Pheromones in entomopathogenic nematodes: effects on dispersal and reproductive behaviours

A thesis submitted to the National University of Ireland, Maynooth for the degree of Doctor of Philosophy

by

Cathryn Jane Hartley, M.Sc

Supervisors: Prof. Christine T. Griffin

and Dr Rebecca Owens

Head of Department: Prof. Paul Moynagh

October 2017

Declaration

This thesis has not been submitted in whole or in part to this or any other university for any other degree and is, except where otherwise stated, the original work of the author.

Signed: ……..…*Cathryn Hartley*……

Date: ……24/10/2017……

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Acknowledgement

This project would not have been possible without the support and guidance of my brilliant supervisor Prof. Christine Griffin, whose wisdom and patience over the past four years mentored me through academic and personal challenges alike. For this I will be forever grateful. Dr Gail Maher at Maynooth University also provided invaluable support and assistance, coaching me through new techniques in the lab and sharing her experiences of her own academic journey to help me feel at ease, earning her the prestigious title of 'Lab Mammy'. Many thanks go to Dr Rebecca Owens for her fantastic help with analytical aspects of the project, as well as Dr Stephan von Reuss, and the Max Planck Institute for Chemical Ecology for the same. Thanks to Maynooth University and the John and Pat Hume scholarship scheme for funding this project and for being such a wonderful host institution.

As well as those mentioned above, I want to thank everyone who helped make Maynooth my home. Sarajane, the best lab assistant a girl could wish for and baker extraordinaire. Nick, the straight talking, darkly hilarious technician with a solution for every situation. Paul, for keeping me fuelled up on coffee and becoming my closest friend.

Finally, to my family, for always believing in me even when I didn't.

This one's for you.

Abbreviations

♀	Female
♂	Male
+ve	Positive
-ve	Negative
µg	Microgram
µl	Microlitre
µM	Micromolar
AI	Attraction index
ascr	Ascaroside
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cm	Centimetre
dd	Double distilled
DA	Dafachronic acid
DF	Degrees of freedom
dhas	Dihydroxy ascaroside derivative
DJ	Dauer juvenile
DNA	Deoxyribonucleic acid
EIC	Extracted ion chromatogram
EPN(s)	Entomopathogenic nematode(s)
eV	Electron volts
fM	Femtomolar
g	Gram
GC-MS	Gas chromatography mass spectrometry
GLM	General linear methods
GPCRs	G-Protein coupled receptors
HPLC	High performance liquid chromatography
h	Hours
icas	Indole ascaroside
IJ(s)	Infective juvenile(s)
KW	Kruskal-Wallis
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LH	Luteinizing hormone
MeOH	Methanol
mg	Milligram
Min	Minutes
ml	Millilitre
mm	Millimetre
mM	Millimolar
Molecule X	Uncharacterised molecule with *m/z* 303.2174 (negative mode LC-MS)
m/z	Mass to charge

N/A	Not applicable
NA	Nutrient agar
NB	Nutrient broth
NCE	Normalised collision energy
NDMM	Nematode-derived modular metobolite
ng	Nanogram
NHR	Nuclear hormone receptor
nM	Nanomolar
NMR	Nuclear magnetic resonance spectroscopy
oscr	Oscaroside
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
pM	Picomolar
pmol	Picomolar
rDNA	Recombinant deoxyribonucleic acid
RP-HPLC	Reversed phase – high performance liquid chromatography
RPM	Revolutions per minute
Rt	Retention time
SPE	Solid phase extraction
Temp	Temperature
TFA	Trifluoroacetic acid
TIC	Total ion chromatogram
UV-vis	Ultra violet visualisation
v/v	Volume/volume
VCLA	Very long fatty acid side chain
x g	Times G-force/relative centrifugal force

Abstract

Entomopathogenic nematodes (EPN) are increasingly gaining favour as model organisms for studying traits relevant to parasitism and for their commercial value as biocontrol agents. The overall project aim is to explore the role of pheromones in coordinating a variety of important behaviours in entomopathogenic nematodes, particularly *Steinernema,* both as primers (longer term developmental effects) and releasers (short term behavioural effects). I investigate both sex pheromones and chemical signals produced by and affecting the infective juvenile (IJ) stage. In this thesis, I both test central hypotheses, and attempt the identification of key pheromone components, using assay-guided fractionation.

As lethal parasites of insects, EPN have been successfully commercialised for pest control, however greater understanding of their ecology and biology is required to improve their efficacy in the field, which is often inconsistent. Infective juveniles (IJs) actively search for hosts within the soil. The IJs enter the host and release symbiotic bacteria which both kill the insect and convert it into a nutritive "soup". The nematodes develop and reproduce inside, and eventually hundreds of thousands of newly-produced IJs emerge and disperse from the spent cadaver. It has previously been shown that chemicals within the spent cadaver stimulate IJ dispersal. In Chapter 3, I show that IJs themselves may also produce chemical signals which promote dispersal in both conspecific and heterospecific IJs. Behavioural assays confirmed that IJs of three species of *Steinernema* (*S. carpocapsae, S. longicaudum* and *S. feltiae*) and one species of *Heterorhabditi*s (*H. megidis*) disperse more when exposed to cadaver extract of pre-infected hosts. More interestingly, dispersal is also promoted by water "conditioned" for 1 or 7 days with IJs which had recently vacated

a spent host, suggesting that they release cues which modify dispersal behaviours. Furthermore, it was found that these cues are not species-specific, with IJs dispersal enhanced by water conditioned with their own species and other species (even between families) to a similar extent. In a longer term storage experiment with *S. carpocapsae* and *H. megidis*, I showed that replacing the water in which IJs were stored reduced the dispersal of these stored IJs when assayed, indicating that chemicals released into the medium by IJs maintained high dispersal. Parallell assays showed that replacing the water did not affect the virulence of the stored IJs.

Chapter 4 addresses the role of chemical signals in mate attraction in dioecious *Steinernema carpocapsae* and *Steinernema longicaudum* adults. It was found that males are attracted to exudates of conspecific females, but not to exudates from females of the other species. The sexual history of the females dictated their level of attractiveness. Exudates of virgin females were highly attractive to males, whereas attractiveness was lost just 1 h post-mating and the loss of attractiveness was sustained for 48 h post-mating. Assay-guided fractionation of female exudates was used to give insight into the chemical properties of the as yet uncharacterised attractant. Males were less attracted to extracted material (using C18 Sep-Pak columns) in comparison to un-extracted female exudates. They also showed some degree of attraction to column flow-through, which indicates that some components may not have bound to the column during the extraction process, perhaps due to high polarity. Furthermore, recombination of column retentate and flow-through did not restore full bioactivity of the original sample, indicating that volatile molecules may be part of the attractant composition and were lost during the extraction process.

Chapter 5 investigated the role of female-emitted chemical cues in sexual priming of males, (inducing production of sperm). This phenomenon, unusual amongst animals,

whereby female presence or odour is essential for sexual maturation of males, was previously described in *S. longicaudum*. Here I show that the same is true for a second species, *S. carpocapsae*. As with female-produced male attractants, maturation signals were also shown to be species-specific, with males only producing sperm on exposure to exudates from females of their own species. Bioassay of C18 extracted female exudates again showed some degree of physiological response to both column retentate and column flow-through, again indicating that some components of the pheromonal blend were not retained by the C18 column resin during extraction. However, unlike in attraction, recombination of these two constituents fully restored bioactivity to the level of the original unextracted female exudates, indicating that there was no essential volatile component involved. Assay-guided fractionation of female exudates showed that the early-eluting fractions (10-30% methanol) were the most biologically active with respect to inducing maturation in males. This finding was supported by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis which revealed the presence of numerous ascarosides, the family of molecules known to be involved in nematode communication, in active fractions. *S. carpocapsae* was previously reported to produce ascarosides at both juvenile and adult stages of the life-cycle; however this study is the first report of ascarosides produced by *S. longicaudum.* Ascr#10 was the dominant ascaroside in both species, with several others found in much lower relative abundance. Although similarities in ascaroside profiles between species were seen (both *S. carpocapsae* and *S. longicaudum* produced ascrs # 9, #1, #14 and #10), differences in ratios and the presence of ascr#14 exclusively in *S. longicaudum* female exudates may contribute to the species-specificity of *Steinernema* sex pheromones. LC-MS/MS analysis also revealed the presence of a

number of female-specific non-ascarosidal molecules in biologically active fractions. Although these were not structurally characterised, their detection may direct future research in the potential role of non-ascarosidal components of chemical signals in nematodes.

The work of this thesis supported two of the three hypotheses proposed: Hypothesis 1, that chemical signals differ between species where recognition is important (sex pheromones) but coincide where different species have a common aim (dispersal from overcrowded conditions), and Hypothesis 2, that chemical signals exuded by IJs after emergence from the cadaver continue to affect IJ behaviour. Full support was not obtained for Hypothesis 3, that the same chemical blend produced by female *Steinernema* is responsible for both behavioural (attraction) and organisational (gonad maturation) effects in males.

Chapter 1 General Introduction

1.1 Introduction

Living organisms, from single celled bacteria to humans, have the ability to perceive the surrounding environment and modify their behaviour or physiology accordingly. Athough behaviour can be defined in many different ways depending on context; in general the term refers to the responses of an organism to perceived changes in the external world and in its internal state (Scott, 2005). Where sensory mechanisms such as vision or hearing are either absent (i.e. in lesser organised species such as nematodes) or inappropriate (such as in dark soil environments), olfaction provides a vital means of information exchange (Wyatt, 2003). In this way, individuals are able to use chemical signals to effectively communicate information about themselves with both conspecifics and heterospecifics. Numerous behaviours and developmental processes including species recognition, sexual attraction and maturation, are mediated by chemical communication in a vast number of species from mammals to bacteria (Wyatt, 2009). Increased understanding of the mechanisms and parameters of such communication is not only of fundamental scientific interest, but may also be useful in commercial ventures, particularly in agriculture, forestry and aquaculture whereby manipulation of stock or pest behaviours and development via chemical means can lead to higher saleable yields. In this thesis, I explore the role of chemical communication in entomopathogenic nematodes, both in the adult and juvenile stages. These nematodes are both important models for symbiosis and parasitism (Dillman et al., 2012) and marketed as biopesticides (Georgis et al., 2006).

1.2 Entomopathogenic nematodes (EPN)

Entomopathogenic ('insect- killing') nematodes (EPN) belong to two main families, the Steinernematidae and Heterorhabditidae each with one main genus, *Steinernema* and *Heterorhabditis* respectively. Reproduction occurs inside the insect host until depleted resources and overcrowding prompt infective juveniles (IJs) to vacate the insect cadaver in search of a new host. With the exception of Antarctica (Griffin et al., 1991), Steinernematidae and Heterorhabditidae have been recovered from every continent (Hominick, 2002), with over 90 species of *Steinernema* known (Nguyen, 2016). The majority of steinernematids show specific continental or national geographic distribution, but some (including *S. carpocapsae* and *S. feltiae* studied in this thesis) have been recorded to occur globally (Hominick et al., 1996). In comparison, only 16 species of Heterorhabditis have been confirmed and described as of 2015 (Nguyen, 2016).

Although individual species differ in infectivity, host range, foraging strategy and tolerance to environmental factors, both Steinernema and Heterorhabditus employ an obligate parasitic life cycle in close association with lethal symbiotic bacteria (family Enterobacteriaceae), reproduce within the insect host and emerge as free living IJs in search of a new host.

1.2.1 Life cycle

Steinernematids and heterorhabditids share the same general life cycle (Figure 1.1) differing mainly in their mechanism of reproduction. While the latter can reproduce both hermaphroditically and by cross-fertilisation of males and females, all but one

species of *Steinernema* are dioecious and exclusively reproduce following cross fertilisation of sexually dimorphic adults, raising fundamental questions of how individuals identify, locate and assess fitness of prospective mates (Griffin, 2012). This thesis focusses mainly on species of the family Steinernematidae, exploring chemically mediated behaviours both at the juvenile (Chapter 3) and reproductive adult stages (Chapters 4-5), whereas *Heterorhabditis* is only examined at the IJ stage (Chapter 3).

In both EPN families, only specialised third stage juveniles, the IJs, occur outside of the insect host as free-living individuals capable of migrating through the soil environment. These IJs exist in a non-feeding state of arrested development comparable to the dauer juvenile (DJ) of *C. elegans* (Viney et al., 2005). IJ foraging strategy varies between species, and they are generally categorised as either 'cruise' foragers, where IJs actively move through the soil in search of a sedentary host, or 'ambush' foragers where IJs lie in wait at the soil surface latching on to mobile hosts as they pass by (Lewis et al., 2006). In reality, this categorization is more of a continuum, with the pure 'cruise' or 'ambush' strategies lying at opposite extremes of the spectrum (Lewis et al., 2006). Once a host is located, IJs enter through natural openings such as the mouth, anus or spiracles. Upon entry into the haemocoel, they release internally stored cells of bacterial symbiont which quickly multiply and induce host death, usually within 48 h of initial infection (Goodrich-Blair, 2007). IJs then recover from their temporary period of arrested development, feeding on the rich supply of bacteria and decaying host tissues to become first generation adults (Nguyen and Smart, 1992).

In *Steinernema*, with the exception of the hermaphroditic species, *S. hermaphroditum* (Griffin et al., 2001), dimorphic males and females reproduce

sexually inside the host and mated females lay fertilised eggs externally from which first stage juveniles hatch (Nguyen and Smart, 1992). In some cases, females internally retain the fertilised eggs which develop into juveniles within the reproductive tract causing subsequent death to the maternal parent, a phenomenon termed *endotokia matricida* (Nguyen and Smart, 1992). Athough *endotokia matricida* has been show to occur in all generations (Baliadi et al., 2004) it is more common in later (second and third) generations within the host. This has been suggested to be an adaptive strategy for ensuring IJ production in response to depleted nutritive resources (Baliadi et al., 2009). After several generations (generally 1-3) within the insect cadaver, specific conditions including overcrowding and resource depletion prompt the formation of IJs capable of locating and infecting a new host (Griffin, 2015). Unlike third stage juveniles from previous generations, these specialised IJs retain cells of the bacterial symbiont required to kill a new host in the intestine, ready for release when a new host is encountered and penetrated.

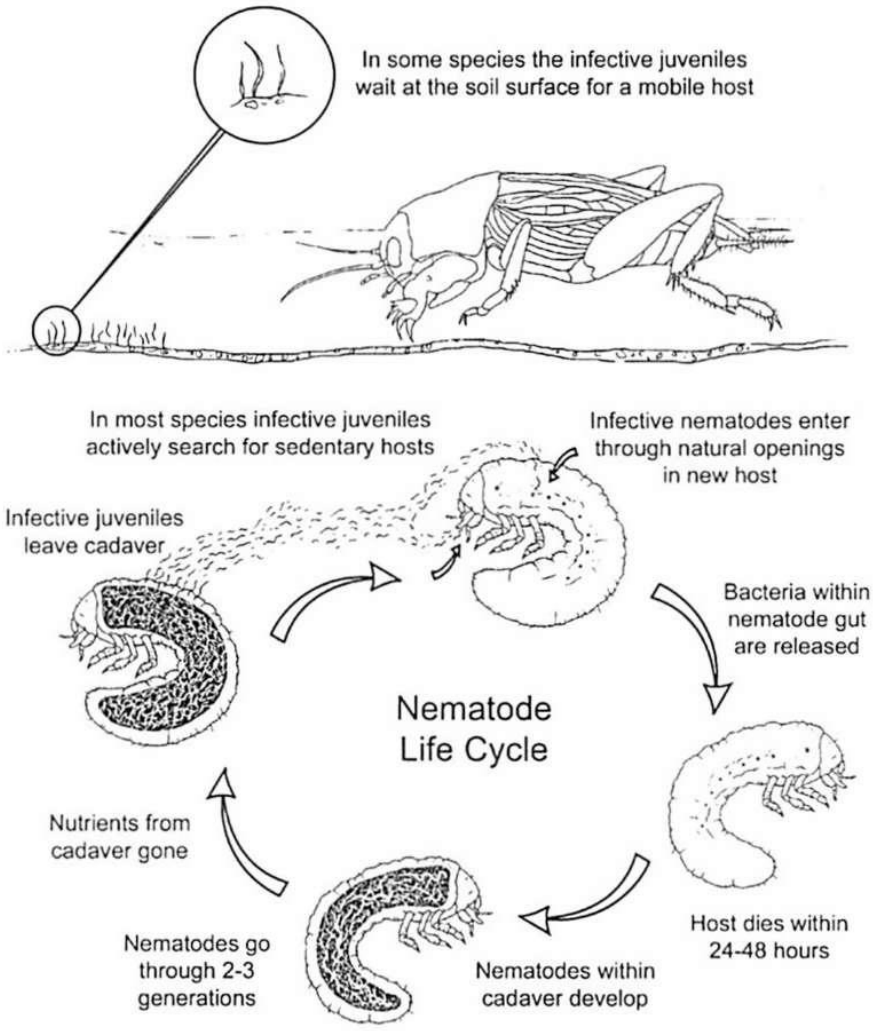


Figure 1.1: Schematic of the EPN life cycle. Diagram credit A. E. Burke (www.pesthoreca.com).

1.2.2 Reproduction and associated behaviours

Although EPN adults are not directly involved in host finding or infection, understanding their reproductive biology is of relevance to commercial mass production and the study of population biology (Griffin, 2012). The most notable difference between *Steinernema* spp. and *Heterorhabditi*s spp. lies in their reproductive strategy. All but a single species of *Steinernem*a (*S. hermaphroditum*) are amphimictic, whereas *Heterorhabditi*s spp. are exclusively hermaphroditic in the

first generation, with males, females and hermaphrodites in subsequent generations (Zioni et al., 1992). As in many nematodes, female *Steinernema* are larger than males (Figure 1.2). Females have an amphidelphic reproductive system consisting of two similar branches, each subdivided into three parts: a reflexed ovary, oviduct and uterus (Zograf et al., 2008). Sperm can be stored in the junction between the ovary and uterus (Zograf et al., 2008). The vulva is located approximately in the middle of the body (Figure 1.2). Male *Steinernema* have a single reflexed testis and paired spicules (Adams and Nguyen, 2002). A seminal vesicle serves as a sperm storage area while the vas deferens opens into the cloaca (Scott, 1996).

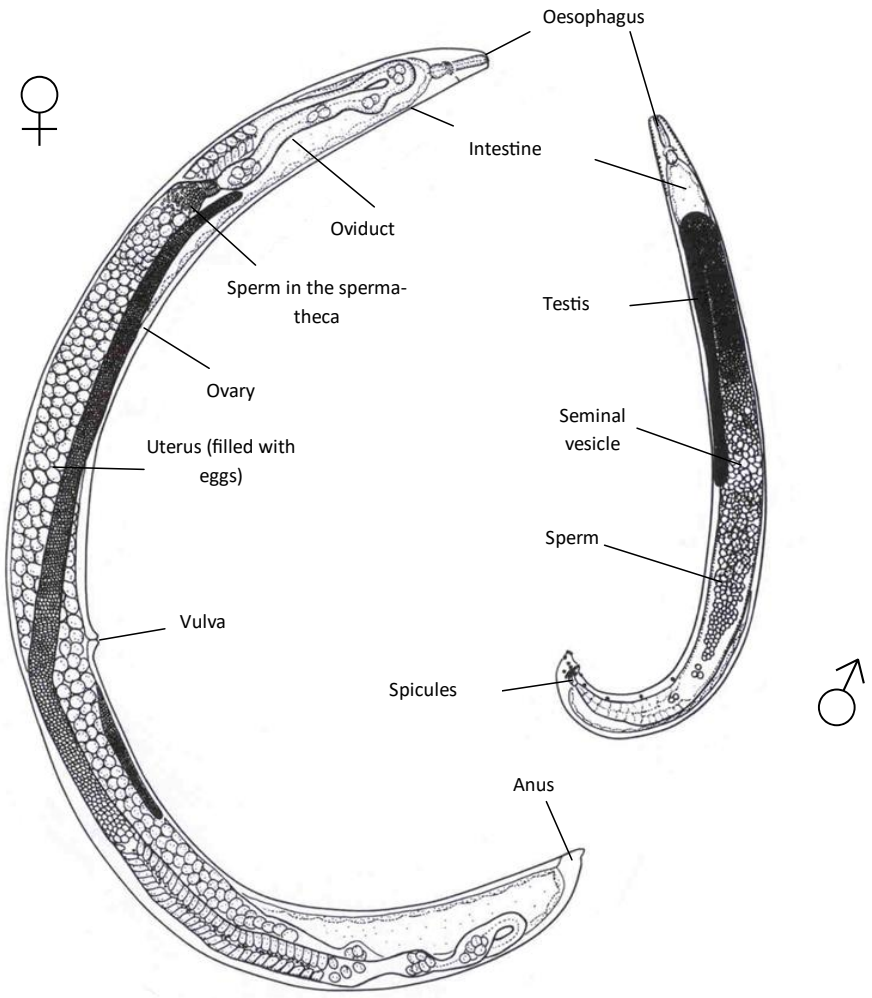


Figure 1.2: General morphology of male and female *Steinernema* reproductive system (Poinar, 1985).

Steinernema spermatozoa are amoeboid and fall into two distinct classes depending on species (Spiridonov et al., 1999). Some species, including *S. affine, S. kraussei* and *S. feltiae*, have relatively small sperm (6-12 µm diameter) which form characteristic chains of up to 25 cells and are similar in morphology and behaviour to *C. elegans* sperm. In contrast, *S. carpocapsae, S. longicaudum* (Figure 1.3) and *S. glaseri* have much larger, macrosperm (up to 110 µm diameter) which do not form chains (Spiridonov et al., 1999). In *Steinernema tami*, sperm dimorphism has been reported whereby large macrosperm serve as a carrier for many smaller microsperm (Yushin et al., 2007).

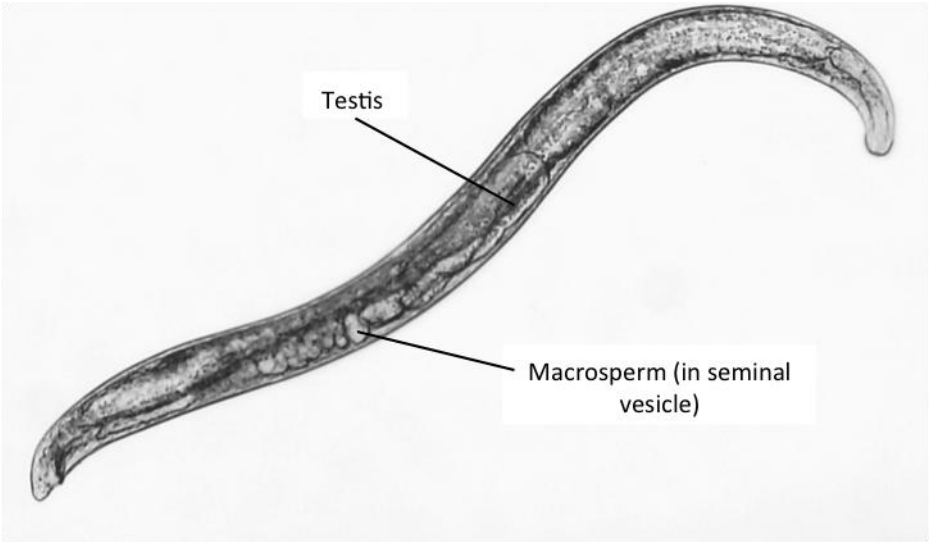


Figure 1.3: Mature male *S. longicaudum* showing macrosperm visible in the seminal vesicle. Photo by Cathryn Hartley.

Copulation behaviour differs between the two families. *Steinernema* males coil around the female whist mating, inserting paired hook-like structures (spicules) on the tail into the vulva to assist in sperm transfer (Strauch et al., 1994; Lewis et al., 2002). In contrast, *Heterorhabditis* males do not coil but align side by side with

hermaphrodites during copulation (Strauch et al., 1994), a behaviour which makes male-hermaphrodite mating in liquid culture impossible.

Once fertilised, eggs are either laid externally or are retained and proceed to develop within the uterus (of either the female or hermaphrodite), a phenomenon known as *endotokia matricida* (Poinar, 1990). Juveniles developing via *endotokia matricida* depend on the mother tissues for nutrition, which may be an adaptive response to limited resources within the host cadaver. It has been suggested that this form of reproduction assists in transmission of symbiotic bacteria from mother to offspring, as in *Heterorhabditis* spp. in particular only juveniles originating from *endotokia matricida* are colonized by bacteria and become IJs capable of infecting a new host (Ciche et al., 2008). Baliadi et al., (2004) report that *Steinernema* spp. IJs developing within the mother's uterus are inferior to those developing outside the mother with regards to ability to invade and kill new hosts (Baliadi et al., 2004).

Colonizing males of *Steinernema* spp. have been suggested to maximise the meeting of the sexes within the host by rendering the host more attractive to females (Grewal et al., 1993), although this 'male colonization hypothesis' is challenged (Stuart et al., 1998; Alsaiyah et al., 2009). Some *Steinernema* spp. may also maximise the potential of encountering a sexual partner by their ability to infect and survive alone within the host for periods of up to 6 weeks (Rolston et al., 2006). It is unknown whether lone residents advertise their presence (e.g by pheromones), and whether IJs can detect the sex of resident nematodes and respond accordingly.

Amphimictic species therefore face challenges in either attracting a partner and potential waiting for long periods for the opportunity to mate. One such challenge is the synchronisation of sexual maturation in partners. In males, production of sperm

in the absence of a female is costly and could mean sperm suffer consequences associated with aging and are not of optimum quality when a female does become available. Optimum rates of fertilisation and successful early stage zygote development are highly dependent on sperm quality (Baker and Aitken, 2004; Tarín et al., 2000). From the instant of activation, sperm cells are subject to oxidative and osmotic stress, temperature damage and depletion of ATP reserves (Reinhardt, 2007); the older the cell, the more its functionality and quality is negatively impacted by these factors. Metabolic processes become less efficient, cell motility is reduced and fertilization ability declines which can result in chromosomal abnormalities, embryo damage, decreased longevity in male offspring and infertility in the next generation (Tarín et al., 2000).

These negative consequences associated with sperm cell senescence (aging), can be combated by producing sperm as close as possible to the point of fertilisation, ensuring they are utilised soon after activation when they are at the highest functional quality. Male *S. longicaudum* have been shown to delay sexual maturation and production of sperm until exposed to female conspecifics, a process likely to be mediated by pheromones as physical contact of partners is not required (Ebssa et al., 2008). The advantages of this are two-fold in that the male prioritizes survival and avoids wasting energy on producing sperm that may never be used in the absence of a reproductive partner along with ensuring sperm are not aged and are of the highest possible quality at the time of fertilization (Reinhardt, 2007).

There is much evidence of sperm competition in nematodes and it is particularly prevalent in gonochoristic species (La Munyon and Ward, 1999). In hermaphroditic species, including *H. bacteriophora* and *C. elegans*, male sperm is often preferred to that of the self-fertile hermaphrodite (Dix et al., 1994; Ward and Carrel, 1979). This

is attributed to the larger size and superior motility of male sperm, allowing the smaller hermaphrodite sperm to be out competed (La Munyon and Ward, 1999). Much like many species of insects and reptiles (Shine et al., 2000), males may also compete for reproductive success by physically blocking entry of competitor sperm through the use of copulatory plugs deposited over the vulva post-mating, as is seen in several *Heterorhabditis* spp. (Strauch et al., 1994). In contrast in *Steinernema* spp. there have been no reports of either the use of mating plugs or sperm competition within the female reproductive tract (Griffin, 2012).

However, pre-copulatory competition has been shown to occur between *Steinernema* males via lethal fighting as a means of eliminating reproductive competitors (Zenner et al., 2014; O'Callaghan et al., 2014). Male *S. longicaudum* coil around male conspecifics causing injury or inducing paralysis and death (Zenner et al., 2014). Furthermore, it was shown that competition is also inter-specific (O'Callaghan et al., 2014) as *Steinernema* spp. males kill both male and female hetero-specifics. The degree of aggression shown varies between species (O'Callaghan et al., 2014) and it has also been shown to be reduced among closely related males (Kapranas et al., 2015) supporting the theory of kin selection.

1.2.3 EPN Phylogeny

The two main families of EPN (Heterorhabditae and Steirnernematidae) are not closely related (Blaxter et al., 1998). Shared traits between them, most notably their association with pathogenic bacteria (Family: Enterobacteriae) are postulated to be a result of convergent evolution (Poinar, 1993). Heterorhabditids are more closely related to the bacterivorous *Caenorhabditis* than the entomopathogenic steinernematids (Figure 1.4, Blaxter et al., 1998) *Heterorhabditis* is closely related to several animal parasites, including *Ostertagia* and *Haemonchus*, while *Steinernema* is quite closely related to the animal parasites *Strongyloides* (Figure 1.4, Blaxter et al., 1998). There are 5 clades within the family *Steinernema* with each of the three species studied in this thesis (*S. longicaudum, S. carpocapsae* and *S. feltiae*) belonging to different ones (V, II and III respectively, see Figure 1.5 and Table 1.1).

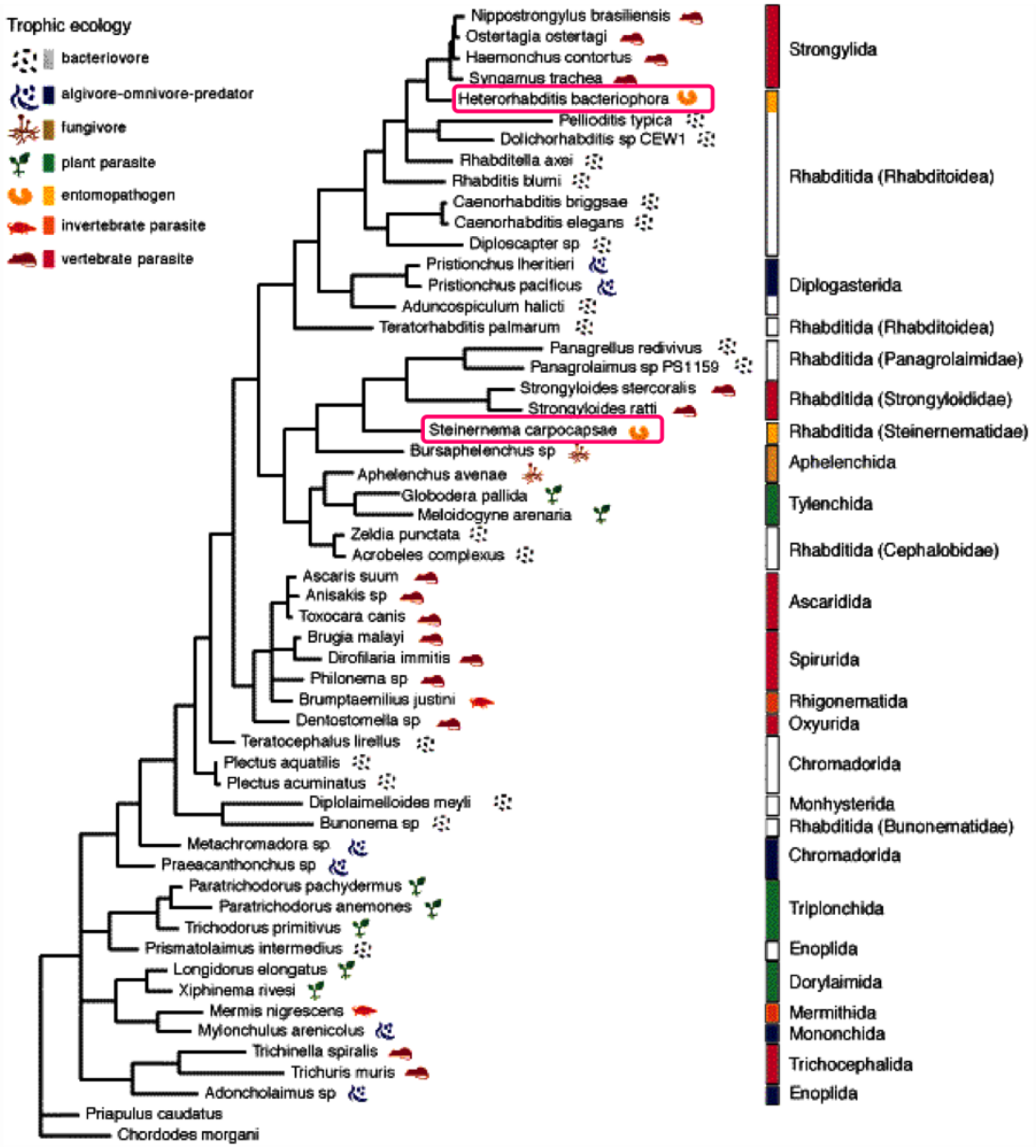


Figure 1.4: Phylogenetic tree highlighting evolutionary relationships of *Steinernema* (respresented by *S. carpocapsae*) and *Heterorhabditis* (represented by *H. bacteriophora*). From Blaxter et al., 1998.

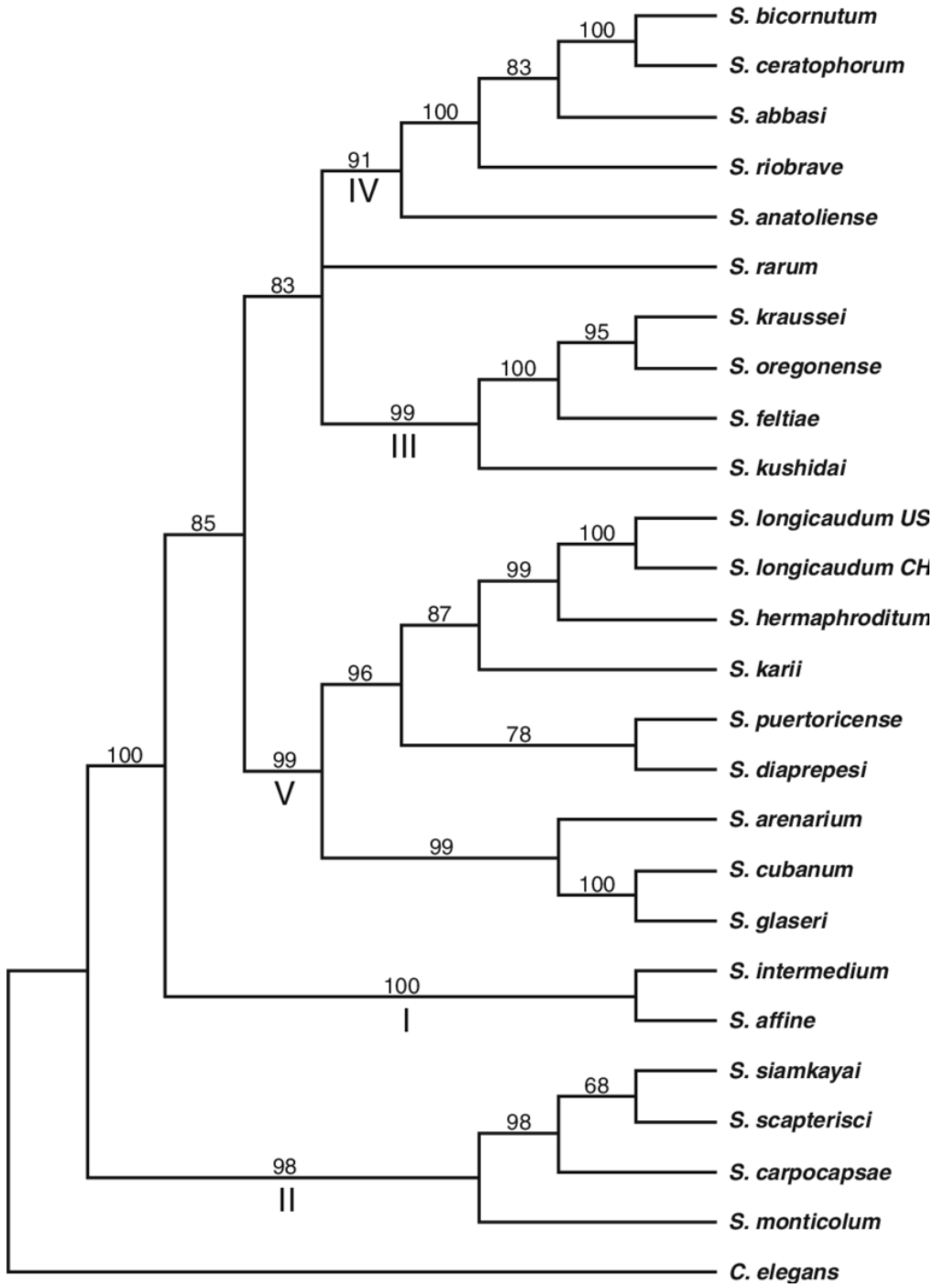


Figure 1.5: Bayesian tree inferred from combined analysis of molecular data. Phylogenetic relationships among 26 Steinernema taxa were estimated using sequences from 2 mitochondrial genes (12S rDNA and cytochrome c oxidase subunit 1) and one nuclear gene (28S rDNA). Numbers shown above internal nodes indicate posterior probabilities resulting from combined likelihood analysis of all molecular data. Roman numerals designate major clades. *(*Nadler et al., 2006).

1.2.4 Bacterial symbionts

The ability of both *Steinernema* spp. and *Heterorhabditis* spp. to kill and subsequently use the host insect cadaver for growth is largely owed to the symbiotic relationship held with bacteria of the Enterobacteria genera *Xenorhabdus* and *Photorhabdus* respectively (Burnell and Stock, 2000). In *Steinernema*, bacterial cells colonising a special intestinal receptacle within the free living IJ (Snyder et al., 2007) are released upon entry to the insect host where they multiply and cause death within 48 hours of infection (Goodrich-Blair, 2007). *Heterorhabditis* spp. employ a similar infection strategy, however carry their bacterial symbiont freely throughout the intestinal tract (Waterfield et al., 2009).In both cases, the bacteria provide nutrition and also offer protection from competitors for the invading nematode. Stationary phase cells secrete metabolites into the host insect haemolymph including exoenzymes, antibacterial and antifungal antibiotics (Maxwell et al., 1994; Webster et al., 2002) insecticidal and nematocidal toxins (Brown et al., 2004; Brown et al., 2006). In this way, the nematode-bacterium complex is able to retain a competitive advantage within the host.

Phenotypic variation in *Xenorhabdus* consisting of two phases (I and II) of the stationary stage of the life cycle has been observed in *in vitro* subcultures (Akhurst and Boemare, 1988). Only Phase I cells (characterised by production of antibiotics and absorption of specific dyes) have been recovered from wild type nematodes (Sugar et al., 2012).

In excess of 90 *Steinernema* species are known (Nguyen, 2016) in contrast to approximately 20 *Xenorhabdus* species (Tailliez et al., 2006; Tailliez et al., 2010). Consequently, a single species of *Xenorhabdus* may colonise multiple species of

Steinernema (Tailliez et al., 2006; Lee and Stock, 2010), however each *Steinernema* species is only capable of retaining a single *Xenorhabdus* species as part of the symbiotic nematode-bacterium complex (Sicard et al., 2004). Nematode-bacterial symbiont complexes to be used in this thesis are shown in Table 1.1.

Table 1.1: *Steinernema* and *Heterorhabditis* strains, identity of associated *Xenorhabdus/Photorhabdus* spp., phylogenetic clade and source of nematodes used in experiments.

Entomopathogenic nematode	Strain	Clade*	Associated bacterial symbiont	Source
Steinernema longicaudum (Shen & Wang, 1992)	CB2B	V	***Xenorhabdus ehlersii*** (Lengyel et al., 2005)	CABI, UK
Steinernema carpocapsae (Wouts et al., 1982)	All	II	***Xenorhabdus nematophila*** (Thomas and Poinar, 1979)	Reading University, UK
Steinernema feltiae (Wouts et al., 1982)	4CFMO	III	***Xenorhabdus bovienii*** (Akhurst and Boemare, 1993)	County Mayo, Ireland (own collection C. Griffin, Maynooth University, Ireland)
Heterorhabditis megidis (Poinar et al., 1987)	UK211		***Photorhabdus temperata temperata*** (Tóth and Lakato, 2008)	CABI, UK

* Nadler et al., 2006

1.2.5 EPN as biocontrol agents

EPN have been used as biocontrol agents against insect pests for several decades (Georgis et al., 2006; Lacey et al., 2015) and can be considered superior to traditional chemical pesticides in several ways. Unlike chemicals which may degrade or be washed away shortly after application, EPN may in some cases be self-propagating in that once applied the life cycle will continue *in vivo* leading to sustained persistence within the environment, reducing the requirement for

reapplication (Peters, 1996; Shields, 2015). Impact on non-target organisms is low as EPN are somewhat host specific and do not have detrimental effects on non-insect animals and plants (Ehlers and Hokannen, 1996, Harvey et al., 2016). Furthermore, direct non-target effects have been shown to be minimised by the targeted application of EPN species (Harvey et al., 2012; Dillon et al., 2012). EPN can be stored for relatively long periods (months) as IJs and are able to withstand mechanical processes necessary for application (Shapiro-Ilan and Dolinski, 2015), making them a legitimately marketable alternative for controlling pests where chemical insecticides either fail or are inappropriate (Ehlers, 2001; Lacey et al., 2015).

EPN used for biocontrol are mass produced in bioreactors as a nematode- bacterium complex (Ehlers, 2001). The life cycle of EPN in liquid culture (*in vitro*) closely resembles the natural life cycle of passage through an insect host (*in vivo*) with the exception that the species-specific symbiotic bacterium (food resource) is pre-cultured in artificial media prior to the inoculation of IJs (Hirao and Ehlers, 2009). Reproduction occurs within the liquid media (usually a single generation) to produce a new generation of IJs as the commercial biocontrol product (Ehlers, 2001). Initial high food availability required for growth is followed by starvation conditions for maximum IJ yield (Hirao and Ehlers, 2010), as natural IJ formation is associated with depletion of food resource. With careful consideration of production parameters to maximise yield, EPN can be produced in large quantities by this method and stored in a dehydrated state in their millions, before application in the field to combat insect pests.

EPN are produced globally by numerous companies (Kaya et al., 2006), and, to date, at least 13 different species have reached commercial development, application, and

sales including *Heterorhabditis bacteriophora*, *H. indica*, *H. marelata*, *H. megidis*, *H. zealandica*, *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, *S. kushidai*, *S. kraussei*, *S. longicaudum*, *S. riobrave*, and *S. scapterisci* (Lacey et al., 2001; Georgis et al., 2006; Kaya et al., 2006: Shapiro-Ilan et al., 2015). Commercial application extends to many economically important pests including the vine weevil, *Otiorhynchus sulcatus* (Lola-Luz et al., 2005), navel orangeworm, *Amyelois transitella* (Siegel et al., 2006) and the pecan weevil, *Curculio caryae* (Shapiro-Ilan et al., 2006). Success of biocontrol initiatives using EPN can be maximised by understanding key behaviours and life strategies of individual species to best exploit them for the purpose of pest control (Lewis et al., 2006; Griffin, 2015). Although EPN are mainly applied innundatively to control existing populations, research has also been directed towards their use in conservation and classical biological control initiatives (Sheilds, 2015).

1.3 IJ behavioural biology and ecology

1.3.1 Dispersal and host finding

Dispersal is particularly important in parasitic species which require a host to complete the life cycle, as in the case of EPN. In the interest of EPN as agricultural pest control agents, optimizing dispersal activity would maximize host-parasite encounters and subsequent pest kill rate. Dispersal and host-finding are distinct behaviours in that IJs must first disperse from the spent insect host before turning focus to seeking a new one to infect (Griffin, 2015).

When IJ are applied in aqueous suspension, typical in EPN biocontrol initiatives, kill rates are lower than when applied inside pre-infected cadavers (Shapiro-Ilan et al., 2003). Laboratory studies indicate that application within the infected host results in improved IJ dispersal (Shapiro-Ilan and Glazer, 1996), along with infectivity (Shapiro-Ilan and Lewis, 1999) and survival (Perez et al., 2003). Chemical compounds, such as the biological waste product ammonia, within EPN infected cadavers stimulate natural dispersal behaviour in emerging IJ (San-Blas et al., 2008; San-Blas et al., 2014). More recently, ascarosidal metabolites have also been implicated in promoting IJ dispersal (Choe et al., 2012a).

Variation in foraging strategy between EPN species may dictate dispersal patterns and mediate to what degree chemical signals influence such behaviour. For example, cruise foragers, the category into which the majority of EPN fall, actively move through the soil in search of a potential (usually immobile) host, whereas ambush foragers (such as *S. carpocapsae*) employ a more passive strategy of waiting for active hosts to move to them (Campbell and Gaugler, 1997). Ambush foragers often engage in specialised behaviours such as nictation ('standing' and waving) and jumping to facilitate attachment to hosts in close proximity (Campbell and Gaugler, 1993). Some species employ an intermediate foraging strategy with characteristics of both cruiser and ambush types, as is seen in *S. feltiae* (Grewal et al., 1994). Cruise foragers rely more heavily on their own movement to locate and ultimately colonize a host, while ambush foragers may take advantage of host mobility and may rely less on their own movement. However, even true 'ambushers' must be mobile and disperse away from a spent host. For example, in *S. carpocapsae* it has been shown that a small proportion of the emerging IJ population behave more akin to cruise foragers, dispersing long distances (Bal et al., 2014). IJs make use of chemicals

emitted by potential hosts to direct host finding behaviour (Dillman et al., 2012). Most notable is the importance of CO_2 in indicating the presence of an insect host, but more host-specific odorants may convey more detailed information required for host location and selection (Dillman et al., 2012).

Foraging strategy is often considered when recommending EPN species for biocontrol, where in simple terms EPN foraging behaviour is matched to the ecological characteristics and habitat of the target host (Gaugler et al., 1997). In reality, however, the story can be much more complex and increasing knowledge on IJ dispersal and foraging behaviour will help better inform future biocontrol programs (Griffin, 2015).

1.3.2 Host selection and assessment

IJs are able to identify host insects via chemical means, typically using cues from the host faeces and cuticle (Grewal et al., 1997; Lewis et al., 1996; Lewis et al., 2006). More interestingly, evidence shows that IJs can assess the quality of a potential host, including whether or not it has already been parasitized by conspecific or heterospecific EPN and which may serve as competition to the invading individual (Griffin, 2012). The mate recruitment hypothesis (Grewal et al., 1993) states that it is in a female IJ's interest to enter a host where conspecific male IJs are already established, therefore maximizing reproductive potential. However prior colonization with high numbers of conspecifics may actually deter a new invader as resource and reproductive competition will be high (Glazer, 1997). IJ production is maximized when an optimum number of IJs initiate infection (Selvan et al., 1993). In most *Steinernema* spp., at least one male and one female must enter a host to

reproduce, unlike the hermaphroditic *Heterorhabditis* spp. in which a single IJ may colonize an insect. Furthermore, it often takes numerous IJs to overcome host defences (Peters and Ehlers, 1994). In several *Steinernema* species (*S. anomali, S. riobrave, S. carpocapsae* and *S. glaseri*) IJs are attracted to and will enter cadavers already infected with conspecifics in some cases up to 72 hours post infection (Christen et al., 2007; Stuart et al., 1998). However, such behaviour is suggested to be a result of low host availability or poor ability of IJs to assess host suitability (Christen et al., 2007). In contrast, entry to a host already infected with conspecifics may actually be detrimental as overcrowding reduces individual reproductive output (Koppenhofer and Kaya, 1995; Ryder and Griffin, 2002). Furthermore, *S. glaseri* IJs are repelled by hosts infected with heterospecifics (*S. riobrave*) suggesting that chemical cues from heterospecifics trigger avoidance of inter-species competition (Grewal et al., 1997).

1.3.3 Behavioural sexual dimorphism

IJ behaviour is suggested to vary between the sexes, with males being more responsive to host chemical cues and subsequently more active dispersers than females (Grewal et al., 1997). In several *Steinernema* species, Grewal et al. (1993) reported that males are the colonizing sex in that they are early emergers; the first to disperse and locate the next insect target. Moreover, parasitism of an insect by males is reported to increase host attraction to females (Grewal et al., 1993), increasing the chance that sexual partners will meet and reproduce. Conflicting evdidence for male-bias in early dispersing IJs is shown in *S. glaseri,* where there was no evidence of male first colonization (Stuart et al., 1998). This finding was supported by Alsaiyah

et al. (2009), who reported that *S. glaseri* and 4 additional *Steinernema* spp. (*S. longicaudum, S. feltiae, S. carpocapsae* and *S. kraussei*) all show a slight female biased sex ratio in first generation nematodes, both *in vivo* and *in vitro*. In *S. feltiae* it has been shown that a greater proportion of females account for initial infection of new hosts (Bohan and Hominick, 1997) and hosts with low IJ establishment (1-5 nematodes) harboured exclusively females in the majority of cases (Scheepmaker et al., 1998).

1.3.4 Social behaviour

It has been reported that IJs engage in social behaviour both with their emerging cohort and on encounters with heterospecifics. For example, *S. carpocapsae* increase nictation behaviour and move more actively toward a host when in the presence of IJs of the competing species, *S. glaseri* (Wang and Ishibashi, 1999), demonstrating the potential for IJs to recognize heterospecifics and modify behaviour accordingly. When dispersing from a spent host, IJs move together in large numbers either through soil or over an agar surface in a laboratory setting (Rolston et al., 2006), particularly in the early stages of emergence. It is proposed that such group movement is an active behavioural decision to offer protection and minimize potential for predation, analogous to shoaling behaviour in fish (El Borai et al., 2011). Alternatively, Shapiro-Ilan et al. (2014) propose that group movement is consistent with group infection behaviour reported by Fushing et al. (2008). According to their paradigm, risk-prone IJs invade the host first followed by risk averse nematodes (Fushing et al., 2008). However, Griffin (2015) suggested that group movement may be purely due to physical constraints of IJs' small size and

subsequent influence from surface tension of the moist environments they inhabit. If IJs do indeed respond behaviourally to each other, this raises questions to how they may achieve this, including whether chemical communication may play a role.

1.3.5 Changes in IJ behaviour during storage

Although IJs may survive for several months whilst waiting to infect a new host, since they do not feed their lipid reserves become depleted and starvation ensues (Patel et al., 1997; Fitters and Griffin, 2006). Qui and Bedding (2000) report a decline in infectivity of *S. carpocapsae* IJs when stored in water for extended periods, and attribute this to the depletion of energy resources (including trehalose and glycogen) and subsequent compromised energy metabolism balance of this non-feeding stage.

There has been much discussion of 'phased infectivity'in EPN, whereby IJ's ability to infect new insect host changes over time post emergence (Lewis et al., 2006). Several reasons have been proposed for this phenomena (Lewis et al., 2006), including that IJs may switch between infectious and non-infectious states following emergence from the host (Bohan and Hominick, 1997) or alternatively that individuals emerge as pre-determined 'risk-takers' eager to colonise new hosts, or 'risk-averse' more suited to secondary entry to an already compromised host (Hay and Fenlon, 1995; Fushing et al., 2008). Changes in proportion of either 'risk taking' or risk averse' individuals with the emerging population would therefore cause fluctuation in the infectivity of the population as a whole (Fushing et al., 2008).

Some studies have shown that IJs may become more infective with age, following short-term storage in tap water at various temperatures (Griffin, 2012) before the

eventual decline due to resource depletion. A progressive increase in infectivity is suppressed temporarily when IJ are stored at low temperature (9˚C), although it recovered over time and reached a peak after about 10 weeks storage (Griffin, 1996, Fan and Hominick, 1991). Alternatively it has been proposed that IJs experience a temporary diapause on emergence from the host cadaver which is terminated once environmental conditions are favourable (Womersley, 1993). Working with steinernematids, Fan and Hominick (1991) proposed that increasing infectivity with age is due to termination of the state of diapause (additional to the state of IJ) due to abiotic factors such as temperature, storage and age (Wormersley, 1993). For *H. megidis*, infectivity increase at high temperature storage (20-25˚C) differs from cold storage (9˚C) in that there is no obvious lag period of low infectivity and the increase proceeds more quickly (Griffin, 1996).

1.4 Pheromones

The term 'pheromone' was coined in 1959 by Peter Karlson and Martin Luscher, (Karlson and Luscher, 1959) to describe externally secreted chemicals used to communicate specific messages to conspecifics. Literally translated from Greek origins, pheromones transport ('*pherin*') information to stimulate ('*hormone*') a response in the receiver. Pheromones are distinguished from hormones in that they are released from an organism into the surrounding environment to act externally on other organisms, whereas hormones are both produced and detected internallly by a single individual (Wyatt, 2003). Historically, studies into chemical signalling have taken a back seat to the other more easily quantifiable means of information transfer such as vision and auditory signals. However, the last 60 years has seen research in

this area boom (Wyatt, 2009), particularly since the identification of the first of many insect pheromones in the 1980s (Hecker and Butenandt, 1984).

1.4.1 Primer and releaser pheromones

The broad term pheromone can be dissected in to several specific sub-groups of semiochemicals with defined function. Primarily, they are categorised as 'primer' or 'releaser' pheromones according to the type of reaction they induce in the receiver (Karlson and Luscher, 1959). Primer pheromones induce long-lasting or permanent physiological changes in the receiver, typically by stimulating hormone release in the endocrine system (Wilson and Bossert, 1963). In contrast, releaser pheromones induce immediate, short-term effects on behaviour of the receiver. In some cases a single pheromone compound or blend can be classified as both a releaser *and* a primer pheromone (Reigner and Law, 1968), highlighting complexities in pheromone classification and more importantly, biological function. As an example, virgin queen honeybees (*Apis mellifera*) produce the sex pheromone, ketodecenoic acid, which acts as a releaser pheromone to attract drones for mating (Gary, 1962). However, the same pheromone acts as an inhibitory primer when produced by newly mated queens (Butler, 1959) preventing female workers from sexually maturing and producing eggs (Butler and Fairey, 1963). If the queen is removed from the hive, workers' ovaries become enlarged and over time they gain the ability to lay fertile eggs (Butler et al., 1961). Originally named 'queen substance' the same pheromone also acts to prevent queen-rearing behaviour in workers (Butler and Gibbons, 1958), ensuring only a single queen remains in the hive, minimising her chance of renouncing the throne. The honeybee model effectively demonstrates the complex

role of pheromones in modifying behaviours and physiology, in particular how a single pheromone may play several roles in the control of both behaviour and developmental processes.

1.4.2 Chemical eavesdropping

Although the strict definition of pheromones states that they are involved in communication between members of the same species (Karlson and Luscher, 1959), they may also be perceived by other species, either to the benefit or detriment of the producer. Predators can benefit from detecting pheromones of prey species, eavesdropping to locate a meal in a similar way that conspicuous brightly coloured displays in prey species may draw unwanted attention from predators. Some species are able to use pheromones to their benefit, deceiving the receiver with an intended false message. Such chemical mimicry is shown in bolas spiders that produce chemicals highly similar to the female sex pheromones of several moth species, luring male moths close enough for capture (Stowe et al., 1987).

1.4.3 Specificity

Species-specificity of pheromones is dependent on the nature of the message being communicated. For example, some fish exhibit anti-predator behaviour when exposed to alarm pheromones released following mechanical damage of both conspecifics and heterospecifics (Golub et al., 2005). If a predator has alarmed an individual of one species, behavioural modification (i.e. fleeing or shoaling in fish) may benefit not only conspecifics, but also members of other species within the local

area. In evolutionary terms, heterospecifics which respond to the alarm cue increase their chance of survival and the response is selected for (Wyatt, 2003). Responding to alarm cues of other species susceptible to similar threats as your own increases the number of individuals on the lookout and maximises the chance of detecting a potential threat. In instances such as this, where members of different species have a common aim, specificity is likely to be relatively low.

In contrast, sex pheromones in particular are generally highly species-specific, resulting from strong sexual selection pressures involved in their evolution (Wyatt, 2003). Correctly identifying and engaging in sexual behaviour with a suitable sexual partner of the same species is key to successful reproduction, while choosing incorrectly wastes energy and could even be fatal, such as in nematodes which attack and kill heterospecifics (O'Callaghan et al., 2014).

The sheer number of species, along with limitations associated with pheromone biosynthesis and reception make difficult for chemically distinct signal molecules to exist in every different species. Although pheromones have been shown to consist of several different types of chemical compounds, including proteins (Wyatt, 2014; Liberles, 2014), ketones and steroids (Sorensen et al., 1998), it is often the case that related species use pheromone molecules of similar chemical structure or origin. In some cases even very distantly related species use the same compound for similar purposes, such as the Asian elephant (*Elephas maximus*) that shares its female sex pheromone, (Z)-7-dodecen-l-yl acetate with over 100 species of moth (Rasmussen et al., 1996). This problem can be overcome as most pheromones exist as mixtures or 'bouquets' of two or more chemical components in defined amounts (Figure 1.6, Wyatt, 2003).

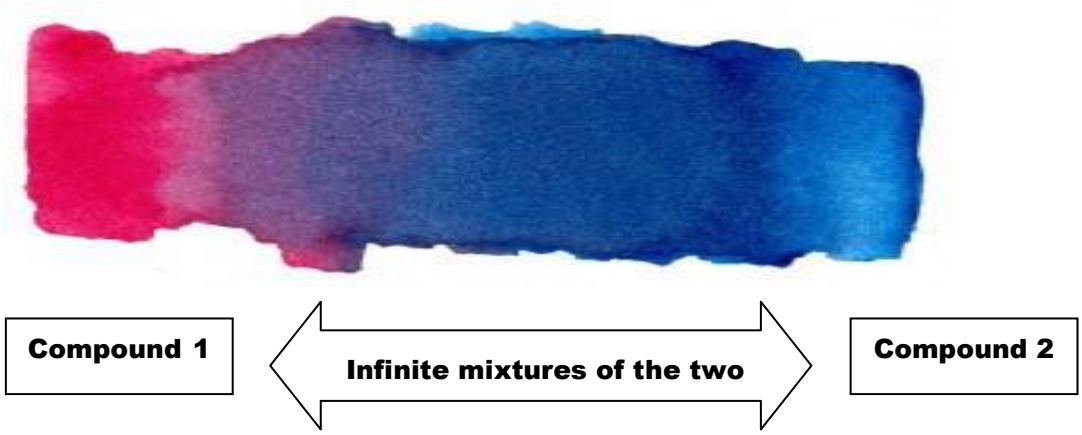


Figure 1.6: A simple visual representation of how two chemical compounds may combine in varied ratios to produce a broad spectrum of specific mixtures. It is important to note that chemical structure of mixed compounds, including polarity, may influence they way different molecules interact when mixed.

1.5 Pheromones as tools for biocontrol

Although pheromonal communication is documented in almost every taxon, from bacteria to mammals, it is insects that have contributed most to the growing body of knowledge in the field of chemical signalling (Wyatt, 2003). The driving force behind research into insect pheromones is directly linked to the economic benefit of controlling insect pests in agriculture, horticulture and forestry. Exploring methods of pest management alternative to chemical insecticides, which often negatively impact non-target organisms in addition to the targeted pest, has been an important research theme over the last 6 decades, leading to the identification of over 100 insect pheromones (Witzgall et al., 2010). The exploitation of sexual and aggregation pheromones in particular is common in modern day pest management schemes as they can be effectively utilised to disrupt key behaviours necessary for population growth and persistence (Agelopoulos et al., 1999; Welter et al., 2005) . The use of pheromones for pest control is not always straightforward, however, and multiple parameters including specificity, accessibility and persistence of chemical cues must be considered before pheromones can be used effectively as behaviour manipulating pest control agents (Foster and Harris, 1997). This highlights how understanding fundamental biological principles can contribute to maximising efficiency of applied agricultural management.

1.5.1 Common pheromonal pest management methodologies

Trap and kill: Initially, application of insect pheromones in agriculture involved mass trapping schemes which aimed to remove target pest organisms from the environment (Burkholder et al., 1985). Aggregation and sex pheromones have been

used in this way to lure problem insects away from the target area, where they are trapped and disposed of. However, the success of insect pheromone-baited traps is extremely variable and efficiency is highly dependent on the species targeted and type of pheromone used (Suckling and Karg, 1999). For example, sexual attractants are generally sex specific, creating difficulty in trapping entire populations rather than males or, less commonly, females exclusively (Witzgall et al., 2010). Sex pheromones are also active only in the adult stage of the life cycle, whereas many insect pests cause extensive damage at the larval stage only. Many insects use aggregation pheromones to gather conspecifics, often in large numbers, for mating, defense against predators or to overcome a host by mass attack (Suckling and Karg, 1999). From as early as the 1980s, pheromone-baited traps have been successfully implemented to suppress populations of insect species across the globe in agricultural, horticultural, forestry and domestic settings (Witzgall et al., 2010). Some of the more notably successful schemes include mass-trapping and killing of bark beetles (Borden, 1989), boll weevils (Hardee and Mitchell, 1997) and numerous Lepidoptera species including citrus flower (Varner et al., 2001) grapevine (Sternlicht et al., 1990) and gypsy moths (Miller et al., 1977).

Behavioural and reproductive disruption: Pheromones can also function as pest control agents without directly attracting insects away from an environment to be killed. Sex pheromones in particular can be used to disrupt reproduction using methods such as flooding the environment with synthetic female pheromone, essentially masking ‘true’ female scent and preventing males from locating a mate (Foster and Harris, 1997).

Population monitoring: Pheromonal attractants can also be utilized, not for removal, but assessment of insect populations. Pheromone traps can be used to attract the target insect to identify which area of an environment is affected by a pest species and in what density (Trematerra, 1997; Witzgall et al., 2010). Defining such parameters prior to an insecticide application, for example, allows for only infested areas to be treated and with an insecticide concentration appropriate to control specific population density (Witzgall et al., 2010). Strategies which involve pheromonal trapping and subsequent re-release of insects can be used to monitor populations without negatively affecting numbers (Wileyto et al., 1994).

1.5.2 Pheromonal control of pest nematodes

Examples of sex pheromones being used to control pest nematodes are rare. The plant-parasitic soybean cyst nematode (*Heterodera glycines*) is a global pest of soybean (*Glycine max*) and is responsible for substantial economic loss annually (Niblack et al., 2006). J2 stage juveniles infect the roots of the plant and feed on the vascular tissues as they develop to adult stage. On maturation, females swell and form cyst-like structures that protrude from the root, giving *H. glycines* its common name.

The female sex pheromone of *H. glycines* was structurally identified as vanillic acid by Jaffe et al. (1989) who removed mature females from infected plants and incubated them in water for 24 h to create a female 'scent' extract. The extract was filtered and lyophilized before being subjected to a number of HPLC (High Performance Liquid Chromatography) steps to identify the active fraction. GC-MS (Gas Chromatography-Mass Spectrometry) technology allowed for final structural

confirmation of the pheromone. The authors also confirmed activity of vanillic acid as a sex pheromone by bioassaying the synthetic version, which both attracted males and induced copulatory behaviour in them (Jaffe et al., 1989). Meyer et al. (1997) trialled vanillic acid as a management agent of *H. glycines*. In initial small scale studies conducted in greenhouses, application of vanillic acid to infected plants succeeded in reducing populations of *H. glycines* (Meyer et al., 1996). Furthermore, a number of chemical analogues of vanillic acid (notably syringic acid, 4-hydroxy-3-methoxybenzonitrile) also reduced the occurrence of nematode cyst on parasitized soybean plants (Meyer et al., 1996). Meyer et al. (1997) replicated the greenhouse results to some extent in the field. Both vanillic acid and a selection of its chemical analogues were able to successfully reduce the number of root cysts caused by *H. glycines*, and as a result pheromone-treated plots saw increased crop yield in comparison to controls (Meyer et al., 1997). As previously shown in insects, their success was concluded to be a result of the applied female sex pheromone (or chemical analogue) disrupting the ability of males to locate females by chemical reception to impact reproductive success and reduce the occurrence of the nematode pest (Meyer et al., 1997). Despite these findings, vanillic acid was never used commercially in pest management.

1.5.3 Advantages and disadvantages of pheromones in biocontrol

Despite major research developments in the use of pheromones in pest control in insects and, to a lesser extent, nematodes, in comparison to more traditional methods (e.g. pesticides) their application globally is still relatively rare, particularly on a large scale. Exclusively pheromonal pest control methods are most commonly

marketed and used in the domestic sector (gardens etc) or smaller horticultural initiatives. In agriculture and forestry, semio-chemicals in pest reduction are almost always used as components of integrated pest management (IPM), complimenting other methods.

Synthetic sex and aggregation pheromones present many advantages, particularly for the environment, in that they are non-toxic, species-specific and active in very low concentrations, minimising detrimental impacts on the wider ecosystem. However, their activity is extremely complex. Furthermore, the species-specific nature of pheromones means that one product is only effective against a single pest species, making them less attractive to commercial companies, or where a pest-complex is present.

1.6 Nematode pheromones

Although chemical recognition of hosts by plant parasitic nematodes was recognised as early as 1925 (Yasuhira et al., 1982), research on chemical communication in nematodes, with particular focus on sex attractants began to emerge in the early 1960s. The first example of sexual attraction via chemical means was reported in the free-living nematode *Panagrolaimus rigidus*, where it was shown that males and females were attracted to each other (Greet, 1964). Within the same decade, further examples of sex attractants were reported in plant-parasitic cyst nematodes, *Globodera rostochiensis* and *Heterodera schactii* (Green, 1966) and the mammalian parasitic hookworm, *Ancylostoma caninum* (Roche, 1966). Lee (2002), reviewing nematode responses to pheromones, noted that over 30 species but relatively few genera of free-living, plant-parasitic and animal-parasitic nematodes had been shown

to exhibit pheromone-mediated behaviour by that date. Furthermore, attempts to characterise these pheromones found they frequently consisted of several components, occasionally including volatiles (Lee, 2002). While early research focussed mainly on sexual attraction between males and females and did not identify the pheromones involved, it is now known that nematodes use pheromones to mediate several sexual and organization behaviours including juvenile dispersal (Kaplan et al., 2012), entry into dauer (Butcher et al., 2007; Butcher et al., 2008) or IJ stage in EPN (Noguez et al., 2012), sexual attraction (Simon and Sternberg, 2002; Lipton et al., 2004) and sexual development (Ebssa et al., 2008). To date, the main body of research concerns the model species *Caenorhabditis elegans* (earlier research reviewed by Edison, 2009), but increasingly covers other nematode genera and species including *Steinernema* spp. (Neves et al., 1998; Choe et al., 2012b), *Heterorhabditis bacteriophora* (Noguez et al., 2012) and *Panagrellus redivivus* (Choe et al, 2012a). Several EPN species have been shown to mediate key behaviours and developmental processes upon reception of chemical cues produced by conspecifics (Neves et al., 1998; Ebssa et al., 2008). Recent research has shown that, like many other nematode species, *Steinernema* spp. produce and modify behaviour and development in response to identified pheromone molecules, ascarosides in both the reproductive adult and IJ stages of the life cycle (Choe et al., 2012b) The first chemical identification of ascarosides as nematode pheromones in 2005 (Jeong et al., 2005), has seen interest in the field boom in recent years. Ascaroside pheromones are reviewed in more depth in Section 1.6.1. In *C. elegans,* it has been shown that additional developmental effects are also mediated by dafachronic acids and nemamides (Butcher, 2017) which regulate adult life span,

timing of entry to dauer and promote survival during starvation-induced larval arrest at the L1 stage (Shou et al., 2016).

1.6.1 Ascarosides

Ascarosides are a family of small molecules, unique to nematodes, responsible for controlling behaviour and regulating development (Edison, 2009). The first example (ascr#1/C7/daumone-1) was identified in 2005, a metabolite of *C. elegans* responsible for inducing juveniles to enter into the non-feeding dauer stage of arrested devlopment (Jeong et al., 2005). Ascaroside synthesis and signalling has since been identified in several phylogenetically distant nematode taxa (Choe et al., 2012b), suggesting that the ascarosidal chemical language of nematodes is highly conserved within the phylum (Braendle, 2012).

The molecules themselves are all similarly composed of the dideoxysugar, ascarylose, covalently linked to fatty-acid based moieties. It is the variation in structure and length of these fatty-acid side chains that distinguish between individual ascarosides and give the molecules their functional diversity as pheromone components (von Reuss et al., 2012). Although numerous ascarosides have been identified from a wide variety of nematode species (Choe et al., 2012b), functionality, particularly outside of *C. elegans,* is poorly understood (Butcher, 2017). Identification is relatively straightforward, however understanding how ascarosides work as finely tuned blends to govern important behaviours and developmental processess is considerably more complex (Braendle, 2012). Relatively soon after the identification of the initial single component ascaroside

pheromone of *C. elegans* (Jeong et al., 2005) it became apparent that the story was far from complete and certainly not straightforward.

The newly identified ascaroside (ascr#1), (-)-(6R)-(3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy)heptanoic acid (Jeong et al., 2005), named by the authors as 'daumone' (Figure 1.7), induced *C. elegans* to enter the dauer stage of the life cycle, a period of arrested development entered naturally in response to high population density and inadequate food resources (Golden and Riddle, 1982). Bioactivity of daumone was confirmed by conducting dauer formation assays with both naturally extracted and synthetic daumone (Jeong et al., 2005).

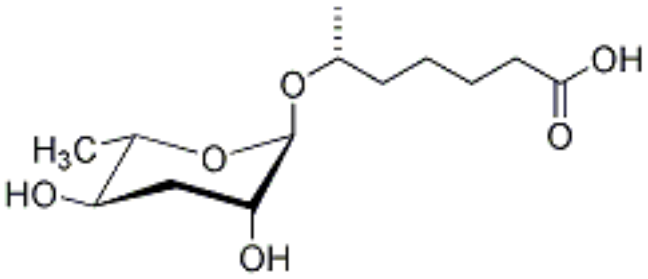


Figure 1.7: Chemical structure of daumone, (-)-6R-(3'R,5'R-dihydroxy-6'S-methyl-(2H)-tetrahydropyran-2'-yloxy)heptanoic acid (www.smid-db.org). Also referred to as ascr#1 or C7.

Within just a few years, two more ascarosides (ascr#2 and ascr#3) were isolated from liquid culture exudate of *C. elegans* that also induced juvenile entry into dauer (Butcher et al., 2007). These were shown individually to be approximately two orders of magnitude more potent than the initially presented ascr#1, and also to act synergistically as part of a three component blend to further promote dauer formation (Butcher et al., 2008). Different combinations of the three molecules gave rise to additive effects on dauer formation (Butcher et al., 2007 and 2008), indicating

daumone was not a single ascaroside but a blend of several. All three molecules are derivatives of 3, 6-dideoxyhexose ascarylose, with the only structural differences occurring on the fatty acid-like side chains. More recently, NMR (Nuclear Magnetic Resonance) spectroscopy and mass spectrometry-based comparative metabolomics, has facilitated the identification of several more ascarosides thought to be implicated in *C. elegans* dauer formation (Figure 1.8), in particular indole containing ascarosides (Butcher et al., 2009a: Srinivasan et al., 2012; von Reuss et al., 2012), though their functional importance has not been fully resolved (Ludewig and Schroeder, 2013).

As with other pheromone molecules and blends, absolute and relative concentrations of nematode pheromone components are key to elicit the intended response. *C. elegans* mate attractants, ascr#3 and ascr#8 are ineffective when presented in concentrations outside of a narrow range, 0.1-1pmol and 1-10 pmol respectively (Srinivasan et al., 2008; Pungaliya et al., 2009). Furthermore, variation in concentration may not only reduce bioactivity, but also modify function. This is seen in the indole ascaroside, icas#9 that induces dauer in *C. elegans* at low concentration but inhibits it at higher concentration (Butcher et al., 2009a).

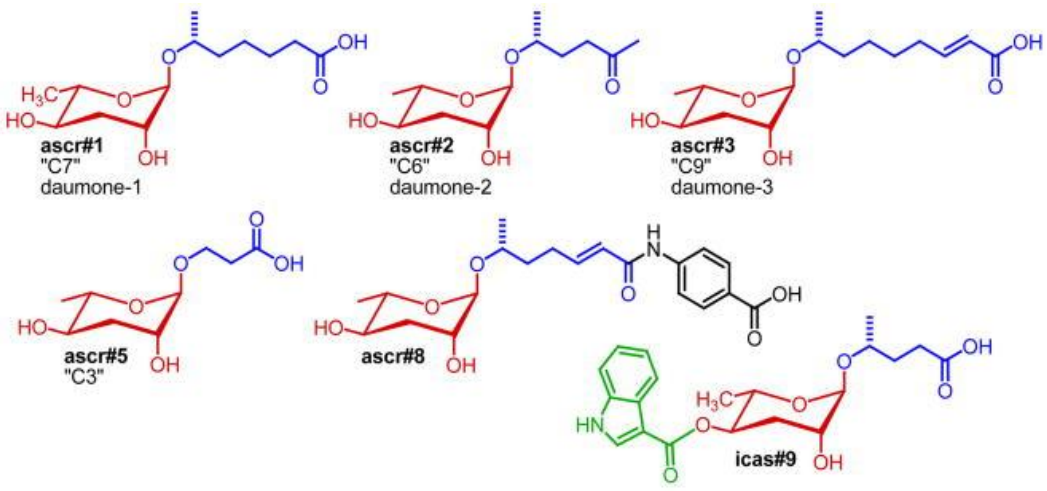


Figure 1.8: Structure of ascarosides shown to induce dauer formation in *C. elegans* (Ludwig and Schroeder, 2013)

Since the initial identification of various ascaroside components of the *C. elegans* dauer pheromone, ascaroside molecules have been found to affect both development and behaviour in nematodes. Though the majority of research has focused on *C. elegans*, evidence of ascaroside signalling has been shown across a range of nematode taxa. In a screen of the composition of liquid culture media of over 15 species of nematodes from 13 different genera, ascarosides were detected in all but 2 species (both *Romanomermis* spp.) (Choe et al., 2012b). The sample contained representatives of many life strategies including entomopathogenic species (*Steinernema* spp., *Oscheius* spp. and *Heterorhabditis* spp.), plant parasites (*Pratylenchus penetrans*), vertebrate parasites (*Ascaris* spp. and *Nippostrongylus* spp.), and both insect-associated (*Pristionchus* spp, *Koernia* spp. and *Oscheius* spp.) and non-insect associated (*C. elegans* and *Rhabditis* spp) bacteriovores and fungivore, suggesting that ascaroside signalling is widely conserved among nematodes (Choe et al., 2012b).

Recent research has shown that ascarylose is not the only carbohydrate forming the structural base of nematode signalling molecules. Paratose-based signal molecules (npar#1 and par#9) have been isolated and shown to induce dauer formation in the necromenic nematode, *Pristionchus pacificus* (Bose et al., 2012), although known ascarosides (asc#1, ascr#9 and ascr#12) were also identified within the exometabolome. This finding shows how structural diversity may arise in areas other than the lipid side chains of nematode signalling molecules and also highlights considerations for the metabolic pathways involved in biosynthesis. As structural diversity of identified nematode signalling molecules grows more complex, they are more recently being referred to as nematode-derived modular metabolites (NDMMs) rather than ascarosides exclusively (von Reuss and Schroeder, 2015).

1.6.2 Perception

It is strongly suggested that ascaroside signals are detected via G protein-coupled receptors (GPCRs) located on chemosensory neurons within amphids (cuticular invaginations) in the head region of nematodes (Ludewig and Schroeder, 2013). In *C.elegans*, specific neurons have been implicated in the expression of GPCRs involved in ascaroside perception (Figure 1.9) with variation in the particular neurons involved in different behaviours (Bargmann, 2006), such as dauer formation and sexual behaviour of males and hermaphrodites (Ludewig and Schroeder, 2013).

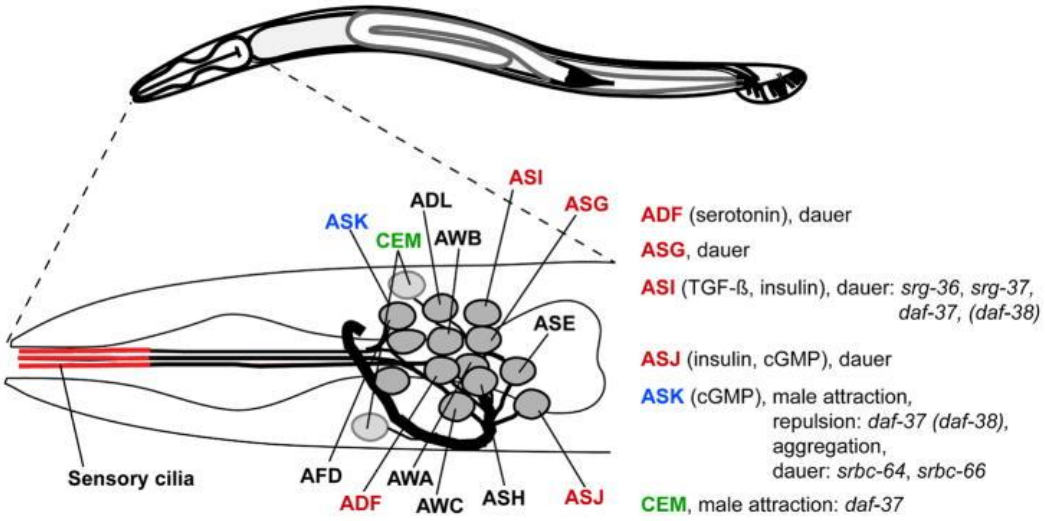


Figure 1.9: Head neurons of *C. elegans* (including the male-specific CEM neurons). Highlighted neurons have so far been implicated in ascaroside perception. Also shown are genes coding for G-protein expression, downstream signaling pathways, and so-far identified receptors (Ludewig and Schroeder, 2013).

The availability of and potential to genetically manipulate mutant daf-d strains of C. *elegans* has provided the most popular model for studying the role of GPCRs in ascaroside reception.

Loss-of-function mutations in the GPCRs *srbc-64* and *srbc-66* have been shown to reduce the occurrence of *C. elegans* dauer formation in response to natural dauer pheromone and also its individual ascaroside components (ascr#1, ascr#2, and ascr#3) in synthetic form (Kim et al., 2009). However, these mutant strains still formed dauer larvae in response to high concentrations of ascr#1, ascr#2, and ascr#3, suggesting that additional GPCRs are involved in perception of these ascarosides. Further studies using introgressive hybridization between daf-f wild type and daf-d mutants have also shed light on the specifics of ascaroside perception (McGrath et al., 2011). Introgression of disrupted GPCRs *(srg-36* and *srg-37*), from ascr#5-resistant mutant strains into non-resistant wild-type strains conferred resistance to

ascr#5, but did not alter sensitivity to other ascarosides, indicating that *srg-36* and *srg-37* are specifically required for perception of ascr#5 (McGrath et al., 2011).

Knowledge on ascaroside perception is still extremely scarce, with relatively few comprehensive studies on the subject (all on *C. elegans*) published to date (Kim et al., 2009; McGrath et al., 2011; Park et al., 2012). The muti-component nature of nematode pheromones along with the occurrence of overlapping functions of receptors, indicates a highly complex signalling system in a relatively simple organism (Ludewig and Schroeder, 2013).

1.6.3 Biosynthesis

The chemical building blocks of all ascarosides are carbohydrates, amino acids and fatty acids. The main unit of the ascarylose ring is ω or ω-1 linked with either saturated, α, β-unsaturated or β-hydroxylated fatty acid side chains of varied length (von Reuss et al., 2012). This variation in side chain length, along with occasional additional linkage to amino acids produces the functional moiety of each different ascaroside unit. Oxygenation of very long fatty acid side chains (VLCAs) occurs prior to attachment to ascarylose (Figure 1.10), which is followed by shortening of the side chains via peroxisomal β-oxidation (von Reuss et al., 2012). Specific enzymes involved in the shortening process are currently unknown (Ludewig and Schroeder, 2013). The acyl-CoA oxidase ACOX-1 is implicated in the early stages of peroxisomal side chain oxidation, introducing α, β-unsaturation (Figure 1.10, von Reuss et al., 2012). Indole attachment in the specific class of indole ascarosides

(containing indole modification at position 4 of the acarylose ring), is thought to involve different biosynthetic pathways, however these are unknown (Ludewig and Schroeder, 2013).

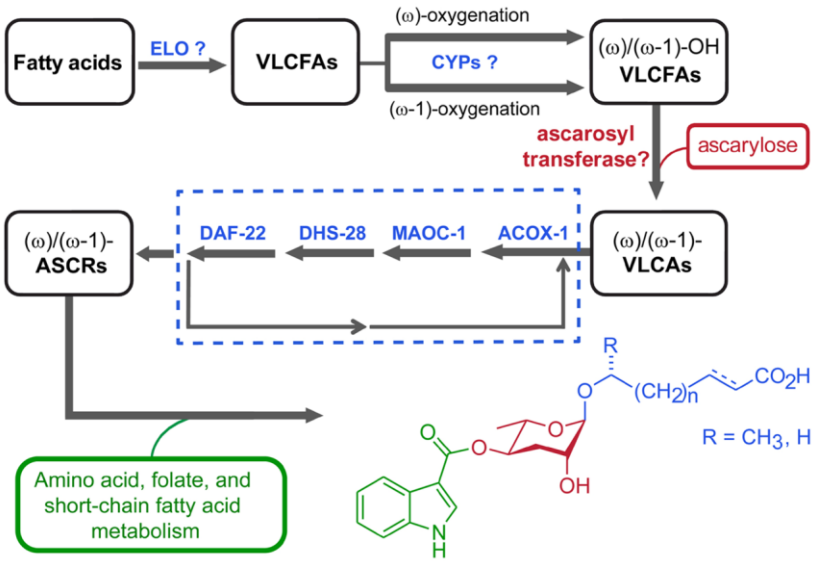


Figure 1.10: Model for ascaroside biogenesis. Chain elongation of fatty acids (by putative elongase homologs elo-1–9 (Agbaga et al., 2008)) is followed by (ω-1)- or (ω)-oxygenation of VLCFAs and ascarylose attachment by yet unidentified enzymes. The resulting VLFCAs enter peroxisomal β-oxidation via ACOX-1, MAOC-1, DHS-28, and DAF-22 producing short chain ascarosides, which are linked to building blocks from several different primary metabolic pathways (von Reuss et al., 2012).

The genes daf-22 and dhs-28 code for proteins involved in β-oxidation of fatty acid side chains of ascarosides present in daumone. Both proteins (DAF-22 and DAF-28) are expressed in the intestine of *C. elegans*, implying this to be the site of ascaroside biosynthesis in nematodes (Butcher et al., 2009a). Ascaroside synthesis is dependent on life stage (Kaplan et al., 2011) and gender (Izrayelit et al., 2012). External factors including diet (von Reuss et al., 2012; Artyukhin et al., 2013), population density (Izrayelit et al., 2012) and temperature (Joo et al., 2010) also affect rates of ascaroside biosynthesis. Screening of ascarosides expressed in the excretome of

several nematode species showed differences in the identity and concentration of ascarosides produced according to life stage (Choe, et al. 2012a), demonstrating how synthesis and functional role is closely related to developmental stage. Similarly, females of the dioecious species, *Caenorhabditis remanei*, only produce male attractant in the adult and late L4 larval stage (immediate adult precursor) of the life cycle where the requirement for sexual behaviour is relevant (Chasnov et al., 2007).

1.6.4 Identification

Ascarosidal pheromone blends in nematodes are commonly characterized by activity guided fractionation of worm conditioned media (or exudates). Put simply, exudates are confirmed to induce a particular behaviour or developmental process using biological assay, before being fractionated into multiple parts to further explore where in the sample bioactivity originates from. Fractionation is commonly carried out using C18 SPE (Solid phase extraction), where fractions are eluted in a stepwise fashion using increasingly strong solvent (such as methanol) in defined increments. The stationary phase of C18 columns has carbon chains (18 carbon atoms in length) bonded to the silica particles inside the column. This has a high affinity for larger non-polar molecules, thus smaller, more polar molecules are eluted first in weak solvent (mobile-phase), whereas less polar molecules require higher solvent concentration to be displaced. Resulting fractions can then be subjected to further assay to find out where activity occurs before being analysed chemically using LC-MS (Liquid Chromatography Mass Spectrometry) which can provide information about the molecularweight, structure, identity and quantity of specific sample components, or NMR (Nuclear Magnetic Resonance Spectroscopy) can either be

used to match detected molecular structures against spectral libraries or to infer the basic structure of unknown sample components directly (Ludewig and Schroeder, 2013).

The process of activity-guided fractionation is not always straight forward. Pheromones commonly occur as blends of multiple molecules which act synergistically (Wyatt, 2003). Separation of different components which synergize can lead to partial or even complete loss, of activity. In such instances, fractions must be assayed in logical combinations, using a process of elimination to determine exactly which combination is required for full activity. Although this process can be laborious (Butcher, 2017), it is often necessary and is a common starting point for characterization of nematode pheromone blends (Butcher et al., 2007; Srinivassan et al., 2008).

1.7 Research objectives

The overall project aim is to explore the role of pheromones in coordinating a variety of important behaviours in entomopathogenic nematodes, particularly *Steinernema,* both as primers (longer term developmental effects) and releasers (short term behavioural effects). I investigate both sex pheromones and chemical signals produced by and affecting the IJ stage. In this, I both test central hypotheses, and attempt the identification of key pheromone components.

Hypothesis 1: chemical signals (a) differ between species where recognition is important (e.g. sex recognition), but (b) coincide where different species have a common aim (e.g. dispersal from overcrowded conditions).

Hypothesis 1a: *Female sex pheromone produced by one species of Steinernema does not attract or have organisational effects on males of another species.* This is expected, since pheromones serve not only to attract a potential mate but also have a role in species identification.

Hypothesis 1b: *Pheromones inducing IJ formation and dispersal from one species will be effective for other species.* Infective juveniles form and disperse in response to crowding; the presence of another species contributes to crowding, thus it would be important to take account of members of all species present. There should be no selection pressure to diversify, and indeed there is advantage to having common signal (as in bird alarm calls). There is already evidence that one species of *Steinernema* responds to the signal for dispersal produced by *C. elegans* (Kaplan et al., 2012).

Hypothesis 2: chemical signals exuded by IJs after emergence from the cadaver continue to affect IJ behaviour.

Hypothesis 3: *The same chemical blend produced by female Steinernema is responsible both for both behavioural (attraction) and organisational (gonad maturation) effects in males.* This would be the most parsimonious solution for the female, avoiding unnecessary duplication of biosynthesis.

Specific objectives that will allow me to test these hypotheses are:

1. Ascertain whether chemicals present in cadaver exudate that promote IJ dispersal (Kaplan et al., 2012) are species-specific in activity (Chapter 3).

2. Ascertain whether IJs that have emerged from cadavers continue to exude dispersal-promoting chemicals (Chapter 3).

3. Ascertain whether chemicals exuded by IJs during storage influence changes in behaviour (dispersal and/or infectivity) of stored IJs (Chapter 3).

4. Confirm that *S. longicaudum* females produce a sex pheromone that attracts males, as is known for *S. carpocapsae* and, for both species, determine optimal parameters, such as female stage and density, for producing attractive female-conditioned exudate (= sex-attractant pheromone) (Chapter 4).

5. Test species specificity of sex-attractant pheromone (Chapter 4).

6. Test whether male *S. carpocapsae* are dependent on female pheromone for sexual maturation, as demonstrated for *S. longicaudum* (Ebssa et al., 2008) and, for both species, determine optimal parameters, such as female stage and

density, for producing effective female-conditioned exudate (= primer pheromone) (Chapter 5).

7. Test species-specificity of primer pheromone for maturation of male *Steinernema* spp. (Chapter 5).

8. Using assay guided fractionation of female-conditioned extract on C18 columns, identify fractions with male-attracting and male maturation-priming activity (Chapters 4 and 5).

9. Identify effective components of female-conditioned exudates (Chapter 5).

The project focusses on three species of *Steinernema*, representing three of the five clades of the genus*: S. carpocapsae, S. feltiae and S, longicaudum.* The first two species are amongst the best studied EPN, are commercially produced and have global distribution, while *S. longicaudum* is included because of its known susceptibility to pheromone priming (Ebssa et al., 2008). In addition, *H. megidis* is included in the work on IJs, both to increase the taxonomic difference between species for interspecific testing, and because of the extent of literature on behavioural changes during storage (see section 1.3.5).

Chapter 2 General methodology

2.1 *In vivo* cultivation of nematodes

Three *Steinernema* spp. were used in the project (*S.longicaudum, S. carpocapsae* and *S. feltiae*) and one *Heterorhabditis* species (*H. megidis*). Nematodes were cycled through larval *Galleria mellonella* (Mealworm Company, Sheffield, UK, stored at 15˚C) on a monthly basis and used approximately 1 month after harvest in all experiments unless otherwise stated. Original origin of all nematode species used is shown in Table 1.1.

2.1.1 Infection of *G. mellonella*

For all nematode species, 8-10 larval *G. mellonella* were added to a 9 cm Petri dish with filter paper lining the lid and base. Filter paper was moistened with 1 ml of nematode suspension (1000 IJ/ml) the dish was closed and was incubated at a temperature appropriate to species (Table 2.1).

Table 2.1: Parameters for passage of all test nematode species through late instar larval *G. mellonella*.

Species	Infection incubation temp (°C)	White trap type	Days to IJ emergence	IJ Storage temp (°C)
S. longicaudum	27	Modified	10-12	20
S. carpocapsae	20	Standard	13-16	9
S. feltiae	20	Standard	13-16	9
H. megidis	20	Standard	22- 25	9

2.1.2 White traps

IJs were harvested from infected *G. mellonella* using White traps (White, 1927), either standard or modified (Woodring and Kaya, 1988). This method allowed IJs to exit the host and migrate into water that was collected and replenished periodically until the host was spent. Standard White traps (White, 1927) were used to harvest *S. carpocapsae, S. feltiae* and *H. megidis.* The lid of a 5.5 cm Petri dish was placed, inverted, inside the base of a 15 cm Petri dish and covered with a 9 cm diameter sheet of filter paper moistened slightly with tap water. Nematode infected *G. mellonella* larvae (8 – 10 per dish, 5-7 days post infection for *S. carpocapsae* and *S. feltiae,* 14 days post infection for *H. megidis*) were transferred onto the filter paper and the base of the 15 cm Petri dish was half filled with tap water, taking care to prevent the 9 cm dish from floating. After replacing the lid of the larger dish, White traps were incubated at 20^0C and checked daily for emergence of IJs. IJs were permitted to accumulate in the surrounding water (without overcrowding) before collection and storage. Refilling the base of the trap with tap water and returning it to incubation allowed single traps to be harvested 2-3 times before disposal.

Modified White traps (Woodring and Kaya, 1988) were used for harvest of *S. longicaudum* only, due to this species emerging initially as pre-IJs which may not develop into IJs capable of infection if emerging directly into water. The lid of a 9 cm Petri dish was lined with moistened filter paper and placed in the base of 15 cm Petri dish. The 9 cm lid was secured within the larger base with small pieces of Blue Tac around the perimeter to prevent movement and leaching of water into the dish. 8-10 infected *G. mellonella* larvae (5-7 days post infection) were placed on the filter

paper, the base filled with tap water half the depth of the lid. After replacing the lid of the larger dish, White traps were incubated at 27^0C and checked daily for emergence of IJs. The water level was also checked daily and topped up whenever necessary. IJs were permitted to accumulate in the surrounding water (without overcrowding) before collection and storage. Refilling the base of the trap with tap water and returning it to incubation allowed single traps to be harvested 2-3 times before disposal.

2.1.3 Harvest and storage

After collection from White traps, nematode suspension was rinsed with tap water by allowing IJs to settle in a graduated cylinder, pouring off old water and refilling with fresh water (process repeated 4-5 times). Rinsed IJs were stored in plastic tubs with lids (9 cm diameter, 250 ml volume) at a density of 1000 IJ/ml in 50 ml aliquots at species-appropriate IJ storage temperature (Table 2.1).

2.2 Surface sterilisation of IJs

Prior to cultivation in hanging drops IJs were surface sterilised in 0.1 % (0.4 mM in H_2O) Hyamine® solution (Aldrich). Five millilitres of 1% Hyamine® solution and 45 ml of nematode suspension were added to a 50 ml sterile Falcon tube and mixed by repeated inversion. The tube was returned to the upright position, allowing IJs to settle in the base (15-20 mins) before pouring off excess water. The settled IJ were rinsed a further four times with sterile water, refilling and inverting the tube each time and pouring off excess water between rinses. Sterilised IJ suspension was used immediately or stored in sterile Falcon tubes laid horizontally (to prevent

sedimentation of IJs) at species appropriate IJ storage temperature (Table 2.1) for up to 7 days.

2.3 *In vitro* cultivation of socially naive nematodes in hanging drops of insect haemolymph

In vitro cultivation of nematodes was carried out in hanging drops of insect blood (haemolymph) based on the methods of Poinar (1967). Unless otherwise stated in the methodology, all drop reared nematodes used for experiments were reared in social isolation; a single worm per drop.

2.3.1 Construction of hanging drops

Haemolymph was extracted from surface sterilised (70% ethanol, v/v) late instar *G. mellonella* larvae by piercing the cuticle with a 0.5 mm diameter needle (25 Gauge, 16 mm length) just below the last pair of prolegs and haemolymph was collected in a sterile Eppendorf tube (1.5 ml) in 1 ml batches (approximately 30 larvae). Haemolymph was used immediately before melanisation occurred.

Haemolymph was pipetted in 10 µl volumes onto the inside surface of a 3.5 cm Petri dish lid. The dish base was filled with 1 ml of sterile water before replacing the lid and the closed dish was placed inside a 15 cm Petri dish which was then sealed with Parafilm (Pechiney Plastic Packaging, Menasha, USA). Each 3.5 cm Petri dish held 7 hanging drops. Individual IJs were picked from suspension using 0.2 mm diameter platinum wire mounted on a glass rod. This method was carried out under a microscope and the wire was flamed between transfers.

2.3.2 Incubation and identification of adult sex

Hanging drops were incubated at species-appropriate infection temperature (Table 2.1). Adults developed within 4-5 days and sex was identified by microscopic observation. Males are characterised by a small body size and the presence of spicules (Figure 1.2). Females are much larger by comparison, do not possess spicules and the vulva is visible in the centre of the body (Figure 1.2).

2.4 Isolation, culture and storage of symbiotic bacteria (*Xenorhabdus* spp.)

Bacterial symbionts *X. ehlersi*, *X. nematophilla* and *X. bovenii* were routinely isolated from *S. longicaudum, S. carpocapsae* and *S. feltiae,* respectively. Isolated bacteria were used to inoculate liquid culture to be used immediately or stored at -20^{0}C.

2.4.1 Isolation of *Xenorhabdus spp.* from infected insect cadavers

Symbiotic bacteria (*Xenorhabdus* spp.) were initially recovered from *Steinernema*-infected *G. mellonella* larvae (Section 2.1.1) 72 h post infection. Cadavers were first surface sterilised by submergence in 70% ethanol (v/v), flamed and extinguished in sterile distilled water. Bacterial symbionts were extracted by creating a small incision on the ventral surface using a sterile scalpel and streaking a loopful of haemolymph onto MacConkey agar plates under aseptic conditions. Plates were

incubated in the dark at 27^0C until single colonies were visible (36-48 h). Single colonies were sub-cultured onto nutrient agar and incubated in the dark at 27˚C (36-48 h).

2.4.2 Liquid culture

Xenorhabdus spp. were cultured in 50 ml of nutrient broth (NB) in 250 ml conical flasks by transferring either a plated colony (Section 2.4.1) into the liquid medium using a sterile pipette tip. Alternatively, NB was inoculated directly with frozen bacterial stock (Section 2.4.3) of 1 % volume of the total culture medium. All species were cultured in the dark at 30^0C and shaken at 210 RPM (New Brunswick Scientific Excella E24 Incubator-Shaker) for 48 h.

2.4.3 Freeze storage (20^0C)

Liquid cultured *Xenorhabdus* spp. were routinely stored (as required) in 40 % glycerol at -20^0C. Eighty percent glycerol was prepared by vortexing 800 µl of sterile glycerol with 200 µl of sterile NB in a 1.5 ml Eppendorf tube. This was mixed in equal volumes with 48 h *Xenorhabdus* spp. culture (Section 2.4.2) and vortexed thoroughly to prevent crystal formation during freezing (crystalisation can reduce cell viability). Cells were stored in this way in 1 ml aliquots (1.5 ml Eppendorf tubes) for up to 6 months.

2.5 Species confirmation of bacterial symbionts

2.5.1 DNA extraction from symbiotic bacteria

Liquid cultures of bacteria were prepared from bacterial isolates (see Section 2.4.1) from *Steinernema longicaudum*, *S. carpocapsae*, *S. feltiae* and *Heterorhabditis megidis* as per Section 2.4.2. Absorbance at λ = 600 nm was checked and cell suspensions were adjusted to give approximately 2 x 10^9 in full strength PBS solution. Cells were collected by spinning 1 ml of suspension (1.5 ml Eppendorf tubes) in a microcentrifuge at 5000 x g for 10 min (room temp.) and discarding the supernatant.

Bacterial cell DNA was extracted according to the Animal Tissue Protocol using a DNeasy Blood and Tissue Mini Spin Column Kit (Qiagen, Crawley, UK). All buffers (AT1, AL, AW1, AW2, and AE), proteinase K, spin columns and collection tubes were supplied with the kit. Details of reagent constituents are available in the DNeasy Blood and Tissue Handbook. Buffer AL was premixed with an equal volume of molecular grade ethanol (Sigma-Aldrich) according to the manufacturer's instructions for the number of reactions being carried out. Buffers AW1 and AW2 were made up with ethanol according to manufacturer's instructions. Centrifugation steps were carried out using a Sigma 1-15 micro-centrifuge (Sigma Laboratory Centrifuges, Osterode, Germany).

Briefly, buffer ATL (180 µ) and proteinase k (20 µl) were added to pelleted bacterial cells (see above) which were then vortexed (5 -10 s) and lysed in a water bath at 56˚C for 1.5 h (light shaking, 5 – 10 s vortexing every 15 min throughout). AL/EtOH (400 µl) was added to the sample, mixed by vortexing, transferred to a spin column (in a 2 ml collection tube) and centrifuged at 6000 x g for 1 min. The collection tube

and flow-through were discarded and replaced with a fresh collection tube. Buffer AW1 (500 µl) was added to the sample and centrifuged at 6000 x g for 1 min before again discarding the collection tube and flow-through and transferring the spin column to a fresh collection tube. Buffer AW2 (500 µl) was added and the sample was centrifuged at 15000 x g for 3 min. The collection tube and flow-through were discarded and the spin column was transferred to a 1.5 ml microcentrifuge tube for DNA elution steps. Buffer AE (30 µl) was added and the sample was incubated at room temperature for 1 min before centrifuging at 6000 x g for 1 min. This step was repeated once. The spin column was discarded and the concentration of DNA in the eluate was quantified using a NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Two replicate extractions were carried out for each isolate and extracted DNA was stored at -20˚C until PCR amplification.

2.5.2 PCR amplification of symbiotic bacteria DNA

Amplification of bacterial DNA was carried out using primers specific for the 16S region of rDNA. All reactions were carried out using an Eppendorf Mastercycler Gradient machine (Eppendorf, Cambridge, UK). All reagents were kept on ice. Sufficient master mix for two replications per isolate was made up in a 1.5 ml microcentrifuge tube. Individual reactions were carried out in 0.5 ml PCR tubes (Fisher Scientific, Ireland) using 22.5 µl of master mix and 2.5 µl of DNA extract (20 ng/µl). A negative control to which no DNA was added was also included. The contents of the tubes were mixed by vortexing. Details of the PCR mixture (Table 2.2), cycling conditions (Table 2.3) and primers used (Table 2.4) are shown below.

Table 2.2: PCR mixture for amplification of 16S region of rDNA from symbiotic bacteria of *Steinernema* and *Heterorhabditis* spp. Reactions were carried out in 25 µl volumes.

Reagent	Volume per rxn	Final conc.	Supplier
5x my Taq reaction buffer	5 µl	1x	Bioline
16S-F primer (10µM)	5 µl	0.1 µM	Eurofin Genomics
16S-R primer (10µM)	5 µl	0.1 µM	Eurofin Genomics
My Taq DNA polymerase (5U/µl)	0.125 µl	0.025 U/µl	Bioline
DNA template (20ng/µl)	2.5 µl	2 ng/µl	N/A
ddH_2O	16.375 ml		N/A

Table 2.3: PCR cycling conditions for amplification of 16S region of rDNA from symbiotic bacteria of *Steinernema* and *Heterorhabditis* spp.

Stage	Cycles	Temperature (˚C)	Duration (min)
Initial denaturation	1	95	5
Denaturation	35	95	1
Annealing		52	1
Extension		72	2
Final extension	1	72	7
Hold	1	4	Indefinate

Table 2.4: Primer sequences for 16S region of rDNA from symbiotic bacteria of *Steinernema* and *Heterorhabditis* spp. (Fischer-Le Saux et al., 1999).

Primer	Sequence
16S (Forward)	5’ GAAGAGTTTGATCATGGCTC 3’
16S (Reverse)	5’ AAGGAGGTGATCCAGCCGCA 3’

For each reaction, 5 µl of PCR product was mixed with 1 µl of 5 x tricolour loading buffer (Bioline) and analysed on a 1 % agarose gel containing 1 g agarose (Life Technologies) dissolved in 100 ml of 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) stained with 1 x SYBR Safe DNA Gel stain (Life Technologies). Gels were run at 100 V for 1.5 hrs in 1 x TAE buffer and photographed using a Syngene G:Box

Chemi HR16 BioImaging System in the dark using GeneSnap 7.12 software (SynGene, Cambridge, UK) to confirm presence of PCR product.

2.5.3 Species identification using 16s gene sequences

The 16S region of DNA obtained in Section 2.5.2 was sequenced by a commercial sequencing company (GATC Biotech, Konstanz, Germany) using 16S forward and reverse primers (Table 2.4). A consensus sequence for each isolate was obtained using CodonCode Aligner (Version 7.1) and homologous nucleotide sequences in GenBank were identified using the blastn algorithm in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Bacterial symbionts were confirmed to be those quoted in Table 1.1 and full BLAST reports and consensus sequences used for identification can be found in Appendix 2.1(a-d).

Chapter 3 IJ Behaviour

3.1 Introduction

As the only free-living stage in the EPN life cycle, IJs play a critical role in the selection and invasion of a new insect host. In this quest they face several challenges, including dispersing through soil to locate a new insect and assessing host suitability when they encounter one. Selecting a suboptimal or impenetrable host wastes valuable time and energy which may ultimately lead to failure to establish. It is therefore important that IJs are able to identify a suitable host where they can reproduce (Griffin, 2012). As organisms that rely mainly on chemo-sensation to perceive the surrounding environment, chemical signalling plays a crucial role in the mediation of EPN infection behaviours. As biocontrol agents, it is the IJ that are of critical importance with regard to suppressing pest insect populations (Ehlers, 2001). Increased understanding of their ecology and behaviour is therefore important to better inform biocontrol initiatives (Lewis et al., 2006; Griffin, 2015), including maximizing infection efficacy, helping predict success of new field applications and assessing potential environmental risk (Ehlers and Hokkanen, 1996; Gaugler et al., 1997). As well as having economic and environmental benefit as tools for biocontrol, EPN are also increasingly being recognised as model organisms for the study of fundamental behavioural and evolutionary biology (Campos-Herrera et al., 2012).

Despite their simple nervous system (just 302 neurons in an adult *C. elegans* (White et al., 1986), it is clear that IJ engage in several behaviours geared towards maximizing host encounter and successful infection. Even in the absence of more sophisticated sensory organs of higher organisms, they are able to perceive and

respond to their environment, conspecifics and competitors by chemical means. EPN are attracted to chemicals which are liberated when plants are damaged by insect feeding (Boff et al., 2002; Rasmann and Turlings, 2008) showing that they perceive information conveyed through plant volatiles that insect hosts are in the vicinity. Insects themselves also produce chemicals, including CO_2 and cues originating from faeces and the cuticle, which help IJs target hosts in the soil (Grewal et al., 1997; Lewis et al., 2006) (See 1.3.2 for further information).

IJs also make use of chemical cues to time dispersal from a spent insect host (Section 1.3.1). Accumulation of nitrogenous nematode waste products (ammonia) have been shown to induce emergence of *S. feltiae* from spent *G. mellonella* (San-Blas, et al. 2008). Furthermore, EPN disperse more efficiently when applied in the field within infected cadavers rather than in aqueous suspension (Shapiro-Ilan et al., 2003), a result which is ascribed to the presence of dispersal-inducing chemical compounds present in the decaying insect cadaver (Kaplan et al., 2012).

To date, relatively little is known about specific IJ-associated chemicals and how they may mediate particular behaviours. Recent research has shown that, similar to their adult counter parts, ascarosidal communication may play a key role in the control of IJ behaviour and development. Numerous ascarosides have been identified in exudates of exclusively juvenile cultures across a range of taxonomical groups (Section 1.6.1), including the species *Ocheius carolinensis, Nippostrongylus brasiliensis* and four *Steinernema* spp. (Choe et al., 2012b). The ascaroside blends of insect-associated nematodes, including *Steinernema,* all have high amounts of ascr#9 (>50% of total composition) amongst other ascaroside components in lesser amounts (Choe et al., 2012b). Such similarities in ascaroside expression according to behaviour and not just genetic relationship, suggests biosynthesis is influenced by

both phylogeny and ecological niche (Choe et al., 2012b). *Steinernema* typically express ascarosides with shorter functional fatty acid side chains when compared with other taxonmical groups (Choe et al., 2012b). *H. bacteriophora* IJs respond differently to ascarosides produced by conspecifics than to those of *C. elegans* DJs (Noguez, et al. 2012). When at high density in the insect host, *H. bacteriophora* IJs produce a pheromone that prevents IJ recovery, facilitating accumulation of IJs in large numbers prior to dispersal (Noguez et al., 2012). Following activity guided fractionation of the crude pheromone, it was found that C11 ethanolamide (asc C11 EA) accounted for the majority of its activity (Noguez et al., 2012). However, assay of individual components of the *C. elegans* dauer pheromone did not inhibit *H. bacteriphora* IJ recovery (Noguez et al., 2012), indicating juvenile nematode signals may be species specific.

IJ behaviour changes during storage in water; in particular, they become more infective before they eventually decline in infectivity due to aging. This has been shown in *H. megidis* (Griffin, 1996; Dempsey & Griffin, 2003) and to a lesser extent in steinernematids (*S. carpocapsae*, Guy et al., 2017). Such behavioural variation is hypothesized to result from an endogenous change (internally programmed) (Griffin et al., 1996), but here I test whether accumulation of externally released chemicals in the storage medium might be a contributing factor to changes in IJ infection and dispersal behaviours. There may be, however, a combination of the two proposed factors. EPN are routinely stored in aqueous suspension both in the laboratory and commercially prior to formulation, making it important to resolve any effect this may have on behaviour and efficacy.

3.1.1 Chapter aims and objectives

This chapter addresses how selected aspects of behaviour in EPN IJ are mediated by chemical cues and whether such cues are species-specific. IJs form and disperse in response to crowding, resource depletion and presence of chemicals in the spent cadaver. Individuals of multiple species may contribute to these dispersal triggers therefore it is expected that chemical dispersal cues are unlikely to be species-specific as there is an advantage to all individuals in having a common signal. There is already evidence that one species of *Steinernema* (*S. feltiae*) responds to the signal for dispersal produced by *C. elegans* (Kaplan et al., 2012). It will also be investigated whether production of dispersal-promoting chemical signals occurs exclusively within the cadaver or whether they continue to be exuded by IJs after emergence (See Hypotheses 1b and 2, Section 1.7).

Specific objectives that will allow me to test these hypotheses are:

1. Based on the findings of Kaplan et al. (2012), who showed that chemicals present in cadaver exudate promote IJ dispersal in *S. feltiae*, I will establish whether this is also the case in other *Steinernema* spp. (*S. carpocapsae* and *S. longicaudum*) and also *H. megidis.*

2. To ascertain whether such chemicals present in cadaver exudate are species-specific in promoting IJ dispersal.

3. To investigate whether dispersal-promoting chemicals continue to be exuded by IJs which have vacated the spent cadaver by preparing IJ conditioned

water and using dispersal assays to determine whether this also promotes dispersal.

4. Ascertain whether chemicals exuded by IJs during storage affect dispersal behaviour and virulence of stored IJs.

For Objectives 1-4 I use representatives of the two main EPN families (Heterorhabditae and Steinermatidae), as listed in Objective 1, while only *Heterorhabditis megidis* and *Steinernema carpocapsae* were used in Objective 4.

3.2 Methodology

3.2.1 Preparation of cadaver extract

Approximately 30 *G. mellonella* larvae were infected with IJ suspension (1 ml of 1000 IJ/ml per 10 insects) and incubated for 5-7 days. Half of the infected cadavers (15) were then White-trapped and observed for the first emergence of IJ, while the rest were maintained in dry conditions until extract collection. Extract was harvested from the un-trapped cadavers 1-2 days post first emergence of IJ from associated White-traps. Cadavers were transferred to 1.5 ml Eppendorf tubes (2 per tube) and punctured multiple times using a sterile needle. 500 µl of sterile, distilled H_2O was added and the tube was vortexed thoroughly to liberate the contents of the cadavers. Tubes were micro-centrifuged (1000 x g for 10 min, Sigma 1-15 Micro- centrifuge) and the supernatant from each tube was collected and combined, before a second micro-centrifugation (1000 x g for 10 min, Sigma 1-15 Microcentrifuge). The final supernatant of all cadavers combined was collected and stored in 1 ml aliquots at 4˚C until use (not exceeding 7 days storage).

3.2.2 Preparation of IJ exudate

IJ were harvested from *G. mellonella* cadavers daily for 4-5 days post first emergence. The first days harvest was discarded and the remaining IJ were pooled, rinsed three times and adjusted to the desired density (0-5000 IJ/ml) in sterile tap water. IJ suspension was then stored in 50 ml aliquots for 1 week at 20˚C or 27˚C (Table 2.1) before removal of IJ by sedimentation. The final exudate was collected, filtered (0.2 µm Acrodisc) and stored in 50 ml aliquots at 4˚C until use (not exceeding 7 days storage).

3.2.3 Dispersal assay

Test IJ were harvested daily for 4-5 days post first emergence. The first days harvest was discarded and the remaining IJ were pooled, rinsed three times in sterile tap water and density adjusted to 1000 IJ/ml. IJ suspension was incubated in 50 ml aliquots at 20˚C or 27˚C (Table 2.1) for 48 h and then concentrated by sedimentation immediately prior to assay.

Dispersal assays were carried out on 2% bacto agar in 5.5 cm petri dishes. Approximately 100-200 IJ (in 5 µl water) were applied to the centre of the dish followed by immediate application of 2 µl of test stimulus (or control H_2O). A circular glass coverslip (19 mm diameter) was placed over the suspension and plates were incubated at 20˚C or 27˚C (Table 2.1) for 10 min. IJ remaining under the coverslip on termination of the assay were recorded as not dispersed and those migrating onto the open agar surface were considered dispersed. Counting was done with the aid of a dissecting microscope.

All experiments using cadaver extract (Figures 3.2 and 3.3) included 10 replicate plates per treatment from a single preparation of cadaver extract per species (extract of 15 cadavers combined, Section 3.2.1). Experiments using IJ exudate (Figures 3.4 - 3.7) included 10 replicate plates per preparation density (0-5000 IJ/ml, Section 3.2.2) or specified treatment (such as time stored) per species.

3.2.4 Storage conditions for storage experiments

Storage conditions were the same for both species used during storage experiments (*S. carpocapsae* and *H. megidis*). IJ to be stored were harvested daily for 4-5 days

post first emergence. The first day's harvest was discarded and the remaining IJ were pooled, rinsed three times in sterile tap water and density adjusted to 1000 IJ/ml. IJ suspension was then stored in 50 ml aliquots (9 cm diameter plastic tubs with lids) in three different treatments at each of two storage temperatures (9˚C and 20˚C):

1. **Undisturbed** – tubs were untouched throughout storage, with the exception of collection of nematodes immediately before assay (tubs were swirled gently to briefly resuspend nematodes and allow 1 ml of suspension to be removed with a pipette).

2. **Resuspended** – tub contents were transferred to a 50 ml Falcon tubes and IJs were allowed to sediment. Tubes were then inverted several times to resuspend IJs, before returning suspension to original storage tub. This was done three times weekly at 20˚C storage, once weekly at 9˚C storage.

3. **Water change** – tub contents were transferred to a 50 ml Falcon tube and IJs were allowed to sediment. The old water was poured off and IJs were resuspended in fresh sterile tap water, before returning suspension to original storage tub. This was done three times weekly at 20˚C storage, once weekly at 9˚C storage.

In this experiment, the main interest was in the effect of chemicals in the medium, which can be detected by comparing treatment 3 (Water change) and 2 (Resuspended), as these differ only in whether the water was changed. Treatment 1 (undisturbed) is included as this is the normal way in which nematode IJs are stored. Comparison of Treatments 1 and 2 will reveal any effect on the IJs of the mechanical disturbance associated with changing the water. There were 5 replicate tubs per storage treatment at each storage temperature (9˚C and 20˚C) per species (*S. carpocapsae* and *H. megidis*) (Figure 3.1).

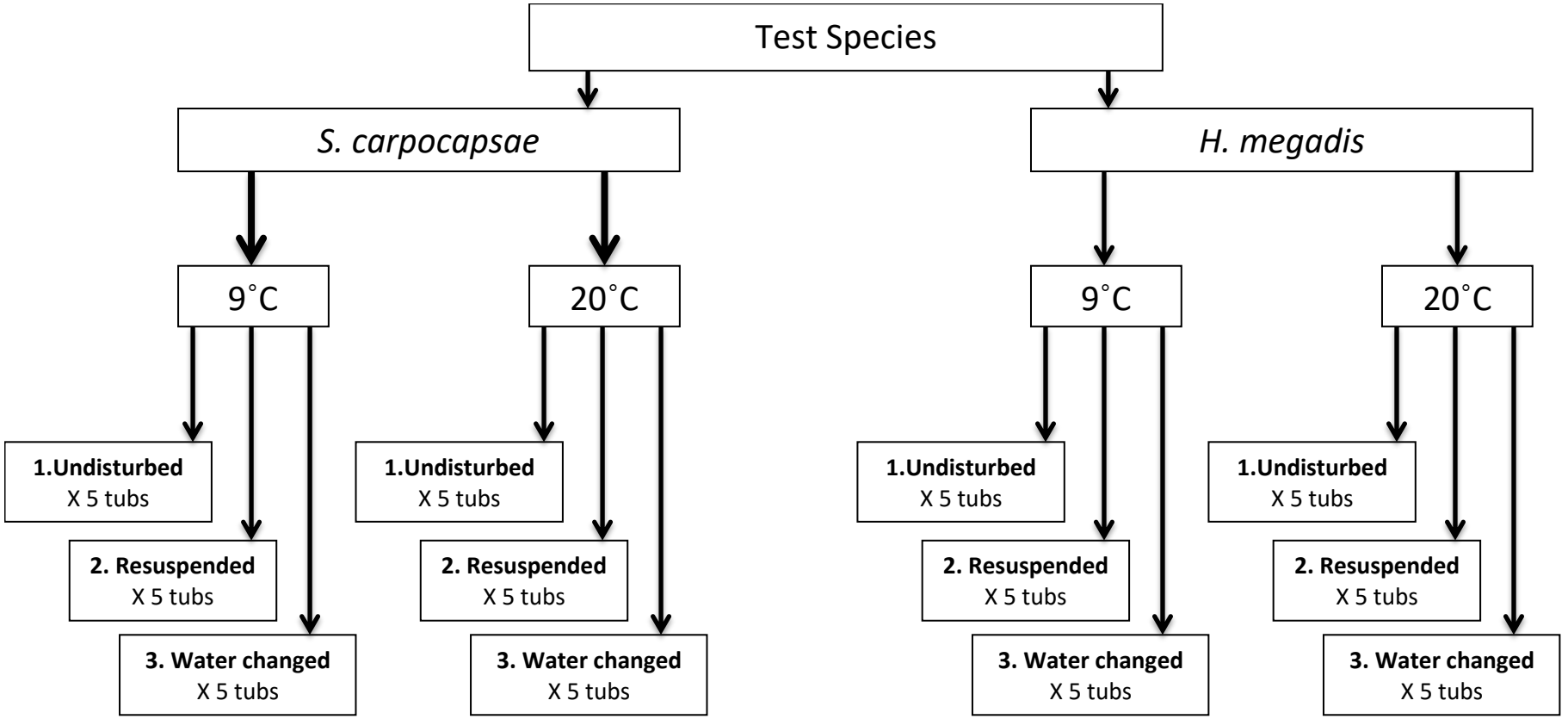


Figure 3.1: Schematic showing storage conditions of test IJs. IJs stored at 9˚C were assayed for dispersal and virulence every 3 weeks and IJs stored at 20˚C were assayed every week.

3.2.5 Dispersal and virulence assay of stored IJ

IJ stored at 20˚C were assayed weekly, and IJ stored at 9˚C were assayed at 3 week intervals for both dispersal and virulence. Test IJ were collected by transferring 1 ml of IJ suspension from each storage tub to separate Eppendorf tubes and allowing them to settle immediately prior to assay (virulence and dispersal). In the undisturbed treatment, tubs were lightly shaken to resuspend IJ before removal of test IJ. In the case of the resuspended and water change treatments, test IJ were removed and assayed immediately after they were resuspended or water was changed in Falcon tubes.

Dispersal assays were carried out using methodology outlined in Section 3.2.3. At each time point (weekly for 20˚C stored IJ, once every 3 weeks for 9˚C stored IJ), 2 replicate assays per storage tub (5 per storage condition) were performed totalling 10 replicates per storage condition.

Nematode virulence was assessed against *T. molitor* in a close-contact assay. As with dispersal, assays were carried out weekly for 20˚C stored IJ, once every 3 weeks for 9˚C stored IJ. Block coconut coir was passed through a 1 mm sieve and soaked overnight with 2.5 times (w/v) tap water. Moistened coir (0.15 g) was lightly compressed into each well of a 24-well cell culture plate (Corning Inc., NY, USA; wells, 1 cm diameter. and 1 cm high). 10 µl of stored IJ suspension (1000 IJ/ml) was added to half (12) of the wells in each plate, while the other 12 wells received tap water only (10 µl) and served as controls. A *T. molitor* larva was added to each well. Plates were closed and sealed with Parafilm (pierced to allow ventilation), wrapped loosely in aluminium foil and incubated at either 9˚C or 20˚C. Plates assayed at 20˚C were assessed every 24 h for insect mortality and plates assayed at

9˚C were assessed once weekly for 5 weeks. One assay plate was used per replicate storage tub (5 tubs per storage condition), thus there were in total 60 nematode-treated insects and 60 control insects assessed per treatment at each time point in each experiment. Mortality in the nematode treatments was corrected for control mortality using Abbotts' formula (Abbott, 1925). Control mortality was generally low in all cases.

3.2.6 Statistical analysis

Data analysis was carried out using Minitab 17. Data were screened for normal (or near normal with normally distributed residuals) distribution using the Anderson-Darling test for normality. Data were arcsine transformed (arcsine($\sqrt{p}$)) to achieve normality where necessary (Experiment 3.3.5, Figure 3.10). Comparison of two data sets used Two-Sample tests. Where three or more data sets were compared, a One-way ANOVA was followed up with Tukeys *post hoc* test when $P < 0.05$ to identify where significant differences occurred. Non-normal data was tested using non-parametric tests, namely Mann Whitney U test for two data sets and Kruskal – Wallis for more than two. In storage experiments, effects of storage method and storage duration were explored using GLM (General Linear Methods). Statistical techniques used are summarised in figure legends and tables in the results. Full test statistics are included in Appendix 6.

3.3 Results

3.3.1 IJ dispersal is promoted by exposure to extract of conspecific EPN infected insect cadavers

Exposure to aqueous extract of *G. mellonella* cadavers infected with nematode conspecifics promoted dispersal of IJs in three *Steinernema* spp. and in *H. megidis* (Figure 3.2). Dispersal in the control treatment (water) was relatively similar across all species (average of 50-60 % IJs dispersed in each). Dispersal behaviour following application of cadaver extract (in comparison to control) was also relatively similar for all species tested, increasing to ~80% in all cases.

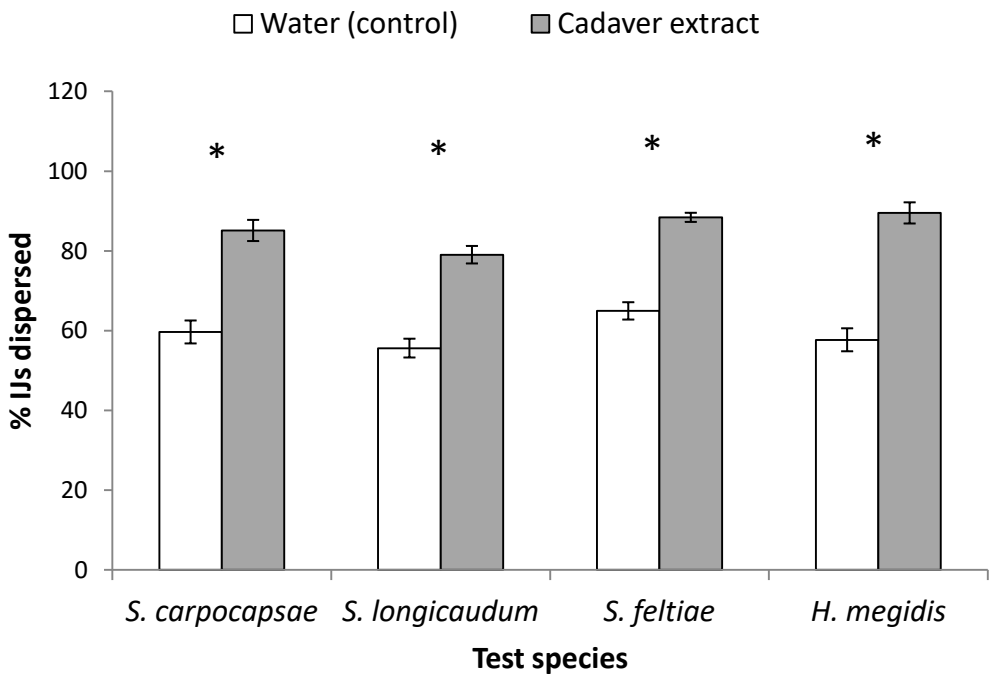


Figure 3.2: Percent of IJs dispersing from 2 µl of conspecific cadaver extract (or 2 µl water control) 10 min after application. N = 10 per condition, error bars show standard error of mean percentage dispersal. * denotes significant difference between treatments in all species tested (Two-sample t test, $p < 0.001$).

3.3.2 Species-specificity of dispersal promoting cadaver extract

Promotion of IJ dispersal by cadaver extract is not species-specific: all 4 species tested increased dispersal to a similar degree regardless of whether they were exposed to conspecific or heterospecific cadaver extract (Figure 3.3).

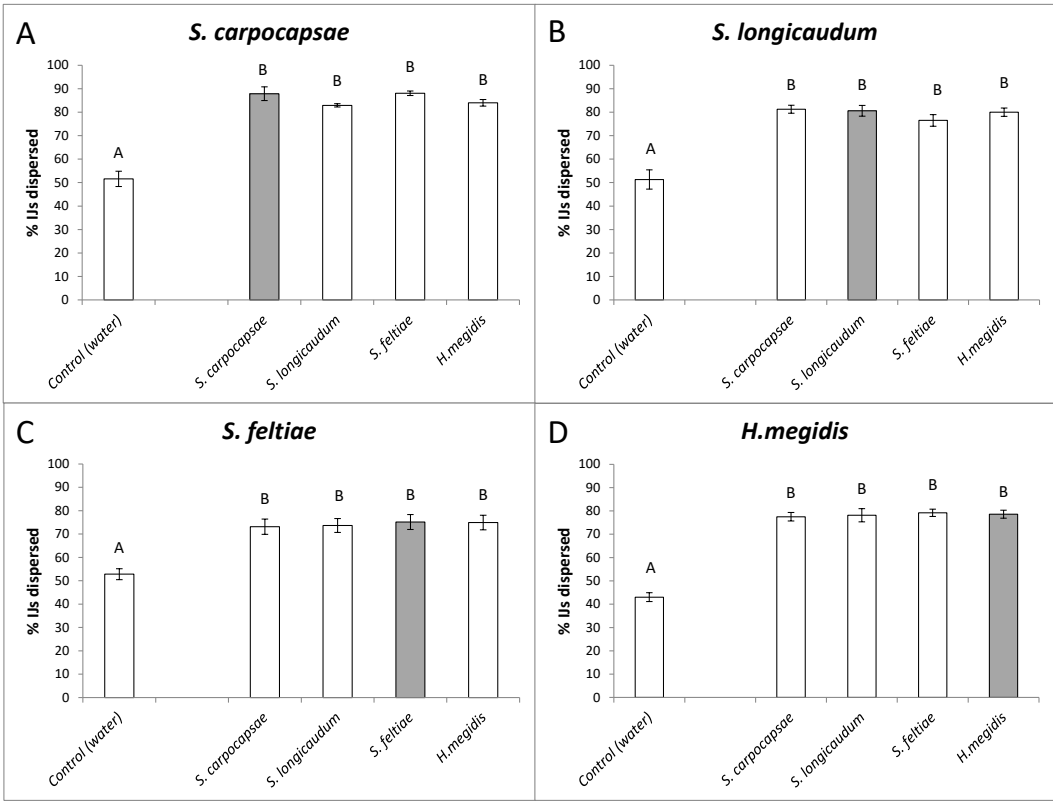


Figure 3.3: Percent of IJs dispersing from 2 µl of either conspecific or heterospecific cadaver extract 10 min after application. Dispersal response was significantly higher than in water control in all cases but response to cadaver extract regardless of species did not differ significantly in all cases (One – way ANOVA $p < 0.01$ followed by Tukeys *post hoc* test). Bars which do not share a letter (within test species) differ significantly. N = 10 per condition.

3.3.3 IJ dispersal is promoted by exposure to exudate of high density conspecific IJs

Three experiments are reported here. In the first, I test the effect of exudate from a high density of IJs stored for 7 days. Exposure to water conditioned with conspecific IJs (5000 IJ/ml) for 7 days promoted dispersal of IJs in three *Steinernema* spp. and in *H. megidis* (Figure 3.4). Dispersal in the control treatment (water) was relatively similar across all species (~ 40 % of IJs dispersed in each case). Dispersal behaviour following application of IJ-conditioned water (in comparison to control) was also relatively similar for all species tested, increasing to ~ 55%. A two – sample t test was used to test for differences between percentage dispersal in treatment and control in all cases except for *S. longicaudum* (data was not normal and normality could not be achieved by transformation). In this case a Mann-Whitney test was used instead.

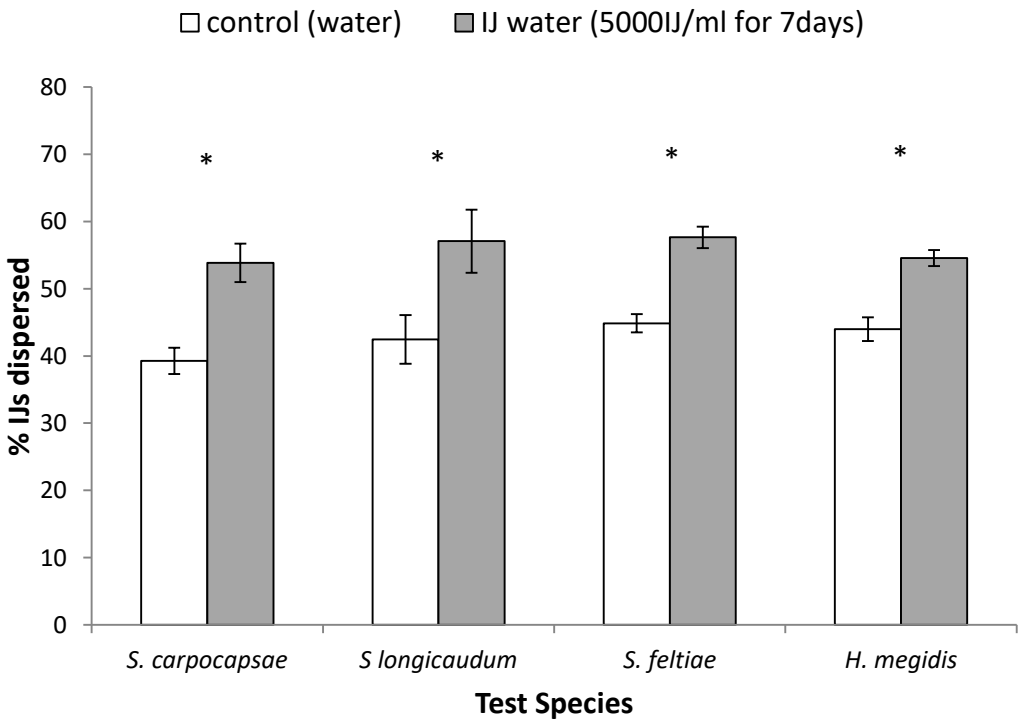


Figure 3.4: Percent of IJs dispersing from 2 µl of water conditioned with conspecific IJs (5000IJ/ml for 7 days) or 2 µl water control 10 min after application. N = 30 per condition, error bars show standard error of mean percentage dispersal. * denotes significant difference between treatments in all species tested (Two – sample t test,$p < 0.05$. Mann-Whitney test for *S. longicaudum* as data was not normal, $p < 0.05$).

In the second experiment, I varied the density of IJs, while keeping the conditioning time constant at 7 days. All four species tested showed an increase in dispersal when exposed to aqueous exudate of conspecific IJs (compared to water control), however there was variation between species with regards to which IJ density used in exudate preparation was required for such an increase to be significant (Figure 3.5). *S. feltiae* required only 500 IJ/ml exudate, whereas both *S. carpocapsae* and *H. megidis* dispersal only increased significantly when exposed to exudate prepared with higher density of IJs (2000 IJ/ml). The response of *S. longicaudum* was intermediate, showing a significant increase in dispersal after exposure to 1000 IJ/ ml exudate, but also showed the greatest increase in percentage dispersal overall. In all species, the

increase in dispersal to aqueous IJ exudate, regardless of preparation density, was less (10 -20% increase) than the equivalent cadaver extract tested in Experiment 3.3.1 (> 30 % increase) (Figure 3.2).

In the third experiment, I varied the time of conditioning while keeping the IJ density constant at 5000 IJs/ml. There was a general trend of increased dispersal of IJs exposed to water conditioned with conspecific IJs (5000 IJ/ml) for either 1 day or 7 days in comparison to the control for all species tested (One – Way ANOVA $p < 0.05$ followed by Tukey *post hoc* analysis) (Figure 3.6). Although an increase in total dispersal was observed in all species when exposed to 1 day IJ-conditioned water, this increase was not significantly different to the control (water). Seven day IJ-conditioned water did increase total dispersal to a significant degree (in comparison to control) in all species except *S. carpocapsae* (Figure 3.6A). However, when examining only IJs which dispersed > 1.5 cm from the assay origin, S. *carpocapsae* results show a similar trend to the other species investigated with 7 day IJ conditioned water increasing dispersal to a significant degree (Figure 3.6B).

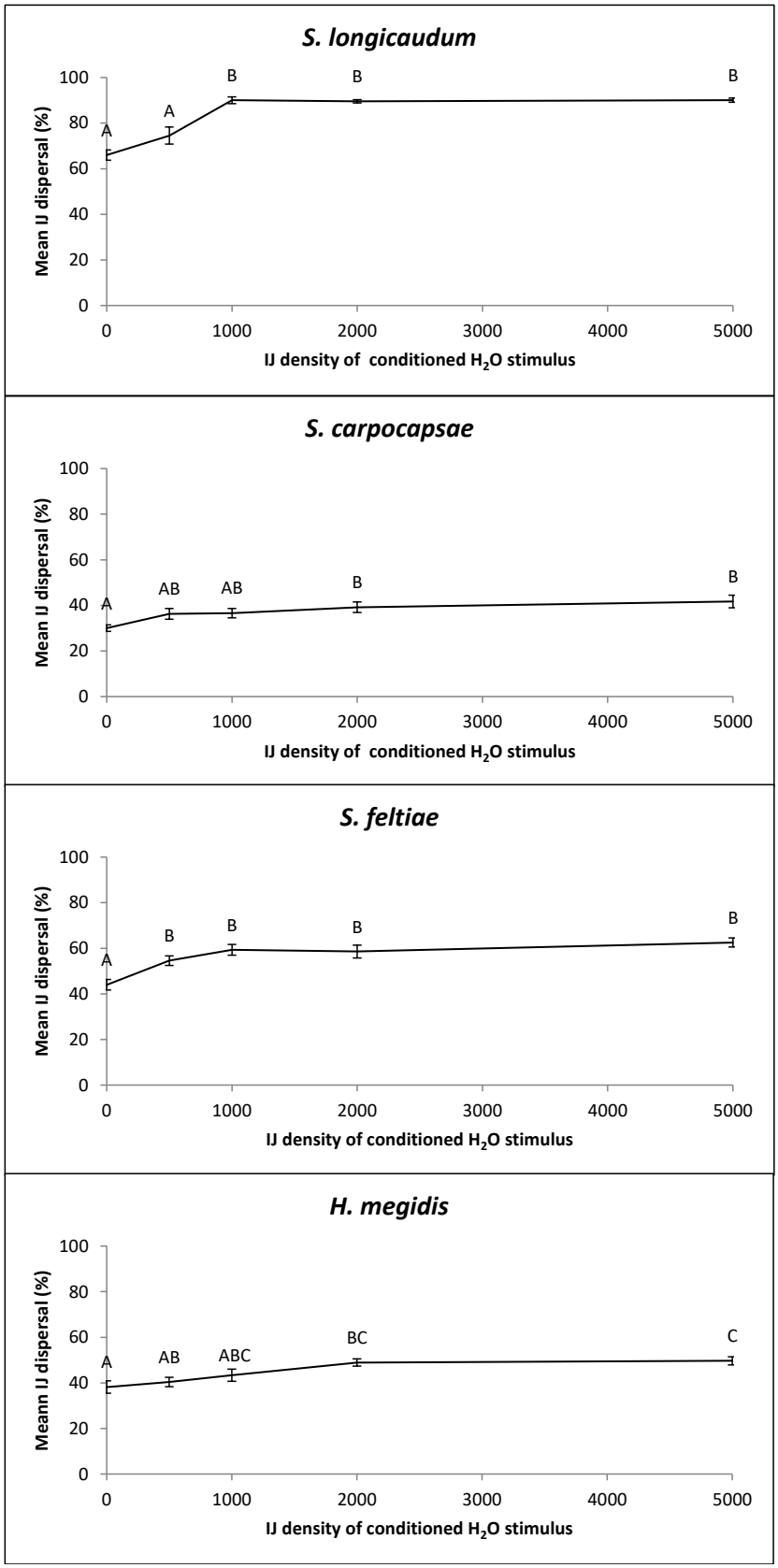


Figure 3.5: Percent of IJs dispersing from 2 µl of water conditioned with conspecific IJs at specific density for 7 days (or 2 µl water control) 10 min after application. N = 10 per condition, error bars show standard error of mean percentage dispersal. One – way ANOVA ($p < 0.05$) followed by Tukeys *post hoc* test . Data points (within species graphs) which do not share a letter differ significantly.

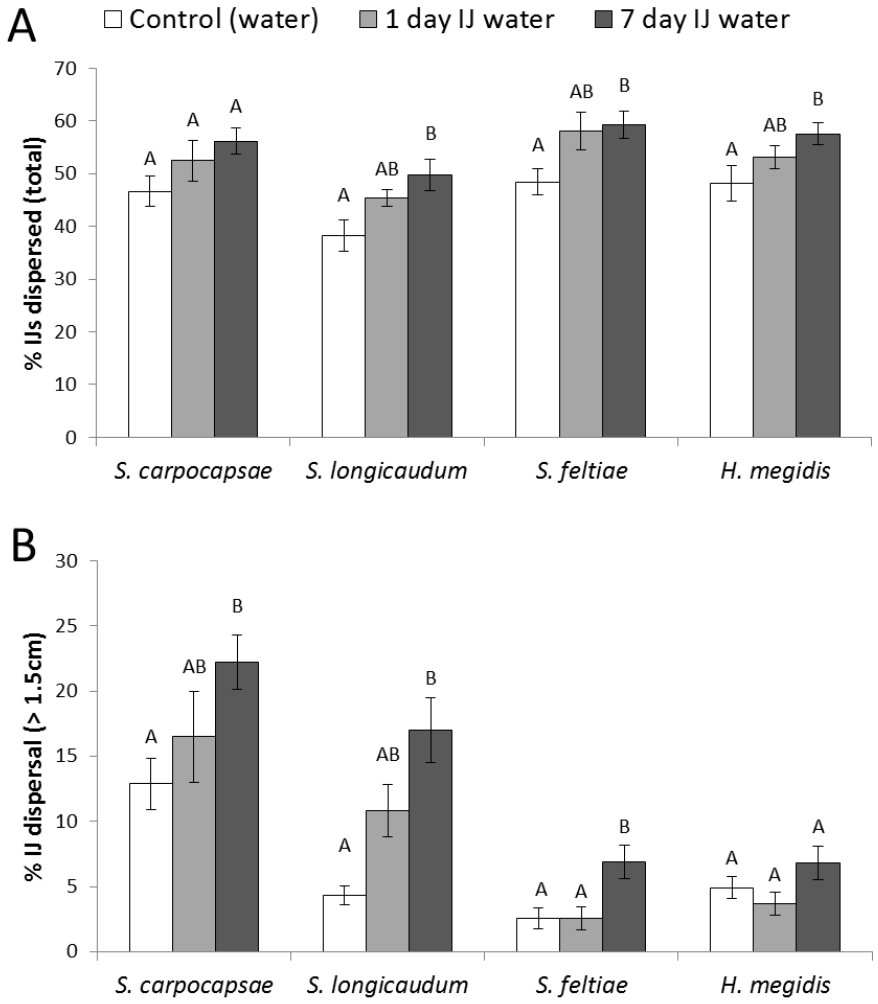


Figure 3.6: Percent of IJs dispersing from 2 µl of water conditioned with conspecific IJs (5000IJ/ml for 1 or 7 days) or 2 µl water control 10 min after application. A: Total % IJs dispersing; B: % IJs dispersing at least 1.5 cm. N = 10 per condition, error bars show standard error of mean percentage dispersal. Bars which do not share a letter (within test species) differ significantly (One – way ANOVA $p < 0.05$ followed by Tukeys *post hoc* test).

In general, promotion of IJ dispersal by IJ-conditioned water (5000 IJ/ml for 7 days) was not species-specific. In the 3 *Steinernema* species tested, dispersal increased to a similar degree regardless of whether IJs were exposed to conspecific or heterospecific IJ-conditioned water (Figure 3.7A-C). For *H. megidis*, *S. longicaudum* IJ-conditioned water promoted dispersal significantly more than all other species conditioned water, including *H. megidis* conspecifics (Figure 3.7D)

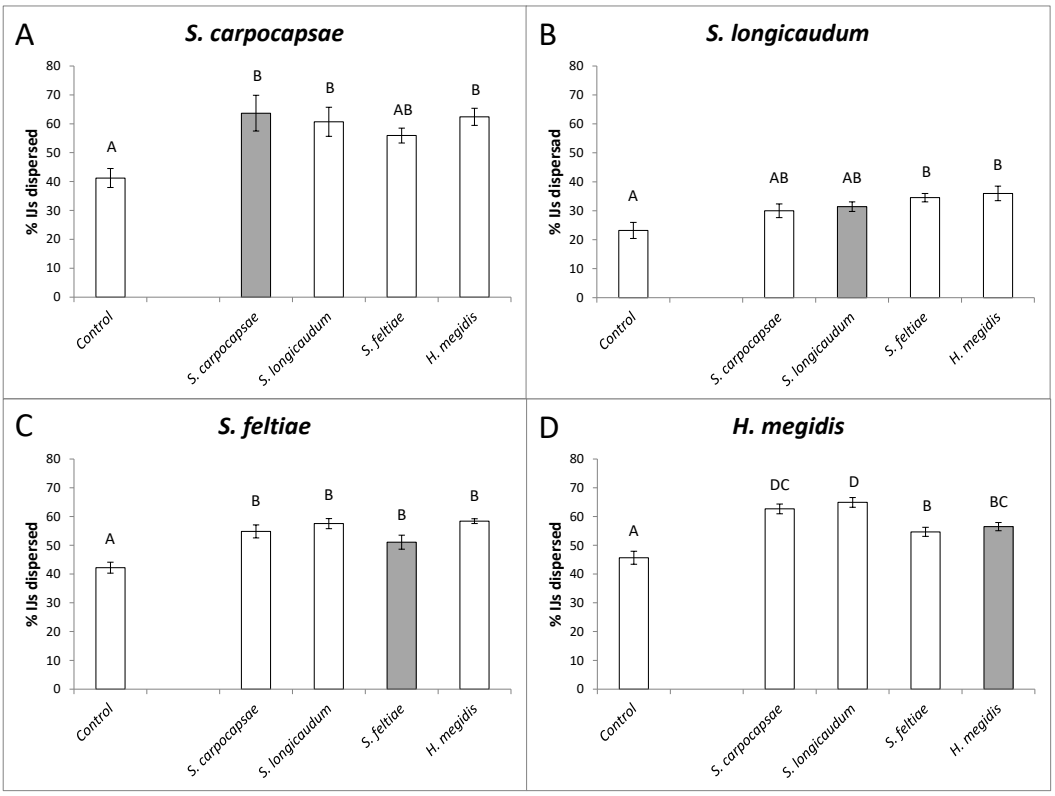


Figure 3.7: Percent of IJs dispersing from 2 µl of either conspecific or heterospecific IJ-conditioned water (5000 IJ/ml for 7 days) 10 min after application. Within test species bars, which do not share a letter differ significantly (One – way ANOVA $p < 0.05$ followed by Tukeys *post hoc* test). N = 10 per condition.

3.3.4 Influence of storage on IJ dispersal and virulence

Both storage treatment and storage duration had a significant effect on dispersal of *H. megidis* IJs stored at both 9˚C and 20˚C. Furthermore, there was a significant interaction between these factors in both cases (Table 3.1). After 3 weeks of storage at either 20˚C (Figure 3.8A) or 9˚C (Figure 3.8B), *H. megidis* which had the storage water changed regularly dispersed significantly less than IJs of the same origin where storage tubs were either undisturbed or the water was not changed (IJs resuspended regularly). This suppression of dispersal in the 'Water changed' treatment was maintained from the 3 week point to the end of both storage conditions (20˚C, 5 weeks and 9˚C, 12 weeks). Dispersal in the 'Resuspended' treatment (involving regular mechanical disturbance of storage water, but not change) did not differ from the Undisturbed treatment at any point during the 20˚C storage condition (Figure 3.8A). A similar trend was seen for IJs stored at 9˚C (Figure 3.8B), with the exception of week 3 (where 'Resuspended' IJ dispersed significantly less than 'Undisturbed') and week 12 (where a higher percentage of 'Resuspended' IJs dispersed than in all other treatments).

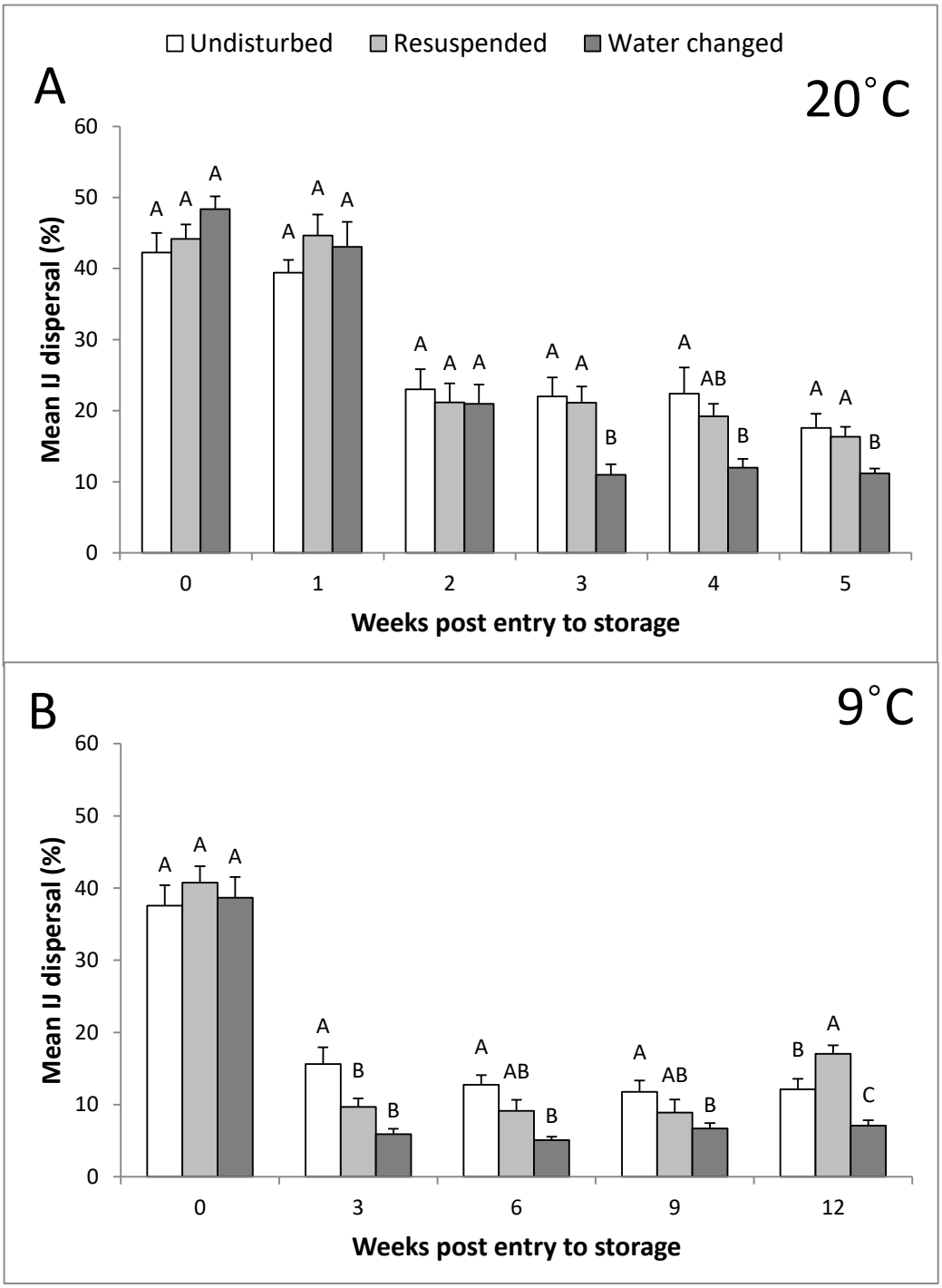


Figure 3.8: Percentage of *H. megidis* IJs stored at 20˚C (A) or 9˚C (B) dispersing. Initial GLM (treatment, week, treatment and week interaction all $p < 0.05$, Table 3.1). Within week one-way ANOVA ($p < 0.05$) followed by Tukeys *post hoc* analysis. Treatments which do not share a letter differ significantly.

Table 3.1: Results of statistical analysis (p values, GLM: General Linear Methods) showing effect of storage treatment and storage duration on dispersal of *H. megidis* IJs stored at 20˚C or 9˚C**.**

H.megidis		Analysis of variance p values			
Storage temp. (˚C)	**Assay temp (˚C)**	**Storage Treatment**	**Storage duration**	**Interaction (treatment x storage duration)**	**Statistical Technique**
20	20	***0.019***	***<0.001***	***0.006***	GLM
9	20	***<0.001***	***<0.001***	***0.008***	GLM

Similar trends to *H. megidis* are seen in *S. carpocapsae*, with both storage treatment and storage duration significantly affecting dispersal of IJs stored at both 9˚C and 20˚C. There was also a significant interaction between these factors in both cases (Table 3.2). IJs from the 'Water changed' treatment dispersed significantly less than 'Undisturbed' IJs from week 4 when stored at 20˚C (Figure 3.9A) and from week 9 when stored at 9˚C (Figure 3.9B). This trend was reversed in week 2 of 20˚C storage where both 'Resuspended' and 'Water changed' treatments showed higher levels of dispersal than 'Undisturbed' IJs (significant only in 'Resuspended' treatment). With this one exception, there is no significant difference in dispersal between 'Undisturbed' and 'Resuspended' treatments at any time point during both 20˚C (Figure 3.9A) and 9˚C (Figure 3.9B) storage experiments.

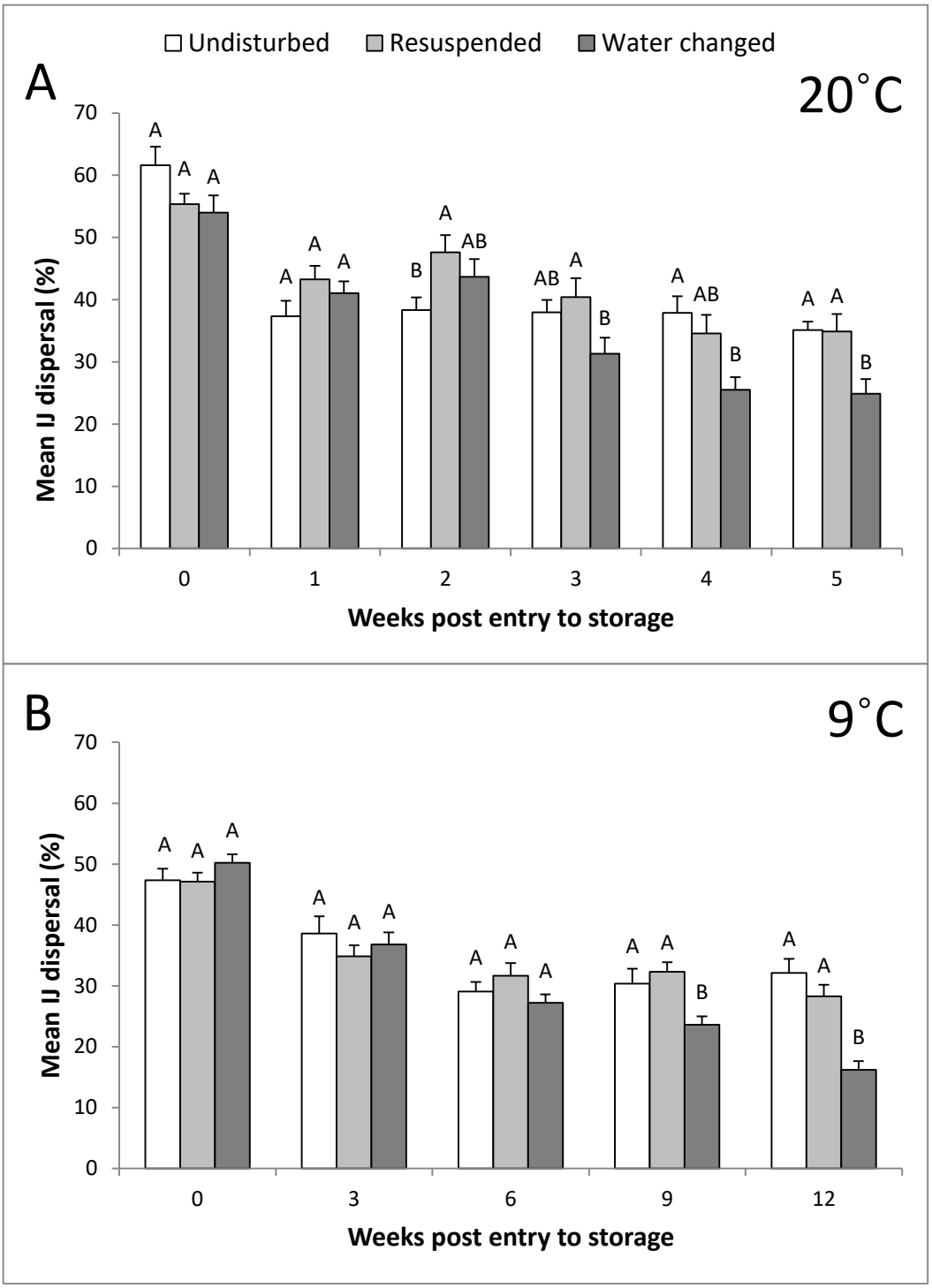


Figure 3.9: Percentage of *S. carpocapsae* IJs stored at 20˚C (A) and 9˚C (B) dispersing. Initial GLM (treatment, week, treatment and week/treatment interaction all $p < 0.05$, Table 2). Treatments which do not share a letter differ significantly (Within week one-way ANOVA ($p < 0.05$) followed by Tukeys *post hoc* test).

Table 3.2: Results of statistical analysis (p values, GLM: General Linear Methods) showing effect of storage treatment and storage duration on dispersal *S. carpocapsae* IJs stored at 20˚C or 9˚C. P values in ***bold*** represent significant difference (α = 0.05) in mortality according to specified variable.

S. carpocapsae		Analysis of variance p values			
Storage temp. (˚C)	**Assay temp (˚C)**	**Storage Treatment**	**Storage duration**	**Interaction (treatment x storage duration)**	**Statistical Technique**
20	20	***<0.001***	***<0.001***	***0.003***	GLM
9	20	***<0.001***	***<0.001***	***<0.001***	GLM

In *S. carpocapsae*, storage treatment had no effect on *T. molitor* mortality in any of the storage and assay temperature combinations, however storage time was significant (or marginally non-significant) in each case (Figure 3.10, Table 3.3).

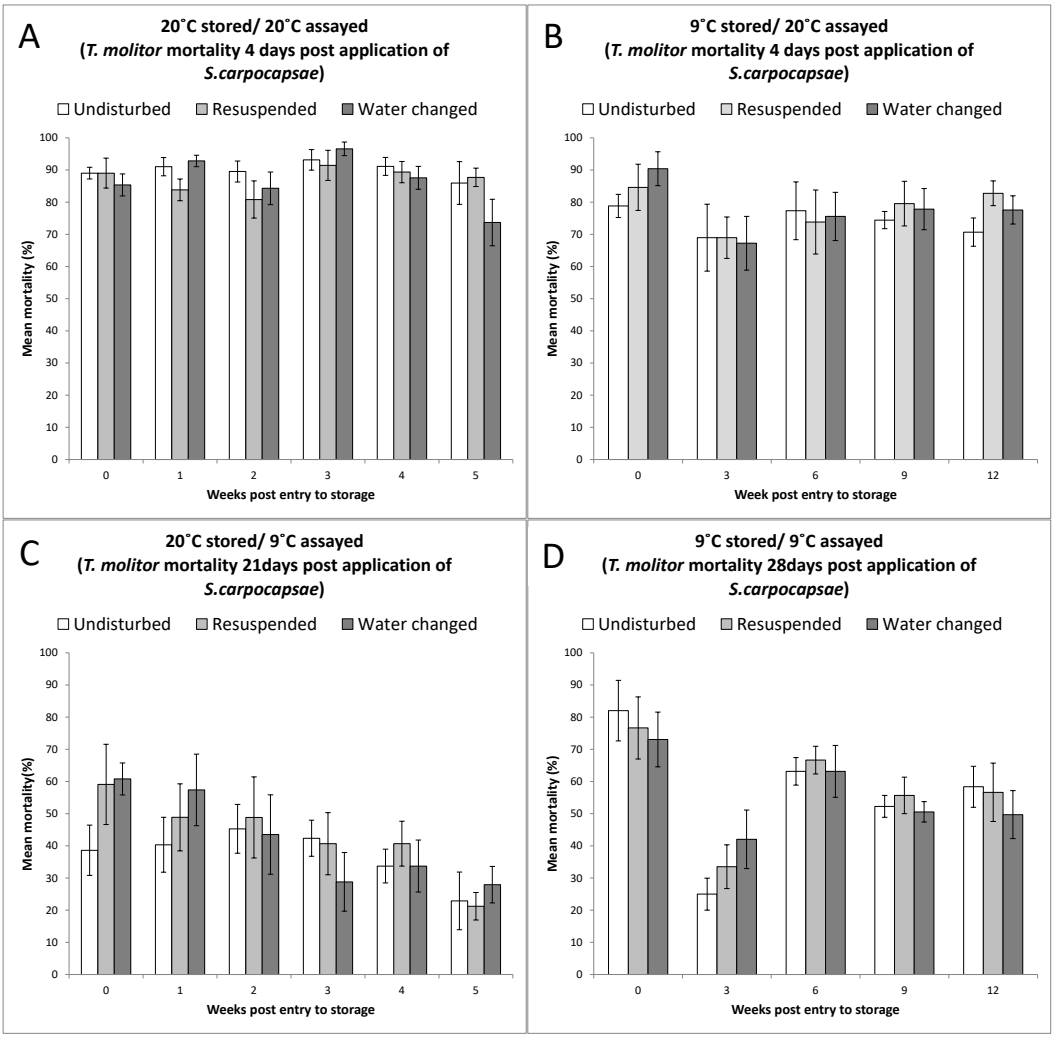


Figure 3.10: Mean *T. molitor* mortality (%) following treatment with *S. carpocapsae* (10 IJ/insect) at either 9˚C or 20˚C. Three different storage treatments of test nematodes were assessed per storage temperature (9˚C and 20˚C) at regular intervals during storage period (5 weeks at 20˚C, 12 weeks at 9˚C). Five replicates of 12 *T. molitor* were assessed per treatment at each time point (Total = 60 insects), corrected for control mortality using Abbotts' formula. Complimentary statistical analysis of data presented in Table 3.3. Error bars show standard error of mean percentage total mortality.

Table 3.3: Results of statistical analysis (p values, GLM: General Linear Methods) showing effect of storage treatment and storage duration on virulence of *S. carpocapsae* IJs stored at 20˚C or 9˚C.Statistical testing carried out on untransformed data (% mortality). P values in ***bold*** represent significant difference (α = 0.05) in mortality according to specified variable.

S. carpocapsae			Analysis of variance p values			
Storage temp. (˚C)	**Assay temp (˚C)**	**Days post infection**	**Storage Treatment**	**Storage duration**	**Interaction (treatment x storage duration)**	**Statistical Technique**
20	20	4	0.314	***0.006***	N/A	KW
20	9	21	0.441	***<0.001***	0.735	GLM
9	9	28	0.882	***<0.001***	0.775	GLM
9	20	4	0.600	0.086	0.961	GLM

Although not the primary aim of this study, it was noted that length of storage had an effect on level of *T. molitor* mortality particularly when assays were carried out at 9˚C. When IJs were assayed at 9˚C after storage at 20˚C, a gradual decline in *T. molitor* mortality over the 5 week storage period was observed (Figure 3.10C). A different trend was seen when IJ were assayed at 9˚C and stored at 9˚C in that there was a steep decrease in mortality from 0 – 3 weeks of storage which gradually recovered over the remainder of the 12 week storage period, although initial level of mortality was not completely restored (Figure 3.10D).

There was a less marked change in *T. molitor* mortality over time when *S. carpocapsae* was assayed at 20˚C for both 20˚C and 9˚C storage temperatures (Figures 3.10A and 3.10B respectively) where mortality was sustained above 60% throughout the experiment.

As no interaction between treatment and storage duration was identified statistically in *S. carpocapsae* (Table 3.3), it was deemed appropriate to pool storage treatment data at each assessment time point for all storage/assay combinations where storage duration significantly affected mortality (Figure 3.11 A- C). When *S. carpocapsae* was stored and assayed at 20˚C, *T. molitor* mortality remained relatively unchanged throughout the 5 week experiment (Figure 3.13A). However, when the same 20˚C stored IJ are assayed at 9˚C, *T. molitor* mortality gradually declined weekly, becoming significant after 5 weeks (Figure 3.11B, One –way ANOVA $p < 0.01$, followed by Tukey *post hoc* analysis). Pooled treatment data for *S. carpocapsae* stored and assayed at 9˚C confirms a significant steep decline in *T. molitor* mortality after 3 weeks storage, however this decline recovers to some degree and remains constant from 6 – 12 weeks post entry to storage (Figure 3.11C, One –way ANOVA $p < 0.001$, followed by Tukey *post hoc* analysis).

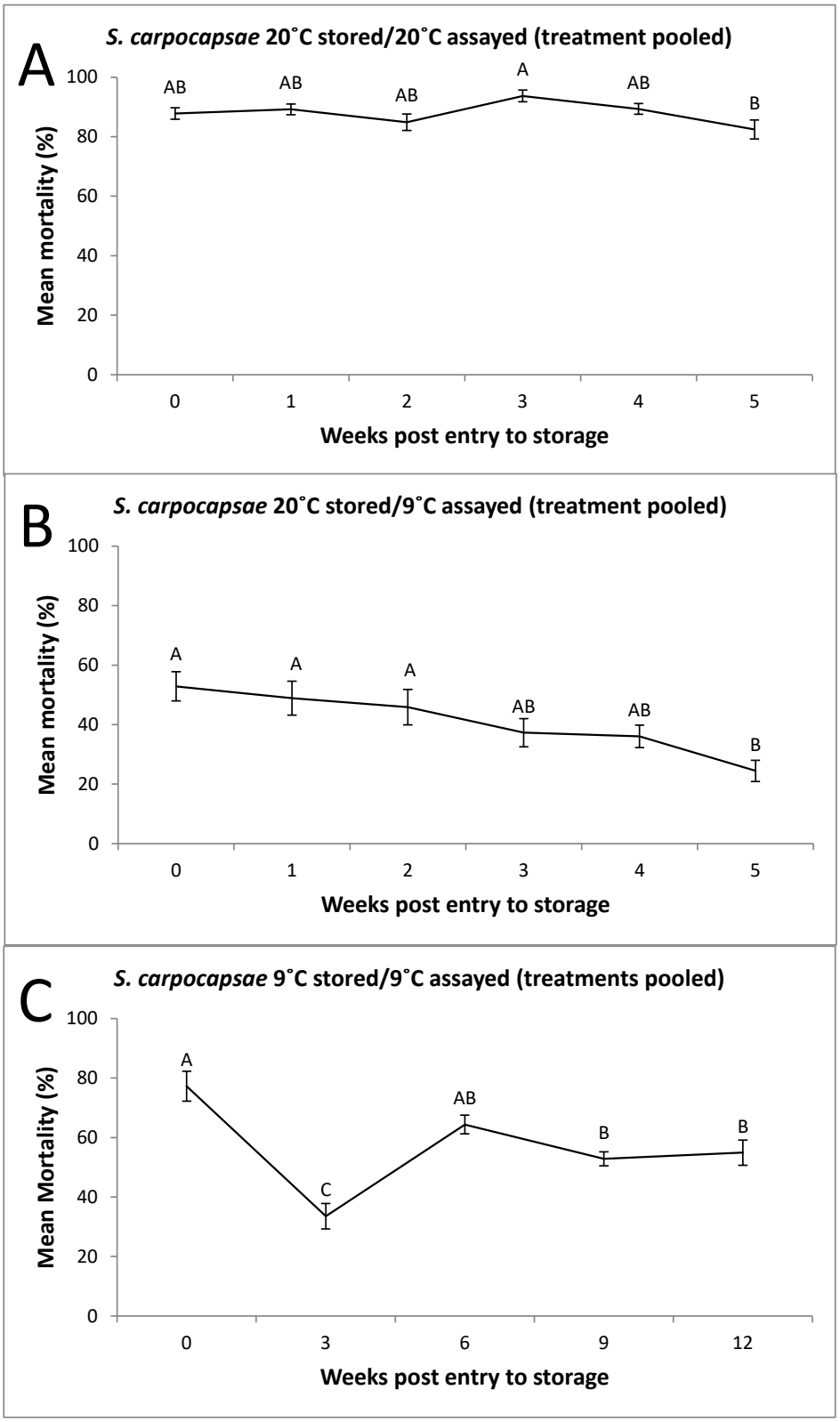


Figure 3.11: Mean *T. molitor* mortality (%) following application of *S. carpocapsae* (10 IJ/insect) at either 20˚C (A) or 9˚C (B-C) after storage at either 20˚C (A-B) or 9˚C (C). Pooled data of all three storage treatments of test nematodes. resulting in a total of 15 replicates of 12 *T. molitor* per treatment corrected for control mortality using Abbotts' formula (Total = 180 insects per time point).. Bars that do not share a letter differ significantly ($\alpha = 0.05$) (One-way ANOVA $p < 0.01$ with Tukeys *post hoc* test). Error bars show standard error of mean percentage mortality.

In *H. megidis*, storage treatment had a significant effect on *T. molitor* mortality only in the 20˚C stored/20˚C assayed experiment (Figure 3.12A, Table 3.4). As there was no interaction shown between storage treatment and storage duration (Table 3.4) it was deemed appropriate to pool data from all assay time points, which identified a significant difference in *T. molitor* mortality between the 'Undisturbed' treatment compared with the other two (Figure 3.13, One-way ANOVA $p < 0.01$ with Tukeys *post hoc* test.).

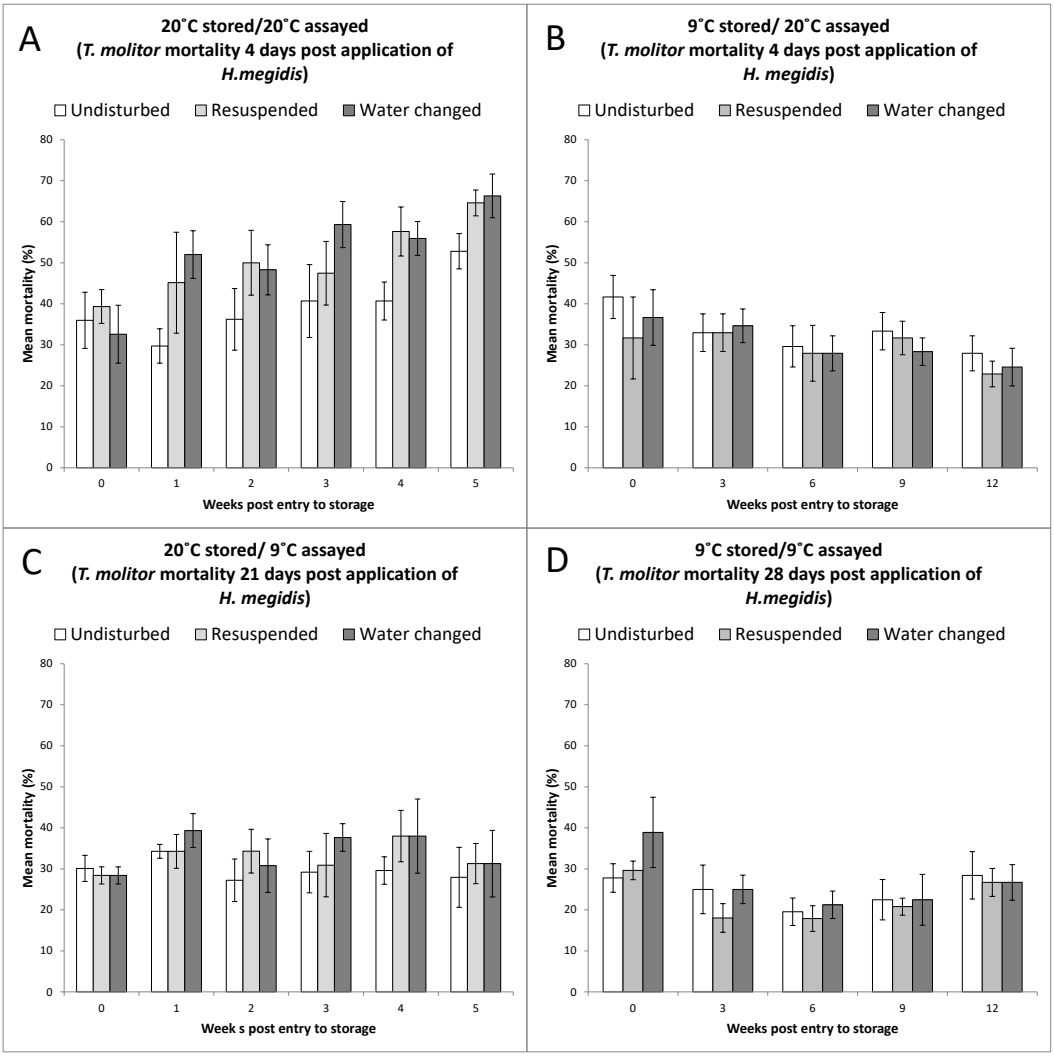


Figure 3.12: Mean *T. molitor* mortality (%) following treatment with *H. megidis* (10 IJ/insect) at either 9˚C or 20˚C. Three different storage treatments of test nematodes were assessed per storage temperature (9˚C and 20˚C) at regular intervals during storage period (5 weeks at 20˚C, 12 weeks at 9˚C). Five replicates of 12 *T. molitor* were assessed per treatment at each time point (Total = 60 insects), corrected for control mortality using Abbotts' formula. Complimentary statistical analysis of data presented in Table 3.4. Error bars show standard error of mean percentage total mortality.

Table 3.4: Results of statistical analysis (p values, GLM: General Linear Methods) showing effect of storage treatment and storage duration on dispersal of *H. megidis* IJs stored at 20˚C or 9˚C. Statistical testing carried out on arcsine transformed data (% mortality). P values in ***bold*** represent significant difference (α = 0.05) in mortality according to specified variable.

H. megidis			Analysis of variance p values			
Storage temp. (˚C)	**Assay temp (˚C)**	**Days post infection**	**Storage Treatment**	**Storage duration**	**Interaction (treatment x storage duration)**	**Statistical Technique**
20	20	4	**0.002**	**>0.001**	0.830	GLM
20	9	21	0.243	0.575	0.983	GLM
9	9	28	0.445	**0.015**	0.957	GLM
9	20	4	0.486	0.114	0.988	GLM

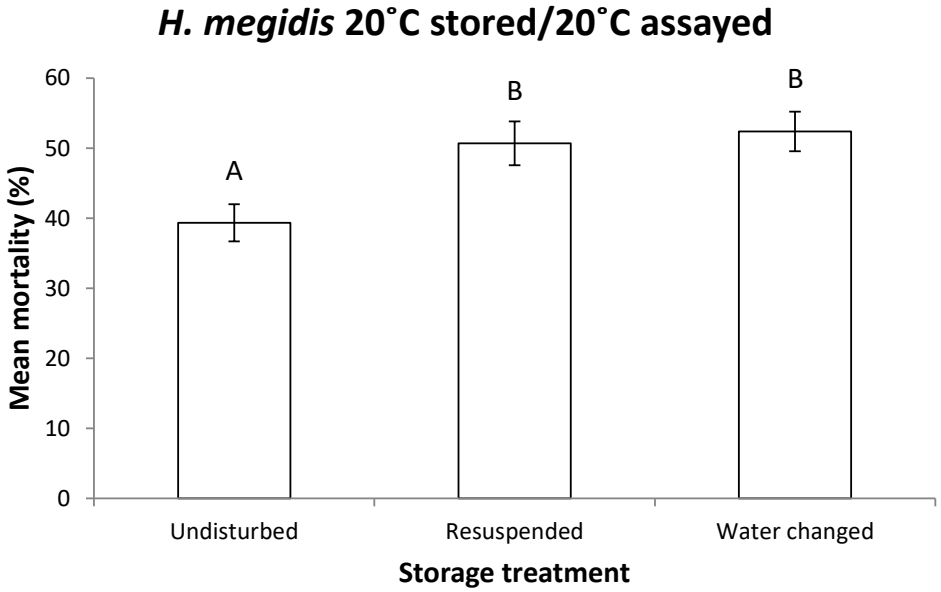


Figure 3.13: Mean *T. molitor* mortality (%) following application of *H. megidis* (10 IJ/insect) at 20˚C. Pooled data of all weekly assessments during 5 week experiment for 3 different storage treatments of test nematodes resulting in a total of 25 replicates of 12 *T. molitor* per treatment, corrected for control mortality using Abbotts' formula (Total = 300 insects). Bars that do not share a letter differ significantly ($\alpha = 0.05$) (One-way ANOVA ($p < 0.01$) with Tukeys *post hoc* test). Error bars show standard error of mean percentage mortality.

Storage duration had a significant effect on mortality in two cases, 20˚C storage/20˚C assay (GLM, $p < 0.001$, Table 3.4) and 9˚C storage/9˚C assay (GLM, $p < 0.05$, Table 3.4), however the trend in mortality changes over time differed between the two. When *H. megidis* were stored and assayed at 20˚C, *T. molitor* mortality steadily increased over time (5 weeks, Figure 3.12A), whereas storage and assay at 9˚C showed an initial decline in mortality after Week 0, with a trend towards recovery after a much longer time period (12 weeks, Figure 3.12D). As no interaction between treatment and storage duration was identified statistically (Table 3.4), pooling data from all storage treatments allowed these trends to be seen more clearly (Figure 3.14).

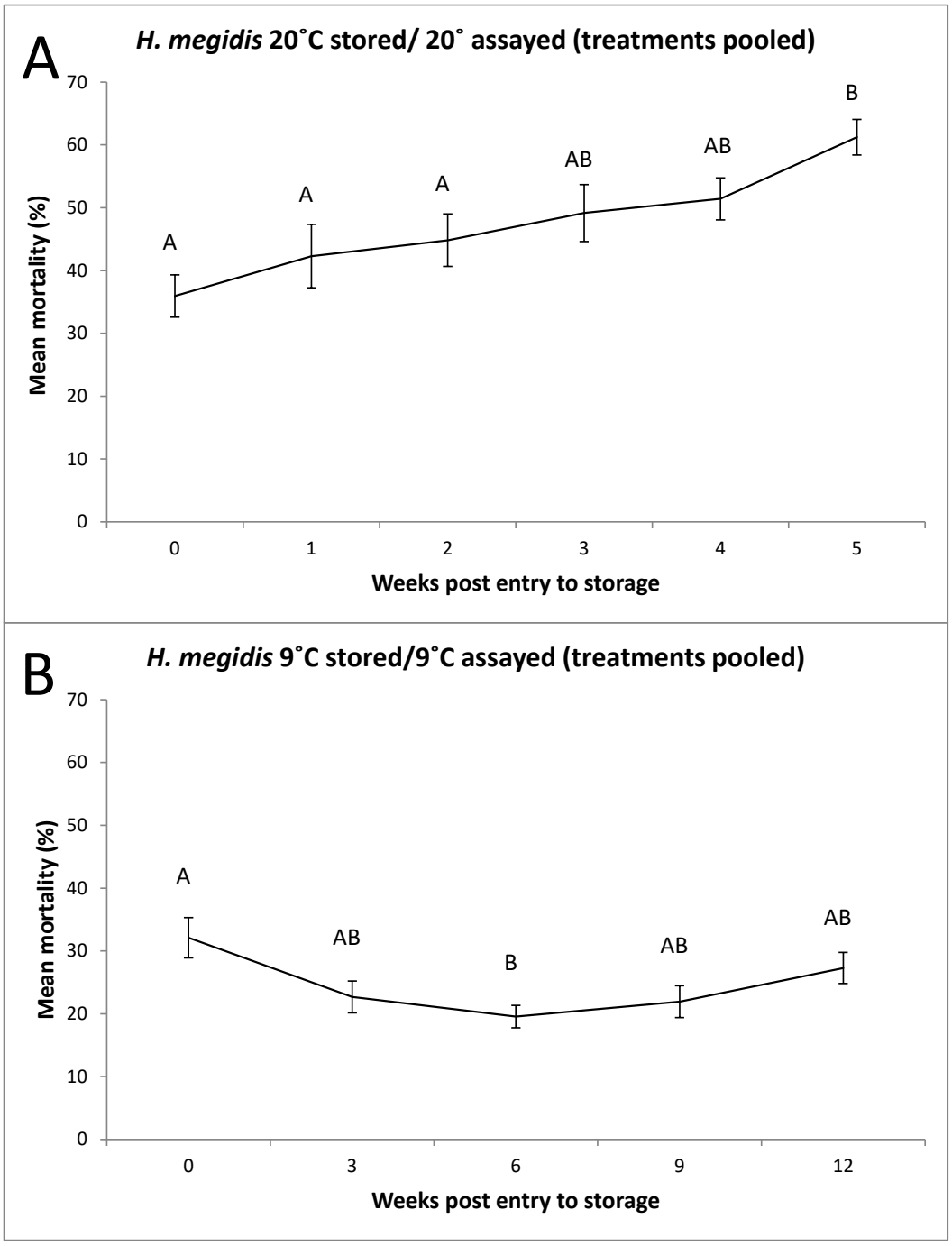


Figure 3.14: Mean *T. molitor* mortality (%) following storage and application of *H.megadis* (10 IJ/insect) at either 20˚C (A) or 9˚C (B). Pooled data of all three storage treatments of test nematodes. resulting in a total of 15 replicates of 12 *T. molitor* per treatment corrected for control mortality using Abbotts' formula (Total = 180 insects per time point).. Bars that do not share a letter differ significantly ($\alpha = 0.05$) (One-way ANOVA $p < 0.01$ with Tukeys *post hoc* test). Error bars show standard error of mean percentage mortality.

Mean time to death was calculated for both *S. carpocapsae* and *H. megidis* at each of the four storage treatments described previously (Figure 3.1). Patterns generally reflected those shown by absolute mortality (Figures 3.10 and 3.12 respectively). Mean time to death results are shown in Appendix 3.1(a-b).

3.4 Discussion

As hypothesised, all 4 species dispersed considerably more when stimulated by extract of *G. mellonella* cadavers infected by either their own or heterospecifics to a similar extent. This was the case both for closely related *Steinernema* spp. and also between more distantly related EPN from the two main families (*Steinernema* spp. and *Heterorhabditus* spp.). Promotion of IJ dispersal from infected cadavers in comparison to aqueous suspension has been widely reported in both laboratory (Shapiro-Ilan and Glazer, 1996) and field settings (Shapiro-Ilan et al., 2003). Increased IJ dispersal from infected cadavers or cadaver extract has been attributed to a repulsion response to increasing levels of toxic ammonia, a product of nematode defecation (San-Blas et al., 2008; San-Blas et al., 2014). Kaplan et al. (2012) demonstrated that ascr#9 plays a role in inducing dispersal in *S. feltiae*. C18 fractionation of infected host cadavers and subsequent bioassay of fraction showed revealed that a combination of all three fractions (A, B, and C) was necessary for activity. LC-MS analysis of fractions revealed the presence of ascr#9 and ascr#11 in fraction A, of which all three (synthetic ascarosides and natural fraction) were inactive on their own. However, when synthetic asc#9 at the biologically relevant concentration (40 pmol/ml) was combined with natural fractions B and C the original dispersal activity was restored, confirming ascr#9 as an active component of the *S. feltiae* dispersal blend (Kaplan et al., 2012). Previous studies report that IJs disperse more readily when applied in infected cadavers than in aqueous solution in a number of *Steinernema* spp. (Shapiro-Ilan and Glazer, 1996) and *Heterorhabditis* spp. (Shapiro-Ilan and Glazer, 1996; Shapiro-Ilan et al., 2003), further suggesting that chemical signals within the infected cadaver may promote dispersal.

Although it has been shown that IJ dispersal is promoted by nematode metabolites produced within the host cadaver, such as ascr #9 (Kaplan et al., 2012), it is unknown whether such dispersal signals are produced on a continual basis even after IJs have vacated the infected cadaver. Here, I found that IJ dispersal activity was increased for 4 EPN species when exposed to water conditioned with high density conspecific IJ, indicating a dispersal cue may be produced by IJs themselves. Choe et al. (2012b) detected several ascarosides in 'worm water' of IJs of 4 *Steinernema* spp. (including *S. carpocapsae*), but did not ascribe any definite function to them with regards to IJ behaviour. In general, IJ and dauer nematodes produce a less diverse set of ascarosides than their adult counterparts (Choe et al., 2012b). However, due to the conserved nature of ascaroside signalling in nematodes (Choe et al., 2012b) it is reasonable to assume ascarosides may be involved in the control of dispersal behaviour, at least to some extent.

It is proposed that in general ascarosides are released through the natural exit points of the mouth and anus (Choe et al., 2012b) especially as *C. elegans* ascarosides are believed to be synthesised in the intestine (Butcher et al.,2009). These non-feeding juveniles have the mouth and anus closed and are covered by the retained cuticle of the J2 stage as a sheath which may prevent the release of ascarosidal metabolites (Choe et al., 2012b). Thus, it is unclear how IJs may release such ascarosidal signals.

It is expected that the species-specificity of signal blends involved in regulating IJ dispersal is considerably less than that involved in mediating sexual behaviour of adults. This hypothesis is based on the understanding that dispersal from overcrowded conditions or a depleted food resource is a common goal of all individuals regardless of species, unlike the sexual encounters of adults where mistakes in mate finding and recognition would considerably impact species

persistence. Both my results and other studies to date support this hypothesis, from observed similarities in ascaroside profiles of *Steinernema* spp. IJ (Choe et al., 2012b), to experimental evidence showing definite behavioural response to exudates from other species and synthetic ascaroside blends (Kaplan et al., 2012). Reception of interspecific chemical signals has been shown to occur even in relatively distantly related species. For example, media conditioned with *C. elegans* dauer juveniles induced dispersal in the entomopathogenic *S. feltiae* as well as plant parasitic *Meloidogyne* spp. (Kaplan et al., 2012). Ascr#9, which is strongly expressed in *Steinernema* spp. is structurally analogous to ascr#2 expressed by *C. elegans* dauer juveniles (Kaplan et al., 2012). Furthermore, both compounds, within their respective species, accumulate in a similar way to ultimately trigger IJ dispersal (*S. feltiae*) or dauer formation (*C. elegans*) in response to overcrowding and food resource depletion (Kaplan et al., 2012).

Long term storage experiments where IJs provided with clean water regularly dispersed significantly less after some weeks when compared to treatments where the original water remained, support the assumption that IJs produce self-stimulating chemical compounds which may enhance dispersal behaviour. This was evident in both *S. carpocapsae* and *H. megidis* at both storage temperatures. This ties in with the earlier experiments which show that IJs secrete into the medium a compound or compounds that stimulate dispersal. In contrast, there was no evidence of an effect of secreted chemicals on virulence.

In all cases, regardless of storage treatment, dispersal of both *S. carpocapsae* and *H. megidis* gradually decreased over time. Dempsey and Griffin (2002) reported similar findings in *H. megidis* and attributed the dispersal decline with time as a waning of the dispersal phase as infectivity increased. In *H. megidis*, it has been shown

previously that infectivity increases steadily in the weeks following emergence but then declines due to age, and this is proposed to be an endogenous process (Griffin, 1996; Dempsey and Griffin, 2002; Griffin, 2012). In my experiments, only *H. megidis* stored at 20˚C and assayed at 20˚C showed the expected steady increase in infectivity over 5 weeks of storage. I found little evidence shown that storage treatment affects virulence in *S. carpocapsae.* Here I tested the possible interaction between storage medium and the putative endogenous improvement in infectivity. *H. megidis* stored in water that was either resuspended or changed regularly performed significantly better than the undisturbed treatment over the 5 week storage period in the 20˚C storage/ 20˚C assay treatment. This indicates that mechanical disturbance, rather than exposure to chemical metabolites, may play a role in improving virulence.

It has also been reported that cold storage of EPN prior to application improves performance at low temperatures. *H. megidis* stored for 12 weeks at 9˚C were significantly more likely to both invade and kill black vine weevil larvae (*Otiorynchus sulcatus*) when applied at 9˚C than at 20˚C (Fitters et al., 2001). Griffin (1996) also reported that *Heterorhabditis* spp. stored and assayed periodically at 9˚C become more infective (towards *G. mellonella*) over time (after 5 – weeks) following an initial 0-4 week period of low infectivity. My data agreed with these previous findings, in cold stored *S. carpocapsae* (9˚C), the characteristic early period of low infectivity was succeeded by gradual improvement from 6 weeks onwards (Figure 3.10D). However, despite this gradual increase in infectivity over time, the final level at week 12 did not exceed the initial level of infectivity at week 0 in both species tested. The impact of cold storage on *S. carpocapsae* infectivity is less well documented than *H. megidis* (Guy et al., 2017). Discrepancies in findings may be

due to variation in methodology among experiments, including target host and how successful infection was assessed, whether it be insect mortality of number of IJs entering a host regardless of kill. For *S. carpocapsae*, the fact that the strain used in my experiments differed from that used by Guy et al. (2016) may also be a factor. The degree of improvement was reported to be variable between *Heterorhabditis* spp. strains (HF85 and UK211, Griffin, 1996). For *H. megidis*, long term lab culture of the strain used may have subjected it to trait deterioration (Wang and Grewal, 2002) which could have affected results.

Chapter 4 Attraction

4.1 Introduction

Sex pheromones are defined as externally released chemicals produced by members of one sex that induce a behavioural response in conspecifics of the opposite sex only (Shorey, 1973). They play an integral role in mate finding and attraction and are particularly relevant in gonochoristic species where the meeting of male and female sexes is vital for reproduction. Typically, mate attractants are female-produced and male-specific. This is the case in *Steinernema carpocapsae*, where females produce a pheromone that is attractive to males (Neves et al., 1998: Lewis et al., 2002). While examples of the opposite exist, male-produced female-specific sex pheromones are considerably less documented. Although pheromones have been implicated in the sexual attraction of over 30 species of nematode, they are recorded in relatively few genera (Lee, 2002).

C. elegans has served as a model species to explore chemical communication in nematodes. Although primarily hermaphroditic, the presence of a small proportion of males in *C. elegans* populations has facilitated the study of chemically mediated sexual behaviours, including mate attraction. Early research, concluding that *C. elegans* hermaphrodites have the ability to attract and hold males (Simon and Sternberg, 2002), used live hermaphrodites on agar to attract conspecific males. These initial indications were supported in a study showing that male *C. elegans* are less likely to vacate an area containing bacterial food source where a hermaphrodite is present (Lipton et al., 2004), than if a male or no worm at all was present. Both

studies used live hermaphrodites as stimuli, however Lipton et al. (2004) noted that physical contact was not required between male and hermaphrodite to prevent the male leaving implying that the putative sex pheromone was externally secreted. Further exploration using supernatant of liquid cultured *C. elegans* as an assay stimulus in place of live worms showed that males spent significantly more time associated with areas conditioned with liquid taken from hermaphrodite cultures than they did with control areas (White et al., 2007), confirming that the cue is externally excreted. Chasnov et al. (2007) showed that males of the androdioecious species *C. elegans* and *Caenorhabditis briggsae* were significantly attracted to female supernatant of the dioecious species, *Caenorhabditis remanei* (Chasnov et al., 2007). Male attraction to female supernatent was sustained when the cue was presented on the agar surface during the assay, but also when applied to the petri dish lid a short distance above the agar surface, showing that nematode sex pheromones are potentially volatile (Chasnov et al., 2007). The study also concluded that attractant production (or release) begins at sexual maturity (male attraction was observed only when adult females were used and not late stage L3 and L4 worms) and that mated females lose attractiveness whereas virgins remain attractive even as they age (Chasnov et al., 2007). Reduction in attraction potential of females post mating is also seen in *S. carpocapsae*, where males were found to be attracted to virgin females but not mated ones (Lewis et al., 2002) suggesting that the pheromone is no longer secreted once the female has mated.

Evidence of sex pheromones in Steinernematids is limited to *S. carpocapsae* (Lewis et al., 2002). Species of the genus *Steinernema* spp. (with one exception) are exclusively dioecious, and so it is likely that gender specific sex attractants may be at play. Neves et al. (1998) showed that *S. carpocapsae* males were significantly more

attracted to assay regions laced with virgin female-conditioned buffer in comparison to buffer alone. Adult females showed no difference in migratory behaviour to female-conditioned buffer or buffer alone, suggesting that the signal is sex-specific, attracting males only. This conclusion was further supported by the finding that males were not significantly attracted to male-conditioned buffer (Neves et al., 1998). The same study also noted that immersion of conspecifics in female-conditioned buffer induced excitory behaviour in males (characterized by rapid undulatory movement) but not females. Two strains of *S. carpocapsae* (Az20 and Breton) were tested and although sex-specific, results were found not to be strain-specific in cross-strain testing (Neves et al., 1998). Further studies on *S. carpocapsae* showed that males placed 2.5 mm away from live females on a bacterial lawn moved towards and spent significantly more time in contact with conspecific virgin females than they did with conspecific mated females, heat-killed females, males or female *Heterorhabditis bacteriophora* (Lewis et al., 2002). To clarify whether the response was mediated by chemical signals or a result of contact cues, the authors performed a follow-up study in which male *S. carpocapsae* were less likely to leave an area of bacterial lawn on agar which had held a virgin female for a period of 3 hrs, but was removed during assay, when compared to a control with no female occupant (Lewis et al., 2002). These results indicated that males were retained by female 'scent' remaining on the agar surface after the female was removed and that physical female presence is not required to induce this response in males.

The elucidation of ascarosides as the active molecules that induce dauer formation in *C. elegans* during periods of environmental stress (Jeong et al., 2005), led to the investigation of whether similar molecules were involved in the control of sexual behaviours, including mate attraction, in nematodes (Srinivassan et al., 2008;

Pungaliya et al., 2009). Several nematode species from a wide variety of taxa have been screened for the production of ascarosides (Choe et al., 2012b), the group of ascarylose based glycosides unique to nematodes implicated in chemically mediated behavioural and developmental organization (Section 1.6.1). Four *Steinernema* species (*S. carpocapsae*, *S. glaseri*, *S. scapterisci* and *S. riobrave*) were included in the study and were found to externally secrete numerous known ascaroside molecules at both juvenile and adult stages of the life cycle. The composition of the ascaroside excretome typically differed according to life stage with IJs producing less varied and numerous ascaroside blends than the corresponding adult population (Choe et al., 2012b), indicating that functionality may change according to life stage. For example, *S. glaseri* IJs and adults both produce similar amounts of ascr#9, but only adults produce large amounts of ascr#1 suggesting the latter may play a role exclusively in adult behaviour. Ascr#9 was common to all insect-associated species tested (*Steinernema* spp., *Oscheius tipulae*, *Oscheius carolinensis*, *Pristontionchus pacificus* and *Heterorhabditis bacteriophora*) regardless of life stage and was found in large amounts relative to other ascarosides identified in *Steinernema* spp (Choe et al., 2012b).

Ascarosides have previously been shown to induce attraction behaviour in several nematode species. In *C. elegans*, ascr#2 and ascr#3 were found to attract males at femtomolar concentrations (Srinivasan et al., 2008), however not to the full extent of supernatant of hermaphroditic culture. Further analysis of the chemical composition of hermaphrodite supernatant led to the identification of ascr#8 as the additional component to those previously tested which restored the level of male attraction to that of the natural exudate (Pungalia et al., 2009). Examining the role of ascarosides in attraction in *Steinernema*, Choe et al. (2012b) conducted retention assays on

bacterial lawns to assess the preference or avoidance of synthetic ascarosides at varied concentrations. Male *S. glaseri* showed significant preference for ascr#1 at pmol concentrations, an ascaroside previously found to be produced by conspecific adults but not IJs. Interestingly, male *S. glaseri* were also retained by ascarosides not produced by their own species (ascr#3, ascr#7 and ascr#10) but higher concentrations were required to facilitate this response (nmol). Athough *S. glaseri* does not produce ascr#10 itself, adult *S. carpocapsae* do, indicating possible overlap between species where behavioural response to ascarosides is concerned (Choe et al., 2012b). *S. glaseri* was the only steinernematid tested in the retention assay used by Choe and colleagues (2012b) and only with solutions of single ascarosides as stimuli. It is well documented that ascarosides act synergistically as multicomponent blends to produce the desired behavioural or developmental response, for example in the dauer inducing pheromone of *C. elegans*, 'daumone' (Butcher et al., 2007; Butcher et al., 2008; Kaplan et al., 2011) and mate attraction in the same species (Pungaliya et al., 2009).

4.1.1 Chapter aims and objectives

This chapter addresses how *Steinernema* spp. utilise chemical signals to identify and attract potential mates and explores how these signals may differ between species in a system where recognition is important (i.e. sex recognition). There is already evidence that females of one species of *Steinernema* (*S.carpocapsae*) produce a male-specific mate attractant (Neves et al., 1998: Lewis et al., 2002) and this will be further characterised along with the examination of sex attractants in the previously unstudied species *S. longicaudum*. Solid Phase Extraction (SPE), High Performance

Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) will be used to try to identify chemicals responsible for attracting conspecific males and highlight differences between *S. carpocapsae* and *S. longicaudum* chemical profiles.

Specific objectives are to:

1. Based on the findings of Neves et al. (1998) and Lewis et al. (2002) who showed that *S. carpocapsae* females attract males via chemical signals, investigate whether *S. longicaudum* behave similarly.

2. Determine optimal parameters, such as female stage and density, for producing attractive female-conditioned exudate in *S. carpocapsae* and *S. longicaudum.*

3. Investigate species-specificity of *Steinernema* sex-attractant pheromones. Species-specificity is expected, since pheromones serve not only to attract a potential mate but also have a role in species identification.

4. Use C18 SPE (Solid Phase Extraction) to extract female-conditioned media and identify chemical components involved in mediation of attraction behaviours through bioassay of extracted material.

4.2 Methodology

4.2.1 Attraction assay

Attraction bioassays were conducted on a lawn of bacterial symbiont appropriate to nematode species: *X. nematophila* or *X. ehlersi* for *S. carpocapsae* and *S. longicaudum* respectively. Assay plates were prepared by evenly spreading 100 µl of liquid cultured bacterial symbiont (Section 2.4.2) over the surface of 3-4 mm thick 2% Nutrient agar in 5.5 cm Petri dishes. Dishes were incubated for 48 h at 27°C prior to assay commencement to produce complete bacterial coverage of the assay arena. In the attraction assays, a mature male was given a choice between a biological or chemical stimulus and control stimulus within a single assay arena. Unless otherwise specified, stimuli were applied in 5 µl volumes (creating a circle of 5 mm diameter on the bacterial lawn) with 10 mm between conditioned and control stimuli boundaries (Figure 4.1). Stimuli were applied to the bacterial lawn 3 h prior to assay commencement to allow a chemical gradient to be established. In all but one experiment (Section 4.3.1), only conditioned (and control) liquid media were used as stimuli during assay. In this exception, a treatment was included where the socially naïve virgin female used to condition insect haemolymph was applied to the plate over the haemolymph stimulus. No female was applied to the control area, which received haemolymph only.

Sexually mature test males (sperm visible within gonad), were dissected from *G. mellonella* cadavers and rinsed in sterile PBS before application (using platinum wire) to the assay arena midway between stimuli and observed at 10 min intervals for 3 h. Males breaching stimulus boundary (either conditioned or control) were

noted as making a choice and assigned a choice category accordingly ('+' and '-', respectively). Males located elsewhere in the assay arena were graded as not making a choice ('0'). Presented data represents the first choice of each male within the 3 h assay period.

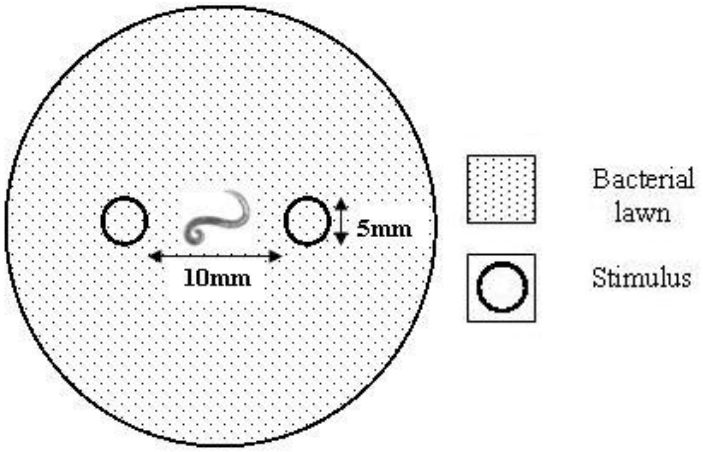


Figure 4.1: Schematic representation of the attraction assay.

4.2.2 Calculation of attraction index

Degree of attraction was expressed as an attraction index (AI) based on that of Bargmann et al. (1993) and later used by Chasnov et al. (2007). Perfect attraction to the putative attractant is signified as +1, while perfect attraction to the control stimulus is signified as -1. Mathematically, the atttraction index (AI) is calculated as:

$$\frac{\text{(No. of males choosing test stimulus – No. of males choosing control)}}{\text{Total No. test males}}$$

4.2.3 Preparation of worm-conditioned insect haemolymph

Female-conditioned haemolymph was accumulated by pooling hanging drops (10 µl per drop) of *G. mellonella* haemolymph in which single females had been reared in isolation from the IJ stage. Single *S. carpocapsae* worms were resident in 10 µl drops for 5 days prior to pooling, developing into visually identifiable adults on day 4 of incubation (20^0C) while *S. longicaudum* worms were resident for 4 days (adults identifiable on day 3, incubated at 27^0C) before pooling. Thus, conditioned haemolymph is estimated to contain externally excreted chemical material from 100 virgin ♀/ml for approximately 24 h prior to collection. Control haemolymph was prepared in the same way, except IJs were removed from drops 24 h after inoculation; sufficient to allow symbiotic bacteria (*Xenorhabdus* spp.) to be released, but prior to development to the adult stage. After IJ removal, control haemolymph was maintained at species appropriate development temperature and harvested at the same time as female-conditioned haemolymph.

4.2.4 Preparation of socially naive worm-conditioned PBS (reared *in vitro*)

All socially naïve virgin adults used for PBS conditioning were reared in hanging drops of *G. mellonella* haemolymph for 4-5 days prior to use as conditioning agents. Adults were rinsed in sterile PBS and transferred (using platinum wire) into wells (24 well plates, containing 500 µl sterile PBS per well) at various specified densities (10, 20 or 50 ♀/ml). Well plates were sealed with Parafilm and incubated at species appropriate development temperature with gentle shaking for 24 h unless specified otherwise. Control condition consisted of sterile PBS only (500 µl/well).

4.2.5 Preparation of socially experienced worm-conditioned PBS (reared *in vivo*)

Socially experienced adults were dissected from infected *G. mellonella* 72 h after infection for *S. carpocapsae* (20^0C) and 48 h post infection (27^0C) for *S. longicaudum* to ensure a yield of adults only. Infected cadavers were opened individually with a scalpel into a 5.5 cm Petri dish half filled with sterile PBS, and shaken gently for 10-15 min to encourage emergence of adults into the surrounding liquid. Large pieces of *G. mellonella* tissues were removed and adult nematode suspension from all cadavers was pooled into a graduated cylinder. The suspension was subsequently rinsed multiple times in sterile PBS by sedimentation to remove the majority of *G. mellonella* tissues. For the collection of large volumes of mixed-sex-conditioned PBS, adult density of the suspension was corrected to the desired amount via addition or removal of sterile PBS. Alternatively, adults were removed manually with platinum wire and added to PBS in well plates for conditioning of small volumes with single or mixed sex adults. Worms were incubated for 24 h (unless otherwise stated) at species appropriate development temperature (shaken gently).

4.2.6 Storage of worm-conditioned media

Immediately after conditioning, all worm-conditioned PBS was passed through a bacterial filter (0.2 µm acrodisc), stored at 4^0C and returned to room temperature before use in assays. Storage prior to assay did not exceed 14 days (with the exception of data presented in Figure 4.6). Insect haemolymph was always used immediately after collection (unfiltered) before significant melanisation could occur.

4.2.7 Controlled mating of females for assessment of the effect of sexual history on attractiveness

Socially naïve females were reared in hanging drops of *G. mellonella* haemolymph (Section 2.3), whereas males were dissected from infected *G. mellonella* cadavers 48 h and 72 h post infection for *S. longicaudum* and *S. carpocapsae* respectively. Males were confirmed as sexually mature prior to mating by microscopic observation of sperm in the gonad. Mating pairs were created by transferring 3 males each to female drops when females were 6 days old, and then incubating for 1 h. After the hour mating period, males were removed and females were retained in their original haemolymph drop for defined time periods (intervals between 0 – 48 h). PBS was conditioned with females (either mated or unmated for controls) in 96 well plates containing a single female and 50 µl PBS per well (equivalent to 20 ♀/ml) for 3 h. Conditioned PBS was used in attraction assays (Section 4.2.1), while females were returned to their original haemolymph drop to continue development. Drops were checked daily for up to 4 days for the presence of offspring as confirmation of successful mating. Only data collected from confirmed mated females (with the exception of unmated controls) was included in results.

4.2.8 C18 extraction, fractionation and resuspension of worm conditioned PBS (Max Planck Institute of Chemical Ecology, Jena, Germany)

Mixed sex adult-conditioned PBS was prepared in 500-600 ml batches (100 adult worms/ml, approx.. 50:50 male:female sex ratio), according to methodology outlined in Section 4.2.5. Following removal of worms and filtration (Section 4.2.6), conditioned PBS was divided into 50 ml aliquots (in 50 ml Falcon tubes), frozen in

liquid nitrogen and lyophilised to dry powder form. It was then sent by post to the Max Planck Institute of Chemical Ecology (Jena, Germany) to be chemically extracted and fractionated in the lab of Dr Stefan von Reuss. C18 extract of the full sample, along with stepwise fractions were returned to Maynooth University in dry form and resuspended in 100 % MeOH to create 20x concentrated stock solutions (stored at -20˚C) which were further diluted in PBS to the original sample concentration for biological assay (Section 4.2.1). For recombination of multiple fractions, equal volumes of concentrated stock fractions were combined, vortexed and dried before resuspension in PBS to restore original sample concentration.

4.2.9 C18 extraction of worm conditioned PBS (Maynooth University, Ireland)

Virgin female-conditioned PBS was prepared as specified in Section 4.2.4 at a concentration of 20 ♀/ml. Solid phase extraction (SPE) was carried out using a C18 Sep-Pak column. The stationary phase was conditioned with 100% MeOH and equilibrated with sterile PBS prior to sample loading. Flow-through was retained and stored at 4˚C in 50 ml Falcon tubes for fraction resuspension and assay purposes (Section 4.2.1). Elution of material bound to the column was carried out using 100 % MeOH. Eluate was dried (Speedy Vac) to the volume required to create 20x concentrated stock solutions (in MeOH) and maintained at -20˚C until use in assay. Prior to assay, the stock solutions were diluted to the original concentration using PBS.

4.2.10 Statistical analysis

All data analysis was carried out using Minitab 17. Data were screened for normal (or near normal with normally distributed residuals) distribution using the Anderson-Darling test for normality. Comparison of two data sets used Two-sample t testing. Where three or more data sets were compared, a One-way ANOVA was followed up either with Tukeys *post hoc* analysis (to identify where any significant differences specifically occurred) or Dunnett *post hoc* analysis to see which treatments (of many) differed significantly from a positive control. Chi-square tests on raw data were used where experiments were not repeated. Statistical techniques used are summarised in figure legends and tables in the results. Full test statistics are included in Appendix 6.

4.3 Results

4.3.1 Verification that physical female presence is not required to elicit attraction response in males and effect of female density in conditioned haemolymph

Attraction assays were carried out with a female either present or absent from the plate during assay (in 5 µl of haemolymph prepared according to Section 4.2.3). In each case haemolymph spiked with bacterial symbiont appropriate to species was used as the control (Section 4.2.3). Male *S. carpocapsae* and *S. longicaudum* were strongly attracted to socially naive female conspecifics in insect haemolymph over the haemolymph only control (Figure 4.2). The attraction response was sustained regardless of whether the female was physically present during the assay or had been removed from the haemolymph prior to assay, with no difference in attraction index between the two conditions (Two-sample t test, $p > 0.05$), indicating that a chemical attractant is produced externally by females.

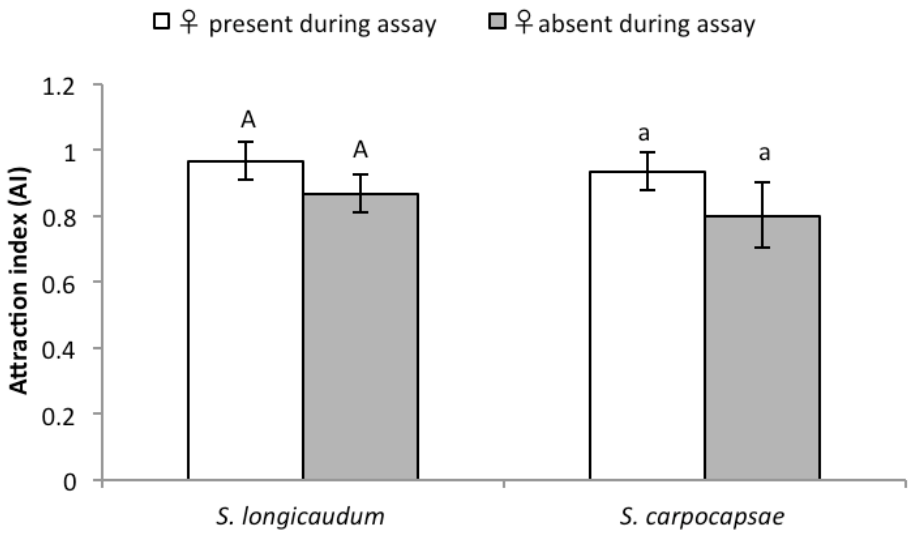


Figure 4.2: Attraction of mature male *Steinernema* spp. to socially naive female conspecifics on a lawn of symbiotic bacteria (N= 20 with 3 experimental repeats per condition). Error bars show standard error of means. Bars which share a letter do not differ significantly, two – sample t test on AI, $p > 0.05$.

Having determined that female presence was not required for attraction, a dilution series of socially naïve female-conditioned haemolymph was prepared (using IJ-inoculated haemolymph as dilutant and control, Section 4.2.3) to estimate the relative concentration of attractant required to elicit an attraction response in males (Figure 4.3). Attraction remained near perfect (AI close to 1.0) when at 1:2 dilution (50 ♀/ml equivalent), and was still strong at 1:5 dilution (20 ♀/ml equivalent) in both species tested (*S. carpocapsae* and *S. longicaudum*). The attraction response was substantially reduced to an AI of 0.3 – 0.4 at 1:10 dilution (10 ♀/ml equivalent) for both species (Figure 4.3).

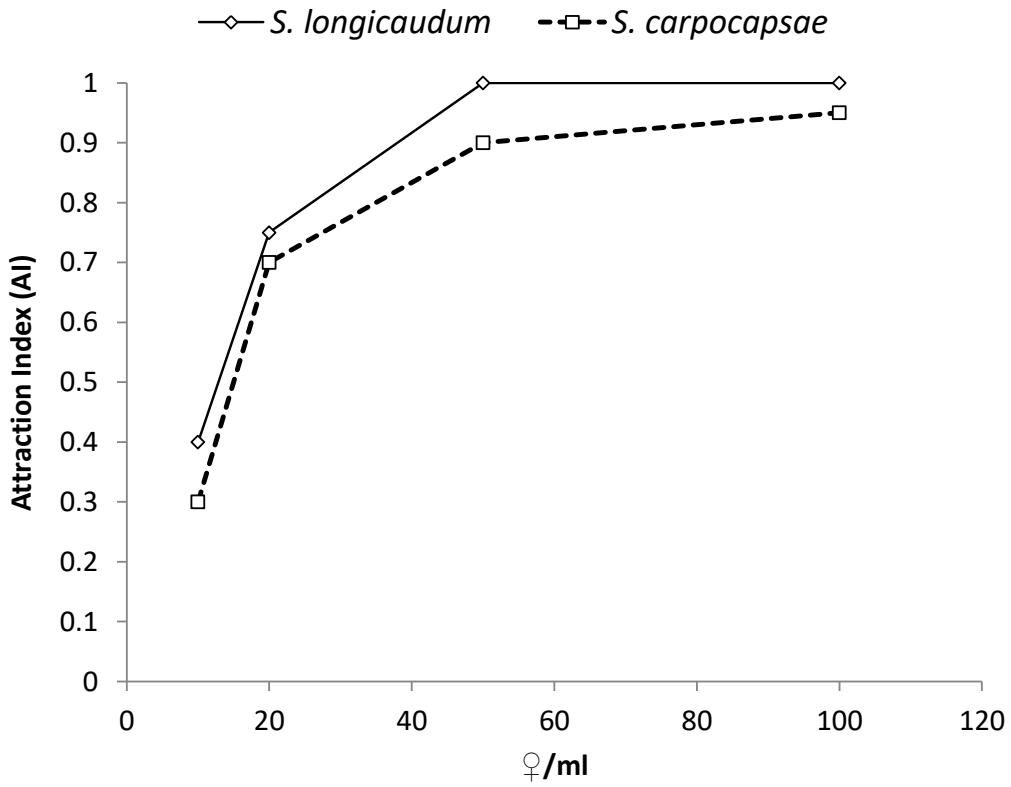


Figure 4.3: Degree of attraction of male *Steinernema* to dilution series of conspecific female conditioned *G. mellonella* haemolymph (original conditioned haemolymph 100 ♀/ml for 24h). N = 15 per dilution (single experimental replicate).

4.3.2 Determination of parameters for attractant production and storage in PBS

A series of experiments was conducted to ascertain the effect of various conditions (female density, PBS conditioning time and effect of storage time on conditioned PBS) to set acceptable parameters for future experiments. As with female-conditioned insect haemolymph, males were attracted to PBS conditioned (for 24 h) with conspecific socially naive female *Steinernema*. The degree of attraction increased with female density used during conditioning in both species tested (*S. carpocapsae* and *S. longicaudum*) and was relatively strong (AI > 0.6) for all densities tested (10, 20 and 50 ♀/ml, Figure 4.4).

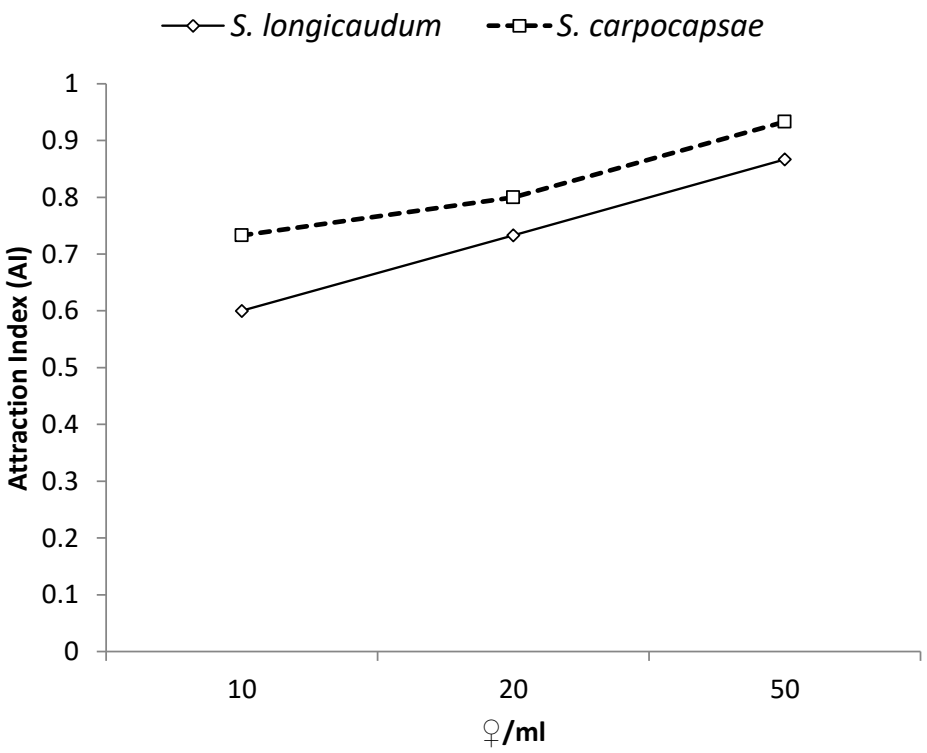


Figure 4.4: Degree of attraction of male *Steinernema* to PBS conditioned with different densities of socially naïve virgin conspecifics for 24h. N = 20 per density (single experimental replicate**).**

To investigate the effect of conditioning time on attraction, a density of 20 ♀/ml was chosen, based on a compromise between attractiveness and labour required for preparation. Both *S. carpocapsae* and *S. longicaudum* males were attracted to PBS conditioned with conspecific females (20 ♀/ml) for 24 h. Attraction remained relatively strong even when conditioning time was reduced to just 1 h (particularly in *S. carpocapsae*), indicating that the attractant accumulates quickly in the surrounding medium (Figure 4.5). Despite this finding, it was decided that PBS in all further experiments would be conditioned for 24 h to be confident of attractant presence and maintain experimental continuity throughout the project.

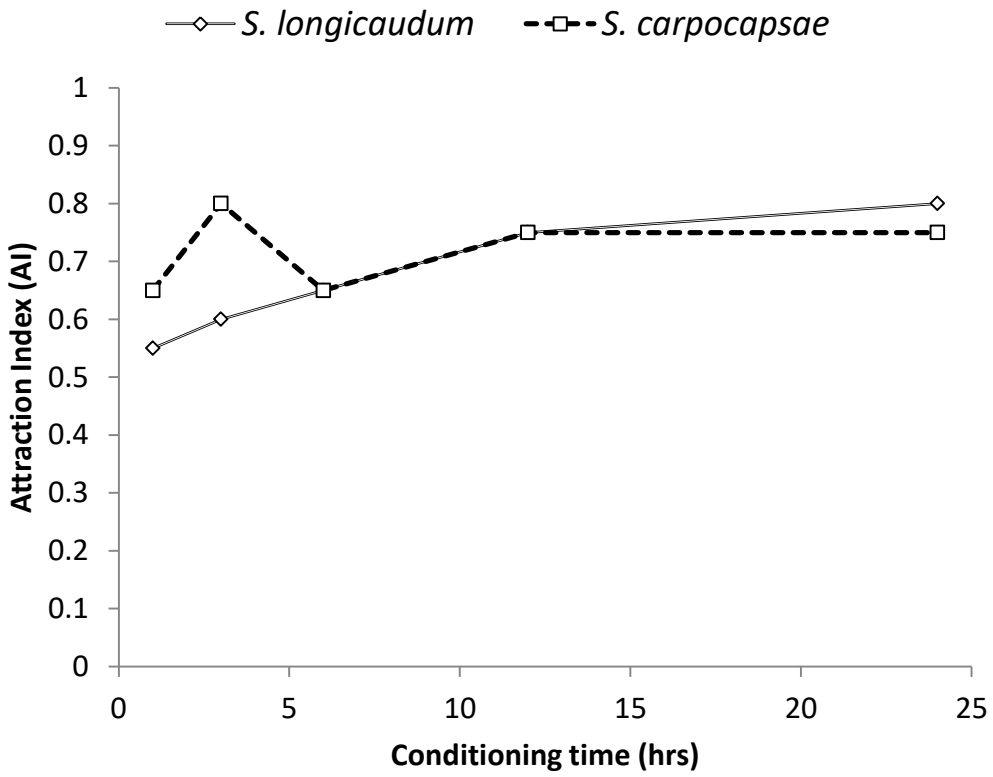


Figure 4.5: Degree of attraction of male *Steinernema* to PBS conditioned with 20♀/ml for varied times. N = 20 per time point, per species (single experimental replicate

Stability of female-produced attractant during storage at 4˚C was verified by storing a single batch of female conditioned PBS (20 ♀/ml) and testing for male attraction repeatedly for 3 weeks (Figure 4.6). A high degree of attraction (AI > 0.8) was maintained throughout 3 week storage in both test species (*S. carpocapsae* and *S. longicaudum*). Although there was little evidence of loss of activity by three weeks, all future experiments used female-conditioned PBS stored for a maximum of 2 weeks.

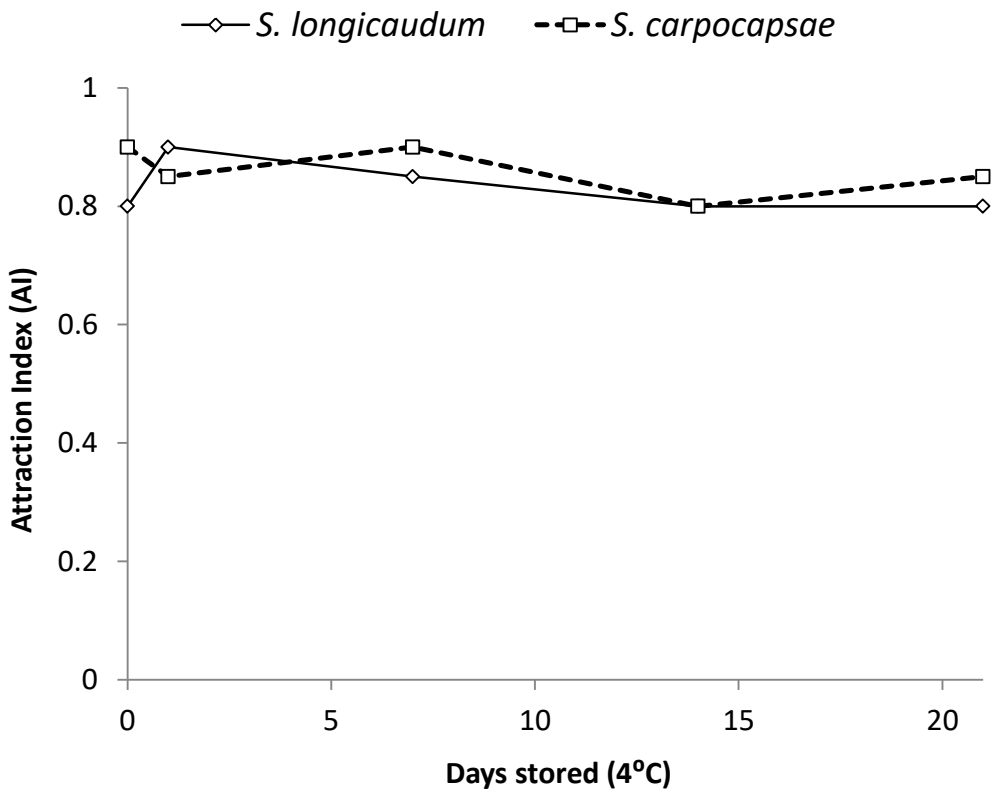


Figure 4.6: Degree of attraction of male *Steinernema* to PBS conditioned with 20 ♀/ml stored at 4˚C (0.2µm filtered) for varied time periods. N = 20 per time point, per species (single experimental replicate).

4.3.3 Influence of female sexual experience on production of attractant

In order to see whether mating influences the production of attractant by females, PBS was conditioned with females that had mated for defined time periods prior to the assay (Section 4.2.7). PBS conditioned with mated females was significantly less attractive to conspecific males than PBS conditioned with socially naïve virgin (unmated) females for both *S. carpocapsae* and *S. longicaudum*. Initial investigation showed that females mated for 24 or 48 h previous to assay were not attractive to males (Figure 4.7) in contrast to virgin females (One –way ANOVA, $p < 0.05$). Indeed, negative attraction indices were recorded to mated *S. longicaudum* (Figure 4.7). Further exploration showed that females became unattractive as quickly as 1 h post mating (Figure 4.8).

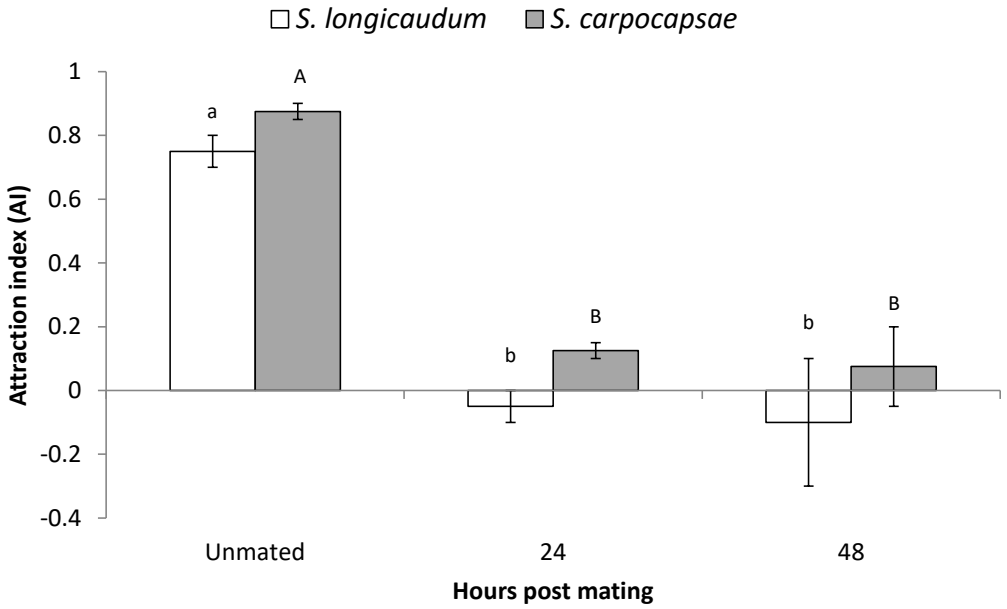


Figure 4.7: Degree of attraction of male *Steinernema* to PBS conditioned with either socially naïve virgin (unmated) or mated female conspecifics (20 ♀/ml). Bars which do not share a letter differ significantly (lower case for *S. longicaudum*, upper case for *S. carpocapsae*) One- way ANOVA, $p < 0.05$ (followed by Tukey *post hoc* test). N = 20, 2 experimental repeats per condition. Error bars show standard error of mean attraction index (AI).

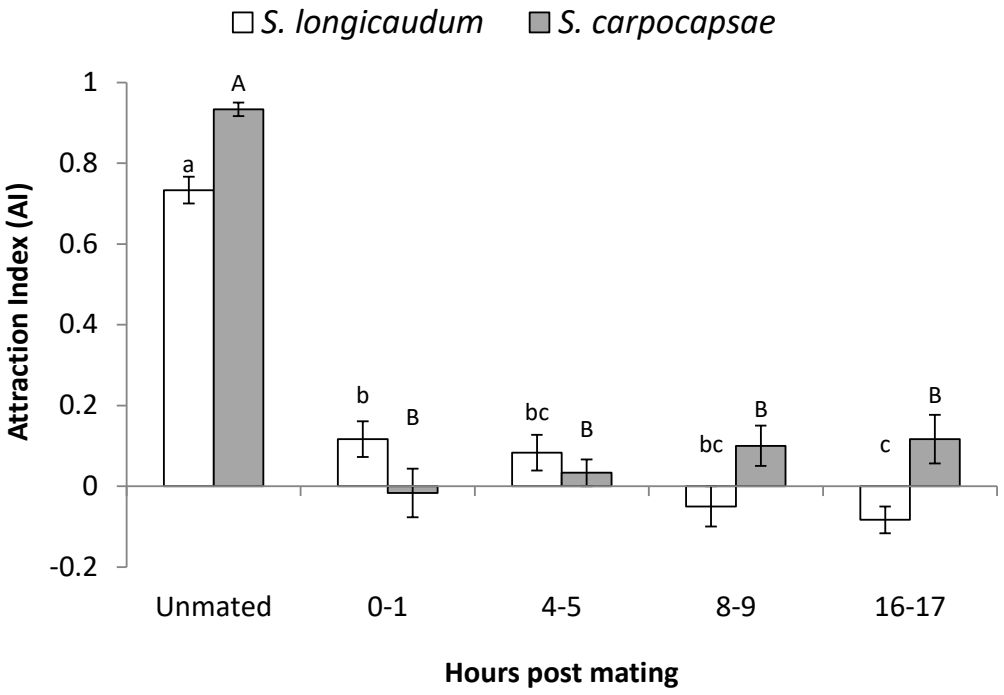


Figure 4.8: Degree of attraction of male *Steinernema* to PBS conditioned with either socially naïve virgin (unmated) or mated female conspecifics (20 ♀/ml). Bars which do not share a letter differ significantly (lower case for *S. longicaudum*, upper case for *S. carpocapsae*) One- way ANOVA, $p < 0.001$ (followed by Tukey *post hoc* test). N = 20, 3 experimental repeats per condition. Error bars show standard error of mean attraction index (AI).

4.3.4 Specificity of attractant: sex and species

PBS conditioned with adult worms dissected from insect cadavers (Section 4.2.5) was used to explore the attractiveness of males, females or a mix of both sexes to conspecific males and the species-specificity of female-produced attractants.

A dilution series of PBS conditioned with cadaver-dissected worms of both sexes (100 worms/ml with approximate 50:50 sex ratio) was assessed for potential to attract conspecific males. This mixed sex adult worm conditioned PBS was attractive to male conspecifics in both *S. carpocapsae* and *S. longicaudum* (Figure 4.9). The attraction index of 'neat' extract, containing approx. 50 ♀/ml was relatively high (AI > 0.7) but declined gradually with dilution, reaching an AI of 0 – 0.2 in a 1 in 10 dilution (containing 5 ♀/ml equivalent, Figure 4.9).

When the sexes were isolated from each other during PBS conditioning, only females were attractive to conspecific males. Male-conditioned PBS at equivalent worm density to female preparation was not attractive in either *S. carpocapsae* (Figure 4.10a) or *S. longicaudum* (Figure 4.10b). The difference in attraction according to stimulant sex was significant in all tested densities (Pearson Chi Square, $p < 0.01$). PBS conditioned by females only at 50 ♀/ml had a similar level of attraction to 'neat' mixed sex conditioned PBS that contained roughly an equivalent number of females (AI > 0.7 in all cases, Figures 4.9 and 4.10). As with the mixed-sex preparation, there was a slight decline in attractiveness with decreasing female density (Figure 4.10). The results showed that males are not attractive to other males (Figures 4.10a and b), and also that male presence (during conditioning of PBS with both sexes present) does not affect female ability to produce male attractant (Figure 4.9).

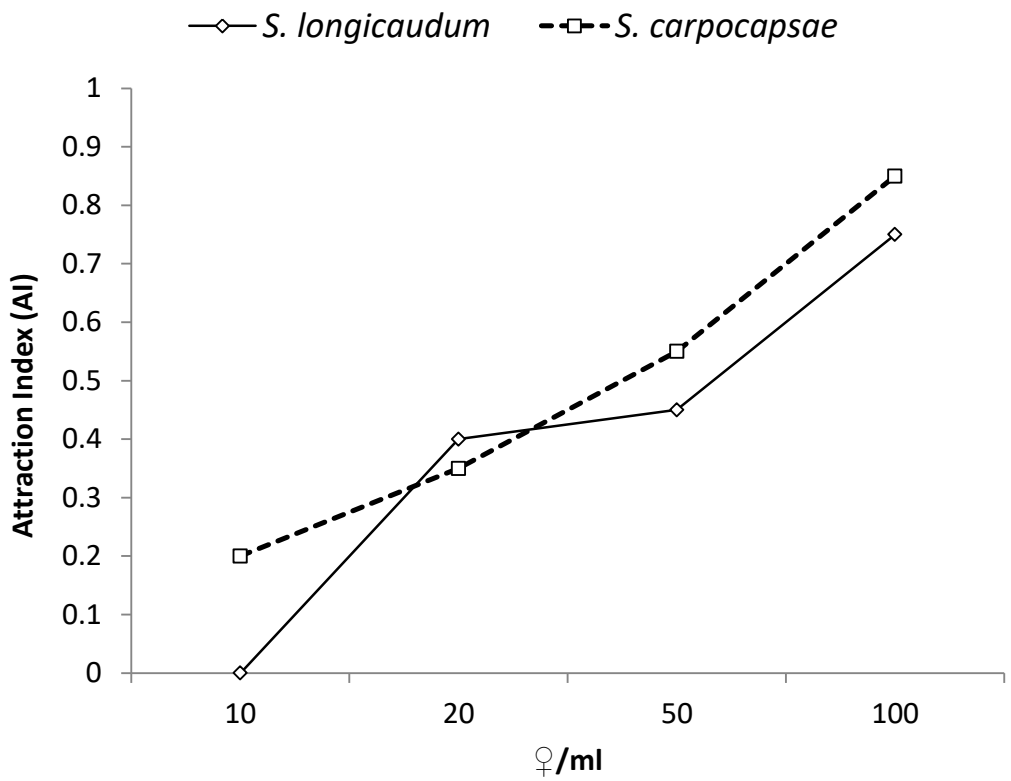


Figure 4.9: Degree of attraction of male *Steinernema* to dilution series of PBS conditioned with mixed sex cadaver dissected adult worms for 24 h. Neat preparation of 100 worms/ml with approximate 50:50 sex ratio. N = 20 per condition; one experimental repeat.

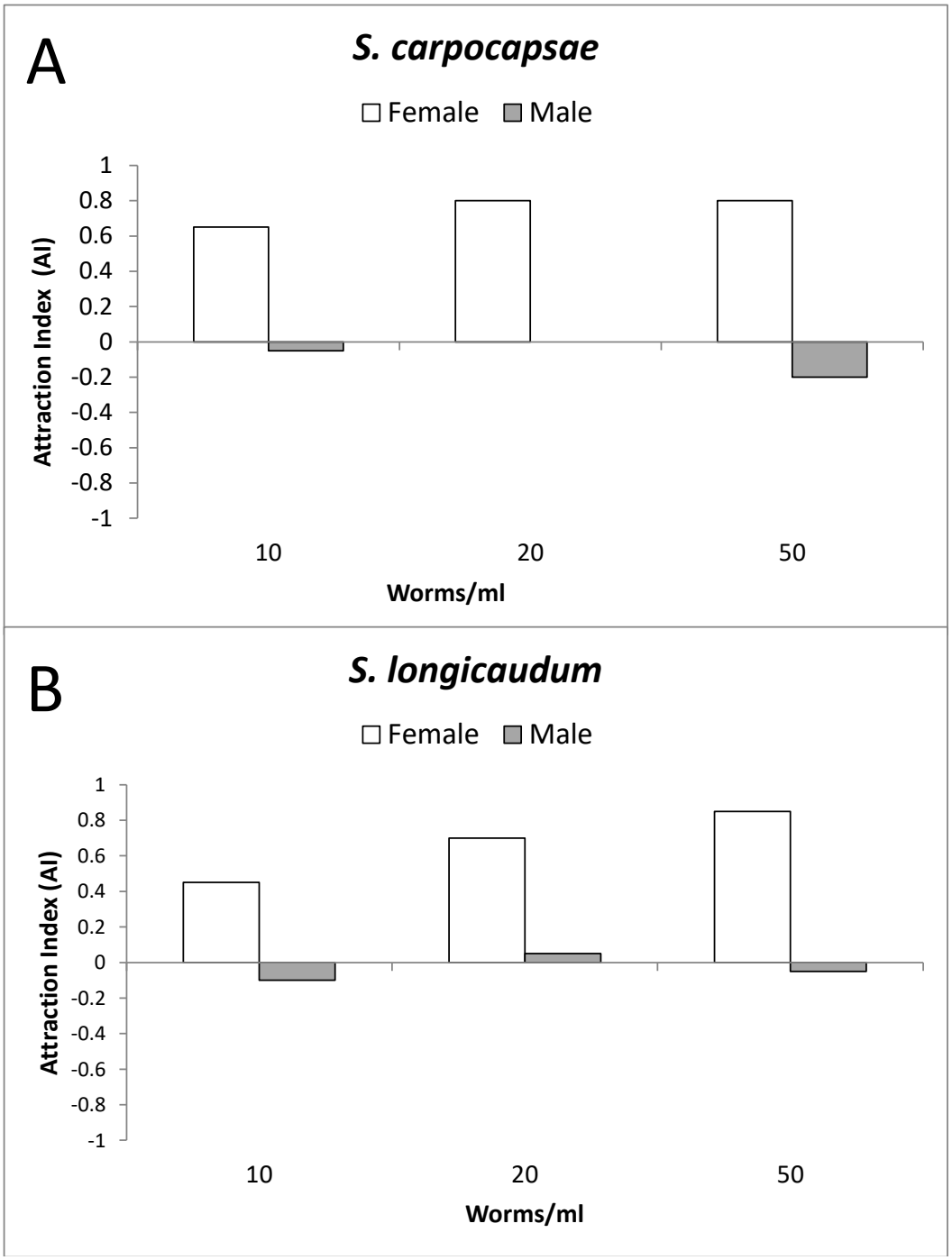


Figure 4.10: Degree of attraction of male *Steinernema* to PBS conditioned with either conspecific females or males dissected from cadavers at varied preparation densities for 24 h. Significant difference between male/female PBS ability to attract conspecific males at all densities (Pearson chi square on raw data at each worm density, $p < 0.01$). N = 20 per condition.

Species-specificity of female-produced male attractant was explored by presenting female-conditioned PBS to conspecific and heterospecific males in separate attraction assays. Male *S. longicaudum* were attracted to PBS conditioned with conspecific virgin females (20/ml) but not PBS conditioned with virgin female *S. carpocapsae* at the same density (Figure 4.11). A similar result was seen in *S. carpocapsae*, with males being attracted to PBS conditioned with conspecific virgin females but not heteropecific (*S. longicaudum*) females (Figure 4.11), showing that mate attraction signals in both test species are species-specific.

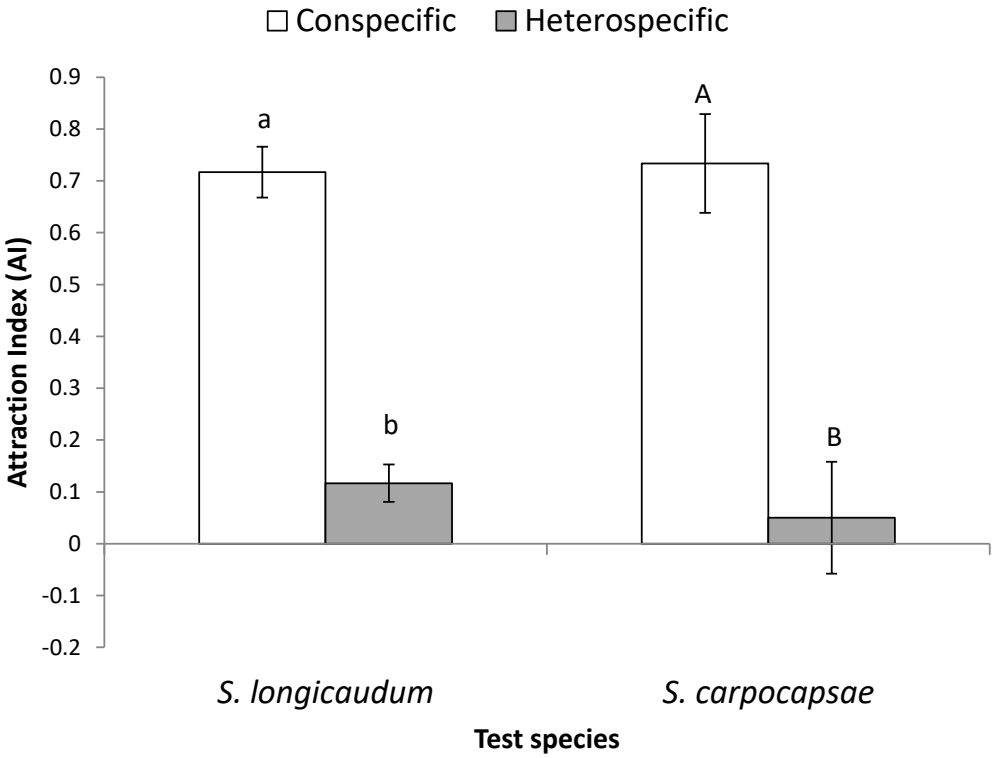


Figure 4.11: Degree of attraction of *S. longicaudum* and *S. carpocapsae* males to PBS conditioned with virgin female conspecifics (20 ♀/ml) or with each other's virgin females (20♀/ml). Means which do not share a letter (within test species) differ significantly (Two – sample t test, $p < 0.05$). N = 20, 3 experimental repeats. Error bars show standard error of mean attraction indices.

4.3.5 Detection and attractiveness of ascarosides in C18-extracted adult worm conditioned PBS

Experiments in this section were carried out using cadaver-dissected, mixed adult-conditioned PBS prepared according to methodology outlined in Sections 4.2.5 – 4.2.6. Chemical analysis and extraction of samples was carried out in collaboration with Dr Stefan von Reuss at the Max Planck Institute of Chemical Ecology, Jena, Germany and extracted samples returned to Maynooth University for behavioural assay (Section 4.2.8).

Using LC-MS/MS and NMR (Nuclear Magnetic Resonance Spectroscopy) analysis (Dr Stefan von Reuss), it was found that both *S. carpocapsae* and *S. longicaudum* adult-conditioned preparations of PBS contained several known ascarosides in varied concentrations (Tables 4.1 and 4.2). Most notable was the high relative concentration of ascr#9 in both species, a molecule confirmed previously to be produced by insect associated nematodes including several *Steinernema* species (Choe et al,. 2012b). Ascarosides #1 and #10 were found to be present in *S. carpocapsae* exudate (although in considerably lower amounts than the dominant ascr#9) also agreeing with previous findings of Choe et al. (2012b) (Table 4.1). Ascr#1 was also detected in *S. longicaudum* exudates (Table 4.2) alongside a number of uncharacterised shorter chain ascarosides in trace amounts.

Table 4.1: Ascarosides identified in *S. carpocapsae* adult (male and female) conditioned PBS (100 adult worms/ml, approx. 50:50 male:female sex ratio, dissected from *G. mellonella* 72 h post infection at 20˚C).

Ascaroside	Formula	Relative Concentration
#9	$C_{11}H_{20}O_6$	High
#1	$C_{13}H_{24}O_6$	Trace
#10	$C_{15}H_{28}O_6$	Trace

Table 4.2: Ascarosides identified in *S. londicaudum* adult (male and female) conditioned PBS (100 adult worms/ml, approx. 50:50 male:female sex ratio, dissected from *G. mellonella* 48 h post infection at 20˚C).

Ascaroside	Formula	Relative Concentration
#9	$C_{11}H_{20}O_6$	High
#10	$C_{15}H_{28}O_6$	Trace
Unspecified short chain ascarosides	-	Trace

Samples of ascr#9 (dried form) were provided by Dr Stefan von Reuss, along with information to enable resuspension to a concentration equal to that detected in the original adult worm exudate preparation (Sections 4.2.5 – 4.2.6). Attraction assays comparing resuspended full exudate extract with purified ascr#9 in the absence of all other chemical components were carried out for both test species (*S. carpocapsae* and *S. longicaudum*, Figures 4.12 and 4.13 respectively) to investigate the role of ascr#9 in attraction. Although ascr#9 was found in high quantities in both *S. carpocapsae* and *S. longicaudum* adult conditioned PBS (Tables 4.1 and 4.2

respectively), ascr #9 alone did not act as strongly as a male attractant in comparison to extract of the full adult worm conditioned PBS in both species (Figures 4.12 and 4.13). In *S. carpocapsae*, males were significantly less attracted to ascr#9 presented at concentration equal to that found in the original female-conditioned PBS than the extracted (resuspended) sample itself (Figure 4.12). However, when both stimulants were presented in concentrated form (x10), this difference was less apparent and marginally insignificant. The x 10 concentration of full extract was slightly more attractive than the original concentration, while the x10 of ascr#9 was no more attractive than the original. However, there was greater variation in response between repeat experiments for this treatment, which likely accounts for the fact that the difference between treatments approaches significance at the higher concentration.

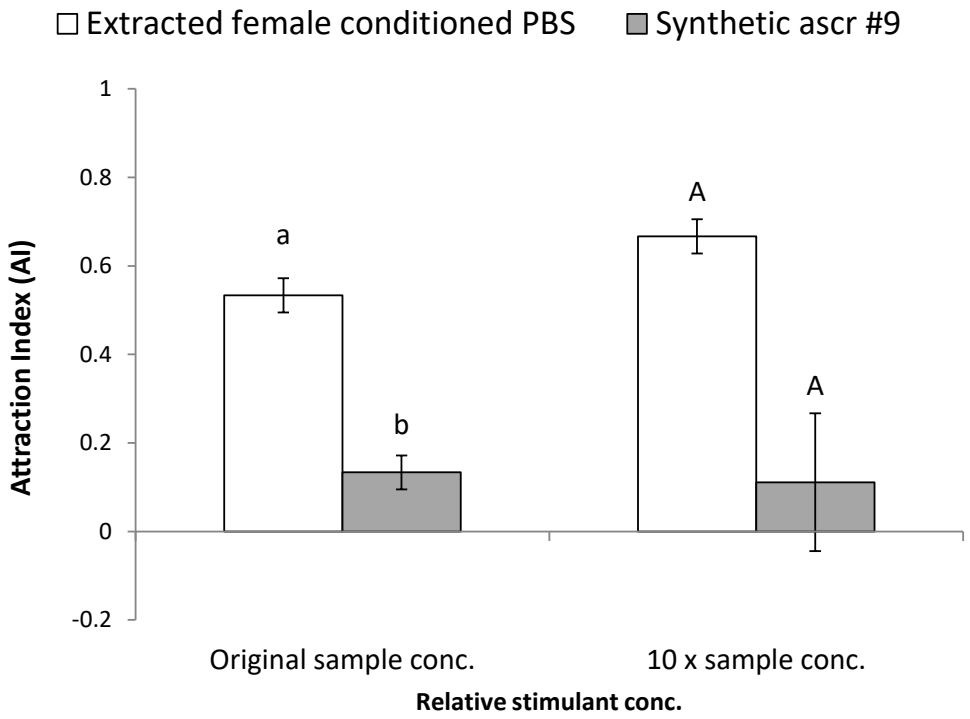


Figure 4.12: Degree of attraction of *S. carpocapsae* males to C18 extracted and resuspended conspecific adult conditioned PBS (100 adult worms/ml) and synthetic ascr#9 at concentration equivalent to that detected in worm conditioned PBS. N = 20, 3 experimental repeats. Means which do not share a letter differ significantly (Two sample t test, $p < 0.05$). Error bars show standard error of mean attraction index.

As in *S. carpocapsae*, *S. longicaudum* males showed a stronger attraction to extracted and resuspended conspecific adult conditioned PBS in comparison to purified ascr#9 alone (Figure 4.13). At a concentration equal to the original sample, the difference in attraction approached significance (Figure 4.13) while more concentrated stimuli (x 10 of original) saw a significant difference between the two (Figure 4.13). In fact, a negative AI (indicating repulsion) was recorded at the higher concentration.

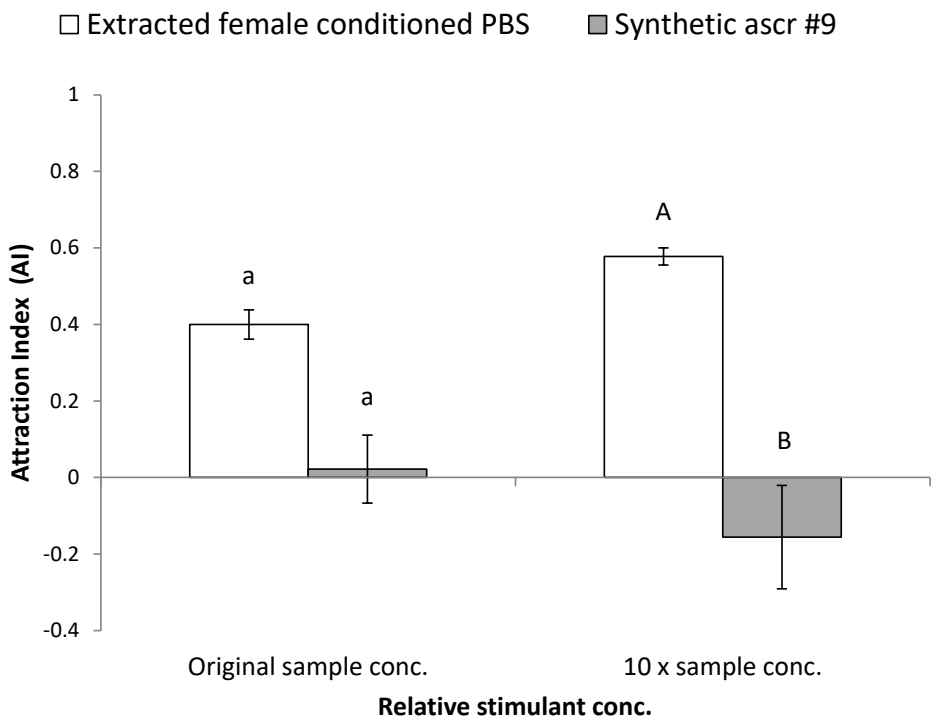


Figure 4.13: Degree of attraction of *S.longicaudum* males to C18 extracted and resuspended conspecific adult conditioned PBS (100 adult worms/ml) and synthetic ascr#9 at concentration equivalent to that detected in worm conditioned PBS. N = 20, 3 experimental repeats. Means which do not share a letter differ significantly (Two sample t test, $p < 0.05$). Error bars show standard error of mean attraction index.

4.3.6 Male attraction to C18-extracted and fractionated adult (male and female) conditioned PBS

PBS was conditioned in large amounts using adult *Steinernema* dissected from *G. mellonella* (see Section 4.2.5) and fractionated stepwise in increasing concentration of MeOH on a C18 SepPak column (Solid phase extraction and fractionation carried out by Dr Stefan von Reuss, see Section 4.2.8). The resulting fractions were dried, resuspended (in 5 % MeOH) and assayed for potential to attract conspecific males. Firstly, in both *S. carpocapsae* and *S. longicaudum*, the resuspended full adult conditioned PBS extract was less attractive than the original untampered sample with attraction index of 0.36 (Figure 4.14) and 0.38 (Figure 4.15), compared to 0.85 and 0.75 respectively, suggesting that the potential attractant may have been lost or compromised during sample processing. In *S. carpocapsae* (Figure 4.14), mean AI of the 20, 50 and 60 % MeOH eluted fractions differed significantly from the full extract (One-way ANOVA $p < 0.05$ followed by Dunnet *post hoc* comparison to 'Full extract'), in that they were significantly less attractive. All other fractions did not differ significantly from the full extract, although fractions eluted at 30, 70 and 90 % MeOH showed the most potential to attract males. Similar conclusions were drawn in *S. longicaudum* (Figure 4.15), mean AI of 40, 40 and 100% MeOH eluted fractions differed significantly from the full extract (One-way ANOVA $p < 0.05$, followed by Dunnet *post hoc* comparison to 'Full extract'). Fractions eluted from 10 – 30 % MeOH showed most potential as attractants, although not as potent as the full extract. It must be noted that due to low sample size (mean AI of 3 experimental repeats, N=20, per fraction), statistical analysis is unlikely to be robust and these results were primarily used to inform further assay development.

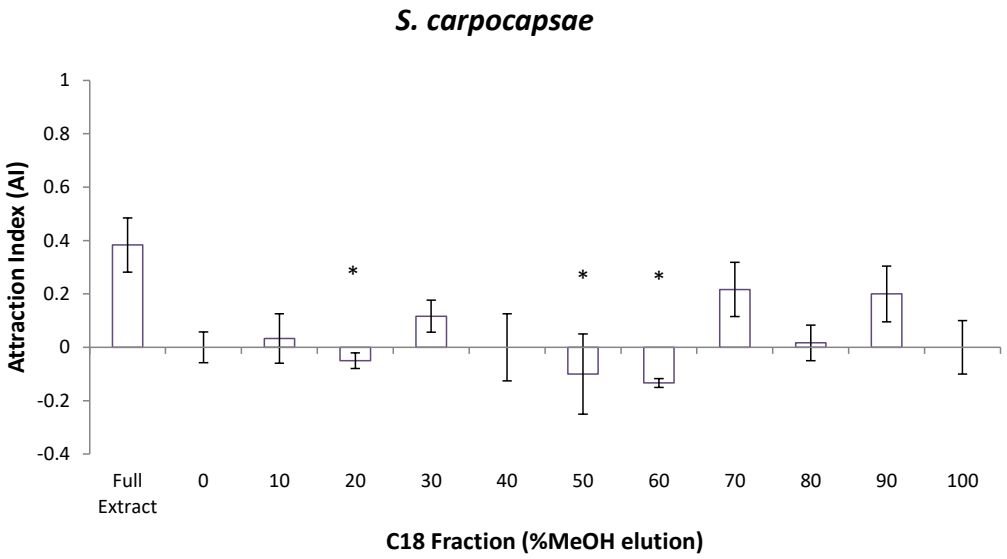


Figure 4.14: Degree of attraction of male *S. carpocapsae* to fractions of extracted adult *S. carpocapsae* exudate, resuspended in H_20 (5% MeOH). Concentrations of all resuspended fractions are equivalent to original sample. Means which differ significantly from 'Full Extract' are indicated by * (One- way ANOVA, $p < 0.05$ followed Dunnet test comparison to 'Full extract') Error bars show standard error of mean attraction index. N = 20 per condition, 3 repeats.

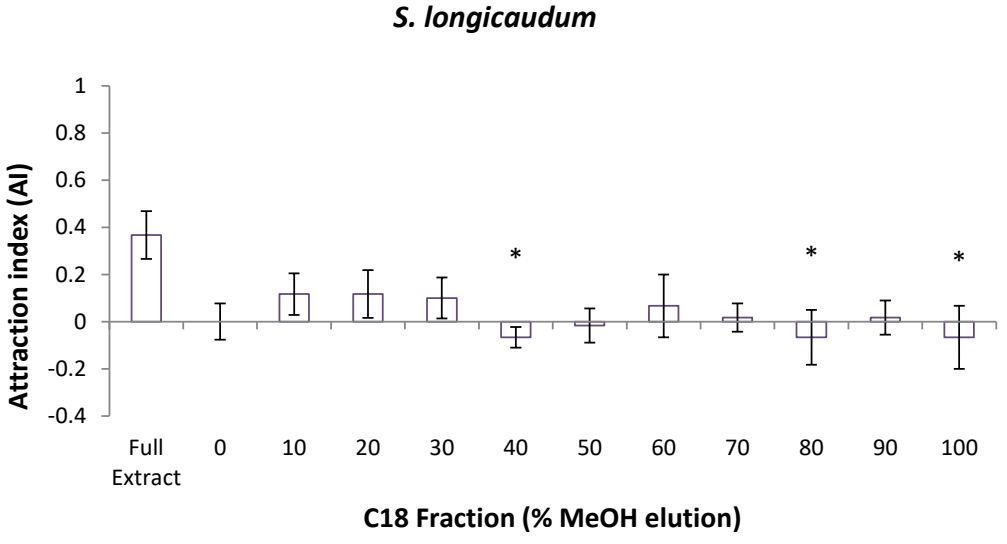


Figure 4.15: Attraction index of individual fractions of extracted adult *S.longicaudum* exudate, resuspended in H_20. Concentrations of all conditions are equivalent to original sample. Means which differ significantly from 'Full Extract' are indicated by * (One- way ANOVA, $p < 0.05$ follwed by Dunnet test comparison to 'Full extract'). Error bars show standard error. N = 20 per condition, 3 repeats.

Following initial screening of individual fractions, fractions were recombined (keeping concentration consistent with original extracted and resuspended conditioned PBS sample) in 4 different combinations (Table 4.3). These were assayed for strength of attraction alongside the unfractionated full sample extract for each species (*S. carpocapsae* and *S. longicaudum*), to investigate whether attraction potential could be restored after recombination of fractionated sample components (Section 4.2.8).

Table 4.3: Schedule for recombination of stepwise MeOH eluted fractions (C18 SepPak column) of adult *S. carpocapsae* and *S. longicaudum* conditioned PBS (100 adult worms/ml).

Stimulus	**Content (recombined fractions)**
Full extract	Un-fractionated adult conditioned PBS extract
All fractions recombined	All fractions eluted from 10 – 100% MeOH
A	Fractions eluted at 0, 10, 20 and 30% MeOH (Early)
B	Fractions eluted at 40, 50, 60 and 70% MeOH (Middle)
C	Fraction s eluted at 80, 90 and 100 % MeOH (Late)

Results were similar for both *S. carpocapsae* (Figure 4.16) and *S. longicaudum* (Figure 4.17). Degree of attraction was significantly lower in all fraction recombinations tested when compared to the unfractioned full extract for both species (One-way ANOVA $p < 0.05$, followed by Tukey pairwise comparison). But perhaps most notable is that recombination of ALL fractions did not fully restore degree of attraction seen in the full extract in either species, despite all components being present. This may be due to loss of content during the fractionation process or during resuspension.

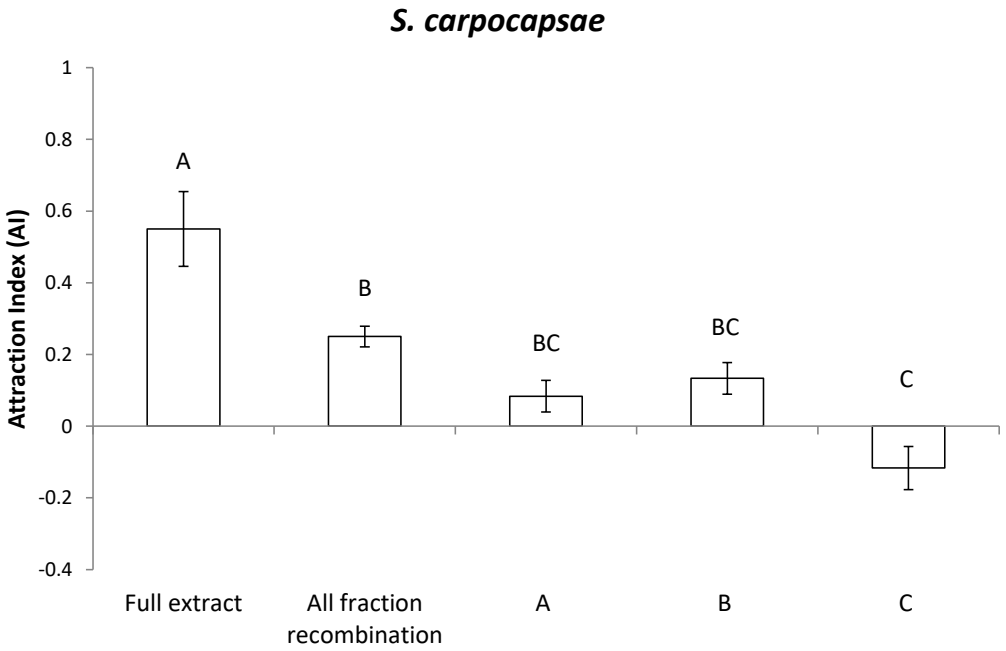


Figure 4.16: Attraction index of extracted adult *S. carpocapsae* exudate, resuspended in 5 % MeOH. Concentrations of all conditions are equivalent to original sample. 'A' = fractions 0% - 30% combined, 'B' = fractions 40% - 70% combined and 'C' = fractions 80% - 100% combined. Means which do not share a letter differ significantly (One way ANOVA $p < 0.05$, followed by Tukey pairwise comparison). N = 20 per condition, 3 repeats. Error bars show standard error of mean attraction index.

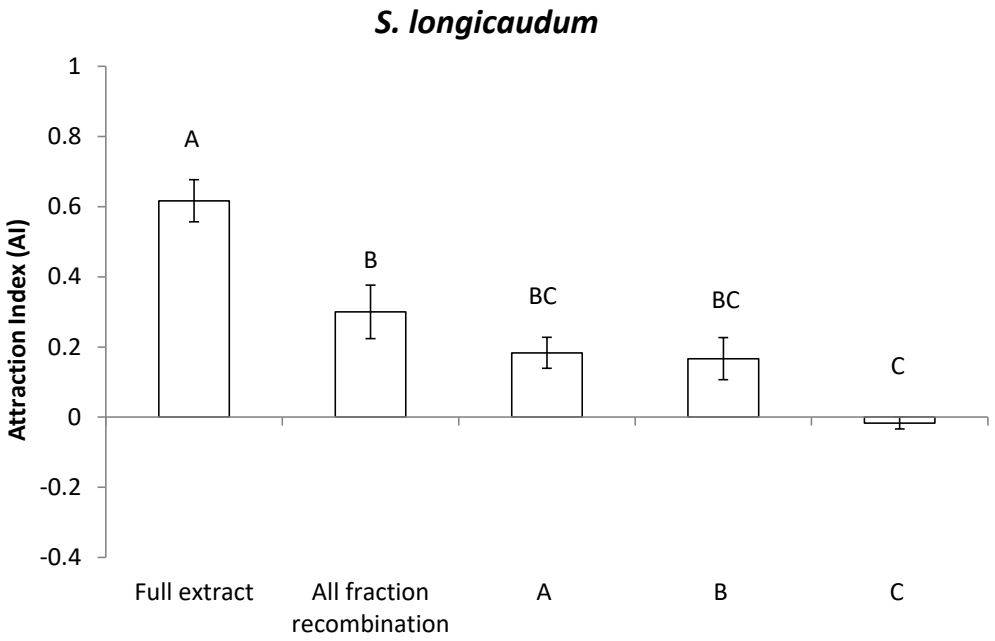


Figure 4.17: Attraction index of extracted adult *S. longicaudum* exudate, resuspended in 5 % MeOH. Concentrations of all conditions are equivalent to original sample. 'A' = fractions 0% - 30% combined, 'B' = fractions 40% - 70% combined and 'C' = fractions 80% - 100% combined. Means which do not share a letter differ significantly (One=way ANOVA $p < 0.05$, followed by Tukey pairwise comparison). N = 20 per condition, 3 repeats. Error bars show standard error of mean attraction index.

4.3.7 Attraction assay of C18-extracted virgin female-conditioned PBS

Following ambiguous results in Section 4.3.6, methodology was revised to minimise sample processing prior to extraction. The following experiments (Section 4.3.7) were carried out in house (Maynooth University) and used exudate of virgin females only (Section 4.2.4) as opposed to mixed adult population used previously. Furthermore, flow through collected after C18-extraction was retained and included in experiments both individually and in combination with extracted material to clarify whether this was where activity may have been lost (Section 4.2.9 for solid phase extraction and resuspension methodology).

Attraction potential of C18-extracted virgin female exudate in PBS (20 ♀/ml) was lower than the original unprocessed sample in both test species, *S. carpocapsae* (Figure 4.18A) and *S. longicaudum* (Figure 4.18B). This agrees with conclusions drawn in Section 4.3.6 that the extraction and resuspension process may compromise the attractant in some way. Novel to this experiment was the retention and behavioural assay of flow-through collected during the extraction process, which interestingly showed a similar level of attraction to extracted material for both species (Figures 4.18A-B). This suggested that some chemical components responsible for male attraction may not bind to the C18 column used. Retentate and flow-through were recombined with the expectation that full activity would be restored, however this was not the case and activity was only partly restored in *S. carpocapsae* (Figure 4.18A) and slightly more so in *S. longicaudum* (Figure 4.18B).

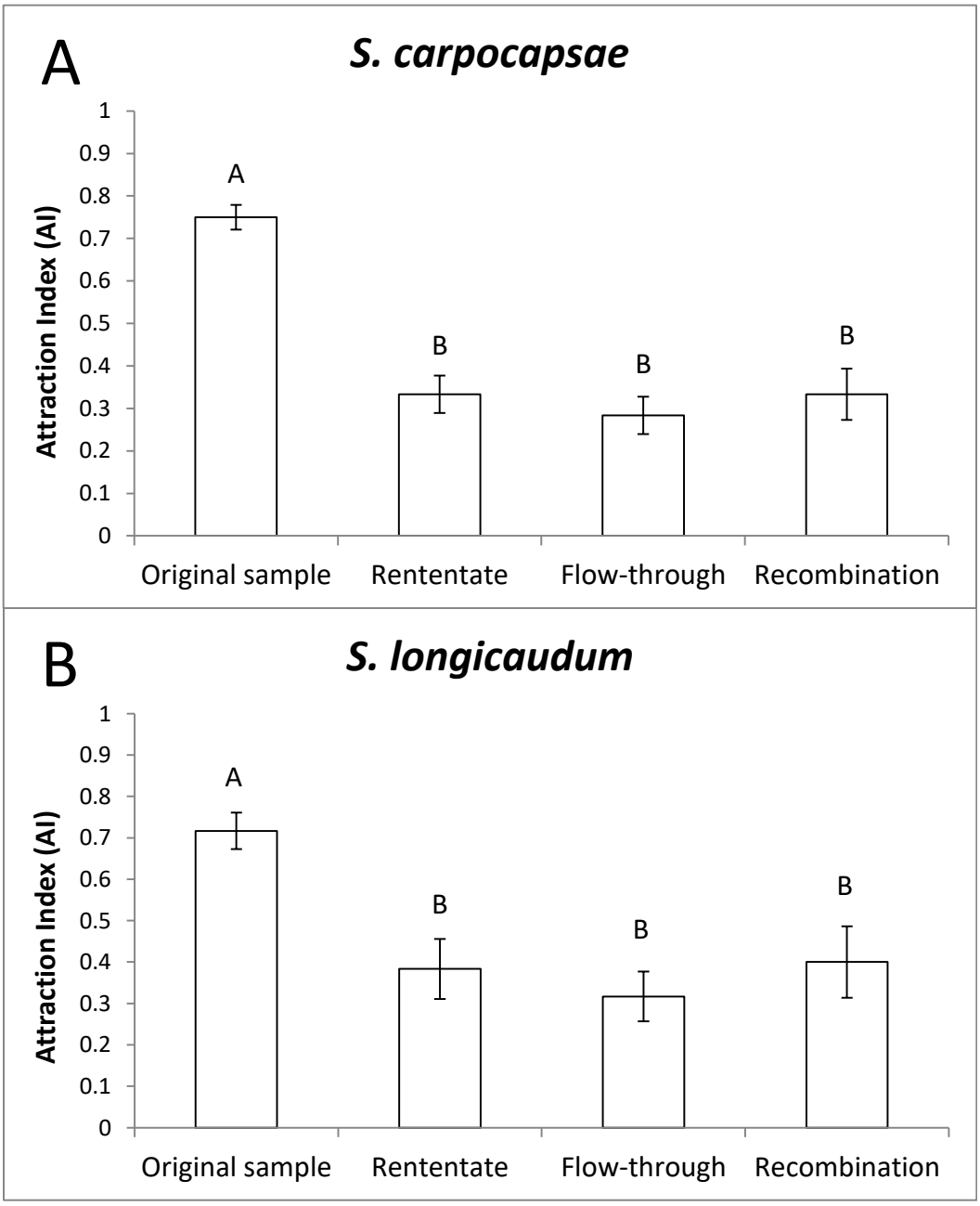


Figure 4.18: Attraction index of virgin female *S. carpocapsae* conditioned PBS (20♀/ml) extracted with 100 % MeOH on a C18 SepPak column (resuspended in PBS). Concentrations of all conditions are equivalent to original sample. Means which do not share a letter differ significantly (One-way ANOVA $p < 0.05$, followed by Tukey pairwise comparison). N = 20 per condition, 3 repeats. Error bars show standard error of mean attraction index.

4.4 Discussion

The knowledge that multiple IJ of different EPN species may enter the same insect host (Stuart and Gaugler, 1994) poses the challenge of reproductive isolation between species. Species-specific mate recognition and attraction between sexual partners via chemical means enables such reproductive isolation within the small physical constraints of a single insect host. Lewis et al. (2002) showed that *S. carpocapsae* males are not attracted to *H. bacteriophora* females, however this is not surprising as the two species are not closely related. Here I show that species-specificity of sex pheromones is also apparent in more closely related species within the *Steinernema* genus. Males of two *Steinernema* species are attracted to exudate of conspecific females, and not to conspecific males or heterospecific females. This confirms previous reports that *S. carpocapsae* males respond to chemical signals produced by conspecific females (Neves et al., 1998), but is the first evidence for a female sex pheromone in *S. longicaudum* and within-genus specificity.

Pheromonal reproductive isolation is perhaps best studied in insects, where moths in particular provide some of the most informative studies to date on sex pheromone discrimination between closely related species (Smadja and Butlin, 2009). Rather than each species using chemically distinct molecules as mate attractants, female pheromones commonly occur as a blend of several chemicals, with species specificity achieved through variation in concentration of multiple components or by minor variation in structure of pheromone compounds (Gomez-Diaz and Benton, 2013). For example, females of two different strains of the corn borer moth (*Ostrina nubilalis*) both produce sex attractants which are comprised of two isomers of 11-14:acetate (Lassance, 2010). However, the ratio in which these two isomers are

produced in each *O. nubilalis* strain is very different, allowing males to identify females of their own strain. There is increasing knowledge of species-specificity in nematodes, some of which indicates that species-specicifity is not always absolute. Bioassays on the attraction of males to females in 10 different species of the plant parasitic *Heterodera* genus showed that most females attracted more than one species of male and most males also responded to females of multiple species (Lee, 2002).

Chasnov et al. (2007) reported that females of two dioecious *Caenorhabditi*s spp. (*C. remanei* and *Caenorhabditis* sp. strain CB5161) secreted a pheromone that attracted male conspecifics but also males of three other *Caenorhabditis* spp. (Chasnov et al., 2007). In depth screening of adult exudates produced by eight *Caenorhabditis* spp. of the elegans clade showed that although all produced similar indole ascarosides (icas), the ratio and blend of components differed between species potentially enabling species-specificity (Dong et al., 2016). However, these authors did not test whether these components of the pheromone blend conferred species-specificity when combined with other ascarosidal components (i.e. not indole derived components). Surprisingly, *C. elegans* males were found to chemotax towards areas conditioned by *Panagrellus redivivus* females but were not attracted by females of *S. carpocapsae*, *Pristionchus pacificus* or *Pelodera strongyloides* (Choe et al., 2012a). More investigation regarding the species-specificity of nematode sex pheromones in sympatric species which share the same ecological niche is required for ecological relevance as it is expected that there should be a greater degree of specificity where species occur together naturally.

The sexual history of females in both *S. carpocapsae* and *S. longicaudum* was found to influence their potential to attract conspecific males. Males were strongly attracted to virgin female exudate but not to exudate collected from females as soon as 1 h post mating and up to 48 h after. Lewis et al. (2002) reported similar findings for *S. carpocapsae*, showing that mated females were not attractive, but virgins were. However, they did not investigate how soon after mating female attractiveness was lost. Examples of reduced 'sex appeal' post mating have been documented in other nematodes, most notably in *C. elegans* whereby males are significantly less attracted to hermaphrodites containing active spermatozoa (either self -produced or following insemination) than those which are sperm free (Morsci et al., 2011; Leighton et al., 2014). Leighton et al. (2014) showed that successful contact between sperm and oocyte in the spermatheca of *C. elegans* hermaphrodite suppresses the production of a volatile pheromone which attracts males, rendering the hermaphrodite temporarily unattractive. Loss of attractiveness in females soon after mating has also been reported in the dioecious *C. remanei* (Chasnov et al., 2007) however, it was shown that female attractiveness was almost fully restored 24 h post mating. There are three main mechanisms whereby female mating status may be advertised chemically, allowing for males to distinguish between mated and unmated females (Thomas, 2011). Firstly, females may begin producing male repellent pheromones post-mating. Secondly, there may be a cessation in production of male attractant by the female as a result of mating. Lastly, and most commonly, males themselves may render the female unattractive through the application of repellent chemicals via direct contact or the transfer of ejaculate or mating plugs (Section 1.2.2). Reduced receptivity of females post mating is commonly seen in many moth species resulting

from mating plugs or molecules transferred in the seminal fluid of males which suppress production of female sex pheromones (Wedell, 2005).

The degree of male discrimination between females according to sexual history and mating status is often dependent on whether the female of a species mates once or multiple times (Thomas, 2011). In species where the female mates only once, there may be a benefit to both male and female parties if males are able to distinguish between mated and non-mated females in that males can avoid wasting energy and resources (e.g. sperm) by courting and copulating with unreceptive females, while already mated females may avoid unnecessary copulation which may be energetically costly (Thomas, 2011). Reduction in hermaphrodite or female lifespan post mating has been shown in several nematode species including *C. elegans* (Gems and Riddle, 1996), *C. remanei* (Diaz et al., 2010) and *P. redivivus* (Duggal, 1978). As well as physical damage to the cuticle inflicted by the male during mating (Woodruff et al., 2014), reduced female lifespan after mating has been suggested to be a result of exposure to male pheromone and transfer of seminal fluid (Leighton and Sternberg, 2016). While males can mate repeatedly (Ebssa et al., 2008), it is unknown whether *Steinernema* females remate or not. However the occurrence of *endotokia matricida* (Baliadi et al., 2009) considerably shortens the female life presenting challenges for repeated mating events.

The concentration of females used for preparation of worm-conditioned media had an effect on its ability to attract males, higher densities elicited stronger attraction responses. Similarly, media prepared with high density females became less attractive when diluted. Differences between how media was prepared (i.e. either using actual worms at varied density for each treatment or a dilution series of media conditioned with high density of worms) may present considerations for direct

comparisons of results from different experiments. For example, little difference in attractiveness was seen between PBS conditioned with 20 ♀/ml or 10 ♀/ml in both *S. carpocapsae* and *S. longicaudum* (Figure 4.4). However, in a dilution series of haemolymph (originally 100 ♀/ml), attractiveness declined by around 50% from 20♀/ml to 10♀/ml equivalent in both species (Figure 4.3). This poses questions of whether there are density dependent effects on pheromone production or release and subsequently whether dilution of conditioned media provides a true representation of actual worms/ml with regards to presence of pheromone. Modification of pheromone production dependent on density is seen in other species, such as the bark beetle *Ips pini*, which produce an aggregation pheromone to recruit conspecifics to a food source (Borden, 1989). However, pioneering individuals are disadvantaged with respect to competition for food if density becomes too high which leads to density-dependent modification in the emitted pheromones to prevent further recruitment (Schlyter et al., 1989). Density-dependent effects on pheromone production have also been shown in nematodes where *C. elegans* males increase ascaroside production in the presence of other males (Izrayelit et al., 2012). Both *S longicaudum* and *S. carpocapsae* males were attracted to PBS conditioned with 20 ♀/ml for only 1 hour and degree of attraction became only mildly stronger when PBS was conditioned for longer periods (Figure 4.5). These results suggest that female produced male attractant is exuded constantly and accumulates relatively quickly in the surrounding medium. Males of both test species were only attracted to medium conditioned with females and not males (Figure 4.10), confirming that the attraction cue is sex specific. This finding informed the decision to used mix sex populations dissected from cadavers to condition large amounts of PBS where required as males themselves do not produce a signal that either attracts or repels other males.

However, it was observed that mating occurred during conditioning with mixed sex adults. As stated previously mating reduces female attractiveness so could limit the amount of pheromone produced/exuded. Mixed sex preparations were used primarily to accumulate larger amounts of exudate required for chemical analysis and as mated females were found to be unattractive, but not repulsive (or maybe only slightly so), mating during conditioning was deemed unlikely to impact results. Mating during mixed sex conditioning could have been minimised by reducing the time period of media conditioning from 24 h to < 3 h as it had already been confirmed that media conditioned with female for a short period still elicted a strong attraction response in males.

Both test species (*S. carpocapsae* and *S. longicaudum*) were found to produce relatively high levels of ascr#9, consistent with reports that ascr#9 is typically exuded by insect-associated nematodes in high amounts (Choe et al., 2012b). However, each produced different profiles of other short chain ascarosides (Tables 4.1 and 4.2) alongside the main ascr#9 component. *S. carpocapsae* adults were found to exude ascr #1 and #10 in low abundance, consistent with findings of Choe et al. (2012b), while *S. longicaudum* adults (previously not investigated) were shown to also exude low amounts of ascr#10 along with a number of other unspecified short chain ascarosides. Ascr#9 alone was only slightly attractive to males of either species, and was even repulsive to male *S. longicadum* males when presented at a concentration ten times higher than that detected in the original exudate. Chaudhuri et al. (2015) found that female *Rhabditis* sp. SB347 also produce ascr#9 and that males were attracted to synthetic formulation of similar concentration to that produced naturally by females (picomolar). However, they further report that high concentrations of ascr #9 (nanomolar) are actually repulsive to males, similar to

results shown here for *S. longicaudum*. Choe at al. (2012b) report that EPN characteristically produce ascr #9 during both juvenile and adult life stages, therefore is is not likely to be involved in mate attraction on its own (i.e. not specific to species without other components)**.** The finding that ascr#9 on its own was not attractive to males of either test species shown here along with the identification of other ascaroside molecules in adult exudates, suggest female-produced male attractants in *S. carpocapsae* and *S. longicaudum* are composed of a specific blend of molecules. It would be apt to test the attraction potential of species-specific ascarosides identified here, both individually and in combination, to further explore the role ascarosides may (or may not) play in mate attraction in *S. carpocapsae* and *S. longicaudum.*

The apparent failure to restore full attraction potential of extracted adult exudate to levels recorded in unprocessed exudates in both *S. carpocapsae* and *S. longicaudum* suggests loss of one or more pheromone components during the extraction process. Although uncommon, there has been evidence that volatile chemicals may play a role in sexual attraction in nematodes (Lee, 2002; Leighton et al., 2014). Attraction of male *C. elegans* to hermaphrodites is due to the detection of a volatile pheromone, production of which is suppressed by the simultaneous presence of mature sperm and activated oocytes in the spermatheca just before fertilization, (Leighton et al., 2014). As previously stated, pheromones often exist as blends and efficacy is highly dependent on synergy and concentration of multiple components (Wyatt, 2003). This presents difficulties in assay-guided fractionation of potential pheromone blends, as incorrect balance of components, however minor, could have implications for behavioural response (Butcher, 2017). This is shown experimentally in *C. elegans* where ascr#9 can either attract or repel males depending on the concentration that it

is presented in (Chaudhuri et al., 2015). Both *S. carpocapsae* and *S. longicaudum* males showed some degree of attraction to material that did not successfully bind to the C18 column used for extraction ('flow-through'). This suggests that some components of the pheromone blend may be highly polar or small molecules and so could have been lost during the extraction process. However, recombination of 'flow-through' with extracted material did not restore full activity of original unextracted exudate in either species. This poses the question of whether, like *C. elegans* (Leighton et al., 2014) there may be a volatile component of *Steinernema* sex pheromones which would also be lost during in extraction.

Although it was shown that male exudate does not attract other males, it was not addressed whether males produce a pheromone which attracts females. Although less well studied than female-produced male attractants, there are some examples of male nematodes producing pheromones which attract mates (Izrayelit et al., 2012; Choe et al., 2012a; Chaudhuri et al., 2015). Male *C. elegans* produce a sex-specific blend of ascarosides, the major component of which, ascr#10, strongly retains and attracts hermaphrodites (Izrayelit et al., 2012). Chaudhuri et al. (2015) showed that virgin but not mated female or hermaphrodite *Rhabditis* sp. SB347 were attracted to supernatant collected from exclusively male cultures. In *Panagrellus redivivus* males, but not females, produce the female-attracting ascr#18 derivative, dhas#18 (10-(3′,5′-dihydroxy-6'-methyltetrahydro-pyran-2'-yloxy)-3,8-dihydroxy-undecanoic acid), further confirming the occurrence of male produced mate attractants in varied nematode species (Choe et al., 2012a). Female *Steinernema* were found to be considerably less active than males and so were not used in attraction assay in this study. Further examination is required to ascertain wherher male *Steinernema* produce mate attractants.

All males used for assay in this study were confirmed as mature (sperm present) prior to use in experiments, as preliminary testing showed immature males (adult but unmated and containing no sperm) to be less responsive to females and female exudates (data not shown). White et al. (2007) report that, in *C. elegans*, the male nervous system undergoes dramatic remodeling during the L4 stage to form adult connections. They further suggest that it is likely that attraction sensory circuits are wired at this point of development. This implies that the ability of nematodes to detect and subsequently respond to sex pheromones may be linked to developmental stage.

Chapter 5 Sexual Development

5.1 Introduction

Unlike releaser pheromones which are fast-acting and initiate changes in behaviour (attraction, aggregation etc.), primer pheromones cause developmental responses and changes in physiological state (Wyatt, 2003). Once detected, they induce changes in levels of substances produced by the endocrine system in the receiver which in turn triggers the onset of important developmental processes such as sexual maturation.

The effects of male pheromones on the stimulation of female sexual development are widespread across the animal kingdom and include species from insects to domesticated livestock such as sheep and cattle (Rekwot et al., 2001). Amongst helminths, male presence also plays a vital role in the induction of female sexual maturation in platyhelminth parasites of the genus *Schistosoma.* The definitive hosts are mammalian, including humans, where the adult stage of the life cycle and sexual reproduction occur (Moné and Boissier, 2004). Schistosomes are unique in that they are the only digenean parasites which are dioecious, not hermaphroditic. They therefore face challenges in identifying a suitable partner of the opposite sex and synchronizing development of sexual structures in order to reproduce successfully. Schistosomes have an unusual mating strategy whereby the male guards the female, holding her in a structure known as gynaecophoral canal (Fulford et al., 1995). Although partner switching is possible, they are typically monogamous and remain coupled with the same partner in the host blood stream for life (Popiel and Basch, 1984). The sexual development of female schistosomes is highly dependent on close male contact, which is required for correct development of the ovaries and vitelline

gland (Shaw, 1987). Moreover, there have been reports of female absence causing differences in male behaviour, physiology and antigenic development (Lo Verde et al., 2004). It has been suggested that induction of female maturation may be due to increased nutrient uptake as a result of a massaging effect associated with actual physical contact from the male (Gupta and Basch, 1987) but also that it is caused by the transfer of biomolecules or chemical cues between partners (Ribeiro-Paes and Rodriguez, 1997).

Studies documenting the reverse phenomenon whereby female chemical exudates stimulate sexual development in males are less common. Examples of female driven modulation in sperm production are seen in insects (Bjork et al., 2007), rodents (Koyama and Kanimura, 2000; Taylor et al., 1987) and teleost fishes (Kobayashi et al., 2002), though for the most part these relate to modulating the amount of sperm produced in relation to social environment, and the stimuli responsible are unknown. Males of several cyprinid species have been shown to increase milt production in the physical presence of females and more importantly when exposed to female odour only (Stacey et al., 2012). In goldfish (*Carassius auratus*), exposure of males to female steroidal sex pheromones triggers sustained elevation of luteinizing hormone (LH) in male blood plasma within just 15 min of exposure, resulting in increased sperm production (Kobayashi et al., 1986). As female *C. auratus* complete vitellogenesis, increased LH levels in the blood induce the production of progestins which stimulate maturation of eggs. These compounds are also released externally, acting as a primer pheromone for sperm production in males (Kobayashi et al., 2002). Sperm production is also increased further in response to prostaglandins released by females at the time of ovulation, ensuring sperm quantity and quality is at an optimum at the time when it is required for fertilisation (Kobayashi et al.,

2002). Further evidence of increased sperm production following male exposure to female pheromones associated with ovulation has been recorded in the crucian carp, *Carassius carassius* (Olsén et al., 2006), common carp, *Cyprinus carpio* (Stacey et al., 1994) and more recently rudd, *Scardinius erythrophthalmus* (Stacey et al., 2012).

In nematodes, Ebssa et al. (2008) showed that female presence is required to induce the production of sperm in males in the entomopathogenic nematode, *S. longicaudum* (Ebssa et al., 2008). Socially naive *S. longicaudum* reared in isolation did not contain sperm in their seminal vesicle. Similarly, males that only experienced social contact with other males also did not develop sperm. The presence of sperm was only recorded when males were paired with females, with macrosperm being visible in the seminal vesicle in 65% of cases after 24 h of contact with a female and 100% of cases after 48 h of contact. The authors also demonstrated that actual physical contact between sexual partners was not necessary to induce sperm production and that single males with no previous social experience, separated from female conspecifics by a permeable barrier also underwent the maturation with regards to sperm production. This implies a pheromone may be involved in inducing the male maturation, a process that is unusual in nematodes.

S. feltiae males and females have been reported to survive alone within the host for periods of up to 6 weeks post first infection (Rolston et al., 2006) before a suitable sexual partner arrives and reproduction can commence. It is therefore important that sexual partners time sexual maturation correctly and avoid detrimental effects associated with maturing too early or too late. For example, premature production of sperm in males if a female is not present is energetically costly and could cause sperm to be of suboptimal quality as a consequence of aging (Reinhardt, 2007);. The

negative consequences associated with sperm cell senescence (aging) are discussed in more detail in Section 1.2.2.

5.1.1 Chapter aims and objectives

This chapter addresses how *Steinernema* spp. utilise chemical signals to synchronise sexual development in preparation for mating. As *Steinernema* may be lone occupants of a host for several weeks, it is important that maturation of both sexes is correctly timed to avoid wasting energy on reproductive development prematurely. There is also risk associated with producing gametes prematurely as they are susceptible to degradation with age and may not be of optimum quality at the time of fertilization. There is already evidence that females of one species of *Steinernema* (*S. longicaudum*) produce a male maturation signal (Ebssa et al., 2008) which induces sperm production in males only when female presence is detected. This will be further characterised along with the examination of maturation signals in an additional species, *S. scarpocapsae*. It is expected that chemical signals differ between species where recognition is important (such as in inducing maturation in sexual partners), therefore species specificity of maturation signals within the *Steinernema* genus will also be explored. Solid Phase Extraction (SPE), High Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) will be used to try to identify chemicals responsible for maturing conspecific males and highlight differences between *S. carpocapsae* and *S. longicaudum* chemical profiles.

Specific objectives are to:

1. Test whether male *S. carpocapsae* are dependent on female pheromone for sexual maturation, as demonstrated previously in *S. longicaudum* (Ebssa et al., 2008).

2. Determine optimal parameters, such as female status and density, for producing female-conditioned exudate effective for maturing males.

3. Test species-specificity of primer pheromone for maturation of male *Steinernema* spp.

4. Test whether ascr #9 previously shown to be exuded by *S. carpocapsae* and *S. longicaudum* (Chapter 4) is biologically active with respect to inducing maturation in males by bioassaying synthetic ascr #9.

5. Use LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry) to screen both female and male exudates for differences in chemical composition.

6. Use assay-guided fractionation of female-conditioned extract on C18 columns to identify fractions (or combinations of fractions) which cause males to mature (see Section 1.6.4 for further detail).

5.2 Methodology

5.2.1 Visual identification of male sexual status

The reproductive tract of *Steinernema* males is a simple tube consisting of the testis (where sperm are produced), the seminal vesicle (where mature sperm are stored) and the vas deferens which leads to the cloaca (where sperm is released) (Ebssa et al., 2008). Both *S. longicaudum* and *S. carpocapsae* produce macrosperm (Spiridonov et al., 2009), which can be easily identified as large spheres within the seminal vesicle when the worm is mature (Figure 5.1). The reproductive tract in immature males is narrower and macrosperm are absent (Figure 5.1). The maturation status of male nematodes was assessed by mounting single males on slides beneath a bridged cover-slip. Presence of sperm could generally be viewed at 10x magnification, but higher levels of magnification were used when necessary. The immature status of all males used for experimentation was confirmed via microscopic observation prior to use.

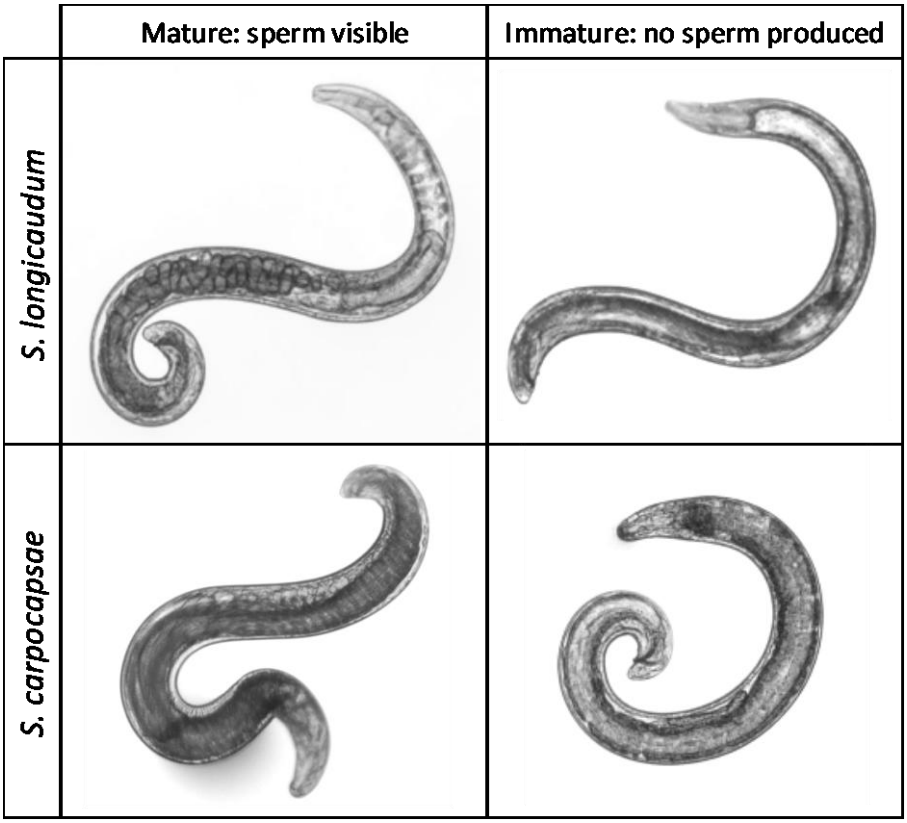


Figure 5.1: Mature and immature male *S. carpocapsae* and *S. longicaudum* showing macrosperm visible in the seminal vesicle (left panels) in sexually mature specimens.

5.2.2 Maturation assays

5.2.2.1 Insect haemolymph

Maturation assays in insect (*G. mellonella*) haemolymph were all carried out with socially naïve adults reared in isolation in hanging drops (Section 2.3). Where live females were used for assay, males were transferred from their own drop using platinum wire (0.2 µm diameter) into a female's. Pairing was always carried out approximately 24 h after worms developed into visibly recognizable males and females, generally 5 days old for *S. longicaudum* and 6 days old in *S. carpocapsae*. Newly created pairs were returned to incubation (see Table 2.1) for 24 h before visually assessing maturation status (Section: 5.2.1). When females were absent during assay, methodology was as above, except females were removed from their

drop immediately prior to the addition of a test male. The control condition consisted of single males (reared in tandem with test males) incubated in their original haemolymph drop for the duration of the assay without any additional social interaction.

5.2.2.2 PBS (live adults present during assay)

Following the methodology of Ebssa et al. (2008), socially naïve males and females were paired in PBS without physical contact using permeable barriers. This allowed the variable of physical contact as a mechanism for sexual maturation to be eliminated, enabling the potential for male maturation via chemical means to be investigated.

500 µl PBS was pipetted into wells of 24 well plates with a single socially naïve (drop-reared) male per well. Inserts with transparent, permeable (0.8 µm) bases (Thincert™, Greiner) were placed in the well with 50 µl of PBS and 3 live females (social experience defined in results) (Figure 5.2). Any air bubbles beneath the base of the insert were removed to ensure direct contact between the two areas. Well plates were sealed with Parafilm and returned to incubation (see Table 2.1) for 48 h before visually assessing maturation status of males (Section 5.2.1).

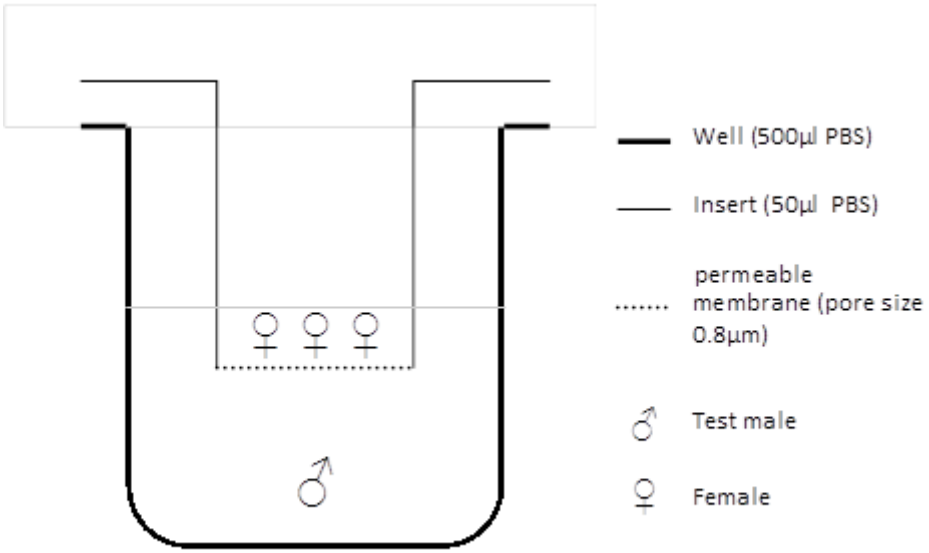


Figure 5.2: Representation of maturation assays in PBS whereby live females are separated from test male by a transparent semi- permeable membrane (pore size 0.8 µm) in the base of inserts in single wells of 24 well plates.

5.2.2.3 Conditioned PBS (live adults absent during assay)

To assess the potential of female- (or male-) produced chemical agents to induce sexual maturation in socially naïve immature males in the absence of females, PBS was conditioned with live adults prior to assay. Adult nematodes (reared either *in vitro* or *in vivo*) were added in defined density (worms/ml) to sterile PBS and incubated (Table 2.1) for 24 h (unless otherwise stated). Small volumes of PBS (1-10 ml) were conditioned in 24 well plates (500 µl per well, pooled) and larger volumes (>10 ml) in 50 ml Falcon tubes or larger vessels as required. Following incubation, live worms were removed either individually using a platinum wire hook (when in wells) or by sedimentation (when in larger vessels). The remaining liquid was collected and filtered through a 0.2 µm Acrodisc syringe filter prior to use in assay. Conditioned PBS was used immediately after filtration unless otherwise stated. Control conditions consisted of 0.2 µm filtered PBS only.

5.2.3 Maturation assay with conditioned PBS

Maturation assays were conducted with 100 µl of conditioned (or control) PBS per well in 96-well plates, with a single immature male in each well (transferred with platinum wire). Plates were sealed with Parafilm and incubated (see Table 2.1) for 48 h before visual assessment of male sexual status (Section 5.2.1).

5.2.4 C18 extraction and fractionation of worm-conditioned PBS

Virgin female-conditioned PBS to be extracted was prepared as specified in Section 4.2.4 at a concentration of 20 adult worms/ml. Solid phase extraction (SPE) was carried out using a C18 Sep-Pak column. The stationary phase was conditioned with 100% methanol (MeOH) and equilibrated with sterile MilliQ H_2O prior to sample loading. Flow-through was retained and stored at 4˚C in 50 ml Falcon tubes for fraction resuspension (Section 5.2.5) and assay purposes (Section 5.2.3). Elution of material bound to the column was carried out using progressively increasing concentration of MeOH (10 – 100% in 10% increments) in 2 ml fractions. Fractions were dried (Speedy Vac) and re-suspended separately in 500 µl of 100% MeOH to create 20 X concentrated stock solutions (maintained at -20˚C until use in assay/chemical analyses).

5.2.5 Resuspension and recombination of C18 fractionated PBS for maturation assay

Concentrated stock of single fractions was diluted using column flow-through originating from the equivalent processed sample (Section 5.2.4) to restore original sample concentration.

For recombination of multiple fractions, equal volumes of concentrated stock fractions were combined, vortexed and dried before dilution with column flow-through originating from the equivalent processed sample (Section 5.2.4) to restore original sample concentration.

5.2.6 Initial LC-MS/MS screening of C18 extracted *S. carpocapsae* virgin female and virgin male exudates

Socially naïve adult worm-conditioned PBS was prepared using 20 worms/ml for 24 h (Section 4.2.4). HPLC was carried out using Shimadzu HPLC with UV-vis detection (4.6 x 150 mm, 5 µm C8 column, 30 min 5-100 % acetonitrile gradient with 0.1 % TFA, 1ml/min flow rate) monitoring all wavelengths from 190 – 800 nm.

Samples were prepared for LC-MS/MS by dilution with 0.1 % (v/v) formic acid. Conditioned extracts and controls were analysed using an Agilent 6340 Ion Trap LC-MS System (Agilent Technologies, Santa Clara, CA), with online reverse phase (RP) separation performed using a ZORBAX 300SB-C18 HPLC Chip (43mm x 75µm, 55 µm) with an Agilent 1200 series HPLC. Samples were loaded in 5 % B (A: 0.1% formic acid, B: 90% acetonitrile, 0.1% formic acid) and a 11 min (5-70 % B)

gradient separation was performed at 0.3 µl/min. MS spectra were recorded in both positive and negative mode. MS/MS spectra were collected using a Top3 method.

5.2.7 High mass accuracy LC-MS/MS analysis of C18 extracted and fractionated female exudates (*S. carpocapsae* and *S. longicaudum*)

Conditioned PBS samples from *S. longicaudum* and *S. carpocapsae* females (Section 4.2.4) were applied to Sep-pak C18 cartridges and fractionated using step-wise elution with increasing concentrations of methanol (10, 20, 30, 40, 50, 60, 70 and 100 % methanol used for elution). Samples were prepared for LC-MS/MS analysis by mixing 1:1 with 0.1 % formic acid. Total conditioned PBS from male and female *S. longicaudum* were included in analysis, following sample desalting using C18 spin cartridges. A blank sample consisted of an injection of Solvent A. Samples were analysed using a Dionex Ultimate 3000 RSLCnano coupled to a Thermo Q-Exactive mass spectrometer. A 10 min method was designed which included a 5-70 % B gradient (A: 0.1 % formic acid, B: 0.1% formic acid in acetonitrile) and 5 µl of sample was injected onto a Thermo Hypersil Gold aQ polar-C18 column (100 x 2.1 mm, 1.9 µm particles). Samples were analysed in positive or negative mode, recording MS only or a Top3 MS/MS method (NCE 20 eV or 30eV).

5.2.8 Bioassay of dafachronic acids for male maturation

(25S)-Δ7-Dafachronic acid (formal name: (5α,25S)-3-oxo-cholest-7-en-26-oic acid) and Δ4-Dafachronic acid (formal name: 3-oxo-cholestenoic acid) were sourced from Cayman Chemical and stored at -20˚C. 1 mM stock solutions were prepared in

molecular grade ethanol and also stored at -20˚C. From this concentrated stock, a 1 in 10 dilution series was carried out using full strength PBS to give concentrations ranging from 10 µM to 1 fM for biological assay (Section 5.2.3). Once diluted in PBS, preparations were used immediately for assay as storage for more than one day in aqueous solution is not recommended (supplier recommendation). PBS conditioned with 20 females/ml for 24 h was used as a positive control, while PBS containing 1% ethanol and PBS alone served as negative controls.

5.2.9 Statistical analysis

All statistical analysis was carried out in Minitab 17. Comparisons between two groups used Chi Square test for association to determine the Pearson Chi square test statistic. In all cases a P value of <0.05 was taken as significant unless indicated otherwise. In experiments testing multiple groups (i.e. extraction and fractionation experiments), maturation success in test conditions were compared individually to a positive control (unprocessed original sample), again using the Chi square test for association method. Statistical analysis was used only where appropriate and not carried out on data sets where results were 'all or nothing' conclusions (i.e. 100 % vs 0% maturation success in two test conditions). Statistical techniques used are summarised in figure legends and tables in the results. Full test statistics are included in Appendix 6.

5.3 Results

5.3.1 Verification that physical female presence is not required to induce maturation in conspecific males

Socially naive male *S. carpocapsae* and *S. longicaudum* developed sexually 100% of the time when exposed to live socially naive females of the same species for 48 h (Table 5.1). Control males exposed to assay medium only (no female present) did not mature (0% maturation success).

Socially naive male *S. carpocasae* and *S. longicaudum* also developed sexually 100% of the time when exposed to female-conditioned media (insect haemolymph or PBS) where socially naive females had been present for 24 h prior to assay (Table 5.2). Control males exposed to assay medium only (no female conditioning) did not mature (0% maturation success). These results showed that physical presence of females is not required to induce sexual maturation in males and that an externally excreted chemical cue is responsible. Concurring results have previously been reported for *S. longicaudum* (Ebssa et al., 2008), however results for *S. carpocapsae* are novel.

Table 5.1: Proportion of socially naive males that sexually matured (development of gonad and macrosperm) when paired with socially naive females in different media. Pairs consist of one adult of each sex, paired for 48 h. Pairing in insect haemolymph conducted in 10 µl hanging drops (males added to female drops). Pairing in PBS conducted in 24 well plates (single male in well, 3 females in permeable insert). Control conditions contain media with male present only. N = 15 per condition.

Test species	**Insect haemolymph**		**PBS**	
	♀ present	No ♀ (control)	♀ present	No ♀ (control)
S. carpocapsae	15/15 (100 %)	0/15 (0 %)	15/15 (100 %)	0/15 (0 %)
S. longicaudum	15/15 (100 %)	0/15 (0 %)	15/15 (100 %)	0/15 (0 %)

Table 5.2: Proportion of socially naive males that sexually mature (development of gonad and macrosperm) when exposed to media where a female had previously been present, but removed prior to assay. Conditioned haemolymph (10 µl) contained a single socially naive female for 24 h (equivalent to 100 ♀/ml). Conditioned PBS contained 50 ♀/ml for 24 h. Unconditioned media unexposed to adult females. N = 15 per condition.

Test species	**Insect haemolymph**		**PBS**	
	♀ conditioned	Unconditioned	♀ conditioned	Unconditioned
S. carpocapsae	15/15 (100 %)	0/15 (0 %)	15/15 (100 %)	0/15 (0 %)
S. longicaudum	15/15 (100 %)	0/15 (0 %)	15/15 (100 %)	0/15 (0 %)

5.3.2 Determination of parameters for production and storage of female conditioned PBS which induces conspecific male maturation

All (100%) socially naive *S. carpocapsae* males matured when exposed to PBS conditioned with ≥ 20 socially naive ♀/ml (Table 5.3). A lower female density during conditioning (10 ♀/ml) resulted in slightly lower percentage of males maturing. A similar trend is shown in *S. longicaudum,* where percentage of males maturing was ≥ 93.3% when PBS was conditioned ≥ 20 ♀/ml and decreased slightly as female density decreased to 10 ♀/ml (Table 5.4). Males of either species did not mature when exposed to PBS conditioned with socially naïve male conspecifics at any density tested (10, 20 and 50 worms/ml), indicating that the maturation signal is produced exclusively by females (Tables 5.3 and 5.4).

A dilution series of PBS conditioned with socially experienced adult worms dissected from infected *G. mellonella* cadavers was assessed for ability to mature immature male conspecifics for both test species, *S. carpocapsae* (Table 5.3) and *S. longicaudum* (Table 5.4). The initial PBS preparation contained 100 adult worms/ml of which approximately 50% were female. In *S. carpocapsae* (Table 5.3), the percentage of males maturing was lower than in PBS conditioned with the equivalent density of socially naïve virgin females at all densities tested, significantly so at 20 ♀/ml and at 10 ♀/ml. However, in *S. longicaudum* (Table 5.4) male maturation success was similar regardless of whether PBS was prepared with socially naïve or socially experienced females at all densities tested.

Table 5.3: Proportion of socially naive ***S. carpocapsae*** males that sexually mature (development of gonad and macrosperm) when exposed to PBS conditioned for 24h with conspecific adults. Treatments using socially naïve worms (drop reared ♀ and ♂) prepared with stated concentration of worms. Variation in concentration of socially experienced (cadaver-dissected) ♀ achieved by dilution of original conditioned PBS containing approximately 50 ♀/ml and 50 ♂/ml . N = 20 per socially experienced condition and 15 per socially naive condition. * denotes significant difference from associated socially naïve female treatment (Pearson Chi Square < 0.05).

Conditioning Worms/ml	**% male maturation in conditioned PBS**		
	Socially naive ♀	Socially experienced ♀	Socially naïve ♂
50	15/15 (100%)	19/20 (95%)	0/15 (0%) *
20	14/15 (93.3%)	18/20 (90%)	0/15 (0%) *
10	13/15 (86.7%)	18/20 (90%)	0/15 (0%) *
5	N/A	2/20 (10%)	N/A
0	0/15 (0%)	0/20 (0%)	0/15 (0%)

Table 5.4: Proportion of socially naive ***S. longicaudum*** males that sexually mature (development of gonad and macrosperm) when exposed to PBS conditioned for 24h with conspecific adults. Treatments using socially naïve worms (drop reared ♀ and ♂) prepared with stated concentration of worms. Variation in concentration of socially experienced (cadaver dissected) ♀ achieved by dilution of original conditioned PBS containing approximately 50 ♀/ml and 50 ♂/ml. N = 20 per socially experienced condition and 15 per socially naive condition. * denotes significant difference from associated socially naïve female treatment (Pearson Chi Square < 0.05).

Conditioning Worms/ml	**% male maturation in conditioned PBS**		
	Socially naïve ♀	Socially experienced ♀	Socially naïve ♂
50	15/15 (100%)	17/20 (85%)	0/15 (0%) *
20	15/15 (100%)	15/20 (75%) *	0/15 (0%) *
10	13/15 (86.7%)	10/20 (50%) *	0/15 (0%) *
5	N/A	1/20 (5%)	N/A
0	0/15 (0%)	0/15 (0%)	0/15 (0%)

5.3.3 Species-specificity of female-produced male maturation signal

When exposed to PBS conditioned with female conspecifics (20 ♀/ml), socially naive male *S. carpocapsae* and *S. longicaudum* matured in > 85% of cases (Table 5.5). In contrast, on exposure to PBS conditioned with females of each other's species (20 ♀/ml), maturation success was dramatically lower (< 7% in *S. longicaudum* males and 0% in *S. carpocapsae* males) indicating that the female emitted chemical cue responsible for inducing male maturation is species-specific.

Table 5.5: Proportion of socially naive *S. carpocapsae* and *S. longicaudum* males that sexually matured (development of gonad and macrosperm) when exposed to PBS conditioned with conspecific or heterospecifc socially naive females at a density of 20♀/ml. N = 15 per condition

Test ♂ species	PBS conditioning ♀ species	Pair type	% Maturation
S. longicaudum	*S. longicaudum*	Conspecific	14/15 (93.3%)
S. carpocapsae	*S. carpocapsae*	Conspecific	13/15 (86.6%)
S. longicaudum	*S. carpocapsae*	Heterospecific	1/15 (6.7%)
S. carpocapsae	*S. longicaudum*	Heterospecific	0/15 (0%)

5.3.4 Effect of ascaroside #9 on male maturation

In Chapter 4, it was reported that ascr#9 was detected in PBS conditioned with mixed sex adults (20 ♀/ml, 24 h) of both *S. carpocapsae* and *S. longicaudum* (Table 4.1). It was therefore deemed appropriate to test whether ascr #9 was biologically active with respect to inducing maturation in males of both species. For *S. carpocapsae,* synthetic ascr#9 resuspended in PBS (5% MeOH) induced male maturation in 5% of cases at low concentration (41.7 ng/ml) and 10% of cases at a 10-fold concentration (417 ng/ml) (Figure 5.3). The % males maturing when exposed to either low or high concentrations of ascr#9 (see above) was significantly less than % males maturing in female-conditioned PBS. Similar was seen in *S. longicaudum*, where again % males maturing in low (41.7 ng/ml) and high (417 ng/ml) concentration of ascr#9 was significantly less than % males maturing in female-condition PBS.

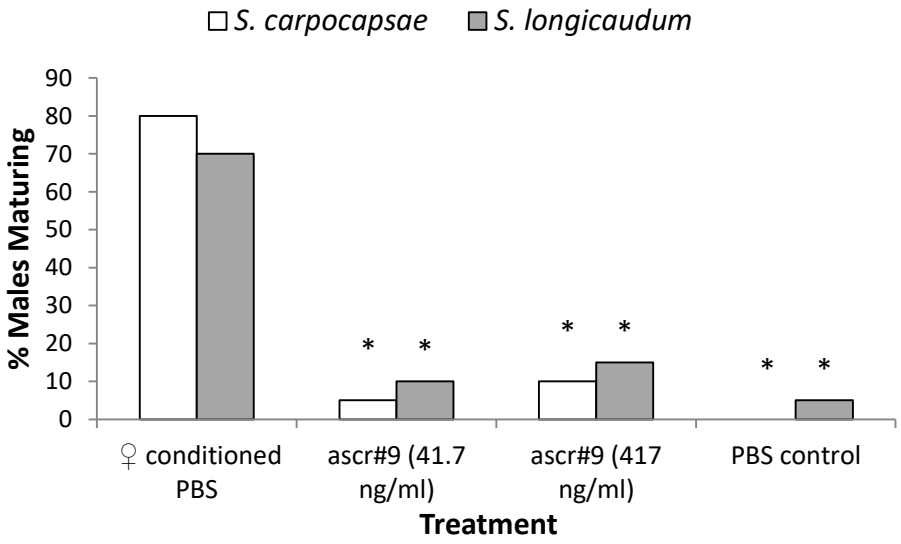


Figure 5.3: Maturation success (% of males maturing) of socially naïve *S. carpocapsae* and *S. longicaudum* males following 48 h exposure to female conditioned PBS (20♀/ml) and synthetic ascr #9 (in PBS, 5% MeOH) at two different concentrations. Control consists of PBS (5% MeOH). N = 20 per treatment. * denotes significant difference in % males maturing from female-conditioned PBS for each species (Pearson Chi-square, p <0.001).

5.3.5 Assay of C18 extracted and fractionated virgin female conditioned PBS

Virgin female *S. carpocapsae* exudate (20 ♀/ml in PBS) was extracted in 100% MeOH on a C18 Sep Pak column, resuspended and assayed for its ability to induce maturation in socially naïve conspecific males. Maturation success (N = 20 per condition) was significantly less than that observed in the original unprocessed sample in both column retentate and flow-through when assayed alone (Figure 5.4A). However, maturation success was restored to almost full activity when these two components were recombined and assayed at a concentration equal to the original sample (Figure 5.4A).

Informed by the above conclusions, stepwise C18 fractionation (Section 5.2.4) was carried out to elucidate where in the sample activity was highest. As it was found that presence of flow-through was required to restore full sample activity (Figure 5.4.A), all fractions were re-suspended in flow-through of the original sample for assay. The number of males maturing (% maturation success) when exposed to fractions eluted in 20 and 50% MeOH did not differ significantly from activity seen in the original untampered sample (Figure 5.4B). Maturation success in all other fractions tested was significantly lower than the original sample (Figure 5.4B). Although significantly lower than activity shown in the original sample, fractions eluted at 10 and 30% MeOH also showed some potential to induce maturation in males (numerical result higher than in pure flow-through, which was used as diluent for all samples).

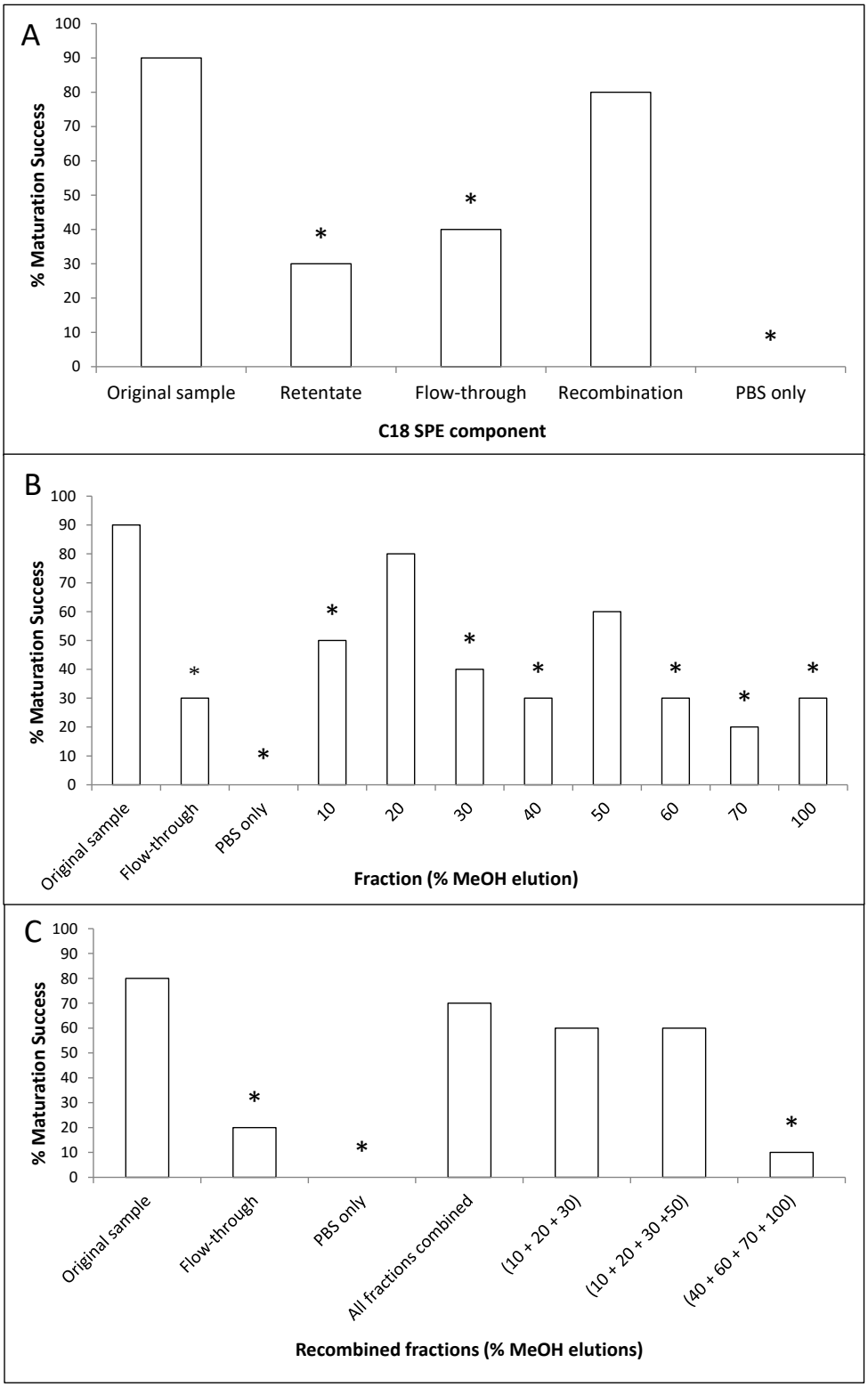


Figure 5.4 (A-C): Maturation success (% of males maturing) of socially naïve *S. carpocapsae* males following 48 h exposure to female conditioned PBS (original sample, 20♀/ml) and components resulting from subsequent C18 SPE of original sample eluted in 100% MeOH (A), on exposure to stepwise C18 fractionation of original sample (0-100% MeOH) (B) and on exposure to various recombinations of individual fractions (C). * indicates significant difference in maturation success between test condition and original sample in each panel (Pearson Chi Square test for association, $p < 0.05$). N = 20 per condition.

Informed by the previous results (Figure 5.4B), fractions were recombined in logical combinations, those that showed potential to mature conspecific males, those which did not and all fractions recombined (Figure 5.4C). This aimed to explore any possible additive effects of individual fractions once recombined and elucidate which components of the female-conditioned sample were responsible for inducing male maturation. As previously, resuspension of combined fractions was carried out using flow-through originating from the sample of interest to ensure any essential chemical components that did not bind to the column were not absent during assay.

As expected, recombination of all fractions restored activity, almost to the full extent shown in the original unprocessed sample (Figure 5.4C). Similarly, recombination of the most successful individual fractions highlighted previously (eluted at 10, 20, 30 and 50% MeOH) also restored the majority of activity and were shown statistically to be as potent in inducing male maturation as the original sample. However, recombination of fractions 10, 20 and 30% MeOH, with fraction 50% MeOH omitted, performs equally as well. Fractions identified previously as not inducing high levels of maturation in conspecific males (eluted in 40, 60, 70 and 100% MeOH), remained biologically inactive even in combination.

These results indicate that chemical components responsible for female-induced male maturation of *S. carpocapsae* conspecifics elute early in the fractionation process, particularly between 10 – 30% MeOH washes.

As previously described for *S. carpocaspsae*, virgin female *S. longicaudum* exudate (20/ml in PBS) was extracted in 100% MeOH on a C18 Sep Pak column, resuspended and assayed for its ability to induce maturation in socially naïve conspecific males. Male maturation success (N = 20 per condition) was significantly less than that observed in the original unprocessed sample in both column retentate and flow-through when assayed alone (Figure 5.5A). Again, maturation success was restored to almost full activity when these two components were recombined and assayed at concentration equal to the original sample (Figure 5.5A).

Informed by the above conclusions, stepwise C18 fractionation (Section 5.2.4) was carried out to elucidate where in the sample activity was highest (Figure 5.5B). Number of males maturing (% maturation success) when exposed to fractions eluted in 10 and 20% MeOH did not differ significantly from activity seen in the original untampered sample (Figure 5.5B). Maturation success in all other fractions tested was significantly lower than the original sample (Figure 5.5B). Although significantly lower than activity shown in the original sample, fractions eluted at 30 and 40% MeOH also showed some potential to induce maturation in males.

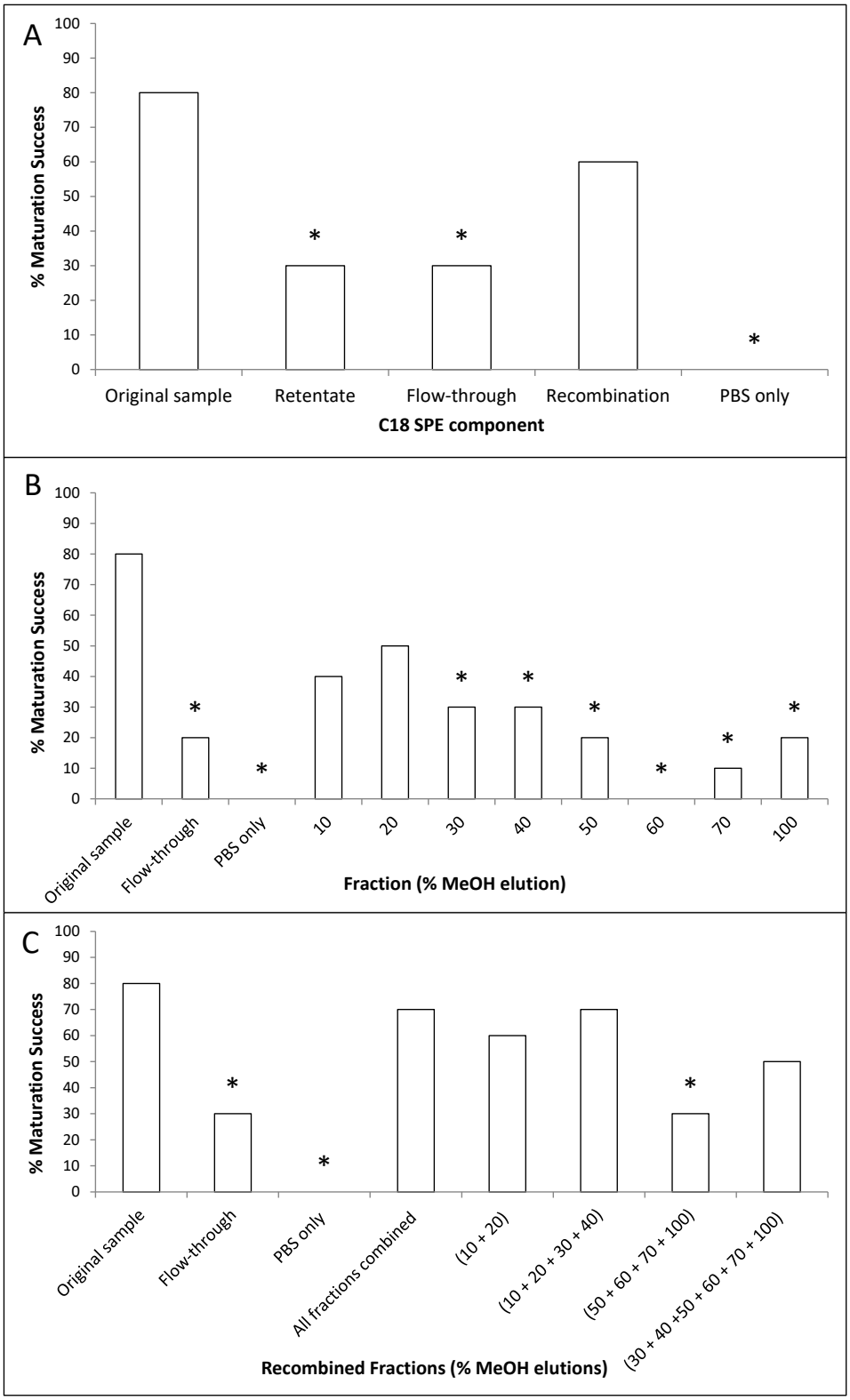


Figure 5.5 (A-C): Maturation success (% of males mauturing) of socially naïve *S. longicaudum* males following 48 h exposure to female conditioned PBS (original sample, 20 ♀/ml) and components resulting from subsequent C18 SPE of original sample eluted in 100% MeOH (A), on exposure to stepwise C18 fractionation of original sample (0-100% MeOH) (B) and on exposure to various recombinations of individual fractions (C). * indicates significant difference in maturation success between test condition and original sample in each panel (Pearson Chi Square test for association, $p < 0.05$). N = 20 per condition.

As for *S. carpocapsae*, results obtained from maturation assay of individual stepwise fractions of *S. longicaudum* virgin female exudate in PBS (Figure 5.5B) were used to inform logical recombination of fractions that showed potential to mature conspecific males (Figure 5.5C). Again, resuspension of combined fractions was carried out using flow-through originating from the sample of interest to ensure any essential chemical components that did not bind to the column were not absent during assay.

Recombination of all fractions restored activity, almost to the full extent shown in the original unprocessed sample (Figure 5.5C). Recombination of the most successful individual fractions highlighted previously (eluted at 10, 20, 30 and 40% MeOH) restored biological activity to the same extent of all fractions together. Early fractions in combination (eluted at 10 and 20% MeOH) were also shown statistically to be as potent in inducing male maturation as the original sample, as were all fractions eluted after and including the 30% MeOH wash. Combination of fractions eluted after and including the 50% MeOH wash exclusively were significantly less active than the original sample allowing it to be deduced that fractions eluted between 10 and 40% MeOH are of most interest for further analysis.

These results indicate that chemical components responsible for female-induced male maturation of *S. longicaudum* conspecifics elute early in the fractionation process, particularly between 10 – 40% MeOH washes.

5.3.6 HPLC and LC-MS/MS screening of socially naïve adult worm conditioned PBS

Virgin female and male exudates in PBS (Section 4.2.4) were screened using HPLC and LC-MS/MS analysis to highlight externally secreted metabolites in both sexes of *S. carpocapsae* and *S. longicaudum.*

Samples were first analysed by RP-HPLC, however no differences were noted between samples using this detection method, indicating that UV-vis detection was insufficient to differentiate between conditioned extracts from male and female worms. Comparison of male and female *S. carpocapsae* exudates using LC-MS/MS however, showed more promising results. MS spectral maps were generated for male and female *S.carpocapsae* exudates (and PBS control), displaying the *m/z* (mass-to-charge ratio) mapped to retention time for each molecule. Relative intensity of each molecular feature is indicated by colour (blue) profile (Figure 5.6). Although *S. longicaudum* male and female exudates were also subjected to the initial screening as described above for *S. carpocapsae,* ongoing technical problems with the equipment used caused the reliability of results to be questioned and so they have not been included in this thesis. Therefore, the following commentary applies to *S. carpocapsae* only until otherwise stated. Comparison of MS spectral maps from negative mode analyses revealed differences between *S. carpocapsae* male and female extracts (Figure 5.6), clustered at 6.8 – 7.6 min. A total ion chromatograph (TIC) further highlights the differences at this time-point (Figure 5.7). Manual inspection of MS spectra was performed and details of molecules with altered relative abundance between the male and female extracts was compiled (Table 5.6). MS profiles (extracted ion chromatographs (EICs)) of all molecules found to show higher intensity in female exudates compared with male can be found in Appendix

5.1(a-f). In particular it was noted that a molecule with *m/z* of 411.0 and retention time (Rt) of 7 mins was detected with substantially increased abundance in female extracts compared to male (Table 5.6, Figure 5.8).

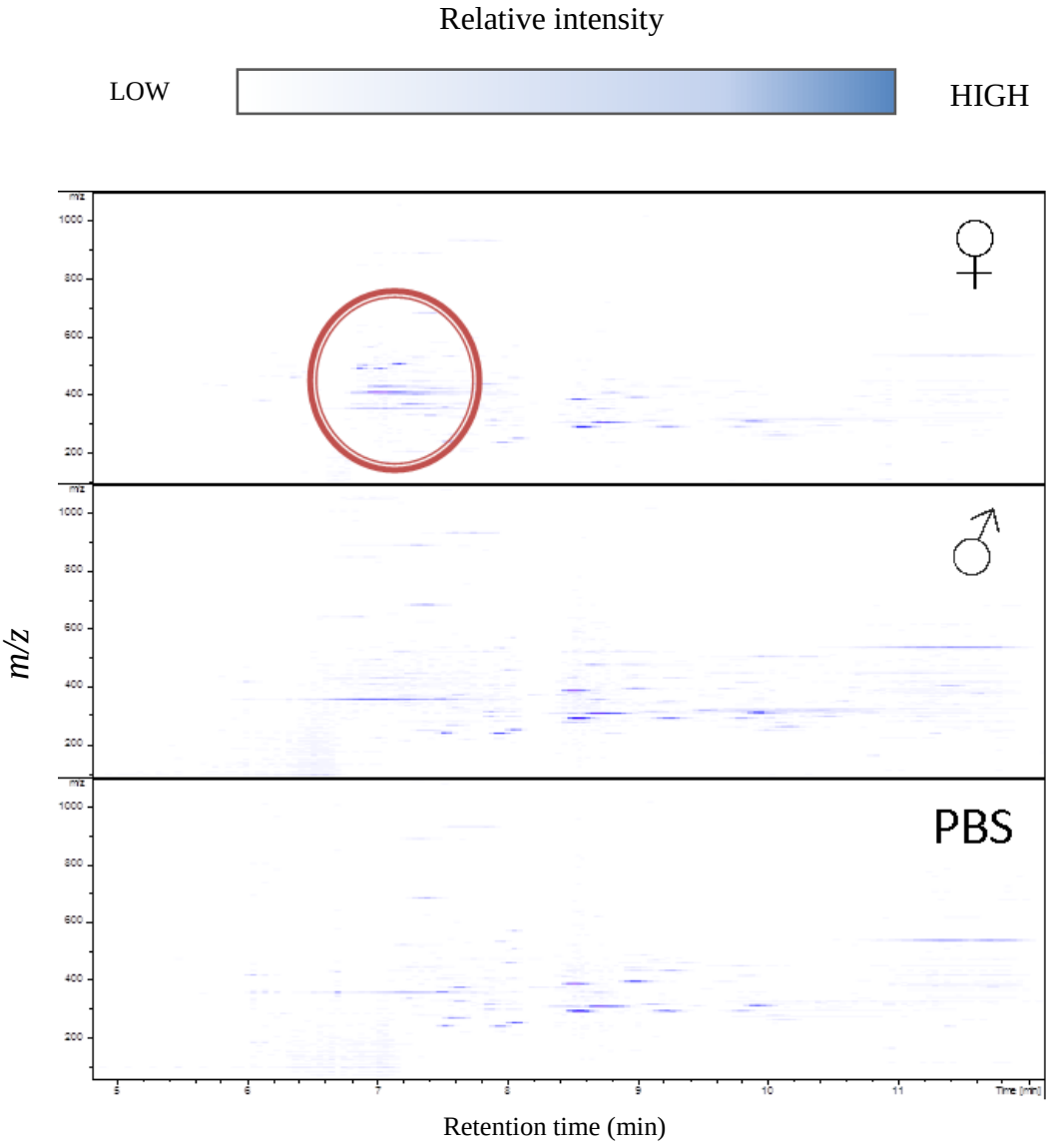


Figure 5.6: MS spectral map of negative mode LC-MS/MS analysis of conditioned extracts from female (top) and male (middle) *S. carpocapsae* (20 worms/ml, 24h). A negative control with the background PBS is included (bottom). Spectral map shows *m/z* of detected features (y-axis) at each retention time (x-axis). Relative intensity of ions is indicated by colour legend (intensity increases with blue colour). The red circle on the top panel highlights that there are molecules present in female exudates which are not seen in male exudates (middle) or control PBS (bottom).

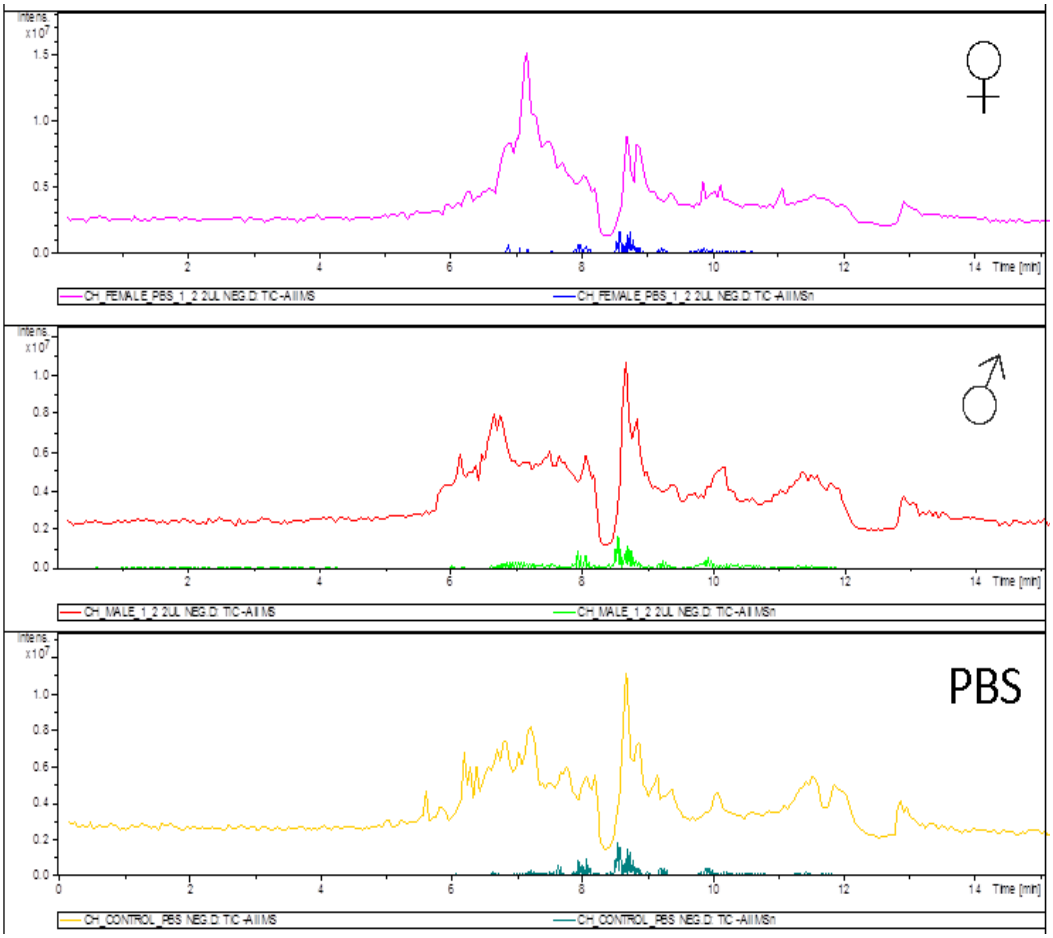


Figure 5.7: Total Ion Chromatograph: TIC of MS (negative mode) and MS/MS of conditioned extracts from female (top panel, MS in pink, MS/MS in dark blue) and male (middle panel, MS in red, MS/MS in green) *S. carpocapsae* (20 worms/ml, 24h), along with PBS control (bottom panel, MS in yellow, MS/MS in light blue).

Table 5.6: Ions detected with altered abundance in extracts from female *S.carpocapsae* compared to male samples. Relative abundance of ions is shown based on peak intensity of each corresponding *m/z*. Mode indicates whether ions were detected in positive mode or negative mode. EICs for each molecule can be found in Appendix 5.1(a-f).

Mode	*m/z*	Retention time (min)	Intensity		
			Female	**Male**	**PBS**
-ve	411.0	7	5×10^6	1×10^5	Trace
-ve	491.1	7	1.25×10^6	1×10^5	Trace
-ve	193.2	6.9	1.2×10^6	Trace	Trace
-ve	506.2	7.2	1.6×10^6	Trace	Trace
+ve	506.3	5.7	5×10^7	1×10^6	Trace
+ve	597	5.2	6.5×10^7	Trace	Trace
+ve	649.4	5.2	3.2×10^7	6×10^6	Trace

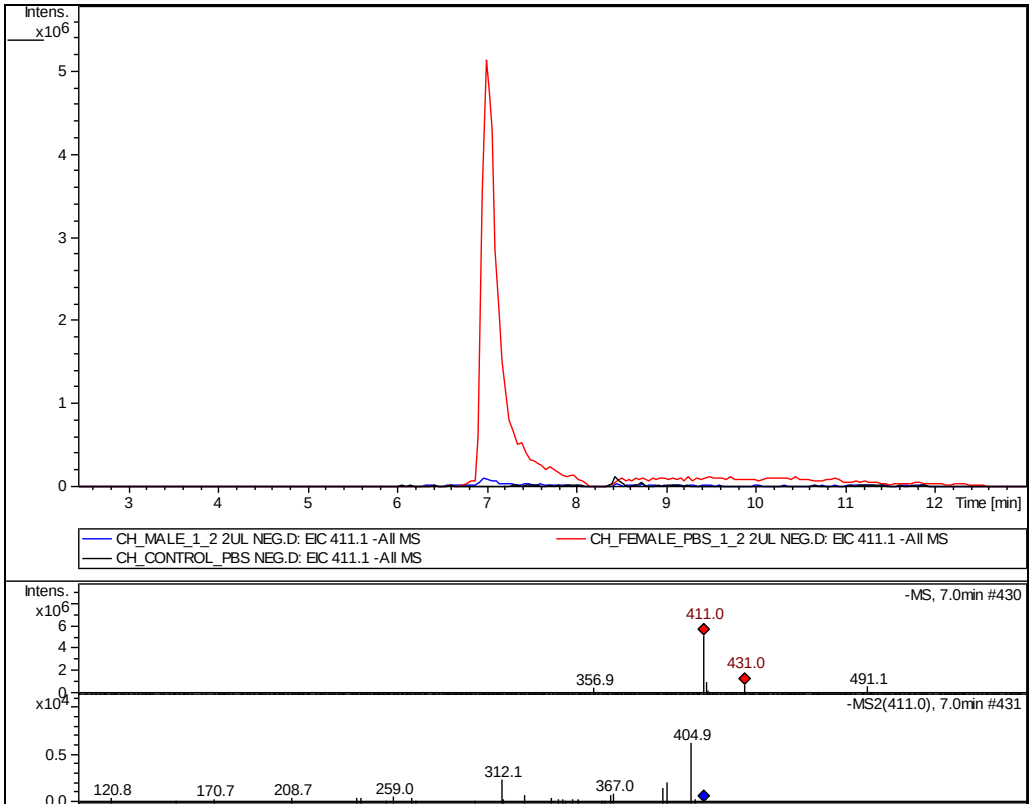


Figure 5.8: MS profile of m/z 411 in conditioned PBS from female and male *S. carpocapsae.* (top) Overlaid extracted ion chromatographs (EICs) at *m/z* 411 for conditioned PBS from female (red) and male (blue) *S. carpocapsae*. MS data was extracted to show only the peak intensity for m/z 411. (middle) MS spectrum at 7 min. (bottom) MS/MS spectrum showing fragmentation pattern of m/z 411. Analysis carried out in negative mode.

The molecule detected in negative mode with $[M\text{-}H]^-$ 411.0 (actual mass 412) (Figure 5.8) was speculated to be (25S)-Δ-1, 7-dafachronic acid ((25S)-Δ-1,7-DA), a steroid hormone with exact mass of 412.297 (Aguilaniu et al., 2016). Based on the fact that (25S)-Δ-1,7-DA) has a similar mass to the molecule detected here and has also been shown to regulate developmental processes in the nematode *C. elegans* (Aguilaniu et al., 2016) it was deemed appropriate to investigate further.

Although (25S)-Δ-1,7-DA was not commercially available, it biosynthetic precursor ((25S)-Δ7-DA) and close relative Δ4-DA were. These have also been implicated in

mediating physiological development in *C. elegans* (Aguilaniu et al., 2016). These two molecules were used for comparative LC-MS analysis and also for biological assay to investigate further. Biological assay of physiologically relevant concentrations (1fM- 10µM) of both (25S)-Δ7-DA and Δ4-DA showed that neither was biologically active with regards to inducing maturation in male *S. longicaudum* or *S. carpocapsae* (see Appendix 5.2 for results).

Furthermore, additional analysis of female exudates (*S. longicaudum* and *S. carpocapsae)* using a high mass accuracy mass spectrometer (Section 5.2.7) revealed an accurate *m/z* of 411.2250 for the candidate molecule. With a mass difference of 0.064, this analysis showed that this molecule is not (25S)-Δ-1,7-DA and instead represents an as yet uncharacterized molecule produced in higher abundance by female *S. carpocapsae* than males. Based on these findings it was deemed appropriate to reject the theorized conclusion that the unknown molecule found in female-conditioned PBS samples was (25S)-Δ-1,7-DA.

5.3.7 LC-MS/MS analysis of C18 stepwise fractionated female exudates

S. longicaudum and *S. carpocapsae* virgin female-conditioned PBS samples were applied to Sep-pak C18 columns and fractionated using step-wise elution with increasing concentrations of methanol (10, 20, 30, 40, 50, 60, 70 and 100 % MeOH) before being subjected to LC-MS analysis (Section 5.2.7). For general respresentation of fraction composition, total ion chromatographs (TICs) of *S. longicaudum* fractions eluted from 10-50 % MeOH are shown (Figure 5.9). As residual trifluoroacetic acid (TFA) present in the system eluted at 1 min and caused signal suppression, figures show retention between 1.5 and 7.5 mins only. TICs of all other fractions analysed (for both and *S. longicaudum* and *S. carpocapsae*) are shown in Appendices 5.3 and 5.4 respectively.

In *S. longicaudum* distinct peaks with retention times of 4.56 and 4.65 mins were present in early eluting fractions (10-30% MeOH), but were much reduced in intensity in later fractions (Figure 5.9). These peaks contain molecules wilth *m/z* of 303.1813 and 303.2176, respectively. These two molecules were also found in early fractions of *S. carpocapsae* female exudates, however in relatively lower abundance (Appendix 5.4). Ascr#10 and oscr#10 both have predicted $[M-H]^-$ of 303.1808, indicating that the molecule with Rt 4.56 min is either ascr#10 or oscr#10. Furthermore, MS/MS analysis revealed a fragmentation pattern matching ascr#10 and oscr#10 molecules, including the m/z 73.027 fragment distinctive to ascarosides (Figure 5.11) (Choe et al., 2012). These two candidate molecules differ in structure by the length (CH_2)n chain, and the presence of a methyl group. While these molecules cannot be differentiated by mass they appear to differ in retention time and show minor differences in their MS/MS signatures (von Reuss et al., 2012).

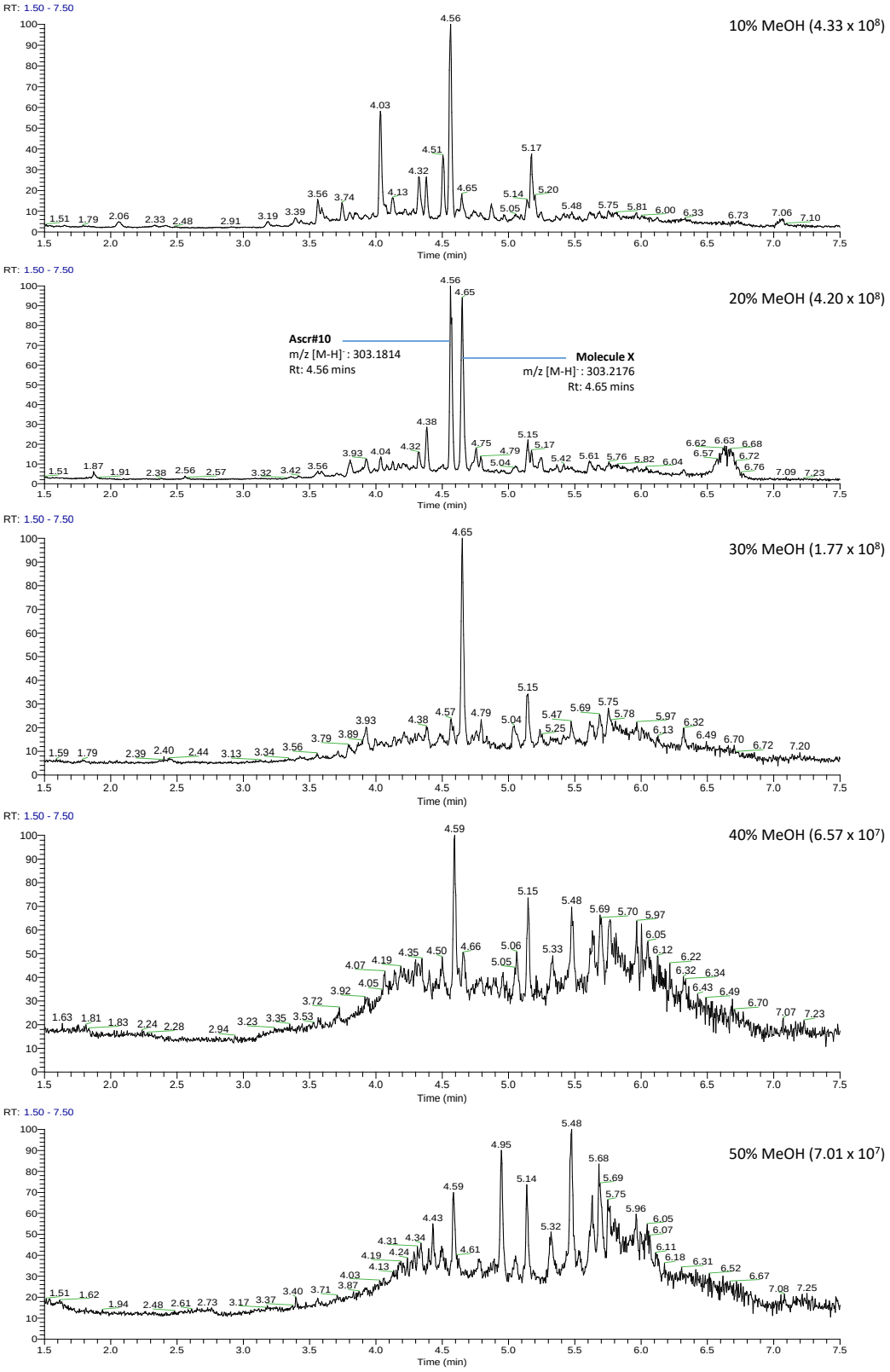


Figure 5.9: Total Ion Chromatographs (TICs) of female *S. longicaudum* exudates following stepwise fractionation (MeOH) on a C18 Sep-Pak column (Rt 1.5 – 7.5 min). LC-MS analysis carried out in negative mode. Peak with Rt 4.56 min putatively identified as ascr#10. TICs of fractions 70-100% shown in Appendix 5.3.

Following the initial indication of the presence of at least one potential ascaroside (ascr#10) in biologically active fractions, the presence of others was investigated by generating extracted ion chromatographs (EICs) which screened for *m/z* 73.027, the signature MS/MS fragment of ascaroside molecules (Choe et al. 2012) (Figure 5.10). Molecules found to contain the *m/z* 73.027 fragment were compared with the SMID database (www.smid-db.org) which details masses of all known ascaroside allowing for the putative identification the presence of several ascarosides in both species tested (Figure 5.10), namely ascr#9, ascr#1, acsr#14 and ascr#10 (found in both *S. carpocapsae* and *S. longicaudum* exudates) and ascr#12 found in *S. longicaudum* only. Further confirmation of these ascarosides was achieved by examining the MS/MS spectra of each candidate (Figure 5.11). Fragmentation patterns were found to match with previously published data (von Reuss et al., 2012) allowing for more confident conclusion that these ascarosides were present in female *S. longicaudum* and *S. carpocaspse* exudates.

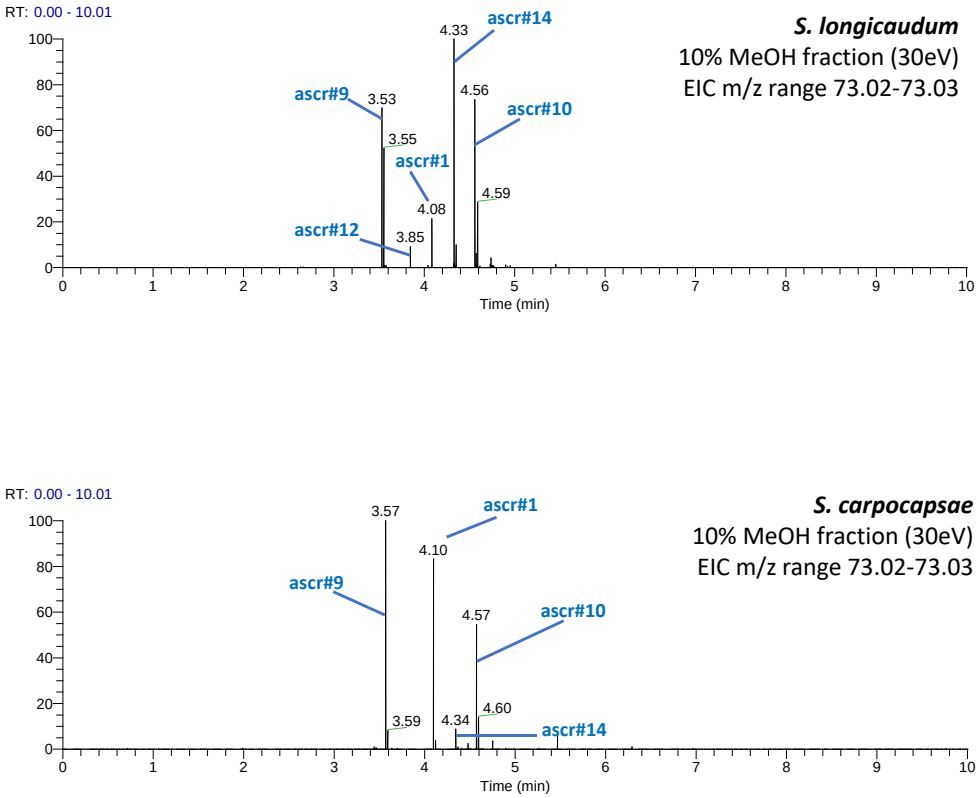
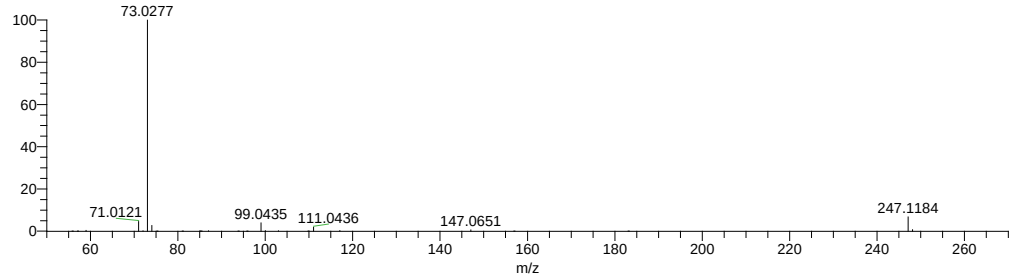
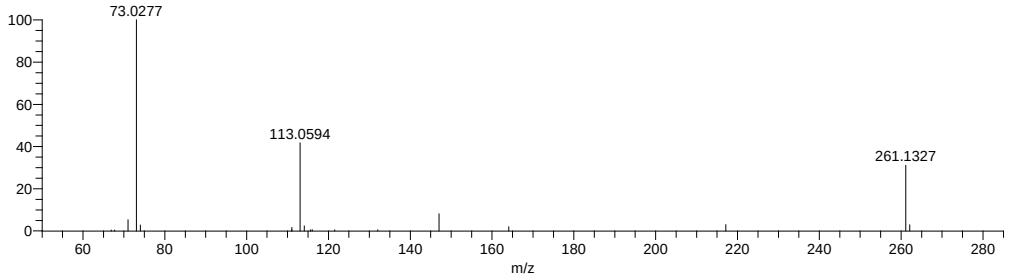


Figure 5.10: Extracted Ion Chromatograph (EIC) of female *S. longicaudum* (top) and *S. carpocapsae* (bottom) exudate sample (*m/z* 73.02-73.03) eluted at 10% MeOH on a C18 Sep-Pak column. LC-MS analysis carried out in negative mode. Putative ascaroside identifications indicated on top of figures.

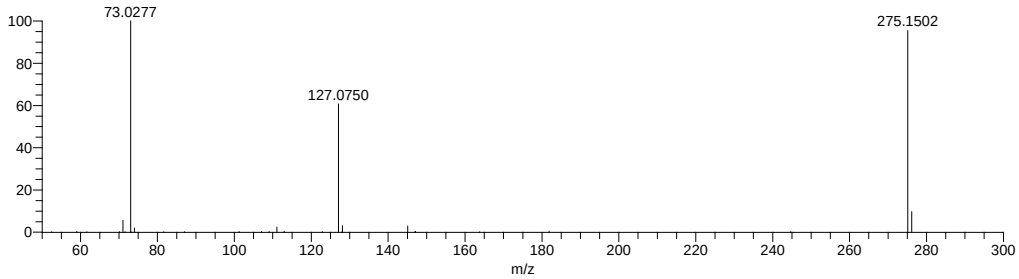
Ascr#9 (precusor ion m/z: 247.1184, Rt: 3.53, NL 3.75 x 10^6)



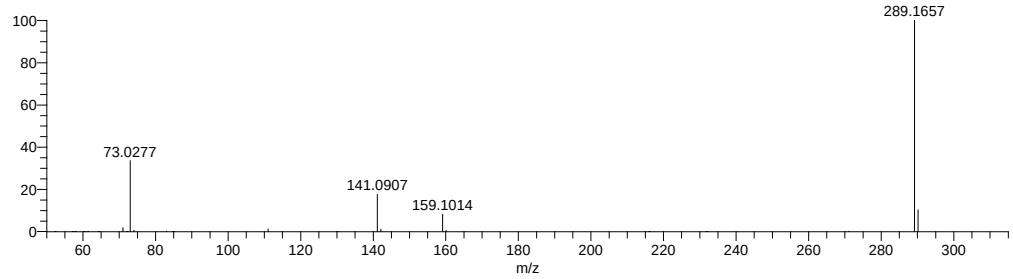
Ascr#12 (precursor ion m/z: 261.1342, Rt: 3.85, NL: 4.88 x 10^5)



Ascr#1 (precursor ion m/z: 275.1501, Rt: 4.08, NL: 1.15 x 10^6)



Ascr#14 (precursor ion m/z: 289.1657, Rt: 4.33, NL: 1.60 x10^7)



Ascr#10 (precursor ion m/z: 303.1814, Rt: 4.56 NL: 2.84 x 10^7)

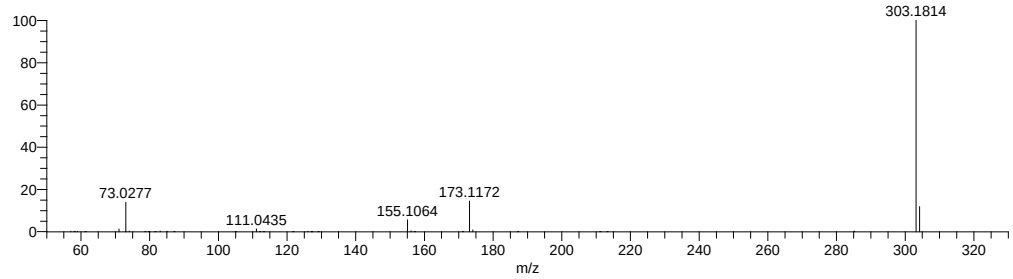


Figure 5.11: MS/MS spectra (negative mode) of molecules containing signature *m/z* 73.027 fragment characteristic of ascarosides recovered from female *S. longicaudum* exudates in PBS. Energy 30eV for fragmentation. Precursor *m/z* indicated on individual panels.

Relative abundance of each of the putatively identified ascarosides was quantified in each fraction by recording the EIC peak area at the reported *m/z* and Rt for each molecule (Figure 5.12). In particular, ascr#10 was found to be the most abundant of all ascarosides detected, eluting in high amounts in the 10 and 20 % MeOH fractions in both species. In *S. longicaudum*, ascr#9, ascr#12, ascr#1 and ascr#14 were all most abundant in the 10 % MeOH fraction, although in much lower amounts than the dominat ascr#10 (Figure 5.12A). In *S. carpocapsae*, ascr#9, ascr#1 and ascr#14 (as well as ascr#10 described previously) were most abundant in the 10 % MeOH fraction, while ascr#12 was absent (Figure 5.12B). Interestingly, the relative abundance of all ascarosides found was far higher in *S. longicaudum* than in *S. carpocapsae* (Figure 5.12).

During initial LC-MS analysis, it was noted that a molecule with a Rt of 4.65 was present in high amounts in *S. longicaudum* 20 and 30 % MeOH fractions (Figure 5.9). For discussion purposes this molecule will hereafter be refered to as Molecule X. The *m/z* of Molecule X (*m/z* 303.2174) was found to be very similar to that of ascr#10 (*m/z* 303.1814), however the differences in Rt (Rt 4.65 mins and Rt 4.56 mins respectively) and mass (0.036) of the two molecules indicated these were indeed two distinct molecules (Figure 5.13). Furthermore, when examining the MS/MS fragmentation pattern of each, it was found that they did not match and more notably Molecule X did not display the *m/z* 73.027 fragment characteristic of ascarosides (Figure 5.13). Relative abundance of Molecule X was found to be high in *S. longicaudum* 20 and 30 % MeOH fractions in particular, but was also present in lower amounts in the 10 % MeOH fraction (Figure 5.12A). It was also detected in early *S. carpocapsae* fractions (10 – 30 % MeOH) with highest abundance seen in

the 20 % MeOH fraction, although overall abundance was much lower than that reported in *S. longicaudum* (Figure 5.12B).

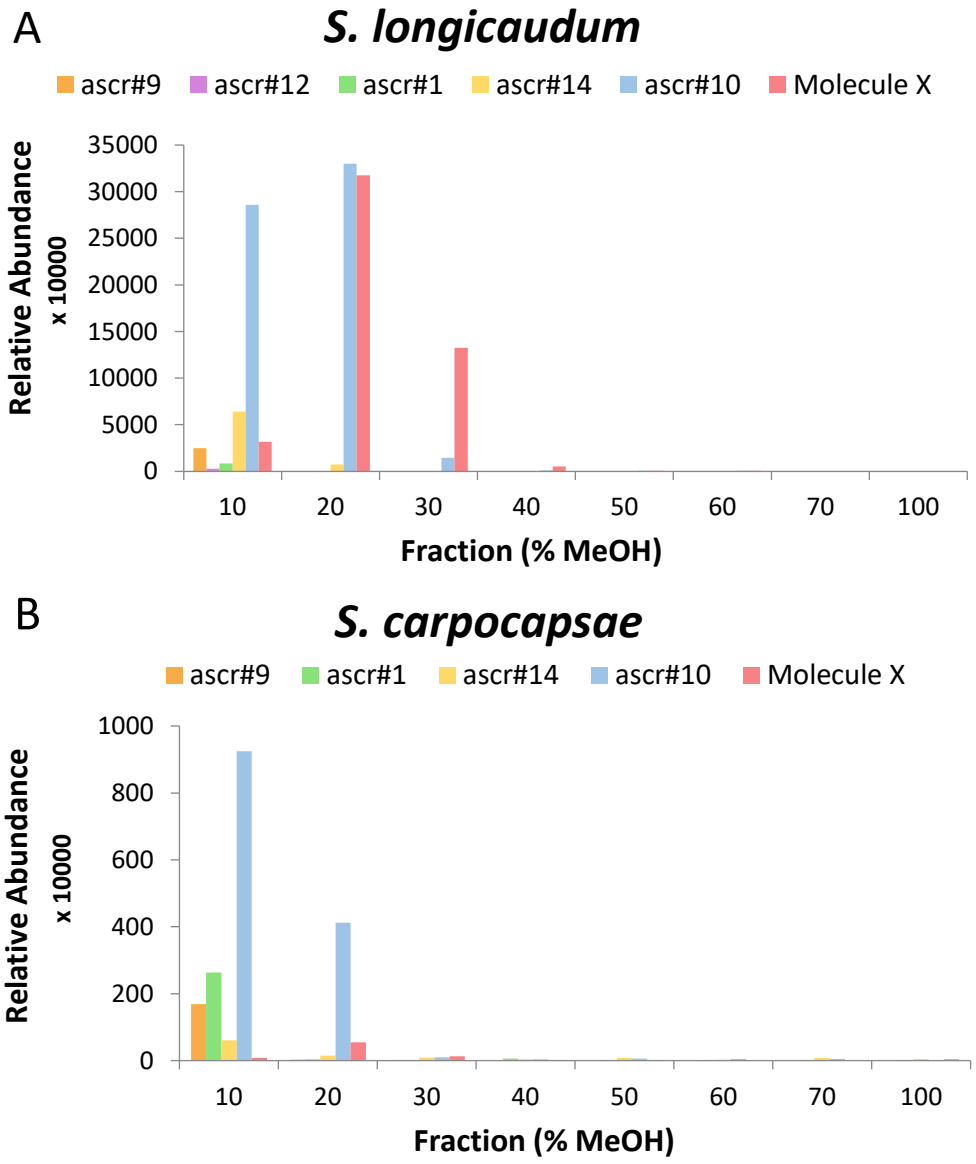
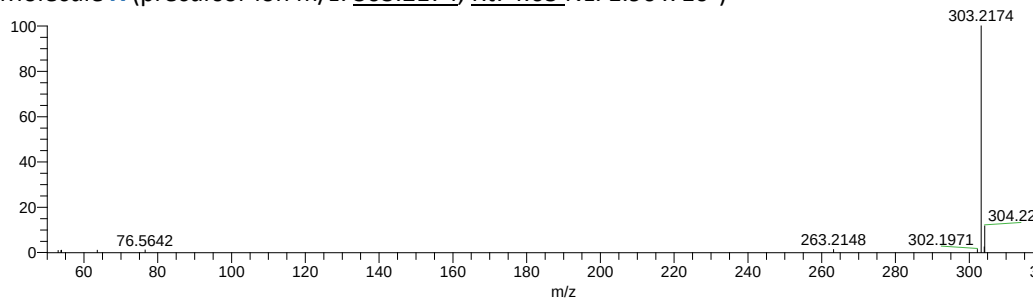


Figure 5.12: Relative abundance (EIC peak area) of putatively identified ascarosides and uncharacterized Molecule X (*m/z* 303.2176, Rt 4.65) in (A) *S. longicaudum* and (B) *S. carpocapsae* virgin female exudates following stepwise fractionation (MeOH) on a C18 Sep-Pak column. LC-MS/MS analysis carried out in negative mode.

Molecule **X** (precursor ion m/z: 303.2174, Rt: 4.65 NL: 1.90 x 10^5)



Ascr**#10** (precursor ion m/z: 303.1814, Rt: 4.56 NL: 2.84 x 10^7)

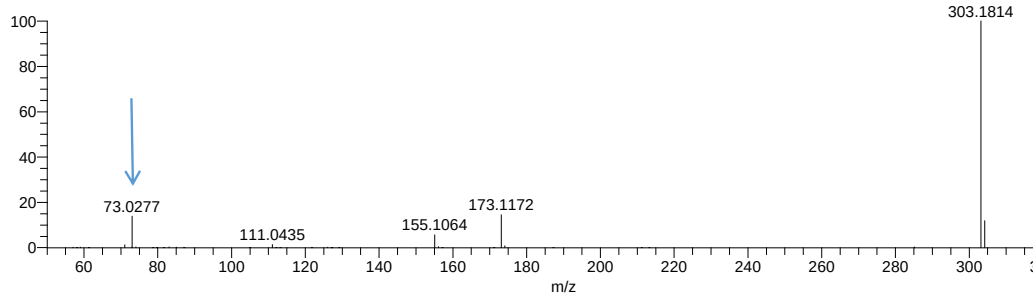


Figure 5.13: MS/MS spectra (negative mode) of molecules found in biologically active fractions of *S. longicaudum* and *S. carpocapsae* female exudates. Note: the similar *m/z* of precursor ions, but differing Rt and fragmentation patterns of each species (most notably the absence of fragment *m/z* 73.027 in Molecule X, top panel). Energy 30eV for fragmentation. Precursor *m/z* indicated on individual panels.

5.4 Discussion

As seen previously *in S. longicaudum* (Ebssa et al., 2008), here I show that male *S. carpocapsae* also only produce sperm when they detect conspecific female presence. Actual physical female presence or contact between partners is not required, as female-conditioned media (PBS and insect haemolymph) was as successful at inducing males to produce sperm in both species. In goldfish (*Carassius auratus*), 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20βp) is released by pre-ovulatory females, stimulating production of luteinizing hormone (LH) in males which in turn increases sperm production and motility (DeFraipont and Sorensen, 1993). This has also been shown to occur in several other carp species including the common carp (Stacey et al., 1994) and crucian carp (Bjerselius et al., 1995).

As was found in female-produced male attractants (Chapter 4), induction of male maturation by female exudates was also species-specific within the *Steinernema* genus (Table 5.5), at least for the two species tested (*S. carpocapsae* and *S. longicaudum*). There is no previous evidence of whether maturation pheromones in *Steinernema* are species-specific as female induced male maturation has only been shown in *S. longicaudum* (Ebssa et al., 2008). More than one species of EPN may enter and proliferate within the same host (Sicard et al., 2006; Bashey et al., 2012), where a male might encounter females of another species while females of his own are absent. It is not known whether the two test species occur sympatrically, but this is likely since *S. carpocapsae* has a global distribution (Hominick, 2006). According to evolutionary principles, it is expected that sympatric species would possess different sex pheromones (Burnard et al., 2008). In freshwater fish, there are examples of the opposite, where species inhabiting the same niche do respond to

each other's sex pheromones. Male Atlantic salmon (*Salmo salar)* respond physiologically to ovarian fluid and urine of both conspecific and heterospecific (*Salmo trutta*) females showing increased sex steroid hormone levels in the blood plasma and also higher milt volume (Olsén et al., 2000). The common carp *Cyprinus carpio* and goldfish *Carassius auratus* appear to share common reproductive steroidal pheromone systems and also show sensitivity to each other's pheromones (Irvine & Sorensen, 1993).

There was a slight reduction in the ability of exudate of socially experienced *S. carpocapsae* females (dissected from cadavers) to induce maturation in males in comparison to socially naïve virgin female exudate (Table 5.3). No significant difference was seen between the two treatments in *S. longicaudum* (Table 5.4). Socially experienced females dissected from cadavers were visually confirmed as mated by the observation of fertilized eggs; however it was not possible to tell how long they had been mated for. It would be apt to assess the maturation potential of exudates from females where mating was more closely controlled (as with attraction assays in Chapter 4) to determine whether mating affects the production or emission of maturation-inducing chemicals. Unlike males which are known to mate repeatedly (Ebssa et al., 2008), it is currently unknown whether *Steinernema* females mate multiple times. Whether they retain the ability to mature conspecific males post mating may be dependent on whether they mate only once or repeatedly. If females do mate several times they may be more likely to continue production and release of chemicals which prime males for successful reproduction, whereas if mating is a once only event, energetic resources may be better spent on survival rather than pheromones for example.

For both *S. carpocapsae* and *S. longicaudum*, female-conditioned PBS extracted on C18 Sep-Pak columns showed a significant reduction (~60%) in activity as a male maturation cue when compared to un-extracted material (Figures 5.4A and 5.5A respectively). However, when extracted material was recombined with sample flow-through (all sample material which did not bind to the column), full biological activity was restored in each case. These results (along with the finding that flow-through alone showed some potential to mature males) suggest that not all chemical components involved in female-induced male-maturation in *S. carpocapsae* and *S. longicaudum* are retained by the C18 Sep-Pak columns used here, perhaps indicating that highly polar or very small molecules are involved. Based on these results assay-guided C18 fractionation on female-conditioned PBS was carried out, with fractions re-suspended and assayed in combination with original sample flow-through so as to ensure any vital chemical components which didn't bind to the column during extraction and fractionation were not emitted from assays. In both species, early fractions eluted at lower MeOH concentrations were the best at inducing male maturation, with activity in 20% and 50% MeOH fractions in *S. carpocapsae* and 10% and 20% MeOH fractions in *S. longicaudum* not differing significantly from the original un-extracted samples. Recombination of individual fractions identified to show potential bioactivity indicated that molecules likely to be involved in female-induced male maturation elute in the region of 10-30% MeOH and 10-40% MeOH in *S. carpocapsae* and *S. longicaudum* respectively. This result was supported by the finding that fractions which showed little activity on their own still performed poorly in combination. This exploratory method of activity-guided fractionation of crude pheromone extract is commonly used in the early stages of pheromone characterisation (Butcher, 2017), allowing for the location of active compounds to be

pinpointed prior to further structural analysis using MS and NMR. The process however, is time intensive and in this case (particularly as flow-through from the original sample was used for re-suspension of fractions and bioassay methodology required relatively large volumes) required quite large amounts of starting material of which preparation was also a lengthy process.

LC-MS/MS analysis of stepwise C18 fractions of female exudates putatively identified the presence of several ascarosides in both *S. carpocapsae* and *S. longicaudum* and more interestingly these occurred in greatest abundance in fractions which were biologically active (Figure 5.12) indicating they may play a role in inducing male maturation. Acarosides #9, #1, #14 and #10 were common to both species, with ascr#10 having the highest relative abundance for both (Figure 5.12). An additional ascaroside (ascr#12) was found in *S. longicaudum* female exudates (Figure 5.12), however in very low abundance relative to the four mentioned above. Choe et al. (2012) previously reported the occurrence of all but one (ascr#14) of the aforementioned ascarosides as well as ascr#11, #12 and #18 adult *S. carpocapsae* exudates, with ascr#9 being the most abundant. Choe et al. (2012b) analysed exudates of mixed sex adults whereas here I analysed exudates from only virgin females. It is possible that the additional ascarosides detected by Choe et al. (2012b) could be exuded by males, explaining why I was unable to detect their presence. *S. longicaudum* adult exudates have not been screened previously for the presence of known ascarosides, however the relatively closely related *Steinernema glaseri* which occurs in the same phylogenetic clade (V) (Figure 1.3) has been shown to produce ascr#9, #12 and #1 (Choe et al., 2012b), all of which I recovered from *S. longicaudum* exudates.

The analytical methods used here could only show the abundance of ascarosides detected relative to each other and not absolute concentration within the collected exudates. Although this provided important information the relative concentration of ascarosides that were produced by *S. carpocapsae* and also in *S. longicaudum* (which previously has not been studied with regards to ascaroside content of exudates), further research is needed to determine exactly the quantities of ascarosides required for bioactivity. This could involve the use of synthetic standards to both confirm identity (as stated previously, it may be possible the molecules detected were not ascarosides but the similar oscarosides) and compare known concentrations to those extracted from exudates. Bioassay of the ascarosides detected both individually and in different combinations is also required to explore synergy between components and establish the exact pheromonal blend required to induce male maturation in each species.

The only synthetic ascaroside that I tested was ascr#9. Ascr#9 on its own did not induce maturation in either *S. carpocapsae* or *S. longicaudum* males at a physiologically relevant concentration (41.7 ng/ml) and a 10 x stronger concentration (417 ng/ml, Figure 5.3). This is not surprising. As ascr#9 is common to EPN and has been found to be produced at both juvenile and adult stages (Choe et al., 2012b), it is unlikely that it would be biologically active on its own, particularly where species recognition is important such as sexual priming or attraction. While it is likely to be involved in pheromonal blends in EPN and may work synergistically with other ascarosidal components to mediate behaviours and developmental processes. Ascr#9 was detected in female exudate of both of my test species, but at a low level relative to other putatative ascarosides, most notably ascr#10.

According to current knowledge on chemical communication in nematodes, it was expected that ascarosides would be detected in *Steinernema* exudates and would play a role in mediating behaviour and developmental processes. However, it was found here that several non-ascarosidal molecules were also present in female exudates, which raises the question of whether molecules from other chemical families may also be involved in nematode communication. Initial LC-MS/MS screening of sex-specific *S. carpocapsae* exudates highlighted several non-ascarosidal molecules which were found to be in high abundance in female exudates. A molecule with *m/z* of 411.1 $[M-H]^-$ was detected with substantially increased abundance in female extracts compared to male (Figure 5.8, Table 5.6), leading to further investigation into whether this may play a role in the female-induced maturation of males**.** Based on its *m/z* (411.1 [M-H]-), it was speculated that this molecule may represent (25S)-Δ1,7-dafachronic acid, a steroid hormone previously shown to regulate development in *C. elegans* (Mahanti et al., 2014). More formally known as (5α,25S)-3-oxo-cholest-7-en-26-oic acid, with an exact mass of 412.19, (25S)-Δ1,7-DA (dafachronic acid) belongs to a wider group of endogenously produced sterol-derived hormones that act as ligands of the DAF-12 nuclear hormone receptor (NHR) which regulates larval diapause, longevity (Cheong et al., 2011) and most notably reproductive development in *C. elegans* (Mahanti et al, 2014). Unfavourable conditions such as overcrowding or lack of food resources suppress synthesis of DAF-12 ligands (such as the dafachronic acids) leading to repression of DAF-12 target genes and entry into dauer diapause (larval arrest). In contrast, favourable conditions trigger upregulation of DAF-12 ligand biosynthesis, promoting expression of DAF-12 target genes to induce rapid development of larvae into reproductive adults (Mahanti et al., 2014).

However, biological assay of two other dafachronic acids ((25S)-Δ7-DA and Δ4-DA, synthetic (25S)-Δ1,7-DA was not available commercially) shown to play a role *in C. elegans* development did not induce maturation in either *S. carpocapsae* or *S. longicaudum* males at a range of physiologically relevant concentrations. Furthermore, when using LC-MS/MS hardware with a higher mass accuracy it was confirmed that the candidate molecule was not (25S)-Δ1,7-DA. Although the identity of this molecule could not be characterized, its presence in female exudate warrants further research as to whether it is of biological importance.

High mass accuracy LC-MS/MS analysis (negative mode) of female *S. longicaudum* exudates also detected a molecule *with m/z* 303.2174. This molecule was also detected in *S. carpocapsae* exudates in lower relative abundance (Figure 5.12). Identified here as 'Molecule X', this molecule could be distinguished from ascr#10 (*m/z* 303.1814) by a very small mass difference of 0.036 and longer Rt of 4.65 mins (compared to 4.56 mins for ascr#10, Figure). Furthermore, when subjected to MS/MS, Molecule X did not fragment as ascarosides would be expected to (there was no characteristic *m/z* 73.027 fragment, Figure 5.13). Most notably, Molecule X was present in high amounts in biologically active fractions of female *S. longicaudum* exudates and was also detected (but in much lower amounts) in active fractions of female *S. carpocapsae* exudates. This finding is good cause to investigate this molecule further, and in particular to question whether it may play a role in inducing maturation in male *Steinernema.*

Chapter 6 General discussion

Here I show evidence that, as hypothesised, species-specificity of chemically mediated EPN behaviours and developmental processes is apparent where species recognition is important (i.e. adult sexual behaviours), but not where different species have a common aim, such as juvenile dispersal from overcrowded conditions. With regards to dispersal behaviour, IJs responded similarly when exposed to cadaver extract and worm-conditioned water originating either from conspecifics or heterospecifics (Chapter 2). This was the case with closely related species (within the *Steinernema* genus) but also between more distantly related species from different genera in different phylogenetic clades (*Steinernema* spp. and *Heterorhabditis megidis*). Different species of EPN may co-infect and successfully reproduce in a single insect host (Kondo, 1989; Koppenhöfer et al., 1995; Sicard et al., 2006; Puza and Mracek, 2010; Bashey et al., 2011), therefore it is beneficial for a nematode to respond to overall crowding levels rather than just accounting for numbers of its own species. There are several possible proximate mechanisms for this lack of species-specificity during the dispersal stage. Firstly, it may due to all species producing universal non-specific chemical cues, advertising unfavourable crowded conditions regardless of species present. Such cues may be produced within the cadaver by population members of all life stages, or indeed continue to be produced by IJs once they have vacated a spent host, as evidence is presented for in this study. Ascaroside #9 has been shown to be produced in high amounts by all insect-associated nematodes (Choe et al., 2012b), and so it is a likely candidate for promoting dispersal. More simply, IJs may be responding to metabolic waste

products such as ammonia which accumulate within the cadaver and indicate overcrowding and may have toxic effects (San-Blas et al., 2008). This however, does not account for the observed non-species-specific response to worm-conditioned water prepared with non-feeding IJs, where metabolic waste is expected to be minimal. Increased dispersal in the presence of another species may give a competitive edge when locating a new host, such as is seen in *S. carpocapsae* which move more actively toward a host when in the presence of IJs of the competing species, *S. glaseri* (Wang and Ishibashi, 1999).

In contrast, in Chapters 4 and 5 I showed that adult behaviours and development processes mediated by chemical signals are species specific, at least for the two *Steinernema* species tested, the potentially sympatric species *S. longicaudum* and *S. carpocapsae* (Stock, 2015). As hypothesised, male *Steinernema* spp. were only attracted to exudates produced by conspecific females and similarly only produced sperm when exposed to exudate from females of their own species. Species recognition is vital in reproductive processes to avoid wasting energy on unnecessary chemotaxis and physiological development as well as avoiding physical damage which may occur during copulation with an incorrect partner. Chemical analysis of female exudates (Chapter 5) showed that females of both *S. carpocapsae* and *S. longicaudum* produce numerous ascarosides, a group of ascarylose based molecules conserved among nematodes and previously implicated in mate attraction (see Chapter 1). Although there were similarities in the ascaroside profiles produced by these two species (both produced ascr #9, ascr#1, ascr #10, ascr # 14), slight differences in blend composition (ascr #12 was only detected in *S. longicaudum*) and relative abundance and ratio of components may be responsible for species specificity.

Male *S. carpocapsae* and *S. longicaudum* were attracted to female-conditioned PBS of conspecifics, however C18 extraction and resuspension of these exudates reduced their ability to attract males to the same extent (Chapter 4). Material which did not bind to the C18 column (flow-through) showed some potential to attract males on its own, suggesting that the attractant may have some highly polar components unable to bind to the column used. More notably, recombination of extracted exudate with flow-through did not restore activity to the full extent of the original female exudate, suggesting some part of the attractant was lost completely during processing. This could indicate the presence of a volatile component required for male attraction. Although uncommon, there is evidence that volatile chemicals may play a role in sexual attraction in nematodes (Lee, 2002; Leighton et al., 2014). More generally, volatiles are often involved in pheromones which induce immediate behaviours, such as alarm and attraction signals, where the signal must convey information in real time and not persist for unnecessarily long periods (Wyatt, 2003). It would not be useful to males trying to locate a female if the attractant remained detectable in one place long after a female had moved location, for example.

Here I show that attractiveness of *Steinernema* spp. females was reduced after mating and showed little sign of recovering up to 48 h post-mating (Section 4.3.3). Although *Steinernema* males may mate multiple times (Ebssa et al., 2008), it is unknown whether *Steinernema* females mate more than once (Griffin, 2012). This raises questions as to how female attractiveness is reduced, whether pheromone production ceases completely, the blend is modified, or emission is blocked by the action of mating itself (use of mating plugs by the male for example). However, there are no reports of mating plugs in *Steinernema* (Griffin, 2012) and none was observed in this research. It also raises the question as to whether this is

manipulation by the male, perhaps components of the seminal fluid turn off female pheromone production to render her unattractive as in *C. elegans* (Leighton et al., 2014) or an adaptive strategy of the female to shut down the costly production of secondary metabolites as she directs her efforts to egg production. *Steinernema* females have a narrow window of opportunity for mating of just a few days, as after this, juveniles hatch and develop in the mother (endotokia matricida), and egg production stops (Baliadi et al., 2001)

Unlike in attraction**,** the activity of male maturation cues associated with female exudates was fully restored after resuspension of C18 SPE retentate in column flow-through (Section 5.3.5). This indicated that all components of the putative pheromone were retained during extraction, either bound to the column or in the flow-through material, and that volatiles were not likely involved (or at least not essential). Activity-guided fractionation showed that active components had a relatively low affinity for the C18 solid phase (being predominantly found in early fractions and in column flow-through) indicating high polarity and potentially low mass. Known ascarosides were putatively identified in female exudates of both test species. Ascarosides identified for *S. carpocapsae* were similar to those previously reported for this species (Choe et al. 2012b) while the data for *S. longicaudum* are novel. Although there were commonalities in the ascaroside profiles seen in both species (i.e. both produced ascrs #9, #1, #14 and #10), minor differences including ratio of components, total abundance and most notably the presence of ascr#12 in *S. longicaudum* exudates only, may contribute to species-specificity of maturation signals between these two species.

It was expected that ascarosides would be detected when analysing exudates which had biologically active properties, as it is well documented that ascarosides play a highly conserved role in nematode communication (see chapter 1). However, LC-MS analysis of female exudates also highlighted several female-associated candidate molecules which could not be matched to currently known ascarosides based on *m/z* (mass: charge ratio) but are certainly of interest for further investigation. Most notably, an uncharacterised molecule with an *m/z* of 303.2174 9[M-H^-]) was detected in biologically active *S. longicaudum* fractions in high abundance and although present in *S. carpocapsae*, relative abundance was much lower (Figure 5.12). Several other non-ascarosidal female-associated molecules were also highlighted by LC-MS which could also be the subject of further research (Table 5.6).

In usual commercial application of IJs in aqueous formulation, natural dispersal cues usually present in the insect cadaver are likely to be absent. In chapter 3, cadaver extract was shown to promote IJ dispersal (Section 3.3.1). This helps explain the finding that IJs emerging from infected cadavers are highly dispersive (Shapiro-Ilan and Glazer, 1996; Shapiro-Ilan et al., 2003) and supports the strategy of field application of IJs within pre-infected cadavers (Dolinski et al., 2015). An alternative strategy which, to my knowledge, has not been tested, would be to apply IJs in suspension spiked with cadaver material to improve the performance of field application. More interestingly, I found that water conditioned with high density IJs for 7 days also had a positive effect on dispersal rate of other IJs. Furthermore, both *S. carpocapsae* and *H. megidis* stored in conditions where the water was not changed dispersed more than when kept in identical conditions but for the regular replacing of storage water. These findings strongly suggest that IJs themselves actively secrete

into the medium chemicals which can accumulate over time and promote dispersal behaviour of both themselves and others. Elucidation, synthesis and application of such cues in combination with mass produced IJ may increase dispersal in the field, but would add to the cost of the nematode product. More simply, it could be beneficial to retain liquid culture material accumulated during production and add to commercial formulations to increase dispersal. EPN are commercially produced in bioreactors and harvested IJs are typically stored in aerated water for a period before formulation, which involves mixing partially desiccated IJs with a carrier (Shapiro Ilan and Gaugler, 2002). Dispersal could be improved by storing formulated IJs in suspension prior to application for adequate time periods to allow dispersal cues to accumulate, assuming that the effect on the IJs would persist during formulation and storage of product. Alternatively, if the secreted molecules could be recovered from the storage water they could be added to the tank mix with IJs prior to field application, instead of the more complex cadaver extract as proposed above. Conversely, it may be desired that dispersal behaviour is kept to a minimum for more targeted applications, therefore the standard procedures of washing prior to formulation and provision of fresh water prior to application may be more appropriate.

The effect of storage method on virulence was less clear, with evidence that mechanical disturbance during storage played a role in improving virulence of *H. megidis* (Figure 3.12), while there was no evidence for an effect of an accumulation of exuded chemicals on virulence of either *H. megidis* or *S. carpocapsae*. Irrespective of storage method, dispersal tendency of IJs declined over time of storage, especially for *H. megidis* and to a lesser extent for *S. carpocapsae*, and infectivity of *H. megidis* increased during storage. Thus my findings, especially for

IJs stored at 20°C, support the 'phased-infectivity' hypothesis (Dempsey and Griffin, 2002) which proposes that *H. megidis* have an initial dispersal phase, during which infectivity is low, followed by an infective phase, during which dispersal tendency is declining (Dempsey and Griffin, 2002). This type of phasing of activities is characteristic of infective stages of other parasites, such as trematode miracidia (Sukhdeo and Sukhdeo 2004), and would lessen competition in the vicinity of the source host.

Greater understanding of how IJ behaviours are mediated through chemical means, (particularly as techniques for IJ application in field settings are somewhat artificial), may help realize their full potential as biocontrol agents and better inform future biocontrol initiatives. In particular, future research might consider practical means of recovering dispersal cues from storage water, spent cadavers or spent bioreactor media to add to EPN at time of application. The research presented here on adult attraction and maturation behaviours has less immediate potential for practical application, but since EPN are such tractable laboratory models for studying fundamental behaviours that may be common to other nematode parasites, there may be potential to use knowledge gained in fundamental research on EPN to devise strategies for control of harmful species parasitic on plants or animals. For example, understanding how spermatogenesis is controlled by pheromones might ultimately lead to strategies for blocking this process in pest nematodes.

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Appendices

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Description	Max score	Total score	Query cover	E value	Ident	Accession
Xenorhabdus nematophila strain Xn 16S ribosomal RNA gene, complete sequence	1051	1051	100%	0.0	99%	KT899945.1
Xenorhabdus nematophila AN6/1 genome assembly XNC2, chromosome : I	1051	7318	100%	0.0	99%	LN681227.1
Xenorhabdus nematophila strain ATCC 19061 16S ribosomal RNA gene, complete sequence	1051	1051	100%	0.0	99%	NR_102822.1
Xenorhabdus nematophila strain Caba02 16S ribosomal RNA gene, partial sequence	1051	1051	100%	0.0	99%	GU293142.1
Xenorhabdus nematophila ATCC 19061 chromosome, complete genome	1051	7318	100%	0.0	99%	FN667742.1
Xenorhabdus nematophila from Peru 16S ribosomal RNA gene, partial sequence	1051	1051	100%	0.0	99%	GU480994.1
Xenorhabdus nematophila from Jordan: Al-Jubiha 16S ribosomal RNA gene, partial sequence	1051	1051	100%	0.0	99%	GU480968.1
Xenorhabdus nematophila strain K102 16S ribosomal RNA gene, partial sequence	1051	1051	100%	0.0	99%	FJ860891.1
Xenorhabdus nematophila strain NC116 16S ribosomal RNA gene, partial sequence	1051	1051	100%	0.0	99%	FJ860890.1
Xenorhabdus nematophila strain ES98 16S ribosomal RNA gene, partial sequence	1051	1051	100%	0.0	99%	FJ860888.1

Appendix 2.1a: Contingency sequence of 16S rDNA (top) of bacterial symbiont isolated from *S. carpocapsae* and top 10 BLASTN results (bottom) identifying isolate as *X. nematophila*.

AAGGCCTTCgGGTTGTaAAGTACtTTCAGcgGGGAGGAAGGCAACAGCGtGaAtaGCGCTGTTG
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GTGAAATCCCCGGGCTCAACCTGGGAACGGCATCTaAAACTGGTTGACTAGAGTCTCGTAGAg
GGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGA
AGGCGGccCCCtGGACGAAGACTGACGCTCAGGTGCGAAAGCGTgGGGAGCAAACAGGATTA
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Description	Max score	Total score	Query cover	E value	Ident	Accession
Xenorhabdus bovienii strain SN52 16S ribosomal RNA gene, partial sequence	1362	1362	100%	0.0	99%	KU297676.1
Xenorhabdus bovienii strain XbZ10Z 16S ribosomal RNA gene, partial sequence	1362	1362	100%	0.0	99%	KU312060.1
Xenorhabdus bovienii strain XbZ82 16S ribosomal RNA gene, partial sequence	1362	1362	100%	0.0	99%	KU312059.1
Xenorhabdus bovienii strain Troitsk 16S ribosomal RNA gene, partial sequence	1362	1362	100%	0.0	99%	KJ413080.1
Xenorhabdus bovienii str. CS03 chromosome, complete genome	1362	9518	100%	0.0	99%	FO818637.1
Xenorhabdus bovienii strain TR03 16S ribosomal RNA gene, partial sequence	1362	1362	100%	0.0	99%	KF945997.1
Xenorhabdus bovienii strain GE02 16S ribosomal RNA gene, partial sequence	1362	1362	100%	0.0	99%	KF945996.1
Xenorhabdus bovienii strain Sk_BU 16S ribosomal RNA gene, partial sequence	1362	1362	100%	0.0	99%	KF437824.1
Xenorhabdus bovienii gene for 16S rRNA, partial sequence, strain: SM	1362	1362	100%	0.0	99%	AB507818.1
Xenorhabdus bovienii strain LB24 16S ribosomal RNA gene, partial sequence	1362	1362	100%	0.0	99%	HM140699.1

Appendix 2.1b: Contingency sequence of 16S rDNA (top) of bacterial symbiont isolated from *S. feltiae* and top 10 BLASTN results (bottom) identifying isolate as *X. bovienii*.

tACgGGagGCAGCagtgGGGAAtattgcnnAATgGGcGCAAGCCTGatGCAGcCAtnccgCGtgtatgaA
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Description	Max score	Total score	Query cover	E value	Ident	Accession
Xenorhabdus ehlersii strain SN22 16S ribosomal RNA gene, partial sequence	1194	1194	100%	0.0	99%	KX870121.1
Xenorhabdus beddingii from China 16S ribosomal RNA gene, partial sequence	1194	1194	100%	0.0	99%	GU480984.1
Xenorhabdus ehlersii strain CN01 16S ribosomal RNA gene, partial sequence	1194	1194	100%	0.0	99%	DQ208307.1
Xenorhabdus ehlersii strain USCA98 16S ribosomal RNA gene, partial sequence	1194	1194	100%	0.0	99%	DQ202312.1
Xenorhabdus ehlersii strain KR03 16S ribosomal RNA gene, partial sequence	1194	1194	100%	0.0	99%	DQ202306.1
Xenorhabdus ehlersii strain DSM 16337 16S ribosomal RNA gene, partial sequence	1194	1194	100%	0.0	99%	NR_042327.1
Xenorhabdus ehlersii strain BDH 16S ribosomal RNA gene, partial sequence	1188	1188	100%	0.0	99%	JQ026406.1
Xenorhabdus ehlersii strain KR02 16S ribosomal RNA gene, partial sequence	1188	1188	100%	0.0	99%	DQ208308.1
Xenorhabdus sp. WS9 16S ribosomal RNA gene, partial sequence	1166	1166	100%	0.0	98%	KT954035.1
Xenorhabdus sp. MY8 NJ gene for 16S rRNA, partial sequence	1155	1155	100%	0.0	98%	AB507811.1

Appendix 2.1c: Contingency sequence of 16S rDNA (top) of bacterial symbiont isolated from *S. longicaudum* and top 10 BLASTN results (bottom) identifying isolate as *X.ehlersii*.

gaAgaaggcctTCgGGTTGTaAAGTACtTTCAGCgGGGaGGAAgGGtTTAGCCTGAAnnnGGTTGA
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CTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA
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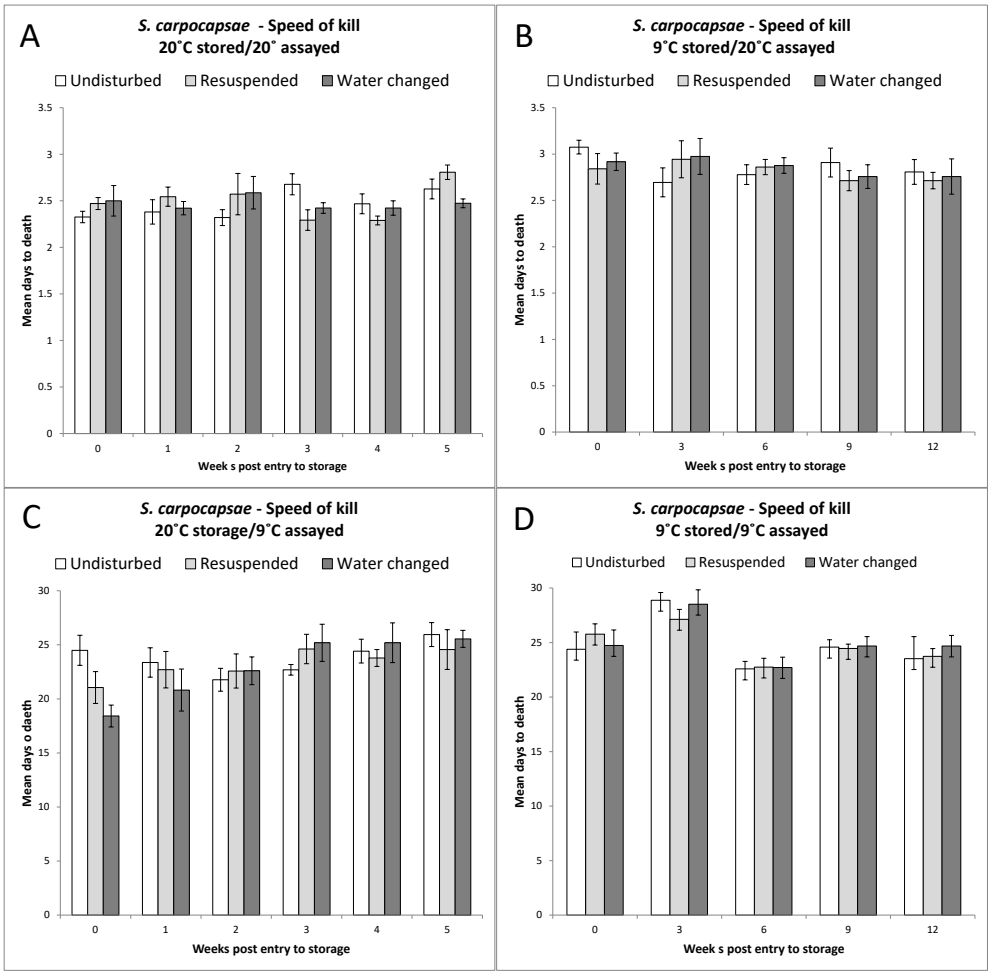
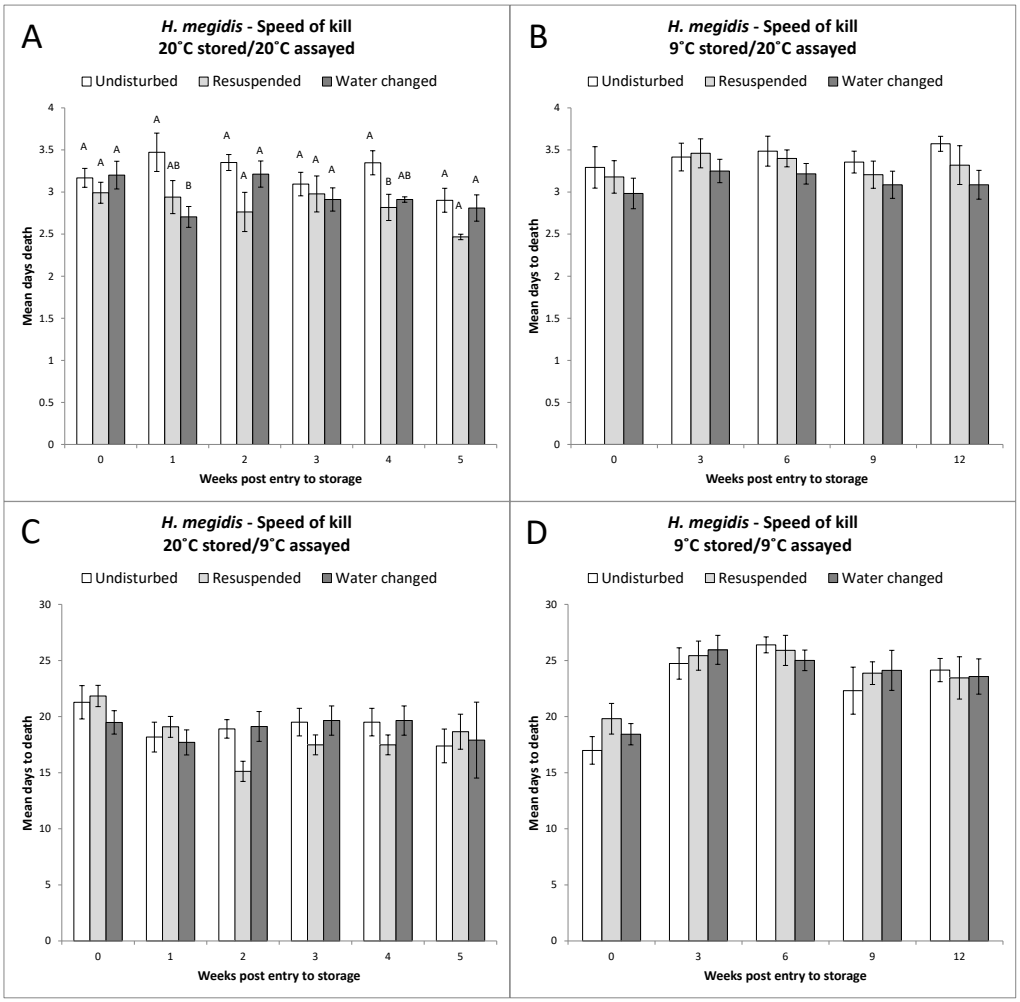
Description	Max score	Total score	Query cover	E value	Ident	Accession
Photorhabdus temperata subsp. temperata strain PT-Hm-N 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	KY290640.1
Photorhabdus temperata strain SN187 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	KU240001.1
Photorhabdus temperata strain Sottinci 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	KJ413081.1
Photorhabdus temperata strain Chuvashia 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	KJ413076.1
Photorhabdus temperata strain J6 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	HQ685757.1
Photorhabdus temperata strain J5 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	HQ685756.1
Photorhabdus temperata strain J7 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	HQ659754.1
Photorhabdus temperata strain J3 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	HQ659753.1
Photorhabdus temperata strain BE09 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	EU930337.1
Photorhabdus sp. IT gene for 16S rRNA, partial sequence	1081	1081	100%	0.0	99%	AB355869.1

Appendix 2.1d: Contingency sequence of 16S rDNA (top) of bacterial symbiont isolated from *H. megidis* and top 10 BLASTN results (bottom) identifying isolate as *P. temperata temperata*.



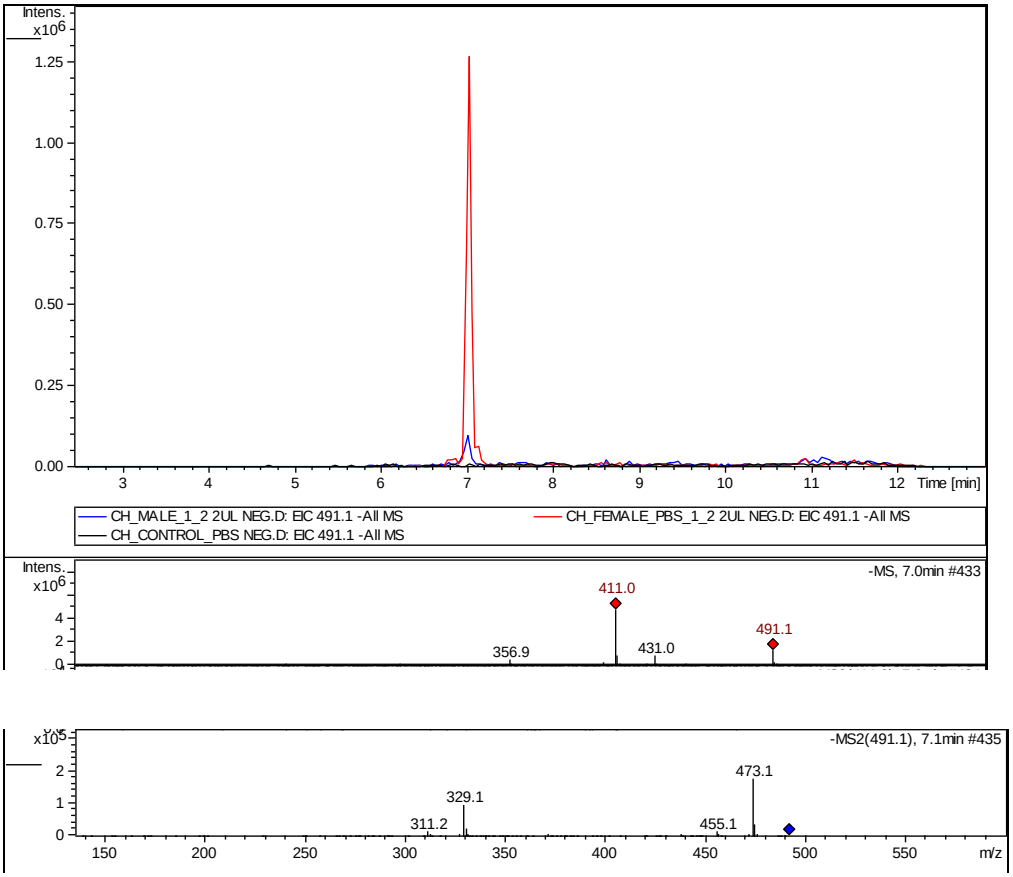
S. carpocapsae			Analysis of variance p values			
Storage temp. (˚C)	**Assay temp (˚C)**	**Days post infection**	**Storage Treatment**	**Storage duration**	**Interaction (treatment x storage duration)**	**Statistical Technique**
20	20	4	0.441	0.302	0.282	GLM
20	9	21	0.359	***0.005***	0.284	GLM
9	9	28	0.939	***<0.001***	0.884	GLM
9	20	4	0.895	0.450	0.699	GLM

Appendix 3.1a: Time to death of *T. molitor* (days) following treatment with *S. carpocapsae* (10 IJ/insect) at either 9˚C or 20˚C. Three different storage treatments of test nematodes were assessed per storage temperature (9˚C and 20˚C) at regular intervals during storage period (5 weeks at 20˚C, 12 weeks at 9˚C). Five replicates of 12 *T. molitor* were assessed per treatment at each time point (Total = 60 insects), corrected for control mortality using Abbotts' formula. Complimentary statistical analysis of data presented in Table (below figure). Error bars show standard error of mean time to death.

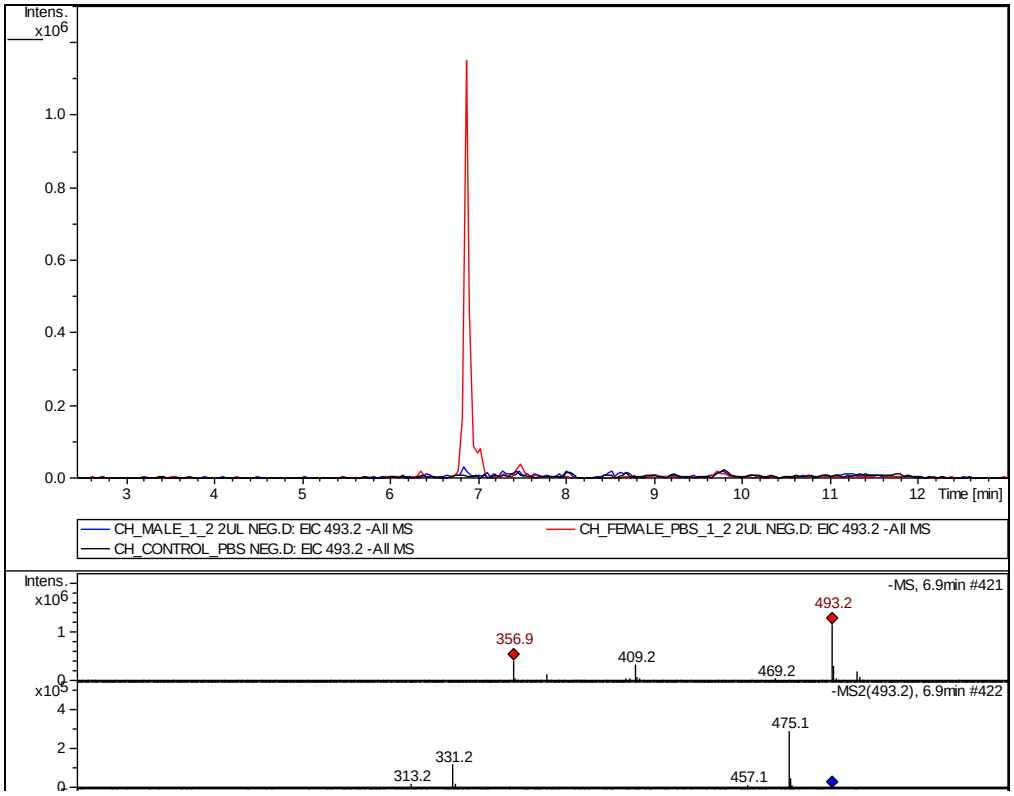


H. megidis			Analysis of variance p values			
Storage temp. (˚C)	**Assay temp (˚C)**	**Days post infection**	**Storage Treatment**	**Storage duration**	**Interaction (treatment x storage duration)**	**Statistical Technique**
20	20	4	***<0.001***	***0.026***	0.128	GLM
20	9	21	0.308	***0.031***	0.312	GLM
9	9	28	0.664	***<0.001***	0.905	GLM
9	20	4	0.073	0.250	0.997	GLM

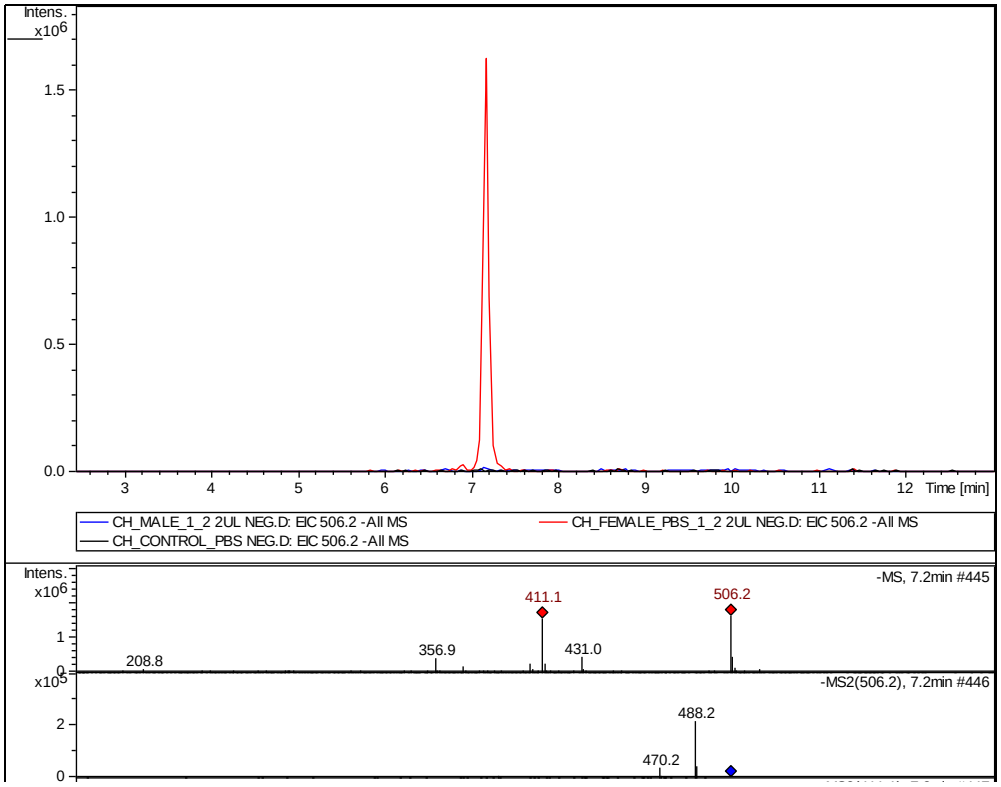
Appendix 3.1b: Time to death of *T. molitor* (days) following treatment with *H. megidis* (10 IJ/insect) at either 9˚C or 20˚C. Three different storage treatments of test nematodes were assessed per storage temperature (9˚C and 20˚C) at regular intervals during storage period (5 weeks at 20˚C, 12 weeks at 9˚C). Five replicates of 12 *T. molitor* were assessed per treatment at each time point (Total = 60 insects), corrected for control mortality using Abbotts' formula. Complimentary statistical analysis of data presented in Table (below figure). Error bars show standard error of mean days to death. Bars which do not share a letter (within week) differ significantly (One-way ANOVA $p < 0.05$, followed by Tukey *post hoc* test.)



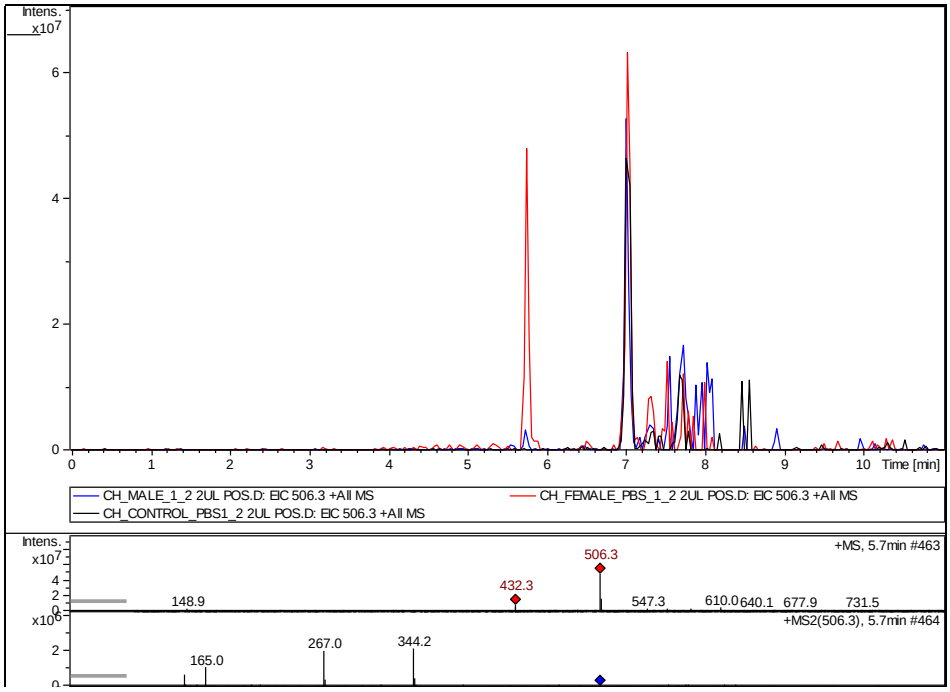
Appendix 5.1a: MS profile of m/z 491 in conditioned PBS from female and male *S. carpocapsae.* (top) Overlaid extracted ion chromatographs (EICs) at *m/z* 491 for conditioned PBS from female (red) and male (blue) *S. carpocapsae*. MS data was extracted to show only the peak intensity for m/z 491. (middle) MS spectrum at 7 min. (bottom) MS/MS spectrum showing fragmentation pattern of m/z 491. Analysis carried out in negative mode.



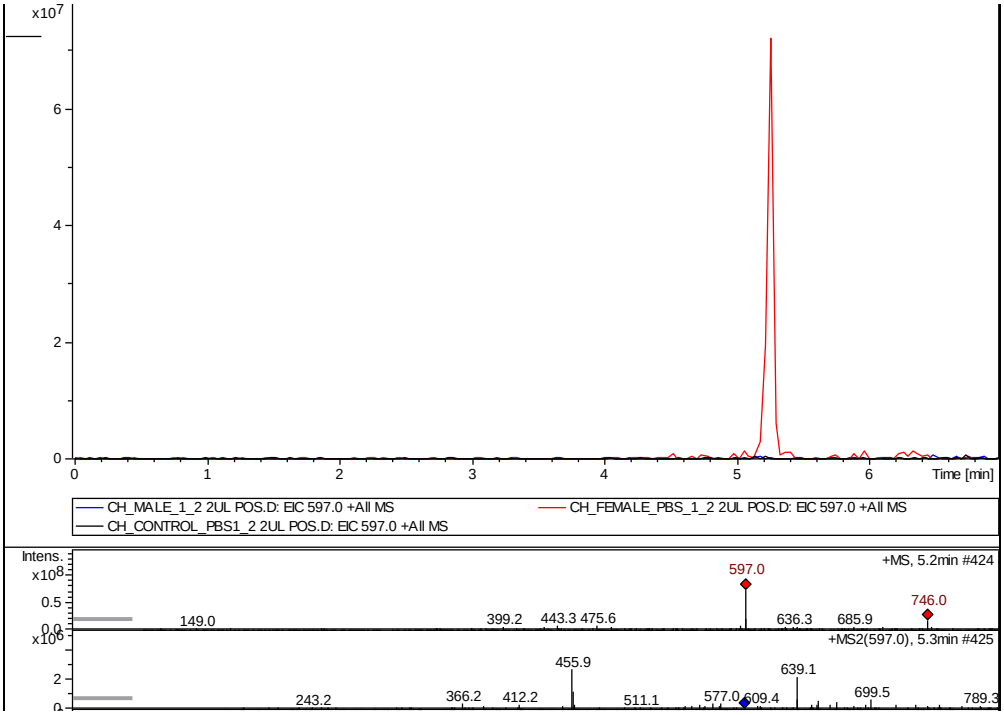
Appendix 5.1b: MS profile of m/z 493.2 in conditioned PBS from female and male *S. carpocapsae.* (top) Overlaid extracted ion chromatographs (EICs) at *m/z* 493.2 for conditioned PBS from female (red) and male (blue) *S. carpocapsae*. MS data was extracted to show only the peak intensity for m/z 493.2. (middle) MS spectrum at 6.9 min. (bottom) MS/MS spectrum showing fragmentation pattern of m/z 493.2. Analysis carried out in negative mode.



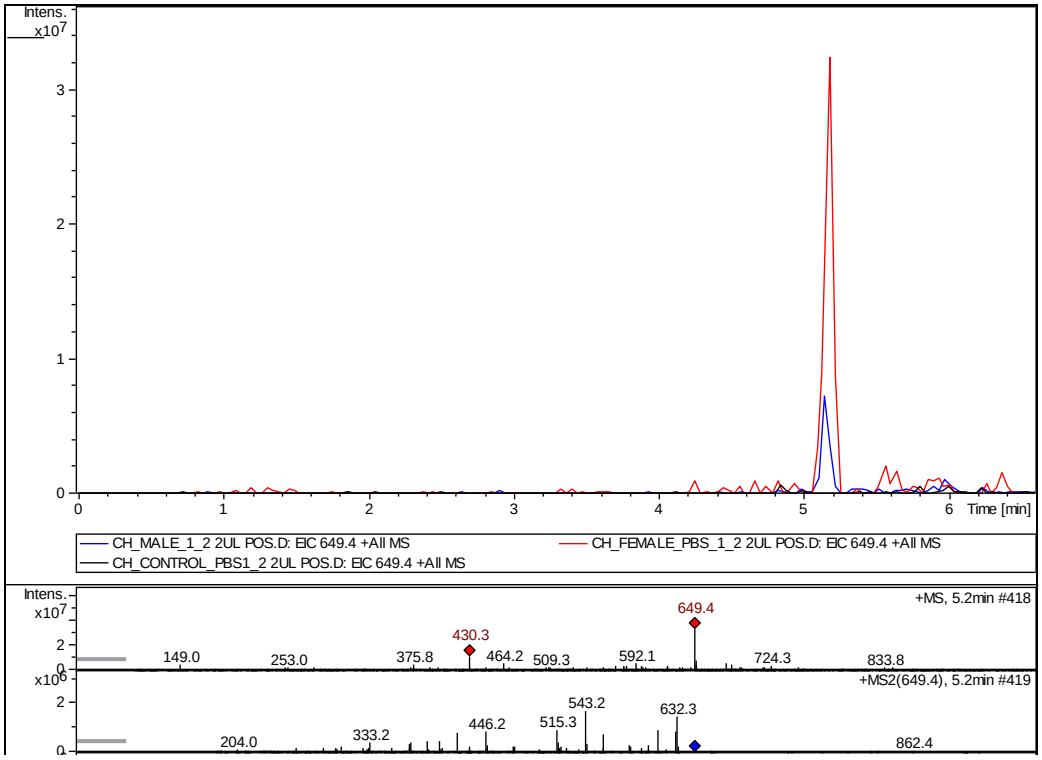
Appendix 5.1c: MS profile of *m/z* 506.2 in conditioned PBS from female and male *S. carpocapsae.* (top) Overlaid extracted ion chromatographs (EICs) at *m/z* 506.2 for conditioned PBS from female (red) and male (blue) *S. carpocapsae*. MS data was extracted to show only the peak intensity for *m/z* 506.2. (middle) MS spectrum at 7.2 min. (bottom) MS/MS spectrum showing fragmentation pattern of *m/z* 506.2. Analysis carried out in negative mode



Appendix 5.1d: MS profile of *m/z* 506.3 in conditioned PBS from female and male *S. carpocapsae.* (top) Overlaid extracted ion chromatographs (EICs) at *m/z* 506.3 for conditioned PBS from female (red) and male (blue) *S. carpocapsae*. MS data was extracted to show only the peak intensity for *m/z* 506.3. (middle) MS spectrum at 5.7 min. (bottom) MS/MS spectrum showing fragmentation pattern of *m/z* 506.3. Analysis carried out in positive mode.



Appendix 5.1e: MS profile of *m/z* 597.0 in conditioned PBS from female and male *S. carpocapsae.* (top) Overlaid extracted ion chromatographs (EICs) at *m/z* 597.0 for conditioned PBS from female (red) and male (blue) *S. carpocapsae*. MS data was extracted to show only the peak intensity for *m/z* 597.0. (middle) MS spectrum at 5.2 min. (bottom) MS/MS spectrum showing fragmentation pattern of *m/z* 597.0. Analysis carried out in positive mode.



Appendix 5.1f: MS profile of *m/z* 649.4 in conditioned PBS from female and male *S. carpocapsae.* (top) Overlaid extracted ion chromatographs (EICs) at *m/z* 649.4 for conditioned PBS from female (red) and male (blue) *S. carpocapsae*. MS data was extracted to show only the peak intensity for *m/z* 649.4 (middle) MS spectrum at 5.2 min. (bottom) MS/MS spectrum showing fragmentation pattern of *m/z* 649.4. Analysis carried out in positive mode.

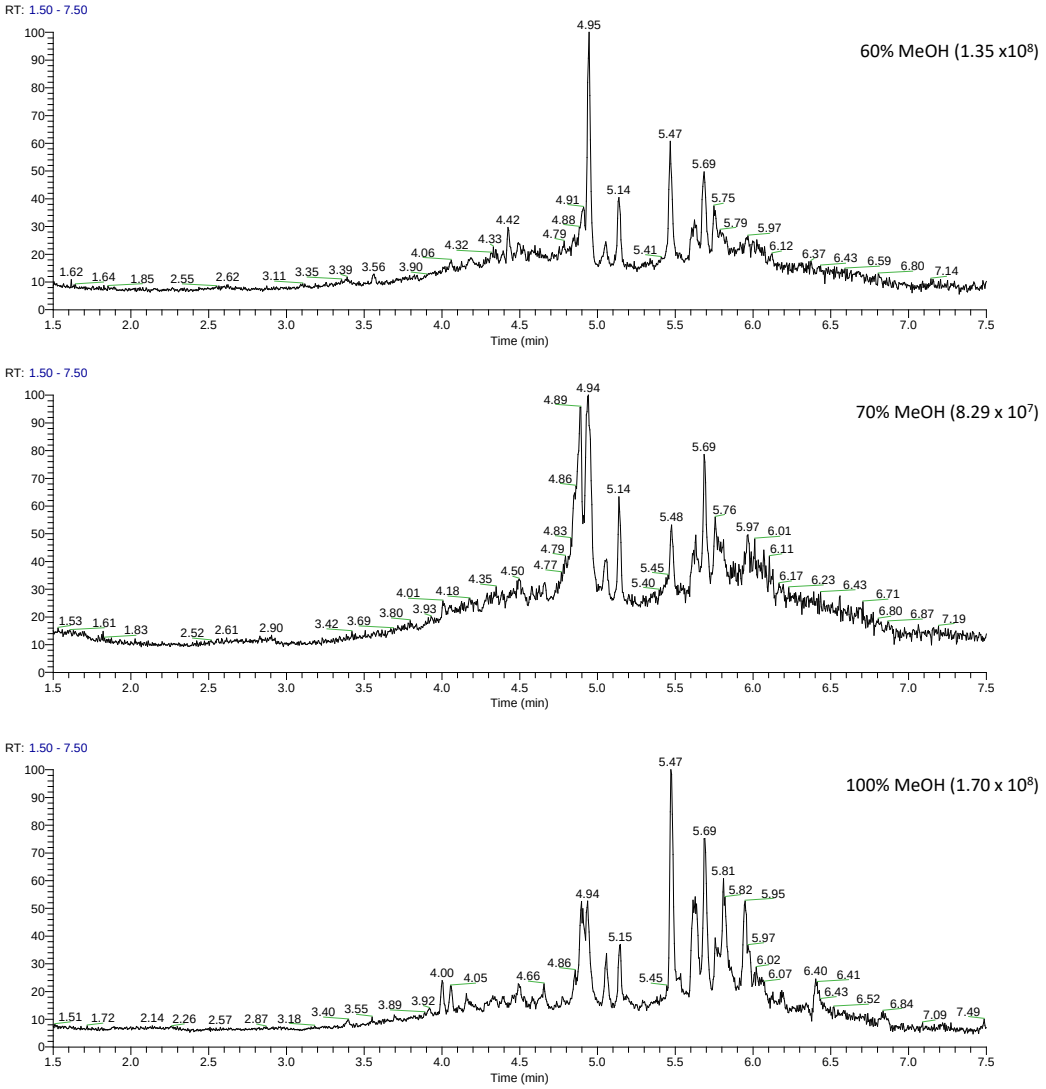
A. *S. carpocapsae*

Concentration	(25S)-Δ-7 dafachronic acid	Δ-4 dafachronic acid
100 µM	1/10 (10%)	0/10 (0%)
100 nM	0/10 (0%)	0/10 (0%)
1nM	0/10 (0%)	0/10 (0%)
10 pM	1/10 (10%)	1/10 (10%)
100 fM	0/10 (0%)	0/10 (0%)
1fM	0/10 (0%)	0/10 (0%)
PBS	0/10 (0%)	
PBS (5% EtOH)	1/10 (10%)	
♀ Exudate	9/10 (90%)	

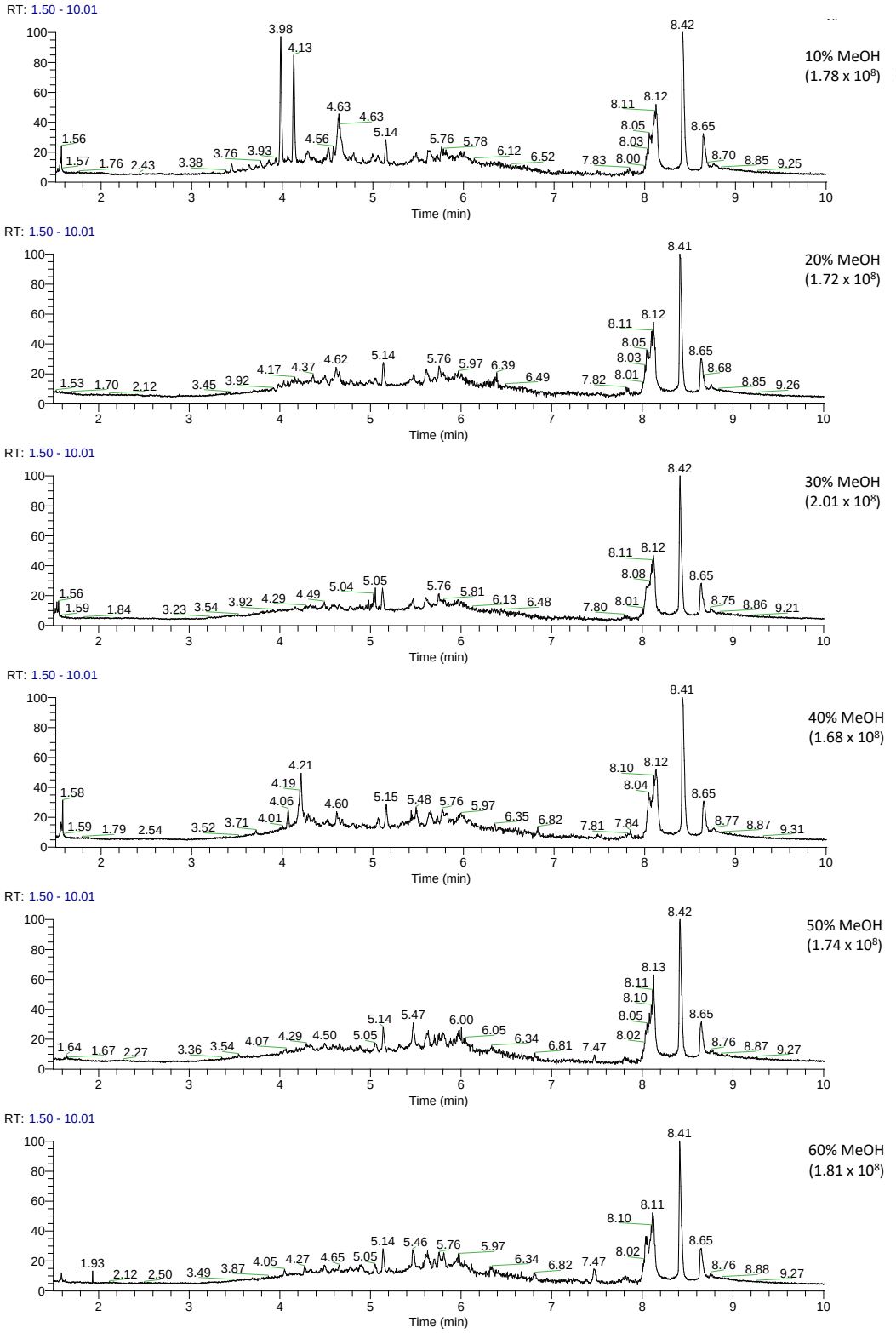
B. *S. longicaudum*

Concentration	(25S)-Δ-7 dafachronic acid	Δ-4 dafachronic acid
100 µM	0/10 (0%)	0/10 (0%)
100 nM	0/10 (0%)	1/10 (10%)
1nM	1/10 (10%)	0/10 (0%)
10 pM	1/10 (10%)	0/10 (0%)
100 fM	0/10 (0%)	1/10 (10%)
1fM	0/10 (0%)	0/10 (0%)
PBS	1/10 (10%)	
PBS (5% EtOH)	1/10 (10%)	
♀ Exudate	10/10 (100%)	

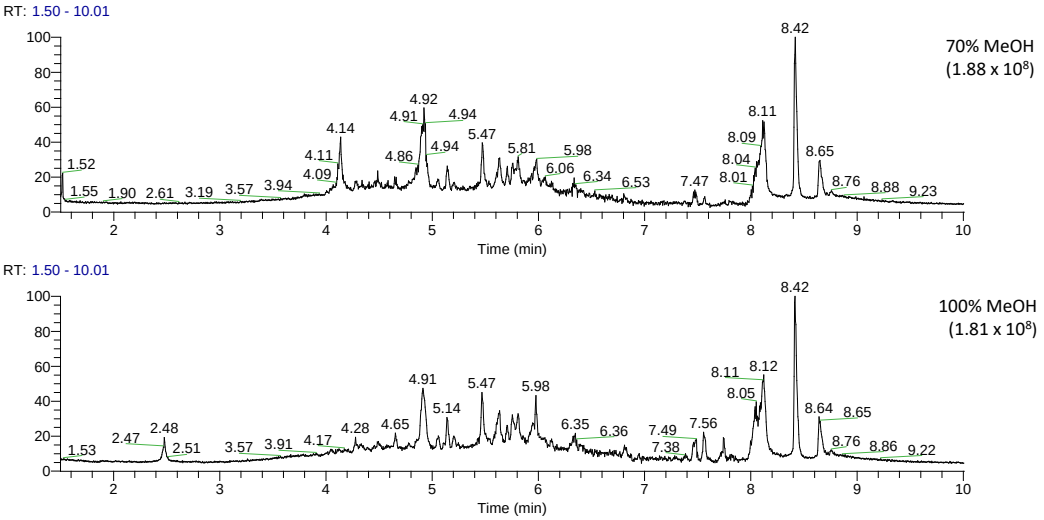
Appendix 5.2: Number (and %) of (A) *S. carpocapsae* and (B) *S. longicaudum* socially naïve males maturing when exposed to varied concentrations of two dafachronic acids ((25S)-Δ-7 DA and Δ-4 DA). Positive (♀ Exudate) and negative (PBS) controls shown in bottom panels of tables. N = 10 per concentration/control treatment.



Appendix 5.3: Total Ion Chromatographs (TICs) of female *S. longicaudum* exudates following stepwise fractionation (60-100% MeOH) on a C18 Sep-Pak column (Rt 1.5 – 7.5min). See figure 5.9 for fractions 10 – 50% MeOH.



Appendix 5.4: Total Ion Chromatographs (TICs) of female *S. carpocapsae* exudates following stepwise fractionation (0-60% MeOH) on a C18 Sep-Pak column (Rt 1.5 – 10min).



Appendix 5.4 (contined): Total Ion Chromatographs (TICs) of female *S. carpocapsae* exudates following stepwise fractionation (70-100% MeOH) on a C18 Sep-Pak column (Rt 1.5 – 10min).

Appendix 6: Statistical analysis of results

Figure numbers correspond to those found in the main body of text (page numbers of corresponding figures in main body are provided in legends). All analysis was carried out using Minitab 17.0.

Figure 3.2: Two-sample T test showing differences in the percentage of IJs dispersing from 2 µl of conspecific cadaver extract (test) or water control 10 mins after stimulus application (see pg. 68).

Test Species	Treatment	N	Mean	StDev	SE	T value	DF	P value
S. carpocapsae	Test	10	85.10	8.39	2.9	-6.50	17	<0.001
	Control	10	59.65	9.10	2.7			
S. longicaudum	Test	10	79.02	6.94	2.2	-7.28	17	<0.001
	Control	10	55.61	7.44	2.4			
S. feltiae	Test	10	88.39	3.63	1.1	-9.55	13	<0.001
	Control	10	69.94	6.87	2.2			
H. megidis	Test	10	83.57	5.22	1.7	-8.72	16	<0.001
	Control	10	59.64	6.93	2.2			

Figure 3.3: One – way ANOVA (followed by Tukey *post hoc* test see pg. 69) showing differences in the percentage of IJs dispersing from 2 µl of conspecific or heterospecific cadaver extract 10 mins after stimulus application.

Null hypothesis: All means are equal.
Alternative hypothesis: At least one mean is different.
Significance level: $\alpha = 0.05$
Equal variances were assumed for the analysis.

S. carpocapsae

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	9542	2385.58	53.01	<0.001
Error	45	2025	45.00		
Total	49	11567			

S. longicaudum

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	6532	1632.93	23.62	<0.001
Error	45	3111	69.12		
Total	49	9642			

S. feltiae

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	3688	922.02	10.32	<0.001
Error	45	4019	89.31		
Total	49	7707			

H. megidis

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	10000	2499.96	60.94	<0.001
Error	45	1846	41.02		
Total	49	11846			

Figure 3.4: Two-sample T test showing differences in the percentage of IJs dispersing from 2 µl of water conditioned with 5000IJ/ml of conspecific IJs for 7days (test) or water control 10 mins after stimulus application (see pg. 71). Note as data was not normal for *S. longicaudum* a Mann-Whitney test was used.

Test Species	Treatment	N	Mean	StDev	SE	T value	DF	P value
S. carpocapsae	Test	30	53.9	15.7	2.9	-4.20	51	<0.001
	Control	30	39.3	10.7	2.0			
S. feltiae	Test	30	57.65	8.68	1.6	-6.14	56	<0.001
	Control	30	44.86	7.39	1.3			
H. megidis	Test	30	54.57	6.48	1.2	-5.01	50	<0.001
	Control	30	43.99	9.59	1.8			

Test Species	Treatment	N	Mean	StDev	SE	W value	DF	P value
S. longicaudum	Test	30	57.1	25.7	3.6	777.0	-	0.0421
	Control	30	42.5	19.8	3.6			

Figure 3.5: One – way ANOVA (followed by Tukey *post hoc* test, see p. 73) showing differences in the percentage of IJs dispersing from 2 µl of water conditioned with conspecific IJs at different densities 10 mins after stimulus application.

Null hypothesis: All means are equal.
Alternative hypothesis: At least one mean is different.
Significance level: $\alpha = 0.05$
Equal variances were assumed for the analysis.

S. longicaudum

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	4988	1246.94	27.33	<0.001
Error	45	2053	45.62		
Total	49	7041			

S. carpocapsae

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	764.0	191.00	3.79	0.010
Error	45	2267.9	50.40		
Total	49	3031.9			

S. feltiae

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	2070	517.45	9.48	<0.001
Error	45	2456	54.59		
Total	49	4526			

H. megidis

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	1039	259.80	5.32	0.001
Error	45	2198	48.85		
Total	49	3238			

Figure 3.6: One – way ANOVA (followed by Tukey *post hoc* test, see pg. 74) showing differences in the percentage of IJs dispersing from 2 µl of water conditioned with conspecific IJs at 5000IJ/ml for either 1 or 7 days 10 mins after stimulus application. Control was water only.

Null hypothesis: All means are equal.
Alternative hypothesis: At least one mean is different.
Significance level: $\alpha = 0.05$
Equal variances were assumed for the analysis.

S. carpocapsae

A: Total dispersed IJs
One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	463.7	231.85	2.37	0.112
Error	27	2639.0	97.74		
Total	29	3102.7			

B: > 1.5 cm dispersed IJs
One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	442.7	221.37	3.22	0.056
Error	27	1858.7	68.84		
Total	29	2301.5			

S. longicaudum

A: Total dispersed IJs
One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	691.8	345.89	5.18	0.013
Error	27	1804.2	66.82		
Total	29	2495.9			

B: > 1.5 cm dispersed IJs
One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	805.7	402.87	11.34	<0.001
Error	27	959.4	35.53		
Total	29	1765.2			

S. feltiae

A: Total dispersed IJs

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	717.5	358.75	4.16	0.027
Error	27	2328.2	86.23		
Total	29	3045.7			

B: > 1.5 cm dispersed IJs

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	123.7	61.84	6.05	0.007
Error	27	275.9	10.22		
Total	29	399.5			

H. megidis

A: Total dispersed IJs

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	443.2	221.62	3.27	0.054
Error	27	1832.3	67.86		
Total	29	2275.5			

B: > 1.5 cm dispersed IJs

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	49.68	24.84	1.66	0.209
Error	27	404.54	14.98		
Total	29	454.21			

Figure 3.7: One – way ANOVA (followed by Tukey *post hoc* test, see pg. 75) showing differences in the percentage of IJs dispersing from 2 µl of water conditioned with either conspecific or heterospecific IJs (5000IJ/ml for 7 days) 10 mins after stimulus application. Control was water only. Also shown for each test species is an additional Dunnett *post hoc* test comparing percentage dispersal of each heterospecific stimulus with conspecific stimulus control. (water control omitted in this analysis).

Null hypothesis: All means are equal.
Alternative hypothesis: At least one mean is different.
Significance level: $\alpha = 0.05$
Equal variances were assumed for the analysis.

S. carpocapsae

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	3366	841.5	4.69	0.003
Error	45	8071	179.4		
Total	49	11438			

Dunnett Multiple Comparisons with a conspecific stimulus control (heterospecific means not labeled with the letter A are significantly different from the control level mean):

Stimulus	N	Mean	Grouping
S. carpocapsae (control)	10	63.66	A
S. longicaudum	10	60.68	A
S. feltiae	10	55.92	A
H. megidis	10	62.40	A

S. longicaudum

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	991.7	247.92	5.05	0.002
Error	45	2207.2	49.05		
Total	49	3198.9			

Dunnett Multiple Comparisons with a conspecific stimulus control (heterospecific means not labeled with the letter A are significantly different from the control level mean):

Stimulus	N	Mean	Grouping
S. carpocapsae	10	29.99	A
S. longicaudum (control)	10	31.38	A
S. feltiae	10	34.53	A
H. megidis	10	35.98	A

S. feltiae

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	1729	432.17	11.65	<0.001
Error	45	1669	37.10		
Total	49	3398			

Dunnett Multiple Comparisons with a conspecific stimulus control (heterospecific means not labeled with the letter A are significantly different from the control level mean):

Stimulus	N	Mean	Grouping
S. carpocapsae	10	54.81	A
S. longicaudum	10	57.55	A
S. feltiae (control)	10	51.07	A
H. megidis	10	58.372	-

H. megidis

One – way ANOVA

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	2286	571.50	18.34	<0.001
Error	45	1402	31.16		
Total	49	3688			

Dunnett Multiple Comparisons with a conspecific stimulus control (heterospecific means not labeled with the letter A are significantly different from the control level mean):

Stimulus	N	Mean	Grouping
S. carpocapsae	10	62.64	-
S. longicaudum	10	64.91	-
S. feltiae	10	54.67	A
H. megidis (control)	10	56.49	A

Figure 3.8A: Influence of storage treatment and storage duration on the percentage of *H.megidis* IJs dispersing when stored at **20°C.** Shown are results of GLM which indicate whether storage treatment or storage duration significantly effect dispersal and whether the two variables show interaction. Also shown are results of within week One – way ANOVA which indicate whether storage treatment had a significant effect on percentage dispersal at specified time points throughout storage (see pg 77 for Tukey *post hoc* test results).

General Linear Model (GLM): Dispersal versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	5	26765.1	5353.03	95.41	<0.001
Storage treatment	2	452.8	226.41	4.04	0.019
Interaction(week*treatment)	10	1467.7	146.77	2.62	0.006

Within week One – way ANOVA:

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

One – way ANOVA (Week 0):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	195.3	97.65	1.97	0.159
Error	27	1339.8	49.62		
Total	29	1535.1			

One – way ANOVA (Week 1):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	144.3	72.17	0.89	0.423
Error	27	2196.2	81.34		
Total	29	2340.6			

One – way ANOVA (Week 2):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	25.54	12.77	0.17	0.846
Error	27	2041.72	75.62		
Total	29	2067.26			

One – way ANOVA (Week 3):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	753.8	376.91	7.84	0.002
Error	27	1298.1	48.08		
Total	29	2051.9			

One – way ANOVA (Week 4):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	570.4	285.20	4.70	0.018
Error	27	1638.2	60.67		
Total	29	2208.6			

One – way ANOVA (Week 5):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	231.1	115.57	5.42	0.010
Error	27	575.2	21.30		
Total	29	806.4			

Figure 3.8B: Influence of storage treatment and storage duration on the percentage of *H.megidis* IJs dispersing when stored at **9°C.** Shown are results of GLM which indicate whether storage treatment or storage duration significantly effect dispersal and whether the two variables show interaction. Also shown are results of within week One – way ANOVA which indicate whether storage treatment had a significant effect on percentage dispersal at specified time points throughout storage (see pg 77 for Tukey *post hoc* test results).

General Linear Model (GLM): Dispersal versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	4	19892.0	4972.99	176.54	<0.001
Storage treatment	2	859.9	429.96	15.26	<0.001
Interaction(week*treatment)	8	613.1	76.64	2.72	0.008

Within week One – way ANOVA:

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

One – way ANOVA (Week 0):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	52.12	26.06	0.37	0.694
Error	27	1900.61	70.39		
Total	29	1952.74			

One – way ANOVA (Week 3):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	482.6	241.29	9.84	0.001
Error	27	662.1	24.52		
Total	29	1144.7			

One – way ANOVA (Week 6):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	293.5	146.73	9.95	0.001
Error	27	398.1	14.74		
Total	29	691.5			

One – way ANOVA (Week 9):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	152.7	76.36	4.37	0.023
Error	27	471.4	17.46		
Total	29	624.1			

One – way ANOVA (Week 12):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	492.1	246.07	17.93	<0.001
Error	27	370.6	13.73		
Total	29	862.7			

Figure 3.9A: Influence of storage treatment and storage duration on the percentage of *S.carpocapsae* IJs dispersing when stored at **20°C.** Shown are results of GLM which indicate whether storage treatment or storage duration significantly effect dispersal and whether the two variables show interaction. Also shown are results of within week One – way ANOVA which indicate whether storage treatment had a significant effect on percentage dispersal at specified time points throughout storage (see pg 79 for Tukey *post hoc* test results).

General Linear Model (GLM): Dispersal versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	5	13044	2608.79	43.20	<0.001
Storage treatment	2	1173	586.34	9.71	<0.001
Interaction(week*treatment)	10	1719	171.86	2.85	0.003

Within week One – way ANOVA:

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

One – way ANOVA (Week 0):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	330.3	165.15	2.59	0.094
Error	27	1723.0	63.82		
Total	29	2053.3			

One – way ANOVA (Week 1):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	180.5	90.27	1.86	0.176
Error	27	1313.3	48.64		
Total	29	1493.8			

One – way ANOVA (Week 2):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	431.9	215.93	3.25	0.054
Error	27	1792.5	66.39		
Total	29	2224.4			

One – way ANOVA (Week 3):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	444.4	222.20	3.40	0.048
Error	27	1765.7	65.40		
Total	29	2210.1			

One – way ANOVA (Week 4):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	823.5	411.73	6.08	0.007
Error	27	1827.8	67.69		
Total	29	2651.2			

One – way ANOVA (Week 5):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	680.7	340.34	6.75	0.004
Error	27	1360.5	50.39		
Total	29	2041.2			

Figure 3.9B: Influence of storage treatment and storage duration on the percentage of *S. carpocapsae* IJs dispersing when stored at **9°C.** Shown are results of GLM which indicate whether storage treatment or storage duration significantly effect dispersal and whether the two variables show interaction. Also shown are results of within week One – way ANOVA which indicate whether storage treatment had a significant effect on percentage dispersal at specified time points throughout storage (see pg 79 for Tukey *post hoc* test results).

General Linear Model (GLM): Dispersal versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	4	9910.6	2477.66	70.45	<0.001
Storage treatment	2	645.3	322.65	9.17	<0.001
Interaction(week*treatment)	8	1381.7	172.72	4.91	<0.001

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

One – way ANOVA (Week 0):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	59.11	29.56	1.13	0.339
Error	27	708.49	26.24		
Total	29	767.60			

One – way ANOVA (Week 3):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	71.52	35.76	0.72	0.495
Error	27	1338.50	49.57		
Total	29	1410.02			

One – way ANOVA (Week 6):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	100.7	50.37	1.74	0.195
Error	27	783.0	29.00		
Total	29	883.8			

One – way ANOVA (Week 9):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	418.3	209.17	6.05	0.007
Error	27	933.1	34.56		
Total	29	1351.5			

One – way ANOVA (Week 12):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	1377.3	688.65	18.89	<0.001
Error	27	984.5	36.46		
Total	29	2361.9			

Figure 3.10: Influence of storage treatment and storage duration of *S.carpocapsae* on ability to cause *T. molitor* mortality (%) following application of 10IJ/insect. Shown are results of GLM or Kruskal Wallis test (where data was not normal), which indicate whether storage treatment or storage duration significantly effected ability to cause *T. molitor* mortality and whether the two variables showed interaction. (see pg. 81).

A: 20°C stored/ 20°C assayed (4days post application of *S. carpocapsae*)

Kruskal Wallis test: Mortality versus Storage treatment

Treatment	N	Median	Ave. Rank	Z	H – value*	DF*	P – value*
Undisturbed	30	1.267	51.4	1.50	2.31	2	0.314
Resuspended	30	1.202	42.0	-0.90			
Water changed	30	1.265	43.1	-0.60			

* adjusted for ties

Kruskal Wallis test: Mortality versus Week of storage

Week of storage	N	Median	Ave. Rank	Z	H – value*	DF*	P – value*
0	15	1.264	34.6	-1.77	16.44	5	0.006
1	15	1.266	44.0	-0.24			
2	15	1.140	42.2	-0.54			
3	15	1.274	68.3	3.71			
4	15	1.268	47.4	0.31			
5	15	1.139	36.5	-1.47			

* adjusted for ties

B: 9°C stored/ 20°C assayed (4days post application of *S. carpocapsae*)
General Linear Model (GLM): Mortality versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	4	0.35324	0.08831	0.94	0.450
Storage treatment	2	0.02100	0.01050	0.11	0.895
Interaction(week*treatment)	8	0.52106	0.06513	0.69	0.699

C: 20°C stored/ 9°C assayed (21 days post application of *S. carpocapsae*)
General Linear Model (GLM): Mortality versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	5	1.05507	0.21101	4.42	0.001
Storage treatment	2	0.07909	0.03955	0.83	0.441
Interaction(week*treatment)	10	0.32707	0.03271	0.69	0.735

D: 9°C stored/ 9°C assayed (28 days post application of *S. carpocapsae*)
General Linear Model (GLM): Mortality versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	4	15532.2	3883.06	15.67	<0.001
Storage treatment	2	62.5	31.25	0.13	0.882
Interaction(week*treatment)	8	1187.6	148.45	0.60	0.775

Figure 3.11: One – way ANOVA (followed by Tukey *post hoc* test, see pg. 84) showing differences in *T. molitor* mortality (%) at specified time points during *S. carpocapsae* storage following application of 10IJ/insect (pooled data from all three storage treatments at each time point).

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

A: 20°C stored/ 20°C assayed

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	1146	229.18	2.89	0.019
Error	84	6670	79.41		
Total	89	7816			

B: 20°C stored/ 9°C assayed

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	8073	1614.6	4.56	0.001
Error	84	29753	354.2		
Total	89	37826			

C: 9°C stored/ 9°C assayed

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	15532	3883.1	16.87	<0.001
Error	70	16114	230.2		
Total	74	31646			

Figure 3.12: Influence of storage treatment and storage duration of *H. megidis* on ability to cause *T. molitor* mortality (%) following application of 10IJ/insect. Shown are results of GLM tests which indicate whether storage treatment or storage duration significantly effected ability to cause *T. molitor* mortality and whether the two variables showed interaction. (see pg. 85).

A: 20°C stored/ 20°C assayed (4 days post application of *H. megidis)*
General Linear Model (GLM): Mortality versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	5	0.6164	0.12327	5.13	<0.001
Storage treatment	2	0.3339	0.16695	6.95	0.002
Interaction(week*treatment)	10	0.1379	0.01379	0.57	0.830

B: 9°C stored/ 20°C assayed (4 days post application of *H. megidis)*
General Linear Model (GLM): Mortality versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	4	0.13606	0.034016	1.95	0.114
Storage treatment	2	0.02552	0.012759	0.73	0.486
Interaction(week*treatment)	8	0.02962	0.003702	0.21	0.988

C: 20°C stored/ 9°C assayed (21 days post application of *H. megidis)*
General Linear Model (GLM): Mortality versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	5	0.06265	0.012531	0.77	0.575
Storage treatment	2	0.04701	0.023503	1.44	0.243
Interaction(week*treatment)	10	0.04590	0.004590	0.28	0.983

D: 9°C stored/ 9°C assayed (28 days post application of *H. megidis)*
General Linear Model (GLM): Mortality versus Week, Treatment

	DF	Adj SS	Adj MS	F - Value	P - value
Week of storage	4	0.19653	0.049132	3.35	0.015
Storage treatment	2	0.02407	0.012037	0.82	0.445
Interaction(week*treatment)	8	0.03698	0.004623	0.32	0.957

Figure 3.13: One – way ANOVA (followed by Tukey *post hoc* test, see pg. 87) showing differences in *T. molitor* mortality (%) following application of H. megidis (10IJ/insect) stored in different ways at 20°C and assayed at 20°C (pooled data from all assay time points during 5 week storage).

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

One – way ANOVA:

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	3024	1512.0	5.88	0.004
Error	87	22372	257.1		
Total	89	25396			

Figure 3.14: One – way ANOVA (followed by Tukey *post hoc* test, see pg. 88) showing differences in *T. molitor* mortality (%) at specified time points during *H. megidis* storage following application of 10IJ/insect (pooled data from all three storage treatments at each time point).

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$
Equal variances were assumed for the analysis.

A: 20°C stored/ 20°C assayed

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	5615	1123.0	4.77	0.001
Error	84	19781	235.5		
Total	89	25396			

B: 9°C stored/ 9°C assayed

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	0.1965	0.04913	3.66	0.009
Error	70	0.9401	0.01343		
Total	74	1.1366			

Figure 4.2: Two-sample T test showing differences between the degree of male attraction (AI) to haemolymph with either a virgin female present or absent during assay (see pg .109).

Test Species	Treatment	N	Mean	StDev	SE	T value	DF	P value
S. carpocapsae	Female present	3	0.9333	0.0577	0.033	2.00	3	0.139
	Female absent	3	0.800	0.100	0.058			
S. longicaudum	Female present	3	0.9667	0.0577	0.033	2.12	4	0.101
	Female absent	3	0.8667	0.0577	0.033			

Figure 4.7: One – way ANOVA (followed by Tukey *post hoc* test, see pg. 114) showing differences between degree of male attraction (AI) to PBS conditioned with either unmated or mated female conspecifics (24 and 48 hrs post mating).

Null hypothesis : All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$

Equal variances were assumed for the analysis.

S. longicaudum

One way ANOVA on AI (Unmated, 24 hrs mated, and 48 hrs mated):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time mated	2	0.91000	0.45500	15.17	0.027
Error	3	0.09000	0.03000		
Total	5	1.00000			

S. carpocapsae

One way ANOVA on AI (Unmated, 24 hrs mated, and 48 hrs mated):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time mated	2	0.80333	0.40167	35.70	0.008
Error	3	0.03375	0.01125		
Total	5	0.83708			

Figure 4.8: One – way ANOVA (followed by Tukey *post hoc* test, see pg. 115) showing differences between degree of male attraction (AI) to PBS conditioned with either unmated or mated female conspecifics (0-17 hrs post mating).

Null hypothesis : All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$

Equal variances were assumed for the analysis.

S. longicaudum

One way ANOVA on AI (Unmated, 24 hrs mated, and 48 hrs mated):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time mated	4	1.31933	0.329833	63.84	<0.001
Error	10	0.05167	0.005167		
Total	14	1.37100			

S. carpocapsae

One way ANOVA on AI (Unmated, 24 hrs mated, and 48 hrs mated):

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time mated	4	1.87167	0.467917	70.19	<0.001
Error	10	0.06667	0.006667		
Total	14	1.93833			

Figure 4.11: Two-sample T test showing differences between male attraction (AI) to either conspecific or heterospecific virgin female conditioned PBS (see pg. 119).

Test Species	Treatment	N	Mean	StDev	SE	T value	DF	P value
S. carpocapsae	Conspecific	3	0.717	0.104	0.060	4.57	2	0.045
	Heterospecific	3	0.117	0.202	0.12			
S. longicaudum	Conspecific	3	0.733	0.076	0.044	4.90	2	0.039
	Heterospecific	3	0.050	0.229	0.13			

Figure 4.12: Two-sample T test showing differences between *S. carpocapsae* male attraction (AI) to either C18 extracted conspecific female conditioned PBS or synthetic ascr#9 (see pg. 123).

Stimulus conc.	Treatment	N	Mean	StDev	SE	T value	DF	P value
Original	Female	3	0.5333	0.0667	0.038	7.35	2	0.002
	ascr#9	3	0.1333	0.0667	0.038			
10 fold	Female	3	0.6667	0.0667	0.038	3.47	2	0.074
	ascr#9	3	0.111	0.269	0.16			

Figure 4.13: Two-sample T test showing differences between *S. longicaudum* male attraction (AI) to either C18 extracted conspecific female conditioned PBS or synthetic ascr#9 (see pg. 124).

Stimulus conc.	Treatment	N	Mean	StDev	SE	T value	DF	P value
Original	Female	3	0.4000	0.0667	0.038	3.90	2	0.060
	ascr#9	3	0.022	0.154	0.089			
10 fold	Female	3	0.5778	0.0385	0.022	5.35	2	0.033
	ascr#9	3	-0.156	0.234	0.14			

Figure 4.14: One – Way ANOVA (followed by Dunnet *post hoc* test comparison to 'Full Extract, see pg. 126) showing differences in *S. carpocapsae* male attraction (AI) to C18 extracted fractions (stepwise in 0 – 100% MeOH).

Null hypothesis : All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$

Equal variances were assumed for the analysis.

One-way ANOVA:

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	13	0.8443	0.06495	2.30	0.032
Error	28	0.7917	0.02827		
Total	41	1.6360			

Figure 4.15: One – Way ANOVA (followed by Dunnet *post hoc* test comparison to 'Full Extract, see pg. 126) showing differences in *S. longicaudum* male attraction (AI) to C18 extracted fractions (stepwise in 0 – 100% MeOH).

Null hypothesis : All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$

Equal variances were assumed for the analysis.

One-way ANOVA:

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time mated	13	0.5783	0.04449	1.71	0.115
Error	28	0.7300	0.02607		
Total	41	1.3083			

Figure 4.16: One – Way ANOVA (followed by Tukey *post hoc* test, see pg. 128) showing differences in *S. carpocapsae* male attraction (AI) to C18 extracted fractions in various recombinations.

Null hypothesis : All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$

Equal variances were assumed for the analysis.

One-way ANOVA:

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	0.7240	0.18100	15.74	<0.001
Error	10	0.1150	0.01150		
Total	14	0.8390			

Figure 4.17: One – Way ANOVA (followed by Tukey *post hoc* test, see pg. 128) showing differences in *S. longicaudum* male attraction (AI) to C18 extracted fractions in various recombinations.

Null hypothesis : All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$

Equal variances were assumed for the analysis.

One-way ANOVA:

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	4	0.65833	0.164583	17.95	<0.001
Error	10	0.09167	0.009167		
Total	14	0.75000			

Figure 4.18: One – Way ANOVA (followed by Tukey *post hoc* test, see pg. 131) showing differences in male attraction (AI) to conspecific female conditioned PBS (original sample, C18 extracted in 100% MeOH, column flow-through and recombination of retentate and flow-through).

Null hypothesis : All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$

Equal variances were assumed for the analysis.

A: *S. carpocapsae*

One-way ANOVA:

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	0.42750	0.142500	22.80	<0.001
Error	8	0.05000	0.006250		
Total	11	0.47750			

B: *S. longicaudum*

One-way ANOVA:

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	0.2873	0.09576	6.96	0.013
Error	8	0.1100	0.01375		
Total	11	0.3973			

Figure 5.3: Pearson Chi Square comparing the proportion of males that sexually mature when exposed to conspecific female conditioned PBS with synthetic ascr#9 at different concentrations (see pg.. 160).

S. carpocapsae

ascr#9 comparison	N	Pearson-Chi Square	DF	P - Value
41.7 ng/ml	20	23.018	1	<0.001
417 ng/ml	20	19.798	1	<0.001

S. longicaudum

ascr#9 comparison	N	Pearson-Chi Square	DF	P - Value
41.7 ng/ml	20	15.000	1	<0.001
417 ng/ml	20	12.379	1	<0.001

Figure: 5.4: Pearson Chi Square comparing the proportion of *S. carpocapsae* males that sexually mature when exposed to conspecific female conditioned PBS with components of C18 fractionation of original female conditioned sample (see pg. 162).

A: C18 SPE in 100% MeOH

Stimulus	N	Pearson-Chi Square	DF	P - Value
Retentate	10	7.500	1	0.006
Flow-through	10	5.495	1	0.019
Recombination	10	0.392	1	0.531
PBS (control)	10	16.364	1	<0.001

B: C18 stepwise fractionation

Stimulus (% MeOH fraction)	N	Pearson-Chi Square	DF	P - Value
Flowthrough	10	7.500	1	0.006
10%	10	3.810	1	0.051
20%	10	0.392	1	0.531
30%	10	5.495	1	0.019
40%	10	7.500	1	0.006
50%	10	2.400	1	0.121
60%	10	7.500	1	0.006
70%	10	9.899	1	0.002
100%	10	7.500	1	0.006
PBS (control)	10	16.364	1	<0.001

C: Fraction recombination

Stimulus (% MeOH fraction)	N	Pearson-Chi Square	DF	P - Value
Flowthrough	10	7.200	1	0.007
All fractions (10-100%)	10	0.267	1	0.606
10 + 20 + 30%	10	0.952		0.329
10 + 20 + 30 + 50%	10	0.952		0.329
40 + 60 + 70 + 100%	10	9.899		0.002

Figure: 5.5: Pearson Chi Square comparing the proportion of *S. longicaudum* males that sexually mature when exposed to conspecific female conditioned PBS with components of C18 fractionation of original female conditioned sample (see pg. 165).

A: C18 SPE in 100% MeOH

Stimulus	N	Pearson-Chi Square	DF	P - Value
Retentate	10	5.051	1	0.025
Flow-through	10	5.051	1	0.025
Recombination	10	0.952	1	0.329
PBS (control)	10	13.333	1	<0.001

B: C18 stepwise fractionation

Stimulus (% MeOH fraction)	N	Pearson-Chi Square	DF	P - Value
Flowthrough	10	7.200	1	0.007
10%	10	3.333	1	0.068
20%	10	1.978	1	0.160
30%	10	5.051	1	0.025
40%	10	5.051	1	0.025
50%	10	7.200	1	0.007
60%	10	13.333	1	<0.001
70%	10	9.899	1	0.002
100%	10	7.200,	1	0.007

C: Fraction recombination

Stimulus (% MeOH fraction)	N	Pearson-Chi Square	DF	P - Value
Flowthrough	10	5.051	1	0.025
All fractions (10-100%)	10	0.267	1	0.606
10 + 20 %	10	0.952	1	0.329
10 + 20 + 30 + 40%	10	0.267	1	0.606
50 + 60 + 70 + 100%	10	5.051	1	0.025
30 + 40 + 50 + 60 + 70 + 100%	10	1.978	1	0.160