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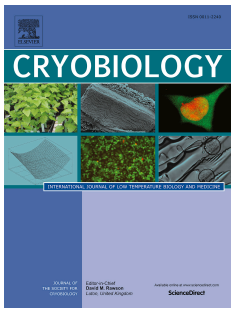

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Molecular snapshot of an intracellular freezing event in an Antarctic nematode

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The Antarctic nematode, *Panagrolaimus* sp. DAW1 (formerly called *Panagrolaimus davidi)*, is the best documented example of an organism able to survive intracellular ice formation in all of its compartments. Not only is it able to survive such extreme physiological disruption, but it is able to produce progeny once thawed from such a state. In addition, under slower rates, or less extreme degrees, of cooling, its body remains unfrozen and the vapour pressure difference between the supercooled body fluids and the surrounding ice leads to a process termed cryoprotective dehydration. In contrast to a fairly large body of work in building up our molecular understanding of cryoprotective dehydration, no comparable work has been undertaken on intracellular freezing. This paper describes an experiment subjecting cultures of *Panagrolaimus* sp. DAW1 to a range of temperatures including a rapid descent to −10°C, in a medium just prior to, and after, freezing. Through deep sequencing of RNA libraries we have gained a snapshot of which genes are highly abundant when *P.* sp. DAW1 is undergoing an intracellular freezing event. The onset of freezing correlated with a high production of genes involved in cuticle formation and subsequently, after 24 hours in a frozen state, protease production. In addition to the mapping of RNA sequencing, we have focused on a select set of genes arising both from the expression profiles, as well as implicated from other cold tolerance studies, to undertake qPCR. Among the most abundantly represented transcripts in the RNA mapping is the zinc-metalloenzyme, neprilysin, which also shows a particularly strong upregulated signal through qPCR once the nematodes have frozen.

keywords: *Panagrolaimus davidi*, Antarctic, nematode, intracellular freezing, neprilysin, leucine-rich repeat.

Introduction

Apart from the work of Salt in describing intracellular freezing in the large fat body cells and labial glands of the goldenrod gall fly, *Eurosta solidaginis,* in the late 50's [44,45,46,50], the study of the phenomenon of intracellular freezing *in vivo* has remained little understood, and little explored. However, some recent physiological studies on the Antarctic nematode, *P.* sp. DAW1 [67,68,38,69,70,72,73,39,40], provides the necessary background to now bring molecular techniques to bear on an extraordinary adaptation.

A free-living bacteriovorous nematode, *Panagrolaimus* sp. DAW1 [61] (previously called *Panagrolaimus davidi*, see Raymond & Wharton [40]), was first isolated from the McMurdo Sound region of the Antarctic in 1989 and cultured at the University of Otago [66]. The culture was found to survive intracellular freezing [68], with the ability to produce progeny afterwards. To date, *P.* sp. DAW1 remains the best documented case of such survival, even among other Antarctic nematodes [51,74,30]. *P.* sp. DAW1 also has the ability to undergo cryoprotective dehydration when subjected to high sub-zero temperatures or slower rates of cooling [71,73]. However, while an increasingly detailed molecular picture is emerging of cryoprotective dehydration [8,9,59,57,18,58], no comparable molecular work has been undertaken on the survival of intracellular freezing at the whole organism level. The study of *P.* sp. DAW1 provides an increasingly detailed physiological understanding of intracellular ice formation on which to build up a molecular picture of the process.

While there are many organisms able to withstand ice formation in extracellular spaces [1,53,64,17,20,16,65,26,10,54], the ability to withstand such disruption within the cells, and survive with the ability to produce progeny afterwards, is much rarer. In *P.* sp. DAW1, although the cuticle is a sufficient barrier to ice, at low sub-zero temperatures inoculative freezing can spread through the excretory pore and other orifices, with the ice seeding the body fluid and freezing all, including intracellular, compartments [68,73]. One of the ideas on the more lethal consequences of this freezing is the physical damage to the cell membranes once the smaller ice crystals that have initially formed, themselves begin to cluster together, or recrystallise [23,38,73]. Traditionally the term antifreeze protein has

been used to designate any protein involved in the inhibition of freezing. Recently, some authors have argued for a more general term for proteins that play a role in ice formation and in combating ice recrystallisation [72,12]. These ice-active proteins (IAP) can then be classed according to one of three types: Ice nucleating proteins (INP), which help to initiate ice formation; antifreeze proteins (AFP), which prevent ice nucleation by creating a thermal hysteresis (TH) between the melting and freezing point of a sample in the presence of an ice crystal; and recrystallisation-inhibition proteins (RIP), which inhibits recrystallisation, the aforementioned process in which larger ice crystals form at the expense of smaller more numerous crystals. Although most RIPs also have at least low levels of thermal hysteresis activity, in some cases extracts of organisms with RIP activity have to be concentrated to a high degree before TH activity can be detected. RIPs tend to be found in freeze tolerant species while AFPs (with more substantive TH) are mostly present in freeze avoiding species. However, in *Panagrolaimus* sp. DAW1, given the extreme freezing disruption that it can experience, and the fact that it is freeze tolerant, it is expected that its IAPs will not include AFPs [38], but rather RIPs. A further clue on the possible importance of RIPs in the survival of intracellular freezing, comes from a recent study by Raymond and Wharton [40], where the smaller the sizes of the ice crystals is correlated positively with the survival rate. But to date, no IAP of any kind has been isolated in *P.* sp. DAW1 [60], even though they have been shown to play a role in freezing tolerance [72].

Among other important classes of genes are those involved in the sugar biosynthetic pathways (such as those producing trehalose), Late Embryogenesis Abundant (LEA) proteins, chaperone proteins (such as the heat shock proteins), and enzymes involved in antioxidant metabolism. These all play a role in the process of cryoprotective dehydration, and have been detected in *P.* sp. DAW1 [60]. While it is not clear what role, if any, these play in the nematodes' survival of intracellular freezing, it would be highly surprising if they were not involved.

The extent of survival of *P.* sp. DAW1 when subjected to intracellular freezing is dependent, however, on at least two properties. Wharton and To [69] have shown decreased survival ability when under either hyper- or hypo-osmotic stress. While Raymond and Wharton [39,40] have shown that nutritional status is an important factor, possibly related to the fact that glycogen levels decrease when under

nourished. These two studies imply that unless in a relatively stress-free state, *Panagrolaimus* sp. DAW1 is unable to endure the extreme physiological disruption of intracellular freezing, suggesting that the physiological conditions that allow for a sudden rate of cooling and intracellular freezing are at the very limit of its physiological endurance.

With all the physiological work that has been undertaken a number of key insights have been gained, yet there remains little understanding at the molecular level. To address this, we have generated sets of transcripts expressed in *P.* sp. DAW1 when the nematodes have been cooled rapidly to a relatively low sub-zero temperature (-10° C), both before and after (immediately and post 24 hours) the medium has frozen. These treatments were compared with controls that include stages in which *P.* sp. DAW1 is expected to undergo both cold acclimation and cryoprotective dehydration. This approach provides a snapshot of highly abundant transcripts from genes novel to the intracellular freezing process and distinct from those generally involved in responses to stress. We have further validated the results through qPCR analysis on genes selected both from within the transcript analysis, as well as those implicated in other cold tolerance studies.

Materials and methods

Culturing and experimental design

The culturing and experimental treatments of the nematodes consisted of control (culturing temperature and cold acclimation), cryoprotective dehydration and intracellular freezing stages. The control and cryoprotective stages consisted of the following:

Treatment 1, culture temperature*: P.* sp. DAW1 were cultured in S medium at 20° C for 3 weeks and fed every 3-4 days with *Escherichia coli* [39].

Treatment 2, cold acclimation*:* After treatment 1, the nematodes were exposed to cold acclimation by keeping them at +5° C for 3 days*.* This duration at +5° C was used because longer periods of acclimation can be detrimental to the ability to survive intracellular freezing, due to the effects of starvation. This acclimation

regime is known to produce about 80% survival in nematodes frozen at −10° C, at which temperature all individuals freeze intracellularly [39, 40].

Nematodes from treatments 1 and 2 were extracted for the subsequent experiments using a modified Baermann technique [19] and transferred to 1.5 ml microcentrifuge tubes in a balanced salt solution (BSS) [36] before snap freezing in liquid nitrogen and storage at −70° C.

Treatment 3, cryoprotective dehydration*:* After cold acclimation, nematodes were extracted and transferred to 1 ml of BSS in 1.5 ml microcentrifuge tubes and placed in an aluminium holder immersed in the bath of a refrigerated circulator. The sample was cooled from +1° C to −1° C at 0.5° C min^{-1}, and the sample frozen by adding a small ice crystal and maintained at −1° C for 24 h. Under these conditions, the medium freezes but not the nematodes.

Treatment 4, cryoprotective dehydration thawing*:* After treatment 3 the nematodes were warmed to +1° C at 0.5° C min^{-1} and allowed to recover for 24 h at 20° C.

The intracellular freezing stages (the stages in which ice is formed inside the cells of P. sp DAW1) consisted of the following:

Treatment 5U, −10° C in unfrozen media: The samples were cooled to −10° C at 0.5° C min^{-1}. When this temperature was reached, it was noted whether the medium had frozen or not, and those in unfrozen medium removed for extraction.

Treatment 5F, −10° C in frozen media: The samples were cooled to −10° C at 0.5° C min^{-1} as with Treatment 5U. When this temperature was reached samples that had frozen spontaneously during cooling were removed and labelled.

Treatment 6, −10° C for 24h: Samples subjected to treatment 5F were kept frozen at −10° C for 24 h.

Treatment 7, thawing and recovery from intracellular freezing: Samples subjected

to treatment 6 were warmed to +1° C at 0.5° C min^{-1} and then kept at 5° C for 24 h. Subsamples (four 2µl samples diluted 4× with BSS and incubated at 5° C) were taken to determine survival and the proportion moving, after a physical stimulus (expelling from a pipette), counted at intervals up to 24 hours, after which no additional increase in survival rate would be expected. The time taken for 50% of the nematodes to recover, and 95% confidence limits (CL), was determined using probit analysis in SPSS after log_{10} transformation of time [31].

After treatment, nematodes were snap frozen in liquid nitrogen before storage at -70° C, ready for RNA extraction.

Illumina library construction and sequencing

Thorne *et al.* [60] describe the process of extraction, library construction, and sequencing on an Illumina HiSeq 2000. After quality control, this resulted in 70,897,520 paired-end reads of 100bp from Treatment 1 (deposited in the NCBI SRA repository (www.ncbi.nlm.nih.gov/sra) under SRR5091936), 73,894,280 from treatment 2 (SRR5092011), 73,249,548 from treatment 3 (SRR5092012), 69,830,160 from treatment 4 (SRR5092013), 72,354,956 from treatment 5U (SRR5092108), 69,940,196 from treatment 5F (SRR5092338), 69,304,572 from treatement 6 (SRR5094587) and 73,423,192 from treatment 7 (SRR5094588).

Separate library assembly and functional analysis

Illumina reads from all the different treatments were assembled separately and annotated to identify any broad patterns of difference in the transcripts. The program Soapdenovo [28] was used to assemble the transcripts using default (genome style) parameters. Contigs greater than 300bp in length were selected from each treatment and annotated against the non-redundant database housed at Genbank (www.ncbi.nlm.nih.gov/genbank). Each transcript set was then functionally annotated using the SEED subsystem [34].

Mapping the RNA libraries and determining the highly abundant transcripts

Using a transcriptome backbone (previously described in [60]), the paired-end

Illumina sequencing reads from the treatments were mapped back using Maq [27] and normalised by a transcripts per million approach. All the treatments taken together have allowed for the comparison of intracellular freezing against not only control cultures of actively reproducing (20° C) and cold acclimated (5° C) nematodes, but also those in which *P* sp. DAW1 would be expected to undergo cryoprotective dehydration (-1° C). We compared each of the intracellular stages (-10° C unfrozen; -10° C frozen; -10° C frozen 24 h; and after freezing to -10° C, thawing and recovery), against all the preceding stages so as to enrich for those transcripts that are more likely to be expressed during intracellular freezing. Each intracellular treatment comparison required at least a 2-fold change in expression vis-a-vis all the previous stages, as well as a ratio test [37] with a multiple correction cutoff of 0.01 [4]. The sets of transcripts from the comparisons were additionally annotated with the *C. elegans* database (wormbase.org), and the results used in STRING [56], which provided not only the gene network analysis, but also the enrichment for various functional representations such as GO, PFAM, KEGG and INTERPRO. Comparison of the neprilysin transcripts was aided by Clustal [25], T-Coffee [32] and Boxshade (sourceforge.net/projects/boxshade/).

qPCR

Primer design

Both the genomic DNA and cDNA sequences from *P.* sp. DAW1 [60] were used to develop the qPCR primers. To identify and target introns, as well as to avoid amplification of any contaminating genomic DNA, both cDNA and genomic DNA sequences were aligned through in-house homology search tools and Spidey (www.ncbi.nlm.nih.gov/spidey). Primers were designed using Primer3web (primer3.ut.ee), and potential primers analyzed for possible dimer formation by Beacon Designer Free Edition (www.premierbiosoft.com) and for specificity by NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast).

Quantitative polymerase chain reaction (qPCR)

Quantitative PCR was performed using the BioRad CFX96 System (Hercules, CA, USA) and theBioRad SSoFast EVA Green Supermix with Low Rox. A 20 μL

reaction contained a 5 μL sample (total of 50 ng cDNA), 10 μL SYBR green mix, 1.2 μL primer mix and 3.2 μL mQ water. Primers were developed as described above and details are shown in Supplementary file 1. Specificity and efficiency assays were performed for all genes and details of these are shown in Supplementary file 1. Of six reference genes tested, the combination of *Pd-gpd-2* and *Pd-tba-1* (see Table 2 in Supplementary file 1) were defined as the most stable and used for all qPCR experiments [7].

Data analysis

The qPCR data were analyzed using the BioRad CFX Manager. Means, standard deviations (s.d.), *P*-values and relative expressions of the normalized expression values were calculated in MS Excel. *P*-values were assessed using a t-test (parametric, two samples, equal variance). The ΔΔCt (Livak) method was used to determine the relative difference in expression level of the target gene in different samples. In the first step, the Ct of the target gene was normalized to that of the reference gene, for both the test sample and the control sample (normalized relative expression value). Then, the ΔCt of the test sample was normalized to that of the control sample (relative expression).

Results and Discussion

Survival after freezing

Survival after freezing at -10° C, followed by thawing and recovery at 5° C was 83.9 ± 3.5% (mean ± 1 s.e., N = 4), and the nematodes took several hours to recover from the freezing stressor (Figure 1), with a 50% recovery time of 7.89 h (95% CL = 7.22, 8.69). These values are in agreement with previous survival analyses post freezing [39].

Functional analysis of separate treatment transcriptome assemblies

Illumina data from each of the treatments were assembled separately using identical criteria. The resulting assemblies contained similar numbers of transcripts and annotation rates: Treatment 1 produced 14,742 transcripts, of which 54.7% were

annotated; treatment 2, 16,349 transcripts with 52.5% annotated; treatment 3, 14,804 transcripts with 53.2% annotated; treatment 4, 14,718 transcripts with 53% annotation; treatment 5U, 13,481 transcripts of which 55% were annotated; treatment 5F 16,670 transcripts with 52% annotated; treatment 6, 14,493 with 54% annotated; and treatment 7, 14,179 transcripts with 54% annotated. The annotations by library can be seen in the Supplementary files 2-9. SEED subsystem analysis was carried out on all the separate treatment assemblies to assess overall functional differences and is depicted in Supplementary Table 1. The breakdown by function is almost identical betweeen the libraries, and indicates a broad distribution of functions. Protein Metabolism has the highest percentage in all the libraries, the same as reflected in an assembly of the entire transcriptome [60]. A subtle difference is that in treatments 2, 3 and 7, there are small representations of the functional category Dormancy and Sporulation, which the others do not have. Apart from this slight functional difference, the overall comparison indicates that the machinery to undergo all the respective physiological changes seems present in all stages of the nematodes that we have looked at. From this, we conclude that it is either the abundance of particular transcripts that plays a role, or that the ability to withstand any of the environmental disruptions tested here is constitutive. This possibility was reinforced by searches in each of the annotation files for selected relevant genes that that are discussed below.

Molecular response to intracellular freezing

Onset of freezing

As the medium temperature was brought down to -10° C, the samples either froze spontaneously or they remained suspended in an unfrozen medium. By sampling the nematodes in the medium that did or did not freeze spontaneously, we were able to look for any large differences in the highly represented genes induced at this particular transitional point. Supplementary Table 2 lists those transcripts that were shown to be highly abundant that were commonly shared both before and after the medium froze, and those unique to each sampling point. The overriding response in both stages is collagen production with 42 (44%) and 36 (55%) transcripts represented in the 5U and 5F treatments respectively. By contrast, after 24 hours at -10° C, only one collagen transcript is represented. Expression

of cuticle genes is not surprising and is a common response in other ecdysozoa, where it has been shown that ecdysis increases cold tolerance [75]. The highly connected relationship between all the annotations, both before and after the medium is frozen, can be seen in Figure 2 (A & B), where the STRING analysis shows a significant connectivity. Other responses evident in the data prior to and immediately after the medium freezes, and which can be seen as smaller clusters or singleton nodes in Figure 2 (A & B), include protection against reactive oxygen species, and changes in lipid and sugar metabolism. There are also three dauer-associated genes present. Functional enrichment of the two gene networks show body morphogenesis is the most significant Biological Process GO term, and the connection to cuticle production is found in all the other enriched functional categories (see Supplementary Table 3).

Post 24 hours

After nematodes were held for 24 hours at -10° C, the response was very different from the immediate freezing stages. Protease genes were the most abundant class (14 transcripts, 35%) expressed. Significant among these is neprilysin, which is represented by a number of different annotated transcripts. Neprilysin [63] is a zinc-dependent metalloprotease gene that in humans is associated with the natural amyloid β-degrading enzyme countering Alzheimer's disease [21]. While its role is not clear in *P.* sp DAW1, a number of different protease genes including neprilysin-like proteins (NEPs, neutral endopeptidases) are assumed to be involved in immune system regulation by degrading immune-specific peptides [3,33]. Evidence for this particular role is provided by the abundance of other immunity related genes (18% of the transcripts). In addition, there are also genes associated with reducing oxidative stress. When represented by the network analysis, and annotated solely against the *C. elegans* dataset, the variety of proteases collapses by homology to a reduced set of *C. elegans* genes, indicating that there is potentially more abundance and variability within these genes in *P. sp* DAW1 than in *C. elegans*. This may explain why the number of different transcripts is represented by a smaller set of nodes in the network analysis (Figure 2C) than might be expected. The Supplementary Table 3 shows that most of the enriched categories point to protease-related functions, while the Cellular Component category is enriched for the membrane region.

Thawing and Recovery

Nematodes initially held at −10° C for 24 h and then warmed from −10° C to +1° C and allowed to recover, show a sizeable increase in the number of highly expressed transcripts (see Figure 2D and Supplementary Table 2). This relative abundance compared with the previous stages indicates 'awakening activity', and the annotation reveals a wide array of functions. Supplementary Table 3 shows, by contrast with the collagen production of the freezing onset stages, and the protease production after being held at −10° C for 24 hours, the variety of enriched functional roles. These range from GTPase signalling, metabolic processes, muscle cell development, endocytosis, among others. This variety of functional roles is also reflected in the STRING network analysis where there is far less transcript connectivity (Figure 2D).

qPCR

Ten genes were chosen for more detailed examination by qPCR. Six of the genes were selected because of their known function and their potential role in freezing and desiccation tolerance: trehalose phosphate synthase, Late Embryonic Abundant protein, heat shock protein 70, desaturase, aquaporin, and glutathione peroxidase. These cover a range of different, yet pertinent, functions: The sugar trehalose has long been known to play an important role in cryoprotection and in general stress responses. It not only plays a role in protecting the membranes and proteins, but also through depressing the supercooling point of a solution via its colligative properties [42,78,30]. LEA proteins, like trehalose, play a number of roles in cryptobiosis, and are viewed as increasingly important in stress responses, and in preventing aggregation of proteins [15,62]; Heat shock proteins are an integral part of the stress response and essential as chaperones that deal with denatured proteins [14,6,54]; Desaturases insert double bonds into fatty acids, increasing the ratio of unsaturated to saturated fatty acids and altering membrane fluidity, a key response in cryoprotection [48]; Aquaporins aid in solute and water transport across the membranes and are associated with changes of osmotic conditions [24,35]; and glutathione peroxidase, one of a number of proteins that help reduce oxidative damage (c.f. [43]). Four other genes were selected because

they were represented in the treatment mappings. Of these, the most intriguing is the neprilysin, some of whose functions have been briefly outlined above. Along with the neprilysin, we have included a leucine-rich repeat protein, lrr-15, a transthyretin-like protein, tlr-5, and clec-49, a c-type lectin carbohydrate binding protein, from which type II antifreeze proteins in fish are derived [11]. The transthyretin-like protein, tlr-5, has been shown to be co-regulated with neprilysin in other systems [22], and since it was represented by a number of different of transcripts after thawing from -10° C (Supplementary Table 2) as well as enriched as a domain in the INTERPRO analysis (Supplementary Table 3), we have included it as a complementary gene to neprilysin. We have also included a leucine rich repeat gene as it has been found that one function of lrr, in large part owing to its repetitive structure, has been implicated in ice recrystallisation [76], and the annotation of different lrr genes shows up in both treatments 6 & 7.

The expression of all these genes in the three treatments of 5U, 5F and 6, when compared to the cold acclimation control treatment (3) roughly breaks down into 5 groups, as can be seen in Figure 3. These can be divided in terms of their general expression behaviour over the three ratios (i.e. whether they remained upregulated, or downregulated or up in one treatment, down in another, etc). Roughly, these are: lea-1, hsp-70, gpx-1, and llr-15 which are down-regulated except in the last treatment (after 24 hours at -10° C); aquaporin, clec-49, and neprilysin, which are upregulated in all three treatments; The remaining genes, tps-2a, desaturase, and tlr-5, all behaved uniquely.

The trehalose synthase (tps-2a) gene, down-regulated in all the treatments compared to the acclimation control suggests that the trehalose pathway, activated and expressed in a constitutive manner in relation to cold acclimation [49], is not playing a vital role in freeze tolerance, a surprising result (although, see [13]). The lea-1, hsp-70, gpx-1 and lrr-15 genes are all upregulated after 24 hours at -10° C, which provides evidence that not only are these genes relevant to the survival of intracellular freezing, but they point to how active the cells are in responding to intracellular freezing after 24 hours. With desaturase, the evidence points to a preparatory role in changing membrane flexibility by inserting double bonds in fatty acids. However, once frozen, desaturase, and presumably its role in the alteration of membrane flexibility, appears to no longer play a role. The picture

of the transthyretin-like protein, tlr-5, is a little confused, in that annotation for this gene showed up in our list of highly abundant genes in treatment 6, yet the qPCR indicate a higher level during treatment 5F, with its expression at treatment 6 lower than during the acclimation control. Three genes, an aquaporin, clec-49, and especially neprilysin, show the strongest signals of a committed response once the nematodes are subjected to -10° C. The aquaporins are familiar genes in the repertoire of those needed for cryptobiosis, and this is not a surprising result, now that we have established transcriptional activity during freezing. The c-type lectin, clec-49, is the same gene upregulated in the dessication induced anhydrobiotic nematode *Aphelenchus avenae* [41], and clearly seems to be playing some role. As already mentioned, c-type lectins gave rise to type II AFPs in fish, and a c-type lectin was annotated and assumed to be an AFP in *Plectus murrayi* [2]. However, a closer examination of the *P. murrayi* AFP suggests it is not functional, since it is highly unlikely that it has any antifreeze properties (C. Marshall, unpublished results). The leucine rich repeat gene lrr-15 is, like LEA-1, HSP-70 and GPx, expressed after the nematodes have been frozen for 24 hours yet not during the onset of freezing, is evidence for the crucial role it must play for survival, and it is quite possible that this lrr-15 plays a role as an IAP, possibly an RIP.

Finally, neprilysin, the protease represented by numerous transcripts among the highly expressed list in Supplementary Table 2, and contributing strongly to the functional enrichment in Supplementary Table 3, shows the strongest representation among all the qPCR genes, where it is increasingly up-regulated in each successive stage. While the annotation describes a gene called neprilysin, this is actually one of a number in a family of zinc-metalloenzymes more generally termed M13, with neprilysin and neprilysin-like genes simply the best characterised [5]. However, while neprilysin has received attention owing to its role in reducing amyloid-β plaque build up, which is correlated with Alzheimer's disease (this is not the first time that a link has been made between Alzheimer's and animals undergoing a form of shutdown owing to their environment or metabolic needs, for example see [55]), the family of M13 proteins, most of which are type II integral membrane proteins, play a number of important roles, and include angiotensin-converting enzymes (ACE), endothelin-converting enzymes (ECE) and thimet oligopeptidases (TOP). While an increasing amount of work is being done in understanding this diverse protein family, the function of many of the

members has yet to be elucidated. Within nematodes, NEP-like proteins, of which over twenty are present [63], seem to play a role in learning as well in pharyngeal activity and motility [52,5,77,47], and a recent study on the expression of dauer *C. elegans* subjected to spaceflight found that neprilysin was among a very small handful of transcripts downregulated by miRNA that were altered when the nematodes were subjected to space radiation and microgravity conditions [79]. Also of possible relevance, the first NEP-like gene found in an invertebrate was in the silkworm, expressed during molting and correlated with ecdysteroid production [80]. The typical characteristic of neprilysin-like genes is a short intracellular domain, a transmembrane domain, and a long extracellular active domain. NEP-like genes have a canonical HExxH zinc-binding motif, as well as two other conserved consensus sites, all of which are involved in catalytic processes [63].

To explore further the neprilysin transcripts that had a high degree of mapping, we compared them to a collection of neprilysin and neprilysin-like sequences that were collated in a study on the family [5] (see Supplementary File 10 for the Fasta file collection of these genes including the *P. sp* DAW1 transcripts). Following the clusters depicted in Bland *et al.* [5], the *P.* sp. DAW1 transcripts separate into two clusters of three and four transcripts respectively (see Supplementary File 11 for the Phylip clustering). The four transcripts clustered together contained the same region (541-652 in human neprilysin) that contain the catalytic residues, while the three other transcripts that clustered elsewhere either did not contain this region, or the transcripts as sequenced are missing them. Of the four with the region containing the catalytically important motifs [63,5], two of the sequences contain the motif region twice (PdU000330_v1.1 and PdU000424_v1.1). This phenomenon of a double domain has also been seen in *C. elegans* and verified through EST sequencing [63]. Aligning these four sequences against the subset of neprilysin and neprilysin-like genes also containing these motif regions (68 of the 111 proteins), we can see that the catalytic motifs are well preserved and the *P.* sp. DAW1 transcripts show the same level of variability among themselves as the other species represented (Supplementary Figure 1 shows the alignment of *P.* sp. DAW1 with just the *C. elegans* and *C. briggsae* genes. The full alignment can be seen in Supplementary File 12), indicating that as far as the active sites that we may investigate *in silico,* there is nothing peculiar about these genes that might shed a light on the role they may play in their role in intracellular freezing.

Conclusion

This study is the first to examine the molecular events during intracellular freezing in an organism capable of surviving the freezing of all of its compartments. We have focused on identifying those genes unique to this process, rather than those shared with the cold avoidance process that *P.* sp. DAW1 is also able to undergo, cryoprotective dehydration. We have shown that there is a moulting reaction during the immediate onset of freezing, after which, having been held frozen for 24 hours, the protease response dominates. We can also see that molecular activity is quite active when frozen, with the expression of a number of genes implicated in aiding survival, such as aquaporins, LEAs, glutathione peroxidases, leucine-rich repeat genes, heat shock proteins, c-type lectins, and neprilysin, among others. Surprisingly, trehalose biosynthetic genes do not seem to be actively expressed once the nematodes are frozen, either because they are not involved, or because they are already prevalent and highly-expressed during cold acclimation. Desaturase, and its role in membrane fluidity also seems to have been active only prior to freezing, but not after. A particularly intriguing result from this study is the expression of the gene for neprilysin, which shows a striking expression profile assessed both by mapping and by qPCR. It is too early to say what role neprilysin might play in the ability of *P.* sp. DAW1 to survive intracellular freezing, and more work will be needed to follow this up. No ice active proteins have yet been isolated [60], despite considerable evidence for recrystallisation inhibition in nematode extracts. Identifying the basis for the recrystallisation inhibition remains critical for understanding the molecular basis of intracellular freezing in *P.* sp. DAW1. The leucine-rich repeat gene looks like a good potential candidate to follow up on, given its possible role as an IAP, and its strong qPCR signal. While this study initiates the process of unravelling the molecular mechanisms involved in whole organism intracellular freezing, further and more detailed expression work is needed, and should also be aimed at exploring molecular behaviour of nematodes frozen to between -15° C and -60° C, the lethal region termed the intermediate zone of temperature [29].

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Legend

Figures

Figure 1 The recovery of *P.* sp. DAW1 after freezing at -10° C for 24h and thawing at 5° C. Vertical lines are standard errors (N = 4).

Figure 2 A schematic of the gene network connectivities produced by STRING [56], of treatments A) 5U -10° C unfrozen, B) 5F -10° C frozen, C) 6 -10° C frozen after 24 h, and D) 7, treatment 6 after thawing.

Figure 3 qPCR of tps-2a, lea-1, hsp-70, gpx-1, aquaporin, clec-49, desaturase, leucine-rich repeat-containing protein (lrr) 15, neprilysin and transthyretin-like protein (tlr) 5 in *Panagrolaimus* sp. samples in response to treatment 5F (cooled to -10° C, unfrozen, white bars), 5U (cooled to -10° C, frozen, hatched bars) and 6 (-10° C frozen, 24h). The control is shown as normalized to a value of 1 and the samples indicate the change relative to the control. Each value represents the mean±s.d. of 4 biological replicates. Significant differential expression (assessed using t-test) is indicated by asterisks (*P = <0.05, **P = <0.01, ***P = <0.001). More detail on the qPCR can be found in Supplementary file 1.

Supplementary files

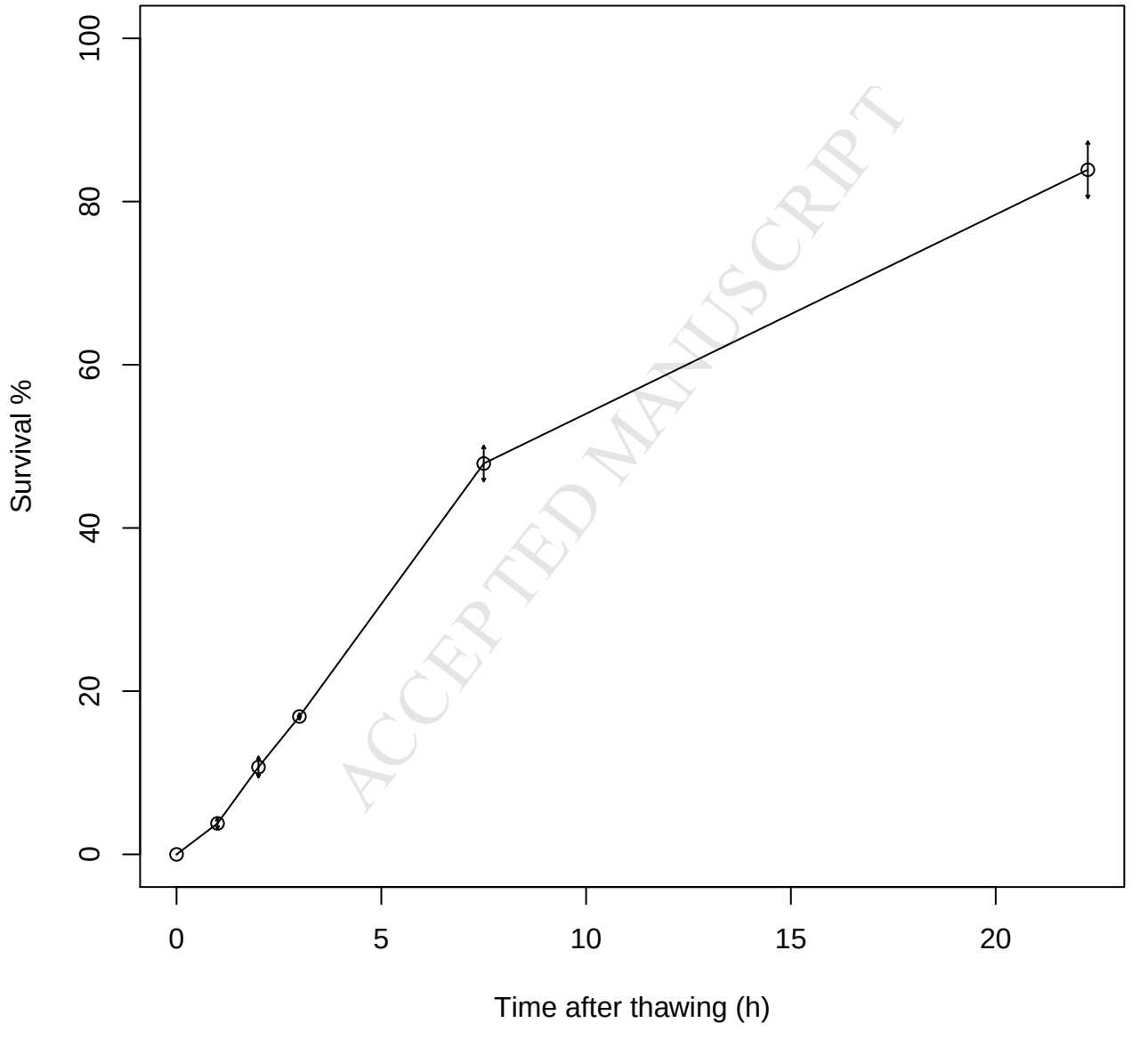
Supplementary Figure 1 A multiple alignment of the catalytic region of *P. sp* DAW1, *C. elegans* and *C. briggsae* neprilysin genes, showing the areas of high consensus.

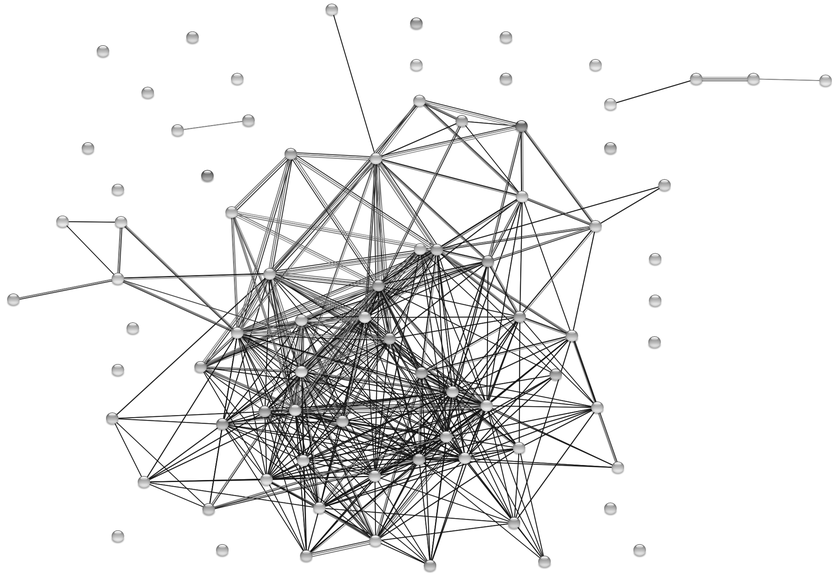
Supplementary Table 1 XLS file of the SEED breakdown of the separate assemblies from each of the treatments. The tables provide the proportion of each functional group.

Supplementary Table 2 XLS file with a table containing the annotations of the transcripts highly expressed in each of the comparisons. The first set are those gene annotations in common between treatments 5U and 5F, unfrozen and frozen at -10° C. The next set are those annotations unique to treatment 5U. The following set are those annotations unique to treatment 5F. The last set are those of treatment 6, when the nematodes have been held at -10°C for 24 h.

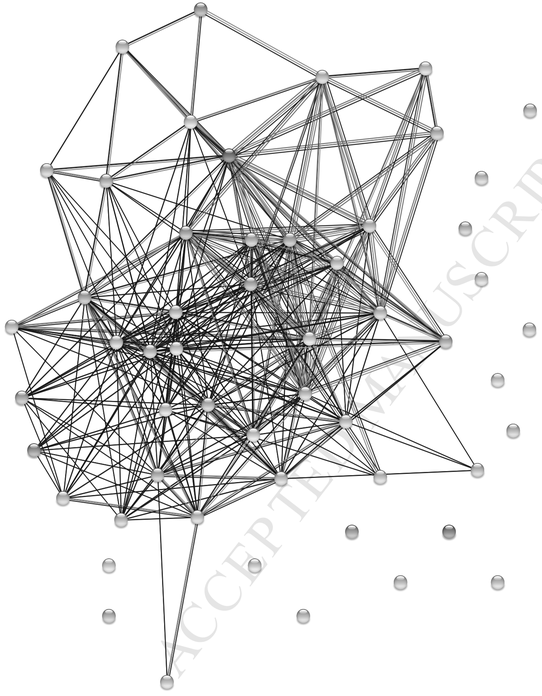
Supplementary Table 3 XLS file showing the enriched functions (from GO, PFAM, KEGG and INTERPRO) found in the highly expressed transcripts for treatment comparisons 5U, 5F, 6 and 7.

870
871 **Supplementary file 1** PDF file on the details of the primers and reference
872 sequences used for the qPCR.
873
874 **Supplementary files 2-9** TXT files of the BLAST annotations from the stage
875 specific assemblies of treatments: culture temperature +20° C, cold acclimated
876 +5° C, cryoprotectively dehydrated −1° C, recovered from −1° C to room
877 temperature, −10° C unfrozen, −10° C frozen, at −10° C for 24h, and thawing
878 and recovery from −10°C to +1°C.
879
880 **Supplementary file 10** Fasta file of all 118 neprilysin sequences used in the
881 comparison, including those of *P.* sp. DAW1.
882
883 **Supplementary file 11** PHYLIP file of the clustering showing the cluster positioning
884 of the *P.* sp. DAW1 neprilysin transcripts in relation to the other 111 neprilysin
885 gene sequences.
886
887 **Supplementary file 12** Clustal ALN file of the alignment of the catalytic region of
888 the neprilysin transcripts.





A



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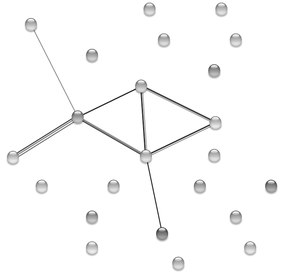

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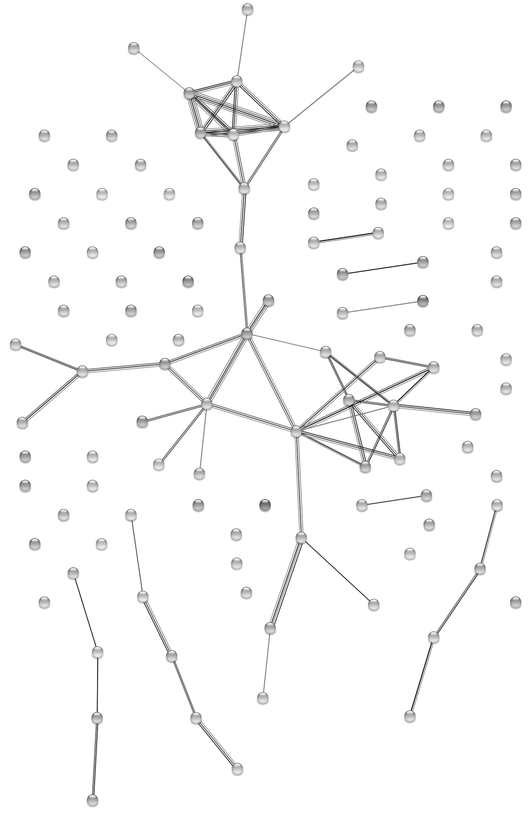
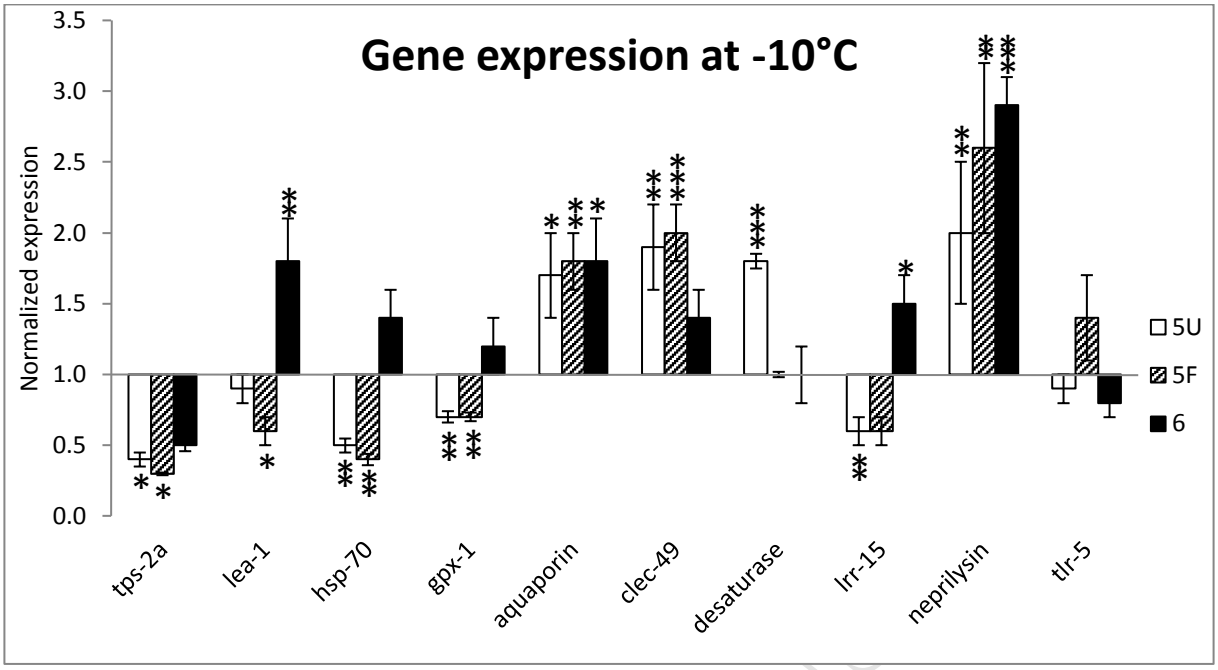


D



qPCR of *tps-2a*, *lea-1*, *hsp-70, gpx-1*, aquaporin, *clec-49*, desaturase, leucine-rich repeat-containing protein (lrr) 15, neprilysin and transthyretin-like protein (tlr) 5 in *Panagrolaimus* sp. samples in response to treatment 5F (cooled to -10°C, unfrozen, white bars), 5U (cooled to -10°C, frozen, hatched bars) and 6 (-10°C frozen, 24h). The control is shown as normalized to a value of 1 and the samples indicate the fold change relative to the control. Each value represents the mean±s.d. of 4 biological replicates. Significant differential expression (assessed using t-test) is indicated by asterisks (**P* = <0.05, ***P* = <0.01, ****P* = <0.001).