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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 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.

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

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toxemia or septicemia within 24–48 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 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 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 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.

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.