

An improved method for nematode infection assays in *Drosophila* larvae

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Abbreviations: AMPs, antimicrobial peptides; EPNs, entomopathogenic nematodes; IJs, infective juveniles; RT, room temperature

The infective juveniles (IJs) of entomopathogenic nematodes (EPNs) seek out host insects and release their symbiotic bacteria into their body cavity causing septicaemia, which eventually leads to host death. The interaction between EPNs and their hosts are only partially understood, in particular the host immune responses appears to involve pathways other than phagocytosis and the canonical transcriptional induction pathways. These pathways are genetically tractable and include for example clotting factors and lipid mediators. The aim of this study was to optimize the nematode infections in *Drosophila melanogaster* larvae, a well-studied and genetically tractable model organism. Here we show that two nematode species namely *Steinernema feltiae* and *Heterorhabditis bacteriophora* display different infectivity toward *Drosophila* larvae with the latter being less pathogenic. The effects of supporting media and IJ dosage on the mortality of the hosts were assessed and optimized. Using optimum conditions, a faster and efficient setup for nematode infections was developed. This newly established infection model in *Drosophila* larvae will be applicable in large scale screens aimed at identifying novel genes/pathways involved in innate immune responses.

Introduction

Entomopathogenic nematodes (EPNs) are soil-dwelling parasitic worms that infect and kill insects.¹ Like many other nematode parasites, they exhibit non-feeding, free-living stages, also known as infective juveniles (IJs) that are tightly associated with their specific symbiotic bacteria. During an infection, IJs enter the insect host through their natural openings or by penetrating the cuticle, and release the symbiotic bacteria that kill and digest host tissues, thereby providing nutrients for the development of both nematodes and bacteria.² To ensure a successful invasion and completion of their life cycle, EPNs and their symbionts employ various strategies to evade or suppress host immune responses, including the secretion of proteases,³ phenoloxidase inhibitors and toxins that interfere for example with phagocytosis.^{4,5} Potential insect hosts rely on both general immune mechanisms and mechanisms that are specific for EPNs.⁶ The first line of defense consists of physical structural barriers, such as the cuticle and the peritrophic membrane of the midgut and the respective epithelia. Once these barriers are breached, the insect defends itself against bacterial or parasitic infection using both cellular and humoral immune responses. Cellular immune reactions are mediated by different types of immune cells, which may contribute to phagocytosis, nodule formation and encapsulation. The nematode *Heterorhabditis bacteriophora* has been observed to

be encapsulated by its insect host *Manduca sexta*.⁷ The humoral effectors recruited by host insects include the inducible antimicrobial peptides (AMPs), cell adhesion molecules, clotting components and the prophenoloxidase system.⁷⁻⁹ Those humoral factors are secreted into the hemolymph by both the fat body and by hemocytes. It has been shown that a set of mRNAs coding for specific pattern recognition proteins in *M. sexta* are upregulated upon the exposure of hosts to *Heterorhabditis* or its symbiont *Photorhabdus luminescens* alone.¹⁰

Besides their wide application as a biological control for insect pests,¹¹ EPNs and their insect hosts are increasingly used as models for human parasitic nematode and vector-borne disease.^{6,12} Although a great deal of knowledge about the interaction between EPNs and their hosts, particularly on host immune responses has been gained from these studies, a more complete picture could be obtained by taking advantage of *Drosophila melanogaster*, a well-studied and genetically tractable model organism. The completion of the *Drosophila* genome sequencing project, microarray analysis and the use of genetic screens have led to a better understanding of host-pathogen interactions¹³ and hold the same promise for studies of EPN infections.

EPN infections of *Drosophila* were performed by Hallem et al. who provided the first insights into the underlying effector mechanisms. The infection of *Drosophila* larvae with *Heterorhabditis*/*Photorhabdus* resulted in the temporary

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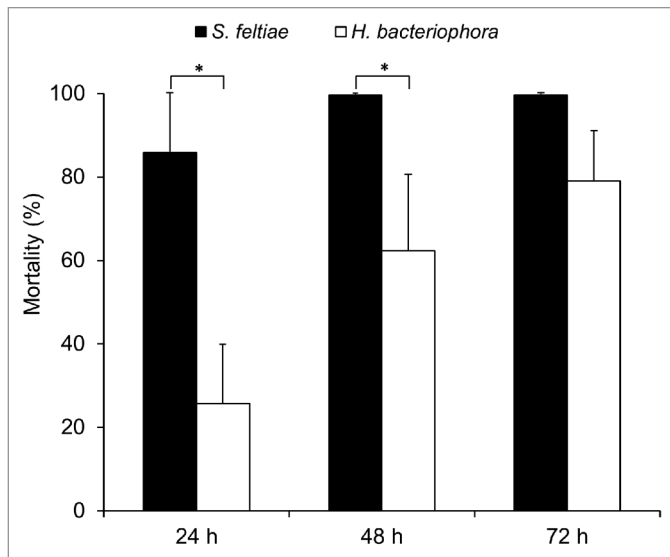


Figure 1. Infectivity of *S. feltiae* or *H. bacteriophora* on *Drosophila* larvae. Larvae were infected with nematodes at an infectious dose of 1,000 IJs/larva in a 96-well assay with tissue paper as described in Material and Methods (mean \pm SD; confidence levels are *: <0.05).

dynamic expression of a subset of antimicrobial peptide genes, a response that is induced specifically by the symbiotic bacterium *Photobacterium*. However, *Drosophila* larvae with defects in the induction of AMPs show the same sensitivity toward EPNs as controls, indicating a dispensable role of AMPs and in fact of both the *imd* and *Toll* pathway in this infection model.¹⁴ In addition, *Drosophila* mutants for phagocytic receptors (*Nimrod1* and *Eater*) survive the *Heterorhabditis*/*Photobacterium* infection which is in line with the previously observed suppression of phagocytosis by *Photobacterium*.^{4,9,15} In contrast, there are indications that several clotting factors help to combat nematode infection and that lipid mediators are involved.^{9,15} Nonetheless, the molecular basis of *Drosophila*-EPN interactions is far from being completely understood.

To take full advantage of the combination of EPN infections and *Drosophila* genetics we wished to optimize the conditions for nematode infections in *Drosophila* larvae. This included the use of two EPN models (*Heterorhabditis bacteriophora* with its bacterium *Photobacterium luminescens* and *Steinernema feltiae* with *Xenorhabdus bovienii*), different supporting media and different doses of IJs. Finally we developed a fast and efficient strategy for nematode infection, which allowed us to continuously monitor the infection process and will be suitable for large-scale screens to identify genes that modulate EPN infections.

Results

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are able to infect a broad range of insect larvae, however, they often display specific host preferences.¹⁷ In *Drosophila melanogaster*, Hallem et al. have shown that *Drosophila* larvae were susceptible to IJs of *Heterorhabditis bacteriophora*,¹⁴ yet no study has been performed to compare the infectivity of

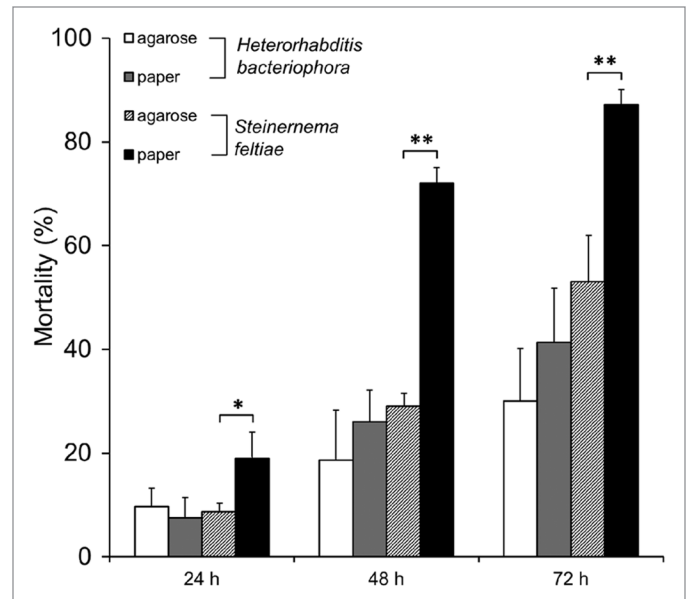


Figure 2. Effect of different supporting media on mortality rate of *Drosophila* larvae in nematode infection with *S. feltiae* or *H. bacteriophora*. Larvae were infected with nematodes at an infectious dose of 100 IJs/larva in a 96-well assay as described in Material and methods (mean \pm SD; confidence levels are *: <0.05; **: <0.01).

the two different nematode species to *Drosophila* larvae. Here we tested the infectivity of *H. bacteriophora* and *S. feltiae* by infecting *Drosophila* larvae with high dose (1,000 IJs/larva). **Figure 1** shows that most of the larvae were killed by *S. feltiae* already 24 h after infection, and all host larvae were dead after 48 h; whereas far fewer larvae succumbed to *H. bacteriophora* after both 24 h and 48 h. Clearly, both nematode species displayed the ability to infect and kill *Drosophila* larvae, but *S. feltiae* is more pathogenic than *H. bacteriophora* against *Drosophila* larvae.

Since different supporting media applied in the nematode infection establishment may have different effect on infectivity, due to differences in host foraging strategies,¹⁸ we next assessed the effect of media (agarose and tissue paper) on nematode infection. Considering the high mortality rate caused by the high dose of nematodes, we conducted the infection with a lower dose IJs suspension (100 IJs/larva). For both nematode species, the infection involving tissue paper soaked with nematode suspension was more effective in killing host larvae than the one involving agarose (**Fig. 2**) but only with *Steinernema*, these effects were significant. For *S. feltiae*, host mortality increased significantly when tissue paper was employed, particularly at 48 h and 72 h post infection ($p < 0.01$ in **Fig. 2**). In addition, the replicates on tissue paper appeared to show somewhat less variation in comparison to that on agarose. Therefore, tissue paper was selected as the better support medium for nematode infection and was used to further studies.

To establish nematode infections for use in genetic screens in *Drosophila*, it is important to identify an optimal dosage, which results in low-to-intermediate mortality rate of the wild-type *Drosophila*, allowing detection of increased mortality caused by loss-of-function or RNAi-mediated knockdowns. Conversely

high mortality rates may decrease in gain-of-function backgrounds. Therefore different doses of IJs from *Heterorhabditis* (25, 100 and 1,000 IJs/larva) were assayed in *W¹¹¹⁸* larvae. The mortality of *Drosophila* larvae showed dose dependence (Fig. 3). With the minimum dose of *H. bacteriophora* tested here, only 30% of the larvae were killed at 72 h post infection; a moderate mortality rate was obtained with a dose of 100 IJs/larva, while with 1,000 IJs/larva, around 80% were dead after 72 h (Fig. 3). We conclude that to identify negative effects on the immune response against EPNs, 100 IJs/larva is the optimal dose for a single host challenged at RT, while the ten times higher concentration may be suitable to identify dominant activators of the immune response.

In addition to allowing detection of modifiers of the immune response, the feasibility of genetic screens also depends on the time and effort required for the phenotypic analysis. Since the 96-well plate assay we have been using so far requires individual inspection of the larvae in each well, we wished to establish an easier and faster assay. Therefore we developed a ‘plastic bag assay’ in which 50 host larvae were mixed with IJs in a transparent resealable plastic bag (Fig. S1). To score the survival easily we used the *H. bacteriophora*/GFP expressing *P. luminescens* complex (*Heterorhabditis bacteriophora* TT01), since both infected and dead hosts could be easily scored as GFP-positive (Figs. 4B and S1). To test the plastic bag assay, we used a previously described line (black cells/*imd* double mutant, *Bc/imd*), for which increased susceptibility toward *Heterorhabditis*/*Photorhabdus* had been described indicating a more subtle role for both crystal cells and the *imd* pathway during EPN infections.¹⁵ Using the novel assay, we could fully confirm the increase in susceptibility. Titration of the nematodes may even indicate that the assay is more robust and less sensitive to variation in nematode concentration (Fig. 4A) since all nematode densities caused significantly higher mortality in the positive control, *Bc/imd*. Both the GFP-expressing bacteria in the hemolymph and the nematodes were visible in infected larva that had been retrieved from the plastic bags and inspected under UV light. Septicaemia with GFP-*Photorhabdus* led to a signal throughout the hemolymph while *Heterorhabditis* nematodes were visible as dark shades in the GFP signal (Fig. 4B).

Discussion

The aim of this study was to optimize the conditions to use *Drosophila melanogaster* as a model host for infection with entomopathogenic nematodes. Our goal was to identify the optimal conditions for a previously described assay that involves 96-well plates and to develop a faster assay that facilitates continuous monitoring of the infected larvae and is suitable for larger screens. A key result is the observation that when *Drosophila* larvae are used as hosts, *Steinernema* appears to be the more effective parasite and therefore parasitisation strategies may differ between the two species. This should be taken into account when modifier screens are designed. For example in order to identify dominant modifiers, based on the work presented here it is possible to use either a high infectious dose of *Heterorhabditis* or a

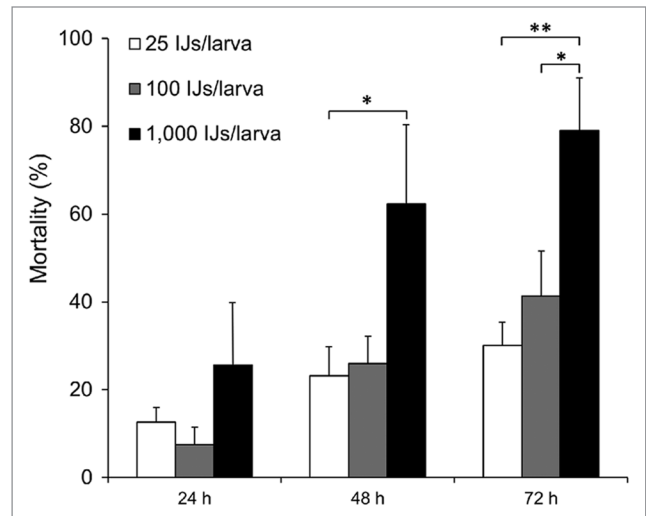


Figure 3. Effect of different doses on mortality rate of *Drosophila* larvae in nematode infection with *H. bacteriophora*. Larvae were infected with nematodes at the indicated infectious doses in a 96-well assay as described in Material and Methods (mean \pm S.D; confidence levels are: * <0.05; ** <0.01).

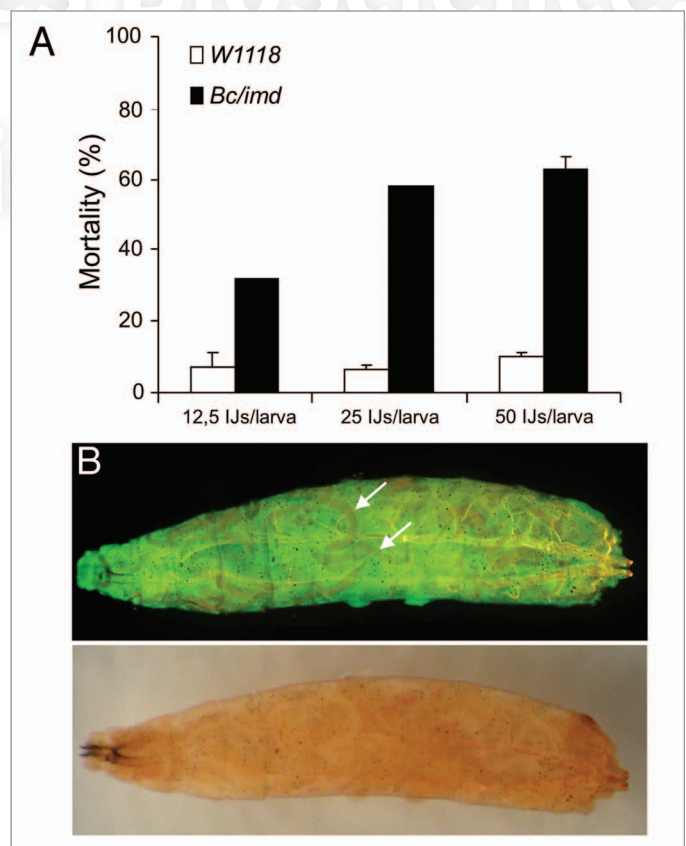


Figure 4. (A) The plastic bag assay recapitulates the results of a 96-well assay in a comparison between wild-type and *Bc/imd* larvae. Mortality of *D. melanogaster* was documented 48 h after the infection with different doses of *H. bacteriophora* in plastic bags at 29°C, (B) a *Bc/imd* larva infected with *H. bacteriophora* harbouring GFP-expressing *P. luminescens*, visualized under UV light (upper part, arrows-note the nematodes which are visible as black shades in a green fluorescent background due to systemic infection with GFP-expressing *Photorhabdus*) and normal light (lower part).

lower concentration of *Steinernema*. Such screens may partially identify the same effector mechanisms but also responses that are specific and may correlate with the level of specialization.

One aspect of this specialization is even apparent in our simple infection setup, where we observe differences in the support medium (paper vs. agarose) with the *Steinernema* and not when we use *Heterorhabditis*. This finding is consistent with previously described differences in the ways the two nematodes seek out their prospective hosts. In case of agarose it is possible for larvae to move to the top of wells and partly avoid the contact with nematodes. Wrinkled tissue paper fills the whole well and the distribution of nematodes is expected to be more equal. The difference in mortality between media is not apparent during *H. bacteriophora* infections. We propose that the explanation may lie in the different ecology and behavior of the two nematode species. *H. bacteriophora* cruise to find hosts and are not dependent on the medium whereas *S. feltiae* can benefit more from tissue paper because it is intermediary in its search behavior sharing some characteristics of both ambush and cruise foragers.^{18,19}

To identify positive regulators and effectors involved in the host immune response, we have established conditions for both the 96-well and the plastic bag assay that lead to moderate mortality of *Drosophila* larvae allowing us to monitor an increase in mortality in hypomorphic or null-mutants. The lower pathogenicity of the *H. bacteriophora* made it a suitable infectious agent for screens (Figs. 1 and 3). To allow larger screens for host immune responses in a systemic and time-efficient manner, we developed the plastic bag assay, by setting up an infection of a group of host larvae sharing the same space, rather than being infected singly. Additionally, the application of the *H. bacteriophora* harbouring GFP expressing *P. luminescens* made it feasible to monitor the infection from a very early time point after infection and in real-time (see Sup. Material). A drawback of this method is that a small fraction of the larvae are injured and may be infected due to crowding and injuring from other larvae rather than the infection with EPNs (i.e., they were not GFP positive). We anticipate that the method in its present version will be suitable for targeted screens with a throughput of dozens of genes/month.

The tripartite model: nematode, bacteria and *Drosophila* larva, has been recently employed in several studies to identify factors involved in the host immune response.^{9,14,15} However, the full picture of the host-pathogen interaction is far from being understood. We expect that screens based on the methods developed here will help in the further characterization of this natural infection model.

Material and Methods

Culture of nematodes. Entomopathogenic nematodes *Steinernema feltiae* (isolated from Prosenice) and *Heterorhabditis bacteriophora* (H222 strain, isolated from Pouzdřany) were kindly provided by Dr. Z. Mracek (Institute of Entomology, Ceske Budejovice, Czech Republic). Both nematodes were cultured

in vivo using larvae of the great wax moth, *Galleria mellonella* at room temperature (RT). *Drosophila* larvae are not suitable hosts for laboratory culture of EPNs as the yields of IJs are too low for routine use in nematode infection assays. Released IJs were collected and stored in tap water with 0.075% formaldehyde at RT.

Culture of *Drosophila melanogaster*. Fly strains were kept under standard condition. Infections of *Drosophila* larvae were conducted with wild-type *W¹¹¹⁸* from Bloomington stock center (stock 6326) and *Bc/imd* double mutant generated by us, as positive control due to its high susceptibility to nematode infection.¹⁵

Infection of *D. melanogaster* larvae. Infection of *D. melanogaster* larvae with IJs was modified after Hallem et al. Second and third instar *D. melanogaster* wild-type larvae (*W¹¹¹⁸*) were rinsed briefly in water and placed in wells of a microtiter plate (96-well, flat bottom, Costar) containing 10 μ l of 1.25% agarose (Lonza) or approx. One cm² of tissue paper. IJs were washed with tap water, diluted to the final density and 10 μ l of required nematode suspension was added to each well containing one *Drosophila* larva. For uninfected controls, 10 μ l of tap water was added to each well containing *Drosophila* larva. The assay plate was covered with Parafilm[®].

In the infection assay in plastic bags (Fig. S1), 50 larvae were placed in a re-sealable transparent plastic bag (6 x 10 cm), in which one layer of tissue paper (with stapled edges preventing larvae from drowning) was soaked with different dose of nematode suspension (12.5, 25, 50 IJs/*Drosophila* larva).

Infections were conducted at RT in the dark. Survival was quantified under a dissecting microscope after 24, 48 and 72 h. Survival rates were determined based on movement, either spontaneous or in response to gentle prodding with forceps and typical reddish coloration of cadavers caused by *Photorhabdus*¹⁶ in case of *H. bacteriophora* or yellow coloration in case of *S. feltiae*. GFP-expressing *Photorhabdus* (a kind gift from Todd Cliche) was used to monitor the infection in the plastic bag assay, see Figure S1 and Movie S1.

All experiments were run in triplicates using 48–100 larvae of *Drosophila* larvae per group. The results are expressed as the mean \pm SD; the level of significance was analyzed by Student's t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material may be found here: www.landesbioscience.com/journals/fly/article/19553

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