

Mechanisms behind differential white grub host susceptibility to entomopathogenic nematodes

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Summary – Control of white grubs, the sporadic root-feeding larvae of scarabid beetles, is difficult due to their cryptic nature and resistance to chemical insecticides. Potential exists for the use of entomopathogenic nematodes (EPN) for such control. The successful infection of white grubs by EPN, however, is limited by the white grubs' innate reduced susceptibility in form of behavioural, morphological and physiological defences. This study aimed to determine the different physiological defence mechanisms of white grubs against attack by EPN. The white grub species, *Pegylis sommeri* and *Schizonycha affinis*, which were previously found to have a low level of susceptibility to EPN, and *Maladera* sp. 4, which had previously shown a relative high level of susceptibility, were examined. The ability of the EPN to penetrate the white grubs and of the EPN symbiotic bacteria to grow inside the insect haemolymph, the encapsulation rates, as well as the nematode growth inside the insect haemolymph, were determined. The results showed that the ability of the nematodes to penetrate white grubs, the encapsulation rates and growth in the haemolymph varied between EPN and white grub species. The different species of the EPN symbiotic bacteria were able to grow in the haemolymph of all the white grub species tested. The results indicate that the white grub species investigated tended to resist the initial penetration of most of the EPN studied, thus avoiding infection.

Keywords – encapsulation, *Pegylis sommeri*, Scarabaeidae, *Schizonycha affinis*, white grub immunity

White grubs (Coleoptera: Scarabaeidae) are the root-feeding larvae of scarabid beetles (Ritcher, 1966). In South Africa and the neighbouring kingdom of Eswatini, white grubs are the most important insect pests in sugarcane and black wattle, *Acacia mearnsii* De Wild (Fabales: Fabaceae), plantations (Echeverri-Molina & Govender, 2016a, b). Their control is difficult, due to their soil-dwelling nature and to their resistance to chemical insecticides (Grewal *et al.*, 2004). The nocturnal nature of the adult further complicates use of the available control options (Jackson & Klein, 2006). Entomopathogenic

nematodes (EPN) have been shown, in some cases, to be able to provide a higher control efficacy against white grubs than chemical insecticides (Koppenhöfer & Fuzy, 2003; Grewal *et al.*, 2004).

EPN are soil-dwelling microscopic roundworms of the order Rhabditida that are known to parasitise various insects (Grewal *et al.*, 2005; Lacey & Georgis, 2012). They kill their insect hosts with the aid of symbiotic bacteria, which they carry in their digestive system and sometimes in a specialised bacterial vesicle (Ishibashi, 2002). Death of the insect hosts normally occurs within 48 h after infection (Kaya *et al.*, 1993; Ishibashi, 2002).

The specialised third instar juvenile of the EPN, known as the infective juvenile (IJ), is the only free-living stage of the EPN. It can move in the soil and locate potential hosts, which it invades through their natural openings (Ishibashi, 2002; Lacey & Georgis, 2012). The soil-dwelling activity of such larvae partly accounts for the suitability of EPN for the control of white grubs. Their suitability is, however, sometimes limited by the white grub's innate defence mechanisms against nematode infection, which is a result of the co-evolution of the white grubs and the nematodes in the soil (Klein *et al.*, 2007; Schmid-Hempel, 2008). The defence mechanisms of insects can generally be separated into primary, secondary and tertiary levels (Smilanich *et al.*, 2009), but the mechanisms can also be classified into behavioural, morphological or physiological defences (Gross, 1993; Klein *et al.*, 2007).

The primary defence mechanisms help the insects to avoid encountering the pathogens. In the case of white grubs, some of the documented primary defences include their cryptic nature (Gaugler, 1988; Gross, 1993; Gaugler *et al.*, 1994), as well as their association with such nematode predatory organisms as mites (Karagoz *et al.*, 2007), their evasive and aggressive behaviour, including grooming (Gaugler *et al.*, 1994; Alvandi *et al.*, 2017), and changes in their respiratory patterns (Gaugler, 1988). The latter defence mechanism involves releasing the respiratory gas in bursts, or infrequently rather than continuously, which assists such grubs to avoid detection by the EPN (Gaugler, 1988; Kaya *et al.*, 1993; Gaugler *et al.*, 1994; Grewal *et al.*, 2005).

The secondary defence mechanisms reduce the energy costs required to get rid of an infection. For white grubs, such mechanisms include the possession of spines and hairs, brushing off invading nematodes, thrashing or dropping, regurgitating and copious defecating (Smilanich *et al.*, 2009). In addition, the gut's dense peritrophic membrane delays EPN penetration, while the food passage removes nematodes from the vulnerable alimentary tract (Forschler & Gardner, 1991; Gaugler *et al.*, 1994; Grewal *et al.*, 2005). The hard exoskeleton and impenetrable sieve plates covering the spiracles (Forschler & Gardner, 1991; Gaugler *et al.*, 1994; Brivio & Mastore, 2018) also limit EPN entry into the white grub's body, which further complicates the infection process.

The tertiary defence of the grub's innate immunity is aimed at providing resistance after an infection. Such defence includes the cellular responses, including phagocytosis, nodulation, and encapsulation mediated by haemocytes (Strand, 2008), and the humoral responses, including production of soluble effector molecules, antimicrobial protein complexes and the activation of phenoloxidase cascades (Hoffmann, 2003; Kanost *et al.*, 2004; Strand, 2008; Tsakas & Marmaras, 2010; Alvandi *et al.*, 2017). Various tertiary resistance mechanisms in white grubs have been identified, including encapsulation and melanisation in *Popillia japonica* Newman (Li *et al.*, 2007; An *et al.*, 2012) and encapsulation and phenyl oxidase activity in *Polyphylla adspersa* Motschulsky (Alvandi *et al.*, 2017). The studies mentioned indicated the importance of nematodes escaping their initial encapsulation to enable successful infection.

In previous studies, the efficacy of various, locally isolated, EPN species was evaluated against five of the predominant white grub species in the Kwa-Zulu Natal province of South Africa (Abate *et al.*, 2019;

Katumanyanne *et al.*, 2023). The white grub species included *Schizonycha affinis* Boheman, *Pegylis sommeri* Burmeister, *Maladera* sp. 4 and *Monochelus* sp., and *Heteronychus* *licas* Klug. *Pegylis sommeri* and *S. affinis* were found to be the least susceptible to the EPN. *Pegylis sommeri* was the least susceptible to most of the tested EPN, with the highest percentage mortality of 30% being obtained with *Heterorhabditis zealandica* Poinar MJ2C, after 14 days of exposure. The mean percentage mortality of *S. affinis*, of greater than 50%, was achieved with six of the seven EPN species tested, but only *H. zealandica* MJ2C achieved a mean percentage mortality of 70% at 21 days post infection. White grubs in South African are generally known to be resistant to EPN (Abate *et al.*, 2019; Katumanyanne *et al.*, 2023). The mechanisms used by the white grub species to avoid EPN infection are not well studied.

The aim of the current study was to identify some of the tertiary or physiological defence mechanisms shown by the white grubs to nematode infection. The ability of two strains of *H. zealandica*, *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers & Hurley and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams to grow and complete their lifecycle in the haemolymph of the white grubs *S. affinis*, *P. sommeri*, *Maladera* sp. 4 and *Galleria mellonella* (Lepidoptera: Pyralidae) was examined. The choice of EPN used in this study was based on the results of Katumanyanne *et al.* (2023), to have a representation of the more efficient EPN *H. zealandica* MJ2C (green infected wax moth cadavers), the less efficient *S. fabii* and *S. yirgalemense*, as well as a second strain of *H. zealandica* SF41, which is known to possess a different species of symbiotic bacteria (blue infected wax moth cadavers). The ability of the three nematodes to infect the third instar larvae of *S. affinis* and *P. sommeri* was also determined. Additionally, *H. bacteriophora* was used in the infection bioassay to have a representation of two heterorhabditid and two steinernematid species. Furthermore, the rates of encapsulation of IJ of the three EPN species by the larvae of *S. affinis*, *P. sommeri* and *Maladera* sp. 4 were established. The ability of four symbiotic bacteria (isolated from the three EPN species and one from a different strain of *H. zealandica* that is known to possess a different species of symbiotic bacteria) to grow in the haemolymph of *S. affinis*, *P. sommeri* and *Maladera* sp. 4 was also determined.

Materials and methods

SOURCE OF INSECTS

Four white grub species, namely *S. affinis*, *P. sommeri*, *Maladera* sp. 4 and an unidentified *Monochelus* sp., were used in the present study. Insect identification was done by the LUCID key, which was previously developed for white grubs in sugarcane plantations in southern African countries (https://keys.lucidcentral.org/keys/v3/sugarcane_white_grubs/sugarcane_white_grubs.html). The white grubs were collected over different seasons in 2018 and 2019, from various locations in wattle and sugarcane plantations in the KwaZulu-Natal province of South Africa (Table 1). White grubs were dug up from the soil at sites where patchy sugarcane growth was observed or in mature wattle plantations with a thick topsoil layer, as prior monitoring indicated such conditions as favouring a relatively high number of grubs. Each white grub was placed in a 30 ml plastic vial, filled with moist autoclaved peat moss (Hygrotech Sustainable Solutions), and provided with a fresh carrot disc as a food source. The vials containing white grubs were placed in cooler boxes for transport. The grubs were reared at the Forestry and Agricultural Biotechnology Institute (FABI) Biocontrol Centre at the University of Pretoria, South Africa, at 23°C and 60-70% relative humidity. The autoclaved peat and carrot discs were replaced weekly, and third instar grubs were used for experiments. White grubs that showed signs of pupation were excluded from the study.

Table 1. Geographical location of sites for white grub (Coleoptera: Scarabaeidae) species collected in the KwaZulu-Natal province of South Africa, indicating the host plant and most dominant grub species collected at the site (underlined).

Site	Dominant grub species	Host plant	GPS coordinates
Dalton1	<i>Pegylis sommeri</i>	Wattle	29°12'40.4"S 30°38'09.0"E
Dalton2	<i>Schizonycha affinis</i>	Sugarcane	29°12'52.8"S 30°37'55.5"E
Dalton3	<i>Maladera</i> sp. 4	Sugarcane	29°12'52.8"S 30°37'55.5"E
Dalton4	<i>Maladera</i> sp. 4	Wattle	29°12'52.8"S 30°37'55.5"E
Dolphin coast1	<i>S. affinis</i>	Sugarcane	29°12'35.0"S 31°28'06.0"E
Dolphin coast2	<i>S. affinis</i>	Sugarcane	29°12'35.0"S 31°28'06.0"E
<u>Hilton1</u>	<i>Maladera</i> sp. 4, <i>S. affinis</i>	<u>Wattle</u>	<u>29°29'30.4"S 30°18'35.9"E</u>
Hilton2	<i>Maladera</i> sp. 4, <i>S. affinis</i>	Wattle	29°28'33.3"S 30°18'42.2"E
Mbalenhle	<i>S. affinis</i>	Sugarcane	29°19'22.7"S 30°48'52.9"E
<u>Piet Retief</u>	<i>Maladera</i> sp. 4	<u>Wattle</u>	<u>27°07'20.0"S 30°57'05"E</u>
<u>Wartburg1</u>	<i>S. affinis</i>	Sugarcane	<u>29°25'51.6"S 30°39'21.6"E</u>
Wartburg2	<i>S. affinis</i>	Sugarcane	29°14'50.7"S 30°39'55.7"E
<u>Wartburg3</u>	<i>P. sommeri</i>	<u>Sugarcane</u>	<u>29°28'47.4"S 30°39'36.3"E</u>

Table 2. *Heterorhabditis* and *Steinernema* isolates used in the study, with their associated host plants, origin, GenBank accession number, and the length and width of the infective juveniles (IJ) in μm .

<i>Heterorhabditis</i> / <i>Steinernema</i>	Isolate	Associated host plant/insect	Origin (town/province)	GenBank accession No. (ITS region)	IJ length (μm)	IJ width (μm)	Reference
<i>H. bacteriophora</i>	SF351	Indigenous forest	KwaZulu-Natal	MF535289	588 (512-671)	23 (18-31)	Abate <i>et al.</i> (2016)
<i>H. zealandica</i> (green)	MJ2C	Citrus orchard	Hex River Valley, Western Cape	MF370073	685 (570-740)	27 (22-30)	James <i>et al.</i> (2018)
<i>H. zealandica</i> (blue)	SF41	Natural vegetation	Patensic, Eastern Cape	EU699436	685 (570-740)	27 (22-30)	Malan <i>et al.</i> (2006)
<i>S. yirgalemense</i>	157-C	Citrus orchard	Friedenheim, Mpumalanga	EU625295	685 (570-740)	29 (24-33)	Malan <i>et al.</i> (2011)
<i>S. fabii</i>	SCH10*	White grub	KwaZulu-Natal	MW618681	641 (590-697)	28 (26-31)	Katumanyanne <i>et al.</i> (2022)

*Isolated during this study.

Table 3. *Photorhabdus* and *Xenorhabdus* symbiotic bacteria associated with entomopathogenic nematodes (EPN) *Heterorhabditis* and *Steinernema* isolates used in the study, with their associated host nematode.

EPN symbiotic bacteria species	Associated nematode species/isolate	Associated host plant/insect	Origin (town/province/ company)	Reference (EPN isolation)
<i>P. thracensis</i>	<i>H. zealandica</i> MJ2C (green)	Citrus orchard	Hex River Valley, Western Cape	James <i>et al.</i> (2018)
<i>P. heterorhabditis</i> subsp. <i>heterorhabditis</i>	<i>H. zealandica</i> SF41(blue)	Natural vegetation	Patensic, Eastern Cape	Malan <i>et al.</i> (2006)
<i>X. indica</i>	<i>S. yirgalemense</i> 157-C	Citrus orchard	Friedenheim, Mpumalanga	Malan <i>et al.</i> (2006)
<i>X. bovienii</i>	<i>S. feltiae</i>	—	e-nema	—

SOURCE OF NEMATODES

Five EPN species (Table 2) were used in the current study. The nematode isolates were obtained from the nematode collections at the FABI Biocontrol Centre and at the Department of Conservation Ecology and Entomology at Stellenbosch University. All the nematodes were reared using the third instar larvae of the greater wax moth, *G. mellonella*, from a laboratory culture. Modified White traps were used to harvest the IJ for a period of 1 week after their emergence. Harvested IJ were stored in distilled water at 12°C, in horizontally placed culture flasks (Woodring & Kaya, 1988). The stored EPN were shaken periodically for ventilation and used within 3 weeks after EPN harvest, to ensure nematode viability. A fresh batch of nematodes was used for each experimental repetition.

EPN SYMBIOTIC BACTERIA ISOLATION

We used the methodology described in Stokwe & Malan (2017) to isolate four EPN symbiotic bacteria species, namely *Xenorhabdus bovienii* Akhurst & Boemare, *Xenorhabdus indica* Somvanshi, Lang, Ganguly, Swiderski, Saxena & Stackebrandt, *Photorhabdus thracensis* Fischer-Le Saux, Viallard, Brunel, Normand & Boemare and *Photorhabdus heterorhabditis* subsp. *heterorhabditis* Ferreira, Reenen, Endo, Tailliez, Pagès, Spröer, Malan & Dicks from *S. feltiae*, *S. yirgalemense*, *H. zealandica* MJ2C and *H. zealandica* SF41, respectively (Table 3). For the bacteria isolation, Petri dishes containing five wax moth larvae were each inoculated with IJ of the different nematode species, at 400 IJ plate⁻¹. When the infected wax moth larvae were moribund, the hind leg of each insect was pricked with a sterile syringe needle, and a drop of haemolymph was streaked on separate NBTA plates (8.0 g nutrient agar; 25 mg bromothymol blue; 40 mg 2,3,5-triphenyltetrazolium chloride l⁻¹). The plates were then incubated at 28°C for 48 h. From each NBTA plate, a single bacterial colony was isolated with a loop and suspended in 30 ml tryptic soy broth (TSB) in an Erlenmeyer flask, which was plugged with non-absorbent cotton. The flasks were then incubated on an orbital shaker on 140 rpm at 28°C for 48 h. The bacterial culture (1 ml) was then pipetted into 1.8 ml cryogenic tubes for preservation at -80°C in 30% glycerol.

NEMATODE GROWTH IN INSECT HAEMOLYMPH

Four EPN, namely *H. zealandica* MJ2C, *H. zealandica* SF41, *S. fabii* and *S. yirgalemense*, were tested for their ability to grow and reproduce in the haemolymph of the white grubs, *S. affinis* and *P. sommeri*. Haemolymph of *G. mellonella* and *Maladera* sp. 4 was used as the positive control, as *Maladera* sp. 4 had shown a higher level of susceptibility than did *G. mellonella* to the EPN in previous experiments (Katumanyane *et al.*, 2023). The aim of this experiment in the haemolymph was to test the hypothesis that bacteria within the host insect may antagonise the EPN symbiotic bacteria thus interfering with the infection process. Thus, this could not be done in the live insect host as there would potentially be antagonising bacteria. Care was taken not to introduce contaminants by working under the lamina flow conditions. The insects, after being surface-sterilised with 75% ethanol, were patted dry with a paper towel. The insects were pierced in the hind leg with a sterile insulin needle to release a drop (ca 50 µl) of the haemolymph onto a sterile Petri dish. Ten IJ of each nematode were transferred to an individual drop of the insect's haemolymph and moist cotton wool was added to the Petri dish to retain 100% relative humidity. The Petri dishes were then gently placed in a plastic container, lined with moist tissue paper and incubated at 25°C for 10 days.

Nematode development was checked and recorded daily for the developmental status of the nematodes. Observations were made for the presence of IJ, recovered IJ (RIJ), live adults (A), dead adults (DA), recovered first-generation IJ (R1GIJ) and second-generation juveniles (2GJ) in the drops of insect haemolymph. The presence of second-generation juveniles in the haemolymph indicated

that the nematodes concerned were able to complete their life cycle within the white grub. Ten white grubs (=ten haemolymph drops) were used for each white grub species *versus* EPN combination and the experiment was repeated twice, with each repeat occurring on a different test date, using a different batch of nematodes.

INFECTION BIOASSAY

An infection bioassay was used to determine the ability and rate of penetration of *H. bacteriophora*, *H. zealandica* MJ2C, *S. fabii* and *S. yirgalemense* into the haemocoel of *S. affinis* and *P. sommeri*, over a period of 72 h. The same number of white grubs (n = 30) were used for each white grub-EPN combination, with 30 also being used for the control experiment, which was inoculated with distilled water only. For each species, single white grubs were placed in the individual cells of 12-well bioassay plates, lined with filter paper discs. Each grub was inoculated with 200 IJ of the EPN, suspended in 50 µl of distilled water. The white grubs in the control experiment were inoculated with distilled water. The plates, after being placed in a plastic container lined with wet paper towels (100% moisture), were kept in a growth chamber, at 25°C for a period of 6-120 h. For each treatment, five grubs were randomly removed after 6, 12, 24, 48, 72 and 120 h, washed with a stream of distilled water and frozen at -40°C, until dissection. Dissection took place with the aid of a light microscope, to enable visual determination of the presence or absence of nematodes. The experiment was repeated twice for each grub species, each on a different test date, using a fresh batch of nematodes.

ENCAPSULATION TEST

The method employed by Li *et al.* (2007) was modified and used to determine the rate of encapsulation of three nematodes by the white grubs, *S. affinis*, *P. sommeri* and *Maladera* sp. 4. The nematodes used included *H. zealandica* MJ2C, *S. fabii* and *S. yirgalemense*. Ten grubs were used for each white grub-EPN species combination. The experiment was repeated twice, using a different batch of nematodes on each occasion.

Ten IJ of each nematode species were injected laterally to the base of a foreleg, in the haemocoel of the insect, using a sterile insulin needle. The inoculated grubs were placed in filter paper-lined bioassay plates and incubated at 25°C.

The nematodes were recovered at 10 h post injection. The duration of incubation was determined from the nematode growth that took place in the haemolymph and melanisation was observed to occur after 10 h, during the pilot study. To recover the nematodes, the grubs were dissected in a Petri dish filled with Ringer's solution. Care was taken not to damage the insect's digestive system, which was removed before recovering the nematodes from the haemolymph. The rest of the tissues were further examined for the presence of nematodes. The amount of encapsulation that occurred was measured by means of counting the proportion of nematodes with haemocytes attached, at 400× magnification. The encapsulated nematodes included only the cellular, but not the melanotic encapsulation, with such nematodes being defined as those nematodes with attached blood cells (Li *et al.*, 2007).

HUMORAL IMMUNITY MEASUREMENT USING INHIBITORY ZONE ASSAYS

The procedure adopted in Stokwe & Malan (2017), Roy & Kim (2020) and Skowronek *et al.* (2020) was modified and used in this experiment to determine the ability of four EPN symbiotic bacteria species to grow in the haemolymph extracts of *P. sommeri*, *S. affinis* and *Maladera* sp. 4. Four bacteria species (Table 3) were isolated, their identity verified and used in this experiment. The insect haemolymph was obtained by means of sterilising white grubs with 75% alcohol, after which they were pricked on

the hind leg, using a sterile needle, to obtain the insect haemolymph. A metal loop was flamed and used to scrape the surface of the frozen bacteria stored at -80°C in Eppendorf tubes and streaked onto NBTA media. The agar plates were then sealed with Parafilm and incubated in the dark at 28°C for 48 h. A single colony was taken with a sterile loop and inoculated on TSB in an Erlenmeyer flask, placed on an orbital shaker on 140 rpm at 28°C in a growth chamber for 48 h. All the available isolates of bacteria were evaluated visually for growth, using the spotting technique suggested by Berkvens *et al.* (2014). A sterile glass spreader was used to distribute 50 μl of 48-h-old TSB bacterial culture from the Erlenmeyer flask evenly onto an NBTA plate. Five filter paper discs (3 mm diam.) were then placed, in a line configuration, on the inoculated medium. Four discs were each spotted with 10 μl of the insect haemolymph, while 10 μl of 5% sodium hypochlorite (JIK) was used to spot the middle disc as control. The bacterial proliferation or inhibition around the filter discs was visually observed and recorded after 2-4 days post treatment. The experiment was repeated three times, each time using a different batch of bacteria. Even though there was no liquid/haemolymph seepage observed during this experiment, volatile seepage could not be ruled out.

STATISTICAL ANALYSIS

The data for the percentage of infected grub bioassays were analysed using the statistical analysis programs R (R Core Team, 2014) and Statistica ver. 14 (StatSoft, 2016). The R function (AOV) was used to perform a one-way ANOVA test, to be able to identify group mean differences and standard errors (SEs). For multiple pairwise-comparison, the Tukey Honest Significant Differences, R function: Tukey (HSD) was used to determine whether the mean difference between specific pairs of groups was statistically significant, with the Tukey (HD) function accepting the fitted ANOVA as its argument. The data obtained during the encapsulation test regarding the proportion of encapsulated nematodes were analysed using the statistical program R. The R function (AOV) was used to perform a one-way ANOVA test, to be able to identify the group mean differences involved. For the multiple pairwise comparison, the Tukey Honest Significant Differences, R function: Tukey (HSD) was used to determine whether the mean difference between specific pairs of groups was statistically significant, with the Tukey (HD) function accepting the fitted ANOVA as its argument.

Results

NEMATODE GROWTH IN INSECT HAEMOLYMPH

The results showed varying ability of the nematodes to grow and reproduce in the insect haemolymph (Table 4). *Maladera* sp. 4 showed the highest level of susceptibility to the tested nematodes, as all the tested EPN species were able to grow in the haemolymph of *Maladera* sp. 4. *Steinernema fabii* particularly preferred the haemolymph of *Maladera* sp. 4, as the nematode completed its life cycle faster in such haemolymph than it did in the positive control experiment using the haemolymph of *G. mellonella*. The second-generation juveniles of *S. fabii* were observed at 4 DAI (days after inoculation), when they were inoculated in the haemolymph of *Maladera* sp. 4.

Pegylis sommeri showed a moderate level of susceptibility to the growth of EPN. Three of the four tested EPN, namely, *H. zealandica* MJ2C, *S. fabii* and *S. yirgalemense*, were able to complete their life cycle in the haemolymph of *P. sommeri*. However, *H. zealandica* SF41 was unable to grow on the haemolymph of *P. sommeri*, as the IJ were observed either to die or not to enter the recovery phase. *Schizonychia affinis* was the least susceptible to the growth of EPN. Only *S. fabii* and *H. zealandica* MJ2C. were able to complete their life cycle in the haemolymph of *S. affinis*. The IJ of *H. zealandica*

Table 4. Growth of the entomopathogenic nematodes (EPN) *Heterorhabditis zealandica* MJ2C, *H. zealandica* SF41, *Steinernema yirgalemense* and *S. fabii* in the haemolymph of three white grub species, over a period of 6 days after inoculation (DAI).

EPN species		DAI										Observed 6 DAI
		2		3		4		5		6		
		I	II	I	II	I	II	I	II	I	II	
<i>Schizonycha affinis</i>	<i>H. zealandica</i> MJ2C	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	A	1GIJ	A + 1GIJ	1GIJ	Successful cycle in one rep.
	<i>H. zealandica</i> SF41	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	IJ never recovered, No ESB observed.
<i>Pegylis somerri</i>	<i>S. fabii</i>	1GIJ	1GIJ	A	R1GIJ	A + 2GJ	A	2GJ	A + 2GJ	2GJ	2GJs	Successful cycle
	<i>S. yirgalemense</i>	1GIJ	1GIJ	R1GIJ	1GIJ	DA + IJ	1GIJ	1GIJ	1GIJ	1GIJ	1GIJ	Cycle unsuccessful
	<i>H. zealandica</i> MJ2C	1GIJ	R1GIJ	R1GIJ	A	A	DA	A	DA	A + 2GJ	DA	Successful cycle
	<i>H. zealandica</i> SF41	1GIJ	D1GIJ	D1GIJ	D1GIJ	D1GIJ	D1GIJ	D1GIJ	D1GIJ	D1GIJ	D1GIJ	Dead IJ
	<i>S. fabii</i>	RIJ	R1GIJ	A	A	A + 2GJ	DA	2GJ	DA	2GJ	DA	Successful cycle in one rep.
<i>Maladera</i> sp. 4	<i>S. yirgalemense</i>	1G IJ	1GIJ	R1GIJ	R1GIJ	A	A	2GJ	2GJ	2GJ	2GJ	Successful cycle
	<i>S. affinis</i>	1GIJ	1GIJ	A	R1GIJ	A + 2GJ	A	2GJ	A + 2GJ	2GJ	2GJ	Successful cycle
	<i>H. zealandica</i> MJ2C	1GIJ + A	1GIJ	A	R1GIJ	A	R1GIJ	A	R1GIJ	A + 2GJ	DA	Successful cycle in one rep.
<i>Galleria mellonella</i>	<i>H. zealandica</i> SF41	RIJ	1GIJ	A	1GIJ	A + 2GJ	1GIJ	2GJ	1GIJ	2GJ	1GIJ	Successful cycle in one rep.
	<i>S. fabii</i>	RIJ	A	A	A	A + 2GJ	A + 2GJ	2GJ	2GJ	2GJ	2GJ	Successful cycle
	<i>S. yirgalemense</i>	RIJ	A	A	A	A + 2GJ	2GJ	2GJ	2GJ	2GJ	2GJ	Successful cycle
	<i>H. zealandica</i> MJ2C	1GIJ	1GIJ	R1GIJ	R1GIJ	A	R1GIJ	A	A	A	2GJ	Successful cycle
	<i>H. zealandica</i> SF41	1GIJ	R1GIJ	R1GIJ	A	A	A	A	A + 2GJ	A + 2GJs	2GJ	Successful cycle
	<i>S. fabii</i>	1GIJ	1GIJ	R1GIJ	1GIJ	A	1GIJ	2G IJ	1GIJ	2GJ	1GIJ	Successful cycle only in one rep.
	<i>S. yirgalemense</i>	1GIJ	1GIJ	R1GIJ	R1GIJ	A	A	A	A + 2GJ	2GJ	2GJ	Successful cycle

I and II: replicates; 1/2 GIJ: first- or second-generation juveniles; ESB: entomopathogenic symbiotic bacteria; A: adults; DA: dead adults; R1GIJ: recovered first-generation infective juveniles. *Galleria mellonella* is the control.

SF41 did not recover, while some IJ of *S. yirgalemense*, despite being observed to recover, died as adults, thus being unable to complete their life cycle.

INFECTION BIOASSAY

The ability and rate of nematode infection differed between EPN species and insect host. For *S. affinis*, the percentage of infected grubs increased over time for *H. zealandica* MJ2C and for *S. yirgalemense* ($P < 0.001$). The percentage of infected grubs varied significantly across EPN species ($P < 0.001$) (Fig. 1A). The highest percentage of infected grubs was obtained by *H. zealandica* MJ2C at 120 h post infection (HPI) (mean percentage infection of grubs of $80 \pm 0\%$). Both *H. bacteriophora* and *S. yirgalemense* moderately infected *S. affinis*. A mean percentage infection of grubs, equal to $50 \pm 10\%$, was achieved by *H. bacteriophora* at 48 HPI. The infection rate declined with time. *Steinernema yirgalemense* achieved a moderate percentage infection of grubs, namely $50 \pm 10\%$ at 120 HPI. The EPN *S. fabii* was not found to infect *S. affinis* throughout most of the trial period, except for a very low infection percentage of $10 \pm 10\%$ at 72 HPI (Fig. 1A).

For *Maladera* sp. 4, the percentage of infection was generally high for all the EPN species concerned, increasing over time for all the EPN species involved ($P > 0.001$). The highest mean percentage infection of $80 \pm 0\%$ was achieved by *H. bacteriophora* at 120 HPI. The presence of *H. zealandica* MJ2C resulted in a mean percentage infection of $70 \pm 10\%$ at 120 HPI. *Steinernema yirgalemense* and *S. fabii* were both able to cause a moderate mean percentage infection of *Maladera* sp. 4 (Fig. 1B).

For *P. sommeri*, the results of the analysis showed that the *S. fabii* and *S. yirgalemense* species were unable to infect the white grub during the trial period, although *H. zealandica* MJ2C and *H. bacteriophora* attained low percentage infection during the trial period. The highest percentage infection for *P. sommeri* of $40 \pm 0\%$ resulted from *H. zealandica* MJ2C at 48 HPI (Fig. 1C).

ENCAPSULATION TEST

The analysis of the results of the current experiment showed varying degrees of rates of nematode encapsulation for the different white grub species (Fig. 2). *Schizonycha affinis* achieved a high encapsulation of *S. yirgalemense*, namely $60 \pm 11.5\%$, but only $23 \pm 3.3\%$ and $3.3 \pm 3.3\%$ for *S. fabii* and *H. zealandica* MJ2C, respectively. *Pegylis sommeri* achieved encapsulation of $43 \pm 3.3\%$ for *S. yirgalemense*, which was slightly higher than, but not significantly different from, that of *H. zealandica* MJ2C at $40 \pm 10\%$ ($P = 0.95$). By contrast, *Maladera* sp. 4 obtained 50% encapsulation for the same nematode, achieving the highest encapsulation rate for *S. yirgalemense*, at $46 \pm 3.3\%$, followed by that for *H. zealandica* MJ2C, at $30 \pm 5\%$, while the lowest was achieved for *S. fabii*, at $13 \pm 3.3\%$.

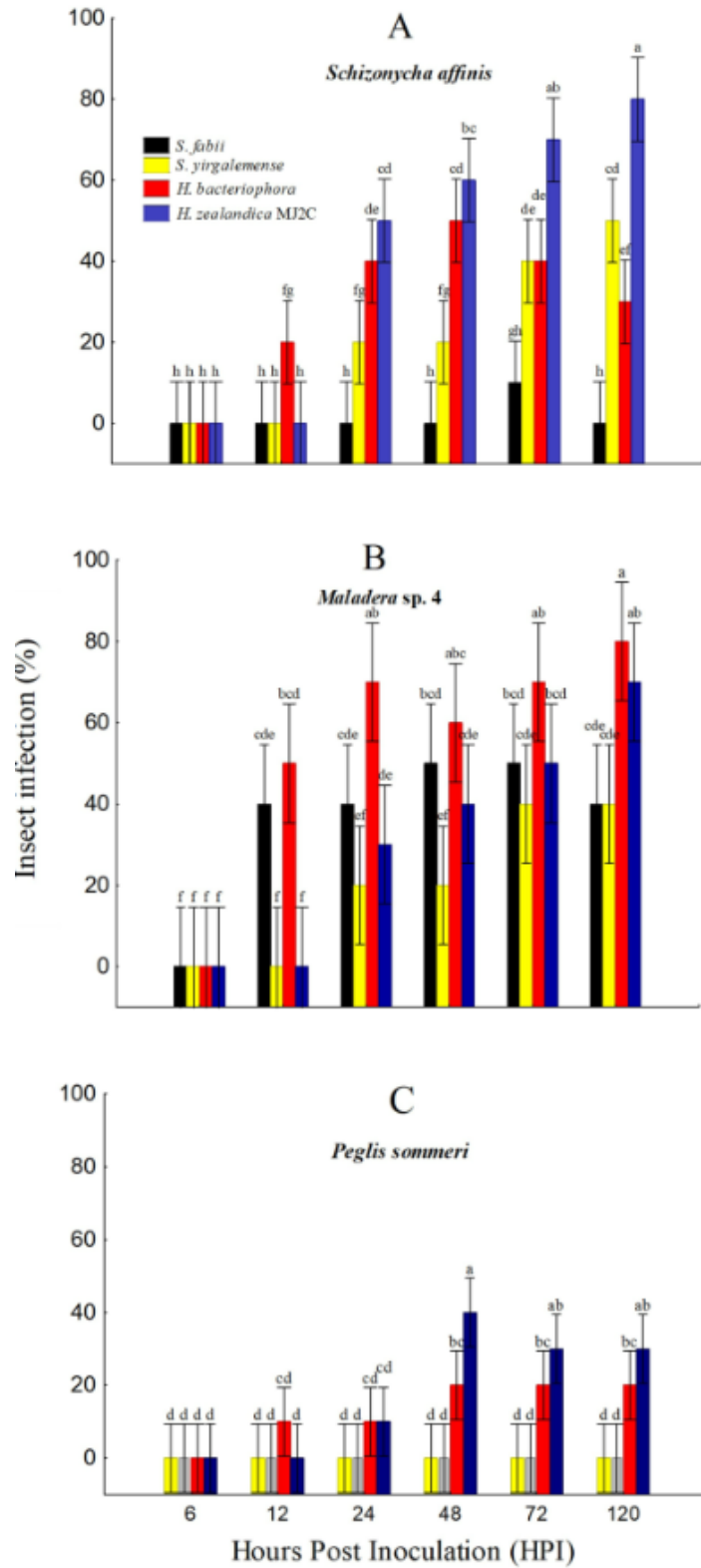


Fig. 1. Mean percentage (95% confidence interval) of infected larvae of A: *Schizonycha affinis*, (one-way ANOVA: $F(15, 24) = 10.044$, $P < 0.01$); B: *Maladera sp. 4* (one-way ANOVA: $F(15, 24) = 3.044$); C: *Pegylis sommeri* (one-way ANOVA: $F(15, 24) = 3.347$, $P < 0.01$). The same letters on the bars indicate no significant difference ($P > 0.05$) between grub species and percentage insect infection. Bars are standard errors

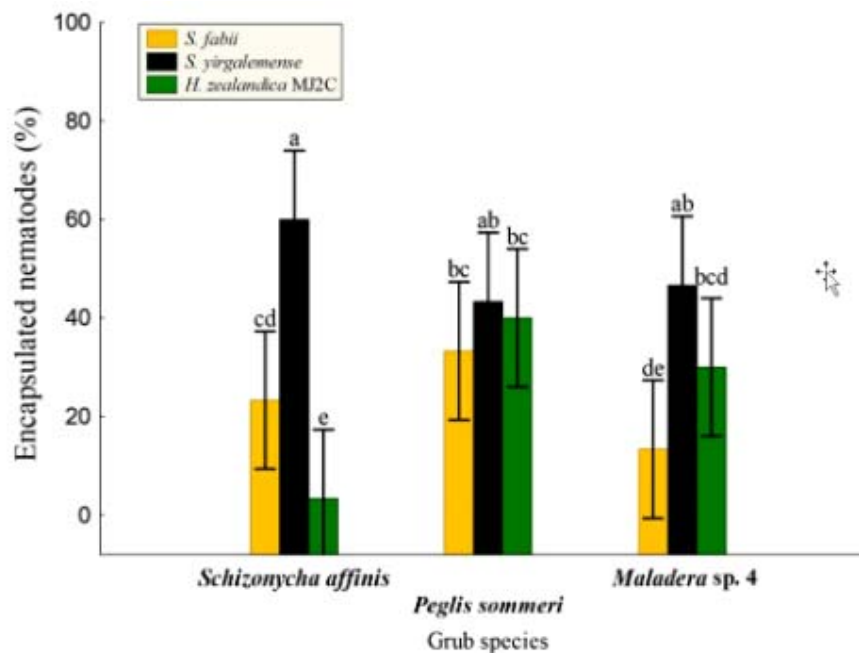


Fig. 2. Mean percentage (95% confidence intervals) of encapsulated nematodes of *Heterorhabditis zealandica* MJ2C, *Steinernema fabii* and *S. yirgalemense* in the haemocoel of *Maladera* sp. 4, *Pegylis sommeri* and *Schizonychia affinis*, at 10 h post infection (one way ANOVA: $F(4, 18) = 5.028$, $P < 0.01$). The same letters on the bars indicate no significant difference ($P > 0.05$) between grub species and percentage nematode encapsulation. Bars are standard errors.

HUMORAL IMMUNITY MEASUREMENT USING INHIBITORY ZONE ASSAYS

The results of the humoral immunity using inhibitory zone bioassays showed that all the symbiotic bacteria tested, including *P. heterorhabditis* subsp. *heterorhabditis*, *P. thracensis*, *X. indica* and *X. bovienii*, were able to grow in the haemolymph of *P. sommeri*, *S. affinis* and *Maladera* sp. 4, as well as in the susceptible control experiment (*G. mellonella*). Visual observation led to no detection of any significant difference in the ability of the different bacteria colonies to colonise the haemolymph, nor to any detection of differences in the colonisation times.

Discussion

The present study showed that the white grubs *S. affinis* and *P. sommeri* tend to be the least susceptible to EPN infection. The finding agrees with the results of the pathogenicity study of Katumanyane *et al.* (2023). The low level of susceptibility was reflected in the low percentage mortality obtained in the screening bioassays and in the limited ability of the EPN both to penetrate the grubs and to grow in their haemolymph, as well as in the high rates of encapsulation of the nematodes by the grubs. Of the white grub species tested, *Maladera* sp. 4 was the most susceptible to attack by the EPN.

Our results showed that some EPN have the ability to grow in the haemolymph of the tested white grubs, while others did not. For example, only *S. fabii* and *H. zealandica* MJ2C were occasionally able to complete their life cycle in the haemolymph of *S. affinis*. Such growth inhibition can be attributed

to the activation of the immunomodulation process of the insect. Various active compounds, including prophenoloxidase, are known to occur in the haemolymph of insects and can mediate the insect's humoral defence (Söderhall & Cerenius, 1998). For example, Roy & Kim (2020) observed that the phenoloxidase activity and the expression of two antimicrobial peptides was higher in the less susceptible *Tenebrio molitor* L. than it was in the more susceptible *Spodoptera exigua* Hübner. El Sadawy *et al.* (2020) observed the presence of additional protein bands in nematode-infected *Rhynchophorus ferrugineus* Olivier, attributing such presence to the ability of the host to release a protein as a form of defence against the parasite.

All the four species of EPN symbiotic bacteria used in the current tests were able to grow separately in the haemolymph of all the insects tested. This suggests that the limitations to EPN growth in the haemolymph is not caused by factors that inhibit the growth of bacteria. Contrary to the rest of the EPN used, *S. fabii* was able to reproduce in the haemolymph of all the insects tested. However, our results may also reflect the undetermined influence of the microbes carried by the polyxenic IJ and insect haemolymph. Future experiments should consider using monoxenic IJ as well as the effect of the haemolymph microbiota on EPN growth.

The results of the infection bioassay correlated with the results obtained in the pathogenicity studies of Katumanyane *et al.* (2023). The nematodes *H. zealandica* MJ2C and *H. bacteriophora*, which caused high mortality among the grubs, were more able to infect the white grubs in the current study than were the other species. The nematode *S. fabii*, which showed little to no ability to infect any of the white grubs, showed minimal ability to penetrate the white grubs. The penetration rate of the nematode contributes significantly to the EPN's virulence (Hominick & Reid, 1990; Roy & Kim, 2020) and is known to differ among different white grub species (Koppenhöfer *et al.*, 2007). Roy & Kim (2020) report that the penetration rate of the IJ into the haemolymph of *S. exigua* was found to be much higher when compared to *T. molitor*, which was found to be less susceptible to the EPN. However, other researchers have found no correlation between penetration rate and mortality. For example, Batalla-Carrera *et al.* (2014) reported that the varied penetration rate of EPN into hazelnut weevil, *Curculio nucum* Linnaeus, based on the life-stage and the increasing nematode dosage concerned, did not necessarily result in higher mortality.

Encapsulation was observed for all the nematode species, as caused by all the white grub species involved, with it being highest for *S. affinis*. *Schizonycha affinis* also showed the most inhibition to nematode growth in the haemolymph. Such observations indicate that the white grub uses encapsulation as a means to avoid EPN attack. Encapsulation involves the use of multiple haemocytes (granulocytes and plasmatocytes) to attach to the invading EPN (Schmidt *et al.*, 2001; Strand, 2008) and to aggregate on the surface coat of the nematode's cuticle. Finally, the encapsulated nematode tends to be killed off by the by-products of phenoloxidase cascade (e.g., quinone and melanin) within a cellular capsule that is entrapped within a necrotic internal layer of haemocytes (Kanost & Gorman, 2008; Strand, 2008; Alvandi *et al.*, 2017). Granulocytes and plasmatocytes have been reported as being the most abundant haemocytes in the white grub *Polyphylla adspersa* Motschulsky (Coleoptera: Scarabaeidae) (Alvandi *et al.*, 2017), with them being involved in the cellular encapsulation that leads to capsule formation (Lavine & Strand, 2002).

For the humoral immunity measurement using inhibitory zone assays, all the EPN symbiotic bacteria tested, including *P. heterorhabditis* subsp. *heterorhabditis*, *P. thracensis*, *X. indica* and *X. bovienii*, were able to grow in the haemolymph of *P. sommeri*, *S. affinis* and *Maladera* sp. 4. These results indicate that the low susceptibility to the EPN might also lie in the inability of the EPN to penetrate the grubs, since the bacteria are able to grow inside the insect haemolymph. The results obtained in the current

research differ from those of Stokwe & Malan (2017) and Javal *et al.* (2019), in whose experiments the haemocoel of the woolly apple aphid, *Eriosoma lanigerum* Hausmann (Homoptera: Pemphigidae), and of the long-horned beetle, *Cacosceles newmannii* Thompson (Coleoptera: Cerambycidae), respectively, were shown to suppress the growth of symbiotic bacteria of some EPN. The inhibition of EPN bacterial growth would, in turn, inhibit EPN growth and reproduction. For example, when Javal *et al.* (2019) tested the three locally isolated *Steinernema* species against the larvae of *C. newmannii*, a very low level of pathogenicity was observed that was attributed to the suppression of the symbiotic bacteria of EPN in the insect's haemocoel, and, thus, to the inability of the IJ to access their main nutrient source. The mechanism of suppression of the bacteria, however, does not appear to play a role in the interactions that were tested in the current trials. The methodology used to determine the humoral immunity using inhibitory zones could have been improved by using the haemolymph of the insects induced by the heat-killed bacterial symbionts of EPN, as it has been noted that the antibacterial activity of the insect haemolymph usually needs to be induced by foreign invaders in the living and healthy insects (Wu *et al.*, 2014).

Coming to understand the patterns of susceptibility specific to each white grub species is important, as doing so should inform future decision-making regarding the application of EPN for the biological control of white grubs. The results described in the present paper attempt to explain some of the mechanisms that South African white grubs use against attack by EPN. However, the study did not exhaust all the possible mechanisms employed. Importantly, future studies should focus on such aspects as the humoral and cellular defence mechanisms used by the white grubs studied to defend themselves from attack by EPN.

Acknowledgements

The authors would like to acknowledge the assistance rendered by Prof. Des Conlong, Tom Webster and Janet Edmonds from the South African Sugarcane Research Institute (SASRI) with the white grub collection. Additionally, our thanks go to Dr Benice Sivparsad, from the Institute for Commercial Forestry Research (ICFR), for assisting with the white grub monitoring data and for providing some of the grubs that were used in the experiments. This work was supported by the Tree Protection Cooperative Programme, TPCP, and the National Research Foundation of South Africa (TP14062571871 and ITR150119112367).

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