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Genomic Analysis of Nematode-Environment Interaction

Bishwo Adhikari
Brigham Young University - Provo

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Bishwo N. Adhikari

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Byron J. Adams, Chair
Chin-Yo Lin
Craig E. Coleman
Gregory F. Burton
Joel S. Griffitts

Department of Biology

Brigham Young University

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ABSTRACT

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The natural environments of organisms present a multitude of biotic and abiotic challenges that require both short-term ecological and long-term evolutionary responses. Though most environmental response studies have focused on effects at the ecosystem, community and organismal levels, the ultimate controls of these responses are located in the genome of the organism.

Soil nematodes are highly responsive to, and display a wide variety of responses to changing environmental conditions, making them ideal models for the study of organismal interactions with their environment. In an attempt to examine responses to environmental stress (desiccation and freezing), genomic level analyses of gene expression during anhydrobiosis of the Antarctic nematode *Plectus murrayi* was undertaken. An EST library representative of the desiccation induced transcripts was established and the transcripts differentially expressed during desiccation stress were identified. The expressed genome of *P. murrayi* showed that desiccation survival in nematodes involves differential expression of a suite of genes from diverse functional areas, and constitutive expression of a number of stress related genes. My study also revealed that exposure to slow desiccation and freezing plays an important role in the transcription of stress related genes, improves desiccation and freezing survival of nematodes.

Deterioration of traits essential for biological control has been recognized in diverse biological control agents including insect pathogenic nematodes. I studied the genetic mechanisms behind such deterioration using expression profiling. My results showed that trait deterioration of insect pathogenic nematode induces substantial overall changes in the nematode transcriptome and exhibits a general pattern of metabolic shift causing massive changes in metabolic and other processes. Finally, through field observations and molecular laboratory experiments the validity of the growth rate hypothesis in natural populations of Antarctic nematodes was tested. My results indicated that elemental stoichiometry influences evolutionary adaptations in gene expression and genome evolution. My study, in addition to providing immediate insight into the mechanisms by which multicellular animals respond to their environment, is transformative in its potential to inform other fundamental ecological and evolutionary questions, such as the evolution of life-history patterns and the relationship between community structure and ecological function in ecosystems.

Keywords: *Plectus murrayi*, *Heterorhabditis bacteriophora*, *Scottnema lindsayae*, stress survival, complementary DNA library, subtractive hybridization, comparative transcriptomics, transcriptional profiling, functional analysis, Antarctic nematode, desiccation, anhydrobiosis, stoichiometry, McMurdo Dry Valleys, quantative real-time polymerase chain reaction, microarray analysis, trait deterioration

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INTRODUCTION

The natural environments of organisms present a multitude of biotic and abiotic challenges that require both short-term ecological and long-term evolutionary responses. These responses have long been the subject of biological interest, yet their intrinsic complexity has made genetic and mechanistic dissection experimentally difficult. These responses include modifications of biochemical, physiological, morphological, or behavioral traits of adaptive significance. To date, most environmental response studies have focused on effects at the ecosystem, community and organismal levels. However, the ultimate controls of these responses are located in the genome of the organism. Thus, genetic and genomic studies of organismal responses to environmental changes are necessary. Recent advances in genome analysis now make such analyses possible (Ungerer et al. 2008). My work begins to span this gap by using molecular tools to characterize the way nematodes respond to environmental changes (stress, growth conditions and nutrient availability).

As changing environments are ubiquitous, one of the greatest challenges in biology is understanding and predicting the effects of environmental changes on the ecology of individual organisms. Organisms respond to environmental changes on both ecological and evolutionary time scales. The magnitude and extent of environmental changes create additional challenges for organisms, including changes to climate, landscape structure and communities. All of these changes lead to novel interactions among species to which, given the rapidity of change, organisms must adapt at an unprecedented pace (Herman et al., 2009). Recent and growing evidence suggests that organisms exhibit remarkable adaptability to various changes like environmental stress, sub-optimal growing conditions and elemental

stoichiometry through morphological, biochemical and genetic changes. Rapid physiological and genetic changes have been reported in response to stress, sub-optimal growing conditions and nutrient availability. Change, abiotic or biotic, elicits organismal responses via mechanisms lodged in the genome; whose study requires an evolutionary and ecological genomic approach.

One of the most important members of belowground communities are nematodes, as they are among the most abundant invertebrates in soils, and are an important component of the microfauna of terrestrial ecosystems. Soil nematode species display a wide variety of responses to changes in environmental conditions, growing conditions and food availability. In addition, nematodes are highly responsive to changing environmental conditions (Freckman and Ettema 1993; Todd et al. 1999), making them ideal organisms to study organismal interactions with their environment. Recent work has demonstrated that soil nematode communities respond strongly to a variety of perturbations, such as nutrient availability, soil moisture, environmental stress, and growing conditions (Adhikari et al., 2009a, b; Adhikari et al., 2010). These studies suggest that some nematode species are better adapted than others to the environmental changes caused by these disturbances (Jones et al., 2006; Herman et al., 2009). However, the environmental changes induced by these disturbances are complex, involving changes in the biotic as well as abiotic environment of the soil. In order to begin to sort out these interactions, I have focused on the genomic responses of nematodes by looking at gene expression in response to changes in abiotic environmental conditions. In this introduction, I will review a few of the interactions taking place during environmental stress survival, trait deterioration and differential nutrient availability to nematodes and interpret them in the contest of results obtained by using different molecular genomics approaches. By reviewing these topics, I will illustrate the

knowledge gap of nematode-environment interactions at the molecular level and highlight the particular questions being addressed in this dissertation.

Environmental stress survival

Stress is not only an attribute of the stressor (the environmental component), but also an attribute of the stressed (the biological component) organisms. Therefore, environmental stress and the level of stress imposed can only be defined in relation to the organism or population experiencing this particular stress. The natural habitat of terrestrial nematodes, the soil, is a harsh environment that poses significant challenges to survival and persistence. As such, nematodes have evolved a myriad of strategies that enable them to respond to environmental stress, including their gross morphology, cell wall constituents, and the expression of a number of genes that encode metabolites, cryoprotectants and signalling compounds. Suites of adaptive traits such as these enable soil-dwelling nematodes to withstand the rigors of fluctuating environmental extremes or survive unfavourable environmental conditions in a dormant state that considerably prolongs their life span. For example, some nematodes can survive freezing conditions and/or exposure to desiccation for long periods of time. The latter are capable of 'anhydrobiosis' or 'life without water' and can survive in a desiccated state for many years.

Different nematode species can also vary in their responses to environmental changes, and this is most likely a function of the habitat in which they have evolved. For example, nematodes that have evolved in desert-like habitats may respond to desiccation stress differently now optimally fit to mesic environments. Terrestrial Antarctic nematodes are part of a community of organisms that are living in one of the harshest environments on Earth. These nematodes have to face the problems of exposure to desiccation, freezing, high radiation, higher soil salinity and frequent freeze-thaw events. Nematodes exposed to such extreme environmental conditions typically show two broad responses. Nematodes like

Plectus antarcticus are adapted to grow and reproduce under conditions that are constantly extreme (extremophiles, displaying capacity adaptation), whilst others like *Scottnema lindsayae* survive extreme conditions in a dormant state, only growing and reproducing when conditions are favourable (cryptobiotes, displaying resistance adaptation).

Our understanding of stress tolerance strategies of nematodes has undergone a number of significant shifts. Substantial information has accumulated on physiological and biochemical mechanisms of stress survival by nematodes. However, research programmes addressing the molecular mechanisms and gene functions involved in desiccation and freeze survival of nematodes are only now beginning to emerge. Because environmental stresses can have a profound effect on the developmental programmes of nematodes, it is expected that the timing and order of gene expression will be the major regulatory mechanisms by which nematodes respond to these external cues. In the following chapters (Chapter 2) I will characterize the molecular mechanisms of Antarctic nematode stress survival and describe these mechanisms in relation to desiccation survival. I will discuss the inducible as well as constitutive mechanisms of stress response along with signal transduction mechanisms. The genetic response of nematodes to stress induced metabolic changes and oxidative stress response will also be discussed. Finally, I will point out interesting conserved mechanisms and cross-talk between stress responses (Chapter 4) that may be used by highly divergent organisms to mediate transcriptional responses to stress. In addition, some of the future areas of research that may be important for our understanding of molecular mechanisms relative to stress survival will be presented.

Desiccation and freeze survival

Organisms have evolved various types of survival strategies against adverse conditions. Some species move away from sites with harsh conditions to conditions more favorable for growth and reproduction. On the other hand, most organisms must cope in the

same place with adverse conditions such as low and high temperature, limited food availability, high salt concentration, anoxia and dehydration (Danks, 1987). Water is the major component of living organisms. Most organisms have only limited ability to survive water loss (Danks, 2000; Wharton, 2002a). Dehydration is one of the most serious stresses in both aquatic and terrestrial organisms. Many organisms have evolved mechanisms to inhibit water loss, but most of them die soon after the water content declines below a critical level, which varies greatly among species. However, some organisms can survive for an extended period even after they are almost completely dehydrated (desiccation tolerance). These desiccation tolerant organisms are able to survive almost complete dehydration; in many cases 95-99% of water content is lost (Danks, 2000). The ability of organisms to survive complete desiccation or complete air dryness is more commonly called anhydrobiosis (Crowe et al., 1992). Anhydrobiotic organisms are found in a variety of biological taxa, including both unicellular and multicellular organisms, and from both the animal and plant kingdom (Clegg, 2001).

Nematodes are ubiquitous residents of soils and sediments in most ecosystems. These microscopic roundworms are widely distributed even in extreme environments where water availability is limited (Porazinska and Wall, 2002; Treonis et al., 1999; Wall and Virginia, 1999). Survival strategies enable nematodes to persist in soils where their activity may be limited for long periods by temperature extremes and/or desiccation (Freckman and Womersley, 1983; Wharton, 1995). Nematode survival strategies have been characterized based on the environmental stress to which the organism is responding (*e.g.*, cryobiosis, anhydrobiosis, osmobiosis, aerobiosis) (Clegg, 2001; Keilin, 1959). Nematodes are also exposed to very low temperatures and to freezing events while in the soil. Cold tolerance strategies have traditionally been either freeze avoidance, where animals maintain their body

fluids as a liquid at temperatures below their melting point (they supercool), or freezing-tolerance, where animals can survive ice forming in their bodies. Freeze tolerant animals tend to show little supercooling ability and cryoprotective dehydration. Instead, even though the organism is surrounded by ice, it dehydrates rather than freezes (Holmstrup et al., 2002; Holmstrup and Westh, 1994).

The McMurdo (MCM) Dry Valleys of Antarctica (77°S 163°E) are one of the most extreme terrestrial environments on Earth (Priscu, 1998). Soils in this cold desert ecosystem are subject to freezing temperatures, desiccation and salt accumulation that affect biological water availability (Campbell et al., 1998; Wall Freckman and Virginia, 1998). Nematodes are the most abundant invertebrate in the MCM Dry Valleys of Antarctica. Nematodes play a disproportionately high role in nitrogen mineralization and carbon cycling (*S. lindsayae* contributes 2-7% of the heterotrophic C flux) (Barrett, et al., 2008; Freckman, 1988) in Dry Valley soils. Antarctic nematodes are continually exposed to environmental extremes (desiccation, cold, higher salinity and wind) and are well adapted to such stresses through tolerance to freezing and anhydrobiosis (Treonis et al., 2000).

Molecular anhydrobiology of Antarctic nematodes

Desiccation tolerance is one of the most fundamental features of terrestrial organisms. Most organisms are homeohydric (ability to restrict cellular water loss regardless of environmental conditions) and avoid desiccation by preventing water loss under dry conditions. However, some organisms (Collembola, nematodes, resurrection plants) are extremely well adapted to dehydration; they are able to survive extended periods of drought in a biological state called anhydrobiosis (Watanabe, 2006). While in an anhydrobiotic state, nematodes are capable of surviving desiccation (Pickup and Rothery, 1991) as well as extreme cold (Wharton and Block, 1993). Laboratory studies of nematodes and other

organisms indicate anhydrobiosis typically is accompanied by the production of large quantities of non-reducing sugars, such as trehalose, which stabilize molecules (proteins, membrane lipids) within the cells of anhydrobiotes (Crowe et al., 2002; Higa and Womersley, 1993). Recent research suggests anhydrobiotes synthesize many other compounds (primarily proteins) that are essential to survival (Browne et al., 2002; Oliver et al., 2002; Solomon et al., 2000; Tunnacliffe and Lapinski, 2003), and our understanding of this complex process is increasing. Several reviews of the physiology and biochemistry of desiccation survival and anhydrobiosis in nematodes have been published (Crowe *et al.,* 1992; Perry, 1999; Wharton, 1986; Womersley *et al.,* 1998).

Despite recent work on behavioural, biochemical and molecular stress response mechanisms (Browne *et al.,* 2004; Gal *et al.,* 2005; Liu and Glazer, 2000) the molecular mechanisms governing anhydrobiosis in nematodes are not fully understood. Studies on desiccation responsive compounds in nematodes have resulted in the identification of many genes that play important roles in stress pre-treatment and cross-tolerance. The molecular mechanisms of anhydrobiosis are surprisingly similar in unrelated species ranging from nematodes to plants. An important process seems to be the formation of intracellular filamentous structures by folding of late embryogenesis abundance (LEA) proteins into superhelical structures (Wise and Tunnacliffe, 2004). Furthermore, the dehydration process is accompanied by induction of genes coding for organic osmolytes (trehalose, glycerol, sorbitol), which prevent membrane damage, scavenge free radicals, and avoid oxidation of proteins and phospholipids (Watanabe, 2006; Adhikari et al., 2010). Heat shock proteins (HSP) serve as chaperones to prevent denaturation of proteins. Induced as well as constitutive expression of these HSP genes has also been reported (Adhikari *et al.,* 2009a). Decreased water content is thought to be the first signal to trigger anhydrobiosis, followed by the

activation of signal transduction cascades, pathways activated by the binding of an extracellular signal molecule (such as a mitogen) to a receptor protein on the cell membrane.

To understand the molecular mechanisms activated during anhydrobiosis, a condition induced by slow dehydration, I initiated a genomic level analysis of gene expression during anhydrobiosis of *P. murrayi* (an Antarctic nematode capable of surviving desiccation as well as freezing conditions) (2009a). Accordingly, an expressed sequence tag library was generated from nematodes that had been slowly desiccated. Desiccation-induced transcripts were sequenced and transcripts differentially expressed during desiccation stress were identified using suppressive subtractive hybridization (SSH). In that study 2,486 ESTs were generated from a cDNA library and the unique transcripts from *P. murrayi* were compared with known sequences. The breakdown of functions was assessed by Gene Ontology (Ashburner *et al.*, 2000) and by assignment to metabolic pathways using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) database. These analyses showed that the desiccation-induced transcriptome encompasses a wide range of functions associated with the EST transcripts, representing many familiar functions that might be expected of a eukaryotic organism. Analyses of the transcripts that were abundantly expressed suggested that metabolic genes and those associated with the processing of environmental information, mainly environmental stresses, were highly expressed. KEGG analysis indicated that gene transcription related to metabolic activity, protein folding sorting and degradation, membrane transport and signal transduction-related activity was high.

Using subtractive hybridization I made a library of *P. murrayi* transcripts present in desiccated nematodes. I sequenced the subtracted library and validated their differential expression by qRT-PCR. A large portion of the expressed genes was considered to be associated with metabolism, followed by environmental information processing, genetic information processing and novel proteins. Many of the differentially expressed genes

showed a significant increase in mRNA expression level after desiccation, including late embryogenesis abundant (LEA) protein, trehalose-6-phosphate synthase (TPS), aldehyde dehydrogenase (ALH) and glycerol kinase (GK). The heat-shock proteins (HSP) of 70 and 90 kda (HSP70 and HSP90) did not alter expression during desiccation, indicating that these HSPs are not induced by environmental challenge but instead are constitutively expressed at high levels. Thus, the expressed genome of *P. murrayi* showed that anhydrobiotic survival in nematodes involves differential expression of a suite of genes from diverse functional areas and constitutive expression of others.

Why an Antarctic nematode for stress studies?

Antarctica offers a unique natural laboratory for fundamental research on the evolutionary processes that shape biological diversity, and the relationships between genome function, physiology and ecology. Physiologists and ecologists have long been attracted to environments that lie at the limits of the physical conditions capable of supporting life. This is because the polar regions demand striking adaptations at the molecular, cellular or whole-organism level to allow organisms living there to survive, grow and reproduce.

Nematodes are the most abundant invertebrate in the MCM Dry Valleys of Antarctica. Five major nematode genera are reported from this region *(*Adams et al., 2006) and their habitat suitability is influenced by soil moisture, carbon and salinity (Treonis et al., 1999). *Plectus murrayi*, a bacteria feeding nematode, inhabits both semi-aquatic and terrestrial biotopes in the MCM Dry Valleys where their distribution is limited by organic carbon and soil moisture. *Plectus* nematodes from the MCM can survive extreme desiccation, freezing conditions, and other multiple types of stress. Ongoing research on the physiological and molecular aspects of stress biology of this nematode, along with availability of genomic resources, such as expressed sequenced tag (EST), cDNA libraries, functional analysis using RNA interference (RNAi) has established this nematode as an excellent invertebrate model

system for studies with extreme environmental survival and could be a potential source of genomic resources for comparative studies in other organism. Moreover, because *P. murrayi* and *C. elegans* share a most recent common ancestor with the rest of the Secernentea, and given the possibility of culturing this nematode at low temperature compared to *C. elegans*, *P. murrayi* could also be an emerging model system for the study of the evolution of environment-sensitive (stress response) alleles in nematodes.

The McMurdo Dry Valleys are ideal for testing hypotheses of ecological amplitude as well as community structure and function because its species diversity is extremely low (communities of more than 5 sympatric metazoan species are rare; Adams et al., 2006) and there is little, if any biotic influence over nutrient cycles (Barrett, et al., 2005). Instead, this environment is unique in that it is one of the few ecosystems on Earth where abiotic factors, (i.e. relative humidity, temperature and wind speed) are more important drivers of population structure than biotic interactions (Convey, 1996a; Hogg et al., 2006). Thus, by controlling for the biotic factors involved in the evolution of the 'structure and functioning of the genome', these ecosystems offer a unique natural laboratory for fundamental research on the evolutionary processes that shape ecological amplitude and the relationships between environmental stress, genome function, physiology and ecology.

Trait deterioration in entomopathogenic nematodes

When a biological control agent is isolated from nature and reared in the laboratory, or mass-produced for commercial purposes, it may lose biocontrol traits due to genetic (drift, inbreeding, or inadvertent selection) processes (Hopper et al. 1993; Roush 1990; Bai et al. 2005). Deterioration in biocontrol traits may also arise from non-genetic factors such as environmental factors (poor nutrition and disease) (Hopper et al. 1993). Trait deterioration under laboratory conditions has been widely documented in various biological control agents

including nematodes (Dulmage and Rhodes, 1971; Geden et al.. 1992; Tanada and Kaya 1993; van Bergeijk et al., 1989).

Entomopathogenic nematodes (genera *Heterorhabditis* and *Steinernema*) are biocontrol agents that kill their invertebrate hosts with the aid of a mutualistic bacterium (Gaugler 2002). The bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids are primarily responsible for killing the host and providing the nematodes with nutrition and defense against secondary invaders (Poinar, 1990). The bacteria depend on the nematode for protection from the external environment (in which the bacteria survive poorly) and for penetration into the host's haemocoel (Poinar, 1990). Infective juveniles (IJs), the only free-living stage, enter hosts through natural openings (mouth, anus, and spiracles), or occasionally through the cuticle (Adams and Nguyen, 2002; Kaya and Gaugler, 1993). After entering the host's haemocoel, the nematodes release their symbiotic bacteria, which reproduce and cause host death through septicemia or toxemia. The nematodes molt and complete 1-3 generations within the host. After about 7-10 days IJs begin to emerge to search out new hosts. Steinernematids (except for one species) are exclusively amphimictic, i.e., have only males and females, whereas heterorhabditids are exclusively hermaphrodites in the first generation and a mix of males, females, and hermaphrodites in subsequent generations (Koltai et al. 1995; Stock et al. 2004).

Loss of biocontrol traits resulting from the culturing of EPNs has been documented. Specifically, significant reductions in environmental tolerance (such as to heat, UV, and desiccation), storage stability, host finding, and reproductive potential have been observed (Bai et al., 2005; Bilgrami et al., 2006; Shapiro et al. 1996; Wang and Grewal 2002). Trait loss can occur rapidly, e.g., Wang and Grewal (2002) observed substantial trait deterioration in *H. bacteriophora* after only three passages in *G. mellonella*.

Recent evidence suggests that genetic processes contribute significantly to changes in biocontrol traits in EPNs and their symbiotic bacteria (Bai et al. 2005; 2006). Yet it is likely that environmental condition (different nutrition regimes, environmental stress, storage conditions) also contributes and may expedite or exacerbate the process. Several previous studies have showed that genetic factors play a significant role in the deterioration process; however, the specific mechanisms behind these genetic processes remain unclear. Additionally, physiological or biochemical effects such as nutritional factors may also contribute to trait deterioration. Therefore, establishing stability in beneficial traits requires an understanding of the mechanisms involved in trait deterioration, specifically, the molecular genetic processes. I investigated the molecular mechanisms of trait deterioration of two experimental lines of an EPN, an inbred line (L5M) (created by sub-culturing different experimental lines of the nematode-bacterium complex over 20 passages in insect hosts) and its original parental line (OHB) (Adhikari et al., 2009). Through generation of transcriptional profiles of the two experimental lines of EPN, I identified and validated the genes that were differentially expressed (DE) in the deteriorated line (Chapter 5).

Evolutionary and ecological stoichiometry of Antarctic nematodes

Despite the striking similarity in the elements involved in basic life processes (i.e. the elemental composition of substrates and enzymes involved in metabolic reactions such as glycolysis) among all organisms, species and genotypes within species vary in their somatic elemental composition (DeMott et al., 2004; Frost et al., 2006). Furthermore, there is evidence of differential performance of species and genotypes under contrasting elemental supply environments. Sources of such variation can be behavioral, physiological or developmental in nature - all of which are orchestrated by a combination of genetic and environmental drivers (e.g. Carroll et al., 2001). For example, recent work (Elser et al., 2006) indicates marked effects of elemental supply environments (relative supply of nitrogen) in

disparate genomes that have generated biases in the usage of various amino acids in proteomes of plants and animals. A similar effect of trace metal availability through geological time has been observed in the metallomes of eukaryotes (Dupont et al., 2006). Finally, there is evidence indicating that organisms may be capable of adapting rapidly to elemental supply environments (Bragg and Wagner (2007). Clearly, genome properties and environmental elemental supply are coupled.

Previous work has shown that there is biologically significant variation in the elemental composition of organisms that relates not only to structural features but also to the very core of biological function, the molecular biology of growth via production of ribosomal RNA. This idea is captured in the "growth rate hypothesis" (GRH), which states that variation in the P content of living things is driven by variation in allocation to P-rich ribosomal RNA that accompanies differences in growth rate, as elevated ribosome allocation is generally needed to meet the protein synthesis demands of rapid growth (Elser et al., 1996a; Elser et al., 2002). Thus, any evolutionary process that results in changes in organismal growth or developmental rate may be manifested in changes in organismal C:N:P ratios, with potential consequences for that organism's sensitivity to stoichiometric food quality constraints and its impacts on nutrient cycling. A genetic basis for such growth-related variations in RNA related P demand has also been proposed (Elser et al., 2002): increased growth rate and associated increases in transcriptional capacity for rRNA production are associated with changes in the rDNA, particularly in the length and content of the rDNA intergenic spacer (IGS) and/or in overall rDNA copy number (Weider et al., 2005a).

A variety of recent findings from work on crustacean zooplankton and herbivorous insects have lent validity to the GRH. Several studies have shown significant positive correlations among various combinations of the proposed growth-RNA-P coupled system

(Gorokhova and Kyle, 2002; Main et al., 1997; Perkins et al., 2004; Schade et al., 2003; Vrede, 1998) which have been reinforced by field observations (Elser et al., 2000b; Schade et al., 2003; Carrillo et al., 2001; DeMott et al., 2001; Ferraro-Filho et al., 2005). Positive associations among growth rate, body RNA and P content, and the presence of long rDNA intergenic spacer variants were observed in studies of various *Daphnia* species (Gorokhova and Kyle, 2002; Weider et al., 2004). Lab experiments have also demonstrated context-dependent success of different rDNA variants in response to conditions of environmental and dietary P supply (Perkins et al., 2004; Weider et al., 2005a). Similarly, a number of studies have surveyed and/or examined natural variation in rDNA genome in number of organisms (Weider et al., 2005a) that links the connection of underlying genetic variation to potentially important environmental variables (Gupta et al., 2002; Zhang et al., 1990). These studies, and several others, suggest that rDNA copy number in a broad range of organisms is influenced by environmental parameters, have ecological significance and can respond to natural selection.

Elemental stoichiometry has provided a useful framework for understanding sources and controls of nutrient availability, and has been widely applied in the study of different ecosystems (Reiners, 1986; Vrede et al., 2004; Hessen et al., 2004; Sterner and Elser, 2002) including those of the Antarctic Dry Valleys (Barrett et al., 2007). It has been suggested that the cellular and biochemical machinery required for divergent life history strategies sets the stoichiometric requirements of individual organisms (Sterner and Elser, 2002). In laboratory experiments, it has been shown that RNA can increase rapidly with increasing P availability under P-limited conditions (Vrede et al., 2002). But, no studies have tested the validity of the GRH in natural populations of nematodes. The correlation between the levels of rRNA transcription, rDNA copy number and body P-content, and nematode growth parameters need to be explored. More specifically, the validity of GRH needs to be tested in natural

environments and whether or not elemental stoichiometry could drive evolutionary changes in gene expression and genome organization demand further exploration.

Genomic analysis of nematode-environment interactions

The effects of environmental changes are evident at multiple levels of biological organization. However, the ultimate controls of biological responses are located in the genome of the organism. Modulation of gene expression has a central role in cellular adaptation to short- or long-term environmental changes, with extensive regulation occurring at both the transcriptional and post-transcriptional level. In this dissertation I used genome-enabled techniques to study aspects of environmental changes using genetically tractable model organisms to identify genes involved in their response to environmental perturbations.

Stress is a fundamental aspect of life and a major aspect of natural selection. Ecologists have studied the responses of plants and animals to environmental stress factors for a long time. Early biochemical studies have showed that there is a surprising degree of uniformity in the stress responses of different species at the cellular level, even to widely different environmental stress factors (Kültz, 2005). Genomic studies have reinforced this idea while at the same time providing new insights into the coherence of the cellular stress response (Van Straalen and Roelofs, 2006). Similarly, deterioration of the physical environment (storage, food and stress) along with genetic factors impacts the longevity, pathogenicity and fecundity of different biological control agents. Rapid deterioration in economically important traits has been reported for several biological control agents in response to sub-optimal environmental conditions. Furthermore, there is evidence of differential performance of species and genotypes under contrasting elemental supply environments. Sources of such variation can be behavioral, physiological or developmental in nature - all of which are orchestrated by a combination of genetic and environmental drivers (Carroll et al., 2001). The application of genome-wide approaches is now providing a global

view on gene expression responses to many different environmental conditions, leading to exciting advances in our understanding of the cellular strategies that are used to stay in tune with environmental conditions. In this dissertation I will highlight some of the studies underlying gene expression responses to different environmental factors (stress, growth conditions and soil elemental supply). The emphasis will be on transcriptional analyses, which have been most intensely studied in many organisms, and for which several recent papers have greatly advanced our understanding. I focus on the general response mechanisms of nematode to different environmental conditions, changes in gene expression in response to changing environments, and the effect of the soil environment on genome evolution of nematodes.

RESEARCH OUTLINE

The ability to constantly sense and respond to environmental changes is important for all organisms to maintain cellular functions. Modulation of gene expression has a central role in cellular adaptation to short- or long-term environmental changes, with extensive regulation occurring at both the transcriptional and post-transcriptional level. In an attempt to examine the response of nematodes to environmental stress (desiccation), chapter two presents a genomic level analysis of gene expression during anhydrobiosis of *P. murrayi*. The first step in this process was to establish an EST collection that is representative of the desiccation-induced transcripts and to identify the transcripts differentially expressed during desiccation stress. Using different molecular tools along with bioinformatics analyses I identified a number of genes differentially expressed during the anhydrobiotic process. The genetic information derived from *P. murrayi* informs the characterization of genes responding to desiccation stress, and is expected to further our understanding of the potential genetic determinants of desiccation tolerance in nematodes and perhaps other metazoans. The third chapter focuses on the Antarctic nematode *P. murrayi*, a bacteria feeding Antarctic nematode

that could be used as an excellent invertebrate model system for studies on extreme environmental survival, and a potential source of genomic resources for comparative studies in other organisms. Considering their exceptional ability to survive extreme environmental conditions, chapter four presents the detailed assessment of the molecular and physiological response to dehydration and freezing of *P. murrayi* under ecologically relevant conditions characteristic of both the austral summer and winter of Antarctica. By investigating the patterns of gene expression and survival of nematodes at RH values similar to those of their natural environment, we tested the hypothesis that physiological adaptations to milder stress promotes desiccation, freezing and cross-tolerance to other stresses.

Deterioration of traits essential for biological control has been recognized in diverse biological control agents including the insect pathogenic nematodes (Adhikari et al., 2009b; Bilgrami et al., 2006; Shapiro et al., 1996; Wang and Grewal, 2002). Genetic and non-genetic processes may be responsible for trait deterioration in laboratory-cultured biocontrol agents and their symbionts. Chapter five presents an expression profiling study to identify the specific genetic mechanisms behind trait deterioration. Results showed that trait deterioration induces substantial overall changes in the nematode transcriptome, and suggests that trait deterioration via inbreeding depression, taking place over a short period of time, can result in massive changes in metabolic processes, cellular transportation and gene translation. Finally, through field observations and molecular laboratory experiments, Chapter six examines the validity of the GRH in natural populations of *Scottnema lindsayae* and *Plectus murrayi* from P-rich and poor environments. The level of rRNA transcription, rDNA copy number and body P-content was related to nematode growth parameters and whether or not elemental stoichiometry could drive evolutionary changes in gene expression and genome organization was explored.

Transcriptome studies are very much underrepresented in soil invertebrates, maybe due to a lack of economic relevance. Still, from an ecological and evolutionary viewpoint there is a strong need for genome-wide analyses of organismal responses to environmental perturbations. There is considerable interaction between the gene expression profiles induced by different environmental conditions. These interactions are of great ecological relevance and ultimately may explain the shape of the ecological niche occupied by a species. Thus, my work, in addition to providing immediate insight into the mechanisms by which multicellular animals respond to environment, is transformative in its potential to inform other fundamental ecological and evolutionary questions, such as the evolution of life-history patterns and the relationship between community structure and ecological function in ecosystems. In combination with the molecular and bioinformatics analyses presented here, further functional genomics analyses are required to generate a more complete picture of the response of metazoans to diverse environmental conditions and determine the biological roles of these genes within the larger context of terrestrial ecosystem.

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CHAPTER 2

Desiccation survival in an Antarctic nematode: Molecular analysis using expressed sequenced tags

Bishwo N Adhikari.[1, 3*], Diana H Wall[2] and Byron J Adams[1, 3]

[1]Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT, USA

[2]Department of Biology and Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO, USA

[3]Present Address: Department of Biology, and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT, USA.

*Corresponding author: adhikaribn@hotmail.com

ABSTRACT

Background

Nematodes are the dominant soil animals in Antarctic Dry Valleys and are capable of surviving desiccation and freezing in an anhydrobiotic state. Genes induced by desiccation stress have been successfully enumerated in nematodes; however we have little knowledge of gene regulation by Antarctic nematodes which can survive multiple environmental stresses. To address this problem we investigated the genetic responses of a nematode species, *Plectus murrayi*, that is capable of tolerating extremes of environmental conditions of the Antarctic continent, in particular the desiccation and freezing. In this study, we provide the first insight into the desiccation induced transcriptome of an Antarctic nematode through the cDNA library construction and suppressive subtractive hybridization.

Results

We obtained 2,486 expressed sequence tags (ESTs) from 2,586 clones derived from the cDNA library of desiccated *P. murrayi*. The 2,486 ESTs formed 1,387 putative unique transcripts of which 523 (38%) had matches in the model-nematode *Caenorhabditis elegans*, 107 (7%) in nematodes other than *C. elegans*, 153 (11%) in non-nematode organisms and 605 (44%) had no significant match to any sequences in the current databases. The 1,387 unique transcripts were functionally classified by using Gene Ontology (GO) hierarchy and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The results indicate that the transcriptome contain a group of transcripts from diverse functional areas. The subtractive library of desiccated nematodes showed 80 transcripts differentially expressed during desiccation stress, of which 28% were metabolism related, 19% were involved in environmental information processing, 28% involved in genetic information processing and 21% novel transcripts. Expression profiling of 14 selected genes by quantitative Real-Time

PCR showed 9 genes significantly up-regulated, 3 down-regulated and 2 continuously expressed in response to desiccation.

Conclusions

The establishment of a desiccation EST collection for *Plectus murrayi*, a useful model in assessing the structural, physiological, biochemical and genetic aspects of multiple stress tolerance, is an important step in understanding the genome level response of nematode to desiccation stress. The type of transcript analysis performed in this study has laid the foundation for more detailed functional and genome level analyses of the genes involved in desiccation tolerance in nematodes.

BACKGROUND

The Dry Valleys of Antarctica are one of the most extreme terrestrial environments on Earth [1]. Soils in this cold desert ecosystem are subjected to freezing temperature, desiccation and salt accumulation that affect biological water availability [2, 3]. Soil communities in Antarctic Dry Valleys are simple and primary production is largely limited to algae and fauna are almost exclusively microbial grazers, i.e., mostly protozoa, rotifers, tardigrades and nematodes [4]. Nematodes are the dominant soil animals and were present in 65% of the 415 soils sampled by Wall Freckman & Virginia [3] across four McMurdo Dry Valleys (MCM). Nematodes have been isolated from soil in an inactive coiled state called anhydrobiosis [5]. Anhydrobiosis is a survival strategy employed by nematodes, rotifers, and tardigrades in response to desiccation [6]. Nematodes in anhydrobiosis lose 95–99% of their body water content and cease metabolic activity at any stage in their life cycle [7]. While in an anhydrobiotic state, nematodes are capable of surviving desiccation [8] as well as extreme cold [9]. Though Antarctic ecosystems are simple and have low species diversity compared to temperate ecosystems, nematodes have the widest distribution and highest diversity of the invertebrates in the Dry Valleys [5], with four major taxa; *Scottnema lindsayae*, *Eudorylaimus antarcticus*, *Plectus antarcticus*, and *Geomonhystera antarcticola* [10]. It has been suggested that specimens identified as *P. antarcticus* de Man 1904 in MCM are *P. murrayi* [11, 12], so the taxonomy of Andrassy [11] being most up-to-date and well-accepted, *P. antarcticus* hereafter will be referred to as *P. murrayi*.

Plectus murrayi inhabits both semi-aquatic and terrestrial biotopes in the Dry Valleys, but is also reported from other parts of the Antarctic [11]. Endemic to the Southern continent, *P. murrayi* has a multiple year life cycle but the exact life span of the different developmental stages is unknown. *Plectus murrayi* from the McMurdo Dry Valleys is freeze tolerant, and

can tolerate repeated freeze-thaw cycles in the laboratory (data not shown). Although, *P. murrayi* appears to be tolerant to extreme environmental conditions of Antarctic continent [12], no detailed molecular studies on the survival and the response of this unique species to environmental extremes have been undertaken.

Despite recent work on behavioral, biochemical and molecular stress response mechanisms [13-15] the molecular mechanisms governing anhydrobiosis in nematodes are not fully understood. Anhydrobiosis in nematodes is reported to involve the biosynthesis of low molecular weight carbohydrates, proteins and glycerol [16, 17]. Recent research suggests anhydrobiotes synthesize many other compounds (primarily proteins) that are essential for survival [18-20]. Studies on these desiccation responsive compounds have resulted in the identification of many genes that play important roles in stress acclimation and survival. These responses include up-regulation of transcriptional regulators, molecular chaperones, antioxidants, hydrophilic proteins, and proteins involved in cell cycle regulation [15, 21-23]. The anhydrobiotic nematode *Aphelenchus avenae* synthesizes large amounts of trehalose in response to desiccation [24]. However, it has become clear that such sugars are not sufficient for anhydrobiosis [25] and, indeed, that some anhydrobiotic organisms seem not to use them [26]. As an effort to identify other adaptations required for anhydrobiosis Goyal *et al*. [27] characterised the genes in the nematode *Aphelenchus avenae* that require a period of preconditioning to enter anhydrobiosis. During this preconditioning period, several genes, including trehalose synthase [27], hydrophilins (highly hydrophilic proteins), anhydrin, and a polypeptide, *Aav-LEA-1,* related to plant Group 3 late embryogenesis abundant (LEA) proteins were induced [19, 15]. Although similar gene classes were found to be associated with desiccation stress in many nematodes, none of the ESTs or proteins detected in these studies were encoded by the same gene [22] and their expression level was quite variable

[28]. To understand such molecular mechanisms activated during anhydrobiosis, a condition induced by slow dehydration, we identified gene expression patterns by gradually desiccating nematodes at relative humidity (RH) values specifically relevant to the Antarctic environment.

To understand the mechanisms of desiccation survival we have initiated a genomic level analysis of gene expression during anhydrobiosis of *Plectus murrayi*. The first step in this process was to establish an EST collection that is representative of the desiccation induced transcripts and to identify the transcripts differentially expressed during desiccation stress. Here we present bioinformatics and molecular analysis of 2,486 ESTs from the gradually desiccated and anhydrobiotically induced nematode *Plectus murrayi* and 80 transcripts differentially expressed during the anhydrobiotic process. The bioinformatics approaches include EST cluster analyses, transcript abundancy estimations, and functional classifications based on Inter-Pro domains, Gene Ontology hierarchy, and KEGG biochemical classifications. The genetic information derived from *P. murrayi* informs the characterization of genes responding to desiccation stress, and is expected to further our understanding of the potential genetic determinants of desiccation tolerance in nematodes and perhaps other metazoans.

RESULTS

Sequencing and assembly of ESTs

A directionally cloned cDNA library of desiccated nematodes was constructed and a total of 2,687 of clones were subjected to single pass sequencing from their 5' ends. Trimming of vector sequences, poly A/T tails, low quality, adaptor, and contaminating sequences provided a data set of 2,486 high quality (hq) ESTs with a minimum length of 100 base pairs (bp) (Table 2.1). Among 2,486 hq ESTs, 1,423 were assembled into a total of 324

contigs, and the remaining 1,063 ESTs were classified as singletons, suggesting a combined total of 1,387 putative unique transcripts (Table 2.1). The number of ESTs in the 324 contigs varied from 2 to 37; 47% of the contig sequences had two ESTs, 37% had 3-5 ESTs, 9% had 6-10 ESTs, 4% had 11-20 ESTs and 2% had more than 21 ESTs (Fig. 2.1). These hq ESTs ranged from 90-1125 bp with average length of 545±156 bp and the average length of the contigs was higher than for singletons. All sequences have been deposited in the dbEST division of DDBJ/EMBL/GenBank under accession numbers [GenBank: FG618921]- [GenBank: FG621295], [GenBank: FG647736]- [GenBank: FG647869].

Comparison against public nematode ESTs

We used the 1,387 unique sequences to search a non-redundant protein data base using BLASTX [29, 30] (Table 2.1) and Wormpep 190 database consisting of extensively curated *C. elegans* proteins from WormBase [31]. A total of 782 unique sequences (56%) matched known proteins including 523 unique sequences (38%) with significant match to *C. elegans* proteins at a cut-off expectation (E)-value of 10^{-5} or below. The remainder of the unique sequences (44%) had no meaningful matches ($E > 10^{-5}$). We compared our unique sequences with the ESTs from other nematodes as well as non-nematodes using BLAST searches. Only 107 unique sequences (7%) matched other nematode ESTs and 153 unique sequences (11%) matched organisms other than nematodes at $E < 10^{-5}$ (Table 1). Of 1,387 unique sequences, 36 had homologues in *C. elegans* which could be silenced by RNAi. The RNAi phenotypes (as described by WormBase) included *mig-15* (migration), *lin-8* (lineage), *unc-16, 89* (uncoordinated), *Dpy-6* (dumpy), *rde-1* (RNAi defective), *drh-2* (dicer related), *nhr-67* (nuclear hormone receptor) and *ard-1* (alcohol/ribitol dehydrogenase).

Identification of differentially expressed genes

To identify transcripts differentially expressed (DE) during desiccation stress, subtractive hybridization was conducted between cDNA from gradually desiccated and fresh active nematodes (control). Two rounds of hybridization were done and DE clones were sequenced which resulted into 80 quality sequences above 100 bp (Table 2). The nucleotide sequences were analyzed and their putative functions identified by searching BLASTX. The DE transcripts included 22 ESTs (28%) similar to metabolism related genes, 15 (19%) were similar to environmental information processing genes, 23 (28%) were similar to genetic information processing genes, 3 (4%) were similar to hypothetical proteins of other organisms and 17 (21%) novel transcripts had no identifiable similarity to known sequences in GenBank [32]. Among the metabolism related genes, 13 ESTs (68%) were involved in carbohydrate metabolism, 2 transcripts (10%) of each in lipid metabolism, amino acid metabolism and protein folding, sorting and degradation. The environmental information processing category was dominated (53%) by stress related proteins. In the genetic information processing category, ribosomal proteins were the most abundant (42%) group followed by translation elongation factor (19%) (Table 2.2). All sequences have been deposited in the dbEST division of DDBJ/EMBL/GenBank under accession numbers [GenBank: FK670236]- [GenBank: FK670315].

The most unexpected discovery among DE ESTs was Type II antifreeze protein (AFP) [GenBank: FK670248] which showed high similarity to that of *Clupea harengus* (Table 2.2), the Atlantic herring. This finding represents the first case of an ice structuring protein from a nematode, suggesting the possibility that Antarctic nematodes may use similar antifreeze proteins for stress adaptation heretofore observed only in some fishes, insects, plants and bacteria.

Abundant transcripts expressed during desiccation

A total of 23 contigs containing 384 ESTs were highly redundant. This accounted for more than 15% of the total high quality ESTs. The minimum and maximum number of ESTs that made up these highly redundant contigs was 7 and 37 respectively (Table 2.3). More than one third (9) of the highly redundant contigs, totalling 127 ESTs, had significant similarity to various genes involved in metabolism. One third (8) of the highly redundant contigs, totalling 139 ESTs, had significant similarity to various environmental information processing related genes, indicating high transcript abundance of stress related genes, as expected. Two of the contigs, totalling 48 ESTs, had significant similarity to ribosomal proteins and three of the contigs, totalling 37 ESTs, had significant similarity to genetic information processing related genes. One of the highly redundant contigs totalling 33 ESTs matched similar sequences derived from the mitochondrial cytochrome oxidase subunit (Table 2.3). The most redundant group of contigs were composed of 37 ESTs and had significant similarity to ribosomal protein from *C. elegans*, indicating higher activities of ribosomal protein genes during desiccation stress.

Functional classification based on gene ontology assignments

To categorize transcripts by putative function, we utilized the GO classification scheme (April 2008 release of GO database, Gene Ontology Consortium). GO provides a dynamic controlled vocabulary and hierarchy that unifies descriptions of biological, cellular and molecular functions across genomes [33]. In this report, we used well-annotated GO information of *C. elegans* and other parasitic nematodes. GO representation of *P. murrayi* clusters is shown for each organizing principle of GO: molecular functions (Additional file 2.2a; Fig. 2.2a), cellular components (Additional file 2.2b; Fig. 2.2b), and biological processes (Additional file 2.2c; Fig. 2.2c). Additional file 2.2 and Fig. 2.2 provide a breakdown of representation by major GO categories. The highest GO term for molecular

functions was protein binding, under 'Ligand binding and carrier' categories, which had 87 unique sequences accounting for 18% of the total unique sequences matched in this category and 6% of the total unique sequences. The highest final GO term in cellular components was mitochondria under the 'cytoplasm' category with a total of 28 unique sequences accounting for 12% of the total in this category. Similarly, the highest final GO term for biological processes was protein metabolism, under 'metabolism' categories, which had 44 unique sequences accounting for 12% of the total in this category and more than 3% of the total unique sequences. We found 13 unique sequences showing significant similarity to *C. elegans* signal transduction factors; 8 of them belonged to the receptor binding group and 5 sequences belonged to receptor and receptor signalling proteins (Additional file 2.2).

Functional classification based on KEGG analysis

As an alternative method of categorizing unique sequences by biochemical functions, sequences were assigned to metabolic pathways via KEGG [34] using EC numbers as the basis for assignment. Only 281 unique sequences (36% of total) were assigned EC numbers and had 158 unique mappings to KEGG biochemical pathways (Table 2.4). The KEGG metabolic pathways that are well represented by *P. murrayi* unique sequences are carbohydrate metabolism (18 enzymes), amino acid metabolism (9 enzymes), lipid metabolism (8 enzymes), xenobiotic and bio-degradation metabolism (5 enzymes), and biosynthesis of secondary metabolites (3 enzymes). Of these, 12% of the unique sequences belonged to the environmental information processing (EIP) category, indicating higher activities of stress and chaperone related genes during desiccation. The KEGG pathways well-represented under EIP are membrane transport (15 enzymes), ligand-receptor interaction (15 enzymes), signal transduction (8 enzymes) and signalling molecules and interaction (9 enzymes). About 11% of the unique sequences belonged to the genetic information

processing (GIP) category with most of them having roles in folding, sorting and degradation. The KEGG pathways well-represented under GIP are folding, sorting and degradation (25 enzymes), transcription (9 enzymes), translation (8 enzymes) and replication and repair (6 enzymes). Most of the sequences (49%) remained unassigned to any known functional pathway and 15% of the sequences were similar to *C. elegans* hypothetical proteins (Table 2.4). The lowest number of sequences mapped to the cellular processes category (3%), suggestive of developmental arrest during anhydrobiosis. The cell growth and death (5 enzymes) and cell communication (4 enzymes) pathways were the well-represented categories under cellular processes (Table 2.4).

Refined gene-specific expression using quantitative real-time PCR

In order to validate our differential gene expression results and obtain more refined gene expression data, we designed gene-specific primers for 14 transcripts selected from Table 2.2 and analyzed their expression using quantitative real-time PCR (qRT-PCR) (Fig. 2.3). These genes were chosen to represent a variety of functional classification. Among these 14 transcripts, 9 were significantly induced (fold-change > 2.0x, *P* value < 0.05) and 3 genes were reduced (fold-change > 2.0x, *P* value < 0.05) in response to desiccation. Significant desiccation-induced gene expression change ranged from -6.50-fold for antifreeze protein to 26.77-fold for trehalose-6-phosphate synthase. Heat shock protein 70 and 90 were also weakly induced (fold-change 1.7 and 1.94x), but lacked significant statistical support (*P* > 0.05). Among the DE transcripts, putative homologs to trehalose 6-phosphate synthase protein showed highest induction (fold-change 26.77x, *P* value < 0.05) followed by the putative homolog to glycerol kinase (fold-change 25.04x, *P* value < 0.05). Interestingly, there was significant reduction and induction on the level of expression of two novel transcripts, [GenBank: FK670306 and FK670310] (fold-change -10.3x and 16.71x respectively, *P* <

0.05), suggestive of their possible roles in desiccation tolerance (Fig. 2.3). The mRNA copy number was calculated using an absolute quantification method [35]. There was no significant difference (*P* value < 0.05) in copy number of transcripts encoding Hsp70 and Hsp90 on desiccated and control nematodes (Additional file 2.3).

DISCUSSION

The expressed genome of *P. murrayi* showed that anhydrobiotic survival in nematodes involves a suite of genes from diverse functional areas, such as hormone signaling transduction, transcription regulation, ROS scavenging, reestablishment of homeostasis, molecular chaperoning and transcriptional regulation of ribosomal proteins and other genes. The 2,486 sequences comprised of 1,387 putative unique sequences from *P. murrayi* presented and represent the first EST analysis from an Antarctic nematode. The number of ESTs analyzed in the study were relatively few; nonetheless, it is apparent that 44% of the unique transcripts (605) expressed by desiccated nematodes represent novel genes that have not been previously isolated from other organisms. Comparison of ESTs presented in this study with ESTs from GenBank showed that 56% of the unique transcripts (1,387) isolated from *P. murrayi* have been previously isolated from other organisms, including the model organism *C. elegans* (Table 2.1). Subtractive hybridization of cDNAs from desiccated and undesiccated nematodes used in library construction was done to enrich for the rare transcripts that are differentially expressed (DE). A total of 80 DE transcripts were identified, some of which are involved in genetic information processing followed by metabolism, and presumably are involved in stress survival of nematodes (Table 2.2). The expression level of 14 transcripts DE during desiccation survival using quantitative real-time PCR (qRT-PCR) indicates that these genes were DE between desiccated and undesiccated nematodes. The results from subtractive hybridization coupled with qRT-PCR showed that we appropriately

enriched for the DE transcripts (Fig. 2.3). Our results showed that expressed sequence tag (EST) analysis coupled with subtractive hybridization is a powerful method to identify the genes involved in nematode desiccation stress. EST analysis is a commonly-used approach to identify genes involved in specific biological functions, especially in organisms where genomic data are not available [36].

Functional analysis of expressed genes

Gene ontology has been widely used to characterize gene function annotation and classification [33]. GO describes gene function using controlled vocabulary and hierarchy, including molecular function, biological processes, and cellular components (Fig. 2.2; Additional file 2.2). A large number of the unique sequences from our study mapped to molecular functions and ligand binding, enzymes, molecular transducer and transporter subcategories. Each one of these subcategories represents catalytic activities that could be argued is important for a cell to survive major metabolic perturbation during desiccation. The second most represented category was biological process, which includes unique sequences associated with cell growth or maintenance, development and cellular communications. Within the cell growth or maintenance category most unique sequences were associated with metabolism, localization, transport and response to stress. This distribution is not surprising for ESTs (unique sequences) derived from an organism undergoing metabolic changes such as desiccation. The subcategories under the least represented category, cellular process, also seem to reflect the nature of the cellular disturbances that result from desiccation. Under this category there is significant representation under intracellular and membrane subcategory. Within these subcategories representation is most significant in mitochondria, cytosol, ribosome, nucleus and ribonucleoprotein complex. The importance of protein synthesis and

cellular homeostastasis during desiccation may explain the preponderance of unique sequences associated with mitochondrial and ribosomal structural components.

As an alternative method of categorizing clusters by biochemical function, clusters were assigned to metabolic pathways using the KEGG database [34] (Table 2.4). Only 36% of the unique sequences mapped to the currently known KEGG pathways with 158 unique mappings. The paucity of EC assignments limits this aspect of analysis but the mapping of unique sequences to the KEGG metabolic and other pathways still presents some useful perspectives on the metabolic, protein folding and degradation and cellular repair emphasis of desiccated cells. Most of the unique transcripts belonged to Genetic information processing (GIP) with protein folding, sorting and degradation. Transcripts encoding a cathespin L-like protease [GenBank: FK670238] enzyme found in this category have long been recognized for their role in intracellular and extracellular protein degradation in a range of cellular processes. In *C. elegans* cathespin L-protease has been demonstrated to play a critical role during embryogenesis, larval development and moulting [37]. Considering the putative homology of the transcript encoding *P. murrayi* cathespin L-protease with *C. elegans*, it might have a similar role in nematode moulting and thus its possible role during desiccation survival needs further investigation.

Metabolism was the second most represented category and pathways well-represented by the *P. murrayi* clusters were carbohydrate metabolism, amino acid metabolism, lipid metabolism, biosynthesis of secondary metabolites, glyoxylate, and decarboxylation metabolism (Table 2.4). The lipid metabolism pathway in anhydrobiotes is one of the most active pathways as lipids are the main reservoir of energy and the most likely source of carbon for the synthesis of trehalose [16]. The GIP category included the pathways involved in protein folding, sorting and degradation, transcription, translation, replication and repair. Desiccation stress coupled with oxidative stress results in lipid and protein damage, leading

to impaired cellular survival and functioning. Nematodes are reported to respond to these damages and repair them with increased expression of stress-protective genes and antioxidant enzymes [38]. The cellular processes category has the least number of transcripts. This finding is suggestive of developmental arrest during anhydrobiosis. Indeed, anhydrobiosis may be an exaptation that allows the individual to survive unfavourable conditions by staying in an arrested state of development and reproduction (Table 2.4).

Abundantly expressed transcripts during anhydrobiosis

A high level of representation in a cDNA library generally correlates with high transcript abundance in the original biological sample [39], although artefacts of library construction can result in selection for or against representation of some transcripts. The genes with the most abundant transcripts were mostly involved in metabolism, molecular chaperones, reactive oxygen species scavenging and genetic information processing (Table 2.3). Ribosomal protein (RP) (Contig_57) was the most abundant transcript expressed during desiccation, suggesting the possible involvement of transcriptional regulation of ribosomal proteins during desiccation stress. Differential expression of plant ribosomal protein genes has been observed during development and following various stress or hormone treatments, including desiccation stress [40]. Interestingly, many ribosomal proteins were DE and one of them was up-regulated, suggesting that RPs may provide a means for selectively translating specific mRNAs required for the desiccation response. Similarly, it may be part of an adaptive response to maintain ribosomal function in dehydrated cytoplasm.

A number of metabolism related transcripts like cytochrome c oxidase (Contig_132), glutamate synthase (Contig_301), NADH-dehydrogenase (Contig_287) and aldehyde dehydrogenase (Contig_29) were also abundantly expressed during desiccation stress. Several lines of evidences have suggested that nematodes activate metabolic pathways in

response to desiccation shortly after exposure to dehydrating conditions [21]. Transcripts of a number of stress related genes were also abundantly expressed, including heat shock proteins, a chaperonin containing sub-unit, multiple stress-responsive zinc finger proteins and oxidative stress responsive genes. Desiccation stress in nematodes is reported to produce a large number of molecular chaperones to facilitate the synthesis, folding, assembly and intracellular transport of proteins, reduce protein denaturation and aggregation, and aid in protein renaturation [14, 41]. Transcripts of several oxidative stress related genes like glutathione S-transferase (Contig_63) and copper/zinc superoxide dismutase (Contig_54) were also abundantly expressed. Reactive oxygen species and other toxins produced by oxidative stress during desiccation of nematodes can damage membrane systems, proteins and nucleic acids. Therefore, the transcript abundance of several proteins that contribute to cellular survival after oxidative damage is not surprising.

Genes of general and secondary metabolism

Nematodes activate their metabolic pathways in response to desiccation shortly after exposure to dehydration [21]. Several transcripts encoding metabolism related genes are found to be differentially expressed by desiccation stress in *P. murrayi*. These genes include aldehyde dehydrogenase [GenBank: FK670237], trehalose-6-phosphate synthase [GenBank: FK670250], thymidylate synthase [GenBank: FK670263], glycerol kinase [GenBank: FK670258], glycogen synthase [GenBank: FK670264], ATP synthase [GenBank: FK670293], ADP/ATP translocase [GenBank: FK670265], and malate dehydrogenase [GenBank: FK670259]. Interestingly, a transcript encoding a bifunctional glyoxylate cycle protein (malate synthase), a distinct and anaplerotic variant of the tricarboxylic acid cycle, was also found to be highly expressed during desiccation (Table 2.2).

Nematodes are found to be unique among animals in utilizing the glyoxylate cycle to generate carbohydrates from the beta-oxidation of fatty acids [42]. The glyoxylate pathway, generally found in plants and micro-organisms, is similar to the citrate cycle, but relies on two critical enzymes, malate synthase and isocitrate lyase, to bypass two decarboxylation steps. Interestingly, the anhydrobiotic nematode *A. avenae* has been reported to use the glyoxylate cycle during induction of anhydrobiosis [43]. One sequence unique to *P. murrayi* mapped to a glyoxylate cycle protein [GenBank: FK670269] includes putative homologs of malate synthase. The abundant expression of malate synthase transcripts in our EST collection and its up-regulation upon desiccation stress may provide experimental support for an active role of glyoxylate cycle proteins during induction of anhydrobiosis by *P. murrayi*.

Transcriptional regulation and signalling affected by desiccation

Transcriptional regulation and intracellular signalling cascades for nematode response in general and secondary metabolism in particular, are poorly understood. A number of desiccation responsive *P. murrayi* ESTs encode putative signalling molecules or transcription factors. One of the *P. antarcticus* ESTs was most similar to the unc-16 gene of *C. elegans*, which encodes a c-Jun N-terminal kinase (JNK)-interacting protein [44], and one of the members of JNK kinase family (JKK-1) [GenBank: FK670239] was DE during desiccation. JNK (also known as stress-activated MAP kinases or SAPK) is a member of the mitogen-activated protein kinases (MAPKs) that regulate cellular responses to a variety of extracellular signals, including desiccation stress [45-47]. Transcripts homologous to genes encoding Zinc finger protein (Contig_198) were abundantly present in the expressed genome of *P. murrayi*. Zinc finger proteins are cellular proteins which play a major role in transcriptional regulation by binding with high affinity to specific regions of DNA. In conjunction with leucine zipper domains these proteins may form hetero- or homodimers and

activate transcription, either constitutively, or in a regulatory manner, through post-translational modifications in response to external stimuli (although some may also be cell specific or developmentally regulated) [48]. The abundance of genes encoding zinc finger protein in response to desiccation suggests that these proteins may regulate further events in the stress-response cascade of *P. murrayi*. Three transcripts from *P. murrayi* were most similar to the *C. elegans* gene encoding a predicted neurotransmitter gated ion-channel (Contig_73) protein. Neuronal signal transduction in response to desiccation stress would be required to initiate the coiling response of desiccating *P. murrayi*.

Stress response genes expressed during anhydrobiosis

Nematodes respond to desiccation stress by synthesizing a conserved set of proteins [18-20]. Our results demonstrate that for *P. murrayi* desiccation stress can significantly elevate stress related genes encoding trehalose 6-phosphate synthase, late embryogenesis abundant proteins, heat shock proteins, ubiquitin, c-type lectins, chaperone related proteins, and other stress responsive genes (Table 2.3). Three transcripts encoding trehalose 6-phosphate synthase, which synthesize the storage carbohydrate trehalose, were expressed during desiccation stress. A characteristic feature of anhydrobiotic organisms is their synthesis of high concentrations of non-reducing sugars during the induction of anhydrobiosis [24, 49]. Trehalose protects membranes and proteins from desiccation damage by replacing structural water [49], and contributes to the formation of an intracellular organic glass [50] which is thought to stabilize the cell's contents. The up-regulation of trehalose during desiccation stress of *P. murrayi* could be a part of an adaptive response to desiccation (Fig. 2.3).

Protein aggregation during desiccation is likely to be a major potential hazard for anhydrobiotes; late embryogenesis abundant (LEA) proteins acting as molecular chaperones

or molecular shields play an important role in prevention of this aggregation [51]. Transcripts similar to plant LEA related family member [GenBank: FK670249] of *C. elegans* were up-regulated during desiccation stress of *P. murrayi*. An LEA group 3 gene *Aav-lea-1* was strongly induced in *A. avenae* during the induction of anhydrobiosis [19]. The *C. elegans* genome encodes three LEA genes [15] and silencing of the *Lea-1* gene by RNA interference (RNAi) caused a marked reduction in desiccation resistance in dauer larvae [52]. We thus assume that LEA proteins contribute to the protection and recovery from desiccation stress in anhydrobiotic nematodes.

The molecular chaperones, such as the Hsp70 family and the Hsp60 chaperonin complexes, are commonly perceived as heat shock proteins (Hsps), being up-regulated by stress. The *P. murrayi* transcripts of 70 [GenBank: FK670245] and 90 kda heat shock protein [GenBank: FK670262] and small heat shock proteins were abundantly expressed following desiccation stress. Heat shock proteins have been implicated in response to desiccation in many nematodes [14], but they appear to be constitutively expressed in *P. murrayi* (Additional file 2.3). Though Hsps may contribute to enhanced stress resistance overall, our results showed no evidence that expression levels of these Hsps were altered by desiccation. It has been shown that other Antarctic organisms constitutively express Hsp*70* and Hsp90, showing no or modest up-regulation of this gene in response to thermal stress [53-55]. This pattern of expression deviates considerably from the well-documented patterns of expression observed in a wide range of organisms from temperate and tropical regions [41]. It may be that *P. murrayi* evolved a mechanism to maintain Hsp function without disrupting normal metabolism and growth that requires synthesis of other proteins. Consistent with the observed constitutive expression, it is possible that desiccation stress did not activate these genes, and the mild desiccation failed to boost Hsp expression. An alternative explanation for the

continuous up-regulation of Hsps is that because Antarctic nematodes are frequently, although unpredictably, exposed to a variety of environmental stressors such as desiccation, high pH, extreme osmotic excursion, freezing, and anoxia as well as temperature [56], their survival depends on maintaining continuous expression of molecular chaperones. Furthermore, because of the unpredictability and the potential rapidity of exposure to diverse environmental stresses, the continuous production of these molecular chaperones may be energetically justified.

Unexpectedly, one of the ESTs encoded a protein similar to the type II antifreeze protein (AFP) [GenBank: FK670248] of Atlantic herring (*Clupea harengus*). The transcript encoding AFP was down-regulated during desiccation stress of *P. murrayi*. Many overwintering organisms, including insects, fish, bacteria, fungi, and plants, accumulate antifreeze proteins (AFPs) that bind to the faces of ice crystals during freezing and inhibit their growth [57]. To our knowledge, no AFP gene has been reported in nematodes or their closest relatives. Based on the data presented here, it is, at present, difficult to unravel the specific role and evolutionary forces that have led to the acquisition of the AFP gene in an Antarctic nematode. In order to better understand the origin and evolution of this gene in an Antarctic nematode, further sequencing and characterization is currently underway.

Oxidative stress genes expressed during anhydrobiosis

Desiccation stress induces the generation of reactive oxygen species (ROS) in nematodes, and therefore it is important for nematodes to have effective ROS-scavenging mechanisms. A number of ESTs encoding proteins which detoxify reactive oxygen species like superoxide dismutase (SOD) [GenBank: FK670240], Ras-realted protein [GenBank: FK670252], and glutathione S-transferase (GST) (Contig_63) were expressed in *P. murrayi* in response to desiccation (Table 2.2). The SOD enzymes are a family of metalloenzymes

responsible for quenching the potentially deleterious effects of superoxide radicals. There was abundant expression of ESTs similar to *C. elegans sod-1* gene that encodes a copper/zinc superoxide dismutase (Table 2.3). Three transcripts encoding glutathione s-transferase 1 were expressed in the ESTs of *P. murrayi* and one of them was up-regulated following desiccation stress. GSTs are a diverse super-family of multifunctional proteins that play prominent roles in detoxification metabolism in nematodes [58]. More than a dozen different GSTs have been isolated from *C. elegans* [59] and these detoxifying enzymes are reported to be involved in several functions, including xenobiotic detoxification and oxidative stress tolerance [58, 60]. Differential expression and up-regulation of detoxifying enzymes like *sod-1* and *gst-1* suggests that *P. murrayi* has efficient ROS scavenging mechanisms under desiccation stress.

Membrane and transport-related protein expressed during desiccation

In this study, we identified several ESTs encoding proteins involved in transport facilitation. A total of 15 genes encoding ion and water transporters included ABC transporter proteins (Contig_241), water channel proteins (Contig_66), ATPase (Contig_55) and lipid transfer proteins (LTP) [GenBank: FK670243]. Though the mechanism of coupling ion and water flow through membrane channels is not well studied, there is evidence that up-regulation of such genes is correlated with sensitivity to different types of stress in nematodes [61]. Sixty ABC transporters have been identified and functionally characterized in *C. elegans*. Members of this protein family are responsible for resistance to heavy metals [62]. In addition to short-term response and regulatory mechanisms, a functional system for reestablishing homeostasis is vital to desiccation tolerance. We found transcripts encoding proteins involved in ion homeostasis, such as vacuolar H^+-ATPase and ATP synthase [GenBank: FK670298], showing that *P. murrayi* has efficient homeostatic pathways. Desiccation tolerance involves changes in the levels and composition of fatty acids of the

major glycerolipids in nematodes [16]. Our data showed that gene encoding non-specific LTP was differentially regulated following desiccation, suggesting an active lipid metabolism and desiccation resistance.

Refined gene-specific expression using quantitative real-time PCR

In general, we observed larger changes in gene expression using qRT-PCR, likely reflecting the greater dynamic range of detection and sensitivity of this method for gene expression profiling. Desiccation stress caused significant up-regulation of transcripts encoding stress related genes like *Lea* (late embryogenesis abundant protein) and *Tps* (trehalose-6-phosphate synthase) and down-regulation of *Afp* (antifreeze protein). Similarly, there was significant up-regulation of the ROS scavenging enzyme *Gst-1*(gluthathione s-transferase 1) and metabolism related genes like *GlPer* (glutathione peroxidise) and *MalSyn* (malate synthase), and down-regulation of *Afp* and *GlySyn* (glycogen synthase). Similar gene expression patterns, except for antifreeze protein, were reported for the free-living mycophagous nematode *A. avenae* [15] and insect parasitic nematode *Steinernema feltiae* [21] during desiccation acclimation and survival (Fig. 2.3). Although the observed changes in gene expression are common to many nematodes (except for the antifreeze protein and heat shock proteins), we observed significant variability on the magnitude of transcript abundance in *P. murrayi*. It is likely that the expression of these genes in response to desiccation stress is influenced by the thermal history of the organism, including seasonal variation experienced in the field, and by phylogenetic constraints.

CONCLUSIONS

Nematodes have an important yet poorly understood place in the study of stress response survival, and desiccation tolerance in particular. Nematodes are the most abundant metazoans on earth [63] and prominent drivers of Antarctic ecosystem functioning [64].

Unlike other Antarctic nematodes studies thus far, *P. murrayi* are desiccation as well as freeze tolerant, which establishes them as a useful model in assessing the structural, physiological, biochemical and genetic aspects of multiple stress tolerance, and the mechanisms by which organisms respond to and survive in extreme environments. To date very few studies have focussed the molecular aspects of desiccation tolerance in Antarctic nematodes and no genomic data is available in existing databases. The progression of Antarctic nematode genomics will be critical to the development of more useful and manipulable models for understanding desiccation tolerance and the nature of extremophiles. It is to these ends that we have initiated this study into the transcriptome of *P. murrayi* as they respond to a major stress event, in this case desiccation.

In the present study of 2,486 ESTs, a significant portion of transcripts had no known homologue in any nematode or other organisms for which sequence data are presently available in public databases. These molecules are particularly interesting as they may represent genes that are species/lineage specific or unique to Antarctic nematodes. There is considerable scope in exploring such molecules in the future and using a combination of genomic and proteomic approaches could provide valuable information on the survival strategy of extremophiles. Although the number of transcripts analyzed under this study is not large, we have identified and validated several genes differentially expressed during desiccation stress. It is crucial to emphasize that changes in mRNA accumulation may not necessarily correlate with protein/enzyme activity level [65]. However, the expression profiles provide starting points for more in-depth studies on candidate genes using additional genetic approaches. Nevertheless, the annotation of transcripts with significant fold change and detection of consistently DE transcripts by SSH and qRT-PCR strongly suggest that these putative genes have an important role in *P. murrayi* desiccation stress response. In

combination with the bioinformatics analysis presented here, further functional genomics analyses are required to generate a more complete picture of the cellular response of a free-living metazoan to extreme abiotic stress and determine the biological roles of these genes within the larger context of its Antarctic ecosystem.

MATERIALS AND METHODS

Nematode culture

Live nematodes (*P. murrayi*) from Antarctica were reared in culture in order to produce sufficient quantities for experiments. Nematodes were cultured in Sand Agar plates containing Bold Modified Basal Freshwater Nutrient Media (BMBFN) (Sigma Aldrich Inc. USA) and stored at 15˚C until used. Briefly, 20 ml/L BMBFN solution was mixed with 15 g/L Bacto-Agar in deionised water and autoclaved. The autoclaved Agar media was poured into Petri dishes and Standard Ottawa Sand (EMD Chemicals Inc., Gibbstown, NJ) was spread over each plate before the media solidified. About 5-10 nematodes were released into each plate and thin layer water was maintained for the duration of culture. The plates were incubated at 26°C for one week followed by 16°C for 3-4 weeks. To harvest the nematodes, the Agar media was cut into small pieces and poured into #40 Standard testing Sieve (Advantech Manufacturing, New Berlin, WI), agar was removed by sieving and nematodes were collected by centrifugation.

Desiccation treatment

Nematodes (about 3,000-5,000) were desiccated in glass desiccation chambers. The relative humidity (RH) was controlled by using saturated salt solutions as described by Winston & Bates [66] with some modifications. The requisite RH was maintained at 23°C±1˚C in the desiccation chamber for 3 days prior to the addition of nematodes for equilibration. Relative humidity was maintained as 100% RH with distilled water vapour;

97% with K_2So_4, 87% RH with HCl and 75% with NaCl. The treatments were an initial desiccation at 97% RH for 3 days followed by exposure to 87% for 2 days. All experiments were repeated under identical conditions using mixed stage populations of *P. murrayi*.

RNA isolation

The desiccated and control nematodes were transferred to 10 volumes of Trizol Reagent (Molecular Research Center Inc., Cincinnati, OH) and exposed to freeze thaw cycles using liquid nitrogen and a 37ºC water bath. The suspension was ground using mortar and pestle and vortexed. RNA was phase separated using chloroform, precipitated by isopropanol and pelleted. Total RNA was quantified and quality checked by spectrophotometer (NanoDrop, ND-1000 Spectrophotometer) and running agarose gel with RNA Century™ Plus Marker (Ambion Inc., Austin, TX).

cDNA Library Construction and Sequencing

Total RNA was extracted from the gradually desiccated nematodes and used as starting material for complementary deoxyribonucleic acid (cDNA) library construction. A full-length cDNA library was prepared using Creator™ SMART™ cDNA library construction kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. Briefly, 2 µg of total RNA was reverse transcribed to cDNA using SMART™ IV Oligonucleotide and PowerScript Reverse Transcriptase and amplified using long distance (LD) PCR. The cycling parameters were 95ºC, 20 s; two 20 cycles of 95ºC, 5 s; and 68ºC, 6 min. DNA was analyzed using agarose gel electrophoresis, proteinase K digested and restriction digested by *SfiI*. DNA was size fractionated using CHROMA SPIN-400 columns and analyzed via agarose gel electrophoresis. The size fractionated DNA was directionally cloned into pDNR-LIB vector and incubated at 16ºC for 15 h. Transformation was done at 37ºC overnight, and colonies were picked and grown for 18 h. Template DNA was extracted

using QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA), amplified, cleaned using ExoSAP-IT® (USB Corporation, Cleveland, Ohio) and sequenced using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, CA) at the DNA Sequencing Center (DNASC) at Brigham Young University (BYU). Sequencing reactions were performed with 2 µl of ABI PRISM BigDye Terminators v3.0 (Applied Biosystems) and 3.5 pmol of primer in 12 µl reaction volumes, followed by a sequencing reaction clean up to remove residual dye and enzyme. Sequencing was with M13 forward (GTAAAACGACGGCCAG) and reverse primers (CAGGAAACAGCTATGAC) for 25 cycles of 96ºC, 10 s; 50ºC, 5 s; and 60ºC, 4 min. The sequenced products were separated and analyzed with Sequencher™ 4.8 (Gene Codes Corporation, Ann Arbor, MI).

Subtractive Hybridization

Subtractive hybridization was performed with the PCR-Select™ cDNA subtraction kit (Clontech, Palo Alto, CA). Random hexamer primers (Clontech) were used to convert mRNA that had been extracted from desiccated and control nematodes into cDNA. The cDNA from desiccated samples were taken as tester while those from the control served as driver. Second strands of driver and tester DNA were synthesized using T4 DNA polymerase and restriction digested by *RsaI* to create blunt-ended double stranded cDNA for subtraction. The tester cDNAs were subdivided into two portions and each group ligated to different adaptor sequence and subjected to two levels of subtractive hybridizations as described in the manufacturer's protocol. The DE cDNAs were selectively amplified by two PCR reactions using 50X Advantage cDNA Polymerase mix. The first PCR amplified double stranded cDNA with different adaptor sequences and the second PCR further reduced background and enriched for DE sequences. The PCR-amplified cDNA fragments generated by SSH were

then ligated into the TOPO® TA Cloning® vector (Invitrogen Corporation, USA) and sequenced using ABI 3730xl DNA Analyzer (Applied Biosystems, CA) at the BYU DNASC.

Sequence analysis and EST clustering

Base calling was performed using PHRED software (versions 0.000925.c) [68, 69] with the quality cut-off set at PHRED 20. Raw sequences were then imported into the Vector NTI Advance™ 10 software (Invitrogen Corporation) and subjected to trimming of vector sequences and 5' adapter sequences (for subtractive hybridization) using default settings. Sequences with less than 100 quality bases (PHRED 20 or better) after trimming were discarded. Sequences having polyA tails of 100 bases or more were eliminated from analysis. EST sequences representing contamination from bacterial, yeast or fungal sources were identified using BLASTN algorithm [29, 30] and removed from further analyses. ESTs were aligned and assembled into contigs using CAP3 software [70] when the criterion of a minimum identity of 95% over 50 bp was met. When an EST could not be assembled with others in a contig, it remained as a "singleton". The contigs and the singletons should thus correspond to sequences of unique genes. The consensus sequences of the contigs and the sequences of the singlets were compared to the sequences in GenBank's non-redundant (nr) and Uniprot database using the TBLASTX and the BLASTX [38, 39] algorithms and *C. elegans* Wormpep 190 database [31] using the BLASTX algorithm [30]. The cut-off for sequence similarity was E-value < 10^{-5} for all analyses.

Functional analysis and pathway assignments

Gene ontology (GO) term annotation and function-based analysis [71] of unique sequences were performed using Blast2go (V 1.6.2) [72], a sequence-based tool to assign GO terms, extracting them for each BLAST hit obtained by mapping to extant annotation associations. GO terms for each of the three main categories (biological process, molecular

function, and cellular component) was obtained from sequence similarity using the application default parameters. From these annotations, pie charts were made using 2nd level GO terms based on biological process, molecular function, and cellular component. Pathway assignments were carried out according to Kyoto encyclopedia of genes and genomes (KEGG) mapping [41]. Enzyme commission (EC) numbers were assigned to unique sequences that had BLASTX scores with a cut-off value of $E = 10^{-5}$ or less upon searching protein databases. The sequences were mapped to KEGG biochemical pathways according to the EC distribution in the pathway database.

Primer Design

All the primers used in quantitative real-time PCR (qRT-PCR) were designed with IDT SciTools (Integrated DNA Technologies, Coralville, IA) by aligning EST sequences with similar sequences from NCBI. All the primers used in this study were synthesized by Operon (Operon Biotechnologies Inc., Huntsville, AL). The sequences of the primers and product sizes are listed in Additional file 2.1.

Quantitative Real-time PCR

Total RNA extracted from dehydrated and control *P. murrayi* nematodes was reverse transcribed using ImProm-II™ reverse transcriptase (Promega corporation, Madison, WI) and subjected to qRT-PCR analysis using Light cycler 480 SYBER Green I mastermix and gene specific primers in a Light cycler 480 RT-PCR system (Roche Applied Science, Mannheim, Germany) equipped with light cycler 480 software. High-resolution gel electrophoresis was used to verify that the qRT-PCR amplification product from each examined gene was a single-band product. Thermal cycling was performed in accordance with the manufacturer's instructions for a total of 40 cycles at an annealing temperature of 58°C for each primer pair. Quantitative RT-PCR analysis was performed with Lightcycler

480 software, the threshold cycle was automatically calculated by the second-derivative maximum method, and the copy number of the specific mRNA in the experimental samples was calculated by extrapolation from the gene-specific standard curve.

Data analyses

The copy number of specific cDNA molecules present in the samples was determined by absolute quantification method of qPCR analysis [35]. A range of six dilutions (10^7–10^2 copies) of the cDNA was made and a gene-specific external standard curve was generated by using cDNA standards that were run simultaneously with the experimental samples. Change in target gene expression was calculated using equation $2^{-\Delta\Delta CT}$ [67]. The fold change in the target gene, normalized to 18S rRNA and relative to the expression of control, was calculated for each sample. A gene with a relative abundance of one is equal to the abundance of 18S rRNA in the same sample. An *F*-test at a significance level of $P < 0.05$ was used to compare the ratio of the mean gene expression of desiccated samples with that of the control. To minimize mRNA quantification errors, genomic DNA contamination biases and to correct for inter-sample variations, we used 18s ribosomal RNAs (rRNAs) of *P. murrayi* as an internal control.

Authors' contributions

BNA carried out most of the work described here including conception and design of experiments, acquisition of data, analysis and interpretation of data and drafting the manuscript. DHW and BJA contributed to sample and culture collection, conception and design of experiments, supervision of the work and critical review of the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 2.1- Distribution of *Plectus murrayi* ESTs by cluster size.

Figure 2.2- Percentage representation of gene ontology (GO) mappings for *Plectus murrayi* clusters.

(A) Molecular functions; (B) Cellular components; and (C) Biological process. More detailed information is provided in Additional files 2a, 2b and 2c. Note that individual GO categories can have multiple mappings.

Figure 2.3- Quantitative Real-time PCR analysis of gene expression in *Plectus murrayi* in response to desiccation.

Values were determined using qRT-PCR and represents relative expression of genes from desiccated to undesiccated nematodes (control). The relative expression of the target gene (*AlDeh*: aldehyde dehydrogenase; *Tps*: trehalose-6-phosphate synthase; *GlPer*: glutathione peroxidise; *Afp*: antifreeze protein; *Hsp70*: heat shock protein 70; *Lea*: late embryogenesis abundant protein; *GlKin*: glycerol kinase; *MalSyn*: malate synthase; *GlySyn*: glycogen synthase; *Hsp90*: heat shock protein 90; *Rpl-4*: ribosomal protein-4; *Nov76*: novel protein I; *Nov80*: novel protein II; *Gst-1*: glutathione s-transferase 1,) normalized to 18S rRNA and relative to the expression of control, was calculated for each sample using the $2^{-\Delta\Delta CT}$ method [67]. Gene expression was determined in each sample using three independent technical replicates. A transcript with relative abundance of one is equivalent to the abundance of 18S rRNA. Bars represent standard errors calculated from three replicates of each experiment. *Significant difference ($P < 0.05$) from control.

TABLES

Table 2.1- *Plectus murrayi* EST summary

Total number of high quality sequences[‡]	2,486
Average length of sequences[†]	545±156
Number of contigs[§]	324
Number of singletons	1,063
Number of putative unique transcripts[¶]	1,387
Unique transcripts with similarity to *C. elegans* database	523 (38%)*
Unique transcripts with similarity to other nematode database	106 (7%)
Unique transcripts with similarity to other organism	153 (11%)
Total unique transcripts with significant similarity	782 (56%)
Unique transcripts with no significant similarity	605 (44%)

[‡]A sequence is considered high quality if it's trimmed PHRED 20 length is >100 bases after vector only, low-quality and contaminating sequences are removed.

[†]Calculated from the total ESTs.

[§]A contig (contiguous sequence) contains two or more ESTs.

[¶]Number of putative unique transcripts equals the number of contigs plus the number of singletons.

*Calculated as percentage of total unique transcripts with similarity at $E < 10^{-5}$.

Table 2.2- Listing of ESTs differentially expressed during desiccation of *Plectus murrayi* and their homologs in GenBank.

GenBank accession number	Homolog accession number	Annotation (Organism)	E-value	Percentage similarity
Metabolism[d] (22)				
FK670236	ref|NP_496237.1|	GPD family member [*Caenorhabditis elegans*]	1e-40	86%
FK670237	ref|NP_498081.2|	Aldehyde dehydrogenase family member [*C. elegans*]	2e-80	74%
FK670241	gb|AAF81283.1|	Glutathione S-transferase [*Haemonchus contortus*]	3e-51	49%
FK670243	ref|NP_496161.1|	Lipid Transfer protein family member [*C. elegans*]	6e-42	72%
FK670244	gb|AAC47996.1|	Aspartyl protease protein 6 [*C. elegans*]	2e-51	53%
FK670249	WBGene00002263	Plant LEA related family member [*C. elegans*]	1e-33	28%
FK670250	gb|EDP32297.1|	Trehalose 6-phosphate synthase protein 1 [*Brugia malayi*]	1e-55	51%
FK670253	gb|EDP36623.1|	Fructose-bisphosphate aldolase 1, putative [*B. malayi*]	1e-73	84%
FK670254	gb|AAC97508.1|	Thymidylate synthase [*C. elegans*]	1e-28	62%
FK670287	WBGene00006975	Zinc finger protein family member [*C. elegans*]	2e-28	53%
FK670258	ref|NP_494721.1|	Probable glycerol kinase [*C. elegans*]	4e-62	71%
FK670259	ref|NP_001006395.1|	Malate dehydrogenase 1, NAD (soluble) [*Gallus gallus*]	1e-71	68%
FK670260	ref|ZP_00056387.1|	Isocitrate dehydrogenases [*Magnetospirillum magnetotacticum*	4e-86	73%
FK670293	ref|NP_498111.2|	ATP synthase subunit family member [*C. elegans*]	8e-110	93%
FK670261	gb|EDP37408.1|	NADH-ubiquinone oxidoreductase 39 kDa subunit [*B. malayi*]	2e-57	60%
FK670264	ref|NP_496736.1|	Glycogen synthase family member [*C. elegans*]	1e-35	50%
FK670265	emb|CAA53718.1|	ADP/ATP translocase [*C. elegans*]	6e-93	90%
FK670269	ref|NP_503306.1|	Bifunctional glyoxylate cycle protein [*C. elegans*]	4e-95	90%
FK670270	gb|AAC19750.1|	Putative glutamate dehydrogenase [*H. contortus*]	9e-111	88%
FK670263	gb|AAC97508.1|	Thymidylate synthase [*C. elegans*]	1e-26	49%
FK670268	WBGene00009165	Glutathione peroxidase [*C. elegans*]	3e-63	79%
FK670298	ref|NP_498111.2|	ATP synthase sub unit family member [*C. elegans*]	8e-110	93%
Environmental information processing (15)				
FK670238	gb|AAM55195.1|	Cathepsin L cysteine protease [*H. contortus*]	2e-81	77%
FK670239	ref|NP_508913.1|	JNK kinase family member jkk-1 [*C. elegans*]	2e-06	41%
FK670240	WBGene00004930	Superoxide dismutase family member [*C.elegans*]	4e-80	81%
FK670242	gb|ABA41369.1|	Type II antifreeze protein [*Clupea harengus*]	2e-35	69%
FK670245	gb|AAN78300.1|	Heat shock protein 70 A [*Heterodera glycines*]	2e-59	89%
FK670248	ref|NP_495536.1|	Small heat shock protein family member [*C. elegans*]	2e-115	90%
FK670251	ref|NP_496549.1|	RAB family member [*C. elegans*]	7e-103	88%
FK670252	gb|EDP28446.1|	Ras-related protein Rab-11B, putative [*B. malayi*]	1e-53	77%
FK670256	ref|NP_509019.1|	Heat shock protein family member [*C. elegans*]	8e-78	91%
FK670257	gb|AAD00182.1|	Inhibitor of apoptosis homolog [*C. elegans*]	5e-53	52%
FK670262	gb|EDP35652.1|	Heat shock protein 90 protein [*B. malayi*]	8e-59	53%
FK670266	ref|NP_499889.2|	DumPY: shorter than wild-type family member [*C.elegans*]	7e-47	63%
FK670294	gb|AAO44907.1|	Collagen protein 170 [*C. elegans*]	4e-30	62%
FK670300	gb|EDP30373.1|	Leucine rich repeat family protein [*B. malayi*]	3e-27	40%
FK670302	gb|EDP31428.1|	Laminin receptor 1 [*Xenopus laevis*]	1e-73	71%
Genetic information processing (23)				
FK670246	gb|AAG50205.1|	Aspartyl protease inhibitor [*Parelaphostrongylus tenuis*]	7e-41	62%
FK670247	emb|CAJ57642.1|	Putative ubc enzyme [*Oesophagostomum dentatum*]	2e-74	97%
FK670267	gb|EDP39185.1|	Histone H2B 2, putative [*B. malayi*]	3e-33	94%
FK670295	gb|AAT28331.1|	Peroxiredoxin [*H. contortus*]	5e-89	83%
FK670308	ref|NP_956267.1|	Ubiquitin specific protease 14 [*D. rerio*]	5e-40	44%
FK670271	ref|NP_491416.1|	Ribosomal protein, large subunit family member [*C. elegans*]	7e-104	85%
FK670272	ref|NP_502794.1|	Ribosomal protein, small subunit family member [*C. elegans*]	8e-73	94%
FK670273	ref|NP_502794.1|	Ribosomal protein, small subunit family member [*C. elegans*]	3e-67	81%
FK670274	ref|NP_501167.1|	Ribosomal protein, small subunit family member [*C. elegans*]	9e-85	84%
FK670275	ref|NP_741371.2|	Ribosomal protein, large subunit family member [*C. elegans*]	5e-57	77%
FK670276	ref|NP_498660.1|	Ribosomal protein, large subunit family member [*C. elegans*]	1e-67	88%
FK670277	ref|NP_740944.1|	Ribosomal protein, small subunit family member [*C. elegans*]	2e-40	82%
FK670278	ref|NP_496375.1|	Ribosomal protein, large subunit family member [*C. elegans*]	9e-51	97%
FK670279	gb|EDP38710.1|	60S ribosomal protein L27a, putative [*B. malayi*]	5e-54	83%
FK670280	gb|EDP38220.1|	60S ribosomal protein L39, putative [*B. malayi*]	9e-21	94%
FK670288	gb|EDP29175.1|	40S ribosomal protein S6, putative [*B. malayi*]	1e-25	86%
FK670281	ref|NP_492457.1|	Elongation factor family member [*C. elegans*]	2e-66	89%
FK670282	gb|EDP34276.1|	Elongation factor 1-alpha, putative [*B. malayi*]	1e-117	88%
FK670283	ref|NP_492457.1|	Elongation factor family member [*C. elegans*]	2e-66	89%
FK670284	ref|NP_524808.2|	Elongation factor 1 beta, isoform A [*Drosophila melanogaster*]	5e-45	68%
FK670285	ref|NP_498520.1|	Elongation factor family member [*C. elegans*]	4e-100	89%
FK670255	WBGene00003623	Nuclear hormone receptor family member [*C. elegans*]	2e-06	38%
FK670289	gb|EDP33960.1|	Transcription factor, putative [*B. malayi*]	9e-16	87%
Hypothetical proteins (3)				
FK670286	ref|XP_001666153.1|	Hypothetical protein [*C. briggsae*]	4e-41	78%
FK670291	ref|XP_001676045.1|	Hypothetical protein [*C. briggsae*]	3e-32	55%

FK670303	ref|XP_001631386.1| Predicted protein [*X. laevis*]		2e-34	42%
Novel proteins (17)				
FK670290	n.a	Novel	n.a.	
FK670292	n.a	Novel	n.a.	
FK670296	n.a	Novel	n.a.	
FK670297	n.a	Novel	n.a.	
FK670299	n.a	Novel	n.a.	
FK670301	n.a	Novel	n.a.	
FK670304	n.a	Novel	n.a.	
FK670305	n.a	Novel	n.a.	
FK670306	n.a	Novel	n.a.	
FK670307	n.a	Novel	n.a.	
FK670309	n.a	Novel	n.a.	
FK670310	n.a	Novel	n.a.	
FK670311	n.a	Novel	n.a.	
FK670312	n.a	Novel	n.a.	
FK670313	n.a	Novel	n.a.	
FK670314	n.a	Novel	n.a.	
FK670315	n.a	Novel	n.a.	

[g]Pathway assignment based on Kyoto Encyclopedia of Genes and Genomes (KEGG) classification.

Table 2.3-The most abundantly represented transcripts in the cDNA library.

Contig No	Tentative annotation[§]	Number of ESTs	Percentage[£] (%)
Contig_57	Ribosomal protein	37	1.48
Contig_132	Cytochrome c oxidase	33	1.32
Contig_9	Small heat shock protein family	27	1.08
Contig_312	Rab-family member	25	1.00
Contig_87	Aquaporin	24	0.96
Contig_231	DNA-binding protein	23	0.92
Contig_32	Y-box family member	22	0.88
Contig_198	Zinc finger protein	18	0.72
Contig_54	Cu/Zn superoxide dismutase	17	0.68
Contig_56	Translation initiation factor	17	0.68
Contig_67	Chaperonin containing subunit	16	0.64
Contig_301	Glutamate synthase	13	0.52
Contig_287	NADH-dehydrogenase	13	0.52
Contig_93	Cathespin b -like cysteine proteinase	13	0.52
Contig_74	Elongation factor 1 alpha	13	0.52
Contig_63	Glutathione S-transferase	12	0.48
Contig_152	Heat shock 70 kda protein	11	0.44
Contig_242	60S ribosomal protein	11	0.44
Contig_131	Elongation factor family member	11	0.44
Contig_29	Aldehyde dehydrogenase	7	0.28
Contig_149	Heat shock protein 90	7	0.28
Contig_313	ATP synthase subunit	7	0.28
Contig_112	Nuclear hormone protein family	7	0.28

[§]Annotation based on most significant BLAST alignment for each cluster.

[£]Percentage based on total number of high quality sequences.

Table 2.4- KEGG biochemical mappings for *Plectus murrayi* clusters.

KEGG categories represented	Unique sequences (Number of enzymes)	Percentage[§]
Metabolism	**84 (52)**	**11%**
Carbohydrate metabolism	29(18)	4%
Amino acid metabolism	14(9)	2%
Lipid metabolism	13(8)	2%
Xenobiotics biodegradation and metabolism	8(5)	1%
Biosynthesis of secondary metabolites	6(3)	<1%
Energy metabolism	5(2)	<1%
Nucleotide metabolism	3(2)	<1%
Metabolism of other amino acids	3(2)	<1%
Glyoxylate and dicarboxylate metabolism	3(3)	<1%
Genetic information processing	**83 (48)**	**11%**
Folding, sorting and degradation	42(25)	6%
Transcription	17(9)	2%
Translation	16(8)	2%
Replication and repair	8(6)	1%
Environmental information processing	**95 (47)**	**12%**
Membrane transport	30(15)	4%
Ligand-receptor interaction	28(15)	4%
Signal transduction	14(8)	2%
Signalling molecules and interaction	13(9)	2%
Cellular processes	**19 (11)**	**3%**
Cell growth and death	8(5)	1%
Cell communication	6(4)	<1%
Cell motility	3(1)	<1%
Development	2(1)	<1%
Unassigned[‡]	**385**	**49%**
Hypothetical	**116**	**15%**

[§]Percentage based on total unique transcripts (782) with significant similarity to sequences in database.
[‡]Unassigned sequences are those that have significant similarity to known sequences whose functions are unclear.

ADDITIONAL FILES

Additional file 2.1- List of the gene specific primer sequences used for quantitative real-time PCR analysis

File name: Additional1_PrimerSequences.doc

File format: Word document

Title: List of the gene specific primer sequences used for quantitative real-time PCR analysis.

Description: Primers were designed by aligning the EST sequences with their putative homologue from GenBank using IDT SciTools (Integrated DNA Technologies, Coralville, IA, USA) and synthesized by Operon (Operon Biotechnologies Inc., Huntsville, AL, USA).

Additional file 2.2- Distribution of (a) molecular functions, (b) cellular components, and (c) biological process categories based on gene ontology for *Plectus murrayi* unique sequences.

File name: Additional2_GOdistribution.doc

File format: Word document

Title: Distribution of (a) molecular functions, (b) cellular components, and (c) biological process categories based on gene ontology for *Plectus murrayi* unique sequences.

Description: More information provided in Figure 2a, 2b and 2c. Note that individual GO categories can have multiple mappings.

Additional file 2.3- Analysis of mRNA copy number ($\times 10^7$) of *Hsp90*: heat shock protein 90 and *Hsp70*: heat shock protein 70 gene in *Plectus murrayi* under desiccated and normal condition

File name: Additional3_mRNAcopyNumber.doc

File format: Word document

Title: Analysis of mRNA copy number ($\times 10^7$) of *Hsp90*: heat shock protein 90 and *Hsp70*: heat shock protein 70 gene in *Plectus murrayi* under desiccated and normal condition.

Description: The experiment was performed using an absolute quantitation method of quantitative real-time PCR analysis with each value represents the mean ± SE of three replicates. Nematode samples were exposed to 97 and 85% RH for 3 and 2 days respectively prior to RNA extraction. Controls received no treatment. *Significant difference ($P < 0.05$) from control.

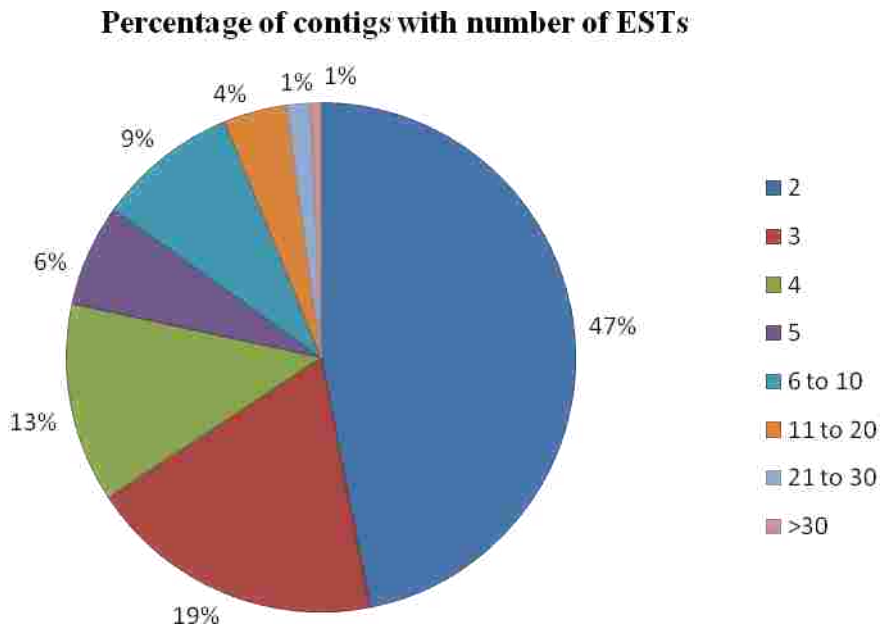
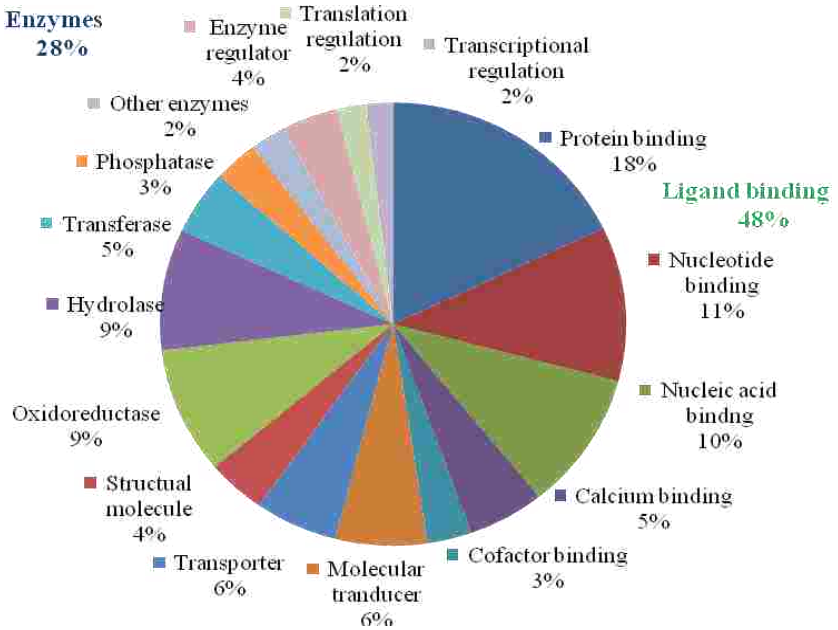
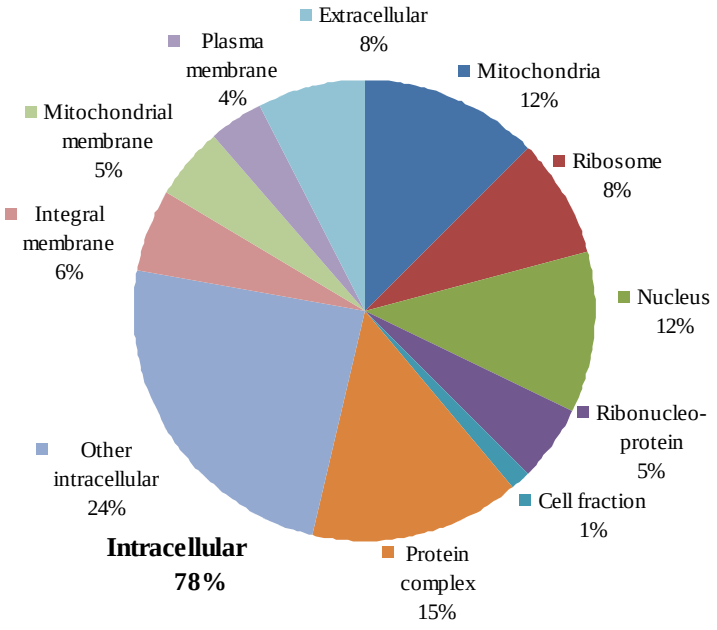

Percentage of contigs with number of ESTs
47%
19%
13%
6%
9%
4%
1%
1%
2
3
4
5
6 to 10
11 to 20
21 to 30
>30

Figure 2.1

(a)


Enzymes
28%
Enzyme
regulator
4%
Translation
regulation
2%
Transcriptional
regulation
2%
Other enzymes
2%
Protein binding
18%
Phosphatase
3%
Ligand binding
48%
Transferase
5%
Nucleotide
binding
11%
Hydrolase
9%
Nucleic acid
bindng
10%
Oxidoreductase
9%
Calcium binding
5%
Structual
molecule
4%
Cofactor binding
3%
Transporter
6%
Molecular
tranducer
6%

(b)


Extracellular
8%
Plasma
membrane
4%
Mitochondria
12%
Mitochondrial
membrane
5%
Ribosome
8%
Integral
membrane
6%
Nucleus
12%
Ribonucleo-
protein
5%
Other
intracellular
24%
Cell fraction
1%
Intracellular
78%
Protein
complex
15%

(c)

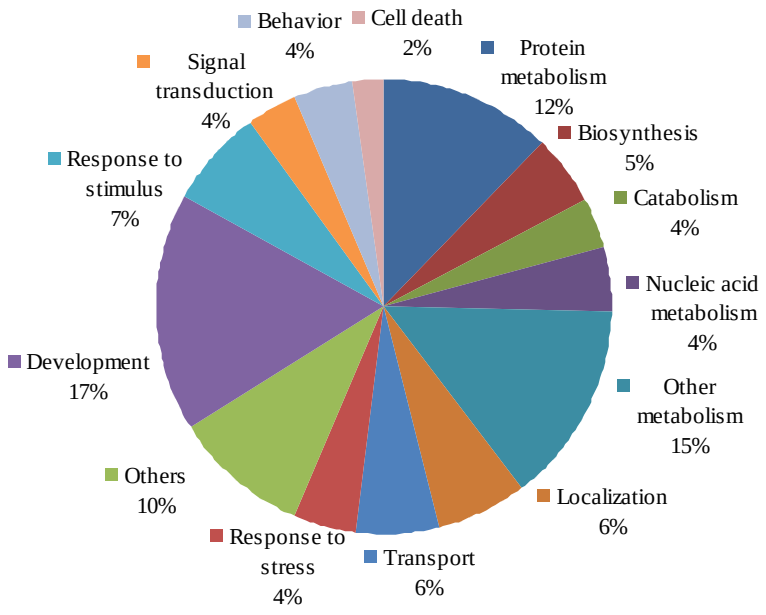

Behavior 4%
Cell death 2%
Protein metabolism 12%
Biosynthesis 5%
Catabolism 4%
Nucleic acid metabolism 4%
Other metabolism 15%
Localization 6%
Transport 6%
Response to stress 4%
Others 10%
Development 17%
Response to stimulus 7%
Signal transduction 4%

Figure 2.2

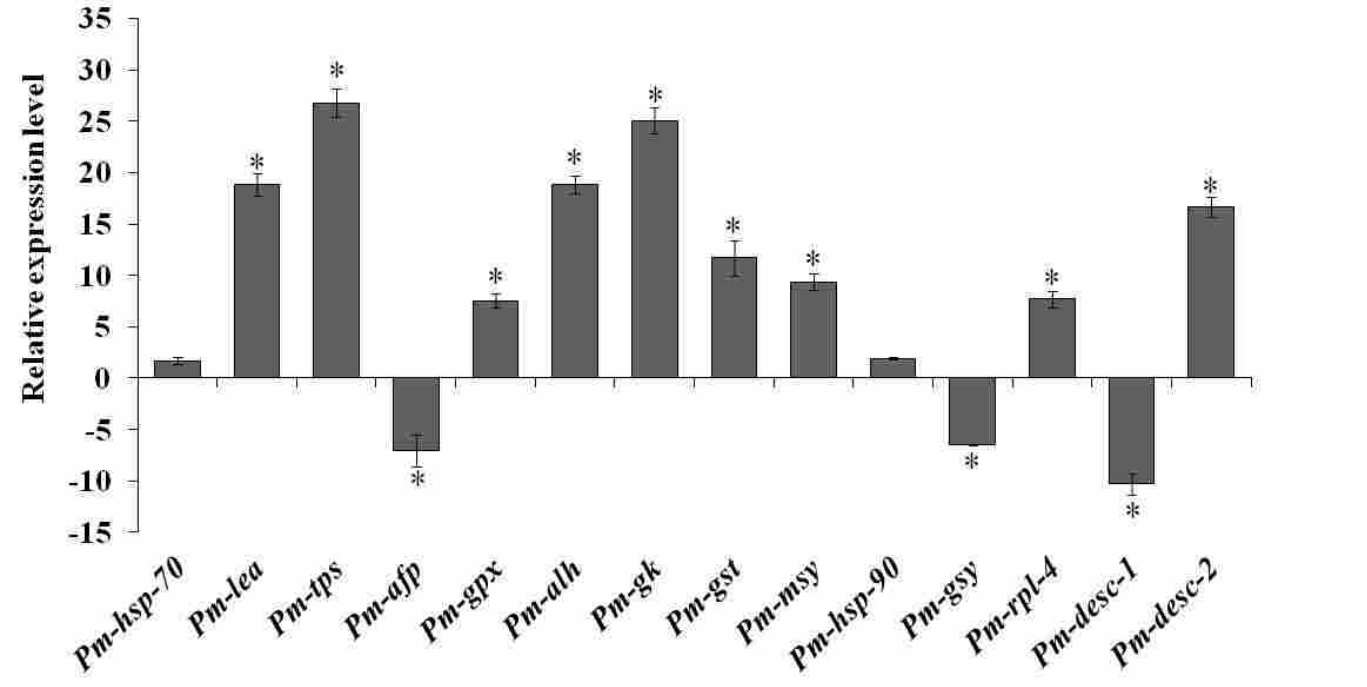

Relative expression level
35
30
25
20
15
10
5
0
-5
-10
-15
Pm-hsp-70
Pm-lea
Pm-tps
Pm-afp
Pm-gpx
Pm-alh
Pm-gk
Pm-gst
Pm-msy
Pm-hsp-90
Pm-gsy
Pm-rpl-4
Pm-desc-1
Pm-desc-2

Figure 2.3

Additional file 2.1- List of the gene-specific primers used in quantitative real-time PCR analysis.

Primer name	Gene	Primer forward 5' to 3'	Primer reverse 5' to 3'	Product length (bp)
AlDeh	Aldehyde dehydrogenase	TGCTTGTGGACCAATCACGCAATC	ATGGAACGTCTGCCCAATACTCGT	222
Tps	Trehalose-6-phosphate synthase	GCACGACAAGCAACGAGTTA	CATGTTCACACCAAGGTTCG	180
GlPer	Glutathione peroxidase	TGCTCATCCACTGTGGCAGTTTC	AGTGAGCTGGGAGATGTTGTTGGA	156
Afp	Antifreeze protein	GAGTTGCAAGTCCAACCCAAACCA	CATTCCAAAGGGTGCCATTGTCGT	192
Hsp70	Heat shock protein 70	AGTTGGGAGCAATCATGGCCAAAG	GCGACTTGATTCTTGGCAGCATCT	255
Lea	LEA protein	ACAACGAGGCCAAGGGAAAGACT	TTTGTCGGAGGCCTTGTCCTTGTA	187
GlKin	Glycerol kinase	TAAGTCAGTGGGCGTGGCTAATCA	CACAATTGCGTTGTAGAGCGGCTT	205
MalSyn	Malate synthase	ACTATCGCTCGTTCGTCAA	CCGGCATCTGTTCTAGTTCC	211
GlySyn	Glycogen synthase	ATGAATGGCAAGCAGGTGTTGGTC	AACGATCCGAGATGTGTAGTCGCA	188
Hsp90	Hsp90	TGCAAACATCTGGAAACCAA	CCAAACTGGCCAATAATGCT	227
Rpl-4	Ribosomal protein	TGAGGTCCCACTTGTTGTTTCCGA	TAACAACTGGTCCGAGCTTCTGCT	235
Nov-76	Novel protein I	CCGCTTATTGGGGTTGTCTA	ATCCGTCCCTACAATCAGCAGCAT	164
Nov-80	Novel protein II	ACTACTCCCGCAGAACAACCAACA	CTCCCGTTGAACGAAACACT	192
Gst-1	Glutathione-S-trans	TGCCTATGGATTCGCTGATGGAGA	AGGGAAAGCAGAGATTGCATTGGG	217

Additional file 2.2- Distribution of (A) molecular functions, (B) cellular components and (C) biological process based on gene ontology for *Plectus murrayi* unique sequences.

A) Molecular Functions

Categories and subcategories	Representation	% Representation‡
Ligand binding/carrier	232	48%
Protein binding	87	18%
Nucleotide binding	55	11%
Nucleic acid binding	49	10%
Calcium binding	26	5%
Cofactor binding	15	3%
Enzyme	138	28%
Oxidoreductase	45	9%
Hydrolase	43	9%
Transferase	23	5%
Phosphatase	15	3%
Isomerase	4	<1%
Helicase	3	<1%
Lyase	3	<1%
Ligase	2	<1%
Molecular transducer	31	6%
Receptor	17	3%
Signal transducer	14	3%
Transporter	28	6%
Substrate specific transporter	19	4%
Transmembrane transporter	9	2%
Structural molecule	20	4%
Enzyme regulator	19	4%
Translation regulation	13	3%
Transcriptional regulation	6	1%

(B) Cellular components

Categories and subcategories	Representation	% Representation‡
Cell	209	92%
Intracellular	176	78%
Cytoplasm	102	45%
Mitochondria	28	12%
Cytosol	25	11%
Ribosome	20	9%
Vesicle	15	7%
Endoplasmic reticulum	5	2%
Cytoskeleton	5	2%
Golgi apparatus	4	2%
Protein complex	33	15%
Nucleus	26	12%
Ribonucleoprotein complex	12	5%
Cell fraction	3	1%
Membrane	33	15%
Integral membrane	13	6%
Mitochondrial membrane	11	5%
Plasma membrane	9	4%

Extracellular	17	8%

(C) Biological process

Categories and subcategories	Representation	% Representation‡
Cell growth and/or maintenance	241	66%
Metabolism	145	40%
Protein metabolism	44	12%
Proteolysis	23	6%
Protein modification	13	4%
Protein folding	8	2%
Biosynthesis	19	5%
Carbohydrate metabolism	18	5%
Nucleic acid metabolism	16	4%
RNA metabolism	11	3%
DNA metabolism	5	1%
Oxygen and reactive oxygen metabolism	15	4%
Catabolism	13	4%
Lipid metabolism	9	2%
Phosphate metabolism	6	2%
Amino acid and derivative metabolism	5	1%
Localization	23	6%
Cell organization and biogenesis	22	6%
Transport	21	6%
Response to stress	16	4%
Cellular respiration	6	2%
Cell cycle	5	2%
Homeostasis	3	<1%
Development	61	17%
Embryonic development	11	3%
Growth	10	3%
Post-embryonic development	10	3%
Reproduction	8	2%
Larval development	8	2%
Cell differentiation	7	2%
Transcription	7	2%
Cell communication	39	11%
Response to stimulus	26	7%
Signal transduction	13	4%
Intracellular signalling cascade	8	2%
Cell surface receptor linked	5	1%
Behavior	15	4%
Programmed cell death	8	2%

‡Percentage representation based on 487, 226 and 364 unique sequences for (A), (B) and (C) respectively.

Additional file 2.3- Analysis of mRNA copy number ($\times 10^7$) of *Hsp90*: heat shock protein 90 and *Hsp70*: heat shock protein 70 gene in *Plectus murrayi* under desiccation and normal condition.

Gene	Treatment	
	Desiccated	Control
Hsp90	6.73±0.58	6.45±1.1
Hsp70	7.20±0.96	7.45±1.19

CHAPTER 3

The Antarctic nematode *Plectus murrayi*: an emerging model to study multiple stress survival

Bishwo N. Adhikari[1*], Cecilia M. de Tomasel[2], Grace Li[2], Diana H. Wall[2], Byron J. Adams[1]

[1]Department of Biology, and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT 84602

[2]Department of Biology and Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO 80523

*Corresponding author: Bishwo N. Adhikari (adhikaribn@hotmail.com)

INTRODUCTION

Plectus murrayi is a representative of the genus *Plectus*, one of the most widely distributed and common nematode taxa of freshwater and terrestrial habitats in the world. The genus *Plectus* is of particular interest because of its phylogenetic position relative to origin of the Secernentean radiation. *Plectus murrayi*, a bacteria feeding nematode, inhabits both semi-aquatic and terrestrial biotopes in the Antarctic McMurdo Dry Valleys (MCM) where their distribution is limited by organic carbon and soil moisture. *Plectus* nematodes from the MCM can survive extreme desiccation, freezing conditions, and other multiple types of stress. Ongoing research on the physiological and molecular aspects of stress biology of this nematode, along with availability of genomic resources, such as expressed sequenced tag (EST), cDNA libraries, functional analysis using RNA interference (RNAi) will establish this nematode as an excellent invertebrate model system for studies with extreme environmental survival and could be a potential source of genomic resources for comparative studies in other organism. Moreover, because *P. murrayi* and *C. elegans* share a most recent common ancestor with the rest of the Secernentea, and given the possibility of culturing this nematode at low temperature compared to *C. elegans*, *P. murrayi* could also be an emerging model system for the study of the evolution of environment-sensitive (stress response) alleles in nematodes.

BACKGROUND INFORMATION

Plectus murrayi is a member of the genus *Plectus* which includes more than 70 described species common in diverse habitats (aquatic and terrestrial; Holovachov and De Ley 2006). This nematode is found in the Dry Valleys and coastal sites in the McMurdo Sound region, and from several other areas of continental East Antarctica, where it is the most widely distributed and

abundant terrestrial nematode. *Plectus murrayi* can be considered an autochthonous species of the continental Antarctica, showing a wide distribution and frequent occurrence. It inhabits both semi aquatic and terrestrial biotopes and can be found across a wide range of altitudes, from the seaside to more than 1,000 meters above sea-level (Andrassy 1998). *Plectus murrayi* has a multiple year life cycle characterized by slow production of eggs relatively few in number. In the laboratory, development from egg to adult female require approximately 6-8 weeks at 15°C (B.N. Adhikari and B.J. Adams, unpubl.).

Plectus murrayi is easily distinguished from other species by their ventrally curved tail (Heyns 1995) (Fig. 3.1A). In spite of its wide distribution and comparatively great abundance, male specimens are very rare, and if present, extremely limited in number. Like other *Plectus* nematodes, parthenogenesis is the dominant mode of reproduction. *Plectus* embryos exhibit a number of developmental similarities with the Secernentea, including *C. elegans*, but also show developmental differences to other Secernentea, supporting their deep divergence (Lahl et al. 2003). This peculiar characteristic, and phylogenetic position, makes *Plectus* an ideal model for exploring the evolution of embryogenesis and other comparative studies in nematodes. The distribution of these nematodes in Antarctica is dependent on organic carbon and soil moisture (Powers et al. 1998). Not surprisingly they are most abundant in stream sediments (Treonis et al. 2000). These nematodes are capable of surviving desiccation and freezing in an anhydrobiotic state (Treonis and Wall 2005), an inactive state that is a survival strategy employed by nematodes, rotifers, and tardigrades in response to desiccation (Crowe and Madin 1975). *Plectus murrayi* from the MCM possess many adaptive characteristics necessary to persist under severe environmental conditions. These traits include protection against desiccation and freezing, ability to survive multiple stresses, and a parthenogenetic life cycle. The ability of *P. murrayi* to

survive extreme Antarctic environment and its role in the Antarctic ecosystem, where abiotic factors are more important drivers of community structure and functioning than biotic interactions, makes this nematode an optimal model for studies of multiple stress survival.

SOURCES AND HUSBANDRY

Plectus murrayi are routinely collected from the MCM and maintained in laboratory culture. Live cultures can be obtained from Dr. Diana Wall's lab at Colorado State University (http://rydberg.biology.colostate.edu/sites/walllab/) or Dr. Byron Adams' lab at Brigham Young University (http://mmbio.byu.edu/faculty/bja43/). A detailed method for *P. murrayi* culture is provided in 'Culturing the Antarctic nematode *Plectus murrayi*'. Although isolated from an extreme environment, *P. murrayi*, grow best at temperatures of 15°C with periodic exposure to 26°C. In Antarctic conditions *P. murrayi* is reported to have a multiple year parthenogenetic life cycle, but the duration of its four larval stages is unknown. Under laboratory conditions egg to egg development takes about 6-8 weeks at 15°C, environmental conditions that may be rare to nonexistent in their natural habitat. *Plectus murrayi* can be cultured in *Escherichia coli* OP50 but their growth rate is comparatively slower and egg to egg development takes longer than sand agar plate culture on bacterial inoculums extracted from their soil.

RELATED SPECIES

Species of the genus *Plectus* are bacterial feeders and among the so-called 'free-living' nematodes. *Plectus* spp. have been collected from the Arctic to the Antarctic, and are reported from all major areas of the world (Maggenti 1961). The genus *Plectus* is of particular interest because of its phylogenetic position relative to origin of the Secernentean radiation (Blaxter et al. 1988; Holterman et al. 2006). *Plectus* sp. (Plectidae) are the sister taxon to the subsequent

lineages of the Secernentean radiation, which produced *Caenorhabditis elegans* and virtually all of the major plant and animal parasitic clades, many of which are scientifically and economically important model species for molecular, developmental and genetic studies. Based on an analysis of a few rDNA sequences, amongst the more than 70 described species in the genus (Holovachov and De Ley 2005), the closest relative of *P. murrayi* appears to be *P. acuminatus*, a free-living species (*i.e.* not associated with plant roots or fungi) ubiquitous in various soils (De Goede et al. 1993). *Plectus acuminatus* is a parthenogenetic, bacterial feeding nematode with a short life cycle, also amenable to laboratory culture. *Plectus acuminatus* can be kept in the laboratory for many generations, and dried cultures can be maintained for several months.

Plectus acuminatus is currently used in international regulatory testing for the determination of chemical hazards and critical loads for toxicants (Van Gestel and Van Straalen 1994) and soil toxicity tests focusing on the sub-lethal effects of toxicants at the population level (Kammenga et al. 1996). It is also used as a model for comparative study of embryogenesis in nematodes (Lahl et al. 2003). Although no genomic data is available for this species, the effects of metal toxicosis have been monitored at the protein level, using unspecific monoclonal antibodies to human stress proteins to evaluate the responses to increasing concentrations of cadmium and copper (Kammenga et al. 1998).

USE OF THE MODEL SYSTEM

Multiple stress survival

The survival by *Plectus* spp. to extreme environmental stress has been the subject of many studies (Hendriksen 1983; Pickup 1990; Treonis et al. 2000; Treonis and Wall 2005; Newsham et al. 2006; Nkem et al. 2006; Islam and Schulze-Makuch 2007, Adhikari et al. 2010).

The current consensus, based on these studies, is that Antarctic *Plectus* spp. can survive extreme desiccation and freezing (Adhikari et al. 2010) along with other types of stress. Several past studies have reported the ability of *Plectus* to survive multiple stresses in Antarctic environments. Hendriksen (1983) reported that *Plectus* sp. can survive total dehydration (0% relative humidity (RH)) even in the absence of induction, and can survive the loss of at least 90% of the internal water realized within one minute. Recently, Adhikari et al. (2009) described changes in gene expression in response to desiccation in *P. murrayi*. Results showed that *P. murrayi* undergoes transcriptional changes during desiccation stress by differential expression of a number of stress related genes and constitutive expression of others (Fig. 3.3). Temporal and functional analyses of desiccation and freezing survival showed that *P. murrayi* exhibits extreme desiccation (0% RH) as well as freeze tolerance (-10°C), activating the expression of a suite of genes involved in various stages of anhydrobiosis and freeze survival. Pre-exposure of nematodes to sub-lethal stress (acclimation) (Fig. 3.1B) promotes desiccation and freeze survival of nematodes, suggestive of cross-stress tolerance mechanisms. Acclimation at higher relative humidity (97%) not only improved the subsequent survival of nematodes at 0% relative humidity but also dramatically increased the survival at -10ºC, relative to the undesiccated controls (Adhikari et al. 2010).

Ecological 'omics'

Plectus murrayi are continually exposed to environmental extremes (desiccation, freezing, osmotic pressures and wind) and are well adapted to such stresses through tolerance to freezing and anhydrobiosis (Treonis et al. 2000, Adhikari et al. 2010), a dry and metabolically inactive state induced by desiccation (Crowe and Madin 1975) and freezing temperatures. The recent revolution in molecular techniques, particularly those in transcriptomics and proteomics,

allows us to link the genome to the environment in entirely new ways (Clark et al. 2007). For example, stress is a major driver of natural selection, and is expected to play a significant role in shaping life-history evolution and trophic ecology. Also, explanations for community structure in the wild may best be inferred from the properties of individual species and their responses to environmental factors (Van Straalen and Roelofs 2002; Hutchinson 1957). *Plectus* nematodes from the MCM are ideal for testing hypotheses of ecological amplitude (niche breadth) as well as community structure and function because overall MCM metazoan biodiversity is extremely low (Adams et al. 2006) and there is little, if any biotic influence over nutrient cycles (Barrett et al. 2005). Instead, this environment is unique in that it is one of the few ecosystems on Earth where abiotic factors (i.e. relative humidity, temperature and soil chemistry) are more important drivers of nematode population structure than biotic interactions (Convey 1996; Hogg et al. 2006). Thus, by controlling for the biotic factors involved in the evolution of the 'structure and functioning of the genome', nematodes from these ecosystems offer a unique natural laboratory model organism for fundamental research on the evolutionary processes that shape ecological amplitude and the relationships between environmental stress, genome function, physiology and ecology.

Source of temperature-sensitive alleles

A potential use of the *P. murrayi* genome will be as a source of temperature-sensitive alleles. These alleles are important components of research programs currently investigating nematode embryogenesis and the inactivation of certain genes at specific points during development. Currently, temperature-sensitive mutations are being used to effectively analyze many essential *C. elegans* genes (i.e. genes necessary for growth to a fertile adult) (Kemphues 2005). As compared to *C. elegans*, which cannot reproduce well at low temperature (below 8°C)

and has a temperature optimum of 20°C (Wood 1988), *P. murrayi* nematodes can thrive in temperatures much lower than that of *C. elegans* and could be a good source of temperature-sensitive alleles. Identification of *P. murrayi* cDNAs of *C. elegans* genes involved in embryogenesis and development could reveal whether the *P. murrayi* cDNAs are functional in *C. elegans* and if they show similar temperature-sensitive characteristics. With the sequencing of the genome and the development of methods to target specific genes, it will be possible to identify such cDNAs that are temperature-sensitive. Furthermore, given its phylogenetic position relative to *C. elegans*, *P. murrayi* offers many opportunities for comparative genomic studies of stress survival, environmental effects on embryogenesis, and the use of stress-related genes as bio-indicators of changes to their physical environment.

GENETICS, GENOMICS, AND ASSOCIATED RESOURCES

At this point in time, genetic tools for gain-of-function and permanent genomic modifications in *P. murrayi* are not developed. *Plectus murrayi* may not be optimal for all classical genetic studies due to its long life cycle and slow growth. However, protocols for gene knockdown using RNAi have been developed (Adams' lab) and used for functional analysis of stress related genes. We advocate following guidelines for gene and protein nomenclature in parasitic and free-living nematodes (Horvitz et al. 1979; Bird and Riddle 1994). Briefly, the prefix "*Pm*" should be attached to the beginning of all gene names; the gene name should be selected to match the convention of the homologous gene and should be lowercase and italicized (e.g., *Pm-afp*). The protein name should be uppercase and not italicized (e.g., PM-AFP).

Genetic and genomic resources developed in other nematodes can be used in *P. murrayi*. A cDNA library of nematodes exposed to mild desiccation was recently sequenced, containing 2,486 ESTs comprising of 1,387 unique transcripts (Adhikari et al. 2009). The *Caenorhabditis elegans* genome comprises about 20,000 genes and the *P. murrayi* genome (87 ± 0.054 Mb by flow cytometry analysis) is smaller than *C. elegans*. The transcripts sequenced are therefore likely to represent only a small proportion of those expressed overall. Additionally, a subtracted library of desiccated nematodes was also prepared and 80 clones differentially expressed in desiccated nematodes were sequenced and analyzed (Adhikari et al. 2009). An initial set of about 2,566 high-quality ESTs has been deposited into GenBank, and this effort continues.

In the study of Adhikari et al. (2009), a significant portion of transcripts had no known homologue in any nematode or other organism for which sequence data are currently available in public databases. There is considerable scope in exploring such transcripts in the future and using a combination of genomic and proteomic approaches could provide valuable information on the survival strategy of extremophiles. Although the number of transcripts analyzed under this study was not large, with the sequencing of the genome and the development of methods to target specific genes, more genes involved in extreme survival will be identified. Despite the considerable advantages of *P. murrayi* as a model system, it has not yet been selected for whole-genome sequencing. At present, the most efficient use of sequencing in *P. murrayi* is for transcriptome analysis. As the price of sequencing declines and the experimental strengths of *P. murrayi* continue to improve, we expect that whole-genome sequencing will occur in the not too distant future.

TECHNICAL APPROACHES

Plectus murrayi is amenable to molecular genetic techniques. Many published protocols from other well-established invertebrate systems such as *C. elegans* have been adapted for *P. murrayi*. Methods used successfully for molecular studies of *P. murrayi* include extraction of RNA from small sample sizes for gene expression analyses (both quantitatively and qualitatively), construction of complementary DNA libraries, subtractive hybridization, and quantitative real-time-polymerase chain reaction (RT-PCR) to evaluate transcript levels,, and RNAi. Methods for culturing *P. murrayi* are described in 'Culturing an Antarctic nematode *Plectus murrayi*'. Since these nematodes can be cultured in *E. coli* OP50, gene knockdown approaches using RNA interference by feeding the nematodes with modified *E. coli* OP50 bacteria have been successful. Systemic RNAi mediated gene silencing has been successfully done on other bacteria feeding, anhydrobiotic nematodes (*Panagrolaimus superbus*; Shannon et al. 2008).

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FIGURE LEGEND

Figure 3.1.

A. Antarctic nematode *Plectus murrayi*. (Photo by Bishwo Adhikari.)

B. *Plectus murrayi* nematode exposed to gradual desiccation. (Photo by Bishwo Adhikari.)

Figure 3.2. Phylogenetic position of *Plectus* among the Nematoda. *Caenorhabditis elegans* is depicted for reference only. Tree modified from De Ley (2006), with permission from WormBook.

Figure 3.3. Quantitative Real-time PCR analysis of gene expression in *Plectus murrayi* in response to desiccation. Values were determined using qRT-PCR and represents relative expression of genes from desiccated to undesiccated nematodes (control). The relative expression of the target gene normalized to *Pm-18s*:18S rRNA and relative to the expression of control, was calculated for each sample. Bars represent standard errors Asterix above the bar indicate difference ($P < 0.05$) from control. (Reproduced from Adhikari et al. 2009, with permission from BioMed Central Ltd.)

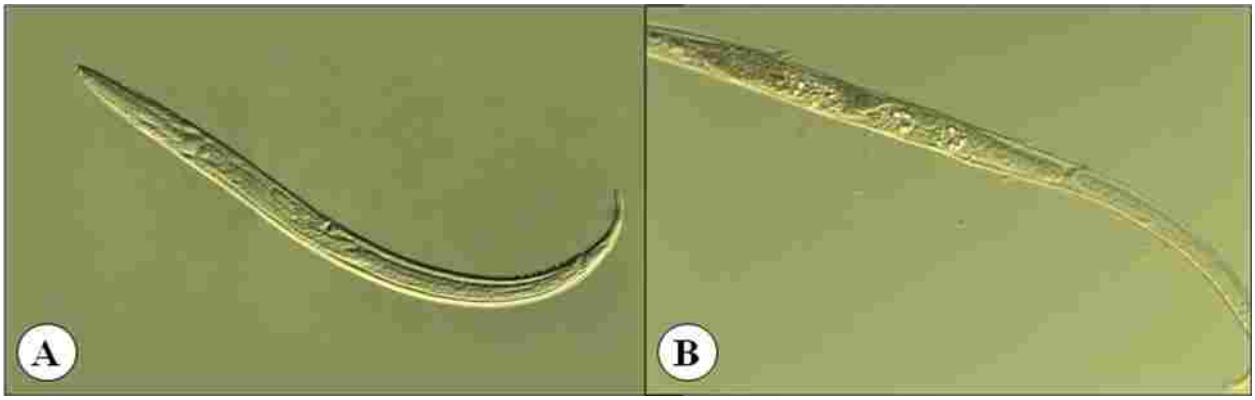

Figure 3.1

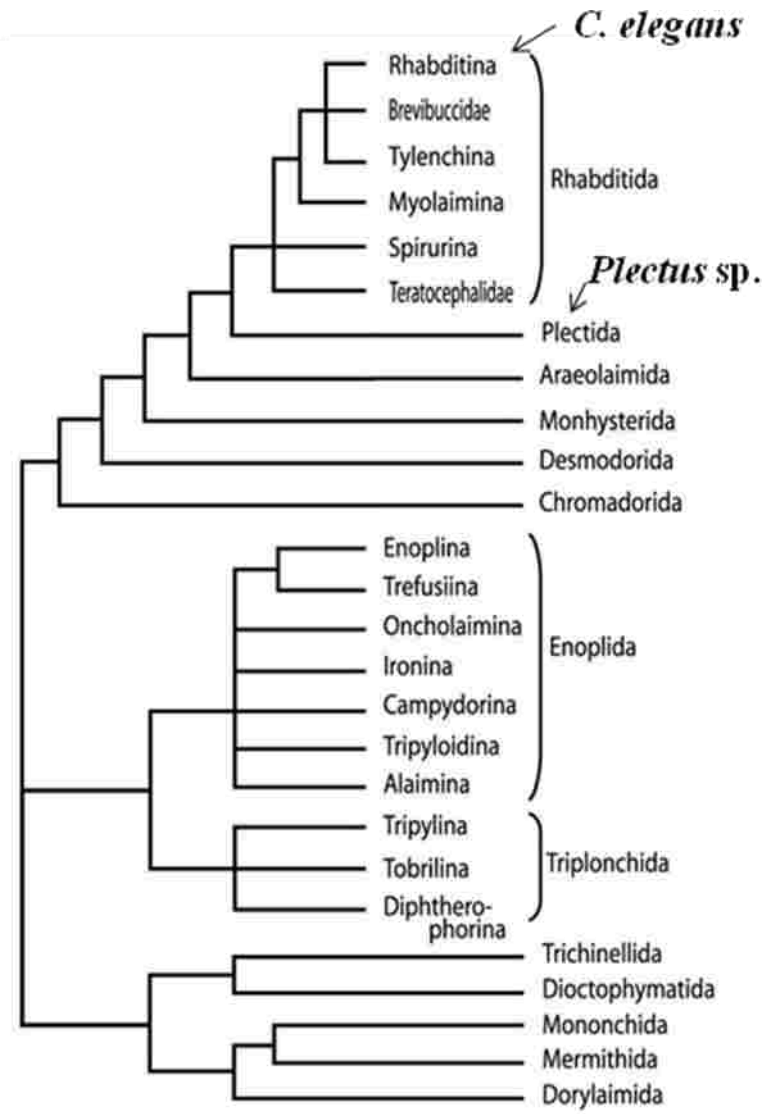
C. elegans
Rhabditina
Brevibuccidae
Tylenchina
Myolaimina
Spirurina
Teratocephalidae
Rhabditida
Plectus sp.
Plectida
Araeolaimida
Monhysterida
Desmodorida
Chromadorida
Enoplina
Trefusiina
Oncholaimina
Ironina
Campydorina
Tripyloidina
Alaimina
Enoplida
Tripylina
Tobrilina
Diphthero-
phorina
Triplonchida
Trichinellida
Dioctophymatida
Mononchida
Mermithida
Dorylaimida

Figure 3.2

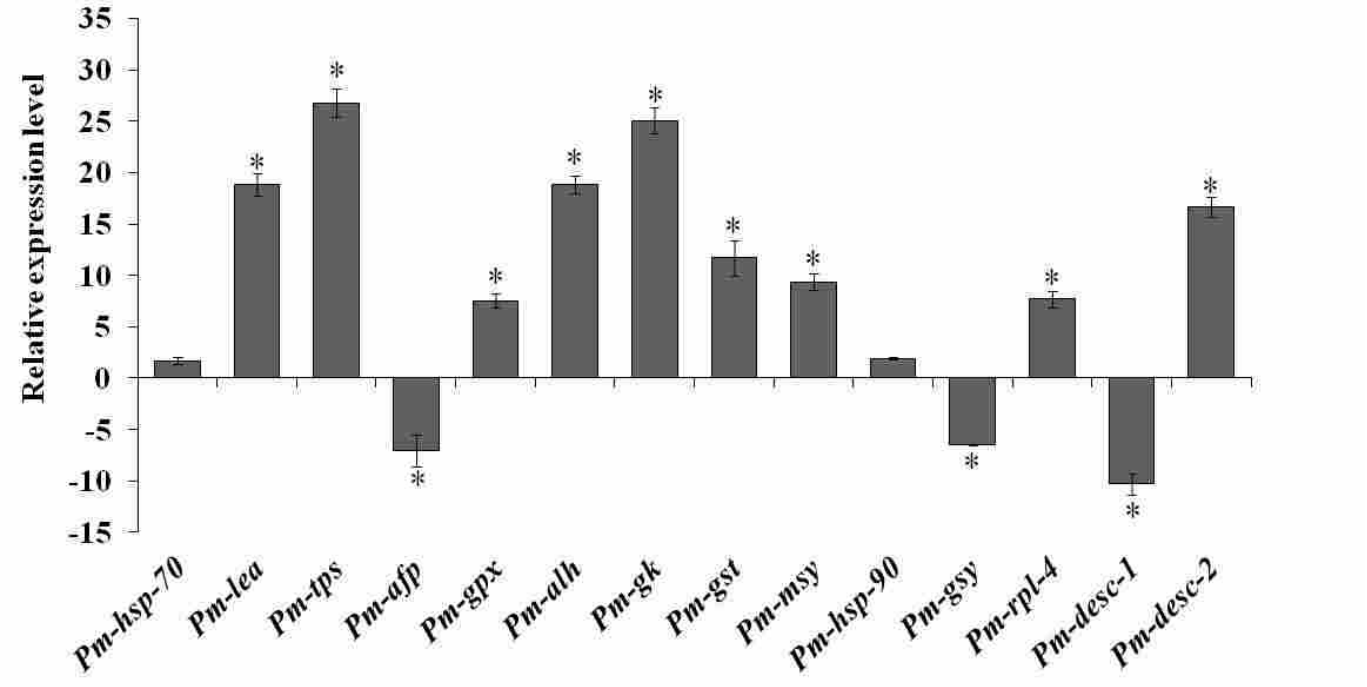
Relative expression level
35
30
25
20
15
10
5
0
-5
-10
-15
Pm-hsp-70
Pm-lea
Pm-tps
Pm-afp
Pm-gpx
Pm-alh
Pm-gk
Pm-gst
Pm-msy
Pm-hsp-90
Pm-gsy
Pm-rpl-4
Pm-desc-1
Pm-desc-2

Figure 3.3

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Culturing the Antarctic nematode *Plectus murrayi*

Bishwo N. Adhikari[1*], Cecilia M. de Tomasel[2], Grace Li[2], Diana H. Wall[2], Byron J. Adams[1]

[1]Department of Biology, and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT 84602

[2]Department of Biology and Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO 80523

*Corrresponding author: adhikaribn@hotmail.com

INTRODUCTION

In this procedure we describe a method for culturing an Antarctic terrestrial nematode, *Plectus murrayi*. *Plectus murrayi* is an excellent model organism for studying the mechanisms and responses to multiple types of environmental stress. Nematodes extracted from soil and sediment samples from the McMurdo (MCM) Dry Valleys of Antarctica are cultured on sand agar plates and incubated at various temperatures. Nematodes feed on bacteria growing on agar media. They can be sub-cultured and stored at 15°C for more than two months. This method is easy to carry out and can produce nematodes in quantities sufficient for ecological and molecular studies.

RELATED INFORMATION

Plectus murrayi, a bacteria-feeding nematode, persists in a harsh environment where it is exposed to the multiple environmental stresses. Laboratory experiments show that these nematodes can survive extreme desiccation (0% relative humidity) as well as freezing (up to -10°C) (Adhikari et al. 2010). In Antarctic conditions, *P. murrayi* appears to have a multiple year life cycle (Porazinska et al. 2002). Endemic to continental Antarctica (Andrassy 1998), they are most commonly found in moist soils and stream sediments where their distribution and abundance is correlated with the presence of organic carbon and soil moisture (Powers et al. 1998; Treonis et al. 2000). This method for culturing *P. murrayi* is adapted from Adhikari et al. (2009) and other unpublished work. For more information about *P. murrayi*, see Andrassy (1998).

MATERIALS

Reagents:

-Soil and sediment samples from Antarctica

-*E. coli* OP50 grown on Luria-Bertani (LB) broth

[Reagent Amount to add

H_2O	950 mL
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g

Combine the reagents and shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 mL). Adjust the final volume of the solution to 1 L with H_2O. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.]

-Bold's modified basal freshwater nutrient (BMBFN) media (Sigma Aldrich Inc. St. Louis, MO)

-Ottawa sand (granular silicon dioxide, SiO_2) (Sigma Aldrich Inc. St. Louis, MO)

-Sugar solution

[Dissolve 454 g of sugar in one liter of deionized water]

-Sand agar plates

[Reagent Amount to add

H_2O	965 mL
Bacto Agar	15 g
BMBFN media	20 mL

Mix Bacto agar in water, add BMBFN media and shake and heat until the agar has dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 mL) or HCl. Adjust the final volume of the solution to 1 L with H_2O. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle. When the medium is removed from the autoclave, swirl it gently to distribute the melted agar evenly throughout the solution. *Be careful!* The fluid may be superheated and may boil over when swirled. Before pouring the plates, allow the medium to cool to 50°C. Pour plates directly from the flask; allow ~30-35 mL of medium per 90-mm plate. Line up 5 poured plates (petridishes) and spread sand (about 0.2 g of sterilized Ottawa sand) evenly over the plates. To remove bubbles from the medium in the plate, flame the surface of the medium with a Bunsen burner before the agar hardens. Leave the petridishes in the laminar flow hood until the media is solid and there is no condensation on the lids. When the medium has hardened completely, invert the plates and store them at 4°C until needed.]

Equipment:

-Petri dishes

-Pasteur pipette (Fisher Scientific, Pittsburgh, PA)

-Erlenmeyer flask

-Microscope, dissecting

-Autoclave

-Scalpel

-Flame (e.g., from Bunsen burner)

-Eyelash with handle (Ted Pella, Inc, Redding, CA)

-Parafilm

-15°C room and 26°C incubator

-Mesh screen- 40, 400 and 500-mesh screen (Hogentogler & Co., Inc, Columbia, MD)

-Beaker

-Stirrer

-50 mL conical tube

-Centrifuge

-Plastic buckets

METHOD

1. Extracting *P. murrayi* from soil and sediment

i. Extract nematodes from Antarctic soil and sediment using the sugar centrifugation method described by Freckman and Virginia (1993).

The purpose of this process is to extract all the nematodes from soil and sediment samples.

ii. Pipette the extracted nematodes to sand agar plates.

If the extracted sample contains nematode species other than P. murrayi*, pick individual* P. murrayi *using an eyelash.* Plectus murrayi *can be easily distinguished from the other co-*

distributed nematodes by their curved tail. If there are no other nematodes they can be pipetted directly to agar plates. For information about morphological identification of P. murrayi *refer to Andrassy (1998).*

Alternatively, nematodes can also be extracted using a soil slurry method.

iii. Take 25 g of soil in a 100 mL beaker and add 50 mL of distilled sterile water.

iv. Stir the soil for about 30 seconds. Thus obtained soil slurry contains soil and nematodes.

The resulting soil slurry with nematodes can be directly pipetted onto the media plates using Pasteur pipette.

2. Inoculating media plates with *P. murrayi*

i. Remove the sand agar plates from storage 1-2 h before they are used.

If the plates have condensation on the lid remove the liquid by shaking the lid with a single, quick motion by holding the open plate in an inverted position.

ii. Inoculate the agar plates by pipetting the water with nematodes (10 nematodes per plate).

iii. Cover the inoculated plates, label and seal them with Parafilm.

iv. Put the plates in a plastic bag face up (lids on the top) and incubate at 26°C for 1 week.

This allows nematodes to grow and lay eggs. Developing eggs can be seen inside the parthenogenetic females at the end of week.

3. Maintenance of *P. murrayi* cultures

i. Remove the *P. murrayi* culture plates from the incubator and incubate at 15°C for 3 weeks.

ii. Before transferring *P. murrayi* cultures to fresh media plates, observe the nematode population on each plate.

For transfer, choose plates with abundant populations (40% of the plate covered) and no contamination.

iii. Transfer the *P. murrayi* cultures to fresh media plates by cutting the media into 4 segments and transferring each segment onto a new media plate.

iv. Seal the plates with Parafilm®, put them in a plastic bag face up and incubate at 26°C for 1 week.

v. Remove the *P. murrayi* culture plates from the incubator and incubate at 15°C for 3 weeks.

vi. One week after inoculation check the culture plates for contamination. Do not leave the culture plates at room temperature for longer than 2 hours.

vii. Repeat steps i-vi to maintain *P. murrayi* cultures until the nematodes are no longer needed.

DISCUSSION

The endemic bacterial-feeding nematode *P. murrayi* is one of the major nematode species found in the McMurdo Dry Valleys where multiple stresses (extreme cold temperatures, desiccated soils, higher ultraviolet radiation and salinity) are common throughout the year (Adams et al. 2006). Though *P. murrayi* is slow-growing relative to some other bacteria-feeding nematodes such as *Caenorhabditis elegans*, this method yields high numbers of individual nematodes that can be used for experimental purposes. Furthermore, this protocol can also be used to culture other bacterivorous nematodes like *P. aquatilis, Panagrolaimus* sp. and Rotifers. Although the purpose of using sand on agar plates is not fully understood, previous attempts to culture *P. murrayi* on bacteria in BMBFN media without sand were unsuccessful. As an alternative method, *P. murrayi* can also be cultured in agar plates with *Escherichia coli* OP50 bacteria like *C. elegans* (Stiernagle 2006). However, growth of *P. murrayi* is very slow and nematodes take longer to complete their life cycle. Our results show that *P. murrayi* cultured in sand agar plates take 5-8 weeks to complete their life cycle as compared to 15-20 weeks in *E.*

coli OP50 (B.N Adhikari and B. J. Adams unpublished work). We also observe lower viability of nematodes when cultured in *E. coli* OP50 as compared to sand agar plates. Our observations show that incubating culture plates at 26°C induces egg laying and when plates are transferred to 15°C eggs hatch and juveniles start growing. In general, this method enables research programs in ecological, physiological and molecular analyses of metazoan stress survival.

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CHAPTER 4

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

Bishwo N Adhikari[1*], Diana H Wall[2] and Byron J Adams[1]

[1]Department of Biology, and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT 84602, USA;

[2]Department of Biology and Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO 80523, USA

*Corresponding author: adhikaribn@hotmail.com

SUMMARY

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

INTRODUCTION

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

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

Wharton and Barclay, 1993), a dry and metabolically inactive state induced by desiccation (Crowe and Madin, 1975).

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

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

Plectus murrayi, a bacterivorous nematode, inhabits both semi-aquatic and terrestrial biotopes in the McMurdo Dry Valleys (76.5–78.5ºS latitude, 160.0–164.0ºE longitude) and a few other regions of continental Antarctica (Adams et al., 2006; Andrassy, 1998). Endemic to Antarctica, *P. murrayi* has a multiple year life cycle but the exact life span of the different developmental stages is unknown. *P. murrayi* from the McMurdo Dry Valleys is capable of surviving desiccation as well as freezing, making it an appropriate model for studying the survival mechanisms of both desiccation and freezing, including the effects of pre-exposure to less extreme conditions. Although *P. murrayi* appears to survive extreme environmental conditions, a detailed molecular assessment of the effect of acclimation on its stress survival response is still lacking. Previous efforts to unravel patterns of gene expression in stress survival

used expressed transcripts (ESTs) from desiccation acclimatized *P. murrayi* to identify genes that are differentially expressed (DE) during entry into anhydrobiosis (Adhikari et al., 2009). Expression profiling of those genes showed that anhydrobiotic survival in *P. murrayi* involves a diverse suite of functional genes. Further validation of those DE genes showed that desiccation stress involves up-regulation of a number of genes including trehalose-6-phosphate synthase, aldehyde dehydrogenase, glycerol kinase, malate synthase, heat shock proteins and a novel protein, and down-regulation of an antifreeze protein (Adhikari et al., 2009).

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

recovery from stress, allowing us to determine the role of slow desiccation and freezing on stress survival of nematodes.

MATERIALS AND METHODS

Nematode rearing

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

Effect of desiccation treatment on survival

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

To assess the survival of nematodes after exposure to different RH conditions, a 100 µl suspension containing approximately 200 nematodes was put in a 35 mm Petri dish and placed in

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

Effect of slow desiccation and freezing on survival

To assess the effect of slow desiccation on nematode survival, a 100 µl suspension containing approximately 200 nematodes was put in a 35 mm Petri dish and placed in desiccation chambers with different RH. Treatments consisted of exposure to 75% RH with (Pretreat+desiccation) or without (Desiccation) pretreatment at 98% (for 12 h) followed by 85% RH (for 12 h) for 3 and 7 days. Nematodes were rehydrated in water at 4˚C for 24 h. Viability was determined by nematode movement. Where nematodes were static a hair probe was used to stimulate movement. Nematodes not moving after this stimulus were recorded as dead. All experiments were repeated at least 3 times under identical conditions using mixed stage populations.

To assess the effect of gradual freezing on nematode survival, a 100 µl suspension containing approximately 200 nematodes was put in a 1.7 ml eppendorf tube and placed in a temperature chamber (Cincinnati Subzero Products Inc.) which can control temperature from -30˚C to 100˚C with a cooling rate of 1˚C hr^{-1}. Nematodes were either directly exposed to -10˚C (Freezing) or gradual cooling from 4˚C to -10˚C (Pretreat+freezing) at the rate of 1˚C hr^{-1} for 3

and 7 days. At least three replicates were performed for each treatment and survival was assessed as previously described.

Stress treatment and gene expression

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

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

Survival of freezing after desiccation

To determine whether prior desiccation enhances the freezing survival of nematodes, a 100 µl suspension containing approximately 200 nematodes was put in a 1.7 ml eppendorf tube and exposed to 98% followed by 85% RH at 4˚C for 12 h each. Those tubes with nematodes were placed in the controlled temperature chamber and exposed to gradual cooling from 4˚C to -

10˚C (Desiccation+freezing) at the rate of 1˚C h^{-1} and left at the final temperature for 3 (short exposure) and 7 (long exposure) days. Nematodes were allowed to recover in water at 4˚C for 24 h and mortality was assessed as previously described.

Primer design and gene selection

Genes were chosen from the list of the transcripts differentially expressed during desiccation stress (Adhikari et al., 2009). Ten different genes with functional roles in stress response, metabolism and signal transduction were selected for the study. Primers were designed from ESTs (selected by subtractive hybridization of desiccated and fresh nematodes) by using PrimerQuest^{SM} from IDT (Coralville, IA, USA) and synthesized by Operon Biotechnologies Inc (Huntsville, AL, USA) (Table 4.1).

RNA extraction and cDNA synthesis

Total RNA for Quantitative real-time PCR (qRT-PCR) was extracted using Trizol reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA) from nematodes exposed to each of the different treatments. Three replications of each stress treatment (plus three groups of controls) were used for RNA extraction, yielding three independent RNA extracts for each different treatment combination. Nematodes exposed to different treatments were directly homogenized in liquid nitrogen, mixed with Trizol Reagent, and the suspension was exposed to three freeze thaw cycles using liquid nitrogen and 37ºC water bath. The suspension was ground using mortar and pestle and vortexed. Forty ml of chloroform was added, the tubes were shaken vigorously for 15 s and then incubated further for 5 min at room temperature. After centrifugation (15 min, 12000 g, 4°C), the aqueous phase containing RNA was separated from

the other phases, which were stored for DNA preparation (see below). The colorless upper aqueous phase was transferred into fresh vials to precipitate the RNA by addition of 100 ml isopropyl alcohol. The samples were incubated for 10 min and centrifuged (20 min, 12000 g, 4°C). The RNA precipitates were then washed twice with 75% ethanol, air-dried, eluted in nuclease-free water, and quantified and quality-checked via spectrophotometer (A260/A280>1.9; NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis.

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

Quantitative real-time PCR

Quantitative real-time PCR was performed with LightCycler 480 SYBER Green I mastermix (three replicate samples for each treatment-time combination) and gene specific

primers in a Light Cycler 480 RT-PCR system (Roche Applied Science, Indianapolis, IN, USA) equipped with LightCycler 480 software with the following program: 3 min at 95°C; 45 repeats of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C followed by a standard melt curve. The real-time PCR reaction mixture contained the following items in a final volume of 10 ml: 3 µl of PCR grade water, .5 µl of PCR primers (20 pmol μl^{-1}), 5 µl of double concentrated SYBR Green Mastermix (LightCycler 480 DNA SYBR Green I Master Mix, Roche Applied Science), and 1 µl of template DNA. Negative control reactions containing water in place of cDNA were included in each batch of PCR reactions to ensure that contamination was not a problem. To minimize mRNA quantification errors, genomic DNA contamination biases, and to correct for inter-sample variation, we used 18S ribosomal RNA (*Pm-18S*) of *P. murrayi* as an internal control.

Statistical Analyses

In qRT-PCR analysis, the absolute number of specific cDNA molecules present in the samples was determined by construction of standard curves. A range of six dilutions (10^7–10^2 copies) of the cDNA was made and a gene-specific external standard curve was generated by using cDNA standards that were run simultaneously with the experimental samples. Real-time PCR analysis was performed with LightCycler 480 software, the threshold cycle was automatically calculated by the second-derivative maximum method, and the copy number of the specific mRNA in the experimental samples was calculated by extrapolation from the gene-specific standard curve. Relative change in target gene expression was calculated using equation $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) (where $\Delta\Delta Ct = (Ct,Target - Ct,18S\ rRNA)_{desiccated} - (Ct,Target - Ct,18S\ rRNA)_{control}$). The fold change in the target gene, normalized to 18S rRNA

and relative to the expression of control, was calculated for each sample. A gene with a relative abundance of one is equal to the abundance of 18S rRNA in the same sample. Analyses were conducted using PROC MIXED on SAS STAT (v.9.1, SAS Institute Inc., Cary, NC, USA). Expression levels significantly (P>0.05) higher and lower than one were considered to be induced and reduced respectively.

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

RESULTS

Changes in gene expression

We observed significant differences in the transcription of genes during various stages of desiccation. When nematodes were exposed to pretreatment, the expression of *Pm-gsy*, *Pm-ms* and *Pm-tps* was significantly up-regulated. Nematodes exposed to pretreat+desiccation showed significant up-regulation of *Pm-ms* and *Pm-tps* along with *Pm-gst-1* and *Pm-jnk-1*. Similarly, when nematodes were allowed to rehydrate in water after Pretreat+desiccation, there was

significant up-regulation of *Pm-gst-1*, *Pm-gsy*, *Pm-ms* and *Pm-tps*. The up-regulation of *Pm-gsy* and *Pm-ms* during Pretreat-rehydration was twice as much as during the pretreatment phase (Fig. 4.1). When nematodes were directly exposed to desiccation, expression level of *Pm-gst-1*, *Pm-jnk-1*, *Pm-gsy*, *Pm-ms* and *Pm-tps* was significantly up-regulated during both desiccation and rehydration. Induction of *Pm-jnk-1* and *Pm-tps* during Pretreat-desiccation was significantly higher than all other treatment stages. Similarly, expression of *Pm-gsy* and *Pm-ms* was significantly higher during Pretreat-rehydration as compared to all other treatment stages. There was no significant difference in induction of genes (except for *Pm-gsy*) between desiccation and rehydration stages. But in the case of *Pm-gsy*, rehydration induced expression was twice as much as during desiccation. There was slight up-regulation of heat shock proteins (*Pm-hsp-70* and *Pm-hsp-90*) and down-regulation of *Pm-afp* during different treatments but the change in expression level was not significant (Fig. 4.1).

We observed significant differences in the transcription of genes during various stages of freezing. When nematodes were exposed to pretreatment, *Pm-gsy*, *Pm-ms* and *Pm-tps* genes were significantly up-regulated while Pretreat-freezing caused up-regulation of all six genes except for heat shock proteins. Similarly, nematodes exposed to Pretreat-recovery significantly induced the expression of all five genes (except for *Pm-jnk-1* and heat shock proteins; Fig. 4.2). Freezing significantly induced the expression of *Pm-gst-1*, *Pm-ms*, *Pm-tps* and *Pm-afp* while recovery significantly induced the expression of *Pm-gst-1*, *Pm-gsy*, *Pm-ms*, *Pm-tps* and *Pm-afp*. The induction of *Pm-ms*, *Pm-tps* and *Pm-afp* was significantly higher during Pretreat-freezing than during freezing. The up-regulation of *Pm-gsy* during Pretreat-recovery and recovery was significantly higher than all other treatment stages. The induction of *Pm-gst-1* and *Pm-afp* during recovery was significantly higher than during recovery and Pretreat+freezing. Heat shock

proteins genes, *Pm-hsp-70* and *Pm-hsp-90* did not show significant change in expression regardless of treatment stage (Fig. 4.2).

mRNA copy number

The mRNA copy number of *Pm-hsp-90* and *Pm-hsp-70* genes was compared at different stages of desiccated and control nematodes. The mRNA copy number ($\times 10^7$) was not significantly different between genes or among different treatments (Pretreatment, Pretreat+desiccation and Pretreat+rehydration) ($P>0.05$, $N=3$) (Table 4.2).

Survival under different relative humidities

There were significant differences in nematode survivability among the different treatments. Nematodes maintained at 98% RH experienced no significant changes in survivability and 89±8.09% (Mean±s.d.) of the nematodes were still alive at the end of the 10-day experimental period. Nematodes exposed to slow desiccation to 0% RH showed gradual decline in mortality throughout the 10-day experimental period (Fig. 4.3). Exposure to 35% and 0% RH caused faster decline in survivability of nematodes as compared to other treatments. After the 10-day experimental period only 42±9.01% and 41±3.65% nematodes survive 35% and 0% RH, respectively, while nematodes exposed to slow desiccation to (0% after exposure to 75% RH) survived significantly higher (54±5.54%) than those exposed to 35% and 0% RH (Fig. 4.3).

Effect of slow desiccation and freezing on survival

To assess the effect of acclimation on desiccation and freeze tolerance, nematode mortality was analyzed after exposure to different treatments. Treatments consisted of exposure to 75% RH with (Pretreat+desiccation) or without (Desiccation) pretreatment at 98% followed

by 85% RH for 3 and 7 days and direct exposure to -10˚C (Freezing) or gradual cooling from 4˚C to -10˚C (Pretreat+ freezing) at the rate of 1˚C hr^{-1} for 3 and 7 days. Pretreatment at higher RH significantly increased the survivorship for both 3 and 7 days of exposure (Fig. 4.4). Similarly, gradual freezing to -10°C significantly increased the survivorship for both 3 and 7 days (Fig. 4.4). Nematodes exposed to fast desiccation treatment survived better than those exposed to fast freezing for both 3 and 7 day-intervals. In contrast, there was no significant difference in nematode survival within and between treatments of slow desiccation with gradual freezing for both 3 and 7 day-intervals. Nematode survival was higher for 3 days for desiccation and freezing treatments (Fig. 4.4).

Effect of desiccation on freeze survival

To determine the effect of prior desiccation on freezing tolerance 200 nematodes were placed in 1.7 ml eppendorf tubes and exposed to 98% followed by 85% RH at 4±0.2˚C for 12 h each. The tubes (with nematodes) were transferred to the controlled cooling chamber and exposed to gradual cooling from 4˚C to -10˚C (Desiccation+freezing) at the rate of 1˚C $h^{-1}$for 3 and 7 days. Nematodes exposed to desiccation and freezing had significantly higher survival than exposure to freezing or Pretreat+freezing for both 3 and 7 days. There was no difference in survivorship of nematodes for 3 and 7 days of exposure to Desiccation+freezing (Fig. 4.4).

DISCUSSION

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

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

Changes in gene expression during desiccation and freezing

Plectus murrayi showed differential expression of a suite of genes and continuous up-regulation of others upon exposure to desiccation and freezing, confirming the results of previous investigations (Adhikari et al., 2009). The free-living mycophagous nematode *Aphelenchus avenae* has been shown to accumulate large amounts of disaccharide trehalose, late embryogenesis abundant (LEA) and a novel protein named anhydrin when exposed to a

moderate reduction in RH (Goyal et al., 2005). The Antarctic nematode *Panagrolaimus davidi* which, like *P. murrayi,* can survive both desiccation and freezing, expresses an ice-active protein that shows recrystallization inhibition during freezing (Wharton et al., 2005). During exposure to dehydration, larvae of the Antarctic midge *Belgica antarctica* constitutively express heat-shock proteins (small *hsp*, *hsp90*, and *hsp70*), and the larval midges maintain a high inherent tolerance to temperature stress (Hayward et al., 2007; Rinehart et al., 2006). Based on our results, it seems reasonable to assume that *P. murrayi* modulates the transcription of genes involved in metabolism, stress survival and signal transduction under strong selection to maintain desiccation tolerance.

We followed the expression of eight different genes from various functional groups at different time points during pretreatment, exposure to and recovery from desiccation and freezing. We show a significant change in the expression of certain genes, while others were constitutively expressed. Trehalose-6-phosphate synthase (*Pm-tps*) was one of the most up-regulated genes during both desiccation and freezing with significant influence of pretreatment on the relative expression: nematodes exposed to a pretreatment had higher levels of mRNA transcripts than those without pretreatment (Fig. 4.1, 2). A characteristic feature of anhydrobiotic organisms is their synthesis of high concentrations of non-reducing sugars during the induction of anhydrobiosis (Goyal et al., 2005). This process protects membranes and proteins from desiccation damage by replacing structural water (Crowe et al., 1992), and formation of an intracellular organic glass (Crowe et al., 1998) to stabilize the cell's contents. Elevated levels of trehalose have also been reported from an Antarctic coastal bacterial feeding nematode *P. davidi* (Wharton et al., 2000) and entomopathogenic nematodes (Grewal and Jagdale, 2002) which improved freezing and desiccation survival. Since trehalose is reported to be the major

compatible solute during multiple stresses (Santos and da Costa, 2002; Yancey, 2005), increased transcription of genes encoding trehalose could be a mechanism to counter multiple stresses (like exposure to high salinity and solar radiation) in addition to desiccation and freezing.

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

Exposure to desiccation and freezing led to marked changes in the expression levels of a gene involved in signal transduction (Fig 4.1, 2). The JNK family, a subgroup of the mitogen-activated protein kinase superfamily, is part of a signal transduction cascade that regulates cellular responses to a variety of extracellular signals, including desiccation and other types of osmotic stress (Davis, 2000). *Pm-jnk-1*, along with a gene encoding a neurotransmitter gated ion-channel protein, were differentially expressed in anhydrobiotic *P. murrayi* (Adhikari et al., 2009), indicating their possible involvement in desiccation survival. Nematodes are unique among animals in utilizing the glyoxylate cycle to generate carbohydrates from the beta-oxidation of fatty acids (reviewed by Barrett and Wright, 1998). Nematodes appear to use this pathway for energy production from stored lipids during starvation or non-feeding stages

(Reversat, 1981; Wadsworth and Riddle, 1989). Relevant to our findings, the anhydrobiotic nematode *A. avenae* has been reported to use the glyoxylate cycle during induction of anhydrobiosis (Madin et al., 1985). Our results support the assumption that *P. murrayi* also uses the glyoxylate cycle, not only during induction of anhydrobiosis, but also for maintenance and recovery from stress.

Our results show that heat shock proteins (*Pm-hsp-70* and *Pm-hsp-90)* are continuously expressed during desiccation and freeze survival (Table 4.2) of *P. murrayi* and neither desiccation nor cold treatment can further up-regulate these genes. *P. murrayi* is exposed to one of the most extreme and unpredictable terrestrial environments on earth (Priscu, 1998) and to combat such extreme conditions, it might have evolved a mechanism to maintain Hsp function without disrupting normal metabolism and the growth that requires synthesis of other proteins. This phenomenon has been reported in many Antarctic organisms, including fish (Buckley et al., 2004), ciliates (La Terza et al., 2001), yeast *Candida psychrophila* (Deeganaars and Watson, 1997) and the Antarctic midge *Belgica antarctica* (Rinehart et al., 2006). Although this has been studied and observed systematically in only a few species, it may be that this is a common adaptation of Antarctic organisms.

Many cold tolerant organisms produce proteins (e.g. antifreeze protein) that assist their survival by interacting with ice in some way (Wharton et al., 2005). Antifreeze protein (AFP) have been identified in many metazoans, including Antarctic marine fish (DeVries, 1971; Duman and Olsen, 1993; Graham et al., 1997; Griffith and Yaish, 2004). An ice-active protein reported in the Antarctic nematode *P. davidi* is thought to play an important role in freezing tolerance, particularly intracellular freezing (Wharton et al., 2005). In *P. murrayi Pm-afp* was significantly

up-regulated during exposure and recovery from freezing but was slightly down-regulated during desiccation stress (Fig.4.1, 2). As AFPs can prevent the potentially injurious process of recrystallization, even at very low concentration (Knight et al., 1984), the induction of *Pm-afp* during freezing of *P. murrayi* could be a possible mechanism of preventing recrystallization of ice and ultimately protecting the nematode from further mechanical damage to its cells.

Desiccation survival

A number of nematodes can tolerate high losses of water, a trait that is especially pronounced in the Antarctic nematode *P. murrayi*. Based on the data presented here, *P. murrayi* exhibits extreme desiccation tolerance among nematodes and showed characteristics features of Anhydrobiotic nematodes (e.g. cuticle, body shape) (Crowe and Madin, 1974; Treonis et al., 2000). However, given the prevalence of soil nematodes with high cuticular permeability in the Dry Valleys, similar physiological attributes may be widespread among nematodes when assessed under appropriate humidity conditions. Nematodes not only survived exposure to 0% RH, a characteristic of anhydrobiotes, but also showed enhanced survival when exposed to gradual desiccation (Fig. 4.3). Such increase in desiccation tolerance at lower water loss is well known in fungivorous nematode *Aphelenchus avenae*, which requires a period of acclimation for successful anhydrobiosis (Goyal et al., 2005). Similarly, the Antarctic midge, *Belgica antarctica*, tolerates a significantly greater loss of body water when dehydrated at higher relative humidities (Benoit et al., 2007). If dehydration occurs at a slow rate, *P. murrayi* induces the gene transcripts coding for sugars and polyols (e.g. trehalose) as demonstrated in this study and as inferred previously (Adhikari et al., 2009). We hypothesize that increases in dehydration tolerance could

likely be due to the replacement of water with trehalose, thus preventing dehydration-induced cellular damage (Goyal et al., 2005).

Effects of slow desiccation and freezing on survival

The ability to survive prolonged periods of desiccation and freezing is an ecologically important trait for many soil invertebrates, especially those inhabiting the extreme terrestrial environment of Antarctic Dry Valleys. In our study we demonstrated that *P. murrayi* survives much better when exposed to slow desiccation and freezing (Fig. 4.4) compared to rapid dehydration and fast freezing. Slow dehydration at 98% followed by 85% RH before exposure to 75% RH conferred significantly higher survival of nematodes as compared to direct exposure to 75% RH. Similarly nematode survival was significantly higher when exposed to gradual freezing from 4°C to -10°C as compared to direct freezing at -10°C (Fig. 4.4). Previous reports have shown that pre-acclimation to a relatively mild desiccation stress can improve severe desiccation tolerance in other soil invertebrates such as nematodes, Collembola and midge larvae (Hayward et al., 2007; Sjursen et al., 2001; Chown et al., 2007; Womersley and Ching, 1989).

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

notably continuous synthesis of chaperoning proteins such as Hsps could be a mechanism in *P. murrayi*.

Desiccation increases freeze survival

A connection between enhanced cold and heat tolerance following acclimation to sub-lethal temperatures has long been established in nematodes (Chen et al., 2005; Jagdale and Grewal, 2003), but the effect of desiccation on freezing tolerance has not been well studied. In *P. murrayi*, 24 h at 98% followed by 85% RH not only resulted in enhanced desiccation survival but also an increase in survival at -10ºC, relative to the undesiccated controls (Fig. 4.4). This result concurs with Bayley et al. (Bayley et al., 2001), who found that 7 days at 98.2% RH significantly increased the cold tolerance of *F. candida*.

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

within a single lifespan, like extremely low moisture, freezing temperatures and salt accumulation (Wall Freckman and Virginia, 1998).

CONCLUSIONS

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

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LEGENDS

Table 4.1. List of gene-specific primers used in quantitative real-time RT-PCR analysis. Primers were designed by aligning differentially expressed ESTs from subtractive hybridization with putative homologous sequences from GenBank using CLUSTALX and PRIMER 3 programs.

Table 4.2. *Pm-hsp-90* and *Pm-hsp-70* expression at different stages of desiccation. The mRNA copy number ($\times 10^7$) was calculated based on 18S rRNA gene copies as described in the text. Treatments were desiccation (Pretreatment, Pretreat+desiccation, and Pretreat+rehydration) and control. Results are presented as means ± s.d.

Figure 4.1. Relative expression of *Plectus murrayi* transcripts during different stages of desiccation. Relative expression level of different genes during pretreatment (Pretreatment), exposure to desiccation with (Pretreat-exposure) and without pretreatment (Desiccation) and rehydration with (Pretreat-rehydration) and without pretreatment (Rehydration). Note the *y*-axis, where values >1 indicate up-regulation and values <1 indicate down-regulation of the transcript. Asterisks indicate values that are significantly different from 1 ($P<0.05$). Mean ± s.e.m. presented, $N=3$ in all cases.

Figure 4.2. Relative abundance of *Plectus murrayi* transcripts during different stages of freezing. Relative expression level of different genes during pretreatment (Pretreatment), exposure to freezing with (Pretreat-freezing) and without pretreatment (Freezing) and recovery with (Pretreat-recovery) and without pretreatment (Recovery). Note the *y*-axis, where values >1 indicate up-regulation and values <1 indicate down-regulation of the transcript. Asterisks indicate values that are significantly different from 1 ($P<0.05$). Mean ± s.e.m. presented, $N=3$ in all cases.

Figure 4.3. Nematode survival (%) maintained under 98%, 75%, 35%, 0% with and without pre-exposure to 98% followed by 85% relative humidity (RH) conditions for up to 10 days. Nematodes were exposed to different relative humidities and rehydrated in water at 5ºC for 24 h. Values shown are Mean ± s.e.m. for three replicates of 200 nematodes.

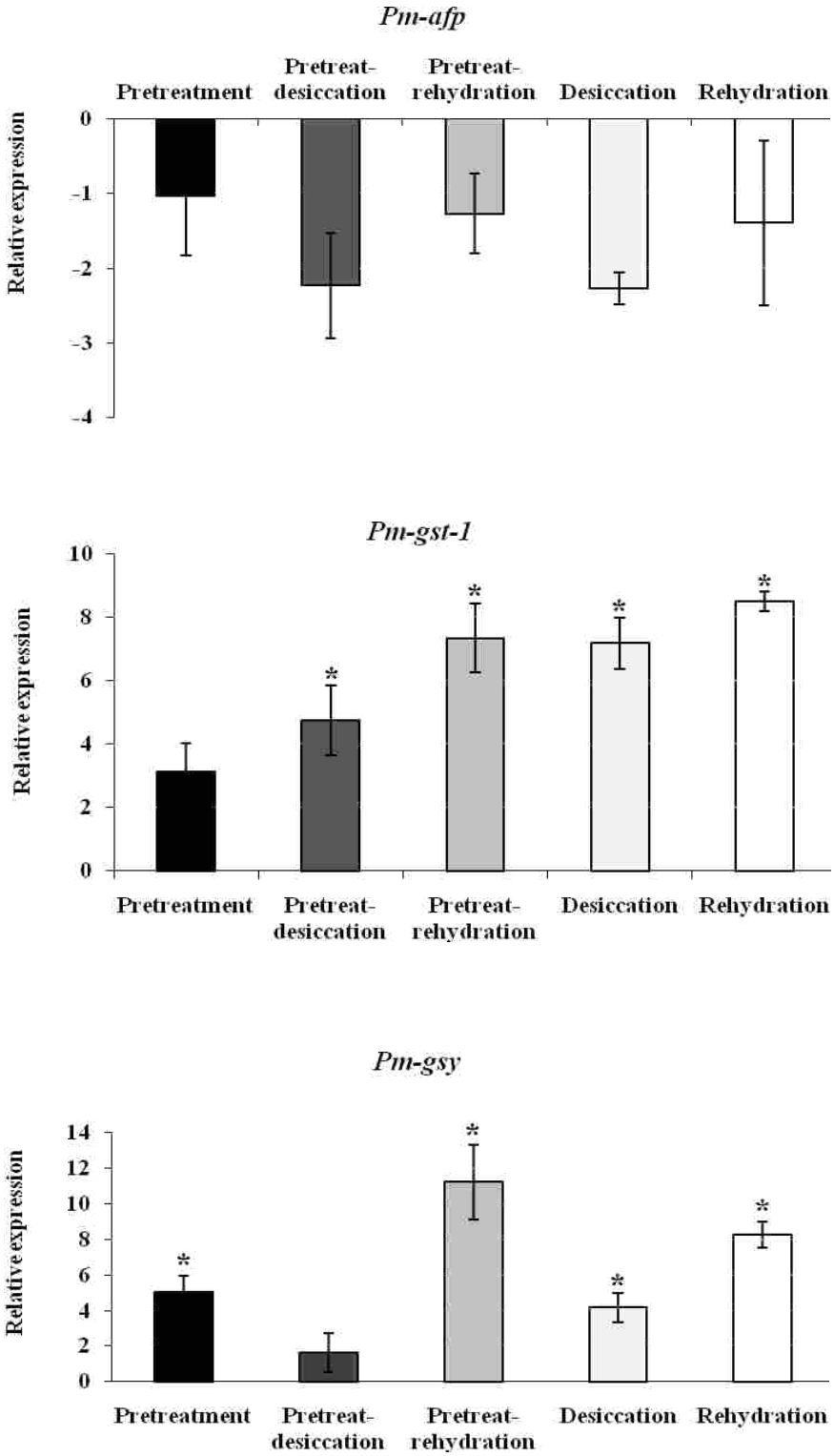
Figure 4.4. Nematode survival (%) under different combinations of stresses. Nematodes were exposed to 75% RH without acclimation to higher RH (Desiccation), 75% RH with acclimation to 98% followed by 85% for 12 h (Pretreat+desiccation), exposure to -10ºC (Freezing), gradual cooling from 4 ºC to -10 ºC (Pretreat+freezing), and gradual cooling from 4 ºC to -10 ºC with pretreatment to 98% RH followed by 85% RH for 24 h (Desic+freez) for 3 (closed bar) and 7 (open bar) days. Bars with different letters denote significant ($P<0.05$) difference in survivorship among treatments. Values are Mean ± s.e.m. based on 3 replicates of 200 nematodes.

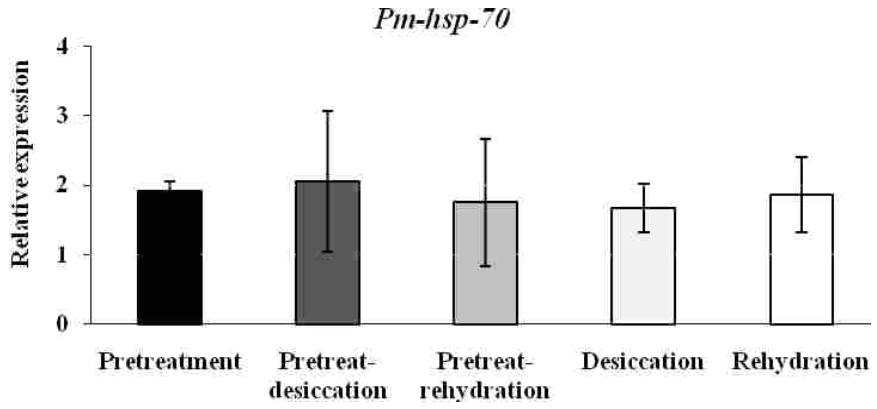
Table 4.1. List of gene-specific primers used in quantitative real-time RT-PCR analysis.

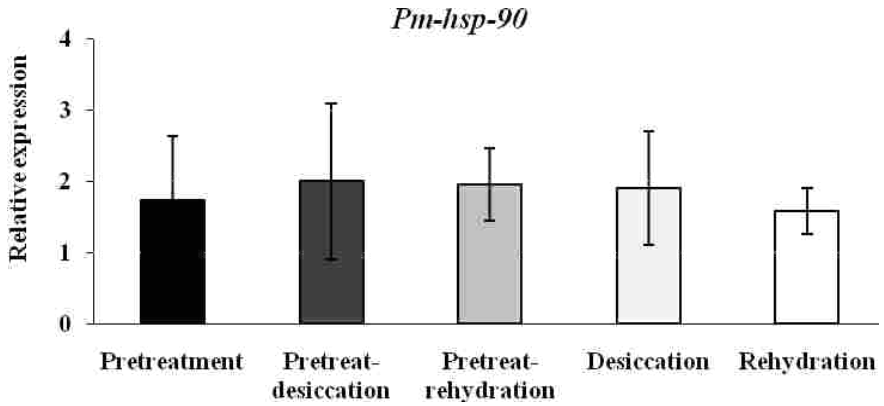
Primer Name	Gene	Primer forward 5' to 3'	Primer reverse 5' to 3'	Product length (bp)
Pm-tps	TPS	GCACGACAAGCAACGAGTTA	CATGTTCACACCAAGGTTCG	180
Pm-afp	Antifreeze protein	GAGTTGCAAGTCCAACCCAAACCA	CATTCCAAAGGGTGCCATTGTCGT	192
Pm-hsp-70	Hsp 70	AGTTGGGAGCAATCATGGCCAAAG	GCGACTTGATTCTTGGCAGCATCT	155
Pm-jnk-1	c-jun kinase	TATGCATGGAGGTCATGGCTCTGT	ACCGCTATACCGATCGCACAATCA	195
Pm-gst-1	Gst- 1	TAAGTCAGTGGGCGTGGCTAATCA	CACAATTGCGTTGTAGAGCGGCTT	205
Pm-ms	Malate synthase	CACTATCGCTCGTTCGTCAA	CCGGCATCTGTTCTAGTTCC	211
Pm-gsy	Glycogen synthase	ATGAATGGCAAGCAGGTGTTGGTC	AACGATCCGAGATGTGTAGTCGCA	188
Pm-hsp-90	Hsp 90	TGCAAACATCTGGAAACCAA	CCAAACTGGCCAATAATGCT	227

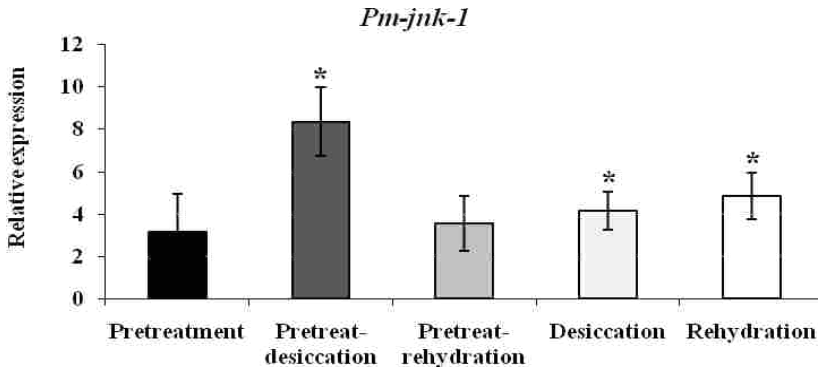
Table 4.2. *Pm-hsp-90* and *Pm-hsp-70* expression at different stages of desiccation.

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


Pm-afp
Pretreatment
Pretreat-desiccation
Pretreat-rehydration
Desiccation
Rehydration
Relative expression
0
-1
-2
-3
-4
Pm-gst-1
Relative expression
10
8
6
4
2
0
*
Pretreatment
Pretreat-desiccation
Pretreat-rehydration
Desiccation
Rehydration
Pm-gsy
Relative expression
14
12
10
8
6
4
2
0
*
Pretreatment
Pretreat-desiccation
Pretreat-rehydration
Desiccation
Rehydration


Pm-hsp-70
Relative expression
4
3
2
1
0
Pretreatment
Pretreat-desiccation
Pretreat-rehydration
Desiccation
Rehydration


Pm-hsp-90
Relative expression
4
3
2
1
0
Pretreatment
Pretreat-desiccation
Pretreat-rehydration
Desiccation
Rehydration


Pm-jnk-1
Relative expression
12
10
8
6
4
2
0
*
*
*
Pretreatment
Pretreat-desiccation
Pretreat-rehydration
Desiccation
Rehydration

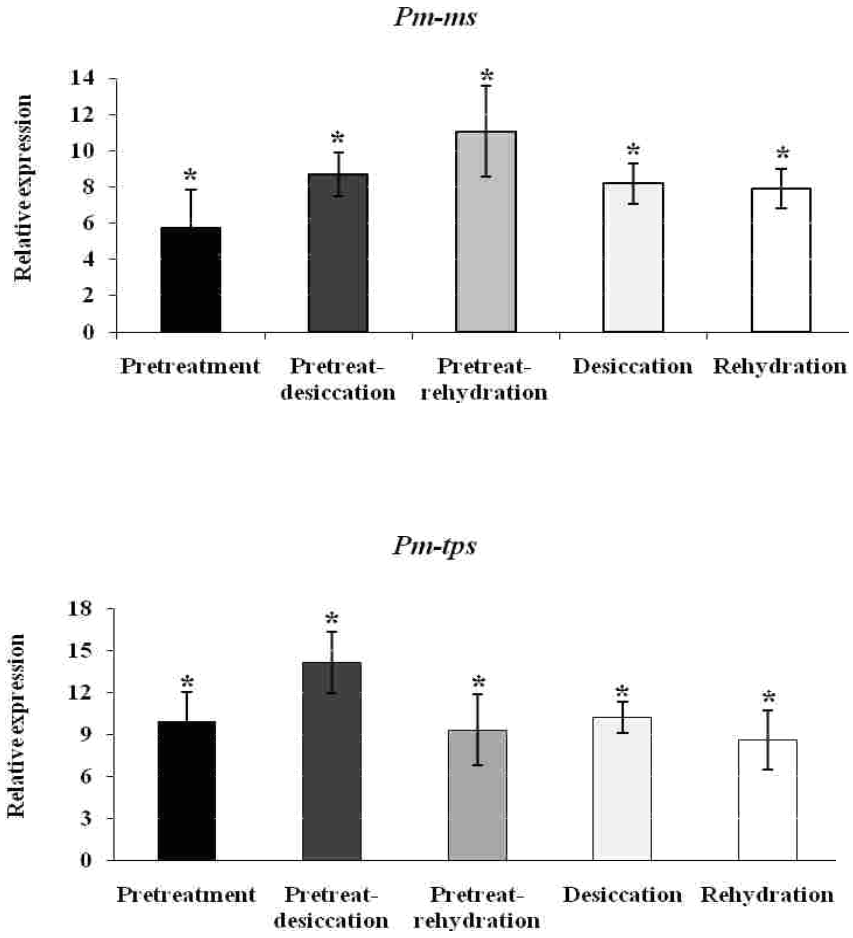
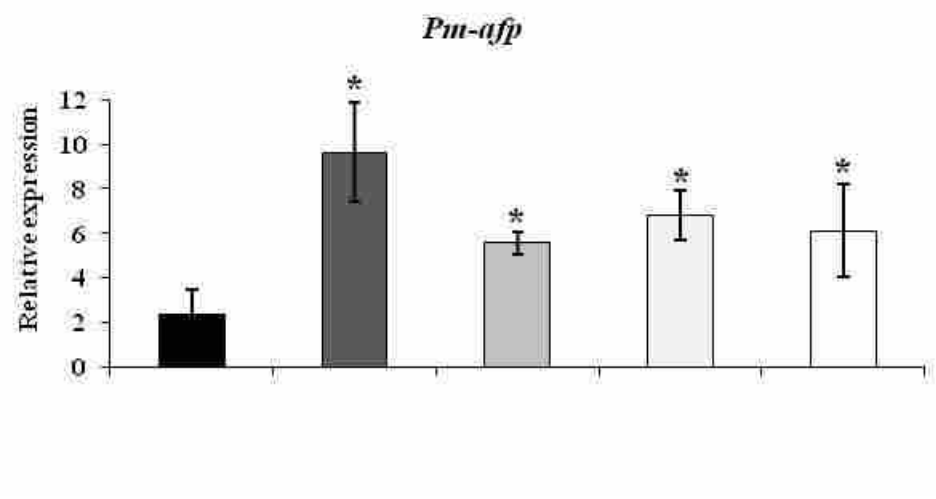
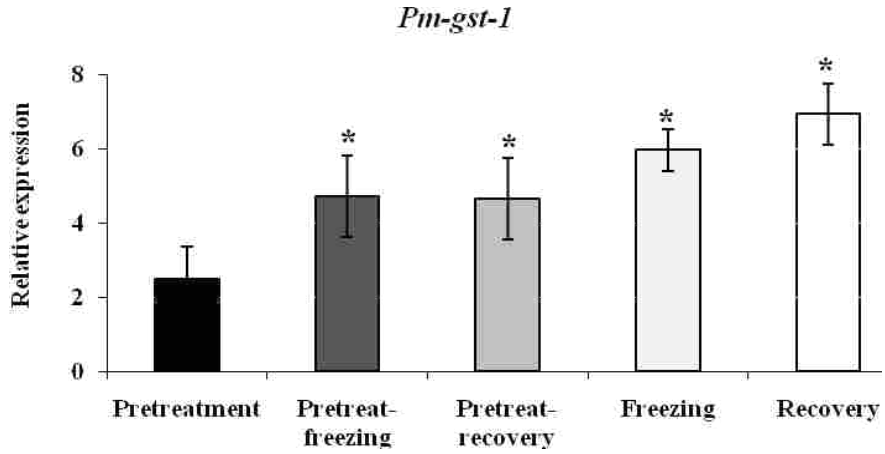
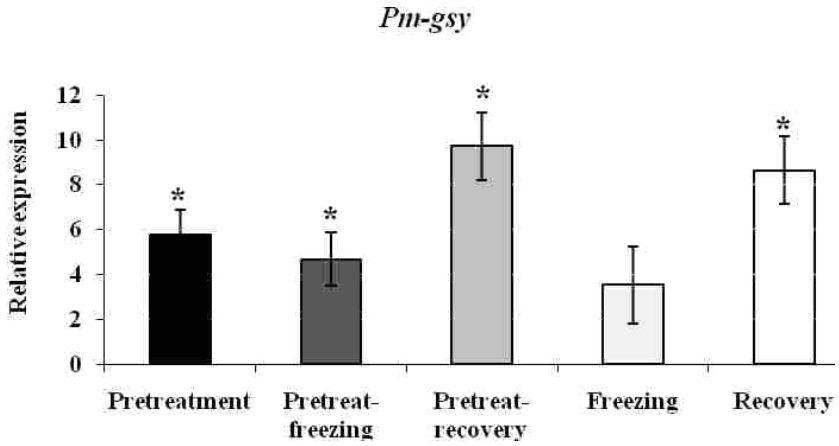
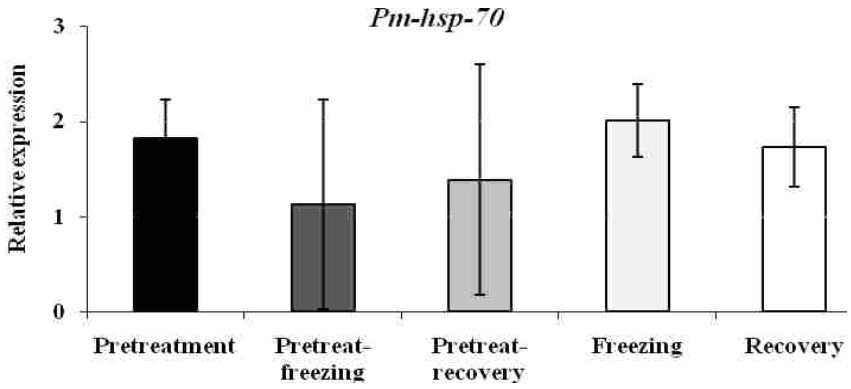

Pm-ms
Relative expression
14
12
10
8
6
4
2
0
Pretreatment
Pretreat-desiccation
Pretreat-rehydration
Desiccation
Rehydration
Pm-tps
Relative expression
18
15
12
9
6
3
0
Pretreatment
Pretreat-desiccation
Pretreat-rehydration
Desiccation
Rehydration

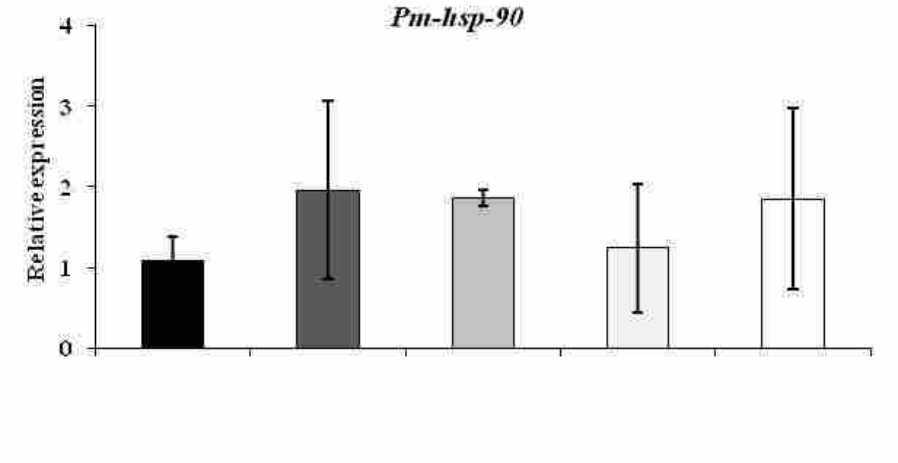
Figure 4.1

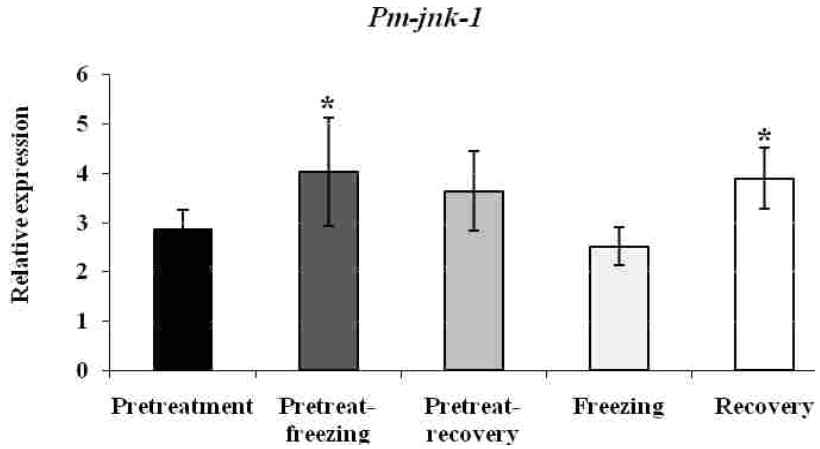

Pm-afp
Relative expression
0
2
4
6
8
10
12
*
*
*
*


Pm-gst-1
Relative expression
0
2
4
6
8
*
*
*
*
Pretreatment
Pretreat-freezing
Pretreat-recovery
Freezing
Recovery


Pm-gsy
Relative expression
0
2
4
6
8
10
12
*
*
*
*
Pretreatment
Pretreat-freezing
Pretreat-recovery
Freezing
Recovery


Pm-hsp-70
Relative expression
3
2
1
0
Pretreatment
Pretreat-freezing
Pretreat-recovery
Freezing
Recovery


Pm-hsp-90
Relative expression
4
3
2
1
0


Pm-jnk-1
Relative expression
6
5
4
3
2
1
0
*
*
Pretreatment
Pretreat-freezing
Pretreat-recovery
Freezing
Recovery

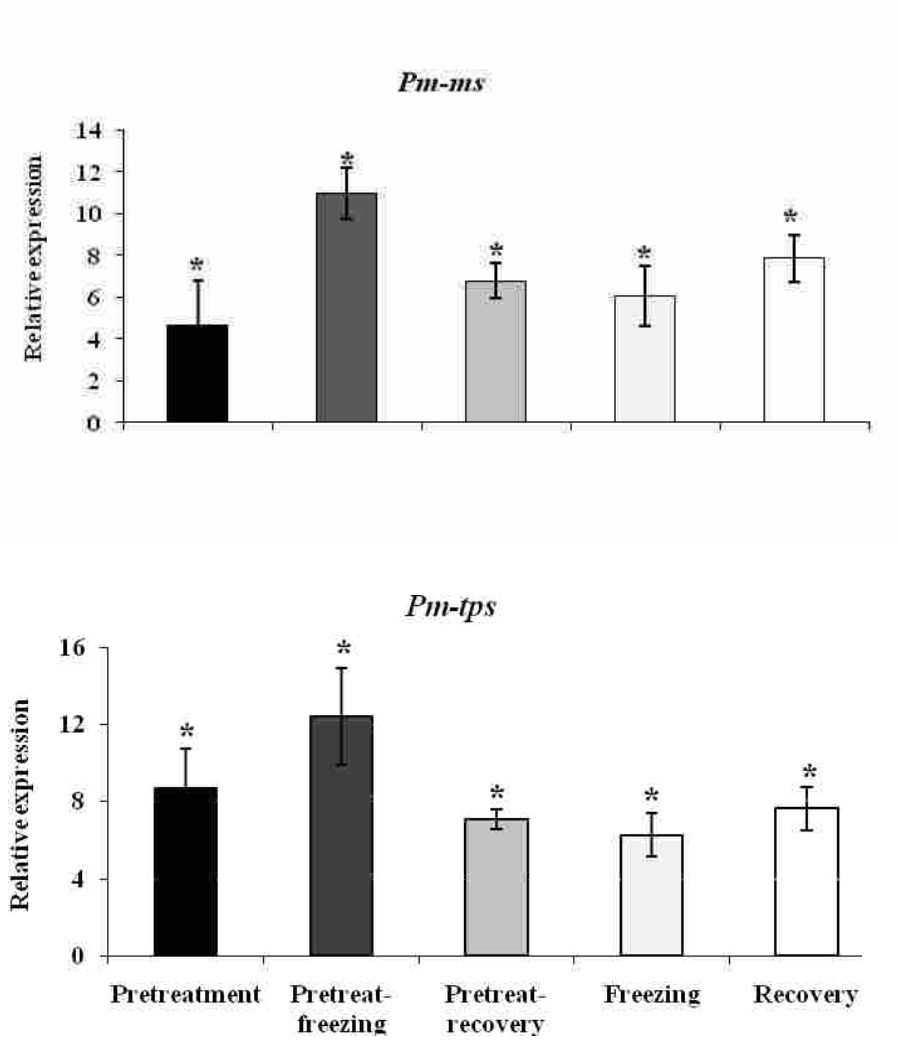

Pm-ms
Relative expression
14
12
10
8
6
4
2
0
Pm-tps
Relative expression
16
12
8
4
0
Pretreatment
Pretreat-freezing
Pretreat-recovery
Freezing
Recovery

Figure 4.2

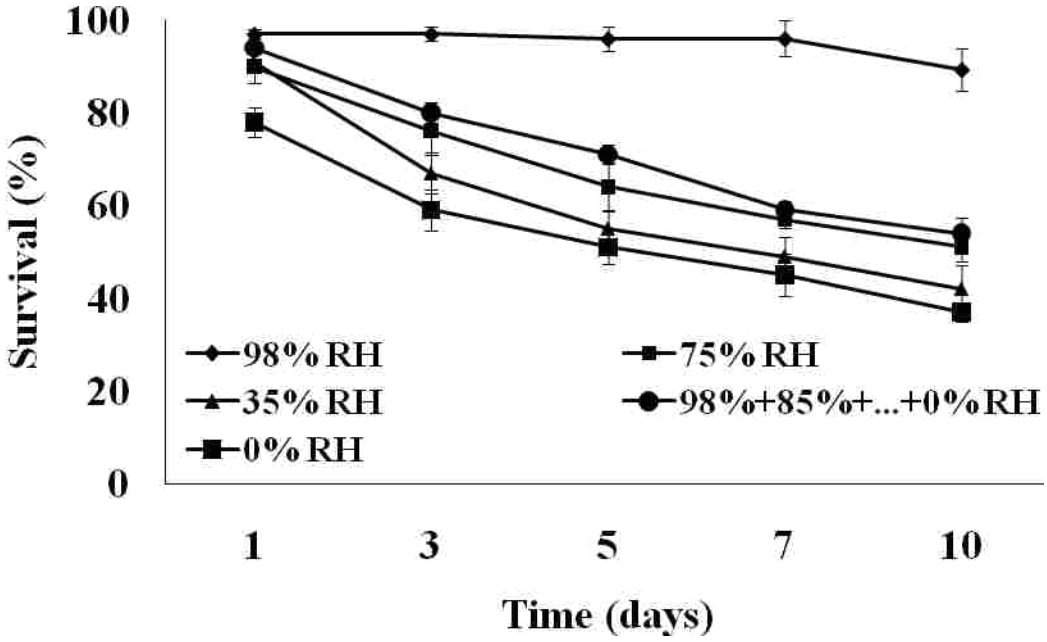

100
80
60
40
20
0
Survival (%)
98% RH
75% RH
35% RH
98%+85%+...+0% RH
0% RH
1
3
5
7
10
Time (days)

Figure 4.3

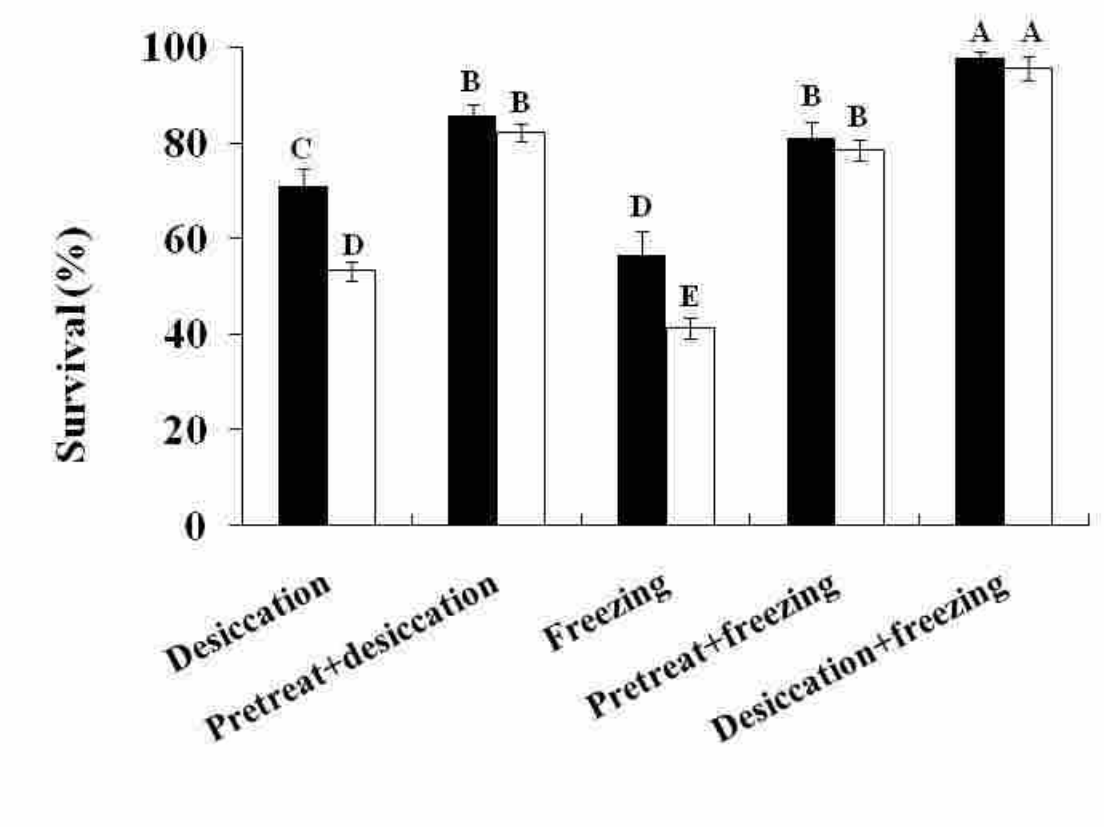

Survival (%)
100
80
60
40
20
0
C
D
B
B
D
E
B
B
A
A
Desiccation
Pretreat+desiccation
Freezing
Pretreat+freezing
Desiccation+freezing

Figure 4.4

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CHAPTER 5

Transcriptional profiling of trait deterioration in the insect pathogenic nematode *Heterorhabditis bacteriophora*

Bishwo N Adhikari[1*], Chin-Yo Lin[2], Xiaodong Bai[3], Todd A Ciche[4], Parwinder S Grewal[3], Adler R Dillman[2,5], John M Chaston[2,6], David I Shapiro-Ilan[7], Anwar L Bilgrami[8], Randy Gaugler[8], Paul W Sternberg[5], Byron J Adams[1]

[1]Department of Biology and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT 84602, USA

[2]Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT 84602, USA

[3]Department of Entomology, The Ohio State University-OARDC, Wooster, OH 44691, USA

[4]Department of Microbiology and Molecular Genetics, Michigan State University, 2215 Biomedical Physical Sciences Building, East Lansing, MI 48824, USA

[5]Division of Biology, California Institute of Technology, 1200 E. California Blvd., Pasadena, CA 91125, USA

[6]Department of Bacteriology, University of Wisconsin, Madison, WI 53076, USA

[7]USDA-ARS, Southeastern Fruit and Nut Research Laboratory, 21 Dunbar Road, Byron, GA 31008, USA

[8]Department of Entomology, Rutgers University, New Brunswick, NJ 08901-8524, USA

*Corresponding author: adhikaribn@hotmail.com

ABSTRACT

Background

The success of a biological control agent depends on key traits, particularly reproductive potential, environmental tolerance, and ability to be cultured. These traits can deteriorate rapidly when the biological control agent is reared in culture. Trait deterioration under laboratory conditions has been widely documented in the entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* (*Hb*) but the specific mechanisms behind these genetic processes remain unclear. This research investigates the molecular mechanisms of trait deterioration of two experimental lines of *Hb*, an inbred line (L5M) and its original parental line (OHB). We generated transcriptional profiles of two experimental lines of *Hb*, identified the differentially expressed genes (DEGs) and validated their differential expression in the deteriorated line.

Results

An expression profiling study was performed between experimental lines L5M and OHB of *Hb* with probes for 15,220 ESTs from the *Hb* transcriptome. Microarray analysis showed 1,185 DEGs comprising of 469 down- and 716 up-regulated genes in trait deteriorated nematodes. Comparative analysis of these DEGs showed that 59% matched *Caenorhabditis elegans* and *C. briggsae* proteins while 19% did not match with genes in existing databases that were designated as parasitic nematode specific. Analysis of the DEGs showed that trait deterioration involves massive changes of the transcripts encoding metabolic and signal transduction enzymes. We observed a pattern of reduced expression of enzymes related to primary metabolic processes and induced secondary metabolism. Expression of ten DEGs in trait deteriorated nematodes was validated by quantitative reverse transcription-PCR (qRT-PCR) which revealed similar expression kinetics for all the genes tested as shown by microarray.

Conclusion

As the most closely related major entomopathogen to *C. elegans*, *Hb* provides an attractive near-term application for using a model organism to better understand interspecies interactions and to enhance our understanding of the mechanisms underlying trait deterioration in biological control agents. This information could also be used to improve the beneficial traits of

biological control agents and better understand fundamental aspects of nematode parasitism and mutualism.

BACKGROUND

Biological control using predators, parasitoids, or pathogens, can be an effective alternative for management of arthropod pests [1, 2]. In contrast to chemical insecticides, biological control agents are generally not harmful to humans or the environment, and have minimal or negligible potential to cause resistance or harm to non-target organisms. The success of a biological control agent depends on key traits, particularly compatibility with the target pest, reproductive potential, host-finding ability, environmental tolerance, and ability to be cultured. These traits, however, can deteriorate rapidly, and substantially when a biological control agent is isolated from nature and reared in the laboratory, or mass-produced for commercial purposes [3-5]. Genetic and non-genetic processes may be responsible for trait deterioration in laboratory-cultured biological control agents. Loss of genetic variation due to inadvertent selection [6, 7], exposure of deleterious recessive genes, increased homozygosity because of inbreeding [8], and disproportionate representation of genotypes in successive generations due to genetic drift [8] during sub-culturing can impair the effectiveness of biological control agents. Trait deterioration may also result from non-genetic factors such as poor nutrition and disease [9].

Entomopathogenic nematodes (EPNs) in the families Heterorhabditidae (Strongyloidea) and Steinernematidae (Strongyloidoidea sensu [10]) are biological control agents that serve as exceptional models for the study of parasitism, pathogenicity, and symbiosis [11-13]. These nematodes form mutualistic symbioses with insect pathogenic bacteria in the family Enterobacteriaceae: heterorhabditids are associated with *Photorhabdus* and steinernematids with *Xenorhabdus*, respectively [14]. The infective juveniles (IJs) or dauer (enduring) juveniles

persist in soil in search of a suitable insect host [15]. Following entry through the cuticle or natural body openings, the IJs release the symbiotic bacteria into the insect hemocoel, which rapidly kill the host, usually within 24-48 h [16]. Nematodes feed on symbiotic bacteria and digested host tissues, complete 1-3 generations in the host cadaver, and as food resources are depleted new IJs are produced which disperse in search of new hosts. In the laboratory, each partner can be cultured separately, but when combined they present a high degree of specificity [14]. These EPNs are cultured for experimental or commercial purpose using in vivo or in vitro methods [17].

Deterioration of traits essential for biological control has been recognized in diverse biological control agents [9, 18-20] including EPNs [21, 22]. Trait deterioration under laboratory conditions has been widely documented in various biological control agents including predators, parasitoids and pathogens [8]. Similarly, microbial control agents such as viruses (e.g. baculoviruses), bacteria (e.g. *Bacillus thuringiensis*) and fungi (e.g. *Beauveria bassiana*) have been reported to lose virulence when sub-cultured in the laboratory [18, 23, 24]. Previous research has shown that traits can deteriorate rapidly in EPNs [21, 22, 25] and in their symbiotic bacteria [26]. Shapiro et al. [27] reported a reduction in heat tolerance of *Heterorhabditis bacteriophora* (*Hb*) under laboratory conditions. Similarly, Wang and Grewal [22] reported rapid deterioration in environmental tolerance and fecundity for *Hb* during laboratory maintenance. Bilgrami et al. [21] showed that genetic factors play a significant role in the deterioration process; however, the specific mechanisms behind these genetic processes remain unclear. Additionally, physiological or biochemical effects such as nutritional factors may also contribute to trait deterioration. Therefore, establishing stability in beneficial traits requires an understanding of the mechanisms involved in trait deterioration, specifically, the molecular

genetic processes. This research investigates the molecular mechanisms of trait deterioration of two experimental lines of an EPN, an inbred line (L5M) (created by sub-culturing different experimental lines of the nematode-bacterium complex over 20 passages in insect hosts) and its original parental line (OHB). These lines differed in their virulence, heat tolerance and fecundity [21]. We generated transcriptional profiles of the two experimental lines of EPN, then identified and validated the genes that were differentially expressed (DE) in the deteriorated line.

RESULTS

To identify genes associated with trait deterioration in the entomopathogenic nematode *Hb*, an expression profiling study was performed using custom Roche NimbleGen expression arrays with probes for 15,220 ESTs from the *Hb* transcriptome. To identify the genes involved in trait deterioration, expression was analyzed between two experimental lines of *Hb*; L5M and OHB. Four biological replicates of each line were used in hybridization experiments, allowing us to identify putative genes involved in the deterioration of important traits in *Hb*.

Microarray analysis

Microarray analysis showed 1,185 genes differentially expressed between L5M and OHB. Of those differentially expressed genes (DEGs) 469 were down-regulated and 716 were up-regulated at $P < 0.05$ (Fig. 5.1). The fold-change in gene expression was from 0.92 to 0.38 (down-regulated) and from 1.07 to 2.45 fold (up-regulated) while considering one (1.0) as the baseline expression level. Differential expression of 253 genes was significant at $P < 0.01$, and 20 genes were significant at $P < 0.001$. A small portion of DEGs (3.29%) have an above 1.5-fold change in expression while the majority (57.13%) has below 1.5-fold change. Our analysis suggests that modest expression changes involving a large number of genes are associated with trait deterioration.

Putative functional identification of differentially expressed ESTs

In order to assess the putative identities, all differentially expressed ESTs (1,185) were subjected to BLASTx sequence similarity searches against GenBank's nr database and WormBase [28] database (WS200) consisting of extensively curated *Caenorhabditis elegans* proteins. Of the 1,185 DEGs, 89% (1,063) had significant matches (E value cutoff 1e-5) to proteins in GenBank's nr database; most of the best matches (95%) were to nematode proteins (Fig. 5.1). A small portion (less than 1%) of the best matches was to prokaryotic proteins. The remaining 4% of the best matches were to other eukaryotes, including humans, insects, and plants. The remaining 122 DEGs had no match with any sequences in the GenBank nr database. The similarity search against the *C. elegans*-specific database WS200 showed 58% (n = 698) of the DEGs had significant matches (E value cutoff 1e-5) to *C. elegans* proteins. In order to identify parasitic nematode-specific DEGs during trait deterioration, a comparison of ESTs to other nematode EST sequences from GenBank was performed. Of the 1,185 DEGs, 7% (n = 82) matched those of animal and human parasitic nematodes (AHPNs) while less than 1% (n = 10) of the ESTs matched other parasitic nematode ESTs. Of the total ESTs, 231 matched parasitic nematode-specific ESTs but did not match AHPNs or other parasitic nematode ESTs which are designated as parasitic nematode-specific (PNS) or *Hb*-specific ESTs (Fig. 5.2). We identified 114 genes that exhibited *C. elegans* RNAi phenotypes (selected phenotypes are listed in Additional file 5.1).

As an important starting point in the prediction of molecules that are secreted or excreted in or during host-parasite interaction, we identified 101 putatively secreted proteins representing a non-redundant catalogue of *Hb* molecules (Additional file 5.2). Examples of such proteins are cysteine proteinase, aspartyl protease, diadenosine tetraphosphatase, Hsp70-interacting protein

and calumenin (calcium-binding protein). In the present data set (= 1,185 DEGs), we identified 101 (9%) putatively secreted proteins with homologies to diverse organisms. Of these, 14 (14%) sequences had no significant similarity to any sequence available in current databases, whereas 87 (86%) had homologues in nematodes and other organisms, with 60 (59%) *C. elegans* and/or *C. briggsae* matches, 11 (11%) AHPNs like *Brugia malayi*, *Ostertagia ostertagi* and *Ancylostoma ceylanicum*, 11 (11%) from eukaryotes other than nematodes (fungi, plants, insects and animals), and 3 (3%) from prokaryotes (like *Burkholderia mallei* and *Neisseria lactamica),* and 2 (2%) other eukaryotes (parasites and vector agents).

Annotation and gene ontology analysis of differentially expressed ESTs

ESTs of DEGs were annotated into different functional groups using Gene Ontology (GO) and mapped to different pathways using the Kyoto encyclopedia of genes and genomes (KEGG) [29]. Gene Ontology [30] has been used widely to predict gene function and classification. GO provides a dynamic vocabulary and hierarchy that unifies descriptions of biological, cellular and molecular functions across genomes. We used Blast2GO [31], a sequence-based tool to assign GO terms, extracting them for each BLAST hit obtained by mapping to extant annotation associations. We found that of the 1,185 DEGs, 28% (n = 334) could be functionally assigned to biological processes (n = 548), cellular components (n = 417) and molecular functions (n = 537) with total of 1,141 GO terms (Fig. 3). Amongst the most common GO categories representing biological processes were: metabolic process (n = 315), developmental process (n = 288), multicellular organismal process (n = 271), cellular process (n = 295) and growth (n = 184). Under cellular components, the higher GO term was for cell (n = 409), cell part (n = 359), organelle (n = 263) and macromolecular complex (n = 168). The largest GO terms in molecular functions were for binding (n = 410) followed by catalytic activity (n =

309), transporter activity (n = 98), structural molecule activity (n = 37) and transcription regulator activity (n = 20) (Fig. 5.3).

Biochemical functionality was predicted by mapping all 1,185 differentially expressed ESTs to pathways, using Blast2GO [31], with an E-value cut-off of 1e-5. Enzyme commission (EC) numbers were used to appraise which sequences pertained to a specific pathway. A total of 19% (n = 224) of the sequences were mapped to 150 KEGG pathways, with 61% (138) of the sequences representing metabolic enzymes characterized by unique EC numbers (Additional file 5.3). The metabolism group was dominated by 'energy' followed by 'carbohydrate' and 'amino acid' metabolism. The complete listing of metabolic enzymes is shown in Additional file 5.4. Metabolic molecules involved in neurodegenerative disease (n=21) and signal transduction mechanisms (n=14) (complete list in Table 5.1) had high representation amongst the sequences mapped to KEGG pathways. Enzymes involved in cellular processes and cell communication were least represented in KEGG pathways (Additional file 3). The most represented enzymes were cytochrome c oxidase (n = 43) followed by H+-transporting two sector ATPase (n = 11), H+-exporting ATPase (n = 10), protein disulfide-isomerase (n = 10) and protein-glutamine gamma-glutamyltransferase (n = 10).

Validation of differential expression with quantitative reverse transcription-PCR

We selected ten genes for validation of the microarray data by quantitative reverse transcription-PCR (qRT-PCR) using gene-specific primers (Additional file 5.5). Four biological replicates of each line were used to determine the effect on metabolism, stress, life span and dauer development-associated candidate gene expression. The values indicated in the bar diagram in figure 4A represent the fold change in the target gene, normalized to 18S ribosomal RNA (*Hb-18S*) and relative to the expression of the control (Fig. 5.4A). A gene with a relative

abundance of one is equal to the abundance of 18S rRNA in the same sample in qRT-PCR analysis. The qRT-PCR analyses confirmed the differential expression of the candidate genes as indicated by microarray analysis. The fold change in gene expression (L5M vs OHB) obtained by using microarray experiments compared to the fold change obtained by using qRT-PCR gives a correlation coefficient (R^2) of 0.88 (Fig. 5.4B). The correlation coefficient obtained in our analysis is very good considering that microarray data are semi-quantitative and subject to error for multigene families where different transcripts could hybridize to similar probes on the array. We obtained significantly higher levels of expression of 6 candidate genes by qRT-PCR as compared to microarray analysis (Fig. 5.4A). Among 10 candidate genes (6 up-regulated and 4 down-regulated), *Hb-co-II* (GenBank: EX007863) showed highest up-regulation by microarray followed by *Hb-rab-33* (GenBank: ES411895). Similar results for the other genes are also shown by qRT-PCR, but change in expression level was significantly higher than indicated by microarray. Among four down regulated genes, qRT-PCR analysis showed significantly higher reduction of *Hb-unc-68* and *Hb-fat-2* as compared to microarray analysis (Fig. 5.4A).

DISCUSSION

Deterioration of traits essential for biological control has been recognized in diverse biological control agents including the insect pathogenic nematode *Hb* [21, 22, 27]. These traits can deteriorate rapidly and substantially when bio-control agents are isolated from nature and reared in the laboratory, or mass-produced for commercial purposes. Genetic and non-genetic processes may be responsible for trait deterioration in laboratory-cultured bio-control agents and their symbionts. However, the specific mechanisms behind these genetic processes remain unclear. To identify genes associated with trait deterioration in the entomopathogenic nematode *Hb*, we undertook an expression profiling study using custom Roche NimbleGen expression

arrays that screened over 15,220 transcripts. To identify the DEGs, expression was compared between two experimental lines of *Hb*; L5M and OHB. Our results showed that trait deterioration of *Hb* induces substantial overall changes in the nematode transcriptome. We observed a few general patterns suggesting that trait deterioration via inbreeding depression, taking place over a short period of time (under 20 generations), can result in massive changes in metabolic processes, cellular transportation and gene translation. In addition, the massive reprogramming of primary and secondary metabolic processes as part of trait-deterioration involved changes in signalling and other regulatory processes. The present study represents the first transcriptional analysis of degradation of beneficial traits in EPNs and highlights several key components of trait deterioration that may be common among biological control agents.

Experimental design and analysis

The advent of microarrays has enabled the screening of thousands of genes in parallel to assist in candidate gene identification. Ideally, one would like to scan the entire genome of a nematode to obtain a more complete picture of transcriptional changes in response to various treatments. However, whole genome sequences are not available for most nematodes, leading to a dependence on collections of ESTs assembled from random cDNA libraries. For *Hb*, we used a set of over 15,220 ESTs to construct a cDNA microarray. The main source of ESTs for this array was derived from *Hb* TT01 that interact symbiotically with *Photorhabdus luminescens* TT01 bacterium. The original parental line (OHB) and the inbred line (L5M) used in our experiments differed in pathogenicity, stress tolerance and fecundity.

The microarray experiments were conducted in a reference design, where tissue samples from the original parental line, OHB, acted as reference against the inbred line, L5M. The results show 1,185 genes were DE, encompassing diverse functions. We validated our microarray

observations by qRT-PCR for several genes that were chosen based on their biological interest as well as spectrum of significance in fold change expression. In general, the results revealed the evolution of altered transcript levels concomitant with trait deterioration, including major changes in the metabolism category, especially in energy, carbohydrate and lipid metabolism.

It is common practice to use an arbitrary transcription differential cut-off (such as two-fold) in order to identify changes that may be biologically significant, but our results showed that the majority of DEGs exhibit a small fold-change in expression level ranging from 0.38 to 2.45. In a large scale microarray experiment that analyzed the response of soybean plants to a pathogen, Zhou et al. [32] reported that the majority of statistically significant transcriptional differences are less than two-fold in magnitude. The low amplitude modulation of gene expression (less than two-fold changes) is suggestive of low-magnitude remodelling of the transcriptome, which may be an integral component of an organism's adaptive response to selection on physiological traits. It is possible that the overall evolutionary response of the nematode, even over very small time scales (a few generations) requires coordinated changes among a wide array of genes, and those changes in turn may require reinforcing changes in an even wider array of functionally connected genetic components. We speculate that numerous genes that function in *Hb* are co-ordinately modulated to support the many physiological changes manifested in the evolution of trait deterioration.

Comparative analysis of differentially expressed genes

We obtained 1,185 genes that were differentially expressed in a deteriorated line of *Hb*. Comparative analysis of these DEGs with those available in various public databases showed that 59% (n = 698) matched *C. elegans* and *C. briggsae* proteins, and 26% (n = 313) matched parasitic nematodes. When these 313 DEGs were compared with a subset of parasitic

nematodes, 7% (n = 82) matched animal and human parasitic nematode (AHPN) proteins, suggesting that these genes may participate in parasitism-related activities. Of the remaining DEGs, 19% (n = 231) did not match AHPN sequences that we designated as parasitic nematode specific. A small portion of DEGs 10% (n = 122) did not appear to match any available sequences, indicative of novel *Hb* genes. These findings suggest the potential of discovering new genes and gene functions, genetic networks, and metabolic pathways specific to *Hb* and other EPNs. Similarly, the identification of putatively secreted proteins and expression profiling of the DEGs shared between *Hb* and other parasitic nematodes could be a valuable resource for conducting in-depth research on gene functions that will ultimately elucidate parasitic nematode-specific biological processes.

We found several *Hb* DEGs that are associated with RNA interference (RNAi) phenotypes of *C. elegans*. Our analysis shows differential expression of genes like *egl-8*, *unc-60*, *daf-8*, *daf-21*, *eat-6* (complete list in Additional file 5.1), each of which exhibit RNAi phenotypes in *C. elegans*. These genes may prove useful candidates in the ongoing RNAi endeavors for functional genomics studies of EPNs. Interestingly, we found 9 DEGs that matched proteins from various prokaryotic organisms. These transcripts encode ATP synthase (GenBank: FF679373) and a DNA-J class molecular chaperone (GenBank: ES409751). The presence of these transcripts could be the result of horizontal gene transfer (HGT) from bacteria encountered by *Hb* during its life cycle. The presence of sequences of putative prokaryotic origin has already been reported in *Hb* [33] as well as in plant parasitic nematodes [34]. Given the similarity of these sequences to prokaryotic sequences, and presence of poly(A) RNA in the transcript, the possibility that these sequences are bacterial contaminants is low. Our findings of *Hb* DEGs with similarity to prokaryotic sequences identified here do not imply that all these

genes have been acquired by HGT, as the null hypothesis remains convergent evolution. However, their presence serves as a first step in identifying a pool of candidates from which parasitism and mutualism-related genes can be explored in the future.

Functional analysis of differentially expressed genes

Gene Ontology (GO) [30] has been used widely to predict gene function and classification. GO provides a dynamic vocabulary and hierarchy that unifies descriptions of biological, cellular and molecular functions across genomes. We used Blast2GO [31], a sequence-based tool, to assign GO terms, extracting them for each BLAST hit obtained by mapping to extant annotation associations. Though GO analysis showed only one third of the DEGs can be assigned to different functional categories, we observed a clear pattern of changes exhibited by deteriorated nematodes. High numbers of DEGs were assigned to biological processes, including metabolic, developmental, cellular processes and cellular stress. We observed a pattern of changes in primary as well as secondary metabolic processes, indicating that our trait-deteriorated nematodes evolved massive metabolic changes. The molecular function category was dominated by binding, catalytic, transporter, transcriptional regulator and enzyme regulator activities. Such a representation of diverse functional areas is suggestive of coordinated modulation of genes from different functional areas to support the changes undergone during the evolution of trait deterioration. Cellular components were least represented in GO analysis, with a significant number of DEGs assigned to cell and cell part, including the mitochondrion, nucleus, ribosome, endoplasmic reticulum and plasma membrane. Cells undergo huge changes in energy dynamics and are constantly under cellular stress resulting from metabolic as well as oxidative stress. So, functional mapping of DEGs to different organelles

(mitochondria, endoplasmic reticulum) and the plasma membrane could be an indication of massive changes in energy dynamics by nematodes under constant cellular stress.

Biochemical functionality was predicted by mapping all DEGs to pathways using KEGG within Blast2GO. Molecules involved in metabolism (energy, amino acid, carbohydrate and lipid metabolism), neurodegenerative diseases and signal transduction had the highest representation amongst the sequences mapped to KEGG pathways. The enzyme cytochrome c oxidase had the highest mapping to both energy metabolism and neurodegenerative disease categories. Similarly, other enzymes well represented in KEGG pathways are vacuolar ATP synthase (GenBank: EX011485), protein disulfide isomerase (GenBank: EX012905), transglutaminase (Tgase) (GenBank: EX012170), phosphoglycerate dehydrogenase (GenBank: EX013716), NADH dehydrogenase (ubiquinone) (GenBank: ES411557, EX010284), aldehyde dehydrogenase (GenBank: ES411128) and aconitate hydratase (GenBank: EX014674, ES741155). We identified predicted proteins with potential roles in host-parasite interactions, MAPK and T-cell receptor signaling pathway and apoptosis. Although at this stage the precise role of such molecules in the nematode-bacteria-insect host interplay is unclear, they could be involved in manipulating the host's immune response or associated with *Hb*'s innate immune response. Furthermore, we identified families of proteins representing serine, cysteine and metallo-proteinases as well as proteinase inhibitors. While these enzymes are inferred to mediate or modulate proteolytic functions, they may in turn, facilitate the nematode's interaction with its host and symbiont, as the proteinase inhibitors may protect the nematodes against its host's immune system.

Genes of general and secondary metabolism

Results obtained from our analysis showed that trait deteriorated nematodes undergo massive changes of the transcripts encoding metabolic enzymes and processes. We observed a

pattern emerging from our studies suggesting that the trait-deteriorated nematodes down-regulate their primary metabolic processes, which at the same time activate secondary metabolic processes. We also identified significant changes in the dynamics of the genes responsible for energy, amino acid, carbohydrate and lipid metabolism. Enzymes involved in xenobiotic biodegradation, glycan biosynthesis and metabolism and biosynthesis of secondary metabolites were also changed. These results show that the evolution of trait deterioration can result in metabolic upheavals that could be responsible for reduced pathogenicity. The biggest change was observed in energy metabolism, involving the up-regulation of cytochrome c oxidases (CCO) (GenBank: EX012198) and down-regulation of vacuolar ATPases (V-ATPases) and NADPH-cytochrome P450 (GenBank: ES411356). Cytochrome c oxidase encodes an important enzyme involved in oxidation phosphorylation pathways and thus energy production. In *Cryptococcus neoformans,* the up-regulation of CCOI was shown to be related to stress response of the yeast, which is vital for survival in its hostile host [35]. It is possible that the up-regulation of this mitochondrial gene might be linked to an increased energy production critically important to the survival of *Hb* in a deteriorated condition. The V-ATPases are ATP-dependent proton pumps present in both intracellular and plasma membranes, and function in processes such as receptor recycling, protein processing and degradation [36]. They also function in a variety of processes, including the coupled transport of small molecules and pH homeostasis [36]. In *C. elegans* H+-V-ATPases are required for development and osmoregulation in animal excretory systems [37] and act as potent lifespan regulators [38]. The down-regulation of V-ATPases is indicative of the deterioration in cellular homeostasis, and general reduction in cellular transportation activities associated with the trait-deteriorated *Hb*.

We observed an interesting transcriptional pattern of genes involved in amino acid, lipid and carbohydrate metabolism, with the majority of the genes being down-regulated. There was up-regulation of sterol metabolism and down-regulation of enzymes in the category of synthases and hydrolases, suggestive of huge shifts in metabolism. Similarly, we observed down-regulation in the category of a dehydrogenase-like aldehyde dehydrogenase, glutamate dehydrogenase, suggesting repression of fermentative pathways. Carbohydrate metabolism was mostly down-regulated with the exception of pyruvate dehydrogenase (GenBank: NP_500340) and phosphoglycolate phosphatase (GenBank: EX910617). Similarly, amino acid metabolism was also mostly down-regulated, except for sorbitol dehydrogenase (SDH) (GenBank: XP_790483), glutathione peroxidase (GenBank: NP_497078) and a few other enzymes. During anhydrobiosis, nematodes reportedly accumulate polyols like sorbitol and glycerol, which are known to protect animal tissues and cells from injuries caused by freezing or dehydration [39]. As anhydrobiosis is an ametabolic stage, the induction of SDH suggests a general reduction in metabolic activities, and nematodes might be using SDH as a stress survival mechanism. We also observed the differential regulation of enzymes involved in the tricarboxylic acid cycle (TCA), including the up-regulation of pyruvate dehydrogenase and down-regulation of citrate synthase (GenBank: ES412521), aconitate hydratase (GenBank: ES741155), and dihydrolipoyl dehydrogenase (GenBank: EX010778). Protein synthesis seems to be induced in deteriorated nematodes as indicated by the up-regulation of valine, threonine and aspartyl tRNA-synthetase. Two DEGs encoding fructose-bisphosphate aldolase (FBPA) (GenBank: EG025510, ES744087) were down-regulated in the deteriorated line relative to original line. FBPA is an early step in the glycolysis pathway. The products of this pathway are ATP and pyruvic acid (PVA). Additionally, an S-adenosylmethionine synthetase (SAMS) transcript was induced in the deteriorated line of *Hb*.

SAMS is known to be involved in the synthesis of polyamines that act as osmolytes and accumulate under osmotic stress. SAMS is a major methyl group donor in the cell and plays a central role in DNA methylation, transcription, and biosynthetic pathways of many secondary metabolites [40].

Potential signal transduction related genes

We identified a set of signal transduction components which likely orchestrate a rapid and general response to a wide range of changes, but also a set of signalling components that may mediate responses more specific to nematode trait deterioration (examples are highlighted in Table 5.1). Transcriptome patterns associated with signalling during trait deterioration of insect parasitic nematodes have not been well established. We observed differential expression of signalling components like stress-induced-phosphoprotein 1(GenBank: ES740228), phospholipase c beta (GenBank: EX009150), cyclophilin-1 (GenBank: ES411663) and sodium/potassium transporting ATPase (GenBank: ES741918), which are involved generally in stress response and transduction.

The stress-induced-phosphoprotein 1 (*sip-1*) or the Hsc70/Hsp90-organizing protein belongs to a group of co-chaperones, which regulate and assist the major chaperones. *sip-1* modulates the chaperone activities of linked proteins and also interacts with other chaperones. The loss of *sip-1* function in *C. elegans* results in embryonic lethality and reduction in life span [41]. Among our DEGs are several transcripts encoding phospholipase c beta (PLCβ), a homolog of the gene encoded by *C. elegans egl-8*. PLCβ in conjunction with *egl-30* acts in motor neurons to directly or indirectly regulate acetylcholine release, thereby modulating locomotion rate and behavior [42]. Also included in our DEGs were two transcripts encoding *C. elegans* cyclophilin (*cyn-1* and *cyn-5*), a class of peptidyl-prolyl *cis-trans* isomerase (PPIase) enzymes which play an

important role in protein folding [43]. These cyclophilins are predicted to be secreted proteins in nematodes that are constitutively expressed [44]. Induced signalling of these genes is likely an attempt on the part of stressed nematodes to combat reduction in life span and maintain the proper functioning of proteins in a changing environment.

A number of transcripts encoding protein tyrosine kinase (GenBank: EX009882), NADH-cytochrome P450 (GenBank: EX009598) were down-regulated and nitric oxide synthase interacting protein (NOSIP) (GenBank: ES412752) was up-regulated in L5M compared to OHB. Protein tyrosine kinases (PTKs) are important for intra- and inter-cellular communication as well as for survival in eukaryotes and play a major role in signal transduction processes. These proteins are also known to be involved in developmental and differentiation processes of cells [45]. In *C. elegans, a*nother signalling molecule, NADH-cytochrome P450 (NADH-CYP), has been shown to be involved in the detoxification of environmental pollutants and synthesis and degradation of signalling molecules [46]. One of the *C. elegans* CYP isoforms plays an important role in regulating lifecycle progression and the adult life span [47]. Na^+/K^+-ATPase (NKA) is a transmembrane protein which plays a significant role in maintaining electrical gradients in the cell [48]. These electrochemical differences not only maintain osmotic balance, but also favor the transport of nutrients and amino acids into the cells. The down-regulation of NKA in the deteriorated *Hb* indicates an impaired ability of these nematodes to maintain osmotic balance.

NOSIP is an enzyme that regulates nitric oxide (NO) production through binding with high affinity to the carboxyl-terminus of the endothelial nitric oxide synthase (eNOS) oxygenase domain and preventing NO synthesis. NO, a signalling molecule produced by nitric oxide synthase (NOS), is part of the immune response and acts as a neurotransmitter and proliferation

signal. It is produced by both parasitic nematodes and their host. The excretory/secretory products from filarial nematodes, which include NO, have been shown to inhibit the proliferation of host cells mediating innate or acquired immunity [49]. Although NO from *Hb* has not been characterized yet, it could play a significant role in invasion and/or suppression of the host immune system. Up-regulation of NOSIP, which negatively regulates NO production, could result in reduced virulence, one of the observed characteristics in the trait-deteriorated *Hb*. In addition to the signalling molecules described here, the discovery of more signalling transcripts from *Hb* adds to the existing knowledge base of dauer-related genes in *C. elegans*, furthering exploration of the importance of signalling components on trait improvement, increased longevity and stress resistance in nematodes.

Stress and defense related genes

With the advent of microarray technology, researchers can now identify a broad range of genes that are involved in trait deterioration in EPNs. While some genes may be developmental stage-specific, others may be part of a general stress response shared across multiple nematode species. A large portion of the DEGs we found are involved with stress and defense response of nematodes, including two up-regulated transcripts encoding a homolog of *C. elegans hsp-12.6* (GenBank: EX007554) and *daf-21*(GenBank: EX007741). The *C. elegans daf-21* gene encodes a member of the HSP90 family of molecular chaperones important for maturation of signal transduction kinases in neurons involved in odorant perception [50]. The post-embryonic phenotype of *Hb* treated with double stranded (ds) *Hba-daf-21* RNA resulted in abnormal gonad morphology [51]. Another up-regulated gene, *hsp-12.6*, which encodes a small heat shock protein (sHSP) in *C. elegans*, is developmentally regulated but is not up-regulated by a wide range of stressors [52]. Despite its lack of chaperone activity, *hsp-12.6* regulates the functions of

other sHSPs, acting as a co-chaperone with other molecular chaperones [53]. It is possible that *hsp-12.6* is also developmentally regulated, specifically during the infective stage. In *Hb* it may play a significant role in driving the chaperone activities of other Hsps, and conferring protection against oxidative stress. Transcripts encoding homologs of the mitochondrial cytochrome c oxidase (CCO) subunits I, II, and III were abundantly expressed and up-regulated in our trait-deteriorated line. Interestingly, the up-regulated DEGs included 4 transcripts (GenBank: ES740428, ES743969, EX013085, NP_492764) encoding ubiquitin conjugating enzymes (UBCs) and one encoding the 26S proteasome regulatory subunit (GenBank: EX009185). UBCs have been shown to be induced under stress conditions in nematodes [54], and nutrient deprivation in plants has shown to induce ubiquitin degradation of proteins and lipids [55]. It has been suggested that cellular stress results in improperly folded proteins, which are targeted for degradation by ubiquitinization. Interestingly, one of the over-expressed genes in the deteriorated line encoded a chaperonin protein (GenBank: ES743704), which are needed for proper folding of nascent proteins.

Among the down-regulated genes, *eat-6* encodes an alpha subunit of sodium/potassium ATPase, which in turn affects the NA^+/K^+-ATPase activity of membranes. It plays a significant role in the relaxation of pharyngeal muscle, fertility, and also affects body length and life span [56]. Transcriptional profiling showed two transcripts (GenBank: ES744087 and EG025510) encoding fructose-1, 6-bisphosphate (FBP) aldolase, which was down-regulated in the trait-deteriorated nematode line. FBP aldolase, a member of the class I aldolase family, is a glycolytic enzyme that catalyzes the cleavage of FBP into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [57]. Predicted proteins with potential roles in T-cell receptor (TCR) and transforming growth factor beta (TGF-beta) signaling were also down-regulated. In

the parasitic nematode *B. malayi,* a *C. elegans daf-7* homolog which encodes a member of the TGF-beta superfamily was reported to be involved in manipulation of the host immune response [58]. Although at this stage the precise role of such molecules in host-parasite interactions is not clear, they could be involved in manipulating the host's immune response.

Dauer and nematode life span regulation

The infective juvenile stage of entomopathogenic nematodes is developmentally similar to the dauer stage in many bacterivorous nematodes, including *C. elegans* and *C. briggsae*. The dauer is a developmentally arrested stage triggered by food deprivation, high population density, and other harsh environmental conditions [59]. Elucidation of this process is of specific interest in the case of entomopathogenic nematodes because the dauer juvenile is the only life stage capable of infecting insects [15]. We observed differential expression of genes which are commonly expressed in dauer and starved adults of *C. elegans.* Transcripts encoding *hsp90* (GenBank: ES743545), *hsp70* (GenBank: FF678037) were up-regulated while a GTP-binding ribosomal protein homolog (GenBank: ES410054) and SH3-domain containing protein (GenBank: FF681443) were down-regulated in the trait-deteriorated line compared to original. These genes are more abundantly expressed in dauer than in non-dauer (L3) larvae of *C. elegans* [60]. One of the down-regulated DEGs that we encountered encodes a serine/threonine protein kinase (*unc-51*) (GenBank: FF681332) that is orthologous to *Saccharomyces cerevisiae* autophagy protein. It is required for normal dauer morphogenesis of the *C. elegans daf-2* mutant [61]. The down-regulation of *unc-51* may limit reallocation of nutrients in starving cells, such as those in dauer juveniles in free-living nematodes, or infective juveniles in entomopathogenic nematodes. Another down-regulated DEG encoded a NADH-ubiquinone oxidoreductase (GenBank: EX010284) whose *C. elegans* homolog is involved in dauer exit [62]. This gene

induces L3 developmental arrest that results in *C. elegans* individuals with longer lifespan [62]. Vacuolar H^+-ATPases (GenBank: EX011485, NP_508711) were another potent lifespan regulator we found differentially expressed in trait-deteriorated nematodes. These proteins acidify intracellular compartments and act in synaptic transmission and the cell death signaling cascade [63]. Another down-regulated *daf-16* dependent gene encodes a glucose-6-phosphate isomerase (GenBank: ES740896) homolog which functions in the insulin/IGF-1 pathway to affect lifespan. In mammals, glucose-6-isomerase functions in glycolysis, which influences aging [64]. The DEGs we recovered included 10 transcripts encoding components of the mitochondrial respiratory chain, including ATP synthase and NADH-ubiquinone oxidoreductase (ES411557, EX010284). RNAi of respiratory-chain components decreases body size and slows movement and eating behavior (pumping) of nematodes [65]. Nematodes exposed to stress induce generation of reactive oxygen species (ROS), and therefore it is important for nematodes to have effective ROS scavenging mechanisms. A transcript which encodes a putative Ras related protein (GenBank: ES411895), a *C. elegans rab-33* homolog, was up-regulated (Fig. 5.4A), suggesting that trait-deteriorated nematodes may have to elevate their ROS scavenging mechanisms.

Refined gene-specific expression using quantitative reverse transcription-PCR

The microarray observations were validated by quantitative reverse transcription-PCR (qRT-PCR) for some representative transcripts (Fig. 4A). Ten genes that were differentially expressed in trait deteriorated nematodes were selected for qRT-PCR validation. The comparison of expression values between the two methods revealed similar expression kinetics for all the genes tested, indicating reliability of the microarray data (Fig. 5.4B). The expression values obtained by qRT-PCR were generally more exaggerated than the corresponding microarray

values, as reported in previous studies [66, 67]. The observed larger changes in gene expression using qRT-PCR could indicate a greater dynamic range of detection and sensitivity of this method for gene expression profiling. Although microarray analyses showed small fold change (0.38 to 2.45) in DEGs, we are able to verify the differential expression by means of qRT-PCR. It is possible that these levels may fall below a technical threshold and therefore do not allow a reliable transcript quantification by using only hybridization-based methods such as microarray analysis. Furthermore, we observed a strong correlation ($R^2 = 0.88$, $P < 0.05$) for all of the 10 transcript-concordant genes that we examined in this study and found that the degree of correlation between microarray and qRT-PCR results was very similar irrespective of the differential regulation of the genes.

The overall physiological response of an organism or cell to a stimulus may require coordinated changes in a wide array of genes. Those changes in turn may require compensating or reinforcing changes in an even wider array of functionally connected genetic components. Our analysis suggests that low magnitude expression changes may be of functional significance. It is possible that some genes may show low magnitude transcriptional modulation but still play a significant role in the resulting physiological response. We speculate that the genes involved in the evolution of EPN trait deterioration are co-ordinately modulated and thus show moderate levels of transcriptional change.

CONCLUSIONS

The present study has given us a first glimpse of the transcriptional analysis of trait deterioration of insect parasitic nematode and represents a starting point for studies in a number of different fundamental and applied areas. In addition to transcriptional profiling using cDNA microarrays, we used comprehensive transcriptional analysis tools for functional annotation at

the DNA and protein level. From this study of 15,220 *Hb* ESTs, we identified 1,185 DEGs which included homologs of *C. elegans* and *C. briggsae*, animal and human parasitic nematodes, prokaryotes, and transcripts specific to parasitic nematodes. These transcripts are particularly interesting, as they may represent genes that are specific to parasitism or to particular EPN species. Many of those DEGs were involved in metabolism while others were involved in signal transduction and transportation. A few were involved in translation, immune response and cellular processes. We also identified a number of potential molecules that are secreted or excreted in the host-parasite interactions, which could serve as a starting point for further experimental analyses. The secreted proteins that lacked homology with other free-living and animal parasitic nematode proteins could be involved in *Hb-P. luminescens* symbiosis-specific processes, or play vital roles in insect parasitism and suppression of host defense mechanisms. The comparison of DEGs with *C. elegans*, *C. briggsae* and other nematodes revealed common, but also parasitic nematode-specific genes. Most of these are homologues of genes previously found to play critical roles in metabolism whilst others are proposed to play metabolism and regulatory roles in trait deterioration specific to *Hb*. As the most closely related major entomopathogen to *C. elegans*, *Hb* provides an attractive near-term application for using a model organism to better understand the origin and evolution of interspecies interactions (e.g. parasitism, mutualism and vector-borne disease) and to enhance our understanding of the mechanisms underlying trait deterioration in biological control agents. Beyond functional analysis of *Hb* genes, clear research avenues are available to apply this information to improve the beneficial traits of bio-control agents and better understand the fundamental aspects of nematode parasitism and mutualism.

METHODS

Nematode culture

A deteriorated population of *Hb* was created by sub-culturing different experimental lines of nematode-bacterium complex over 20 passages in larvae of the greater wax moth, *Galleria mellonella* [21]. The original parental strain (OHB) was maintained in Ringer's solution without sub-culturing while the inbred line (L5M) was continuously cultured in *G. mellonella*. Both OHB and L5M were cultured identically in *G. mellonella* larvae [68] and emerging infective juveniles (IJs) were collected using White traps [69]. The IJs of both lines were stored in Ringer's solution at 16ºC for not more than one day before used for RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated from four biological replicates of IJs of each strain. The IJs (~8-10,000) were transferred to 10 volumes of Trizol Reagent (Molecular Research Center Inc., Cincinnati, OH) and exposed to freeze thaw cycles using liquid nitrogen and 37ºC water bath. The suspension was ground using mortar and pestle and vortexed. RNA was phase separated using chloroform, precipitated by isopropanol and pelleted. At least three sub-samples from each biological replicates were used for RNA extraction and total RNA was pooled. Total RNA was converted to double stranded cDNA using SuperScript double-stranded cDNA synthesis kit (Invitrogen Corporation, Carlsbad, CA). Double-stranded cDNA was quantified and quality checked by using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA).

Array design and data analysis

Microarrays containing probes against 15,220 *Hb* ESTs assembled from ESTs under GenBank accession numbers [GenBank: EG025323] - [GenBank: EG025806], [GenBank: ES408468] - [GenBank: ES414355], [GenBank: ES738967] - [GenBank: ES744677], [GenBank: EX006911] - [GenBank: EX015306], [GenBank: EX910019] - [GenBank: EX916843], and

[GenBank: FF678120] - [GenBank: FF681586] were designed and manufactured by Roche NimbleGen. Double-stranded cDNA extracted from four biological replicates of each strain of *Hb* were shipped to Roche NimbleGen for labelling, hybridization, and data collection and normalization according to established manufacturer's protocols. Briefly, single color (Cy3) fluorescently labelled cDNA samples were hybridized to the arrays, and signal intensities were obtained on a microarray scanner. Data from all eight arrays were then normalized using the Robust Multichip Average (RMA) algorithm. A two-group (inbred vs. parental strain) statistical analysis using a two-tailed student Student's t-test was performed to identify differentially expressed genes. The complete set of microarray data is accessible through the Gene Expression Omnibus at the National Center for Biotechnology Information (NCBI) under accession number GSE19152.

Sequence analysis

The differentially expressed (DE) EST sequences representing contamination from bacterial, yeast or fungal sources were identified using the BLASTN algorithm [70] and removed from further analyses. ESTs were compared to the sequences in GenBank's non-redundant (nr) and Uniprot database using tBLASTX and BLASTX [71] algorithms, and *C. elegans* WS200 (11th release of WormBase [28]) database using the BLASTX algorithm [70, 72]. ESTs were also compared to the available animal and human parasitic nematodes (AHPNs) and plant parasitic nematode (PPNs) ESTs using tBLASTX. The DE ESTs with no significant matches to proteins of AHPNs and PPNs but matched to *Hb* and other EPNs were designated as parasitic nematode-specific ESTs, which were further characterized.

In order to minimize the number of false positive predictions from the peptides inferred from DE ESTs, secreted proteins were predicted using a combination of two programs,. Firstly,

SignalP 3.0 [73] was used to predict the presence of secretory signal peptides (SPs) for each predicted DE EST proteins. A signal sequence was considered present when it was predicted both by the artificial neural network and the hidden Markov model prediction approaches (SignalPNN and SignalP-HMM). In order to exclude the erroneous prediction of putative Transmembrane (TM) sequences as signal sequences, TMHMM [74], a membrane topology prediction program, was then applied. Identification of sequence similarity was performed using BLAST analyses against nr (non-redundant) databases.

Functional analysis and pathway assignment

Gene ontology (GO) term annotation and function-based analysis of DEGs were performed using Blast2GO (V 1.6.2) [31]. GO terms for each of the three main categories (biological process, molecular function, and cellular component) was obtained from sequence similarity using the application default parameters. From these annotations, pie charts were made using 2nd level GO terms based on biological process, molecular function, and cellular component. Pathway assignments were carried out according to Kyoto encyclopedia of genes and genomes (KEGG) [29] mapping. Enzyme commission (EC) [75] numbers were assigned to DE sequences that had BLASTX scores with a cut-off value of $E = 10^{-5}$ or less upon searching protein databases. The sequences were mapped to KEGG biochemical pathways according to the EC distribution in the pathway database.

Primer design

A set of DEGs from different functional areas were selected and gene specific primers were designed. All the primers used in quantitative real-time PCR (qRT-PCR) were designed using IDT SciTools (Integrated DNA Technologies, Coralville, IA) by aligning EST sequences with similar sequences from NCBI. All the primers used in this study were synthesized by

Operon (Operon Biotechnologies Inc., Huntsville, AL). The sequences of the primers and product sizes are listed in Additional file 5.5.

Validation of differential expression by quantitative reverse transcription-PCR

Total RNA extracted from L5M and OHB nematodes was reverse transcribed using ImProm-II™ reverse transcriptase (Promega corporation, Madison, WI) and subjected to qRT-PCR analysis using LightCycler 480 SYBER Green I mastermix and gene specific primers in a LightCycler 480 RT-PCR system (Roche Applied Science, Mannheim, Germany) equipped with LightCycler 480 software. High-resolution gel electrophoresis was used to verify that the qRT-PCR amplification product from each examined gene was a single-band product. Thermal cycling was performed in accordance with the manufacturer's instructions for a total of 45 cycles at an annealing temperature of 58°C for each primer pair. Quantitative RT-PCR analysis was performed with LightCycler 480 software, the threshold cycle was automatically calculated by the second-derivative maximum method.

Data analysis

In qRT-PCR experiments, changes in target gene expression were calculated using equation $2^{-\Delta\Delta CT}$ [76]. The fold change in the target gene, normalized to 18S rRNA (*Hb-18s*) and relative to the expression of control, was calculated for each sample. A gene with a relative abundance of one is equal to the abundance of 18S rRNA in the same sample. An F-test at a significance level of $P < 0.05$ was used to compare the ratio of the mean gene expression of L5M samples with that of OHB. To minimize mRNA quantification errors, genomic DNA contamination biases and to correct for inter-sample variations, we used 18s ribosomal RNAs (rRNAs) of *Hb* as an internal control. The correlation coefficient between qRT-PCR and microarray data was calculated using NCSS [77]. For microarray experiments, gene expression

above and below one (1.0) was considered as up- and down-regulation for further analysis. Significant differential expression between two lines was calculated using the student t-test ($P < 0.05$).

Author's contributions

BNA carried out most of the work described here including conception of experiments, analysis and interpretation of data, functional characterization and validation of differentially expressed genes and drafting the manuscript. CYL contributed to conception and design of microarray experiments, acquisition of data and statistical analyses. XB, TAC, PSG, PWS, DIS and BJA contributed to EST sequencing, sequence annotation and assembly. ARD, JMC, DIS, ALB, RG and BJA performed breeding experiments and stress tolerance, fecundity and pathogenicity assays. BJA contributed to conception and design of experiments, supervision of the work and critical review of the manuscript. All authors critically reviewed and approved the final manuscript.

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FIGURE LEGENDS

Figure 5.1-Summary of microarray analysis and results.

(A) Experimental design of the microarray experiments included four biological replicates from both the inbred (L5M4, 2, 6 and 5) and the original parental line (OHB4, 3, 6 and 5). RNA from each replicate sample was fluorescently labeled and hybridized to a custom microarray containing probes for 15,220 *Heterorhabditis bacteriophora* transcripts identified in an EST library. Statistical analysis of microarray data identified 1,185 transcripts with significant ($p < 0.05$) differential expression between the two lines. (**B)** Clustergram of the profiles of the 1,185 differentially expressed transcripts (rows) in the eight microarray experiments (columns). Higher expression levels, relative to the mean expression levels for a given transcript, are indicated by red features and lower expression levels are indicated in green. Differences in intensity reflect gradations of over- or under-expression. Transcripts were hierarchically clustered into those with similar profiles.

Figure 5.2- Categories of organisms with significant protein matches to distinct *Heterorhabditis bacteriophora* ESTs.

The percentage was calculated considering the total number of *Heterorhabditis bacteriophora* differentially expressed ESTs having significant matches (E value < 1e-5) as 100%.

Figure 5.3- Percent representation of gene ontology (GO) mappings for *Heterorhabditis bacteriophora* differentially expressed genes.

Distribution of (A) Molecular functions; (B) Cellular components; and (C) Biological process categories based on gene ontology for *Heterorhabditis bacteriophora* differentially expressed ESTs. Analysis was based on GO terms assigned to 712 differentially expressed sequences. Note that individual GO categories can have multiple mappings.

Figure 5.4-Comparison of expression of representative genes selected from microarray data with qRT-PCR.

(A) Comparison of fold-change values from microarray data with expression ratios calculated from qRT-PCR. Values were determined using qRT-PCR and represents relative expression of genes between L5M and OHB. The relative expression of the target gene (*Hb-sec-23*: Yeast sec homolog, *Hb-co-II*: Cytochrome c oxidase II, *Hb-dao-3*: Dauer or aging adult overexpression, *Hb-unc-68*: Uncoordinated, *Hb-asp-3*: Aspartyl protease, *Hb-hid-1*: High temperature induced dauer formation, *Hb-fat-2*: Fatty acid desaturase, *Hb-daf-21*: Abnormal dauer formation, *Hb-rab-33*: RAB family member, *Hb-spl-1*: Sphingosine-1- phosphate lyase) normalized to *Hb-18s*:18S rRNA and relative to the expression of control. Bars represent standard errors calculated from 4 replicates of each experiment. *Significant difference ($P < 0.05$) between qRT-PCR and microarray data. **(B)** Correlation coefficient between the fold-change values from microarray and the expression ratios calculated from qRT-PCR presented as level of gene expression.

Table 5.1-Signal transduction-related transcripts exhibiting differential expression between original parental line (OHB) and trait-deteriorated (inbred) line (L5M) in *Heterorhabditis bacteriophora*.

GenBank Accession number	Enzyme	Signalling pathway	NrSeq[€]	Fold change	*P*- value[£]
ES740228	Stress-induced-phosphoprotein 1	Calcium signalling	1	1.32	0.021
		Wnt signalling	1		
		VEGF signalling	1		
		TGF-beta signalling	1		
		MAPK signalling	1		
EX009882	Protein-tyrosine kinase	Calcium signalling	1	0.87	0.044
		Jak-STAT signalling	1		
		ErbB signalling	1		
		VEGF signalling	1		
EX009150	Phospholipase C beta homolog	Calcium signalling	1	1.26	0.043
		Wnt signalling	1		
		VEGF signalling	1		
		ErbB signalling	1		
		Phosphatidylinositol sign	1		
EX009598	NADPH-cytochrome P450	Calcium signalling	1	0.88	0.019
ES741918	Sodium/Potassium ATPase	Calcium signalling	1	0.64	0.039
		Two component system	1		
EX007037	Peptidylprolyl isomerase	Calcium signalling	1	0.87	0.013
ES740428	Ubiquitin conjugating enzyme	Jak-STAT signalling	1	1.54	0.006
		ErbB signalling	1		
		Wnt signalling	1		
		TGF-beta signalling	1		
ES743969	Ubiquitin conjugating enzyme	Jak-STAT signalling	1	1.52	0.011
		Wnt signalling	1		
		ErbB signalling	1		
		TGF-beta signalling	1		
ES411663	Cyclophilin-1	Jak-STAT signalling	1	1.25	0.043
		MAPK signalling	1		
ES740900	Glycogen synthase kinase 3 beta	ErbB signalling	1	0.80	0.014
		Wnt signalling	1		
		Hedgehog signalling	1		
EX007896	DNA-directed RNA polymerase	Two component system	1	0.79	0.011
EX012170	Glutamine synthetase	Two component system	1	0.61	0.046
XP849696	K+-transporting ATPase	Two component system	1	1.35	0.011
		Two component system	1		
EDP31097	Protein-tyrosine-phosphatase	MAPK signalling	1	1.25	0.043
		Jak-STAT signalling	2		
		TGF-beta signalling	1		

[€]Number of differentially expressed sequences mapped to a given signalling pathway.
[£]According to student t-test; P<0.05.

Additional files

Additional file 5.1- Differentially expressed genes in trait deteriorated *Heterorhabditis bacteriophora* exhibiting RNAi phenotype similar to *Caenorhabditis elegans*.
File name: Additional1_RNAiPhenotypes.doc

File format: Word document

Title: Differentially expressed genes in trait deteriorated *Heterorhabditis bacteriophora* exhibiting RNAi phenotype similar to *Caenorhabditis elegans*.

Description: The RNAi phenotypes were identified by comparison of differentially expressed ESTs with *Caenorhabditis elegans* database (WS200). The table also provides corresponding RNAi phenotypes in *C. elegans* and their annotations.

Additional file 5.2- Secreted proteins predicted from differentially expressed ESTs from trait deteriorated *Heterorhabditis bacteriophora.*
File name: Additional2_SecretedProteins

File format: Word document

Title: Secreted proteins predicted from differentially expressed ESTs from trait deteriorated *Heterorhabditis bacteriophora.*

Description: Signal sequence was considered present when predicted both by SignalPNN and SignalP-HMM [68]. Putative transmembrane (TM) sequences were excluded by applying a topology prediction program TMHMM [69].

Additional file 5.3- KEGG biochemical mappings for *Heterorhabditis bacteriophora* differentially expressed ESTs.
File name: Additional3_KEGGMapping.xls

File format: Microsoft excel

Title: KEGG biochemical mappings for *Heterorhabditis bacteriophora* differentially expressed ESTs.

Description: Differentially expressed ESTs were mapped to different biochemical pathways via Kyoto encyclopedia of genes and genomes (KEGG) [24].

Additional file 5.4- Metabolism related genes exhibiting differential expression between inbred (L5M) and original parental line (OHB) in *Heterorhabditis bacteriophora*.
File name: Additional4_MetabolismGenes.doc

File format: Word document

Title: Metabolism related genes exhibiting differential expression between the trait-deteriorated, inbred line (L5M) and its original parental line (OHB) in *Heterorhabditis bacteriophora*.

Description: The table provides the most represented metabolism related genes which were differentially expressed in the deteriorated line as compared to original line of *Heterorhabditis bacteriophora*. These genes were mapped to different biochemical pathways via Kyoto encyclopedia of genes and genomes (KEGG) [24].

Additional file 5.5- List of gene-specific primer sequences used for quantitative reverse transcription-PCR analysis.
File name: Additional5_PrimerSequences.doc

File format: Word document

Title: Gene specific primer sequences used for quantitative reverse transcription-PCR analysis.

Description: Primers were designed by aligning the EST sequences with their putative homologue from GenBank.

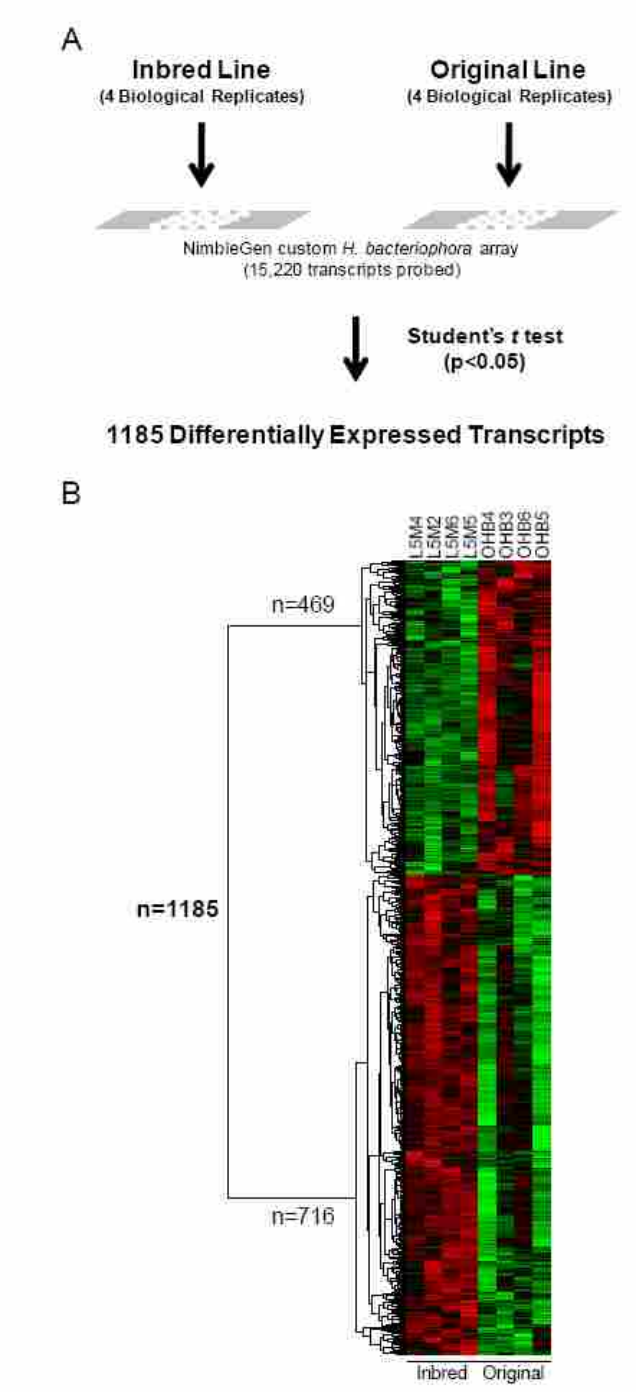

A
Inbred Line
(4 Biological Replicates)
Original Line
(4 Biological Replicates)
NimbleGen custom H. bacteriophora array
(15,220 transcripts probed)
Student's t test
(p<0.05)
1185 Differentially Expressed Transcripts
B
L5M4
L5M2
L5M6
L5M5
OHB4
OHB3
OHB6
OHB5
n=469
n=1185
n=716
Inbred
Original

Figure 5.1

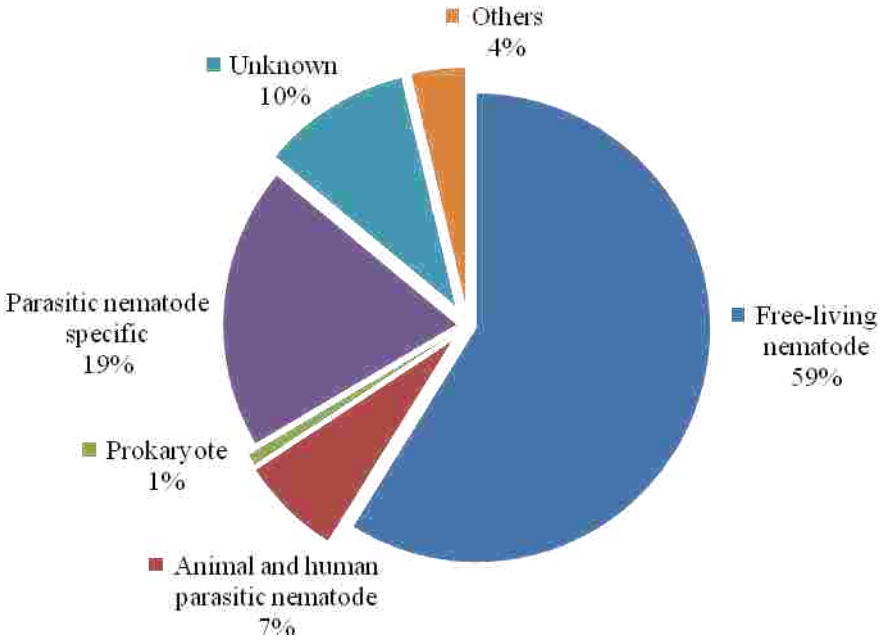
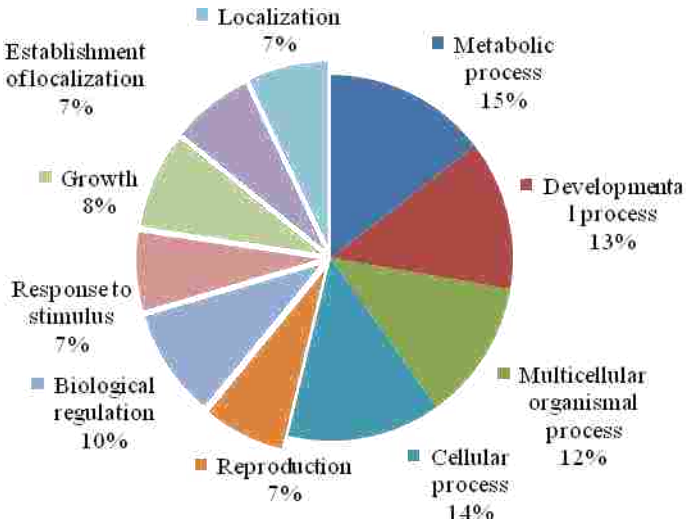
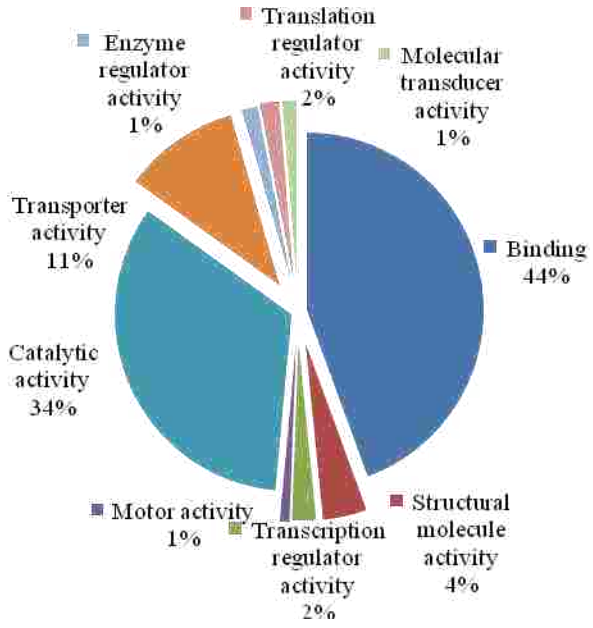

Others
4%
Unknown
10%
Parasitic nematode
specific
19%
Free-living
nematode
59%
Prokaryote
1%
Animal and human
parasitic nematode
7%

Figure 5.2

A.


Localization 7%
Metabolic process 15%
Establishment of localization 7%
Growth 8%
Developmental process 13%
Response to stimulus 7%
Biological regulation 10%
Multicellular organismal process 12%
Reproduction 7%
Cellular process 14%

B.


Translation regulator activity 2%
Enzyme regulator activity 1%
Molecular transducer activity 1%
Transporter activity 11%
Binding 44%
Catalytic activity 34%
Motor activity 1%
Transcription regulator activity 2%
Structural molecule activity 4%

C.

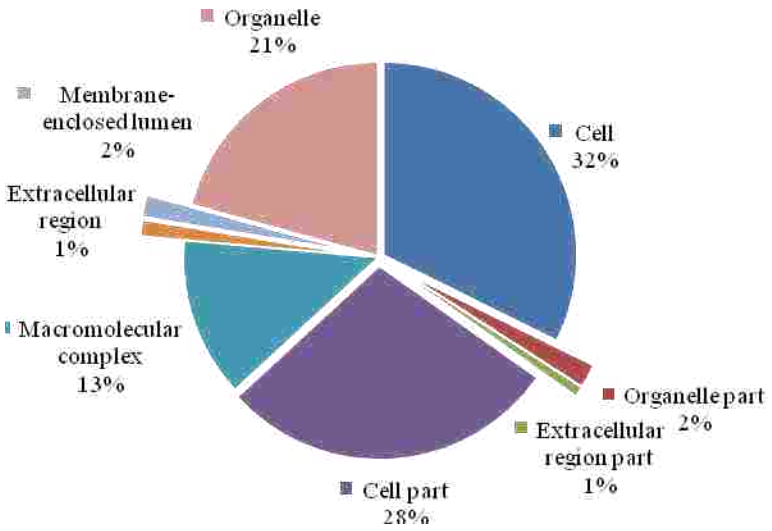
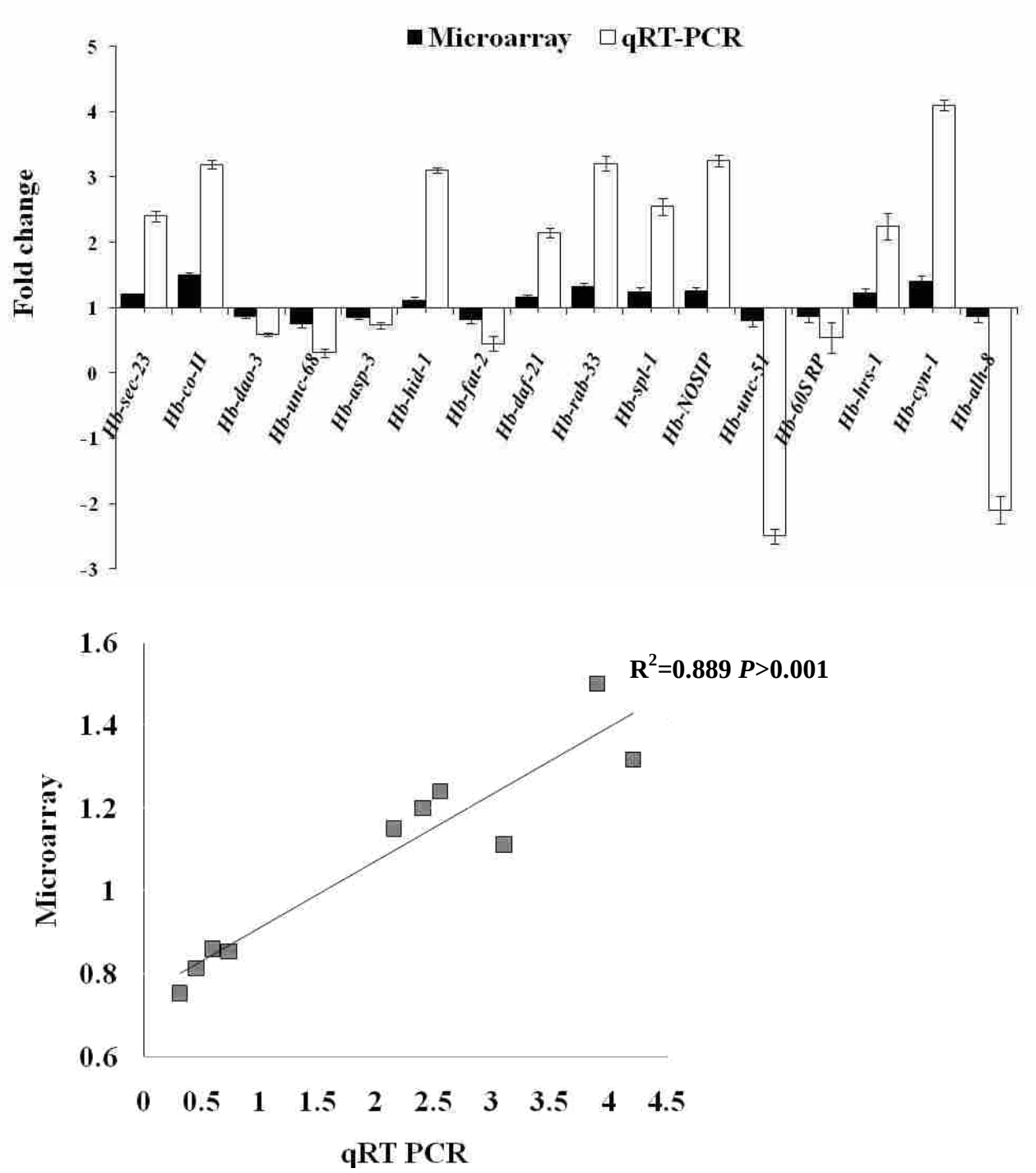

Organelle
21%
Membrane-
enclosed lumen
2%
Extracellular
region
1%
Macromolecular
complex
13%
Cell
32%
Organelle part
2%
Extracellular
region part
1%
Cell part
28%

Figure 5.3


Microarray
qRT-PCR
Fold change
5
4
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1
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-1
-2
-3
Hb-sec-23
Hb-co-II
Hb-dao-3
Hb-unc-68
Hb-asp-3
Hb-hid-1
Hb-fat-2
Hb-daf-21
Hb-rab-33
Hb-spl-1
Hb-NOSIP
Hb-unc-51
Hb-60S RP
Hb-hrs-1
Hb-cyn-1
Hb-alh-8
R2=0.889 P>0.001
Microarray
1.6
1.4
1.2
1
0.8
0.6
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
qRT PCR

Figure 5.4

Additional file 5.1- Differentially expressed genes in trait deteriorated *Heterorhabditis bacteriophora* exhibiting RNAi phenotype similar to *Caenorhabditis elegans*.

Accession number	*C. elegans* homolog	Annotation	E-value	Fold change (P value)	RNAi phenotype[£]
EX009150	WBGene00001177 (egl-80	Phospholipase C beta	1e-38	0.89 (0.004)	Egg laying defective
EX915077	WBGene00006794 (unc-60)	Actin depolymerizing factor 1	2e-66	0.87 (0.005)	Uncoord
ES411502	WBGene00006810 (unc-78)	Actin-interacting protein 1	1e-42	0.81 (0.006)	Uncoord
ES744585	WBGene00006810 (unc-78)	Actin-interacting protein 1	4e-47	0.85 (0.048)	Uncoord
FF681332	WBGene00006786 (unc-51)	Serine/threonine protein kinase	5e-63	0.80 (0.007)	Uncoord
EX007741	WBGene00000915 (daf-21)	Heat shock protein 90	5e-63	1.14 (0.024)	Abn dauer formation
ES743277	WBGene00006801 (unc-68)	Ryanodine receptor family	1e-90	0.75 (0.033)	Uncoord
ES741918	WBGene00001137 (eat-6)	Sodium/potassium ATPase	3e-94	0.63 (0.039)	Abn phar pumping
ES743931	WBGene00006789 (unc-54)	Myosin class II heavy chain	1e-104	0.73 (0.041)	Uncoord
ES741407	WBGene00006754 (unc-15)	Paramyosin	5e-77	0.69 (0.044)	Uncoord
ES412571	WBGene00005026 (sqv-8)	Glucuronosyl transferase	1e-72	1.15 (0.044)	Squashed vulva
EX010968	WBGene00019322 (dpy-14)	S-adenosylhomocysteine hyd	1e-101	0.79 (0.047)	Dumpy
NP_492321*	WBGene00000904 (daf-8)	SMAD protein	5e-52	1.18 (0.007)	Abn dauer formation
NP_001021093*	WBGene00006819 (unc-87)	C-terminal repeat of calponin	1e-174	1.12 (0.020)	Uncoord

*Homolog accession number.
[£]Phenotype according to WormBase database.

Additional file 5.2- Secreted proteins predicted from differentially expressed ESTs from trait deteriorated *Heterorhabditis bacteriophora*.

Number	Sequence ID	Residue	SP	Description (top NR hit)	E-value	% Identity (aa)	RNAi phenotype in *C. elegans*
1	149396870	322	18	Hypothetical protein CBG20736 (*Caenorhabditis elegans*)	7e-87	162/201 (80%)	None
2	156785065	266	18	40S ribosomal protein S13 (*Salmo salar*)	3e-15	15/20 (75%)	-
3	149399353	231	19	Cytochrome c oxidase subunit II (*Heterorhabditis bacteriophora*)	1e-27	62/68 (92%)	-
4	145974548	593	18	Hypothetical protein CBG20880 (*Caenorhabditis briggsae* AF16)	2e-08	62/192 (32%)	-
5	183184914	607	15	Serine/threonine-protein kinase (*unc-51*) (*Caenorhabditis elegans*)	2e-104	182/220 (82%)	dumpy (Dpy), paralyzed (Prl), egg laying defective (Egl_D), locomotion abnormal (Unc)
6	145970758	173	15	Hyptothetical protein CBG14277 (*Caenorhabditis briggsae* AF16)	2e-19	57/173 (32%)	expulsion defective (aex-3)
7	156781521	163	15	Hypothetical protein KO7B1.6 (*Caenorhabditis elegans*)	1e-14	57/121 (47%)	-
8	156785987	169	18	RIKEN cDNA 2610029G23 (*Brugia malayi*)	3e-26	55/107 (76%)	-
9	145974570	62	26	None	None	None	None
10	156786049	183	19	Tropomyosin family protein (*Brugia malayi*)	3e-25	59/73 (80%)	-
11	158948474	325	18	Carbonic anhydrase isoform III (*Ostertagia ostertagi*)	4e-97	167/207 (80%)	None

12	145972366	88	19	60S ribosomal protein L31 (*Salmo salar*)	2e-34	15/16 (93%)	maternal sterile (Ste), embryonic lethal (Emb), lethal (Let), molt defect (Mlt), larval lethal (Lvl), protruding vulva (Pvl)
13	156784162	420	19	Proteasome regulatory particle, non-ATPase-like (*Caenorhabditis elegans*)	1e-49	113/150 (75%)	embryonic lethal (Emb), maternal sterile (Ste), sick (Sck), lethal (Let), transgene expression increased
14	183184800	231	20	Cytochrome c oxidase subunit II (*Heterorhabditis bacteriophora*)	4e-13	35/42 (83%)	embryonic lethal (Emb), extended life span (Age), larval arrest (Lva), sterile progeny (Stp)
15	149396570	127	18	Hypothetical protein CBG20110 (*Caenorhabditis briggsae* AF16)	5e-28	62/111 (55%)	-
16	158952769	339	19	Hypothetical protein ZC477.5 (*Caenorhabditis elegans*)	3e-10	53/201 (26%)	None
17	145971764	540	18	Hypothetical protein F56F10.1 (*Caenorhabditis elegans*)	3e-59	127/209 (60%)	None
18	156782545	216	19	Thioredoxin-like protein p19 precurson (*Brugia malayi*)	1e-56	128/152 (84%)	None
19	145974548	151	19	Hypothetical protein CBG20880 (*Caenorhabditis briggsae* AF16)	2e-08	62/192 (32%)	-
20	145971378	82	15	T Cell Lineage defect family member (*tcl-2*) (*Caenorhabditis elegans*)	2e-16	19/49 (38%)	protruding vulva (Pvl)
21	145971524	88	19	None	None	None	None
22	145973325	395	19	Phosphoglucomutase/phosphomanno-mutase (*Brugia malayi*)	1e-36	86/173 (49%)	-
23	156784130	91	25	U6 snRNA-associated Sm-like protein LSm5 (*Brugia malayi*)	1e-33	71/74 (95%)	-
24	145974038	285	18	U2AF splicing factor family member (*uaf-2*)	9e-73	127/141 (90%)	maternal sterile (Ste), embryonic lethal (Emb), protruding vulva (Pvl),

				(*Caenorhabditis elegans*)			locomotion abnormal (Unc), molt defect (Mlt), clear (Clr)
25	156783078	709	19	High temperature-induced Dauer formation family member (*hid-1*) (*Caenorhabditis elegans*)	2e-104	182/220 (82%)	Maternal sterile (Ste), slow growth (Gro)
26	156779371	152	16	Arp2/3 complex component family member (*arx-7*) (*Caenorhabditis elegans*)	3e-52	100/152 (65%)	larval arrest (Lva), sick (Sck), lethal (Let), sterile progeny (Stp), locomotion abnormal (Unc), sterile (Ste), embryonic lethal (Emb)
27	183182159	61	25	None	None	None	None
28	149397618	93	18	Ferritin, middle subunit (*Salmo salar*)	1e-22	18/29 (62%)	-
29	183185634	107	20	None	None	None	-
30	149396192	248	18	Histidyl tRNA synthetase (*hrs-1*) (*Caenorhabditis elegans*)	6e-36	47/52 (90%)	embryonic lethal (Emb), slow growth (Gro), reduced brood size, larval arrest (Lva), sterile progeny (Stp), small
31	149396972	543	27	Chaperonin containing TCP-1 family member (*cct-3*) (*Caenorhabditis elegans*)	8e-75	138/181 (76%)	larval arrest (Lva), lethal (Let), sterile progeny (Stp), embryonic lethal (Emb), microtubule polymerization abnormal
32	149399615	606	18	Hypothetical protein CB04632 (*Caenorhabditis briggsae* AF16)	5e-106	188/196 (95%)	-
33	183184503	114	16	None	None	None	None
34	183185079	214	21	G protein-coupled receptor 89 (*Apis melifera*)	7e-22	39/44 (97%)	-
35	156779517	243	18	Hypothetical protein CBG13057 (*Caenorhabditis briggsae* AF16)	2e-45	110/201 (54%)	None
36	145970704	318	19	Hypothetical protein BRAFLDRAFT_113873 (*Branchiostoma floridae*)	2e-19	48/122 (39%)	-
37	145970758	184	15	Synaptosomal-associated protein 25 (*Oikopleura*	3e-12	43/143	-

				dioica)		(30%)	
38	158951523	150	27	Hypothetical protein CBG00524 (*Caenorhabditis briggsae* AF16)	3e-08	30/36 (83%)	None
39	149400747	452	18	Hypothetical protein M60.7 (*Caenorhabditis elegans*)	2e-77	142/205 (69%)	None
40	156783801	159	18	Adenosine deaminases acting on RNA (*adr-1*) (*Caenorhabditis elegans*)	2e-09	48/142 (47%)	larval arrest (Lva), reduced brood size, molt defect (Mlt)
41	145972572	513	19	Amino acid transporter family member (*aat-6*) (*Caenorhabditis elegans*)	8e-59	111/149 (74%)	None
42	156780887	387	19	Hypothetical protein F13H8.7 (*Caenorhabditis elegans*)	1e-66	130/191 (68%)	maternal sterile (Ste)
43	156779893	418	18	Dehydrogenases, short chain family member (*dhs-6)* (*Caenorhabditis elegans*)	1e-94	169/198 (85%)	None
44	156780513	156	19	Hypothetical protein F44E2.3 (*Caenorhabditis elegans*)	6e-09	45/81 (55%)	None
45	156779401	82	19	60S ribosomal protein L10 (*Salmo salar*)	1e-21	17/23 (73%)	-
46	145974364	537	15	Hypothetical protein (*Caenorhabditis briggsae* AF16)	5e-90	176/191 (92%)	-
47	183184910	474	16	Hypothetical protein ZC434.3 (*Caenorhabditis elegans*)	7e-46	87/95 (91%)	None
48	183182897	504	17	Hypothetical protein T20B12.3 (*Caenorhabditis elegans*)	5e-29	71/127 (55%)	maternal sterile (Ste), slow growth (Gro), larval arrest (Lva), early larval lethal (Lvl), embryonic lethal (Emb), locomotion abnormal (Unc), nicotine hypersensitive, sterile (Ste)
49	183182197	133	16	None	None	None	None
50	149399416	196	18	Calumenin (calcium-binding protein) homolog family member (*calu-1*) (*Caenorhabditis elegans*)	1e-13	37/41 (90%)	shortened life span (Age), locomotion abnormal (Unc), lethal (Let), molt defect (Mlt), larval arrest (Lva), clear (Clr), Sterile (Ste), larval lethal (Lvl)

51	158947668	131	17	Transthyretin-related family domain family member (*ttr-17*) (*Caenorhabditis elegans*)	1e-41	81/125 (64%)	None
52	156784100	216	15	Thioredoxin-like protein p19 precursor (*Brugia malayi*)	5e-60	134/160 (83%)	-
53	156779174	463	18	Hypothetical protein CBG16828 (*Caenorhabditis briggsae* AF16)	9e-105	188/190 (98%)	None
54	158953609	211	28	None	None	None	-
55	183183646	81	24	None	None	None	-
56	158953311	305	18	Synthetic lethal with Mec family member (*sym-3*) (*Caenorhabditis elegans*)	3e-61	125/206 (60%)	None
57	183183887	680	16	Hypothetical protein NELLACOT_O1747 (*Neisseria lactamica*)	1e-19	21/54 (38%)	-
58	149400942	333	18	Hypothetical protein (*Brugia malayi*)	2e-29	82/188 (43%)	-
59	145972366	88	18	60S ribosomal protein L31 (*Salmo salar*)	1e-34	15/16 (93%)	-
60	156783907	606	15	Hypothetical protein CBG04632 (*Caenorhabditis briggsae* AF16)	3e-105	172/180 (95%)	None
61	145974496	188	15	Hypothetical protein CBG22129 (*Caenorhabditis briggsae* AF16)	7e-47	93/124 (75%)	None
62	156780714	477	18	K-Voltage-gated sensory channel family member (*kvs-1)* (*Caenorhabditis elegans*)	5e-42	88/167 (52%)	None
63	156783198	373	17	Hypothetical protein F15B9.10 (*Caenorhabditis elegans*)	2e-80	142/177 (80%)	fat content increased
64	149400838	523	19	Aldehyde dehydrogenase family member (*alh-8*) (*Caenorhabditis elegans*)	1e-75	149/182 (81%)	None
65	149398966	288	18	Hsp70-interacting protein, putative	1e-34	79/171 (46%)	-

				(*Aedes aegypti*)			
66	145974137	309	20	Hypothetical protein Y39F10A.3 (*Caenorhabditis elegans*)	5e-38	78/180 (43%)	None
67	145969064	172	24	None	None	None	None
68	156784479	222	18	60S ribosomal protein L18a (*Salmo salar*)	4e-06	23/24 (95%)	-
69	183183626	341	16	Cysteine proteinase (*Ancylostoma ceylanicum*)	3e-11	40/98 (40%)	-
70	156785471	948	15	CBR-UNC-52 (*Caenorhabditis briggsae*)	9e-101	170/221 (76%)	None
71	156781116	398	18	Aspartyl protease family member (asp-3) (*Caenorhabditis elegans*)	3e-71	128/212 (60%)	cell death abnormal (Ced), life span abnormal (Age)
72	149398893	101	18	Hypothetical protein F32D1.9 (*Caenorhabditis elegans*)	1e-15	25/66 (37%)	
73	183182275	110	15	None	None	None	None
74	156779387	155	18	60S ribosomal protein L36 (*Salmo salar*)	1e-27	14/15 (93%)	embryonic lethal (Emb)
75	149398775	248	19	Mitochondrial 2-oxoglutarate/malate carrier protein (*Salmo salar*)	8e-16	51/161 (31%)	-
76	149396248	484	18	Putative protein disulfide isomerase 1 (*Dictyocaulus viviparous*)	4e-98	168/211 (79%)	-
77	183184854	150	18	None	None	None	None
78	156786713	286	18	None	None	None	-
79	156786506	216	19	3'-5' exoribonuclease CSL4 homolog (*Brugia malayi*)	4e-54	106/207 (51%)	-
80	183182980	208	22	Hypothetical protein CBG16402 (*Caenorhabditis briggsae* AF16)	5e-15	49/105 (46%)	None
81	156779132	192	18	Cyclophilin family member (*cyn-1*) (*Caenorhabditis elegans*)	3e-66	131/178 (73%)	None
82	156779631	231	24	Cytochrome c oxidase subunit II (*Heterorhabditis*	4e-53	138/174	-

				bacteriophora)		(79%)	
83	contig_1263	282	15	Diadenosine tetraphosphatase (*Burkholderia mallei*)	1e-37	24/98 (24%)	-
84	contig_1631	530	21	Hypothetical protein CBG11507 (*Caenorhabditis briggsae* AF16)	5e-77	165/303 (54%)	None
85	contig_1678	436	17	Hypothetical protein CBG19706 (*Caenorhabditis briggsae* AF16)	2e-41	79/123 (64%)	None
86	contig_2035	443	18	Similar to KIAA1379 protein (*Monodelphis domestica*)	2e-42	104/235 (44%)	-
87	contig_2070	352	16	Hypothetical protein CBG21051 (*Caenorhabditis briggsae* AF16)	2e-38	78/98 (79%)	None
88	contig_230	255	24	Hypothetical protein CBG03983 (*Caenorhabditis briggsae* AF16)	5e-103	192/255 (75%)	None
89	contig_2418	269	15	Protein K10D6.2b (*Caenorhabditis elegans*)	2e-65	120/170 (70%)	-
90	contig_2448	217	16	SJCHGC04950 protein (*Schistosoma japonicum*)	1e-22	36/141 (25%)	-
91	contig_2455	373	18	Hypothetical protein CBG09043 (*Caenorhabditis briggsae* AF16)	7e-21	52/117 (44%)	None
92	contig_2463	89	31	None	None	None	None
93	contig_2547	296	21	Hypothetical protein (*Caenorhabditis elegans*)	1e-42	19/27 (70%)	-
94	contig_259	152	23	Hypothetical protein CO6A5.1 (*Caenorhabditis elegans*)	1e-08	33/135 (24%)	None
95	contig_2597	332	19	Similar to Not3 (*Ciona intestinalis*)	1e-11	16/34 (47%)	-
96	contig_2633	165	16	Hypothetical protein F23H12.3 (*Caenorhabditis elegans*)	5e-54	104/142 (73%)	-
97	contig_3009	569	15	Hypothetical protein (*Paramecium tetraurelia*)	1e-13	35/157 (22%)	-

98	contig_304	612	16	Hypothetical protein CBG02033 (*Caenorhabditis briggsae* AF16)	1e-10	31/55 (56%)	None
99	contig_546	382	18	Ce protein C41C4.6 confirmed by transcript (*ulp-4*) (*Caenorhabditis elegans*)	8e-64	113/174 (64%)	aldicarb resistant (Ric), lethal (Let), embryonic lethal (Emb), patchy coloration (Pch), egg laying abnormal (Egl), dumpy (Dpy), sterile (Ste)
100	contig_860	56	20	None	None	None	None
101	contig_864	585	15	Hypothetical protein CBG14624 (*Caenorhabditis briggsae* AF16)	2e-158	208/310 (67%)	None

Additional file 5.3- KEGG biochemical mappings for *Heterorhabditis bacteriophora* differentially expressed ESTs.

Pathway	KEGG pathway	Seqs in Pathway	Enzyme
Parkinson's disease	Neurodegenerative diseases	61	ubiquitin---protein ligase
Parkinson's disease	Neurodegenerative diseases	61	H+-transporting two-sector ATPase
Parkinson's disease	Neurodegenerative diseases	61	NADH dehydrogenase (ubiquinone)
Parkinson's disease	Neurodegenerative diseases	61	peptidylprolyl isomerase
Parkinson's disease	Neurodegenerative diseases	61	cytochrome-c oxidase
Parkinson's disease	Neurodegenerative diseases	61	tyrosine 3-monooxygenase
Oxidative phosphorylation	Energy metabolism	57	H+-exporting ATPase
Oxidative phosphorylation	Energy metabolism	57	H+-transporting two-sector ATPase
Oxidative phosphorylation	Energy metabolism	57	NADH dehydrogenase (ubiquinone)
Oxidative phosphorylation	Energy metabolism	57	cytochrome-c oxidase
Vibrio cholerae infection	Infectious diseases	22	phosphoinositide phospholipase C
Vibrio cholerae infection	Infectious diseases	22	H+-transporting two-sector ATPase
Vibrio cholerae infection	Infectious diseases	22	protein disulfide-isomerase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	aspartate-semialdehyde dehydrogenase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	L-threonine 3-dehydrogenase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	phosphoglycerate dehydrogenase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	glycine---tRNA ligase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	serine---tRNA ligase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	betaine-aldehyde dehydrogenase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	aminomethyltransferase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	sarcosine oxidase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	threonine---tRNA ligase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	choline kinase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	phosphoserine phosphatase
Glycine, serine and threonine metabolism	Amino acid metabolism	18	dihydrolipoyl dehydrogenase
Epithelial cell signaling in Helicobacter pylori infection	Infectious diseases	15	non-specific protein-tyrosine kinase
Epithelial cell signaling in Helicobacter pylori infection	Infectious diseases	15	phosphoinositide phospholipase C
Epithelial cell signaling in Helicobacter pylori infection	Infectious diseases	15	H+-transporting two-sector ATPase
Epithelial cell signaling in Helicobacter pylori infection	Infectious diseases	15	protein-tyrosine-phosphatase
Photosynthesis	Energy metabolism	13	ferredoxin---NADP+ reductase
Photosynthesis	Energy metabolism	13	H+-transporting two-sector ATPase
Huntington's disease	Neurodegenerative diseases	13	protein-glutamine gamma-glutamyltransferase
Huntington's disease	Neurodegenerative diseases	13	ubiquitin---protein ligase
Huntington's disease	Neurodegenerative diseases	13	GAPDH (phosphorylating)
Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	pyruvate dehydrogenase (acetyl-transferring)
Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	fructose-bisphosphate aldolase
Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	glucose-6-phosphate isomerase
Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	hexokinase

Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	GAPDH (phosphorylating)
Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	aldehyde dehydrogenase [NAD(P)+]
Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	aldehyde dehydrogenase (NAD+)
Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	pyruvate kinase
Glycolysis / Gluconeogenesis	carbohydrate metabolism	11	dihydrolipoyl dehydrogenase
Type III secretion system	Genetic information processing	11	H+-transporting two-sector ATPase
Flagellar assembly	Genetic information processing	11	H+-transporting two-sector ATPase
Arginine and proline metabolism	Amino acid metabolism	11	1-pyrroline-5-carboxylate dehydrogenase
Arginine and proline metabolism	Amino acid metabolism	11	nitric-oxide synthase
Arginine and proline metabolism	Amino acid metabolism	11	proline---tRNA ligase
Arginine and proline metabolism	Amino acid metabolism	11	procollagen-proline dioxygenase
Arginine and proline metabolism	Amino acid metabolism	11	ornithine aminotransferase
Arginine and proline metabolism	Amino acid metabolism	11	argininosuccinate lyase
Arginine and proline metabolism	Amino acid metabolism	11	glutamate dehydrogenase [NAD(P)+]
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	methylmalonate-semialdehyde dehydrogenase (acylating)
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	enoyl-CoA hydratase
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	acetyl-CoA C-acetyltransferase
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	aldehyde dehydrogenase (NAD+)
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	3-hydroxyacyl-CoA dehydrogenase
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	3-hydroxyisobutyrate dehydrogenase
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	acetyl-CoA C-acyltransferase
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	dihydrolipoyl dehydrogenase
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	acyl-CoA dehydrogenase
Valine, leucine and isoleucine degradation	Amino acid metabolism	11	propionyl-CoA carboxylase
Antigen processing and presentation	Immune system	10	protein disulfide-isomerase
Complement and coagulation cascades	Immune system	10	protein-glutamine gamma-glutamyltransferase
Purine metabolism	Nucleotide metabolism	10	DNA-directed RNA polymerase
Purine metabolism	Nucleotide metabolism	10	IMP cyclohydrolase
Purine metabolism	Nucleotide metabolism	10	AICAR formyltransferase
Purine metabolism	Nucleotide metabolism	10	purine-nucleoside phosphorylase
Purine metabolism	Nucleotide metabolism	10	ribose-phosphate diphosphokinase
Purine metabolism	Nucleotide metabolism	10	pyruvate kinase
Purine metabolism	Nucleotide metabolism	10	adenylosuccinate lyase
Purine metabolism	Nucleotide metabolism	10	bis(5'-nucleosyl)-tetraphosphatase (asymmetrical)
Purine metabolism	Nucleotide metabolism	10	nucleoside-triphosphatase
Purine metabolism	Nucleotide metabolism	10	ribonucleoside-diphosphate reductase
Propanoate metabolism	carbohydrate metabolism	9	methylmalonate-semialdehyde dehydrogenase (acylating)
Propanoate metabolism	carbohydrate metabolism	9	enoyl-CoA hydratase
Propanoate metabolism	carbohydrate metabolism	9	malonate-semialdehyde dehydrogenase (acetylating)
Propanoate metabolism	carbohydrate metabolism	9	acetyl-CoA C-acetyltransferase
Propanoate metabolism	carbohydrate metabolism	9	aldehyde dehydrogenase (NAD+)
Propanoate metabolism	carbohydrate metabolism	9	4-aminobutyrate transaminase
Propanoate metabolism	carbohydrate metabolism	9	methylmalonyl-CoA decarboxylase

Propanoate metabolism	carbohydrate metabolism	9	acyl-CoA dehydrogenase
Propanoate metabolism	carbohydrate metabolism	9	propionyl-CoA carboxylase
Glyoxylate and dicarboxylate metabolism	carbohydrate metabolism	9	phosphoglycolate phosphatase
Glyoxylate and dicarboxylate metabolism	carbohydrate metabolism	9	methenyltetrahydrofolate cyclohydrolase
Glyoxylate and dicarboxylate metabolism	carbohydrate metabolism	9	citrate (Si)-synthase
Glyoxylate and dicarboxylate metabolism	carbohydrate metabolism	9	aconitate hydratase
Glyoxylate and dicarboxylate metabolism	carbohydrate metabolism	9	2-hydroxy-3-oxopropionate reductase
Glyoxylate and dicarboxylate metabolism	carbohydrate metabolism	9	formate---tetrahydrofolate ligase
Glyoxylate and dicarboxylate metabolism	carbohydrate metabolism	9	tartronate-semialdehyde synthase
Butanoate metabolism	carbohydrate metabolism	8	pyruvate dehydrogenase (acetyl-transferring)
Butanoate metabolism	carbohydrate metabolism	8	enoyl-CoA hydratase
Butanoate metabolism	carbohydrate metabolism	8	acetyl-CoA C-acetyltransferase
Butanoate metabolism	carbohydrate metabolism	8	aldehyde dehydrogenase (NAD+)
Butanoate metabolism	carbohydrate metabolism	8	acetolactate synthase
Butanoate metabolism	carbohydrate metabolism	8	4-aminobutyrate transaminase
Butanoate metabolism	carbohydrate metabolism	8	acetoacetyl-CoA reductase
Butanoate metabolism	carbohydrate metabolism	8	3-hydroxyacyl-CoA dehydrogenase
Butanoate metabolism	carbohydrate metabolism	8	3-hydroxybutyrate dehydrogenase
Citrate cycle (TCA cycle)	carbohydrate metabolism	8	pyruvate dehydrogenase (acetyl-transferring)
Citrate cycle (TCA cycle)	carbohydrate metabolism	8	citrate (Si)-synthase
Citrate cycle (TCA cycle)	carbohydrate metabolism	8	aconitate hydratase
Citrate cycle (TCA cycle)	carbohydrate metabolism	8	fumarate hydratase
Citrate cycle (TCA cycle)	carbohydrate metabolism	8	dihydrolipoyl dehydrogenase
beta-Alanine metabolism	Metabolism of other amino acids	8	enoyl-CoA hydratase
beta-Alanine metabolism	Metabolism of other amino acids	8	malonate-semialdehyde dehydrogenase (acetylating)
beta-Alanine metabolism	Metabolism of other amino acids	8	aldehyde dehydrogenase (NAD+)
beta-Alanine metabolism	Metabolism of other amino acids	8	4-aminobutyrate transaminase
beta-Alanine metabolism	Metabolism of other amino acids	8	beta-ureidopropionase
beta-Alanine metabolism	Metabolism of other amino acids	8	acyl-CoA dehydrogenase
Cell cycle	Cellular process	8	non-specific protein-tyrosine kinase
Cell cycle	Cellular process	8	ubiquitin---protein ligase
Cell cycle	Cellular process	8	tau-protein kinase
Cell cycle	Cellular process	8	cyclin-dependent kinase
Cell cycle	Cellular process	8	protein-tyrosine-phosphatase
T cell receptor signaling pathway	Cellular process	8	phosphoprotein phosphatase
T cell receptor signaling pathway	Cellular process	8	non-specific protein-tyrosine kinase
T cell receptor signaling pathway	Cellular process	8	ubiquitin---protein ligase
T cell receptor signaling pathway	Cellular process	8	cyclin-dependent kinase
T cell receptor signaling pathway	Cellular process	8	protein-tyrosine-phosphatase
Glutamate metabolism	Amino acid metabolism	7	1-pyrroline-5-carboxylate dehydrogenase
Glutamate metabolism	Amino acid metabolism	7	glutamate---ammonia ligase
Glutamate metabolism	Amino acid metabolism	7	4-aminobutyrate transaminase
Glutamate metabolism	Amino acid metabolism	7	glutamate dehydrogenase [NAD(P)+]
Glutamate metabolism	Amino acid metabolism	7	glutamate dehydrogenase

Calcium signaling pathway	Signal transduction	7	phosphoprotein phosphatase
Calcium signaling pathway	Signal transduction	7	non-specific protein-tyrosine kinase
Calcium signaling pathway	Signal transduction	7	phosphoinositide phospholipase C
Calcium signaling pathway	Signal transduction	7	nitric-oxide synthase
Calcium signaling pathway	Signal transduction	7	Ca2+-transporting ATPase
Calcium signaling pathway	Signal transduction	7	peptidylprolyl isomerase
Chronic myeloid leukemia	Human disease	7	non-specific protein-tyrosine kinase
Chronic myeloid leukemia	Human disease	7	ubiquitin---protein ligase
Chronic myeloid leukemia	Human disease	7	cyclin-dependent kinase
Chronic myeloid leukemia	Human disease	7	protein-tyrosine-phosphatase
Tyrosine metabolism	Amino acid metabolism	7	4-hydroxyphenylpyruvate dioxygenase
Tyrosine metabolism	Amino acid metabolism	7	fumarylacetoacetase
Tyrosine metabolism	Amino acid metabolism	7	aldehyde dehydrogenase [NAD(P)+]
Tyrosine metabolism	Amino acid metabolism	7	tyrosine 3-monooxygenase
Insulin signaling pathway	Cellular process	7	phosphoprotein phosphatase
Insulin signaling pathway	Cellular process	7	ubiquitin---protein ligase
Insulin signaling pathway	Cellular process	7	tau-protein kinase
Insulin signaling pathway	Cellular process	7	pyruvate kinase
Insulin signaling pathway	Cellular process	7	protein-tyrosine-phosphatase
One carbon pool by folate	Metabolism of cofactors and vitamins	7	methenyltetrahydrofolate cyclohydrolase
One carbon pool by folate	Metabolism of cofactors and vitamins	7	AICAR formyltransferase
One carbon pool by folate	Metabolism of cofactors and vitamins	7	aminomethyltransferase
One carbon pool by folate	Metabolism of cofactors and vitamins	7	formate---tetrahydrofolate ligase
One carbon pool by folate	Metabolism of cofactors and vitamins	7	formyltetrahydrofolate dehydrogenase
One carbon pool by folate	Metabolism of cofactors and vitamins	7	methylenetetrahydrofolate reductase [NAD(P)H]
Fatty acid metabolism	Lipid metabolism	6	enoyl-CoA hydratase
Fatty acid metabolism	Lipid metabolism	6	acetyl-CoA C-acetyltransferase
Fatty acid metabolism	Lipid metabolism	6	aldehyde dehydrogenase (NAD+)
Fatty acid metabolism	Lipid metabolism	6	3-hydroxyacyl-CoA dehydrogenase
Fatty acid metabolism	Lipid metabolism	6	acetyl-CoA C-acyltransferase
Fatty acid metabolism	Lipid metabolism	6	glutaryl-CoA dehydrogenase
Fatty acid metabolism	Lipid metabolism	6	acyl-CoA dehydrogenase
Lysine biosynthesis	Amino acid metabolism	6	homoaconitate hydratase
Lysine biosynthesis	Amino acid metabolism	6	L-aminoadipate-semialdehyde dehydrogenase
Lysine biosynthesis	Amino acid metabolism	6	aspartate-semialdehyde dehydrogenase
Lysine biosynthesis	Amino acid metabolism	6	lysine---tRNA ligase
Pentose phosphate pathway	carbohydrate metabolism	6	fructose-bisphosphate aldolase
Pentose phosphate pathway	carbohydrate metabolism	6	glucose-6-phosphate isomerase
Pentose phosphate pathway	carbohydrate metabolism	6	ribose-phosphate diphosphokinase
Pentose phosphate pathway	carbohydrate metabolism	6	phosphogluconate dehydrogenase (decarboxylating)
Tryptophan metabolism	Amino acid metabolism	6	enoyl-CoA hydratase
Tryptophan metabolism	Amino acid metabolism	6	acetyl-CoA C-acetyltransferase
Tryptophan metabolism	Amino acid metabolism	6	aldehyde dehydrogenase (NAD+)
Tryptophan metabolism	Amino acid metabolism	6	3-hydroxyacyl-CoA dehydrogenase
Tryptophan metabolism	Amino acid metabolism	6	glutaryl-CoA dehydrogenase
Tryptophan metabolism	Amino acid metabolism	6	tryptophan 5-monooxygenase
Phenylalanine metabolism	Amino acid metabolism	6	4-hydroxyphenylpyruvate dioxygenase

Phenylalanine metabolism	Amino acid metabolism	6	peroxidase
Phenylalanine metabolism	Amino acid metabolism	6	aldehyde dehydrogenase [NAD(P)+]
Valine, leucine and isoleucine biosynthesis	Amino acid metabolism	6	3-isopropylmalate dehydratase
Valine, leucine and isoleucine biosynthesis	Amino acid metabolism	6	pyruvate dehydrogenase (acetyl-transferring)
Valine, leucine and isoleucine biosynthesis	Amino acid metabolism	6	acetolactate synthase
Valine, leucine and isoleucine biosynthesis	Amino acid metabolism	6	valine---tRNA ligase
Valine, leucine and isoleucine biosynthesis	Amino acid metabolism	6	isoleucine---tRNA ligase
Valine, leucine and isoleucine biosynthesis	Amino acid metabolism	6	leucine---tRNA ligase
p53 signaling pathway	Cellular process	6	phosphoprotein phosphatase
p53 signaling pathway	Cellular process	6	ubiquitin---protein ligase
p53 signaling pathway	Cellular process	6	cyclin-dependent kinase
p53 signaling pathway	Cellular process	6	ribonucleoside-diphosphate reductase
Nitrogen metabolism	Energy metabolism	6	glutamate---ammonia ligase
Nitrogen metabolism	Energy metabolism	6	aspartate ammonia-lyase
Nitrogen metabolism	Energy metabolism	6	carbonate dehydratase
Nitrogen metabolism	Energy metabolism	6	aminomethyltransferase
Nitrogen metabolism	Energy metabolism	6	glutamate dehydrogenase [NAD(P)+]
Nitrogen metabolism	Energy metabolism	6	glutamate dehydrogenase
Lysine degradation	Amino acid metabolism	5	enoyl-CoA hydratase
Lysine degradation	Amino acid metabolism	5	acetyl-CoA C-acetyltransferase
Lysine degradation	Amino acid metabolism	5	aldehyde dehydrogenase (NAD+)
Lysine degradation	Amino acid metabolism	5	3-hydroxyacyl-CoA dehydrogenase
Lysine degradation	Amino acid metabolism	5	glutaryl-CoA dehydrogenase
Natural killer cell mediated cytotoxicity	Cellular process	5	phosphoprotein phosphatase
Natural killer cell mediated cytotoxicity	Cellular process	5	non-specific protein-tyrosine kinase
Natural killer cell mediated cytotoxicity	Cellular process	5	phosphoinositide phospholipase C
Natural killer cell mediated cytotoxicity	Cellular process	5	protein-tyrosine-phosphatase
Jak-STAT signaling pathway	Signal transduction	5	non-specific protein-tyrosine kinase
Jak-STAT signaling pathway	Signal transduction	5	ubiquitin---protein ligase
Jak-STAT signaling pathway	Signal transduction	5	protein-tyrosine-phosphatase
Alanine and aspartate metabolism	Amino acid metabolism	5	malonate-semialdehyde dehydrogenase (acetylating)
Alanine and aspartate metabolism	Amino acid metabolism	5	aspartate ammonia-lyase
Alanine and aspartate metabolism	Amino acid metabolism	5	asparagine---tRNA ligase
Alanine and aspartate metabolism	Amino acid metabolism	5	aspartate---tRNA ligase
Alanine and aspartate metabolism	Amino acid metabolism	5	4-aminobutyrate transaminase
Alanine and aspartate metabolism	Amino acid metabolism	5	adenylosuccinate lyase
Alanine and aspartate metabolism	Amino acid metabolism	5	argininosuccinate lyase
Pyrimidine metabolism	Nucleotide metabolism	5	DNA-directed RNA polymerase
Pyrimidine metabolism	Nucleotide metabolism	5	purine-nucleoside phosphorylase
Pyrimidine metabolism	Nucleotide metabolism	5	beta-ureidopropionase
Pyrimidine metabolism	Nucleotide metabolism	5	bis(5'-nucleosyl)-tetraphosphatase (asymmetrical)
Pyrimidine metabolism	Nucleotide metabolism	5	ribonucleoside-diphosphate reductase
ErbB signaling pathway	Signal transduction	5	non-specific protein-tyrosine kinase
ErbB signaling pathway	Signal transduction	5	ubiquitin---protein ligase
ErbB signaling pathway	Signal transduction	5	phosphoinositide phospholipase C

ErbB signaling pathway	Signal transduction	5	tau-protein kinase
Pyruvate metabolism	carbohydrate metabolism	5	pyruvate dehydrogenase (acetyl-transferring)
Pyruvate metabolism	carbohydrate metabolism	5	acetyl-CoA C-acetyltransferase
Pyruvate metabolism	carbohydrate metabolism	5	aldehyde dehydrogenase (NAD+)
Pyruvate metabolism	carbohydrate metabolism	5	pyruvate kinase
Pyruvate metabolism	carbohydrate metabolism	5	dihydrolipoyl dehydrogenase
Aminoacyl-tRNA biosynthesis	Translation	5	asparagine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	histidine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	proline---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	glycine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	aspartate---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	serine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	methionine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	valine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	lysine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	isoleucine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	leucine---tRNA ligase
Aminoacyl-tRNA biosynthesis	Translation	5	threonine---tRNA ligase
Axon guidance	Development	5	phosphoprotein phosphatase
Axon guidance	Development	5	non-specific protein-tyrosine kinase
Axon guidance	Development	5	tau-protein kinase
Axon guidance	Development	5	cyclin-dependent kinase
Small cell lung cancer	Cancers	5	non-specific protein-tyrosine kinase
Small cell lung cancer	Cancers	5	nitric-oxide synthase
Small cell lung cancer	Cancers	5	cyclin-dependent kinase
Prostate cancer	Cancers	5	ubiquitin---protein ligase
Prostate cancer	Cancers	5	tau-protein kinase
Prostate cancer	Cancers	5	cyclin-dependent kinase
Glioma	Cancers	5	ubiquitin---protein ligase
Glioma	Cancers	5	phosphoinositide phospholipase C
Glioma	Cancers	5	cyclin-dependent kinase
Long-term depression	Nervous system	5	phosphoprotein phosphatase
Long-term depression	Nervous system	5	non-specific protein-tyrosine kinase
Long-term depression	Nervous system	5	phosphoinositide phospholipase C
Long-term depression	Nervous system	5	nitric-oxide synthase
Histidine metabolism	Amino acid metabolism	5	histidine---tRNA ligase
Histidine metabolism	Amino acid metabolism	5	aldehyde dehydrogenase [NAD(P)+]
Histidine metabolism	Amino acid metabolism	5	aldehyde dehydrogenase (NAD+)
Wnt signaling pathway	Signal transduction	5	phosphoprotein phosphatase
Wnt signaling pathway	Signal transduction	5	ubiquitin---protein ligase
Wnt signaling pathway	Signal transduction	5	phosphoinositide phospholipase C
Wnt signaling pathway	Signal transduction	5	tau-protein kinase
B cell receptor signaling pathway	Immune system	5	phosphoprotein phosphatase
B cell receptor signaling pathway	Immune system	5	non-specific protein-tyrosine kinase
B cell receptor signaling pathway	Immune system	5	tau-protein kinase
B cell receptor signaling pathway	Immune system	5	protein-tyrosine-phosphatase
Fatty acid elongation in mitochondria	Lipid metabolism	4	enoyl-CoA hydratase
Fatty acid elongation in mitochondria	Lipid metabolism	4	trans-2-enoyl-CoA reductase (NADPH)
Fatty acid elongation in mitochondria	Lipid metabolism	4	3-hydroxyacyl-CoA dehydrogenase
Fatty acid elongation in mitochondria	Lipid metabolism	4	acetyl-CoA C-acyltransferase
Selenoamino acid metabolism	Metabolism of other amino	4	selenide, water dikinase

	acids		
Selenoamino acid metabolism	Metabolism of other amino acids	4	adenosylhomocysteinase
Selenoamino acid metabolism	Metabolism of other amino acids	4	methionine---tRNA ligase
Selenoamino acid metabolism	Metabolism of other amino acids	4	methionine adenosyltransferase
Benzoate degradation via CoA ligation	Xenobiotic biodegradation and metabolism	4	enoyl-CoA hydratase
Benzoate degradation via CoA ligation	Xenobiotic biodegradation and metabolism	4	acetyl-CoA C-acetyltransferase
Benzoate degradation via CoA ligation	Xenobiotic biodegradation and metabolism	4	3-hydroxyacyl-CoA dehydrogenase
Benzoate degradation via CoA ligation	Xenobiotic biodegradation and metabolism	4	glutaryl-CoA dehydrogenase
Biosynthesis of unsaturated fatty acids	Lipid metabolism	4	enoyl-CoA hydratase
Biosynthesis of unsaturated fatty acids	Lipid metabolism	4	3-oxoacyl-[acyl-carrier-protein] reductase
Biosynthesis of unsaturated fatty acids	Lipid metabolism	4	trans-2-enoyl-CoA reductase (NADPH)
Biosynthesis of unsaturated fatty acids	Lipid metabolism	4	acetyl-CoA C-acyltransferase
PPAR signaling pathway	Endocryne system	4	enoyl-CoA hydratase
PPAR signaling pathway	Endocryne system	4	3-hydroxyacyl-CoA dehydrogenase
PPAR signaling pathway	Endocryne system	4	acetyl-CoA C-acyltransferase
PPAR signaling pathway	Endocryne system	4	acyl-CoA dehydrogenase
Bladder cancer	Cancers	4	ubiquitin---protein ligase
Bladder cancer	Cancers	4	cyclin-dependent kinase
Melanoma	Cancers	4	ubiquitin---protein ligase
Melanoma	Cancers	4	cyclin-dependent kinase
Gap junction	Cell communication	4	non-specific protein-tyrosine kinase
Gap junction	Cell communication	4	phosphoinositide phospholipase C
Gap junction	Cell communication	4	cyclin-dependent kinase
Tight junction	Cell communication	4	phosphoprotein phosphatase
Tight junction	Cell communication	4	non-specific protein-tyrosine kinase
Tight junction	Cell communication	4	cyclin-dependent kinase
Progesterone-mediated oocyte maturation	Cellular process	4	cyclin-dependent kinase
Progesterone-mediated oocyte maturation	Cellular process	4	protein-tyrosine-phosphatase
Leukocyte transendothelial migration	Immune system	4	non-specific protein-tyrosine kinase
Leukocyte transendothelial migration	Immune system	4	phosphoinositide phospholipase C
Leukocyte transendothelial migration	Immune system	4	protein-tyrosine-phosphatase
Two-component system - General	Signal transduction	4	DNA-directed RNA polymerase
Two-component system - General	Signal transduction	4	glutamate---ammonia ligase
Two-component system - General	Signal transduction	4	acetyl-CoA C-acetyltransferase
Two-component system - General	Signal transduction	4	K+-transporting ATPase
Reductive carboxylate cycle (CO2 fixation)	Energy metabolism	4	aconitate hydratase
Reductive carboxylate cycle (CO2 fixation)	Energy metabolism	4	fumarate hydratase
Cell cycle - yeast	Cell division	4	cyclin-dependent kinase
Cell cycle - yeast	Cell division	4	protein-tyrosine-phosphatase
Limonene and pinene degradation	Biosynthesis of secondary metabolites	3	enoyl-CoA hydratase
Limonene and pinene degradation	Biosynthesis of secondary metabolites	3	aldehyde dehydrogenase (NAD+)
Methionine metabolism	Amino acid metabolism	3	adenosylhomocysteinase
Methionine metabolism	Amino acid metabolism	3	methionine---tRNA ligase

Methionine metabolism	Amino acid metabolism	3	methionine adenosyltransferase
Starch and sucrose metabolism	carbohydrate metabolism	3	glucose-1-phosphate adenylyltransferase
Starch and sucrose metabolism	carbohydrate metabolism	3	glucose-6-phosphate isomerase
Starch and sucrose metabolism	carbohydrate metabolism	3	hexokinase
Neurodegenerative Diseases	Human dieseases	3	ubiquitin---protein ligase
Neurodegenerative Diseases	Human dieseases	3	GAPDH (phosphorylating)
Fructose and mannose metabolism	carbohydrate metabolism	3	fructose-bisphosphate aldolase
Fructose and mannose metabolism	carbohydrate metabolism	3	hexokinase
Dentatorubropallidoluysian atrophy (DRPLA)	Neurodegenerative diseases	3	ubiquitin---protein ligase
Dentatorubropallidoluysian atrophy (DRPLA)	Neurodegenerative diseases	3	GAPDH (phosphorylating)
alpha-Linolenic acid metabolism	Lipid metabolism	3	enoyl-CoA hydratase
alpha-Linolenic acid metabolism	Lipid metabolism	3	3-hydroxyacyl-CoA dehydrogenase
alpha-Linolenic acid metabolism	Lipid metabolism	3	acetyl-CoA C-acyltransferase
Inositol metabolism	carbohydrate metabolism	3	methylmalonate-semialdehyde dehydrogenase (acylating)
Inositol metabolism	carbohydrate metabolism	3	malonate-semialdehyde dehydrogenase (acetylating)
Drug metabolism - cytochrome P450	Xenobiotic biodegradation and metabolism	3	aldehyde dehydrogenase [NAD(P)+]
Metabolism of xenobiotics by cytochrome P450	Xenobiotic biodegradation and metabolism	3	aldehyde dehydrogenase [NAD(P)+]
VEGF signaling pathway	Signal transduction	3	phosphoprotein phosphatase
VEGF signaling pathway	Signal transduction	3	non-specific protein-tyrosine kinase
VEGF signaling pathway	Signal transduction	3	phosphoinositide phospholipase C
MAPK signaling pathway	Signal transduction	3	phosphoprotein phosphatase
MAPK signaling pathway	Signal transduction	3	protein-tyrosine-phosphatase
Non-small cell lung cancer	Cancers	3	phosphoinositide phospholipase C
Non-small cell lung cancer	Cancers	3	cyclin-dependent kinase
TGF-beta signaling pathway	Signal transduction	3	phosphoprotein phosphatase
TGF-beta signaling pathway	Signal transduction	3	ubiquitin---protein ligase
Pancreatic cancer	Cancers	3	non-specific protein-tyrosine kinase
Pancreatic cancer	Cancers	3	cyclin-dependent kinase
Renal cell carcinoma	Cancers	3	fumarate hydratase
Renal cell carcinoma	Cancers	3	protein-tyrosine-phosphatase
Amyotrophic lateral sclerosis (ALS)	Neurodegenerative diseases	3	phosphoprotein phosphatase
Amyotrophic lateral sclerosis (ALS)	Neurodegenerative diseases	3	nitric-oxide synthase
Adipocytokine signaling pathway	Cell communication	3	non-specific protein-tyrosine kinase
Adipocytokine signaling pathway	Cell communication	3	protein-tyrosine-phosphatase
Adherens junction	Cell communication	3	non-specific protein-tyrosine kinase
Adherens junction	Cell communication	3	protein-tyrosine-phosphatase
Caprolactam degradation	Xenobiotic biodegradation and metabolism	3	enoyl-CoA hydratase
Caprolactam degradation	Xenobiotic biodegradation and metabolism	3	3-hydroxyacyl-CoA dehydrogenase
Ubiquitin mediated proteolysis	Folding, sorting and degradation	3	ubiquitin---protein ligase
Ubiquitin mediated proteolysis	Folding, sorting and degradation	3	peptidylprolyl isomerase
Primary immunodeficiency	Folding, sorting and degradation	3	non-specific protein-tyrosine kinase
Primary immunodeficiency	Folding, sorting and degradation	3	protein-tyrosine-phosphatase
Focal adhesion	Cell communication	3	phosphoprotein phosphatase
Focal adhesion	Cell communication	3	non-specific protein-tyrosine kinase
Focal adhesion	Cell communication	3	tau-protein kinase

Ubiquinone biosynthesis	Metabolism of cofactors and vitamins	3	NADH dehydrogenase (ubiquinone)
Streptomycin biosynthesis	Biosynthesis of secondary metabolites	3	dTDP-4-dehydrorhamnose reductase
Streptomycin biosynthesis	Biosynthesis of secondary metabolites	3	glucose-1-phosphate thymidylyltransferase
Streptomycin biosynthesis	Biosynthesis of secondary metabolites	3	hexokinase
Glutathione metabolism	Metabolism of other amino acids	3	glutamate---cysteine ligase
Glutathione metabolism	Metabolism of other amino acids	3	phosphogluconate dehydrogenase (decarboxylating)
Glutathione metabolism	Metabolism of other amino acids	3	ribonucleoside-diphosphate reductase
Geraniol degradation	Xenobiotic biodegradation and metabolism	3	enoyl-CoA hydratase
Geraniol degradation	Xenobiotic biodegradation and metabolism	3	3-hydroxyacyl-CoA dehydrogenase
Geraniol degradation	Xenobiotic biodegradation and metabolism	3	acetyl-CoA C-acyltransferase
Carbon fixation in photosynthetic organisms	Energy metabolism	3	fructose-bisphosphate aldolase
Carbon fixation in photosynthetic organisms	Energy metabolism	3	pyruvate kinase
D-Glutamine and D-glutamate metabolism	Energy metabolism	2	glutamate dehydrogenase [NAD(P)+]
Synthesis and degradation of ketone bodies	Lipid metabolism	2	acetyl-CoA C-acetyltransferase
Synthesis and degradation of ketone bodies	Lipid metabolism	2	3-hydroxybutyrate dehydrogenase
Nucleotide excision repair	Replication and repair	2	cyclin-dependent kinase
Fatty acid biosynthesis	Lipid metabolism	2	3-oxoacyl-[acyl-carrier-protein] reductase
Fatty acid biosynthesis	Lipid metabolism	2	[acyl-carrier-protein] S-malonyltransferase
Regulation of actin cytoskeleton	Cell motility	2	phosphoprotein phosphatase
Regulation of actin cytoskeleton	Cell motility	2	non-specific protein-tyrosine kinase
MAPK signaling pathway - yeast	Signal transduction	2	protein-tyrosine-phosphatase
Urea cycle and metabolism of amino groups	Amino acid metabolism	2	aldehyde dehydrogenase (NAD+)
Urea cycle and metabolism of amino groups	Amino acid metabolism	2	argininosuccinate lyase
Urea cycle and metabolism of amino groups	Amino acid metabolism	2	glutamate-5-semialdehyde dehydrogenase
Pantothenate and CoA biosynthesis	Metabolism of cofactors and vitamins	2	acetolactate synthase
Pantothenate and CoA biosynthesis	Metabolism of cofactors and vitamins	2	beta-ureidopropionase
Sphingolipid metabolism	Lipid metabolism	2	sphingomyelin phosphodiesterase
Glycerophospholipid metabolism	Lipid metabolism	2	glycerol-3-phosphate dehydrogenase
Glycerophospholipid metabolism	Lipid metabolism	2	choline kinase
Type I diabetes mellitus	Metabolic disorders	2	protein-tyrosine-phosphatase
Benzoate degradation via hydroxylation	Xenobiotic biodegradation and metabolism	2	3-carboxy-cis,cis-muconate cycloisomerase
Benzoate degradation via hydroxylation	Xenobiotic biodegradation and metabolism	2	acetyl-CoA C-acyltransferase
Alzheimer's disease	Neurodegenerative diseases	2	GAPDH (phosphorylating)
Alzheimer's disease	Neurodegenerative diseases	2	tau-protein kinase
Long-term potentiation	Nervous system	2	phosphoprotein phosphatase
Long-term potentiation	Nervous system	2	phosphoinositide phospholipase C
Melanogenesis	Endocryne system	2	phosphoinositide phospholipase C
Melanogenesis	Endocryne system	2	tau-protein kinase
GnRH signaling pathway	Endocryne system	2	non-specific protein-tyrosine kinase
GnRH signaling pathway	Endocryne system	2	phosphoinositide phospholipase C

Aminosugars metabolism	carbohydrate metabolism	2	UDP-N-acetylglucosamine diphosphorylase
Aminosugars metabolism	carbohydrate metabolism	2	hexokinase
Cell adhesion molecules (CAMs)	carbohydrate metabolism	2	protein-tyrosine-phosphatase
Polyketide sugar unit biosynthesis	Biosynthesis of polyketides	2	dTDP-4-dehydrorhamnose reductase
Polyketide sugar unit biosynthesis	Biosynthesis of polyketides	2	glucose-1-phosphate thymidylyltransferase
Nucleotide sugars metabolism	Carbohydrate metabolism	2	dTDP-4-dehydrorhamnose reductase
Nucleotide sugars metabolism	Carbohydrate metabolism	2	glucose-1-phosphate thymidylyltransferase
Methane metabolism	Energy metabolism	2	peroxidase
Methane metabolism	Energy metabolism	2	methylenetetrahydrofolate reductase [NAD(P)H]
Fc epsilon RI signaling pathway	Immune system	2	non-specific protein-tyrosine kinase
Fc epsilon RI signaling pathway	Immune system	2	phosphoinositide phospholipase C
Bile acid biosynthesis	Lipid metabolism	2	aldehyde dehydrogenase (NAD+)
Bile acid biosynthesis	Lipid metabolism	2	acetyl-CoA C-acyltransferase
Porphyrin and chlorophyll metabolism	Metabolism of cofactors and vitamins	1	glutamate-1-semialdehyde 2,1-aminomutase
C5-Branched dibasic acid metabolism	carbohydrate metabolism	1	acetolactate synthase
Asthma	Human disease	1	peroxidase
RNA polymerase	Transcription	1	DNA-directed RNA polymerase
Ascorbate and aldarate metabolism	carbohydrate metabolism	1	aldehyde dehydrogenase (NAD+)
Galactose metabolism	carbohydrate metabolism	1	hexokinase
Styrene degradation	Xenobiotic biodegradation and metabolism	1	fumarylacetoacetase
Ethylbenzene degradation	Xenobiotic biodegradation and metabolism	1	acetyl-CoA C-acyltransferase
3-Chloroacrylic acid degradation	Xenobiotic biodegradation and metabolism	1	aldehyde dehydrogenase (NAD+)
Pentose and glucuronate interconversions	carbohydrate metabolism	1	2-deoxy-D-gluconate 3-dehydrogenase
1,2-Dichloroethane degradation	Xenobiotic biodegradation and metabolism	1	aldehyde dehydrogenase (NAD+)
Drug metabolism - other enzymes	Xenobiotic biodegradation and metabolism	1	beta-ureidopropionase
Apoptosis	Cell growth and death	1	phosphoprotein phosphatase
Biotin metabolism	Metabolism of cofactors and vitamins	1	adenosylmethionine-8-amino7-oxononanoate transaminase
Nicotinate and nicotinamide metabolism	Metabolism of cofactors and vitamins	1	purine-nucleoside phosphorylase
Phenylalanine, tyrosine and tryptophan biosynthesis	Amino acid metabolism	1	phenylalanine 4-monooxygenase
Inositol phosphate metabolism	carbohydrate metabolism	1	phosphoinositide phospholipase C
Basal cell carcinoma	Cancers	1	tau-protein kinase
Glycerolipid metabolism	Lipid metabolism	1	aldehyde dehydrogenase (NAD+)
Glycan structures - biosynthesis 1	Glycan biosynthesis and metabolism	1	mannosyl-oligosaccharide 1,2-alpha-mannosidase
Endometrial cancer	Cancers	1	tau-protein kinase
Colorectal cancer	Cancers	1	tau-protein kinase
Hedgehog signaling pathway	Signal transduction	1	tau-protein kinase
Type II diabetes mellitus	Metabolic disorders	1	pyruvate kinase
Peptidoglycan biosynthesis	Glycan biosynthesis and metabolism	1	glutamate---ammonia ligase
Androgen and estrogen metabolism	Lipid metabolism	1	estradiol 17beta-dehydrogenase
Phenylpropanoid biosynthesis	Biosynthesis of secondary metabolites	1	peroxidase
Lipopolysaccharide biosynthesis	Glycan biosynthesis and metabolism	1	acyl-UDP-N-acetylglucosamine O-acyltransferase
Thiamine metabolism	Metabolism of cofactors and	1	nucleoside-triphosphatase

	vitamins		
Phosphatidylinositol signaling system	Signal transduction	1	phosphoinositide phospholipase C
Pathogenic Escherichia coli infection - EPEC	Infectious diseases	1	non-specific protein-tyrosine kinase
Pathogenic Escherichia coli infection - EHEC	Infectious diseases	1	non-specific protein-tyrosine kinase
ABC transporters - General	Membrane transport	1	polyamine-transporting ATPase
High-mannose type N-glycan biosynthesis	Glycan biosynthesis and metabolism	1	mannosyl-oligosaccharide 1,2-alpha-mannosidase
N-Glycan biosynthesis	Glycan biosynthesis and metabolism	1	mannosyl-oligosaccharide 1,2-alpha-mannosidase
Total		**3100**	

Additional file 5.4-Metabolism related genes exhibiting differential expression between inbred (L5M) and original parental line (OHB) in *Heterorhabditis bacteriophora*.

Sequence ID	GenBank accession number	Annotation	Fold change	P- value[1]
Energy metabolism (56)				
183183314	FF679373	ATP synthase beta subunit	0.91	0.004
156783580	EX011485	Vacuolar ATP synthase subunit H 2	0.65	0.013
149396932	ES740341	H+-transporting two-sector ATPase	0.73	0.013
145973706	ES413394	H+-transporting two-sector ATPase	0.77	0.020
149399615	ES743024	Vacuolar ATP synthase subunit H 2	0.66	0.022
115342469	EG025722	H+-transporting two-sector ATPase	0.88	0.023
149400813	ES744222	Vacuolar ATPase subunit H 2	0.84	0.027
145974364	ES414052	H+-transporting two-sector ATPase	0.85	0.031
156783907	EX011812	Vacuolar ATPase subunit H 2	0.64	0.034
156783658	EX011563	H+-transporting two-sector ATPase	0.76	0.047
contig_1679	NP_508711*	Vacuolar H ATPase family member	1.10	0.011
158948474	EX910693	Carbonate dehydratase	1.07	0.012
156786782	EX014687	Glutamate dehydrogenase	0.80	0.009
156784293	EX012198	Cytochrome c oxidase II	1.52	0.001
156779958	EX007863	Cytochrome c oxidase II	1.58	0.002
158949666	EX911878	Cytochrome c oxidase II	1.48	0.004
145972807	ES412495	Cytochrome c oxidase I	1.63	0.004
156783031	EX010936	Cytochrome c oxidase III	1.37	0.004
183184677	FF679196	Cytochrome c oxidase II	1.44	0.005
149396629	ES740038	Cytochrome c oxidase I	1.62	0.006
156781873	EX009778	Cytochrome c oxidase I	1.49	0.006
156783305	EX011210	Cytochrome c oxidase II	1.56	0.006
149400917	ES744326	Cytochrome c oxidase I	1.47	0.006
156780157	EX008062	Cytochrome c oxidase I	1.63	0.007
156779528	EX007433	Cytochrome c oxidase II	1.61	0.008
145974553	ES414241	Cytochrome c oxidase II	1.36	0.008
156782059	EX009964	Cytochrome c oxidase II	1.35	0.008
156786865	EX014770	Cytochrome c oxidase II	1.52	0.009
149397515	ES740924	Cytochrome c oxidase II	1.48	0.009
145972076	ES411764	Cytochrome c oxidase II	1.57	0.011
156784525	EX012430	Cytochrome c oxidase II	1.53	0.011
156786949	EX014854	Cytochrome c oxidase II	1.54	0.012
149400317	ES743726	Cytochrome c oxidase II	1.47	0.012
156784522	EX012427	Cytochrome c oxidase II	1.57	0.012
145971774	ES411462	Cytochrome c oxidase I	1.51	0.012
149398997	ES742406	Cytochrome c oxidase II	1.45	0.013
183184800	FF681870	Cytochrome c oxidase II	1.20	0.013
158948829	EX910557	Cytochrome c oxidase III	1.60	0.013
115342293	EG025546	Cytochrome c oxidase I	1.54	0.014
156781873	EX009778	Cytochrome c oxidase I	1.49	0.006
156780089	EX007994	Cytochrome c oxidase II	1.46	0.015
158951243	EX913568	Cytochrome c oxidase III	1.26	0.015
149399597	ES743006	Cytochrome c oxidase III	1.52	0.016
156786450	EX014355	Cytochrome c oxidase III	1.60	0.018
156783557	EX011462	Cytochrome c oxidase II	1.39	0.020
156786456	EX014361	Cytochrome c oxidase III	1.55	0.022
115342293	EG025546	Cytochrome c oxidase I	1.45	0.022

156785587	EX013492	Cytochrome c oxidase II	1.48	0.026
156786492	EX014397	Cytochrome c oxidase II	1.43	0.028
156787076	EX014981	Cytochrome c oxidase III	1.46	0.029
158951856	EX914214	Cytochrome c oxidase II	1.48	0.029
145970526	ES410214	Cytochrome c oxidase I	1.49	0.031
156779126	EX007031	Cytochrome c oxidase II	1.46	0.043
156782590	EX010495	Cytochrome c oxidase I	1.35	0.043
156779631	EX007536	Cytochrome c oxidase II	1.26	0.046
156786480	EX014385	Cytochrome c oxidase VIb	0.85	0.049
Amino acid metabolism (29)				
149397203	ES740612	Aspartate-semialdehyde dehydrogenase	0.47	0.043
149396870	ES740279	Phosphoglycerate dehydrogenase	0.70	0.001
156784717	EX012622	Phosphoglycerate dehydrogenase	0.69	0.002
149398107	ES741516	Phosphoglycerate dehydrogenase	0.75	0.005
149400313	ES743722	Phosphoglycerate dehydrogenase	0.68	0.005
158948008	EX910309	Phosphoglycerate dehydrogenase	0.74	0.009
156781460	EX009365	Phosphoglycerate dehydrogenase	0.80	0.023
149397957	ES741366	Serine-tRNA ligase	0.75	0.038
145973942	ES413630	Betaine-aldehyde dehydrogenase	0.88	0.005
149397016	ES740425	Aminomethyltransferase	0.81	0.020
149397016	ES740425	Sarcosine oxidase	1.22	0.041
145970789	ES410477	Threonine-tRNA ligase	1.09	0.037
156782597	EX010502	Phosphoserine phosphatase	0.81	0.032
156782873	EX010778	Dihydrolipoyl dehydrogenase	0.77	0.047
145973064	ES412752	Nitric-oxide synthase interacting protein	1.25	0.019
156780809	EX008714	Proline dioxygenase	0.65	0.044
149400835	ES744244	Ornithine aminotransferase	1.28	0.046
156786720	EX014625	3-hydroxyisobutyrate dehydrogenase	1 .17	0.003
149400838	ES744247	1-pyrroline-5-carboxylate dehydrogenase	0.86	0.037
156784265	EX012170	Glutamate-ammonia ligase	0.60	0.046
183185277	FF680848	Glutamate dehydrogenase [NAD(P)+]	0.85	0.035
145970682	ES410370	4-hydroxyphenylpyruvate dioxygenase	0.46	0.009
149396404	ES739813	4-hydroxyphenylpyruvate dioxygenase	0.48	0.042
145973110	ES412798	Fumarylacetoacetase	0.76	0.015
contig_832	NP_495863*	Phenylalanine hydroxylase	1.47	0.005
contig_762	NP_499089*	Aspartyl- tRNA synthetase	1.18	0.031
contig_1024	NP_497078*	Glutathione peroxidase	1.25	0.007
contig_1164	NP_501914*	Valine-tRNA ligase	1.19	0.029
149396192	ES739601	Histidine-tRNA ligase	1.22	0.021
Carbohydrate metabolism (17)				
contig_2070	NP_500340*	Pyruvate dehydrogenase	1.24	0.024
149396498	ES739907	Methylmalonyl-CoA decarboxylase	0.71	0.008
156780356	EX008261	Methylmalonyl-CoA decarboxylase	0.72	0.015
156780356	EX008261	Propionyl-CoA carboxylase	0.72	0.015
158948398	EX910617	Phosphoglycolate phosphatase	1.18	0.009
145971363	ES411051	Citrate (Si)-synthase	0.67	0.010
145972833	ES412521	Citrate (Si)-synthase	0.70	0.045
156779893	EX007798	Acetoacetyl-CoA reductase	0.77	0.030
156786769	EX014674	Aconitate hydratase	0.64	0.038
149397746	ES741155	Aconitate hydratase	0.83	0.043
149397487	ES740896	Glucose-6-phosphate isomerase	0.75	0.044
115342257	EG025510	Fructose-bisphosphate aldolase	0.54	0.036
149400678	ES744087	Fructose-bisphosphate aldolase	0.60	0.049
145971975	ES411663	Protein-tyrosine-phosphatase	0.87	0.007
contig_2231	XP_790483*	Sorbitol dehydrogenase	1.30	0.046

contig_540	Q8HXX4*	Acetyl-CoA C-acetyltransferase	1.34	0.011
145970127	ES409815	Aconitate hydratase	0.78	0.019
Nucleotide metabolism (9)				
156779991	EX007896	DNA-directed RNA polymerase	0.78	0.011
149400704	ES744113	AICAR formyltransferase	0.58	0.042
145972757	ES412445	Ribose-phosphate diphosphokinase	0.76	0.004
156782981	EX010886	Ribose-phosphate diphosphokinase	0.81	0.003
145968881	ES408569	Pyruvate kinase	0.86	0.007
156783533	EX011438	Adenylosuccinate lyase	0.79	0.001
149398352	ES741761	Bis(5'-nucleosyl)-tetraphosphatase	1.33	0.035
156784077	EX011982	Nucleoside-triphosphatase	1.29	0.049
156780887	EX008792	Beta-ureidopropionase	0.89	0.029
Metabolism of cofactors and vitamins (6)				
149395588	ES739189	Formate-tetrahydrofolate ligase	0.88	0.003
149398099	ES741508	Methylenetetrahydrofolate reductase	1.23	0.026
115342189	EG025442	NADH dehydrogenase (ubiquinone)	1.83	0.007
145971869	ES411557	NADH dehydrogenase (ubiquinone)	1.52	0.007
156782379	EX010284	NADH dehydrogenase (ubiquinone)	0.78	0.023
156786466	EX014371	Acetolactate synthase	0.63	0.028
Lipid metabolism (9)				
145971440	ES411128	Aldehyde dehydrogenase (NAD+)	0.86	0.010
149396422	ES739831	Acyl-CoA dehydrogenase	0.82	0.005
183182916	FF679618	Trans-2-enoyl-CoA reductase (NADPH)	0.86	0.046
156785500	EX013405	Sphingomyelin phosphodiesterase	1.27	0.016
145974076	ES413764	Sphingomyelin phosphodiesterase	1.34	0.023
156785811	EX013716	Glycerol-3-phosphate dehydrogenase	0.85	0.038
contig_2386	NP_492417*	Fatty acid synthase	0.74	0.025
158950797	EX913393	Choline kinase	0.81	0.033
145971668	ES411356	NADPH-cytochrome P450	0.88	0.019
Metabolism of other amino acids (5)				
149401243	ES744652	Selenide, water dikinase	1.11	0.015
156783063	EX010968	Adenosylhomocysteinase	0.79	0.047
158952481	EX914791	Methionine adenosyltransferase	0.78	0.027
156781630	EX009535	Glutamate-cysteine ligase	1.25	0.012
156784452	EX012357	Ribonucleoside-diphosphate reductase	1.18	0.028
Xenobiotic biodegradation and metabolism (3)				
145971662	ES411350	Enoyl-CoA hydratase	0.85	0.030
145970639	ES410327	Acetyl-CoA C-acetyltransferase	1.17	0.003
156779520	EX007425	Glutaryl-CoA dehydrogenase	0.81	0.002
Biosynthesis of secondary metabolites (3)				
contig_2884	ABF95280*	dTDP-4-dehydro rhamnose reductase	1.12	0.006
145974196	ES413884	Glucose-1-phosphate thymidylyltransferase	0.73	0.005
156780031	EX007936	Hexokinase	1.23	0.046
Glycan biosynthesis and metabolism (1)				
Contig_864	XP_001676681*	Man(9)-alpha-mannosidase	0.81	0.014

[1]According to student t-test; $P<0.05$.
*Homolog accession number.

Additional file 5.5- List of gene-specific primer sequences used for quantitative real-time PCR analysis.

Target gene	Abbreviated name	Accession number	Forward sequence/ Reverse sequence (5' to 3') (3' to 5')	Amplicon size
Yeast sec homolog	*Hb-sec-23*	EX012534	AAGTTGGTTTGAGCCACGAT TTGCCTTACAACTGGCCTTT	318
Cytochrome c oxidase II	*Hb-co-II*	EX007863	GGTGAACCCCGTTTGTTAGA AGCACCACAAATCTCAGAACA	221
Dauer overexpression	*Hb-dao-3*	ES739189	TGATCAAGTTGCCCGATACA TCTCACGCGTTAAACCATCA	202
Uncoordinated	*Hb-unc-68*	ES743277	AGTACGAGCTGGTGGAGGAA AAGGCATCGATGATCAAACC	266
Aspartyl protease	*Hb-asp-3*	EX009021	TTTGGGTTCCATGCAAAAAT CAGGCGAAACCTTGTGTTTT	214
High temperature-induced DF	*Hb-hid-1*	EX010983	TCCGAATTCCTCAAAGAAGG TGCATCCGTAATGTGGAAAA	270
Fatty acid desaturase	*Hb-fat-2*	ES744357	CAACTGTGGATGACGTGAGG ACAATCATGTCCGACCACAA	224
Abnormal dauer formation	*Hb-daf-21*	EX007741	AGGAGCCTCAGTCACATGCT ACGAGATGACGCAGAGATCA	186
RAB family	*Hb-rab-33*	ES411895	ATGCTGGTGTAGGGAAAACG GCTCCTGTCCTGCAGTATCC	147
Sphingosine-1-phosphate lyase	*Hb-spl-1*	FF679861	TGGAACCCCCTTTTACACTG GAATGGCATTTGGGTTTTGT	311

RNAi mediated functional analysis of stress related transcripts in the insect parasitic nematode *Heterorhabditis bacteriophora*

Bishwo N Adhikari*, Byron J Adams

Department of Biology, and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT 84602, USA

*Corresponding author: adhikaribn@hotmail.com

ABSTRACT

Background

Heterorhabditis bacteriophora is applied throughout the world for the biological control of insects and is an animal model to study interspecies interactions, e.g. mutualism, parasitism and vector-borne disease. *H. bacteriophora* nematodes are mutually associated with the insect pathogen, *Photorhabdus luminescens*. RNA interference is an excellent reverse genetic tool to study gene function in *C. elegans*, and we attempted to use RNAi to demonstrate the importance of stress related genes differentially expressed in trait deteriorated *H. bacteriophora*.

Results

Soaking L1 stage *H. bacteriophora* with dsRNAs of five genes whose *C. elegans* orthologs had severe RNAi phenotypes resulted in highly penetrant and obvious developmental and reproductive abnormalities. The efficacy of RNAi was evident by abnormal gonad morphology and sterility of adult *H. bacteriophora* and *C. elegans*. The penetrance of RNAi phenotypes in *H. bacteriophora* was high for five genes (62–91%; *Hba-daf-21*, *Hba-egl-8*, *Hba-gpx-1*, *Hba-jnk-1*, and *Hba-tol-1*). Specific and severe reduction in transcript levels in RNAi treated L1s was determined by quantitative real-time qRT-PCR. Of 5 *H. bacteriophora* ESTs screened, a significant reduction in survival of desiccation in treated *H. bacteriophora* populations was observed with one sequence which encoded a glutathione peroxidase. To confirm a role for glutathione peroxidases in desiccation survival, RNAi with dsRNA sequence from *H. bacteriophora* was performed and was also shown to reduce the desiccation tolerance in this species.

Conclusion

These results demonstrate the potential of RNAi for the analysis of stress related genes and provides genetic data to underline the importance of effective antioxidant systems in metazoan desiccation tolerance. This greatly enhances the utility of *H. bacteriophora* as a model system for the study of the molecular biology of stress survival and as a possible model nematode for comparative and functional genomics.

INTRODUCTION

Heterorhabditis bacteriophora is a rhabditid entomopathogenic nematode (EPN) symbiotic with the enteric bacterium *Photorhabdus luminescens*, a dangerous liaison lethal to many insect hosts (Ciche et al., 2006). EPNs are applied globally for the biological control of insects. The nematode is also a potentially powerful animal model to study interspecies interactions such as mutualism, parasitism, and vector borne disease. Genomics and genetics are available for the symbiont, and because it is a close relative to *C. elegans*, are being developed for the nematode. *Heterorhabditis bacteriophora* is closely related to *C. elegans* (Rhabditidae) and can be propagated outside of insect hosts on agar based media and in liquid culture provided that a monoculture of symbiotic *P. luminescens* is present.

RNA interference (RNAi) was first described in the nematode *C. elegans* (Fire et al., 1998; Montgomery et al., 1998), when it was found that the injection of double-stranded RNA (dsRNA) into a hermaphrodite worm resulted 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. Potent RNAi effects can also be achieved in *C. elegans* by soaking the nematodes in a dsRNA solution (Tabara et al., 1998) or by feeding the nematodes on bacteria engineered to express dsRNA of the target gene (Timmons and Fire, 1998).

RNAi is a powerful molecular genetic tool to elucidate gene function. In *C. elegans*, RNAi was originally performed by injecting dsRNA into the body of L4 animals (Guo et al., 1995; Fire et al., 1998) and also shown to be effective by soaking (Tabara et la., 1998; Kuroyanagi et al., 2000), on lawns of bacteria expressing dsRNA (Timmons et al., 2001) or by expressing dsRNA in *C. elegans* cells (Tavernarakis et al., 2000). RNAi by feeding (Kamath et

al., 2003; Simmer et al., 2003), soaking (Maeda et al., 2001) and injection (Sonnichsen et al., 2005) are amenable to high throughput methodologies. The dependence of *H. bacteriophora* for symbiotic bacteria for growth and reproduction makes RNAi by feeding problematic but RNAi by soaking has been reported to be potent and specific (Ciche and Sternberg, 2006). It has been demonstrated that RNAi can be used effectively in *H. bacteriophora* and can be applied for analyses of nematode genes involved in symbiosis and parasitism (Ciche and Sternberg, 2006).

Previous research has shown that RNAi can be used in the free living bacterial feeding nematode *Panagrolaimus superbus* (Shannon et al., 2008) and anhydrobiotic nematode *Aphelenchus avenae* (Reardon et al., 2010) using methods developed for *C. elegans* (Brenner, 1974) and cross-species RNAi (Reardon et al., 2010) respectively. Work done by Reardon et al. (2010) has confirmed a role for glutathione peroxidases in anhydrobiosis and RNAi with cognate sequences was shown to reduce desiccation tolerance *A. avenae*. Their work also demonstrated the potential of RNAi for the analysis of anhydrobiosis and provides the first genetic data to underline the importance of effective antioxidant systems in metazoan desiccation tolerance.

In this study, we tested the applicability of a soaking protocol for RNAi of stress related genes in *H. bacteriophora*. Using this protocol we detected highly penetrant and obvious phenotypes in *H. bacteriophora* for five *C. elegans* orthologs, previously reported to have highly penetrant and obvious phenotypes in *C. elegans*. The stress related genes were selected from the genes differentially expressed in trait deteriorated *H. bacteriophora* from our previous experiment (Adhikari et al. 2009a). Our results show that potent and specific RNAi effects can be achieved in *H. bacteriophora* by soaking in dsRNA solution for genes with stress related functions. Our findings also indicate that glutathione peroxidases represent a key class of

enzymes required for desiccation tolerance in *H. bacteriophora* and emphasize the need for redox balancing in desiccation survival.

RESULTS AND DISCUSSION

Efficacy of RNAi by soaking in *Heterorhabditis bacteriophora*

Among 1,185 genes differentially expressed in trait deteriorated *H. bacteriophora*, five orthologs that had obvious and highly penetrant RNAi phenotypes in *C. elegans*, were chosen for RNAi by soaking in *H. bacteriophora*. RNAi by soaking L1s in dsRNA corresponding to *Hba-daf-21, Hba-egl-8, Hba-jnk-1, Hba-gpx-1* and *Hba-tol-1*were successful as evidenced by highly penetrant RNAi phenotypes (62-91%) including sterility and defective gonad development in adult animals (Figure 5.5). L1s soaked in Ringer's solution with no dsRNA added, resulted in adults with normal fertility and gonad morphology.

RNAi of *Heterorhabditis bacteriophora*

The *tol-1* encodes a predicted transmembrane protein that is the sole *C. elegans* Toll-like receptor (TLR). TOL-1 is required for proper embryonic development and for pathogen avoidance behavior, and is expressed primarily in the nervous system (Pujol et al., 2001). The *tol-1* mutants are defective in their avoidance of pathogenic *S. marcescens*, although other chemosensory behaviors are wild type. In *C. elegans*, *tol-1* is important for development and pathogen recognition. In *C. elegans*, published RNAi experiments of *tol-1* resulted into larval arrest and embryonic lethal phenotypes (Pujol et al., 2001). Soaking L1 *H. bacteriophora* in double stranded *Hba-tol-1* RNA resulted in a highly penetrant (62%) embryonic lethal phenotype (Figure 5.5).

The *jnk-1* encodes a serine/threonine kinase that is the sole *C. elegans* member of the c-Jun N-terminal kinase (JNK) subgroup of mitogen-activated protein (MAP) kinases. It is required for normal coordinated locomotion as well as for normal adult lifespan and response to heat and oxidative stress. The JNK signaling pathway serves as a molecular sensor for various stresses. Upon detecting environmental cues, JNK-1 might deliver the signal to DAF-16 by direct interaction and modulation of its nuclear translocation (King et al., 2009). Published RNAi results of *jnk-1* in *C. elegans* resulted in embryonic lethal phenotype (King et al., 2009). Soaking of *H. bacteriophora* L1 in double stranded *jnk-1* RNA resulted in a highly penetrant (74%) lethal phenotype (Figure 5.5).

The *egl-8* encodes a phospholipase C beta homolog that affects pharyngeal pumping, defecation, and activity levels; it is genetically downstream of *egl-30* with respect to aldicarb-induced paralysis, and is expressed in most or all neurons of *C. elegans*. EGL-8 plays a more important role in interneurons and sensory neurons than in the motor neurons that comprise most of the ventral cord. In *C. elegans* published RNAi results include sterility and embryonic lethal (Lehner et al., 2006). In *H. bacteriophora*, L1 soaked in double strand *Hba-egl-8* resulted in highly penetrant (86%) sterile phenotype (Figure 5.5). Such sterility could be due to defective gonad development and absence of germ cells.

The *daf-21* encodes a member of the Hsp90, a chaperone with numerous specific protein targets. DAF-21 activity is required for larval development, negative regulation of dauer larva formation, and a number of specific chemosensory behaviors, such as the response to chemicals and odorants detected by the ASE and AWC sensory neurons (Piano et al., 2000). In *C. elegans* down-regulation of *daf-21* via RNAi results in a small but reproducible reduction in *age-1* life

span, suggesting that *daf-21* is part of a chaperone network required for the extended life span seen in *age-1* mutant animals. In *C. elegans daf-21* null phenotype is early larval lethality and maternal sterility (Piano et al., 2000). In *H. bacteriophora*, L1 soaked in double stranded *Hba-daf-21* resulted in highly penetrant (79%) maternal sterility (Figure 5.5).

Quantification of RNAi by Real-time RT-qPCR

To determine the extent of RNAi silencing in *H. bacteriophora*, quantitative real-time RT-qPCR experiments were performed. Expression levels of *Hba-daf-21, Hba-egl-8, Hba-gpx-1, Hba-jnk-1* and *Hba-tol-1*were quantified relative to *Hba-18S* when treated with the specific (Figure 5.6) double stranded RNAs. Relative amounts of mRNA were determined using the $\Delta\Delta C_t$ method. RNAi of all the genes tested resulted in reduced mRNA levels relative to the untreated control (Figure 5.6). From these data we conclude that RNAi by soaking in *H. bacteriophora* is potent and specific.

Effect of gene silencing on desiccation survival

Silencing of specific gene targets by RNAi has been invaluable in nematodes, in particular *C. elegans*, as an alternative to mutagenesis that allows gene function to be probed on a large scale (Kamath et al., 2003; Maeda et al., 2001). Such high throughput functional genomics would be ideal for identifying the stress responsive gene set in a nematode such as *H. bacteriophora*. In this study, by soaking L1s in double stranded solution of glutathione peroxidase, we are able to demonstrate that *Hba-gpx-1* plays important role in desiccation survival of *H. bacteriophora* (Figure 5.7).

An oxidative stress response forms part of the environmental stress response defined by Gasch et al. (2000) in yeast and is part of the minimal stress response described by Kültz (2005).

Desiccation stress is no exception and it has long been recognized that antioxidants are likely to play a role in desiccation tolerance (reviewed in Leprince et al., 1993; Kranner et al., 2002; Franca et al., 2007). The demonstration of a role for glutathione peroxidase in nematode anhydrobiosis is consistent with, and validates, the many correlative studies which have previously implicated antioxidants. In nematodes, for instance, several previous studies have identified dehydration induced genes encoding antioxidant enzymes (Gal et al., 2003; Browne et al., 2004; Adhikari et al., 2009b; Wharton and Marshall, 2009). The present study takes our understanding of desiccation survival by *H. bacteriophora* a step further by showing a reduced level of desiccation tolerance after silencing glutathione peroxidase transcripts, thus clearly demonstrating a stress-combative role for antioxidants.

CONCLUSIONS

Using RNAi by soaking we have identified a panel of genes implicated in the response to desiccation in the insect parasitic nematode, *H. bacteiophora*. The gene set includes stress response genes which were differentially regulated in trait deteriorated nematodes. We also presented evidence for RNAi of glutathione peroxidase implicated in desiccation survival of many nematodes including *H. bacteriophora*. RNAi of glutathione peroxidase reduced survival of desiccation of *H. bacteriophora*. Our results demonstrate the involvement of glutathione peroxidase in desiccation survival and emphasize the need for effective antioxidant systems in desiccated nematodes. The results reported here are promising to the applicability of RNAi to study gene function in *H. bacteriophora* and this will greatly enhances the utility of *H. bacteriophora* as a model system for the study of the molecular biology of stress survival and as a possible model nematode for comparative and functional genomics.

METHODS

Strains and growth conditions

Heterorhabditis bacteriophora strain TTO1 was kindly provided by Todd Ciche (Michigan State University, East Lansing). Glycerol stocks of *Photorhabdus luminescens* bacterium were stored at -80°C. The nematodes were maintained by infecting Greater Waxmoth larvae, *Galleria mellonella* (Rainbow Mealworms, Compton, CA) or propagated on lawns of symbiotic bacteria as follows: the primary phase variant of *P. luminescens* was grown overnight at 28°C in 3 ml of PP3 after which 50 (MICRO) ul were spread on NA+chol (1.5×Nutrient Broth, 1.5% agar (Difco, Detroit, MI) and 10 µg/ml cholesterol), the inoculated plates were incubated at 28°C overnight, after which 50-100 surface sterilized IJs were added. The nematode cultures were grown at 28°C and eggs were collected ~ 86 h or IJs ~ 10 d after inoculation. *Caenorhabditis elegans* N2 were maintained on *E. coli* OP50 seeded NGM agar as previously described (Brenner, 1974).

Generation of dsRNA

The transcripts were selected from the list of the differentially expressed genes from trait deteriorated *H. bacteriophora* from our previous experiment (Adhikari et al., 2009a) and checked for the presence of *C. elegans* orthologs by BLASTX analysis (Altschul et al., 1990) and for gene function and RNAi phenotypes using gene annotations and WormBase (Schwarz et al., 2006). Five ESTs were chosen that had severe RNAi phenotypes in *C. elegans* (*Hba-daf-21*, *Hba-egl-8*, *Hba-gpx-1*, *Hba-jnk-1*, *Hba-tol-1*). Oligonucleotide primers were designed using the EST sequence and Primer3 (Rozen and Skaletsky, 2000). The T7 RNA polymerase promoter

sequence taatacgactcactatagggaga (T7) was added to each of the 5' ends of the PCR primers for in vitro transcription to generate dsRNA. The primer sequences are listed in Table 5.2.

Genomic DNA was purified from *H. bacteriophora* IJs collected from *P. luminescens* containing NA+chol plates using DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) after which bacterial DNA was digested with DpnI (New England Biolabs, Bedford, MA). 100-200 ng of template was added to a PCR reaction containing 20 pM of each primer, in a 50 µl vol using the standard reaction provided for Taq (Promega, Madison, WI). The PCR condition used was: 1.94°C for 3 min, 2.94°C 45 sec, 3. 57°C 30 sec, 4. 72°C 45 sec, 5. repeat steps 2-4 30× then 6.72°C 10 min. The PCR reactions were analyzed for a single band of predicted size on a 1.2% agarose gel. 5 µl of the PCR reaction was then used directly for *in vitro* transcription using the T7 RiboMax (Promega) according to the instructions provided, except the transcription reactions were incubated for >10 h at 37°C. The DNA templates were removed by DNAse treatment and then dsRNA precipitated by adding 1/10 vol. of 5 M ammonium acetate and 2.5 vol. of 100% ethanol for >1 h at 4°C. The precipitated dsRNA was centrifuged for 30 min at 16,000 × *g* and then washed with 70% ethanol prepared in RNAse free water. After air drying for 5 min, the pellet was dissolved in 25 µl of RNAse free water. The quality of the transcribed RNA was determined by running 1 µl on a 1.2% agarose gel and quantified (A260) using a NanoDrop (Nanodrop Technologies, Wilmington, DE).

RNAi of *Heterorhabditis bacteriophora*

Heterorhabditis bacteriophora eggs were harvested from NA+chol. containing *P. luminescens* usually 82–86 h after the addition of 50–100 IJs when grown at 28-29°C. Washing 1-2 week old IJs three times in 15 ml of Ringer's solution (100 mM NaCl, 1.8 mM KCl, 2 mM

CaCl2, 1 mM MgCl2, 5 mM HEPES pH 6.9) improved the synchrony of IJ recovery. At this time most of the eggs were at the pretzel stage of embryonic development. The eggs were harvested by washing the plates 3× with 2 ml of sterile Ringer's solution and bacteria removed by filtering on a 10 μm TCTP membrane (Millipore, Billerica, MA) with a gentle vacuum applied. Eggs and adult hermaphrodites were washed 3 times with 10 ml of Ringer's solution after which eggs were purified from hermaphrodites by their different settling rates. The eggs were concentrated by centrifugation, 2,000 × g for 1-2 min and resuspended in Ringer's to a concentration of ~ 5 eggs per μl. 4 μl of 5-7.5 mg/ml dsRNA was then added to eggs at total volume of 20 μl. The eggs hatch in the dsRNA solution while they are incubated for >24 h at 28°C. The resulting L1s were then placed on 18–24 h lawns of *P. luminescens* on NA+chol plates. Postembryonic abnormalities caused by the dsRNA were observed 2-5 days post RNAi treatment.

Quantification of RNAi by Real-time qRT-PCR

RNAi was performed as described above except 150-250 L1s were soaked with 4 ul of 5-7.5 mg/ml dsRNA in a total volume of 15 μl. After 24 h, the L1s were washed 3× in Ringer's solution. The RNA was extracted by adding 500 ul Trizol (Invitrogen, Carlsbad, CA) and stored at -80°C before extracting according to the manufacturer's instructions. 80-100 ng total RNA treated with 10 U DNAse I for 15 min at 37°C. The following primers were used for qRT-PCR: *Hba-daf-21*: (For) agaagatggctgatttcctccgct, (Rev) gggtctgattatccttcatgcgag; *Hba-egl-8*: (For) atgaagaacagttgacaacgcgcc, (Rev) taccacagatgcgggtttcgatga; *Hba-gpx-1*: (For) gaaggcaggtttaccaatggctca (Rev) gtttccgtttccaccctactgcat; *Hba-jnk-1*: (For) tcagtccattctcggtggcttcaa, (Rev) gcacaaacaattccctgagcacca and *Hba-tol-1*: (For) tttccgttgcgactgttcattgcg, (Rev) accaacgttaggcggatatgctga, 50 ng of total RNA was reverse

transcribed using, ThermoScript Reverse Transcriptase (Invitrogen) according to the Manufacturer's instruction, 56°C annealing temperature, using antisense primers. Realtime RT-qPCR was performed according to the manufacturer's instructions using Syber Green PCR Master Mix (Applied Biosystems Incorporated, Foster City, CA) performed using a Light Cycler 480 System (Roche Applied Science). Using *Hba-18S* as an internal standard, *Hba-cct-2* and *Hba-nol-5* mRNAs were quantified either in RNAi experiments using specific dsRNA. Non-RT samples were used as negative controls. The experiments were repeated three times.

RNAi by feeding of *Caenorhabditis elegans*

RNAi by feeding was done as published (Kamath et al., 2003), except that eggs were harvested by alkaline hypochlorite lysis of gravid hermaphrodites and added to the HT115 expressing dsRNA. The following HT115 (DE3) clones expressing dsRNA in the feeding vector L440 were obtained from the Gene Service's feeding library: *cct-2*, *daf-21*, *ben-1*. Sterility of adults, abnormal gonad development and protruding vulva phenotypes were scored 72–80 h after eggs were placed on dsRNA expressing bacteria.

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FIGURE LEGEND

Figure 5.5. **qRT-PCR to assess transcript levels for *Hba-daf-21, Hba-egl-8, Hba-jnk-1, Hba-gpx-1* and *Hba-tol-1* genes in control and RNAi treated *Heterorhabditis bacteriophora* nematodes**.

RNA was isolated from nematodes whose progeny showed a positive RNAi phenotype and from individual control nematodes. Transcript levels were determined by qRT-PCR for the target gene and for the *rRNA 18S* gene (which served as an internal control).

Figure 5.6. **Percent penetrance of different RNAi phenotypes observed in *Heterorhabditis bacteriophora* after soaking in solution containing double stranded RNAs.**

Figure 5.7. RNAi of glutathione peroxidase (*Hba-gpx-1*) transcripts in *Heterorhabditis bacteriophora* reduces desiccation tolerance.

Soaking of *H. bacteriophora* in dsRNA corresponding glutathione peroxidase (*Hba-gpx-1*) sequence, followed by assessment of desiccation tolerance. Nematodes soaked in water were used as a negative control. Mixed stage worms were subjected to desiccation at 90% RH for 24 h (grey bars). All samples were also subjected to mock desiccation at 100% RH (white bars). Mean values of three replicates, together with standard error, are shown; a one-way ANOVA with Tukey post-hoc test was performed: *, significance at $P < 0.05$.

Table 5.2. List of primers used to generate dsRNA strands in *Heterorhabditis bacteriophora*.

Gene name	Primer
Hba-tol-1T7F	taatacgactcactatagggagaCCGCTCAATTGTACGAAGGT
Hba-tol-1T7R	taatacgactcactatagggagaTCGAAATATTCAGCCAAAAGC
Hba-jnk-1T7F	taatacgactcactatagggagaGTCTTTTCTCGACCGTTTGC
Hba-jnk-1T7R	taatacgactcactatagggagaATCTAAGGCCGGGTCGTACT
Hba-daf-21T7F	taatacgactcactatagggagaTCGATTGCAGCCTTGCTAGT
Hba-daf-21T7R	taatacgactcactatagggagaAGTTCTGCTGGCTGGAAAAA
Hba-egl-8T7F	taatacgactcactatagggagaAACTTTCTGGGCTTCGACAA
Hba-egl-8T7R	taatacgactcactatagggagaAACACCAGAAGCAGCGAGAT
Hba-gpx-1T7F	taatacgactcactatagggagaAAGGAGGAGCACAAGGTCAA
Hba-gpx-1T7R	taatacgactcactatagggagaCTCCTGCAGGGACTCCTTC

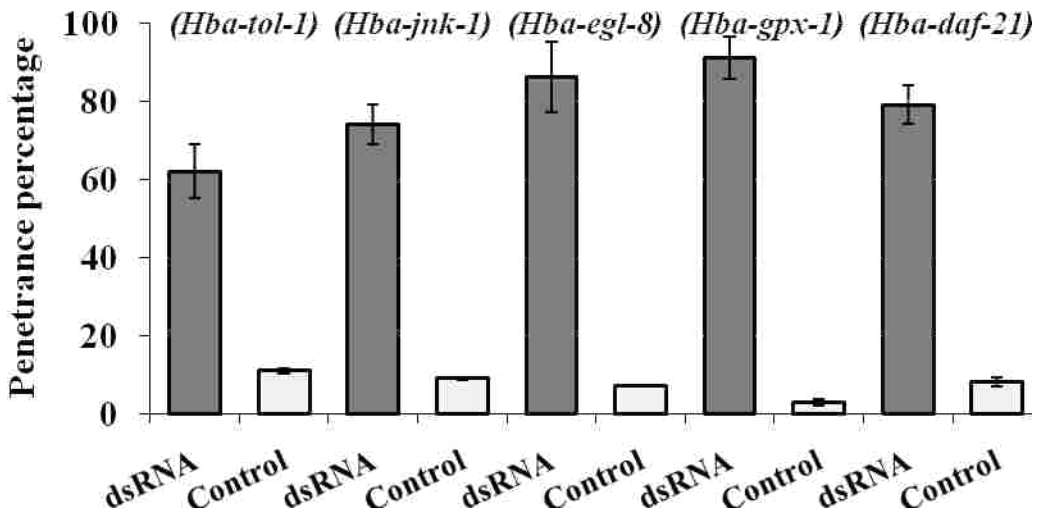
Penetrance percentage
100
80
60
40
20
0
(Hba-tol-1)
(Hba-jnk-1)
(Hba-egl-8)
(Hba-gpx-1)
(Hba-daf-21)
dsRNA
Control
dsRNA
Control
dsRNA
Control
dsRNA
Control
dsRNA
Control

Figure 5.5

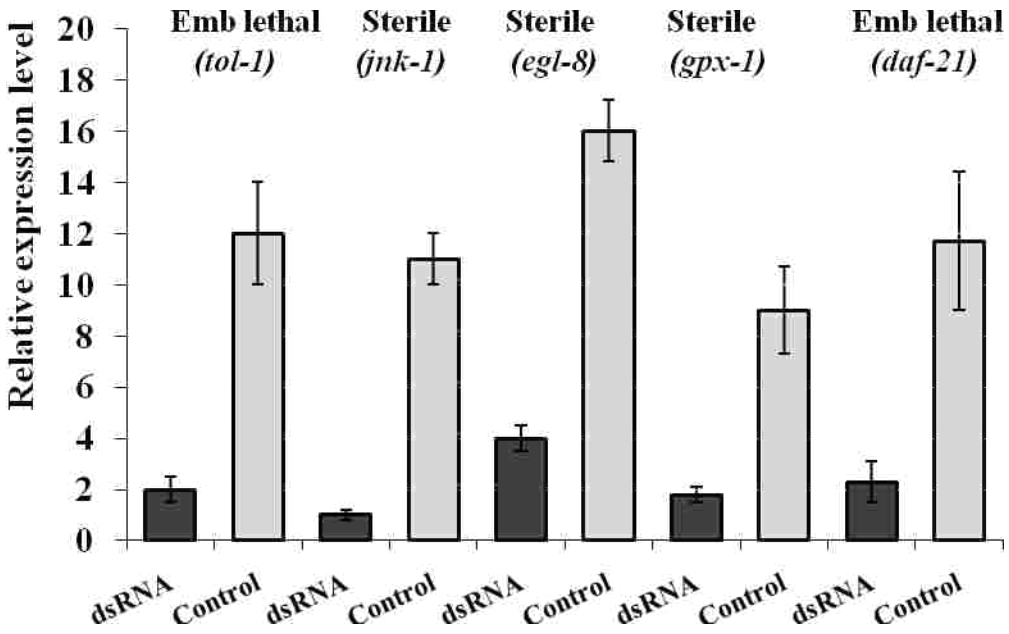
Relative expression level
20
18
16
14
12
10
8
6
4
2
0
Emb lethal
(tol-1)
Sterile
(jnk-1)
Sterile
(egl-8)
Sterile
(gpx-1)
Emb lethal
(daf-21)
dsRNA
Control
dsRNA
Control
dsRNA
Control
dsRNA
Control
dsRNA
Control

Figure 5.6

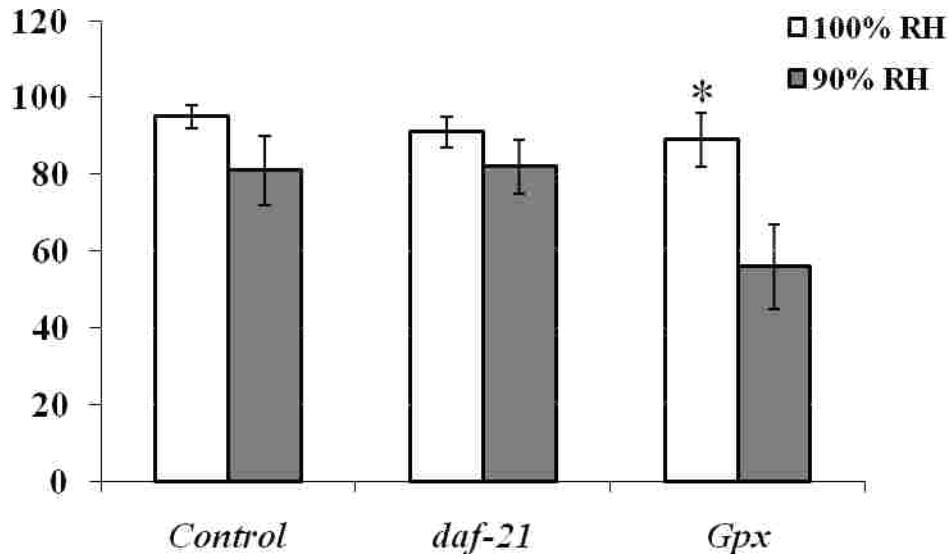

Figure 5.7

Manuscript to be submitted to *Molecular Ecology*

CHAPTER 6

Evolutionary and Ecological Stoichiometry of Phosphorus Content in Antarctic Nematodes

ADHIKARI, B. N.[1*], SIMMONS, B. L.[2], BALL, B. A.[3], WALL, D. H.[2] AND ADAMS, B. J.[1]

[1]Department of Biology, and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT 84604, USA

[2]Department of Biology, and Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO 80523, USA

[3]Environmental Studies Program, Dartmouth College, 6182 Steele Hall, Hanover, NH 03755, USA

*Corresponding author: Bishwo N. Adhikari (adhikaribn@hotmail.com)

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INTRODUCTION

All organisms are composed of multiple elements held together by various chemical bonds that are formed and broken during primary biotic processes (Williams 1997). During development organisms differ, for reasons not yet fully established, in their somatic elemental composition (e.g. carbon (C), phosphorus (P)). Such elemental ratios reflect underlying biochemical allocations that are produced to meet particular biological functions (e.g. growth & development). Despite the striking similarity in the elements involved in basic life processes (i.e. the elemental composition of substrates and enzymes involved in various metabolic reactions) all organisms, species and genotypes within species vary in their somatic elemental composition (DeMott *et al.* 2004; Forst *et al.* 2006). Furthermore, there is evidence of differential performance of species and genotypes under contrasting elemental supply environments. Sources of such variation can be behavioral, physiological or developmental in nature and all of which are coordinated by a combination of genetic and environmental drivers (Carroll *et al.* 2001).

Previous work has shown that there is biologically significant variation in the elemental composition of organisms that relates not only to structural features but also to the very core of biological function, the molecular biology of growth via production of ribosomal RNA. This idea is captured in the "growth rate hypothesis" (GRH), which states that variation in the P content (and thus C:P and N:P ratio) of living things is driven by variation in allocation to P-rich ribosomal RNA that accompanies differences in growth rate, as elevated ribosome allocation is generally needed to meet the protein synthesis demands of rapid growth (Elser *et al.* 1996a; Elser *et al.* 2002). Thus, any evolutionary process that results in changes in organismal growth or developmental rate may be manifested in changes in organismal C:N:P ratios, with potential consequences for that organism's sensitivity to stoichiometric food quality constraints and its

impacts on nutrient cycling. A genetic basis for such growth-related variations in RNA related P demand has also been proposed (Elser *et al.* 2002): increased growth rate and associated increases in transcriptional capacity for rRNA production are associated with changes in the rDNA, particularly in the length of the rDNA intergenic spacer (IGS) and/or in overall rDNA copy number (Weider *et al.* 2005a). Thus, a mechanistic thread has been proposed that extends from the organization of a major gene family (rDNA) through cellular allocation, to physiological nutritional demands, to trophic interactions and nutrient cycling in food webs.

A variety of recent findings from work on crustacean zooplankton and herbivorous insects have lent validity to the GRH. Several studies have shown significant positive correlations among various combinations of the proposed growth-RNA-P coupled system (Dobberfuhl & Elser 1999; Gorokhova & Kyle 2002; Main *et al.* 1997; Perkins *et al.* 2004; Schade *et al.* 2003; Vrede 1998) which have been reinforced by field observations (Elser *et al.* 2000b; Schade *et al.* 2003; Carrillo *et al.* 2001; DeMott *et al.* 2001; Ferraro-Filho *et al.* 2005). Positive associations among growth rate, body RNA and P content, and the presence of long rDNA intergenic spacer variants were observed in studies of various *Daphnia* species (Gorokhova & Kyle 2002; Weider *et al.* 2004). Lab experiments have also demonstrated context-dependent success of different rDNA variants in response to conditions of environmental and dietary P supply (Perkins *et al.* 2004; Weider *et al.* 2005a). Similarly, a number of studies have surveyed and/or examined natural variation in rDNA genome in number of organisms (i.e. Weider *et al.* 2005a) that links the connection of underlying genetic variation to potentially important environmental variables. Work on cultivated and wild barley has shown strong correlations between rDNA length variant and important environmental factors such as temperature and moisture availability (Allard *et al.* 1990; Gupta *et al.* 2002; Saghai-Maroof *et al.*

1990; Zhang *et al.* 1990). Similar work on wild emmer wheat shows that natural variation among rDNA loci are significantly correlated with important environmental parameters (Flavell *et al.* 1986). These studies, and several others, have suggested that rDNA copy number in a broad range of organisms is influenced by environmental parameters, have ecological significance, can affect key life processes including life-history traits (reviewed by Weider *et al.* 2005).

In this study we tested the GRH in natural populations of nematodes from the McMurdo Dry Valleys (MDVs) of Antarctica and analyzed the influence of elemental stoichiometry as an agent of selection on rDNA gene expression and genome evolution. The MDVs of Antarctica are an ice-free terrestrial landscape consisting of glaciers, ephemeral streams, ice-covered lakes and arid soils that are among the coldest and driest on Earth (Bockheim 1997), and where carbon and biologically available water are major limiting factors for life (Barrett *et al.* 2007; Kennedy 1993). The harsh environment and low availability of carbon and water support a simplified belowground mesofaunal community of rotifers, tardigrades, nematodes, and microarthropods near lakes and ephemeral streams, and even simpler communities in the arid soils that occupy the majority of the landscape (Adams *et al.* 2006; Wall 2005; Wall & Virginia 1999). Nematodes are the most widely distributed and biologically diverse invertebrates in the Dry Valleys (Treonis *et al.* 2000). *Scottnema lindsayae*, a microbial feeder and the most abundant and widely distributed metazoan invertebrate, often occurs as the sole metazoan species in the arid soils of the McMurdo Dry Valleys (Bamforth *et al.* 2005; Powers *et al.* 1998, Wall 2005; Wall & Virginia 1999). *Plectus murrayi*, a bacteria feeding nematode (Freckman & Virginia 1997), inhabits both semi-aquatic and terrestrial biotopes in the Dry Valleys. The distribution of *P. murrayi* in Antarctica is dependent on organic carbon and soil moisture (Powers *et al.* 1998) with high abundance in stream sediments (Treonis *et al.* 2000). In addition to the harsh physical

environment, the distribution of nematode communities is highly limited by nutrient availability (Freckman & Virginia 1997; Poage *et al.* 2008; Powers *et al.* 1998).

Elemental stoichiometry has provided a useful framework for understanding sources and controls of nutrient availability, and has been widely applied in the study of different ecosystems (Reiners 1986; Vrede *et al.* 2004; Redfield 1958; Hessen *et al.* 2004; Sterner & Elser 2002) including those of the Antarctic Dry Valleys (Barrett *et al.* 2007). Stoichiometric approaches are based on the general premise that organisms influence, and are influenced by the chemical composition of their environment, especially the relative availability of essential nutrients. It has been suggested that the cellular and biochemical machinery required for divergent life history strategies sets the stoichiometric requirements of individual organisms (Sterner & Elser 2002). In natural systems, P limitation may constrain the sizes of populations, and/or limit P sequestration by individuals. In laboratory experiments, it has been shown that RNA can increase rapidly with increasing P availability under P-limited conditions (Vrede *et al.* 2002). Higher RNA concentrations may in turn allow individual organisms to sustain higher rates of protein synthesis for reproduction and growth.

For our study we used field observations and molecular laboratory experiments with Antarctic nematodes to explore the validity of the GRH in natural populations of *Scottnema lindsayae* and *Plectus murrayi* from P-rich and poor environments. We quantified the level of rRNA transcription, rDNA copy number and body P-content, and relate that to nematode growth parameters. More specifically, the following questions were addressed: (i) Is there any effect of soil P-content on nematode body P-content and growth? (ii) Does variation in soil P-content have any effect on nematode gene expression (rRNA expression) and genome (rDNA) evolution? Our

goal was to test the GRH in a natural experiment and explore whether or not elemental stoichiometry could drive evolutionary changes in gene expression and genome organization.

MATERIALS AND METHODS

Study site

The study sites are in the McMurdo Dry Valleys, located in southern Victoria Land, Antarctica, and the principle region of study for the McMurdo Dry Valleys Long Term Ecological Research program (MCM-LTER; Fig. 6.1). Average annual temperatures range from -17 to -20°C, with an average precipitation of less than 100 mm (Doran *et al.* 2002). The soils are sandy, alkaline and typically saline (Campbell & Claridge 1987) with soil organic matter largely derived from contemporary and paleolake sediments (Burkins *et al.* 2000; Elberling *et al.* 2006). Samples were collected during 2008-2009 austral summer from two hydrologically distinct lake basins of Taylor Valley: Bonney (SSLB) (77°43' S, 162°18' E) and Fryxell (F6) (77°38' S, 163°06' E). These specific basins were chosen because they represent a gradient of soil properties and suitable habitat for biota, with F6 being more suitable than SSLB (Barrett *et al.* 2004; Wall & Virginia 1999). Lake Bonney lies at approximately 200 m in elevation and 25 km from the coast near the terminus of Taylor glacier; Lake Fryxell lies at approximately 50 m in elevation and 5 km from the coast. Soils around Lake Bonney tend to be slightly drier and warmer than soils around Lake Fryxell (Fountain et al., 1999) and less biologically productive in terms of nematode abundance and CO_2 respiration (Parsons *et al.* 2004).

Soil sampling and nematode isolation

Soil samples were collected using standard aseptic protocols developed specifically for MCM LTER soil studies (Freckman & Virginia 1997). Samples were placed in an insulated chest for transport to Crary Laboratory at McMurdo Station, Antarctica. Soils were sub-sampled

under a laminar flow hood to provide material for invertebrate and chemical analyses. Ortho-phosphate (PO_4^{2-}) content was measured by extracting 10 g of soil in 50 ml of 0.5M $NaHCO_3$ at pH 8.5. Extracts were shaken for 90 min at 170 rpm, then decanted into centrifuge tubes and spun at 27,216xg for 10 min. Supernatant was poured into Nalgene bottles and acidified to pH~2. The acidified filtrate was stored at -20 ºC, shipped to Dartmouth College, and analyzed on a Lachat QuikChem 8500 (Lachat Instruments).

RNA extraction and real-time polymerase chain reaction

Nematodes were extracted from sieved soil samples using a sugar-centrifugation technique modified for Antarctic soils (Freckman & Virginia 1997), identified to species under light microscopy based upon morphology and picked into RNAlater® (Ambion Inc.) solution. Nematodes preserved in RNAlater® were shipped to Brigham Young University, Provo for RNA extraction and further analyses. Nematodes stored in RNAlater solution were washed twice with 5% solution of Phosphate buffer saline (PBS) before RNA extraction. Total RNA for Quantitative real-time PCR (qRT-PCR) was extracted using Trizol reagent (Molecular Research Center Inc.). Three replications of each sample were used for RNA extraction, yielding three independent RNA extracts for each bulk sample. Nematodes were directly homogenized in liquid nitrogen, mixed with Trizol Reagent, and the suspension was exposed to three freeze thaw cycles using liquid nitrogen and 37ºC water bath. The suspension was ground using mortar and pestle and vortexed phase separated by using chloroform. After centrifugation (15 min, 12000 g, 4°C), the aqueous phase containing RNA was separated from the other phases, which were stored for DNA preparation (see below). The colorless upper aqueous phase was transferred into fresh vials to precipitate the RNA by addition of 100 ml isopropyl alcohol. The samples were incubated for 10 min and centrifuged (20 min, 12000 g, 4°C). The RNA precipitates were then washed twice

with 75% ethanol, air-dried, eluted in nuclease-free water, and quantified and quality-checked via spectrophotometer (A260/A280>1.9; NanoDrop ND-1000, NanoDrop Technologies, Thermo Fisher Scientific Inc.) and agarose gel electrophoresis.

Reverse transcription (RT) was performed with 1µg of total RNA from each specimen. RT reaction of polyadenylated mRNA to cDNA was done using the ImPromp-II™ reverse transcriptase (Promega Corporation) and random hexamer primer. Total RNA was incubated with 20 picamole (pmol) random hexamer primer at 70°C for 5 min and quickly chilled on ice. The reverse transcription mixture (20 µl) was mixed with RNA template and incubated at 25°C for 5 min for annealing and the first strand was extended for 60 min at 42°C. The cDNA was precipitated in 100% ethanol and washed twice with 75% ethanol, air-dried and dissolved in DEPC-treated water.

Quantitative real-time PCR was performed with LightCycler 480 SYBER Green I Mastermix (three replicate samples for each extraction) and gene specific primers in a Light Cycler 480 RT-PCR system (Roche Applied Science) equipped with LightCycler 480 software with the following program: 3 min at 95°C; 45 repeats of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C followed by a standard melt curve. The real-time PCR reaction had a final volume of 10 ml including SYBR Green Mastermix (Roche Applied Science), and template DNA. Negative control reactions containing water in place of cDNA were included in each batch of PCR reactions to monitor potential contamination. To minimize mRNA quantification errors, genomic DNA contamination biases, and to correct for inter-sample variation, we used nematode ß-actin, ß-tubulin and GAPDH genes as internal controls (Table S6.1).

Nematode body Phosphorus content

Nematode samples preserved in RNAlater were washed twice with 5% solution of PBS. Nematode samples were shipped to ALS Laboratory Group at Salt Lake City for total phosphorus analysis. The total nematode body phosphorus content was measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) with external calibrator according to company's standard protocol. ICP-MS is capable of extremely low detection limits for most elements. To test if preservation in RNAlater® had any effect on P-content of nematodes, samples containing 10, 50, and 100 *Heterorhabditis bacteriophora* nematodes stored in distilled water and RNAlater for two weeks were analyzed and compared for body P-content

Nematode biomass measurement

For biomass measurements, up to 10 adult *Scottnema lindsayae* of each sex from each plot at all experimental sites (N = 1,464) in 1999/2000 and 2004/2005 were photographed using SPOT 3.0 imaging software for microscope digital cameras (Diagnostics Instruments). The images were measured (lengths from tail to mouth, widths from just behind the basal pharyngeal bulb) using Carnoy 2.0 digital image analysis program (Biovolution). Biomass was calculated by determining average individual dry weights from lengths and width and corrected for water content using standard calculations (Andrassy 1956). Measurement was done on nematodes collected over several years to account for seasonal and annual variation.

Statistical analyses

We performed conventional statistical analyses in SAS 9.1 (SAS Institute Inc.) to test for variation in soil P-content, relative expression level, rDNA copy number, nematode body P-content and nematode biomass. Total biomass calculations were analyzed as mixed model in SAS 9.1. In qRT-PCR experiments, changes in target gene expression were calculated using

equation $2^{-\Delta\Delta CT}$ (Livak & Schmittgen 2001). The fold change in expression of the rRNA gene in nematodes from F6, normalized to the housekeeping genes and relative to those from SSLB, was calculated for each sample. The fold change in expression of rRNA in samples from SSLB was set as equal to one. An *F*-test at a significance level of $P < 0.05$ was used to compare the ratio of the mean gene expression of F6 samples with that of SSLB. For the relative change in target gene expression, the geometric average of the three internal control genes was used (Vandesompele *et al.* 2002).

Ribosomal DNA gene copy number was determined by absolute quantification. In order to determine the copy number of 18S rDNA, a single-copy reference gene, ribosomal protein L3, (*rpl-3*) was used. Since the *rpl-3* is a single copy gene that is highly conserved in nematodes (Moore *et al.* 1995), we selected this gene to use as the single-copy reference probe in the qRT-PCRs. Confirmation was done by using relative quantification (ratios of one gene to another) to determine the number of copies present per genome. Quantification standards were run in conjunction with each set of samples after primers and probes for the *rpl-3* and 18S rDNA genes were optimized for PCR amplification efficiency and relative efficiency of target and reference gene. Six serial 1:2 dilutions (20, 10, 5, 2, 1.25, and 0.625 ng/l) of genomic DNA were used to generate standard curves of *CT* (threshold cycle) value against the DNA concentration on each PCR plate for the *rpl-3* and 18S rDNA genes. Each experiment was performed three separate times from one DNA preparation and run in duplicate. *CT* values were determined and then converted into template quantity.

Absolute quantification requires that absolute quantities of standards first be determined by some independent means. In our study, nematode DNA was used to make absolute standards. Concentration and DNA quality were measured by determining the A260 and by gel

electrophoresis and converted to the number of copies by use of the molecular weight of the DNA. The equation $CT=m$ (log quantity) + b from the equation for a line ($y = mx + b$) was constructed by plotting the standard curve of log quantity versus its corresponding *CT* value. After the creation of standard curves, the copy number of each gene was determined by DNA quantification. PCR cycle numbers were plotted against the value of fluorescence signal, and then threshold values were plotted against the copy number of the template DNA that was used to generate standard curves. Comparative copy numbers were determined using the relative quantification ($\Delta\Delta$CT) $2^{-\Delta\Delta CT}$ method. The 18S rDNA copy numbers were determined by the absolute quantitation method, by which total copies were calculated using the following equation: total 18S rDNA copies = $10([CT - b]/m)$. The number of 18S rDNA copies per genome was then determined by the following equation: 18S rDNA copies per genome = (total copies of 18S rDNA)/ (total copies of *rpl-3*). Copy number was calculated as the ratio of template quantity for 18S rDNA to the template quantity for *rpl-3*.

RESULTS

Soil P-content variation

Six bulk samples were collected from each study site and analyzed for orthophosphate (P) content. The two sites exhibited significant differences in P-content ($P = 0.025$) while there was no variation within the site ($P > 0.05$). Bulk samples from F6 had higher P-content (4.36±1.91), around 8 times as much as the samples from SSLB (0.55±0.12) (Fig. 6.2).

Ribosomal RNA expression level

Expression of rRNA in both *S. lindsayae* and *P. murrayi* was measured by qRT-PCR method and expression level was normalized by using housekeeping genes. The relative change

in rRNA expression in nematodes from F6 relative to SSLB and normalized to housekeeping genes was calculated. Accordingly, the $2^{-\Delta\Delta CT}$ method single fold change in relative expression of rRNA in F6 is equal to the expression level of nematodes from SSLB. *Scottnema lindsayae* from F6 showed 5.42±0.41 fold change in rRNA expression while *P. murrayi* from the same location showed 5.93±0.18 fold change. There was no inter- or intra-generic variation in rRNA expression ($P > 0.05$) (Fig. 6.3).

Ribosomal DNA copy number

The rDNA copy number of both *S. lindsayae* and *P. murrayi* was estimated using qRT-PCR by with a single copy gene (Ribosomal protein L3 (*rpl-3*)) as a reference. We found significant differences in the rDNA copy number of *S. lindsayae* from F6 versus SSLB ($P = 0.007$). *Scottnema lindsayae* from F6 had 2.45 times as many copies of rDNA as those from SSLB ($P < 0.001$) (Fig. 6.4). Similarly, *P. murrayi* from F6 had 2.71 times as many copies of rDNA as those from SSLB ($P < 0.001$). There was a significant interaction between nematode species and sampling sites ($P < 0.001$). Nematodes from F6 exhibited strong inter-generic variation, and *P. murrayi* had significantly more copies of rDNA than *S. lindsayae*, but no intra-generic variation was observed (Fig. 6.4).

Nematode body P-content

Results of the test to see if preservation in RNAlater® had any effect on P-content showed that *H. bacteriophora* tend to have size dependent P-content, but that the preservation media had no effect on the body P-content of nematodes (Fig. S6.1). Results of the ICP-MS measurements indicated that *S. lindsayae* from F6 had 1.23 times greater body P-content than

those from SSLB (P = 0.007). Similarly, *P. murrayi* from F6 had 1.13 times more body P-content than those from SSLB (P = 0.002) (Fig. 6.5). No significant difference in body P-content was observed among bulk extractions from a particular site (P = 0.79) or for particular nematode genera (P = 0.660). The percent P-content of *S. lindsayae* was measured by dividing the corrected biomass by nematode body P-content. Nematodes from F6 (2.31-2.54%) had higher P percentage (P = 0.001) as compared to those from SSLB (1.01-1.22%; Fig. S6.1).

Nematode biomass measurements

The average body size of individual adult *S. lindsayae* was inversely proportional to the P-content of the basin, and nematodes were smaller in higher P-content soil at F6 compared to low P-content soil at SSLB. Individual biomass decreased over time; on average, nematodes were smaller in 2004 compared to those collected in 1999 (P < 0.01). While individual nematodes tend to be smaller at F6 compared to SSLB, this trend was only significant in 1999 (P < 0.01). Nematode biomass was higher at F6 for both years (P < 0.01) (Table 6.1).

DISCUSSION

Phosphorus content and growth rate

The link between cellular P and organismal growth as postulated by the GRH infers that high biomass P-content reflects an increased allocation to P-rich ribosomal RNA that is needed to meet the protein synthesis demands of increased growth rates. The physico-chemical properties of P predispose it to be a critical component of many key compounds (Westheimer 1987) that carry out basic biochemical processes. These central roles of P in the structure and functioning of primary biological components suggest that the lack of P and the subsequent

structural and functional consequences in these basic cellular components could affect individual fitness.

Organisms have evolved a variety of responses to P deficiency, which can be observed at multiple levels of organization. While we know that certain genotypes or species perform better under high P conditions (Jeyasingh & Weider 2005), we know little about the standing genetic variation in natural populations for growth performance under contrasting P regimes. In this study, we found a significant variation in the genome of two populations of Antarctic nematodes under contrasting P-supply environments, which is correlated with an altered growth rate and body size of these nematodes. This finding suggests that the amount of available P can strongly impact ontogeny and genome organization. Nematodes from F6 were comparatively bigger than those from SSLB. Possibly nematodes from F6 grew faster, took comparatively shorter time to reach maturity and started laying earlier as compared to those from SSLB. Although such a variation in growth rate and body size could be driven by factors other than those considered in this study, the differential demand for P in populations of Antarctic nematodes could be driven by geographical and climatic factors (Elser *et al.* 2000a). But based on our data, it is reasonable to hypothesize that such variation is due to considerable standing genetic variation in phosphorus acquisition and/or use efficiency (PUE) in nematode populations that is subsequently acted upon by natural selection.

Ribosomal DNA genome evolution

Ribosome biogenesis is one of the most central processes in cellular biology from a functional perspective because of its close connections to the pace of growth and development. Furthermore, rRNA synthesis represents a large energetic and nutrient sink for all growing organisms, representing ~80% of cellular RNA content and 20% of total cell dry weight

(Neidhardt *et al.* 1990). The rDNA repeat unit is composed of regions that are under different levels of selective constraint, and thus they vary substantially in their rates of divergence among species and in their levels of intraspecific polymorphism. For example, highly conserved core regions of the rRNA genes (the rDNA) are interspersed with variable domains or expansion segments that show high levels of divergence between species, as well as variation within populations and individuals. In our study, nematodes from SSLB tend to have significantly more copies of rDNA and total biomass, indicating strong coupling between soil P-content and rDNA genome duplication. Furthermore, nematodes from the P-rich environment had higher levels of rDNA transcription, as indicated by higher rRNA expression, which could be a mechanism to support faster growth rate. The association of P-rich rRNA and growth rate has drawn attention to the role of rRNA genes (the rDNA) in supporting elevated production of rRNA for rapid growth (Elser *et al.* 2000b). For example, variations in rDNA genotype, i.e. rDNA copy number (White & McLaren 2000) have been implicated as responsible for differences in rRNA synthetic capacity and concomitant variation in P content and growth rate (Kay *et al*. 2005).

In eukaryotic organisms, the increase in repetitive DNA, and in particular rDNA, contributes largely to genome size variation (Prokopowich *et al.* 2003). Because duplicate genes both increase the diversity of gene expression in an organism and evolve faster than do single-copy genes (Gu *et al.* 2004), the increase in repetitive rDNA may play an important role in the evolution of growth rates and ecological tolerances related to nutrient requirements. Indeed, genome size variation is correlated with environmental conditions and geographic distributions in a variety of species (Bottini *et al.* 2000). Our results indicate that the evolution of rDNA copy is correlated with nematode biomass, soil P-content and growth rate (as indicated by rRNA expression). Additionally, we show that rDNA variation can be connected not only to growth

itself but also to the elemental composition of living biomass. It is clear from our results that there may be an intrinsic link between the stoichiometric demands of an organism and its critical molecular structures (i.e., nucleic acids such as RNA) that affect key organismal processes, including life history traits.

Soil P-content variation

Phosphorus concentrations were greater in Fryxell basin compared with the Lake Bonney basin (Figure 6.2) yet, no difference in P-content was observed within each basin, indicating uniformity of soil sediments within the lake basin. The differences in P-content may be explained by the differences in *in situ* physical, chemical and biological processes. The lower P content at SSLB may be due to the less intense weathering and generally lower biological activity as compared with F6, or less retention of weathered P fractions in older tills (Barrett *et al.* 2004). In addition, soils in the Fryxell basin are reported to have higher concentrations of weathered P fractions relative to the Lake Bonney basin (Blecker *et al.* 2006) and thus have the higher bulk soil P and biologically available P.

Phosphorus is an essential nutrient for terrestrial and aquatic ecosystems and is widely limiting to primary productivity. Our data indicate that Antarctic nematodes exhibit strong stoichiometric homeostasis and thus most variation in biomass P content seen under natural conditions is probably a reflection of variation in soil P-content even under differential P availability. We observed that nematodes from P-limiting conditions had lower rRNA expression and fewer copies of rDNA as compared to those from P-rich environments.

Ribosomal RNA as a proxy for Growth rate

For several P-rich and fast-growing organisms, RNA constitutes a major fraction of body P, suggesting a close association between the P content and growth rate (Andersen & Hessen

1991; Sterner & Hessen 1994; Elser *et al.* 1996b). RNA is especially interesting in the light of recent work on the biochemistry of growth. Elser et al. (2000) have proposed direct links between growth rate and cellular contents of ribosomes and rRNA. Ribosomes, the protein synthesis centers of the cell, are required in large numbers for fast protein synthesis and growth (Lewin 1994). RNA is almost 10% phosphorus, meaning that ribosomes are the most phosphorus-rich major cellular component (Elser *et al.* 1996a; Lewin 1994). We observed strong coupling of P availability with rDNA copy number and ultimately to rRNA gene expression in both nematode species we assayed. Nematodes from P-rich conditions also had higher body P content, indicating the possibility that observed changes in body phosphorus content are partially caused by increases in ribosome numbers through increased expression of rRNA, which could have resulted in an increased growth rate. Several studies have shown that rRNA is a particularly attractive proxy for growth rate because the efficiency of ribosomal protein synthesis is thought to vary relatively little; therefore cellular ribosome (or rRNA) content can be expected to reflect cellular protein synthesis rate (Bremer & Dennis 1996). This synthesis rate should in turn be closely related to growth rate as reported in many organisms (Bremer & Dennis 1996; Keener & Nomura 1996). If such an assumption holds true, our results support rRNA expression as a strong proxy for growth rate measurement in nematodes.

Growth rate hypothesis and assumptions

The growth rate hypothesis (GRH) (Elser *et al.* 2000b; Elser *et al.* 1996b) proposes that elevated demands for increased allocation to P-rich ribosomal RNA under rapid growth drives variation in the P content of many biota (Elser *et al.* 2000a). Specifically, the GRH predicts the following: (1) faster growing organisms should have higher RNA content and (2) as a result,

rapidly growing organisms should have higher levels of P (i.e., low C: P and N: P ratios) in their bodies than slower growing organisms because of the increased allocation to P-rich RNA. Elser *et al*. (2000b) have extended the hypothesis to the genetic level, arguing that fast growth rate and high RNA allocation demand high rates of RNA production, and thus variation in the genes coding for ribosomal RNA (the rDNA) are to be expected. In particular, rDNA copy number should be positively correlated with growth rate because the higher the rDNA copy numbers the greater the potential for transcription (i.e., rRNA production) supporting ribosome production is needed for rapid growth. These assumptions seem to be met for a wide range of organisms (Acharya *et al.* 2004a,b; Elser *et al.* 2003b; Vrede *et al.* 2002; Weider *et al.* 2005a) and our data from a natural experiment also showed that the populations of Antarctic nematodes evolve as predicted by these assumptions.

Patterns of variation in body and soil P indicate that Antarctic nematode body P was strongly influenced by spatial variation in soil P availability. Positive correlations between body and soil P within both P-limited and P-rich sites suggest that the nematodes responded strongly to spatial variation in soil P with changes in rDNA copy number. Patterns of P and rDNA copy number in both Antarctic nematodes reflected patterns in soil available P and rRNA expression as predicted by the GRH. *Plectus* exhibited higher rDNA copy number than *Scottnema lindsayae* indicating the possibility of differential response of nematodes to different stoichiometric conditions. Such differences between genera could be due to differences in mode of reproduction (*Scottnema* is amphimictic while *Plectus murrayi* is strictly parthenogenetic), niche differences and differential stress survivability. Our results met all the assumptions of the GRH including rRNA content and body P-content. But the nematodes from F6 were larger than those from SSLB which could sound contradictory to the assumption of GRH. But it is possible that the

cells in the larger animals are simply larger- perhaps they are compensating for a lower concentration of P for cellular functions. Nematodes from F6 could have grown faster, reached maturity, and started laying eggs earlier than those from SSLB. Nematodes from likely SSLB grew slower and took a comparatively longer time to reach maturity. These results indicate that adaptations to specific habitats and environmental conditions may underlie differences in nematode body size. As the Antarctic environment is severely limited by different types of stress and essential nutrient availability, the combined effect of these factors strongly influences stoichiometric elemental requirements and utilization by nematodes.

Ecological and evolutionary implications

Previous studies have highlighted how structural and regulatory features of rDNA operate to affect key aspects of organism function, such as the life history traits of diverse biota (Weider *et al.* 2005b). In particular, it has been shown that variations in rDNA structure and expression are commonly linked to the challenge of maintaining a high rate of rRNA production associated with rapid cellular proliferation. By employing the perspective of biological stoichiometry we showed how ecological forces, such as the supply of the key limiting nutrient P, impinge on evolutionary change involving rDNA, as well as potential feedbacks generated by the coupling of rDNA to growth and rRNA expression. Similar to our findings, other workers have noted genera-specific and intra-population differences in the growth rates and stoichiometric ratios of various zooplankton taxa. Specifically, growth rate was positively correlated with %P, body size and rDNA copy number (Elser *et al*. 2000b; Gorokhova *et al*. 2002; Kay *et al*. 2005). However in our case, these same relationships did not hold true for the body size of the nematodes. Thus, it appears that different organisms have different approaches for allocating resources among growth and biochemical pools containing P and adaptations to specific habitats and

environmental conditions may underlie differences in body size. It is conceivable, then, that genotypes within individual populations of Antarctic nematodes are influenced by the stoichiometric food quality limitation in the field.

We also document significant variation in P-content among different populations of nematodes. Previous studies have shown that different populations can have different %P levels in their body tissues, with some values of %P varying by as much as 40% between populations (Elser *et al.* 2007). Such findings suggest that populations with differing P demands may be favored in environments that differ in stoichiometric food quality, as has been postulated and documented by past studies. Such differences suggest evolutionary constraints among Antarctic nematodes and require further study with additional species. Likewise, we detected intraspecific differences in P-content in *P. murrayi*. Such genetic variation fuels the microevolutionary process leading to potential shifts in the competitive dominance of different genotypes and ultimately to population genetic changes through time. As pointed out by (Elser *et al.* 2000a), these types of links between sub-organismal processes (i.e., growth rate) and higher level (i.e., population, ecosystem) processes influence the evolutionary potential of genotypes and, thus, establish a basis of reciprocal feedbacks between ecosystem conditions and microevolutionary change.

It is clear from the present study that much additional work is needed to clarify the potential associations among growth, body size, rDNA evolution, and P-content that our limited data have suggested. More species need to be examined to determine the robustness of these findings. More sophisticated work is also needed to unravel the specific mechanisms connecting rDNA evolution, and rRNA expression ultimately leading to impacts on organismal growth rates. Future work should therefore focus on study of the mechanisms underlying rDNA evolution and

rRNA expression, which would involve examination of a number of taxa from different environments. Appreciation of the mechanisms generating genomic variation and the consequences of that variation permit us to understand the role of stoichiometric relations in the organization of trophic interactions and nutrient cycling over evolutionary time.

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FIGURE LEGENDS

Figure 6.1 Location of study area in the McMurdo Dry Valleys, Antarctica. The filled circles denote the sampling sites.

Figure 6.2 Ortho-phosphate (PO_4^{2-}) content of the soils from Lake Fryxell (F6) and South Side Lake Bonney (SSLB). The bars show mean ± SE of two pooled samples of three replicates each of the bulk samples from two sites. Asterix indicate significant difference (*P<0.05*; N=3)

Figure 6.3 The relative expression of rRNA of *Scottnema lindsayae* and *Plectus murrayi* from Lake Fryxell (F6) normalized to housekeeping genes and relative to nematodes from South Side Lake Bonney (SSLB). Fold change in expression of rRNA in samples from SSLB is equal to one. Bars represent mean ± SE of three replicates.

Figure 6.4 Ribosomal DNA (Small subunit) copy number variation in Antarctic nematodes *Scottnema lindsayae* and *Plectus murrayi* from Lake Fryxell (F6) and South Side Lake Bonney (SSLB). Each bar represent the mean ± S.E. of three replicates and bars with different numbers of Asterix indicate significant difference (P<0.05).

Figure 6.5 The PO_4 content of *Scottnema lindsayae* and *Plectus murrayi* from Lake Fryxell (F6) and South Side Lake Bonney (SSLB) as measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). The bars show mean ± SE of two pooled samples of three replicates each and Asterix indicate significant difference between samples from F6 and SSLB.

Supporting information

Table S6.1 Primers used for qRT-PCR analysis.

Figure S6.1 Percentage phosphorus content of *Scottnema lindsayae* from Lake Fryxell (F6) and South Side Lake Bonney (SSLB).

Figure S6.2 The PO_4 content of bulk samples of *Heterorhabditis bacteriophora* stored in RNAlater® (RL) and washed with water after 2 weeks storage in RNAlater®, versus those washed only with water (W). The bars show mean ± SE of three replicates of bulk samples of 10, 50 and 100 nematodes.

Table 6.1 Size and biomass of the nematodes extracted from F6 and SSLB.

	SSLB			**F6**		
Year	Length (µm)	Diameter (µm)	Biomass (µg)	Length (µm)	Diameter (µm)	Biomass (µg)
1999	655.11±2.63	34.91±0.16	0.13±0.001	610.93±1.82	32.51±0.13	0.098±0.001
2004	638.87±3.78	32.15±0.002	0.11±0.002	612.86±2.53	32.19±0.16	0.086±0.001

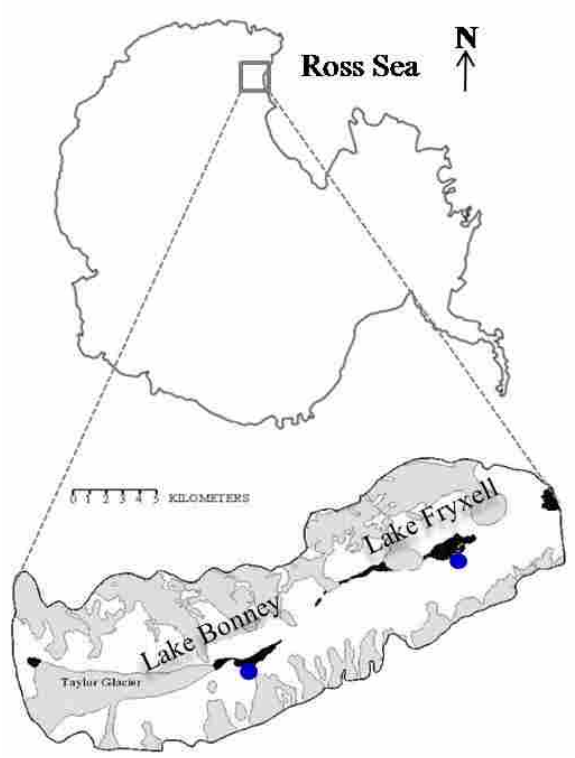
Ross Sea
N
KILOMETERS
Lake Fryxell
Lake Bonney
Taylor Glacier

Figure 6.1

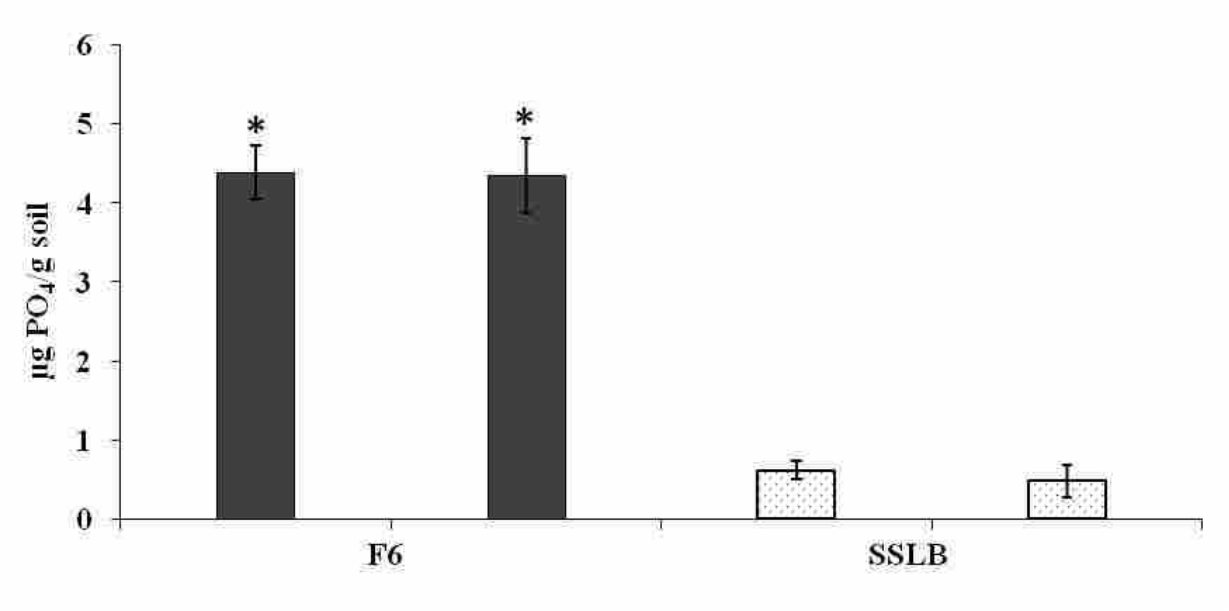

Figure 6.2

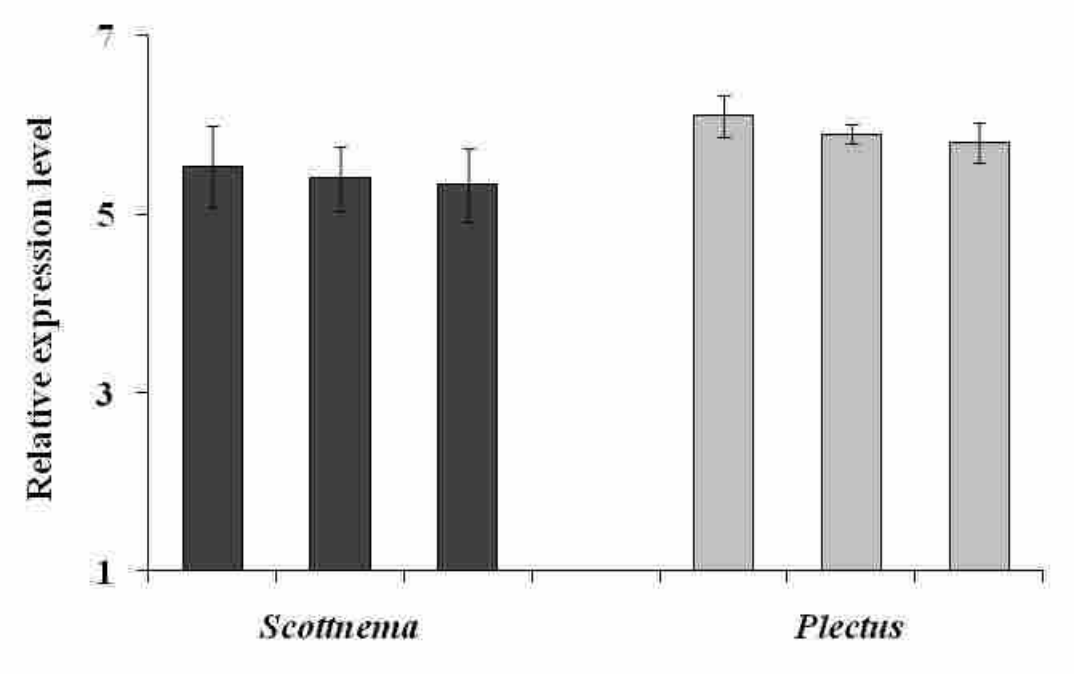

Figure 6.3

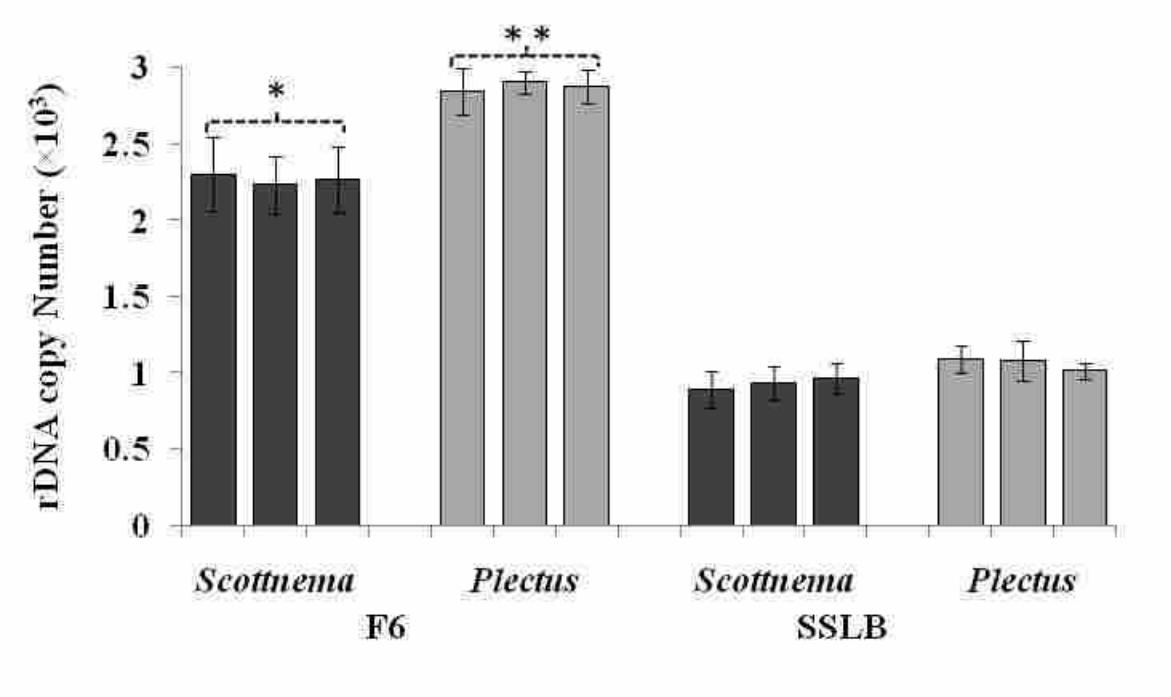
rDNA copy Number (×10³)
3
2.5
2
1.5
1
0.5
0
*
**
Scottnema
Plectus
Scottnema
Plectus
F6
SSLB

Figure 6.4

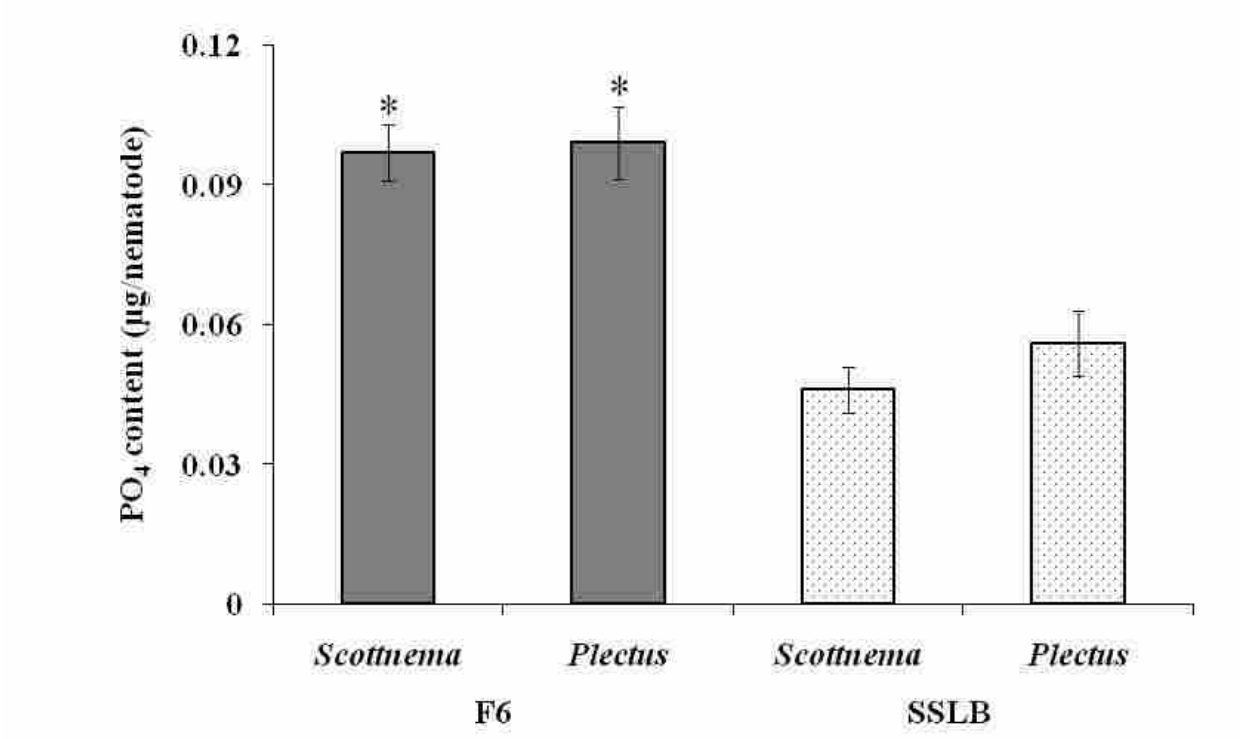
0.12
0.09
0.06
0.03
0
PO4 content (µg/nematode)
*
*
Scottnema
Plectus
Scottnema
Plectus
F6
SSLB

Figure 6.5

Table S6.1 Primers used for qRT-PCR analysis

Coding regions	Forward primer (5'-3')	Reverse primer (5'-3')
18S Ribosomal	ACTTTGAAGAGAGAGTTCAAGAG	TCGGAAGGAACCAGCTACTA
β-actin	GTGCCGTATTCCCTTCTATCGT	GATACCGTGCTCAATTGGGTACT
ß-tubulin	TGAATCAGATCTTCAACTTGAAAGGA	TTCCAGGCTCGAGATCAACAA
GAPDH	AAAGTACGCACAAAATCAAAGTTCA	TTCTTGGCACCACCCTTTAAA

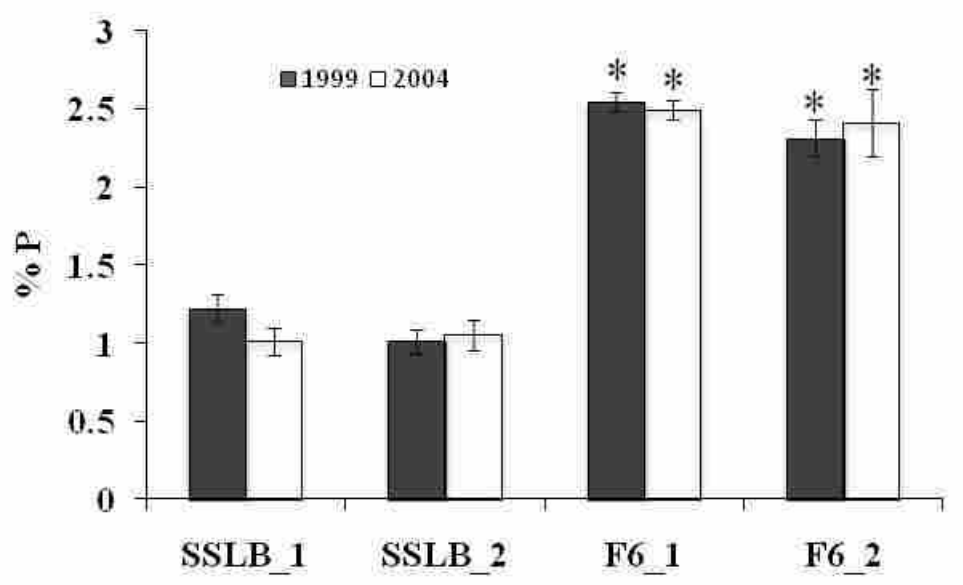

Figure S6.1

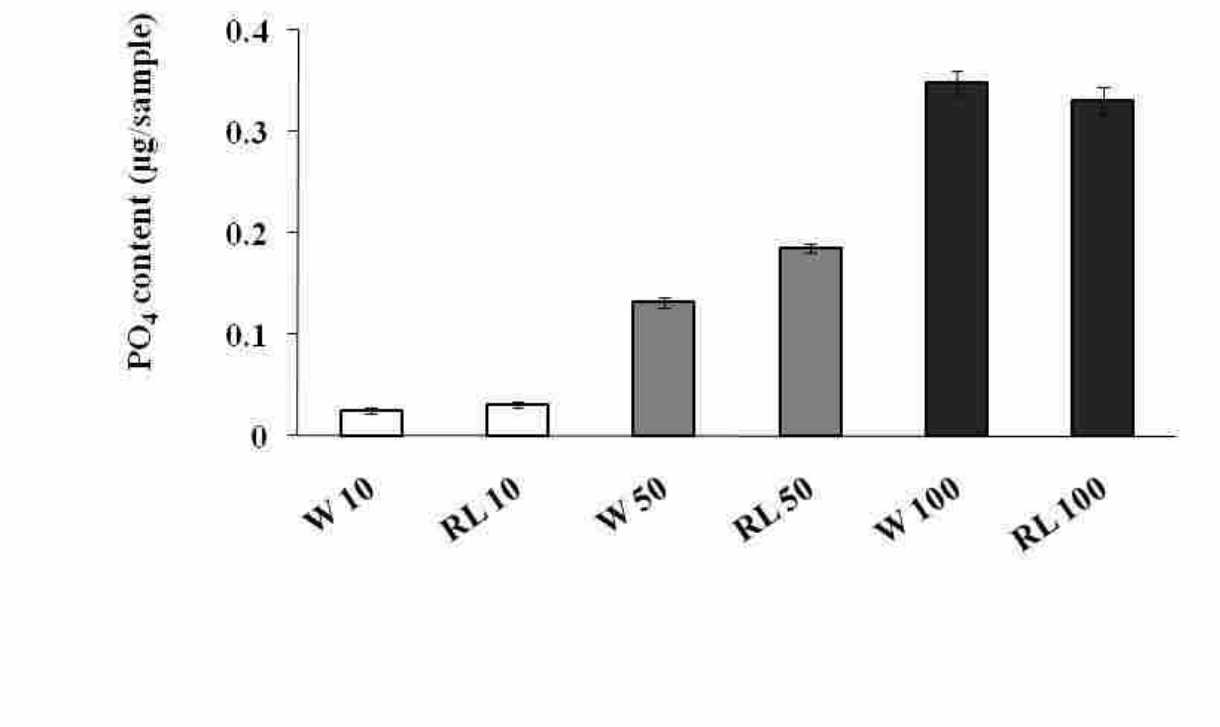
PO_4 content (µg/sample)
0.4
0.3
0.2
0.1
0
W 10
RL 10
W 50
RL 50
W 100
RL 100

Figure S6.2