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ORIGINAL ARTICLE

Susceptibility of *Chironomus plumosus* larvae (Diptera: Chironomidae) to entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae): Potential for control

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Abstract. Chironomidae, or non-biting midges, are found worldwide in a wide variety of aquatic habitats. During periods of mass adult eclosion they can become a nuisance and health hazard. Current control methods target the aquatic larval stage and include the use of insect growth regulators or insecticides, which may be prohibited in certain environments or affect non-target organisms. The aim of this study was to investigate whether entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae, currently employed for control of terrestrial crop pests, could be used as a viable biocontrol for the aquatic larval stages of the Chrionomidae, offering an alternative to current chemical methods. We demonstrate that *Steinernema feltiae* (Filipjev, 1934), *Steinernema carpocapsae* (Weiser, 1955), *Steinernema kraussei* (Steiner, 1923) and *Heterorhabditis bacteriophora* (Poinar, 1975) are able to survive in water up to 96 h and are able to parasitize and kill *Chironomus plumosus* (Linnaeus, 1758) larvae, with mortality observed after just 24 h exposure and with < 20% survival after 4 days. We also show that following application to the water column, EPNs sink to the bottom of the lentic water body and can remain alive for more than 96 h. Taken together, we believe that several EPN species could be developed as a valid form of biocontrol for Chironomidae.

INTRODUCTION

The Chironomidae is a large family of Diptera with a cosmopolitan range (Usher & Edwards, 1984; Oliver & Corbet, 1966 in Lin & Quek, 2011) including two species found on the Antarctic continent (Oliver, 1971). Their ubiquitous nature may be in part due to the organism's tolerance of a wide range of environmental conditions including water temperature, and heavy metal pollution (Pinder, 1986). The Chironomidae is a holometabolous insect family, with the vast majority of the life cycle spent in an aquatic larval stage. With few exceptions, the short-lived adult stage of the organism does not feed, as the main purpose is to mate and oviposit (Oliver, 1971). To that end, Chironomidae exhibit adult mass emergence, which is thought to be a strategy evolved to increase the likelihood of finding a suitable mate and provide a sufficient quantity of individuals to allow sexual selection (Oliver, 1971; Lin & Quek, 2011). The ubiquitous presence of Chironomidae in natural and man-made freshwater systems brings them into contact with human habitations where their mass emergence and aerial swarming can cause a nuisance (Failla et al., 2015).

The presence of all life stages of Chironomidae close to human activity causes a number of issues ranging from public health concerns to environmental and economic problems (Ali, 1980; Baur et al., 1982; Cranston et al., 1983; Sakai et al., 1993; Failla et al., 2015). Exposure to swarming adult stages can result in allergic reactions (Baur et al., 1982) leading to asthma attacks (Sakai et al., 1993), rhinitis (Cranston et al., 1981), discomfort and distress at adults flying into eyes, mouth or nose (Ali, 1980) and even asphyxia (Cranston et al., 1983). Other reported nuisances include causing traffic accidents through adults covering car windshields and lights, and dead Chironomidae causing road and airport runway surfaces to become slippery (Ali, 1980; Armitage et al., 1995; Failla et al., 2015), defacing of paintwork through meconium deposits from fresh emergents (Sakai et al., 1993, Ferrington, 2008) and general restriction of outdoor activity (Ali et al., 2008). The larvae can also be significant pests of agriculture, causing damage to rice seeds and plants if they enter the irrigated growing environment (Ali, 1980; Failla et al., 2015).

Current control methods take a number of forms including chemical control using insecticides and insect growth regulators (Tabaru et al., 1987; Stevens, 1992; Giddings et al., 2009; Failla et al., 2015). Physical control methods such as the use of highly-polarised light in traps and attraction of adults with sound (Hirabayashi & Ogawa, 2000; Failla et al., 2015), rotational flooding and drying of breed-

ing areas, increasing depth of water bodies and removal of substrate in concrete storm drains (Ali, 1980) have also been used. The effectiveness of these treatments is related to the species targeted and the places in which they are breeding, with not all species as susceptible as others (Failla et al., 2015). The use of these control treatments raises concerns as to the effects on non-target species (Failla et al., 2015) and increasing resistance to chemical control is a significant issue for future control programmes. Biological control methods, using natural enemies of Chironomidae larvae have included the use of Odonate nymphs (Arena & Calver, 1996) or carp (Bay & Anderson, 1965). It has also been previously documented that parasitic nematodes from the family Mermithidae are capable of parasitizing the aquatic larval stages of Chironomidae (Poinar, 1964; de Doucet & Poinar, 1984; Johnson & Kleve, 1996) but there is no research to date that explores the capability of entomopathogenic nematodes (EPNs) from the genera *Steinernema* and *Heterorhabditis* to parasitize Chironomidae.

EPNs are obligate parasites of insects (Poinar, 1979; Gaugler, 2002; Kaya et al., 2006). The only free-living stage; the infective juvenile (IJ) kills insects by penetrating through the cuticle, anal opening, spiracles or mouth and releasing their symbiotic bacteria: *Xenorhabdus* spp. for *Steinernema* spp. and *Photorhabdus* spp. for *Heterorhabditis* spp. (Forst et al., 1997; Shapiro-Ilan & Gaugler, 2002). The host insect typically dies within 48 h and the EPNs then feed and reproduce, until the bacterial food supply within the decaying cadaver has been depleted, then the IJs move into the soil looking for new hosts (Gaugler & Kaya, 1990; Gaugler, 2002).

Laboratory studies have shown that EPNs are able to penetrate and kill the larval stages of up to 200 species of arthropod (Laumond et al., 1979; Grewal et al., 1994; Georgis et al., 2006) including many vector and nuisance biting genera. Since the 1980s, their natural pathogenicity towards arthropods has been utilised through their use as biological control agents for a range of insect pests. EPNs are currently used widely by growers of commercial or ornamental plant crops such as citrus, soft fruits and in greenhouse floriculture and forestry (Nguyen & Duncan, 2002; Georgis et al., 2006; Cuthbertson & Audsley, 2016). As EPNs are a 'natural enemy' of many pest species they can be considered to be a more environmentally friendly control method and as such are widely used in food crops where the use of traditional pesticides is banned or where growers wish to retain an 'organic' status (Kaya et al., 2006).

There have been some studies that have used EPNs to kill pestiferous aquatic insects. Poinar & Kaul (1982) showed that *Heterorhabditis bacteriophora* could kill *Culex pipiens* larvae and Welch & Bronskill (1962) found that *Steinernema carpocapsae* can kill and reproduce in *Aedes aegypti* larvae. Based on this knowledge this study sought to understand whether EPNs (*Heterorhabditis* and *Steinernema*) could kill and reproduce inside *Chironomus plumosus* and whether EPNs could therefore have the potential for development as a realistic biocontrol agent for chironomids.

MATERIALS AND METHODS

Source of invertebrates

Pelagial larvae of *Chironomus plumosus* were purchased from Andy's Aquatics, Wirral, U.K. and Chico's Pets, Liverpool, U.K. After purchase, larvae were immediately transported back to the laboratory where they were stored at 10°C in their original packaging. No food was provided for the *C. plumosus* larvae. Entomopathogenic nematodes (*Steinernema feltiae*, *Steinernema kraussei*, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*) were supplied by BASF Agricultural Specialities in a proprietary storage matrix and kept at 8°C until use. All EPNs were used within 28 days of receipt and all chironomid experimentation was initiated within 2 days of receipt of larvae.

Survival of *C. plumosus* to different doses of EPNs and establishment of dose-response relationships

EPN challenge was conducted in 200 ml plastic-cups (70 mm diameter top × 44 mm diameter base × 80 mm tall). Following guidelines from the World Health Organisation (2005) for larval testing twenty-five *C. plumosus* larvae were placed in separate disposable drinking cups containing 100 ml of distilled water (5 cm depth). To the cups, 0, 1000, 2000, 4000, 8000 or 16000 of each EPN species were added. These doses were based on testing multiples of the field application rate of 50 nematodes per cm^2 (Campos-Herrera, 2015). The area of the cups (38.46 cm^2) would warrant a dose of 1923 nematodes and we used a selection of doses that had previously been shown to kill a range of insects (Gaugler, 2002; Campos-Herrera, 2015). This process was repeated for each of the four tested EPN species. Each cup was covered with netting secured with an elastic band to avoid loss through emergence, and were incubated at 10°C to recreate the lower temperature water that *C. plumosus* would ordinarily inhabit (Hilsenhoff, 1966), yet be within the active range of the EPN species. Larval survival was recorded daily for 21 days. Larvae were adjudged to be dead if they were unresponsive when touched with a seeker. Each condition was tested 12 times. Any *C. plumosus* that died were examined for presence of nematodes.

Potential pathogenicity of EPN transport medium

EPNs are stored in a water dispersible gel carrier (proprietary formulation). To determine if this affected the survival of *C. plumosus*, EPN formulations were autoclaved at 121°C for 20 min to kill all EPNs and associated bacteria and allowed to cool to room temperature before being added to the plastic cups containing the Chironomidae larvae. It should be noted that the researchers were unable to separate the formulation from all the nematodes present therefore had to kill the nematodes via autoclaving and this may have changed the properties of the gel. The same amount of autoclaved gel formula as non-autoclaved formula with alive nematodes was added. This was repeated for each EPN species and repeated 3 times. The survival of *C. plumosus* was monitored daily for 21 days.

Survival of EPNs in water column

Soil-dwelling EPNs inhabit the water film between soil pores and the genera *Steinernema* and *Heterorhabditis* are not considered aquatic organisms. As such, it was important to discover if their survival would be affected when applied to water over time. Thirty plastic measuring cylinders (1 litre) were filled with 830 ml distilled water (a depth of 30 cm). Each cylinder was marked at 5 cm intervals. Ten thousand *S. feltiae* were added to each cylinder. *S. feltiae* was used as it had the longest survival in prelimi-

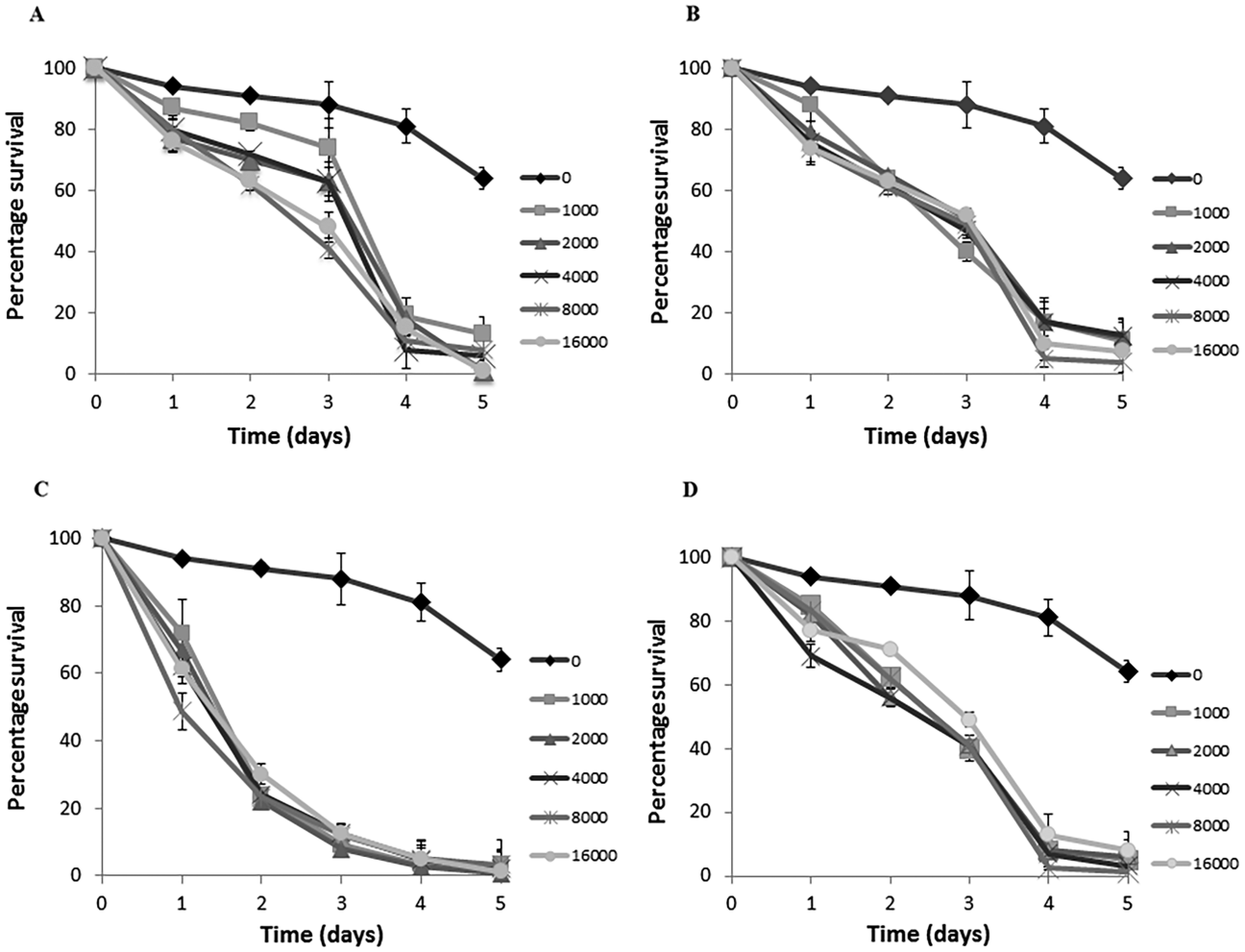

Fig. 1. Survival of *C. plumosus* exposed to 0, 1000, 2000, 4000, 8000 and 16000 S. *feltiae* (A), *H. bacteriophora* (B), *S. kraussei* (C) and *S. carpocapsae* (D) for 5 days. Bars represent ± one standard error.

nary, unpublished experiments. The cylinders were then covered with fine netting and secured with an elastic band. After 6 h, 150 ml was removed at 5 cm intervals from the top of the cylinder using a rota-filler pipette, ensuring minimal disturbance to position of EPNs in the water column. The survival of nematodes was quantified from 3 cylinders. This process was repeated at 12, 24, 48 and 96 h after application of S. *feltiae.* Experiments were done in triplicate.

Statistical analyses

The survival of *C. plumosus* exposed to each EPN species and potential pathogenicity of the transport medium were analysed using Log-rank tests using OASIS software (Yang et al., 2011). As multiple comparisons were carried out the P value was adjusted to 0.01 using the Bonferroni correction.

RESULTS

Survival of *C. plumosus* to difference doses of EPNs

All four EPN species were effective in killing *C. plumosus*. At four days, survival was < 20% for treated larvae in comparison to > 80% for control larvae unexposed to EPNs. The survival of untreated *C. plumosus* was significantly different from those that were exposed to different doses of *S. kraussei*, *S. feltiae*, *H. bacteriophora* and *S. carpocapsae* after 4 days exposure ($P < 0.01$) (Figs 1A–D). All nematodes (*S. feltiae*, *H. bacteriophora*, *S. kraussei* and *S. carpocapsae*) showed no significant dose-response with 1000–16000 being equally effective at killing chironomids (Figs 1A–D) ($P > 0.01$) (for complete results of statistical analysis see Supplementary Table S1). *H. bacteriophora*, *S. kraussei* and *S. carpocapsae* showed clear larvicidal effects from day two onwards whilst significant effects were not seen until day four for *S. feltiae* ($P < 0.01$). *S. kraussei* killed *C. plumosus* fastest with < 70% survival after just 2 days (Fig. 1) ($P < 0.001$). When dead *C. plumosus* were examined for the presence of nematodes after 5 days exposure, infective juveniles were found that had penetrated inside from the initial experimental dose of nematodes (Fig. 2A) and that had developed into adults and reproduced via endotokia matricida of the first generation *S. kraussei* females (Fig. 2B).

Potential pathogenicity of EPN transport medium

EPNs are stored and transported in a transport matrix. It is possible that this substance has larvicidal effect either through toxicity or through interference with respiration. To rule out the possibility that this killed *C. plumosus*, experiments were conducted under conditions identical

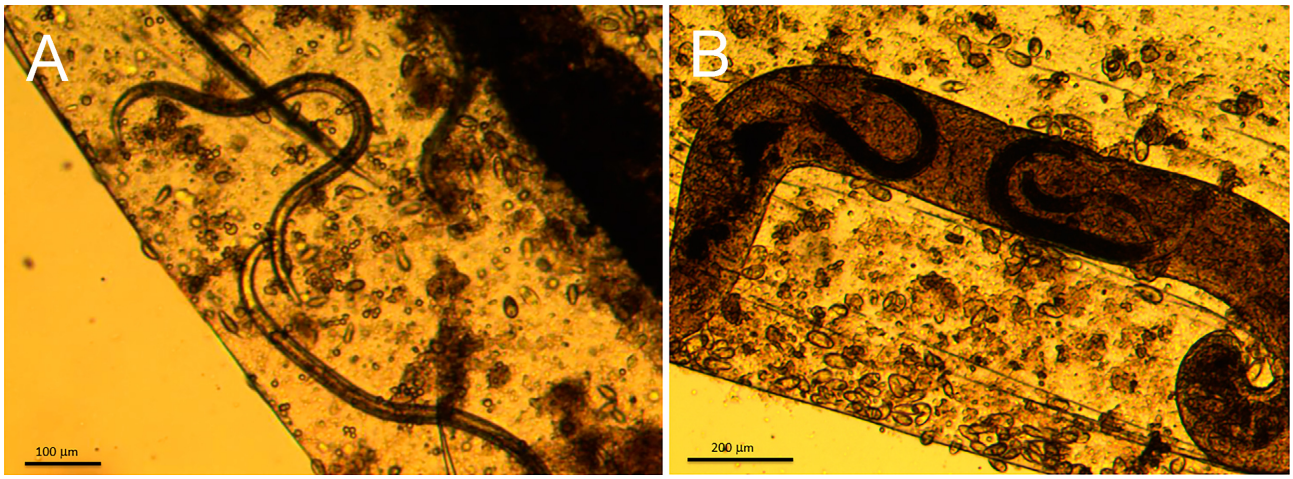

Fig. 2. Over 5 days *S. kraussei* were able to penetrate into *C. plumosus* (A) and reproduce via endotokia matricida in first generation mother (B). Scale bar represents 100 µm in A and 200 µm in B.

Table 1. Numbers of EPN (*Steinernema feltiae*) found at different water depths in a 30 cm column of water over time (up to 96 h) following the addition of N = 10,000 nematodes.

Depth (cm)	Mean number of *Steinernema feltiae* found ± SE				
	6 h	12 h	24 h	48 h	96 h
0–5	47.11 ± 14.64	36.67 ± 9.99	14.67 ± 3.64	2.00 ± 0.97	5.11 ± 0.73
5–10	59.56 ± 18.61	34.44 ± 9.49	36.67 ± 12.38	2.44 ± 0.99	6.33 ± 1.12
10–15	87.78 ± 22.33	70.33 ± 22.91	83.11 ± 36.92	5.44 ± 1.79	6.33 ± 2.06
15–20	90.67 ± 19.5	87.00 ± 30.26	122.56 ± 58.59	27.22 ± 14.91	8.11 ± 2.04
20–25	119.56 ± 25.65	99.56 ± 30.40	122.56 ± 58.59	27.22 ± 14.91	9.44 ± 2.04
25–30	9596.33 ± 89.63	9672.00 ± 97.65	9627.44 ± 8.33	9955.44 ± 8.33	9964.67 ± 6.44

to the pathogenicity experiments except that nematodes and symbiotic bacteria were killed by autoclaving prior to commencement of the experiment. In contrast to the experiments using live EPNs there were no significant differences in the survival of *C. plumosus* exposed to different doses of gel transport medium form untreated control chironomids for *S. kraussei* (P = 1.00); *S. feltiae* (P = 1.00); *H. bacteriophora* (P = 1.00) or *S. carpocapsae* (P = 1.00) throughout the 7 days of exposure (Fig. 3).

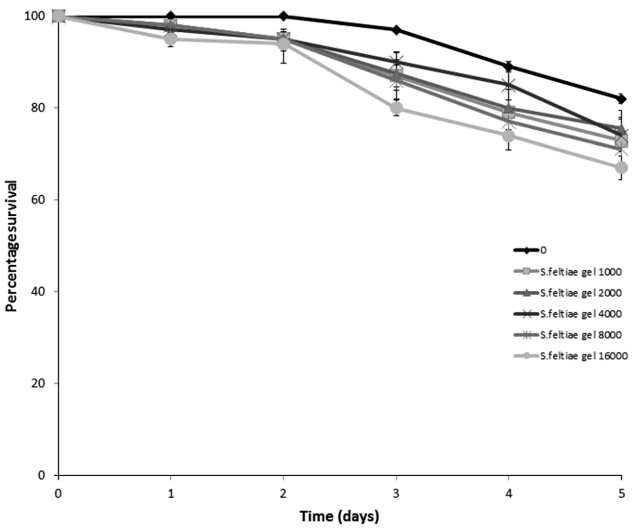

Fig. 3. The survival of *C. plumosus* exposed to 0, 1000, 2000, 4000, 8000 and 16000 autoclaved *S. feltiae* for 5 days. Bars represent ± one standard error.

Survival of EPNs in water column

Chironomid larvae are bottom dwelling and therefore it is important that EPNs are indeed able to sink and survive at depth. Following just 6 h in a 30 cm water column the majority of nematodes were found at 25–30 cm depth of water and from 6–96 h > 96% of nematodes were found alive and at the bottom of the water column (Table 1). Those found in the top of the water column after the first day were dead, suggesting that they had floated to the surface post-mortem.

DISCUSSION

This study found that aquatic larval stages of *C. plumosus* were parasitized and killed by four species of EPNs from two genera, with significant mortality observable after 2 days and the clearest mortality across all species visible after 4 days of exposure. *S. kraussei* exhibited the greatest pathogenic effect against *C. plumosus* larvae and required fewer nematodes to kill faster with only approximately 20% of the larvae alive after 24 h exposure. *S. kraussei* is known to be a cold tolerant EPN (Dolmans, 1983; Willmott et al., 2002; Laznik et al., 2009) with an effective thermal niche breadth of 6–10°C (Mráček et al., 1999; Long et al., 2000). The assays for this experiment took place at 10°C, within the infection thermal niche for that species (Wilmott et al., 2002). Previous studies have shown that *S. feltiae* has an infective thermal niche breadth of 10–30°C, *S. carpocapsae* 12–32°C and *H. bacteriophora* 12–32°C (Grewal et al., 1994) so they would not be inactive at the incubation temperature but may be more

biologically active at warmer temperatures. The successful pathogenic effect of *S. kraussei* at a relatively low temperature indicates that it may be more suitable for practical application in colder or deeper water bodies and its quicker mortality rate against the *C. plumosus* larvae may enhance its effectiveness in water.

The nematodes utilised in this study were supplied in a proprietary gel-based matrix. The assay with the gel carrier medium was used to determine whether it had any mortality effect on the target organisms. We used exactly the same amount of autoclaved formulation as used in experiments using live nematodes with un-autoclaved media. All results for this were not significant in comparison to the control suggesting that there was no effect from the carrier medium. This might be expected since larval chironomids are apneustic (Armitage et al., 1995) and therefore the medium would not block the breathing tubes.

In order to effectively control benthic larvae EPNs must sink to the bottom of the water column and survive for the period required to exhibit mortality effects. The depth assay shows that over a 96 h period *S. feltiae* remained at the bottom of the water body. Those found in the top parts of the column after 12 h were dead, whereas those in the lower segment (20–30 cm) were alive suggesting that those higher in the water column had floated to the top following death. The results from this experiment indicate that EPNs sink to the bottom of the water body over 6–12 h as they cannot actively swim through the water column (Lewis et al., 1998) due to the lack of friction (Koppenhofer et al., 1995). When at the bottom of the cup they are presumably able to locate and penetrate through the mouth, anus, spiracles or even directly through the cuticle as in terrestrial insects (Gaugler, 2002). This behaviour was observed in a similar experiment by Poinar & Kaul (1982) who described how *H. bacteriophora* penetrated directly through the anterior portion of the alimentary tract of *C. pipiens* larvae. The larval stages of the Chrionomidae life cycle are benthic (Prat & Rieradevall, 1995) therefore a lack of swimming ability may not hamper the EPNs attempts to seek a Chironomidae host. The pathogenicity assays show that at the most effective EPN doses nematodes were able to penetrate and kill more than 90% of the Chironomidae within 4 days. After 96 h the majority of the EPNs were still alive, which demonstrates that although these obligate parasitic nematodes are not usually found in aquatic media, they could survive long enough to potentially be useful as a biocontrol agent for Chironomidae. If an application of EPNs could be timed precisely to coincide with the later instars of the larvae this would be more effective. Much research has been undertaken at sites where Chironomidae cause a particular nuisance and environmental parameters related to swarming behaviour measured (Lin & Quek, 2011), so theoretically it may be possible to accurately time the introduction of EPNs to target later instars of the Chironomidae before emergence occurs.

The ability of the nematode to proliferate in insects in the soil habitat is key to their appeal as a biocontrol agent (Gaugler, 1988; Lacey & Georgis, 2012). This study showed that a range of EPNs can kill chironomids and, crucially, not only the infective stages were observed within the chironomid carcasses but there was also evidence of reproduction. These results therefore show that EPNs can infect, kill and reproduce in *C. plumosus*, however, it is unknown whether the infective juvenile stage would survive emergence from the host in a deep aquatic environment and this requires further study if they are to be applied in the field, for example in reservoirs.

In conclusion, field trials of the use of EPNs to combat nuisance populations of Chironomidae are an essential step forward in the validity of their use as an effective biocontrol but the results from these laboratory studies are highly promising. The advantage of using EPNs as biocontrol are many, including that they are already produced on an industrial scale with a large body of research on best practice and effectiveness. Our data also raise the tantalising possibility that such EPNs could be developed for use in control of other insect species with aquatic larval stages such as mosquito and *Simulium* vectors.

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CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

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Supplementary file:

Table S1 (http://www.eje.cz/2017/067/S01.xlsx). Statistical analysis of survival of *C. plumosus* exposed to 0, 1000, 2000, 4000, 8000 and 16000 S. *feltiae*, *H. bacteriophora*, *S. kraussei* and *S. carpocapsae* after 5 days exposure.