# The Molecular Signatures of Adaptive Plasticity in Parasitic Nematodes


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

By

**Peter Evan Lillis, BSc**

**Supervisor:**

Prof. Christine T. Griffin

**Co. Supervisor:**

Dr James C. Carolan

**Head of Department:**

Prof. Paul Moynagh

Ph.D. 2021

## Declaration of Authorship

This thesis has not been previously submitted in whole or in part to this or any other university for any other degree. This thesis is the sole work of the author, with the exception of bioinformatics work performed by Dr Jamie McGowan, Dr Charley McCarthy, and Dr David Fitzpatrick, and the previous work of Dr Cathryn Hartley who shares first-authorship on the first paper.

**Signed** _____

**Date** _____

# Table of Contents

# Figures

# Tables

# List of Presentations and Publications

**2022** ***Draft*** Peter E. Lillis, Ian P. Kennedy, James C. Carolan, Christine T. Griffin. Low temperature exposure has immediate and lasting effects on the behaviour and proteome of entomopathogenic nematodes

**2022** Lillis, P., Griffin, C. and Carolan, J. The effect of temperature conditioning (9°C and 20°C) on the proteome of entomopathogenic nematode infective juveniles. PLOS ONE, 17(4), p.e0266164.

**2019** **Publication:** Hartley, C., Lillis, P., Owens, R. and Griffin, C. Infective juveniles of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) secrete ascarosides and respond to interspecific dispersal signals. Journal of Invertebrate Pathology, 168

**2019** **Presentation:** Lillis. P., Griffin. C., Carolan. J. Proteomic profiling of Proteotoxic Stress on two related species of EPN (Irish Society of Parasitology Meeting, Belfast).

**2019** **Presentation:** Lillis, P., Carolan, J., Griffin, C. Proteomic profiling of *Steinernema carpocapsae* and *Heterorhabditis megidis* in 20°C and 9°C (SIP Conference, Valencia)

**2019** **Presentation:** Lillis. P., Griffin. C., Carolan. J. Proteomic profiling of environmental diapause in entomopathogenic nematodes (Research day, MU)

**2018** **Presentation:** Lillis. P., Griffin. C., Carolan. J. Proteomic profiling of the effect of conditioning on the proteome of *H. megidis* (AAB, Linnean Society of London)

**2018** **Presentation:** Lillis. P. EPN as alternatives to Chemical pesticides (MU Ignite)

# Acknowledgments

I would like to thank my supervisor Professor Christine Griffin, for the amazing level of support and patience she's provided over the last few years. Initially for hiring me as a research assistant with an inordinate fascination of parasites and for keeping me on as a PhD student so I could continue my research into the strangest organisms in the animal kingdom. I could not have done any of this work without her guidance. I am also indebted to my co-supervisor Dr Jim Carolan for all the help he has provided, especially with the proteomics side of this project. Thank you to Dr Rebecca Owens for her help with my metabolomic work. The bioinformatics lab was instrumental in helping me with my bioinformatics work, and I couldn't have done this without their help. I could not have pursued this PhD without Maynooth University and their John and Pat Hume Scholarship, which funded this project.

I'd also thank all of Callan for keeping me sane, especially Ciara, Dean, Felipe, Eoin, Merissa and Sarah, and my lab mates, Cathy, Sarajane and Maria. You all made the last few years great craic and were always around to help. A special thanks to those who helped me chair the Postgrad/Postdoc Committee, without your help I would not have been able to get any of my own work done.

Thanks to all the staff in the biology department, from the admin staff to the technicians, who work tirelessly to ensure that everything is working perfectly. Everything you do is greatly appreciated, and I could not have completed this project without your help.

Finally, I'd like to thank my family and my partner for all their love, and most importantly, for tolerating me. I will be forever grateful for their endurance.

# Abbreviations

| | |
|---|---|
| °C: | Degrees Celsius |
| µg: | Microgram |
| µl: | Microlitre |
| µm: | Micrometer |
| µM: | Micromolar |
| Ach: | Acetylcholine |
| ANOVA: | Analysis of variance |
| Ascr: | Ascaroside |
| ATP: | Adenosine triphosphate |
| cDNA: | Complementary DNA |
| CI: | Chemotaxis Index |
| Cm: | Centimetres |
| $CO_2$: | Carbon Dioxide |
| DA: | Differentially abundant |
| DNA: | Deoxyribonucleic acid |
| DTT: | Dithiothreitol |
| EPN: | Entomopathogenic nematodes |
| ESTs: | Expressed sequence tags |
| eV: | Electronvolts |
| FAR: | Fatty acid and retinoid binding proteins |
| FDR: | False discovery rate |
| Fig.: | Figure |
| GC-MS: | Gas chromatography-mass spectrometry |
| GLM: | General linear model |
| GO: | Gene ontology |
| GPCRs: | G protein coupled receptors |
| GST: | Glutathione S-transferase |
| H: | Hours |

| | |
|---|---|
| Hbas: | Hydroxybenzoyl ascaroside |
| HPLC: | High performance liquid chromatography |
| HSP: | Heat shock protein |
| IAA: | Iodacetamide |
| ICAS: | Indole ascaroside |
| IJ: | Infective juvenile |
| J1/J2: | $1^{st}/2^{nd}$ stage juvenile nematodes |
| kDa: | Kilodalton |
| LC-MS/MS: | Liquid chromatography tandem mass spectrometry |
| LEA: | Late embryogenesis abundant |
| LFQ: | Label free quantitation |
| m/z: | Mass to charge |
| M: | Molar |
| ml: | Millilitre |
| mm: | Millimetre |
| mRNA: | Messenger ribonucleic acid |
| MS/MS: | Tandem mass spectrometry |
| MS: | Mass spectrometry |
| nL: | Nanolitres |
| nm: | Nanometres |
| NPA: | Nematode polyprotein allergens/antigens |
| nrD: | Nerve ring dorsal |
| nrV: | Nerve ring ventral |
| PCA: | Principal component analysis |
| Pfam: | Protein family |
| RH: | Relative humidity |
| RNA: | Ribonucleic acid |
| ROS: | Reactive oxygen species |
| SD: | Standard deviation |
| ± SE: | Plus or minus the standard error |

| | |
|---|---|
| sHSPs: | Small heat shock proteins |
| SOLid: | Sequencing by oligo ligation detection |
| SS: | Statistically significant |
| SSDA: | Statistically significant differentially abundant |
| TCA cycle: | Tricarboxylic acid cycle |
| TFA: | Trifluoroacetic acid |
| UV: | Ultraviolet |
| v/v: | Volume per volume |
| VLCAs: | Very long chain ascarosides |
| w/v: | Weight per volume |
| wks: | Weeks |
| x g: | Times gravity |

# Abstract

Entomopathogenic nematodes (EPN; *Steinernema* spp. and *Heterorhabditis* spp.) are parasites which kill and reproduce within insects. The infective juvenile (IJ) is a developmentally arrested, nonfeeding, stress tolerant stage which forages in the soil for new insects to infect. IJs carry symbiotic bacteria which aid killing the insect and converts the host's tissues to a nutritive liquid which the EPN can consume. EPN are promising biocontrol agents for the control of insect pests and are also model organisms for the study of parasitism. The objective of this project was to characterise the plasticity of IJ behaviour, and to investigate some of the associated molecular processes. There are two broad components: firstly, the identification of molecules (ascarosides) externally secreted by IJs and their effects on dispersal, and secondly changes in behaviour and stress tolerance of IJs over time during storage at different temperatures, and associated changes in the proteome.

When the host cadaver becomes overcrowded, there is a build-up of signals which encourage newly formed IJs to disperse, thus reducing competition between the many IJs arising from the same cadaver. Among these chemicals are members of a class of pheromones, ascarosides, which have profound effects on IJ behaviour and development. Whether or not these IJs continue producing ascarosides into the surrounding medium, the composition of the mixture, the abundance of these pheromones and whether they affect the behaviour of conspecifics and heterospecifics was investigated. The IJs of four EPN species, *Steinernema carpocapsae, Steinernema longicaudum, Steinernema feltiae,* and *Heterorhabditis megidis*, were collected and stored in water at 5000 IJs/ml. The worm conditioned water was analysed with LC-MS/MS after storage in 20°C for specific timepoints. Various ascarosides were detected, generally increasing in abundance the longer the IJs were stored in the water, indicating that these IJs secrete ascarosides

consistently after emergence from the host. The composition of ascarosides detected was species specific however the most abundant ascaroside detected in all species was Ascr#9, similar to many other insect-associated species. The worm-conditioned water from both conspecifics and heterospecifics was shown to induce dispersal behaviour in each of the species in agar plate assays. The species-specificity and complexity of ascaroside secretion, despite the similar effects on dispersal, implies that it has other ecological or biological functions.

The second part of the thesis investigates the effects of time and temperature on stress tolerance, behaviour (chemotaxis) and the proteome of *S. carpocapsae* and *H. megidis* IJs. Previously it has been shown that conditioning IJs in low temperatures (<10°C) enhances not only their freezing tolerance, but their tolerance to other abiotic stressors. This cross tolerance is presumed to be due to the accumulation of non-specific protectants within the IJs. The IJs of *H. megidis* and *S. carpocapsae* were stored in 20°C and 9°C for up to 12 weeks, and at specific timepoints, assays were performed, and their proteins were extracted and analysed via LC-MS/MS.

More proteins were detected within *S. carpocapsae* IJs (2422) than in *H. megidis* IJs (1582). The *S. carpocapsae* proteome was more strongly affected by low temperature storage (9°C), whereas the *H. megidis* proteome changed over time in a similar manner, less influenced by the ambient temperature. Compared to freshly emerged IJs, the highest abundance proteins detected within *S. carpocapsae* IJs at 20°C were proteins related to the cytoskeleton, cell signalling, and infection proteins such as proteases, protease inhibitors and a chitinase. The proteins that were detected at the highest abundance after conditioning at 9°C were late embryogenesis abundant proteins, heat shock proteins, and proteins related to stress tolerance. The proteins which were decreased in abundance to the greatest extent after conditioning at 20°C and 9°C were those related to the cytoskeleton and stress

related proteins. The highest abundance proteins detected in *H. megidis* after conditioning in both 20°C and in 9°C were those related to the cytoskeleton, cell signalling, and carbon metabolism. The proteins decreased to the greatest extent after conditioning the IJs in 20°C and 9°C were those related to metabolism, heat shock proteins and ribosomal proteins.

Storage of IJs at low temperatures prolongs their survival. *S. carpocapsae* IJs exhibited increased molecular chaperones over time, and to a greater extent in 9°C. The *H. megidis* IJs exhibited decreased abundance of proteins associated with translation over time. These two responses may represent a species-specific response to proteostatic collapse as the IJs age. Proteostatic collapse is one of the consequences of aging in cells and likely a contributing factor to nematode longevity, as misfolded and damaged proteins and toxic aggregations of proteins begin to accumulate within the cells. Similarly, as nematodes age, reactive oxygen species (ROS) accumulate. Both species demonstrated an increase in abundance of proteins which enhance ROS tolerance, and to a greater extent in 9°C.

Storage of EPN IJs in low temperatures also affects various behaviours such as dispersal, infectivity, and response to host volatiles. If these behaviours are adversely affected, then cold storage of nematodes will be a trade-off between longevity of the nematodes in storage and the efficacy of these parasites when applied to the field as biocontrol agents. After conditioning at 20°C, IJs of both species were attracted to hexanol, methyl salicylate and acetone, and were repelled by prenol (an odour associated with infected hosts). The chemotaxis responses of *H. megidis* to each odorant was enhanced after storage in 9°C, becoming more strongly attracted or repelled to each odour than after storage at 20°C. *S. carpocapsae* IJs tended to act in the opposite manner after exposure to low temperatures, becoming repelled by attractants and strongly attracted to the repellent, prenol. Storage of *H. megidis* IJs at temperatures below the culture temperature of 20°C resulted in IJs with a gradual change in chemotaxis from 9°C to 12°C to 15°C, whereas *S. carpocapsae* showed

a binary response, as all temperatures below culture temperature tested resulted in IJs with similar responses.

To investigate whether IJs retain these altered chemotaxis responses following a return to culture temperature, and how long a period of cold it takes to induce them, IJs from both species were stored at 9°C for brief periods, 3 hours, 1 day and 1 week, and were transferred to 20°C for the duration of the experiment (3 weeks). At the 3-week timepoint, all of the conditioned IJs were exposed to a strong attractant or a strong repellent, and their responses were compared to that of IJs which were stored at 9°C or 20°C for the full 3 weeks. *H. megidis* did not retain cold-altered chemotaxis responses after transfer to 20°C, while *S. carpocapsae* IJs stored at 9°C and transferred to 20°C after just 3 hours retained their altered chemotaxis responses for 3 weeks.

Storage at 9°C for a week enhanced the freezing (-10°C for 6 hours) and desiccation (75% relative humidity) tolerance of *S. carpocapsae* IJs, and this enhanced stress tolerance was retained by those IJs which were transferred to 20°C when tested after 2 weeks at the temperature.

I next explored whether the cold-induced changes in the *S. carpocapsae* proteome were also maintained following return to 20°C, as was found for behaviour and stress tolerance. IJs which were stored at 9°C for the full 3 weeks, and those stored for just for 1 week and then transferred to 20°C for 2 weeks had similar proteomes and were both different to that of IJs stored at 20°C for the full 3 weeks. The first two groups displayed hundredfold increases in the abundance of many molecular chaperones when compared to IJs that were stored at 20°C for the full duration. Conversely, the highest abundance protein in the IJs stored at 20°C for 3 weeks relative to the IJs stored at 9°C was a chitinase, implicated in many roles in nematodes including fungal defence and infection.

The plasticity of EPN IJs enables them to adapt to diverse and changing environments. Proteomic profiling is a useful guide for further elucidating the molecular mechanisms behind these phenotypes, which will facilitate their use as biocontrol agents, and as models for parasitism.

# Chapter 1 Introduction

## 1.1 General introduction

All organisms must be able to dynamically respond to the many challenges they face every day. Stress may be biotic or abiotic in nature. Biotic stressors are derived from one organism towards another organism, such as predation, parasitism, or competition for resources. Abiotic stressors are those caused by the environment, including freezing, desiccation, drought, and osmotic stress. Both sources of stress affect the fitness of organisms, and organisms have evolved a huge range of adaptations to deal with the stressors encountered in their evolutionary history. Adaptations may be constitutive- such as the spine of hedgehogs or cacti- or may consist of being able to alter their behaviour and physiology to cope with these stresses dynamically, as they arise. This thesis investigates the phenotypic plasticity of the infective stage juveniles of entomopathogenic (insect-killing) nematodes (EPN). Despite being relatively simple organisms, nematodes are capable of complex behaviour and physiological adaptations that vary in response to internal and external factors. In this work, I explore how the phenotypes expressed by the nematode changes over time (endogenous factors) and are influenced by the temperature (exogenous factor) experienced during that time.

Organisms may avoid certain biotic stresses, particularly intraspecific competition, with honest chemical communication, producing pheromones or other metabolites. These signalling molecules may communicate the organism's internal state such as sexual status, or environmental information such as food availability. Pheromones mediate biotic stress in a variety of invertebrate species and in nematodes certain pheromones can not only influence behaviour (Kaplan et al., 2012) but also the development of conspecifics,

redirecting their development to a nonfeeding abiotic stress tolerant stage (Noguez et al., 2012) which disperse in search of food, thus avoiding competition.

Organisms must either avoid or endure these stressors, or else they will not survive. Nematodes may avoid abiotic stress by adopting behaviours such as aggregation, clumping which slows desiccation (Burnell and Tunnacliffe, 2011) and thermotaxing away from harmful temperatures (Devaney, 2011), or physiological changes which prevent the stress from damaging the nematode such as losing water slowly to prevent harm from freezing (Wharton et al., 2005) and by ridding their body of "ice nucleating" molecules (Wharton, 2011). Enduring abiotic stress involves the accumulation of protectants which reduce the effect of stressors on the organism (Hibshman et al., 2020).

In addition to their olfaction of pheromones which influence their behaviour, EPN must be sensitive to the volatiles associated with insect hosts. Temperature strongly affects the chemotaxis of these organisms (Lee et al., 2016). These EPN must balance stress tolerance adaptations and behaviours, such as clumping or quiescence and their need to infect an insect before their internal lipid stores are fully utilised. Therefore, EPN behaviour exists on a spectrum which may be altered by both their external environment and their internal state, and the extent to which is affected by either factor is unclear.

In this thesis I document changes in the behaviours and stress tolerance of EPN alongside the accompanying changes in the metabolome and proteome, with the aim of shedding light on some of the molecular mechanisms. Entomopathogenic nematodes are promising biocontrol agents and understanding these mechanisms may enhance their use as alternatives to chemical pesticides, as well as helping to understand how simple organisms may alter their phenotype adaptively.

## 1.2 Entomopathogenic nematode life cycle and omics

Entomopathogenic nematodes are a diverse group of nematodes belonging to two main families, Steinernematidae and Heterorhabditidae. These parasites are found ubiquitously in nature, on all continents (Hominick et al., 1996) except Antarctica (Griffin et al, 1990). The widespread distribution of these species attests to their ability to adapt to a wide array of climates (Grewal and Gaugler, 1994; Shapiro-Ilan et al., 2014a).

### 1.2.1 Life cycle

While both Steinernematidae and Heterorhabditidae may have superficially similar life cycles, these similarities are due to convergent evolution rather than direct ancestry (Poinar, 1993; Blaxter 1998). Both exhibit a stress resistant, nonfeeding, non-reproductive stage of their life cycle known as the infective juvenile (IJ). The IJ is free-living, existing in the soil in search of an insect host to colonise. This stage is analogous to the dauer state of free-living nematodes such as the model organism *Caenorhabditis elegans.* After the successful invasion of an insect host, EPN IJs egest symbiotic bacteria which reproduce and kill the insect via sepsis. Steinernematids associate with *Xenorhabdus* spp., and heterorhabditids associate with *Photorhabdus* spp. (Griffin., 2012). The IJs "recover", and mature to become the first-generation adults, feeding on the necrotic host tissues digested by the bacterium. These adults reproduce, producing successive generations of offspring (figure 1.1). Steinernematids are amphimictic, with few exceptions (Griffin et al., 2001) whereas heterorhabditids are hermaphroditic. While *Heterorhabditis* is exclusively hermaphroditic in the first generation, they do produce XO males in later generations, which can mate with the females/hermaphrodites and enhance the genetic diversity of the offspring.

When the nutrition from the host's tissues is nearing depletion, the offspring of these nematodes undergo an alternative pathway to produce IJs. The early juvenile (J1 and J2) stages feed, grow and arrest their development at the non-feeding IJ stage. In addition to providing nutrition and laying down the lipid reserves which the IJ will rely on for the duration of the stage until it can find a new host, the feeding by the developing juveniles enables the symbiotic bacteria to establish a new association with the juveniles. *Xenorhabdus* bacteria colonise the receptacle, a specialised pouch in the intestine in *Steinernema* spp. (Kim et al., 2012) whereas the *Photorhabdus* bacteria colonise the intestinal lumen of *Heterorhabditis* spp. (Ciche et al., 2008).

EPN IJs are reliant on their bacterial symbiont for breaking down host tissues into a medium the nematodes can consume, along with the bacteria themselves. The IJs themselves produce a complex mix of proteases and inhibitors which disrupt and supress the insect immune system (Lu et al., 2017a), facilitating the bacterial infection and subsequent sepsis (Dziedziech et al., 2020). In addition to their role in killing and digesting the host insect, the symbionts also produce an array of secondary metabolites which protect the cadaver against scavengers and competing microbes (Raja et al., 2021). *Steinernema* and *Heterorhabditis* developed a mutualistic relationship with bacteria independently, however it is unknown exactly when this relationship became obligatory. Axenic *Heterorhabditis* spp. are unable to kill insects however axenic steinernematids can (Burman, 1982; Han and Ehlers, 2000), but the IJs arising from these infections will contain bacteria from the insect's microflora and not their symbionts (Sicard et al., 2003), which will likely impair their fitness (McMullen et al., 2017).

As a member of the superphylum Ecdysozoa, entomopathogenic nematodes moult at each stage of their life cycle. Infective juveniles retain their second stage moults, referred to as a

sheath (Adams and Nguyen., 2002). This helps protect the IJ against abiotic stresses in the soil (see section 1.5) and is normally shed before or at the time of entry into a host.

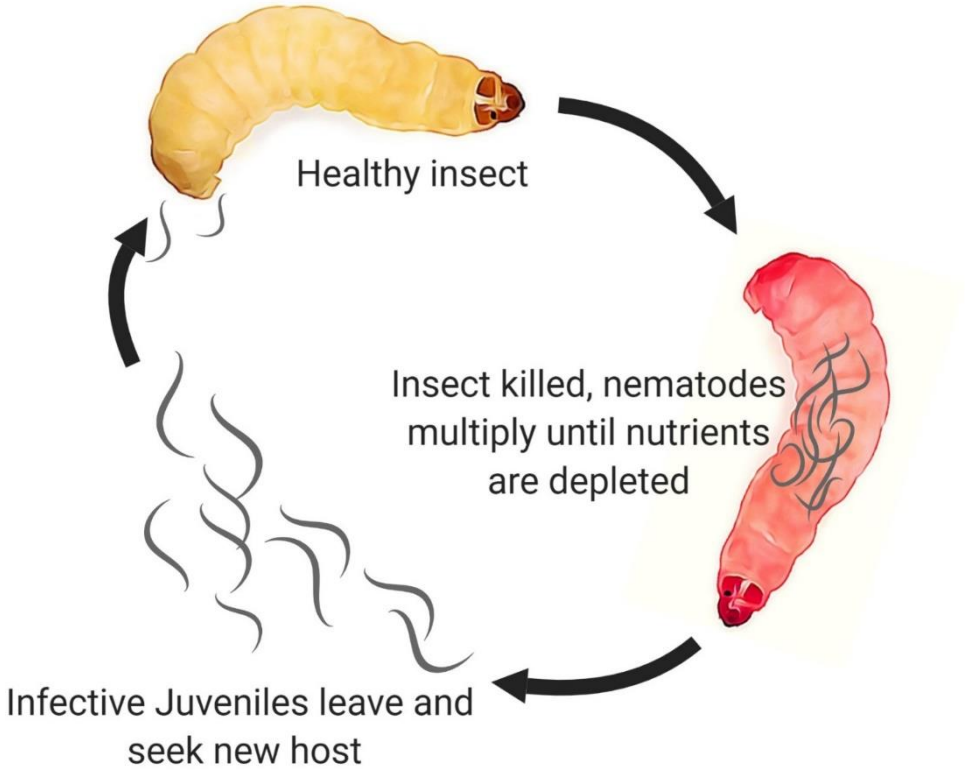



**Figure 1.1:** The general entomopathogenic nematode life cycle.

### 1.2.2 Phylogeny

The life cycle, behaviour, and close association with insect-pathogenic bacteria of *Steinernema* spp. and *Heterorhabditis* spp. may appear to be strikingly similar. However, Poinar (1993) speculated these similarities are due to convergent evolution, and not from shared ancestry. Poinar (1993) concluded that *Heterorhabditis* probably evolved from a *Pellioditis*-like ancestor whereas *Steinernema* were suggested to be descended from a "proto-*Rhabditonema"*, due to similarities in their mouthparts, tails, habitats, and phasmids among other characteristics (figure 1.2). These hypotheses were confirmed by Blaxter's (1998) analysis (figure 1.3). A database of small subunit sequences was

constructed utilising sequences from 53 taxa aligned based on similarity. This analysis placed *S. carpocapsae* closest to *Strongyloides stercoralis* and *Heterorhabditis bacteriophora* was placed closer to *Pellioditis typica.* Around 90 species of steinernematids and 20 species of heterorhabditids have been described (Labaude and Griffin, 2018) and the differences and similarities between both families are becoming more apparent as molecular approaches become more sophisticated.

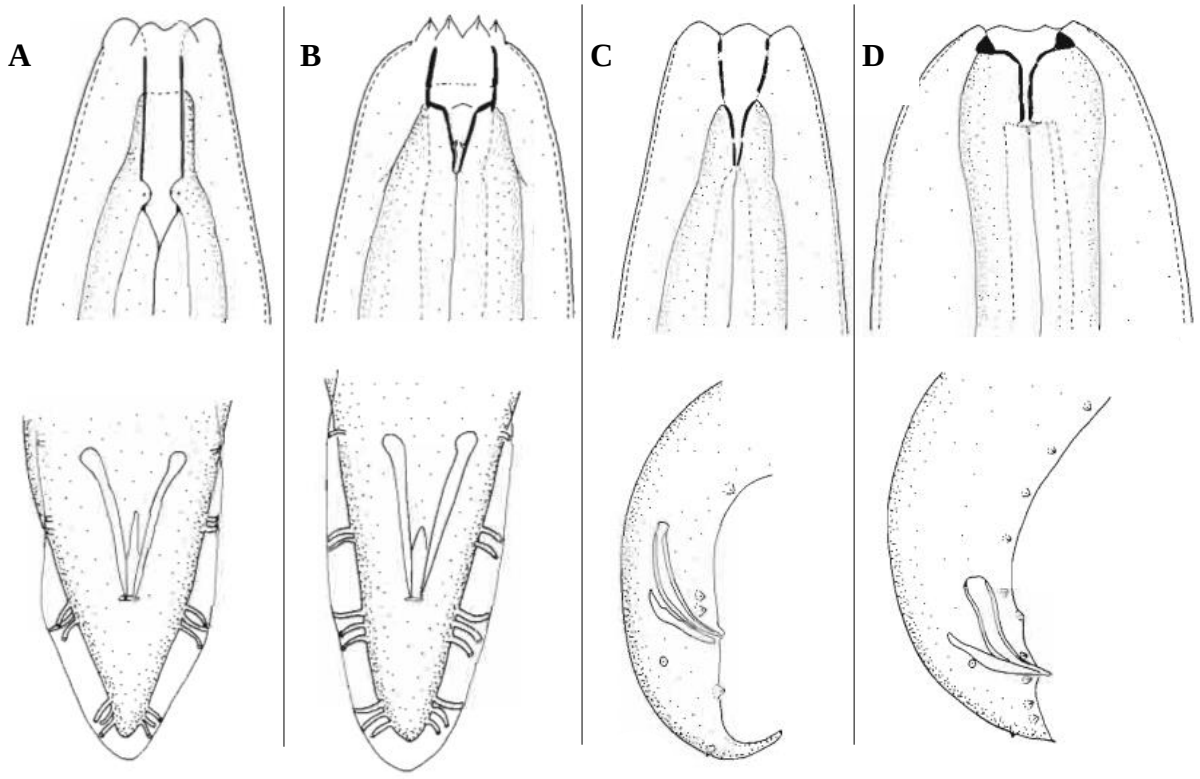



**Figure 1.2:** Mouth parts (top) and tail region (bottom) of *Pellioditis marina* (A), *Heterorhabditis bacteriophora* (B), *Rhabditophanes schneideri* (C), and *Steinernema carpocapsae* (D). These images show the likely closest living relatives of both EPN species, based on phenetics. Adapted from Poinar (1993).

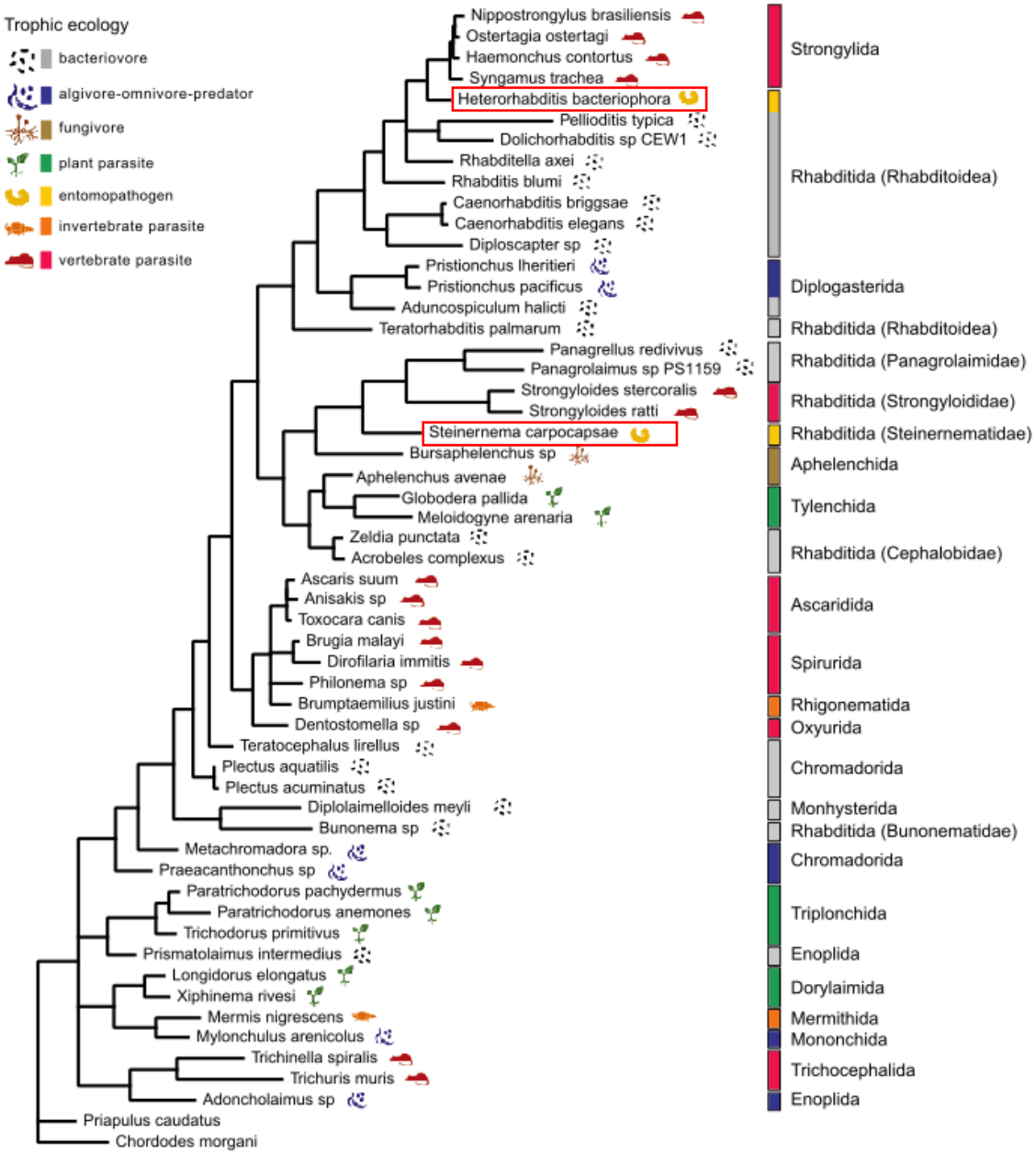



**Figure 1.3:** The phylogenetic tree of Nematoda. This tree was assembled from small subunit sequences from 53 nematode taxa, with representatives from Heterorhabditidae and Steinernematidae highlighted. From Blaxter et al. (1998).

### 1.2.3 Genomics

Nematodes have been a focal point of the genomics community since the first sequencings of a metazoan animal, *C. elegans,* in 1998 (*C. elegans* Sequencing Consortium, 1998). This first genome paved the way for all subsequent analyses and revolutionised the field. The genome of *H. bacteriophora* was sequenced by Bai et al. (2013) and its annotation was improved by McLean et al. (2018). This genome utilised Sanger and Roche 454 sequencing from adult and infective juvenile *H. bacteriophora.* The genome was constructed with 1,263 scaffolds, predicted 20,964 coding genes in a genome of 77 Mb and has an N50 of 3.12 Mb (Bai et al., 2013). The reannotation of this genome analysed the Roche 454 transcriptome with the BRAKER 1 pipeline (McLean et al., 2018; Hoff et al., 2016). The *H. bacteriophora* genome contains an expanded family of mariner transposase motifs when compared to that of the *C. elegans* genome, indicative of a "mobile genome" (Bai et al., 2013). In contrast, the genome contains fewer C-type lectins (Bai et al., 2013), which are involved in the functional immune system in *C. elegans* (Schulenberg et al., 2008) and in immune evasion (Loukas and Maizels, 2000). The new annotation (McLean et al., 2018) significantly improved our understanding of *H. bacteriophora*. Only 603 predicted proteins were originally identified as secreted proteins, much lower than that of free-living nematodes (Bai et al., 2013). The paucity of secreted proteins was thought to have been exhibitive of *H. bacteriophora* IJs' acquisition of and reliance on its symbiont. The subsequent re-annotation of this genome revealed that 1,023 proteins (6.5% of the genome) are secreted, not significantly lower than free living nematodes as previously reported (McLean et al., 2018).

The *S. carpocapsae* All genome and transcriptome were first assembled alongside those of *S. monticolum, S. scapterisci, S. feltiae and S. glaseri* (Dillman et al., 2015). This simultaneous study of five closely related species allowed for the direct comparison of

these genomes, investigation of their phylogenetic relationships and the extent of gene conservation between the species (Dillman et al., 2015). The *S. carpocapsae* genome was assembled with 1,578 contigs, predicted 2,313 coding genes in a genome of 86.1Mb and has an N50 of 2.99 Mb. A subsequent genomic investigation of the *S. carpocapsae* Breton strain not only enhanced the genome for this economically important parasite, but it allowed for the analysis of strain-specific alterations in the genome (Rougon-Cardoso et al., 2016). This genome was assembled with 347 scaffolds, predicted 16,333 genes in a genome of 84.6 Mb, and has an N50 of 1.25 Mb. The newest and most complete *S. carpocapsae* genome was assembled with 16 scaffolds, predicted 30,957 gene models from the *S. carpocapsae* embryo to adult in a genome of 84.5 Mb and has an N50 of 7.36 Mb (Serra et al., 2019). Previous draft genomes were assembled using the 454 GS FLX and SOLiD 5500xl platforms (Rougan-Cardoso et al., 2016), and the Illumina GenomeAnalyzer IIx and Illumina HiSeq 2000 platforms (Dillman et al., 2015). The latest genome utilised a hybrid approach between PacBio long reads and Illumina reads (Illumina Nextseq 500 platform) with subsequent BioNano optical mapping (Serra et al., 2019) and represents the most complete view of the *S. carpocapsae* available to date.

### 1.2.4 Comparisons between the heterorhabditid and steinernematid genomes

The *S. carpocapsae* genome is enriched in metallo- and serine-proteases, protease inhibitors, G protein coupled receptors (GPCRs) and fatty acid and retinol binding (FAR) proteins (Serra et al., 2019; Dillman et al., 2015). These protein families have various functions such as signal transduction, energy storage and digestion within the nematode, however their expansion in the parasitic *Steinernema* spp. relative to that of free-living species suggests that they have a role in the species' novel parasitic lifestyle. Serine proteases and their inhibitors (serpins) are involved in the immune suppression of the

insect pro-phenoloxidase system (Toubarro et al., 2013) and inducing apoptosis within insect cells (Toubarro et al., 2009). While FAR proteins are ubiquitous among animals, their structure in nematodes is altered, suggesting nematode-specific functions (Kennedy et al., 2013). GPCRs are sensory receptors involved in chemoreception and foraging behaviour in nematodes (Thomas and Robertson, 2008). *S. carpocapsae* contains 604 GPCRs, whereas *H. bacteriophora* contains 82 (Bai et al., 2013). The *S. carpocapsae* genome appears to have upwards of 41 genes which encode FAR proteins (Dillman et al., 2015), whereas *H. bacteriophora* contains just 3 (Bai et al., 2013; Lu et al., 2016). While the updated *H. bacteriophora* annotation (McLean et al., 2018) has increased the number of genes identified, especially those which encode secreted proteins, the large difference makes it clear that *H. bacteriophora* produce fewer of these proteins.

High quality genomes are valuable for many reasons. In particular, the improvement of EPN genomes can identify potential gene targets for manipulation to improve desirable traits in EPN, enhancing their use as model organisms, and biocontrol agents (Glazer, 2015; Sumaya et al., 2018; see section 1.6.6). In addition, high quality genomes and particularly the predicted protein sequences are necessary to enable the characterisation of an organism's proteome, which represents the functional component of the genome. Genomics is vital for the understanding of the blueprint of an organism; however transcriptomics, proteomics and metabolomics are essential for the visualisation of how that genome is expressed. For example, many of the genes identified within the *S. carpocapsae* genome were expanded relative to free living nematodes, and therefore assumed to be involved in parasitism (Dillman et al., 2015). While the number of genes common to one function can certainly indicate crucial information about an organism, only by studying the mRNA produced, the resulting protein and molecule products from these

genes, when they are produced and to what extent, can elucidate their function and purpose.

### 1.2.5 Transcriptomics

The transcriptome is the initial product of genome expression, composed of all RNA molecules produced by an organism. Protein biosynthesis is regulated and induced via mRNA production, processing, translation and subsequent modifications. Expressed sequence tags (ESTs) are fragments of mRNA sequences derived from the sequencing of selected clones from a cDNA library such as GenBank, which can aid gene discovery, transcript profiling and proteomics (Parkinson and Blaxter, 2009). Of 1,246 ESTs from *H. bacteriophora* infective juveniles, 1,072 were categorised into functional GO categories. Only 459 were similar to sequences in GenBank, of which 417 matched those found in *C. elegans* (Sandhu et al., 2006). Most of these predicted proteins, 613 (57%), had no similarity to known proteins, indicating a high degree of novelty in the genome. This novelty may be attributed to the divergence of *H. bacteriophora* from the bacterivorous lifestyle of its closest ancestors to its entomopathogenic lifestyle. Subsequently, an analysis of 10,886 distinct ESTs generated from *H. bacteriophora* showed that 72% of the ESTs had significant matches to proteins in GenBank (Bai et al., 2009), identifying ESTs involved in parasitism, RNA interference and stress resistance (Sandhu et al., 2006). Hao et al. (2010) analysed 1,592 unique ESTs from *S. carpocapsae*, 63% of which had significant matches in GenBank. Similar to *H. bacteriophora*, most of the genes identified were predicted to be involved in metabolism, however a significant number of ESTs were predicted to be involved in parasitism. Predicted to encode secreted proteins, 119 of these ESTs were overrepresented by proteases which are involved in infection (Hao et al., 2010; Toubarro et al., 2009; Toubarro et al., 2013).

### 1.2.6 Proteomics

While mRNA is responsible for regulating the production of novel proteins, the abundance of mRNA and proteins are not always directly corelated, and the abundances of both vary between different cells and tissues (Franks et al., 2017) and between species (Laurent et al., 2010). Therefore, it cannot be assumed that high abundances of mRNA for certain proteins will directly translate to higher abundances of those proteins. Proteomics gives real time and accurate insights into protein distribution and abundance are present within a cell/tissue/organism at the time of extraction. Analysing the proteins produced in response to stimuli can give insights into the molecular mechanisms behind a wide variety of behaviours and responses. The proteome of *S. feltiae* IJs under evaporative stress revealed the proteins involved in their resistance against desiccation (Chen et al., 2006). *S. carpocapsae* IJs produce a wide array of immune suppressing and toxic proteins and molecules (Burman, 1982; Lu et al., 2017a; Chang et al., 2019). There were 472 "venom proteins" identified from activated *S. carpocapsae* IJs (Lu et al., 2017a), most of which were proteases, protease inhibitors and FAR proteins. Most of these proteins are encoded by gene families which were identified as expanded within the *S. carpocapsae* genome and are implicated in infection and immune evasion (Dillman et al., 2015), however, their presence upon activation of IJs with host tissues gives concrete evidence for their role in parasitism (Lu et al., 2017a). The mRNA for 266 excreted products from *S. feltiae* was detected and tracked over time-course of infection. Upon activation, 96 of these excreted products were upregulated, enriched with hydrolases and peptidases (Chang et al., 2019). By collecting the secreted proteins from *S. feltiae* and *S. carpocapsae* IJs after infection, it was found that these parasites produced small quantities of these so-called "venom proteins" upon initial contact with host tissues (0-6 hours), however after 6-12 hours the

venom proteins spiked in quantity, and then decreased after 18-30 hours (Chang et al., 2019).

### 1.2.7 Metabolomics

In addition to proteomics and transcriptomics, the study of molecules, either derived from the primary or secondary metabolism, is referred to as metabolomics. These small molecules, whether composed of sugars, fats, or other components, play vital roles in cell signalling, stress resistance and communication. One such group of small molecules produced by nematodes that has received a lot of attention in recent years are ascarosides, and these are described in the next section.

## 1.3 Ascarosides

Ascarosides are a modular class of small molecules which act as nematode pheromones, affecting nematode development, behaviour, and many life history traits. They are composed of a dideoxy sugar, ascarylose, with a long lipid side chain which may be cleaved and modified to a wide variety of structures (Butcher et al., 2009; figure 1.4). Ascarosides were first detected in *Ascaris lumbricoides,* and subsequently were identified as the protective coatings in eggshells of members of the order Ascaridida (Flury et al., 1912; Jezyk and Fairbairn, 1967; in Ludewig and Schroeder, 2013). The first discovery of the chemical signalling role of these molecules was in the study of the *C. elegans* "daumone", a pheromone that induces the formation of the dauer stage in crowded or adverse conditions (Jeong et al., 2005; Butcher et al., 2007; Edison, 2009). It is now clear that ascarosides have roles in many other aspects of nematode physiology and behaviour such as chemotaxis (Yamada et al., 2010) and social behaviour even at femtomolar concentrations (Srinivasan et al., 2012). Despite their name, ascarosides are highly conserved across both free living and parasitic nematode species (Choe et al., 2012b; Butcher et al., 2017).

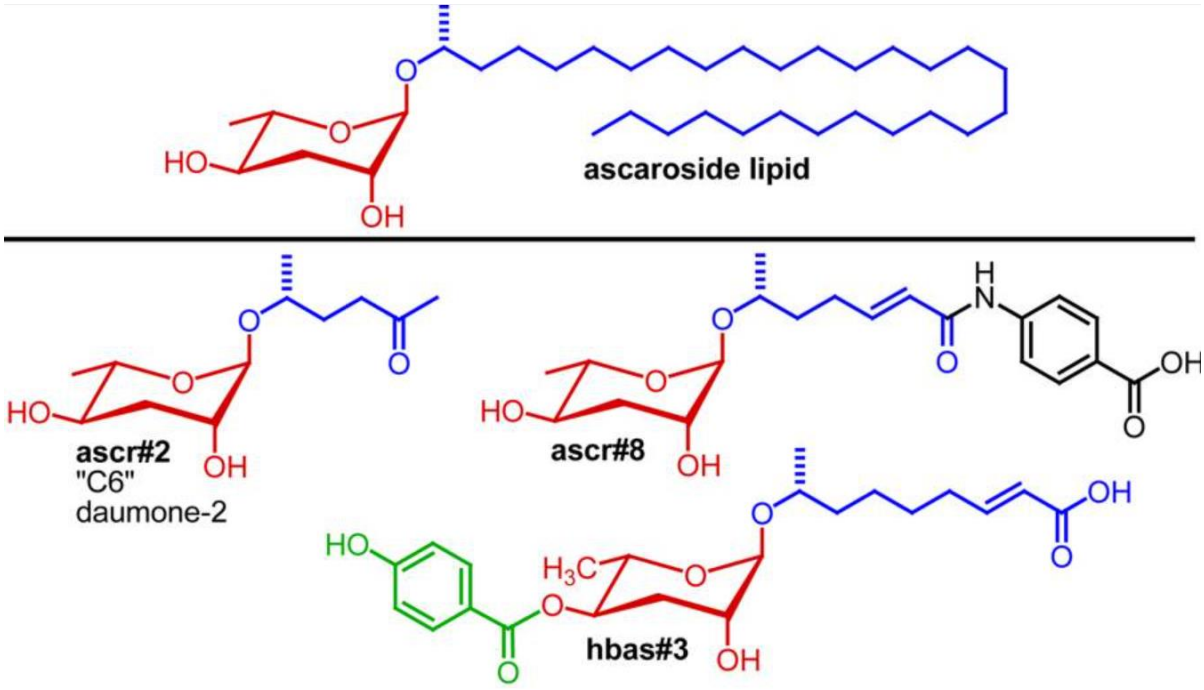



**Figure 1.4:** Structure of ascarosides in nematodes. Long side chained ascarosides (top) are found as a protective coating in *Ascaris suum* eggs, and shorter side chained ascarosides are indicative of signalling molecules within *C. elegans* (Bottom). From Ludewig and Schroeder, (2013).

### 1.3.1 Diversity and nomenclature

Initially named ascarosides A, B and C (Fouquey et al., 1957; in Ludewig and Schroeder, 2013), the naming of these small metabolites has been updated as their structure and diversity became better understood. The influence of ascarosides on dauer formation begat the term “daumones”, differentiated by numbering daumones 1, 2 and 3 (Jeong et al., 2009) however not all ascarosides are involved in dauer formation, and while these terms are still used interchangeably for ascaroside 1,2 and 3, they are incomplete as a naming system for the entire family. Ascarosides were subsequently named based on the number of carbons in the side chain (Butcher et al., 2007); however, a study using LC-MS/MS identified 146 ascarosides, 124 of which were previously unreported (Von Reuss et al, 2012) and many of these ascarosides share the same number of carbons in their side chain (figure 1.5).

Due to the complexity of ascarosides, their sheer number and modular nature, a more consistent nomenclature was suggested, whereby 4 lower case letters followed by a hash symbol and unique number would be attributed to each unique ascaroside. Ascarosides in the strict sense (ascr#X) are generally composed of ascarylose and a lipid side chain. Modifications to the side chain or its precursor result in a different class of ascaroside; such modifications include hydroxylation (hbas#X), oxygenation (oscr#X), succinylation (ocas#X) and addition of tryptophan-derived indole units as a moiety (icas#X), where X is an arbitrary number (Srinivasan et al., 2012; Artyukhin et al., 2013). Ascaroside 9 has the identifier “ascr#9”, and the indole ascaroside formerly known as indole ascaroside C5 or IC-asc-C5 is identified with the code icas#9 (Butcher et al., 2017). The codes for each ascaroside, as well as their structures, chemical formulas and other information can be found in the Small Molecule Identifiers Database (https://www.smid-db.org/).

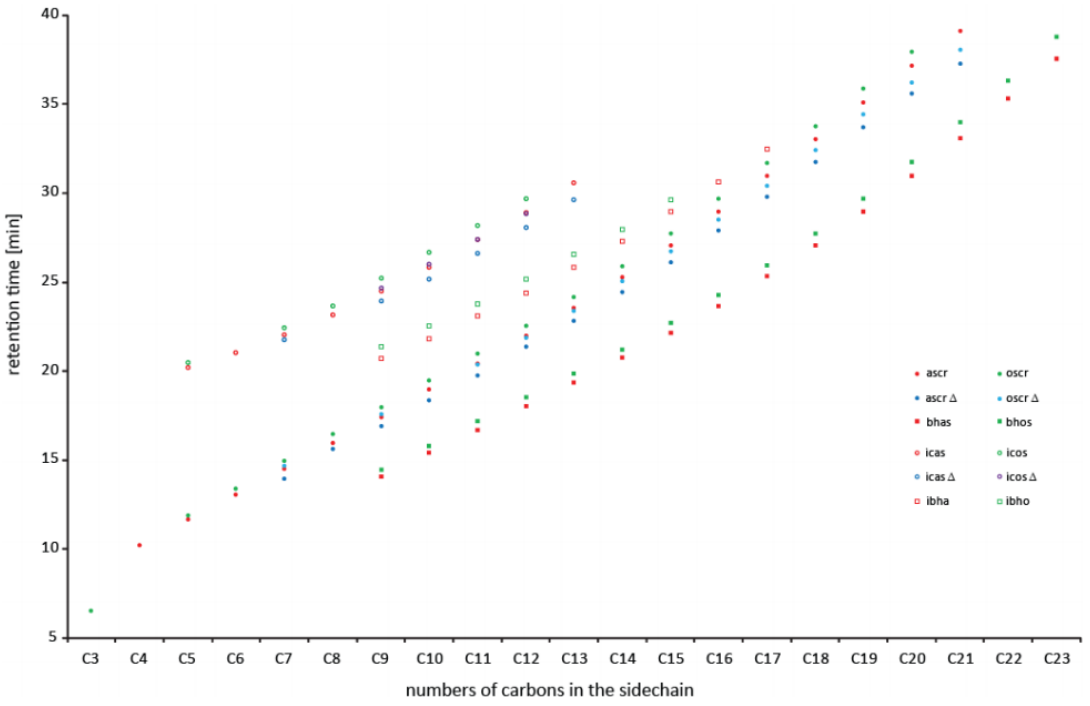



**Figure 1.5:** HPLC elution profiles of ascarosides identified in *C. elegans*, demonstrating the overlap in the number of carbons in the side chain of ascarosides. From Von Reuss et al., 2012.

### 1.3.2 Biosynthesis of ascarosides

The nematode intestine appears to be integral to ascaroside production (Butcher et al., 2009). *C. elegans* produce ascarosides with long side chains, which are subsequently cleaved and modified into their functional forms (Butcher et al., 2017; figure 1.6). The acyl-CoA oxidase ACOX-1 is responsible for the first step in the conversion of very long-chain ascarosides (VLCAs) to a functional ascaroside, whereby the enzyme is responsible for the β-oxidation of the side chain (Von Reuss et al., 2012). Subsequently, MAOC-1 acts as an enoyl-CoA hydratase and catalyses the hydration of the double bond of the long chain α,β-unsaturated side chains. The hydroxyacyl-CoA dehydrogenase DHS-28 is

responsible for converting β-hydroxyacyl-CoA-derivatives into the corresponding β-ketoacyl-CoA intermediates (Von Reuss et al., 2012). *Dhs-28* may act upstream of *daf-22*, in the same manner as their mammalian homologs. The gene *daf-22* (abnormal dauer Formation) encodes a thiolase which engages in the β-oxidation of the side chains. Deletion of either *dhs-28* or *daf-22* results in a worm unable to produce ascarosides with a lipid side chain shorter than 12 carbon atoms, resulting in the formation of VLCAs rather than functional ascaroside pheromones (Butcher et al., 2009). *Daf-22* knockout *C. elegans* worms cannot produce ascr#2 and ascr#3 (Srinivasan et al., 2008b).

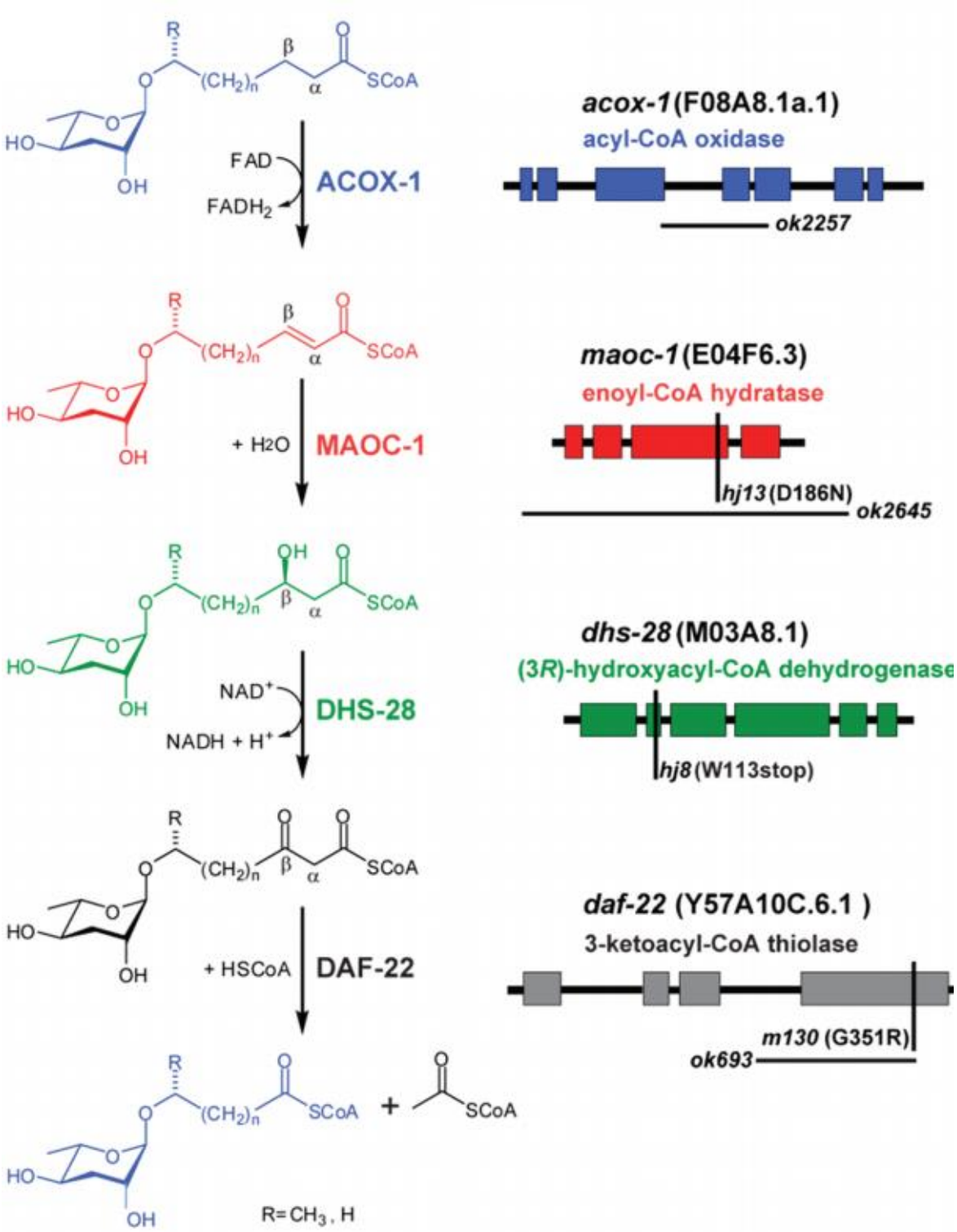



**Figure 1.6:** Biosynthesis of ascarosides in *C. elegans*. Peroxisomal B-oxidation enzymes ACOX-1, MAOC-1, DHS-28 and DAF-22 involved in the cleavage of the side chain which leads to the formation of diverse chemical structures. Adapted from Von Reuss et al., (2012)

### 1.3.3 Ascarosides as nematode pheromones

Ascarosides have been shown to affect the behaviour and physiology of a wide variety of nematodes (Choe et al., 2012a). The specific combination and concentrations of ascarosides vary from species to species and blends with different compositions can have profoundly different effects on the physiology and behaviour of nematodes (Srinivasan et al., 2012; Kaplan et al., 2012). Most of what is known about the function of ascarosides comes from *C. elegans.* In addition to their role in signalling worm density and stimulating dauer formation, as mentioned above, they also have been shown to have a role in mate attraction, suppressing foraging, enhancing aggregation, or causing avoidance in *C. elegans* (Butcher, 2019).

Ascarosides may be produced in a sex specific manner, in keeping with their roles as sex attractants. Hermaphroditic *C. elegans* worms produce large quantities of ascr#3 whereas males produce greater amounts of ascr#10 (Izrayelit et al., 2012). The blend of ascarosides produced by males was shown to be attractive to hermaphrodites (Izrayelit et al., 2012) whereas other combinations were specifically attractive to males and repulsive to hermaphrodites (Srinivasan et al., 2008b). *Panagrellus redivivus* males were strongly attracted to the female-produced ascr#1, whereas it repelled females (Choe et al., 2012b). Conversely, females were strongly attracted to the male-produced dhas#18, which was repulsive to males.

*C. elegans* males were highly attracted to Ascr#8 but only within a short range, whereas they were attracted to Ascr#2 and Ascr#3 from long distances. Therefore Ascr#2 and 3 may function to attract males from a distance and then Ascr#8 keeps them close to the hermaphrodite (Choe et al., 2012b). Various nematode species are attracted by the same set of ascarosides (ascr#1, ascr#3, ascr#7, ascr#8, and ascr#10), however, the species and

even the sex of the nematode influenced their preferred different combinations and concentrations of each ascaroside (Choe et al., 2012a).

### 1.3.4 Ascarosides in EPN

Ascarosides have been shown to have both developmental and behavioural effects on entomopathogenic nematodes (Kaplan et al., 2012; Noguez et al., 2012; Choe et al., 2012b). A unique ascaroside with an ethanolamide moiety (asc c11 EA), produced by *H. bacteriophora*, inhibited IJ recovery (Noguez et al., 2012). High densities of nematodes in the host may increase the concentration of this pheromone to a threshold which prevents recovery, which then may facilitate the IJs' accumulation before exiting the natal host and dispersing (Noguez et al., 2012). Older IJs were less sensitive to this pheromone. Multiple ascarosides (ascr#1, ascr#3, ascr#7 and ascr#10) were shown to be attractive to *Steinernema glaseri* males whereas ascr#2 and icas#9 were repellent (Choe et al., 2012b). *C. elegans* produce ascarosides in food-scarce conditions to induce dauer formation (Srinivasan et al., 2008a; Butcher et al., 2007) and the diet of *C. elegans* affects their ascaroside production (Kaplan et al, 2011). Entomopathogenic nematodes associate with symbiotic bacteria, which digest and breakdown the host tissues for the nematodes. Production of ascarosides in *S. carpocapsae* was not significantly affected by the presence of the symbiont and similar results were found for *S. feltiae* (Roder et al., 2019). Ascaroside blends enhanced the dispersal of IJs of various EPN species (Kaplan et al., 2012). The modular side chain of ascarosides share structural similarity to acyl homoserine lactones, quorum sensing molecules utilised by bacteria (Choe et al., 2012b). Quorum sensing is a method by which bacteria signal the density of bacteria in their vicinity, which regulates their growth and facilitates biofilm formation. As the insect cadaver becomes crowded with nematodes, there is a build-up of certain ascarosides which may act as a

signal of overcrowding and a dispersal signal (Oliveira-Hofman et al., 2019; Kaplan et al., 2020). The role of ascarosides in EPN behaviour is further discussed in chapter 1.5.

### 1.3.5 Ascarosides in interactions with other organisms

Ascarosides are highly conserved across many kinds of nematodes (Choe et al., 2012b), and other organisms have evolved to respond to, and even produce, ascarosides themselves. The plant parasitic pine wilt nematode *Bursaphelenchus xylophilus* produces a variety of ascarosides which affect the behaviour and reproduction of its vector beetle *Monochamus* (Zhao et al., 2016). Surprisingly, the beetle itself produces ascarosides, which affects its own gene expression and pupation, and attracts L4 nematodes, possibly facilitating the transfer of these nematodes between trees (Zhao et al., 2016). Ascarosides significantly increase the growth of ophiostomatoid fungi, such as *Leptographium pini-densiflorae* and *Sporothrix* sp. 1 which are symbiotic to *B. xylophilus*. Application of ascr#9 caused a fivefold increase in the spore production of *L. pini-densiflorae,* possibly facilitating dispersal by insect vectors (Zhao et al., 2018).

The nematophagous fungus *Arthrobotrys oligospora, A. musiforms, A. javanica* and *Dactylella gampsospora* may also be capable of "eavesdropping" on nematode communication. After exposure to ascarosides, especially ascr#1, ascr#3, ascr#7 and ascr#9, these species switch to their nematode-trapping morphology (Hsueh et al., 2013). A wide variety of plant species can recognise and respond to ascr#18, the primary ascaroside associated with plant parasitic nematodes, as well as ascr#1, and ascr#9 (Manosalva et al., 2015; Ning et al., 2020). Exposure to these ascaroside enhances the plant's resistance to pathogens.

### 1.3.6 Neuronal basis of behaviour

The infective juvenile stage of entomopathogenic nematodes is analogous to the dauer stage of *C. elegans. C. elegans* are simple organisms, containing just 302 neurons (White et al., 1986), however they are capable of relatively complex decision making (Bono and Maricq, 2005). The *C. elegans* connectome, the description of the entire nervous system, has been mapped out in detail (White et al., 1986; Cook et al., 2019; Bhattacharya et al., 2019). *C. elegans* undergo extensive neuronal remodelling upon their transition to the dauer stage (Bhattacharya et al., 2019), resulting in worms with behaviours that differ from other stages, such as nictation (waving their body in the air) and reduced movement and pharyngeal pumping (Cassada and Russell, 1975).

Complex behaviour, such as foraging, is achieved by relatively simple nervous feedback systems. One such mechanism, outlined by Liu et al. (2018), illustrates how sensory stimuli are perceived and translated to locomotion within *C. elegans* (see figure 1.7). A sensory neuron, AWC, releases glutamate, supressing AIY which in turn supresses the RIA neuron via acetylcholine (Ach). The RIA neuron connects to a looped nerve ring with two nerve domains, nrD (dorsal), and nrv (ventral). RIA may receive excitatory Ach signals in either nrD or nrV from motor neurons as the worm waves its head. Therefore, the worm will move in the direction of an odorant by the asymmetric suppression of these nerve rings (figure 1.7; Liu et al., 2018; Kaplan and Zimmer, 2018).

While the neuronal basis for nematode behaviour may be relatively simple, depending on few neurons, the resulting behaviours and the plasticity of these behaviours are highly sophisticated and an active area of research.

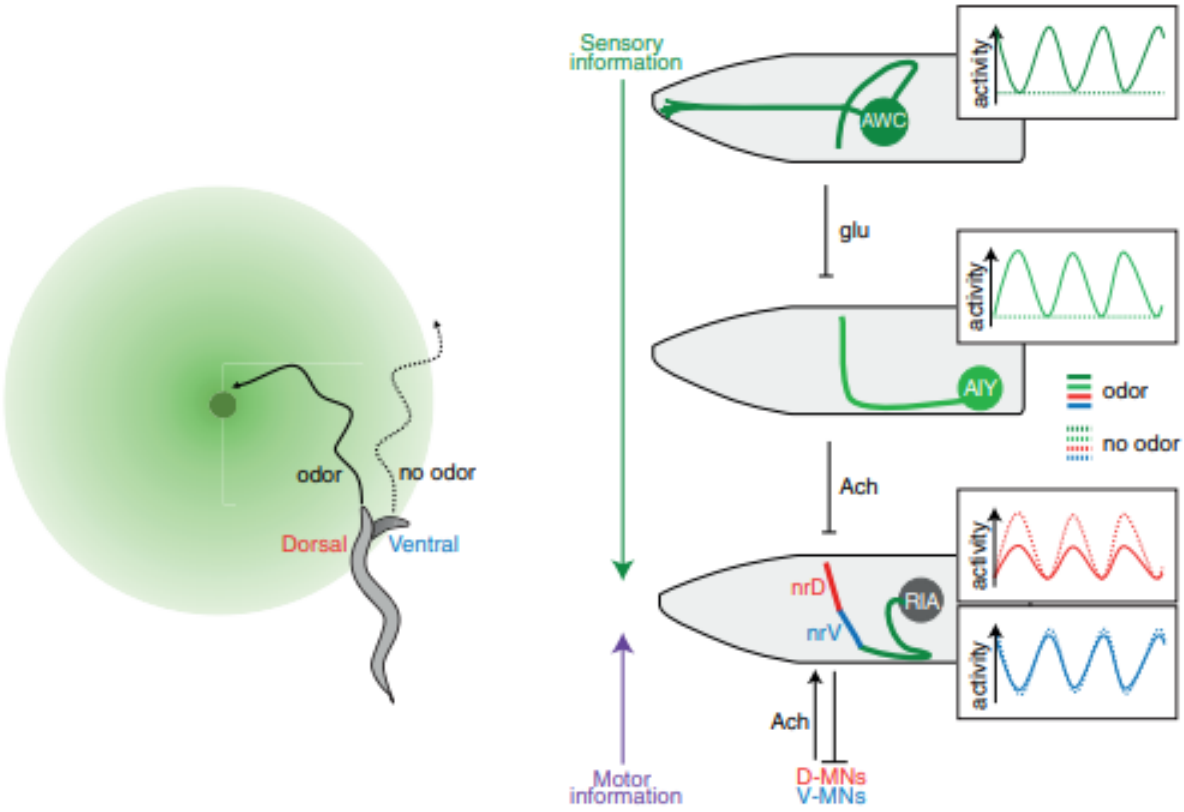



**Figure 1.7:** Diagram of worm movement regulated by environmental volatile cues. The odorant, isoamyl alcohol, is sensed by AWC neurons, and the activity of AWC fluctuates in exposure to the odorant as the worm swings its head, altering the neuron-mediated release of glutamate which supresses the interneuron AIY. In this way, the suppression and disinhibition of AIY is modulated by the worm's sensation of the odorant, which supresses RIA interneurons via acetylcholine (Ach). RIA may be excited by Ach signals received from Ach receptor on either side of a nerve ring domain, nrD (dorsal) and nrV (ventral) from motor neurons as the worm is swinging its head. In this example, the asymmetrical suppression of the RIA nrD nerve ring leads to diminished dorsal movement and maintains ventral movement, leading the worm to move in the dorsal direction towards the odorant. When no environmental sensory cues are available, there is no asymmetry in AWC signals and downstream suppression, leading to the worm continuing in a relatively straight direction (dotted lines). Adapted from Kaplan and Zimmer (2018).

### 1.3.7 Chemosensory neurons and apparatus

Chemosensory organs in nematodes, including sensilla and amphids, are replete with sensory neurons and are responsible for detecting odorants (Figure 1.8; Hart and Chao, 2010). Nematodes contain many different sensory neurons which facilitate chemotaxis and host finding, though little is known about their function in EPN compared to *C. elegans*.

$CO_2$ is primarily detected via head and tail neurons (Hallem and Sternberg, 2008; Bretscher et al., 2011; Hallem et al., 2011a; Hallem et al., 2011b) and $CO_2$ sensing in nematodes is facilitated by BAG neurons (Hallem and Sternberg, 2008; Hallem et al., 2011a), and its deletion inhibits *H. bacteriophora* and *S. carpocapsae* chemotaxis towards $CO_2$ (Hallem et al., 2011a). Carbon dioxide is one of the primary chemical cues utilised by host seeking parasites and brief exposure can stimulate nictation and jumping behaviours in entomopathogenic nematodes (Hallem et al., 2011a) however many other host associated volatiles can play a role in host seeking (see section 1.4.4).

Diversity of signalling molecules and receptors can further contribute to the complexity of behaviour in nematodes. Warnock et al. (2019) investigated the transcriptional basis of diverse host finding behaviours in IJs of three *S. carpocapsae* strains (All, Breton and UK1), with a focus on neuronal genes known to influence behaviour in other nematode species. The IJs of *S. carpocapsae* Breton display more refined host finding abilities than *S. carpocapsae* All or UK1, which corelates with an upregulation of GPCR and sodium transporter genes, implicated in chemosensation. Conversely, the UK1 strain nictates and jumps less than All and Breton, which corelates with the upregulation of the neuropeptide-like protein 36 (Warnock et al., 2019). It is likely that many neuropeptides work in tangent to facilitate the accurate identification and chemotaxis towards potential hosts. Knockdown of the expression of the FMRFamide-like gene inhibited host finding, dispersal, nictation and jumping in *S. carpocapsae* IJs (Morris et al., 2017). In these ways, relatively complex behaviour may be mediated by relatively simple nervous systems.

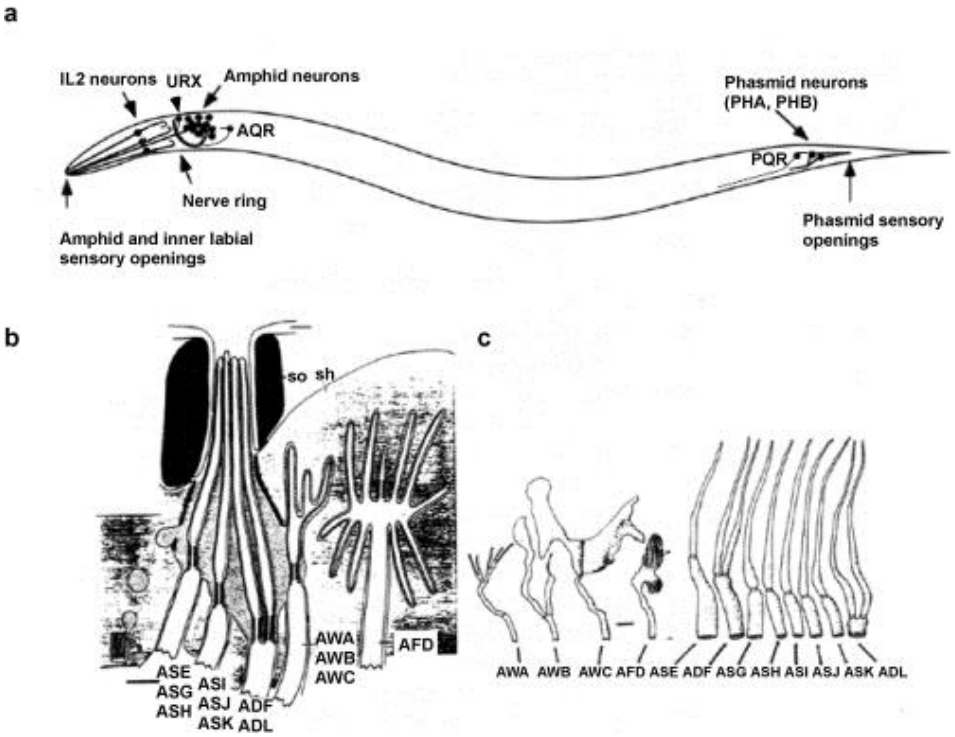



**Figure 1.8:** Basic layout of amphids and phasmids in the model organism *C. elegans.*

A.) The diagram displays amphids and phasmids, presentative of the general nematode chemosensory anatomy. B.) Detailed structure of the amphid sensory opening including ciliated nerve endings. C.) Detailed structure of cilia in 12 of the classes of amphid neurons. From Bargmann (2006).

## 1.4 Behaviour

Infective Juveniles leave their natal host in high numbers and must disperse far from the insect cadaver to avoid competition and find a new insect host to continue their life cycle. Their movement through the soil may be random (typical of the initial dispersal from the host), or directed, utilising host volatiles and environmental cues to find hosts (Griffin, 2015). IJ behaviour upon emergence can be broadly categorised into different stages, dispersal, host-finding, and infection.

### 1.4.1 Dispersal

IJs dispersing from the natal host are in direct competition with the thousands of other emerging IJs. This competition is further complicated by the life history of each species; the hermaphroditic heterorhabditids are not reliant on other IJs for reproduction within the new host, whereas while a single steinernematid may kill an insect, it cannot reproduce on its own (Griffin, 2015). It is speculated that in amphimictic EPNs, the males may act as a "colonising sex" (Grewal et al., 1993a), and exhibit greater dispersal abilities than females (Lewis and Gaugler, 1994). Therefore, the IJs emerging from a host must balance their need to move far from the host to reduce competition and their need to infect with conspecifics in a species-specific manner.

Upon first emergence from their host, *H. megidis* IJs disperse widely and exhibit low infectivity, facilitating wide dispersal, reducing competition between highly related IJs emerging from the same natal host (Dempsey and Griffin, 2002). The first of these IJs to leave the host exhibit good host finding abilities and poor dispersal, and IJs which disperse later have poor host finding abilities and have a greater propensity to disperse (O'Leary et

al., 1998). A similar, initially high, dispersal rate and hundredfold decrease weeks after was found in *S. scarabaei* (Koppenhöfer et al., 2013).

Chemical cues may play a role in stimulating dispersal. Cadaver macerate from EPN infected hosts increased the dispersal of IJs in lab tests (Shapiro and Glazer, 1996; Shapiro et al., 2000; Wu et al., 2018a). High levels of ascarosides build up within the cadaver, some of which such as Ascr#9 cause IJs to disperse (Kaplan et al., 2012; Kaplan et al., 2020). Other chemical signals are present within the cadavers which may act as dispersal agents, such as ammonia (San-Blas et al., 2008; San-Blas et al., 2014) and prenol (Baiocchi et al., 2017). Higher concentrations of prenol induced higher dispersal in IJs, and freshly emerged IJs were more sensitive to this cue than older IJs (Kin et al., 2019). Indeed, IJs applied to a field within infected cadavers exhibited higher dispersal than those applied in aqueous suspension (Shapiro and Glazer, 1996; Shapiro and Lewis, 1999), possibly due to the signals received from the cadaver. The dispersal of IJs is strongly affected by time (see section 1.4.5), however it is unclear whether declining dispersal is primarily affected by the physiological aging of the IJ, or these chemical cues from the natal host wearing off.

### 1.4.2 Foraging strategies

Entomopathogenic nematodes are loosely defined as either cruisers or ambushers. After IJs have emerged from the host, they may stay on the soil surface and nictate, waiting for potential new hosts to come near. Nictation is when IJs raise their body from the substrate and wave it in the air, facilitating their ability to attach to passing insects (Campbell and Gaugler, 1993). These IJs are designated as ambushers, such as *S. carpocapsae* (Grewal et al. 1994; Lewis et al., 1995b; Campbell and Gaugler 1997). Mobile IJs that move within the soil actively looking for a new host, are classified as cruisers, such as *H. bacteriophora*

and *H. megidis* (Bal et al., 2015; Kruitbos et al., 2010). Some EPN such as *S. feltiae* and *S. riobrave* adopt an intermediate tactic between both cruising and ambushing (Campbell and Gaugler, 1997). EPN IJs which are classified as ambushers are reported to be more effective against mobile insects, whereas cruisers are more effective at infecting stationary insects, such as larvae or pupae beneath the soil (Bal and Grewal, 2015).

While *S. carpocapsae* is generally defined as an ambusher, a small percentage of their population disperse faster, and further than others (Grewal et al., 1993a; Bal et al., 2014; Baiocchi et al., 2017). It is suggested that the 4% of the *S. carpocapsae* population which "sprint" may do so in order to maximise their chances of infection in the absence of hosts (Bal et al., 2014). The presence of moving hosts increased the dispersal speed and distance of *S. carpocapsae* sprinters, implying that they modulate this behaviour based on cues, likely $CO_2$ given off by the host (Bal and Grewal, 2015). Another hypothesis is that these sprinters are males (Grewal et al., 1993a), and that they may act as colonisers, which disperse widely to find suitable hosts, and signal females which follow after (Grewal et al., 1993a).

*S. carpocapsae* is widely considered an ambush forager, and generally does not disperse as widely as cruiser foragers, though this assumption is questionable (Wilson et al., 2012). The media upon which EPN IJs are placed may affect the nictation rates of "ambusher" IJs, indicating that their observed ambushing behaviour may be a product of their immediate environment as they ambush on sand but were mobile in peat (Kruitbos et al., 2010). The extent to which these species can be categorised into strict foraging tactics is likely overstated (Griffin, 2012).

### 1.4.3 Host-finding and infection

The IJs of EPN must seek out and infect new insects to mature to a reproductive stage and feed, and infection of an unsuitable host represents a "dead end" for the IJ. Therefore, EPN IJs rely, to a large extent, on host volatiles to find potential hosts, and respond to a wide variety of odours to assess the quality of a potential host (section 1.4.1). EPN which require specific hosts use volatiles to identify these hosts, such as the preference *S. scapterisci* IJs exhibit for cricket related odorants and indifference to odours associated with other insects (Dillman et al., 2012).

Following their attraction to a potential host, EPN utilise a number of host recognition cues, which include insect excretory products, their cuticle or gut contents (Grewal et al., 1993b; Grewal et al., 1993c; Griffin, 2015). Part of this assessment may also involve ascertaining if there are con- or heterospecifics already within the host. While steinernematids are amphimictic with only one known notable exception (Griffin et al., 2001), and require at least 2 (a male and a female) to enter the host in order reproduce, heterorhabditids are hermaphroditic and do not require a mate to reproduce. While low numbers of IJs may not be able to kill an insect host on their own, coinfection by conspecifics will increase the intraspecific competition within the host. Therefore, honest displays of the infection status of a host benefits all IJs. Such mechanisms have been proposed, such as the build-up of a repulsive chemical prenol within the host (Baiocchi et al., 2017) or ammonia by-products (San-Blas et al., 2008; San-Blas et al., 2014) which may deter coinfection. EPN may act as scavengers or infect insects infected with EPN, although it diminishes their fitness (Blanco-Pérez et al., 2019) however hosts offer a refuge from freezing and desiccation (Lewis and Shapiro-Ilan., 2002; Perez et al., 2003) which may offset the reproductive costs.

Upon finding a suitable host, the EPN IJs penetrate it via the spiracles, mouth or anus, and *Heterorhabditis* spp. may also enter through the cuticle itself (Bedding and Molyneux, 1982). Once in the haemolymph, the EPN release their symbiotic bacteria, which multiply and kill the host via sepsis. These bacteria constitute the food source for the subsequent generations of nematodes, and possibly produce chemicals which warn off late infectors (Baiocchi et al., 2017). Infectivity is one of the most desirable traits for EPN IJs as biocontrol agents, and one way in which this might be improved is that cadaver macerate or extracts from EPN infected hosts increased the infectivity of *H. bacteriophora, S. feltiae* and *S. carpocapsae* in lab conditions (Wu et al 2018a; Shapiro-Ilan et al., 2019). Understanding how to maximise IJ host finding and infectivity will enhance their use as biocontrol agents.

### 1.4.4 Chemotaxis

Nematodes are responsive to a wide range of odorants and chemical stimuli. Bacterivorous nematodes such as *C. elegans* are attracted towards short-chain alcohols associated with bacterial metabolic by-products, whereas the entomopathogenic *H. bacteriophora* are attracted to long-chain alcohols associated with insects (O'Halloran and Burnell., 2003). Infective juveniles of various EPN species respond to a wide variety of signals while foraging for hosts, as outlined above. While they are most attracted to $CO_2$ (Hallem et al., 2011a; Dillman et al., 2012), both *Steinernema* and *Heterorhabditis* IJs are attracted to a wide range of host volatiles, and their responses towards these volatiles are remarkably similar (Chaisson and Hallem, 2012), despite the phylogenetic distance between the 2 families (Blaxter et al., 1998; Figure 1.9). Host-specific nematodes, such as the cricket parasite *S. scapterisci,* were only strongly attracted by cricket-associated volatiles (Dillman et al., 2012). Many factors affect the chemotaxis response exhibited by EPN IJs.

Host volatiles alone are attractive to EPN IJs, however when accompanied by vibration, the attraction of the IJs is stronger (Torr et al., 2004). Exposure to live insect cuticle enhanced the chemotaxis of *S. carpocapsae* IJs towards host related volatiles (Baiocchi et al., 2019).

Many of the insects that are infected by EPN feed on plant roots, and so chemotaxis towards the appropriate plant-related volatiles can aid in host finding. *H. megidis* can respond to plant volatile cues such as (E)-β-caryophyllene, which are secreted by plants in response to insect larvae feeding on their roots (Rasmann et al., 2005). *H. megidis* IJs were more strongly attracted to *Thuja* roots which were being fed on by weevils, than to intact *Thuja* roots, those mechanically damaged by researchers or weevils on their own (Van Tol et al., 2001; Boff et al., 2002). *H. megidis* are just as attracted to plant volatile cues as they are to $CO_2$, and when combined the two create a more attractive stimulus than any individual cue (Turlings et al., 2012).

Volatiles associated with infected hosts are also valuable signals for EPN both as indicators of occupancy and as dispersal signals. Solid-phase microextraction and GC-MS analysis identified host chemical cues which *S. glaseri* (cruiser forager), *S. carpocapsae* (ambusher), *S. riobrave* (intermediate) and *S. feltiae* (intermediate) use to distinguish between uninfected hosts, and infected hosts, and even between hosts with conspecific or heterospecific infections (Baiocchi et al., 2017).

Prenol was identified as a volatile cue which increases in concentration in the late stages of host infection. This odour is highly repulsive to EPN IJs (Kin et al., 2019) and may encourage newly emerged IJs to disperse and discourage IJs from coinfecting an infected host (Baiocchi et al., 2017). Dimethyl disulphide was identified as the highest abundant volatile given off by infected *G. mellonella*, regardless of the species examined (*H. bacteriophora, S. feltiae, S. carpocapsae* and *S. rarum*) and prenol was not detected (Fu et

al, 2021). However, since there were no uninfected insects as controls, the source of the dimethyl disulphide cannot be unambiguously attributed to infection by EPN.

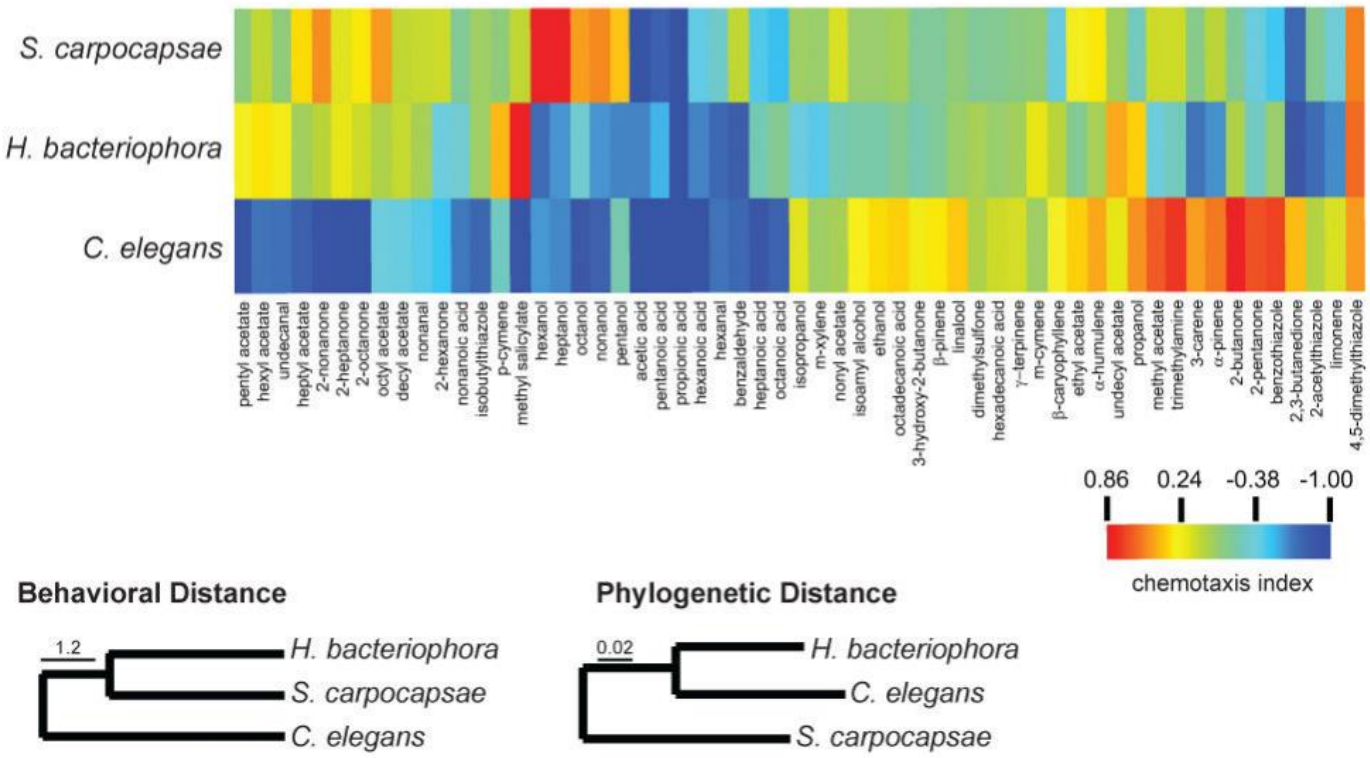



**Figure 1.9:** The chemotaxis response of *S. carpocapsae* and *H. bacteriophora* compared to that of the free-living, bacterivorous nematode *C. elegans*. Despite their phylogenetic distance, both of the entomopathogenic species of nematodes are attracted to the same odours, those associated with insects, due to convergent evolution. Adapted from Chaisson and Hallem, (2012).

### 1.4.5 Effects of age and storage temperature

The IJ is a non-feeding stage, which relies on its stored lipids and glycogen for energy. IJs that do not find a host may eventually die of starvation, but before that time their ability to find and infect hosts declines (e.g., Lewis et al 1995b; Patel et al., 1997). The rate of decline depends on the ambient temperature, but lower temperatures generally prolong life,

and there is an optimum storage temperature for each species (Grewal, 2002). Because shelf life is an important feature of organisms used for biocontrol, the decline in infectivity over time at different temperatures, and ways to prolong it, has been the subject of much research (see section 1.6.5). However, before the general decline in EPN quality, there may be other more subtle changes in behaviour, including the phenomena that can be loosely termed "phased infectivity", and these will be addressed here.

While infectivity has received most attention, Lee et al. (2016) showed that culture and storage temperature have a strong effect on the chemotaxis of EPN IJs. *H. bacteriophora* and *S. carpocapsae* IJs stored at and swapped between 15-25°C both showed altered responses to odorants (acetone and 1-hexanol) and altered host-seeking behaviour (Lee et al., 2016). *S. carpocapsae* IJs cultured at low temperature (15°C) were strongly attracted to acetone, and neutral towards 1-hexanol whereas IJs cultured at a higher temperature (25°C) were repulsed by acetone, and strongly attracted towards 1-hexanol. Chemotaxis was also affected by the age of the IJ. The chemotaxis response of *H. bacteriophora* was affected by age more than by temperature (Lee et al., 2016). In the cricket-specific nematode, *S. scapterisci,* young IJs were repelled by $CO_2$, an important host volatile cue, whereas older IJs were attracted to $CO_2$ (Lee et al., 2016). This may be advantageous as it facilitates the dispersal of the nematodes after emergence from the natal host as the emerging nematodes will not super-parasitise the first host in their vicinity.

Fan and Hominick (1991) showed that two steinernematids, including *S. feltiae,* exhibited fluctuations in infectivity during storage in moist sand at 5°C but not at 15°C. This is generally referred to as phased infectivity, as discussed by Hominick and Reid (1990). Phased infectivity in Steinernematidae has been contested (Campbell et al., 1999) however it has been well documented in *Heterorhabditis* spp., such as *H. bacteriophora* (Campbell et al., 1999; Perez et al., 2003) and *H. megidis* (Griffin, 1996; Fitters et al., 2001; Dempsey

and Griffin, 2002; Ryder and Griffin, 2003). The infectivity of *H. megidis* IJs increased over time as dispersal decreased, suggesting that IJs switch their focus from dispersal to infection (Dempsey and Griffin, 2002). *Heterorhabditis* spp. showed an increase in infectivity, especially after cold-storage, before the eventual age-related decline sets in (Griffin, 1996; Fitters et al., 2001; Perez et al., 2003). There is also evidence that infectivity of steinernematids can be improved by storage conditions. The virulence of *S. kraussei* increased in assays at both optimal and sub-optimal temperatures following pre-conditioning at 9-12°C (Guy et al., 2009). Similarly, storage at 8°C increased the infectivity of *S. scarabaei* IJs (Koppenhöfer et al., 2013), and cold storage at 9°C improved the infectivity of *S. carpocapsae* and *H. megidis* against the black vine weevil, *Otiorhynchus sulcatus* at 9°C (Fitters et al., 2001; Guy et al., 2017)

Similar to its effects on chemotaxis, temperature has a strong effect on the infectivity of many IJs. *S. rarum* IJs reared at 23°C showed weekly fluctuations in infectivity whereas those stored at 5°C showed an initial increase and subsequent decline (Cagnolo and Campos, 2008). Cold stored (15°C) *S. carpocapsae* adopted "cruising tactics", whereas those stored at 25°C nictated more (Lee et al., 2016).

Temperature and aging also affect the dispersal of EPN IJs. Aging *S. carpocapsae* IJs nictated less, and dispersed more (Lewis et al., 1995b). *Steinernema scarabaei* IJs stored at 8°C dispersed less than IJs stored at room temperature (Koppenhöfer et al., 2013). No significant increase in transparency was detected in this study, indicating that the lipid levels of the IJs remained relatively high.

## 1.5 Stress tolerance

EPN belonging to both Heterorhabditidae and Steinernematidae can be found on all continents except Antarctica (Griffin et al., 1990), including tropical, temperate, and desert conditions (Hominick, 2002). Organisms tolerate stress in species-specific ways, either constitutively or by induction of stress resistance behaviours or molecules after exposure to stress. Nematodes with constitutive stress tolerance include *Panagrolaimus superbus*, which can tolerate rapid desiccation with the constitutive expression of protectants and the induction of repair pathways upon rehydration (Tyson et al., 2012). Other organisms require prior exposure to abiotic stress to survive prolonged periods or more severe stress (Hibshman et al., 2020). EPN IJs are found in the soil all year round and are exposed to a wide array of stressors such as UV light, desiccating conditions, and temperature extremes. EPN IJs are relatively resistant against these stressors (especially compared to other stage of the life cycle) and have a variety of stress tolerance mechanisms. Here I will focus mainly on tolerance to freezing and desiccation, and the role of acclimation in protection.

### 1.5.1 Acclimation

Oncoming winter or summer is indicated by the gradual reduction or increase in temperature, respectively. These conditions may act as cues for both a change in the IJ's behaviour, and for the production of protective molecules. Acclimation to low temperatures enhances freezing tolerance in many species of nematode (McGill et al., 2015; Seybold et al., 2017; Thorne et al., 2020; Liu et al., 2019; Wu et al., 2018b; Okahata et al., 2016). Similarly, preconditioning nematodes in relatively low relative humidity (RH) enhances nematodes' survival in desiccating conditions (Womersley et al., 1989; Solomon et al., 1999; Erkut et al., 2011). Cold and warm storage enhanced *S. carpocapsae*

and *S. riobrave* tolerance to UV light (340nm; Jagdale and Grewal, 2007). Acclimation to stressful conditions induces the accumulation within the nematode of protectants, such as trehalose (Jagdale and Grewal, 2003; Erkut et al., 2011; Ali and Wharton, 2015) and glycerol (Ali and Wharton, 2015), and the upregulation of chaperones such as heat shock proteins (HSPs; Xie et al., 2020), late embryogenesis (LEA) proteins (Solomon et al., 2000; Hibshman et al., 2020) and proteins involved in reactive oxygen species (ROS) reactions (Hibshman et al., 2020).

These mechanisms have been demonstrated in the IJ stage of EPN. Acclimation to low temperatures greatly enhances freezing resistance in *S. feltiae, S. riobrave, S. carpocapsae,* and *H. bacteriophora* (Grewal and Jagdale., 2002; Jagdale and Grewal., 2003; Brown and Gaugler 1996; Ali and Wharton, 2013). Acclimation to lower temperatures induces the production of metabolites and proteins and other responses which can enhance freezing tolerance; many of these adaptations also have roles in desiccation tolerance (Chen et al., 2005; Solomon et al., 2000; Grewal and Jagdale, 2002).

It is unknown how long IJs retain their freezing tolerance after exposure to low temperatures. While it may be advantageous for an organism to retain resistance to stress indefinitely, the production of proteins (stress proteins or enzymes responsible for the synthesis of small molecule protectants) is energetically expensive, and represent an opportunity cost for the nonfeeding IJ, as it cannot replenish the amino acids used to assemble proteins by feeding. IJs may instead dynamically respond to their environment to resist immediate stresses and not retain that resistance long term.

Freezing of extracellular water increases the concentration of extracellular solutes drawing water out of the cells via osmosis (Muldrew and McGann, 1994; Dumont et al., 2003). Cells can lose water due to increased membrane permeability (Mazur, 1963). Nematodes within the soil which are highly permeable may be dehydrated in this manner upon

exposure to freezing temperatures (Forge and MacGuidwin, 1992; Wharton et al., 2003; Wharton et al., 2005; Ali and Wharton, 2013), as is the case with other small soil dwelling organisms (Sømme and Birkemoe, 1997; Scholander et al., 1953; Holmstrup et al., 1994) and plants (Lenné et al., 2010; Daskalova et al., 2010).

### 1.5.2 Freezing resistance

EPN are generally classed as either freezing-tolerant, able to survive intracellular ice formation, or freezing-susceptible, avoiding ice nucleation and maintaining their body fluids as a liquid below their melting point (Glazer, 2002). *S. feltiae, H. bacteriophora* and *S. anomali* are all freezing tolerant, capable of withstanding temperatures down to −22°C, −19°C and −14°C respectively (Brown and Gaugler, 1996). *H. zealandica*'s sheath prevents inoculative freezing, facilitating supercooling to -32°C, though exsheathed IJs freeze above −6°C, which kills the IJ, classifying them as freezing-susceptible (Wharton and Surry, 1994; in Glazer, 2002). To achieve this supercooling of body fluids, IJs accumulate protectants such as trehalose, glycerol and polyols upon exposure to cold or freezing temperatures (Ali and Wharton, 2015). Indeed, conditioning *S. glaseri, S. riobrave* and *S. carpocapsae* in glycerol enhanced their resistance to freezing (Brown and Gaugler, 1998). Storage of EPN at low temperatures increased the concentration of unsaturated fatty acids, which are implicated in increasing membrane fluidity in low temperatures (Grewal et al., 2006)

Freezing negatively affects living cells in several ways, however the exact mode of action is unclear. Counter-intuitively, the danger freezing poses to living cells occurs not at the lowest temperatures, but at the intermediate zone between their optimum temperature and the temperature at which they freeze, which is generally between −15°C and −60°C,

however this differs between organisms. Cells must traverse this “danger zone” twice, initially when freezing and upon their thawing. (Mazur, 1963; Mazur, 1984).

The two-factor hypothesis proposed by Mazur (1972) explains how freezing rates which are too fast or slow can negatively affect cells. Cells which are gradually introduced to lower temperatures at a constant rate fare better than those which are exposed to extremely low temperatures immediately. Exposure to extreme low temperatures can cause the formation of intracellular ice crystals, which almost always causes mortality due to the mechanical distress they place upon the cell, as well as puncturing of the cell membrane (Mazur 1960; Arora, 2018). The puncturing of the plasma membrane may be the cause of ROS stress within the cells, as repeated freeze thaw cycles increase the rate of ROS in winter wheat crowns, and those that were acclimated to low temperatures has increased tolerance to ROS (Arora, 2018). Gradual freezing prevents the formation of intracellular ice (Mazur, 1984), lessens the chance of cell membrane rupturing (McGann et al., 1988) and induces the accumulation of cryoprotectants within the cells (Jagdale and Grewal, 2003; Ali and Wharton, 2015). Cooling too slowly can fully dehydrate the cells affecting their viability (Gao and Critser, 2000). Subsequent return to hypotonic solutions can kill thawing organisms due to osmotic shock (Mazur, 1984; Gao and Critser, 2000).

### 1.5.3 Desiccation resistance

Some organisms are constitutively anhydrobiotic and can readily desiccate without prior exposure to dehydrating conditions. These organisms can endure this state of desiccation for long periods of time and can rehydrate readily with few negative effects after years of anhydrobiosis, such as the resurrection plants (Scott, 2000) or the Antarctic nematode *P. superbus* (Tyson et al., 2012), which is classified as a fast dehydration strategist. Other organisms (including EPN IJs) must be exposed to brief or mild abiotic stress to induce

protectants or behaviours which increase their tolerance of that stress e.g., some days at a high RH before exposure to lower RH; these are termed slow desiccation strategists. Other terms have been proposed for slow and fast, such as external dehydration strategists (which have little control over their water loss) and innate dehydration strategists which have intrinsic adaptations to control their water loss (Perry and Moens, 2011). The desiccation tolerance of EPN varies from species to species however in general their tolerance of desiccation is improved by prior exposure to high RH. No EPN species is capable of true anhydrobiosis, which is described as survival with loss of almost all water and cessation of metabolic activity (Madin and Crowe, 1975; Wharton, 2015). Preconditioning *S. feltiae, H. bacteriophora* and various *Heterorhabditis* spp. in desiccating conditions (97% RH) improves the IJs' survival in desiccative conditions (Solomon et al., 1999; Liu and Glazer, 2000). Desiccative conditions induce the accumulation of various protective molecules such as glycerol and trehalose (Qiu and Bedding, 2000; Qiu and Bedding, 2002) and LEA proteins (Solomon et al., 2000). Conditioning *H. megidis* and *H. indica* IJs at 98% RH induced the synthesis of glycerol but not trehalose (O'Leary et al., 2001), whereas similarly conditioned *S. carpocapsae* IJs produced trehalose (Womersley 1990). Physical factors such as cuticle structure also affect EPN IJs, and loss of their sheath makes *H. megidis* IJs susceptible to desiccation stress (O'Leary et al., 1998), presumably as the sheath slows the rate of water loss from the IJ.

### 1.5.4 Accumulating sugars

Sugars as a group appear to be protective against abiotic stress; such sugars include sucrose (Carpenter et al., 1987), maltose (Carpenter et al., 1987), and glucose (Koster et al., 1994). Trehalose, a disaccharide composed of two glucose molecules and found in many organisms, appears to be especially protective against abiotic stressors, and has been

shown to be superior to other saccharides in this function (Crowe et al., 1987). Trehalose is the primary sugar found in nematodes, with multiple vital roles in energy storage and stress resistance (Behm et al., 1997; Elbein et al., 2003). Trehalose is involved in the formation of glass, an amorphous solid which retains the relative structure and position of the cellular contents upon desiccation, enhancing the cell's survival (Crowe et al., 1996). Many species of nematode (Madin and Crowe, 1975; Behm, 1997; Adhikari et al., 2009; Łopieńska-Biernat et al., 2020), and other stress resistant organisms such as tardigrades (Westh and Ramløv, 1991; Hengherr et al., 2008), yeast (D'Amore et al., 1991; Eleutherio et al., 1993), brine shrimp (Glasheen and Hand, 1988: Hand and Menze 2015) and plants (Scott, 2000; Iturriaga et al., 2000; Wingler, 2002; Williams et al., 2015) accumulate trehalose upon exposure to desiccative, temperature or osmotic stress.

### 1.5.5 Protein and chaperone response

Exposure to abiotic stress induces the expression of protective proteins within nematodes. Nematodes accumulate intrinsically disordered proteins called late embryogenesis abundant (LEA) proteins which were first discovered in plant seeds (Cuming and Lane, 1979; Dure et al., 1981). LEA proteins have been found in many species of nematodes including *C. elegans* (Gal et al., 2004), *Steinernema feltiae* (Solomon et al., 2000) and *Aphelenchus avenae* (Browne et al., 2004), and other organisms renowned for their stress tolerance including brine shrimp (Wang et al., 2007), rotifers (Pouchkina-Stantcheva et al., 2007), tardigrades (Boothby et al., 2017) and certain plants (Cuming and Lane, 1979; Dure et al., 1981; Ndong et al., 2002). LEA proteins are involved in protecting cells from oxidative stress (Zheng et al., 2019), freezing (Anderson et al., 2015; Reyes et al., 2008), and desiccation (Solomon et al., 2000).

Heat shock proteins (HSPs) are produced by nematodes in response to abiotic and biotic stress (Wharton, 2011). HSPs are ATP-dependent molecular chaperones which surround proteins and prevent their misfolding and aggregation (Borges and Ramos, 2005; Clare and Saibil, 2013). HSPs are differentiated by their size - HSP70 is roughly 70 kDa, and HSP90 is ~90kDa - and each have different functions.

HSP70 mediates protein chaperoning activity, receiving proteins directly from ribosomes (James et al., 1997; Siegers et al., 1999) or from other chaperones for correct folding, and can transfer misfolded proteins to HSP60 for refolding (Langer et al., 1992) or to HSP90 via the cochaperone HOP (Frydman and Höhfeld, 1997). HSP70 also facilitates the degradation of improperly folded proteins (Connell et al., 2001; Murata et al., 2001). HSP70 and its cochaperones are implicated in resistance to abiotic stress (Lee and Schöffl, 1996; Alvim et al., 2001; Sung and Guy, 2003). HSP70 works synergistically with chaperonins to facilitate de-novo protein folding of nascent polypeptides (Siegers et al.,1999).

Members of the HSP60 family are also called chaperonins. Chaperonins are grouped into two classes, group I and group II chaperonins. Group I chaperonins fold unfolded proteins transported into the mitochondria from the cytosol (Levy-Rimler et al., 2002) and are also found within the chloroplast. Group II chaperonins work synergistically with prefoldins to bind and fold actin and tubulin (Vainberg et al., 1998)

HSP90 is involved in various signal transduction pathways as it ensures the correct folding of various kinases and hormone receptors (Richter and Buchner, 2001; Frydman, 2001). HSP90 can prevent the aggregation of misfolded proteins (Bose et al., 1996), mark misfolded proteins for degradation (McClellan et al., 2005), facilitate the recovery of heat damaged proteins and refold misfolded proteins (Nathan et al., 1997)

Proteins in the HSP100 family are involved in preventing and reversing toxic aggregates of proteins (Palleros et al., 1991; Glover and Lindquist, 1998), mediated by HSP70 and its cochaperone HSP40 (Rampelt et al., 2012).

Small heat shock proteins (sHSPs) are, as their name suggests, smaller than their counterparts at around 12-43kDa (De Jong et al., 1998; Franck et al., 2004; Haslbeck et al., 2005). These proteins are implicated in protecting organisms against low temperature or freezing stress (Sabehat et al., 1998; Pacheco et al., 2009) and against heat stress (Raman et al., 1995). Small heat shock proteins prevent protein misfolding and aggregations (Jakob et al., 1992; Raman et al., 1995; Ungelenk et al., 2016). Unlike most other HSPs, sHSPs can act independently of ATP hydrolysis (Jakob et al., 1992).

The molecular mechanisms involved in stress tolerance in EPN are not well understood, though the *C. elegans* dauer's tolerance to abiotic stress has been extensively studied (Erkut and Kurzchalia et al., 2015) and several recent studies have utilised transcriptomic and proteomic approaches to investigate the molecular response of EPN IJs upon abiotic stress exposure. Yaari et al. (2016) analysed transcriptome expression in *Steinernema* spp. following heat and desiccation treatments. Many stress genes were expressed upon stress exposure including HSPs, LEA proteins, superoxide dismutase, ubiquitin and various enzymes involved in dehydrogenation and cell signalling. Heat shock in *S. carpocapsae* induced the expression of HSPs, proteins involved in stress and ROS tolerance and transcription factor genes which are related to longevity pathways (Xie et al., 2020). *H. bacteriophora* IJs exposed to oxidative stress exhibited similar upregulation of stress response in both the stress sensitive and stress tolerant strains tested, whereas the stress sensitive strain downregulated a far greater number of genes than the tolerant strain (Sumaya et al., 2020). The proteome of *S. feltiae* in desiccating conditions revealed an

accumulation of a number of protectants, which may also enhance the IJs' tolerance of freezing conditions (Chen et al., 2005) via cross tolerance.

### 1.5.6 Cross tolerance

Acclimation to one stressor may confer resistance to another, due to the general protective effects of stress induced proteins, molecules and behaviours (such as coiling and clumping) and the fact that individual stressors involve part of another stress i.e. freezing stresses cells by removing free water, and by the physical stress of expansion and shrinkage (Pegg, 1987), and these stresses are also caused by desiccation. Therefore, mechanisms which enhance the IJ's tolerance of freezing stress likely confer resistance to desiccation, and vice versa. Exposure to desiccation enhanced EPN IJs' freezing tolerance (Chen et al., 2005; Adhikari et al., 2010), and exposure to cold temperatures enhances their tolerance to desiccation, likely due to the accumulation of trehalose, a desiccation protectant (Solomon et al., 2000; Grewal and Jagdale, 2002; Jagdale and Grewal., 2007). The phenomenon of cross tolerance is found in many organisms. Both cold shock and heat shock induce heat shock protein activity in brine shrimp (Gbotsyo et al., 2020), indicating that these proteins may confer non-specific resistance against cell damage. Of 416 identified mass spectral tags detected in temperature-shocked *Arabidopsis*, 93 were increased or decreased in abundance in a similar manner to both cold and heat shocked plants, indicating a dual function to these molecules (Kaplan et al., 2004).

## 1.6 Biocontrol agents

At least 13 species of EPN (Heterorhabditidae and Steinernematidae) are used in biocontrol against many pest targets, often achieving above 75% efficacy in field or greenhouse conditions (Lacey et al., 2015). EPN are effective killers of insects and populations can survive for successive generations within a field giving lasting protection against insect pests (Kapranas et al., 2017). EPN have approached, or indeed surpassed chemical pesticides in terms of efficacy in many cases (Jansson et al., 1993), with few of the negatives associated with chemical pesticides (Lacey et al., 2015; Harvey et al., 2016). In some cases, however, EPN applications fail to control pest populations, prompting research into the various factors which may affect their success (Georgis et al., 2006; Lacey et al., 2015).

The most common EPN used as biocontrol agents are *S. carpocapsae*, *S. feltiae* and *H. bacteriophora* partially due to their wide host and temperature ranges (Kung et al., 1991; Saunders and Webster, 1999; Chen et al., 2003; Hazir et al., 2001; Lortkipanidze et al., 2019). Other EPN species have also been tested, such as *S. glaseri*, and *S. scarabaei* against beetles (Gaugler et al., 1992b; Koppenhöfer et al., 2006) and heterorhabditids such as *H. megidis* against weevils (Guy et al., 2017).

### 1.6.1 Safety and regulation

From the earliest days of their introduction to the markets, entomopathogenic nematodes have been recognised as “exceptionally safe” and safer than chemical pesticides by the Organisation for Economic Cooperation and Development (OECD; Ehlers and Hokkanen, 1996). EPN based biocontrol agents are safer for humans and non-target organisms than chemical pesticides (Akhurst and Smith, 2002). However, care must be taken to ensure

that exotic nematodes are not introduced to areas to which they are not endemic (Jansson, 1993). Native species should be considered first, as they do not involve the risk of introducing novel species to an ecosystem, are adapted to the climate of application and may be more effective against endemic insect pests (Kepenekci et al., 2015; Navarez et al., 2021; Şahin et al., 2021).

### 1.6.2 Choice of species to use

One of the most important factors to consider in choosing which species of EPN to use for a particular pest is its virulence for that pest. *S. carpocapsae, S. feltiae, H. bacteriophora,* and *H. megidis* are classified as generalist entomopathogens, as they infect and kill a wide range of insects. In contrast, *S. scapterisci* is not only ineffective against insects other than their preferred cricket host (Nguyen and Smart, 1991) but the IJs of this species preferentially activate upon exposure to cricket homogenate (Lu et al., 2017b). EPN IJs are attracted towards odours associated with their preferred hosts (section 1.4.1), enhancing their efficacy as biocontrol agents against those pests in particular (Castelletto et al., 2014; Hallem et al., 2011a). In addition, the selection of appropriate EPN for the control of specific pests can reduce off-target effects of those EPN.

Off-target effects could exacerbate pest populations in the long run, as they can adversely affect beneficial insects, including parasitoids, or local entomopathogenic nematodes. Repeated applications of EPN have been shown to reduce populations of local nematode species, which may represent a loss in natural predators and biodiversity after long term application (Duncan et al., 2003a; Duncan et al., 2003b). Using host-specific species, such as *S. scapterisci*, or targeted application can reduce off target effects (Nguyen and Smart, 1991; Harvey et al., 2012). Parasitoid wasps are able to detect EPN infected cadavers and avoid them, reducing the risk of their populations being adversely affected by EPN

application (Harvey and Griffin., 2012). Understanding these specific interactions between IJs and their hosts can enhance the manipulation of these IJs as biocontrol agents.

Another crucial factor in selecting an EPN species for application is the consideration of how each species responds to the abiotic and biotic factors present at the site of application (Shapiro-Ilan et al., 2006). Temperature is one of the most important abiotic factors (Griffin, 1993). The survival, infectivity, and chemotaxis of EPN IJs is strongly affected by temperature (Georgis and Gaugler, 1991; Andaló et al., 2011; Kepenekci et al., 2015; Baimey et al., 2015; Lee et al., 2016; El Khoury et al., 2018), and therefore anticipating the temperatures likely to prevail in the host's target habitat following application and ensuring the IJ will be effective at that temperature is crucial. *S. carpocapsae*, *S. glaseri*, *H. bacteriophora*, and *H. megidis* all have an optimum temperature somewhere between 5- and 25°C (Kung et al., 1991; Saunders and Webster, 1999; Chen et al., 2003). *S. feltiae* may be considered a cold-adapted species, showing high efficacy against insect pests between 8°C and 25°C (Hazir et al., 2001; Kepenekci et al., 2015). At temperatures above 25°C however, its efficacy falls off gradually (Jagdale et al, 2004) limiting the use of this species in greenhouses. The ability to infect and kill at low soil temperatures is a desirable trait when targeting certain pests, such as the black vine weevil, that are active under such conditions (Guy et al., 2017).

EPN species also vary in their tolerance to the physical stresses in spray equipment (Wright et al., 2005; section 1.7.5), as well as various environmental stresses encountered during and following application. These include high temperatures and UV radiation (Gaugler et al., 1992a), and IJs may also be exposed to low temperatures, and freezing conditions post application. The effects of stressors such as heat, UV and desiccation are exacerbated during foliar application, and EPN's success when applied as a biocontrol

agent is reduced on exposed foliage but impacts are less severe during application to longer foliage where the canopy may help protect the IJs (Griffin, 2015).

The behaviour of both the parasites and the pest must also be considered. EPN IJs which employ an "ambush" strategy are more effective against mobile pests, whereas "cruisers" can actively seek out immobile larvae below the soil level (Bal and Grewal, 2015; see section 1.4.2). These classifications aid the selection of EPN IJs against certain pests, however the extent to which these species employ one strategy over another is often overestimated (Griffin, 2012). Although regarded as ambushers due their propensity to "nictate" on the soil surface, *S. carpocapsae* are still able to infect and kill soil pests efficiently 40cm and below soil level (Dillon et al., 2007; Kapranas et al., 2017).

### 1.6.3 Mass production

Entomopathogenic nematodes can be produced *in-vivo* within insect hosts, especially if they are to be applied to the field within cadavers, however this process is labour intensive and costly. Most commercial production of EPN is in liquid culture in large scale bioreactors containing nutrition dense liquid media (Gaugler and Han, 2002). A key step in this *in-vitro* production process is the inoculation of the appropriate bacterial symbiont into the nutritive medium before the addition of the nematodes (Shapiro-Ilan et al., 2014b). Successive generations of nematodes may develop within this bioreactor until the nematode population peaks and the bacterial populations are consumed, initiating the production of IJs (Ehlers, 2001). Once the population contains a high proportion of IJs, the nematodes are separated from the spent medium and washed.

### 1.6.4 Formulation and application

Entomopathogenic nematodes may be kept in aqueous suspension at 4-15°C for 3-6 months for heterorhabditids and for 6-12 months for steinernematids (Hazir et al., 2003). The refrigeration requirement for IJs in this form is expensive and the bulk of this form also hinders transportation (Grewal, 2002). Embedding IJs in a medium can enhance their lifespan and reduce the need for specific storage conditions. Therefore, in most EPN products, IJs are formulated using ingredients that either physically prevent movement (e.g., gels) or induce partial anhydrobiosis, thereby reducing metabolism and increasing stress tolerance (Grewal, 2002).

Storage in alginate beads prolongs the survival of EPN species at room temperature (Hussein and Abdel-Aty, 2012; Chen and Glazer, 2005). The addition of glycerol to these beads improved *S. feltiae* survival after 6 months of storage at room temperature, and they retained their infectivity after this time (Chen and Glazer, 2005). These IJs had entered a quiescent state within the bead, which may further enhance their longevity as they avoid energy expensive behaviours while dormant. Many EPN species can be kept in this state at room temperature for months at a time, and for longer below 10°C (Grewal, 2002). Alginate beads may be applied to the field directly and can be supplemented with attractants to draw insects towards the beads themselves, facilitating their infection (Hiltpold et al., 2012). Most formulations are dissolved in water prior to application to the field (Grewal et al., 2002), "reactivating" the IJs and ending this quiescence (Kim et al., 2021)

EPN can be applied using a wide variety of sprayer equipment (Georgis, 1990). As with all abiotic stressors, some species are more resistant to the water pressure within the sprayer than others. *S. feltiae, S. carpocapsae* and *H. bacteriophora* can withstand pressures of up

to 2,000 kPa (Nilsson and Gripwall, 1999; Fife et al., 2003), while *H. megidis* is recommended to be used at less than 1380 kPa instead (Fife et al., 2003).

IJ-infected cadavers may be directly applied to fields. This method protects the IJs from desiccation and can increase their dispersal (Shapiro & Glazer 1996), efficacy (Shapiro-Ilan et al. 2003) and infectivity (Shapiro & Lewis 1999). The infected cadaver contains many IJs as well as nematode-secreted products. These phenomena may be explained by the build-up of ascarosides and other chemicals within host cadavers, which affect IJs' behaviours (see section 1.3.4; Wu et al., 2018a). Infected cadavers can be desiccated to decrease their weight and size to facilitate transport and also prolong storage life (Shapiro-Ilan et al., 2001). These cadavers can be formulated, coated in chemical agents which can enhance the shelf-life and desiccation tolerance of the products; however these formulated cadavers present other challenges. During transport and storage formulated cadavers can clump or rupture, which reduces their efficacy (Shapiro-Ilan et al., 2001).

### 1.6.5 Genetic improvement

The selection of desirable traits in EPN can improve their use as biocontrol agents, and reduce costs associated with their production. EPN can be selected for desirable traits such as tolerance of cold (Grewal et al., 1996), heat (Ehlers et al., 2005; Anbesse et al., 2013), desiccation (Strauch et al., 2004), nematicides (Glazer et al., 1997), oxidative stress (Sumaya et al., 2018) as well as host finding (Gaugler et al., 1989), and longevity (Sumaya et al., 2018). Conventional breeding and selection may be combined with molecular techniques to refine and enhance the selection of traits (Sumaya et al., 2018). Desirable traits such as high virulence against insects that IJs display in the lab may not be effective in the field due to a variety of abiotic and biotic factors (Shapiro-Ilan et al., 2014a). Furthermore, selection of specific traits may diminish other desirable traits (Gaugler 1987;

Gaugler et al., 1990). Genetic screening may enable the selection of beneficial traits in wild nematodes (Segal and Glazer, 2000) and enhance the selection of certain traits without losing other (Sumaya et al., 2018). Whether the traits are introduced by conventional or molecular methods, IJs tend to lose beneficial traits when produced in liquid cultures (Bilgrami et al., 2006; Adhikari et al., 2009).

Trait deterioration involves decreased expression of genes related to metabolism, signal transduction, longevity and virulence and increased expression of stress genes (Adhikari et al., 2009). Heterorhabditids do not mate in liquid cultures, and therefore all offspring are a result of self-fertilisation, creating inbred lines. Pooling multiple inbred lines initially can reduce trait deterioration in heterorhabditids (Anbesse et al., 2013). Trait deterioration in *Steinernema* spp. appears to be largely due to inbreeding depression, rather than inadvertent selection for negative traits (Chaston et al., 2011). Trait deterioration may be reduced in steinernematids by utilising inbred lines without deleterious alleles (Chaston et al., 2011).

## 1.7 Aims and objectives

Storage conditions affect the behaviour of EPN IJs and their tolerance of abiotic stress. The overall aim of this project is to identify some of the molecular mechanisms associated with various phenotypes that *Steinernema* and *Heterorhabditis* IJs exhibit in varied storage conditions. In this project, IJs were stored in water, as is common in laboratories and prior to formulation during mass production, though findings are also expected to have implications for IJs under more natural soil conditions.

There are two main strands to the project. The first investigates ascarosides or other metabolites secreted into the storage water by the IJs. The second strand explores the effect of storage temperature and age on behaviour, stress tolerance and proteome of IJs. Two species, *S. carpocapsae* All and *H. megidis* UK211, are included throughout this work, while the secreted metabolites are also investigated for two other species, *S. feltiae* and *S. longicaudum.*

Disentangling the relation between secreted or internally expressed products and their effect on the IJ can improve our understanding of these parasites and enhance their use as biocontrol agents.

**Ascarosides**

Ascarosides are a class of nematode signalling molecules with effects on behaviour and development (section 1.3). Choe et al (2012b) identified several ascarosides secreted by *Steinernema* IJs for up to 6 hours, leading to my first hypothesis.

**Hypothesis 1.** Storage of IJs in water will lead to increasing concentrations over time of signalling molecules, which affects IJ behaviour.

**Effects of storage temperature and time on behaviour, stress tolerance and proteomics**

Storage temperature has strong effects on the behaviour (Lee et al., 2016) and stress tolerance (Chen et al., 2005; Solomon et al., 2000; Grewal and Jagdale, 2002) of EPN IJs (see sections 1.4.5 and 1.5). Freezing and desiccation tolerance is partially due to the accumulation of trehalose and other small molecule cryoprotectants as well as stress proteins. Therefore proteins, including stress proteins and enzymes involved in the synthesis of protectants, are likely to be affected by conditioning as an organism adapts.

**Hypothesis 2.** Storage of IJs in water at various temperatures will affect their behaviour and stress tolerance in a temperature specific manner.

**Hypothesis 3.** Effects of storage regimes (time and temperature) on IJs will be reflected in their proteome, and the accumulation of protectant related proteins will be detectable.

**Hypothesis 4:** Changes in behaviour, stress tolerance and proteome induced by cold-storage will be maintained following a return to culture temperature.

Science is driven by the scientific method, which involves hypothesis testing as a central, focal point of enquiry, however hypothesis testing can be problematic. The Duhem-Quine thesis states hypothesis formation involves researchers relying on assumptions to form hypotheses, and they may avoid those which seem implausible despite their veracity (Harding, 1976). With the availability of data-driven, high throughput methods to investigate molecular systems, specifically the "omics", the investigative process is not necessarily hypothesis-led, and instead should be "model-independent" (Brown and Botstein, 1999). In agreement with Kell and Olver (2003), I believe that hypothesis-led and discovery based experimental approaches are complementary, and not oppositional.

Discovery-based research may resolve the molecular determinants of complex behaviours or phenotypes and may enable the formation of hypotheses for further testing.

These hypotheses are addressed in the following chapters:

1. At intervals over time, water is removed from high density (5000 IJs/ml) suspensions of *Steinernema* and *Heterorhabditis* IJs and applied to conspecific and heterospecific IJs on agar plates. Their dispersal is assessed to ascertain the effect of this worm conditioned water on the behaviour of conspecific and heterospecific IJs (Chapter 2; Hypothesis 1).
2. The worm conditioned water is also analysed using LC-MS/MS, using retention time and mass to charge ration (m/z) to identify which metabolites, especially ascarosides, are present in each species' exometabolome (Chapter 2; Hypothesis 1).
3. *Steinernema carpocapsae* All and *Heterorhabditis megidis* UK211 IJs are stored at 9°C and 20°C for up to 9 weeks. Label free quantitative proteomics is used to identify temperature dependent changes in the proteome of both species (Chapter 3; Hypothesis 3).
4. The effect of storage temperature and time on the behaviour and stress tolerance of conditioned IJs is assessed using chemotaxis, desiccation, and freezing assays (Chapter 4; Hypothesis 2).
5. To assess whether the effect of temperature on the IJs' behaviours and proteome are transient or lasting, IJs are conditioned in 9°C for brief periods of time and then transferred to 20°C for the duration of the experiment. Then, these IJs are subjected to the same assays as those kept in constant temperatures, and their proteomes are compared (Chapter 4; Hypothesis 4).

# Chapter 2 Infective juveniles of Entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) secrete ascarosides and respond to interspecific dispersal signals

**Published as:**

Hartley[1], C.J., Lillis[1], P.E., Owens, R.A., & Griffin, C.T. 2019. Infective juveniles of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) secrete ascarosides and respond to interspecific dispersal signals. *Journal of Invertebrate Pathology*, 168, 107257.

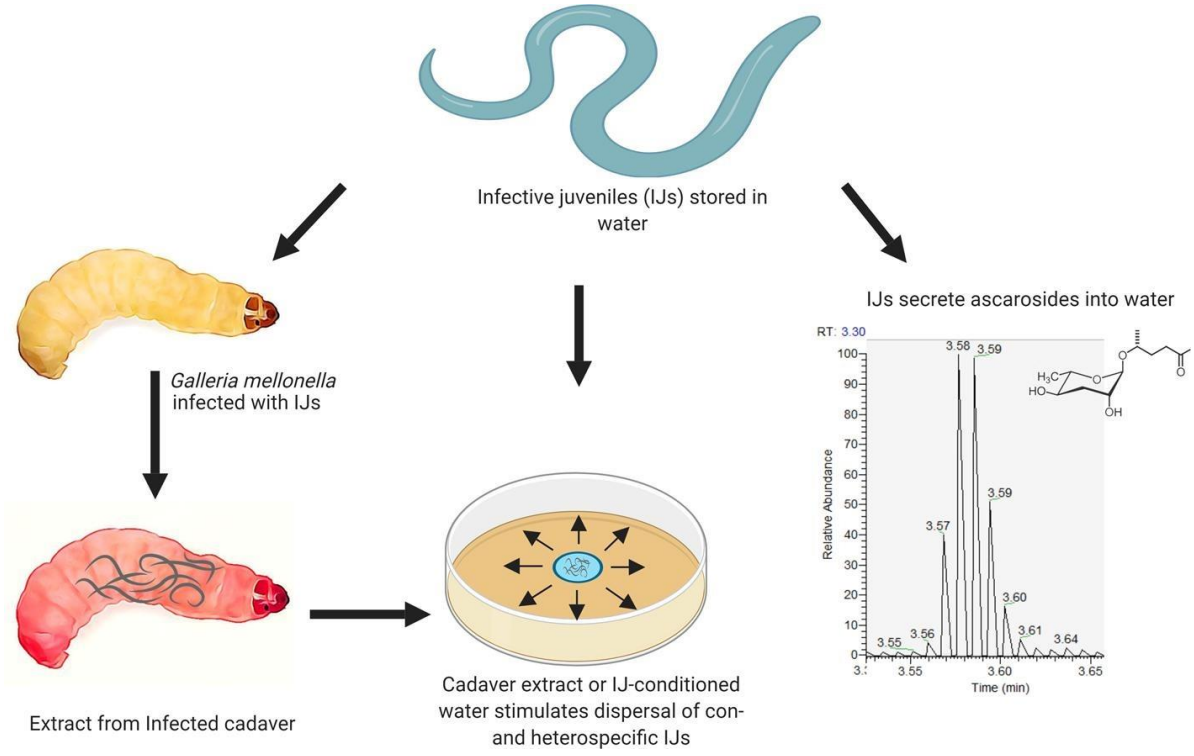
[1]Joint first authors

## Abstract

Ascarosides are a modular series of signalling molecules that are widely conserved in nematodes where they function as pheromones with both behavioural and developmental effects. Here we show that the developmentally arrested infective juvenile (IJ) stage of entomopathogenic nematodes (EPN) secrete ascarosides into the surrounding medium. The exometabolome of *Steinernema carpocapsae* and *Heterorhabditis megidis* was examined at 0, 1, 7 and 21 days of storage. The concentration of several ascarosides (ascr#11, ascr#9, ascr#12, ascr#1 and ascr#14 for both species, plus ascr#10 for *H. megidis*) showed a progressive increase over this period, while the concentration of longer chain ascarosides increased up to day 7, with an apparent decline thereafter. Ascr#9 was the main ascaroside produced by both species. Similar ascarosides were found over a 7-day period for *Steinernema longicaudum* and *S. feltiae*. Ascaroside blends have previously been shown to promote nematode dispersal. *S. carpocapsae* and *H. megidis* IJs were stored for up to 12 weeks and assayed at intervals. IJs where exometabolome was allowed to accumulate showed higher dispersal rates than those where water was changed frequently, indicating that IJ exometabolome maintained high dispersal. Infectivity was not affected. IJ exometabolome accumulated over 7 days promoted dispersal of freshly harvested IJs, both of their own and other EPN species. Similarly, extracts of nematode-infected cadavers promoted dispersal of con- and heterospecific IJs. Thus, IJs are encouraged to disperse from a source cadaver or from other crowded conditions by public information cues, a finding that may have application in enhancing biocontrol. However, the complexity of the ascaroside blend produced by IJs suggests that it may have ecological functions other than dispersal.

**Keywords**: pheromone, biocontrol, biological pest control, interspecific communication, metabolomics

## Graphical Abstract



## 2.1 Introduction

Ascarosides are a family of small signalling molecules with both behavioural and developmental effects that are widely conserved in nematodes (Edison, 2009; Butcher, 2017). They are composed of the dideoxy sugar, ascarylose, covalently linked to a fatty acid side chain. Variation in structure and length of the fatty acid side chain, together with various modifications of the ascarylose, results in a modular system with great potential variety (Butcher, 2017; von Reuss, 2018). The signalling role of ascarosides was first recognised when they were identified as constituents of the *Caenorhabditis elegans* dauer pheromone (Jeong et al., 2005; Butcher et al., 2007; Srinivasan et al., 2008). Ascaroside synthesis and signalling has since been identified in several phylogenetically distant nematode taxa apart from *C. elegans*, including entomopathogenic nematodes (Choe et al., 2012; Noguez et al., 2012; Butcher, 2017).

Entomopathogenic nematodes (EPN), principally *Steinernema* spp. and *Heterorhabditis* spp., are a clade of "beneficial" nematodes with a wide natural distribution in soils and other cryptic habitats. The infective juvenile (IJ) - a stage analogous to the *C. elegans* dauer juvenile - is a non-feeding, stress resistant stage that actively seeks out insect hosts. IJs invade insects which they rapidly kill with the aid of their symbiotic bacteria. IJs resume development ("recover") and multiply within the host cadaver, and eventually a new generation of IJs emerges and disperses from the spent resource to seek out fresh hosts to infect. As a result of their active host-seeking and rapid mortality for insects, several EPN species have been commercialised for use as a biological insecticide. For this purpose, they are produced in large scale bioreactors and IJs are applied to crops, normally in liquid suspension. Noguez et al. (2012) identified an ascaroside produced by *Heterorhabditis bacteriophora* at high densities (such as those found within a host) that prevented IJs from resuming development. Two other studies have reported ascaroside

production and/or function in EPN (Choe et al. 2012; Kaplan et al., 2012). As part of a broader survey of free-living and parasitic nematodes, Choe et al. (2012) identified several ascarosides produced by *H. bacteriophora* adults and by adult and IJ stages of three *Steinernema* species. Limited functional analysis included in that study showed that *Steinernema glaseri* males were retained by a selection of ascarosides in single-chemical assays (Choe et al., 2012).

Kaplan et al. (2012) focussed on responses of IJs to ascarosides. Since IJs are the active ingredient of biopesticides, their behaviour is of considerable applied interest and has been intensively researched for several decades (see reviews by Lewis et al., 2006; Griffin, 2015). Kaplan et al. (2012) studied the effects of ascarosides on the dispersal of IJs. Firstly, they found that *C. elegans* dispersal signal (a blend of ascarosides) promoted dispersal of *Steinernema feltiae* IJs (and also of J2 of the plant parasite *Meloidogyne* spp.) leading to the conclusion that interspecific nematode signals regulate dispersal. Secondly, in a more ecologically relevant investigation, they showed that extract of insect cadavers infected by *S. feltiae* promoted dispersal of *S. feltiae* IJs and that ascarosides (especially ascr#9) accumulated in the cadaver, with accumulation highest immediately prior to the dispersal of IJs from the cadavers. The hypothesis that ascarosides produced within the host cadaver are part of a dispersal pheromone for *S. feltiae* IJs was supported by dispersal assays of fractionated cadaver extract and synthetic ascarosides. Two ascarosides (ascr#9 and ascr#11) were detected in the ascaroside-rich fraction of cadaver extract, and synthetic versions of either of these at physiological concentrations stimulated dispersal - but only when combined with the other two fractions of natural cadaver extract (Kaplan et al., 2012). Finally, ascarosides were detected in cadavers infected by three other species of *Steinernema* and by three species of *Heterorhabditis*. All extracts contained ascr#9, suggesting that ascr#9 may be used by a broad range of EPN as part of their dispersal

blends, while ascr#11 was found in all species of *Steinernema*, but not in *Heterorhabditis*, suggesting that ascr#11 might be specific for *Steinernema* (Kaplan et al., 2012).

Here we investigate the secretion by IJs of ascarosides and/or other behaviour-affecting substances, and the responses of IJs to dispersal signals derived from nematode-infected cadavers and from IJs. 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. We test three hypotheses: (1) that IJs continue to secrete ascarosides beyond the 6 hours reported by Choe et al. (2012); (2) that exometabolome (ascarosides and/or other chemicals secreted by IJs) will accumulate in the water in which they are stored and affect the behaviour both of the stored IJs and of other IJs exposed to the exometabolome, and (3) that dispersal cues derived from IJs and from infected host cadavers are not species specific, but instead are public information, indicating crowding. This hypothesis that interspecific nematode signals regulate dispersal is based on the findings of Kaplan et al. (2012). If cadaver macerate or extracts prepared from cadavers are used to promote success of EPN in biocontrol as has been suggested (Wu et al., 2018; Oliveira-Hofman et al., 2019), it would be of obvious commercial benefit if preparations made from insects infected with one EPN species would also promote dispersal of other EPN species.

To test our hypotheses, we store IJs in water for up to 21 days and identify secreted ascarosides in the exometabolome. We then test whether preventing the accumulation of exometabolome during long-term storage by frequently removing and replacing the water affects the behaviour (dispersal and virulence) of the stored IJs themselves, relative to IJs where the water is not changed. We further test whether water in which IJs were stored for a week (IJ-conditioned water) stimulates dispersal when applied to other nematodes. Finally, we explore whether the dispersal signal of IJ-conditioned water and of nematode-

infected cadavers is species-specific by assaying preparations of each of four EPN species on each of the other species. We use members of both EPN genera, *Heterorhabditis megidis* and three species of *Steinernema* (*S. carpocapsae, S. feltiae* and *S. longicaudum*). Each of the three *Steinernema* species belongs to a different clade of the genus (clades II, III and V, respectively) (Nadler et al., 2006).

## 2.2 Materials and methods

### 2.2.1 Nematode culture and storage

The nematodes used were *S. longicaudum* CB2B, *S. carpocapsae* All, *S. feltiae* 4CFMO and *H. megidis* UK211. Nematodes were cultured in last instar larvae of *Galleria mellonella* (Mealworm Company, Sheffield, UK) using standard procedures (Woodring and Kaya, 1988), at 20ºC (27 ˚C for *S. longicaudum*), with an inoculum density of 100 IJ/insect. Cadavers were placed in White traps and IJs were harvested daily. IJs that emerged 2-5 days after emergence began were pooled, rinsed three times by sedimentation, and stored in sterile tap water in 50 ml aliquots in lidded plastic dishes (9 cm diam.).

### 2.2.2 Chemical profiling of IJ-conditioned water

Freshly harvested IJs were washed by sedimentation successively in the following: tap water; 50:50 tap water: milliQ water, and finally milliQ water. The final wash water was retained and used to control for residual ascarosides produced within the cadaver (time 0). IJs (5000/ml) were stored in milliQ water at 20˚C. After 1 or 7 (and 21 days in the case of *S. carpocapsae* and *H. megidis*), conditioned water containing IJ exometabolome was

separated from IJs by sedimentation followed by filtration through a 0.22 µm filter. All filters and tubes were rinsed with milliQ water prior to use. Samples (20 ml) were frozen at -20 °C overnight and were transferred to liquid nitrogen and lyophilised. Lyophilised material was stored at 4°C until mass spectrometry (MS) analysis. Samples were resuspended in HPLC-grade methanol (1 ml) and placed in a sonication bath for 2 min. Samples were dried using a vacuum concentrator and resuspended in 100 µl methanol. Samples were prepared for LC-MS/MS analysis by mixing 1:1 with 0.1 % formic acid. 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 (equivalent to the secretion of 5,000 IJs) was injected onto a Thermo Hypersil Gold aQ polar-C18 column (100 x 2.1 mm, 1.9 µm particles). Samples were analysed in negative mode, using a Top3 MS/MS method (NCE 40 eV). For each species, there were three biological replicates (each prepared from a different culture batch of nematodes). For each ascaroside detected, a regression of relative abundance on time was performed.

### 2.2.3 Effect of IJ conditioned water on dispersal of IJs

IJ suspension (5000 IJs/ml) was stored at 20°C (27°C for *S. longicaudum*) for 7 days. Following this, IJs were removed by sedimentation and the supernatant was filtered through a 0.2 µm syringe filter (Acrodisc). The filtered exudate was used to test the dispersal of freshly harvested IJs (48 h since harvest). IJs were concentrated by sedimentation immediately prior to use in the assay. Approximately 100-200 IJs in 5 µl water were applied to the centre of an agar plate (2% bacto agar in 5.5 cm Petri dish) followed by immediate application of 2 µl of IJ-conditioned water (equivalent to the

secretion of 10 IJs) or water only (controls). A circular glass coverslip (19 mm diameter) was placed over the suspension, delimiting a central application zone. Plates were incubated at 20˚C (or 27˚C for *S. longicaudum*) for 10 minutes. IJs remaining under the coverslip were recorded as not dispersed and those migrating onto the open agar surface were recorded as dispersed. Counting was done with the aid of a dissecting microscope. There were two experiments. In the first, IJs of each species were tested with water conditioned by IJs of their own species. This experiment was run three times with independent preparations of IJ conditioned water and test IJs, and 10 assay plates per treatment in each experiment. Data were analysed for each species using GLM with treatment as a fixed factor and experiment as a random factor. In the second experiment, IJs of each species were tested with water conditioned by IJs of their own and the other three species. This experiment was run four times, once for each test species, with freshly prepared IJ-conditioned water of all four species and 10 assay plates per treatment in each experiment.

### 2.2.4 Influence of replacing storage water on IJ dispersal and virulence (*H. megidis* and *S. carpocapsae*)

IJ suspension (1000 IJ/ml) was stored at 20˚C, and the water was either changed at intervals (to limit the accumulation of exometabolome) or left unchanged. To change the water, the contents of a dish were transferred to a 50 ml centrifuge tube and IJs were allowed to sediment. The water was then decanted and replaced with fresh sterile tap water and the suspension was returned to the dish. For the unchanged treatment (control), IJs were allowed to sediment in a centrifuge tube which was then inverted to resuspend the IJs in the original water before returning to the storage dish. The dispersal and virulence of IJs

was assayed at intervals during storage. IJs for assay were collected immediately after the treatment (resuspension or change of water). Dispersal was assayed as described above. Virulence was assessed against mealworms, *Tenebrio molitor,* in a close-contact assay. Wells of a 24-well cell culture plate were lined with moistened coconut coir and 10 IJs in 10 µl were added to 12 of the wells in each plate, while the other 12 wells received 10 µl tap water only and served as controls. A *T. molitor* larva was added to each well. Plates were closed, sealed with Parafilm and incubated for 4 days in the dark at 20˚C. One assay plate was used per replicate storage dish. Mortality in the nematode treatments was corrected for control mortality using Abbotts' formula.

The experiment was run twice with independent batches of nematodes for each of *H. megidis* and *S. carpocapsae* five storage dishes per treatment in each experiment. In the first experiment IJs were stored at 20$^{o}$C (which is within the optimum temperature range for activity of these species). Water was replaced every 2-3 days and assays were conducted at time 0 and after 1, 2, 3, 4 and 5 weeks. In the second experiment IJs were stored at 9°C (a temperature below the optimum temperature range for activity, with prolonged longevity). Water was replaced every 7 days and assays were conducted at time 0 and after 3, 6, 9 and 12 weeks. All assays were at 20°C. Data were subjected to regression analysis with time as a continuous variable and treatment as categorical.

### 2.2.5 Effect of cadaver extract on dispersal

Cadaver extract was prepared using infected wax moths 1-2 days after IJ emergence had commenced. Cadavers were punctured several times using a sterile needle in 1.5 ml Eppendorf tubes (2 per tube), then 500 µl of sterile distilled $H_2O$ was added to the tube which was vortexed thoroughly to liberate the contents of the cadavers. Tubes were

centrifuged at 800 x g for 10 mins. The supernatant was centrifuged a second time (800 x g, 10 mins) and the resulting supernatant (equivalent to 4 cadavers/ml) was used to test the dispersal of freshly harvested IJs as described above, using 2 µl of cadaver extract (equivalent to 0.008 cadavers). There were two experiments. In the first, IJs of each species were tested with their own cadaver extract. In the second, IJs of each of the four nematode species were assayed with extract from cadavers infected with each of the four species. This experiment was conducted once for each of the four nematode species, with cadaver extract freshly prepared for each experiment. There were 10 replicate assay plates for each treatment in each experiment. For intraspecific comparison of cadaver extract with control, two-sample t tests were used. In the cross-species cadaver experiments, a one-way ANOVA was followed by Tukey's *post hoc* test when $P < 0.05$ to identify where significant differences occurred.

## 2.3 Results

### 2.3.1 Identification of ascarosides in IJ-conditioned water

The exometabolome of *S. carpocapsae* and *H. megidis* IJs was analysed for up to 21 days of storage. The relative concentration of several ascarosides (ascr#11, ascr#9, ascr#12, ascr#1 and ascr#14, and ascr#10 for *H. megidis*) showed a progressive increase over this period, and the regression of relative abundance on time was significant ($P < 0.05$) or approaching significance ($P < 0.10$) in each case (Fig. 2.1; Appendix 2.3, Appendix 2.4). The concentration of longer chain ascarosides ascr#16 and ascr#18, and ascr#10 in the case of *S. carpocapsae*, increased up to day 7, followed by an apparent decline between day 7 and day 21 (Fig. 2.1). Ascr #9 was the main ascaroside detected for both species, while ascr#18 was also prominent in the *H. megidis* exometabolome (Fig. 2.1). Seven-day

exometabolome of IJs of two other species, *S. longicaudum* and *S. feltiae* showed a similar profile to that of *S. carpocapsae*, with ascr#9 as the main signature and trace amounts of ascr#11, ascr#12, ascr#1, ascr#14 ascr#16 and ascr#18.

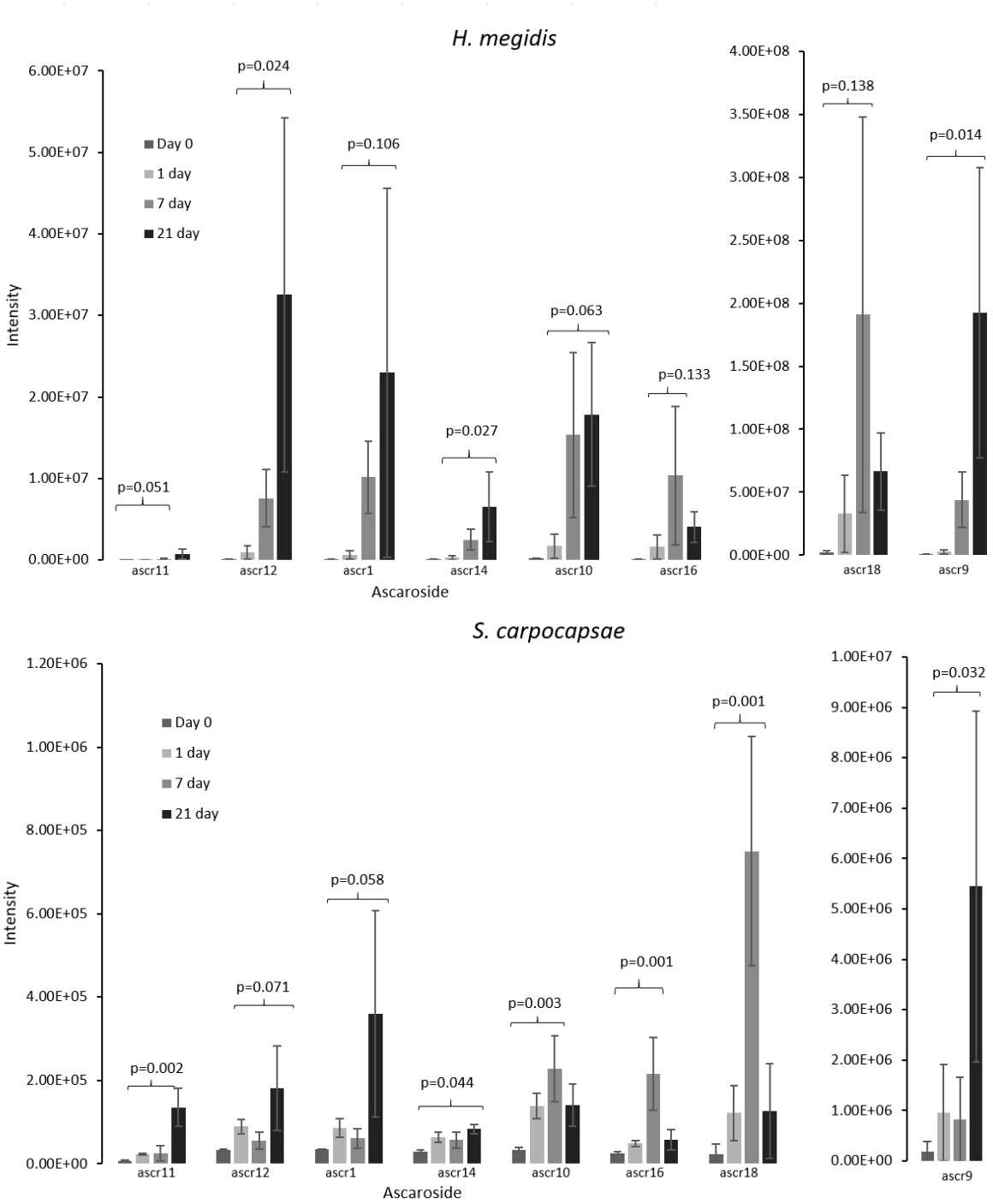



**Figure 2.1:** Relative abundance of ascarosides in water conditioned by infective juveniles of *Steinernema carpocapsae* and *Heterorhabditis megidis* over 21 days at 20°C. Values are for 5000 IJs worm equivalence. P values indicate the significance of the regression on time either over 7 or 21 days as indicated by brackets (see Appendix 2.4).

### 2.3.2 IJ dispersal is promoted by exposure to water conditioned by conspecific and heterospecific IJs

Water conditioned with IJs (5000/ml) for 7 days stimulated dispersal of conspecific IJs relative to the control and the difference was significant for each of the four species tested (*H. megidis*: $F_{1,56} = 31.41$, $P < 0.001$; *S. carpocapsae*: $F_{1,56} = 25.39$, $P < 0.001$; *S. feltiae*: $F_{1,56} = 43.93$, $P < 0.001$; *S. longicaudum*: $F_{1,56} = 51.26$, $P < 0.001$) (Fig. 2.2).

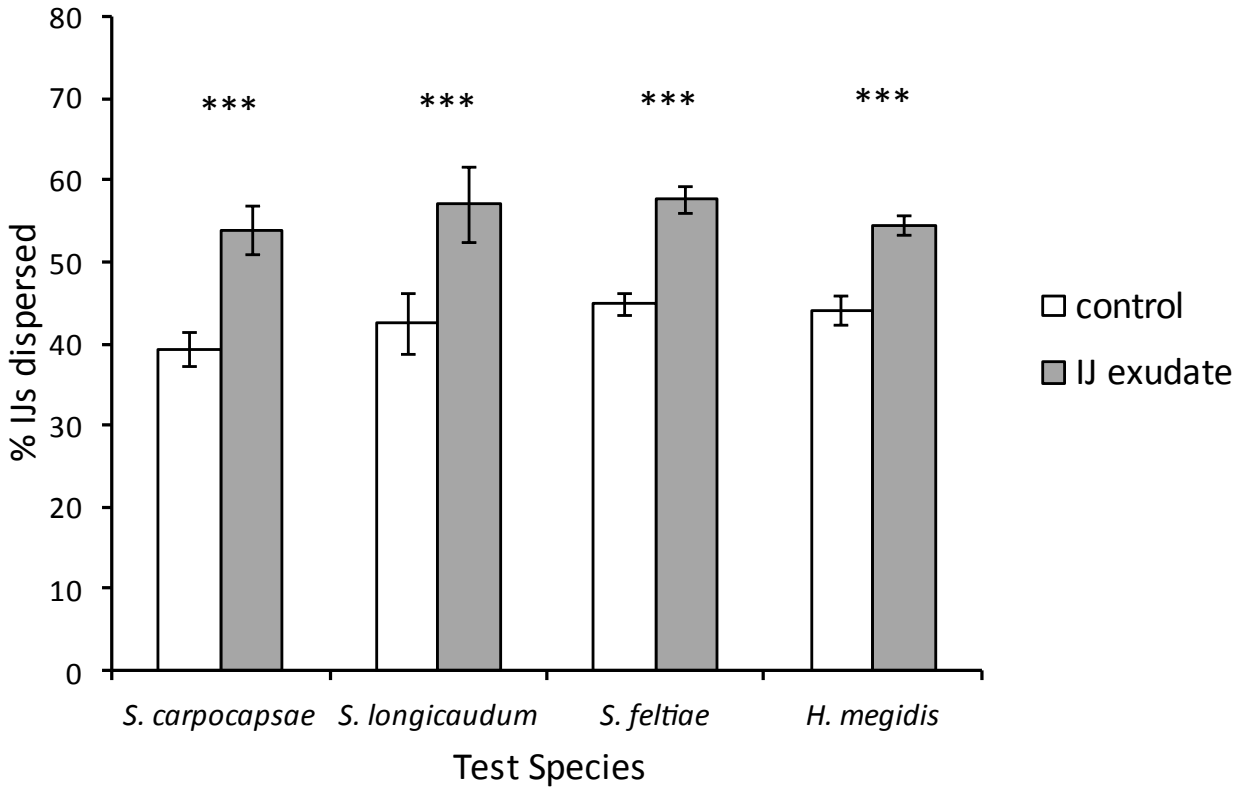



**Figure 2.2:** Percentage (mean ± SE) of IJs dispersing from 2 µl water conditioned with conspecific IJs (5000 IJ/ml for 7 days; i.e. 10 IJs worm equivalence). Mean of three independent experiments. Within species, *** = $P < 0.001$.

The storage concentration of 5000 IJs/ml was chosen based on the results of preliminary assays with concentrations ranging from 500 to 5000 IJs/ml. The minimum storage concentration for which dispersal differed significantly from the control ranged from 500 (*S. feltiae*) to 5000 (*H. megidis*), with the other two species showing intermediate levels of 1000 (*S. longicaudum*) or 2000 (*S. carpocapsae*) IJs/ml (data not shown). The promotion of IJ dispersal by IJ exudate was not species-specific (Fig. 2.3). For each test species, there was a significant difference due to treatment (*H. megidis*: $F_{4,45} = 18.34$, $P < 0.001$; *S. carpocapsae*: $F_{4,45} = 4.69$, $P = 0.003$; *S. feltiae*: $F_{4,45} = 11.65$, $P < 0.001$; *S. longicaudum*: $F_{4,45} = 5.05$, $P = 0.002$). In general, the percentage of IJs dispersing differed to that in the control irrespective of the species used to condition the water (Tukey test) (Fig. 2.3). For each of the three *Steinernema* species, there was no difference in the percentage of IJs dispersing due to the species used to condition the water. While there were significant differences in the response of *H. megidis* to the various exudates, the proportion of IJs dispersing in the presence of exudate from *Steinernema* spp. was at least as high as in response to exudate of conspecific IJs (Fig. 2.3).

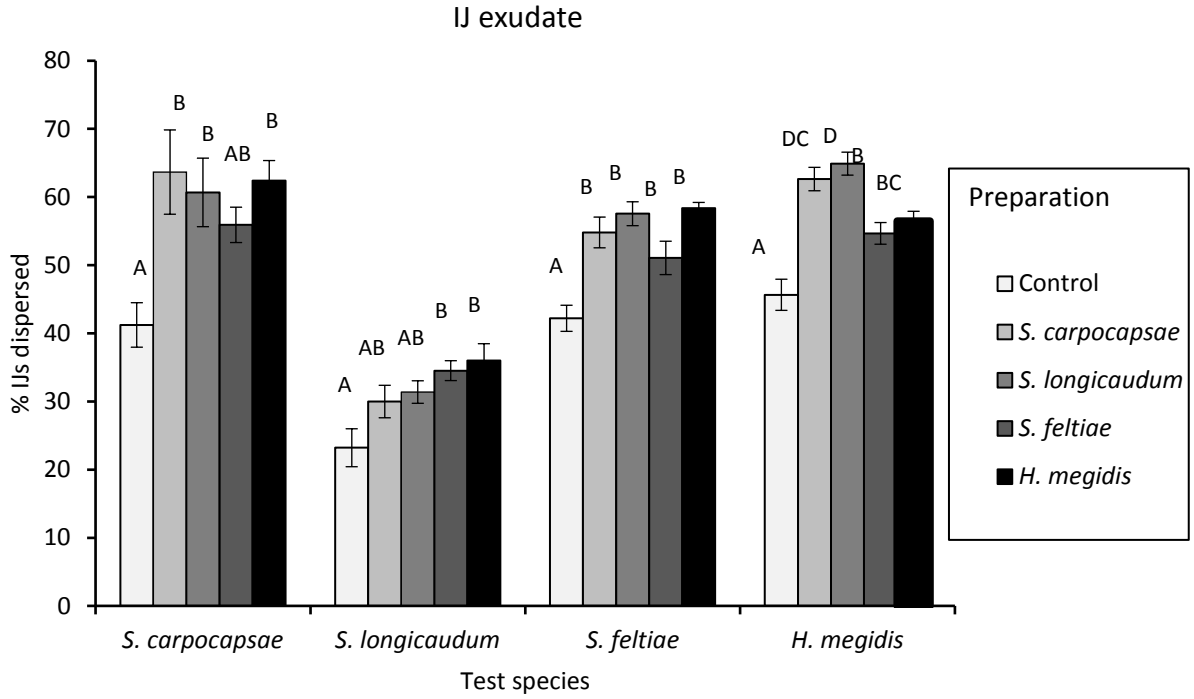



**Figure 2.3:** Percentage (mean + SE) of infective juveniles of four entomopathogenic nematode species dispersing from 2 µl water conditioned by IJs (5000 IJ/ml for 7 days; i.e. 10 IJs worm equivalence) of each of the four species. Within test species, bars which do not share a letter differ significantly.

Replacing water during storage impacts dispersal but not virulence of stored IJs

The dispersal rate of both *H. megidis* and *S. carpocapsae* IJs declined over time and the effect of time was highly significant ($P < 0.001$) for both species at both storage temperatures (Fig. 2.4). There was a significant interaction between storage treatment and storage duration for both species when stored at 20°C (*H. megidis*: $F_{1, 116} = 6.29$, $P = 0.014$; *S. carpocapsae*: $F_{1, 116} = 4.85$, $P = 0.030$) and for *S. carpocapsae* stored at 9°C ($F_{1, 96} = 24.57$, $P < 0.001$). At later time points, IJs where the storage water was changed regularly dispersed less than IJs where the water was not changed (Fig. 2.4). There was no effect of storage treatment on virulence for either species stored at either 9 or 20°C ($P > 0.05$; data not shown).

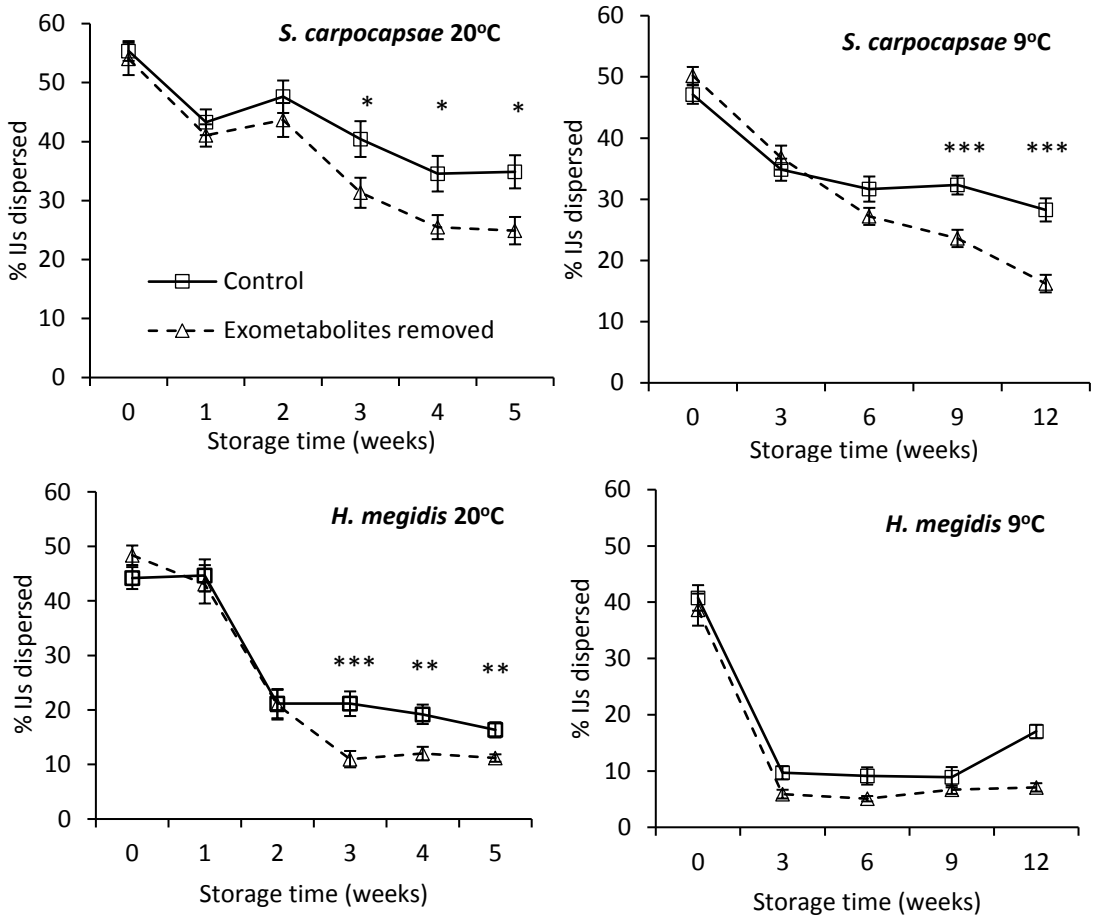



**Figure 2.4:** Percentage of *H. megidis* or *S. carpocapsae* IJs dispersing following storage (5000 IJs/ml) at 20˚C. Water in the storage dishes was regularly either removed and placed back in the dish ("control"), or removed and replaced by fresh water ("exometabolites removed"). Within time point, *, **, *** indicates difference between treatments significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$.

### 2.3.3 Effect of infected cadaver extract on dispersal is not species-specific

In the cadaver extract assay, treatment had a significant effect on dispersal of IJs of each of the four species tested (*H. megidis*: $F_{4,45} = 60.94$, $P < 0.001$; *S. carpocapsae*: $F_{4,45} = 53.01$, $P < 0.001$; *S. feltiae*: $F_{4,45} = 10.32$, $P < 0.001$; *S. longicaudum*: $F_{4,45} = 23.62$, $P < 0.001$). For each species, the percentage of IJs dispersing in the presence of cadaver extract was higher than in the control, but was no difference between the four cadaver extracts (Tukey test) (Fig. 2.5).

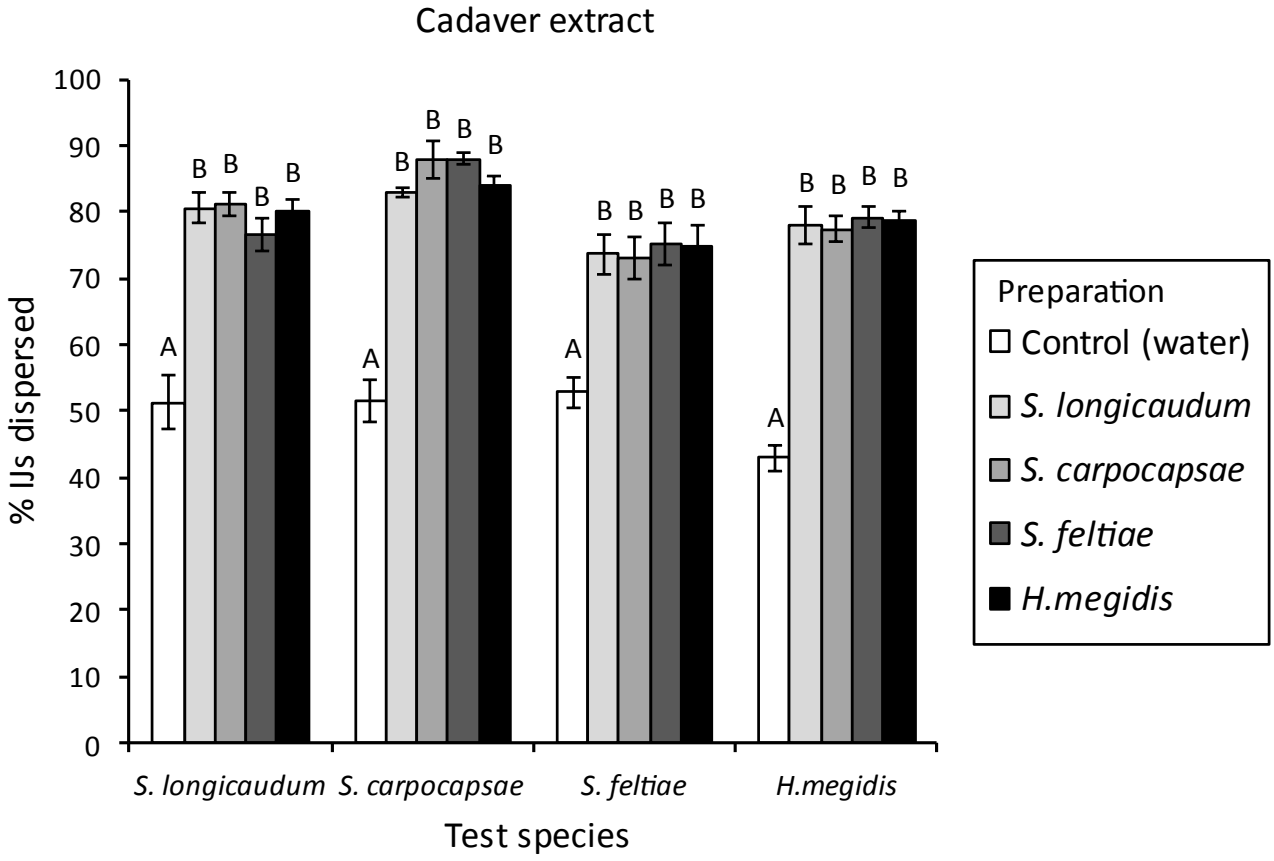



**Figure 2.5:** Percentage (mean + S.E.) of IJs of four entomopathogenic nematode species dispersing from cadaver extract of each of the four species. Within test species, bars which do not share a letter differ significantly.

## 2.4 Discussion

Here we show that ascarosides accumulate in the water in which IJs of *Steinernema* and *Heterorhabditis* are stored, and this accumulation continued for more than a week. Ascarosides are important in inducing dauer juvenile formation in *C. elegans*, and it was in this context that their importance as pheromones was first detected (Jeong et al., 2005; Butcher et al., 2007). However, rather little attention has been paid to the production or release of ascarosides by dauer juveniles or the analogous developmentally arrested IJs of parasitic nematodes. (Kaplan et al., 2011) did not detect ascarosides in "worm water" conditioned by *C. elegans* dauers, though small quantities of two ascarosides were found within the dauer bodies, while Choe et al. (2012) detected several ascarosides in worm water from dauers of *Caenorhabditis* species. Ascarosides have also been identified in worm water of IJs of diverse taxonomic and ecological groups including the animal parasite *Nippostrongylus brasiliensis* (Choe et al., 2012), various plant parasites (Manosalva et al., 2015; Zhao et al., 2018) and the insect-associated *Ocheius carolinensis* and *Steinernema* species (*S. carpocapsae, S. scapterisci, S. riobrave and S. glaseri*) (Choe et al., 2012)). Our results are the first to show that IJs or dauers continue to release ascarosides to the external medium for longer than 24 hours.

Ascr#9 was the dominant signal detected in the exo-metabolome of the three *Steinernema* species tested here, with smaller signals for several other ascarosides. This is in line with the findings that the ascaroside blends of various insect-associated nematodes (the entomopathogens *Steinernema* spp., *H. bacteriophora* and *Oscheius tipuli*, the necromenic *Pristionchus pacificus*, and the beetle-vectored pine wilt nematode *Bursaphelenchus xylophilus)* (Choe et al. 2012; Zhao et al., 2018) all had high amounts of this ascaroside. Choe et al. (2012) noted that ascaroside biosynthesis patterns appear in part to correlate with lifestyle or ecological niche as well as with phylogeny. Similarly, the plant parasites

*Meloidogyne* spp., *Heterodera glycines* and *Pratylenchus brachyuris* all had high expression of ascr#18 (Manosalva et al., 2015). In our study, the signal for ascr#18 was higher for *H. megidis* than for the *Steinernema* spp., consistent with Choe et al.'s (2012) findings of a relatively high signal for ascr#18 worm water from adult *H. bacteriophora*.

Our experiments show that IJ exometabolome (water conditioned by IJs for 7 days) stimulated dispersal of other, freshly washed IJs. Based on Kaplan et al.'s work there is a strong probability that the dispersal cue of IJ-conditioned water is at least partly due to the ascarosides detected in it, though other unidentified metabolites and excretory products of IJs may also be involved. We also confirm the previous finding (Kaplan et al., 2012; Wu et al., 2018; Oliveira-Hofman et al. 2019) that extract of nematode-infected cadaver promotes dispersal of IJs, and extend this to two other species (*H. megidis* and *S. longicaudum*). Nitrogenous waste products of nematodes accumulating within the cadaver have been implicated in repulsion of IJs from infected hosts, and in stimulating emergence of IJs developing within the hosts (Shapiro et al., 2000; San-Blas et al., 2008; San-Blas et al., 2014). Kaplan et al. (2012) demonstrated that ascr#9 accumulates in cadavers and plays a role in inducing dispersal in *S. feltiae*, but that other components of the cadaver exudate also played a role. Many chemicals are repulsive to IJs in single-agent chemotaxis assays (Lee et al., 2016; Baiocchi et al., 2017). Baiocchi et al. (2017) identified several odorant molecules from *Steinernema*-infected wax moth larvae that were repulsive to *Steinernema* IJs. These included prenol and acetyl methyl carbinol, which according to Baiocchi et al. (2017) could conceivably be produced by the nematodes themselves, their symbiotic bacteria, or as a by-product of the decay of the insect cadaver. Thus, the insect cadaver and its extract represent a complex chemical mixture potentially derived not only from the nematodes themselves, but also from a range of bacteria including their symbiont, and products of the cadaver itself. It would be adaptive for IJs to respond to any stimulus,

alone or in combination, that signifies a depleted resource, and it is therefore probable that several components of cadaver extract contribute to the dispersal effect on IJs.

Given that any of several different chemicals accumulating in a depleted host cadaver may signify poor resource quality, our finding that IJs of all four species dispersed in response to extract of cadavers infected by each of the other species, as well as their own, is not unexpected. It is perhaps more surprising that the IJs were also stimulated to disperse by water conditioned by IJs of other species as well as their own. This was the case both for the three *Steinernema* species, and also between *Steinernema* spp. and the more distantly related *H. megidis*. In this case, the medium is expected to contain a simpler blend of molecules, including ascarosides, but derived from IJs only. The lack of species-specificity means that both cadaver extract and IJ conditioned water can be considered public information, produced by one species but similarly responded to by several species with similar lifestyle (Danchin et al., 2004; Gil et al., 2018). Unlike aggregation and mating signals, there is little advantage to have species-specificity in dispersal cues, and indeed there is an advantage in having common indicators of crowding. Our results support this hypothesis, as do other studies including the observed similarities in ascaroside profiles of *Steinernema* spp. IJs (Choe et al., 2012), and the experimental evidence showing dispersal of EPN and plant parasites in response *C. elegans* ascaroside blends (Kaplan et al., 2012).

The finding that dispersal cues are not species-specific is of potential commercial significance, since it means that a single preparation could be used to stimulate IJs of various EPN species- a significant factor in any possible commercialisation of such an approach. EPN are typically produced in bioreactors, and IJs are cleaned and formulated for distribution, following which they are resuspended in water for application (Ehlers, 2001; Shapiro-Ilan and Gaugler, 2002). The use of insect cadavers to enhance the biocontrol efficacy of EPN in an approach that has been developed in recent decades.

Laboratory studies showed that IJs emerging from infected hosts had enhanced dispersal, infectivity and survival compared to IJs applied in water (Shapiro and Glazer, 1996; Shapiro and Lewis, 1999; Perez et al., 2003), and superior efficacy of IJs applied in cadavers was demonstrated in the greenhouse and field (Shapiro-Ilan et al., 2003; Ansari et al., 2009). Application of EPN in cadavers has been advocated as a way of improving field efficacy though this approach faces practical challenges (Dolinski et al., 2015). With the growing evidence that chemical compounds associated with the cadaver stimulate dispersal, as discussed above, and Kaplan et al.'s (2012) demonstration that cadaver exudate stimulated dispersal of *S. feltiae* on agar plate assays, there is potential for addition of dispersal-stimulating compounds to tank mix of EPN prior to application, rather than applying cadavers themselves. IJs of three EPN species (*S. feltiae*, *S. carpocapsae* and *H. bacteriophora*) were stimulated by macerate of cadavers (infected by their own species) to disperse through columns of sand and infect hosts at the bottom (Wu et al., 2018). Subsequently, Oliveira-Hofman et al. (2019) showed that methanol extract of infected cadaver was more effective than cadaver macerate, and that such preparations enhanced the efficacy of EPN against insect pests in greenhouse trials.

In the studies by Wu et al. (2018) and Oliveira-Hofman et al. (2019) it is unclear whether the enhanced infection of insects by treating IJs with cadaver macerate or extract was due to enhanced dispersal of IJs, with host-seeking behaviour remaining unaffected, or whether both untargeted movement (dispersal) and targeted host-finding were both impacted. Indeed, it may be difficult to distinguish between the two processes. Behaviours involved in host-finding in EPN have received considerable attention; such studies indicate that IJs may employ active responses including to chemical cues emanating from insects themselves (Grewal et al., 1997; Lewis et al., 2006) and from insect-damaged (Rasmann and Turlings, 2008; Ali et al., 2012). Less attention has been paid to dispersal as a

phenomenon separate from that of host-finding. Dispersal is an essential feature in the life cycle of many animals, serving to move away from depleted resources and/or high numbers of competitors and to avoid inbreeding (Clobert et al., 2001). There is evidence for at least some EPN species, that innate dispersal tendency of IJs is high shortly after they emerge from the cadaver, but declines with time. In *H. megidis*, dispersal declines as infectivity rises, resulting in a characteristic phasing of activity (Dempsey and Griffin, 2002). While the ultimate goal of the IJ is to find and invade another host, the first task must be to disperse away from the spent natal cadaver and the densely crowded soil surrounding it. Even species classified as sit-and-wait or ambush foragers, such as *S. carpocapsae*, must disperse from the spent host before or while searching for hosts (Bal et al., 2014). In our long-term storage experiments, there was a decline in dispersal tendency of both *H. megidis* and *S. carpocapsae* over time, irrespective of treatment. In these experiments, IJs provided with clean water on a regular basis to limit the accumulation of IJ exometabolome in the medium dispersed significantly less after some weeks when compared to treatments where the original water remained. This is further evidence that IJs secrete into the medium a compound or compounds that enhance dispersal. Hence the observed level of dispersal (but not virulence) is an interaction of the IJs' endogenous tendency and self-produced exogenous stimuli. Since IJs of entomopathogenic nematodes are routinely stored in aqueous suspension in laboratories (and also between harvesting and formulation in commercial enterprises), this information may help in the interpretation or design of laboratory experiments. The ecological significance is unclear, but there may be circumstances in which a group of IJs remain together for long enough to accumulate sufficient exometabolome in the surrounding soil water film to promote dispersal when conditions favouring movement return.

While we have shown that the exometabolome promotes IJ dispersal, this may not be the primary function. Indeed, since any single chemical could effectively stimulate dispersal (Baiocchi et al., 2017), the complex mix of molecules in the IJ exometabolome would be unnecessarily expensive for this purpose, and hence evolutionarily unstable. There is evidence that EPN IJs exhibit group movement (Fushing et al., 2008; Shapiro-Ilan et al., 2014; Ruan et al., 2018); ascarosides may play a role in intraspecific communication to promote aggregation. There is increasing evidence that ascarosides can be detected by other trophic groups (Hsueh et al., 2013; Manosalva et al., 2015; Zhao et al., 2016; Zhao et al., 2018). This may be to the advantage of the nematodes; for example, ascarosides produced by *B. xylophilus* juveniles promoted metamorphosis of their beetle vector (and the prevalence of its fungal symbiont (Zhao et al., 2016, 2018). Other soil organisms may "eavesdrop" on nematode communication, to their disadvantage, for example triggering plant defences (Manosalva et al., 2015), and trap formation by nematophagous fungi (Hsueh et al., 2013).

## 2.5 Conclusion

In conclusion, our study builds on the previous findings of Choe et al. (2012) and Kaplan et al. (2012). Its novelty is three-fold: firstly, in demonstrating that the secretion of ascarosides beyond the 6 hours of Choe et al. (2012); secondly, showing that IJ conditioned water (IJ exometabolome) promotes dispersal of IJs, and thirdly, that the dispersal signals of both IJ-conditioned water and EPN-infected cadavers are public information, detected by members of their own and other species.

**Acknowledgements**

CJH and PEL were funded by John and Pat Hume scholarships from Maynooth University. Liquid chromatography and high mass accuracy mass spectrometry facilities were funded by Science Foundation Ireland (12/RI/2346 (3)).

## References

ALI, J.G., ALBORN, H.T., CAMPOS-HERRERA, R., KAPLAN, F., DUNCAN, L.W., RODRIGUEZ-SAONA, C., KOPPENHOFER, A.M., STELINSKI, L.L. 2012. Subterranean, herbivore-induced plant volatile increases biological control activity of multiple beneficial nematode species in distinct habitats. *PLoS One,* 7, e38146.

ANSARI, M.A., HUSSAIN, M., MOENS, M. 2009. Formulation and application of entomopathogenic nematode-infected cadavers for control of *Hoplia philanthus* in turfgrass. *Pest Management Science,* 65, 367-374.

BAIOCCHI, T., LEE, G., CHOE, D.H., DILLMAN, A.R. 2017. Host seeking parasitic nematodes use specific odors to assess host resources. *Scientific Reports,* 7, 6270.

BAL, H.K., TAYLOR, R.A.J., GREWAL, P.S. 2014. Ambush foraging entomopathogenic nematodes employ 'sprinting emigrants' for long distance dispersal in the absence of hosts. *Journal of Nematology,* 44, 450-451.

BUTCHER, R.A. 2017. Decoding chemical communication in nematodes. *Natural Product Reports,* 34, 472-477.

BUTCHER, R.A., FUJITA, M., SCHROEDER, F.C., CLARDY, J. 2007. Small-molecule pheromones that control dauer development in *Caenorhabditis elegans. Nature Chemical Biology,* 3, 420-422.

BUTCHER, R.A., RAGAINS, J.R., KIM, E., CLARDY, J. 2008. A potent dauer pheromone component in *Caenorhabditis elegans* that acts synergistically with other components. *Proceedings of the National Academy of Sciences, USA.* 105, 14288-14292.

CHOE, A., VON REUSS, S.H., KOGAN, D., GASSER, R.B., PLATZER, E.G., SCHROEDER, F.C., STERNBERG, P.W. 2012. Ascaroside signaling is widely conserved among nematodes. *Current Biology,* 22, 772-780.

CLOBERT, E.L., DANCHIN, E., DHONDT, A.A., NICHOLS, J.D. 2001. Dispersal. *Oxford University Press, Oxford,* U.K.

DANCHIN, E., GIRALDEAU, L.A., VALONE, T.J., WAGNER, R.H. 2004. Public information: From nosy neighbors to cultural evolution. *Science,* 305, 487-491.

DEMPSEY, C.M., GRIFFIN, C.T. 2002. Phased activity in *Heterorhabditis megidis*. *Parasitology,* 124, 605-613.

DOLINSKI, C., SHAPIRO-ILAN, D., LEWIS, E.E. 2015. Insect cadaver applications: pros and cons, in: Campos-Herrera, R. (Ed.), Nematode pathogenesis of insects and other pests. *Springer, London,* 207-229.

EDISON, A.S. 2009. *Caenorhabditis elegans* pheromones regulate multiple complex behaviors. *Current Opinion in Neurobiology,* 19, 378-388.

EHLERS, R.-U. 2001. Mass production of entomopathogenic nematodes for plant protection. *Applied Microbiology and Biotechnology,* 56, 623-633.

FUSHING, H., ZHU, L., SHAPIRO-ILAN, D.I., CAMPBELL, J.F., LEWIS, E.E. 2008. State-space based mass event-history model I: many decision-making agents with one target. *Annals of Applied Statistics,* 2, 1503-1522.

GIL, M.A., HEIN, A.M., SPIEGEL, O., BASKETT, M.L., SIH, A. 2018. Social information links individual behavior to population and community dynamics. *Trends in Ecology and Evolution,* 33, 535-548.

GREWAL, P.S., LEWIS, E.E., GAUGLER, R. 1997. Response of infective stage parasites (Nematoda: Steinernematidae) to volatile cues from infected hosts. *Journal of Chemical Ecology*, 23, 503-515.

GRIFFIN, C.T. 2015. Behaviour and population dynamics of entomopathogenic nematodes following application, in: Campos-Herrera, R. (Ed.), Nematode pathogenesis of insects and other pests, *Springer, London*, 57-95.

HSUEH, Y.P., MAHANTI, P., SCHROEDER, F.C., STERNBERG, P.W. 2013. Nematode-trapping fungi eavesdrop on nematode pheromones. *Current Biology,* 23, 83-86.

JEONG, P.Y., JUNG, M., YIM, Y.H., KIM, H., PARK, M., HONG, E.M., LEE, W., KIM, Y.H., KIM, K., PAIK, Y.K. 2005. Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. *Nature,* 433, 541-545.

KAPLAN, F., ALBORN, H.T., VON REUSS, S.H., AJREDINI, R., ALI, J.G., AKYAZI, F., STELINSKI, L.L., EDISON, A.S., SCHROEDER, F.C., TEAL, P.E. 2012. Interspecific nematode signals regulate dispersal behavior. *PLoS One,* 7, e38735.

KAPLAN, F., SRINIVASAN, J., MAHANTI, P., AJREDINI, R., DURAK, O., NIMALENDRAN, R., STERNBERG, P.W., TEAL, P.E.A., SCHROEDER, F.C., EDISON, A.S., ALBORN, H.T. 2011. Ascaroside expression in *Caenorhabditis elegans* is strongly dependent on diet and developmental stage. *PLoS One,* 6, e17804

LEE, J.H., DILLMAN, A.R., HALLEM, E.A. 2016. Temperature-dependent changes in the host-seeking behaviors of parasitic nematodes. *BMC Biology*. 14, 36.

LEWIS, E.E., CAMPBELL, J., GRIFFIN, C., KAYA, H., PETERS, A. 2006. Behavioral ecology of entomopathogenic nematodes. *Biological Control,* 38, 66-79.

MANOSALVA, P., MANOHAR, M., VON REUSS, S.H., CHEN, S.Y., KOCH, A., KAPLAN, F., CHOE, A., MICIKAS, R.J., WANG, X.H., KOGEL, K.H., STERNBERG, P.W., WILLIAMSON, V.M., SCHROEDER, F.C., KLESSIG, D.F. 2015. Conserved nematode signalling molecules elicit plant defenses and pathogen resistance. *Nature Communications*, 6, 7795.

NADLER, S.A., BOLOTIN, E., STOCK, S.P. 2006. Phylogenetic relationships of *Steinernema* Travassos, 1927 (Nematoda : Cephalobina : Steinernematidae) based on nuclear, mitochondrial and morphological data. *Systematic Parasitology*, 63, 161-181.

NOGUEZ, J.H., CONNER, E.S., ZHOU, Y., CICHE, T.A., RAGAINS, J.R., BUTCHER, R.A. 2012. A novel ascaroside controls the parasitic life cycle of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *ACS Chemical Biology*, 7, 961-966.

OLIVEIRA-HOFMAN, C., KAPLAN, F., STEVENS, G., LEWIS, E., WU, S.H., ALBORN, H.T., PERRET-GENTIL, A., SHAPIRO-ILAN, D.I. 2019. Pheromone extracts act as boosters for entomopathogenic nematodes efficacy. *Journal of Invertebrate Pathology*, 164, 38-42.

PEREZ, E.E., LEWIS, E.E., SHAPIRO-ILAN, D.I. 2003. Impact of the host cadaver on survival and infectivity of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) under desiccating conditions. *Journal of Invertebrate Pathology*, 82, 111-118.

RASMANN, S., TURLINGS, T.C.J. 2008. First insights into specificity of belowground tritrophic interactions. *Oikos*, 117, 362-369.

RUAN, W.B., SHAPIRO-ILAN, D., LEWIS, E.E., KAPLAN, F., ALBORN, H., GU, X.H., SCHLIEKELMAN, P. 2018. Movement patterns in entomopathogenic nematodes: continuous vs. temporal. *Journal of Invertebrate Pathology,* 151, 137-143.

SAN-BLAS, E., GOWEN, S.R., PEMBROKE, B. 2008. *Steinernema feltiae*: Ammonia triggers the emergence of their infective juveniles. *Experimental Parasitology,* 119, 180-185.

SAN-BLAS, E., PIRELA, D., GARCIA, D., PORTILLO, E. 2014. Ammonia concentration at emergence and its effects on the recovery of different species of entomopathogenic nematodes. *Experimental Parasitology,* 144, 1-5.

SHAPIRO-ILAN, D.I., GAUGLER, R. 2002. Production technology for entomopathogenic nematodes and their bacterial symbionts. *Journal of Industrial Microbiology and Biotechnology,* 28, 137-146.

SHAPIRO-ILAN, D.I., LEWIS, E.E., SCHLIEKELMAN, P. 2014. Aggregative group behavior in insect parasitic nematode dispersal. *International Journal for Parasitology*, 44, 49-54.

SHAPIRO-ILAN, D.I., LEWIS, E.E., TEDDERS, W.L. 2003. Superior efficacy observed in entomopathogenic nematodes applied in infected-host cadavers compared with application in aqueous suspension. *Journal of Invertebrate Pathology*, 83, 270-272.

SHAPIRO, D.I., GLAZER, I. 1996. Comparison of entomopathogenic nematode dispersal from infected hosts versus aqueous suspension. *Environmental Entomology*, 25 1455-1461.

SHAPIRO, D.I., LEWIS, E.E. 1999. Comparison of entomopathogenic nematode infectivity from infected hosts versus aqueous suspension. *Environmental Entomology,* 28, 907-911.

SHAPIRO, D.I., LEWIS, E.E., PARAMASIVAM, S., MCCOY, C.W. 2000. Nitrogen partitioning in *Heterorhabditis bacteriophora*-infected hosts and the effects of nitrogen on attraction/repulsion. *Journal of Invertebrate Pathology,* 76, 43-48.

SRINIVASAN, J., KAPLAN, F., AJREDINI, R., ZACHARIAH, C., ALBORN, H.T., TEAL, P.E.A., MALIK, R.U., EDISON, A.S., STERNBERG, P.W., SCHROEDER, F.C. 2008. A blend of small molecules regulates both mating and development in *Caenorhabditis elegans*. *Nature,* 454, 1115-U1146.

VON REUSS, S.H. 2018. Exploring modular glycolipids involved in nematode chemical communication. *Chimia,* 72, 297-303.

WOODRING, J. L. & KAYA, H. K. 1988. Steinernematid and heterorhabditid nematodes: a handbook of biology and techniques. *Southern cooperative series bulletin (USA).*

WU, S.H., KAPLAN, F., LEWIS, E., ALBORN, H.T., SHAPIRO-ILAN, D.I. 2018. Infected host macerate enhances entomopathogenic nematode movement towards hosts and infectivity in a soil profile. *Journal of Invertebrate Pathology,* 159, 141-144.

ZHAO, L.L., AHMAD, F., LU, M., ZHANG, W., WICKHAM, J.D., SUN, J.H. 2018. Ascarosides promote the prevalence of ophiostomatoid fungi and an invasive pathogenic nematode, *Bursaphelenchus xylophilus*. *Journal of Chemical Ecology,* 44, 701-710.

ZHAO, L.L., ZHANG, X.X., WEI, Y.A., ZHOU, J., ZHANG, W., QIN, P.J., CHINTA, S., KONG, X.B., LIU, Y.P., YU, H.Y., HU, S.N., ZOU, Z., BUTCHER, R.A., SUN,

J.H. 2016. Ascarosides coordinate the dispersal of a plant-parasitic nematode with the metamorphosis of its vector beetle. *Nature Communications*, 7, 12341.

# Chapter 3 The effect of temperature conditioning (9°C and 20°C) on the proteome of entomopathogenic nematode infective juveniles

**Published as:**

Lillis, P., Griffin, C. and Carolan, J. (2022). The effect of temperature conditioning (9°C and 20°C) on the proteome of entomopathogenic nematode infective juveniles. PLOS ONE, 17(4), p.e0266164.

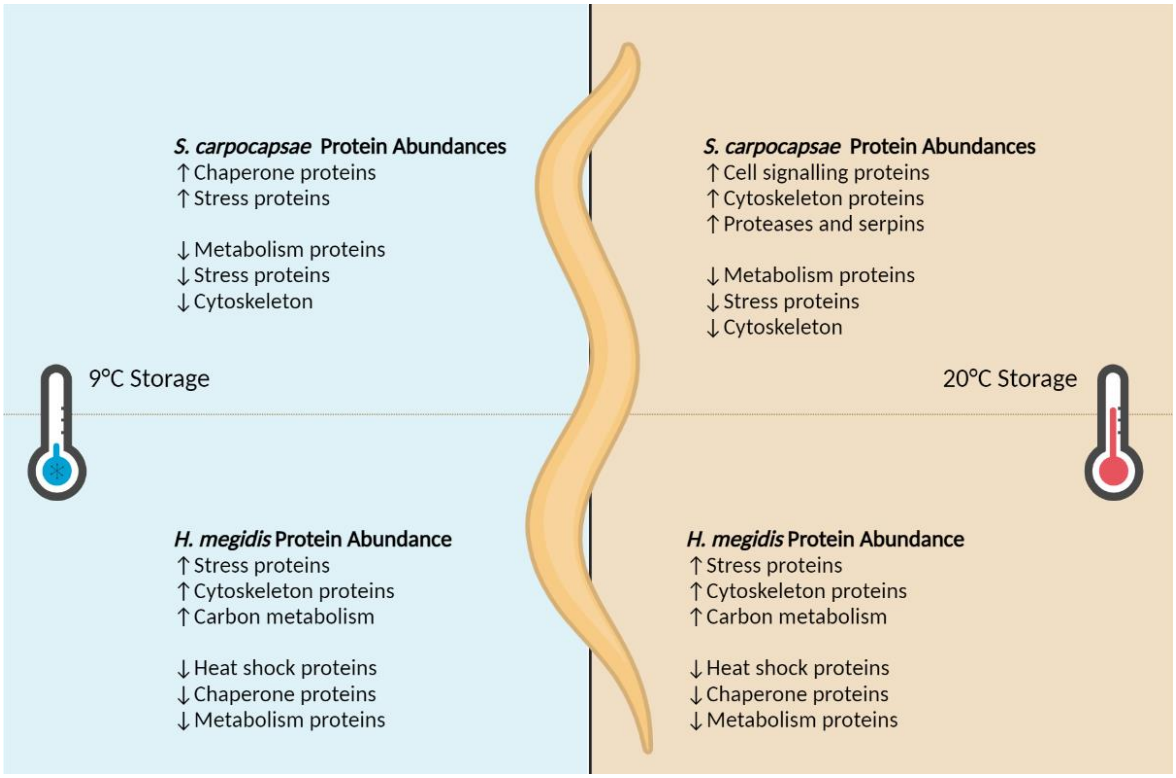
# Abstract

Entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis* are parasites which kill and reproduce within insects. While both have life cycles centred around their developmentally arrested, nonfeeding and stress tolerant infective juvenile (IJ) stage, they are relatively distantly related. These IJs are promising biocontrol agents, and their shelf life and stress tolerance may be enhanced by storage at low temperatures. The purpose of this study was to investigate how the proteome of the IJs of two distantly related EPN species is affected by storage at 9°C (for up to 9 weeks) and 20°C (for up to 6 weeks), using label-free quantitative proteomics. Overall, more proteins were detected in *S. carpocapsae* (2422) than in *H. megidis* (1582). The *S. carpocapsae* proteome was strongly affected by temperature, while the *H. megidis* proteome was affected by both time and temperature. The proteins which increased in abundance to the greatest extent in *S. carpocapsae* IJs after conditioning at 9°C were chaperone proteins, and proteins related to stress. The proteins which increased in abundance the most after storage at 20°C were proteins related to the cytoskeleton, cell signalling, proteases and their inhibitors, which may have roles in infection. The proteins which decreased in abundance to the greatest extent in *S. carpocapsae* after both 9°C and 20°C storage were those associated with metabolism, stress and the cytoskeleton. After storage at both temperatures, the proteins increased to the greatest extent in *H. megidis* IJs were those associated with the cytoskeleton, cell signalling and carbon metabolism, and the proteins decreased in abundance to the greatest extent were heat shock and ribosomal proteins, and those associated with metabolism. As the longest-lived stage of the EPN life cycle, IJs may be affected by proteostatic stress, caused by the accumulation of misfolded proteins and toxic aggregates. The substantial increase of chaperone proteins in *S. carpocapsae,* and to a greater extent at 9°C, and the general decrease in ribosomal and chaperone proteins in *H.*

*megidis* may represent species-specific proteostasis mechanisms. Similarly, organisms accumulate reactive oxygen species (ROS) over time and both species exhibited a gradual increase in proteins which enhance ROS tolerance, such as catalase. The species-specific responses of the proteome in response to storage temperature, and over time, may reflect the phylogenetic distance and/or different ecological strategies.

**Keywords**: temperature, conditioning, biological pest control, proteomics, chaperones

## Graphical Abstract



## 3.1 Introduction

Entomopathogenic nematodes (Rhabditida; Steinernematidae and Heterorhabditidae) are insect parasites which are of economic importance due to their use as biocontrol agents. The third larval stage, infective juveniles (IJs) leave the natal host and move in the soil to locate a new insect host. Once inside, IJs (at least in Steinernema) release a wide array of proteins, which supress the immune system of, and can kill, the insect (Lu et al., 2017; Balasubramanian et al., 2010; Han et al., 2010). Both families are associated with mutualistic bacteria, which are released into the haemolymph of the host insect, helping to kill the insect and provide nutrition for nematode development and reproduction (Adams and Nguyen, 2002), and are thus categorised as entomopathogenic nematodes (EPN). Similarities between these two families are due to convergent evolution associated with this lifestyle, rather than common ancestry (Poinar, 1993; Blaxter and Koutsovoulos, 2015). Heterorhabditids are closely related to the vertebrate parasites Strongylida (Adams and Nguyen, 2002) and to *Caenorhabditis elegans*, whereas steinernematids are more closely related to Strongyloididae (Blaxter et al., 1998). The IJ stage of parasitic nematodes is analogous to the dauer stage of *C. elegans*; both are developmentally arrested, stress resistant stages that disperse to colonise new hosts or food resources, respectively.

The IJs of entomopathogenic nematodes persist in the soil year-round (Preisser et al., 2005), surviving harsh weather conditions. Falling temperatures are an indication of the onset of winter and/or freezing conditions. Exposure to low temperatures improves *Steinernema* and *Heterorhabditids* IJs survival in freezing conditions (Brown and Gaugler., 1996; Ali and Wharton., 2013) indicating that there is an acclimatisation mechanism in these IJs. Cold storage also improves the longevity of EPN (Fan and Hominick, 1991; Grewal, 2000). IJs are nonfeeding, relying for energy on their internal

lipid reserves, which vary in composition amongst EPN species (Grewal and Georgis, 1999). Dauers and IJs already have lower levels of metabolic activity than other stages of their life cycle (Vanfleteren and De Vreese, 1996; Houthoofd et al., 2002) and *C. elegans* dauers exhibit reduced activity of enzymes involved in glycolytic, gluconeogenic, TCA cycle and oxidative phosphorylation pathways relative to adults (O'Riordan and Burnell, 1989). This is referred to as hypometabolism, whereby the organism shuts down all non-essential metabolic activities and redirects their limited resources to essential functions only (Lant and Storey, 2010). In cold conditions, metabolism in EPN IJs slows down further, and their lipids are utilised at a slower rate (Andaló et al., 2011), which is thought to prolong survival. Lack of caloric intake, leading to starvation, is a stressor to which these nonfeeding organisms are inevitably subject, affecting their behaviour and, ultimately, survival (Fitters and Griffin., 2004)

While both steinernematids and heterorhabditids have convergently evolved similar life cycles, there are differences between species in behaviour. For example, of the two species studied here, *Steinernema carpocapsae* IJs are designated as ambushers, waiting on the soil surface for new insect hosts, whereas *Heterorhabditis megidis* IJs are regarded as cruisers, which move within the soil to actively seek out insects (Lewis et al., 2006). Such differences in the behaviour and in other ecological strategies of the species may be mirrored by molecular differences between them, including their responses to stress. The dauer stage of *C. elegans* and IJs of steinernematids and heterorhabditids often considered non-aging as their lifespan as adults is not significantly affected by the length of time spent developmentally arrested (Kenyon et al., 2010). Nevertheless, these organisms are long-lived when compared to their other developmental stages, and therefore challenges at the molecular level associated with aging may occur. These include protein aggregation and misfolded proteins, problems that may be exacerbated in this stage (David et al., 2010;

Walther et al., 2015). The overall number of proteins expressed in the dauer/IJ stage of nematodes is lower than in other stages (Wang et al., 2019) and investigations into *C. elegans* confirm that dauers reduce protein synthesis (Pan et al., 2007) and upregulate the expression of gene families associated with preserving and maintaining cellular components rather than the synthesis of proteins (Jones et al., 2010; Kagan and Clarke, 1995). Much of the research on developmentally arrested juveniles is conducted on dauers of the free-living *C. elegans*, and there is relatively little molecular data regarding IJs of parasites (Wang et al., 2019; Nuamtanong et al.,2019) including those of EPN (Chen et al., 2006; Rougon-Cardoso et al., 2016; Lu et al., 2017).

By investigating the molecular mechanisms and consequences of temperature acclimation and time in EPN IJs, insights into survival and the changes induced by low temperature exposure may be gained. Tandem mass spectrometer-based proteomics facilitates the identification and quantification of thousands of proteins in a single run. Such data allows for the comparison of the proteome of EPN IJs after conditioning via gene ontology mapping and functional enrichment analysis. Understanding of how the IJ proteome is affected by temperature and time may elucidate the molecular mechanisms underlying the phenotypic plasticity of EPN IJs (Griffin, 1996; Lee et al., 2016; Griffin, 2012). This study aims to provide proteomic data for two distantly related species with contrasting ecological strategies, *S. carpocapsae* and *H. megidis*, stored at 9°C and 20°C for up to 9 weeks.

## 3.2 Materials and methods

### 3.2.1 Nematode Culturing and conditioning

*Heterorhabditis megidis* UK211 and *Steinernema carpocapsae* All were used. Nematodes were cultured in last instar *Galleria mellonella* larvae (Mealworm Company, Sheffield, UK) using methods outlined in Woodring and Kaya (1988), at 20°C, with an inoculum density of 100 IJs/insect. Cadavers were placed on White traps and monitored daily. After first emergence of IJs, the White trap water was replaced with fresh sterile water. IJs were allowed to emerge into the water for 3-4 days and collected. IJs from successive harvests were pooled, rinsed 3 times by sedimentation and stored at 1000 IJs/ml in sterile tap water in 35 ml aliquots in lidded plastic tubs (9 cm diameter). Tubs were placed at 20°C and 9°C temperature-controlled rooms and sampled at intervals (3 or 6 weeks at 20$^{o}$C and 3, 6 or 9 weeks at 9$^{o}$C). In addition, unconditioned IJs (time 0) were also sampled. There were 5 replicate tubs for each storage time and temperature.

### 3.2.2 Sample preparation

The contents of a tub were sedimented in a 50 ml Falcon tube in their conditioning temperature. The pelleted IJs in approx. 150 µl were transferred to a 1.5 ml Eppendorf tube and snap frozen in liquid nitrogen.

Each sample was homogenised in lysis buffer, containing 6M urea, 2M thiourea, and a Protease Inhibitor Cocktail (cOmplete, Mini Protease Inhibitor Cocktail, Merck), centrifuged at 10000 x g for 1 minute, and snap frozen. This step was repeated 3 times to ensure complete homogenisation. Protein content was then quantified using Qubit (Invitrogen), following the manufacturer's instructions. Protein (100 µg) was purified

using a 2D Clean Up Kit (GE Healthcare) according to the manufacturer's instructions. The resulting pellets were stored in the kit's wash solution at -20°C until the last samples were collected, then all were centrifuged at 13000 x g for 5 minutes and the resulting pellets were resuspended in 50 µl of resuspension buffer (6M urea, 2M thiourea, 0.1M TrisHCl, pH8). A 20 µl aliquot was removed from each sample for reduction, alkylation and digestion. One hundred and five µls of ammonium bicarbonate (50 mM) and 1 µl of dithiothreitol (DTT) were added and samples were incubated at 56°C for 20 minutes. Once cooled, samples were alkylated with 2.7 µl of iodoacetamide (IAA) in dark conditions.

One µl of a 1% (w/v) solution of ProteaseMax (Promega) and 0.5µg/µl trypsin (Promega) were added to the samples and incubated at 37°C for a minimum of 16 hours. Samples were removed from 37°C, centrifuged briefly and acidified with 1 µl of trifluoroacetic acid (TFA) for 5 minutes at room temperature (20-25°C). Samples were centrifuged at 13000 x g for 10 minutes and the supernatant was purified using C18 Spin Columns (Pierce, Thermo Fisher Scientific) following the manufacturer's instructions and then lyophilised in a Speedyvac concentrator (Thermo Scientific Savant DNA120). Samples were then resuspended in a loading buffer, (2% v/v acetonitrile, 0.05% v/v TFA) and 1 µg was loaded from each of 4 biological replicates per samples were run on a QExactive (Thermo Fisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated over a 2% to 40% gradient of acetonitrile on a Thermo Fisher EASY-Spray, PepMap RSLC C18 column (500mm length, 75mm ID), using a reverse-phase gradient at a flow rate of 250nL $min^{-1}$ over 125 minutes. All data were acquired over 105 minutes with the mass spectrometer operating in automatic data dependent switching mode. A full MS scan at 140,000 resolution and range of 300–1700 m/z was followed by an MS/MS scan,

resolution 17,500 and range of 200–2000 m/z, selecting the 15 most intense ions prior to MS/MS.

### 3.2.3 Data processing

Protein identification and LFQ normalisation of MS/MS data was performed using MaxQuant v1.6.3.3 (http://www.maxquant.org) following the general procedures and settings outlined in Hubner et al. (2010). The Andromeda search algorithm (Cox et al., 2011) incorporated in the MaxQuant software was used to correlate MS/MS data for *S. carpocapsae* and *H. megidis* against the predicted protein data sets derived from the *S. carpocapsae* (Serra et al., 2019) and *H. bacteriophora* (Bai et al., 2013) genomics initiatives, respectively.

Normalised LFQ intensities were used to quantify protein abundances, and the data was filtered to remove contaminants. The LFQ intensities were $\log_2$ transformed, and each replicate was renamed to their respective groups (3wks9°C for proteins from IJs stored at 9°C for 3 weeks). Only proteins found in 3 replicates of at least 1 group were retained. A data imputation step replaced missing values with values of low abundant proteins chosen randomly from a distribution specified by a downshift of 2 times the mean standard deviation (SD) and a width of 0.3 times the SD.

A principal component analysis (PCA) was initially performed on the normalised intensity values of all replicates. However, a number of outliers were identified, resulting in 3 replicates in each sample in the final datasets for analysis.

An analysis of variance (ANOVA) was performed on all groups using a Benjamini-Hochberg false discovery rate (FDR) of <5% to select proteins for z-score normalisation. These ANOVA significant proteins were used for hierarchical clustering of samples using

Euclidean distance and average linkage pre-processed with K means. Gene Ontology (GO) term enrichment was performed in Blast2Go v5.2 using a Fishers exact test ($p<0.05$) on each cluster relative to all ANOVA significant proteins.

Pairwise Student's t-tests were performed for all samples relative to the week 0 samples to visualise the effect of time and temperature conditioning on the IJs proteome. Volcano plots were generated in Perseus by plotting negative log p values of the y axis and $log_2$fold transformed differences on the x axis for each comparison. Statically significant (SS; $p< 0.05$) and differentially abundant (DA; fold change of 1.5) proteins were identified as SSDAs and selected for further analysis.

All statistically significant proteins identified in pairwise-t-tests were grouped using Bioedit (v7.0.5.3) and uploaded in FASTA format to STRING: Protein-Protein Interaction Networks Functional Enrichment Analysis v11.0 with the highest confidence setting (0.9) and disconnected nodes were removed, to identify protein-protein interactions which were increasing and decreasing in IJs after storage at 9°C or 20°C over time.

### 3.2.4 Bioinformatics

The *H. megidis* genome has not been sequenced, and therefore there is a paucity in molecular data available for the species. The genome of the closely related *H. bacteriophora* is available (Bai et al., 2013) and allows for the detection of similar peptides from *H. megidis.* The genome of *S. carpocapsae* has been recently sequenced (Serra et al., 2019).

The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium (Côté et al., 2012) via the PRIDE partner repository with the dataset identifier PXD027608.

## 3.3 Results and discussion

In total, 2422 proteins were detected in *S. carpocapsae* IJs, of which 2381 remained after filtering and processing, while 1582 proteins were detected in *H. megidis* IJs and 653 remained after filtering and processing. The lower number of proteins detected in *H. megidis* may be partly a result of using a congeneric (*H. bacteriophora)* rather than the subject species as a reference proteome.

A PCA for *S. carpocapsae* (figure 3.1B) showed two distinct groupings of samples, with those stored at 9°C for 3-9 weeks clearly separated from those stored at 20°C or freshly harvested (week 0). There is evidence of progressive change at 20°C from week 0 through week 3 to week 6, while there is less clear differentiation between samples stored at 9°C for different periods. In contrast to *S. carpocapsae*, in the PCA for *H. megidis* (figure 3.1A) there is less of a distinction due to temperature. As in *S. carpocapsae*, there is evidence of a progression from 0 to 6 weeks at 20°C, but there is no clear temporal progression for IJs stored at 9°C. Components 1 and 2 for the *S. carpocapsae* PCA accounts for 41.5% of the data's variability. Components 1 and 2 for the *H. megidis* PCA account for 25.9% of the data's variability.

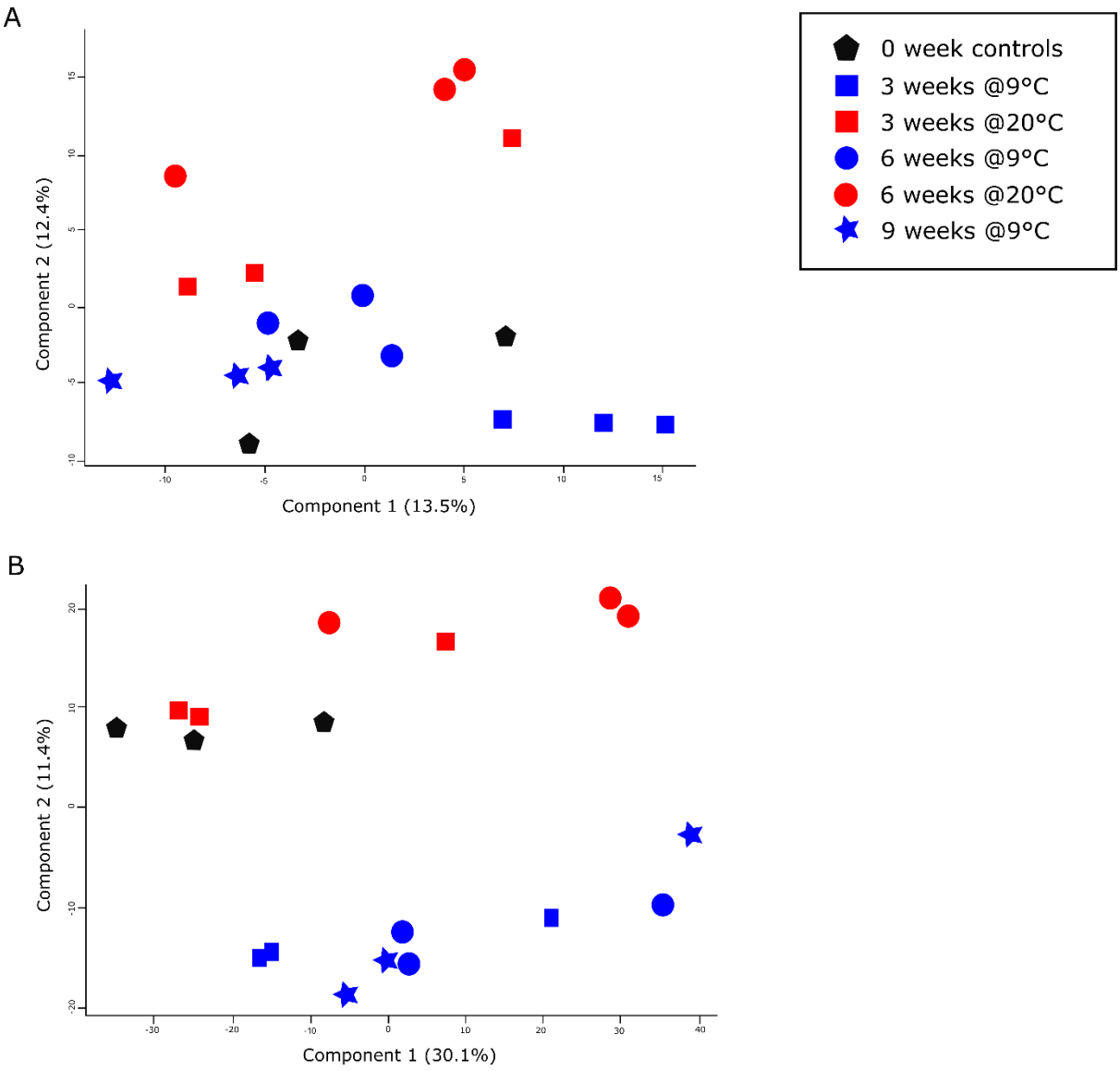



**Figure 3.1:** Principal component analysis (PCA) of the *H. megidis* (A) and *S. carpocapsae* (B) proteomes at time 0, or after storage at either 9 °C or 20 °C for up to 9 weeks. A clear distinction can be seen between IJs stored at 20 °C and 9°C.

Temporal changes in proteins are shown in more detail as heatmaps (figures 3.2 and 3.3), revealing 5 clusters of proteins in *S. carpocapsae*, and 7 clusters in *H. megidis.* The clusters group proteins which are detected with similar abundance-profiles in each group. Distinct time and temperature-dependent responses are seen in *S. carpocapsae;* for example, cluster A increases over time at 20°C while remaining rather stable at 9°C, while

in contrast clusters C and D decrease over time at 20°C (figure 3.2). Patterns are more complex in *H. megidis* (figure 3.3).

Pairwise comparisons to time 0 allowed the numbers of proteins changed in each stored sample (SSDA) to be quantified (Table 1). There were 724 SSDA proteins for *S. carpocapsae* and 175 for *H. megidis*, representing 30.4 and 26.8%, respectively, of the (filtered) proteome for each species. The identity and fold change of a selection of these proteins is given in appendices 3.1A, 3.1B and 3.2. In *S. carpocapsae*, there was a tendency for twice as many proteins to be increased in abundance as were decreased in abundance in each storage treatment, while in *H. megidis* the numbers increased and decreased in abundance tended to be more equal (table 1).

**Table 1. Number of significantly significant (P < 0.05) differentially abundant (SSDA) proteins showing increased or decreased activity (fold change +/- 1.5-fold) relative to time 0 following storage at 9°C or 20°C of infective juveniles of *S. carpocapsae* or *H. megidis*.**

| **Storage temp (°C)** | **Storage duration (wks)** | ***S. carpocapsae*** | | ***H. megidis*** | |
|---|---|---|---|---|---|
| | | Up | Down | Up | Down |
| 20 | 3 | 80 | 46 | 27 | 22 |
| | 6 | 229 | 105 | 57 | 49 |
| 9 | 3 | 104 | 51 | 25 | 24 |
| | 6 | 225 | 85 | 21 | 13 |
| | 9 | 167 | 80 | 21 | 48 |

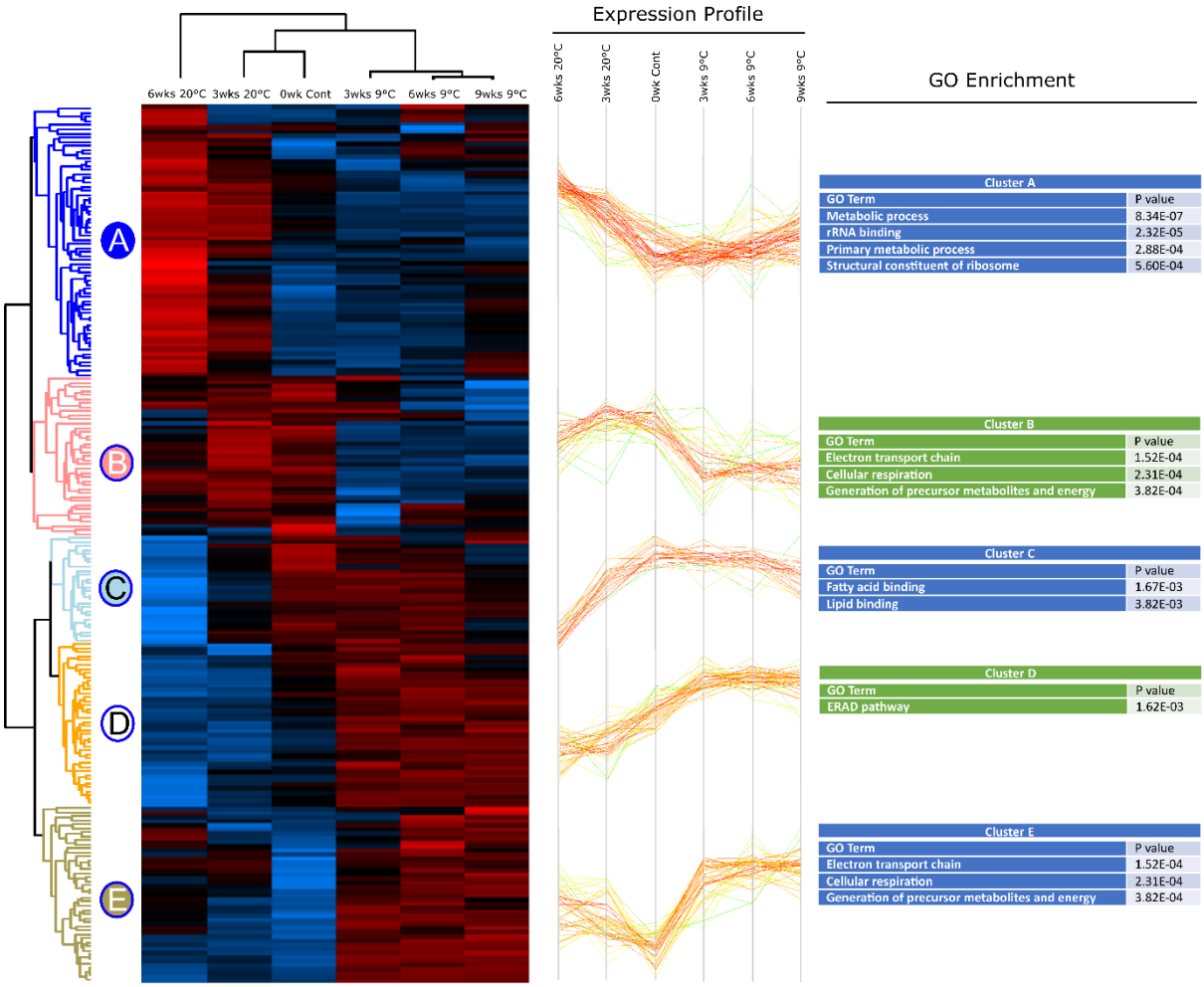



**Figure 3.2:** Heat map of *S. carpocapsae* statistically significant proteins: Two-way unsupervised hierarchical clustering of the median Z-score normalised label-free quantification (LFQ) intensity values of all statistically significant proteins (n=214) for freshly emerged IJs, IJs stored at 9°C for 3, 6 and 9 weeks or IJs stored at 20°C for 3 and 6 weeks. Hierarchical clustering resolved 5 distinct clusters. Differences in protein abundance are indicated by colour changes from low (blue) to high (red) protein abundance representative of changes in Z-score normalised $log_2$-fold transformed LFQ intensity values. Selected GO terms enriched in each cluster are displayed (right), along with the P value for that category.

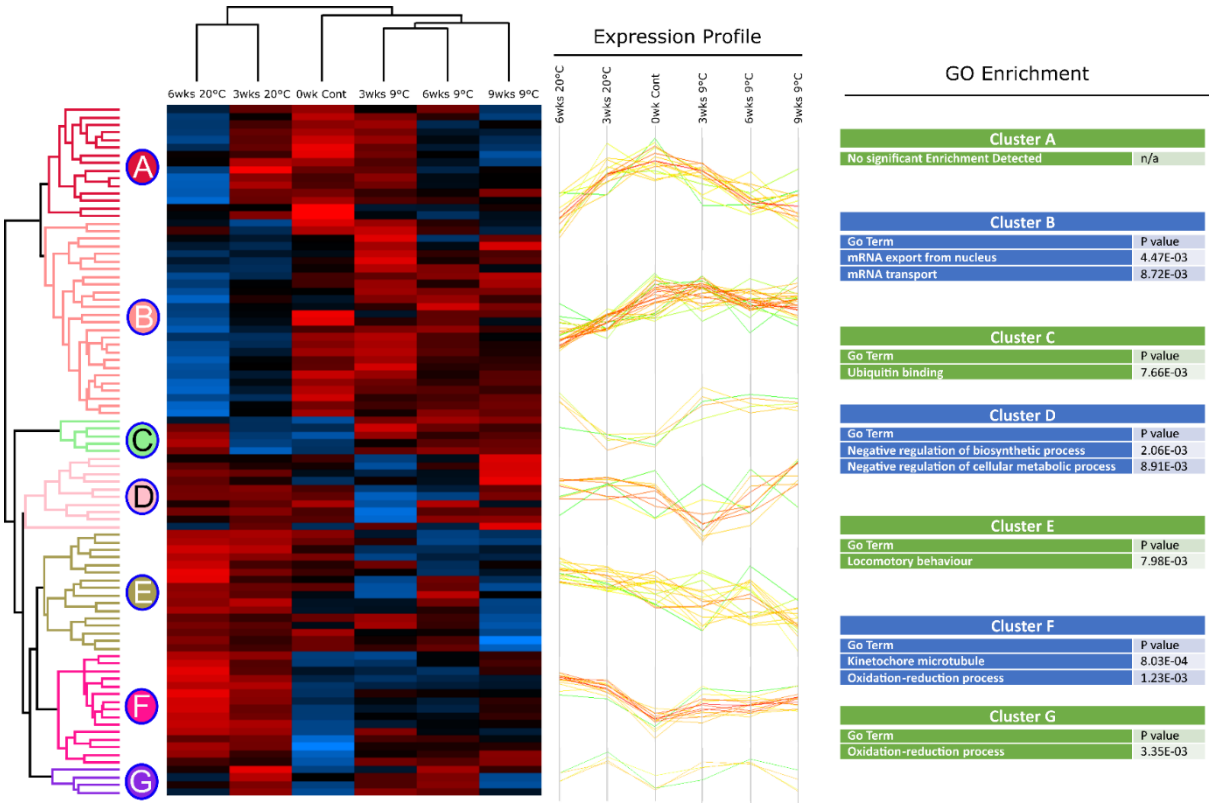



**Figure 3.3:** Heat map of *H. megidis* statistically significant proteins: Two-way unsupervised hierarchical clustering of the median Z-score normalised label-free quantification (LFQ) intensity values of all statistically significant proteins (n=91) for freshly emerged IJs, IJs stored at 9°C for 3, d 9 weeks or IJs stored at 20°C for 3 and 6 weeks. Hierarchical clustering resolved 5 distinct clusters. Differences in protein abundance are indicated by colour changes from low (blue) to high (red) protein abundance representative of changes in Z-score normalised $log_2$-fold transformed LFQ intensity values. Selected GO terms enriched in each cluster are displayed (right), along with the P value for that category.

In general, SSDA proteins demonstrated a greater fold change in *S. carpocapsae* than in *H. megidis*. The greatest change in *S. carpocapsae* was for chaperone proteins which showed up to 90-fold increase after conditioning at 9°C for 9 weeks (appendix 3.1.A), while all chaperone proteins detected in *H. megidis* were decreasing in abundance (appendix 3.2). In *H. megidis*, the greatest change in any single protein was that of UDP-glucoronosyl and UDP-glucosyl transferase which increased ~20 fold at 20°C, and ~7 times at 9°C (appendix 3.2).

### 3.3.1 Translation

The most prominent difference between the two species is in the response of proteins related to translation, which were decreased in *H. megidis* after storage in both 9°C and 20°C (figure 3.4) and were generally increased in *S. carpocapsae* (figure 3.5; appendix 3.1.A). All ribosomal proteins decreased in abundance over time in *H. megidis* IJs (appendix 3.2) regardless of storage temperature. Protein production is energetically expensive, costing up to 75% of the cell's energy (Lane and Martin, 2010). As metabolic activity is reduced, reduction of energetically expensive processes would be advantageous. Walther et al., (2015) reported extensive proteome remodelling in aging *C. elegans* worms, with reduced ribosomes and increased proteasome complexes. Most proteasome related proteins detected were increased at both temperatures in both species. Reduction of translation and mRNA production in has shown to extend the lifespan of *C. elegans* (Pan et al., 2007; Hansen et al., 2007). *S. carpocapsae* IJs did not exhibit a general decrease in ribosomal proteins over time, and translation related proteins were generally increased in abundance, regardless of storage temperature (appendix 3.1.A; figure 3.5).

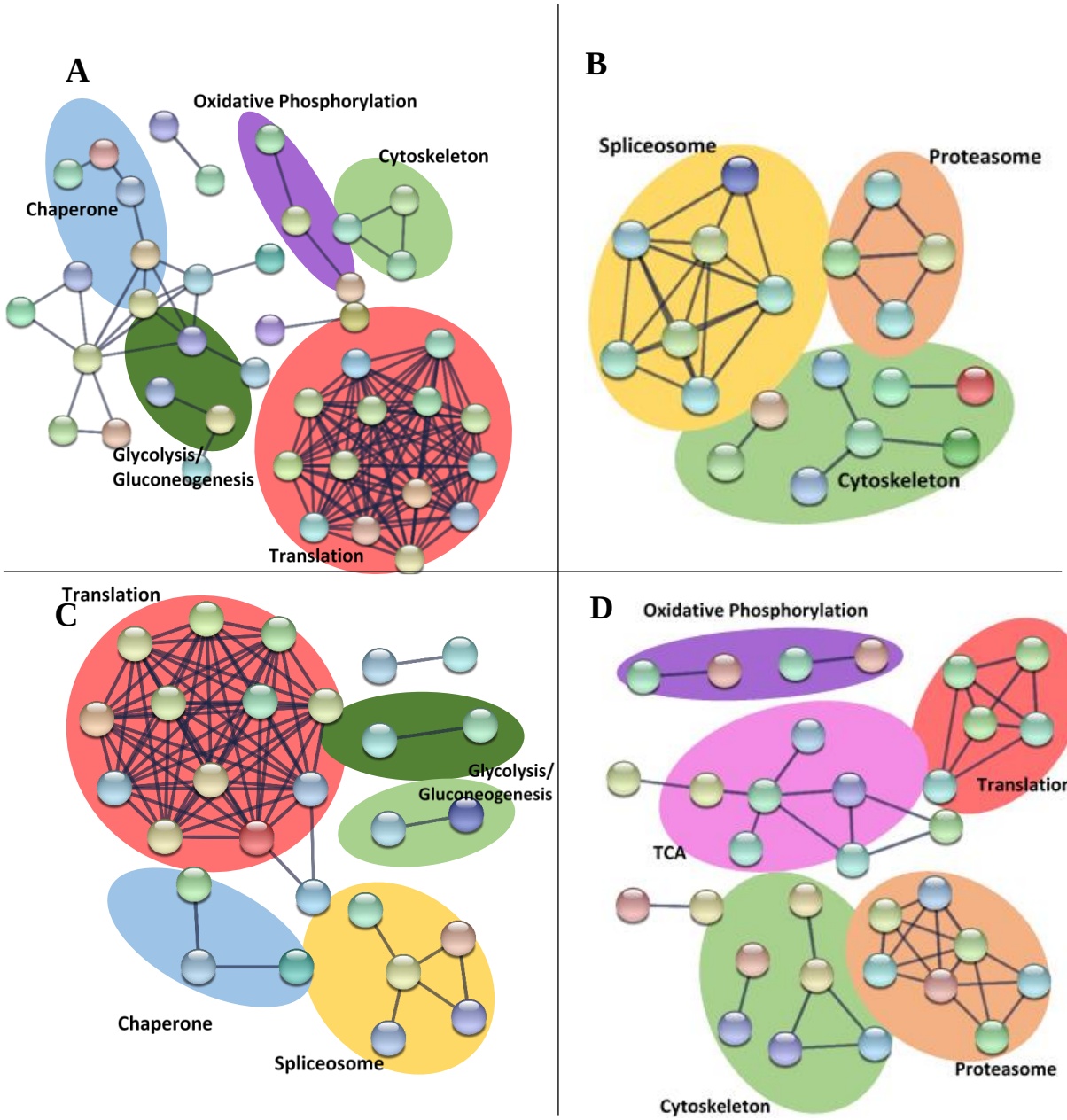



**Figure 3.4:** String interactions of *H. megidis* proteins which are decreased (left) and increased (right) in abundance after storage at 9°C (top) and 20°C (bottom) for 6 weeks.

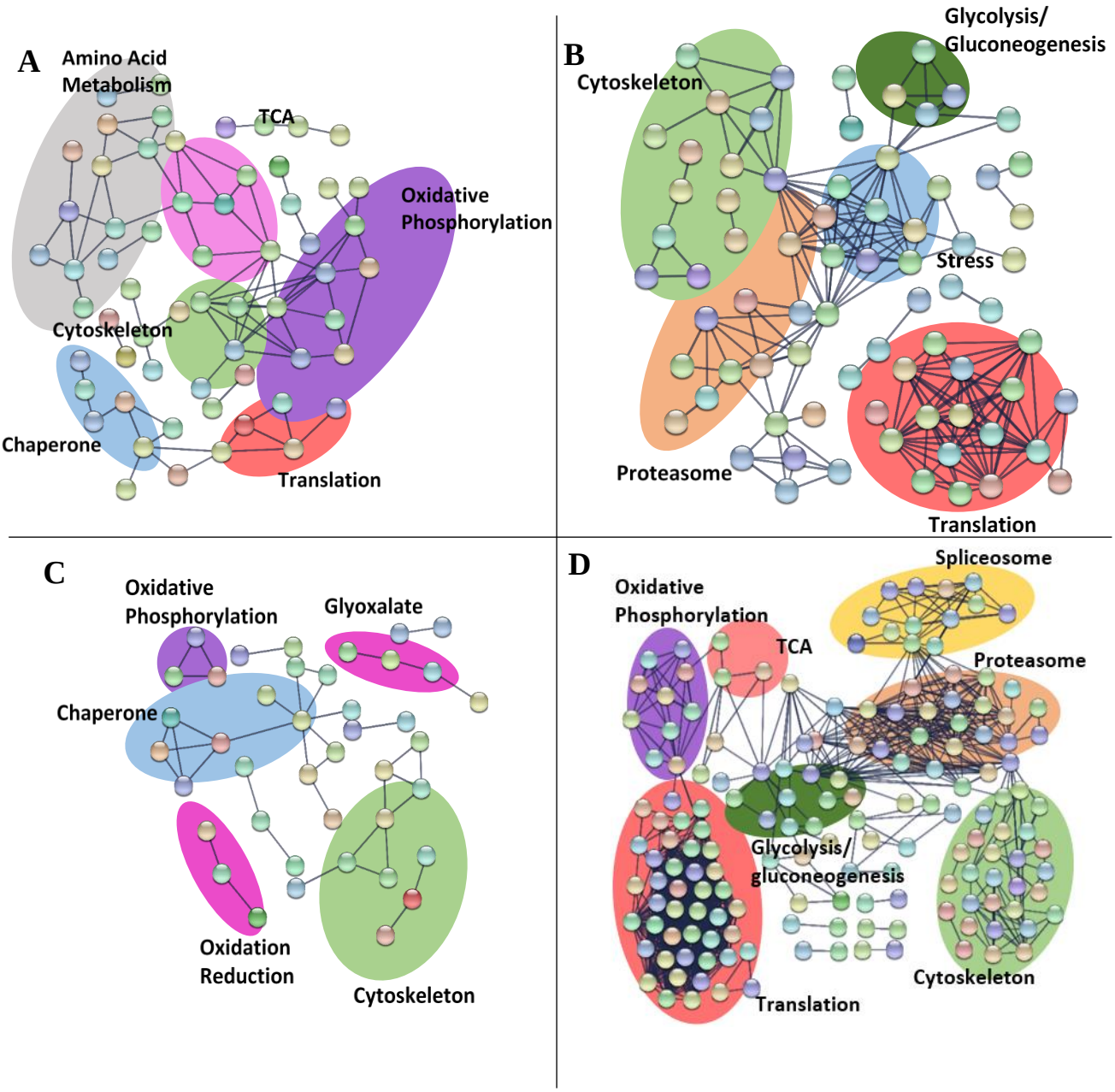



**Figure 3.5:** String interactions of *S. carpocapsae* proteins which are decreased (left) or increased (right) in abundance after storage at 9°C (top) and 20°C (bottom) for 6 weeks.

### 3.3.2 Chaperones

Most chaperone proteins that were detected were increased in abundance in *S. carpocapsae* IJs, and to a greater extent after storage at 9°C (appendix 3.1.A). These include chaperonins, prefoldins, heat shock proteins and late embryogenesis (LEA)

proteins. One chaperonin was detected in *S. carpocapsae,* increasing in abundance at 20°C, and in *H. megidis,* decreasing at 9°C. Chaperonins are ATP-dependent chaperones which assist in the folding of nascent polypeptides (Clare and Saibil, 2013). Various heat shock proteins (HSPs) were detected in *S. carpocapsae* (appendix 3.1.A) and generally increased in abundance over time, and to a greater extent at 9°C. HSPs are molecular chaperones which aid folding of proteins, prevent stress induced aggregation or misfolding of proteins, and can revert misfolded proteins to their native conformation (Walter and Buchner., 2002). HSPs, despite their name, are protective against a wide array of stresses and can also have roles in maintaining cellular components such as the cytoskeleton (Whitley et al., 1999). Small heat shock proteins are typified by a conserved α-crystallin C terminal domain (Walter and Buchner., 2002) and are involved in the prevention of toxic aggregates of protein (Borges and Ramos, 2005), and have higher binding affinities than the larger, classical HSPs (Walter and Buchner, 2002). Prefoldins are molecular chaperones which detect, bind to and deliver unfolded proteins, especially actin, to cystolic chaperonins (Vainberg et al., 1998). Prefoldin proteins were amongst the few chaperone proteins decreased in abundance in *S. carpocapsae* IJs, at both 9°C and 20°C.

Many late embryogenesis abundant (LEA) proteins were detected in *S. carpocapsae* IJs, and most were temperature specific: increasing by up to ~90 times abundance after storage at 9°C, but mostly decreased by ~2-4 times if stored at 20°C (appendix 3.1.A). LEA proteins were first discovered in the seeds of plants (Cuming and Lane, 1979; Dure et al., 1981) and confer desiccation tolerance to the seeds. Since their initial discovery, these proteins have been found in a variety of nematodes such as *C. elegans* (Gal et al., 2004), *Steinernema feltiae* (Solomon et al., 2000) and *Aphelenchus avenae* (Browne et al., 2004). LEA proteins are atypical molecular chaperones which can protect the structure of proteins (Tantos et al., 2009), if present before the exposure to stress. Unlike heat shock proteins,

LEA proteins alone cannot protect proteins from heat stress and cannot revert misfolded proteins back to their native state (Reyes et al., 2005; Olvera-Carrillo et al., 2011). LEA proteins enhance the organism's survival in response to cold and freezing conditions (Anderson et al., 2015; Reyes et al., 2008), oxidative stress (Zheng et al., 2019) and salt stress (Han et al., 2017). LEA proteins, in conjunction with trehalose, facilitate the formation of "glass" (Browne et al., 2004), which is protective against desiccation (Solomon et al., 2000) and freezing (NDong et al., 2002). Trehalose-6-phosphate synthase which was increased at 6 weeks in *S. carpocapsae* regardless of storage temperature (appendix 3.1.B), may be involved in this bioglass formation or could be a response to starvation.

All chaperone proteins detected as SSDAs in *H. megidis* were decreased in abundance, regardless of storage temperature (figure 3.4; appendix 3.2). With the sharp decrease in translation, chaperone proteins may be redundant to *H. megidis* IJs without an immediate stressor such as heat or desiccation triggering their expression. Decreased translation would also reduce the level of misfolded proteins and toxic aggregates, which may reduce the need for protein chaperone activities.

### 3.3.3 Metabolism

Proteins associated with gluconeogenesis are amongst the few groups of metabolism related proteins which were increased in abundance in *S. carpocapsae* IJs (figure 3.5; appendix 3.1.B) and their increase may be related to starvation of the worm. Gluconeogenesis is responsible for the generation of monosaccharides used to generate energy in subsequent metabolic processes. Sugars formed in gluconeogenesis are converted to pyruvate during glycolysis. Pyruvate formed by glycolysis is transported to

mitochondria to be converted to acetyl-CoA in the citric acid cycle, an aerobic process which utilises acetyl-CoA to reduce NAD+ to NADH, producing carbon dioxide as a by-product. The NADH is then used in oxidative phosphorylation. While glucose is the classic sugar transport unit formed in gluconeogenesis, nematodes and insects also form trehalose, a disaccharide composed of 2 glucose molecules. This sugar is functionally similar but also plays a crucial role in nematodes' resistance against desiccation, freezing (Qiu and Bedding, 2002), and other stresses (Behm et al., 1997). This sugar, along with LEA proteins facilitates the formation of bioglass, which enhances nematodes' freezing tolerance (Browne et al., 2004).

Few glycolysis-related proteins were detected as SSDAs in *H. megidis* and these were generally decreasing in abundance at both temperatures (figure 3.4; appendix 3.2). Most glycolysis proteins detected in *S. carpocapsae* did not change significantly over time at either temperature (figure 3.5; appendix 3.1.B). The glyoxylate pathway is an alternative pathway to the TCA cycle, which is not normally found in metazoans, but is present in nematode dauers (O'Riordan and Burnell, 1990). Isocitrate lyase and malate synthase catalyse the conversion of coenzyme A to succinate and malate (Wright and Perry, 2002), which may then be processed by succinate dehydrogenase to form malate, which can then be processed by malate dehydrogenase. This pathway allows for the generation of glucose molecules from the β-oxidation of fatty acids. There is significant overlap between the enzymes in these two metabolic pathways, and most enzymes were increased in abundance during storage at 20°C and decreased at 9°C in *S. carpocapsae* IJs (appendix 3.1.B). All SSDA proteins detected as part of both the citric acid cycle and the glyoxylate pathway were decreased in abundance regardless of storage temperature in *H. megidis* IJs (appendix 3.2).

Few oxidative phosphorylation enzymes were detected in *H. megidis* (appendix 3.2), but those that were detected increased in abundance at 20°C and decreased in abundance at 9°C (figure 3.4; appendix 3.2). The exception to this was NADH-ubiquinone oxidoreductase ASHI subunit, which was increased by ~11 times at both temperatures. Proteins related to oxidative phosphorylation were abundant in a temperature specific manner in *S. carpocapsae* IJs, increasing at 20°C and decreased in abundance at 9°C (figure 3.5; appendix 3.1.B).

Many fatty acid- and retinol-binding proteins (FARs) and nematode polyprotein allergens/antigens (NPAs) were detected in *S. carpocapsae* (appendix 3.1.A), most decreasing in abundance at both storage temperatures. FARs are a diverse family of proteins that are expanded in the *S. carpocapsae* genome (Dillman et al., 2015). NPAs are spliced to form many copies of nematode FARs (McDermott et al., 1999). Nematode FARs have structures similar to FARs found in other animals but have structures unique to nematodes, and therefore probably have nematode specific functions. FAR proteins may transport and store small quantities of lipids (Kennedy et al., 2013).

In general, *S. carpocapsae* proteins associated with metabolism decreased at 9°C and increased at 20°C (appendix 3.1.B). Proteins related to both gluconeogenesis and glycolysis were increased in abundance in *S. carpocapsae* IJs stored at 9°C, whereas proteins related to intermediary metabolism and oxidative phosphorylation were decreased to a greater extent at 9°C (figure 3.5). This may be due to IJs being more active at higher temperatures and requiring more energy (Andaló et al., 2011) and may be partially responsible for the IJs' enhanced longevity at low temperatures (Fan and Hominick, 1991; Grewal, 2000).

### 3.3.4 Cytoskeleton

Cytoskeletal proteins such as actin, myosin, collagen, and tubulin were amongst the highest abundance proteins detected in both species by raw LFQ intensity. Proteins associated with the cytoskeleton were generally decreased in abundance in both species over time (figures 3.4 and 3.5; appendices 3.1.A and 3.2). EPN IJs tend to become less active over time (Fitters and Griffin., 2004), and therefore proteins associated with locomotion may be degraded. Collagen remodelling is also reported to be associated with lifespan-lengthening in *C. elegans,* regulated by the stress pathway SKN-1 (Ewald et al., 2014). Collagen was detected as decreased in abundance in *S. carpocapsae* IJs stored at 20°C, however it was increased in those stored at 9°C (appendix 3.1.A).

### 3.3.5 Stress/Detoxification Proteins

String analysis identified a network of stress proteins which increased in abundance in *S. carpocapsae* at 9°C (figure 3.5), although there was a tendency for stress/detoxification proteins to also increase in abundance at 20°C. Many stress proteins detected as SSDAs in this analysis in both species such as short chain dehydrogenases, thioredoxins, GSTs, catalase, oxidoreductases, aldehyde dehydrogenases (appendices 3.1.B and 3.2), are known to be regulated by SKN-1 (Choe et al., 2012), the pathway implicated in collagen remodelling mentioned above (Ewald et al., 2014). Proteins associated with the cell's response to reactive oxygen species, such as catalase in *H. megidis,* and both catalase and copper oxide dismutase in *S. carpocapsae,* were increased to a greater extent at 9°C than at 20°C. Thioredoxin, an antioxidant, was decreased in abundance at both temperatures at all timepoints in both *H. megidis* and *S. carpocapsae*. Other stress proteins such as aldehyde dehydrogenase, which is involved in the stress response against by-products of anaerobic

fermentation were increased to a greater extent at 20°C than at 9°C in *S. carpocapsae* (appendix 3.1.B).

Xenobiotic detoxification is generally divided into three distinct stages. Short chain dehydrogenases and reductases render xenobiotics less stable and represent phase I. Glutathione S transferase is involved in stage II, and transfers glutathione (an antioxidant) onto xenobiotics, increasing their solubility and facilitating its breakdown. Glutathione can also reduce free radicals generated during the stress response. UDP-glucoronosyl and UDP-glucosyl transferase increased in abundance in both *H. megidis* and *S. carpocapsae* IJs, at most timepoints and both temperatures (appendices 3.1.B and 3.2). UDP-glucoronosyl transferases add glucuronic acid to a xenobiotic, which may render it harmless, or aid in its excretion (Bock and Köhle, 2005). Glucosyltransferases may also be involved in the synthesis of trehalose (Teramoto et al., 2008), a sugar which enables survival in harsh conditions (Behm, 1997).

Fewer stress-related proteins were detected in *H. megidis*, and many of them were decreased in abundance after conditioning at both temperatures, except for catalase, which increased after storage at 9°C (appendix 3.2). Amongst these proteins, one of the few SSDA proteins that was constitutively increased in abundance over time was an autophagy related protein, and it increased to a greater extent at 9°C than at 20°C (appendix 3.2). Selective autophagy has been shown to improve *C. elegans* lifespan at low temperatures (Chen et al., 2019), and may have a similar role in *H. megidis* IJs.

## 3.4 Conclusion

When infective juveniles of *H. megidis* UK211 and *S. carpocapsae* All were conditioned at 9°C or 20°C, the proteome of these two species changed in radically different manners. The change in the *H. megidis* proteome was characterised by a decrease in proteins associated with metabolism and protein synthesis, while the change in *S. carpocapsae* was characterised by increases in proteins associated with protein chaperoning activities and responses to stress which increased over time, and to a greater extent at 9°C. The difference in proteostasis may be due to several factors.

Firstly, since the two species are not closely related, their strategies may be legacy of their ancestry. Secondly, it may relate to differences in the behavioural (foraging) strategies of the two species. *H. megidis* IJs are defined as "cruisers", IJs which actively move through soil to find their host, whereas *S. carpocapsae* IJs are defined as "ambushers", IJs which wait until a potential host comes near enough to infect (Lewis et al., 2006) although this is likely an oversimplification of their complex behaviours (Griffin, 2012). As protein production is energetically expensive, requiring up to 75% of the cell's energy (Lane and Martin, 2010), a reduction in protein synthesis may free up energy for locomotion in *H. megidis* and may enable the IJ to avoid proteotoxic stress such as misfolded or aggregating proteins (Walther et al., 2015). All chaperone proteins were decreased in abundance in *H. megidis*, regardless of storage temperature. *S. carpocapsae,* described as more of a sedentary ambusher, may not require this extra energy. Without reducing protein synthesis, *S. carpocapsae* IJs may be affected by proteotoxic stress. This may be why chaperone proteins were increased to such an extent in *S. carpocapsae,* regardless of storage temperature. Thirdly, the differences between the two species may relate to differences in broader ecological strategies. *H. megidis* IJs disperse widely and all are hermaphroditic, characteristics of r-strategists or a colonising species, and so perhaps this species spends

relatively little time as IJs in soil (Downes and Griffin, 1996). *S. carpocapsae* IJs are relatively long-lived compared to *H. megidis* and would thus be better suited to persist in soil during periods when hosts are unavailable such as winter. Storage temperature had a clear effect on the *S. carpocapsae* proteome, which showed a much greater increase in chaperone abundance at 9°C, especially in LEA proteins. As these IJs are present in the soil year-round, exposure to low temperatures may indicate the onset of freezing conditions, and the IJ's proteome may adapt to this. LEA proteins, which are important for freezing resistance in nematodes were increased in a temperature specific manner.

Oxidative phosphorylation produces the most energy of all metabolic processes, but it also produces detrimental reactive oxygen species. Free radicals damage cells and contribute towards aging. *Steinernema* and *Heterorhabditis* IJs generally live longer at 9°C than at 20°C, which is generally attributed to their metabolism slowing down at lower temperatures (Andaló et al., 2011; Santra et al., 2019). Catalase, the stress enzyme which breaks down hydrogen peroxide, and copper oxide dismutase were both increased in abundance after storage at 9°C in *S. carpocapsae.* IJs may enhance their survival in colder conditions by reducing metabolic activities which produce these free radicals and increasing their stress response against them. Further studies on a broader range of species (including species of *Steinernema* that adopt cruise-foraging strategies) would help elucidate to what extent the patterns detected in the two species studied here reflect their ancestry and/or their ecological strategies.

## References

ADAMS, B. & NGUYEN, K. B. 2002. Taxonomy and Systematics In: GAUGLER, R. (Ed). Entomopathogenic Nematology. *CABI, Wallingford UK*, 1-33.

ALI, F. & WHARTON, D. A. 2013. Cold tolerance abilities of two entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *Cryobiology,* 66**,** 24-29.

ANDALÓ, V., MOINO, A., MAXIMINIANO, C., CAMPOS, V. & MENDONÇA, L. 2011. Influence of temperature and duration of storage on the lipid reserves of entomopathogenic nematodes. *Revista Colombiana de Entomología.,* 37**,** 203-209.

ANDERSON, D., FERRERAS, E., TRINDADE, M. & COWAN, D. 2015. A novel bacterial Water Hypersensitivity-like protein shows in vivo protection against cold and freeze damage. *FEMS Microbiology Letters,* 362.

BAI, X. D., ADAMS, B. J., CICHE, T. A., CLIFTON, S., GAUGLER, R., KIM, K., SPIETH, J., STERNBERG, P. W., WILSON, R. K. & GREWAL, P. S. 2013. A Lover and a Fighter: The Genome Sequence of an Entomopathogenic Nematode *Heterorhabditis bacteriophora*. *PLOSE ONE,* 8**,** 13.

BALASUBRAMANIAN N, TOUBARRO D, SIMÕES N. 2010. Biochemical study and in vitro insect immune suppression by a trypsin-like secreted protease from the nematode Steinernema carpocapsae. *Parasite Immunology.* 32(3):165-75.

BEHM, C. A. 1997. The role of trehalose in the physiology of nematodes. *International Journal for Parasitology*, 27, 215-229

BLAXTER, M. & KOUTSOVOULOS, G. 2015. The evolution of parasitism in Nematoda. *Parasitology,* 142 Suppl 1**,** S26-39.

BLAXTER, M. L., DE LEY, P., GAREY, J. R., LIU, L. X., SCHELDEMAN, P., VIERSTRAETE, A., VANFLETEREN, J. R., MACKEY, L. Y., DORRIS, M., FRISSE, L. M., VIDA, J. T. & THOMAS, W. K. 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature,* 392**,** 71-75.

BOCK, K. W. & KÖHLE, C. 2005. UDP-glucuronosyltransferase 1A6: structural, functional, and regulatory aspects. *Methods in Enzymology,* 400**,** 57-75.

BORGES, J. C. & RAMOS, C. H. 2005. Protein folding assisted by chaperones. *Protein & Peptide Letters,* 12**,** 257-261.

BROWN, I. M. & GAUGLER, R. 1996. Cold tolerance of steinernematid and heterorhabditid nematodes. *Journal of Thermal Biology,* 21**,** 115-121.

BROWNE, J. A., DOLAN, K. M., TYSON, T., GOYAL, K., TUNNACLIFFE, A. & BURNELL, A. M. 2004. Dehydration-specific induction of hydrophilic protein genes in the anhydrobiotic nematode *Aphelenchus avenae*. *Eukaryotic cell,* 3**,** 966-975.

CHEN, S., GLAZER, I., GOLLOP, N., CASH, P., ARGO, E., INNES, A., STEWART, E., DAVIDSON, I. & WILSON, M. J. 2006. Proteomic analysis of the entomopathogenic nematode *Steinernema feltiae* IS-6 IJs under evaporative and osmotic stresses. *Molecular and Biochemical Parasitology,* 145**,** 195-204.

CHEN, Y.-L., TAO, J., ZHAO, P.-J., TANG, W., XU, J.-P., ZHANG, K.-Q. & ZOU, C.-G. 2019. Adiponectin receptor PAQR-2 signaling senses low temperature to promote *C. elegans* longevity by regulating autophagy. *Nature Communications,* 10**,** 2602.

CHOE, K. P., LEUNG, C. K. & MIYAMOTO, M. M. 2012. Unique structure and regulation of the nematode detoxification gene regulator, SKN-1: implications to

understanding and controlling drug resistance. *Drug metabolism reviews,* 44**,** 209-223.

NDONG, C., DANYLUK, J., WILSON, K.E., POCOCK, T., HUNER, N.P. & SARHAN, F. 2002. Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. *Plant Physiology,* 129, 1368-1381

CLARE, D. K. & SAIBIL, H. R. 2013. ATP-driven molecular chaperone machines. *Biopolymers,* 99**,** 846-859.

COX, J., NEUHAUSER, N., MICHALSKI, A., SCHELTEMA, R. A., OLSEN, J. V. & MANN, M. 2011. Andromeda: a peptide search engine integrated into the MaxQuant environment. *Journal of Proteome Research,* 10**,** 1794-1805.

CUMING, A. C. & LANE, B. G. 1979. Protein Synthesis in Imbibing Wheat Embryos. *European Journal of Biochemistry,* 99**,** 217-224.

CÔTÉ, R. G., GRISS, J., DIANES, J. A., WANG, R., WRIGHT, J. C., VAN DEN TOORN, H. W. P., VAN BREUKELEN, B., HECK, A. J. R., HULSTAERT, N., MARTENS, L., REISINGER, F., CSORDAS, A., OVELLEIRO, D., PEREZ-RIVEVOL, Y., BARSNES, H., HERMJAKOB, H. & VIZCAÍNO, J. A. 2012a. The PRoteomics IDEntification (PRIDE) Converter 2 Framework: An Improved Suite of Tools to Facilitate Data Submission to the PRIDE Database and the ProteomeXchange Consortium*. *Molecular & Cellular Proteomics,* 11**,** 1682-1689.

DAVID, D. C., OLLIKAINEN, N., TRINIDAD, J. C., CARY, M. P., BURLINGAME, A. L. & KENYON, C. 2010. Widespread Protein Aggregation as an Inherent Part of Aging in *C. elegans*. *PLOS Biology,* 8**,** e1000450.

DILLMAN, A. R., MACCHIETTO, M., PORTER, C. F., ROGERS, A., WILLIAMS, B., ANTOSHECHKIN, I., LEE, M.-M., GOODWIN, Z., LU, X., LEWIS, E. E., GOODRICH-BLAIR, H., STOCK, S. P., ADAMS, B. J., STERNBERG, P. W. & MORTAZAVI, A. 2015. Comparative genomics of *Steinernema* reveals deeply conserved gene regulatory networks. *Genome Biology,* 16**,** 200.

DOWNES M.J., GRIFFIN C.T. 1996. Dispersal behaviour and transmission strategies of the entomopathogenic nematodes *Heterorhabditis* and *Steinernema. Biocontrol Science and Technology,* 6, 347–356.

DURE, L., 3RD, GREENWAY, S. C. & GALAU, G. A. 1981. Developmental biochemistry of cottonseed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by in vitro and in vivo protein synthesis. *Biochemistry,* 20**,** 4162-4168.

EWALD, C. Y., LANDIS, J. N., ABATE, J. P., MURPHY, C. T. & BLACKWELL, T. K. 2015. Dauer-independent insulin/IGF-1-signalling implicates collagen remodelling in longevity. *Nature,* 519**,** 97-101.

FAN, X. & HOMINICK, W. Effects of low storage temperature on survival and infectivity of two *Steinernerna* species (Nematoda : Steinernematidae). *Revue of Nématology.* 14, 407–412.

FITTERS, P. & GRIFFIN, C. 2004. Spontaneous and induced activity of *Heterorhabditis* megidis infective juveniles during storage. *Nematology,* 6**,** 911-917.

GAL, T. Z., GLAZER, I. & KOLTAI, H. 2004. An LEA group 3 family member is involved in survival of *C. elegans* during exposure to stress. *FEBS Letters,* 577**,** 21-26.

GREWAL, P., AND GEORGIS, R. 1999. Entomopathogenic nematodes. In: HALL, F.R.,

MENN, J.J. Biopesticides: Use and Delivery. *The Quarterly Review of Biology,* 5**,** 271-299.

GREWAL, P. S. 2000. Anhydrobiotic potential and long-term storage of entomopathogenic nematodes (Rhabditida: Steinernematidae). *International Journal for Parasitologyy*, 30, 995-1000.

GRIFFIN, C. 1996. Effects of prior storage conditions on the infectivity of *Heterorhabditis* sp. (Nematoda: Heterorhabditidae). *Fundamental and Applied Nematology,* 19, 95-102.

GRIFFIN, C. T. 2012. Perspectives on the behavior of entomopathogenic nematodes from dispersal to reproduction: traits contributing to nematode fitness and biocontrol efficacy. *Journal of nematology,* 44, 177-184.

HAN R, EHLERS RU. 2000. Pathogenicity, development, and reproduction of Heterorhabditis bacteriophora and Steinernema carpocapsae under axenic in vivo conditions. *Journal of Invertebrate Pathology*.75(1):55-8.

HAN, X. 2017. Overexpression of protein gene of stress-tolerant *Chlorella* confers tolerance to NaCl stress in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*. *International Journal of Agriculture and Biology*, 19**,** 1452-1456.

HANSEN, M., TAUBERT, S., CRAWFORD, D., LIBINA, N., LEE, S. J. & KENYON, C. 2007. Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*. *Aging Cell,* 6**,** 95-110.

HOUTHOOFD, K., BRAECKMAN, B. P., LENAERTS, I., BRYS, K., DE VREESE, A., VAN EYGEN, S. & VANFLETEREN, J. R. 2002. Ageing is reversed, and metabolism is reset to young levels in recovering dauer larvae of *C. elegans*. *Experimental Gerontology,* 37**,** 1015-1021.

HUBNER, N. C., BIRD, A. W., COX, J., SPLETTSTOESSER, B., BANDILLA, P., POSER, I., HYMAN, A. & MANN, M. 2010. Quantitative proteomics combined with BAC TransgeneOmics reveals in vivo protein interactions. *Journal of Cell Biology,* 189**,** 739-754.

JONES, L. M., STAFFA, K., PERALLY, S., LACOURSE, E. J., BROPHY, P. M. & HAMILTON, J. V. 2010. Proteomic analyses of *Caenorhabditis elegans* dauer larvae and long-lived daf-2 mutants implicates a shared detoxification system in longevity assurance. *Journal of Proteome Research,* 9**,** 2871-2881.

KAGAN, R. M. & CLARKE, S. 1995. Protein L-isoaspartyl methyltransferase from the nematode *Caenorhabditis elegans*: genomic structure and substrate specificity. *Biochemistry,* 34**,** 10794-10806.

KENYON, CJ. 2010. The genetics of ageing. *Nature*.464(7288):504-12.

KENNEDY, M. W., CORSICO, B., COOPER, A. & SMITH, B. 2013. The unusual lipid-binding proteins of nematodes: NPAs, nemFABPs and FARs. *Parasitic nematodes: molecular biology*, 397-412.

LANE, N. & MARTIN, W. 2010. The energetics of genome complexity. *Nature,* 467**,** 929-934.

LANT, B. & STOREY, K. B. 2010. An overview of stress response and hypometabolic strategies in *Caenorhabditis elegans*: conserved and contrasting signals with the mammalian system. *International Journal of Biological Sciences,* 6**,** 9-50.

LEWIS, E. E., CAMPBELL, J., GRIFFIN, C., KAYA, H. & PETERS, A. 2006. Behavioral ecology of entomopathogenic nematodes. *Biological Control,* 38**,** 66-79.

LU, D., MACCHIETTO, M., CHANG, D., BARROS, M. M., BALDWIN, J.,

MORTAZAVI, A. & DILLMAN, A. R. 2017. Activated entomopathogenic nematode infective juveniles release lethal venom proteins. *PLOS Pathogens,* 13**,** e1006302.

MCDERMOTT, L., COOPER, A. & KENNEDY, M. W. 1999. Novel classes of fatty acid and retinol binding protein from nematodes. *Molecular and Cellular Biochemistry,* 192**,** 69-75.

NUAMTANONG, S., REAMTONG, O., PHUPHISUT, O., CHOTSIRI, P., MALAITHONG, P., DEKUMYOY, P. & ADISAKWATTANA, P. 2019. Transcriptome and excretory-secretory proteome of infective-stage larvae of the nematode *Gnathostoma spinigerum* reveal potential immunodiagnostic targets for development. *Parasite,* 26**,** 34.

O'RIORDAN, V. B. & BURNELL, A. M. 1989. Intermediary metabolism in the dauer larva of the nematode *Caenorhabditis elegans*— 1. Glycolysis, gluconeogenesis, oxidative phosphorylation and the tricarboxylic acid cycle. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 92**,** 233-238.

O'RIORDAN, V. B. & BURNELL, A. M. 1990. Intermediary metabolism in the dauer larva of the nematode *Caenorhabditis elegans*—II. The glyoxylate cycle and fatty-acid oxidation. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 95**,** 125-130.

OLVERA-CARRILLO, Y., LUIS REYES, J. & COVARRUBIAS, A. A. 2011. Late embryogenesis abundant proteins: versatile players in the plant adaptation to water limiting environments. *Plant Signaling Behavior,* 6**,** 586-9.

PAN, K. Z., PALTER, J. E., ROGERS, A. N., OLSEN, A., CHEN, D., LITHGOW, G. J. & KAPAHI, P. 2007. Inhibition of mRNA translation extends lifespan in

*Caenorhabditis elegans*. *Aging Cell,* 6**,** 111-119.

POINAR, G. 1993. Origins and phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and *Steinernema*. *Fundamental & Applied Nematology,* 16.

PREISSER EL, DUGAW CJ, DENNIS B, STRONG DR. 2005. Long-Term Survival of the Entomopathogenic Nematode *Heterorhabditis marelatus*. Environmental Entomology.34(6):1501-6.

QIU, L. & BEDDING, R. A. 2002. Characteristics of protectant synthesis of infective juveniles of *Steinernema carpocapsae* and importance of glycerol as a protectant for survival of the nematodes during osmotic dehydration. *Comparative Biochemistry & Physiology Part B: Biochemical and Molecular Biology,* 131**,** 757-765.

REYES, J. L., CAMPOS, F., WEI, H., ARORA, R., YANG, Y., KARLSON, D. T. & COVARRUBIAS, A. A. 2008. Functional dissection of hydrophilins during in vitro freeze protection. *Plant, Cell & Environment,* 31**,** 1781-1790.

REYES, J. L., RODRIGO, M.-J., COLMENERO-FLORES, J. M., GIL, J.-V., GARAY-ARROYO, A., CAMPOS, F., SALAMINI, F., BARTELS, D. & COVARRUBIAS, A. A. 2005. Hydrophilins from distant organisms can protect enzymatic activities from water limitation effects in vitro. *Plant, Cell & Environment,* 28**,** 709-718.

ROUGON-CARDOSO, A., FLORES-PONCE, M., RAMOS-ABOITES, H. E., MARTÍNEZ-GUERRERO, C. E., HAO, Y.-J., CUNHA, L., RODRÍGUEZ-MARTÍNEZ, J. A., OVANDO-VÁZQUEZ, C., BERMÚDEZ-BARRIENTOS, J. R., ABREU-GOODGER, C., CHAVARRÍA-HERNÁNDEZ, N., SIMÕES, N. & MONTIEL, R. 2016. The genome, transcriptome, and proteome of the nematode *Steinernema carpocapsae*: evolutionary signatures of a pathogenic lifestyle.

*Scientific Reports,* 6**,** 37536.

SANTRA, M., DILL, K. A. & DE GRAFF, A. M. R. 2019. Proteostasis collapse is a driver of cell aging and death. *Proceedings of the National Academy of Sciences,* 116**,** 22173.

SERRA, L., MACCHIETTO, M., MACIAS-MUÑOZ, A., MCGILL, C. J., RODRIGUEZ, I. M., RODRIGUEZ, B., MURAD, R. & MORTAZAVI, A. 2019. Hybrid Assembly of the Genome of the Entomopathogenic Nematode *Steinernema carpocapsae* Identifies the X-Chromosome. *G3 (Bethesda),* 9**,** 2687-2697.

SOLOMON, A., SALOMON, R., PAPERNA, I. & GLAZER, I. 2000. Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable protein. *Parasitology,* 121**,** 409-416.

TANTOS, A., FRIEDRICH, P. & TOMPA, P. 2009. Cold stability of intrinsically disordered proteins. *FEBS Letters,* 583**,** 465-469.

TERAMOTO, N., SACHINVALA, N. D. & SHIBATA, M. 2008. Trehalose and trehalose-based polymers for environmentally benign, biocompatible and bioactive materials. *Molecules,* 13**,** 1773-1816.

VAINBERG, I. E., LEWIS, S. A., ROMMELAERE, H., AMPE, C., VANDEKERCKHOVE, J., KLEIN, H. L. & COWAN, N. J. 1998. Prefoldin, a Chaperone that Delivers Unfolded Proteins to Cytosolic Chaperonin. *Cell,* 93**,** 863-873.

VANFLETEREN, J. R. & DE VREESE, A. 1996. Rate of aerobic metabolism and superoxide production rate potential in the nematode *Caenorhabditis elegans*. *Journal of Experimental Zoology,* 274**,** 93-100.

WALTER, S. & BUCHNER, J. 2002. Molecular chaperones--cellular machines for protein

folding. *Angewandte Chemie International Edition in English,* 41**,** 1098-1113.

WALTHER, DIRK M., KASTURI, P., ZHENG, M., PINKERT, S., VECCHI, G., CIRYAM, P., MORIMOTO, RICHARD I., DOBSON, CHRISTOPHER M., VENDRUSCOLO, M., MANN, M. & HARTL, F. U. 2015. Widespread Proteome Remodeling and Aggregation in Aging *C. elegans. Cell,* 161**,** 919-932.

WANG, T., MA, G., ANG, C. S., KORHONEN, P. K., XU, R., NIE, S., KOEHLER, A. V., SIMPSON, R. J., GREENING, D. W., REID, G. E., WILLIAMSON, N. A. & GASSER, R. B. 2019. Somatic proteome of *Haemonchus contortus*. *International Journal for Parasitology,* 49**,** 311-320.

WHITLEY, D., GOLDBERG, S. P. & JORDAN, W. D. 1999. Heat shock proteins: a review of the molecular chaperones. *Journal of Vascular Surgery,* 29**,** 748-751.

WOODRING, J. L. & KAYA, H. K. 1988. Steinernematid and heterorhabditid nematodes: a handbook of biology and techniques. *Southern cooperative series bulletin (USA).*

WRIGHT, D., Perry, R. 2002. Physiology and Biochemistry In*:* GAUGLER, R. (Ed). Entomopathogenic Nematology. *CABI, Wallingford UK,* 145-168.

ZHENG, J., SU, H., LIN, R., ZHANG, H., XIA, K., JIAN, S. & ZHANG, M. 2019. Isolation and characterization of an atypical LEA gene (IpLEA) from Ipomoea pes-caprae conferring salt/drought and oxidative stress tolerance. *Scientific Reports,* 9**,** 14838.

# Chapter 4 Low temperature exposure has immediate and lasting effects on the stress tolerance, chemotaxis, and proteome of entomopathogenic nematodes

**For Submission as:**

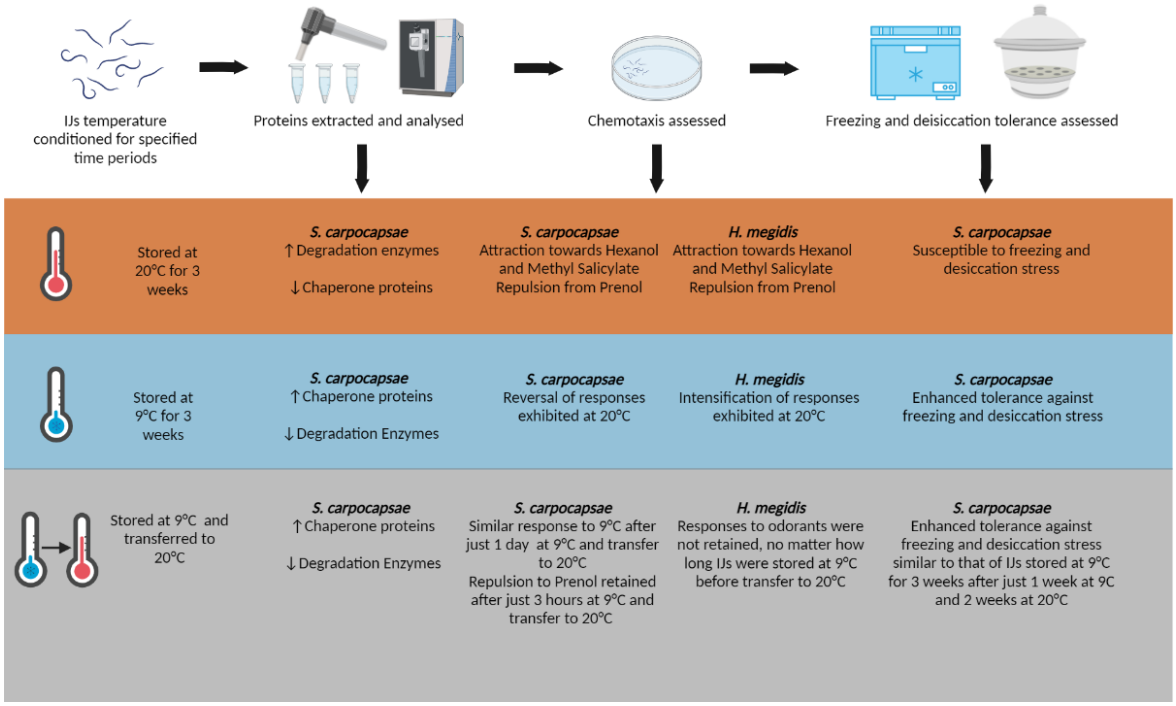
Lillis, P.E., Kennedy, I.P., Carolan, J.C., Griffin, C.T. 2021. Low temperature exposure has immediate and lasting effects on the stress tolerance, chemotaxis, and proteome of entomopathogenic nematodes.

## Abstract

Entomopathogenic nematodes (EPN), *Steinernema* spp. and *Heterorhabditis* spp., are obligate parasites of insects and effective biocontrol agents. Infective juveniles (IJs) of *Steinernema carpocapsae* and *Heterorhabditis megidis* are distantly related and exhibit different foraging behaviours but utilise similar volatiles to find hosts. These IJs live in the soil year-round and are exposed to a range of temperatures and associated stressors. Here we explore how exposure to low temperatures, and the duration of this exposure, affects the behaviour and stress resistance of these IJs. In chemotaxis assays at 20°C, *S. carpocapsae* and *H. megidis* had similar responses to odorants when freshly emerged from their natal host. The chemotaxis of *H. megidis* was generally enhanced by prior storage at lower temperatures, whereas the chemotaxis of *S. carpocapsae* tended to be reversed by this treatment. Exposure to temperatures (9°C, 12°C and 15°C) below culture temperature (20°C) affected the chemotaxis of *H. megidis* in a graded manner, and in a binary manner for *S. carpocapsae*. Cold storage (9°C) of *S. carpocapsae* enhanced their resistance to freezing and desiccation. Alteration in chemotaxis and stress tolerance induced by brief exposure to 9°C were maintained for weeks afterwards. Label free quantitative proteomics showed that exposure to 9°C for 1 week elicited dramatic increases in chaperone proteins in *S. carpocapsae* IJs, which were retained at high levels for 2 weeks after their transfer to 20°C. The proteome of *S. carpocapsae* IJs exposed to 9°C for 1 week and subsequently transferred to 20°C for 2 weeks conformed closely to that of IJs kept at 9°C for 3 weeks. Thus, *S. carpocapsae* IJs display a high degree of plasticity, and their behaviour and stress resistance may be manipulated by brief controlled exposure to low temperatures.

**Keywords**: temperature, conditioning, biological pest control, chemotaxis, proteomics

# Graphical Abstract



## 4.1 Introduction

Temperature profoundly affects the successful transmission of parasites, in three main ways: extreme temperatures are lethal or damaging, while for parasites with active stages, dispersal or host-finding may only take place within an optimal range. Thirdly, temperature may affect the persistence of transmission stages in the environment, for example by slowing metabolism or by inducing diapause or related phenomena. Thus, temperatures experienced prior to a crucial activity such as host infection may affect the outcome of that activity. This is particularly important in the case of parasites with relatively long-lived transmission stages, such as the infective juveniles (IJs) of many plant and animal parasitic nematodes. Amongst these, the entomopathogenic nematodes (EPN) *Steinernema* and *Heterorhabditis* have received much attention due to their usefulness both as model parasites, and as biological agents.

EPN have a developmentally arrested stage known as the IJ. The IJ leaves their natal host, seeks out and infects insects, which become the food-source for the next generation of nematodes. The IJs of EPN are promising biocontrol agents, which are mass-produced and then stored until applied against insect pests. The IJ is a nonfeeding stage and has a sealed mouth and anus. These IJs depend solely upon their lipid and glycogen stores for nutrition and are resistant to abiotic stressors such as UV radiation, desiccation, and extreme temperatures. The IJs invade the mouth, anus, and spiracles of insects, and once inside the haemocoel they egest their symbiotic bacteria into the host haemolymph. Heterorhabditids have a mutualistic relationship with *Photorhabdus* spp., and steinernematids have a mutualistic relationship with *Xenorhabdus* spp. The bacteria multiply and kill the insect via sepsis. The activated IJs consume the bacteria, develop to sexual maturity, and reproduce within the insect cadaver.

Effects of storage temperature and time (age) on behaviours such as dispersal and infection have been documented for *Steinernema* spp. (Fan and Hominick, 1991; Guy et al., 2017; Kaplan et al., 2020) and for *Heterorhabditis* spp. (Griffin, 1996; Fitters and Griffin, 2004). More recently it has been shown that chemotaxis of IJs of both EPN and animal parasites is altered by culture and storage at temperatures (Lee et al., 2016), which could affect their host finding abilities and foraging behaviour. Prior exposure to low temperatures can also enhance the IJ's resistance to freezing (Ali and Wharton., 2013; Brown and Gaugler., 1996) and desiccation (Jagdale and Grewal, 2007).

This study aims to investigate the effect of temperature conditioning and aging on the behaviour, proteome and stress resistance of EPN, using two economically important species, *S. carpocapsae* and *H. megidis. S. carpocapsae* is well studied and commercially used as a biocontrol agent, and the behaviour of *H. megidis* has been extensively studied with respect to storage temperature and time (Griffin, 1996; O'Leary et al., 1998; Fitters and Griffin, 2004; Guy et al., 2017). While *Heterorhabditis* and *Steinernema* are distantly related (Blaxter et al., 1998), they have convergently evolved remarkably similar entomopathogenic life cycles including the IJ stage (Poinar., 1993). Their response towards host volatiles is also similar (Chaisson and Hallem., 2012).

We have previously shown, using quantitative label-free proteomics, that the proteomes of *S. carpocapsae* and of *H. megidis* IJs showed different responses to temperature. When transferred from a culture temperature of 20°C to 9°C, the proteome of *S. carpocapsae* underwent a profound and immediate changes that was maintained during further storage at that temperature, while that of *H. megidis* underwent more gradual changes over time at both 20°C and 9°C (Lillis et al., 2022; Chapter 3). Here we extend our exploration of the effects of storage temperature and time on these two species using similar conditions but extending our study to include chemotaxis and stress tolerance, and investigating whether

cold-induced changes in each of these parameters are retained when IJs are returned to their culture temperature.

Specifically, (1) Following Lee et al., (2016) we explore how olfactory responses of both species change over time at different storage temperatures (2) we test how 9°C storage protects *S. carpocapsae* IJs against stress (freezing and desiccation) (3) we ask whether changes in olfaction and stress tolerance induced in *S. carpocapsae* by short-term storage at 9°C are maintained following return to culture temperature, and (4) we compare the proteome of transferred IJs with that of IJs maintained in constant conditions.

## 4.2 Materials and methods

### 4.2.1 Nematode culturing and conditioning

*Heterorhabditis megidis* UK211 and *Steinernema carpocapsae* All were cultured in last instar *Galleria mellonella* larvae (Mealworm Company, Sheffield, UK) using methods outlined in Woodring and Kaya (1988), at 20°C, with an inoculum density of 100 IJs/insect. Cadavers were placed on White traps and monitored daily. After first emergence of IJs, the White trap water was replaced with fresh sterile tap water. IJs that emerged into the water for 3-4 days were collected and rinsed 3 times by sedimentation. The IJs were stored at 1000 IJs/ml in sterile tap water in 35 ml aliquots in lidded plastic tubs (9cm diameter). IJs were placed into constant temperatures stated and assayed at intervals or transferred between temperatures as specified. Each tub of IJs was used once only.

### 4.2.2 Chemotaxis assays

Chemotaxis assays (Bargmann et al, 1993) were conducted on 60mm Petri dishes with 20mls of 2% nutrient agar. Test stimulus (5 µl) was added to a circle (1cm diameter) on one side of the plate, and 5 µl of diluent was added to a control circle on the opposite side (see appendix 4.1). Sodium azide (2 µl) was added to each circle, to anesthetise nematodes arriving there. IJs were concentrated by sedimentation and approximately 100-250 IJs in 2 µl were added to the centre of the plate. Plates were orientated in random directions at 20°C and IJs were allowed to migrate for 1 hour. The chemotaxis index (CI) was calculated as the number of IJs in the treatment circle, minus the IJs in the control circle, divided by the total number of IJs in both circles. Plates which had fewer than 5 IJs in either scoring region were discounted to prevent small numbers from skewing the data.

Putative attractants and repellents were chosen based on the literature: methyl salicylate (Chaisson and Hallem, 2012), acetone (O'Halloran and Burnell., 2003), prenol (Baiocchi et al., 2017; Kin et al., 2019; Baiocchi et al., 2019), and hexanol (O'Halloran and Burnell, 2003; Chaisson and Hallem., 2012). Prenol (3-methyl-2-buten-1-ol) was diluted to 2M, by mixing 203 µl of 99.9% prenol (Sigma) with 797 µl of ethanol as per Baiocchi et al. (2017). Dilutions (1 in 10) of acetone, hexanol and methyl salicylate were made in paraffin oil. Sodium azide (1M) was prepared by transferring 0.06501 g of crystalline sodium azide to 1 ml of MilliQ water.

There were three experiments utilising chemotaxis assays. In experiment 1, IJs of *S. carpocapsae* and *H. megidis* were assayed at time 0, and after conditioning for 1, 3, 6, and 9 weeks at 9°C, and for 1, 3 and 6 weeks at 20°C. An additional treatment was included for *S. carpocapsae*: after IJs were stored at 9°C for 1 week, they were transferred to 20°C, and assayed after 1 day, and 2, 5 and 8 weeks (corresponding to a total time of 3, 6 or 9 weeks from time 0).

In experiment 2, odours which elicited significantly different chemotaxis indices at the 3-week timepoint in experiment 1 were chosen to assay the effect of brief exposure to low temperatures on the chemotaxis index of IJs, both immediately and after subsequent storage at 20°C. Methyl salicylate and prenol were used for *H. megidis* IJs and hexanol and prenol were used for *S. carpocapsae* IJs. IJs were assayed at time 0 and after storage for 1 day, 1 week, or 3 weeks at 9°C, or 3 weeks at 20°C. In addition, after 1 day or 1 week at 9°C, IJs were transferred to 20°C and assayed 3 weeks from the start of the experiment. *S. carpocapsae* IJs were also assayed and transferred to 20°C after 3 hours, due to the plasticity of their chemotaxis responses.

In experiment 3, IJs were assayed at time 0, and after 1 and 3 weeks at 9°C, 12 °C, 15°C and 20°C. As above, chemotaxis was assessed against odours which give strong responses,

methyl salicylate and prenol for *H. megidis*, and hexanol and prenol for *S. carpocapsae* IJs.

In each experiment there were at least 3 culture batches of each species with either 5 (experiment 1) or 10 (experiments 2 and 3) assay plates per batch to give a total of 15 or 30 technical replicates per treatment at each timepoint. An exception was for IJs transferred from 9°C to 20°C in experiment 1, where there was a total of 5 technical replicates.

### 4.2.3 Freezing and desiccation stress assays

Sterile tap water (50 µl) containing approximately 50 IJs was pipetted onto Whatman filter paper (1 cm diameter) in a 3 cm petri dish. Plates (without lids) were transferred to either -10°C for 6 hours or 75% RH for 5 days. These conditions were chosen to give approximately 50% survival in unconditioned IJs. Desiccation chambers were maintained at 75% relative humidity using supersaturated sodium chloride solutions (Winston and Bates, 1960). After the assay time, 20 mls of sterile tap water was added to the IJs and their survival was assessed after 24 hours. There were six culture batches of nematodes, and 5 plates were tested per batch of nematodes, to give 30 technical replicates per conditioning treatment.

### 4.2.4 Statistical analysis

Statistics were carried out in Graphpad Prism v9.0.1. Kruskal Wallis tests were performed on data at the significance level of $p < 0.05$, with post hoc Dunn's multiple comparisons tests to demonstrate groups which were significantly different.

### 4.2.5 Protein sample preparation

The contents of a tub of IJs were sedimented in a 50 ml Falcon tube in their conditioning temperature. The pelleted IJs (150 µl) were transferred to a 1.5 ml Eppendorf tube and snap frozen in liquid nitrogen. Each sample was homogenised in lysis buffer, containing 6M urea, 2M thiourea, and a Protease Inhibitor Cocktail (cOmplete, Mini Protease Inhibitor Cocktail, Merck), centrifuged at 10000 x g for 1 minute, and snap frozen. This step was repeated 3 times to ensure complete homogenisation. Protein content was then quantified using Qubit (Invitrogen), following the manufacturer's instructions. Protein (100 µg) was purified using a 2D Clean Up Kit (GE Healthcare) according to the manufacturer's instructions. The resulting pellets were stored in the kit's wash solution at -20°C until the last samples were collected, then all were centrifuged at 13000 x g for 5 minutes and the resulting pellets were resuspended in 50 µl of resuspension buffer (6M urea, 2M thiourea, 0.1M TrisHCl, pH8). A 20 µl aliquot was removed from each sample for reduction, alkylation and digestion. One hundred and five µls of ammonium bicarbonate (50 mM) and 1 µl of dithiothreitol (DTT) were added and samples were incubated at 56°C for 20 minutes. Once cooled, samples were alkylated with 2.7 µl of iodoacetamide (IAA) in dark conditions.

One µl of a 1% (w/v) solution of ProteaseMax (Promega) and 0.5 µg/µl trypsin (Promega) were added to the samples and incubated at 37°C for a minimum of 16 hours. Samples were removed from 37°C, centrifuged briefly and acidified with 1 µl of trifluoroacetic acid (TFA) for 5 minutes at room temperature (20-25°C). Samples were centrifuged at 13000 x g for 10 minutes and the supernatant was purified using C18 Spin Columns (Pierce, Thermo Fisher Scientific) following the manufacturer's instructions and then lyophilised in a Speedyvac concentrator (Thermo Scientific Savant DNA120). Samples were then resuspended in a loading buffer (2% v/v acetonitrile, 0.05% v/v TFA) and 1 µg was loaded

from each of 4 biological replicates per samples were run on a QExactive (Thermo Fisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated over a 2% to 40% gradient of acetonitrile on a Thermo Fisher EASY-Spray, PepMap RSLC C18 column (500mm length, 75mm ID), using a reverse-phase gradient at a flow rate of 250nL $min^{-1}$ over 125 minutes. All data were acquired over 105 minutes with the mass spectrometer operating in automatic data dependent switching mode. A full MS scan at 140,000 resolution and range of 300–1700 m/z was followed by an MS/MS scan, resolution 17,500 and range of 200–2000 m/z, selecting the 15 most intense ions prior to MS/MS.

### 4.2.6 Proteomic data processing

Protein identification and LFQ normalisation of MS/MS data was performed using Max-Quant v1.6.3.3 (http://www.maxquant.org) following the general procedures and settings outlined in Hubner et al. (2010). The Andromeda search algorithm (Cox et al., 2011) incorporated in the MaxQuant software was used to correlate MS/MS data for *S. carpocapsae* against the predicted protein data sets derived from the *S. carpocapsae* (Serra et al., 2019).

Normalised LFQ intensities were used to quantify protein abundances, and the data was filtered to remove contaminants. The LFQ intensities were $log_2$ transformed, and each replicate was renamed to their respective groups (3wks9°C for proteins from IJs stored at 9°C for 3 weeks). Only proteins found in 3 replicates of at least 1 group were retained. A data imputation step replaced missing values with values of low abundant proteins chosen randomly from a distribution specified by a downshift of 2 times the mean standard deviation (SD) and a width of 0.3 times the standard deviation.

A principal component analysis (PCA) was initially performed on the normalised intensity values of all replicates. However, a number of outliers were identified, resulting in 3 replicates in each sample in the final datasets for analysis.

An analysis of variance (ANOVA) was performed on all groups using a Benjamini-Hochberg false discovery rate (FDR) of <1% to select proteins for z score normalisation. These ANOVA significant proteins were used for hierarchical clustering of samples using Euclidean distance and average linkage pre-processed with K means.

Volcano plots were generated in Perseus by plotting negative log p values of the y axis and $log_2$fold transformed differences on the x axis for each comparison. Pairwise t-tests were performed between the *S. carpocapsae* IJs conditioned at 9°C for 1 week and transferred to 20°C for 2 weeks and the IJs stored at 9°C and 20°C for 3 weeks to visualise the effect of temperature swapping on the IJs proteome (table 2). Statically significant (SS; $p< 0.05$) and differentially abundant (DA; fold change of 1.5) proteins were identified as SSDAs and selected for further analysis.

The genome of *S. carpocapsae* has been recently sequenced (Serra et al., 2019), and the protein file was downloaded from Wormbase Parasite (https://parasite.wormbase.org/Steinernema_carpocapsae_prjna202318/Info/Index) and used for detection of peptides.

The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium (Côté et al., 2012) via the PRIDE partner repository with the dataset identifier PXD027609.

## 4.3 Results

### 4.3.1 Effect of conditioning at constant temperatures (9°C and 20°C) on chemotaxis

*Steinernema carpocapsae* IJs were initially (week 0) strongly attracted to hexanol and methyl salicylate, strongly repulsed by prenol, and weakly attracted to acetone (figure 4.1). When stored at 9°C for one week, initially strong responses (CI > $\pm$ 0.8) were completely reversed and remained so for the remainder of the 9°C storage period: IJs became repulsed by hexanol (figure 4.1C) and methyl salicylate (figure 4.1B) and highly attracted towards prenol (figure 4.1A). In contrast, the initially weak attraction (CI 0.3) towards acetone was intensified after 1 week at 9°C (figure 4.1D). The responses of IJs which remained at 20°C generally followed the same trend as those at 9°C but the change was more gradual: over time, IJs slowly became attracted to prenol (figure 4.1A), repulsed by methyl salicylate (figure 4.1B), and less attracted to hexanol (figure 4.1C). The response to acetone did not follow this trend; IJs were repulsed after 1 week at 20°C but the response returned to week 0 levels thereafter (figure 4.1D).

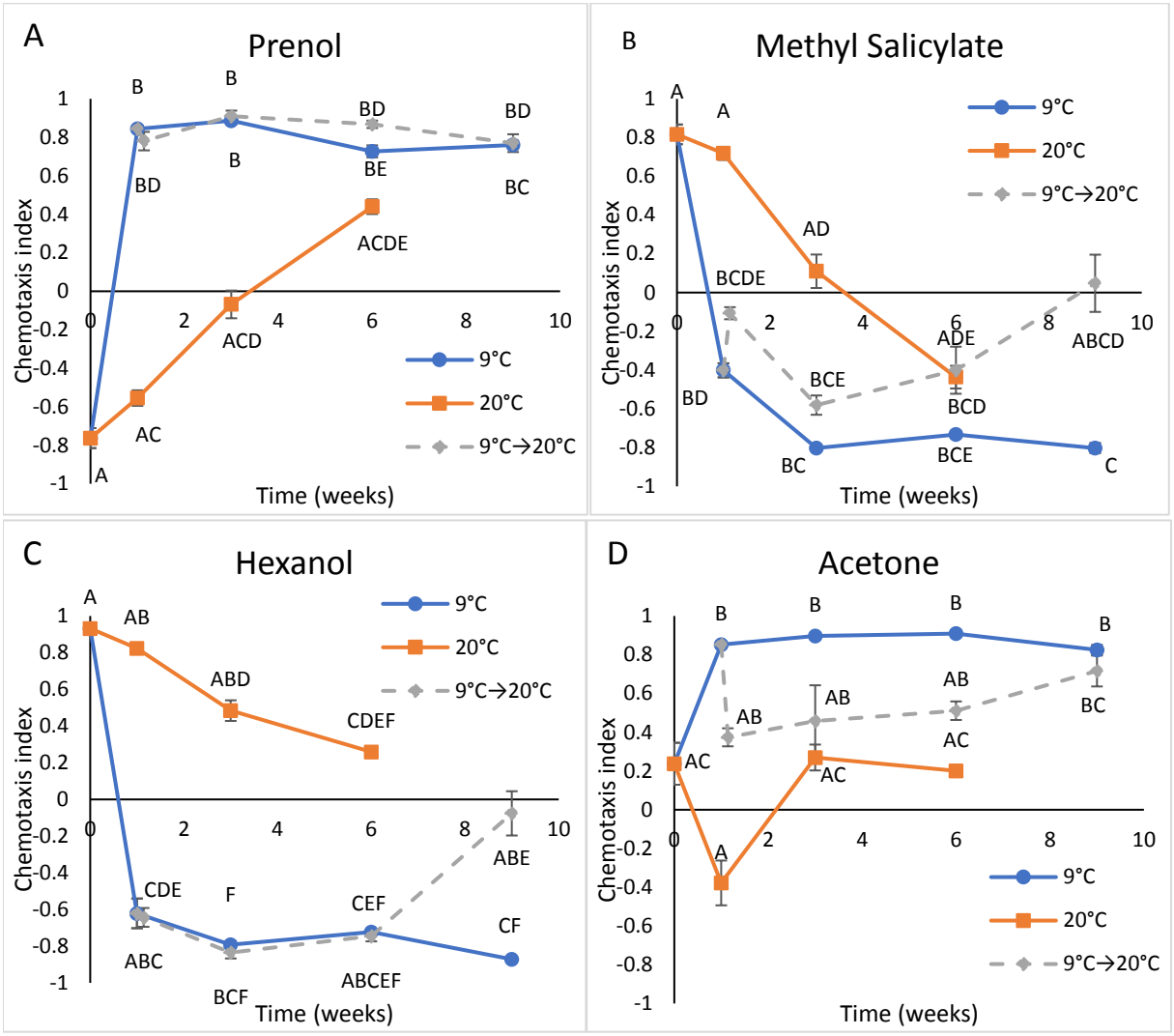



**Figure 4.1:** Chemotaxis Index of *S. carpocapsae* IJs conditioned at 9°C (●), 20°C (■), and those placed at 9°C for 1 week and transferred to 20°C (◆) in response to odorants.

The reversal of chemotaxis observed following 1 week at 9°C seen in *S. carpocapsae* IJs in experiment 1 was explored over shorter time periods in experiment 2. Exposure to 9°C for only 3 hours had a significant effect on the response to prenol, which changed from strong repulsion at time 0 to weak attraction after just 3 h at 9°C (figure 4.3B). A significant effect on the response to hexanol was first seen after 1 day at 9°C, and the response was reversed from positive to negative following 3 days at 9°C (figure 4.3A).

*Heterorhabditis megidis* IJs were initially (time 0) weakly attracted to methyl salicylate and hexanol and repulsed by prenol and acetone (figure 4.2). Storage at 9°C tended to enhance the response of IJs, which became more attracted to methyl salicylate (figure 4.2B) and more repulsed by prenol (figure 4.2A) and acetone (figure 4.2D). Exploration of shorter-term exposure to 9°C in exp 2 showed that increased repulsion to prenol was seen already after just one day (figure 4.3D). Storage at 20°C tended to have the opposite effect to 9°C, and the greatest difference between storage temperatures was seen in the first 1-3 weeks (figure 4.2). The greatest difference between temperatures was for prenol, which had the strongest week 0 response (CI -0.6; figure 4.2A) while little divergence was seen for hexanol which elicited a weak response (CI 0.1; figure 4.2C) at week 0.

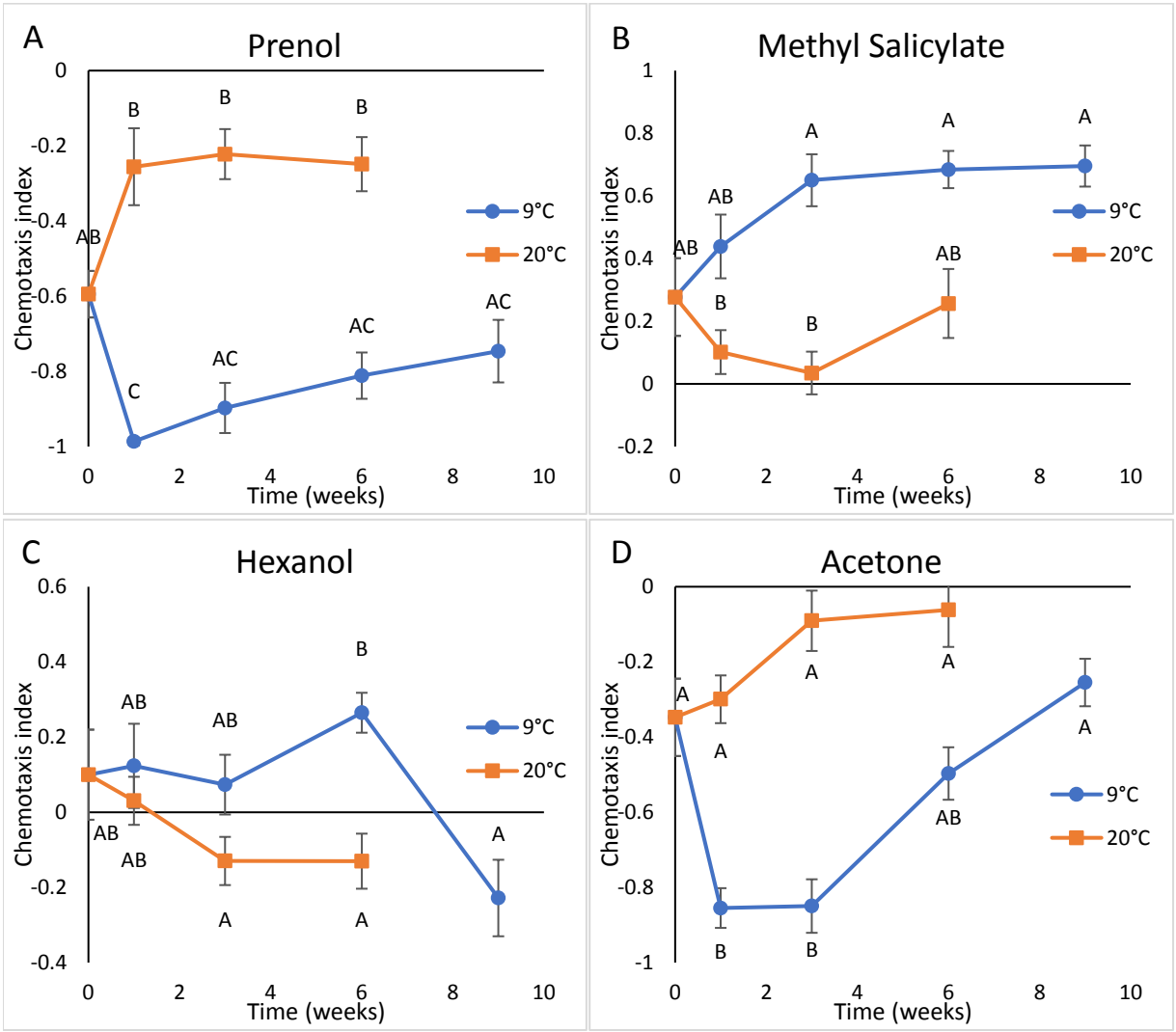



**Figure 4.2:** Chemotaxis Index of *H. megidis* IJs after temperature conditioning at 9°C (●) and 20°C (■) in response to odorants.

### 4.3.2 Chemotaxis of IJs stored for up to 1 week at 9°C and then transferred to 20°C

In experiment 1, the chemotaxis of *S. carpocapsae* IJs which were conditioned at 9°C for 1 week and then transferred to 20°C for the rest of the experiment tended to remain similar to the CI of IJs which remained at 9°C throughout (figure 4.1). The similarity persisted for the full 9 weeks in the case of prenol (figure 4.1A), but the responses began to diverge at 6 weeks in the case of methyl salicylate (figure 4.1C) and at 9 weeks for hexanol (Figure 4.1B). The response to acetone of transferred IJs was intermediate between that of IJs stored exclusively at 9 or 20°C (figure 4.1D).

Cold-induced changes in chemotaxis towards prenol that were induced in *S. carpocapsae* by 3 hours at 9°C were maintained and even intensified following transfer to 20°C (figure 4.3B). When tested after 3 weeks, the IJs which were transferred to 20°C after 3 hours, 1 day or 1 week at 9°C had the same response as IJs left at 9°C for the full 3 weeks, differing significantly from the CI of IJs kept at 20°C for 3 weeks (figure 4.3B).

In contrast, although *H. megidis* IJs which were exposed to 9°C for 1 day or 1 week had an altered chemotaxis response towards prenol, this was not maintained after subsequent storage at 20°C; the CI differed from that of IJs maintained for 3 weeks at 9°C but not from that of IJs that remained at 20°C throughout (figure 4.3D). A similar pattern was seen in response to methyl salicylate (figure 4.3C).

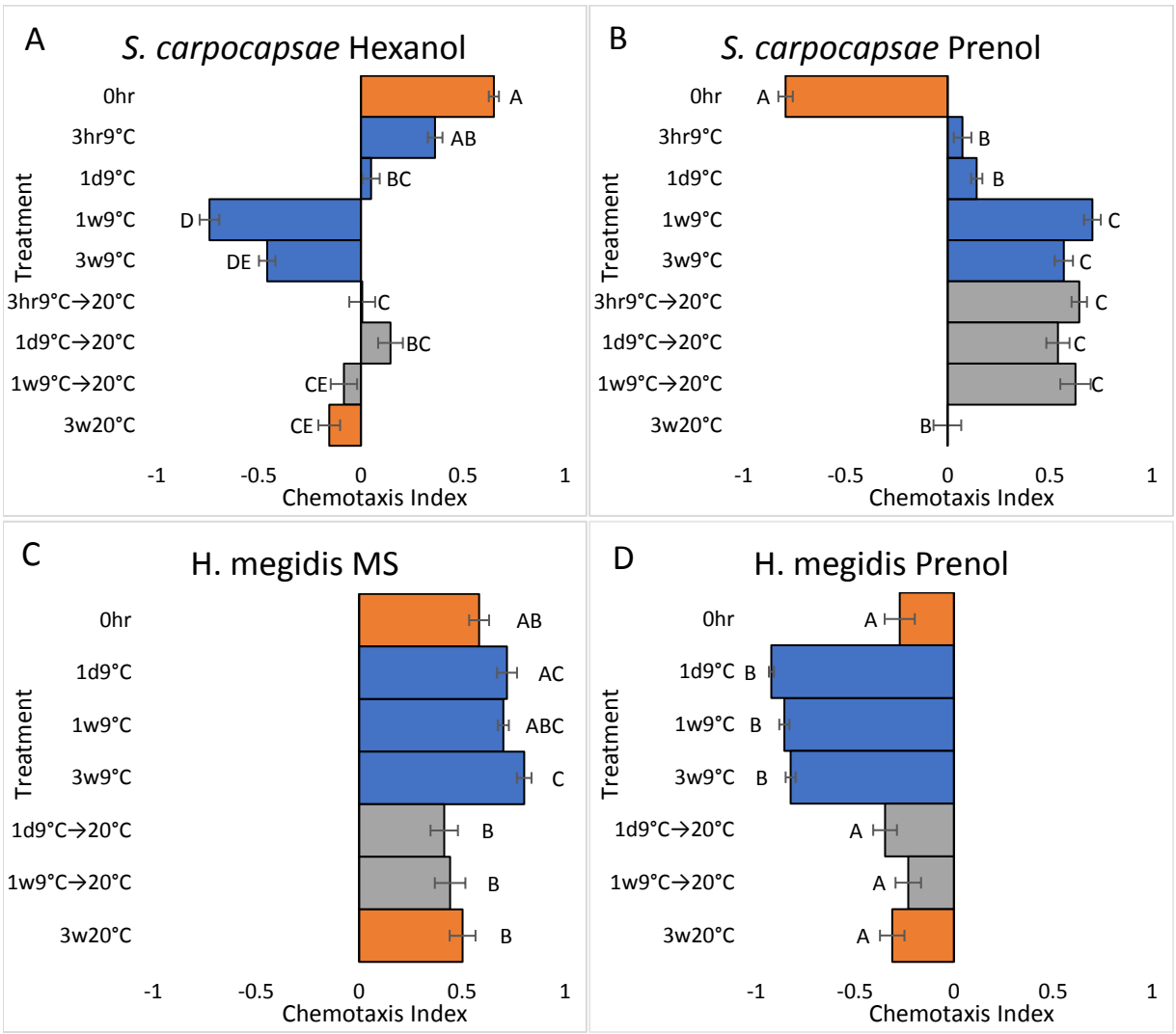



**Figure 4.3:** Chemotaxis indexes of *S. carpocapsae* (top) and *H. megidis* (bottom) IJs stored at 9°C for brief periods and transferred into 20°C for the duration of the experiment. Control IJs were left at 9°C and 20°C for the duration and tested at intervals stated.

### 4.3.3 Do all temperatures below culture temperature affect chemotaxis similarly?

For *S. carpocapsae,* IJs stored at 9°C, 12°C and 15°C all showed a similar CI, differing significantly from that of IJs stored 20°C after one (hexanol) or three (prenol) weeks (figure 4C and D). Similarly, *H. megidis* IJs stored at 9°C, 12°C or15°C for three weeks had a similar CI for prenol, that differed significantly from that of IJs stored at 20°C. However, chemotaxis towards methyl salicylate showed a more graded effect of storage temperature on *H. megidis*. After either one or three weeks, there was a significant difference between IJs stored at 9°C and 20°C, with IJs stored 12°C and 15°C showing intermediate chemotaxis indices (figure 4.4A and B).

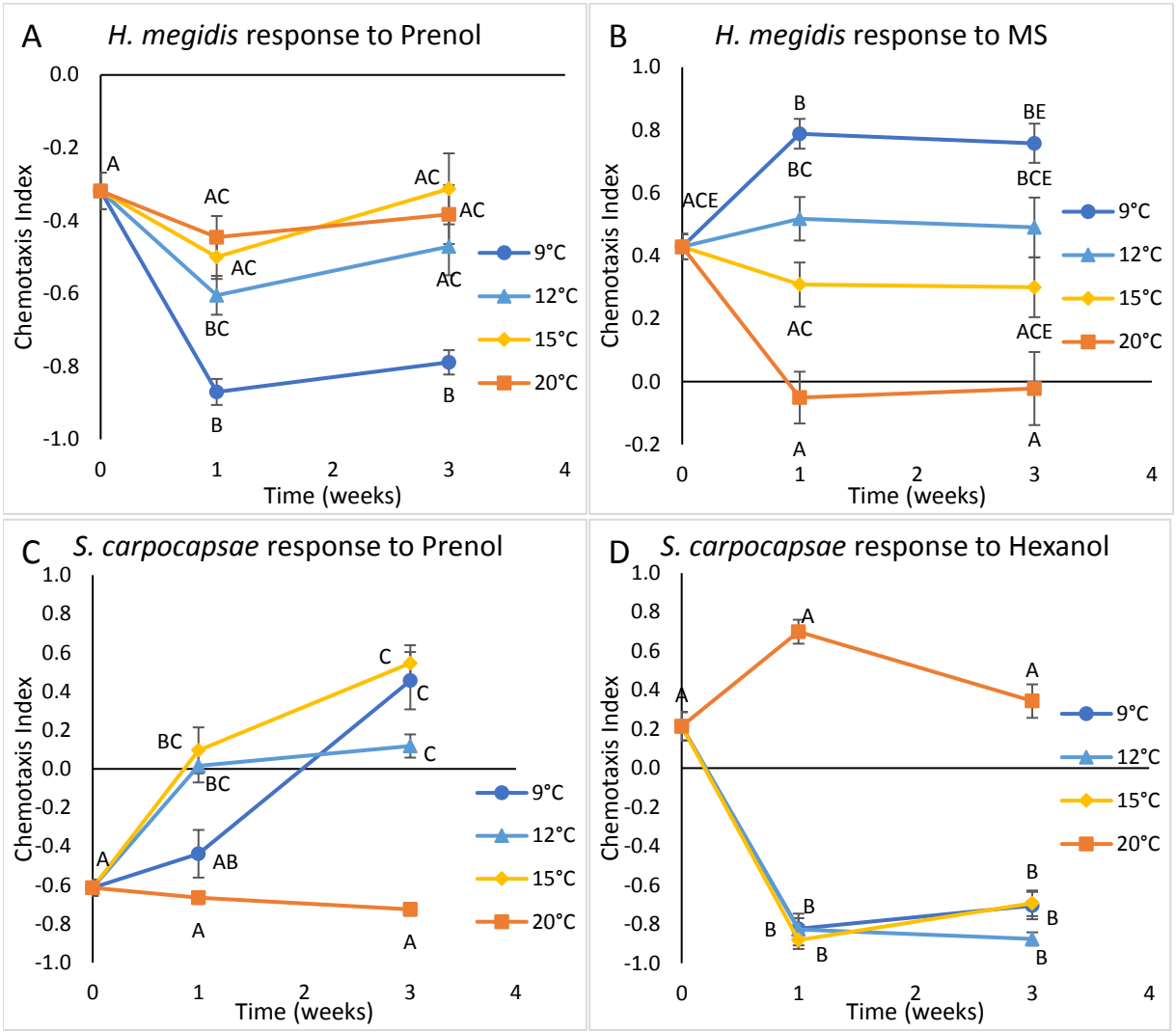



**Figure 4.4:** The Chemotaxis Index of *H. megidis* IJs (top) and *S. carpocapsae* IJs (bottom) against an attractant (left), and a repellent (right) upon emergence from the host, after storage at 9°C, 12°C, 15°C, and 20°C for 1 or 3 weeks.

### 4.3.4 Effect of conditioning on freezing and desiccation tolerance of *S. carpocapsae*

*S. carpocapsae* IJs stored at 9°C showed increased survival in both freezing (-10°C for 6 hours) and in desiccation (75% RH for 5 days) assays relative to freshly emerged IJs and those stored at 20°C, with significant differences in both cases (figure 4.5). IJs which were stored at 9°C for 1 week and transferred to 20°C for 2 weeks showed similarly high survival rates and were not statistically significantly different from those of IJs stored at 9°C for 1 or 3 weeks (figure 4.5).

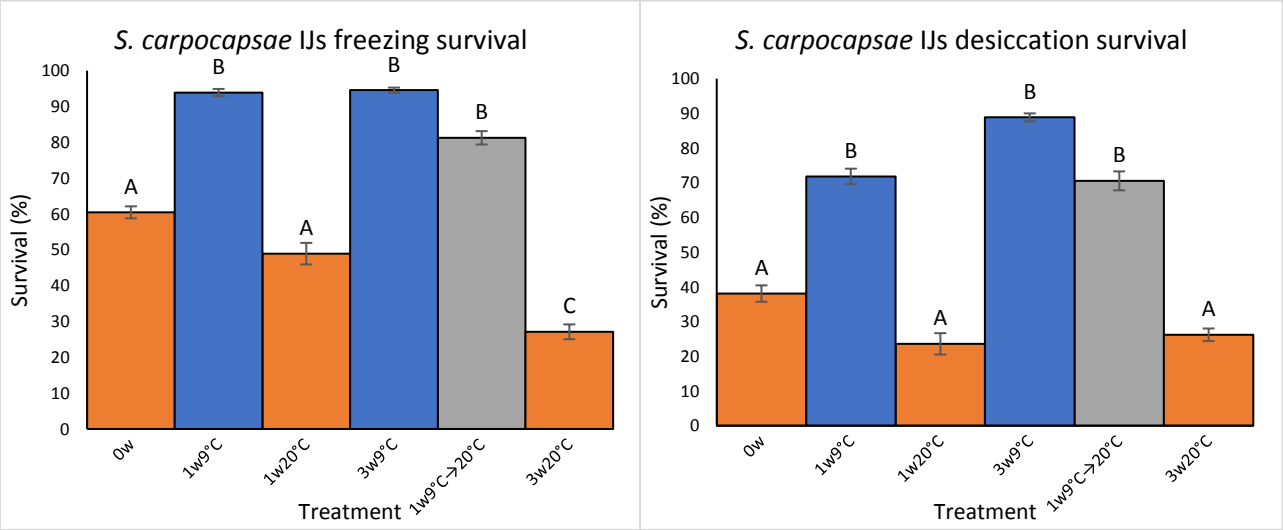



**Figure 4.5:** Survival of *S. carpocapsae* IJs freshly emerged, stored at 9°C for 1 and 3 weeks, at 9°C for 1 week and then swapped to 20°C for 2 weeks, and at 20°C for 1 and 3 weeks exposed to freezing stress (-10°C for 6 hours) or desiccation stress (75% RH for 5 days).

### 4.3.5 Response of *S. carpocapsae* proteome to conditioning

In general, the proteome of *S. carpocapsae* IJs which were conditioned at 9°C for 1 week and transferred to 20°C for 2 weeks (1wk9°C-20°C; temperature-swapped) was more similar to the proteome of IJs stored for 3 weeks at 9°C than those stored for 3 weeks at 20°C (figures 4.6 and 4.7).

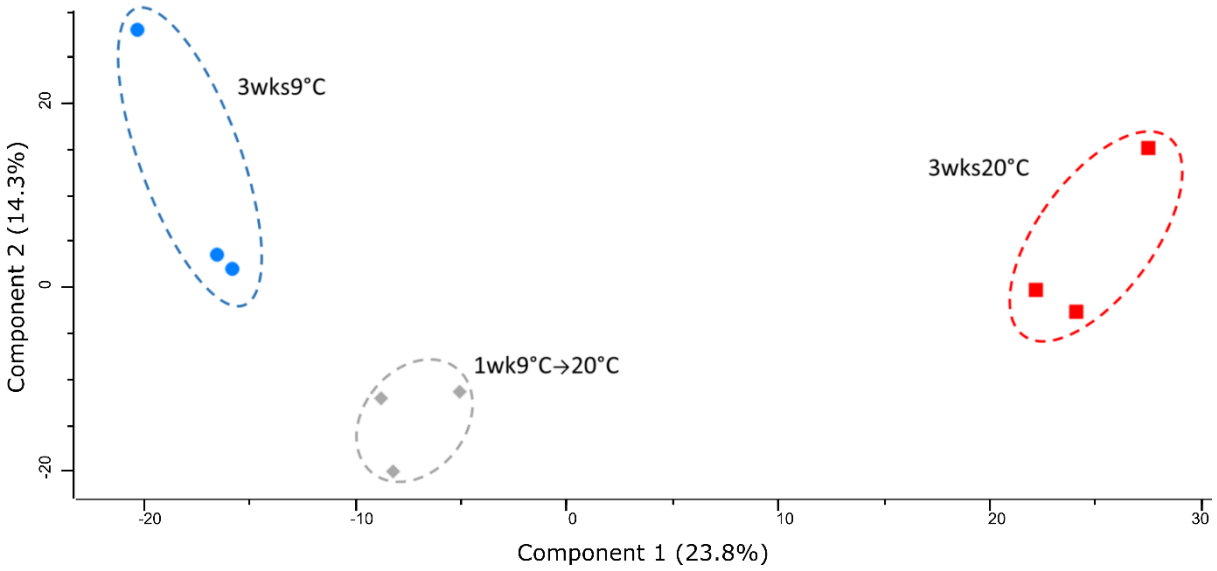



**Figure 4.6:** PCA of proteins from *S. carpocapsae* IJs stored at 9°C for 3 weeks (blue circles), at 9°C for 1 week and then swapped to 20°C for 2 weeks (grey diamonds), and at 20°C for 3 weeks (red squares).

In total, 2359 proteins were detected in the *S. carpocapsae* IJs, 36 of which were identified as statistically significant in a one-way ANOVA (Benjamini Hochberg FDR 0.01). These 36 proteins were grouped by unsupervised hierarchical clustering in Perseus, as shown in the heat map (a visualisation of the high and low abundance proteins present in the IJs; figure 4.7) with details shown in table 4.1.

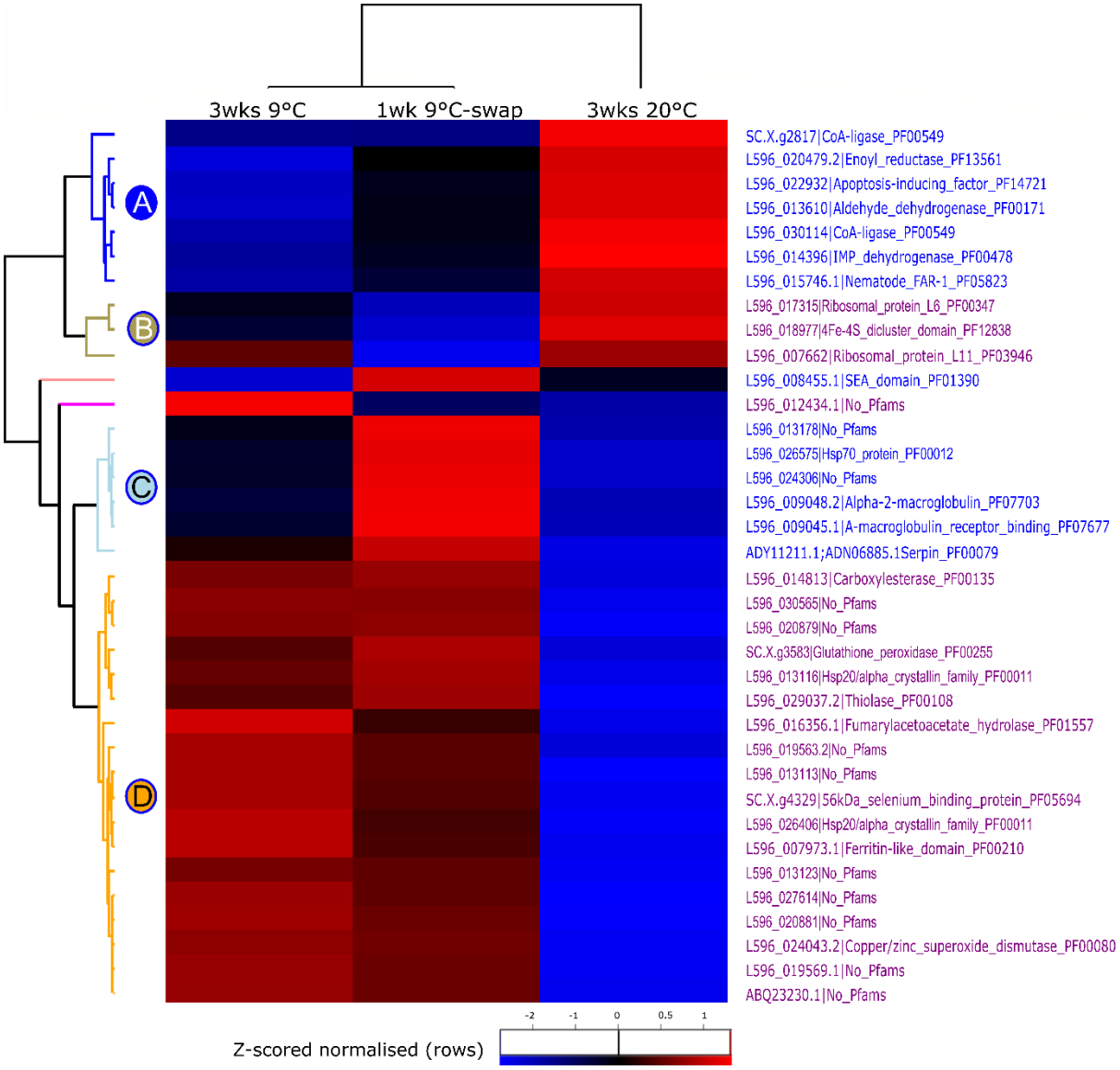



**Figure 4.7:** Heat map of *Steinernema carpocapsae* All statistically significant proteins: Two-way unsupervised hierarchical clustering of the median Z-score normalised label-free quantification (LFQ) intensity values of all statistically significant proteins IJs stored at 9°C for 3 weeks (left), at 9°C for 1 week and then swapped to 20°C for 2 weeks (middle), and at 20°C for 3 weeks (right). Differences in protein abundance are indicated by colour changes from low (blue) to high (red) protein abundance representative of changes in Z-score normalised $log_2$fold transformed LFQ intensity values.

Cluster D consists of proteins which are detected at high abundance in the IJs stored at 9°C and in the temperature swapped IJs (1wk9°C-20°C), and low abundance in 20°C stored IJs. This cluster is the largest of those detected, and consists primarily of molecular chaperones, such as heat shock proteins (HSPs) and late embryogenesis abundant (LEA) proteins, and stress proteins such as glutathione peroxidase and copper superoxide dismutase involved in reactive oxygen species protection.

Clusters A and B are comprised of 10 proteins, which show the reverse pattern to cluster D: being detected in increased abundance at 20°C and decreased abundance in both the 9°C, and temperature swapped IJs. Cluster A contains proteins related to fat binding and stress proteins while Cluster B contains proteins which related to the ribosome.

Cluster C consists of proteins which are highest in the temperature swapped IJs, lowest in the 20°C stored IJs, and intermediate in the 9°C stored IJs. This cluster contains a heat shock protein, a serine protease inhibitor, macroglobulin related proteins and various hypothetical proteins (table 4.1). There were 2 "clusters" which contained 1 protein each, without a clear function (table 4.1).

**Table 4.1:** Proteins identified in two-way unsupervised hierarchical clustering of the median Z-score normalised label-free quantification (LFQ) intensity values of all statistically significant proteins (Benjamini-Hochberg false discovery rate 0.01)

| Z-scored abundance | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 3wks9°C | 1wk9°C→20°C | 3wks20°C | Cluster | ID | Blast annotation | Peptides | Mol. weight [kDa] | Intensity | MS/MS count |
| -0.72 | -0.69 | 1.27 | A | SC.X.g2817 | Succinate-coa ligase, alpha subunit | 15 | 34.181 | 3.52E+09 | 229 |
| -1.19 | 0.01 | 1.10 | A | L596_020479.2 | Hypothetical protein L596_020479 | 7 | 28.93 | 2.87E+08 | 49 |
| -1.03 | -0.15 | 1.14 | A | L596_022932 | Programmed cell death 8 | 10 | 70.283 | 2.46E+08 | 65 |
| -1.07 | -0.11 | 1.15 | A | L596_013610 | Aldehyde dehydrogenase | 29 | 57.781 | 3.30E+09 | 297 |
| -0.92 | -0.11 | 1.26 | A | L596_030114 | Succinate--coa ligase [GDP-forming] subunit beta, mitochondrial | 7 | 46.012 | 2.96E+08 | 58 |
| -0.84 | -0.17 | 1.34 | A | L596_014396 | Inosine-5'-monophosphate dehydrogenase 1 | 7 | 34.475 | 3.51E+08 | 55 |
| -0.89 | -0.29 | 1.09 | A | L596_015746.1 | Fatty acid and retinol-binding protein | 11 | 21.296 | 1.67E+09 | 90 |
| -0.14 | -1.01 | 1.06 | B | L596_017315 | 60S ribosomal protein L9 | 11 | 21.254 | 2.93E+09 | 122 |
| -0.27 | -1.11 | 1.16 | B | L596_018977 | Putative NADH-quinone oxidoreductase subunit I | 7 | 23.184 | 1.39E+09 | 59 |
| 0.49 | -1.28 | 0.82 | B | L596_007662 | 60S ribosomal protein L12 | 1 | 20.089 | 2.23E+09 | 63 |
| -1.12 | 1.10 | -0.17 | X | L596_008455.1 | SEA domain containing protein | 5 | 84.611 | 3.38E+08 | 52 |
| 1.28 | -0.52 | -0.90 | Y | L596_012434.1 | Hypothetical protein L596_012434 | 3 | 51.288 | 2.26E+08 | 18 |
| -0.13 | 1.23 | -0.92 | C | L596_013178 | Hypothetical protein L596_013178 | 6 | 18.033 | 9.70E+07 | 21 |
| -0.22 | 1.18 | -1.08 | C | L596_026575 | Heat shock protein | 25 | 72.459 | 8.42E+09 | 342 |
| -0.21 | 1.21 | -1.08 | C | L596_024306 | Hypothetical protein L596_024306 | 8 | 33.817 | 6.07E+08 | 62 |
| -0.32 | 1.24 | -0.97 | C | L596_009048.2 | Alpha-2-macroglobulin family protein | 30 | 86.756 | 2.28E+09 | 301 |
| -0.25 | 1.25 | -1.00 | C | L596_009045.1 | A-macroglobulin complement component | 21 | 99.788 | 7.21E+08 | 127 |
| 0.15 | 1.02 | -1.24 | C | ADY11211.1 | Serine proteinase inhibitor | 22 | 43.674 | 2.30E+09 | 203 |
| 0.63 | 0.78 | -1.19 | D | L596_014813 | Carboxylesterase, type B domain-containing protein | 34 | 77.629 | 8.35E+09 | 432 |
| 0.72 | 0.70 | -1.29 | D | L596_030565 | Hypothetical protein L596_030565 | 5 | 14.902 | 7.41E+08 | 59 |
| 0.69 | 0.73 | -1.35 | D | L596_020879 | LEA5 protein | 3 | 7.4813 | 8.29E+08 | 28 |
| 0.44 | 0.89 | -1.17 | D | SC.X.g3583 | Glutathione peroxidase | 7 | 26.194 | 1.16E+10 | 119 |
| 0.52 | 0.84 | -1.27 | D | L596_013116 | Hypothetical protein L596_013116 | 7 | 18.748 | 1.62E+09 | 62 |
| 0.44 | 0.81 | -1.37 | D | L596_029037.2 | Acetyl-coa C-acetyltransferase | 11 | 42.561 | 5.83E+08 | 92 |
| 1.08 | 0.28 | -1.27 | D | L596_016356.1 | CRE-FAH-1 protein | 13 | 46.228 | 1.77E+09 | 118 |
| 0.85 | 0.46 | -1.17 | D | L596_019563.2 | Hypothetical protein L596_019563 | 1 | 17.438 | 2.27E+09 | 102 |
| 0.86 | 0.46 | -1.38 | D | L596_013113 | Hypothetical protein L596_013113 | 3 | 15.175 | 4.95E+08 | 22 |
| 0.87 | 0.42 | -1.31 | D | SC.X.g4329 | Selenium-binding protein 1 | 15 | 50.706 | 8.80E+08 | 115 |
| 0.95 | 0.35 | -1.36 | D | L596_026406 | CRE-HSP-12.1 protein | 5 | 23.854 | 1.44E+09 | 73 |
| 0.96 | 0.39 | -1.29 | D | L596_007973.1 | Ferritin-like protein | 11 | 20.251 | 6.96E+09 | 130 |
| 0.61 | 0.49 | -1.32 | D | L596_013123 | Hypothetical protein L596_013123 | 5 | 35.902 | 8.98E+09 | 195 |
| 0.80 | 0.50 | -1.38 | D | L596_027614 | Hypothetical protein L596_027614 | 5 | 14.179 | 4.55E+08 | 44 |
| 0.82 | 0.54 | -1.37 | D | L596_020881 | LEA5 protein | 2 | 10.094 | 6.11E+08 | 20 |
| 0.73 | 0.59 | -1.31 | D | L596_024043.2 | Extracellular superoxide dismutase [Cu-Zn] | 5 | 18.899 | 2.41E+09 | 57 |
| 0.79 | 0.55 | -1.31 | D | L596_019569.1 | Hypothetical protein L596_019569 | 42 | 68.331 | 7.08E+09 | 370 |
| 0.77 | 0.55 | -1.28 | D | ABQ23230.1 | LEA1 protein | 5 | 9.7546 | 5.25E+09 | 75 |

### 4.3.6 Highest and lowest abundance proteins

Volcano plots were used to identify proteins which were present in the highest and lowest abundances amongst each cohort of IJs. Statistically significant ($p<0.05$ t test) annotated proteins which were at least fivefold increased or decreased in abundance between the 9°C stored IJs, and the temperature swapped IJs compared to the 20°C IJs (table 4.2). The highest abundance proteins identified in both the 9°C and temperature-swapped treatments were chaperone proteins, such as CRE-HSP and LEA proteins which were increased between 140.5-fold and 13.4-fold higher than in the IJs at 20°C for 3 weeks. Various enzymes were decreased in abundance relative to the 20°C conditioned IJs including chitinase which was 25.6-39.2 times lower in the 9°C conditioned IJs (table 4.2). There were a number of proteins identified as SSDAs which were not annotated (appendix 4.2), and these proteins may have important roles in temperature adaptation.

**Table 4.2:** Annotated Statistically significant proteins fivefold changed in abundance in IJs stored at 9°C for 3 weeks and those stored at 9°C for 1 week and transferred to 20°C, compared to IJs stored at 20°C for 3 weeks. Proteins which were identified but could not be annotated were excluded but can be found in appendix 4.2.

| Protein ID | Annotation | Fold change vs 3wks20°C | | Peptides | Mol. Weight | Intensity | MS/MS Count |
|---|---|---|---|---|---|---|---|
| | | 3wks9°C | 1wk9°C→20°C | | | | |
| L596_026406 | CRE-HSP-12.1 protein | 140.5 | 35.9 | 5 | 23.854 | 1.44E+09 | 73 |
| L596_020879 | LEA5 protein | 93.6 | 95.8 | 3 | 7.4813 | 8.29E+08 | 28 |
| L596_020881 | LEA5 protein | 61.2 | 33.5 | 2 | 10.094 | 6.11E+08 | 20 |
| L596_015330 | SaPosin-like Protein family | 60.0 | 43.9 | 4 | 10.214 | 1.16E+09 | 37 |
| L596_019565.1 | LEA2 protein | 56.8 | na | 4 | 10.539 | 2.20E+09 | 45 |
| L596_012217 | PhosphoGlycolate Phosphatase Homolog | 23.4 | na | 5 | 38.207 | 2.28E+08 | 18 |
| L596_024791 | Cystathionine beta-synthase | 17.9 | na | 16 | 77.905 | 1.31E+08 | 43 |
| ABQ23230.1 | LEA1 protein | 17.1 | 13.4 | 5 | 9.7546 | 5.25E+09 | 75 |
| SC.X.g2587.2 | Putative cystathionine gamma-lyase 2 | 7.4 | 8.7 | 5 | 42.673 | 1.07E+08 | 30 |
| SC.X.g3323 | Myosin regulatory light chain 1 | 7.3 | na | 4 | 18.941 | 8.40E+07 | 17 |
| L596_019840 | C. briggsae CBR-OSM-11 protein | 7.2 | na | 3 | 28.814 | 2.74E+08 | 22 |
| SC.X.g3305 | C-1-tetrahydrofolate synthase, cytoplasmic | 6.8 | na | 8 | 102.42 | 1.34E+08 | 48 |
| L596_030616 | Protein LSM12 -like protein A | 6.8 | 3.2 | 3 | 24.9 | 4.87E+07 | 17 |
| L596_017887.4 | ADP-ribose pyrophosphatase, mitochondrial precursor | 5.7 | 5.7 | 4 | 17.112 | 1.22E+08 | 28 |
| L596_024012 | thiamin pyrophosphokinase | na | 45.7 | 9 | 30.405 | 2.58E+08 | 43 |
| L596_028041 | Serine/threonine-protein phosphatase PP1-alpha | na | 7.0 | 2 | 19.921 | 1.13E+08 | 11 |
| SC.X.g1861 | Ani s 9 allergen precursor | na | 5.3 | 2 | 14.755 | 9.04E+07 | 13 |
| L596_026200 | Ancylostoma secreted protein | na | -5.6 | 3 | 29.665 | 1.94E+08 | 26 |
| SC.X.g6035 | Ras protein let-60 | na | -11.5 | 4 | 21.122 | 1.06E+08 | 15 |
| L596_019533 | medium-chain specific acyl-CoA dehydrogenase, mitochondrial | -5.2 | na | 7 | 45.266 | 5.91E+07 | 27 |
| L596_028677 | piwi domain protein | -6.0 | na | 5 | 100 | 5.03E+07 | 20 |
| L596_017378 | trypsin-like serine protease | -6.8 | -4.5 | 3 | 32.864 | 1.34E+08 | 22 |
| L596_018471 | acetyl-Coenzyme A synthetase 2, putative | -6.9 | -4.0 | 4 | 78.513 | 7.81E+07 | 21 |
| SC.X.g5447 | probable H/ACA ribonucleoprotein complex subunit 1-like protein | -8.2 | -5.0 | 4 | 24.466 | 1.50E+08 | 23 |
| SC.X.g3201 | Short-chain dehydrogenase/reductase | -8.2 | -3.6 | 2 | 29.235 | 7.42E+07 | 14 |
| L596_023970 | NAC domain containing protein | -11.8 | na | 4 | 21.879 | 1.57E+08 | 31 |
| L596_015314 | chitinase class I | -39.2 | -25.6 | 9 | 101.39 | 3.12E+08 | 33 |

## 4.4 Discussion

Exposure to low temperatures had profound effects on the chemotaxis and stress tolerance of *S. carpocapsae* IJs, similar to previous findings (Lee et al., 2016; Jagdale and Grewal, 2007). We also showed that these changes were accompanied by major proteomic reorganisation and that the changes in chemotaxis, stress tolerance and proteome induced by low-temperature storage were largely maintained on return to culture temperature for two weeks. These findings have implications for how we understand the seasonal adaptations of these nematodes in their natural environment, but also how cold storage of mass-produced nematodes may impact their subsequent behaviour and survival following application for biocontrol purposes.

Conditioning at 9°C for 1- or 3-weeks enhanced *S. carpocapsae* IJs' tolerance to both freezing and desiccation. Cold acclimation is known to enhance the survival of nematodes, including *Steinernema* IJs, at sub-zero temperatures (Smith et al., 2008; Ali and Wharton., 2013; Brown and Gaugler., 1996), and there are also reports of cold acclimation enhancing desiccation tolerance (Jagdale and Grewal, 2007). Freezing and desiccation are closely linked environmental stressors for nematodes, both in the nature of the stress and the adaptations involved, with considerable cross-tolerance between the two stresses (Adhikari et al., 2010). The protective effect of cold acclimation has been attributed in part to the accumulation of trehalose and other low molecular weight polyols which play a protective role (Jagdale and Grewal 2007; Ali and Wharton 2015), while stress proteins are also important (Seybold et al., 2017; Phadtare et al., 1999). Recently, Wang et al. (2021) reported widespread transcriptional programming in eggs of the plant parasite *Meloidogyne incognita* acclimated at 4°C, including genes involved in lipid and carbohydrate metabolism, and also HSPs.

In our study, label free quantitative proteomics was used to compare proteins in cold-acclimated IJs (3 weeks at 9°C) with those maintained at the culture temperature (3 weeks at 20°C) as well as those exposed to 9°C for 1 week and transferred to 20°C for 2 weeks. The proteins which exhibited the most activity in the IJs stored at 9°C for 3 weeks or transferred to 20°C were chaperone proteins. The highest abundance chaperone was CRE-HSP, a sHSP found in *Caenorhabditis remanei.* Members of the sHSP family enhance cold and freezing stress tolerance (Sabehat et al., 1998; Pacheco et al., 2009; Wang et al., 2011). High levels of LEA proteins were detected in the 9°C stored IJs and transferred IJs. LEA proteins have been identified in several nematode species (Gal et al., 2004; Goyal et al., 2005; Solomon et al., 2000; Browne et al., 2004) and are disordered molecular chaperones which enhance an organism's survival under freezing stress (Solomon et al., 2000; Browne et al., 2002; NDong et al., 2002; Reyes et al., 2008; Anderson et al., 2015). The increased abundance of LEA proteins and HSPs induced by low temperature storage may also have enhanced *S. carpocapsae* IJs' tolerance to desiccation (Close, 1996; Hand et al 2011; Adhikari et al., 2009; Mizrahi et al 2010).

The protein which was decreased to the greatest extent in cold stored IJs was chitinase, the enzyme responsible for degrading chitin. This protein's abundance was decreased over 25-fold in the IJs in the 9°C stored IJs relative to the IJs stored at 20°C for 3 weeks. Chitinases have been detected in *S. riobrave* infected *G. mellonella* and are speculated to play a role in antifungal activity (Isaacson and Webster, 2002). Fungal infection may pose less of a problem to IJs at low temperatures. Chitinases may also facilitate penetration of the insect cuticle and damages the host, expediting its death (Brandt et al, 1978; Osman et al., 2004; Hao et al., 2012).

The ecological significance of increased stress resistance and associated proteomic remodelling in response to cold is clear: a drop in temperature may indicate the onset of

winter and the increased probability of unfavourable conditions including freezing. The changes in chemotaxis seen in response to cold are less easy to interpret, in particular the reversal of valance in which previously attractive substances become repulsive and vice versa. Similar phenomena were documented in detail by Lee et al. (2016) who showed that both culture and storage temperature affect the olfactory responses of *S. carpocapsae*, including a reversal of valance for several of the tested substances. In our experiments, the initial repulsion of *S. carpocapsae* by prenol and attraction by methyl salicylate and hexanol was completely reversed by one week at 9°C. Hexanol and methyl salicylate are released by plant roots (Roberts et al., 2019) and so the initial attraction might help bring IJs to the rhizosphere and associated insects. Prenol was identified in *G. mellonella* infected with *Steinernema* spp. (Baiocchi et al., 2017), and was highly repulsive to several species of *Steinernema* and to *Heterorhabditis indica* (Kin et al., 2019; Baiocchi et al., 2017; Baiocchi et al., 2019), and so the initial repulsion to prenol could help IJs avoid an already infected insect. When maintained at 20°C, the response of *S. carpocapsae* IJs to these three odours declined gradually, and achieved reversal of valence after 6 weeks in the case of prenol and methyl salicylate. This age-related change is exceeded by that of cold-stored IJs within a week. The attraction of *S. carpocapsae* IJs to prenol with age or after short-term exposure to cold may indicate the adoption of a more risk-prone strategy, with IJs prepared to enter an already infected insect. While infection of an already-occupied host lowers fitness as a result of competition (Koppenhöfer and Kaya, 1996; Ryder and Griffin, 2003; Blanco-Pérez et al., 2019), the increased protection from freezing and desiccation a cadaver confers may offset those costs (Lewis and Shapiro-Ilan., 2002; Perez et al., 2003). Similarly, as an older IJ fails to find on uninfected host, invading an already occupied host may be preferable to continuing to wait for a fresh host. However, while prenol is an odorant given off by *Steinernema*-infected hosts, an infected host will

likely release a complex variety of volatile and non-volatile chemicals, and an IJ's decision to infect will likely be the product of the blend rather than individual constituents.

Intriguingly, the changes in stress resistance, protein expression and olfaction induced in *S. carpocapsae* by low temperature exposure were not reversed following return to the culture temperature. IJs which were exposed to 9°C for 1 week retained their enhanced freezing and desiccation resistance following storage at 20°C for 2 weeks, and the proteome of these temperature shifted IJs was more similar to that of IJs maintained at 9°C than to that of IJs kept at 20°C. Similarly, a return of 9°C-stored IJs to 20°C did not revert their cold-induced attraction towards prenol back to the original repulsion. Just 3 hours at 9°C induced a significant change in response to prenol, from strong repulsion to ambivalence when IJs were tested immediately, and these IJs showed a strong attraction towards prenol when tested after 3 weeks at 20°C. This shows that even a brief exposure to 9°C can have long term effects on the chemotaxis response of IJs, and suggests that the reversal of valence that was triggered by 3 hours in the cold was completed during the subsequent period of warm storage. Such rapid shifts in response valence are indicative of neuromodulatory changes rather than synaptic rewiring (Guillermin et al., 2017). In contrast to our findings with prenol, Lee et al (2016) found that the alteration in olfactory response induced in of *S. carpocapsae* IJs by a drop in temperature was reversed following return to the culture temperature. This may reflect differences in the temperatures used: 25° and 15°C in Lee at al. (2016) compared to 20° and 9°C in our experiments – as well as the specific odorants tested. In our assays, the maintenance of the cold-induced response following return to warm conditions was more clear-cut and distinct for prenol than for the other odours tested. Moreover, other factors such as age of the nematodes at the time of temperature shift can affect their olfactory plasticity (Lee et al., 2016).

Like *S. carpocapsae*, *H. megidis* IJs were initially repelled by prenol and attracted (though weakly) by hexanol and methyl salicylate. However, in contrast to *S. carpocapsae*, the response of *H. megidis* IJs to odours was generally accentuated rather than reversed by storage at 9°C, and cold-induced alterations were not retained by IJs after transfer to 20°C. The two species also differed in the way that different storage temperatures affected their olfactory response: while for *S. carpocapsae*, all storage temperatures lower than the culture temperature of 20°C had a similar effect on olfaction, resulting in a reversal of valence, the effect of different storage temperatures on *H. megidis* IJs was more graded: IJs stored at 12°C and 15°C exhibited an intermediate response between IJs stored at 20°C or 9°C. As for *H. megidis* in our study, a shift to a lower temperature did not result in a change in valance of *H. bacteriophora* IJs (Lee et al., 2016).

Our results support the conclusion of the more comprehensive study by Lee et al. (2016) that plasticity in olfactory responses dependent on temperature and age is a broadly conserved trait across species of entomopathogenic nematodes, but that the exact nature of the changes varies between species and odorants. However, the ecological relevance of these changes remains to be understood. The behaviour of animals in response to single compounds in controlled laboratory assays may not be indicative of their response to blends of volatile and non-volatile chemicals encountered in the field (e.g., Webster et al., 2010). For IJs, the universally produced $CO_2$ is the most important odour (Hallem et al., 2011; Dillman et al., 2012) and this remained attractive to most of the EPN species tested including *S. carpocapsae* and *H. bacteriophora*, irrespective of culture and storage conditions (Lee et al., 2016). Responses to live or infected insects and associated cues are the ultimate measure of how the age and the prior temperature conditions affect IJs in way that is both ecologically relevant and of importance to biocontrol. While the ability of IJs to find and kill insects generally declines with age (Lewis et al., 1995; Patel et al., 1997),

there is also evidence of more complex effects of age and prior temperature conditions. For *H. megidis*, conditions similar to those used in the present study have been shown to profoundly influence infectivity (a compound trait of movement towards and entry into insects), which increased with age, especially in IJs maintained at 9°C (Griffin, 1996; Fitters et al., 2001). Similar effects have been documented for steinernematids (Guy et al., 2017; Koppenhöfer et al., 2013; Yadav and Eleftherianos, 2018). Host finding and infection in soil involve a series of steps, including dispersal, host-finding and host-recognition and acceptance (Griffin 2015; Lewis et al., 1995). Odour blends may increase the probability of IJs finding an insect but undirected dispersal and attraction to $CO_2$ will result in many IJs arriving at it anyway, after which the decision is made as to whether to infect. A decreased responsiveness to host volatiles during cold storage of heterorhabditids and steinernematids can occur concomitantly with an increase infectivity, which possibly indicates increased tendency to penetrate into a host following arrival (Dempsey and Griffin, 2002; Koppenhöfer et al., 2013).

While alterations in response to any specific odorant may be difficult to interpret meaningfully in an ecological context, they are indicative of profound physiological changes brought about by temperature and by age as evidenced also in the proteomic data (present study and Lillis et al. 2022; Chapter 3). Moreover, it is clear that assays of chemo-attraction reflect the current status of the IJs, a product of their age, culture and storage conditions (present study; Lee et al. 2016) as well as learning (Willett et al., 2015). This extreme plasticity of olfactory responses needs to be taken into account in defining the role of specific volatiles for a species or strain of EPN.

## 4.5 Conclusion

Storage of *S. carpocapsae* and *H. megidis* IJs at temperatures lower than culture temperature affected each species in profoundly different ways. *S. carpocapsae* IJs underwent reversals of valence in olfactory response while *H. megidis* IJs' chemotaxis response to odours was generally intensified by exposure to low temperatures. Together with the proteomic profiling of Lillis et al., 2022 (Chapter 3), it appears that while both species are affected by storage time and temperature, *H. megidis* undergoes more gradual changes, in contrast to the dramatic changes demonstrated for *S. carpocapsae* when placed at 9°C. The proteome of *S. carpocapsae* IJs exhibited extensive remodelling upon exposure to 9°C and these changes were retained for weeks after their transfer to 20°C, as were changes in stress tolerance and chemotaxis. The most dramatic changes were found in chaperone proteins such as HSPs and LEA proteins which improve organisms' survival of freezing and desiccation. The demonstration that *S. carpocapsae* IJs' altered olfactory responses and enhanced stress resistance induced by brief exposure to low temperature can be retained for weeks after their return to higher temperatures, has implications both for laboratory testing and for their use as biocontrol agents. Moreover, induction in a nematode by brief cold exposure of profound changes that are maintained following the return of favourable conditions has resonances with diapause and may share some common pathways with these little understood phenomena.

## References

ADHIKARI, B. N., WALL, D. H. & ADAMS, B. J. 2009. Desiccation survival in an Antarctic nematode: molecular analysis using expressed sequenced tags. *BMC Genomics,* 10**,** 69.

ADHIKARI, B. N., WALL, D. H. & ADAMS, B. J. 2010. Effect of slow desiccation and freezing on gene transcription and stress survival of an Antarctic nematode. *Journal of Experimental Biology,* 213**,** 1803-1812.

ALI, F. & WHARTON, D. 2015. Infective Juveniles of the Entomopathogenic Nematode, *Steinernema feltiae* Produce Cryoprotectants in Response to Freezing and Cold Acclimation. *PLOS ONE,* 10**,** e0141810.

ALI, F. & WHARTON, D. A. 2013. Cold tolerance abilities of two entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *Cryobiology,* 66**,** 24-29.

ANDERSON, D., FERRERAS, E., TRINDADE, M. & COWAN, D. 2015. A novel bacterial Water Hypersensitivity-like protein shows in vivo protection against cold and freeze damage. *FEMS Microbiology Letters,* 362.

BAIOCCHI, T., BRAUN, L. & DILLMAN, A. R. 2019. Touch-stimulation increases host-seeking behavior in *Steinernema carpocapsae*. *Journal of Nematology,* 51**,** 1-5.

BAIOCCHI, T., LEE, G., CHOE, D.-H. & DILLMAN, A. R. 2017. Host seeking parasitic nematodes use specific odors to assess host resources. *Scientific Reports,* 7**,** 6270.

BARGMANN, C. I., HARTWIEG, E. & HORVITZ, H. R. 1993. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell,* 74**,** 515-527.

BLANCO-PÉREZ, R., BUENO-PALLERO, F., VICENTE-DÍEZ, I., MARCO-

MANCEBÓN, V. S., PÉREZ-MORENO, I. & CAMPOS-HERRERA, R. 2019. Scavenging behavior and interspecific competition decrease offspring fitness of the entomopathogenic nematode *Steinernema feltiae*. *Journal of Invertebrate Pathology,* 164**,** 5-15.

BLAXTER, M. L., DE LEY, P., GAREY, J. R., LIU, L. X., SCHELDEMAN, P., VIERSTRAETE, A., VANFLETEREN, J. R., MACKEY, L. Y., DORRIS, M., FRISSE, L. M., VIDA, J. T. & THOMAS, W. K. 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature,* 392**,** 71-75.

BRANDT, C. R., ADANG, M. J. & SPENCE, K. D. 1978. The peritrophic membrane: ultrastructural analysis and function as a mechanical barrier to microbial infection in *Orgyia pseudotsugata*. *Journal of Invertebrate Pathology,* 32**,** 12-24.

BROWN, I. M. & GAUGLER, R. 1996. Cold tolerance of steinernematid and heterorhabditid nematodes. *Journal of Thermal Biology,* 21**,** 115-121.

BROWNE, J., TUNNACLIFFE, A. & BURNELL, A. 2002. Plant desiccation gene found in a nematode. *Nature,* 416**,** 38.

BROWNE, J. A., DOLAN, K. M., TYSON, T., GOYAL, K., TUNNACLIFFE, A. & BURNELL, A. M. 2004. Dehydration-specific induction of hydrophilic protein genes in the anhydrobiotic nematode *Aphelenchus avenae*. *Eukaryotic cell,* 3**,** 966-975.

CHAISSON, K. E. & HALLEM, E. A. 2012. Chemosensory behaviors of parasites. *Trends in parasitology,* 28**,** 427-436.

CLOSE, T. J. 1996. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum,* 97**,** 795-803.

COX, J., NEUHAUSER, N., MICHALSKI, A., SCHELTEMA, R. A., OLSEN, J. V. &

MANN, M. 2011. Andromeda: a peptide search engine integrated into the MaxQuant environment. *Journal of Proteome Research,* 10**,** 1794-1805.

CÔTÉ, R. G., GRISS, J., DIANES, J. A., WANG, R., WRIGHT, J. C., VAN DEN TOORN, H. W. P., VAN BREUKELEN, B., HECK, A. J. R., HULSTAERT, N., MARTENS, L., REISINGER, F., CSORDAS, A., OVELLEIRO, D., PEREZ-RIVEVOL, Y., BARSNES, H., HERMJAKOB, H. & VIZCAÍNO, J. A. 2012. The PRoteomics IDEntification (PRIDE) Converter 2 Framework: An Improved Suite of Tools to Facilitate Data Submission to the PRIDE Database and the ProteomeXchange Consortium*. *Molecular & Cellular Proteomics,* 11**,** 1682-1689.

DEMPSEY, C. M. & GRIFFIN, C. T. 2002. Phased activity in *Heterorhabditis* megidis infective juveniles. *Parasitology,* 124**,** 605-613.

DILLMAN, A. R., GUILLERMIN, M. L., LEE, J. H., KIM, B., STERNBERG, P. W. & HALLEM, E. A. 2012. Olfaction shapes host–parasite interactions in parasitic nematodes. *Proceedings of the National Academy of Sciences,* 109**,** E2324.

FAN, X. & HOMINICK, W. Effects of low storage temperature on survival and infectivity of two Steinernerna species (Nematoda : Steinernematidae). *Revue of Nématology.* 14, 407–412.

FITTERS, P. & GRIFFIN, C. 2004. Spontaneous and induced activity of *Heterorhabditis* megidis infective juveniles during storage. *Nematology,* 6**,** 911-917.

FITTERS, P. F. L., DUNNE, R. & GRIFFIN, C. T. 2001. Vine Weevil Control in Ireland with Entomopathogenic Nematodes: Optimal Time of Application. *Irish Journal of Agricultural and Food Research,* 40**,** 199-213.

GAL, T., GLAZER, I. & KOLTAI, H. 2004. A LEA3 family member is involved in

survival of C. *FEBS letters,* 577**,** 21-26.

GOYAL, K., WALTON, L. J. & TUNNACLIFFE, A. 2005. LEA proteins prevent protein aggregation due to water stress. *Biochemical Journal,* 388**,** 151-157.

GRIFFIN, C. 1996. Effects of prior storage conditions on the infectivity of *Heterorhabditis* sp. (Nematoda: Heterorhabditidae). *Fundamental and Applied Nematology,* 19**,** 95-102.

GRIFFIN, C.T. 2015. Behaviour and population dynamics of entomopathogenic nematodes following application, in: Campos-Herrera, R. (Ed.), Nematode pathogenesis of insects and other pests, *Springer, London*, 57-95.

GUILLERMIN, M. L., CARRILLO, M. A. & HALLEM, E. A. 2017. A single set of interneurons drives opposite behaviors in *C. elegans*. *Current Biology,* 27**,** 2630-2639.e6.

GUY, A., GAFFNEY, M., KAPRANAS, A. & GRIFFIN, C. T. 2017. Conditioning the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis* megidis by pre-application storage improves efficacy against black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) at low and moderate temperatures. *Biological Control,* 108**,** 40-46.

HALLEM, E. A., DILLMAN, A. R., HONG, A. V., ZHANG, Y., YANO, J. M., DEMARCO, S. F. & STERNBERG, P. W. 2011. A sensory code for host seeking in parasitic nematodes. *Current Biology,* 21**,** 377-83.

HAND, S. C., MENZE, M. A., TONER, M., BOSWELL, L. & MOORE, D. 2011. LEA proteins during water stress: not just for plants anymore. *Annual Review of Physiology,* 73**,** 115-134.

HAO, Y. J., MONTIEL, R., LUCENA, M. A., COSTA, M. & SIMOES, N. 2012. Genetic

diversity and comparative analysis of gene expression between *Heterorhabditis bacteriophora* Az29 and Az36 isolates: uncovering candidate genes involved in insect pathogenicity. *Experimental Parasitology,* 130**,** 116-125.

HUBNER, N. C., BIRD, A. W., COX, J., SPLETTSTOESSER, B., BANDILLA, P., POSER, I., HYMAN, A. & MANN, M. 2010. Quantitative proteomics combined with BAC TransgeneOmics reveals in vivo protein interactions. *Journal of Cell Biology,* 189**,** 739-754.

ISAACSON, P. J. & WEBSTER, J. M. 2002. Antimicrobial activity of Xenorhabdus sp. RIO (Enterobacteriaceae), symbiont of the entomopathogenic nematode, *Steinernema riobrave* (Rhabditida: Steinernematidae). *Journal of Invertebrate Pathology,* 79**,** 146-153.

JAGDALE, G. B. & GREWAL, P. S. 2007. Storage temperature influences desiccation and ultra violet radiation tolerance of entomopathogenic nematodes. *Journal of Thermal Biology,* 32**,** 20-27.

KAPLAN, F., PERRET-GENTIL, A., GIURINTANO, J., STEVENS, G., ERDOGAN, H., SCHILLER, K. C., MIRTI, A., SAMPSON, E., TORRES, C., SUN, J., LEWIS, E. E. & SHAPIRO-ILAN, D. 2020. Conspecific and heterospecific pheromones stimulate dispersal of entomopathogenic nematodes during quiescence. *Scientific Reports,* 10**,** 5738.

KIN, K., BAIOCCHI, T. & DILLMAN, A. R. 2019. Dispersal and Repulsion of Entomopathogenic Nematodes to Prenol. *Biology,* 8.

KOPPENHÖFER, A. M., EBSSA, L. & FUZY, E. M. 2013. Storage temperature and duration affect *Steinernema scarabaei* dispersal and attraction, virulence, and infectivity to a white grub host. *Journal of Invertebrate Pathology,* 112**,** 129-137.

KOPPENHÖFER, A. M. & KAYA, H. K. 1996. Coexistence of two steinernematid nematode species (Rhabditida: Steinernematidae) in the presence of two host species. *Applied Soil Ecology,* 4**,** 221-230.

LEE, J. H., DILLMAN, A. R. & HALLEM, E. A. 2016. Temperature-dependent changes in the host-seeking behaviors of parasitic nematodes. *BMC Biology,* 14**,** 36.

LEWIS, E. & SHAPIRO-ILAN, D. 2002. Host cadavers protect entomopathogenic nematodes during freezing. *Journal of invertebrate pathology,* 81**,** 25-32.

LEWIS, E. E., GREWAL, P. S. & GAUGLER, R. 1995a. Hierarchical order of host cues in parasite foraging strategies. *Parasitology,* 110**,** 207-213.

LEWIS, E. E., SELVAN, S., CAMPBELL, J. F. & GAUGLER, R. 1995b. Changes in foraging behaviour during the infective stage of entomopathogenic nematodes. *Parasitology,* 110**,** 583-590.

LILLIS, P., GRIFFIN, C. AND CAROLAN, J. 2022. The effect of temperature conditioning (9°C and 20°C) on the proteome of entomopathogenic nematode infective juveniles. *PLOS ONE,* 17(4), p.e0266164.

MIZRAHI, T., HELLER, J., GOLDENBERG, S. & ARAD, Z. 2010. Heat shock proteins and resistance to desiccation in congeneric land snails. *Cell stress & chaperones,* 15**,** 351-363.

NDONG, C., DANYLUK, J., WILSON, K.E., POCOCK, T., HUNER, N.P. & SARHAN, F. 2002. Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. *Plant Physiology.* 129, 1368-1381

O'HALLORAN, D. M. & BURNELL, A. M. 2003. An investigation of chemotaxis in the insect parasitic nematode *Heterorhabditis bacteriophora*. *Parasitology,* 127**,** 375-

385.

O'LEARY, S. A., STACK, C. M., CHUBB, M. A. & BURNELL, A. M. 1998. The effect of day of emergence from the insect cadaver on the behavior and environmental tolerances of infective juveniles of the entomopathogenic nematode *Heterorhabditis megidis* (strain UK211). *Journal of Parasitology,* 84**,** 665-672.

OSMAN, G., HUSSEIN, E. & ABDALLAH, N. 2004. Characterization and Purification of a Chitinolytic Enzyme Active against *Sesamia cretica* (pink borer). Arab. *Journal of Biotechnology,* 7**,** 65-74.

PACHECO, A., PEREIRA, C., ALMEIDA, M. J. & SOUSA, M. J. 2009. Small heat-shock protein Hsp12 contributes to yeast tolerance to freezing stress. *Microbiology (Reading),* 155**,** 2021-2028.

PATEL, M. N., STOLINSKI, M. & WRIGHT, D. J. 1997. Neutral lipids and the assessment of infectivity in entomopathogenic nematodes: observations on four *Steinernema* species. *Parasitology,* 114**,** 489-496.

PEREZ, E. E., LEWIS, E. E. & SHAPIRO-ILAN, D. I. 2003. Impact of the host cadaver on survival and infectivity of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) under desiccating conditions. *Journal of Invertebrate Pathology,* 82**,** 111-118.

LILLIS, P. E., CAROLAN, J. C. & GRIFFIN, C. T. 2021. The Effect of Temperature Conditioning (9°C and 20°C) on the Proteome of *Steinernema carpocapsae a*nd H*eterorhabditis megidis* Infective Juveniles. *Unpublished manuscript.*

PHADTARE, S., ALSINA, J. & INOUYE, M. 1999. Cold-shock response and cold-shock proteins. *Current Opinion in Microbiology,* 2**,** 175-180.

POINAR, G. 1993. Origins and phylogenetic relationships of the entomophilic rhabditids,

*Heterorhabditis* and Steinernema. *Fundamental & Applied Nematology*, 16.

REYES, J. L., CAMPOS, F., WEI, H., ARORA, R., YANG, Y., KARLSON, D. T. & COVARRUBIAS, A. A. 2008. Functional dissection of hydrophilins during in vitro freeze protection. *Plant, Cell & Environment,* 31**,** 1781-1790.

ROBERTS, J. M., KUNDUN, J., ROWLEY, C., HALL, D. R., DOUGLAS, P. & POPE, T. W. 2019. Electrophysiological and Behavioral Responses of Adult Vine Weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), to Host Plant Odors. *Journal of Chemical Ecology,* 45**,** 858-868.

RYDER, J. J. & GRIFFIN, C. T. 2003. Phased infectivity in *Heterorhabditis megidis*: the effects of infection density in the parental host and filial generation. *International Journal for Parasitology*, 33**,** 1013-1018.

SABEHAT, A., LURIE, S. & WEISS, D. 1998. Expression of small heat-shock proteins at low temperatures. A possible role in protecting against chilling injuries. *Plant physiology,* 117**,** 651-658.

SERRA, L., MACCHIETTO, M., MACIAS-MUÑOZ, A., MCGILL, C. J., RODRIGUEZ, I. M., RODRIGUEZ, B., MURAD, R. & MORTAZAVI, A. 2019. Hybrid Assembly of the Genome of the Entomopathogenic Nematode *Steinernema carpocapsae* Identifies the X-Chromosome. *G3 (Bethesda),* 9**,** 2687-2697.

SEYBOLD, A. C., WHARTON, D. A., THORNE, M. A. S. & MARSHALL, C. J. 2017. Investigating trehalose synthesis genes after cold acclimation in the Antarctic nematode *Panagrolaimus* sp. DAW1. *Biology open,* 6**,** 1953-1959.

SMITH, T., WHARTON, D. A. & MARSHALL, C. J. 2008. Cold tolerance of an Antarctic nematode that survives intracellular freezing: comparisons with other nematode species. *Journal of Comparative Physiology B,* 178**,** 93-100.

SOLOMON, A., SALOMON, R., PAPERNA, I. & GLAZER, I. 2000. Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable protein. *Parasitology,* 121**,** 409-416.

WANG, H., LEI, Z., LI, X. & OETTING, R. D. 2011. Rapid Cold Hardening and Expression of Heat Shock Protein Genes in the B-Biotype *Bemisia tabaci*. *Environmental Entomology,* 40**,** 132-139.

WANG, Y., CHEN, Z., YANG, Y. & ZHANG, F. 2021. Transcriptional reprogramming caused by cold acclimation in *Meloidogyne incognita* eggs. *Genes & Genomics,* 43**,** 533-541.

WEBSTER, B., BRUCE, T., PICKETT, J. & HARDIE, J. 2010. Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. *Animal Behaviour,* 79**,** 451-457.

WILLETT, D. S., ALBORN, H. T., DUNCAN, L. W. & STELINSKI, L. L. 2015. social networks of educated nematodes. *Scientific Reports,* 5**,** 14388.

WINSTON, P. W. & BATES, D. H. 1960. Saturated solutions for the control of humidity in biological research. *Ecology,* 41**,** 232-237.

WOODRING, J. L. & KAYA, H. K. 1988. Steinernematid and heterorhabditid nematodes: a handbook of biology and techniques. *Southern cooperative series bulletin (USA).*

YADAV, S. & ELEFTHERIANOS, I. 2018. Prolonged Storage Increases Virulence of *Steinernema* Entomopathogenic Nematodes Toward *Drosophila* Larvae. *Journal of Parasitology,* 104**,** 722-725.

# Chapter 5 General Discussion

Soil, the natural environment for EPN IJs, is a complex, variable and changing environment. In this thesis I have focussed on two soil parameters that are important for IJs: informative chemicals (including those from spent hosts, fresh hosts and possibly each other) and ambient temperature, which profoundly influences the behaviour and survival of the IJs. For commercial use as a biopesticide, EPN are reared in large bioreactors, and IJs are briefly stored at low temperatures prior to formulation. Moreover, they are routinely stored at low temperatures in laboratories. The storage conditions of these IJs may affect their lifespan, freezing tolerance and their behaviour. The objective of this project was to identify how storage conditions affect the behaviour, stress tolerance, and physiology of EPN IJs.

The first step for newly formed IJs is dispersal from the spent cadaver, and signals derived from it may enhance their natural tendency to disperse. When EPN IJs are applied in the field inside a cadaver, their dispersal is greater than those applied in an aqueous medium (Shapiro and Lewis, 1999). It has been speculated that ascaroside pheromones produced by nematodes within the host increase the dispersal of the IJs from the host, thereby reducing competition between emerging IJs, and these signals may accumulate within a cadaver. In Chapter 2, I showed that secretion of ascarosides continues after IJs leave the host, and that the water conditioned by IJs also stimulates dispersal.

IJs of four species of EPN, *S. carpocapsae, S. feltiae, S. longicaudum,* and *H. megidis* were stored in sterile water at high densities at 20°C, and this water was analysed with LC-MS/MS after specific storage times. Ascaroside #9 was the dominant ascaroside detected in all species of EPN, similar to the results found for other insect-associated nematode species including *Bursaphelenchus xylophilus, Oscheius tipulae, Oscheius carolinensis,*

*Tipula paludosa, Pristionchus pacificus, Heterorhabditis bacteriophora* and *Steinernema* spp. (Zhao et al., 2018; Choe et al., 2012b). The presence of high levels of this ascaroside, which is conserved amongst many insect-associated species, may indicate its importance as a chemical signal. The composition and especially the ratio of ascarosides detected was species-specific, and therefore may have species-specific roles. The greatest difference in ascaroside profiles was found between that of the *Steinernema* spp. and *H. megidis. H. megidis* had higher levels of ascaroside 18 than the other species and was the only species in which ascaroside 10 was detected.

In Chapter 2 I also showed that the dispersal of all 4 nematode species increased upon exposure to the worm conditioned water, when compared to their dispersal after an equivalent amount of water was added. This increased dispersal was not species-specific. The dominant ascaroside detected in water conditioned by IJs of all four species was ascr#9, and this signature of insect-associated species has previously been shown to increase the dispersal of EPN IJs (Kaplan et al., 2012). These pheromones may play the same role as acyl homoserine lactones produced by a wide range of bacteria which facilitates quorum sensing and avoiding overcrowding. Cadaver extracts from all 4 species also increased the dispersal of each species, and to an even greater extent than the worm conditioned water. Depleted cadavers may not only contain a build-up of dispersal-inducing ascarosides such as ascr#9, but also chemicals which may facilitate dispersal, such as prenol (Baiocchi et al., 2017) and ammonia by-products (San-Blas et al., 2008; San-Blas et al., 2014).

The worm conditioned water from 4 species of EPN IJs induced dispersal in each species. This water contained high levels of ascarosides, a pheromone which has been shown to increase dispersal in EPN (Kaplan et al., 2012). These ascarosides are now being investigated as potential “boosters” for enhancing the activity of EPN. The species

specificity of the ascaroside profiles of each EPN may indicate a role more specific to each species than the general dispersal shown here. They may play a role as specific cues which facilitate the aggregation of conspecifics to coinfect an insect together, facilitating infection and sexual reproduction, however this would also increase the competition between each individual IJ. Conversely, ascarosides may play a role in recognition of closely related kin, enabling the IJs to avoid inbreeding. A balance between the attraction of conspecifics and the repulsion of closely related IJs may be optimal for these IJs, and the complexity of this task may be mirrored in the complexity of the ascaroside profiles between each species.

Temperature has profound effects on all aspects of nematode biology, influencing development, behaviour, and survival. Most attention focusses on the effects of current temperature on nematode activities. But prior temperatures such as those experienced over time in soil, or during storage, may influence the subsequent behaviour or state of a nematode, and that was the focus of Chapters 3 and 4. Nematode IJs or dauers live for longer if stored at low temperatures than at higher temperatures, which may be attributed to lower activity and therefore lower lipid utilisation at lower temperatures (Andaló et al., 2011), or to lower levels of proteostatic stress (Santra et al., 2019). In Chapter 3 I showed that storage at 9°C and 20°C had different effects on the proteome of two distantly related species of EPN. There was a stark decrease in metabolic related proteins in both *H. megidis* and *S. carpocapsae* when stored at a low temperature (9°C), compared to those kept at 20°C. The only exception were proteins related to gluconeogenesis and glycolysis. Many of these proteins involved in carbon metabolism, such as trehalose-6-phosphate synthase, are involved in the synthesis of trehalose, a sugar which confers freezing and desiccation tolerance to nematodes (Qiu and Bedding, 2002; Ali and Wharton, 2015).

Oxidative phosphorylation produces high levels of energy from stored fats; however, it produces relatively high levels of reactive oxygen species (ROS) as a by-product. High levels of these oxidants affect the survival of nematodes (Santra et al., 2019). As noted above, IJs utilise their lipids more slowly at lower temperatures (Andaló et al., 2011). In both species, there was a general increase in abundance of proteins related to oxidative phosphorylation after storage at 20°C, and a decrease in these proteins after storage at 9°C. Accompanying the decrease of proteins related to oxidative phosphorylation, there was an increase in the levels of catalase and super oxide dismutase in *S. carpocapsae* IJs after storage at 9°C, indicating that these organisms may prioritise mitigating the effects from ROS over energy production in cold conditions.

Protein production is energetically expensive, consuming up to 75% of the cell's energy (Lane and Martin, 2010) which may be costly for the long lived and nonfeeding IJ. Proteins related to translation were decreased in both 9°C and 20°C in *H. megidis* IJs, and to a greater extent over time, and proteins associated with the proteasome were generally increased in abundance over time in both temperatures. The proteasome is involved with the targeted degradation of proteins, which may facilitate the degradation of misfolded or aggregating proteins. Decreased translation (Pan et al., 2007; Hansen et al., 2007) and overexpression of chaperones or protein degradation machinery (Santra., 2019) extends the lifespan of nematodes. There was no evidence of decreased translation in *S. carpocapsae* IJs, as these IJs exhibited a general increase in the abundance of proteins associated with translation in both storage temperatures. Over time, organisms may accumulate misfolded proteins which can form toxic aggregations within the cell. With no apparent decrease in translation, *S. carpocapsae* IJs may be subjected to proteotoxic stress over time. *S. carpocapsae* IJs stored at 9°C displayed hundredfold increases in chaperone proteins, specifically HSPs and LEA proteins. Chaperone proteins attach to and protect proteins

from misfolding or aggregating, especially in stressful situations. These stark differences between the proteomes of these two species may represent a species-specific response to proteostatic collapse in their aging cells.

In Chapter 4, I confirm that the proteome of IJs is strongly affected by temperature and show that after brief exposure to 9°C, 1 week in the case of their proteome and stress tolerance, and as short as 3 hours in the case of their behaviour, affects IJs for weeks after return to 20°C. *S. carpocapsae* IJs stored at low temperatures showed a general reversal in odour preference. IJs stored at 9°C for 3 hours began to become less attracted to odours they were previously attracted to and become attracted to odours they were previously repelled by. This phenomenon was demonstrated for methyl salicylate, acetone, prenol and hexanol. A similar trend for *S. carpocapsae* was found in all temperatures tested below the culture temperature of 20°C (9°C, 12°C, and 15°C). In contrast to these results, *H. megidis* IJs showed an intensification of their preferences, i.e., if they were repelled to prenol when stored at 20°C, they were more strongly repelled by this odour after storage at 9°C. The effect of different temperatures on attraction and repulsion towards prenol and methyl salicylate tended to be graded, whereby the chemotaxis response of the *H. megidis* IJs was gradually intensified by each incremental drop in temperature.

*S. carpocapsae* and *H. megidis* IJs were exposed to low temperatures (9°C) for short timepoints, such as a week, a day and even as brief a period as 3 hours in the case of *S. carpocapsae*, and then these IJs were transferred to 20°C. In the case of *H. megidis,* any change in chemotaxis due to this brief cold exposure were not retained after the IJs were stored at 20°C for the duration of the experiment. In contrast, *S. carpocapsae* IJs retained their cold-induced change in chemotaxis towards prenol for weeks after return to 20°C. Indeed, in the case of the IJs stored at 9°C for just 3 hours, which became indifferent towards the previously repellent prenol after just 3 hours, became intensely attracted

towards the odour after storage at 20°C for 3 weeks. This finding may indicate that the neurological changes which underly the alterations in IJ behaviour are triggered upon exposure to 9°C continue after their return to 20°C. While the data regarding *H. megidis* chemotaxis after exposure to relatively low temperatures match previous studies with *H. bacteriophora* (Lee et al., 2016), the retention of behavioural changes found in *S. carpocapsae* IJs was not previously found for that species by Lee et al. (2016). This may be attributed to differences in the temperatures investigated, 9°C and 20°C in this study compared to 12°C and 25°C in the previous study (Lee et al., 2016). Many other factors may affect this alteration, highlighting the importance of replication and reproduction of experiments in science.

Chemotaxis is one of the primary methods that IJs find new insect hosts. These profound changes in chemotaxis in response to the IJ's ambient temperature, or the temperature they have previously encountered, may help the IJ optimise their foraging tactics. If temperatures are dropping, insects are likely to become less plentiful, especially at the onset of freezing conditions or Winter. In the case of *S. carpocapsae*, becoming repelled by normally attractive odours may prevent the IJ from pursuing insects until warmer conditions in which they can kill the host return. Their sudden attraction to normally repulsive conditions may facilitate coinfection of already infected cadavers. Being in a cadaver enhances freezing tolerance (Lewis and Shapiro-Ilan., 2002) at the cost of reproductive fitness (Blanco-Pérez et al., 2019). *H. megidis* IJs became more attracted to host related odours, and more repelled by repellent odours. This may represent the adoption of a risk prone foraging approach, to infect any insect regardless of quality to survive harsh conditions. While it is tempting to explain the cold-induced changes in chemotaxis as ecologically relevant, as I have done here, it is also possible that they are a by-product of altered thermotactic behaviour, such as migration to warmer regions of the

soil. There is some overlap of neurons which mediate chemotaxis and thermotaxis, and these neurons may "memorise" sensed temperatures (Clark et al., 2006; Kimata et al., 2012).

Cold conditioning at 9°C for just 1 week increased *S. carpocapsae* IJ survival at -10°C from ~30% at week 0 to ~90%. Their desiccation tolerance was enhanced similarly. IJs stored at 9°C for 1 week were transferred to 20°C for 2 weeks retained this tolerance to freezing and desiccation. Low temperature conditioning has previously been shown to enhance desiccation tolerance in nematodes (Jagdale and Grewal, 2003; Jagdale and Grewal, 2007) via cross tolerance. Exposure to moderately stressful conditions induces the accumulation of general protectants such as trehalose (Solomon et al., 2000; Jagdale and Grewal, 2007) and molecular chaperones (Solomon et al., 2000; Chen et al., 2005; Wharton, 2011). In chapter 3 I showed that storage of *S. carpocapsae* IJs at 9°C increases the abundance of trehalose-6-phosphate synthase, an enzyme which facilitates the conversion of sugars to trehalose, the protective molecule. Furthermore, in chapter 4, I showed that *S. carpocapsae* IJs stored at 9°C, whether for 1 week or 3 weeks, had hundredfold times the quantity of HSPs and LEA proteins relative to IJs stored at 20°C for 3 weeks. This substantial increase in chaperone proteins was retained by the IJs which were transferred back to 20°C for 2 weeks after their exposure to 9°C. This considerable increase of chaperone proteins, and their subsequent retention, is likely responsibly for the increased tolerance of both freezing and desiccation tolerance in the *S. carpocapsae* IJs stored at low temperatures and those which were transferred back to 20°C for 2 weeks.

These findings build upon previous work which showed that EPN IJ's chemotaxis towards volatiles were affected by storage temperatures (Lee et al., 2016) and work that shows that storage at low temperatures can enhance IJ's tolerance of freezing (Jagdale and Grewal., 2003; Brown and Gaugler 1996; Ali and Wharton, 2013) and desiccation (Grewal and

Jagdale., 2002; Chen et al., 2005). My findings add that stark changes in the proteome accompanies these phenotypic changes. I investigated the extent to which these changes may occur and how long they may be retained after brief exposure to 9°C. After just 3 hours at 9°C, *S. carpocapsae* exhibited altered chemotaxis behaviours, and during their subsequent storage at 20°C for the remaining 3 weeks these altered behaviours were enhanced. Similarly, storage at 9°C for 1 week had significant effects on the proteome. It has previously been shown that temperature storage can not only affect the behaviour of nematodes such as *C. elegans* (Parida et al., 2014) but can also affect their offspring for generations after (Klosin et al., 2017). These data indicate the importance of controlled conditions in lab trials and experiments. There is often difficulty in the replication and reproducibility of behavioural experiments, and the demonstration of how many seemingly minor factors may affect not only the organisms in an assay but their offspring will encourage efforts to improve reproducibility of these assays through stricter control of storage conditions.

### 5.1.1 Limitations of this research

The research presented here relies primarily on correlation of the behaviours and stress tolerance with the changing proteome and metabolome of EPN. This discovery-based proteomics approach may identify the molecular mechanisms by which EPN's physiology and behaviour may be mediated. This work enables the broad look at almost all proteins in the EPN proteome after specific culture conditions, however it can only corelate the conditions with the resulting proteome.

The lowest temperature looked at in this investigation was 9°C, however IJs are often stored at 4°C in industrial settings to enhance their shelf life. It is possible that this further

drop in temperature may have stronger effects on the IJs and may be retained for longer. In addition, IJs in this study were stored in water when exposed to low temperatures, however the effects of low temperature conditioning on IJs which are formulated in gels or other media was not examined.

### 5.1.2 Future work

Future work may involve the knockout of genes which encode the proteins identified in this study, and identify which proteins identified in this study are essential for survival, and possible targets for genetic screening, breeding or genetic modification. Furthermore, as new genomes are sequenced and as genetic tools become more refined, these data may be reanalysed and elucidate more information.

As mentioned above, the specimens studied here were retained in water while subjected to temperature conditioning, which is not how IJs are typically formulated for field application. Formulated IJs are often maintained in a state of partial anhydrobiosis, with reduced metabolism (Grewal, 2002), and therefore IJs in that state may not be as responsive to their ambient temperature. Comparing the effects of temperature conditioning on IJs stored in water to that of IJs stored in formulations for field application would further the field relevancy of this research. Further studies investigating these factors may enhance the survival, shelf life and efficacy of EPN IJs as biocontrol agents.

Many genes and proteins in nematodes are highly conserved amongst distantly related species. Insect associated parasites are fantastic model organisms for mammal associated parasites as they are cheap, may be produced rapidly, and require fewer ethical considerations. The data presented in this thesis indicates that insect associated nematodes

response drastically to temperature conditioning and in a species-specific manner. These data may be applied to our understanding of mammal parasites in future studies.

### 5.1.3 Conclusion

A greater understanding of the behaviour and physiology of IJs may enhance our use of these insect parasites as biocontrol agents, or as models for parasitism. Specifically, the findings presented here highlights the importance of the storage conditions in which IJs are maintained, influencing their proteome, stress tolerance, and behaviour for weeks after their return to 20°C. EPN IJs may be exposed to low temperatures after in-vitro culture to prolong their shelf-life. My work clearly shows that in addition to storage conditions affecting stress tolerance and proteome of these parasites, their behaviour is also affected, and this behavioural change can be retained for weeks after a return from cold to culture temperature. Furthermore, I have demonstrated the extreme plasticity of both the behaviour and proteome of these economically important parasites.

## References

ADAMS, B. & NGUYEN, K. B. 2002. Taxonomy and Systematics, In: GAUGLER, R. (Ed). Entomopathogenic Nematology. *CABI, Wallingford UK, 1-33*.

ADHIKARI, B. N., LIN, C.-Y., BAI, X., CICHE, T. A., GREWAL, P. S., DILLMAN, A. R., CHASTON, J. M., SHAPIRO-ILAN, D. I., BILGRAMI, A. L., GAUGLER, R., STERNBERG, P. W. & ADAMS, B. J. 2009a. Transcriptional profiling of trait deterioration in the insect pathogenic nematode *Heterorhabditis bacteriophora*. *BMC Genomics,* 10**,** 609.

ADHIKARI, B. N., WALL, D. H. & ADAMS, B. J. 2009b. Desiccation survival in an Antarctic nematode: molecular analysis using expressed sequenced tags. *BMC Genomics,* 10**,** 69.

ADHIKARI, B. N., WALL, D. H. & ADAMS, B. J. 2010. Effect of slow desiccation and freezing on gene transcription and stress survival of an Antarctic nematode. *Journal of Experimental Biology,* 213**,** 1803-1812.

AKHURST, R. & SMITH, K. 2002. Regulation and Safety. In: GAUGLER, R. (Ed). Entomopathogenic Nematology. *CABI, Wallingford UK,* 311-332.

ALVIM, F. C., CAROLINO, S. M., CASCARDO, J. C., NUNES, C. C., MARTINEZ, C. A., OTONI, W. C. & FONTES, E. P. 2001. Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. *Plant physiology,* 126**,** 1042-1054.

ANBESSE, S., SUMAYA, N. H., DÖRFLER, A. V., STRAUCH, O. & EHLERS, R.-U. 2013. Stabilisation of heat tolerance traits in *Heterorhabditis bacteriophora* through selective breeding and creation of inbred lines in liquid culture.

*BioControl,* 58**,** 85-93.

ANDALÓ, V., MOINO, A., MAXIMINIANO, C., CAMPOS, V. & MENDONÇA, L. 2011. Influence of temperature and duration of storage on the lipid reserves of entomopathogenic nematodes. *Revista Colombiana de Entomología,* 37**,** 203-209.

ANDERSON, D., FERRERAS, E., TRINDADE, M. & COWAN, D. 2015. A novel bacterial Water Hypersensitivity-like protein shows in vivo protection against cold and freeze damage. *FEMS Microbiology Letters,* 362.

ARORA, R. 2018. Mechanism of freeze-thaw injury and recovery: A cool retrospective and warming up to new ideas. *Plant Science,* 270**,** 301-313.

BAI, X., ADAMS, B. J., CICHE, T. A., CLIFTON, S., GAUGLER, R., HOGENHOUT, S. A., SPIETH, J., STERNBERG, P. W., WILSON, R. K. & GREWAL, P. S. 2009. Transcriptomic analysis of the entomopathogenic nematode *Heterorhabditis bacteriophora* TTO1. *BMC Genomics,* 10**,** 205.

BAI, X., ADAMS, B. J., CICHE, T. A., CLIFTON, S., GAUGLER, R., KIM, K.-S., SPIETH, J., STERNBERG, P. W., WILSON, R. K. & GREWAL, P. S. 2013a. A Lover and a Fighter: The Genome Sequence of an Entomopathogenic Nematode *Heterorhabditis bacteriophora*. *PLOS ONE,* 8**,** e69618.

BAI, X. D., ADAMS, B. J., CICHE, T. A., CLIFTON, S., GAUGLER, R., KIM, K., SPIETH, J., STERNBERG, P. W., WILSON, R. K. & GREWAL, P. S. 2013b. A Lover and a Fighter: The Genome Sequence of an Entomopathogenic Nematode *Heterorhabditis bacteriophora*. *PLOSE ONE,* 8**,** 13.

BAIMEY, H., ZADJI, L., AFOUDA, L., MOENS, M. & DECRAEMER, W. 2015. Influence of pesticides, soil temperature and moisture on entomopathogenic nematodes from southern Benin and control of underground termite nest

populations. *Nematology,* 17**,** 1057-1069.

BAIOCCHI, T., BRAUN, L. & DILLMAN, A. R. 2019. Touch-stimulation increases host-seeking behavior in *Steinernema carpocapsae*. *Journal of Nematology,* 51**,** 1-5.

BAIOCCHI, T., LEE, G., CHOE, D.-H. & DILLMAN, A. R. 2017. Host seeking parasitic nematodes use specific odors to assess host resources. *Scientific Reports,* 7**,** 6270.

BAL, H. K. & GREWAL, P. S. 2015. Lateral Dispersal and Foraging Behavior of Entomopathogenic Nematodes in the Absence and Presence of Mobile and Non-Mobile Hosts. *PLOS ONE,* 10**,** e0129887.

BAL, H. K., TAYLOR, R. A. & GREWAL, P. S. 2014. Ambush foraging entomopathogenic nematodes employ 'sprinters' for long-distance dispersal in the absence of hosts. *Journal of Parasitology,* 100**,** 422-432.

BARGMANN. C. 2006. Chemosensation in *C. elegans*. *WormBook***,** 1-29.

BEDDING, R. & MOLYNEUX, A. S. 1982. Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp. (Heterorhabditidae: Nematoda). *Nematologica,* 28**,** 354-359.

BEHM, C. A. 1997. The role of trehalose in the physiology of nematodes. *International Journal for Parasitology.* 27, 215-229

BHATTACHARYA, A., AGHAYEVA, U., BERGHOFF, E. G. & HOBERT, O. 2019. Plasticity of the Electrical Connectome of *C. elegans*. *Cell,* 176**,** 1174-1189

BILGRAMI, A., GAUGLER, R., SHAPIRO-ILAN, D. & ADAMS, B. 2006. Source of trait deterioration in entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* during in vivo culture. *Nematology,* 8**,** 397-409.

BINDESBØL, A.-M., HOLMSTRUP, M., DAMGAARD, C. & BAYLEY, M. 2005.

Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. *Environmental Toxicology and Chemistry,* 24**,** 1462-1467.

BLANCO-PÉREZ, R., BUENO-PALLERO, F. Á., VICENTE-DÍEZ, I., MARCO-MANCEBÓN, V. S., PÉREZ-MORENO, I. & CAMPOS-HERRERA, R. 2019. Scavenging behavior and interspecific competition decrease offspring fitness of the entomopathogenic nematode *Steinernema feltiae*. *Journal of Invertebrate Pathology,* 164**,** 5-15.

BLAXTER, M. L., DE LEY, P., GAREY, J. R., LIU, L. X., SCHELDEMAN, P., VIERSTRAETE, A., VANFLETEREN, J. R., MACKEY, L. Y., DORRIS, M., FRISSE, L. M., VIDA, J. T. & THOMAS, W. K. 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature,* 392**,** 71-75.

BOFF, M. I. C., VAN TOL, R. H. W. M. & SMITS, P. H. 2002. Behavioural response of *Heterorhabditis* megidis towards plant roots and insect larvae. *BioControl,* 47**,** 67-83.

BOOTHBY, T. C., TAPIA, H., BROZENA, A. H., PISZKIEWICZ, S., SMITH, A. E., GIOVANNINI, I., REBECCHI, L., PIELAK, G. J., KOSHLAND, D. & GOLDSTEIN, B. 2017. Tardigrades Use Intrinsically Disordered Proteins to Survive Desiccation. *Molecular cell,* 65**,** 975-984.

BORGES, J. C. & RAMOS, C. H. 2005. Protein folding assisted by chaperones. *Protein & Peptide Letters.* 12**,** 257-2561.

BOSE, S., WEIKL, T., BÜGL, H. & BUCHNER, J. 1996. Chaperone Function of Hsp90-Associated Proteins. *Science,* 274**,** 1715.

BROWN, I. & GAUGLER, R. 1998. Survival of steinernematid nematodes exposed to

freezing. *Journal of thermal biology,* 23**,** 75-80.

BROWN, I. M. & GAUGLER, R. 1996. Cold tolerance of steinernematid and heterorhabditid nematodes. *Journal of Thermal Biology,* 21**,** 115-121.

BROWN, P. O. & BOTSTEIN, D. 1999. Exploring the new world of the genome with DNA microarrays. *Nature Genetics,* 21**,** 33-37.

BROWNE, J. A., DOLAN, K. M., TYSON, T., GOYAL, K., TUNNACLIFFE, A. & BURNELL, A. M. 2004. Dehydration-specific induction of hydrophilic protein genes in the anhydrobiotic nematode *Aphelenchus avenae*. *Eukaryotic cell,* 3**,** 966-975.

BURMAN, M. 1982. *Neoaplectana carpocapsae*: Toxin production by axenic insect parasitic nematodes. *Nematologica.,* 28**,** 62-70.

BURNELL, A. & TUNNACLIFFE, A. 2011. Gene induction and desiccation stress in Nematodes In: PERRY, R. N. & WHARTON, D. Molecular and physiological basis of nematode survival. *CABI.* 126-146.

BUTCHER, R. A. 2017. Decoding chemical communication in nematodes. *Nat Prod Rep,* 34**,** 472-477.

BUTCHER, R. A., FUJITA, M., SCHROEDER, F. C. & CLARDY, J. 2007. Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. *Nature Chemical Biology,* 3**,** 420-422.

BUTCHER, R. A., RAGAINS, J. R., LI, W., RUVKUN, G., CLARDY, J. & MAK, H. Y. 2009. Biosynthesis of the *Caenorhabditis elegans* dauer pheromone. *Proceedings of the National Academy of Sciences,* 106**,** 1875.

CAGNOLO, S. & CAMPOS, V. 2008. Effect of storage temperature on survival and

infectivity of *Steinernema rarum* (OLI strain) (Rhabditida: Steinernematidae). *Journal of Invertebrate Pathology,* 98**,** 114-115.

CAMPBELL, J., F & GAUGLER, R., R 1997. Inter-specific variation in entomopathogenic nematode foraging strategy: dichotomy or variation along a continuum? *Fundamental & Applied Nematology.* 20, 393-398

CAMPBELL, J. & GAUGLER, R. 1993. Nictation Behaviour and Its Ecological Implications in the Host Search Strategies of Entomopathogenic Nematodes (Heterorhabditidae and Steinernematidae). *Behaviour,* 126**,** 155-169.

CAMPBELL, J. F., KOPPENHÖFER, A. M., KAYA, H. K. & CHINNASRI, B. 1999. Are there temporarily non-infectious dauer stages in entomopathogenic nematode populations: a test of the phased infectivity hypothesis. *Parasitology,* 118**,** 499-508.

CARPENTER, J. F. & CROWE, J. H. 1988. Modes of stabilization of a protein by organic solutes during desiccation. *Cryobiology,* 25**,** 459-470.

CASSADA, R. C. & RUSSELL, R. L. 1975. The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Developmental Biology,* 46**,** 326-342.

CASTELLETTO, M. L., GANG, S. S., OKUBO, R. P., TSELIKOVA, A. A., NOLAN, T. J., PLATZER, E. G., LOK, J. B. & HALLEM, E. A. 2014. Diverse host-seeking behaviors of skin-penetrating nematodes. *PLoS Pathogens,* 10**,** e1004305.

CHAISSON, K. E. & HALLEM, E. A. 2012. Chemosensory behaviors of parasites. *Trends in parasitology,* 28**,** 427-436.

CHANG, D. Z., SERRA, L., LU, D., MORTAZAVI, A. & DILLMAN, A. R. 2019. A core set of venom proteins is released by entomopathogenic nematodes in the genus Steinernema. *PLOS Pathogens,* 15**,** e1007626.

CHASTON, J. M., DILLMAN, A. R., SHAPIRO-ILAN, D. I., BILGRAMI, A. L., GAUGLER, R., HOPPER, K. R. & ADAMS, B. J. 2011. Outcrossing and crossbreeding recovers deteriorated traits in laboratory cultured *Steinernema carpocapsae* nematodes. *International Journal for Parasitologyy,* 41**,** 801-809.

CHEN, S. & GLAZER, I. 2005. A novel method for long-term storage of the entomopathogenic nematode *Steinernema feltiae* at room temperature. *Biological Control,* 32**,** 104-110.

CHEN, S., GLAZER, I., GOLLOP, N., CASH, P., ARGO, E., INNES, A., STEWART, E., DAVIDSON, I. & WILSON, M. J. 2006. Proteomic analysis of the entomopathogenic nematode *Steinernema feltiae* IS-6 IJs under evaporative and osmotic stresses. *Molecular and Biochemical Parasitology.* 145**,** 195-204.

CHEN, S., LI, J., HAN, X. & MOENS, M. 2003. Effect of temperature on the pathogenicity of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp.) to *Delia radicum*. *BioControl,* 48**,** 713-724.

CHOE, A., CHUMAN, T., VON REUSS, S. H., DOSSEY, A. T., YIM, J. J., AJREDINI, R., KOLAWA, A. A., KAPLAN, F., ALBORN, H. T., TEAL, P. E. A., SCHROEDER, F. C., STERNBERG, P. W. & EDISON, A. S. 2012a. Sex-specific mating pheromones in the nematode *Panagrellus redivivus*. *Proceedings of the National Academy of Sciences,* 109**,** 20949.

CHOE, A., VON REUSS, S. H., KOGAN, D., GASSER, R. B., PLATZER, E. G., SCHROEDER, F. C. & STERNBERG, P. W. 2012b. Ascaroside signaling is widely conserved among nematodes. *Current Biology,* 22**,** 772-780.

CICHE, T. A., KIM, K. S., KAUFMANN-DASZCZUK, B., NGUYEN, K. C. & HALL, D. H. 2008. Cell Invasion and Matricide during *Photorhabdus luminescens*

Transmission by *Heterorhabditis bacteriophora* Nematodes. *Appl Environ Microbiol,* 74**,** 2275-87.

CLARE, D. K. & SAIBIL, H. R. 2013. ATP-driven molecular chaperone machines. *Biopolymers,* 99**,** 846-859.

CLARK, D. A., BIRON, D., SENGUPTA, P. & SAMUEL, A. D. T. 2006. The AFD Sensory Neurons Encode Multiple Functions Underlying Thermotactic Behavior in *Caenorhabditis elegans*. *The Journal of Neuroscience,* 26**,** 7444.

CONNELL, P., BALLINGER, C. A., JIANG, J., WU, Y., THOMPSON, L. J., HÖHFELD, J. & PATTERSON, C. 2001. The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nature Cell Biology,* 3**,** 93-96.

CONSORTIUM, C. E. S. 1998. Genome Sequence of the Nematode *C. elegans*: A Platform for Investigating Biology. *Science,* 282**,** 2012.

COOK, S. J., JARRELL, T. A., BRITTIN, C. A., WANG, Y., BLONIARZ, A. E., YAKOVLEV, M. A., NGUYEN, K. C. Q., TANG, L. T. H., BAYER, E. A., DUERR, J. S., BÜLOW, H. E., HOBERT, O., HALL, D. H. & EMMONS, S. W. 2019. Whole-animal connectomes of both *Caenorhabditis elegans* sexes. *Nature,* 571**,** 63-71.

CROWE, J. H., CROWE, L. M., CARPENTER, J. F. & AURELL WISTROM, C. 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. *The Biochemical journal,* 242**,** 1-10.

CROWE, L. M., REID, D. S. & CROWE, J. H. 1996. Is trehalose special for preserving dry biomaterials? *Biophysical journal,* 71**,** 2087-2093.

CUMING, A. C. & LANE, B. G. 1979. Protein Synthesis in Imbibing Wheat Embryos.

*European Journal of Biochemistry*, 99**,** 217-224.

D'AMORE, T., CRUMPLEN, R. & STEWART, G. G. 1991. The involvement of trehalose in yeast stress tolerance. *Journal of Industrial Microbiology,* 7**,** 191-195.

DASKALOVA, E., DONTCHEVA, S., YAHUBYAN, G., MINKOV, I. & TONEVA, V. 2010. Ecological Characteristics and Conservation of the Protected Resurrection Species *Haberlea Rhodopensis Friv*. as In Vitro Plants Through a Modified Micropropagation System. *Biotechnology & Biotechnological Equipment,* 24**,** 213-217.

DE BONO, M. & MARICQ, A. V. 2005. Neuronal substrates of complex behaviors in *C. elegans*. *Annual Review of Neuroscience,* 28**,** 451-501.

DE JONG, W. W., CASPERS, G.-J. & LEUNISSEN, J. A. M. 1998. Genealogy of the α-crystallin—small heat-shock protein superfamily. *International Journal of Biological Macromolecules,* 22**,** 151-162.

DEMPSEY, C. M. & GRIFFIN, C. T. 2002. Phased activity in *Heterorhabditis megidis* infective juveniles. *Parasitology,* 124**,** 605-613.

DEVANEY, E. 2011. Thermobiotic survival In: PERRY, R. N. & WHARTON, D. Molecular and physiological basis of nematode survival. *CABI,* 233-249.

DILLMAN, A. R., GUILLERMIN, M. L., LEE, J. H., KIM, B., STERNBERG, P. W. & HALLEM, E. A. 2012. Olfaction shapes host–parasite interactions in parasitic nematodes. *Proceedings of the National Academy of Sciences,* 109**,** E2324.

DILLMAN, A. R., MACCHIETTO, M., PORTER, C. F., ROGERS, A., WILLIAMS, B., ANTOSHECHKIN, I., LEE, M.-M., GOODWIN, Z., LU, X., LEWIS, E. E., GOODRICH-BLAIR, H., STOCK, S. P., ADAMS, B. J., STERNBERG, P. W. & MORTAZAVI, A. 2015. Comparative genomics of *Steinernema* reveals deeply

conserved gene regulatory networks. *Genome Biology,* 16**,** 200.

DILLON, A. B., DOWNES, M. J., WARD, D. & GRIFFIN, C. T. 2007. Optimizing application of entomopathogenic nematodes to manage large pine weevil, *Hylobius abietis L*. (Coleoptera: Curculionidae) populations developing in pine stumps, *Pinus sylvestris*. *Biological Control,* 40**,** 253-263.

DUMONT, F., MARECHAL, P.-A. & GERVAIS, P. 2003. Influence of cooling rate on *Saccharomyces cerevisiae* destruction during freezing: unexpected viability at ultra-rapid cooling rates. *Cryobiology,* 46**,** 33-42.

DUNCAN, L. W., DUNN, D. C., BAGUE, G. & NGUYEN, K. 2003a. Competition Between Entomopathogenic and Free-Living Bactivorous Nematodes in Larvae of the Weevil *Diaprepes abbreviatus*. *Journal of nematology,* 35**,** 187-193.

DUNCAN, L. W., GRAHAM, J. H., DUNN, D. C., ZELLERS, J., MCCOY, C. W. & NGUYEN, K. 2003b. Incidence of Endemic Entomopathogenic Nematodes Following Application of *Steinernema riobrave* for Control of *Diaprepes abbreviatus*. *Journal of Nematology,* 35**,** 178-186.

DURE, L., 3RD, GREENWAY, S. C. & GALAU, G. A. 1981. Developmental biochemistry of cottonseed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by in vitro and in vivo protein synthesis. *Biochemistry,* 20**,** 4162-4168.

DZIEDZIECH, A., SHIVANKAR, S. & THEOPOLD, U. 2020. High-Resolution Infection Kinetics of Entomopathogenic Nematodes Entering *Drosophila melanogaster*. *Insects,* 11**,** 60.

EDISON, A. S. 2009. *Caenorhabditis elegans* pheromones regulate multiple complex behaviors. *Current opinion in neurobiology,* 19**,** 378-388.

EHLERS, R.-U., OESTERGAARD, J., HOLLMER, S., WINGEN, M. & STRAUCH, O. 2005. Genetic Selection for Heat Tolerance and Low Temperature Activity of the Entomopathogenic Nematode–bacterium Complex *Heterorhabditis bacteriophora–Photorhabdus Luminescens*. *BioControl,* 50**,** 699-716.

EHLERS, R. U. 2001. Mass production of entomopathogenic nematodes for plant protection. *Applied Microbiology and Biotechnology,* 56**,** 623-633.

EHLERS, R. U. & HOKKANEN, H. M. T. 1996. Insect Biocontrol with Non-endemic Entomopathogenic Nematodes (*Steinernema* and *Heterorhabditis* spp.): Conclusions and Recommendations of a Combined OECD and COST Workshop on Scientific and Regulatory Policy Issues. *Biocontrol Science and Technology,* 6**,** 295-302.

EL KHOURY, Y., ORESTE, M., NOUJEIM, E., NEMER, N. & TARASCO, E. 2018. Effect of temperature on the pathogenicity of mediterranean native entomopathogenic nematodes (steinernematidae and heterorhabditidae) from natural ecosystems. *REDIA*, 101**,** 123-127.

ELBEIN, A. D., PAN, Y. T., PASTUSZAK, I. & CARROLL, D. 2003. New insights on trehalose: a multifunctional molecule. *Glycobiology,* 13**,** 17R-27R.

ELEUTHERIO, E. C., ARAUJO PS FAU - PANEK, A. D. & PANEK, A. D. 1993. Role of the trehalose carrier in dehydration resistance of *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta,* 1156, 263-266.

ERKUT, C. & KURZCHALIA, T. V. 2015. The *C. elegans* dauer larva as a paradigm to study metabolic suppression and desiccation tolerance. *Planta,* 242**,** 389-396.

ERKUT, C., PENKOV, S., KHESBAK, H., VORKEL, D., VERBAVATZ, J.-M., FAHMY, K. & KURZCHALIA, TEYMURAS V. 2011. Trehalose Renders the

Dauer Larva of *Caenorhabditis elegans* Resistant to Extreme Desiccation. *Current Biology,* 21**,** 1331-1336.

FAN, X. & HOMINICK, W. Effects of low storage temperature on survival and infectivity of two *Steinernerna* species (Nematoda : Steinernematidae). *Revue of Nématology.* 14, 407–412.

FIFE, J. P., DERKSEN, R. C., ERDAL OZKAN, H. & GREWAL, P. S. 2003. Effects of pressure differentials on the viability and infectivity of entomopathogenic nematodes. *Biological Control,* 27**,** 65-72.

FITTERS, P. F. L., DUNNE, R. & GRIFFIN, C. T. 2001. Vine Weevil Control in Ireland with Entomopathogenic Nematodes: Optimal Time of Application. *Irish Journal of Agricultural and Food Research,* 40**,** 199-213.

FLURY, F. 1912. Zur Chemie und Toxikologie der Ascariden. *Archiv für experimentelle Pathologie und Pharmakologie,* 67**,** 275-392.

FORGE, T. A. & MACGUIDWIN, A. E. 1992. Effects of water potential and temperature on survival of the nematode *Meloidogyne hapla* in frozen soil. *Canadian Journal of Zoology,* 70**,** 1553-1560.

FRANCK, E., MADSEN, O., VAN RHEEDE, T., RICARD, G., HUYNEN, M. A. & DE JONG, W. W. 2004. Evolutionary Diversity of Vertebrate Small Heat Shock Proteins. *Journal of Molecular Evolution,* 59**,** 792-805.

FRANKS, A., AIROLDI, E. & SLAVOV, N. 2017. Post-transcriptional regulation across human tissues. *PLOS Computational Biology,* 13**,** e1005535.

FRYDMAN, J. 2001. Folding of Newly Translated Proteins In Vivo: The Role of Molecular Chaperones. *Annual Review of Biochemistry,* 70**,** 603-647.

FRYDMAN, J. & HÖHFELD, J. 1997. Chaperones get in touch: the Hip-Hop connection. *Trends Biochem Sci,* 22**,** 87-92.

FU, Y., WANG, W., CHEN, C., SHAN, S., WEI, X., LIU, Y., SHAPIRO-ILAN, D., GU, X., HU, B., YOSHIGA, T. & RUAN, W. 2021. Chemotaxis behaviour of *Steinernema carpocapsae* in response to *Galleria mellonella* (L.) larvae infected by con- or hetero-specific entomopathogenic nematodes. *Biocontrol Science and Technology,* 31**,** 299-313.

GAL, T. Z., GLAZER, I. & KOLTAI, H. 2004. An LEA group 3 family member is involved in survival of *C. elegans* during exposure to stress. *FEBS Letters,* 577**,** 21-26.

GAO, D. & CRITSER, J. K. 2000. Mechanisms of Cryoinjury in Living Cells. *ILAR Journal,* 41**,** 187-196.

GAUGLER, R., BEDNAREK, A. & CAMPBELL, J. F. 1992a. Ultraviolet inactivation of heterorhabditid and steinernematid nematodes. *Journal of Invertebrate Pathology,* 59**,** 155-160.

GAUGLER, R., CAMPBELL, J. F. & MCGUIRE, T. R. 1989. Selection for host-finding in *Steinernema feltiae*. *Journal of Invertebrate Pathology,* 54**,** 363-372.

GAUGLER, R., CAMPBELL, J. F. & MCGUIRE, T. R. 1990. Fitness of a genetically improved entomopathogenic nematode. *Journal of Invertebrate Pathology,* 56**,** 106-116.

GAUGLER, R., CAMPBELL, J. F., SELVAN, S. & LEWIS, E. E. 1992b. Large-scale inoculative releases of the entomopathogenic nematode *Steinernema* glaseri: Assessment 50 years later. *Biological Control,* 2**,** 181-187.

GAUGLER, R. & HAN, R. 2002. Production technology In: Entomopathogenic

Nematology. *CABI, Wallingford UK,* 289-310.

GBOTSYO, Y. A., ROWARTH, N. M., WEIR, L. K. & MACRAE, T. H. 2020. Short-term cold stress and heat shock proteins in the crustacean *Artemia franciscana*. *Cell Stress and Chaperones,* 25**,** 1083-1097.

GEORGIS, R. 1990. Formulation and Application Technology. In*:* GAUGLER, R and KAYA, H. K. 1990. Entomopathogenic nematodes in Biological Control. *CRC Press*, 173-191

GEORGIS, R. & GAUGLER, R. 1991. Predictability in Biological Control Using Entomopathogenic Nematodes. *Journal of Economic Entomology,* 84**,** 713-720.

GEORGIS, R., KOPPENHÖFER, A. M., LACEY, L. A., BÉLAIR, G., DUNCAN, L. W., GREWAL, P. S., SAMISH, M., TAN, L., TORR, P. & VAN TOL, R. W. H. M. 2006. Successes and failures in the use of parasitic nematodes for pest control. *Biological Control,* 38**,** 103-123.

GLASHEEN, J. & HAND, S. C. 1988. Anhydrobiosis in embryos of the brine shrimp *Artemia*: characterization of metabolic arrest during reductions in cell-associated water. *Journal of experimental biology,* 135**,** 363-380.

GLAZER, I. 2002. Survival biology In*:* Gaugler, R. (Ed). Entomopathogenic Nematology. *CABI, Wallingford UK,* 169-187.

Glazer, I. (2015). Improvement of Entomopathogenic Nematodes: A Genetic Approach. In: Campos-Herrera, R. (Ed.), Nematode pathogenesis of insects and other pests, *Springer, London*, 29-55.

GLAZER, I., SALAME, L. & SEGAL, D. 1997. Genetic Enhancement of Nematicide Resistance in Entomopathogenic Nematodes. *Biocontrol Science and Technology,* 7**,** 499-512.

GLOVER, J. R. & LINDQUIST, S. 1998. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell,* 94**,** 73-82.

GREWAL, P., SELVAN, S., LEWIS, E. & GAUGLER, R. 1993a. Male insect-parasitic nematodes: a colonizing sex. *Experientia,* 49**,** 605-608.

GREWAL, P. S. 2002. Formulation and Application Technology, In: Gaugler, R. (Ed). Entomopathogenic Nematology. *CABI, Wallingford UK,* 265-287.

GREWAL, P. S., BORNSTEIN-FORST, S., BURNELL, A. M., GLAZER, I. & JAGDALE, G. B. 2006. Physiological, genetic, and molecular mechanisms of chemoreception, thermobiosis, and anhydrobiosis in entomopathogenic nematodes. *Biological Control,* 38**,** 54-65.

GREWAL, P. S., GAUGLER, R. & LEWIS, E. E. 1993b. Host Recognition Behavior by Entomopathogenic Nematodes during Contact with Insect Gut Contents. *The Journal of Parasitology,* 79**,** 495-503.

GREWAL, P. S., GAUGLER, R. & SELVAN, S. 1993c. Host recognition by entomopathogenic nematodes: Behavioral response to contact with host feces. *Journal of Chemical Ecology,* 19**,** 1219-1231.

GREWAL, P. S., GAUGLER, R. & WANG, Y. I. 1996. Enhanced cold tolerance of the entomopathogenic nematode *Steinernema feltiae* through genetic selection. *Annals of Applied Biology,* 129**,** 335-341.

GREWAL, P. S. & JAGDALE, G. B. 2002. Enhanced Trehalose Accumulation and Desiccation Survival of Entomopathogenic Nematodes Through Cold Preacclimation. *Biocontrol Science and Technology,* 12**,** 533-545.

GREWAL, P. S., SELVAN, S. & GAUGLER, R. 1994. Thermal adaptation of entomopathogenic nematodes: Niche breadth for infection, establishment, and

reproduction. *Journal of Thermal Biology,* 19**,** 245-253.

GRFFIN, C. T. 1993. Temperature responses of entomopathogenic nematodes: implications for the success of biological control programmes. In: Bedding, R., AKHURST, R. & KAYA, H.K. Nematodes and the Biological Control of Insect Pests, *CSIRO* Publishing, 178

GRIFFIN, C. 1996. Effects of prior storage conditions on the infectivity of *Heterorhabditis* sp. (Nematoda: Heterorhabditidae). *Fundamental and Applied Nematology,* 19**,** 95-102.

GRIFFIN, C.T. 2015. Behaviour and population dynamics of entomopathogenic nematodes following application, in: Campos-Herrera, R. (Ed.), Nematode pathogenesis of insects and other pests, *Springer, London*, 57-95.

GRIFFIN, C. T. 2012. Perspectives on the behavior of entomopathogenic nematodes from dispersal to reproduction: traits contributing to nematode fitness and biocontrol efficacy. *Journal of nematology,* 44**,** 177-184.

GRIFFIN, C. T., DOWNES, M. J. & BLOCK, W. 1990. Tests of Antarctic soils for insect parasitic nematodes. *Antarctic Science,* 2**,** 221-222.

GRIFFIN, C. T., O'CALLAGHAN, K. M. & DIX, I. 2001. A self-fertile species of *Steinernema* from Indonesia: further evidence of convergent evolution amongst entomopathogenic nematodes? *Parasitology,* 122**,** 181-186.

GUY, A., GAFFNEY, M., KAPRANAS, A. & GRIFFIN, C. T. 2017. Conditioning the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis* megidis by pre-application storage improves efficacy against black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) at low and moderate temperatures. *Biological Control,* 108**,** 40-46.

GUY, A. G., WRIGHT, D., GAFFNEY, M. & GRIFFIN, C. 2009. Conditioning of *Steinernema kraussei* and *S. carpocapsae* through storage: Enhancing their performance over wide temperature ranges. *IOBC/wprs Bulletin,* 45**,** 349-352.

HALLEM, E. A., DILLMAN, A. R., HONG, A. V., ZHANG, Y., YANO, J. M., DEMARCO, S. F. & STERNBERG, P. W. 2011a. A sensory code for host seeking in parasitic nematodes. *Current Biology,* 21**,** 377-383.

HALLEM, E. A., SPENCER, W. C., MCWHIRTER, R. D., ZELLER, G., HENZ, S. R., RÄTSCH, G., MILLER, D. M., HORVITZ, H. R., STERNBERG, P. W. & RINGSTAD, N. 2011b. Receptor-type guanylate cyclase is required for carbon dioxide sensation by *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences,* 108**,** 254.

HALLEM, E. A. & STERNBERG, P. W. 2008. Acute carbon dioxide avoidance in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences, USA,* 105**,** 8038-8043.

HAN, R. & EHLERS, R. U. 2000. Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic in vivo conditions. *Journal of Invertebrate Pathology,* 75**,** 55-58.

HAND, S. C. & MENZE, M. A. 2015. Molecular approaches for improving desiccation tolerance: insights from the brine shrimp *Artemia franciscana*. *Planta,* 242**,** 379-388.

HANSEN, M., TAUBERT, S., CRAWFORD, D., LIBINA, N., LEE, S. J. & KENYON, C. 2007. Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*. *Aging Cell,* 6**,** 95-110.

HAO, Y.-J., MONTIEL, R., ABUBUCKER, S., MITREVA, M. & SIMÕES, N. 2010.

Transcripts analysis of the entomopathogenic nematode *Steinernema carpocapsae* induced in vitro with insect haemolymph. *Molecular and Biochemical Parasitology,* 169**,** 79-86.

HARDING, S. G. 1976. Can Theories Be Refuted? Essays on the Duhem-Quine Thesis. *D. Reidel Publishing Company.*

HARVEY, C. D., ALAMEEN, K. M. & GRIFFIN, C. T. 2012. The impact of entomopathogenic nematodes on a non-target, service-providing longhorn beetle is limited by targeted application when controlling forestry pest *Hylobius abietis*. *Biological Control,* 62**,** 173-182.

HARVEY, C. D. & GRIFFIN, C. T. 2012. Host activity and wasp experience affect parasitoid wasp foraging behaviour and oviposition on nematode-infected larvae of the forestry pest *Hylobius abietis*. *Ecological Entomology,* 37**,** 269-282.

HARVEY, C. D., WILLIAMS, C. D., DILLON, A. B. & GRIFFIN, C. T. 2016. Inundative pest control: How risky is it? A case study using entomopathogenic nematodes in a forest ecosystem. *Forest Ecology and Management,* 380**,** 242-251.

HASLBECK, M., FRANZMANN, T., WEINFURTNER, D. & BUCHNER, J. 2005. Some like it hot: the structure and function of small heat-shock proteins. *Nat Struct Mol Biol,* 12**,** 842-846.

HAZIR, S., KAYA, H., STOCK, S. P. & KESKIN, N. 2003. Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests. *Turkish Journal of Biology,* 27**,** 181-202.

HAZIR, S., STOCK, S. P., KAYA, H. K., KOPPENHÖFER, A. M. & KESKIN, N. 2001. Developmental Temperature Effects on Five Geographic Isolates of the Entomopathogenic Nematode *Steinernema feltiae* (Nematoda: Steinernematidae).

*Journal of Invertebrate Pathology,* 77**,** 243-250.

HENGHERR, S., HEYER, A. G., KÖHLER, H.-R. & SCHILL, R. O. 2008. Trehalose and anhydrobiosis in tardigrades – evidence for divergence in responses to dehydration. *The FEBS Journal,* 275**,** 281-288.

HIBSHMAN, J. D., CLEGG, J. S. & GOLDSTEIN, B. 2020. Mechanisms of Desiccation Tolerance: Themes and Variations in Brine Shrimp, Roundworms, and Tardigrades. *Frontiers in Physiology,* 11.

HILTPOLD, I., HIBBARD, B. E., FRENCH, B. W. & TURLINGS, T. C. 2012. Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. *Plant and Soil,* 358**,** 11-25.

HOFF, K. J., LOMSADZE, A., BORODOVSKY, M. & STANKE, M. 2019. Whole-Genome Annotation with BRAKER. *Methods in molecular biology (Clifton, N.J.),* 1962**,** 65-95.

HOMINICK, W. M. 2002. Biogeography *In:* Gaugler, R. (Ed). Entomopathogenic Nematology. *CABI, Wallingford UK,* 115-143.

HOMINICK, W. M., REID, A. P., BOHAN, D. A. & BRISCOE, B. R. 1996. Entomopathogenic Nematodes: Biodiversity, Geographical Distribution and the Convention on Biological Diversity. *Biocontrol Science and Technology,* 6**,** 317-332.

HSUEH, Y.-P., MAHANTI, P., SCHROEDER, F. C. & STERNBERG, P. W. 2013. Nematode-trapping fungi eavesdrop on nematode pheromones. *Current biology,* 23**,** 83-86.

HUSSEIN, M. & ABDEL-ATY, M. A. 2012. Formulation of two native entomopathogenic nematodes at room temperature. *Journal of Biopesticides,* 5**,** 23-

27.

ITURRIAGA, G., GAFF, D. & ZENTELLA, R. 2000. New desiccation-tolerant plants, including a grass, in the central highlands of Mexico, accumulate trehalose. *Australian Journal of Botany*, 48**,** 153-158.

IZRAYELIT, Y., SRINIVASAN, J., CAMPBELL, S. L., JO, Y., VON REUSS, S. H., GENOFF, M. C., STERNBERG, P. W. & SCHROEDER, F. C. 2012. Targeted metabolomics reveals a male pheromone and sex-specific ascaroside biosynthesis in *Caenorhabditis elegans*. *ACS chemical biology*, 7**,** 1321-1325.

JAGDALE, G. B., CASEY, M. L., GREWAL, P. S. & LINDQUIST, R. K. 2004. Application rate and timing, potting medium, and host plant effects on the efficacy of *Steinernema feltiae* against the fungus gnat, *Bradysia coprophila*, in floriculture. *Biological Control,* 29**,** 296-305.

JAGDALE, G. B. & GREWAL, P. S. 2003. Acclimation of entomopathogenic nematodes to novel temperatures: trehalose accumulation and the acquisition of thermotolerance. *International Journal for Parasitologyy,* 33**,** 145-52.

JAGDALE, G. B. & GREWAL, P. S. 2007. Storage temperature influences desiccation and ultra violet radiation tolerance of entomopathogenic nematodes. *Journal of Thermal Biology,* 32**,** 20-27.

JAKOB, U., GAESTEL, M., ENGEL, K. & BUCHNER, J. 1993. Small heat shock proteins are molecular chaperones. *Journal of Biological Chemistry,* 268**,** 1517-1520.

JAMES, P., PFUND, C. & CRAIG, E. A. 1997. Functional Specificity Among Hsp70 Molecular Chaperones. *Science*, 275**,** 387.

JANSSON, R. K. 1993. Introduction of Exotic Entomopathogenic Nematodes (Rhabditida:

Heterorhabditidae and Steinernematidae) for Biological Control of Insects: Potential and Problems. *The Florida Entomologist,* 76**,** 82-96.

JANSSON, R. K., LECRONE, S. H. & GAUGLER, R. 1993. Field Efficacy and Persistence of Entomopathogenic Nematodes (Rhabditida: Steinernematidae, Heterorhabditidae) for Control of Sweetpotato Weevil (Coleoptera: Apionidae) in Southern Florida. *Journal of Economic Entomology,* 86**,** 1055-1063.

JEONG, P. Y., JUNG, M., YIM, Y. H., KIM, H., PARK, M., HONG, E., LEE, W., KIM, Y. H., KIM, K. & PAIK, Y. K. 2005. Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. *Nature,* 433**,** 541-545.

JEONG, P. Y., KWON, M. S., JOO, H. J. & PAIK, Y. K. 2009. Molecular time-course and the metabolic basis of entry into dauer in *Caenorhabditis elegans*. *PLoS One,* 4**,** e4162.

JEZYK, P. F. & FAIRBAIRN, D. 1967. Ascarosides and ascaroside esters in Ascaris lumbricoides (Nematoda). *Comparative Biochemistry and Physiology,* 23**,** 691-705.

KAPLAN, F., ALBORN, H. T., VON REUSS, S. H., AJREDINI, R., ALI, J. G., AKYAZI, F., STELINSKI, L. L., EDISON, A. S., SCHROEDER, F. C. & TEAL, P. E. 2012. Interspecific Nematode Signals Regulate Dispersal Behavior. *PLOS ONE,* 7**,** e38735.

KAPLAN, F., KOPKA, J., HASKELL, D. W., ZHAO, W., SCHILLER, K. C., GATZKE, N., SUNG, D. Y. & GUY, C. L. 2004. Exploring the temperature-stress metabolome of Arabidopsis. *Plant Physiology,* 136**,** 4159-4168.

KAPLAN, F., PERRET-GENTIL, A., GIURINTANO, J., STEVENS, G., ERDOGAN, H., SCHILLER, K. C., MIRTI, A., SAMPSON, E., TORRES, C., SUN, J., LEWIS, E.

E. & SHAPIRO-ILAN, D. 2020. Conspecific and heterospecific pheromones stimulate dispersal of entomopathogenic nematodes during quiescence. *Scientific Reports,* 10**,** 5738.

KAPLAN, F., SRINIVASAN, J., MAHANTI, P., AJREDINI, R., DURAK, O., NIMALENDRAN, R., STERNBERG, P. W., TEAL, P. E. A., SCHROEDER, F. C., EDISON, A. S. & ALBORN, H. T. 2011. Ascaroside Expression in *Caenorhabditis elegans* Is Strongly Dependent on Diet and Developmental Stage. *PLOS ONE,* 6**,** e17804.

KAPLAN, H. S. & ZIMMER, M. 2018. Sensorimotor Integration for Decision Making: How the Worm Steers. *Neuron,* 97**,** 258-260.

KAPRANAS, A., MALONE, B., QUINN, S., O'TUAMA, P., PETERS, A. & GRIFFIN, C. T. 2017. Optimizing the application method of entomopathogenic nematode suspension for biological control of large pine weevil *Hylobius abietis*. *BioControl*, 62**,** 659-667.

KARTHIK RAJA, R., ARUN, A., TOURAY, M., HAZAL GULSEN, S., CIMEN, H., GULCU, B., HAZIR, C., AISWARYA, D., ULUG, D., CAKMAK, I., KAYA, H. K. & HAZIR, S. 2021. Antagonists and defense mechanisms of entomopathogenic nematodes and their mutualistic bacteria. *Biological Control,* 152**,** 104452.

KELL, D. B. & OLIVER, S. G. 2004. Here is the evidence, now what is the hypothesis? The complementary roles of inductive and hypothesis-driven science in the post-genomic era. *BioEssays,* 26**,** 99-105.

KENNEDY, M. W., CORSICO, B., COOPER, A. & SMITH, B. 2013. The unusual lipid-binding proteins of nematodes: NPAs, nemFABPs and FARs. *Parasitic nematodes: molecular biology***,** 397-412.

KEPENEKCI, İ., HAZIR, S. & ÖZDEM, A. 2015. Evaluation of native entomopathogenic nematodes for the control of the European cherry fruit fly *Rhagoletis cerasi* L. (Diptera: Tephritidae) larvae in soil. *TURKISH JOURNAL OF AGRICULTURE AND FORESTRY,* 39**,** 74-79.

KIM, J., HILTPOLD, I., JAFFUEL, G., SBAITI, I., HIBBARD, B. E. & TURLINGS, T. C. J. 2021. Calcium-alginate beads as a formulation for the application of entomopathogenic nematodes to control rootworms. *Journal of Pest Science*.

KIM, S. K., FLORES-LARA, Y. & PATRICIA STOCK, S. 2012. Morphology and ultrastructure of the bacterial receptacle in *Steinernema* nematodes (Nematoda: Steinernematidae). *Journal of Invertebrate Pathology,* 110**,** 366-374.

KIMATA, T., SASAKURA, H., OHNISHI, N., NISHIO, N. & MORI, I. 2012. Thermotaxis of *C. elegans* as a model for temperature perception, neural information processing and neural plasticity. *Worm,* 1**,** 31-41.

KIN, K., BAIOCCHI, T. & DILLMAN, A. R. 2019. Dispersal and Repulsion of Entomopathogenic Nematodes to Prenol. *Biology,* 8.

KLOSIN, A., CASAS, E., HIDALGO-CARCEDO, C., VAVOURI, T. & LEHNER, B. 2017. Transgenerational transmission of environmental information in *C. elegans*. *Science,* 356**,** 320.

KOPPENHÖFER, A. M., EBSSA, L. & FUZY, E. M. 2013. Storage temperature and duration affect *Steinernema scarabaei* dispersal and attraction, virulence, and infectivity to a white grub host. *Journal of Invertebrate Pathology*, 112**,** 129-137.

KOPPENHÖFER, A. M., GREWAL, P. S. & FUZY, E. M. 2006. Virulence of the entomopathogenic nematodes *Heterorhabditis bacteriophora, Heterorhabditis zealandica,* and *Steinernema scarabaei* against five white grub species

(Coleoptera: Scarabaeidae) of economic importance in turfgrass in North America. *Biological Control,* 38**,** 397-404.

KOSTER, K. L., WEBB, M. S., BRYANT, G. & LYNCH, D. V. 1994. Interactions between soluble sugars and POPC (1-palmitoyl-2-oleoylphosphatidylcholine) during dehydration: vitrification of sugars alters the phase behavior of the phospholipid. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1193**,** 143-150.

KRUITBOS, L. M., HERITAGE, S., HAPCA, S. & WILSON, M. J. 2010. The influence of habitat quality on the foraging strategies of the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis* megidis. *Parasitology,* 137**,** 303-309.

KUNG, S.-P., GAUGLER, R. & KAYA, H. K. 1991. Effects of soil temperature, moisture, and relative humidity on entomopathogenic nematode persistence. *Journal of Invertebrate Pathology,* 57**,** 242-249.

LABAUDE, S. & GRIFFIN, C. T. 2018. Transmission Success of Entomopathogenic Nematodes Used in Pest Control. *Insects,* 9.

LACEY, L. A., GRZYWACZ, D., SHAPIRO-ILAN, D. I., FRUTOS, R., BROWNBRIDGE, M. & GOETTEL, M. S. 2015. Insect pathogens as biological control agents: Back to the future. *Journal of Invertebrate Pathology,* 132**,** 1-41.

LANE, N. & MARTIN, W. 2010. The energetics of genome complexity. *Nature,* 467**,** 929-934.

LANGER, T., LU, C., ECHOLS, H., FLANAGAN, J., HAYER, M. K. & HARTL, F. U. 1992. Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature,* 356**,** 683-689.

LAURENT, J. M., VOGEL, C., KWON, T., CRAIG, S. A., BOUTZ, D. R., HUSE, H. K., NOZUE, K., WALIA, H., WHITELEY, M., RONALD, P. C. & MARCOTTE, E. M. 2010. Protein abundances are more conserved than mRNA abundances across diverse taxa. *Proteomics,* 10**,** 4209-4212.

LEE, J. H., DILLMAN, A. R. & HALLEM, E. A. 2016. Temperature-dependent changes in the host-seeking behaviors of parasitic nematodes. *BMC Biology,* 14**,** 36.

LEE, J. H. & SCHÖFFL, F. 1996. An Hsp70 antisense gene affects the expression of HSP70/HSC70, the regulation of HSF, and the acquisition of thermotolerance in transgenic Arabidopsis thaliana. *Mol Gen Genet,* 252**,** 11-9.

LENNÉ, T., BRYANT, G., HOCART, C. H., HUANG, C. X. & BALL, M. C. 2010. Freeze avoidance: a dehydrating moss gathers no ice. *Plant, Cell & Environment,* 33**,** 1731-1741.

LEVY-RIMLER, G., BELL, R. E., BEN-TAL, N. & AZEM, A. 2002. Type I chaperonins: not all are created equal. *FEBS Letters,* 529**,** 1-5.

LEWIS, E. & SHAPIRO-ILAN, D. 2002. Host cadavers protect entomopathogenic nematodes during freezing. *Journal of invertebrate pathology,* 81**,** 25-32.

LEWIS, E. E. & GAUGLER, R. 1994. Entomopathogenic nematode (Rhabdita: Steinernematidae) sex ratio relates to foraging strategy. *Journal of Invertebrate Pathology,* 64**,** 238-242.

LEWIS, E. E., SELVAN, S., CAMPBELL, J. F. & GAUGLER, R. 1995. Changes in foraging behaviour during the infective stage of entomopathogenic nematodes. *Parasitology,* 110**,** 583-590.

LIU, H., YANG, W., WU, T., DUAN, F., SOUCY, E., JIN, X. & ZHANG, Y. 2018. Cholinergic Sensorimotor Integration Regulates Olfactory Steering. *Neuron,* 97**,**

390-405.e3.

LIU, Z., LI, Y., PAN, L., MENG, F. & ZHANG, X. 2019. Cold adaptive potential of pine wood nematodes overwintering in plant hosts. *Biology Open,* 8.

ŁOPIEŃSKA-BIERNAT, E., STRYIŃSKI, R., POLAK, I., PAWLIKOWSKI, B., PAWLAK, J. & PODOLSKA, M. 2020. Effect of freezing on the metabolic status of L3 larvae of Anisakis simplex s. s. *Infection, Genetics and Evolution,* 82**,** 104312.

LORTKIPANIDZE, M., HWSEYNOV, K., KOKHIA, M., GORGADZE, O. & KUCHAVA, M. 2019. Effect of Temperature on the Virulence of Entomopathogenic Nematodes. *Environment and Development Economics,* Vol.4, January 2019**,** 32-38.

LOUKAS, A. & MAIZELS, R. M. 2000. Helminth C-type Lectins and Host–Parasite Interactions. *Parasitology Today,* 16**,** 333-339.

LU, D., BAIOCCHI, T. & DILLMAN, A. R. 2016. Genomics of Entomopathogenic Nematodes and Implications for Pest Control. *Trends in parasitology,* 32**,** 588-598.

LU, D., MACCHIETTO, M., CHANG, D., BARROS, M. M., BALDWIN, J., MORTAZAVI, A. & DILLMAN, A. R. 2017a. Activated entomopathogenic nematode infective juveniles release lethal venom proteins. *PLoS Pathogens,* 13**,** e1006302.

LU, D., SEPULVEDA, C. & DILLMAN, A. R. 2017b. Infective Juveniles of the Entomopathogenic Nematode *Steinernema scapterisci* Are Preferentially Activated by Cricket Tissue. *PLOS ONE,* 12**,** e0169410.

LUDEWIG, A. H. & SCHROEDER, F. C. 2013. Ascaroside signaling in *C. elegans*. *WormBook***,** 1-22.

MADIN, K. A. C. & CROWE, J. H. 1975. Anhydrobiosis in nematodes: Carbohydrate and lipid metabolism during dehydration. *Journal of Experimental Zoology,* 193**,** 335-342.

MANOSALVA, P., MANOHAR, M., VON REUSS, S. H., CHEN, S., KOCH, A., KAPLAN, F., CHOE, A., MICIKAS, R. J., WANG, X., KOGEL, K.-H., STERNBERG, P. W., WILLIAMSON, V. M., SCHROEDER, F. C. & KLESSIG, D. F. 2015. Conserved nematode signalling molecules elicit plant defenses and pathogen resistance. *Nature Communications,* 6**,** 7795.

MAZUR, P. 1960. PHYSICAL FACTORS IMPLICATED IN THE DEATH OF MICROORGANISMS AT SUBZERO TEMPERATURES*. *Annals of the New York Academy of Sciences,* 85**,** 610-629.

MAZUR, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *The Journal of general physiology,* 47**,** 347-369.

MAZUR, P. 1984. Freezing of living cells: mechanisms and implications. *American Journal of Physiology,* 247**,** 125-142.

MAZUR, P., LEIBO, S. P. & CHU, E. H. Y. 1972. A two-factor hypothesis of freezing injury: Evidence from Chinese hamster tissue-culture cells. *Experimental Cell Research,* 71**,** 345-355.

MCCLELLAN, A. J., TAM, S., KAGANOVICH, D. & FRYDMAN, J. 2005. Protein quality control: chaperones culling corrupt conformations. *Nature Cell Biology,* 7**,** 736-741.

MCGANN, L. E., YANG, H. Y. & WALTERSON, M. 1988. Manifestations of cell damage after freezing and thawing. *Cryobiology,* 25**,** 178-85.

MCGILL, L. M., SHANNON, A. J., PISANI, D., FÉLIX, M.-A., RAMLØV, H., DIX, I., WHARTON, D. A. & BURNELL, A. M. 2015. Anhydrobiosis and freezing-tolerance: adaptations that facilitate the establishment of *Panagrolaimus* nematodes in polar habitats. *PLOS ONE,* 10**,** e0116084-e0116084.

MCLEAN, F., BERGER, D., LAETSCH, D. R., SCHWARTZ, H. T. & BLAXTER, M. 2018. Improving the annotation of the *Heterorhabditis bacteriophora* genome. *GigaScience,* 7.

MCMULLEN, J. G., PETERSON, B. F., FORST, S., BLAIR, H. G. & STOCK, S. P. 2017. Fitness costs of symbiont switching using entomopathogenic nematodes as a model. *BMC Evolutionary Biology,* 17**,** 100.

MORRIS, R., WILSON, L., STURROCK, M., WARNOCK, N. D., CARRIZO, D., COX, D., MAULE, A. G. & DALZELL, J. J. 2017. A neuropeptide modulates sensory perception in the entomopathogenic nematode *Steinernema carpocapsae*. *PLOS Pathogens,* 13**,** e1006185.

MULDREW, K. & MCGANN, L. E. 1994. The osmotic rupture hypothesis of intracellular freezing injury. *Biophysical Journal,* 66**,** 532-541.

MURATA, S., MINAMI, Y., MINAMI, M., CHIBA, T. & TANAKA, K. 2001. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep,* 2**,** 1133-1138.

NATHAN, D. F., VOS, M. H. & LINDQUIST, S. 1997. In vivo functions of the *Saccharomyces cerevisiae* Hsp90 chaperone. *Proceedings of the National Academy of Sciences, USA,* 94**,** 12949-12956.

NAVAREZ, M. L., SANGCOPAN, R., ARYAL, S., SUMAYA, N. P. D., BHAT, A. H. & SUMAYA, N. H. 2021. Native Philippine *Heterorhabditis indica* isolates from

banana and rice fields and preliminary results of their virulence against the larvae of super worm (*Zophobas morio* Fabricius Coleoptera: Tenebrionidae). *Egyptian Journal of Biological Pest Control,* 31**,** 10.

NDONG, C., DANYLUK, J., WILSON, K.E., POCOCK, T., HUNER, N.P. & SARHAN, F. 2002. Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. *Plant Physiology*. 129, 1368-1381

NGUYEN, K. B. & SMART, G. C. 1991. Pathogenicity of *Steinernema scapterisci* to Selected Invertebrates. *Journal of nematology,* 23**,** 7-11.

NILSSON, U. & GRIPWALL, E. 1999. Influence of application technique on the viability of the biological control agents *Verticillium lecanii* and *Steinernema feltiae*. *Crop Protection,* 18**,** 53-59.

NING, S., ZHANG, L., MA, J., CHEN, L., ZENG, G., YANG, C., ZHOU, Y., GUO, X. & DENG, X. 2020. Modular and scalable synthesis of nematode pheromone ascarosides: implications in eliciting plant defense response. *Organic & Biomolecular Chemistry,* 18**,** 4956-4961.

NOGUEZ, J. H., CONNER, E. S., ZHOU, Y., CICHE, T. A., RAGAINS, J. R. & BUTCHER, R. A. 2012. A Novel Ascaroside Controls the Parasitic Life Cycle of the Entomopathogenic Nematode *Heterorhabditis bacteriophora*. *ACS Chemical Biology,* 7**,** 961-966.

O'HALLORAN, D. M. & BURNELL, A. M. 2003. An investigation of chemotaxis in the insect parasitic nematode *Heterorhabditis bacteriophora*. *Parasitology,* 127**,** 375-385.

O'LEARY, S. A., STACK, C. M., CHUBB, M. A. & BURNELL, A. M. 1998. The effect

of day of emergence from the insect cadaver on the behavior and environmental tolerances of infective juveniles of the entomopathogenic nematode *Heterorhabditis megidis* (strain UK211). *Journal of Parasitology,* 84**,** 665-672.

OKAHATA, M., OHTA, A., MIZUTANI, H., MINAKUCHI, Y., TOYODA, A. & KUHARA, A. 2016. Natural variations of cold tolerance and temperature acclimation in *Caenorhabditis elegans*. *Journal of Comparative Physiology B,* 186**,** 985-998.

OLIVEIRA-HOFMAN, C., KAPLAN, F., STEVENS, G., LEWIS, E., WU, S., ALBORN, H. T., PERRET-GENTIL, A. & SHAPIRO-ILAN, D. I. 2019a. Pheromone extracts act as boosters for entomopathogenic nematodes efficacy. *Journal of Invertebrate Pathology,* 164**,** 38-42.

OLIVEIRA-HOFMAN, C., KAPLAN, F., STEVENS, G., LEWIS, E., WU, S., ALBORN, H. T., PERRET-GENTIL, A. & SHAPIRO-ILAN, D. I. 2019b. Pheromone extracts act as boosters for entomopathogenic nematodes efficacy. *Journal of Invertebrate Pathology,* 164**,** 38-42.

PACHECO, A., PEREIRA, C., ALMEIDA, M. J. & SOUSA, M. J. 2009. Small heat-shock protein Hsp12 contributes to yeast tolerance to freezing stress. *Microbiology (Reading),* 155**,** 2021-2028.

PALLEROS, D. R., WELCH, W. J. & FINK, A. L. 1991. Interaction of hsp70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding. *Proceedings of the National Academy of Sciences of the United States of America,* 88**,** 5719-5723.

PAN, K. Z., PALTER, J. E., ROGERS, A. N., OLSEN, A., CHEN, D., LITHGOW, G. J. & KAPAHI, P. 2007. Inhibition of mRNA translation extends lifespan in

*Caenorhabditis elegans*. *Aging Cell,* 6**,** 111-119.

PARIDA, L., NEOGI, S. & PADMANABHAN, V. 2014. Effect of Temperature Pre-Exposure on the Locomotion and Chemotaxis of *C. elegans*. *PLOS ONE,* 9**,** e111342.

PARKINSON, J. & BLAXTER, M. 2009. Expressed sequence tags: an overview. *Methods in Molecular Biology,* 533**,** 1-12.

PATEL, M. N., STOLINSKI, M. & WRIGHT, D. J. 1997. Neutral lipids and the assessment of infectivity in entomopathogenic nematodes: observations on four *Steinernema* species. *Parasitology,* 114**,** 489-496.

PEGG, D. E. 1987. Mechanisms of freezing damage. *Symposia of the Society for Experimental Biology,* 41**,** 363-78.

PEREZ, E. E., LEWIS, E. E. & SHAPIRO-ILAN, D. I. 2003. Impact of the host cadaver on survival and infectivity of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) under desiccating conditions. *Journal of Invertebrate Pathology,* 82**,** 111-118.

PERRY, R. N. & MOENS, M. 2011. Survival of Parasitic Nematodes outside the Host. In: PERRY, R.N and WHARTON, D. Molecular and physiological basis of nematode survival. *CABI*, 1-22

POINAR, G. 1993. Origins and phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and Steinernema. *Fundamental & Applied Nematology,* 16.

POUCHKINA-STANTCHEVA, N. N., MCGEE, B. M., BOSCHETTI, C., TOLLETER, D., CHAKRABORTEE, S., POPOVA, A. V., MEERSMAN, F., MACHEREL, D., HINCHA, D. K. & TUNNACLIFFE, A. 2007. Functional Divergence of Former Alleles in an Ancient Asexual Invertebrate. *Science,* 318**,** 268.

QIU, L. & BEDDING, R. A. 2000. Energy Metabolism and Survival of the Infective Juveniles of *Steinernema carpocapsae* under Oxygen-Deficient Conditions. *Journal of nematology,* 32**,** 271-280.

QIU, L. & BEDDING, R. A. 2002. Characteristics of protectant synthesis of infective juveniles of *Steinernema carpocapsae* and importance of glycerol as a protectant for survival of the nematodes during osmotic dehydration. *Comparative Biochemistry & Physiology Part B: Biochemical and Molecular Biology,* 131**,** 757-65.

RAMAN, B., RAMAKRISHNA, T. & MOHAN RAO, C. 1995. Temperature dependent chaperone-like activity of alpha-crystallin. *FEBS Letters,* 365**,** 133-136.

RAMPELT, H., KIRSTEIN-MILES, J., NILLEGODA, N. B., CHI, K., SCHOLZ, S. R., MORIMOTO, R. I. & BUKAU, B. 2012. Metazoan Hsp70 machines use Hsp110 to power protein disaggregation. *Embo Journal,* 31**,** 4221-4235.

RASMANN, S., KÖLLNER, T. G., DEGENHARDT, J., HILTPOLD, I., TOEPFER, S., KUHLMANN, U., GERSHENZON, J. & TURLINGS, T. C. J. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature,* 434**,** 732-737.

REYES, J. L., CAMPOS, F., WEI, H., ARORA, R., YANG, Y., KARLSON, D. T. & COVARRUBIAS, A. A. 2008. Functional dissection of hydrophilins during in vitro freeze protection. *Plant, Cell & Environment,* 31**,** 1781-90.

RICHTER, K. & BUCHNER, J. 2001. Hsp90: Chaperoning signal transduction. *Journal of Cellular Physiology,* 188**,** 281-290.

RODER, A. C., WANG, Y., BUTCHER, R. A. & STOCK, S. P. 2019. Influence of symbiotic and non-symbiotic bacteria on pheromone production in *Steinernema*

nematodes (Nematoda, Steinernematidae). *The Journal of Experimental Biology*, 222**,** jeb212068.

ROUGON-CARDOSO, A., FLORES-PONCE, M., RAMOS-ABOITES, H. E., MARTÍNEZ-GUERRERO, C. E., HAO, Y.-J., CUNHA, L., RODRÍGUEZ-MARTÍNEZ, J. A., OVANDO-VÁZQUEZ, C., BERMÚDEZ-BARRIENTOS, J. R., ABREU-GOODGER, C., CHAVARRÍA-HERNÁNDEZ, N., SIMÕES, N. & MONTIEL, R. 2016. The genome, transcriptome, and proteome of the nematode *Steinernema carpocapsae*: evolutionary signatures of a pathogenic lifestyle. *Scientific Reports,* 6**,** 37536.

RYDER, J. J. & GRIFFIN, C. T. 2003. Phased infectivity in *Heterorhabditis megidis*: the effects of infection density in the parental host and filial generation. *International Journal for Parasitology*, 33**,** 1013-1018.

SABEHAT, A., LURIE, S. & WEISS, D. 1998. Expression of small heat-shock proteins at low temperatures. A possible role in protecting against chilling injuries. *Plant physiology,* 117**,** 651-658.

ŞAHIN, Ç. & GÖZEL, U. 2021. Efficacy and persistence of native entomopathogenic nematodes against *Capnodis tenebrionis* in peach (*Prunus persica*) orchard in Turkey. *Phytoparasitica,* 49, 613–621.

SAN-BLAS, E., GOWEN, S. R. & PEMBROKE, B. 2008. *Steinernema feltiae*: ammonia triggers the emergence of their infective juveniles. *Experimental Parasitology,* 119**,** 180-185.

SAN-BLAS, E., PIRELA, D., GARCÍA, D. & PORTILLO, E. 2014. Ammonia concentration at emergence and its effects on the recovery of different species of entomopathogenic nematodes. *Experimental Parasitology,* 144**,** 1-5.

SANDHU, S. K., JAGDALE, G. B., HOGENHOUT, S. A. & GREWAL, P. S. 2006. Comparative analysis of the expressed genome of the infective juvenile entomopathogenic nematode, *Heterorhabditis bacteriophora*. *Molecular and Biochemical Parasitology,* 145**,** 239-244.

SANTRA, M., DILL, K. A. & DE GRAFF, A. M. R. 2019. Proteostasis collapse is a driver of cell aging and death. *Proceedings of the National Academy of Sciences,* 116**,** 22173.

SAUNDERS, J. E. & WEBSTER, J. M. 1999. Temperature Effects on *Heterorhabditis megidis* and *Steinernema carpocapsae* Infectivity to *Galleria mellonella*. *Journal of Nematology,* 31**,** 299-304.

SCHOLANDER, P. F., FLAGG, W., HOCK, R. J. & IRVING, L. 1953. Studies on the physiology of frozen plants and animals in the arctic. *Journal of Cellular and Comparative Physiology,* 42**,** 1-56.

SCHULENBURG, H., HOEPPNER, M. P., WEINER, J., 3RD & BORNBERG-BAUER, E. 2008. Specificity of the innate immune system and diversity of C-type lectin domain (CTLD) proteins in the nematode *Caenorhabditis elegans*. *Immunobiology,* 213**,** 237-250.

SCOTT, P. 2000. Resurrection Plants and the Secrets of Eternal Leaf. *Annals of Botany,* 85**,** 159-166.

SEGAL, D. & GLAZER, I. 2000. Genetics for improving biological control agents: the case of entomopathogenic nematodes. *Crop Protection,* 19**,** 685-689.

SERRA, L., MACCHIETTO, M., MACIAS-MUÑOZ, A., MCGILL, C. J., RODRIGUEZ, I. M., RODRIGUEZ, B., MURAD, R. & MORTAZAVI, A. 2019. Hybrid Assembly of the Genome of the Entomopathogenic Nematode *Steinernema*

*carpocapsae* Identifies the X-Chromosome. *G3 (Bethesda, Md.),* 9**,** 2687-2697.

SEYBOLD, A. C., WHARTON, D. A., THORNE, M. A. S. & MARSHALL, C. J. 2017. Investigating trehalose synthesis genes after cold acclimation in the Antarctic nematode *Panagrolaimus* sp. DAW1. *Biology open,* 6**,** 1953-1959.

SHAPIRO, D. I. & GLAZER, I. 1996. Comparison of Entomopathogenic Nematode Dispersal from Infected Hosts Versus Aqueous Suspension. *Environmental Entomology,* 25**,** 1455-1461.

SHAPIRO, D. I. & LEWIS, E. E. 1999. Comparison of Entomopathogenic Nematode Infectivity from Infected Hosts Versus Aqueous Suspension. *Environmental Entomology,* 28**,** 907-911.

SHAPIRO-ILAN, D. I., BROWN, I. & LEWIS, E. E. 2014a. Freezing and desiccation tolerance in entomopathogenic nematodes: diversity and correlation of traits. *Journal of nematology,* 46**,** 27-34.

SHAPIRO-ILAN, D. I., GOUGE, D. H., PIGGOTT, S. J. & FIFE, J. P. 2006. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biological Control,* 38**,** 124-133.

SHAPIRO-ILAN, D. I., HAN, R. & QIU, X. 2014b. Production of Entomopathogenic Nematodes. In*:* MORALES-RAMOS, J. A., ROJAS, M. G. & SHAPIRO-ILAN, D. I. (eds.) Mass Production of Beneficial Organisms. *San Diego: Academic Press.*

SHAPIRO-ILAN, D. I., LEWIS, E. E., BEHLE, R. W. & MCGUIRE, M. R. 2001. Formulation of Entomopathogenic Nematode-Infected Cadavers. *Journal of Invertebrate Pathology,* 78**,** 17-23.

SHAPIRO-ILAN, D. I., LEWIS, E. E., SON, Y. & TEDDERS, W. L. 2003. Superior efficacy observed in entomopathogenic nematodes applied in infected-host

cadavers compared with application in aqueous suspension. *Journal of Invertebrate Pathology,* 83**,** 270-272.

SICARD, M., LE BRUN, N., PAGES, S., GODELLE, B., BOEMARE, N. & MOULIA, C. 2003. Effect of native *Xenorhabdus* on the fitness of their *Steinernema* hosts: contrasting types of interaction. *Parasitology Research,* 91**,** 520-524.

SIEGERS, K., WALDMANN, T., LEROUX, M. R., GREIN, K., SHEVCHENKO, A., SCHIEBEL, E. & HARTL, F. U. 1999. Compartmentation of protein folding in vivo: sequestration of non-native polypeptide by the chaperonin-GimC system. *The EMBO journal,* 18**,** 75-84.

SOLOMON, A. & GLAZER, I. 1999. Desiccation survival of the entomopathogenic nematode *Steinernema feltiae*: induction of anhydrobiosis. *Nematology,* 1**,** 61-68.

SOLOMON, A., SALOMON, R., PAPERNA, I. & GLAZER, I. 2000. Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable protein. *Parasitology,* 121**,** 409-416.

SRINIVASAN, J., KAPLAN, F., AJREDINI, R., ZACHARIAH, C., ALBORN, H., TEAL, P., MALIK, R., EDISON, A., STERNBERG, P. & SCHROEDER, F. 2008b. A blend of small molecules regulates both mating and development in *Caenorhabditis elegans*. *Nature,* 454**,** 1115-1118.

SRINIVASAN, J., KAPLAN, F., AJREDINI, R., ZACHARIAH, C., ALBORN, H. T., TEAL, P. E., MALIK, R. U., EDISON, A. S., STERNBERG, P. W. & SCHROEDER, F. C. 2008a. A blend of small molecules regulates both mating and development in *Caenorhabditis elegans*. *Nature,* 454**,** 1115-1118.

SRINIVASAN, J., VON REUSS, S. H., BOSE, N., ZASLAVER, A., MAHANTI, P., HO, M. C., O'DOHERTY, O. G., EDISON, A. S., STERNBERG, P. W. &

SCHROEDER, F. C. 2012. A modular library of small molecule signals regulates social behaviors in *Caenorhabditis elegans*. *PLoS biology,* 10**,** e1001237-e1001237.

STRAUCH, O., OESTERGAARD, J., HOLLMER, S. & EHLERS, R.-U. 2004. Genetic improvement of the desiccation tolerance of the entomopathogenic nematode *Heterorhabditis bacteriophora* through selective breeding. *Biological Control,* 31**,** 218-226.

SUMAYA, N. H., GOHIL, R., OKOLO, C., ADDIS, T., DOERFLER, V., EHLERS, R.-U. & MOLINA, C. 2018a. Applying inbreeding, hybridization and mutagenesis to improve oxidative stress tolerance and longevity of the entomopathogenic nematode *Heterorhabditis bacteriophora. Journal of Invertebrate Pathology,* 151**,** 50-58.

SUMAYA, N. H., GOHIL, R., OKOLO, C., ADDIS, T., DOERFLER, V., EHLERS, R. U. & MOLINA, C. 2018b. Applying inbreeding, hybridization and mutagenesis to improve oxidative stress tolerance and longevity of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology,* 151**,** 50-58.

SUMAYA, N. H., OLAREWAJU, O., AMBAW, G., YALI, A., GODINA, G., VANDENBOSSCHE, B., BARG, M., DOERFLER, V., EHLERS, R.-U. & MOLINA, C. 2020. Gene expression analysis of oxidative stress tolerance in the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology,* 23**,** 15-31.

SUNG, D. Y. & GUY, C. L. 2003. Physiological and Molecular Assessment of Altered Expression of Hsc70-1 in Arabidopsis. Evidence for Pleiotropic Consequences.

*Plant Physiology,* 132**,** 979.

SØMME, L. & BIRKEMOE, T. 1997. Cold tolerance and dehydration in Enchytraeidae from Svalbard. *Journal of Comparative Physiology B,* 167**,** 264-269.

THOMAS, J. H. & ROBERTSON, H. M. 2008. The *Caenorhabditis* chemoreceptor gene families. *BMC Biology,* 6**,** 42.

THORNE, M. A. S., BRITOVŠEK, N. K., HAWKINS, L., LILLEY, K. S. & STOREY, K. 2020. Proteomics of intracellular freezing survival. *PLOS ONE,* 15**,** e0233048.

TORR, P., HERITAGE, S. & WILSON, M. 2004. Vibration as novel signal for host location by parasitic nematodes. *International journal for parasitology,* 34**,** 997-9.

TOUBARRO, D., AVILA, M., HAO, Y., BALASUBRAMANIAN, N., JING, Y., MONTIEL, R., FARIA, T., BRITO, R. & SIMÕES, N. 2013. A Serpin Released by an Entomopathogen Impairs Clot Formation in Insect Defense System. *PLOS ONE,* 8**,** e69161.

TOUBARRO, D., LUCENA, M., NASCIMENTO, G., COSTA, G., MONTIEL, R., COELHO, A. & SIMÕES, N. 2009. An apoptosis-inducing serine protease secreted by the entomopathogenic nematode *Steinernema carpocapsae*. *International journal for parasitology,* 39**,** 1319-1330.

TURLINGS, T. C. J., HILTPOLD, I. & RASMANN, S. 2012. The importance of root-produced volatiles as foraging cues for entomopathogenic nematodes. *Plant and Soil,* 358**,** 51-60.

TYSON, T., O'MAHONY ZAMORA, G., WONG, S., SKELTON, M., DALY, B., JONES, J. T., MULVIHILL, E. D., ELSWORTH, B., PHILLIPS, M., BLAXTER, M. & BURNELL, A. M. 2012. A molecular analysis of desiccation tolerance mechanisms in the anhydrobiotic nematode *Panagrolaimus superbus* using

expressed sequenced tags. *BMC Research Notes,* 5**,** 68.

UNGELENK, S., MOAYED, F., HO, C.-T., GROUSL, T., SCHARF, A., MASHAGHI, A., TANS, S., MAYER, M. P., MOGK, A. & BUKAU, B. 2016. Small heat shock proteins sequester misfolding proteins in near-native conformation for cellular protection and efficient refolding. *Nature Communications,* 7**,** 13673.

VAINBERG, I. E., LEWIS, S. A., ROMMELAERE, H., AMPE, C., VANDEKERCKHOVE, J., KLEIN, H. L. & COWAN, N. J. 1998. Prefoldin, a Chaperone that Delivers Unfolded Proteins to Cytosolic Chaperonin. *Cell,* 93**,** 863-873.

VAN TOL, R. W. H. M., VAN DER SOMMEN, A. T. C., BOFF, M. I. C., VAN BEZOOIJEN, J., SABELIS, M. W. & SMITS, P. H. 2001. Plants protect their roots by alerting the enemies of grubs. *Ecology Letters,* 4**,** 292-294.

VON REUSS, S. H., BOSE, N., SRINIVASAN, J., YIM, J. J., JUDKINS, J. C., STERNBERG, P. W. & SCHROEDER, F. C. 2012. Comparative Metabolomics Reveals Biogenesis of Ascarosides, a Modular Library of Small-Molecule Signals in *C. elegans*. *Journal of the American Chemical Society,* 134**,** 1817-1824.

WANG, W., MENG, B., CHEN, W., GE, X., LIU, S. & YU, J. 2007. A proteomic study on postdiapaused embryonic development of brine shrimp (*Artemia franciscana*). *PROTEOMICS,* 7**,** 3580-3591.

WARNOCK, N. D., COX, D., MCCOY, C., MORRIS, R. & DALZELL, J. J. 2019. Transcriptional variation and divergence of host-finding behaviour in *Steinernema carpocapsae* infective juveniles. *BMC Genomics,* 20**,** 884.

WESTH, P. & RAMLØV, H. 1991. Trehalose accumulation in the tardigrade *Adorybiotus coronifer* during anhydrobiosis. *Journal of Experimental Zoology,* 258**,** 303-311.

WHARTON, D. 2015. Anhydrobiosis. *Current Biology,* 25**,** R1114-R1116.

WHARTON, D. & SURREY, M. 1994. Cold tolerance mechanisms of the infective larvae of the insect parasitic nematode, *Heterorhabditis zealandica* Poinar. *Cryo letters*.

WHARTON, D. A. 2011. Cold tolerance in Perry, R. N. & Wharton, D. A. Molecular and physiological basis of nematode survival. *CABI,* 182-198

WHARTON, D. A., DOWNES, M. F., GOODALL, G. & MARSHALL, C. J. 2005. Freezing and cryoprotective dehydration in an Antarctic nematode (*Panagrolaimus davidi*) visualised using a freeze substitution technique. *Cryobiology,* 50**,** 21-28.

WHARTON, D. A., GOODALL, G. & MARSHALL, C. J. 2003. Freezing survival and cryoprotective dehydration as cold tolerance mechanisms in the Antarctic nematode *Panagrolaimus davidi*. *Journal of Experimental Biology,* 206**,** 215-221.

WHITE, J. G., SOUTHGATE, E., THOMSON, J. N. & BRENNER, S. 1986. The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 314**,** 1-340.

WILLIAMS, B., NJACI, I., MOGHADDAM, L., LONG, H., DICKMAN, M. B., ZHANG, X. & MUNDREE, S. 2015. Trehalose Accumulation Triggers Autophagy during Plant Desiccation. *PLoS Genetics,* 11**,** e1005705.

WILSON, M., EHLERS, R.-U. & GLAZER, I. 2012. Entomopathogenic nematode foraging strategies - Is *Steinernema carpocapsae* really an ambush forager? *Nematology,* 14**,** 389-394.

WINGLER, A. 2002. The function of trehalose biosynthesis in plants. *Phytochemistry,* 60**,** 437-440.

WOMERSLEY, C. & CHING, C. 1989. Natural dehydration regimes as a prerequisite for the successful induction of anhydrobiosis in the nematode *Rotylenchulus reniformis*. *Journal of Experimental Biology,* 143**,** 359-72.

WOMERSLEY, C. Z. 1990. Dehydration survival and anhydrobiotic potential. Entomopathogenic nematodes in biological control. *CRC Press Boca Raton, FL*.

WU, S., KAPLAN, F., LEWIS, E., ALBORN, H. T. & SHAPIRO-ILAN, D. I. 2018a. Infected host macerate enhances entomopathogenic nematode movement towards hosts and infectivity in a soil profile. *Journal of Invertebrate Pathology,* 159**,** 141-144.

WU, X., ZHU, X., WANG, Y., LIU, X., CHEN, L. & DUAN, Y. 2018b. The cold tolerance of the northern root-knot nematode, *Meloidogyne hapla*. *PLOS ONE,* 13**,** e0190531.

XIE, Y.-F., WANG, X.-D., ZHONG, W.-H., ZHU, D.-H. & HE, Z. 2020. Transcriptome profile changes associated with heat shock reaction in the entomopathogenic nematode, *Steinernema carpocapsae*. *Frontiers in Physiology,* 11.

YAARI, M., DORON-FAIGENBOIM, A., KOLTAI, H., SALAME, L. & GLAZER, I. 2016. Transcriptome analysis of stress tolerance in entomopathogenic nematodes of the genus Steinernema. *International Journal for Parasitologyy*, 46**,** 83-95.

YAMADA, K., HIROTSU, T., MATSUKI, M., BUTCHER, R. A., TOMIOKA, M., ISHIHARA, T., CLARDY, J., KUNITOMO, H. & IINO, Y. 2010. Olfactory plasticity is regulated by pheromonal signaling in *Caenorhabditis elegans*. *Science,* 329**,** 1647-1650.

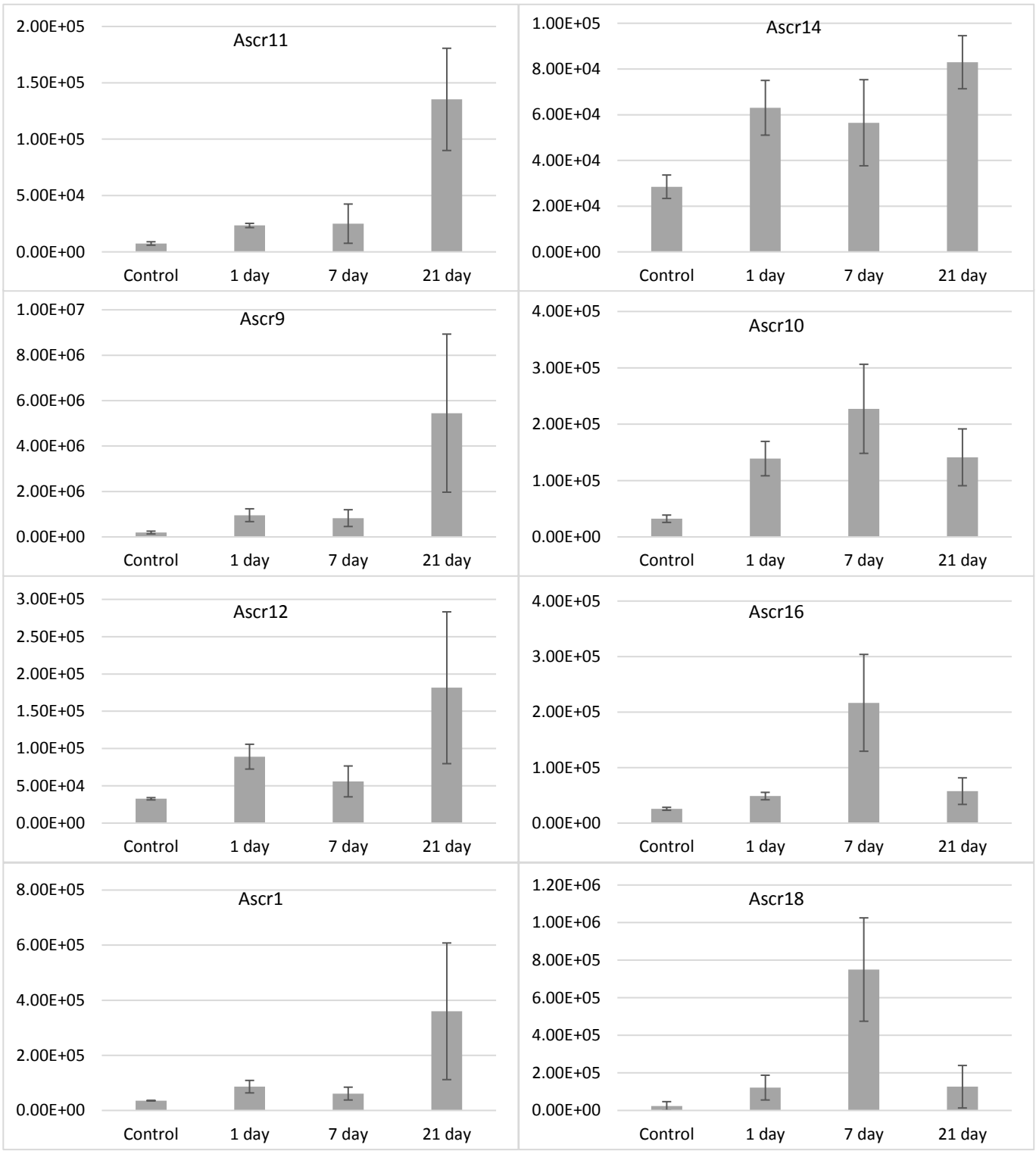
ZHAO, L., AHMAD, F., LU, M., ZHANG, W., WICKHAM, J. D. & SUN, J. 2018. Ascarosides Promote the Prevalence of Ophiostomatoid Fungi and an Invasive

Pathogenic Nematode, *Bursaphelenchus xylophilus*. *Journal of Chemical Ecology,* 44**,** 701-710.

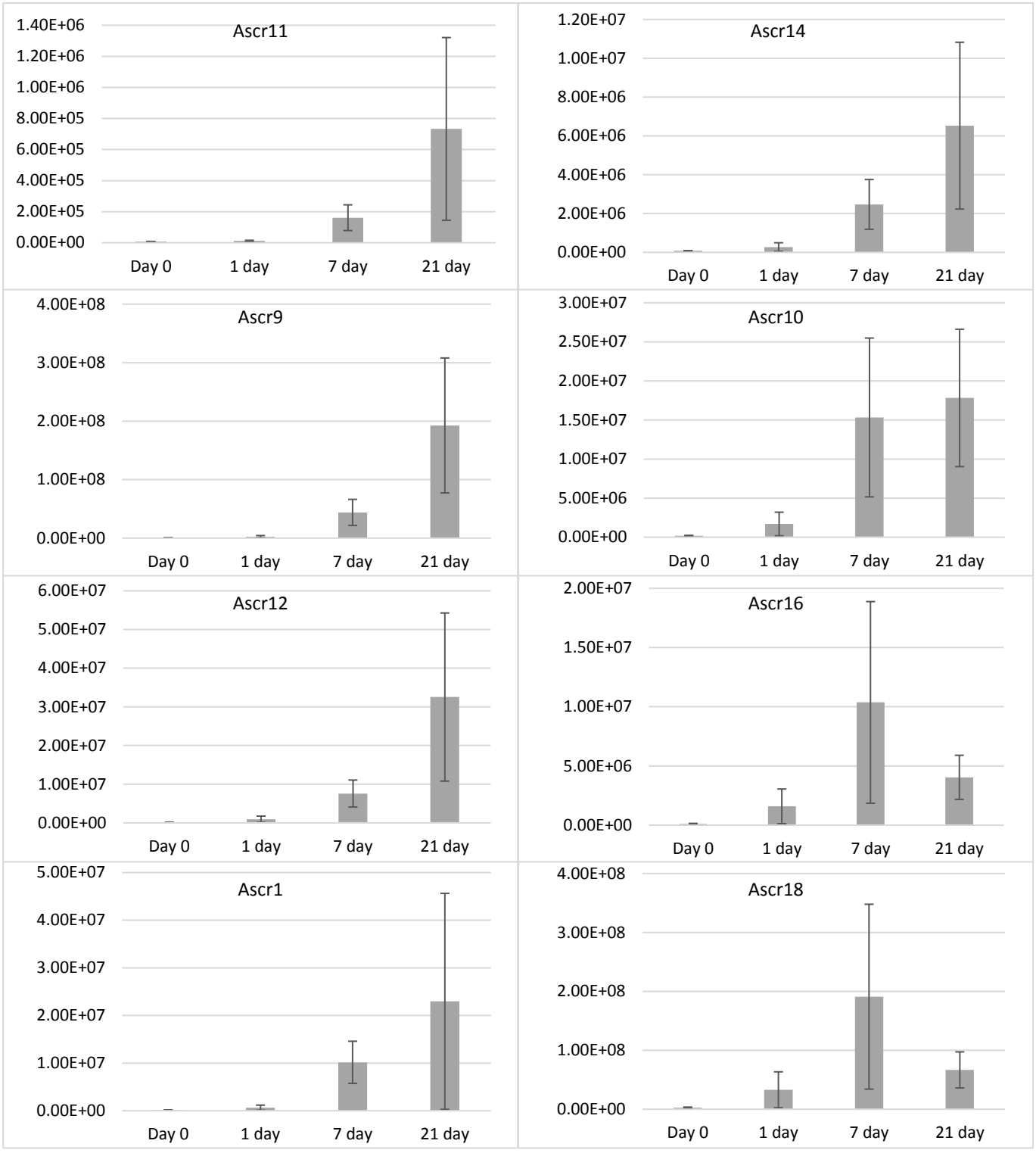
ZHAO, L., ZHANG, X., WEI, Y., ZHOU, J., ZHANG, W., QIN, P., CHINTA, S., KONG, X., LIU, Y., YU, H., HU, S., ZOU, Z., BUTCHER, R. A. & SUN, J. 2016. Ascarosides coordinate the dispersal of a plant-parasitic nematode with the metamorphosis of its vector beetle. *Nature Communications,* 7**,** 12341.

ZHENG, J., SU, H., LIN, R., ZHANG, H., XIA, K., JIAN, S. & ZHANG, M. 2019. Isolation and characterization of an atypical LEA gene (IpLEA) from *Ipomoea pes-caprae* conferring salt/drought and oxidative stress tolerance. *Scientific Reports,* 9**,** 14838.

# Appendices

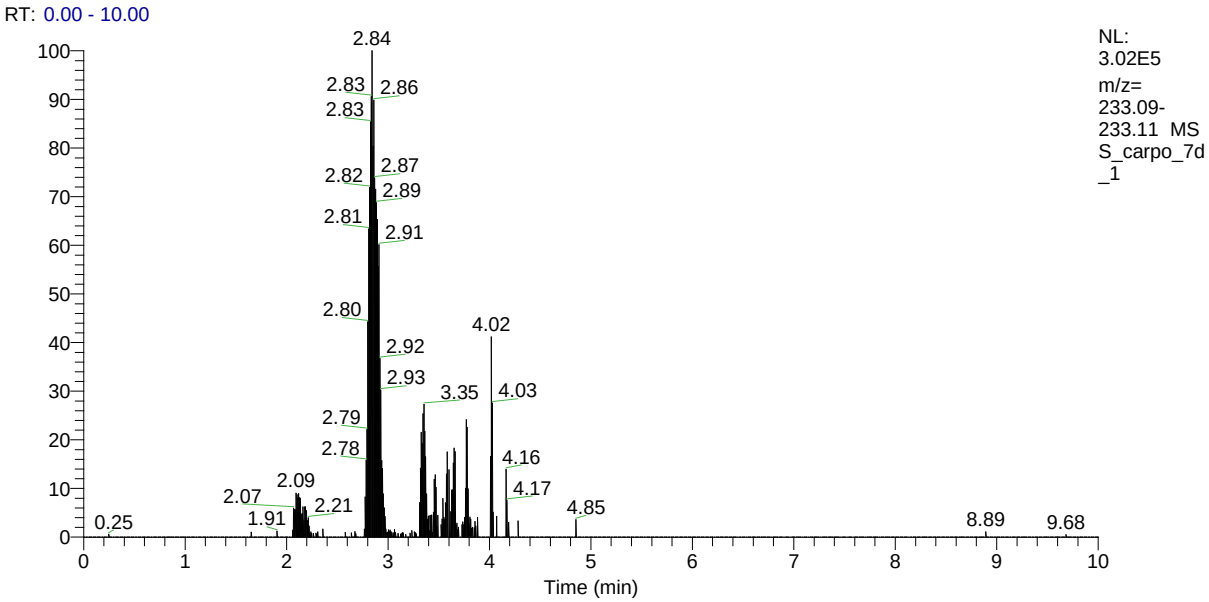


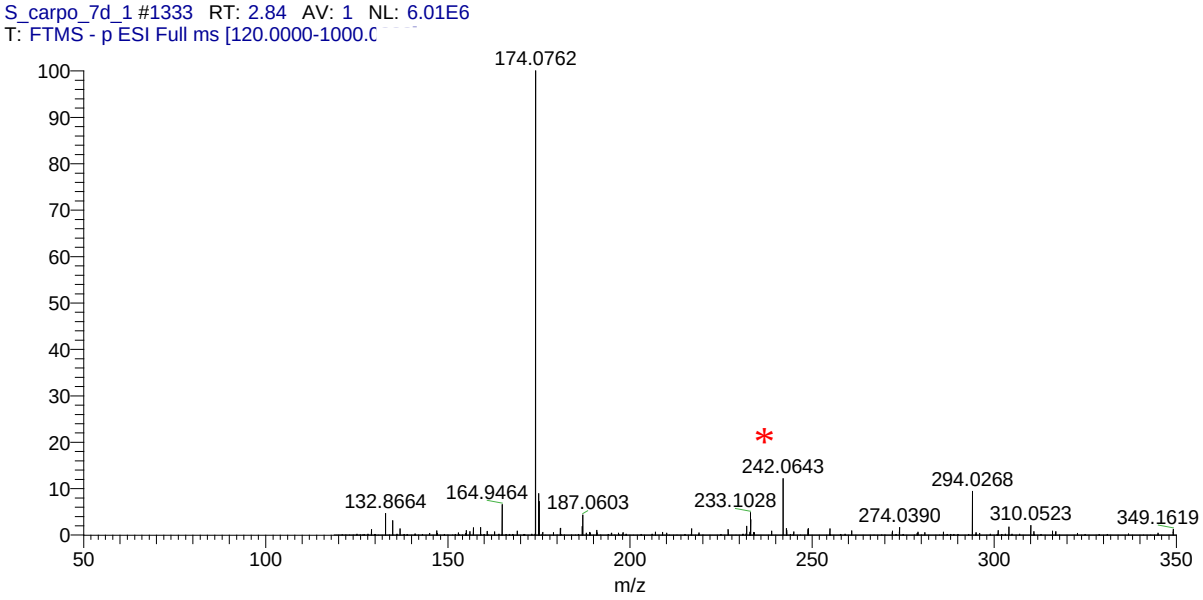

**Appendix 2.1.** Relative abundance (mean + SE) for each ascaroside detected in the exometabolome of *Steinernema carpocapsae* infective juveniles. Values are for 5000 IJs worm equivalence.

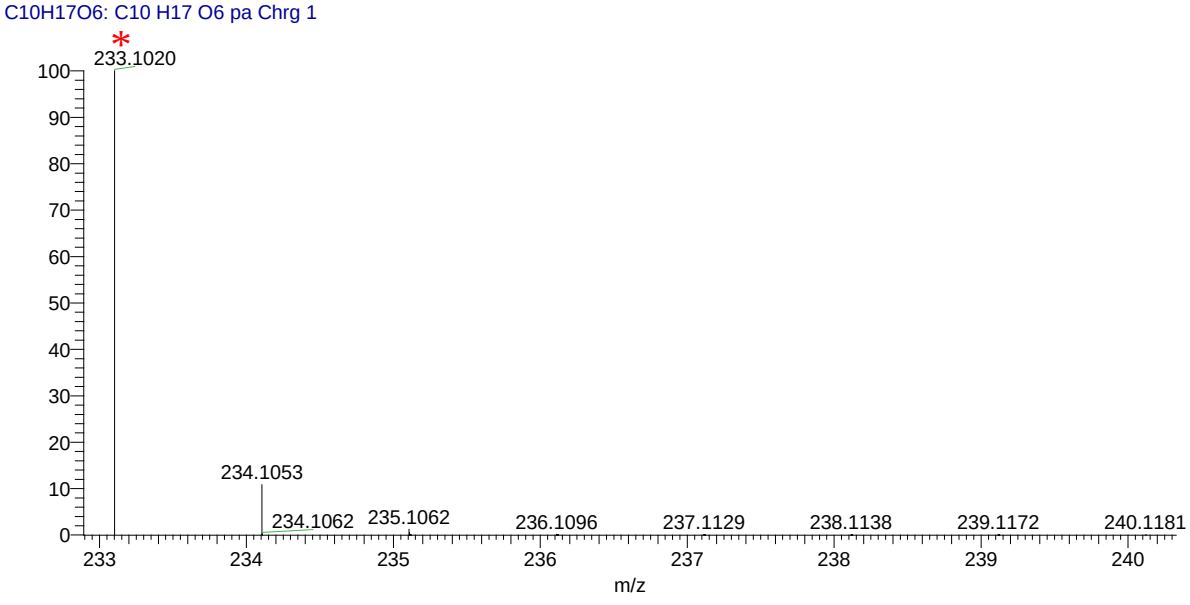



**Appendix 2.2**. Relative abundance (mean ± SE) for each ascaroside detected in the exometabolome of *Heterorhabditis megidis* infective juveniles. Values are for 5000 IJs worm equivalence.

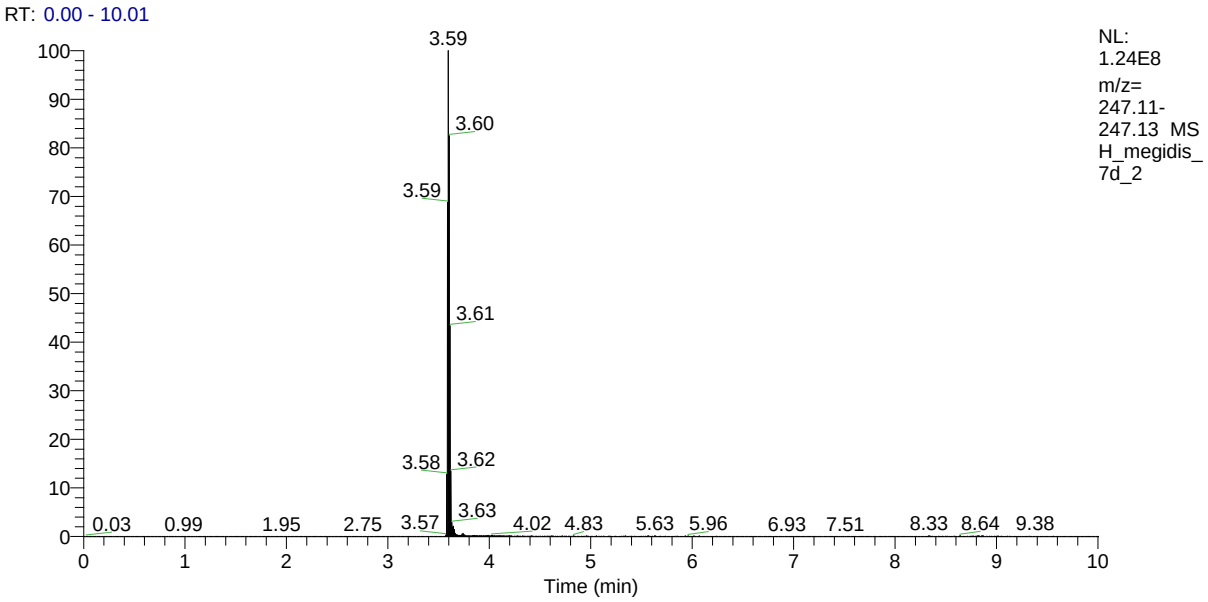
Representative Ascaroside 11 chromatogram (*S. carpocapsae)*

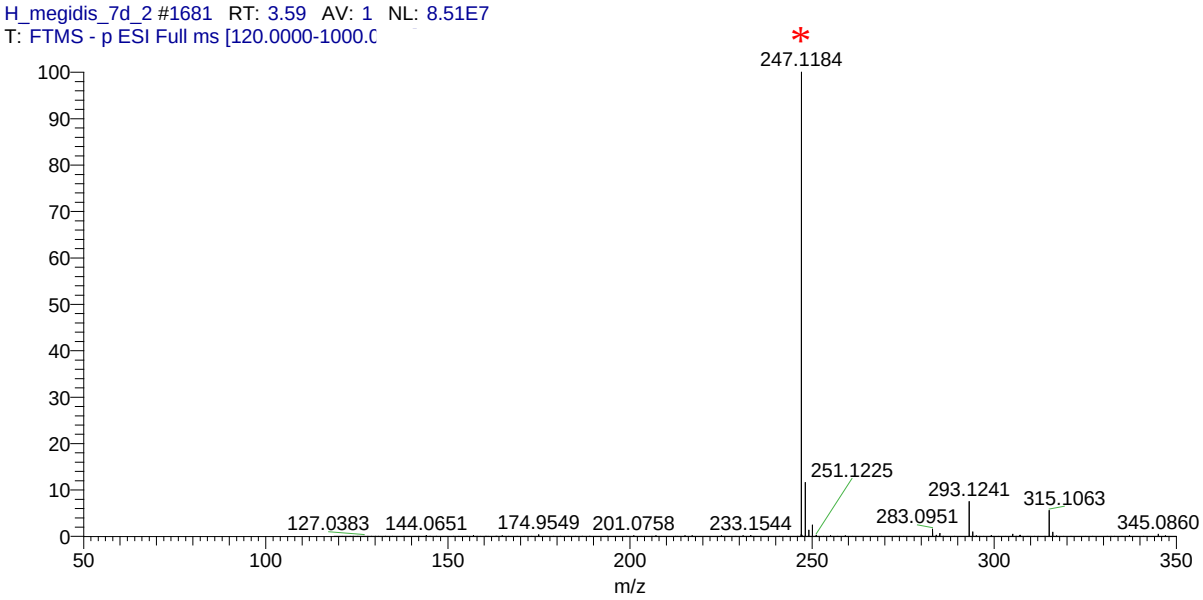


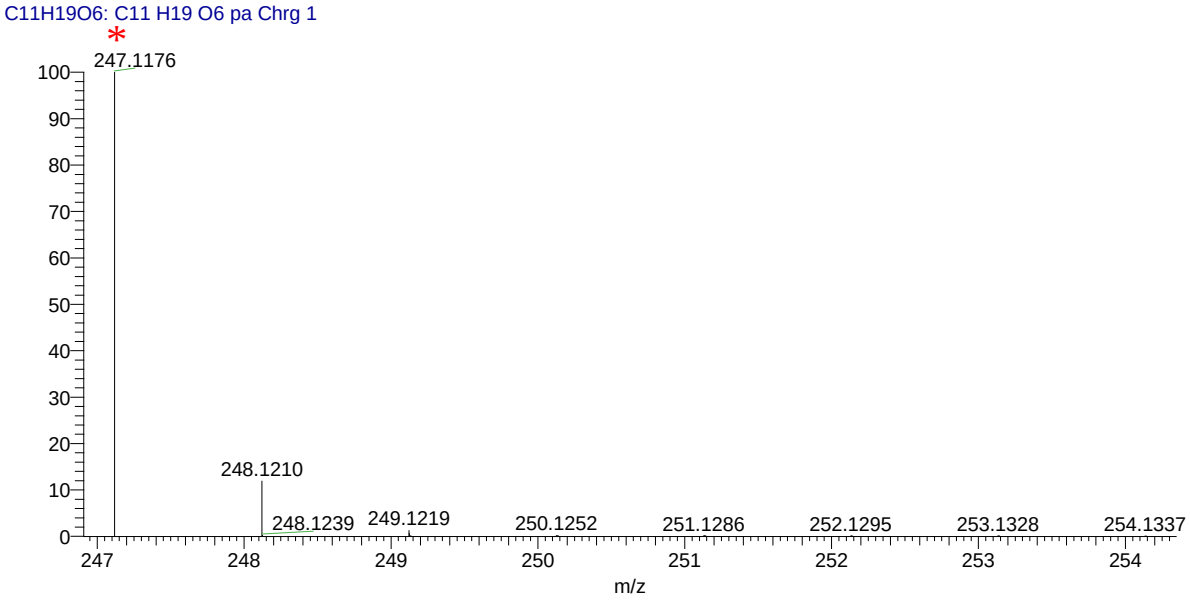



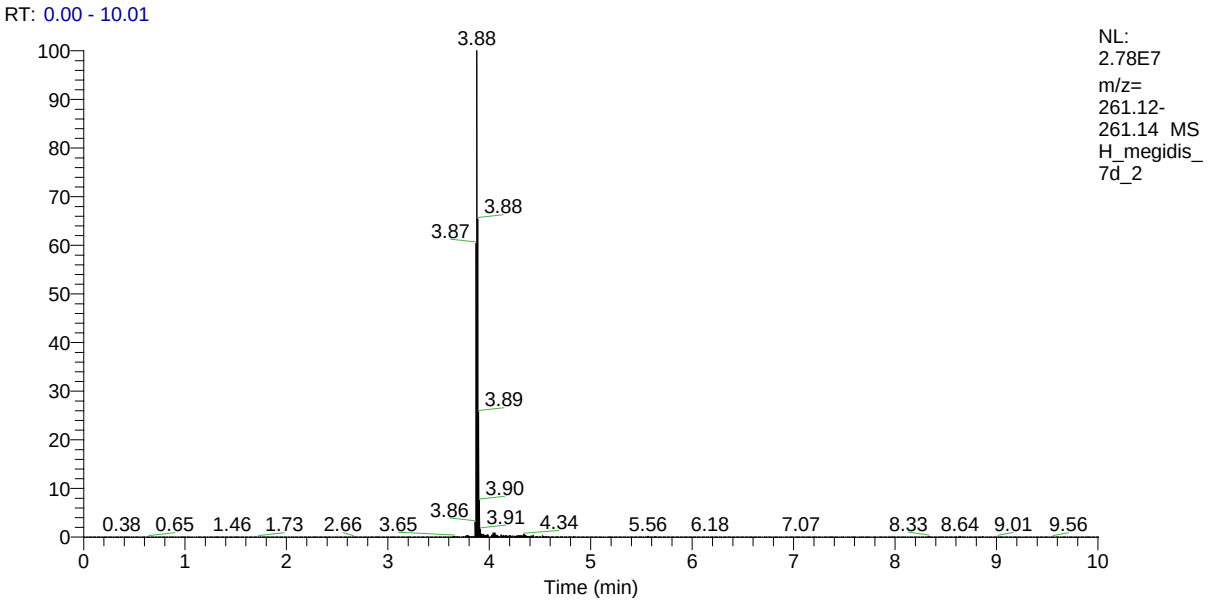
Representative Ascaroside 9 chromatogram (*H. megidis)*

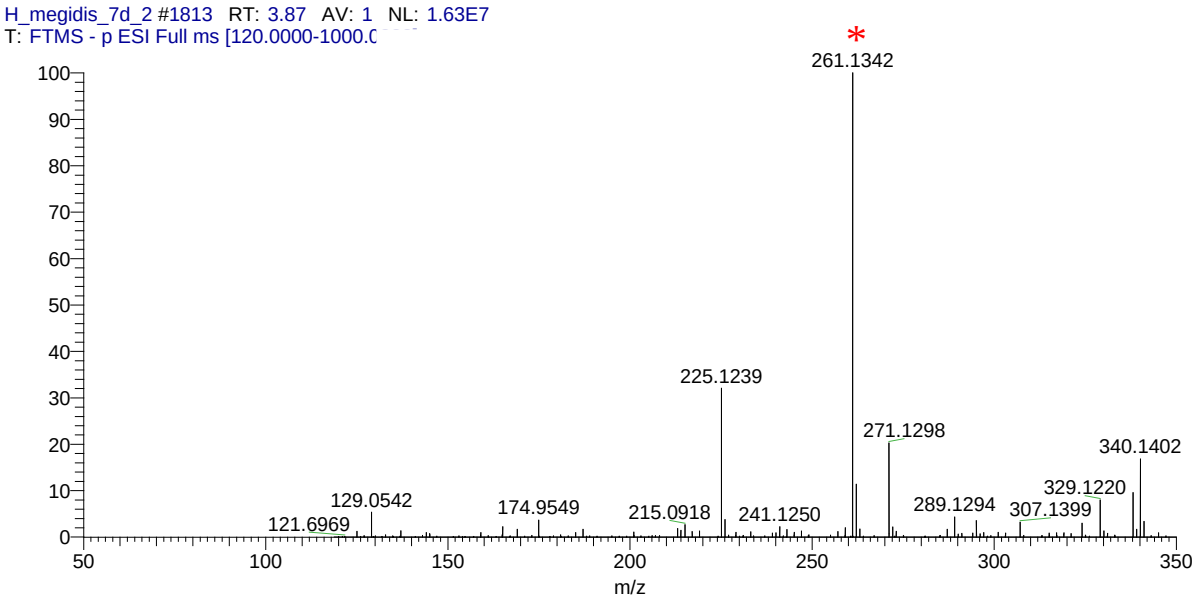


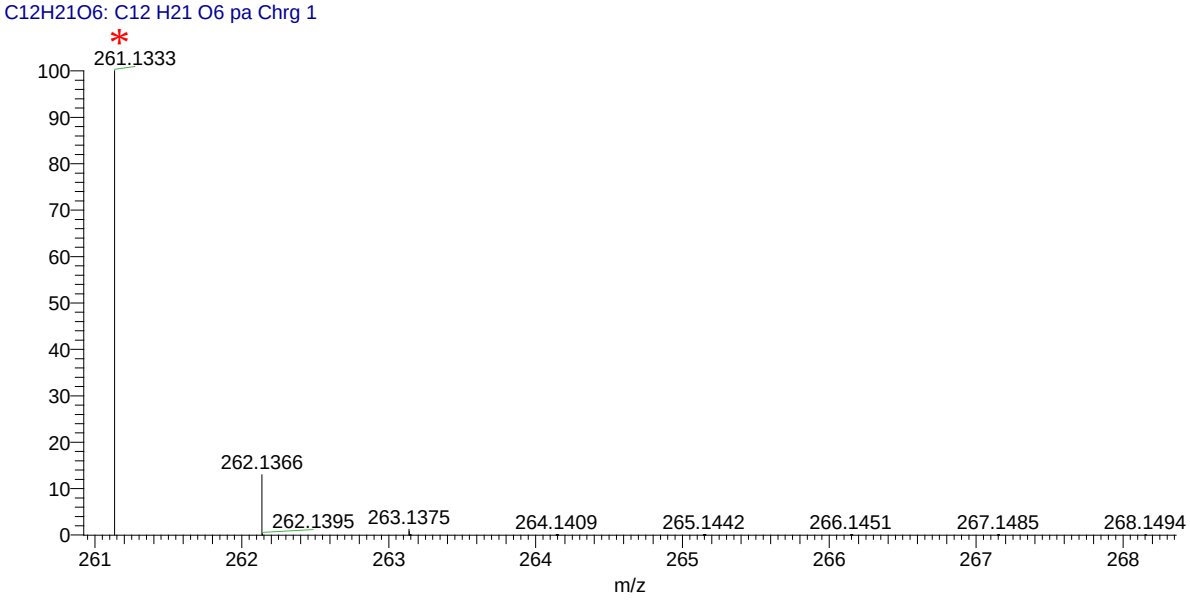



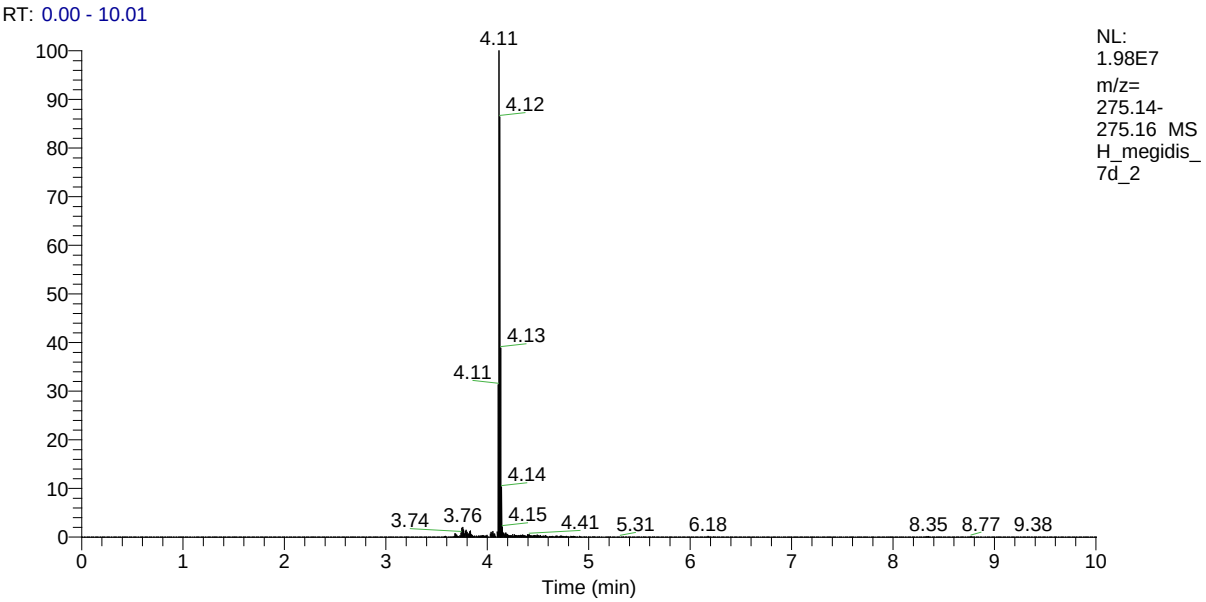
Representative Ascaroside 12 chromatogram (*H. megidis)*

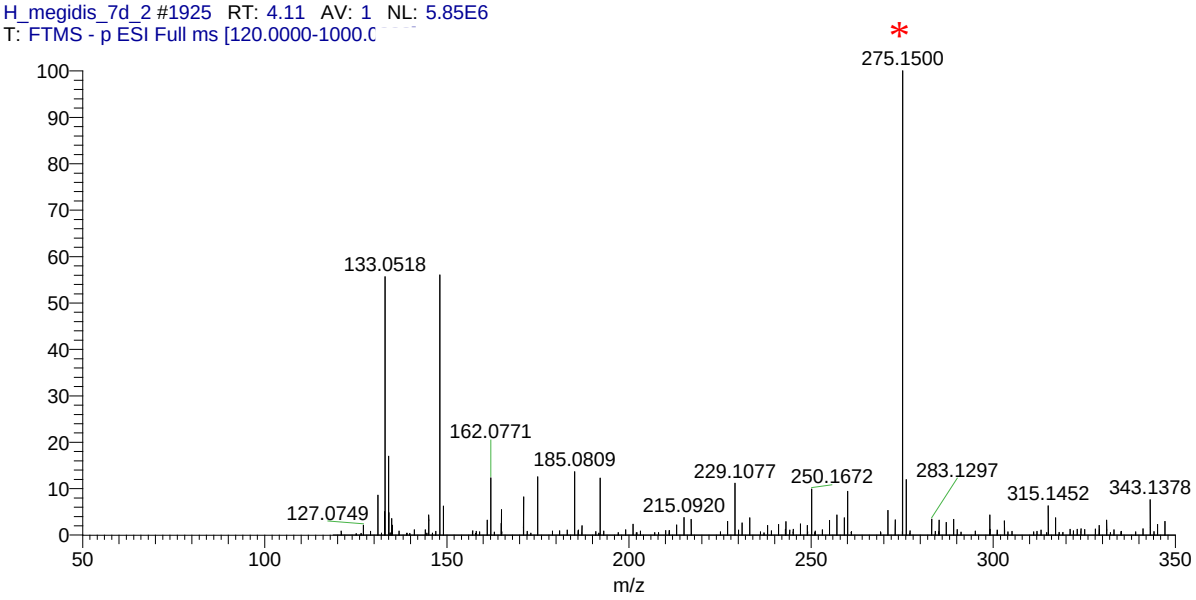


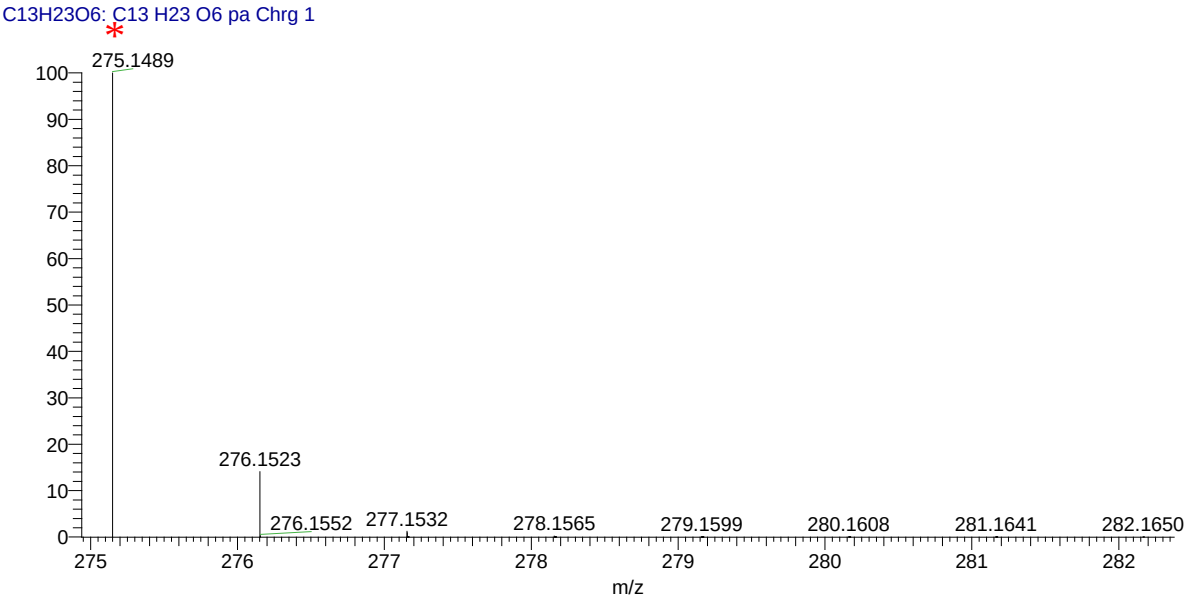



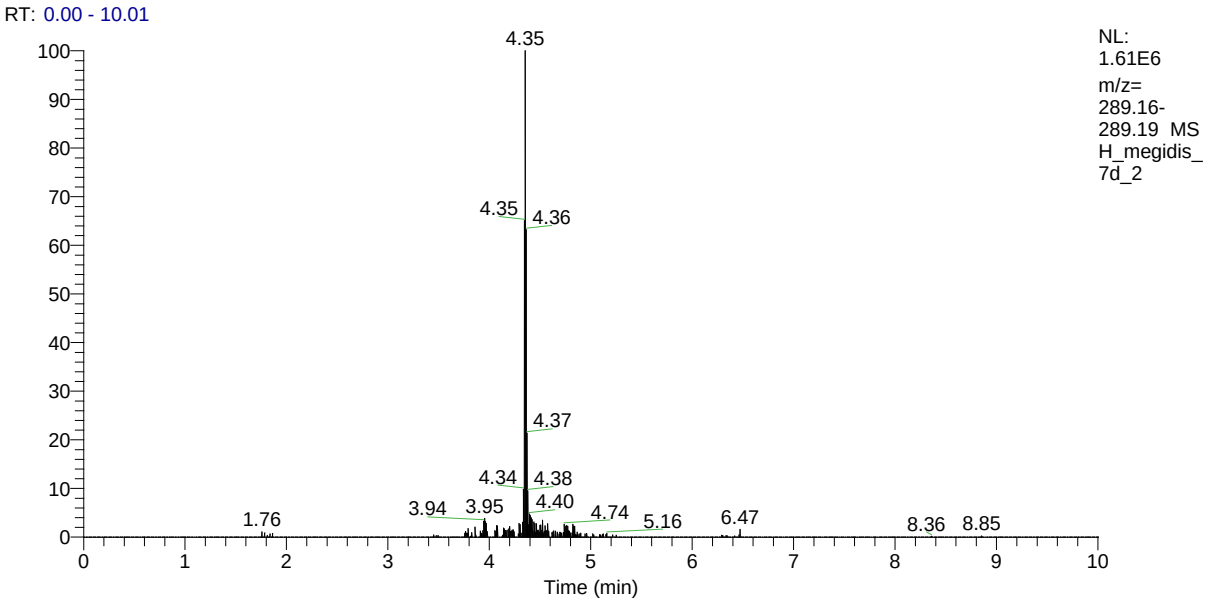
Representative Ascaroside 1 chromatogram (*H. megidis)*

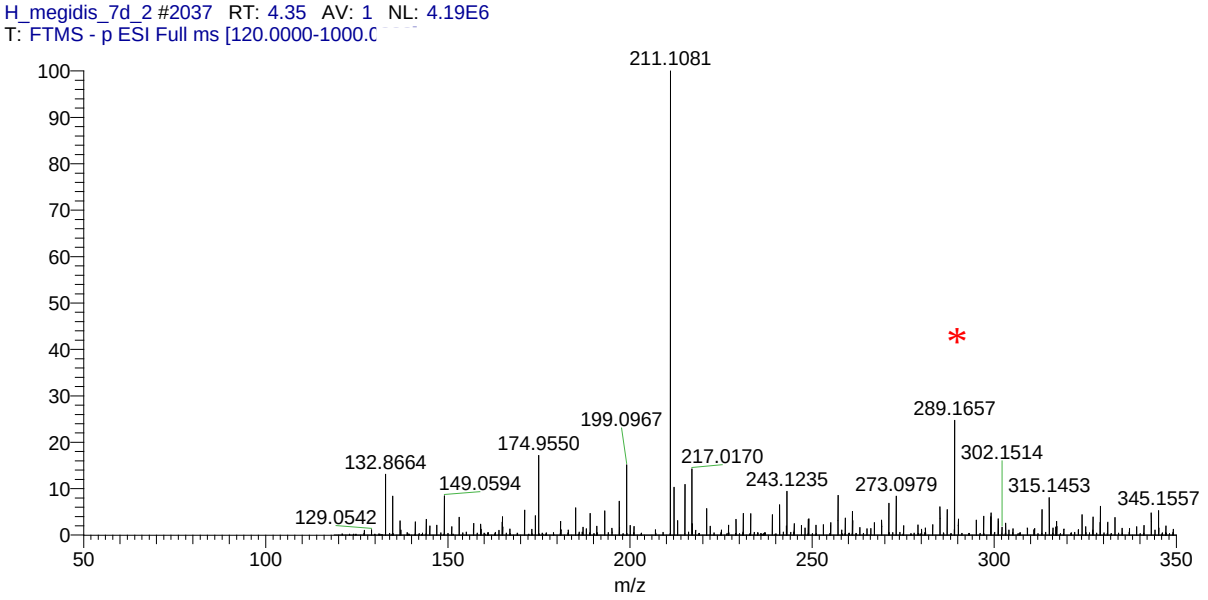


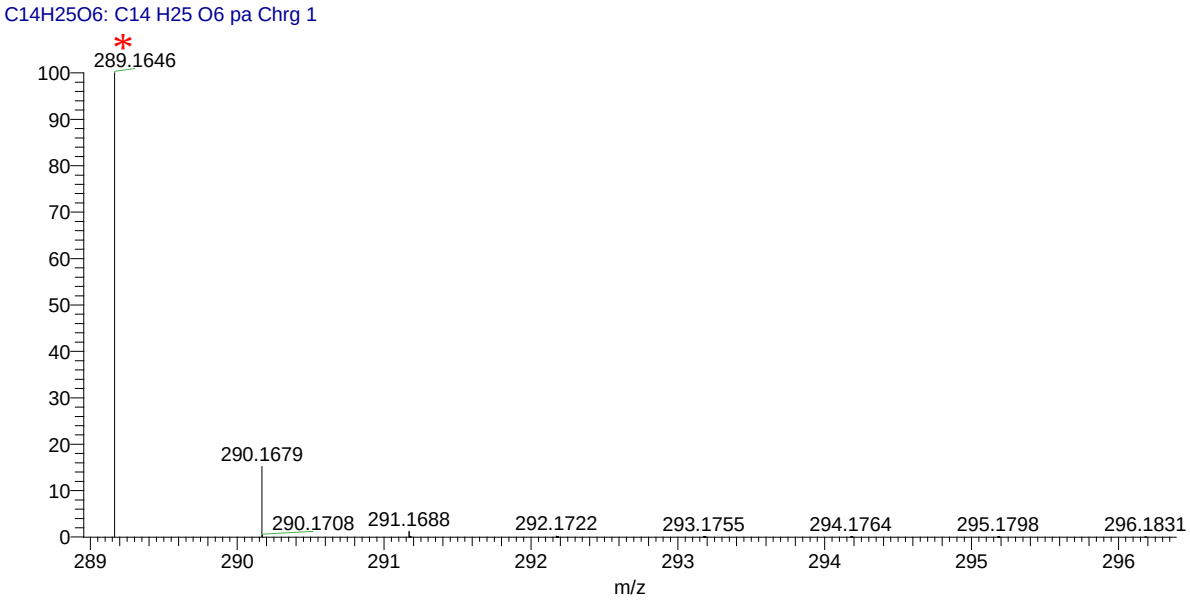



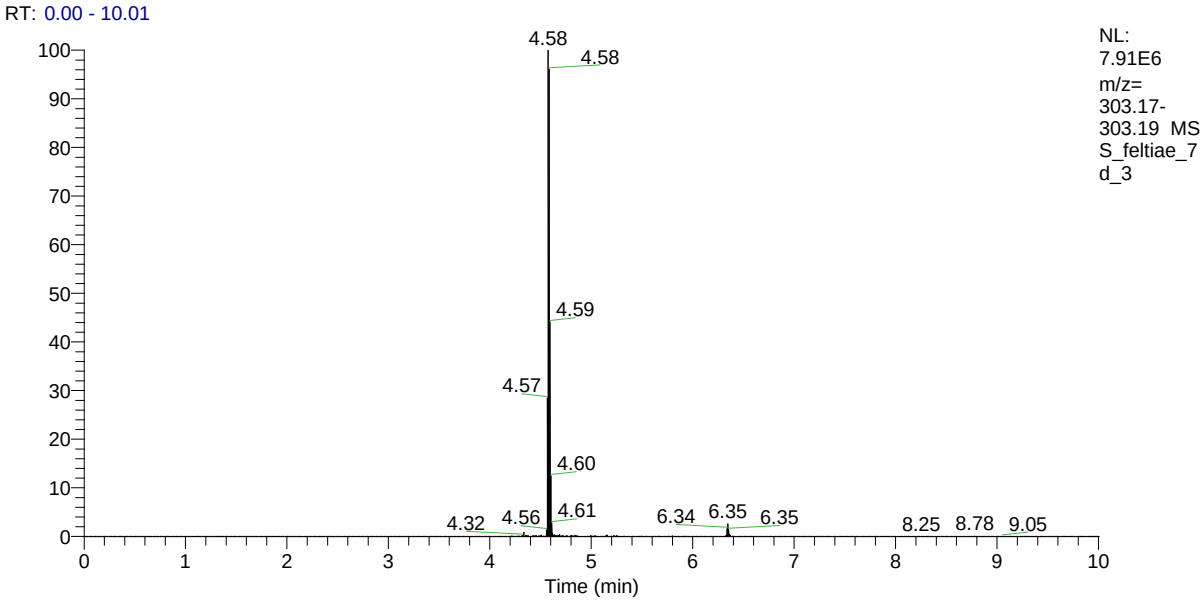
Representative Ascaroside 14 chromatogram (*H. megidis)*

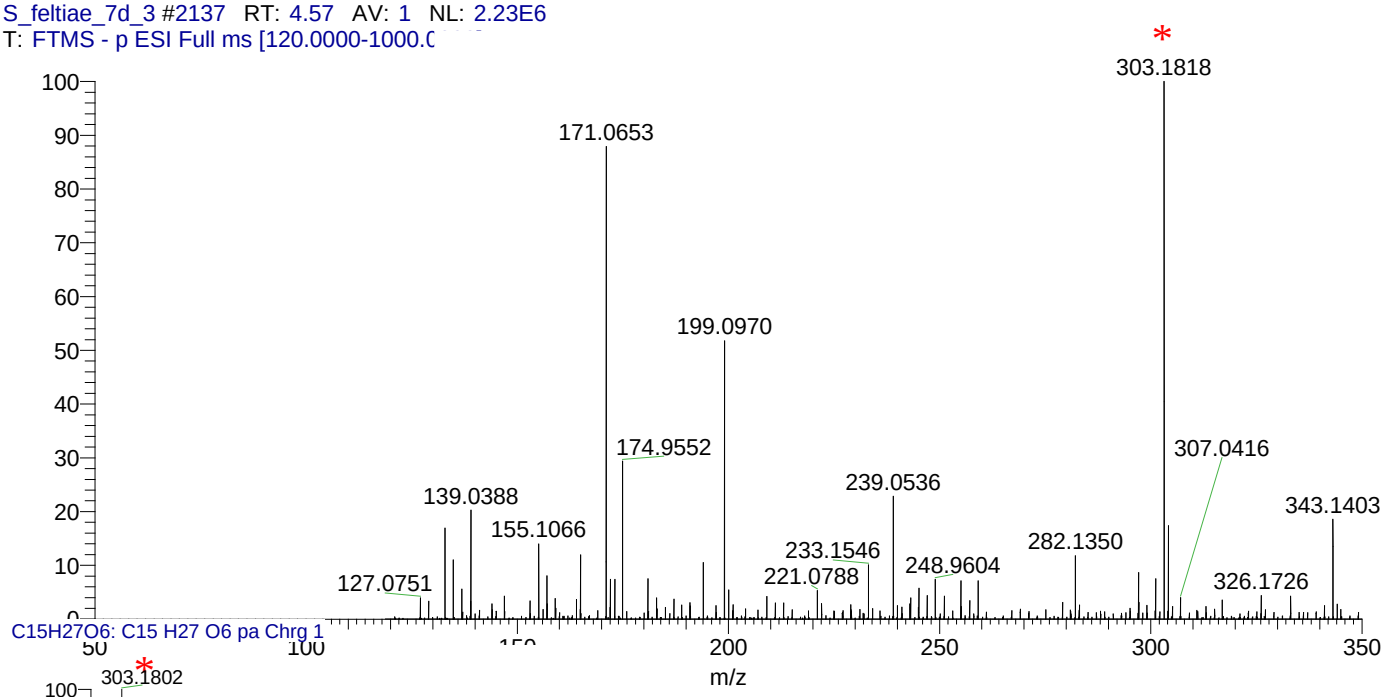


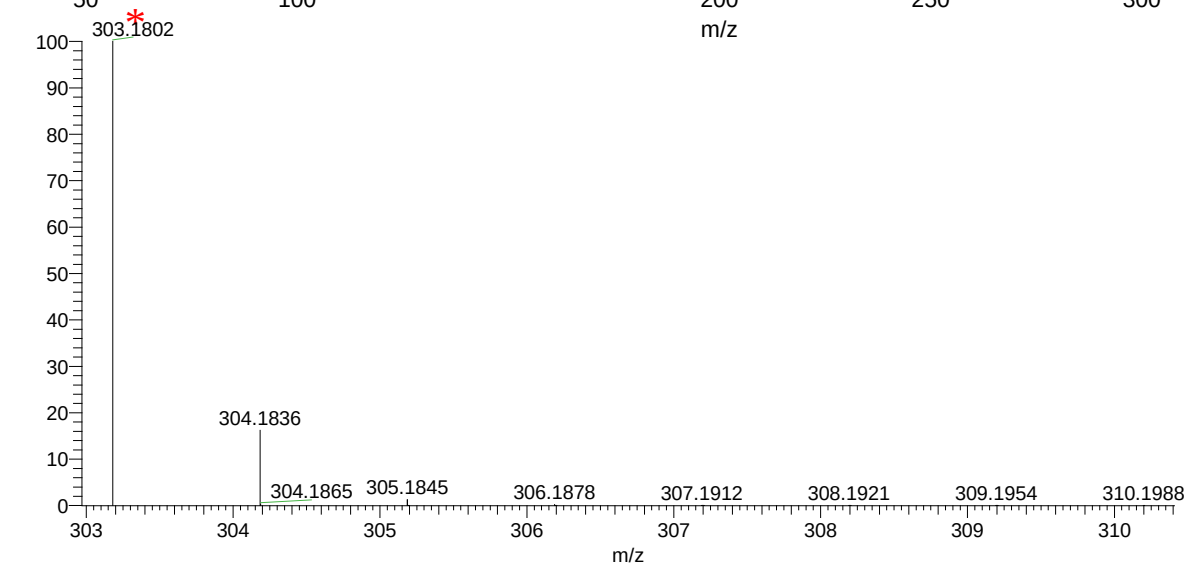



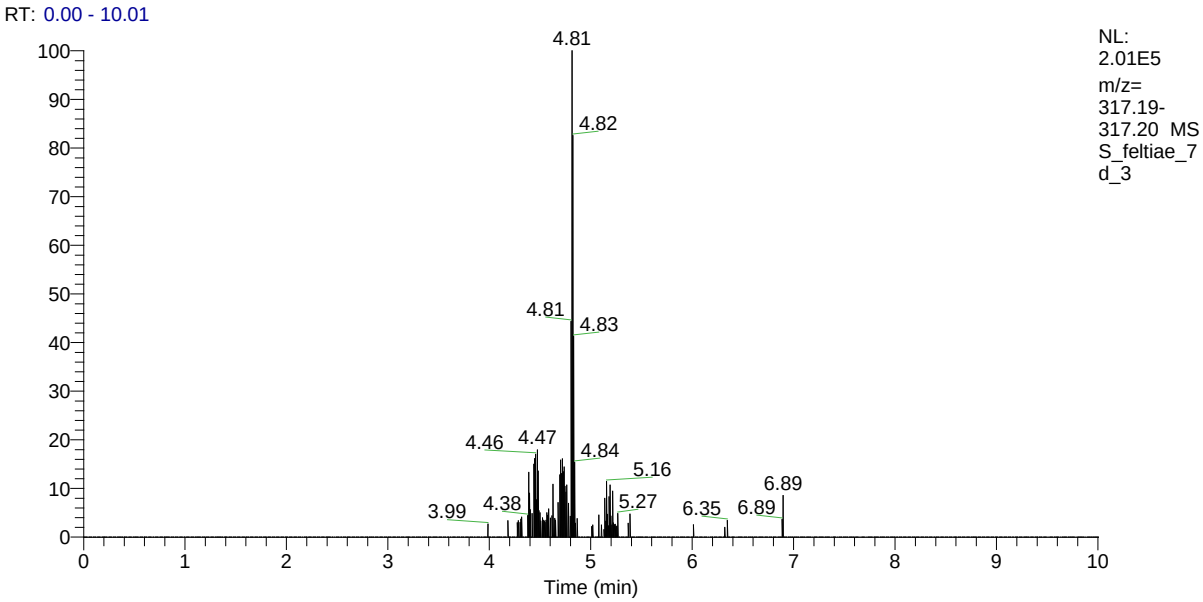
Representative Ascaroside 10 chromatogram (*S. feltiae)*

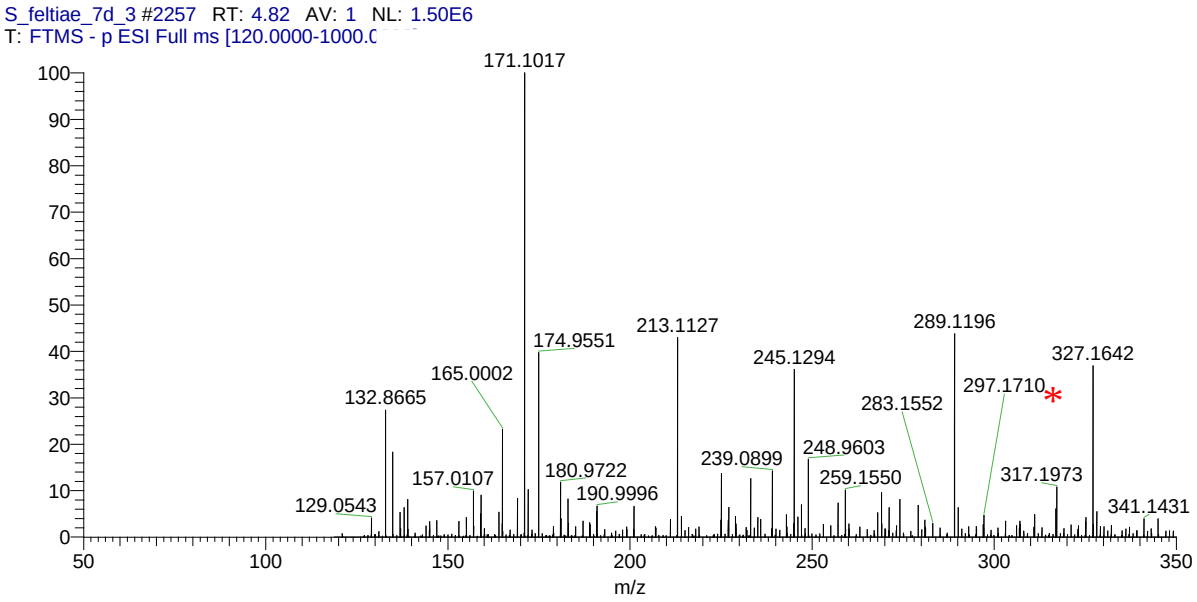


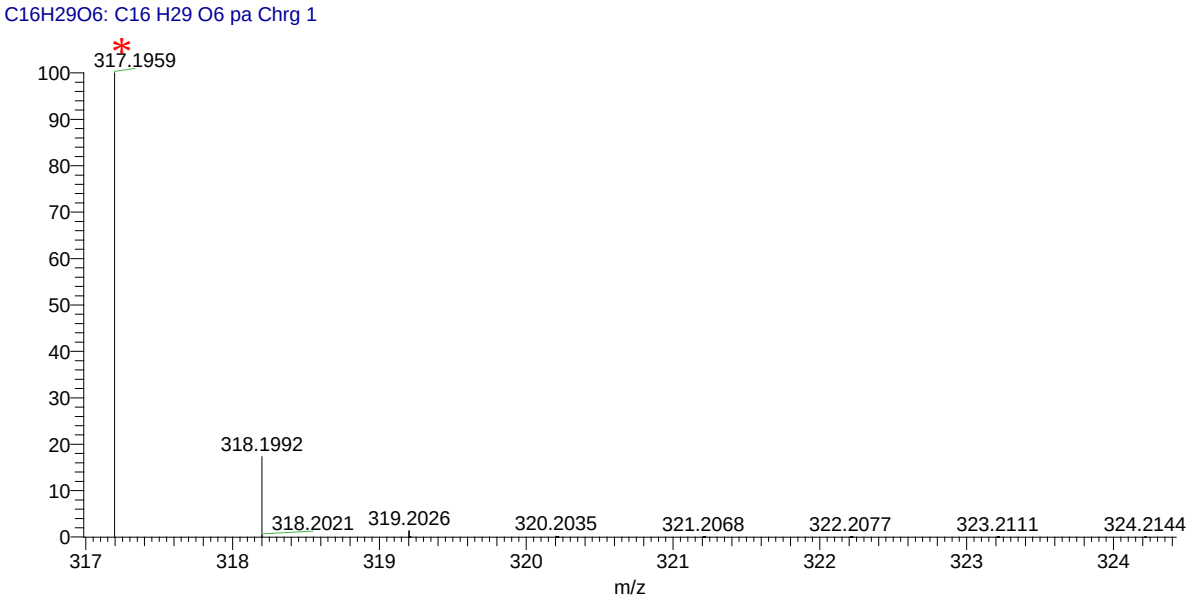



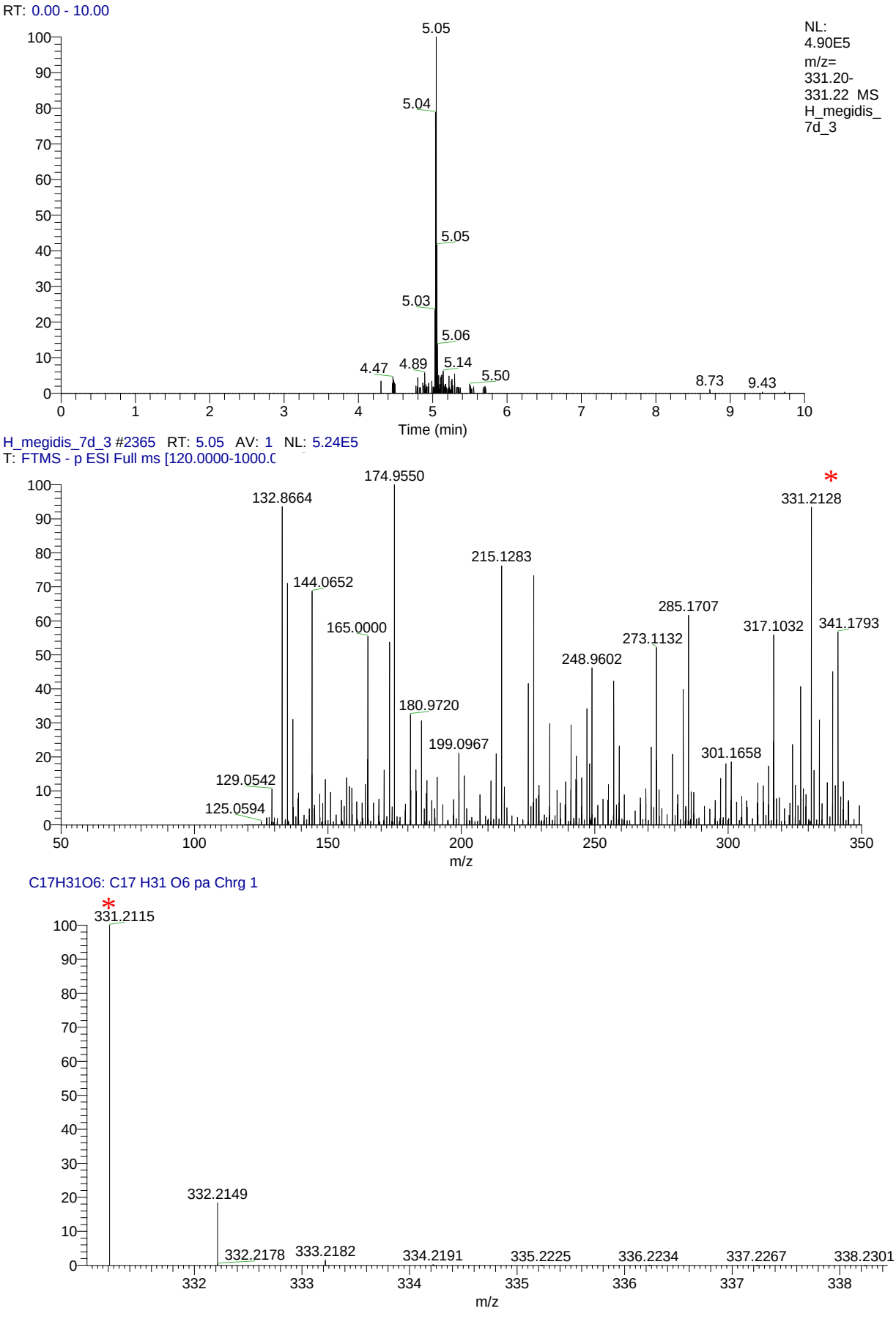
Representative Ascaroside 16 chromatogram (*S. feltiae)*



Representative Ascaroside 18 chromatogram (*H. megidis)*



**Appendix 2.3.** Representative chromatograms and mass spectra of ascarosides quantified in this study. Top: Extracted ion chromatogram (EICs) of ascaroside based on the parent mass/charge (*m/z*) of the deprotonated molecule. Base peak in each chromatogram indicates the retention time of each ascaroside. Middle: Mass spectra of respective ascarosides with measured m/z of parent molecules indicated (*). Bottom: Simulated mass spectra of respective ascarosides based on the molecular formula of deprotonated ascarosides, with predicted *m/z* indicated (*).

| Ascaroside | *Steinernema carpocapsae* | | *Heterorhabditis megidis* | |
|---|---|---|---|---|
| | 7 days | 21 days | 7 days | 21 days |
| Ascr#11 | | **0.002** | | **0.051** |
| Ascr#9 | | **0.032** | | **0.014** |
| Ascr#12 | | **0.071** | | **0.024** |
| Ascr#1 | | **0.058** | | **0.106** |
| Ascr#14 | | **0.044** | | **0.027** |
| Ascr#10 | **0.003** | 0.457 | | **0.063** |
| Ascr#16 | **0.001** | 0.819 | **0.133** | 0.595 |
| Ascr#18 | **0.001** | 0.873 | **0.138** | 0.659 |

**Appendix 2.4.** Probability values for regression of ascaroside relative abundance on time for *Steinernema carpocapsae* and *Heterorhabditis megidis* infective juveniles stored in water for 7 or 21 days. Where the P value for the regression over the full 21-day period was > 0.10, the regression was run for the first 7 days only. Values in bold are the values shown in Figure 2.1.

# Appendix 3.1.A. Log$_2$fold transformed abundances of proteins identified as statistically significant (P<0.05 in student t-tests) relative to week 0 in *S. carpocapsae* IJs after conditioning.

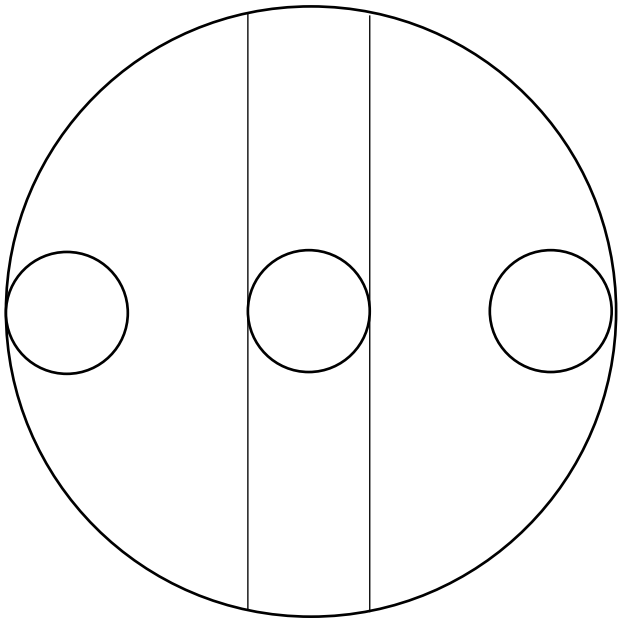
| Protein IDs | Blast Annotation | PFAM Annotation | Log$_2$fold change | | | | |
|---|---|---|---|---|---|---|---|
| | **Ribosome related proteins** | | 3w20°C | 6w20°C | 3w9°C | 6w9°C | 9w9°C |
| L596_007088 | large subunit ribosomal protein 32 | Ribosomal_protein_L32_PF01655 | 0.6 | 0.9 | | 0.6 | 0.7 |
| L596_007094 | ribosomal protein L19 | Ribosomal_protein_L19_PF01245 | | | | | 1.6 |
| L596_007400.2 | putative ribosomal protein L24 | KOW_motif_PF00467 | | | -0.8 | | |
| L596_007886 | 60S acidic ribosomal protein P1 | 60s_Acidic_ribosomal_protein_PF00428 | | 0.6 | | | |
| L596_008044 | 28S ribosomal protein S22, mitochondrial | Mitochondrial_28S_ribosomal_protein_S22_PF10245 | | 1.9 | | | |
| L596_008394 | 60S ribosomal protein L27 | Ribosomal_L27e_protein_family_PF01777 | 0.5 | 0.5 | 0.3 | 0.8 | 0.6 |
| L596_008523 | ribosomal protein S2 | Ribosomal_protein_S2_PF00318 | | | | -1.7 | |
| L596_009145.2 | hypothetical protein L596_009145 | 50S_ribosome-binding_GTPase_PF01926 | | | 3.5 | 3.7 | 4.6 |
| L596_009212 | 60S ribosomal protein L17 | Ribosomal_protein_L22p/L17e_PF00237 | | 0.6 | | | |
| L596_009715 | 40S ribosomal protein S11 | Ribosomal_protein_S17_PF00366 | | | | -0.6 | |
| L596_010756 | putative 50S ribosomal protein L7Ae | Ribosomal_protein_L7Ae/L30e/S12e/Gadd45_family_PF01248 | | | -4.3 | | |
| L596_013726 | 40S ribosomal protein S9 | S4_domain_PF01479 | 0.6 | 0.8 | | 0.8 | |
| L596_014863.2 | ribosomal protein L5 | ribosomal_L5P_family_C-terminus_PF00673 | 0.3 | 0.6 | | | |
| L596_014864.2 | 60S ribosomal protein L3 | Ribosomal_protein_L3_PF00297 | 0.4 | 0.6 | | | |
| L596_014937 | Ribosomal family S4e | Ribosomal_family_S4e_PF00900 | 0.3 | 0.6 | | | |
| L596_015768 | 60S acidic ribosomal protein P0 | 60s_Acidic_ribosomal_protein_PF00428 | | 0.6 | | | |
| L596_016621 | S25 ribosomal protein | S25_ribosomal_protein_PF03297 | | 1.1 | | | |
| L596_016864 | large subunit ribosomal protein 1 | Ribosomal_protein_L1p/L10e_family_PF00687 | 0.6 | 1.0 | | | |
| L596_017116 | Ribosome maturation protein SBDS | SBDS_protein_C-terminal_domain_PF09377 | | | | 1.3 | |
| L596_017949 | small subunit ribosomal protein 1 | Ribosomal_S3Ae_family_PF01015 | | 1.1 | | | |
| L596_018442 | 60S ribosomal protein L22 | Ribosomal_L22e_protein_family_PF01776 | | 0.8 | | | |
| L596_018948 | ribosomal protein L35Ae | Ribosomal_protein_L35Ae_PF01247 | | 1.0 | | | |
| L596_022937 | 60S ribosomal protein L12 | Ribosomal_protein_L11,_N-terminal_domain_PF03946 | | 1.0 | | | |
| L596_023590 | small subunit ribosomal protein 1 | Ribosomal_S3Ae_family_PF01015 | | 0.8 | | | |
| L596_024435 | ribosomal protein S15 | Ribosomal_S13/S15_N-terminal_domain_PF08069 | 0.6 | | 0.4 | 0.8 | 0.5 |
| L596_026004 | ribosomal protein S19 | Ribosomal_protein_S19_PF00203 | | | | 2.7 | |
| SC.X.g4331 | 40S ribosomal protein S23 | Ribosomal_protein_S12/S23_PF00164 | | | | 1.4 | |
| SC.X.g5301.1 | 60S ribosomal protein L23a 2-like | Ribosomal_protein_L23,_N-terminal_domain_PF03939 | | 1.1 | | | 1.2 |
| SC.X.g5303 | small subunit ribosomal protein 7 | Ribosomal_protein_S7e_PF01251 | | 1.0 | | | |
| | **Chaperone related proteins** | | | | | | |
| L596_022153 | Body wall muscle protein HR-29 | Hsp20/alpha crystallin family PF00011 | 0.7 | 0.4 | 0.7 | 1.0 | 1.0 |
| L596_022428 | CDC-37 protein | Cdc37 Hsp90 binding domain PF08565 | | | | 2.4 | |
| L596_021416 | chaperone protein DnaK | Hsp70 protein PF00012 | | | -0.5 | | -0.6 |
| L596_026406 | CRE-HSP-12.1 protein | Hsp20/alpha crystallin family PF00011 | | 1.3 | 6.2 | 6.6 | 6.5 |
| L596_017588.1 | DNA gyrase/topoisomerase IV, A subunit | HSP90-like ATPase PF02518 | | 2.0 | | 1.5 | 1.8 |
| L596_026575 | Heat Shock Protein | Hsp70 protein PF00012 | 1.6 | 1.1 | 1.8 | 2.0 | 2.2 |
| L596_022874 | heat shock protein 90 | Hsp90 protein PF00183 | | | | 0.9 | |
| L596_018762.1 | Hsp20/alpha crystallin family protein | Hsp20/alpha crystallin family PF00011 | 2.1 | | 3.4 | 4.0 | 3.9 |
| L596_026859 | Hsp20/alpha crystallin family protein | Hsp20/alpha crystallin family PF00011 | -0.6 | -0.7 | 0.5 | 0.6 | 0.5 |
| L596_013116 | hypothetical protein L596_013116 | Hsp20/alpha crystallin family PF00011 | 0.8 | | 2.9 | 3.2 | 3.0 |
| L596_013176.1 | hypothetical protein L596_013176 | Hsp20/alpha crystallin family PF00011 | | 1.7 | | | |
| L596_007237 | prefoldin subunit | Prefoldin subunit PF01920 | -1.5 | | | -1.6 | -1.6 |
| L596_007980 | prefoldin subunit 3-like | Prefoldin subunit PF02996 | -0.3 | -1.6 | | 0.5 | 0.5 |
| SC.X.g4770 | prefoldin subunit 4 | Prefoldin subunit PF01920 | | 1.2 | | | |
| L596_013656 | putative chaperone protein DnaK | Hsp70 protein PF00012 | | | -1.9 | | |
| L596_023431 | T-complex protein 1, alpha subunit | TCP-1/cpn60 chaperonin family PF00118 | | 0.9 | | | |
| | **Late embryogeneis abundant proteins** | | | | | | |
| L596_019460 | Late embryogeneis abundant protein | No Pfams | | | | | 1.1 |
| ABQ23230.1 | LEA1 protein | LEA1 protein | -1.2 | -3.9 | 1.9 | 2.0 | 1.6 |
| L596_019566 | LEA1 protein | No Pfams | | | 1.1 | | |
| L596_019565.1 | LEA2 protein | No Pfams | | -1.2 | 3.7 | 3.7 | 3.4 |
| L596_019567.1 | LEA2 protein | No Pfams | 1.0 | | 1.0 | 1.5 | 1.3 |
| L596_020881 | LEA5 protein | No Pfams | | | 4.7 | | 4.6 |
| L596_020883 | LEA5 protein | No Pfams | | 2.4 | 3.1 | 3.1 | 3.3 |
| L596_020879 | LEA5 protein | No Pfams | -2.1 | -2.7 | | 2.2 | 1.9 |
| | **Cytoskeleton related proteins** | | | | | | |
| L596_009226 | actin | Actin_PF00022 | -2.27 | | -2.17 | -2.35 | -2.55 |
| SC.X.g2005.1 | Basement membrane proteoglycan | Laminin_B_(Domain_IV)_PF00052 | | | | -2.79 | -1.75 |
| SC.X.g6296 | CAP-Gly domain-containing protein | Dynein_associated_protein_PF12455 | | 0.98 | | | |
| SC.X.g4223 | Collagen alpha-1(XX) chain | Fibronectin_type_III_domain_PF00041 | | | | 1.74 | |
| L596_022545 | Collagen alpha-5(IV) chain | Collagen_triple_helix_repeat_(20_copies)_PF01391 | | -3.81 | | | |
| L596_018662 | collagen triple helix repeat protein | Collagen_triple_helix_repeat_(20_copies)_PF01391 | | 2.02 | | | 1.74 |
| L596_025641.4 | CRE-LET-805 protein | Fibronectin_type_III_domain_PF00041 | | -2.59 | -2.02 | | |
| L596_012863 | CRE-SCPL-4 protein | NLI_interacting_factor-like_phosphatase_PF03031 | | 1.05 | | | |
| L596_017840 | dynein light chain 1, cytoplasmic | Dynein_light_chain_type_1_PF01221 | | 1.85 | | | |
| L596_007728 | dynein light intermediate chain | Dynein_light_intermediate_chain_(DLIC)_PF05783 | | 1.35 | | | |
| L596_030886 | f-actin capping protein, beta subunit | F-actin_capping_protein,_beta_subunit_PF01115 | | -1.04 | | -1.73 | |
| L596_023292 | FERM central domain protein | FERM_central_domain_PF00373 | | -0.96 | | -1.14 | |
| L596_006890.2 | FERM domain (protein4.1-ezrin-radixin-moesin) family | No_Pfams | | -0.76 | | | |
| L596_007912 | FERM domain (protein4.1-ezrin-radixin-moesin) family | No_Pfams | | -1.49 | | | |
| L596_014002 | FiLamiN (actin binding protein) homolog | FilaminaBP280_repeat_PF00630 | | -0.67 | | | |
| L596_007064.1 | Growth-Arrest-Specific Protein 2 Domain containing protein | Growth-Arrest-Specific_Protein_2_Domain_PF02187 | | 1.78 | 1.28 | 1.32 | |
| L596_017115 | Kinesin heavy chain | Kinesin_motor_domain_PF00225 | -0.61 | -1.99 | | | |
| L596_022381 | Kinesin light chain | Tetratricopeptide_repeat_PF13374 | | 2.52 | | | 2.26 |
| L596_029252.2 | laminin alpha 1 chain | Laminin_B_(Domain_IV)_PF00052 | | | 0.85 | 1.35 | |
| L596_030045 | Laminin-like protein C54D1.5 precursor, putative | Laminin_EGF_domain_PF00053 | | | | | -0.63 |
| L596_010020.1 | Muscle M-line assembly protein unc-89 | Fibronectin_type_III_domain_PF00041 | 1.43 | 1.89 | | | 2.48 |
| L596_030030 | myosin head | Myosin_tail_PF01576 | | -0.64 | | -0.69 | -0.54 |
| SC.X.g3323 | Myosin regulatory light chain 1 | EF-hand_domain_PF13405 | 1.08 | 1.95 | 2.90 | 2.79 | 2.98 |
| L596_022347 | nematode cuticle collagen domain protein | Collagen_triple_helix_repeat_(20_copies)_PF01391 | | | | 1.02 | |
| L596_007058 | nematode cuticle collagen domain protein | Nematode_cuticle_collagen_N-terminal_domain_PF01484 | -3.19 | -2.31 | | -1.96 | -2.62 |
| L596_025642.3 | Phosphatidylinositol phosphatase PTPRQ | Fibronectin_type_III_domain_PF00041 | | 1.58 | | | |
| L596_008491 | predicted protein | Actin_PF00022 | | | | | 1.10 |
| L596_008221 | PSME3-interacting protein isoform X1 | N-terminal_domain_of_NEFA-interacting_nuclear_protein_NIP30_PF10187 | | 1.03 | | | |
| L596_027373.1 | Sensory AXon guidance | Bravo-like_intracellular_region_PF13882 | -0.35 | -0.62 | -0.17 | | -0.37 |
| L596_016805 | talin 1 | Talin,_middle_domain_PF09141 | | -0.82 | | | |
| L596_021930.1 | Titin, putative | Fibronectin_type_III_domain_PF00041 | | -2.33 | | | |
| L596_007346 | Tropomyosin | No_Pfams | | | | | 0.87 |
| SC.X.g3838 | troponin family protein | Troponin_PF00992 | | | | 1.04 | |
| SC.X.g3415 | troponin T family protein | Troponin_PF00992 | | -0.96 | | | -0.68 |
| L596_027733 | Tubulin alpha chain | Tubulin/FtsZ_family,_GTPase_domain_PF00091 | 0.73 | 1.03 | 0.49 | 0.41 | 0.63 |
| SC.X.g3907 | tubulin alpha chain | Tubulin/FtsZ_family,_GTPase_domain_PF00091 | | 0.64 | | | 0.63 |
| L596_015436 | Tubulin beta-1 chain | Tubulin/FtsZ_family,_GTPase_domain_PF00091 | | | | -2.19 | |
| L596_011798.1 | tubulin-folding cofactor B | CAP-Gly_domain_PF01302 | | 1.26 | 1.52 | 1.69 | |
| L596_009753 | tubulin-specific chaperone A | Tubulin_binding_cofactor_A_PF02970 | | -2.45 | | -1.85 | -2.62 |
| SC.X.g194 | Tubulin-tyrosine ligase family protein | Tubulin-tyrosine_ligase_family_PF03133 | 2.79 | 3.45 | 2.16 | 2.18 | |
| | **Fatty acid and retinol binding related proteins** | | | | | | |
| L596_023264 | fatty acid retinoid binding protein precursor | Nematode_fatty_acid_retinoid_binding_protein_(Gp-FAR-1)_PF05823 | 0.33 | | -0.92 | -1.00 | -1.69 |
| L596_009693 | Fatty acid-binding protein homolog 3 | No_Pfams | | -0.96 | -1.47 | | -1.40 |
| L596_019606.2 | Fatty acid-binding protein homolog 9 | Lipocalin_/_cytosolic_fatty-acid_binding_protein_family_PF00061 | -1.70 | | | | |
| L596_009677 | fatty acid-binding protein, heart | No_Pfams | -0.40 | -0.67 | | | |
| SC.X.g5564 | Fatty-acid amide hydrolase 1 | Amidase_PF01425 | | | | 1.55 | |
| SC.X.g2753 | Fatty-acid amide hydrolase 2 | Amidase_PF01425 | | | | | -1.19 |
| L596_015746.2 | hypothetical protein L596_015746 | Nematode_fatty_acid_retinoid_binding_protein_(Gp-FAR-1)_PF05823 | 0.86 | 1.20 | -1.02 | | -0.93 |
| L596_023208 | hypothetical protein L596_023208 | Nematode_fatty_acid_retinoid_binding_protein_(Gp-FAR-1)_PF05823 | | | -0.52 | -0.56 | -1.08 |
| L596_012161.2 | ladder protein | Nematode_polyprotein_allergen_ABA-1_PF16469 | -0.48 | -1.15 | -0.26 | | -0.65 |
| L596_012162 | ladder protein | Nematode_polyprotein_allergen_ABA-1_PF16469 | -0.52 | -1.36 | | | -0.80 |
| L596_008490 | lipocalin / cytosolic fatty-acid binding protein | Lipocalin_/_cytosolic_fatty-acid_binding_protein_family_PF00061 | | -3.12 | | | |
| L596_008504 | lipocalin / cytosolic fatty-acid binding protein | Lipocalin_/_cytosolic_fatty-acid_binding_protein_family_PF00061 | -0.59 | -1.04 | | | -0.31 |
| L596_023295 | Nematode fatty acid retinoid binding family-containing protein | Nematode_fatty_acid_retinoid_binding_protein_(Gp-FAR-1)_PF05823 | | -2.37 | | | |
| L596_023261 | nematode fatty acid retinoid binding protein | Nematode_fatty_acid_retinoid_binding_protein_(Gp-FAR-1)_PF05823 | -0.52 | -0.94 | -1.43 | | |
| L596_023215 | nematode fatty acid retinoid binding protein | Nematode_fatty_acid_retinoid_binding_protein_(Gp-FAR-1)_PF05823 | | 0.89 | | | |
| L596_023212 | nematode fatty acid retinoid binding protein | Nematode_fatty_acid_retinoid_binding_protein_(Gp-FAR-1)_PF05823 | | | | 0.79 | 0.44 |

## Appendix 3.1.B. $Log_2$fold transformed abundances of proteins identified as statistically significant (P<0.05 in student t-tests) relative to week 0 in *S. carpocapsae* IJs after conditioning.

| Protein IDs | Blast Annotation | PFAM Annotation | Log.fold change | | | | |
|---|---|---|---|---|---|---|---|
| | **Gluconeogenesis related proteins** | | **3w20°C** | **6w20°C** | **3w9°C** | **6w9°C** | **9w9°C** |
| L596_009845 | pyruvate carboxylase | Conserved_carboxylase_domain_PF02436 | 0.3 | 0.5 | | | |
| L596_007895 | Phosphoenolypyruvate CarboxyKinase | Phosphoenolpyruvate_carboxykinase_N-terminal_domain_PF17297 | | | 0.2 | | 0.4 |
| ADH95417.1 | enolase | enolase [Steinernema carpocapsae] | | | | 0.3 | 0.3 |
| L596_010468.1 | phosphoglycerate mutase family protein | 6-phosphofructo-2-kinase_PF01591 | | 0.6 | | | |
| SC.X.g3982.1 | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | BPG-independent_PGAM_N-terminus_(iPGM_N)_PF06415 | | 0.4 | | | |
| L596_007008 | glyceraldehyde-3-phosphate dehydrogenase, type I | Glyceraldehyde_3-phosphate_dehydrogenase | -0.3 | | | | 0.4 |
| SC.X.g1801 | Glyceraldehyde-3-phosphate dehydrogenase, testis-specific | Glyceraldehyde_3-phosphate_dehydrogenase | -0.2 | | | | |
| SC.X.g2348 | Triosephosphate isomerase | Triosephosphate_isomerase_PF00121 | | | 0.6 | | |
| L596_016332 | Transaldolase | Transaldolase/Fructose-6-phosphate_aldolase_PF00923 | | | 0.4 | | 0.4 |
| L596_017098 | fructose-bisphosphate aldolase class-I | Fructose-bisphosphate_aldolase_class-I_PF00274 | -0.3 | -0.3 | 0.2 | | |
| L596_007259 | Glucose-6-phosphate isomerase | Phosphoglucose_isomerase_PF00342 | 0.3 | 0.2 | | | |
| SC.X.g3330 | trehalose-6-phosphate synthase domain protein | Trehalose-6-phosphate_phosphatase_N-terminal_helical_bundle_domain_PF18572 | | 1.2 | | 1.3 | |
| | **Glycolysis related proteins** | | | | | | |
| SC.X_g5232 | Hexokinase | Hexokinase_PF03727 | | 0.6 | | | |
| L596_007259 | Glucose-6-phosphate isomerase | Phosphoglucose_isomerase_PF00342 | 0.3 | 0.2 | | | |
| L596_017098 | fructose-bisphosphate aldolase class-I | Fructose-bisphosphate_aldolase_class-I_PF00274 | -0.3 | -0.3 | 0.2 | | |
| SC.X.g2348 | Triosephosphate isomerase | Triosephosphate_isomerase_PF00121 | | | 0.6 | | |
| L596_007008 | glyceraldehyde-3-phosphate dehydrogenase, type I | Glyceraldehyde_3-phosphate_dehydrogenase,_NAD_binding_domain_PF00044 | -0.3 | | | | 0.4 |
| SC.X.g1801 | Glyceraldehyde-3-phosphate dehydrogenase, testis-specific | Glyceraldehyde_3-phosphate_dehydrogenase,_NAD_binding_domain_PF00044 | -0.2 | | | | |
| L596_010468.1 | phosphoglycerate mutase family protein | 6-phosphofructo-2-kinase_PF01591 | | 0.6 | | | |
| SC.X.g3982.1 | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | BPG-independent_PGAM_N-terminus_(iPGM_N)_PF06415 | | 0.4 | | | |
| ADH95417.1 | enolase | enolase [Steinernema carpocapsae] | | | | 0.3 | 0.3 |
| L596_007652.3 | pyruvate kinase | Pyruvate_kinase,_alpha/beta_domain_PF02887 | | | 1.8 | | |
| L596_026845 | pyruvate kinase | Pyruvate_kinase,_alpha/beta_domain_PF02887 | | 1.4 | | | |
| L596_007652.1 | pyruvate kinase | Pyruvate_kinase,_alpha/beta_domain_PF02887 | | | | -0.3 | |
| L596_007697.3 | pyruvate kinase | Pyruvate_kinase,_alpha/beta_domain_PF02887 | | | | | 0.4 |
| SC.X.g3082 | pyruvate dehydrogenase E1 component, alpha subunit | Dehydrogenase_E1_component_PF00676 | 0.2 | 0.7 | | | |
| L596_021831 | pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase | e3_binding_domain_PF02817 | | | | | -0.3 |
| L596_030323 | Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | Transketolase,_C-terminal_domain_PF02780 | 0.2 | | | | |
| | **Glyoxalate pathway/Citric Acid Cycle related proteins** | | | | | | |
| L596_029737 | ATP-citrate synthase | Citrate_synthase,_C-terminal_domain_PF00285 | 0.6 | 0.4 | | | |
| SC.X.g3964 | Probable aconitate hydratase, mitochondrial | Aconitase_C-terminal_domain_PF00694 | 0.3 | | -0.4 | -0.5 | -0.3 |
| SC.X.g5397 | putative isocitrate dehydrogenase, NAD-dependent | Isocitrate/isopropylmalate_dehydrogenase_PF00180 | | 1.8 | | | |
| L596_007375 | Isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial | Isocitrate/isopropylmalate_dehydrogenase_PF00180 | 0.3 | 0.6 | | | |
| L596_007457 | isocitrate dehydrogenase, NAD-dependent | Isocitrate/isopropylmalate_dehydrogenase_PF00180 | 0.4 | 0.4 | | | |
| L596_019319.1 | Probable isocitrate dehydrogenase [NAD] subunit beta, mitochondrial | Isocitrate/isopropylmalate_dehydrogenase_PF00180 | | 0.4 | -0.3 | | |
| L596_011794 | malate synthase A | Malate_synthase_PF01274 | | -0.1 | -0.3 | | -0.2 |
| SC.X.g2793 | Lactoylglutathione lyase | Glyoxalase/Bleomycin_resistance_protein/Dioxygenase_superfamily_PF00903 | | 0.4 | 0.5 | 0.5 | 0.4 |
| SC.X.g163 | L-lactate dehydrogenase | lactate/malate_dehydrogenase,_alpha/beta_C-terminal_domain_PF02866 | | | | -2.7 | |
| L596_022993 | Malate dehydrogenase, cytoplasmic | lactate/malate_dehydrogenase,_alpha/beta_C-terminal_domain_PF02866 | | -0.2 | | | |
| L596_015397 | Choline/Carnitine O-acyltransferase | Choline/Carnitine_o-acyltransferase_PF00755 | 0.4 | 0.6 | | | |
| SC.X.g5090.2 | Acyl-CoA dehydrogenase family member 11 | Acyl-CoA_dehydrogenase | 1.5 | 1.6 | | 1.3 | |
| L596_020086 | Acyl-CoA dehydrogenase | Acyl-CoA_dehydrogenase,_C-terminal_domain_PF00441 | | | | | -0.4 |
| SC.X.g1509 | acyl-CoA dehydrogenase family member 9 | Acyl-CoA_dehydrogenase,_C-terminal_domain_PF00441 | | | -0.5 | -0.4 | |
| L596_028977 | fatty acid oxidation complex, alpha subunit | Enoyl-CoA_hydratase/isomerase_PF00378 | | 2.2 | | | |
| SC.X.g4209 | enoyl-CoA hydratase/isomerase family protein | Enoyl-CoA_hydratase/isomerase_PF00378 | | | | 1.5 | 1.2 |
| L596_016210 | enoyl-CoA hydratase/isomerase family protein | Enoyl-CoA_hydratase/isomerase_PF00378 | -0.4 | -0.6 | -0.3 | -0.2 | -0.4 |
| L596_023219 | enoyl-CoA hydratase/isomerase family protein | Enoyl-CoA_hydratase/isomerase_PF00378 | | | -0.5 | | |
| L596_028976 | fatty acid oxidation complex, alpha subunit | Enoyl-CoA_hydratase/isomerase_PF00378 | 0.4 | 0.5 | | | 0.3 |
| SC.X.g2384 | enoyl-CoA hydratase/isomerase family protein | Enoyl-CoA_hydratase/isomerase_PF00378 | | 0.4 | -0.3 | -0.3 | |
| SC.X.g2800 | enoyl-CoA delta isomerase 2, mitochondrial-like | Enoyl-CoA_hydratase/isomerase_PF00378 | | | -0.4 | -0.5 | |
| SC.X.g4351 | Methylglutaconyl-CoA hydratase, mitochondrial | Enoyl-CoA_hydratase/isomerase_PF00378 | -0.2 | | -0.3 | | -0.5 |
| L596_018770 | ACetyl-CoA Acyltransferase 2 homolog | Thiolase,_N-terminal_domain_PF00108 | | 0.4 | | -0.4 | |
| L596_030114 | Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial | ATP-grasp_domain_PF08442 | 0.7 | | | | |
| L596_014776 | Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial | 2Fe-2S_iron-sulfur_cluster_binding_domain_PF13085 | | | -0.6 | -1.4 | -0.9 |
| L596_029720 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | FAD_binding_domain_PF00890 | 0.1 | 0.1 | | | |
| | **Oxidative phosphorylation proteins related proteins** | | | | | | -1.3 |
| L596_019629 | NADH dehydrogenase [ubiquinone] 1 domain containing protein | NADH:ubiquinone_oxidoreductase,_NDUFB6/B17_subunit_PF09782 | | | -0.6 | | -0.4 |
| SC.X.g4180 | NADH dehydrogenase subunit D | Respiratory-chain_NADH_dehydrogenase,_49_Kd_subunit_PF00346 | | 0.7 | | | |
| L596_007275 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial | NADH_ubiquinone_oxidoreductase,_20_Kd_subunit_PF01058 | | | | -1.2 | -0.9 |
| L596_014693 | putative NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial | Thioredoxin-like_[2Fe-2S]_ferredoxin_PF01257 | | | | | |
| SC.X.g4436 | putative NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12 | NADH_ubiquinone_oxidoreductase_subunit_NDUFA12_PF05071 | 0.3 | | | | |
| L596_014776 | Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial | 2Fe-2S_iron-sulfur_cluster_binding_domain_PF13085 | | | -0.6 | -1.4 | |
| L596_029720 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | FAD_binding_domain_PF00890 | 0.1 | 0.1 | | | -0.6 |
| L596_007725 | NADH Ubiquinone Oxidoreductase | Respiratory-chain_NADH_dehydrogenase,_30_Kd_subunit_PF00329 | 0.4 | 0.3 | | | |
| L596_011131 | NADH-ubiquinone oxidoreductase ASHI subunit | NADH-ubiquinone_oxidoreductase_ASHI_subunit_(CI-ASHI_or_NDUFB8)_PF05821 | | | -0.5 | | |
| L596_011440 | NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial | Molybdopterin_oxidoreductase_PF00384 | | | | -0.4 | -0.4 |
| L596_009678 | NADH-ubiquinone oxidoreductase B12 subunit family protein | NADH-ubiquinone_oxidoreductase_B12_subunit_family_PF08122 | | | -2.5 | | -0.5 |
| L596_023111 | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial | Electron_transfer_flavoprotein-ubiquinone_oxidoreductase,_4Fe-4S_PF05187 | 0.4 | 0.6 | | | -0.6 |
| L596_009365 | NADH-ubiquinone oxidoreductase B15 subunit | NADH-ubiquinone_oxidoreductase_B15_subunit_(NDUFB4)_PF07225 | 0.4 | | -0.7 | | |
| SC.X.g4325 | electron transfer flavoprotein subunit beta | Electron_transfer_flavoprotein_domain_PF01012 | -0.2 | -0.2 | | | |
| SC.X.g5343 | Cytochrome b-c1 complex subunit Rieske, mitochondrial | Ubiquinol_cytochrome_reductase_transmembrane_region_PF02921 | | | -0.6 | -0.5 | |
| L596_015395 | Cytochrome b-c1 complex subunit 7 | Ubiquinol-cytochrome_C_reductase_complex_14kD_subunit_PF02271 | 0.4 | | | | -0.7 |
| L596_007818 | cytochrome c oxidase subunit Vb | Cytochrome_c_oxidase_subunit_Vb_PF01215 | | 1.2 | | | -1.2 |
| L596_029294 | cytochrome c oxidase subunit VIIc | Cytochrome_c_oxidase_subunit_VIIc_PF02935 | | | | -1.3 | |
| L596_023716 | cytochrome c oxidase subunit Va | Cytochrome_c_oxidase_subunit_Va_PF02284 | | -0.4 | | | |
| L596_025594 | cytochrome c oxidase subunit VIa | Cytochrome_c_oxidase_subunit_VIa_PF02046 | | | -0.8 | -0.9 | |
| L596_007262 | cytochrome c oxidase subunit IV | Cytochrome_c_oxidase_subunit_IV_PF02936 | 0.2 | | -0.6 | -0.3 | |
| L596_007757 | CYtochrome C | Cytochrome_C1_family_PF02167 | | 0.6 | -0.4 | | |
| | **Stress related proteins** | | | | | | |
| L596_012580 | Aldehyde dehydrogenase | Aldehyde dehydrogenase family PF00171 | | | | | -1.17 |
| L596_012582 | Aldehyde dehydrogenase | Aldehyde dehydrogenase family PF00171 | 1.83 | 1.97 | | | |
| L596_013610 | ALdehyde deHydrogenase | Aldehyde dehydrogenase family PF00171 | 0.52 | 0.64 | | -0.15 | |
| L596_012539.1 | aldehyde dehydrogenase family 3 member B1 isoform X3 | Aldehyde dehydrogenase family PF00171 | 1.60 | 2.20 | 1.31 | | |
| L596_020877 | antioxidant, AhpC/TSA family | Thioredoxin-like PF13905 | -1.55 | -0.82 | | | |
| L596_018168.1 | apoptosis-inducing factor 3-like isoform X1 | Reductase C-terminal PF14759 | | 0.92 | | | |
| SC.X.g1189 | Catalase | Catalase PF00199 | | | 0.67 | | 0.77 |
| L596_009096 | cold-shock DNA-binding domain protein | Cold-shock' DNA-binding domain PF00313 | | -2.12 | | -1.32 | -0.74 |
| SC.X.g2721 | copper/zinc superoxide dismutase | Copper/zinc superoxide dismutase (SODC) PF00080 | -0.55 | | | | 0.66 |
| L596_007731 | Dihydroorotate dehydrogenase (quinone), mitochondrial | Ferrochelatase PF00762 | | | | 1.39 | |
| L596_024043.2 | Extracellular superoxide dismutase [Cu-Zn] | Copper/zinc superoxide dismutase (SODC) PF00080 | -0.35 | | 0.81 | 1.08 | 1.05 |
| L596_018193 | hypoxia induced protein region | Hypoxia induced protein conserved region PF04588 | 1.14 | 1.11 | 0.86 | 1.14 | 1.01 |
| L596_018208 | hypoxia induced protein region | Hypoxia induced protein conserved region PF04588 | | 1.48 | | 1.62 | |
| L596_007213 | oxidoreductase, short chain dehydrogenase/reductase family protein | No_Pfams | | -1.87 | | -1.78 | |
| L596_009334 | oxidoreductase, short chain dehydrogenase/reductase family protein | Enoyl-(Acyl_carrier_protein)_reductase_PF13561 | | 1.01 | 0.32 | 0.68 | |
| L596_026869 | oxidoreductase, short chain dehydrogenase/reductase family protein | short_chain_dehydrogenase_PF00106 | | 1.53 | | | 1.41 |
| SC.X.g6352 | oxidoreductase, short chain dehydrogenase/reductase family protein | Enoyl-(Acyl_carrier_protein)_reductase_PF13561 | 1.29 | 1.93 | | 1.54 | |
| L596_014247 | Probable aldehyde oxidase gad-3 | [2Fe-2S] binding domain PF01799 | | | | 0.90 | |
| L596_021103 | protein disulfide-isomerase domain protein | Thioredoxin PF00085 | | -2.09 | | -1.17 | |
| L596_014733 | Sulfhydryl oxidase | Erv1 / Alr family PF04777 | | | | | 0.97 |
| L596_029281 | Thioredoxin domain-containing protein | Thioredoxin-like domain PF13848 | | | | -2.25 | |
| L596_007977 | Thioredoxin family protein | PITH domain PF06201 | | | -2.59 | | -1.33 |
| L596_014868 | UDP-glucose:Glycoprotein Glucosyltransferase containing protein | Glucosyltransferase 24 PF18404 | | 1.60 | 1.57 | 1.53 | |
| L596_013399 | UDP-glucuronosyl UDP-glucosyltransferase domain containing protein | UDP-glucoronosyl and UDP-glucosyl transferase PF00201 | | 2.71 | | 2.06 | 2.00 |
| L596_022536.1 | UDP-glucuronosyl/UDP-glucosyltransferase family-containing protein | UDP-glucoronosyl and UDP-glucosyl transferase PF00201 | 1.02 | 1.45 | | 1.39 | |
| L596_007599 | glutathione S-transferase-like | Glutathione_S-transferase,_N-terminal_domain_PF02798 | | | | 1.37 | |
| L596_011219 | serine/threonine-protein phosphatase | Calcineurin-like_phosphoesterase_PF00149 | | | | 1.72 | |
| L596_020572 | glutathione transferase | Glutathione_S-transferase,_N-terminal_domain_PF02798 | 0.38 | 0.63 | | | |
| SC.X.g2364.2 | glutathione S-transferase protein | Glutathione_S-transferase,_N-terminal_domain_PF02798 | | | | 1.79 | |
| SC.X.g2368 | glutathione S-transferase protein | Glutathione_S-transferase,_N-terminal_domain_PF02798 | | 0.58 | | | |
| SC.X.g3583 | glutathione peroxidase | Glutathione_peroxidase_PF00255 | 0.61 | | 1.79 | 2.05 | 2.10 |
| L596_015243 | Glutathione S-transferase | Glutathione_S-transferase,_N-terminal_domain_PF02798 | | | | | 1.48 |
| SC.X.g6648 | putative phospholipid hydroperoxide glutathione peroxidase | Glutathione_peroxidase_PF00255 | | | 0.57 | 0.89 | 0.88 |
| L596_013446.3 | Gamma-glutamyltranspeptidase 1 | Gamma-glutamyltranspeptidase_PF01019 | | | | | -1.97 |
| L596_006932 | Glutathione S-transferase 1 | Glutathione_S-transferase,_N-terminal_domain_PF02798 | | | 0.29 | | 0.51 |
| SC.X.g2793 | Lactoylglutathione lyase | Glyoxalase/Bleomycin_resistance_protein/Dioxygenase_superfamily_PF00903 | | 0.38 | 0.49 | 0.52 | 0.40 |

## Appendix 3.2. Log$_2$fold transformed abundances of proteins identified as statistically significant (P<0.05 in student t-tests) relative to week 0 in *H. megidis* IJs after conditioning

| Protein IDs | Blast Annotation | PFAM Annotation | Log$_2$fold Difference | | | | |
|---|---|---|---|---|---|---|---|
| | **Ribosome related proteins** | | 3w20°C | 6w20°C | 3w9C | 6w9C | 9w9C |
| Hba_18986 | Ribosomal protein S10 domain containing protein | Ribosomal protein S10p/S20e | -0.5 | -0.7 | | -0.4 | -0.8 |
| Hba_11568 | ribosomal protein S11 | Ribosomal protein S11 | -0.4 | | | | -0.6 |
| Hba_02354 | unnamed protein product | Ribosomal protein L4/L1 family | | -0.8 | | | |
| Hba_09213 | ribosomal protein S21e | Ribosomal protein S21e | | -0.7 | | -0.7 | -0.7 |
| Hba_12622 | unnamed protein product | Ribosomal S17 | | | | | -1.7 |
| Hba_16924 | hypothetical protein Y032_0013g1999 | Ribosomal protein L14 | | -2.5 | | | -2.0 |
| Hba_17253 | Ribosomal Proteins L2, RNA binding domain protein | Ribosomal Proteins L2, RNA binding domain | | -0.7 | | -0.4 | -0.6 |
| Hba_17797 | hypothetical protein Y032_0283g1331 | Ribosomal protein S15 | | -0.8 | | | -0.9 |
| Hba_18018 | ribosomal protein S17 | Ribosomal protein S17 | | | | | -0.8 |
| Hba_21570 | hypothetical protein WR25_18482 | Ribosomal protein L5 | | -0.5 | -0.6 | | |
| Hba_21082 | unnamed protein product | KOW motif | | -0.6 | | | |
| | **Chaperone Proteins** | | | | | | |
| Hba_02553 | hsp-1 | Hsp70 protein | | -0.2 | | -0.4 | -0.3 |
| Hba_08949 | unnamed protein product | Hsp70 protein | | | -0.7 | | |
| Hba_09040 | unnamed protein product | Hsp70 protein | -1.2 | -1.3 | -0.7 | -0.5 | |
| Hba_19207 | hypothetical protein Y032_0044g1081 | Hsp90 protein | | | | | -0.3 |
| Hba_19685 | Hsp20/alpha crystallin family protein | Hsp20/alpha crystallin family | | | -4.1 | -3.4 | |
| Hba_19878 | Heat shock protein Hsp-12.2 | Hsp20/alpha crystallin family | | -0.7 | | | |
| Hba_09309 | hypothetical protein FL81_08775 | TCP-1/cpn60 chaperonin family | | | -0.6 | | -0.7 |
| | **Gluconeogenesis related proteins** | | | | | | |
| Hba_06687 | unnamed protein product | Phosphoenolpyruvate carboxykinase | -0.7 | -1.1 | | | -0.5 |
| Hba_06688 | hypothetical protein Y032_0043g885 | Phosphoenolpyruvate carboxykinase | | -0.8 | | | |
| Hba_17763 | Phosphoenolpyruvate carboxykinase domain containing protein | Phosphoenolpyruvate carboxykinase | 0.5 | | | 0.4 | 0.5 |
| Hba_17764 | unnamed protein product | Phosphoenolpyruvate carboxykinase | 0.4 | 0.4 | 0.3 | 0.2 | |
| Hba_13336 | fructose-bisphosphate aldolase class-I | Fructose-bisphosphate aldolase class-I | | | | 0.5 | |
| | **Glycolysis related proteins** | | | | | | |
| Hba_13336 | fructose-bisphosphate aldolase class-I | Fructose-bisphosphate aldolase class-I | | | | 0.5 | |
| Hba_15591 | hypothetical protein ANCDUO_08568 | Enolase, C-terminal TIM barrel domain | -0.5 | -0.8 | | | |
| Hba_15739 | phosphogluconate dehydrogenase | 6-phosphogluconate dehydrogenase, C-terminal domain | | | | | -0.6 |
| | **Glyoxalate Pathways/Citric Acid Cycle related proteins** | | | | | | |
| Hba_07181 | isocitrate dehydrogenase, NADP-dependent | Isocitrate/isopropylmalate dehydrogenase | -0.5 | -0.4 | | | -0.3 |
| Hba_09075 | hypothetical protein B9Z55_005615 | lactate/malate dehydrogenase, NAD binding domain | | | | -0.5 | -0.7 |
| Hba_17373 | hydrolase, alpha/beta domain protein | lactate/malate dehydrogenase, NAD binding domain | -0.4 | -0.7 | | | |
| Hba_07788 | acyl-CoA dehydrogenase family member 9 | Acyl-CoA dehydrogenase, C-terminal domain | | 0.1 | | | |
| Hba_21014 | Acyl-CoA-binding protein and Crotonase domain containing protein | Enoyl-CoA hydratase/isomerase | | -0.3 | | | |
| Hba_07181 | isocitrate dehydrogenase, NADP-dependent | Isocitrate/isopropylmalate dehydrogenase | -0.5 | -0.4 | | | -0.3 |
| | **Oxidative Phosphorylation related proteins** | | | | | | |
| Hba_17947 | hypothetical protein CAEBREN_25478 | ETC complex I subunit conserved region | | | -0.3 | | -0.6 |
| Hba_00533 | hypothetical protein Y032_0014g2237 | NADH-ubiquinone oxidoreductase ASHI subunit (CI-ASHI or NDUFB8) | 3.6 | | 3.4 | 3.2 | |
| Hba_08447 | hypothetical protein Y032_0058g2851 | NADH-ubiquinone oxidoreductase B18 subunit (NDUFB7) | | 0.5 | | | |
| Hba_14632 | hypothetical protein Y032_0334g2847 | Electron transfer flavoprotein-ubiquinone oxidoreductase, 4Fe-4S | | | -0.4 | | -0.5 |
| | **Cytoskeleton related proteins** | | | | | | |
| Hba_17725 | Actin actin domain containing protein | Actin | | | | | -1.8 |
| Hba_06391 | PLaSTin (actin bundling protein) homolog | Calponin homology (CH) domain | | | -0.6 | | -0.6 |
| Hba_15288 | c-terminal tandem repeated domain in type 4 procollagen | Collagen triple helix repeat (20 copies) | | 1.0 | | | |
| Hba_19067 | FERM central domain protein | FERM central domain | | | | 0.9 | |
| Hba_20268 | UNC-22 | Fibronectin type III domain | | 0.2 | 0.2 | 0.1 | 0.2 |
| Hba_15377 | Sec23/Sec24 helical domain protein | Gelsolin repeat | | | | | -0.6 |
| Hba_03189 | unnamed protein product | Laminin B (Domain IV) | | 1.9 | | | |
| Hba_18045 | hypothetical protein CAEBREN_32818 | LIM domain | | -0.7 | | -0.2 | |
| Hba_18044 | hypothetical protein Y032_0123g1176 | LIM domain | | -1.0 | | | |
| Hba_16775 | RNA recognition motif and Zinc finger domain containing protein | LIM domain | | | | | 1.0 |
| Hba_20578 | fibronectin type III domain protein | NonAnnotated | | 0.7 | | | |
| Hba_16474 | BMA-ATN-1 | Spectrin repeat | | | -1.0 | | 1.0 |
| Hba_17919 | hypothetical protein NECAME_08305 | Spectrin repeat | | 0.6 | | | |
| Hba_14066 | spectrin repeat-containing domain protein | Spectrin repeat | | 0.9 | | 0.6 | |
| Hba_09949 | unnamed protein product | Villin headpiece domain | | -0.9 | | | |
| Hba_01885 | Vinculin alpha-catenin domain containing protein | Vinculin family | | | | | -0.6 |
| | **Stress related proteins** | | | | | | |
| Hba_05836 | hypothetical protein ANCCAN_14388 | Thioredoxin-like | | | | -1.9 | -1.5 |
| Hba_21175 | hypothetical protein NECAME_15400 | Catalase | -1.0 | -1.3 | | 0.6 | 0.8 |
| Hba_12519 | hypothetical protein WR25_20906 | MSP (Major sperm protein) domain | | | | -2.1 | |
| Hba_00606 | hypothetical protein Y032_0003g1609 | Autophagy-related protein C terminal domain | 0.5 | 0.6 | 0.7 | 0.6 | 0.8 |
| Hba_00533 | hypothetical protein Y032_0014g2237 | NADH-ubiquinone oxidoreductase ASHI subunit (CI-ASHI or NDUFB8) | 3.6 | | 3.4 | 3.2 | |
| Hba_09772 | hypothetical protein Y032_0412g982 | Thioredoxin | | -1.6 | | -1.2 | |
| Hba_09734 | protein disulfide-isomerase domain protein | Thioredoxin | | -0.7 | | | -0.4 |
| Hba_09453 | PUL domain protein | WD domain, G-beta repeat | | 1.2 | 0.9 | 0.8 | |
| Hba_08155 | pyrroline-5-carboxylate reductase | NADP oxidoreductase coenzyme F420-dependent | | | | | -1.2 |
| Hba_14944 | unnamed protein product | Spermine/spermidine synthase | | -1.6 | | -1.4 | -2.0 |
| Hba_03214 | WD domain, G-beta repeat protein | WD domain, G-beta repeat | | 0.2 | | | |
| Hba_03215 | WD domain, G-beta repeat protein | WD domain, G-beta repeat | 0.5 | 0.8 | 0.9 | | 0.4 |
| Hba_03387 | WD domain, G-beta repeat protein | WD domain, G-beta repeat | | | 0.8 | | 0.5 |



**Appendix 4.1. Outline of chemotaxis plate.** Chemotaxis assays are carried out on a 60mm petri dish, with a 1cm zone on the left and right of the plate demarking the treatment and control zones. The middle circle is the application point where 2 µl of IJs are applied, and the 2 lines in the centre of the plate demark the middle zone.

## Appendix 4.2. Statistically significant proteins changed at least fivefold in abundance in IJs stored at 9°C for 3 weeks, or for 1 week and transferred to 20°C, compared to IJs stored at 20°C for 3 weeks.

| Protein ID | Annotation | Fold change vs 3wks20°C | | Peptides | Mol. Weight | Intensity | MS/MS Count |
|---|---|---|---|---|---|---|---|
| | | 3wks9°C | 1wk9°C→20°C | | | | |
| L596_019569.1 | Hypothetical protein | 312.3 | 179.7 | 42 | 68.331 | 7.08E+09 | 370 |
| L596_026406 | CRE-HSP-12.1 protein | 140.5 | 35.9 | 5 | 23.854 | 1.44E+09 | 73 |
| L596_020879 | LEA5 protein | 93.6 | 95.8 | 3 | 7.4813 | 8.29E+08 | 28 |
| L596_020881 | LEA5 protein | 61.2 | 33.5 | 2 | 10.094 | 6.11E+08 | 20 |
| L596_015330 | SaPosin-like Protein family | 60.0 | 43.9 | 4 | 10.214 | 1.16E+09 | 37 |
| L596_019565.1 | LEA2 protein | 56.8 | na | 4 | 10.539 | 2.20E+09 | 45 |
| L596_030565 | Hypothetical protein | 36.2 | 32.7 | 5 | 14.902 | 7.41E+08 | 59 |
| L596_013113 | Hypothetical protein | 35.7 | 16.8 | 3 | 15.175 | 4.95E+08 | 22 |
| L596_012434.1 | Hypothetical protein | 29.9 | 2.2 | 3 | 51.288 | 2.26E+08 | 18 |
| L596_027614 | Hypothetical protein | 28.2 | 19.3 | 5 | 14.179 | 4.55E+08 | 44 |
| L596_012217 | PhosphoGlycolate Phosphatase Homolog | 23.4 | na | 5 | 38.207 | 2.28E+08 | 18 |
| L596_009145.1 | Hypothetical protein | 20.7 | 16.7 | 5 | 95.72 | 2.72E+08 | 89 |
| L596_024791 | Cystathionine beta-synthase | 17.9 | na | 16 | 77.905 | 1.31E+08 | 43 |
| ABQ23230.1 | LEA1 protein | 17.1 | 13.4 | 5 | 9.7546 | 5.25E+09 | 75 |
| L596_019563.2 | Hypothetical protein | 15.3 | 9.8 | 1 | 17.438 | 2.27E+09 | 102 |
| L596_027831.1 | Hypothetical protein | 10.1 | 7.8 | 8 | 51.877 | 6.22E+08 | 60 |
| SC.X.g2587.2 | Putative cystathionine gamma-lyase 2 | 7.4 | 8.7 | 5 | 42.673 | 1.07E+08 | 30 |
| SC.X.g3323 | Myosin regulatory light chain 1 | 7.3 | na | 4 | 18.941 | 8.40E+07 | 17 |
| L596_019840 | C. briggsae CBR-OSM-11 protein | 7.2 | na | 3 | 28.814 | 2.74E+08 | 22 |
| SC.X.g3305 | C-1-tetrahydrofolate synthase, cytoplasmic | 6.8 | na | 8 | 102.42 | 1.34E+08 | 48 |
| L596_030616 | Protein LSM12 -like protein A | 6.8 | 3.2 | 3 | 24.9 | 4.87E+07 | 17 |
| L596_013116 | Hypothetical protein | 6.5 | 9.5 | 7 | 18.748 | 1.62E+09 | 62 |
| L596_019570 | Hypothetical protein | 6.3 | na | 2 | 12.129 | 1.22E+08 | 12 |
| L596_017887.4 | ADP-ribose pyrophosphatase, mitochondrial precursor | 5.7 | 5.7 | 4 | 17.112 | 1.22E+08 | 28 |
| L596_024012 | Thiamin pyrophosphokinase | na | 45.7 | 9 | 30.405 | 2.58E+08 | 43 |
| L596_013178 | Hypothetical protein | na | 17.1 | 6 | 18.033 | 9.70E+07 | 21 |
| L596_028041 | Serine/threonine-protein phosphatase PP1-alpha | na | 7.0 | 2 | 19.921 | 1.13E+08 | 11 |
| SC.X.g1861 | Ani s 9 allergen precursor | na | 5.3 | 2 | 14.755 | 9.04E+07 | 13 |
| L596_017776 | Hypothetical protein | -5.0 | -13.0 | 2 | 14.45 | 1.19E+08 | 16 |
| L596_019533 | medium-chain specific acyl-CoA dehydrogenase, mitochondrial | -5.2 | na | 7 | 45.266 | 5.91E+07 | 27 |
| L596_012246 | Hypothetical protein | -5.5 | na | 4 | 13.671 | 8.92E+07 | 28 |
| L596_028677 | piwi domain protein | -6.0 | na | 5 | 100 | 5.03E+07 | 20 |
| L596_017378 | trypsin-like serine protease | -6.8 | -4.5 | 3 | 32.864 | 1.34E+08 | 22 |
| L596_018471 | acetyl-Coenzyme A synthetase 2, putative | -6.9 | -4.0 | 4 | 78.513 | 7.81E+07 | 21 |
| SC.X.g5447 | probable H/ACA ribonucleoprotein complex subunit 1-like protein | -8.2 | -5.0 | 4 | 24.466 | 1.50E+08 | 23 |
| SC.X.g3201 | Short-chain dehydrogenase/reductase | -8.2 | -3.6 | 2 | 29.235 | 7.42E+07 | 14 |
| L596_015745 | Hypothetical protein | -11.6 | -2.5 | 6 | 22.75 | 1.93E+08 | 26 |
| L596_023970 | NAC domain containing protein | -11.8 | na | 4 | 21.879 | 1.57E+08 | 31 |
| L596_020964 | Hypothetical protein | -13.4 | -11.0 | 5 | 19.028 | 3.80E+08 | 44 |
| L596_027332 | Hypothetical protein | -15.7 | na | 3 | 17.455 | 2.08E+08 | 22 |
| L596_015314 | Chitinase class I | -39.2 | -25.6 | 9 | 101.39 | 3.12E+08 | 33 |