any nematodes attached to the exterior of the flies (use the microscope). Dissect flies in 1x PBS using a pair of clean forceps to determine the number of nematodes present in each fly.

Nematode development: 5 d. To determine whether Drosophila adult flies are suitable hosts for supporting the development of *Heterorhabditis* nematodes, we infect flies with symbiotic nematodes (50 IJ/fly), as described above. We have observed that infection with a high number of nematodes slows down nematode development, probably due to crowding conditions in the fly.

(27) Set up: 15 min. Infect Drosophila adult flies as previously described and incubate insects at RT.

(28) Dissection and fixation: 1–2 h. Five days after infection, dissect flies in sterile 1x PBS. Incubate dissected cadavers in cold 4% paraformaldehyde diluted in 1x PBS, in a 96-well plate for 1 h. This treatment eliminates any remaining live nematodes. Gently remove the fixative solution and wash samples three times in 1x PBS. Carefully mount the dissected flies on a glass slide using 50% glycerol diluted in 1x PBS solution, and cover the samples with a coverslip. Specimens are ready for viewing under the microscope. Slides can be stored at 4°C for a few days.

(29) Imaging: flexible. Time needed for viewing depends on the number of prepared samples and experience of the researcher with microscopy techniques. Images are assembled and illustrations prepared using Adobe Illustrator and Photoshop software packages (Adobe CS5 suite).

Results and Further Studies

We first infected Drosophila WT adult flies with *Heterorhabditis* nematodes carrying or lacking *Photorhabdus* bacteria and estimated the ability of flies to survive infection over time (Fig. 3). We found that in both cases flies succumbed to nematode infection after 5–6 d. Survival of flies infected with axenic or symbiotic nematodes was highly significant (p < 0.05; Chi-square test) compared with uninfected controls. High fly mortality caused by axenic nematodes merits further investigation and it will be the focus of our future studies. Injection of *Drosophila* with *Photorhabdus* (positive control) also

![Figure 3](image-url)
killed all flies within the same length of time. Injection of flies with *E. coli* bacteria or uninfected flies (negative controls) produced minimum insect mortality.

We then infected *Drosophila* adult flies with different numbers of axenic or symbiotic *Heterorhabditis* IJ. Interestingly, we found that flies infected with 25 or 100 axenic *Heterorhabditis* died slightly faster (3 d after infection) compared with those infected with 10 or 50 axenic worms (4 d after infection) (Fig. 4A). Different number of axenic nematodes caused mortality that was significantly higher compared with uninfected controls (*p* < 0.05; Dunnett’s multiple comparison test). One explanation for this discrepancy could be variation in nematode seeking behavior or increased fly activity. We have found that hyperactive flies significantly lower *Heterorhabditis* infectivity (unpublished data). However, we observed that all flies infected with different numbers of symbiotic *Heterorhabditis* succumbed within 4 d of infection (Fig. 4B). Again, mortality of uninfected control flies was significantly lower (*p* < 0.05; Dunnett’s multiple comparison test) compared with mortality of flies infected with symbiotic nematodes.

We further looked at the persistence of axenic and symbiotic *Heterorhabditis* in *Drosophila* WT flies at 24 and 48 h after infection (Fig. 5). We found that both groups of nematodes were present in flies at both time-points, which suggests that *Drosophila* adults can serve as valuable model for studying host-parasite interactions and nematode parasitism. We did not observe any significant difference in the number of axenic or symbiotic worms between the two time-points (*p* < 0.5, *df* = 3; one-way ANOVA).

Finally, we asked whether *Drosophila* adult flies permit nematode development. For this, we used *Heterorhabditis* IJ to infect WT flies that were dissected 5 d later (Fig. 6A). We found that infected flies contained nematodes at different stages of development; these included pre-hermaphrodite nematodes (Fig. 6B), hermaphrodites harboring newly hatched L1 nematodes (Fig. 6C), and pre-IJ worms that disrupt the nematode cuticle and move to the fly hemocoel (Fig. 6D). We also observed cuticles of hermaphrodites

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**Figure 4.** Dose-response survival results for *Drosophila* wild-type (WT) adult flies infected with (A) axenic *Heterorhabditis* (nematodes lacking *Photorhabdus*) and (B) symbiotic *Heterorhabditis* (nematodes carrying *Photorhabdus*). We used 10, 25, 50 and 100 IJ per fly. Ten Oregon 4–6-d-old flies were used for each treatment. Uninfected flies served as controls. Survival of flies was estimated daily and up to day seven following infection. Data represent the percent survival of infected and control flies. The averages from at least three experiments are shown.

**Figure 5.** Persistence of axenic and symbiotic *Heterorhabditis* in *Drosophila* adult flies. Ten Oregon 4–6-d-old flies were used in each replicate. Flies were infected with approximately 1,000 nematodes (100 nematodes per fly), and dissected at 24 and 48 h after infection. Nematode numbers were counted under a dissecting microscope. Colored horizontal lines indicate the arithmetic means of nematode numbers and error bars indicate standard errors. Data represent the averages from at least three independent experiments.
(or mother worms) that were left empty after the new generation of nematodes had escaped to the insect body cavity (Fig. 6E). The various stages of nematode life cycle were not observed outside flies. These results suggest that Drosophila adult flies, despite their small size, are able to support the development of Heterorhabditis nematodes.

Future studies will use this infection protocol to test the ability of Drosophila gain-of-function or loss-of-function immune mutant flies to survive infection with axenic or symbiotic Heterorhabditis nematodes. Results from fly survival experiments combined with information on the number of nematodes that persist over-time in Drosophila immune mutants will provide important clues about the pathological events that take place in the adult fly as well as the physiological processes that determine whether the fly lives or dies following Heterorhabditis infection. As there is literally no information if and how entomopathogenic nematodes are detected by the insect immune system, further studies using this protocol together with quantitative real-time RT-PCR can be readily used to monitor the transcriptional levels of a wide range of immune recognition genes in flies at various times following infection with axenic or symbiotic worms. Similar studies could potentially lead to the identification of particular immune pathways that are activated in Drosophila adult flies following immune recognition of Heterorhabditis as well as to the number and nature of signaling genes that participate in these processes. Otherwise, WT or immune mutant flies infected with axenic or symbiotic nematodes can provide the starting material to examine the global transcription response to Heterorhabditis infection in Drosophila adults using transcriptomics.

Information on insect anti-nematode cellular responses has only recently started to emerge. Our nematode infection assay can be used to investigate the Drosophila cellular immune reactions (e.g., encapsulation, nodulation responses) in adult flies against Heterorhabditis, and to determine whether (and how) these reactions interact and coordinate with the humoral immune response that is also directed against the nematode parasites. Finally, it is possible that the current method can be applied to evaluate the interaction between host hemolymph clotting factors with the presence of Heterorhabditis in adult flies, as it has recently been shown that the clotting response plays a protective role against nematode infection in Drosophila larvae.

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