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

Peter E. Lillis, Ian P. Kennedy, James C. Carolan and Christine T. Griffin

Introduction

Many nematode parasites of plants and animals achieve transmission by means of a free-living infective larva or infective juvenile (IJ). Temperature can profoundly impact the success of IJs in many ways, with moderate temperatures affecting the rates of dispersal or host finding, while extreme temperatures are lethal or damaging (Grewal et al., 1994; O’Connor et al., 2006; Bryant and Hallem, 2018; Aleuy and Kutz, 2020). Less extreme temperatures may also affect survival, for example, by slowing metabolism (Andaló et al., 2011). Conditions experienced in the past may affect current activities; for species occurring at higher latitudes, the most widely documented effects are those of low temperature which may protect against subsequent more damaging conditions through acclimatization, or act to induce diapause or quiescence (Sommerville and Davey, 2002; McCorley, 2003; Zhao et al., 2007; Evans and Perry, 2009; Aleuy and Kutz, 2020). Here, we explore the effects of prior temperature conditions on entomopathogenic nematodes (EPN).

EPN Steinernema and Heterorhabditis have received much attention in recent years due to their usefulness both as model parasites and as biological control agents (Hallem et al., 2007; Koppenhöfer et al., 2020). Their IJs seek out and infect living insects, typically in soil. The IJ of EPN is similar to that of other plant and animal parasites (where present), and to the dauer juvenile of free-living nematodes such as Caenorhabditis elegans (Bubrig and Fierst, 2021), being a developmentally arrested, non-feeding stage with sealed mouth and anus, depending on lipid and glycogen stores for survival. The IJ, being a dauer stage, is more resistant than other life stages to abiotic stressors such as ultraviolet radiation, desiccation and extreme temperatures (Gaugler et al., 1994; Hibshman et al., 2020). EPN have a mutualistic relationship with insect pathogenic bacteria (Xenorhabdus spp. for steinernematids and Photorhabdus spp. for heterorhabditids), which are released by the IJ once inside a host (Forst et al., 1997). The bacteria multiply, killing the insect (in conjunction with the nematodes) and converting it to a food source for 1 or more generations of nematodes; newly developed IJs emerge from depleted cadavers and disperse in search of new hosts. Heterorhabditis and Steinernema are not closely related (Blaxter et al., 1998), and have convergently evolved the entomopathogenic lifestyle (Poinar, 1993). EPN occur widely in nature, and several species are promising biocontrol agents, which are mass-produced and then stored until applied against insect pests (Lacey et al., 2001).

The effects of storage temperature and time (‘age’) on behaviours such as dispersal and infection have been documented for several EPN species (Fan and Hominick, 1991; Griffin, 1996; Yoder et al., 2004; Koppenhöfer et al., 2013). More recently it has been shown that culture and storage temperatures have profound effects on the chemotaxis of both EPN and
animal parasites, including reversals of valence between attraction and repulsion to the same chemical (Lee et al., 2016), which could impact host-finding and foraging behaviour. Prior exposure to low temperatures can also enhance IJs’ resistance to freezing (Brown and Gaugler, 1996; Ali and Wharton, 2013) and desiccation (Jagdale and Grewal, 2007).

This study aims to investigate the effects of temperature and time on the behaviour, stress tolerance and proteome of *Steinernema carpocapsae*, the best studied and most economically important EPN species. We also include *Heterorhabditis megidis* in the behavioural assays; the behaviour of this species 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). We have recently shown, using quantitative label-free proteomics, that the proteomes of *S. carpocapsae* and *H. megidis* IJs differ in their response to temperature: when newly emerged IJs were transferred from a culture temperature of 20 to 9°C, the proteome of *S. carpocapsae* underwent profound changes that were maintained during further storage at that temperature, while the proteome of *H. megidis* underwent more gradual changes over time at both 20 and 9°C (Lillis et al., 2022). Here, we extend our exploration of the effects of storage temperature and time on these 2 species, using similar conditions to those of Lillis et al. (2022) but including phenotypic assays (chemotaxis and, for *S. carpocapsae*, stress tolerance), and investigating whether cold-induced changes are retained when IJs are returned to their culture temperature. Specific objectives were (1) following Lee et al. (2016), to explore how olfactory responses of both species change over time at different storage temperatures; (2) to test how 9°C storage protects *S. carpocapsae* IJs against stress (freezing and desiccation); (3) to investigate 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) to compare the proteome of such temperature-swapped IJs with that of IJs maintained in constant conditions.

**Materials and methods**

**Nematode culturing and conditioning**

*Heterorhabditis megidis* UK211 and *S. carpocapsae* All (both of which had been maintained in laboratory culture for several years) 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 per insect. The larvae died after 2–3 days, and cadavers were placed on White traps (White, 1927) and monitored daily. After the first emergence of IJs, the White trap water was replaced with fresh sterile tap water. IJs that emerged into the water over 3 h was used once only.

**Chemotaxis assays**

Chemotaxis assays (Bargmann et al., 1993) were conducted on 60 mm Petri dishes with 20 mL of 2% nutrient agar. Test stimulus (5 μL) was added to a circle (1 cm diameter) on one side of the plate, and 5 μL of the appropriate diluent was added to a control circle on the opposite side. Sodium azide (2 μL) was added to each circle, to anaesthetize nematodes arriving there (Bargmann et al., 1993). IJs were concentrated by sedimentation and approximately 100–250 IJs in 2 μL were added to the centre of the plate. IJs were allowed to migrate for 1 h at 20°C. The chemotaxis index (CI) was calculated as (number of IJs in treatment circle–number of IJs in control circle)/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 (Hallem et al., 2011).

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, 2019; Kin et al., 2019) and hexanol (O’Halloran and Burnell, 2003; Chaisson and Hallem, 2012). Prenol (3-methyl-2-buten-1-ol) was diluted to 2 μM by mixing 203 μL of 99.9% prenol (Sigma-Aldrich, UK) 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 (1 μL) was prepared by transferring crystalline sodium azide to 1 mL MilliQ water.

There were 3 experiments utilizing chemotaxis assays. In experiment 1, IJs of *S. carpocapsae* and *H. megidis* were assayed at time 0 (immediately after collection and washing), and after 1, 3, 6 and 9 weeks at 9°C, and 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, 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 CIs at the 3-week timepoint in experiment 1 were chosen to assay the effect of brief exposure to low temperatures on the CI, both immediately and after subsequent storage at 20°C. Methyl salicylate and prenol were used for *H. megidis* and hexanol and prenol were used for *S. carpocapsae*. IJs were assayed at time 0 and after 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. *Steinernema carpocapsae* IJs were also assayed and transferred to 20°C after 3 h, due to the plasticity of their chemotactic responses.

In experiment 3, IJs were assayed at time 0, and after 1 and 3 weeks at 9, 12, 15 and 20°C. As above-mentioned, 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 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 plates per treatment at each timepoint, respectively. An exception was for IJs transferred from 9 to 20°C in experiment 1, where there was a total of 5 plates per timepoint. Experiment 2 was repeated with 3 culture batches and 10 plates per batch in each run.

**Freezing and desiccation assays**

Assays were adapted from O’Leary et al. (1998) and Adhikari et al. (2010). 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 h or 75% RH for 5 days. These conditions were chosen to give approximately 50% survival in freshly harvested (time 0) IJs. Desiccation chambers were maintained at 75% RH using supersaturated sodium chloride solutions (Winston and Bates, 1960). After the assay time, 20 mL of sterile tap water was added and IJ survival was assessed after 24 h. Experiments were conducted twice, with 3 culture batches of nematodes in each repeat of the experiment. Five plates were tested per batch of nematodes, to give a total of 30 plates per storage treatment.
**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 Dunn’s multiple comparison post hoc tests to identify groups which were significantly different.

**Protein sample preparation**

The contents of a tube of IJs were sedimented in a 50 mL Falcon tube at their storage temperature. The pelleted IJs (150 \( \mu \)L) were transferred to a 1.5 mL Eppendorf tube and snap frozen in liquid nitrogen. Each sample was homogenized in lysis buffer, containing 6 \( \mu \)M urea, 2 \( \mu \)M thiourea and a protease inhibitor cocktail (cOmplete, Mini Protease Inhibitor Cocktail, Merck), centrifuged at 1 \( 000 \times g \) for 1 min, and snap frozen. This step was repeated 3 times to ensure complete homogenization. Protein content was then quantified using Qubit (Invitrogen), following the manufacturer’s instructions. Protein (100 \( \mu \)g) 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 \(-20^\circ C\) until the last samples were collected, then all were centrifuged at 1 \( 000 \times g \) for 5 min and the resulting pellets were resuspended in 50 \( \mu \)L of resuspension buffer (6 \( \mu \)M urea, 2 \( \mu \)M thiourea, 0.1 \( \mu \)M Tris-HCl, pH 8). A volume of 20 \( \mu \)L was aliquoted from each sample for reduction, alkylation and digestion. One hundred and five microtubes of ammonium bicarbonate (50 mM) and 1 \( \mu \)L of dithiothreitol were added and the samples were incubated at 56°C for 20 min. Once cooled, the samples were alkylated with 2.7 \( \mu \)L of iodoacetamide in the dark.

One microlitre of 1% (w/v) solution of ProteaseMax (Promega) and 0.5 \( \mu \)gL\(^{-1}\) trypsin (Promega) were added to the samples and incubated at 37°C for a minimum of 16 h. Samples were removed from 37°C, centrifuged briefly and acidified with 1 \( \mu \)L of trifluoroacetic acid (TFA) for 5 min at room temperature (20–25°C). Samples were centrifuged at 1 \( 000 \times g \) for 10 min and the supernatant was purified using C18 Spin Columns (Pierce, Thermo Fisher Scientific) following the manufacturer’s instructions and then lyophilized in a Speedyvac concentrator (Thermo Scientific Savant DNA120). Samples were resuspended in a loading buffer (2% v/v acetonitrile, 0.05% v/v TFA) and 1 \( \mu \)g per sample was loaded 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 2–40% gradient of acetonitrile on a Thermo Fisher EASY-Spray, PepMap RSLC C18 column (500 mm length, 75 mm ID), using a reverse-phase gradient at a flow rate of 250 nL min\(^{-1}\) over 125 min. All data were acquired over 105 min with the mass spectrometer operating in automatic data-dependent switching mode. A full mass spectrometry (MS) scan at 140 000 resolution and a range of 300–1700 m/z was followed by an MS/MS scan, resolution 17 500 and a range of 200–2000 m/z, selecting the 15 most intense ions prior to MS/ MS. There were 4 biological replicates (tubs of IJs) per storage treatment.

**Proteomic data processing**

Protein identification and label-free quantification (LFQ) normalization of MS/MS data were 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 datasets derived from the \( S.\ carpocapsae \) genome (Serra et al., 2019).

Normalized LFQ intensities were used to quantify protein abundances, and the data were filtered to remove contaminants. The LFQ intensities were log\_transformed, and each replicate was renamed to their respective groups (e.g. 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 the values of low abundant proteins chosen randomly from a distribution specified by a downshift of 2 times the mean standard deviation (s.d.) and a width of 0.3 times the s.d.

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

An analysis of variance (ANOVA) was performed on all groups using a Benjamini–Hochberg false discovery rate of \(< 0.1\%\) to select proteins for Z-score normalization. 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 on the y-axis and log\_2-fold transformed differences on the x-axis for each comparison. Pairwise t-tests were performed comparing each of the cold-stored treatments (IJs stored at 9°C for 1 week and transferred to 20°C for 2 weeks, and IJs stored at 9°C for 3 weeks) to the IJs maintained at 20°C for 3 weeks, to visualize the effect of temperature change on the IJ proteome. Statistically 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.

**Results**

**Effect of storage at constant temperatures (9 and 20°C) on chemotaxis**

The results of chemotaxis experiments 1 and 2 are shown in Figs 1–3, respectively. \( S.\ carpocapsae \) IJs were initially (time 0) strongly attracted to hexanol and methyl salicylate, strongly repulsed by prenol, and weakly attracted to acetone (Fig. 1). When stored at 9°C for 1 week, initially strong responses (CI ≥0.8) were completely reversed and remained so for the remainder of the 9°C storage period: IJs became repulsed by hexanol (Fig. 1C) and methyl salicylate (Fig. 1B) and highly attracted towards prenol (Fig. 1A). In contrast, the initially weak attraction (CI 0.3) towards acetone was intensified after 1 week at 9°C (Fig. 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 (Fig. 1A). In contrast, the initially weak attraction (Fig. 1A) strongly attracted to hexanol and methyl salicylate, strongly repulsed by prenol, and weakly attracted to acetone (Fig. 1). When stored at 9°C for 1 week, initially strong responses (CI ≥0.8) were completely reversed and remained so for the remainder of the 9°C storage period: IJs became repulsed by hexanol (Fig. 1C) and methyl salicylate (Fig. 1B) and highly attracted towards prenol (Fig. 1A). In contrast, the initially weak attraction (CI 0.3) towards acetone was intensified after 1 week at 9°C (Fig. 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 (Fig. 1A), repulsed by methyl salicylate (Fig. 1B), and less attracted to hexanol (Fig. 1C). The response to acetone did not follow this trend; IJs were repulsed after 1 week at 20°C but the response returned to time 0 levels thereafter (Fig. 1D).

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 h 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 (Fig. 3A). 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 (Fig. 3B).

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

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

In experiment 1, the CI of *S. carpocapsae* IJs which were stored 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 (Fig. 1). The similarity persisted for the full 9 weeks in the case of prenol (Fig. 1A), but the responses began to diverge at 6 weeks in the case of methyl salicylate (Fig. 1C) and at 9 weeks for hexanol (Fig. 1B). The response to acetone of transferred IJs was intermediate between that of IJs stored exclusively at 9 or 20°C (Fig. 1D).

Cold-induced changes in chemotaxis towards prenol seen in *S. carpocapsae* by 3 h at 9°C were maintained and even intensified following transfer to 20°C (Fig. 3A). When tested after 3 weeks, the IJs which were transferred to 20°C after 3 h, 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 (Fig. 3A).

In contrast, although *H. megidis* IJs which were at 9°C for 1 day or 1 week had an altered (intensiﬁed) chemotactic 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 (Fig. 3C). A similar pattern was seen in response to methyl salicylate (Fig. 3D).

**Do all temperatures below culture temperature affect chemotaxis similarly?**

For *S. carpocapsae*, IJs stored at 9, 12 and 15°C all showed a similar CI, differing signiﬁcantly (and with opposite valence) from...
that of IJs stored 20°C after 1 (hexanol) or 3 (prenol) weeks (Fig. 4A and B). Similarly, *H. megidis* IJs stored at 9, 12 or 15°C for 3 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 1 or 3 weeks, there was a significant difference between IJs stored at 9 and 20°C, with IJs stored at 12 and 15°C showing intermediate CIs (Fig. 4C and D).

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

*Steinernema carpocapsae* IJs stored at 9°C showed increased survival in both freezing (−10°C for 6 h) and in desiccation (75% RH for 5 days) assays relative to freshly emerged (time 0) IJs and those stored at 20°C, with significant differences in both cases (Fig. 5). IJs stored at 9°C for 1 week and transferred to 20°C for 2 weeks showed similarly high survival rates which were not statistically significantly different from those of IJs stored at 9°C for 1 or 3 weeks (Fig. 5).

**Response of *S. carpocapsae* proteome to storage conditions**

In general, the proteome of *S. carpocapsae* IJs which were stored at 9°C for 1 week and transferred to 20°C for 2 weeks (temperature-swapped) was more similar to the proteome of IJs stored at 9°C than at 20°C for the full 3 weeks (Fig. 6). In total, 2359 proteins were detected in the *S. carpocapsae* IJs, 36 of which were identified as statistically significant in a 1-way ANOVA. These 36 proteins (grouped by unsupervised hierarchical clustering in Perseus) are shown in a heat map (a visualization of the high and low abundance proteins present in the IJs; Fig. 6B) with further details shown in Table 1.

Cluster D consists of proteins which were detected at high abundance in the IJs stored at 9°C and in the temperature-swapped IJs, 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-stored and temperature-swapped IJs. Cluster A contains proteins related to fat binding and stress proteins while cluster B contains proteins 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 HSP, a serine...
protease inhibitor, macroglobulin-related proteins and various hypothetical proteins (Table 1). There were 2 'clusters' which contained 1 protein each, without a clear function (Table 1).

**Highest and lowest abundance proteins**

Statistically significant annotated proteins which were at least 5-fold increased or decreased in abundance (SSDAs) in the 9°C-stored and/or in the temperature-swapped IJs relative to the 20°C IJs are shown in Table 2. The proteins that showed the greatest increase in abundance in both the 9°C and temperature-swapped treatments were chaperone proteins, such as (*Caenorhabditis remanii* heat shock protein) CRE-HSP and LEA proteins which were 13.4- to 140.5-fold higher than in the IJs at 20°C for 3 weeks. Various enzymes were decreased in abundance relative to the 20°C-stored IJs including chitinase which was 25.6–39.2 times lower in the temperature-swapped and 9°C-stored IJs (Table 2). A number of proteins identified as SSDAs were not annotated (Supplementary Table 1), and these proteins may have important but unknown roles in temperature adaptation.

**Discussion**

Exposure to low temperatures had profound effects on the chemotaxis and stress tolerance of *S. carpocapsae* IJs, as expected based on previous research (Jagdale and Grewal, 2007; Lee et al., 2016). We also showed that these changes were accompanied by major proteomic reorganization and that the changes in chemotaxis, stress tolerance and proteome induced by low-temperature storage were largely maintained on return to culture temperature for 2 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.

Storage 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 (Brown and Gaugler, 1996; Smith et al., 2008; Ali and Wharton, 2013), 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 2 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

---

Fig. 3. Chemotaxis index (mean ± S.E.) of *S. carpocapsae* (A, B) and *H. megidis* (C, D) IJs stored at 9°C for periods of up to 1 week and transferred into 20°C for the remainder of a 3-week period. Control IJs were kept at 9 or 20°C and tested at intervals stated. Within a panel, values accompanied by the same letter are not significantly different ($P < 0.05$, Dunn’s multiple comparisons).
important (Phadtare et al., 1999; Seybold et al., 2017). 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 HSPs.

In our study, the proteins which exhibited the greatest increase in abundance in cold-stored and temperature-swapped *S. carpocapsae* IJs relative to those maintained at the culture temperature were chaperone proteins. The highest abundance chaperone was CRE-HSP, a short heat shock protein (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 both 9°C-stored IJs and temperature-swapped IJs. LEA proteins, which have been identified in several nematode species (Solomon et al., 2000; Browne et al., 2004; Gal et al., 2004; Goyal et al., 2005), are disordered molecular chaperones which enhance an organism’s survival under freezing stress (Solomon et al., 2000; Browne et al., 2002; N'Dong et al., 2002; Reyes et al., 2008; Anderson et al., 2015). LEA proteins interact with trehalose to facilitate the formation of bioglasses (Browne et al., 2004) which confer resistance to desiccation (Solomon et al., 2000) and freezing (N'Dong et al., 2002). 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; Adhikari et al., 2009; Mizrahi et al., 2010; Hand et al., 2011).

The protein which was decreased to the greatest extent (at least 25-fold) in cold-stored relative to 20°C-stored IJs was chitinase, the enzyme responsible for degrading chitin. Chitinases have been detected in *Steinernema riobrave*-infected *G. mellonella* and are speculated to play a role in antifungal activity (Isaacson and Webster, 2002). Chitinases may also facilitate penetration of the insect cuticle and damage the host, expediting its death (Brandt et al., 1978; Osman et al., 2004; Hao et al., 2012). Thus, relatively low levels of chitinase in cold relative to warm-stored IJs may reflect the lower probability of IJs at low temperatures being infected by fungal pathogens, or of infecting an insect host, or both.

The ecological significance of increased stress tolerance 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 valence 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 affected the olfactory responses of *S. carpocapsae*, including a reversal of valence for several of the tested substances. In our experiments, the initial attraction of *S. carpocapsae* by hexanol and methyl salicylate, and repulsion by prenol, was completely reversed by 1 week at 9°C. Hexanol and methyl salicylate are released by plant roots (Roberts et al., 2019) and so 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* (Baiocchi et al., 2017, 2019; Kin et al., 2019), and so the initial repulsion by prenol could help IJs avoid an already infected insect. When maintained at 20°C, the response of *S. carpocapsae* IJs to these 3 odours declined gradually, and reached reversal of valence after 6 weeks in the case of prenol and methyl salicylate. These age-related changes were exceeded by those 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 due to competition (Koppenhöfer and Kaya, 1996; Ryder and Griffin, 2003; Blanco-Pérez et al., 2019), the increased protection from freezing and desiccation a cadaver confers (Lewis and Shapiro-Ilan, 2002; Perez et al., 2003) may offset those costs. Similarly, as an older IJ fails to find an 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 encountered in the field (e.g. Webster et al., 2010). For IJs, the universally produced CO$_2$ is the most

![Fig. 5. Survival (mean ± S.E.) of *S. carpocapsae* IJs exposed to freezing stress (−10°C for 6 h) or desiccation stress (75% RH for 5 days). IJs were either freshly emerged (time 0), stored at 9 or 20°C for 1 and 3 weeks, or stored at 9°C for 1 week and then swapped to 20°C for 2 weeks. Within a panel, values accompanied by the same letter are not significantly different (P = 0.05, Dunn’s multiple comparisons).](image-url)
Fig. 6. (A) PCA of proteins from *S. carpocapsae* IJs stored at 9°C for 3 weeks, at 9°C for 1 week and then swapped to 20°C for 2 weeks, and at 20°C for 3 weeks. (B) Heat map of *S. carpocapsae* All statistically significant proteins: 2-way unsupervised hierarchical clustering of the median Z-score normalized label-free quantification (LFQ) intensity values of all statistically significant proteins from 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 normalized log2-fold transformed LFQ intensity values.
Table 1. Proteins identified in 2-way unsupervised hierarchical clustering of the median Z-score normalized label-free quantification (LFQ) intensity values of all statistically significant proteins (Benjamini–Hochberg false discovery rate 0.01), for *S. carpocapsae* IJs stored for 3 weeks, either at 9°C (3w9C) or 20°C (3w20C) throughout, or swapped from 9 to 20°C after 1 week (1w9C → 20C).

| Relative abundance | 3w9C | 1w9C → 20C | 3w20C | 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         | 3.5 × 10^9 | 229         |
| −1.19              |      | 1.10        | A     | LS96_020479.2 | Hypothetical protein LS96_020479 | 7     | 28.93          | 2.9 × 10^8 | 49          |
| −1.03              | −0.15| 1.14        | A     | LS96_022932 | Programmed cell death 8 | 10    | 70.283         | 2.5 × 10^8 | 65          |
| −1.07              | −0.11| 1.15        | A     | LS96_013610 | Aldehyde dehydrogenase | 29    | 57.781         | 3.3 × 10^6 | 297         |
| −0.92              | −0.11| 1.26        | A     | LS96_030114 | Succinate-CoA ligase [GDP-forming] subunit beta, mitochondrial | 7     | 46.012         | 3.0 × 10^6 | 58          |
| −0.84              | −0.17| 1.34        | A     | LS96_014396 | Inosine-5′-monophosphate dehydrogenase 1 | 7     | 34.475         | 3.5 × 10^8 | 55          |
| −0.89              | −0.29| 1.09        | A     | LS96_015746.1 | Fatty acid and retinol-binding protein | 11    | 21.296         | 1.7 × 10^9 | 90          |
| −0.14              | −1.01| 1.06        | B     | LS96_017315 | 60S ribosomal protein L9 | 11    | 21.254         | 2.9 × 10^9 | 122         |
| −0.27              | −1.11| 1.16        | B     | LS96_018977 | Putative NADH-quinone oxidoreductase subunit I | 7     | 23.184         | 1.4 × 10^9 | 59          |
| 0.49               | −1.28| 0.82        | B     | LS96_007662 | 60S ribosomal protein L12 | 1     | 20.089         | 2.2 × 10^9 | 63          |
| −1.12              | 1.10 | −0.17       | X     | LS96_008455.1 | SEA domain containing protein | 5     | 84.111         | 3.4 × 10^6 | 52          |
| 1.28               | −0.52| −0.90       | Y     | LS96_012434.1 | Hypothetical protein LS96_012434 | 3     | 51.288         | 2.3 × 10^8 | 18          |
| −0.13              | 1.23 | −0.92       | C     | LS96_013178 | Hypothetical protein LS96_013178 | 6     | 18.033         | 9.7 × 10^7 | 21          |
| −0.22              | 1.18 | −1.08       | C     | LS96_026575 | Heat-shock protein | 25    | 72.459         | 8.4 × 10^6 | 342         |
| −0.21              | 1.21 | −1.08       | C     | LS96_024306 | Hypothetical protein LS96_024306 | 8     | 33.817         | 6.1 × 10^8 | 62          |
| −0.32              | 1.24 | −0.97       | C     | LS96_009048.2 | Alpha-2-macroglobulin family protein | 30    | 86.756         | 2.3 × 10^9 | 301         |
| −0.25              | 1.25 | −1.00       | C     | LS96_009045.1 | a-Macroglobulin complement component | 21    | 99.788         | 7.2 × 10^8 | 127         |
| 0.15               | 1.02 | −1.24       | C     | ADY11211.1 | Serine proteinase inhibitor | 22    | 43.674         | 2.3 × 10^9 | 203         |
| 0.63               | 0.78 | −1.19       | D     | LS96_014613 | Carboxylesterase, type B domain-containing protein | 34    | 77.629         | 8.4 × 10^9 | 432         |
| 0.72               | 0.70 | −1.29       | D     | LS96_030565 | Hypothetical protein LS96_030565 | 5     | 14.902         | 7.4 × 10^8 | 59          |
| 0.69               | 0.73 | −1.35       | D     | LS96_020879 | LEAS protein | 3     | 7.4813         | 8.3 × 10^6 | 28          |
| 0.44               | 0.89 | −1.17       | D     | SC.X.g3583 | Glutathione peroxidase | 7     | 26.194         | 1.2 × 10^10 | 119        |
| 0.52               | 0.84 | −1.27       | D     | LS96_013116 | Hypothetical protein LS96_013116 | 7     | 18.748         | 1.6 × 10^9 | 62          |
| 0.44               | 0.81 | −1.37       | D     | LS96_029037.2 | Acetyl-CoA C-acetyltransferase | 11    | 42.561         | 5.8 × 10^8 | 92          |
| 1.08               | 0.28 | −1.27       | D     | LS96_016356.1 | CRE-FAH-1 protein | 13    | 46.228         | 1.8 × 10^9 | 118         |
| 0.85               | 0.46 | −1.17       | D     | LS96_019563.2 | Hypothetical protein LS96_019563 | 1     | 17.438         | 2.3 × 10^9 | 102         |
| 0.86               | 0.46 | −1.38       | D     | LS96_013113 | Hypothetical protein LS96_013113 | 3     | 15.175         | 4.9 × 10^8 | 22          |
| 0.87               | 0.42 | −1.31       | D     | SC.X.g4329 | Selenium-binding protein 1 | 15    | 50.706         | 8.8 × 10^6 | 115         |
| 0.95               | 0.35 | −1.36       | D     | LS96_026406 | CRE-HSP-12.1 protein | 5     | 23.854         | 1.4 × 10^9 | 73          |
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 prior storage temperature and time affect IJs in a way that is both ecologically relevant and of importance to biocontrol. The sophistication of the nematodes' responses to variables and stimuli applied singly under the highly artificial conditions of laboratory assays can only suggest the level of variation to be expected between individuals experiencing diverse combinations of factors in the soil environment.

While the ability of IJs to find and kill insects generally declines with age (Lewis et al., 1995b; Patel et al., 1997), there is also evidence of more complex effects of prior 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 time, especially in IJs maintained at 9°C (Griffin, 1996; Fitters et al., 2001). Similar effects have been documented for steinernematids (Koppenhöfer et al., 2013; Guy et al., 2017; Yadav and Eleftherianos, 2018). Host-finding and infection in soil involve a series of steps, including dispersal, host-finding and host-recognition and acceptance (Lewis et al., 1995a; Griffin, 2015). 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 in infectivity, which possibly indicates increased tendency to penetrate into a host following arrival (Dempsey and Griffin, 2002; Koppenhöfer et al., 2013).

While it is tempting to explain the cold-induced changes in chemotaxis as ecologically relevant, it is also possible that they are a by-product of altered thermotactic behaviour, such as migration to warmer regions of the soil, and/or tracking seasonal vertical movements of hosts within the soil (Villani and Wright, 1990). There is some overlap of neurons which mediate chemotaxis and thermotaxis, and these neurons may 'memorize' sensed temperatures (Clark et al., 2006; Kimata et al., 2012). While alterations in response to any specific odourant are 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; Lillis et al., 2022). Moreover, it is clear that the assays of chemo-attraction reflect the current status of the IJs, a product of their 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.

Storage of *S. carpocapsae* and *H. megidis* IJs at temperatures lower than culture temperature affected each species in profoundly different ways. Steinernema carpocapsae IJs underwent reversals of valence in olfactory response while *H. megidis* IJs' chemotactic response to odours was generally intensified by exposure to low temperatures. Together with the proteomic profiling of Lillis et al. (2022), it appears that while both species are affected by storage time and temperature, *H. megidis* undergoes more graduated changes, in contrast to the dramatic changes demonstrated for *S. carpocapsae* when placed at 9°C. It is unclear to what extent the differences between the 2 species are a result of phylogeny or of species-specific adaptation to ecological factors. Similar studies on a larger number of species of each genus could help resolve this.
The proteome of *S. carpocapsae* IJs exhibited extensive remodelling upon exposure to 9°C, as previously shown by Lillis et al. (2022) with the novel finding that 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 tolerance induced by brief cold 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. The rapid phenotypic adaptation of IJs also has implications for the interpretation of experiments testing genetic adaptation to different temperatures (Grewal et al., 1996). While we only explored the effects of storage conditions on the individuals that were exposed to those conditions, possible transgenerational (including epigenetic) effects of thermal history (Klosin et al., 2017; McCaw et al., 2020) should also be investigated. Moreover, the induction by brief cold exposure of profound changes that are maintained following the return of favourable conditions has resonances with certain forms of diapause and quiescence in parasitic nematodes and may share some common pathways with these poorly understood phenomena.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0031182022001445.

**Author’s contributions.** P. E. L. and C. T. G. conceived and designed the study. P. E. L. and I. P. K. did the experimental work. J. C. C. supervised the proteomic analysis. P. E. L. carried out the statistical analysis and wrote the original draft manuscript. P. E. L., J. C. C. and C. T. G. contributed to writing the manuscript. The proteomic data and output files for the present study

### Table 2. Annotated statistically significant proteins 5-fold changed in abundance in *S. carpocapsae* IJs stored at 9°C for 3 weeks (3w9C) and those stored at 9°C for 1 week and transferred to 20°C (1w9C → 20C), relative to IJs stored at 20°C for 3 weeks

| Protein ID | Blast annotation | Relative fold change vs 3w20C | Peptides | Mol. weight | Intensity | MS/MS count |
|------------|------------------|-----------------------------|----------|-------------|-----------|-------------|
| L596_026406 | CRE-HSP-12.1 protein | 140.5 | 35.9 | 5 | 23.854 | 1.44 × 10^8 | 73 |
| L596_020879 | LEA5 protein | 93.6 | 95.8 | 3 | 7.4813 | 8.29 × 10^8 | 28 |
| L596_020881 | LEA5 protein | 61.2 | 33.5 | 2 | 10.094 | 6.11 × 10^8 | 20 |
| L596_015330 | SaPosin-like protein family | 60.0 | 43.9 | 4 | 10.214 | 1.16 × 10^9 | 37 |
| L596_019565.1 | LEA2 protein | 56.8 | na | 4 | 10.539 | 2.20 × 10^9 | 45 |
| L596_012217 | PhosphoGlycolate phosphatase homolog | 23.4 | na | 5 | 38.207 | 2.28 × 10^8 | 18 |
| L596_024791 | Cystathionine beta-synthase | 17.9 | na | 16 | 77.905 | 1.31 × 10^8 | 43 |
| ABQ23230.1 | LEA1 protein | 17.1 | 13.4 | 5 | 9.7546 | 5.25 × 10^8 | 75 |
| SC.X.g25872 | Putative cystathionine gamma-lyase 2 | 7.4 | 8.7 | 5 | 42.673 | 1.07 × 10^8 | 30 |
| SC.X.g3323 | Myosin regulatory light chain 1 | 7.3 | na | 4 | 18.941 | 8.40 × 10^7 | 17 |
| L596_019840 | C. briggsae CBR-OSM-11 protein | 7.2 | na | 3 | 28.814 | 7.40 × 10^8 | 22 |
| SC.X.g3305 | C-1-tetrahydrololate synthase, cytoplasmic | 6.8 | na | 8 | 102.42 | 1.34 × 10^8 | 43 |
| L596_030616 | Protein LSM12-like protein A | 6.8 | 3.2 | 3 | 24.9 | 4.87 × 10^7 | 17 |
| L596_017887.4 | ADP-ribose pyrophosphatase, mitochondrial precursor | 5.7 | 5.7 | 4 | 17.112 | 1.22 × 10^8 | 28 |
| L596_024012 | Thiamin pyrophosphokinase | na | 45.7 | 9 | 30.405 | 2.58 × 10^8 | 48 |
| L596_028041 | Serine/threonine-protein phosphatase PP1-alpha | na | 7.0 | 2 | 19.921 | 1.13 × 10^8 | 11 |
| SC.X.g1861 | Ani s 9 allergen precursor | na | 5.3 | 2 | 14.755 | 9.04 × 10^7 | 13 |
| L596_026200 | Anclyostoma secreted protein | na | −5.6 | 3 | 29.665 | 1.94 × 10^8 | 26 |
| SC.X.g6035 | Ras protein let-60 | na | −11.5 | 4 | 21.122 | 1.06 × 10^8 | 15 |
| L596_019533 | Medium-chain specific acyl-CoA dehydrogenase, mitochondrial | −5.2 | na | 7 | 45.266 | 5.91 × 10^7 | 27 |
| L596_028677 | Piwi domain protein | −6.0 | na | 5 | 100 | 5.03 × 10^7 | 20 |
| L596_017378 | Trypsin-like serine protease | −6.0 | −4.5 | 3 | 32.864 | 1.34 × 10^8 | 22 |
| L596_018471 | Acetyl-coenzyme A synthetase 2, putative | −6.9 | −4.0 | 4 | 78.513 | 7.81 × 10^7 | 21 |
| SC.X.g4477 | Probable H/ACA ribonucleoprotein complex subunit 1-like protein | −8.2 | −5.0 | 4 | 24.466 | 1.50 × 10^8 | 23 |
| SC.X.g3201 | Short-chain dehydrogenase/reductase | −8.2 | −3.6 | 2 | 29.235 | 7.42 × 10^7 | 14 |
| L596_023970 | NAC domain containing protein | −11.8 | na | 4 | 21.879 | 1.57 × 10^8 | 31 |
| L596_015314 | Chitinase class I | −39.2 | −25.6 | 9 | 101.39 | 3.12 × 10^8 | 33 |

Proteins which were identified but could not be annotated can be found in Supplementary Table 1.
are available at https://www.ebi.ac.uk/pride/archive/projects/PXD027609. Other datasets generated during this study are available from the corresponding author on reasonable request.

**Financial support.** P. E. L. was funded by a Maynooth University John and Pat Hume Doctoral Fellowship. The Maynooth University Q-Exactive Quantitative Mass Spectrometer was funded under the SFI Research Infrastructure Call 2012; Grant Number: 12/RI/2346 (3).

**Conflict of interest.** None.

**Ethical standards.** Not applicable.

**References**

Adikari BN, Wall DH and Adams BJ (2009) Desiccation survival in an Antarctic nematode: molecular analysis using expressed sequenced tags. *BMC Genomics* 10, 69.

Adikari BN, Wall H and Adams BJ (2010) Effect of slow desiccation and freezing on gene transcription and stress survival of an Antarctic nematode. *Journal of Experimental Biology* 213, 1803–1812.

Aleyu OA and Kutz S (2020) Adaptations, life-history traits and ecological mechanisms of parasites to survive extremes and environmental unpredictability in the face of climate change. *International Journal for Parasitology: Parasites and Wildlife* 12, 308–317.

Ali F and Wharton DA (2013) Cold tolerance abilities of two entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *Cryobiology* 66, 24–29.

Ali F and Wharton D (2015) Infective juveniles of the entomopathogenic nematode, *Steinernema feltiae* produce cryoprotectants in response to freezing and cold acclimation. *PLOS ONE* 10, e0141810.

Andaló V, Moino A, Mazinimiano C, Campos V and Mendonça I (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 and Cowan D (2015) A novel bacterial water hypersensitivity-like protein shows in vivo protection against cold and freeze damage. *FEBS Microbiology Letters* 362, fnv110.

Baicăoici T, Lee G, Choe D-H and Dillman AR (2017) Host seeking parasitic nematodes use specific odors to assess host resources. *Scientific Reports* 7, 6270.

Baicăoici T, Braun I and Dillman AR (2019) Touch-stimulation increases host-seeking behavior in *Steinernema carpocapsae*. *Journal of Nematology* 51, 1–5.

Bargmann CI, Hartwig E and Horvitz HR (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 VS, Bargmann CI, Hartwieg E and Horvitz HR (2017) Conditioning the *Steinernema* and *Heterorhabditis* bacteria: bugs that kill bugs. *Annual Review of Microbiology* 51, 47–72.

Boyce MD, Barracca IG, Corti A, Di Bartolo L, Di Bari R and Cingolani A (2020) Pyrosequencing of *Steinernema* and *Heterorhabditis* bacteria. *Journal of Invertebrate Pathology* 157, 1–15.

Bubrig LT and Fierst JL (2021) Review of the dauer hypothesis: what non-parasitic species can tell us about the evolution of parasitism. *Journal of Parasitology* 107, 717–725.

Chaisson KE and Hallem EA (2012) Chemosensory behaviors of parasites. *Trends in Parasitology* 28, 427–436.

Clark DA, Biron D, Sengupta P and Samuel ADT (2006) The AF2 sensory neurons encode multiple functions underlying thermotactic behavior in *Caenorhabditis elegans*. *The Journal of Neuroscience* 26, 7444.

Close TJ (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum* 97, 795–803.

Côté RG, Griss J, Dianes JA, Wang R, Wright JG, van den Toorn HWP, van Breukelen B, Heck AJR, Hulstaert N, Martens L, Reisinger F, Coardas A, Ovelleiro D, Perez-Rivevol Y, Barsnes H, Hermjakob H and Vizcaino JA (2012) The Phoebotixms 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.

Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV and Mann M (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *Journal of Proteome Research* 10, 1794–1805.

Dempsey CM and Griffin CT (2002) Phased activity in *Heterorhabditis megidis* insective juveniles. *Parasitology* 124, 605–613.

Dillman AR, Guillermin ML, Lee JH, Kim B, Sternberg PW and Hallem EA (2012) Olfaction shapes host–parasite interactions in parasitic nematodes. *Proceedings of the National Academy of Sciences* 109, E2324.

Evans AAF and Perry RN (2009) Survival mechanisms. In Perry RN, Moens M and Starr JL (eds), *Root-Nematode*.s. Wallingford, UK: CAB International. Publishing, pp. 201–222.

Fan X and Hominick W (1991) Effects of low storage temperature on survival and infectivity of two *Steinernema* species (*Nematoda : Steinernematidae*). *Revue de Nematologie* 14, 407–412.

Fitters PFL and Griffin CT (2004) Spontaneous and induced activity of *Heterorhabditis megidis* insective juveniles during storage. *Nematology* 6, 911–917.

Fitters PFL, Dunne R and Griffin CT (2001) Vine weevil control in Ireland with entomopathogenic nematodes: optimal time of application. *Irish Journal of Agricultural and Food Research* 40, 199–213.

Forst S, Dowds B, Boemare N and Stackebrandt E (1997) *Xenorhabdus and Photorhabdus spp.: bugs that kill bugs*. Annual Review of Microbiology 51, 47–72.

Gal T, Glazer I and Kolta H (2004) A LEA3 family member is involved in survival of *C. elegans* during exposure to stress. *FEBS Letters* 577, 21–26.

Gaugler R, Bednarek A and Campbell JF (1992) Ultraviolet inactivation of *Heterorhabditis* and *steinernematid* nematodes. *Journal of Invertebrate Pathology* 59, 155–160.

Goyal K, Walton IJ and Tunnacchile A (2005) LEA proteins prevent protein aggregation due to water stress. *Biochemical Journal* 388, 151–157.

Grewal PS, Lewis EE, Gaugler R and Campbell JF (1994) Host finding behaviour as a predictor of foraging strategy in entomopathogenic nematodes. *Parasitology* 108, 207–215.

Grewal PS, Gaugler R and Shape C (1996) Rapid changes in thermal sensitiviy of entomopathogenic nematodes in response to selection at temperature extremes. *Journal of Invertebrate Pathology* 68, 65–73.

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

Griffin CT (2015) Behaviour and population dynamics of entomopathogenic nematodes following application. In Campos-Herrera R (ed.), *Nematode Pathogenesis of Insect and Other Pests*. Cham, Switzerland: Springer International Publishing, pp. 57–95.

Guillemín ML, Carrillo MA and Hallem EA (2017) A single set of interneurons drives opposite behaviors in *C. elegans*. *Current Biology* 27, 2630–2639.e2636.

Guy A, Galfney M, Kapranas A and Griffin CT (2017) Conditioniing 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 EA, Rengarajan M, Ciche TA and Sternberg PW (2007) Nematodes, bacteria, and flies: a tripartite model for nematode parasitism. *Current Biology* 17, 898–904.

Hallem EA, Dillman AR, Hong AV, Zhang Y, Yano JM, DeMarco SF and Sternberg PW (2011) A sensory code for host seeking in parasitic nematodes. *Current Biology* 21, 377–383.

Hand SC, Menze MA, Toner M, Boswell L and Moore D (2011) LEA proteins during water stress: not just for plants anymore. *Annual Review of Physiology* 73, 115–134.

Hao YJ, Montiel R, Lucena MA, Costa M and Simeo N (2012) Genetic diversity and comparative analysis of gene expression between *Heterorhabditis bacteriophora Az29* and *Az26* isolates: uncovering candidate genes involved in insect pathogenicity. *Experimental Parasitology* 130, 116–125.

Parasitology
Patel MN, Stolinski M and Wright DJ (1997) Neutral lipids and the assessment of infectivity in entomopathogenic nematodes: observations on four Steinernema species. Parasitology 114, 489–496.

Perez EE, Lewis EE and Shapiro-Ilan DI (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.

Phadare S, ALSina J and inouye M (1999) Cold-shock response and cold-shock proteins. Current Opinion in Microbiology 2, 175–180.

Poinar GO (1993) Origins and phylogenetic relationships of the entomophilic rhabditids, Heterorhabditis and Steinernema. Fundamental & Applied Nematology 16, 333–338.

Reyes JL, Campos F, Wei H, Arora R, Yang Y, Karlson DT and Covarubbia AA (2008) Functional dissection of hydrophins during in vitro freeze protection. Plant, Cell & Environment 31, 1781–1790.

Roberts JM, Kondun J, Rowley C, Hall DR, Douglas P and Pope TW (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 JJ and Griffin CT (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 and 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, Machietto M, Macias-Muñoz A, McGill CJ, Rodriguez IM, Rodriguez B, Murad R and Mortazavi A (2019) Hybrid assembly of the genome of the entomopathogenic nematode Steinernema carpocapsae identifies the X-Chromosome. Genes, Genomes, Genetics 9, 2687–2697.

Sebald AC, Wharton DA, Thorne MAS and Marshall CJ (2017) Investigating trophalage synthesis genes after cold acclimation in the Antarctic nematode Panagrolaimus sp. DAW1. Biology Open 6, 1953–1959.

Smith T, Wharton DA and Marshall CJ (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 and Glazer I (2000) Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable protein. Parasitology 121, 409–416.

Sommerville RI and Davey KG (2002) Diapause in parasitic nematodes: a review. Canadian Journal of Zoology 80, 1817–1840.

Villani MG and Wright RJ (1990) Environmental influences on soil microarthropod behavior in agricultural systems. Annual Review of Entomology 35, 249–269.

Wang H, Lei Z, Li X and Oetting RD (2011) Rapid cold hardening and expression of heat shock protein genes in the B-histotype Bemisia tabaci. Environmental Entomology 40, 132–139.

Wang Y, Chen Z, Yang Z and Zhang F (2021) Transcriptional reprogramming caused by cold acclimation in Meloidogyne incognita eggs. Genes & Genomics 43, 533–541.

Webster B, Bruce T, Pickett J and Hardie J (2010) Volatiles functioning as host cues in a blend become non-host cues when presented alone to the black bean aphid. Animal Behaviour 79, 451–457.

White GF (1927) A method for obtaining infective nematode larvae from culture. Science 66, 302–303.

Willet DS, Alborn HT, Duncan LW and Stelinski LL (2015) Social networks of educated nematodes. Scientific Reports 5, 14388.

Winston PW and Bates DH (1960) Saturated solutions for the control of humidity in biological research. Ecology 41, 232–237.

Woodring JL and Kaya HK (1988) Steinernematid and Heterorhabditid Nematodes: A Handbook of Biology and Techniques. Fayetteville, AR, USA: Southern Cooperative Series Bulletin 331.

Yadav S and Eleftherianos I (2018) Prolonged storage increases virulence of Steinernema entomopathogenic nematodes toward Drosophila larvae. Journal of Parasitology 104, 722–725.

Yoder CA, Grewal PS and Taylor RAJ (2004) Rapid age-related changes in infection behavior of entomopathogenic nematodes. Journal of Parasitology 90, 1229–1234.

Zhao LL, Wei W, Kulhavy DL, Zhang XY and Sun JH (2007) Low temperature induces two growth-arrested stages and change of secondary metabolites in Bursaphelenchus xylophilus. Nematology 9, 663–670.