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Response of Three Cyprinid Fish Species to the Scavenger Deterrent Factor Produced by the Mutualistic Bacteria Associated with Entomopathogenic Nematodes

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ABSTRACT

The symbiotic bacteria, *Photorhabdus* and *Xenorhabdus* associated with entomopathogenic nematodes (EPNs) in the genera *Heterorhabditis* and *Steinernema*, respectively, produce a compound(s) called the Scavenging Deterrent Factor (SDF). SDF deters a number of terrestrial insect scavengers and predators and one bird species from feeding on host insects killed by the nematode-bacterium complex but has not been tested against aquatic vertebrates. Moreover, the *Heterorhabditis*-*Photorhabdus* association is believed to have evolved in an aquatic environment. Accordingly, we hypothesized that SDF will deter fish from feeding on nematode-killed insects and tested the responses of three omnivorous fresh water fish species, *Devario aequipinnatus*, *Alburnoides bipunctatus,* and *Squalius pursakensis*, to SDF in the laboratory. When the fish were exposed to *Galleria mellonella* larvae killed by the *Heterorhabditis*- or *Steinernema*-bacterium complex at 2 or 4 days post-infection, all three fish species made several attempts to consume the cadavers but subsequently rejected them. However, all fish species consumed freeze-killed control larvae. In a choice test, when *D. aequipinnatus* or *A. bipunctatus* were offered a pair of nematode-killed larvae, both fish species rejected these cadavers; when offered a nematode-killed larva and a freeze-killed larva, both fish species consumed the freeze-killed larva but not the nematode-killed one. In further tests with *D. aequipinnatus*, there was no significant difference in the number of 2-day-old *Bacillus thuringiensis* subsp. *kurstaki*-killed (*Btk*) larvae consumed compared to freeze-killed larvae, but significantly fewer 4-day-old *Btk*-killed larvae were consumed compared to freeze-killed larvae. When *D. aequipinnatus* was fed *G. mellonella* larvae killed by the symbiotic bacteria, the fish rejected the cadavers. When given freeze-killed or

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nematode-killed mosquito (*Aedes aegypti*) larvae, the fish consumed significantly more of the former larvae (99%) compared to the latter (55%). When *D. aequipinnatus* was placed in a symbiotic cell-free supernatant for 18 h, a significant reduction in consumption of freeze-killed larvae compared to cell-free *Btk* or control broth supernatant was observed. We showed that SDF protects the nematode-killed insects from being consumed by omnivorous fishes and suggests that they will have minimal effects on recycling of EPNs in the aquatic environment.

Keywords: *Photorhabdus, Xenorhabdus,* Entomopathogenic nematodes; Scavenger Deterrant Factor; Warning signal.

1. Introduction

Entomopathogenic nematodes (EPNs) in the families Steinernematidae and Heterorhabditidae are lethal insect parasites that are associated with mutualistic bacteria in the genus *Xenorhabdus* or *Photorhabdus,* respectively (Hazir et al., 2003; Lewis and Clarke, 2012). These EPNs have adapted specific mechanisms to transmit the bacteria to their insect hosts (Dillman et al., 2012). The infective juveniles (IJs) of the nematodes, the only free-living stage, infect an insect host through natural openings (the mouth, anus, or spiracles), or in some cases, through the soft, thin cuticle. After entering the host's hemocoel, the IJs release their bacterial symbionts which are primarily responsible for killing the host by toxemia or septicemia within 24 to 48 h. The multiplying mutualistic bacteria not only provide nutrition to the nematodes but also degrade the host's tissues and protect the insect cadavers against secondary microbial invaders by producing immune-suppressive and antibiotic compounds (Dowds and Peters, 2002; Shapiro-Ilan et al., 2015). After the death of the

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host, the nematodes feed on the host tissues and the mutualistic bacteria, mature and reproduce. The EPNs can complete up to three generations within the host cadaver depending on the available resources and exit as IJs in 1-3 weeks post-infection (Gaugler and Kaya, 1990).

Both *Xenorhabdus* and *Photorhabdus* spp. produce a chemical compound(s) that can affect the behavior of scavengers to the insect cadaver (Zhou et al., 2002; Griffin, 2012; Gulcu et al., 2012). This compound(s) from the bacterial symbionts within the cadaver can serve as a repellent to scavengers including ants (Baur et al., 1998; Zhou et al., 2002), crickets, cockroaches, springtails, wasps (Gulcu et al., 2012; Ulug et al., 2014), and predatory insects (Foltan and Puza, 2009; Jones et al., 2016). Moreover, *Heterorhabditis bacteriophora*-killed insects were not consumed by the insectivorous European robin, *Erithacus rubecula*, and this behavior was attributed to the red color produced by *Photorhabdus* that may have been reinforced by the unpalatable taste when the cadavers with the nematode-bacterium complex were sampled (Fenton et al., 2011). The deterrent chemical compound(s) was initially called the “Ant Deterrent Factor” (ADF) (Zhou et al., 2002) and then re-named as the “Scavenger Deterrent Factor” (SDF) by Gulcu et al. (2012) because it deterred other insect scavengers. Recently, Jones et al. (2016) demonstrated that a warning odor produced by *H. bacteriophora*-killed insects is a key strategy in colony defense for EPNs. That is, this “parasite-induced aposematism” or warning signal served as a deterrent against the nocturnal, soil-inhabiting beetle, *Pterschusis madidus*, which did not feed on cadavers colonized by *H. bacteriophora*, thus serving as a means to protect the developing nematodes in the cadaver. According to Gamberale-Stille and Guilford (2004), aposematism signaling is a common defensive mechanism by

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organisms using color, odor or movement to avoid or prevent consumption by predators.

Although the natural habitat of EPNs is the soil (Kaya and Gaugler, 1993), EPNs can also infect aquatic insects (Begley, 1990). Welch (1961) and Welch and Bronskill (1962) were the first to demonstrate that EPNs infect the larvae of the mosquito, *Aedes aegypti*, in the laboratory and field. Studies by Welch (1961), Poinar and Leutenegger (1971), Finney and Harding (1981), Poinar and Kaul (1982) and Molta and Hominick (1989) generally showed that EPNs infected and killed mosquito larvae, but a number of factors such as damage to the IJs during ingestion, immune responses, and spatial separation of the host and EPNs affected their efficacy. More recently, Cagnolo and Almiron (2010) reported that 75% of *Ae. aegypti* were killed by *Steinernema rarum* at a rate of 400 IJs/larva, and Peschiutta et al. (2014) stated that 84% of *Ae. aegypti* were killed by *H. bacteriophora* and that the nematodes could develop and reproduce and had the potential for the continuity of its life cycle in an aquatic environment. Finally, Cardoso et al. (2015) demonstrated that the EPNs, *H. baujardi* and *H. indica,* were highly virulent to *Ae. aegypti* larvae under laboratory conditions, whereas *S. carpocapsae* was avirulent to this mosquito species.

All previous studies with SDF or nematode-killed insects were conducted with terrestrial invertebrate scavengers and predators and an insectivorous bird. In an aquatic environment, EPN-killed mosquito larvae will be exposed to many omnivorous or scavenging fish species which may reduce EPN survival and affect recycling of the nematode. Moreover, Poinar (1993) proposed that *Heterorhabditis* evolved from a marine ancestor, and Boemare (2002) suggested that the symbiosis between *Heterorhabditis* and *Photorhabdus* may have originated at the seashore interface. Thus, we evaluated the effect of SDF against aquatic vertebrate

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omnivores/scavengers. We hypothesized that SDF produced by the EPN symbiotic bacteria also deters aquatic omnivores/scavengers. Accordingly, our objective was to evaluate the response of vertebrate omnivores/scavengers in the aquatic environment against EPN-killed insects. Here, we conducted experiments with the fresh water, omnivorous, cyprinid fishes, *Devario aequipinnatus*, *Alburnoides bipunctatus*, and *Squalius pursakensis* using EPN-killed *Galleria mellonella*. We conducted further research to evaluate whether *D. aequipinnatus* consumed (1) *Bacillus thuringiensis*-killed *G. mellonella* larvae, (2) 2-day-old *G. mellonella* larvae injected with the symbiotic bacterium, *Photorhabdus luminescens* or *Xenorhabdus stockiae*, (3) nematode-killed *Ae. aegypti* larvae, and (4) first-generation females of *Steinernema siamkayai* or hermaphroditic adults of *Heterorhabditis indica*. In addition, we conducted an experiment to assess whether *P. luminescens* or *X. stockiae* produce sufficient deterrent compound(s) to adversely affect the behavior of *D. aequipinnatus*.

2. Material and methods

2.1. Source of insects

The experimental insects used in this study were larvae of the greater wax moth, *G. mellonella* and *Ae. aegypti*. *G. mellonella* was reared on an artificial diet (22% maize meal, 22% wheat germ, 11% dry yeast, 17.5% bee wax, 11% honey and 11% glycerin) at 28°C in the dark according to Han and Ehlers (2000). The last instar *G. mellonella* weighing between 190 and 220 mg were used for all experiments. For *Ae. aegypti*, fourth instars were obtained from the National Center for Disease Control (NCDC), Mettupalayam, Tamil Nadu, India. These larvae were maintained in partitioned trays containing deionized water (Chanthini et al., 2015) before being used in experiments.

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2.2. Nematode cultures and test insects

Two- and four-day-old nematode-killed *G. mellonella* experiments were conducted both in India and Turkey. The EPNs, *S. siamkayai* (KPR-4) and *H. indica* (KPR-8) isolated from Tamil Nadu Province, India (Raja et al., 2011) and *S. feltiae* (09-20) and *H. bacteriophora* (09-38) isolated from Turkey, were used in the experiments. All nematodes were reared in the last instar of *G. mellonella* according to Kaya and Stock (1997), and the IJs were stored in distilled water at 15°C incubator for no more than 3 weeks before they were used.

To obtain nematode-killed *G. mellonella*, 1000 IJs of a given nematode species were pipetted with 1 ml distilled water on the surface of double filter paper lined in 90 mm diam. Petri dish. Then, 10 last instar *G. mellonella* were added to each Petri dish and incubated in the dark at room temperature (25°C ±2°C). The cadavers were used after 2 or 4 days for the experiments.

When freeze-killed *G. mellonella* larvae were used as controls in experiments, they were placed at -18°C for 1 h. After removal from the freezer, they were kept at 30°C for at least 1 h for the development of normal gut bacterial flora inside the cadaver and for melanization to occur.

For the mosquito experiments, 20 last instar *Ae. aegypti* larvae were infected using 50 IJs/larva of *S. siamkayai* or *H. indica* in plastic cups having 150 ml of water. The nematode-killed larvae were used after 48 h for the experiments. Before using the mosquito cadavers for the experiments, they were examined under a dissecting microscope to confirm nematode infection. Freeze-killed mosquito larvae that were placed at -18°C for 1 h were used as controls.

For the adult nematode experiments, first-generation females of *S. siamkayai* or hermaphrodites of *H. indica* were obtained by dissecting 4-day-old *G. mellonella*

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cadavers (Kaya and Stock, 1997). Adult nematodes were picked out one by one and washed in Ringer's solution and used for the experiments immediately.

2.3. Bacterial cultures

An established culture of *B. thuringiensis* subspecies *kurstaki* HD1 (*Btk*) was obtained from Department of Biotechnology, Periyar University, India and maintained in nutrient broth. *B. thuringiensis* has a number of subspecies that infects and kills insects (Jurat-Fuentes and Jackson, 2012) and was included in our study to determine if the fish species were deterred from feeding on *Btk*-killed *G. mellonella*.

Bacterial strains from EPNs used in the experiments were *P. luminescens* (isolated from *H. indica* KPR-8), *X. stockiae* (isolated from *S. siamkayai* KPR-4). *P. luminescens* and *X. stockiae* were obtained from the hemolymph of *G. mellonella* infected with IJs of *H. indica* and *S. siamkayai,* respectively (Akhurst, 1980). Briefly, five last instar *G. mellonella* were placed on the surface of filter paper in 90 mm Petri dishes. IJs of *H. indica* or *S. siamkayai* were released on the surface of the filter paper at a rate of 400 IJs/Petri dish. After 24 h, *G. mellonella* larvae were removed, rinsed in sterile distilled water, surface sterilized with 70% ethanol and placed in a Laminar flow cabinet for the integument to dry. The hemolymph was collected aseptically by dissecting dorsally between the 5th and 6th interstitial segments and using a sterile loop, the hemolymph was streaked on NBTA plates (nutrient agar with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue medium) (Hazir et al., 2004). The bacterial colonies were grown for 48 h at 28°C. Single colonies showing morphological differences (color, shape, and size) were removed using a sterile needle and transferred to fresh NBTA plates. Both the genera *Xenorhabdus* and *Photorhabdus* have two distinct forms, namely, phase I and phase II (primary and

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secondary forms, respectively) (Akhurst, 1980; Dybvig, 1993; Owuama, 2001). Phase I variants provide essential nutrients for the nematodes by killing and metabolizing the insect host and producing a range of antimicrobial agents whereas phase II variants are less effective in providing growth conditions for the nematodes (Akhurst, 1980, 1982; Akhurst and Boemare, 1990). The phase I variants (i.e., primary form) of symbiotic bacteria identified as *Xenorhabdus* or *Photorhabdus* was determined by using the absorption of bromothymol blue from NBTA agar plates as an indicator, and these primary bacterial forms were used for the experiments (Boemare and Akhurst, 1988).

After 48 h, a single putative primary form bacterial colony of either *P. luminescens* or *X. stockiae* was transferred into Tryptic Soy Broth (TSB) (HiMedia, Mumbai, India) and placed in a shaking incubator set at 28°C and 180 rpm for 24 h. Each bacterial isolate was deposited in skim milk agar medium (peptone from casein 5 g, yeast extract 2.5 g, skim milk powder 1 g, glucose 1 g; agar 10.5 g) (HiMedia, Mumbai, India) and stored at -80°C (Sinha et al., 1974; Barbaree et al., 1982; Gulcu et al., 2012). As needed, the bacterial cells were taken from the frozen stock cultures and transferred directly to NBTA medium. The growth of the bacteria and colony morphology were checked after 48 h incubation to ensure that there was no contamination. The identity of the *Photorhabdus* and *Xenorhabdus* species was verified by using the 16S rRNA of each bacterial isolate for molecular analysis according to Tailliez et al. (2006). Resulting sequences were compared to sequences available in gene bank at the National Center for Biotechnology Information (NCBI) confirming that they were indeed *P. luminescens* and *X. stockiae*.

2.4. Bacteria-killed insects

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A single colony of *Btk* culture was inoculated in 50 ml of nutrient broth and incubated at 37°C for 24 h at 110 rpm. Bacterial suspensions were injected into the hemocoel of *G. mellonella* larvae using a 5 µl Hamilton syringe. Insects were pre-chilled on ice for 5 min and injected with 5 µl of bacterial suspensions of *P. luminescens* or *X. stockiae* or *Btk* and held 2 or 4 days before use in the experiments.

2.5. Fish culture

The fresh water tropical cyprinid fish species, the giant danio *D. aequipinnatus*, was used in experiments in India. It was collected from Stanley Reservoir, Tamil Nadu, India by using a drag net. The live fish were transported to the laboratory in aerated bags and disinfected by treating with 0.05% potassium permanganate ($KMnO_4$) for 2 min. They were maintained in a 700-litre aquarium tank with artificial, continuous aeration at room temperature (27-30°C). All fish were acclimatized to constant laboratory environmental conditions (14 h light : 10 h dark photoperiod) for 10-14 days and fed twice a day with commercial fish feed (Optimum-Perfect Companion Group Co. Ltd, Thailand) until the beginning of the experiments (Raja et al., 2015). All fish used in the experiments were adults ranging in length from 60 to 90 mm.

The other two fresh water fish species, the spirlin chub *A. bipunctatus* and the pursak chub *S. pursakensis,* were collected from Melen Creek, Duzce, Turkey. The fishes were transferred to the laboratory in aerated 46-litre ice boxes. Each fish species was maintained in a 200-litre aquarium with artificial, continuous aeration at room temperature (22-23°C). They were acclimatized to constant laboratory environmental conditions (14 h light: 10 h dark photoperiod) and fed live, 2nd-3rd

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instar *G. mellonella* or *Tenebrio molitor* larvae daily until the experiment. The fish ranged in length from 50 to 100 mm.

2.6. Experimental design

The experiments with *D. aequipinnatus* were conducted at Periyar University, Tamil Nadu, India and included *S. siamkayai*-, *H. indica*-, and *Btk*-killed *G. mellonella* and *S. siamkayai*- and *H. indica*-killed *Ae. aegypti* mosquito larvae, first-generation females of *S. siamkayai* or hermaphroditic adults of *H. indica*, and the bacterium, *P. luminescens* or *X. stockiae*. The experiments with *A. bipunctatus* and *S. pursakensis* were carried out in Duzce University, Duzce, Turkey and involved only *S. feltiae*- and *H. bacteriophora*-killed *G. mellonella* larvae. Fish were distributed individually in 40-liter glass aquaria and starved for 24 h before the experiments. Each set of experiments usually had 5 or 10 replicates and was conducted two or three times on different dates. For each experiment, the details of the number of replicates and number of time it was repeated are provided.

Experiment 1: Response of D. aequipinnatus *to 2-or 4-day-old nematode-killed* G. mellonella *larvae*

Two sets of experiments were carried out. In the first set, 2-day-old *S. siamkayai* or *H. indica* killed or *Btk*-killed or freeze-killed *G. mellonella* were introduced to the fish. In the second set, 4-day-old cadavers were used to observe the response of *D. aequipinnatus*. On the first day, each fish was given a freeze-killed larva and the third day, the same fish was given a 2-day-old cadaver with *S. siamkayai* and on the fifth day, it was fed a 2-day-old cadaver with *H. indica*. Lastly, on the seventh day, the fish was given a 2-day-old cadaver with *Btk.* Observations on the response of the fish to the cadavers were recorded with a hand-held video camera. The number of attacks/attempts of the fish to consume the cadaver and the

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consumption time and consumption rates were recorded. Each fish was observed until the cadaver was consumed or if the cadaver was not consumed in 15 minutes (900 seconds), the experiment was terminated. There were 10 replicates and this experiment was conducted three times on different dates.

The same experiment described above was carried out but the order of cadaver presentation was changed. On the first day, each fish was given a 2-day-old cadaver with *S. siamkayai*, on the third day, the same fish was provided with a freeze-killed larva, on the fifth day, it was given a 2-day-old cadaver with *H. indica* and on the seventh day, the fish was given a 2-day-old cadaver with *Btk.* The same experimental design was conducted for 4-day-old cadaver with nematodes or *Btk* or freeze-killed. There were 10 replicates and the experiments were conducted two times on different dates.

Experiment 2: Response of A. bipunctatus *and* S. pursakensis *to 2- and 4-day-old nematode-killed* G. mellonella *larvae*

The experiments with the *A. bipunctatus* and *S. pursakensis* followed the same protocol as described for *D. aequipinnatus* except that the nematodes, *S. feltiae* and *H. bacteriophora*, were used and *Btk*-killed larvae were not included in the treatments. Freeze-killed *G. mellonella* were used as controls. For *A. bipunctatus*, there were 10 replicates and for *S. pursakensis*, there were 5 replicates and the experiments were conducted two times on different dates.

Experiment 3: Choice test of feeding 2-day-old nematode-killed or freeze-killed or Btk-killed G. mellonella *larvae to* D. aequipinnatus

D. aequipinnatus was given a choice of two 2-day-old nematode-killed or *Btk*-killed or freeze-killed *G. mellonella* larvae to observe its feeding behavior. The fish

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was exposed to the same or different pairs of cadavers simultaneously as follows: (1) *S. siamkayai*-killed vs freeze-killed larvae; (2) *H. indica*-killed vs freeze-killed larvae; (3) *Btk*-killed vs freeze-killed larvae; (4) freeze-killed vs freeze-killed larvae; (5) *S. siamkayai*-killed vs *S. siamkayai*-killed larvae; (6) *H. indica*-killed vs *H. indica*-killed larvae; and (7) *Btk*-killed vs *Btk*-killed larvae. Observations on the response of the fish to the cadavers were recorded with a hand-held video camera. The consumption rates were recorded. Each fish was observed until the cadaver was consumed or if the cadaver was not consumed in 15 minutes (900 seconds), the experiment was terminated. Each set of experiments was carried out with 10 replicates on different dates and the experiment was conducted two times on different dates.

Experiment 4: Choice test of feeding 2-day-old nematode-killed or freeze-killed G. mellonella *larvae to* A. bipunctatus

The fish, *A. bipunctatus,* was given a choice of 2-day-old nematode-killed and freeze-killed *G. mellonella* larvae to observe its feeding behavior. Each fish was given different or same pairs of cadavers simultaneously as follows: (1) *S. feltiae*-killed vs freeze-killed larvae; (2) *H. bacteriophora*-killed vs freeze-killed larvae; (3) freeze-killed vs freeze-killed larvae; (4) *S. feltiae*-killed vs *S. feltiae*-killed larvae; and (5) *H. bacteriophora*-killed vs *H. bacteriophora*-killed larvae. Each set of experiments was carried out with 10 replicates on different dates and the experiment was conducted two times on different dates.

Experiment 5: Response of D. aequipinnatus *to 2-day-old cadavers with* P. luminescens *or* X. stockiae *or Btk*

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An experiment similar to the Experiment 1 was designed with *G. mellonella* larvae injected with a bacterial suspension of *P. luminescens* or *X. stockiae* or *Btk*. Cadavers with the bacteria had been injected 2 days prior to the experiments. The cadavers were introduced to *D. aequipinnatus* as described above and the number of fish attacks/attempts at consumption of the cadavers and consumption time and the consumption rates were recorded. This experiment was conducted three times on different dates.

Experiment 6: Response of D. aequipinnatus *to 2-day-old mosquito cadavers with* S. siamkayai *or* H. indica

The consumption rate of nematode-killed *Ae. aegypti* mosquito larvae by *D. aequipinnatus* was determined. The experiment was conducted in a 2-litre glass beaker with 1600 ml of tap water and a single *D. aequipinnatus* fish was introduced and allowed to acclimatize for 1 week before initiating the experiment. The fish was starved for 24 h before the experiment. On the first day, each fish was given 20 two-day-old mosquito cadavers with *H. indica* and on the third day, the same fish was provided 20 freeze-killed mosquito larvae and on the fifth day, the fish was fed with 20 two-day-old larvae with *S. siamkayai*. The number of unconsumed mosquito cadavers was counted after 20 minutes. Observation on the response of each fish to the cadaver was recorded with a hand-held video camera. There were 5 replicates and the experiment was conducted three times on different dates.

Experiment 7: Response of D. aequipinnatus *to adult nematodes of* S. siamkayai *or* H. indica

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In this experiment, consumption of first-generation females of *S. siamkayai* or hermaphrodite adults of *H. indica* was assessed. The experiment was conducted in 2-litre glass beakers as described in experiment 6. Twenty first generation nematode females or hermaphrodites of either *S. siamkayai* or *H. indica* that were free of insect tissues on their integument were given to each fish. After 20 minutes, the fish was removed from the beaker, the nematodes allowed to settle to the bottom of the beaker for 5 minutes, and the water was checked carefully with a stereo microscope and the unconsumed nematodes were counted. The control experiment was conducted without fish and the nematodes were placed in the water and counted after 20 minutes. There were five replicates of each experimental group and the experiment was conducted three times on different dates.

Experiment 8: Effect of P. luminescens *or* X. stockiae *or Btk cell-free supernatants on* D. aequipinnatus *feeding behavior*

The potential for *P. luminescens* or *X. stockiae* to produce sufficient deterrent compound(s) to adversely affect the behavior of *D. aequipinnatus* was evaluated. For this purpose, a dialysis tube containing 3 ml of *P. luminescens* or *X. stockiae* or *Btk* cell-free supernatant in TSB (48 h post inoculation) or sterile TSB alone (control) was placed in a 2-litre glass beaker containing 1600 ml of tap water. After 1 h, a 24-h starved fish was placed into the beaker and allowed to acclimate. After another hour, a freeze-killed *G. mellonella* larva was introduced into the beaker and the feeding behavior of the fish was observed as described in Experiment 1. The feeding behavior was checked again after 18 h. The fish was left in the beaker for an additional 24 h and any aberrant fish behavior such as erratic swimming was recorded. There were

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five replicates of each experimental group and the experiment was conducted three times on different dates.

2.7. Statistical analysis

The response variables (mean consumption rate, the mean number of attacks and the mean number of consumed adult nematodes) by the fish were analyzed using analysis of variance (ANOVA). Means were compared at the $P = 0.05$ level and Tukey's test was used to separate means. The data of mean consumption rate were arcsine transformed before statistical analysis (SPSS 13.0). In the choice tests, differences in numbers of *D. aequipinnatus* and *A. bipunctatus* selecting the cadavers were analyzed using a replicated *G*-test for goodness of fit (Sokal and Rohlf, 1995).

3. Results

General observations

In experiments 1 and 2, when only one cadaver was introduced, we observed that starved *D. aequipinnatus*, *A. bipunctatus* and *S. pursakensis* immediately attacked the freeze-killed *G. mellonella* larvae when they were introduced into the aquarium. Generally, the fish consumed the cadaver within 60 seconds after 1 to 5 attacks (Supplementary material: Video-1). However, the fish did not consume the nematode-killed cadavers even after 1 to 45 attacks made during the course of the 15 minute experimental duration (Supplementary material: Video-2 and 3). Interestingly, *D. aequipinnatus* and *A. bipunctatus* did multiple attacks on the nematode-killed insects without consumption, whereas *S. pursakensis* did 1 or 2 attacks and then ceased attacks completely. Basically, the fishes rejected the cadavers leaving them to settle to the bottom of the aquarium. In Experiments 3 and 4, starved *D. aequipinnatus* or *A.*

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bipunctatus were given a choice of two cadavers to consume. When one pair was the nematode-killed larva and the other was the freeze-killed larva, we observed that if the freeze-killed larva was attacked first, it was consumed and the fish subsequently attempted to eat the nematode-killed larva, but desisted after a single attack. On the other hand, if the fish attacked the nematode-killed larva first, after one or two attacks, the fish desisted and showed no further interest in either the nematode-killed or freeze-killed larvae.

Experiment 1: Response of D. aequipinnatus *to 2-or 4-day-old nematode-killed* G. mellonella *larvae*

For 2-day-old cadavers, *D. aequipinnatus* consumed more than 95% and 90% of freeze-killed and *Btk*-killed *G. mellonella* larvae, respectively. However, less than 10% or no consumption was observed of any nematode-killed larvae. Significant differences in cadaver consumption were detected between the control groups (freeze-killed and *Btk*-killed) and nematode-killed insect groups (F = 211.06; df = 3, 116; P < 0.05) (Fig. 1A). The mean number of fish attacks was 3 or less for freeze-killed and *Btk*-killed insects before cadaver consumption, whereas the number of fish attacks for *S. siamkayai*-killed and *H. indica*-killed insects was >7 (Fig. 1B). Significant differences in the number of attacks were observed between control and nematode-killed insect groups (F = 12.08; df = 3, 116; P < 0.05). In a few instances, the fish completely rejected the nematode-killed *G. mellonella* larvae. If the fish consumed the control freeze-killed and *Btk*-killed insects, they consumed the cadavers within 42 ± 29.6 and 89 ± 40.5 seconds, whereas based on the number of attacks, the fish did show interest in the nematode-killed insects but did not consume the cadavers during the 900 seconds observation period.

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Changing the sequence of cadaver presentation to *D. aequipinnatus* did not change the outcome. For example, with the 2-day-old nematode-, freeze-, or *Btk*-killed insects, the fish consumed the freeze-killed and *Btk*-killed insects within 1.5±0.1 and 5.5±0.9 mean number of attacks, respectively, but the fish did not consume nematode-killed insects even after 12.0±1.8 (*S. siamkayai*) and 11.4±1.7 (*H. indica*) mean number of attacks. Significant differences in number of attacks were observed between freeze-killed, *Btk*-killed and nematode-killed larval groups (F = 54.524; df = 3, 76; P<0.05) (data not shown). In addition, significant differences in cadaver consumption were observed between control groups and nematode-killed groups (F = 13.12; df = 3, 76; P<0.05).

For 4-day-old cadavers, the overall consumption rate of freeze-killed larvae and 4-day-old *Btk*-killed was 100% and 66%, respectively, but none of the nematode-killed insects was consumed by *D. aequipinnatus*. Significant differences in cadaver consumption were observed among freeze-killed, *Btk*-killed and nematode-killed insects (F = 130.5; df = 3, 116; P < 0.05). There were also significant differences in the number of attacks between control groups and nematode-killed insect groups (F = 31.94; df = 3, 116; P < 0.05) (Fig. 2). Fish consumed freeze-killed larvae within an average of 5±0.5 seconds, but it took 307±77 seconds to consume 66% of the *Btk*-killed insects. No consumption was observed for the nematode-killed insect group even after 900 seconds. Significant differences were observed among freeze-killed, *Btk*-killed and nematode-killed groups (F = 131.92; df = 3, 116; P < 0.05). Similar results were observed when changing the order of presentation of cadavers to the fish (data not shown).

Experiment 2: Response of A. bipunctatus *and* S. pursakensis *to 2- and 4 -day-old nematode-killed* G. mellonella *larvae*

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Both *A. bipunctatus* and *S. pursakensis* tested in Turkey consumed all of the freeze-killed *G. mellonella* larvae, whereas neither 2-day-old *S. feltiae-* nor *H. bacteriophora*-killed *G. mellonella* were consumed (data not shown). For *A. bipunctatus*, significant differences in cadaver consumption were observed between freeze-killed and nematode-killed insects (F=2E+034; df = 2, 27; P<0.05). *A. bipunctatus* exhibited 2.7±0.5 mean number of attacks to freeze-killed insects, whereas 37.2±4.3 and 41.0±4.6 mean number of attacks was observed for cadavers with *S. feltiae* and *H. bacteriophora*, respectively. Significant differences in number of attacks were observed between nematode-killed insect and the freeze-killed insect groups (F = 32.97; df = 2, 57; P<0.05) (Fig. 3). Similarly, *S. pursakensis* consumed freeze-killed larvae within 1.1±0.1 mean number of attacks, but this fish species had only 1.7±0.2 mean number of attacks for *S. feltiae*- and *H. bacteriophora*-killed insects, and thereafter the attacks stopped and no consumption occurred. In terms of number of attacks, there was no significant difference observed between nematode-killed and freeze-killed larval groups for *S. pursakensis* (F = 2.92; df = 2, 27; P>0.05).

For 4-day-old cadavers with *S. feltiae* or *H. bacteriophora*, *A. bipunctatus* rejected 100% of these cadavers even after averaging 34±5.6 and 45±5.5 attacks, respectively, whereas all freeze-killed larvae were consumed within 1.6±0.2 attacks. Significant differences were observed between nematode-killed insect and freeze-killed insect groups for the mean number of attacks (F = 24.919; df = 2, 57; P<0.05). *S. pursakensis* also rejected 100% of the cadavers with nematodes, but all the freeze-killed larvae were consumed within 1.3±0.1 mean numbers of attacks. *S. pursakensis* attacked an average of 2.0±0.1 and 1.6±0.1 times for *H. bacteriophora*- and *S. feltiae*-killed insects, respectively, and thereafter showed no interest in these cadavers.

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Significant differences in the number of attacks were observed between freeze-killed and *H. bacteriophora*-killed insects ($F = 5.123$; df = 2, 27; $P<0.05$), but not for freeze-killed and *S. feltiae*-killed insects.

Experiment 3. Choice test of feeding 2-day-old nematode-killed or freeze-killed or Btk-killed G. mellonella *larvae to* D. aequipinnatu**s**

Based on the results of Experiments 1 and 2 where the fishes were given only one cadaver at a time, we conducted Experiments 3 and 4 to offer the fishes a pair of cadavers. In a choice test, when offered a choice between 2-day-old nematode-killed larva and freeze-killed larva, the fish showed a significant preference for freeze-killed compared to nematode-killed larva. Fish consumed more than 65% of freeze-killed larvae whereas, only 10% of *S. siamkayai*-killed larvae were consumed. However, the fish did not consume any of the *H. indica*-killed larvae. The pooled data were significantly different (Fig. 4). When offered a choice between nematode-killed vs nematode-nematode larvae, none of the fish consumed any of the cadavers. There was no significant difference observed when the fish was offered a choice between freeze-killed (100%) vs freeze-killed (95%) or freeze-killed (80%) vs *Btk*-killed (55%) or *Btk*-killed (65%) vs *Btk*-killed (60%) larvae (Fig. 4).

Experiment 4: Choice test of feeding 2-day-old nematode-killed or freeze-killed G. mellonella *larvae to* A. bipunctatus

A. bipunctatus had a significant preference for freeze-killed larvae compared to nematode-killed larvae. The fish consumed more than 75% of freeze-killed larvae, whereas it consumed only 5% and 5% of *S. feltiae*- and *H. bacteriophora*-killed larvae, respectively. The pooled data were significantly different (Fig. 5). *A.*

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bipunctatus did not show significant difference in preference of choice between freeze-killed and freeze-killed larvae. When offered a choice between nematode-killed and nematode-killed larvae, none of the fish consumed any of the cadavers (Fig. 5).

Experiment 5: Response of D. aequipinnatus *to 2-day-old cadavers with* P. luminescens *or* X. stockiae *or Btk*

The fish, *D. aequipinnatus*, consumed 100% of freeze-killed insects within 5±0.5 seconds and 86% of *Btk*-injected cadavers within 132±55.8 seconds, whereas they completely rejected the *P. luminescens*-injected insects and consumed < 7% of *X. stockiae*-injected insects during the 900 seconds experimental duration (Fig. 6A). It took 842 ± 40 seconds for the fish to consume the *X. stockiae*-killed insects. Thus, there were significant differences in cadaver consumption between symbiotic bacteria-injected insects and the control groups (F = 178.59; df = 3, 116; $P < 0.05$). In addition, there were significant differences in the number of attacks among the freeze-killed, *Btk*-killed and symbiotic bacteria-killed insects (F = 183.886; df = 3, 116; $P < 0.05$) (Fig. 6B).

The same experiment was conducted again, except that the order of presentation to *D. aequipinnatus* was changed. The results were comparable to the preceding paragraph with similar statistical differences. That is, the fish consumed 100% of freeze-killed and 95% of *Btk*-injected insects after a mean attack rate of 1.4±0.1 and 7.8±1.3, respectively, and the fish did not consume *P. luminescens*-injected insects, even after 11.0±1.4 mean number of attacks, whereas the fish consumed only 5% of the *X. stockiae*-injected insects after 13.5±1.5 mean number of attacks.

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Experiment 6: Response of D. aequipinnatus *to 2-day-old mosquito cadavers with* S. siamkayai *or* H. indica

Fish consumed only 53.6% of *H. indica*-killed and 58% of *S. siamkayai*-killed mosquito larvae, but in the control group, the fish consumed 99% freeze-killed mosquito larvae. Significant differences were observed between consumption of freeze-killed and nematode-killed mosquito larvae (F=34.641; df= 2, 42; P<0.05) (Fig. 7).

Experiment 7: Response of D. aequipinnatus *to adult nematodes of* S. siamkayai *or* H. indica

Fish consumed an average of 12±1 of *S. siamkayai* (60%) and 11±1 of *H. indica* (55%) adult nematodes, whereas in the controls, 19 of 20 (95%) nematodes were recovered after the experiment period. There were significant differences between the control and the various treatments (F = 68.08; df = 3, 56; P < 0.05).

Experiment 8: Effect of P. luminescens *or* X. stockiae *or Btk cell-free supernatants on* D. aequipinnatus *feeding behavior*

D. aequipinnatus consumed more than 90% of freeze-killed larvae after 1 h exposure to the cell-free supernatant of *P. luminescens* or *X. stockiae* or *Btk* (Fig. 8). There was no significant difference observed between sterile TSB alone group and bacterial supernatant groups (F = 0.333; df = 3, 36; P>0.05). However, after 18 h of exposure to the cell-free supernatant of *P. luminescens* or *X. stockiae*, the fish demonstrated an adverse effect on the feeding behavior as they consumed only 20% of freeze-killed larvae in *P. luminescens* supernatant exposed group, whereas they consumed 90% of the freeze-killed insects in the sterile TSB alone group (control).

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Significant differences were observed between *P. luminescens* and sterile TSB group (F=5.455; df= 3, 36; P<0.05), but no significant difference was observed between *X. stockiae* group and control groups (*Btk* or sterile TSB alone group) (Fig. 8). Except for the change in feeding behavior, we did not detect any aberrant behavior in the fish such as erratic swimming during the course of the experiment.

4. Discussion

The fish species, *D. aequipinnatus*, *A. bipunctatus*, and *Squalius* spp. including S. *pursakensis*, can be classified as omnivores because they will consume a wide variety of food including zoo- and phytoplanktons, and live and dead invertebrates (http://badmanstropicalfish.com/profiles/profile206_Devario_aequipinnatus.html; Blanco-Garrido et al., 2003; Gomes-Ferreira et al., 2005a; Gomes-Ferreira et al., 2005b; Treer et al., 2006). We have demonstrated that these fishes will consume freeze-killed insects but *D. aequipinnatus* consumed, on average, less than one 2-day-old cadaver with *S. siamkayai* suggesting that the consumed 2-day-old cadavers with *S. siamkayai* did not have a sufficient titer of SDF. Apart from this aberrant observation, all three fish species did not consume any cadavers colonized with the other nematode-bacterium complex species. Thus, we can add these three omnivorous fish species to the list of arthropod scavengers and predators and a bird species that will avoid consuming cadavers already colonized with the nematode-bacterium complex.

We noted that there were differences in the behavior of the three fishes in attacking the cadavers with the nematode-bacterium complex. *D. aequipinnatus*, *A. bipunctatus* and S. *pursakensis* showed similar behavior in that they attacked a freeze-killed control *G. mellonella* and in 1 or 2 attacks consumed it. Thus, the three fish species in our study found that the freeze-killed insects are palatable. In terms of an

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EPN-killed insect, *D. aequipinnatus* and *A. bipunctatus* attacked the cadaver, placed it into its mouth, rejected it, and repeated this sequence a number of times over the 900 second observational period. Except for a few rare instances with 2-day-old *S. siamkayai*-killed *G. mellonella*, the cadavers were not consumed. However, *S. pursakensis* attacked the cadaver with the nematode-bacterium complex 1 or 2 times without consuming it and subsequently showed no further interest in the cadaver during the observational period. In choice test experiments, our data showed that both *D. aequipinnatus* and *A. bipunctatus* had a significant preference in consumption between nematode-killed and freeze-killed insects. However, both the fish consumed the freeze-killed insects and *D. aequipinnatus* consumed considerable *Btk*-killed insect. Both fish species rejected cadavers obtaining the nematode-bacterium complex.

Kasumyan and Doving (2003) state that olfaction and gustation are the main chemosensory systems in fish and that both systems play important roles in fish feeding behavior. In contrast, *D. aequipinnatus* and *A. bipunctatus* made numerous attempts to feed on the EPN-killed insects without consuming them suggesting that a gustatory deterrent was present resulting in rejection of the cadaver. S. *pursakensis*, on the other hand, may have been deterred gustatorily after 1 or 2 feeding attempts or by an olfactory chemical that was being released by the cadaver after an attack resulting in complete rejection of the cadaver with no further feeding attempts on the EPN-killed insect.

Insect cadavers colonized by the EPN-bacterium complex do not deteriorate but remain intact because of the presence of antimicrobial compounds synthesized by the symbiotic bacteria (Clarke, 2008). This preservation of the cadavers allows the nematodes to develop and reproduce because the production of IJs may take from 7 to

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more than 20 days depending on environmental conditions and nematode species. Interestingly, Fenton et al. (2011) showed that the European robin approached *H. bacteriophora*-killed larvae and pecked at them but subsequently rejected them and selected and consumed uninfected larvae. The induced color changes may act as a signal to predators or scavengers, which is reinforced by their unpalatable taste. They hypothesized that the *H. bacteriophora*-killed larvae contained a distasteful substance(s) that deterred consumption, which supports our study. Foltan and Puza (2009) showed that a scavenger beetle's (*Pterostichus melanarius*) response to nematode-killed invertebrates may be intermediate. In a feeding preference experiment of the grey slug killed by the slug nematode, *Phasmarhabditis hermaphrodita* and *G. mellonella* larvae killed by *Steinernema affine*, they observed that *P. melanarius* consumed significantly more freeze-killed *G. mellonella* larvae than those killed by the nematode/bacterium complex and were more likely to attack control slugs than the nematode-killed slugs. Additionally, the beetles were attracted more to non-infected *G. mellonella* larvae than to nematode-killed larvae suggesting that the beetles avoided the nematode-killed larvae.

More recently, Jones et al. (2016) demonstrated that a novel form of odour-based host manipulation by EPNs deters the beetle, *P. madidus,* from consuming the insect cadaver with the nematode-bacterium complex. They demonstrated that the olfactory cues from the nematode-killed insects can provide substantial protection, keeping the beetles away from hosts containing the nematode-bacterium complex. In a study with two wasp species, *Vespa orientalis* and *Paravespula* sp. were offered a choice of lamb liver or meat treated with *P. luminescens* supernatant or *Escherichia coli* supernatant or untreated control liver or meat (Gulcu et al., 2012). The researchers found that the two vespid species fed on meat treated with *E. coli*

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supernatant and control liver or meat but did not feed on lamb liver or meat treated with *P. luminescens* supernatant. Similarly, calliphorid flies did not oviposit on lamb meat treated with *P. luminescens* supernatant but did oviposit on non-treated meat (control). This study strongly suggests that olfactory cues were involved as a feeding and ovipostional deterrent to these insects. Thus, an olfaction response may work as a first level of defense to avoid or prevent consumption by scavengers as well as predators and serves a common defensive mechanism by organisms (Gamberale-Stille and Guilford, 2004).

We conducted more in-depth studies with *D. aequipinnatus* that included (1) *Btk*-killed *G. mellonella*, (2) *G. mellonella* larvae injected with the symbiotic bacterium, *X. stockiae* or *P. luminescens* or *Btk*, (3) mosquito (*Ae. aegypti*) larvae with the nematode-bacterium complex, (4) adult *S. siamkayai* and *H. indica*, and (5) the use of a dyalysis tube containing the supernatant of cultured *X. stockiae* or *P. luminescens* or *Btk.* With 2-day-old *Btk*-killed *G. mellonella*, *D. aequipinnatus* showed no preference in terms of number of attacks or time to consume the cadaver compared with freeze-killed insects. However, with 4-day-old *Btk*-killed *G. mellonella*, the fish showed significant differences in percentages consumed, number of attacks and time to consume the cadaver compared with freeze-killed insects. These data indicate that 4-day-old *Btk*-killed *G. mellonella* were less “attractive” as food for this fish species. In contrast, the experiment with *D. aequipinnatus* and 2-day-old *G. mellonella* larvae with the symbiotic bacterium provided data that were similar to those found with 2-day-old nematode-killed *G. mellonella* demonstrating that the symbiotic bacterium produces SDF as shown by Zhou et al. (2002).

The experiment with *D. aequipinnatus* and 20 *Ae. aegypti* with the nematode-bacterium complex demonstrated that the fish consumed less than 60% (53% of *H.*

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indica and 58 % of *S. siamkayai*) of the 2-day-old nematode-killed mosquito larvae compared to nearly all of the freeze-killed mosquitoes. It seems that after consuming about 60% of the small mosquito larvae, the fish lost interest in the remaining nematode-killed mosquito larvae. Conceivably, the presence of SDF in the ingested nematode-killed mosquito larvae changed the feeding behaviour of the fish. On the other hand, due to lack of adequate control treatments, it was not possible to draw conclusion with the experiment using *D. aequipinnatus* and 20 adult nematodes (*S. siamkayai* or *H. indica*). That is, we showed that the fish will consume about 60% of the nematode adults. Perhaps, they did not consume the remaning nematode adults because they could not locate them or because of the presence of SDF as we speculated for the *Ae. aegypti* experiment. In future experiments of this type, we need to include a treatment of free-living nematode adults that are of similar size as EPN adults and provide them to *D. aequipinnatus*. We did not have access to any free-living nematode species when we were conducting this experiment with the adult EPNs, but we report these data because we did show that *D. aequipinnatus* will consume some adult EPNs.

The dialysis tube experiment with 48-h-old *X. stockiae* or *P. luminescens* or *Btk* supernatant or sterile TSB provided interesting results. At 1 h post introduction of the dialysis tube with the supernatant showed no difference in consumption of control freeze-killed *G. mellonella* by *D. aequipinnatus*. However, after 18 h post introduction, significantly lower consumption was observed in *P. luminescens* treatments compared to the *X. stockiae*, *Btk* or TSB treatments. These data indicate that after 18 h of dialysis time with *P. luminescens* that SDF was present in the water with the fish and consequently affected their feeding behavior. Assuming that SDF from *P. luminescens* and *X. stockiae* are the same or very similar, the data suggest that

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P. luminescens produced a higher titer of SDF during the 48 h bacterial growth period. Our data confirm and extend previous observations by Zhou et al. (2002) that the Ant Deterrent Factor [later renamed as scavenging deterrent factor (SDF) by Gulcu et al. (2012) and Ulug et al. (2014)] is present in the supernatant of bacterial cultures and can pass through a 0.45µm-pore-size Millipore filter, indicating the compound(s) is extracellular and a small molecule. We speculate that SDF is soluble, passes through the dialysis tube and reaches a sufficiently high enough concentration in an aquatic environment after several hours to serve as an aposematic warning signal (olfactory or gustatory or both) to adversely affect the feeding behavior of *D. aequipinnatus* on freeze-killed control insects. Further studies are needed to elucidate the mechanism by which the deterrent factor present in EPN-killed insects affects fish feeding behavior.

EPNs are soil-dwelling organisms and are commonly found in soils throughout much of the world (Hazir et al., 2003; Kaya and Gaugler, 1993), and we can ask why does SDF serve to deter terrestrial and aquatic scavengers, omnivores and even predators? Poinar (1993) and Boemare (2002) have proposed the marine origin of the *Heterorhabditis/Photorhabdus* association, and Blaxter et al. (1998) constructed a preliminary evolutionary tree based on 18S ribosomal DNA suggesting that *Heterorhabditis* may have evolved from a free-living bacterial feeding ancestor. On the other hand, *Steinernema* evolved from a primitive terrestrial rhabditid ancestor (Poinar, 1993), but their ancestral food source is not clear. Accordingly, Poinar (1993) hypothesized that *Heterorhabditis* and *Steinernema* had a convergent evolution with similar morphology and life history. We suggest that SDF also is the result of convergent evolution after both genera became entomopathogens as a

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mechanism to protect the developing nematodes in a cadaver from scavengers, omnivores and predators.

How would fishes encounter nematode-killed insects? Considerable research has been carried out using EPN as biocontrol agents against immature stages of several mosquito and black fly species [see Begley (1990) for the early literature; Cagnolo and Almiron (2010); Cardoso et al. (2015); Peschiutta et al. (2014)]. Although it is unlikely that EPNs will be widely used, if at all, in the aquatic environment for controlling insects (Lewis et al., 1998), we have demonstrated that *D. aequipinnatus* consumed less than 60% of *H. indica*-killed *Ae. aegypti* larvae suggesting that some of the nematode-killed larvae will remain and have the potential to recycle and produce IJs to infect a new generation of mosquito larvae. However, some fish species may not be affected by SDF and the majority of the nematode-killed larvae may be consumed. Conversely, some fish species may respond in a manner similar to S. *pursakensis* and the majority of the nematode-killed larvae will not be consumed. Clearly, if EPNs are to be used as biocontrol agents in an aquatic environment, the impact of the resident fish species on nematode-killed insects should be assessed.

In conclusion, our study suggests that the warning signal (SDF) produced by the symbiotic bacteria works not only against the terrestrial scavengers but also against omnivorous freshwater fish species. By the symbiotic bacteria producing SDF, the nematode-killed insect becomes a distasteful carcass which serves to protect the developing nematode in the cadaver. Our data strongly suggest that both olfaction and gustation chemosensory responses of the fishes are involved as the protection mechanism against the nematode-killed insects from being consumed. Yet, there are some interesting questions that can be asked. Is SDF produced by any other

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microorganisms as a deterrent or is it restricted to *Photorhabdus* and *Xenorhabdus*? What is the molecular structure of SDF and is it the same in *Heterorhabditis* and *Steinernema*? Are there any practical applications of our findings in the terrestrial or aquatic environment?

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References

Akhurst, R.J., 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. J. Gen. Microbiol. 121, 303-309.

Akhurst, R.J., 1982. Antibiotic activity of *Xenorhabdus* spp. bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J. Gen. Microbiol. 128, 3061-3065.

Akhurst, R.J., Boemare, N.E., 1990. Biology and taxonomy of *Xenorhabdus*. In: Gaugler, R., Kaya, H.K. (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press, Boston, pp. 75-90.

Barbaree, J.M., Thompson, F., Smith, S.J., 1982. Use of thermal stability studies to compare *Bacteroides fragilis* lyophilized in skim milk and polyvinylpyrrolidone solutions. Cryobiology 19, 92-98.

Baur, M.E., Kaya, H.K., Strong, D.R., 1998. Foraging ants as scavengers on entomopathogenic nematode-killed insects. Biol. Control. 12, 231-236.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Begley, J.W., 1990. Efficacy against insects in habitats other than soil. In: Gaugler, R., Kaya, H.K. (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press. Boston, pp. 215-231.

Blanco-Garrido, F., Sánchez-Polaina, F.J., Prenda, J., 2003. Summer diet of the Iberian chub (*Squalius pyrenaicus*) in a Mediterranean stream in Sierra Morena (Yeguas stream, Córdoba, Spain). Limnetica 22, 99-106.

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M., Vida, J.T., Thomas, W.K., 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 392: 71-75.

Boemare, N., 2002. Biology, taxonomy, and systematics of *Photorhabdus* and *Xenorhabdus*. In: Gaugler, R. (Ed.), Entomopathogenic Nematology.CABI Publishing, Wallingford, UK. pp. 35-56.

Boemare, N.E., Akhurst, R.J., 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). J. Gen. Microbiol. 134, 751-761.

Cagnolo, S.R., Almiron, W.R., 2010. Capacity of the terrestrial entomopathogenic nematode *Steinernema rarum* (Rhabditida: Steinernematidae) to parasite *Culex apicinus* larvae (Diptera: Culicidae). Rev. Soc. Entomol. Argent. 69, 141-145.

Cardoso, D.O., Gomes, V.M., Dolinski, C., Souza, R.M., 2015. Potential of entomopathogenic nematodes as biocontrol agents of immature stages of *Aedes aegypti*. Nematoda 2:e092015. http://dx.doi.org/10.4322/nematoda.09015.

Chanthini, A.B., Balasubramani, G., Ramkumar, R., Sowmiya, R., Balakumaran, M.D., Kalaichelvan, P.T., Perumal, P., 2015. Structural characterization, antioxidant and *in vitro* cytotoxic properties of seagrass, *Cymodocea serrulata* (R.Br.) Asch. & Magnus mediated silver nanoparticles. J. Photochem. Photobiol. B: Biol. 153, 145-152.

Clarke, D.J., 2008. *Photorhabdus*: a model for the analysis of pathogenicity and mutualism. Cell Microbiol. 10, 2159-2167.

Dillman, A.R., Chaston, J.M., Adams, B.J., Ciche, T.A., Goodrich-Blair, H., Stock, S.P., Sternberg, P.W., 2012. An entomopathogenic nematode by any other name. PLoS Pathog. 8, 8-11.

Dowds, B.C.A., Peters, A., 2002. Virulence mechanisms. In: Gaugler, R. (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford, UK. pp. 79-98.

Dybvig, K., 1993. DNA rearrangements and phenotypic switching in prokaryotes. Mol. Microbiol. 10, 465-471.

ACCEPTED MANUSCRIPT

Fenton, A., Magoolagan, L., Kennedy, Z., Spencer, K.A., 2011. Parasite-induced warning coloration: a novel form of host manipulation. Anim. Behav. 81, 417-422.

Finney, J.R., Harding, J.B., 1981. Some factors affecting the use of *Neoaplectana* sp. for mosquito controls. Mosq. News 41, 798-800.

Foltan, P., Puza, V., 2009. To complete their life cycle, pathogenic nematode-bacteria complexes deter scavengers from feeding on their host cadaver. Behav. Proc. 80, 76-79.

Gamberale-Stille, G., Guilford, T., 2004. Automimicry destabilizes aposematism: predator sample-and-reject behaviour may provide a solution. Proc. R. Soc. B: Biol. Sci. 271, 2621-2625.

Gaugler, R., Kaya, H.K., 1990. Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, FL.

Gomes-Ferreira, A., Ribeiro, F., Moreira da Costa, L., Cowx, I.G., Collares-Pereira, M.J., 2005a. Variability in diet and foraging behaviour between sexes and ploidy forms of the hybridogenetic *Squalius alburnoides* complex (Cyprinidae) in the Guadiana River basin, Portugal. J. Fish Biol. 66, 454-467.

Gomes-Ferreira, A., Ribeiro, F., Moreira da Costa, L., Cowx, I.G., Collares-Pereira, M.J., 2005b. Diet composition of chub, *Squalius cephalus* (Teleostei: Cyprinidae), in Lake Tödürge, Sivas, Turkey. J. Appl. Ichthyol. 27, 1350-1355.

Griffin, C.T., 2012. Perspectives on the behavior of entomopathogenic nematodes from dispersal to reproduction: Traits contributing to nematode fitness and biocontrol efficacy. J. Nematol. 44, 177-184.

Gulcu, B., Hazir, S., Kaya, H.K., 2012. Scavenger deterrent factor (SDF) from symbiotic bacteria of entomopathogenic nematodes. J. Invertebr. Pathol. 110, 326-333.

Han, R.C., Ehlers, R.U., 2000. Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic in vivo conditions. J. Invertebr. Pathol. 75, 55-58.

Hazir, S., Stackebrandt, E., Lang, E., Schumann, P., Ehlers, R., Keskin, N., 2004. Two new subspecies of *Photorhabdus luminescens*, isolated from *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae): *Photorhabdus luminescens* subsp. *kayaii* subsp. nov. and *Photorhabdus luminescens* subsp. *thracensis* subsp. nov. Syst. Appl. Microbiol. 27, 36-42.

Hazir, S., Stock, S.P., Keskin, N., 2003. Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests. Turkish J. Biol. 27, 181-202.

ACCEPTED MANUSCRIPT

Jones, R.S., Fenton, A., Speed, M.P., 2016. "Parasite-induced aposematism" protects entomopathogenic nematode parasites against invertebrate enemies. Behav. Ecol. 27, 645-651.

Jurat-Fuentes, J.L., Jackson, T.A., 2012. Bacterial entomopathogens. In: Vega, F.E., Kaya, H.K. (Eds.), Insect Pathology. 2nd ed. Academic Press, Amsterdam, The Netherlands. pp.265-349.

Kasumyan, A.O., Doving, K.B., 2003. Taste preferences in fishes. Fish Fish. 4, 289-347.

Kaya, H.K., Gaugler, R., 1993. Entomopathogenic nematodes. Ann. Rev. Entomol. 38, 181-206.

Kaya, H.K., Stock, S.P., 1997. Techniques in insect nematology. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, London UK. pp. 281-324.

Lewis, E.E., Clarke, D.J., 2012. Nematode parasites and entomopathogens. In: Vega, F.E., Kaya, H.K., (Eds.), Insect Pathology, 2nd ed. Academic Press, Amsterdam, The Netherlands. pp. 395-424.

Lewis, E., Campbell, J., Gaugler, R., 1998. A conservation approach to using entomopathogenic nematodes in turf and landscapes. In: Barbosa, P. (Ed.), Perspectives on the Conservation of Natural Enemies of Pest Species. Academic Press, New York. pp. 235-254.

Molta, N.B., Hominick, W.M., 1989. Dose-and time-response assessments of *Heterorhabditis heliothidis* and *Steinernema feltiae* (Nematoda: Rhadbitida) against *Aedes aegypti* larvae. Entomophaga 34, 485-493.

Owuama, C.I., 2001. Entomopathogenic symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* of nematodes. World J. Microbiol. Biotechnol. 17, 505-515.

Peschiutta, M.L., Cagnolo, S.R., Almiron, W.R., 2014. Susceptibility of larvae of *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) to entomopathogenic nematode *Heterorhabditis bacteriophora* (Poinar) (Rhabditida: Heterorhabditidae). Rev. Soc. Entomol. Argent. 73, 99-108.

Poinar, G.O. Jr., 1993. Origin and phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and *Steinernema*. Fund. Appl. Nematol. 16:333-338.

Poinar, G.O. Jr., Kaul, H.N., 1982. Parasitism of the mosquito *Culex pipiens* by the nematode *Heterorhabditis bacteriophora*. J. Invertebr. Pathol. 39, 382-387.

Poinar, G.O. Jr., Leutenegger, R., 1971. Ultrastructual investigation of the melanization process in *Culex pipiens* in response to a nematode. J. Ultrastruc. Res. 30, 149-158.

ACCEPTED MANUSCRIPT

Raja, M., Kavitha, M., Ramkumar, R., Anandhi, M., Perumal, P., 2015. The 'Giant Danio' (*Devario aequipinnatus*) - Another model for diabetic retinopathy study: Induction of hyperglycemia and resultant retinal impairment. Endocrinol. Diabetes Res. 1:2. doi:http://dx.doi.org/10.4172/ecdr.1000108.

Raja, R.K., Sivaramakrishnan, S., Hazir, S., 2011. Ecological characterization of *Steinernema siamkayai* (Rhabditida: Steinernematidae), a warm-adapted entomopathogenic nematode isolate from India. BioControl 56, 789-798.

Shapiro-Ilan, D.I., Cottrell, T.E., Mizell, R.F., Horton, D.L., Zaid, A., 2015. Field suppression of the peachtree borer, *Synanthedon exitiosa*, using *Steinernema carpocapsae*: Effects of irrigation, a sprayable gel and application method. Biol. Control 82, 7-12.

Sinha, R.N., Dudani, A.T., Ranganathan, B., 1974. Effect of individual ingredients of fortified skim milk as suspending media on survival of freeze-dried cells of *Streptococcus lactis*. Cryobiology 11, 368-370.

Sokal, R.R., Rohlf, F.J., 1995. Replicated goodness-of-fit tests (G statistics). Biometry, 3rd ed. Freeman, New York. pp. 716-722.

SPSS., 2004. SPSS v.13.0 for Windows. SPSS Inc., Chicago, Illinois.

Tailliez, P., Pages, S., Ginibre, N., Boemare, N., 2006. New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. Int. J. Syst. Evol. Microbiol. 56, 2805-2818.

Treer, T., Piria, M., Aničić, I., SafneR, R., Tomljanović, T., 2006. Diet and growth of spirlin, *Alburnoides bipunctatusin* the barbel zone of the Sava River. Folia Zool. 55, 97-106.

Ulug, D., Hazir, S., Kaya, H.K., Lewis, E., 2014. Natural enemies of natural enemies: the potential top-down impact of predators on entomopathogenic nematode populations. Ecol. Entomol. 39, 462-469.

Welch, H.E., 1961. Nematodes as agents for insect control. Proc. Entomol. Soc. Ont. 92, 11-19.

Welch, H.E., Bronskill, J.F., 1962. Parasitism of mosquito larvae by the nematode DD-136 (Nematoda: Neoaplectanidae). Can. J. Zool. 40, 1263-1268.

Zhou, X.S., Kaya, H.K., Heungens, K., Goodrich-Blair, H., 2002. Response of ants to a deterrent factor(s) produced by the symbiotic bacteria of entomopathogenic nematodes. Appl. Environ. Microbiol. 68, 6202-6209.

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Figure Legends:

Fig. 1. A. Percentage consumption (mean ± SE) of 2-day-old *Steinernema siamkayai*-killed or *Heterorhabditis indica*-killed *Galleria mellonella* larvae by the fish, *Devario aequipinnatus*. Freeze-killed or *Bacillus thuringiensis*-killed *G. mellonella* were included as controls. B. The number of attacks (mean ± SE) of cadavers by *D. aequipinnatus* of the same treatments as in Fig. 1A. Different letters above SE bars indicate significant differences (P<0.05; Tukey's test).

Fig. 2. The number of attacks (mean ± SE) on 4-day-old *Steinernema siamkayai*-killed or *Heterorhabditis indica*-killed *Galleria mellonella* larvae by the fish, *Devario aequipinnatus*. Different letters above SE bars indicate significant differences (P<0.05; Tukey's test).

Fig. 3. Number of attacks (mean ± SE) on 2-day-old *Steinernema feltiae*-killed and *Heterorhabditis bacteriophora*-killed *Galleria mellonella* larvae by the fish *Alburnoides bipunctatus*. Different letters above SE bars indicate significant differences (P<0.05; Tukey's test).

Fig. 4. The response of *Devario aequipinnatus* when offered a choice between 2-day-old nematode-killed or freeze-killed or *Bacillus thuringiensis* subsp. *kurstaki*-killed (*Btk*) *Galleria mellonella* larvae. Each bar represents the choice of 20 cadavers. **P<0.01.

Fig. 5. The response *of Alburnoides bipunctatus* when offered a choice between 2-day-old nematode-killed or freeze-killed *Galleria mellonella* larvae. Each bar represents the choice of 20 cadavers. **P<0.01.

Fig. 6. A. Percentage consumption (mean ± SE) of 2-day-old *Galleria mellonella* larvae that were killed by injection with the symbiotic bacterium, *Xenorhabdus stockiae* or *Photorhabdus luminescens* or *Bacillus thuringiensis* by the fish, *Devario aequipinnatus*. Freeze-killed *G. mellonella* larvae were included as controls. B. The number of attacks (mean ± SE) on cadavers by *D. aequipinnatus* of the same treatments as in Fig. 6A. Different letters above SE bars indicate significant differences (P<0.05; Tukey's test).

Fig. 7. Percentage consumption (mean ± SE) of 2-day-old *Aedes aegypti* larvae killed by the nematode, *Steinernema siamkayai* or *Heterorhabditis indica* by the fish, *Devario aequipinnatus.* Freeze-killed *Ae. aegypti* larvae were included as controls. Different letters above SE bars indicate significant differences (P<0.05; Tukey's test).

Fig. 8. Percentage consumption (mean ± SE) of freeze-killed *Galleria mellonella* larvae by the fish, *Devario aequipinnatus*, after 1 and 18 h after continuous exposure to the symbiotic bacteria, *Xenorhabdus stockiae* or *Photorhabdus luminescens* or *Bacillus thuringiensis* culture supernatant. Different lower case

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(1 h) and upper case letters (18 h) above the SE bars indicate significant differences (P<0.05; Tukey's test).

FIGURES

Fig. 1A.

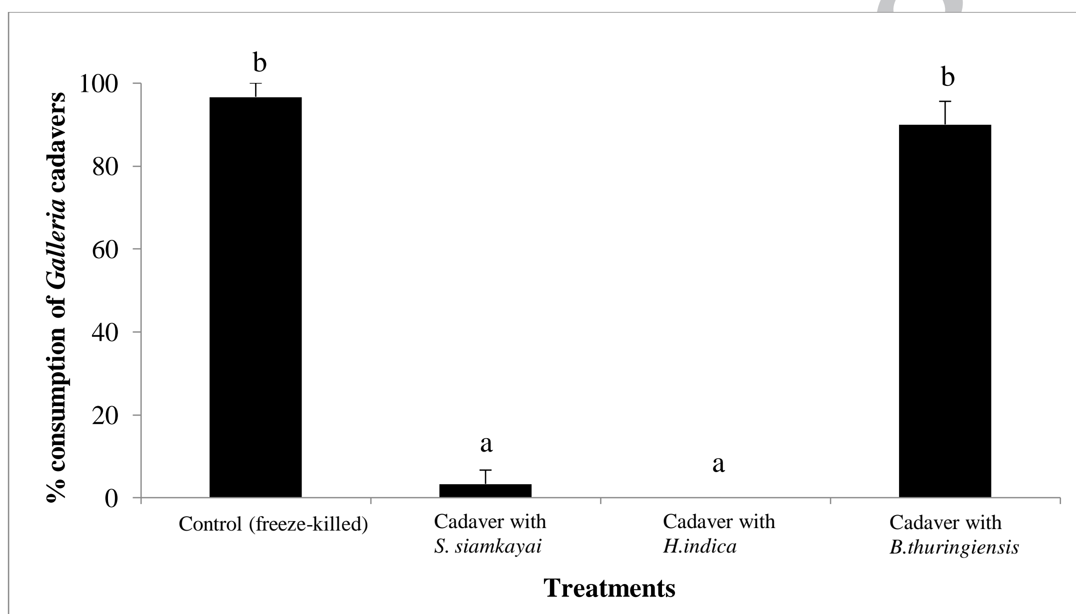
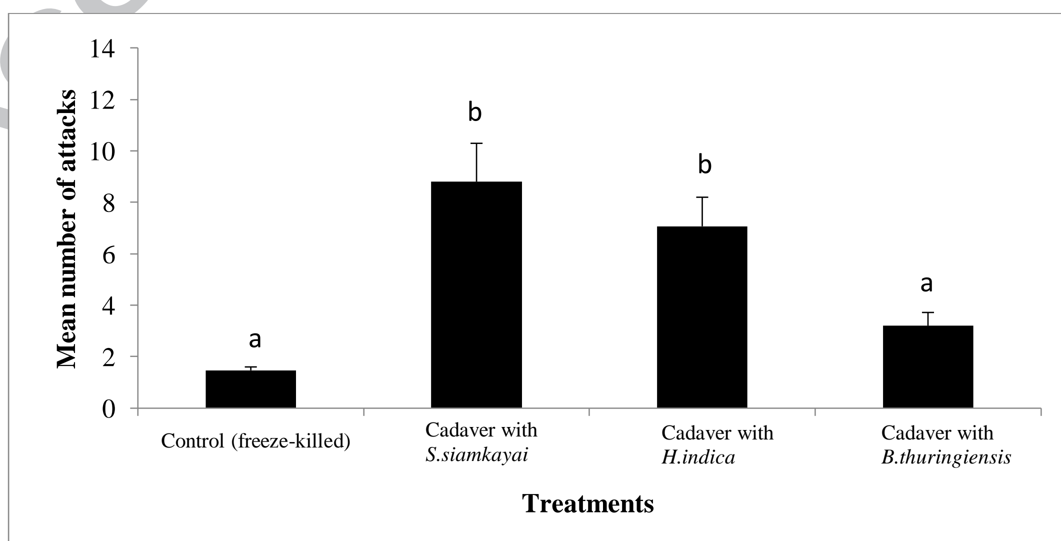


Fig. 1B.



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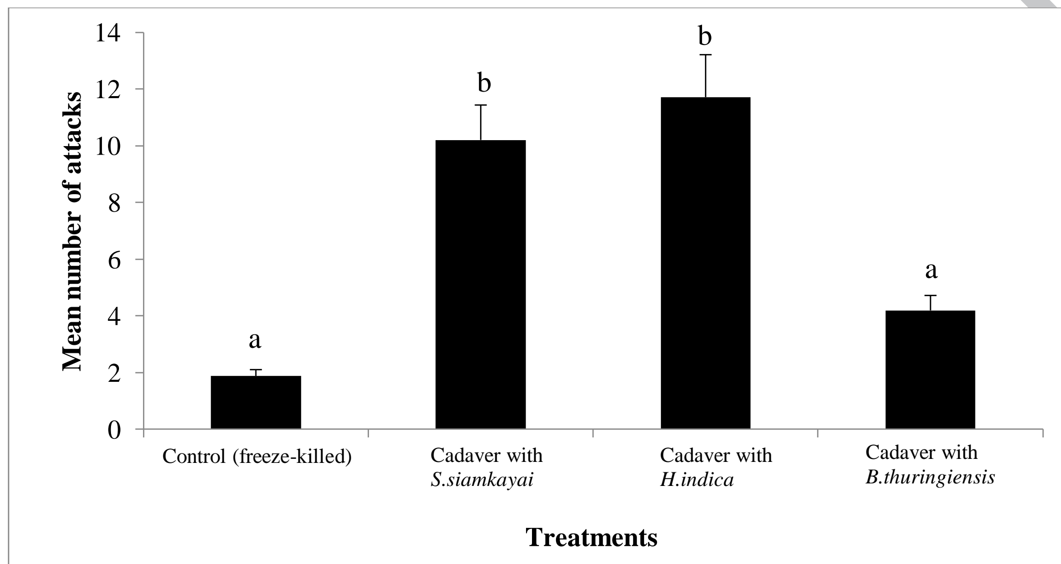
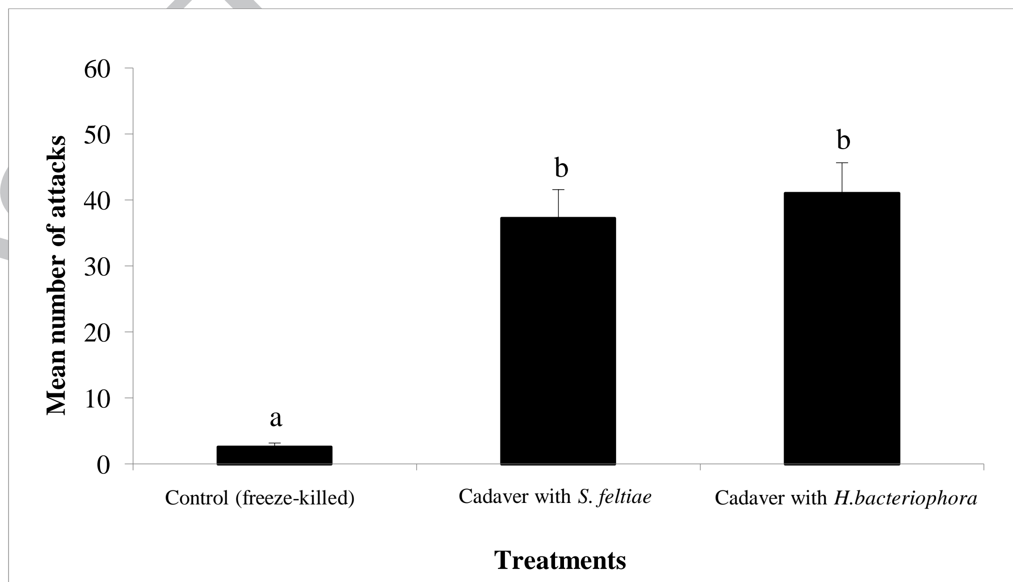


Fig. 3.



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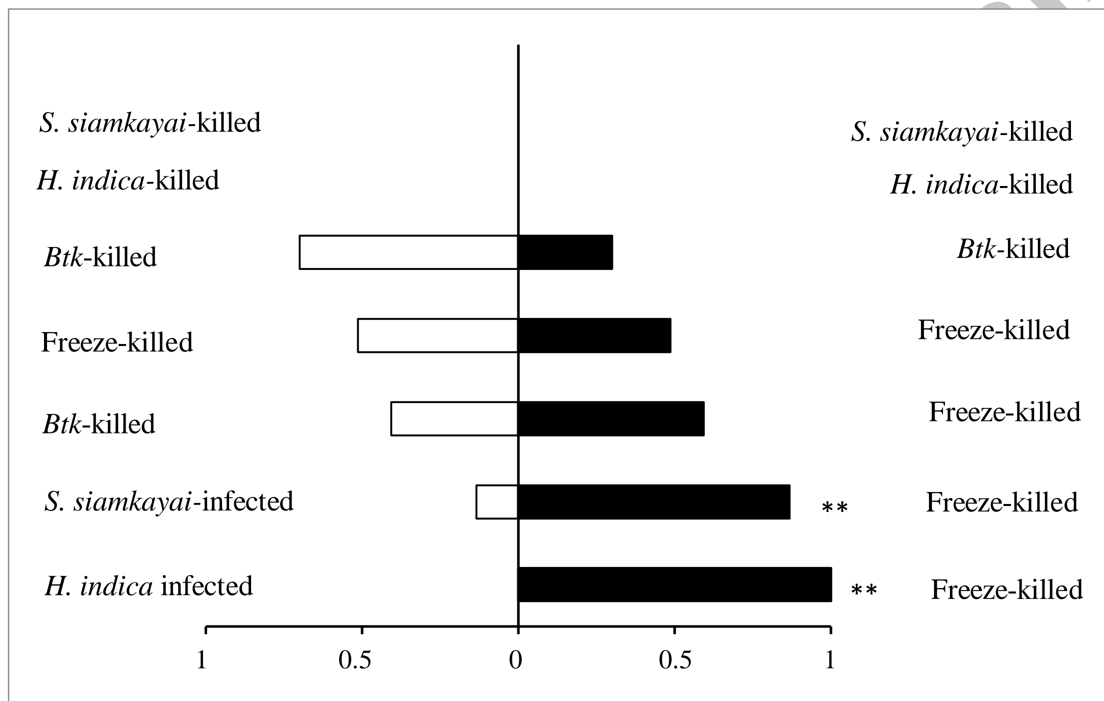

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Fig. 5.

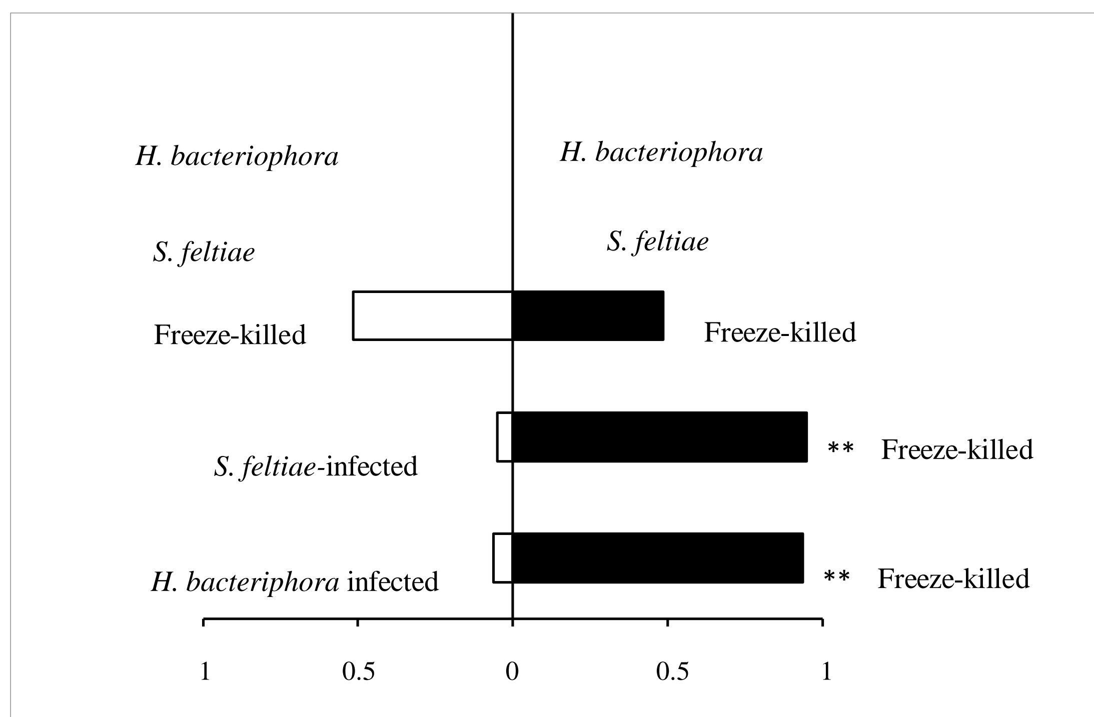


Fig. 6A.

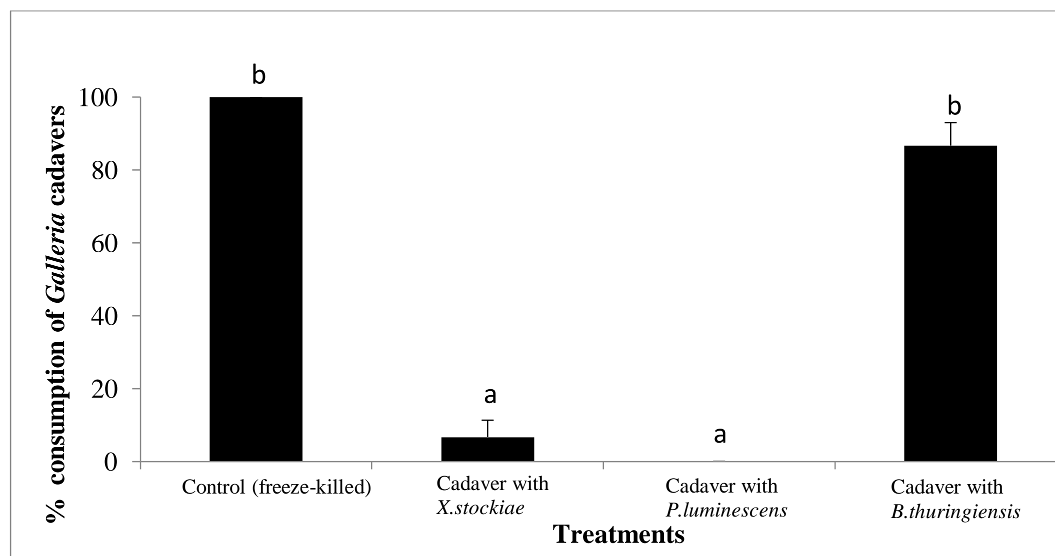
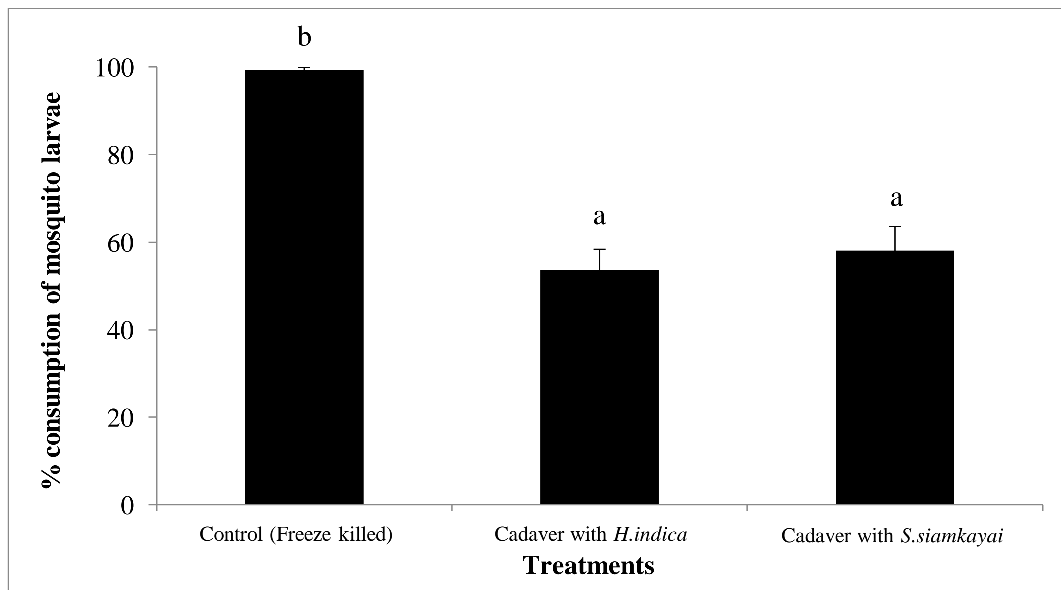


Fig. 6B.

Fig. 7.



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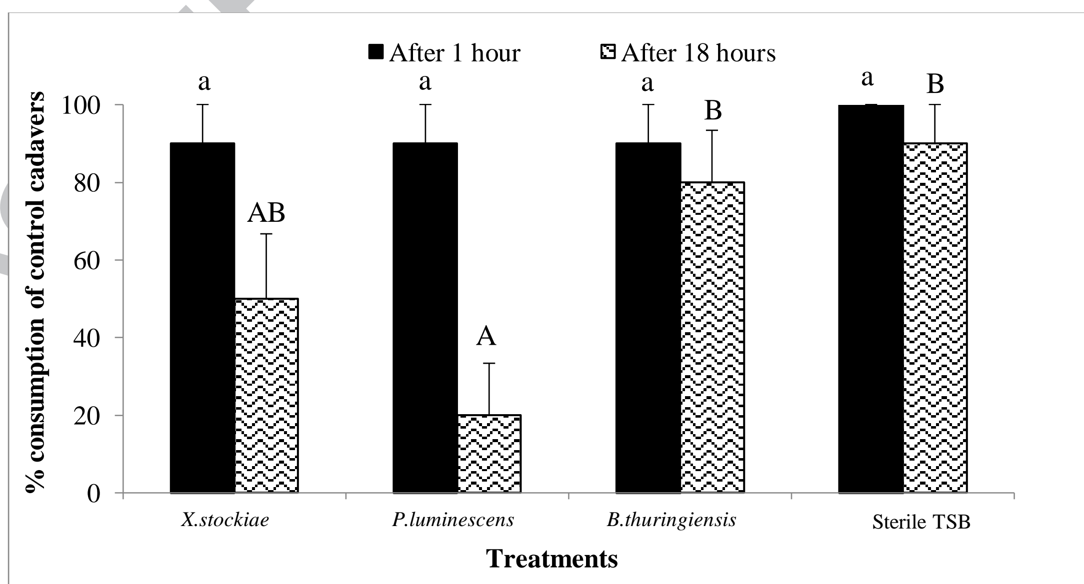

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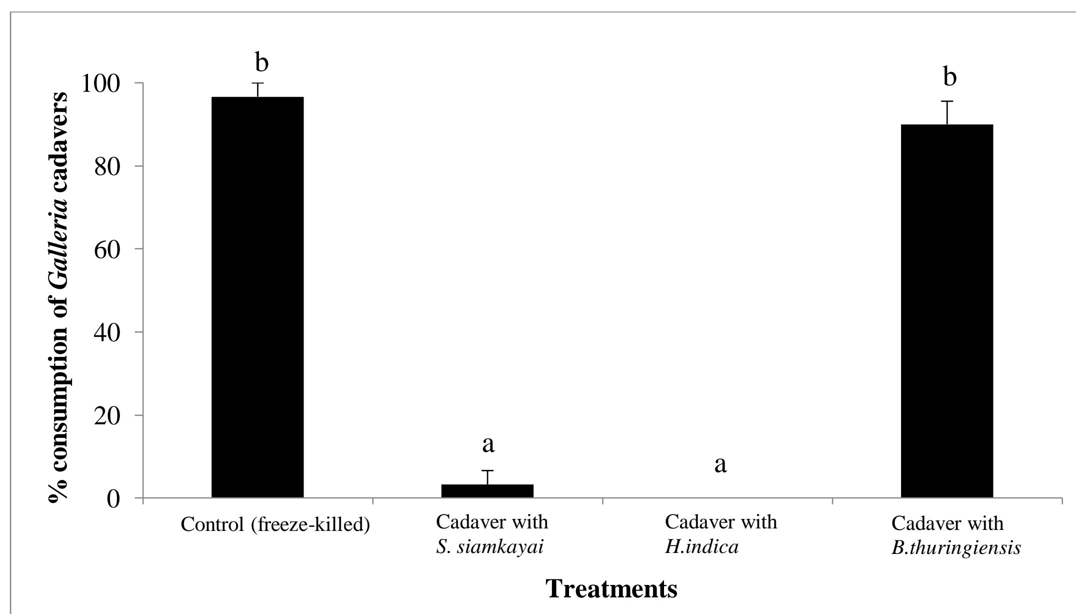
Graphical abstract



Percentage consumption (mean ± SE) of 2-day-old *Steinernema siamkayai*-killed or *Heterorhabditis indica*-killed *Galleria mellonella* larvae by the fish, *Devario aequipinnatus*.

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Highlights

-The bacterium of insect-parasitic nematodes produces a Scavenger Deterrent Factor (SDF).

-SDF deters not only terrestrial scavengers but also omnivorous freshwater fish.

- SDF protects the nematode-killed insect from being consumed by omnivorous fishes.

- Fish consumed significantly less dead mosquito larvae with SDF than control.

- SDF may make the cadaver unpalatable to protect the developing nematodes.

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