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Effect of slow desiccation and freezing on gene transcription and stress survival of an Antarctic nematode

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SUMMARY

Nematodes are the dominant soil animals of the Antarctic Dry Valleys and are capable of surviving desiccation and freezing in an anhydrobiotic state. Genes induced by desiccation stress have been successfully enumerated in nematodes; however, little is known about gene regulation by Antarctic nematodes that can survive multiple types and incidences of environmental stress. In order to reveal the molecular response of anhydrobiotic survival, we investigated the genetic response of an Antarctic nematode, *Plectus murrayi*, which can survive desiccation and freezing. Using molecular approaches, we provide insight into the regulation of desiccation-induced transcripts during different stages of stress survival under conditions characteristic of the Antarctic Dry Valley environment. Our study revealed that exposure to slow desiccation and freezing plays an important role in the transcription of stress, metabolism and signal transduction-related genes and improves desiccation and freezing survival compared with nematodes exposed to fast desiccation and freezing. Temporal analyses of gene expression showed that pre-exposure to mild stress promotes survival of harsher stress. Our results further show that exposure to slow dehydration not only improves extreme desiccation survival but also promotes enhanced cold tolerance. We also provide evidence that slow dehydration can enhance freeze tolerance in an Antarctic nematode. Expression profiling of *P. murrayi* transcripts is an important step in understanding the genome-level response of this nematode to different environmental stressors.

Key words: pretreatment, desiccation, anhydrobiosis, freezing, transcription, *Plectus murrayi,* quantitative real-time PCR.

INTRODUCTION

The abundance and activity of many soil-dwelling organisms depends upon the moisture characteristics of their environment. This is especially true in the Antarctic where the availability of liquid water, even more so than temperature, is recognized as the most important determinant of the distribution of Antarctic terrestrial organisms (Kennedy, 1993). Most of the time moisture is likely to be limited, as water is biologically unavailable in the form of ice. Similarly, during the austral summer, terrestrial microhabitats may dry depending on the vagaries of precipitation, wind, temperature and insulation in relation to soils and sediments (Kennedy, 1993). Therefore, desiccation tolerance and/or survival of varying relative humidity (RH) conditions are likely to be as important as cold tolerance for the survival of terrestrial organisms in polar environments (Block, 2002; Ring and Danks, 1994).

Nematodes are the most abundant invertebrate in the McMurdo Dry Valleys of Antarctica, the coldest and driest desert on Earth, inhabiting sandy soils and sediments that are commonly high in salinity and low in carbon and nitrogen (Burkins et al., 2000). Four nematode taxa are reported from this region (*Scottnema lindsayae*, *Plectus murrayi*, *Eudorylaimus antarcticus* and *Monhystera villosa*) (Adams et al., 2006) and their habitat suitability is influenced by soil moisture, carbon and salinity (Treonis et al., 1999). These nematodes play important roles in nitrogen mineralization and carbon cycling (*S. lindsayae* contributes 2–7% of the heterotrophic carbon flux) (Barrett et al., 2008; Freckman, 1988) in Dry Valley soils. Antarctic nematodes are continually exposed to environmental extremes (desiccation, cold, higher salinity and wind) and are well adapted to such stresses through anhydrobiosis (Treonis et al., 2000; Wharton and Barclay, 1993), a dry and metabolically inactive state induced by desiccation (Crowe and Madin, 1975).

Traditionally, studies on the anhydrobiotic survival of Antarctic nematodes have focused on desiccation stress and physiological mechanisms used to reduce water loss (Pickup, 1990a; Pickup, 1990b; Pickup, 1990c; Pickup and Rothery, 1991; Wharton, 2003). The mechanisms of cold and desiccation survival by an Antarctic nematode *Panagrolaimus davidi* have been extensively enumerated (Wharton, 2003; Wharton et al., 2003; Wharton et al., 2005) but the molecular mechanisms are poorly understood. A connection between desiccation and cold tolerance has been identified in several invertebrate species, including nematodes (Forge and MacGuidwin, 1992; Holmstrup and Zachariassen, 1996; Somme, 1996; Worland et al., 1998) and insects (Ring and Danks, 1994; Williams et al., 2004). Dehydration seems to be an important freeze avoidance strategy used by invertebrates in cold deserts, including Arctic and Antarctic environments (Holmstrup et al., 2002a; Ring and Danks, 1994; Treonis and Wall, 2005; Wharton, 2003). This has led to the conclusion that many of the physiological and molecular responses to cold may have originally been adaptations for desiccation stress (Danks, 2000). Among many environmental stresses that terrestrial nematodes encounter, low temperature and desiccation are thought to be particularly closely linked (Crowe et al., 1983; Forge and MacGuidwin, 1992) both in the nature of the stress and in the adaptations that allow nematodes to survive them. In addition, nematodes that survive freezing utilize osmotic dehydration to convert a cold stress into a desiccation stress at the cellular level, and there is considerable cross-tolerance between cold and desiccation stress effects (Bayley et al., 2001; Block, 2002; Ramlov

and Lee, 2000; Williams et al., 2004). At least one of the cold tolerance strategies utilized by soil invertebrates (cryoprotective dehydration) is based upon this cross-tolerance (Holmstrup et al., 2002b).

Like cold tolerance, desiccation tolerance can be increased by pre-exposure (acclimation) to a non-lethal stress. For example, in the soil nematode *Aphelenchus avenae*, exposure to 97% RH causes accumulation of high levels of trehalose (Madin and Crowe, 1975; Womersley and Smith, 1981), which acts to preserve membrane and protein integrity (Crowe et al., 1992). Similarly, pre-exposure to mild stress can significantly increase subsequent resistance to the same challenge in many invertebrates including nematodes (Holmstrup et al., 2002a). Trehalose and other sugars accumulated during desiccation stress in many invertebrates can also act as cryoprotectants (Ring and Danks, 1994). Pre-exposure to milder stress is important not only in the context of stress survival but also because, as for the desiccation response, a period of acclimation can allow modulation of metabolic and biochemical processes crucial for the successful induction of anhydrobiosis (reviewed by Barrett, 1991; Womersley, 1990). However, very little is known of the molecular and physiological changes induced by desiccation stress in Antarctic Dry Valley nematodes.

Plectus murrayi, a bacterivorous nematode, inhabits both semi-aquatic and terrestrial biotopes in the McMurdo Dry Valleys (76.5–78.5°S latitude, 160.0–164.0°E longitude) and a few other regions of continental Antarctica (Adams et al., 2006; Andrassy, 1998). Endemic to Antarctica, *P. murrayi* has a multiple-year life cycle but the exact life span of the different developmental stages is unknown. *Plectus murrayi* from the McMurdo Dry Valleys is capable of surviving desiccation as well as freezing, making it an appropriate model for studying the survival mechanisms of both desiccation and freezing, including the effects of pre-exposure to less extreme conditions. Although *P. murrayi* appears to survive extreme environmental conditions, a detailed molecular assessment of the effect of acclimation on its stress survival response is still lacking. Previous efforts to unravel patterns of gene expression in stress survival used expressed transcripts (ESTs) from desiccation-acclimatized *P. murrayi* to identify genes that are differentially expressed during entry into anhydrobiosis (Adhikari et al., 2009). Expression profiling of these genes showed that anhydrobiotic survival in *P. murrayi* involves a diverse suite of functional genes. Further validation of these differentially expressed genes showed that desiccation stress involves up-regulation of a number of genes including those encoding trehalose-6-phosphate synthase, aldehyde dehydrogenase, glycerol kinase, malate synthase, heat shock proteins and a novel protein, and down-regulation of a gene encoding an antifreeze protein (Adhikari et al., 2009).

In this study, we report the first detailed assessment of the molecular and physiological response to dehydration and freezing of *P. murrayi* under ecologically relevant conditions characteristic of both the austral summer and winter. By investigating the patterns of gene expression and survival of nematodes at RH values similar to those of their natural environment, we have identified changes that play a major role in response to stress exposure and recovery. In addition, we tested the hypothesis that physiological adaptations to milder stress promote desiccation, freezing and cross-tolerance to other stresses. Accordingly, we investigated eight genes which were shown to be differentially expressed in response to desiccation: glutathione S-transferase (*Pm-gst-1*), trehalose-6-phosphate synthase (*Pm-tps*), c-Jun N-terminal kinase (*Pm-jnk-1*), heat shock protein 70 (*Pm-hsp-70*), malate synthase (*Pm-ms*), glycogen synthase (*Pm-gsy*), heat shock protein 90 (*Pm-hsp-90*) and antifreeze protein (*Pm-afp*). These genes are reported to be directly implicated in the regulation of metabolism and cellular responses by nematodes during environmental stress (Adhikari et al., 2009; Davis, 2000; Duman, 2001; Gal et al., 2003; Tyson et al., 2007). Therefore, we designed an experiment that examines changes in gene expression and nematode survival during pretreatment, exposure to cold and desiccation, and a period of recovery from stress, allowing us to determine the role of slow desiccation and freezing on stress survival of nematodes.

MATERIALS AND METHODS

Nematode rearing

Plectus murrayi Yeates 1970 collected from soils and sediments in Taylor Valley (77°S latitude, 163°E longitude), Antarctica, site of the NSF McMurdo Dry Valley Long Term Ecological Research site, were reared in mass culture to generate sufficient quantities for experimental and control treatments. Nematodes were cultured in sand agar plates (15 g l^{-1} of Bacto-agar) containing 20 ml l^{-1} Bold's Modified Basal Freshwater (BMBF) nutrient media (Sigma Aldrich Inc., St Louis, MO, USA) as described previously (Adhikari et al., 2009). The sand agar plates with nematodes were incubated at 26°C for 1–2 weeks followed by 15°C for 3–5 weeks. Nematodes were stored at 4°C for 1–2 weeks before being harvested for experiments.

Effect of desiccation treatment on survival

RH was controlled using saturated salt solutions (Winston and Bates, 1960) in glass desiccation chambers (Ginsberg Scientific Inc., Fort Collins, CO, USA). Required RH was maintained at 4±0.2°C in the desiccation chamber for 3 days for equilibration prior to the addition of nematodes. Humidity was maintained as 100% RH with distilled water vapor, and as 98%, 85%, 75%, 50% and 35% RH with different saturated salt solutions, while 0% RH was maintained with Drierite desiccant (Drierite Co. Ltd, Xenia, OH, USA).

To assess the survival of nematodes after exposure to different RH conditions, a 100 µl suspension containing approximately 200 nematodes was put in a 35 mm Petri dish and placed in desiccation chambers maintained at 4±0.2°C. Treatments consisted of exposure to 98%, 75%, 35%, 98%+85%+…+0% (exposed to a gradual decrease in RH from 98% to 85%, 75%, 50% and 35% RH for 6 h each before exposure to 0% RH) and 0% RH for 10 days. Nematodes were rehydrated in water at 4°C for 24 h. Viability was determined by nematode movement. Where nematodes were static a hair probe was used to stimulate movement. Nematodes not moving after this stimulus were recorded as dead. All experiments were repeated at least 3 times under identical conditions using mixed stage populations.

Effect of slow desiccation and freezing on survival

To assess the effect of slow desiccation on nematode survival, a 100 µl suspension containing approximately 200 nematodes was put in a 35 mm Petri dish and placed in the desiccation chambers with different RH. Treatments consisted of exposure for 3 and 7 days to 75% RH with (pretreat+desiccation) or without (desiccation) pretreatment at 98% (for 12 h) followed by 85% RH (for 12 h). Nematode viability and survival was determined as mentioned above.

To assess the effect of gradual freezing on nematode survival, a 100 µl suspension containing approximately 200 nematodes was put in a 1.7 ml Eppendorf tube and placed in a temperature chamber (Cincinnati Subzero Products Inc., Cincinnati, OH, USA) which can control temperature from –30°C to 100°C with a cooling rate of

1°C h^{-1}. Nematodes were either directly exposed to –10°C (freezing) or exposed to gradual cooling from 4°C to –10°C (pretreat+freezing) at a rate of 1°C h^{-1} for 3 and 7 days. At least three replicates were performed for each treatment and survival was assessed as described above.

Stress treatment and gene expression

The effect of desiccation treatment on gene expression was measured by putting a 200 µl suspension containing approximately 1000 nematodes in a 35 mm Petri dish and exposing them to different RH. Treatments consisted of pretreatment (exposure to 98% RH followed by 85% RH for 12 h each), pretreat+desiccation (exposure to 75% RH for 3 days after pretreatment), desiccation (direct exposure to 75% RH for 3 days without pretreatment), pretreat+rehydration (rehydration in water at 4°C after pretreat+desiccation) and rehydration (rehydration in water after desiccation).

To assess the effect of freeze treatment on gene expression, a 200 µl suspension containing approximately 1000 nematodes was put in a 1.7 ml Eppendorf tube and placed in a temperature chamber. Treatments were pretreatment (exposure to 4°C for 24 h), freezing (direct exposure to –10°C for 3 days), pretreat+freezing (exposure to gradual cooling from 4°C to –10°C at a rate of 1°C h^{-1} and left at the final temperature for 3 days), pretreat+recovery (recovery in water at 4°C after pretreat+freezing) and recovery (recovery in water at 4°C after freezing).

Survival of freezing after desiccation

To determine whether prior desiccation enhances the freezing survival of nematodes, a 100 µl suspension containing approximately 200 nematodes was put in a 1.7 ml Eppendorf tube and exposed to 98% followed by 85% RH at 4°C for 12 h each. Those tubes with nematodes were placed in the controlled temperature chamber and exposed to gradual cooling from 4°C to –10°C (desiccation+freezing) at a rate of 1°C h^{-1} and left at the final temperature for 3 (short exposure) and 7 (long exposure) days. Nematodes were allowed to recover in water at 4°C for 24 h and mortality was assessed as described above.

Primer design and gene selection

Genes were chosen from the list of transcripts differentially expressed during desiccation stress (Adhikari et al., 2009). Eight different genes with functional roles in stress response, metabolism and signal transduction were selected for the study. Primers were designed from ESTs (selected by subtractive hybridization of desiccated and fresh nematodes) using PrimerQuest™ from IDT (Coralville, IA, USA) and synthesized by Operon Biotechnologies Inc. (Huntsville, AL, USA) (Table 1).

RNA extraction and cDNA synthesis

Total RNA for quantitative real-time PCR was extracted using Trizol reagent (Molecular Research Center Inc., Cincinnati, OH, USA) from nematodes exposed to each of the different treatments. Three replicates of each stress treatment (plus three groups of controls) were used for RNA extraction, yielding three independent RNA extracts for each different treatment combination. Nematodes exposed to different treatments were directly homogenized in liquid nitrogen, mixed with Trizol reagent, and the suspension exposed to three freeze–thaw cycles using liquid nitrogen and a 37°C water bath. The suspension was ground using mortar and pestle, and vortexed; 40 ml of chloroform was added, and the tubes were shaken vigorously for 15 s and then incubated further for 5 min at room temperature. After centrifugation (15 min, 12,000 ***g***, 4°C), the aqueous phase containing RNA was separated from the other phases, which were stored for DNA preparation (see below). The colorless upper aqueous phase was transferred into fresh vials to precipitate the RNA by addition of 100 ml isopropyl alcohol. The samples were incubated for 10 min and centrifuged (20 min, 12,000 ***g***, 4°C). The RNA precipitates were then washed twice with 75% ethanol, air dried, eluted in nuclease-free water, and quantified and quality checked *via* a spectrophotometer (A_{260}/A_{280}>1.9; NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis.

Reverse transcription (RT) was performed with 1 µg of total RNA from each specimen. RT reaction of polyadenylated mRNA to cDNA was done using ImPromp-II™ reverse transcriptase (Promega Corporation, Madison, WI, USA) and an oligo(dT) primer. Total RNA was incubated with 20 pmol oligo(dT) primer at 70°C for 5 min and quickly chilled on ice. The reverse transcription mix (20 µl) was prepared by mixing 4 µl of ImPromp-II 5× reaction buffer, 2.4 µl (3 mmol l^{-1}) $MgCl_2$ and 1 µl dNTP mix (10 mmol l^{-1} each dNTP). Nuclease-free water (6.6 µl) was added, vortexed, and 1 µl of ImPromp-II reverse transcriptase was added. The reverse transcription mixture was mixed with RNA template and incubated at 25°C for 5 min for annealing and the first strand was extended for 60 min at 42°C. Reverse transcriptase was inactivated by heating to 70°C for 15 min. The cDNA was precipitated in 100% ethanol and washed twice with 75% ethanol, air-dried and dissolved in DEPC-treated water.

Quantitative real-time RT-PCR

Quantitative real-time PCR was performed with LightCycler 480 SYBER Green I mastermix (three replicate samples for each treatment–time combination) and gene-specific primers in a Light Cycler 480 RT-PCR system (Roche Applied Science, Indianapolis, IN, USA) equipped with LightCycler 480 software, with the

Table 1. List of gene-specific primers used in quantitative real-time RT-PCR analysis

Primer name	Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Product length (bp)
Pm-tps	Trehalose-6-phosphate synthase	GCACGACAAGCAACGAGTTA	CATGTTCACACCAAGGTTCG	180
Pm-afp	Antifreeze protein	GAGTTGCAAGTCCAACCCAAACCA	CATTCCAAAGGGTGCCATTGTCGT	192
Pm-hsp-70	Heat shock protein 70	AGTTGGGAGCAATCATGGCCAAAG	GCGACTTGATTCTTGGCAGCATCT	155
Pm-jnk-1	c-Jun N-terminal kinase	TATGCATGGAGGTCATGGCTCTGT	ACCGCTATACCGATCGCACAATCA	195
Pm-gst-1	Glutathione S-transferase 1	TAAGTCAGTGGGCGTGGCTAATCA	CACAATTGCGTTGTAGAGCGGCTT	205
Pm-ms	Malate synthase	CACTATCGCTCGTTCGTCAA	CCGGCATCTGTTCTAGTTCC	211
Pm-gsy	Glycogen synthase	ATGAATGGCAAGCAGGTGTTGGTC	AACGATCCGAGATGTGTAGTCGCA	188
Pm-hsp-90	Heat shock protein 90	TGCAAACATCTGGAAACCAA	CCAAACTGGCCAATAATGCT	227

Primers were designed by aligning differentially expressed ESTs from subtractive hybridization with putative homologous sequences from GenBank using CLUSTALX and PRIMER 3 programs.

following program: 3 min at 95°C; 45 repeats of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C followed by a standard melt curve. The real-time PCR reaction mixture contained the following items in a final volume of 10 ml: 3 µl PCR grade water, 5 µl PCR primers (20 pmol µl^{-1}), 5 µl double concentrated SYBR Green mastermix and 1 µl template DNA. Negative control reactions containing water in place of cDNA were included in each batch of PCR reactions to ensure that contamination was not a problem. To minimize mRNA quantification errors and genomic DNA contamination biases, and to correct for inter-sample variation, we used 18S ribosomal RNA (*Pm-18S*) of *P. murrayi* as an internal control.

Statistical analyses

In quantitative real-time PCR analysis, the absolute number of specific cDNA molecules present in the samples was determined by construction of standard curves. A range of six dilutions (10^7–10^2 copies) of the cDNA was made and a gene-specific external standard curve was generated by using cDNA standards that were run simultaneously with the experimental samples. Real-time PCR analysis was performed with LightCycler 480 software, the threshold cycle was automatically calculated by the second-derivative maximum method, and the copy number of the specific mRNA in the experimental samples was calculated by extrapolation from the gene-specific standard curve. Relative change in target gene expression was calculated using the $2^{-\Delta\Delta CT}$ equation (Livak and Schmittgen, 2001), where $\Delta\Delta Ct = (Ct_{Target}–Ct_{18S\ rRNA})_{desiccated}–(Ct_{Target}–Ct_{18S\ rRNA})_{control}$. The fold change in the target gene, normalized to 18S rRNA and relative to the expression of the control, was calculated for each sample. A gene with a relative abundance of one is equal to the abundance of 18S rRNA in the same sample. Analyses were conducted using PROC MIXED on SAS STAT (v.9.1, SAS Institute Inc., Cary, NC, USA). Expression levels significantly ($P>0.05$) higher and lower than one were considered to be induced and reduced, respectively.

In nematode survival experiments, statistical analyses were performed using SAS 9.1, fitting the GLM model after logit transformation of the survival data ($\log[p/1–p]$, where p is the percentage of surviving nematodes). The overall slopes of the curves were compared by an F-test and in pairs by t-tests (significant at $P<0.05$). In addition, the percentage survival at the end of each experiment was compared by one-way analysis of variance (ANOVA) after arcsine transformation. Whenever data did not fit the logit model, we used a two-way ANOVA after arcsine transformation. For the effect of acclimation on desiccation and freezing survival analysis, significant differences within each treatment series were determined by one-way ANOVA ($P<0.05$) with Tukey's honestly significant difference tests.

RESULTS

Changes in gene expression

We observed significant differences in the transcription of genes during various stages of desiccation. When nematodes were exposed to pretreatment, the expression of *Pm-gsy*, *Pm-ms* and *Pm-tps* was significantly up-regulated. Nematodes exposed to pretreat+desiccation showed significant up-regulation of *Pm-ms* and *Pm-tps* along with *Pm-gst-1* and *Pm-jnk-1*. Similarly, when nematodes were allowed to rehydrate in water after pretreat+desiccation, there was significant up-regulation of *Pm-gst-1*, *Pm-gsy*, *Pm-ms* and *Pm-tps*. The up-regulation of *Pm-gsy* and *Pm-ms* during pretreat+rehydration was twice as much as during the pretreatment phase (Fig. 1). When nematodes were directly exposed to desiccation, the expression level of *Pm-gst-1*, *Pm-jnk-1*, *Pm-gsy*, *Pm-ms* and *Pm-tps* was significantly up-regulated during both desiccation and rehydration. Induction of *Pm-jnk-1* and *Pm-tps* during pretreat+desiccation was significantly higher than that for all other treatment stages. Similarly, expression of *Pm-gsy* and *Pm-ms* was significantly higher during pretreat+rehydration than that for all other treatment stages. There was no significant difference in the induction of genes (except for *Pm-gsy*) between desiccation and rehydration stages. But in the case of *Pm-gsy*, rehydration-induced expression was twice as much as during desiccation. There was slight up-regulation of heat shock proteins (*Pm-hsp-70* and *Pm-hsp-90*) and down-regulation of *Pm-afp* during different treatments but the change in expression level was not significant (Fig. 1).

We observed significant differences in the transcription of genes during various stages of freezing. When nematodes were exposed to pretreatment, *Pm-gsy*, *Pm-ms* and *Pm-tps* genes were significantly up-regulated while pretreat+freezing caused up-regulation of all genes except those for heat shock proteins. Similarly, nematodes exposed to pretreat+recovery significantly induced the expression of five genes (except those for *Pm-jnk-1* and heat shock proteins; Fig. 2). Freezing significantly induced the expression of *Pm-gst-1*, *Pm-ms*, *Pm-tps* and *Pm-afp* while recovery significantly induced the expression of *Pm-gst-1*, *Pm-gsy*, *Pm-jnk-1*, *Pm-ms*, *Pm-tps* and *Pm-afp*. The induction of *Pm-ms*, *Pm-tps* and *Pm-afp* was significantly higher during pretreat+freezing than during freezing. The up-regulation of *Pm-gsy* during pretreat+recovery and recovery was significantly higher than at all other treatment stages. The induction of *Pm-gst-1* during recovery was significantly higher than during pretreat+recovery and pretreat+freezing. Heat shock protein genes *Pm-hsp-70* and *Pm-hsp-90* did not show a significant change in expression regardless of treatment stage (Fig. 2).

mRNA copy number

The mRNA copy number of *Pm-hsp-90* and *Pm-hsp-70* genes was compared for nematodes at different stages of desiccation and control nematodes. The mRNA copy number ($\times 10^7$) was not significantly different between genes or among different treatments (pretreatment, pretreat+desiccation and pretreat+rehydration) ($P>0.05$, $N=3$) (Table 2).

Survival under different RH

There were significant differences in nematode survival among the different treatments. Nematodes maintained at 98% RH experienced no significant changes in survival and 89±8.09% (mean ± s.d.) of the nematodes were still alive at the end of the 10 day experimental period. Nematodes exposed to slow desiccation to 0% RH showed a gradual decline in mortality throughout the 10 day experimental period (Fig. 3). Exposure to 35% and 0% RH caused a faster decline in survival of the nematodes compared with other treatments. After the 10 day experimental period only 42±9.01% and 41±3.65% of nematodes survived 35% and 0% RH, respectively, while nematodes exposed to slow desiccation (0% after exposure to 75% RH) showed significantly higher survival (54±5.54%) than those exposed to 35% and 0% RH (Fig. 3).

Effect of slow desiccation and freezing on survival

To assess the effect of acclimation on desiccation and freeze tolerance, nematode mortality was analyzed after exposure to different treatments. Treatments consisted of exposure for 3 and 7 days to 75% RH with (pretreat+desiccation) or without (desiccation) pretreatment at 98% (12 h) followed by 85% RH (12 h) and direct exposure for 3 and 7 days to –10°C (freezing) or gradual

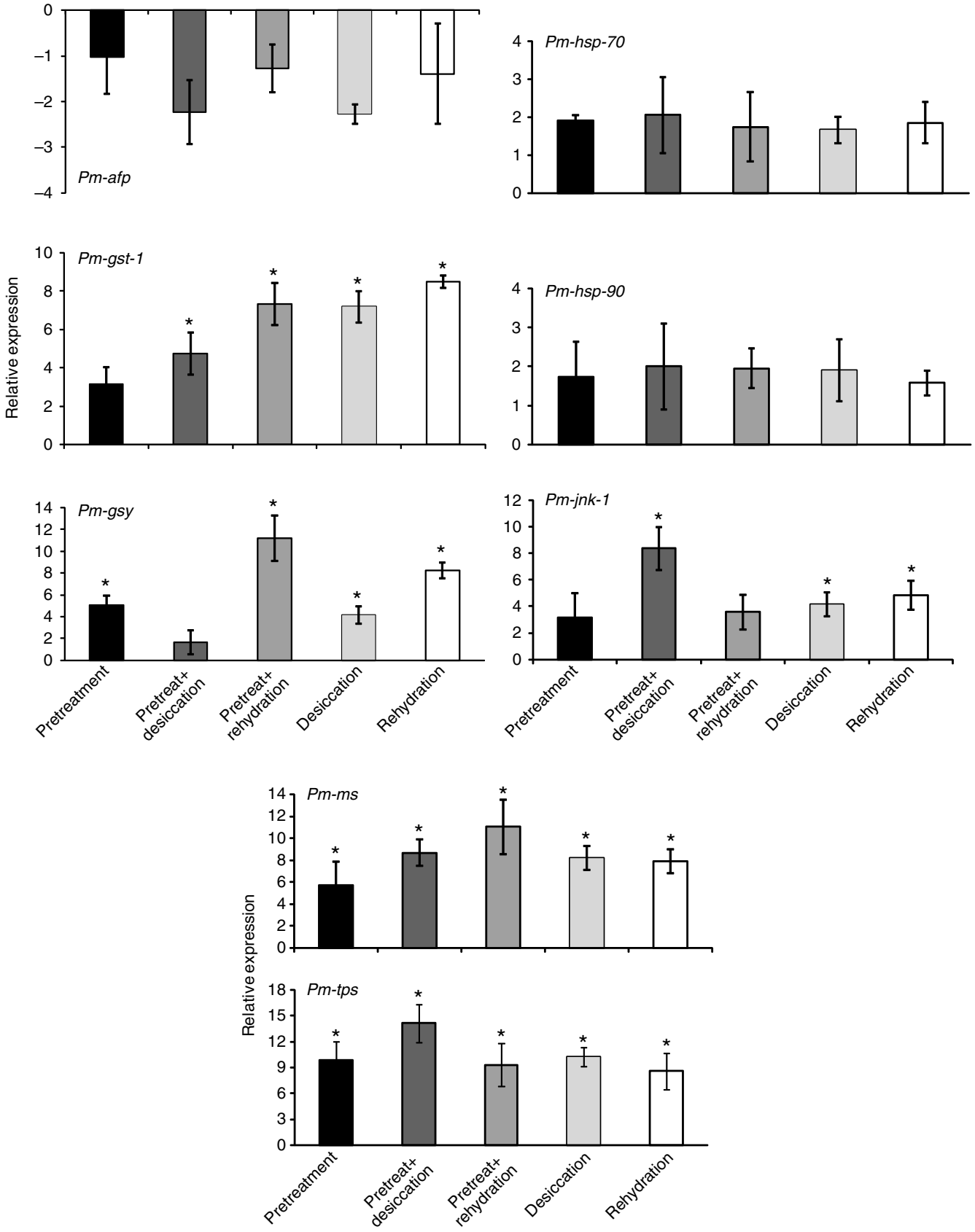

Fig. 1. Relative expression of *Plectus murrayi* transcripts during different stages of desiccation. Relative expression level of different genes during pretreatment (Pretreatment), exposure to desiccation with (Pretreat+desiccation) and without pretreatment (Desiccation) and rehydration with (Pretreat+ rehydration) and without pretreatment (Rehydration). Note, on the *y*-axis, values >1 indicate up-regulation and values <1 indicate down-regulation of the transcript. Asterisks indicate values that are significantly different from 1 (P<0.05). Means ± s.e.m. are presented, N=3 in all cases.

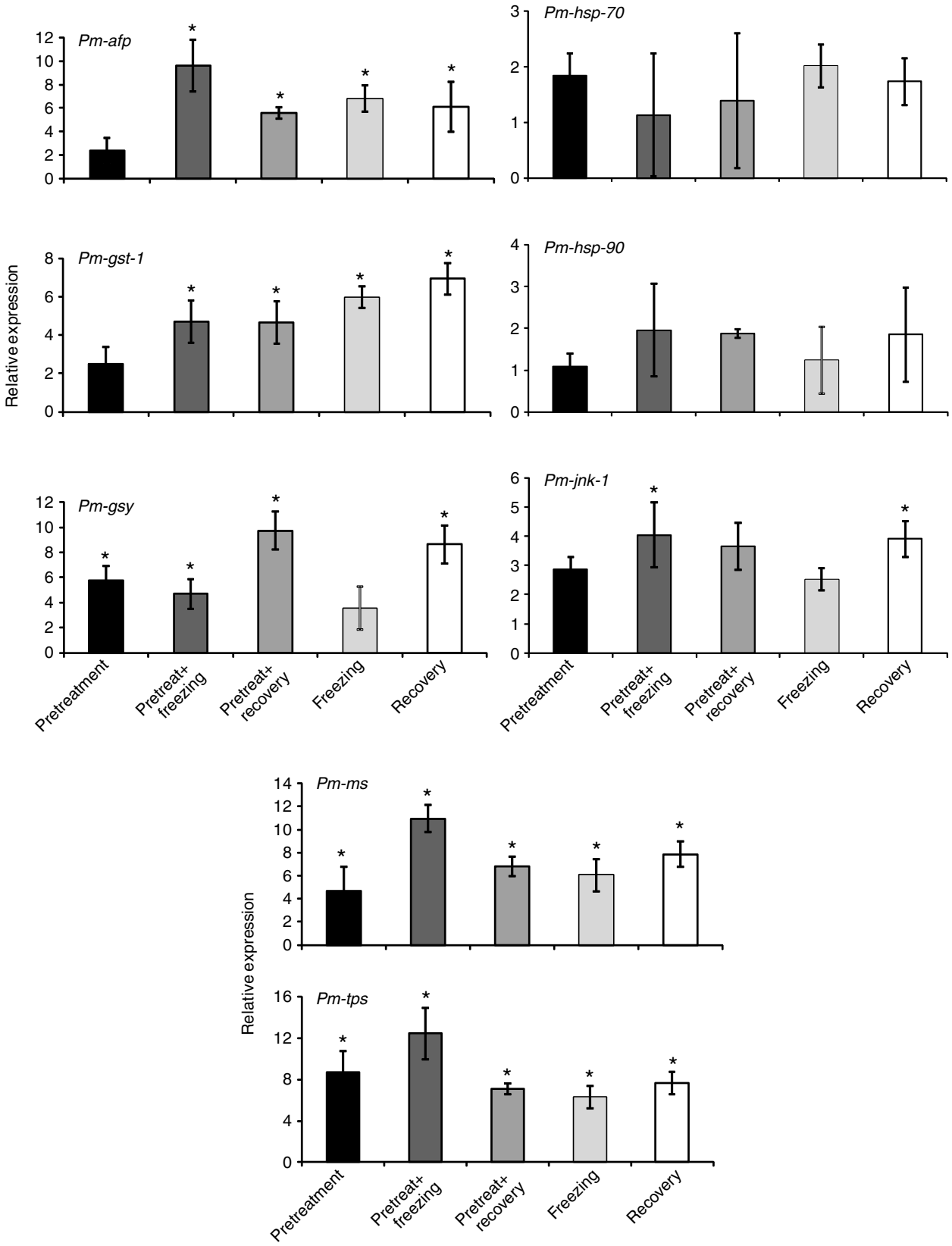

Fig. 2. Relative abundance of *P. murrayi* transcripts during different stages of freezing. Relative expression level of different genes during pretreatment (Pretreatment), exposure to freezing with (Pretreat+freezing) and without pretreatment (Freezing) and recovery with (Pretreat+recovery) and without pretreatment (Recovery). Note, on the *y*-axis, values >1 indicate up-regulation and values <1 indicate down-regulation of the transcript. Asterisks indicate values that are significantly different from 1 (P<0.05). Means ± s.e.m. are presented, N=3 in all cases.

Table 2. *Pm-hsp-90* and *Pm-hsp-70* expression at different stages of desiccation

Treatment/stage	*Pm-hsp-90*	*Pm-hsp-70*
Pretreatment	7.03±1.28	6.97±1.89
Pretreat+desiccation	7.73±1.18	6.55±0.81
Pretreat+rehydration	8.27±1.00	6.72±1.54
Control	7.45±1.80	6.15±1.92

The mRNA copy number ($\times 10^7$) was calculated based on 18S rRNA gene copies as described in the text. Treatments were desiccation (pretreatment, pretreat+desiccation and pretreat+rehydration) and control. Results are presented as means ± s.d.

cooling from 4°C to –10°C (pretreat+freezing) at a rate of 1°C h^{-1}. Pretreatment at higher RH significantly increased survival of desiccation for both 3 and 7 days of exposure (Fig. 4). Similarly, gradual freezing to –10°C significantly increased survival for both 3 and 7 days (Fig. 4). Nematodes exposed to fast desiccation treatment survived better than those exposed to fast freezing for 3 days. In contrast, there was no significant difference in nematode survival within and between treatments of slow desiccation with gradual freezing for both 3 and 7 day intervals. Nematode survival was higher for 3 days than for 7 days for desiccation and freezing treatments (Fig. 4).

Effect of desiccation on freeze survival

To determine the effect of prior desiccation on freeze tolerance, 200 nematodes were placed in 1.7 ml Eppendorf tubes and exposed to 98% followed by 85% RH at 4±0.2°C for 12 h each. The tubes (with nematodes) were transferred to the controlled cooling chamber and exposed to gradual cooling from 4°C to –10°C (desiccation+freezing) at a rate of 1°C h^{-1} for 3 and 7 days. Nematodes exposed to desiccation and freezing showed significantly higher survival than those exposed to freezing or pretreat+freezing for both 3 and 7 days. There was no difference in survival of nematodes at 3 and 7 days of exposure to desiccation+freezing (Fig. 4).

DISCUSSION

The Antarctic Dry Valley nematodes are known for their ability to survive desiccation and freezing conditions in an anhydrobiotic stage (Treonis and Wall, 2005). Mechanisms that control the entry into, maintenance and recovery from anhydrobiosis are poorly

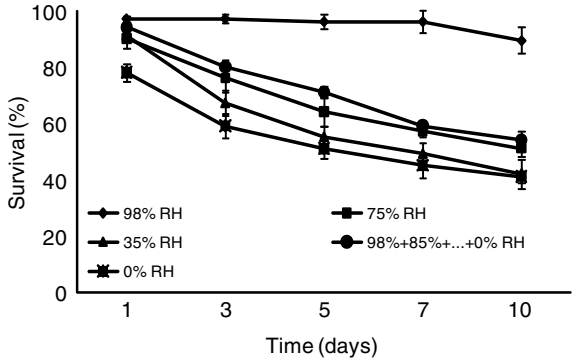

Fig. 3. Survival of nematodes maintained under 98%, 75%, 35% and 0% relative humidity (RH) with and without pre-exposure to 98% followed by 85% RH conditions for up to 10 days. Nematodes were exposed to different RH and rehydrated in water at 5°C for 24 h. Values shown are means ± s.e.m. for three replicates of 200 nematodes.

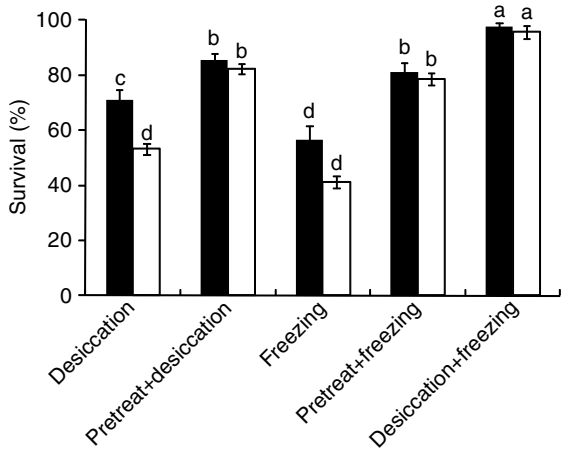

Fig. 4. Nematode survival under different combinations of stresses. Nematodes were exposed to 75% RH without acclimation to higher RH (Desiccation), 75% RH following acclimation to 98% then 85% RH for 12 h (Pretreat+desiccation), exposure to –10°C (Freezing), gradual cooling from 4°C to –10°C (Pretreat+freezing), and gradual cooling from 4°C to –10°C with pretreatment to 98% RH followed by 85% RH for 24 h (Desiccation+freezing) for 3 (filled bars) and 7 (open bars) days. Bars with different letters denote significant (P<0.05) differences in survival among treatments. Values are means ± s.e.m. based on three replicates of 200 nematodes.

understood. In our study, we sought to characterize the desiccation and freeze survival of *P. murrayi* under ecologically relevant conditions characteristic of both austral summers and winters of the Antarctic Dry Valleys, and also to identify molecular and physiological mechanisms that may take place during different stages of stress survival. Our results show that *P. murrayi* exhibits extreme desiccation tolerance, activating the expression of a suite of genes involved in various stages of anhydrobiosis and freeze tolerance. Exposure to a slow rate of desiccation significantly increased the survival of *P. murrayi* compared with those desiccated directly at lower RH. Exposure to gradually decreasing RH enabled more than 50% of the nematodes to survive extreme desiccation (0% RH) while significantly increasing their freeze survival. Pre-exposure of nematodes to sub-lethal stress (pretreatment) promoted the desiccation as well as freeze survival of nematodes, suggestive of cross-stress tolerance mechanisms. Transcription of a number of genes was induced by pretreatment and their expression varied significantly at different stages of stress while heat shock proteins were constitutively expressed and showed no further up-regulation upon freezing and desiccation. We discuss these findings below in the context of our current knowledge regarding the gene expression, physiology and ecology of this nematode.

Changes in gene expression during desiccation and freezing

Plectus murrayi showed differential expression of a suite of genes and continuous up-regulation of others upon exposure to desiccation and freezing, confirming the results of previous investigations (Adhikari et al., 2009). The free-living fungivorous nematode *A. avenae* has been shown to accumulate large amounts of the disaccharide trehalose, late embryogenesis abundant (LEA) protein and a novel protein named anhydrin when exposed to a moderate reduction in RH (Goyal et al., 2005). The Antarctic nematode *P. davidi* which, like *P. murrayi*, can survive both desiccation and freezing, expresses an ice-active protein that shows recrystallization

inhibition during freezing (Wharton et al., 2005). During exposure to dehydration, larvae of the Antarctic midge *Belgica antarctica* constitutively express heat shock proteins (small *hsp*, *hsp90* and *hsp70*), and the larval midges maintain a high inherent tolerance to temperature stress (Hayward et al., 2007; Rinehart et al., 2006). Based on our results, it seems reasonable to assume that *P. murrayi* modulates the transcription of genes involved in metabolism, stress survival and signal transduction under strong selection to maintain desiccation tolerance.

We followed the expression of eight different genes from various functional groups at different time points during pretreatment, exposure to and recovery from desiccation and freezing. We found a significant change in the expression of certain genes, while others were constitutively expressed. Trehalose-6-phosphate synthase (*Pm-tps*) was one of the most up-regulated genes during both desiccation and freezing, with a significant influence of pretreatment on its relative expression: nematodes exposed to pretreatment had higher levels of mRNA transcripts than those without pretreatment (Figs 1 and 2). A characteristic feature of anhydrobiotic organisms is their synthesis of high concentrations of non-reducing sugars during the induction of anhydrobiosis (Goyal et al., 2005). This process protects membranes and proteins from desiccation damage by replacing structural water (Crowe et al., 1992), and formation of an intracellular organic glass (Crowe et al., 1998) to stabilize the cell's contents. Elevated levels of trehalose have also been reported from an Antarctic coastal bacterial feeding nematode *P. davidi* (Wharton et al., 2000) and entomopathogenic nematodes (Grewal and Jagdale, 2002) which improved freezing and desiccation survival. Since trehalose is reported to be the major compatible solute during multiple stresses (Santos and da Costa, 2002; Yancey, 2005), increased transcription of genes encoding trehalose could be a mechanism to counter multiple stresses (like exposure to high salinity and solar radiation) in addition to desiccation and freezing.

We observed an inverse relationship between the transcription of trehalose and glycogen synthase (*Pm-gsy*), the rate-limiting enzyme in the synthesis of glycogen and the primary storage form of glucose in higher eukaryotes including nematodes (reviewed by Behm, 1997). Such a reduction in glycogen synthase transcription levels suggests a shift from glycogen to trehalose synthesis during exposure and, perhaps, a shift from trehalose to glycogen synthesis during rehydration/recovery. Adjustments of biochemical pathways and changes in kinetics such as those described above may play a major role in the induction and maintenance of stress tolerance, allowing nematodes, and perhaps other metazoans, to persist in one of the Earth's harshest environments.

Exposure to desiccation and freezing led to marked changes in the expression levels of a gene involved in signal transduction (Figs 1 and 2). The JNK family, a subgroup of the mitogen-activated protein kinase superfamily, is part of a signal transduction cascade that regulates cellular responses to a variety of extracellular signals, including desiccation and other types of osmotic stress (Davis, 2000). *Pm-jnk-1*, along with a gene encoding a neurotransmitter-gated ion channel protein, were differentially expressed in anhydrobiotic *P. murrayi* (Adhikari et al., 2009), indicating their possible involvement in desiccation survival. Nematodes are unique among animals in utilizing the glyoxylate cycle to generate carbohydrates from the beta-oxidation of fatty acids (reviewed by Barrett and Wright, 1998). Nematodes appear to use this pathway for energy production from stored lipids during starvation or non-feeding stages (Reversat, 1981; Wadsworth and Riddle, 1989). Relevant to our findings, the anhydrobiotic nematode *A. avenae* has been reported to use the glyoxylate cycle during induction of anhydrobiosis (Madin et al., 1985). Our results support the assumption that *P. murrayi* also uses the glyoxylate cycle, not only during induction of anhydrobiosis but also for maintenance and recovery from stress.

Our results show that heat shock proteins (*Pm-hsp-70* and *Pm-hsp-90*) are continuously expressed during desiccation and freeze survival (Table 2) of *P. murrayi* and neither desiccation nor cold treatment can further up-regulate these genes. *Plectus murrayi* is exposed to one of the most extreme and unpredictable terrestrial environments on earth (Priscu, 1998) and to combat such extreme conditions it might have evolved a mechanism to maintain heat shock protein function without disrupting normal metabolism and the growth that requires synthesis of other proteins. This phenomenon has been reported in many Antarctic organisms, including fish (Buckley et al., 2004), ciliates (La Terza et al., 2001), yeast *Candida psychrophila* (Deeganaars and Watson, 1997) and the Antarctic midge *B. antarctica* (Rinehart et al., 2006). Although this has been studied and observed systematically in only a few species, it may be that this is a common adaptation of Antarctic organisms.

Many cold-tolerant organisms produce proteins (e.g. antifreeze protein) that assist their survival by interacting with ice in some way (Wharton et al., 2005). Antifreeze proteins (AFPs) have been identified in many metazoans, including Antarctic marine fish (DeVries, 1971; Duman and Olsen, 1993; Graham et al., 1997; Griffith and Yaish, 2004). An ice-active protein reported in the Antarctic nematode *P. davidi* is thought to play an important role in freeze tolerance, particularly intracellular freezing (Wharton et al., 2005). In *P. murrayi*, *Pm-afp* was significantly up-regulated during exposure and recovery from freezing but was slightly down-regulated during desiccation stress (Figs 1 and 2). As AFPs can prevent the potentially injurious process of recrystallization, even at very low concentration (Knight et al., 1984), the induction of *Pm-afp* during freezing of *P. murrayi* could be a possible mechanism for preventing recrystallization of ice and ultimately protecting the nematode from further mechanical damage to its cells.

Desiccation survival

A number of nematodes can tolerate high losses of water, a trait that is especially pronounced in the Antarctic nematode *P. murrayi*. Based on the data presented here, *P. murrayi* exhibits extreme desiccation tolerance among nematodes and shows characteristic features of anhydrobiotic nematodes (e.g. cuticle, body shape) (Crowe and Madin, 1974; Treonis et al., 2000). However, given the prevalence of soil nematodes with high cuticular permeability in the Dry Valleys, similar physiological attributes may be widespread among nematodes when assessed under appropriate humidity conditions. Nematodes not only survived exposure to 0% RH, a characteristic of anhydrobiotes, but also showed enhanced survival when exposed to gradual desiccation (Fig. 3). Such an increase in desiccation tolerance at lower water loss is well known in the fungivorous nematode *A. avenae*, which requires a period of acclimation for successful anhydrobiosis (Goyal et al., 2005). Similarly, the Antarctic midge, *B. antarctica*, tolerates a significantly greater loss of body water when dehydrated at higher RH (Benoit et al., 2007). If dehydration occurs at a slow rate, *P. murrayi* induces the transcription of genes coding for sugars and polyols (e.g. trehalose) as demonstrated in this study and as inferred previously (Adhikari et al., 2009). We hypothesize that increases in dehydration tolerance could be due to the replacement

of water with trehalose, thus preventing dehydration-induced cellular damage (Goyal et al., 2005).

Effects of slow desiccation and freezing on survival

The ability to survive prolonged periods of desiccation and freezing is an ecologically important trait for many soil invertebrates, especially those inhabiting the extreme terrestrial environment of Antarctic Dry Valleys. In our study we demonstrated that *P. murrayi* survives much better when exposed to slow desiccation and freezing (Fig. 4) compared with rapid dehydration and fast freezing. Slow dehydration at 98% followed by 85% RH before exposure to 75% RH conferred significantly higher survival of nematodes than direct exposure to 75% RH. Similarly, survival was significantly higher when nemotodes were exposed to gradual freezing from 4°C to –10°C compared with direct freezing at –10°C (Fig. 4). Previous reports have shown that pre-acclimation to a relatively mild desiccation stress can improve severe desiccation tolerance in other soil invertebrates such as nematodes, Collembola and midge larvae (Hayward et al., 2007; Sjursen et al., 2001; Chown et al., 2007; Womersley and Ching, 1989).

The higher survival was probably linked to the higher water content in the gradually exposed nematodes which in turn may be linked to the accumulation of osmolytes and induced transcriptional changes during pretreatment. An increased transcription of genes encoding sugars and/or polyols is likely to enhance cellular and membrane integrity during both desiccation and freezing by replacing the primary water of hydration and through the formation of amorphous glasses (vitrification), thus stabilizing the structure of macromolecules and membranes (Crowe et al., 1992). However, mechanisms other than the production of trehalose, notably continuous synthesis of chaperone proteins such as heat shock proteins, could be a mechanism in *P. murrayi*.

Desiccation increases freeze survival

A connection between enhanced cold and heat tolerance following acclimation to sub-lethal temperatures has long been established in nematodes (Chen et al., 2005; Jagdale and Grewal, 2003), but the effect of desiccation on freeze tolerance has not been well studied. In *P. murrayi*, 12 h at 98% RH followed by 12 h at 85% RH resulted not only in enhanced desiccation survival but also in an increase in survival at –10°C, relative to the non-desiccated controls (Fig. 4). This result concurs with those of Bayley and colleagues (Bayley et al., 2001), who found that 7 days at 98.2% RH significantly increased the cold tolerance of *Folsomia candida*.

Our study supports the idea that exposure to desiccation stress promotes enhanced cold tolerance, and provides evidence that gradual desiccation can enhance the lower limit of freeze tolerance in this Antarctic nematode. Although limited data exist regarding molecular mechanisms of the cross-stress tolerance of Antarctic nematodes, the observed enhanced tolerance could partly be due to an increased transcription of genes encoding sugars and/or polyols, which is likely to enhance cellular and membrane integrity during both desiccation and freezing (Crowe et al., 1984; Crowe et al., 1992; Takagi et al., 2000), or by induction of *Pm-afp* and *Pm-gsy* during exposure to freezing. The moisture content of the Antarctic Dry Valley soils averages around 1.7% (Treonis and Wall, 2005) and temperatures can vary from –20 to 10°C (Doran et al., 2002). Such cross-stress tolerance is very important with regard to nematodes, which are frequently exposed to multiple stress factors that are encountered multiple times within a single lifespan, like extremely low moisture, freezing temperatures and salt accumulation (Wall Freckman and Virginia, 1998).

CONCLUSIONS

The capacity to survive prolonged periods of low moisture availability and freezing temperatures is of considerable adaptive significance to some Antarctic terrestrial nematodes including *P. murrayi*. Our study supports the idea that pretreatment plays an important role in the adaptation of nematodes under the harsh conditions of Dry Valleys through enhanced transcription of stress-related genes. The survival of nematodes encompasses differential expression of a suite of genes from different functional groups, and constitutive expression of others. The results presented here also support the idea that adaptations to desiccation stress can promote enhanced freeze survival, and provide the first evidence that slow dehydration can enhance the lower limit of freeze tolerance in an Antarctic nematode. Exposure to the slow rate of dehydration and freezing in our study is as relevant to the Antarctic condition as it is to rehydration in water where nematodes face sudden soil wetting due to rare precipitation pulses, flood and snowmelt. Furthermore, as demonstrated here, such conditions can influence the survival of significant water loss, and permit the identification of more subtle desiccation and cold tolerance strategies employed by Antarctic biota.

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