Transcriptomic and proteomic analysis of anhydrobiosis in *Panagrolaimus superbus* and *Caenorhabditis elegans* dauer larvae

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List of Abbreviations

2-DE	Two-dimensional gel electrophoresis
A	Adenine
Ala	Alanine
ANOVA	Analysis of variance
BER	Base-excision repair
BSA	Bovine serum albumin
C	Cytosine
Ca^{2+}	Calcium
cDNA	Complementary DNA
Cys	Cysteine
ddH_20	Distilled deionised water
DE	Differential expression
DEPC	Diethylpyrocarbonate
DSB	Double strand break
dsRNA	double stranded RNA
dH_20	Distilled water
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
ETOH	Ethanol
G	Guanine
G6P	Glucose 6 phosphate
gDNA	Genomic DNA
Gly	Glycine
h	Hour
HPLC	High performance liquid chromatography
HR/AM	High resolution/accurate mass
HSF	Heat shock factor
HSP	Heat shock protein
IPTG	Isopropyl-beta-D-thiogalactopyranoside
K_2HPO_4	Dipotassium phosphate
KEGG	Kyoto Encyclopedia of Genes and Genomes
KH_2PO_4	Monopotassium phosphate
L	Litre
LB	Liquid broth
LC-MS/MS	Liquid chromatography-mass spectrometry mass spectrometry
LEA	Late embryogenesis abundant
LFQ	Label free quantitation
LTQ	Linear trap quadrupole
min	Minute
ml	Millilire
mRNA	Messenger RNA
MS	Mass spectrometry
NaCl	Sodium Chloride
NaOCL	Sodium hypochlorite
NaOH	Sodium hydroxide

NER	Nucleotide-excision repair
NGM	Nematode growth media
^{o}C	Degrees Celsius
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RH	Relative humidity
RNA	Reoxyribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	Reactive oxygen species
RPM	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
Ser	Serine
siRNA	Small interfering RNA
sHsp	Small Hsp
SOD	Superoxide dismutase
sp.	Species
T	Thymine
TPS	Trehalose 6 phosphate synthase
TAE	Tris Acetate EDTA buffer
Thr	Threonine
TPP	Trehalose-phosphatase
Tris	Tris (hydroxy-methyl) amino methane
U	Uracil
UV	Ultra violet
μL	Microlitre

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Declaration

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

Signed _____

Eoin Mulvihill

Abstract

Many organisms are able to survive the loss of up to 95% of their cellular fluid by entering a state of suspended animation known as anhydrobiosis. The mechanisms which allow these organisms to survive desiccation are poorly understood. The nematodes *Panagrolaimus superbus* and *Caenorhabditis elegans* are able to survive extreme desiccation. These nematodes have contrasting strategies for surviving desiccation perhaps defined by the habitats in which they evolved. *P. superbus* was originally isolated in 1981 from a gull's nest in Surtsey, Iceland. In contrast *C. elegans* adults inhabit rotting fruit, a stable environment, less susceptible to desiccation. However, *C. elegans* does possess a stress resistant dauer larval stage in which the larvae are able to survive desiccation. The aim of this project was to identify the genes and proteins involved in desiccation tolerance in *P. superbus* and *C. elegans* dauer larvae.

The genes which were differentially expressed in response to desiccation and recovery in *P. superbus* and *C. elegans* dauer larvae were identified using RNA-seq technology. The mechanisms of desiccation tolerance were further investigated in *P. superbus* and *C. elegans* dauer larvae by using quantitative proteomic methods. In *C. elegans* dauer larvae and *P. superbus*, the following processes and pathways were implicated in the response to desiccation at both the transcriptome and proteome level: molecular chaperone activity, molecular shield activity, antioxidants, compatible solutes, the ubiquitin proteasome system, autophagy, DNA repair, cellular repair and response to pathogens. While there was much overlap in the identities of the genes and proteins which were up-regulated in response to dehydration, differences were also observed, which could be a reflection of the differences in the adaptive strategies used by these two nematode species to combat desiccation. Desiccation responsive gene candidates were selected for functional analyses using RNAi in *C. elegans* dauer larvae. This RNAi study revealed the importance of genes encoding hydrophilic proteins, molecular chaperones, trehalose phosphate synthase and glycerol kinase, as well as previously uncharacterised genes in enabling *C. elegans* dauer larvae to successfully survive desiccation.

The data presented in this thesis show that substantial reorganisation of cellular structures and mobilization of cellular protection repair systems occurs in *C. elegans* dauer larvae and *P. superbus* in response to desiccation, indicating that anhydrobiotic survival requires a major, integrated organismal response.

1 Chapter I General introduction

1.1 Anhydrobiosis

Considering that the Earth's surface is two thirds water and that most life on the planet began in the oceans, it is little surprise that water is the main constituent of living organisms. Water plays an essential role in the biochemistry of life, it is vital both as a solvent in which many of the body's solutes dissolve and as an essential part of many metabolic processes and enzymatic reactions within cells. Even though water is crucial for life, some organisms have adapted to survive in the absence of water. These organisms are capable of entering a state of suspended animation when water loss occurs. This state of suspended animation is called anhydrobiosis (Crowe *et al.*, 1992; Clegg, 2001). Anhydrobiosis is an extreme form of dehydration in which metabolism ceases and water content falls below 5% and in some cases as low as 2% of body weight (Danks, 2000). Anhydrobiotic organisms can survive in this dormant ametabolic state for long time periods and upon rehydration they resume normal biological activity and continue on in their development (Barrett, 1991). The ability to undergo anhydrobiosis is a remarkable example of evolutionary adaptation when we consider that the average percentage of water by weight in both vertebrates and invertebrates is estimated at 70% (Edney, 1977; Danks, 2000; McNab, 2002) and that most organisms cannot survive a loss of 50% of their water content (Watanabe, 2006). Not all water in living organisms is equivalent; therefore not all of it is lost with equal readiness. Much of the internal water in living organisms is influenced by its association with molecules and biological surfaces (Danks, 2000). Water associated with cellular components (normally 10-30%), termed bound water, is difficult to remove (Block and Harrisson, 1995). Even more resistant to removal is water incorporated into matrix molecules which normally comprised less than 0.4 % of the total (Danks, 2000).

There are species of animals, plants, and microbes that tolerate complete desiccation. Desiccation tolerance is common in nematodes (Wharton, 2003; Shannon *et al.*, 2005), tardigrades (Wright, 2001) and rotifers (Ricci *et al.*, 2003). Drought remains a major cause of famine as no crop tolerates severe water loss (Alpert, 2005). Complete desiccation seems to be always accompanied by the cessation of measurable metabolism (Danks, 2000).

Complete desiccation is defined as water content below 0.1 g H_2O g^{-1} dry mass (Clegg, 2001). Desiccation-sensitive seeds die before they dry to 20% water content whereas desiccation-tolerant seeds survive below 7% (Tweddle *et al.*, 2003). The threshold of 10% water content seems to have biological significance, since it may correspond to the point at which there is no longer enough water to form a monolayer around macromolecules, as a result stopping enzymatic reactions and thus metabolism (Alpert, 2005).

The ability to undergo anhydrobiosis has been linked to a high tolerance to a variety of other stresses. For example, desiccated tardigrades can survive treatment with x-rays and UV and temperatures from near zero to over 100^0C (Jonsson and Bertolani, 2001). When in an anhydrobiotic state nematodes, rotifers, and tardigrades can also survive fumigation with methyl bromide which damages their nucleic acids (Jonsson and Guidetti, 2001).

1.2 Applications of anhydrobiosis

Commercial applications from research into desiccation tolerance have been further explored in recent years. The potential to confer this anhydrobiotic ability onto non-desiccation tolerant animals, plants or tissues is increasingly seen as a potentially important research area (Bartels and Nelson, 1994; Panek, 1995; Tunnacliffe *et al.*, 2001). Attempts have been made to induce tolerance in human cells for medical purposes and to engineer tolerance in crop plants to make them less susceptible to drought (Alpert, 2005). Additionally attempts have been made to induce tolerance in bacteria and nematodes used for biological control. While no success has been made to improve the desiccation tolerance of plants, the disaccharide trehalose has been successfully used to induce desiccation tolerance in mammalian cells. Freeze drying blood platelets in trehalose can greatly improve their shelf life (Wolkers *et al.*, 2002). Products such as vaccines and blood products such as platelets which require constant refrigeration could be engineered to maintain their structural stability without the need for costly refrigerated transport. It is also likely that knowledge of the mechanisms of desiccation tolerance will lead to improved technologies in seed storage and in the preservation of dry foods and pharmaceutical products (Franca *et al.*, 2007).

1.3 Nematodes

Phylum Nematoda is an extremely successful and diverse group. There are approximately 26,000 described nematode species (Hugot *et al.*, 2001), but estimates of the actual number in existence range from 40,000 to 10 million (Blaxter *et al.*, 1998). Phylum Nematoda is thought to have arisen in marine habitats during the Cambrian explosion (600-650 million years ago). Nematodes are present in huge numbers in virtually all marine, freshwater and terrestrial environments (Van Megen *et al.*, 2009). This omnipresence is attributed to the evolution of a life history strategy to respond to changing environments (Sommer and Ogawa, 2011).

1.3.1 Habitat and ecology

Nematodes are structurally simple organisms. They have no circulatory or respiratory systems, instead using diffusion to exchange respiratory gasses. Free-living forms such as *Caenorhabditis elegans* and *Panagrolaimus superbus* as well as plant parasitic nematodes are typically only a few millimetres long, whereas animal parasitic nematodes tend to be larger. They are thin and are round in cross section, though they are actually bilaterally symmetrical. Although nematodes are one of the simplest animal groups, they have a complete digestive system, with a separate oral cavity for food intake and anus or cloaca for waste excretion. Nematodes do not have a true coelom, the body cavity is a pseudocoelom and the intestine lacks the necessary muscles to force food through their gut. Food is forced down the digestive tract by external and internal pressures on the nematode and by the movement of the nematodes. The pseudocoelom is the area between the body wall and the digestive tract. It is filled with fluid that bathes all of the tissues and serves as a transport system for oxygen, food and metabolic products.

1.3.2 The model nematode *Caenorhabditis elegans*

The free living bacterial feeding nematode *Caenorhabditis elegans* was selected by Brenner (1974) as a simple eukaryotic model system for genetic studies because of its ease of culture in the laboratory, its short life cycle and relatively simple morphology and organ systems.

1.3.2.1 Life cycle of *C. elegans*

The basic life cycle of *C. elegans* consists of embryogenesis (which includes fertilisation and hatching) which normally lasts 14 h and four larval stages, known as L1-L4. Each

larval phase is separated by a moult before the final moult occurs producing an adult nematode (Figure 1.1). In favourable conditions, the life cycle of *C. elegans* takes approximately 3 days whereas in unfavourable conditions, *C. elegans* arrests at the dauer diapause stage. Dauer larvae arise from a long-lived stress resistant stage which forms as an alternative to the L3 larval stage. When returned to favourable environments, dauer larvae rapidly recover to enter into a post-dauer L3/L4 stage where they will develop into adults with normal life spans and reproductive capabilities. This shows that longevity in *C. elegans* is plastic and under both environmental and genetic control. Extended life span likely arises as a secondary consequence of increased protection, hormesis, or thrifty metabolism (Fielenbach and Antebi, 2008).

1.3.2.2 The *C. elegans* dauer larva

In unfavourable environmental conditions (low food supply, high temperature and/or high population density), L1 or early L2 *C. elegans* worms enter the dauer larva developmental programme and at the L2 larval moult stress resistant dauer larvae are formed (Cassada and Russell, 1975). *C. elegans* secretes dauer pheromones in response to overcrowding or starvation conditions and it is this pheromone signal which leads to dauer formation (Golden and Riddle, 1984a; b). Dauer larvae do not feed; their mouth and anus are sealed; their cuticle is resistant to harsh chemicals and they have resistance to the environmental stresses of heat, cold and desiccation; their metabolic activity is reduced and their metabolism is dependent on internal energy stores (Cassada and Russell, 1975; Riddle *et al.*, 1997; Burnell *et al.*, 2005). Entry into the dauer stage represents the major life history response of nematodes to escape unfavourable environmental conditions (Sommer and Ogawa, 2011). However not all nematode taxa form dauer larvae. This phenomenon is most characteristic of the nematode order Rhabditida and the free living dauer larval stage is analogous to the infective larval stage of nematode parasites (Bird and Bird, 1991). Recently, the four major components of the dauer pheromone which induces *C. elegans* dauer formation were identified: daumone, ascaroside C3, ascaroside C6 and ascaroside C9 (Jeong *et al.*, 2005; Butcher *et al.*, 2007; Butcher *et al.*, 2008). They are all derivatives of 3,6-dideoxyhexose ascarilose (Jeong *et al.*, 2005; Butcher *et al.*, 2007; Butcher *et al.*, 2008).

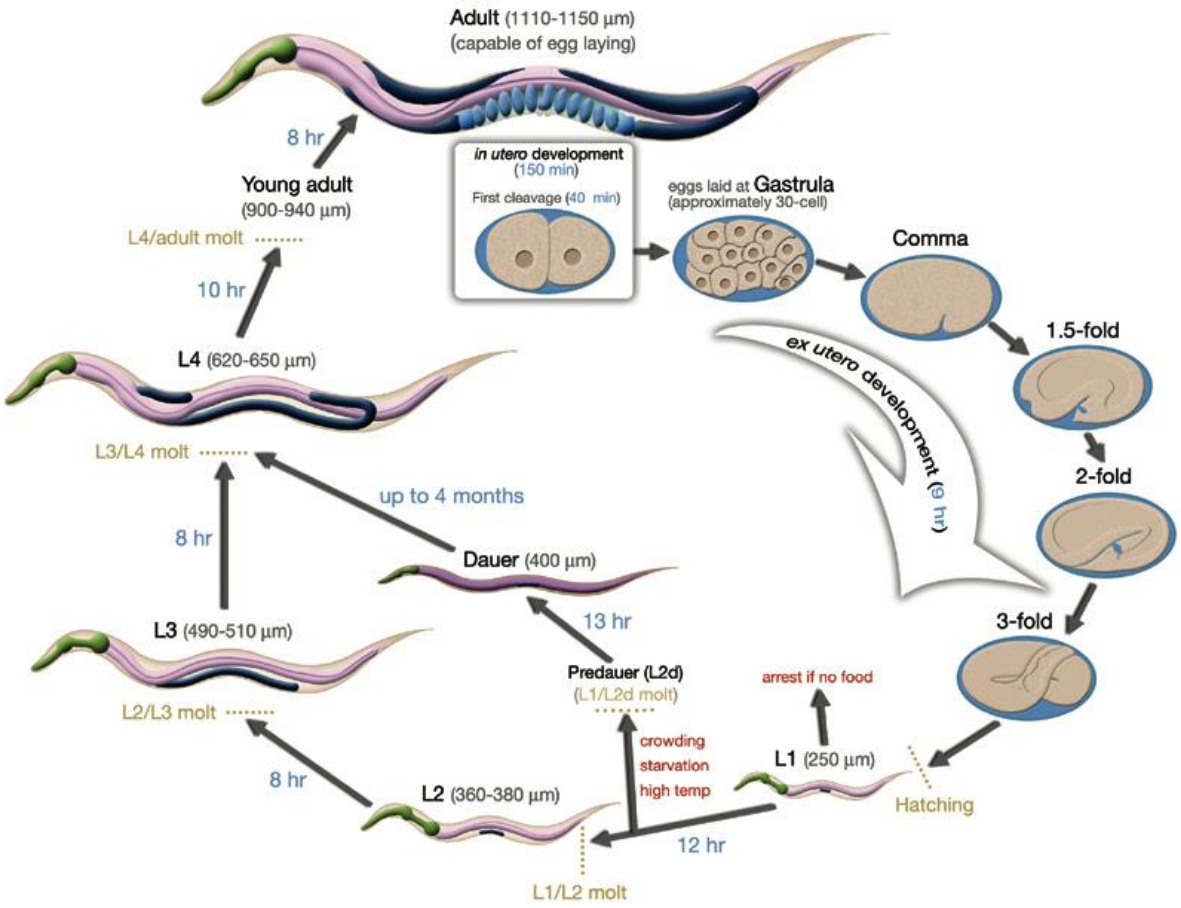


Figure 1.1 The life cycle of *C. elegans* at 22°C. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. The first cleavage occurs at about 40 min. postfertilization. Eggs are laid at about 150 min. postfertilization during the gastrula stage. The length of the animal at each stage (in micrometers) is marked next to the stage name. The source of this illustration is http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm

1.3.2.3 *C. elegans* insulin signalling

Two parallel signal transduction pathways are involved in dauer formation: the *daf-7*/transforming growth factor beta (TGF-ß) pathway and the *daf-2*/insulin-like growth factor 1 receptor (IGF1R) pathway (Figure 1.2). DAF-9, a cytochrome P450 steroid hydroxylase, lies at the convergence of these two signalling pathways in the dauer formation pathway. DAF-9 is postulated to produce a cholesterol-derived hormone with DAF-12 the putative target (Gerisch and Antebi, 2004; Matyash *et al.*, 2004; Burnell *et al.*, 2005). Hormone activated DAF-12 promotes reproductive development, while in the absence of this hormone DAF-12 activates the dauer formation programme (Gerisch and Antebi, 2004; Burnell *et al.*, 2005).

The insulin signalling pathway has been extensively studied in *C. elegans* due to its links with ageing and dauer larva formation. In *C. elegans* a mutation in the *daf-2* gene, which encodes an insulin/IGF receptor ortholog (Kimura *et al.*, 1997), was found to double the lifespan of the animal (Kenyon, 2005). Insulin/IGF-1 signalling appears to be only one step in a signalling cascade that affects lifespan as *daf-16*, which encodes a FOXO (forkhead box) family transcription factor, is also required for the longevity phenotype (Lin *et al.*, 1997; Kenyon, 2005). There are many genes whose expression is correlated with insulin/IGF-1 activity. Some of these genes were found to affect lifespan. These include antioxidant genes superoxide dismutase, catalase and glutathione S-transferase, chaperones particularly small heat shock proteins, and antibacterial genes (Honda and Honda, 1999; Wang *et al.*, 2004). Overexpression of superoxide dismutase and small heat shock proteins was even found to increase lifespan in *Drosophila melanogaster* (Sun *et al.*, 2002; Wang *et al.*, 2004). One of the theories of the evolution of age related pathways, called the insulin/IGF longevity regulatory module, links longevity to enduring harsh environmental stress. Many longevity genes downstream of *DAF-2*/DAF-16 not only extend life but also protect against environmental stress (Kenyon, 2005). Further evidence of the link between longevity and stress tolerance is seen in the fact that the insulin/IGF-1 pathway regulates entry into the dauer alternative life-stage which is stress resistant (Riddle *et al.*, 1997; Kenyon, 2005).

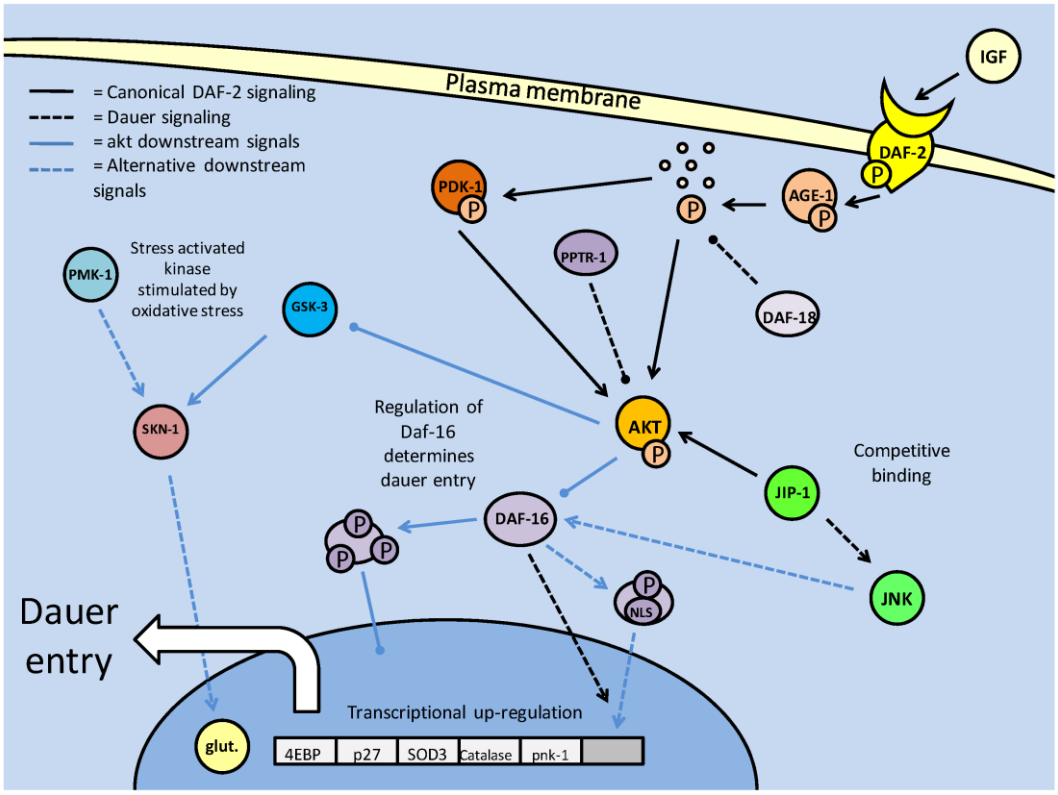


Figure 1.2 The Insulin/IGF signaling pathway which controls entry of *C. elegans* larvae into the dauer stage. Dauer entry is prevented through AKT mediated phosphorylation of the DAF-16 (FOXO homolog) transcription factor. AKT phosphorylation is the key to canonical DAF-2 signaling, and is regulated by protein kinases (PDK-1) and phosphatases (DAF-18 and PPTR-1). The source of this illustration is Lant and Storey (2010). The abbreviations used in the diagram are as follows: AGE-1 (age=AGEing alteration), a phosphoinositide 3-kinase catalytic subunit; AKT is a serine/threonine kinase (encoded by a homolog of the viral oncogene, *v-akt* first isolated in strain AK mouse thymocytes); DAF-2 (DAF=abnormal DAuer Formation, DAF-2 is a receptor tyrosine kinase that is the *C. elegans* insulin/IGF receptor); DAF-16 is a forkhead box O (FOXO) transcription factor; DAF-18 is a lipid phosphatase homologous to the human PTEN tumor suppressor; GSK-3 (gsk=Glycogen Synthase Kinase); JIP-1 (jip=JNK Interacting Protein), a scaffold protein; JNK (jnk=Jun N-terminal Kinase); PDK-1 is a 3-phosphoinositide-dependent kinase; PMK-1 (pmk=P38 Map Kinase); *pnk-1* (pnk=PaNtothenate Kinase), required for coenzyme A synthesis; PPTR-1 (pptr=Protein Phosphatase Two A Regulatory subunit); SKN-1 (skn=SKiNhead) is a bZip family transcription factor; 4E-BPI (4e-bpi=4E binding protein 1) is a phosphoprotein with an important role in the regulation of translation; P27 is a kinase inhibitory protein which has a role in inducing cell cycle arrest; *sod-3* encodes a iron/manganese superoxide dismutase.

1.3.2.4 Increased gene expression associated with *C. elegans* dauer larvae formation

Recent studies have shown increased expression of genes encoding antioxidants and molecular chaperones in the *C. elegans* dauer larvae stage. Dauer larvae have increased activity of the antioxidants *sod-3* (sod=superoxide dismutase) and *ctl-1* (ctl=catalase) (Vanfleteren and Devreese, 1996; Jones *et al.*, 2001; Houthoofd *et al.*, 2002; Wang and Kim, 2003). The *sod* genes encode metalloenzymes which are responsible for quenching the potentially deleterious effects of superoxide free radicals by converting them to hydrogen peroxide (Bowler *et al.*, 1992). The *ctl* genes encode enzymes that deactivate hydrogen peroxide, another potent oxidising agent (Lindblom and Dodd, 2006). The induction of these antioxidant genes may explain the increased tolerance of desiccation observed in the *C. elegans* dauer larvae stage.

As well as an increase in transcription of genes encoding antioxidants, transcripts encoding molecular chaperones are highly expressed in dauers. The up-regulated genes encode small heat shock proteins (*hsp-12.6)*, HSP-70 (*hsp-70*) as well as HSP-90 (*daf-21*). Recently, Sinha *et al.* (2012) compared the change of gene expression in the conserved dauer stage versus dauer exit stage of the nematodes *Pristionchus pacificus* and *C. elegans*. They found that the dauers of the two nematodes differed in the dynamics and function of the genes which were differentially expressed. In both *C. elegans* and *P. pacificus*, genes were induced that contained domains of the HSP-20 family of heat shock proteins. In contrast, genes containing domains corresponding to proteasome proteins were up-regulated in *P. pacificus* but not in *C. elegans*. Since proteasome function has been implicated in the regulation of longevity (Ghazi *et al.*, 2007; Yun *et al.*, 2008), the increase in expression of genes with proteasome domains may be linked with the increased longevity of *P. pacificus* compared to *C. elegans*.

1.3.2.5 Increased protein expression associated with *C. elegans* dauer larvae formation

Various proteomic analyses have been carried out in *C. elegans* in the last few years focusing on proteins which are up-regulated in the dauer stage. One such study by Madi *et al.* (2008) compared the proteins which were differentially expressed between the dauer and L3 developmental stages in *C. elegans* using 2-DE (two-dimensional gel electrophoresis). Interestingly, they found that the protein profile of the two stages were nearly identical, with only eight proteins differentially expressed. The following proteins

were found to be up-regulated in the dauer larval stage; inorganic pyrophosphatase; phosphatidylethanolamine-binding protein; alcohol dehydrogenase and aldehyde dehydrogenase. Another study by Jeong *et al.* (2009) also utilised 2-DE to analyze the whole-body proteome of mixed-stage worms and dauer larvae at different pH values. They found that proteins belonging to diverse functional categories were up-regulated in the dauer larvae including oxidative stress-defense (HSP-12.6, GST-1), muscle proteins (levamisol-resistant proteins, troponin C, and ACT-3, etc.), and energy generation (ATP synthase and NADH-ubiquinone oxidoreductase). The most recent proteomic comparison of dauer versus L3 larvae, again using 2-DE, found that small heat shock proteins (HSP-12.3 and HSP-12.6) and antioxidants were up-regulated in the dauers while ribosomal protein 12 (RPS-12) and nucleoside diphosphate kinase were down-regulated relative to the L3 stage (Jones *et al.*, 2010). They also found significantly higher GST activity in the dauer larvae stage versus the L3 stage in a GST subproteome analysis. Additionally, three isoforms of GST-5 and two isoforms of GST-7 were up-regulated in the dauer larvae stage compared to the L3 stage (Jones *et al.*, 2010). Interestingly, an increase in proteins involved in anaerobic fermentation pathways was seen in all three proteomic studies. This up-regulation of anaerobic metabolism is also supported at the transcript level (Wang and Kim, 2003; McElwee *et al.*, 2004) indicating a suppression of aerobic respiration in favour of glycolysis and fermentation (reviewed by Burnell *et al.*, 2005).

1.3.3 The nematode *Panagrolaimus superbus*

Panagrolaimus species are free-living organisms, inhabiting the soils of temperate, tropical, arid and polar geographical regions. *Panagrolaimus superbus* was isolated from co-culture in a seagull's nest in a lava cavity on an exposed nunatak on the volcanic island of Surtsey which is located just to the south of Iceland. *Panagrolaimus* species are bacteriovores and can be readily cultured in the laboratory on a lawn of *Escherichia coli*. Its anhydrobiotic survival and ease of culture were perhaps the first indication that *Panagrolaimus* could become a model organism for studying anhydrobiosis in nematodes. The life cycle of these nematodes is completed in approximately 8 days at 20^{o}C. Members of the genus *Panagrolaimus* exhibit different reproductive modes including gonochoristic, hermaphroditic and parthenogenetic (Lewis *et al.*, 2009). The *Panagrolaimus* species used in this project was *Panagrolaimus superbus* which is gonochoristic i.e. two distinct sexes exist.

Various *Panagrolaimus* species are capable of anhydrobiosis (Shannon *et al.*, 2005). Shannon *et al.* (2005) found that within the genus *Panagrolaimus* slow and fast dehydration strategists exist. *P. superbus* is a fast dehydration strategist which is capable of surviving immediate exposure to 0% RH. The Antarctic nematode *P. davidi* is a slow dehydration strategist nematode (Wharton and Barclay, 1993) which requires preconditioning at high relative humidity in order to mobilise the adaptive changes necessary to enable it to survive subsequent exposure to lower relative humidities. Freezing tolerance has also been found in the genus *Panagrolaimus.* This phenomenon has been observed in both *P. davidi* (Wharton and Brown, 1991) and *P. superbus* (Wharton, 1996; McGill, unpublished work).

1.4 Evolutionary context

The lack of clearly defined homologous characters and the absence of any fossil record have hampered efforts to clearly identify an evolutionary framework for the phylum Nematoda (Blaxter *et al.*, 1998). Thus classifications of the nematode phyla have traditionally relied on morphological and ecological traits (Dorris *et al.*, 1999). Two nematode classes have traditionally been recognised, the predominantly marine Adenophorea and terrestrial Secernentea (Chitwood, 1933).

A bioinformatic analysis of the SSU ribosomal RNAs has shed more light on the evolutionary history of the nematodes. This analysis has shown no evidence of an adenophorean ancestry, as was previously thought (Blaxter *et al.*, 1998). From this work five major nematode clades have been identified (Figure 1.3). Clades I-II have been shown to be strictly adenophorean. Clade I contains members such as the human parasitic *Trichinella spiralis* and the free living *Miconchoides studeri*, while representatives of clade II are the free-living marine dweller *Enoplus brevis* a member of the Enolpia subclass and *Trichodorus* which is a plant parasitic nematode. Three major clades, III-V, have been found within Secernentea (Dorris *et al.*, 1999). Clade III comprises animal parasites with members such as the filarial worm *Brugia malayi* whose genome has been sequenced (Ghedin *et al.*, 2007). Clade IV is more genetically diverse than clade III, and as a result has been split into two parts IVa and IVb (Dorris *et al.*, 1999). Among the IV clade are representatives from animal-parasitic, plant-parasitic, fungal feeding and free-living groups. The nematode *P. superbus*, which was studied in this project, falls within

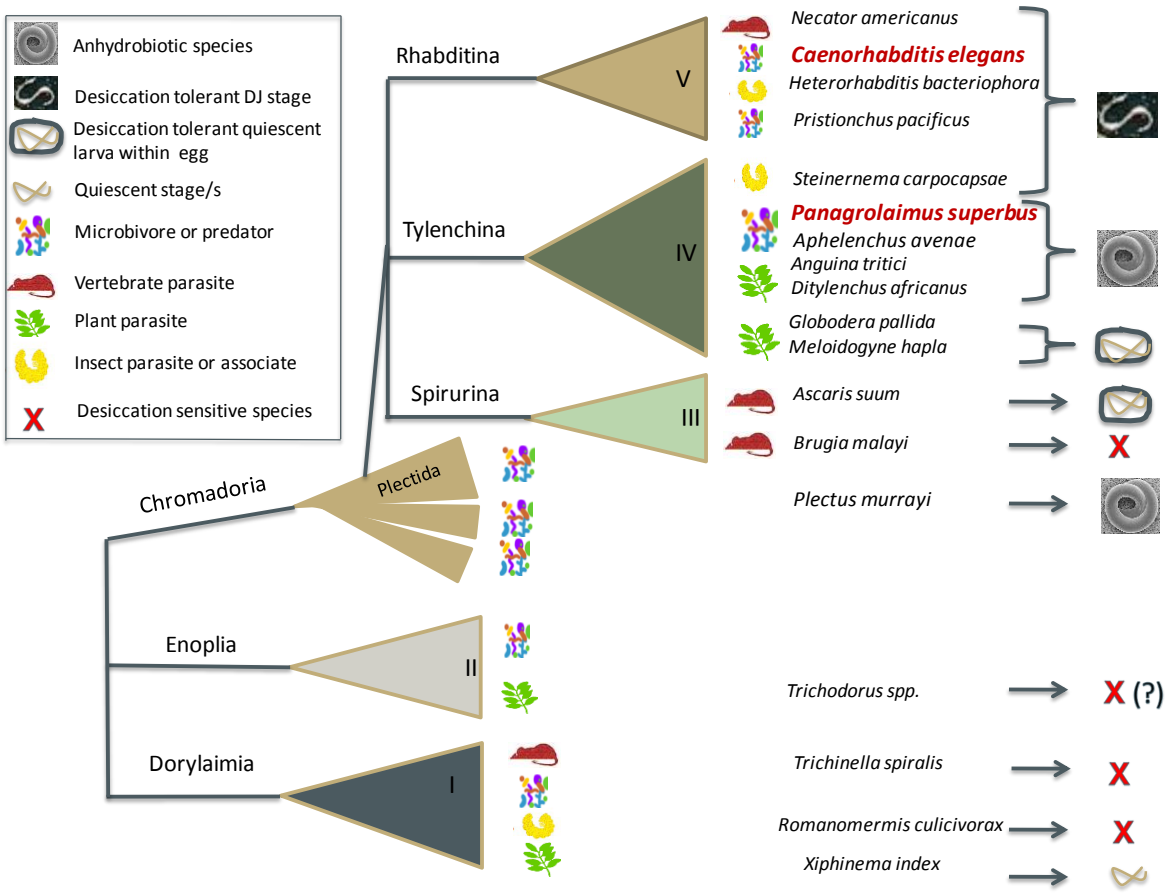


Figure 1.3 A phylogenetic tree of the Phylum Nematoda based on the phylogeny of Blaxter *et al.* (1998) showing the distribution of anhydrobiotic and desiccation tolerant taxa, along with their trophic ecology, across the phylum. The nematodes *Caenorhabditis elegans* and *Panagrolaimus superbus*, which were used in this study, are highlighted (red font). The source of this illustration is Tyson *et al.* (2012).

this clade. Finally, clade V contains free-living (including *C. elegans*), microbivores and vertebrate parasitic members.

The distribution of anhydrobiotic phenotypes within the Nematoda is presented in Figure 1.3. Anhydrobiosis is not an uncommon trait in nematodes. Many free-living nematodes such as *P. superbus* and *A. avenae* can undergo anhydrobiosis at all stages of their life cycle, and in addition, a large number of plant and animal parasitic nematodes have anhydrobiotic egg, cyst or infective juvenile stages (Shannon *et al.*, 2005). In clade V, Erkut *et al.* (2011) have recently shown that *C. elegans* dauer larvae can survive exposure to low relative humidity following preconditioning at 98% RH for 4 days. The best described anhydrobiotic nematodes to date occur in clade IV. These include members of the genera *Panagrolaimus, Aphelenchus* and the plant parasitic/entomopathogenic order Tylenchida which includes *Meloidogyne* and *Globodera* which often exhibit anhydrobiotic encysted infective stages. Clade III contains four animal parasitic orders (Blaxter, 1998). Within this clade lies the order *Spirurida*. This order contains members such as the human filarial nematode *B. malayi,* which forms encysted infective stages. All remaining nematode clades contain some terrestrial and parasitic species, thus it is possible that more nematode species with anhydrobiotic capabilities may occur within these taxa (Shannon *et al.*, 2005).

1.5 Adaptive strategies used by anhydrobiotic organisms to combat desiccation stress

1.5.1 Coiling and clumping

When exposed to desiccation stress many individual nematodes coil their bodies and aggregate with other nematodes to form clumps. This strategy reduces or protects the exposed surfaces so the rate of further water loss is slowed. For example in the anhydrobiotic nematode *D. dipsaci* the rate of water loss is controlled during desiccation by narrowing the groove between the body annulations (Wharton, 1996). While, shrinkage to the "tun" stage of tardigrades (Somme, 1996) and contraction of rotifers (Gilbert, 1974) upon dehydration also reduces the rate of water loss. Clumping is also an effective strategy for nematodes to reduce the rate of water loss. This has been extensively studied in *Ditylenchus dispaci* (Bird and Buttrose, 1974) where large clumps of the fourth stage juveniles have been observed to survive after 23 years. The outermost cuticle of

individuals in these clumps fuse together to form a water tight barrier, protecting individual nematodes at the centre against excessive water loss. This effect has been seen in *A. avenae*, *S. feltiae*, and *T. semipenetrans* and correlation between clump size and survival has also been observed (Crowe and Madin, 1974; Tsai and Van Gundy, 1989). Another mechanism which may reduce water loss is the secretion of lipid on the surface of nematodes. Wharton *et al.* (2008) observed a lipid composed of triglyceride in the plant parasitic nematode *D. dipsaci* which may decrease the permeability of the cuticle during the early stage of desiccation.

1.5.2 Compatible solutes

In many species of plants, animals and microorganisms dehydration leads to the accumulation of a variety of soluble, low molecular weight water-replacement molecules that are non-toxic and do not interfere with cellular metabolism (Yancey, 2005). Many of these solutes have cytoprotective properties such as antioxidative activity and protein stabilization. The non-reducing disaccharides, trehalose (in plants, animals and fungi) and sucrose (in plants), which accumulate in diverse anhydrobiotes have been proposed to function as either water-replacement molecules or vitrification agents (Hoekstra *et al.*, 2001).

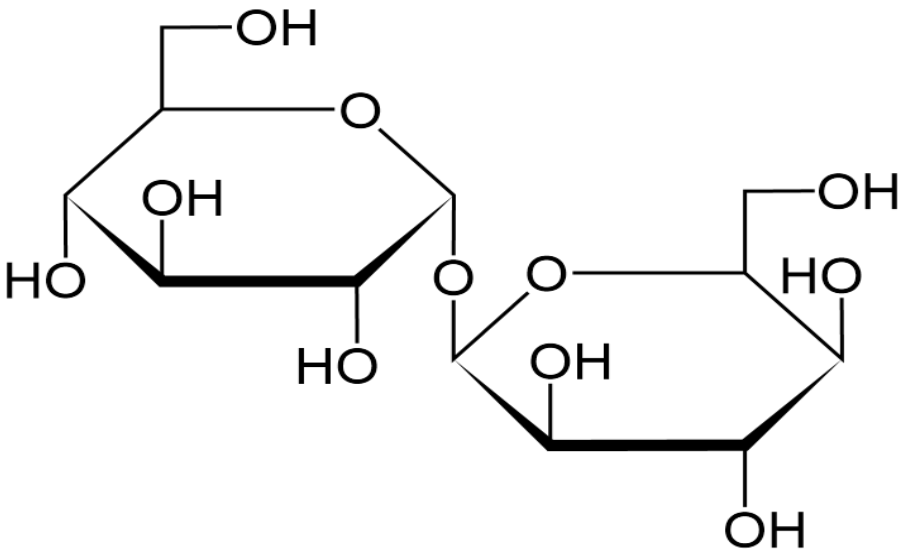
1.5.2.1 Trehalose

Trehalose has been shown to constitute circa 15% and 9% of the dry weight of anhydrobiotic *Artemia salina* cysts and *A. avenae* nematodes, respectively (Clegg, 1965; Madin and Crowe, 1975). Trehalose defective mutants of *E. coli* have been found to have an impaired osmotic tolerance and an impaired stationary-phase-induced heat tolerance (Strom and Kaasen, 1993). Trehalose 6-phosphate synthase is the rate limiting enzyme for the synthesis of trehalose in nematodes (Behm, 1997). The *C. elegans* genome encodes two trehalose-6-phosphate (*tps*) genes: *tps-1* (ZK430.3) and *tps-2* (F19H8.1). Erkut *et al.* (2011) showed that a *C. elegans* deletion strain, lacking both trehalose phosphate synthase genes was to be unable to synthesize trehalose and that the capacity of dauer larvae from this strain to survive desiccation was substantially impaired.

Trehalose is a non-reducing sugar formed from two glucose units joined by the bonding the 1,1 carbon atoms of the two glucopyranose rings (Figure 1.4a). Trehalose is synthesized by two enzymes trehalose-6-phosphate phosphatase and trehalose-6-phosphate synthase (Figure 1.4b), discussed further below. As the energy required for the bonding of trehalose is low, the molecule is very stable resulting in a relatively unreactive molecule. Trehalose can also be readily broken down into its component monosaccharides, glucose, meaning that it can function in cells as a less chemically reactive energy store in the form of the compound glucose.

Trehalose has been shown to be an extremely efficient carbohydrate for protecting proteins (Prestrelski *et al.*, 1995). Trehalose is able to stabilise the enzyme phosphofructokinase which normally becomes irreversibly damaged when dried (Carpenter *et al.*, 1987). Several mechanisms have been proposed as to how trehalose stabilises such proteins. Trehalose may stabilize the protein by hydrogen bonding through its OH groups to the polar residues of the protein during dehydration (Carpenter, 1993). Trehalose may also protect proteins by a process called preferential exclusion (Carpenter and Crowe, 1988). It is hypothesized the addition of certain co-solutes (such as sugars) to a protein causes a thermodynamically unfavourable effect. The result of this is the co-solutes become excluded from the immediate vicinity of the protein and the protein is more likely to exist in a compact folded form in order to reduce the surface area for interaction with the co-solute (Timasheff, 1992). Trehalose may also act as a chemical chaperone (Singer and Lindquist, 1998; Crowe, 2007). Unlike traditional molecular chaperones which promote correct folding, chemical chaperones may influence the rate or fidelity of the folding reaction by stabilising the properly folded form (Welch and Brown, 1996). Trehalose has also been shown to act as an antioxidant in yeast. In *S. cerevisiae*, it accumulates in response to oxidative stress and it protects against lipid peroxidation (Herdeiro *et al.*, 2006). Increased levels of protein oxidation damage within the cells of trehalose deficient *S. cerevisiae* mutants was also observed when the in mutants were exposed to ROS (Benaroudj *et al.*, 2001).

(a)



(b)

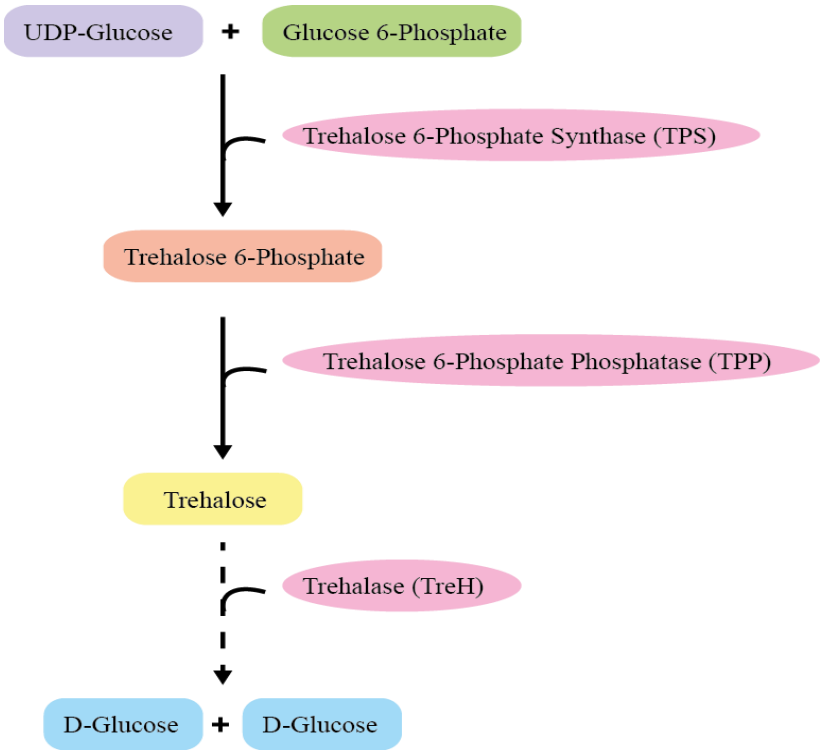


Figure 1.4 (a) The structure of trehalose. Trehalose is a disaccharide sugar bonded at the 1,1-glucoside bond between the two α-glucose units. The source of this illustration is http://commons.wikimedia.org/wiki/File:Trehalose.svg. (b) The trehalose-6-phosphate synthase (TPS)/trehalose-phosphatase (TPP) pathway for trehalose biosynthesis. Trehalose is synthesised via the enzymes TPS and TPP, and is degraded by trehalase. This image was modified from Avonce *et al.* (2006).

1.5.2.2 The trehalose water-replacement theory

During desiccation water molecules are removed from their phospholipid bilayers which results in a phase transition in the plasma membranes from the liquid crystalline state to a gel phase (Figure 1.5). It is believed that the stabilising effect of trehalose in anhydrobiotic organisms lies in its ability to form hydrogen bonds. By hydrogen bonding to the phosphate heads of the bilayer, replacing water, there is a prevention of increased van der Waal interactions among the phosphate groups of desiccated membranes. Therefore the presence of trehalose in drying cells leads to a decrease in phase transition temperature in the phospholipid bilayer (Crowe *et al.*, 1984). Due to this substitution of water molecules by trehalose the membrane remains in a liquid crystalline state and does not undergo a phase transition during desiccation or upon rehydration as shown in Figure 1.5 (Crowe *et al.*, 1996).

This 'water replacement hypothesis' is supported by high-resolution proton NMR studies where it was observed that the hydrocarbon chains of phospholipids exist in a gel phase when dried without trehalose, but remain in a liquid phase when dried with trehalose (Lee *et al.*, 1986). Trehalose is also seen to be much more efficient than other disaccharides at preventing membrane phase transitions. For example sucrose is required at three times the concentration in order to achieve the same effect as trehalose (Crowe *et al.*, 1987).

1.5.2.3 Vitrification

Sugars such as trehalose and sucrose possess the ability to form glasses. A glass is a liquid of high viscosity formed when a liquid transforms into an amorphous solid driven by a change in temperature, chemical composition or pressure. The formation of an intracellular glass is thought to play an important role in anhydrobiosis (Burke, 1986; Bruni and Leopold, 1991; Crowe *et al.*, 1992; Sun and Leopold, 1997; Crowe *et al.*, 1998). When a cell loses water the cytoplasm becomes increasingly concentrated with solutes which then tend to crystallise out of solution or the cytoplasm becomes supersaturated causing a noticeable increase in viscosity. When the viscosity reaches a certain point the solution takes on the properties of a plastic solid. At this point the solution is referred to as a 'glass' (Hirsh, 1987). Vitrification can have many advantages for organisms undergoing dehydration.

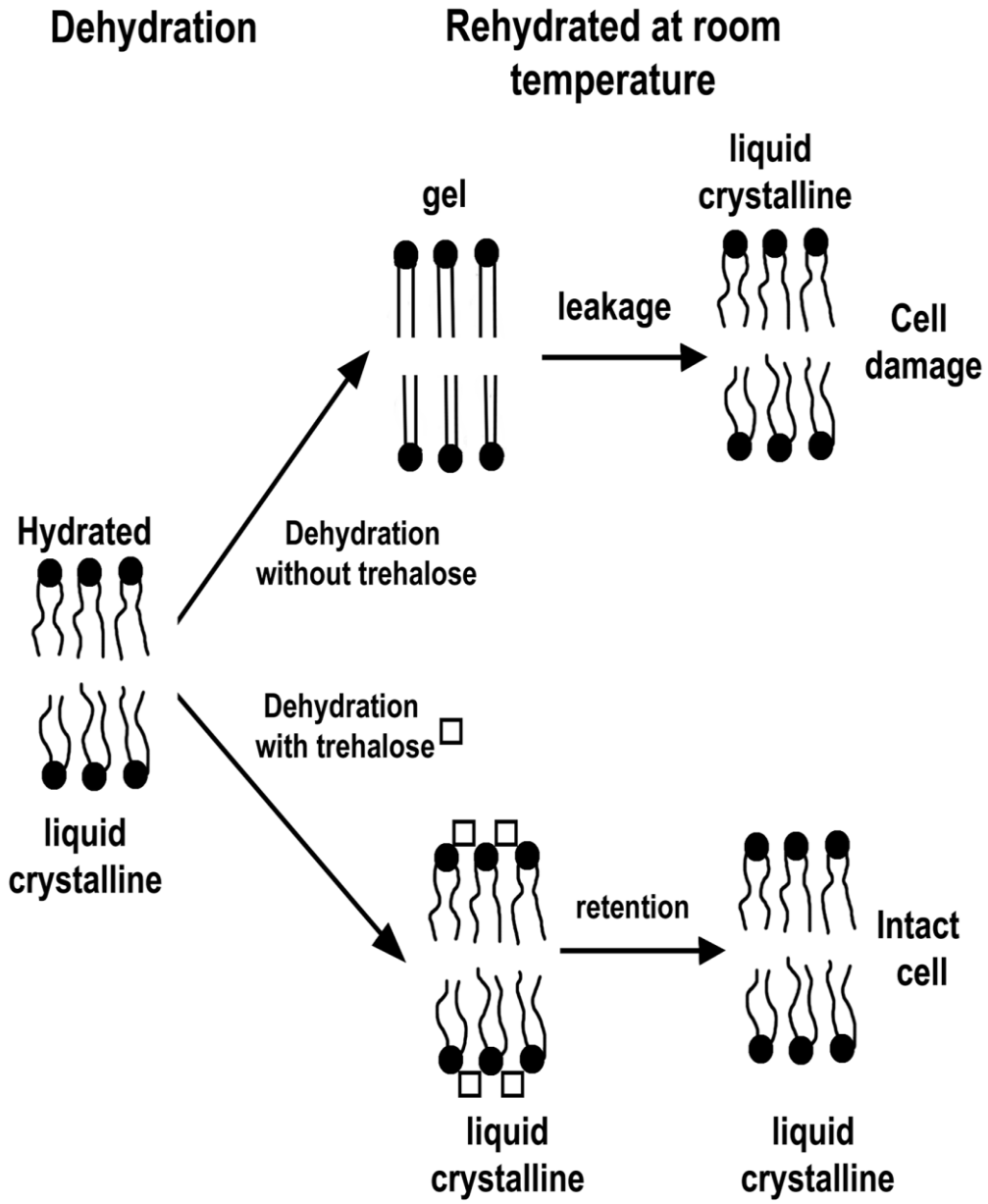


Figure 1.5 The mechanism of membrane protection by the sugar trehalose. This sugar prevents phospholipid fusion during desiccation and lowers transition temperature. This helps avoid phase transition and stabilises the membrane structure (Taken from Crowe *et al*, 1992).

Firstly, the glass is able to fill intracellular spaces preventing cellular collapse and membrane fusion. The high viscosity of the glass also prevents chemical reactions that rely on diffusion for completeness ensuring long-term stability. Also, chaotropic solutes (those with the ability to destabilize hydrogen bonding and hydrophobic interactions) become trapped in the matrix of the glass where they are protected during drying (Crowe *et al.*, 1998).

The sugars that make up the intracellular glass would also be able to form stabilising hydrogen bonds with labile macromolecules offering similar protection to that of trehalose (Koster, 1991). Formation of a glass would also provide hydroxyl groups for the formation of hydrogen bonds in order to stabilise proteins that would not be present on a crystalline sugar (Crowe *et al.*, 1998). It is likely that this process works alongside trehalose accumulation, i.e. the 'water replacement hypothesis' to protect the cell during drying.

Studies of glassy states and glass transitions in nature have traditionally focused on plant systems. In soybean seeds for example, it was shown that at water contents of <10% a glassy state exists. No glassy state was observed in controls performed on desiccation intolerant seeds (Bruni and Leopold, 1991). Other studies have found glasses in dried pollen (Buitink *et al.*, 1996) and the seeds of other plant species such as corn (Williams and Leopold, 1989) and the common bean (Leprince and Walters-Vertucci, 1995), in resurrection plants (Wolkers *et al.*, 1998) and in bacterial spores (Sapru and Labuza, 1993; Potts, 1994). More recently glasses were also found to occur in the anhydrobiotic larvae of the insect *Polypedilum vanderplanki* (Sakurai *et al.*, 2008).

1.5.2.4 Trehalose biosynthetic pathway

Trehalose is a common soluble compound involved in basic metabolism, and so can be converted to other uses more readily than new substances can be elaborated (Danks, 2000). Several biosynthetic pathways have been described for the synthesis of trehalose. However, there is only one pathway which is conserved across eubacteria, archaea, fungi, animals and plants. This pathway, first proposed by Cabib in 1957, involves two enzymatic steps catalysed by trehalose-6-phosphate synthase (TPS) and trehalose-phosphatase (TPP), as shown in Figure 1.4b. The TPS catalyses the transfer of glucose

from uridine diphosphoglucose (UDPG) to glucose-6-phosphate, this generates trehalose-6-phosphate (T6P) and uridine diphosphate (UDP). The TPP dephosphorylates T6P to trehalose and inorganic phosphate. The TPS and TPP protein domains appear to have co-evolved and have likely undergone several gene duplications and lateral gene transfer events (Avonce *et al.*, 2006). Trehalase breaks down trehalose by hydrolysis, forming two molecules of D-glucose.

1.5.2.5 Other compatible solutes

Trehalose is not universally used in anhydrobiotic protection as is evident from its absence in dehydrated anhydrobiotic bdelloid rotifers (Lapinski and Tunnacliffe, 2003). Other biomolecules known to protect organisms against dehydration, include sorbitol (Wolfe *et al.*, 1998), glycerol (Cannon, 1986), ribitol (Cannon, 1986), and inositol (Bayley and Holmstrup, 1999).

1.5.3 Amphiphilic metabolites

An amphiphile is a chemical compound that possesses both hydrophobic and hydrophilic elements. The amphiphilic compound rutin and arbutin are both found in anhydrobiotic plants as well as plant seed and pollen (Oliver *et al.*, 1996). Studies of pollen and seeds have shown that amphiphilic molecules localise in the membranes upon dehydration (Golovina *et al.*, 1998; Golovina and Hoekstra, 2002). Upon dehydration the concentration of amphiphilic metabolites increases in the cell until they partition from the cytoplasm into the lipid phase (reviewed by Hoekstra *et al.*, 2001). There is evidence that amphiphile partitioning into the membrane causes membrane disturbance (Golovina *et al.*, 1998). However, partitioning of cytoplasmic amphiphiles into membranes during desiccation may also have a positive effect by assisting the automatic insertion of antioxidants or phospholipase inhibitors with amphiphilic properties (Hoekstra *et al.*, 2001). Additionally, similar to sugars such as trehalose, amphiphiles can affect membrane fluidity and depress the phase transition temperature of membranes (Golovina *et al.*, 1998; Golovina and Hoekstra, 2002).

1.5.4 Late embryogenesis abundant proteins

Late embryogenesis abundant (LEA) proteins are so called because they were first discovered in the maturation stages of *Gossypium hirsutum* (cotton) embryos. LEA

protein homologs have been found in nematodes and bacteria (Goyal *et al.*, 2003; Browne *et al.*, 2004; Wise and Tunnacliffe, 2004), pollen (Wolkers *et al.*, 2001), and bryophytes (Alpert and Oliver, 2002) and in the anhydrobiotic insect *P. vanderplanki* (Hinton, 1951). Genes encoding LEA proteins are particularly numerous and heterogenous in plant genomes, with *Arabidopsis thaliana* encoding 51 *lea* genes placed into 9 different Pfam domains (Bies-Etheve *et al.*, 2008; Hundertmark and Hincha, 2008). In animal genomes *lea* genes are less abundant and mainly belong to the Group 3 LEA family (Tunnacliffe and Wise, 2007). LEA proteins are, in general, highly resistant to heat denaturation and are highly hydrophilic (Dure, L. *et al.*, 1989; Russouw *et al.*, 1997). It is widely accepted that due to their extremely biased amino acid composition and from predicted 3D structures that these proteins are naturally unfolded and are not catalytic (Goyal *et al.*, 2003). Several hypotheses have been proposed concerning the function of LEAs, these include: hydration buffer, molecular chaperone, ion sink, membrane stabiliser, the participation in the formation of cytoplasmic glass and in the formation of a stabilizing microfibril lattice (Dure, 1993; Wolkers *et al.*, 2001; Wise and Tunnacliffe, 2004).

Following their initial discovery and characterization cotton LEA proteins were named with the prefix D- and the groups were simply numbered in order of discovery. As the number of LEA proteins discovered in other plants increased it became apparent that they could be classified according to the presence of conserved sequence motifs and their similarity to the original cotton seed LEA proteins (Dure *et al.*, 1989). More recently, Wise (2003) introduced a naming scheme based on amino acid composition. The majority of LEA proteins fall within groups 1, 2 and 3. However some unusual LEA proteins have since been discovered which cannot be placed within these groupings. Hence more comprehensive classification systems have been suggested containing 5, 6, or 7 groups (Ingram and Bartels, 1996; Garay-Arroyo *et al.*, 2000; Kim *et al.*, 2005). A bioinformatic analysis of these groups has led to the division of some of the larger groups into two parts (e.g. 3a and 3b) and the reallocation of some of the more unusual LEAs into other groups (Wise, 2003). Wise's data has also designated separate additional groups. More recently the LEA family has been split into superfamilies using the POPP (protein or oligonucleotide probability profile) bioinformatics program (Wise and Tunnacliffe, 2004). This superfamily system has allowed for Pfam groupings to be created and has also categorised the LEA proteins according to their predicted protein function.

1.5.5 Antioxidants

Oxygen is a highly oxidizing molecule which is important for its role in aerobic respiration. However, oxygen can have deleterious effects on the cell through the production or conversion into free radicals (Finkel and Holbrook, 2000), many of which are directly associated with degenerative processes and diseases (Marx, 1987; Skulachev, 2000; Kranner and Birtic, 2005). Oxygen radicals are extremely reactive as they contain unpaired electrons which are readily donated to other substance/atoms (Finkel and Holbrook, 2000). Oxygen radicals include singlet oxygen, superoxide anion, the hydroxyl radical and nitric oxide. It is believed that the mitochondria convert 1-2% of the oxygen consumed into superoxide anions alone (Boveris and Chance, 1973). These oxygen free radicals, along with hydrogen peroxide (also highly reactive but not a free radical), are referred to as reactive oxygen species (ROS) (Kranner and Birtic, 2005). ROS accumulate in cells as a result of cellular dehydration (Kranner and Birtic, 2005; Franca *et al.*, 2007). ROS cause oxidative damage to proteins, lipids, DNA and other macromolecules. Therefore proteins with antioxidant properties are required to rapidly neutralise ROS as soon as they are formed. During desiccation ROS formation is greatly enhanced. This effective antioxidant machinery is bound to be an essential trait of anhydrobiotes (Kranner and Birtic, 2005). In yeast a 10-fold increase in intracellular oxidation was observed after dehydration (Pereira *et al.*, 2003).

Free radical production appears to be linked to respiration in a process involving desiccation-induced impairment of the mitochondrial electron transport chain in plants (Leprince *et al.*, 1994) as well as in animals (Glasheen and Hand, 1989). This decrease in metabolism has also been correlated with an increase in survival during desiccation (Glasheen and Hand, 1989; Leprince *et al.*, 2000). Apart from affecting energy metabolism, dehydration causes other changes that can promote ROS. It reduces the cytoplasm volume and causes shrinkage of cells (which concentrates all cellular molecules), decreases intracellular transport, increases ionic strength and alters the pH of the cytoplasm (Senaratna *et al.*, 1987; Franca *et al.*, 2007).

1.5.6 Molecular chaperones

Water is important to the structure, stability, dynamics, and function of biological macromolecules, including proteins (Levy and Onuchic, 2006). Thus the desiccation of proteins frequently leads to their misfolding and aggregation, as demonstrated by Chakrabortee *et al.* (2007) for the soluble proteomes of the nematode *A. avenae* and two mammalian cell lines. Misfolded protein aggregates may cause cellular damage by exposing the flexible hydrophobic surfaces that can mediate aberrant interactions with other proteins. This results in their functional impairment and sequestration (Chiti and Dobson, 2006). In addition by engaging and depleting the chaperone machinery, protein aggregates may interfere with central protein quality control and clearance mechanisms (Balch *et al.*, 2008).

Heat shock proteins (HSPs) are essential for the correct folding and maturation of a great diversity of client proteins and for protecting proteins from stress-induced unfolding and aggregation (Morimoto, 2008; Richter *et al.*, 2010). Eukaryotic HSP families contain multiple genes, which may be either constitutively expressed or stress inducible and targeted to specific cellular compartments (Kabani and Martineau, 2008; Vos *et al.*, 2008). The HSP expression repertoire of an anhydrobiotic organism may thus be important in maintaining the integrity of the proteome during the dehydration and recovery phases of anhydrobiosis (Sales *et al.*, 2000; Jonsson and Schill, 2007; Cho and Choi, 2009; Hu *et al.*, 2010).

The HSP-70 family is the largest group of heat shock proteins, involved in inhibiting protein aggregation as well as promoting folding in newly synthesized and denatured proteins (Jonsson and Schill, 2007). As well as HSP-70, small heat shock proteins (sHSPs) seem to play an important role in the response to desiccation. Although sHSPs are one of the least understood classes of molecular chaperones, their importance is shown in the fact they are ubiquitously expressed, are dramatically up-regulated in response to stress and are implicated in a number of diseases. Small HSPs protect cells from protein losses and toxicity caused by aggregation (Sun and Macrae, 2005). sHSP are the major “holding” chaperones, retaining unfolding proteins in a conformation suitable for subsequent refolding, thus preventing their irreversible aggregation (Eyles and Gierasch, 2010; Stengel *et al.*, 2010). Anhydrobiotic encysted larvae of the brine shrimp

Artemia franciscana accumulate large quantities of an sHSP known as p26 which constitutes ~15% of the non-yolk protein in these larvae (Liang *et al.*, 1997). *A. franciscana* cysts are resistant to desiccation, high temperature, γ-irradiation and anoxia and the chaperoning activity of p26 is likely to be a very significant factor in this resistance (Sun *et al.*, 2006).

The unfolded protein response (UPR) is a transcription and translational intracellular pathway activated by the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER). This pathway may therefore be important in desiccated organisms when the number of denatured unfolded proteins increases as water content decreases. In *C. elegans*, as in other eukaryotic organisms, cells have three different mechanisms for dealing with an accumulation of unfolded proteins in the ER: transcriptional induction, translational attenuation, and degradation (Shen *et al.*, 2001). Activation of the UPR results in the expression of genes encoding catalysts and protein degrading complexes as well as proteins which augment the ER folding capacity (Shen *et al.*, 2001).

1.5.7 Removal of damaged proteins

The rate of protein misfolding is significantly increased by desiccation and eventually it may exceed the organismal chaperone capacities (Kubota, 2009). Essential cellular systems can be damaged by the accumulation of misfolded proteins which occurs once the cellular chaperone capacity is surpassed. Such misfolded proteins are degraded by either the autophagy or ubiquitin-proteasome systems. However, the major system to degrade proteins in the cytosol is the ubiquitin-proteasome system. This system involves the covalent attachment of ubiquitin to the target protein before degradation (Kubota, 2009). In desiccated nematodes the cellular chaperone capacity may be insufficient to protect misfolded proteins against aggregation. The nematodes would then come to rely on the ubiquitin-proteasome systems as well as autophagy systems to remove these damaged proteins.

Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation, development, and homeostasis. Autophagy is also activated as an adaptive catabolic process in response to different forms of stress, including desiccation (Lamark

and Johansen, 2010). A recent study by Ratnakumar *et al.* (2011) has revealed that autophagy plays a major role in the desiccation tolerance of *Saccharomyces cerevisiae*. Autophagy differs from the proteasomal degradation as it can degrade aggregates that are not monomeric and unfolded, as required by the proteasome. If the production of misfolded proteins exceeds the capacity of the cellular degradation systems, misfolded monomers and small soluble aggregates are actively collected into large aggregates that eventually are degraded by autophagy (Lamark and Johansen, 2010). The knockdown of autophagy in *C. elegans* results in an increase in aggregate formation and toxicity of polyQ expansion proteins (Jia *et al.*, 2007). This is strong evidence for the important role that autophagy plays in the degradation of misfolded proteins. The amino acids released by autophagy are most likely used for the *de novo* synthesis of proteins that could be essential for stress adaptation (Levine and Kroemer, 2008). Autophagy is also responsible for life-extending effects of dietary restriction in *C. elegans*. Furthermore, autophagy may also have a role in limiting DNA damage and chromosomal instability, as has been shown in immortalized epithelial cells that are ATG gene-deficient (Mathew *et al.*, 2007).

1.5.8 DNA repair

In metabolically active cells, DNA is believed to exist in the fully hydrated B form and to have properties similar to those measured for DNA in solution. Therefore under desiccation stress, the chemical stability of DNA is postulated to be a major determinant of cell survival (Potts, 1994; Potts *et al.*, 2005). Browning reactions, metal-catalyzed Haber-Weiss and Fenton reactions, reactive oxygen species and free radicals contribute to DNA modification and ultimately to the killing of cells (Potts, 1994). Various repair mechanisms are conserved across different organisms to repair DNA damage (Dizdaroglu, 2012). The major mechanisms used by organisms to repair DNA damage are base-excision repair (BER), nucleotide-excision repair (NER) and repair of double strand breaks (DSBs).

BER is the main process involved in the recognition and repair of single-base DNA damage. This type of damage can occur spontaneously, or be induced by ROS (Fortini *et al.*, 2003; Escargueil *et al.*, 2008) which are known to accumulate during desiccation. Single base DNA lesions do not cause sufficiently significant distortion of the DNA to stall transcription machineries but can be highly mutagenic (Girard and Boiteux, 1997).

The NER pathway is capable of eliminating a wide variety of DNA lesions caused by UV irradiation or toxins (Reardon and Sancar, 2005). It recognizes chemical modifications of the DNA that are usually accompanied by significant helical distortion (Hoeijmakers, 2001; Fortini *et al.*, 2003).

DSBs are thought to be the most harmful type of DNA lesions. They can be caused by environmental stresses such as desiccation as well as by chemicals or the stalling of the DNA replication fork. In eukaryotes such as nematodes, DSBs are repaired either by homologous recombination (HR) or by non-homologous end-joining (NHEJ). NHEJ directly ligates broken DNA ends while HR uses the genetic sequence of the undamaged sister chromatid or chromosomal homologue to repair damaged DNA (Fortini *et al.*, 2003). As well as the mechanisms mentioned above DNA histone modification and cell cycle events also have a role to play in DNA repair (Fortini *et al.*, 2003; Sonoda *et al.*, 2006).

Studies in the anhydrobiotic chironomid *P. vanderplanki* (Gusev *et al.*, 2010) and anhydrobiotic tardigrades (Neumann *et al.*, 2009; Rebecchi *et al.*, 2009) show that DSBs accumulate with time in the desiccated state in these organisms. Additionally, DSBs also accumulate during desiccation in the anhydrobiotic and radiation resistant bacterium *Deinococcus radiodurans*. Similar to *P. vanderplanki* (Gusev *et al.*, 2010) and anhydrobiotic tardigrades (Neumann *et al.*, 2009; Rebecchi *et al.*, 2009), *D. radiodurans* has acquired the ability to rapidly repair DNA damage when rehydrated (Mattimore and Battista, 1996).

1.6 Systems biology and its application to studies in anhydrobiosis

Systems biology can be defined as the study of phenomena in terms of how biological objects are related rather than what they are composed of. The principle challenge facing systems biology is the complexity of living organisms and their ecosystems. At the heart of this challenge is the need for a new approach, a shift from reductionism to a holistic perspective (Mesarovic *et al.*, 2004). Systems biology has been revolutionised by modern genomics and proteomics technologies, as well as by computer-assisted theoretical and modelling biology. These combined methodologies allow us to obtain a holistic overview of the regulatory processes and reactions of an organism in response to environmental

changes such as desiccation by studying the roles of different molecular components (i.e. genes and proteins) and their complex interactions (Fukushima *et al.*, 2009; Weckwerth, 2011; Jogaiah *et al.*, 2013). The recent shift from a reductionist to a more holistic approach was born out of necessity in this post-genome era. For example it is important to understand not only how many genes are involved in a process but also how they interact to result in the observed behaviour of the overall system (Mesarovic *et al.*, 2004). The proteomic and transcriptomic techniques used in this project were only recently developed and already have had a major impact.

A recent example of systems biology being successfully employed is in a study by Ratnakumar *et al.* (2011) where two complementary genome-wide approaches, phenomics and transcriptomics, were used to define genes involved in desiccation tolerance in *Saccharomyces cerevisiae*. Both methods implicated similar processes including autophagy pathways in conferring desiccation tolerance in *S. cerevisiae*.

In this study we used RNA-seq and proteomic technologies to identify genes and proteins involved in desiccation tolerance in the anhydrobiotes *P. superbus* and *C. elegans* dauer larvae. Focusing on these desiccation responsive genes and proteins we subsequently used RNAi gene silencing to further investigate their involvement in desiccation tolerance in *C. elegans* dauer larvae. The techniques RNA-seq, differential proteomics and RNAi gene silencing were used in a systems biology context and are further discussed below.

1.6.1 RNA-seq

The study of transcriptomics is essential for discovering the functional elements of the genome as well as understanding organismal development disease states and stress responses (Wang *et al.*, 2009). The introduction of high-throughput next-generation sequencing (NGS) technologies has revolutionized transcriptomics by allowing RNA analysis through cDNA sequencing on a massive scale. The major commercial pioneers of the NGS platforms are Roche (454) (Margulies *et al.*, 2005) and Illumina (Bentley *et al.*, 2008). Prior to NGS, automated Sanger sequencing dominated the industry for almost two decades. Automated Sanger sequencing is now referred to as ‘first generation’ technology, while newer sequencing methods are described as NGS. The major difference

between NGS and the earlier described Sanger method is the ability of NGS technologies to produce large amount of data cheaply (Metzker, 2010).

The characterization and quantification of an organism's transcriptome utilizes either hybridization or sequence based approaches (Wang *et al.*, 2009). Before the development of RNA-seq technologies, Sanger sequencing approaches had been adapted for gene expression studies, with the development of techniques such as Serial Analysis of Gene Expression (SAGE) and Massively Parallel Signature Sequencing (MPSS) (Wang *et al.*, 2009). The SAGE method of gene expression has such drawbacks as being laborious, expensive and not particularly sensitive (Lockhart *et al.*, 1996). Hybridization based techniques, such as microarrays, measure the signal intensity for each probe on the array (Lockhart *et al.*, 1996). Microarray technologies were a major breakthrough in providing a systems biology approach to transcriptomics studies but when compared to more recent RNA-seq technologies they have several drawbacks which include: reliance upon existing knowledge about genome and transcriptome sequences; high background levels caused by cross-hybridization; and a limited dynamic range of detection owing to both background noise and saturation of signals (Cheng *et al.*, 2005; Okoniewski and Miller, 2006; Wang *et al.*, 2009). Additionally, comparing expression levels across different experiments is often difficult and can require complicated normalisation methods (Wang *et al.*, 2009).

RNA-seq protocols have many advantages over hybridization and older sequence based approaches. RNA-seq has very low background noise unlike other techniques such as DNA microarrays. This is because DNA sequences can be unambiguously mapped to unique regions of the genome. In RNA-seq the number of transcripts which are present in an experimental sample is counted, thus the dynamic range is unlimited and saturation effects do not occur. In addition, qPCR experiments have shown expression values produced by RNA-seq to be highly accurate (Wang *et al.*, 2009).

The method by which each of the NGS platforms produces RNA-seq data are different, but they all have similar work-flows for the preparation of sequencing libraries. Briefly, the RNA sample is initially fragmented to reduce possible secondary structures and to generate fragment sizes which are compatible with the sequencing system to be used. The RNA is converted into double stranded cDNA and ligated to unique adaptors for

sequencing and amplification. The adaptors will allow the cDNA fragments to be singled out, either on beads (as in the Roche/454system) or on a slide (as in the Illumina system) to be sequenced in parallel.

The NGS platforms each use different sequencing chemistry and methodological procedures for RNA-seq. The sequencing method employed by the Illumina platform (Costa *et al.*, 2010) will be described, as it is was used in this study. The prepared cDNA library is placed on one of the lanes of a flow-cell (slide). Individual cDNA fragments attach onto the surface of the lane (used to separate different samples) and undergo an amplification step where they are converted into clusters of double stranded cDNA. The flow-cell is placed in the sequencing machine and each cluster is sequenced in parallel. Illumina uses the Cyclic Reversible Termination (CRT) method of sequencing, meaning that it uses reversible terminators in a cyclic manner. A DNA polymerase bound to the primed template adds one fluorescently modified nucleotide per cycle. The remaining unincorporated nucleotides are washed away and image capturing is performed. A cleavage step occurs before the next cycle to remove the terminating group and the fluorescent dye; this is then followed by another washing step. For each flow-cell this process is repeated for a given number of cycles. The fluorescence intensities are converted into base calls. The number of cycles the sequencer is capable of completing determines the length of the reads and the number of clusters determines the number of reads. With the Illumina sequencing method substitutions are the most common error type and there may also be an underrepresentation of AT-rich and GC-rich regions (Dohm *et al.*, 2008).

When the reads have been obtained from the sequencer they are generally assessed for quality before they are mapped to a reference genome. There are several programs for mapping reads to the reference genome or transcriptome, including Bowtie, SOAP2, MAQ, BWA, Mosaik, Novoalign and ZOOM reviewed by (Trapnell and Salzberg, 2009) (2009). In this study Bowtie was used to perform the alignments (Langmead *et al.*, 2009). NGS sequencers can produce billions of reads in one run, which makes mapping the reads to a reference genome or transcriptome using traditional alignment algorithms very computationally expensive. The Bowtie and Tophat programs use a computational strategy known as “indexing” to speed up the mapping process. Like the index in a book,

an index of the large reference sequences allows shorter sequences to be found within it rapidly. This approach involves the use of a technique known as the Burrows-Wheeler transform that was originally developed for compressing large files (Burrows and Wheeler, 1994). This memory-efficient data structure allows the Bowtie program to scan reads in a very fast and efficient manner on a standard computer. The alignment of the RNA sequence reads to the reference genome or transcriptome results in three categories of sequence: those that map uniquely to the reference, those that map to multiple regions in the reference genome or transcriptome and those that do not map to the reference. Reads that map to multiple locations are typically removed, as the precise region they were sequenced from cannot be determined. The use of paired-end protocols reduces this problem.

1.6.2 Differential quantitative proteomics

Proteomics was largely a qualitative discipline until a decade ago (Schulze and Usadel, 2010). In the past, proteomic experiments typically identified proteins in a tissue or protein complex without information about abundance, distribution, or stoichiometry (Schulze and Usadel, 2010). Nowadays, quantitative measurements are at the heart of practically every proteomic study (Bantscheff *et al.*, 2012). Investigating living systems at the protein level is continuing to provide important insights into many biological processes. The increased technical advances in proteomics have resulted in the growth to many diverse fields. This technology also allows the massive multiplexing of primary data acquisition with better quality than established quantitative methods such as western blots. Furthermore, MS based proteomics is challenging traditional hypothesis-driven research in biology where researchers focused on one protein at a time. Similarly, LC-MS methods are challenging traditional assays such as ELISAs in protein biomarker discovery (Whiteaker *et al.*, 2011).

The major commercial pioneers of the mass spectrometry platforms today are orbital traps, QTOF (Quadrupole Time-of-Flight) instruments, and triple-quadrupole instruments, mostly using electrospray ionization as an interface for chromatographic systems along with collision-induced dissociation as the peptide fragmentation technique. Generally LC separations using reverse-phase chromatography systems are directly coupled to the mass spectrometer. In the experiments described in this thesis, the Thermo Scientific Q

Exactive hybrid quadrupole-orbitrap mass spectrometer was used for protein identification and quantification. The orbitrap has proven to be a robust analyzer that can routinely deliver high resolving power and mass accuracy. Differing from other mass spectrometers such as ion traps, the orbitrap uses only electrostatic fields to confine and analyse injected ion populations (Perry *et al.*, 2008).

The new mass spectrometer technologies allow label-free quantitative methods which are able to quantify peptides and proteins without the use of stable-isotope labels. Label free approaches have the advantages of being inexpensive and yield high proteome coverage because every protein that is identified by one or more spectra can be quantified (Schulze and Usadel, 2010). Furthermore, label-free based mass spectrometry results in high analytical depth and dynamic range (Old *et al.*, 2005). Therefore this method suits experiments in which global proteome changes (i.e. large numbers of proteins) are expected as may perhaps occur in response to desiccation in anhydrobiotic nematodes.

There are two main label free quantitative methods: spectrum count and intensity based approaches. Spectral count approaches estimate quantity indirectly by comparing the number of peptide-to-spectrum matches for each protein (Liu *et al.*, 2004). Several factors affect spectral count approaches including the number of peptide-to-spectrum matches, the number of distinct peptides identified, and the sequence coverage obtained for a protein of interest (Liu *et al.*, 2004). The spectral count method suffers from a number of drawbacks: the relative quantification of very short proteins tends to be more variable than that for an equal amount of larger proteins and the dynamic range of detectable protein expression is affected by saturation (Bantscheff *et al.*, 2012). Intensity-based label-free quantification uses the MS signal response of intact peptides and proteins for quantification. The height or volume of a peak with a given mass-to-charge ratio (*m/z*) is a measure of the number of the ions of that particular mass detected within a given time interval (Schulze and Usadel, 2010). Studies by Bondarenko *et al.* (2002) have shown that the signal response is linear over four orders of magnitude and has excellent correlation with the concentration of measured peptides. Differences in protein abundances are measured by the sum of the differences measured for all peptides matching the protein of interest. In the experiments described in this thesis an intensity-based label-free quantification approach was used, given the clear advantages it offers.

One advantage of proteomic over RNA-seq based analyses relates to the poor correlation between mRNA and protein abundance (reviewed by Hegde *et al.*, 2003; Abreu *et al.*, 2009). In addition posttranslational modifications are not captured by the analysis of transcript abundances (Schulze and Usadel, 2010). In mammalian hematopoetic and liver cells (Tian *et al.*, 2004) only 40% of the variation in protein expression could be attributed to differential expression of mRNA whereas data from a more recent study in three functionally different human cell lines concluded that "transcript changes between cell lines are accompanied with similar changes on the protein level" (Lundberg *et al.*, 2010). Other recent studies have also reported stronger relationships between mRNA abundance and protein abundance (e.g. Lee *et al.*, 2011; Lackner *et al.*, 2012). Some of the reported discrepancies between transcriptomes and proteomics in earlier studies may have arisen because of technical problems in accurately quantifying the expression of some mRNA transcripts using microarrays and because of limitations in the efficiency of MS-based proteomics systems available at that time. Nevertheless variations in translational control between different mRNAs do contribute to the poor correlations which are often observed between the transcriptome and the proteome, particularly during early development and in response to cellular stress (Abreu *et al.*, 2009). Since proteins are the entities that ultimately perform the cellular processes it is important to utilise proteomic techniques to gain a deeper understanding of processes such as anhydrobiosis. As Hoog and Mann (2004) point out "biological function is not carried out by the static genome but mainly by the dynamic population of proteins determined by an interplay of gene and protein regulation with extracellular influences". This sums up why both transcriptomic and proteomic methods are required to study diverse biological processes, including desiccation tolerance and anhydrobiosis.

1.6.3 RNAi gene silencing

C. elegans is a suitable model organism for studying gene function using RNAi because of its genetic simplicity (Kamath and Ahringer, 2003). The potency of RNAi is shown in the fact that exposure of a *C. elegans* adult to only a few molecules of dsRNA per cell results in a systematic spreading of the RNAi effect (Fire *et al.*, 1998; Hannon, 2002). dsRNA can be introduced by injection, soaking or feeding in *C. elegans* (Kamath and Ahringer, 2003). RNAi by feeding is regularly used because it is less labour-intensive

than the other two methods and a large number of worms can be screened in a single experiment. Nematodes are fed bacteria expressing dsRNA identical in sequence to that of the target gene of interest, thus silencing the gene. The RNAi system may have developed in organisms as a defence mechanism against dsRNA viruses and transposons (Buchon and Vaury, 2006). The systematic effect of RNAi would be appropriate for a role in antiviral defence. Furthermore, silencing of genes involved in the RNAi mechanism in nematodes causes the activation of multiple transposable elements lending further support to the hypothesis that their function is to stop the spreading of transposons within the genome (Plasterk, 2002). RNAi mechanisms have also been implicated in the regulation of gene expression. It was discovered in plants that small endogenous microRNAs (ca. 22 nucleotides long) have a role in gene expression by guiding the cleavage of sequence-complementary mRNAs (Bartel, 2004; Meister and Tuschl, 2004).

When dsRNA is introduced into *C. elegans* by feeding, injection or soaking it is processed by the RNase III enzyme Dicer into RNA duplexes of about 21 nucleotides in length (Figure 1.6). In *C. elegans* the Dicer protein, DCR-1, cooperates with the dsRNA-binding domains protein RDE-4 to unwind the short dsRNAs into short interfering RNA (siRNA). The siRNAs are subsequently rearranged into the RNA-induced silencing complex (RISC) which contains a member of the Argonaute protein family. The Argonaute protein binds directly with the siRNA in these complexes, while the conserved PIWI domain is thought to interact with Dicer (Meister and Tuschl, 2004). Argonaute proteins may have evolved an intrinsic sequence-specificity that allows them to bind preferentially to small RNAs of specific sequence. The single-stranded siRNA in RISC guides sequence-specific degradation of complementary or near-complementary target mRNAs. The RNAi effect can also be amplified by cellular RNA-directed RNA polymerase (RdRP) on mRNAs that are being targeted by the RNAi mechanism (Sijen *et al.,* 2001). This combined with the amplification derived from the cleavage of dsRNA into siRNAs results in a very potent effect.

The discovery of RNAi knockdown in *C. elegans* led to testing of other organisms to see if they are amenable to RNAi. This led to the expansion of the RNAi silencing tool to *Drosophila* cell lines (Caplen *et al.,* 2000). However, it had not been possible to detect potent and specific RNA interference in mammalian cell culture systems using full length

dsRNAi molecules. The introduction of dsRNA in the cytoplasm of mammalian cell triggered physiological reactions that lead to the induction of interferon synthesis that caused the death of the treated cells. To combat this, it was discovered that 21- nucleotide siRNA duplexes can be used for efficient sequence specific mRNA degradation in mammalian cell lines (Elbashir *et al.*, 2001).

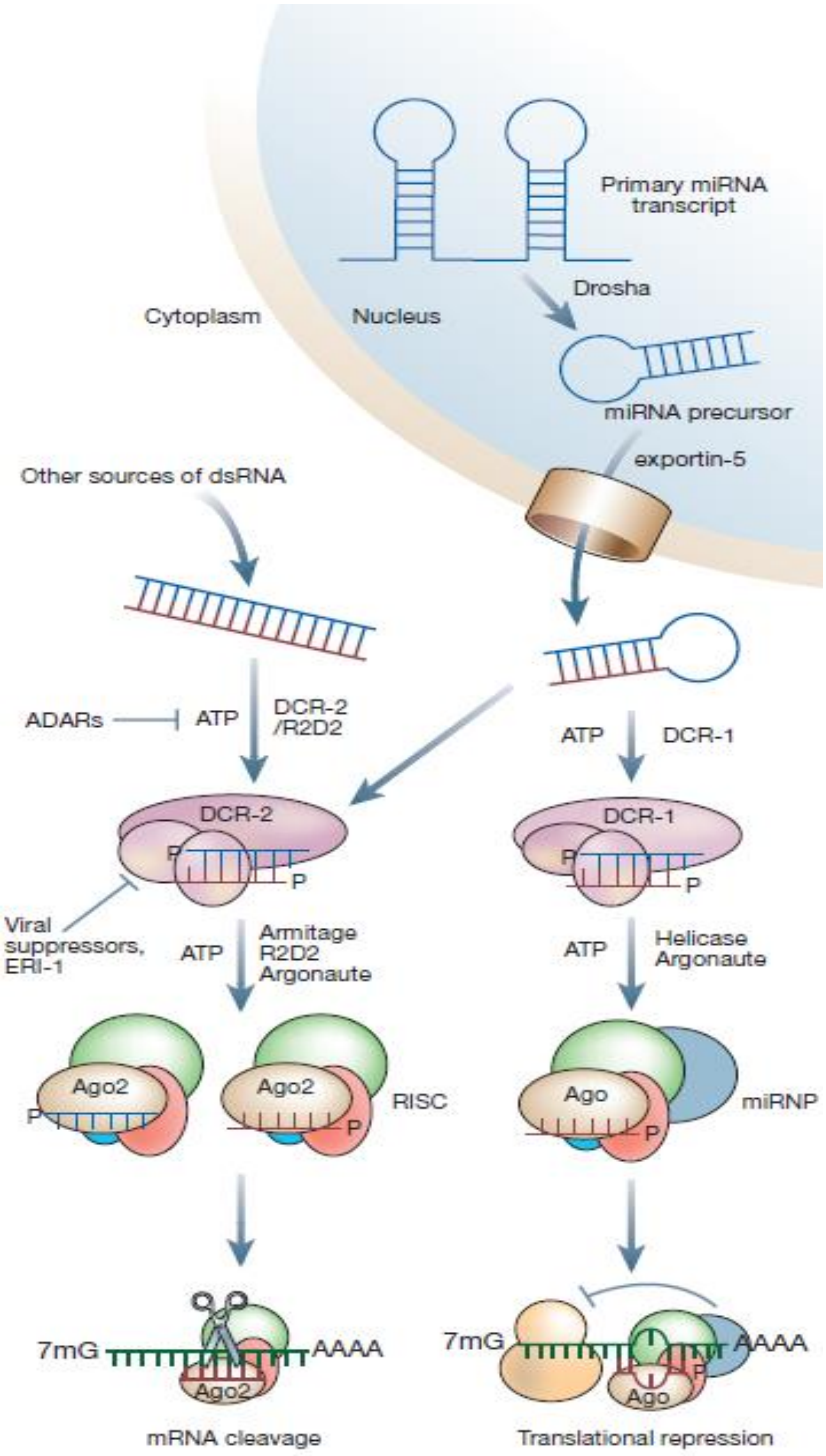


Figure 1.6 Model of small-RNA-guided post-transcriptional regulation of gene expression. Long dsRNA is processed by the RNase III enzyme Dicer into 21–23 nucleotide dsRNA intermediates. Assisted by the RNA helicase Armitage and R2D2, the single-stranded siRNA-containing RISC is formed. The source of this illustration is Meister and Tuschl (2004).

1.7 Objectives of this research project

The main objective of this project was to obtain a better understanding of the molecular mechanisms used by *P. superbus* (all life stages) and *C. elegans* dauer larvae to survive severe desiccation. Both *P. superbus* and *C. elegans* dauer larvae have contrasting strategies for surviving desiccation perhaps defined by the habitats in which they evolved. The nematode *Panagrolaimus superbus* was originally isolated in 1981 from a gull's nest in Surtsey, Iceland (Bostrom, 1988). The family Panagrolaimidae contains nematodes which are variously resistant to diverse stresses including desiccation, freezing and high temperatures (Wharton and Brown, 1991; Shannon *et al.*, 2005; Borgonie *et al.*, 2011). Since Surtsey island was formed by volcanic eruptions during the 1970s *P. superbus* nematodes are presumed to have transported there in an anhydrobiotic state. On Surtsey island, *P. superbus* is subjected to a frequent cycle of freeze-thaw action and desiccation. Their rapid dehydrating, anhydrobiotic phenotype, as well as their cold tolerance, allows them to adapt to the harsh environmental conditions on the Island. *C. elegans* adults inhabit rotting fruit, a stable environment, less susceptible to desiccation. However, *C. elegans* does possess a stress resistant dauer larval stage in which the larvae are able to survive desiccation (their ability to survive extreme desiccation is much less than that of *P. superbus*, as discussed in Chapter V). The desiccation survival strategies used by *C. elegans* dauer larvae and *P. superbus* are analysed in this thesis using RNA seq and proteomics technologies.

As this research project developed, its specific objectives became focused on the following:

1. To investigate the genes differentially expressed in response to desiccation and recovery from desiccation in *P. superbus*.
2. To investigate the proteins differentially expressed in response to desiccation and recovery from desiccation in *P. superbus*.
3. To investigate the genes differentially expressed in response to desiccation and recovery from desiccation in *C. elegans* dauer larvae.
4. To investigate the proteins differentially expressed in response to desiccation and recovery from desiccation in *C. elegans* dauer larvae.

5. To use RNAi gene silencing in *C. elegans* dauer larvae to further investigate desiccation tolerance.

6. The identification of the similarities and differences that underlie the desiccation tolerance strategies of by *P. superbus* (larvae and adults) and *C. elegans* dauer larvae.

2 Chapter II – Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals were obtained from the following companies: Amershan Bioscience/GE heathcare (Little Chalfont, UK); Sigma-Aldrich Co. Ltd. (Gillingham, UK); Thermo Fischer Scientific Ltd. (Massachusetts, US); Novagen – Division of Merck/EMD (Wisconsin, US); BioRad Laboratories Ltd. (Hemel Hempstead, UK); Invitrogen Ltd. (Paisley, UK); Gibco BRL – Division of Invitrogen Ltd.; Promega UK Ltd., (Southampton, UK); Amersham Biosciences – Division of GE Healthcare (Buckinghamshire, UK); Bioline Ltd., (London, UK); Millipore (Carrigtwohill, IRE); Molecular BioProducts Inc.- Division of Thermo Fischer Scientific Ltd (California, US); Fermentas (Maryland, US); or Pierce – Division of Thermo Fischer Scientific Ltd., (Cramlington, UK). Enzymes were purchased from New England Biolabs (NEB) (Beverly, MA, US), Roche (Clarecastle, IRE), Promega or Novagen. Oligonucleotide primers were purchased from Eurofins MWG Operon (Ebersberg, DE). Sterile plastic ware was purchased from Sartorius AG (Goettingen, DE). Molecular biology kits were purchased from Qiagen (Cologne, Germany).

2.1.2 Nematode and bacterial strains

Panagrolaimus superbus used in this study was maintained in the Nematode Genetics strain collection at NUI Maynooth. *P. superbus* was isolated from a gull's nest Surtsey island, Iceland (Bostrom, 1988) and the NUIM culture was obtained from Prof. Bjorn Sohlenius, Swedish Natural History Museum. *Caenorhabditis elegans* strain N2, *C. elegans* strain *daf-2* (e1370) III, *C. elegans* strain *eri-1* and *C. elegans* strain *rrf-3* were also obtained from the Caenorhabditis Genetics Center (CGC). *E. coli* strain HB101 (streptomycin resistant) was used for nematode feeding. The *E. coli* strain HT115 (DE3) transformed with L4440 vector was used for RNAi experiments. The genotype for HT115 (DE3) is as follows: F-, mcrA, mcrB, IN(rrnD-rrnE)1, lambda -, rnc14::Tn10 (DE3 lysogen: lavUV5 promoter –T7 polymerase) (IPTG-inducible T7 polymerase) (RNase III minus).

2.1.3 Plasmids

The L4440 feeding vector was used as a basis for cloning PCR products. This vector contains T7 promoter sites flanking each side of the multiple cloning site (MCS). After a gene-specific DNA fragment is cloned into this vector, dsRNA can be produced in bacteria by transcription with T7 polymerase. Plasmids were transformed into bacterial strain HT115 (DE3), an RNase III-deficient strain of *E. coli* in which expression of T7 RNA polymerase is induced by addition of isopropyl-ß-D thiogalactopyranoside (IPTG). The RNase III deficiency improves the efficacy of RNAi by feeding (Kamath and Ahringer, 2003).

2.2 Culturing nematodes and bacteria

2.2.1 Culturing *Panagrolaimus superbus* and *Caenorhabditis elegans*

Nematode growth medium (NGM) plates were prepared by dissolving 3 g NaCl, 2.5 g peptone, and 17 g agar in 970 ml of H_2O. The solution was autoclaved and upon cooling 1 ml cholesterol in ethanol (5 mg/ml), 25 ml 1 M potassium phosphate buffer pH 6.0, 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$ and streptomycin to a final concentration of 10 µg/ml was added. The plates were poured and left to set. Upon setting 40 µl of an *E. coli* strain HB101 liquid culture was spread on the plates and the plates were incubated overnight at 37^{o}C. A 1 cm^2 chunk of agar from an established NGM nematode culture was transferred onto the plates. The plates were cultured in the dark at 22^{o}C for approximately 8 days. The nematodes were regularly subcultured (~every 8 days) using 1x1cm agar squares.

2.2.2 Harvesting *P. superbus* and *C. elegans*

Nematodes were rinsed off the NGM plates with sterile H_2O. The plates were left to gently agitate on a shaker for 30 min in order to digest any bacteria in the nematode gut. The liquid was then poured into sterile 50 ml Falcon tubes and left to settle at RT for 10 min. The supernatant was then removed and fresh sterile H_2O was added. This settling process was repeated a total of three times in order to obtain bacteria free nematodes. When harvesting nematodes for protein or RNA extraction the pellet was resupended in either protein lysis buffer (Section 2.4.1) or Trizol Reagent® (Invitrogen) (Section 2.3.1). The samples were snap frozen in liquid nitrogen prior to storage at -80^{o}C. For stress experiments the nematodes were resuspended in a suitable volume of H_2O. The number of nematodes per ml was then estimated by counting the number of nematodes in 25 µl (10

replicates). The volume of the nematodes suspended in H_2O was adjusted to give the required nematode density for each experiment (normally 2,000 nematodes /ml).

2.2.3 Cleaning nematode stocks

Harvested nematodes were brought to a final volume of 30 ml with S Basal buffer [100 mM NaCl, 5 mM K_2HPO_4, 50 mM KH_2PO_4]. The nematodes were treated with a freshly prepared solution comprising 8 ml 1:3 NaOCl solution and 2 ml 10 M NaOH. This solution was shaken vigorously for 4 min to disintegrate the adult nematodes and to allow the release of their eggs. Released eggs were pelleted by centrifugation (2,000 x *g* for 2 min). The supernatant was removed and fresh sterile S Basal buffer was added. This process was repeated a total of three times to obtain sterile NaOCl/NaOH/bacteria free eggs. To evaluate the quality of the egg isolation procedure and to estimate the number of eggs recovered, aliquots of the eggs were examined under the microscope. The remaining eggs were resuspended in S Basal buffer to a final volume of approximately 4,000 eggs per ml. The eggs were then pipetted (50 µl) onto fresh NGM plates to start new sterile cultures.

2.2.4 Desiccation stress experiments

2.2.4.1 *P. superbus*

Nematodes were harvested (Section 2.2.2) and adjusted to a concentration of approximately 2,000 nematodes per ml. Control samples were pelleted and flash frozen and stored at -80^{o}C in either Trizol or protein lysis buffer. One ml of the worm suspension was vacuum filtered onto a Supor®-450 filter membrane disc (Pall, Life Sciences, Port Washington, NY, USA), transferred to 3 cm Petri dishes without lids and placed in a 10.0 L desiccation chamber containing approximately 300 ml of saturated potassium dichromate ($K_2Cr_2O_7$) which maintained a relative humidity (RH) of 98% for either 24 or 48 h (Winston and Bates, 1960). Humidity indicator cards (Sigma, Z163457-10EA) were used to ensure the appropriate humidity was reached in the desiccation chambers. The nematodes were washed off the filters with distilled H_2O at the specified time-points and were transferred to a 15 ml Falcon tube. The nematodes were then pelleted by centrifugation at 5,000 g for 3 min. The nematode pellets were immediately flash frozen in either 1 ml of Trizol or protein lysis buffer and stored at -80^{o}C. After this preconditioning period the remaining nematodes were transferred to a desiccation chamber containing freshly activated silica gel for 24 h. Following this desiccation period

the nematodes were rehydrated in distilled water and allowed to recover for 120 min at 20^{o}C with shaking at 50 RPM. These samples were pelleted and flash frozen in either Trizol or protein lysis buffer and stored at -80^{o}C. Percentage survival was assessed by microscopic observation of movement and a minimum of five hundred nematodes were counted. Three biological replicates were prepared for each time point.

2.2.4.2 *C. elegans daf-2* (e1370) mutants

C. elegans daf-2 (e1370) mutants are conditional temperature sensitive dauer constitutive nematodes. These worms propagate normally at 15 ºC but when grown at 25 ºC they form dauer larvae (Kenyon *et al.*, 1993). Eggs were harvested from *daf-2* (e1370) mutants grown at 15 ºC as outlined in Section 2.2.3. These eggs were placed on fresh NGM plates containing a lawn of bacteria and were incubated at 25 ºC. At this temperature, *C. elegans daf-2* (e1370) larvae enter the dauer developmental programme. After 3-4 days, an aliquot of the nematodes was treated with 1% SDS solution for 1 h with shaking at 50 RPM (Cassada and Russell, 1975). The percentage survival was then calculated upon microscopic observation of movement. This test was carried out, before harvesting these *daf-2* (e1370) nematodes from 25 ºC cultures, to confirm all the nematodes had reached the dauer stage. Control samples were flash frozen in Trizol or protein lysis buffer and stored -80^{o}C at this point. The dauers were washed off, pelleted and placed onto 3 cm Petri dishes in droplets. The Petri dishes, without lids, were placed in a 10.0 L desiccation chamber containing approximately 300 ml of saturated potassium dichromate ($K_2Cr_2O_7$) which maintained a relative humidity (RH) of 98% (Winston and Bates, 1960). They were monitored until the water droplet had evaporated (≈16 h). Humidity indicator cards (Sigma, Z163457-10EA) were used to ensure the appropriate humidity was reached in the desiccation chambers. The Petri dishes were left in the desiccation chamber for an additional for 48 h. These worms were washed off with Trizol or protein lysis buffer (as described in Section 2.2.4.1), flash frozen and stored -80^{o}C. The remaining plates were left in the desiccation chamber set at 98% RH for 96 h after the droplet evaporated and then transferred to a desiccation chamber set at 32.5% RH (using a saturated solution of magnesium chloride) (Winston and Bates, 1960) for 24 h. Samples were washed off with Trizol or protein lysis buffer and flash frozen as before. The final batch of dauers was treated as before (98% RH for 96 h followed by 32.5% RH for 24 h) followed by rehydration in distilled H_2O for 30 min with shaking at 50 RPM. The nematodes were transferred to a 15 ml falcon tube and pelleted by centrifugation at 5,000 g for 3 min. The

nematode pellets were immediately flash frozen in either 1 ml of Trizol or protein lysis buffer and stored at -80^{o}C. A survival assay was carried out on an aliquot to ensure reproducibility.

2.3 General molecular methods

2.3.1 RNA extraction

Nematodes were cultured and harvested as described in Section 2.2.4. Pelleted nematodes (~100 µl) were resuspended in 500 µl of Trizol® reagent (Invitrogen) and snap frozen in liquid nitrogen and were then stored at –80^{o}C until required. All areas used for RNA preparation, as well as the pipettes, were thoroughly cleaned with EtOH and RNase Zap solution (Invitrogen) before usage. Sterile filtered tips (Molecular BioProducts Inc.) and certified DNase and RNase free Eppendorf tubes were used for all RNA work. Tubes containing frozen nematodes were defrosted as required at room temperature and the nematodes ground into a fine powder (≥3 times) using liquid nitrogen in a cooled pestle and mortar, which had been baked O/N 200^{o}C. The homogenised material was incubated at room temperature (RT) for 10 min. Two hundred µl of chloroform was then added, shaken vigorously for 10 sec and incubated at RT for 15 min. The tubes were then spun at 12,000 x *g* for 15 min at 4^{o}C in a microcentrifuge (Eppendorf). The aqueous phase containing the RNA was removed to a new tube and the RNA precipitated with 500 µl of isopropyl alcohol. The samples were incubated at RT for 15 min and centrifuged for 10 min as previously described. The supernatant was removed and the pellet resuspended in 1 ml 75% EtOH and centrifuged at 7,500 x *g* for 5 min. The supernatant was then removed and the pellet air-dried. The pellet was resuspended in DEPC treated H_2O (Invitrogen). All RNA was treated with RQI DNase (Promega) according to the manufacturer's protocol. The solution was then extracted with 1 vol of phenol:chloroform:isoamyl alcohol (24:24:1) at room temperature for 10 min to remove protein contaminants and any salts left by RQI DNase reaction buffer. The tubes were spun in a cooled microcentrifuge (Eppendorf) at 4 ^{o}C at 12,000 x *g* for 10 min. The aqueous layer was then transferred to a new tube and 1/30th vol of 3 M sodium acetate and 2.5 vol of ice-cold 100% ethanol were added. The solution was stored at -20^{o}C for 30 min. The RNA was pelleted by centrifugation at 12000 x *g* for 20 min at 4^{o}C. The supernatant was removed and the pellet washed with 1 ml of 75% EtOH and spun for 5 min at 7,500 x *g*. The supernatant was removed and the pellet air-dried. The pellet was

then resuspended in 30 µl of DEPC treated H_20 and the RNA concentration and integrity were determined as described in Section 2.3.2, 2.3.3 and 2.3.4. The integrity of RNA used for RNA-seq experiments was determined using the Agilent Bioanalyzer according to manufacturer's instructions.

2.3.2 Determination of DNA/RNA concentration

DNA/RNA concentration and purity were determined by reading the absorbance of the sample at 260 and 280 nm using a Nanodrop 2000 spectrophotometer (Thermo Fischer Scientific) or by visual comparison to standards of known concentration on ethidium bromide stained gels. RNA for use in qPCR experiments was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fischer Scientific).

2.3.3 Agarose Electrophoresis

Electrophoresis was carried out using 0.7-1% agarose gels (depending on the degree of separation required) in 1X Tris Acetate EDTA buffer (TAE): 40 mM Tris, 20 mM acetic acid and 2 mM EDTA, pH 8.1. Agarose powder was dissolved by heating in 1X TAE buffer. When RNA was to be electrophoresed all buffers were made using DEPC treated H_2O. Upon cooling to 60^oC ethidium bromide was added (10 mg/ml). The solution was then poured into a casting tray and allowed to solidify. Samples were mixed with loading buffer [5 mg/ml bromophenol blue, 5 mg/ml xylene cyanol, 50% glycerol] at a ratio of 5:1. Six µl of 1 kb or 100 bp bench top DNA ladders (Promega) and HyperLadder I (Bioline) was loaded on each gel. Gels were typically run at 100 V using BioRad electrophoresis equipment.

2.3.4 Visualizing DNA and RNA on agarose gels

The ethidium bromide stained DNA or RNA gels were visualized under UV light using a UV transilluminator at 365 nm. Gels were photographed using an AlphaImager® gel documentation system (Proteinsimple, Santa Clara Ca. USA).

2.3.5 cDNA synthesis

cDNA for qPCR experiments was synthesized using a Transcriptor High Fidelity cDNA synthase kit (Roche) according to the manufacturer's instructions. cDNA was prepared using 1 µg of RNA and Oligo dT primer to a final concentration of 2.5 µM. Oligo dT primers are specific to poly $(A)^+$ RNA and produces an RT-PCR product more

consistently than random hexamers or gene-specific primers as discussed in the manufacturer's instructions manual.

2.3.6 Polymerase chain reaction (PCR)

All PCR reactions were carried out in 50 µl volumes using an Eppendorf PCR Thermal Cycler or G-Storm GS1 Thermal Cycler. PCR reactions were carried out using Promega's GoTaq. The PCR reaction conditions used can be seen in Table 2.1. The different cycling parameters required are shown in Table 2.2. Denaturing, annealing and extension steps were repeated for 25-40 cycles. Annealing temperatures were estimated as ~5^{o}C below the melting temperature (T_m) of the primers used. The extension times used were 1 min/kb of DNA to be synthesised.

Table 2.1 Components required for PCR reactions using Promega GoTaq.

Reagent	Volume per reaction
GoTaq®	
5X Green or Colourless reaction buffer	10 µl
PCR Nucleotide Mix (10 mM each)	1 µl
$MgCl_2$	5 µl
Primer forward (10 pmol)	1 µl
Primer reverse (10 pmol)	1 µl
GoTaq Polymerase	0.25 µl
Template DNA	100 ng-0.5 µg/50 µl
PCR grade H_2O	To total of 50 µl

Table 2.2 PCR cycling conditions required for the various commercial Taq polymerases.

	Temperature/Time
Denaturation/Activation	95°C/2 min
Denaturation	95°C/0.5-1 min
Annealing	5°C below T_m/0.5-1 min
Extension	72°C/1 min
Final Extension	72°C/10 min

2.3.7 Quantitative PCR

RNA was isolated as described in Section 2.3.1 and cDNA synthesis was performed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) as described in Section 2.3.5 Real time quantitative PCR (qPCR) reactions were performed using a Roche Lightcycler 480 thermocycler and SYBR green master mix (Roche). Each reaction had a total volume of 10 µl and was comprised of: 5 µl SYBR green master mix, 1.875 µl PCR grade H_2O, 0.5 µl forward primer (5 pM), 0.5 µl reverse primer (5 pM), 0.125 µl Lightcycler Uracil-DNA Glycosylase (Roche) and 2 µl cDNA. The PCR program used consisted of 40 °C for 10 min, 95°C 10 min followed by 45 cycles of 95°C for 10 sec, 58°C for 20 sec, 72°C for 10 sec. Melting curve analysis was carried out for each experiment. The *P. superbus* RNA polymerase II and 60S ribosomal subunit primers were used as reference primers (Tyson *et al.*, 2012) and the *C. elegans* primers CE*pmp-3* (Acyl-CoA transporter) and CE*cdc-42* (RHO GTPase) were used as reference primers (Hoogewijs *et al.*, 2008). The primers used in the qPCR experiments are shown in Table 2.3. Negative controls containing no cDNA were also performed. Each reaction was replicated a minimum of 4 times on each plate. By comparison of the cycle threshold values of the control (housekeeping genes) to the sample, the relative concentration of the template was determined using the second derivative maximum method for relative quantification as described in the Roche Lightcycler manual. Statistically significant differences between the control and treatments were confirmed by comparing the normalised mean crossing points using ANOVA ($P<0.001$) using Graphpad (PRISM) statistical software.

Table 2.3 Primer sequences used in qPCR experiments.

Primer name	5' Sequence 3'
CEpmp-3qpcrF	GTTCCCGTGTTCATCACTCAT
CEpmp-3qpcrR	ACACCGTCGAGAAGCTGTAGA
CEcdc-42qpcrF	CTGCTGGACAGGAAGATTACG
CEcdc-42qpcrR	CTCGGACATTCTCGAATGAAG
CEtre-1qpcrF	TGCAATGGACCAATTCTTCA
CEtre-1qpcrR	AGTTCGATCACCAAGCTCGT
CEpyk-1qpcrF	CCAAAGTTCTTTCGGTTGGA
CEpyk-1qpcrR	TTCCCAACATTCCACCATTT
CEcrn-7qpcrF	GCGTGGAAAATCTGTGGACT
CEcrn-7qpcrR	TTTCCACGTGTCCAATCTGA
CErpt-2qpcrF	GAAGCTCGAAAAAGCACCAC
CErpt-2qpcrF	CGGATTCCCATCTCTTCGTA
CEprx-12qpcrF	TCGACTGGAGTGACAGATCG
CEprx-12qpcrR	CTGGTCGAAGTAGTGCGTCA
CEced-3 qpcrF	TTCACCGGATGCTCTTCTCT
CEced-3 qpcrR	CGAAAACACGGCTTATGGTT
CEcox-1 qpcrF	ACCACCACATGCAAAGATCA
CEcox-1 qpcrR	TCTGCCCCTGTCTTCTCACT
PST17504 qpcrF	GTCCTGGTCGAATTGATCGT
PST17504 qpcrR	TCTTTTGCAAGGGTCATTCC
PST09334 qpcrF	CCATCTGGCCGAAATGTACT
PST09334 qpcrR	AGTTTTCCTTTTGCGGGTTT
PST13772 qpcrF	ATTTGTGTGGTCGCCATTTT
PST13772 qpcrR	AATGCTTGTCCATTGGCTTC
PST03988 qpcrF	GTTGCTGCAATTTTTCGTGA
PST03988 qpcrR	GGAAAAGTTGCTCCACATGC
PST12235 qpcrF	ATGGCACAAGATTTCCTTGG
PST12235 qpcrR	CCGGAATTTTCACAACTGCT
PST12692 qpcrF	GTGGCGATTGGGGTAGTTTA
PST12692 qpcrR	TGGAATTGTTGGCACTGAGA
PST01758 qpcrF	GCCATTTCAAGGCCATTTTA
PST01758 qpcrR	TGGATCATTGTTTGGATTTGC

Table 2.3 (continued) Primer sequences used in qPCR experiments.

Primer name	5' Sequence 3'
PST02298 qpcrF	TGGCCCACATTTTAATCCAT
PST02298 qpcrR	CCCAAATTGTTGGTTTGCTT
PSC02494 qpcrF	TGATGATGCAGCACCACTTT
PSC02494 qpcrR	TGGAGCGAAACGTTTAACAA
PShsp-70 qpcrF	ACGTGCAATGACCAAAGACA
PShsp-70 qpcrR	ACCATTGGCATCAACATCAA
PShsp-40 qpcrF	AAACAAGCCGTTGAAGCACT
PShsp-40 qpcrR	GCAGGCGATACTCCAAGAAC
PShsp-12 qpcrF	ACTCCAACATGGACGGAAAA
PShsp-12 qpcrR	ACGGTTGCCAATTTGCTATT
PShsp-21 qpcrF	GTTCATTCCTTCGTCGGGTA
PShsp-21 qpcrR	AGGCTTTGGAGCAAAGATGA
PSlea-2 qpcrF	TGGAATCCTCATCTCCAACA
PSlea-2 qpcrR	GCAGCATCATAGGCATCAGA
PSlea-5 qpcrF	GGAGCTGCAAAGGTTAAAGC
PSlea-5 qpcrR	ATGGCATCTTGTTGTTCACG
PSlea-8 qpcrF	GCTGGTAAAGCTAAGGATGTTATG
PSlea-8 qpcrR	GAACATTATCCCATGTTTCTTCAGC
PScyp-3 qpcrF	TATCTGCACTGCCGTTACCA
PScyp-3 qpcrR	TCGGCAGAAGTTTTTCCACT
PS00740 qpcrF	GCAAAACTGGAGCTGGTCTC
PS00740 qpcrR	AAACAGGCAATTTGCGTACA
PS001029 qpcrF	TTAGGAAATGCCCTCATTGG
PS001029 qpcrR	CAAGAACAAGGAAGGCAAGG
PScys-2 qpcrF	TGGGGCTTAAACTTGGTGAC
PScys-2 qpcrR	GTTGTGCAGACAGGCGTAAA
PSric-1 qpcrF	CCCCGATTATGTTGCTCTGT
Psric-1 qpcrR	ATCCGGGGATATAACCCAAA
PSdj-1 qpcrF	AGCGCCAGTTATTTTTGCAC
PSdj-1 qpcrR	CCTGGAGCTCGACTCGTTAC

Table 2.3 (continued) Primer sequences used in qPCR experiments.

Primer name	5' Sequence 3'
PSC02494 qpcrF	TGATGATGCAGCACCACTTT
PSC02494 qpcrR	TGGAGCGAAACGTTTAACAA
PSC04819 qpcrF	TCAAGAACCTGCGGAAAATC
PSC04819 qpcrR	GCCGTTGACTTCAAGCTTTC
PSC02624 qpcrF	CCCCAAGAATGATTTTGCAT
PSC02624 qpcrR	TTTGCCATCAACTTCAAGGA
PSC04040 qpcrF	GGAGCTCCATGGTTTGTCAT
PSC04040 qpcrR	ATGGGCTCCAACAAAATCAA
PSC01063 qpcrF	GTTGCACGTCGAATTGTTTG
PSC01063 qpcrR	CAAGTTCATCACGCTTTGGA
PSC01095 qpcrF	TGATTTCGCTGTAGGCCTTT
PSC01095 qpcrR	AACCCCAACAACACCAAGAG
PSRNAPOLII qpcrF	GATGACTTTATGGAAGAAGATGAGG
PSRNAPOLII qpcrR	CTATGATCACAATTTCGGCAAG
PS60SL32 qpcrF	GTTCGTAGACGTTTCAAGGGTACT
PS60SL32 qpcrR	TCGAGATCTCTGACATTATTGACG

2.4 Protein methods

2.4.1 Protein extraction

The nematodes were harvested as described in Section 2.2.4. The nematodes were then transferred to a 15 ml Falcon tube and centrifuged at 5,000 xg for 3 min (nematode pellet ≈200 µl). The supernatant was removed and 1 ml of lysis buffer [9.5 M urea, 4% (w/v) CHAPS, 10 mM Tris-base], and protease inhibitor cocktail (Roche, 1 tablet for each 10 ml of buffer) was added. The sample was then ground with a pestle and mortar under liquid nitrogen. The homogenates were then added to a 1.5 ml Eppendorf tube with 0.25 g of glass beads (< 106 µm, Sigma (G4649)) and were further homogenised for five 30 sec pulses with cooling on ice for one min between each round. The protein concentration was then determined using the BCA protein assay kit as in Section 2.4.2.

2.4.2 Determination of protein concentration

Concentrations of mixed protein extracts were determined using the BCA (bicinchoninic acid) protein assay kit (Novagen) according to the manufacturer's instructions. Dilutions of BSA from 0.1–1.5 mg/ml were used to produce a standard curve for each assay.

2.4.3 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (Laemmili, 1979) was carried out in a Biorad Mini Protean 3 gel tank according to the manufacturer's instructions. A 12% (w/v) gel with a 4% (w/v) stacking gel (see Table 2.4) was used, unless otherwise stated. Protein samples were diluted 1:4 in 4X SDS sample buffer (Novagen) and were heated to 95°C for 5 min and cooled on ice for 3 min prior to loading. Approximately 40 µg of crude protein extract was loaded on each lane using a Hamilton syringe and BioRad Precision Plus All Blue Standards were used as a marker. Gels were stained in 10% (v/v) acetic acid, 45% (v/v) methanol, and 0.2% (w/v) Coomassie Brilliant Blue (R250) for 1 h followed by destaining in 10% (v/v) acetic acid and 30% (v/v) methanol.

Table 2.4 SDS-PAGE gel components.

	Stacking gel	Separating gel
Tris-glycine SDS-PAGE		
30% (w/v) Acrylamide/bis (ml)	1.3	11.3
1.5M TrisHCl pH 8.8 (ml)	-	7.0
0.5M TrisHCl pH 6.8 (ml)	2.5	-
Deionised water (ml)	6.1	9.3
10% (w/v) SDS (µl)	100	280
10% (w/v) APS (µl)	200	100
TEMED (µl)	20	23

2.4.4 Preparation of protein sample for shotgun proteomics

Nematode protein samples were extracted as described in Section 2.4.1. The extracted protein was placed in a fresh 1.5 ml Eppendorf tube. TCA precipitation was then carried out by adding TCA to a final concentration of 10% (w/v) and precipitating the proteins overnight at 4°C. The protein pellet was centrifuged at 13,000 g for 5 min at 4 ^{o}C and the supernatant was removed, leaving the protein pellet intact. The sample was then washed with ice cold acetone and resuspended in 100 µl of 50 mM ammonium bicarbonate pH 8.0. The protein concentration was then determined using the BCA protein assay kit as described in Section 2.4.2.

Proteins were reduced by adding 5 µl DTT [100 mM in ammonium bicarbonate] to the samples and incubating at 95 ^{o}C for 5 min. The samples were then alkylated by adding 4 µl of iodoacetamide (IAA) [1 M in ammonium bicarbonate (50 mM)] and incubated for 25^{o}C for 45 min in the dark. Following alkylation 20 µl of DTT [100 mM in ammonium bicarbonate (50 mM)] solution was added and the samples were incubated at 25^{o}C for 45 min. The samples were then trypsinized by adding molecular grade trypsin (Promega) in a ratio of 1:40 ([concentration trypsin]: [concentration protein sample]). The samples were then reduced to dryness using a ThermoScientific DNA120 SpeedVac concentrator. To purify the trypsin digested protein peptides for mass spectrometry the ZipTip procedure

was used (Millipore). In brief, trypsin digested peptides were resuspended in 20 µl Resuspension Buffer [0.5% TFA in LCMS grade H_2O]. The peptide samples were sonicated for 2 min, to help resuspend the pellet, followed by a brief centrifugation. The peptide samples were then processed using Zip-tips according to the manufacturer's instructions. The peptide samples were then reduced to dryness using a ThermoScientific DNA120 SpeedVac concentrator and resuspended in 12 µl of loading buffer (0.05% trifluoroacetic acid, 98% LCMS grade H_2O, 2% LCMS grade acetonitrile).

2.4.5 Mass spectrometric analysis

Peptide samples were run on a Thermo Scientific Q Exactive mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system in the UCD Conway Institute of Biomolecular and Biomedical Research, Mass Spectrometry Resource. Each sample was loaded onto Biobasic Picotip Emitter (120 mm length, 75 µm ID) packed with Reprocil Pur C18 (1.9 µm) reverse phase media and was separated by an increasing acetonitrile gradient over 43 min at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220 °C, and with a potential of 2000V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution (70,000) MS scan (300-2000 Dalton) was performed using the Q Exactive to select the 15 most intense ions prior to MS/MS analysis using HCD (higher-energy collisional dissociation).

Protein identification and label free quantitative (LFQ) analysis was conducted using MaxQuant (version 1.2.2.5; http://maxquant.org/) supported by the Andromeda database search engine to correlate MS/MS data against the Caenorhabditis_elegans.WBcel215.70 protein database (ftp://ftp.ensembl.org/pub/release-70/fasta/caenorhabditis_elegans/pep/)**.** For protein identification the following search parameters were used: precursor-ion mass tolerance of 1.5 Da, fragment ion mass tolerance of 6 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) were set to 0.01 for both peptides and proteins and only peptides with minimum length of six amino acid length were considered for identification. Proteins were considered indentified when a minimum of two peptides for each parent protein was observed.

Differentially abundant proteins among experimental groups were determined by LFQ (label-free quantification) using MaxQuant and Perseus software (version 1.2.0.17). Peptides were matched across samples based on mass to charge ratio, elution time (within a 2 minute boundary) and spectral features. Protein intensities were determined using unique and razor (those most likely to have originated from a protein based on the principle of parsimony) peptides. Protein intensities were normalised across runs to account for variation in sample loading. Then, pair-wise ratios of all peptides of a particular protein group were calculated across all samples and the protein intensities were corrected in order to reflect the median peptide ratios. The data matrix containing the normalised intensities (presented as LFQ intensities) for each individual sample was imported into Perseus for quantitative analysis. LFQ intensities measured for individual runs were grouped based on their experimental treatment. The data was then log2 transformed and an ANOVA ($p<0.05$) was performed between the control and individual treatment samples to identify differentially abundant proteins. Proteins with statistically significant differential expression were extracted and these were used to generate heat maps of expression. To improve visual representation of differentially abundant proteins mean values were generated for each treatment and used to build the heat maps.

A qualitative assessment of differential expression was also conducted. This involved the identification of proteins that were completely lacking from a specific treatment. Those proteins that were completely missing from all replicates of a particular group but present in other groups were determined manually from the data matrix. These proteins are not considered statistically significant as the values for absences are given as NaN (not a number) which is not a valid value for an ANOVA analysis. However the complete absence of a protein from a group may be biologically significant and these proteins were reported as qualitatively differentially expressed. To scale the LFQ intensity differences from zero a corrective constant was subtracted from each LFQ intensity. This value (hereafter the zero corrective value) was determined as the lowest log2 transformed intensity value minus one and essentially represents a background intensity value. Considering that all intensities had already been normalised to account for variability among samples the same zero corrective value was subtracted from the intensities for all samples (rather than subtracting the lowest intensity minus 1 for each individual sample run). NaN values were then substituted with a zero in the final LFQ intensity matrix.

Hierarchical clustering was performed in Perseus using Euclidian distance and average linkage clustering to group proteins with similar intensity and expression levels.

2.4.6 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE)

2.4.6.1 Labelling protein samples with cyanine dyes Cy3 and Cy5

Proteins from control and desiccated (36 h 98% RH) *P. superbus* nematodes for three biological replicates were prepared, extracted and quantified as described in Sections 2.2.4.1, 2.4.1 and 2.4.2 respectively. The two dyes, Cy3 and Cy5, were reconstituted in freshly prepared DMF (Dimethylformamide) and diluted to a 0.2 mM solution prior to use. Protein samples (525 µg per gel) were placed in 1 ml of DIGE lysis buffer [9.5 M urea, 4% w/v CHAPS, 30 mM Tris-Cl, pH 8.5]. Each protein sample being studied was labelled with 200 pmol of Cy3 fluorescent dye per 50 µg protein sample. The Cy5 dye was used as the pooled internal standard. The labelling reaction was carried out on ice in the dark, and then quenched by incubation with 10 mM lysine for 10 min on ice. The labelled protein extracts were mixed with an equal volume of 2X buffer [9.5M urea, 4% CHAPS, 2% IPG buffer pH 3.0-10.0 and 100 mM DTT] and this suspension was left on ice for 10 min prior to electrophoresis separation. The samples were loaded onto Immobilized pH gradient (IPG) strips with an equal volume of 2x sample buffer (7 M urea, 2 M thiourea, 65 mM CHAPS, 2% (v/v) ampholytes and 2% (w/v) dithiothreitol).

2.4.6.2 2-D gel electrophoresis

IPG strips (GE Healthcare, Bucks, UK) used for isoelectric focusing were rehydrated for 12 h in rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 3% (v/v) ampholytes, 2% (v/v) DTT and 0.05% (w/v) bromophenol blue as tracking dye]. The sample (450 µl) was added to a re-swelling tray (Amersham Biosciences/GE Healthcare) containing 24 cm IPG strips. Following rehydration for 9 h at room temperature in the IPGphor machine, the following running conditions were used for the pH 3.0-10.0 24 cm strips: 120 min at 100 V (step and hold), 90 min at 500 V (step and hold), 60 min at 1000 V (step and hold), 60 min at 2000 V (step and hold), 60 min at 4000 V (step and hold), 120 min at 6000 V (step and hold), 240 min at 8000 V (step and hold), 180 min at 500 V (step and hold), 240 min at 8000 V (step and hold) for a total of 70,000 Vh. Before the 2-D separation step the gel strips were equilibrated in buffer (75 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002 % bromophenol blue) containing 100 mM DTT followed by 10 min of equilibration buffer containing 0.25 M iodoacetamide.

Electrophoretic separation in the second dimension was performed using 12.5% (w/v) slab gels that were made in the lab with at least 12 h of polymerisation. After washing in SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS pH 8.3) the strips placed on top of the second dimension gel and held in place using 1% (w/v) agarose sealing gel. SDS-PAGE gels were run at a constant voltage of 100 V for 1 h, followed by 300 V for 3 h.

2.4.6.3 DIGE Image Acquisition and Analysis

Typhoon Trio variable mode imager (Amersham Biosciences/GE Healthcare) was used to visualize the labelled proteins at a scanning wavelength of λ=550 nm for Cy3 and λ=650 nm for Cy5 labelled proteins. The Photomultiplier tubes (PMT) values for the gels analysed were between 500 V and 700 V and the maximum pixel volume was between 85,000 and 95,000. Scanning was performed at 50 µm resolution for DIGE gels. The gel images were then analysed using Progenesis Same Spot Software version 3.2.3 from NonLinear Dynamics (Newcastle Upon Tyne, UK). All gels in an experiment were aligned to a reference gel. Images were separated into groups (control vs. desiccation) and a list was made of protein spots with changed abundance. A power score was given to each spot above 0.8 and any spots below 0.8 were excluded from consideration. Power can be defined as the probability of finding a real difference if it exists. Eighty percent or 0.8 is considered an acceptable value for power. The Power Analysis is performed independently for each feature, using the expression variance, sample size and difference between the means. Similarly, spots with a *P*-value less than 0.05 were taken as significant and those greater than 0.05 were excluded from the experiment. An ANOVA score was determined using the one way ANOVA test. Any spot with an ANOVA score above 0.05 were excluded from consideration. Selected protein spots that met all criteria were identified by Liquid chromatography-Mass spectrometry.

2.4.6.4 Protein identification using mass spectrometry

The identification of proteins which were found to be differentially expressed was carried out using mass spectrometry. Samples which were excised from 2-D gels were desalted and destained to remove contaminants. The samples were then digested using trypsin to cleave the protein at lysine and arginine amino acid residues.

Peptides were obtained by removing supernatants from trypsin digested gel plugs. Further gel recovery was achieved by adding 30% acetonitrile/0.2% trifluoroacetic acid to the gel plugs for 10 min at 37 ºC with gentle agitation. The mass spectrometry analysis of peptides was carried out on a 6340 Model Ion Trap LC/MC apparatus from Agilent. Five microliters of sample was loaded into the enrichment at a capillary flow set to 2 µl/min with a mix of 0.1% formic acid and 50% acetonitrile and formic acid at a ratio of 19:1. Database searches were carried out with Agilent technologies Spectrum Mill Mass Proteomics software (http://spectrummill.mit.edu/). The amino acid sequences found by the mass spectrometer were compared to a *P. superbus* protein database which had being theoretically digested using Protparam tools (McGill, unpublished work).

2.4.7 Statistical analysis

Statistically significant differences in expression were confirmed using ANOVA with a confidence level of 99.9% and Dunnett's comparison test with $\alpha = 1$ using Graphpad (PRISM) statistical software.

2.5 RNA-seq methods for *C. elegans* experiment

2.5.1 RNA extraction

Nematodes were grown as described in Section 2.2.1 and exposed to desiccation stress as described in Section 2.2.4.2. Three biological replicates were prepared for each stress treatment. Following desiccation stress the nematodes were harvested and placed in Trizol Reagent® (Invitrogen) before freezing, as described in Section 2.2.4.2. RNA was prepared as described in Section 2.3.1. RNA quality was analysed using Qubit® 2.0 Fluorometer and the Agilent Bioanalyzer according to manufacturer's instructions. Total RNA of 12 samples (4 treatments with 3 biological replicates) with at least 5 µg for each samples was sent to BGI for library construction.

2.5.2 Preparation of sequencing library for *C. elegans* samples

The cDNA library construction for each treatment was performed by Beijing Genomics Institute (Shenzhen, China) according to the Illumina TrueSeq RNA sample preparation protocol. The libraries were quantified using a Qubit dsDNA HS (high sensitivity) assay kit with the Qubit® 2.0 Fluorometer and the Agilent qPCR NGS library quantification assay. Following quantification samples were pooled for sequencing. At BGI mRNA was

isolated by oligo dT enrichment and a 200 bp short insert cDNA library was prepared for 91 paired end 4 Gigabyte (GN) sequencing.

2.5.3 RNA-sequencing

Paired-end RNA-sequencing was performed on an Illumina HiSeq^{TM} 2000 sequencing system by BGI. The raw sequencing data were filtered by BGI to remove adapters and poor quality sequences. At NUIM the filtered sequence data was uploaded onto the SGI Altix 8200 ICE cluster (Sioc) on the high performance computing facility.

2.5.4 Quality control

Sequencing reads from the Illumina sequencer were exported in FASTQ format with the corresponding Phred quality scores. The program FastQC was used to perform quality control (www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). This program performs a series of analyses that produce the following graphs and tables to give an overall assessment of the data: basic statistics, per base sequence quality, per sequence quality scores, per base sequence content, per base sequence content, per base GC content, per sequence GC content, per base N content, sequence length distribution, duplicate sequences, overrepresented sequences and overrepresented kmers. This analysis indicated that the sequences were of high quality (Section 4.2.4). However, the analysis also indicated there was a high degree of duplication which requires duplicate removal discussed in Section 2.5.6

2.5.5 Alignment of sequencing reads to the *C. elegans* genome

A critical step in an RNA-Seq experiment is that of mapping the short sequence 'reads' to the reference genome or transcriptome. Alignment of RNA-Seq reads to a reference transcriptome is achieved using the TopHat2 pipeline (Kim *et al.*, 2013) which incorporates the Bowtie2 program (Langmead *et al.*, 2009; Langmead and Salzberg, 2012). Bowtie is an ultrafast memory-efficient short read aligner for aligning short DNA sequence reads to transcriptomes or genomes. It uses the Burrows-Wheeler algorithm (Burrows and Wheeler, 1994) for compressing text which is then indexed based on the full-text minute-space index (Ferragina and Manzini, 2005). Bowtie can be used to efficiently find the number of occurrences of a pattern within the compressed text, as well as to locate the position of each occurrence. Further details of processing the RNA-seq data including the commands used can be found in the Supplementary methods file.

2.5.6 Removal of PCR duplicates

Removal of PCR duplicates (identical sequence reads that map to the same location with the same orientation) was performed using Picard program (http://picard.sourceforge.net). The presence of duplicates is a major issue in paired short reads from NGS platforms. The purpose of removing duplicates is to mitigate the effect of PCR amplification bias introduced during library construction (Kozarewa *et al.*, 2009). These duplicates might have a serious impact on downstream analysis (Xu *et al.*, 2012), therefore they were removed in this experiment. Picard provides various utilities for manipulating alignments in the SAM format. The alignments were viewed and converted from binary (BAM) format (output of TopHat) to SAM (sequence alignment map) format using a command in SAM tools (Li *et al.*, 2009).

2.5.7 Sorting files using SAM tools

The reads need to be converted from SAM format to BAM format and sorted in order to map them correctly to the transcriptome. This was carried out using SAM tools (Li *et al.*, 2009).

2.5.8 Counting mapped reads

The number of reads that mapped to a transcriptome identified gene sequence were counted with HTSeq-count, part of the Python package HTSeq available at www.huber.embl.de/users/anders/HTSeq/doc/overview.html (Anders and Huber, 2010). This program requires a SAM alignment input format, so BAM files were converted back to SAM format with SAM tools. The HTSeq-count program took the reads and counted the number of times they mapped to a given gene annotation in the transcriptome (gtf file).

2.5.9 Normalisation of counts

A single text file was constructed containing all counts for each biological replicate for each transcriptome gene id. The counts for different treatments and replicates were normalised to adjust for varying sequencing depths using the R package DEseq. This normalisation is based on the hypothesis that most genes are not differentially expressed (DE) (Anders and Huber, 2010). A DEseq scaling factor for a given sample is computed as the median of the ratio, for each gene, of its read count over its geometric mean across all samples. The underlying idea is that non-DE genes should have similar read counts

across samples, leading to a ratio of 1. Assuming most genes are not DE, the median of this ratio for the lane provides an estimate of the correction factor that should be applied to all read counts of this sample to fulfil the hypothesis (Anders and Huber, 2010).

2.5.10 Multidimensional scaling plot

The R package edgeR (Robinson *et al.*, 2010) was used to make a multidimensional scaling plot (MDS) from the counts data for each condition. This gives an initial overall estimation of differences in the count data between each condition and each replicate before any statistical testing.

2.5.11 Estimating variance and resulting plots

The core assumption of the model employed by DEseq is that the mean is a good predicator of the variance. For each condition a function that allows the variance to be predicted from the mean must be computed.

2.5.12 Calling differential expression

Having estimated the variance-mean dependence (Section 2.5.11), the number of differentially expressed genes may be calculated. To compare two conditions to see whether there is any differential gene expression the function nbinomTest is called. The nbinom test uses a negative binomial model i.e. the number of counts for a given gene in sample j come from a negative binomial distribution with the mean $s_j\mu_p$ and the variance $s_j\mu_p + s_j^2 v(\mu_p)$ where s_j is the relative size of library *j*, μ_p is the mean value for condition p and $v(\mu_p)$ is the fitted variance for the mean μ_p. The null hypothesis for the nbionom test is that the experimental condition has no influence on the expression of the gene under consideration (i.e. $\mu_{p1} = \mu_{p2}$) (Anders and Huber, 2010).

2.5.13 Gene ontology and Pathway analysis

The *C. elegans* transcriptome was mapped to Uniprot IDs and the Reactome overrepresentation analysis tool (www.reactome.org) was used to find pathways, with an unadjusted *P*-value of 0.01, which are strongly enriched in response to the different conditions (Matthews *et al.*, 2009). Cytoscape (Lopes *et al.*, 2010) (version 3.0.2) plugin BiNGO (Maere *et al.*, 2005) (v.2.44) was used for GO enrichment analysis. All the genes identified in this study were used as reference set for the enrichment analysis. A hypergeometric test was used to identify overrepresented GO and KEGG pathway terms

with a significance level at 0.05 and the Benjamini & Hochberg method was used for multiple comparisons (Maere *et al.*, 2005). The DAVID bioinformatics tool was also used to cluster functionally related annotations into groups (Huang *et al.*, 2009). A cut-off enrichment score of >1.3 was set, as this is equivalent to a non-log scale of 0.05 (Huang *et al.*, 2009).

2.6 RNA-seq *P. superbus*

The methods for RNA-seq for *P. superbus* were as in Section 2.5 with the following changes. Nematodes were prepared as described in Section 2.2.4.1. The cDNA library construction for each treatment was performed by TrinSeq (TCD, Dublin) according to the Illumina TrueSeq RNA sample preparation low throughput protocol. The libraries were quantified using a Qubit dsDNA HS (high sensitivity) assay kit with the Qubit® 2.0 Fluorometer and the Agilent qPCR NGS library quantification assay. Following quantification samples were pooled for sequencing. Paired-end RNA-sequencing was performed on an Illumina Genome Analyzer II (GAII) over seven lanes of a flow-cell by TrinSeq. The resulting data was uploaded onto the Darwin cluster on the high performance computing facility at NUIM.

The RNA-seq reads were mapped to the *P. superbus* reference transcriptome which was prepared by Dr. Georgina O'Mahony Zamora at NUIM (O'Mahony Zamora, 2013).

The bioinformatic analysis of the RNA-Seq data was carried out under the guidance of Dr. Chris Creevey (Animal and Grassland Research and Innovation Centre, Teagasc, Dunsany, Co. Meath).

2.7 RNAi feeding in *C. elegans daf-2* (e1370) worms

2.7.1 PCR amplification of genes of interest

Primers were designed to amplify genes of interest (Table 2.5) and PCR was carried out using cDNA as a template as described in Sections 2.3.5 and 2.3.6.

2.7.2 Purification of DNA from agarose gels

PCR products were separated on an agarose gel and visualised as described in Sections 2.3.3 and 2.3.4. The band corresponding to the gene of interest of interest was cut from

the ethidium bromide stained agarose gel and purified using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions.

2.7.3 Ligation and transformation

Purified PCR products were ligated to the L4440 vector by TA cloning using Promega T4 DNA ligase according to the manufacturer's instructions. Plasmids were transformed into the *E. coli* strain HT115(DE3) as described by Kamath and Ahringer (2003).

2.7.4 Colony PCR

Bacterial colonies obtained by transformation were directly screened for the presence of the desired insert by PCR. Using aseptic techniques, an isolated colony was removed, and a sterile tip placed in a sterile 0.2 ml tube containing PCR reagents (Section 2.2.5). The primer sets used for the PCR were L4440F/L4440R (Table 2.5). These primers flank each side of the multiple cloning site (MCS). Each colony was also streaked onto a reference LB agar plate containing carbenicillin at a concentration of 30 µg/ml to select for the ampicillin resistant vector. DNA-free controls were included and PCR was carried out as described in Section 2.3.6, with the initial denaturing step increased to 10 min at 95^{o}C to allow the bacterial cells to lyse.

2.7.5 *E. coli* growth conditions

Clones which tested positive by PCR were picked from a single colony and were inoculated with LB broth at 37^{o}C with shaking at 180 rpm. A concentration of 30 µg/ml carbenicillin was used to select for the ampicillin resistant vector.

2.7.6 Storage of *E. coli* strains

E. coli strains containing the various plasmids were stored at -80^{o}C. One ml of culture Section 2.7.6) was mixed with 500 µl sterile glycerol and frozen in cryovials.

2.7.7 Plasmid purification

Plasmids were purified from overnight 5 ml cultures of *E. coli* inoculated with a single transformed colony using a Qiagen Miniprep kit (Qiagen) according to the manufacturer's instructions.

2.7.8 DNA Sequencing

The purified plasmids were sent for DNA sequencing to Agowa (www.agowa.de). The DNA sequences obtained were vector screened using the VecScreen program at NCBI (http://www.ncbi.nlm.nih.gov/Vecscreen). The sequences were then analyzed using BLAST http://www.ncbi.nlm.nih.gov/BLAST against the GenBank database.

2.7.9 RNAi feeding protocol

C. elegans daf-2 (e1370) eggs were isolated as described in Section 2.2.3. The sterilised eggs were allowed to hatch overnight in S buffer at 20ºC with shaking (50 RPM). The hatched L1 worms were then aliquoted onto NGM IPTG (1 mM) carbenicillin (30 µg/L) agar plates (9 cm) containing either a lawn of *E. coli* strain HT115(DE3) transformed with an empty L4440 plasmid or a lawn of HT115(DE3) transformed with an L4440 plasmid containing a target gene fragment. The RNAi L4440 constructs were cultured as in Section 2.7.5. The culture was then spread on NGM plates as described above. The bacterial lawn was allowed to dry for 5 days at 20ºC in a laminar flow cabinet before the eggs were added. Worms were grown on the L4440 bacteria at 25ºC for several days until they entered the dauer developmental programme as described in Section 2.2.4.2. An aliquot of worms from each condition is treated with 1% SDS solution for 1 h with shaking at 50 (Cassada and Russell, 1975). This test was carried out, before harvesting these *daf-2* nematodes, to confirm all the nematodes had reached the dauer larval stage.

The *C. elegans daf-2* (e1370) dauer larvae were washed off, pelleted and placed onto 3 cm Petri dishes in droplets. The Petri dishes, without lids, were placed in a 10.0 L desiccation chamber containing approximately 300 ml of saturated potassium dichromate ($K_2Cr_2O_7$) which maintained a relative humidity (RH) of 98% at 25ºC (Winston and Bates, 1960). The dauer larvae were monitored until the water droplet had evaporated (≈16 h). The Petri dishes were then left in the desiccation chamber for an additional for 96 h. The dauer larvae were desiccated at 32.5% RH (using a saturated solution of magnesium chloride) (Winston and Bates, 1960) for 24 h. Finally, the dauer larvae were then allowed to recover in sterile H_2O for 16 h before percentage survival was assessed by microscopic observation of movement and a minimum of five hundred nematodes were counted. Five biological replicates were prepared for each RNAi L4440 construct.

Table 2.5 Primers used to amplify inserts for ligation into L4440 vector.

Primer name	5' Sequence 3'
L4440-F	ATAGGGAGACCGGCAGATCTGATT
L4440-R	TCGACGGTATCGATAAGCTTGATT
CEdjr-1.2 F	CAGAAGACGCCGAAGAAATC
CEdjr-1.2 R	CCAAACACAGTGGCTTCAAA
CEdjr-1.1 F	AATCATATTGGCGGCCGAAG
CEdjr-1.1 R	TCCTTTCCCTCAAGCAGCTC
CEfipr-24 F	TCGTTTCTCTATTCCTCCTTGC
CEfipr-24 R	ACCGTATCCACCGTATCCTC
CEcdr-3 F	TCCTGTGCAATCAAAGTTGTG
CEcdr-3 R	GATAAATCTCTTTGCGAACACG
CEaqp-1 F	ACGGCCGAGGAAGATACTTT
CEaqp-1 R	TCAGGATTGTGCATTCCAAG
CEaqp-11 F	TGGCAGATCACTTATTCGACA
CEaqp-11 R	GCCTTCTTTTCTTTTGCCGC
CEsip-1 F	ACTTCGAGGACATGATGCCA
CEsip-1 R	CTGGATGGCTTCTGGGTGA
CEhsp12.6 F	TGATGAGCGTTCCAGTGATG
CEhsp12.6 R	TTCCATGTGAATCCAAGTTGCT
CElbp-1 F	CTCGCATTTTGTGGAGCTAC
CElbp-1 R	TCTTGTAGAATCTGCGGCCT
CEcey-2 F	CCAAGCAACAGCGAAGAGAA
CEcey-2 R	ATCTGAGCATCGATTGGCTG
CEoxi-1 F	CGTCGCTGCATTTCTGAATA
CEoxi-1 R	CTCGCCGTCTTCGTATTCTC
CEgpdh-2 F	CCGTATGTGGGTTTTCGAGG
CEgpdh-2 R	TGAACGGCTGTGAACAATGG
CEglykin F	TGGTTCTGTTGGCTGCAATC
CEglykin R	CCCGTCCCATAATATCCGCT
CElbp-2 F	CTCGCATTTTGTGGAGCTAC
CElbp-2 R	TCTTGTAGAATCTGCGGCCT
CEcey-2 F	CCAAGCAACAGCGAAGAGAA
CEcey-2 R	ATCTGAGCATCGATTGGCTG
CEoxi-1 F	AGCCCTGAGAAATGTGCTCT
CEoxi-1 R	TTGGAAGTCGAGTAGCTGCA
CEtps-2 F	CCCGGTGAGCATTAAGAACG
CEtps-2 R	TTCCTTGAGCCTCGGTGATT
CEtps-2 F	CCCGCAAATCTACAACTCGG
CEtps-2 R	CGGTCCGGATACTTCTCGAA
CEtre-3 F	ACCTGACGATTGGCAAGAGA
CEtre-3 R	GATCCTGGTTGTGGGTAGCT
CEgpdh-2 F	CCGTATGTGGGTTTTCGAGG

CEgpdh-2 R	TGAACGGCTGTGAACAATGG
CEglykin F	TGGTTCTGTTGGCTGCAATC
CEglykin R	CCCGTCCCATAATATCCGCT
C44B7.10.1 F	TTCCCTCATCCAGGTCTCAC
C44B7.10.1 R	ATGCAGCCTTGTCGAGAGAT
F49E2.5 F	AGCCGAACAGGTTCAAGAGA
F49E2.5 R	TGGCTCCTCAACTGCTTTTT
K07H8.10 F	ATGGGTTTCGACTCACCAAG
K07H8.10 R	GCTGGAGTTTTAGCGACAGG
B0491.5 F	ATCGGTCTTTGGAGTGATCG
B0491.5 R	CAGTCTCCGAGTGTCGTTGA
F21C10.10 F	CAATCGTTGTTGGAAGTGGA
F21C10.10 R	CTGGAGCATTGAGTGGTGTG
Y17G7B.17 F	GGGCGGTATGCTACGAAATA
Y17G7B.17 R	CGACACGGTGAGATGTTGAG
F55C12.6 F	TTCATGGGGAGATGCTTACC
F55C12.6 R	TCGGATGAGCATTCACAAAA

3 Chapter III RNA-seq analysis of desiccation survival in *Panagrolaimus superbus*

3.1 Introduction

P. superbus is a free living bacterial feeding nematode that is able to survive extreme desiccation by entering into a state of suspended animation known as anhydrobiosis. During anhydrobiosis, *P. superbus* nematodes can survive for indefinite periods until rehydration allows them to resume normal metabolism (Shannon *et al.*, 2005). In this chapter, the molecular mechanisms of desiccation tolerance are investigated in *P. superbus* using an RNA-seq transcriptomics approach. This RNA-seq approach was made possible because of the existence of a complete transcriptome dataset derived from high throughput Roche 454 sequencing of cDNA sequences prepared from pooled nematodes which had been exposed to heat, cold, desiccation and osmotic stress. There are a number of previous studies which have used transcriptomics to understand the molecular mechanisms involved in desiccation tolerance. These studies have largely focused on desiccation tolerance in plants. Studies to quantify transcriptional mechanisms of desiccation tolerance in the Antarctic midge *Belgica antarctica* (Teets *et al.*, 2012), the Antarctic nematode *Plectus murrayi* (Adhikari *et al.*, 2010) and *Saccharomyces cerevisiae* (Ratnakumar *et al.*, 2011) have also recently been carried out. Teets *et al.* (2012) observed a number of transcripts up-regulated in response to desiccation that were associated with stress response, ubiquitin-dependent proteasome, actin organisation and signal transduction. There were also a number of genes which encode heat shock proteins (HSPs) which showed increased expression in response to desiccation such as small *hsps* (3 genes), *hsp-70* (8 genes) and *hsp-90* (1 gene). Increased expression in *hsf* (heat shock factor), the transcription factor that regulates *hsp* expression was also observed. They also noted a concurrent down-regulation of genes involved in general metabolism and ATP production. This metabolic depression is a common adaptation in dehydration-tolerant insects, presumably to minimize the loss of water bound to glycogen and other carbohydrates. Adhikari *et al.* (2010) used qPCR to focus on the expression of 8 genes during desiccation in the Antarctic nematode *P. murrayi*. When *P. murrayi* nematodes were exposed to preconditioning (mild desiccation) and desiccation (harsh desiccation), there was increased expression in *ms* (malate synthase), *tps* (trehalose phosphate synthase), *gst-1* (glutathione S-transferase), *jnk-1* (C-Jun N-terminal kinase), *hsp-70* and

hsp-90. This study also revealed that exposure to slow desiccation plays an important role in the transcription of stress, metabolism and signal transduction genes. Slow desiccation also improved the desiccation survival of *P. murrayi* compared with nematodes exposed to fast dehydration. This suggests the increased expression of certain genes during preconditioning may enhance desiccation tolerance in *P. murrayi* (Adhikari *et al.*, 2010). Ratnakumar *et al.* (2011) have identified the genes differentially expressed in response to desiccation in *Saccharomyces cerevisiae* using microarray technology. This study found that desiccation alters 12% of the yeast genome, which includes the induction of 484 genes upon dehydration. Some of the processes which were up-regulated in response to desiccation in *S. cerevisiae* include: the autophagy system, antioxidants and nitrogen metabolism. Additionally, a reduction in the expression of genes encoding ribosomal proteins was also observed. Ratnakumar *et al.* also carried out desiccation survival analyses on almost 5,000 mutant strains of *S. cerevisiae*, each deleted for a different non-essential gene. Interestingly, these phenotypic analyses also implicated autophagic processes as an important part of the response to desiccation in yeast.

Recently, there has been an increasing interest in the molecular mechanisms of desiccation tolerance in plants. These studies have generally focused on desiccation tolerance in resurrection plants and the acquisition of desiccation tolerance during seed development. Maia *et al.* (2011) looked at the transcriptomic mechanisms underlying the loss and gain of desiccation tolerance in *Arabidopsis thaliana*. They discovered that the incubation of desiccation sensitive *Arabidopsis thaliana* seeds in polyethylene glycol (PEG) solution, re-induces the mechanisms necessary for expression of desiccation tolerance. Using microarray analysis, they discovered increased expression in transcripts encoding LEA (late embryonic abundant) as well as seed storage and dormancy proteins in the desiccation tolerant seeds. Additionally, genes associated with photosynthesis, cell wall modification and energy metabolism were down-regulated in the desiccation tolerant seeds. Although *Arabidopsis thaliana* seeds can confer tolerance to desiccation, the vegetative tissues of the plant are desiccation sensitive (Maia *et al.*, 2011). Strategies for desiccation tolerance in non-vascular plants and in the seed, pollen and spores of truncheophytes are common. However, in vegetative tissues of higher plants, the ability to recover from extreme desiccation is very rare. The plants which confer this exceptional trait are called resurrection plants (Gaff, 1971; Rodriguez *et al.*, 2010). RNA-seq has been

used to monitor gene expression in the leaves of the resurrection plant *Craterostigma plantagineum* during preconditioning, desiccation and rehydration (Rodriguez *et al.*, 2010). The preconditioning dataset of *C. plantagineum* was associated with transcripts related to carbohydrate metabolism (sucrose synthase) as well as dehydrins and other groups of LEA proteins. In desiccated *C. plantagineum* leaves, there was a higher prevalence of transcripts encoding LEA proteins, cysteine proteases and amino-acid metabolism associated proteins. Finally, the rehydration dataset was associated with an increase in transcripts encoding proteins related to defense responses and oxidative stress. A consideration to take into account in transcriptomic studies is the possibility that messenger RNA may be stored during desiccation and only translated during rehydration. This has proved to be the case in certain resurrection plants (Gasulla *et al.*, 2013) and perhaps it occurs in the desiccation tolerant nematode *P. superbus*. Additionally, a perceived increase in the abundance of certain transcripts may only be attributed to the increased stability of that transcript. However, these transcripts could also be important during desiccation tolerance as they have been selected to be stabilized, so they can be transcribed possibly upon rehydration. In the moss *Tortula ruralis*, select transcripts, presumably required upon rehydration, are stabilized by sequestration in messenger ribonucleoprotein complexes (mRBPs) during slow drying (Wood and Oliver, 1999).

3.2 Results and Discussion

3.2.1 Selection of conditions for RNA-seq

3.2.1.1 Primers for qPCR experiment

Quantitative PCR (qPCR) was used to estimate the preconditioning time points that would yield the highest level of differential gene expression for desiccated and recovering *P. superbus* nematodes. The gene expression profiles of six putative desiccation-response genes were investigated for four preconditioning time points. The desiccation treatments were as follows: exposure to 98% RH for 12, 24, 36 or 48 h. The worms were then harvested, washed, pelleted and frozen at -80^{o}C in Trizol (Chapter 2 Section 2.2.4.2). Control worms which were not desiccated were also collected. Each sample was thawed, RNA was extracted and converted into cDNA before analysis by qPCR (as described in Sections 2.3.1, 2.3.5, 2.3.7). The genes tested were *lea-1* (lea=late embryogenesis abundant), *lea-5* and *lea-8*; *tps-2* which encodes an enzyme for trehalose synthesis and *dj-1* which encodes a putative stress responsive molecular chaperone.

To select appropriate time points at which to measure gene expression in nematodes that were recovering from desiccation stress, the gene expression profiles of four putative recovery genes were investigated. For this experiment control *P. superbus* mixed population nematodes which had not been desiccated were harvested as well as nematodes which were desiccated at 98% RH for 36 h followed by exposure to 0% RH for 24 h, with the nematodes being subsequently rehydrated for 0 h, 2 h, 3 h or 4 h. These samples were collected, stored and processed as above. The genes tested were *tre-1* (tre=trehalase), *pyk-1* (pyk=pyruvate kinase), *bec-1* (bec=beclin homolog) and *idhb-1* (idhb=isocitrate dehydrogenase beta). The gene *bec-1* is orthologous to the mammalian autophagy gene beclin and may be important in removing proteins damaged during desiccation. *tre-1* encodes the enzyme trehalase which may be up-regulated during recovery to degrade the trehalose which has accumulated during desiccation. Finally, *pyk-1* and *idhb-1* are involved in metabolism. The resumption of metabolism after desiccation may be an indication of recovery in *P. superbus*.

3.2.1.2 Results of qPCR experiment

The results obtained show that of the five putative desiccation response genes three (*lea-5*, *lea-8* and *dj-1*) were over 2 fold up-regulated in response to preconditioning (Figure 3.1). The expression of *lea-8* in response to 98% RH for 12, 24, 36 or 48 h was constant. This was in contrast to the expression of the genes *lea-5* and *dj-1* which increased as the time at 98% RH progressed. The time points of 12 h at 98% RH and 36 h at 98% RH were selected for RNA-seq. These two time points were selected to get a good overall picture of the response of *P. superbus* to dehydration, at both early and late preconditioning intervals. The relative expression values of the putative recovery responsive genes during the rehydration of desiccated *P. superbus* nematodes are presented in Figure 3.2. Increased gene expression was only observed for the genes *tre-1* and *pyk-1* at the 2 h rehydration time point. Thus, the 2 h recovery time point was selected for the RNA-seq analysis.

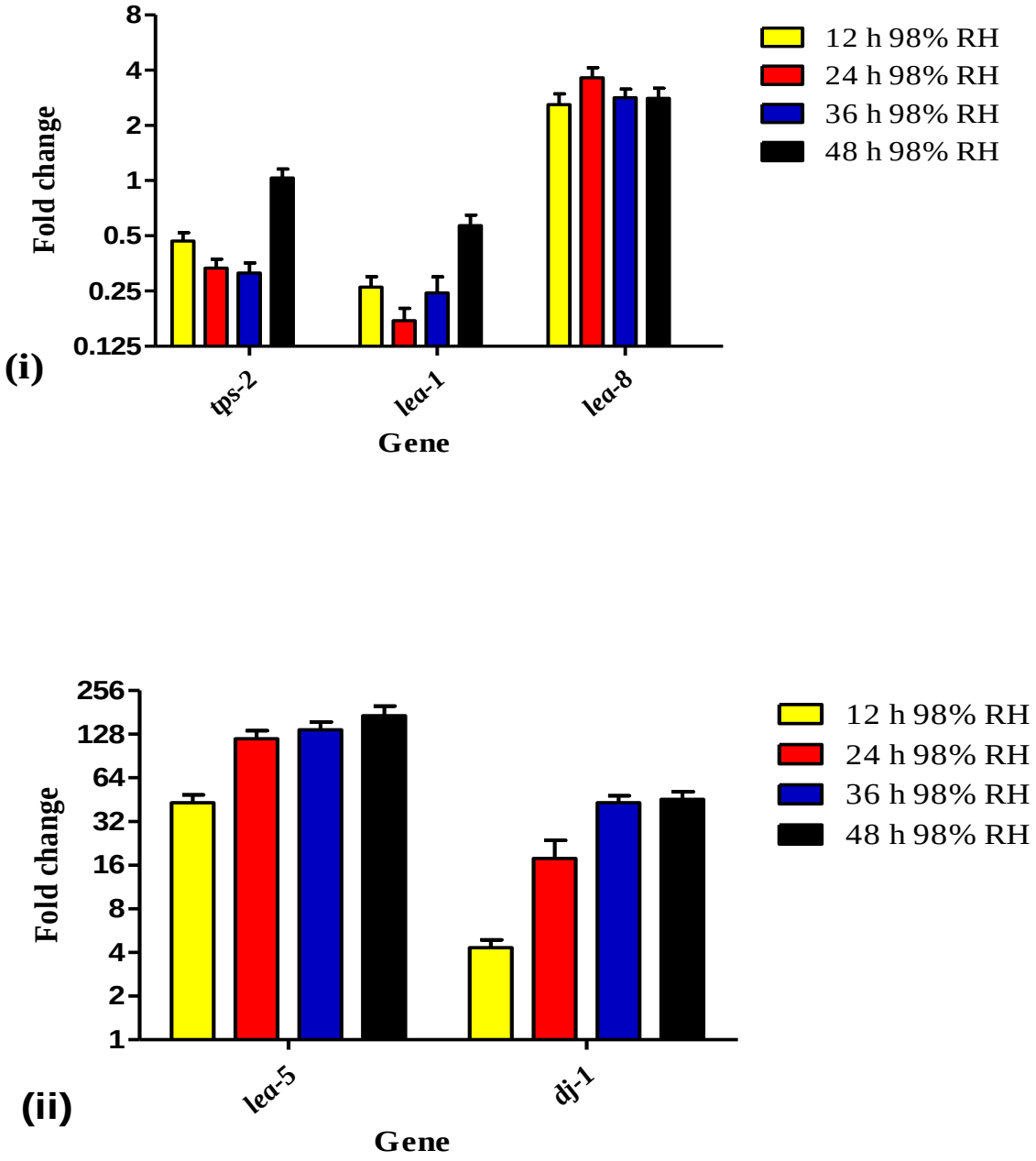


Figure 3.1 Relative expression (measured using qPCR) of putative desiccation-responsive genes in *P. superbus* in response to four desiccation treatments of preconditioning at 98 % RH for 12 h (yellow), 24 h (red), 36 h (blue) and 48 h (black). The genes shown are (i) *tps-2* (trehalose phosphate synthase), *lea-1* (late embryonic abundant), *lea-8* (ii) *lea-5* and *dj-1* There is a log2 scale on the y-axis in order to clearly display the large range of relative expression values. There was an up-regulation of gene expression in response to preconditioning in *lea-5*, *lea-8* and *dj-1*.

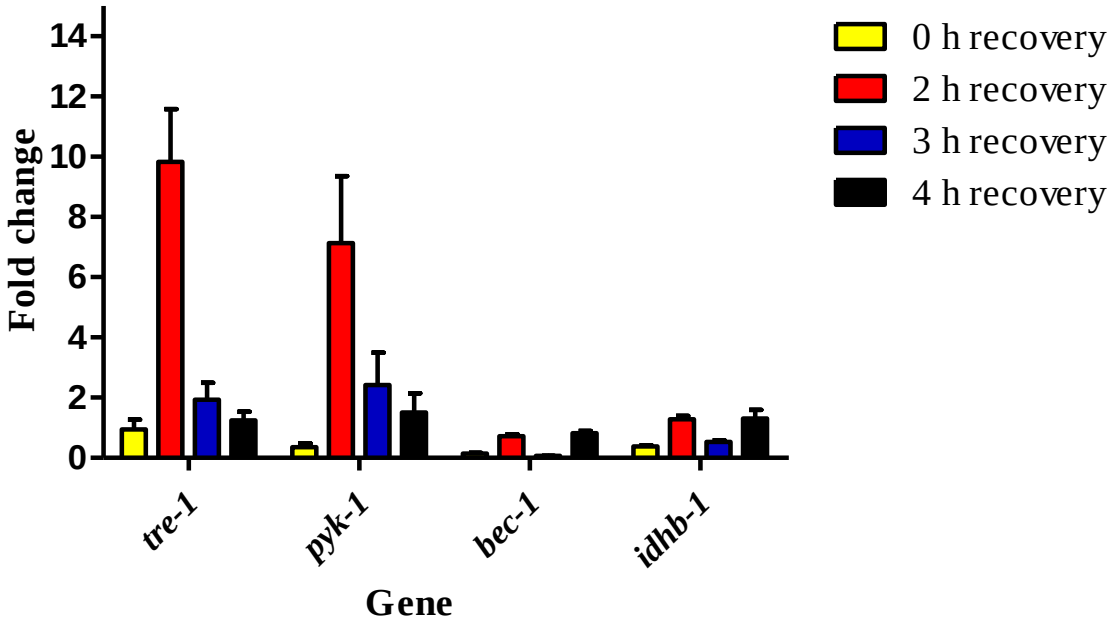


Figure 3.2 Relative expression (measured using qPCR) of putative recovery-response genes in *P. superbus* during recovery from desiccation. The worms were desiccated by exposing them to 98% RH for 36 h and 0% RH for 24 h. The worms were then allowed to recover in water for 0 h (yellow), 2 h (red), 3 h (blue) and 4 h (black). The genes used were *tre-1* (trehalase), *pyk-1* (pyruvate kinase), *bec-1* (beclin) and *idhb-1* (isocitrate dehydrogenase beta). Increased gene expression for *tre-1* and *pyk-1* was observed at 2 h recovery.

3.2.2 RNA extraction and RNA quality tests

Four conditions were selected for RNA-seq, a control (*P. superbus* mixed population) and three treatments: preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery from desiccation (98% RH for 36 h followed by 0% RH for 24 h and 2 h recovery in water). The RNA was extracted as described in Section 2.3.1. Following extraction the RNA concentration was calculated using the Qubit® fluorometer (Invitrogen). The integrity of the RNA was assessed using a Bioanalyzer (Agilent) (Figure 3.3). The Bioanalyzer software assigns an RNA integrity number (RIN) value of 1 to 10, where a value of 10 indicates completely intact RNA (Schroeder *et al.,* 2006). The samples used to construct the RNA-seq library all had a RIN value of 7.0 or more. These RNA samples were brought to a final volume of 50 µl; each sample contained more than 10 µg of RNA. The TrinSeq laboratory at Trinity College Dublin performed library preparation and sequencing for RNA-seq using an Illumina genome analyzer II.

3.2.3 Quality control analysis of the RNA-seq sequence data

The program Fast QC (www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) was used to perform quality control analyses on the raw sequence data from the Illumina sequencing runs. Figure 3.4 (i) shows the quality scores across all bases and Figure 3.4 (ii) shows the quality score distribution for the Control Replicate 1 sample. Data are presented for this sample only, as all samples gave similar results. The Phred quality score across the read including the error was within 30 and 40 across all bases, this means that the probability of calling an incorrect base was very low (between 1 in 1000 and 1 in 10000). The quality of the calls reduces slightly as the run progresses. This is common across most sequencing platforms but the quality score is still within the quality range registered as "good" (Figure 3.4 (i), green zone). The skewed distribution of the quality scores towards high Phred scores (Figure 3.4 (ii)) further demonstrates that all of the reads are of high quality (Ewing *et al.*, 1998).

Across each position in a read the percentages of each of the four bases remains constant (Figure 3.5). Although the quality scores at the start of the read are high, there were spikes in the percentage of the bases content across the first 10 positions in the read. A slight GC bias and a non-random base composition is normally observed over the first 10-12 bases of Illumina RNA-seq reads. This bias in nucleotide composition at the start of Illumina

RNA-seq reads does not result from sequencing errors. It results from non-random priming by the hexamers used in the construction of ds cDNA during Illumina RNA-seq library construction – the hexamer primers tend to bind preferentially at GC sites on the mRNA (Hansen *et al.*, 2010).

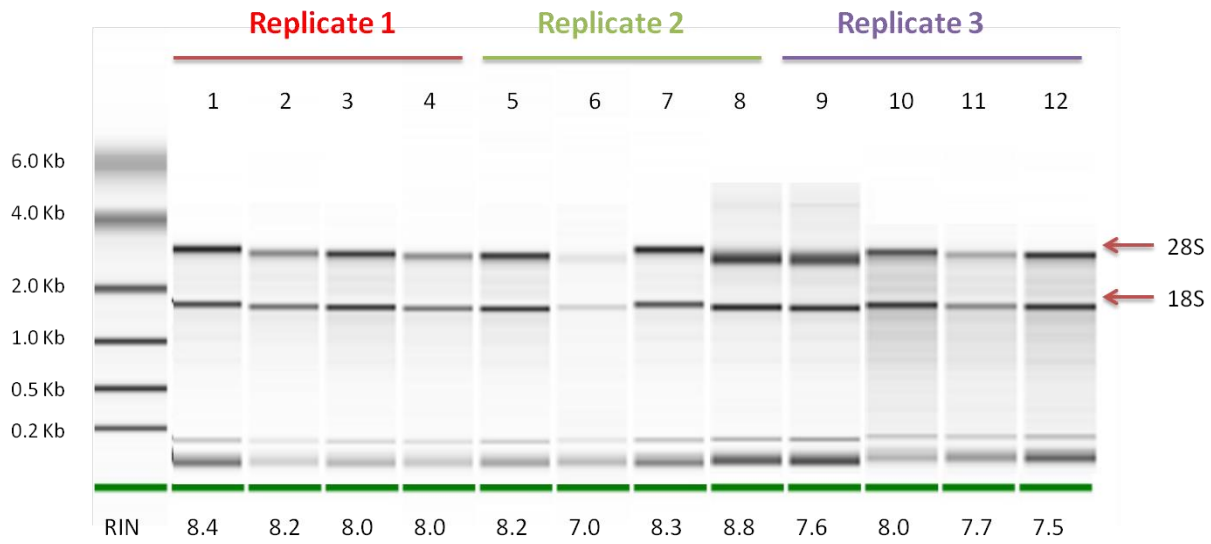
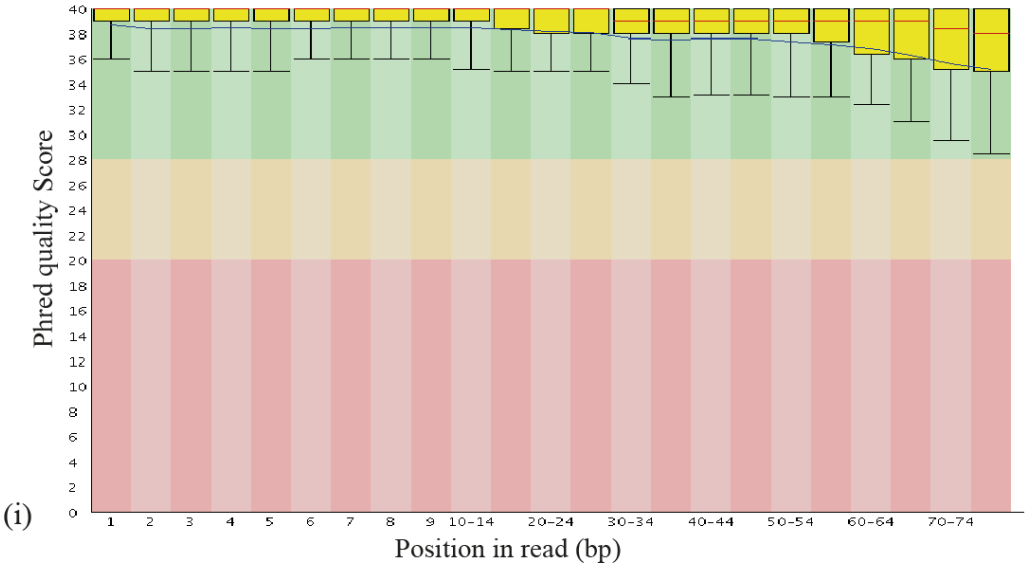
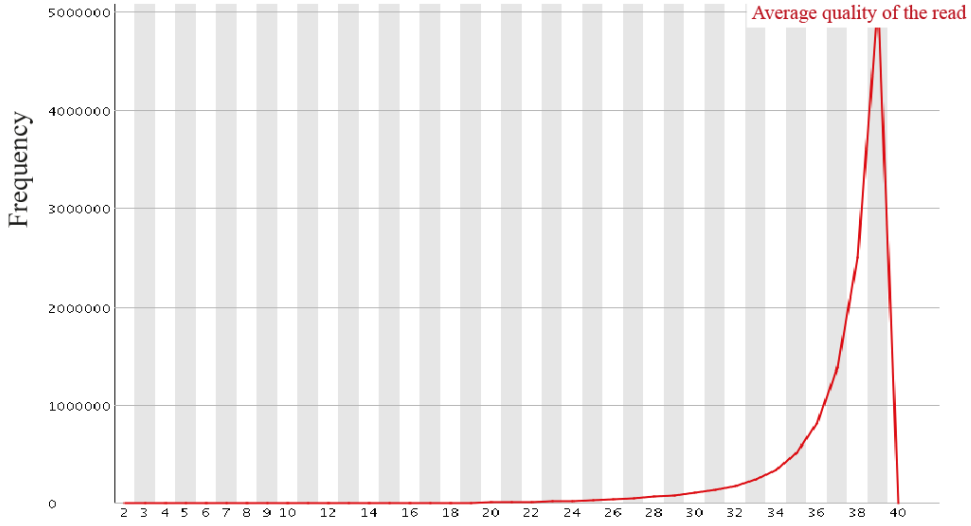


Figure 3.3 Digital image and RNA integrity numbers (RIN) of *P. superbus* samples used for RNA-seq experiments. The location of the bands corresponding to the 18S and 28S rRNA are indicated in the image. The layout of the image is follows: Lane 1, RNA 6000 ladder (Agilent); Lane 2, control replicate 1; Lane 3, preconditioning 1 replicate 1; Lane 4, preconditioning 2 replicate 1; Lane 5, recovery replicate 1; Lane 6, control replicate 2; Lane 7, preconditioning 1 replicate 2; Lane 8, preconditioning 2 replicate 2; Lane 9, recovery replicate 2; Lane 10, control replicate 3; Lane 11, preconditioning 1 replicate 3; Lane 12, preconditioning 2 replicate 3; Lane 13, recovery replicate 3.



(i)



(ii)

Figure 3.4 FastQC analysis on quality of RNA-seq data. (i) Quality scores calculated across all bases for each position in the reads. The whiskers represent the 1st lower quartile, and the 4th upper quartile (the upper quartile is not visible here because of the tight distribution at the top of the graph) and the yellow box is the range of the 2nd and 3rd quartile. The line in the middle is the mean. The Phred quality score across the reads including the error was within 30 and 40 across all bases, this means that the probability of calling an incorrect base was very low. (ii) Distribution of the quality score over all sequences.

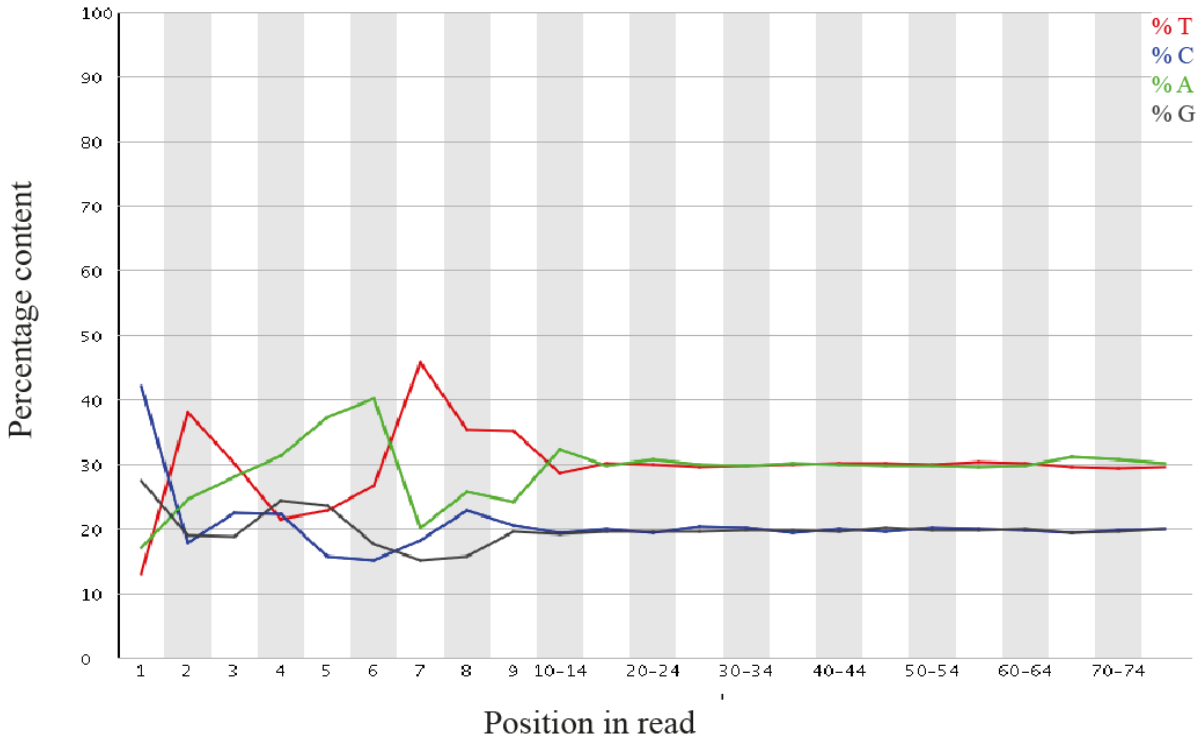


Figure 3.5 FastQC analysis on base composition in RNA-seq data. Bases are represented by the colours red (T), blue (C), green (A) and black (G).

3.2.4 Alignment of RNA-seq reads to the *P. superbus* reference transcriptome

Additional processing of the data was carried out at NUI Maynooth and this resulted in a slight reduction in the number of reads which were carried forward for further processing. Indexing of the *P. superbus* reference transcriptome sequences was done using the Bowtie program (Langmead *et al.*, 2009). The *P. superbus* reference transcriptome was prepared by Dr. Georgina O'Mahony Zamora at NUIM (O'Mahony Zamora, 2013). Alignment of the RNA-seq reads to the *P. superbus* transcriptome was done using Tophat software (Kim *et al.*, 2013). Tophat also removed reads that mapped to multiple regions on the reference *P. superbus* transcriptome. PCR duplicates were also removed using the Pichard program (Li *et al.*, 2009). Further details of the indexing of the *P. superbus*, alignment of the RNA-seq reads and PCR duplicate removal is given in Chapter II Materials and Methods (Sections 2.5 and 2.6). An overview of the RNA-seq workflow used for the *P. superbus* samples is presented in Figure 3.6.

3.2.5 Counting, normalisation and statistical analysis

The number of unique reads that mapped to a given annotated gene in the *P. superbus* transcriptome was counted by the program HTSeq-count (Anders, Simon and Huber, Wolfgang, 2010). The data were combined into one master table that contained all of the counts, conditions and replicates. Normalisation and differential expression analysis were carried out using the R package DEseq (Anders and Huber, 2010). After normalisation (to adjust for varying sample sequencing depths) multidimensional scaling (MDS) analysis was carried out using the edgeR package (Robinson *et al.*, 2010). MDS analysis gives an overview of differences between treatments and replicates (Figure 3.7), which shows the datasets for the biological replicates for treatment 1 (control), treatment 2 (preconditioning 1) and treatment 3 (preconditioning 2) cluster together. In the recovery dataset (treatment 3), the samples T4R1 and T4R3 cluster together while T4R2 clusters closer to treatment 2. The reason that the preconditioning 1 and recovery samples did not cluster tightly may be explained by the fact that the nematodes were not given sufficient time to respond to the conditions. Since the replicates of each treatment clustered separately from the control, all the treatment sample datasets were retained for further analysis.

The data was also visualised as a heat map, based on a distance matrix generated using the edgeR package (Robinson *et al.*, 2010), showing the distances between samples as calculated from the variance-stabilising transformation of the count data (Figure 3.8). This analysis shows treatment 2 (preconditioning 1) and treatment 3 (preconditioning 2) clustering together which is explained by the similarity between the conditions. Additionally the treatment 1 (control) and treatment 4 (recovery) cluster together which suggests the recovery samples are returning to normality after desiccation.

Differential expression data for each gene for different pairs of treatments were calculated using the negative binomial test in the DEseq package as described in Section 2.5.9. The proportion of genes differentially expressed was visualised by plotting the fold change against the mean expression level (Figure 3.9). As can be seen from Figure 3.9, the power to detect differential expression depends on the overall counts for each gene. According to Anders and Huber (2010) this effect results from the strong shot noise for low counts, which causes the testing procedure to call only very high fold changes as significant. It can also be seen that for counts below approximately 100 even a small increase in count levels reduces the impact of shot noise and hence the fold change requirement, while at higher counts, when shot noise becomes unimportant the fold-change cut-off depends only weakly on count level.

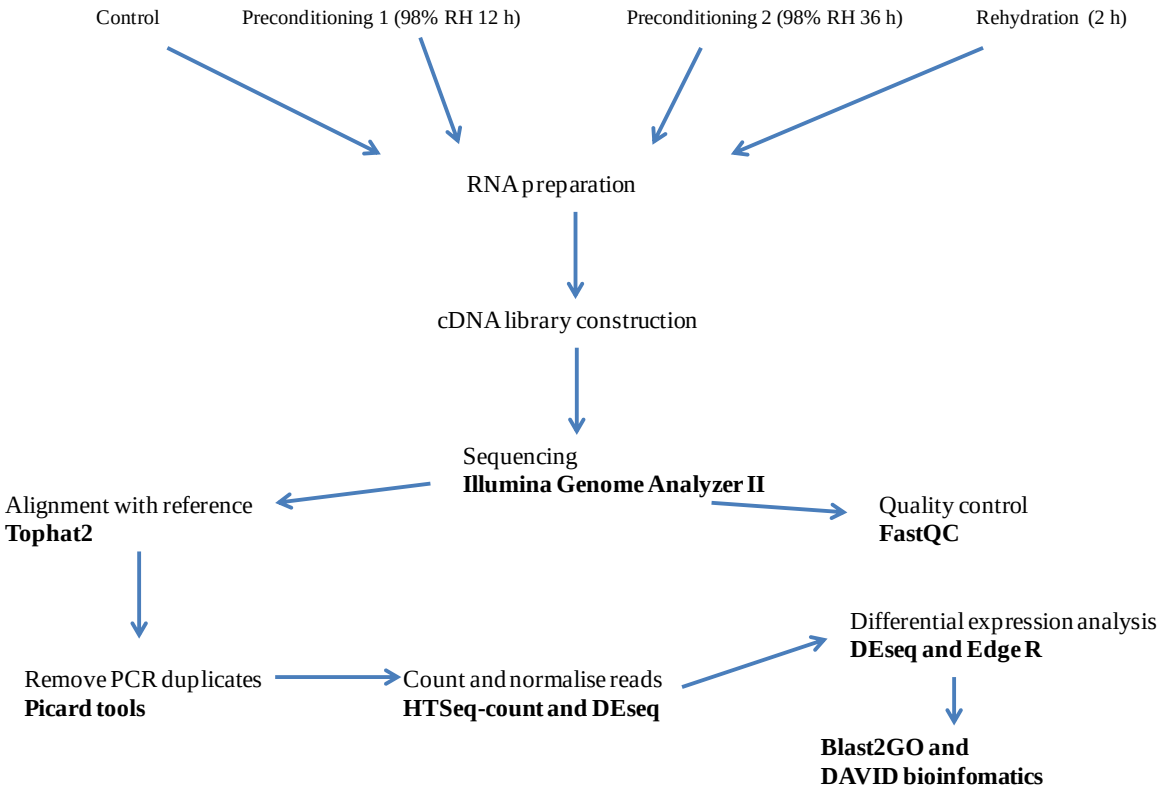


Figure 3.6 Overview of RNA-seq method for *P. superbus* samples. Software/protocols used in RNA preparation and sequence analysis.

T1R1-3: Control
T2R1-3: 98% RH for 12 h
T3R1-3: 98% RH for 36 h
T4R1-3: 2 h recovery

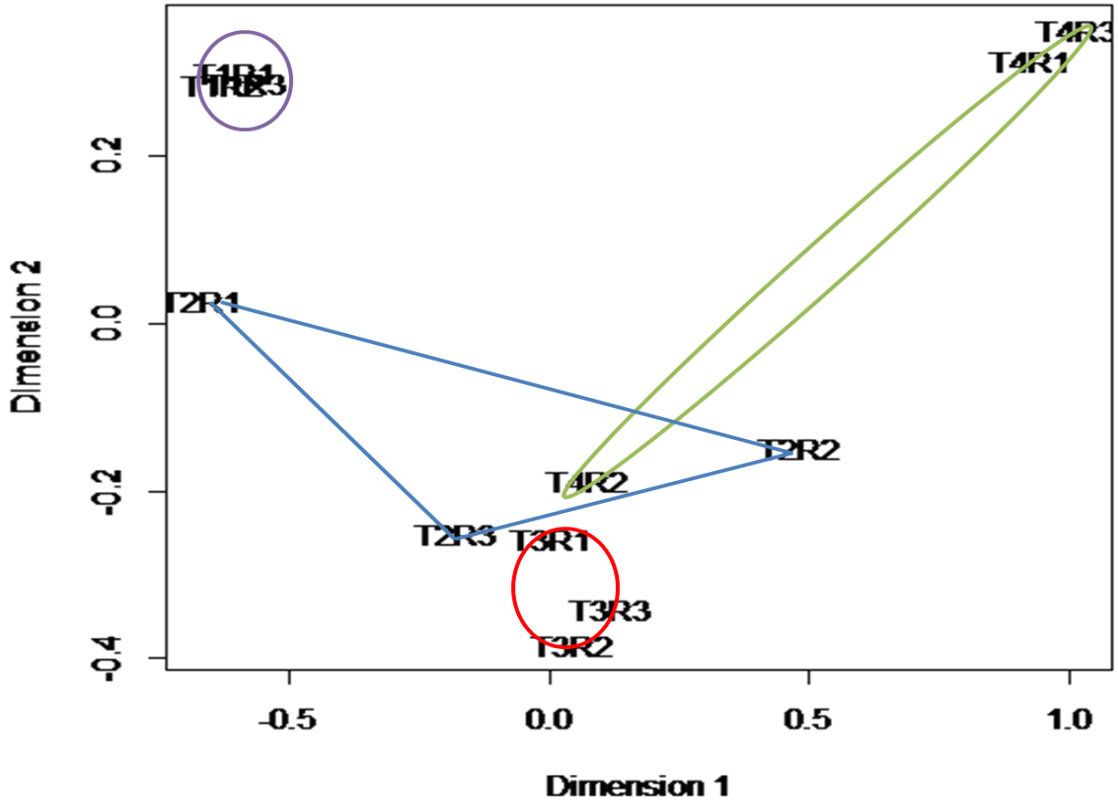


Figure 3.7 Multidimensional scaling (MDS) plot for the *P. superbus* RNA-seq count data showing the relations between the samples in two dimensions. The four treatments are indicated in purple [control (T1R1-T1R3)], blue [preconditioning 1 (T2R1-T2R3)], red [preconditioning 2 (T3R1-T3R3)] and green [recovery (T4R1-T4R3)].

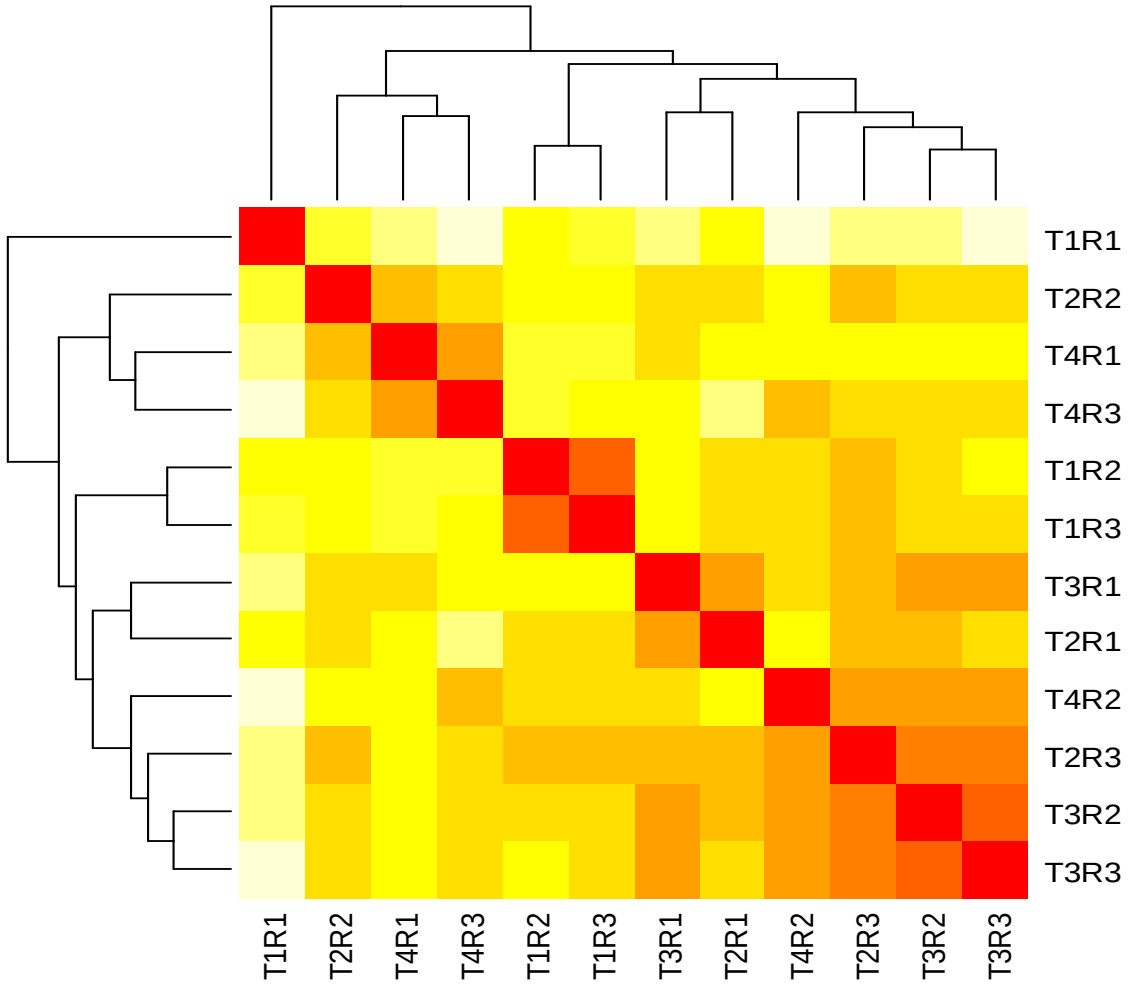


Figure 3.8 A heat map showing the distances between *P. superbus* RNA-seq count data for all samples, as calculated from the variance-stabilising transformation of the count data using the edgeR package (Robinson *et al.*, 2010). The heat map shows a representation of the Euclidean distance matrix and the dendrogram represents a hierarchical clustering of the treatment replicates. T1=control, T2=98% RH for 12 h, T3=98% RH for 36 h and T4=98% RH for 36 h, 0% RH for 24 h followed by rehydration in water for 2 h.

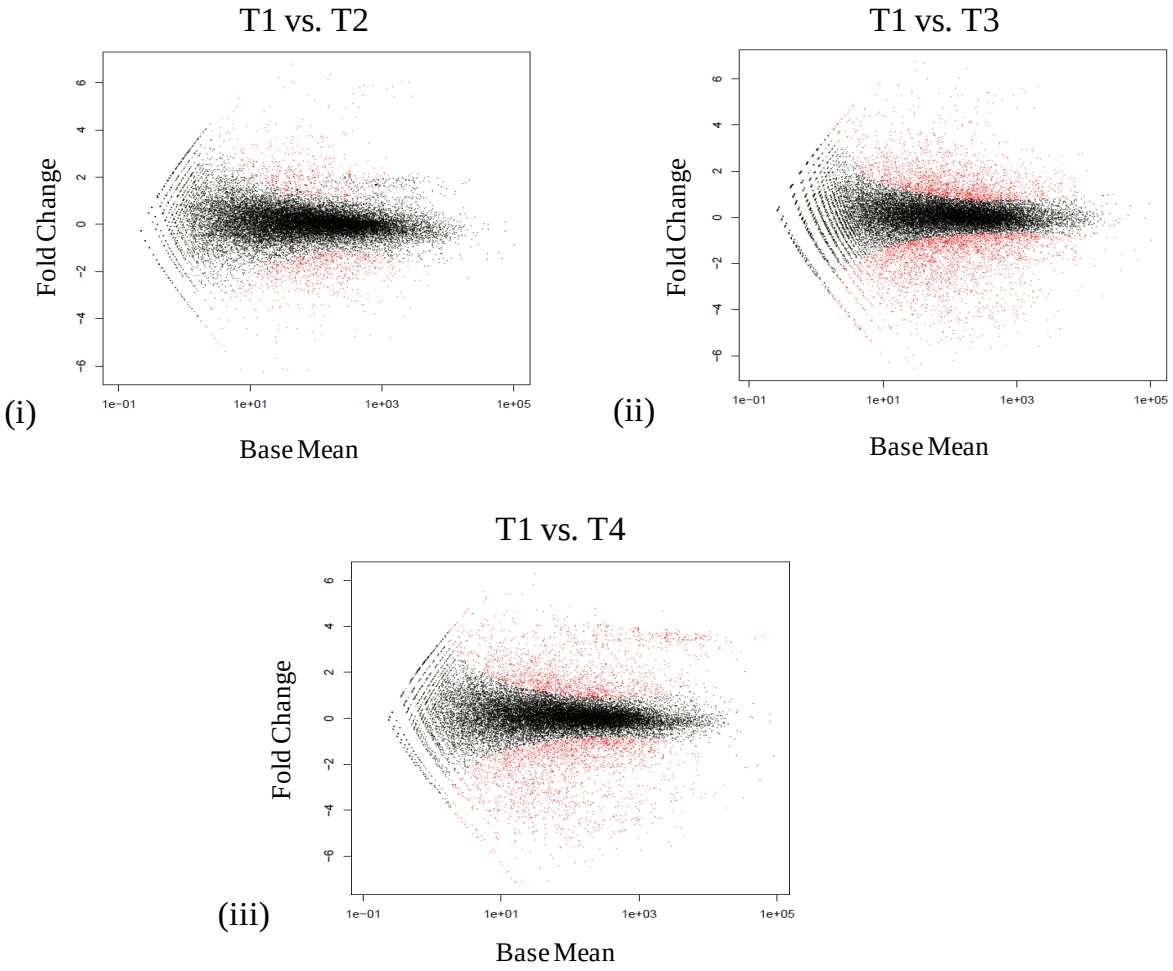


Figure 3.9 Testing for differential expression between the read count data of the RNA-seq samples from *P. superbus*. The treatments compared were: (i) control (T1) and preconditioning 1 (98% RH for 12 h=T2); (ii) control and preconditioning 2 (98% RH for 36 h=T3) and (iii) control and recovery (36 h at 98% RH, 24 h 0% and recovery in S. basal for 2 h=T4). The data represent the read counts for individual gene responses plotted as log2 fold change versus Base Mean (i.e the mean of the normalized counts for each gene averaged over all replicates for each pair of treatments under consideration). The red colour marks those genes detected as differentially expressed at a 10% false discovery rate, when the Benjamini-Hochberg multiple testing adjustment is used. Negative fold change values represent down-regulated genes and positive change values represent up-regulated genes.

3.2.6 Differentially expressed genes

The identities of the *P. superbus* transcripts and the results of the statistical analyses were exported from DEseq into an Excel file. Further analyses were concentrated on those differentially expressed genes which had a >2 fold change in expression and a *p*-adjusted cut-off value <0.01 to ensure a minimal level of false positives. Using these selection parameters a total of 615 genes were differentially expressed in response to the preconditioning treatment 1, 254 of which were up-regulated. In response to preconditioning treatment 2, a total of 2,655 were differentially expressed, 979 of these were up-regulated. Finally, there were 2,344 genes which were differentially expressed during recovery compared to the control, 979 of which were up-regulated (Table 3.1). In this chapter, I have focused on those transcripts which were up-regulated in response to the different treatments, since the genes and gene products which these transcripts represent are likely to have important roles in desiccation tolerance in *P. superbus* nematodes. The *P. superbus* transcripts were identified from BLAST searches against the NCBI database. Using the Blast2GO program (Conesa *et al.*, 2005) Gene Ontology terms were obtained for the differentially expressed genes. The Gene Ontology consortium has developed a vocabulary of defined terms that describe gene products in the context of three domains – Biological Process, Molecular Function and Cellular Component in a species-independent manner (Ashburner *et al.*, 2000). In Figure 3.10, a Venn diagram displays (i) the number of genes significantly up-regulated in response to preconditioning, desiccation and recovery when compared to the control and (ii) the number of gene significantly down-regulated in *P. superbus* in response to preconditioning, desiccation and recovery when compared to the control.

Table 3.1 The number of *P. superbus* genes which had a >2 fold change in expression at a Benjamini-Hochberg *P*-adjusted cut-off value of 0.01 in response to various desiccation-related treatments.

***P*- adjusted cut-off**	**Preconditioning 12 h versus Controls**	**Preconditioning 36 h versus Controls**	**Recovery versus Controls**
>2 fold increase, $p<0.01$	254	979	979
>2 fold decrease, $p<0.01$	361	1,675	1,365
Total	615	2,654	2,344
Number of genes represented*	24,526	24,856	24,914

* The total number of transcripts detected in the two conditions being compared.

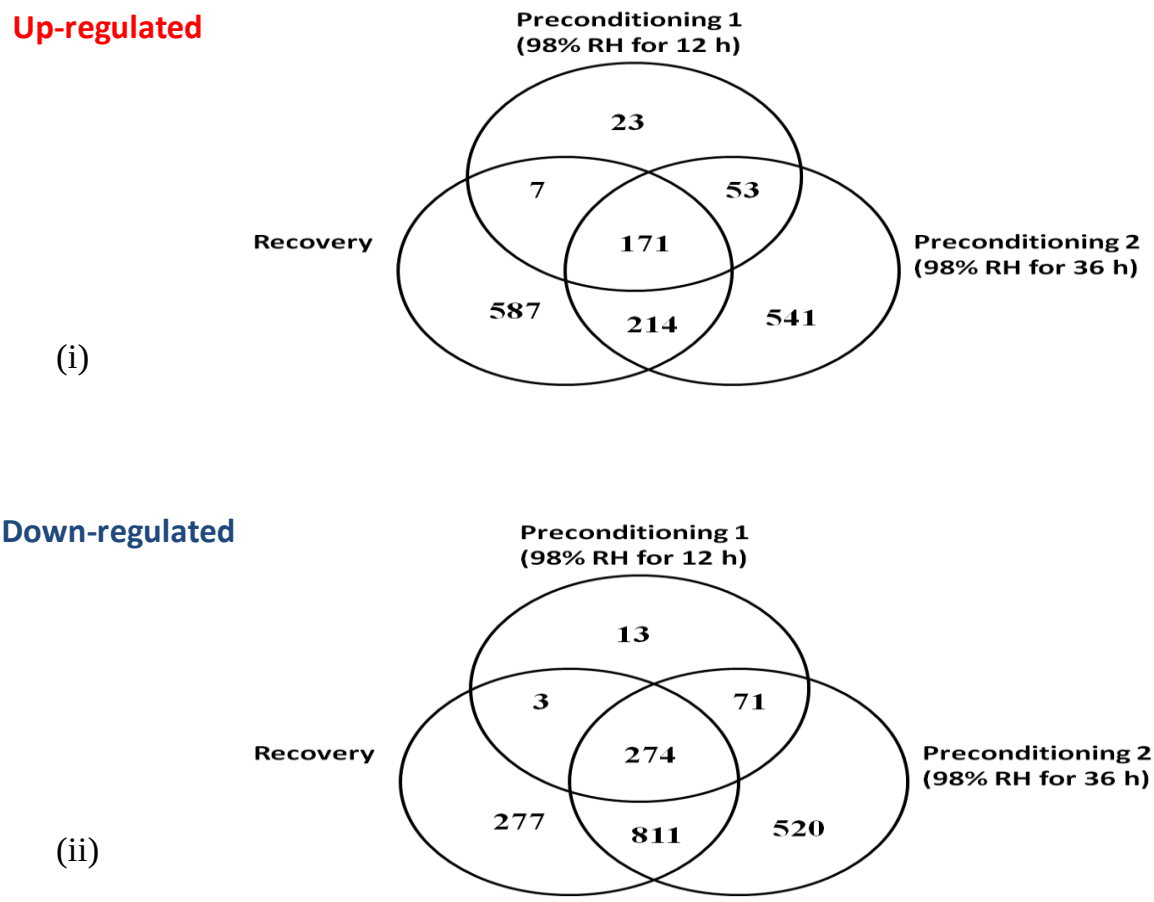
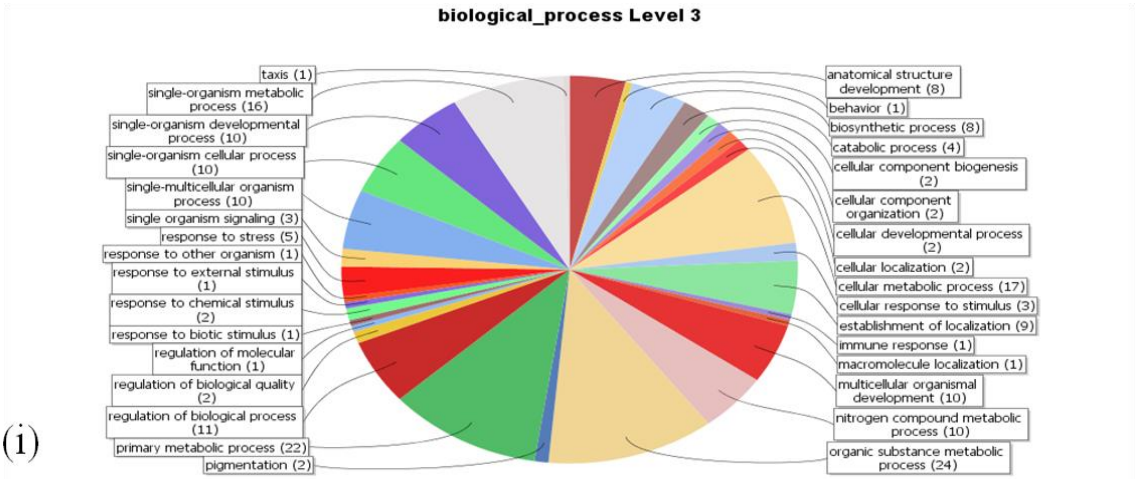


Figure 3.10 (i) the number of *P. superbus* genes significantly up-regulated in response to preconditioning 1, preconditioning 2 and recovery when compared to the control nematodes (ii) the number of *P. superbus* genes significantly down-regulated in response to preconditioning 1, preconditioning 2 and recovery when compared to the control nematodes.

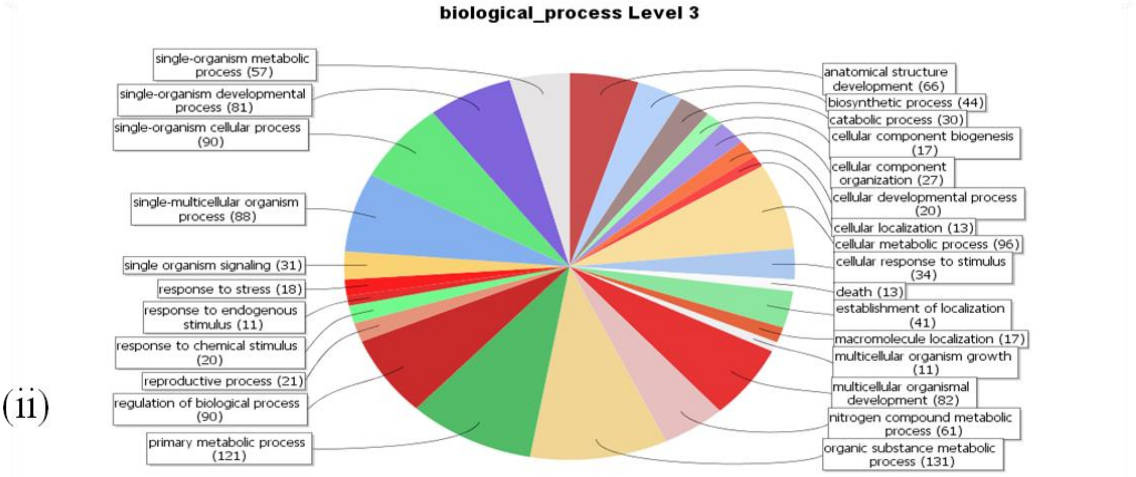
3.2.7 Differentially expressed pathway analysis and functional annotation clustering

Blast2GO is a functional annotation tool and pipeline that was used to analyze RNA-seq differentially expressed genes (Conesa *et al.*, 2005). Blast2GO allows homologous mapping using BLAST. It also integrates the Gene Ontology database, Enzyme Commission database and InterPro database. The results of the BLAST2GO analysis is shown in the supplementary file named "Supplementary data *P. superbus* RNA-seq" and summarised in Figures 3.11-3.13. In order to carry out the DAVID bioinformatics analysis, the gene IDs must be in a form it can recognise. To do this a BLASTX alignment was carried out between the *P. superbus* transcriptome and *C. elegans* transcriptome (version 190 downloaded from Ensembl at www.ensembl.org). Those with hits to *C. elegans* were used for the DAVID bioinformatics analysis. However this approach does mean that *P. superbus* genes which did not have homologs in *C. elegans* were not included in the DAVID analysis (51% of the genes in the *P. superbus* transcriptome lacked a *C. elegans* homolog). DAVID bioinformatics tool was also used to cluster functionally related annotations into groups (Huang *et al.*, 2009). A cut-off enrichment score of >1.3 was set, as this is equivalent to non-log scale of 0.05 (Huang *et al.*, 2009). The results of the DAVID analysis are shown in Tables 3.10-3.15. *C. elegans* gene names are used for *P. superbus* homologs in this chapter because the *C. elegans* genome is well annotated and *C. elegans* gene names are more informative than *P. superbus* transcript names which consist of numbers. In instances where multiple *P. superbus* transcripts had the same *C. elegans* homolog, an additional letter was added to the gene name to distinguish them (e.g. the *P. superbus* transcripts *hsf-1a* and *hsf-1b* are homologs of the *C. elegans hsf-1* gene) For *P. superbus* transcripts with no *C. elegans* homolog, a gene name was assigned based on the top BLAST2GO analysis hit.

(i)



(ii)



(iii)

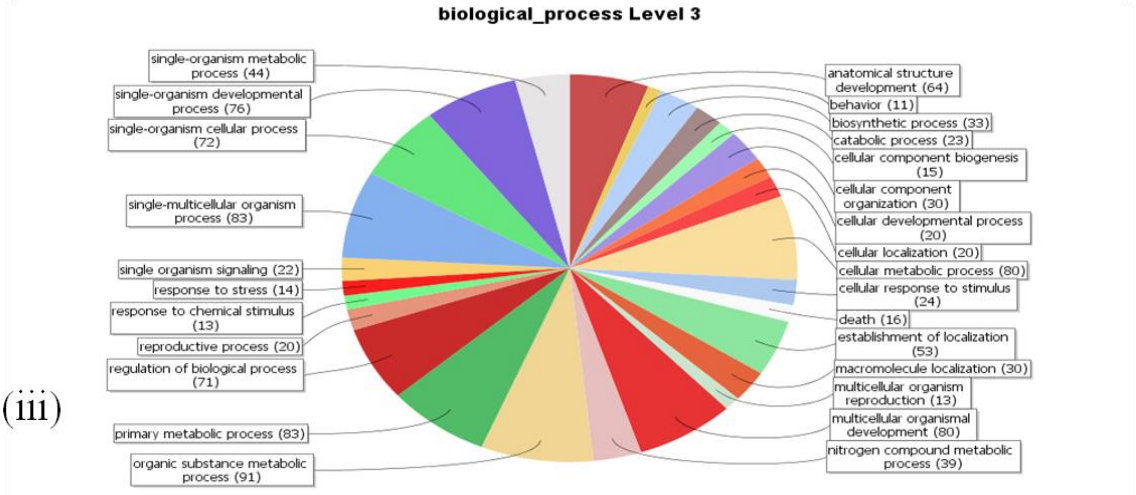


Figure 3.11 Blast2GO biological processes annotation analysis of genes up-regulated in response to (i) preconditioning 1 vs. control, (ii) preconditioning 2 vs. control and (iii) recovery vs. control.

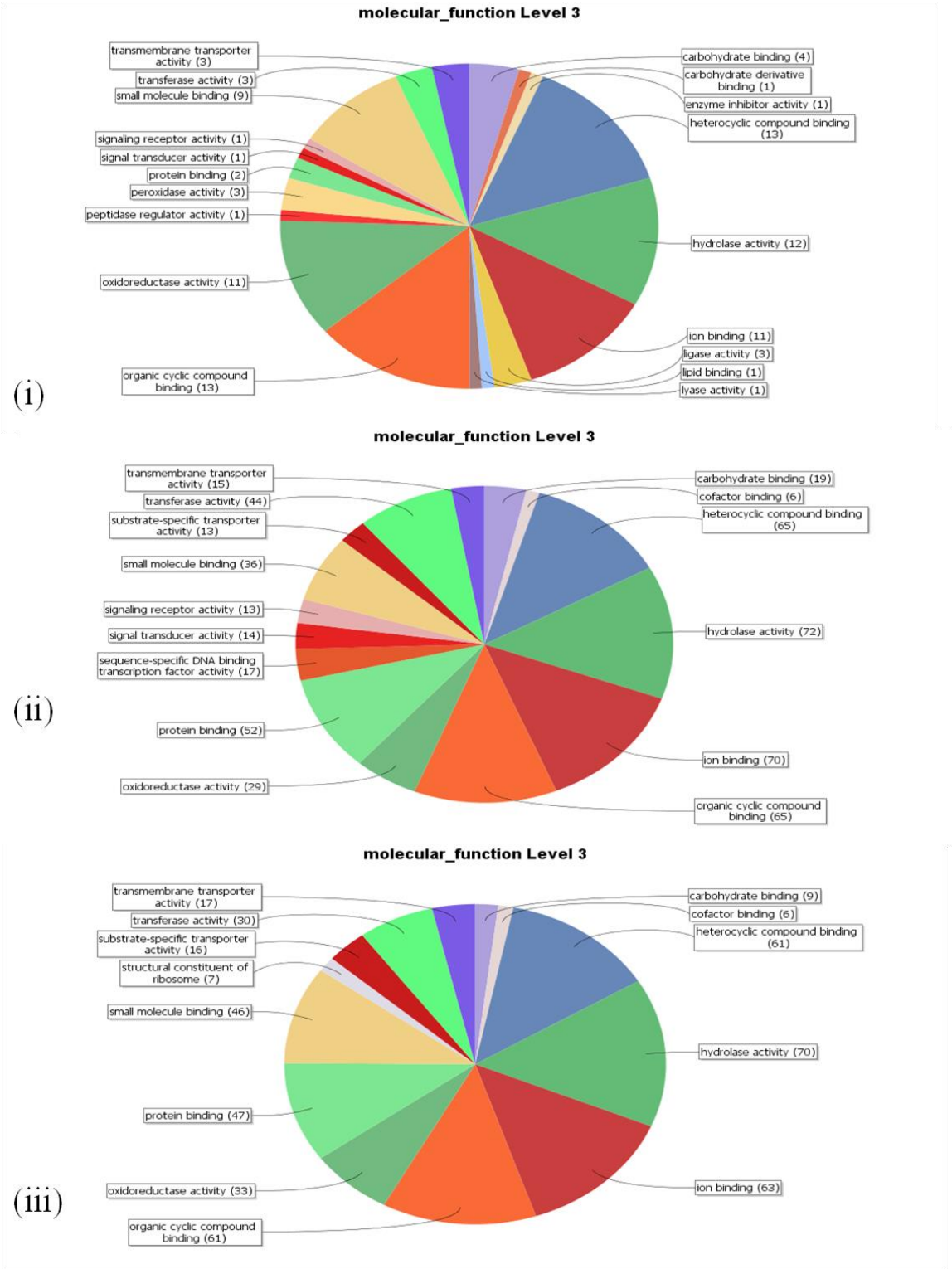


Figure 3.12 Blast2GO molecular function annotation analysis of genes up-regulated in response to (i) preconditioning 1 vs. control, (ii) preconditioning 2 vs. control and (iii) recovery vs. control.

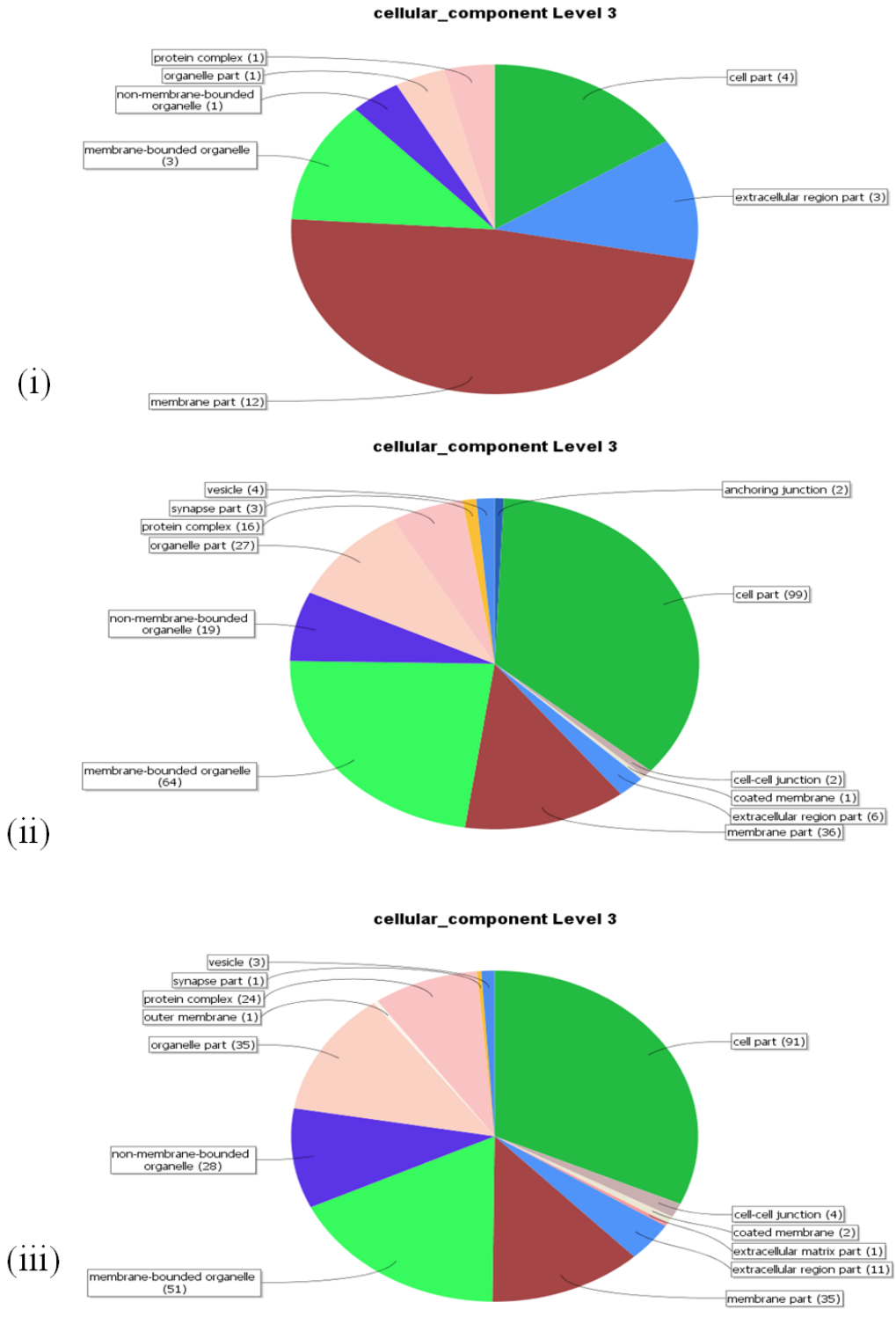


Figure 3.13 Blast2GO cellular component annotation analysis of genes up-regulated in response to (i) preconditioning 1 vs. control, (ii) preconditioning 2 vs. control and (iii) recovery vs. control.

3.2.8 Overview of the genes up-regulated in response to desiccation-related stress in *P. superbus* nematodes.

In this RNA-seq study, a great number of genes were found to be differentially expressed in response to preconditioning and recovery from desiccation in *P. superbus*. The identities of the up-regulated genes are summarised in this section as well as the biological significance of these up-regulated genes. The main pathways and processes implicated by this RNA-seq analysis in the response of *P. superbus* nematodes to desiccation were as follows: signal transduction, translation, cellular organisation, DNA metabolic processes and repair, removal of damaged proteins, antioxidants, molecular chaperones, transport and lipid metabolism (Sections 3.2.8.1-3.2.8.8).

In addition to the processes described in this section a number of individual genes in other pathways were also up-regulated in response to desiccation or during recovery from desiccation, but due to space constraints it was not possible to present this data set in this section. A table listing all the genes which were differentially expressed in response to desiccation-related stress is presented in the supplementary CD appended to this thesis (Supplementary data *P. superbus* RNA-seq).

3.2.8.1 Signal transduction

There were a number of genes up-regulated in *P. superbus* in response to the preconditioning and recovery that map to GO terms associated with signalling and neuron development (Table 3.2). The GO analysis indicates that genes associated with the GO term Signal Transduction are most abundant in the preconditioning 2 time point. However, genes associated with some child terms of Signal Transduction such as Intracellular Signal Transduction and Small GTPase Mediated Signal transduction are more active in the recovery time point. Moreover, genes involved in Neuron Development showed higher expression in the recovery dataset.

The RNA-seq analysis has revealed that insulin signalling may be important response to desiccation in *P. superbus*. The *daf-2* gene, which encodes an insulin receptor, was up-regulated in the preconditioning 1 dataset (98% RH for 12 h). Strikingly, a number of genes downstream of *daf-2* were also up-regulated, as dehydration progressed in the precondition 2 dataset (98% RH for 36 h) which were *age-1*, *hsf-1a*, *hsf-1b*, *aak-2a*, *aak-*

2b, *sir-2.1* and *sir-2.4*. In the recovery dataset, only the *aak-2a*, *aak-2b*, *sir-2.1* and *sir-2.4* genes were up-regulated in *P. superbus*. These AMP activated kinase and sirtuin genes act downstream of *daf-2* and *age-1* which may explain why they are still up-regulated in the recovery dataset. The proposed *C. elegans* insulin signalling pathway is shown in Figure 3.14. The genes relating to this pathway that are up-regulated in *P. superbus* are shown in Figure 3.15. In *C. elegans* a mutation in *daf-2* was found to double the lifespan of the animal (Kenyon, 2005). Insulin/IGF-1 signalling appears to be only one step in a signalling cascade that affects lifespan as *daf-16*, which encodes a FOXO (forkhead box) family transcription factor, is also required (Lin *et al.*, 1997; Kenyon, 2005).

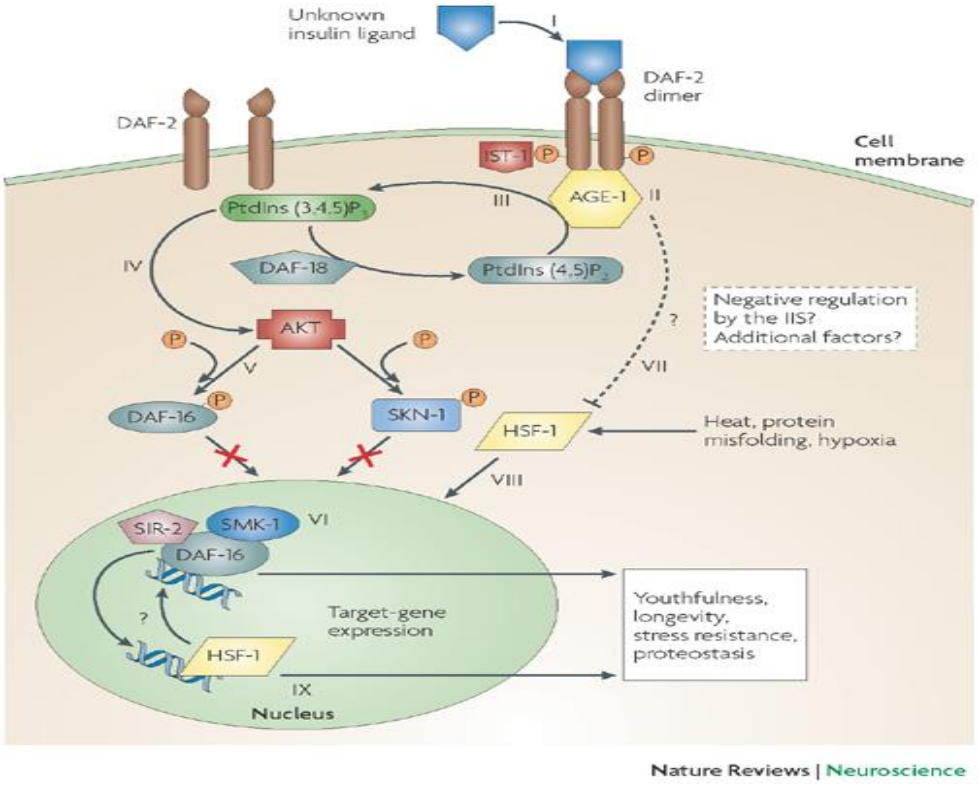


Figure 3.14 Proposed pathway of DAF-2 insulin signalling in *C. elegans* (Cohen and Dillin, 2008).

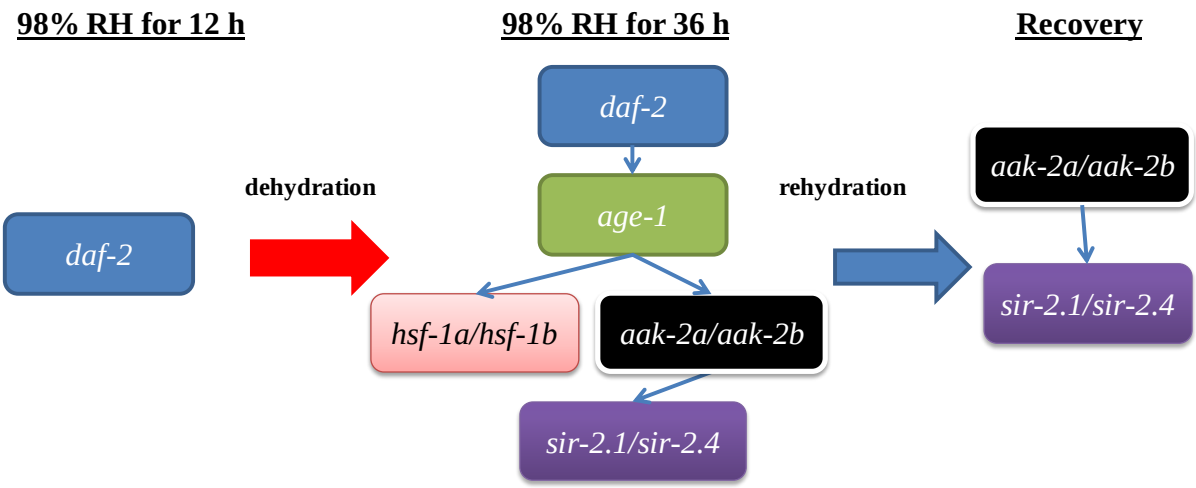


Figure 3.15 Up-regulated genes associated with insulin signalling in *P. superbus* in response to precondition 1 (98% RH for 12 h), preconditioning 2 (98% RH for 12 h) and recovery (98% RH for 36 h, 0% RH for 24 h and rehydration for 2 h).

Along with *daf-2*, the genes *flp-18* (FMRF-like peptide) and *osm-12* (bardet-biedl syndrome related) were up-regulated in the preconditioning 1 time point and are also associated with signal transduction. *flp-18* encodes a FMRFamide-related neuropeptide which associates with NPR-1 and changes social feeding behaviour in *C. elegans* (Rogers *et al.*, 2003). The RNA-seq analysis also revealed that two putative *osm-12* genes were up-regulated in response to preconditioning 1, preconditioning 2 and recovery. *osm-12* is homologous to a gene involved in Bardet-Biedl syndrome (BBS) in humans. BBS is a multi-systemic disorder characterised by obesity, blindness, polydactyly, renal abnormalities and cognitive impairment (Baker and Beales, 2009). In *C. elegans*, *osm-12* is required for cilia function as well as for chemotaxis and is expressed exclusively in ciliated sensory neurons (Blacque *et al.*, 2004; Tan *et al.*, 2007). In the preconditioning 2 dataset, another BBS related gene called *arl-6* was significantly up-regulated along with *osm-12*. In *C. elegans*, *arl-6* encodes a member of the Ras superfamily. Similar to *osm-12*, *arl-6* has been implicated in ciliary transport and is expressed in ciliated sensory neurons (Fan *et al.*, 2004; Tan *et al.*, 2007). In *P. superbus*, *osm-12* and *arl-6* may play a sensory role in recognising desiccation stress and initiating a signal transduction cascade. This may result in the production of effector proteins to enhance the nematode's tolerance to desiccation.

In the preconditioning 2 dataset, eleven nuclear hormone receptor (NHR) genes are up-regulated which are homologous to six *C. elegans* genes called *nhr-19*, *nhr-36*, *nhr-45*, *nhr-46*, *nhr-64* and *nhr-141*. Moreover, *nhr-64* is the only NHR to be up-regulated in both preconditioning 2 and recovery. NHRs are transcription factors that respond to lipophilic molecules to regulate the expression of target genes involved in metabolism, reproduction and development (Liang *et al.*, 2010). There are a total of 284 NHRs in the *C. elegans* genome, several of which have been implicated in lipid metabolism. Interestingly, a number of genes associated with lipid metabolism are up-regulated in the preconditioning 2 and recovery datasets which may have been activated by the *nhr* genes.

3.2.8.2 Translation

There were a number of genes up-regulated in *P. superbus* in response to preconditioning and recovery that map to GO terms associated with translation as shown in Table 3.3. The genes up-regulated in the recovery dataset encode proteins involved in translation including eukaryotic peptide chain release factor proteins and ribosomal proteins (Figure 3.16). In both the GO analysis and the expression profile of genes encoding proteins involved in translation, there is a clear trend, showing that the expression values for these genes peak during recovery.

There are obvious differences in the strategies used by *P. superbus* and *C. elegans* to combat desiccation. The RNA-seq study of desiccation tolerance in *C. elegans* (Chapter V) reveals that there were a great number of genes involved in translation that were up-regulated at the 98% RH for 48 h time point (87 genes map to the translation GO term) with similar numbers of genes up-regulated in the recovery time point (90 genes map to the translation GO term). In contrast, there were few *P. superbus* genes involved in translation up-regulated at the 98% RH for 36 h time point. This suggests that in *P. superbus* the proteins required to survive desiccation are already synthesized. Further evidence, supporting this possibility is the observation that *P. superbus* nematodes can survive harsh desiccation without any prior preconditioning (98% RH) while *C. elegans* dauers require extensive preconditioning to enable them to survive mild desiccation.

3.2.8.3 Cellular organisation

The number of genes up-regulated in *P. superbus* in response to desiccation-related treatments that map to GO terms related to cellular organisation is shown in Table 3.4. Strikingly, the number of up-regulated genes associated with cellular organisation increases substantially from precondition 1 to preconditioning 2 which is maintained into recovery from desiccation. However, the up-regulated genes which map to GO terms concerned with cellular organisation differ greatly between the preconditioning and desiccation datasets. Genes up-regulated in response to preconditioning 2 and recovery which map to the GO term anatomical structure development include *fat-6* (fatty acid desaturase), *rpn-7* (proteasome regulatory particle), *osm-12* (relate to bardet-biedl syndrome-7 gene) and *cht-1* (chitinase).

3.2.8.4 DNA metabolic processes and repair

The number of genes up-regulated in *P. superbus* in response to desiccation-related treatments that map to GO terms related to DNA metabolic processes and repair is shown in Table 3.5. There were 3 genes significantly up-regulated in the preconditioning 2 dataset that were associated with the GO term DNA repair *viz. sprt* (spartan), *ck-1* (casein kinase) and *sirt-6* (sirtuin) (Figure 3.17). In the recovery dataset, *sirt-6* was the only gene that mapped to the GO term DNA repair that was up-regulated. Interestingly, *ck-1* and *sirt-6* exhibit a similar expression pattern in response to desiccation and recovery in *P. superbus*. Additionally, a number of genes which are most closely related to *chk-1* (check-point kinase) were also up-regulated. While *chk-1* is not associated with the GO term DNA repair, previous studies have linked this gene to DNA repair (Patil *et al.*, 2013). In total 23 *P. superbus* genes related to *chk-1* were up-regulated with expression clearly peaking in the recovery dataset (Figure 3.17).

Caesein kinase 1 (*ck-1*) is a ubiquitous serine/threonine-specific protein kinase and comprises the majority of kinase activity in the eukaryotic cell (Issinger, 1993; Kusuda *et al.*, 1996). The link between *ck-1* and DNA damage was first revealed in yeast. It was discovered that that mutations in the *ck-1* gene in *S. cerevisiae* and *S. pombe*, resulted in severely reduced growth when treated with DNA damaging agents (Hoekstra *et al.*, 1991; Demaggio *et al.*, 1992; Dhillon and Hoekstra, 1994). Moreover, studies in *Drosophilia*

melanogaster embryos have shown that cytoplasmic CK-1 relocalizes to the interphase nucleus upon irradiation which causes DNA breakages.

Recent studies in mammalian cells subjected to oxidative stress have found that SIRT-6 is recruited to the sites of DNA double-stranded breaks and stimulates repair by activating PARP-1 (poly[adenosine diphosphate (ADP)–ribose] polymerase 1) (Mao *et al.*, 2011). Repair of DNA double stranded breaks is essential for longevity (Gorbunova and Seluanov, 2005). Evidence for this is seen in the fact that mice deficient for SIRT-6 exhibit a premature aging phenotype associated with base repair excision (Mostoslavsky *et al.*, 2006). Moreover, overexpression of SIRT-6 in human fibroblast cells strongly stimulates double-stranded break repair leading to increased lifespan in the cells (Mao *et al.*, 2012). Another gene called *sprt* (spartan) was up-regulated in response to preconditioning 2 in *P. superbus*. SPRT plays an important role in the cellular resistance to UV radiation. It does this by recognizing ubiquitylated PCNA (proliferating cell nuclear antigen) which serves as a molecular marker to orchestrate post replication repair (Centore *et al.*, 2012). Specific to the recovery dataset, a gene involved in nucleotide excision repair (NER) called *rhp-16* was significantly up-regulated in *P. superbus*. NER is a versatile mechanism capable of removing a large variety of lesions from the genome (Lombaerts *et al.*, 1999). The mutation of *rad-16* (homolog of *rhp-16*) in *S. cerevisiae* results in increased sensitivity to UV light (Tijsterman *et al.*, 1997).

3.2.8.5 Removal of damaged proteins

There were a number of genes that map to GO terms relating to the removal of damaged proteins, which were up-regulated in *P. superbus* in response to preconditioning 2 and recovery as outlined in Table 3.6. Moreover, there were a greater number of up-regulated genes associated with removal of damaged proteins in the recovery dataset compared to the preconditioning 2 dataset. The expression of some of these up-regulated genes involved in protein degradation is shown in Figures 3.18 and 3.19. In the recovery dataset, genes encoding proteasome proteins (*rpn-7* and *rpn-4*) and ubiquitin proteins (*rnf-14*, *arih-2* and *uch*) were significantly up-regulated. The gene *rpn-7* is predicted to encode a non-ATPase subunit of the 19S regulatory complex of the proteasome. RNAi knockdown of this gene in *C. elegans* affects fertility and embryonic viability (Kamath and Ahringer, 2003; Simmer *et al.*, 2003). Another gene predicted to encode a proteasome regulatory

particle called *rpn-4* (26s proteasome regulatory chain 4) had increased expression in the recovery dataset. There was a substantial increase in expression of certain ubiquitin proteins (*rnf-14*, *arih-2* and *uch*) specifically in the recovery dataset. The two genes predicted to encode ubiquitin ligase proteins called *arih-2* and *rnf-14* had top BLASTX hits in the sea squirt *Ciona intestinalis* and seed grass *Aegilops tauschii* respectively. The E3 ligase ARIH-2 family have a unique mechanism of elongating ubiquitin chains that distinguishes them from other ligases (Wenzel *et al.*, 2011). The gene *rnf-14* is predicted to encode an E3 ubiquitin protein ligase which accepts ubiquitin from specific E2 ubiquitin-conjugating enzymes and then transfers it to substrates (Xu *et al.*, 2009).

The two genes *pdcd-6a* (programmed cell death protein) and *pdcd-6b* are both up-regulated only in the recovery dataset and are predicted to encode a calcium-binding protein required for apoptotic cell death (Lee *et al.*, 2005). Other genes involved in apoptosis, which have increased expression in *P. superbus* in response to recovery from desiccation, include *aif-1* (apoptosis-inducing factor) and *hipr-1* (huntingtin-interacting protein related). Studies in mammalian cell lines have revealed that AIF-1 has an important role in caspase-independent apoptosis. In response to apoptotic stimuli, AIF-1 is released from the mitochondrion intermembrane space into the cytosol and to the nucleus. In the nucleus, it functions as a proapoptotic factor in a caspase-independent pathway (Kim *et al.*, 2006). The HIP-1 (Huntingtin interacting protein 1) was first identified through its interaction with the Huntington's disease protein HTT (Huntingtin) (Parker *et al.*, 2007). Huntington's disease is a neurodegenerative disorder, associated with the expansion of a polyglutamine stretch at the amino terminus of the HTT protein (Gervais *et al.*, 2002). This results in neuronal loss in the striatum, which seems to occur by apoptosis (Porteracailliau *et al.*, 1995). In *C. elegans*, knockout studies of the *hip-1* homolog (*hipr-1*) revealed that it modulates presynaptic activity and the abundance of synaptobrevin, a protein involved in synaptic vesicle fusion (Parker *et al.*, 2007). Another gene associated with degenerative cell death in neurons is *clp-1* (calpain) (Syntichaki *et al.*, 2002) and was also found to be up-regulated in *P. superbus* in response to recovery from desiccation.

3.2.8.6 Antioxidants

The expression of genes up-regulated in *P. superbus* in response to desiccation-related treatments that are associated with antioxidant activity is shown in Figure 3.20. Genes which encode antioxidant proteins including superoxide dismutase (*sod-4a*, *sod-4b* and *sod-5*), animal heme peroxidase (*ahp-a* and *ahp-b*), glutathione S-transferase (*gst-8*) and nucleoredoxin (*nrx-a* and *nrx-b*) showed increased expression mainly in the preconditioning 1 and preconditioning 2 datasets. Additionally, the *glrx* (glutaredoxin) gene showed increased expression in the recovery dataset only. The superoxide dismutase transcripts (*sod4a*, *sod-4b* and *sod-5*) which are up-regulated in response to preconditioning 1 and preconditioning 2 in *P. superbus*, encode metalloenzymes responsible for quenching the potentially deleterious effects of superoxide free radicals by converting them to hydrogen peroxide. In *C. elegans*, the *sod-4* gene expresses an extracellular Cu/Zn SOD. Additionally, knocking-out the *sod-4* gene in *C. elegans* enhances *daf-2* longevity and constitutive diapauses, suggesting a signalling role for *sod-4*. The SOD-4 antioxidant activity might generate H_2O_2, which could then cross into the cell and promote insulin signalling by inhibiting signal quenching phosphatases (Doonan *et al.*, 2008). In *C. elegans*, *sod-1* and *sod-5* encode Cu/Zn SODs that are present in the cytosol (Yanase *et al.*, 2009).

In *C. elegans* there are 44 annotated glutathione S-transferase (GST) genes. In this RNA-seq study, the gene *gst-8* was up-regulated in the preconditioning 2 dataset only. In *C. elegans* knockdown of *gst-8* (along with *gst-5*, *gst-6* and *gst-10*) results in sensitivity to electrophilic stress elicited by exposure to 4-hydroxynon-2-enal (4-HNE). The lipid peroxidation product 4-HNE, forms as a consequence of oxidative stress and acts as a signalling molecule or, at higher concentrations, as a toxin. The steady state concentration of 4-HNE reflects the balance between its generation and metabolism, mostly through glutathione conjugation (Ayyadevara *et al.*, 2005; Ayyadevara *et al.*, 2007).

Nucleoredoxin is a member of the thioredoxin family of proteins, that are involved in various biological process by regulating the response to oxidative stress (Funato *et al.*, 2007). It has been found that nucleoredoxin protein is mainly localized in the nucleus of cells which separates it from other thioredoxin proteins (Kurooka *et al.*, 1997). The

transcripts *nrx-a* and *nrx-b*, which encode nucleoredoxin proteins, were up-regulated in *P. superbus* in response to preconditioning 1 and preconditioning 2.

3.2.8.7 Cellular Protection

The expression of genes up-regulated in *P. superbus* in response to desiccation-related treatments that encode heat shock proteins (HSPs) or are associated with HSPs is shown in Figure 3.21. These genes encode proteins associated with chaperone activity such as HSP-70, *(hsp-70a*, *hsp-70b*, *hsp-70c*, *hsp-70d*, *hsp-70e*, *hsp-70f*, *hsp-70g* and *hsp-1*), small HSP (*hsp-12.2* and *hsp-20*) and heat shock transcription factor (*hsf-1a* and *hsf-1b*). The expression of the majority of the up-regulated genes encoding HSPs peaks during the preconditioning 2 time point (Figure 3.22 (i)). However there were several HSP genes whose expression peaked in the recovery dataset in *P. superbus* (Figure 3.21 (ii)). Most of the *hsp* genes up-regulated in response to desiccation in *P. superbus* (8 genes) encode members of HSP-70 family. A phylogenetic tree of *P. superbus hsp-70* sequences up-regulated in response to preconditioning, desiccation and recovery is shown in Figure 3.22. The DAVID bioinformatics analysis shows that signal transduction in response to unfolded proteins may contribute to desiccation tolerance in *P. superbus*. The up-regulated transcripts in the recovery dataset were significantly enriched with functional annotations relating to the unfolded protein response (UPR) pathway (Table 3.12). There were three genes associated with the UPR pathway that were up-regulated in the recovery dataset in *P. superbus*. These were: heat shock protein (*hsp-70*); a sarco-endoplasmic reticulum protein (*sca-1*) and a protein involved in apoptosis signalling (*ced-1*).

It was first discovered by Yochem *et al.* (1978) that a protein called DnaJ stimulates the ATPase activity of the bacterial HSP-70 homolog DnaK (Yochem *et al.*, 1978; Liberek *et al.*, 1988). In the RNA-seq analysis of desiccation tolerance in *P. superbus*, there was a single gene encoding a DnaJ protein that was up-regulated in response to preconditioning 2 in *P. superbus* (Figure 3.21 (i)).

As well as HSP-70, small heat shock proteins (sHSPs) seem to play an important role in the response to desiccation. In *P. superbus*, two genes which encode small HSPs (*hsp-12.2* and *hsp-20*) were up-regulated in response to preconditioning and recovery. *hsp-12.2* encodes a small heat-shock protein that forms heterotetramers with HSP-12.3 and

displays no chaperone-like activity (Kokke *et al.*, 1998). HSP-12.2 is mainly associated with reproductive tissue in *C. elegans* nematodes (Ding and Candido, 2000). The small heat shock protein HSP-20 was first isolated from rat and human skeletal muscle (Kato *et al.*, 1994). HSP-20 is not normally induced by stress as it is already constitutively expressed but is classified as a heat shock protein based on sequence similarity (Sugiyama *et al.*, 2000). Moreover, HSP-20 shares considerable sequence homology to other heat shock proteins, such as the myotonic dystrophy kinase binding protein HSP-27 and the alpha crystallins. Similar to HSP-12.2, HSP-20 is a poor molecular chaperone in terms of reconstituting enzymatic activity (Van De Klundert *et al.*, 1999). HSP-20 is highly and constitutively expressed in skeletal, cardiac and smooth muscle (Kato *et al.*, 1994). Much of the research into the function of HSP-20 has focused on its association with actin and the modulation of smooth-muscle relaxation (Tessier *et al.*, 2003; Matsushima-Nishiwaki *et al.*, 2011). Studies by Tessier *et al.* (2003) have suggested that phosphorylation of HSP-20 may lead to the relaxation of vascular smooth muscles through a dynamic association with the cytoskeleton. As phosphorylation of HSP-20 increases the association of HSP-20 with α-actinin decreases, suggesting that HSP-20 is dynamically associated with this actin-binding protein (North *et al.*, 1994; Tessier *et al.*, 2003). Furthermore, diverse cellular processes such as cell adhesion, cytokinesis, cell motility, migration and contraction all require dynamic reorganisation of the cytoskeleton (North *et al.*, 1994; Tessier *et al.*, 2003).

The RNA-seq analysis revealed that two genes called *hsf-1a* (heat shock factor) and *hsf-1b* were up-regulated in response to preconditioning 2 in *P. superbus*. In *C. elegans*, HSF-1 acts downstream of DAF-2/DAF-16 in defense response and confers protection through a system of HSPs, independently of the p38 MARK pathway (Singh and Aballay, 2006). RNAi knockdown of *hsf-1* in *C. elegans* results in reduced mRNA expression in genes which encode small HSPs (*hsp-16.2* and *sip-1*) as well as HSP-70 (Chiang *et al.*, 2012). Additionally, HSF-1 along with DAF-16 is required for longevity of *daf-2* mutants (Garigan *et al.*, 2002). Conversely, animals carrying additional *hsf-1* gene copies lived 40% longer than normal (Hsu *et al.*, 2003). HSF-1 is also part of a multipathogen defense pathway and is required for immunity against *Pseudomonas aeruginosa*, *Salmonella enterica*, *Yersinia pestis*, and *Enterococcus faecalis* in *C. elegans* (Kim *et al.*, 2002; Aballay *et al.*, 2003; Kim *et al.*, 2004; Young and Dillin, 2004).

The RNA-seq study also revealed that in *P. superbus* two *lea* (late embryogenesis protein) genes and a *djr-1.1* gene were significantly up-regulated across precondition, desiccation and recovery as shown in Figure 3.23. LEA proteins are extremely hydrophilic and are thought to provide protection to other proteins through molecular shield activity (Chakrabortee *et al*., 2012). Mutations in human DJ-1 are the cause of a form of recessive familiar Parkinson's disease (Park7). The human DJ-1 protein has been seen shown *in vitro* to function as a redox dependent molecular chaperone (Zhou *et al.*, 2006).

3.2.8.8 Transport and lipid metabolism

The RNA-seq analysis in *P. superbus* revealed that transport and lipid metabolism may be important during desiccation. Genes associated with transport are up-regulated in *P. superbus*, particularly in response to preconditioning 2 and recovery. The expression of genes associated with transport peaks during recovery, with 53 genes mapping to the GO term Transport up-regulated in the recovery dataset in *P. superbus* (Table 3.8). Some of the genes up-regulated in response to precondition 2 and recovery which are associated with transport are shown in Figure 3.24.

The *haf-2* (half-type ABC transporter) and *haf-4* genes encode a half-molecular ATP-binding cassette (ABC) transporter and are up-regulated in response to preconditioning and recovery. ABC transporters are one of the largest families of transport proteins. ABC transporters are collectively able to accommodate an unusually large array of different substrates (Sheps *et al.*, 2004). The *haf-2* and *haf-4* ABC transporters may be important in the transport of water to and from the cell during desiccation and rehydration. A member of the aquaporin (AQP) gene family, *aqp-3* was also up-regulated in response to preconditioning and recovery. Aquaporin channels facilitate transport of water, glycerol, and other small solutes across cell membranes. Studies carried out in *Xenopus* oocytes have increased the understanding of the function of specific *C. elegans* aquaporins. It was found that expression of *aqp-1* and *aqp-3* increased the glycerol permeability three to sevenfold, while the expression of *aqp-3* also increased the water permeability five- to sevenfold (Huang *et al.*, 2007). Interestingly, in the RNA-seq analysis of desiccation in *C. elegans* (Chapter V), *aqp-1* showed increased expression in the recovery dataset. The role of *aqp-3* could be essential in *P. superbus* during dehydration and rehydration. In

response to desiccation, proteins such as AQP-3 may facilitate the import of protective osmolytes such as the disaccharide trehalose into *P. superbus* cells. Trehalose has been found to stabilize proteins in their native state and to preserve the integrity of membranes during dehydration (Crowe, 2002). *pept-1* encodes a oligopeptide transporter and is up-regulated in response to preconditioning and recovery in *P. superbus*. In *C. elegans*, *pept-1* is required for uptake of intact peptides from the intestine and thus for normal development, growth and reproduction. Additionally, *pept-1* interacts with both the daf-2/insulin and let-363/TOR signalling pathways in *C. elegans*. *pept-1* may be synthesized in preparation for recovery in *P. superbus* when normal development and growth returns.

There are a number of genes which encode proteins involved in lipid metabolic processes that are up-regulated in *P. superbus* particularly in response to preconditioning 2 and recovery. These include genes encoding fatty acid desaturase (*fat-6* and *des-2*), abc transporters (*hal-2* and *hal-4*) and cytochrome P450 (*cyp-33C9*). The integrity of the lipid membrane is believed to be important in organisms conferring tolerance to desiccation. The gene encoding phosphatidylserine decarboxylase (*psd-1*) in *P. superbus* was up-regulated in response to all three conditions of preconditioning 1, preconditioning 2 and recovery. Studies in the root cells of *Avena sativa* (oat) have found that phosphatidylserine is up-regulated in response to desiccation stress (Larsson *et al.*, 2006).

Table 3.2 The number of up-regulated genes in *P. superbus* in response to preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery that map to GO terms associated with signalling. The Benjamini-Hochberg method was used for the correction of the *p*-values. The BLAST2GO program was used to map genes to GO terms.

GO-ID	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child of (Go ID)**
		Preconditioning 1	**Preconditioning 2**	**Recovery**	
7165	Signal transduction	3	29	21	
35556	Intracellular signal transduction	0	6	9	7165
23051	Regulation of signaling	0	4	11	
16055	Wnt receptor signaling pathway	0	3	1	
7264	Small GTPase mediated signal transduction	0	3	4	

7399	Nervous system development	1	9	9	
22008	Neurogenesis	0	5	8	7399
48666	Neuron development	0	2	7	
31175	Neuron projection development	0	1	6	
7268	Synaptic transmission	0	2	2	
48488	Synaptic vesicle endocytosis	0	2	0	

Table 3.3 The number of up-regulated genes in *P. superbus* in response to preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery that map to GO terms associated with Translation. The Benjamini-Hochberg method was used for the correction of the *p*-values. The BLAST2GO program was used to map genes to GO terms.

GO-ID	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test** $p<0.05$**)**			**child of (Go ID)**
		Preconditioning 1	**Preconditioning 2**	**Recovery**	
6412	Translation	1	3	11	
6413	Translational initiation	0	1	2	6412
42254	Ribosome biogenesis	0	3	4	
33750	Ribosome localization	0	1	2	

Table 3.4 The number of up-regulated genes in *P. superbus* in response to preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery that map to GO terms associated with cellular organisation. The Benjamini-Hochberg method was used for the correction of the *p*-values. The BLAST2GO program was used to map genes to GO terms.

GO-ID	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test** $p<0.05$**)**			**child of (Go ID)**
		Preconditioning 1	**Preconditioning 2**	**Recovery**	
48856	Anatomical structure development	8	66	64	
9653	Anatomical structure morphogenesis	4	28	22	48856
51234	Establishment of localization	9	41	53	
51649	Establishment of localization in cell	2	12	20	51234
72663	Establishment of protein localization to peroxisome	0	2	0	51234
7010	Cytoskeleton organization	0	9	8	
30036	Actin cytoskeleton organization	0	7	7	
31032	Actomyosin structure organization	0	5	5	30036
60429	Epithelium development	2	6	9	
7017	Microtubule-based process	1	3	2	
2009	Morphogenesis of an epithelium	2	6	8	
61061	Muscle structure development	0	8	9	

61024	Membrane organization	0	5	9	
10324	Membrane invagination	0	2	5	
6643	Membrane lipid metabolic process	2	3	2	

Table 3.5 The number of up-regulated genes in *P. superbus* in response to preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery that map to GO terms associated with DNA packaging and repair. The Benjamini-Hochberg method was used for the correction of the *p*-values. The BLAST2GO program was used to map genes to GO terms.

GO-ID	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child of (Go ID)**
		Preconditioning 1	**Preconditioning 2**	**Recovery**	
6259	DNA metabolic process	1	6	5	
6281	DNA repair	0	3	1	6259
6260	DNA replication	0	1	3	6259
51170	Nuclear import	0	1	3	

Table 3.6 The number of up-regulated genes in *P. superbus* in response to preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery that map to GO terms associated with cell death, apoptosis and ubiquitin dependent processes. The Benjamini-Hochberg method was used for the correction of the *p*-values. The BLAST2GO program was used to map genes to GO terms.

GO-ID	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child of (Go ID)**
		Preconditioning 1	**Preconditioning 2**	**Recovery**	
8219	Cell death	0	13	16	
12501	Programmed cell death	0	9	13	8219
6915	Apoptotic process	0	8	11	8219
97190	Apoptotic signaling pathway	0	1	3	8219
43161	Proteasomal ubiquitin-dependent protein catabolic process	0	1	2	
6898	Receptor-mediated endocytosis	0	7	10	
6511	Ubiquitin-dependent protein catabolic process	0	1	3	
43652	Engulfment of apoptotic cell	0	2	5	
43277	Apoptotic cell clearance	0	2	5	
6508	Proteolysis	1	14	9	

Table 3.7 The number of up-regulated genes in *P. superbus* in response to preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery

that map to GO terms associated with response to stress and aging. The Benjamini-Hochberg method was used for the correction of the *p*-values. The BLAST2GO program was used to map genes to GO terms.

GO-ID	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child of (Go ID)**
		Preconditioning 1	**Preconditioning 2**	**Recovery**	
7568	Aging	2	21	21	7568
6950	Response to stress	5	18	14	
33554	Cellular response to stress	0	6	2	6950
6952	Defense response	2	5	5	6950
6970	Response to osmotic stress	0	2	0	6950
6979	Response to oxidative stress	3	4	3	6950
80134	Regulation of response to stress	0	3	2	6950
72593	Reactive oxygen species metabolic process	1	3	0	
50832	Defense response to fungus	1	1	2	
2376	Immune system process	1	5	3	
45087	Innate immune response	0	2	1	2376

Table 3.8 The number of up-regulated genes in *P. superbus* in response to preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery that map to GO terms associated with transport. The Benjamini-Hochberg method was used for the correction of the *p*-values. The BLAST2GO program was used to map genes to GO terms.

GO-ID	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child of (Go ID)**
		Preconditioning 1	**Preconditioning 2**	**Recovery**	
6810	Transport	9	41	53	
46907	Intracellular transport	2	9	14	6810
6811	Ion transport	0	10	9	6810

Table 3.9 The number of up-regulated genes in *P. superbus* in response to preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h) and recovery that map to GO terms associated with lipid metabolism. The Benjamini-Hochberg method was used for the correction of the *p*-values. The BLAST2GO program was used to map genes to GO terms.

GO-ID	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child of (Go ID)**
		Preconditioning 1	**Preconditioning 2**	**Recovery**	

6629	Lipid metabolic process	4	19	16	
6631	Fatty acid metabolic process	2	6	7	6629
6633	Fatty acid biosynthetic process	2	5	4	6631
19915	Lipid storage	0	9	8	
6869	Lipid transport	0	1	6	
1676	Long-chain fatty acid metabolic process	0	2	4	

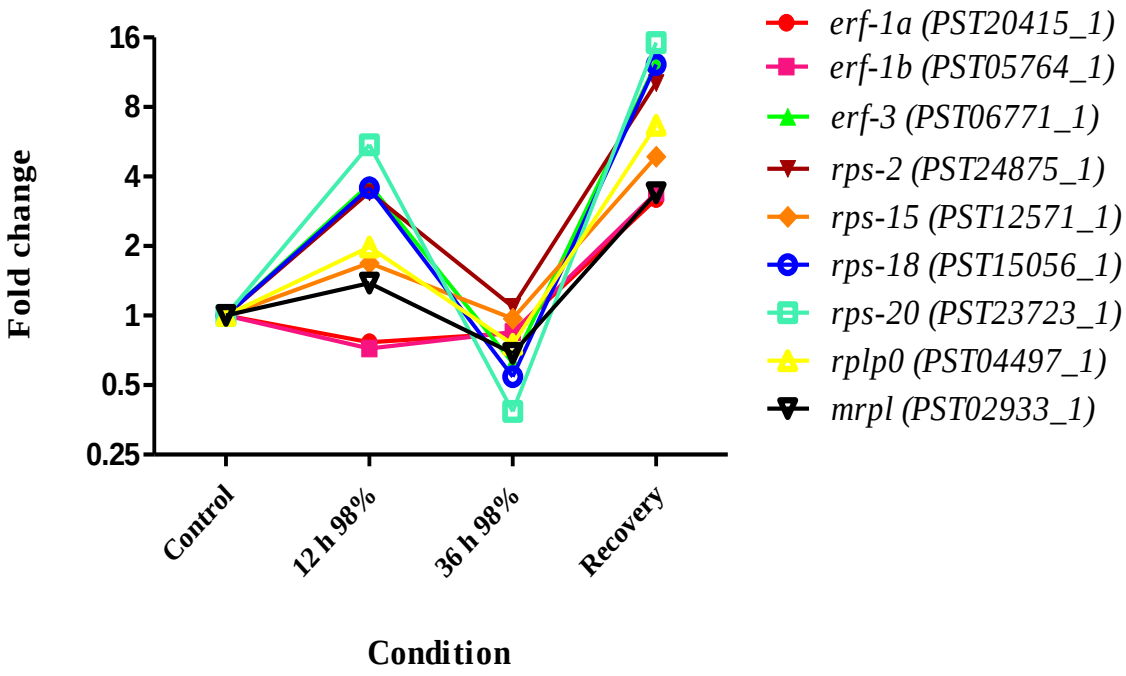


Figure 3.16 Change in expression of genes (RNA-seq transcript counts) encoding proteins involved in translation in response to preconditioning 1 (98% for 12 h), preconditioning 2 (98% for 36 h) and recovery compared to the control. The expression of the following genes is shown: *erf-1a* (eukaryotic peptide chain release factor), *erf-1b*, *erf-3*, *rps-2* (small ribosomal subunit), *rps-15*, *rps-18*, *rps-20*, *rplp0* (acidic ribosomal protein p0) and *mrpl* (mitochondrial ribosomal protein). These genes associated with translation were identified from manual evaluation of the tables of differentially expressed genes.

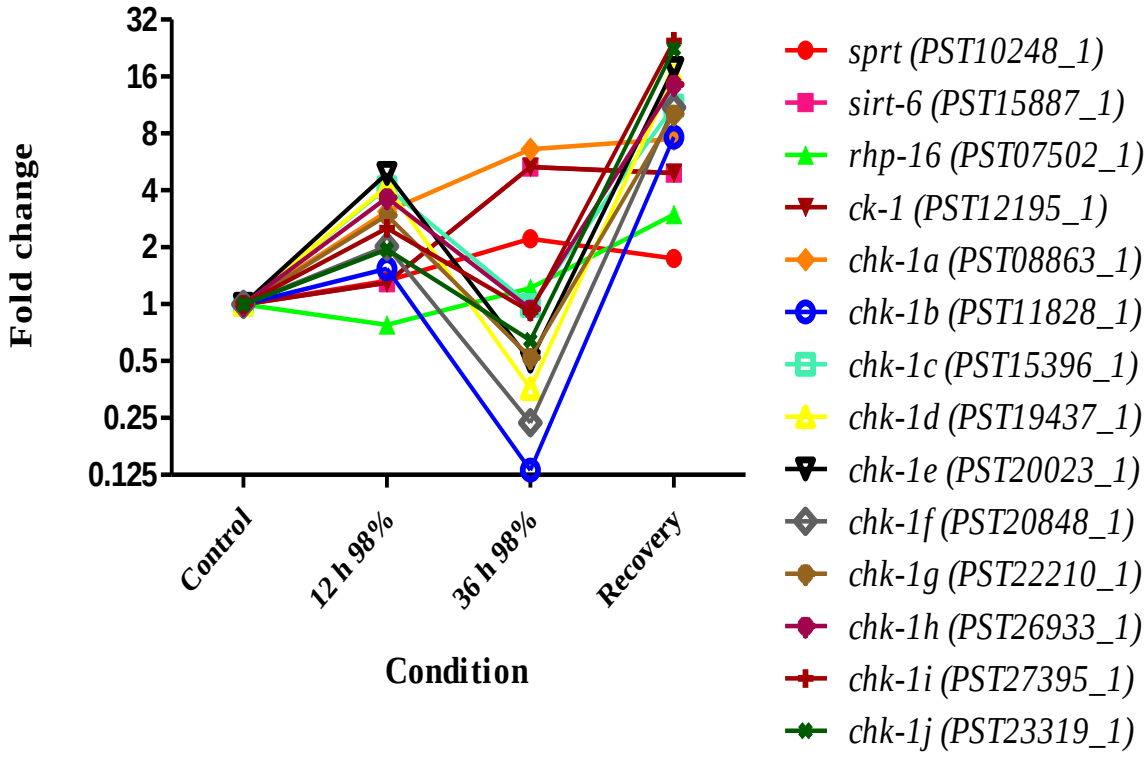


Figure 3.17 Change in expression of genes (RNA-seq transcript counts) encoding proteins involved in DNA repair in response to preconditioning 1 (98% for 12 h), preconditioning 2 (98% for 36 h) and recovery compared to the control. The expression of the following genes is shown: *sprt* (spartan), *sir-2.4* (sirtuin), *rhp-16* (ATP-dependent helicase), *kin-20* (protein kinase), *chk-1a* (checkpoint kinase), *chk-1b*, *chk-1c*, *chk-1d*, *chk-1e*, *chk-1f*, *chk-1g*, *chk-1h*, *chk-1i*, and *chk-1j*.

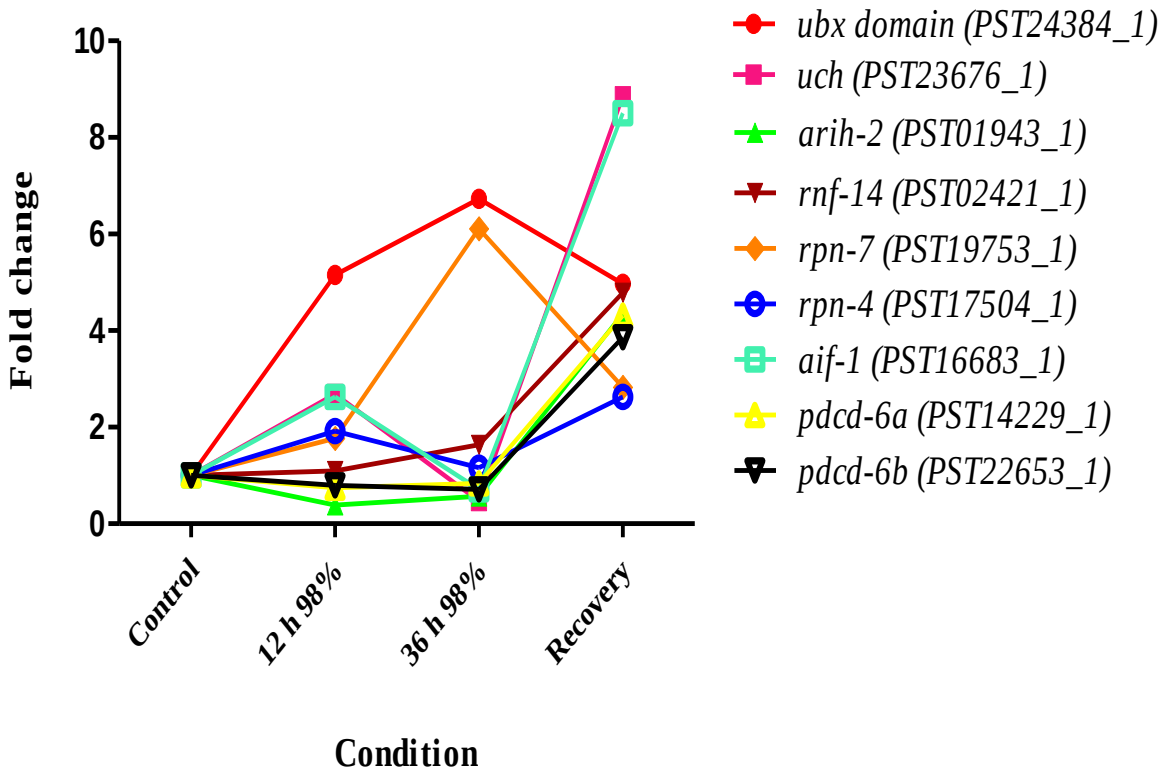


Figure 3.18 Change in expression of genes (RNA-seq transcript counts) encoding proteins involved in protein degradation in response to preconditioning 1 (98% for 12 h), preconditioning 2 (98% for 36 h) and recovery compared to the control. The expression of the following genes is shown: *ubx domain* (produces an ubx domain containing protein), *uch* (ubiquitin carboxyl-terminal hydrolase family protein), *arih-2* (e3 ubiquitin-protein ligase *arih-2* like), *rnf-14* (e3 ubiquitin-protein ligase *rnf-14*), *rpn-4* (26s proteasome regulatory chain 4), *rpn-7* (proteasome Regulatory Particle, Non-ATPase-like), *aif-1* (apoptosis-inducing factor), *pdcd-6a* (programmed cell death protein) and *pdcd-6b*.

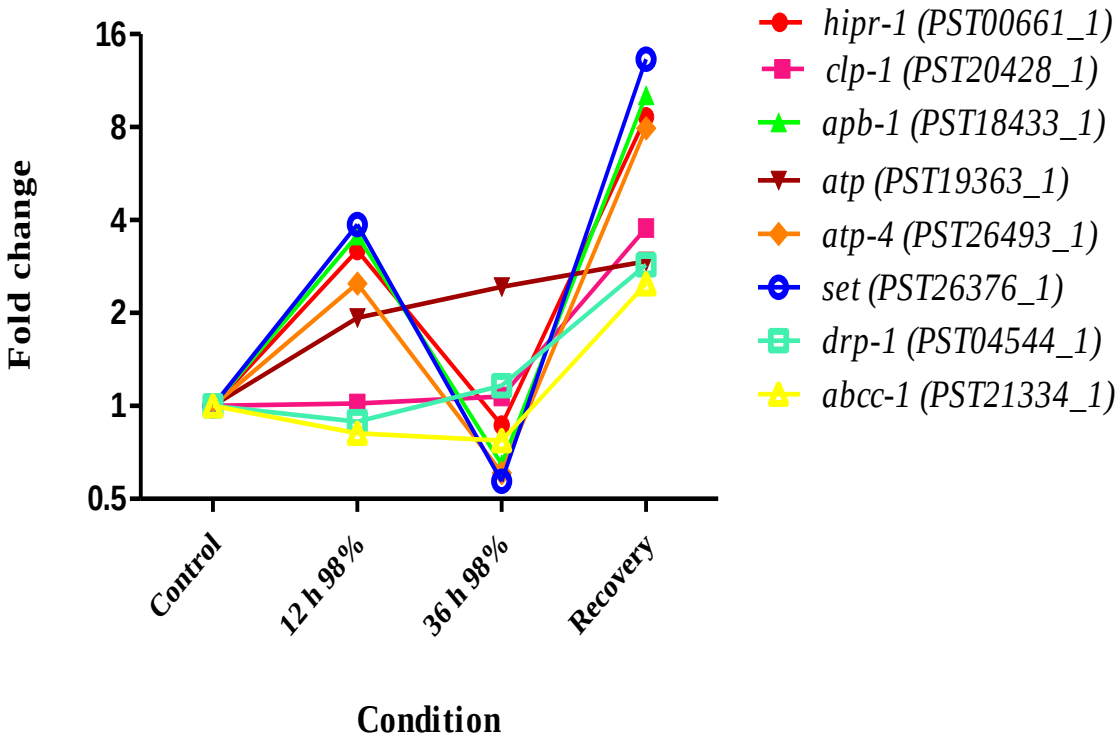


Figure 3.19 Change in expression of genes (RNA-seq transcript counts) encoding proteins involved in protein degradation in response to preconditioning 1 (98% for 12 h), preconditioning 2 (98% for 36 h) and recovery compared to the control. The expression of the following genes is shown: *hipr-1* (huntingtin-interacting protein), *clp-1* (calpain), *apb-1* (adaptin), *atp* (phospholipid-transporting atpase iib), *atp-4* (ATP synthase subunit), *set* (SET (trithorax/polycomb) domain containing), *drp-1* (Dynamin-Related Protein), *abcc-1* (multidrug resistance-associated protein).

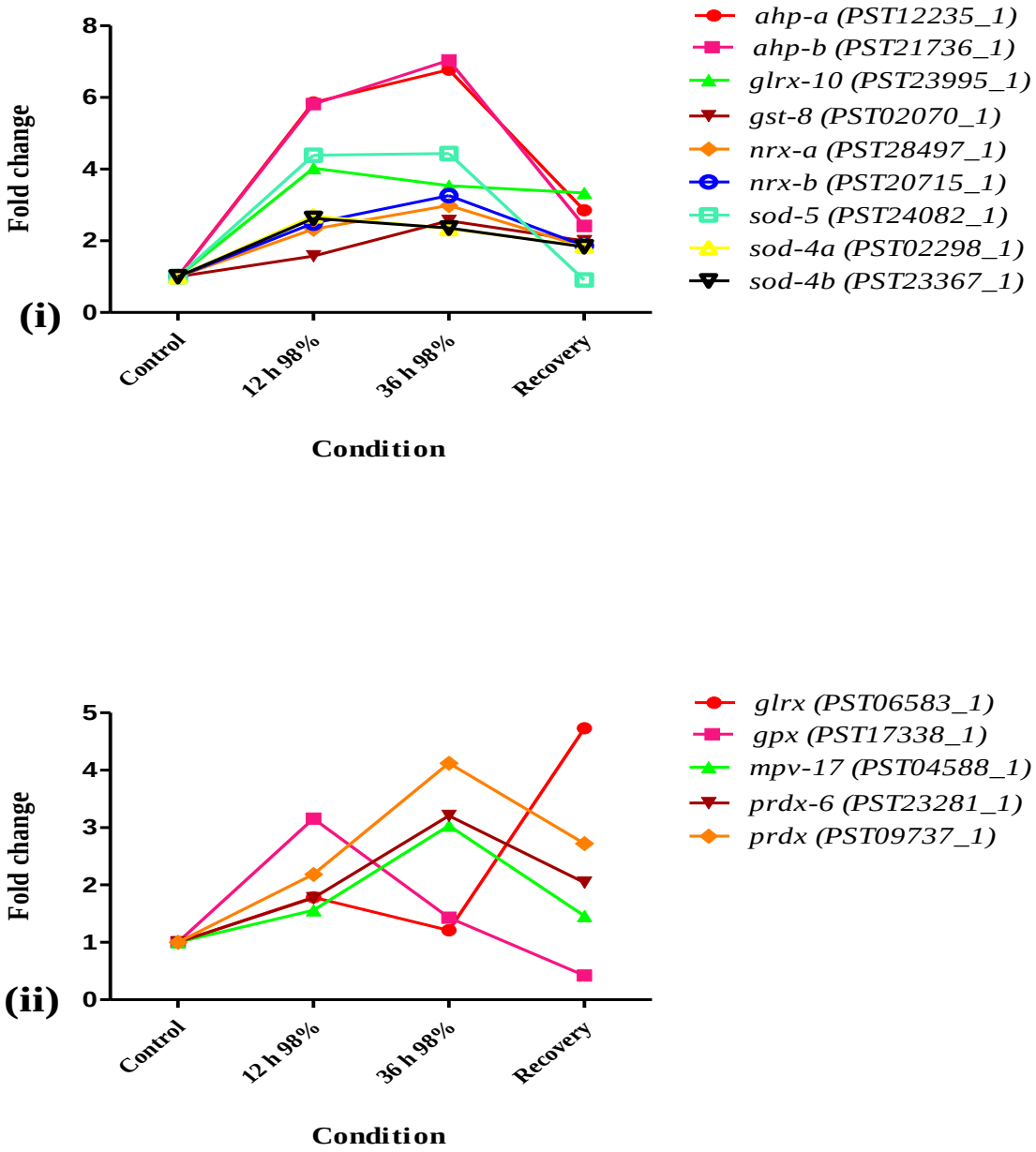


Figure 3.20 Change in expression of genes (RNA-seq transcript counts) encoding antioxidant proteins in response to preconditioning 1 (98% for 12 h), preconditioning 2 (98% for 36 h) and recovery compared to the control. The expression of the following genes is shown: (i) *ahp-a* (animal haem peroxidise), *ahp-b*, *glrx-10* (glutaredoxin), *gst-8* (gluatathione S-transferase), *nrx-a* (nucleoredoxin), *nrx-b*, *sod-5* (superoxide dismutase), *sod-4a*, *sod-4b*, (ii) *glrx* (glutaredoxin), *gpx* (glutathione peroxidise), *mvp-17* (related human *mvp-17* gene), *prdx-6* (peroxiredoxin) and *prdx*.

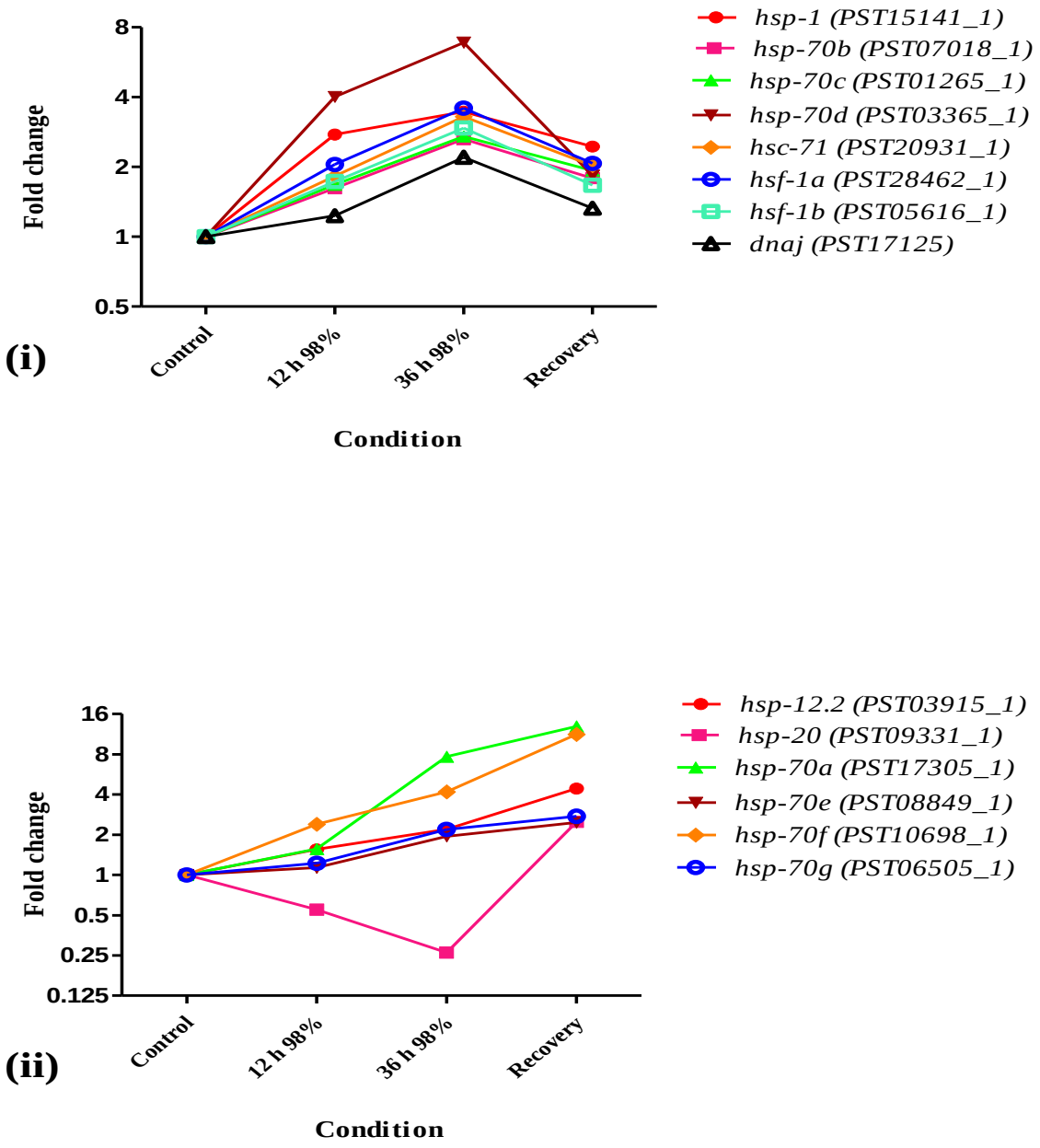


Figure 3.21 Change in expression of genes (RNA-seq transcript counts) encoding HSPs and other molecular chaperones in response to preconditioning 1 (98% for 12 h), preconditioning 2 (98% for 36 h) and recovery compared to the control. The expression of the following genes is shown: (i) *hsp-1* (heat shock protein), *hsp-70b*, *hsp-70c*, *hsp-70d*, *hsc-7* (heat shock cognate), *hsf-1a* (heat shock factor), *hsf-1b*, *dnaj*, (ii) *hsp-a*, *hsp-70a*, *hsp-70e*, *hsp-70f* and *hsp-70g*. Relative expression values were plotted on a log-2 scale.

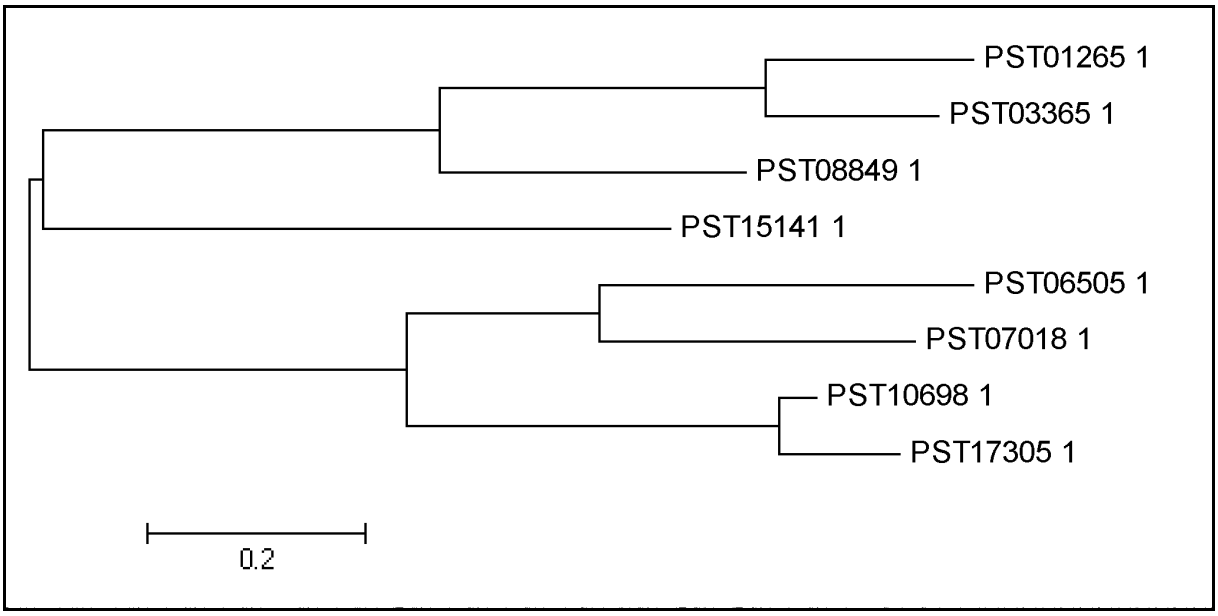


Figure 3.22 Maximum likelihood phylogenetic of *P. superbus hsp-70* sequences up-regulated in response to preconditioning, desiccation and recovery. This tree was generated using Mega5 under the Jones-Taylor-Thornton model with all other default settings.

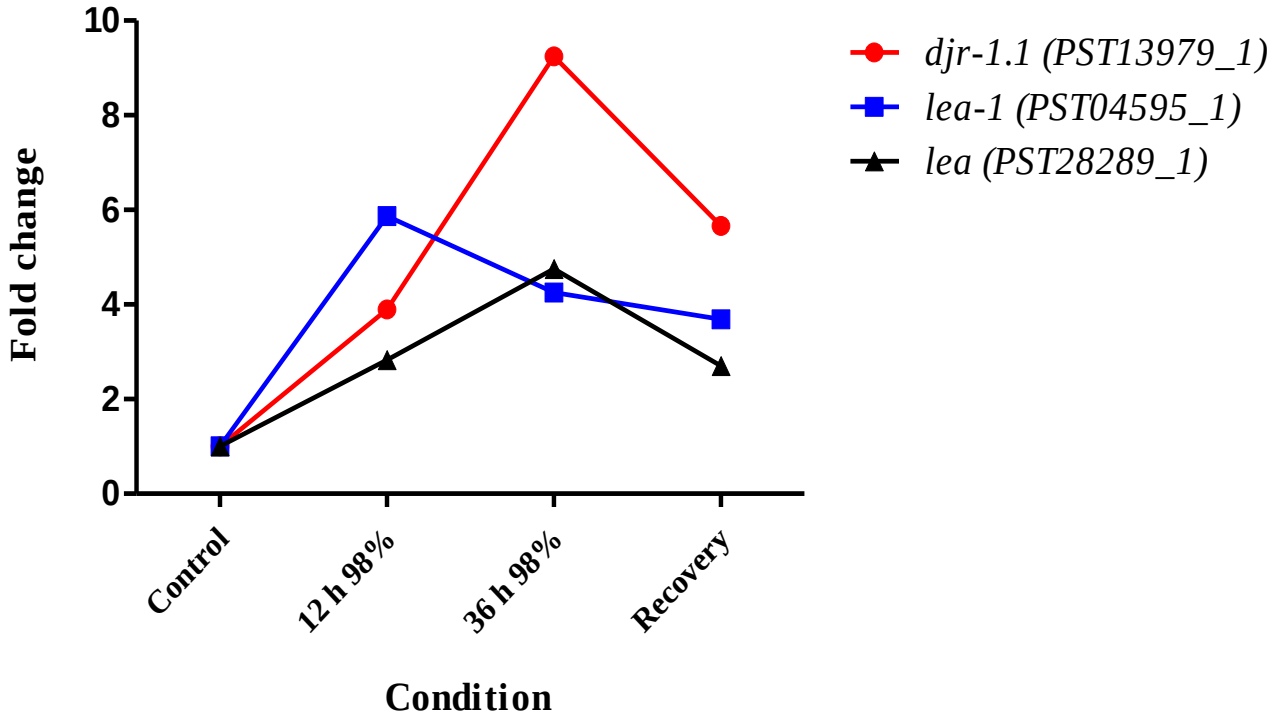


Figure 3.23 Change in expression of genes (RNA-seq transcript counts) encoding proteins *lea-1* (late embryogenesis abundant) and *djr-1.1* (DJ-1) in response to preconditioning 1 (98% for 12 h), preconditioning 2 (98% for 36 h) and recovery compared to the control.

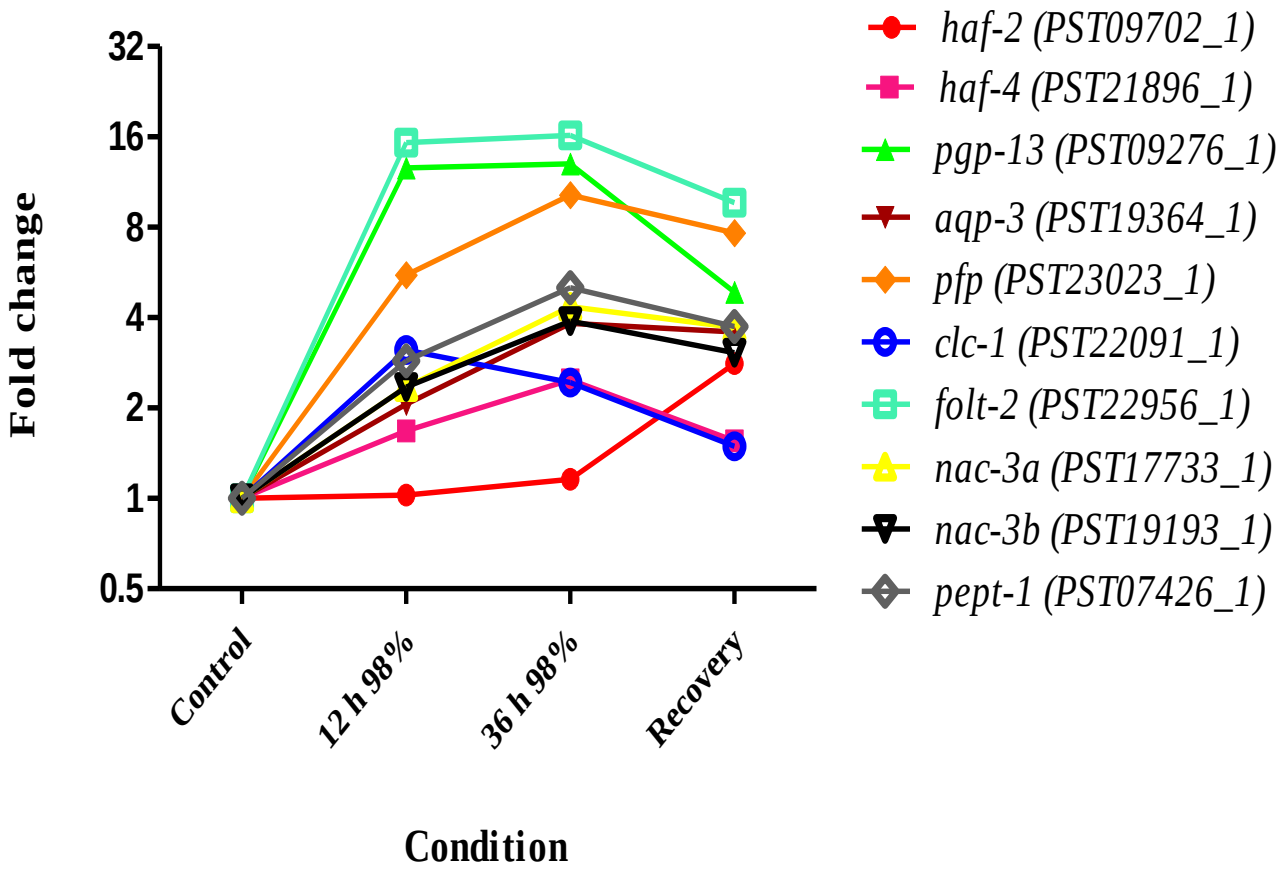


Figure 3.24 Change in expression of genes (RNA-seq transcript counts) encoding proteins involved in transport in response to preconditioning 1 (98% for 12 h), preconditioning 2 (98% for 36 h) and recovery compared to the control. The expression of the following genes is shown: *haf-2* (half transporter), *haf-4*, *pgp-13* (P-glycoprotein), *aqp-3* (aquaporin), *pfp* (pore forming protein), *clc-1* (chloride channel), *folt-2* (folate transporter family), *nac-3a* (Na^+-coupled dicarboxylate transporter), *nac-3b*, *pept-1* (peptide transporter family). Relative expression values were plotted on a log-2 scale.

3.3 Conclusion

In this chapter the results of an RNA-seq analysis to identify alterations in the *P. superbus* transcriptome in response to desiccation are presented. An overview of the genes up-regulated in response to desiccation and recovery is given in Figure 3.25. The data revealed a large transcriptomic response by the nematode in response to the treatments. In *P. superbus*, genes responsive to desiccation encode proteins associated with signal transduction, antioxidant activity, DNA repair, ubiquitin-proteasome degradation, chaperone activity, cellular organisation, transport, and lipid metabolism as discussed in sections 3.2.8.1-3.2.8.8.

The gene expression changes in response to desiccation have been previously studied in a wide range of organisms including bacteria, plants, insects and nematodes. Strikingly many of the genes up-regulated in previous studies were also up-regulated in *P. superbus* in response to desiccation. Teets *et al.* (2012) used RNA-sequencing to quantify transcriptomic mechanisms of desiccation in the Antarctic midge *Belgica antarctica*. Similar to *P. superbus,* these authors found a great number of genes differentially expressed in response to desiccation in *B. antarctica*. Comparing the up-regulated genes in response to desiccation in *B. antarctica* and *P. superbus*, there is good correlation in the two expression profiles. In both datasets there was an up-regulation of genes which encode proteins involved in signal transduction, the ubiquitin-mediated proteasome, chaperone activity and cellular organisation. There were also distinct differences in the desiccation responses of *B. antarctica* and *P. superbus*. For example genes encoding LEA (late embryogenesis abundant) proteins were only up-regulated in *P. superbus* in response to desiccation. Additionally, genes encoding proteins involved in antioxidant activity, DNA repair, transport and lipid metabolism were up-regulated in *P. superbus* but not in *B. antarctica* in response to desiccation. These differences could be attributed to the fact that *P. superbus* can tolerate extreme desiccation, while *B. antarctica* can only survive mild desiccation. The data also revealed that transcripts associated with translation, protein degradation and DNA repair are up-regulated in response to recovery from desiccation. This suggests metabolism is returning to normal and repair mechanisms are activated.

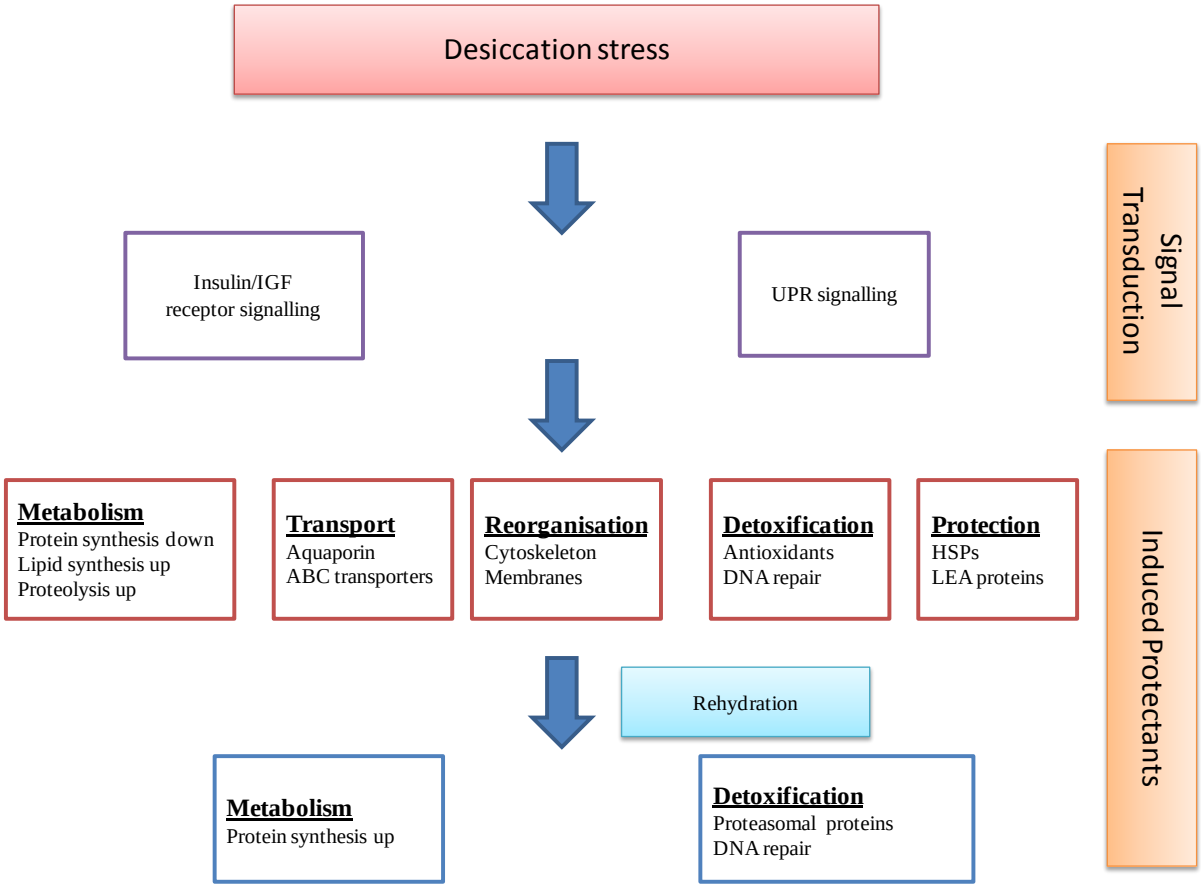


Figure 3.25 Overview of expression of genes in response to desiccation and subsequent rehydration in *P. superbus* nematodes.

Table 3.10 DAVID bioinformatics output showing significantly enriched annotation clusters for up-regulated genes in response to preconditioning for 12 h.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	0.51	Glycoprotein/signal peptide	6	0.06	0.99
2	0.25	Cytoskeleton	3	0.28	1.00
3	0.18	Protein tyrosine kinase activity	3	0.46	1.00
4	0.14	Cation binding	8	0.66	1.00
5	0.02	Positive regulation of growth rate	6	0.94	1.00

Table 3.11 DAVID bioinformatics output showing significantly enriched annotation clusters for up-regulated genes in response to preconditioning for 36 h.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	2.56	Multicellular organismal aging	17	0.00	0.82
2	2.01	Proteolysis	20	0.03	0.89
3	1.91	Glycoprotein/signal peptide	24	0.00	0.38
4	1.82	Protease	10	0.12	0.79
5	1.76	Lipid biosynthetic process	10	0.01	0.79
6	1.61	Lipid biosynthetic process	10	0.01	0.79
7	1.57	Dauer larval development	7	0.02	0.76
8	1.50	Serine-type peptidase activity	6	0.08	0.69
9	1.49	Lipase activity	6	0.01	0.39
10	1.49	Metal ion binding	60	0.01	0.46

Table 3.12 DAVID bioinformatics output showing significantly enriched annotation clusters for up-regulated genes in response to recovery.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	1.96	Ribonucleotide binding	36	0.01	0.28
2	1.87	Cysteine-type endopeptidase activity	6	0.00	0.39
3	1.84	Multicellular organismal aging	11	0.01	0.60
4	1.68	Chk kinase-like	3	0.02	0.85
5	1.66	Respiratory electron transport chain	5	0.00	0.35
6	1.39	Cellular response to unfolded protein	3	0.01	0.63

Table 3.13 DAVID bioinformatics output showing significantly enriched annotation clusters for down-regulated genes in response to preconditioning for 12 h.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	3.89	Nematode cuticle collagen	16	4.56E-10	1.25E-07
2	2.95	Oxidoreductase/cytochrome p450/iron ion binding	21	5.97E-07	5.49E-05
3	2.80	Glycolysis /gluconeogenesis/alcohol dehydrogenase	7	1.70E-04	0.004504
4	2.53	Pan-1 domain	4	0.005596	0.142524
5	2.39	C-type lectin/carbohydrate binding	6	0.004044	0.129585
6	1.71	Nitrogen compound biosynthetic process	11	0.004612	0.542174
7	1.41	Protein folding	4	0.163571	0.943574
8	1.36	Glutathione s-transferase	4	0.031351	0.420438
9	1.29	Heat shock protein hsp20	3	0.026534	0.409222

Table 3.14 DAVID bioinformatics output showing significantly enriched annotation clusters for down-regulated genes in response to preconditioning for 36 h.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	31.36	Structural constituent of cuticle/collagen	58	2.74E-45	1.04E-42
2	7.52	Cuticle collagen/cuticle development	28	2.91E-21	6.42E-19
3	6.87	Signal peptide/disulfide bond/secreted	58	1.76E-15	8.78E-13
4	4.59	Oxidoreductase/cytochrome p450	47	1.29E-07	6.36E-06
5	4.24	Metabolism of xenobiotics by cytochrome p450	10	4.04E-06	1.58E-04
6	4.12	C-type lectin	13	2.28E-04	0.017682
7	3.51	Egf-like region, conserved site	22	1.36E-04	0.011842
8	3.23	Pan-1 domain	7	1.62E-04	0.003388
9	2.93	Carbohydrate binding/lectin	22	2.66E-08	3.36E-06
10	2.65	Lipid glycosylation	5	0.005508	0.470634
11	2.48	Glutathione transferase activity	6	9.45E-04	0.049781
12	2.38	Cofactor binding	29	0.00208	0.083724
13	2.14	Acyl-coa dehydrogenase activity/fad binding	7	9.81E-04	0.045299
14	2.06	Heat shock protein hsp20	6	0.001053	0.048158
15	1.95	Aromatic amino acid family metabolic process	6	9.60E-04	0.198492

Table 3.15 DAVID bioinformatics output showing significantly enriched annotation clusters for down-regulated genes in response to recovery.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	36.29	Collagen/structural constituent of cuticle	37	4.79E-29	8.87E-27
2	6.69	Signal peptide/disulfide bond/secreted	50	2.37E-14	1.06E-11
3	6.31	C-type lectin-like	15	2.73E-06	3.46E-04
4	5.81	Pan-1 domain	9	3.76E-06	3.97E-04
5	5.36	Metabolism of xenobiotics by cytochrome p450	11	9.72E-08	3.45E-06
6	5.07	Cuticle development	12	0.012332	0.79924
7	3.85	Egf-like region, conserved site	23	5.18E-06	4.69E-04
8	3.53	Oxidation reduction/iron ion binding	47	7.98E-09	5.16E-06
9	3.17	Cysteine-rich repeat/peptidase inhibitor activity	10	1.01E-04	0.006361
10	2.96	Electron carrier activity/cytochrome p450	27	5.19E-06	6.26E-04
11	2.96	Cofactor binding	29	2.13E-04	0.015328
12	2.89	Glutathione s-transferase	10	1.48E-04	0.007801
13	2.82	Molting cycle	28	0.001499	0.384414
14	2.55	Carbohydrate binding/lectin	17	1.16E-05	0.001045
15	2.45	Von willebrand factor, type a	8	0.018793	0.305452

4 Chapter IV Label Free Quantification based proteomic analysis of desiccation survival in *Panagrolaimus superbus*

4.1 Introduction

In this chapter the mechanisms of desiccation tolerance are further investigated in *P. superbus* by using quantitative proteomic methods. Proteomics can reveal the actively translated portion of the genome that performs the enzymatic, regulatory and structural functions of the cell at a particular moment (Gasulla *et al.*, 2013). There have been many studies which have used quantitative proteomics to understand the mechanisms underlying desiccation tolerance. Most of these studies have been carried out on plants. Typically there is an up-regulation of molecular chaperones, LEA proteins and antioxidants and a down-regulation of proteins involved in metabolism in plants, as well as animals, in response to desiccation. However, there is normally variation in the specific proteins up-regulated, as well as additional proteins outside these categories that are responsive to desiccation. For example, Gasulla *et al.* (2013) found 13 proteins up-regulated in response to desiccation in the isolated lichen phycobiont *Asterochloris erici*. The up-regulated proteins were involved in cellular protection (HSP), the cytoskeleton (ß-tubulin) and degradation (proteases) but no proteins involved in ROS scavenging were detected. In contrast, in response to desiccation the antioxidants superoxide dismutase (SOD), catalase and glutathione reductase as well as LEA proteins were up-regulated in the resurrection spike moss *Selaginella tamariscina* (Wang *et al.*, 2010). However, the majority of the proteins that were differentially expressed in both the spike moss and the lichen studies were down-regulated.

Many studies have focused on the molecular mechanisms of desiccation tolerance in seeds. One such study was carried out to find the proteins differentially expressed in response to desiccation in maize embryos (Huang *et al.*, 2012). There was good correlation with the studies previously mentioned with increased expression of HSPs, LEA proteins and antioxidants. Additionally, small HSPs and proteasomal subunits were also up-regulated. Generally, it is believed that plants can overcome drought-induced mechanical, oxidative, and destabilizing stress by relying on their morphological adaptations, hormone regulation, antioxidant protection and the accumulation of osmolytes (Wang *et al.*, 2010). Similar to plants, animals such as Antarctic midges

(*Belgica antarctica*), rotifers (*Macrotrachela quadricornifera*) and nematodes (*Steinernema feltiae*) are predicted to rely on morphological adaptation, antioxidant protection and the accumulation of osmolytes to survive desiccation (Ricci *et al.*, 2003; Chen *et al.*, 2006; Teets *et al.*, 2012). When the bdelloid rotifer *M. quadricornifera* perceives a sufficient decrease in relative humidity, they withdraw their head and foot into the trunk and contract into a compact shape called a tun. Although nematodes are incapable of shortening, some desiccation tolerant nematodes such as *P. superbus* can coil into a tight spiral shape in response to desiccation. These morphological adjustments reduce the surface to volume ratio which may result in a decreased rate of internal water loss during the process of desiccation (Ricci *et al.*, 2003). In plants, morphological changes are also observed which reduce the rate of water loss during desiccation such as leaf curling (Wang *et al.*, 2010).

Proteomic analyses have revealed a high degree of conservation in the mechanisms evolved for desiccation tolerance in plants and animals. Li *et al.* (2009) used 2-D electrophoresis and LC/MS to find the proteins differentially expressed in the Antarctic midge *Belgica antarctica* in response to dehydration and recovery. This analysis found that the majority of proteins up-regulated in response to desiccation and recovery had contractile and cytoskeletal functions. Although there was an overlap in the differentially expressed proteins in the two conditions, there were also distinct changes in response to dehydration and rehydration. For example catalase was up-regulated specifically during desiccation while HSPs were only up-regulated in response to recovery from desiccation. This demonstrates the need to analyse the different stages of desiccation as well as the proteomes of organisms during recovery from desiccation to understand the underlying mechanisms of desiccation tolerance. Thus, in the experiments described in this chapter the *P. superbus* proteome was monitored throughout preconditioning, desiccation and recovery. Chen *et al.* (2006) used the same approach of 2-D electrophoresis and LC/MS to study the proteomics of desiccation in the entomopathogenic nematode *Steinernema feltiae*. The proteins which were up-regulated in *S. feltiae* include the chaperone HSP-60, the antioxidant coenzymes q and the stress related proteins inositol monophosphatase and fumarate lyase.

The 2-D electrophoresis and LC/MS methods used in these two analyses are less sensitive than currently available proteomic techniques. In this chapter, desiccation tolerance in *P. superbus* was analysed using both gel-based and gel-free differential proteomics. Gel-free differential proteomics involved a label free quantitation (LFQ) approach using a high resolution/accurate mass (HR/AM) orbitrap mass spectrometer. Differing from ion traps, the orbitrap uses only electrostatic fields to confine and analyse injected ion populations (Perry *et al.*, 2008). The differences between these two approaches are further discussed later in the chapter.

To my best knowledge, the only previous proteomic study of desiccation tolerance in animals using HR/AM orbitrap LC-MS/MS was carried out in *Milnesium tardigradum* (Schokraie *et al.*, 2011). The drawback in working with tardigrades and other non-model organisms is that their transcriptome and genome sequence data may be lacking or if available their annotation may be incomplete. Therefore, the identification of peptides can be limited, as in *M. tardigradum* only 53 protein entries are recorded in the publicly available NCBInr database. Furthermore, the identification of proteins is based predominantly on high homologies between tardigrade sequences and sequences from related species of other taxa. The analysis was therefore somewhat restricted to conserved proteins that can be identified with high sequence coverage. Schokraie *et al.* (2011) focused on the activity of the HSPs, which are highly conserved across different species. In this proteomic study a Thermo Fisher Quadrapole Orbitrap (Q Exactive) was used which is more accurate than the linear orbitrap. Identifying the proteins that are differentially expressed in *P. superbus* was facilitated by searching MS/MS data against the predicted protein set, derived from the fully sequenced transcriptome (O'Mahony Zamora, 2013).

4.2 Results and Discussion

4.2.1 Selection of desiccation treatments, protein extraction and data analysis

The treatment conditions selected were identical to those used for the *P. superbus* RNA-seq transcriptomic experiments as described in Chapter III so the data from the two experiments could be compared directly. However, one extra condition was added to the proteomic experiments which represents the *P. superbus* proteome in its most desiccated state. Therefore, the *P. superbus* mixed stage nematodes were exposed to the following

treatments prior to proteome analysis: control; preconditioning 1 (98% RH for 12 h); preconditioning 2 (98% RH for 36 h); desiccation (98% RH for 36 h, 0% RH for 24 h) and recovery (98% RH for 36 h, 0% RH for 24 h, rehydration in water for 2 h). Details of sample preparation, protein and peptide separation, and MS analysis can be found in Sections 2.4.4 and 2.4.5.

Protein identification and LFQ analysis were conducted using the MaxQuant quantitative proteomics software package supported by the Andromeda database search engine (Cox and Mann, 2011; Cox *et al.*, 2011), to correlate MS/MS data against the *P. superbus* protein database. Proteins were considered identified when more than one peptide for each parent protein was observed. Differentially abundant proteins among experimental groups were determined by LFQ using MaxQuant and Perseus a software package designed to perform all downstream bioinformatics and statistics analyses on MaxQuant output tables (Cox and Mann, 2011). Details of the data analysis methods can be found in Section 2.4.5, while an overview of the label-free differential proteomics analysis in *P. superbus* is shown in Figure 4.1.

4.2.2 Differentially expressed proteins

There were 1,197 different *P. superbus* proteins detected across all the samples with a total of 76, 165, 228 and 230 proteins differentially expressed (ANOVA, $p>0.05$) in response to preconditioning 1, preconditioning 2, desiccation and recovery respectively relative to the control as outlined in the supplementary data file "Supplementary data *P. superbus* LFQ" and summarised in Table 4.1. The number of differentially expressed proteins in *P. superbus* relative to the control undesiccated worms increased continuously as desiccation progressed and into recovery from desiccation (Table 4.1). However, the number of up-regulated proteins peaked during preconditioning 2 (36 h at 98% RH), with 63 proteins showing increased expression. The smallest number of proteins were up-regulated in the recovery dataset when compared to the control.

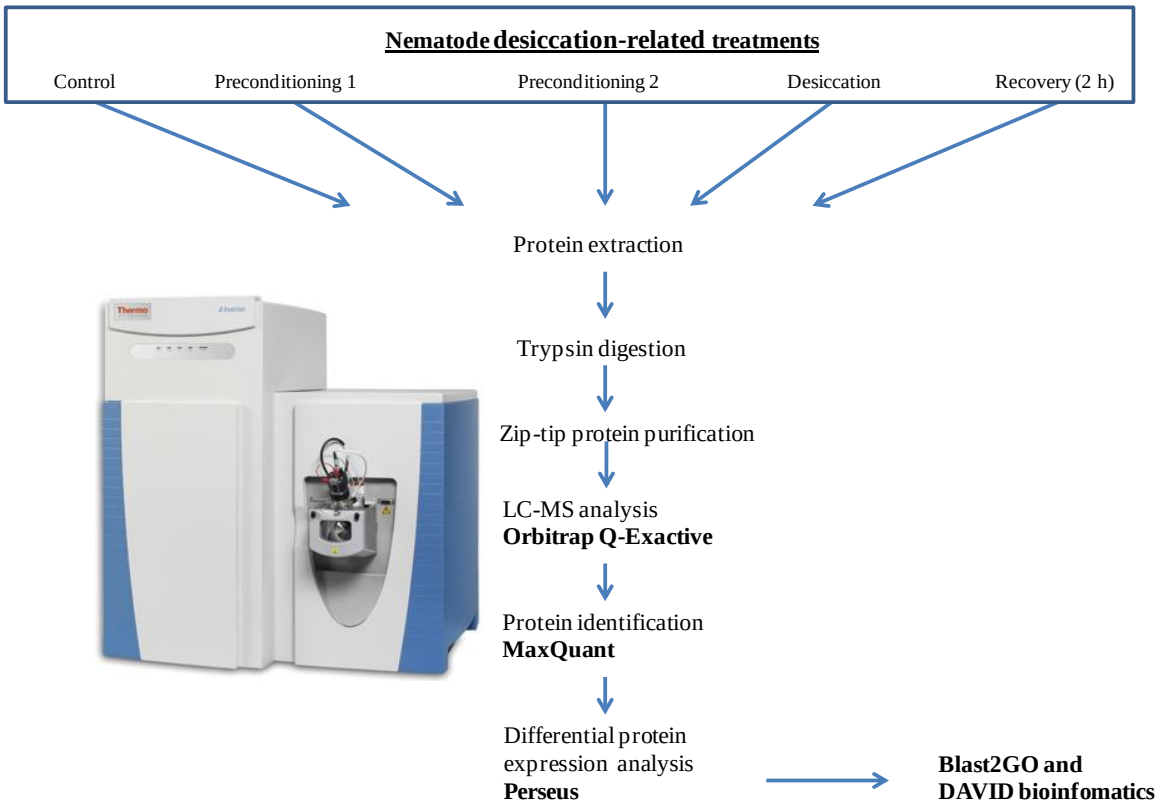


Figure 4.1 Overview of label-free differential proteomics analysis in *P. superbus*. The analysed proteomes were from nematodes which had been exposed to the following desiccation regimes: control, preconditioning 1 (98% RH for 12 h), preconditioning 2 (98% RH for 36 h), desiccation (98% RH for 36 h, 0% RH for 24 h) and recovery (98% RH for 36 h, 0% RH for 24 h, rehydration in water for 2 h).

Table 4.1 The number of proteins which were up-regulated and down-regulated in *P. superbus* in response to preconditioning 1, preconditioning 2, desiccation and recovery. The data for the different conditions are compared to the control unless stated.

	Preconditioning 1	**Preconditioning 2**	**Desiccation**	**Recovery**	**Recovery versus Desiccation**
Up-regulated	53	63	53	40	127
Down-regulated	23	102	175	190	152
Total	76	165	228	230	279
Number of proteins represented*	1,197	1,181	1,178	1,170	1,121

*The total number of proteins detected in the two conditions being compared.

Only four of the up-regulated proteins were common to all treatments compared to the control, which were predicted to function as a 26S protease regulatory subunit (PST03869_1), a 40S ribosomal protein (PST12500_1), a lipoamide acyltransferase component (PST03614_1) and a KH domain containing protein (PST10572_1) (The KH domain is present in a wide variety of RNA-binding proteins). The up-regulated proteins are categorized and discussed in Sections 4.2.5-4.2.11.

4.2.3 Blast2GO and DAVID analyses

Blast2GO is a functional annotation tool and pipeline (Conesa *et al.*, 2005) and was used to analyze the differentially expressed proteins. Blast2GO allows homologous mapping of query sequences to the GenBank database using BLAST. It also integrates the Gene Ontology database, Enzyme Commission database and InterPro database. The results of the BLAST2GO analysis are shown in the supplementary data file "Supplementary data *P. superbus* LFQ" and summarised in Figures 4.2-4.7. The BLAST2GO analysis revealed that over 20 proteins involved in anatomical structure developments are up-regulated in *P. superbus* in response to each of the treatments. The BLAST2GO analysis also identified protein binding, organic cyclic compound binding, heterocyclic compound binding, hydrolase activity and ion binding as the major categories of molecular function in the proteins up-regulated in response the desiccation-related treatments.

In order to carry out the DAVID bioinformatics analysis the gene IDs must be in a form the program software can recognise. To do this a BLASTX alignment was carried out between the *P. superbus* transcriptome and *C. elegans* transcriptome (version 190 downloaded from Ensembl at www.ensembl.org). Those *P. superbus* sequences with hits to *C. elegans* were used for the DAVID bioinformatics analysis. The DAVID bioinformatics tool was also used to cluster functionally related annotations into groups (Huang *et al.*, 2009). The results of the DAVID analysis are shown in Tables 4.9-4.18. Additionally, *C. elegans* gene names are used for *P. superbus* homologs in this chapter for clarity as well as comparison reasons.

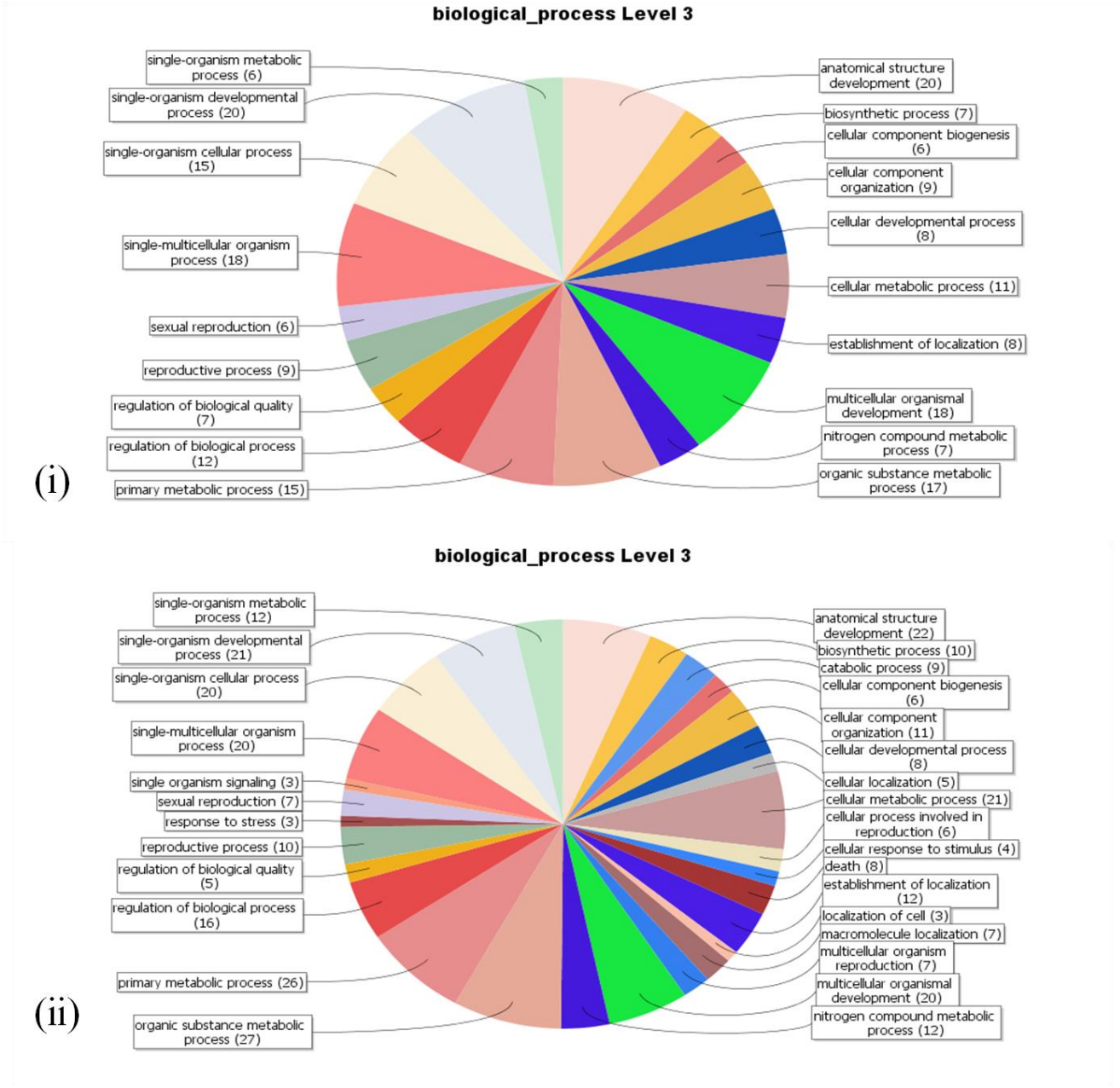


Figure 4.2 Blast2GO biological processes annotation analysis of proteins up-regulated in response to (i) preconditioning 1 (98% RH for 12 h) vs. control and (ii) preconditioning 2 (98% RH for 36 h) vs. control in *P. superbus* nematodes. A minimum of 3 up-regulated proteins in a category was required for inclusion.

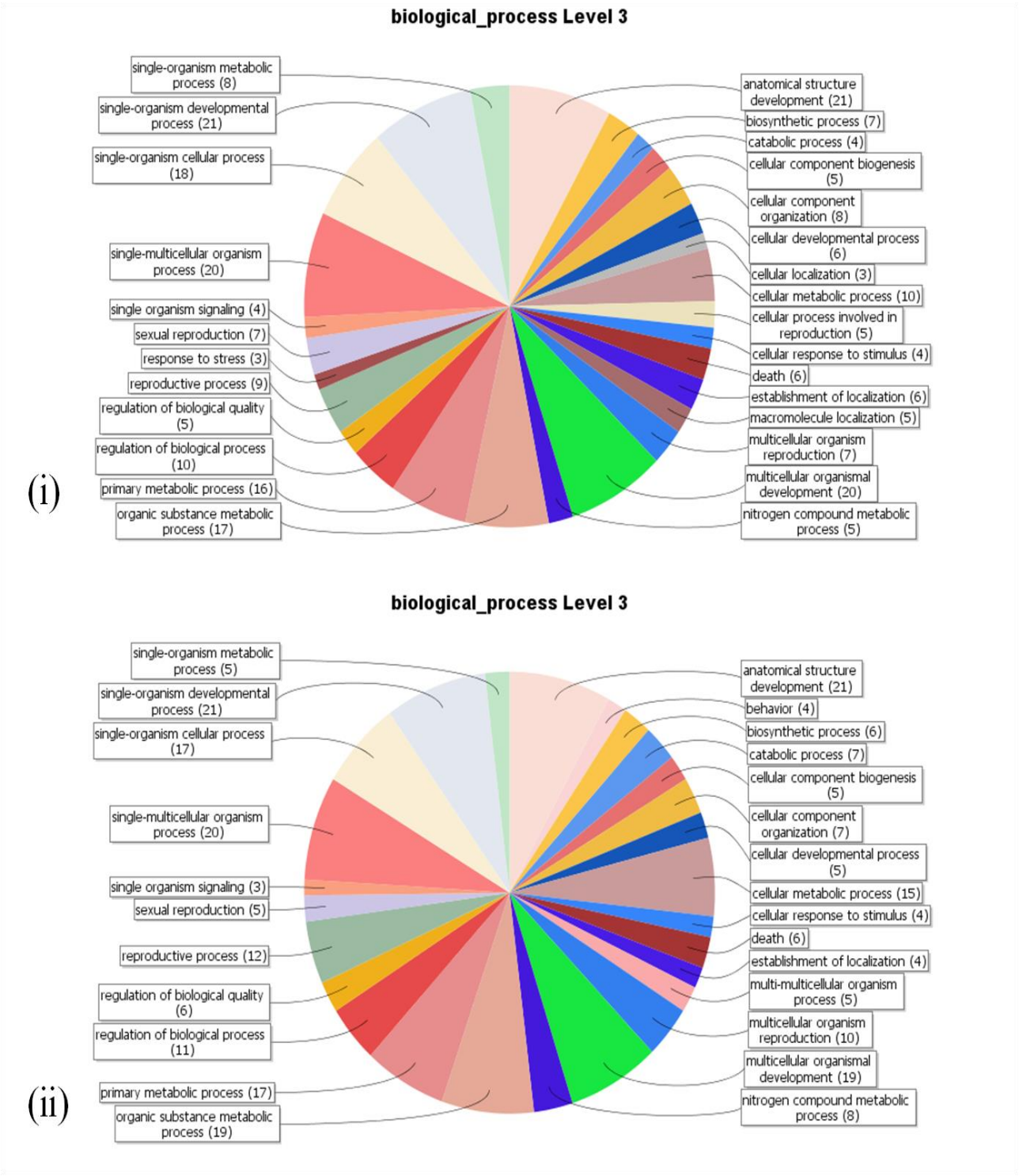


Figure 4.3 Blast2GO biological processes annotation analysis of proteins up-regulated in response to (i) desiccation (98% RH for 36 h, 0% RH for 24 h) vs. control and (ii) recovery (98% RH for 36 h, 0% RH for 24 h, rehydration in water for 2 h) vs. control in *P. superbus* nematodes. A minimum of 3 up-regulated proteins in a category was required for inclusion.

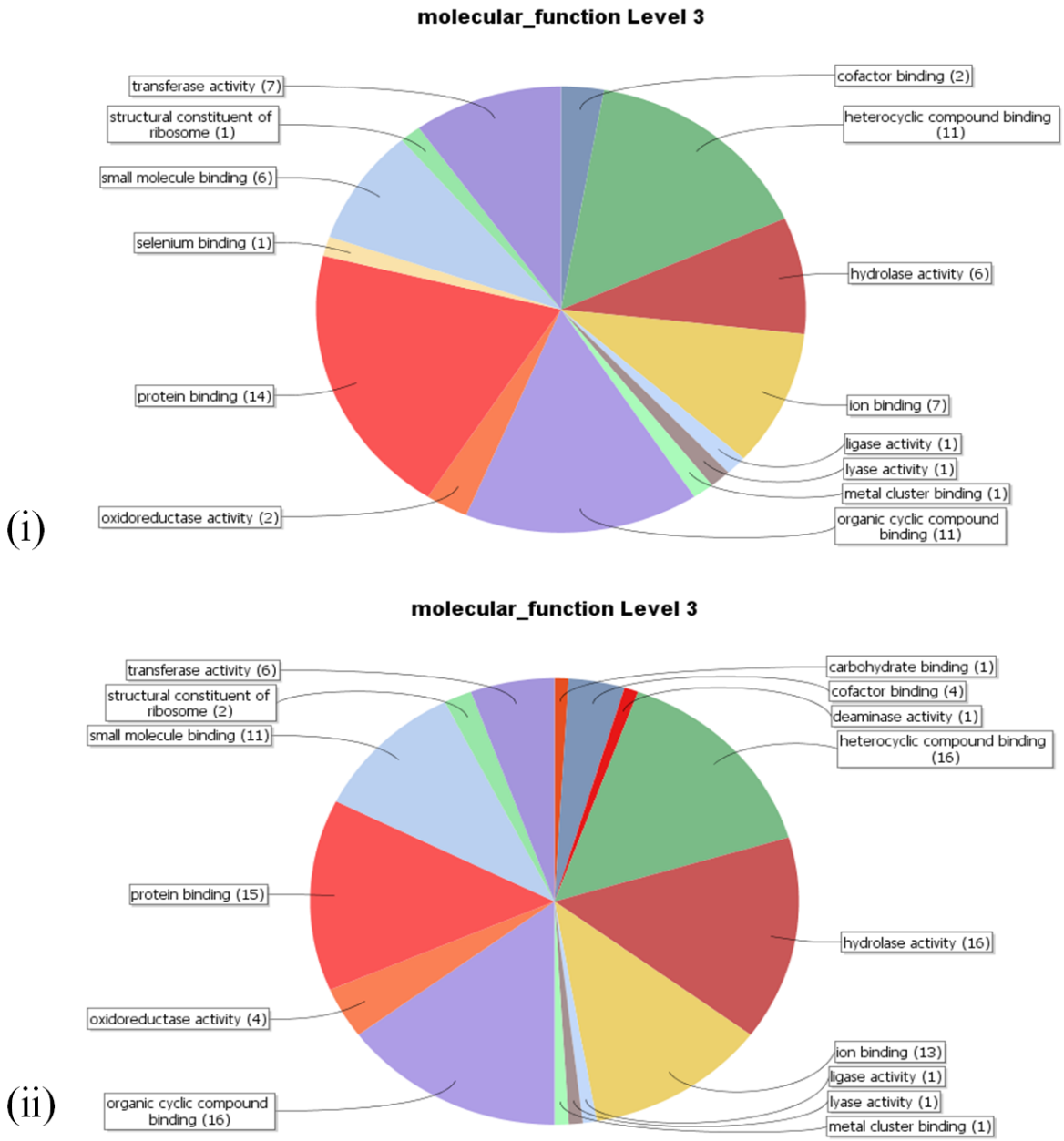


Figure 4.4 Blast2GO molecular function annotation analysis of proteins up-regulated in response to (i) preconditioning 1 (98% RH for 12 h) vs. control and (ii) preconditioning 2 (98% RH for 36 h) vs. control in *P. superbus* nematodes.

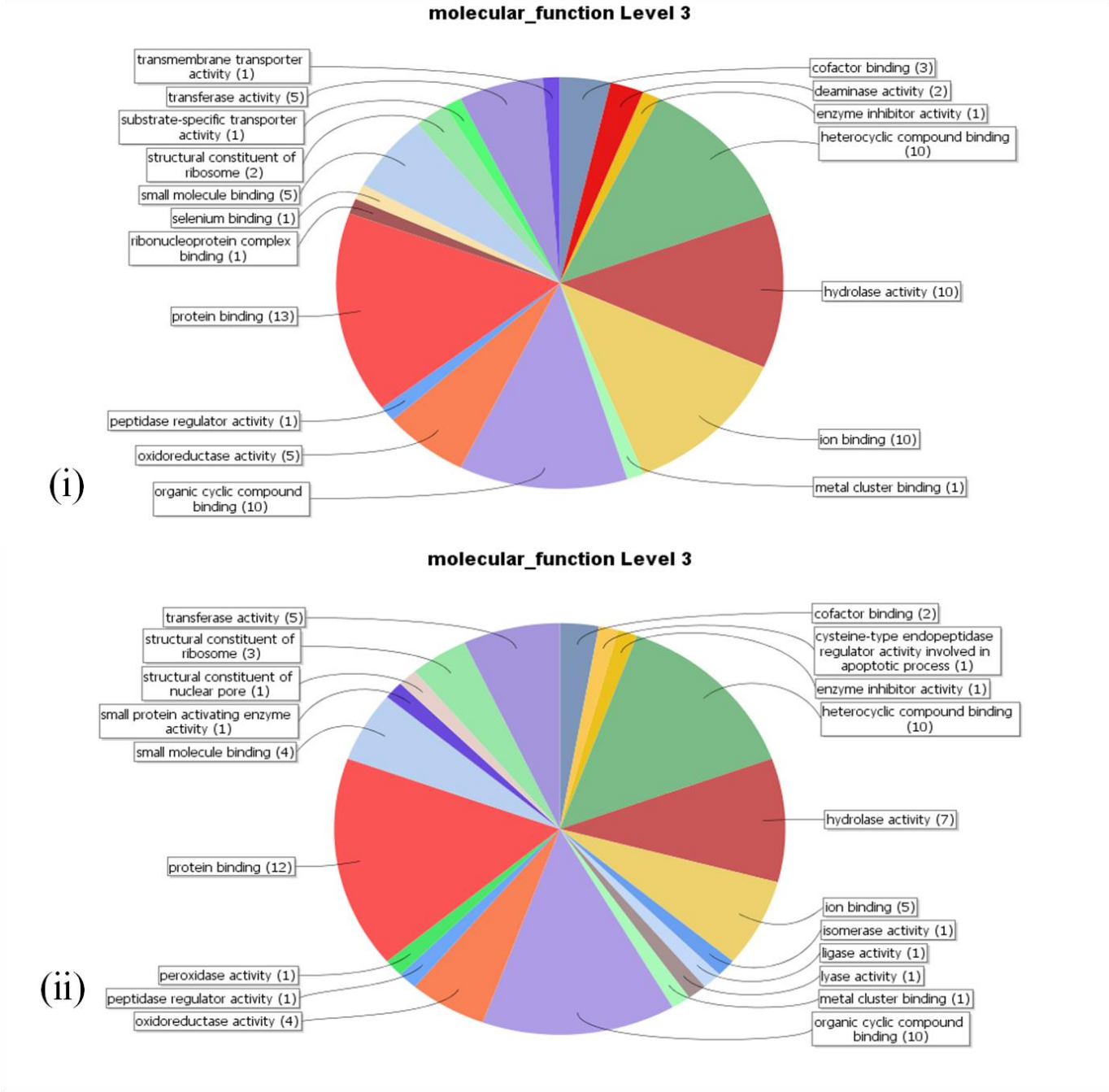


Figure 4.5 Blast2GO molecular function annotation analysis of proteins up-regulated in response to (i) desiccation (98% RH for 36 h, 0% RH for 24 h) vs. control and (ii) recovery (98% RH for 36 h, 0% RH for 24 h, rehydration in water for 2 h) vs. control in *P. superbus* nematodes.

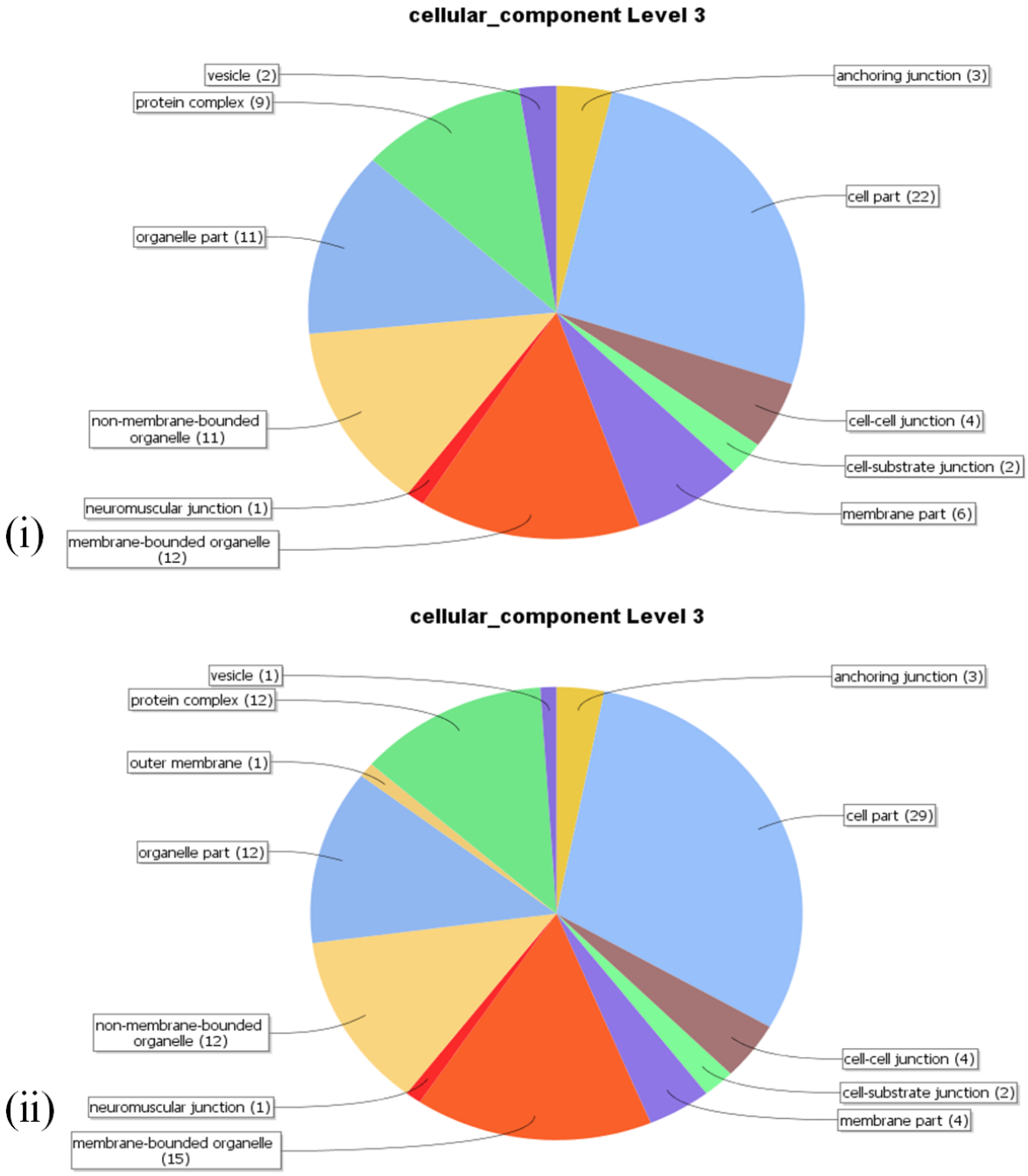


Figure 4.6 Blast2GO cellular component annotation analysis of proteins up-regulated in response to (i) preconditioning 1 (98% RH for 12 h) vs. control and (ii) preconditioning 2 (98% RH for 36 h) vs. control in *P. superbus* nematodes.

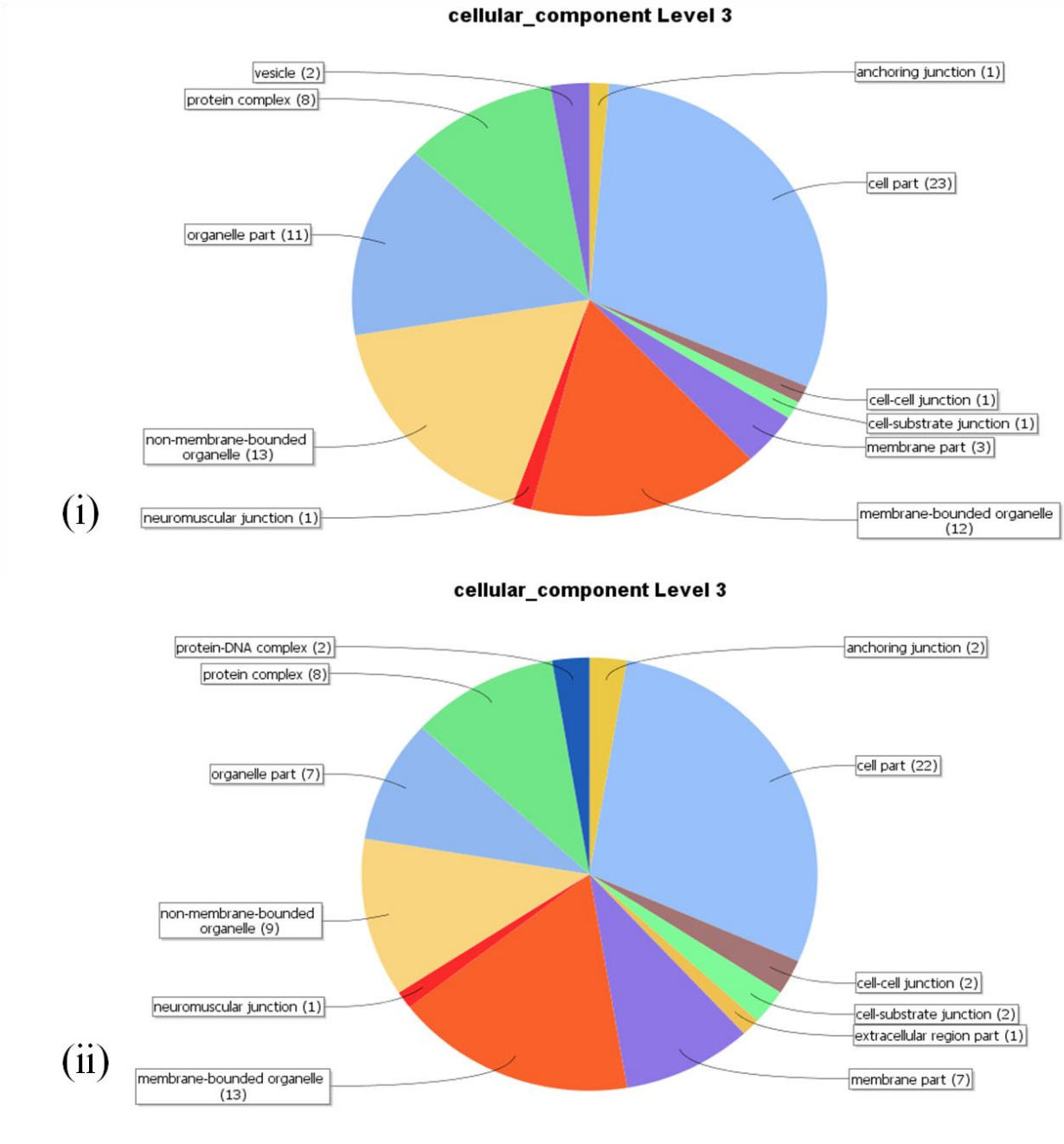


Figure 4.7 Blast2GO cellular component annotation analysis of proteins up-regulated in response to (i) desiccation (98% RH for 36 h, 0% RH for 24 h) vs. control and (ii) recovery (98% RH for 36 h, 0% RH for 24 h, rehydration in water for 2 h) vs. control in *P. superbus* nematodes.

4.2.4 Overview of the proteins up-regulated in response to desiccation-related stress in *P. superbus* nematodes

A large number of proteins were found to be differentially expressed in response to desiccation and recovery from desiccation in *P. superbus*. The identities of the up-regulated proteins are summarised in this section, as well as their biological significance. The main pathways and processes implicated by this differential proteomic analysis in the response of *P. superbus* nematodes to desiccation include translation, cellular organisation and morphogenesis, removal of damaged proteins, protection, DNA packaging and transport, and proteases (Sections 4.2.4.1-4.2.4.7). In addition to the processes described in this section a number of individual proteins in other pathways were also up-regulated in response to desiccation or during recovery from desiccation, but due to space constraints it is not possible to present this data set in this section. A table listing all the proteins which were differentially expressed in response to desiccation-related stress is presented in the supplementary CD appended to this thesis (Supplementary data *P. superbus* LFQ).

4.2.4.1 Translation

There are several proteins up-regulated in response to preconditioning, desiccation and recovery associated with protein synthesis including ribosomal proteins (RPS-2, RSP-23, RSP-24 and RPL-13), elongation factor (EEF-1G) and eukaryotic translation initiation factor (EIF-6) (Table 4.2). The DAVID analysis shows the up-regulated proteins in the recovery dataset were enriched with annotations associated with translation (Table 4.12). This indicates proteins synthesized during recovery may be required for processes involved in recovery and repair in *P. superbus*. Additionally, the proteins down-regulated in response to desiccation were enriched with annotations associated with protein biosynthesis (Table 4.16). This supports the hypothesis formed from the RNA-seq analysis in *P. superbus* (Chapter III) that protein synthesis is actively reduced during desiccation and activated upon rehydration.

The eukaryotic translation initiation factor (EIF-6) was up-regulated in *P. superbus* only in response to desiccation. EIF-6 is a 60S ribosome-associated factor that prevents the assembly of a translationally competent (80S) ribosome (Russell and Spremulli, 1979; Raychaudhuri *et al.*, 1984; Ceci *et al.*, 2003). Additionally, the association of EIF-6 with

RISC in human cell lines suggests a role for EIF-6 in miRNA-mediated post-translational silencing. It also appears that its role in post-translational silencing is conserved in *C. elegans* as EIF-6 is required for *lin-4* (abnormal cell lineage) miRNA-mediated repression of *lin-14* and *lin-28* mRNA and protein levels (Chendrimada *et al.*, 2007). The fact that EIF-6 is only detected in the desiccation dataset is unusual. This protein may have a conserved role in silencing protein synthesis during desiccation in *P. superbus*.

Elongation factor-1 (EF-1) is a cellular protein that plays a role in protein synthesis by mediating the transfer of aminoacyl-tRNA to 80S ribosomes. The EF-1G subunit (up-regulated in *P. superbus* in response to preconditioning) contains an N-terminal glutathione S transferase domain, which may be involved in regulating the assembly of multisubunit complexes containing this elongation factor and aminoacyl-tRNA synthetases (Mathur *et al.*, 1998).

4.2.4.2 Cellular organisation and morphogenesis

The DAVID bioinformatic analysis shows that the proteins which are up-regulated in response to preconditioning and desiccation are enriched for annotations associated with the cytoskeleton and body morphogenesis (Table 4.9-4.11). Additionally, BLAST2GO analysis shows that structural development is the largest GO category of up-regulated proteins in response to all of the condition (Figure 4.2-4.3). Most of the proteins relating to cellular organisation that were up-regulated during preconditioning and desiccation, decrease in expression upon rehydration. This indicates that cellular organisation is most important in *P. superbus* during desiccation. Morphogenesis may also be important during desiccation. In *P. superbus*, there were 15 proteins associated with cytoskeleton organisation and morphogenesis which were up-regulated in response to preconditioning, desiccation and recovery in *P. superbus* (Table 4.3). In this section, I will discuss these proteins and their possible roles in desiccation tolerance. The expression profiles of six of these proteins are presented in Figure 4.8.

VAB-10 (variable abnormal morphology) is a spectraplakin. Spectraplakins are cytoskeleton crosslinkers, which may also be involved in morphogenetic processes (Kim *et al.*, 2011). *C. elegans vab-10* mutants that lack VAB-10B reveal a possible role for spectraplakin in linking F-actin and the microtubule cytoskeleton. Thus spectraplakin may

be important in cytoskeleton reorganisation and/or stabilization during desiccation. In the *vab-10* mutant nematodes the alignment of filamentous F-actin and microtubules was severely disorganised which resulted in failure to translocate the distal tip cell nucleus (Kim *et al.*, 2011). Spectraplakins such as MACF/ACF7 in mammals and Kakapo/Shot in flies have roles in cell migration and also regulate the cytoskeleton. In endodermal cells from the ACF7 knockout mouse, microtubules do not grow along actin bundles. Moreover, the actin bundles are not tethered to the actin cortex, resulting in defective migration in response to wounding (Wu *et al.*, 2008). Studies in *Drosophila* have revealed that axon extension requires the presence of binding sites for F-actin and microtubules in the same Shot (i.e. spectraplakin) molecule (Lee and Kolodziej, 2002). *vab-10* is the sole spectraplakin gene in *C. elegans* and it encodes several protein isoforms with distinct functions related either to plectin (VAB-10A) or to microtubule actin cross-linking factor plakin (VAB-10B) through alternative splicing (Bosher *et al.*, 2003). It has been suggested that a combination of VAB-10A and VAB-10B protects cells against tension that builds up in the epidermis. Previous studies in *C. elegans* have found loss of VAB-10A impairs the integrity of fibrous organelles leading to epidermal detachment from the cuticle and muscles. Lack of VAB-10B leads to increased epidermal thickness during embryonic morphogenesis, when epidermal cells change shape (Bosher *et al.*, 2003). In *P. superbus* two proteins which are up-regulated in response to preconditioning and recovery show sequence similarity to both VAB-10A (PST04832_1) and VAB-10B (PST29612_1). Additionally, they appear to be displaying an inverse expression pattern (Figure 4.8). VAB-10A (PST04832_1) is only up-regulated during preconditioning 1 while VAB-10B (PST29612_1) is up-regulated in preconditioning 2 and recovery.

Two putative GEI-15 (gei=gex interacting protein) proteins are up-regulated in response to preconditioning and desiccation. GEI-15 has been found to interact with GEX-3 in *C. elegans* in yeast 2-hybrid analyses (Tsuboi *et al.*, 2002). In *C. elegans*, GEX-3 activities are necessary for tissue morphogenesis and cell migrations. It is believed that GEX-3 may function at cell boundaries to regulate cell migrations and cell shape changes required for proper morphogenesis and development (Soto *et al.*, 2002). It is interesting that four proteins, two VAB-10 and two GEI-15, associated with cell migration and morphogenesis were up-regulated in response to preconditioning, desiccation and recovery in *P. superbus*.

The protein LAM-1 (laminin) was up-regulated in response to preconditioning and desiccation in *P. superbus*. Laminin is a component of the basement membrane that affects protein integrity of the muscle cells which they support (Jensen *et al.*, 2012). In *C. elegans*, *lam-1* encodes a laminin beta that affects degenerin-induced cell death. RNAi knockdown of both *lam-1* and *lam-2* genes resulted in animals arresting during morphogenesis. Therefore, *lam-1* is required redundantly with *lam-2* for morphogenesis in *C. elegans* (Kao *et al.*, 2006). Another protein involved in cellular organisation called DLC-1 (dynein light chain) was also up-regulated in response to preconditioning and desiccation in *P. superbus*. Cytoplamic dynein is a microtubule-dependent motor protein that has roles in diverse cellular processes including meiotic and mitotic spindle assembly and function, neuronal transport, and organelle positioning (Vallee *et al.*, 2004; O'Rourke *et al.*, 2007). In *C. elegans*, DLC-1 is required for normal embryonic and larval viability, embryonic pronuclear migration, growth speed, fertility, brood size, body shape, and cuticular integrity. CAP-2 (CAP-z protein), also up-regulated in preconditioning and desiccation, has been predicted to interact with DLC-1. CAP-2 is the beta subunit of actin capping protein that regulates actin cytoskeleton assembly and establishment of initial asymmetry in the embryo in *C. elegans* (Zhong and Sternberg, 2006).

Interestingly two proteins associated with the apical junction called AJM-1 (apical junction molecule) and DLG-1 (drosophila disc large homolog) were up-regulated in response to preconditioning, desiccation and recovery in *P. superbus*. Apical junction proteins such as AJM-1 and DLG-1 are crucial for these epithelial functions. Apical junctions play a role in providing the paracellular barrier required to separate tissue spaces (Miyoshi and Takai, 2008). The apical cell-cell junctions mediate strong adhesive linkages between adjacent epithelial cell and form connections to the cytoskeleton (Goodwin and Yap, 2004; Lockwood *et al.*, 2008). Apical junctions also provide a cellular permeability barrier that regulates diffusion of solutes between epithelial cells (Anderson *et al.*, 2004). In *C. elegans*, recent yeast two hybrid and RNAi studies have found that AJM-1 and DLG-1 form a complex which is essential for embryonic elongation (Koppen *et al.*, 2001; Lockwood *et al.*, 2008). DLG-1 is a member-associated guanylate kinase (MAGUK) which acts as a scaffolding molecule. MAGUKs contain multiple protein-protein interaction domains that integrate specialized proteins found at cell-cell junctions. These specialized proteins are necessary for the movement and

integrity of epithelia (Lockwood *et al.*, 2008). Epithelial tissues undergo morphogenetic movements that protect the organism from its external and internal environments. These morphogenetic movements may be important in *P. superbus* to control water loss and stabilise the integrity of the epithelia during desiccation. Additionally, these apical junction proteins may be important during rehydration, when nematodes need to efficiently regain the water lost during desiccation (Casella *et al.*, 1987).

Similar to AJM-1 and DLC-1, both MLC-4 (myosin light chain) and NMY-1 (non-muscle myosin) activity is required for proper embryonic elongation and normal morphology in *C. elegans* (Shelton *et al.*, 1999). MLC-4 and NMY-1 were both up-regulated in response to preconditioning in *P. superbus*. The proteins UNC-44 (uncoordinated) and UNC-94 were up-regulated in response to dehydration in *P. superbus*. UNC-94 is a tropomodulin protein which caps the 'minus' end of actin regulating the length of actin filaments in muscle and non-muscle cells (Stevenson *et al.*, 2007). UNC-44 is an ankyrin-like protein that is required for proper sex myoblast and axonal guidance during development in *C. elegans*. UNC-44 interacts with another protein called SAX-7 to maintain neuronal positioning (Zhou *et al.*, 2008).

Thus, the proteins related to cellular organisation and morphogenesis which were up-regulated in response to desiccation and recovery in *P. superbus* show the importance of adjustments to the cytoskeleton and cellular structures during anhydrobiosis.

4.2.4.3 Removal of damaged proteins

A number of up-regulated proteins were associated with the ubiquitin-proteasome degradation system and phagocytosis, particularly in response to recovery from desiccation. The DAVID analysis also shows that the proteins up-regulated in response to recovery were enriched with annotations associated with the ubiquitin conjugation pathway and the proteasome. The up-regulated proteins include proteasome subunits (PBS-6 and PAS-4), proteasome regulatory subunits (RPT-6, RPT-2 and PST00761_1), ubiquitin (UBQ-1), ubiquitin activating enzyme (UBA-1) and Rab GTPase protein (RAB-7) (Table 4.4).

RAB-7 activity is required for normal apoptotic cell clearance, embryogenesis, locomotion and body morphology in *C. elegans*. RAB-7 also acts in endosome and endosome to lysosome trafficking (Mukhopadhyay *et al.*, 2007). Finally, RAB-7 is

involved in tub-1/tubby mediated endocytic pathway for the regulation of fat storage (Mukhopadhyay *et al.*, 2007). In *C. elegans* RAB-2, RAB-7 and RAB-14 function cooperatively and act in sequential steps to regulate phagolysosome formation (Guo *et al.*, 2010). RAB-7 acts downstream of RAB-5 to control the late steps of phagosome maturation (Kinchen *et al.*, 2008; Yu *et al.*, 2008). *P. superbus* nematodes rely on the ubiquitin-proteasome systems as well as autophagy systems to remove damaged proteins (Kubota, 2009) that accumulate during desiccation. The role of RAB-7 in autophagy may be useful in degrading damaged proteins which could accumulate during desiccation (Hyttinen *et al.*, 2013). The ubiquitin-proteasome and autophagy systems are likely to play an important role in removing and recycling damaged proteins which may accumulate in response to desiccation damage.

Ubiquitin has many functions in the cell apart from tagging proteins for degradation by the proteasomal system. Ubiquitin has diverse roles in protein trafficking, DNA repair and transcription (Welchman *et al.*, 2005; Howard *et al.*, 2007). Ubiquitin and the ubiquitin-proteasome system can also control transcription in several ways. One such mechanism, discovered in yeast, is through the ubiquitylation of histone H2B which in turn mediates the methylation of histone H3 resulting in altered gene expression (Sun and Allis, 2002). Interestingly, two putative H2B proteins (PST08533 and PST21462_1, discussed further in Section 4.2.4.5) were up-regulated along with ubiquitin (UBQ-1) and ubiquitin activating enzyme (UBA-1) during recovery from desiccation, suggesting a possible role for chromatin remodelling in the response to desiccation stress in *P. superbus*.

4.2.4.4 Protection

There were two LEA proteins that had increased expression in response to the two preconditioning conditions and also to desiccation. The *P. superbus* LEA-1 protein had increased expression in preconditioning at 98% RH for 12 h and 36 h as well as desiccation. The other LEA protein (PST28289_1) was up-regulated in the two preconditioning datasets (Table 4.5).

A subunit of the TCP-1 chaperonin complex (CCT-7) was up-regulated in response to preconditioning. TCP-1 was originally characterized as being responsible for only actin and tubulin folding (Gao *et al.*, 1992; Yaffe *et al.*, 1992). However, as time has progressed the importance of TCP-1 in the folding of the newly synthesized polypeptides

from a wide range of proteins has become apparent (Young *et al.*, 2004). TCP-1 has a double ring-shaped architecture with each ring containing 8 subunits (Valpuesta *et al.*, 2002). TCP-1 may function by enclosing substrate polypeptides in a cavity, where unfolded polypeptides are folded in an ATP-dependent manner. The opening and closing of the cage is mediated by the α-helical extension of its apical domain (Meyer *et al.*, 2003). Previous studies in bacteria, yeast and mammals have found that that TCP-1 co-operates with HSP-70 and DnaK proteins to assist in the folding of newly synthesized polypeptides (Young *et al.*, 2004). Furthermore, in mammals TCP-1 and GimC/prefoldin have been shown to work as an integrated team for the purpose of protein folding (Valpuesta *et al.*, 2002). Interestingly, PFD-2 (prefoldin) and PFD-5 were up-regulated in the *P. superbus* recovery dataset, compared to desiccation. Remarkably, in the *C. elegans* RNA-seq analysis (Chapter V), the actin/tubulin:prefoldin complex, which associates with CCT/TriC pathway, was up-regulated in response to preconditioning, desiccation and recovery.

A putative HSP-20 (PST13335_1) was up-regulated in the recovery dataset and shows greatest sequence similarity (BLASTX) to an alpha crytallin family protein in *Haemonchus contortus* (nematode sheep parasite). Strikingly, the RNA-seq analysis of desiccation tolerance in *P. superbus* (Chapter III) found that a putative *hsp-20* (PST09331_1) gene was up-regulated also in the recovery dataset. Finally, the DAO-2 (dauer or aging adult overexpression, PST18855_1) protein was up-regulated in the desiccated samples. In *C. elegans*, the expression of the *dao-2* gene is controlled by the DAF-2 signalling cascade. The *daf-2* insulin-like receptor pathway regulates development and lifespan (Yu and Larsen, 2001). Interestingly, there is much overlap between the genes implicated in both aging and stress tolerance in *C. elegans* (Zhou *et al.*, 2011). In the RNA-seq analysis of desiccation tolerance in *P. superbus* two genes that are homologs of *hsf-1* (PST28462_1 and PST05616_1) were up-regulated in response to preconditioning and recovery. Both *dao-2* and *hsf-1* encode proteins that act down-stream of DAF-2.

4.2.4.5 DNA packaging and transport

The proteins associated with DNA packaging and transport that are up-regulated in *P. superbus* in response to preconditioning and recovery are shown in Table 4.6. Two proteins (PST08533_1 and PST21462_1) that were up-regulated specifically in response

to recovery from desiccation are both homologs of *C. elegans* histone H2B proteins. Additionally, the histone HIL-2, which is a putative regulator of chromosome condensation, was up-regulated in recovery compared to desiccation. Interestingly, the differential proteomic analysis of desiccation tolerance in *C. elegans* dauer larvae (Chapter VI) also revealed that two histones, H3 and H2A, were up-regulated in response to desiccation and recovery. The nucleosome, the functional structural unit of chromatin, contains an octamer of core histones H3, H4, H2A and H2B (Bonenfant *et al.*, 2006). In addition to their well-known function in DNA packaging these core histones are also involved in DNA replication, transcription and repair by controlling the accessibility of the different macromolecular machineries to their substrate (Escargueil *et al.*, 2008). Furthermore, studies in yeast have revealed that histone H2B is required for the DNA damage checkpoint response. In *S. cerevisiae*, ubiquitination of histone H2B by the Rad6-Bre1 complex is necessary for activation of Rad53 kinase and cell cycle arrest in response to genotoxic stresses (Giannattasio *et al.*, 2005). The fact that different types of histones were up-regulated in response to desiccation and recovery from desiccation across two nematode taxa, indicates that these proteins may be important in surviving desiccation, possibly through their auxiliary roles in DNA replication, transcription and repair.

4.2.4.6 Proteases

Seven different proteases were up-regulated across preconditioning, desiccation and recovery (Table 4.7). These include aspartic proteases (ASP-2 and ASP-3), metallopeptidases (NEP-17, LAP-1 and APP-1) and cysteine proteases (CPZ-1). Additionally, the DAVID analysis also revealed the proteins up-regulated in response to preconditioning 2 and recovery were enriched with annotations associated with peptidase and proteolysis activity (Table 4.10 and 4.11). NEP-17 is a member of the neprilysin metallopeptidase family and was up-regulated in *P. superbus* in response to preconditioning 2. Neprilysins, found in the outer surface of animal cells, negatively regulate small signalling peptides such as insulin by cleaving them (Turner, 2003). In *P. superbus*, NEP-17 may regulate the insulin signalling pathway, which appears to be important during desiccation. The majority of these proteases were up-regulated during preconditioning 2. Interestingly, similar changes were observed in the *P. superbus* transcriptome in response to preconditioning (Chapter III). The RNA-seq analysis of *P.*

superbus revealed that 9 protease genes were up-regulated in response to preconditioning 2 while only one protease showed increased expression in the recovery dataset.

Proteolysis plays vital roles in cellular processes from fertilization through to cell death (Turner, 2003). The transcriptomic and proteomic analyses of *P. superbus* also reveal that proteases may have a role in surviving desiccation. The proteases may work in combination with the ubiquitin-proteasome machinery to degrade proteins that have become damaged during desiccation. In plants, the degradation of proteins and an increase in proteolytic enzymes are common responses to desiccation (Gasulla *et al.*, 2013). These authors hypothesize that the induction of proteases may play a role in the reallocation of resources for biosynthesis of proteins involved in dehydration tolerance mechanisms.

4.2.4.7 Proteins up-regulated in recovery compared to desiccation

The results show that in response to recovery (compared to desiccation) there was an up-regulation of proteins involved in translation, antioxidant activity, protein degradation, protein folding, lipid storage and DNA packaging. Three peroxiredoxin antioxidants were found to have increased expression in response to recovery (compared to desiccation) which were PRDX-3, PRDX-6 and the redox-regulatory protein PST16499_1. Additionally, PST16499_1 was also up-regulated in response to preconditioning 1 compared to the control. Similarly, in the RNA-seq analysis of *P. superbus*, four peroxiredoxins were up-regulated mainly in the preconditioning 2 dataset. Furthermore, a previous qPCR analysis carried out by our group has shown that a gene which encodes 1-Cys peroxiredoxin was up-regulated in *P. superbus* in response to exposure to 98% RH for 12 h (Tyson *et al*., 2012). Two proteins, ACBP-1 and ACBP-3, required for lipid droplet morphology and lipid storage function (Elle *et al.*, 2011), were up-regulated in response to recovery compared to desiccation. This suggests that metabolism which slowed down during desiccation and is now returning to normal in recovery by breaking down lipid stores to release energy. The prefoldin proteins PFD-2 and PFD-5 were both up-regulated in the recovery versus desiccation dataset and are required for normal microtubule growth in *C. elegans* (Lundin *et al.*, 2008). TCP-1 and prefoldin have been found to work as an integrated team for the purpose of protein folding (Valpuesta *et al.*, 2002). Moreover, TCP-1 was up-regulated in response to preconditioning in *P. superbus* as discussed in Section 4.2.4.4.

Table 4.2 Proteins associated with translation which were up-regulated in response to preconditioning 1, preconditioning 2, desiccation and recovery compared to the control in *P. superbus* nematodes. The protein description, *P. superbus* gene name (PS ID), closest *C. elegans* homolog ID (CE ID), closest *C. elegans* homolog gene name (CE gene name), ANOVA (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Protein description	**PS ID**	**CE ID**	**CE gene name**	**ANOVA**	**LFQ intensity**				
					Control	**Preconditioning 1**	**Preconditioning 2**	**Desiccation**	**Recovery**
Four conditions									
40S ribosomal protein	PST12500_1	F28D1.7	*rps-23*		NaN	22.34	18.72	20.11	19.33
Two condition									
Elongation factor 1 gamma	PST04351_1	F17C11.9	*eef-1G*		NaN	20.47	19.64	NaN	NaN
One condition									
Small subunit ribosomal protein	PST14217_1	T07A9.11	*rps-24*	+	24.35	-	25.28	-	-
Eukaryotic translation initiation factor	PST06038_1	C47B2.5	*eif-6*	+	23.20	-	-	23.89	-
40S ribosomal protein s19s	PST04494_1	C23G10.2	*C23G10.2*	+	25.27	-	-	26.47	-
40S ribosomal protein s2	PST28714_1	C49H3.11	*rps-2*	+	23.00	-	-	-	23.92
60S ribosomal protein l13	PST08115_1	C32E8.2	*rpl-13*	+	26.89	-	-	-	27.27

Table 4.3 Proteins associated with cellular organisation and morphogenesis which were up-regulated in response to preconditioning 1, preconditioning 2, desiccation and recovery compared to the control in *P. superbus* nematodes. The protein description, *P. superbus* gene name (PS ID), closest *C. elegans* homolog ID (CE ID), closest *C. elegans* homolog gene name (CE gene name), ANOVA (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Protein description	**PS ID**	**CE ID**	**CE gene name**	**ANOVA**	**LFQ intensity**				
					Control	**Preconditioning 1**	**Preconditioning 2**	**Desiccation**	**Recovery**
Three conditions									
Laminin subunit	PST22320_1	W03F8.10	*lam-1*		NaN	22.83	23.05	22.54	NaN
Cre-GEI-15	PST18347_1	M03A8.4	*gei-15*		NaN	21.49	21.79	21.11	NaN
Dynein light chain	PST06626_1	T26A5.9	*dlc-1*		NaN	23.39	22.07	21.45	NaN
Two conditions					NaN				
Protein AJM- isoform b	PST02611_1	C25A11.4	*ajm-1*		NaN	19.92	19.92	NaN	NaN
Guanylate kinase family protein	PST01510_1	C25F6.2	*dlg-1*		NaN	22.34	NaN	NaN	22.39
Tropomodulin family protein	PST14993_1	C06A5.7	*unc-94*		NaN	19.49	NaN	21.36	NaN
VAB-10B	PST29612_1	ZK1151.1	*vab-10*		NaN	22.27	NaN	NaN	20.98
Capping protein (actin filament)	PST26749_1	M106.5	*cap-2*		NaN	23.44	NaN	22.19	NaN
Myosin	PST03269_1	C56G7.1	*mlc-4*	+	23.1026	-	24.06	23.76	-
One condition									
Protein nmy-1	PST02173_1	F52B10.1	*nmy-1*	+	21.77217	-	23.42	-	-
Ankyrin-related UNC-44	PST12197_1	B0350.2	*unc-44*	+	22.7179	-	23.62	-	-
VAB-10a protein	PST04832_1	ZK1151.1	*vab-10*	+	22.7686	-	23.75	-	-
Profilin family protein	PST05820_1	F35C8.6	*pfn-2*	+	26.45367	-	-	27.20	-
Cre-GEI-15 protein	PST09472_1	M03A8.4	*gei-15*	+	22.67375	-	-	24.38	-
Reticulon family protein	PST21085_1	W06A7.3	*ret-1*	+	21.381	22.00235	-	-	-

Table 4.4 Proteins associated with protein degradation which were up-regulated in response to preconditioning 1, preconditioning 2, desiccation and recovery compared to the control in *P. superbus* nematodes. The protein description, *P. superbus* gene name (PS ID), closest C. elegans homolog ID (CE ID), closest *C. elegans homolog* gene name (CE gene name), ANOVA (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Protein description	**PS ID**	**CE ID**	**CE gene name**	**ANOVA**	**LFQ intensity**				
					Control	**Preconditioning 1**	**Preconditioning 2**	**Desiccation**	**Recovery**
Four conditions									
26s protease regulatory subunit 8	PST03869_1	Y49E10.1	*rpt-6*		NaN	20.33	22.57	23.70	21.24
Three conditions									
Ras-related protein RAB-7a-like	PST09042_1	W03C9.3	*rab-7*		NaN	22.2212	23.3859	23.6453	NA
Signal transducing adapter molecule	PST29322_1	C34G6.7	*stam-1*		NaN	20.47495	21.4568	21.0989	NA
Two conditions									
26s proteasome non-atpase regulatory subunit 10	PST00761_1	F40G9.17	*F40G9.17*		NaN	21.08	NaN	NaN	22.08
One condition									
Proteasome subunit alpha	PST17875_1	C36B1.4	*pas-4*	+	23.7344	-	24.54	-	-
26s protease regulatory subunit 4	PST03604_1	F29G9.5	*rpt-2*	+	24.15	-	-	-	24.51
Proteasome subunit beta type 1	PST22121_1	C02F5.9	*pbs-6*	+	22.85	-	-	-	23.48
Protein UBQ- isoform c	PST16555_1	F25B5.4	*ubq-1*	+	24.77	-	-	-	25.42
Ubiquitin-activating enzyme e1	PST07374_1	C47E12.5	*uba-1*	+	21.88	-	-	-	23.06

Table 4.5 Proteins associated with protection which were up-regulated in response to preconditioning 1, preconditioning 2, desiccation and recovery compared to the control in *P. superbus* nematodes. The protein description, *P. superbus* gene name (PS ID), closest *C. elegans* homolog ID (CE ID), closest *C. elegans* homolog gene name (CE gene name), ANOVA (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Protein description	**PS ID**	**CE ID**	**CE gene name**	**ANOVA**	**LFQ intensity**				
					Control	**Preconditioning 1**	**Preconditioning 2**	**Desiccation**	**Recovery**
Three conditions									
Group 3 lea protein	PST15499_1	K08H10.1	*lea-1*		NaN	22.19	23.71	23.73	NaN
Two conditions									
LEA	PST28289_1	NA	NA		NaN	19.48	18.89	NaN	NaN
T-complex protein 1 subunit eta	PST09789_1	T10B5.5	*cct-7*		NaN	21.87	22.36	NaN	NaN
One condition									
Dauer or aging overexpression	PST18855_1	M03A1.7	*dao-2*	+	24.7244	-	-	25.68	-
Small heat shock protein (HSP-20)	PST13335_1	Y55F3BR.6	*Y55F3BR.6*	+	24.13893	-	-	-	24.45

Table 4.6 Proteins associated with transport and DNA packaging which were up-regulated in response to preconditioning 1, preconditioning 2, desiccation and recovery compared to the control in *P. superbus* nematodes. The protein description, *P. superbus* gene name (PS ID), closest *C. elegans* homolog ID (CE ID), closest *C. elegans* homolog gene name (CE gene name), ANOVA (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Protein Description	**PS ID**	**CE ID**	**CE gene name**	**ANOVA**	**LFQ intensity**				
					Control	**Preconditioning 1**	**Preconditioning 2**	**Desiccation**	**Recovery**
Two conditions									
Chloride intracellular channel EXC-4	PST11761	NA	NA		NaN	21.27	20.55	NaN	21.04
One condition									
Protein HIS-44	PST08533_1	F55G1.3	*his-62*	+	27.699	-	-	-	28.21
Histone H2B	PST21462_1	K06C4.12	*his-22*		NaN	NaN	NaN	NaN	21.13
Nuclear pore glycoprotein p62	PST14143_1	NA	NA		NaN	NaN	NaN	NaN	22.22

Table 4.7 Proteins associated with protease activity which were up-regulated in response to preconditioning 1, preconditioning 2, desiccation and recovery compared to the control in *P. superbus* nematodes. The protein description, *P. superbus* gene name (PS ID), closest *C. elegans* homolog ID (CE ID), closest *C. elegans* homolog gene name (CE gene name), ANOVA (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Protein description	**PS ID**	**CE ID**	**CE gene name**	**ANOVA**	**LFQ intensity**				
					Control	**Preconditioning 1**	**Preconditioning 2**	**Desiccation**	**Recovery**
All four conditions									
Lipoamide acyltransferase	PST03614_1	ZK669.4	*ZK669.4*		NaN	21.40	22.78	22.75	22.18
Three conditions									
Probable aminopeptidase npepl1	PST13277_1	ZK353.6	*lap-1*		NaN	21.57	23.28	23.22	NaN
Two conditions									
Aspartic protease	PST06647_1	T18H9.2	*asp-2*		NaN	19.92	21.51	NaN	NaN
One condition									
X-prolyl aminopeptidase	PST23530_1	W03G9.4	*app-1*	+	23.8468	-	25.07	-	-
NEPrilysin metallopeptidase family	PST22676_1	F54F11.2	*nep-17*	+	25.19657	-	26.45	-	-
Lysosomal aspartic protease-like	PST14638_1	H22K11.1	*asp-3*	+	25.6533	-	-	26.66	-
Cathepsin z precursor	PST04470_1	F32B5.8	*cpz-1*		NaN	NaN	NaN	NaN	23.74

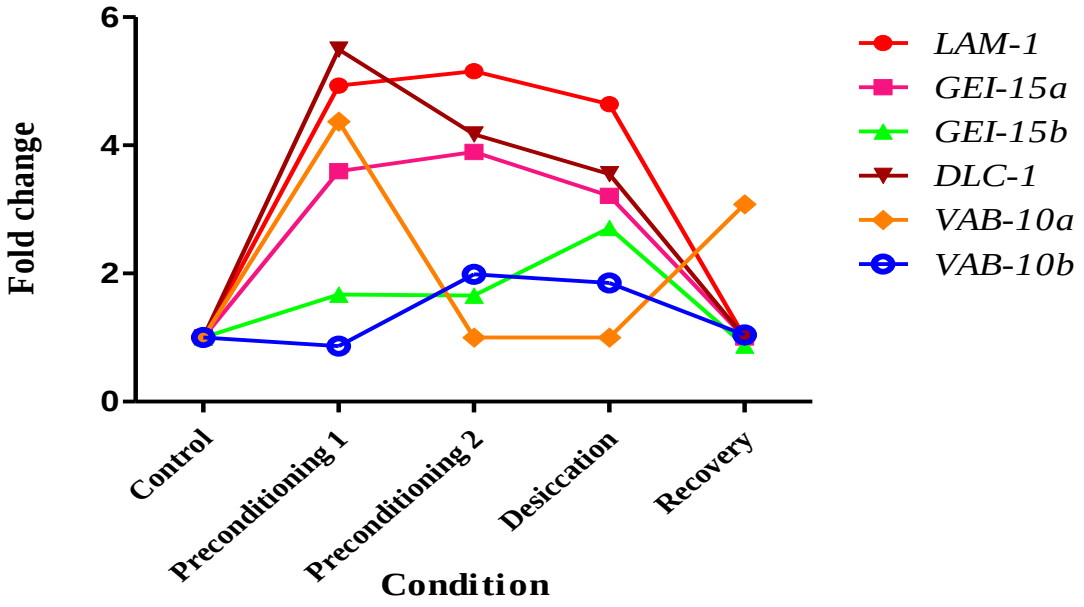


Figure 4.8 Change in expression of proteins (based on label-free quantification intensities) involved in cellular organisation in response to preconditioning, desiccation and recovery compared to the control in *P. superbus* (LAM-1, GEI-15a, GEI-15b, DLC-1, VAB-10a and VAB-10b). When proteins were not identified in protein extracts from a specific treatment, a base LFQ value (lowest recorded LFQ intensity across the experiment) was applied.

4.2.5 Using 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) to identify proteins up-regulated in response to dehydration in *P. superbus*

The treatments used for the 2D-DIGE analysis were control and desiccated (98% RH for 36 h) *P. superbus* nematodes. Protein extraction, labelling, 2D gel electrophoresis, image acquisition and protein identification are described in Sections 2.4.1 and 2.4.6. The two-dimensional reference gel of differentially expressed *P. superbus* protein in response to 98% RH for 36 h is shown in Figure 4.9. The DIGE analysis revealed that 17 proteins were differentially expressed in *P. superbus* in response to preconditioning (Table 4.8).

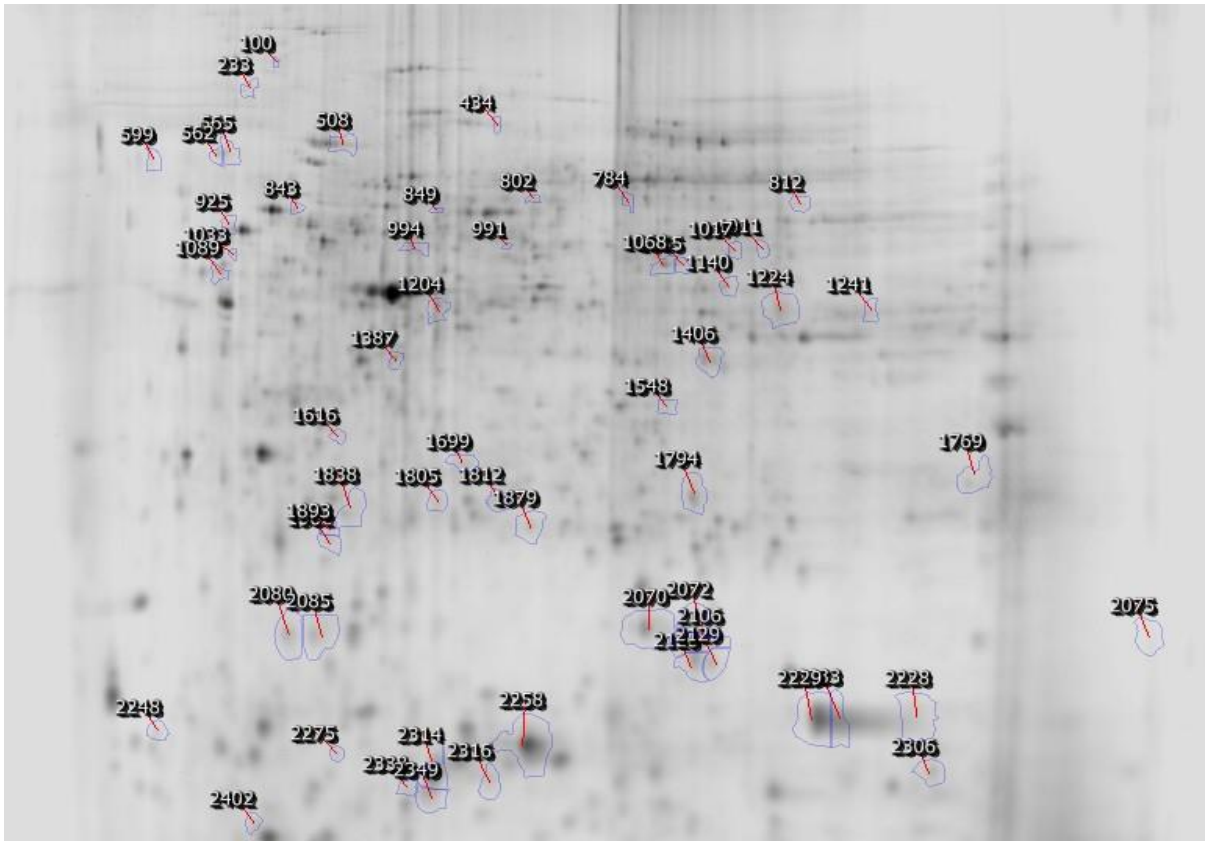


Figure 4.9 Two-dimensional reference gel of *P. superbus* protein. The proteins which were differentially expressed in response to preconditioning (98% RH for 36 h) compared to the control are circled and identified by the reference number assigned to them by the Progenesis software.

Table 4.8 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE): Differentially expressed proteins which were identified in response to preconditioning (98% RH for 36 h) in *P. superbus* nematodes. The fold change, top *P. superbus* hit, protein function, Progenesis number, (ANOVA ($p>0.05$), peak intensity and the orbitrap analysis result for each of the differentially expressed proteins is shown.

Fold change	Top PS hit	Protein function	Progenesis number	ANOVA	Peak intensity (%)	Orbitrap analysis
2	PST15764_1	Intermediate filament protein 1	802	0.000169	25.48	
1.9	PST13945_1	Polyprotein	2314	0.007	88.9	
1.5	PST01119_1	Glyceraldehyde-3 phosphate dehydrogenase	2349	0.008	81.5	
1.4	PST04351_1	Elongation factor 1 gamma	1204	0.01	84	Statistically up-regulated
1.3	PST21738	ATP synthase subunit gamma	1805	0.007	97.1	
1.2	PST18525_1	Proteasome subunit alpha type 4	1548	0.014	86.9	
1.1	PST02672_1	Pyruvate Kinase	1224	0.004	92.6	Statistically up-regulated
1.1	PST05289_1	Triosephosphate isomerase	1794	0.009	92.7	
-1.2	PST08585_1	Peptidl-prolyl cis-trans isomerase	2275	0.011	94.1	
-1.5	PST15667_1	Glucodidase 2 subunit beta	565	0.006	17.2	
-1.6	PST04009_1	Heat shock protein 90	508	0.003	80.37	
-1.6	PST07466_1	Calreticulin	1033	0.003	44.31	
-1.7	PST16105_1	Major sperm protein	2229	0.011	86.1	Not represented
-1.7	PST15989_1	Major sperm protein	2228	0.006	88.9	Not represented
-1.7	PST22470_1	Major sperm protein	2233	0.01	94.4	Not represented
-1.9	PST22679_1	Translationally controlled tumour protein	1893	0.007	95.7	
-2.3	PST21219_1	Myosin regulatory light chain	2080	0.018	92.97	Not represented

4.2.6 Comparing 2-D DIGE and gel-free proteomics for proteins up-regulated in *P. superbus* in response to 98% RH for 36 h versus the control

Two-dimensional gel electrophoresis (2-DE) is widely applied in proteomics. However, state of the art quantitative mass spectrometry based techniques have recently become the preferred method of choice in quantitative proteomics. The main reasons behind the decrease in 2-DE applications are issues relating to reproducibility (Lilley *et al.*, 2002), poor representation of low abundance proteins (Gygi *et al.*, 2000), or highly acidic/basic proteins, or extremely large proteins or hydrophobicity (Ong and Pandey, 2001), and difficulties in automation of the gel based techniques (Tonge *et al.*, 2001; Abdallah *et al.*, 2012).

The 2-D DIGE analysis of differentially expressed proteins in *P. superbus* in response to 98% RH for 36 h revealed that 17 proteins were differentially expressed (Table 4.8). Strikingly, this is in contrast to the LFQ analysis derived from HR/AM spectrometry, comparing the same conditions in *P. superbus* (98% RH for 36 h versus the control) which found 165 proteins to be differentially expressed. The 2-D DIGE analysis found that 8 proteins were up-regulated and 9 proteins were down-regulated in *P. superbus*. From the 8 proteins up-regulated in the 2-D DIGE analysis, 2 were also statistically up-regulated in the LFQ analysis which were pyruvate kinase (PST02672_1) and elongation factor 1 gamma (PST04351_1). Moreover, of the remaining 6 proteins up-regulated in the 2-D DIGE analysis, 4 showed an increase in expression (although not statistically significant) in the LFQ analysis. This indicates that some of the differences between the MS-based LFQ and 2D-DIGE analyses could be associated with the different statistical tests used.

Comparing the down-regulated proteins in 2-D DIGE analysis and the orbitrap experiment there was no overlap of proteins. This could be attributed to the fact that four of the proteins found to be down-regulated in the 2-D DIGE analysis were not represented in the LFQ analysis (PST16105_1, PST15989_1, PST22470_1 and PST21219_1). Additionally, heat shock protein 90 (PST04009_1) which was down-regulated in the 2-D DIGE analysis, showed decreased expression in the LFQ analysis (although not significant). Moreover, there was a different heat shock protein 90 (PST08952_1) down-regulated in the LFQ analysis. The same heat shock protein may have been down-

regulated in both the 2-D DIGE and LFQ analysis, with the difference in result down to different protein identification software. In the 2-D DIGE analysis, database searches were carried out with Agilent technologies Spectra Mill Mass Proteomics software. While, MaxQuant software supported by the Andromeda database search engine was used in the LFQ analysis. The advantages of gel-free proteomics coupled to HR/AM spectrometry relative to 2-D DIGE approaches are apparent in this chapter as considerable more differentially expressed proteins were identified.

4.3 Conclusion

In this differential proteomic study, a great number of proteins were differentially expressed in response to desiccation and recovery from desiccation in *P. superbus* nematodes. An overview of the proteins up-regulated in response to preconditioning, desiccation and recovery is shown in Figure 4.10. In *P. superbus*, the proteins up-regulated in response to dehydration and rehydration are mainly involved in processes associated with translation, antioxidant activity, DNA repackaging, ubiquitin-proteasome degradation, chaperone activity, cellular organisation and transport as discussed in section 4.2.4.

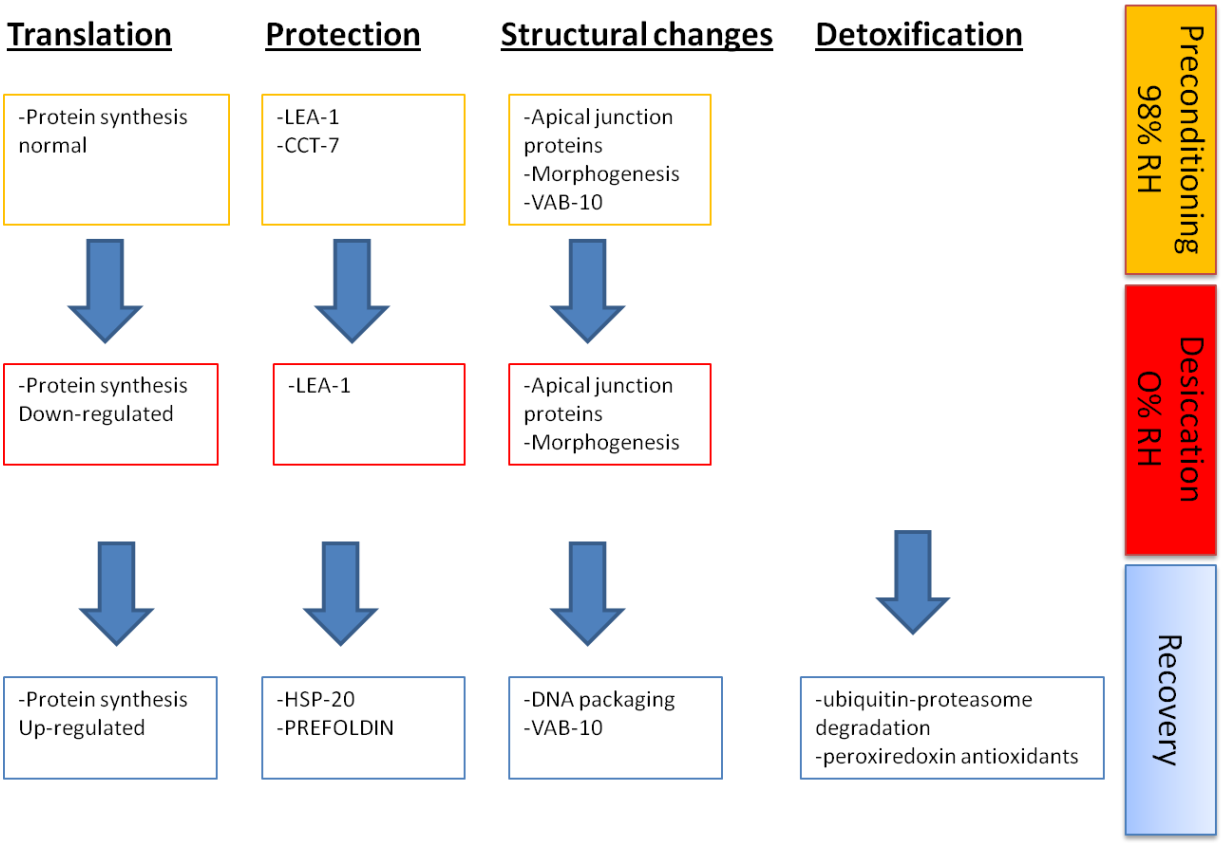


Figure 4.10 Overview of expression of proteins in response to preconditioning, desiccation and rehydration in *P. superbus* nematodes.

Table 4.9 DAVID bioinformatics functional annotation output showing enriched annotation clusters for up-regulated proteins in response to preconditioning 1.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	0.88	Cytoskeletal protein binding	4	0.06	0.99
2	0.61	Body morphogenesis	6	0.15	1
3	0.27	Nucleoside binding	6	0.34	0.99
4	0.26	Growth/reproduction	9	0.82	1
5	0.19	Body morphogenesis/growth	6	0.15	1
6	0.06	Cation binding	4	0.88	0.99

Table 4.10 DAVID bioinformatics functional annotation output showing enriched annotation clusters for up-regulated proteins in response to preconditioning 2.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	0.57	Peptidase activity	6	0.26	0.99
2	0.46	Cytoskeleton	3	0.51	1.000
3	0.40	Nucleotide-binding	9	0.15	0.99
4	0.37	Morphogenesis of an epithelium	4	0.26	1.00
5	0.27	Guanyl nucleotide binding	3	0.57	0.99
6	0.07	Ion binding	6	0.88	1.00
7	0.06	Regulation of growth	12	0.91	1

Table 4.11 DAVID bioinformatics functional annotation output showing enriched annotation clusters for up-regulated proteins in response to desiccation.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	0.67	Proteolysis	6	0.11	1
2	0.64	Cytoskeleton	5	0.04	0.69
3	0.13	Body morphogenesis/growth	6	0.25	1
4	0.09	Ion binding	6	0.75	0.99
5	0.03	Nucleotide binding	6	0.94	0.99

Table 4.12 DAVID bioinformatics functional annotation output showing significantly enriched annotation clusters for up-regulated genes in response to recovery.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	1.89	Ubl conjugation/isopeptide bond	3	0.01	0.37
2	0.77	Growth	15	0.07	0.98
3	0.44	Proteasome/protein catabolic process	3	0.20	0.93
4	0.15	Translation	4	0.76	1.00
5	0.09	Body morphogenesis/growth	5	0.36	1.00

6	0.07	Reproductive developmental process	3	0.89	1.00

Table 4.13 DAVID bioinformatics functional annotation output showing significantly enriched annotation clusters for up-regulated genes in response to recovery compared to desiccation.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	1.29	tRNA biosynthesis/nucleotide binding/translation	6	0.00	0.17
2	1.03	Molting cycle, collagen and cuticulin-based cuticle	10	0.09	0.95
3	0.93	Regulation of organism growth	8	0.20	0.97
4	0.66	Protein localization/protein transport	7	0.15	0.97
5	0.53	Atp-binding	10	0.48	1.00
6	0.50	Growth	36	0.09	0.97
7	0.47	Secondary metabolites biosynthesis/Short-chain dehydrogenase/reductase SDR	3	0.09	0.66
8	0.45	Nucleotide-binding	5	0.23	1.00
9	0.42	Sexual reproduction	7	0.22	0.98
10	0.41	Sexual reproduction	7	0.22	0.98
11	0.39	Chromosome organization	3	0.29	0.98

Table 4.14 DAVID bioinformatics functional annotation output showing enriched annotation clusters for down-regulated proteins in response to preconditioning 1.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	1.33	Protein transport/nucleotide binding	4	0.01	0.20
2	0.04	Embryonic development/growth	5	0.88	1
3	0.01	Growth/larval development	3	0.94	1

Table 4.15 DAVID bioinformatics functional annotation output showing enriched annotation clusters for down-regulated proteins in response to preconditioning 2.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	1.54	Integral to membrane	16	0.026	0.75
2	0.99	Ion binding	16	0.19	0.98
3	0.68	Metabolism of xenobiotics by cytochrome P450	3	0.33	0.99
4	0.62	Nucleotide binding	19	0.33	0.98
5	0.62	Transmembrane/transport	6	0.03	0.97

Table 4.16 DAVID bioinformatics functional annotation output showing enriched annotation clusters for down-regulated proteins in response to desiccation.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	0.86	Acyl-coa binding/fatty acid binding	3	0.11	0.99
2	0.85	Trna aminoacylation/protein biosynthesis	5	0.09	0.99
3	0.64	Protein transport/nuclear transport	9	0.03	0.99
4	0.64	Nucleotide binding	31	0.12	0.99
5	0.59	Atpase, AAA+ type, core	5	0.14	1

Table 4.17 DAVID bioinformatics functional annotation output showing enriched annotation clusters for down-regulated proteins in response to recovery.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	2.04	Metal ion binding	32	0.001	0.20
2	0.91	WD40 repeat, conserved site	4	0.03	1
3	0.91	Negative regulation of growth	5	0.07	1
4	0.85	Transmembrane	7	0.09	0.97
5	0.83	Metabolism of xenobiotics by cytochrome P450	4	0.19	0.98

Table 4.18 DAVID bioinformatics functional annotation output showing enriched annotation clusters for down-regulated proteins in response to recovery.

Annotation Cluster	Enrichment Score	Term	Count	*P* Value	Benjamini-Hochberg test
1	2.77	Peptidase activity	19	9.99E-05	0.01
2	2.10	Ion binding	27	0.002	0.08
3	1.27	Metallopeptidase activity	7	0.008	0.15
4	0.78	Short-chain dehydrogenase	5	0.14	0.99
5	0.70	Exopeptidase activity/Aminopeptidase	5	0.10	0.71
6	0.65	Actin binding/cytoskeleton organization	8	0.018	0.27

5 Chapter V RNA-seq analysis of desiccation survival in dauer larvae of *Caenorhabditis elegans*

5.1 Introduction

In this chapter the ability of *C. elegans* to survive desiccation is further investigated. Erkut *et al.* (2011) reported that the desiccation tolerance of *C. elegans* dauer larvae is substantially improved following preconditioning at 98% relative humidity which is further discussed later. This improved capacity for desiccation tolerance may occur due to the expression or up-regulation of genes that render the nematode more resistant to the damaging effects of desiccation. This chapter describes the identification and characterisation of the genes that are differentially expressed in response to preconditioning, desiccation and recovery from desiccation in *C. elegans* dauer larvae. The differentially expressed genes were identified using the next generation sequencing method RNA-seq.

Dauer larvae of *Caenorhabditis elegans* can survive desiccation if appropriately preconditioned

Although Ohba and Ishibashi (1981) observed that dauer larvae of *C. elegans* were more resistant to desiccation at 97% RH than any of the other life stages of this nematode, *C. elegans* worms were only recently used as a model for desiccation tolerance. The ability of *C. elegans* dauer larvae to survive desiccation at high RH was confirmed by Gal *et al.* (2004) who reported that following exposure to 98% RH for 8 or 24 h 78% and 69%, respectively of the treated dauer larvae survived. Moreover, they demonstrated that when *lea-1* (late embryonic abundant) gene was knocked down using RNAi, it resulted in reduced survival when exposed to 98% RH. This showed that, with the great variety of molecular techniques applicable to *C. elegans*, this nematode could be a suitable model for studying molecular aspects of desiccation tolerance. More recently Erkut *et al.* (2011) showed that when *C. elegans* dauers were preconditioned at 98% RH, they could subsequently survive exposure to much harsher desiccation at 30% RH for 24 h as shown in Figure 5.1. The protocol developed by Erkut *et al.* was to allow a droplet of water containing dauer larvae to slowly evaporate at 98% RH and to follow this evaporation step by preconditioning the dauers for 4 days at 98% RH before subjecting the worms to

harsher desiccation, as shown in Figure 5.1. The genetics of *C. elegans* dauer larva formation has been studied extensively (reviewed by Hu, 2007). Several temperature sensitive dauer constitutive mutants of *C. elegans* have been isolated. When grown at the restrictive temperature these mutants form dauer larvae in conditions suitable for optimum growth and reproduction. Such mutants provide a convenient and efficient means of generating uniform populations of dauer larvae and eliminate the need to maintain nematode cultures until starvation and overcrowding conditions lead to the formation of dauer larvae. Erkut *et al.* (2011) tested a number of different strains of *C. elegans* which were *daf-2*, *daf-7* and wild-type (N-2) and concluded that *daf-2* dauers had the highest survival after exposure to desiccation.

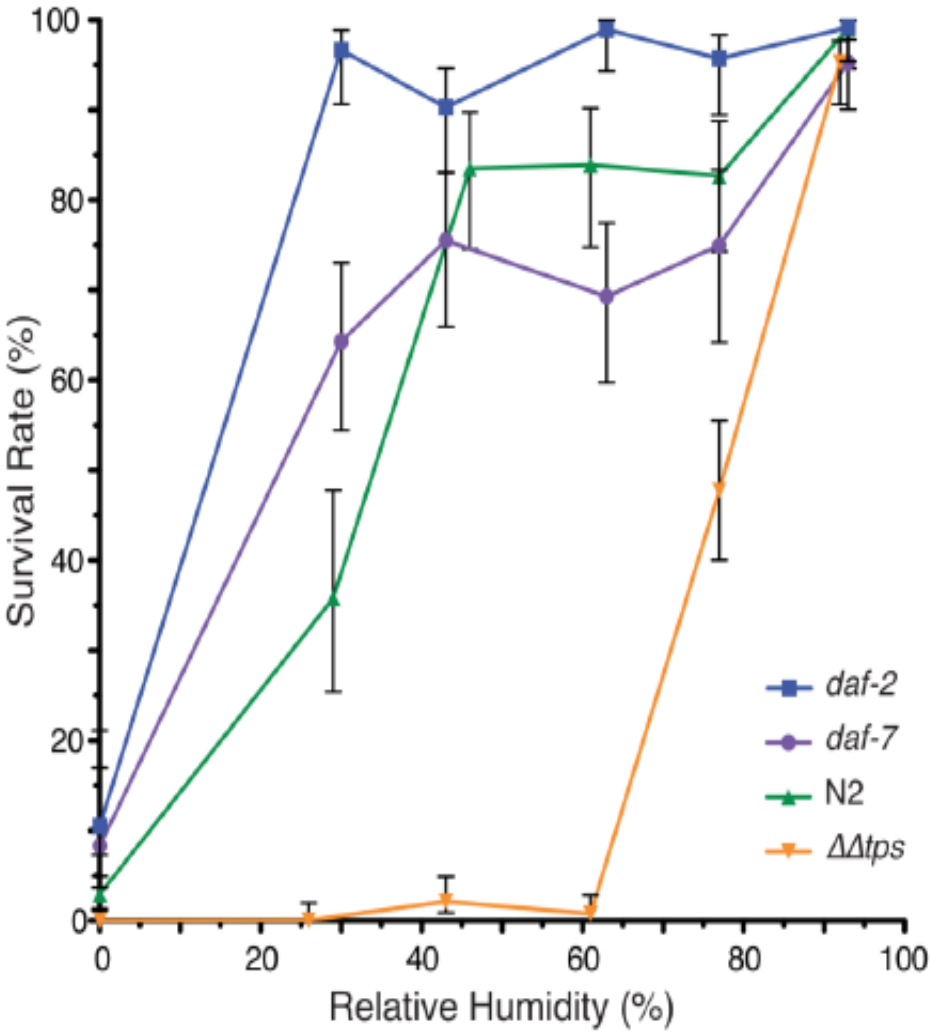


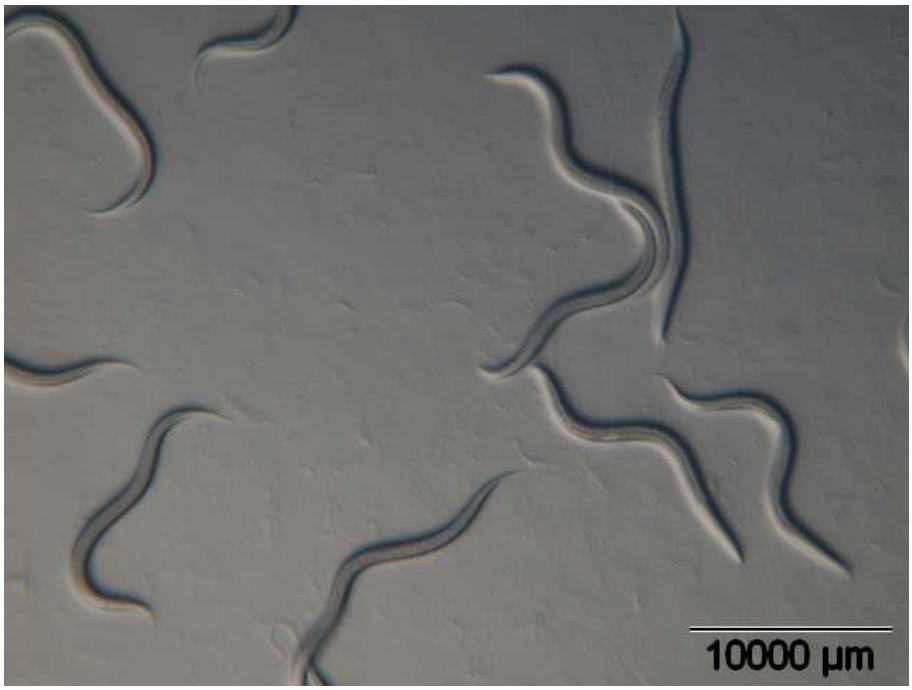
Figure 5.1 Survival rates of preconditioned *daf-2*, *daf-7*, wild-type (N-2) and Δ*tps* dauer larvae of *C. elegans* after desiccation in lower humidity. The Δ*tps* mutant strain lacks two isoforms of the enzyme trehalose 6-phosphate synthase which catalyzes the first step of trehalose biosynthesis. The source of this illustration is Erkut *et al*. (2011).

5.2 Results

5.2.1 Confirmation dauer larvae of *C. elegans* can survive desiccation if appropriately preconditioned

To confirm the finding of Erkut *et al.* (2012) *daf-2* dauer larvae were preconditioned at 98% RH for 96 h, desiccated at 32.5% RH for 24 h and assessed for survival as described in section 2.2.4.2. This resulted in a comparably high survival rate of 87.76% survival. Control and desiccated (98% RH for 96 h followed by 32.5% RH for 24 h) *C. elegans daf-2* dauer larvae are shown in Figure 5.2. These results show that although the survival values of preconditioned *C. elegans* dauer larvae over-activated silica are much lower than other anhydrobiotic nematodes such as *P. superbus* (Shannon *et al.*, 2005), they have a substantially improved survival to desiccation after preconditioning. This implies that metabolic adjustments are occurring during the preconditioning phase and that it may be possible to detect these metabolic changes using transcriptomic or proteomic tools. The identity of genes or proteins which are up-regulated in response to desiccation stress in *C. elegans* dauer larvae would be particularly valuable because of the large amount of genetic, molecular and biochemical data which have been generated for this nematode and because of the extensive suite of molecular tools which have been developed to manipulate and explore gene function and expression in this nematode.

(i)



(ii)

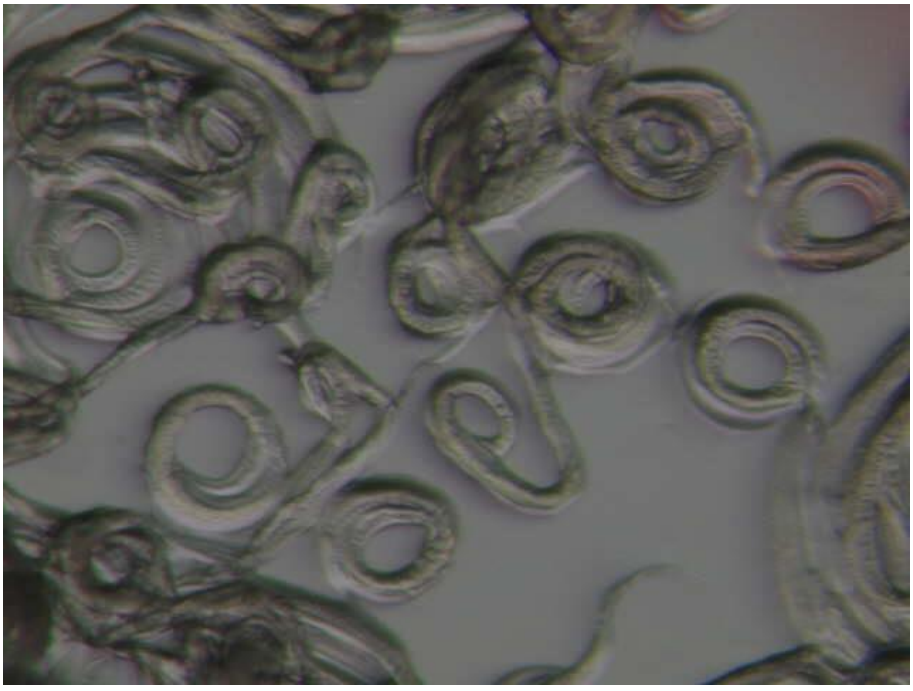


Figure 5.2 Images of control and desiccated *C. elegans daf-2* dauer larvae. (i) Control *C. elegans* dauer larvae grown on HT115(DE3) bacteria at 25^{o}C. (ii) *C. elegans* dauer larvae placed on a petri dish and exposed to 98% RH for 96 h (after evaporation) followed by 32.5% RH for 24 h. Coiling of the *C. elegans daf-2* dauers was observed which reduces the rate of water loss during desiccation.

5.2.2 Selection of conditions for RNA-seq

Quantitative PCR (qPCR) was used to select the preconditioning time points that would be suitable for the detection of differential gene expression for the desiccated and recovering *C. elegans daf-2* dauer larvae. The gene expression profiles of six putative desiccation-response genes were investigated for two desiccation regimes. The desiccation treatments were exposure to 98% RH for 24 or 48 h. The worms were then harvested washed and pelleted and frozen at -80^{o}C in Trizol. Control worms which were not desiccated were also collected (Chapter II Section 2.2.4.2). Each sample was thawed, RNA was extracted and converted into cDNA before analysis by qPCR (as described in Sections 2.3.1, 2.3.5, 2.3.7). The genes tested were the molecular chaperones *hsp-60* (hsp=heat shock protein) and *hsp-70*; the antioxidants *gst-4* (gst=glutathione S-transferase) and *sod-2* (sod=superoxide dismutase) and the natively unfolded proteins *lea-1* (lea=late embryogenesis abundant) and *dur-1* (dur=dauer up-regulated).

To select appropriate time points at which to measure gene expression in nematodes which were recovering from desiccation stress, the gene expression profiles of six putative recovery genes were investigated. For this experiment control dauer larvae which had not been desiccated were harvested, as well as dauer larvae which were desiccated at 98% RH for 96 h followed by exposure to 32.5% RH for 24 h, with the nematodes being subsequently rehydrated for 30 min, 60 min or 90 min. These samples were collected, stored and processed as above. The genes tested were *ced-3* (ced=cell death abnormality), *prx-1* (prx=peroxisome assembly factor), *rpt-2* (rpt=proteasome regulatory Particle, ATPase like), *crn-7* (crn=cell death related nuclease), *tre-3* (tre=trehalase) and *pyk-1* (pyk=pyruvate kinase). The putative recovery genes were selected because of their roles in autophagy (*ced-3* and *crn-7*), proteasomal degradation of damaged proteins (*rpt-2*) and metabolism (*prx-1*, *pyk-1* and *tre-3*). Peroxisomes (prx) are involved in a variety of metabolic reactions, including several aspects of energy metabolism such as the ß-oxidation of long chain fatty acids (Cooper, 2000). Pyruvate kinase is an important regulatory enzyme in aerobic glycolysis, while trehalase is required to release α-glucose molecules from the trehalose molecules (which tend to accumulate in response to desiccation in nematodes). The *C. elegans* genes *pmp-3* (pmp=peroxisome membrane protein related, an Acyl-CoA transporter) and *cdc-42* (cdc=cell division cycle related, a RHO GTPase) were used as reference genes. These genes had been previously shown to

be stably expressed (at the same level) across different developmental stages in *C. elegans* including the dauer larval stage (Hoogewijs *et al.*, 2008).

The results obtained show that of the six putative desiccation response genes two, *hsp-70* and *lea-1*, were strongly up-regulated in response to desiccation stress following exposure to 98% RH for 24 or 48 h (Figure 5.3). In addition, the *dur-1* gene was two-fold up-regulated following 24 h at 98% RH, but its expression levels were similar to that of the control nematodes following exposure to 98% RH for 48 h. There were very slight increases in the relative expression of *sod-2* in nematodes exposed to 98% RH for 24 or 48 h. The expression of the remaining two genes *hsp-60* and *gst-4* was decreased relative to the control nematodes following exposure to 98% RH for 24 and 48 h (Figure 5.3). Although higher up-regulation of desiccation responsive genes was observed in the nematodes exposed to 98% RH for 24 h, the 48 h at 98% RH time point was chosen to ensure there was reproducibility amongst biological replicates. Selecting the 48 h time point ensures the variable time in which the droplets evaporate (up to 8 hours difference was observed) had a limited effect on reproducibility by increasing the time.

The relative expression values of the putative recovery responsive genes during the rehydration of desiccated *C. elegans* dauer larvae are presented in Figure 5.4. These results show that three genes *rpt-2*, *tre-3* and *pyk-1* have the highest relative expression levels following 30 min recovery, while the other putative recovery genes did not show increased transcription levels during recovery as compared to the control nematodes. On the basis of this experiment the 30 min recovery time point was selected for the RNA-seq experiments. As indicated above the regime selected for the RNA-seq desiccation preconditioning experiment was exposure of the nematodes to 98% RH 48 h.

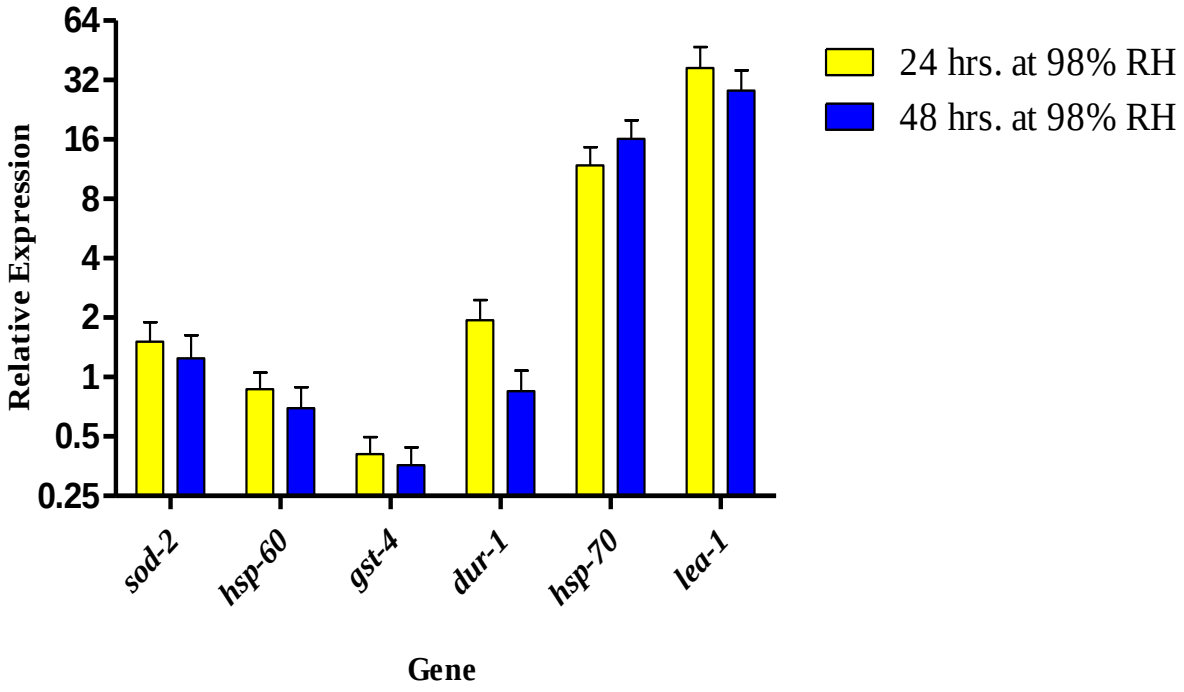


Figure 5.3 Relative expression (measured using qPCR) of putative desiccation-responsive genes in dauer larvae of *C. elegans* in response to two desiccation treatments of preconditioning at 98 % RH for 24 h (yellow) and preconditioning at 98% RH for 48 h (blue). A log2 scale has been used on the y-axis in order to clearly display the large range of relative expression values.

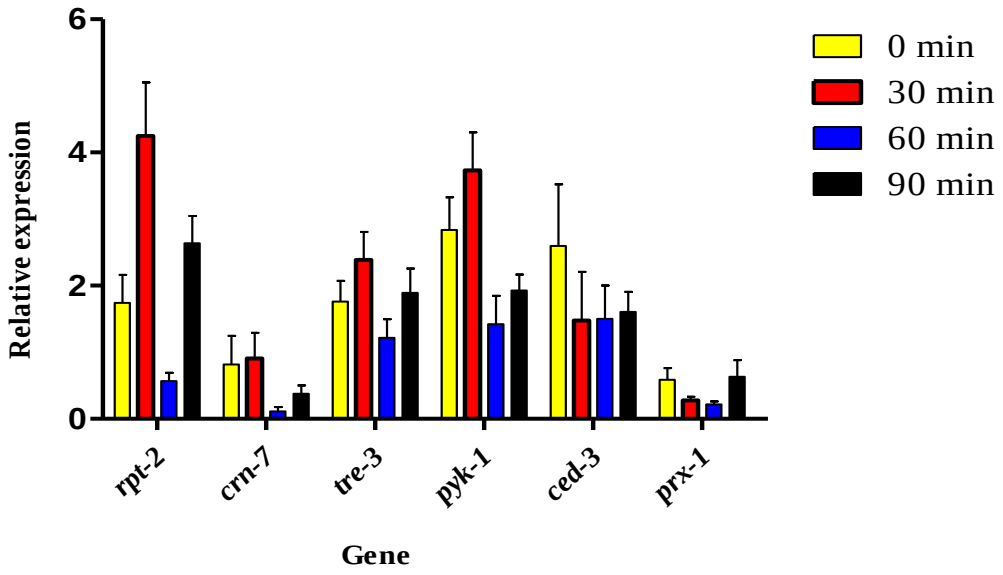


Figure 5.4 Relative expression (measured using qPCR) of putative recovery-response genes in dauer larvae of *C. elegans* during recovery from desiccation. The worms were desiccated by exposing them to 98% RH for 36 h followed by exposure to 32.5% RH for 24 h. These desiccated worms were then allowed to recover in water for 0 min, 30 min, 60 min or 90 min prior to RNA extraction.

5.2.3 RNA extraction and RNA quality tests

Four conditions were selected for RNA-seq as shown in the flow chart in Figure 5.5. These were: T1, a control (*C. elegans daf-2* dauer larvae grown at 25^{o}C); T2, preconditioning (98% RH for 48 h); T3, desiccation (98% RH for 96 h followed by 32.5% RH for 24 h) and T4, recovery from desiccation (98% RH for 96 h followed by 32.5% RH for 24 h and 30 min recovery in sterile water).

The RNA was extracted as described in section 2.3.1. Following extraction, the RNA concentration was measured using the Qubit® fluorometer (Invitrogen). The integrity of the RNA was assessed using a Bioanalyzer (Agilent) (Figure 5.6). The Bioanalyzer software assigns an RNA integrity number (RIN) value of 1 to 10, where a value of 10 indicates completely intact RNA (Schroeder *et al.*, 2006). Samples used to construct a RNA-seq library required a RIN value of 7.0 or more. Three out of the twelve samples, all in the second replicate had RIN values below 7; their RIN values were 6.6, 6.1 and 6.5. This was considered high enough for use. It was difficult to obtain high quantity and quality RNA samples as the yield of mRNA from dauer larvae is 20 times less than that obtained from other developmental stages of the worm (Sinha *et al.*, 2012).

These RNA samples were brought to a final volume of 50 μl, each sample contained more than 5 μg of RNA. The RNA samples were frozen in liquid nitrogen and were sent by courier under dry ice to BGI Ltd. Hong Kong who performed library preparation and sequencing for RNA-seq using an Illumina HiSeq2000 sequencing system.

5.2.4 Quality control analysis of the RNA-seq sequence data

The program Fast QC (www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) was used to perform quality control analyses on the raw sequence data from the Illumina sequencing runs. The results of this analysis revealed that the sequences were of high quality (Figures 5.7 and Figure 5.8). Further details of this analysis can be found in Section 3.2.3 as similar results were obtained in the *P. superbus* RNA-seq analysis.

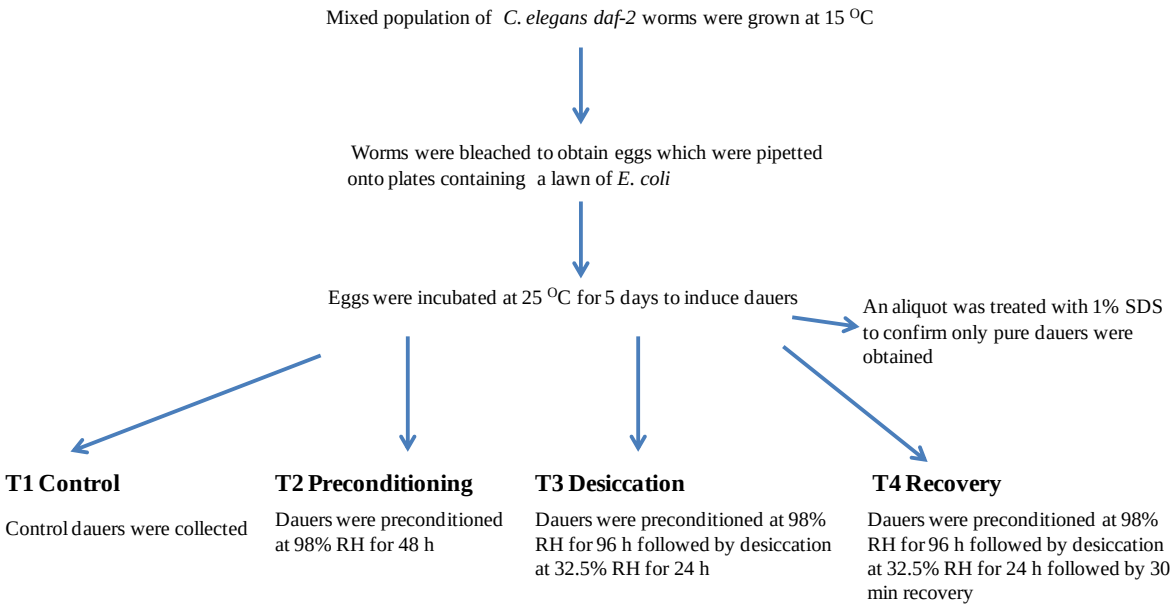


Figure 5.5 Flow chart of the experimental conditions to which the *C. elegans daf-2* dauer samples were exposed prior to RNA-seq analysis of desiccation tolerance.

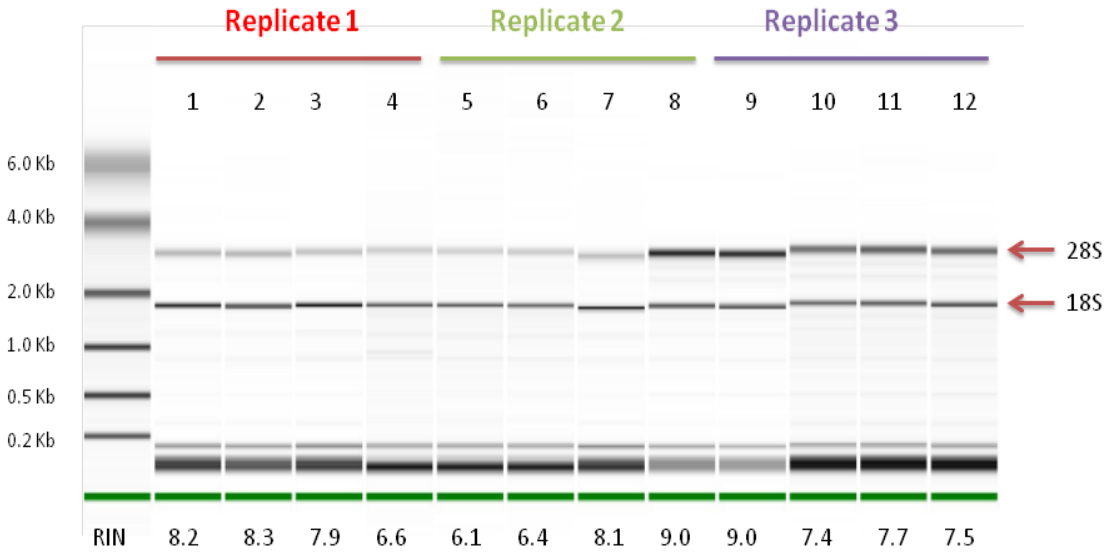


Figure 5.6 Digital image and RNA integrity numbers (RIN) of the *Caenorhabditis elegans* samples used for RNA-seq experiments. The location of the bands corresponding to the 18S and 28S are indicated in the image. The layout of the image is follows: Lane 1, RNA 6000 ladder (Agilent); Lane 2, control replicate 1; Lane 3, preconditioning replicate 1; Lane 4, desiccation replicate 1; Lane 5, recovery replicate 1; Lane 6, control replicate 2; Lane 7, preconditioning replicate 2; Lane 8, desiccation replicate 2; Lane 9, recovery replicate 2; Lane 10, control replicate 3; Lane 11, preconditioning replicate 3; Lane 12, desiccation replicate 3; Lane 13, recovery replicate 3.

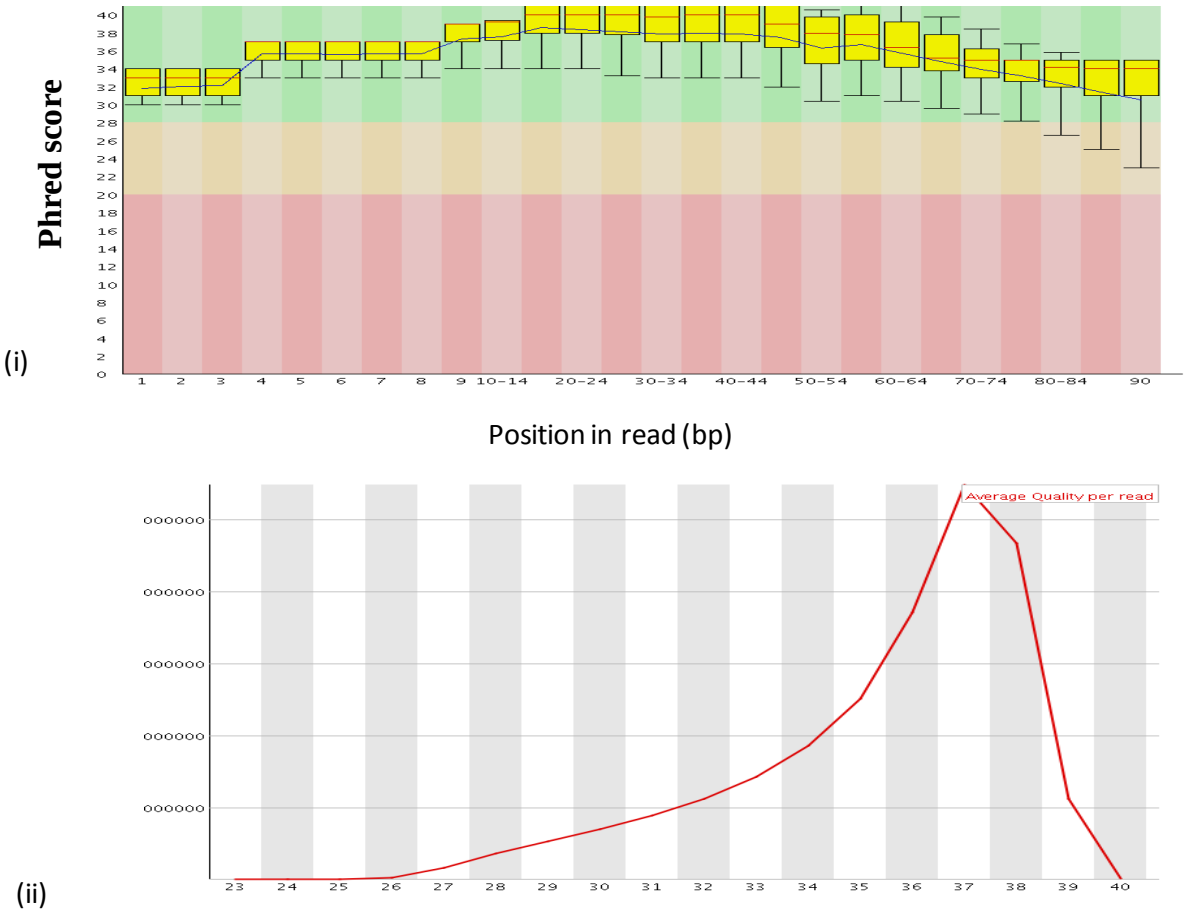


Figure 5.7 FastQC analysis on quality of RNA-seq data. (i) Quality scores calculated across all bases for each position in the reads. The whiskers represent the 1st lower quartile, and the 4th upper quartile (not visible for some positions because of the tight distribution at the top of the graph) and the yellow box is the range of the 2nd and 3rd quartile. The line in the middle is the mean. The Phred quality score across the reads was within 30 and 40 across all bases, this means that the probability of calling an incorrect base was very low. (ii) Distribution of the quality score over all sequences.

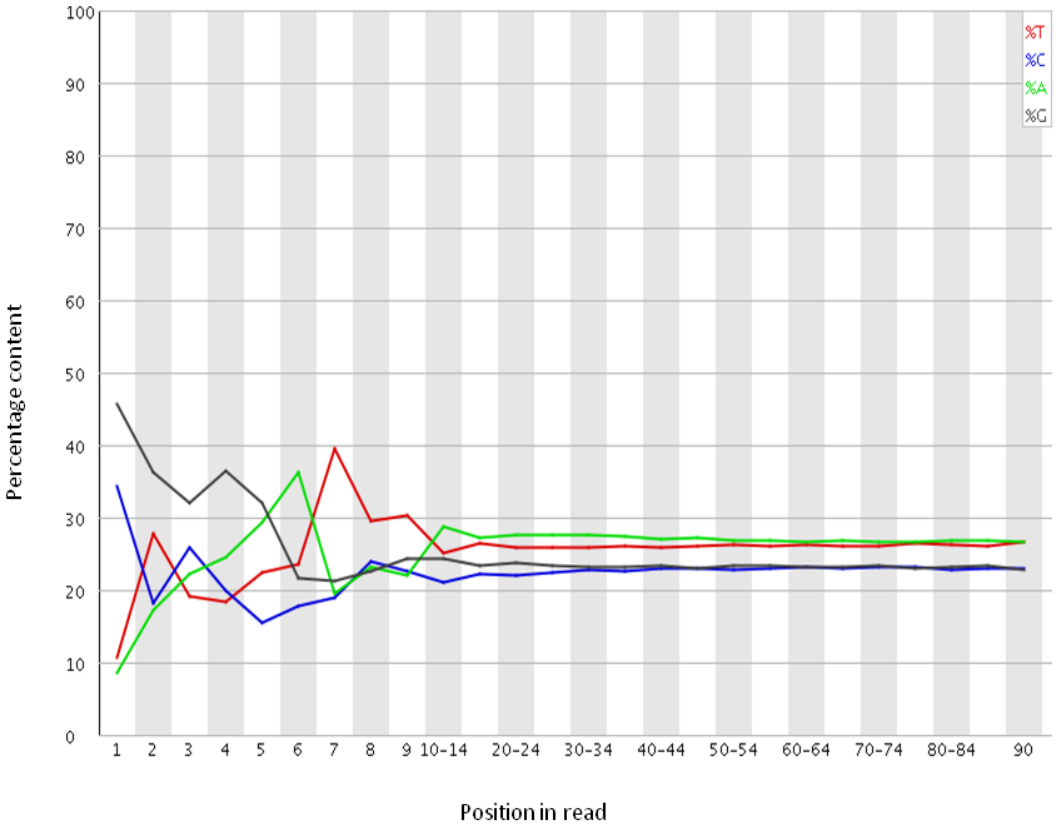


Figure 5.8 FastQC analysis on base composition in RNA-seq data. Bases are represented by the colours red (T), blue (C), green (A) and black (G).

5.2.5 Alignment of RNA-seq reads to the *C. elegans* reference genome

The number of clean reads that were obtained for each of the replicates after initial filtering by BGI is shown in Table 5.1. Additional processing of the data was carried out at NUI Maynooth and this resulted in a slight reduction in the number of reads which were carried forward for further processing. Indexing of the *C. elegans* reference genome sequences was done using Bowtie (Langmead *et al.*, 2009). Alignment of the RNA-seq reads to the *C. elegans* genome was done using Tophat2 (Kim *et al.*, 2013). Tophat was able to align 100% of the reads to the reference *C. elegans* genome. Tophat also removed reads that mapped to multiple regions on the reference *C. elegans* genome (Table 5.1). The number of reads was further reduced by the removal of reads that mapped to multiple regions in the *C. elegans* reference genome. PCR duplicates were also removed using the Picard program (Li *et al.*, 2009). Further details of the indexing of the *C. elegans* genome, alignment of the RNA-seq reads and PCR duplicate removal is given in Chapter II Material and Methods (Section 2.5)

5.2.6 Counting, normalisation and statistical analysis

The number of unique reads that mapped to a given annotated gene in the *C. elegans* genome was counted by the program HTSeq-count (Anders and Huber, 2010). The data were combined into one master table that contained all of the counts, conditions and replicates. Normalisation and differential expression analysis were carried out using the R package DEseq (Anders and Huber, 2010). After normalisation (to adjust for varying sample sequencing depths) multidimensional scaling (MDS) analysis was carried out using the edgeR package (Robinson *et al.*, 2010). MDS analysis gives an overview of differences between treatments and replicates (Figure 5.9). This figure shows that the datasets for the biological replicates for treatment 1 (T1, control), treatment 2 (T2, preconditioning), treatment 3 (T3, desiccation) and treatment 4 (T4, recovery) cluster together and that the data for T1 and T2 are clearly distinct from all the other treatments. However, the data for T3 sample 7 and T4 sample 12 lie close together on the MDS plot.

The data were also visualised as a heat map, based on a distance matrix generated using the edgeR package (Robinson *et al.*, 2010), showing the distances between samples as calculated from the variance-stabilising transformation of the count data (Figure 5.10). This analysis shows that all the biological replicates cluster together for T1 (control), T2 (preconditioning) and T4 (recovery). This analysis also shows that T3 sample 7

(desiccation) is clustering between T2 (preconditioning) and T4 (recovery). However the MDS plot (Figure 5.9) shows that all the T3 data samples cluster together (although T3S7 is adjacent to T4S12 on the MDS plot). Thus, the T3S7 data set was retained for further analysis.

Differential expression data for each gene for the different pairs of treatments were calculated using the negative binomial test in the DEseq package as described in Section 2.5.9. The proportion of genes differentially expressed was visualised by plotting the fold change against the mean expression level (Figure 5.11). Further details describing the significance of this graph can be found on Section 3.2.5.

Table 5.1 Quality control analysis of the RNA-seq sequence data. The number of *C. elegans* RNA-seq reads (clean data) obtained for each experimental condition after initial filtering by BGI are presented, together with the number of *C. elegans* reads obtained for each experimental condition after pre-processing by Tophat, the number of reads properly paired (in situations where a forward and reverse read align exactly they are said to be "properly paired"), the percentage of singletons, the percentage duplication estimated by the Picard program (http://picard.sourceforge.net/) and the final number of molecules (paired reads) retained for statistical analysis.

Experimental condition	**Replicate**	**Clean data (no. of reads)**	**Number of reads after preprocessing**	**Reads that paired properly (%)**	**Singeltons (%)**	**Duplication (%)**	**Final number of unique molecules**
Control	1	48,344,338	43,616,520	87.25	8.91	68.79	6,961,624
	2	49,730,636	44,650,889	87.12	9.02	67.85	7,386,185
	3	48,863,668	44,333,535	86.94	8.76	60.84	9,469,256
Preconditioning	1	48,285,970	43,221,359	88.04	8.33	76.63	4,992,750
	2	49,303,726	44,010,414	87.57	8.58	75.47	5,362,701
	3	49,083,396	43,371,862	87.08	9.17	78.78	4,505,818
Desiccation	1	49,485,170	44,087,715	87.8	8.77	74.36	5,635,150
	2	49,207,864	44,158,623	88.32	8.03	63.64	8,585,885
	3	48,792,532	43,406,152	87.24	8.65	62.04	8,957,533
Recovery	1	49,227,542	42,723,657	86.35	10.06	82.91	3,526,446
	2	48,961,038	43,200,691	86.28	9.67	77.23	4,833,365
	3	48,122,714	42,528,746	88.25	8.57	77.75	4,658,446

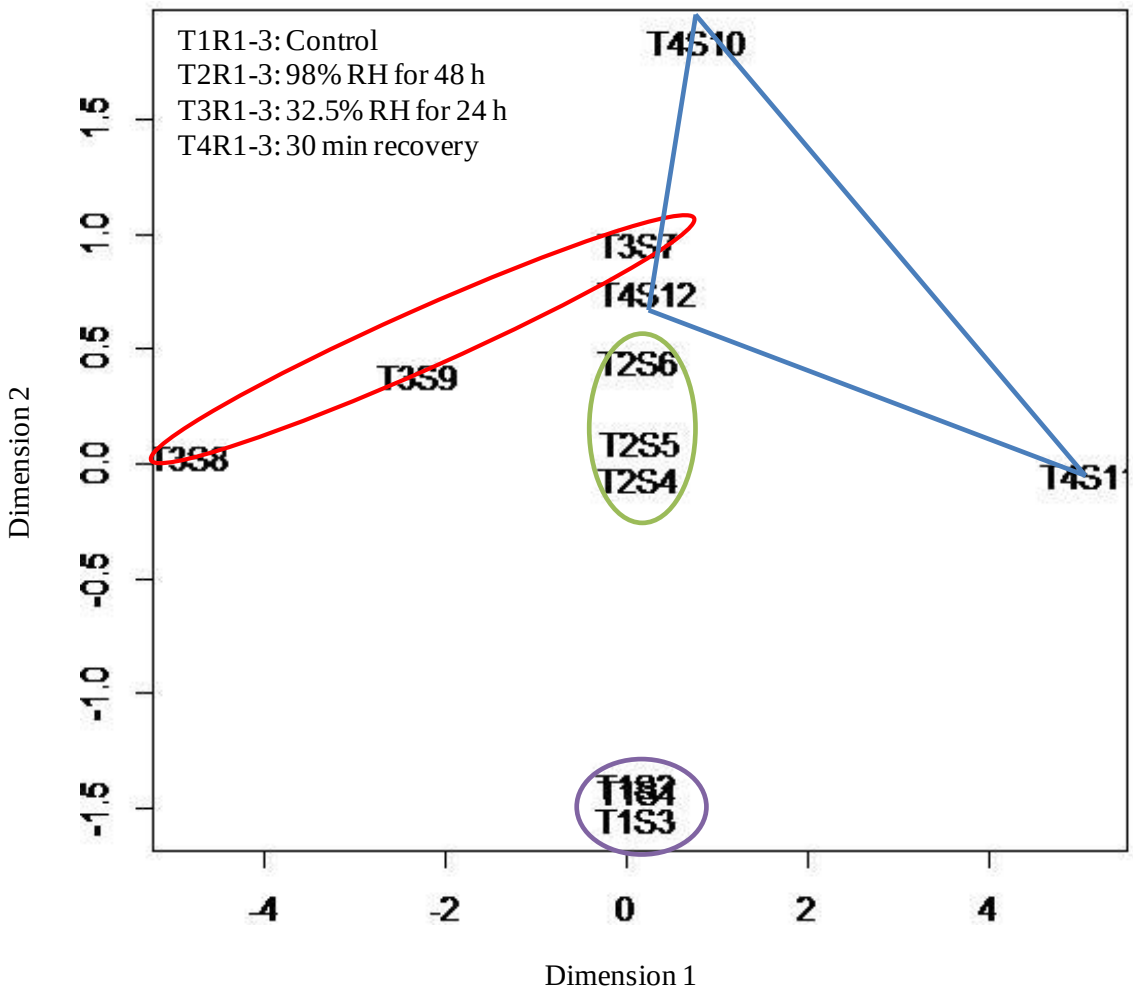


Figure 5.9 Multidimensional scaling (MDS) plot for the *C. elegans* RNA-seq read count data showing the relations between the samples in two dimensions. The four treatments are indicated in purple [control (T1S1-T1S3)], green [preconditioning (T2S4-T2S6)], red [desiccation (T3S7-T3S9)] and blue [recovery (T4S10-T4S12)].

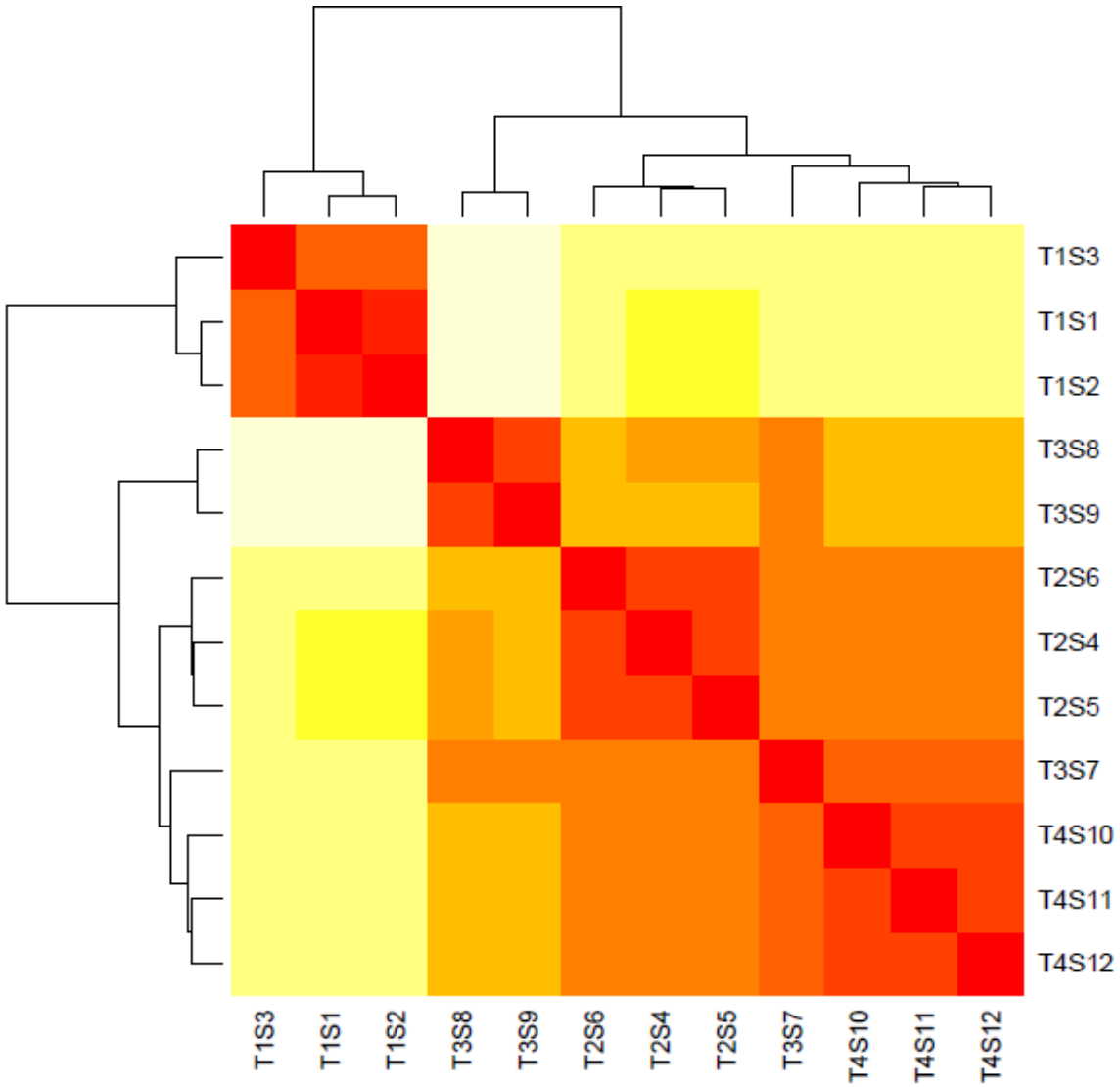


Figure 5.10 A heat map showing the distances between *C. elegans* dauer larvae RNA-seq count data for all samples, as calculated from the variance-stabilising transformation of the count data using the edgeR package (Robinson *et al.*, 2010). The heat map shows a representation of the Euclidean distance matrix and the dendrogram represents a hierarchical clustering of the treatment replicates. T1=control, T2= 98% RH for 48 h, T3= 98% RH for 96 h followed by 32.5% for 24 h and T4=98% RH for 96 h, 32.5% for 24 h followed by rehydration in water for 30 min.

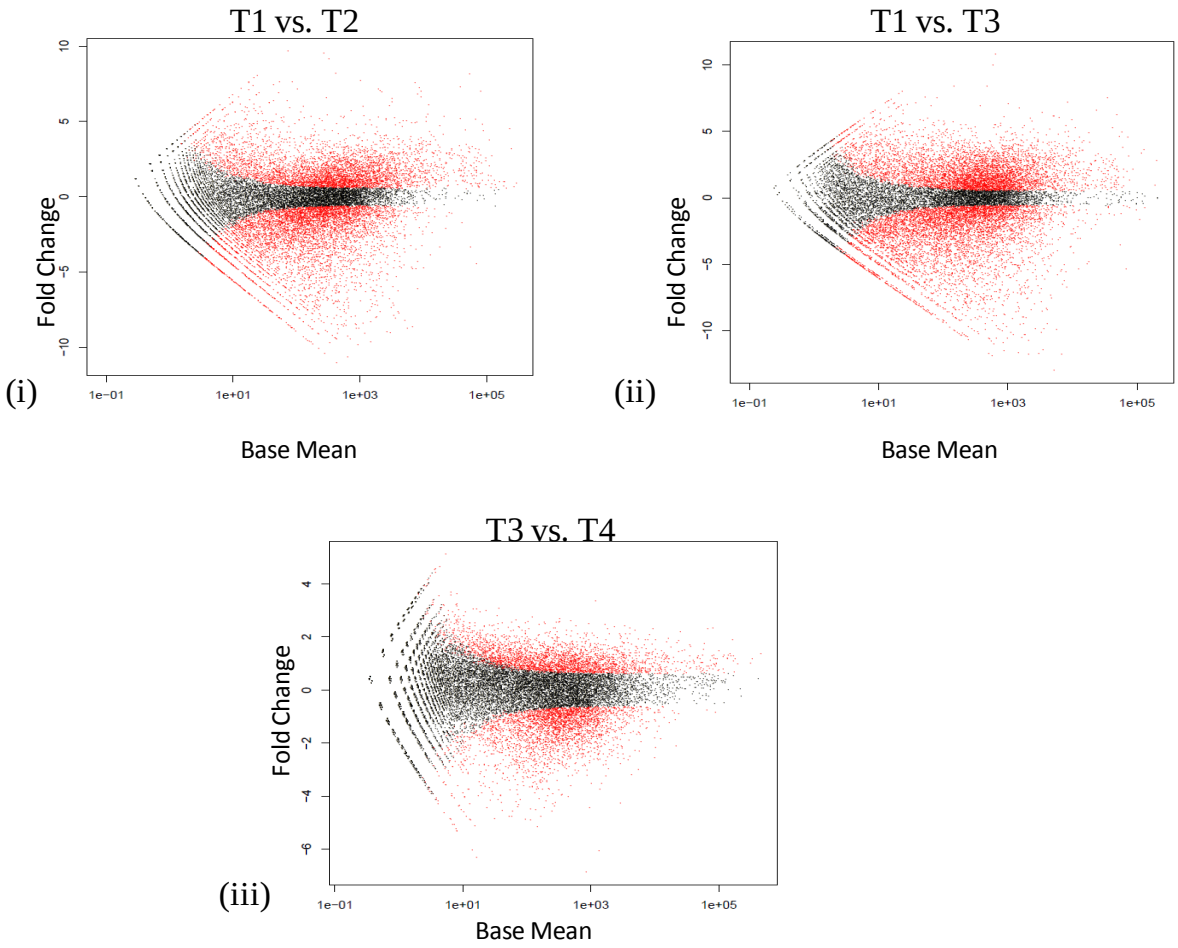


Figure 5.11 Testing for differential expression between the read count data of the RNA-seq samples from *C. elegans* dauer larvae. The treatments compared were: (i) control (T1) and preconditioning (98% RH for 24 h=T2); (ii) control and desiccation (96 h at 98% RH, 24 h at 32.5% RH=T3) and (iii) desiccation and recovery (96 h at 98% RH, 24 h 32.5%, recovery in water for 30 min=T4). The data represent the read counts for individual gene responses plotted as log2 fold change versus Base Mean (i.e the mean of the normalized counts for each gene averaged over all replicates for each pair of treatments under consideration). The red colour marks those genes detected as differentially expressed at a 10% false discovery rate, when the Benjamini-Hochberg multiple testing adjustment is used. Negative fold change values represent down-regulated genes and positive change values represent up-regulated genes.

5.2.7 Differentially expressed genes

The identities of the *C. elegans* transcripts and the results of the statistical analyses were exported from DEseq into an Excel file. Further analyses were concentrated on those differentially expressed genes which had a >2 fold change in expression and a Benjamini-Hochberg *p*-adjusted cut-off value of <0.01. The Benjamini-Hochberg procedure as used to correct for multiple comparisons to ensure a minimal level of false positives. Using these selection parameters a total of 6,860 genes were differentially expressed in response to preconditioning, 2,763 of which were up-regulated. In response to desiccation, a total of 8,155 were differentially expressed, 3,634 of these being up-regulated. Finally, there were 6,459 genes which were differentially expressed during recovery compared to the control, 2,685 of which were up-regulated. In this chapter, I have focused on those transcripts which were up-regulated in response to the different treatments since the genes and gene products which these transcripts represent are likely to have important roles in desiccation tolerance in *C. elegans* dauer larvae. The *C. elegans* transcripts were identified from BLAST searches against the NCBI database in combination with searches of the Wormbase database (www.wormbase.org). Using the Blast2GO program (Conesa *et al.*, 2005) Gene Ontology terms were obtained for the differentially expressed genes. The Gene Ontology consortium has developed a vocabulary of defined terms that describe gene products in the context of three domains – Biological Process, Molecular Function and Cellular Component in a species-independent manner (Ashburner *et al.*, 2000).

In Figure 5.12, a Venn diagram displays (i) the number of *C. elegans* genes significantly up-regulated in response to preconditioning, desiccation and recovery when compared to the control nematodes and (ii) the number of genes significantly down-regulated in response to preconditioning, desiccation and recovery when compared to the control.

Table 5.2 The number of *Caenorhabditis elegans* genes which had a >2 fold change in expression at Benjamini-Hochberg *P*-adjusted cut-off values of 0.05, 0.01 and 0.001 in response to various desiccation-related treatments

***P*- adjusted cut-off**	**Preconditioning versus Controls**	**Desiccation versus Controls**	**Recovery versus Control**	**Recovery versus Desiccation**
>2 fold increase, $p<0.05$	3,018	3,920	2,930	1,098
>2 fold increase, $p<0.01$	2,763	3,634	2,686	878
>2 fold increase, $p<0.001$	2,437	3,295	2,511	622
>2 fold decrease, $p<0.05$	4,677	5,029	4,302	1,937
>2 fold decrease, $p<0.01$	3,907	4,521	3,773	1,733
>2 fold decrease, $p<0.001$	3,405	3,934	3,157	1,435
Number of genes represented*	18,618	18,690	18,053	18,676

*The total number of transcripts detected in the two conditions being compared.

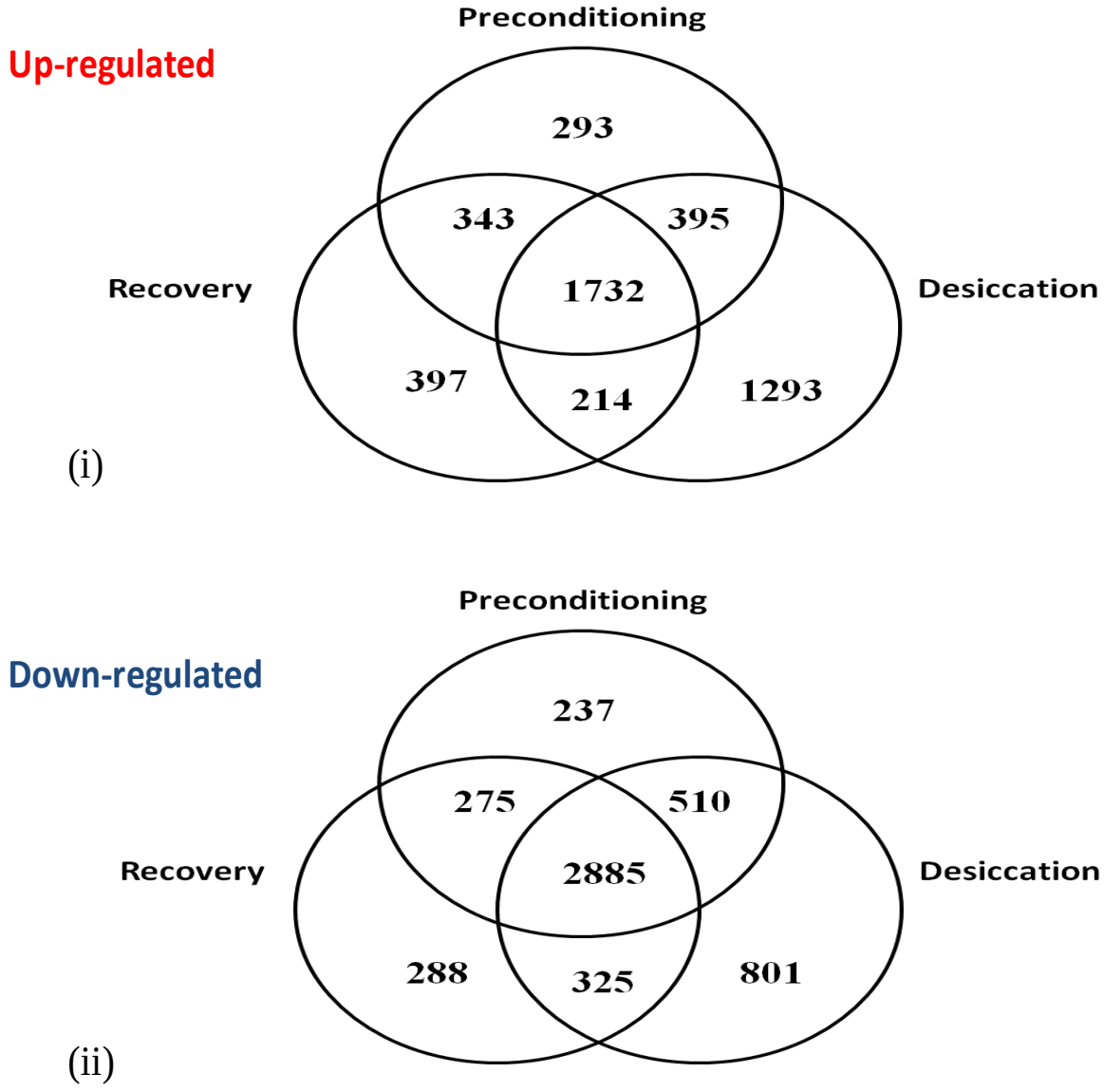


Figure 5.12 (i) The number of *C. elegans* genes significantly up-regulated in response to preconditioning, desiccation and recovery when compared to the control nematodes. (ii) The number of *C. elegans* genes significantly down-regulated in response to preconditioning, desiccation and recovery when compared to the control nematodes.

5.2.8 Differentially expressed pathway analysis and functional annotation clustering

The *C. elegans* transcriptome was mapped to Uniprot IDs and the Reactome overrepresentation analysis tool (Matthews *et al.*, 2009) was used to find pathways which are strongly enriched in response to the different conditions. The Reactome is an open source, open access, manually curated, database of human pathways and processes. Cytoscape (version 3.0.2) plugin BiNGO (v.2.44) was used for GO enrichment analysis as described in section 2.5.13.

The DAVID bioinformatics tool (Huang *et al.*, 2009) was also used to cluster functionally related annotations into groups. A cut-off enrichment score of >1.3 was set, as this is equivalent to non-log scale of 0.05 (Huang *et al.*, 2009). DAVID clustered 26 groups in response to preconditioning, 88 groups in response to desiccation, 21 groups in response to recovery compared to the control and 12 groups in response to recovery compared to desiccation as shown in Tables 5.24-5.27.

5.2.9 Overview of the genes, GO terms and Reactome Pathways which are up-regulated in response to desiccation-related stress in *C. elegans* dauer larvae

In this RNA-seq study a great number of genes, GO terms and Reactome Pathways were found to be differentially expressed in response to desiccation and recovery from desiccation in *C. elegans* dauer larvae. The identities of the up-regulated genes, processes and pathways are summarised in this section, and the biological significance of these processes in anhydrobiotic protection is discussed in Section 5.3. The main pathways and processes implicated by RNA-seq in the response of *C. elegans* dauer larvae to desiccation were as follows: signal transduction; gene expression, transcription and translation; ribosomal proteins; energy metabolism; cellular and cytoskeletal organization; repair of DNA damage; antioxidants; molecular chaperones, defense against pathogens and removal of damaged proteins. In addition to the processes described in this section a number of individual genes in other pathways were also up-regulated in response to desiccation or during recovery from desiccation, but due to space constraints it is not possible to present this data set in this section. A table listing all the genes which were differentially expressed in response to desiccation-related stress is presented in the supplementary CD appended to this thesis (Supplementary data *C. elegans* RNA-seq).

This table also lists the fold change in expression for each gene and their Benjamini-Hochberg *P*-adjusted probability score.

5.2.9.1 Signal transduction

There were a number of GO terms associated with signal transduction and signalling which are significantly up-regulated in response to preconditioning and desiccation as shown in Table 5.3. The number of individual genes which are up-regulated in each category (Benjamini-Hochberg test $p<0.05$) is also indicated in this table. There are clearly more GO terms associated with signalling which are differentially expressed in the desiccation dataset, which has 17 significantly up-regulated GO terms, as compared to just 4 GO terms which have significantly higher expression in the preconditioning dataset. Althought the hypergeometric test did not identify any GO terms which were up-regulated in the recovery samples, it is clear that several individual genes which are involved in signal transduction are up-regulated during recovery from desiccation, most notably genes involved in Response to Stimulus (125 genes), Signal Transduction (37 genes) and Intracellular Signalling Pathway (41 genes).

The GO enrichment analysis map (Figure 5.13) shows the relationship between parent-child terms and indicates that there are major differences between the GO terms associated with signal transduction and signalling pathways, their enrichment scores and the numbers of up-regulated genes in the preconditioning and desiccation datasets. The signalling pathways which were differentially up-regulated in response to desiccation include general signal transduction which encompasses Ras and mitogen-activated protein kinases (MAPKs), Ras protein signal transduction, response to unfolded protein, and insulin receptor signalling. The significantly up-regulated genes which mapped to the MAPK and Ras signalling pathways obtained from the KEGG database are shown in Figure 5.14 and Figure 5.15 respectively. As well as the GO terms significantly up-regulated associated with signalling, the desiccation dataset was also enriched for several GO terms associated with the nervous system, such as response to stimulus, neuron development and neuron projection morphogenesis.

5.2.9.2 Gene expression and transcription

A number of GO terms and Reactome pathways associated with gene expression and transcription are significantly up-regulated in response to preconditioning, desiccation and recovery as shown in Table 5.4 and Table 5.5. The GO term "Gene Expression" is significantly up-regulated in all three experimental treatments, relative to the control nematodes, but a higher number of individual genes are up-regulated in the preconditioning and recovery samples than in the desiccated nematodes. The preconditioning and recovery samples also have several significantly up-regulated GO terms which are associated with translation and translation elongation activity which are not significantly up-regulated in the desiccated nematodes. Similarly the GO term "Structural constituent of ribosome" is significantly up-regulated in the preconditioning and recovery samples but not in the desiccated nematodes. This increased transcription of ribosomal protein genes in the preconditioned nematodes can also be seen in the KEGG pathway map (Figure 5.16), showing that 63 genes encoding ribosomal proteins are significantly up-regulated in response to preconditioning whereas 24 ribosomal protein genes are up-regulated in the desiccation dataset.

In the desiccated nematodes there are many GO terms associated with regulation of gene expression which have significantly higher expression which are not up-regulated in the preconditioning and recovery samples. These include Negative Regulation of Gene Expression, Epigenetic Regulation of Gene Expression, Posttranscriptional Regulation of Gene Expression, Posttranscriptional Gene Silencing and Negative Regulation of Translation. Similarly, Reactome pathway analysis shows that the preconditioning and recovery datasets are enriched for pathways associated with mRNA translation while the desiccated dataset is enriched for mRNA processing pathways. These mRNA processing components function in the regulation of gene expression. In the desiccation dataset, there are 28 genes which are up-regulated that map to the "mRNA Processing" Reactome pathway. This includes genes such as *cpsf-1* (cpsf=cleavage polyadenlylation specificity factor) which encodes a protein involved in cleavage and polyadenylation in a process that produces mRNA (Cui *et al.*, 2008). The DAVID bioinformatics analysis also indicates that the most significantly enriched annotation cluster for the up-regulated genes in the preconditioned nematode dataset is related to the ribosome and translation (Table 5.24). A GO enrichment analysis map (Figure 5.17) which maps the relationship between

parent–child terms, also shows that genes encoding proteins required to regulate mRNA processing and translation are enriched in the desiccated dataset while genes required for mRNA translation are enriched in the preconditioning and recovery datasets.

The data presented in this chapter show that activation of the signal transduction pathways in response to desiccation associated stress in dauer larvae of *C. elegans* leads to the transcription of an extensive panel of genes encoding diverse effector proteins. During preconditioning at 98% RH many of these desiccation response genes are likely to be translated into protein products since many GO terms and Reactome pathways associated gene expression, transcription and translation are significantly up-regulated in response to preconditioning. A similar response is also apparent in the rehydrating nematodes. Here, too, the emphasis is on protein synthesis of recovery genes involved in repair and detoxification. However it is likely that the desiccated nematodes, while still engaged in mRNA synthesis may not translate the mRNA until rehydration.

5.2.9.3 Intermediary metabolism

Intermediary metabolism describes all reactions concerned with the storage and generation of metabolic energy required for the biosynthesis of low-molecular mass compounds and energy storage compounds (Mathews *et al.*, 2000). The intermediary metabolism of *C. elegans* dauer larvae is adapted to utilize internal energy reserves in the form of triglycerides and glycogen (Burnell *et al.*, 2005; Braeckman *et al.*, 2009), however dauer larvae are characterised by their low levels of oxidative metabolism compared to other developmental stages in the life cycle (Burnell *et al.*, 2005). The data presented in Table 5.6 show that transcripts encoding enzymes required for the β-oxidation of fatty acids, for glycolysis and for oxidative metabolism are synthesized by *C. elegans* dauer larvae in response to desiccation stress and during recovery from desiccation. Further details concerning the identities and fold changes of a selection of the up-regulated transcripts are presented in a supplementary file (Supplementary data *C. elegans* RNA-seq intermediate metabolism). These data suggest that an increase in intermediary metabolism occurs in the desiccated and recovering nematodes in order to obtain the metabolic energy required to mobilize and synthesise the effector molecules required for desiccation protection.

5.2.9.4 Cellular and cytoskeletal organisation

The GO enrichment analysis (Table 5.9) shows that several terms associated with cytoskeleton organisation are enriched in the desiccation dataset. These terms include Cytoskeleton Organisation, Microtubule Organizing Center and Cytoskeletal Protein Binding. The GO term Microtubule Cytoskeleton Organization is enriched in both the preconditioned and desiccated nematodes, with 31 and 67 genes respectively up-regulated in response to these treatments. The GO enrichment analysis map (Figure 5.18), also shows the relationship between the parent child terms associated with cytoskeleton organisation. These associations are enriched in the desiccation dataset, and to a lesser extent the preconditioning dataset. Although several individual genes associated with cytoskeleton organisation are up-regulated in the recovery dataset none of the relevant GO terms show significant enrichment in the recovering nematodes.

The GO term Morphogenesis of an Epithelium and several other terms associated with morphogeneis and tissue development were also significantly enriched in the desiccation dataset. Changes in the epithelium during desiccation may reduce water loss, while modifications to the epithelium as well modifications to cell, tissue and organ morphogenesis may be important in maintaining the structural integrity of the desiccated nematodes. Membrane organisation also has significantly higher expression across precondition, desiccation and recovery. However, many more genes are up-regulated in the desiccation dataset that are associated with membrane organisation. The child term which contributes most to Membrane Organisation is Membrane Invagination (i.e. the infolding of a membrane resulting in the formation of a vesicle). Invagination is a morphogenetic process involved in embryo formation (Munoz *et al.*, 2007), endocytosis (Shimada *et al.*, 2007) as well as structural changes in the inner membrane of the mitochondrion (Arechaga, 2013). Additionally, phagocytosis engulfment is a child term of membrane invagination which correlates protein degradation with desiccation. Interestingly, GO terms relating to endocytic membrane transport were significantly up-regulated in response to all of the conditions and is further discussed in Section 5.3.

The GO analysis revealed that there are hundreds of individual genes associated with transport that were up-regulated in response to preconditioning, dehydration and rehydration (Table 5.10). The majority of the genes relating to transport which were up-

regulated are involved in endocytosis, with 151, 229 and 129 individual genes up-regulated relative to the control nematodes in response to preconditioning, desiccation and recovery, respectively.

Several genes which encode nuclear pore complex protein were found to be highly up-regulated in response to desiccation as shown in Figure 5.19. The nuclear pore complex may have a role in repairing DNA damage inflicted by severe desiccation. Moreover, DNA repair mechanisms were up-regulated specifically in the desiccation dataset (Section 5.2.9.5).

5.2.9.5 Repair of DNA damage

In the desiccation dataset there are a number of GO terms associated with response DNA damage and repair which are up-regulated that are shown in Table 5.11. The GO enrichment analysis map shown in Figure 5.20 visualises the GO terms associated with DNA damage and repair as well as the response to stress pathway. The GO terms response to DNA damage stimulus and DNA repair show significantly higher expression in the desiccated datasets with 62 and 37 genes up-regulated respectively for these GO terms. In contrast, no GO terms related to DNA damage and repair are up-regulated in the preconditioning and recovery datasets. Reactome pathway analysis also indicates that DNA repair and nucleotide excision repair processes are up-regulated in the desiccated nematodes (Table 5.12). The up-regulated genes that are associated with nucleotide excision repair are shown in the KEGG map in Figure 5.21.

5.2.9.6 Removal of damaged proteins

There were a number of GO terms and Reactome pathways associated with the removal of damaged proteins which were up-regulated, particularly in response to preconditioning and desiccation as outlined in Table 5.13 and Table 5.14. The GO terms Programmed Cell death and Apoptosis are enriched in both the preconditioning and desiccation datasets while Proteasome Complex genes show significantly higher expression in the preconditioning dataset.

As well as ubiquitin-proteasome complex genes, several genes which encode autophagy proteins were up-regulated in response to preconditioning, desiccation and recovery compared to the control is shown in Figure 5.22.

5.2.9.7 Antioxidants

A number of genes which encode antioxidant enzymes were up-regulated in *C. elegans* dauer larvae during preconditioning, desiccation and recovery. The changes in expression of genes encoding the antioxidants enzymes superoxide dismutase, catalase, glutathione peroxidise and glutathione S-transferase are presented in Figure 5.23.

5.2.9.8 Molecular Chaperones and Protein Protection

A number of GO terms associated with protection of proteins were significantly up-regulated in response to preconditioning, desiccation and recovery. The GO terms Response to Stress and its child term Response to Heat showed higher expression relative to the control nematodes across preconditioning, desiccation and recovery. The GO terms Response to Unfolded Protein and Endoplasmic Reticulum Unfolded Protein Response, were up-regulated in the desiccation dataset only. These GO terms are comprised of genes which encode HSPs and are child terms of Response to Stress. Changes in expression of genes encoding HSPs that were significantly up-regulated in at least one of the experimental conditions are shown in Figure 5.24. The up-regulated genes encoding HSPs in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery are as follows: the small HSP genes *hsp-12.6*, *hsp-43* (a member of the HSP-20 family), members of the DnaJ/HSP-40 family (*dnj-8, dnj-12, dnj-13, dnj-19 dnj-28 dnj-29*) and members of the DNAK/HSP-70 family (*hsp-1, hsp-3, hsp-6* and *hsp-70*). This diagram shows that genes which encode HSPs generally have higher expression in the preconditioning and recovery datasets. The GO enrichment map in Figure 5.20 shows relationship between differentially up-regulated parent and child GO terms associated with Responses to Stress across the different treatment conditions. Reactome analysis (Table 5.16) shows that the actin/tubulin prefoldin complex pathway was significantly up-regulated in response to preconditioning, desiccation and recovery. Prefoldin is required for the facilitated folding of actin and tubulin to form microtubules (Lundin *et al.*, 2010). The DAVID analysis shows that the up-regulated genes in the preconditioning, desiccation and recovery datasets in *C. elegans* dauer larvae are significantly enriched with annotations associated with the CPN-60/TCP-1 chaperonin family.

The RNA-seq study also revealed the gene *lea-1*, which encodes a member of the late embryogenesis abundant protein family, was significantly up-regulated during

preconditioning, desiccation and recovery in the dauer larvae (Figure 5.25). In *C. elegans* trehalose 6-phosphate synthase is encoded by the genes *tps-1* and *tps-2*. The *tps-2* gene is significantly up-regulated in preconditioning, desiccation and recovery, while *tps-1* shows significantly higher expression in response to desiccation and recovery (Figure 5.25). The *djr-1.2* gene was also substantially up-regulated in response to preconditioning and desiccation in *C. elegans* dauer larvae. Several individual biotic and abiotic stress response genes were up-regulated in the *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery, as shown in Table 5.17. These include fungus-induced protein-response genes (*fipr*), putative antimicrobial genes (*cnc* = caenacins) and cadmium responsive genes (*cdr*).

5.2.9.9 Recovery compared to desiccation

The RNA-seq recovery dataset has already been compared to that of the undesiccated control nematodes in the previous sections. However, comparing the recovery dataset to the desiccated dataset also gives an important insight into biological processes involved in recovery in *C. elegans* dauer larvae. The GO terms which are significantly up-regulated in recovery compared to desiccation include Integral to Membrane, Response to Fungus, Immune Response, Lipid Binding and Cellular Amino Acid Biosynthetic Process, as shown in Table 5.18. The GO enrichment analysis map of the Response to Fungus and the Immune Response shows the relationship between parent-child terms in the recovery dataset when compared to desiccation as shown in Figure 5.26. Reactome analysis shows the pathways Peroxisomal Lipid Metabolism and Metabolism of Polyamines, amongst others, have higher expression in the recovery dataset as shown in Table 5.19. The superoxide dismutase transcript *sod-2* was found to be up-regulated in recovery compared to desiccation (Table 5.20). Two putative genes, *aqp-1* and *aqp-11*, which encode aquaporin proteins were up-regulated in recovery compared to desiccation (Table 5.20). Aquaporins channels facilitate transport of water, glycerol, and other small solutes across cell membranes and thus may have an important role in desiccation tolerance.

5.2.10 Statistically significant down-regulated GO terms and pathways in response to preconditioning, desiccation and recovery compared to the control

There are a number of GO terms which were significantly down-regulated in response to preconditioning, desiccation and recovery as shown in Table 5.21. These GO terms with

significantly lower expression include histidine family amino acid metabolic process, lipid metabolism as well as several GO terms relating to channel activity.

Table 5.3 GO terms relating to signal transduction which were significantly up-regulated in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (*=significantly up-regulated GO terms). A hypergeometric test was used to identify overrepresented GO terms with a significance level at 0.05 and the Benjamini-Hochberg method was used for the correction of the *p*-values. The Cytoscape plugin BiNGO was used to calculate overrepresented GO terms.

GO-ID	Category	Description	Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)			child of/ subset of (Go ID)
			Preconditioning	Desiccation	Recovery	
7165	BP	Signal transduction	40	*80	37	
23034	BP	Intracellular signalling pathway	45	*79	41	subset of 7165
35466	BP	Regulation of signalling pathway	31	*52	22	regulates 7165
9966	BP	Regulation of signal transduction	*29	*40	20	child of 7165
51056	BP	Regulation of small GTPase mediated signal transduction	*25	*36	16	child of 9966
46578	BP	Regulation of Ras protein signal transduction	*21	*31	13	child of 51056
51058	BP	Negative regulation of small GTPase mediated signal transduction	6	*9	5	child of 46578
46580	BP	Negative regulation of Ras protein signal transduction	6	*9	5	child of 51058
5099	MF	Ras GTPase activator activity	8	*12	5	
23051	BP	Regulation of signalling process	*29	*40	20	
23057	BP	Negative regulation of signalling process	8	*10	7	child of 23051
8286	BP	Insulin receptor signalling pathway	4	*6	3	
31175	BP	Neuron projection development	21	*42	21	
48666	BP	Neuron development	23	*44	22	
48667	BP	Cell morphogenesis involved in neuron differentiation	20	*39	20	
48812	BP	Neuron projection morphogenesis	20	*40	20	
50896	BP	Response to stimulus	144	*239	125	

Table 5.4 GO terms relating to gene expression and translation which were significantly up-regulated in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (*=significantly up-regulated GO terms).

GO-ID	Category	Description	Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)			child/ subset of (Go ID)
			Preconditioning	Desiccation	Recovery	
10467	BP	Gene expression	*133	*122	*133	

10468	BP	Regulation of gene expression	151	*231	129	child of 10467
40029	BP	Regulation of gene expression, epigenetic	30	*62	26	child of 10468
10628	BP	Positive regulation of gene expression	12	*26	12	child of 10468
10629	BP	Negative regulation of gene expression	40	*70	32	child of 10468
10608	BP	Posttranscriptional regulation of gene expression	33	*66	30	child of 10468
16458	BP	Gene silencing	29	*52	25	child of10629
16441	BP	Posttranscriptional gene silencing	28	*52	24	child of16458
31047	BP	Gene silencing by RNA	28	*52	24	child of16458
35194	BP	Posttranscriptional gene silencing by RNA	28	*52	24	child of16458
6417	BP	Regulation of translation	6	*15	6	child of 6412
17148	BP	Negative regulation of translation	2	*6	2	child of 6412
6412	BP	Translation	*87	49	*90	
6414	BP	Translational elongation	*8	5	*8	child of 6412
6413	BP	Translational initiation	6	6	*9	child of 6412
3746	MF	Translation elongation factor activity	*7	5	*7	
3743	MF	Translation initiation factor activity	6	6	*9	
8135	MF	Translation factor activity, nucleic acid binding	12	11	*15	
3735	MF	Structural constituent of ribosome	*72	30	*72	

Table 5.5 Reactome analysis showing significantly up-regulated pathways related to gene expression and translation in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae compared to the control nematodes.

	Name of this Event	**Identifier**	**Unadjusted *P*-value**	**Number of genes**	
				mapping to this Event	**involved in this Event**
Preconditioning	Peptide chain elongation	REACT_32702	3.10E-29	52	86
	Translation	REACT_81833	5.72E-22	59	141
	Metabolism of mRNA	REACT_142358	1.62E-21	58	139
	Gene Expression	REACT_108313	1.57E-15	99	419
Desiccation	Gene Expression	REACT_108313	3.12E-09	84	419
	mRNA Processing	REACT_80866	0.00014	28	124
Recovery	Eukaryotic Translation Initiation	REACT_33969	1.20E-25	54	112
	Peptide chain elongation	REACT_32702	2.06E-25	47	86
	Gene Expression	REACT_108313	3.38E-15	93	419

Table 5.6 The numbers of up-regulated genes which encode enzymes involved in intermediary metabolism, as identified by KEGG orthology mapping.

Kegg pathway	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**

Carbohydrate metabolism	T1/T2	T1/T3	T1/T4
KO_00010 Glycolysis/Gluconeogenesis	10	9	11
KO_00020 Citrate cycle (TCA cycle)	10	12	8
Lipid metabolism			
KO_00071 Fatty acid degradation	5	5	6
Energy metabolism			
KO_00010 Oxidative phosphorylation	21	8	14

Table 5.7 GO terms relating to energy metabolism which were significantly up-regulated in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (*=significantly up-regulated GO terms).

GO-ID	**Category**	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**		
			Preconditioning	**Desiccation**	**Recovery**
6091	BP	Generation of precursor metabolites and energy	*28	20	20
6006	BP	Glucose metabolic process	*14	*15	*14
15980	BP	Energy derivation by oxidation of organic compounds	*12	10	10

Table 5.8 Reactome analysis showing significantly up-regulated pathways related to energy metabolism in *C. elegans* dauer larvae in response to preconditioning and recovery compared to the control nematodes.

	Name of this Event	**Identifier**	**Unadjusted *P*-value**	**Number of genes**	
				mapping to this Event	**involved in this Event**
Preconditioning	The citric acid (TCA) cycle and respiratory electron transport	REACT_108279	0.002359	11	38
Recovery	Pyruvate metabolism and Citric Acid (TCA) cycle	REACT_157878	0.007854	9	35

Table 5.9 GO terms relating to cellular organisation which were significantly up-regulated in *C. elegans* dauer larvae in response to at least one of the treatment conditions (*=significantly up-regulated GO terms).

GO-ID	**Category**	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child/subset of (Go ID)**
			Preconditioning	**Desiccation**	**Recovery**	
7010	BP	Cytoskeleton organization	43	*94	28	
226	CC	Microtubule cytoskeleton organization	*31	*67	18	child of 7010
30036	BP	Actin cytoskeleton organization	10	*19	7	child of 7010
5815	BP	Microtubule organizing center	14	*29	6	

32886	BP	Regulation of microtubule-based process	4	*5	3	
32970	BP	Regulation of actin filament-based process	4	*9	3	
8092	MF	Cytoskeletal protein binding	17	*30	12	
48513	BP	Organ development	*213	*347	*179	
9887	BP	Organ morphogenesis	62	*126	52	child of 48513
9888	BP	Tissue development	60	*123	50	
60429	BP	Epithelium development	56	*113	47	child of 9888
48729	BP	Tissue morphogenesis	55	*115	48	
2009	BP	Morphogenesis of an epithelium	55	*112	47	child of 48729
10171	BP	Body morphogenesis	*107	*180	94	
902	BP	Cell morphogenesis	29	*51	27	
904	BP	Cell morphogenesis involved in differentiation	21	*40	21	child of 902
48858	BP	Cell projection morphogenesis	21	*44	20	child of 902
60429	BP	Epithelium development	56	*113	47	
2009	BP	Morphogenesis of an epithelium	55	*112	47	child of 60429
61024	BP	membrane organization	*153	*232	*130	
6900	BP	Membrane budding	0	2	1	
61025	BP	Membrane fusion	3	2	0	
10324	BP	Membrane invagination	*151	*229	*129	
6911	BP	Phagocytosis, engulfment	4	6	4	child of 10324
48475	CC	Coated membrane	7	*13	9	child of 48475
43621	BP	Protein self-association	0	*5	0	

Table 5.10 GO terms relating to transport which were significantly up-regulated in *C. elegans* dauer larvae in response to at least one of the treatment conditions (*=significantly up-regulated GO terms).

GO-ID	**Category**	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child/subset of (Go ID)**
			Preconditioning	**Desiccation**	**Recovery**	
6810	BP	Transport	302	*422	279	
51050	BP	Positive regulation of transport	2	*6	0	child of 6810
51049	BP	Regulation of transport	5	11	4	child of 6810
16192	BP	Vesicle-mediated transport	*166	*253	*143	child of 6810

32456	BP	Endocytic recycling	2	2	1	child of 6810
6887	BP	Exocytosis	3	6	4	child of 6810
48193	BP	Golgi vesicle transport	2	4	2	child of 6810
6900	BP	Membrane budding	0	2	1	child of 6810
60627	BP	Regulation of vesicle-mediated transport	0	3	0	child of 6810
48489	BP	Synaptic vesicle transport	7	*13	7	child of 6810
48278	BP	Vesicle docking	0	3	2	child of 6810
6906	BP	Vesicle fusion	1	1	0	child of 6810
6897	BP	Endocytosis	*151	*229	*129	child of 6810
6898	BP	Receptor-mediated endocytosis	*140	*214	*120	child of 6897
6909	BP	Phagocytosis	8	10	7	child of 6897
45807	BP	Positive regulation of endocytosis	1	3	0	child of 6897
30100	BP	Regulation of endocytosis	0	3	0	child of 6897
48488	BP	Synaptic vesicle endocytosis	1	3	1	child of 6897
22832	BP	Voltage-gated channel activity	9	16	9	
5635	CC	Nuclear envelope	7	*18	7	
5643	CC	Nuclear pore	1	*7	1	child of 5635

Table 5.11 GO terms relating to DNA damage and repair, DNA replication, chromosome component and nuclear component which were significantly up-regulated in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (*=significantly up-regulated GO terms).

GO-ID	**Category**	**Description**	**Number of individual genes up-regiated in each GO category (Benjamini-Hochberg test** $p<0.05$**)**			**child/subset of (Go ID)**
			Preconditioning	**Desiccation**	**Recovery**	
6974	BP	Response to DNA damage stimulus	25	*62	19	
6281	BP	DNA repair	12	*37	9	child of 6974
6260	BP	DNA replication	0	*28	0	
6265	BP	DNA topological change	0	*4	0	
3684	MF	damaged DNA binding	2	*9	0	
3678	MF	DNA helicase activity	7	*19	0	
793	CC	Condensed chromosome	7	*27	0	
794	CC	Condensed nuclear chromosome	5	*16	3	
5635	CC	Nuclear envelope	7	*18	7	
5643	CC	Nuclear pore	1	*7	1	child of 5635

Table 5.12 Reactome analysis showing significantly up-regulated pathways related to DNA repair in response to desiccation in *C. elegans* dauer larvae compared to the control nematodes.

	Name of this Event	Identifier	Unadjusted *P*-value	Number of genes	
				mapping to this Event	involved in this Event
Desiccation	DNA Repair	REACT_102449	7.48E-06	19	58
	Nucleotide Excision Repair	REACT_110428	4.91E-05	13	35

Table 5.13 GO terms relating to cell death, apoptosis and ubiquitin which significantly up-regulated in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (*=significantly up-regulated GO terms).

GO-ID	Category	Description	Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)			child/subset of (Go ID)
			Preconditioning	Desiccation	Recovery	
12501	B.P	Programmed cell death	*26	*39	20	
6915	B.P	Apoptosis	*20	*34	13	
6916	B.P	Anti-apoptosis	1	*4	0	child of 6915
6921	B.P	cellular component disassembly involved in apoptosis	5	*7	4	child of 6915
502	B.P	proteasome complex	*10	8	8	
4842	B.P	ubiquitin-protein ligase activity	4	*11	4	
19787	B.P	small conjugating protein ligase activity	14	*21	*15	

Table 5.14 Reactome analysis showing significantly up-regulated pathways related to removal of damaged proteins in *C. elegans* dauer larvae in response preconditioning, desiccation and recovery compared to the control.

	Name of this Event	Identifier	Unadjusted P-value	Number of genes	
				mapping to this Event	involved in this Event
Preconditioning	Proteasomal cleavage of substrate	REACT_129051	0.000141	12	33
Desiccation			0.025198	8	33
Recovery			0.01721	8	33

Table 5.15 GO terms relating to protection which were significantly up-regulated in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (*=significantly up-regulated GO terms).

GO-ID	Category	Description	Number of individual genes up-regiated in each GO category (Benjamini-Hochberg test $p<0.05$)			child/subset of (Go ID)
			Preconditioning	Desiccation	Recovery	
6950	B.P	Response to stress	*79	*120	*72	
9408	B.P	Response to heat	*15	*19	*15	child of 6950
50896	B.P	Response to stimulus	144	*239	125	
6986	B.P	Response to unfolded protein (HSPs)	5	*8	4	child of 6950
30968	B.P	Endoplasmic reticulum unfolded protein response	5	*7	4	child of 6986
9607	B.P	Response to biotic stimulus	12	*17	11	child of 50896
9266	B.P	Response to temperature stimulus	*18	*24	*18	
9628	B.P	Response to abiotic stimulus	25	*36	20	
33554	B.P	Cellular response to stress	33	*73	26	

Table 5.16 Reactome analysis showing pathways related to protein folding which are significantly up-regulated in *C. elegans* dauer larvae in response preconditioning, desiccation and recovery compared to the control nematodes.

	Name of this Event	Identifier	Unadjusted P-value	Number of genes	
				mapping to this Event	involved in this Event
Preconditioning	Actin/tubulin:prefoldin complex associates with CCT/TriC	REACT_94082	0.002627	8	23
Desiccation			0.002401	8	23
Recovery			0.00028	9	23

Table 5.17 Expression of individual fungus induced protein related (*fipr*), caenacin (*cnc*) and cadmium responsive (*cdr*) genes in *C. elegans* dauer larvae relative to control nematodes preconditioning, desiccation and recovery (*not significantly up-regulated).

Gene Name	Gene ID	Preconditioning		Desiccation		Recovery	
		Fold change	*P*-value	Fold change	*P*-value	Fold change	*P*-value
fipr-22	C37A5.2	5.55	5.86E-07	3.63	9.37E-05	6.19	3.3E-07
fipr-23	C37A5.4	3.25	0.000445	*1.69	0.130134	3.50	0.000252
fipr-24	C37A5.8	7.58	5.65E-17	2.43	0.000225	4.53	3.13E-09
fipr-26	F53B6.8	12.41	1.68E-35	9.27	1.21E-30	18.98	1.15E-43
cnc-5	R09B5.10	3.61	3.62E-05	5.32	7.33E-08	25.08	7.72E-30
cnc-6	Y46E12A.1	5.82	1.44E-16	*1.43	0.336059	5.27	2.07E-13
cnc-11	R09B5.13	30.40	1.21E-44	16.95	2.92E-34	36.73	1.46E-47

cdr-3	C54D10.2	39.42	4.53E-64	22.42	1.04E-49	60.90	1.95E-74
cdr-4	K01D12.11	5.52	1.87E-17	4.37	3.07E-14	8.39	1.48E-24

Table 5.18 GO terms which are significantly enriched in *C. elegans* dauer larvae during recovery from desiccation when compared to desiccated nematodes.

GO-ID	Description	corrected value	Number of genes associated
16021	Integral to membrane	4.08E-06	301
16020	Membrane	7.82E-06	312
16491	Oxidoreductase activity	3.85E-04	47
51213	Dioxygenase activity	1.74E-03	6
50832	Defense response to fungus	2.20E-03	6
9620	Response to fungus	2.20E-03	6
55114	Oxidation reduction	2.55E-03	36
51707	Response to other organism	2.55E-03	8
45087	Innate immune response	2.55E-03	7
19752	Carboxylic acid metabolic process	2.55E-03	20
43436	Oxoacid metabolic process	2.55E-03	20
6082	Organic acid metabolic process	2.55E-03	20
2376	Immune system process	2.88E-03	7
6955	Immune response	2.88E-03	7
6952	Defense response	2.88E-03	12
42180	Cellular ketone metabolic process	3.96E-03	20
9309	Amine biosynthetic process	6.58E-03	8
6725	Cellular aromatic compound metabolic process	7.51E-03	11
16053	Organic acid biosynthetic process	7.75E-03	9
46394	Carboxylic acid biosynthetic process	7.75E-03	9
8289	Lipid binding	1.05E-02	12
8652	Cellular amino acid biosynthetic process	2.75E-02	6
16702	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	2.75E-02	4
6790	Sulfur metabolic process	2.75E-02	5
9607	Response to biotic stimulus	2.81E-02	8
6519	Cellular amino acid and derivative metabolic process	2.81E-02	14
44106	Cellular amine metabolic process	3.03E-02	13
16701	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	3.50E-02	4

Table 5.19 Reactome analysis showing significantly up-regulated pathways in response to recovery compared to the desiccation dataset.

Name of this Event	Identifier	Unadjusted *P*-value	Number of genes	
			mapping to this Event	involved in this Event
Peroxisomal lipid metabolism	REACT_82818	0.001702	5	37
Metabolism of polyamines	REACT_109915	0.00255	3	12
Metabolism of amino acids and derivatives	REACT_104914	0.003147	10	153

Metabolism	REACT_113610	0.004328	31	852
Synthesis of very long-chain fatty acyl-CoAs	REACT_103020	0.011216	2	7

Table 5.20 Expression of genes that encode aquaporin proteins as well the antioxidant (*sod-2*) in response to recovery compared to desiccation.

Gene name	**Gene ID**	**Fold change**	***P*-value**
aqp-1	F32A5.5	5.093237	1.77E-10
aqp-11	ZK525.2	2.338828	6.58E-05
sod-2	F10D11.1	2.109256	0.000366

Table 5.21 GO terms which are significantly down-regulated in *C. elegans* dauer larvae relative to control nematodes in response to exposure to preconditioning, desiccation and recovery (*= significantly up-regulated GO terms).

GO-ID	**Category**	**Description**	**Number of individual genes up-regulated in each GO category (Benjamini-Hochberg test $p<0.05$)**			**child/subset of (Go ID)**
			Preconditioning	**Desiccation**	**Recovery**	
9075	B.P	Histidine family amino acid metabolic process	*10	*11	*12	
30258	B.P	Lipid modification	*47	*54	*44	
15267	B.P	Channel activity	*121	*112	*105	
22836	B.P	Gated channel activity	*89	*77	*74	child of 15267
5230	B.P	Extracellular ligand-gated ion channel activity	*70	*62	*52	child of 22836
5216	M.F	Ion channel activity	*117	*107	*101	
15276	M.F	Ligand-gated ion channel activity	*74	*63	*55	
5506	M.F	Iron ion binding	*60	*71	*57	
5509	M.F	Calcium ion binding	*63	*56	*56	
6811	B.P	Ion transport	*147	*136	*128	

Figure 5.13 Enrichment analysis for GO terms associated with signal transduction and signalling which are up-regulated in response to (i) preconditioning and (ii) desiccation in *C. elegans* dauer larvae. The size of circles is proportional to the number of up-regulated genes associated with the GO term. The arrows represent the relationship between parent-child terms. The colour scale indicates corrected *p*-value of enrichment analysis.

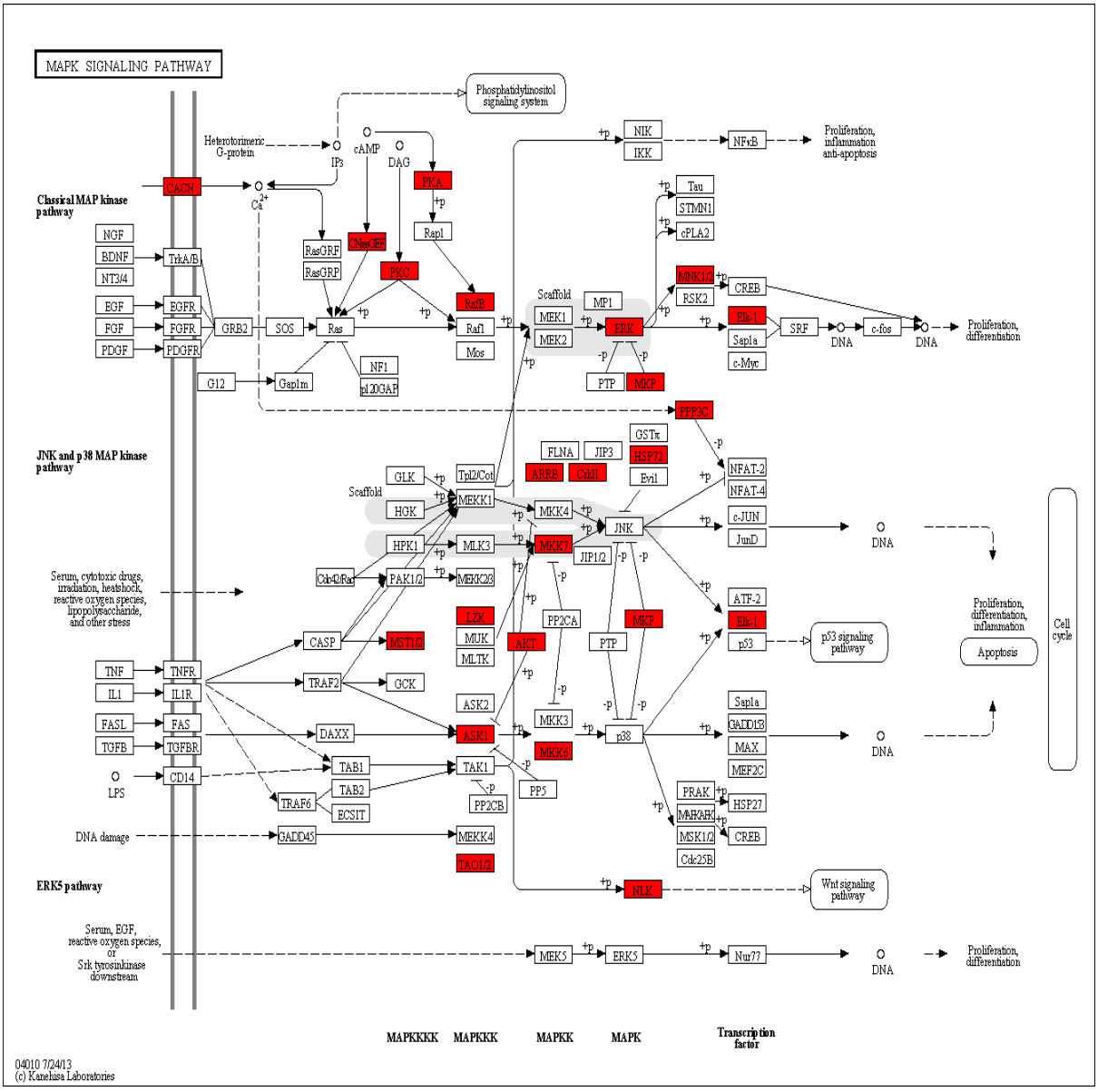


Figure 5.14 KEGG pathway map showing MAPK signalling in response to desiccation stress in *C. elegans* dauer larvae. The genes which are up-regulated in response to desiccation are shown in red.

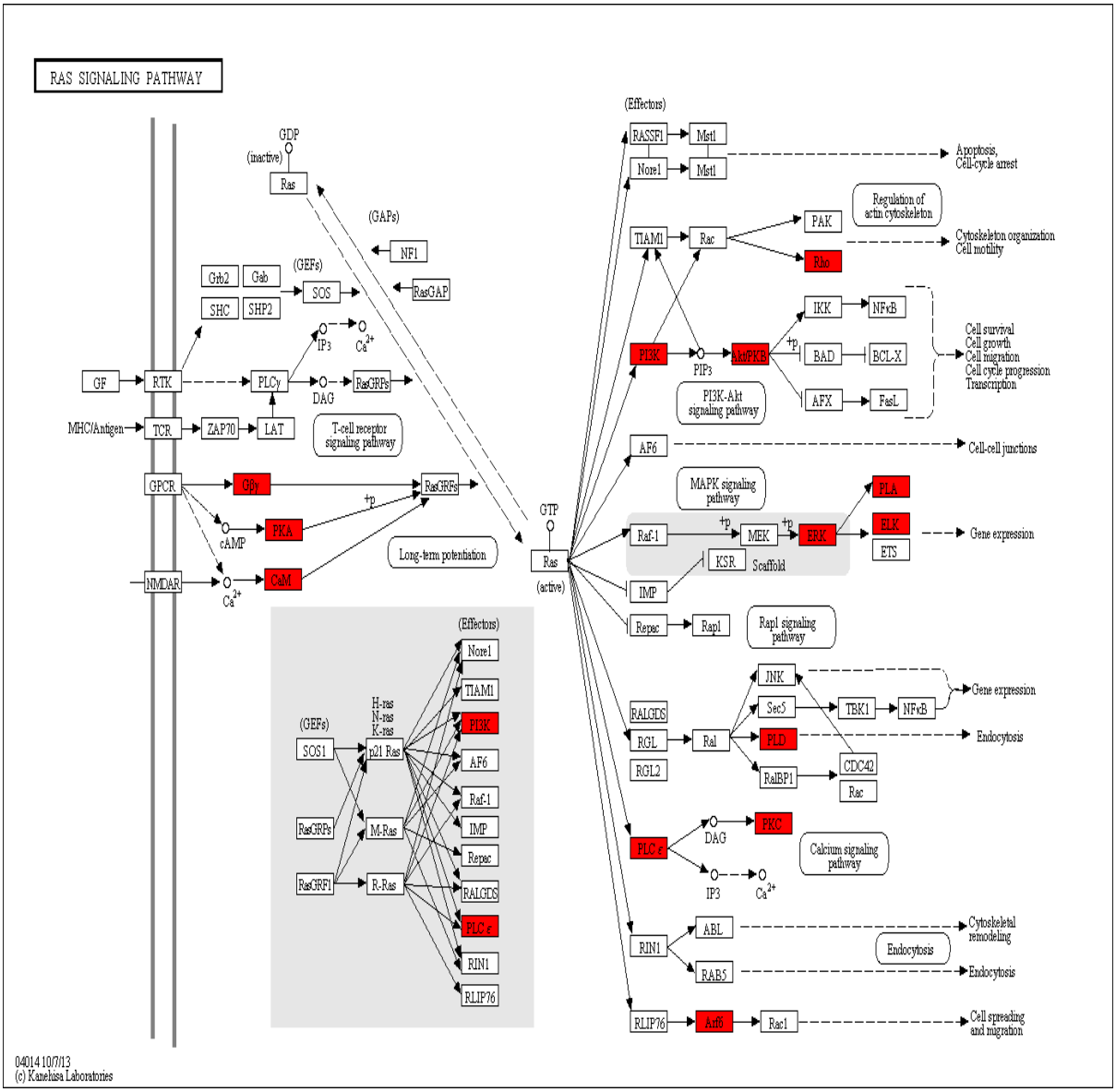
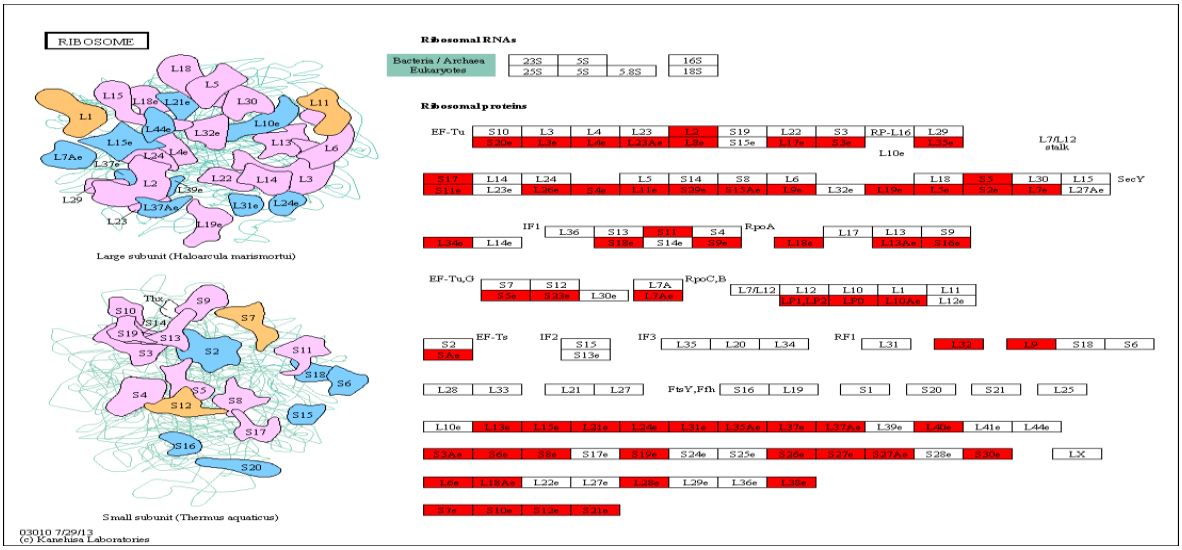


Figure 5.15 KEGG pathway map showing Ras signalling in response to desiccation stress stress in *C. elegans* dauer larvae. The genes which are up-regulated in response to desiccation are shown in red.

(i)



(ii)

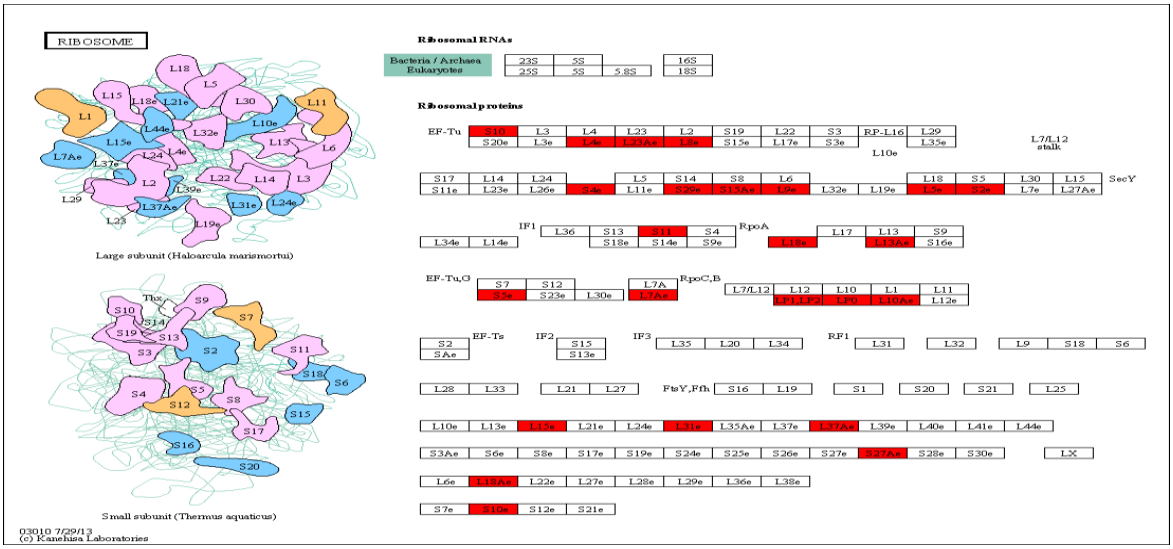
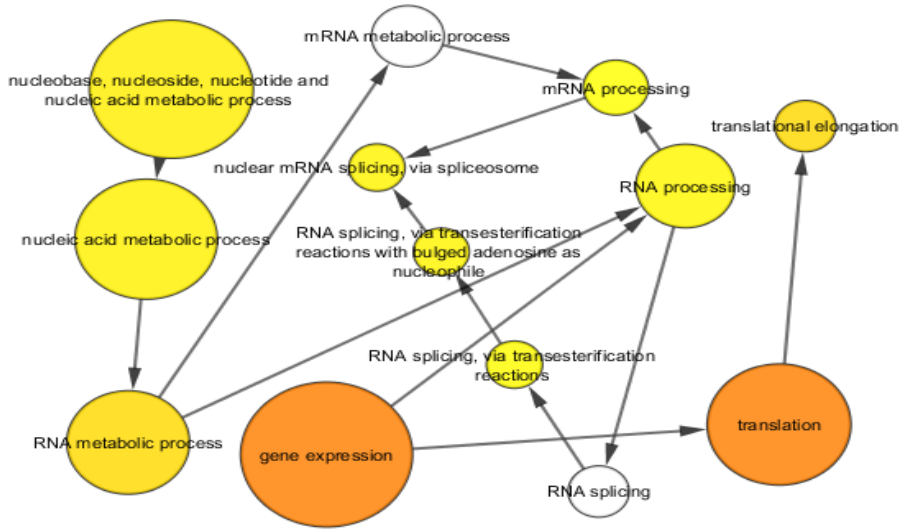


Figure 5.16 KEGG pathway map showing genes encoding ribosomal proteins that are significantly up-regulated in response to (i) preconditioning (ii) desiccation in *C. elegans* dauer larvae. The genes which are up-regulated are shown in red.

(i)



(ii)

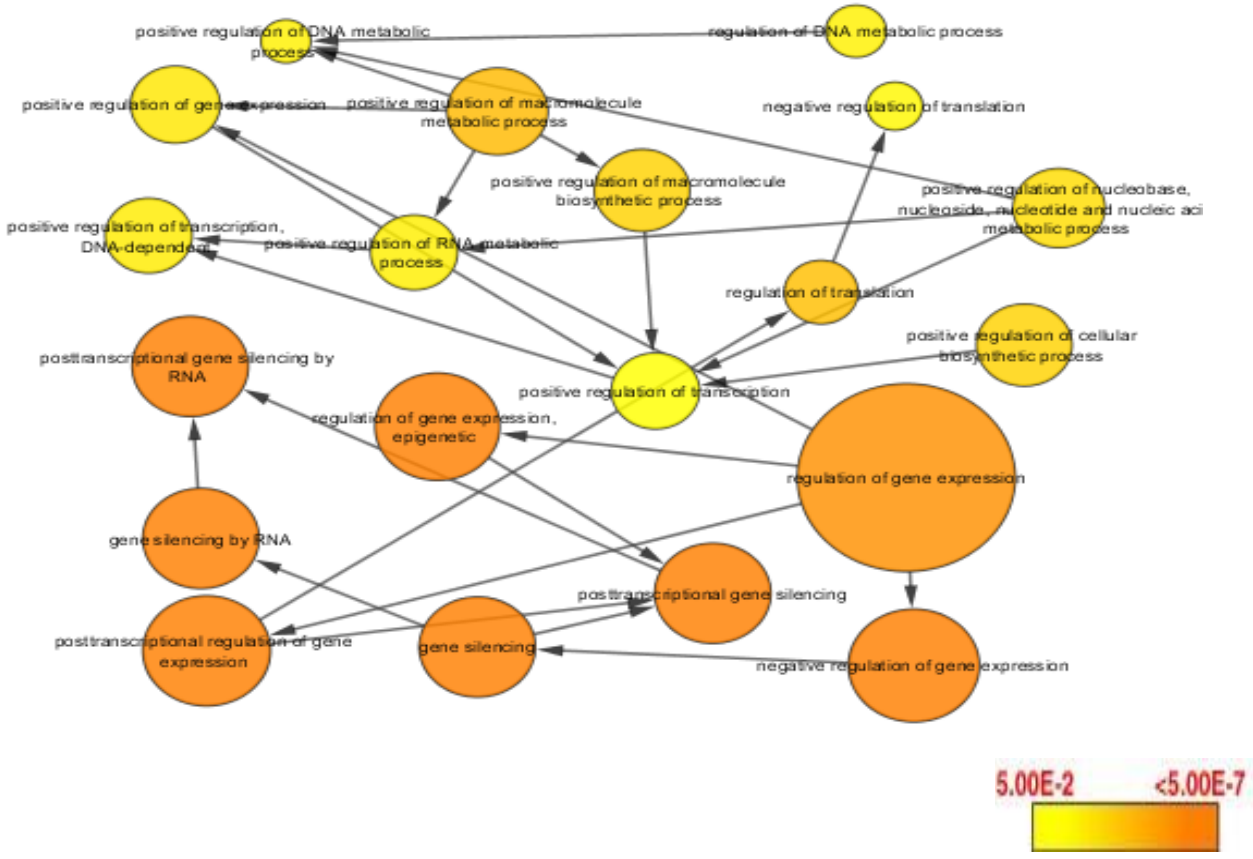


Figure 5.17 Enrichment analysis for GO terms associated with signal transduction and signalling which are up-regulated in response to (i) preconditioning and (ii) desiccation in *C. elegans* dauer larvae. GO terms associated with RNA metabolism and gene expression are shown. The size of circles is proportional to the number of genes associated with each GO term. The arrows represent the relationship between parent–child terms. The colour scale indicates corrected p-value of enrichment analysis.

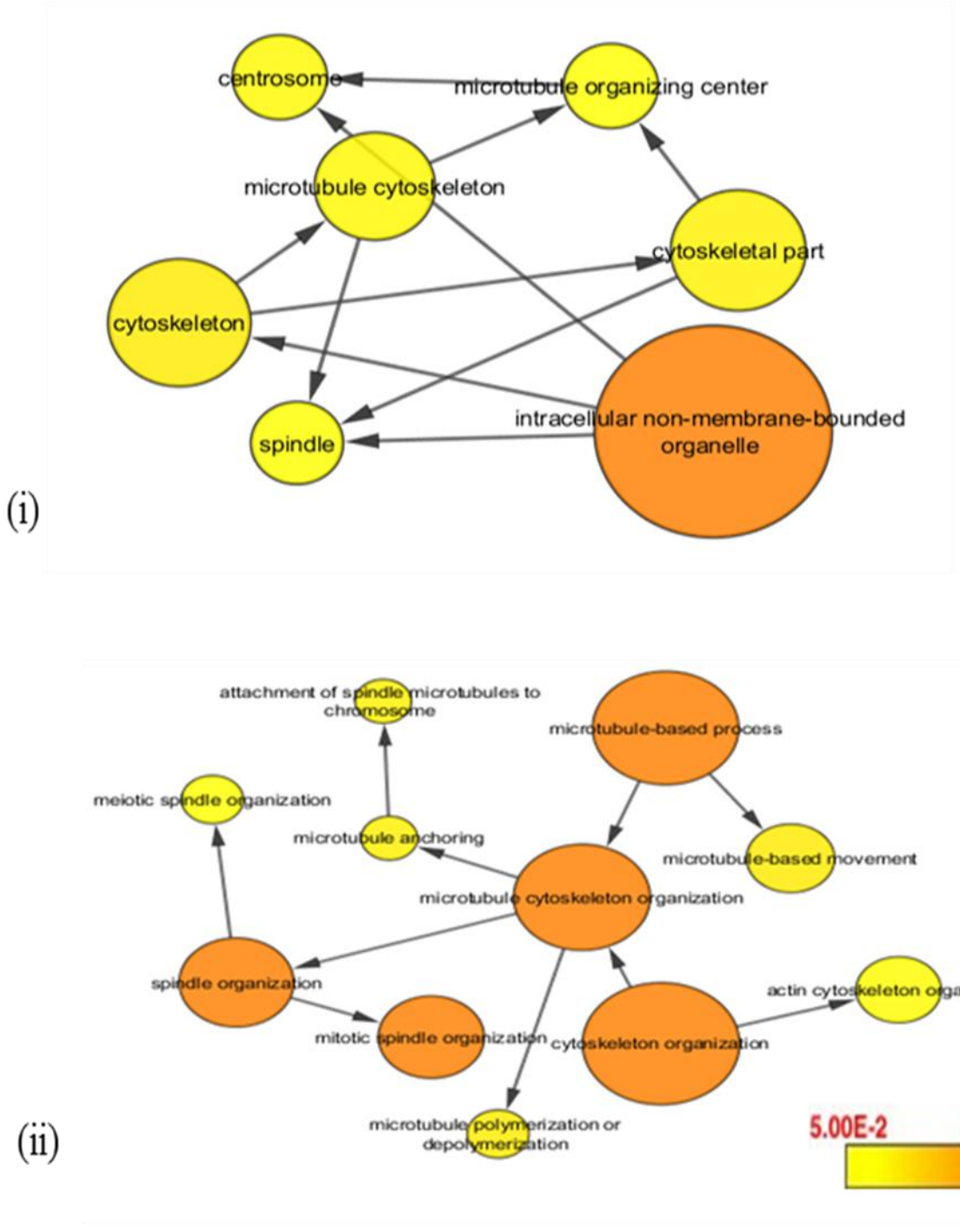


Figure 5.18 Enrichment analysis for GO terms associated with cytoskeleton organisation which are up-regulated in response to (i) preconditioning and (ii) desiccation. The size of circles is proportional to the number of genes associated with the GO term. The arrows represent the relationship between parent–child terms. The colour scale indicates corrected *p*-value of enrichment analysis.

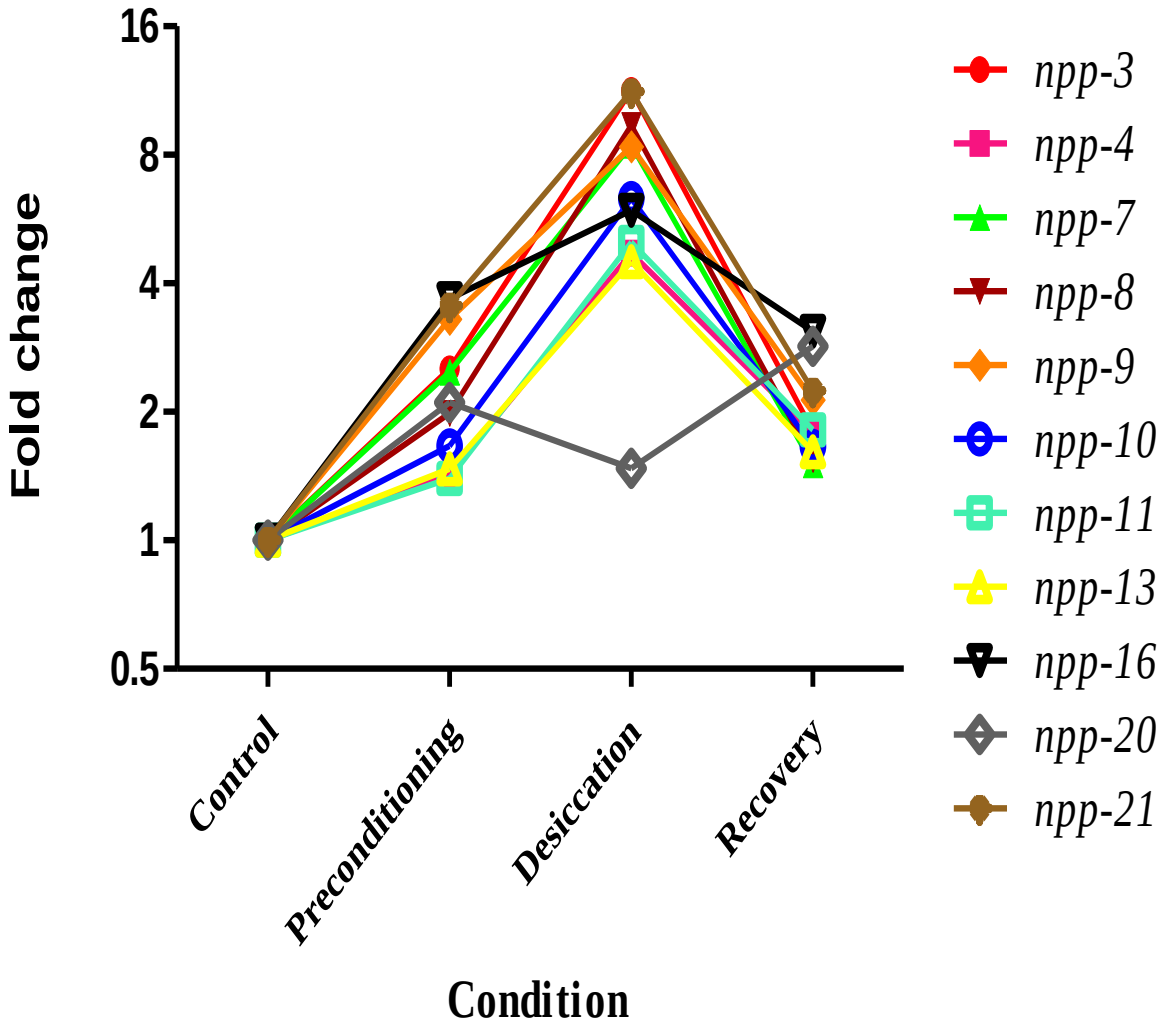


Figure 5.19 Change in expression of genes (RNA-seq transcript counts) encoding nuclear pore complex proteins in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery compared to the control (*nnp-3*, *nnp-4*, *nnp-7*, *nnp-8*, *nnp-9*, *nnp-10*, *nnp-11*, *nnp-13*, *nnp-16*, *nnp-20*, *nnp-21*). Relative expression values (determined from RNA-seq transcript counts) were plotted on a log-2 scale.

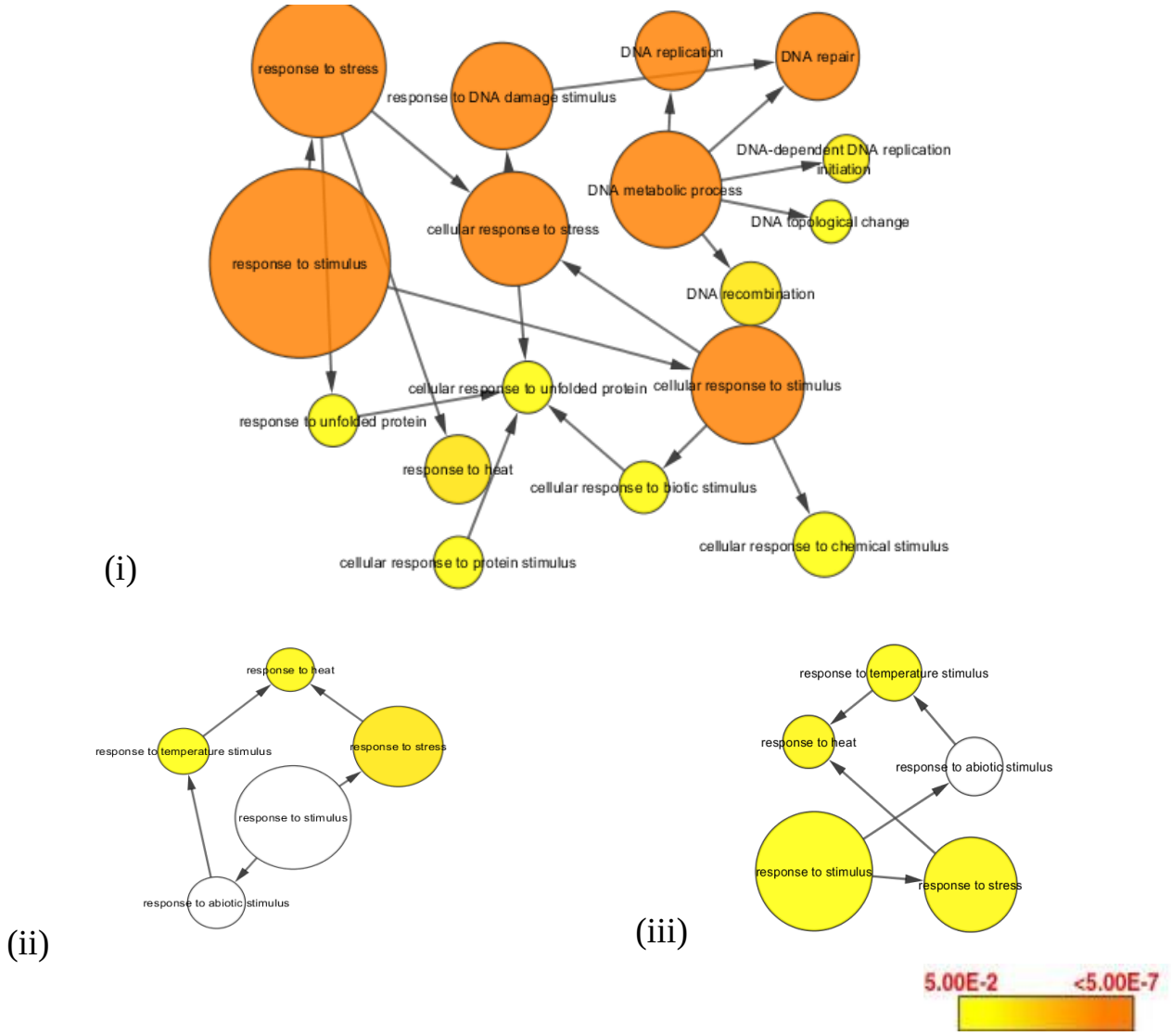


Figure 5.20 GO Enrichment analysis for GO terms associated with DNA damage, repair and replication, which are up-regulated in response to desiccation (i). Cytoscape mapping of comparable GO terms for preconditioning (ii) recovery from desiccation (iii) shows no evidence of enrichment for terms associated with DNA damage, repair and replication. The size of circles is proportional to the number of genes associated with the GO term. The arrows represent the relationship between parent–child terms. The colour scale indicates corrected *p*-value of the enrichment analysis.

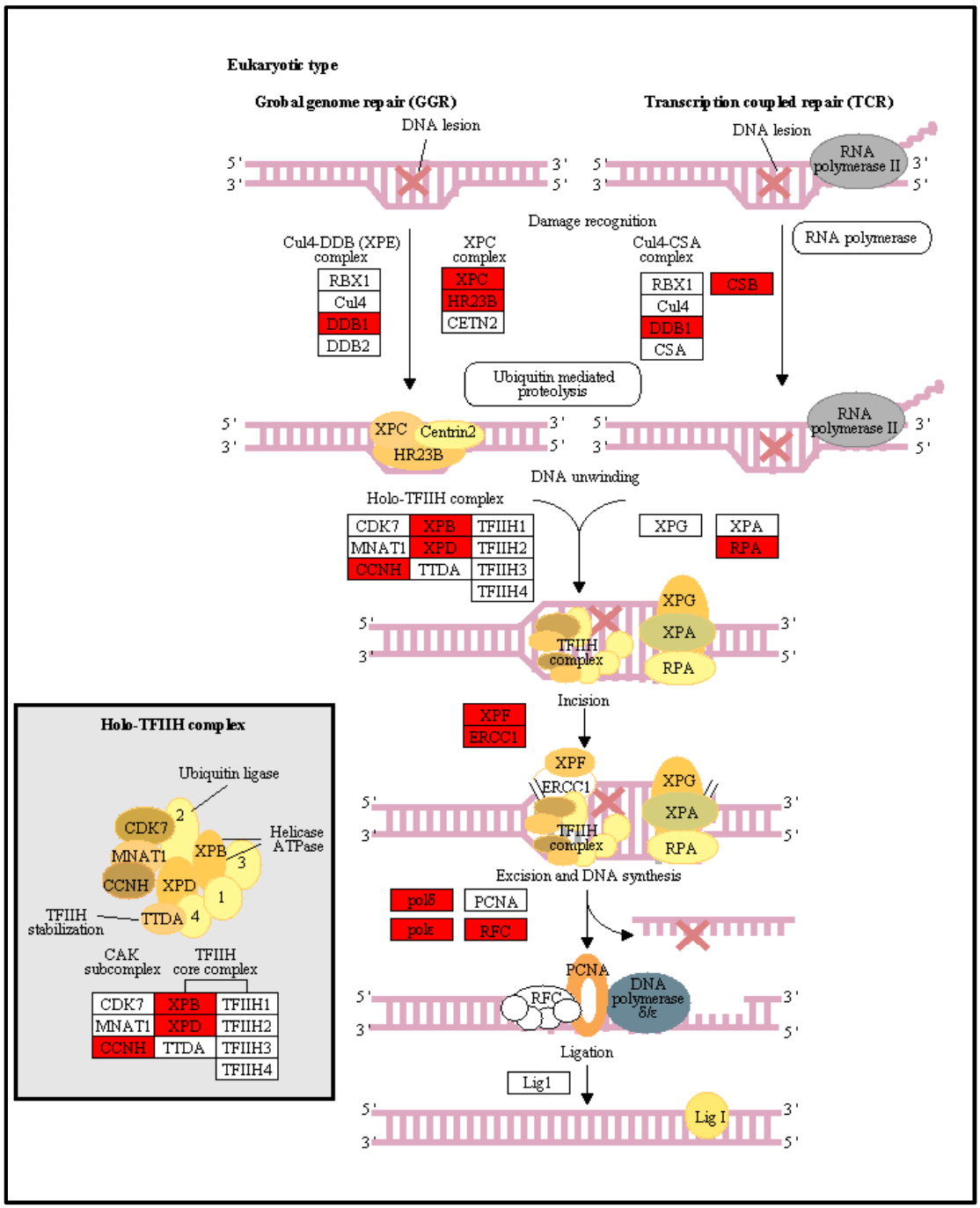


Figure 5.21 KEGG pathway map showing significantly up-regulated genes encoding proteins involved in nucleotide excision repair in response to desiccation in *C. elegans* dauer larvae. The genes up-regulated in response to desiccation are shown in red.

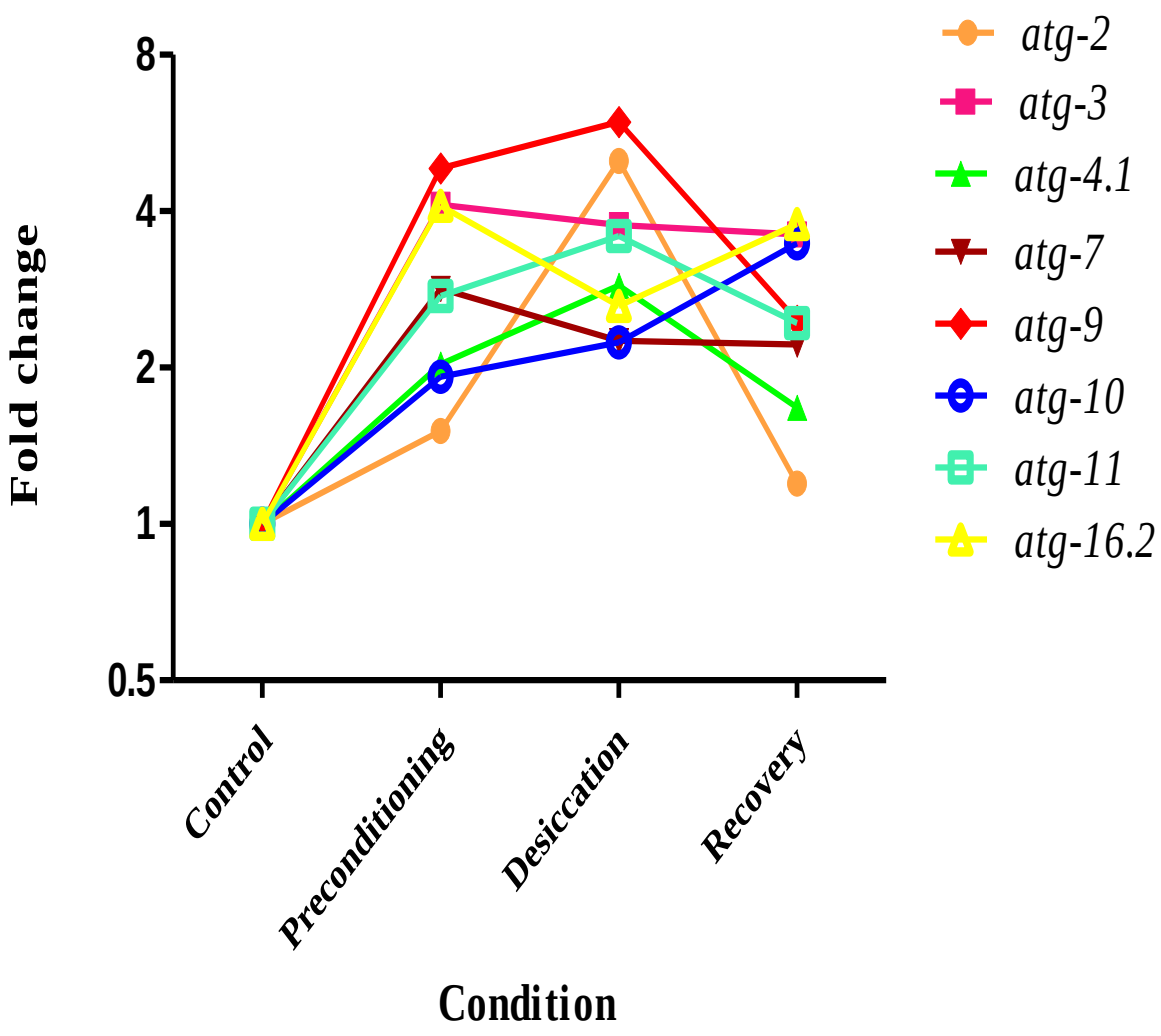


Figure 5.22 Change in expression of genes (RNA-seq transcript counts) encoding autophagy proteins in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery compared to the control (*atg-2*, *atg-3*, *atg-4.1*, *atg-7*, *atg-9*, *atg-10*, *atg-11*, *atg-16.2*). Relative expression values (determined from RNA-seq transcript counts) were plotted on a log-2 scale.

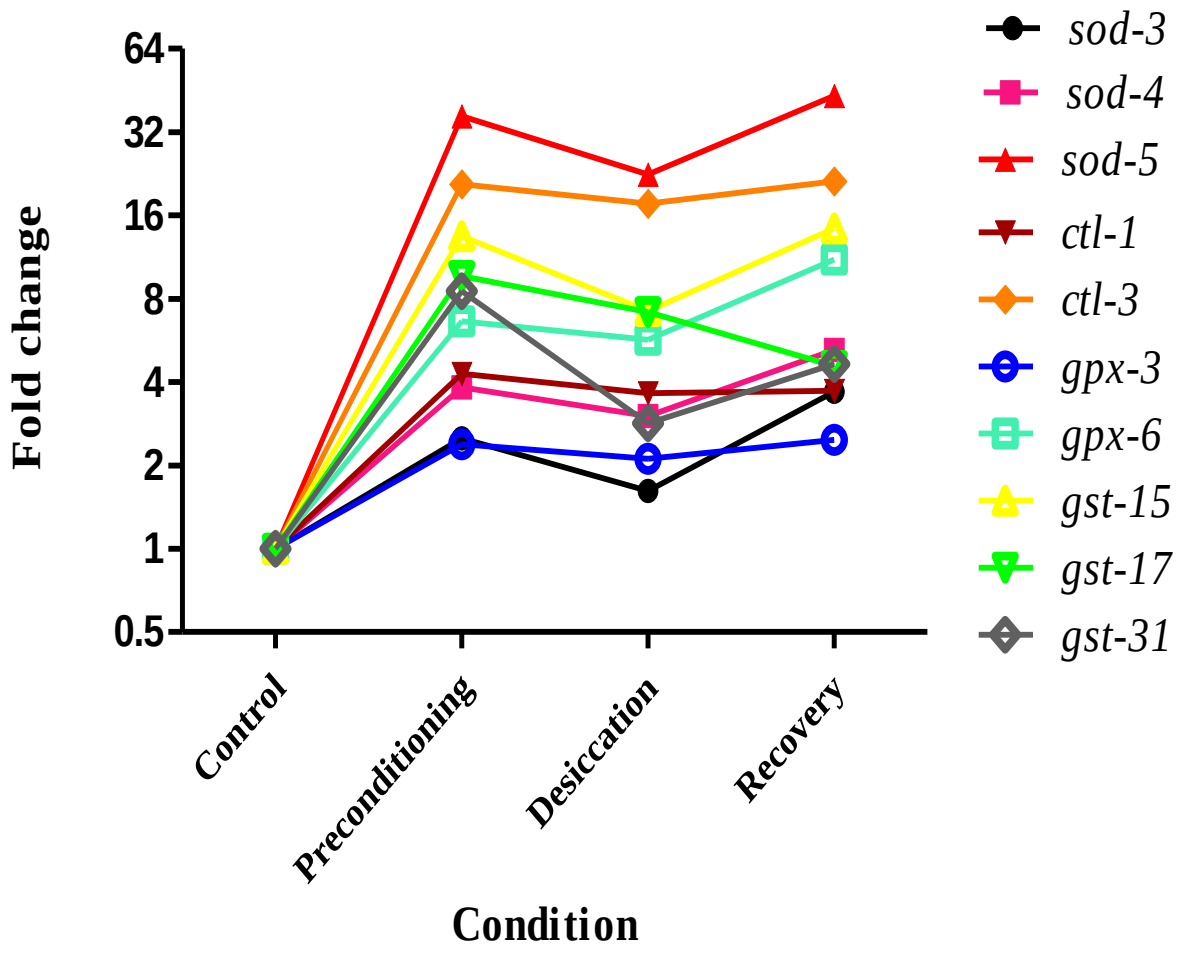


Figure 5.23 Changes in expression of genes encoding antioxidant enzymes in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery compared to the control. Relative expression values (determined from RNA-seq transcript counts) were plotted on a log-2 scale. These genes encode superoxide dismutase (*sod-3*, *sod-4*, *sod-5*), catalase (*ctl*-1, ctl-*3*), glutathione peroxidase (*gpx-3*, *gpx-6*) and glutathione S-transferase (*gst-15*, *gst-17*, *gst-31*) enzymes.

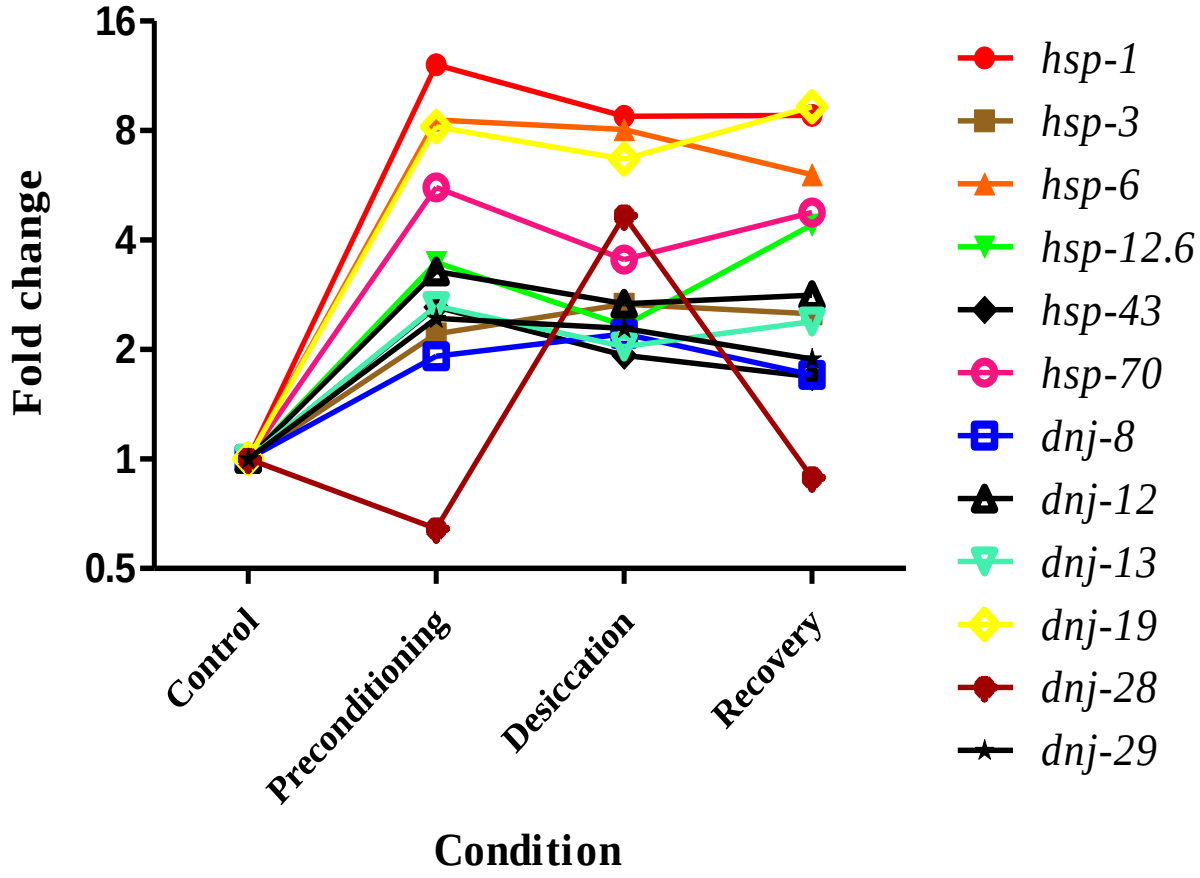


Figure 5.24 Change in expression of genes encoding HSPs in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery relative to their expression in control nematodes The genes are as follows: the small HSP genes *hsp-12.6*, *hsp-43* (a member of the HSP-20 family), members of the DnaJ/HSP-40 family (*dnj-8*, *dnj-12*, *dnj-13*, *dnj-19 dnj-28 dnj-29*) and members of the DNAK/HSP-70 family (*hsp-1*, *hsp-3*, *hsp-6* and *hsp-70*). Relative expression values (determined from RNA-seq transcript counts) were plotted on a log-2 scale.

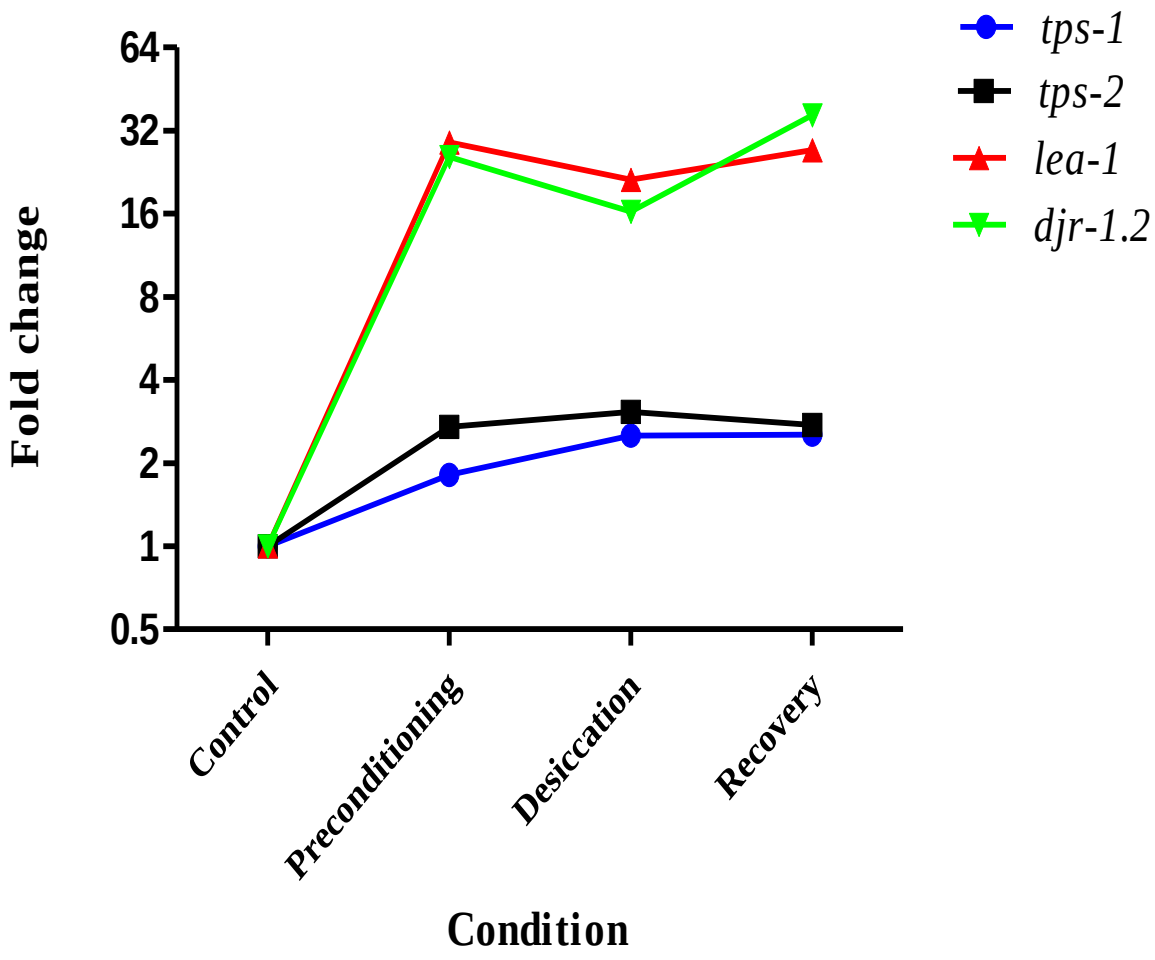


Figure 5.25 Change in expression of genes encoding LEA protein (*lea-1*), trehalose 6-phosphate synthase enzymes (*tps-1* and *tps-2*) and DJR-1.2 (*djr-1.2*) in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery compared to the control. Relative expression values (determined from RNA-seq transcript counts) were plotted on a log-2 scale.

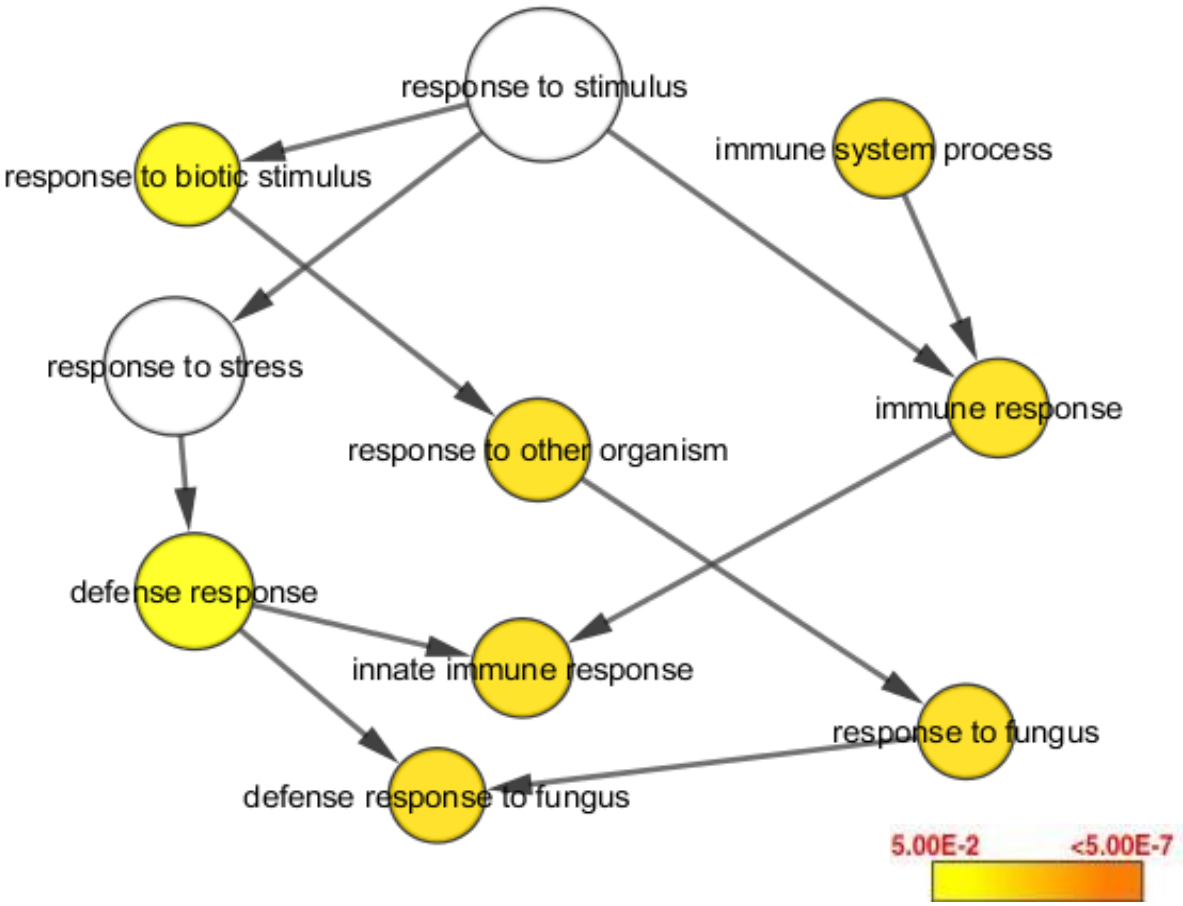


Figure 5.26 Enrichment analysis for GO terms associated with defense which are up-regulated in *C. elegans* dauer juveniles during recovery from desiccation relative to desiccated nematodes. The size of circles is proportional to the number of genes associated with the GO term. The arrows represent the relationship between parent–child terms. The colour scale indicates corrected p-value of enrichment analysis.

5.2.11 Comparing RNA-seq and qPCR results for the expression of genes in response to desiccation in *C. elegans* dauer larvae

Quantitative PCR (qPCR) was used to select the optimum desiccation-related time points to use for the RNA-seq experiment (Section 5.2.2). Comparing the gene expression values between the RNA-seq and qPCR experiments is a good way to validate the results. There was very good correlation between the RNA-seq and qPCR as shown in Tables 5.22 and 5.23. The fold-change in expression was not the same for both experiments. This is expected since the RNA sample used in this experiment was not the same as that used in the sequencing run.

Table 5.22 Comparison of RNA-seq and QPCR (quantitative PCR) gene expression values in response to preconditioning (98% RH for 48 h).

Gene name	**Preconditioning**	
	RNA-seq (fold change)	**qPCR fold change (fold change)**
hsp-70	5.59	16.10
lea-1	29.05	28.31
sod-2	0.70	1.25
hsp-60	0.48	0.70
gst-4	0.72	0.36
dur-1	0.87	0.85

Table 5.23 Comparison of RNA-seq and QPCR (quantitative PCR) gene expression values in response to desiccation (98% RH for 96 h followed by 0% RH 24 h) and recovery (98% RH for 96 h, 0% RH 24 h followed by 2 h rehydration).

Gene name	**Desiccation**		**Recovery**	
	RNA-seq (fold change)	**qPCR fold change (fold change)**	**RNA-seq (fold change)**	**qPCR fold change (fold change)**
rpt-2	2.75	1.74	2.87	4.25
crn-7	0.49	0.82	0.59	0.91
pyk-1	6.87	2.84	5.87	3.73
ced-3	15.34	2.60	2.43	1.48
prx-1	0.83	0.59	0.91	0.28

5.3 Discussion

The development of the RNA-seq technique has revolutionised the field of transcriptomics. RNA-seq has the power to detect differences in gene expression levels between different cell types, growth stages or environmental conditions across the entire transcriptome of an organism. However this technique requires the availability of a fully sequenced and annotated genome or a complete transcriptome dataset, which tends to restrict its use in non-model organisms. I have used RNA-seq to identify the transcripts which are differentially expressed in response to desiccation in *C. elegans* dauer larvae. *C. elegans* nematodes are not normally desiccation tolerant but *C. elegans* dauer larvae can be preconditioned to survive desiccation, discovered by Erkut *et al.* (2011) as described in Section 5.2.1. A pilot qPCR study indicated that up-regulation of some *C. elegans* transcripts was occurring in response to a preconditioning, desiccation and recovery. The length of the preconditioning period required to give optimal expression of the genes tested was also determined. Time points were selected based on the results of the pilot qPCR study, as described in Section 5.2.2, and a flow chart of experimental conditions is shown in Figure 5.5.

In this RNA-seq study a great number of genes were found to be differentially expressed in response to desiccation and recovery from desiccation in *C. elegans* dauer larvae as described in Section 5.2.9. An overview of the genes up-regulated in response to desiccation and recovery is given in Figure 5.27. In this discussion section the up-regulated genes have been divided into the following categories based on the characteristics of the proteins they encode and the biological pathways in which they function: signalling pathways, translation, molecular chaperones, compatible solutes, defense against pathogens, cellular organization; antioxidants, DNA damage repair and removal of damaged proteins.

5.3.1. Signalling pathways

All organisms respond to different types of environmental stress such as desiccation with coordinated adjustments to deal with the consequences of the stress (Lant and Storey, 2010). In *C. elegans* a number of important signalling pathways regulate stress responses. These include Ras signalling, mitogen-activated protein kinase (MARKs) as well as insulin receptor signalling (Inoue *et al.*, 2005; Neumann-Haefelin *et al.*, 2008; Perrin *et*

al., 2013). A number of GO terms associated with these signalling pathways were significantly up-regulated in response to preconditioning and desiccation as outlined in Table 5.3. However, from the GO pathway enrichment analysis, there is clearly a stronger association with signalling in the desiccation dataset with 17 GO terms up-regulated compared to just four terms up-regulated in response to preconditioning. These signalling pathways which were up-regulated in response to desiccation include general signal transduction which encompasses Ras and mitogen-activated protein kinases (MAPKs), Ras protein signal transduction, response to unfolded protein, and insulin receptor signalling. The significantly up-regulated genes which mapped to the MAPK and Ras signalling pathways obtained from the KEGG database are shown in Figure 5.14 and Figure 5.15 respectively. As well as significantly up-regulated GO terms associated with signalling, GO terms associated with the nervous system such as neuron development and neuron projection morphogenesis were also up-regulated.

A number of GO terms which are significantly up-regulated in the desiccation dataset are related to Ras and MAPKs signalling such as signal transduction and regulation of Ras protein signal transduction. Ras functions as a relay switch that is positioned downstream of cell surface receptor tyrosine kinases and upstream of a cytoplasmic cascade of kinases that includes the mitogen-activated protein kinases (MAPKs). Activated MAPKs in turn regulate the activities of nuclear transcription factors (Campbell *et al.*, 1998). Ras proteins act as molecular switches to transduce extracellular ligand-mediated stimuli into the cytoplasm to control signal transduction pathways that influence cell growth, differentiation and apoptosis (Campbell *et al.*, 1998).

Four pathways relating to neurons and neuron development are specifically up-regulated in response to desiccation (Table 5.3). Thermosensory neurons have been previously shown to regulate the heat shock and protein folding response in *C. elegans* (Prahlad *et al.*, 2008; Prahlad and Morimoto, 2011). Furthermore, it has been hypothesized that neuronal signalling can serve as a homeostatic switch for the control of chaperone expression in response to environmental stresses such as desiccation (Prahlad and Morimoto, 2011).

5.3.2. Translation

As discussed above, environmental stresses such as desiccation can activate signalling cascades that result in the downstream expression of genes which encode effector proteins. The GO terms and Reactome pathways associated gene expression, transcription and translation which are significantly up-regulated in response to preconditioning, desiccation and recovery have been described in Section 5.2.9.2. Several of these up-regulated GO terms are associated with RNA processing in the desiccation dataset, while translation and protein synthesis GO terms are up-regulated in the preconditioning and recovery datasets. This trend is also observed in the Reactome analysis with such pathways as peptide chain elongation significantly up-regulated in the preconditioning and recovery dataset while the RNA processing pathway has significantly higher expression in the desiccation dataset. The expression of genes involved in protein synthesis in the preconditioning dataset may be a result of an effort to produce proteins that enable the nematode to survive exposure to desiccation. In the desiccation dataset there is a significantly higher expression for genes involved RNA processing, but not for genes encoding proteins required for RNA translation. The reason for this could be that protein synthesis does not occur during desiccation stress but instead the stressed nematode produces transcripts to be translated upon recovery from desiccation. This hypothesis is supported by the fact that genes associated with translation have increased expression in the recovery dataset. Further evidence, alluding to the fact that transcription is important during desiccation is that more genes are significantly up-regulated in the desiccation dataset than in the preconditioning or recovery datasets.

5.3.3. Molecular chaperones

In our RNA-seq study, there is a major response of chaperone pathways to desiccation at the transcriptomic level, with many HSP transcripts being significantly up-regulated (Figure 5.24). The general trend for the expression of the HSP transcripts in this study was for a peak in transcription to occur during preconditioning and recovery. The higher expression of HSP genes leads to the enrichment of GO terms response to stress and response to heat (Table 5.15). The HSP-70 family is the largest group of heat shock proteins, involved in inhibiting protein aggregation as well as promoting folding in newly synthesized and denatured proteins (Jonsson and Schill, 2007). In the RNA-seq analysis,

three transcripts which encode HSP-70 proteins were up-regulated which are *hsp-1*, *hsp-6* and *hsp-70* in response to all three conditions (Figure 5.24). The DAVID bioinformatics tool, which was used to cluster functionally related annotations into groups, clustered 9 genes related to HSP-70 in the preconditioning dataset (Table 5.24). The DAVID analysis indicates that the up-regulated genes in the preconditioning dataset are enriched for annotations relating to HSP-70 as compared to the desiccation dataset. In plant studies, that hydrogen peroxide can induce *hsp-70* expression which in turn significantly increases the expression of glutathione peroxidase, glutathione reductase, catalase, and superoxide dismutase (Hu *et al.*, 2010). This trend was also seen in our data set with the increased transcription of antioxidant genes (Section 5.2.9.7).

As well as HSP-70, small heat shock proteins (sHSPs) seem to play an important role in the response to desiccation. In our RNA-seq analysis, the sHSPs *hsp-12.6* and *hsp-43* (a member of the HSP-20 family) were up-regulated in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery. Mohri-Shiomi and Garsin (2008) have recently shown a reduction of *hsp-12.6* expression by RNAi resulted in a statistically significant increase in the number of protein aggregates in the *C. elegans* intestine caused by infection (Mohri-Shiomi and Garsin, 2008). Therefore, HSP-12.6 may protect proteins against aggregation during desiccation in *C. elegans* dauers.

As well as HSPs, there are a number of up-regulated genes encoding chaperonins that also prevent protein aggregation. The increased expression of these chaperonins has resulted in significantly up-regulation in the prefoldin and TriC/CCT chaperonin pathways in the preconditioning, desiccation and recovery datasets (Table 5.16). The up-regulation of the eukaryotic TRiC chaperonin complex is essential for cell survival, employing ATP hydrolysis to fold 10% of the proteome including many essential proteins, such as the cytoskeletal components actin and tubulin (Yam *et al.*, 2008).

In addition to transcripts encoding molecular chaperones and chaperonins other transcripts which were up-regulated in the *C. elegans* dauer larvae encode proteins that are believed to stabilize and protect other proteins from denaturation. These include members of the LEA and DJ-1 protein families. LEA proteins are classified as natively unfolded proteins. These are a group of proteins that are intrinsically unstructured and

constitute an important family of proteins whose members are up-regulated during the cellular response to dehydration and are thus inducible in response to freezing, saline conditions, or drying (Ingram and Bartels, 1996; Tunnacliffe and Wise, 2007). Mutations in the human *dj1* gene give rise to autosomal recessive Parkinson's disease (Bonifati *et al.*, 2003). DJ1 is a member of a large, functionally diverse protein family (Lucas and Marin, 2007), with roles in protection from oxidative stress and protein denaturation (Shendelman *et al.*, 2004).

In this study the *lea-1* gene was up-regulated over 29 fold in response to preconditioning and 21 fold in response to desiccation in *C. elegans* dauer larvae (Figure 5.25). This correlates well with previous studies which showed that *lea-1* transcripts increased by 21 fold in response desiccation in *C. elegans* (Gal *et al.*, 2004). LEA proteins are multifunctional proteins that are known to have a role in desiccation tolerance (Gal *et al.*, 2004; Tunnacliffe and Wise, 2007; Hand *et al.*, 2011).

The *C. elegans* genome contains two homologs of the human *dj-1* gene viz. *djr-1.1* (B0432.2) and *djr-1.2* (C49G7.11) (*djr* = DJ-1 related) (Castro *et al.*, 2010). The *djr-1.2* gene was substantially up-regulated in response to preconditioning and desiccation in *C. elegans* dauer larvae (Figure 5.25), but transcripts of the *djr-1.1* gene were not differentially expressed in any of the conditions. Knockout studies in *C. elegans* have revealed that *djr-1.1* and *djr-1.2* have a role in ubiquitin-proteasomal systems, immunity and response to oxidative stress (Ved *et al.*, 2005). *C. elegans* DJR-1.1 and DJR-1.2 have recently been shown to have glyoxalase activity, with DJR-1.2 specifically protecting dopaminergic neurons (Lee *et al.*, 2012). Increased gene and protein expression of *djr-1.2* has been observed in the dauer larvae, relative to other life stages, in *C. elegans***.** The up-regulation of *djr-1.1* was also observed in the *C. elegans* dauer larvae stage, although protein expression remained unchanged (Lee *et al.*, 2013). Thus the up-regulation of *djr-1.2* in unstressed *C. elegans* dauer larvae and in desiccated dauer larvae is consistent with its potential role as an antioxidant and in the prevention of protein aggregation.

5.3.4. Compatible solutes

In many species of plants, animals and microorganisms dehydration leads to the accumulation of a variety of soluble, low molecular mass water-replacement molecules

that are non-toxic and do not interfere with cellular metabolism such as trehalose (Yancey, 2005). The *C. elegans* genome encodes two trehalose-6-phosphate (*tps*) genes: *tps-1* (ZK430.3) and *tps-2* (F19H8.1). In our RNA-seq study, we found both *tps-1* and *tps-2* to have increased expression in response to desiccation and recovery while *tps-2* was also up-regulated in response to preconditioning (Figure 5.25).

In our RNA-seq study two members of the aquaporin (*aqp*) gene family, *aqp-1* and *aqp-11*, were specifically up-regulated in recovering nematodes as compared to desiccated nematodes (Table 5.20). Aquaporin channels facilitate transport of water, glycerol, and other small solutes across cell membranes. Aquaporins (AQPs) can be divided into three subfamilies based on their primary AA sequences: classical AQPs; aquaglyceroporins and unorthodox AQPs (Ishibashi *et al.*, 2011). Classical AQPs and aquaglyceroporins function respectively, as water and glycerol channels. The best characterized unorthodox AQP HsAQP11 was also found to be a highly efficient water transporter (Yakata *et al.*, 2007). The *C. elegans* genome contains 11 *aqp* genes, encoding 3 classical AQPs 5 aquaglyceroporins and 3 unorthdox AQPs (Huang *et al.*, 2007; Ishibashi *et al.*, 2011). Studies carried out in *Xenopus* oocytes have increased the understanding of the function of specific aquaporins. Expression of the *C. elegans* genes *aqp-1, aqp-3* and *aqp-7* in *Xenopus* oocytes increased the glycerol permeability of these oocytes three to sevenfold, while expression of *aqp-2, aqp-3, aqp-4, aqp-6 and aqp-7* increased oocyte water permeability (Huang *et al.*, 2007). In our RNA-seq study, the transcripts *aqp-1* and *aqp-11* were up-regulated 5-fold and 2-fold, respectively, in recovering nematodes. Functional characterisation has not been carried out on *Ce-aqp-11*, (which is classed as an unorthodox AQP), but its human ortholog AQP-11 functions as a water channel (Yakata *et al.*, 2007). AQP-1 is expressed on the intestinal basolateral membrane (Huang *et al.*, 2007), but the expression pattern of AQP-11 has not been determined. The up-regulation of transcripts encoding a putative water channel in recovering dauer larvae reflects the importance of water transport in recovering dauer larvae. The up-regulation of the glycerol channel gene *aqp-1* suggests that glycerol efflux from cells may be required during recovery. Glycerol accumulation in desiccated dauer larvae may result as a by-product of lipid metabolism during gluconeogenesis.

The sugar *myo*-inositol has been reported to accumulate in plants during desiccation (Oliver *et al.*, 2011) and may act as a compatible solute. Additionally, *myo*-inositol is an effective antioxidant that can neutralise hydroxyl radicals that are known to accumulate during desiccation (Kranner and Birtic, 2005; Franca *et al.*, 2007). *myo*-inositol is synthesized from glucose-6-phosphate (G-6-P) in two steps. First, G-6-P is isomerised by an inositol-3-phosphate synthase enzyme (called ISYNA1) to *myo*-inositol 1-phosphate, which is then dephosphorylated by an inositol monophosphatase enzyme (called IMPase 1) to give free *myo*-inositol. In *C. elegans*, *inos-1* (INOsitol-3-phosphate Synthase) encodes the *C. elegans myo*-inositol-1-phosphate which is predicted to function in de nova inositol biosynthesis (Torabinejad and Gillaspy, 2006). In our RNA-seq study, *inos-1* was up-regulated in *C. elegans* dauer larvae in response to preconditioning and recovery.

5.3.5. Defence against pathogens

In *C. elegans* the infection of barrier epithelia results in the up-regulation of genes encoding antimicrobial peptides (AMPs) such as fungus induced proteins and caenacin proteins (Pujol *et al.*, 2008). A number of individual genes which encode fungus induced proteins and caenacin proteins were up-regulated in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae (Table 5.17). GO enrichment analysis shows that the terms Defence Response, Defence Response to Fungus and Immune Response are up-regulated in dauer larvae recovering from desiccation (Table 5.18). Many AMPs act by disrupting microbial cell membranes. The up-regulation of transcripts that encode AMPs could possibly be a mechanism to prevent fungal infection. In the desiccated state, *C. elegans* dauer larvae could be more prone fungal infection due to increased susceptibility to damage in its epithelial barriers. The extent of involvement of pathogenesis-related proteins in the protection of anhydrobiotic animals during desiccation is unknown, but in plants several pathogenesis-related genes are up-regulated in response to abiotic stresses, including desiccation (Huang *et al.*, 2008; Gao *et al.*, 2013).

5.3.6. Cellular organisation

The GO enrichment analysis shows there is a clear association in the desiccation dataset with the synthesis of transcripts associated with cytoskeleton and membrane organisation (Table 5.9). The Reactome analysis shows that pathways associated with the folding of actin and tubulin are up-regulated in response to preconditioning and desiccation (Table 5.16) and the DAVID bioinformatics analysis also indicates that reorganisation of the cytoskeleton may be important to survive desiccation. The up-regulated transcripts for the desiccation dataset are particularly enriched with functional annotations relating to the cytoskeleton and microtubules. The top annotation cluster for the up-regulated genes in the desiccation dataset relates to cytoskeleton reorganisation with an enrichment value of 24 and up to 70 up-regulated genes (Table 5.25). The up-regulated genes for the preconditioning time point were also enriched for cytoskeleton and microtubule terms but not to the same extent as in the desiccation dataset (Table 5.24). This preconditioning annotation cluster has an enrichment value of 1.55. A substantial amount of evidence shows that under normal conditions, microtubules constantly remodel their arrangement in order to adjust cell growth (De Forges *et al.*, 2012). Studies have shown that microtubule organisation in radicular cells of maize roots is responsive to osmotic stress, and osmotic stress altered the dynamic balance of α-tubulin proteins from depolymerisation to polymerization (Lue *et al.*, 2007). Slow drying elicits controlled dismantling of the cytoskeleton in protonemal cells of the moss *Physcomitrella patens* (Pressel *et al.*, 2006) and up-regulation of actin genes was also detected in desiccated midge *Belgica antarctica* (Teets *et al.*, 2012). Cytoskeletal reorganisation is also likely to be important in *C. elegans* dauer larvae during desiccation when there is dramatic change in the morphology of the worm.

5.3.7. Antioxidants

During preconditioning, desiccation and recovery in *C. elegans* dauers there is an up-regulation of several transcripts which encode proteins capable of detoxifying ROS (Figure 5.23). The superoxide dismutase transcripts (*sod-3*, *sod-4*, *sod-5*) which are up-regulated in response to preconditioning, desiccation and recovery encode metalloenzymes responsible for quenching the potentially deleterious effects of superoxide free radicals by converting them to hydrogen peroxide (Bowler *et al.*, 1992). Transcripts encoding the enzymes catalase and glutathione peroxidase were also up-

regulated in response to preconditioning, desiccation and recovery (Figure 5.23). The expression of catalase and glutathione peroxidase predominately peaks during preconditioning and recovery. These enzymes deactivate hydrogen peroxide, another potent oxidising agent. Glutathione S-transferases (GSTs) are a diverse super-family of multifunctional proteins that play prominent roles in detoxification in nematodes (Lindblom and Dodd, 2006). Transcripts encoding GSTs were up-regulated in *C. elegans* dauer larvae in response to the three experimental conditions. A number of transcripts corresponding to cadmium-responsive genes were up-regulated during preconditioning, desiccation and recovery. There are six cadmium responsive genes in *C. elegans*, two of which (*cdr-3* and *cdr-4*) are up-regulated in preconditioning, desiccation and recovery (Table 5.17). Cadmium exposure is correlated with the production of ROS and lipid peroxidation (Dong *et al.*, 2005). Furthermore, previous studies in mammalian cell lines have found that exposure to cadmium can reduce glutathione levels that can result in increased levels of oxidative stress (Ochi *et al.*, 1987). The build up of ROS species which occurs during desiccation could potentially cause damage to proteins, lipids, DNA and other macromolecules. Therefore, the increased expression of these antioxidant genes may play an important protective role during desiccation in *C. elegans* dauer larvae.

5.3.8. DNA damage repair

GO terms and Reactome pathways relating to DNA damage response and DNA repair are specifically up-regulated in the desiccation dataset as described in section 5.2.9.5. In the desiccated dataset, there was an up-regulation of 37 genes that are associated with the GO "DNA repair" category. The DAVID bioinformatics tool also indicated that transcripts up-regulated in response to desiccation were significantly enriched with terms relating to DNA repair (see annotation cluster 17 in Table 5.25). These up-regulated pathways include DNA repair and nucleotide excision repair (NER) (Table 5.11). The majority of the DNA repair genes up-regulated in the desiccation dataset are involved in NER. NER removes bulky DNA lesions that distort the double helix. For the NER Reactome pathway, thirteen out of a possible thirty-five genes were found to up-regulated during desiccation. Among the up-regulated genes were those encoding proteins involved in DNA damage recognition such as xeroderma pigmentosum group C (XPC) and DNA damage binding protein 1 (DDB1). The XPC complex mediates the recruitment of the transcription initiation factor TFIIH to the damaged DNA, and this promotes opening of

the DNA in an ATP-dependent manner. The unwinding of the DNA requires the helicase activity of XPD and the ATPase activity of XPB. Also up-regulated were the genes encoding the ERCC1-XPF complex, an endonuclease which cuts 5' to the damaged DNA and the gene encoding the Cockayne syndrome B protein (CSB*)* which is required for coupling DNA excision repair to transcription.

Although NER appears to be the most common DNA repair mechanism in the desiccated dataset, there are also genes associated with base excision repair (BER) and double strand break repair up-regulated in this dataset. The *ung-1* (ung=Uracil DNA N-Glycosylase) gene, which is up-regulated in response to desiccation, encodes the only known uracil-DNA glycosylase in *C. elegans* and is involved in base excision repair. BER is the predominant mechanism that handles single-base DNA damage induced by ROS.

Previous studies have found that anhydrobiotic tardigrades in a desiccated state are unable to repair damaged macromolecules or proteins since water is absent and their metabolism arrested (Neumann *et al.*, 2009). Therefore, it is likely such accumulated damage is repaired upon rehydration. In the desiccation dataset the most DNA repair transcripts are up-regulated; this could be in preparation for the synthesis of proteins during recovery from desiccation.

In desiccated tardigrades, it has been shown that DNA damage occurs and accumulates with time (Rebecchi *et al.*, 2009). DNA is a biologically important target of reactive oxygen species (ROS) (Gros *et al.*, 2002). The up-regulation of transcripts encoding antioxidants was observed in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery and was discussed in section 5.3.7.

GO terms related to chromosome and nuclear pore complex (NPC) components were also significantly up-regulated in response to desiccation (Table 5.10). Several transcripts which encode NPC proteins are up-regulated (Figure 5.19). NPC proteins function as gatekeepers of the nucleus, performing the essential cellular role of mediating the exchange of molecules between the nucleoplasm and the cytoplasm. Through the SUMO (small ubiquitin-related modifiers) pathway, NPCs are indirectly involved in the regulation of numerous cellular processes such as gene transcription, DNA replication,

DNA damage and repair, chromosome segregation, genome stability, cell death and senescence (D'angelo and Hetzer, 2008). Thus, NPCs may play a role in the exchange of molecules between the nucleoplasm and the cytoplasm to repair damaged DNA and other nuclear cytoplasmic molecules.

5.3.9. Removal of damaged proteins

A number of GO terms and Reactome pathways associated with the ubiquitin-proteasome system for the removal of damaged proteins were significantly up-regulated in the *C. elegans* dauer larvae in response to preconditioning and desiccation as outlined in Table 5.13 and 5.14. The DAVID bioinformatics functional annotation clustering tool shows that there are more genes related to the ubiquitin-proteasome system up-regulated in response to desiccation than preconditioning (Tables 5.24 and 5.25). Several autophagy pathway genes show increased expression in response to preconditioning, desiccation and recovery (Figure 5.22).

Some recent studies have illustrated that the removal and recycling of unfolded proteins and protein aggregates is important for the survival of desiccation. A whole genome RNA interference (RNAi) screen in *C. elegans* identified 40 genes that are essential for survival during acute hypertonic stress (Choe and Strange, 2008). Half of these genes encode proteins that function to detect, transport, and degrade damaged proteins (Choe and Strange, 2008). In the yeast *Saccharomyces cerevisiae* a microarray study of desiccation responsive genes, revealed two of the four most over represented GO terms were associated with autophagy (Ratnakumar *et al.*, 2011). Similarly in RNA-seq study of the dehydration tolerant midge *Belgica antarctica*, Teets and Denlinger (2013) found that that cell recycling pathways are essential for extreme dehydration tolerance, with autophagy serving as the focal point and that genes involved in the ubiquitin-mediated proteasomal pathway were also enriched among the dehydration-up-regulated genes (Teets and Denlinger, 2013).

5.3.10. Down-regulated genes

Fifty four percent of the 5,321 down regulated genes were common to preconditioning, desiccation and recovery datasets, as shown in Figure 5.11 (b). The down-regulated Reactome pathways which were common to all three treatments include biological

oxidation reactions, extracellular matrix organisation, peroxisomal lipid metabolism and xenobiotics metabolism. The biological oxidation reactions and xenobiotics pathways were significantly down-regulated mainly because of transcripts corresponding to 31 cytochrome P450 genes having lower expression. The peroxisomal lipid metabolism pathway was down-regulated in response to preconditioning, desiccation and recovery compared to the control. However, this pathway was up-regulated when the recovery dataset was compared to the desiccation dataset. This suggests that metabolism has slowed down during desiccation and is now returning to normal in recovery by breaking down lipid stores to produce energy. The down-regulation of the extracellular matrix organisation pathways is intrinsically associated with the decrease in expression of genes encoding collagen. In the extracellular matrix reorganisation pathway, eleven out of the nineteen down-regulated genes are associated with collagen.

5.3.11 Conclusion

Desiccation tolerance in *C. elegans* dauer larvae is a complex multifaceted process which involves the transcription of upwards of 4,667 genes. In addition to the up-regulated genes which were involved in signal transduction, cellular protection and reorganization, an additional 3,907 genes were down-regulated. These latter genes were mainly involved in biological oxidation reactions, extracellular matrix organisation, peroxisomal lipid metabolism and xenobiotics metabolism. An overview of expression of genes and processes which were up-regulated in response to desiccation and subsequent rehydration in dauer larvae is presented in Figure 5.27.

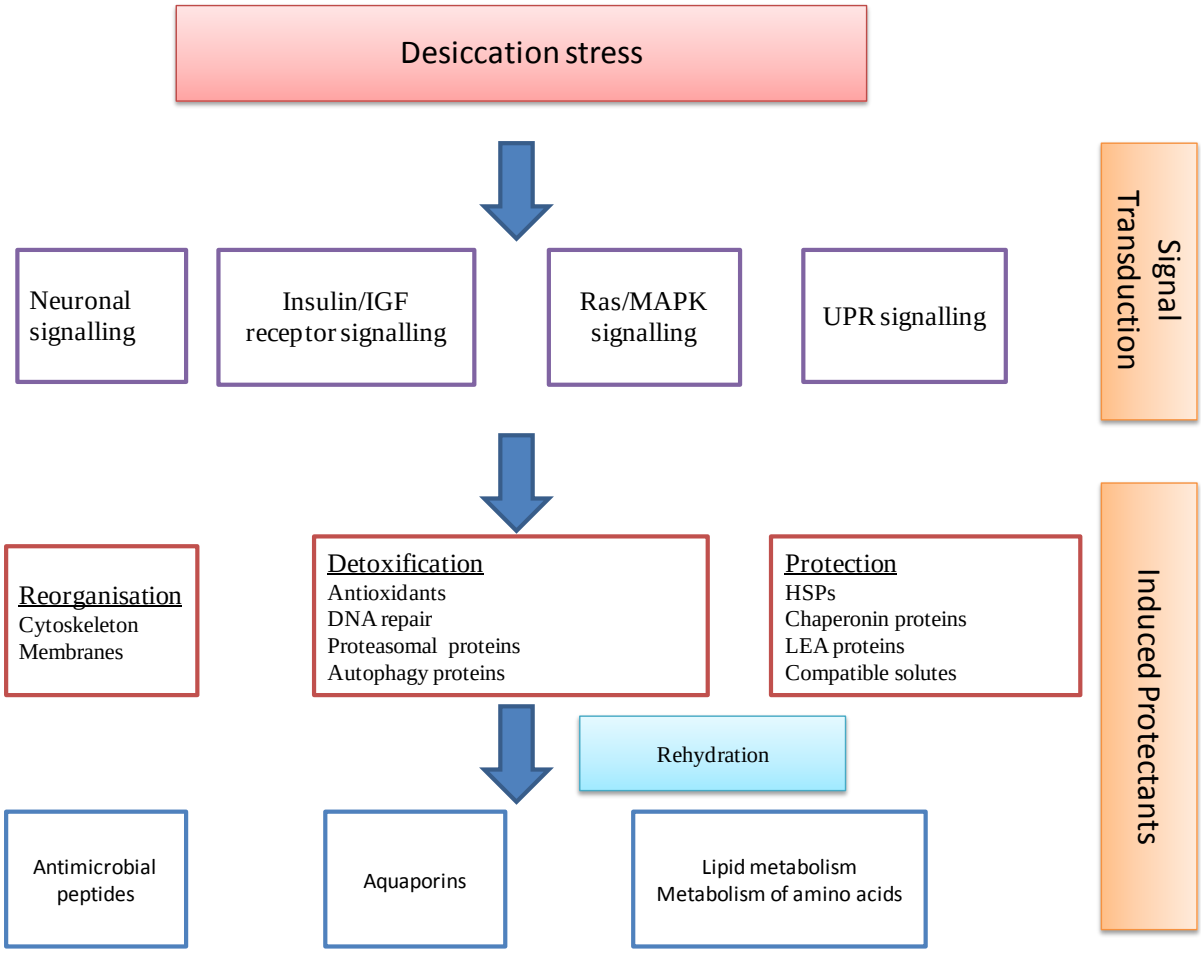


Figure 5.27 Overview of expression of genes and processes which were up-regulated in response to desiccation and subsequent rehydration in *C. elegans* dauer larvae.

Table 5.24 DAVID bioinformatics output showing significantly enriched annotation clusters for up-regulated genes in response to preconditioning (Huang *et al.*, 2009).

Annotation Cluster	Enrichment Score	Cluster summary	Count	*P* Value	Benjamini-Hochberg test
1	21.08	Ribosome/translation	77	3.34E-29	7.97E-27
2	16.01	Embryonic and larval development	616	5.30E-20	3.70E-17
3	9.87	Regulation of growth rate	405	3.81E-11	7.60E-09
4	6.17	Determination of adult life span/aging	77	6.78E-07	7.88E-05
5	5.56	Reproductive developmental process	208	4.04E-07	5.13E-05
6	5.23	Nucleotide-binding	37	1.24E-06	0.002505
7	3.97	Protein biosynthesis	32	3.84E-09	2.13E-07
8	3.75	Nucleotide-binding	153	2.09E-10	1.74E-08
9	3.67	Chaperonin Cpn60/TCP-1/protein folding	8	1.77E-05	0.011808
10	3.22	Mitochondrion	50	2.05E-05	9.82E-04
11	2.94	Heat shock protein 70	9	1.10E-04	0.043364
12	2.72	Proteasome complex	14	1.72E-04	0.004569
13	2.62	Generation of precursor metabolites and energy	42	6.36E-05	0.005536
14	2.52	Intracellular organelle lumen/mitochondrial matrix	36	1.55E-04	0.004625
15	2.38	Mitochondrial matrix	50	2.05E-05	9.82E-04
16	2.15	Ubiquitin	7	0.006254	0.999997
17	2.04	Translational elongation	8	0.00267	0.170215
18	1.80	ATP binding site	39	4.39E-05	0.04885
19	1.67	Glycolysis	12	3.12E-04	0.007392
20	1.55	Cytoskeleton/microtubule	51	8.06E-06	4.82E-04
21	1.50	Ubiquitin-conjugating enzyme	12	0.004662	0.575713
22	1.40	Helix-loop-helix DNA-binding	11	0.034547	0.934702
23	1.38	Cell death	19	0.01408	0.451106
24	1.38	Cell cycle switching	10	0.041619	0.653443
25	1.32	K homology	12	0.006205	0.648929
26	1.31	Zinc finger	4	0.031277	0.92308
27	1.30	Proteasome component	7	0.022098	0.906829
28	1.30	Aminopeptidase activity	7	0.015276	0.412043

Table 5.25 DAVID bioinformatics output showing significantly enriched annotation clusters for up-regulated genes in response to desiccation.

Annotation Cluster	Enrichment Score	Cluster summary	Count	*P* Value	Benjamini-Hochberg test
1	24.56	Non-membrane-bounded organelle/cytoskeleton	156	2.45E-30	7.13E-28
2	23.54	Nucleotide-binding	240	3.44E-40	1.13E-37
3	22.82	Reproductive developmental	331	6.68E-30	3.56E-27
4	16.60	Cell cycle	170	5.81E-30	4.65E-27
5	16.52	Nematode larval development/growth	547	4.98E-19	7.97E-17
6	14.60	RNA recognition motif/Nucleotide-binding	59	1.23E-17	9.17E-15
7	11.83	Regulation of growth	569	1.98E-13	2.12E-11
8	11.48	Helicase activity	47	5.9E-19	1.32E-15
9	10.05	Mitotic cell cycle/microtubule cytoskeleton organization	76	3.76E-15	4.65E-13
10	8.08	Organelle fission/nuclear division	45	1.56E-13	7.35E-12
11	7.49	Nucleotide phosphate-binding region:ATP/protein kinase	102	1.68E-21	2.56E-18
12	7.40	Zinc finger, PHD-type	20	1.77E-10	3.59E-08
13	6.36	Nuclear lumen	33	3.64E-08	1.76E-06
14	5.92	Cell cycle switching	19	1.18E-06	3.94E-05
15	5.72	Morphogenesis of an epithelium/tissue	115	1.14E-06	3.88E-05
16	5.60	Helicase C-terminal/DNA/RNA helicase	23	1.58E-09	4.83E-07
17	5.23	Response to DNA damage stimulus/DNA repair	73	8.12E-12	5E-10
18	5.02	Cytoskeleton/microtubule	70	3.44E-15	5.01E-13
19	4.81	Embryonic morphogenesis	58	1.95E-06	6.11E-05
20	4.73	Chromatin binding/chromatin organization	45	1.8E-13	1.75E-11
21	4.68	Pleckstrin homology	31	2.85E-06	0.000318
22	4.38	RNA processing	37	7.41E-07	2.58E-05
23	4.26	Organelle localization	43	6.13E-08	2.58E-06
24	4.19	Sexual reproduction	94	1.15E-11	6.83E-10
25	4.07	Regulation of cell cycle	34	5.06E-07	1.84E-05
26	4.02	Negative regulation of vulval development	46	3.62E-05	0.000982
27	3.74	Atpase, AAA+ type, core	41	2.99E-06	0.000318
28	3.49	Zinc finger, C2H2-like	64	9.18E-06	0.000892
29	3.33	DNA-dependent atpase activity	12	0.000147	0.006491
30	3.29	K homology	16	0.000142	0.008562
31	3.18	Chromatin regulation	17	7.47E-05	0.00091
32	3.18	Ubiquitin-associated/translation elongation factor EF1B	9	0.000325	0.017171
33	3.16	Zinc finger, CCHC-type	17	3.1E-05	0.002569
34	2.95	Chaperonin Cpn60/TCP-1/protein folding	9	4.22E-05	0.003247
35	2.94	Regulation of small gtpase mediated signal transduction	39	8.74E-06	0.000245

36	2.63	Bromodomain	11	5.47E-05	0.003942
37	2.63	SANT, DNA-binding	13	2.11E-05	0.001891
38	2.62	Establishment or maintenance of cell polarity	20	0.000395	0.00887
39	2.59	Ubiquitin/proteolysis	35	1.9E-06	3.67E-05
40	2.57	Apoptosis/cell death	22	0.000651	0.013441
41	2.53	RNA splicing	21	0.000918	0.017136
42	2.47	Aging	77	0.003371	0.049239
43	2.46	Regulation of translation	17	0.001715	0.029091
44	2.42	Nucleoplasm/transcription initiation	23	7.67E-06	0.000149
45	2.41	Protein complex biogenesis	31	0.000764	0.015175
46	2.38	Cell division	13	0.000575	0.012039
47	2.35	Nuclear envelope/nuclear pore	14	1.55E-06	4.11E-05
48	2.34	Multicellular organism reproduction/reproductive behaviour	145	2.83E-06	8.53E-05
49	2.31	Cell fate determination	12	0.00148	0.02571
50	2.26	Metal-binding	178	1.51E-06	3.3E-05
51	2.23	WD40 repeat	41	1.92E-05	0.000274
52	2.23	Src homology-3 domain	22	0.001732	0.074642
53	2.12	DNA replication	32	5.98E-07	2.13E-05
54	2.09	Chromosome condensation/structural maintenance of chromosomes protein	9	0.000287	0.006644
55	2.00	Zinc finger	44	0.000674	0.034478
56	1.97	Nuclease/exonuclease activity	11	0.001529	0.014284
57	1.97	Microtubule/centrosome organiziation	11	1.96E-05	0.000317
58	1.86	Cytokinesis	9	0.000833	0.015747
59	1.82	Heat shock protein 70	7	0.007773	0.238728
60	1.74	Negative regulation of signal transduction/ cell communication	9	0.001977	0.032114
61	1.72	Protein biosynthesis	22	0.004485	0.037206
62	1.72	Positive regulation of gene expression	21	0.007894	0.097962
63	1.69	Tetratricopeptide TPR-1	12	0.005728	0.046152
64	1.68	Sex determination	14	0.010348	0.118461
65	1.68	Endocytosis	21	0.000918	0.017136
66	1.64	Vesicle	16	0.000132	0.001596
67	1.62	Short sequence motif: Q motif, Dead motif, zinc finger region	8	0.0005	0.067055
68	1.5	Negative regulation of macromolecule metabolic process/rnai silencing	30	0.001749	0.029357
69	1.58	Reproductive structure development	33	0.021432	0.208811
70	1.58	ATP-dependent DNA helicase activity	9	0.000728	0.02313
71	1.50	Mitochondrion	36	2.7E-05	0.000375
72	1.49	Protein kinase C-like	13	0.008855	0.256929
73	1.45	Carboxyl transferase	4	0.021684	0.462468
74	1.41	Regulation of multicellular organism growth	118	0.00707	0.09099
75	1.41	Kinetochore assembly	5	0.01301	0.140864
76	1.40	Nucleoplasm part	21	1.88E-05	0.000322
77	1.39	Proteasome complex	11	0.006764	0.05487
78	1.38	Glycolysis	12	0.000851	0.008719

79	1.38	Ubiquitin-protein ligase activity	11	0.008446	0.186804
80	1.37	Kinetochore/condensed chromosome	6	0.012698	0.086711
81	1.36	Meiosis I	12	0.000793	0.015364
82	1.36	Glycolysis	12	0.000851	0.008719
83	1.33	Transcription regulator cyclin related	10	0.005779	0.194344
84	1.32	Double-stranded RNA-binding-like	7	0.001983	0.083374
85	1.31	Negative regulation of macromolecule metabolic process	30	0.001749	0.029357
86	1.30	Nucleotide binding	16	0.002294	0.01969
87	1.30	Post-SET zinc-binding region/methyltransferase	14	0.002078	0.085599
88	1.30	Nucleus/DNA binding/transcription	223	1.24E-10	3.7E-09

Table 5.26 DAVID bioinformatics output showing significantly enriched annotation clusters for up-regulated genes in response to recovery versus desiccation.

Annotation Cluster	**Enrichment Score**	**Cluster summary**	**Count**	***P* Value**	**Benjamini -Hochberg test**
1	5.08	Oxidation reduction	35	1.31E-08	6.62E-06
2	3.34	Organic acid biosynthetic process	13	1.33E-07	3.35E-05
3	2.74	Signal peptide	33	1.68E-05	0.003696
4	2.29	Fatty acid metabolic process	7	0.003162	0.147797
5	2.05	Ves allergen/SCP-like extracellular	5	0.002117	0.152383
6	1.99	Lipid-binding	5	0.007298	0.240183
7	1.83	Defense response	3	0.013939	0.340973
8	1.82	Lipid glycosylation	12	5.20E-04	0.057318
9	1.78	Sapb	5	0.002476	0.092143
10	1.72	Phosphate transport/anion transport	4	0.001589	0.085377
11	1.71	Short-chain dehydrogenase/reductase SDR/peptide metabolic process	12	2.92E-04	0.053678
12	1.60	Metabolism of xenobiotics by cytochrome P450/Thioredoxin fold/glutathione transferase	6	0.004332	0.245859

Table 5.27 DAVID bioinformatics output showing significantly enriched annotation clusters for up-regulated genes in response to recovery versus control.

Annotation Cluster	**Enrichment Score**	**Cluster summary**	**Count**	***P* Value**	**Benjamini-Hochberg test**
1	20.07	Ribosome/translation	76	2.57E-29	5.59E-27
2	8.98	Growth/larval development	319	1.93E-12	1.26E-09
3	4.94	Aging	69	1.16E-05	0.001259
4	4.34	Chaperonin Cpn60/TCP-1/protein folding	9	9.04E-06	0.017798
5	3.94	Protein biosynthesis/translational initiation	32	1.12E-09	9.01E-08
6	3.77	Nucleotide-binding	32	6.58E-05	0.032161

7	3.33	Mitochondrion	53	2.99E-06	1.63E-04
8	3.09	Mitochondrion/organelle envelope	53	2.99E-06	1.63E-04
9	3.07	Nucleotide-binding	164	8.78E-07	5.66E-05
10	2.91	Glucose metabolic process/glycolysis	23	5.85E-06	6.94E-04
11	2.77	Genitalia development/sex differentiation	149	0.001017	0.061327
12	2.43	Organelle lumen	34	4.05E-04	0.01253
13	2.36	Molting cycle	59	0.004687	0.174476
14	2.31	Heat shock protein 70	7	0.002744	0.541524
15	2.25	Ubl conjugation pathway/proteolysis	25	0.001765	0.042814
16	2.18	Elongation factor	7	0.004636	0.08426
17	2.07	Ubl conjugation pathway	25	0.001765	0.042814
18	1.58	GO:0006399~trna metabolic process	27	0.001408	0.076884
19	1.45	IPR001993:Mitochondrial substrate carrier	12	0.009056	0.835944
20	1.38	Glucosyltransferase activity/ carbohydrate biosynthetic process	4	0.040858	0.667008
21	1.34	Iron-sulfur cluster binding	12	0.057193	0.737711

6 Chapter VI: Label Free Quantification based proteomic analysis of desiccation survival in *Caenorhabditis elegans* dauer larvae

6.1 Introduction

Mass spectrometry (MS) based proteomics has fundamentally changed the way in which biological systems are interrogated because of its ability to measure thousands of proteins and posttranslational modifications in parallel (Bantscheff *et al.*, 2012). The development of low cost, high throughput DNA sequencing technologies, outlined in the previous chapters, enables proteomic research on practically any species. In parallel with these developments in DNA sequencing, the field of MS-based proteomics is changing rapidly with the development of new and more sensitive instrumentation almost on a yearly basis (Schulze and Usadel, 2010). These modern liquid chromatography mass spectrometry (LC-MS) systems are capable of identifying and quantifying 2,000-5,000 proteins from a given proteome in a single run (Brunner *et al.*, 2007; Baerenfaller *et al.*, 2008; De Godoy *et al.*, 2008; Beck *et al.*, 2011). The performance of these mass spectrometers in relation to sensitivity, speed, mass accuracy, and resolution is continuing to rise (Brunner *et al.*, 2007; Baerenfaller *et al.*, 2008; De Godoy *et al.*, 2008; Beck *et al.*, 2011).

6.2 Results

6.2.1 Selection of desiccation treatments, protein extraction and data analysis

The treatment conditions selected for the proteomic analysis were identical to those used for the *C. elegans* RNA-seq transcriptomic experiment as described in Chapter V (Figure 5.5), so the two datasets could be compared directly. The four conditions selected were a control (*C. elegans daf-2* dauer larvae grown at 25^{o}C) and three treatments: preconditioning (98% RH for 48 h), desiccation (98% RH for 96 h followed by 32.5% RH for 24 h) and recovery from desiccation (98% RH for 96 h followed by 32.5% RH for 24 h and 30 min recovery in sterile water). Details of sample preparation, protein and peptide separation, and MS can be found in Sections 2.4.4 and 2.4.5.

Protein identification and label free quantitative (LFQ) analysis were conducted using the MaxQuant quantitative proteomics software package supported by the Andromeda database search engine (Cox and Mann, 2011; Cox *et al.*, 2011) to correlate MS/MS data

against the Caenorhabditis_elegans.WBcel215.70 protein database**.** Proteins were considered identified when more than one peptide from each parent protein was observed. Differentially abundant proteins among experimental groups were determined by label-free quantification (LFQ) using MaxQuant and Perseus a software package designed to perform all downstream bioinformatics and statistics on MaxQuant output tables (Cox and Mann, 2011). Details of the data analysis methods can be found in Section 2.4.5.

6.2.2 Hierarchical clustering and heat maps of differentially expressed proteins

Hierarchical clustering and heat maps of qualitative and statistically significant protein abundances identified between the various conditions based on mean and log2 transformed LFQ peptide/protein intensities was carried out as shown in Figures S6.2-6.9 (Supplementary data *C. elegans* LFQ heat maps).

6.2.3 Differentially expressed proteins

Further analyses were performed on statistically differentially expressed (ANOVA, $p>0.05$) and qualitatively differentially expressed proteins (Section 2.4.5). There were 721 different *C. elegans daf-2* dauer proteins detected across all the samples, with a total of 114, 148 and 165 proteins differentially expressed in response to preconditioning, desiccation and recovery respectively. The identities of these differentially expressed proteins are displayed in the supplementary file "Supplementary data *C. elegans* LFQ". The following information is displayed in this supplementary file: the function of the protein, a description of the protein, the gene ID, the protein ID, the LFQ intensity value for the control obtained from the orbitrap mass spectrometer, the LFQ intensity value for the treatment obtained from the orbitrap mass spectrometer, the number of peptides on which the intensity value was based, the number of peptides that were unique, the Posterior error probability (PEP) which is calculated using Bayesian statistics as the probability of a false hit using the peptide identification score and length of the peptide.

The *C. elegans* proteins were identified from BLAST searches against the NCBI database in combination with searches of the Wormbase database (www.wormbase.org). Gene Ontology terms were obtained for the differentially expressed proteins using the Blast2GO program (Conesa *et al.*, 2005). In Figure 6.1, a Venn diagram displays (i) the

number of *C. elegans* proteins up-regulated in response to preconditioning, desiccation and recovery when compared to the control nematodes and (ii) the number of proteins down-regulated in response to preconditioning, desiccation and recovery when compared to the control. While there were differences in protein expression patterns between the experimental conditions, 54 proteins were up-regulated in response to all three conditions-preconditioning, desiccation and recovery. These include the small heat shock protein HSP-12.6, the antioxidant CTL-1, the proteasome regulatory particle RPN-11 and the cytoskeleton related proteins FLN-1 (filamin), GSNL-1 (gelsolin) and TBA-2 (α-tubulin). A large number of proteins involved in metabolism and translation were also up-regulated in response to preconditioning, desiccation and recovery in *C. elegans daf-2* dauer larvae.

Table 6.1 Number of proteins up-regulated and down-regulated in response to preconditioning, desiccation and recovery in *C. elegans daf-2* dauer larvae. The data for the different conditions are compared to control unstressed larvae unless otherwise stated. In this proteomic analysis a total of 721 *C. elegans* proteins were detected.

	Preconditioning	**Desiccation**	**Recovery**	**Recovery vs. desiccation**	**Preconditioning vs. desiccation**
Up-regulated	94	80	104	61	12
Down-regulated	20	68	61	34	77
Total	114	148	165	95	89

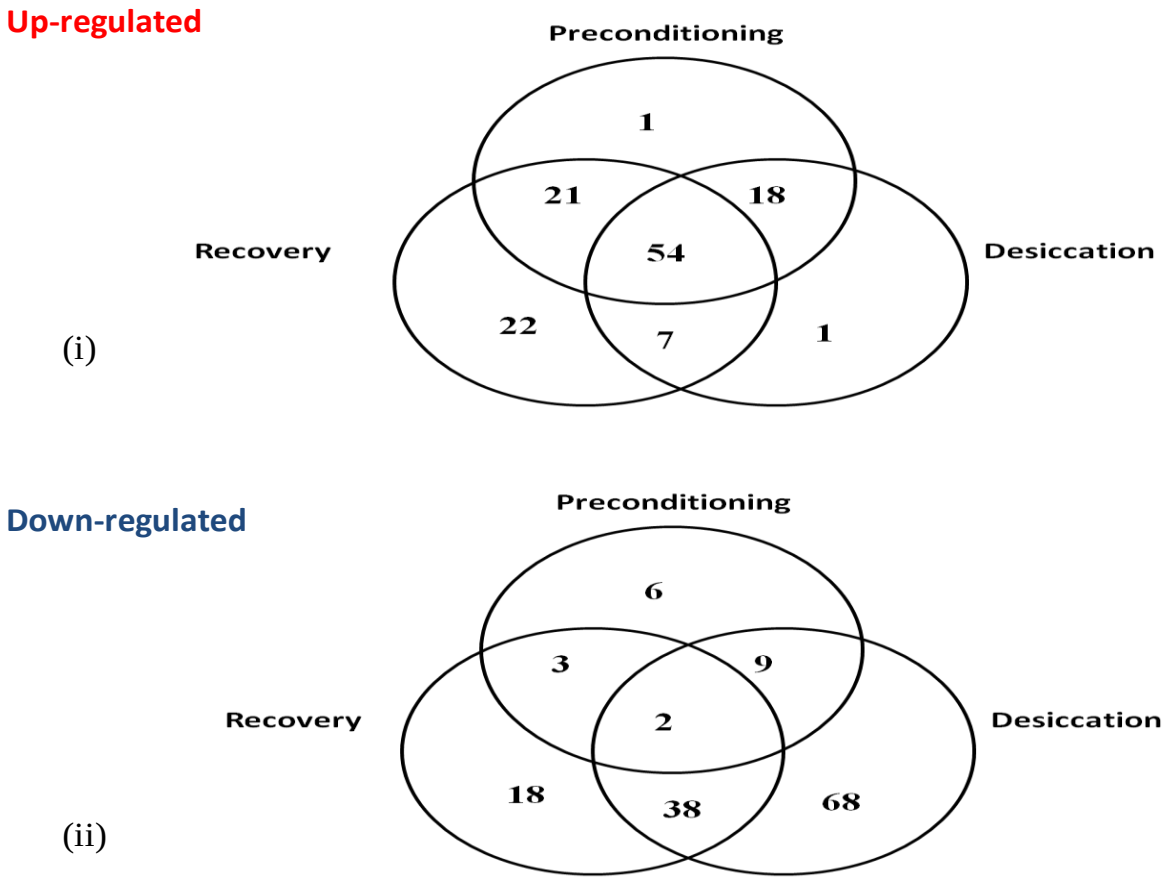


Figure 6.1 Venn diagram showing (i) the number of proteins up-regulated in response to preconditioning, desiccation and recovery in dauer larvae of *C. elegans* when compared to the control larvae and (ii) the number of proteins down-regulated in response to preconditioning, desiccation and recovery when compared to the control larvae.

6.2.4 Overview of the proteins up-regulated in response to desiccation-related stress in *C. elegans daf-2* dauer larvae

In this proteomic analysis a total of 721 proteins were detected of which 144 were up-regulated and 124 were down-regulated (Table 6.1 and Figure 6.1) in response to desiccation and/or recovery from desiccation in *C. elegans* dauer larvae. A table listing all the proteins which were differentially expressed in response to desiccation-related stress is presented in the supplementary CD appended to this thesis (Supplementary data *C. elegans* LFQ). Additionally, a table showing all proteins which were detected in each of the treatments is also included in this supplementary CD (Supplementary data *C. elegans* LFQ total proteins detected).

The identities of the up-regulated proteins are summarised in this section, while the biological significance of these up-regulated proteins is discussed in section 6.3. The main pathways and processes implicated by this differential proteomic analysis in the response of *C. elegans* dauer larvae to desiccation were as follows: signal transduction, translation, metabolism, cellular organisation, DNA packaging and repair, the removal of damaged proteins, antioxidants and stress induced proteins. In addition to the processes described in this section a number of individual proteins in other pathways were also up-regulated in response to desiccation or during recovery from desiccation, but due to space constraints it is not possible to present these data in this section.

6.2.4.1 Signal transduction and signalling

A number of up-regulated proteins in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae map to GO terms associated with Signalling and Neuron development (Table 6.2). More proteins associated with Signalling were up-regulated in response to recovery than in any other treatments; two of these proteins UNC-32 and UNC-89 belong to the Cell surface receptor linked signalling pathway – of these UNC-89 was also up-regulated in response to preconditioning. HMG-1.2 (hmg=high-motility group – a DNA binding protein) was also up-regulated in response to preconditioning and desiccation and is involved in the Wnt Receptor signalling pathway.

6.2.4.2 RNA processes

Proteins associated with RNA processes which were up-regulated in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae are shown in Table 6.3. The biological significance of these proteins is further discussed in Section 6.3.2.

6.2.4.3 Translation

Proteins which were up-regulated in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae that map to GO terms associated with translation are shown in Table 6.4. This GO analysis reveals that the majority (>20%) of proteins up-regulated in the preconditioning and recovery datasets are involved in translation. Furthermore, there are more ribosomal proteins up-regulated in the preconditioning and recovery datasets compared to desiccation datasets (Table 6.5).

Proteins associated with the ribosome that are up-regulated in response to (i) desiccation and (ii) recovery are shown in the diagram in Figure 6.2. This KEGG pathway shows that 23 up-regulated proteins map to the ribosome in the recovery dataset while 18 up-regulated proteins map to the ribosome in the desiccation dataset. The change in average LFQ (label free quantification) intensities of up-regulated ribosomal proteins in response to preconditioning, desiccation and recovery compared to the control is shown in Figure 6.3. This protein expression graph follows the same trend as the GO analysis with ribosomal protein expression peaking in response to the preconditioning and recovery treatments.

6.2.4.4 Metabolism

There were a number of up-regulated proteins in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae that map to GO terms associated with metabolism and the cell cycle (Table 6.6). The up-regulated proteins associated with metabolism which contributed to these GO terms in response to preconditioning, desiccation and recovery are shown in Table 6.7. The KEGG pathways of metabolism showing proteins that are up-regulated in response to precondition, desiccation and recovery are shown in Figures 6.4, 6.5 and 6.6 respectively.

C. elegans dauer larvae are characterised by their low levels of oxidative metabolism compared to other developmental stages in its life cycle (Burnell *et al.*, 2005). The data presented in Table 6.6 show that dauer larvae recruit proteins required for glycolysis and oxidative metabolism in response to desiccation stress and during recovery from desiccation. Among the proteins for intermediary metabolism which were up-regulated were the following: phosphoglycerate kinase, a crucial enzyme in glycolysis and gluconeogenesis; pyruvate kinase, which catalyzes the last step of glycolysis generating pyruvate, which is then converted to acetyl CoA by pyruvate dehydrogenase linking glycolysis to Krebs Cycle. The Krebs Cycle enzyme fumarase was up-regulated in response to all three treatments and citrate synthase, the key regulatory enzyme of the cycle was up-regulated in recovering nematodes. Several components of the mitochondrial electron transport chain were also up-regulated in the treated nematodes, most notably in the recovering nematodes.

6.2.4.5 Cellular organisation

Several proteins that map to GO terms related to cellular organisation were up-regulated in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae as shown in Tables 6.8 and Table 6.9. The highest numbers of up-regulated proteins associated with cellular organisation were from the preconditioning and recovery datasets (including GO terms such as Cellular Organisation, Cellular Membrane Organization, Anatomical Structure Development and Body Morphogenesis). The changes in expression of a selection of these proteins are illustrated in Figure 6.7. Increased expression of proteins associated with cellular organization may be required during preconditioning and recovery possibly because the nematodes undergo major structural changes going into desiccation and upon rehydration after desiccation. Five proteins have increased expression in response to all of the conditions and include FLN-1 (fln=filamin), GSNL-1

(gsnl=gelosin-like), TBA-2 (tba=tubulin, alpha), UNC-22 (unc=uncoordinated) and UNC-70. The change in expression of the genes which encode these proteins in *C. elegans* dauer larvae measured by RNA-seq (Figure 6.8) reveals *tba-2*, *fln-1* and *unc-70* are up-regulated in response to desiccation.

6.2.4.6 Detoxification and protection

DNA packaging and repair

The number of proteins up-regulated in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae that map to GO terms related to DNA packaging and repair is shown in Table 6.10. The proteins involved in DNA packaging which are up-regulated in preconditioning and desiccation include histones HIS-45 (his=histone), HIL-1 (hil=histone H1 like) and HTZ-1 (htz=histone variant H2AZ homolog) as shown in Table 6.11. The proteins involved in Response to DNA Damage Stimulus which are up-regulated in the preconditioning dataset were HIL-1 (hil=HIstone H1 Like) and RPT-3 (proteasome Regulatory Particle, ATPase-like).

Removal of damaged proteins

A number of proteins involved in the removal of damaged proteins and cell death are up-regulated in response preconditioning, desiccation and recovery in *C. elegans* dauer larvae compared to the control nematodes (Table 6.12). These include proteasome regulatory particles RPN-11 (proteasome Regulatory Particle, Non-ATPase-like) and RPT-3 (proteasome Regulatory Particle, ATPase-like) as well as cell death related LAM-1 (LAMinin).

6.2.4.7 Antioxidants and stress induced proteins

The number of up-regulated proteins that map to GO terms associated with stress and aging in *C. elegans* dauer larvae in response to exposure to desiccation-related experimental conditions are presented in Table 6.13.

The up-regulated proteins associated with antioxidant activity, such as the catalase CTL-1 as well as proteins associated with stress such the small heat shock protein HSP-12.6 and the LEA protein DUR-1 are shown in Table 6.14. Moreover, the up-regulated proteins CTL-1 and HSP-12.6 map to the GO categories response to stress and aging as shown in Table 6.13. Proteasomal proteins such as RPT-3 and ribosomal proteins such as RPS-6 also map to the response to Stress and Aging GO terms. Two antioxidants, superoxide dismutase SOD-1 and glutathione peroxidase GST-7, were up-regulated only in the recovery dataset. The change in protein expression of antioxidant enzymes in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae is shown in Figure 6.9.

The relative expression of *hsp-12.6* transcripts (RNA-seq) as well as HSP-12.6 protein is shown in Figure 6.10. At the transcript level *hsp-12.6* peaks during recovery while at the protein level HSP-12.6 peaks during desiccation. The fact this sHSP is up-regulated across all conditions compared to the control at both the transcriptomic and proteomic level indicates it may play an important role in the survival of desiccated *C. elegans* dauer larvae.

Table 6.2 The number of up-regulated proteins that map to biological process (BP) GO terms associated with signalling in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (BP= biological processes).

GO-ID	Description	Number of up-regulated proteins			child/subset of (Go ID)
		Preconditioning	Desiccation	Recovery	
23052	Signalling	2	1	3	
23033	Signalling pathway	2	1	2	child of 23052
7166	Cell surface receptor linked signalling pathway	2	1	1	child of 23052
16055	Wnt receptor signalling pathway	1	1	0	child of 23052
48666	Neuron development	3	3	5	

Table 6.3 Up-regulated proteins associated with RNA processes in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	LFQ intensity			
				Control	Preconditioning	Desiccation	Recovery
All three condition							
RNAi component	Tudor Staphylococcal Nuclease homolog	*tsn-1*	F10G7.2	NaN	23.0763	23.66666	23.5988
RNA processing	FIBrillarin family	*fib-1*	T01C3.7	NaN	24.486	23.97369	23.7867
	NucleOLar protein	*nol-5*	W01B11.3	NaN	22.0829	23.67142	24.1689
RNA transport	Ref/ALY RNA export adaptor family	*aly-2*	F23B2.6	NaN	24.2827	24.60162	23.8327
	Ref/ALY RNA export adaptor family	*aly-3*	M18.7	NaN	26.5254	26.1189	25.8383
	RNA recognition motif domain related	*T04A8.6*	T04A8.6	NaN	22.6181	21.94714	22.4925
	RNA recognition motif domain related	*K07H8.10*	K07H8.10	NaN	24.7644	26.17794	24.898
Two conditions							
	RNA binding domain related	*K07C5.4*	K07C5.4	NaN	22.1966	23.75124	NaN
	Endoribonuclease domain related	*C23G10.2*	C23G10.2	NaN	22.4696	NaN	21.7699

Table 6.4 The number of up-regulated proteins that map to GO terms associated with translation and protein modification in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (B.P= biological processes).

GO-ID	Category	Description	Number of up-regulated proteins		
			Preconditioning	Desiccation	Recovery
6412	B.P	Translation	21	17	21
6464	B.P	Protein modification process	2	1	2

Table 6.5 Up-regulated proteins associated with translation in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	LFQ intensity			
				Control	Preconditioning	Desiccation	Recovery
Three conditions	Ribosomal Protein, Large subunit	*rpl-10*	F10B5.1	NaN	24.7805	24.14492	25.1566
	Ribosomal Protein, Large subunit	*rpl-15*	K11H12.2	NaN	23.036	23.35555	24.3865
	Ribosomal Protein, Large subunit	*rpl-20*	E04A4.8	NaN	24.1413	23.45023	23.2935
	Ribosomal Protein, Large subunit	*rpl-25.2*	F52B5.6	NaN	24.3783	21.84491	22.7847
	Ribosomal Protein, Large subunit	*rpl-28*	R11D1.8	NaN	25.1102	24.87159	25.4911
	Ribosomal Protein, Large subunit	*rpl-3*	F13B10.2	NaN	24.7601	25.34052	25.8341
	Ribosomal Protein, Large subunit	*rpl-41*	C09H10.2	NaN	24.392	22.33606	22.6477
	Ribosomal Protein, Large subunit	*rpl-7A*	Y24D9A.4	NaN	23.7347	22.30666	22.3097
	Ribosomal Protein, Small subunit	*rps-10*	D1007.6	NaN	23.6767	22.30922	21.7636
	Ribosomal Protein, Small subunit	*rps-11*	F40F11.1	NaN	25.1164	24.00033	24.2831
	Ribosomal Protein, Small subunit	*rps-13*	C16A3.9	NaN	26.4112	25.03959	24.9512
	Ribosomal Protein, Small subunit	*rps-16*	T01C3.6	NaN	24.5654	25.6133	25.0596
	Ribosomal Protein, Small subunit	*rps-4*	Y43B11AR.4	NaN	26.7747	27.42886	27.707
	Ribosomal Protein, Small subunit	*rps-6*	Y71A12B.1	NaN	25.4557	24.89749	25.4009
	Ribosomal Protein, Small subunit	*rps-9*	F40F8.10	NaN	25.2681	24.34746	23.9877

Table 6.5 (continued) Up-regulated proteins associated with translation in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID, Anova (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	Anova	LFQ intensity			
					Control	Preconditioning	Desiccation	Recovery
Two conditions								
Translation	Ribosomal Protein, Large subunit	*rpl-21*	C14B9.7		NaN	24.1852	23.45576	NaN
	Ribosomal Protein, Large subunit	*rpl-23*	B0336.10		NaN	21.9123	20.84161	NaN
	Ribosomal Protein, Large subunit	*rpl-43*	Y48B6A.2		NaN	23.1642	22.40242	NaN
	rla-0 (Ribosomal protein, Large subunit, Acidic)	*rla-0*	F25H2.10		NaN	21.4924	NaN	23.3292
	Ribosomal Protein, Large subunit	*rpl-16*	M01F1.2		NaN	22.6575	NaN	22.9534
	Ribosomal Protein, Large subunit	*rpl-32*	T24B8.1		NaN	23.6522	NaN	22.2301
	Ribosomal Protein, Small subunit	*rps-0*	B0393.1		NaN	22.6653	NaN	22.1226
	Ribosomal Protein, Small subunit	*rps-24*	T07A9.11		NaN	26.5626	NaN	28.5205
	Ribosomal Protein, Small subunit	*rps-25*	K02B2.5		NaN	NaN	24.60851	24.407
One condition								
Translation	Ribosomal Protein, Large subunit	*rpl-7*	F53G12.10	+	25.4782	-	-	26.59157
	Ribosomal Protein, Large subunit	*rpl-17*	Y48G8AL.8	+	24.65265	-	-	26.23965
	Ribosomal Protein, Small subunit	*rps-18*	Y57G11C.16	+	24.213	-	-	26.3582
	Ribosomal Protein, Large subunit	*rpl-7*	F53G12.10	+	25.4782	-	-	26.59157

Table 6.6 The number of up-regulated proteins that map to GO terms associated with metabolism in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (CC= cell component, BP= biological processes).

GO-ID	Category	Description	Number of up-regulated proteins			child/subset of (Go ID)
			Preconditioning	Desiccation	Recovery	
5739	CC	Mitochondrion	1	3	6	
8152	BP	Metabolic process	39	33	44	
15980	BP	Energy derivation by oxidation of organic compounds	2	3	4	child of 8152
6006	BP	Glucose metabolic process	4	4	3	child of 8152
6807	BP	Nitrogen compound metabolic process	6	5	8	child of 8152
6091	BP	Generation of precursor metabolites and energy	5	6	7	child of 8152
6096	BP	Glycolysis	3	3	2	child of 6091
6099	BP	Tricarboxylic acid cycle	2	3	3	child of 6091
6139	BP	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5	3	6	
6753	BP	Nucleoside phosphate metabolic process	2	1	3	child of 6139
6810	BP	Transport	9	8	15	
279	B.P	M phase	3	3	7	
87	B.P	M phase of mitotic cell cycle	1	1	4	child of 279
22402	B.P	Cell cycle process	4	3	8	
22403	B.P	Cell cycle phase	3	3	7	
51726	B.P	Regulation of cell cycle	1	0	2	

Table 6.7 Up-regulated proteins associated with metabolism in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	LFQ intensity			
				Control	Preconditioning	Desiccation	Recovery
All three condition							
Nitrogen metabolism	GLutamiNe synthetase	*gln-3*	Y105C5B.28	NaN	22.3719	22.60914	23.3784
Iron	FerriTiN	*ftn-1*	C54F6.14	NaN	24.9237	23.3652	23.7618
	PhosphoGlycerate Kinase	*pgk-1*	T03F1.3	NaN	23.8123	24.15653	26.2489
Mitochondrial	Pyruvate DeHydrogenase Alpha subunit	*pdha-1*	T05H10.6	NaN	23.5659	23.59901	23.3684
Glycolysis	PYruvate Kinase	*pyk-1*	F25H5.3	NaN	25.264	25.45025	25.545
Two conditions							
Metabolism	Malate DeHydrogenase	*mdh-1*	F46E10.10	NaN	23.463	22.81364	NaN
Electron transport	Succinate DeHydrogenase complex subunit A	*sdha-1*	C03G5.1	NaN	21.7931	NaN	23.7797
	Probable adenylate kinase isoenzyme F38B2.4	*F38B2.4*	F38B2.4	NaN	24.1937	NaN	23.8699
Mitochondrial	FUMarase	*fum-1*	H14A12.2	NaN	22.776	NaN	24.1432
	Propionyl Coenzyme A Carboxylase Beta subunit	*pccb-1*	F52E4.1	NaN	24.6421	NaN	25.0091
	GABA TransAminase family	*gta-1*	K04D7.3	NaN	NaN	23.3065	24.2239

Table 6.7 (continued) Up-regulated proteins associated with metabolism in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID, Anova (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	Anova	LFQ intensity			
					Control	Preconditioning	Desiccation	Recovery
Only one condition								
Lipid binding protein	Lipid Binding Protein	*lbp-2*	F40F4.2	+	25.68558	26.45447	26.45447	
	Adenine Nucleotide Translocator	*ant-1.1*	T27E9.1	+	24.88363	-	-	26.82063
Electron transport	NADH Ubiquinone Oxidoreductase	*nuo-1*	C09H10.3	+	23.06065	-	-	24.4193
	Inner Membrane of MiTochondria protein	*immt-1*	T14G11.3		NaN	NaN	NaN	24.6729
Kreb cycle	CiTrate Synthase	*cts-1*	T20G5.2		NaN	NaN	NaN	24.1215

Table 6.8 The number of up-regulated proteins that map to GO terms associated with cellular organisation in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (C.C= cell component, B.P= biological processes).

GO-ID	Category	Description	Number of up-regulated proteins			child/subset of (Go ID)
			Preconditioning	Desiccation	Recovery	
5856	C.C	Cytoskeleton	4	5	7	
15629	C.C	Actin cytoskeleton	2	3	4	child of 5856
16043	B.P	Cellular component organization	17	15	24	
16044	B.P	Cellular membrane organization	7	5	10	child of 16043
48856	B.P	Anatomical structure development	20	16	23	
9653	B.P	Anatomical structure morphogenesis	13	10	16	child of 48856
9888	B.P	Tissue development	5	3	2	
10171	B.P	Body morphogenesis	9	7	10	child of 48856

Table 6.9 Up-regulated proteins associated with cellular organisation in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID, Anova (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	Anova	LFQ intensity			
					Control	Preconditioning	Desiccation	Recovery
All three condition								
Cytoskeleton	FiLamiN (actin binding protein) homolog	*fln-1*	Y66H1B.2		NaN	25.557	24.75873	24.9732
	GelSoliN-Like	*gsnl-1*	K06A4.3		NaN	22.9222	23.34557	24.5628
	TuBulin, Alpha	*tba-2*	C47B2.3		NaN	26.037	26.25395	27.2059
Muscle organisation	UNCoordinated	*unc-22*	ZK617.1		NaN	23.6426	24.70841	24.8391
	UNCoordinated	*unc-70*	K11C4.3		NaN	22.4569	21.82026	22.2979
Two conditions								
Cytoskeleton	ProFiliN	*pfn-2*	F35C8.6		NaN	22.3562	21.96102	NaN
	CAP-z protein	*cap-1*	D2024.6		NaN	NaN	23.0862	23.5596
	KETtiN homolog	*ketn-1*	F54E2.3	+	24.98641	NaN	25.92518	27.03233
Spectraplakin	Variable ABnormal morphology	*vab-10*	ZK1151.1	+	24.0065	24.96473	-	24.66957
Muscle organisation	UNCoordinated	*unc-89*	C09D1.1		NaN	21.4046	NaN	22.8137

Table 6.9 (continued) Up-regulated proteins associated with cellular organisation in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID, Anova (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	Anova	LFQ intensity			
					Control	Preconditioning	Desiccation	Recovery
One condition								
Cytoskeleton	Intermediate Filament, A	*ifa-1*	F38B2.1		NaN	NaN	NaN	22.848
	FERM domain family	*frm-1*	ZK270.2	+	24.05705	-	-	25.53935
Muscle	MYOsin heavy chain structural genes	*myo-3*	K12F2.1	+	25.57947	-	-	27.9555
Muscle myosin	UNCoordinated	*unc-54*	F11C3.3	+	30.80377	-	-	32.0589
Nervous system	UNCoordinated	*unc-32*	ZK637.8		NaN	NaN	NaN	21.6529
Extracellular fibronectin	LEThal	*let-805*	H19M22.2		NaN	NaN	NaN	25.431

Table 6.10 The number of up-regulated proteins that map to GO terms associated with DNA packaging and repair in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (C.C=cell component, B.P= biological processes).

GO-ID	Category	Description	Number of up-regulated proteins		
			Preconditioning	Desiccation	Recovery
6323	B.P	DNA packaging	3	3	1
6325	B.P	Chromatin organization	4	4	2
6974	B.P	Response to DNA damage stimulus	2	1	1
5634	C.C	Nucleus	7	7	4

Table 6.11 Up-regulated proteins associated with DNA packaging and repair in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID, Anova (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	Anova	LFQ intensity			
					Control	Preconditioning	Desiccation	Recovery
All three condition								
Histone	HIStone	*his-45*	B0035.10		NaN	24.7051	23.73789	23.4174
	C. Elegans Chromodomain protein	*cec-5*	F32E10.6		NaN	26.8206	27.3724	26.1852
Nuclear transport	Associated with RAN (nuclear import/export) function	*ran-4*	R05D11.3		NaN	23.8076	24.1141	24.388
Two conditions								
	HIstone H1 Like	*hil-1*	C30G7.1		NaN	23.0793	22.44993	NaN
	HisTone variant H2AZ homolog	*htz-1*	R08C7.3		NaN	23.6723	22.87044	NaN
Nuclear binding related	Homologous to Drosophila SQD (squid) protein	*sqd-1*	Y73B6BL.6		NaN	22.6438	25.84814	NaN
One condition								
	Nuclear LaMiN	*lmn-1*	DY3.2	+	25.32207	-	-	27.1522

Table 6.12 Up-regulated proteins associated with removal of damaged proteins and cell death in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	LFQ intensity			
				Control	Preconditioning	Desiccation	Recovery
Up in all three condition							
Proteasome	Proteasome Regulatory Particle, Non-ATPase-like	*rpn-11*	K07D4.3	NaN	21.4178	22.08184	22.503
Cell death	LAMinin related	*lam-1*	W03F8.5	NaN	24.2567	24.07455	23.9731
Apoptosis	ACINus homolog	*acin-1*	C43E11.1	NaN	22.5299	23.48395	23.1639
Up in two conditions							
Proteasome	Proteasome Regulatory Particle, ATPase-like	*rpt-3*	F23F12.6	NaN	22.3645	NaN	23.1223

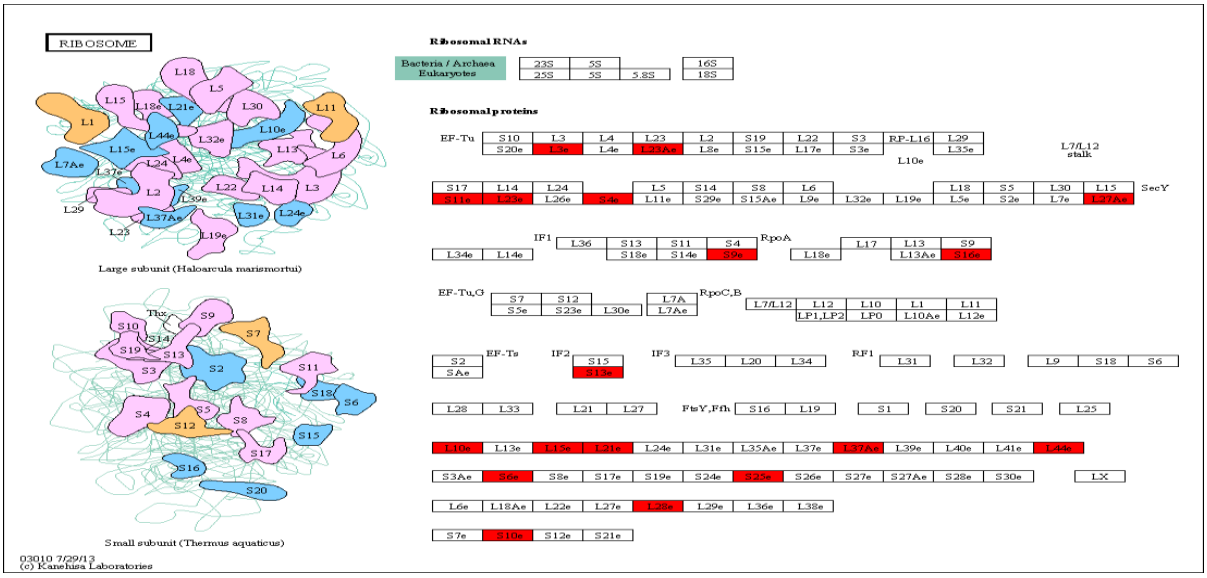
Table 6.13 The number of up-regulated proteins that map to GO terms associated with stress and aging in *C. elegans* dauer larvae relative to control nematodes in response to exposure to desiccation-related experimental conditions (B.P= biological processes).

GO-ID	Category	Description	Number of up-regulated proteins			child/subset of (Go ID)
			Preconditioning	Desiccation	Recovery	
6950	B.P	Response to stress	5	4	5	
9408	B.P	Response to heat	2	2	2	child of 6950
7568	B.P	Aging	11	10	15	

Table 6.14 Up-regulated proteins associated with removal of protection and detoxification in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. The function, description, gene name, Protein ID, Anova (+=positive, p>0.05) and average LFQ intensities for each of the conditions are shown. NaN indicates a protein not identified in all samples for a specific treatment.

Function	Description	Gene name	Protein	Anova	LFQ intensity			
					Control	Preconditioning	Desiccation	Recovery
All three condition								
Antioxidant	CaTaLase	*ctl-1*	Y54G11A.6		NaN	23.9325	23.78835	24.9035
HSP	Heat Shock Protein	*hsp-12.6*	F38E11.2	+	29.40573	31.4812	32.27488	31.89447
Two conditions								
Antioxidant	GLutaRedoXin	*glrx-10*	Y34D9A.6		NaN	22.3347	21.57487	NaN
	GLutaRedoXin	*glrx-5*	Y49E10.2		NaN	22.8103	NaN	21.7894
Stress related	Dauer Up-Regulated	*dur-1*	F25H8.5		NaN	24.4794	NaN	22.597
One condition								
Antioxidant	Glutathione S-Transferase	*gst-7*	F11G11.2	+	25.2357	-	-	26.7564
	Superoxide dismutase	*sod-2*	F10D11.1		NaN	NaN	NaN	23.4505

(i)



(ii)

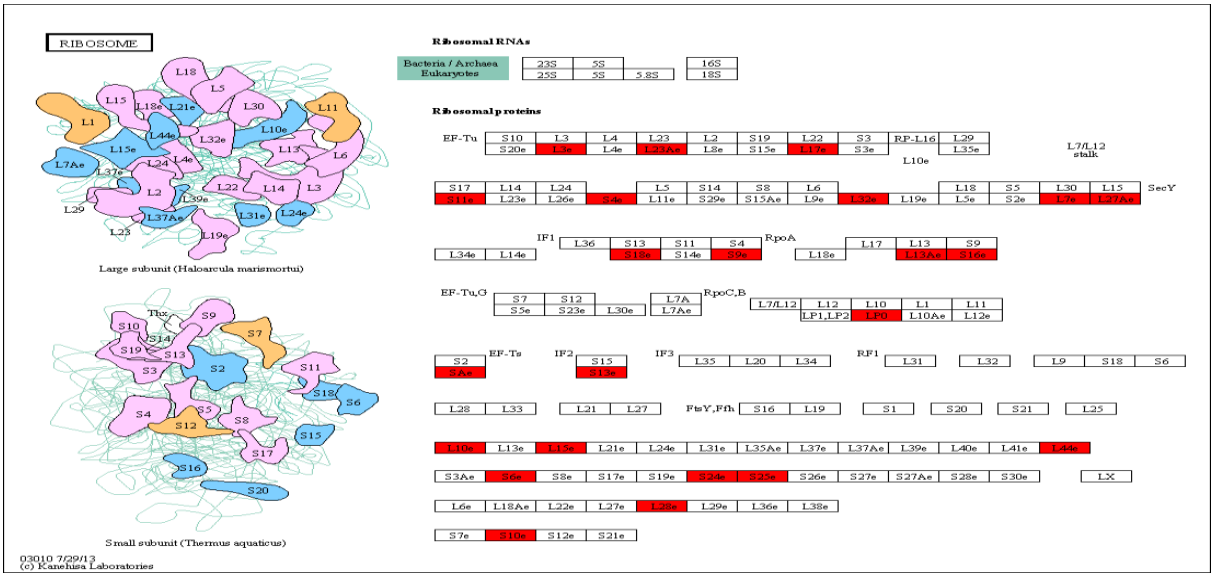


Figure 6.2 KEGG diagram showing ribosomal proteins that are up-regulated in response to (i) desiccation and (ii) recovery compared to the control in *C. elegans* dauer larvae. The proteins that are up-regulated are shown in red.

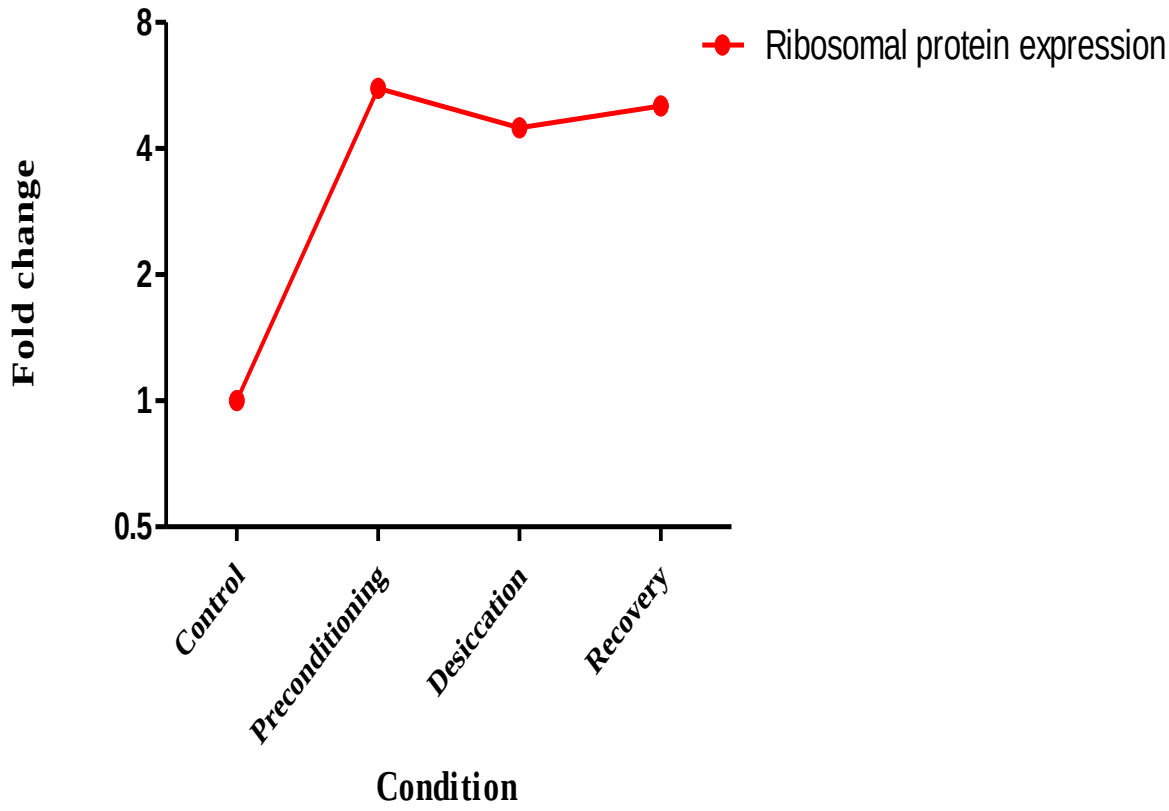


Figure 6.3 The average change in expression of up-regulated ribosomal proteins in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae. Log-2 of relative expression values (based on label-free quantification intensities) were plotted on a log-2 scale. When proteins were not identified in protein extracts from a specific treatment, a base LFQ value (lowest recorded LFQ intensity across the experiment) was applied.

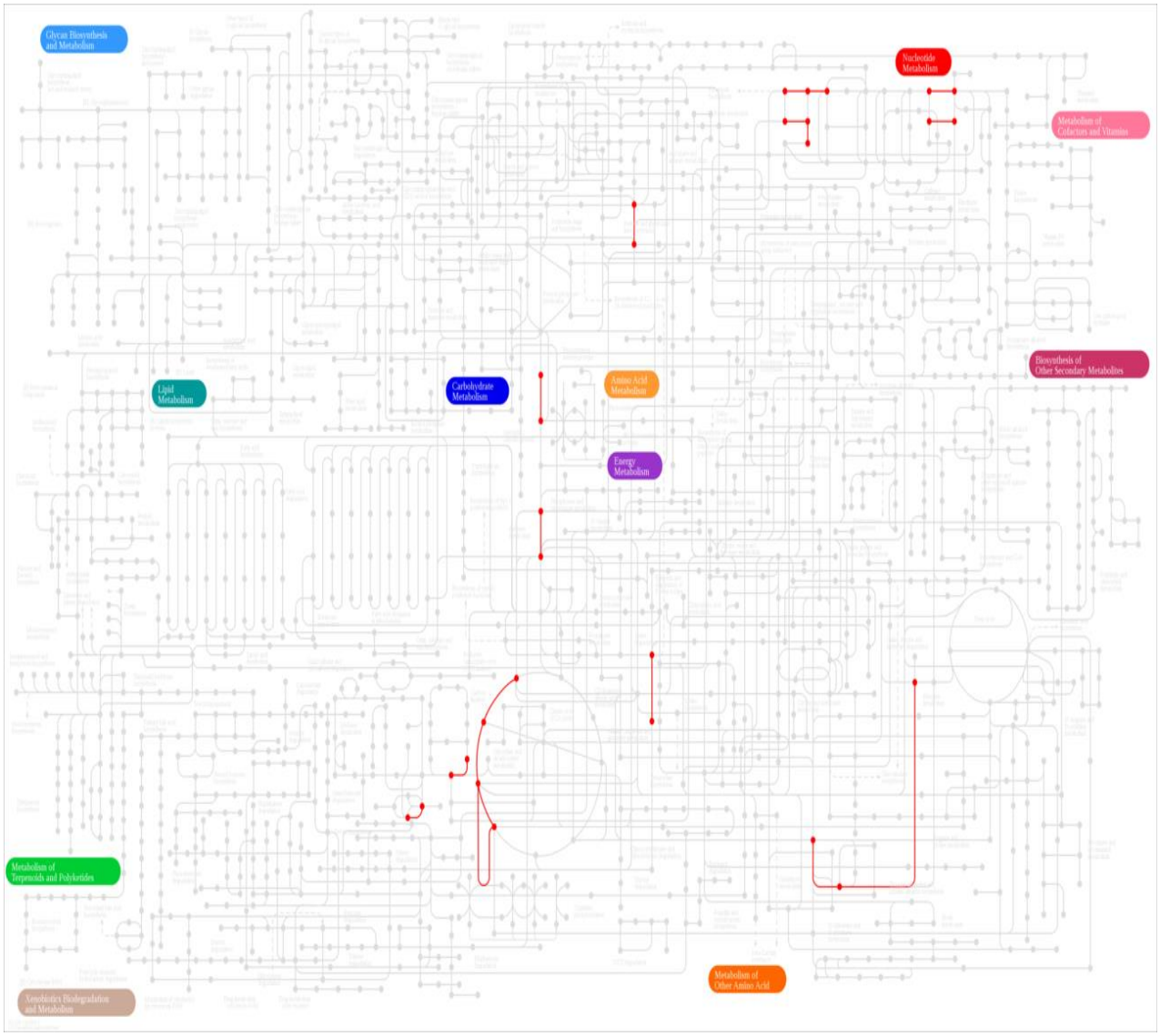


Figure 6.4 KEGG pathway map of metabolism showing proteins that are up-regulated in response to preconditioning in *C. elegans* dauer larvae. The up-regulated proteins in response to preconditioning were involved in pathways such as the citrate cycle, carbon fixation and nucleotide metabolism. Up-regulated proteins are shown in red.

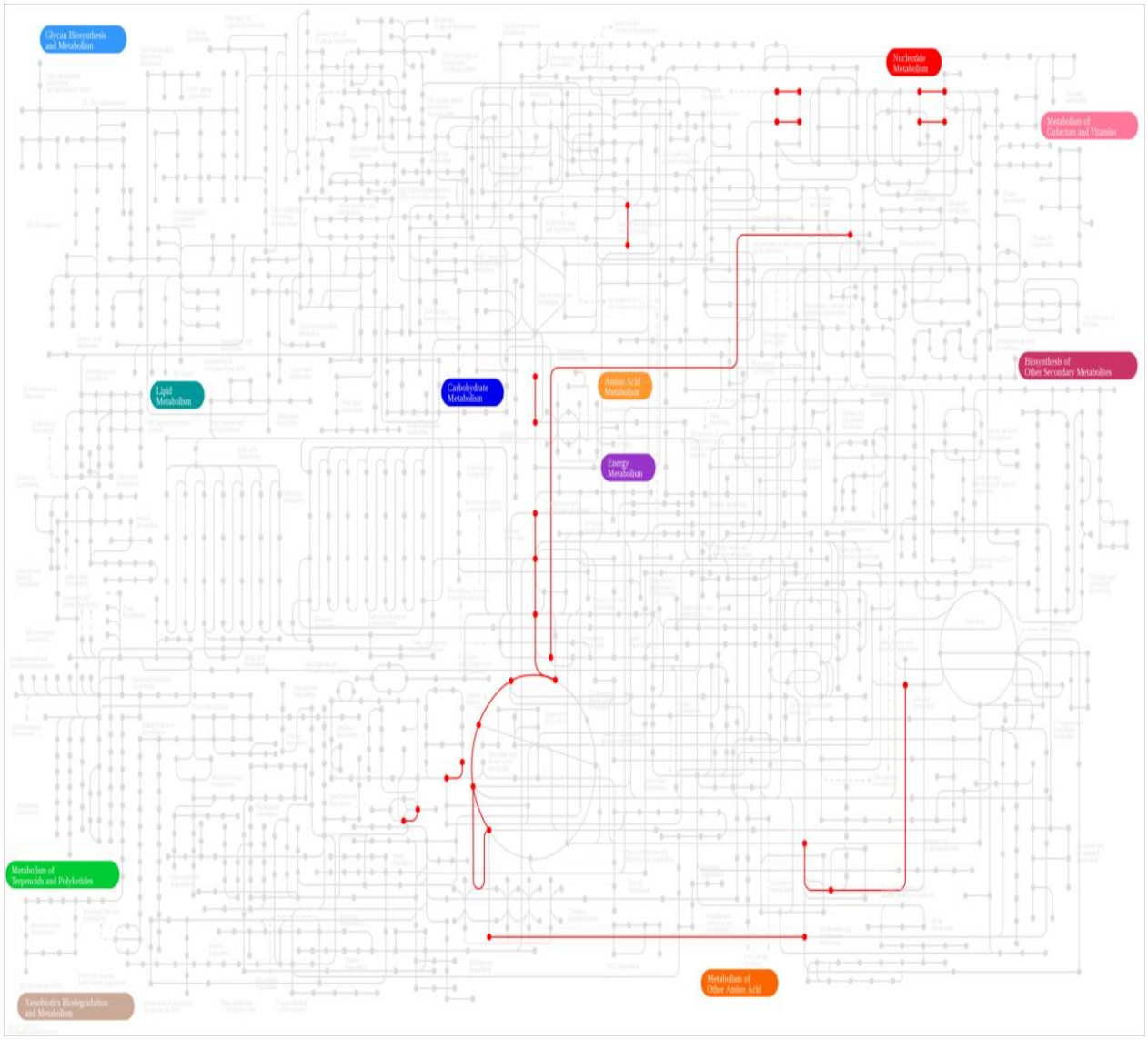


Figure 6.5 KEGG pathway map of metabolism showing proteins that are up-regulated in response to desiccation in *C. elegans* dauer larvae. The up-regulated proteins in response to desiccation were involved in pathways such as the citrate cycle, carbon fixation, nucleotide metabolism, pyruvate metabolism, pyrimidine metabolism and oxidative phosphorylation. Up-regulated proteins are shown in red.

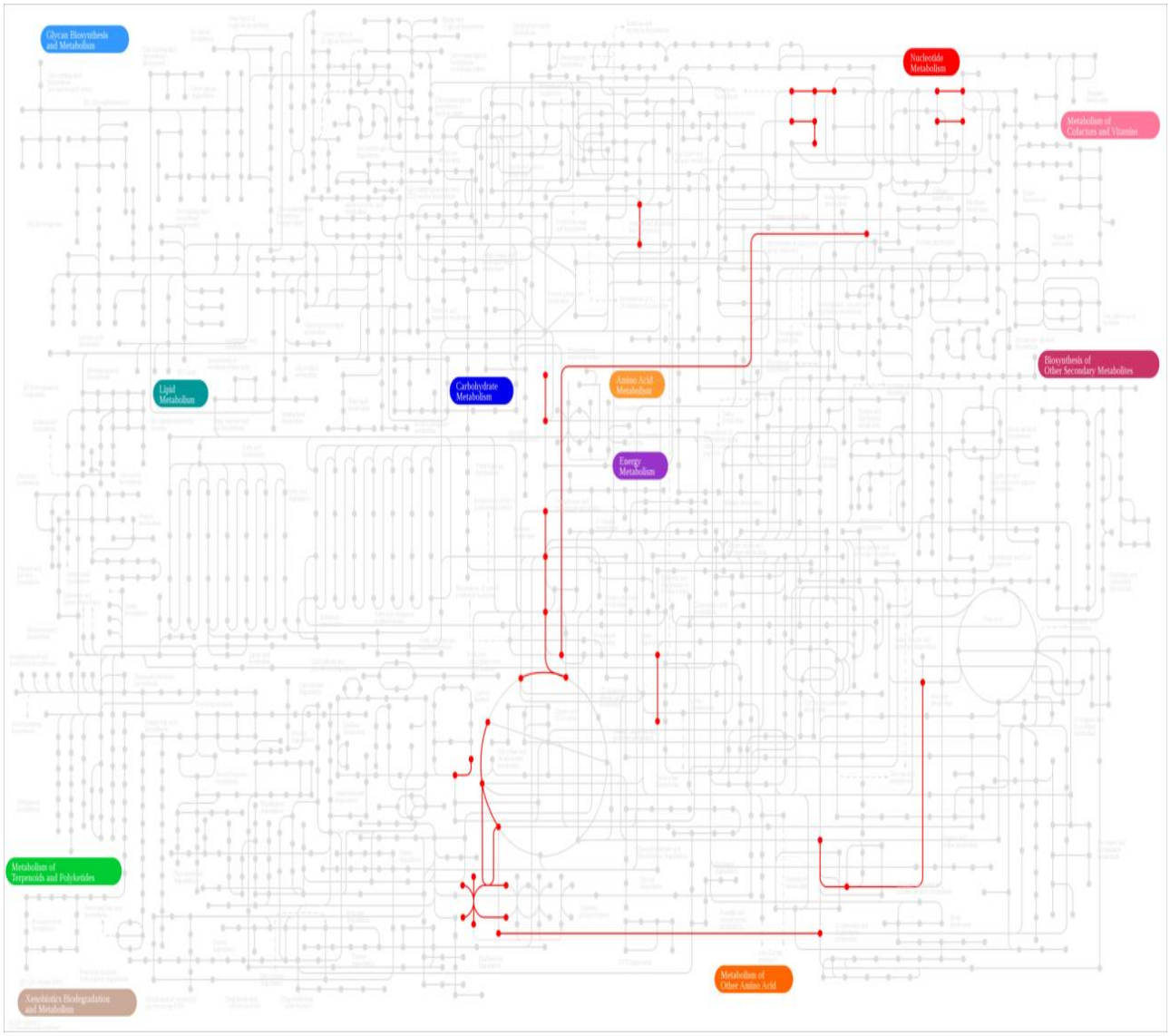


Figure 6.6 KEGG pathway map of metabolism showing proteins that are up-regulated in response to recovery in *C. elegans* dauer larvae. The up-regulated proteins in response to recovery were involved in pathways such as the citrate cycle, carbon fixation, nucleotide metabolism, pyruvate metabolism and pyrimidine metabolism. Up-regulated proteins are shown in red.

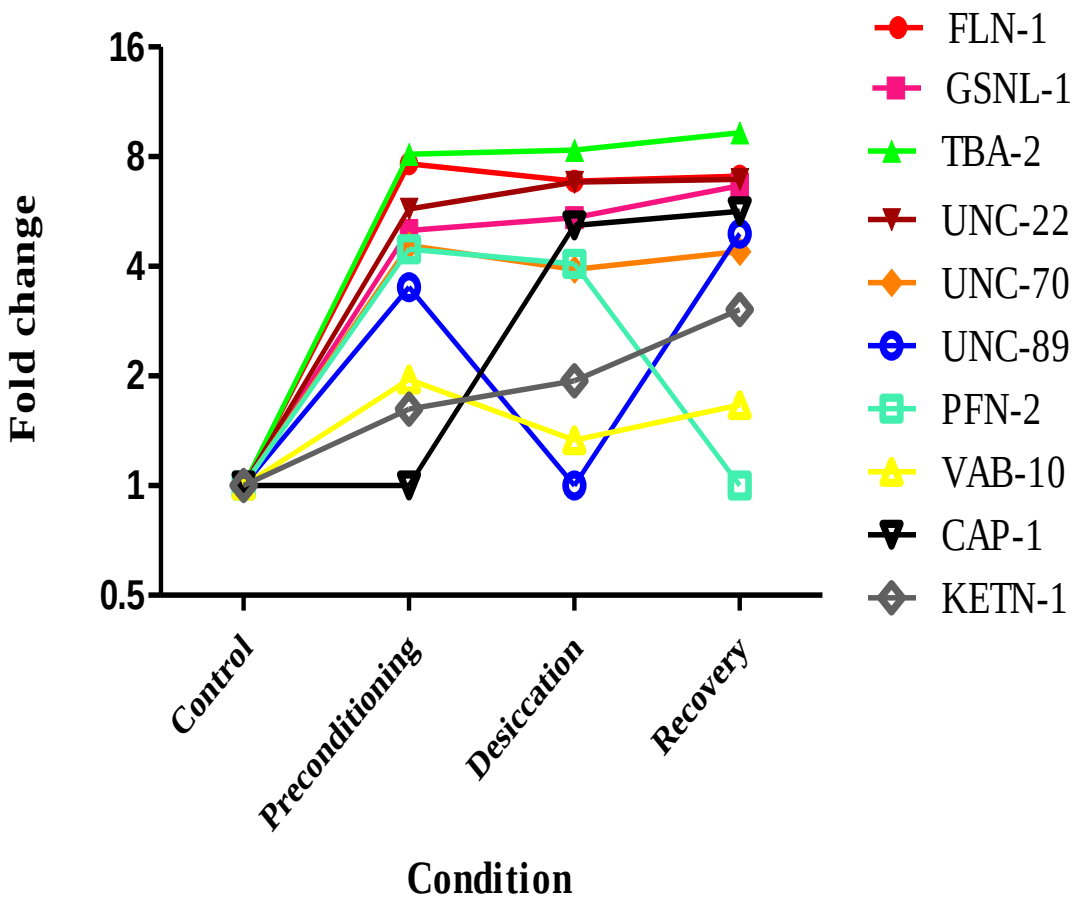


Figure 6.7 Change in expression of proteins associated with cellular organisation in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae (FLN-1, GSNL-1, TBA-2, UNC-22, UNC-70, UNC-89, PFN-2, VAB-10, CAP-1, KETN-1). Log-2 of relative expression values (based on label-free quantification intensities) were also plotted on a log-2 scale. When proteins were not identified in protein extracts from a specific treatment, a base LFQ value (lowest recorded LFQ intensity across the experiment) was applied.

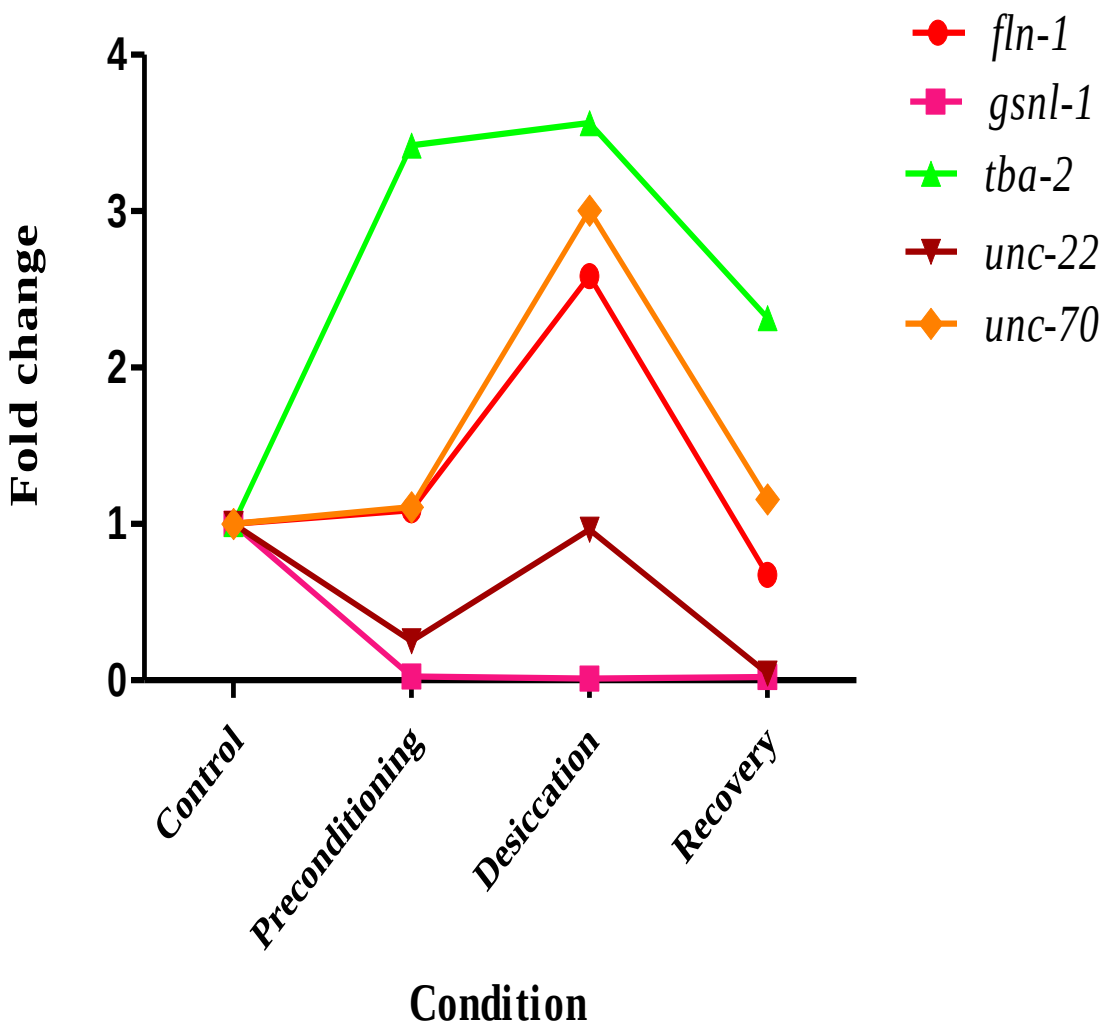


Figure 6.8 Change in expression of genes (RNA-seq transcript counts) encoding proteins associated with cellular organisation in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery compared to the control (*fln-1*, *gsnl-1*, *tba-2*, *unc-22* and *unc-70*). Relative expression values (determined from RNA-seq transcript counts) were plotted on a log-2 scale. The proteins which these genes encode were found to be up-regulated in response to all the conditions in *C. elegans* dauer larvae.

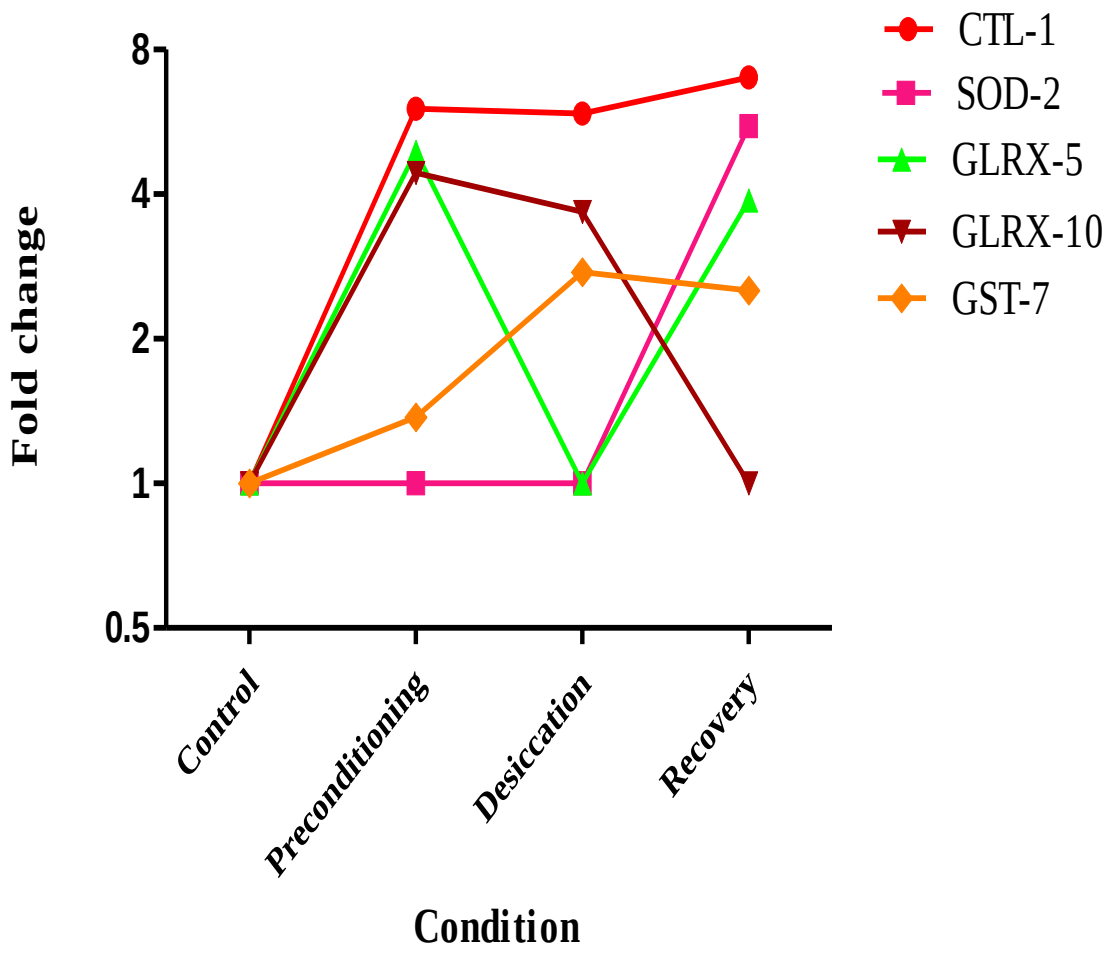


Figure 6.9 Change in protein expression of antioxidant enzymes in response to preconditioning, desiccation and recovery compared to the control in *C. elegans* dauer larvae (CTL-1, SOD-2, GLRX-5, GLRX-10, GST-7). Log-2 of relative expression values (based on label-free quantification intensities) were also plotted on a log-2 scale. When proteins were not identified in protein extracts from a specific treatment, a base LFQ value (lowest recorded LFQ intensity across the experiment) was applied.

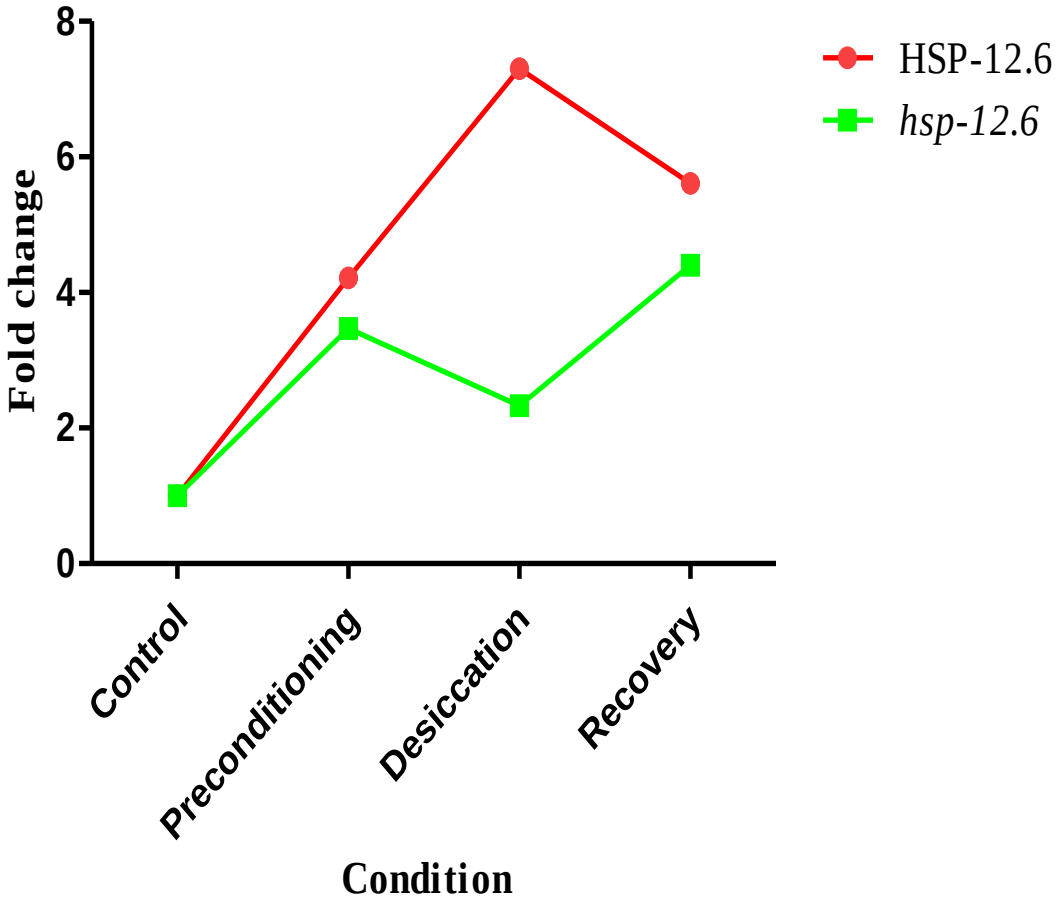


Figure 6.10 The relative expression of *hsp-12.6* transcripts (RNA-seq transcript counts) as well as HSP-12.6 protein (based on label-free quantification intensities) in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery is shown. HSP-12.6 protein and *hsp-12.6* transcript were statistically up-regulated in response to preconditioning, desiccation and recovery when compared to the control. When proteins were not identified in protein extracts from a specific treatment, a base LFQ value (lowest recorded LFQ intensity across the experiment) was applied.

6.2.5 Comparison of RNA-seq and LFQ differential proteomics in *C. elegans* dauer larvae

The overlap between genes and proteins that are up-regulated in *C. elegans* dauer larvae in response to (i) at least one condition and (ii) across all conditions is presented in the Venn diagram in Figure 6.11. This diagram shows good correlation between the transcriptomic and proteomic datasets with 96 of the total 153 proteins up-regulated in the proteomic dataset also showing increased expression in at least one of the transcriptomic conditions. Additionally, the genes and proteins that are up-regulated in response to all three conditions were compared and 14 from a total of 54 sequences were common to both datasets. These genes and proteins include the antioxidant *ctl-1*, small heat shock protein *hsp-12.6*, proteasomal protein *rpn-11* as well as a number of ribosomal proteins (Table 6.15). One of the up-regulated sequences B0491.5 encodes a novel nematode specific protein. No motifs were detected for this protein in the Prosite database (http://prosite.expasy.org), but four iterations of psiBLAST gave a putative hit to a NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11 protein (42% coverage, 19% identity and E value $1e^{-49}$).

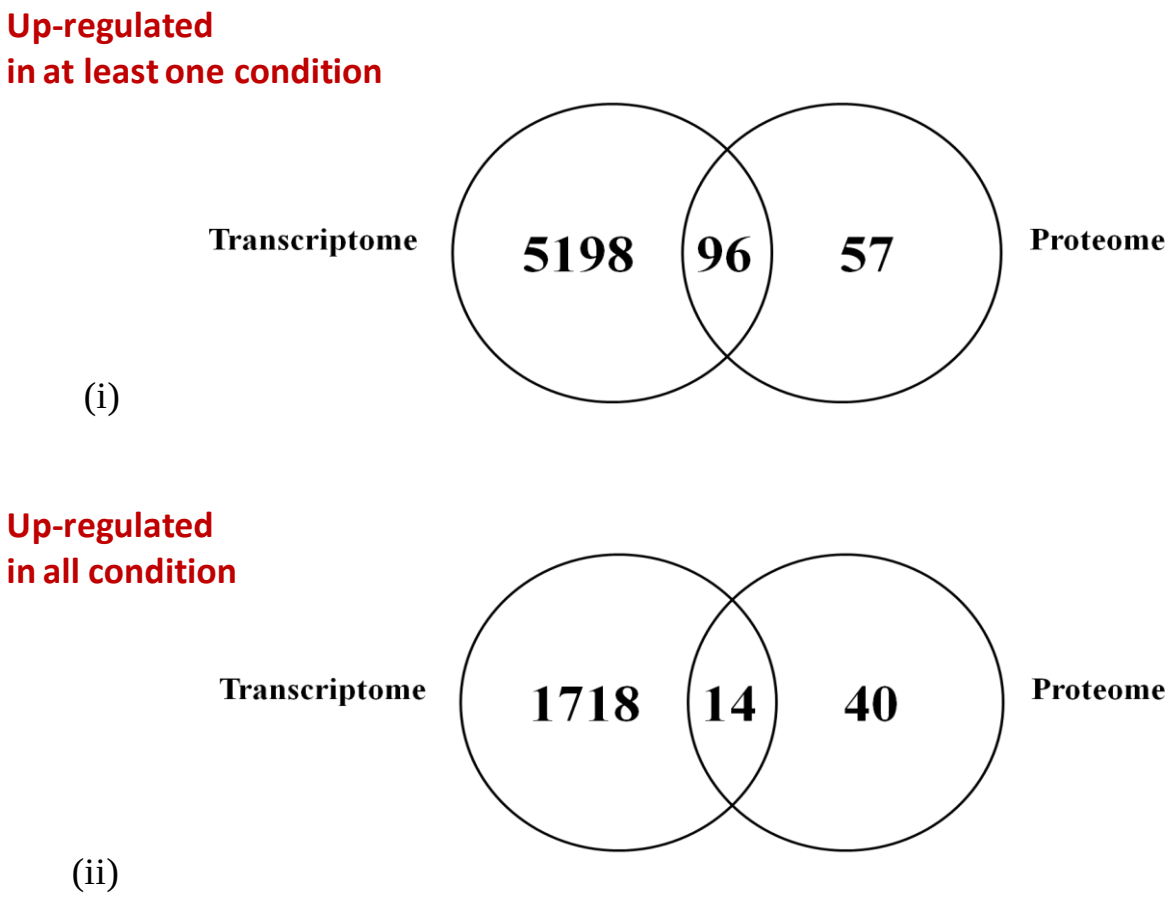


Figure 6.11 Venn diagram showing (i) overlap of genes and proteins that are up-regulated in response to at least one condition (preconditioning, desiccation and recovery compared to the control) and (ii) genes and proteins that are up-regulated in response to all three conditions (preconditioning, desiccation and recovery compared to the control) in *C. elegans* dauer larvae.

Table 6.15 Genes and proteins that are up-regulated in response to in all three conditions (preconditioning, desiccation and recovery compared to the control) in *C. elegans* dauer larvae based on RNA-seq and LFQ differential proteomic data.

Description	**Gene name**	**Sequence ID**	**Transcript (fold change based on RNA-seq data)**			**Protein expression (log2 fold change based on LFQ intensities)**		
			Preconditioning	**Desiccation**	**Recovery**	**Preconditioning**	**Desiccation**	**Recovery**
Heat shock protein	*hsp-12.6*	F38E11.2	3.47	2.33	4.41	3.08	3.87	3.49
Catalase	*ctl-1*	Y54G11A.6	4.29	3.65	3.72	6.03	5.89	7.00
Proteasome Regulatory Particle	*rpn-11*	K07D4.3	3.72	3.55	3.03	3.52	4.18	4.60
Phosphoglycerate kinase	*pgk-1*	T03F1.3	3.64	2.14	3.44	5.91	6.26	8.35
Pyruvate kinase	*pyk-1*	F25H5.3	7.14	6.87	5.87	7.36	7.55	7.65
TuBulin, Alpha	*tba-2*	C47B2.3	3.42	3.57	2.32	8.14	8.35	9.31
Ubiquinol-Cytochrome c oxidoReductase	*ucr-1*	F56D2.1	3.63	2.93	2.65	5.87	6.25	7.34
NucleOLar protein	*nol-5*	W01B11.3	2.96	3.34	2.33	4.18	5.77	6.27
Ribosomal Protein, Large subunit	*rpl-7A*	Y24D9A.4	4.77	3.29	4.36	5.83	4.41	4.41
Ribosomal Protein, Large subunit	*rpl-15*	K11H12.2	3.56	2.08	3.38	5.14	5.46	6.49
Ribosomal Protein, Large subunit	*rpl-20*	E04A4.8	3.61	2.23	3.06	6.24	5.55	5.39
Ribosomal Protein, Small subunit	*rps-4*	Y43B11AR.4	4.86	3.24	3.88	8.87	9.53	9.81
Ribosomal Protein, Small subunit	*rps-10*	D1007.6	4.49	2.67	3.48	5.78	4.41	3.86
Unknown	B0491.5	B0491.5	4.76	2.86	3.02	5.77	5.68	6.76

6.3 Discussion

In this differential proteomic analysis several proteins were found to be differentially expressed in response to desiccation and recovery from desiccation in *C. elegans* dauer larvae. The identities and potential anhydrobiotic significance of the up-regulated proteins are discussed in this section under the following headings: signalling pathways, translation, protection, cellular organisation and the cytoskeleton, and detoxification and repair mechanisms. Additionally, the genes and proteins which were up-regulated in all conditions in both the transcriptomic and proteomic studies in *C.elegans* dauers larvae are further discussed.

6.3.1 Signalling pathways

There are a number of proteins with increased expression, particularly in response to recovery after desiccation, that have a role in signal transduction (Table 6.2) and may be involved in regulating the nematodes' response to desiccation stress. However the number of differentially expressed signalling proteins is substantially less than the number of up-regulated signalling pathway genes (Table 5.3). This may be partly because of the comparatively small number of proteins in our LFQ MS dataset as compared with the RNA-seq data and because many signalling proteins are effective at relatively low concentrations.

UNC-89, which is up-regulated in preconditioning and recovery is required for proper organization of A bands in striated muscle and thus for fully normal locomotion, pharyngeal muscle contractions, and body size (Benian *et al.*, 1996; Small *et al.*, 2004). UNC-89 may also activate a Rho family member in nematode muscle through its signal transducing DH domains (Small *et al.*, 2004). This may activate a specific Rho GTPase leading to a signalling cascade which could produce effector proteins in response to desiccation stress. Transcripts encoding UNC-89 are over 5 fold up-regulated in the desiccation dataset, but show no increase in expression in response to preconditioning or recovery (Chapter V).

Another protein called UNC-54, with higher expression during recovery, has been found to interact with a key component of the calcium signalling network called the IP_3 receptor (Walker *et al.*, 2002). UNC-54 is the major myosin heavy chain which is required for locomotion and egg-laying, but importantly the interaction between UNC-54 and the IP_3 receptor may provide a link between calcium signalling and the cytoskeleton. Finally,

UNC-32 was also found to be up-regulated in recovery and is essential for normal synaptic vesicle morphology in motoneurons. The RNA-seq study (Chapter V) found that transcripts encoding UNC-32 are up-regulated in response to all three conditions. The *unc-32* gene encodes a set of V-ATPase 100-kDa subunits which are essential for neurotransmitter trafficking in *C. elegans* (Pujol *et al.*, 2001).

6.3.2 RNA processes and mRNA Translation

Environmental stresses such as desiccation can activate signalling cascades that result in the downstream translation of proteins. The largest group of proteins up-regulated in precondition, desiccation and recovery are involved in protein synthesis and many of these are ribosomal proteins as outlined in Section 6.2.4.3. The GO and KEGG analyses indicate that protein expression is most active in the preconditioning and recovery datasets (Table 6.4 and Figure 6.2).

There are a number of proteins associated with RNA processes that are up-regulated in *C. elegans* dauer larvae in response to desiccation-related treatments (Table 6.3). The protein TSN-1 (tsn=Tudor Staphylococcal Nuclease) which is a component of the RNA-induced silencing complex (RISC) was up-regulated in all three datasets. TSN is a multifunctional protein that has been identified in mammals, fish, *Drosophila*, ciliates, yeast as well as *C. elegans* and is implicated in a variety of cellular processes including gene transcription and pre-mRNA splicing (Li *et al.*, 2008; Gao *et al.*, 2012). The proteins FIB-1 (fib=fibrillarin), NOL-5 (nol=nucleolar) and K07C5.4 were also up-regulated in all three datasets. FIB-1, a *C. elegans* homolog of the yeast Nap1p (Macqueen and Villeneuve, 2001), is an essential factor for 18S rRNA processing (Tollervey *et al.*, 1991; Tollervey *et al.*, 1993). Interestingly, NOL-5, FIB-1 and K07C5.4 are predicted to be members of the box C/D snoRNP. Box C/D snoRNPs function in the nucleolus and methylate pre-rRNAs during ribosome maturation (Reichow *et al.*, 2007; Sheaffer *et al.*, 2008). In *C. elegans*, RNAi inactivation of both *fib-1*, *nol-5* and *K07C5.4* results in a larval arrest with excess epidermal granules and small nucleoli (Sheaffer *et al.*, 2008). The *C. elegans* proteins ALY-2 and ALY-3, which are orthologous to human THOC4, were both up-regulated in all three datasets. ALY-2 is a RRM motif-containing protein (RRM = RNA recognition motif) required for normal export of TRA-1/*tra-2* mRNA complexes which is associated with normal hermaphroditism (Kuersten *et al.*, 2004). ALY-3, also an RRM motif-containing protein is thought to promote recruitment of mRNA export factor to mRNAs; has no obvious effect on nuclear retention of *tra-2* mRNA in the absence of TRA-1

(Fortes *et al.*, 2007). Strikingly, the genes which encode the proteins NOL-5, FIB-1 T04A8.6, K07C3.4 and K07H8.10 (up-regulated in response to desiccation in *C. elegans* dauer larvae) are predicted to form genetic interactions with each other, i.e. are likely to have similar expression patterns and phenotypes (Zhong and Sternberg, 2006). These proteins may work in concert during desiccation to control gene expression. The up-regulation of proteins involved in protein synthesis in the preconditioning dataset may be a result of an effort to produce proteins that enable the nematode to survive exposure to desiccation. When the *C. elegans* dauer larvae are rehydrated after desiccation, the expression of proteins involved in translation increases again.

6.3.3 Protection

HSPs which are essential for the correct folding and maturation of a great diversity of client proteins and for protecting proteins from stress induced unfolding and aggregation (Lamark and Johansen, 2010). HSP-12.6 protein levels as well as *hsp-12.6* transcript levels (discussed in Chapter V) were statistically up-regulated in response to preconditioning, desiccation and recovery when compared to the control (Figure 6.10). Previous studies using, 2-D differential proteomics, have found HSP-12.6 to be up-regulated in *C. elegans* dauer larvae relative to the analogous L3 stage (Jones *et al.*, 2010). Similarly *hsp-12.6* has been found to be one of the most abundant transcripts in *C. elegans* dauer larvae (Jones *et al.*, 2001). A reduction of *hsp-12.6* expression by RNAi in *C. elegans* resulted in decreased survival in response to anoxia (Mendenhall *et al.*, 2005) and in another experiment *hsp-12.6* RNAi resulted in a statistically significant increase in the number of protein aggregates in the *C. elegans* intestine caused by infection (Mohri-Shiomi and Garsin, 2008).

In addition to its role in preventing protein aggregation during desiccation the up-regulation of HSP-12.6 may also play a role in regulating actin filament dynamic and stabilising microfilaments. Studies carried out on Chinese hamster CCL39 cell lines have found that small HSP-27 increased the stability of microfilaments (Guay *et al.*, 1997). Moreover, phosphorylation of small HSP-25 is induced in vascular endothelial cells *in vitro* by fluid shear stress, which is accompanied by extensive rearrangements of the microfilament cytoskeleton (Li *et al.*, 1996). The role of small HSPs in cytoskeleton stabilisation may also be important in desiccation tolerance in *C. elegans* dauer larvae. Furthermore, there are also a number of proteins related to cytoskeletal organization and

stability that were up-regulated across all three treatments, as discussed below.

The HSPs ENPL-1 and C30C11.4, were shown to be up-regulated specifically in response to recovery from desiccation when compared to the desiccation time-point. The RNA-seq analysis, discussed in the last chapter, revealed the transcripts of ENPL-1 and C30C11.4 were significantly up-regulated throughout preconditioning and desiccation. This could be to provide transcripts for protein synthesis during recovery from desiccation. ENPL-1 encodes an ortholog of the HSP-90 and endoplasmic reticulum (ER) chaperone GRP94/GP96 (Kapulkin *et al.*, 2005). Loss of *enpl-1* via RNAi results in slow growth, larval arrest, uncoordinated locomotion, and weak endocytosis defects (Kamath and Ahringer, 2003; Kapulkin *et al.*, 2005; Sonnichsen *et al.*, 2005). Furthermore, knockdown of *enpl-1* expression results in an increase in *hsp-4* expression, revealing compensatory regulation amongst ER chaperones (Kapulkin *et al.*, 2005). C30C11.4 encodes a member of the HSP-70 family of heat shock proteins. In large-scale RNAi screens loss of C30C11.4 activity results in a variety of defects including embryonic and larval lethality, slow growth rates, and locomotory and morphological defects (Kamath and Ahringer, 2003; Simmer *et al.*, 2003).

The protein DUR-1 was up-regulated in *C. elegans* dauer larvae in response to preconditioning as well as recovery. Originally identified as a transcript which was up-regulated in *C. elegans* dauer larvae relative to the L3 larval stage of this nematode (Cherkasova *et al.*, 2000), DUR-1 is a member of the LEA protein family (Browne *et al.*, 2004). RNAi studies have revealed that knockdown of *dur-1* results in an increase in fat storage (Ashrafi *et al.*, 2003). Therefore, *dur-1* may play a role in balancing energy levels with the effective utilisation of fat storage during desiccation. At the transcript level, *dur-1* had significantly higher expression only in the recovery dataset.

6.3.4 Cellular organisation and the cytoskeleton

The cytoskeleton regulates cell mechanics and is also involved in many basic cell processes ranging from cytoplasmic transport of proteins to stress response regulation (Carberry *et al.*, 2009). These events require coordinated responses of all three major cytoskeleton networks which are the actin filaments, the tubulin-based microtubules as well intermediate filament proteins (Carberry *et al.*, 2009). The differential proteomic

data identified a number of proteins associated with the cytoskeleton which were up-regulated in response to preconditioning, desiccation as well as recovery when they are compared to the control nematodes (Table 6.9). The highest number of up-regulated proteins mapping to GO terms associated with cellular organisation were found in the recovery dataset (Table 6.8). This indicates that cellular organisation may be a more important physiological response in the recovery stage upon rehydration after desiccation. There are only five proteins that are up-regulated in response to all of the conditions: FLN-1, GSNL-1, TBA-2, UNC-22 and UNC-70. The RNA-seq analysis discussed in Chapter V reveals that *tba-2*, *fln-1* and *unc-70* are up-regulated in response to desiccation (Figure 6.8).

From Figure 6.7 it can be seen that the cytoskeletal proteins TBA-2 (alpha tubulin), FLN-1 (filamin A, an actin cross-linking protein) and GSNL-1 (gelsolin, an actin regulatory protein) increased in abundance by four to eight fold in response to all three treatments and that CAP-1 (F-actin capping protein) and KETN-1 (kettin, an actin binding protein) showed a pattern of increasing abundance from preconditioning through dessication, with maximum protein expression being detected in the recovering nematodes. The actin monomer-binding protein PFN-2 (profilin) peaked in abundance in the preconditioned and desiccated samples, but its level had returned to control values in the recovering nematodes. These results imply a role for microtubules and the actin cytoskeleton in the desiccation response in *C. elegans* dauer larvae. The microtubule system determines the positions of membrane-enclosed organelles in cells, while the actin cytoskeleton lies under the plasma membrane, providing strength and shape to the cells' surface (Deward and Alberts, 2008). Dynamic reorganisation of the actin cytoskeleton transforms cell shapes. This may be important during desiccation when the cytoskeleton is put under extreme strain as cells dehydrate.

The gelsolin family is one of the largest classes of actin-filament severing proteins (Ono, 2007). *C. elegans* GSNL-1 has four gelsolin like domains (Liu and Ono, 2013). Gelsolin's potent severing activity allows it to modulate actin dynamics by dismantling existing polymers to produce a pool of capped shorter oligomers. These are primed to either nucleate rapid and directed filament growth upon dissociation of gelsolin by an uncapping agent, or to completely depolymerize and supplement the cytoplasmic pool of actin monomers (reviewed by Nag *et al.,* 2013). Profilin, which peaked in abundance in the preconditioned and desiccated samples, binds actin monomers in a 1:1 complex (Lodish *et*

al., 2000) and profilin-bound actin can participate in filament elongation (Xue and Robinson, 2013). CAP-1 an actin capping protein was up-regulated in response to desiccation and recovery. Actin filaments are crosslinked by filamin. Filamins stabilize delicate three-dimensional actin webs and link them to cellular membranes (Stossel *et al.*, 2001). FLN-1 was up-regulated in the *C. elegans* dauer larvae in response to all of the experimental conditions. Filamins (FLN) are log, flexible, multi-domain dimeric proteins, with each an actin-binding domain at each N-terminus (Demaso *et al.*, 2011). There are two filamin genes in *C. elegans*, of which *fln-1* encodes 3 isoforms. FLN-1 is structurally very similar to vertebrate filamins, particulary in the actin binding domain, and its isoforms have a wide distribution in muscle, epidermis and gonad tissue (Demaso *et al.*, 2011).

C. elegans contains a single spectraplakin gene, *vab-10.* Spectraplakins are evolutionarily conserved multifunctional cytoskeletal proteins that coordinate cytoskeletal elements through direct binding to different cytoskeletal networks (Suozzi *et al.*, 2012). VAB-10 was up-regulated in the preconditioning and recovery datasets. In *C. elegans* VAB-10 exists in two main isoforms VAB-10A and VAB-10B. Bosher *et al.* (2003) found that VAB-10A and VAB-10B have distinct distributions and functions in the nematode epidermis. Studies with mutants led them to propose that VAB-10A protects epidermal cells against forces external to the epidermis while VAB-10B protects cells against tension that builds up within the epidermis. UNC-70, which was up-regulated in response to all three treatments is a homolog of beta-spectrin, a protein found at the plasma membrane of most animal cells. Loss of beta-spectrin in *C. elegans* leads to disorganization of the myofilament lattice, discontinuities in the dense bodies, and a reduction or loss of the sarcoplasmic reticulum - defects which are consistent with beta-spectrin function in anchoring proteins at cell membranes (Hammarlund *et al.*, 2000). FRM-1, a member of the ezrin/radixin/moesin (ERM) family of actin-binding proteins was up-regulated in the recovering nematodes (Table 6.9). ERM proteins act both as linkers between the actin cytoskeleton and plasma membrane proteins and as signal transducers in responses involving cytoskeletal remodelling (Tsukita and Yonemura, 1999). ERM proteins are activated by osmotic shrinkage in mouse ascites cells (Rasmussen *et al.*, 2008). Myotactin which is encoded by *let-805* is a transmembrane protein containing extracellular fibronectin repeats (Hresko *et al.*, 1999). Genetic analyses indicate that LET-805 is essential for proper association between hypodermal fibrous

organelles and the contractile apparatus of muscle cells.

IFA-1 which is a intermediate filament (IF) protein is one of the proteins up-regulated specifically in response to recovery. Intermediate filaments are required provide tensile strength for cells. In humans, point mutations in IF genes lead to diseases associated with skin fragility, which indicates a role in cellular resistance to mechanical stress (Fuchs and Weber, 1994). The *C. elegans* genome contains 11 genes coding cytoplasmic IFs and of these IFA-1 is required for survival past the L1 larva stage, and a normal intestine (Karabinos *et al.*, 2001).

KETN-1, an ortholog of kettin in *D. melanogaster*, showed a pattern of increasing abundance from preconditioning through desiccation, with maximum protein expression being detected in the recovering nematodes. It is expressed in the reproductive system as well as in several muscle types in *C. elegans* and is up-regulated in response to hypoxia (Shen *et al.*, 2005). KETN-1 colocalizes with actin to I-bands in muscle cells (Ono *et al.*, 2005). RNAi knockdown of *ketn-1* reveals that it may be important in myofibrillar organisation and providing mechanical stability to the myofibrils during contraction (Ono *et al.*, 2006). The following proteins which are important in myofibrillar organisation were also up-regulated: UNC-89, up-regulated in response to preconditioning and recovery, is required for proper organization of A bands in striated muscle and thus for fully normal locomotion, pharyngeal muscle contractions, and body size (Qadota *et al.*, 2008); UNC-54 the major myosin heavy chain expressed in *C. elegans* was up-regulated in recovering nematodes and and is required for locomotion and UNC-22, up-regulated in response to all three treatments is a giant intracellular protein with multiple fibronectin- and immunoglobulin-like domains which is required in muscle for regulation of the actomyosin contraction-relaxation cycle and for maintenance of normal muscle morphology (Benian *et al.*, 1989).

Previous proteomic studies, in the moss *Physcomitrella patens* have revealed many cytoskeleton proteins differentially expressed in response to desiccation (Wang *et al.*, 2009). These proteins associated with actin, tubulin and myosin heavy chain were mainly down-regulated in response to desiccation in contrast to our study in *C. elegans* dauer larvae. This group hypothesized that the cytoskeleton is disassembled in response to

desiccation to limit the damage caused by the stress. An experiment carried out in another moss, *Polytrichum formosum*, using scanning electron microscopy has also revealed a breakdown in the cytoskeleton endoplasmic microtubules in response to desiccation (Pressel *et al.*, 2006). GO Enrichment for Actin Filament Organization was also detected in a recent study on the dehydration tolerant midge *Belgica antarctica* (Teets *et al.*, 2012), but the identities of the up-regulated genes were not provided.

6.3.5 Detoxification and repair mechanisms

Reactive oxygen species (ROS) accumulate in the cells as a result of cellular dehydration as discussed in Chapter V. ROS cause oxidative damage to proteins, lipids, DNA and other macromolecules (Temple *et al.*, 2005). In response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae there was an up-regulation of several proteins capable of detoxifying ROS (Table 6.14). The catalase protein CTL-1 was up-regulated in response to preconditioning and desiccation. This correlates well with expression at the transcript level as *ctl-1* is up-regulated in response to preconditioning, desiccation and recovery (Figure 5.23). Moreover, the expression of *ctl-1* messenger RNA has been found to be increased in *C. elegans* dauer larvae when compared with non dauers (Waterston *et al.*, 1992). *ctl-1* encodes one of three *C. elegans* catalases. Catalase may provide protection against hydrogen peroxide that is generated as a by-product of peroxisomal fatty-acid ß-oxidation or offer a more general antioxidant defence. CTL-1 does not possess a C-terminal peroxisomal targeting signal, therefore it is predicted to be a cytosolic catalase. The most active form of catalase is CTL-2 which accounts for 80% of total catalase activity. The cytosolic catalase, CTL-1, is responsible for the rest of the catalase activity (Petriv and Rachubinski, 2004).

In the recovery samples there was an up-regulation of SOD-2 which was also seen at the transcript level (in response to recovery when compared to desiccation). SOD-2 was not even detected in any of the other samples. SOD is a detoxification enzyme that converts superoxide to hydrogen peroxide, which can subsequently be converted to water and oxygen by catalase (Fridovich, 1995). Knockout studies of the mitochondrial SOD-2 have resulted in increased oxidation stress in mice (Carlsson *et al.*, 1995) and a reduction in lifespan in flies (Kirby *et al.*, 2002; Duttaroy *et al.*, 2003). In *C. elegans* the knockout of

SOD-2 showed no reduction in lifespan but an increased sensitivity to oxidative stress was observed (Van Raamsdonk and Hekimi, 2009).

Also up-regulated specifically in the recovery dataset was GST-7. Glutathione S-transferases (GSTs) are a diverse super-family of multifunctional proteins that play prominent roles in detoxification in nematodes (Lindblom and Dodd, 2006). GSTs catalyzing the conjugation of glutathione with a wide range of endogenous and xenobiotic alkylating agents, including environmental toxins and endogenous products of oxidative stress. Adhikari *et al.* (2009) have previously found that a GST transcript was up-regulated in response to desiccation stress in the nematode *Plectus murrayi*. Glutathione is also used as a co-factor by glutaredoxin (Grx) proteins. Grxs are ubiquitous antioxidants enzymes present in most living organisms from viruses and bacteria to mammals (Sagemark *et al.*, 2007). In this study GLRX-5 was up-regulated in response to preconditioning and recovery while GLRX-10 was up-regulated in response to preconditioning and desiccation. RNAi knockdown studies of the *glrx-5* gene have resulted in embryonic and larval lethality as well as slow growth (Kamath and Ahringer, 2003; Sonnichsen *et al.*, 2005). Up-regulation of a *glrx* transcript in the dauer stage of the the nematode *Steinernema carpocapsae* in response to desiccation was also observed by Tyson *et al.* (2007). A previous study by Reardon *et al.* (2010) using expressed sequence tags has found that transcripts encoding glutathione peroxidase are up-regulated in response to preconditioning in the nematode *A. avenue*. Moreover, Reardon *et al*. knocked down the expression of glutsthione peroxidase in *P. superbus* nematodes using RNAi and this was shown to reduce desiccation tolerance in this species.

The GO analysis, shown in Table 6.10, indicates that a number of proteins involved in chromosome organisation and DNA packaging are up-regulated mainly in response to preconditioning and desiccation. In these two datasets, four up-regulated proteins map to the chromatin organization GO term which include histone related proteins HIS-45, HIL-1, HTZ-1 as well as the chromosome modifier CEC-5. HIS-45 and HTZ-1 are predicted to function as core histones H3 and H2A respectively. In addition to their well-known function in DNA packaging these core histones are also involved in DNA replication, transcription and repair by controlling the accessibility of the different macromolecular machineries to their substrate (Escargueil *et al.*, 2008). Moreover, the histone variant HTZ-1 promotes transcription by modifying how DNA is packaged within cells (Updike and Mango, 2006). The transcripts encoding HTZ-1 were also found to be up-regulated in

the RNA-seq desiccation dataset (Chapter V). HIL-1 protein contains similarities to linker histones which are associated with interphase chromatin as well as with condensed chromosomes (Jedrusik *et al.*, 2002).

As discussed in the last chapter, *C. elegans* nematodes rely on the ubiquitin-proteasome systems as well as autophagy systems to remove the damaged proteins that may accumulate during desiccation. Proteins involved in the proteasome were up-regulated in response to preconditioning, desiccation and recovery (Table 6.12). RPN-11, which is an ATPase-like proteasome regulatory particle, was up-regulated in response to all three treatments (at both the transcript and protein level) while RPT-3, a non ATPase-like proteasome regulatory particle is up-regulated in the preconditioning and recovery datasets. Interestingly, yeast two-hybrid assays carried out by Davy *et al.* (2001) have revealed that RPT-11 interacts with itself, RPN-9 and F55A11.3. RPT-11 and RPN-9 may work together to remove damaged proteins during desiccation. *rpt-3* encodes a triple A ATPase subunit of the 26S proteasome's 19S regulatory particle base subcomplex. The 26S proteasome is formed by the interaction of two sub complexes, a 20S particle which is the protealytic core and a 19S regulatory particle which caps the 20S at both ends. RPT-3 is required for embryonic, larval and germline development in *C. elegans*. RPT-3 by homology is predicted to function in unfolding protein substrates and translocating them into the core proteolytic particle of the proteasome (Davy *et al.*, 2001). RPN-11 is a predicted non-ATPase subunit of the 19S regulatory complex of the proteasome. According to RNAi studies, knockdown of this gene affects adult viability, osmoregulation, embryonic viability, movement and increased protein aggregation (Kamath and Ahringer, 2003; Nollen *et al.*, 2004).

Two ubiquitin related proteins UBH-4 and SKR-1 were also up-regulated in response to recovery when compared with the desiccation dataset. Transcripts which encode SKR-1 were also up-regulated in the preconditioning dataset (Chapter V). UBH-4 is a ubiquitin C-terminal hydrolase while SKR-1 is part of an ubiquitin ligase family involved in recognition and ubiquitination of specific target proteins for degradation by the proteasome. SKR-1 binds the substrate-recognition subunit through an F-box motif of the SRS (the substrate recognition subunit) (Nayak *et al.*, 2002; Zheng *et al.*, 2002). In *C. elegans* RNAi studies have shown that *skr-1* is required for the restraint of cell proliferation, progression through the pachytene stage of meiosis, and the formation of

bivalent chromosomes at diakinesis (Nayak *et al.*, 2002). Interestingly there are 21 SKR genes in *C. elegans* relative to just one in yeast (Yamanaka *et al.*, 2002).

6.3.6 Comparing transcriptomic and proteomic studies

The genes and proteins which were up-regulated in all conditions in both the transcriptomic and proteomic studies are shown in Section 6.2.5. Ribosomal proteins made up 5 out of the total 14 genes and proteins up-regulated. This shows the importance of increased translation of proteins during desiccation in the low metabolism *C. elegans* dauer larvae developmental stage. Other genes found to be up-regulated in both lists were *hsp-12.6*, antioxidant *ctl-1*, proteasome regulatory particle *rpn-11* and cytoskeletal tubulin *tba-2*. These genes are a good overall representation of the categories of genes up-regulated in both the transcriptomic and proteomic datasets.

7 Chapter VII RNAi gene silencing of *C. elegans* dauer larvae and *P. superbus* nematodes to investigate desiccation tolerance

7.1 Introduction

RNAi (RNA interference) has become one of the most widely used tools in biology to study gene function (Boutros and Ahringer, 2008). Fire *et al.* (1998) first discovered that the injection of double-stranded RNA (dsRNA) into a hermaphrodite worm results in the degradation of endogenous mRNA corresponding in sequence to the injected dsRNA. This resulted in a loss of function phenotype for the target gene. Prior to these experiments, the less effective method of introducing sense and antisense RNA had been used to manipulate gene expression (Ecker and Davis, 1986; Fire *et al.*, 1998; Boutros and Ahringer, 2008). In the original RNAi experiment using dsRNA, expression of the *unc-22* gene, which encodes an abundant but nonessential myofilament protein, was knocked down in *C. elegans*. This resulted in a strong twitching phenotype in the injected worms, as well as their progeny. In RNAi gene silencing experiments, the dsRNA introduced into the organism leads to the degradation of messenger RNA (mRNA) that has an identical sequence to it. RNAi is initiated by the cleavage of exogenous dsRNA into short interfering RNAs (siRNAs) by a dsRNA-specific RNAse enzyme Dicer. These siRNAs activate a multiprotein RNA-induced silencing complex (RISC) that cleaves RNA molecules identical to the siRNA (Hammond *et al.*, 2000; Nykanen *et al.*, 2001).

The RNAi experiments used in a functional analysis of desiccation response genes in *C. elegans daf-2* dauer larvae

RNAi has been used in the past to further study genes involved in desiccation tolerance in *C. elegans*. Gal *et al.* (2004) used RNAi feeding to knockdown mRNA corresponding to *lea-1* transcripts and found a functional role for this gene in relation to desiccation tolerance. In this study, control *C. elegans* dauer larvae and *lea-1* RNAi treated *C. elegans* dauer larvae were exposed to 97% RH for 8 h and 24 h. The partial silencing of *lea-1* transcription reduced the survival of the dauer larvae under mild desiccation. Gal *et al.* also found that silencing of *lea-1* transcription resulted in reduced dauer larvae survival in response to osmotic and heat stress. However, the survival was only tested under mild desiccation of 97% RH as harsher desiccation resulted in low survival for control dauer

larvae. Using the method of preconditioning developed by Erkut *et al.* (2010) outlined in Chapter V allows *C. elegans* dauer larvae to survive harsher desiccation. Therefore, in the RNAi experiments described in this chapter we have been able to use RNAi to conduct a functional analysis of desiccation response genes in *C. elegans daf-2* dauer larvae under harsh desiccation conditions.

7.2 Results

7.2.1 Selection of genes for RNAi investigation of desiccation tolerance *C. elegans* in dauer larvae

The candidate genes selected for the RNAi experiment encode proteins associated with diverse functions, which include: molecular chaperones (*hsp-12.6* and *sip-1*), molecular shield activity (*lea-1* and *dur-1*), antioxidants (*cdr-3*, *djr-1.1* and *djr-1.2*), pathogen defence (*fipr-24*), compatible solutes (*tps-1*, *tps-2* and *tre-3*), glycerol synthesis and metabolism (*gpdh-2* and *R11F4.1*), and transport (*aqp-1*, *aqp-11*, and *lbp-1*) as well as ten novel uncharacterised genes. The genes selected for the RNAi gene silencing experiment (Table 7.1) were based on the results of the transcriptomic and proteomic analyses of desiccation tolerance in *C. elegans* dauer larvae (Chapter V and Chapter VI). The current literature on genes involved in desiccation tolerance was also taken into account. Ten novel genes were also selected based on transcriptomic and proteomic data only. Many of the up-regulated genes and proteins from the proteomic and RNA-seq analyses carry out essential functions and consequently their RNAi phenotypes are lethal or have viability effects. Therefore, the selection of candidate genes for the RNAi study was curtailed to genes that either had no previously reported phenotype in large scale RNAi studies or had mild phenotypes which would not affect the desiccation survival results.

A total of 30 RNAi feeding constructs were generated, encompassing 28 different genes. Since multiple isoforms exist in the late embryogenesis abundant gene, *lea-1*, and the dauer up-regulated gene, *dur-1*, two constructs were made for different regions of these genes to include homology to most of the isoforms. Gene models of the *C. elegans* genes *lea-1* and *dur-1* displaying the various isoforms are shown in Figures 7.1 and 7.2. In the selection process, the genes with known functions were chosen first. The only gene which was significantly up-related in all conditions, relative to the control, at both the

transcriptomic and proteomic level was *hsp-12.6* which encodes a small heat shock protein. This

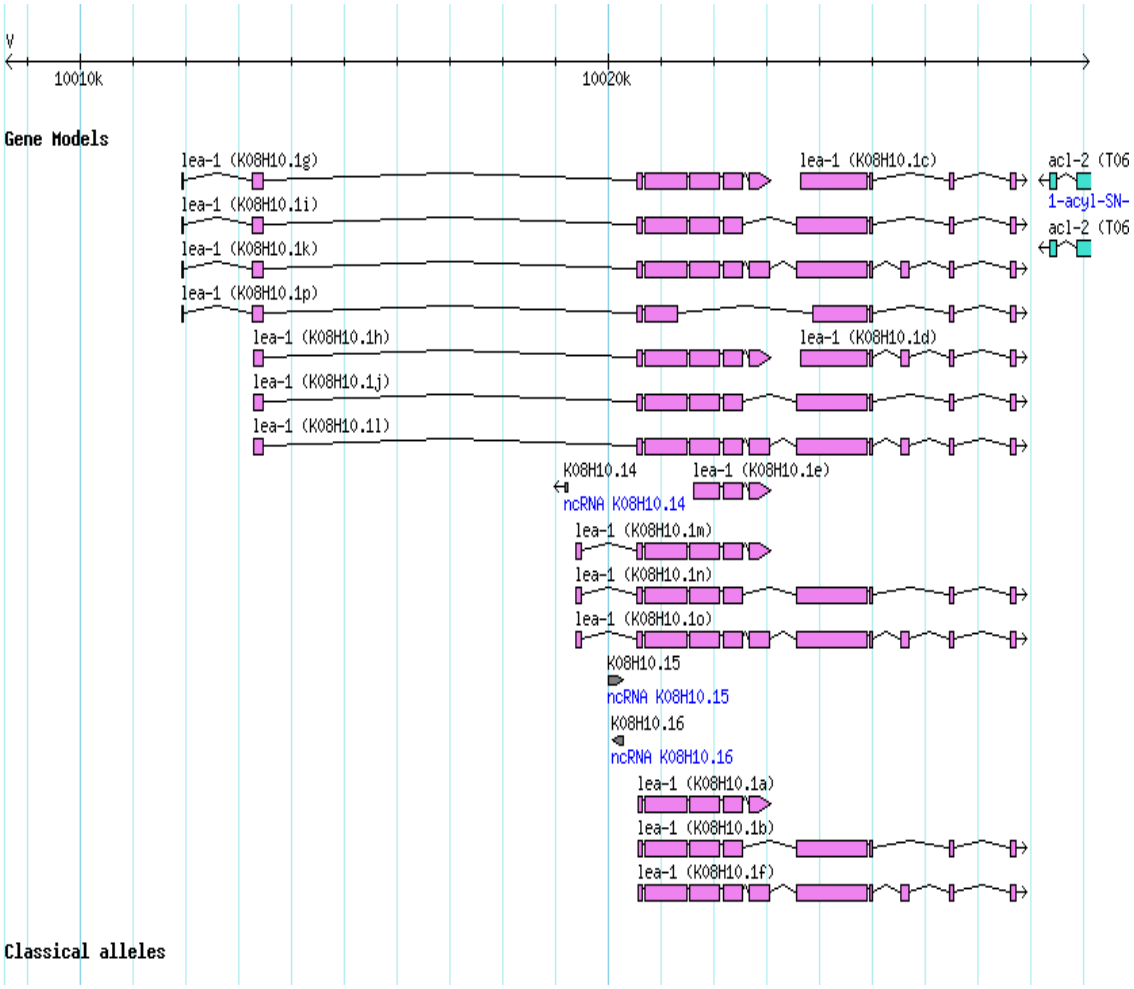


Figure 7.1 Gene model for the *C. elegans lea-1* gene (www.wormbase.org). Two RNAi feeding constructs were made for *lea-1* from the spliced sequences to encompass most of the isoforms.

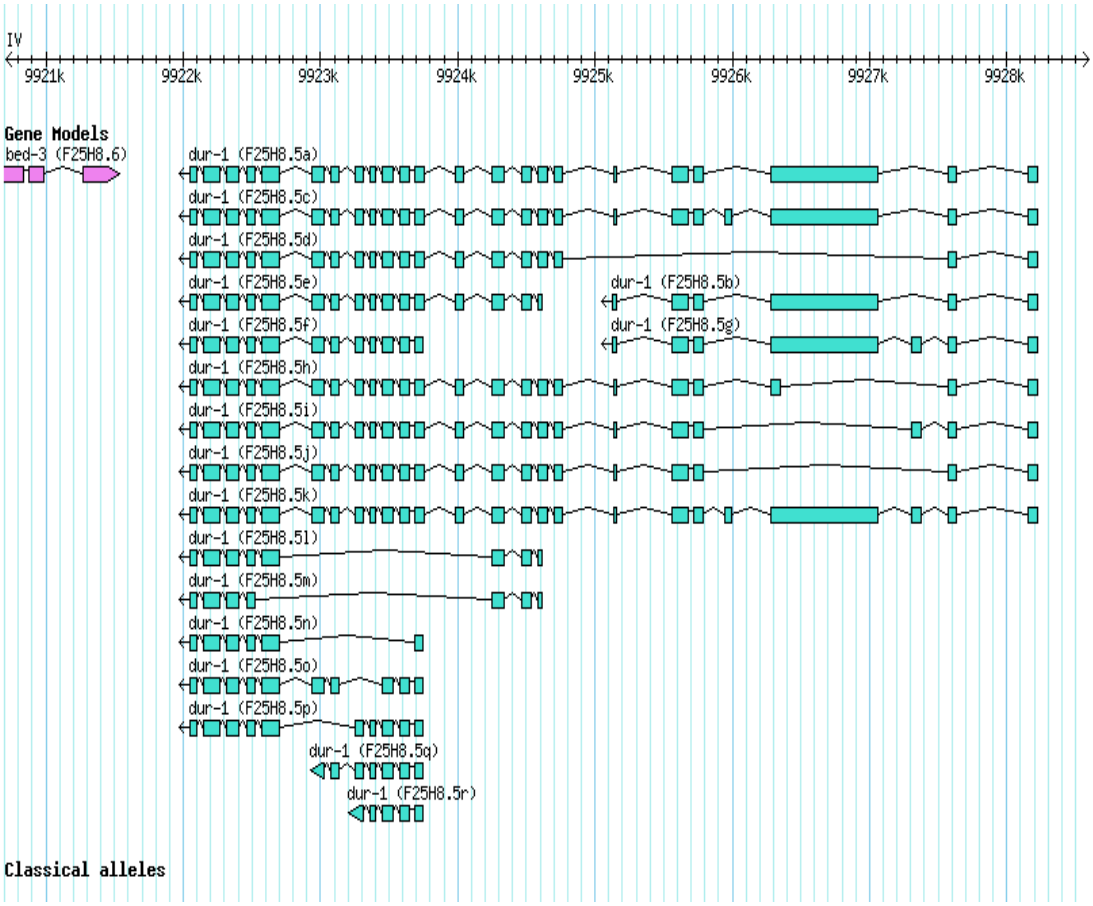


Figure 7.2 Gene model for the *C. elegans dur-1* gene (www.wormbase.org). Two RNAi feeding constructs were made for *dur-1* from the spliced sequences to encompass most of the isoforms.

warranted inclusion in the RNAi study in itself. Furthermore, Mohri-Shiomi and Garsin (2008) have recently shown a reduction of *hsp-12.6* expression by RNAi resulted in a statistically significant increase in the number of protein aggregates in the intestine cells of bacteria-infected nematodes. Therefore, *hsp-12.6* may also have a role in preventing aggregation which may occur during desiccation when proteins become damaged. Another gene called *sip-1*, which also encodes a small heat shock protein (BLASTp data show that it is a homolog of ß-alpha crystallin), was also selected for the RNAi study of desiccation in *C. elegans* dauer larvae. The cadmium responsive gene, *cdr-3*, and the dauer up-regulated gene, *dur-1*, were also included in the RNAi study based on up-regulation during preconditioning, desiccation and recovery at the transcript level and the up-regulation of CDR-3 protein in recovering nematodes. As discussed in Chapter V, cadmium exposure is correlated with the production of reactive oxygen species and lipid peroxidation (Dong *et al.*, 2005). Since ROS also accumulate during desiccation *cdr-3* may be implicated in desiccation tolerance. The dauer up-regulated gene (*dur-1*) is both heat resistant and hydrophilic and has been previously linked to anhydrobiosis (Browne *et al.*, 2004).

In *C. elegans*, two putative trehalose-6-phosphate genes (known as *tps-1* and *tps-2*) encode the enzymes required for trehalose synthesis. These genes were up-regulated in response to desiccation and recovery in *C. elegans* dauer larvae. Previous studies, using a double knockout mutant of trehalose phosphate synthase genes have shown reduced resistance to desiccation in *C. elegans* dauer larvae (Erkut *et al.*, 2011). Thus RNAi feeding constructs were made for both *tps-1* and *tps-2* genes. Another gene called *tre-3* (trehalase) was selected for the RNAi investigation of desiccation tolerance in *C. elegans* dauer larvae. In *C. elegans*, five putative trehalase genes encode enzymes which catalyse the hydrolysis of trehalose (Pellerone *et al.*, 2003). The *tre-3* gene was found to have significantly increased expression in the recovery dataset when compared to the desiccation in the *C. elegans* RNA-seq analysis of desiccation (Chapter V). In recent studies carried out in *Arabidopsis thaliana*, it was discovered that overexpression of a trehalase gene resulted in increased drought stress tolerance (Van Houtte *et al.*, 2013). Therefore, the effect of *tre-3* RNAi on desiccation tolerance in *C. elegans* dauer larvae was investigated in this project.

Several different fungus induced protein (FIP) transcripts were up-regulated in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae. The up-regulation of transcripts that encode FIPs could possibly be a mechanism to prevent fungal infection which *C. elegans* dauers may be more susceptible to during desiccation (Pujol *et al.*, 2008). The *fipr-24* (fungus induced protein related) gene was significantly up-regulated in response to each of the desiccation related conditions, which justified its inclusion in the RNAi investigation of desiccation-tolerance in *C. elegans*. Novel genes with no definitive function in *C. elegans* were also selected based on up-regulation in response to desiccation and recovery in our transcriptomic and proteomic investigation. The novel gene and corresponding protein of B0491.5 was up-regulated in *C. elegans* dauer larvae in response to preconditioning, desiccation and recovery both at the transcriptional level and proteomic level. In contrast, the genes *F11F1.4, Y56A3A.33, F55C12.6* and *Y17G7B.17* are only up-regulated at the transcriptional level with up-regulation occurring in response to all conditions relative to the control. Also at the transcriptional level, the genes *K07H8.10* and *F49E2.5* are up-regulated in response to desiccation while the gene *F21C10.10* has increased expression during recovery from desiccation. The proteins R13H4.2 and C44B7.10 were both up-regulated in response to two or more conditions as shown in Table 7.1.

7.2.2 RNAi by feeding in *C. elegans daf-2* (e1370) dauer larvae

C. elegans RNAi feeding constructs were designed, created and sequenced as described in Section 2.7. An *unc-22* feeding construct was used as a positive control to evaluate the effectiveness of each RNAi experiment. RNAi knockdown of the *C. elegans unc-22* gene causes a pronounced body surface twitch associated with impaired movement and disruption of muscle structure (Fire *et al.*, 1998). RNAi silencing of genes and subsequent desiccation survival assays were carried out as described in Section 2.7.9. A schematic of the workflow for the *C. elegans daf-2* RNAi experiment is shown in Figure 7.5. Additionally, diagrams displaying different stages of the experimental procedure are shown in Figures 7.6-7.8. Figure 7.6 (i) shows L1 larvae feeding on an *E. coli aqp-11* dsRNA expression construct; Figure 7.6 (ii) shows the RNAi-*aqp-11* treated dauer larvae obtained when treated *daf-2* (e1370) L1 larvae are incubated at 25^{o}C. Figure 7.7 shows RNAi treated *C. elegans* dauer larvae which have been preconditioned at 98% RH for 96 h and desiccated at 32.5% RH for 24 h. The larvae form loose clumps, with the nematodes

at the edges of the clumps tending to be loosely coiled. Figure 7.8 shows the importance of preconditioning at 98% RH for 96 h on the desiccation tolerance of *C. elegans daf-2* (e1370) dauer larvae. Lack of this preconditioning step causes 100% mortality of the dauer larvae when they are exposed to activated silica gel.

Table 7.1 Genes selected for RNAi silencing experiments to investigate desiccation tolerance in *C. elegans* dauer larvae. The gene name, gene ID, gene description, result of the RNA-seq analysis of desiccation (Chapter V), the result of the proteomic LFQ analysis of desiccation (Chapter VI), the RNAi construct length and the result of previous RNAi experiments for undesiccated *C. elegans* nematodes are shown. (NA = not detected; LFQ = Label Free Quantification).

Gene name	**Gene ID**	**Description**	**RNA-seq analysis**	**Orbitrap proteomic analysis**	**RNAi construct gene length**	**Previously discovered RNAi phenotype**
lea-1A	K08H10.1	Late embryonic abundant protein	Up in all conditions	NA	858/723	Hypersensitive to desiccation, osmotic stress response variant, thermotolerance reduced
djr-1.2	C49G7.11	DJ-1 related, a putative redox related molecular chaperone	Up in all conditions	NA	516	Protein aggregation variant, shortened lifespan
fipr-24	C37A5.8	Fungus induced protein related	Up in all conditions	NA	200	Non-detected
cdr-3	C54D10.2	Cadmium responsive	Up in all conditions	Up in recovery	808	Non-detected
hsp-12.6	F38E11.2	Heat shock protein	Up in all conditions	Up in all conditions	300	Life span variant, osmotic stress response variant, physiology variant
tps-2	F19H8.1	Trehalose 3-phosphate synthase	Up in all conditions	NA	1607	Intraflagellar transport variant, life span variant, organism osmotic stress response variant, shortened lifespan, thermotolerance reduced
F11F1.4	F11F1.4	Novel	Up in all conditions	NA	622	Non-detected
Y56A3A.33	Y56A3A.33	Novel	Up in all conditions	NA	856	Non-detected
F55C12.6	F55C12.6	Novel	Up in all conditions	NA	815	Non-detected
Y17G7B.17	Y17G7B.17	Novel	Up in all conditions	NA	704	Non-detected
B0491.5	B0491.5	Novel	Up in all conditions	Up in all conditions	817	Non-detected

Table 7.1 (continued) Genes selected for RNAi silencing experiments to investigate desiccation tolerance in *C. elegans* dauer larvae. The gene name, gene ID, gene description, result of the RNA-seq analysis of desiccation (Chapter V), the result of the orbitrap proteomic analysis of desiccation (Chapter VI), the gene length and the result of previous RNAi experiments for undesiccated *C. elegans* nematodes is shown. (NA = not detected; LFQ = Label Free Quantification).

Gene name	**Gene ID**	**Description**	**RNA-seq analysis**	**Orbitrap proteomic analysis**	**RNAi construct gene length**	**Previously discovered RNAi phenotype**
tps-1	ZK54.2	Trehalose 3-phosphate synthase	Up in desiccation and recovery	NA	593	Life span variant, organism osmotic stress response variant, Protein aggregation variant, shortened lifespan, thermotolerance reduced
cey-2	F46F11.2	Cold-shock/Y-box domain-containing protein	Up in preconditioning, desiccation	NA	710	Non-detected
R11F4.1	R11F4.1	Glycerol kinase	Up in preconditioning, recovery	NA	1299	Non-detected
sip-1	F43D9.4	Stress Induced Protein, an sHSP protein	Up in desiccation	NA	406	Life span variant, embryonic lethality
oxi-1	Y39A1C.2	Oxidative stress induced	Up in desiccation	NA	861	Non-detected
K07H8.10	K07H8.10	Novel (RNA recognition domain)	Up in desiccation	Up in all conditions	971	Non-detected
F49E2.5	F49E2.5	Novel	Up in desiccation	Up in preconditioning/ recovery	916	Non-detected
dur-1	F25H8.5	Dauer up-regulated	Up in recovery	Up in preconditioning/ recovery	400/600	Fat content increased
aqp-1	F32A5.5	Aquaporin	Up in recovery	NA	809	Shortened life span, transgene expression variant

Table 7.1 (continued) Genes selected for RNAi silencing experiments to investigate desiccation tolerance in *C. elegans* dauer larvae. The gene name, gene ID, gene description, result of the RNA-seq analysis of desiccation (Chapter V), the result of the orbitrap proteomic analysis of desiccation (Chapter VI), the gene length and the result of previous RNAi experiments for undesiccated *C. elegans* nematodes is shown. (NA = not detected; LFQ = Label Free Quantification).

Gene name	**Gene ID**	**Description**	**RNA-seq analysis**	**Orbitrap proteomic analysis**	**RNAi construct gene length**	**RNAi phenotype**
aqp-11	ZK525.2	Aquaporin	Up in recovery	NA	730	Non-detected
lbp-1	F40F4.3	Lipid binding protein	Up in recovery	NA	451	Non-detected
tre-3	W05E10.4	Trehalase	Up in recovery	NA	1209	Non-detected
F21C10.10	F21C10.10	Novel	Up in recovery	NA	633	Non-detected
djr-1.1	B0432.2	DJ-1 related, a putative redox related molecular chaperone	Not up-regulated	NA	501	Shortened lifespan
gpdh-2	K11H3.1	Glycerol 3-phosphate dehydrogenase	Not up-regulated	NA	873	Non-detected
R13H4.2	R13H4.2	Novel	Not up-regulated	Up in all conditions	552	Non-detected
C44B7.10	C44B7.10	Novel	Not up-regulated	Up in preconditioning/ recovery	935	Non-detected

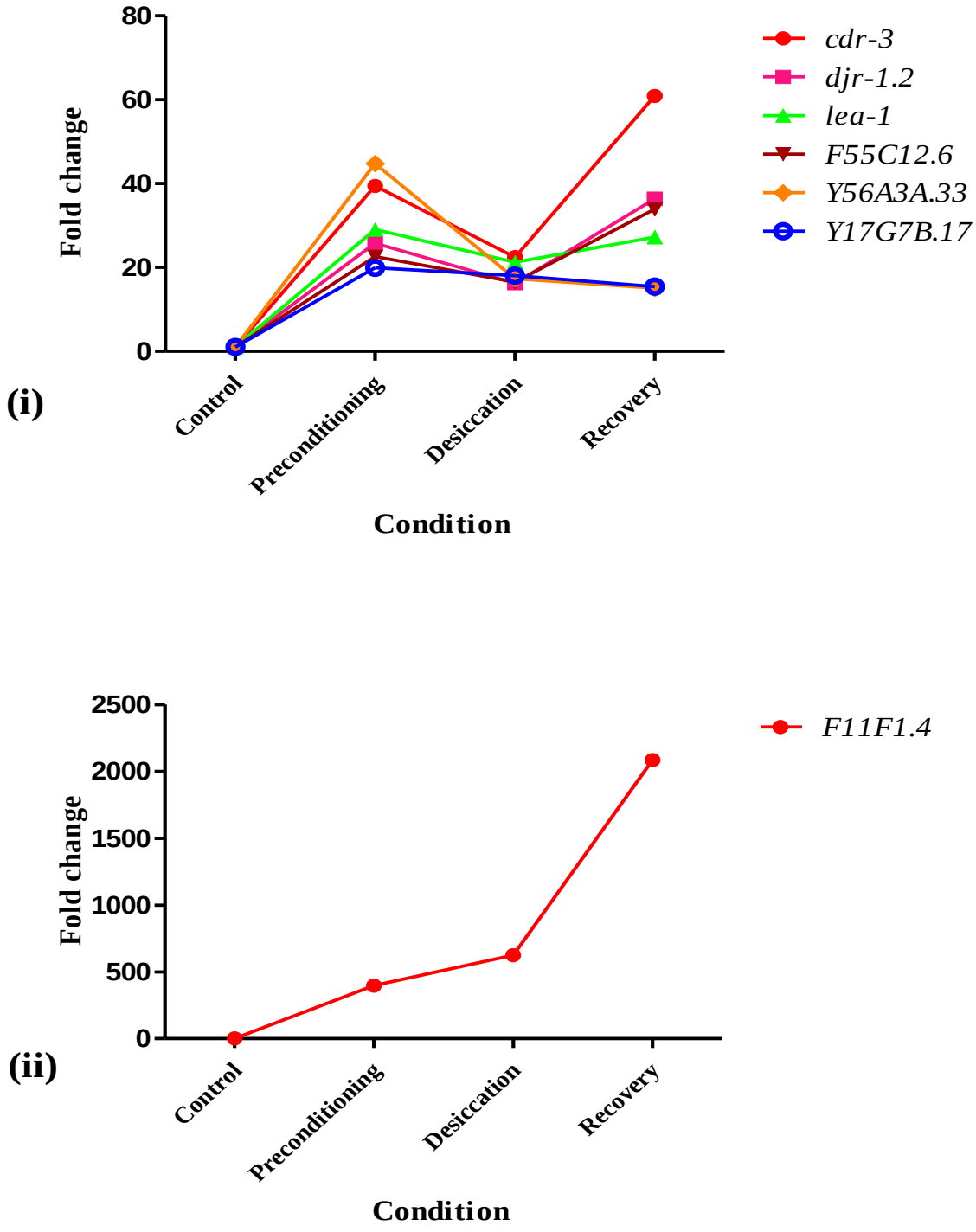


Figure 7.3 Change in expression of the genes (RNA-seq transcript counts) used in the RNAi feeding experiment. Values were obtained from RNA-seq analysis discussed in Chapter V. The genes shown are (i) *cdr-3*, *djr-1.2*, *lea-1*, *F55C12.6*, *Y56A3A.3*, *Y17G7B.17* (ii) and *F11F1.4*.

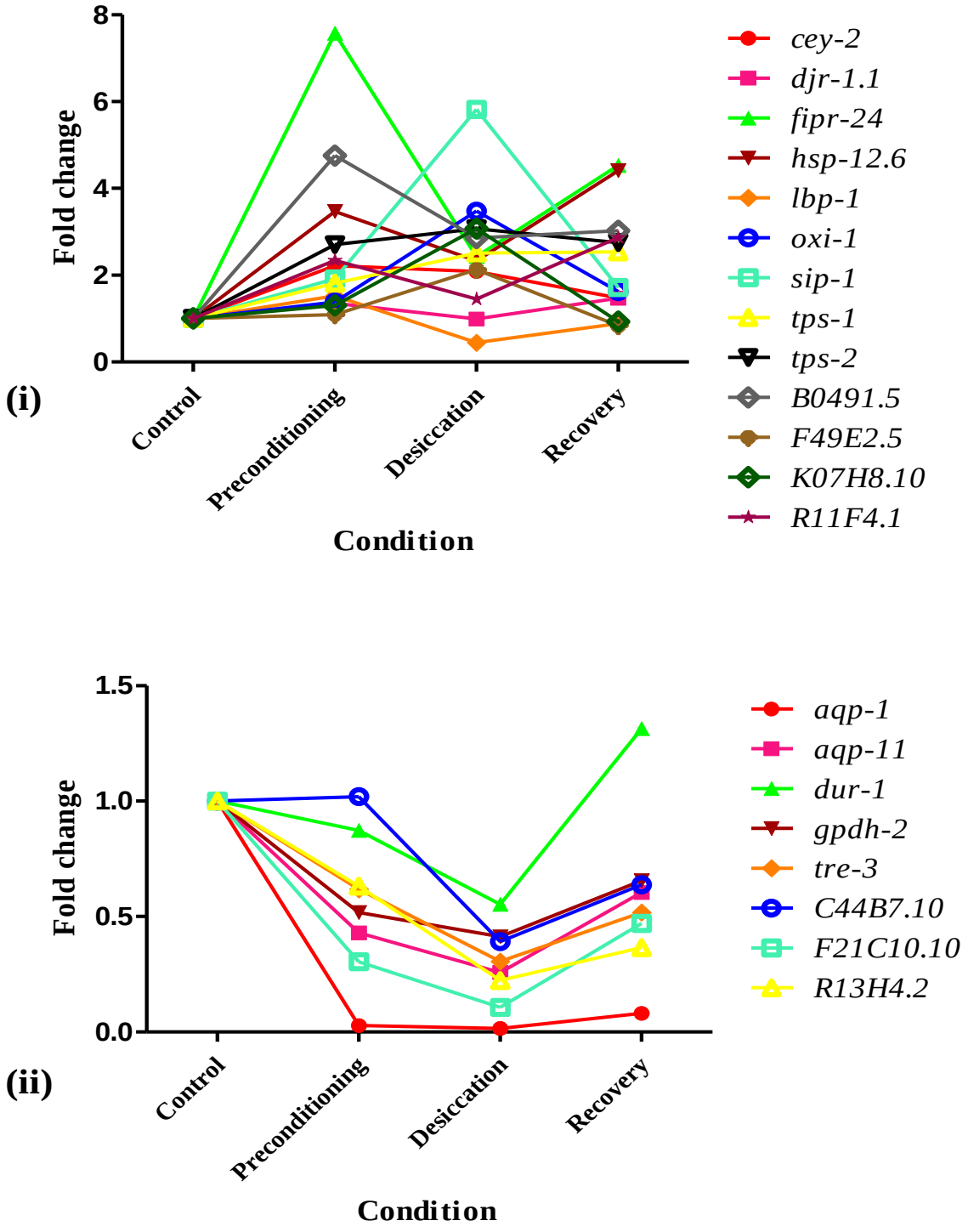


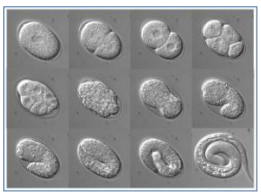
Figure 7.4 Change in expression of the genes (RNA-seq transcript counts) used in the RNAi feeding experiment. Values were obtained from RNA-seq analysis discussed in Chapter V. The genes shown are (i) *cey-2*, *djr-1.1*, *fipr-24*, *hsp-12.6*, *lbp-1*, *oxi-1*, *sip-1*, *tps-1*, *tps-2*, B0491.5, *F49E2.5*, *K07H8.10*, *R11F4.1* (ii) *aqp-1*, *aqp-11*, *dur-1*, *gpdh-2*, *tre-3*, *C44B7.10*, *F21C10.10* and *R13H4.2*.

7.2.3 *C. elegans* RNAi results

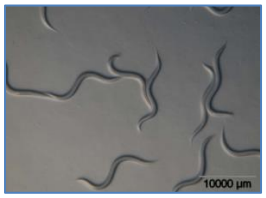
C. elegans L1s fed with an *E. coli* clone expressing *unc-22* dsRNA expressed a twitching phenotype, confirmed the effectiveness of the RNAi feeding protocol. This twitching phenotype was maintained as the *C. elegans* L1s developed into dauer larvae and when the dauer larvae recovered from desiccation (98% RH for 96 h, 32.5% RH for 24 h and rehydration for 16 h). The RNAi knockdown of *lea-1*, *dur-1*, *hsp-12.6*, *tps-1/2*, glycerol kinase, *F21C10.10* and *C44.B7.10* resulted in a statistically significant reduction in survival when exposed to desiccation compared to the control (Figure 7.9). The most significant reduction in survival was observed in the knockdown of *lea-1* (late embryonic abundant), *dur-1* (dauer up-regulated) and *tps-1/2* (trehalose phosphate synthase) (P<0.001, Dunnett's multiple comparison test). Knockdown of both *lea-1* and *tps-1/2* genes in previous studies has resulted in reduced survival in response to desiccation in *C. elegans* dauer larvae (Gal *et al.*, 2004; Erkut *et al.*, 2011). Interestingly, RNAi knockdown of *tps-1* and *tps-2* individually did not significantly reduce survival but when the two genes were knocked down simultaneously, a highly significant reduction in survival was observed. This indicates that redundancy exists between *tps-1* and *tps-2* in *C. elegans* nematodes exposed to desiccation. RNAi knockdown of the novel genes *F21C10.10* and *C44.B7.10* resulted in reduced survival in response to desiccation.

Mixed population of *C. elegans daf-2* worms were grown at 15 ^{O}C.

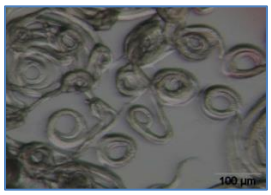
Worms were bleached to obtain eggs and hatched overnight in S Basal buffer to produce worms in the first larval stage (L1).



L1 worms were plated on different HT-115 RNAi feeding constructs and were incubated at 25 ^{O}C to induce dauer larvae.



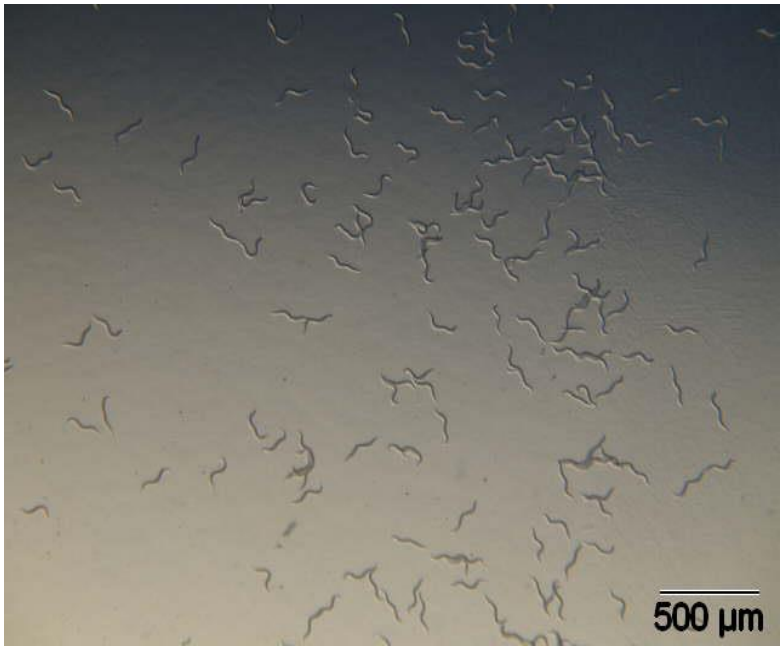
Dauer larvae were washed off and preconditioned at 98% RH for 4 days followed by desiccation at 32.5% RH for 24 h.



Dauer larvae were rehydrated for 16 h and % survival was assessed.

Figure 7.5 Schematic of the workflow for the RNAi experiments used in a functional analysis of desiccation response genes in *C. elegans daf-2* dauer larvae.

(i)



(ii)

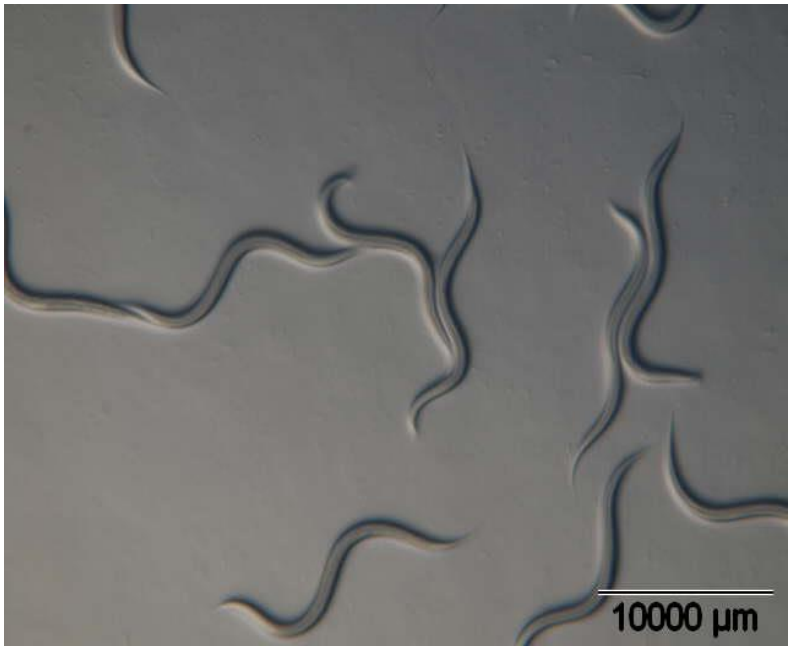
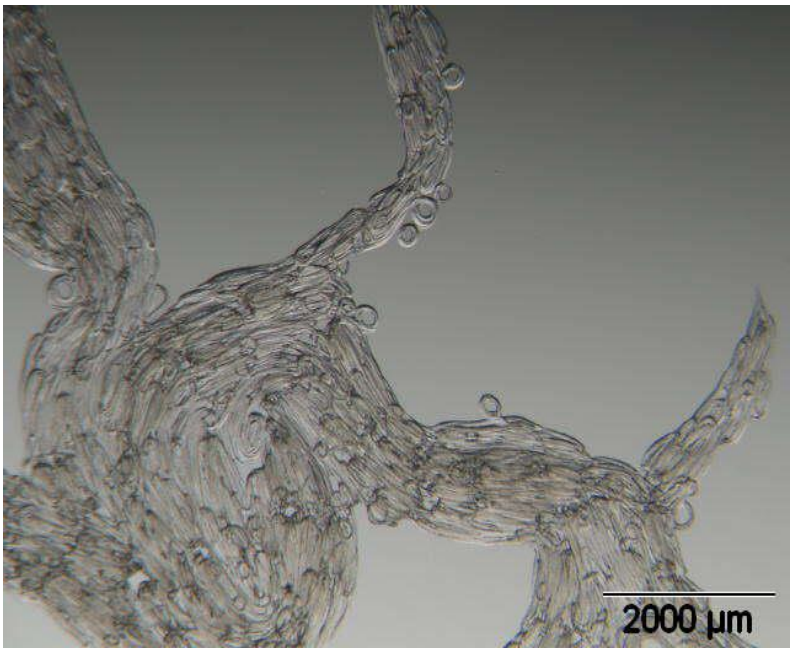


Figure 7.6 (i) *C. elegans* L1 stage worms feeding on HT115(DE3) bacteria expressing dsRNA for *aqp-11* (aquaporin), incubated at 25^{o}C to produce dauer larvae. (ii) *C. elegans* dauer larvae produced after feeding on HT115(DE3) bacteria expressing dsRNA for a target gene of interest for 5 days. No differences between the control *C. elegans* dauer larvae and the RNAi treated dauer larvae were observed at this point. Photographic data are only presented for RNAi-*aqp-11* treated dauer larvae as all RNAi treated and control dauer larvae gave similar results.

(i)



(ii)

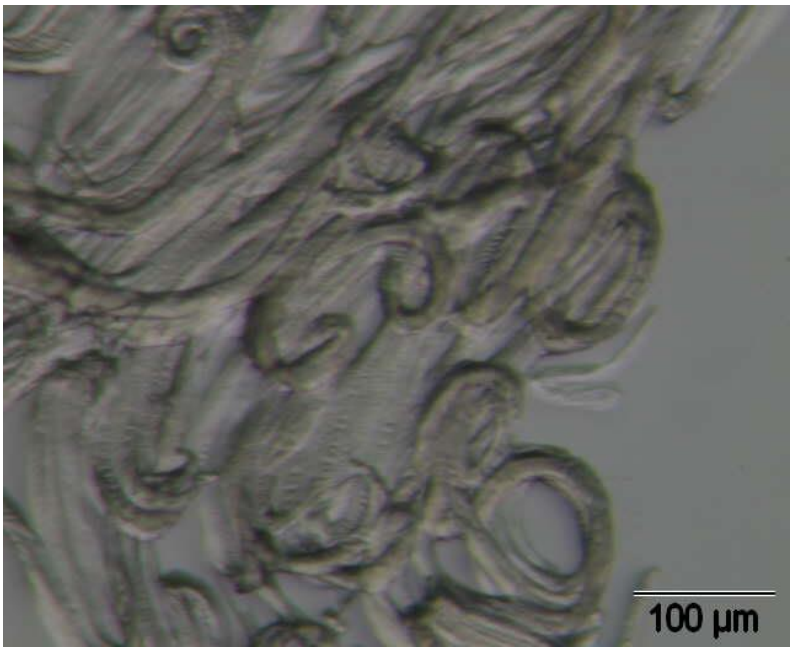


Figure 7.7 RNAi treated *C. elegans* dauer larvae which have been preconditioned at 98% RH for 96 h and desiccated at 32.5% RH for 24 h. Shown at (i) low magnification (ii) high magnification. No differences between the control *C. elegans* dauer larvae and the RNAi treated dauer larvae were observed at this point. Photographic data are only presented for RNAi-*aqp-11* treated dauer larvae as all RNAi treated and control dauer larvae gave similar results.

(i)

(ii)

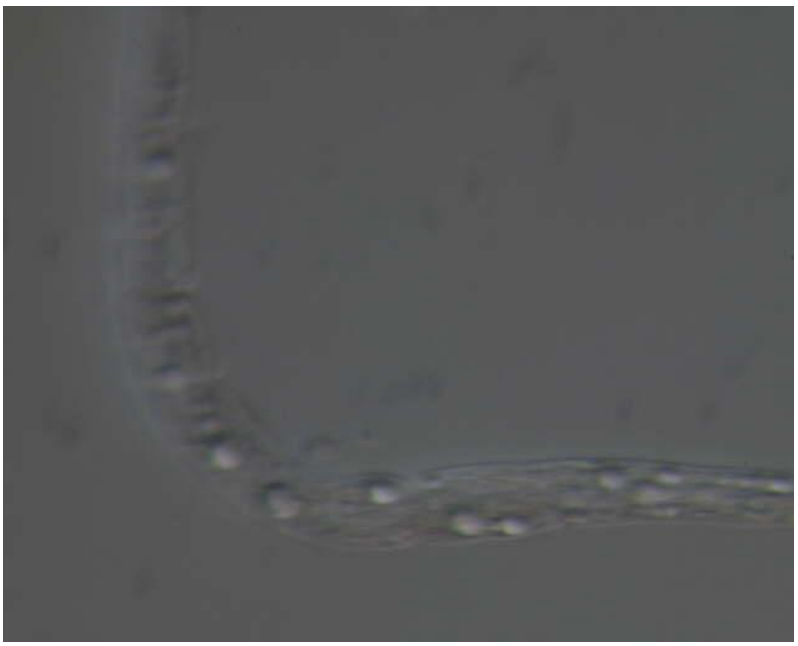


Figure 7.8 (i) RNAi-*aqp-11* (aquaporin) treated *C. elegans* dauer larvae which have been preconditioned for at 98% RH for 96 h, desiccated at 32.5% RH for 24 h and rehydrated in dH_2O for 30 min (ii) *C. elegans* dauer larvae which have been desiccated over activated silica for 24 h and rehydrated in dH_2O for 30 min. The lack of a preconditioning step resulted in 0% survival and lipid droplets forming upon rehydration.

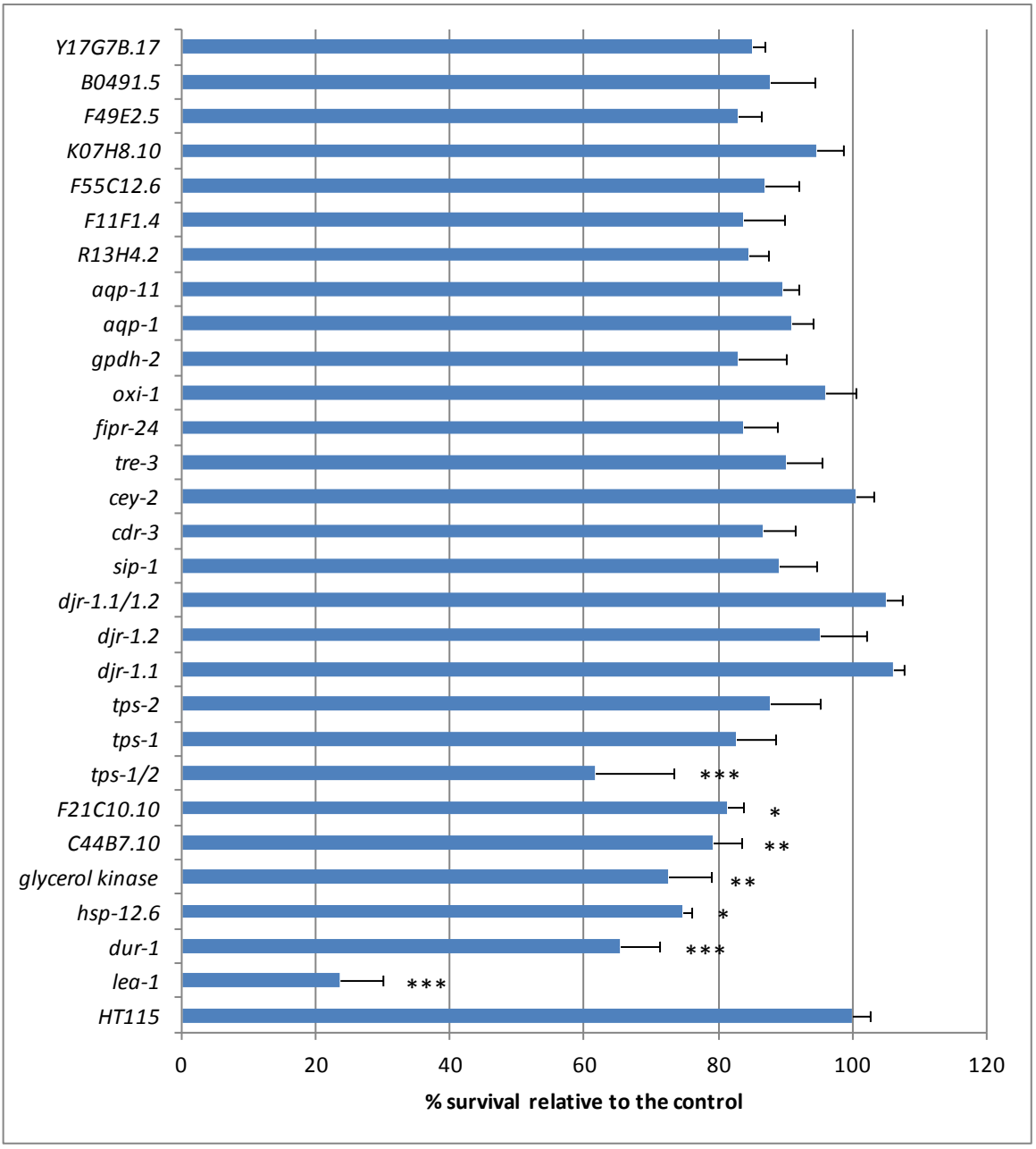


Figure 7.9 Survival (%) of *C. elegans* dauer larvae control, RNAi-*lea-1*, RNAi-*dur-1*, RNAi-*hsp-12.6*, RNAi-*glycerol kinase*, RNAi-*C44B7.10*, RNAi-*F21C10.10*, RNAi-*tps-1*/*tps-2*, RNAi-*tps-1*, RNAi-*tps-2*, RNAi-*djr1.1*, RNAi-*djr-1.2*, RNAi-*djr-1.2*/*djr-1.2*, RNAi-*sip-1*, RNAi-*cdr-3*, RNAi-*cey-2*, RNAi-*tre-3*, RNAi-*fipr-24*, RNAi-*oxi-1*, RNAi-*gpdh-2*, RNAi-*aqp-1*, RNAi-*aqp-11*, RNAi-*R13H4.2*, RNAi-*F11F1.4*, RNAi-*F55C12.6*, RNAi-*K07H8.10*, RNAi-*F49E2.5*, RNAi-*B0491.5* and RNAi-Y17G7B.17 following exposure to 32.5% RH for 24 h. and rehydration for 16 h. The % survival values shown are relative to the control. Data are presented as the mean and standard error of the mean of a minimum of 5 biological replicates ***P<0.001; **P<0.01 ;*P<0.05 as compared to the control using Dunnett's multiple comparison test.

7.2.4 RNAi gene silencing of *P. superbus* nematodes to investigate mechanisms of desiccation tolerance

7.2.4.1 Selection of positive controls and genes for RNAi investigation of desiccation tolerance

The genes in Table 7.2 were selected as candidates to use as possible RNAi positive controls in *P. superbus*. Previous studies have shown that RNAi knockdown of each of these genes in *C. elegans* results in embryonic lethal and larval arrest phenotypes as they are essential genes. The candidate genes selected encode essential proteins involved in translation (*rps-2*, *eft-1* and *eftu-2*), the cytoskeleton (*ifb-1*, *act-2* and *let-75*), proteasome (*rpn-3*), embryonic development (*ama-1*, *sma-1* and *emb-4*) and DNA packaging (*his-3*) Additionally, genes were selected that may have a role in desiccation for further investigation using RNAi in *P. superbus* (Table 7.3). The desiccation related genes which were selected encode proteins associated with chaperone activity (*lea-1*, *lea-7* and *lea-8*), antioxidant activity (*gst-41*, *gst-92* and *gst-114*), phosphorylation (*kin-7*, *kin-11*, *kin-13* and *kin-25*) and compatible solutes (*tps*). The closest homolog for these *C. elegans* genes was found in *P. superbus* using BLAST analysis of the *P. superbus* transcriptome described in Chapter III. RNAi clones were constructed for each of these genes in *P. superbus* using the primers sets as outlined (Table 7.2 and 7.3). Some of the genes were also amplified from *P. superbus* cDNAs cloned into pDNR-lib vectors. *C. elegans* RNAi clones were also constructed to confirm the effectiveness of RNAi (Table 7.4). RNAi knockdown of these *C. elegans* genes has resulted in twitching (*unc-22*) and embryonic lethal/larval arrest (*sumo-1*, *ran-4* and *lmn-1*) phenotypes in previous studies (Fire *et al.*, 1998; Fraser *et al.*, 2000; Liu *et al.*, 2000; Piano *et al.*, 2000). *P. superbus* and *C. elegans* RNAi feeding constructs were designed, created and sequenced as described in Section 2.7.

7.2.4.2 *P. superbus* RNAi feeding results

The RNAi knockdown of the *P. superbus* genes, selected as positive controls to confirm the effectiveness of RNAi, resulted in no reproducible embryonic lethal or larval arrest phenotypes in *P. superbus*. However, the RNAi knockdown of CE-*lmn-1* (lamin), Figure 7.10B, CE-*ran-4* (nuclear import/export), Figure 7.10C and CE-*sumo-1* (ubiquitin related), not shown, in *C. elegans* resulted in strong embryonic lethal and larval arrest phenotypes. Additionally, *C. elegans* nematodes fed with a clone expressing *P. superbus*

act-2 ds-RNA resulted in embryonic lethal and larval arrest phenotypes (Figure 7.10D). The fact that *P. superbus* nematodes exposed to *P. superbus act-2* ds-RNA did not result in a similar embryonic and larval arrest phenotype shows the difference in effectiveness of RNAi between the two species of nematode.

Table 7.2 *P. superbus* RNAi constructs selected as possible positive controls. These genes are essential in *C. elegans* and successful RNAi knockdown in *P. superbus* would be expected to result in embryonic lethal and larval arrest phenotypes. *PCR amplified from a *P. superbus* cDNAs using pDNR-lib primers.

Primer name	Description	5' Sequence 3'
PSifb-1F(isotig05256)	Intermediate Filament, B	GACCAATTTGAGTTGTCTTTTGTG
PSifb-1R(isotig05256)		TGAATCTAAGAATTACTCAACTTGAAA
PSrps-2 F(isotig10985)	Ribosomal Protein	TGAAAAGGAATGGGTTCCAG
PSrps-2R(isotig10985)		GGACTTCATCAGTTCTAATCCCTTT
PSeftu-2F(isotig09718)	Elongation Factor 2	TTTTGCCAGTTCCATAAGCA
PSeftu-2R(isotig09718)		TGCCGCAGAACCCTTATATC
PSemb-4F(contig03157)	Abnormal embryogenesis	TTCGAGCTTTTGAATTGTTACG
PSemb-4R(contig03157)		CACCATTAAAATTTGGTACATCAAT
PSact-2F(isogroup00214)	Actin	CATCAAGGTGTCATGGTTGG
PSact-2R(isogroup00214)		GGCGAGATGGTGATGACTTT
PSama-1F(isotig14623)	Amanitin resistant	AAGAAGGACGTATTCGTGGAAA
PSama-1R(isotig14623)		TCATGTCCCAATTCAAGTGC
*PSeef-1bF	Translation elongation factor	GCATAACTTCGTATAGCATAC
*PSeef-1bR		AAACAGCTATGACCATGTTC
*PSsma-1 F	Beta-H spectrin	GCATAACTTCGTATAGCATAC
*PSsma-1 R		AAACAGCTATGACCATGTTC
*PShis-3 F	Histone	GCATAACTTCGTATAGCATAC
*PShis-3 R		AAACAGCTATGACCATGTTC
*PSlet-75 F	Myosin heavy chain	GCATAACTTCGTATAGCATAC
*PSlet-75 R		AAACAGCTATGACCATGTTC
*PSrpn-3 F	Proteasome regulatory particle	GCATAACTTCGTATAGCATAC
*PSrpn-3 R		AAACAGCTATGACCATGTTC

Table 7.3 *P. superbus* genes associated with desiccation that were selected for further investigation using RNAi.

Primer name	Description	5' Sequence 3'
PSlea-1F	LEA	GCATAACTTCGTATAGCATAC
PSlea-1R		AAACAGCTATGACCATGTTC
PSlea-7F	LEA	GCATAACTTCGTATAGCATAC
PSlea-7R		AAACAGCTATGACCATGTTC
PSlea-8F	LEA	GCATAACTTCGTATAGCATAC
PSlea-8R		AAACAGCTATGACCATGTTC
PStps-F	Trehalose phosphate synthase	GCATAACTTCGTATAGCATAC
PStps-R		AAACAGCTATGACCATGTTC
PSgst-41 F	Glutathione *S*-transferase	GCATAACTTCGTATAGCATAC
PSgst-41 R		AAACAGCTATGACCATGTTC
PSgst-92 F	Glutathione *S*-transferase	GCATAACTTCGTATAGCATAC
PSgst-92 R		AAACAGCTATGACCATGTTC
PSgst-114 F	Glutathione *S*-transferase	GCATAACTTCGTATAGCATAC
PSgst-114 R		AAACAGCTATGACCATGTTC
PSkin-7 F	Protein kinase	GCATAACTTCGTATAGCATAC
PSkin-7 R		AAACAGCTATGACCATGTTC
PSkin-11 F	Protein kinase	GCATAACTTCGTATAGCATAC
PSkin-11 R		AAACAGCTATGACCATGTTC
PSkin-13 F	Protein kinase	GCATAACTTCGTATAGCATAC
PSkin-13 R		AAACAGCTATGACCATGTTC
PSkin-25 F	Protein kinase	GCATAACTTCGTATAGCATAC
PSkin-25 R		AAACAGCTATGACCATGTTC

Table 7.4 *C. elegans* RNAi constructs used as positive controls to confirm the effectiveness of RNAi. RNAi knockdown of these genes has resulted in twitching (*unc-22*) and embryonic lethal (*sumo-2*, *ran-4* and *lmn-1*) phenotypes in previous studies in *C. elegans*.

Primer name	Description	5' Sequence 3'
CEunc-22 F	Uncoordinated	TTAATGAATGCGGACTCTCT
CEunc-22 R		TGTATTTTCCCTTGTTGTCC
CEsumo-1F	Ubiquitin-related	ACTCCCGCTATAAACGATGG
CEsumo-1R		TGATTCGAAATGTGGAAAATAGA
CEran-4F	Associated with ran (nuclear import/export) function	GTGTTGCAAAGGCCTTCATT
CEran-4R		TTCTCGATATCTCGGAAAGAAAA
CElmn-1F	Nuclear lamin	GATCGAGTATGACGCTGCAA
CElmn-1R		GCGCCTCCTGAGTAAGATTG

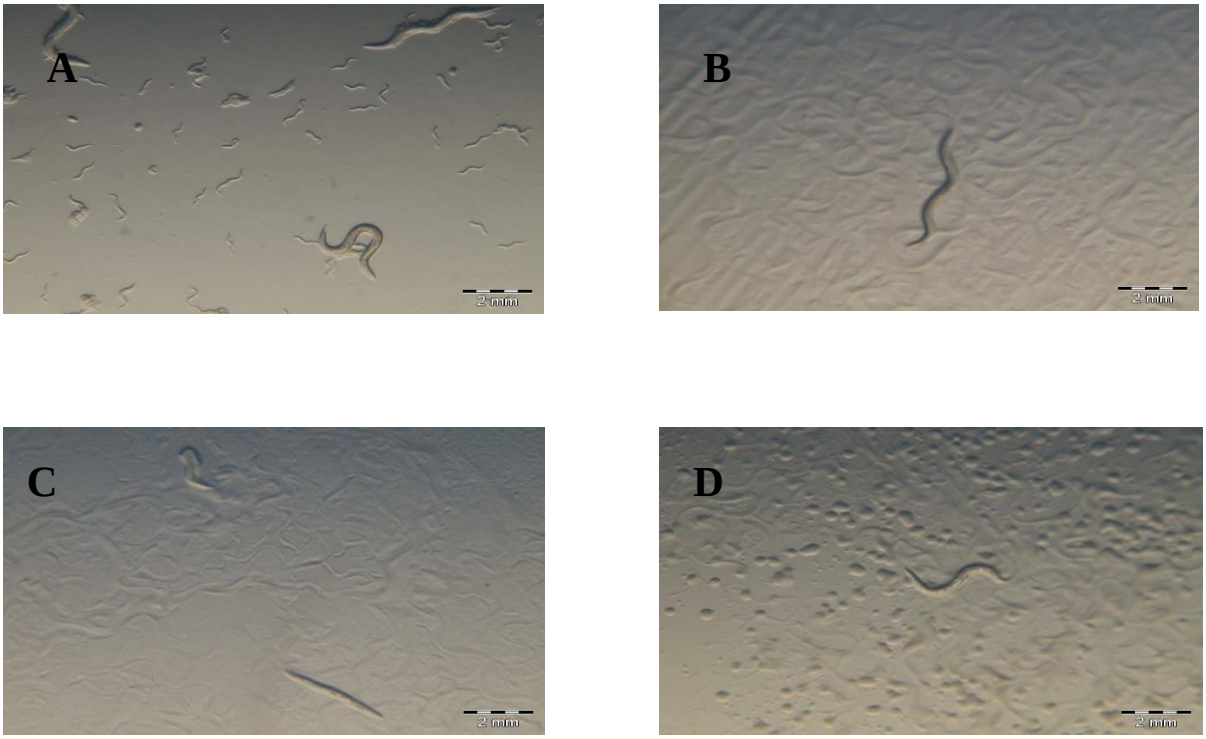


Figure 7.10 Embryonic lethal and larval phenotypes observed by plating *C. elegans* eggs onto agar plates containing a lawn of *E. coli* expressing dsRNA for *C. elegans* and *P. superbus* genes. The images were taken 6 days after the eggs were plated. (A) Control *C. elegans* nematodes plated onto agar plates containing a lawn of *E. coli* containing the empty L4440 plasmid vector. A mixed population of adults and their progeny can be seen. (B) dsRNAi embryonic lethal and larval arrest phenotype of CE-*lmn-1* (nuclear lamin). (C) dsRNAi embryonic lethal and larval arrest phenotype of CE-*ran-4* (nuclear import/export). (D) dsRNAi embryonic lethal and larval arrest phenotype of PS-*act-2* (actin). Note the lack of larval progeny in panels B, C and D.

7.3 Discussion

RNAi was used to further investigate desiccation tolerance in *C. elegans* dauer larvae. This RNAi study revealed the importance of genes encoding hydrophilic proteins (*lea-1* and *dur-1*), molecular chaperones (*hsp-12.6*), trehalose phosphate synthase (*tps-1* and *tps-2*), glycerol kinase (*R11F4.1*) as well as previously uncharacterised genes (*F21C10.10* and *C44.B7.10*) in enabling *C. elegans* dauer larvae to successfully survive desiccation (Figure 7.9). The biological significance of the genes that had an impact on desiccation survival in *C. elegans* dauer larvae is further discussed in this section.

Although RNAi feeding has many advantages for studying gene function there are some disadvantages. These include incomplete gene knockdown, off target effects and genetic redundancy caused by multiple gene families. Genetic redundancy occurs when two or more genes perform the same function such that inactivation of one of these genes has little or no effect on the biological phenotype (Nowak *et al.*, 1997). Although, knockdown of two genes by RNAi feeding can be achieved using a mixture of bacteria that each contain individual dsRNA species, this is not as effective as knocking a single gene (Min *et al.*, 2010). However, the double knock-down of the *C. elegans tps-1* (trehalose phosphate synthase) and *tps-2* resulted in a reduction in the survival rate in response to desiccation.

7.3.1 Trehalose phosphate synthase

In *C. elegans*, two putative trehalose-6-phosphate (tps) genes encode the trehalose synthesis enzymes *tps-1* (ZK430.3) and *tps-2* (F19H8.1). In our RNA-seq study, we found that *tps-1* and *tps-2* had an increased expression in response to desiccation (Figure 5.25). In this experiment, the RNAi knockdown of *tps-1* and *tps-2* individually in *C. elegans daf-2* dauer larvae did not have an impact on the survival from desiccation. However, the simultaneous RNAi knockdown of both *tps-1* and *tps-2* in *C. elegans daf-2* dauer larvae resulted in a highly significant drop in survival after exposure to desiccation. This indicates possible redundancy between the *tps-1* and *tps-2* genes since the knockdown of both genes is required for a reduction in survival in response to desiccation. The sugar trehalose, a disaccharide of glucose, is thought to act as a stress protectant by preserving lipid membranes and stabilising proteins in their native state (Carpenter and Crowe, 1988;

Singer and Lindquist, 1998). In a recent study, a strain of *C. elegans* mutant, which had both trehalose phosphate synthase genes knocked out (by deletion) was produced which could not synthesize trehalose. This strain had a reduced ability to survive desiccation (Erkut *et al.*, 2011).

7.3.2 LEA-1 (late embryogenesis abundant)

The transcript *lea-1*, which encodes the LEA-1 protein, was found to be up-regulated in response to preconditioning and desiccation in *C. elegans* dauer larvae (Figure 5.25). Additionally, *lea-1* expression has also been found to be up-regulated in response to desiccation at both the transcriptomic and proteomic level in the anhydrobiotic nematode *P. superbus* (Chapter III and Chapter IV). For these reasons, the role of *lea-1* during desiccation was further investigated using RNAi. RNAi knockdown of *lea-1* in *C. elegans daf-2* dauer larvae resulted in a 75% decrease in survival, compared with the control, upon rehydration after exposure to desiccation. Of the seven genes found to have an effect on desiccation survival in *C. elegans daf-2* dauer larvae, the RNAi knockdown of *lea-1* had the greatest impact on survival.

LEAs are multifunctional proteins that are known to have a role in desiccation tolerance (Gal *et al.*, 2004; Tunnacliffe and Wise, 2007; Hand *et al.*, 2011). LEA proteins are classified as natively unfolded proteins (Goyal *et al*., 2003). Late embryonic abundant (LEA) proteins are extremely hydrophilic proteins whose intracellular accumulation is tightly correlated with the acquisition of desiccation tolerance in drought tolerant resurrection plants, anhydrobiotic nematodes, tardigrades and the anhydrobiotic larvae of the chironomid Polypedilum (Bartels, 2005; Tunnacliffe *et al.*, 2005; Kikawada *et al.*, 2006; Hand *et al.*, 2011). Suggested roles of LEA proteins include the protection of proteins through chaperone activity and/or molecular shielding, interaction with and stabilization of phospholipid membranes and reinforcement of vitrified sugar glasses (reviewed by Hand *et al.*, 2011).

7.3.3 DUR-1 (dauer up-regulated)

RNAi knockdown of the *dur-1* gene significantly reduced survival due to desiccation in *C. elegans daf-2* dauer larvae. Interestingly, DUR-1 shows similarity to LEA-1 with a

Blast-p e-value score of 4e-12. Additionally, DUR-1 is predicted to be hydrophilic and to have 11-mer repeats typical of a Group Three LEA protein (Browne *et al.*, 2004). Therefore, DUR-1 may have similar roles to LEA in the protection of proteins during desiccation in *C. elegans daf-2* dauer larvae. DUR-1 was found to be up-regulated in *C. elegans* dauer larvae in response to preconditioning as well as in recovery. Moreover, at the transcript level, *dur-1* had significantly higher expression in *C. elegans* dauer larvae in the recovery dataset.

7.3.4 HSP-12.6 (small heat shock protein)

HSP-12.6 protein levels as well as *hsp-12.6* transcript levels (discussed in Chapter VI) were statistically up-regulated in response to preconditioning, desiccation and recovery when compared to the control in *C. elegans daf-2* dauer larvae (Chapter VI, Figure 6.10). The RNAi knockdown of *hsp-12.6* resulted in a statistically significant reduction in survival in response to desiccation when compared to the control. The importance of sHSPs is shown in the fact they are ubiquitously expressed, are dramatically up-regulated in response to stress and are implicated in a number of diseases. Small HSPs protect cells from protein losses or toxicity caused by aggregation (Sun and Macrae, 2005). Mohri-Shiomi and Garsin (2008) have recently shown a reduction of *hsp-12.6* expression by RNAi resulted in a statistically significant increase in the number of protein aggregates in the *C. elegans* intestine caused by infection (Mohri-Shiomi and Garsin, 2008). Stresses such as desiccation have been shown to increase protein aggregation (Jonsson and Schill, 2007). The knockdown of *hsp-12.6* in RNAi treated *C. elegans* dauer larvae reduced their tolerance to desiccation. This could, most likely, be attributed to the increased aggregation of proteins caused by desiccation and a lack of HSP-12.6 protection, resulting in reduced survival.

7.3.5 Glycerol kinase

Fat is a major source of energy and biosynthetic precursors in dauer larvae and it accumulates in the intestine and hypodermis during dauer formation in *C. elegans*. The majority of fat in the dauer is found in the form of triacylglycerides (Narbonne and Roy, 2009) consisting of three fatty acids attached to a glycerol backbone. The metabolism of fats for energy also results in the concurrent break-down of glycerol. Glycerol can be converted into glyceraldehyde 3-phosphate, a metabolic intermediate of glycolysis by the

two enzymes: glycerol kinase and glycerol 3-phospate dehydrogenase (Wang and Kim, 2003). Glycerol kinase is also involved in the synthesis of phospholipids and triacylglycerols which are essential components of the lipid membrane (Castelein *et al.*, 2008). In this experiment, RNAi knockdown of the gene encoding glycerol kinase in *C. elegans* dauer larvae resulted in significantly reduced tolerance to desiccation (Figure 7.9). Two possible explanations for this result could be a reduced ability of the recovering nematodes to metabolise their triacylglyceride stores or a reduced ability to synthesize essential phospholipids required for repairing lipid membranes. In contrast to our findings, the knockout of glycerol kinase in *Arabidopsis thaliana* leads to the accumulation of glycerol and enhanced resistance to a variety of abiotic stress such as desiccation (Eastmond, 2004). Glycerol has conserved role in maintaining intracellular water balance as well as possible roles in stabilising proteins, protein complexes, membranes and protecting against oxidative damage. The contrasting effects of glycerol kinase knockdown in *C. elegans* and *Arabidopsis* in response to desiccation indicate differing strategies to survive desiccation. *Arabidopsis* plants may be reliant on the accumulation of the osmolyte glycerol to stabilise proteins and membranes during desiccation. Recent studies in the mouse model for glycerol kinase deficiency have revealed roles for glycerol kinase in apoptosis and insulin signalling (Maclennan *et al.*, 2009). In *C. elegans daf-2* dauer larvae, apoptosis and insulin signalling pathways were up-regulated in response desiccation at the transcriptome level (Chapter V). These roles could be conserved in *C. elegans* which may have resulted in reduced survival in response to desiccation in glycerol kinase deficient dauer larvae.

7.3.6 C44B7.10.1

The C44B7.10.1 protein was up-regulated in response to preconditioning and recovery in *C. elegans* dauer larvae. RNAi knockdown of the *C44B7.10.1* gene resulted in a significant reduction in survival ($P<0.01$) of *C. elegans* dauer larvae in response to desiccation. Using BLAST analysis, the translated sequence of *C44B7.10.1* was found to have 57% similarity to an *Artemia franciscana* protein called p49 which cross-links microtubules *in vitro*. The brine shrimp *A. franciscana*, has attracted much attention for its ability to produce encysted embryos wrapped in a protective shell when exposed to extremely harsh environmental conditions such as desiccation. These anhydrobiotic cysts can resume development when more favourable conditions prevail (Clegg, 1978; Liu *et*

al., 2009). The protein p49, which has a molecular mass of 49 kDa was purified and was found to produce cross-linked microtubules when the protein was incubated with *A. franciscana* tubulin (Oulton *et al.*, 2003). Interestingly, the proteomic and transcriptomic studies of desiccation tolerance in *C. elegans daf-2* dauer larvae (see Chapter V and VI) found a number of up-regulated genes and protein associated the cytoskeleton and microtubules.

7.3.7 F21C10.10

BLAST searches of the translated sequence of *F21C10.10* revealed similarity to a member of the Crotonase/Enoyl-Coenzyme A (CoA) hydratase superfamily. This superfamily contains a diverse set of enzymes including enoyl-CoA hydratase, napthoate synthase, methylmalonyl-CoA decarboxylase, 3-hydoxybutyryl-CoA dehydratase, and dienoyl-CoA isomerase. Many of these play important roles in fatty acid metabolism (Mullernewen *et al.*, 1995). Our results show that RNAi knockdown of *F21C10.10* gene expression in *C. elegans daf-2* dauer larvae resulted in reduced survival in response to desiccation. The possible role this gene plays in fatty acid metabolism may be important in conferring desiccation tolerance in *C. elegans* dauer larvae: a reduction in fatty acid metabolism could result in a decrease in the synthesis of effector proteins needed for desiccation survival.

7.3.8 RNAi feeding in *P. superbus*

Previous studies published by our group have shown that *P. superbus* as well as *Panagrolaimus* sp. 1159 display embryonic lethal RNAi phenotypes following ingestion of *E. coli* expressing dsRNA for the *C. elegans* embryonic lethal genes *Ce-lmn-1* and *Ce-ran-4* (Shannon *et al*., 2008). Shannon *et al.* (2008) also observed lethal RNAi phenotypes upon ingestion of dsRNA for the *Panagrolaimus* genes *ef1b* (ef1b=elongation factor 1-beta) and *rps-2* (rps=ribosomal protein small subunit) and locomotion and growth defects upon ingestion of dsRNA for the *Panagrolaimus* genes *Psu-ifb-1* (ifb=intermediate filament, B) and *Psu-act-2* (act=actin). Furthermore, RT-PCR showed a reduction in mRNA transcript levels for the target *ef1b* and *rps-2* genes in RNAi treated *Panagrolaimus* sp. 1159 nematodes (Shannon *et al*., 2008). Cross-species RNAi using *A. avenue* sequences (glutathione peroxidase sequence and a novel sequence) in *P. superbus*

was subsequently found to cause a significant reduction in post-desiccation survival (Reardon *et al*., 2010).

The constructs used in the papers described above as well as additional positive controls (Section 7.2.4) to confirm the effectiveness of RNAi, resulted in no reproducible RNAi phenotypes in *P. superbus*. This indicated that *P. superbus* was not as amenable to high-throughput RNAi as *C. elegans*. However, further optimization of RNAi in *P. superbus* may have resulted in the successful use of this nematode in high-throughput experiments. *C. elegans* dauer larvae were instead used to further investigate the involvement of certain genes in desiccation as positive controls resulted in visible phenotypes without optimisation.

As part of this PhD project the constructs used in the papers described above as well as additional positive controls (Section 7.2.4) were tested to confirm the effectiveness of RNAi in *P. superbus*, prior to initiating a large-scale RNAi screening study in this nematode. However these experiments failed to generate reproducible RNAi phenotypes in *P. superbus.* The efficacy of experimental protocol being used for RNAi was tested using *C. elegans*, where it was found that ingestion of *E. coli* expressing dsRNA for the *C. elegans* genes CE-*lmn-1* (lamin), CE-*ran-4* (nuclear import/export) and CE-*sumo-1* (ubiquitin related) in *C. elegans* resulted in strong embryonic lethal and larval arrest phenotypes. Additionally, *C. elegans* nematodes fed with a clone expressing *act-2* ds-RNA from *P. superbus* resulted in embryonic lethal and larval arrest phenotypes in *C. elegans.* The fact that *P. superbus* nematodes exposed to *P. superbus act-2* ds-RNA did not result in a similar embryonic and larval arrest phenotype shows the difference in effectiveness of RNAi between the two species of nematode. Further optimization of the RNAi protocol for *P. superbus*, such as microinjection of dsRNA or of small double-stranded siRNAs (Elbashir *et al.*, 2001) for the target gene, or of soaking (Tabara *et al*., 1998) the desiccated nematodes in dsRNA or siRNAs may have resulted in reproducible phenotypes and facilitated its use in high throughput RNAi screens. However because of time constrains it seemed more prudent to use RNAi experiments in *C. elegans* dauer larvae to further investigate the involvement of certain genes in desiccation, as positive controls resulted in visible RNAi phenotypes without further optimisation in this nematode.

7.4 Conclusion

RNAi is a straightforward approach, which knocks down the expression of target genes to induce a phenotype that may indicate gene function. The results presented in this chapter show that the RNAi knockdown of *lea-1*, *dur-1*, *hsp-12.6*, *tps-1/2*, glycerol kinase, *F21C10.10* and *C44.B7.10* resulted in a statistically significant reduction in desiccation survival of *C. elegans daf-2* (e1370) dauer larvae. This shows that *C. elegans* dauer larvae can be used as a model to study the mechanisms of desiccation tolerance. The results obtained also reveal the existence of a multifaceted approach to desiccation survival in *C. elegans*, involving a variety of genes encoding proteins with diverse functions as described in section 7.3.1-7.3.7. The RNAi knockdown of novel genes also reduced the tolerance of *C. elegans* dauer larvae to desiccation. Additionally, high-throughput RNAi could be used to identify other genes which are involved in the mechanisms of desiccation tolerance in *C. elegans* dauer larvae, utilising the *C. elegans* RNAi libraries which cover the majority of the protein encoding genes in *C. elegans* for screening. Focusing on the other desiccation responsive genes and proteins which were up-regulated in this RNA-seq and proteomics study in the first instance should provide an enriched set of target genes.

8 Chapter VIII General Discussion

The genes and proteins which are differentially expressed in response to desiccation and recovery were identified and characterised in the both *C. elegans* dauer larvae and *P. superbus* (mixed population containing all life stages). Both *P. superbus* and *C. elegans* dauer larvae have contrasting strategies for surviving desiccation perhaps defined by the habitats in which they evolved. The nematode *P. superbus* was originally isolated in 1981 from a gull's nest in Surtsey, Iceland (Bostrom, 1988). The family Panagrolaimidae contains nematodes which are variously resistant to diverse stresses including desiccation, freezing and high temperatures (Wharton and Brown, 1991; Shannon *et al.*, 2005; Borgonie *et al.*, 2011). Since Surtsey island was formed by volcanic eruptions during the 1970s *P. superbus* nematodes are presumed to have transported there in an anhydrobiotic state. On Surtsey Island, *P. superbus* is subjected to a frequent cycle of freeze-thaw action and desiccation. Their rapid dehydrating, anhydrobiotic phenotype, as well as their cold tolerance, allows them to adapt to the harsh environmental conditions on the island. *C. elegans* adults inhabit rotting fruit, a stable environment, less susceptible to desiccation. However, *C. elegans* does possess a stress resistant dauer larval stage in which the larvae are able to survive desiccation. This larvae stage is formed in response to high population density, scarce food resources and/or high temperatures. In *C. elegans* dauer larvae and *P. superbus*, the following processes and pathways were implicated in the response to desiccation at both the transcriptome and proteome level: molecular chaperone activity, antioxidants, compatible solutes, the ubiquitin proteasome system, autophagy, DNA repair, cellular repair and response to pathogens. While there was much overlap in the identities of the genes and proteins which were up-regulated in response to dehydration, differences were also observed, which could be a reflection of the differences in the adaptive strategies of these two nematode species to desiccation.

8.1 Physical and behavioural adaptations to desiccation stress

The environmental niches in which nematodes are found are likely to determine their strategies of desiccation survival. *P. superbus* nematodes are subjected frequent cycles of dehydration, freezing and thawing in their natural habitat which may have driven adaptation to extreme desiccation. Interestingly, there appears to be a correlation between desiccation tolerance and freezing tolerance of *P. superbus* and other *Panagrolaimus*

strains from temperate, polar, subpolar and continental regions of the world (McGill, unpublished work). In contrast *C. elegans* adults and L1-L4 larvae, which inhabit a more stable environment, are unable to survive desiccation. However, *C. elegans* dauer larvae are able to survive desiccation. Sealed at all orifices, *C. elegans* dauer larvae have thickened cuticles that are more resistant to desiccation (Riddle *et al.*, 1997; Gal *et al.*, 2004). Interestingly**,** it appears that *P. superbus* do not have a dauer larval stage in their life cycle (Burnell, unpublished). However, *P. superbus* unlike *C. elegans* can undergo anhydrobiosis at all stages of their life cycle.

Some desiccation tolerant nematodes can coil into a tight spiral shape in response to desiccation. This morphological adjustment reduces the surface to volume ratio**,** which may result in a decreased rate of internal water loss during the process of desiccation (Ricci *et al.*, 2003). Both *C. elegans* dauer larvae and *P. superbus* can coil into a spiral shape in response to desiccation. Interestingly, *P. superbus* nematodes appear to form a more compact spiral shape (reducing the exposed surface area) which may result in a further decreased rate of internal water loss compared to the loosely coiled *C. elegans* dauer larvae (Figure 8.1).

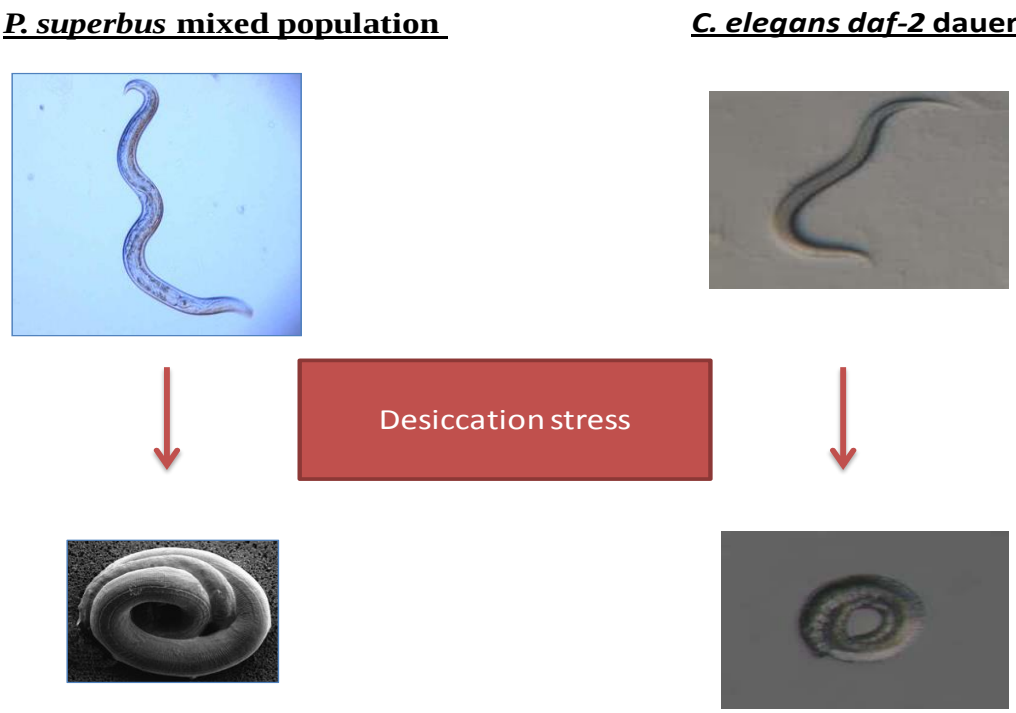


Figure 8.1 Images of *P. superbus* adults and *C. elegans* dauer larvae before and after exposure to preconditioning (98% RH for 24 h). Both nematodes coil into a spiral shape in response to desiccation stress which may result in a decreased rate of internal water loss.

8.2 Genes and proteins which were up-regulated in response to desiccation-related treatments in *C. elegans* dauer larvae and *P. superbus*

There were 5,294 and 1,598 genes up-regulated in *C. elegans* dauer larvae and *P. superbus* respectively, in response to desiccation-related treatments. Although the same desiccation treatments were not selected for both nematode species, it appears that considerably more genes were induced in *C. elegans* compared to *P. superbus*. A possible explanation could be that the genes required to confer desiccation tolerance in *P. superbus* are already constitutively expressed. Interestingly, in *P. superbus* only 4% of the proteins up-regulated in response to desiccation and recovery have a correlated up-regulation of their mRNA transcripts. While in *C. elegans* dauer larvae 63% of the proteins up-regulated in response to desiccation-related treatments have a correlated induction at the transcript level (Figure 8.2). This further suggests that the genes required for desiccation tolerance in *P. superbus* nematodes are already constitutively expressed. *P. superbus* nematodes are able to survive extreme desiccation without a period of preconditioning (Shannon *et al*., 2005). However, preconditioning does improve their desiccation survival which suggests that some *de novo* synthesis of proteins required for desiccation tolerance does occur. In *C. elegans* dauer larvae, preconditioning at 98% RH is essential for them to survive harsh desiccation (Erkut *et al*., 2011). Additionally, even with preconditioning *C. elegans* dauers have relatively poor survival in response to extreme desiccation (Chapter V).

A good correlation was observed between the desiccation-responsive genes and proteins identified in both *C. elegans* dauer larvae and *P. superbus,* despite their differing abilities to undergo anhydrobiosis (Figure 8.3). Strikingly, 37% of the genes up-regulated in *P. superbus* (that had a *C. elegans* homolog) were also induced in *C. elegans* dauers in response to desiccation-related treatments. Furthermore, 46% of the proteins up-regulated in response to desiccation and recovery in the *P. superbus* were up-regulated at the transcript level in *C. elegans* dauer larvae (Figure 8.3).

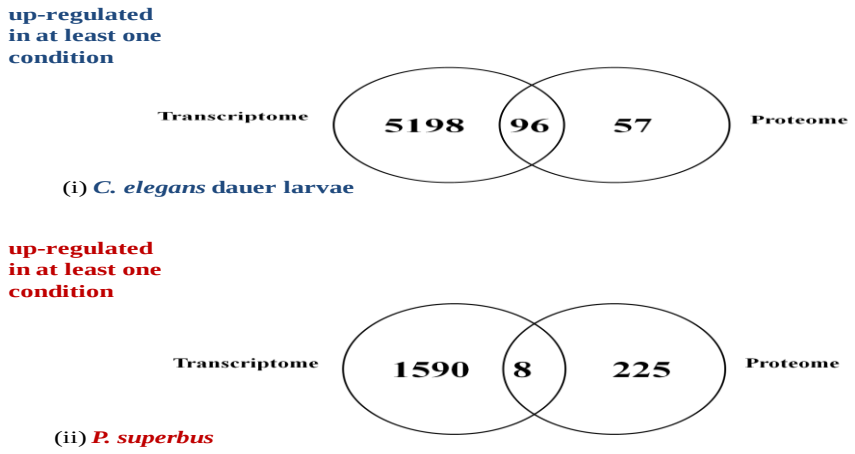


Figure 8.2 (i) The number of *C. elegans* genes and proteins up-regulated in response to desiccation and recovery. (ii) The number of *P. superbus* genes and proteins up-regulated in response to desiccation and recovery.

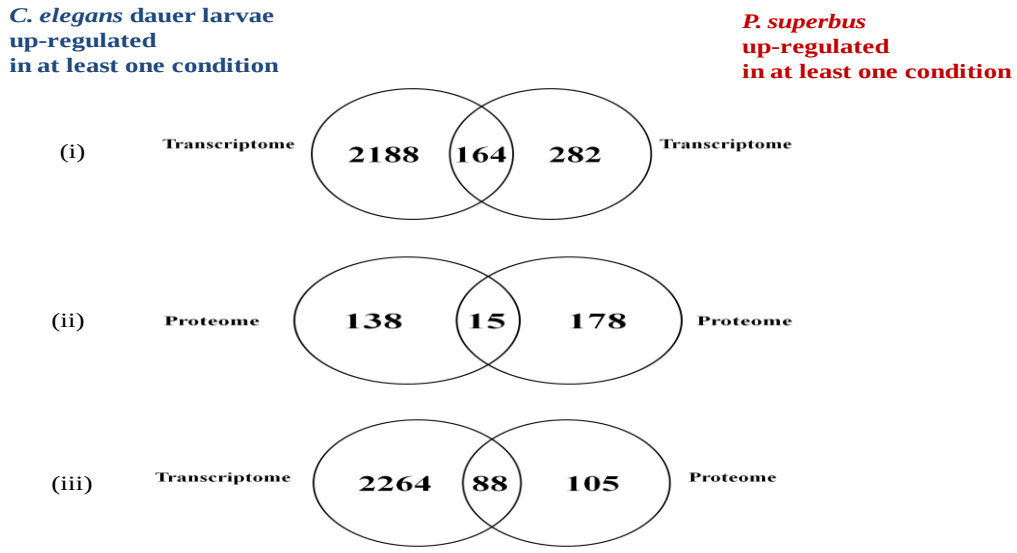


Figure 8.3 (i) The number of homologous *C. elegans* and *P. superbus* genes up-regulated in response to desiccation and recovery. (ii) The number of homologous *C. elegans* and *P. superbus* proteins up-regulated in response to desiccation and recovery. (iii) The number of *C. elegans* genes and *P. superbus* protein homologs up-regulated in response to desiccation and recovery. To identify homologs a BLASTX alignment was carried out between the *P. superbus* transcriptome and *C. elegans* transcriptome (version 190 downloaded from Ensembl at www.ensembl.org).

8.3 Comparison of the different strategies of desiccation tolerance in *C. elegans* dauer larvae and *P. superbus*

The main pathways and processes involved in desiccation tolerance in *C. elegans* dauer larvae and *P. superbus* are discussed in this section. These include insulin signalling, translation, metabolic adjustments, trehalose biosynthesis, antioxidants, molecular chaperones, stress related genes, cellular re-organisation, DNA damage repair, removal of damaged proteins and response to pathogens. While there was much overlap in the identities of the genes and proteins which were up-regulated in response to dehydration and during recovery from dehydration in the two nematode species, differences were also observed, which could be a reflection of the differences in their adaptive strategies. In this section the changes in the proteomic and transcriptomic profiles in *C. elegans* dauer larvae and *P. superbus* in response to desiccation and rehydration is compared and contrasted.

8.4 Insulin signalling

The insulin signalling pathway has been extensively studied in *C. elegans* due to its links with ageing and dauer larva formation (as discussed in Chapter I). Genes which encode multiple components of the insulin-signalling pathway were up-regulated in response to desiccation in *P. superbus* and *C. elegans*. Two parallel signal transduction pathways are involved in dauer formation: the *daf-7*/transforming growth factor beta (TGF-ß) pathway and the *daf-2*/insulin-like growth factor 1 receptor (IGF1R) pathway. DAF-9 is postulated to produce a cholestrol-derived hormone with DAF-12 the putative target (Gerisch and Antebi, 2004; Matyash *et al.*, 2004). Hormone activated DAF-12 promotes reproductive development, while in the absence of this hormone DAF-12 activates the dauer formation programme (Gerisch and Antebi, 2004). In *C. elegans* a mutation in the *daf-2* gene, which encodes an insulin/IGF receptor ortholog (Kimura *et al.*, 1997), was found to double the lifespan of the animal (Kenyon, 2005). Insulin/IGF-1 signalling appears to be only one step in a signalling cascade that affects lifespan as *daf-16*, which encodes a FOXO (forkhead box) family transcription factor, is also required for the longevity phenotype (Lin *et al.*, 1997; Kenyon, 2005).

Interestingly, in both nematodes the greatest up-regulation of genes encoding proteins involved in insulin signalling was observed at the most desiccated time-point (Figure 8.4). The insulin-signalling genes *daf-2* (abnormal dauer formation), *age-1* (age alteration),

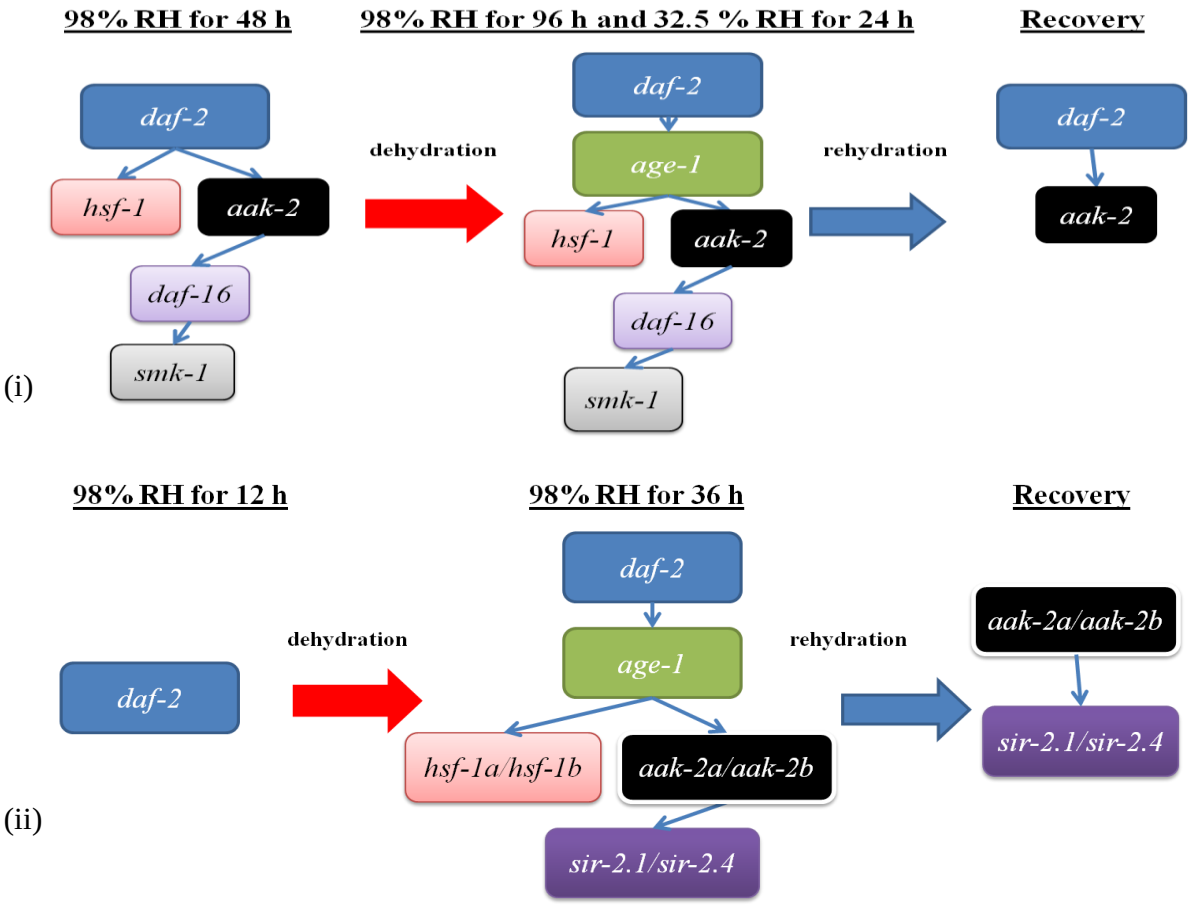


Figure 8.4 Up-regulated genes associated with insulin-signalling in (i) *C. elegans* dauer larvae and (ii) *P. superbus* in response to desiccation-related conditions and recovery from desiccation.

aak-2 (aak=AMP-activated kinase) and *hsf-1* (hsf=heat shock factor) were up-regulated in response to desiccation in *C. elegans* dauer larvae and in *P. superbus*. HSF-1 is activated by stress and promotes the expression of *hsps* in collaboration with DAF-16 (Hsu *et al.*, 2003; Morley and Morimoto, 2004). The increased expression of *hsps* is further discussed latter in this chapter. As well as *hsps*, DAF-2 regulated genes encoding antioxidants (*sod-4* and *sod-5*) and pathogen defence proteins (*lys-8*) were up-regulated in *C. elegans* dauer larvae and *P. superbus* in response to desiccation.

Another signalling pathway that is known to respond to environmental stress is the mitogen-activated protein kinase (MAPK) signalling pathway. In *C. elegans*, the three types of MAPKs (ERK, p38 and JNK) have been implicated in stress responses (Sakaguchi *et al.*, 2004; Lant and Storey, 2010). A recent study by Banton and Tunnacliffe (2012) determined the phosphorylation status of the Mitogen-activated protein kinases (MAPK) p38, JNK and ERK in the nematode species *C. elegans*, *Aphelenchus avenae* and *P. superbus* in response to desiccation. Additionally, the anhydrobiotic potential of the three nematodes was compared. *C. elegans* young adults (non-dauers), which do not survive harsh desiccation, were used in these experiments. The results of the study show that differing MAPK phosphorylation patterns in the three nematodes are linked to strategies in surviving desiccation. In the nematodes, *C. elegans* and *A. avenae*, which both require a period of preconditioning to survive desiccation, increased MAPK phosphorylation was observed upon drying. In contrast, *P. superbus*, which is able to survive extreme desiccation without prior preconditioning, MAPK phosphorylation was observed in the control and then its phosphorylation status decreased upon dehydration. Moreover, in *P. superbus* MAPK phosphorylation returned to pre-stress levels 30 min after rehydration (Banton and Tunnacliffe, 2012). The expression patterns of the genes encoding three subgroups of the MAPK superfamily (ERK, JNK and p38) was monitored in *C. elegans* dauer larvae and *P. superbus* using the RNA-seq data generated in this project. The gene *jnk-1* (jnk=c-Jun N-terminal Kinase), which encodes a serine/threonine kinase that is the sole member of the JNK MAPKs, was down-regulated in *C. elegans* dauer larvae in response to all desiccation-related conditions (preconditioning, desiccation and recovery). While in *P. superbus jnk-1* gene was only down-regulated in the preconditioning time-point, (although there was no equivalent desiccation time point in the *P. superbus* RNA-seq dataset). There was no significant

change in the expression of *pmk-1* (pmk-1=p38 MAPK), which encodes a MAPK orthologous to human p38 MAPK, in either *C. elegans* dauer larvae or *P. superbus* in response to desiccation or recovery. Finally, the gene *mpk-1* (mpk=map kinase), which is an ortholog of ERK (erk=extracellular signal-regulated kinases) was up-regulated in *C. elegans* dauer larvae in response to desiccation. An additional gene involved in ERK signalling called *mek-1* (mek=MAP kinase kinase or Erk Kinase) was up-regulated in response to preconditioning, desiccation and recovery in *C. elegans* dauer larvae but was up-regulated only in response to recovery in *P. superbus* (two *mek-1* related genes up-regulated=PST19932_1 and PST00940_1). This RNA-seq analysis suggests ERK signalling may be important to survive desiccation in *P. superbus* and *C. elegans* dauer larvae and that it may also have a role in the recovery response in *C. elegans* dauer larvae. Huang *et al*. (2010) have previously shown that chemical inhibition of ERK reduces the expression of desiccation-responsive genes in a human embryonic kidney cell line while Banton and Tunnacliffe (2012) have found that ERK phosphorylation is elevated during preconditioning and rehydration of *C. elegans* and the anhydrobiotic nematode *Aphelenchus avenae.*

The genes *nhr-19* (nhr=nuclear hormone receptor), *nhr-33* and *nhr-64* were up-regulated in both *C. elegans* dauer larvae and *P. superbus* in response to desiccation. NHRs are transcriptional factors that respond to lipophilic molecules to regulate the expression of target genes involved in metabolism, reproduction and development. There are a total of 284 NHRs in the *C. elegans* genome, several of which have been implicated in lipid metabolism (Liang *et al.*, 2010).

8.5 Translation

The RNA-seq analysis of *C. elegans* dauer larvae and *P. superbus* has provided insights into how protein synthesis is altered in response to dehydration and subsequent rehydration. In *C. elegans* dauer larvae the GO term Translation was up-regulated in response to preconditioning but not desiccation. *C. elegans* dauer larvae require preconditioning at 98% RH in order to survive harsher desiccation (Erkut *et al.*, 2011). This suggests that *C. elegans* dauer larvae may need to synthesize proteins during preconditioning in order to survive exposure to lower relative humidity, and this hypothesis is supported by the RNA-seq data. In contrast to the *C. elegans* RNA-seq data, transcripts encoding proteins involved in translation are not induced in *P. superbus* in response to preconditioning. This indicates that *P. superbus* nematodes may already have synthesized a pool of stress related mRNA transcripts or may be constitutively expressing the effector proteins needed to survive desiccation. The fact that *P. superbus* nematodes can survive harsh desiccation without preconditioning gives further credence to this hypothesis. Additionally, the differential proteomic data also depicted a similar trend with ribosomal proteins showing increased expression in response to dehydration in *C. elegans* dauer larvae but decreased expression of ribosomal proteins was observed during preconditioning in *P. superbus*. In both *C. elegans* dauer larvae and *P. superbus*, the RNA-seq analyses shows that translation is up-regulated during recovery from desiccation. In recovery from desiccation, the nematodes may require the synthesis of proteins which are involved in cellular repair. It is also possible that several of the transcripts involved in cellular protection which were synthesized during preconditioning or desiccation are translated upon recovery from desiccation.

8.6 Metabolism

The DAVID analysis shows that the down-regulated genes in the preconditioning 1 and preconditioning 2 treatments of *P. superbus* are significantly enriched with annotations associated with oxidoreductase activity and glycolysis. This indicates that metabolism is reduced in response to dehydration in *P. superbus*. Oxidoreductase enzymes play key roles in both aerobic and anaerobic metabolism. They are involved in glycolysis, TCA cycle, oxidative phosphorylation, and in amino acid metabolism (Hatefi, 1999). In contrast, the up-regulated genes in the recovery dataset are significantly enriched with annotations associated with the respiratory electron transport chain. This indicates that

metabolism is returning to normal after a reduction of transcripts involved in intermediate metabolism. Transcripts encoding enzymes required for the β-oxidation of fatty acids, for glycolysis and for oxidative metabolism are synthesized by *C. elegans* dauer larvae in response to desiccation stress and during recovery from desiccation. Enzymes involved in glycolysis were also up-regulated at the protein level in *C. elegans* dauer larvae.

The differences observed in metabolism in the response to dehydration may reflect the synchronized and mixed stage populations used in *C. elegans* dauer larvae and *P. superbus* respectively. *C. elegans* dauer larvae are characterised by their low levels of oxidative metabolism, compared to other developmental stages in the life cycle (Burnell *et al.*, 2005). Therefore, an increase in intermediary metabolism may be needed in order to obtain the metabolic energy required to mobilize and synthesise the effector molecules required for desiccation protection. A mixed population of *P. superbus* nematodes was used in the RNA-seq and differential proteomic experiments. At the transcript level, there appears to be a reduction in intermediate metabolism in *P. superbus* in response to dehydration. Recent studies have given support to the hypothesis that reduction of metabolism coincides with survival of desiccation (Leprince *et al.*, 1999; Pammenter and Berjak, 1999). The ability to reduce metabolism prior to environmental stresses such as desiccation (Clegg, 2005), anoxia (Storey, 2007) and freezing (Clegg, 2001) improves the survival of many species. A coordinated control of energy metabolism upon dehydration appears to be essential in avoiding oxidative stress conditions (Hoekstra *et al.*, 2001). In anhydrobiosis, dormant organisms maintain low but significant levels of metabolism. Dormant organisms can sequester substantial reserves, mainly lipids (Williams-Howze *et al.*, 1998; Danks, 2000). An example of down-regulation of general metabolism in response to dehydration is observed in the Antarctic midge *Belgica antarctica* (Teets *et al.*, 2012). Larvae of *B. antarctica* significantly depress oxygen consumption rates in response to desiccation (Benoit *et al.*, 2007). This reduction in metabolic activity could be an adaptation to minimize respiratory water loss and to minimize the loss of water bound to glycogen and other carbohydrates (Marron *et al.*, 2003; Teets *et al.*, 2012).

8.7 Trehalose biosynthesis

Trehalose is a non-reducing disaccharide that has been found to be induced in response to desiccation and is thought to act as a stress protectant (further discussed in Chapter I)

(Singer and Lindquist, 1998). Trehalose has been shown to constitute circa 15% and 9% of the dry weight of anhydrobiotic *Artemia salina* cysts and *A. avenae* nematodes, respectively (Clegg, 1965; Madin and Crowe, 1975). Trehalose defective mutants of *E. coli* have been found to have an impaired osmotic tolerance and an impaired stationary-phase-induced heat tolerance (Strom and Kaasen, 1993). The RNA-seq analysis shows the transcripts encoding proteins involved in trehalose synthesis (trehalose 6-phosphate synthase and trehalose 6-phosphate phosphatase) were up-regulated in *C. elegans* dauer larvae in response to all desiccation-related treatments (Figure 8.5). Additionally, the transcripts encoding the protein trehalase, which breaks down trehalose by hydrolysis, were down-regulated in *C. elegans* dauer larvae in response to desiccation. Therefore, it appears that *C. elegans* may accumulate trehalose in response to dehydration. In *P. superbus*, there was no increase in expression of genes encoding trehalose 6-phosphate synthase and trehalose 6-phosphate phosphatase. However, the genes involved in trehalose metabolism could be constitutively expressed in *P. superbus*. The *P. superbus* gene *PST1465_1*, which is homologous to the *C. elegans* gene *tps-2* (tps=trehalose phosphate synthase), is constitutively expressed in the undesiccated control nematodes with an average of over 1,500 reads identified for this gene in each control sample. A study by Shannon *et al*. (2005) found that *P. superbus* nematodes accumulate trehalose when exposed to preconditioning at high RH. *P. superbus* nematodes had an initial concentration of 10.8% trehalose and this value increased to 15.9% after 96 h of preconditioning at 98% RH. However, *P. superbus*, which demonstrates 93% survival when transferred directly to silica gel, did not demonstrate improved desiccation survival after preconditioning (Shannon *et al*., 2005). The high trehalose levels observed in fully hydrated *P. superbus* (10.8% dry mass) suggest that constitutive expression of high levels of trehalose may pre-adapt this nematode to combat desiccation (Shannon *et al*., 2005). Other *Panagrolaimus* species termed "slow dehydration strategists" require preconditioning at high RH to improve their tolerance to desiccation which correlates with increased trehalose accumulation (Shannon *et al*., 2005). The mean initial concentration of these "slow dehydration strategists" was 4.5% dry mass and this value was found to rise to 10.3% dry mass after 96 h preconditioning which is similar to the trehalose levels observed in fully hydrated *P. superbus* (Shannon *et al*., 2005).

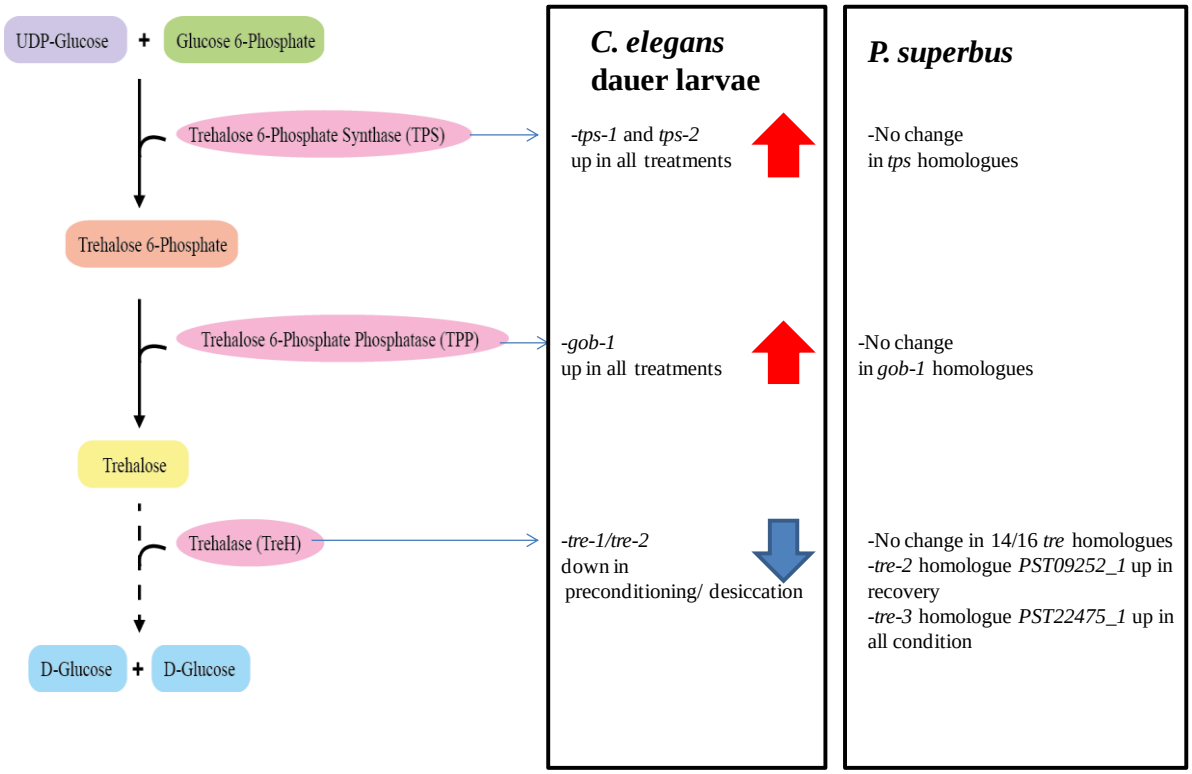


Figure 8.5 The trehalose-6-phosphate synthase/trehalose-phosphatase pathway for trehalose biosynthesis. The expression of genes (statistically up or down-regulated) encoding proteins involved in the trehalose biosynthesis pathway are shown in response to desiccation-related treatments in *C. elegans* dauer larvae and *P. superbus*. These include genes encoding trehalose 6-phosphate synthase (*tps-1* and *tps-2*), trehalose 6-phosphate phosphatase (*gob-1*) and trehalase (*tre-1*, *tre-2*, *tre-3* and *tre-4*).

8.8 Antioxidants

Reactive oxygen species (ROS) accumulate in the cells as a result of cellular dehydration and can cause damage to proteins, lipids, DNA and other macromolecules (Bowler *et al.*, 1992; Franca *et al.*, 2007; Kranner and Birtic, 2005). In *P. superbus* and *C. elegans* dauer larvae a substantial number of genes which encode antioxidants are up-regulated in response to desiccation and during recovery from desiccation. However, there was a greater variety of antioxidant genes that were up-regulated in response to desiccation in *P. superbus*, as compared to *C. elegans* dauer larvae. This includes up-regulation of *P. superbus* genes which encode antioxidants such as superoxide dismutase, animal haem peroxidase, peroxiredoxin, glutaredoxin, nucleoredoxin, glutathione peroxidase and glutathione *S*-transferase. In *C. elegans* genes encoding the antioxidants superoxide dismutase, glutathione *S*-transferase, glutathione peroxidase and catalase were up-regulated in response to desiccation. Interestingly, several gene homologs encoding superoxide dismutase (*sod-4* and *sod-5*), glutathione peroxidise (*C11E4.1*), glutathione *S*-transferase (*gst-8*) and peroxiredoxin (*prdx-6*) were up-regulated in response to desiccation in *C. elegans* dauer larvae and *P. superbus*. Additionally, up-regulation of *gst-34* and *prdx-6* was observed in *C. elegans* dauer larvae at the transcript level, as well as in *P. superbus* at the protein level in response to desiccation.

An up-regulation of genes encoding various antioxidants was observed in response to desiccation in the anhydrobiotic nematode *A. avenae* (Browne *et al.*, 2004; Reardon *et al*., 2010), in the entomopathogenic nematode *S. carpocapsae* (Tyson *et al*., 2007) and in the Antarctic nematode *P. murrayi* (Adhikari *et al.*, 2010), as well as in several other anhydrobiotic organisms such as *S. cerevisiae* (Ratnakumar *et al.*, 2011) and the resurrection plant *C. plantagineum* (Rodriguez *et al.*, 2010). Recent proteomic studies have also revealed an increased abundance of antioxidants in response to desiccation in the entomopathogenic nematode *S. feltiae* (Chen *et al.*, 2006), the Antarctic midge *B. antarctica* (Li *et al.*, 2009) as well as in maize embryos (Huang *et al.*, 2012) and the spike moss *S. tamariscina* (Wang *et al.*, 2010).

8.9 Molecular chaperones

There was an increase in the expression of HSPs at both the transcriptomic and proteomic level in both *C. elegans* dauer larvae and *P. superbus* in response to desiccation. The greatest overlap of up-regulated HSP homologues was seen at the transcriptomic level.

The *hsf-1* (heat shock factor) gene was up-regulated in *C. elegans* and *P. superbus* in response to desiccation. In *C. elegans*, HSF-1 acts downstream of DAF-2/DAF-16 in defense response and confers protection through a system of HSPs, independently of the p38 MARK pathway (Singh and Aballay, 2006). RNAi knockdown of *hsf-1* in *C. elegans* results in reduced mRNA expression in genes which encode small HSPs (*hsp-16.2* and *sip-1*) as well as HSP-70 (Chiang *et al.*, 2012). Additionally, HSF-1 is also involved in longevity (Garigan *et al.*, 2002) as well pathogen defence (Aballay *et al.*, 2003). This transcription factor may be responsible for the increased transcription of HSPs observed *P. superbus* and *C. elegans* dauer larvae in response to desiccation.

A large number of genes encoding HSP-70 proteins were up-regulated in both nematodes in response to desiccation. HSP-70 chaperones bind selectively to unfolded hydrophobic regions of proteins and their activity is controlled by the cycle of ATP binding, hydrolysis and nucleotide exchange (Minami *et al.*, 1996). Five desiccation responsive *hsp-70* genes were up-regulated in *C. elegans* (*hsp-1, hsp-3, hsp-6, hsp-70* and *F44E5.5*) and nine in *P. superbus* nematodes *(hsp-70a, hsp-70b, hsp-70c, hsp-70d, hsp-70e, hsp-70f, hsp-70g* and *hsp-1* and *F44E5.5*). Seven *hsp* genes were up-regulated in response to desiccation in *P. superbus* which were homologous to the *C. elegans hsp-70* gene. Thus a lineage expansion of the *hsp-70* gene family may have occurred in *P. superbus* as an adaptation to increase its tolerance to desiccation. The HSP-70 family is the largest group of heat shock proteins, involved in inhibiting protein aggregation as well as promoting folding in newly synthesized and denatured proteins (Jonsson and Schill, 2007). Previous studies have found an increased expression of genes encoding various HSP proteins such as in the Antarctic midge *B. antarctica* (Teets *et al.*, 2012) and the Antarctic nematode *P. murrayi* (Adhikari *et al.*, 2010). HSP proteins have also been shown to increase in response to desiccation in the entomopathogenic nematode *S. feltiae* (Chen *et al.*, 2006), Antarctic midge *B. antarctica* (Li *et al.*, 2009) as well a number of plants including the lichen phycobiont *A. erici* (Gasulla *et al.*, 2013) and maize embryos (Huang *et al.*, 2012).

Small HSPs (sHSPs) protect cells from protein losses or toxicity caused by aggregation (Sun and Macrae, 2005). sHSPs are the major "holding" chaperones, retaining unfolding proteins in a conformation suitable for subsequent refolding thus preventing their irreversible aggregation (Eyles and Gierasch, 2010; Stengel *et al.*, 2010). In *P. superbus*

and *C. elegans* dauer larvae *hsp-43*, which encodes a sHSP from the HSP-20 family, was up-regulated in response to desiccation. Interestingly, *hsp-20* was up-regulated across desiccation and recovery in *P. superbus* but was only up-regulated in response to recovery in *C. elegans* dauer larvae. The small heat shock protein HSP-12.6 was also up-regulated in *C. elegans* dauer larvae at the transcript and proteome level in response to desiccation, as revealed by the RNA-seq and the LFQ based proteomic analyses. A correlation between the accumulation of transcripts encoding sHSP and desiccation tolerance has been observed in larvae of *A. franciscana* (Liang *et al.*, 1997) and the Antarctic midge *B. Antarctica* (Teets *et al*., 2012). An increased abundance of sHSP proteins was also observed in maize embryos in response to desiccation (Huang *et al.*, 2012). Finally, in both nematodes *dnaj-8* was up-regulated in response to desiccation. Proteins containing a DnaJ domain are either known, or suspected, to be co-chaperones that guide HSP-70 proteins to specific substrates and control their binding to substrates by control of their ATPase cycle (Vos *et al.*, 2008).

At the transcript level in *C. elegans* dauer larvae and at the protein level in *P. superbus* *cct-7* (cct=chaperonin containing TCP-1) and *pfd-5* (pfd=prefoldin) were up-regulated in response to desiccation and recovery from desiccation. *cct-7* encodes a protein involved in the folding of newly synthesized polypeptides in a wide variety of proteins (Young *et al.*, 2004). Furthermore, in mammals TCP-1 and GimC/prefoldin have been shown to work as an integrated team for the purpose of protein folding (Valpuesta *et al.*, 2002). Moreover, PFD-2 is also up-regulated in *C. elegans* dauer larvae and *P. superbus* nematodes in response to desiccation.

8.10 Stress related genes

In both the *C. elegans* dauer larvae and the *P. superbus* studies up-regulation of the stress related genes *dj-1* and *lea-1* was observed in response to desiccation and recovery from desiccation. *lea-1* was up-regulated 6 fold and 30 fold in response to desiccation in *P. superbus* and *C. elegans* dauer larvae respectively. Additionally, the LFQ proteomic analysis found that LEA-1 is up-regulated in response to desiccation in *P. superbus*, while LEA-1 was not detected in any *C. elegans* dauer larvae protein samples, including the control. LEA proteins are multifunctional proteins that are known to have a role in desiccation tolerance (Gal *et al.*, 2004; Tunnacliffe and Wise, 2007; Hand *et al.*, 2011).

While, there is only one *lea* gene in *C. elegans*, there appear to be multiple genes related to *lea* in *P. superbus* (11 genes with homology to the *C. elegans lea-1* gene in the *P. superbus* transcriptome). The possible expansion of the *lea* genes in *P. superbus* may be linked to its ability to survive extreme desiccation. The relative abundance of *lea* genes in *P. superbus* as compared to *C. elegans*, along with their constitutive expression, suggest that LEA proteins are an important component of the anhydrobiotic protection repertoire of *P. superbus* and that the *lea* gene family may have undergone lineage-specific expansion in this species (Tyson *et al.*, 2012). Recent studies have found that genes encoding LEA proteins increase in abundance upon desiccation in *Arabidopsis thaliana* (Maia *et al*., 2011) as well as in the leaves of *Craterostigma plantagineum* (Rodriguez *et al*., 2010). Moreover, increased abundance of LEA proteins was observed in the spike moss *Selaginella tamariscina* (Wang *et al*. 2010) and maize embryos (Huang *et al*., 2012) in response to desiccation.

In *C. elegans* dauer larvae and *P. superbus*, up-regulation of *dj-1* (mammalian transcription factor) homologs was observed in response to desiccation and recovery. The *C. elegans* genome contains two homologs of the human *dj-1* gene viz. *djr-1.1* (B0432.2) and *djr-1.2* (C49G7.11) (*djr* = DJ-1 related) (Castro *et al.*, 2010). The *djr-1.2* gene was substantially up-regulated in response to desiccation in *C. elegans* dauer larvae. In contrast, a gene homologous to the *C. elegans* gene *djr-1.1* was up-regulated in *P. superbus* in response to desiccation. In the *P. superbus* transcriptome, there are three genes which show homology to *djr-1.1*. Knockout studies in *C. elegans* have revealed that *djr-1.1* and *djr-1.2* have a role in ubiquitin-proteasomal systems, immunity and response to oxidative stress (Ved *et al.*, 2005). *C. elegans* DJR-1.1 and DJR-1.2 have recently been shown to have glyoxalase activity, with DJR-1.2 specifically protecting dopaminergic neurons (Lee *et al.*, 2012).

8.11 Cellular organisation

The RNA-seq and proteomic analyses of *C. elegans* dauer larvae and *P. superbus* show the importance of adjustments to the cytoskeleton and cellular structures during anhydrobiosis. In *C. elegans* dauer larvae, there were 94 genes involved in cytoskeleton organisation that had increased expression in response to desiccation. In *P. superbus*, there were only 9 genes involved in cellular organisation that are up-regulated in response

to desiccation. However, there were fifteen proteins associated with cellular organisation and morphogenesis that were up-regulated in response to desiccation-related treatments in this nematode. Interestingly, the structural proteins VAB-10 (vab=variable abnormal morphology), UNC-94 (unc=uncoordinated), AJM-1 (ajm=apical junction molecule) and CAP-2 (cap=cap-z protein) were up-regulated in response to desiccation at the protein level in *P. superbus* as well at the transcript level in *C. elegans* dauer larvae. Of these, VAB-10 and UNC-94 were also up-regulated in *C. elegans* dauer larvae at the protein level. This indicates that the role of certain genes and proteins in desiccation tolerance may be conserved between *C. elegans* dauer larvae and *P. superbus*. Interestingly, Teets *et al*. (2012) found that the genes up-regulated in response to desiccation in the Antarctic midge *B. antarctica* were enriched with terms related to actin organisation. Additionally Li *et al*. (2009) concluded that the major protein responses elicited by both desiccation and rehydration in *B. antarctica* are linked to body contraction and cytoskeleton rearrangement. Finally, in the moss *Polytrichum formosum* the cytoskeleton also plays a significant role in both desiccation and rehydration (Proctor *et al*., 2007). Cytoskeletal reorganisation is also likely to be important in during desiccation when there is a dramatic change in the morphology of the *C. elegans* dauer larvae and of *P. superbus*.

8.12 DNA damage repair and packaging

There are desiccation-responsive genes in both *C. elegans daf-2* dauer larvae and *P. superbus* nematodes that are associated with DNA repair. Moreover, there appear to be differences in the types DNA repair mechanisms undertaken by *P. superbus* and *C. elegans* in response to desiccation stress. The RNA-seq studies revealed that there were clearly many more DNA repair genes up-regulated in response to desiccation in *C. elegans daf-2* dauer larvae compared with *P. superbus* nematodes. Most of these DNA repair genes were up-regulated in *C. elegans* in response to harsh desiccation (4 days at 98% RH and 1 day at 32.5% RH). There was no equivalent harsh desiccation time-point in the *P. superbus* data. This may explain why there were so few genes relating to DNA repair up-regulated in *P. superbus* in response to desiccation. Alternatively, DNA repair mechanisms may be constitutively expressed in *P. superbus*. The contrast in the DNA repair mechanisms utilised may contribute to the differing tolerances to desiccation between *C. elegans* dauer larvae and *P. superbus*.

Various repair mechanisms are conserved across different organisms to repair DNA damage (Dizdaroglu, 2012). DNA damage can lead to genetic instability which may ultimately result in death. The major mechanisms used by a variety of organisms to repair DNA damage are base-excision repair (BER), nucleotide-excision repair (NER) and repair of double strand breaks. In *C. elegans daf-2* dauer larvae, the RNA-seq analysis revealed that NER is the major DNA repair pathway up-regulated in response to desiccation. In total, thirteen out of a possible thirty-five genes in the NER pathway were up-regulated during desiccation in *C. elegans daf-2* dauer larvae. Furthermore, *cku-70* (cku=caenorhabditis KU), which is thought to be involved in non-homologous end joining of double-stranded breaks in DNA (Clejan *et al.*, 2006), was also up-regulated in *C. elegans daf-2* dauer larvae in response to desiccation. *crn-1* which encodes a protein which promotes apoptotic DNA degradation (Parrish *et al.*, 2003) was also up-regulated in response to desiccation. In contrast, the genes up-regulated in *P. superbus* in response to desiccation-related treatments were mainly associated with double strand breaks (*ck-1*, *sirt-6* and *sprt*).

In desiccated tardigrades, DNA damage occurs and accumulates with time. Additionally, DNA damage which occurs during anhydrobiosis can lead to the death of tardigrades (Rebecchi *et al*., 2006). Damaging effects of oxidation by reactive oxygen species (ROS) could be one of the reasons for the decrease in survival rate after long periods of desiccation (Neumann *et al*., 2009). Anhydrobiotic nematodes in a desiccated state are unable to repair damaged macromolecules or proteins since water is absent and their metabolism arrested (Neumann *et al*., 2009). Therefore, DNA repair transcripts which are up-regulated in *P. superbus* and *C. elegans* in response to desiccation are likely to be transcribed only upon rehydration.

Histone proteins that are involved in DNA packaging were up-regulated in both *C. elegans* dauer larvae and *P. superbus* in response to desiccation and recovery from desiccation. In *C. elegans* dauer larvae, the histones HIS-45, HIL-1 and HTZ-1 were up-regulated in response to desiccation-related treatments. While in *P. superbus* the histones HIS-62 and HIS-22 were up-regulated in response to desiccation and recovery. In addition to their well-known function in DNA packaging these core histones are also involved in

DNA replication, transcription and repair by controlling the accessibility of the different macromolecular machineries to their substrate (Escargueil *et al.*, 2008).

8.13 Removal of damaged proteins

The RNA-seq and differential proteomic analysis of *C. elegans* dauer larvae and *P. superbus* has revealed that pathways involved in the removal of damaged proteins were enriched in response to desiccation-related stresses. Essential cellular systems can be affected if the accumulation of misfolded proteins exceeds the cellular chaperone capacity. Misfolded proteins are degraded by either the autophagy or ubiquitin-proteasome systems (Lamark and Johansen, 2010). A substantial number of genes associated with the ubiquitin-proteasome system and autophagic programmed cell death were significantly up-regulated in response to desiccation and recovery in the RNA-seq datasets from both nematodes. There were considerably more genes up-regulated in response to desiccation in *C. elegans* dauer larvae as compared to *P. superbus*. Moreover, the number of up-regulated genes associated with protein degradation peaked in the *C. elegans* desiccation dataset. In *P. superbus*, the greatest up-regulation of genes which encode proteins involved in the removal of damaged proteins was seen in the recovery dataset. The genes *ced-1* (ced=cell death abnormality) and *lgg-2* (lgg=LC3, GABARAP and GATE-16 family) which encode proteins associated with autophagic programmed cell death were up-regulated in both *P. superbus* and *C. elegans* dauer larvae in response to desiccation-related time-points. CED-1 is a component of the apoptotic pathway and functions to initiate a signaling pathway in phagocytic cells that promotes cell corpse engulfment and phagosome maturation (Yu *et al.*, 2008), while *lgg-2* encodes an ortholog of the autophagic budding yeast Atg8p (Khan *et al.*, 2008). The gene *rpn-7* (proteasome Regulatory Particle, Non-ATPase-like), which is associated with proteosomal degradation was also up-regulated in response to dehydration in *C. elegans* dauer larvae and *P. superbus*. In the yeast *S. cerevisiae* a microarray study of desiccation responsive genes, revealed that GO terms associated with autophagy were overrepresented (Ratnakumar *et al.*, 2011). Similarly in the dehydration tolerant midge *B. antarctica*, Teets and Denlinger (2013) found that that cell recycling pathways are essential for extreme dehydration tolerance, with autophagy serving as the focal point and that genes involved in the ubiquitin-mediated proteasomal pathway were also enriched among the dehydration-up-

regulated genes. A recent proteomic study in maize embryos also found a higher abundance of proteasomal proteins in response to dehydration (Huang *et al*. 2012).

8.14 Response to pathogens

A number of individual genes which encode proteins involved in pathogen defense were up-regulated in response desiccation and recovery in both *C. elegans* dauer larvae and *P. superbus*. The genes *fip-6* (fip=fungus induced protein) and *lys-8* (lys=lysosome) were up-regulated in response to desiccation and recovery in both nematodes. Both *fip-6* and *lys-8* have been implicated in pathogen defence in *C. elegans* (O'Rourke *et al.*, 2006; Pujol *et al.*, 2008; Pujol *et al.*, 2012). In *C. elegans* the infection of barrier epithelia results in the up-regulation of genes encoding antimicrobial peptides (AMPs) such as fungus induced proteins (Pujol *et al.*, 2008).

8.15 Summary

The data show that substantial reorganisation of cellular structures and mobilizaton of cellular protection repair systems occurs in *C. elegans* dauer larvae and *P. superbus* in response to desiccation, indicating that anhydrobiotic survival requires a major, integrated organismal response.

8.16 Future work

The data presented in this thesis provide a system-wide overview of the organismal responses of *C. elegans* dauer larvae and mixed population stages of *P. superbus* to desiccation and recovery from desiccation. Although differences exist in the timing of the expression profiles of specific genes and pathways during the induction of anhydrobiosis in *C. elegans* dauer larvae and *P. superbus* (possibly as a result of the extent to which a specific pathway is inducible or constitutive), nevertheless it is clear that both nematodes share a similar repertoire of anhydrobiotic protection mechanisms. Thus this information provides a framework for functional studies on the molecular mechanisms of specific molecules and pathways in anhydrobiotic protection.

8.16.1 High-throughput RNAi

The RNAi experiments carried out in this project show that in many cases the importance of specific up-regulated genes or proteins in anhydrobiotic protection can be confirmed. Thus a more extensive high-throughput RNAi could be used to identify other genes which are involved in the mechanisms of desiccation tolerance in *C. elegans* dauer larvae. This task would be made easier because of the commercial availability of *C. elegans* RNAi libraries which cover the majority of the protein encoding genes in *C. elegans*. Focusing on the other desiccation-responsive genes and proteins which were up-regulated in this RNA-seq and proteomics study in the first instance should provide an enriched set of target genes.

8.16.2 *C. elegans* mutants

There exists a large collection of *C. elegans* mutants which could also be utilised to investigate the involvement of specific genes in desiccation tolerance. The National Bioresource project in Japan (www.shigen.nig.ac.jp/c.elegans/mutants) houses an ever-expanding library of deletion mutants of *C. elegans*. *C. elegans* mutants are also being generated in a project called "the million mutation project" **(**Thompson *et al.***,** 2013). These projects generate *C. elegans* knockouts using the mutagens such as ethyl methanesulfonate (EMS) or N-ethyl-N-nitrosourea (ENU). Whole genome sequencing is then carried out to confirm successful knockouts. The *C. elegans* mutants of interest could be integrated into the *C. elegans daf-2* strain by genetic crossing and used to investigate the effect of specific gene knockouts on the ability of dauer larvae to survive desiccation. Gene knockout studies do not always yield conclusive data on the importance of a specific gene in a molecular process. Some of the genes and proteins may function redundantly (e.g. some of the HSPs or antioxidants) so that knocking out a single gene may not yield a mutant phenotype. However, the role of a key gene or protein in a particular pathway may be essential and in this case the significance of a particular pathway can be confirmed by RNAi or with knockdown mutants. Having identified such key proteins further characterization of the role of the protein in anhydrobiosis could be done *in vitro* using recombinant proteins.

8.16.3 Immunocytochemistry

Immunocytochemistry could also be used to visualise the distribution of target proteins in *C. elegans* dauer larvae during desiccation. Immunocytochemistry provides the most straightforward method for identifying both the cellular and subcellular distribution of a protein by targeting specific peptides. Immunocytochemistry is more reliable than GFP (gfp=green fluorescent protein) or other translational or transcriptomic constructs which suffer from a number of potential problems. These include abnormal regulation of expression from multi-copy transgenic arrays and abnormal localisation of the protein due to over-expression or the presence of the tag. In contrast the antibody-based immunocytochemistry does not experience these problems. Moreover, specific antibodies also allow you to examine protein modifications such as phosphorylation or glycosylation *in vivo* (Duerr, 2006).

The complexity and extent of the cellular and metabolic reorganisation which occurs during the induction of anhydrobiosis makes it difficult to envisage how the data obtained in this project could be used to direct the anhydrobiotic engineering of a desiccation sensitive organism. However there may exist a hierarchy of anhydrobiotic protection mechanisms which could be identified. Once these key protection mechanisms have been found it may be possible to use this information to genetically improve desiccation-sensitive organisms to be able to withstand desiccation stress, even if their full-scale anhydrobiotic engineering remains intractable.

Additional notes in relation to the publication by Erkut *et al.* (2013)

In the final stages of the preparation of this PhD thesis Erkut *et al.* (on December 04 2013) published the results of their study investigating the mechanisms underlying desiccation in the *C. elegans* dauer larvae using microarray transcriptomics and proteomics methods. Moreover, Erkut *et al.* further investigated the role of candidate genes in desiccation tolerance using *C. elegans* mutant strains as well as RNAi. The experimental design for the investigation of desiccation tolerance in *C. elegans* by Erkut *et al.* (2013) is shown in Figure 1 while the experimental design used in the experiments described in this thesis is summarized in Figure 2. The results of our analysis of desiccation tolerance in the *C. elegans* dauer larvae in the context of the work carried out by Erkut *et al.* (2013) are further discussed below.

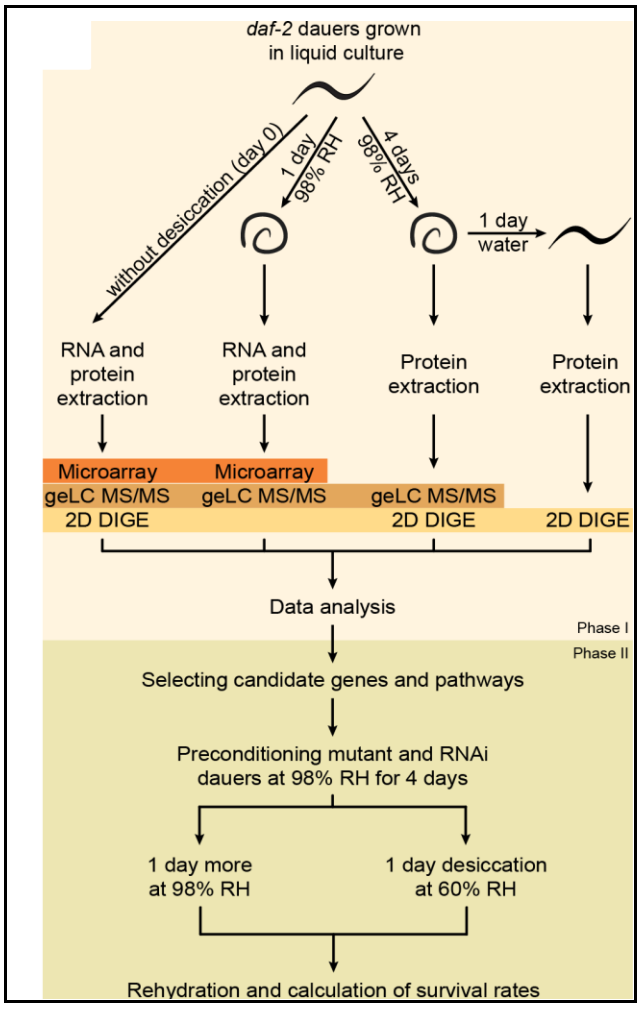


Figure 1 Experimental design of the investigation of desiccation tolerance in *C. elegans* dauer larvae by Erkut *et al.* (2013).

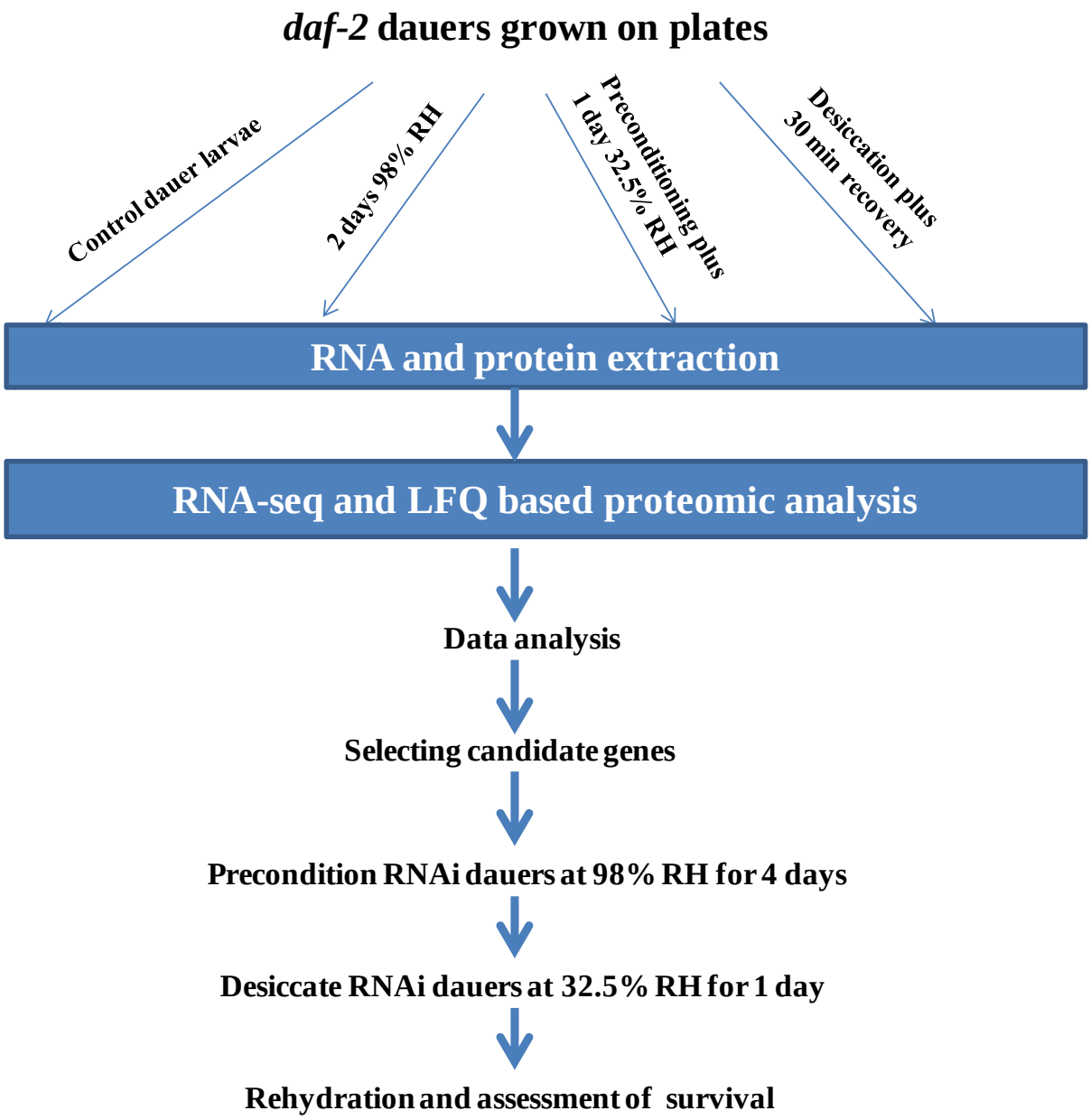


Figure 2 Experimental design of the investigation of desiccation tolerance in *C. elegans* dauer larvae by Mulvihill *et al.* (2014).

Transcriptomic analyses of desiccation survival in dauer larvae of *Caenorhabditis elegans*

C. elegans daf-2 (e1370) dauer larvae were used in both studies. These mutants are temperature-sensitive dauer-constitutive nematodes. *C. elegans daf-2* (e1370) worms propagate normally at 15 ºC but when grown at 25 ºC they form dauer larvae (Kenyon *et al.*, 1993). The time points for our RNA-seq experiment were as follows (1) control dauer larvae, (2) dauer larvae exposed to two days of preconditioning (98% RH), (3) dauer larvae exposed to four days of preconditioning followed by one day at 32.5% RH and (4) dauer larvae exposed to condition 3 followed by a 30 min recovery period in water. These desiccation treatments were selected based on desiccation survival data (Section 5.2.1) and qPCR expression analysis of putative anhydrobiotic genes in response to different desiccation treatments (Section 5.2.2). The time points for the microarray carried out by Erkut *et al.* (2013) were as follows: (1) control dauer larvae and (2) dauer larvae exposed to one day preconditioning (98% RH). The most comparable treatments in the RNA-seq and transcriptome datasets are: dauer larvae exposed to one day preconditioning at 98% RH (microarray analysis) and dauer larvae exposed to two days preconditioning at 98% RH (RNA-seq analysis). In both studies significantly up-regulated genes were identified relative to their expression in undesiccated control dauer larvae.

In our RNA-seq analysis, we found that 2,763 genes were significantly up-regulated and 3,907 genes were significantly down-regulated in *C. elegans dauer* larvae in response to preconditioning. In the microarray analysis Erkut *et al*. found that 1,833 genes showed significantly increased expression and 2,433 genes had significantly decreased in expression. A comparison of the RNA-seq dataset (two days preconditioning at 98% RH) with the microarray preconditioning dataset (one day preconditioning at 98% RH) shows that 762 genes (42%) were up-regulated and 1,318 genes (54%) were down-regulated in common in both datasets. Looking at the genes with the highest increase in expression, referred to as the high fold change cluster in the analysis by Erkut *et al.* (2013), 48 of a total 65 genes (75%) were up-regulated and 80 out of 103 genes (78%) were down-regulated in both the RNA-seq and microarray analyses. Therefore, it appears that the genes which were substantially differentially expressed in response to preconditioning were very similar in both transcriptomic analyses, although the nematodes used for the RNA-seq experiment had been preconditioned for two days while the microarray dataset was based on larvae preconditioned for one day. However, when comparing all the

differentially expressed genes from low, medium and high fold change clusters of Erkut *et al*. (2013) there are major differences as only 42% of the up-regulated genes and 54% of the down-regulated genes overlap. There were 930 more genes up-regulated in the response to preconditioning in the RNA-seq analysis compared to the microarray analysis. Several factors may be responsible for the differences in the number of differentially expressed genes detected in the two datasets: 1) the differences in the length of preconditioning at 98% RH; 2) the methods used to detect differential expression (i.e. RNA-seq versus microarray); 3) the statistical methods used to identify differentially expressed genes – using k-means clustering Erkut *et al.* eliminated genes with less than 1.82 fold up-regulation and 1.92 down-regulation from their dataset while in this study we concentrated on those differentially expressed genes which had a >2 fold change in expression and a Benjamini-Hochberg *p*-adjusted cut-off value of <0.01; and 4) the culturing methods to produce dauer larvae (microarray analysis=grown in liquid culture, RNA-seq analysis=grown on agar plates).

Genes involved in several pathways and processes were up-regulated in both datasets: genes encoding molecular chaperones, antioxidants, intrinsically disordered proteins (*lea-1* and *dur-1*), as well as enzymes required for trehalose biosynthesis and lipid metabolism. The following pathways were also down-regulated in both the microarray and RNA-seq analyses: cuticle and extracellular matrix proteins, lipid glycosylation enzymes and proteases. However genes involved in some other pathways and processes which were only found to be activated in our RNA-seq preconditioning dataset include signal transduction, programmed cell death, apoptosis, endocytosis, and body morphogenesis. There were also pathways and processes that were only identified in our desiccation and recovery time points such as insulin-signalling, DNA-repair, autophagy, ubiquitin-proteasome processes and antimicrobial peptides. The microarray analysis by Erkut *et al.* focussed on dauer larvae which had been preconditioned at 98% RH for 24 hours – these authors did not include desiccated or recovering dauer larvae in their analysis. Thus these recovery and desiccation pathways were not detected in their microarray study and interestingly these pathways were also not detected by Erkut *et al.* in their proteomic studies of dauer larvae which had been exposed to 98% RH for four days, or in rehydrated nematodes as discussed below.

Proteomic analyses of desiccation survival in dauer larvae of *Caenorhabditis elegans*

Erkut *et al.* (2013) also investigated the proteins differentially expressed in response to preconditioning using label-free protein quantification method geLC-MS/MS where one dimensional gel electrophoresis is combined with label free protein quantification (Vasilj *et al.*, 2012). In our analysis of proteins induced in the *C. elegans* dauer larvae in response to preconditioning we used a label-free gel-free method as described in sections 2.2.4 and 2.2.5. In our label-free analysis a Thermo Scientific Q Exactive Orbitrap mass spectrometer was used while a Thermo Scientific LTQ Orbitrap Velos mass spectrometer was used in the experiment by Erkut *et al.* The LTQ Orbitrap Velos contains a linear ion trap system while the Q Exactive Orbitrap contains a quadropole mass filter. In both experiments the mass spectrometer was used in conjunction with a Dionex Ultimate 3000 (RSLCnano) chromatography system. Linear elution gradients of 150 minutes were used by Erkut *et al.* while shorter 43 minutes gradients were used in our gel-free proteomic analysis. There were also differences between the downstream software packages used to identify peptides and to determine whether proteins are differentially expressed. MaxQuant (version 1.2.2.5; http://maxquant.org/) quantitative proteomics software package supported by the Andromeda database search engine (Cox and Mann, 2011; Cox *et al.*, 2011) was used to correlate MS/MS data against the WormBase WS215 protein database in our gel-free analysis while database searches were performed using Mascot 2.2.04 software (Matrix Science, UK) against the WormBase WS228 protein sequence database in the analysis by Erkut *et al.*. Additionally, differentially abundant proteins among experimental groups were determined by label-free quantification (LFQ) using MaxQuant and Perseus software packages (Cox and Mann, 2011) in our gel-free analysis while Erkut *et al.* used SuperHirn 0.03 and DanteR 1.0.1.1 for the same function.

The conditions used for our proteome analysis were identical to the ones used in the RNA-seq experiment described above i.e. undesiccated control, preconditioning (two days at 98% RH), desiccation (four days of preconditioning at 98% RH followed by one day at 32.5% RH) and recovery (dauer larvae exposed to desiccation, followed by a 30 min recovery period in water). In the proteomic analysis by Erkut *et al.* undesiccated control dauer larvae as well as dauer larvae preconditioned for one or four days were used in the geLC MS/MS analysis. A total of 721 *C. elegans* proteins were detected across the conditions in our analysis while 1,058 proteins were detected in the label-free analysis

preformed by Erkut *et al.* In our analysis 94 proteins increased in abundance and 20 proteins decreased in abundance in response to preconditioning (98% RH for two days) compared to the control. In the label-free proteomic analysis carried out by Erkut *et al.* the abundance of 17 proteins was significantly increased response to preconditioning at 98% RH for one day and none were reported as being down-regulated for this treatment (Erkut *et al*., 2013, dataset S3). Following four days preconditioning at 98% RH Erkut *et al*. found that 39 proteins were up-regulated and 9 proteins were down-regulated in response to preconditioning. Four proteins were up-regulated in response to preconditioning at 98% RH in both studies: HSP-12.6 (hsp=heat shock protein), DUR-1 (dur=dauer up-regulated), DIG-1 (dis=displaced Gonad) and novel protein C16E9.1. However, when the up-regulated proteins in the Erkut *et al.* preconditioning dataset are compared to the proteins up-regulated in response to all desiccation-related treatments (preconditioning, desiccation and recovery versus the control) in our dataset two more proteins occur in common: GPX-5 (gpx=glutathione peroxidase) and TTR-17 (ttr=transthyretin-Related family domain). SOD (sod=super oxide dismutase) proteins, IF (if=intermediate filament) proteins as well as multiple members of the TTR (TransThyretin-Related family domain) family were also up-regulated in both studies. Possible reasons for the differences in the results of the label-free analyses of desiccation tolerance in *C. elegans* dauer larvae observed could be attributed to: the desiccation treatment conditions used; sample preparation e.g. the use of 1-D electrophoresis in the Erkut *et al*. study could preferentially exclude large proteins, as well as poorly soluble and hydrophobic proteins; the lengths of the linear elution gradients; the different mass spectrometers used (Q Exactive orbitrap versus LTQ) and the different software packages used to identify proteins and to determine whether proteins were differentially expressed.

To investigate the protein profiles of rehydrated dauer larvae Erkut *et al*. compared the 2D DIGE protein profiles of desiccated (four days at 98% RH) and recovering dauer larvae (24 hours in water). They found that “many DTR (desiccation tolerance relevant) proteins were down-regulated to their basal levels, although the level of some proteins remained elevated”. They also found that “desiccation-induced post-translational modifications were also reversed in many cases upon rehydration”. However their overlay of 2D-DIGE images of preconditioned dauer proteomes before and after rehydration (Erkut *et al*., Figure S2) do not show evidence of differentially up-regulated proteins at this time point. In our LFQ analysis some of the proteins up-regulated specifically in response to recovery

from desiccation include antioxidants (SOD-2 and GST-7), structural proteins (IFA-1, MYO-3) and ribosomal proteins (RPL-17 and RPL-18).

Functional investigation of the involvement of candidate genes in conferring desiccation tolerance in *C. elegans* dauer larvae

Erkut *et al.* (2013) selected candidate desiccation tolerance relevant genes for functional analysis based on the results of the microarray transcriptomic and label-free proteomic analyses described above. They investigated the involvement of candidate genes using 37 different *C. elegans* mutants obtained from the *Caenorhabditis* Genetics Center, USA and the National Bioresource Project, Japan. Erkut *et al.* compared the survival of control dauer larvae and gene knockout dauer larvae in response to 98 % RH and 60% RH for 24 h. In contrast, we compared the survival of control dauer larvae and RNAi treated dauer larvae in response to 32.5% RH for 24 h. Although, Erkut *et al.* used *C. elegans* dauer larvae carrying mutations in candidate genes in their functional analyses, they also used RNAi to investigate the involvement of *lea-1* in *C. elegans* dauer larvae desiccation tolerance.

Some of the genes selected by Erkut *et al.* were eliminated from our candidate list of genes for further investigation by RNAi as they had been shown to produce deleterious phenotypes in previous studies. These include genes which encode HSPs (*F08H9.3*, *F08H9.4* and *hsp-70*), superoxide dismutase (*sod-1*, *sod-3* and *sod-5*) and catalase (*ctl-1* and *ctl-3*). However the involvement of the six genes in desiccation tolerance in *C. elegans* dauer larvae was investigated in both studies: *dur-1*, *lea-1*, *hsp-12.6*, *djr-1.1/djr-1.2* and *cdr-3* (Table 1). Reduced expression of *dur-1* and *lea-1* significantly reduced survival in response to desiccation both analyses (Table 1). In our analysis, we also found that RNAi knockdown of *hsp-12.6* reduced desiccation survival rates, however a reduction in *C. elegans hsp-12.6* knockout dauers survival upon desiccation was not observed by Erkut *et al.* The conflicting results could be attributed to the low number of replicates used in the Erkut *et al.* (n=2) study versus our RNAi study (n=5) which would have reduced the power of their statistical analysis. Erkut *et al.* found that *C. elegans* dauer larvae carrying *djr-1.1/djr-1.2* and *cdr-3* mutations had significantly reduced desiccation survival rates compared to the controls. However, a significant reduction in desiccation survival rates of *djr-1.1/djr-1.2* and *cdr-3* in *C. elegans* dauer larvae in comparison to the control was not observed in our RNAi knockdown studies. The reason

for this could be attributed to the different methods used i.e. RNAi and genomic lesions. In addition, in relation RNAi-*djr-1.1/djr-1.2* treated *C. elegans* dauer larvae, simultaneous knockdown of two genes by RNAi feeding is not as effective as knocking a single gene (Min *et al.*, 2010). In the RNAi analysis, we also found RNAi knockdown of three additional genes not tested by Erkut *et al*. (2013) (viz. glycerol kinase, C44B7.10 and F21C10.10) reduced the survival in response to desiccation in *C. elegans* dauer larvae (Chapter VII).

In conclusion, the two analyses investigating desiccation tolerance in *C. elegans* dauer larvae described in this thesis and by Erkut *et al.* (2013) are complementary. There, was considerable overlap between the differentially expressed genes and proteins detected in both studies, particularly in the high fold-change genes detected in the microarray analysis. Moreover, our RNA-seq and proteomic analyses identified additional pathways and process which were up-regulated in response to desiccation and rehydration. Thus the results presented in this thesis confirm and extend the work of Erkut *et al*. (2013) on the molecular strategies of *C. elegans* dauer larvae to survive and recover from extreme desiccation.

Table 1 Comparison of results of desiccation survival assays in *C. elegans* dauer larvae. The experiments conducted by Erkut *et al.* (2013) utilised dauers carrying mutations with the exception of *lea-1* where RNAi was used to alter gene expression. RNAi gene silencing was used in our analysis to further investigate the involvement of candidate genes in desiccation tolerance in *C. elegans* dauer larvae. (The symbol '+' indicates that there was a statistical difference in survival in response to desiccation compared to control dauer larvae while the symbol '-' indicates there was no statistical difference ($P<0.05$).

	Erkut *et al.*		**Our RNAi analysis**
Desiccation treatment	**98% RH**	**60% RH**	**32.5% RH**
dur-1	+	+	+
lea-1	-	+	+
hsp-12.6	-	-	+
djr-1.1/djr-1.2	-	+	-
cdr-3	-	+	-

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