Resource defence by the entomopathogenic nematodes Steinernematidae and Heterorhabditidae

Thesis submitted to Maynooth University for the degree of Doctor of Philosophy by

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Declaration of Authorship

This thesis has not been previously submitted in whole or in part to this or any other university for any other degree. This thesis is the sole work of the author except where otherwise indicated.

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List of Presentations and Publications

Publication: Cassells, M. D., Treanor, N., Muñoz-Adalia, E. J. & Griffin, C. T. Scavenger damage to the host cadaver affects fitness of EPN species differentially. In preparation.

Publication: Cassells, M. D., Campos-Herrera, R. & Griffin, C. T. Co-infection and competition of *Heterorhabditis* and *Steinernema* entomopathogenic nematodes. In preparation.

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Oral Presentation: "Mechanisms of defence in entomopathogenic nematodes: Lethal fighting." 3-minute thesis competition, International Federation of Nematology Societies, virtual, December 2021.

Oral Presentation: "The effects of female pheromone exposure on lethal fighting in *Steinernema carpocapsae* males." International Conference of Nematology, Antibes, France, May 2022.

Oral Presentation: "Sex and death: How copulation makes male nematodes more lethal against rivals" Irish Society of Parasitology, Tralee, Ireland, June 2023

Oral Presentation: "Competition between *Steinernema* and *Heterorhabditis* nematodes co-infecting a host." 55th International Congress on Invertebrate Pathology

and Microbial Control, Maryland, USA, August 2023. Funded by the Maynooth University Postgraduate Research Travel Fund.

Poster Presentation: "Lethal fighting in *Steinernema carpocapsae* males: Advantages of being mated". Society of Invertebrate Pathology international conference (virtual), June 2021.

Poster Presentation: "Mating makes a better killer: The effects of female pheromone exposure on lethal fighting in *Steinernema carpocapsae* males". Maynooth University Department of Biology Research Day, Maynooth University, May 2022.

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Abbreviations

°C:	Degrees celsius
µg:	Microgram
µl:	Microlitre
ABS:	Animal Behavior Society
AIC:	Akaike´s Information Criteria
AMC:	Hydroxy-2-butanone
ANOVA:	Analysis of variance
ASAB:	Association for the Study of Animal Behaviour
Ascr:	Ascaroside
Cm:	Centimetres
CO_2:	Carbon dioxide
DNA:	Deoxyribonucleic acid
DPI:	Days post infection
EPN:	Entomopathogenic nematodes
Fig.:	Figure
FK:	Freeze-killed
FLBN:	Free-living bacterivorous nematodes
GLM:	General linear model
HB:	*Heterorhabditis bacteriophora*
HD:	*Heterorhabditis downesi*
hrs:	Hours
Hpi:	Hours post infection
IJ:	Infective juvenile
J1/J2/J3:	$1^{st}/2^{nd/}$ 3^{rd} stage juvenile nematodes

LED:	Light emitting diode
LSD:	Least significant difference
m:	Meter
mad:	Maternal adherence defective
Mb	Mega-base pair
Mcf:	Make-caterpillar-floppy toxin
min:	Minutes
ml:	Millilitre
mm:	Millimetre
NF:	Nemataphagous fungi
PBS:	Phosphate buffer saline
RH:	Relative humidity
RHP:	Resource holding potential
ROS:	Reactive oxygen species
RV:	Resource value
SC:	*Steinernema carpocapsae*
SCP:	Surface coat proteins
SDF:	Scavenger deterrent factor
± SE:	Plus or minus the standard error
SF:	*Steinernema feltiae*
Spp.:	Species
TCA:	Trans-cinnamic acid
Tcs:	Toxin complexes
T6SS:	Type VI secretion systems
UV:	Ultraviolet
v.:	Version

v/v:	Volume per volume
w/v:	Weight per volume
wks:	Weeks

Abstract

Entomopathogenic nematodes (EPN) are insect parasites that depend on the limited resources available within the host for development and reproduction. They carry symbiotic bacteria that aid in killing the host and provide nutrition for the nematodes. Several generations of EPN are produced within a single host and when the resources are depleted stress-resistant, free-living infective juveniles (IJs) leave the insect cadaver in search of a new host. This thesis focuses on how EPN of the genera *Heterorhabditis* and *Steinernema* defend resources within the host cadaver from interspecific and intraspecific competitors, scavengers, and abiotic factors.

Steinernema males fight and kill male competitors in interspecific competition for females. In this thesis, it is shown that *S. carpocapsae* males that have mated are better killers than unmated males, independent of size or sexual development.

IJs of the two genera can co-occur geographically, but species from one genus will usually dominate over the other genus when found within the same host. This thesis demonstrates that this dominance is mainly due to failure of one genus to develop in a host infected by the other genus, and not due to a lack of attraction to infected hosts.

The insect cadaver is an attractive source of nutrition for scavengers. *Heterorhabditis* associates with the bioluminescent bacteria *Photorhabdus*. Here it is shown that the bioluminescence produced by *Photorhabdus* helps to deter scavengers from feeding on *Heterorhabditis*-infected cadavers.

The host cadaver protects EPN from abiotic stresses. It is demonstrated here that damage to the host's cuticle, imitating scavenging, leads to desiccation of the cadaver, and reduces both the quantity and size of IJs that emerge from the host. This effect is

more severe for *Heterorhabditis* than for *Steinernema*, presumably due to the slower development of *Heterorhabditis* within the host. *Heterorhabditis* are better than *Steinernema* at deterring scavengers from feeding on the cadaver, preventing damage from occurring.

This thesis demonstrates the various defensive mechanisms adapted by *Steinernema* and *Heterorhabditis*, highlighting their distinct phylogenies and the relative importance of their symbionts.

Chapter 1. Introduction

1.1 General introduction

Competition for resources imposes a selective pressure in favour of organisms with greater defence mechanisms. Organisms that are better able to defend themselves and their resources from predators, antagonists, and competitors will survive and reproduce, passing on their genes to the next generation. Entomopathogenic nematodes (EPN) and their symbiotic bacteria are an interesting system for studying the development of defences in a multi-trophic system in the soil. Both EPN and their symbionts can only develop and reproduce within insect hosts. The EPN rely on their bacteria as a source of nutrition, and to aid in host mortality, while the bacteria require the nematode to vector them between hosts (Poinar, 1979). Both the EPN and their symbiont must compete for hosts in the soil, defend themselves from predators and other antagonists, and defend the resources within the host from competitors and scavengers (Kaya, 2002; Raja et al., 2021). The two main genera of EPN, *Steinernema* and *Heterorhabditis,* are not closely related, and the similar life cycle/ bacterial symbiosis of the two is the result of convergent evolution (Blaxter et al., 1998; Ahmed et al., 2022). In this thesis I study the defence mechanisms of representatives of the two EPN genera that have evolved in response to similar selection pressures.

1.2 Entomopathogenic nematodes

EPN are obligate parasites that reside in soil environments throughout the world. While EPN can exist in a free-living state temporarily, as stress resistant infective juveniles, they must invade an insect host in order to develop and reproduce. The two main families of EPN are Steinernematidae (genera *Steinernema* and *Neosteinernema)*

and Heterorhabditidae (genus *Heterorhabditis*) (Poinar, 1976; Nguyen and Smart, 1994). *Steinernema* and *Heterorhabditis* carry symbiotic Enterobacterales bacteria, *Xenorhabdus* and *Photorhabdus* respectively, that aid in killing the insect host. The nematode/bacteria complexes are dependent on the limited resources available within the insect host for survival. There are more than 100 species of *Steinernema,* belonging to 12 clades (*Affine*, *Bicornutum*, *Cameroonense*, *Carpocapsae*, *Costaricense*, *Feltiae*, *Glaseri*, *Karii*, *Khoisanae*, *Kushidai*, *Longicaudum* and *Monticola*) and 20 *Heterorhabditis* species, identified worldwide (Spiridonov et al., 2016; Bhat et al., 2020). Despite having similar life cycles and associations with bacteria, Heterorhabditidae are more closely related to vertebrate parasites in the Strongylida order than to Steinernematidae, which group with the Strongyloididea and Panagrolaimidea (free-living bacterivores) families of the Rhabditida order (Blaxter, 1998; Ahmed et al., 2022) (Fig. 1.1). This divergence is reflected in the genomes of the two genera; the genome of *Heterorhabditis bacteriophora* (77 Mb) is smaller than that of *Steinernema carpocapsae* (85.6 Mb) and *Steinernema feltiae* (82.4Mb), with both *Steinernema* species' encoding ten times the number of proteases and G protein-coupled receptors (important proteins in host immune suppression and olfaction) (Dillman et al., 2015; Lu et al., 2016).

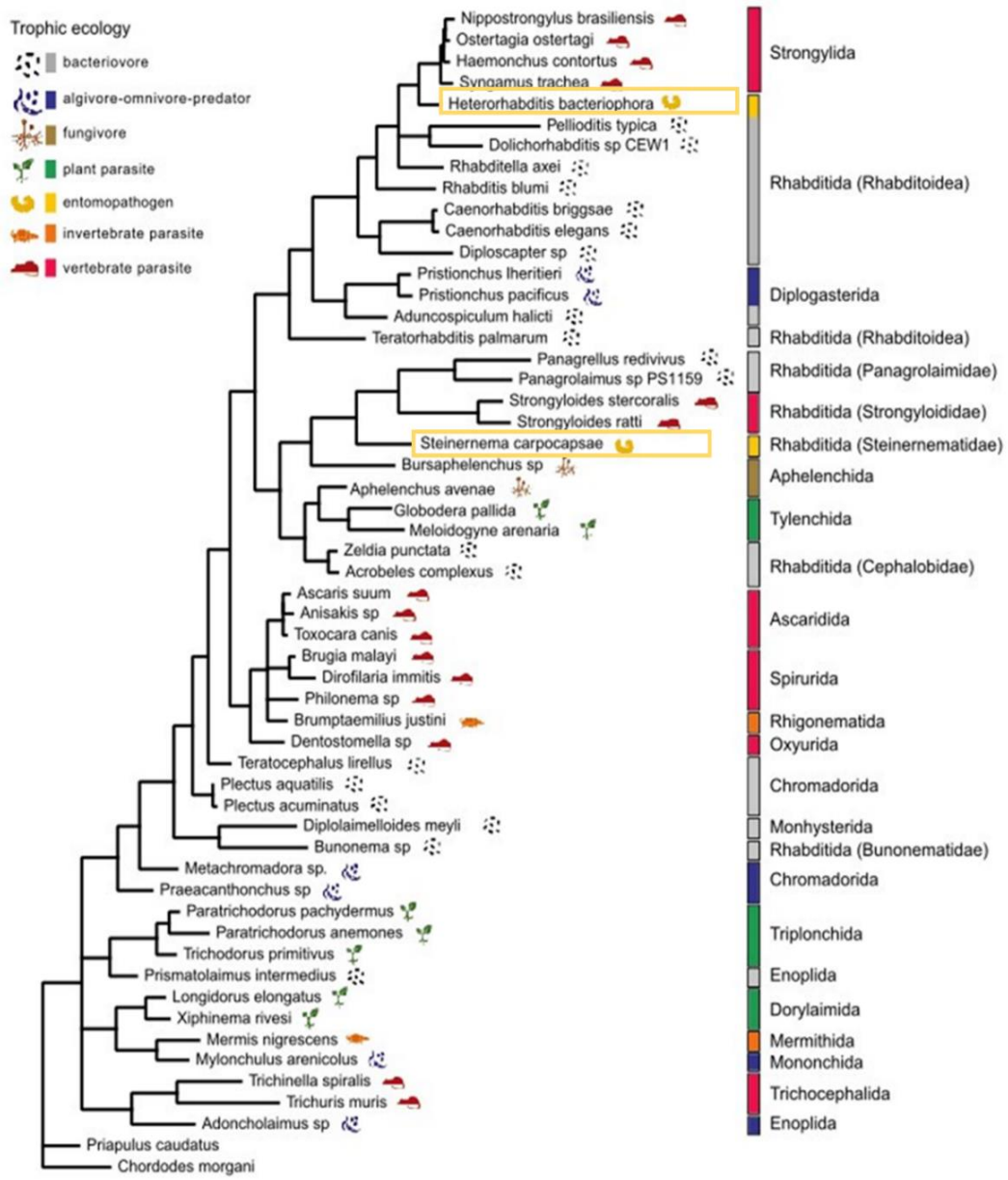

Figure 1.1. Phylogeny of the Nematoda. The Steinernematidae and Heterorhabditidae families, represented by *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* here, are highlighted in yellow. Taken from Blaxter et al. (1998).

1.2.1 Life cycle

Both *Steinernema* and *Heterorhabditis* nematodes follow a similar life cycle with some physiological and behavioural differences. Non-feeding developmentally arrested infective juveniles (IJs) dwell in the soil and seek out insects, either as

"ambush" foragers, that attach onto passing insects, or as "cruisers" that use odour cues to detect and move towards potential hosts (Campbell and Gaugler, 1993; Grewal et al., 1994; Bal et al., 2014). They enter the host through natural openings and in the case of *Heterorhabditis*, through the cuticle (Bedding and Molyneux, 1982). Once inside, the nematodes release their symbiotic bacteria, which proliferate and kill the host within 48-72 hrs. The nematodes then feed on the bacteria and available nutrients and develop to adult (Poinar, 1979). Heterorhabditids are self-fertile hermaphrodites in the first generation, and only require one invading nematode to reproduce, whereas the majority of *Steinernema* species are amphimictic. Each maternal nematode produces dozens of offspring. If conditions are good, and nutrients are available, the offspring develop through four juvenile stages (J1-J4), moulting at each stage, before reaching adulthood. The cycle is repeated, and several generations can be produced within a single host cadaver. When the nutrients are depleted, the juveniles develop into IJs instead, diverging at the J3 stage (Fig.1.2). On this pathway the juveniles reassociate with their symbiont. *Xenorhabdus* are carried by steinernematids in specialised intestinal receptacles, while *Photorhabdus* colonises the intestinal lumen of heterorhabditids (Boemare et al., 1996; Goodrich-Blair and Clarke, 2007). Overcrowding, leading to the accumulation of nematode waste products such as ammonia, and depletion of nutrients signal the IJs to leave the cadaver in search of a new host (Ryder & Griffin, 2002; San-Blas et al., 2008; Dillman and Sternberg, 2012). The IJs retain their J2 cuticle as a sheath, which protects against natural enemies and environmental stress, and aids in survival outside of a host for several months (Poinar, 1979; Timper and Kaya, 1989).

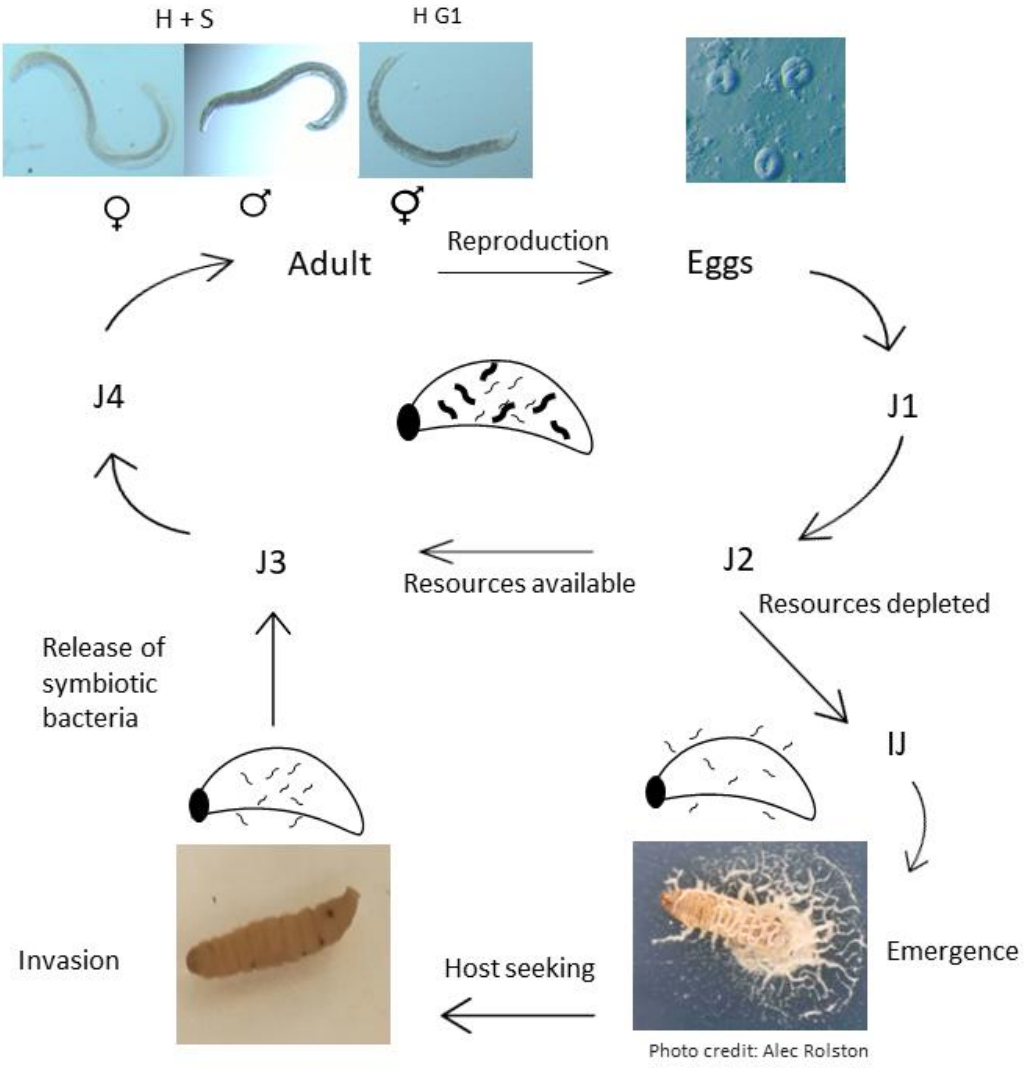

Figure 1.2. Life cycle of entomopathogenic nematodes. *Heterorhabditis* (H) IJs develop to hermaphroditic adults in the 1st generation (G1), with subsequent generations producing both males and females, whereas *Steinernema* (S) produces both males and females in every generation.

1.2.2 Bacterial symbionts

Xenorhabdus and *Photorhabdus* are bacteria of the Enterobacterales order that kill insects and proliferate within them. Both bacterial species are vulnerable to desiccation, UV radiation and extreme temperatures, and are not found free-living in

nature but depend on their nematode symbiont to vector them between hosts (Morgan et al., 1997).

Xenorhabdus and *Photorhabdus* exhibit both pathogenic and mutualistic phases (Goodrich-Blair and Clarke, 2007). After their nematode symbiont delivers them into the insect host, they exhibit pathogenicity and overcome the host immune system (Forst et al., 1997). They release insecticidal toxins (such as toxin complexes (Tcs) and Make-caterpillar-floppy (Mcf)), proteases and lipases that aid in killing the host (Bowen et al., 1998; Daborn et al., 2002; Ffrench-Constant and Waterfield, 2006; Brown et al., 2004, 2006a). The host's immune response is supressed through inhibition of phenoloxidase, phospholipases and phagocytic cells (Eleftherianos et al., 2007; Clarke, 2008). The bacteria then exhibit mutualism, playing a key role in providing a suitable environment for the IJs to recover and develop. *Photorhabdus* and *Xenorhabdus* produce antibiotics and antifungals, including carbapenems, stilbenes and chitinases, which inhibit the growth of competing fungi and bacteria, and prevent putrefaction of the host cadaver (Chen et al., 1996; Isaacson, and Webster, 2002; Hu et al., 2006; Eleftherianos et al., 2007; Ullah et al., 2015; Tobias et al., 2018). Both *Steinernema* and *Heterorhabditis* also inhibit the host's immune system, aiding in their symbiont's proliferation. Secretions from the nematodes reduce phenoloxidase activity, preventing melanisation of the host, and suppress anti-microbial peptide activity (Brivio et al., 2002; Eleftherianos et al., 2010; Kenney et al., 2019; Jones et al., 2022).

Despite *Xenorhabdus* and *Photorhabdus* being closely related, their symbiosis with EPN is the result of convergent evolution (Poinar, 1993; Goodrich-Blair and Clarke, 2007; Chaston et al., 2011). The symbiosis is specific, with *Photorhabdus* only

associating with heterorhabditids and *Xenorhabdus* associating only with steinernematids (Adams et al., 2006; Lewis & Clarke, 2012). There are at least 26 species of *Xenorhabdus* and 20 species of *Photorhabdus* recorded to date (Machado et al., 2018; Sajnaga & Kazimierczak, 2020; Castaneda-Alvarez et al., 2022).

While several species of *Xenorhabdus* can provide nutrition for a single species of *Steinernema*, allowing for development and reproduction, each *Steinernema* species can only associate with and carry one species of *Xenorhabdus* e.g. *S. carpocapsae* can feed on *Xenorhabdus bovienii*, *Xenorhabdus beddingii* and *Xenorhabdus nematophila*, but will only be colonised by and carry *X. nematophila* (Stock and Goodrich-Blair, 2008; Cowles and Goodrich-Blair, 2008). Some species of *Xenorhabdus* are vectored by several species of *Steinernema* e.g. *X. bovienii* is carried by *S. feltiae, S. kraussei, S. affine* and others, and *X. nematophila* is carried by both *S. carpocapsae* and *S. websteri* (Stock and Goodrich-Blair, 2008). There is strain specificity, with each species of *Steinernema* associating with a specific strain of *Xenorhabdus*, and symbiont switching has a negative impact on the reproductive success and virulence of the nematode (Murfin et al., 2015; McMullen et al., 2017a). For *Photorhabdus* the association is less specific. A *Heterorhabditis* species can associate with several species of *Photorhabdus* (e.g. *H. bacteriophora* can associate with both *P. luminescens* and *P. temperata* (Stock and Goodrich-Blair, 2008). There is less strain specificity, and it can be advantageous to a *Heterorhabditis* species to associate with more than one *Photorhabdus* species or strain, allowing for niche expansion (Maher et al., 2017).

The specificity of these associations is maintained through complex colonisation processes regulated by species-specific genes (Goodrich-Blair, 2007; Clarke, 2008; Cowles and Goodrich-Blair, 2008; Somvanshi et al., 2010). *Xenorhabdus* that has

proliferated in the insect haemocoel is ingested by *Steinernema* IJs. A few bacterial cells bind to matrix structures within specialised receptacles in the anterior of the IJ's intestine, where they multiply until maximum population density within the vesicle is achieved and maintained (Martens et al., 2003; Martens and Goodrich-Blair, 2005; Synder et al., 2007). This colonisation is species-specific e.g. *nilABC* genes, found only in *X. nematophila*, are essential for colonising *S. carpocapsae,* possibly through the production of adherence proteins (Cowles and Goodrich-Blair, 2008). The IJs carry their symbiont in these receptacles and when they encounter the haemolymph of a new host, release them into the lumen of the intestine, from where they are egested through the anus. In comparison, *Photorhabdus* colonises *Heterorhabditis* IJs through the mother. The *Photorhabdus* bacteria are ingested by the maternal nematode where they occupy the mother's intestine before invading the rectal glands. Juveniles hatch within the mother (*endotokia matricida*) and as the rectal glands are lysed the *Photorhabdus* encounters and colonises the prospective IJs within the mothers' body cavity (Somvanshi et al., 2010). *Photorhabdus* colonise the intestine of the IJ and are released by regurgitation. Attachment of *Photorhabdus* to the maternal intestine and colonisation of the IJ is genetically regulated. *Photorhabdus* possess maternal adhesion defective (mad) genes that, when activated by a promoter, encode adhesive fimbriae that allow the bacteria to attach to the nematode (Bennett and Clarke, 2005; Easom et al., 2010; Somvanshi et al., 2010).

Heterorhabditis nematodes are dependent on the presence of *Photorhabdus* for recovery from IJ and further development (Strauch and Ehlers, 1998; Han and Ehlers, 2000; Aumann and Ehlers, 2001). While *Steinernema* nematodes can recover and develop in axenic conditions, their reproduction is affected (Han and Ehlers, 2000;

Sicard et al., 2006). Their infectivity is also reduced (Poinar and Thomas, 1966; Han and Ehlers, 2000) as the next generation of IJs that emerge from hosts where the symbiont did not proliferate will not be carrying the bacteria. *Steinernema* IJs produce toxins and venom proteins that aid in the killing of the host and are not reliant on their symbionts for pathogenicity (Ehlers et al., 1997; Hans and Ehlers, 2000; Lu et al., 2017) but they are less effective at killing hosts without *Xenorhabdus* (Dunphy and Webster, 1985).

1.2.3 Commercial use

Entomopathogenic nematodes, particularly *H. bacteriophora*, *S. carpocapsae* and *S. feltiae*, are used as a biocontrol for crop pests (Grewal and Georgis, 1999; Lacey et al., 2015). They have a wide range of host insects and have been successfully implemented against vine weevil, root weevil, white grubs, thrips, and borers (Peters, 1996; Shapiro-Ilan et al., 2002; Georgis et al., 2006; Lacey and Georgis, 2012). For example, *S. carpocapsae* ($0.75–3.75 \times 10^8$ IJs/ha) is used to protect peach trees in North America from the borer *Synanthedon exitiosa* with 78-100% successful control, while *H. bacteriophora* (5×10^9 IJs/ha) has control rate of 54-79% against cranberry rootworm *Rhadopterus picipes* (Shapiro-Ilan et al., 2016; Koppenhofer et al., 2020). Their relative specificity to target hosts is an advantage over traditional chemical pesticides that negatively impact non-target insect populations (Lacey et al., 2015). They are a sustainable alternative as they can survive in the soil for several months after application and propagate naturally (Kaya and Gaugler, 1993; Lacey et al., 2015; Helmberger et al., 2017).

Commercialisation of EPN has increased with advancements in mass production of IJs using bioreactors (Woodring and Kaya, 1988; Ehlers, 2001; Shapiro-Ilan et al., 2002; Dunn et al., 2021). The most common application method is in suspension, or as pellets, directly onto the soil (Georgis et al., 1995, 2006). The efficacy of these methods varies depending on the target host and environmental conditions e.g. applications of *S. carpocapsae* as pellets results in high mortality of the Colorado potato beetle (*Lepinotarsa decemlineata)* but the nematodes do not persist in the environment (Georgis et al., 2006), while foliar applications of *S. feltiae* are effective against leaf miners but only if a high humidity is maintained (Williams and Walters, 2000; Georgis et al., 2006). IJs are vulnerable to extreme temperatures, desiccation, and UV damage (Gaugler et al., 1992; Glazer, 2002), and numerous studies have been conducted on application methods to improve the survival and efficacy of IJs in the field (Shapiro-Ilan et al., 2006a, 2006b, 2012). IJs emerging directly from infected hosts are more efficacious than liquid applications of IJs (Shapiro-Ilan et al., 2003). EPN that emerge directly from cadavers have a higher survival rate, with a greater tolerance to dry conditions and extreme temperatures, compared to those that have been stored in aqueous suspensions (Shapiro and Lewis, 1999; Perez et al., 2003; Gulzar et al., 2020). A better understanding of how EPN defend their host resources can aid in their development as a successful biocontrol, as well as provide new avenues for research into antimicrobial and anti-scavenger applications.

1.3 Defence

The proliferation and survival of EPN, including their symbiotic bacteria, is dependent on the limited resources of an insect host. The nematode/bacteria complex must

compete for, and defend, the resources of the insect host, on multiple levels (Kaya, 2002; Raja et al., 2021). As IJs in the soil, EPN employ a number of mechanisms to find hosts and avoid natural enemies. Once they invade a host, they must defend against microbes, competing nematodes (both free-living and entomopathogenic) and scavengers that feed on the cadavers. Abiotic factors, such as moisture and temperature, can also negatively affect EPN, necessitating adaption for survival.

1.3.1 Natural enemies of IJs in the soil

The nematode/symbiont complex must defend against natural enemies in the soil environment, such as nemataphagous fungi, microsporidia, and predators. In cases where EPN are not killed by the enemy, their virulence can be reduced, affecting the population as a whole (Campos-Herrera et al., 2006).

Some nemataphagous fungi (NF) attack the IJs of *Steinernema* and *Heterorhabditis* by trapping the nematodes in hyphae and releasing proteases to degrade their cuticle (Zhang et al., 2016). Other endoparasitic fungi attach to the cuticle of IJs as spores and use germ tubes to invade, feeding on the nematode (Jaffee et al, 1992; Soares et al., 2018). Defensive mechanisms against these NFs include shedding the outer cuticle to escape the traps and spores. As *Heterorhabditis* IJs retain their sheaths for longer they may be less vulnerable to NFs compared to *Steinernema* IJs (Timper and Kaya, 1989), while *S. carpocapsae* are better than *Heterorhabditis indica* at detecting NFs and avoiding them (Arêdesa et al., 2017) indicating the two genera may have adopted different strategies to defend against the same threat.

Mites, earthworms, springtails, and other predators feed on the IJs (Ulug et al., 2014; Helmberger et al., 2017). Several mite species (*Macrobiotus richtersi*, *Gamasellodes vermivorax, Alycus roseeus*) have been found to feed on *Steinernema* IJs (Kaya, 2002). A cruising foraging strategy, used by *S. feltiae* and *H. bacteriophora*, has been suggested to be an advantage in the avoidance of mites in the soil (Kaya, 2002). The bacterial symbiont may also make EPN less attractive as a food source to mites (Heidemann et al., 2011).

1.3.2 Defence against microbes

Once the nematodes have invaded an insect, the nematode/symbiont complex must defend their resources against bacteria, fungi, and free-living nematodes. Insects in the soil are an attractive source of nutrition for microbes such as bacteria and fungi, particularly when the insect's immune system is no longer active. *Photorhabdus* and *Xenorhabdus*, along with their symbiotic nematodes, must compete with microbes that reside in the gut of the insect, as well as those from the soil environment. Insects such as *Galleria mellonella* and *Tenebrio molito*r harbour a wide range of bacteria in their guts and on their cuticle, including gram-positive bacteria (*Staphylococcus aureus, Enterococcus* spp.) and gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli*) (Isaacson and Webster, 2002; Cambon et al., 2020). Bacteria (*Bacillus thuringiensis, Bacillus subtilis, Bacillus cereus, Micrococcus luteus)* and fungi (including *Beauveria bassiana*), present in soil can invade insect hosts through the cuticle or natural openings (Jensen et al., 2003; Akhoundi et al., 2012; Ortiz-Urquiza & Keyhani, 2016). Some bacteria can also be carried into the host by attaching to cuticle of invading IJs (Enright and Griffin, 2004; El-Borai et al., 2005; Ogier et al.,

2023; Loulou et al., 2023). Competing species of *Photorhabdus* and *Xenorhabdus* can also be vectored into the host by other species of EPN.

Infection of a host with competing bacteria, like *B. thuringiensis* can negatively affect development of *Xenorhabdus* and *Photorhabdus* (Poinar et al., 1990). Competition with fungal infections also prevent the growth of *Photorhabdus*/*Xenorhabdus,* affecting the development and progeny production of the EPN (Barbercheck and Kaya, 1990; Poinar et al., 1990; Ansari et al., 2005). IJs show adaptive avoidance of live insects infected with *B. bassiana* (Barbercheck and Kaya, 1991).

The growth of *B. bassiana* can be inhibited by *Xenorhabdus* and *Photorhabdus* through antibiosis, as long as the bacteria are established before the fungus (Barbercheck and Kaya, 1990; Chen et al., 1994; Ansari et al., 2005; Orozco et al., 2016). *Xenorhabdus* also outcompetes *B. subtilis* and *Botrytis cinerea,* and inhibits the growth of *Enterococcus* spp., when established first (Isaacson and Webster, 2002). *Photorhabdus* inhibits the growth of *B. cinerea* by inhibiting conidial germination (Chen et al., 1996).

There is a rapid increase in antimicrobial activity in the initial 24-48 hrs of infection (Chen et al., 1996; Hu and Webster, 2000) as *Photorhabdus* and *Xenorhabdus* proliferate and produce antibiotics and antifungals that inhibit the growth of competitors (Chen et al.,1994; Forst et al., 1997; Isaacson, and Webster, 2002; Hu et al., 2006; Ullah et al., 2015; Heryanto and Eleftherianos, 2020). *Photorhabdus* and *Xenorhabdus* differ in their antimicrobial products (Chaston et al., 2011). *Xenorhabdus* produces bacteriocins, xenorhabdins, xenocoumacins and nematophin. The bacteriocins, xenocin and xenorhabdicin, kill competing microbes, including

competing species of *Xenorhabdus* and *Photorhabdus* (Boemare et al., 1992; Thaler et al., 1995; Thappeta et al., 2020). Immunity proteins (e.g. Xenocin-immunity protein complex) produced by *Xenorhabdus* protects it from its own bacteriocins (Singh et al., 2013). Xenorhabdins and xenocoumacins are active against gram-positive bacteria, such as *E. coli*, *P. aeruginosa* and *S. aureus*, as well as several fungi (McInerney et al., 1991a, 1991b; Yang et al., 2011) while nematophin is active against the bacteria *S. aureus* and *B. subtilis* and fungi (Li and Webster, 1997; Zhang et al., 2019c).

In comparison *Photorhabdus* produces stilbenes, anthraquinones, photobactin, epoxides and carbapenem antibiotics that have antimicrobial activity (Paul et al., 1981; Hu et al., 2006; Clarke, 2008). Stilbenes and hydroxystilbene derivatives produced by *Photorhabdus* are active against gram-negative and gram-positive bacteria, and fungi (Paul et al., 1980; Eleftherianos et al., 2007; Orozco et al., 2016; Wollenberg et al., 2016). *Trans*-cinnamic acid (TCA), a precursor for stilbene biosynthesis, also has antifungal activity (Bock et al., 2014). Anthraquinones have antimicrobial activity, although the mechanism of action is not known (Eleftherianos et al., 2007). Photobactin is a siderophore that has antibiotic activity against *M. luteus*, *S. aureus* and *B. cereus* (Ciche et al., 2003) while epoxides 1 & 2 are active against *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* (Hu et al., 2006).

Both *Photorhabdus* and *Xenorhabdus* produce chitinases that lyse fungal mycelia (Chen et al., 1996; Isaacson and Webster, 2002; Son et al., 2024). Chitinases produced by *X. nematophila* have a stronger antifungal activity than those produced by *X. bovienii, P. luminescens* (Chen et al., 1996) or *P. temperata* (Son et al., 2024). These chitinases may also assist in the breakdown of the insect cuticle, aiding IJ emergence (Chen et al., 1996). Indoles produced by *Photorhabdus* and *Xenorhabdus* are active

against bacteria and fungi. While they have been detected in cultures in vitro, their production in infected cadavers is less certain (Hu et al., 1999). Some *Photorhabdus* and *Xenorhabdus* species also use bacterial type VI secretion systems (T6SS) to directly inject competing bacteria with toxins (McMullen et al., 2017b; McQuade and Stock, 2018). Species possessing T6SS outcompete those without it (McMullen et al., 2017b).

1.3.3 Defence against free-living nematodes

Free-living bacterivorous nematodes (FLBN) can feed on the bacteria and nutrients in dead insects in the soil. Prenol, a chemical which accumulates in EPN infected cadavers, is an attractant for FLBN (Baiocchi et al., 2017). FLBN can opportunistically invade insects infected with EPN and avail of the resources released by *Xenorhabdus/ Photorhabdus* when the host dies. Co-infection of a host with both EPN and FLBN increases the likelihood of mortality of the host and is beneficial to the FLBN population but is detrimental to the EPN population (Duncan et al., 2003; Campos-Herrera et al., 2019). The presence of FLBN in a cadaver drastically decreases the number of emerging EPN IJs (Duncan et al., 2003; Blanco-Perez et al., 2019). EPN species with faster developmental times are better equipped to outcompete FLBN as they appropriate resources at a faster rate (Duncan et al., 2003). Secondary metabolites produced by *Photorhabdus* and *Xenorhabdus,* such as stilbenes and indoles, have nematicidal activity and can kill free-living nematodes (Hu et al., 1999; Sicard et al., 2006; Orozco et al., 2016).

For FLBN, attraction, repulsion and dispersal are regulated by small molecule chemicals known as ascarosides (Srinivasan et al., 2012). EPN also use ascarosides for chemical signalling and produce many ascarosides that are analogous with those produced by FLBN. For example, ascr#2 produced by FLBN, and ascr#9 produced by EPN are analogous and both signal dispersal, allowing for possible cross-communication between the two groups (Kaplan et al., 2012). *S. feltiae* can recognise and are deterred by ascarosides produced by the free-living nematode *Caenorhabditis elegans*, and vice versa (Kaplan et al., 2012).

1.3.4 Defence against competing EPN

Hosts are a limited resource for IJs in the soil and a single insect can be infected with multiple EPN, both of the same species (intraspecific competition) and of differing species from the same or differing genera (interspecific competition). IJs use several chemical cues produced by insects (e.g. faeces and carbon dioxide) to identify suitable hosts for invasion (Lewis et al., 1996, 2006; Grewal et al., 1997; Dillman et al., 2012; Zhang et al., 2021). IJs of some species are repulsed by chemicals, such as prenol and ammonia, that accumulate in hosts infected with conspecific/ heterospecific EPN (Grewal et al., 1997; Baiocchi et al., 2017; Kin et al., 2019; Grunseich et al., 2021). The ability to detect and avoid infected hosts benefits both the established nematodes and the potential invaders. Overcrowding of a host increases competition for resources and negatively impacts EPN populations in a host (Selvan et al., 1993a; Koppenhöfer and Kaya, 1995; Ryder and Griffin, 2002). Higher numbers of invaders leading to overcrowding in the host decreases both the number and size of IJs that emerge (Selvan et al., 1993a; Koppenhöfer and Kaya, 1995; Boff et al., 2000; Ryder and Griffin, 2002),

though the severity of this decrease is dependent of the species of EPN (Selvan et al., 1993a). However, there are circumstances where it is beneficial to IJs to invade a host that is already occupied by conspecific/ heterospecific nematodes e.g. to avoid starvation/ poor conditions, to overcome host defences and, for conspecific infections, to increase the likelihood of finding a mate.

EPN may invade hosts that are already infected/ dead to avoid starvation. Generally, IJs invade living hosts but they can also invade and develop in hosts that have already died, indicating that they are facultative scavengers (Pye and Burman, 1978; San Blas and Gowen, 2008; Blanco-Pérez et al., 2017) with *Steinernema* being more disposed to this behaviour than *Heterorhabditis* (San Blas and Gowen, 2008). Scavenging is not an ideal pathway for EPN as it increases the likelihood of competition and reduces survival and reproduction (Blanco-Pérez et al., 2019). IJs are non-feeding and rely on lipid and glycerol stores for nutrition outside of the host (Selvan et al., 1993b; Qiu and Bedding, 2000; Fitters & Griffin, 2006). The symbiotic bacteria are reliant on the limited resources within the IJ between hosts and can further shorten the lifespan of IJs as those resources are used up (Emelianoff et al., 2007). IJs with less reserves may be more inclined to invade and settle for a less suitable host. An increase in infectivity has also been see in older nematodes for *Heterorhabditis megidis* and *S. feltiae* but not *S. carpocapsae* (Dempsey and Griffin, 2002; Yoder et al., 2004).

The greatest risk to IJs during invasion is to initial invaders, as the host immune system is still active (Wang et al., 1994; Peters and Ehlers, 1997). EPN do not immediately release their symbiont upon invasion and some initial invaders can be killed by encapsulation before the bacteria disarm the host's immune system (Wang et al., 1995; Ciche & Ensign, 2003). Some EPN species avoid host responses independently of their

symbiont through the production of surface coat proteins (SCP) and through immune suppression (Blaxter et al., 1992; Peters et al., 1997; Brivio et al., 2002; Maizels et al., 2004; Kenney et al., 2019). Resistance of IJs to the host's immunity varies widely depending on the species of nematode and the host insect (Wang et al., 1995; Peters et al., 1997; Li et al., 2007). Investment in these anti-immunity measures is costly to the parasite (Hurford & Day, 2013). If a host is already infected with EPN then the symbiotic bacteria will already be established, and the host immunity poses less of a threat to invading IJs. It could be particularly beneficial to species that are more susceptible to a host's immunity to take advantage and invade after a more resistant species has already overcome the host's immune response. The benefits are time dependent however, as the initial invaders will have begun development and reproduction before secondary invaders do.

The initial EPN invaders and their symbiotic bacteria must defend their hosts resources from secondary invaders either by deterring invasion or outcompeting them within the host. Resource competition between EPN within the host can take the form of scramble competition, where the individuals focus on exploiting the resources, or interference competition, where one individual will prevent another from accessing a resource.

1.3.4.1 Intraspecific competition

While a single IJ is capable of killing an insect host, usually multiple IJs of the same species will invade at once. Invasion by multiple IJs increases the chances of host mortality (Koppenhöfer et al., 2006; Li et al., 2007) so it is beneficial to invade in groups (Shapiro-Ilan et al., 2014). A number of chemical signals, such as ammonia

and ascarosides, encourage IJs to disperse after emergence from a depleted host (San-Blas et al., 2008; Kaplan et al., 2012), however there are several records of IJs moving in aggregates after emergence (Shapiro-Ilan et al., 2014; Ruan et al., 2018) which may lead to mass infection of a new host by several individuals of the same species. Some species of EPN are attracted to insect cadavers already infected with conspecific EPN (Hay and Fenlon, 1995; Grewal et al., 1997; Christen et al., 2007; Zhang et al., 2019b). However, the longer a host has been infected, the less attractive it becomes for invasion by further IJs (Glazer 1997; Christen et al., 2007, Baiocchi et al., 2017). Both *S. carpocapsae* and *S. feltiae* IJs were attracted to hosts 3 days after infection but were repulsed by day 16 (Baiocchi et al., 2017). Each insect host has a limited supply of resources that can be exploited (i.e. the carrying capacity) and the more IJs that invade the less potential nutrition each individual receives (Dobson, 1985). This can result in decreased reproductive output for each nematode (Boff et al., 2000; Koppenhofer and Kaya, 1995; Ryder and Griffin, 2002) as well as a potential decrease in the size of their offspring (Boff et al., 2000). There is evidence of lower invasion rates of IJs into hosts at higher inoculum densities (Selvan et al., 1993a; Koppenhofer and Kaya, 1995; Boff et al., 2000) and Lewis et al., (2006) suggested that some IJs may adaptively avoid invading overcrowded hosts.

For *Steinernema*, infecting a host together with conspecifics could also be an adaptation to increase mate finding. As *Steinernema* requires both males and females for reproduction, and the ratio of males to females is approximately 40:60, invading a cadaver already occupied by conspecifics increases the likelihood of finding a mate (Poinar, 1990; Alsaiyah et al., 2009). Grewal et al. (1993) proposed that males of *Steinernema* spp. are less risk averse than females and are early invaders of insects to

attract conspecific females, however this hypothesis was not supported for all *Steinernema* species (Bohan and Hominick, 1997; Stuart et al., 1998).

There is intense competition for mating opportunities in the first generation as there are relatively few invaders, and females generally die shortly after mating due to *endotokia matricida* (Baliadi et al., 2004) making them a limited resource. Males of *Steinernema* engage in lethal fighting, a form interference competition, to defend their resources from other *Steinernema* males (Zenner et al., 2014; Kapranas et al., 2016). Males wrap around each other and compress, resulting in paralysis or death of their opponent (Fig. 1.3). They may also use their spicules (an appendage used for insemination) to rupture the opponent's cuticle. This behaviour is seen more commonly in first generation invaders than in subsequent generations (Zenner et al., 2014), likely due to the more limited mating opportunities in the first generation. Kapranas et al. (2020) showed that prior mating experience in *S. longicaudum* males increased their killing capabilities, but the reason for this advantage was not determined.

Figure 1.3. Photograph of lethal fighting in *Steinernema* males. The attacking male wraps around and compresses the opponent male.

1.3.4.2 Interspecific competition

Co-infection of a host with two or more species within the same genus has been demonstrated experimentally for *Steinernema* IJs (Koppenhofer et al., 1995; Kondo, 1989) but not *Heterorhabditis* IJs. The outcome of competition between two *Steinernema* spp. within a single host is dependent on their ability to engage in both exploitative and interference competition (Bashey et al., 2013). Generally, one species of *Steinernema* will dominate depending on which species invades first, the developmental time of the species, the proficiency of their symbiont, and the dependence of the nematode on their symbiont (Kondo, 1989; Koppenhofer et al., 1995; Koppenhofer and Kaya, 1996a; Sicard et al., 2006). Species which invade and develop faster will reproduce earlier and gain more resources (Koppenhofer et al., 1995; Bashey et al., 2013). Interference competition occurs between *Xenorhabdus* symbionts (Bashey et al., 2012, 2013). Some species of *Xenorhabdus* produce bacteriocins that prevent the proliferation of their competitor (See section 1.3.2), which in turn affects the development of the *Steinernema* IJs that feed on them (Sicard et al., 2006; Bashey et al., 2013). *Steinernema* males of each species may also engage in lethal fighting to kill both male and female competitors (O'Callaghan et al., 2014).

Despite attraction to the same host, co-infection of EPN from different genera is not common and is rarely found in nature (Alatorre-Rosas and Kaya, 1991; Hatting et al., 2009; Kanga et al., 2012; Campos-Herrera et al., 2015). The foraging techniques of different species can affect their co-occurrence in hosts. As "cruisers" *Heterorhabditis* and *S. feltiae* IJs can travel further from their point of emergence to find hosts in the soil than stationary "ambushers" such as *S. carpocapsae*, with only a small percentage of *S. carpocapsae* IJs acting as "sprinters" that can outpace *Heterorhabditis* IJs

(Campbell and Kaya 2002; Bal et al., 2014). While *S. carpocapsae* are more likely to encounter mobile hosts near the soil surface, *Heterorhabditis* spp. and *S. feltiae* are better suited to infect immobile hosts in the soil (Campbell and Gaugler 1993; Campbell et al., 1996). Temperature can also affect the infectivity of EPN, with *Steinernema* showing greater infectivity than *Heterorhabditis* when in competition at temperatures of 20-25°C (Dzięgielewska et al., 2023). The outcome of direct competition for hosts is dependent on location and temperature, as well as species (Kruitbos et al., 2010; Dzięgielewska et al., 2023).

In experiments where nematodes of both genera of EPN are directly applied to a single host, one species usually dominates and outcompetes the other depending on time of infection. In experiments by Alatorre-Rosas and Kaya (1991) *Steinernema* IJs developed and outcompeted *H. bacteriophora* IJs if they entered the host 3 hrs earlier, while *H. bacteriophora* were dominant if inoculated 6 hrs before the steinernematids. Co-infections of both genera will result in bacterial competition (Thaler et al., 1997). *Photorhabdus* and *Xenorhabdus* co-infecting a host will directly compete for resources and also produce bacteriocins to kill the other genera of bacteria (see section 1.3.2), and as neither *Heterorhabditis* nor *Steinernema* IJs can feed on the bacterial symbiont of the other genus, their development will be impeded if their symbiont is killed. *Heterorhabditis* in particular are dependent on *Photorhabdus* for development in the insect cadaver and are unable to reproduce in its absence. Therefore, this time dependent dominance may be due to an inability of the secondary invader to develop and reproduce due the proliferation of the symbiotic bacteria of the already established genus.

Generally, it is in the interest of IJs to avoid hosts that are already infected with heterospecific nematodes. Some EPN species can detect the presence of heterospecific nematodes and alter their foraging behaviour in response (Wang & Ishibashi, 1999). Species-specific olfactory cues are released from EPN-infected hosts (Baiocchi et al., 2017; Zhang et al., 2019b; Grunseich et al., 2021), with cadavers infected by different *Steinernema* species' producing more similar volatile blends, that differ from those of *H. bacteriophora*-infected cadavers (Grunseich et al., 2021). *H. bacteriophora*-infected *Acalymma vittatum* cadavers produced volatiles such as sesquiterpenes and 1-dodecene but *Steinernema-infected* cadavers did not (Grunseich et al., 2021). 3-Hydroxy-2-butanone (AMC), which is repulsive *to S. glaseri* and *S. riobrave*, was detected in *S. glaseri*-infected *G. mellonella* cadavers but not *S. riobrave*-infected cadavers (Baiocchi et al., 2017). *S. carpocapsae* were repelled by cadavers infected with other species of *Steinernema* or *H. bacteriophora,* while *S. feltiae* were neither attracted to nor repelled by cadavers infected with heterospecific EPN (Grewal et al., 1997). *H. bacteriophora* were attracted to insect cadavers infected with *S. carpocapsae* and *S. riobrave* (Grunseich et al., 2021). *Steinernema* were also repelled by some heterospecific bacterial symbionts (McMullen et al., 2017a). *S. feltiae* but not *S. carpocapsae* or *H. bacteriophora* IJs were repelled by stilbenes produced by *P. luminescens* (Hu et al., 1999).

1.3.5 Defence against scavengers

It takes several days for nutrients to be depleted in the insect host and more than one generation of EPN are produced during this time. IJs do not emerge from the host cadaver until 5-25 days after infection, depending on the size of the host, the species

of nematode and the environmental conditions (Koppenhofer et al., 1997; Boff et al., 2000). During this time, omnivores and scavengers may consume or damage the insect cadaver, directly killing the nematodes and their symbionts, or negatively impacting their fitness by removing valuable cadaver biomass and exposing them to harsh environmental conditions.

Ants, wasps, crickets, birds, beetles, and fish have all been shown to be deterred from feeding on EPN/symbiont-infected cadavers (Baur et al., 1998; Kaya, 2002; Foltan and Puza, 2009; Gulcu et al 2012; Raja et al., 2017; Grunseich et al., 2021). Both *Photorhabdus* and *Xenorhabdus* produce an as yet unidentified chemical scavenger deterrent factor (SDF) that discourages scavengers from feeding on the host cadaver (Zhou et al., 2002; Raja et al., 2017). Time post infection is an important factor in scavenger deterrence. Ants (*Lepisiota frauenfeldi*), crickets (*Gryllus bimaculatus*) and wasps (*Vespa orientalis L.* and *Paravespula spp.*) were deterred from feeding on 2-day but not 1-day EPN-infected *Galleria mellonella* larvae (Gulcu et al., 2012). Similarly, 10-day EPN-infected cadavers were scavenged less than 4-day infected cadavers by ants (*Linepithema humile*) (Baur et al., 1989). Ulug et al. (2014) reported lower incidence of feeding on EPN-infected cadavers 2-3 days after infection as opposed to 1 day for both cockroaches (*P. americana*) and crickets (*G. bimaculatus*). We assume that as the symbiotic bacteria proliferate the intensity of deterrence is increased due to an accumulation of metabolites.

In general, *Heterorhabditis*-infected cadavers tend to be better protected from scavengers than *Steinernema*-infected cadavers (Baur et al., 1998; Gulcu et al., 2012; Ulug et al., 2014). In addition, chemical cues produced by *H. bacteriophora*-infected cadavers, but not *S. carpocapsae* or *S. feltiae*-infected cadavers, deterred herbivorous

beetle larvae (*Acalymma vittatum*) from approaching (Grunseich et al., 2021). This may be due to additional deterrence factors produced by *Photorhabdus* compared to *Xenorhabdus*. Each species of EPN/ bacteria produces a unique volatile blend that may act as deterrent (Grunseich et al., 2021). Anthraquinones produced by *Photorhabdus* give the *Heterorhabditis* infected cadavers a characteristic red/yellow pigmentation (Richardson et al., 1988; Li et al., 1995). Both Fenton et al. (2011) and Jones et al. (2017) found that birds (*Erithacus rubecula, Parus major*) were deterred from feeding on *H. bacteriophora*-infected larvae and suggested that the bright pigmentation of the cadaver may act as an aposematic signal, warning daytime scavengers of the unpalatability of the host.

Photorhabdus are also bioluminescent (Poinar et al., 1980). They possess a *lux* operon that codes for luciferase, an enzyme which acts on a luciferin, producing a visible luminescence (Kasai et al., 2007). From approximately 20 hrs post infection, a glow emits from the host cadaver (Poinar et al., 1980) reaching a peak wavelength of ~490 nm (Maher et al., 2021). It has been suggested that this bioluminescence could be either a deterrent for scavengers (Akhurst and Boemare, 1990; Baur et al., 1998; Jones et al., 2015; Maher et al., 2021) or an attractant for potential hosts (Poinar et al., 1980; Patterson et al., 2015) but there is little experimental evidence for either of these functions (See section 4.1).

1.3.6 Defence against abiotic factors

EPN face numerous abiotic factors that can reduce their survival, including desiccation, extreme temperatures, and osmotic stress (Kung et al., 1991; Liu &

Glazer, 2000, 2002; Shapiro-Ilan et al., 2015). Defensive mechanisms, both behavioural and physiological, allow the nematodes to survive unfavourable conditions.

The infective juvenile stage is adapted for survival under harsh conditions. IJs in the free-living state can persist in dry conditions in the soil for weeks, through a number of physiological and behavioural adaptations that reduce moisture loss including aggregation (Womersley, 1990), dormancy (Chen and Glazer, 2004) and entrance into an anhydrobiotic state where metabolic activity is reduced (Womersley, 1990; Solomon et al., 1991; O'Leary et al., 2001; Chen and Glazer, 2004; Ali and Wharton, 2013). Stress resistance varies between IJs of different EPN species. *H. bacteriophora* are more vulnerable to osmotic and desiccation stress than *S. carpocapsae* or *S. feltiae* and take longer to recover when conditions improve (Chen and Glazer, 2004; Somvanshi et al., 2008). The synthesis of glycogen and trehalose in response to desiccation may account for the higher survival of *Steinernema* IJs in dry conditions (O'Leary et al., 2001). Trehalose accumulates in *S. feltiae* IJs in dry conditions (Solomon et al., 1999; Jagdale and Grewal, 2003). Clumping of IJs in large groups helps to slow water loss of individual IJs by minimising surface exposure (Glazer, 2002). While aggregation occurs in both genera of EPN, *Steinernema* IJs also respond to desiccation by coiling (Womersley, 1990). Retention of the cuticle from the second moult provides *Heterorhabditis* but not *Steinernema* IJs with an additional layer of protection from desiccation (Patel et al., 1997a; Perry et al., 2012). EPN IJs deal with osmotic stress by entering a dormant state (Chen and Glazer, 2004). IJs have a limited lipid and glycogen reserves on which they survive between hosts (Selvan et al., 1993b;

Wright et al., 1997) and dormancy reduces the metabolic rate of IJs allowing them to survive for longer (Evans, 1987; Chen and Glazer, 2004; Ali and Wharton, 2013).

IJs can be freeze tolerant or freeze avoidant. *Steinernema anomali*, *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* were all found to be freezing tolerant (Brown and Gaugler, 1996, 1998; Ali and Wharton, 2013, 2015). *S. feltiae* and *H. bacteriophora* can survive temperatures as low as -22°C and -19°C respectively (Brown and Gaugler, 1996). The production of trehalose and glycogen aid in anti-freezing response of IJs to extreme cold temperatures (Glazer, 2002; Ali and Wharton, 2015). IJs that retain their sheath are freeze avoidant as the sheath prevents freezing, as seen in *H. zealandica* (Wharton and Surrey, 1994). Several species of EPN also tolerate extreme temperatures through the production of heat shock proteins (Glazer, 2002). Trehalose, which, as previously stated, aids desiccation and freezing survival in IJs, also accumulates in response to heat shock (Jagdale and Grewal, 2003). The similar physiological responses to osmotic, desiccation and heat stress means pre-conditioning of the nematodes under one environmental stress allows greater survival when encountering another. For example, IJs exposed to osmotic stress have a greater heat tolerance (Glazer and Salame, 2000) and *S. carpocapsae* and *S. feltiae* IJs acclimated to cold temperatures showed enhanced heat tolerance (Jagdale and Grewal, 2003).

Outside of the IJ stage, EPN are less stress resistant. The host cadaver protects the nematodes inside from environmental stress (Koppenhofer et al., 1997; Serwe-Rodriguez et al., 2004). Studies have been conducted to determine the effects of freezing and desiccation of the host cadaver on EPN, both while in the host and after emergence (Lewis and Shapiro-Ilan, 2002; Serwe-Rodriguez et al., 2004; Spence et al., 2011; Maher et al., 2017). Lewis and Shapiro-Ilan (2002) demonstrated that the

host cadaver protects EPN from freezing (Lewis and Shapiro-Ilan, 2002). Freezing conditions were more detrimental for *S. carpocapsae* than *H. bacteriophora* in the host (Lewis and Shapiro-Ilan, 2002). Depending on the species of nematode, desiccation of the host cadaver can reduce the overall number of IJs that emerge from infected cadavers (Koppenhofer et al., 1997; Serwe-Rodriguez et al., 2004; Spence et al., 2011; Maher et al., 2017) and delay their emergence (Koppenhofer et al., 1997). Spence et al., (2011) found that *H. bacteriophora* had lower emergence success than *S. carpocapsae* or *S. riobrave* from desiccated cadavers while Koppenhofer et al. (1997) found *H. bacteriophora* shows better persistence in cadavers in dry soil than *S. carpocapsae*. In contrast, Serwe-Rodriguez et al. (2004) found increased *S. carpocapsae* IJ emergence from desiccated cadavers. Exposure of the host to desiccating conditions increased virulence of *S. carpocapsae* IJs (Serwe-Rodriguez et al., 2004) while desiccation stress reduces infectivity in *H. bacteriophora* (Mukuka et al., 2010). The cuticle of the cadaver may act as a buffer in dry conditions, preventing desiccation inside the host (Koppenhofer et al., 1997). The bacterial symbiont may also affect rate of desiccation of the host (Koppenhofer et al., 1997; Maher et al., 2017; Spence et al., 2011). Maher et al., (2017) demonstrated that *P. cinerea*-infected hosts had a slower desiccation rate than *P. temperata*-infected hosts, which led to a higher number of *Heterorhabditis downesi* emerging. Scavengers feeding on the insect cadavers will breach the cuticle, which may lead to enhanced desiccation within the host (Zhou et al., 2002).

1.4 Research aims

The highly competitive environment, limited resources and short life cycle of EPN make them ideal models to study defensive mechanisms in multi-tiered ecological

systems. This thesis focuses on how entomopathogenic nematodes along with their symbiotic bacteria defend themselves and their limited resources within the host cadaver from other EPN (inter and intra-specifically) and from scavenging enemies (Table 1.1).

1.4.1 Intraspecific competition

Males of *S. longicaudum* engage in lethal fighting to minimise resource competition and those with prior mating experience are better killers than males that are naïve (Kapranas et al., 2020). Mated males differ from naïve males in experience but also in physiology. Exposure to females leads to sexual development in *S. longicaudum* males (Ebssa et al., 2008) which may lead to an increase in size. Larger males tend to have an advantage in contest behaviour (Archer, 1988; Hughes, 1996; Brown et al., 2006b; Hsu et al., 2006;). This sexual development in *Steinernema* males may lend an advantage in lethal fighting. To investigate this the following hypotheses were tested using *S. carpocapsae* males (Chapter 2):

Hypothesis 1a. Exposure to female pheromone increases the size of *S. carpocapsae* males and promotes sexual development.

Hypothesis 1b. This increase in size gives sexually developed males an advantage in lethal fighting.

1.4.2 Interspecific competition

Despite frequently occupying the same territory *Steinernema* and *Heterorhabditis* nematodes do not commonly co-occur within a host (Alatorre-Rosas and Kaya, 1991; Hatting et al., 2009; Kanga et al., 2012; Campos-Herrera et al., 2015). It is presumed that one species outcompetes the other within the host however this has not been confirmed (See section 1.3.4.2). To determine the mechanism behind this dominance the following hypotheses were tested using combinations of *S. feltiae/ S. carpocapsae* and *H. bacteriophora*/ *H. downesi* at various timepoints (Chapter 3):

Hypothesis 2a. IJs of one genus of EPN will not succeed in developing and reproducing in an insect host that is already infected with a species of the other genus.

Hypothesis 2b. Success of the species that invades second will decline the longer the first species has been established in the insect.

Hypothesis 2c. IJs of the second species will show adaptive behaviour and either not be attracted to or not invade insects in which they have a low probability of developing and reproducing due to the presence of a competing species.

1.4.3 Scavengers

Defence against scavengers was the subject of two investigations: (1) bioluminescence as a scavenger deterrent and (2) the impact of host damage on the fitness of emerging EPN.

(1) Bioluminescence has been proposed as a deterrent that discourages scavengers from feeding on *Heterorhabditis/ Photorhabdus*-infected insects. To determine the

importance of bioluminescence as a defence mechanism the following hypothesis was tested using *H. downesi* (Chapter 4):

Hypothesis 3: Bioluminescence contributes to the protection of *Photorhabdus*-infected cadavers against nocturnal scavengers.

(2) Scavengers feeding on EPN infected cadavers can directly kill the nematodes through consumption and can also expose the nematodes to unfavourable conditions including desiccation and competing microbes. To determine the effect on EPN of scavenger damage to the host cadaver the following hypothesis was tested using *S. feltiae* and *H. downesi* (Chapter 5):

Hypothesis 4: Damage to the cadaver cuticle, imitating scavenging, will lead to reduced nematode fitness in terms of number of IJs that emerge and IJ size.

Table 1.1. Summary of investigations.

	Hypothesis	Nematode species (and strain) tested	Symbiont(s)	Chapter
1	Sexual development in *Steinernema* males will increase their size and affect their fighting capabilities.	*S. carpocapsae* (All)	*X. nematophila*	2
2	IJs of one genus of EPN will not invade/ develop in an insect host that is already infected with a species of the other genus.	*S. carpocapsae* (All) *S. feltiae* (4cfmo) *H. bacteriophora* (VM21) *H. downesi* (K122)	*X. nematophila* *X. bovenii* *P. thracensis* *P. temperata*	3
3	Bioluminescence contributes to the protection of *Photorhabdus*-infected cadavers against scavengers.	*H. downesi* (K122)	*P. temperata*	4
4	Scavenger damage to the cadaver cuticle will lead to reduced nematode fitness in terms of number and size of IJs that emerge.	*S. feltiae* (4cfmo) *H. downesi* (K122)	*X. bovenii* *P. temperata*	5

Chapter 2. Mating status, independent of size, influences in an entomopathogenic nematode.

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2.0 Summary

Males of many species compete over access to females through physical contests. Previous experience of, and opportunity to mate with a female may influence the motivation of males to engage in contests and the outcome of such contests. Often, prior mating results in increased aggression and probability of success. This is mediated by the effects on the male's subjective evaluation, both of the resource value (RV) and of his own ability to acquire the resource (resource holding potential: RHP). Moreover, having mated may also affect a male's actual fighting ability. In *Steinernema* nematodes, mated males paired with naïve males are more likely to win contests. Here we show that this advantage in mated males cannot be explained solely by the physical changes brought about by prior female contact, since males exposed to female pheromone alone developed sperm and increased in size like mated males but did not have the same advantage as them in contest with naïve males. Effects of mating on other components of RHP, such as skill or motivation, or on RV may explain the greater probability of mated males winning. We also show that mating had differential effects on the probability of a male initiating attack, depending on whether the opponent was a mated male (and thus ready to mate again) or naïve, consistent with the relative threat of the opponent as a competitor for copulations.

2.1 Introduction

Individuals of many animal taxa compete for resources such as food or mates through contest behaviour of various kinds, sometimes resulting in injury or even death (Alexander, 1961; Enquist & Leimar, 1987; Kravitz & Huber, 2003; Moore et al., 2008; Hardy & Briffa, 2013). For example, honeybee queens (*Apis mellifera ligustica*) engage in lethal stinging duels for dominance of the colony (Kravitz & Huber, 2003), pumpkinseed sunfish (*Lepomis gibbosus*) will defend territory by biting and butting contestants (Beacham and Newman, 1987), while male hermit crabs (*Pagurus* spp.) will guard potential female mates by using an enlarged cheliped to grapple opponents (Yasuda et al., 2015). Who wins a contest is largely dependent on the relative ability of opponents to acquire the contested resource (resource-holding potential, RHP), although the value of the contested resource to the opponents affects their motivation and hence also the outcome of the contest (Parker, 1974; Enquist & Leimar, 1987; Arnott & Elwood, 2008; Vieira & Peixoto, 2013). Body size and strength, weaponry, skill, and physiological condition all contribute to an animal's RHP (Arnott & Elwood, 2009; Briffa & Lane, 2017; Palaoro & Peixoto, 2022), but size is frequently the major factor, with larger size correlating with higher RHP in many animals (Archer, 1988; Hughes, 1996; Petersen & Hardy, 1996; Hack et al., 1997; Neat et al., 1998; Morand, 2000; Hsu et al., 2006; Brown et al., 2006b). Contest behaviour can be affected by contestants' assessment of their own RHP (self-assessment) and of their opponents' RHP in comparison (mutual assessment; Arnott & Elwood, 2009; Chapin et al., 2019).

Prior experience, including social experience, can influence the outcome of a contest (Beacham & Newman, 1987; Jacques et al., 1996; Kimberly & Rowland, 2000).

Among the best documented effects of social experience are the ‘winner effect’, whereby winning a contest increases an animal's probability of winning a subsequent contest by altering its self-assessment of RHP (Rutte et al., 2006; Hsu et al., 2006), and the finding that residents also generally win contests, explained by their greater familiarity with the contested resource (Kokko et al., 2006; Kapranas et al., 2020). Many other social experiences, including those of early life, may also affect the outcome of contests (Lee et al., 2014; Colella et al., 2019; Favati et al., 2021).

For males competing for mating opportunities, prior mating or other social experience of females can affect subsequent contest behaviour (Innocent et al., 2011; Yasuda et al., 2015; Yuan et al., 2014; Baxter & Dukas, 2017; Zhang et al., 2019a). There are sound theoretical arguments to predict either an increase or a decrease in aggression following mating (Judge et al., 2010; Baxter & Dukas, 2017). On the one hand, unmated males may be more motivated to engage in fighting because future mating opportunities are of greater value to them, while mated males may be less willing to engage in costly fighting (Kemp, 2006). On the other hand, having mated may change a male's evaluation of the contested resource increasing his willingness to fight for a more highly valued prize (Kemp, 2006; Brown et al., 2006b, 2007; Innocent et al., 2011). Mating may also increase the male's self-assessment of his own RHP (Yasuda et al., 2015), in a manner similar to the ‘winner’ effect whereby males that win a contest become more aggressive and likely to fight (Hsu et al., 2006). In empirical studies across a range of invertebrates and vertebrates, mating and/or contact with a female tends to increase rather than decrease a male's aggression and success in contests (Killian & Allen, 2008; Bergman et al., 2010; Judge et al., 2010; Kralj-Fišer et al., 2011; Guevara-Fiore et al., 2012; Yasuda et al., 2015; Zhang et al., 2019a), while

for house crickets, *Acheta domesticus*, males with restricted prior access to females were more aggressive (Brown et al., 2006b, 2007).

In studies where mating or sexual experience had no effect on aggression, this may be due to the counterbalancing effects on the males' motivation; future expectations of reproductive success versus subjective assessments of RV or RHP (Innocent et al., 2011; Baxter & Dukas, 2017). Since mating, even for males, may incur costs in terms of energy (Perry & Tse, 2013; Scharf et al., 2013; Cargnelutti et al., 2022), it may also alter the outcome of aggressive encounters by reducing RHP. For example, while male field crickets, *Gryllus pennsylvanicus*, were more aggressive after experiencing a female (whether they mated with her or not), those that copulated were less likely to win a contest than those that did not (Judge et al., 2010). In the nematode *Steinernema longicaudum* (a species with lethal male combat; see 'Background biology' below) mated males were more successful in terms of survival and killing than naïve males when the two were paired together in controlled fights (Kapranas et al., 2020). Interpreting these findings is complicated by the fact that mating involves both exposure to female pheromone and the act of copulation. In *Steinernema*, female pheromone induces physiological changes in males, stimulating sexual maturation and sperm production (Ebssa et al., 2008; Hartley, 2017). In some animals, aggression of males can be influenced by sexual development. Gonadal development increased aggression in cichlid fish (*Tilapia zillii*), while exposure to female pheromone increased aggression in crickets (*A. domesticus)* (Neat et al., 1998; Killian & Allen, 2008). In *Steinernema*, the gonads are large relative to somatic body size (Ebssa et al., 2008), and therefore their maturation might be accompanied by an increase in overall body size that could contribute to RHP.

Here we tested whether the physiological change induced by exposure to female pheromone alone, without the opportunity to mate, in male steinernematids could explain their higher RHP when they engage other competitors in lethal fights. We first assessed whether male sexual maturation induced by pheromone exposure is associated with an increase in their body size. We then experimentally distinguished the effects of female pheromones and the act of mating on contest (fighting) behaviour and outcome.

2.2 Methods

2.2.1 Background Biology

In *Steinernema* spp., a free-living infective juvenile seeks out and invades an insect. Once inside the insect, infective juveniles release symbiotic bacteria which assist in rapidly killing the host and digesting its tissues. The nematodes feed on the bacteria and digested host tissue and develop to adult. A single dead host may support two to three generations and result in tens of thousands of infective juvenile progeny. In most *Steinernema* species, infective juveniles develop into either males or females. Although the sex ratio is slightly female biased (Alsaiyah et al., 2009), females die shortly after mating as juveniles hatch inside their mother (*endotokia matricida*; Baliadi et al., 2004), resulting in a male-biased operational sex ratio. Males of some *Steinernema* engage in lethal fighting, wrapping around and compressing their opponent, which can cause paralysis or death (Zenner et al., 2014). Lethal fighting has evolved in *Steinernema* spp. in response to the limited mating opportunities and to

secure the valuable resource of a host for the developing offspring (Innocent et al., 2011; Kapranas et al., 2016). The incidence of fighting is much higher in males that develop from infective juveniles and colonize a host in small numbers than in males of subsequent generations which typically occur in crowded conditions (Zenner et al., 2014). In at least some *Steinernema* species, laboratory studies have shown that males that develop in isolation have underdeveloped testes and the seminal vesicle contains no sperm (Ebssa et al., 2008). The state of reproductive development can be clearly discerned by microscopic examination through the nematode's transparent cuticle. Gonadal development of solitarily reared males can be stimulated by exposure to female pheromone (Ebssa et al., 2008; Hartley, 2017). Of the close to 100 species of *Steinernema* described, *Steinernema carpocapsae* is the best studied and is also widely commercialized as a biological control agent (Murfin et al., 2012; Lacey et al., 2015; Koppenhöfer et al., 2020).

2.2.2 Nematode Culture

Steinernema carpocapsae (All strain) cultures were routinely maintained using standard procedures by passage through late-instar *Galleria mellonella* (wax moth) larvae (Kaya & Stock, 1997) at 20 °C. Infective juveniles were stored in tap water at 9 °C. Adults for experiments were obtained by placing surface-sterilized (0.1 % v/v hyamine) infective juveniles individually in 20 µl hanging drops of haemolymph from *Galleria mellonella* larvae, which provides a suitable environment for development to adulthood (Kaya & Stock, 1997; Zenner et al., 2014). The hanging drops were placed on the under surface of a Petri dish lid which was placed over a water-filled Petri dish (3.5 cm diameter) to prevent desiccation. The infective juveniles in the hanging drops developed to adulthood within 3 days at 20 °C. Adult males can be distinguished by

their copulatory spicules and their smaller size whereas females are larger and bear a vulva. We used 3-day-old adult males and females in our experiments. At this time a minority of males had spontaneously developed sperm and were discarded.

2.2.3 Conditioning

Males without sperm were randomly assigned to create three categories: mated, pheromone-exposed or naïve. (1) For the mated category a male was placed in a drop with an adult female. Only males for which mating was confirmed by subsequent production of progeny were included in the results. (2) For the pheromone-exposed category a male was placed in a drop that had previously been occupied by an adult female. (3) For the naïve category a male was removed from and placed back into its own drop. After 48 hrs sperm development was recorded. Only males that had developed sperm in the mated and pheromone treatments (indicating successful exposure to female pheromone), and males without sperm in the naïve treatment (the condition of the majority of naïve males), were used in experiments. Males were photographed, and their area and length were measured using Image J 1.53e (http://imagej.nih.gov/ij) as an index of their size.

2.2.4 Fighting assays

Each male, including the controls, was transferred to a fresh drop of *G. mellonella* haemolymph using a sterile platinum wire and was allowed to acclimatize for 1 hr. Males were then paired in drops symmetrically within category (mated with mated; pheromone-exposed with pheromone-exposed; naïve with naïve) or asymmetrically

(mated with naïve; pheromone-exposed with naïve). There were thus five treatments. Control males of each category were taken out of and immediately replaced into their own drops. Pairs were observed for 30 min at 26 °C. Fighting was recorded when one male wrapped around the other and tightened (Kapranas et al., 2020). Not all fights resulted in injury or death, and some pairs engaged in more than one fight during observation. Incidence of fighting, the identity of the initiator (in asymmetric pairs), number of fights, duration of fights and time at which fights occurred were recorded for each pair. A total of 33–59 pairs per treatment and 37–66 control individuals per category were observed. After 24 hrs at 20 °C, mortality (including severe injury or paralysis) was recorded for each pair and control.

2.2.5 Data analysis

The area and length of males from each category were compared using one-way analysis of variance (ANOVA) with significance at $P < 0.05$, followed by Tukey post hoc tests. For pairs in which fighting was observed, the number of fights, median duration and latency were analysed using ANOVA or the Kruskal–Wallis test, with significance at $P < 0.05$. For asymmetric fights, a one-sample proportion test was used to compare initiation of fights between conditioned (mated or pheromone-exposed) and naïve males with a hypothesized proportion initiated by naïve males of 0.5. The identity of the victim in these fights was also similarly tested for deviation from 0.5. To assess whether a mated male assesses its opponent, the probability of a mated male initiating an attack against a naïve male or against another mated male was compared using chi-square analysis. All the above data analysis was performed using Minitab 20.3 statistical software (Minitab, LLC, 2021) (or RStudio version 2022.12.0 (RStudio

Team, 2020)). The incidence of fighting and death as influenced by different worm pairs (treatments) was explored with a binary logistic analysis. We used backward stepwise procedures and aggregation of factor levels (akin to a post hoc test) to obtain the parsimonious 'minimal adequate model' by model simplification (Briffa and Hardy, 2013). In the subsets of observations for which worm measurements were available for asymmetric pairings (pheromone-exposed versus naïve: $N = 24$; mated versus naïve: $N = 19$), we tested whether the initiation of fighting and the winning of fights by naïve worms was influenced by their opponent's status and the size (area) difference between them using a binary logistic regression (Briffa and Hardy, 2013). These analyses were performed with GENSTAT v. 22 (VSN International Ltd, Hemel Hempstead, U.K.). The same analysis could not be carried out for symmetrical pairings as the individuals were indistinguishable after pairing.

2.2.6 Ethical note

No ethical approval was required for the species used in this research. The methodology used in this study was in keeping with the ASAB/ABS guidelines for the treatment of animals in behavioural research, as well as the suggestions by Drinkwater et al. (2019) for ethical treatment of invertebrates, with a focus on reduction and refinement. The number of *G. mellonella* used for haemolymph extraction was minimized by calculation of exact required volumes and the number of nematodes used in staged fights was reduced through refinement of the protocol.

2.3 Results

2.3.1 Size

There was a significant difference in area between the three categories of males (ANOVA: $F_{2, 237} = 19.1$, $P < 0.001$). Males with sperm (mated or pheromone-exposed) were larger than naïve males, but there was no difference in area between the mated and pheromone-exposed males (Fig. 2.1). There was no significant difference in length between naïve males (1.80 ± 0.04 mm, $N = 83$), mated males (1.87 ± 0.05 mm, $N = 88$) or pheromone-exposed males (1.89 ± 0.05 mm, $N = 74$; ANOVA: $F_{2, 237} = 1.14$, $P = 0.320$).

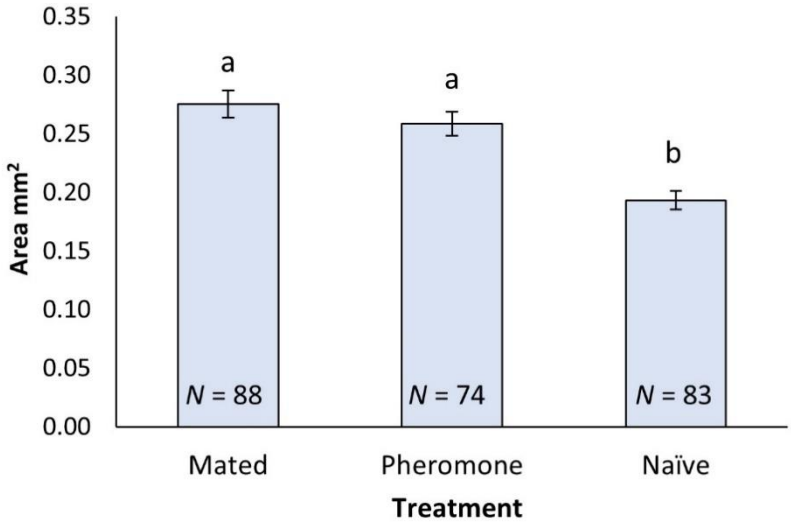

Figure 2.1. Area (mean +/- SE) of males that were conditioned for 48 hrs in drops with a female (mated), in drops previously occupied by a female (pheromone) and in their own drops (naïve). The number of males measured in each category is indicated on the bars. Bars with differing letters are significantly different ($P < 0.001$, Tukey's post hoc test). Measured males in the mated and pheromone-exposed categories all had sperm present while none of the naïve males had sperm.

2.3.2 Fighting and mortality

There was a significant difference in incidence of fighting across treatments (logistic analysis: $G_4 = 3.04$, $P = 0.016$). Incidence of fighting was highest in symmetric mated pairs and lowest in symmetric pheromone-exposed pairs compared to all other treatments (Fig. 2.2). For pairs in which fighting was recorded, there was no difference across treatments in the number of fights per pair (Kruskal–Wallis: $H_4 = 2.72$, $P = 0.605$), duration of fighting (Kruskal–Wallis: $H_4 = 4.37$, $P = 0.358$) or time until the first fight (ANOVA: $F_{4, 63} = 1.93$, $P = 0.117$; Appendix Table 2.1).

After 24 hrs, the overall incidence of damage or death in the pairs was 26% (61/234). There was no difference across treatments in incidence of damage or death in the pairs (logistic analysis: $G_4 = 1.17$, $P = 0.326$; Appendix Table 2.1). Control mortality was low (3%; 10/300) and did not differ between categories of male (Pearson chi square: $X^2_2 = 1.8$, $P = 0.405$).

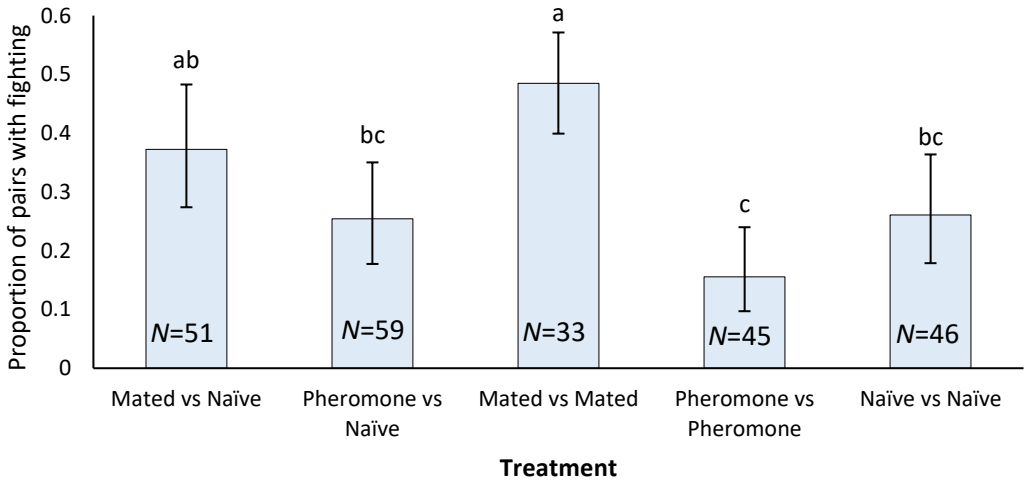

Figure 2.2. The proportion (+/- SE) of pairs of *S. carpocapsae* males in which fighting was observed during 30 min observation. Conditioned males (Mated or pheromone-exposed) were paired symmetrically with a similarly conditioned male, or asymmetrically with a naïve male. Pairings of a naïve male vs a naïve male were also observed. Bars with differing letters are significantly different ($P < 0.05$). The number of pairs measured in each treatment is shown on the bars.

2.3.3 Identity of Initiator and Victim of Fighting in Asymmetric Pairings

When a fight occurred in asymmetric pairings, it was more likely to be the naïve male than the conditioned male that initiated fighting. This was consistent for both the mated versus naïve and pheromone-exposed versus naïve treatments (one-sample proportion test: $X^2_1 = 6.368$, $P = 0.012$ and $X^2_1 = 5.4$, $P = 0.020$, respectively; hypothesized proportion = 0.5; Fig. 2.3, Appendix Table 2.2). In mated versus naïve pairs, where one male was damaged or dead after 24 hr it was more likely to be the naïve male than the mated male (one-sample proportion test: $X^2_1 = 7.118$, $P = 0.008$; hypothesized proportion = 0.5), but in the case of pheromone-exposed versus naïve males, there was no deviation from equality (one-sample proportion test: $X^2_1 = 0.474$, $P = 0.491$; hypothesized proportion = 0.5; Fig. 2.4).

When mated versus naïve and pheromone-exposed versus naïve male pairs were compared, naïve males were as likely to initiate a fight when paired with a mated male as with a pheromone-exposed male (logistic analysis: $G_1 = 1.21$, $P = 0.271$). Analysis of the subsets for which worm size measurements were available confirmed that the probability of naïve worms initiating a fight was not influenced by competitor status (mated or pheromone-exposed) or size (opponent's status: $G_1 = 0.34$, $P = 0.562$; size: $G_1 = 1.45$, $P = 0.228$; interaction between opponent's status and size: $G_1 = 1.36$, $P = 0.244$).

Naïve males had a marginally higher but not significant tendency to win when paired against pheromone-exposed than when paired with mated males (logistic analysis: $G_1 = 3.31$, $P = 0.069$). However, in the subsets for which worm size measurements were available, competitor status (mated or pheromone-exposed) and size were not

significant (opponent's status: G_1 = 1.17, P = 0.279; size: G_1 = 1.22, P = 0.269; interaction between opponent's status and size: G_1 = 0.02, P = 0.902).

Of the mated males tested in asymmetric pairings, only 8% (4/51) initiated fighting, while in symmetric mated–mated pairings, fighting was initiated by 24% (16/66) of the individuals tested, a significant difference (chi-square test: X^2_1 = 5.459, P = 0.019).

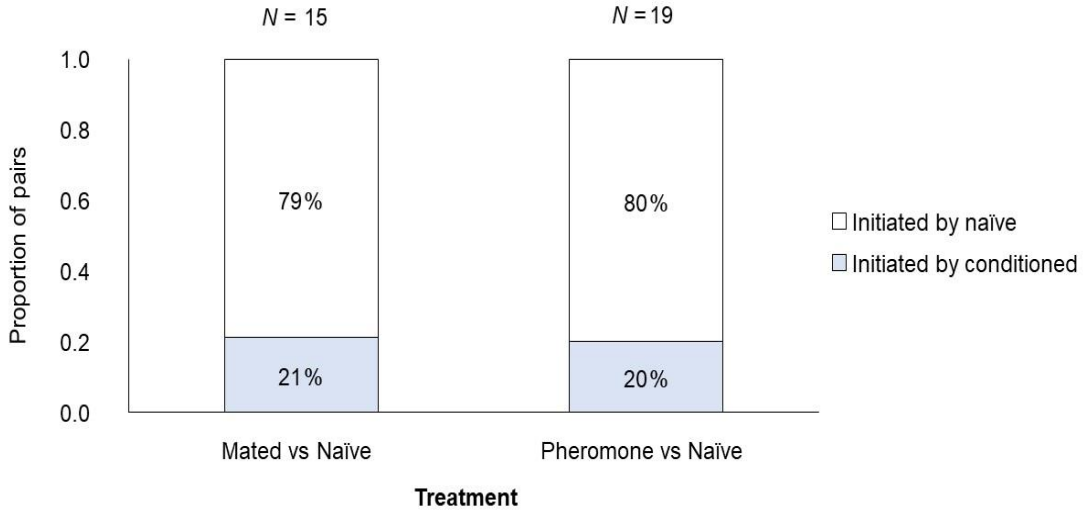

Figure 2.3. Proportion of pairs in which fighting was initiated by conditioned (mated or pheromone-exposed) or naïve males in asymmetric pairings of conditioned versus naïve. Data shown only for pairs that engaged in fighting. The number of pairs measured in each treatment is shown above the bars.

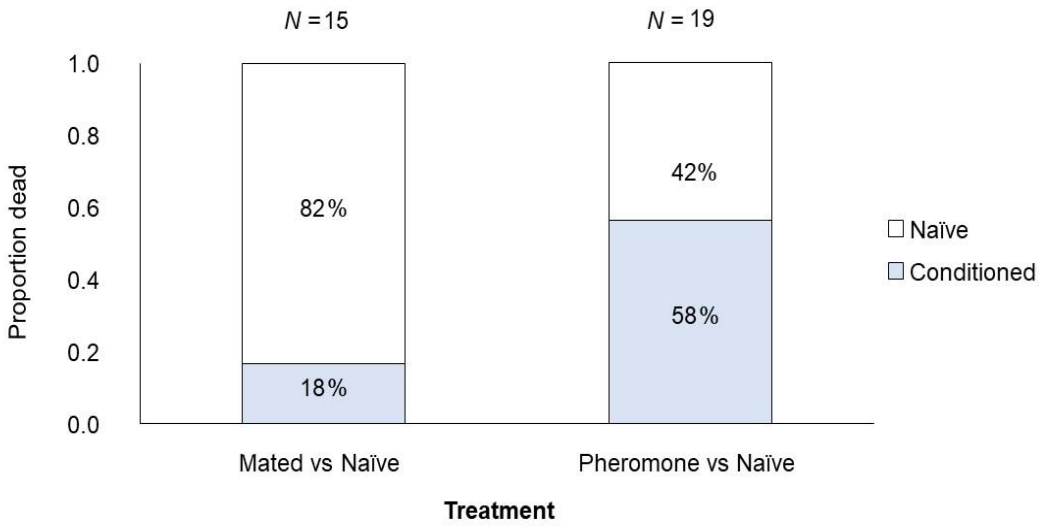

Figure 2.4. Proportion of conditioned (mated or pheromone-exposed) or naïve males dead or damaged after 24 hrs in asymmetric pairings. Data shown only for pairs that engaged in fighting. The number of pairs measured in each treatment is shown above the bars.

2.4 Discussion

As seen previously in *S. longicaudum* males (Kapranas et al., 2020), mated *S. carpocapsae* males had a killing advantage when paired against naïve males. Contrary to our hypothesis, however, this advantage could not be explained by the physiological effects induced by exposure to female pheromone, such as sperm production and the associated increase in size, since males that received pheromone-exposure treatment alone did not have a similar advantage over naïve males. Both mated and pheromone-exposed males were similar in size, being larger than naïve males, presumably due to the sperm stored in the seminal vesicle increasing the worms' diameter. Despite the similarity in size, mated and pheromone-exposed males differed in the outcome of interactions with naïve males, in that mated but not pheromone-treated males were

more likely to be the victor over the naïve male. Moreover, analyses on asymmetric pairs showed that the size difference between opponents did not affect the outcome of the interaction. Body size is an important determinant of RHP: in general, larger animals are more likely to win (Archer, 1988; Brown et al., 2006b; Hsu et al., 2006; Hughes, 1996) but this is not always the case. For example, Elwood et al. (1998) found that relative size affected neither the cost nor the probability of victory in fighting in the hermit crab *Pagurus bernhardus.* Although both the mated and pheromone-exposed males had developed sperm and increased in diameter, they differed in their experience with a female. In many animal taxa, previous mating has been shown to increase a male's self-assessment of RHP, thus increasing their confidence and likelihood of fighting (Guevara-Fiore et al., 2012; Killian & Allen, 2008; Kralj-Fišer et al., 2011; Yasuda et al., 2015). This is similar to the 'winner effect': animals that have succeeded in combat are more likely to succeed again (Hsu et al., 2006; Kasumovic et al., 2009; Kou & Hsu, 2013). Experience gained during mating may also increase skills that contribute to an advantage in fighting (Briffa & Lane, 2017). In *Steinernema*, the male wraps its tail end around the female at the vulva and uses a pair of copulatory spicules to assist insemination (Lewis et al., 2002). This behaviour resembles the coiling used in fighting, and the spicules may assist in inflicting injuries either by puncturing or by focusing the pressure delivered by squeezing (Zenner et al., 2014). Mating behaviour of *Caenorhabditis elegans* male nematodes is controlled by a specialized posteriorly located 'connectome', a network of neurons, muscles and gonad (Jarrell et al., 2012). In *C. elegans,* mating experience results in the rewiring of specific synapses in the male connectome, with evidence that these changes are translated into finer coordination of muscle contraction, such as those involved in

spicule protraction (Hart & Hobert, 2018). A similar physiological change may occur in *Steinernema* males.

In our experiments, we exposed males to female pheromone to induce physiological maturation, to explore the importance of the mated male's physical status in winning a fight. In other animals, experience of a female (without mating) may or may not mimic the effects of actual copulation on male aggression (Bergman et al., 2010; Judge et al., 2010; Killian & Allen, 2008; Rillich et al., 2019). For example, male crickets, *A. domesticus*, that were allowed to contact a female but not to copulate with her showed the same increased aggression as males that were allowed to copulate, suggesting that chemotactile cues from the female were sufficient to elicit this change in aggression (Killian & Allen, 2008). While female pheromone gives an indication to *S. carpocapsae* males of female presence, it is unclear whether it can be considered equivalent to the 'female experience' treatment of studies in other animals. In nematodes, the response of males to female pheromone represents the first step in mate finding, but there is also a second step involving contact cues at the female's body surface (Barr & Garcia, 2006; Sakai et al., 2013).

Pheromones are widely implicated in modulating aggression in insects and mammals (Itakura et al., 2022; Sengupta et al., 2022). In nematodes, pheromones are involved in numerous social behaviours including mate attraction, aggregation, and dispersal (Edison, 2009; Hartley et al., 2019; Muirhead & Srinivasan, 2020). Nematode pheromones mainly belong to a class of molecules called ascarosides, a modular library of potent molecules with both developmental and behavioural effects (Choe et al., 2012; Butcher, 2017; Park et al., 2019). Media conditioned by *S. carpocapsae* females contain a blend of ascarosides and both attract conspecific males and initiate

their gonadal maturation (Choe et al., 2012; Hartley, 2017). Exposure to female pheromone in our experiments clearly induced the physical effects (sperm development and concomitant increase in size) but not the behavioural effects of mating. Indeed, pairs of pheromone-treated males showed the lowest incidence of fighting, in contrast to the elevated fighting in pairs of mated males. Female steinernematids have a short window of availability, becoming less attractive (Lewis et al., 2002; Hartley, 2017) and of lower RV (Kapranas et al., 2020) for males shortly after mating. In the closed environment of a dead insect, residual pheromone, in the absence of available females, would indicate that there were no more available females in the founding generation, and the only way for a male to achieve at least some reproductive success would be to survive until females of the filial generation become available (Zenner et al., 2014). Thus, the reduced aggression of pheromone-matured males would be adaptive. Internal state, social interaction and other experiences interact in a complex manner to influence the translation of stimuli into behaviours, even in a relatively simple organism such as a nematode (Hashikawa et al., 2018; Park et al., 2019). The impact on a male steinernematids of experiencing pheromone only, without encountering a female, warrants further exploration.

Although mated males tended to win fights against naïve males, it was the naïve male that was more likely to initiate fighting in these pairings. Naïve males are expected to value a mating opportunity more highly than a male that has just mated (Kemp, 2006) and will be more aggressive as a result (Brown et al., 2006b, 2007). This may be a case of contradictory asymmetry where the opponent with the lower RHP has more to gain from the fight (Parker & Rubinstein, 1981). It is expected that if males can assess relative RHP, the male with lower RHP will terminate the fight before sustaining

injury. This is based on mutual assessment (Enquist & Leimar, 1987). However, if the male with the lower RHP is in a position where it cannot reproductively succeed by quitting the fight then the 'desperado effect' comes into play and the male will continue to attack due to the lower potential cost (Grafen, 1987). Naïve males in this case exist in a divisive asymmetry where they will always lose out to mated males and pheromone-exposed males for mating opportunities. This is because naïve males have not developed sperm and are not ready to mate. By the time the naïve males are sexually developed the mature males may have taken all the available females. Therefore, it is to the naïve male's advantage to always risk the fight. Pheromone-exposed males do not have the same disadvantage as they are sexually developed and are therefore less motivated to fight and risk their future reproductive success.

Comparison of symmetric and asymmetric pairings involving mated males suggests that males are able to assess their opponents' state (using physiological and/or behavioural clues) and thus their relative RHP; mated males were three times more likely to initiate fighting in mated–mated than in mated–naïve pairings. This may be related to the threat that the opponent poses as a competitor in reproduction. A mated male will risk attacking another mated male, as it is ready to mate, but may decide that attacking a naïve male is not worth it, as it is not ready to mate. Despite the high incidence of fighting in mated–mated pairs relative to all other pairings, the mortality rate was not similarly elevated in this treatment. Fighting should end when one male has died or gives up the attack. When opponents are equally matched the cost of fighting is highest due to continued escalation (Arnott & Elwood, 2009; Payne and Pagel, 1996). If males can assess each other's RHP they may decide to terminate fights before injury occurs when equally matched (Enquist & Leimar, 1990).

Adding to the previously described effects of competitor density and relatedness (Zenner et al., 2014; Kapranas et al., 2016), residency and objective RV (Kapranas et al., 2020), this study further shows the value of the entomopathogenic nematodes in testing theoretical predictions in contest behaviour and in behavioural ecology in general (Lewis et al., 2022).

2.5 Conclusion

In conclusion, there are considerable asymmetries due to mating status between *Steinernema* males in conflict behaviour, in their tendency both to initiate and to win a fight. The advantage of mated *Steinernema* males over naïve opponents cannot be explained by larger size, in contrast to findings reported for many animal taxa, but may be due to other elements of RHP or RV.

Chapter 3. Co-infection and competition of *Heterorhabditis* and *Steinernema* entomopathogenic nematodes

In preparation as:

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3.0 Summary

Steinernema and *Heterorhabditis* occupy a similar niche and therefore may compete for host insects when co-occurring. Species of one genus will usually dominate over the other genus when co-infecting a single host at the same site, but the reason for this dominance is unknown. Both *Steinernema* and *Heterorhabditis* rely on their specific symbiotic bacteria (*Xenorhabdus* and *Photorhabdus,* respectively) to provide a suitable environment within the host for development, by providing nutrition and killing microbial competitors (including bacteria of the other genus). *Xenorhabdus* and *Photorhabdus* produce bacteriocins and other antimicrobials that inhibit the growth of the other genus. Competition between the two bacterial species could affect the recovery and development of IJs that invade the host, depending on the reliance of the EPN species on their symbiont. In this chapter the competition between *Heterorhabditis* and *Steinernema* within a single insect was explored using *H. downesi, H. bacteriophora, S. carpocapsae* and *S. feltiae* as representatives of each genus. The invasion by IJs and development to adult of one genus in a host insect infected with the nematodes of the other genus 24, 48 or 72 hrs earlier was investigated. For *S. carpocapsae, S. feltiae* and *H. bacteriophora* the rate of invasion of IJs into hosts infected with the other genus varied depending on the species of nematode occupying the host and the time since infection but was generally lower than into healthy hosts. *H. downesi* IJs showed no variation in the rate of invasion into infected host compared to un-infected hosts. The time since infection affected the likelihood of *Steinernema* IJs developing to adult in *Heterorhabditis*-infected hosts, whereas *Heterorhabditis* IJs were less likely to develop to adulthood in hosts infected with *Steinernema* IJs at all timepoints tested.

IJs may be expected to show adaptive behaviour and not be attracted to hosts occupied by nematodes of the other genus, where development could be impeded. The attraction of IJs of one genus to an insect infected with the other genus 72 hrs earlier was investigated. IJs of all species tested were attracted to insects infected with the other genus of EPN, except for *H. downesi*-infected hosts. There was no evidence of repulsion of IJs of any species to hosts infected with the other genus of EPN.

In conclusion, EPN of one genus will usually dominate over the other genus when co-infecting a single host due a failure of the secondary invading species to develop and not due to a lack of attraction or invasion into the host (with the exception of *H. downesi*-infected hosts).

3.1 Introduction

Entomopathogenic nematodes (EPN) are parasites that invade and kill insects, where they develop and reproduce. The genera *Steinernema* and *Heterorhabditis* are phylogenetically distinct (Blaxter et al., 1998; Ahmed et al., 2022), but follow similar life cycles. The free-living infective juvenile (IJ) stage is tolerant of environmental stress and dwells in the soil. IJs can actively seek out their host as "cruisers" (*Heterorhabditis downesi, Heterorhabditis bacteriophora*) or attach to passing insects as "ambushers" (*Steinernema carpocapsae*) or use a combination of the two strategies (*S. feltiae*) (Campbell and Gaugler, 1993; Grewal et al., 1994; Bal et al., 2014). They then invade the insects through orifices or alternatively, in the case of *Heterorhabditis*, by puncturing the cuticle (Bedding and Molyneux, 1982). Upon invasion they release their symbiotic bacteria into the host insect. *Heterorhabditis* nematodes carry the *Photorhabdus* bacteria in their guts, while *Steinernema* carry *Xenorhabdus* bacteria in specialised receptacles (Goodrich-Blair and Clarke, 2007). The bacteria proliferate and produce a wide array of chemicals, including toxins, enzymes, and antimicrobials, which kill the host insect and break it down into available nutrients, as well as prevent the growth of competitive fungi and bacteria (Maxwell et al., 1994; Webster et al., 2002; Brown et al., 2004; 2006a).

If conditions are good, the IJs receive a recovery signal, feed on the bacteria and available nutrients, and develop to adulthood before mating and producing the next generation of offspring (Poinar, 1979). *Heterorhabditis* are self-fertile in the first generation and hence require only one IJ to enter a host, whereas *Steinernema* are amphimictic for all generations. Several generations are produced within a host; as nutrients are depleted and the host becomes crowded, the next generation of nematodes

switch to Infective IJ development and leave the cadaver in search of a new host insect (Poinar, 1990; Kaplan et al., 2012).

Despite sharing a similar host range, and co-occurring geographically, there have only been a few documented cases of nematodes from the two genera co-infecting the same individual (Alatorre-Rosas and Kaya, 1990; Hatting et al., 2009; Kanga et al., 2012; Campos-Herrera et al., 2015). Previous studies have shown that when competing for a host one genus will outcompete the other, with the outcome depending on the combination of species, time of infection and distance to host insect (Alatorre-Rosas and Kaya, 1991; Koppenhofer and Kaya, 1996b; Fu et al., 2020). However, it is not clear at what stage this outcome is determined.

The lack of successful co-infections of *Heterorhabditis* and *Steinernema* could be due to (1) IJs of the *Heterorhabditis* and *Steinernema* genera not being attracted to hosts occupied by members of the other genus, (2) the IJs deeming the host unsuitable after contact and not invading the infected hosts or (3) an inability to recover or survive in a host already occupied by a population of the other genus due to proliferation of the initial colonisers' symbiotic bacteria.

IJs use several signals, including carbon dioxide, faecal matter, and host specific chemicals to find host insects at a distance (Lewis et al., 1996; Grewal et al., 1997; Bargmann, 2006; Lewis et al., 2006; Dillman et al., 2012). They can detect the presence of heterospecific and conspecific EPN infections in the insects at this stage, which can alter their behaviour (Gaugler, 1997; Glazer, 1997; Grewal et al., 1997; Baiocchi et al., 2017; Maher et al., 2021). Then, when in the vicinity of the insect, they

use chemical signals to recognise whether that host is suitable for development before invasion occurs (Lewis et al., 2006).

Photorhabdus and *Xenorhabdus* produce nematicidal proteins and toxins that can kill competing nematodes or prevent their development (Hu et al., 1999; Sicard et al., 2006; Orozco et al., 2016). The two genera of bacteria also compete through the production of antibiotics and bacteriocins (Akhurst, 1982; Thaler et al., 1997; Clarke, 2008; Thappeta et al., 2020). *Heterorhabditis* are dependent on *Photorhabdus* for nutrition and their development is hindered if the bacteria are not present in a host (Strauch and Ehlers, 1998; Aumann and Ehlers, 2001). *Steinernema* IJs can survive without *Xenorhabdus*, but their recovery, reproduction and infectivity are reduced (Poinar and Thomas, 1966; Dunphy and Webber, 1985; Han and Ehlers, 2000; Sicard, 2006; Hirao & Ehlers, 2009). Competition between *Photorhabdus* and *Xenorhabdus* in a host could hinder the development of *Steinernema* and *Heterorhabditis*.

This study proposes the following hypotheses: (1) that a species of *Steinernema* will not succeed in developing and reproducing in an insect host that is already infected with a *Heterorhabditis* species, and vice versa, (2) that the probability of success of the second species will decline the longer the first species has been established in the insect and (3) that IJs of the second species will show adaptive behaviour, and either not be attracted to or not invade insects in which they have a low probability of developing and reproducing in due to the presence of a competing species.

To investigate these hypotheses, we tested (1) the movement of IJs on agar plates in response to larvae infected with the other genus of EPN 72 hrs prior and (2) the invasion rate and development of IJs in larvae infected with the other genus of EPN

24, 48 or 72 hrs prior through dissection of the cadaver. The nematodes (and symbionts) used for this study were *H. downesi (P. temperata* symbiont*), H. bacteriophora (P. thracensis* symbiont), *S. carpocapsae* (*X. nematophila* symbiont) and *S. feltiae* (*X. bovienii* symbiont).

3.2 Methods

3.2.1 Species and storage

Galleria mellonella L. larvae (late instar) were supplied by Peregrine Live Foods (Chipping Ongar, UK) and Live Foods Direct (North Anston, UK).

Heterorhabditis downesi Stock, Griffin & Burnell (K122), *Heterorhabditis bacteriophora* Poinar (VM21), *Steinernema feltiae* Filipjev (4cfmo) and *Steinernema carpocapsae* Weiser (All) cultures were maintained through *G. mellonella* larvae (Kaya & Stock, 1997). Infective juveniles (IJs) were stored in aqueous suspension at 9 °C. As controls, *G. mellonella* were frozen for 24 hrs and thawed 2 hrs prior to use in experiments.

3.2.2 The attractiveness of *Heterorhabditis* and *Steinernema*-infected hosts for IJs of the other genus

The movement of IJs towards or away from EPN-infected larvae was tested in an agar plate assay. *G. mellonella* larvae were infected by exposure to 100 IJs 72 hrs prior to use in the assay.

The assay was conducted in 5.5 cm Petri dishes (5 mls of 2 % w/v bacteriological agar) with lids modified with two Eppendorf tubes, one of which contained an insect and the other of which was empty. The bottom 3 mm of the 1.5 ml tubes was removed, providing a 4 mm aperture, and the tubes were inserted into 0.5 cm diameter holes in the lid, 2 cm either side of the centre, and secured with glue (Fig. 3.1A). To start the assay, 50 IJs of the test species were applied in 10 µl to the centre of the plate and the water was allowed to evaporate for 5 minutes. After 2-3 hrs the number of IJs in each section of the dish was recorded as: Middle (within 0.5 cm of the central line of the dish), Insect side (> 0.5 cm from the central line towards the insect), Non-insect side (> 0.5 cm from the central line towards the empty Eppendorf) (Fig. 3.1B). The number of IJs in the Insect side was then analysed as a proportion of all those that moved beyond the Middle section. For each test species there were 4 insect treatments. For *Steinernema* IJs (*S. carpocapsae* or *S. feltiae*), the treatments were either a single live (Live), freeze-killed (FK), *H. downesi*-infected, or *H. bacteriophora*-infected larva placed in one Eppendorf while the other was left empty. For *Heterorhabditis* IJs (*H. downesi* and *H. bacteriophora*), there were Live, FK, *S. carpocapsae*-infected or *S. feltiae*-infected larvae. A further treatment with no larvae (None) in either Eppendorf was also included in each experiment as a control for directional bias.

The experiment took place twice for each of the test species (IJs) with a total of 12-16 plates per treatment, per test species.

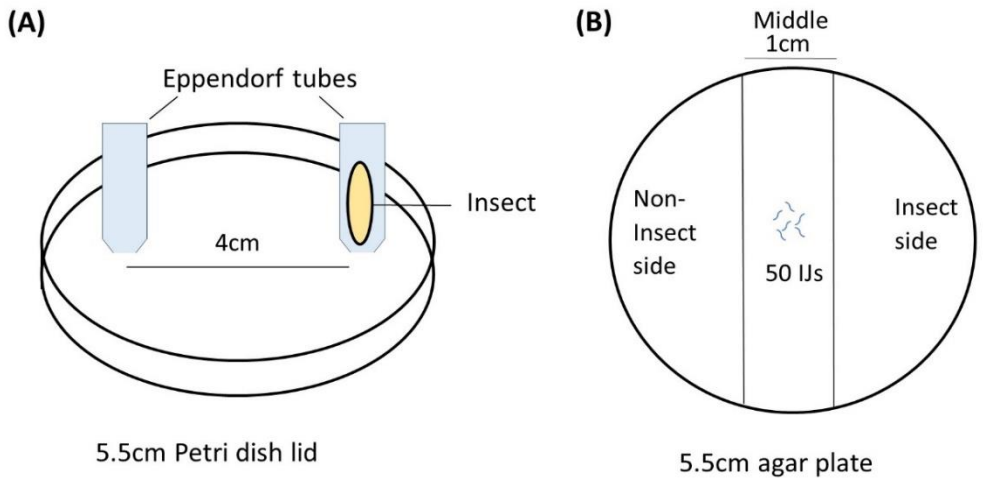

Figure 3.1. Attraction assay on agar plates **(A)** Petri dish lid (5.5 cm) modified to allow gas exchange between the dish and 1.5ml Eppendorfs containing larvae of various treatments. **(B)** Application and scoring of IJs for attraction assay.

3.2.3 Co-infections of *Heterorhabditis* and *Steinernema* in a single host

In this experiment, insects previously infected by one genus of EPN (primary infection) were exposed to IJs (termed "invaders") of the other genus of EPN and the success of each species in the co-infection was assessed by dissection of the insect. To set up the primary *Heterorhabditis* infections, 10 *H. bacteriophora* or *H. downesi* IJs were applied in 10 µl drops directly onto individual *G. mellonella* larvae and incubated at 20°C in 24 well plates. Each well contained a single larva and two 1.5 cm filter papers dampened with 50 µl of sterile tap water. IJs were removed 24 hrs after application by washing the insects with sterile tap water. The insects were dried and placed in fresh wells. After 24, 48 or 72 hrs since primary infection (hpi), one-third of the *H. bacteriophora* (HB) and *H. downesi* (HD) exposed larvae, along with uninfected living (Live) and freeze-killed (FK) larvae as controls were transferred to fresh 24-well plates with 1.5cm filter papers and 100 IJs of either *S. carpocapsae* or *S. feltiae,* in 100 µl of water, were applied to each larva. Plates were covered with vented

parafilm and tinfoil and incubated at 20°C. The time of death of each larva was recorded daily. The larval cadavers were dissected in phosphate buffered saline (PBS) 72 hrs after application of the *Steinernema* IJs. Nematode species were identified based on tail shape, tail length relative to body size, and relative spicule size (Adams and Nguyen, 2002; Himani et al., 2021). For each nematode species the following were recorded: total number of individuals present, their developmental stage and whether they were alive or dead. Larvae for which the primary infection failed were excluded from analysis (104/468; 22%).

The experiment was repeated using *H. downesi/ H. bacteriophora* IJs applied as invaders to *S. carpocapsae* (SC) and *S. feltiae* (SF) infected larvae.

For the both the Live and FK conditions, larvae from the 3 timepoints (24, 48 and 72 hrs) were combined for analysis. Thus, for each invading species of *Heterorhabditis* there were 8 host treatments: Live, FK, SC (24, 48 and 72 hpi) and SF (24, 48, 72 hpi). Similarly, there were 8 treatments for each *Steinernema* invading species: Control, FK, HD (24, 48 and 72 hpi) and HB (24, 48 and 72 hpi).

There were 2-3 repeats of the experiment for each of the four invading species, with a minimum total of 7 larvae analysed per treatment (Appendix Table 3.1).

3.2.4 Data analysis

Statistical analyses were performed in R programming environment (R Core Team, 2022) or Minitab 20.3 statistical software (Minitab, LLC, 2021)

Attraction assay: For each species (*H. bacteriophora, H. downesi, S. feltiae* and *S. carpocapsae*) attraction of IJs within each treatment was analysed using One-sample t-Test, by comparing the proportion of IJs that moved towards the "Insect Side" with a hypothesised proportion of 0.5, assuming no attraction.

Co-infection assay: For *S. carpocapsae* and *S. feltiae* invaders, the proportion of IJs that were found to have invaded larvae (Invasion) (transformed by square root), and the proportion that developed to live adults (LiveAdult) were analysed separately using generalized linear models (GLMs) with Treatment (Live, FK, HD 24, HD 48, HD 72, HB 24, HB 48, and HB 72) as the explanatory variable. For each test species an analysis of variance (ANOVA) was performed followed by Dunn's post hoc analysis ($P < 0.05$ adjusted with Bonferroni correction for multiple comparisons)

A subset consisting of only the HB and HD treatments was further analysed by GLMs to explain the variation in Invasion of *Steinernema* IJs, using the species of the initial *Heterorhabditis* coloniser (Het species), the number of *Heterorhabditis* present (HetTotal), how long they were established in the host (Timepoint of infection; 24, 48 or 72 hpi) and state of the larvae at time of invasion (State of host; Living = 0, Dead = 1) as the explanatory variables.

Similar analyses were carried out for *H. bacteriophora* and *H. downesi* invaders using the variables Treatment (Live, FK, SC 24, SC 48, SC 72, SF 24, SF 48, and SF 72) for the full dataset, and species of initial *Steinernema* coloniser (Stein species), the number of *Steinernema* present (SteinTotal), Timepoint of infection (24, 48 or 72 hpi) and State of host (Living = 0, Dead = 1) for the restricted dataset (SC and SF infected hosts only).

The quality of fit of the models was evaluated using the package "DHARMa" in R (Hartig, 2022).

3.3 Results

3.3.1 The attractiveness of *Heterorhabditis* and *Steinernema*-infected hosts for IJs of the other genus

In the attraction assays, if the IJs were not attracted to the host larvae we would expect only half the population to move towards the bait side, as seen in the "None" treatments (Fig. 3.2). In our experiments IJs were not deterred from approaching cadavers already infected with EPN of the other genus and, in some cases, were attracted to them.

S. carpocapsae IJs were attracted to Live, FK and *H. bacteriophora*-infected larvae but not *H. downesi*-infected larvae (Fig. 3.2A). *S. feltiae* IJs were only significantly attracted to *H. bacteriophora*-infected larvae (Fig. 3.2B). *H. bacteriophora* IJs were attracted to Live, FK, *S. feltiae*-infected and *S. carpocapsae*-infected larvae (Fig. 3.2C). *H. downesi* IJs were attracted to larvae infected with *S. feltiae* or *S. carpocapsae* but not to Live or FK larvae (Fig. 3.2D).

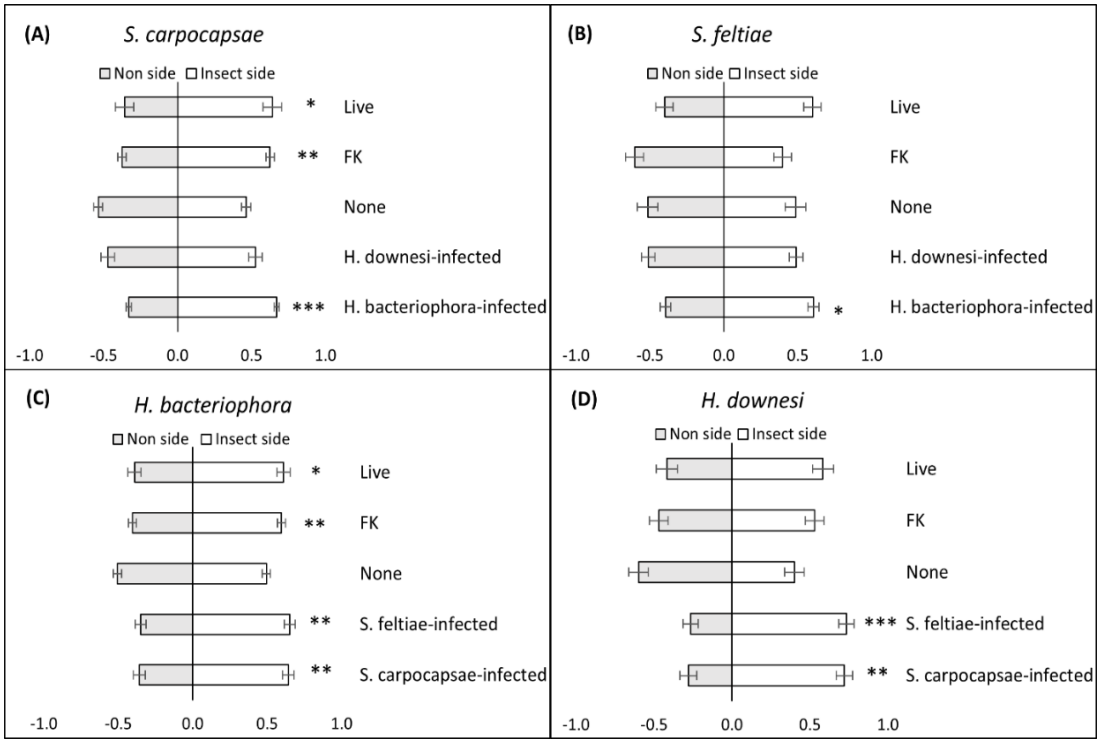

Figure 3.2. The proportion (Mean ± SE) of **(A)** *S. carpocapsae*, **(B)** *S. feltiae*, **(C)** *H. bacteriophora* and **(D)** *H. downesi* IJs (of those that moved from the middle zone) that moved towards or away from the larvae in each treatment. Bars marked with one or more * are significantly different from a hypothesised mean of 0.5 (One sample t-Test: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$)

3.3.2 Co-infections of *Heterorhabditis* and *Steinernema* in a single host

3.3.2.1 Invasion into EPN-infected cadavers

For *S. carpocapsae,* treatment was a significant factor affecting the proportion of IJs that invaded a larva (ANOVA: $F_{7, 242} = 9.44$, $P < 0.001$). A lower proportion of *S. carpocapsae* IJs invaded FK larvae and HD-infected larvae, but not HB-infected larvae, compared to the Live treatment (Fig. 3.3A).

For the GLM investigating invasion of *S. carpocapsae* into only the *Heterorhabditis*-infected larvae, the species of *Heterorhabditis* occupying the larvae was a significant

predictor of the proportion of *S. carpocapsae* IJs that invaded (Table 3.1). There was a trend for lower invasion into HD-infected larvae compared to HB-infected larvae (Fig. 3.3A). The number of *Heterorhabditis* nematodes in the larvae, how long they had been established, or whether the larvae were living or dead at time of invasion had no effect on the number of IJs that invaded (Table 3.1).

For *S. feltiae*, treatment was a significant factor affecting the proportion of IJs that invaded a larvae (ANOVA: $F_{7, 224} = 16.00$, $P < 0.001$). A lower proportion of S. *feltiae* IJs invaded FK and HD-infected larvae compared to the Live treatment (Fig. 3.3B). There was also a lower proportion of IJs invading larvae infected with *H. bacteriophora* 72 hpi compared to the Live treatment, but not 24 or 48 hpi.

For the GLM comparing invasion of *S. feltiae* IJs into *Heterorhabditis*-infected larvae, the proportion of IJs that invaded was lower if the larvae were dead at the time of application (Table 3.1). Neither the species nor number of *Heterorhabditis* nematodes in the larvae, were factors affecting the proportion of IJs that invaded (Table 3.1). There was trend for lower invasion into larvae that were infected with *Heterorhabditis* for longer (Fig. 3.3B).

For *H. bacteriophora*, treatment was a significant factor affecting the proportion of IJs that invaded a larvae (ANOVA: $F_{7, 203} = 5.20$, $P < 0.001$). A lower proportion of *H. bacteriophora* IJs invaded larvae infected with *S. feltiae* 48 hpi, compared to the Live treatment (Fig. 3.3C). There was a trend for lower invasion into larvae infected with *S. feltiae* 24 hpi and 72 hpi, as well as *S. carpocapsae*-infected larvae, compared to larvae in the Live treatment, but these differences were not significant. There was no

difference in the proportion of IJs invading larvae in the FK treatment compared to Live (Fig. 3.3C).

For the GLM comparing the invasion of *H. bacteriophora* IJs into *Steinernema*-infected larvae, neither the species of *Steinernema* occupying the larvae, how many *Steinernema* nematodes were in the larvae, how long they had been established nor whether the larvae was living or dead at time of invasion, were significant factors affecting the proportion of IJs that invaded the larvae (Table 3.1).

For *H. downesi*, treatment was not a significant factor affecting the proportion of IJs that invaded a larvae (ANOVA: $F_{7, 147} = 1.26$, $P = 0.276$). There was a trend for increased invasion of *H. downesi* IJs into *Steinernema*-infected larvae compared to Live larvae (Fig. 3.3D).

For the GLM comparing the invasion of *H. downesi* IJs into *Steinernema*-infected larvae, the number of *Steinernema* nematodes established in the larvae was a significant factor affecting the proportion IJs that invaded (Table 3.1), with a higher proportion of *H. downesi* IJs invading larvae that had higher numbers of *Steinernema* nematodes established in the host (Fig. 3.4). The species of *Steinernema* occupying the larvae, how long they had been established or whether the larvae was living or dead at time of invasion were not significant factors for invasion of *H. downesi* IJs (Table 3.1).

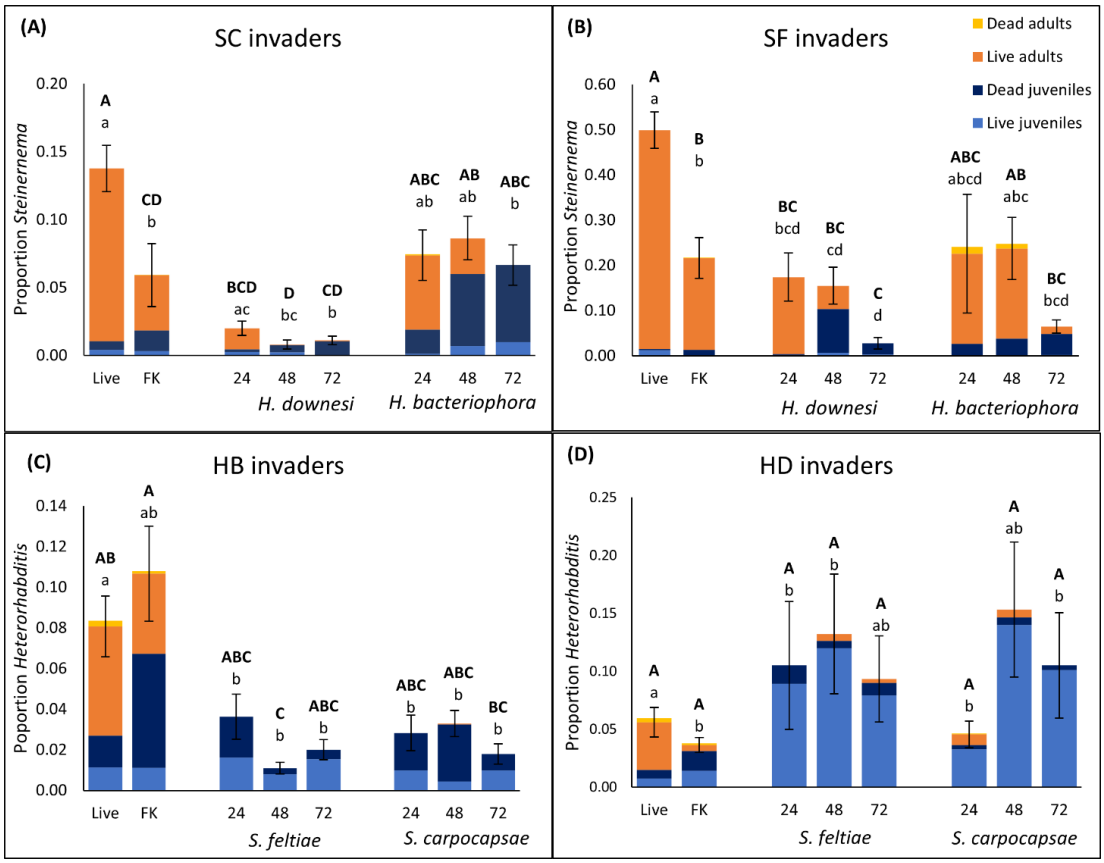

Figure 3.3. The proportion (Mean ± SE) of **(A)** *S. carpocapsae*, **(B)** *S. feltiae*, **(C)** *H. bacteriophora* and **(D)** *H. downesi* IJs, of 100 applied, that invaded *G. mellonella* larvae that were live, freeze-killed (FK) or infected with nematodes of the other genus 24, 48 or 72 hrs prior. Development and mortality of the IJs is indicated by coloration of the bars. Bars with the same letter within a panel are not significantly different ((Capital letters) Proportion of IJs that invaded, (Lower case letters) Proportion of live adults; Dunn's post hoc with Bonferroni correction, $P < 0.05$).

3.3.2.2 Development in EPN-infected cadavers

For *S. carpocapsae*, treatment was a significant factor affecting the proportion of live adults in larvae at time of dissection (ANOVA: $F_{7, 242} = 5.42$, $P < 0.001$). There were fewer live adult *S. carpocapsae* in FK larvae, 48 hpi and 72 hpi HD-infected larvae, and 72 hpi HB-infected larvae compared to the Live treatment. The proportion of *S. carpocapsae* developing to adult in *H. downesi*-infected cadavers was lower the longer the primary infection had been established (Fig. 3.3A). A similar trend was observed for *H. bacteriophora*-infected larvae; however, the differences were not significant.

Many of the *S. carpocapsae* IJs that remained as juveniles in *Heterorhabditis*-infected larvae were dead at the time of dissection (Fig. 3.3A).

For *S. feltiae,* treatment was a significant factor affecting the proportion of live adults in larvae at time of dissection (ANOVA: $F_{7, 224} = 11.11$, $P < 0.001$). There were fewer live adult *S. feltiae* in FK larvae, HD-infected larvae, and 72 hpi HB-infected larvae, compared to the Live treatment (Fig. 3.3B). There was a trend for a lower number of adult *S. feltiae* in *Heterorhabditis*-infected cadavers the longer the primary infection had been established, but these differences were not significant (Fig. 3.3B). Like *S. carpocapsae,* the majority of *S. feltiae* IJs that remained as juveniles in *Heterorhabditis*-infected larvae were dead at the time of dissection (Fig. 3.3A, B).

For *H. bacteriophora,* treatment was a significant factor affecting the proportion of live adults in larvae at time of dissection (ANOVA: $F_{7, 203} = 3.27$, $P = 0.003$). There were fewer live adult *H. bacteriophora* in SF-infected and SC-infected larvae, regardless of time since infection, compared to the Live treatment (Fig. 3.3C). The majority of *H. bacteriophora* IJs that invaded the *Steinernema*-infected larvae remained as juveniles at the time of dissection (Fig. 3.3C).

For *H. downesi,* treatment was a significant factor affecting the proportion of live adults in larvae at time of dissection (ANOVA: $F_{7,147} = 2.86$, $P = 0.008$). There were fewer live adult *H. downesi* in FK larvae, 24 hpi and 48 hpi SF-infected larvae, and 24 hpi and 72 hpi SC-infected larvae, compared to the Live treatment (Fig. 3.3D). The *H. downesi* IJs that invaded the *Steinernema*-infected insects mostly remained as juveniles. The majority of those *H. downesi* juveniles were still alive at the time of dissection (Fig. 3.3D).

Table 3.1. Results of models (GLMs) for each species, testing the proportion of IJs that invaded larvae infected with the other genus, using the species of the initial coloniser (Het species : *H. bacteriophora* or *H. downesi*/ Stein species: *S. carpocapsae* or *S. feltiae*), the number of initial colonisers present (HetTotal/ SteinTotal), how long they were established in the host (Timepoint of infection; 24, 48 or 72 hrs) and the state of the host larvae at time of invasion (State of Host: Living/Dead) as the explanatory variables. Significant variables highlighted in bold.

		Proportion invaded	
Invader	**Factors**	**T value**	**Pr(>|t|)**
S. carpocapsae	**Het species**	-7.602	**< 0.001**
	Timepoint of infection	0.151	0.880
	State of Host	-1.134	0.260
	HetTotal	0.116	0.908
S. feltiae	Het species	-1.306	0.195
	Timepoint of infection	-0.066	0.948
	State of Host	-2.928	**0.004**
	HetTotal	0.292	0.771
H. bacteriophora	Stein species	-0.892	0.374
	Timepoint of infection	-1.901	0.060
	State of Host	-1.537	0.127
	SteinTotal	1.346	0.181
H. downesi	Stein species	-0.432	0.667
	Timepoint of infection	0.791	0.432
	State of Host	-0.570	0.571
	SteinTotal	2.024	**0.047**

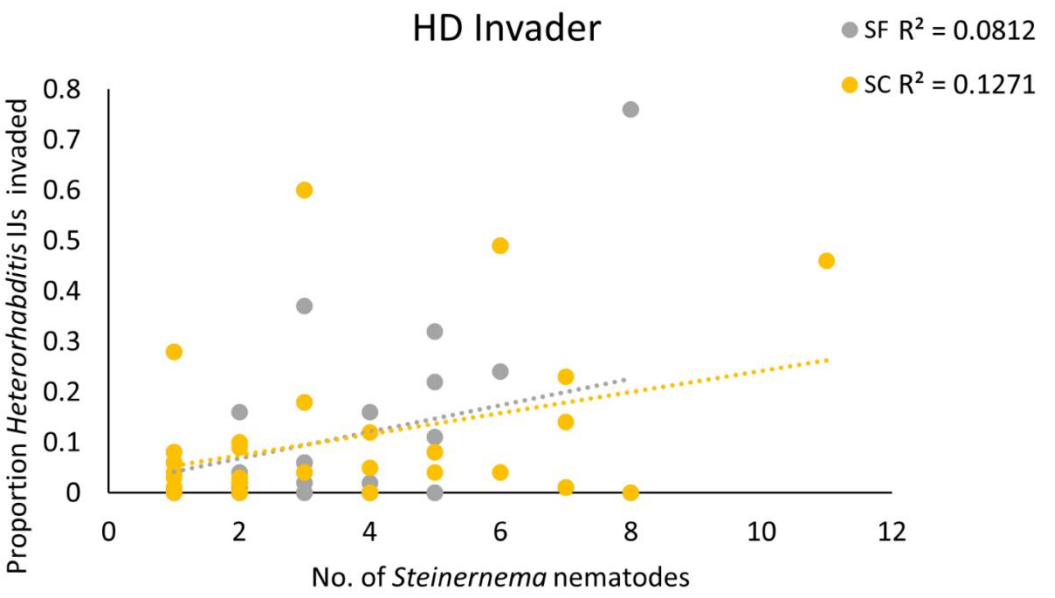

Figure 3.4. Regression for the proportion of *H. downesi* IJs, of 100 applied, that invaded *G. mellonella* larvae harbouring varying numbers of *S. feltiae* (SF) or *S. carpocapsae* (SC) nematodes.

3.4 Discussion

A host can be defined as "suitable" for a parasite if the parasite can (1) successfully infect the host and (2) produce fertile offspring that can develop within the host (Salt, 1938). In the experiments described here, *Steinernema* and *Heterorhabditis* killed healthy, uninfected *G. mellonella* larvae within 72 hrs of exposure. The proportion of EPN invaders that developed to live adults in each larva by 72 hrs is an indicator of the suitability of the host in these experiments. Invasion into an unsuitable host is generally believed to be a "dead end" for EPN as they are unlikely to leave the host after entry. It is therefore an advantage to EPN to be able to determine if a host is suitable for infection before invading. In these experiments invasion of the IJs into larvae infected with EPN of the other genus varied depending on the species of the

invader, the species occupying the host and the stage of the infection but was generally lower than invasion into uninfected larvae.

Steinernema IJs were attracted to *H. bacteriophora*-infected larvae but not *H. downesi*-infected larvae. This was also reflected by the reduced invasion rate of both *Steinernema* species into larvae infected with *H. downesi* as early as 24 hrs prior. While both *Heterorhabditis* species carry *Photorhabdus,* differences in the metabolites produced by the two species, *P. thracensis* and *P. temperata,* could affect the attractiveness of the host (Maher et al., 2021). It is unclear whether the *Steinernema* IJs are "eavesdropping" on a signal produced by *H. downesi/ P. temperata* or whether the *H. downesi/ P. temperata* are masking signals produced by *G. mellonella* that make it attractive to IJs. The number of live, adult *Steinernema* in larvae infected with *H. downesi* for 48 hrs or 72 hrs, was lower than for live larvae. In liquid cultures, recovery and development of both *S. carpocapsae* and *S. feltiae* to adult is slower when cell density of *Xenorhabdus* is low (Hirao and Ehlers, 2009). By 48 hrs *Photorhabdus* would have proliferated in *Heterorhabditis*-infected cadavers, potentially preventing the proliferation of *Xenorhabdus* and slowing the development of the *Steinernema* nematodes. The ability of *Steinernema* IJs to perceive these signals and adapt their behaviour would be beneficial to both the *Steinernema* IJs and the *H. downesi* nematodes in the host as it prevents *Steinernema* IJs from invading a host where they are less likely to develop, and for *H. downesi,* it avoids increasing competition for resources.

In this experiment, *Steinernema* were attracted to larvae infected for 72 hrs with *H. bacteriophora*. Previous findings of *S. carpocapsae* IJs being deterred from larvae infected with *H. bacteriophora* (HP88 strain) for 6 hrs (Grewal et al., 1997), indicate

that time of infection may play an important role in attraction (Baiocchi et al., 2017). In the co-infection assays, invasion of *Steinernema* IJs into larvae infected with *H. bacteriophora* for 72 hrs was not beneficial to either *Steinernema* species as the majority of these nematodes died as juveniles. The production of nematicidal toxins by the *H. bacteriophora/ Photorhabdus* complex could have led to the death of the *Steinernema* juveniles before they developed to adult (Hu et al., 1999; Orozco et al., 2016).

While *S. carpocapsae* continued to invade *H. bacteriophora*-infected larvae regardless of the stage of infection, there was a tendency for fewer *S. feltiae* to invade larvae that had been infected with *H. bacteriophora* for 72 hrs. This indicates that *S. feltiae* IJs may be more sensitive to signs of unsuitability of the host for invasion compared to *S. carpocapsae* IJs. Fewer *S. feltiae* IJs, but not *S. carpocapsae* IJs, invaded dead hosts compared to live hosts. The low invasion rate of *S. feltiae* into the larvae infected with *Heterorhabditis* for 72 hrs may have been due to the death of the host at this time. When a host larva dies, it no longer produces some of the signals that are attractive to IJs e.g. Carbon dioxide, which may affect their invasion rate. Alternatively, changes in the composition and quantity of metabolites produced by *Photorhabdus* at this stage may act as deterrents for invasion.

H. bacteriophora IJs were attracted towards *S. feltiae* and *S. carpocapsae*-infected larvae. This is in keeping with previous findings of *H. bacteriophora*'s attraction to *S. carpocapsae*-infected cadavers (Grunseich et al., 2021). *H. bacteriophora* IJs invaded all *Steinernema*-infected larvae, except 48 hr *S. feltiae* infections, at the same level as healthy living larvae. However, the number of *H. bacteriophora* developing to live adults in larvae infected with *S. feltiae* or *S. carpocapsae* at all timepoints was low,

indicating unsuitable conditions; with many of the IJs dying as juveniles. For *Heterorhabditis* IJs recovery from the juvenile stage is aided by the presence of a food signal produced by *Photorhabdus* (Poinar and Thomas 1966; Strauch & Ehlers, 1998; Han and Ehlers, 1998, 2002). Therefore, the lack of development of *H. bacteriophora* IJs in larvae infected with *Steinernema* nematodes is likely due to a lack of *Photorhabdus* bacteria, as evidenced also by a lack of yellow/orange pigmentation and no bioluminescence of these cadavers (data not shown) (Poinar et al., 1980; Richardson et al., 1988; Li et al., 1995; Maher et al., 2021; Chapter 4). Death of the juveniles could be a result of starvation, as *Heterorhabditis* are reliant on *Photorhabdus* for nutrition while in the host (Han and Ehlers, 2000). *Xenorhabdus* bacteria also produce nematicidal proteins that are effective against free living nematodes (Abebew et al., 2022). These nematicidal proteins may be lethal to *H. bacteriophora* IJs as well.

H. downesi IJs were attracted towards and invaded *Steinernema*-infected larvae, regardless of stage of infection, at the same level as healthy live larvae and freeze-killed larvae. Invasion of *H. downesi* IJs increased with increasing number of *Steinernema* nematodes already established. IJs can be attracted to larvae infected with heterospecific EPN and it has been suggested this is a means of overcoming the host's immune system (Grewal et al., 1997; Grunseich, et al., 2021; See section 1.3.4). However, the lack of development to adult indicates that *Steinernema*-infected larvae are unsuitable hosts for *H. downesi* and that there is no advantage in co-infecting. These larvae may have been particularly attractive for unrelated reasons, hence why more of the initial S*teinernema* invaders colonised them.

The majority of *H. downesi* IJs that invaded *Steinernema*-infected larvae remained in the juvenile state but were still live and active. It is possible that the lack of a suitable environment, including a lack of available nutrients, ensured the IJs did not begin recovery (Nguyen and Smart, 1994). Remaining as non-feeding IJs may protect the EPN in unsuitable hosts, with the possibility of later emerging in search of a more suitable host. However, invasion is energy intensive and IJs have a limited lipid supply so starvation may occur if the IJs do not recover and feed in the host they have invaded (Patel et al., 1997b; Fitters and Griffin, 2006). In the case of *H. downesi* in *Steinernema*-infected larvae, further studies on the outcome of these live juveniles would be required to determine if this is an effective strategy for survival, an inability to develop, or a delay in development. To this end, a set of larvae from each set of infections were not dissected but were incubated at 20° C for 40 days to allow the nematodes to continue development and reproduction. The IJs that emerged from these larvae were collected and their DNA was extracted and stored at – 4° C. Future analysis will show if any of the secondary invaders in the co-infected larvae were ultimately successful in producing progeny.

Previous studies have shown that virulence and reproduction of IJs is reduced in freeze-killed hosts (San Blas and Gowen, 2008; Blanco-Pérez et al., 2017; Blanco-Pérez et al., 2019). Once the larvae are killed by freezing, saprophytic bacteria and fungi can develop in the host unchallenged, which could later impede the proliferation of *Xenorhabdus* and *Photorhabdus*. For both *Steinernema* species, invasion into freeze-killed larvae was lower than for healthy living insects, indicating that the IJs recognise the host as potentially unsuitable. The majority of *Steinernema* IJs that did invade freeze-killed larvae developed to living adults. In contrast, *H. downesi* invaded

freeze-killed larvae at a similar rate to healthy living insects but produced fewer live adults. Similarly, *H. bacteriophora* IJs were not deterred from invading freeze-killed larvae despite a large proportion of the invaders dying as juveniles. The differences in the ability of *Heterorhabditis* and *Steinernema* to develop in freeze-killed hosts is likely due to the differential reliance on their symbionts for recovery and development as *Steinernema* juveniles can develop without the presence of *Xenorhabdus* whereas *Heterorhabditis* juveniles are dependent on *Photorhabdus* for nutrition.

The use of EPNs as effective biocontrols depends on their ability to reproduce within a host and persist in the environment. Our studies show that dual applications of different species of EPN can lead to redundancy, as the secondary invader is less likely to develop and reproduce. *S. feltiae* IJs showed the greatest avoidance of unsuitable hosts, highlighting their potential advantage over other species in field applications.

3.5 Conclusion

This study shows that IJs of both EPN genera, *Heterorhabditis* and *Steinernema,* were attracted to hosts occupied by the other genus of EPN for 72 hrs, with the exception of *H. downesi* occupied hosts. In general, all the EPN species had reduced success in developing to adult in larvae that were infected with the other genus of EPN for 72 hrs. *Steinernema* IJs were less likely to develop to adult the longer that *Heterorhabditis* nematodes had been established in the host, whereas *Heterorhabditis* IJs were unlikely to develop to adult in hosts infected with *Steinernema* regardless of time since infection.

Heterorhabditis IJs appeared less capable of determining the suitability of a host, and invaded insect hosts in which they did not develop, whereas both *S. feltiae* and *S. carpocapsae* IJs showed some selectivity when invading hosts. *Steinernema* IJs avoided *H. downesi*-infected cadavers in which they could not develop, but not *H. bacteriophora*-infected cadavers, indicating *H. downesi* or its symbiont *P. temperata,* may produce chemical signals to deter competing nematodes from invading.

Chapter 4. Bioluminescence of nematode symbiont *Photorhabdus* protects nematode-infected host cadavers from nocturnal scavenging.

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Christine T. Griffin: Conceptualization; Supervision; Writing - review & editing.

4.0 Summary

Photorhabdus spp. are the only known terrestrial bioluminescent bacteria. In this chapter we show that the bioluminescence produced by these bacteria reduces scavenging activity on the insect cadavers they colonize. *Photorhabdus* spp. are the symbiont of the insect pathogenic nematodes *Heterorhabditis* spp. Together they kill insects and colonize the cadaver. The function of their bioluminescence has been the subject of debate, but here for the first time we demonstrate an ecological benefit. In our experiments, fewer *Photorhabdus* temperata-infected cadavers than uninfected cadavers were scavenged, but only in dark conditions where their bioluminescence would be visible. This was the case both in the field and in laboratory experiments with *Lehmannia valentiana* slugs (the primary scavengers found in our field tests). We also show that *L. valentiana* is innately deterred from scavenging on uninfected cadavers in proximity to light imitating the bioluminescence of *Photorhabdus*, indicating that luminescence can be a deterrent independent of chemical cues. We propose a multimodal defence where bioluminescence works together with the chemical defences also produced by *Photorhabdus* to deter scavengers, such as slugs, from feeding on the host cadaver, with the potential for aposematism.

4.1 Introduction

Bioluminescence is the biochemical production and emission of light by a living organism (Widder, 1999), usually by the oxidation of a light-emitting molecule -a luciferin - by a luciferase enzyme (Haddock et al., 2010). Bioluminescence has evolved many times (Haddock et al., 2010; Lau and Oakley, 2021) in prokaryotes and eukaryotes including bacteria, fungi, dinoflagellates, arthropods, and fish (Herring, 1994; Forey and Patterson 2006; Dunlap and Kita-Tsukamoto, 2006; Baker et al., 2008). The original function of luciferin-luciferase systems may have been for detoxification of reactive oxygen species (McElroy and Seliger, 1962; Seliger, 1975; Rees et al., 1998; Labas et al., 2001), but bioluminescence now serves diverse ecological functions including defence, attraction of prey, and intraspecific communication (Herring, 1989; Buck and Case, 2002; Lewis and Cratsley, 2008). Distraction or deterrence of nocturnal predators and scavengers has been proposed as the function of bioluminescence in Dinoflagellata, Copepoda, Cnidaria and Coleoptera (Morin, 1983; Buskey and Swift, 1985; Latz et al., 1988; Branham and Wenzel, 2001; Herring and Widder, 2004).

In bacteria, while the mechanism of luminescence is well characterised (Meighen and Szittner, 1992; Forst and Nealson, 1996; Zavilgelsky and Shakulov, 2018), its functions are less well understood than in higher organisms (Nealson and Hastings, 1979; Timsit et al., 2021). Amongst the biochemical benefits that have been suggested are the detoxification of reactive oxygen species (Timmins et al., 2001) or the promotion of DNA repair (Czyz et al., 2000), but the likely importance of ecological functions involving perception by some other organisms is increasingly acknowledged (Nealson and Hastings, 1979; Zavilgelsky and Shakulov, 2018; Timsit et al., 2021).

Many marine species of bioluminescent bacteria reside in specialised light organs of higher organisms (fish and squid) where they clearly provide light in return for nutrients from their host, whilst other marine species are postulated to use light to attract consumers that provide a medium for growth and a means of dispersal (Nealson and Hastings, 1979; Widder, 2010; Tanet et al., 2020, Ramesh and Bessho-Uehara, 2021). *Photorhabdus* spp. are the only known terrestrial bioluminescent bacteria; the function of the bioluminescence in this genus is debated (Peat and Adams, 2008; Waterfield et al., 2009; Zavilgelsky and Shakulov, 2018). *Photorhabdus* spp. are insect pathogens that occur as obligate symbionts of the *Heterorhabditis* spp. of nematodes. (Boemare, 2002; Clarke, 2008; Waterfield et al., 2009). The stress resistant, free-living infective juvenile stage of the nematode travels through soil and enters a living insect, releasing the bacteria from its gut. When the bacteria have grown to a certain density, they emit light at a wavelength of around 490 nm (Poinar et al., 1980; Maher et al., 2021). Pigmentation of the cadaver can shift the light emitted by 20-30 nm depending on the species of *Photorhabdus* and its characteristic colouration (Maher et al., 2021). Luminescence can start as early as 20 hours post infection and is visible to the human eye by 72 hrs (Poinar et al., 1980), but only in darkness. Buried in the soil, and/or at night, these luminescent insect cadavers would stand out as a source of light. Within 2-3 days of infection the bacteria help to kill the insect and enzymatically break down the cadaver, resulting in a bacteria-rich nutrient soup on which the nematodes feed (Waterfield et al., 2009; Dillman and Sternberg, 2012). The nematodes then develop to adulthood and reproduce within the host. Several generations may be produced over 2-3 weeks, until resources are depleted, after which the next generation of infective juveniles with their symbiont leave the cadaver in search of new hosts (Adams and

Nguyen, 2002). Only the infective juvenile stage is capable of survival in soil conditions (Poinar, 1979).

Functions that have been ascribed to bioluminescence in *Photorhabdus* can be divided into general biochemical functions, roles specialised to its ecological niche, and redundancy (Poinar et al., 1980; Akhurst and Boemare, 1990; Peat and Adams, 2008; Waterfield et al., 2009; Peat et al., 2010; Zavilgelsky and Shakulov, 2018; Timsit et al., 2021). The *lux* operon containing the genes responsible for bacterial bioluminescence had a single evolutionary origin and spread by horizontal gene transfer (Kasai et al., 2006); many of the common functions that are ascribed to the cellular processes that result in bacterial bioluminescence, including protection against free radicals and stimulation of DNA repair, have also been ascribed to *Photorhabdus* (Nealson and Hastings, 1979; Peat and Adams, 2008; Zavilgelsky and Shakulov, 2018). Amongst the adaptive functions related to *Photorhabdus*'s specialised niche that have been proposed are: signalling to the nematode to synchronise symbiosis (Waterfield et al., 2009); cell-to-cell communication with other bacteria (Timsit et al., 2021); attracting new insect hosts to the cadaver (Poinar et al., 1980; Patterson et al., 2015) or deterring scavengers from it (Akhurst and Boemare, 1990; Baur et al., 1998; Jones et al., 2015; Maher et al., 2021). It has also been suggested that since the acquisition by *Photorhabdus* of the *lux* operon from a marine bacterium, bioluminescence is a nonfunctional trait in the genus that is declining over time, due to lack of selection pressure in the terrestrial environment (Peat and Adams, 2008). The most widely hypothesised possible ecological function proposed for bioluminescence in *Photorhabdus* is deterring scavengers from damaging the host cadaver, but to date there is no evidence supporting this or any other proposed function

(Poinar et al., 1980; Akhurst and Boemare, 1990; Peat and Adams, 2008; Peat et al., 2010; Waterfield et al., 2009; Zavilgelsky and Shakulov, 2018; Timsit et al., 2021; Cimen, 2023).

There is ample evidence that *Photorhabdus* spp. invest heavily in defence of the cadaver against both microbes and animals (reviewed by Raja et al., 2021), protecting both themselves and their essential nematode mutualist. They produce a wide variety of molecules (Bode et al., 2009; Cimen et al., 2023), some of which have antimicrobial activity and suppress competing bacteria and fungi - including agents of putrefaction (Li et al., 1995; Eleftherianos et al., 2007; Ullah et al., 2015; Tobias et al., 2018). Insect cadavers infected by *Heterorhabditis* and their symbionts are also protected against feeding by a variety of scavengers including ants, wasps, crickets and beetles, and this is attributed to the production of an as yet unidentified chemical called "scavenger deterrent factor" by *Photorhabdus* (Baur et al., 1998; Zhou et al., 2002; Gulcu et al., 2012; Ulug et al., 2014; Gulcu et al., 2018; Raja et al., 2021). There is some evidence that olfactory cues contribute to scavenger deterrence (Jones et al., 2015), and that the distinct red colouration typical of many *Photorhabdus*-infected cadavers can serve as a warning signal of unpalatability for daytime scavengers such as birds (Fenton et al., 2011; Jones et al., 2017). Jones et al. (2017) proposed that a multimodal defence – chemical and visual – due to *Photorhabdus* may protect the cadaver, either by acting in concert and/or by being effective against different scavengers.

Here, we test the hypothesis that bioluminescence contributes to the protection of *Photorhabdus*-infected cadavers against nocturnal scavengers (Akhurst and Boemare, 1990), using *Photorhabdus temperata* Fischer-Lesaux, Villard, Brunnel, Normand & Boemare, carried by the nematode *Heterorhabditis downesi* Stock, Griffin & Burnell.

P. temperata emits light at a high intensity relative to other species of *Photorhabdus* tested (Hyrsl et al., 2004; Maher et al., 2021). We tracked the luminescence profile produced by *P. temperata* infected cadavers over the course of the infection to determine at what stage the luminescence would have its highest impact. In field trials at dusk, we compare scavenging on infected and uninfected insect cadavers under conditions either of ambient light, where only chemical defence is expected, or in darkness, where bioluminescence may be visible and contribute to cadaver defence. We conduct similar experiments in the laboratory using the scavenger encountered most commonly in the field trials, the slug *Lehmannia valentiana* Férussac. Most slugs are omnivorous, nocturnal scavengers that feed on dead vegetation, fungi, dead animals, and other detritus (Barnes and Weil, 1945; Jennings and Barkham, 1975; Keller and Snell, 2002). While they possess simple eyes (and certain light sensitive areas of the brain) that can detect light intensity, they do not form clear images (Zieger et al., 2009; Nishiyama et al., 2019) and seek food using olfactory cues (Gelperin, 1974, 1975; Kiss, 2017). Where organisms are protected in more than one modality, it can be difficult to disentangle the contribution of each modality to defence; artificial light sources are an important tool in demonstrating a defensive role of light where chemical defences are also effective (Underwood et al., 1997; Marek at al., 2011). We use light emitting diodes (LEDs) producing artificial light simulating that of a *Photorhabdus*-infected cadaver at peak luminescence in a choice experiment to test the effect of light alone on *L. valentiana* orientation and scavenging of freeze-killed insects.

4.2 Methods

4.2.1 Species, storage and conditioning

Late instar *Galleria mellonella* L. larvae were supplied by Peregrine Live Foods (Chipping Ongar, UK) and Live Foods Direct (North Anston, UK). They were infected with *H. downesi* K122 (a native Irish strain carrying *Photorhabdus temperata* that imparts a yellow-orange colour to the infected cadaver) by applying 100 infective juveniles to each larva. The insects were incubated at 20°C and died 3 days later, and these freshly dead cadavers were used in experiments. Relative light intensity of the cadavers was recorded using 5-minute exposure in a Syngene G:Box Chemi HR16 BioImaging System using GeneSnap 7.12 software (SynGene, Cambridge, UK). Images (16 bit) were exported to Image J (v1.5) and analysed for mean grey value/pixel. To determine peak luminescence over the course of infection, the luminescence produced by two sets of infected *G. mellonella* (N = 13, 19) were measured at alternating 2 hr intervals from 1-19 days post infection.

Freshly dead cadavers at day 3 of infection (relative light intensity 24,723 $\pm$ 643.58 (mean $\pm$ SE) mean grey scale units/pixel) were used in experiments. As controls, *G. mellonella* were frozen for 24 hrs and thawed 2 hrs prior to use.

Slugs *(Lehmannia valentiana;* synonym *Limax valentianus)* were collected from mature deciduous woodland. Identification of the collected slugs was confirmed by dissection and examination of genitalia (Forsyth, 2004) of a proportion (~10 %) of the population. The slugs were maintained in plastic containers with a thin layer of damp soil at 15°C and 16:8 Light: Dark for 1 week, during which time they were fed fresh

vegetables and dog food kibble (Bakers Small Dog, Purina, UK). Slugs used in experiments were 5-6 cm in length (fully extended).

4.2.2 Does bioluminescence deter nocturnal scavenging? (Field study)

Field trials were conducted at dusk (starting 2 hrs before sunset), in open deciduous woodland with ivy/grass ground cover. Ambient light levels were < 200 lux at soil level (recorded using an RT MT30 digital lux meter). Soil temperatures averaged 16°C in Summer and 11°C in Winter (Appendix Table 4.1). A single *H. downesi*-infected or freeze-killed cadaver was placed in a Petri dish (3.5 cm) with a hole (4 mm diam.) in the base to allow drainage. Each dish was placed in a shallow (2-3 cm deep) hole in the soil which was covered with a rigid polypropylene sheet (5 x 6 cm) resting on the soil surface. The cover was either translucent or opaque (black) to allow or prevent, respectively, light from entering the test arena below. There were thus four treatments, infected or freeze-killed cadavers in light or dark conditions (Fig. 4.1A). Dishes were arranged in groups of 4 (1 dish of each treatment), with all dishes and all groups 1 m apart. After 2 hrs the number and identity of invertebrates in the dishes were recorded and the dishes were returned to the laboratory. Signs of scavenging were recorded and classed as: cadaver fully consumed, partly consumed, or small punctures in the cuticle. In addition, each cadaver was weighed at the start and end of the experiment and the proportion of weight lost was recorded.

The were 6 trials for this field study at two woodland locations in County Kildare: Maynooth University Campus (53.377876, -6.600679) and Carton Demesne (53.383668, -6.573001). Trials were repeated in Summer (June-August) and Winter

(October-November) (Appendix Table 4.1). Overall, a total of 426 cadavers were tested, 103-108 per treatment.

4.2.3 Does bioluminescence deter slugs (*L. valentiana*) from feeding? (Lab study)

Based on the findings of the field trials, the slug *L. valentiana* was used as a model scavenger in lab trials. The slugs were allowed to feed on either a *H. downesi*-infected or freeze-killed cadaver under conditions in which the bioluminescence would either be visible (i.e., in darkness) or not visible (i.e. in low level ambient light (8-20 lux) provided by an LED bench lamp. Slugs which had been starved for 24 hrs before the experiment were placed individually in 9 cm Petri dishes, 2 cm from the centre of the dish. A cadaver (either infected or freeze-killed) was then introduced to the centre of each dish. As in the field trial, there were thus four treatments, infected or freeze-killed cadavers in light or dark conditions (Fig. 4.1B). Slugs were observed for 2 hrs, and their behaviour was recorded using the following categories: moving (within or outside a 2 cm radius of the cadaver), feeding (rasping the cadaver), tasting (touching the cadaver with mouth parts), searching (rotation of the tentacles), or none of the above. Red light was used for observations in the dark condition. The experiment was conducted at 16°C. After 5 hrs the cadavers were removed, and signs of scavenging were noted. The weight of each cadaver was recorded before and after the trial and the proportion weight loss was calculated. There were 18 trials for this lab study. Overall, a total of 569 slugs were tested, 141-144 slugs per treatment.

4.2.4 Does cadaver-mimicking light attract or repel slugs, or affect their feeding?

In order to separate the effects of light from those of chemical defences, a *L. valentiana* slug was given a choice between two freeze-killed insects; one of which was paired with artificial light of a similar wavelength and intensity to that of a *Photorhabdus* infected insect, and the other was not. The experiment was carried out in the dark, at 16°C. A freeze-killed *G. mellonella* was placed at the end of each arm of a Y shaped tube directly in front of a 5 mm-diameter LED (HLMP-AB64/65, Avago Technologies Inc., USA) which was either lit or unlit (Fig. 4.1C). The lit diodes emitted light at 470 nm and the relative light intensity (measured as described for cadavers) was adjusted to fall within the range 15,000-35,000 (mean 24303) mean grey scale units/pixel to mimic the intensity of light emitted by bioluminescent cadavers at days 3-6 of infection (Fig. 4.2). The sides of each arm were covered with opaque tape and tubes were orientated in different directions to avoid positional effects. A single *L. valentiana* slug that had been starved for 24-48 hrs was placed in the entrance chamber of the Y-tube, and its behaviour was observed for 2 hrs under red light. The time spent in each of the following behavioural categories was recorded for each arm: moving, feeding, tasting, searching or none of the above. After 5 hrs the cadavers were removed and examined for signs of scavenging. The proportion of weight loss of each cadaver was calculated. The experiment was conducted across 13 trials with a total of 98 slugs tested.

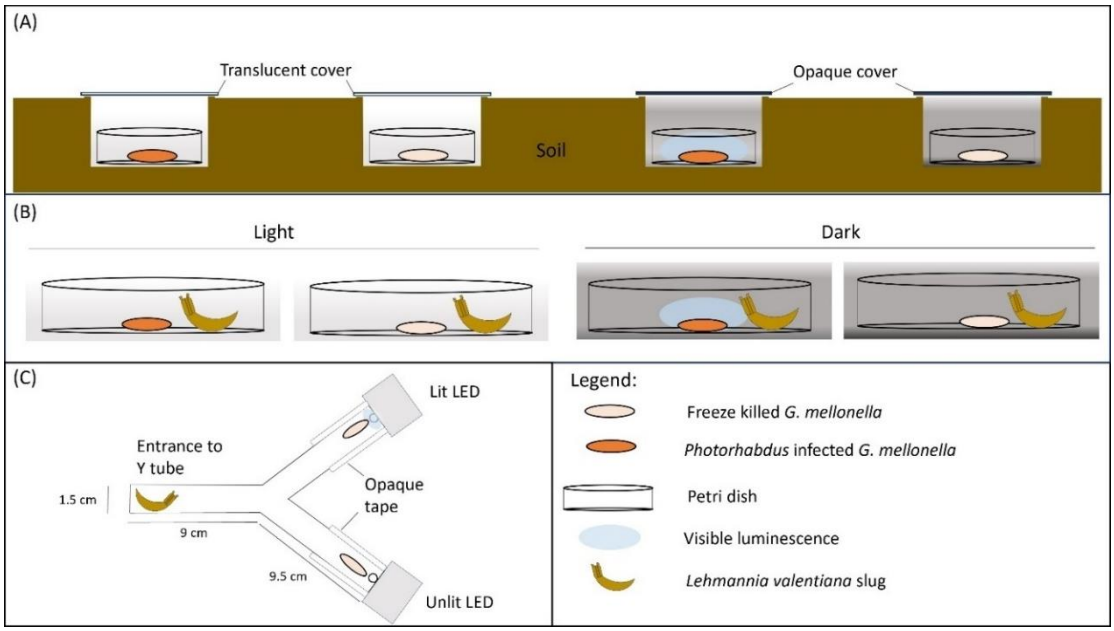

Figure 4.1. Experimental set up. (**A**) and (**B**): No-choice trials in which scavenging is recorded on *Photorhabdus temperata*-infected insect cadavers under conditions where bioluminescence is visible or not. Freeze-killed insects are included as controls. (**A**) **Field trial:** Insect cadavers are in soil pits covered with either translucent or black polypropylene squares, to allow or block light from entering the pit. (**B**) **Lab trial**: Insect cadavers are exposed to a scavenger slug (*L. valentiana*) under conditions of either light or dark. (**C**) Two-choice apparatus with either a lit LED (emitting at 470 nm) or unlit LED light positioned behind a freeze-killed insect at the end of each arm. A slug is placed in the entrance chamber at the start of the experiment.

4.2.5 Data analysis

For each experiment, the results for repeated trials were pooled prior to analysis. The incidence of scavenging was analysed using Pearson's Chi square with significance set at $P < 0.05$, and with Bonferroni correction for multiple post hoc comparisons. Weight loss of cadavers in field and lab trials, and proportion of time spent feeding, moving, tasting, and searching in the non-choice lab test were compared between treatments using Kruskal-Wallis tests with significance at $P < 0.05$. Where significance was detected, this was followed by Mann-Whitney U tests with Bonferroni correction applied for multiple comparisons. For the choice experiment, slugs that did not move into either arm were excluded from the analysis, resulting in 69 replications. A

Wilcoxon signed rank test was used to compare time spent in each arm of the Y tube, with a hypothesised proportion of 50 %. The proportion weight loss of the cadavers and slug behaviours for this experiment were analysed using a two-sample t-test and Mann-Whitney U tests, respectively. All data analysis was performed using Minitab v. 20.3 statistical software or R studio v. 2022.12.0+353.

4.2.6 Ethical statement

The species used in this study did not require formal ethical approval. The methodology used for these experiments was in keeping with recommendations for the ethical treatment of invertebrates (Drinkwater et al., 2019). Slugs collected for use in lab trials were monitored for 3 weeks after experimentation and healthy slugs were released at their collection point. Ecological impact during field trials was minimised by recording invertebrate activity in situ without removal.

4.3 Results

The average luminescence of *H. downesi*-infected *G. mellonella* increased from day 2 of infection, peaked at day 3 at 28924.3 $\pm$ 1132.482 (mean $\pm$ SE, N = 19) mean grey scale units/pixel and decreased from then, ceasing completely by day 19 (Fig. 4.2).

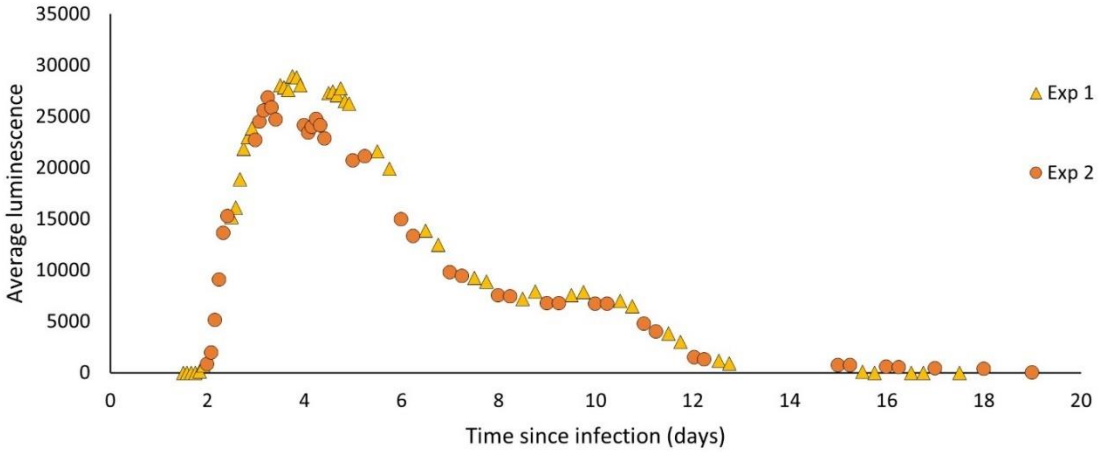

Figure 4.2. The average luminescence (mean grey scale units/pixel) produced by *P. temperata* infected *G. mellonella* from time of exposure to time of emergence. Larvae infected with 100 IJs of *H. downesi* were measured at 2-4 hr intervals for two sets of infections (Experiment 1 and 2).

4.3.1 Does bioluminescence deter nocturnal scavenging (Field study)?

Slugs were the most frequently recorded potential scavengers, accounting for 70% (68/97 individuals) of all invertebrates found in the dishes, with *L. valentiana* being the most common slug species, accounting for 23% (22/97) of all invertebrates found. Other potential invertebrate scavengers recorded in the dishes included earwigs (Dermaptera) and beetles (Coleoptera) (Appendix Table 4.4).

There were significant differences between treatments in scavenging rate (the proportion of cadavers showing signs of biting or feeding) by nocturnal scavengers (Chi square: $X^2_3 = 10.39$, $P = 0.0155$, $N = 103\text{-}108$) (Fig. 4.3A; Appendix Table 4.2) and in the weight loss of cadavers, used as a proxy for amount consumed (Kruskal-Wallis: $H_3 = 18.09$, $P < 0.001$, $N = 103\text{-}108$) (Fig. 4.3B; Appendix Table 4.3). Scavenging rate and consumption were lower for *P. temperata*-infected than for freeze-killed cadavers, but the differences were significant only for those under dark

covers, where luminescence would be apparent, but not for cadavers under translucent covers, indicating that bioluminescence is effective in reducing scavenging (Fig. 4.3A and 4.3B). The majority of those cadavers that had evidence of scavenging had only small puncture wounds in both the infected treatments (Dark: 10/15; 66.7%, Light: 23/28; 82.1%) and the controls (Dark: 25/37; 67.6%, Light: 17/31; 54.8%). Of the cadavers that were attacked, the proportion that were fully consumed was approximately 3.6 times lower in the infected treatments than in the controls both in the dark (1/15; 6.7% vs 9/37; 24.3%) and in the light (3/28; 10.7% vs 12/31; 38.7%).

There was no difference between treatments in their association with invertebrates, either in incidence (the proportion of dishes containing at least one invertebrate along with the cadaver) (Pearsons's chi square: X^2_3= 1.382, P = 0.710, N = 103-108) or in numbers of individuals recorded (Kruskal Wallis: H_3 = 0.85, P = 0.838, N = 103-108).

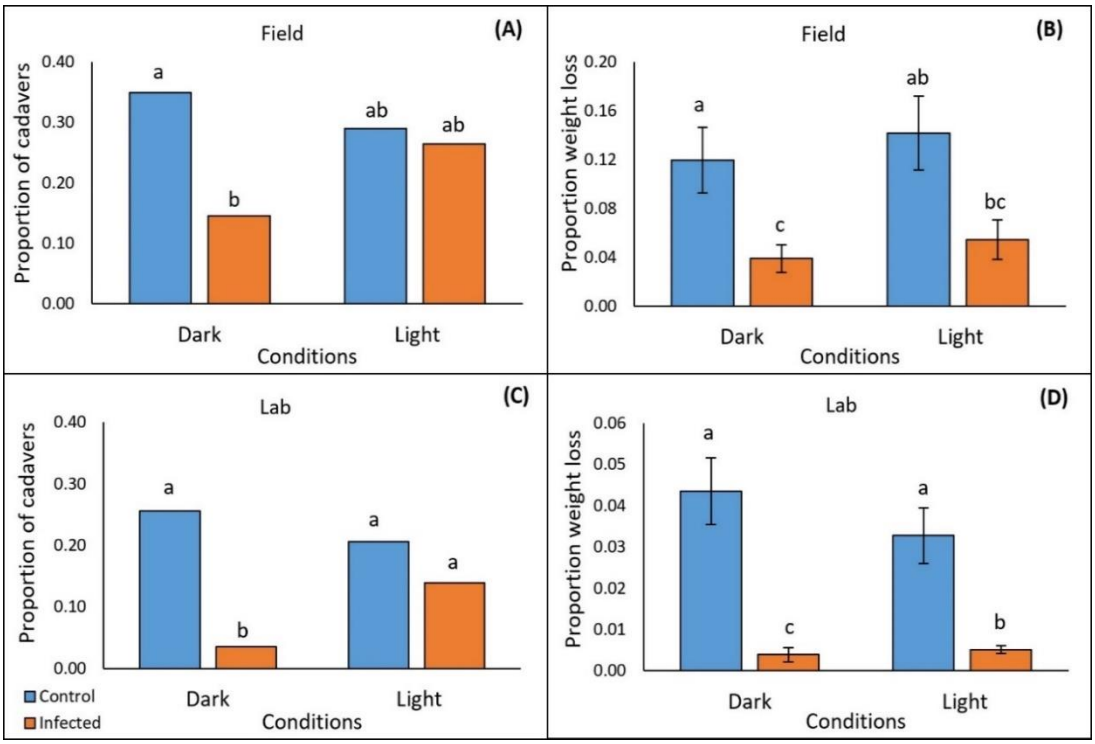

Figure 4.3. Scavenging on *P. temperata*- infected and freeze-killed (control) *G. mellonella* cadavers under conditions of either light or dark, after 2 hrs in the field (**A**)(**B**), and after 5 hrs exposure to slugs (*L. valentiana*) in the laboratory (**C**)(**D**). (**A**)(**C**) The proportion of cadavers tested that showed incidence of scavenging in the field and laboratory trials respectively (columns accompanied by the same letters are not significantly different at $P < 0.05$, Chi square with Bonferroni correction). (**B**)(**D**) Weight loss as a proportion of the cadaver (mean +/- SE) of *G. mellonella* tested in the field and laboratory respectively (Columns accompanied by the same letters are not significantly different at $P < 0.05$, Mann Whitney with Bonferroni correction).

4.3.2 Does bioluminescence deter slugs (*L. valentiana*) from feeding (Lab study)?

In laboratory tests with *L. valentiana* there were significant differences between treatments in the proportion of cadavers showing signs of scavenging (Pearsons's chi square: $X^2_3 = 33.968$, $P < 0.0001$, $N = 141$-144) (Fig. 4.3C; Appendix Table 4.5), in weight loss of cadavers- a proxy for amount consumed (Kruskal-Wallis: $H_3 = 65.71$, $P < 0.001$, $N = 141$-144)(Fig. 4.3D; Appendix Table 4.6), and in the amount of time the *L. valentiana* slugs spent feeding on the cadavers during the 2 hr observation (Kruskal-Wallis: $H_3 = 10.26$, $P = 0.016$, $N = 141$-144) (Fig. 4.4; Appendix Table 4.7) . There

was less scavenging (attack and consumption) by *L. valentiana* on *P. temperata* infected than on control cadavers. The difference between infected and control cadavers in the incidence of scavenging (Fig. 4.3C) and in time spent feeding (Fig. 4.4) was significant only in the dark when luminescence would be visible, and not in the light, when luminescence would not be apparent, indicating a defensive role for bioluminescence. In the light conditions, the amount of cadaver consumed was lower for infected than for freeze-killed, indicating the operation of chemical defences. The amount of infected cadaver consumed in the dark was lower than in ambient light, clearly showing the additive effect of both defence modalities (Fig. 4.3D). Time spent on behaviours other than feeding (moving close to the cadaver, moving at a distance from the cadaver, searching, tasting) did not differ between treatments (Appendix Table 4.8).

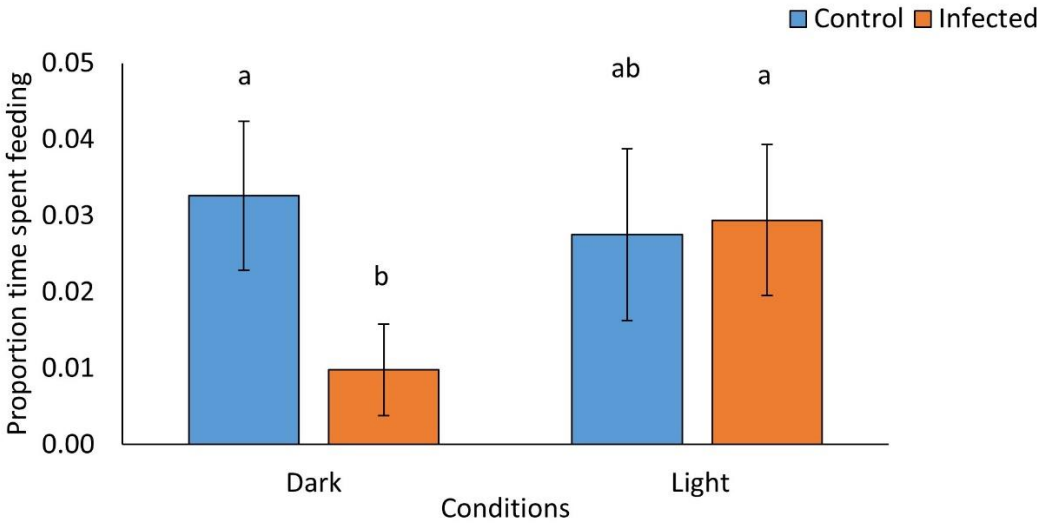

Figure 4.4. Proportion of time *L. valentiana* slugs spent feeding on *P. temperata*- infected or freeze-killed (control) *G. mellonella* cadavers under conditions of either light or dark in no-choice laboratory experiments. Values accompanied by the same letter are not significantly different (Mann Whitney tests with Bonferroni correction, $P < 0.05$)

4.3.3 Does cadaver-mimicking light attract, repel, or affect feeding of slugs?

There was no evidence that the slug *L. valentiana* was either repelled or attracted by artificial light of similar wavelength and intensity to a nematode-infected insect. When given a choice of two arms each with a freeze-killed *G. mellonella* as bait either paired or not paired with a light source, they spent an equal proportion of time in each arm (Wilcoxon signed rank test: Z = 1152.50, P = 0.980; N = 69), and there was no difference in the amount of time spent moving, searching, tasting, or feeding in the two arms (Appendix Table 4.7). However, there was a lower incidence of scavenging on the bait insect paired with a light than on the bait insect without a light (Pearson's Chi square: X^2_1 = 3.881, P = 0.049; N = 69) (Fig. 4.5). There was no difference in the percentage weight loss of cadavers in the two conditions (Mann Whitney: W= 4989, P = 0.411; N = 69).

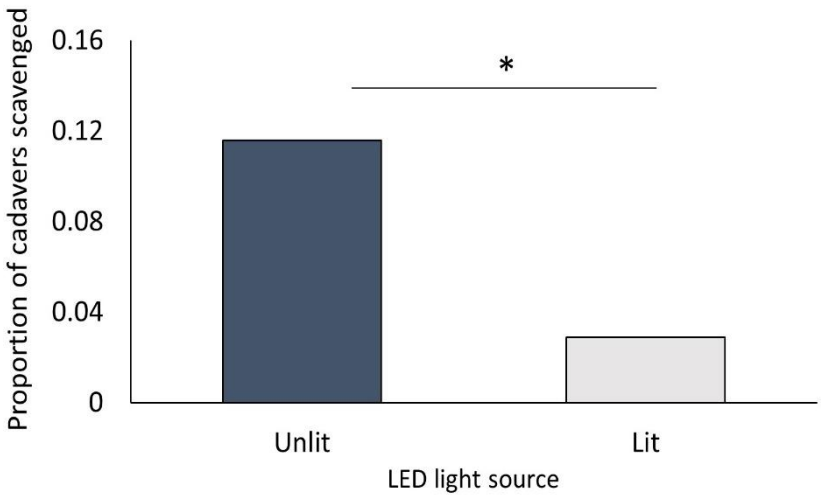

Figure 4.5. Proportion of freeze-killed *G. mellonella* cadavers scavenged by *L. valentiana* when close to either a lit or an unlit LED in a choice test. Slugs that did not move into either arm of the Y-tube were excluded from analysis. Significant differences between treatments in scavenging rate is indicated by * (Chi square, $P < 0.05$).

4.4 Discussion

Our findings support the scavenger-deterrence hypothesis (Akhurst and Boemare, 1990) for bioluminescence in *Photorhabdus*. In both our field and laboratory trials, a lower rate of scavenging on *Photorhabdus*-infected cadavers compared to controls was significant only under dark conditions, where bioluminescence would be visible, providing strong evidence that bioluminescence contributes to scavenger deterrence, while the experiment with an artificial light source indicates that bioluminescence alone could protect cadavers. Cadavers were not completely undefended in ambient light conditions, since the amount of cadaver consumed was reduced in the light as well as the dark, indicating the operation of a chemical "scavenger deterrent factor" (Zhou et al., 2002; Gulcu et al., 2012). We suggest that the two defences operate slightly differently, with chemical factors reducing the amount of cadaver consumed while light serves to reduce the probability of a cadaver being damaged in the first place. Preventing breaches of the cadaver cuticle is important; even a small lesion without further consumption may completely compromise the success of the nematode-bacterial complex developing inside, by increasing vulnerability to desiccation or competing organisms (Koppenhöfer et al., 1997; Baur et al., 1998, Serwe-Rodriguez et al., 2004).

The scavenger-deterrent hypothesis contrasts with the earlier suggestion by Poinar et al. (1980) that light could attract insects which could then be infected by, or serve to transport, the next generation of nematodes as they emerge from the cadaver into the soil. While there is some evidence that insects are attracted by the light emitted by *P. luminescens* (Patterson et al., 2015), attraction of new hosts is implausible as an ecological function of bioluminescence (as indeed was recognised by Poinar et al.

(1980)). As we show, it peaks within days of infection, long before the new infective stage nematodes have been produced and are ready to emerge from the host. In *H. downesi*, for example, the first infective juveniles do not emerge until at least 15 days after infection, by which time luminescence has declined to a low level. In contrast, peak luminescence occurs at a time when its role in defence against scavengers would be important: the nematodes developing inside are at a vulnerable stage (Poinar, 1979) and cadavers are otherwise poorly defended (Baur et al., 1998; Gulcu et al., 2012; Ulug et al., 2014; Fenton et al., 2011; Jones et al., 2015; Jones et al., 2017). Bioluminescence is produced once *Photorhabdus* reaches a critical population density (Meighen, 1999), while defences based on unpalatable chemicals and/or pigmentation intensify over time (Baur et al., 1998; Gulcu et al., 2012; Ulug et al., 2014; Fenton et al., 2011; Jones et al., 2015, 2017), presumably as the products responsible for them accumulate within the cadaver. Although not tested here, it may be that bioluminescence as an independent defence is particularly important early in the infection, helping during a period of vulnerability while chemical defence is less efficient.

Variation amongst scavenger species in the extent to which they are deterred from *Photorhabdus*-infected cadavers (Gulcu et al., 2012, 2018; Ulug et al., 2014) may explain why trends in the field, with a diverse assemblage of scavengers, were less clearcut than in the laboratory trials with just a single species of photophobic nocturnal scavenger - the slug *L. valentiana*. Slugs move away from areas of light and are particularly sensitive to blue light in the 400 - 520 nm range (Suzuki et al., 1979; Nishiyama et al., 2019) which spans the range in which *Photorhabdus* emits luminescence (Peat et al., 2010). Their negative response to diffuse light is mainly associated with habitat selection (South 1992; Zieger et al., 2009). In our experiments,

there was no evidence that a point source of light deterred the approach of *L. valentiana* – there was no difference in the category "moving close" to a cadaver in the no-choice experiment, or in the frequency of entry into lit or unlit arms containing a cadaver-mimicking LED in the choice experiment. Slugs seek and recognise food using olfaction (Gelperin, 1974; Gelperin, 1975; Kiss, 2017); again, the lack of effect on "moving close" may indicate that slugs were not deterred at a distance by the odour of infected cadavers. There is evidence that the odour of *P. luminescens* infected cadavers deters attack by beetle and avian scavengers (Jones et al., 2015, 2017), but it would appear that for *L. valentiana*, both chemical and light defences operate at close contact, serving to deter feeding, but neither modality deters their approach.

The unpalatability of *Photorhabdus*-infected cadavers to scavengers appears to be an innate (unlearned) response, as it is displayed in laboratory tests by untrained animals (Baur et al., 1998; Zhou et al., 2002; Gulcu et al., 2012, 2018; Ulug et al., 2014; Lordan et al., 2014; Raja et al., 2017; Cimen et al., 2023). Similarly, the aversive response of slugs to the luminescence of *Photorhabdus* shown here seems also to be innate, as it was demonstrated by naïve (untrained) individuals at first exposure.

Bioluminescence is produced by *Photorhabdus* as a constant glow. In general, while sudden flashes of bioluminescence are repellent, bioluminescent glows are thought to function as attractant signals (Haddock et al., 2010). It is argued that without additional chemical defences, light would only serve to make the emitting organism more obvious to predators (De Cock & Matthysen, 1999). Slugs may be unusual in having an innate dislike of feeding on glowing food, though few terrestrial animals have been tested for this response. For toads and small mammals, the possibility of

an innate deterrent response to the bioluminescence of glow worms or millipedes could not be excluded (De Cock & Matthysen, 1999, 2003; Marek et al., 2011).

While not demonstrated here, the bioluminescence produced by *Photorhabdus*-infected cadavers could act more generally as an aposematic signal for nocturnal scavengers, as has been suggested for the red colour of *P. luminescens*-infected cadavers for diurnal scavengers (Fenton et al., 2011). Aposematism is the use of warning signals to advertise unprofitability (Breed and Moore, 2016) epitomised by the conspicuous colours used by insects and frogs to advertise toxicity or unpalatability (Guilford, 1990; Ruxton et al., 2018). Like conspicuous colours, bioluminescence is frequently associated with unpalatability, leading to the suggestion that it functions as an aposematic signal in a range of organisms, including algae (Dinoflagellata), annelids, brittle stars (Ophiuroidea), millipedes, glow worm larvae (Coleoptera) and fish (lanternsharks: Etmopteridae) (Grober, 1988 a, b; de Cock and Mathysen, 1999; Marek et al., 2011; Verdes and Gruber, 2017; Duchatelet et al., 2019; Cusick and Widder, 2020). This suggestion is not always supported by empirical data, but there is good experimental evidence both for brittle stars (Grober, 1988 a, b; Jones and Mallefet, 2013) and for glow worms (De Cock and Matthysen, 1999, 2003). Toads (*Bufo bufo*) were deterred by the bioluminescence of the common glow worm, after learning to associate the glow with the noxious smell/taste of the larvae (De Cock and Matthysen, 1999, 2003). Naïve wild-caught toads demonstrated some aversion to the bioluminescence, which was interpreted as evidence either of prior learning in the field or of neophobia (rejection of novel food), but the aversion was strengthened by the associative learning (De Cock and Matthysen, 2003). Similarly, when paired with chemical defences, the bioluminescence of *Photorhabdus* may strengthen the innate

aversion of slugs to feeding on luminous food, and in addition deter other nocturnal scavengers from feeding, even those that are initially indifferent to the light or even attracted by it. Associative learning - required for aposematism to work- is well documented in insects (Dethier, 1980; Duerr and Quinn, 1982; Matsumoto and Mizunami, 2000; Giurfa, 2007, 2015) and in gastropods (Delaney and Gelperin, 1986; Balaban, 1993; Farley et al., 2004) including *L. valentiana* which can form a long-term association between chemical and visual stimuli (Fujisaki and Matsuo, 2017).

4.5 Conclusion

In conclusion, our experiments provide support for an ecological function for the bioluminescence of *Photorhabdus* in line with the scavenger deterrence hypothesis (Akhurst and Boemare, 1990), and show that light acts in concert with chemical defences, as previously shown for colour and chemical defences in daylight (Fenton et al., 2011; Jones et al., 2017). Deterrence is unlikely to have been the function of bioluminescence in the bacteria from which *Photorhabdus* acquired its *lux* operon, as there is evidence that in the marine environment, a constant glow is attractive (Zavilgelsky and Shakulov, 2018). A plausible scenario is that the gene transfer occurred within a crustacean; bioluminescent marine bacterial species occur as pathogens of many crustacea (Nealson and Hastings, 1979; Ramesh and Bessho-Uehara, 2021), and *Photorhabdus* can infect littoral amphipods and isopods (Mauléon et al., 2006). Bioluminescence may have been maintained by in the terrestrial environment by protecting infected hosts against innately photophobic scavengers such as slugs, and/or by aposematically reinforcing pre-existing chemical defences of *Photorhabdus*. Support for the scavenger deterrence in *P. temperata* does not rule out

the possibility that bioluminescence is maintained in *Photorhabdus* by more than one selection pressure, either in the same or different species, or is being lost in some species in which it offers less advantage (Peat et al., 2010).

Chapter 5. Damage to the host cadaver, simulating the effects of scavenging, affects fitness of EPN species differentially.

In preparation as:

Cassells, M. D., Treanor, N., Muñoz-Adalia, E. J. & Griffin, C. T. Scavenger damage to the host cadaver affects fitness of EPN species differentially.

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5.0 Summary

Entomopathogenic nematodes are reliant on their host insects to provide nutrients and protect them from environmental stresses such as desiccation when developing. Damage to the host cadavers by scavengers has been proposed to negatively affect the nematode populations within but has not been tested until now. In this chapter scavenging activity on cadavers infected with either *Heterorhabditis downesi* or *Steinernema feltiae* was quantified in the field and in lab experiments with crickets, and in separate experiments, the fitness of EPN populations that emerged from cadavers with simulated scavenger damage was investigated.

In both lab and field trials, there was less scavenger damage to EPN infected cadavers than controls, with *H. downesi*-infected cadavers being damaged the least. For the majority of the infected cadavers that showed signs of feeding only small lesions were made to the cuticle. Scavenging damage for *S. feltiae* and *H. downesi*-infected cadavers was simulated shortly after death by piercing the cuticle 1, 3 or 5 times and placing the damaged cadavers in moist or dry conditions. For *H. downesi* infections the proportion weight loss (due to loss of moisture) of the host cadaver increased significantly with damage, exacerbated by dry conditions. The number of emerging IJs was negatively correlated with this weight loss, and the average size of the emerging IJs decreased with increasing damage to the host. For *S. feltiae*, damage to the host decreased the number of emerging IJs to a lesser extent. The reduction was not completely explained by weight/moisture loss, indicating that for this species, some factor other than desiccation (perhaps competition with opportunistic microbes) impacts the nematodes when the cuticle is damaged. In conclusion, *H. downesi* nematodes are more vulnerable than *S. feltiae* nematodes to scavenger damage to the

host cadaver, possibly due to *Heterorhabditis*' longer generational time in the host resulting in longer exposure to desiccating conditions. The greater susceptibility of *Heterorhabditis* nematodes to this scavenger damage may select for greater defences to deter scavengers from feeding on their host insects, compared to *Steinernema* nematodes.

5.1 Introduction

Entomopathogenic nematodes (EPN) are parasites that invade and kill soil dwelling insects in which they develop and reproduce. Nematodes of the genera *Steinernema* and *Heterorhabditis* have a similar life cycle with some variations in infection strategy, development, and reproduction. Both *Steinernema* and *Heterorhabditis* enter the insects as infective juveniles (IJ) and release symbiotic bacteria (*Xenorhabdus* or *Photorhabdus* respectively) that kill the host. The nematodes then feed on the proliferating bacteria and host nutrients, develop to adult, and produce progeny (Poinar, 1979). The offspring develop through four juvenile stages (J1-J4) before reaching adulthood. Several generations are produced within a single host cadaver. When the nutrients are depleted, a stress resistant IJ generation diversifies at the J3 stage and leaves the cadaver in search of a new host (Kaya, 1990; Dillman and Sternberg, 2012). The host cadaver offers protection as both EPN and their symbiotic bacteria are vulnerable to stresses such as desiccation, high temperatures and UV radiation (Glazer, 2002). Only the IJs are capable of survival outside of the host (Poinar, 1979). The symbiotic bacteria produce a wide variety of antimicrobials to kill competing bacteria and fungi, thereby preventing putrefaction of the host (Akhurst, 1982; Li et al., 1995; Eleftherianos et al., 2007; Ullah et al., 2015; Tobias et al., 2018). Development and reproduction of the nematodes is dependent on the limited nutrient availability of the host and proliferation of their symbiotic bacteria (Van zyl & Malan, 2015; Rahoo et al., 2019).

Scavengers feeding on the host insect cadavers not only kill the EPN through consumption but also expose the remaining nematode population, and their symbiotic bacteria, to unfavourable conditions by breaching the insect cuticle, which otherwise

acts as a barrier. EPN and their mutualistic bacteria produce a number of chemical and visual deterrents to prevent scavengers from damaging the host cadavers (Baur et al., 1998; Zhou et al., 2002; Ulug et al., 2014; Raja et al., 2021; Cimen et al., 2023). Both *Photorhabdus* and *Xenorhabdus* invest in the production of a 'scavenger deterrent factor' that reduces the incidence of invertebrates feeding on the host cadaver (Gulcu et al., 2012; Gulcu et al., 2018). In the case of *Photorhabdus,* the bacteria also cause red-orange pigmentation and bioluminescence of the host, which act as visual deterrents for scavengers (Jones et al., 2015; Chapter 4).

Steinernema-infected cadavers are more vulnerable than *Heterorhabditis*-infected cadavers to scavenging damage by ants (*Linepithema humile, Veromessor andrei, Pheidole vistana, Formica pacifica, Monomoriom ergatogyna)* and cockroaches (*Periplenata americana*) (Baur et al., 1998; Gulcu et al., 2012; Ulug et al., 2014). It was noted that scavenger damage led to rapid desiccation of the host, and it was suggested that the nematodes, now exposed to unsuitable conditions and competing microorganisms, would die (Baur et al., 1998; Ulug et al., 2014). This exposure could also negatively affect the symbiotic *Xenorhabdus/Photorhabdus* bacteria, leading to reduced nutrient availability for the nematodes.

We hypothesise that scavenger damage to the cadaver cuticle will lead to reduced nematode fitness. The aims of this study were to determine (1) the vulnerability of *Heterorhabditis downesi* and *Steinernema feltiae*-infected cadavers to damage by invertebrates, under both lab and field conditions, and (2) the effect that this damage to the host (simulated in the lab under both moist and dry conditions) has on the fitness of the nematode population in terms of number of IJs that emerge and IJ size.

5.2 Methods

5.2.1 Insects and Infections

Galleria mellonella L. larvae (late instar), supplied by Peregrine Live Foods (Chipping Ongar, UK) and Live Foods Direct (North Anston, UK) were infected with either *Heterorhabditis downesi* Stock, Griffin & Burnell (strain K122) or *Steinernema feltiae* Filipjev (strain 4cfmo) by application of 100 IJs in aqueous suspension to each larva, and incubation at 20 °C. Larvae died after 2 days (*S. feltiae)* or 3 days (*H. downesi*) exposure to IJs (days post infection: DPI). As controls, *G. mellonella* were frozen for 24 hrs and thawed 2 hrs prior to use in experiments.

Field crickets (*Gryllus bimaculatus* De Geer) were reared and supplied by Peregrine Live Foods at the late nymphal stage. The crickets were maintained for a week at 18°C, 12: 12 Light: Dark and were fed a mixture of fresh fruit and vegetables. Crickets were starved for 24 hrs before use in experiments.

5.2.2 Scavenging on nematode-infected cadavers

5.2.2.1 Nocturnal scavenging on nematode-infected cadavers in the field

EPN-infected *G. mellonella* cadavers were placed in open woodland conditions shortly after sunset and the incidence of damage by nocturnal scavengers was recorded. A single freshly dead (3 DPI for *H. downesi*, 2 DPI for *S. feltiae*, or freeze-killed as control) larva was placed in the base of a 3.5 cm Petri dish that had been modified with a 4 mm diameter hole to allow staking. Dishes were then staked to the ground 1 m equidistant to each other in groups of three, randomly alternating treatments within a

group. After 12 hrs (Trials 1 & 2) or 2 hrs (Trial 3) the dishes were photographed and the presence of invertebrates in the dishes was noted, with slugs (*Lehmannia* spp. and *Arion* spp.) being the most common. Dishes were returned to the laboratory where the cadavers were checked for signs of feeding. Level of feeding damage to the cadavers was classed as: fully consumed, partly consumed, or bitten (small cuts in the cuticle). Cadavers were weighed before and after the trial, with proportion weight loss (due either directly to consumption of insect tissue and/or to enhanced rate of moisture loss through the damaged cuticle) being used as another measure of damage. The field trials were conducted in 3 locations: Navan (53.648917, -6.697855), Maynooth (53.375893, -6.5981235) and Carton Demesne (53.382781, -6.571821) (Appendix Table 5.1), with a total of 60 cadavers per treatment.

5.2.2.2 Scavenging on nematode-infected cadavers by *Gryllus bimaculatus*

Crickets were placed individually in 9 cm Petri dishes with either a freshly dead EPN-infected (3 DPI for *H. downesi,* 2 DPI for *S. feltiae*) or a freeze-killed larva. After 2 hrs in the dark, the incidence and level of feeding damage to each cadaver was recorded. Proportion weight loss of each cadaver was calculated as a proxy for scavenging intensity, as above. There were 3 runs of this experiment, with a total of 52-53 cadavers per treatment across the 3 runs.

In a separate trial, EPN-infected cadavers were tested at 5 DPI. Feeding damage was recorded after 2 hrs and 24 hrs and cadavers were weighed after 24 hrs. There were 3 runs of this experiment with a total of 52-60 cadavers per treatment across the 3 runs.

5.2.3 Effect of simulated scavenger damage on EPN fitness

5.2.3.1 Effect of damage an ambient humidity on IJ emergence (Experiment 1)

Scavenging damage was simulated by cutting the cuticle of freshly dead EPN-infected *G. mellonella* larvae (3 DPI for *H. downesi*, 2 DPI for *S. feltiae).* Damage was applied at four intensities: No Damage, Low Damage, Medium Damage and High Damage with 0, 1, 3 or 5 cuts, respectively (Fig. 5.1A). A curved scalpel (No. 12 blade) was used to perform ~3 mm incisions, evenly spaced along the abdomen of the insect.

Cadavers (each on a 3.5 cm Petri dish lids) were maintained at 20°C inside 15 cm Petri dishes with either moist (100 % relative humidity (RH)) or dry conditions (60-70 % RH). Humidity was measured using a ThermoPro TP50 Digital Thermo-Hygrometer and 100 % RH was maintained using damp tissue paper. When IJs first began emerging (7-8 DPI for *S. feltiae*, 16-17 DPI for *H. downesi*) the cadavers were transferred on the Petri dish lids (to reduce handling of the damaged cadavers) to modified White traps (Stock and Goodrich-Blair, 2012). IJs were harvested at intervals until emergence ceased (46 days for *S. feltiae*, 64 days for *H. downesi*). IJs were stored at 9°C and the total number of emerged IJs per cadaver was assessed through counts. Cadavers were weighed immediately after damage, and again before being placed in White traps, and the proportion weight loss of each cadaver was calculated. Since this experiment did not involve removal of cadaver biomass, weight loss is assumed to be due to moisture loss. There were 8 treatments per EPN species (4 damage levels at each of two moisture conditions) with a minimum of 8 cadavers per treatment.

5.2.3.2 Effect of simulated scavenger damage on IJ emergence and size (Experiment 2)

In this experiment, RH was maintained at 100 % for all cadavers and the size of IJs was recorded as well as numbers emerging. The protocol was similar to that used in experiment 1, with minor differences: cadavers were placed on glass slide coverslips instead of 3.5 cm Petri dish lids, and these were then placed in unmodified White traps (Stock and Goodrich-Blair, 2012) for collection of IJs. For *S. feltiae,* harvesting began 7 DPI and ended at 28 days, while for *H. downesi* harvesting began at 23 DPI and ended at 41 days. The juveniles collected in the first harvest (7/23 DPI) were exposed to 0.1 % v/v sodium dodecyl sulfate (Sigma) to confirm IJ status (Cassada and Russell, 1975). A sample of confirmed IJs were then straightened using water at 70°C and photographed. The length of 30 randomly selected IJs from each cadaver was measured using Image J 1.53e (Rasband, 2015). There were 4 treatments per species: No Damage, 1 cut, 3 cuts, 5 cuts with 9-10 cadavers per treatment.

To compare the rate of weight loss from cadavers infected by *S. feltiae* and *H. downesi* over the same time period, cadavers at each of the four damage levels were weighed immediately after damage and again 5 and 6 days later. There were 8-10 cadavers per treatment.

5.2.4 Data analysis

All statistical analyses developed in this study were performed in R programming environment (R Core Team, 2022) or Minitab 20.3 statistical software (Minitab, LLC, 2021)

Incidence of feeding on EPN-infected cadavers was analysed using Pearson's chi-square (significance at $P < 0.05$) with Bonferroni correction applied for multiple comparisons. Proportion weight loss of the cadavers after exposure to nocturnal scavengers was analysed using Kruskal-Wallis's test at significance $P < 0.05$, followed by Mann-Whitney post hoc comparisons carried out with Bonferroni correction applied for non-parametric data, or using one way analysis of variance (ANOVA) at significance $P < 0.05$ followed by Tukey's post hoc test for parametric data.

For the simulated damage experiments, time until first emergence from cadavers was analysed using Kruskal-Wallis followed by Mann-Whitney post hoc comparisons with Bonferroni correction.

Effects of simulated damage on cadaver weight loss, numbers of IJs emerging and IJ size were investigated using general linear models (GLMs) as follows:

Exp 1: seventeen GLMs with Gaussian distribution for errors were fitted separately for *S. feltiae* and *H. downesi* considering the proportion weight loss (Weightloss) of the cadavers as dependent variable. The treatment applied to the cadaver before incubation either as the number of cuts (DamageLevel : 0,1,3,5) or as the corresponding qualitative scale (DamageScale : Control/Low/Medium/High), the initial weight of the host larva in grams (Hostsize) and the incubation conditions (Conditions: Moist/Dry) were included in the models as explanatory variables as well as their interactions when they retained biological meaning. Model selection was performed using the Akaike´s Information Criteria (AIC) using the "AICcmodavg" package in R (Mazzerolle, 2023). The most parsimonious fitted GLMs (that is, those that showed lower AIC values) were compared using the Chi-square test whenever

their AIC values differed by less than 3 units. The quality of fit of the models was then evaluated using the package "DHARMa" in R (Hartig, 2022), and fitted values of the model were visualized using the package "ggeffects" (Lüdecke, 2018). The models with the best fit were selected for further analysis (Table 5.1). Post hoc analysis was performed when a qualitative variable was included in the selected models as explanatory by computing the Fisher's least significant difference (LSD) test using the "agricolae" package of R (de Mendiburu, 2023). Dunn's post hoc test was performed on significant variables with multiple levels e.g. (DamageLevel, DamageScale) with Bonferroni correction applied for multiple comparisons using the "rstatix" package of R (Kassambara, 2023).

An additional 25 GLMs (with Gaussian distribution of errors) were fitted separately for *S. feltiae* and *H. downesi* modelling the variation in the number of emerged IJs (Emergence) considering Hostsize, Weightloss, Conditions, DamageLevel and DamageScale as explanatory variables.

Exp 2: Six GLMs were fitted separately for *S. feltiae* and *H. downesi* to investigate the variation of Weightloss considering Hostsize, DamageScale and DamageLevel as explanatory variables. Thirteen GLMs were fitted separately for *S. feltiae* and *H. downesi* to investigate the variation in IJ Emergence considering Hostsize, Weightloss, DamageScale and DamageLevel as explanatory variables. In addition, fifteen GLMs were fitted separately for *S. feltiae* and *H. downesi* to investigate the possible variation of the length (mm) of emerging IJs (I) considering Emergence, Hostsize, Weightloss and DamageLevel as explanatory variables. For each investigation explanatory variables were evaluated separately in different models, in combination, and as interactions whenever they retained biological meaning, and model selection, fitness

quality comparison and evaluation were performed as described in experiment 1 (Table 5.1).

In a separate experiment, the area under the curve for the proportion weight loss over 6 days (AUCPWT) was calculated for each species, for each damage level, as the sum of areas of the corresponding trapezoids (Muñoz-Adalia et al. 2018). A GLM was fitted in R for AUCPWT using DamageLevel (qualitative scale, see above) and EPN species as the explanatory variables.

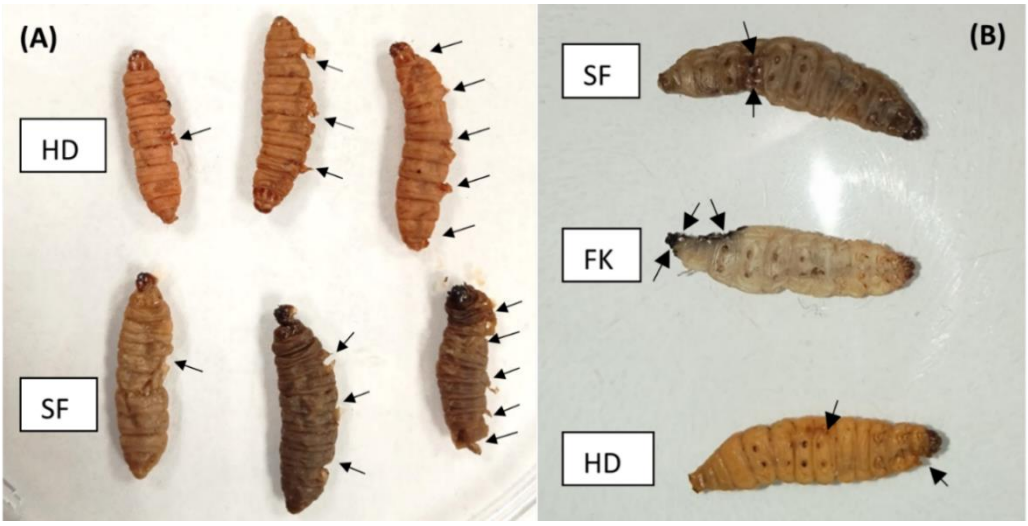

Figure 5.1. **(A)** Feeding damage simulated by a scalpel at three intensities: Low damage (1 cut), Medium damage (3 cuts) and High damage (5 cuts) and **(B)** examples of typical feeding damage incurred by *G. bimaculatus* on: *S. feltiae*-infected cadavers (SF), freeze-killed cadavers (FK) and *H. downesi*-infected cadavers (HD). Damage indicated by black arrows.

5.3 Results

5.3.1 Scavenging on nematode-infected cadavers

In the field trial, there were significant differences in scavenging between treatments, both the incidence of scavenging (Pearsons Chi square: $X^2_2 = 15.953$, $P < 0.001$) and its intensity as measured by weight loss (Kruskal Wallis*:* $H_2 = 16.08$, $P < 0.001$). *H.*

downesi-infected cadavers were less likely than freeze-killed cadavers to be damaged by scavengers (Fig. 5.2A) and all EPN-infected cadavers had a lower proportion weight loss compared to freeze-killed cadavers (Fig. 5.2B). There was no significant difference between the two EPN species in terms of scavenging or weight loss in the field trials.

In laboratory feeding trials with *G. bimaculatus* there was significant differences between treatments in incidence of feeding after 2 hrs, regardless of time since infection (2-3 DPI: $X^2_2 = 6.024$, $P = 0.049$; 5 DPI: $X^2_2 = 69.332$, $P < 0.001$). At 2-3 DPI *H. downesi*-infected but not *S. feltiae*-infected cadavers were fed on less than freeze-killed cadavers (Fig. 5.2.C). At 5 DPI both species of EPN-infected cadavers were fed on less than freeze-killed cadavers (Fig. 5.2E). After 24 hrs, the majority of cadavers had been damaged and incidences of feeding for infected cadavers (5 DPI) were not significantly different to freeze-killed cadavers (Pearsons chi square: $X^2_2 = 5.994$, $P = 0.05$). The level of damage was at a lower intensity for EPN-infected cadavers than for freeze-killed cadavers, most of which had been fully consumed (Fig. 5.1B, 5.2F). The lower intensity of damage on EPN-infected cadavers was also shown by less weight loss in EPN-infected cadavers compared to freeze-killed (2-3 DPI after 2 hr: Kruskal Wallis*:* $H_2 = 23.09$, $P < 0.001$, and 5 DPI after 24 hr: Kruskal Wallis: $H_2 = 66.97$, $P < 0.001$) (Fig. 5.2D, G). When comparing the two EPN species, there was a trend of less scavenging on cadavers infected with *H. downesi* compared to *S. feltiae* across all lab trials and time points, but differences between the species were not significant. *H. downesi*-infected cadavers suffered only bites, while some *S. feltiae*-infected cadavers were classed as partially consumed (Fig. 5.2A, C, E, F). This was

reflected by a lower weight loss in 5 DPI *H. downesi*-infected cadavers compared to 5 DPI *S. feltiae*-infected cadavers (Fig. 5.2G).

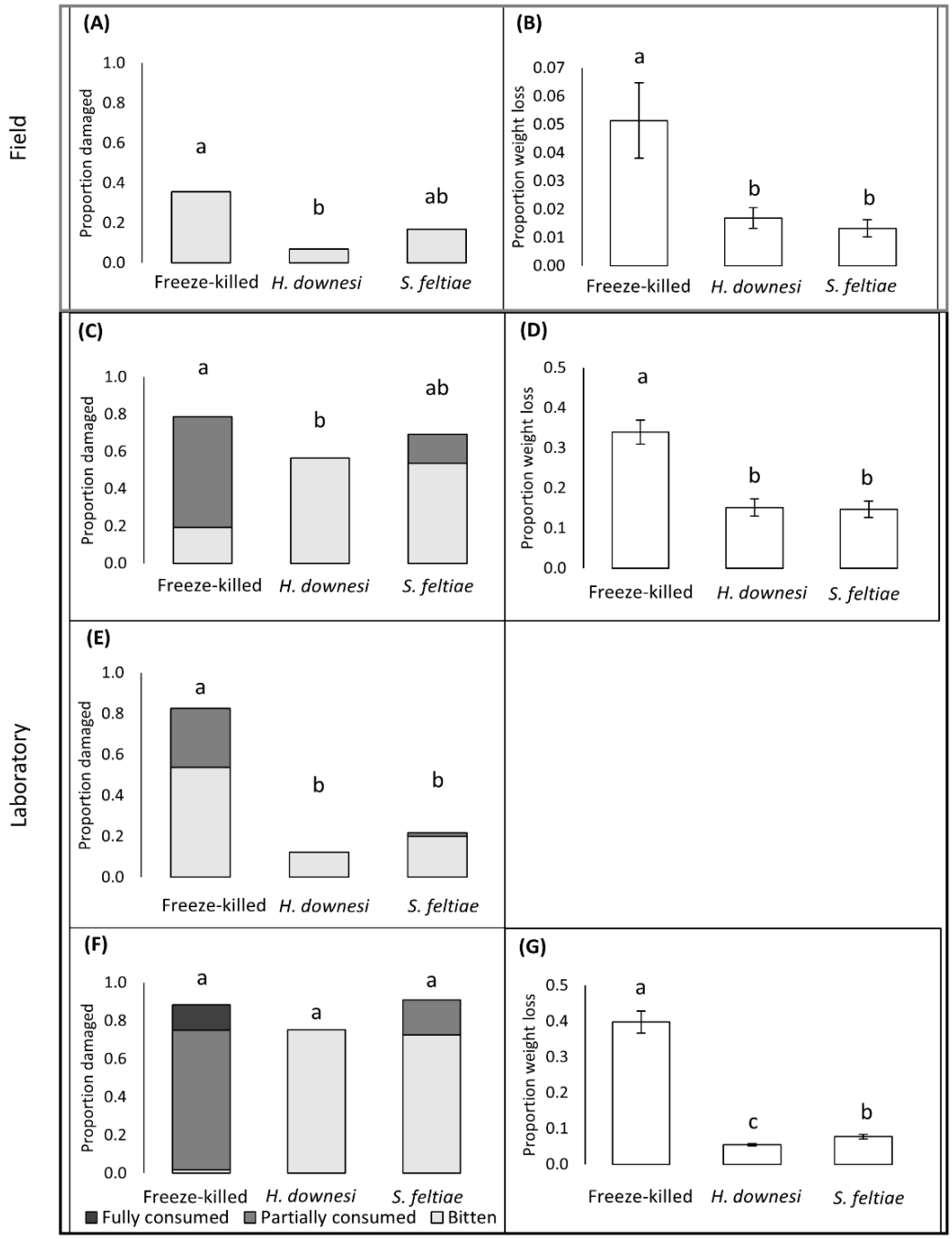

Figure 5.2. Scavenging on EPN-infected and freeze-killed (control) cadavers **(A, B)** in the field and **(C-G)** in the laboratory by field crickets. The proportion of cadavers showing signs of feeding damage (Left panels) and the proportion of weight lost by cadavers (mean ± SE) (Right panels) of: **(A-B)** Freshly infected (2-3 DPI) cadavers after 2-12 hrs in the field, (**C-D**) Freshly infected (2-3 DPI) cadavers after 2 hrs with crickets, (**E**) Infected cadavers (5 DPI) after 2 hrs with crickets and (**F-G**) and infected cadavers (5 DPI) after 24 hrs with crickets. Within a panel, bars accompanied by the same letter (lowercase) are not significantly different ($P < 0.05$), (**A, C, E, F**) Chi square, (**B, D, G**) Mann Whitney with Bonferroni correction).

5.3.2 Effect of simulated scavenger damage on EPN fitness

For each tested variable (Weight loss, Emergence and IJ length) the quality of fit of the most parsimonious GLMs, indicated by low ΔAIC values, were compared, and those with the best fit were selected for further analysis (Highlighted in Table 5.1).

Table 5.1. Results of models GLMs describing the variation for *S. feltiae* (SF) and *H. downesi* (HD) of **(1)** Weight loss (WL) of cadavers with Damage Level (DL), Damage Scale (DS), Host size (HS) and incubation Conditions (C) as explanatory variables. **(2)** Emergence (E) with DL, DS, HS, C and WL as explanatory variables, **(3)** IJ length (I) with DL, HS, E and WL as explanatory variables. The selected models are highlighted.

Experiment	*N*[1]	Model	Description	K[2]	LL[3]	AICc[4]	ΔAIC[5]
Weight loss							
SF Exp 1	**115**	**MZw15**	**WL ~ C * DS**	**9**	**118.30**	**-218.59**	**0.00**
		MZw17	WL ~ C *DS + HS	10	118.75	-217.50	1.50
SF Exp 2	**39**	**MAW1**	**WL ~ DS**	**5**	**57.91**	**-105.81**	**0.00**
		MAW5	WL ~ DS + HS	**6**	58.13	-104.26	2.36
HD Exp 1	**95**	**MZv11**	**WL ~ C + DS**	**6**	**70.03**	**-128.06**	**0.00**
		MZv14	WL ~ C + DS + HS	7	70.30	-126.60	1.79
HD Exp 2	**38**	**MXW5**	**WL ~ HS + DS**	**6**	**46.96**	**-81.93**	**0.00**
		MXW1	WL ~ DS	5	45.35	-80.707	0.39
Emergence							
SF Exp 1	**110**	**MZ10**	**E ~ WL**	**3**	**-1274.09**	**2554.20**	**0.00**
		MZ16	E~ WL + HS	4	-1273.14	2554.30	0.27
SF Exp 2	**39**	**MAE13**	**E ~ DS + HS**	**6**	**-416.61**	**845.23**	**0.00**
		MAE9	E~ DS + HS +WL	7	-415.88	845.75	1.52
HD Exp 1	**95**	**MX3**	**E ~ DL + WL**	**4**	**-1089.39**	**2186.80**	**0.00**
		MX13	E~ C*WL +HS	6	-1087.70	2187.40	1.12
HD Exp 2	**36**	**MXE4**	**E ~ WL**	**3**	**-383.43**	**772.85**	**2.93**
		MXE8	E ~ WL + HS	4	-380.69	769.38	0.00
		MXE7	E ~ DL + WL + HS	5	-380.60	771.21	2.53
IJ length							
HD Exp 2	**27**	**MX2t**	**I ~ DL**	**3**	**65.78**	**-125.56**	**0.00**
		MX9t	I ~ DL + HS	5	68.59	-127.18	0.19
SF Exp 2	**28**	**MA6**	**I ~ HS**	**3**	**33.07**	**-60.132**	**0.00**
		MA5	I ~ E	3	32.82	-59.65	0.48

[1]Number of observations (cadavers) of the tested variable, [2] the number of estimated parameters for each model, [3]the log-likelihood of each model, [4]Akaike's information criterion (corrected for small sample sizes),[5] the difference in AIC score between the lowest scoring model and the model being compared.

5.3.2.1 Host weight loss

Weight loss of cadavers infected with either *S. feltiae* or *H. downesi* increased with increasing levels of damage in both experiments, and this was exacerbated by dry conditions in experiment 1 (Fig. 5.3). For both species in both experiments, the qualitative variable "damage scale" was a predictor of cadaver weight loss and, where moisture was varied (Exp 1), this also made a significant contribution to the models (Table 5.2). For *S. feltiae*-infected cadavers in Exp 1 the interaction between damage scale and moisture conditions was also a significant predictor of cadaver weight loss. For *H. downesi*-infected cadavers in Exp 2 initial host size was a non-significant predictor of cadaver weight loss (Table 5.2).

Table 5.2. Results of models (GLMs) for *S. feltiae* (SF) and *H. downesi* (HD) describing the variation of proportion Weight loss (WL) of cadavers with simulated scavenger damage.

Experiment/species	Model	Factors	T value	Pr(>|t|)
SF Exp 1	MZw15	Conditions	-2.552	0.012
		Damage Scale Low	5.476	< 0.001
		Damage Scale Medium	9.727	< 0.001
		Damage Scale High	11.974	< 0.001
		Conditions: Damage Scale Low	-1.765	0.081
		Conditions: Damage Scale Medium	-4.426	< 0.001
		Conditions: Damage Scale High	-6.439	< 0.001
SF Exp 2	MAW1	Damage Scale Low	6.840	< 0.001
		Damage Scale Medium	9.707	< 0.001
		Damage Scale High	14.745	< 0.001
HD Exp 1	MZv11	Conditions	-7.69	< 0.001
		Damage Scale Low	6.184	< 0.001
		Damage Scale Medium	9.340	< 0.001
		Damage Scale High	11.53	< 0.001
HD Exp 2	MXW5	Host size	-1.709	>0.050
		Damage Scale Low	6.796	< 0.001
		Damage Scale Medium	10.831	< 0.001
		Damage Scale High	10.342	< 0.001

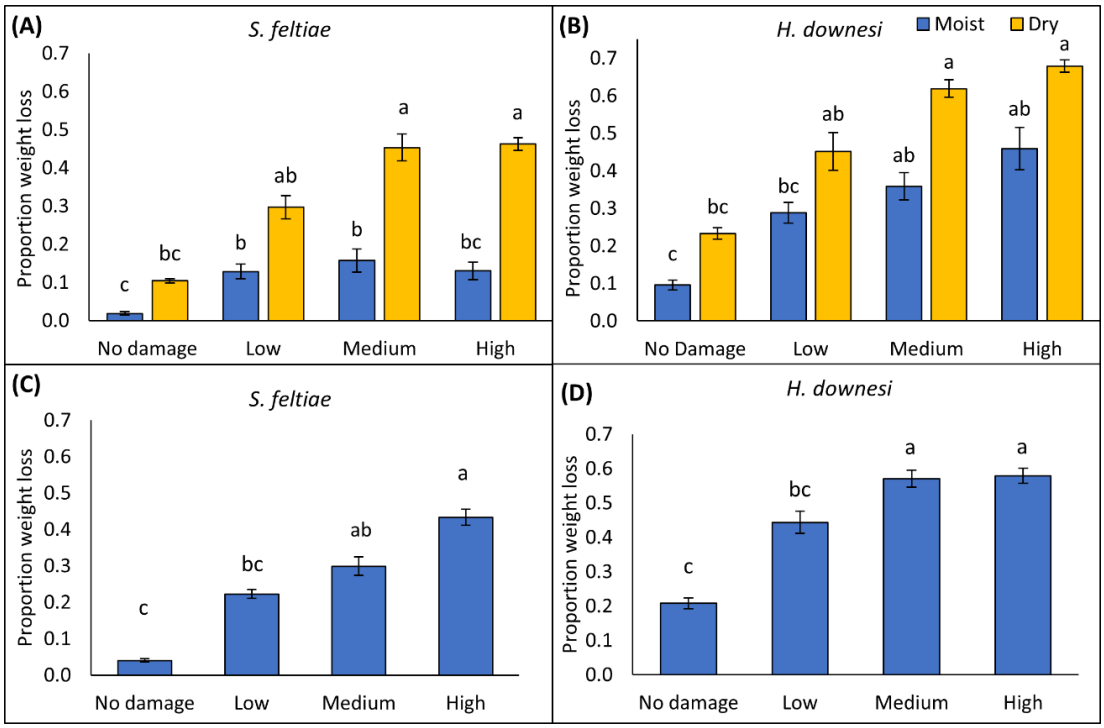

Figure 5.3. The proportion weight loss (Mean ± SE) at time of emergence of *S. feltiae*-infected cadavers **(A, C)** and *H. downesi*-infected cadavers **(B, D)**, under moist/ dry conditions with varying levels of damage. Exp 1 **(A, B)** and Exp 2 **(C, D)**. Bars accompanied by the same letter are not significantly different ($P < 0.05$, Dunn's test post hoc with Bonferroni correction).

Weight loss tended to be higher from cadavers infected with *H. downesi* compared to *S. feltiae* in both experiments. For example, undamaged cadavers in moist conditions infected with *H. downesi* lost ~5 times as much weight as those infected with *S. feltiae*, for both experiment 1 and 2, and undamaged cadavers in dry conditions infected with *H. downesi* lost ~2 times as much weight as those infected with *S. feltiae* (Fig. 5.3). The proportion of weight lost was calculated for each cadaver by weighing immediately after damage and again just before first IJ emergence (7-8 DPI for *S. feltiae*, 16-17 DPI for *H. downesi*). In order to determine if this difference between species in weight loss was due to differences in incubation time, or due to a difference in the rate of moisture loss, a separate set of cadavers infected with *S. feltiae* and *H.*

downesi at the four damage levels were weighed immediately after damage and again at day 5 and day 6 post infection. The rate of weight loss of infected cadavers increased with damage ($P < 0.001$) but did not differ between species (GLM, $P = 0.439$) (Appendix Fig. 5.1).

5.3.2.2 Effect of simulated scavenger damage on IJ emergence

For *S. feltiae*, no factor significantly affected the number of IJs that emerged in Exp 1. In Exp 2, however, the level of damage to the host, as well as the initial host weight were significant predictors for IJ numbers (Table 5.3). Fewer *S. feltiae* IJs emerged from cadavers with low and high levels of damage than from undamaged cadavers (Fig. 5.4). *S. feltiae* IJs emerged earlier from damaged cadavers than from undamaged cadavers under all conditions (Appendix Table 5.2). Thus, breaching of the cuticle of *S. feltiae*-infected cadavers, regardless of the level of damage, encourages early emergence and reduces the number of IJs that emerge from the host.

For *H. downesi*, weight loss was a significant predictor of IJ numbers in both experiments, with damage level also being a significant factor for Exp 1 (Table 5.3). The number of IJs per cadaver decreased with increased weight loss in both experiments (Fig. 5.5). In contrast to *S. feltiae*, there was a delayed time of first emergence of *H. downesi* IJs from damaged hosts in experiment 2 compared to undamaged hosts (Appendix Table 5.2).

Table 5.3. Results of models (GLMs) for *S. feltiae* (SF) and *H. downesi* (HD) describing the variation of total number of IJs emerged (E) from hosts with simulated scavenger damage

Experiment/species	Model	Factors	T value	Pr(>|t|)
SF Exp 1	MZ10	Proportion weight loss	-2.55	0.182
SF Exp 2	MAE13	Damage Scale Low	-4.92	< 0.001
		Damage Scale Medium	-2.93	0.006
		Damage Scale High	-4.35	< 0.001
		Host size	2.37	0.023
HD Exp 1	MX3	Damage Level	-3.09	0.003
		Proportion weight loss	-5.84	< 0.001
HD Exp 2	Mxe4	Proportion weight loss	-9.24	< 0.001

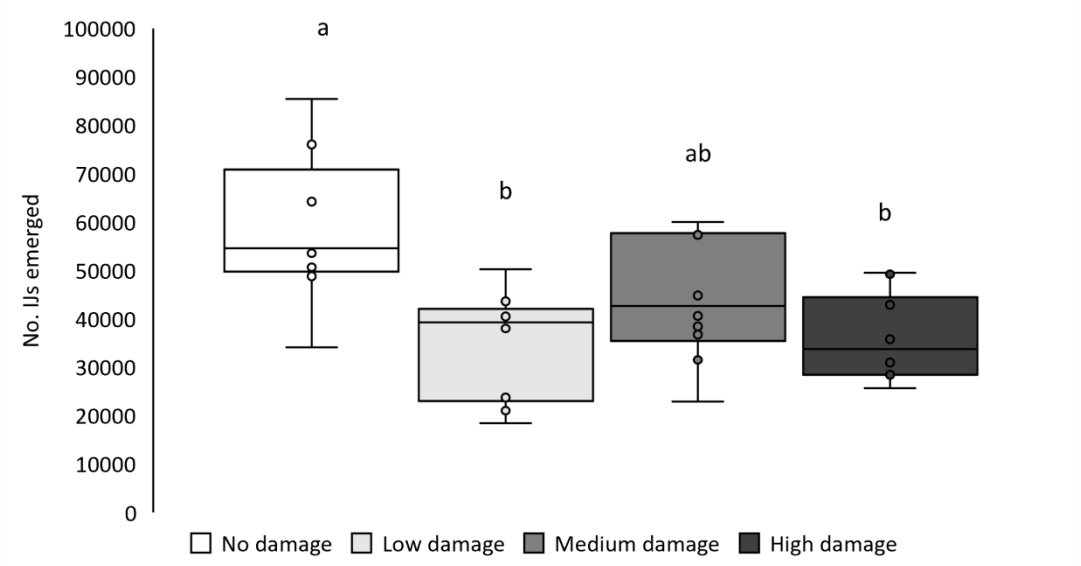

Figure 5.4. The number of *S. feltiae* IJs that emerged from cadavers that had their cuticle damaged in experiment 2. Data points are shown as circles, while whiskers indicate the highest and lowest values recorded. Boxes represent the interquartile range with the median indicated by a centre line. Bars accompanied by the same letter are not significantly different ($P < 0.05$, Dunn's test post hoc with Bonferroni correction).

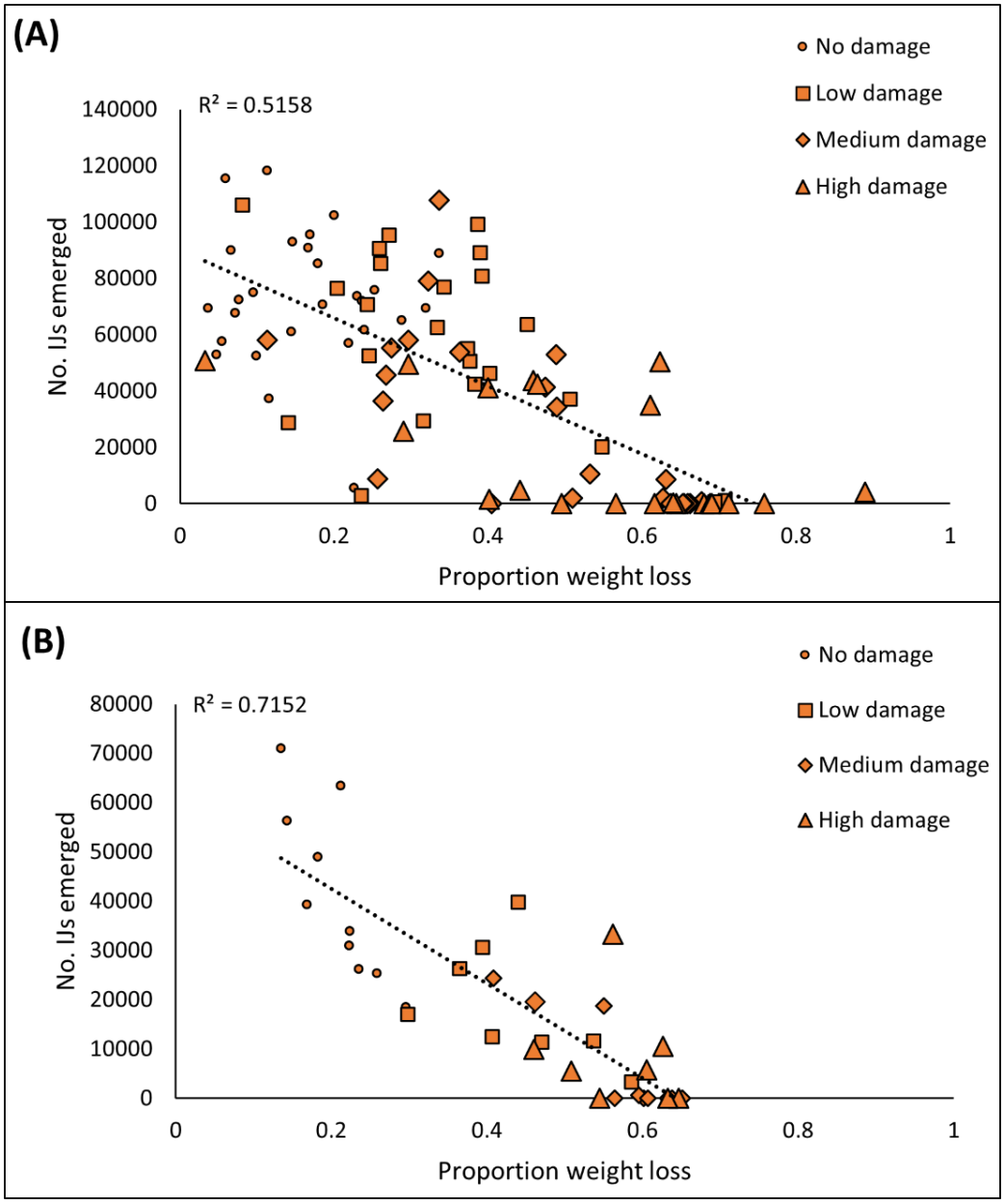

Figure 5.5. The relationship between the number of *H. downesi* IJs that emerged from cadavers that had their cuticle damaged and the proportion weight loss of the cadavers in experiment 1 **(A)** and experiment 2 **(B)**.

5.3.2.3 Effect of simulated scavenger damage on IJ length

The level of damage to the host was a significant predictor of IJ length for *H. downesi* (Table 5.4). There was a trend for decreasing length of *H. downesi* IJs emerging from cadavers with greater levels of damage (Fig. 5.6). For *S. feltiae*, none of the factors

tested affected the length of IJs; host size was a contributing factor, but it was not a significant predictor of length (Table 5.4).

Table 5.4. Results of models (GLMs) for *S. feltiae* (SF) and *H. downesi* (HD) describing the variation of mean length of IJs (I) from hosts with simulated scavenger damage

Species	Model	Factors	T value	Pr(>|t|)
SF Exp 2	MA6	Host size	1.821	0.0801
HD Exp 2	MX2t	Damage Level	-3.208	0.0036

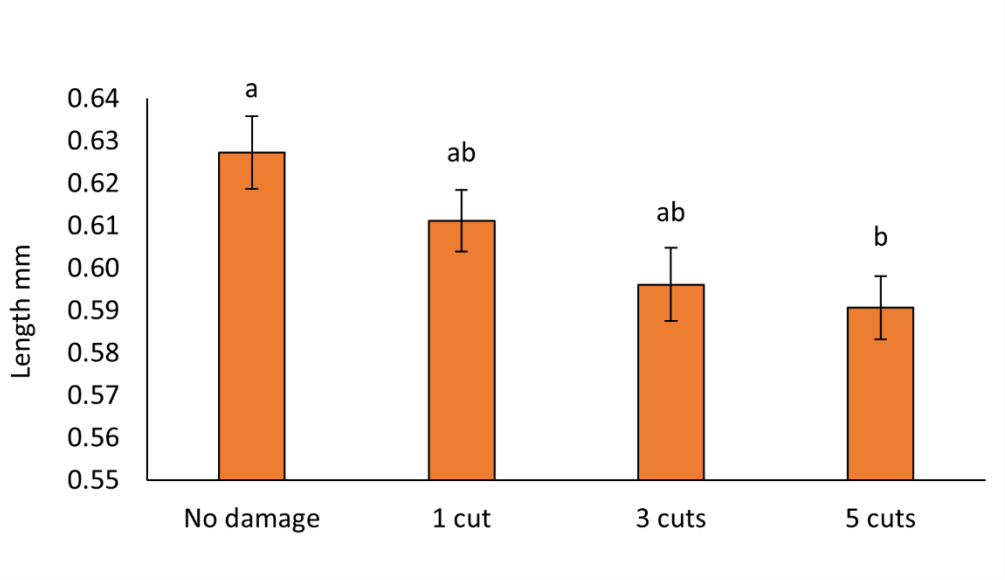

Figure 5.6. The length (Mean +/- SE) of *H. downesi* IJs that emerged from cadavers that had their cuticle damaged (1, 3 or 5 cuts). Bars accompanied by the same letter are not significantly different ($P < 0.05$, Dunn's test post hoc with Bonferroni correction).

5.4 Discussion

5.4.1 Scavenging on nematode-infected cadavers

Fewer EPN-infected insect cadavers than uninfected cadavers were fed on by scavengers in the field trials and by crickets in the lab trials. The ability of the

EPN/symbiont complex to deter feeding on the host cadaver has been noted for several scavenger species, including crickets (reviewed by Raja et al., 2021). This deterrence is proposed to be a combination of visual deterrents and a chemical "Scavenger deterrent factor" (SDF). As in previous studies (Baur et al., 1989; Gulcu et al., 2012; Ulug et al., 2014), there was a trend for a lower incidence of feeding on *H.* downesi-infected cadavers compared to *S. feltiae*-infected cadavers, however in our experiments this difference was not significant. This deterrence is mediated by the symbiotic *Xenorhabdus/ Photorhabdus* bacteria (Zhou et al., 2002; Raja et al., 2017). The SDF produced by *Photorhabdus* has been shown to be more effective than *Xenorhabdus* in some (Baur et al., 1998; Gulcu et al, 2012) but not all cases (Zhou et al., 2002) and varies depending on the symbiont species.

Visual and olfactory deterrents produced by the nematode-symbiotic bacteria act as a warning signal of unpalatability (Fenton et al., 2011; Jones et al., 2015, 2017). *Photorhabdus* has an additional visual deterrent of bioluminescence, which provides additional protection from nocturnal scavengers in the early stages of infection (Chapter 4).

In the case of either species, the majority of infected cadavers that showed feeding damage by scavengers had only small lesions to the cuticle. This was the case even when left with crickets for 24 hrs. There was also less weight loss, a proxy for consumption, in EPN-infected cadavers compared to uninfected cadavers. This indicates that the scavengers that pierced the cuticle did not continue feeding after tasting the infected cadavers. The production of foul-tasting chemicals or repulsive chemicals, such as SDF by the symbiotic bacteria would make the cadaver unappealing as a source of nutrition for the scavenger (Zhou et al., 2002; Gulcu et al., 2012, 2018).

This effect also appears to be more prominent in *H. downesi* infections than *S. feltiae* infections as all cadavers infected with *H. downesi* were only bitten, whereas some of the *S. feltiae*-infected cadavers were partially consumed.

In our experiments, cadavers infected with *H. downesi* / *S. feltiae* for 5 days had lower incidence of scavenging by 2 hrs than those infected for 3 days. The production of deterrent factors by *Photorhabdus* and *Xenorhabdus* appears to increase over the course of infection, leaving the cadavers more vulnerable to damage during the early stages of infection (Fenton et al., 2011; Gulcu et al., 2012; Ulug et al., 2014).

5.4.2 Effect of simulated scavenger damage on EPN fitness

For either species of nematode, lesion damage (simulating the effect of scavenger biting) to the host cadaver 3 DPI increased the proportion weight loss of the cadaver, indicating increased loss of moisture from the cadaver. This weight loss was exacerbated by dry conditions, presumably as result of increased desiccation. While cadavers infected with either species lost weight at the same rate, cadavers infected with *H. downesi* had undergone a greater proportional weight loss at the time of first emergence than cadavers infected with *S. feltiae*. This can be explained by the fact that *H. downesi* IJs emerge from their host much later than *S. feltiae,* presumably due to the slower development and longer generational time of these nematodes.

For *H. downesi,* the number of IJs that ultimately emerged from the cadavers was predicted by the level of damage to the cadaver, as well as the proportion of weight lost by the cadaver. Damage to the host cuticle (1, 3 or 5 cuts) increased the rate of desiccation of the cadaver and reduced the number of *H. downesi* IJs that emerged, in

a linear manner. Freezing and desiccation of the host have previously been found to reduce IJ yields (Koppenhofer et al., 1997; Lewis & Shapiro-Ilan, 2002). Drying of the host cadavers can lead to the death of progeny inside the host (Koppenhofer et al., 1997). Loss of moisture from the host would increase osmotic stress and reduce the physical space in the host. EPN are reliant on their symbiotic bacteria for nutrition (Poinar, 1979). Desiccation of the host would also increase stress on the symbiotic bacteria and likely reduce the bacterial titre, reducing the availability of nutrients. Overcrowding in the host can lead to reduced IJ emergence for EPN species (Koppenhofer and Kaya, 1995) and can also affect developmental time, time of first emergence and duration of emergence (Zervos et al., 1991; Flanders et al., 1996).

Damage level was also a predictor for the length of the emerging *H. downesi* IJs, which decreased with increasing host damage in a linear manner. This was likely due to the reduced availability of nutrients in damaged hosts. Unlike *H. downesi*, the level of damage to the host cadaver was not a predictor of IJ length for *S. feltiae* nematodes, but host size was a non-significant predictor. Poor conditions, such as increased resource competition, can lead to smaller individuals (Selvan et al., 1993; Nguyen and Smart, 1995). For example, IJ length decreased for *S. carpocapsae* and *H. bacteriophora* with increasing population density (Selvan et al., 1993).

For *S. feltiae* the number of emerging IJs was not predicted by cadaver weight loss but was lower in damaged than in undamaged host cadavers. Unlike *H. downesi,* increasing damage levels did not continue to decrease the number of emerging IJs. This indicates that while increasing levels of damage increased desiccation of the host cadaver, this was not a factor impacting *S. feltiae* IJ emergence. Different species of symbiont vary in their ability to survive desiccation (Maher et al., 2017) and

Xenorhabdus may be more resilient to drying than *Photorhabdus*. The reduced IJ emergence from damaged cadavers must be explained instead by some factor other than desiccation, such as reduced nutrient availability through competition. Opportunistic microbes compete with the EPN/symbiont complex for the nutrients in insect cadavers (Raja et al., 2021; Section 1.3.2). For example, *Bacillus thuringiensis* can compete with *Xenorhabdus* for resources and affect the development of *Steinernema* (Poinar et al., 1990). The *G. mellonella* larvae were not sterilised prior to use in these experiments and could have been carrying bacteria such as *Pseudomonas*, *Streptophyta* and *Enterococcus* spp. on their cuticle (Allonsius et al., 2019). Breaching the cuticle of the insect cadaver removes a barrier for these microbes and increases the risk of competition to the symbiont.

S. feltiae IJs also emerged earlier from the damaged host cadavers. Early development of IJs has been recorded in *Steinernema* nematodes developing in desiccated hosts (Serwe-Rodriguez et al., 2004). A proportion of juveniles from each generation develop into IJs, with a higher proportion developing per generation with limited resources (Nguyen and Smart, 1990; Ryder & Griffin, 2002). It is possible that a reduction in available resources led to the production of more IJs in earlier generations as a result. Dispersal of IJs from the host occurs in response to limited resources and overcrowding. It is stimulated by ascarosides produced by EPN as well as by the build of nitrogenous waste products in the host (San-Blas et al., 2008; Kaplan et al., 2012; Hartley et al., 2019). Early emergence of IJs from the host cadaver could also be a result of increased dispersal signalling due to poor conditions. Alternatively, early emergence may be due to easier escape through lesions in the host cadaver.

In contrast, *H. downesi* emerged later from damaged cadavers. The host cadavers for *H. downesi* were more desiccated as a result of their longer incubation. It has previously been shown that IJs may delay emergence from the host insect in dry conditions as the cadaver typically offers protection, however there is a risk of becoming trapped in the desiccated host (Brown & Gaugler, 1997). It is possible this was the case for *H. downesi* as several cadavers that experienced higher levels of damage showed no emergence of IJs. Dissection of the desiccated cadavers did not yield any living IJs.

As already mentioned, *Steinernema* and *Heterorhabditis* IJs that emerge later in the infection tend to be smaller than earlier emerging IJs (Nguyen and Smart, 1995), likely due to decreased nutrient availability and overcrowding. Larger, earlier emerging nematodes are more successful colonisers of new hosts compared to smaller, later emerging IJs (Therese and Bashey, 2012). While not tested here, the reduced size of the *H. downesi* IJs that emerged from damaged cadavers may negatively affect their ability to colonise new hosts, representing a further reduction in fitness on top of the reduced numbers. Reduced size is often a trade-off for more individuals in conditions with limited resources (Selvan et al., 1993; Nguyen and Smart, 1995). In this case, the reduced size, and therefore fitness, of the *H. downesi* IJs in damaged cadavers could be a compromise for survival of a greater number of IJs than if their size were not reduced.

Nematodes that are exposed to unfavourable conditions such as desiccation and freezing become more tolerant of these stresses (Womersley, 1990; Brown & Gaugler 1995; Serwe-Rodriguez et al., 2004; Mukaka et al., 2010). The stress tolerance of IJs

that emerged from damaged hosts was not investigated here but should be addressed in future studies.

Damage to the host's cuticle, simulating scavenger damage, at day 2-3 of infection adversely affected *H. downesi* more severely than *S. feltiae* in terms of IJ size and numbers. *H. downesi* nematodes have a longer development time and rely on the host cadaver for protection from environmental stresses for longer than *S. feltiae* nematodes do. As a result, if the host cadaver is damaged early in the infection, *H. downesi* nematodes are exposed to poor conditions for longer than *S. feltiae* nematodes are. It is possible that the *H. downesi /Photorhabdus* complex have evolved stronger scavenger deterrent defences, such as luminescence and bright pigmentation (Fenton et al., 2011; Jones et al. 2015, 2017), early in the infection time course, as a result of selection pressure due to this vulnerability. It is also possible that *Steinernema* nematodes have adapted a faster developmental cycle, in place of stronger scavenger deterrents, as a response to the threat of scavenger damage.

5.5 Conclusion

In conclusion, the fitness of EPN - in particular the number of emerging IJs - is greatly reduced when the cuticle of the host cadaver is damaged (simulating scavenging) during the early stages of infection, and this is likely due to desiccation of the host cadaver and increased entry of microbial contaminants. This effect is more severe for *H. downesi* than *S. feltiae,* including a reduction in the size of *H. downesi* IJs with increasing levels of damage. This selection pressure has resulted in both EPN species

and their symbiotic bacteria evolving a number of mechanisms to deter scavengers from feeding on and damaging the host cadaver.

Chapter 6. Discussion

6.1 General discussion

The availability of carrion helps shape ecosystem structure and species interactions (Selva & Fortuna, 2007; Wilson & Wolkovich, 2011; Moleón et al., 2014). The act of killing an organism is energy intensive and scavenging can be an attractive alternative (Mellard et al., 2021). Competition with scavengers can negatively affect a predators' ability to exploit the resources of its kill (Krofel et al., 2012; Bothma, & Walker, 2013; Elbroch & Wittmer, 2013). Similarly, scavengers can compete for a single resource, leading to the evolution of defences against competitors (Burkepile et al., 2006). For EPN the host cadaver represents not only a source of nutrition but also a territory that must be defended. Investment in defensive traits is costly and there is a trade-off between enacting defensive responses instead of mating or foraging. Defensive traits will only be conserved if beneficial to the survival and/or reproduction.

6.2 Genus specific adaptations

Heterorhabditis and *Steinernema* represent an interesting model for the study of resource defence for several reasons, including: (1) their existence as part of a multi-trophic system where the nematodes and their symbiotic bacteria co-evolve and (2) the evolution of the symbiont-nematode-insect system occurred twice, independently, and differences in the phylogeny (and consequent variations in morphology), as well as the chance events resulting in symbiotic associations, may have led to different defence mechanisms in the two genera in response to similar selection pressures. A limited number of representatives for each genus are studied in this thesis but the general comparisons of defensive traits show clear genus-specific adaptations.

6.2.1 Reproduction

The differing reproductive anatomy and behaviour of *Heterorhabditis* and *Steinernema* affect their competitive behaviours. *Heterorhabditis* males possess a caudal bursa, a feature commonly seen in the Strongylida sub-order, that allows them to attach to the female while parallel to each other to facilitate mating (Machado et al., 2015; Ahmed et al., 2022). *Steinernema* males do not have a bursa (Wright, 1990). They maintain their position while mating by coiling around the females. While this curling behaviour in *Steinernema* originated for the purposes of mating (Strauch et al., 1994; Lewis et al., 2002), it has been adapted for resource defence through lethal fighting, a form of interference competition (Zenner et al., 2014; O'Callaghan et al., 2014; Kapranas et al., 2016). In chapter 2, I show that like *S. longicaudum* males (Kapranas et al., 2020), *S. carpocapsae* males with previous mating experience are more efficient killers. As females are a limited resource for the first generation of *Steinernema* males that invade the host, this ability to kill competitors is an advantage in securing mating partners. Those males that are successful killers and survive encounters will have a greater opportunity to reproduce, passing on their genes and selecting for this trait. This lethal fighting is also beneficial when defending against other species of *Steinernema* (O'Callaghan et al., 2014), and potentially other nematodes.

Male *Heterorhabditis* do not engage in lethal fighting. As heterorhabditids are hermaphroditic in the first generation there is no selection pressure for securing a mate in this generation. In subsequent generations while many females are available, the presence of many males makes fighting less profitable (Zenner et al., 2014). Though

the hermaphroditic females can self-fertilise, the sperm from males has an advantage during fertilisation over the hermaphrodite's own sperm (Dix et al., 1994). Interference competition does occur in *Heterorhabditis* males in the form of sperm plugs, where males deposit material in the female's vulva after mating to block it (Dix et al., 1994; Machado et al., 2015). While this does not completely prevent other males from inseminating, it can delay them in finding the vulva, which discourages the male from mating (Barker, 1994; Machado et al., 2015). The use of copulatory plugs has not been observed in *Steinernema* to date (Lewis et al., 2002; Griffin, 2012; Machado et al., 2015).

6.2.2 Symbiosis

Both *Steinernema* and *Heterorhabditis* formed symbiotic associations with Enterobacterales bacteria at some point in their evolutionary history (Chaston et al., 2011; Sajnaga & Kazimierczak, 2020). Association with a symbiont represents a cost to the IJ as the bacterial cells must be sustained within the IJ between hosts (Emelianoff et al., 2007). IJs that carry fewer bacterial cells survive for longer indicating a detrimental effect of carrying the symbiont (Emelianoff et al., 2008). In order for this association to be maintained, this cost must be outweighed by the benefits of vectoring the symbiont into a host.

For *Heterorhabditis*, the bacterial symbiont *Photorhabdus* are essential for host mortality and providing the nutrition required for reproduction. It also inhibits microbial and FLBN competitors within the host (See sections 1.2.2; 1.3). In this thesis, the importance of *Photorhabdus* to *Heterorhabditis* for scavenger deterrence is

also demonstrated (Chapter 4). Insects usually die within 48-72 hrs after infection and are vulnerable to opportunistic scavengers after death. *Heterorhabditis* can occupy the host for at least 14 days (Chapter 5). Aside from being directly eaten, damage to the host cadaver leaves both *Photorhabdus* and *Heterorhabditis* within the cadaver vulnerable to desiccation, starvation, and microbial competition. While not tested here, we can assume that the earlier that this damage occurs the more detrimental the effect will be for the EPN. In this thesis I show that the fitness of *H. downesi* is greatly reduced in host cadavers that are damaged 72 hrs after infection, with greater levels of damage reducing both the size and number of emerging IJs (Chapter 5). I also show that the bioluminescence produced by *Photorhabdus* increases from 48 hrs, reaching its peak 72 hrs after infection, and deters invertebrate scavenges from damaging the host cadaver at this stage (Chapter 4). *Photorhabdus* also produces chemical deterrents, termed SDF, unpalatable metabolites that reduce the amount of damage caused by scavengers when feeding (Zhou et al., 2002; Gulcu et al., 2012; Raja et al., 2017; Chapter 4). In my experiments most scavengers that did feed on insects infected with *Heterorhabditis* for 72 hrs did not feed much, presumably deterred by the taste, and caused only low levels of damage (Chapter 4, 5). While the bioluminescence of infected hosts decreased after 72 hrs, these chemical defences continued to deter scavengers 120 hrs after infection (Chapter 5). Those bacteria whose bioluminescence are more effective as a deterrent, and the nematodes that vector those bacteria, would have an increased survival rate in the presence of scavengers. This scavenger pressure may select for *Photorhabdus* spp. with greater bioluminescence (Hyrsl et al., 2004; Maher et al., 2021). The volatiles prenol and butylated hydroxytoluene (BHT; a prenol derivative) produced by *Heterorhabditis*-infected insects act as an attractant for potential host insects (Zhang et al., 2019b; Baiocchi et al., 2017) and some of these

insects may also act as opportunistic scavengers. The ability to attract hosts in the soil, while deterring any potential scavengers from feeding, would be a major advantage to the *Heterorhabditis*/*Photorhabdus* complex.

While *Xenorhabdus* does produce SDF and can deter scavengers, it is not as effective as *Photorhabdus* at preventing scavengers from feeding on the host cadaver during the early stages of host death (as shown in Chapter 5; Baur et al., 1998; Gulcu et al., 2012; Ulug et al., 2014). This may partly be due to a lack of bioluminescence, as *Xenorhabdus* does not possess a *lux* operon, or due to production of different, less powerful chemical deterrents and/or lower concentrations of them. Based on the findings of Chapter 5, using *S. feltiae* and *H. downesi*, *Steinernema* species may not be as vulnerable to the negative impacts of damage to the host cadaver at time of death as *Heterorhabditis.* This is likely due to *Steinernema's* shorter generational time within the host (~7 days). It is reasonable to conclude that the selection pressure for *Xenorhabdus* to deter scavengers may not be as intense as that for *Photorhabdus.*

The bacterial symbiont also plays a key role in interspecific competition, both as a source of nutrition and by killing competitors. *Xenorhabdus* produces bacteriocins that are lethal to *Photorhabdus* (Boemare et al., 1992; Thappeta et al., 2020). As *Heterorhabditis* are reliant on *Photorhabdus* for nutrition this bacterial competition could be a contributing factor that reduces the likelihood of *Heterorhabditis* developing in a cadaver previously infected with *Steinernema* (Chapter 3). Similarly, *Photorhabdus* produces stilbenes and hydroxystilbene derivatives that are active against gram-negative bacteria and may be lethal to *Xenorhabdus* (Paul et al., 1980; Eleftherianos et al., 2007; Wollenberg et al., 2016). *Steinernema* are not completely reliant on *Xenorhabdus* for development, however their likelihood of developing in

hosts infected with *Heterorhabditis* 72 hrs earlier is reduced compared to healthy hosts, indicating bacterial competition is detrimental. Both *Photorhabdus* and *Xenorhabdus* may also kill competing nematodes through the production of nematicidal proteins (Hu et al., 1999; Sicard et al., 2006; Orozco et al., 2016).

In general, *Steinernema* appears less reliant on their symbiont *Xenorhabdus* for host mortality and nutrition compared to *Heterorhabditis'* reliance on *Photorhabdus*, yet the symbiosis is more selective, with each *Steinernema* species strictly associating with only one *Xenorhabdus* species, while *Heterorhabditis* can switch *Photorhabdus* symbionts (Stock and Goodrich-Blair, 2008; Murfin et al., 2015; McMullen et al., 2017a; Maher et al., 2017). The exact mechanism behind the relative specificity of the symbionts is unknown (Chaston et al., 2011; Murfin et al., 2012).

6.2.3 Chemical cues and communication

The ability to produce, recognise and respond to chemical cues aids EPN in avoiding competition for resources, both in terms of foraging (Grewal et al., 1997), and mating (Lewis et al., 2002). *Steinernema* and *Heterorhabditis* show differing responsiveness to chemical signals. Like Grunseich et al., (2021), this thesis shows that *Heterorhabditis* IJs were attracted to *Steinernema*-infected hosts (Chapter 3). Host insects are attracted to prenol and to BHT which accumulate in infected cadavers during infection (Baiocchi et al., 2017; Zhang et al., 2019b). Zhang et al., (2019b) demonstrated that BHT was also attractive to *H. bacteriophora* IJs and this, combined with the attraction of potential host insects, increased the infection success of these IJs.

If potential hosts are also attracted to *Steinernema*-infected cadavers, this may be an adaptation in *Heterorhabditis* for host finding.

Steinernema species show varying degrees of attraction to potential hosts based on their ability to reproduce within the host (Lewis et al., 1996). In this thesis I demonstrate that *Steinernema* IJs show beneficial avoidance of unsuitable hosts and are less likely to invade a host in which they cannot develop compared to *Heterorhabditis* IJs (Chapter 3). The chemical profile produced from cadavers infected by each species of EPN is distinct (Zhang et al., 2019b, Grunseich et al., 2021). Previous studies show varying response of *Steinernema* IJs to hosts infected with conspecific or heterospecific EPN depending on the combination of species (Grewal et al., 1997; Hu et al., 1999; McMullen et al., 2017a; Fu et al., 2020). In this thesis, *Steinernema* IJs were not attracted to *H. downesi*-infected cadavers but were attracted to *H. bacteriophora*-infected cadavers. *H. downesi/ P. temperata* may produce a chemical deterrent, which *H. bacteriophora/ P. thracensis* does not, that discourages competitors from invading (Chapter 3; Hu et al., 1999; McMullen et al., 2017a). This is beneficial to *H. downesi* as it reduces competition, and to the *Steinernema* IJs as their likelihood of developing to adult in these *H. downesi*-infected cadavers is reduced.

Steinernema males engage in lethal fighting to defend resources in the cadaver from both heterospecific and conspecific competitors. These males can differentiate between females of their own species and those of other species, presumably through the use of species-specific chemical cues (O'Callaghan et al., 2014). Within their own species *Steinernema* males can recognise females and determine their mating status (Lewis et al., 2002; Kapranas et al., 2020). In chapter 2, I show that they can also

determine the mating status of competing males. Attacking opponents to defend resources is costly, in terms of both time and energy, and risky as the attacker itself may be harmed. This ability to recognise both the species, as well as the mating status, of other nematodes allows *Steinernema* males to efficiently engage in aggressive behaviours only where it would be beneficial (Chapter 2). There is little evidence of *Heterorhabditis* using chemical cues for mate finding however there has been limited investigation into this area (Machado et al., 2015).

6.3 Applications of this work

6.3.1 Biocontrol

A major advantage of EPN as an alternative to chemical pesticides is their ability to reproduce and persist, reducing pest insect populations continuously after a single application (Kaya, 1990; Kaya and Gaugler, 1993). However, their persistence is highly variable (Duncan et al., 1996; Parkman et al., 1993; Smits, 1996; Dillon et al., 2008; Harvey et al., 2016) due to differences in environmental conditions as well as species-specific traits, including stress tolerance (Kung et al., 1991; Liu and Glazer, 2000; Molyneux, 1985; Shapiro-Ilan et al., 2006a). To date, the majority of studies on EPN as biocontrol agents have focused on IJs, including their survival, virulence and efficacy. As demonstrated in this thesis, many factors can also affect EPN survival within the host. This is especially pertinent as the focus for EPN application methods shifts from aqueous suspension to the use of infected host cadavers (Shapiro-Ilan et al., 2003). The differing ability of various EPN species and their symbionts to defend

the host cadaver and reproduce must be accounted for when choosing an appropriate species to apply in the field.

Heterorhabditis spp. have been found to have a poorer persistence in soil compared to *Steinernema* spp. (Molyneux 1985; Shapiro-Ilan et al., 2006). This is likely due, at least in part, to poorer tolerance to environmental stress. *Steinernema* IJs show greater tolerance than *H. bacteriophora* to desiccation and osmotic stress outside of the host (O'Leary et al., 2001; Chen and Glazer, 2004; Somvanshi et al., 2008). While the host cadaver offers protection from environmental stresses, it can be damaged by scavengers. This thesis demonstrates that *H. downesi* are better able to deter scavengers and protect the host cadaver from damage (Chapter 5, 4), while *S. feltiae* are better able to tolerate desiccation in hosts that are damaged compared to *H. downesi* (Chapter 5). While no difference in the rate of desiccation was observed for *H. downesi* and *S. feltiae*-infected hosts in this thesis (Chapter 5), the species/strain of the symbiont may affect the rate of desiccation of an infected host (Koppenhofer et al., 1997; Spence et al., 2011; Maher et al., 2017). For example, *G. mellonella* infected with *P. cinerea* show a slower rate of desiccation compared to those infected with *P. temperata* (Maher et al., 2017).

While co-infection of a host with multiple EPN can increase host mortality, competition reduces progeny production (Selvan et al., 1993a; Koppenhöfer and Kaya, 1995; Ryder and Griffin, 2002; Duncan et al., 2003; Campos-Herrera et al., 2019) and could affect the persistence of a species applied for biocontrol. Avoiding hosts already infected with EPN, both conspecific and heterospecific, is a desirable trait for biocontrol as it avoids dead-ending and redundancy. Of the species tested in the co-

infection study, *S. feltiae* IJs showed the greatest avoidance of unsuitable hosts (Chapter 3).

6.3.2 Conservation

An important consideration in the use of EPN for biocontrol is their effect on non-target organisms. The introduction of natural enemies for the purpose of biocontrol carries the risk of disrupting the native biota (Van Lenteren et al., 2006; Hajek et al., 2016). While some studies have investigated the impact of EPN on non-target organisms and found minimal effect (Somasekhar et al., 2002; Hodson et al., 2012), few have considered the impact of introducing non-native species of EPN on the native population (Ehlers and Hokkanen, 1996; Millar and Barbercheck 2001; Dillon et al., 2008; Harvey et al., 2016). In this thesis I have demonstrated that some species have advantages in interspecific competition. While EPN applied for biocontrol do not persist in the soil long term (< 5 years) (Smits, 1996; Dillon et al., 2008; Griffin, 2015; Harvey et al., 2016) they may compete with native EPN and other microbes in the soil during this time, supressing their populations. Millar and Barbercheck (2001) reported the suppression of endemic *H. bacteriophora* in soil after application of non-native *S. riobrave.* Native *S. feltiae* strains persist longer than non-native strains, presumably due to previous adaptation to the environment (Dillon et al., 2008). Ehlers and Hokkanen (1996) recommended the regulation of exotic species of EPN at the species level but not the strain level, and the unregulated use of indigenous species for biocontrol. Different strains of a single *Heterorhabditis* species can associate with different species/strains of *Photorhabdus* (Stock and Goodrich-Blair, 2008; Maher et al., 2017). The species of symbiont associated with a nematode can have an impact on

interspecific competition for EPN (Chapter 3) as well as scavenger deterrence (Chapter 4; Baur et al., 1988). Therefore, the use of exotic strains of an indigenous species of EPN, carrying non-native symbionts, could have unforeseen consequences on the native population, including the possibility of symbiont switching (Maher et al., 2017). An extensive study of the native soil biota and climate of a region should be taken into account before choosing a species for use in biocontrol, with a focus on using native EPN strains (Ehlers and Hokkanen, 1996; Van Lenteren et al., 2003; Abate et al., 2017) carrying native symbionts.

6.4 Contributions to the field and future work

This thesis offers a glimpse into some of the adaptations that *Heterorhabditis* and *Steinernema* have evolved in order to defend the resources of the host cadaver in a multi-trophic system. While several novel findings have been produced, they have raised many more questions, such as those listed below.

Lethal fighting is an uncommon in nature due to the high risk of mortality. Kapranas et al., (2020) demonstrated that mated *S. longicaudum* males are more likely to kill naïve males than vice-versa when paired in fights, raising the question of whether this advantage is due to an increase in the male's resource holding potential. In chapter 2, it is demonstrated for the first time that mated *S. carpocapsae* males kill more compared to virgin males when engaged in lethal fighting, independent of size or sexual maturity. It is still unclear whether this advantage is due to increased skill, increased confidence or other changes in motivation. Further investigations could elucidate this. It is also shown here that the aggressiveness of males varied depending

both on their own mating status and that of their opponent, which suggests that *S. carpocapsae* males can recognise if another male has mated. This has implications for the mating strategies of *S. carpocapsae*- if females can recognise this as well, it may influence their mate selection. Overall, this chapter also contributes to the study of contest behaviour, highlighting the relative importance of motivation vs ability in determining the outcome of lethal fighting.

It has previously been noted that when competing for a host, one genus of EPN will dominate over the other (Alatorre-Rosas and Kaya, 1991; Koppenhofer and Kaya, 1996b; Fu et al., 2020) but the reason for this dominance was unknown. Chapter 3 of this thesis is the first investigation into what happens to the invading IJs inside the host cadaver when two genera of EPN compete. It is shown here that the development of EPN in a host is impeded if the host was infected with EPN from another genus 24 hrs earlier. Whether the IJs that do develop to adult can reproduce or not has yet to be elucidated. This work is planned for the near future.

As the only terrestrial bioluminescent bacteria, *Photorhabdus* is an oddity. The function of this bioluminescence in *Photorhabdus*-infected hosts has long been debated (Poinar et al., 1980; Akhurst and Boemare, 1990; Peat and Adams, 2008; Peat et al., 2010; Waterfield et al., 2009; Zavilgelsky and Shakulov, 2018; Timsit et al., 2021; Cimen, 2023). This thesis demonstrates for the first time a plausible ecological explanation for this bioluminescence in *Photorhabdus*. In chapter 4, it is demonstrated that bioluminescence acts as a deterrent for scavengers, including the slug *L. valentina,* protecting *H. downesi*-infected cadavers from feeding damage. This bioluminescence may also act as an aposematic signal for scavengers who are not innately deterred, a

possibility which could have further implications for resource defence in *Heterorhabditis*-infected hosts.

While it has been proposed that low level scavenger damage to the host cadaver could impact the survival and fitness of EPN (Baur et al., 1998; Ulug et al., 2014), it has not been explicitly shown until now. In chapter 5, it is shown that *Steinernema* and *Heterorhabditis* are differentially affected by damage to the host cadaver. The number and size of emerging IJs was reduced in both cases. In future work, it would be interesting to see whether the infectivity of these IJs is also impacted.

As EPN exist in a multitrophic system their interactions can depend on the species and strain, their associated symbiont, the host insect used and the competitors they are exposed to in their natural environment (Lewis et al., 2006; Griffin, 2012; Maher et al., 2017). Due to time constraints a limited number of species were examined for this project across various studies. For a more comprehensive understanding of EPN defensive behaviour these investigations should be repeated with a greater variety of species and strains.

6.5 Conclusions

Competition for the limited resources within the host cadaver has resulted in the evolution of a variety of defence mechanisms in EPN, with genus-specific advantages depending in part on morphology/physiology and symbiotic partner. The adaptation of coiling, a mating behaviour in males, as a killing technique in lethal fighting reduces both intraspecific and interspecific competitors for *Steinernema* males. Chemical cues, indicating species, gender and mating status of other EPN, aid in regulating this

behaviour. Both genera of EPN can inhibit the invasion and development of competing EPN in a host in which they are established, reducing intraspecific competition. *Steinernema* can recognise insects as unsuitable and avoid invading hosts in which they cannot develop. *Heterorhabditis'* association with *Photorhabdus* aids in its protection of the host cadaver from scavengers, while *Steinernema's* faster developmental cycle limits the negative effects of damage to the host cadaver on the fitness of emerging IJs.

The findings of this thesis have implications for the use of EPN as biocontrol agents, as well as for the study of resource defence in multi-trophic systems. The limited number of species studied in this thesis offer a glimpse into how varied the response to resource competition is between *Steinernema* and *Heterorhabditis*, and further investigations should be carried out to determine the extent to which these defensive traits are conserved or modified across the two genera of EPN.

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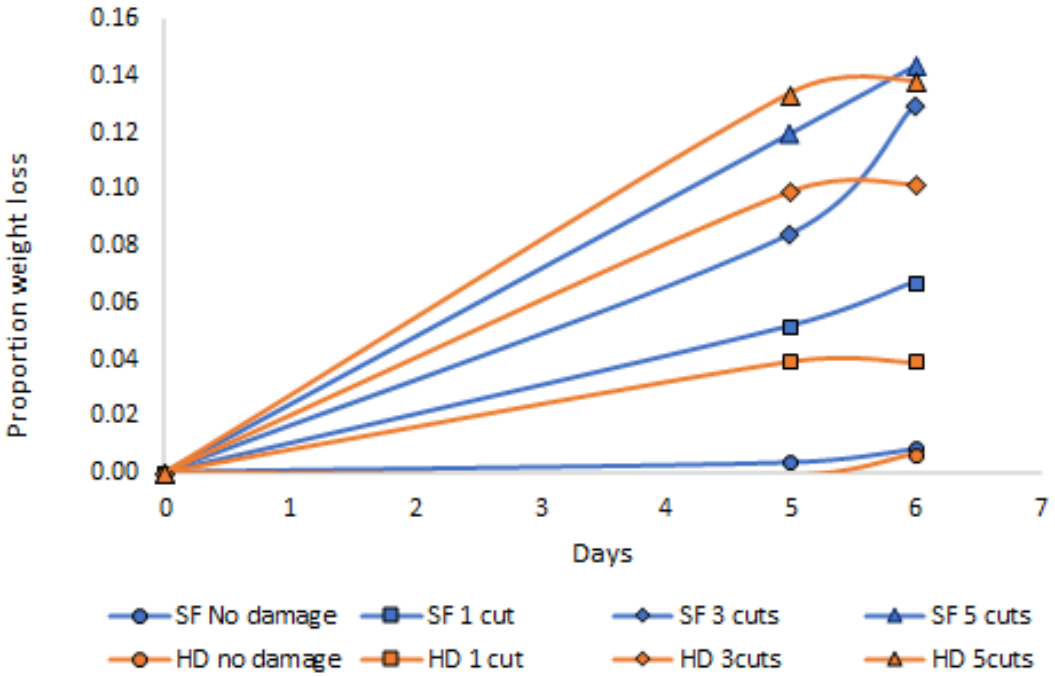
Appendices



Appendix Figure 5.1. The rate of weight loss over 6 days of *H. downesi* or *S. feltiae*-infected *G. mellonella* cadavers with varying levels of damage.

Appendix Table 2.1. Fighting behaviour in males that fought from each treatment in symmetric and asymmetric pairs.

Treatment	*N*	No. of pairs with fighting	No. of fights/pair (Median)	Duration of fights (secs) (Median)	Time till 1st fight (mins) (Mean + SE)	% Pairs with at least one male damaged or dead
Mated vs Mated	33	16	2	13	16.6 + 7.7	21
Pheromone vs Pheromone	45	7	1	13	15.4 + 11.7	18
Naïve vs Naïve	46	12	1	11.5	14.8 + 9.0	22
Mated vs Naïve	51	19	1	26.5	10.2 + 7.8	33
Pheromone vs Naïve	59	15	1	20	10.1 + 8.0	32

Appendix Table 2.2. Initiator in asymmetric pairings between a naïve and a conditioned (mated or naïve) male.

Treatment	*N*	No. of pairs with attacks	Attacker		Total no. of attacks		Median no. of attacks	
			Conditioned	Naïve	Conditioned	Naïve	Conditioned	Naïve
Mated vs Naïve	51	19	4	15	10	19	2	1
Pheromone vs Naïve	59	15	3	12	9	13	2	1

Appendix Table 3.1. Invasion of *Steinernema* and *Heterorhabditis* IJs into live (Live), freeze-killed (FK), *S. carpocapsae*-infected (SC), *S. feltiae*-infected (SF), *H. bacteriophora*-infected (HB) and *H. downesi*-infected (HD) *G. mellonella larvae* 24, 48 or 72 hrs since infection.

	S. carpocapsae invader			*S. feltiae* invader		
Treatment	*N*	*N* infected	% infected	*N*	*N* infected	% infected
Live	85	81	95	92	92	100
FK	54	35	65	50	49	98
HD 24	18	14	78	15	14	93
HD 48	21	7	33	21	20	95
HD 72	19	11	58	18	12	67
HB 24	13	11	85	10	9	90
HB 48	14	14	100	12	11	92
HB 72	12	12	100	12	9	75
	H. downesi invader			*H. bacteriophora* invader		
Live	35	35	100	41	40	98
FK	40	35	88	47	39	83
SF 24	14	10	71	22	17	77
SF 48	14	11	79	23	13	57
SF 72	13	8	62	20	14	70
SC 24	11	9	82	19	14	74
SC 48	12	10	83	22	16	73
SC 72	10	8	80	20	15	75

Appendix Table 4.1. Dates of sampling and environmental conditions of field sites, Carton House Demesne (53.383668, -6.573001) and Maynooth University Campus (53.377876, -6.600679).

Field site	Date	Temperature °C		Rainfall	Ground cover (%)				
		Soil	Air		Grass	Ivy	Bare	Litter	Other
Carton	24/6/21	14.1	15	Heavy	12	42	9	25	12
Carton	6/7/21	14.2	16.1	Light	2	51	15	22	10
Carton	22/7/21	19.7	23.5	Dry	5	35	20	35	5
Carton	17/8/21	14.6	16.4	Light	9	38	24	24	5
Maynooth	22/10/21	12.0	12.5	Dry	0	63	5	30	2
Maynooth	16/11/21	10.8	11.6	Dry	0	64	0	33	3

Appendix Table 4.2. Proportion of *Heterorhabditis*-infected (Infected) or freeze-killed (Control) cadavers fed on under conditions of light or dark for each field trial date.

Date	*N*	Dark		Light	
		Control	Infected	Control	Infected
24/06/2021	20	0.25	0.10	0.15	0.25
06/07/2021	10	0.40	0.16	0.30	0.30
22/07/2021	20	0.40	0.37	0.60	0.52
17/08/2021	11	0.55	0.00	0.36	0.09
22/10/2021	20	0.40	0.15	0.25	0.20
16/11/2021	26	0.23	0.08	0.15	0.15

Appendix Table 4.3. Mean proportion of weight loss of *Heterorhabditis*-infected (Infected) or freeze-killed (Control) cadavers in each field trial.

Date	*N*	Dark		Light	
		Control	Infected	Control	Infected
24/06/2021	20	0.035	0.013	0.046	0.014
06/07/2021	10	0.259	0.009	0.042	0.029
22/07/2021	20	0.321	0.151	0.579	0.156
17/08/2021	11	0.118	0.003	0.102	0.002
22/10/2021	20	0.041	0.024	0.040	0.026
16/11/2021	26	0.034	0.012	0.011	0.055

Appendix Table 4.4. Invertebrates found in dishes in field trials with *Heterorhabditis*-infected (Infected) or freeze-killed (Control) bait under conditions of light or dark. Numbers in parentheses represent the number of invertebrates found in dishes with signs of feeding.

Taxon		Dark		Light		**Total**
		Control	Infected	Control	Infected	
Gastropoda*	*Lehmannia valentiana*	7 (6)	5 (2)	6 (3)	4 (1)	**22 (12)**
	Arion fasciatus	2 (2)	5 (3)	2 (0)	6 (5)	**15 (10)**
	Arion (other)	2 (0)	5 (2)	4 (2)	4 (2)	**15 (6)**
	Limacus maculatus	2 (2)	0 (0)	0 (0)	0 (0)	**2 (2)**
	Tandonnia	1 (1)	0 (0)	1 (1)	1 (0)	**3 (2)**
	Zonitoides nitidus	0 (0)	0 (0)	1 (1)	1 (0)	**2 (1)**
	Other	1 (0)	3 (1)	3 (1)	2 (1)	**9 (3)**
Insecta	Diptera (Flies)	0 (0)	0 (0)	6 (6)	0 (0)	**6 (6)**
	Coleoptera (Beetles)	5 (3)	0 (0)	2 (2)	1 (1)	**8 (6)**
	Dermaptera (Earwigs)	0 (0)	0 (0)	1 (1)	0 (0)	**1 (1)**
Arachnida	Araneae (Spiders)	5 (3)	0 (0)	1 (0)	1 (0)	**7 (3)**
Malacostraca	Isopoda (Woodlice)	0 (0)	1 (1)	0 (0)	0 (0)	**1 (1)**
Unidentified		1 (1)	2 (0)	3 (2)	0 (0)	**6 (3)**
Total		26	21	30	20	**97 (56)**

[*Slug species were identified using a Key (Rowson, B., Turner, J., Anderson, R. & Symondson, B. (2013). Slugs of Britain & Ireland. Shropshire, UK: Field Studies Council.)]

Appendix Table 4.5. Proportion of cadavers fed on by *L. valentiana* slugs in each run of no-choice lab trials with *Heterorhabditis*-infected (Infected) or freeze-killed (Control) bait under conditions of light or dark.

Date	*N*	Dark		Light	
		Control	Infected	Control	Infected
15/09/2020	5	0.40	0.00	0.00	0.40
16/09/2020	4	0.00	0.00	0.00	0.20
21/09/2020	5	0.20	0.00	0.00	0.20
22/09/2020	5	0.00	0.00	0.00	0.20
29/09/2020	10	0.20	0.00	0.40	0.20
06/10/2020	10	0.10	0.00	0.20	0.00
15/10/2020	10	0.00	0.00	0.20	0.10
16/10/2020	10	0.30	0.00	0.10	0.00
24/10/2020	10	0.40	0.00	0.10	0.11
26/10/2020	10	0.30	0.00	0.10	0.00
30/10/2020	10	0.00	0.00	0.40	0.10
03/06/2021	5	0.80	0.20	0.20	0.40
16/06/2021	4	0.50	0.00	0.25	0.33
15/07/2021	10	0.29	0.00	0.38	0.10
19/07/2021	9	0.33	0.00	0.38	0.22
04/12/2021	9	0.22	0.00	0.11	0.11
08/12/2021	8	0.13	0.25	0.13	0.13
15/12/2021	10	0.60	0.18	0.40	0.20

Appendix Table 4.6. Mean proportion of weight loss of cadavers in each run of no-choice lab trials with *Heterorhabditis*-infected (Infected) or freeze-killed (Control) bait under conditions of light or dark.

Date	*N*	Dark		Light	
		Control	Infected	Control	Infected
15/09/2020	5	0.081	0.004	0.003	0.019
16/09/2020	4	0.011	-0.005	0.005	0.006
21/09/2020	5	0.009	0.002	0.003	0.010
22/09/2020	5	0.002	0.001	0.000	0.003
29/09/2020	10	0.050	0.005	0.049	0.008
06/10/2020	10	0.016	0.001	0.012	0.000
15/10/2020	10	0.008	0.002	0.061	0.001
16/10/2020	10	0.097	0.003	0.030	0.005
24/10/2020	10	0.052	-0.003	0.027	0.005
26/10/2020	10	0.040	0.002	0.006	0.005
30/10/2020	10	0.004	-0.001	0.047	0.003
03/06/2021	5	0.217	0.010	0.016	0.015
16/06/2021	4	0.066	-0.011	0.064	0.019
15/07/2021	10	0.011	0.000	0.121	0.000
19/07/2021	9	0.004	0.001	0.121	0.005
04/12/2021	9	0.016	0.003	0.002	0.000
08/12/2021	8	0.032	0.015	0.001	0.007
15/12/2021	10	0.108	0.026	0.069	0.005

Appendix Table 4.7. Mean proportion of time spent by *L. valentiana* slugs performing different behaviours in no-choice feeding trials with *Heterorhabditis*-infected (Infected) or freeze-killed (Control) bait under conditions of light or dark.

Treatment		*N*	Moving outside 2cm radius of cadaver	Moving within 2cm radius of cadaver	Searching	Tasting	Feeding	Null
Dark	Control	141	0.57	0.07	<0.01	0.03	0.03	0.29
	Infected	143	0.64	0.09	<0.01	0.02	0.01	0.23
Light	Control	141	0.59	0.08	0.01	0.03	0.03	0.26
	Infected	144	0.57	0.09	<0.01	0.01	0.03	0.29
P value (Kruskal Wallis)			0.177	0.268	0.119	0.929	0.016	0.216

Appendix Table 4.8. Proportion of time spent by slugs *L. valentiana* (*N* = 69) performing different behaviours in lit and dark chambers of a Y tube in a choice test. Each arm contained a freeze-killed *G. mellonella* bait and a light-emitting diode that was either lit or unlit.

Side arm	Moving	Searching	Tasting	Feeding
Lit LED	0.116	0.002	0.004	0.005
Unlit LED	0.120	0.002	0.001	0.000
P value (Mann Whitney U test)	0.798	0.349	0.20	0.11

Appendix Table 5.1. Field sites used for scavenging on *G. mellonella* cadavers infected with entomopathogenic nematodes.

Field site	Date	Location	Rainfall	Description of canopy
Navan Garden	21.11.19	Clogherboy, Navan, Co. Meath, 53.648917, -6.697855	Dry	Coniferous trees/ shrubs, grass
Maynooth Garden	21.11.19	Collegeland, Maynooth, Co. Kildare. 53.375893,-6.5981235	Dry	Hedgerow/shrubs, litter
Carton House	02.12.20	Carton Demesne, Railpark street, Co. Kildare. 53.382781, -6.571821	Light	Trees, ivy

Appendix Table 5.2. Emergence of *H. downesi* and *S. feltiae* IJs from manually damaged cadavers in all experiments.

Treatment		Cadavers infected	Cadavers with emergence	Day emerged (Median)	Kruskal-wallis H_3 value	*P*
H. downesi Exp 1 moist	No damage	14	14	18	7.82	0.05
	1 cut	15	15	22		
	3 cuts	14	13	22		
	5 cuts	13	13	23		
H. downesi Exp 1 dry	No damage	13	13	23	5.63	0.131
	1 cut	10	10	24		
	3 cuts	9	6	31		
	5 cuts	8	1	37		
H. downesi Exp 2	No damage	10	10	23	16.83	0.001
	1 cut	10	8	23		
	3 cuts	10	4	24		
	5 cuts	10	5	25		
S. feltiae Exp 1 moist	No damage	18	18	14	21.33	<0.001
	1 cut	20	20	9		
	3 cuts	20	20	9		
	5 cuts	18	18	9		
S. feltiae Exp 1 dry	No damage	18	16	15	21.00	<0.001
	1 cut	17	17	9		
	3 cuts	14	14	10		
	5 cuts	17	17	9		
S. feltiae Exp 2	No damage	9	9	10	14.95	0.002
	1 cut	10	10	8		
	3 cuts	10	10	7		
	5 cuts	10	10	8		

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Beware glowing cadavers: bioluminescence of nematode symbiont *Photorhabdus* protects nematode-infected host cadavers from nocturnal scavengers

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Photorhabdus spp. are the only known terrestrial bioluminescent bacteria. We show that the bioluminescence produced by these bacteria reduces scavenging activity on the insect cadavers they colonize. *Photorhabdus* spp. are the symbiont of the insect pathogenic nematodes *Heterorhabditis* spp. Together they kill insects and colonize the cadaver. The function of their bioluminescence has been the subject of debate, but here for the first time we demonstrate an ecological benefit. In our experiments, fewer *Photorhabdus temperata* -infected cadavers than uninfected cadavers were scavenged, but only in dark conditions where their bioluminescence would be visible. This was the case both in the field and in laboratory experiments with *Lehmannia valentiana* slugs (the primary scavengers found in our field tests). We also show that *L. valentiana* is innately deterred from scavenging on uninfected cadavers in proximity to light imitating the bioluminescence of *Photorhabdus*, indicating that luminescence can be a deterrent independent of chemical cues. We propose a multimodal defence where bioluminescence works together with the chemical defences also produced by *Photorhabdus* to deter scavengers, such as slugs, from feeding on the host cadaver, with the potential for aposematism.

KEYWORDS

bioluminescent bacteria, aposematism, *Heterorhabditis downesi*, *Lehmannia valentiana*, invertebrates

1 Introduction

Bioluminescence is the biochemical production and emission of light by a living organism (Widder, 1999), usually by the oxidation of a light-emitting molecule -a luciferin - by a luciferase enzyme (Haddock et al., 2010). Bioluminescence has evolved many times (Haddock et al., 2010; Lau and Oakley, 2021) in prokaryotes and eukaryotes including

bacteria, fungi, dinoflagellates, arthropods, and fish (Herring, 1994; Dunlap and Kita-Tsukamoto, 2006; Forey and Patterson, 2006; Baker et al., 2008). The original function of luciferin-luciferase systems may have been for detoxification of reactive oxygen species (McElroy and Seliger, 1962; Seliger, 1975; Rees et al., 1998; Labas et al., 2001), but bioluminescence now serves diverse ecological functions including defence, attraction of prey, and intraspecific communication (Herring, 1989; Buck and Case, 2002; Lewis and Cratsley, 2008). Distraction or deterrence of nocturnal predators and scavengers has been proposed as the function of bioluminescence in Dinoflagellata, Copepoda, Cnidaria and Coleoptera (Morin, 1983; Buskey and Swift, 1985; Latz et al., 1988; Branham and Wenzel, 2001; Herring and Widder, 2004).

In bacteria, while the mechanism of luminescence is well characterised (Meighen and Szittner, 1992; Forst and Nealson, 1996; Zavilgelsky and Shakulov, 2018), its functions are less well understood than in higher organisms (Nealson and Hastings, 1979; Timsit et al., 2021). Amongst the biochemical benefits that have been suggested are the detoxification of reactive oxygen species (Timmins et al., 2001) or the promotion of DNA repair (Czyz et al., 2000), but the likely importance of ecological functions involving perception by some other organisms is increasingly acknowledged (Nealson and Hastings, 1979; Zavilgelsky and Shakulov, 2018; Timsit et al., 2021). Many marine species of bioluminescent bacteria reside in specialised light organs of higher organisms (fish and squid) where they clearly provide light in return for nutrients from their host, whilst other marine species are postulated to use light to attract consumers that provide a medium for growth and a means of dispersal (Nealson and Hastings, 1979; Widder, 2010; Tanet et al., 2020; Ramesh and Bessho-Uehara, 2021). *Photorhabdus* spp. are the only known terrestrial bioluminescent bacteria; the function of the bioluminescence in this genus is debated (Peat and Adams, 2008; Waterfield et al., 2009; Zavilgelsky and Shakulov, 2018). *Photorhabdus* spp. are insect pathogens that occur as obligate symbionts of the *Heterorhabditis* spp. of nematodes. (Boemare, 2002; Clarke, 2008; Waterfield et al., 2009). The stress resistant, free-living infective juvenile stage of the nematode travels through soil and enters a living insect, releasing the bacteria from its gut. When the bacteria have grown to a certain density, they emit light at a wavelength of around 490 nm (Poinar et al., 1980; Maher et al., 2021). Pigmentation of the cadaver can shift the light emitted by 20-30 nm depending on the species of *Photorhabdus* and its characteristic colouration (Maher et al., 2021). Luminescence can start as early as 20 hours post infection and is visible to the human eye by 72 hrs (Poinar et al., 1980), but only in darkness. Buried in the soil, and/or at night, these luminescent insect cadavers would stand out as a source of light. Within 2-3 days of infection the bacteria help to kill the insect and enzymatically break down the cadaver, resulting in a bacteria-rich nutrient soup on which the nematodes feed (Waterfield et al., 2009; Dillman and Sternberg, 2012). The nematodes then develop to adulthood and reproduce within the host. Several generations may be produced over 2-3 weeks, until resources are depleted, after which the next generation of infective juveniles with their symbiont leave the cadaver in search of new hosts (Adams and Nguyen, 2002). Only the infective juvenile stage is capable of survival in soil conditions (Poinar, 1979).

Functions that have been ascribed to bioluminescence in *Photorhabdus* can be divided into: general biochemical functions, roles specialised to its ecological niche, and redundancy (Poinar et al., 1980; Akhurst and Boemare, 1990; Peat and Adams, 2008; Waterfield et al., 2009; Peat et al., 2010; Zavilgelsky and Shakulov, 2018; Timsit et al., 2021). The *lux* operon containing the genes responsible for bacterial bioluminescence had a single evolutionary origin and spread by horizontal gene transfer (Kasai et al., 2006); many of the common functions that are ascribed to the cellular processes that result in bacterial bioluminescence, including protection against free radicals and stimulation of DNA repair, have also been ascribed to *Photorhabdus* (Nealson and Hastings, 1979; Peat and Adams, 2008; Zavilgelsky and Shakulov, 2018). Amongst the adaptive functions related to *Photorhabdus*'s specialised niche that have been proposed are: signalling to the nematode to synchronise symbiosis (Waterfield et al., 2009); cell-to-cell communication with other bacteria (Timsit et al., 2021); attracting new insect hosts to the cadaver (Poinar et al., 1980; Patterson et al., 2015) or deterring scavengers from it (Akhurst and Boemare, 1990; Baur et al., 1998; Jones et al., 2015; Maher et al., 2021). It has also been suggested that since the acquisition by *Photorhabdus* of the *lux* operon from a marine bacterium, bioluminescence is a nonfunctional trait in the genus that is declining over time, due to lack of selection pressure in the terrestrial environment (Peat and Adams, 2008). The most widely hypothesised possible ecological function proposed for bioluminescence in *Photorhabdus* is deterring scavengers from damaging the host cadaver, but to date there is no evidence supporting this or any other proposed function (Poinar et al., 1980; Akhurst and Boemare, 1990; Peat and Adams, 2008; Waterfield et al., 2009; Peat et al., 2010; Zavilgelsky and Shakulov, 2018; Timsit et al., 2021; Cimen, 2023).

There is ample evidence that *Photorhabdus* spp. invest heavily in defence of the cadaver against both microbes and animals (reviewed by Raja et al., 2021), protecting both themselves and their essential nematode mutualist. They produce a wide variety of molecules (Bode, 2009; Cimen, 2023), some of which have antimicrobial activity and suppress competing bacteria and fungi - including agents of putrefaction (Li et al., 1995; Eleftherianos et al., 2007; Ullah et al., 2015; Tobias et al., 2018). Insect cadavers infected by *Heterorhabditis* and their symbionts are also protected against feeding by a variety of scavengers including ants, wasps, crickets and beetles, and this is attributed to the production of an as yet unidentified chemical called "scavenger deterrent factor" by *Photorhabdus* (Baur et al., 1998; Zhou et al., 2002; Gulcu et al., 2012; Ulug et al., 2014; Gulcu et al., 2018; Raja et al., 2021). There is some evidence that olfactory cues contribute to scavenger deterrence (Jones et al., 2015), and that the distinct red colouration typical of many *Photorhabdus*-infected cadavers can serve as a warning signal of unpalatability for daytime scavengers such as birds (Fenton et al., 2011; Jones et al., 2017). Jones et al.

(2017) proposed that a multimodal defence – chemical and visual – due to *Photorhabdus* may protect the cadaver, either by acting in concert and/or by being effective against different scavengers.

Here, we test the hypothesis that bioluminescence contributes to the protection of *Photorhabdus*-infected cadavers against nocturnal scavengers (Akhurst and Boemare, 1990), using *Photorhabdus temperata* Fischer-Lesaux, Villard, Brunnel, Normand & Boemare, carried by the nematode *Heterorhabditis downesi* Stock, Griffin & Burnell. *Photorhabdus temperata* emits light at a high intensity relative to other species of *Photorhabdus* tested (Hyrsl et al., 2004; Maher et al., 2021). We tracked the luminescence profile produced by *P. temperata* infected cadavers over the course of the infection to determine at what stage the luminescence would have its highest impact. In field trials at dusk, we compare scavenging on infected and uninfected insect cadavers under conditions either of ambient light, where only chemical defence is expected, or in darkness, where bioluminescence may be visible and contribute to cadaver defence. We conduct similar experiments in the laboratory using the scavenger encountered most commonly in the field trials, the slug *Lehmannia valentiana* Férussac. Most slugs are omnivorous, nocturnal scavengers that feed on dead vegetation, fungi, dead animals, and other detritus (Barnes and Weil, 1945; Jennings and Barkham, 1975; Keller and Snell, 2002). While they possess simple eyes (and certain light sensitive areas of the brain) that can detect light intensity, they do not form clear images (Zieger et al., 2009; Nishiyama et al., 2019) and seek food using olfactory cues (Gelperin, 1974; Gelperin, 1975; Kiss, 2017). Where organisms are protected in more than one modality, it can be difficult to disentangle the contribution of each modality to defence; artificial light sources are an important tool in demonstrating a defensive role of light where chemical defences are also effective (Underwood et al., 1997; Marek et al., 2011). We use light emitting diodes (LEDs) producing artificial light simulating that of a *Photorhabdus*-infected cadaver at peak luminescence in a choice experiment to test the effect of light alone on *L. valentiana* orientation and scavenging of freeze killed insects.

2 Methods

2.1 Species, storage and conditioning

Late instar *Galleria mellonella* L. larvae were supplied by Peregrine Live Foods (Chipping Ongar, UK) and Live Foods Direct (North Anston, UK). They were infected with *H. downesi* K122 (a native Irish strain carrying *Photorhabdus temperata* that imparts a yellow-orange colour to the infected cadaver) by applying 100 infective juveniles to each larva. The insects were incubated at 20°C and died 3 days later, and these freshly dead cadavers were used in experiments. Relative light intensity of the cadavers was recorded using 5-minute exposure in a Syngene G:Box Chemi HR16 BioImaging System using GeneSnap 7.12 software (SynGene, Cambridge, UK). Images (16 bit) were exported to Image J (v1.5) and analysed for mean grey value/pixel. To determine peak luminescence over the course of infection, the luminescence produced by two sets of infected *G. mellonella* (N = 13, 19) were measured at alternating 2 hr intervals from 1-19 days post infection.

Freshly dead cadavers at day 3 of infection with relative light intensity 24,723 ± 643.58 (mean ± SE) mean grey scale units/pixel were used in experiments. As controls, *G. mellonella* were frozen for 24 hours and thawed 2 hours prior to use.

Slugs *(Lehmannia valentiana*; synonym *Limax valentianus)* were collected from mature deciduous woodland. Identification of the collected slugs was confirmed by dissection and examination of genitalia (Forsyth, 2004) of a proportion (~10%) of the population. The slugs were maintained in plastic containers with a thin layer of damp soil at 15°C and 16:8 Light: Dark for 1 week, during which time they were fed fresh vegetables and dog food kibble (Bakers Small Dog, Purina, UK). Slugs used in experiments were 5-6 cm in length (fully extended).

2.2 Does bioluminescence deter nocturnal scavenging (field study)

Field trials were conducted at dusk (starting 2 hrs before sunset), in open deciduous woodland with ivy/grass ground cover. Ambient light levels were <200 lux at soil level (recorded using an RT MT30 digital lux meter). A single *H. downesi*-infected or freeze-killed cadaver was placed in a Petri dish (3.5 cm) with a hole (4 mm diam.) in the base to allow drainage. Each dish was placed in a shallow (2-3 cm deep) hole in the soil which was covered with a rigid polypropylene sheet (5 x 6 cm) resting on the soil surface. The cover was either translucent or opaque (black) to allow or prevent, respectively, light from entering the test arena below. There were thus four treatments, infected or freeze-killed cadavers in light or dark conditions (Figure 1A). Dishes were arranged in groups of 4 (1 dish of each treatment), with all dishes and all groups 1 m apart. After 2 hrs the number and identity of invertebrates in the dishes were recorded and the dishes were returned to the laboratory. Signs of scavenging were recorded and classed as: cadaver fully consumed, partly consumed, or small punctures in the cuticle. In addition, each cadaver was weighed at the start and end of the experiment and the proportion of weight lost was recorded.

The were 6 trials for this field study, from June to November 2021, at two woodland locations in County Kildare: Maynooth University Campus and Carton Demesne (Supplementary Table 1). Overall, a total of 426 cadavers were tested, 103-108 per treatment.

2.3 Does bioluminescence deter slugs (*L. valentiana*) from feeding? (lab study)

Based on the findings of the field trials, the slug *L. valentiana* was used as a model scavenger in lab trials. The slugs were allowed to feed on either a *H. downesi*-infected or freeze killed cadaver under conditions in which the bioluminescence would either be visible (i.e., in darkness) or not visible (i.e. in low level ambient light

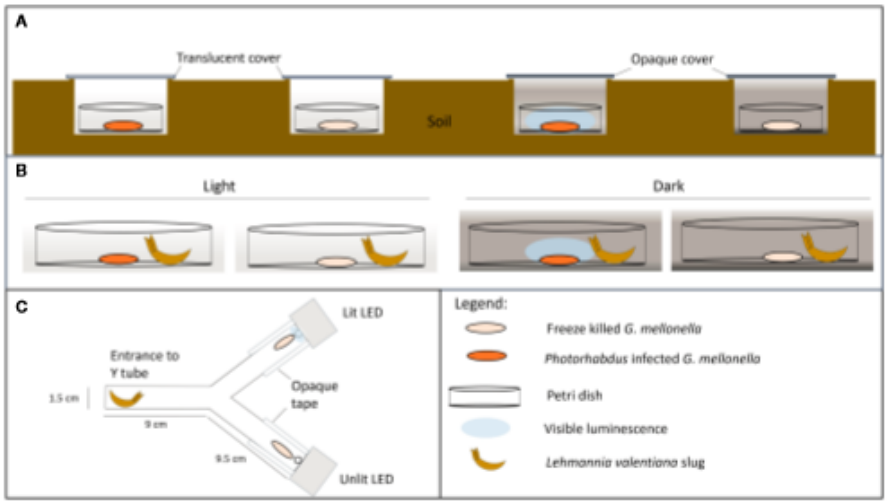

FIGURE 1
Experimental set up. **(A, B)** No-choice trials in which scavenging is recorded on *Photorhabdus temperata*-infected insect cadavers under conditions where bioluminescence is visible or not. Freeze-killed insects are included as controls. **(A)** Field trial: Insect cadavers are in soil pits covered with either translucent or black polypropylene squares, to allow or block light from entering the pit. **(B)** Lab trial: Insect cadavers are exposed to a scavenger slug (*L. valentiana*) under conditions of either light or dark. **(C)** Two-choice apparatus with either a lit LED (emitting at 470 nm) or unlit LED light positioned behind a freeze-killed insect at the end of each arm. A slug is placed in the entrance chamber at the start of the experiment.

(8-20 lux) provided by an LED bench lamp. Slugs which had been starved for 24 hrs before the experiment were placed individually in 9 cm Petri dishes, 2 cm from the centre of the dish. A cadaver (either infected or freeze-killed) was then introduced to the centre of each dish. As in the field trial, there were thus four treatments, infected or freeze-killed cadavers in light or dark conditions (Figure 1B). Slugs were observed for 2 hours, and their behaviour was recorded using the following categories: moving (within or outside a 2 cm radius of the cadaver), feeding (rasping the cadaver), tasting (touching the cadaver with mouth parts), searching (rotation of the tentacles), or none of the above. Red light was used for observations in the dark condition. The experiment was conducted at 16°C. After 5 hrs the cadavers were removed, and signs of scavenging were noted. The weight of each cadaver was recorded before and after the trial and the proportion weight loss was calculated. There were 18 trials for this lab study. Overall, a total of 569 slugs were tested, 141-144 slugs per treatment.

2.4 Does cadaver-mimicking light attract or repel slugs, or affect their feeding?

In order to separate the effects of light from those of chemical defences, a *L. valentiana* slug was given a choice between two freeze-killed insects; one of which was paired with artificial light of a similar wavelength and intensity to that of a *Photorhabdus* infected insect, and the other was not. The experiment was carried out in the dark, at 16°C. A freeze-killed *G. mellonella* was placed at the end of each arm of a Y shaped tube directly in front of a 5 mm-diam. LED (HLMP-AB64/65, Avago Technologies Inc., USA) which was either lit or unlit (Figure 1C). The lit diodes emitted light at 470 nm and the relative light intensity (measured as described for cadavers) was adjusted to fall within the range 15,000-35,000 (mean 24303) mean grey scale units/pixel to mimic the intensity of light emitted by bioluminescent cadavers at days 3-6 of infection (Figure 2). The sides of each arm were covered with opaque tape and tubes were orientated in different directions to avoid positional effects. A single *L. valentiana* slug that had been starved for 24-48 hrs was placed in the entrance chamber of the Y-tube, and its behaviour was observed for 2 hrs under red light. The time spent in each of the following behavioural categories was recorded for each arm: moving, feeding, tasting, searching or none of the above. After 5 hrs the cadavers were removed and examined for signs of scavenging. The proportion of weight loss of each cadaver was calculated. The experiment was conducted across 13 trials with a total of 98 slugs tested.

2.5 Data analysis

For each experiment, the results for repeated trials were pooled prior to analysis. The incidence of scavenging was analysed using Pearson's Chi square with significance set at $P < 0.05$, and with Bonferroni correction for multiple *post hoc* comparisons. Weight loss of cadavers in field and lab trials, and proportion of time spent feeding, moving, tasting, and searching in the non-choice lab test were compared between treatments using Kruskal-Wallis tests with significance at $P < 0.05$. Where significance was detected, this was followed by Mann-Whitney U tests with Bonferroni correction applied for multiple comparisons. For the choice experiment, slugs that did not move into either arm were excluded from the analysis, resulting in 69 replications. A Wilcoxon signed rank test was used to

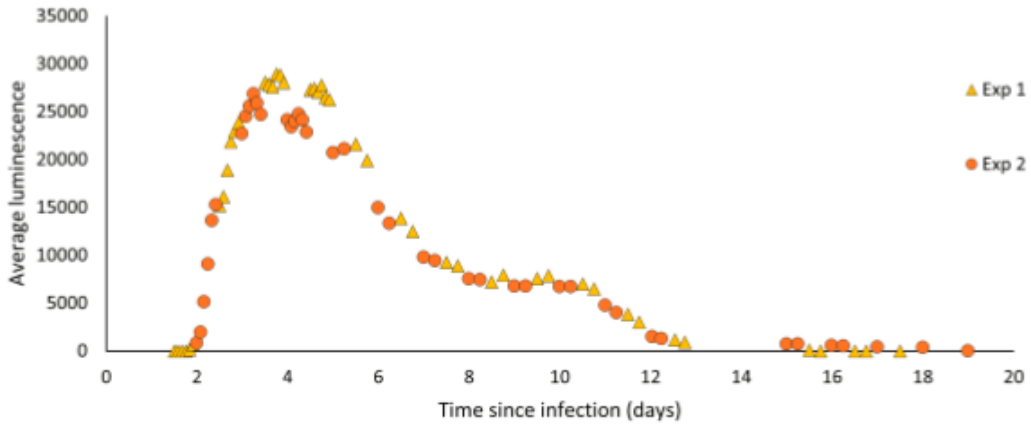

FIGURE 2
The average luminescence (mean grey scale units/pixel) produced by *P. temperata*-infected *G. mellonella* from time of exposure to time of emergence. Larvae infected with 100 IJs of *H. downesi* were measured at 2–4 hr intervals for two sets of infections (Experiment 1 and 2).

compare time spent in each arm of the Y tube, with a hypothesised proportion of 50%. The proportion weight loss of the cadavers and slug behaviours for this experiment were analysed using a two-sample t-test and Mann-Whitney U tests, respectively. All data analysis was performed using Minitab v. 20.3 statistical software or R studio v. 2022.12.0 + 353.

3 Results

The average luminescence of *H. downesi* infected *G. mellonella* increased from day 2 of infection, peaked at day 3 at 28924.3 ± 1132.482 (mean ± SE, N = 19) mean grey scale units/pixel and decreased from then, ceasing completely by day 19 (Figure 2).

3.1 Does bioluminescence deter nocturnal scavenging (field study)?

Slugs were the most frequently recorded potential scavengers, accounting for 70% (68/97 individuals) of all invertebrates found in the dishes, with *L. valentiana* being the most common slug species, accounting for 23% (22/97) of all invertebrates found. Other potential invertebrate scavengers recorded in the dishes included earwigs (Dermaptera) and beetles (Coleoptera) (Supplementary Table 4).

There were significant differences between treatments in scavenging rate (the proportion of cadavers showing signs of biting or feeding) by nocturnal scavengers (Chi square: X^2_3 = 10.39, P = 0.0155, N = 103-108) (Figure 3A; Supplementary Table 2) and in the weight loss of cadavers, used as a proxy for amount consumed (Kruskal-Wallis: H_3 = 18.09, P < 0.001, N = 103-108) (Figure 3B; Supplementary Table 3). Scavenging rate and consumption were lower for *P. temperata*-infected than for freeze-killed cadavers, but the differences were significant only for those under dark covers, where luminescence would be apparent, but not for cadavers under translucent covers, indicating that bioluminescence is effective in reducing scavenging (Figures 3A, B). The majority of those cadavers that had evidence of scavenging had only small puncture wounds in both the infected treatments (Dark: 10/15; 66.7%, Light: 23/28; 82.1%) and the controls (Dark: 25/37; 67.6%, Light: 17/31; 54.8%). Of the cadavers that were attacked, the proportion that were fully consumed was approximately 3.6 times lower in the infected treatments than in the controls both in the dark (1/15; 6.7% *vs* 9/37; 24.3%) and in the light (3/28; 10.7% *vs* 12/31; 38.7%).

There was no difference between treatments in their association with invertebrates, either in incidence (the proportion of dishes containing at least one invertebrate along with the cadaver) (Pearsons's chi square: X^2_3 = 1.382, P = 0.710, N = 103-108) or in numbers of individuals recorded (Kruskal Wallis: H_3 = 0.85, P = 0.838, N = 103-108).

3.2 Does bioluminescence deter slugs (*L. valentiana*) from feeding (lab study)?

In laboratory tests with *L. valentiana* there were significant differences between treatments in the proportion of cadavers showing signs of scavenging (Pearsons's chi square: X^2_3 = 33.968, P < 0.0001, N = 141-144) (Figure 3C; Supplementary Table 5), in weight loss of cadavers- a proxy for amount consumed (Kruskal-Wallis: H_3 = 65.71, P < 0.001, N = 141-144)(Figure 3D; Supplementary Table 6), and in the amount of time the *L. valentiana* slugs spent feeding on the cadavers during the 2 hr observation (Kruskal-Wallis: H_3 = 10.26, P = 0.016, N = 141-144) (Figure 4; Supplementary Table 7). There was less scavenging (attack and consumption) by *L. valentiana* on *P. temperata*-infected than on control cadavers. The difference between infected and control cadavers in the incidence of scavenging (Figure 3C) and in time spent feeding (Figure 4) was significant only in the dark when luminescence would be visible, and not in the light, when luminescence would not be apparent, indicating a

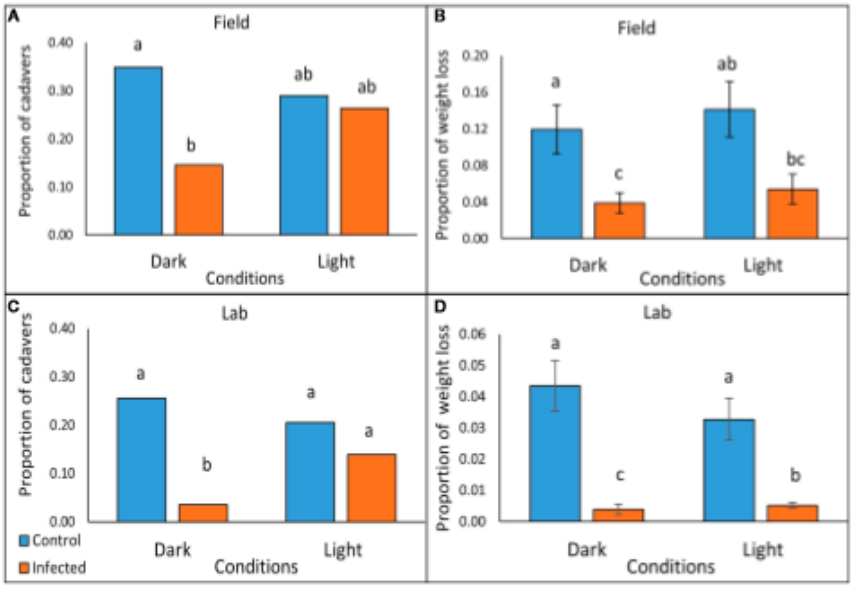

FIGURE 3
Scavenging on *P. temperata*- infected and freeze-killed (control) *G. mellonella* cadavers under conditions of either light or dark, after 2 hrs in the field **(A, B)**, and after 5 hrs exposure to slugs (*L. valentiana*) in the laboratory **(C, D)**. **(A, C)** The proportion of cadavers tested that showed incidence of scavenging in the field and laboratory trials respectively (columns accompanied by the same letters are not significantly different at $P < 0.05$, Chi square with Bonferroni correction). **(B, D)** Weight loss as a proportion of the cadaver (mean +/- SE) of *G. mellonella* tested in the field and laboratory respectively (Columns accompanied by the same letters are not significantly different at $P < 0.05$, Mann Whitney with Bonferroni correction).

defensive role for bioluminescence. In the light conditions, the amount of cadaver consumed was lower for infected than for freeze-killed, indicating the operation of chemical defences. The amount of infected cadaver consumed in the dark was lower than in ambient light, clearly showing the additive effect of both defence modalities (Figure 3D). Time spent on behaviours other than feeding (moving close to the cadaver, moving at a distance from the cadaver, searching, tasting) did not differ between treatments (Supplementary Table 8).

3.3 Does cadaver-mimicking light attract, repel, or affect feeding of slugs?

There was no evidence that the slug *L. valentiana* was either repelled or attracted by artificial light of similar wavelength and intensity to a nematode-infected insect. When given a choice of two arms each with a freeze-killed *G. mellonella* as bait either paired or not paired with a light source, they spent an equal proportion of time in each arm (Wilcoxon signed rank test: $Z = 1152.50$, $P = 0.980$; $N = 69$), and there was no difference in the amount of time spent moving, searching, tasting, or feeding in the two arms (Supplementary Table 7). However, there was a lower incidence of scavenging on the bait insect paired with a light than on the bait insect without a light (Pearson's Chi square: $X^2_1 = 3.881$, $P = 0.049$; $N = 69$) (Figure 5). There was no difference in the percentage weight loss of cadavers in the two conditions (Mann Whitney W= 4989, $P = 0.411$; $N = 69$).

4 Discussion

Our findings support the scavenger-deterrence hypothesis (Akhurst and Boemare, 1990) for bioluminescence in *Photorhabdus*. In both our field and laboratory trials, a lower rate of scavenging on *Photorhabdus*-infected cadavers compared to controls was significant only under dark conditions, where bioluminescence would be visible, providing strong evidence that bioluminescence contributes to scavenger deterrence, while the experiment with an artificial light source indicates that bioluminescence alone could protect cadavers. Cadavers were not completely undefended in ambient light conditions, since the amount of cadaver consumed was reduced in the light as well as the dark, indicating the operation of a chemical "scavenger deterrent factor" (Zhou et al., 2002; Gulcu et al., 2012). We suggest that the two defences operate slightly differently, with chemical factors reducing the amount of cadaver consumed while light serves to reduce the probability of a cadaver being damaged in the first place. Preventing breaches of the cadaver cuticle is important; even a small lesion without further consumption may completely compromise the success of the nematode-bacterial complex developing inside, by increasing vulnerability to

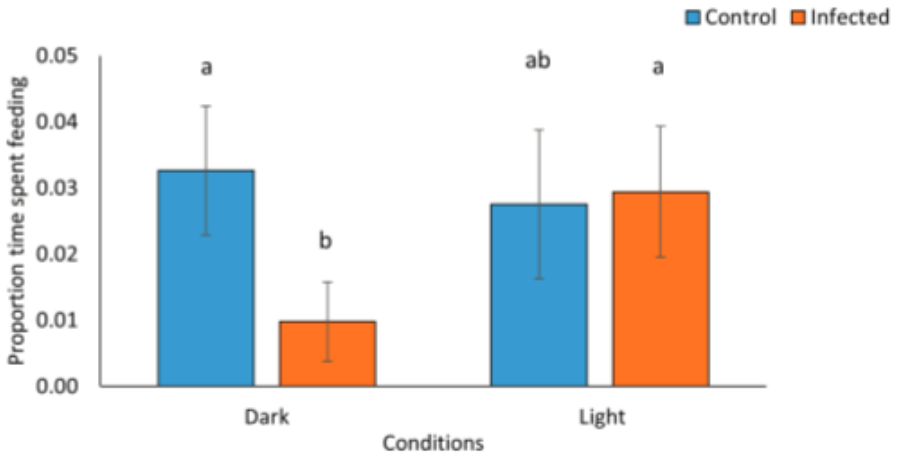

FIGURE 4
Proportion of time *L. valentiana* slugs spent feeding on *P. temperata*- infected or freeze killed (control) *G. mellonella* cadavers under conditions of either light or dark in no-choice laboratory experiments. Values accompanied by the same letter are not significantly different (Mann Whitney tests with Bonferroni correction, $P < 0.05$).

desiccation or competing organisms (Koppenhöfer et al., 1997; Baur et al., 1998; Serwe-Rodriguez et al., 2004).

The scavenger-deterrent hypothesis contrasts with the earlier suggestion by Poinar et al. (1980) that light could attract insects which could then be infected by, or serve to transport, the next generation of nematodes as they emerge from the cadaver into the soil. While there is some evidence that insects are attracted by the light emitted by *P. luminescens* (Patterson et al., 2015), attraction of new hosts is implausible as an ecological function of bioluminescence [as indeed was recognised by Poinar et al. (1980)]. As we show, it peaks within days of infection, long before the new infective stage nematodes have been produced and are ready to emerge from the host. In *H. downesi*, for example, the first infective juveniles do not emerge until at least 15 days after infection, by which time luminescence has declined to a low level. In contrast, peak luminescence occurs at a time when its role in defence against scavengers would be important: the nematodes developing inside are at a vulnerable stage (Poinar, 1979) and cadavers are otherwise poorly defended (Baur et al., 1998; Fenton et al., 2011; Gulcu et al., 2012; Ulug et al., 2014; Jones et al., 2015, Jones et al., 2017). Bioluminescence is produced once *Photorhabdus* reaches a critical population density (Meighen, 1999), while

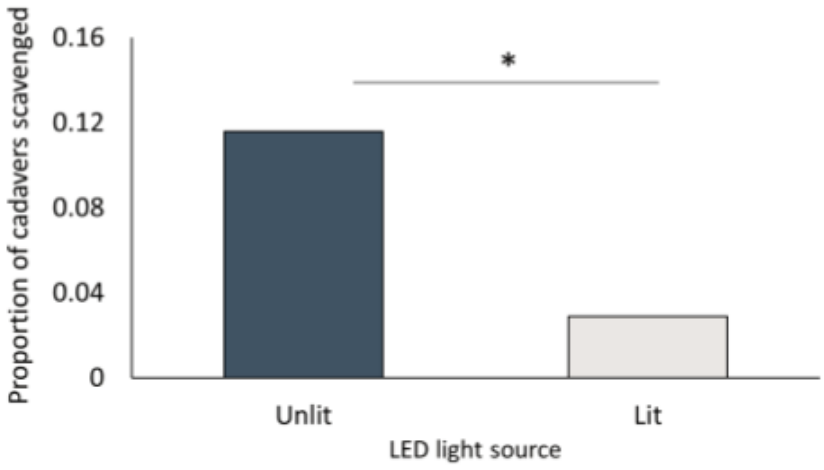

FIGURE 5
Proportion of freeze-killed *G. mellonella* cadavers scavenged by *L. valentiana* when close to either a lit or an unlit LED in a choice test. Slugs that did not move into either arm of the Y-tube were excluded from analysis. Significant differences between treatments in scavenging rate is indicated by * (Chi square, $P < 0.05$).

defences based on unpalatable chemicals and/or pigmentation intensify over time (Baur et al., 1998; Gulcu et al., 2012; Ulug et al., 2014; Jones et al., 2015; Jones et al., 2017), presumably as the products responsible for them accumulate within the cadaver. Although not tested here, it may be that bioluminescence as an independent defence is particularly important early in the infection, helping during a period of vulnerability while chemical defence is less efficient.

Variation amongst scavenger species in the extent to which they are deterred from *Photorhabdus*-infected cadavers (Gulcu et al., 2012; Ulug et al., 2014; Gulcu et al., 2018) may explain why trends in the field, with a diverse assemblage of scavengers, were less clearcut than in the laboratory trials with just a single species of photophobic nocturnal scavenger - the slug *L. valentiana*. Slugs move away from areas of light and are particularly sensitive to blue light in the 400 - 520 nm range (Suzuki et al., 1979; Nishiyama et al., 2019) which spans the range in which *Photorhabdus* emits luminescence (Peat et al., 2010). Their negative response to diffuse light is mainly associated with habitat selection (South, 1992; Zieger et al., 2009). In our experiments, there was no evidence that a point source of light deterred the approach of *L. valentiana* – there was no difference in the category "moving close" to a cadaver in the no-choice experiment, or in the frequency of entry into lit or unlit arms containing a cadaver-mimicking LED in the choice experiment. Slugs seek and recognise food using olfaction (Gelperin, 1974; Gelperin, 1975; Kiss, 2017); again, the lack of effect on "moving close" may indicate that slugs were not deterred at a distance by the odour of infected cadavers. There is evidence that the odour of *P. luminescens*-infected cadavers deters attack by beetle and avian scavengers (Jones et al., 2015; Jones et al., 2017), but it would appear that for *L. valentiana*, both chemical and light defences operate at close contact, serving to deter feeding, but neither modality deters their approach.

The unpalatability of *Photorhabdus*-infected cadavers to scavengers appears to be an innate (unlearned) response, as it is displayed in laboratory tests by untrained animals (Baur et al., 1998; Zhou et al., 2002; Gulcu et al., 2012; Lordan et al., 2014; Ulug et al., 2014; Raja et al., 2017; Gulcu et al., 2018; Cimen, 2023). Similarly, the aversive response of slugs to the luminescence of *Photorhabdus* shown here seems also to be innate, as it was demonstrated by naïve (untrained) individuals at first exposure. Bioluminescence is produced by *Photorhabdus* as a constant glow. In general, while sudden flashes of bioluminescence are repellent, bioluminescent glows are thought to function as attractant signals (Haddock et al., 2010). It is argued that without additional chemical defences, light would only serve to make the emitting organism more obvious to predators (De Cock and Matthysen, 1999). Slugs may be unusual in having an innate dislike of feeding on glowing food, though few terrestrial animals have been tested for this response. For toads and small mammals, the possibility of an innate deterrent response to the bioluminescence of glow worms or millipedes could not be excluded (De Cock and Matthysen, 1999; De Cock and Matthysen, 2003; Marek et al., 2011).

While not demonstrated here, the bioluminescence produced by *Photorhabdus*-infected cadavers could act more generally as an aposematic signal for nocturnal scavengers, as has been suggested for the red colour of *P. luminescens*-infected cadavers for diurnal scavengers (Fenton et al., 2011). Aposematism is the use of warning signals to advertise unprofitability (Breed and Moore, 2016) epitomised by the conspicuous colours used by insects and frogs to advertise toxicity or unpalatability (Guilford, 1990; Ruxton et al., 2018). Like conspicuous colours, bioluminescence is frequently associated with unpalatability, leading to the suggestion that it functions as an aposematic signal in a range of organisms, including algae (Dinoflagellata), annelids, brittle stars (Ophiuroidea), millipedes, glow worm larvae (Coleoptera) and fish (lanternsharks: Etmopteridae) (Grober, 1988a; Grober, 1988b; De Cock and Matthysen, 1999; Marek et al., 2011; Verdes and Gruber, 2017; Duchatelet et al., 2019; Cusick and Widder, 2020). This suggestion is not always supported by empirical data, but there is good experimental evidence both for brittle stars (Grober, 1988a; Grober, 1988b; Jones and Mallefet, 2013) and for glow worms (De Cock and Matthysen, 1999; De Cock and Matthysen, 2003). Toads (*Bufo bufo*) were deterred by the bioluminescence of the common glow worm, after learning to associate the glow with the noxious smell/taste of the larvae (De Cock and Matthysen, 1999; De Cock and Matthysen, 2003). Naïve wild-caught toads demonstrated some aversion to the bioluminescence, which was interpreted as evidence either of prior learning in the field or of neophobia (rejection of novel food), but the aversion was strengthened by the associative learning (De Cock and Matthysen, 2003). Similarly, when paired with chemical defences, the bioluminescence of *Photorhabdus* may strengthen the innate aversion of slugs to feeding on luminous food, and in addition deter other nocturnal scavengers from feeding, even those that are initially indifferent to the light or even attracted by it. Associative learning - required for aposematism to work- is well documented in insects (Dethier, 1980; Duerr and Quinn, 1982; Matsumoto and Mizunami, 2000; Giurfa, 2007; Giurfa, 2015) and in gastropods (Delaney and Gelperin, 1986; Balaban, 1993; Farley et al., 2004) including *L. valentiana* which can form a long-term association between chemical and visual stimuli (Fujisaki and Matsuo, 2017).

In conclusion, our experiments provide support for an ecological function for the bioluminescence of *Photorhabdus* in line with the scavenger deterrence hypothesis (Akhurst and Boemare, 1990), and show that light acts in concert with chemical defences, as previously shown for colour and chemical defences in daylight (Fenton et al., 2011; Jones et al., 2017). Deterrence is unlikely to have been the function of bioluminescence in the bacteria from which *Photorhabdus* acquired its *lux* operon, as there is evidence that in the marine environment, a constant glow is attractive (Zavilgelsky and Shakulov, 2018). A plausible scenario is that the gene transfer occurred within a crustacean; bioluminescent marine bacterial species occur as pathogens of many crustacea (Nealson and Hastings, 1979; Ramesh and Bessho-Uehara, 2021), and *Photorhabdus* can infect littoral amphipods and isopods (Mauléon et al., 2006). Bioluminescence may have been maintained by in the terrestrial environment by protecting infected hosts against innately photophobic scavengers such as slugs, and/or by aposematically reinforcing pre-existing chemical defences of *Photorhabdus*. Support for the scavenger deterrence in *P. temperata* does not rule out the possibility that

bioluminescence is maintained in *Photorhabdus* by more than one selection pressure, either in the same or different species, or is being lost in some species in which it offers less advantage (Peat et al., 2010).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The species used in this study did not require formal ethical approval. The methodology used for these experiments was in keeping with recommendations for the ethical treatment of invertebrates (Drinkwater et al., 2019). Slugs collected for use in lab trials were monitored for 3 weeks after experimentation and healthy slugs were released at their collection point. Ecological impact during field trials was minimised by recording invertebrate activity *in situ* without removal.

Author contributions

MC: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. SL: Data curation, Investigation, Methodology, Writing – review & editing. CG: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1264251/full#supplementary-material

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Mating status, independent of size, influences lethal fighting in an entomopathogenic nematode

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Males of many species compete over access to females by physical contests. Previous experience of mating and the opportunity to mate with a female may influence the motivation of males to engage in contests and the outcome of such contests. Often, prior mating results in increased aggression and probability of success. This is mediated by the effects on the male's subjective evaluation of both the resource value (RV) and his own ability to acquire the resource (resource-holding potential, RHP). Moreover, having mated may also affect a male's actual fighting ability. In *Steinernema* nematodes, mated males paired with naïve males are more likely to win contests. Here we show that this advantage in mated males of *Steinernema carpocapsae* cannot be explained solely by the physical changes brought about by prior female contact, since males exposed to female pheromone alone developed sperm and increased in size like mated males but did not have the same advantage as them in contests with naïve males. Effects of mating on other components of RHP, such as skill or motivation, or on RV may explain the greater probability of mated males winning. We also show that mating had differential effects on the probability of a male initiating attack, depending on whether the opponent was a mated male (and thus ready to mate again) or naïve, consistent with the relative threat of the opponent as a competitor for copulations.

Individuals of many animal taxa compete for resources such as food or mates through contest behaviour of various kinds, sometimes resulting in injury or even death (Alexander, 1961; Enquist & Leimar, 1987; Hardy & Briffa, 2013; Kravitz & Huber, 2003; Moore et al., 2008). Who wins a contest is largely dependent on the relative ability of opponents to acquire the contested resource (resource-holding potential, RHP), although the value of the contested resource to the opponents affects their motivation and hence also the outcome of the contest (Arnott & Elwood, 2008; Enquist & Leimar, 1987; Parker, 1974; Vieira & Peixoto, 2013). Body size and strength, weaponry, skill and physiological condition all contribute to an animal's RHP (Arnott & Elwood, 2009; Briffa & Lane, 2017; Palaoro & Peixoto, 2022), but size is frequently the major factor, with larger size correlating with higher RHP in many animals (Archer, 1988; Brown et al., 2006; Hack et al., 1997; Hsu et al., 2006; Hughes, 1996; Morand, 2000; Neat et al., 1998; Petersen & Hardy, 1996). Contest behaviour can be affected by contestants' assessment of their own RHP (self-assessment) and of their opponents' RHP in comparison (mutual assessment; Arnott & Elwood, 2009; Chapin et al., 2019).

Prior experience, including social experience, can influence the outcome of a contest (Beacham & Newman, 1987; Jacques et al., 1996; Kimberly & Rowland, 2000). Among the best documented effects of social experience are the 'winner effect', whereby winning a contest increases an animal's probability of winning a subsequent contest by altering its self-assessment of RHP (Rutte et al., 2006; Hsu et al., 2006), and the finding that residents also generally win contests, explained by their greater familiarity with the contested resource (Kokko et al., 2006; Kapranas et al., 2020). Many other social experiences, including those of early life, may also affect the outcome of contests (Colella et al., 2019; Favati et al., 2021; Lee et al., 2014).

For males competing for mating opportunities, prior mating or other social experience of females can affect subsequent contest behaviour (Baxter & Dukas, 2017; Innocent et al., 2011; Yasuda et al., 2015; Yuan et al., 2014; Zhang et al., 2019). There are sound theoretical arguments to predict either an increase or a decrease in aggression following mating (Baxter & Dukas, 2017; Judge et al., 2010). On the

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one hand, unmated males may be more motivated to engage in fighting because future mating opportunities are of greater value to them, while mated males may be less willing to engage in costly fighting (Kemp, 2006). On the other hand, having mated may change a male's evaluation of the contested resource increasing his willingness to fight for a more highly valued prize (Brown et al., 2006, 2007; Innocent et al., 2011; Kemp, 2006). Mating may also increase the male's self-assessment of his own RHP (Yasuda et al., 2015), in a manner similar to the 'winner' effect whereby males that win a contest become more aggressive and likely to fight (Hsu et al., 2006). In empirical studies across a range of invertebrates and vertebrates, mating and/or contact with a female tends to increase rather than decrease a male's aggression and success in contests (Bergman et al., 2010; Guevara-Fiore et al., 2012; Judge et al., 2010; Killian & Allen, 2008; Kralj-Fišer et al., 2011; Yasuda et al., 2015; Zhang et al., 2019), while for house crickets, *Acheta domesticus*, males with restricted prior access to females were more aggressive (Brown et al., 2006, 2007).

In studies where mating or sexual experience had no effect on aggression, this may be due to the counterbalancing effects on the males' motivation; future expectations of reproductive success versus subjective assessments of RV or RHP (Baxter & Dukas, 2017; Innocent et al., 2011). Since mating, even for males, may incur costs in terms of energy (Cargnelutti et al., 2022; Perry & Tse, 2013; Scharf et al., 2013), it may also alter the outcome of aggressive encounters by reducing RHP. For example, while male field crickets, *Gryllus pennsylvanicus*, were more aggressive after experiencing a female (whether they mated with her or not), those that copulated were less likely to win a contest than those that did not (Judge et al., 2010). In the nematode *Steinernema longicaudum* (a species with lethal male combat; see 'Background biology' below) mated males were more successful in terms of survival and killing than naïve males when the two were paired together in controlled fights (Kapranas et al., 2020). Interpreting these findings is complicated by the fact that mating involves both exposure to female pheromone and the act of copulation. In *Steinernema*, female pheromone induces physiological changes in males, stimulating sexual maturation and sperm production (Ebssa et al., 2008; Hartley, 2017). In other species, aggression of males may be influenced by gonadal development (Neat et al., 1998) or by exposure to female pheromone (Killian & Allen, 2008). In *Steinernema*, the gonads are large relative to somatic body size (Ebssa et al., 2008), and therefore their maturation might be accompanied by an increase in overall body size that could contribute to RHP.

Here we tested whether the physiological change induced by exposure to female pheromone alone, without the opportunity to mate, in male steinernematids could explain their higher RHP when they engage other competitors in lethal fights. We first assessed whether male sexual maturation induced by pheromone exposure is associated with an increase in their body size. We then experimentally distinguished the effects of female pheromones and the act of mating on contest (fighting) behaviour and outcome.

METHODS

Background Biology

In *Steinernema* spp., a free-living infective juvenile seeks out and invades an insect. Once inside the insect, infective juveniles release symbiotic bacteria which assist in rapidly killing the host and digesting its tissues. The nematodes feed on the bacteria and digested host tissue and develop to adult. A single dead host may support two to three generations and result in tens of thousands of infective juvenile progeny. In most *Steinernema* species, infective juveniles develop into either males or females. Although the sex ratio is slightly female biased (Alsaiyah et al., 2009), females die shortly after mating as juveniles hatch inside their mother (endotokia matricida; Baliadi et al., 2004), resulting in a male-biased operational sex ratio. Males of some *Steinernema* engage in lethal fighting, wrapping around and compressing their opponent, which can cause paralysis or death (Zenner et al., 2014). Lethal fighting has evolved in *Steinernema* spp. in response to the limited mating opportunities and to secure the valuable resource of a host for the developing offspring (Innocent et al., 2011; Kapranas et al., 2016). The incidence of fighting is much higher in males that develop from infective juveniles and colonize a host in small numbers than in males of subsequent generations which typically occur in crowded conditions (Zenner et al., 2014). In at least some *Steinernema* species, laboratory studies have shown that males that develop in isolation have underdeveloped testes and the seminal vesicle contains no sperm (Ebssa et al., 2008). The state of reproductive development can be clearly discerned by microscopic examination through the nematode's transparent cuticle. Gonadal development of solitarily reared males can be stimulated by exposure to female pheromone (Ebssa et al., 2008; Hartley, 2017). Of the close to 100 species of *Steinernema* described, *Steinernema carpocapsae* is the best studied and is also widely commercialized as a biological control agent (Koppenhöfer et al., 2020; Lacey et al., 2015; Murfin et al., 2012).

Nematode Culture

Steinernema carpocapsae (All strain) cultures were routinely maintained using standard procedures by passage through late-instar *Galleria mellonella* (wax moth) larvae (Kaya & Stock, 1997) at 9 °C. Infective juveniles were stored in tap water at 20 °C. Adults for experiments were obtained by placing surface-sterilized (0.1 % hyamine) infective juveniles individually in 20 µl hanging drops of haemolymph from *G. mellonella* larvae, which provides a suitable environment for development to adulthood (Kaya & Stock, 1997; Zenner et al., 2014). The hanging drops were placed on the under surface of a petri dish lid which was placed over a water-filled petri dish (3.5 cm diameter) to prevent desiccation. The infective juveniles in the hanging drops developed to adulthood within 3 days at 20 °C. Adult males can be distinguished by their copulatory spicules and their smaller size whereas females are larger and bear a vulva. We used 3-day-old adult males and females in our experiments. At this time a minority of males had spontaneously developed sperm and were discarded.

Conditioning

Males without sperm were randomly assigned to create three categories: mated, pheromone-exposed or naïve. (1) For the mated category a male was placed in a drop with an adult female. Only males for which mating was confirmed by subsequent production of progeny were included in the results. (2) For the pheromone-exposed category a male was placed in a drop that had previously been occupied by an adult female. (3) For the naïve category a male was removed from and placed back into its own drop. After 48 h sperm development was recorded. Only males that had developed sperm in the mated and pheromone treatments (indicating successful exposure to female pheromone), and males without sperm in the naïve treatment (the condition of the majority of naïve males), were used in experiments. Males were photographed, and their area and length were measured using Image J 1.53e (http://imagej.nih.gov/ij) as an index of their size.

Fighting Assays

Each male, including the controls, was transferred to a fresh drop of *G. mellonella* haemolymph using a sterile platinum wire

and was allowed to acclimatize for 1 h. Males were then paired in drops symmetrically within category (mated with mated; pheromone-exposed with pheromone-exposed; naïve with naïve) or asymmetrically (mated with naïve; pheromone-exposed with naïve). There were thus five treatments. Control males of each category were taken out of and immediately replaced into their own drops. Pairs were observed for 30 min at 26 °C. Fighting was recorded when one male wrapped around the other and tightened (Kapranas et al., 2020). Not all fights resulted in injury or death, and some pairs engaged in more than one fight during observation. Incidence of fighting, the identity of the initiator (in asymmetric pairs), number of fights, duration of fights and time at which fights occurred were recorded for each pair. A total of 33–59 pairs per treatment and 37–66 control individuals per category were observed. After 24 h at 20 °C, mortality (including severe injury or paralysis) was recorded for each pair and control.

Data Analysis

The area and length of males from each category were compared using one-way analysis of variance (ANOVA) with significance at $P < 0.05$, followed by Tukey post hoc tests. For pairs in which fighting was observed, the number of fights, median duration and latency were analysed using ANOVA or the Kruskal–Wallis test, with significance at $P < 0.05$. For asymmetric fights, a one-sample proportion test was used to compare initiation of fights between conditioned (mated or pheromone-exposed) and naïve males with a hypothesized proportion initiated by naïve males of 0.5. The identity of the victim in these fights was also similarly tested for deviation from 0.5. To assess whether a mated male assesses its opponent, the probability of a mated male initiating an attack against a naïve male or against another mated male was compared using chi-square analysis. All the above data analysis was performed using Minitab 20.3 statistical software (Minitab, LLC, 2021) (or RStudio version 2022.12.0 (RStudio Team, 2020)). The incidence of fighting and death as influenced by different worm pairs (treatments) was explored with a binary logistic analysis. We used backward stepwise procedures and aggregation of factor levels (akin to a post hoc test) to obtain the parsimonious 'minimal adequate model' by model simplification (Briffa and Hardy, 2013). In the subsets of observations for which worm measurements were available for asymmetric pairings (pheromone-exposed versus naïve: $N = 24$; mated versus naïve: $N = 19$), we tested whether the initiation of fighting and the winning of fights by naïve worms was influenced by their opponent's status and the size (area) difference between them using a binary logistic regression (Briffa and Hardy, 2013). These analyses were performed with GENSTAT v. 22 (VSN International Ltd, Hemel Hempstead, U.K.). The same analysis could not be carried out for symmetrical pairings as the individuals were indistinguishable after pairing.

Ethical Note

No ethical approval was required for the species used in this research. The methodology used in this study was in keeping with the ASAB/ABS guidelines for the treatment of animals in behavioural research, as well as the suggestions by Drinkwater et al. (2019) for ethical treatment of invertebrates, with a focus on reduction and refinement. The number of *G. mellonella* used for haemolymph extraction was minimized by calculation of exact required volumes and the number of nematodes used in staged fights was reduced through refinement of the protocol.

RESULTS

Size

There was a significant difference in area between the three categories of males (ANOVA: $F_{2,\ 237} = 19.1$, $P < 0.001$). Males with sperm (mated or pheromone-exposed) were larger than naïve males, but there was no difference in area between the mated and pheromone-exposed males (Fig. 1). There was no significant difference in length between naïve males (1.80 ± 0.04 mm, $N = 83$), mated males (1.87 ± 0.05 mm, $N = 88$) or pheromone-exposed males (1.89 ± 0.05 mm, $N = 74$; ANOVA: $F_{2,\ 237} = 1.14$, $P = 0.320$).

Fighting and Mortality

There was a significant difference in incidence of fighting across treatments (logistic analysis: $G_4 = 3.04$, $P = 0.016$). Incidence of fighting was highest in symmetric mated pairs and lowest in symmetric pheromone-exposed pairs compared to all other treatments (Fig. 2). For pairs in which fighting was recorded, there was no difference across treatments in the number of fights per pair (Kruskal–Wallis: $H_4 = 2.72$, $P = 0.605$), duration of fighting (Kruskal–Wallis: $H_4 = 4.37$, $P = 0.358$) or time until the first fight (ANOVA: $F_{4,\ 63} = 1.93$, $P = 0.117$; Appendix Table A1).

After 24 h, the overall incidence of damage or death in the pairs was 26% (61/234). There was no difference across treatments in incidence of damage or death in the pairs (logistic analysis: $G_4 = 1.17$, $P = 0.326$; Appendix Table A1). Control mortality was low (3%; 10/300) and did not differ between categories of male (Pearson chi square: $X^2_2 = 1.8$, $P = 0.405$).

Identity of Initiator and Victim of Fighting in Asymmetric Pairings

When a fight occurred in asymmetric pairings, it was more likely to be the naïve male than the conditioned male that initiated fighting. This was consistent for both the mated versus naïve and pheromone-exposed versus naïve treatments (one-sample proportion test: $X^2_1 = 6.368$, $P = 0.012$ and $X^2_1 = 5.4$, $P = 0.020$, respectively; hypothesized proportion = 0.5; Fig. 3, Appendix Table A2). In mated versus naïve pairs, where one male was damaged or dead after 24 h it was more likely to be the naïve male than the mated male (one-sample proportion test: $X^2_1 = 7.118$, $P = 0.008$; hypothesized proportion = 0.5), but in the case of

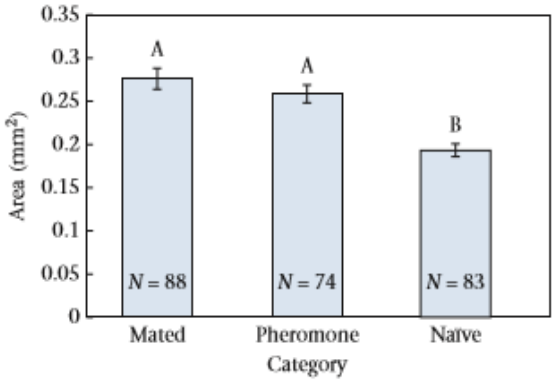

Figure 1. Area (mean ± SE) of *S. carpocapsae* males that were conditioned for 48 h in haemolymph drops with a female (mated), in drops previously occupied by a female (pheromone) and in their own drops (naïve). The number of males measured in each category is indicated on the bars. Bars with differing letters are significantly different ($P < 0.001$, Tukey's post hoc test). Measured males in the mated and pheromone-exposed categories all had sperm present while none of the naïve males had sperm.

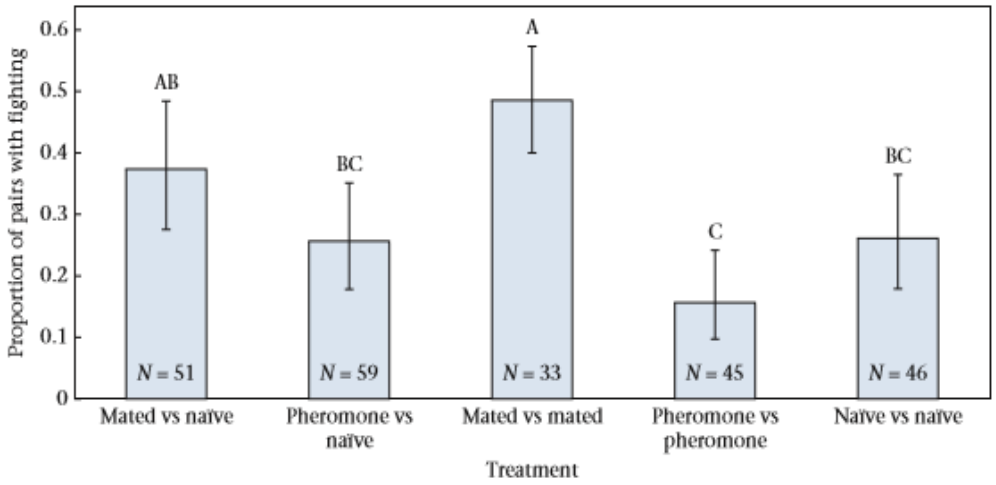

Figure 2. The proportion (± SE) of pairs of *S. carpocapsae* males in which fighting was observed during the 30 min observation. Conditioned males (mated or pheromone-exposed) were paired symmetrically with a similarly conditioned male, or asymmetrically with a naïve male. Pairings of a naïve male versus a naïve male were also observed. Bars with differing letters are significantly different ($P < 0.05$). The number of pairs measured in each treatment is shown on the bars.

pheromone-exposed versus naïve males, there was no deviation from equality (one-sample proportion test: $X^2_1 = 0.474$, $P = 0.491$; hypothesized proportion = 0.5; Fig. 4).

When mated versus naïve and pheromone-exposed versus naïve male pairs were compared, naïve males were as likely to initiate a fight when paired with a mated male as with a pheromone-exposed male (logistic analysis: $G_1 = 1.21$, $P = 0.271$). Analysis of the subsets for which worm size measurements were available confirmed that the probability of naïve worms initiating a fight was not influenced by competitor status (mated or pheromone-exposed) or size (opponent's status: $G_1 = 0.34$, $P = 0.562$; size: $G_1 = 1.45$, $P = 0.228$; interaction between opponent's status and size: $G_1 = 1.36$, $P = 0.244$).

Naïve males had a marginally higher but not significant tendency to win when paired against pheromone-exposed than when paired with mated males (logistic analysis: $G_1 = 3.31$, $P = 0.069$). However, in the subsets for which worm size measurements were available, competitor status (mated or pheromone-exposed) and size were not significant (opponent's status: $G_1 = 1.17$, $P = 0.279$; size: $G_1 = 1.22$, $P = 0.269$; interaction between opponent's status and size: $G_1 = 0.02$, $P = 0.902$).

Of the mated males tested in asymmetric pairings, only 8% (4/51) initiated fighting, while in symmetric mated–mated pairings, fighting was initiated by 24% (16/66) of the individuals tested, a significant difference (chi-square test: $X^2_1 = 5.459$, $P = 0.019$).

DISCUSSION

As seen previously in *S. longicaudum* males (Kapranas et al., 2020), mated *S. carpocapsae* males had a killing advantage when paired against naïve males. Contrary to our hypothesis, however, this advantage could not be explained by the physiological effects induced by exposure to female pheromone, such as sperm production and the associated increase in size, since males that received pheromone-exposure treatment alone did not have a similar advantage over naïve males. Both mated and pheromone-exposed males were similar in size, being larger than naïve males, presumably due to the sperm stored in the seminal vesicle

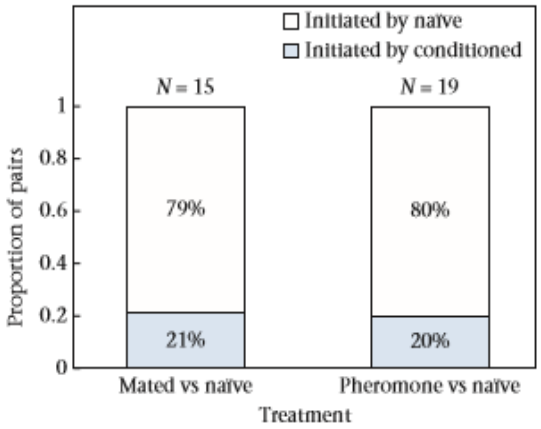

Figure 3. Proportion of pairs in which fighting was initiated by conditioned (mated or pheromone-exposed) or naïve males in asymmetric pairings of conditioned versus naïve males. Data are shown only for pairs that engaged in fighting. The number of pairs measured in each treatment is shown above the bars.

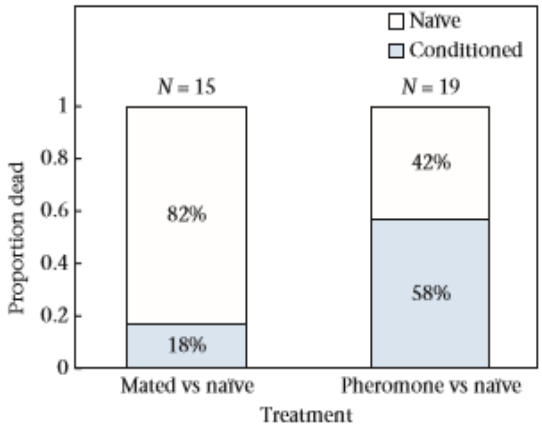

Figure 4. Proportion of conditioned (mated or pheromone-exposed) or naïve males dead or damaged after 24 h in asymmetric pairings. Data are shown only for pairs that engaged in fighting. The number of pairs measured in each treatment is shown above the bars.

increasing the worms' diameter. Despite the similarity in size, mated and pheromone-exposed males differed in the outcome of interactions with naïve males, in that mated but not pheromone-treated males were more likely to be the victor over the naïve male. Moreover, analyses on asymmetric pairs showed that the size difference between opponents did not affect the outcome of the interaction. Body size is an important determinant of RHP: in general, larger animals are more likely to win (Archer, 1988; Brown et al., 2006; Hsu et al., 2006; Hughes, 1996) but this is not always the case. For example, Elwood et al. (1998) found that relative size affected neither the cost nor the probability of victory in fighting in the hermit crab *Pagurus bernhardus*. Although both the mated and pheromone-exposed males had developed sperm and increased in diameter, they differed in their experience with a female. In many animal taxa, previous mating has been shown to increase a male's self-assessment of RHP, thus increasing their confidence and likelihood of fighting (Guevara-Fiore et al., 2012; Killian & Allen, 2008; Kralj-Fišer et al., 2011; Yasuda et al., 2015). This is similar to the 'winner effect': animals that have succeeded in combat are more likely to succeed again (Hsu et al., 2006; Kasumovic et al., 2009; Kou & Hsu, 2013). Experience gained during mating may also increase skills that contribute to an advantage in fighting (Briffa & Lane, 2017). In *Steinernema*, the male wraps its tail end around the female at the vulva and uses a pair of copulatory spicules to assist insemination (Lewis et al., 2002). This behaviour resembles the coiling used in fighting, and the spicules may assist in inflicting injuries either by puncturing or by focusing the pressure delivered by squeezing (Zenner et al., 2014). Mating behaviour of male nematodes is controlled by a specialized posteriorly located 'connectome', a network of neurons, muscles and gonad (Jarrell et al., 2012) that may also have a role in fighting behaviour in *Steinernema*. In *Caenorhabditis elegans*, mating experience results in the rewiring of specific synapses in the male connectome, with evidence that these changes are translated into finer coordination of muscle contraction, such as those involved in spicule protraction (Hart & Hobert, 2018).

In our experiments, we exposed males to female pheromone to induce physiological maturation, to explore the importance of the mated male's physical status in winning a fight. In other animals, experience of a female (without mating) may or may not mimic the effects of actual copulation on male aggression (Bergman et al., 2010; Judge et al., 2010; Killian & Allen, 2008; Rillich et al., 2019). For example, male crickets, *A. domesticus*, that were allowed to contact a female but not to copulate with her showed the same increased aggression as males that were allowed to copulate, suggesting that chemotactile cues from the female were sufficient to elicit this change in aggression (Killian & Allen, 2008). While female pheromone gives an indication to *S. carpocapsae* males of female presence, it is unclear whether it can be considered equivalent to the 'female experience' treatment of studies in other animals. In nematodes, the response of males to female pheromone represents the first step in mate finding, but there is also a second step involving contact cues at the female's body surface (Barr & Garcia, 2006; Sakai et al., 2013).

Pheromones are widely implicated in modulating aggression in insects and mammals (Itakura et al., 2022; Sengupta et al., 2022). In nematodes, pheromones are involved in numerous social behaviours including mate attraction, aggregation and dispersal (Edison, 2009; Hartley et al., 2019; Muirhead & Srinivasan, 2020). Nematode pheromones mainly belong to a class of molecules called ascarosides, a modular library of potent molecules with both developmental and behavioural effects (Choe et al., 2012; Butcher, 2017; Park et al., 2019). Media conditioned by *S. carpocapsae* females contain a blend of ascarosides and both attract conspecific males and initiate their gonadal maturation (Choe et al., 2012; Hartley, 2017). Exposure to female pheromone in our experiments clearly induced the physical effects (sperm development and concomitant increase in size) but not the behavioural effects of mating. Indeed, pairs of pheromone-treated males showed the lowest incidence of fighting, in contrast to the elevated fighting in pairs of mated males. Female steinernematids have a short window of availability, becoming less attractive (Lewis et al., 2002; Hartley, 2017) and of lower RV (Kapranas et al., 2020) for males shortly after mating. In the closed environment of a dead insect, residual pheromone, in the absence of available females, would indicate that there were no more available females in the founding generation, and the only way for a male to achieve at least some reproductive success would be to survive until females of the filial generation become available (Zenner et al., 2014). Thus, the reduced aggression of pheromone-matured males would be adaptive. Internal state, social interaction and other experiences interact in a complex manner to influence the translation of stimuli into behaviours, even in a relatively simple organism such as a nematode (Hashikawa et al., 2018; Park et al., 2019). The impact on a male steinernematid of experiencing pheromone only, without encountering a female, warrants further exploration.

Although mated males tended to win fights against naïve males, it was the naïve male that was more likely to initiate fighting in these pairings. Naïve males are expected to value a mating opportunity more highly than a male that has just mated (Kemp, 2006) and will be more aggressive as a result (Brown et al., 2006, 2007). This may be a case of contradictory asymmetry where the opponent with the lower RHP has more to gain from the fight (Parker & Rubinstein, 1981). It is expected that if males can assess relative RHP, the male with lower RHP will terminate the fight before sustaining injury. This is based on mutual assessment (Enquist & Leimar, 1987). However, if the male with the lower RHP is in a position where it cannot reproductively succeed by quitting the fight then the 'desperado effect' comes into play and the male will continue to attack due to the lower potential cost (Grafen, 1987). Naïve males in this case exist in a divisive asymmetry where they will always lose out to mated males and pheromone-exposed males for mating opportunities. This is because naïve males have not developed sperm and are not ready to mate. By the time the naïve males are sexually developed the mature males may have taken all the available females. Therefore, it is to the naïve male's advantage to always risk the fight. Pheromone-exposed males do not have the same disadvantage as they are sexually developed and are therefore less motivated to fight and risk their future reproductive success.

Comparison of symmetric and asymmetric pairings involving mated males suggests that males are able to assess their opponents' state (using physiological and/or behavioural clues) and thus their relative RHP; mated males were three times more likely to initiate fighting in mated–mated than in mated–naïve pairings. This may be related to the threat that the opponent poses as a competitor in reproduction. A mated male will risk attacking another mated male, as it is ready to mate, but may decide that attacking a naïve male is not worth it, as it is not ready to mate. Despite the high incidence of fighting in mated–mated pairs relative to all other pairings, the mortality rate was not similarly elevated in this treatment. Fighting should end when one male has died or gives up the attack. When opponents are equally matched the cost of fighting is highest due to continued escalation (Arnott & Elwood, 2009; Payne and Pagel, 1996). If males can assess each other's RHP they may decide to terminate fights before injury occurs when equally matched (Enquist & Leimar, 1990).

Adding to the previously described effects of competitor density and relatedness (Zenner et al., 2014; Kapranas et al., 2016), residency and objective RV (Kapranas et al., 2020), this study further shows the value of the entomopathogenic nematodes in testing

theoretical predictions in contest behaviour and in behavioural ecology in general (Lewis et al., 2022).

In conclusion, there are considerable asymmetries due to mating status between *Steinernema* males in conflict behaviour, in their tendency both to initiate and to win a fight. The advantage of mated *Steinernema* males over naïve opponents cannot be explained by larger size, in contrast to findings reported for many animal taxa, but may be due to other elements of RHP or RV.

Author Contributions

Maria D. Cassells: Conceptualization; Data curation; Formal analysis; Investigation; Writing–original draft; Writing–review & editing. **Apostolos Kapranas**: Formal analysis, Writing-review & editing. **Christine T. Griffin:** Conceptualization; Supervision; Writing–review & editing.

Data Availability

Data are available as Supplementary material.

Declaration of Interest

None.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.anbehav.2023.04.016.

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Appendix

Table A1
Fighting behaviour in males that fought from each treatment in symmetric and asymmetric pairs

Treatment	*N*	No. of pairs with fighting	No. of fights/pair (median)	Duration of fights (s) (median)	Time until 1st fight (min) (mean + SE)	% Pairs with at least one male damaged or dead
Mated versus mated	33	16	2	13	16.6 + 7.7	21
Pheromone versus pheromone	45	7	1	13	15.4 + 11.7	18
Naïve versus naïve	46	12	1	11.5	14.8 + 9.0	22
Mated versus naïve	51	19	1	26.5	10.2 + 7.8	33
Pheromone versus naïve	59	15	1	20	10.1 + 8.0	32

Table A2
Initiator in asymmetric pairings between a naïve and a conditioned (mated or naïve) male

	Category	Mated versus naïve	Pheromone versus naïve
N		51	59
No. of pairs with attacks		19	15
Attacker	Conditioned	4	3
	Naive	15	12
Total no. of attacks	Conditioned	10	9
	Naive	19	13
Median no. of attacks	Conditioned	2	2
	Naive	1	1