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METHODS AND TECHNICAL ADVANCES

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Monitoring the effect of pathogenic nematodes on locomotion of *Drosophila* larvae

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ABSTRACT

One of the key factors that determine the interaction between hosts and their parasites is the frequency of their interactions, which depends on the locomotory behavior of both parts. To address host behavior we used natural infections involving insect pathogenic nematodes and *Drosophila melanogaster* larvae as hosts. Using a modified version of a recently described method (FIMTrack) to assess several parameters in larger sets of animals, we initially detected specific differences in larval food searching when comparing *Drosophila* strains. These differences were further influenced by the presence of nematodes. Given a choice, *Drosophila* larvae clearly avoided nematodes irrespective of their genetic background. Our newly developed methods will be useful to test candidate genes and pathways involved in host/pathogen interactions in general and to assess specific parameters of their interaction.

Introduction

Insect pathogenic nematodes (entomopathogenic nematodes, EPNs) are used to control pest species in an environmentally sustainable way.[1] They also provide insight into the mechanisms that protect the insect hosts from being infected.[2] In addition to insects of agricultural importance, hosts include the genetically tractable fruitfly *Drosophila melanogaster*.[3] In a landmark study it was shown that the canonical immune pathways Toll and Imd are induced but dispensable for protection against nematodes.[3] Instead, the hemolymph clotting system was shown to delay EPN infections with a contribution from complement-like components.[4-6] This was further confirmed when a *Drosophila* member of the chitinase-like proteins (imaginal disk growth factor 3, IDGF3) was shown to localize to hemolymph clots and *idgf3* mutants showed increased sensitivity toward infections with *Heterorhabditis bacteriophora*, one of the most commonly used EPNs.[7] Whole genome transcription profiling identified genes that are induced

upon EPN infections, both in *idgf3* mutants and in wild type animals, providing a substantial array of targets for further studies.[6-8]

In addition to physiologic- and immune-protective mechanisms, potential hosts may also avoid being infected by preventing or reducing contact with the pathogen in the first place. While nematode behavior has been shown to differ between parasitic nematode species,[9,10] less is known about the potential avoidance behavior of their insect hosts. Similar to parasites, host avoidance is expected to show substantial variability and therefore, to obtain statistical significance, a method that allows large throughput screening of individuals is desirable.

To study whether the behavior of *Drosophila* larvae was influenced by the presence of EPNs, we used a recently developed method, which allows to simultaneously track the migration of several larvae.[11,12] The method relies on the detection of crawling larvae by frustrated internal reflection of infrared light (FTIR). Using FIM (FTIR-based imaging method) larvae are

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individually tracked over a given period and their movement analyzed.[12] FIMTrack allows the extraction of several parameters including the larval distance traveled, larval velocity, the rate of larval bending, the rate of time spent on moving, as well as the fraction of larvae that coil up.[13] Using FIM, we assessed inter-strain variation, the influence of nematodes on larval locomotion and avoidance behavior toward nematodes.

Material and methods

Fly strains and handling

Fly strains were maintained under standard condition with potato mash as the main ingredient. Two different genotypes of *Drosophila melanogaster* were used: w^{1118} and Canton S. Both w^{1118} and Canton S strains were obtained from the Bloomington stock center. The cross between w^{1118} x Canton S was used to determine the effect of the 2 genetic backgrounds. Eggs were collected on fly food with addition of yeast at 25°C. Randomly selected larvae at 72 – 76 h after egg deposition were washed out from the fly food with room temperature tap water and used unless noted otherwise. Larvae were starved for 2 h before the start of the experiments. 8–10 replicates, which contained 120 larvae in total, were used per condition for each experiment. Each replicate consisted of 12–15 larvae, which could be successfully traced during the complete recording period.

Nematode culture

Two different species of EPNs were used - *Heterorhabditis bacteriophora* and *Steinernema feltiae* which harbor the symbiotic bacteria *Photorhabdus luminescens* TT01 and *Xenorhabdus bovienii*, respectively. Both EPNs were cultured in the Greater Wax Moth *Galleria mellonella* at room temperature. Infective juveniles (IJ) of EPNs were maintained in tap water with wet sponges. EPNs were diluted in tap water to a density of 50 IJ/10μl for both species. The age of the nematodes ranged between 15–50 d after the emergence from the hosts' cadaver. *C. elegans* (N2) were used as negative control. Maintenance was performed according to standard protocols obtained from www.wormbook.org.[14] Different developmental stages (L1 – L4) were used, due to the fact that these stages are more motile compared with the relatively inactive

dauer stages and therefore more similar in their behavior to EPN infective juveniles (refs.[15,16]; see also discussion of EPN foraging strategies below).

Gel preparation

A 0.8% agarose gel of 2mm thickness was used as a crawling surface. To improve illumination, the gel was moisturized with tap water. A salt barrier was poured to prevent larvae from escaping the experimental area by adding 5M NaCl in 2.5% agarose gel in deionized wate.[12] To prevent drying out, the gels were surrounded by wet tissue paper.

Image capture

Images were captured in a dark room without any additional light source except the built-in infrared light, which was generated by the FIMTrack. The size of the images was 1000 ×1000 pixels and 1200×1000 pixels for circular and rectangular arena, respectively. Images were captured with a frequency of 1 FPS (frame per second) for 720 s. The scale factor was 100 pixels/cm.

Processing of the pictures

For acquiring and processing images, Basler A601f camera coupled with FIMTrack v2 Windows (X86) software[12] (downloaded from http://fim.uni-muenster.de/) was used. All the larval locomotion tracks were initially recorded and processed with the software. Later, all tracks were manually verified so that data for each track belonged to a given larval trajectory. Data gathered from software were processed and visualized in Prism 6 (USA GraphPad). A Fisher's LSD test by GraphPad Prism 6 was used to determine statistical significance.

Results

The genetic background influences larval food searching behavior

During preliminary experiments, we observed that *Drosophila* larvae moved from a food source onto a water-soaked filter paper (without nematodes). In contrast, if the filter paper was soaked in a solution containing nematodes (*H. bacteriophora*), fewer larvae left the food. This observation inspired us to establish methods that allowed us to quantitatively assess food-

searching behavior in the presence and absence of nematodes. We placed larvae in a circular fashion on an agarose based crawling platform that was covered with water containing EPNs, and we assessed the chemotactic behavior that required larvae to reach a food source in the center of the platform (Fig. 1).

To assess the influence of the genetic background on the locomotory behavior, we tested the chemotactic behavior toward a food source using larvae from a w^{1118} and a Canton S strain, as well as respective heterozygotes, which revealed significant differences in chemotactic behavior (Fig. 1B-E). Canton S larvae were most successful in reaching the food while w^{1118} larvae showed a lower tendency to move toward food and heterozygotes had an intermediate phenotype regardless of whether *H. bacteriophora* or *Steinernema feltiae* was present on the platform (Fig. 1C and D). When only the successful attempts to reach the food source were analyzed, a trend toward a positive influence of EPNs on food searching behavior was observed (Fig. 1E). This was most obvious with larvae from the crosses between w^{1118} and Canton S, more of which reached the food source in the presence of both EPNs. Taken together, this indicates that both the genetic background and the presence of EPNs may influence larval food searching.

Drosophila *larvae adjust their locomotory behavior in the presence of nematodes*

To account for genetic differences during infection experiments, we individually compared the locomotion of larvae on way to the food source with and without EPNs (Fig. 2 A and B) using FIM (representative tracks are shown in Fig. 2 C-E"). In the presence of nematodes, Canton S larvae traveled shorter distances while w^{1118} seemed unaffected when compared with the setting without nematodes. For Canton S larvae the effect was more significant with *S. feltiae* than with *H. bacteriophora* while for the heterozygotes it was only significant with *S. feltiae*. The shorter distances resulted from a combination of reduced speed (Fig. 2B) and shorter periods of locomotion (shorter Go-phase, Fig. 3A), in Canton S larvae. Some of these features are retained in the crosses while w^{1118} larvae are again unaffected. Conversely, the bending rate and the incidence of coiled-up larvae increase in w^{1118} but not in Canton S larvae (Fig. 3B and C). Here, too, the heterozygotes showed an intermediate phenotype in

some combinations. Bending preferences (left vs. right) remained the same irrespective of genetic background or mixing with nematodes (Fig. 3D). Altogether this shows that *Drosophila* larvae change their locomotion in the presence of nematodes but differ in locomotory parameters depending on the genetic background.

Despite the differences in avoidance behavior between the different strains, the net outcome may still be comparable, i.e. nematodes may still be avoided to similar extents. To test this we used a setup where we allowed larvae to choose between a nematode-free and a nematode-infested area on their way to food (Fig. 4A). In this setting, larvae from both strains fared equally well and avoided the nematodes (Fig. 4B and C). This was true for both EPNs and was also reflected in the different distances the larvae had traveled in nematode-containing and nematode-free areas (Fig. 4D and E). This means that given a choice, larvae with different genetic backgrounds sense the presence of nematodes and prefer to obtain food via a nematode-free area.

When given a choice between an area containing EPNs and non-pathogenic *Caenorhabditis elegans* to obtain food, *Drosophila* larvae of both genotypes prefer *C. elegans* to *H. bacteriophora* while in response to *S. feltiae*, only Canton S showed a preference toward *C. elegans* (Fig. 5). *Drosophila* larvae preference was assessed using both the time spent and the area covered in nematode-containing areas (Fig 5 A and C).

Discussion

We have adapted a described previously method (FIM[11]) to track larval locomotion for use in infection studies. In this study we focused on an ecologically relevant natural infection system that involves 2 *Drosophila* strains (Canton S and w^{1118}) as hosts and 2 species of EPNs. The software that had previously been developed to analyze FIM data allowed us to assess different aspects of larval behavior. We found variations in larval responses both dependent on the host strain and the EPN used. In the presence of nematodes, Canton S larvae appeared to reduce their locomotory activity while w^{1118} larvae show increased bending frequencies. The rationale behind using the 2 strains was that they are commonly used as control strains and many transgenic lines are in a w^{1118} background. Of note, w^{1118} is a null allele of
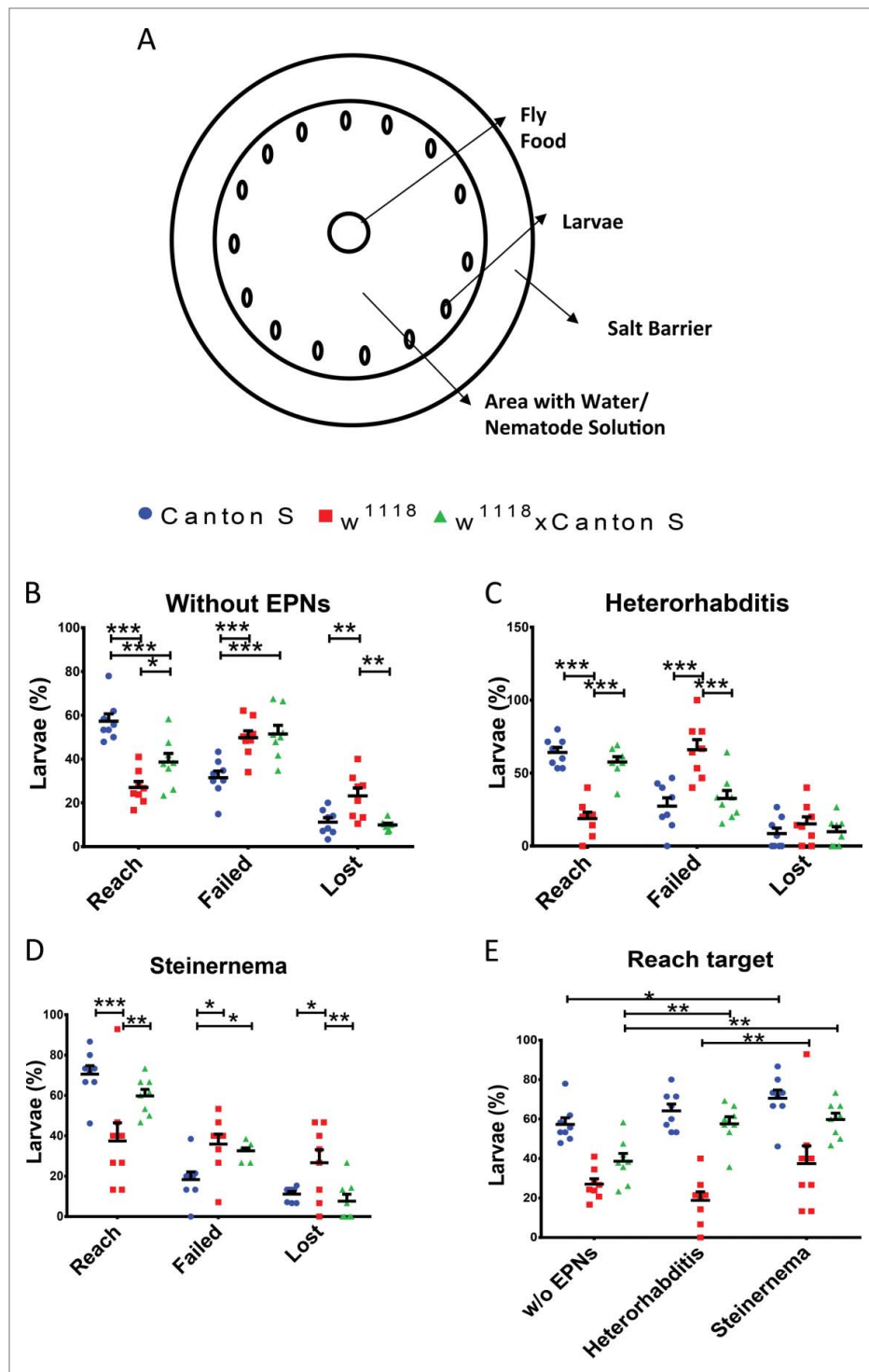
Figure 1. Locomotion of larvae toward a food source differs depending on genetic background. Larvae were placed in a ring-like manner in a circular arena with a centrally located food source (A) The percentage of larvae reaching the food source, staying outside the food and escapers from the arena was scored (B-D) for 3 genetic backgrounds (Canton S, w[1118], and a cross between the 2). Either water or one of 2 nematode species (GFP-positive *H. bacteriophora* and *S. feltiae*) were used to cover the arena. (E) Successful attempts to reach the food source ("reach" in parts B-C) were compared between absence/presence of the 2 EPNs. Each dot represents the mean value for a replicate and the middle line represent the mean of the replicates. Error bar represents SEM; sample size was at least 115 ($n_{min} = 115$ larvae) (B-E).
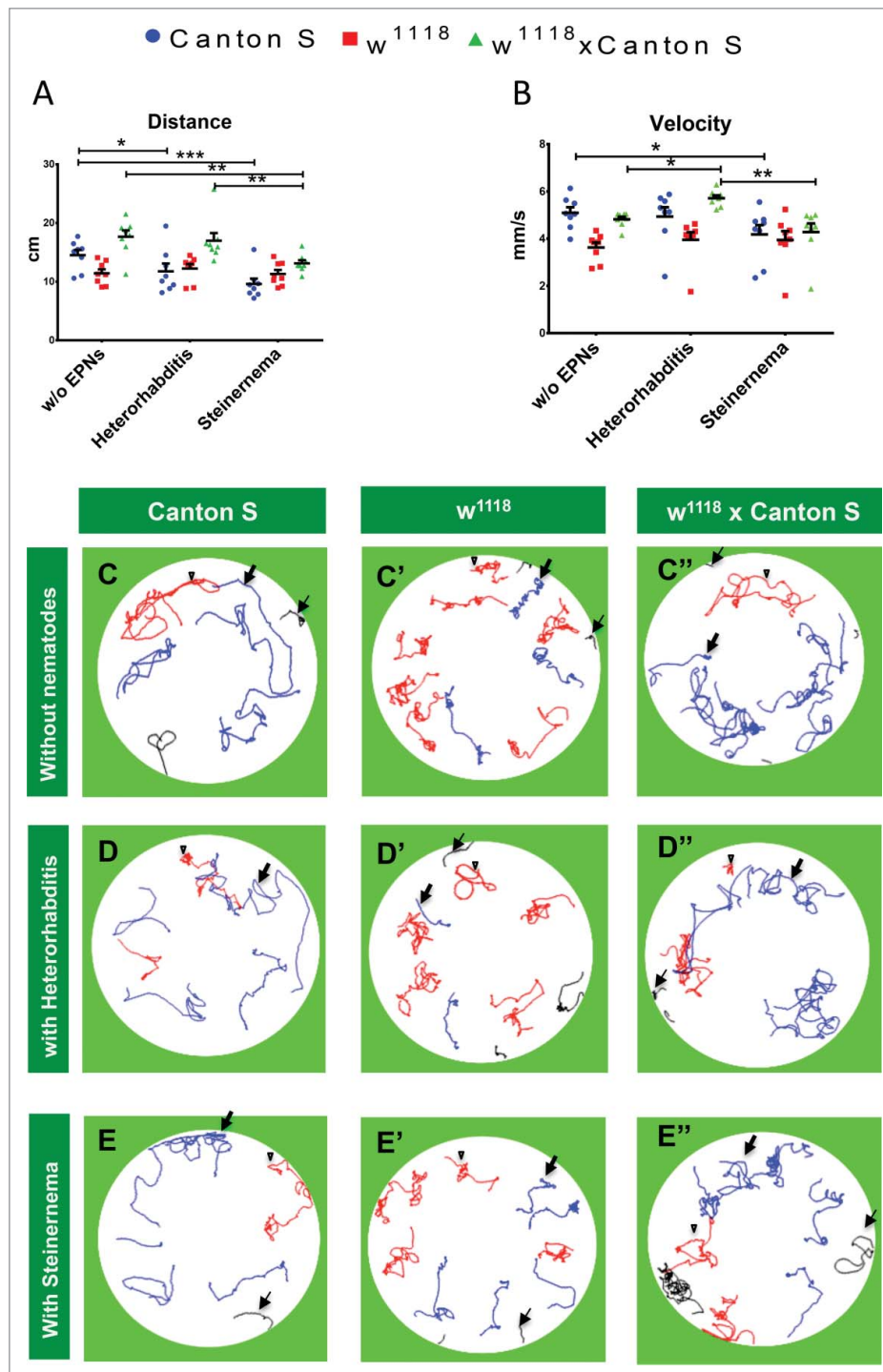
Figure 2. Canton (S)but not w[1118] larvae reduce the distance and speed of travel in the presence of nematodes. Individual larval tracks were recorded in the absence of nematodes or upon co-incubation with one of 2 EPNs (*H. bacteriophora* or *S. feltiae*) using FIM. (A−B) the distance covered by individual larva as well as its velocity was extracted using FIMTrack. Each dot represents the mean value for a replicate and the middle line represent the mean of the replicates. Error bar represents SEM; sample size was at least 115 (n_{min} = 115 larvae). (C−E) examples for individual tracks using different setups (fly strains and with/without nematodes) are shown. Three exemplary tracks are indicated by arrowheads, thin arrows and thick arrows, which represent larvae that failed to reach food, reach food and lost larvae (escaped from the experimental area), respectively. Often several tracks were connected to one-another; therefore, it was necessary to manually validate the data by tracking larvae individually.
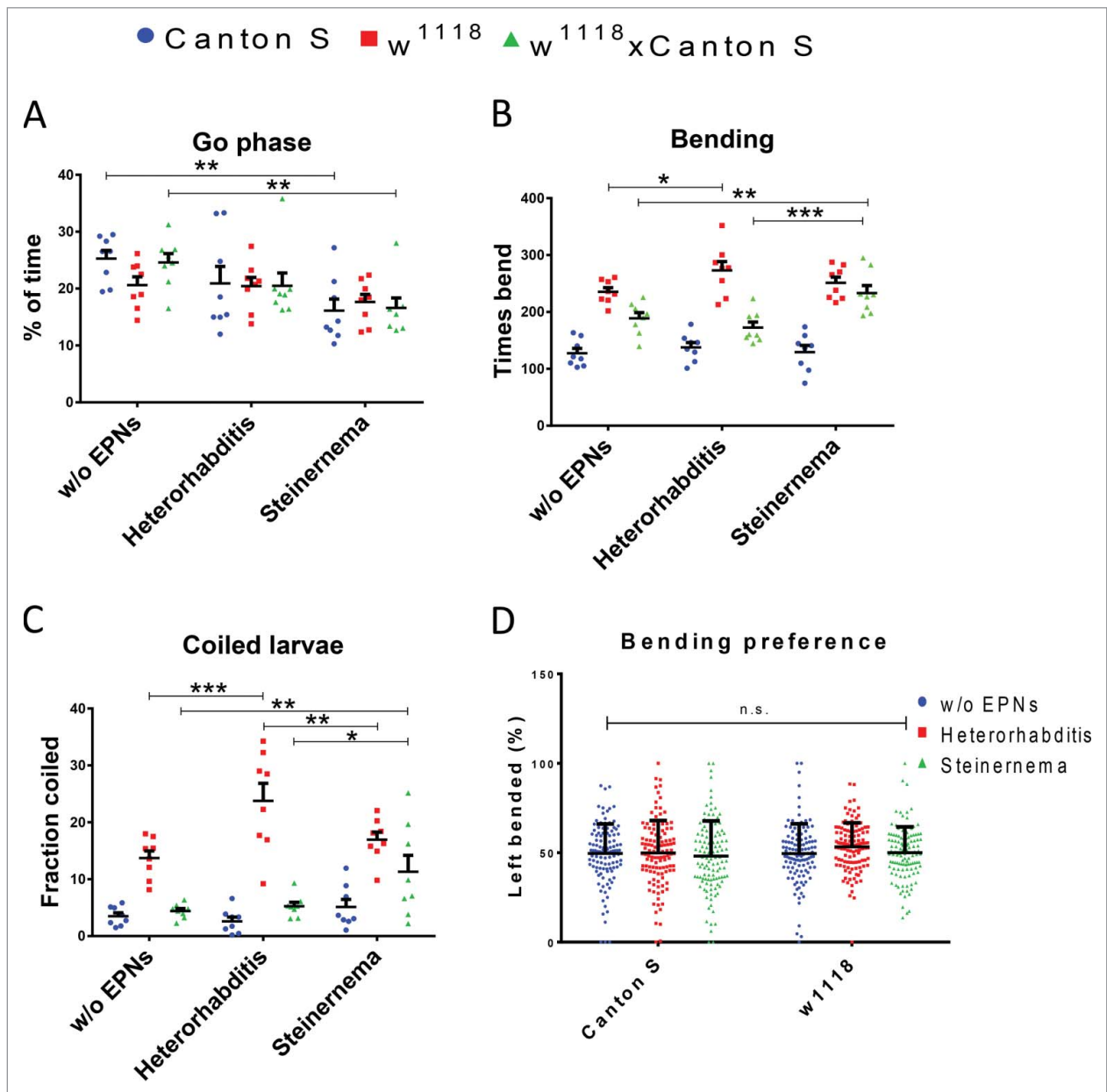
Figure 3. Canton (S)and w[1118] differ in their response toward the presence of nematodes. Several parameters were extracted from the FIM-based data set using FIMTrack, including the number of recorded frames where the larvae moved (Go-phase, A), the frequency of larval bending (B), the frequency of coiled-up larvae (C) and the bending preference (left vs. right). Note that in line with the data from Figure 2, Canton S larvae reacted to the presence of *S. feltiae* by lowered activity while both bending and the incidence of coiled larvae were increased in w[1118]. Each dot represents the mean value for a replicate and the middle line represent the mean of the replicates. Error bar represents SEM; sample size was at least 115 ($n_{min} = 115$ larvae) (A-C). (D) Each dot represents one larvae and the middle line represents the mean of the replicates. Error bar represents SD; sample size was at least 115 ($n_{min} = 115$ larvae).

the *white* gene, which encodes a transmembrane ABC transporter. Loss of the white protein is associated with changes in pigmentation of the eye and results in impaired vision[17] but affects additional biologic processes including locomotion and courtship behavior in adults,[17-19] in line with our observations. On a practical note these differences have to be taken into account when mutant phenotypes are analyzed,

for example when knockdowns are performed using crosses with w[1118]. When larvae were given the choice to avoid nematodes, the difference between w[1118] and Canton S seems less important leading to robust avoidance behavior irrespective of the strain used. Thus, analysis of different locomotory parameters appears to address different aspects of the genetic differences in *Drosophila* hosts. We also
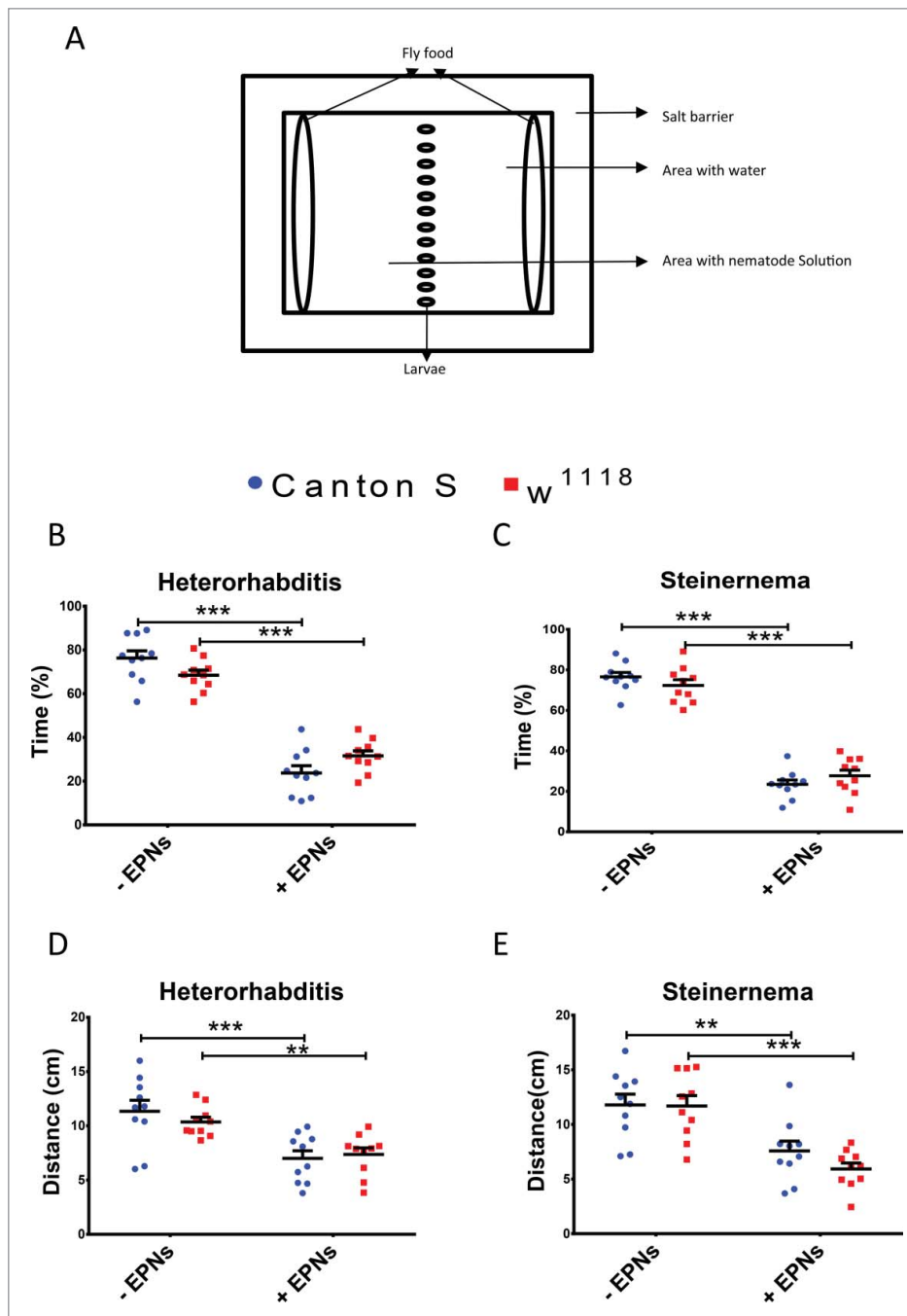
Figure 4. Both w[1118] and Canton (S)control strains show nematode avoidance behavior. (A) A schematic overview of the setup used to analyze nematode avoidance behavior: larvae were placed in the middle of an arena where they could choose to travel to the food source via either nematode-free or nematode-containing area. (B-C) shows the time spent and (D-E) the distance covered in the respective areas. Significant differences were observed with both EPNs. Each dot represents the mean value for a replicate and the middle line represent the mean of the replicates. Error bar represents SEM; sample size was at least 115 ($n_{min} = 115$ larvae) (B-E).

observe that some parameters differ more significantly when *S. feltiae* is used instead of *H. bacteriophora*, for example in the reduction of the distance covered by larvae (Fig. 2A). Since *S. feltiae* is more pathogenic for *Drosophila* larvae,[5] stronger avoidance would indeed make biologic sense. In addition,

for dispersal and host searching, *S. feltiae* prefers an ambushing strategy in which the nematode remains more stationary while waiting for potential hosts.[10] Thus, a reduction in locomotion is expected to minimize the risk for *Drosophila* larvae to encounter *S. feltiae* infective juveniles.[10] This is in contrast to *H.*
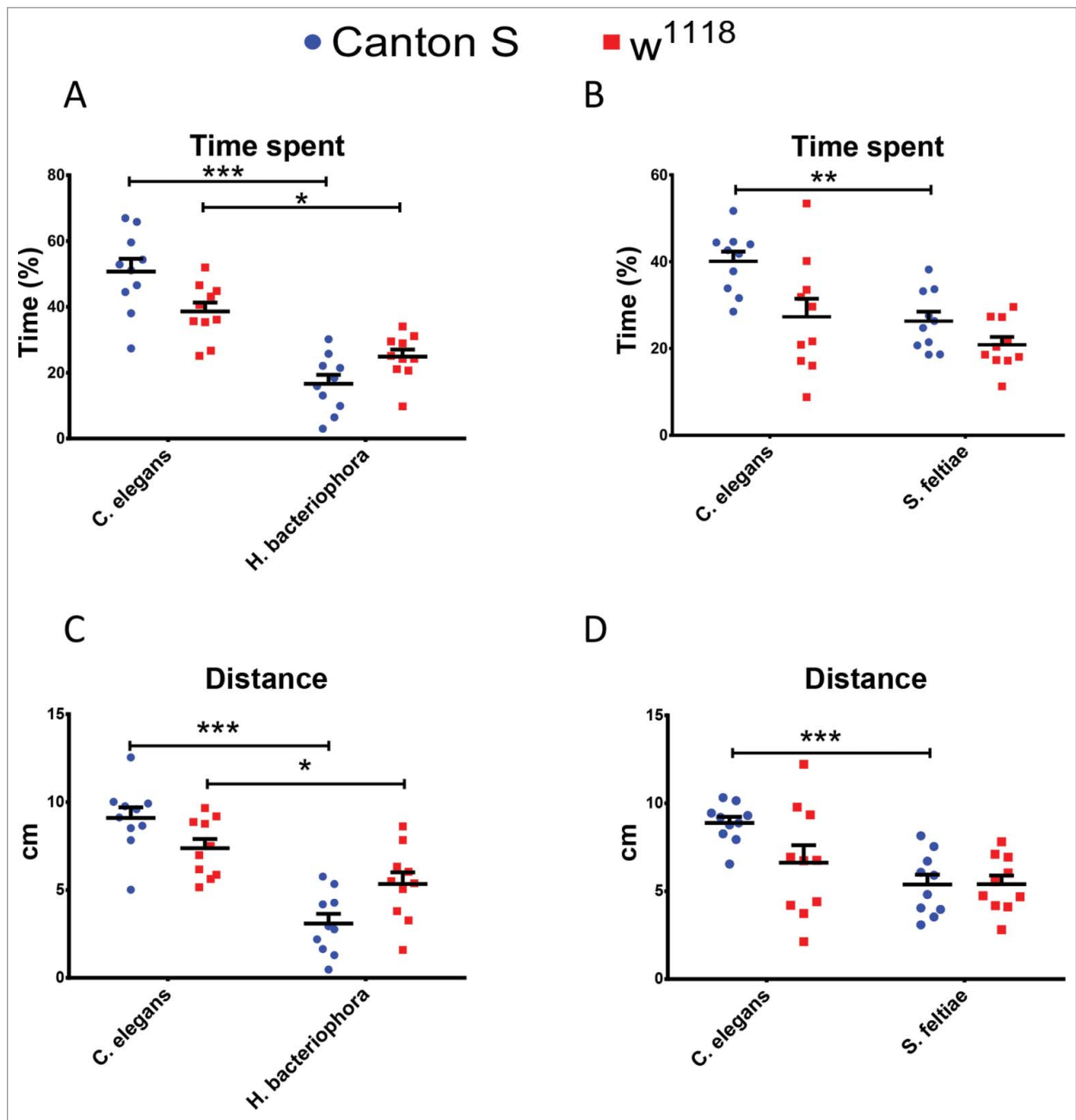
Figure 5. Larvae avoid EPNs in the presence of *C. elegans*. w[1118] and Canton S were placed in an arena such as in Figure 4 except that one half of the arena contained *C. elegans* and the other half EPNs. Both genotypes prefer non-pathogenic *C. elegans* over *H. bacteriophora* assessed by the time spent and the distance covered among EPNs while only Canton S avoids *S. feltiae*. Each dot represents the mean value for a replicate and the middle line represent the mean of the replicates. Error bar represents SEM, sample size was at least (n_{min} = 115 larvae).

bacteriophora, which prefers a cruising strategy for host searching. It should be noted that the non-pathogenic control species *C.elegans* includes different developmental stages, which - although more motile than dauer stages - may differ in their locomotory behavior. In addition, differences in the chemotactic behavior of *C.elegans* and *H. bacteriophora*[20] are expected to influence the interaction between nematodes and insect larvae.

Taken together, we present a high throughput tracking method (FIM), which was previously developed to study larval behavior and which we adapted to analyze modifications of host behavior in an infection context. The modified method will

be suitable for both ecological studies on different species/populations and for genetic studies of mutant hosts and their parasites.[21,22] It will be useful for targeted secondary screens of candidate genes identified in transcriptome studies[6-8] as well as for testing both non-pathogenic nematodes and EPNs that use different foraging strategies and rely on different combinations of virulence factors.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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