Reproductive efficiency of entomopathogenic nematodes as scavengers. Are they able to fight for insect’s cadavers?

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Entomopathogenic nematodes (EPNs) and their bacterial partners are well-studied insect pathogens, and their persistence in soils is one of the key parameters for successful use as biological control agents in agroecosystems. Free-living bacteriophagous nematodes (FLBNs) in the genus *Oscheius*, often found in soils, can interfere in EPN reproduction when exposed to live insect larvae. Both groups of nematodes can act as facultative scavengers as a survival strategy. Our hypothesis was that EPNs will reproduce in insect cadavers under FLBN presence, but their reproductive capacity will be severely limited when competing with other scavengers for the same niche. We explored the outcome of EPN-*Oscheius* interaction by using freeze-killed larvae of *Galleria mellonella*. The differential reproduction ability of two EPN species (*Steinernema kraussei* and *Heterorhabditis megidis*), single applied or combined with two FLBNs (*Oscheius onirici* or *Oscheius tipulae*), was evaluated under two different infective juvenile (IJ) pressure: low (3 IJs/host) and high (20 IJs/host). EPNs were able to reproduce in insect cadavers even in the presence of potential scavenger competitors, although EPN progeny was lower than that recorded in live larvae. Hence, when a highly susceptible host is available, exploiting cadavers by EPN might limit the adaptive advantage conferred by the bacteria partner, and might result in an important trade-off on long-term persistence. Contrary to our hypothesis, for most of the combinations, there were not evidences of competitive relationship between both groups of nematodes in freeze-killed larvae, probably because their interactions are subject to interference by the microbial growth inside the dead host. Indeed, evidences of possible beneficial effect of FLBN presence were observed in certain EPN-FLBN treatments compared with single EPN exposure, highlighting the species-specific and context dependency of these multitrophic interactions occurring in the soil.

Key words: *Heterorhabditis megidis*; multitrophic interactions; *Oscheius onirici*; *Oscheius tipulae*; scavenging; *Steinernema kraussei*
1. Introduction

Entomopathogenic nematodes (EPNs) are well-studied insect pathogens (Stock, 2015) and important agents for the biological control of soil insect pests in agroecosystems (Denno et al., 2008). This group of nematodes traditionally includes two phylogenetically distant families, Steinernematidae and Heterorhabditidae (Blaxter et al., 1998), which share similarities in their life cycles and behaviour as the result of convergent evolution (Poinar, 1993). For both families, one stage of life cycle comprises a free-living stage called infective juvenile (IJ), which can survive in the soil until it locates, penetrates and rapidly kills the host (48-72 hours) with the aid of obligate bacterial partners, transmitted from one generation to another (Dillman et al., 2012).

A better understanding of EPN soil food web dynamics, particularly antagonistic interactions, is critical to achieving a long-term EPN persistence in crops. The soil is a complex, species-rich environment and thus, various organisms have the potential to influence the survival and reproduction of EPNs. The survival of both naturally occurring IJs and those augmented for biocontrol action, is affected by biotic as much as abiotic factors (Ishibashi & Kondo, 1987; Griffin, 2015; Lewis et al., 2015). Moreover, introduced EPNs may alter the naturally occurring microbiota (nematode fauna included) in the soil (Duncan et al., 2007; Ishibashi & Kondo, 1986; Lewis et al., 2015). Nevertheless, as with most soil organisms, the natural habitats and behavioural plasticity of EPNs are still poorly known. A better understanding of ecological associations in the soil, such as competitive relationships and mutualism associations, is required to effectively use EPNs as biological control agents in agroecosystems (Stuart et al., 2015).

Campos-Herrera et al. (2015a) studied the competition for the insect larva as resource (live host) between two EPN species, *Heterorhabditis megidis* (Rhabditida: Heterorhabditidae) and *Steinernema kraussei* (Rhabditida: Steinernematidae), and two free-living bacteriophagous nematode (FLBN) species in the genus *Oscheius* (Rhabditida: Rhabditidae), *O. onirici* and *O. tipulae*. The selection of these nematode species was based on their co-occurrence in field experiments to evaluate the presence and activity of selected members of the nematode food web (Campos-Herrera et al., 2015b). They observed that the interaction between these two different
groups of nematodes depended on the number of IJs in the initial inoculum and on the nematode species combination. However, little attention was conferred to the Oscheius reproductive success nor the prevalence of larvae allowing single or mixed progeny, to evaluate the full extent of the competition.

The two species in the genus Oscheius used in previous experiments (Campos-Herrera et al., 2015a) are hermaphroditic and easy to isolate from soil samples (Félix et al., 2001). Oscheius tipulae was described initially as a saprophagous organism (Lam & Webster, 1971), insofar as they are able to feed on insect cadavers and decaying organic matter. Sudhaus (1993) reported that their use of cadavers can involve necromeny, process that implies to latch onto an insect, wait for its death and then, exploit it to complete the life cycle (Sudhaus and Schulte, 1989). Although Félix et al. (2001) suggested that O. tipulae is too common and ubiquitous to be associated with the life cycle of a particular insect, other Oscheius spp. were reported to display necrometric associations (Stock et al., 2005). Necromeny might be the intermediate evolutionary stage between parasitism and entomopathogenicity (Dillman et al., 2012). In fact, entomopathogenic behaviour has been ascribed to several species of nematodes in the genus Oscheius (Zhang et al., 2008; Ye et al., 2010; Torres-Barragan et al., 2011), O. onirici included (Torrini et al. 2015). Because of this possible transitional stage, the degree of entomopathogenic capability might differ among populations of the same species. For example, contrary to the Italian isolate described by Torrini et al. (2015), Swiss isolates did not exhibit entomopathogenic activity, but behaved as facultative kleptoparasites that compete for insect cadavers killed by EPNs (Campos-Herrera et al. 2015a).

Both EPN families had been considered as obligate parasites or pathogens of insects (Poinar, 1979), although some evidence was reported early on the use of insect cadavers by EPNs as a source of food and development (Jackson and Moore, 1968; Pye and Burman, 1978). Even if EPNs have never been reported as scavenger organisms in nature, laboratory experiments had shown that EPNs are able to colonise (San Blas et al., 2008) and produce offspring (San Blas & Gowen, 2008; Půža & Mráček, 2010) in freeze-killed insects. San Blas & Gowen (2008) reported evidences of EPN attraction to cadavers in olfactometer assays, and observed that certain species
can complete their life cycles when exposed to cadavers up to 240 hours-post-frozen old. However, these studies were performed in absence of other possible competitors of the cadavers as a resource.

Depending on the status of the available host (alive or dead), theoretically, the IJs can follow the usual entomopathogenic development (live host) or act as facultative scavengers (dead host). However, still is unknown to which extent each path will influence the net efficiency when more naturalized conditions are considered, such as presence of other scavengers that can compete for the cadaver. Various studies have addressed the EPN-FLBN interaction using live hosts (Duncan et al., 2003; Campos-Herrera et al., 2012, 2015a); however, whether the nature of these interactions could change using freeze-killed larvae instead of live larvae as hosts, and if EPNs could still reproduce in cadavers under scavenger competition by FLBNs remains completely unknown. We speculate that EPNs will be able to reproduce in insect cadavers under FLBN presence, but with certain limitations than when following their entomopathogenic behaviour. In addition, we expect that when EPN presence is restricted (low concentration), the reduction of their efficiency by FLBN-co-occurrence will be magnified, in a species-specific, density-dependent manner. Therefore, the aim of this study was to investigate the efficiency of EPN acting as scavengers in the presence of other possible competitors, and to evaluate how the initial inocula of EPN might contribute to modulate this interaction.

2. Material and Methods

2.1. Nematode cultures

The species of EPNs and FLBNs were selected on the basis of previous co-occurrence in field experiments (Campos-Herrera et al., 2015b). In particular, we evaluated two EPN species (S. kraussei OS population and H. megidis commercial, Andermatt Biocontrol AG), and two FLBN species (Oscheius onirici MG-67 and O. tipulae MG-68). EPNs were cultured in larvae of Galleria mellonella (Lepidoptera: Pyralidae) reared at University of Algarve (Portugal), IJs recovered in tap water upon emergence, stored at 10-12 °C, and used within 2 weeks of harvest.
FLBN species were mass-produced in Petri dishes containing a thin layer of 1.0% nutrient agar (NA, Fluka Analytical, Sigma–Aldrich), for 7–10 days at room temperature (20–22 °C) in the dark (Campos-Herrera et al., 2015a). For each trial, several plates were rinsed in M9 buffer (Herrmann et al., 2006), producing a suspension of mainly juvenile nematodes with possibly some adults.

2.2. Scavenging behaviour of entomopathogenic nematodes and their competition with Oscheius spp. for cadavers

The EPN scavenger activity under FLBN competition was evaluated following the experimental design proposed by Campos-Herrera et al. (2015a), but using freeze-killed G. mellonella larvae as hosts. Briefly, we assigned one 24-well plate (Falcon Multiwell, 24 well Polystyrene, Corning Incorporated-Life Sciences, Duham, USA) per each of the 12 treatments considered per trial (Table 1). In each of the 16 wells per treatment, we added 1.0 g of sterile sand (neograd, Migros, Switzerland) and the suspension of nematodes/control adjusted to final volume of 200 µl/well. The concentration of FLBNs was a constant variable (500 nematodes per well, equivalent to 282.5 nematodes/cm²), whereas the EPN density was adjusted to a low concentration (3 IJs per well each EPN species, hand-picked, equivalent to 1.7 IJs/cm²) and a high concentration (20 IJs per well each EPN species, equivalent to 11.3 IJs/cm²). Low numbers of IJs were applied in order to minimize the use of a model insect as G. mellonella, especially sensitive to infections by EPNs (Dutky et al., 1962). All the treatments (single application and combination) were inoculated at the same time, followed by the introduction of the freeze-killed host. After 4 days of incubation (21 °C in the dark) all cadavers were thoroughly washed and placed individually in White traps (White, 1929). Nematode emergence was observed every 2-3 days over a period of 30 days, and final progeny (number of IJs and/or FLBNs, depending on the treatment) was counted 9–10 days after the onset of emergence. Both low and high EPN concentration experiments were performed at 2 different times, with freshly produced nematodes and hosts.
2.3. Statistical analysis

We analysed variables related to the EPN infectivity and reproduction as well as the EPN impact on the FLBN activity. For EPNs, the variables were (i) frequency of larvae producing only IJs (pure EPN emergences), (ii) frequency of larvae producing IJs (even when mixed with FLBN emergences), and (iii) number of IJs produced per larva. Similarly, for Oscheius spp. we evaluated: (iv) frequency of larvae producing only Oscheius progeny (pure FLBN emergences), (v) frequency of larvae producing Oscheius progeny (even when mixed with EPN emergences), and (vi) number of Oscheius produced per larva. Prior to statistical analysis, all variables expressed as percentage were arcsine transformed, and quantitative variables were log (x + 1) transformed. We confirmed that the data of the independent trials could be pooled by two ways ANOVA, and thereafter, we employed t-student and one-way ANOVA for subsequent analysis (SPSS 21.0, SPSS Statistics, SPSS Inc., Chicago, IL, USA). For each of the variables described above, we consider the following factors: EPN species (H. megidis, S. kraussei), FLN species (O. tipulae, O. onirici), the initial IJ concentration (low with 3 IJs, high with 20 IJs), and the corresponding combinations. All data are presented as mean ± SEM of untransformed values.

3. Results

3.1. Scavenging activity of entomopathogenic nematodes

In general, irrespective of the EPN species studied (applied alone or combined with FLBN) or the initial IJ inoculum (3 IJs or 20 IJs), Oscheius spp. presence does not affect the frequency of larvae producing IJs as progeny (Fig. 1). The only exception was the combination of 3 IJs - H. megidis and O. onirici, which recorded a significantly higher frequency of larvae producing IJs (0.41 ± 0.09) compared with the single EPN application (0.13 ± 0.09, P = 0.040, Fig. 1A). Differences in the initial IJ inoculum did not affect the larvae producing IJs in H. megidis treatments. However, in the case of S. kraussei, in the low concentration experiment, the frequency of larvae producing IJs was 0.06 ± 0.04 for all the treatments (EPNs single applied or combined with Oscheius spp.), but this frequency increased in the high concentration experiment.
to 0.25 ± 0.08 for single EPN application \((P = 0.039)\), 0.31 ± 0.08 when combined with \textit{O. onirici} \((P = 0.002)\), and 0.38 ± 0.09 when combined with \textit{O. tipulae} \((P = 0.010, \text{Fig. 1B})\). Similarly, when \textit{H. megidis} and \textit{S. kraussei} were combined, higher frequencies of larvae producing IJs were observed in the high IJ inoculum than in the low concentration experiment, but this increase was only significant (marginally) in the presence of \textit{Oscheius} spp. \((P = 0.075 \text{ when combined with } \textit{O. onirici}; P = 0.049 \text{ when combined with } \textit{O. tipulae}, \text{Fig. 1C})\). Number of IJs emerged per larva was not affected neither by the presence of \textit{Oscheius} spp. nor by the differences on the initial IJ inoculum (Fig. 2).

### 3.2. Scavenging activity of free-living bacterivorous nematodes

The frequency of larvae producing FLBNs for \textit{Oscheius} spp. single applications was not different of these observed when combined with EPNs, for both low and high initial inoculum (Fig. 3). Overall, differences on the initial IJ inoculum did not affect the frequency of larvae producing FLBNs in pair-treatment comparison; however, some exceptions were observed when \textit{O. onirici} was involved. When the initial inoculum was increased from 3 IJs to 20 IJs, we observed 29% reduction in the incidence when combined with \textit{H. megidis} \((P = 0.012)\), and increased it by 10% when combined with \textit{S. kraussei} \((P = 0.083, \text{Fig. 3A})\). Similarly, in some cases, the presence of EPNs affected the number of \textit{Oscheius} emerging per larva. Specifically, when \textit{O. onirici} was involved, a statistically significant reduction of FLBN emergence occurred when combined with \textit{H. megidis}. The EPN caused 32% reduction \((P = 0.011)\) at the low concentration, and 44% reduction \((P = 0.001)\) in the high concentration experiment (Fig. 4A). When \textit{O. tipulae} was involved, statistically significant reduction of FLBN emergence only occurred in the high concentration experiment, but for all treatments: 37% off for \textit{H. megidis} \((P = 0.001)\), 31% off for \textit{S. kraussei} \((P = 0.001)\), and 50% off when both EPN species were combined \((P = 0.001, \text{Fig. 4B})\).

In pair-treatment comparison of the FLBN production between high and low initial IJ application, the only significant reductions was observed when \textit{Oscheius} spp. was combined with \textit{S. kraussei}: 27% reduction in the case of \textit{O. onirici} \((P = 0.010)\) and 31% reduction in the presence of \textit{O. tipulae} \((P = 0.018, \text{Fig. 4})\).
4. Discussion

In agreement with our first hypothesis, EPNs were able to complete their life cycles in insect cadavers even in the presence of potential scavenger competitors such as *Oscheius* spp. In the study by San-Blas & Gowen (2008), EPN species differed in their scavenging ability in old insect cadavers and fresh cadavers (24 h). Heterorhabditids were less successful in completing their life cycles than steinernematids in old cadavers. Both San Blas & Gowen (2008) and Půža & Mráček (2010) reported that IJs emerged from the majority of freshly freeze-killed *G. mellonella* larvae, independently of the EPN species. In agreement with those studies, our results did not reflect interspecific differences in the frequency of larvae producing IJs in single EPN applications. However, in our experiments, considerably fewer cadavers supported IJ emergence than the previous studies (San Blas & Gowen, 2008; Půža & Mráček, 2010). Without considering the methodological differences among experiments, these differences are likely due to the reduced starting IJ inocula: 3 IJs per larva (1.7 IJs/cm$^2$) or 20 IJs per larva (11.3 IJs/cm$^2$) in our experiments, compared with 100 or 200 IJs per larva (12.6 IJs/cm$^2$ or higher) the earlier works. Because few insect cadavers produced IJ offspring, our results should be viewed with caution; nevertheless, in contrast to the findings by San Blas & Gowen (2008), the IJ production was, in all cases, higher for *H. megidis* than for *S. kraussei*. The fact that the first generation adults of *H. megidis* are hermaphroditic (Forst & Clarke, 2002; Stock, 2015) may help to partially explain its biological advantage when initial IJ inocula were so limited, since *S. kraussei* needs the presence of at least one female and one male to complete its life cycle and produce progeny. Additional studies including more EPN species of both *Heterorhabditis* and *Steinernema* genera in combination of different species of host (San Blas 2012; Půža & Mráček, 2010) are necessary to establish whether there is a common predisposition for scavenging activity in each genus or if it is species-specific and context dependent ecological scenario.

Our study revealed how exploiting cadavers by EPN might limit their final progeny, highlighting the context-dependency (initial inoculum, host species) on the critical adaptive
advantage conferred by the bacteria partner, and hence, finding in the bacteria dynamic other plausible reasons for these interspecific differences. For example, not all EPN species release their symbiont bacteria within the same period of time after entering the insect’s hemocoel (Lewis et al., 2015). *Steinernema glaseri* releases its symbiotic bacteria *Xenorhabdus poinarii* (Enterobacteriales: Enterobacteriaceae) around 8 hours after entering the host hemocoel, whereas *Heterorhabditis bacteriophora* requires just 30 minutes to release its own bacteria *Photorhabdus luminescens* (Enterobacteriales: Enterobacteriaceae) (Wang et al. 1994). Upon release, symbiont bacteria multiply rapidly, killing the host and producing antibiotics with antifungal and antibacterial activities to obtain the ideal conditions for growth and reproduction of their associated EPNs, protecting the specificity of the symbiosis by eliminating microbial competitors (Boemare, 2002). A delay in the release of the symbiont bacteria in cadavers could benefit the growth of the intestinal microflora already present on the dead host, which can be detrimental to the best possible conditions for the establishment and development of the nematode-symbiotic bacterium complex (Kaya, 2002). However, it remains unknown whether the EPNs release their bacteria at the same time when acting as entomopathogens or scavengers. Growth by microbial competitors could explain why, according to our results and supposition, both EPN species were less skilful behaving like scavengers than performing as insect parasites (Campos-Herrera et al., 2015a). Further investigations are required to unravel the extent to which the presence of microbial competitors reduce the EPN progeny when acting as scavengers. Phylogenetic studies support that entomopathogenic activity of *Heterorhabditis* and *Steinernema* nematodes is an adaptation from ancestral trophic behaviour by FLBNs (Blaxter et al., 1998; Poinar, 1993). Moreover, according to the dauer hypothesis, which holds that the similarities in physiology and role of the dauer stage of free-living nematodes with the IJs of parasitic nematodes (Rogers and Sommerville, 1963; Hawdon and Schad, 1991) suggest a pre-adaptation to parasitism (Crook, 2014; Hotez et al., 1993). Thus, facultative scavenging by EPNs could simply be a reminiscence of its past as FLBNs. Additional studies are required to evaluate the impact of the hosts with different degree of susceptibility to EPN attack might help understanding these context-dependent scenarios (Půža & Mráček, 2010; San Blas et al., 2012).
Contrary to our expectations, the presence of Oscheius spp. did not affect much the EPN reproductive ability when acting as scavengers. Perhaps the competitive pressure of exogenous scavengers was lower, to the point of being negligible, compared with that exerted by the endogenous bacterial growth. Indeed, in a few cases we observed an opposite trend to that expected. In the low IJ inoculum experiment, the frequency of larvae producing IJs in the H. megidis single application treatment was significantly lower than when combined with Oscheius spp. Although the application of 3 IJs of amphimictic S. kraussei was too low for the successful colonization of the nematode-bacterium complex into the cadaver, increasing to 20 IJs we obtained a similar pattern as observed for 3 IJs-H. megidis. It seems plausible that if the symbiont bacteria is able to settle within the insect's cadaver, but in too low amounts to compete against hostile environment, the presence of bacteriophagous nematodes could assist EPN reproduction, simply by feeding on other bacteria. Conversely, when the EPN-symbiont complex is able to establish strongly (regardless whether the insect was killed or not by the EPN), other opportunists such as Oscheius spp. did not seem to interfere much with EPN fitness, while their own fitness was impaired. Such mechanisms could explain why FLBN production of O. onirici was significantly lower when combined with H. megidis than with S. kraussei, while O. tipulae reproductive success was significantly reduced for all treatments when initial inocula was increased from 3 IJs to 20 IJs. Similar trends were observed when live hosts were exposed to different EPN-Oscheius spp. combinations (Campos-Herrera et al., 2015a). Fewer larvae produced FLBN progeny and fewer FLBNs emerged per larva when insects were killed by H. megidis than by S. kraussei or the combination of both EPN species, especially at high inoculum concentration (Supplementary data 1-3), when presumably the EPN-bacterium complex competitive pressure was the highest for FLBNs.

Some evidences of competition by FLBNs towards EPNs were observed when live larvae were used as hosts (Campos-Herrera et al., 2015a), but only under low EPN-bacteria complex concentration conditions. In the current and previous studies, both laboratory experiments (Campos-Herrera et al., 2015a) and bait field soil samples (Campos-Herrera et al., 2015b; Jaffuel
et al., 2016, 2017), recorded progeny of both heterorhabditids and steinernematids leaving the same cadaver. However, Alatorre-Rosas & Kaya (1990) observed that, even if *Heterorhabditis* and *Steinernema* dual infection occasionally occurred, and development of both EPN species inside the insect cadaver is possible, their progeny eventually died. What may happen inside the insect cadaver is an interspecific competition between the two different EPN species, probably mediated by the symbiotic bacteria (Sicard et al., 2006), which would limit the final IJ production.

In general, if two *Steinernema* species co-infect an individual host, one species predominates in the emerging progeny (Koppenhöfer et al., 1995; Půža & Mráček, 2009). Recently, *Steinernema*-males were observed to physically injure and even kill both males and females of other *Steinernema* species when competing for the same host (O’Callaghan et al., 2014; Zenner et al. 2014). Campos-Herrera et al. (2015a) expected that the FLBNs would take the advantage of the EPN interspecific competition, which would result in a reduction of the final IJ production. Effectively, the IJ outcome was lower when two EPN species were combined with *Oscheius* spp. than in the treatment with two EPN species applied alone. This trade off could not been confirmed when freeze-killed insect larvae were used as hosts. Production of IJs was also reduced, but only in the high inoculum concentration treatments and too moderate to be significant. The low number of larvae producing EPN offspring in these particular treatments could be insufficient to complete an accurate statistical analysis, but it could also be that the competitive pressure of FLBNs is much lower than that exerted on EPNs by endogenous bacterial growth.

Our study illustrates the complexity of the EPN fight for the cadaver under more naturalized conditions. The results indicated that compared with the EPN traditional natural path (entomopathogenic), scavenging activity is less productive in a highly susceptible host scenario. It is plausible that the type of host (susceptible versus resistant to EPN attack) modulates this interaction (Půža & Mráček, 2010), and hence, additional studies are recommended. The fight between FLBN and EPN for the cadaver resources depends on species identity, and is modulated by ecological context; for example, a low numbers of IJs were sufficient for *H. megidis* to overcome the competition, whereas *S. kraussei* suffered strong competition even for higher initial IJ inocumum. Also, it is plausible that the type of host (susceptible versus resistant to EPN attack)
modulates this interaction (San Blas et al., 2012; Půža & Mráček, 2010), and hence, additional studies are recommended. In addition, EPN successful reproduction in the cadaver may sometimes be more a question of bacterial competition than nematode interaction, and in this scenario, the presence of FLBNs might alleviate the unfavourable bacterial conditions. Futures studies might investigate the extent to which these patterns are consistent for species with various life histories traits and behaviours, and particularly whether the presence of FLBN might be beneficial under certain conditions. By addressing various ecological contexts of natural pressure, we can better understand multitrophic interactions affecting EPNs, and we can identify key factors modulating their efficiency and long-term persistence.

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References


Duncan, L.W., Graham, J.H., Zellers, J., Bright, D., Dunn, D.C., El-Borai, F.E., Porazinska, D.L.,


Jaffuel, G., Blanco-Pérez, R., Büchi, L., Mäder, P., Fliessbach, A., Charles, R., Degen, T.,


Rhabditidae), a novel member of the entomopathogenic nematodes. J. Invertebr. Pathol. 98, 153–168.
Figure legends

Fig. 1. Frequency of frozen-killed larvae producing infective juveniles (IJ), including when they are mixed with *Oscheius* spp. emergences. **A.** Addition of either 3 infective juveniles (IJ) or 20 IJs of *Heterorhabditis megidis* (Hme) alone or in combination of *Oscheius onirici* (Ooni) or *Oscheius tipulae* (Otip). **B.** Addition of either 3 IJs or 20 IJs of *Steinernema kraussei* (Skr) alone or in combination of Ooni or Otip. **C.** Addition of either 3 infective juveniles (IJ) or 20 IJs of Hme and Skr mixed, alone or in combination of Ooni or Otip. Letters indicate significant differences among treatments (One-way ANOVA, \( P < 0.05 \)). Pair-treatment compassion between initial inoculum is represented with lines above the columns (Student's t-test (t): * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), ns, no significant). Data are average ± SEM.

Fig. 2. Number of infective juveniles (IJ) produced per frozen-killed larva. **A.** Addition of either 3 infective juveniles (IJ) or 20 IJs of *Heterorhabditis megidis* (Hme) alone or in combination of *Oscheius onirici* (Ooni) or *Oscheius tipulae* (Otip). **B.** Addition of either 3 IJs or 20 IJs of *Steinernema kraussei* (Skr) alone or in combination of Ooni or Otip. **C.** Addition of either 3 infective juveniles (IJ) or 20 IJs of Hme and Skr mixed, alone or in combination of Ooni or Otip. Letters indicate significant differences (One-way ANOVA, \( P < 0.05 \)). Pair-treatment compassion between initial inoculum is represented with lines above the columns (Student's t-test (t): * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), ns, no significant). Data are average ± SEM.

Fig. 3. Frequency of frozen-killed larvae producing free-living bacteriophagous nematodes (FLBNs), including when they are mixed with infective juvenile (IJ) emergence. **A.** Addition of 500 *Oscheius onirici* (Ooni) by single application (sum of low and high concentration experiments represented in the first column) or in combination of either *Heterorhabditis megidis* (Hme), *Steinernema kraussei* (Skr), or Hme and Skr mixed, in high and low concentration experiments. **B.** Addition of 500 *Oscheius tipulae* (Otip) by single application (sum of low and high concentration experiments represented in the first column) or in
combination of either Hme, Skr, or both mixed, in high and low concentration experiments. Letters indicate significant differences (One-way ANOVA, $P < 0.05$). Pair-treatment compassion between initial inoculum is represented with lines above the columns (Student's t-test (t): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, no significant). Data are average ± SEM.

**Fig. 4.** Number of free-living bacteriophagous nematodes (FLBNs) produced per frozen-killed larva. **A.** Addition of 500 *Oscheius onirici* (Ooni) by single application (sum of low and high concentration experiments) or in combination of either *Heterorhabditis megidis* (Hme), *Steinernema kraussei* (Skr), or Hme and Skr mixed, in high and low concentration experiments. **B.** Addition of 500 *Oscheius tipulae* (Otip) by single application (sum of low and high concentration experiments) or in combination of either Hme, Skr, or both mixed, in high and low concentration experiments. Letters indicate significant differences (One-way ANOVA, $P < 0.05$). Pair-treatment compassion between initial inoculum is represented with lines above the columns (Student's t-test (t): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, no significant). Data are average ± SEM.
**B**

*S. kraussei* treatments

![Graph showing frequency of larvae producing IJs for different treatments](image)

**C**

Hme and Skr combined treatments

![Graph showing frequency of larvae producing IJs for different treatments](image)
B

*S. kraussei* treatments

C

Hme and Skr combined treatments
B

**O. tipulae** treatments

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<th>Frequency of larvae producing FLBNs</th>
<th>Ooni</th>
<th>+ Hme</th>
<th>+ Skr</th>
<th>+ Hme+Skr</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 IJs</td>
<td>a</td>
<td>A</td>
<td>a</td>
<td>A</td>
</tr>
<tr>
<td>20 IJs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**ns**

No significant difference.
B

O. tipulae treatments
Table 1. Experimental design of the experiment to evaluate the scavenging behaviour of entomopathogenic nematodes (EPN) and their competition with *Oscheius* spp.

<table>
<thead>
<tr>
<th>Treatment code</th>
<th>EPN species</th>
<th><em>Oscheius</em> species</th>
<th>EPN applied (IJs)/well</th>
<th><em>Oscheius</em> applied/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hme</td>
<td><em>H. megidis</em></td>
<td>-</td>
<td>3 or 20</td>
<td>-</td>
</tr>
<tr>
<td>Skr</td>
<td><em>S. kraussei</em></td>
<td>-</td>
<td>3 or 20</td>
<td>-</td>
</tr>
<tr>
<td>Ooni</td>
<td>-</td>
<td><em>O. onirici</em></td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>Otip</td>
<td>-</td>
<td><em>O. tipulae</em></td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>Hme + Ooni</td>
<td><em>H. megidis</em></td>
<td><em>O. onirici</em></td>
<td>3 or 20</td>
<td>500</td>
</tr>
<tr>
<td>Hme + Otip</td>
<td><em>H. megidis</em></td>
<td><em>O. tipulae</em></td>
<td>3 or 20</td>
<td>500</td>
</tr>
<tr>
<td>Skr + Ooni</td>
<td><em>S. kraussei</em></td>
<td><em>O. onirici</em></td>
<td>3 or 20</td>
<td>500</td>
</tr>
<tr>
<td>Skr + Otip</td>
<td><em>S. kraussei</em></td>
<td><em>O. tipulae</em></td>
<td>3 or 20</td>
<td>500</td>
</tr>
<tr>
<td>Hme + Skr</td>
<td><em>H. megidis</em> + <em>S. kraussei</em></td>
<td>-</td>
<td>3 + 3 or 20 + 20</td>
<td>-</td>
</tr>
<tr>
<td>Hme + Skr + Ooni</td>
<td><em>H. megidis</em> + <em>S. kraussei</em></td>
<td><em>O. onirici</em></td>
<td>3 + 3 or 20 + 20</td>
<td>500</td>
</tr>
<tr>
<td>Hme + Skr + Otip</td>
<td><em>H. megidis</em> + <em>S. kraussei</em></td>
<td><em>O. tipulae</em></td>
<td>3 + 3 or 20 + 20</td>
<td>500</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Supplementary data 1. Frequency of larvae producing nematode progeny in the following categories: only infective juvenile (IJ) emergences, only free-living bacteriophagous nematode (FLBN) emergences, and both kind of nematodes mixed. Comparative of the competition experiments using live (L) or dead (D) insect larvae as host. A. Addition of either 3 infective juveniles (IJs) or 20 IJs of *Heterorhabditis megidis* (Hme) alone or in combination of *Oscheius onirici* (Ooni) or *Oscheius tipulae* (Otip). B. Addition of live or dead bacteria *S. kraussei* (Skr). C. *Hme* and *Skr* combined treatments.
**Supplementary data 2.** Differences between entomopathogenic nematode (EPN) reproduction ability between live and freeze-killed insects used as hosts. Data from the live host were taken from Campos-Herrera et al. (2015a). Treatments: *Heterorhabditis megidis* (Hme) applied alone or in combination of *Oscheius onirici* (Ooni) or *Oscheius tipulae* (Otip), *Steinernema kraussei* (Skr) applied alone or in combination of Ooni or Otip, and Hme and Skr mixed, applied alone or in combination of Ooni or Otip; for initial inoculum (No. IJs) of 3 and 20 infective juveniles (IJs) (data are average ± SEM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Species</th>
<th>No. IJs</th>
<th>Frequency of larvae producing any IJs</th>
<th>Frequency of larvae producing only IJs</th>
<th>Number of IJs produced per larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Live Larvae</td>
<td>↑/↓</td>
<td>Live Larvae</td>
<td>↑/↓</td>
</tr>
<tr>
<td>Hme</td>
<td>3</td>
<td>0.42 ± 0.04</td>
<td>↓70%</td>
<td>0.42 ± 0.04</td>
<td>↓70%</td>
</tr>
<tr>
<td>Hme + Ooni</td>
<td>3</td>
<td>0.50 ± 0.09</td>
<td>↓19%</td>
<td>0.36 ± 0.08</td>
<td>↓100%</td>
</tr>
<tr>
<td>Hme + Otip</td>
<td>3</td>
<td>0.53 ± 0.08</td>
<td>↓47%</td>
<td>0.36 ± 0.08</td>
<td>↓91%</td>
</tr>
<tr>
<td>Skr</td>
<td>3</td>
<td>0.19 ± 0.07</td>
<td>↓68%</td>
<td>0.19 ± 0.07</td>
<td>↓68%</td>
</tr>
<tr>
<td>Skr + Ooni</td>
<td>3</td>
<td>0.17 ± 0.06</td>
<td>↓63%</td>
<td>0.03 ± 0.03</td>
<td>↓100%</td>
</tr>
<tr>
<td>Skr + Otip</td>
<td>3</td>
<td>0.08 ± 0.05</td>
<td>↓25%</td>
<td>0.03 ± 0.03</td>
<td>↓100%</td>
</tr>
<tr>
<td>Hme + Skr</td>
<td>3+3</td>
<td>0.50 ± 0.09</td>
<td>↓74%</td>
<td>0.50 ± 0.09</td>
<td>↓74%</td>
</tr>
<tr>
<td>Hme + Skr + Ooni</td>
<td>3+3</td>
<td>0.31 ± 0.08</td>
<td>↓10%</td>
<td>0.11 ± 0.05</td>
<td>↓100%</td>
</tr>
<tr>
<td>Hme + Skr + Otip</td>
<td>3+3</td>
<td>0.28 ± 0.08</td>
<td>↓43%</td>
<td>0.17 ± 0.06</td>
<td>↓100%</td>
</tr>
<tr>
<td>Hme</td>
<td>20</td>
<td>0.88 ± 0.05</td>
<td>↓71%</td>
<td>0.88 ± 0.05</td>
<td>↓71%</td>
</tr>
<tr>
<td>Hme + Ooni</td>
<td>20</td>
<td>0.75 ± 0.07</td>
<td>↓71%</td>
<td>0.65 ± 0.08</td>
<td>↓100%</td>
</tr>
<tr>
<td>Hme + Otip</td>
<td>20</td>
<td>0.95 ± 0.04</td>
<td>↓77%</td>
<td>0.78 ± 0.07</td>
<td>↓88%</td>
</tr>
<tr>
<td>Skr</td>
<td>20</td>
<td>0.53 ± 0.08</td>
<td>↓52%</td>
<td>0.53 ± 0.08</td>
<td>↓52%</td>
</tr>
<tr>
<td>Skr + Ooni</td>
<td>20</td>
<td>0.70 ± 0.07</td>
<td>↓55%</td>
<td>0.05 ± 0.04</td>
<td>↓100%</td>
</tr>
<tr>
<td>Skr + Otip</td>
<td>20</td>
<td>0.55 ± 0.08</td>
<td>↓32%</td>
<td>0.08 ± 0.04</td>
<td>↓100%</td>
</tr>
<tr>
<td>Hme + Skr</td>
<td>20+20</td>
<td>0.80 ± 0.06</td>
<td>↓65%</td>
<td>0.80 ± 0.06</td>
<td>↓65%</td>
</tr>
<tr>
<td>Hme + Skr + Ooni</td>
<td>20+20</td>
<td>0.78 ± 0.07</td>
<td>↓36%</td>
<td>0.13 ± 0.05</td>
<td>↓100%</td>
</tr>
<tr>
<td>Hme + Skr + Otip</td>
<td>20+20</td>
<td>0.55 ± 0.08</td>
<td>↓31%</td>
<td>0.13 ± 0.05</td>
<td>↓77%</td>
</tr>
</tbody>
</table>

*Relative increment (↑) or decrease (↓) of the frequency of larvae producing any IJ (mixed or not with FLBNs) for freeze-killed larvae respect live larvae used as hosts.*

b *Relative increment (↑) or decrease (↓) of the frequency of larvae producing only IJs (no mixed with FLBNs) for freeze-killed larvae respect live larvae used as hosts.*

c *Relative increment (↑) or decrease (↓) of the number of IJs emerged per larva for freeze-killed larvae respect live larvae used as hosts.*
Supplementary data 3. Differences on the free-living bacteriophage nematode (FLBN) reproduction ability between live and freeze-killed insects used as hosts. Data from the live host were taken from Campos-Herrera et al. (2015a). Treatments: Oscheius onirici (Ooni) applied in combination of Heterorhabditis megidis (Hme), Steinernema kraussei (Skr) or Hme and Skr mixed, and Oscheius tipulae (Otip) applied in combination of Hme, Skr or Hme and Skr mixed; for initial inoculum (No. IJs) of 3 and 20 infective juveniles (IJ) (data are average ± SEM).

<table>
<thead>
<tr>
<th>Species</th>
<th>No. IJs</th>
<th>Frequency of larvae producing any FLBNs</th>
<th>Frequency of larvae producing only FLBNs</th>
<th>Number of FLBNs produced per larva</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Frequency of larvae</td>
<td>↑/↓</td>
<td>Frequency of larvae</td>
</tr>
<tr>
<td>Ooni + Hme</td>
<td>3</td>
<td>0.19 ± 0.07</td>
<td>↑414%</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>Ooni + Skr</td>
<td>3</td>
<td>0.28 ± 0.08</td>
<td>↑226%</td>
<td>0.14 ± 0.06</td>
</tr>
<tr>
<td>Ooni + Hme + Skr</td>
<td>3+3</td>
<td>0.42 ± 0.08</td>
<td>↑138%</td>
<td>0.22 ± 0.07</td>
</tr>
<tr>
<td>Otip + Hme</td>
<td>3</td>
<td>0.17 ± 0.06</td>
<td>↑481%</td>
<td>0.00</td>
</tr>
<tr>
<td>Otip + Skr</td>
<td>3</td>
<td>0.14 ± 0.06</td>
<td>↑575%</td>
<td>0.08 ± 0.05</td>
</tr>
<tr>
<td>Otip + Hme + Skr</td>
<td>3+3</td>
<td>0.14 ± 0.06</td>
<td>↑550%</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>Ooni + Hme</td>
<td>20</td>
<td>0.15 ± 0.06</td>
<td>↑442%</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>Ooni + Skr</td>
<td>20</td>
<td>0.80 ± 0.06</td>
<td>↑25%</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td>Ooni + Hme + Skr</td>
<td>20+20</td>
<td>0.75 ± 0.07</td>
<td>↑29%</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>Otip + Hme</td>
<td>20</td>
<td>0.18 ± 0.06</td>
<td>↑400%</td>
<td>0.00</td>
</tr>
<tr>
<td>Otip + Skr</td>
<td>20</td>
<td>0.60 ± 0.08</td>
<td>↑41%</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>Otip + Hme + Skr</td>
<td>20+20</td>
<td>0.68 ± 0.08</td>
<td>↑43%</td>
<td>0.23 ± 0.07</td>
</tr>
</tbody>
</table>

* Relative increment (↑) or decrease (↓) of the frequency of larvae producing any FLBN (mixed or not with IJs) for freeze-killed larvae respect live larvae used as hosts.

b Relative increment (↑) or decrease (↓) of the frequency of larvae producing only FLBNs (no mixed with IJs) for freeze-killed larvae respect live larvae used as hosts.

c Relative increment (↑) or decrease (↓) of the number of FLBNs emerged per larva for freeze-killed larvae respect live larvae used as hosts.
EPN – Bacteria Complex

Dead Insect Larvae as Host

Low frequency of larvae producing IJs
High frequency of larvae producing FLBNs

Exogenous FLBNs
Research Highlights

- Entomopathogenic nematodes (EPNs) co-occur with free-living nematodes (FLNs) in soils
- EPNs were able to reproduce in insect cadavers in the presence of scavenger FLNs
- EPN reproductive success is lower when acting as scavengers
- Using cadavers by EPNs might limit the advantage conferred by the bacteria partner
- Scavenging EPN-FLN interaction is species-specific and context dependency