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Establishing the Microcrustacean *Daphnia* as a Model System for Research on Aging

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ESTABLISHING THE MICROCRUSTACEAN *DAPHNIA* AS A MODEL SYSTEM FOR RESEARCH ON AGING

by

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Dedication

To my family, Melanie Schumpert, Charles B. Schumpert, Bridgett Schumpert,
Carly Dickey, Brendan Dickey, Tony Hunt, Fran Hunt, Alicia Lawson, and Grippie Lawson.

Acknowledgements

First I would like to thank my advisor Dr. Patel who has been beyond helpful in providing constructive criticism, brilliant ideas and making sure that I was on track in my studies. She was always open to new ideas and allowed me to follow my own interests within my research project, which has helped me develop as a scientist over the course of my graduate student career. Not only was she dedicated to making sure I became the best scientist I could possibly be, she also cared that I excelled in other aspect of my life as well, such as teaching and mentoring undergraduates in the laboratory. Overall, Dr. Patel has been the best mentor I could have ever hoped for and I'll forever be grateful for the guidance and opportunities that she has given me over the years.

My committee members have also been extremely helpful in providing wonderful insight for my project. Dr. Dudycha has provided large amounts of *Daphnia* over the years as well as provided crucial feedback on many manuscripts including help with statistical analysis of several datasets. Dr. Reisman, Dr. Smith and Dr. Outten have all provided much needed feedback during committee meetings and have always been willing to help (even by providing needed reagents or equipment). All of my committee members have gone above and beyond their duty to help me succeed as a graduate student and a scientist.

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for years to come. Not only were we able to support one another through trials and tribulations (repeat after repeat of experiments), we were able to enjoy our time in the lab while also learning what it takes to be a successful part of a scientific laboratory. From Maddios Monday to trips to Cool Beans for a “Perfect Woman” the time spent together has made for an exciting and enjoyable graduate student career.

Lastly, I would like to thank the other graduate students in the department that have helped out in various ways, either through equipment use, needed reagents, or just through support and ideas.

Abstract

Aging is a ubiquitous process pertaining to all biological systems around the planet. Although much has been learned from studies so far on the molecular mechanisms that lead to aging, a complete understanding of a healthy life span and longevity still eludes us. In this dissertation, we will examine the use of a freshwater microcrustacean *Daphnia* as a model system for studies on the biology of aging. The Introduction chapter presents a review of the general molecular alterations associated with cellular and organismal aging, and discusses the core model organisms currently used to study the aging process. The introduction chapter includes a brief discussion of the advantages offered by *Daphnia* as a model system. The chapters 2-5 present the results of our experiments using *Daphnia* that examine the pathways established to play a regulatory role in the aging process. The Chapter 2 presents a comparison of the telomere length, telomerase activity, and telomerase processivity in the two ecotypes, the short-lived *Daphnia pulex* and the long-lived *Daphnia pulicaria*. The Chapter 3 presents a study of the heat shock responses in both short- and long-lived ecotypes. Chapter 5 describes a new method for fast and effective RNA interference in *Daphnia*. This method is expected to be widely useful for all *Daphnia* biologists, as no method was yet available for RNAi in adult *Daphnia*. Chapter 4 describes a characterization *Daphnia* Sir2 mRNA levels and activity during life span and examines the effects of RNA interference mediated Sir2 knockdown on lifespan and survival following proteotoxic

stress. Overall, in this thesis we establish *Daphnia* as a new model organism for research on aging and offer novel insights into stress response and telomerase pathways in *Daphnia*.

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List of Abbreviations

3L2-1.....	Three Lakes Two Clone One
Bp	Base Pairs
CE	Cellular Extract
dsRNA.....	Double Stranded RNA
EMSA	Electrophoretic Mobility Shift Assay
EV	Empty Vector
FRTA	Free Radical Theory of Aging
GAPDH.....	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
HI	Heat Inactivated
HSE	Heat Shock Element
HSF	Heat Shock Factor
Hsp70	Heat Shock Protein 70
HSR	Heat Shock Response
IIS.....	Insulin and IGF-1 signaling
IPTG	Isopropyl Beta-D-1-Thiogalactopyranoside
Kb	Kilobases
Kd	KiloDalton
mTOR.....	mammalian Target of Rapamycin

NM.....	Non-melanin
ORF.....	Open Reading Frame
PANTHER.....	Protein Analysis Through Evolutionary Relationships
RNAi.....	RNA Interference
RW20.....	RoughWood 20
Sir2	Sirtuin 2
TCO.....	The Chosen One
TERC	Telomeric RNA Component
TERT	Telomerase Reverse Transcriptase
TPG.....	Total Product Generated
TRAP	Telomeric Repeat Amplification Protocol
TRBP	TAR RNA Binding Protein
TRF.....	Terminal Restriction Fragment
XVI-11.....	Lake Sixteen Clone 11

Chapter 1: Introduction¹

¹ Schumpert C, Dudycha J, Patel R. 2015. *Daphnia* as a Model Organism for Research on Aging. In Preparation.

1.1 General Introduction

From antiquity, the process of aging has captivated humans. Aging can be defined as the process of growing old and over the course of our own lives we develop a personal understanding of what it means to age but defining aging in scientific terms is not as straightforward. Aging usually refers to the biological process of growing older in a deleterious sense. It represents the gradual and irreversible changes in an organism that normally occur in a time-dependent manner, are intrinsic to the particular species, cause a decline in overall function thereby increasing the probability of death. Aging is a multifaceted biological process (Kenyon 2010, Lopez-Otin et al 2013) and is defined as a process of cellular senescence that results in compromised stress response, greater homeostatic imbalance, and elevated risk of disease (Rakyan et al 2010). Collectively, aging results in a gradual biological dysfunction resulting in the eventual death of the organism (Rakyan et al 2010, Lopez-Otin et al 2013).

Aging can be studied at multiple levels, including organismal, cellular, and mechanistic (Kirkwood 2005, Gems and Partridge 2013). The organismal and cellular of aging obviously originates from the gradual time-dependent changes in basic molecular functions. Study of such molecular alterations that are associated with aging in various model organisms has indicated that a decrease in mitochondrial efficiency leading to a decline in ATP production (Johnson et al 1999, Kenyon 2010, Lopez-Otin et al 2013), an increase in aberrant mRNA maturation (caused in part by a deregulation of alternative splicing), an increase in mutations in the nuclear as well as the mitochondrial genome, increased production of reactive oxygen species, and an increase in altered or misfolded

proteins with age (Bahar et al 2006, Harries et al 2011, Green et al 2011, Nicholas et al 2010, Cui et al 2012, Powers et al 2009, Morimoto and Cuervo 2009) are some of the characteristics of cells in aged organisms. Thus, there are numerous molecular changes that occur either as a result of aging or are causative in the aging process.

Recently, Lopez-Otin et al have named particular molecular hallmarks that signify the aging process (Lopez-Otin et al 2013). These nine hallmarks encompass various aspects of aging and include genomic instability, telomere attrition, epigenetic alteration, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (reviewed extensively in Lopez-Otin et al 2013). The hallmarks of aging were formulated by exhaustive work on various model organisms each with particular strengths and weakness in relation to human aging (Lopez-Otin et al 2013, Tissenbaum and Leonard 2002, Partridge and Tower 2008, Vanhooren and Libert 2013). In our current work, we provide a comprehensive review of the nine described hallmarks of aging, various model organisms, and the major theories of aging, followed by an examination of *Daphnia* as a model organism in aging research (Dudycha and Tessier 1999, Schumpert et al 2014, Kim et al 2014, Schumpert et al 2015). As *Daphnia* becomes an established model organism, potentially novel aspects of the molecular processes involved in aging could be elucidated using the small freshwater crustacean as a model.

1.2 Hallmarks of Aging

1.2.1 Genomic Instability:

Genomic damage and as a result genomic instability increases with age and thus far is associated with the aging process of every organism surveyed (Lopez-Otin et al 2013, Hoeijmakers 2009). An organism's DNA, both in the nucleus as well as mitochondria, is constantly being bombarded by various insults from outside and inside the cell (Hoeijmakers 2009, Moskalev et al 2012). These insults lead to different kinds of damages and mutations that accumulate with age including but not limiting to point mutations, potential translocations and pyrimidine dimers (Hoeijmakers 2009). Normally genomic mutations are coped with by a variety of repair mechanisms; however, the repair mechanisms become less efficient with age and are unable to effectively cope with these insults over time and the resulting accumulation of mutations can lead to altered gene expression and compromised cellular function. (Moskalev et al 2012).

1.2.2 Telomere Attrition:

The ends of linear chromosomes, termed telomeres, are essential for proper chromosome stability and are added and maintained by the enzyme telomerase (Morgan 2013). DNA polymerases cannot replicate DNA in a 3' to 5' direction and hence in cells that do not contain active telomerase, each DNA replication event leads to a progressive shortening of these protective structures (Shay and Wright 2000). Once telomeres erode, genomic instability ensues often resulting in cellular senescence and eventual death of the cell (Shay and Wright 2000). Hence, the presence and proper

maintenance of telomeres is essential for cellular health and life span in an organism. In mice, shortening of telomeres leads to shortened lifespan while lengthening telomeres extends lifespan (Blasco et al 1997, Armanios et al 2009). Correlative studies have also demonstrated that human telomeres shorten with age (Blasco 2007a).

1.2.3 Epigenetic Alterations:

The epigenome of an organism changes with age. In general, DNA methylation, histone modifications (along with chromatin structure), and microRNA profiles have been studied with respect to aging. Global DNA methylation, which occurs on cytosines within CpG islands, becomes dysregulated as an organism ages; some regions of the genome become hypomethylated with other regions exhibit hypermethylation (Maegawa et al 2010). Histone modification patterns, including methylation and acetylation marks, change in an age-dependent manner and this change is implicated in the aging process with many chromatin-remodeling proteins (such as the Polycomb complex) showing decreased expression with age (Pegoraro et al 2009, Pollina and Brunet 2011). MicroRNA profiles also change with age, with expression of many miRNAs correlated to aging (their targets are often longevity factors/genes) and are hence termed gero-miRs (Boulias and Horvitz 2012, Ugalde et al 2011).

1.2.4 Loss of Proteostasis:

Protein homeostasis (proteostasis) has been heavily investigated in the context of aging and numerous studies from various model organisms indicate an age-dependent increase in altered proteins in the cells with a concomitant decrease in the function of protective mechanisms that maintain protein homeostasis (Morimoto and

Cuervo 2009, Powers et al 2009). One such protective mechanism, the heat shock response, induces the expression of molecular chaperones that renature damaged proteins as well as target proteins for degradation (if the damage is too great to effectively renature the protein). As the heat shock response declines with age, there is an accumulation of damaged and otherwise altered proteins resulting in compromised cellular function (Powers et al 2009). Multiple studies indicate that the dysregulation of protein homeostasis leads to the phenotypes and pathologies associated with aging (Clancy and Birdsall 2013, Cui et al 2011, Powers et al 2009). There is also an associated decline in the proteasome and autophagy systems with age, providing another mechanism for the age-associated accumulation of altered and damaged proteins (Rubinsztein et al 2011, Tomaru et al 2012).

1.2.5 Deregulated Nutrient Sensing:

Multiple signaling pathways regulate the response of cells in unicellular organisms as well as in various organs of multicellular organisms to nutrients. These pathways are evolutionarily conserved and help the organism respond to their changing environmental status in regards to food availability (Barzilai et al 2012, Fontana et al 2010, Kenyon 2010). These pathways include the insulin and IGF-1 signaling (IIS) pathway, the mTOR pathway, AMPK pathway and sirtuins (Barzilai et al 2012, Fontana et al 2010, Kenyon 2010). The IIS pathway and mTOR pathway are both activated when nutrients are relatively in abundance and signal anabolism while AMPK and sirtuins are activated when the resources are limited, signaling catabolism (Fontana et al 2010). In almost every model examined thus far, caloric restriction extends lifespan of organisms

(which results in inhibition of both IIS and mTOR pathways, depending on the method of caloric restriction) (Fontana et al 2010). In general, a reduction in anabolic nutrient sensing signaling leads to healthy aging and an overall extension of lifespan (activation of catabolic pathways have also been implicated in extending lifespan as well) (Fontana et al 2010).

1.2.6 Mitochondrial Dysfunction:

The mitochondrion is an essential organelle charged with energy production through generation of ATP for all biological processes that take place in the cell (Green et al 2011). There is evidence that the efficiency of the electron transport chain located in the mitochondria and responsible for ATP generation, declines with age (Green et al 2011). This results in sub-optimal levels of ATP generation as well as an increase in reactive oxygen species, which damage other macromolecular components of the cell by oxidation (Harman 1956, Green et al 2011). The reduction in ATP also leads to lower turnover of damaged, inefficient mitochondria and leads to an accumulation of dysfunctional mitochondria resulting in generation of even more reactive oxygen species (Wang and Klionsky 2011). In mammals, an increase in mitochondrial dysfunction leads to a shorter lifespan (Kujoth et al 2005). Although mitochondrial dysfunction is implicated in the aging process, the mechanistic details are not clearly worked out thus far (Kujoth et al 2005).

1.2.7 Cellular Senescence:

The numbers of senescent cells in an organism increase with age. Senescence is defined as an irreversible arrest in the cell cycle and Hayflick was the first to describe

senescence in cultured primary cells (Hayflick and Moorhead 1961, Campisi and d'Adda di Fagagna 2007, Lopez-Otin et al 2013). Cells can become senescent from 50 different mechanisms that include telomere attrition (briefly described previously) and DNA damage (via p53 activation) (Gorgoulis and Halazonetis 2010). Tissues from multiple model organisms exhibit an increase in senescent cells, however not all tissues display this characteristic with age (Hoenicke and Zender 2012, Kang et al 2011). An accumulation of senescent cells in tissues may not directly contribute to the aging process but may be a protective mechanism adopted by organisms to shield the non-senescent cells and the tissue/organ function from the deleterious effects of damaged cells in the tissue (Lopez-Otin 2013). This challenging issue is yet to be addressed experimentally.

1.2.8 Stem Cell Exhaustion:

Studied in aging mice and noted in humans, there is an age-associated decline in the regeneration of adult tissues (Lopez-Otin et al 2013). Stem cells responsible for the regeneration of adult tissues become quiescent and non-functional with age as a result of an accumulation of DNA damage, telomere erosion, and potentially other aging related mechanisms listed above (Rossi et al 2007, Flores et al 2005, Sharpless and Depinho 2007). An age-associated increase in quiescent stem cells in bone, muscle, forebrain and many other tissues has been documented in mammals (Molofsky et al 2006, Gruber et al 2006, Conboy and Rando 2012). Some suggest that the overall cause of organismal aging is mainly from the loss of stem cell function in these essential tissues (Rando and Chang 2012).

1.2.9 Altered Intercellular Communication:

The communication between cells within an organism is also altered with aging. Studies have found that inflammatory signaling increases with age while hormonal and neuronal signaling declines with age (Laplane and Sabatini 2012, Rando and Chang 2012, Zhang et al 2013). Immune related signaling, particularly the pathogen recognition related pathways, is also diminished with aging (Zhang et al 2013). This dysregulation of intercellular communication contributes to various phenotypes associated with organismal aging and offers another potential target for therapeutic intervention for extending the healthspan of humans (Lopez-Otin et al 2013, Conboy et al 2005).

Of these aforementioned hallmarks of aging, genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis are considered to be the molecular alterations that cause the age-associated molecular damage (Lopez-Otin et al 2013). The deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence are thought to be the cellular changes in response to the macromolecular damage (Lopez-Otin et al 2013). These responses may be beneficial for coping with the macromolecular damages at first, but turn detrimental if continually used to cope with the molecular damages (Lopez-Otin et al 2013). Finally, stem cell exhaustion and altered intercellular communication is what leads to the biological decline at the organismal level, which we know as aging (Lopez-Otin et al 2013).

1.3 Theories of Aging

Based on the research on various model organisms listed in the next section, several theories of aging have been proposed that can be divided into two sets: theories

that postulate (i) biological decline known as aging is due to accumulation of damaged cellular components, and (ii) aging is a programmed biological event (Sergeiv et al 2015).

We first review several theories in the damage accumulation set and then of the programmed event set. Originally published in 1956, Denham Harman postulated the 'free radical theory of aging' (FRTF), which states that free radicals produced by normal cellular respiration cause damaged macromolecules and this damage leads to aging (Harman 1956). Later the original theory was altered to include various reactive oxygen species that are produced as byproducts of normal cellular respiration in addition to free radicals and was termed as the 'oxidative damage theory of aging' (also known as the oxidative stress theory of aging) (Harman 1981). Another theory, termed the 'mitochondrial theory of aging', focuses on the fact that the mitochondria produce up to 90% of all reactive oxygen species in the cell. Due to this, the theory suggests that mutations in the mitochondria lead to larger accumulation of damage and ultimately lead to even more damage, phenotypes, and pathologies of aging (Harman 1965). Another theory originally conceived in 2002, focuses on the accumulation of cellular metabolic wastes as being a cause for aging (Gladyshev 2012,2013). Some cells are extremely long lived (i.e. brain cells, cardiomyocytes) and are thus more vulnerable to accumulating metabolic wastes products (Sergeiv et al 2015). The inability to effectively deal with these waste products from the various biochemical reactions within the cell leads to eventual cellular senescence and cell death (Gladyshev 2012). Other theories include the 'DNA damage theory of aging', attribute the pathologies and phenotypes of aging to mutations and damage that accumulate with age in the genome (Freitas and de

Magalhaes 2011), and the 'inflamm-aging theory' which postulates that inflammation early in life can have negative effects later in life span (Franceschi et al 2000).

The second set of theories of aging focus on aging as a programmed biological phenomenon. Proposed in 1882 by Weismann, the 'programmed death theory of aging' states that there is a genetically programmed component to aging that triggers death of an organism as an overall benefit for the species (Goldsmith 2004). Another theory of aging in this category is termed the 'developmental theory of aging', which proposes that aging occurs as a byproduct of reproduction (de Magalhaes and Church 2005). A newer theory proposed by Skulachev termed 'biochemical mechanism of slow aging' postulates that the biological reactions and buildup of ROS over time leads to the slow death of the organism (Skulachev 2012). Certain biological phenomena are cited as examples supporting these theories of aging: Salmon travel upstream to reproduce and following this life event they perish within days (studies demonstrate a rapid increase in phenotypes of aging, such as an increase in cholesterol and fatty acids in the blood, within days of salmon reproduction (Sergeiv et al 2015)). (Note there are some other examples not listed here).

Although between the two mentioned sets of theories of aging there is an overlap with respect to the underlying molecular/cellular causes of aging, the two sets of theories are postulated with different views of the aging process. In general, the damage accumulation theories of aging look at the aging process as a biological side effect of normal cellular processes while the programmed theories of aging consider aging as the direct consequence of genetic pathways (Sergeiv et al 2015). Overall, no

single theory of aging has been able to fully explain the extremely complex biological process of aging that is common to all living organisms and thus aging can be a net result of multiple causes (Lopez-Otin et al 2013, Gems and Partridge 2013, Kenyon 2010, Kirkwood 2005, Vijg and Campisi 2008). In view of this, the nine hallmarks of aging that have been identified offer a more definite road map to the study of aging at cellular/molecular level as these are measurable outcomes of a complex biological process.

1.4 Model Organisms Used in Study of Aging

Many studies have investigated mechanisms of aging at molecular levels using various model organisms including (but not limiting to) *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus* (Tissenbaum and Leonard 2002, Partridge and Tower 2008, Vanhooren and Libert 2013). These model systems have helped build much of the knowledge we currently have about the molecular mechanisms of aging, yet they are not perfect models for such a complex biological process as aging. The ultimate goal of aging research is to develop a comprehensive understanding of the aging process and the underlying molecular mechanisms so that possible therapeutic interventions can extend the healthy life span of human beings (Tissenbaum and Leonard 2002, Austad 2010, Selman and Wither 2010). Here we provide highlights of contribution of each model organism to our understanding of the aging processes, noting the advantages and disadvantages of each model.

Often considered one of the first model organisms of aging, *S. cerevisiae* helped launch a more defined, and molecular approach to aging research. This small budding yeast lives about 3 days (reproductive age) and its life cycle encompasses three stages: a logarithmic growth phase in which the organism can use glucose for growth, a reproductive phase, and then a final stationary phase in which the organism becomes resistant to many stressors and can survive in this state for several months (Tissenbaum and Leonard 2002, Partridge and Tower 2008). Although the studies on budding yeast led to the discovery of the role of target of rapamycin (mammalian homolog known as mTOR) in aging and life span, it has certain limitations for being used as a model in relation to human aging. The first major limitation is that it's a unicellular organism. Obviously the intricate aging process involving intercellular signaling cannot be examined or replicated in a single celled organism. Related to that, the lifespan of *S. cerevisiae* is represented in two distinct manners: replicative lifespan which is essentially how many cell divisions the yeast cell can undergo and chronological lifespan which is the duration for which yeast cells are viable in the stationary phase cultures (Mortimer and Johnson 1959, Muller et al 1980, Tissenbaum and Leonard 2002). These measures of lifespan are in stark contrast from the biology of human lifespan and hence pose a limitation in correlating findings from study of this unicellular model to the human aging process. Another major limitation is that some of the genes affecting the aging process in yeast are not well conserved in human genome (Tissenbaum and Leonard 2002). With these limitations, *S. cerevisiae*, while an excellent model system for other aspects of

molecular biology, isn't optimal as a major model organism in aging research at least in relation to human aging.

C. elegans is a small nematode that is an important developmental and molecular cell biology model. The developmental processes of the organism are extremely well studied with the fate of each individual cell being mapped during the growth and development of the nematode (Riddle et al 1997). Under stressful conditions, such as limited resources, larval *C. elegans* can enter a dauer state that is alternative to the reproductive larval stages (Riddle et al 1997, Tissenbaum and Leonard 2002). During this dauer larval state which can last for months, the organism undergoes minimal aging (normal *C. elegans* lifespan is on average between 15 and 21 days depending on the strain); and when conditions return to being optimal for the survival of *C. elegans*, the larvae exit the dauer state to complete a normal adult lifespan (Riddle et al 1997, Partridge and Tower 2008). Insulin signaling being involved in life span extension from caloric restriction was the biggest and most valuable contribution for *C. elegans* to the knowledge of aging mechanisms. Ability to generate gene knock down by an easy feeding method makes this model organism suitable to study contribution of individual genes to aging and life span regulation (Riddle et al 1997). One limitation of this organism as a model in aging is that it is post-mitotic with all somatic cells being in the G₀ phase and not undergoing active cellular proliferation and renewal similar to certain human cell types (Tissenbaum and Leonard 2002).

Another quintessential model organism, *D. melanogaster* has become a valuable model for a multitude of molecular and developmental fields. The fruit fly has become a

popular aging model for multiple reasons, one of which is the availability of a number of excellent analytical tools (Helfand and Rogina 2003, Tower 2011, Eleftherianos and Castillo 2012). The ability to knockdown and overexpress genes in *Drosophila* along with many other core molecular techniques available for use in the fruit fly have made it the founding model organism in the field of genetics and developmental biology (Morgan 1910). The lifespan of *Drosophila* spans for on average of 3 months and many of the evolutionarily conserved molecular pathways associated with aging function in *Drosophila* including the insulin signaling pathway (Helfand and Rogina 2003, Tower 2011, Eleftherianos and Castillo 2012). *D. melanogaster* has been used to investigate various evolutionary theories of aging (accumulation of mutations, aging is a time dependent side effect of mutation pressure; and pleiotropy, certain genes are beneficial during early life but become detrimental in late life) (Partridge and Tower 2008). Beginning in the 1980s, *D. melanogaster* was used to demonstrate that artificial selection of individuals that are long lived can lead to different populations exhibiting short and long lifespan (Luckinbill et al 1984, Partridge and Tower 2008). The small fruit fly is limited as an aging model, however, since it also is a post-mitotic organism without cellular renewal, except for a limited capacity in the gut (Partridge and Tower 2008, Helfand and Rogina 2003). There is also no easy RNAi via feeding system available for *D. melanogaster* (unlike *C. elegans*) to achieve targeted transient gene knockdown (Partridge and Tower 2008). Although RNAi methods are available, they require technical expertise such as microinjection of the embryos as well as adult organisms.

The mouse is a widely used model for various diseases and is an essential model organism for studies involving potential human therapeutics (Selman and Wither 2011, Vanhooren and Libert 2013). The mouse model is well suited for aging research partly because it's a mammalian vertebrate with many similarities and conserved pathways with humans (Selman and Wither 2011, Vanhooren and Libert 2013). Studies have also shown that mice and humans are very similar in their immune system, digestive system, and even musculoskeletal system (Vanhooren and Libert 2013). The mouse also exhibits similar physiological declines with age as humans, making it an optimal mammalian model system for aging (Selman and Wither 2011, Vanhooren and Libert 2013). The mouse lives on average 2 years and in terms of mammalian research is extremely cost effective for being a mammalian vertebrate model system (Nadon 2004, Hasty and Vijg 2004). With all the advantages of the mouse model for aging research, there are also some limitations. Although 2 years of life is considered an advantage compared to other mammalian vertebrates, it's still a relatively long life span in relation to other model organisms of aging and thus the experimental time span is significantly longer (Vanhooren and Libert 2013). Mouse telomeres are much longer than human telomeres and any correlation of mouse studies with telomere erosion in relation to cellular and organismal aging may not be directly applicable to humans. Mice are also able to synthesize vitamin C, a crucial part of human diet as humans can't generate vitamin C, and vitamin C has been implicated in various aging mechanisms (Gershoff 1993, Vanhooren and Libert 2013, Gershoff 1993, Park et al 2009). Nevertheless, *M. musculus*,

along with the rat, has helped establish the effects of caloric restriction in extending lifespan.

Although these models suffer from some limitations that may not make them ideal model systems in relation to human aging, there are important molecular mechanisms of aging that have been elucidated using these models. Sir2, the first sirtuin gene to be discovered in *S. cerevisiae*, is an NAD⁺-dependent deacetylase and an important regulator of cell survival, nutrient sensing and aging (Ivy et al 1986). Perhaps the most influential discovery in aging research has been that of dietary restriction extending lifespan which was identified initially using rats and have since been verified in all of the aforementioned model organisms of aging (Osbourne et al 1917, McCay et al 1935, Masoro 2005). From this, *C. elegans* was used to further elucidate the fact that insulin-signaling pathway was responsible for the life extending effect of dietary restriction (Tissenbaum and Leonard 2002). From extensive studies using these model organisms, we know over 200 single gene manipulations that can provide lifespan extension in *C. elegans* and *D. melanogaster* (Austad 2010, Ladiges et al 2009).

Of note is the concept that we are at the basis interested in the molecular mechanisms of aging to help influence and understand human aging, yet we are using organisms with dramatically shorter lifespans than humans. Although the base molecular mechanisms are likely similar, not all mechanisms of aging are fully understood; therefore, there may be unknown mechanisms that govern human aging but may not affect the aging of small, short lived eukaryotes. Thus, research trends are currently trying to utilize some long lived organisms that are outliers in terms of life

span and their size as well as using other species as model organisms of aging that may be better suited than the existing models (Austad 2010, de Magalães 2014, Petralia et al 2014, Harel et al 2015). Of particular interest are the naked mole rats (live on average about 32 years) and the little brown bat (with most species living between 20 and 30 years with one species, *Myotis brandti*, with an average lifespan of 41 years) that have a long lifespan for their small body size (Austad 2009, Austad 2010, Buffenstein 2008, Wilkinson and South 2002). Obvious practical limitations will prevent exhaustive intervention type research on such long-lived model organisms (for example, the addition of dietary supplements to examine if lifespan is extended would be extremely difficult to perform using these long lived species). Due to such limitations, it is essential to utilize model organisms that have a characteristically short lifespan; however, the more closely related to humans evolutionarily, the more useful these models will be in relation to illuminating the mechanisms related to human aging.

1.5 *Daphnia* as a Model Organism for Research on Aging

In my dissertation, I present *Daphnia*, a freshwater microcrustacean, as a model for research on aging. These microcrustaceans are filter feeders found in bodies of freshwater around the world and are primary consumers in their ecosystem, consuming algae and other small organisms in water (Benzie 2005). *Daphnia* are currently used extensively in ecotoxicology, ecology, and population genetics and have been a central model organism in these fields as they can be cultured easily in the lab and also studied easily in the field (Benzie 2005). *Daphnia* is just beginning to be established as a model system for molecular studies involving neurobiology (Ungerer et al 2011; McCoole et al

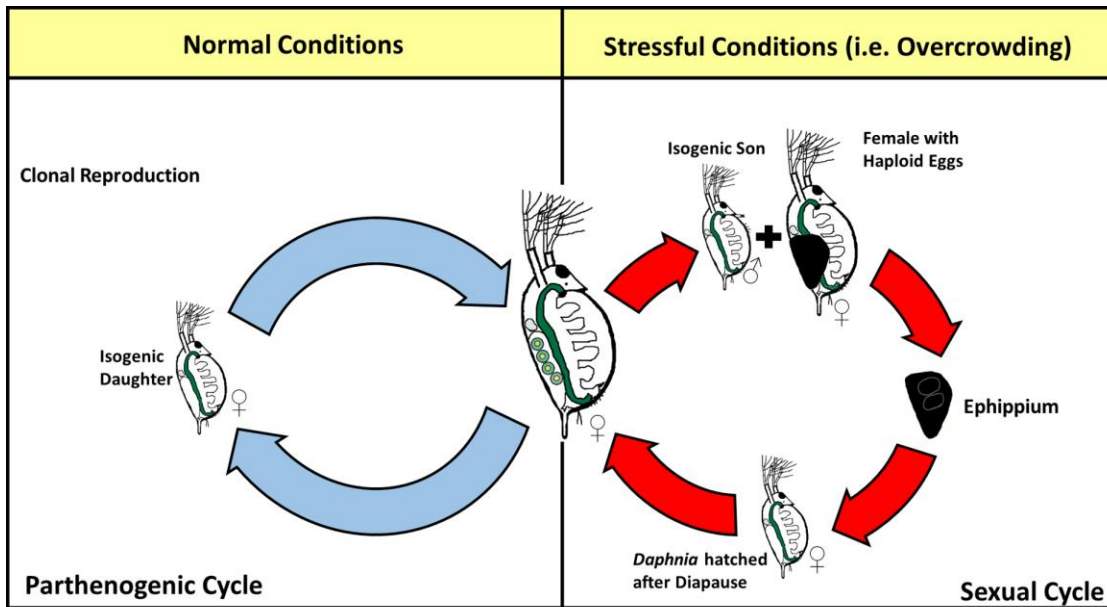


Figure 1.1 Reproduction via cyclic parthenogenesis. See text for detailed description of *Daphnia* reproduction. Symbols denote male or female *Daphnia*.

2012; Christie and McCool 2012, Weiss et al 2012; Toyota et al 2015) and biology of aging (Schumpert et al 2014, Kim et al 2014, Schumpert et al 2015, Murthy and Ram 2014).

We study two ecotypes of *Daphnia* in relation to aging: *Daphnia pulex* and *Daphnia pulicaria*. *D. pulex* inhabits small transitory ponds and live about 20 to 25 days on average while *D. pulicaria* occupies stratified lakes and live about 60 to 65 days on average (Dudycha 2000). Due to this large variation in life spans, these ecotypes have been used to examine environmental effects on longevity as well as the effect of naturally occurring genetic variations on longevity (Dudycha 2001,2003; Steinberg et al 2010, Dudycha and Hassel 2013). These two ecotypes, although labeled as separate species, are able to interbreed to produce viable hybrids and gene flow between the two ecotypes has been documented (Cristescu et al 2012, Heier and Dudycha 2009, Xu et al 2013). The estimates of divergence between the two ecotypes are only 82,000 years, which is shorter than various human populations (Omillian and Lynch 2009). These two ecotypes exhibit variations in age-specific mortality rates, reproduction rate (including reproductive decline rate), and growth rate of juveniles (Dudycha and Tessier 1999). Initial studies also suggest that the long-lived *D. pulicaria* has higher efficiency of DNA damage repair via a photoenzymatic mechanism (Connelly et al 2009) as compared to the short-lived *D. pulex*.

Daphnia make an attractive model for research on aging and life span regulation based on certain unique characteristics. These freshwater microcrustaceans reproduce via cyclic parthenogenesis, which is clonal reproduction that can be interrupted by

sexual reproduction during conditions that challenge survival of the organisms (Benzie 2005). In the laboratory, clonal reproduction is easily supported with proper care and maintenance of *Daphnia*, leading to the production of large populations of isogenic individuals without the need for a regulated breeding by setting up crosses. Being able to perform experiments on genetically identical organisms reduces the amount of aging and life span variations that can arise from sexual reproduction due to changes in genotypes. Clonogenicity of *Daphnia* also enables an accurate comparison of life spans and aging between different genetic backgrounds or to study the effects of different environments or treatments on the same genetic background. In addition, the two ecotypes offer significant advantages, as other model organisms do not have two naturally occurring subtypes that are closely related to interbreed and produce viable offspring and yet have such a divergent lifespan. These microcrustaceans are also very easy to culture in the lab and have relatively short lifespans to allow multiple life span experiments to be performed in a relatively short amount of time. *Daphnia* have transparent bodies, allowing for examination of internal structures and organs in living organisms (for example, examination of the heart and the associated beating rate is easily performed with a light microscope). A major difference between *Daphnia* and other model organisms of aging is that *Daphnia* have regenerative capabilities with continued cell renewal and proliferation, and have indeterminate growth throughout their lifespan (Benzie 2005). Whereas *C. elegans* and *D. melanogaster* are considered to be post-mitotic organisms, *Daphnia* cells are constantly replicating throughout their lifespan. *Daphnia* also have a large genome composed of 30,907 protein-coding genes

and share the most genes in common with humans among any other arthropod (Colborne et al 2011). In comparison, widely used molecular and aging models *D. melanogaster* and *C. elegans* have roughly 12,000 and 20,704 protein-coding genes respectively (Adams et al 2000, The *C. elegans* Sequencing Consortium 1998). Thus, *Daphnia* have a larger genome with more gene homologs in humans than other established invertebrate model organisms of aging.

In recent years, *Daphnia* has become increasingly amenable to genetic manipulations by using molecular tools. First described by Kato et al in 2011, gene knockdown by RNA interference is possible in *Daphnia* using microinjection in the embryos of both *D. pulex* and *D. magna* (Kato et al 2011, Hiruata et al 2013). We have recently developed an RNAi via feeding method to effectively study the transient knockdown of genes in adult *Daphnia*. These RNAi methods will allow a direct analysis of the genes that are predicted regulators of life span or aging. For a thorough analysis of genetic and molecular mechanisms involved in life span regulation, it is essential to have the ability to create transgenic organisms for overexpression of genes as well as the ability to create gene knockouts to totally ablate the expression of genes under study. Several different technologies have been successfully used for *Daphnia* including targeted genome editing with TALEN endonucleases as well as the CRISPR/Cas9 system (Hiruta et al 2014, Nakanishi et al 2014). With the ability to create gene knockouts or specific mutations as well as to transiently knockdown the expression of specific genes, these freshwater microcrustaceans are poised to make an impact on the aging field.

We have studied several different molecular processes known to be involved in the aging process to compare the short-lived *D. pulex* and long-lived *D. pulicaria*. Such comparison is expected to shed light on important regulators of the aging process. The first process we examined was telomere length and telomere attrition during the aging process, as genomic instability due to telomere attrition is observed in old, senescent cells in many organisms (Lopez-Otin et al 2013). In **Chapter 2**, we examine the telomeres and the activity of telomerase, the enzyme that imparts *de novo* telomeric repeats, in the ecotypes of *D. pulex* and *D. pulicaria* (Schumpert et al 2015). Our results indicate that in the short lived *D. pulex*, telomere length is maintained throughout the life span of the organism while in the long lived *D. pulicaria*, telomeres erode with age. Coupled with this, telomerase activity was found to stay constant with age in *D. pulex* and declined with age in *D. pulicaria*. Therefore, our data suggests that at the characteristically short life span of *D. pulex*, does not result from telomere attrition or the potential genomic stability associated with it. In **Chapter 3**, we examined the heat shock response of *D. pulex* and *D. pulicaria* at equivalent ages (Schumpert et al 2014). The ability to respond to proteotoxic stress, termed the heat shock response, has been demonstrated in multiple model organisms to decline with age (Ankar and Sistonen 2011). The heat shock response includes the induction of several molecular chaperones that are involved in renaturation of damaged and altered proteins (or to target them for degradation if damage is beyond repair) (Morimoto and Cuervo 2009, Ankar and Sistonen 2011). Heat shock protein 70 (Hsp70) is the main molecular chaperone involved in this renaturation process and has been studied extensively with respect to

the aging process (Morimoto and Cuervo 2009). Our results establish that as *Daphnia* age, they exhibit a decline in the heat shock response (assayed via Western Blot Analysis of Hsp70 levels following proteotoxic insult). Interestingly, the short lived *D. pulex* stops responding to the heat shock by induction of Hsp70 by middle age whereas the long lived *D. pulicaria* can still appropriately induce the expression of Hsp70 following proteotoxic stress at an equivalent middle age. *D. pulicaria* also showed age-dependent decline in HSR, albeit at a much later age as compared to *D. pulex*. Thus, the early demise of *D. pulex* may be attributed to the proteotoxicity caused by an accumulation of misfolded, and inactive proteins due to the lack of an efficient chaperone response as *D. pulex* ages. Since both *D. pulex* and *D. pulicaria* exhibited age-dependent decline in HSR, we further investigated the mechanism responsible for the age-associated decline in the HSR by investigating the DNA binding ability of the transcription factor (heat shock factor -1) that regulates induction of Hsp70 in response to heat shock heat shock. Heat shock factor-1 undergoes trimerization upon proteotoxic stress and binds to the heat shock element found in the promoters of heat shock induced genes leading to their robust expression in a relatively short amount of time after proteotoxic stress (Ankar and Sistonen 2011). Our results indicate that the lack of HSR in old *D. pulicaria* can be attributed to the lack of DNA-binding activity of HSF-1 in old organisms. Our research into the cause for the age associated decline in HSF's ability to bind to DNA in *D. pulicaria* continues further in **Chapter 4** which focuses on the NAD⁺-dependent protein deacetylase named Sir2, which deacetylates HSF1. Previous studies have shown that HSF1 is unable to bind DNA when acetylated on K80 and that Sir2 deacetylates HSF1 at

this particular residue and renders it active (Westerheide et al 2009, Raynes et al 2012). We first demonstrated that *Daphnia* Sir2 is functionally conserved, being able to protect cells from proteotoxic stress and functionally deacetylate HSF1 in order to induce a heat shock reporter. We examine overall transcript levels of Sir2, Sir2 activity level, and the level of the essential cofactor NAD⁺ in *D. pulicaria* at young, middle and old ages. Our findings suggest that *Daphnia* Sir2 activity declines with age and may potentially play a causative role in the inability to respond to proteotoxic stimuli in old *Daphnia*. We further demonstrate the overall negative effect of Sir2 knockdown on the lifespan of *D. pulicaria* by using our newly developed RNAi via feeding method. The final chapter, **Chapter 5** of this dissertation presents the experimental details of the RNAi via feeding method for gene knockdown in adult *Daphnia* that I developed. This method gives us the ability to easily knock down specific genes at any age in adult *Daphnia*, thereby offering direct means for testing contribution of various genes to the physiology, development, and ecotoxicology, in addition to study of longevity, aging, and life span. Thus, this method is expected to be widely applicable and useful for many *Daphnia* biologists.

Overall, as a model organism *Daphnia* provides unique characteristics that make it an attractive model for aging research. This small water flea has been used for over a century as a model organism mainly for the ease in culturing and maintaining large populations in a laboratory setting and also for the feasibility of studies in the field under various environmental conditions (Benzie 2005). Recently, the published genome of *Daphnia pulex* revealed it to have an extremely large genome that is the most

homologous to the human genome out of any other published arthropod genomes making it an attractive model to study complex biological processes in relation to human biology (Colbourne et al 2011). *Daphnia* also have continual cell proliferation and renewal, unlike *C. elegans* and *D. melanogaster* which are both considered post-mitotic (Benzie 2005). With the recent genome editing technologies being applied to *Daphnia*, the model is set to quickly become a major keystone model system in aging, development, and molecular cell biology.

Chapter 2:
Telomerase Activity and Telomere Length in *Daphnia*²

² Schumpert C, Nelson J, Kim E, Dudycha J, Patel R. 2015. Telomerase Activity and Telomere Length in *Daphnia*. PLoS ONE 10(5):e0127196. Reprinted with permission.

2.1 Abstract

Telomeres, comprised of short repetitive sequences, are essential for genome stability and have been studied in relation to cellular senescence and aging. Telomerase, the enzyme that adds telomeric repeats to chromosome ends, is essential for maintaining the overall telomere length. A lack of telomerase activity in mammalian somatic cells results in progressive shortening of telomeres with each cellular replication event. Mammals exhibit high rates of cell proliferation during embryonic and juvenile stages but very little somatic cell proliferation occurs during adult and senescent stages. The telomere hypothesis of cellular aging states that telomeres serve as an internal mitotic clock and telomere length erosion leads to cellular senescence and eventual cell death. In this report, we have examined telomerase activity, processivity, and telomere length in *Daphnia*, an organism that grows continuously throughout its life. Similar to insects, *Daphnia* telomeric repeat sequence was determined to be TTAGG and telomerase products with five-nucleotide periodicity were generated in the telomerase activity assay. We investigated telomerase function and telomere lengths in two closely related ecotypes of *Daphnia* with divergent lifespans, short-lived *D. pulex* and long-lived *D. pulicaria*. Our results indicate that there is no age-dependent decline in telomere length, telomerase activity, or processivity in short-lived *D. pulex*. On the contrary, a significant age dependent decline in telomere length, telomerase activity and processivity is observed during life span in long-lived *D. pulicaria*. While providing the first report on characterization of *Daphnia* telomeres and telomerase activity, our

results also indicate that mechanisms other than telomere shortening may be responsible for the strikingly short life span of *D. pulex*.

2.2 Introduction

Telomeres, the ends of linear chromosomes, have been studied extensively in relation to cellular aging and senescence (Hayflick 1965, Shay et al 2000, de Lange 2002). Composed of repetitive nucleotide sequences (TTAGGG for mammals) associated with proteins, telomeres protect important genetic information of linear chromosomes from deletion arising due to the “end replication” problem (Shay et al 2000, de Lange 2002). The process of DNA replication leads to progressive shortening of linear chromosomes at the telomeres due to the fact that DNA polymerases can only polymerize in a 5’ to 3’ direction and require a primer with a free 3’-OH group (Shay et al 2000, de Lange 2002). This inability to replicate linear DNA on the lagging strands all the way to ends necessitates the telomerase, an enzyme responsible for *de novo* addition of telomeric repeats to chromosomal ends (Greider and Blackburn 1985). Telomerase is a ribonucleoprotein complex, comprised of a protein catalytic subunit TERT (Telomeric Reverse Transcriptase), and an RNA template termed TERC (telomeric RNA Component) (Shay et al 2000, de Lange 2002). Telomerase activity is essential for maintaining telomere length throughout cellular lifespan. Early in human development, telomerase is constitutively active in cells but after birth it is active predominately in stem cells and germ cells with most somatic tissues having no telomerase activity (Liu et al 2007, Morgan 2013). Each individual DNA replication event of human telomerase-negative somatic cells leads to a loss of 100 bp of telomeric sequence, resulting in a progressive

decline in telomere length with each cellular division (Raices et al 2005). Because of this progressive telomere shortening, human somatic cells can only undergo approximately 50 to 80 cellular replication events before becoming senescent (Raices et al 2005). Thus, telomere length is essential for normal cellular function and proliferation as well as chromosome stability. In the absence of proper telomere complex formation, the double-stranded break repair pathway can be initiated resulting in apoptosis or senescence (d'Adda et al 2003, Takai et al 2003). Thus, telomeres serve a protective molecular role by shielding the loss of important genetic information as well as by maintaining chromosome stability throughout the cellular lifespan. Telomerase has also been implicated in nuclear DNA damage repair and plays a protective role for mitochondrial DNA during oxidative stress response during which telomerase shuttles from the nucleus to the mitochondria (Bommer et al 2002, Geserick and Blasco 2006, Ahmed et al 2008, Haendeler et al 2009, Indran et al 2011, Singhapol et al 2013).

In this study we investigated telomerase activity, the telomeric repeat sequence, and telomere lengths in *Daphnia*, a freshwater crustacean, and an emerging model in aging liuresearch. *Daphnia* has been used extensively as a model in ecotoxicology studies (Benzie 2005) and with a fully sequenced genome of *D. pulex*, it is an emerging model in biomedical research (Colborne et al 2011, Murthy and Ram 2014). Two ecotypes of *Daphnia* are of interest in relation to aging, *D. pulex* and *D. pulicaria* (Dudycha and Tessier 1999, Dudycha 2001, 2003, 2004, Dudycha and Hassel 2013, Schumpert et al 2014). *D. pulex* is found in small transitory ponds, in which selection favors short longevity due to the limited time the ponds have water. In a laboratory

environment *D. pulex* exhibits a lifespan on average of about 20 days (Dudycha and Tessier 1999, Dudycha 2003). *D. pulicaria* lives primarily in a stable environment of stratified lakes that are present all year long. In the lab *D. pulicaria* exhibits lifespans on average of about 70 days (Dudycha and Tessier 1999, Dudycha 2003). Genetically, the two ecotypes are almost identical and are capable of interbreeding with viable offspring in the wild (Dudycha 2004). *Daphnia* can be easily cultured and undergo cyclic parthenogenesis (Benzie 2005), thus enabling creation of clonal lineages without the genetic variation normally associated with sexually reproducing organisms. Being crustaceans, *Daphnia* constantly shed their outer carapace and have regenerative cellular capacities (Benzie 2005). Due to these unique characteristics *Daphnia* is an interesting model organism for understanding cellular processes associated with aging.

We present characterization of the telomere length, telomerase activity and processivity in the two ecotypes, the short-lived *D. pulex* and the long lived *D. pulicaria*. In the short-lived *D. pulex*, telomere length did not decline with age; however, in the long-lived *D. pulicaria*, telomere length decreased with age. Accordingly, telomerase activity in *D. pulex* is relatively constant throughout the life span, whereas in *D. pulicaria*, it declines considerably with age. In addition, the telomerase processivity increased with age in *D. pulex*, whereas in *D. pulicaria* it declined with age. This is an important initial study to investigate telomere length and telomerase activity in a newly emerging model system for research on aging.

2.3 Materials and Methods

2.3.1 *Daphnia* Cultures

Daphnia pulex (clone RW 20) and *Daphnia pulicaria* (clone Lake XVI-11) were isolated from populations in southwest Michigan in 2008 and have since been cultured in the lab. No specific permissions are required to collect zooplankton from these public-access waterbodies in Michigan. *D. pulex* and *D. pulicaria* are neither endangered nor protected. For further details on the source populations, see Dudycha (Dudycha 2004).

Daphnia were maintained at 20⁰ C with a photoperiod of 12:12 L:D (12 hours of light followed by 12 hours of dark) within a Percival growth chamber. *Daphnia* were maintained at 3 to 5 animals per 250 mL beaker in 200 mL of filtered lake water.

Daphnia were cleared of young and transferred to a new beaker with fresh water on alternate days. They were fed every day with vitamin supplemented algae

Ankistrodesmus falcatus at a concentration of 20,000 cells/mL. To generate experimental animals, even-aged cohorts were obtained by placing neonates individually in 100 mL of hardwater COMBO, or artificial lake water. Experimental animals were otherwise maintained as in the source cultures.

All *Daphnia* were maintained clonally in the lab and all *Daphnia* used for experimentation are diploid females. Once individuals were collected from the field, they were placed in beakers of fresh lake water and allowed to clonally reproduce. In this manner, each isolate, or clone, of *Daphnia* was produced (for example, individuals from the pond named Rough Wood were placed individually into beakers and designated as clones RW followed by a number which corresponds to the original

analysis of the collected *Daphnia* back in the lab, i.e. RW20 which corresponds to the 20th individual analyzed). After each clone was established, *Daphnia* were maintained by constant feeding of algae and changing of the lake water to create an isogenic clonal line of *Daphnia*. Care was taken not to induce stress for the *Daphnia* such that sexual reproduction would not occur in the clonal lines.

2.3.2 Telomeric Repeat Amplification Protocol (TRAP) Assay

Telomeric repeat amplification protocol (TRAP) Assay was performed using the TRAPeze Telomerase Detection kit (Millipore). *D. pulex* and *D. pulicaria* were collected at different ages. For *D. pulex* (RW20), we used 1, 2, and 3 week old individuals and for *D. pulicaria* (LakeXVI-11) we used 1, 4, and 8 week old individuals. To prepare cell extracts from *D. pulex* and *D. pulicaria*, 25 to 30 individual *Daphnia* were drained of all residual water, and homogenized in 200 µl of 1X CHAPS Lysis Buffer on ice. The homogenate was incubated on ice for 30 min, centrifuged at 12000 X g for 20 min at 4^o C, 160 µl of extract was transferred to a new tube, and the total protein concentration in the extract was determined using a Bradford Assay. Samples were stored at -80^o C.

The TRAP Assay was performed as per the instructions in the TRAPeze kit. A 50 µl reaction was performed for all samples and controls. A positive control of human cancer cell line extract was provided with the kit. Each reaction contained 5 µl of 10X TRAP reaction buffer, 1 µl of 50X dNTP mix, 1 µl of TS Primer, 1 µl of TRAP primer mix, 0.4 microliters (2 units) of Taq polymerase, 39.6 µl of deionized water, and 2 µl of extract (either control or sample). Tubes containing each reaction were placed in the thermocycler and heated to 30^o C for 30 min. Then 30-33 cycles of 94^o C for 30 sec, 59^o C

for 30 sec, and 72⁰ C for 1 min were performed. The heat-inactivated control samples, were incubated at 85⁰ C for 10 min prior to TRAP assay.

Following PCR amplification, 5 µl of loading dye (with bromophenol blue (0.25% in 50% glycerol/50 mM EDTA) and xylene cyanol (0.25% in 50% glycerol/50 mM EDTA) was mixed with the TRAPeze product. 25 µl was loaded into a 10% non-denaturing polyacrylamide gel and the gel was run at 400 V for 1.5 hours in 0.5 X TBE buffer, stained using Sybr Green for 30 min before scanning in a Typhoon FLA 7000.

2.3.3 Quantification of the TRAP Assay Results

TRAP Assay results were quantified using a formula described in TRAPeze kit manual. The formula takes into account the signal measured in each reaction/lane (x) as well as the heat inactivated control (x₀), the no-template control (r₀), and the TSR8 quantitation control (r) reactions/lanes. All of these reactions contain an internal standard (36 bp band) engineered into the assay, which was also used in the formula (c for samples, c_r for TSR8 control). The resulting quantification was in units of Total Product Generated (TPG), which corresponds to the number of TS primers extended by at least 4 telomeric repeats in 30 min at 30⁰ C.

$$TPG = \frac{(x - x_0)/c}{(r - r_0)/c_r} \times 100$$

2.3.4 Sequencing of the TRAP Product

A TRAP Assay reaction that contained a strong positive result from the *Daphnia* samples was used to determine the telomeric repeat sequence for *Daphnia*. 5 µl of the

reaction products was purified using a Qiagen PCR Purification kit, and ligated into the vector pGEMT Easy (Promega), and sequenced.

2.3.5 Survivorship studies

We compared survivorship of the XVI-11 and RW20 clones using standard life table methods for *Daphnia* (Kim et al 2014). Experimental conditions were 20°C, a 12:12 L:D photoperiod, with animals kept in individual 150-ml pyrex beakers in 100 ml COMBO hard water artificial lake water (Kilham et al 1998). Two generations were maintained under experimental conditions prior to initiating the life table to minimize maternal effects variation. Experimental individuals were fed 20,000 cells/ml *Ankistrodesmus falcatus* daily, a food level that produces normal aging processes in *Daphnia* (Dudycha 2003). Individuals were transferred to fresh beakers and COMBO every other day, and survivorship was observed daily until all experimental animals died. For each clone, $n = 60$ females.

2.3.6 Terminal Restriction Fragment (TRF) Assay

We performed a Terminal Restriction Fragment Assay using a previously established protocol by Herbert *et al.* (Herbert et al 2003). Genomic DNA was extracted from *D. pulex* and *D. pulicaria* at various ages using a CTAB (Hexadecyltrimethylammonium Bromide) based protocol optimized for use with *Daphnia*. For each aged set of *Daphnia*, 25 individuals were pooled for the extraction of genomic DNA. Each individual *Daphnia* was cleared of all embryos before the genomic DNA was extracted to ensure the results were reflective of the adult telomere length. One μg of genomic DNA was digested at 37°C overnight with 1 U/ μl of the following

restriction enzymes: HinfI, RsaI, MspI, CfoI, HaeII and AluI. Digests were run on a 0.7% agarose gel. Samples were run for 4 h at 120 V in 1X TAE Buffer to achieve desirable separation in size range 1 kb- 25 kb. The gel was denatured for 20 min in denaturing solution (0.5 M NaOH, 1.5M NaCl), and rinsed in distilled water for 10 min. The gel was then dried upside down between 2 sheets of Whatman 3MM filter paper under vacuum for 1 hour at 50⁰ C. After removing the gel from the dryer, the gel was neutralized for 15 min in neutralizing solution (1.5 M NaCl, 0.5 M Tris-Cl, pH 8.0) and then rinsed with distilled water for 10 min. The gel was then soaked in 10 ml of prehybridization buffer (5X SSC Buffer, 5X Denhardt solution, 10 mM Na₂HPO₄, 1 mM Na₂H₂P₂O₇) for 10 min. The gel was then transferred to hybridization solution (5X SSC Buffer, 5X Denhardt solution, 10 mM Na₂HPO₄, 1 mM Na₂H₂P₂O₇) containing the radiolabeled telomeric sequence probe (see below). The gel was hybridized overnight at 42⁰ C. After hybridization, the gel was washed once in 2X SSC for 15 min at 22⁰ C, washed four times for 10 min each in 0.1X SSC/0.1% SDS. Following washes, the gel was exposed to a phosphor screen overnight and scanned on a Typhoon FLA 7000 phosphor imager and visualized with ImageQuant software.

2.3.7 *Daphnia* telomeric probe design and labeling

Based on the telomeric repeat sequence for *Daphnia*, a telomeric probe was designed as 6 repeats of the telomeric repeat: 5'-TTAGGTTAGGTTAGGTTAGGTTAGGTTAGG-3'. The probe was 5' end-labeled with γ -P³² ATP using polynucleotide kinase. To label the probe, 2 μ l of 20 pmol/ μ l of oligonucleotide, 24 μ l of γ -P³² ATP (3000 Ci/mmol), 10 μ l 5X forward reaction buffer, 2 μ l

10 U/ μ l T4 polynucleotide kinase, and 12 μ l H₂O were added to the reaction mix and incubated at 37⁰ C for 30 min. A QIAquick nucleotide removal kit was used to remove the unincorporated radioactivity. The labeling and specific activity of probe was determined by counting total cpm in a 1 μ l aliquot (total 200 μ l) using a scintillation counter.

2.3.8 Statistics

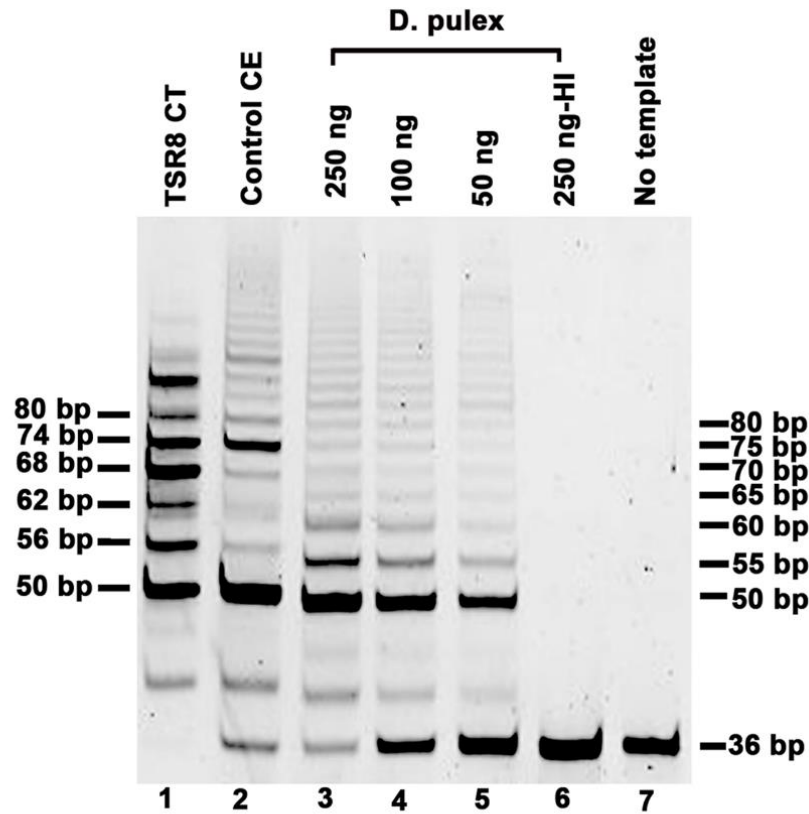
To determine statistical significance of results of the TRAP assay, as well as TRF Assay, a two-tailed Student's T-test was performed, assuming equal variance. Each figure legend denotes p values as denoted by brackets and special characters. Note that our alpha level was p=0.05. A nonparametric log-rank test was performed for significance of the survival curves.

2.4 Results

2.4.1 Telomerase activity is present in *Daphnia* samples

To detect and assay for telomerase activity from adult *Daphnia*, we performed a telomerase repeat amplification protocol (TRAP) assay, using the TRAPeze (Millipore) kit. The kit is optimized for human telomerase activity, thus we tested it for use with *Daphnia* extracts. As represented in figure 2.1A, we could detect strong telomerase activity in extracts prepared from adult *Daphnia* (lanes 3-5). Telomerase activity showed dose-dependence with amount of total protein in extracts. Each TRAP reaction mixture contains primers as well as a template for amplification of a 36 bp internal control, which also serves to determine false negative results due to the presence of a telomerase inhibitor in the extracts. *Daphnia* extracts did not show any inhibition of the

A.



B.

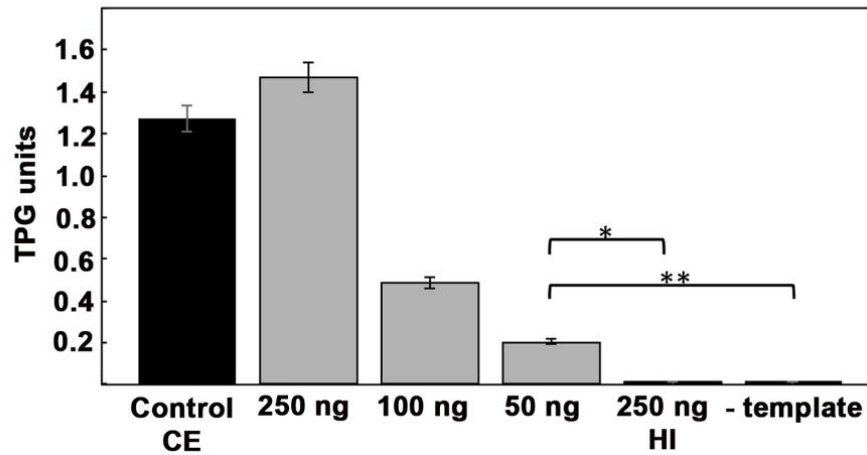


Figure 2.1. Telomerase activity in *Daphnia*. A) Telomeric repeat amplification protocol (TRAP) assay of *D. pulex* extracts. The amounts of cell extract used is indicated above each lane. HI: Heat Inactivated, CE: Cell Extract, TSR8 CT: Positive control for PCR step. B) Quantification of the TRAP Assay in 1A. Student T-test was performed, p values are as follows: $\ast=6.15 \times 10^{-6}$, $\ast\ast=6.63 \times 10^{-6}$ (n=3).

telomerase activity compared to the positive control (lane 2) provided with the kit. The dependence of the band ladder on telomerase activity was confirmed by heat treatment of the *Daphnia* extract (lane 6), which completely eliminated the ladder due to inactivation of telomerase, thus confirming the presence of telomerase activity in *Daphnia* extract. Absence of bands with no template added (lane 7), indicated no contamination in the PCR step of the assay and further confirmed the presence of telomerase activity in *Daphnia* extract. The periodicity of the bands generated using *Daphnia* extract (lanes 3-5) was different than the periodicity obtained with extract from telomerase positive human cell line HEK293 (positive control- lane 1). Figure 2.1B represents quantification of telomerase activity.

Human telomeres have a six-nucleotide telomeric repeat sequence (TTAGGG). To determine the telomeric repeat length and sequence in *Daphnia*, we cloned and sequenced the *Daphnia* TRAP reaction products. As seen in figure 2.2A, the sequence analysis of the clones revealed that the telomeric repeat was TTAGG, a sequence identical to crustaceans *H. americanus* and *G. pulex* (Klapper et al 1998). The five nucleotide repeat sequence corresponds to the observed difference in the periodicity of bands in TRAP assay (Fig. 2.1A, lanes 3-5), as the bands are expected to be shorter by one nucleotide as compared to TRAP assay products obtained with human cell extract (Fig. 2.1A, lane 2). When telomerase (TERT) sequences across various species were compared, the similarity between the *D. pulex* TERT sequence and those of other organisms is relatively low for the entire protein (Figure 2.2B). The highest homology is found in the essential functional domains of the protein: the RNA binding domain and

A.

Species	Telomeric Repeat
<i>D. pulex</i>	TTAGG
<i>H. americanus</i>	TTAGG
<i>G. pulex</i>	TTAGG
<i>B. mori</i>	TTAGG
<i>A. viridus</i>	TTAGG
<i>M. musculus</i>	TTAGGG
<i>H. sapiens</i>	TTAGGG

B.

Species	Similarities	Identities
<i>D. pulex</i>	100%	100%
<i>M. musculus</i>	35.6%	19%
<i>R. norvegicus</i>	35.8%	19.1%
<i>H. sapiens</i>	35.0%	18.7%
<i>M. mulatta</i>	34.9%	18.7%
<i>D. rerio</i>	36.6%	18.7%

C.

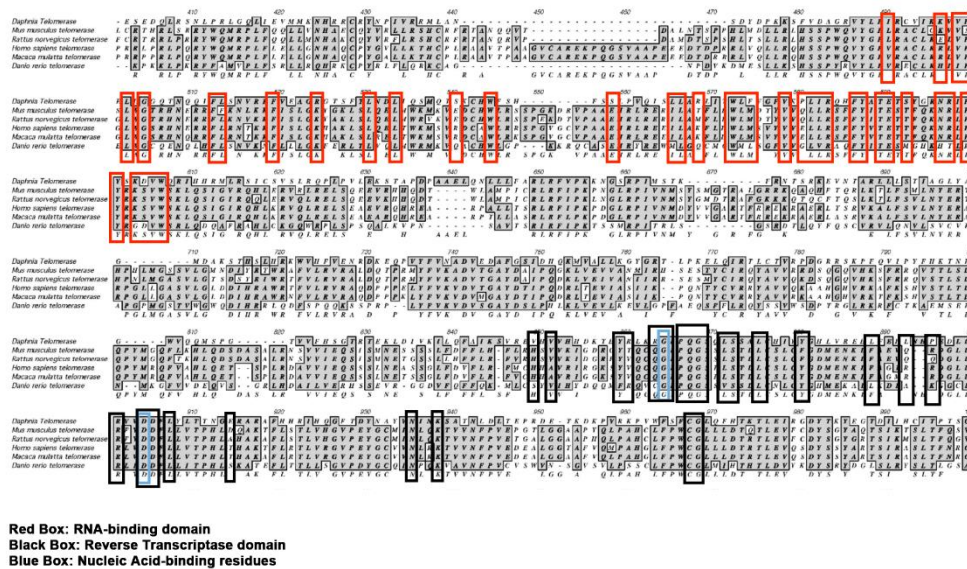


Figure 2.2. Telomeric repeat sequence from *Daphnia* and sequence alignment of *Daphnia* telomerase (dTERT) with telomerase proteins from other species. A) Telomeric repeat sequence of *Daphnia* and other species. *H. americanus*: *Homarus americanus*—Lobster, *G. pulex*: *Gammarus pulex*—Freshwater amphipod, *B. mori*: *Bombyx mori*—Silkworm/moth, *A. viridus*: *Amaranthus viridus*—Beetle, *M. musculus*: *Mus musculus*—Mouse, *H. sapiens*: *Homo sapiens*—Human. B) Identity and similarity percentages of dTERT with TERT from other species. C) *D. pulex* telomerase reverse transcriptase (dTERT) sequence alignment for the RNA-binding and reverse transcriptase domains. Green Boxes: conserved residues within the RNA binding domain. Black Boxes: conserved residues within the reverse transcriptase domain. Blue boxes: residues within the reverse transcriptase domain that are essential for nucleic acid binding.

the reverse transcriptase domain (Figure 2.2C), with high degree of sequence conservation.

2.4.2 Telomerase in *Daphnia* embryos is more processive than in adult organisms

In order to compare the relative telomerase activities in embryos and adult organisms we assayed the extracts from embryos and 1 week-old adults of two ecotypes, *D. pulex* (RW20) and *D. pulicaria* (LakeXVI-clone11). As is shown in figure 2.3, 1 week old *D. pulex* (lane 1) shows more telomerase activity than *D. pulicaria* of the same age (lane 2). On the other hand, both ecotypes have very comparable levels of telomerase activity at the embryonic stage (lanes 3 and 4). *D. pulex* adults at 1 week (lane 1) show some increase in telomerase activity (as measured by total intensity of the bands in entire lane) as compared to embryos (lane 3). In contrast to this, *D. pulicaria* adults at 1 week show a significant decrease in total telomerase activity (lane 2) as compared to embryos (lane 4). Figure 2.3B shows quantification of total telomerase activity in figure 2.3A. In addition to determining total telomerase activity based on band intensities, the TRAP assay can also measure the processivity of telomerase, which is the ability of an enzyme to catalyze multiple reactions without releasing the substrate. The greater the processivity, the greater the number of repeats added by the enzyme during the time interval of the assay. This can be visualized as the ladder reaching to higher molecular weights. As seen in figure 2.3C, a decline in telomerase processivity is observed from embryo stage to 1 week-old stage of both ecotypes.

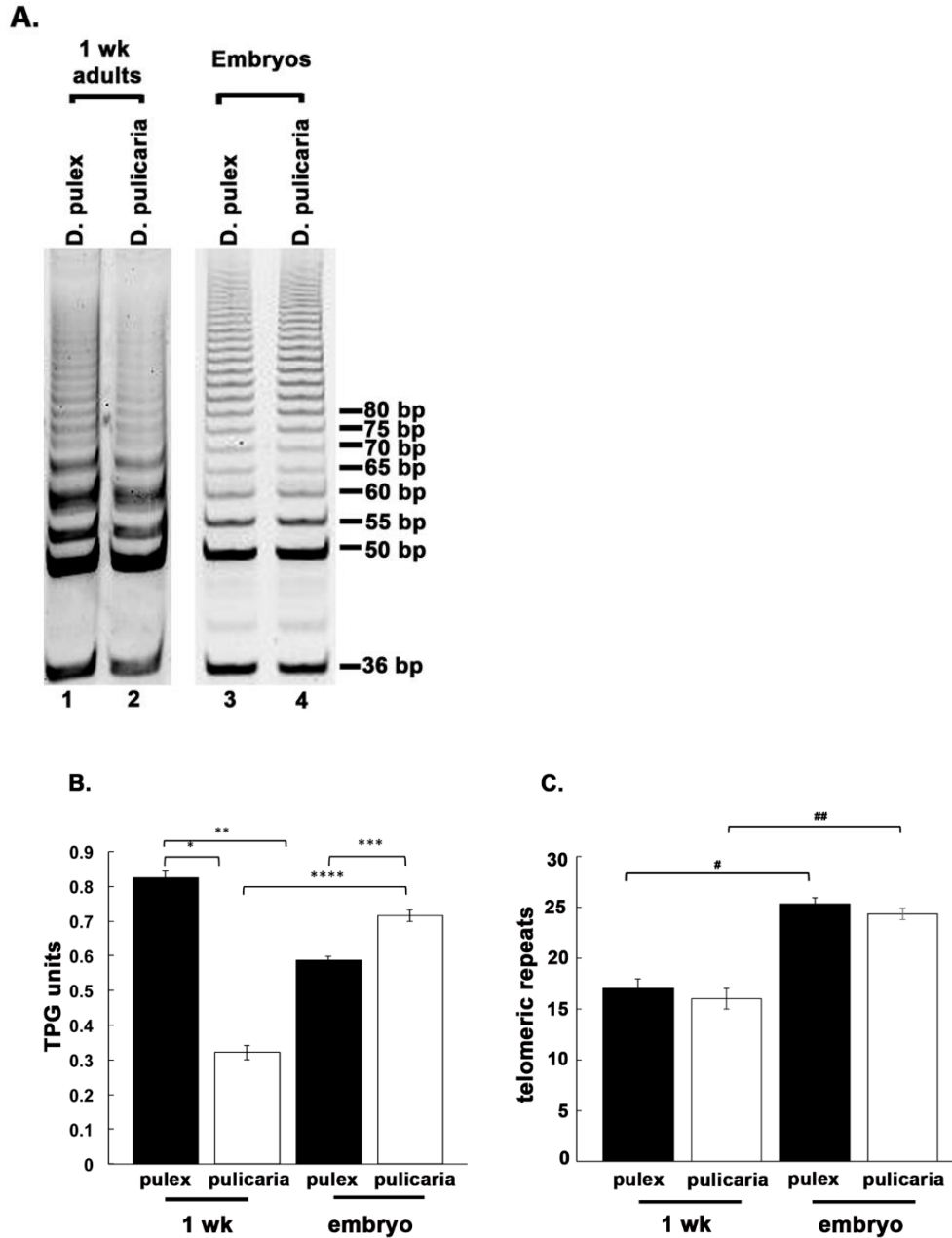


Figure 2.3. Telomerase from *Daphnia* embryos shows high processivity. A) TRAP assay from egg stage 1 embryos and 1 week old adults of *D. pulex* and *D. pulicaria*. 250 ng of *Daphnia* extract was used in each lane. B) Quantification of the TRAP assay displayed in 2 A. C) Quantification of the processivity of telomerase from each displayed sample. The p values calculated from student T-tests are as follows: * = 6.15×10^{-6} , ** = 5.56×10^{-6} , *** = 0.0005, **** = 1.5×10^{-5} ; # = 0.0002, ## = 0.0002 (n = 4).

2.4.3 Comparison of telomerase activity from *D. pulex* and *D. pulicaria* at different ages

We further investigated the telomerase activity of *D. pulex* (RW20 ecotype) and *D. pulicaria* (LakeXVI-clone11) at equivalent points in their respective life spans. We performed the TRAP Assay with extracts from 1, 2, and 3 week-old *D. pulex* and 1, 4, and 8 week-old *D. pulicaria* which were previously determined to correspond to equivalent time points in their respective life spans (Schumpert et al 2014). As seen in figure 2.4 A and B, *D. pulex* (lanes 1-3) showed an increase in telomerase activity from 1 week to 2 week but showed a marginal decline in 3-week-old organisms. In contrast, *D. pulicaria* displayed a steady decline of telomerase activity with age, telomerase activity being the highest at 1 week and the lowest at 8 weeks (Fig. 2.4A, lanes 4-6). The telomerase activity was quantified using the Imagequant software and is shown in figure 2.4B, which shows a 50% increase in telomerase activity from 1 week-old to 2 week-old organisms in *D. pulex*. In contrast, *D. pulicaria* showed an age-dependent decline in telomerase activity with about 30% decrease from ages 1 week to 4 week and another 30% decrease from ages 4 week to 8 week. The processivity of the telomerase from these samples was determined and is represented in figure 2.4C. In *D. pulex*, the processivity of telomerase increased considerably from 1 week to 2 week and showed a small but consistent increase from 2 week to 3 week. In contrast, the processivity of telomerase in *D. pulicaria* samples showed a steady decline with age from 1 week to 8 week.

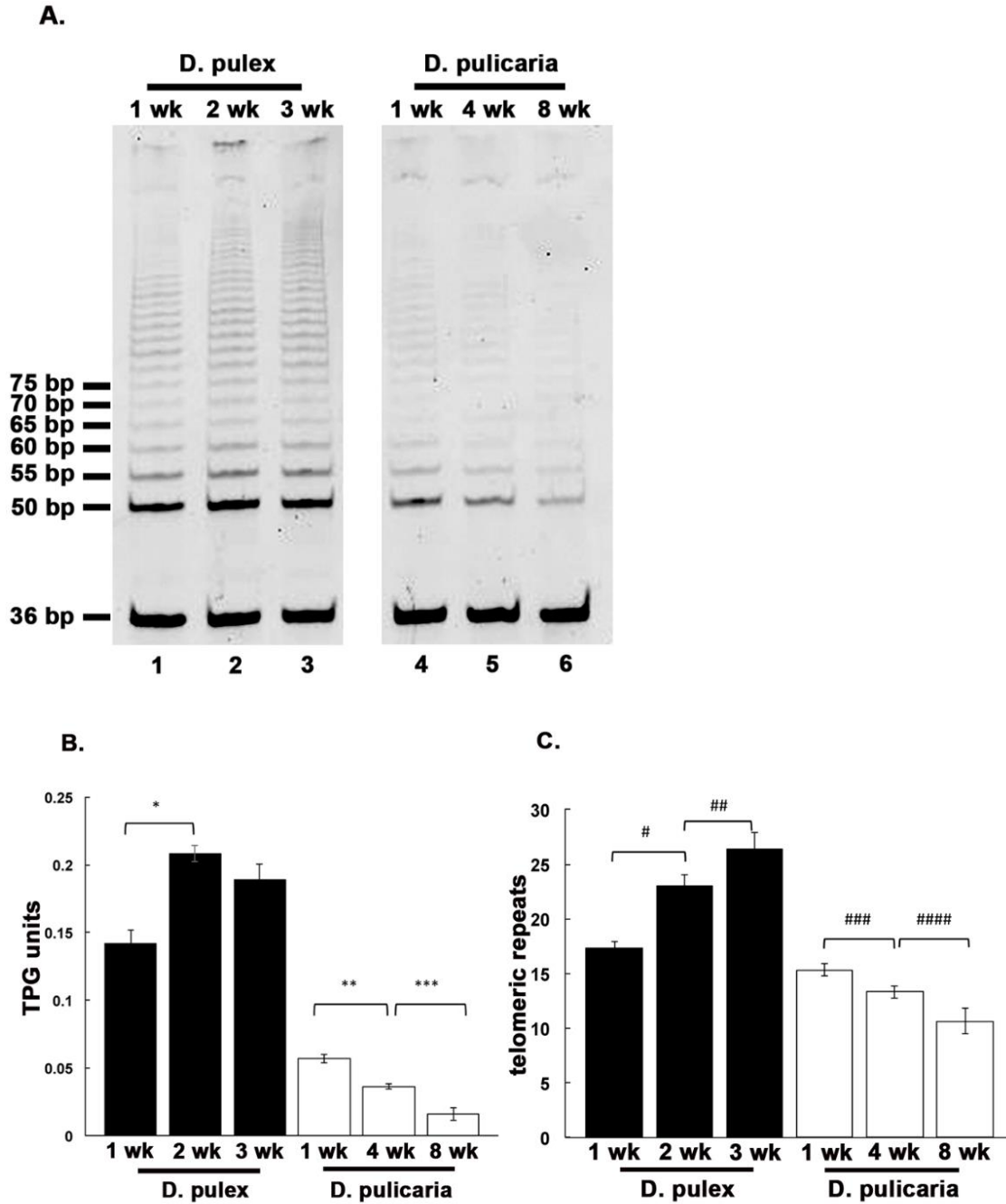


Figure 2.4. Comparison of telomerase activity at different ages in *D. pulex* and *D. pulicaria*. A) TRAP assay was performed using *Daphnia* extracts prepared at indicated ages. Lanes 1–3: *D. pulex*, lanes 4–6: *D. pulicaria*. B) Quantification of the TRAP assay displayed in 4 A. C) Quantification of the processivity of telomerase from each displayed sample. Student T-tests were performed, and p values are as follows * = 0.0005, ** = 0.0006, *** = 0.002, # = 0.001, ## = 0.034, ### = 0.0132, #### = 0.029 (n = 4)

2.4.4 Telomere length in *D. pulex* and *D. pulicaria* at various ages

As shown in figure 2.5A, survivorship patterns are substantially different between *D. pulex* (clone RW20) and *D. pulicaria* (clone Lake XVI-11). RW20 had a median lifespan of 16 d (maximum = 56 d), whereas XVI-11 had a median lifespan of 79 d (maximum = 112 d). A nonparametric log-rank test showed that the survival curves are significantly different ($\chi^2 = 120.894$, $df = 1$, $p < 0.0001$). To analyze the average telomere lengths during life span in these two *Daphnia* clones that display markedly different lifespans as well as significantly different telomerase activities (Fig. 2.4B), we performed a terminal restriction fragment (TRF) assay using genomic DNA isolated from 1, 2, and 3 week old *D. pulex* and 1, 4, and 8 week old *D. pulicaria*. As seen in Figure 2.5B, the telomere-specific probe detected the TRFs at various ages in both ecotypes. The average telomere length was calculated and is represented in Fig. 2.5B. *D. pulex* and *D. pulicaria* telomeres at age of 1 week were quite similar in length with *D. pulex* at 4.9 kb and *D. pulicaria* at 4.5 kb. In *D. pulex*, the telomere length did not shorten with age, with the 2 week old and 3 week old telomeres averaging at 4.9 kb and 5.0 kb respectively. In contrast to this, *D. pulicaria* telomeres exhibited age-dependent shortening with telomere lengths being 3.5 kb and 3.1 kb at 4 weeks and 8 weeks of age respectively. Therefore, at all equivalent ages, *D. pulex*'s average TRF is longer than *D. pulicaria*'s average TRF. Thus, the short-lived ecotype *D. pulex* displayed no shortening of telomere length with age while the long-lived ecotype *pulicaria* showed an age-dependent decline of telomere length.

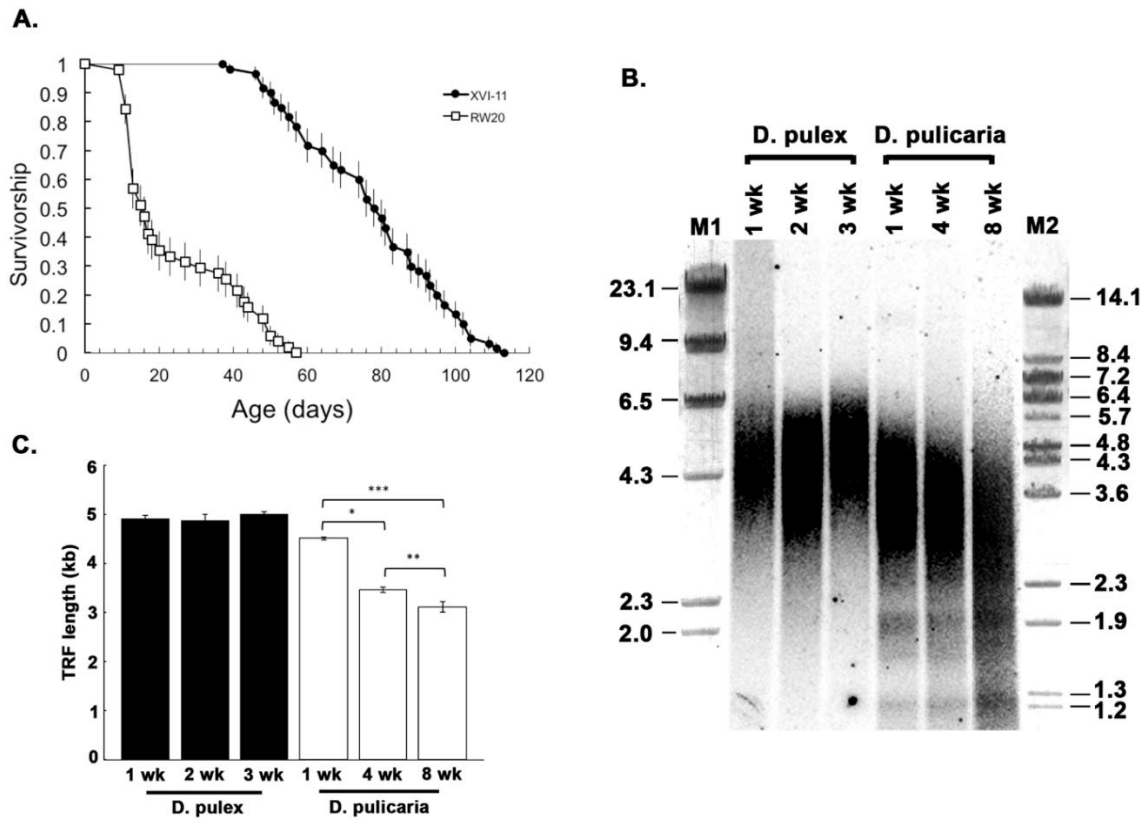


Figure 2.5. Life spans and telomere lengths in *D. pulex* (RW20) and *D. pulicaria* (LakeXVI-clone11). A) Survivorship curves of clones RW20 (open squares) and XVI-11 (black circles). Error bars show age-specific standard errors from Kaplan-Meier survival probability estimates. B) Terminal Restriction Fragment (TRF) assay was performed to estimate the average telomere length of the *Daphnia* at various ages. The age in weeks is indicated above each lane. Lanes 1–3: *D. pulex*, lanes 4–6: *D. pulicaria*. M1 and M2: molecular weight markers; lambda HindIII digest and lambda BstEII digest respectively. C) Quantification of 5 A, error bars indicate standard deviation. Student T-tests were performed and p values are as follows: * = 1.03×10^{-5} , ** = 0.007, *** = 2.48×10^{-5} (n = 3).

2.5 DISCUSSION

Aging is associated with a progressive deterioration of cellular functions leading to a functional decline of organs and tissues. Molecular processes that are considered as characteristics of aged organisms include loss of telomere function, epigenetic genomic changes, declining protein homeostasis, increased cellular senescence, depletion of the stem cell pool, and altered intercellular communication (Lopez-Otin et al 2013).

Telomerase deficiency in humans is associated with premature onset of diseases that are typical of old age (Armanios and Blackburn 2012). There is evidence of a causal link between telomere loss, cellular senescence and organismal aging that emerged from genetically-modified animal models. Mice with shortened or lengthened telomeres exhibit decreased or increased lifespan, respectively (Rudolph et al 1999, Tomas-Loba et al 2008, Armanios et al 2012) and aging could be reverted by telomerase activation in telomerase deficient mice (Jaskelioff et al 2011). In humans, recent meta-analyses have indicated a strong correlation between short telomeres and mortality risk (Boonekamp et al 2013). Along with its telomere-associated function, telomerase is also involved in DNA damage response and shuttles from the nucleus to the mitochondria upon oxidative stress to protect the mitochondrial DNA from sustaining oxidative damage (Ahmed et al 2008, Haendeler et al 2009, Indran et al 2011, Singhapol et al 2013).

Daphnia telomerase protein shows a high degree of homology in the essential functional domains of the protein: the RNA binding domain and the reverse transcriptase domain. *Daphnia* telomeric repeat sequence is TTAGG, a sequence identical to crustaceans *H. americanus* and *G. pulex* (Klapper et al 1998). Shelterin, a

complex formed by six telomere-specific proteins (TRF1, TRF2, TIN2, Rap1, TPP1, and POT1) binds to the telomeric repeats and protects the chromosome ends in mammalian cells (de Lange 2005). Without the shelterin complex, telomeres are not protected from being recognized by the DNA damage surveillance and are inappropriately processed by DNA repair pathway (de Lange 2005). Although the shelterin complex is not found in some organisms, there are functional orthologs of the shelterin component proteins. Using the data and tools in PANTHER (a comprehensive, curated database of protein families, trees, subfamilies, and functions)(Mi et al 2013) we could identify a *Daphnia* homolog of POT1. POT1-like proteins are present in nearly all eukaryotes (Palm and de Lange 2008), and thus it may be of interest in future to identify other proteins that complex with *Daphnia* POT1 since such proteins may be involved in telomere maintenance and chromosome integrity in *Daphnia*.

In our present study, we characterized the telomerase activity, telomerase processivity, and telomere length during the lifespan in two ecotypes of *Daphnia*, the short-lived *D. pulex* and the long-lived *D. pulicaria*. Our results demonstrate a clear age-associated decline in telomerase activity, telomerase processivity, and telomere length in the long-lived ecotype *D. pulicaria*. Surprisingly, the short-lived ecotype *D. pulex* showed no decline in telomerase activity and telomere length but an age-dependent increase in processivity of telomerase. The telomere hypothesis of cellular aging states that the telomeres shorten with each cellular replication event until the telomeres are completely eroded and the resulting genomic instability leads to the cellular death in telomerase negative mammalian somatic cells (Harley et al 1990, Allsopp et al 1992,

Harley et al 1992). Organismal aging, however, is more complex with several different factors in addition to the telomere length and telomerase activity playing a role in the overall lifespan of an organism. Supporting this multifaceted organismal aging process, our results indicate that in the *D. pulex* ecotype, telomere length does not decline with age and thus is not the main cause of its short life span.

Overall, our results are in agreement with previous studies in other model organisms with respect to the relationship between the telomerase activity and corresponding telomere length (Hemann and Greider 2000, Joeng et al 2004, Raices et al 2005, Lund et al 2009). In *D. pulex*, which has high telomerase activity throughout lifespan, there is no decline in overall telomere length. For *D. pulicaria*, a decrease in telomerase activity coincides with a decrease in telomere length with age. Comparing telomere lengths of *D. pulex* and *D. pulicaria* at equivalent points in their lifespan, *D. pulex* always has longer telomeres than *D. pulicaria* corresponding to higher telomerase activity in *D. pulex*. The processivity of *Daphnia* telomerase could be of biological significance in terms its impact on the length of telomeres. Although the telomerase activity may be high in terms of high rate of telomeric repeat addition in TRAP assay, if the enzyme is not processive, the overall telomere length may not be maintained. Thus, in *D. pulex* with high telomerase activity and processivity, individual cellular replication events do not effectively erode the telomeres since telomerase can add *de novo* telomeric repeats to the ends of telomeres. However, in *D. pulicaria* with telomerase activity and processivity decreasing with age, each cellular replication event leads to shortening of telomeres with age. This is particularly relevant in *Daphnia* as it shows

indeterminate overall growth during the entire life span. Although telomerase processivity as a function of aging has not been explored in the past, several different factors are known to contribute to telomerase processivity. These include telomerase RNA template structure, telomere structure, and various proteins that stabilize telomeres (Evans and Lundblad 2000, Lue 2004, Smogorzewska and de Lange 2004). It's possible that there is a difference in any of these three factors between the two ecotypes of *D. pulex* and *D. pulicaria*, which could be investigated in future studies. While our study provides an initial characterization of telomerase activity, processivity, and telomere length in *D. pulex* and *D. pulicaria*, this work utilizes one isolate or clone from each ecotype. Investigating telomerase activity and telomere lengths in other *Daphnia* species and more isolates or clones of *D. pulex* and *D. pulicaria* will be of value.

Although much work has been done investigating cellular aging and telomere length, the connection between telomere length and organismal longevity is not straightforward (Hemann and Greider 2000, Joeng et al 2004, Raices et al 2005, Lund et al 2009). Formulated by Harley *et al*, the telomere hypothesis of cellular aging postulated that telomeres serve as an internal mitotic clock in telomerase negative mammalian somatic cells and when telomere length is exhausted cellular senescence and the eventual death ensues (Harley et al 1990, Allsopp et al 1992, Harley et al 1992, Morin et al 1997). Multiple studies have found varying results in terms of telomere length and overall organismal longevity (Hemann and Greider 2000, Raices et al 2005, Lund et al 2009). Studies involving *C. elegans*, a well-established model for molecular biology of aging, have demonstrated that overall telomere length does not affect or

predict the organismal longevity (Raices et al 2005). *Danio rerio* shows high levels of telomerase activity throughout its lifespan and maintains telomere length even into late stages of life (Lund et al 2009). No telomere shortening with increasing age is seen in wild-derived mouse strains, as well as in the marine bird *Oceanodroma leucorhoa* (Hemann and Greider 2000, Nakagawa et al 2004). The crustacean, *Homarus americanus* (lobster), was investigated to determine overall telomere length and telomerase activity during aging. These organisms display such extensive lifespans that some predict would be nearly immortal if provided with optimal environment (Klapper et al 1998b, Krupp et al 2000). Lobsters display telomerase activity throughout their lifespan with a relatively unchanged telomere length (Klapper et al 1998b). Although cellular aging may be well defined by the telomere hypothesis of cellular aging, organismal aging is a multifactor process with a more complicated relationship between telomere length and organismal longevity (Hemann and Greider 2000, Joeng et al 2004, Raices et al 2005, Lund et al 2009).

The above results lead to the question that if telomere erosion and hence genomic instability is not the cause of the characteristic short life span of *D. pulex* what may be causing this remarkably short life span in *D. pulex*? Organismal aging is a multifaceted process and the ability to respond to and survive the proteotoxic stress is an important factor in determining the longevity of an organism (Morimoto and Cuervo 2009, Anckar and Sistonen 2011). There are several theories of aging; however, studies have shown that the pathologies and phenotypes associated with aging may stem from damaged proteins and the inability to repair or eliminate these damaged molecules

from cells (Morimoto and Cuervo 2009, Anckar and Sistonen 2011, Clancy and Birdsall 2013). Our previous work has established that the induction of chaperone HSP70 in response to heat shock in *D. pulex* declines rapidly with age making it highly susceptible to proteotoxicity. In contrast, *D. pulicaria* continues to show a robust heat shock response and chaperone HSP70 induction past the midpoint in its life span, thus enabling it to survive proteotoxicity (Schumpert et al 2014). Although there are many different aspects of the aging process, it is possible that the ability to respond to proteotoxic stress is a better determinant of organismal life span instead of overall length of the telomeres. In other words, the cellular damage arising from being unable to respond appropriately to proteotoxic stress may lead to death before the telomere length declines. In addition to heat stress, oxidative damage to proteins also causes proteotoxicity and in multiple organisms investigated, better ability to respond to oxidative stress is correlated with longer lifespans (Lopez-Otin et al 2013). In the marine crustacean, *Acartia tonsa*, oxidative damage was found to be greater in the shorter lived male individuals of the species indicating that oxidative damage may play a role in the overall lifespan of this crustacean (Rodriguez-Gana et al 2010). A study done in honeybees identified vitellogenin as a protein that protects the organism from oxidative stress and contributes to longevity (Seehuus et al 2006). *Daphnia* contain a vitellogenin homolog that could be investigated for any differences in *D. pulex* vs *D. pulicaria* for its role in protection from oxidative damage (Colbourne et al 2011). As *Daphnia* is emerging as a model system in aging studies our results reveal a non-concordance between telomerase activity, telomere length and the overall organismal aging and

indicate that factors other than telomere length maintenance contribute to strikingly short life span in *D. pulex*.

Chapter 3:
Relationship between heat shock protein 70 expression and life span in *Daphnia*³

³ Schumpert C, Handy I, Dudycha J, Patel R. Relationship between heat shock protein 70 expression and life span in *Daphnia*. *Mechanisms of Ageing and Development* 139:1-10. Reprinted with permission.

3.1 Abstract

The longevity of an organism is directly related to its ability to effectively cope with cellular stress. Heat shock response (HSR) protects the cells against accumulation of damaged proteins after exposure to elevated temperatures and also in ageing cells. To understand the role of Hsp70 in regulating life span of *Daphnia*, we examined the expression of Hsp70 in two ecotypes that exhibit strikingly different life spans. *D. pulicaria*, the long lived ecotype, showed a robust Hsp70 induction as compared to the shorter lived *D. pulex*. Interestingly, the short-lived *D. pulex* isolates showed no induction of Hsp70 at the midpoint in their life span. In contrast to this, the long-lived *D. pulicaria* continued to induce Hsp70 expression at an equivalent age. We further show that the Hsp70 expression was induced at transcriptional level in response to heat shock. The transcription factor responsible for Hsp70 induction, heat shock factor-1 (HSF-1), although present in aged organisms did not exhibit DNA-binding capability. Thus, the decline of Hsp70 induction in old organisms could be attributed to a decline in HSF-1's DNA-binding activity. These results for the first time, present a molecular analysis of the relationship between HSR and life span in *Daphnia*.

3.2 Introduction

Ageing is a universal property of multicellular organisms that causes functional decline of all biological systems. Proteotoxicity due to misfolded proteins is a central component of ageing and the ability of organisms to deal with misfolded proteins is crucial for longevity (Taylor and Dillin 2011). As an organism ages, the free radicals increase within cells due to mitochondrial malfunctions and inefficiency, which in turn

leads to an increase in altered proteins (Cui et al. 2012). The pathologies and phenotypes of ageing are caused primarily by the inability to deal with proteotoxic stress and an accumulation of altered proteins (Clancy and Birdsall 2013). In order to appropriately respond to proteotoxic stress, a physiological response termed the heat shock response (HSR) is induced, which involves a rapid and transient increase in expression of molecular chaperones such as heat shock proteins (Hsps) following a proteotoxic stimulus (Calderwood et al. 2009). HSR enables an organism to handle proteotoxic conditions and survive without an extended, damaging imbalance in protein homeostasis. Molecular chaperones, such as Hsp70, act to renature the denatured or misfolded proteins or trigger their degradation if renaturation is not possible (Bukau et al. 2006). The Hsp gene expression is induced at transcriptional level and is mainly regulated by the transcription factor Heat Shock Factor-1 (HSF-1) (Anckar and Sistonen 2011).

Among molecular chaperones, Hsp70 plays a regulatory role in the ageing process since it mitigates the effects of proteotoxic stress (Calderwood et al. 2009; Kim et al. 2013). Overexpression of Hsp70 by knocking in more copies of the gene in *C. elegans* led to an overall increase in lifespan (Yokoyama et al. 2002). Consistent with this, knockdown of Hsp70 led to accelerated aging including premature death (Kimura et al. 2007). Hsp70 and its transcriptional regulator HSF-1 are both important in coping with proteotoxic stress as well as the general increase of altered and misfolded proteins in aged organisms (Morley and Morimoto 2004; Morimoto and Cuervo 2009; Anckar and Sistonen 2011). Hsp70 protein levels can be used as a predictor of total life span in *C.*

elegans and *D. melanogaster*, because the organisms that display a more robust HSR and a higher expression of Hsp70 also have longer lifespans (Rea et al. 2005; Tower 2011). In the present study we have investigated the HSR of two different ecotypes of *Daphnia* that have very different life spans by examining the expression of Hsp70.

Daphnia are small freshwater crustaceans that are an important model system in ecology, evolutionary biology, and ecotoxicology (Benzie 2005). These organisms are easy to maintain, reproduce via cyclic parthenogenesis, have transparent carapaces, and have relatively short life-span, thus making them a good experimental model. In cyclic parthenogenesis, wild populations reproduce mainly via ameiotic cloning (parthenogenesis), but periodic environmental stress induces sexual reproduction. Parthenogenetic reproduction can be enforced in the lab, making it possible to maintain isogenic individuals from one generation to the next that reflect naturally occurring genomes. These unique properties have made them a useful model for research on naturally occurring patterns of variation in ageing (Dudycha 2001; Dudycha and Hassel 2013) and on the effect of environmental variation on ageing (Dudycha 2003; Steinberg et al. 2010). We focus on two ecotypes of the *D. pulex* species that are adapted to distinct habitats, and thus have evolved sharply divergent lifespans (Dudycha and Tessier 1999). *D. pulex* inhabits temporary ponds, where it faces high extrinsic mortality risk and has a short lifespan with an average being about 25-30 days (Dudycha 2001; Dudycha 2004). In contrast, *D. pulicaria* inhabits large stratified lakes, encounters low extrinsic mortality, and has a long lifespan with an average being about 60-65 days (Dudycha 2001, 2004). Though the ecotypes have different names, they are not fully

distinct species, and abundant evidence supports ongoing genetic exchange (Dudycha 2004; Cristescu et al. 2012). Estimated divergence time is only ~82,000 years (Omilian and Lynch 2009), a relatively short evolutionary span that is lower than the divergence among some human populations. The complete genome sequence of *D. pulex* was recently published (Colbourne et al. 2011). For simplicity, throughout the manuscript, we use the specific terminology *D. pulex* and *D. pulicaria* to distinguish the pond- and lake- ecotypes with short and long life spans respectively. All of the clones used in this study have been acclimated to the lab for >3 years (i.e., >75 asexual generations).

It is clear that these named taxa, though ecologically separate, are not evolutionarily distinct species, and gene flow continues to occur between the habitats at a local scale. Heier & Dudycha (2009) showed that experimental crosses between the ecotypes were fully fertile, and recent population genetic and genome-scale analyses confirm that gene flow and introgression is widespread (Daniel et al. 1995; Cowan et al. 1996; Vergilino et al. 2011; Cristescu et al. 2012; Tucker et al. 2013; Xu et al. 2013). The two ecotypes face different patterns of temperature variation in nature, but the ranges of temperatures they experience are similar. In a study of field demography (Dudycha 2004) populations in southwest Michigan, including the sources of our study clones, were monitored for a year, confirming that these populations experience similar temperature ranges. In both habitats, *Daphnia* will experience temperatures ranging from 4°C to the >28°C, but the physics of water bodies dictates that they experience them differently (Wetzel 2001). In temporary ponds, the largest change is seasonal, with cold temperatures as snow melts shortly before the population emerges from

overwintering dormant eggs, and warmer temperatures as the population produces dormant eggs in summer. There is some daily variation with changes in air temperature, but small ponds are primarily insulated by and reflect ground temperatures. Lakes also have seasonal dynamics, but the populations are active year round, and in winter experience a continuous temperature of 4°C. By summer, lakes stratify into a warm upper layer (typically ranging from 24° - 28°) and a cool bottom layer (~10°C), with *Daphnia* migrating between the two temperature zones on a daily basis.

Previous reports have shown that despite the limited genetic divergence between the ecotypes that there is substantial differentiation of life history and related traits. They differ in lifespan, age-specific mortality rates, the timing and rate of age-dependent increases in mortality rates, maximum reproductive rate, the rate of reproductive decline with aging, and juvenile growth rate (Dudycha and Tessier 1999). This study included naturally occurring hybrids between the ecotypes which showed intermediate characteristics, and the results were confirmed in later studies (Dudycha 2001, 2003). Other direct comparisons have indicated there are differences in investment in dormancy and sexual reproduction (Caceres and Tessier 2011). Physiological studies on *Daphnia* are very few in number; we are unaware of any comparisons of respiration or metabolic rate, though there is limited evidence that *D. pulicaria* has superior photoenzymatic repair of DNA damage (Connelly et al. 2009).

In our present work we establish a foundation for the use of *Daphnia* as a model system in molecular biology of ageing. We evaluate age-dependence of the HSR in *D. pulex* and *D. pulicaria*, testing its relationship to their life span. Our results show that

the two ecotypes respond differently to heat stress, as measured by analysis of Hsp70 protein expression. *D. pulicaria*, the long lived ecotype, responds better than the shorter lived *D. pulex*, supporting a role for HSR in differential ageing of the ecotypes. For the first time, these results present a molecular analysis of the relationship between stress response and regulation of life span in *Daphnia*. In addition, our findings offer a mechanistic insight for the lack of HSR in older organisms by demonstrating that the transcription factor HSF-1 responsible for transcriptional induction of Hsp70 loses its ability to bind DNA as *Daphnia* age.

3.3 Materials and Methods

3.3.1 Daphnia Cultures

D. pulex and *D. pulicaria* ecotypes used in this study were isolated from ponds in southwest Michigan, USA in 2008 (except for clone “TCO” which was isolated from Oregon, USA) and have since been cultured in the lab. *Daphnia* are maintained at a temperature of 20° C with a photoperiod of 12:12 L:D (12 hours of light followed by 12 hours of dark) within a Percival growth chamber. *Daphnia* were maintained at a concentration of 3 to 5 animals per 250 ml beaker in 200 ml of filtered lake water. *Daphnia* were cleared of young and transferred to a new beaker with fresh water on alternate days. They were fed every day with vitamin-supplemented algae *Ankistrodesmus falcatus* at a concentration of 20,000 cells/ml. To generate experimental animals, even-aged cohorts were begun by placing neonates individually in 100 ml of filtered lake water. Experimental animals were otherwise maintained as in the source cultures.

3.3.2 Heat Shock Treatment:

Daphnia of specific ages were placed into groups of 25-30 organisms in 1 ml of lake water in a microcentrifuge tube. The tubes were placed in a heating block at 32°C for 30 minutes. *Daphnia* were allowed to recover at room temperature (22° C) for 4 or 6 hr. The control samples were also separated into groups of 25-30 but were kept at room temperature for the duration of the experiment and then harvested.

3.3.3 Western Blot Analysis:

D. pulex and *D. pulicaria* total protein extracts were prepared from either treated or untreated organisms. All water was removed from the tubes containing *Daphnia*, the organisms were washed once with 1 ml cold PBS. The PBS was removed and the organisms were homogenized using a tight-fitting pestle in RIPA buffer (150 mM NaCl, 1.0% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitor cocktails (Sigma and Calbiochem). The samples were chilled on ice for 5 minutes, centrifuged at 13,000 x *g* for 4 minutes and supernatant was saved as total protein extract. The protein concentration was determined immediately using the BCA kit (Pierce), Laemmli's sample loading buffer (4X) was immediately added to the extract following protein concentration determination and samples were heated at 95° C for 5 minutes to further inactivate any residual proteases from the *Daphnia*. This protocol ensures efficient inactivation of gut proteases. 50 µg of total protein were separated by SDS-PAGE and a western blot analysis was performed using ECL plus (Pierce) chemifluorescence detection. The primary antibodies used are as follows: Hsp70 (Fisher MAB3-007, 1:1000), β-actin (Sigma A5441, clone AC-15, 1:5000), α-tubulin (Sigma,

T5168, 1:5000). Chemifluorescent signal was detected using a Molecular Dynamics Storm 860 Phosphor Imager.

3.3.4 Statistics

For the analysis of fold-inductions in western blot and Real Time-PCR analyses a two-tailed Student's t test was performed with equal variance comparing values as indicated by brackets. A P value of 0.01 or lower was considered statistically significant.

3.3.5 Reverse Transcriptase PCR

RNA was isolated from *Daphnia* using RNazol B reagent (TelTest). Between 25 and 30 individuals were separated into a microcentrifuge tube, subjected to heat shock or left untreated, all water was removed after heat shock, 1 wash with 1 ml cold PBS was given, and 0.8 ml of RNazol B was added. The samples were homogenized and total RNA was isolated as per the manufacturer's instructions. cDNA was synthesized at 42°C for 1 h using random hexamer primers, 1 µg total RNA, M-MuLV reverse transcriptase, 500 µM dNTPs, and RNase Inhibitor RNasin (Promega). For each PCR reaction, 2 µl of total cDNA and 50 pmoles each of the forward and reverse primers designed to amplify a 418 bp region of Hsp70 transcript or a 500 bp region of β-actin transcript were used with the Promega GoTaq PCR kit. The following conditions were used for PCR: 95° C for 5 minutes (initial denaturation), denaturation at 95° C for 30 seconds, annealing at 52° C for 30 seconds, extension at 72° C for 30 seconds for 20 cycles in order to stay within linear range of amplification. The linear range was determined by varying cycle numbers and performing a densitometric analysis of the amplified product.

3.3.6 Real Time PCR

RNA was isolated from *Daphnia* after heat shock after indicated recovery times (as well as a control, non-heat shocked sample). cDNA was synthesized as described before using random hexamers. We first standardized our cDNA by performing real time PCRs with serial dilutions of cDNA to ensure appropriate efficiency and correlation. Every reaction was performed in triplicate in a total volume of 20 μ l. This included 4 μ l cDNA, 250nM Hsp70 or β -actin primers, and SsoFast EvaGreen Supermix (BioRad). β -actin was used for normalization. All reactions were run on a BioRad CFX96 Real Time System C1000 Thermal cycler machine with the following conditions: 95° C for 30 seconds, 95° C for 5 seconds, 52° C for 5 seconds (the last three steps repeated for 40 cycles), 65° C for 5 seconds, and then 95° C for 5 seconds. We analyzed our data using the Bio-Rad CFX Manager Software and used the $2^{-\Delta\Delta Ct}$ method to compare Hsp70 expression in heat shocked versus the non-heat shocked samples. Note that 3 separate RNA isolations were utilized from 3 separate groups of *Daphnia* to serve as biological replicates for each clone for each treatment.

3.3.7 Electrophoretic Mobility Shift Assay (EMSA)

The probe used was heat shock element (HSE) as previously described previously (Akerfelt et al. 2010), sequence of the upper strand: GGGCAGAAATTTCTAGAATCAGC. The double-stranded synthetic oligonucleotide corresponding to HSE sequence was end labeled with [γ -³²P] ATP using T4 polynucleotide kinase. To determine specificity of interaction, HSE oligonucleotide and a consensus Oct1 oligonucleotide (upper strand sequence: TGTCGAATGCAAATCACTAGAA) were used as unlabeled competitors in 50

molar fold excess. HSE oligonucleotide serves as the specific competitor and Oct-1 oligonucleotide, which is the specific binding sequence for Oct-1 (octamer-binding transcription factor -1) transcription factor serves as the non-specific competitor for HSF-1. Whole cell extracts were prepared as described previously with some modifications (Murata et al. 1999). For analysis of HSF-1 DNA-binding activity after heat shock, whole cell extracts have been used widely as HSF-1 undergoes post-translational modification and trimerization leading to its activation in response to heat shock. Prior to heat shock activation, HSF-1 is non active and exhibits no DNA-binding activity. *Daphnia* were heat shocked as described before and homogenized in 3 volumes of extraction buffer (20mM HEPES pH 7.9, 0.4 M KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 25% glycerol and 0.2 mM phenylmethylsulfonyl fluoride), extracts were centrifuged at 13,000 xg for 5 minutes. The supernatants were diluted with an equal volume of the extraction buffer lacking KCl and were stored at -80 C. Binding reaction mixture included 18 mM HEPES pH 7.9, 80 mM KCl, 2 mM MgCl₂, 10 mM DTT, 10% glycerol, 0.2 mg/ml poly (dl-dC), 15 fmol of ³²P-labeled probe, and 5 µg of whole cell extract in 12.5 µl. The reaction mixtures were incubated at 25° C for 20 minutes and analyzed on a 5% native polyacrylamide gel via PAGE. The gel was dried and radioactivity was visualized via Phosphor Imager analysis (Molecular Dynamics Storm 860).

3.4 Results

3.4.1 Induction of Hsp70 in *Daphnia*

In order to characterize the Hsp70 induction in response to heat shock, we analyzed the induction of Hsp70 at different temperatures in *D. pulicaria* clone Lake XVI-11 (Fig. 3.1A). *D. pulicaria* were normally grown and maintained at 20° C and in order to determine the temperature at which Hsp70 would be induced optimally we performed a western blot analysis on protein extracts prepared from *Daphnia* heat shocked for 30 minutes at each of the indicated temperatures and allowed to recover for 4h. As seen in Fig. 3.1A, there was a basal level of Hsp70 expression in control sample without heat shock (lane 1), which increased in a temperature dependent manner (lanes 2-5). Heat shock at 34° C resulted in Hsp70 induction at the same level as at 32° C (data not shown), but exhibited some lethality in the organisms and thus, we decided on using 32° C as the temperature for all future experiments. We tested several anti-Hsp70 antibodies, of which only one detected a heat-shock inducible band at the appropriate molecular weight in *Daphnia* extracts. As seen in Fig. 3.1A, the antibody detected a single band on western blots and was used for all further analyses. Both anti- β -actin and anti- α -tubulin antibodies were used as loading controls because at present there is limited information on which antibodies recognize corresponding *Daphnia* proteins. *Daphnia* β -actin protein has a predicted molecular weight of 41.8 kd and exhibits a high degree of homology to human β -actin with 367 amino acids being identical out of the total 376 amino acids (97 % sequence conservation). *Daphnia* α -tubulin protein has a predicted molecular weight of 49.9 kd and shows a high degree of homology to human

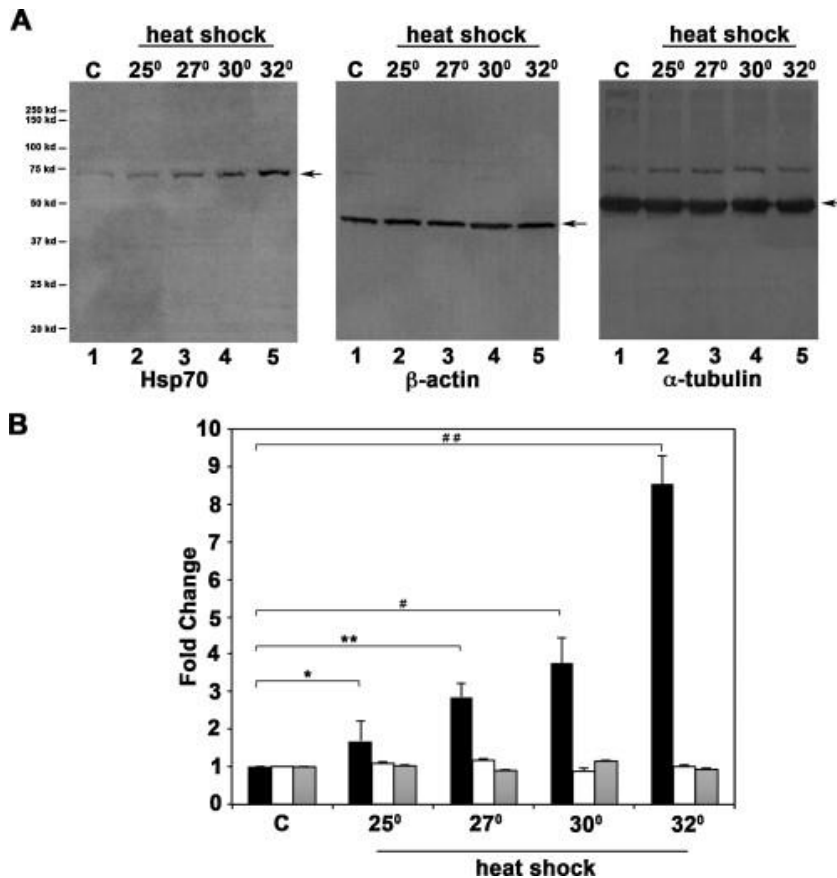


Figure 3.1. Induction of Hsp70 in *D. pulicaria* in response to heat shock. A) Western blot: *D. pulicaria* (lake XVI-clone11) were subjected to heat shock at indicated temperatures for 30 minutes and allowed to recover for 4h. Western Blot analysis was performed using 50 µg of total protein using anti-Hsp70 antibody. C lanes indicate control samples with no heat shock treatment. The blot was re-probed with anti-β actin and anti-α tubulin antibodies to ensure equal amounts of protein were loaded in each lane. The positions of molecular weight markers are as indicated on the left. The arrows indicate bands corresponding to Hsp70, β-actin, and α-tubulin. The antibody used in each panel is indicated at the bottom of the blot. B) Quantification of western blot data: The chemifluorescent band intensities were quantified using STORM phosphorimager and the averages from 4 independent biological replicates is represented as a bar graph and the error bars represent standard deviations. Fold-changes are calculated with respect to signals in control lanes. The black bars represent Hsp70, the white bars represent β-actin, and the grey bars represent α-tubulin bands. As shown above bars, *, **, #, and ## symbols indicate P values that indicate a significant difference (0.0019, 0.0009, 0.0008, and 0.0007 resp.). There was no significant difference observed in signals from β-actin or α-tubulin bands in various lanes with significant values being < 0.01.

β -tubulin with 419 amino acids being identical out of the total 450 amino acids (93% sequence conservation). Based on the percentage homologies between human and *Daphnia* proteins, both antibodies that were raised against the human homologs were able to detect bands at expected molecular weights in *Daphnia* extracts as seen in Fig.3.1A. Our results establish that either one of these proteins could be used as a loading control to normalize western blot analysis since both antibodies detect corresponding *Daphnia* proteins. The western blot signals were quantified using STORM phosphorimager and Imagequant software and the data is represented as bar graphs in Fig. 3.1B. The levels of β -actin and β -tubulin did not vary after heat shock, whereas the Hsp70 protein was significantly induced in response to heat shock. The P values are indicated in the figure legend and were obtained by a statistical analysis of data obtained from 4 independent biological replicates.

3.4.2 Long-lived *D. pulicaria* displays a more robust HSR than short-lived *D. pulex*

Since the two ecotypes differ substantially in lifespan we examined the induction of Hsp70 in response to heat shock in *D. pulex* and *D. pulicaria*. As seen in Fig. 3.2A, *D. pulicaria*, the long-lived ecotype, displayed a more robust induction of Hsp70 (4.1-fold and 7.5-fold induction of Hsp70 in lanes 6 and 7) in response to heat shock than *D. pulex* at the age of 1 week at both 4h and 6h recovery time points (3.2-fold and 1.5-fold induction of Hsp70 in lanes 3 and 4). In addition, the basal level of Hsp70 expression was also higher in *D. pulicaria* than in *D. pulex* (lanes 5 and 2 respectively). The band intensities were quantified using STORM phosphorimager and Imagequant software and the data is represented as a bar graph in Fig. 3.2C. The levels of β -actin and β -tubulin

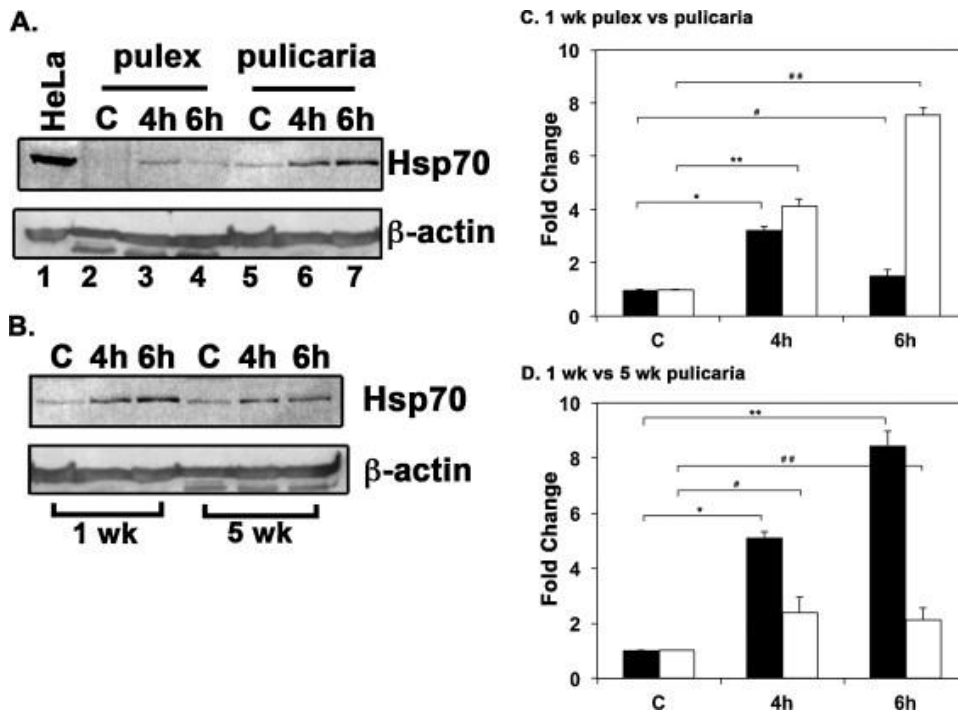


Figure 3.2. A. Comparison of HSR in *D. pulex* and *D. pulicaria*. One week old *D. pulex* (TCO) and *D. pulicaria* (lake XVI-clone11) were subjected to heat shock at 32° C for 30 minutes, extracts were prepared after 4h or 6h recovery period. Western Blot analysis was performed using 50 μ g total protein; anti-Hsp70 antibody. The source of the extract and recovery periods after heat shock are as indicated above each lane. C: control extract from organisms not subjected to heat shock. HeLa extract from heat-shocked cells was used as a positive control. The blot was re-probed with anti- β actin antibody to ensure equal loading. B. Comparison of HSR in young versus old *D. pulicaria*. One and five week old *D. pulicaria* (lake XVI-clone11) were subjected to heat shock at 32° C for 30 minutes, allowed to recover for indicated time, and protein was extracted. Western Blot analysis was performed using 50 μ g of total protein with anti-Hsp70 antibody. Lane C: non-heat shocked control, 4h: 4h recovery period, and 6h: 6h recovery. The age of *Daphnia* is indicated below lanes. Blot was re-probed with anti- β actin antibody to ensure even loading. C and D. Quantification of western blot in Fig. 3.2 A and Fig 3.2 B respectively: The chemifluorescent band intensities were quantified and the averages from 5 and 3 independent biological replicates (resp.) are represented as bar graphs; error bars represent standard deviations. C: The black bars represent *D. pulex*, and the white bars represent *D. pulicaria*. As shown above bars, *, **, #, and ## symbols indicate P values that indicate a significant difference C: (0.0009, 0.0007, 0.0018, 0.0008 resp.). D: *, #, **, and ## are associated with respective p values (0.0013, 0.0027, 0.0018, 0.0038.). The black bars represent samples from 1 wk old *D. pulicaria*, and the white bars represent samples from 5 wk old *D. pulicaria*. As shown above bars, symbols indicate P values that indicate a significant difference.

did not vary after heat shock, whereas the Hsp70 protein was significantly induced in response to heat shock. The P values are indicated in the figure legend and were obtained by a statistical analysis of data obtained from 5 independent biological replicates.

3.4.3 Young Daphnia exhibit a more robust HSR than old Daphnia

Next, we wanted to examine the ability to respond to heat stress in relation to age. We thus characterized the induction of Hsp70 in response to heat shock at two different ages of *D. pulicaria*. As seen in Fig. 3.2B, the 1 week-old *D. pulicaria* exhibited a more robust HSR (5.1-fold and 8.1-fold induction of Hsp70 in lanes 2 and 3) than the 5 week-old organisms (2.3-fold and 2.1-fold induction of Hsp70 in lanes 5 and 6). The band intensities were quantified using STORM phosphorimager and Imagequant software and the data is represented as a bar graph in Fig. 3.2D. The levels of β -actin and β -tubulin did not vary after heat shock, whereas the Hsp70 protein was significantly induced in response to heat shock. The P values are indicated in the figure legend and were obtained by a statistical analysis of data obtained from 3 independent biological replicates.

3.4.4 HSR decreases with age in multiple clones of D. pulex and D. pulicaria

Two of the isolated clones within the *D. pulex* ecotype were chosen for further analysis, TCO (The Chosen One, Fig. 3.3A) and RW20 (Roughwood 20, Fig. 3.3B) and two of the isolated clones of *D. pulicaria* were investigated further, 3L2-1 (Three Lakes Two-1, Fig. 3.3C) and XVI-11 (Lake Sixteen-11, Fig. 3.3D). Both *D. pulex* clones exhibit short lifespans (~ 4 weeks) as compared to *D. pulicaria* clones, which display longer lifespans

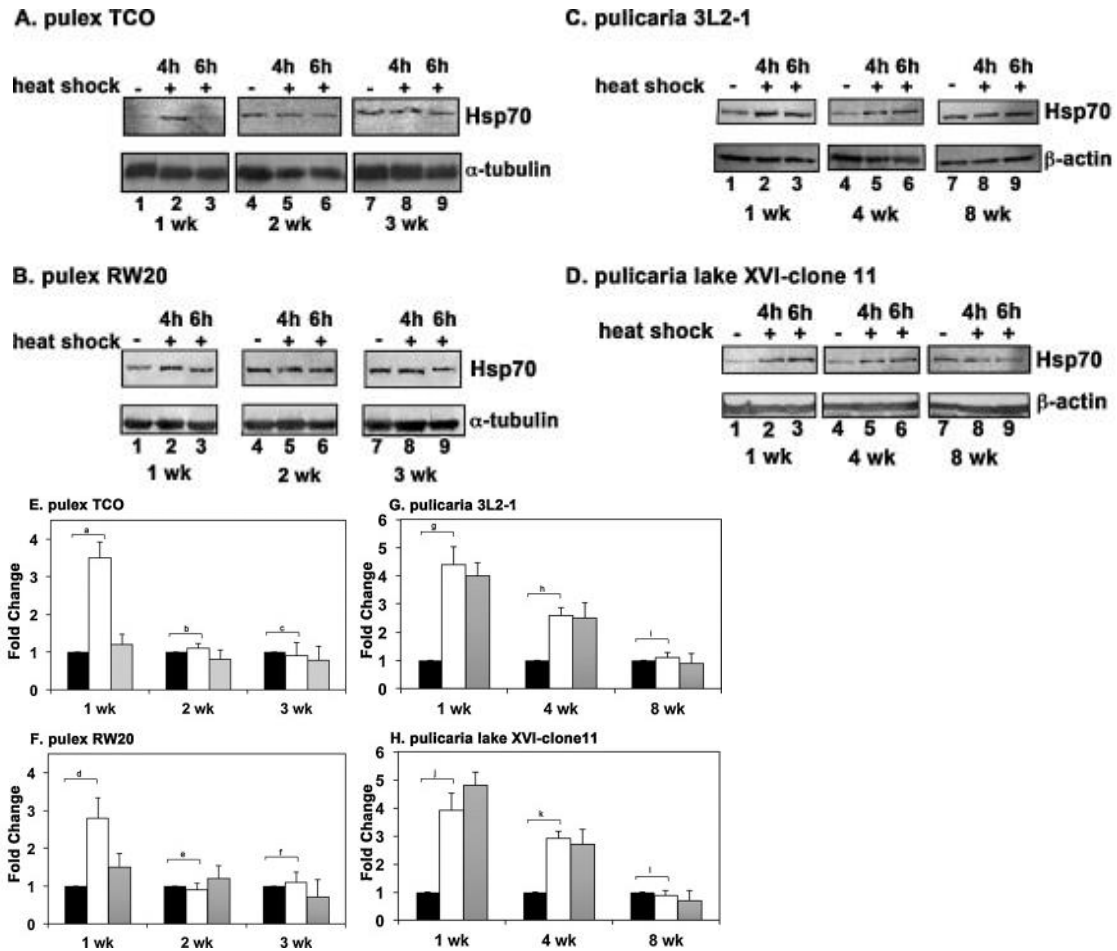


Figure 3.3. Comparison of HSR at different ages in two isolates each of *D. pulex* and *D. pulicaria*. A. HSR in *D. pulex* isolate TCO. B. HSR in *D. pulex* isolate RW20. C. HSR in *D. pulicaria* isolate 3L2-1. D. HSR in *D. pulicaria* isolate lake XVI-clone 11. *Daphnia* were subjected to heat shock at 32° C for 30 minutes, allowed to recover for indicated time periods, and protein extracted. Western Blot analysis was performed using 50 µg total protein; anti-Hsp70 antibody. The recovery periods after heat shock are as indicated above the lanes and the age of the *Daphnia* are indicated in weeks below the panels. Blots were re-probed with anti-α tubulin antibody to ensure even loading. E-F. Quantification of western blot in Figs. 3 A-D: The chemifluorescent band intensities were quantified and the averages from several independent biological replicates (3 replicates for pulex ecotypes and 4 replicates for pulicaria ecotypes) is represented as bar graphs; error bars represent standard deviations. The black bars: control samples, the white: 4 h recovery, and grey bars: 6 h recovery. As shown above bars, a, d, g, h, j and k labels indicate P values that show a significance (0.0010, 0.0018, 0.0014, 0.0008, 0.0021, and 0.0038 resp.) compared to controls. The P values indicated by labels b, c, e, f, i, and l exhibited no significant difference (0.034, 0.045, 0.086, 0.052, 0.11, and 0.21) compared to controls. There was no significant difference observed in signals for β-actin in various lanes with significant values being < 0.01

(~ 9 weeks). The difference in their lifespans is more than two-fold. We examined the induction of Hsp70, in response to heat shock at different ages in two clones for each ecotype. In all clones, the HSR as indicated by induction of Hsp70 expression, decreases with age (Fig. 3.3A-D) while the basal level of Hsp70 protein increases with age (lanes 1, 4, 7). It is interesting to note that in RW20, the Hsp70 induction persisted for a longer duration compared to TCO, since at 6h recovery, there was significant amount of Hsp70 expression in RW20 (Fig. 3.3B lane 3), whereas for TCO Hsp70 expression returned to basal levels by that time (Fig. 3.3A, lane 3). Any significance of this observation to ageing related physiology remains to be explored in future. In addition to a more robust HSR in *D. pulicaria* (Fig. 3.3C and D, lanes 1-3) as compared to *D. pulex* at 1 week (Fig. 3.3A and B, lanes 1-3), there were other significant differences observed in HSR of short-lived ecotypes as compared to long-lived ecotypes. Both TCO and RW20 ecotypes of *D. pulex* stopped responding to heat shock at the age of 2 weeks and showed no induction of Hsp70 (Fig. 3.3A and B, lanes 4-6). Both these ecotypes also showed increased basal expression of Hsp70 at 2 weeks of age as compared to 1 week (Fig. 3.3A and B, lanes 1,4, and 7). In contrast to this, both 3L2-1 and lake XVI-11 ecotypes of *D. pulicaria* showed an induction of Hsp70 levels in response to heat shock at the age of 4 weeks (Fig. 3.3C and D, lanes 4-6). Since the average life spans of *D. pulex* and *D. pulicaria* are 4 weeks and 9 weeks, these ages are midpoints of their average life span and thus are equivalent time points in their respective life spans. Thus, it can be concluded that the long-lived *pulicaria* ecotypes continue to respond to heat shock by inducing Hsp70 expression until their middle age but the short-lived *pulex* ecotypes cease to respond to

heat shock by their corresponding middle age. These results are highly significant since it is known that induction of Hsp70 in response to heat shock protects against proteotoxicity and limits damage to the cellular function.

The data in western blot analyses (Fig. 3.3A-D) was quantified using STORM phosphorimager and Imagequant software and is represented as bar graphs in Fig. 3.3E-H. The P values are indicated in the figure legend and were obtained by a statistical analysis of data obtained from 3 independent biological replicates for *D. pulex* ecotypes and 4 independent biological replicates for *D. pulicaria*.

3.4.5 The *Daphnia* Hsp70 protein is highly homologous to known Hsp70 sequences

There are five different Hsp70 isoforms that can be identified in *D. pulex* genome based on sequence homology with mammalian Hsp70. In order to determine which of these genes represents the isoform primarily induced by heat shock, we performed RT-PCR using primers corresponding to the conserved regions of the five Hsp70 nucleotide sequences so that they could amplify all possible Hsp70 sequences if they were expressed in response to heat shock. RT-PCR was performed on RNA isolated from control and heat shocked organisms. Sequence analysis of these products revealed that the control as well as heat shocked samples contained only the sequence from genomic scaffold 3. An alignment of the predicted Hsp70 protein sequence from this chromosomal location is represented in Fig. 3.4. The protein sequence is highly homologous to Hsp70 sequence from *C. elegans*, *D. melanogaster*, as well as human and murine sequences. Based on this information, we designed specific primers for *Daphnia*

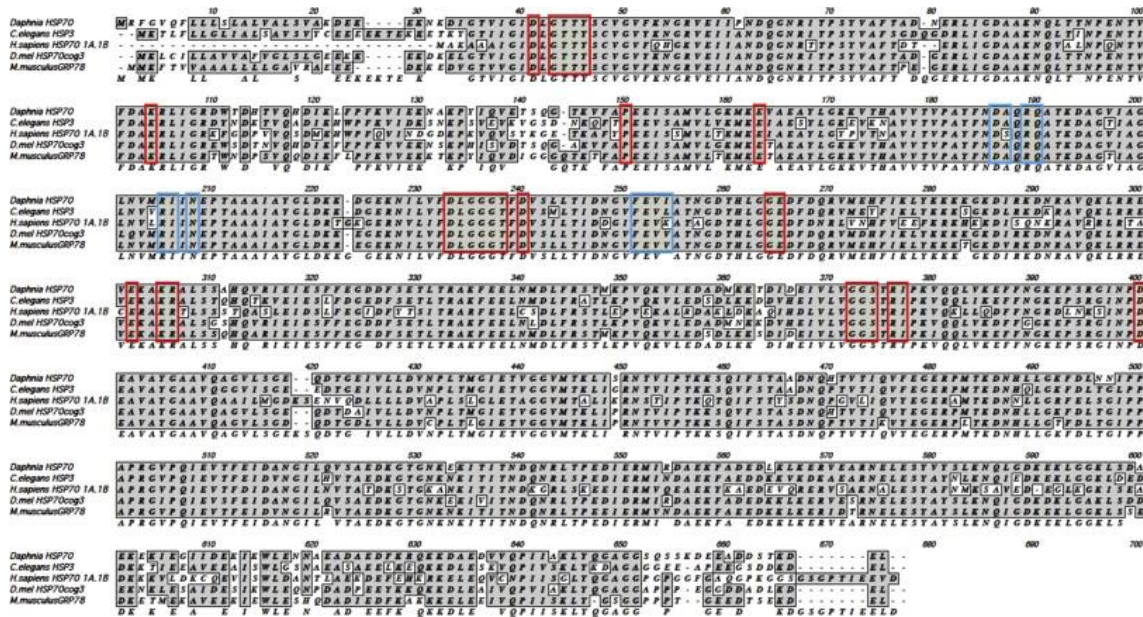


Figure 3.4. Sequence alignment of *Daphnia* Hsp70. *D. pulex* Hsp70 protein sequence is aligned with Hsp70 sequence from other organisms. Gray shaded boxes indicate identity or similarity. Red boxes indicate residues that are involved in nucleotide binding while blue boxes indicate residues that are involved in substrate binding. Gaps (designated as dashes) were entered for the best alignment. *C. elegans*: *Caenorhabditis elegans*. *D. mel*: *Drosophila melanogaster*. *H. sapiens*: *Homo sapiens*. *M. musculus*: *Mus musculus*.

Hsp70 sequence from scaffold 3 to determine if the Hsp70 induction in response to heat shock resulted from an increase in hsp70 mRNA levels

3.4.6 Hsp70 transcript levels increase after heat shock

Hsp70 induction after heat shock is regulated primarily at the level of transcription by the transcription factor HSF-1 (Morimoto et al. 1992). Various proteotoxic stimuli lead to trimerization of HSF, which translocates to the nucleus to induce the transcription of Hsp70 and consequently leads to an increase in Hsp70 mRNA and protein levels (Akerfelt et al. 2010; Anckar and Sistonen 2011). We next asked if heat shock in *Daphnia* led to an increase in mRNA levels for Hsp70. We performed RT-PCR and qRT-PCR analysis to quantify Hsp70 mRNA levels in total RNA isolated from control and heat shocked *D. pulicaria* (lakeXVI-clone 11) at age of 1 week. Using RT-PCR, we found that the Hsp70 mRNA levels were induced after heat shock at a 4 hours recovery time point (Fig.3.5A lane 2) and returned to basal levels at 6 hour recovery time point (Fig. 3.5A lane 3) although the Hsp70 protein levels remained high at 6 hours (Figs. 3.2, and 3.3 D). The qRT-PCR analysis also showed a 8.5-fold elevation of Hsp70 mRNA levels at 4 hours recovery and return to basal levels at 6 hours recovery (Fig. 3.5B). Thus, similar to other organisms, in *Daphnia* the Hsp70 mRNA levels are elevated in response to heat shock.

3.4.7 The ability of HSF-1 to bind DNA decreases with age in Daphnia

Transcriptional induction of Hsp70 is primarily regulated by transcription factor HSF in all organisms studied for HSR (Akerfelt et al. 2010). In order to gain an insight about why the Hsp70 induction declines with age, we investigated if the HSF levels or activity

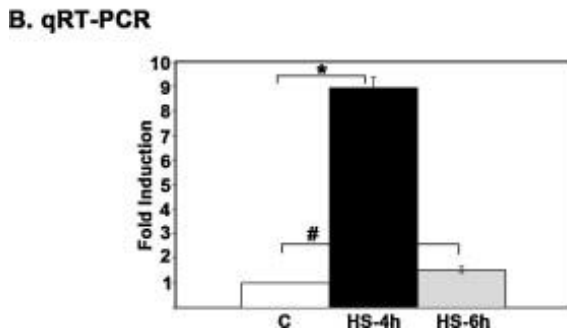
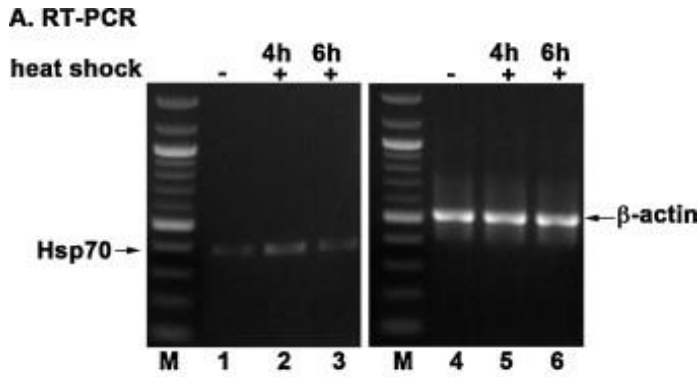
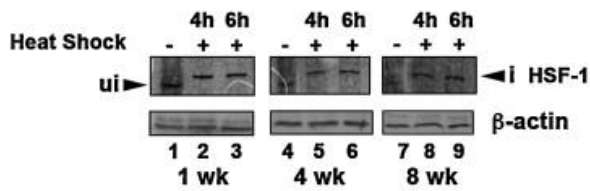


Figure 3.5. Heat shock leads to an increase in Hsp70 mRNA levels in *D. pulicaria*. A. RT-PCR analysis. Total RNA was isolated from 1 week-old *D. pulicaria* (lake XVI-clone 11) either after heat shock or non- heat shock (control) conditions. Hsp70 mRNA levels were analyzed via reverse-transcriptase PCR and visualized on a 1% agarose gel. β-actin mRNA levels were utilized as a control for normalization. The labels above the lanes indicate control or heat-shocked samples and the length of recovery periods after heat shock. B: qRT-PCR analysis. qRT-PCR analysis was performed to examine the fold-change in expression between non-heat shocked and heat shocked samples from one week old *D. pulicaria* (lake XVI-clone 11). Data represents the average from nine replicate experiments from three different RNA isolations. C: without heat shock, HS-4h: 4h recovery after heat shock, and HS-6h: 6h recovery after heat shock. All results are normalized to β-actin. Error bars indicate standard deviation calculated from the nine replicates. Statistical analysis was performed to calculate P values and the symbol * represents P value (0.0012) that indicated significant difference compared to control and the symbol # represents P value (0.032) that indicates no significant difference compared to control values.

also shows similar decline with age. In response to heat shock, HSF is phosphorylated and trimerizes to form a functional complex that translocates to nucleus (Akerfelt et al 2010). We first examined the expression of HSF in *Daphnia* by western blot analysis. Since there is a single HSF gene in *Daphnia* and it is most homologous to human HSF-1, we used anti-human HSF-1 antibody for our analysis. As seen in Fig. 3.6A, HSF-1 is activated in response to heat shock at 1 week age as indicated by the characteristic upward mobility shift (lanes 2 and 3), that is a likely consequence of HSF-1 phosphorylation. HSF-1 activation is not compromised with increasing age as indicated by the same characteristic upward mobility shift in response to heat shock at ages 4 weeks (lanes 5 and 6) and 8 weeks (lanes 8 and 9). HSF-1 phosphorylation in response to heat shock leads to an increase in the apparent molecular weight of the monomer on SDS-PAGE, by as much as 12 kDa, which has been described previously in literature (Westwood and Wu 1993). We also observe an increased apparent molecular weight of HSF-1 after heat shock on SDS-PAGE, which corresponds to ~10 kDa increase in its apparent molecular weight (Fig. 3.6A, lanes 2, 3, 5, 6, 8, and 9). Thus, it can be concluded that activation of HSF-1 is not compromised with age in *D. pulicaria*. It is worth noting that there is a slight reduction in the abundance of HSF-1 protein at 4 and 8 weeks of age as seen in lanes 4-9 in comparison to age of 1 week (lanes 1-3).

We next examined if HSF-1 DNA binding activity is reduced with age and can explain the decline in Hsp70 expression in 4 and 8 week old organisms. We analyzed DNA-binding activity of HSF-1 by performing EMSAs with heat shock element (HSE) oligonucleotide using nuclear extracts from 1 week and 8 week-old *D. pulicaria* (Lake

A. HSF-1 western blot



B. HSF-1 EMSA

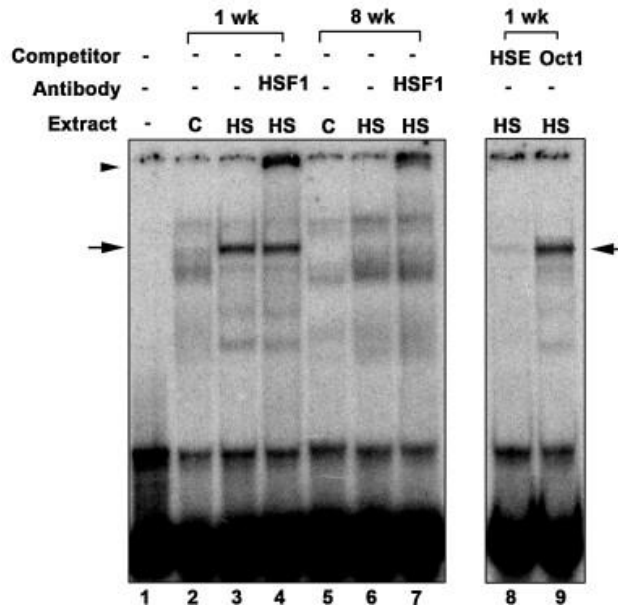


Figure 3.6. Activation of HSF-1 in response to heat shock at different ages in *D. pulicaria*. A. Western blot analysis. *D. pulicaria* (lake XVI-clone 11) were subjected to heat shock at 32° C for 30 minutes, allowed to recover for indicated time periods, and protein was extracted. Western Blot analysis was performed using 50 µg of total protein with anti-HSF-1 antibody. The recovery periods after heat shock are as indicated above the lanes and the age of the *Daphnia* are indicated in weeks below the panels. Blots were re-probed with anti-β actin antibody to ensure even loading. Arrowhead marked “ui” indicates HSF-1 position from non-heat shocked samples, and an arrowhead marked “i” indicates upward mobility shift of HSF-1 in heat shocked samples. B. Electrophoretic mobility shift analysis. EMSA performed using nuclear extracts from *D. pulicaria* (XVI-clone 11) with (HS) or without heat shock (C) at different ages as indicated. 5 µg of nuclear extracts were incubated with P³²-labeled HSE probe. Above each lane, the age of the *Daphnia* in weeks, addition of a competitor (HSE: specific, Oct1: nonspecific), or addition of an antibody (HSF-1) is as indicated. The ‘-’ lane indicates probe alone lane without any added nuclear extract. Arrows indicate the position of HSF-1 containing complex, and arrowhead indicates position of complex after antibody super-shift. Specific and non-specific non-radioactive competitor oligonucleotides were used in 50-fold molar excess to confirm the specificity of the bound complex.

XVI-clone 11). As seen in Fig. 3.6B, extract from the 1 week old individuals showed a strong mobility shifted band (indicated by an arrow) with HSE after heat shock (lane 3). This complex was missing with the extract from control (non-heat shocked) individuals (lane 2) thereby indicating that that this is the complex that is strongly induced in response to heat shock. Part of this complex could also be super-shifted (lane 4, indicated by an arrowhead) with HSF-1 antibody thereby demonstrating the presence of HSF-1 transcription factor in the complex. Competition with unlabeled specific (HSE oligo, lane 8) and the lack of competition with unlabeled non-specific oligonucleotides (Oct1 oligo, lane 9) confirmed that the complex indicated by an arrow resulted from specific recognition of the HSE probe. 8 week old individuals showed no complex at the equivalent position of HSF-1 complex (lane 6), thereby indicating that HSF-1 is not competent to bind DNA in aged organisms. It is interesting to note the presence of two much weaker complexes in lane 6. These complexes did not exhibit any significant super shift with HSF-1 antibody (lane 7), thereby indicating lack of HSF-1 in these complexes. The composition and significance of these complexes for transcriptional regulation of Hsp70 expression in older organisms remains to be explored in future studies. Our results establish that the DNA-binding ability of HSF-1 is diminished in older animals.

In summary, we have presented results which establish that the freshwater crustacean *Daphnia* responds to heat shock by inducing the expression of Hsp70 and the ability to induce Hsp70 in response to heat shock decreases with age in both short-lived and long-lived ecotypes. We also show that the long lived ecotype, *D. pulicaria*, exhibits a better induction of Hsp70 in response to heat shock than the short lived *D. pulex* at a

young age. In both short- and long-lived species the ability to respond to heat shock by inducing Hsp70 decreases with age. Furthermore, the short-lived *pulex* ecotypes stop responding at about midpoint of their average life span, but the long-lived *pulicaria* ecotypes show HSR at an age that is equivalent of the midpoint in their longer life span. In addition, we demonstrate that the lack of HSR in older organisms is not due to lack of HSF-1 activation, but rather can be attributed to a lack of DNA-binding by HSF-1 in older organisms. These results for the first time characterize HSR and Hsp70 induction in *Daphnia* and this is first report to study ageing mechanisms at molecular level using *Daphnia* as a model organism.

3.5 Discussion

3.5.1. Daphnia as a new model organism for research on ageing

Organisms sense and respond to physiological and environmental stress by inducing specific stress response pathways that function to protect the core biological processes by promoting protein folding, and triggering degradation of damaged proteins (Kim et al. 2013). The HSR pathway protects the proteome of all cells against acute damage resulting from exposure to elevated temperatures, oxidative damage, heavy metals, and ageing related accumulation of misfolded and aggregated proteins (Calderwood et al. 2009). Induction of HSR leads to a rapid and robust expression of Hsps that function as molecular chaperones, which prevent protein misfolding and aggregation to promote recovery from stress. In this report, we investigated the regulation of Hsp70 expression in relation to ageing in *D. pulex* and *D. pulicaria*. These two *Daphnia* ecotypes vary about 2-fold in their relative life spans, thereby making it possible to identify and study

molecular pathways that regulate ageing and longevity (Dudycha and Tessier 1999).

Daphnia have numerous characteristics that make them a novel and versatile model for ageing research. The parthenogenetic mode of reproduction in *Daphnia* is particularly useful because large numbers of genetically identical individuals can be easily produced without creating artificially homozygous genetic backgrounds. They have short lifespans, tractable genetics, an available completed *D. pulex* genome sequence with additional genome sequences in progress, and adult tissue regeneration. They have sexual and asexual phases to their life cycle, permitting crosses as well as straightforward generation of many genetically identical individuals. Furthermore, *Daphnia* have significantly more genes than *Drosophila* or *C. elegans*, raising the possibility that additional genes with relevance to human ageing could be identified in them (Colbourne et al. 2011). Currently the biomedical research community uses *Daphnia* primarily in the contexts of ecotoxicology. In order to establish *Daphnia* as a model system for ageing, we have analyzed the well-established HSR pathway to determine if the longer lived ecotype, *D. pulicaria* displayed a better HSR in comparison to the short lived *D. pulex*. There are relatively few molecular studies involving *Daphnia*, none published on the relationship between HSR and molecular biology of ageing. Our results presented here for the first time present an analysis of regulation of Hsp70 expression during *Daphnia* life span.

3.5.2. HSR in short-lived ecotype versus the long-lived ecotype

Our results establish that the long-lived *D. pulicaria* displays a more robust induction of Hsp70 in response to elevated temperatures than the short-lived *D. pulex*.

Within each ecotype, the young organisms display a more robust HSR than the old organisms, which is in accordance with data from studies in mammals (Gagliano et al. 2007; Kayani et al. 2008). In addition, the short lived *D. pulex* show no induction of Hsp70 by the average midpoint in their life span (2 weeks), but the long-lived *D. pulicaria* continues to exhibit good induction of Hsp70 at the average midpoint in their life span (4 weeks). Our results indicate that the lack of Hsp70 induction in older organisms results from a lack of DNA-binding by HSF-1. These results are similar to data from other model organisms that the ability of HSF-1 to bind to DNA decreases with age (Heydari et al. 2000; Westerheide et al. 2009). Thus, *Daphnia* have shown similar characteristics about HSR in relation to ageing as other model organisms, while offering a direct comparison between HSR in ecotypes that have evolved short and long life spans, which is unique to our system.

3.5.3. Basal versus heat-induced Hsp70 expression during ageing

Both *D. pulicaria* and *D. pulex* display an increase in basal Hsp70 expression as they age, with the oldest organisms displaying the highest basal expression of Hsp70. The basal expression level of Hsp70 in the absence of any proteotoxic signal such as heat shock is known to increase as the organisms age in *D. melanogaster* (Wheeler et al. 1995). Tissue-specific induction of stress proteins in mammals is also observed during normal ageing (Schultz et al. 2001) indicating that induction of Hsp expression during ageing is evolutionarily conserved across a broad range of taxa. Ageing-associated up-regulation of Hsps has also been documented including some recent genome-wide studies of gene expression changes (Wheeler et al. 1995; King and Tower 1999; Wheeler

et al. 1999; Landis et al. 2004). Although some studies indicate that a functional HSE is required in the Hsp promoters for this age-dependent induction of basal transcription, posttranscriptional regulation at the level of Hsp translation or stability has also been documented (Wheeler et al. 1995). This could explain the age-dependent upregulation of Hsp70 protein levels in *Daphnia* while HSF-1 is not competent for DNA-binding in old organisms. In contrast to this, induction of Hsp70 expression in response to proteotoxic stimuli (HSR) has been shown to decrease with age in other model organisms, which is also the case in two isolates of each of the two ecotypes of *Daphnia* that we investigated (Morley and Morimoto 2004; Rea et al. 2005; Tower 2011).

3.5.4. Mechanism of reduced HSR in older Daphnia

The functional role of HSF-1 as a transcription factor that regulates stress-induced expression of Hsps including Hsp70 has been unambiguously established (Akerfelt et al. 2010). Given the central role played by HSF-1 in inducing Hsp expression in response to proteotoxic stimuli and the need for proteome maintenance for increased organismal life span, it is not surprising that HSF-1 is required for longevity. In *C. elegans* a reduction in HSF-1 levels by RNAi reduces life span by 30-40% (Morley and Morimoto 2004) and worms carrying additional copies of HSF-1 gene are resistant to elevated temperatures and live about 40% longer than their wild type counterparts (Hsu et al. 2003). Extensive studies done in *C. elegans* also indicate HSF-1 as a longevity factor affected by insulin-regulated signaling pathways (Barna et al. 2012). Previous studies have found that HSF-1's ability to bind to DNA decreases with age; therefore, causing an age-dependent decrease in HSR (Fawcett et al. 1994; Jurivich et al. 1997). In our present study, we find

that the *Daphnia* HSF-1 also shows a decrease in its DNA-binding activity with age. Many post translational modifications such as phosphorylation, sumoylation, and acetylation occurring at several sites are known to affect HSF-1 DNA binding (Anckar and Sistonen 2011). The molecular mechanism of the observed age dependent decrease in DNA-binding of HSF-1 in *Daphnia* remains to be determined in future studies.

Traditionally, *Daphnia* have been studied as an ecological model. The ability of *Daphnia* to respond to environmental stressors and toxins is crucial for the survival and propagation of the species, especially the short lived *D. pulex*, which lives in temporary ponds that may only exist for a few weeks. In addition, predation is one of the major components of a changing, unstable environment (Lampert 1987; Pijanowska and Kloc 2004). Some of the antipredator defenses in *Daphnia* involve a variety of morphological, behavioral, and life-history adaptations that may not be life-saving, but may enable organisms to complete reproduction before predation. Most of these phenotypic changes are induced by chemical compounds (kairomones) released by a predator into water. Exposure of *Daphnia* to kairomones results in a change in motion, behavior, and life history (Miyakawa et al. 2010). At the molecular level, the response also involves induction of Hsps. Since some of the kairomone-induced effects are transgenerational, i.e., they are passed on from the mother to her offspring, it may be interesting to test if the short lived *D. pulex* exposed to kairomones at a young age may show extension of life span under laboratory setting which may be attributed to induction of Hsp70 expression.

3.5.5 Conclusions

We examined the HSR, and more specifically the expression of Hsp70 in two *Daphnia* ecotypes that exhibit strikingly different life spans. *D. pulicaria*, the long lived ecotype, showed a robust Hsp70 induction as compared to the shorter lived *D. pulex*. The short-lived *D. pulex* isolates showed no induction of Hsp70 at the midpoint in their life span. In contrast to this, the long-lived *D. pulicaria* continued to induce Hsp70 expression at an equivalent age. The Hsp70 expression was induced at transcriptional level in response to heat shock and the decline of Hsp70 induction in old organisms could be attributed to a decline in the transcription factor HSF-1's DNA-binding activity. This is a first report of the molecular analysis of relationship between HSR and life span in *Daphnia*.

Chapter 4:
Involvement of *Daphnia pulex* Sir2 in Regulation of Stress Response and Life Span⁴

⁴ Schumpert C, Anderson C, Dudycha J, Patel R. Involvement of *Daphnia pulex* Sir2 in Regulation of Stress Response and Life Span. In Preparation.

4.1 Abstract

The ability to appropriately respond to proteotoxic stimuli is a major determinant of longevity. This involves induction of various heat shock response (HSR) genes, which are essential to cope with cellular and organismal insults throughout lifespan. There is extensive evidence for the central role of the HSR in regulation of aging. The activity of NAD⁺-dependent deacetylase Sir2, originally discovered in yeast, is known to be essential for effective HSR and longevity. Our previous work on HSR in *Daphnia pulicaria* indicated a drastic reduction of the HSR in older organisms. In this report we investigate the role of Sir2 in regulating HSR during the life span of *D. pulicaria*. We first cloned *Daphnia* Sir2 open reading frame (ORF) to characterize the enzyme activity and confirmed that the overall function of Sir2 was conserved in *Daphnia*. We further characterized the steady state levels of sir2 transcripts and enzyme activity in young, middle and old age *Daphnia pulicaria*. The sir2 mRNA levels increased while the enzyme activity declined with age in correlation with the previously observed age-dependent decline in HSR. Finally, we tested the effect of Sir2 knockdown throughout adult life by using our new RNA interference (RNAi) method by feeding. Sir2 knockdown severely reduced both the median life span as well as significantly increased mortality following heat shock. Our study provides the first characterization and functional study of *Daphnia* Sir2 and indicates its involvement in regulation of HSR and aging.

4.2 Introduction

The ability to respond to proteotoxic stress has proven to be a key regulator in the aging process in several model organisms (Powers et al 2008, Morimoto and Cuervo 2009, Ankar et al 2011). Aging is a universal biological process that leads to a decline in multiple functions at the organismal and cellular levels (Johnson *et al* 1999, Kenyon 2010, Lopez-Otin *et al* 2013). Although several pathways responsible for the aging process have been described, the crosstalk between such regulatory pathways is not fully understood. Aging is an unavoidable biological process that is a culmination of multiple cellular pathways being rendered dysfunctional over time (Lopez-Otin et al 2013). One well-studied mechanism that regulates aging process involves cellular responses to proteotoxic stress, also referred to as proteostasis (Soti and Csermely 2003, Morimoto and Cuervo 2009). Clancy and Birdsall, among others, attribute the pathologies and phenotypes associated with aging to the loss of proteostasis and the resulting inability to respond to various proteotoxic damages (Soti and Csermely 2003, Clancy and Birdsall 2013). The predominant cellular response to proteotoxic stimuli is the heat shock response (HSR), which has been studied extensively in numerous species (Ankar and Sistonen 2011). The HSR involves the induction of molecular chaperones, termed heat shock proteins (Hsps) (Ankar and Sistonen 2011) for relieving the molecular damage. The Hsps either renature the misfolded or damaged proteins following stress or target them for degradation in case the damage is too severe (Morimoto and Cuervo 2009, Ankar and Sistonen 2012). Heat shock protein 70 (Hsp70) is one of the main Hsps responsible for such remedial action (Bukau et al 2006).

Following a proteotoxic insult (heat shock, exposure to heavy metals, oxidative stress, or exposure to extreme pH), Hsp70 is induced transcriptionally by the transcription factor heat shock factor (HSF) (Morimoto and Cuervo 2009, Anckar and Sistonen 2011). Upon heat shock or another proteotoxic event HSF undergoes trimerization and binds to specific sequences within the Hsp70 promoter to induce rapid and robust transcription to combat proteotoxic stress.

In multiple model organisms, previous studies have established that the HSR declines with age (Tower 2011, Schumpert et al 2014). Studies in *C. elegans* as well as *D. melanogaster* have demonstrated the inability of old organisms to respond to proteotoxic stress (Rea et al 2005, Tower 2011). Previous studies have also demonstrated that the inability to mount a strong HSR is due to the inability of HSF to bind to DNA in old organisms (Fawcett et al 1994, Jurivich et al 1997, Schumpert et al 2014). One mechanism leading to this decline of HSF's DNA-binding activity during aging is attributed to the post-translational modifications of HSF (Westerheide et al 2008, Rayne et al 2012). In order for HSF to trimerize and become active for DNA-binding, HSF has to be deacetylated by the NAD⁺-dependent histone deacetylase Sir2 (named Sirt1 in mammals, Sir2 in yeast and *Daphnia*). Sir2 was first discovered in yeast and has since been investigated for its role in deacetylation of multiple targets including p53, FoxO, NFκB and HSF (Raynes et al 2013, Rovollo and Li 2013, Imai and Guarente 2014). Previous work has also demonstrated that overexpression of Sir2 homologs in yeast, *C. elegans*, and *D. melanogaster* leads to a lifespan extension (30% increase in median life span) while a knockdown in Sir2 results in a decrease in lifespan (Kaeberlein et al 1999,

Rogina and Helfand 2004, Wang and Tissenbaum 2006, Frankel et al 2011, Imai and Guarente 2014). In terms of the HSR, Westerheide *et al* demonstrated that Sirt1 deacetylates HSF1 at K80 and that in WI-38 fibroblasts, expression of Sirt1 declines with passage number or age of fibroblasts (Westerheide et al 2009). Several studies reported that Sir2/Sirt1 deacetylase activity declines with age, without a corresponding definitive decline in Sir2/Sirt1 protein expression (Ramsey et al 2008, Quintas et al 2012, Gomes et al 2013, Chang and Guarente 2013, Gong et al 2014, Kwon et al 2015). Thus, a decline in HSF's ability to bind to DNA in old organisms may result from inactive, acetylated HSF due to a decrease in Sir2/Sirt1 activity.

We use *Daphnia*, a freshwater microcrustacean, in our studies on aging and life span regulation. In particular, we examine the differences in life span and aging between two ecotypes *Daphnia pulex* and *Daphnia pulicaria*. These two ecotypes diverged only ~82,000 years ago with *D. pulicaria* life span being more than twice as long as *D. pulex* (Omilian and Lynch 2009). The short lived *D. pulex* naturally inhabits small transitory ponds that are found around the world and exhibit a median life span of about 20-25 days (Dudycha and Tessier 1999, Dudycha 2003, 2004). The closely related, yet long lived *D. pulicaria* inhabits larger, more stable, stratified lakes and has a median life span of about 65-70 days (Dudycha and Tessier 1999, Dudycha 2003, 2004). *Daphnia* is a useful model organism for research on aging especially due to its unique characteristics. (Schumpert et al 2014, 2015). *Daphnia* are easily cultured in the lab and they reproduce via cyclic parthenogenesis making it easy to establish a population of isogenic individuals (Benzie 2005). The *D. pulex* genome is fully sequenced with

estimated 30,907 protein coding genes, and has the highest number of genes homologous to the human genome among all sequenced arthropods (Colbourne et al 2011). Although the list of molecular techniques to make *Daphnia* amenable to molecular studies is still growing, multiple techniques have been established including an RNA interference system, and a gene replacement and targeted mutagenesis system using TALEN and CRISPR/Cas9 systems (Kato et al 2011, Hiruta et al 2013, Nakanishi et al 2014, Hiruta et al 2014, Schumpert *et al* 2015).

We have previously studied the HSR of *D. pulex* and *D. pulicaria* in relation to aging (Schumpert *et al* 2014). Our results showed that the short-lived *D. pulex* stop responding to proteotoxic stress by middle age whereas the long-lived *D. pulicaria* can still mount a strong HSR at an equivalent age. In both ecotypes, the ability to respond to proteotoxic stimuli was abrogated at old age (Schumpert *et al* 2014). We further investigated the possible mechanism for this decline in the HSR and found that although the HSF protein levels were equal throughout lifespan, its ability to bind DNA in old *D. pulicaria* declined (Schumpert *et al* 2014). Due to the established role of Sir2 in activation of HSF and the known decrease in its enzymatic activity with age in other organisms, we wanted to investigate the potential role of *Daphnia* Sir2 in regulation of HSR and longevity in *D. pulicaria*.

In the present study, we cloned *D. pulicaria* Sir2 open reading frame (ORF), examined Sir2 transcript and activity levels during its life span, and investigated its functional role in HSR and life span regulation by performing gene-specific RNA interference (RNAi). We demonstrate that Sir2 ORF cloned from *D. pulicaria* (Clone:

LakeXVI-11) produces a functional protein that has similar overall functions to mammalian Sirt1. Cell viability experiments examining the effects of *Daphnia* Sir2 overexpression following a severe heat shock showed that similar to mammalian Sirt1, *Daphnia* Sir2 confers a protective effect resulting in a markedly reduced cell death following proteotoxic stress. *Daphnia* Sir2 overexpression in mammalian cells also exhibits an enhanced HSR as measured by a transcriptional reporter assay. Although the transcript levels for Sir2 increased with age in *D. pulicaria*, the enzymatic activity of Sir2 showed significant decrease with age. Finally, utilizing our newly developed RNAi via feeding system we knocked down Sir2 in adult *Daphnia* throughout their life span. A knockdown of Sir2 expression in adult *Daphnia*, led to a drastic shortening of lifespan by 64 +/- 2% compared to wild-type control organisms. This is a first report demonstrating a decrease in *Daphnia* lifespan as a result of a targeted knockdown of an established longevity gene by using RNAi via feeding method.

4.3 Materials and Methods

4.3.1 Cloning of *Daphnia* Sir2 ORF

Primers were designed to clone the *Daphnia pulicaria* Sir2 ORF using MacVector based on the published *D. pulex* genome (Colbourne et al 2011). We designed four primers such that the two PCR products would cover the entire ORF in two overlapping pieces, the “5’ half” (883 bp) and the “3’ half” (1065 bp) with an overlap of 46 bp (Figure 1B) between the two halves around an internal MscI restriction enzyme site. The primers were designed with a restriction site NdeI for the 5’ end of the 5’half and BamHI for the 3’ end of the 3’half (sequence of primers is given in the next section). The

PCR products were cloned into pGEMT-EZ (Promega) and sequenced. We utilized the internal MscI restriction enzyme cut site to join each half of the Sir2 PCR product from their respective 5' half Sir2/pGEMT EZ and 3' half Sir2/pGEMT EZ constructs. A three-piece ligation was performed to join the two halves of the *Daphnia* Sir2 ORF thereby sub-cloning the full length ORF into the pBSIKS+ (Stratagene) that contained an in-frame FLAG tag on the amino terminus of Sir2. A final sub-cloning in pcDNA3.1- (Invitrogen) resulted in the FLAG- Sir2/pcDNA 3.1- expression construct used for this study.

4.3.2 Reverse Transcriptase PCR

Total RNA was isolated from *D. pulicaria* using RNazol B reagent (TelTest). cDNA was synthesized at using random hexamer primers, 1 µg total RNA, M-MuLV reverse transcriptase, 500 µM dNTPs, and RNase Inhibitor RNasin (Promega) as previously described (Schumpert et al 2014). For each PCR reaction, 2 µl of total cDNA and 50 pmoles each of the forward and reverse primers were used with the Promega GoTaq PCR kit. The following conditions were used for PCR: 95⁰ C for 5 minutes (initial denaturation), denaturation at 95⁰ C for 30 seconds, annealing at 56⁰ C for 5' half Sir2, 58⁰ C for 3' half Sir2, or 59⁰ C for GAPDH for 30 seconds, extension at 72⁰ C for 60 seconds. We ran 40 cycles for the initial amplification of *Daphnia* Sir2 5' and 3' halves for cloning, and 27 cycles for the analysis of sir2 mRNA knockdown in the RNAi experiment. The linear range was determined by varying cycle numbers and performing a densitometric analysis of the amplified product for the reverse transcriptase PCR in

the RNAi experiment. Note the Sir2 PCR product shown in Figure 6B is the 3' half of *Daphnia* Sir2 ORF. Primer sequences used were as follows:

5' half Sir2 Forward: 5'-CATATGACCATGGCCGACGAACAAGGCGAG-3'

Reverse: 5'-ATGTTCTGCGTGTAGTTGCG-3'

3' half Sir2 Forward: 5'-CGACCGTTCTTTAAATTCGC-3'

Reverse: 5'-TCAATCCATCGCTTTCCTTTTAC-3'

GAPDH Forward: 5'-TTATCACCTCCTCAACTTC-3'

Reverse: 5'-CTTCTTCCTTCACTTCTCC-3'

4.3.3 Western Blot Analysis

Cos-1 cells were transfected with 100 ng FLAG-Sir2/pcDNA3.1- or control FLAG-TRBP/pcDNA3.1-. 24 hours post-transfection, Cos-1 cells were harvested, washed with 1 ml of cold PBS twice and lysed in RIPA Buffer (150 mM NaCl, 1.0% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), the lysates were centrifuged at 13000 Xg for 5 minutes at 4°C and the supernatants were used as total protein extracts. Total protein concentrations in the extracts were determined using the BCA kit (Pierce). 50 µg of protein per sample was resolved by SDS-PAGE on an 8% polyacrylamide gel. The primary antibody used was FLAG M2 from Sigma at a dilution of 1:2000. ECL plus (Pierce) chemifluorescence detection was used to detect signals on a GE Typhoon LAS4000.

4.3.4 In vitro Translation of *Daphnia* Sir2

Daphnia Sir2 was *in vitro* translated using the TNT T7 coupled reticulocyte system (Promega). We incorporated S³⁵ methionine during translation to radioactively

label *Daphnia* Sir2. Following *in vitro* translation, an SDS-PAGE was analysis was performed with an 8 % polyacrylamide gel that confirmed the presence of a radioactively labeled protein the same size as *Daphnia* Sir2. *In vitro* translated protein was then used in the Sirt1 Activity Assay.

4.3.5 Sir2 Activity Assay

We assayed for the deacetylase activity of *D. pulicaria* Sir2 using a commercially available kit from Abcam (ab156065). The kit is designed to measure the activity of any sirtuin; however, we used the Sirt1/Sir2 specific inhibitor EX-527 to determine the relative contribution of Sir2 activity present in *Daphnia* samples isolated from 1, 4, and 8 week-old organisms as well as *in vitro* translated *Daphnia* Sir2. The assay is based upon a proprietary substrate which is contains a fluorophore coupled to an acetylated peptide that also contains a quencher. Upon deacetylation, the quencher is cleaved by a peptidase included in the kit, and the fluorophore fluoresces, which is measured using a plate reader. In general, the higher the amount of fluorescence emitted, the higher the amount of Sirt1/Sir2 activity. All assays were performed using an opaque clear bottomed 96 well plate (UVstar from Greiner). Ten *Daphnia*, having embryos removed, were homogenized in lysis buffer (10 mM Tris HCl (pH 7.5), 10 mM NaCl, 15 mM MgCl₂, 250 mM sucrose, 0.5% NP-40 and 0.1 mM EGTA). The samples were incubated on ice for 15 minutes and spun through 4 ml sucrose cushion (30% sucrose, 10 mM Tris HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂) at 1300 X g for 10 minutes at 4°C. The supernatant was discarded and the nuclear pellet was resuspended in ice-cold solution of 10 mM Tris HCl and 10 mM NaCl and pelleted via centrifugation at 1,300 X g for 10 minutes at 4°C. The

washed, pelleted nuclei were lysed in 50 μ l of extraction buffer (50 mM Hepes-KOH, pH 7.5, 420 mM NaCl, 0.5 mM EDTA Na₂, 0.1 mM EGTA, 10% glycerol) by gentle homogenization. The samples were incubated on ice for 30 minutes and centrifuged for 10 minutes at 20000 x g. The supernatant, the crude nuclear extract, was stored at -80⁰ C until use in the assay. Protein concentration of the extracts was determined using the BCA kit (Pierce). The Sirt1 Activity Assay was performed as per the manufacturer's directions. We used human recombinant Sirt1 as a positive control and EX-527 to ensure Sirt1 specificity. All plates were read on a Molecular Devices Gemini EM Microplate reader with an excitation wavelength of 340 nm and emission wavelength of 460 nm for the duration of 30 minutes with reads occurring every minute.

4.3.6 Mammalian cell viability assay

Cos-1 cells were transfected with 500 ng *Daphnia* Flag-Sir2/ pcDNA3.1- expression construct or 500 ng empty vector pcDNA3.1- using Effectene (Qiagen). 24h after transfection the cells were subjected to a severe heat shock for either 20 minutes or 30 minutes at 42⁰ C. The control cells (without heat shock) remained at 37⁰ C for the duration of the entire experiment with no changes in viability (data not shown). 24 hours following the heat shock, cell viability was determined via Trypan Blue viability assay. Trypan Blue staining was done by mixing 200 μ l of trypsinized cell suspensions with 200 μ l of Trypan Blue followed by an incubation at room temperature for 3 minutes. The cell mixture was mixed briefly and 20 μ l of this suspension was loaded onto a hemacytometer slide and cell viability was determined by counting the blue cells

(dead cells) and total cells visualized by a light microscope. The percentage viability was calculated by counting at least 300 cells in each sample.

4.3.7 Luciferase Reporter Assays

Cos-1 cells were co-transfected with *Daphnia* Flag-Sir2/pcDNA3.1- expression construct along with 1 ng pRL null (Promega) and 250 ng pGL4.41 (Promega) using Effectene (Qiagen) as per the manufacturer's instructions. pGL4.41 has the firefly luciferase gene under the control of 4 tandem copies of a consensus mammalian heat shock element (HSE). 24h after transfection the cells were treated with 30 μ M CdCl₂ to induce proteotoxic stress. For treatment with CdCl₂, the culture medium was removed and replaced with DMEM with charcoal stripped FBS containing 30 μ M CdCl₂. Six hours following the addition of CdCl₂, the cell extracts were harvested and the luciferase activity was measured using the Promega dual luciferase reporter system as per the manufacturer's instructions. The firefly luciferase activity was normalized to the Renilla luciferase activity to account for differences in transfection efficiencies in different samples. Fold induction following cadmium chloride treatment was calculated for each set by dividing the normalized luciferase activity (firefly luciferase activity divided by the Renilla luciferase activity) in the cadmium treated samples by the normalized luciferase activity in untreated samples. The control represents the fold induction obtained with pGL4.41 reporter co-transfection with empty vector pcDNA3.1- following cadmium chloride treatment.

4.3.8 Real Time PCR

RNA was isolated from *D. pulicaria* at ages 1 week, 4 week, and 8 week using RNazol B (Teltest). cDNA was synthesized as described before using random hexamers (Schumpert *et al* 2014). We standardized our real time PCR reactions by performing real time PCRs with serial dilutions of cDNA to ensure appropriate reaction efficiencies. In order for primer sets to be used we set a threshold of 95% efficiency and both primer sets used were within this range (97% for Sirt1, 98% for GAPDH). All reactions were performed in triplicates in a total volume of 20 μ l. This included 4 μ l cDNA, 250 nM Sirt1 or GAPDH primers, and SsoFast EvaGreen Supermix (BioRad). GAPDH was used for normalization and the primers were same as described by Scoville *et al* (Scoville and Pfrender 2010). All reactions were run on a BioRad CFX96 Real Time System C1000 Thermal cycler machine with the following conditions: 95⁰ C for 30 seconds, 95⁰ C for 5 seconds, 52⁰ C for 5 seconds (the 95 C for 5 seconds and 52 C for 5 second steps are repeated for 40 cycles), 65⁰ C for 5 seconds, and then 95⁰ C for 5 seconds. The data was analyzed using the Bio-Rad CFX Manager Software and used the $2^{-\Delta\Delta Ct}$ method to compare Sir2 expression in 1, 4, and 8 week RNA samples. Note that 3 separate RNA isolations were used from 3 separate groups of *Daphnia* to serve as biological replicates. Primer sequences used were as follows:

Real Time Amplicon Sir2	Forward: 5'-GCAGCGAGGATGAAAATCTC-3'
	Reverse: 5'-CTCCCAGTCATGATTGCTCA-3'
GAPDH	Forward: 5'-TTATCACCTCCTCAACTTC-3'
	Reverse: 5'-CTTCTTCCTTCACTTCTCC-3'

4.3.9 RNAi of *Sir2*

We created a dsRNA expression construct for the RNAi feeding regimen using the plasmid vector L4440 (AddGene #: 1654, originally a gift to Addgene from Dr. Andrew Fire). We sub-cloned the 5' half of *Daphnia* Sir2 ORF in L4440 to express a Sir2 dsRNA of corresponding length (Fig. 5A). The Sir2/L4440 plasmid was transformed into BL21 (DE3) bacteria, which contain an integrated T7 RNA polymerase under the control of lacUV5 promoter. The bacteria were grown in Luria broth containing 2mM IPTG to induce the expression of the T7 RNA polymerase and consequent production of the Sir2 dsRNA as both strands of the Sir2 ORF are expressed from two opposing T7 promoters in L4440 plasmid. Bacterial cells from 1 ml of the overnight culture ($OD_{600} = 2.6$) were pelleted and resuspended in 1 ml of filtered lake water. 500 μ l of this suspension was then added daily to the beakers containing the experimental *Daphnia*. For setting up the experiments, *Daphnia* neonates were collected and grouped as ten individuals per 100 ml of filtered lake water. Bacterial feed was administered each day, along with 20,000 cells/ml of the algae *Ankistrodesmus falcatus*. The death and reproductive output of *Daphnia* were recorded every day until all organisms had perished. Water was changed every other day to ensure no additional growth of bacteria was occurring in beakers and the amount of bacteria consumed was equal each day throughout the course of the experiment. We used two controls, a wild-type control that was not fed on bacteria and a dsRNA control that encodes GFP dsRNA (L4417, AddGene #: 1649, originally a gift to AddGene from Dr. Andrew Fire). GFP dsRNA serves as a control for any effect the presence of dsRNA can potentially exhibit, as there is not endogenous target for GFP

dsRNA. To investigate if the knockdown of Sir2 was occurring properly, a separate set of *Daphnia* of the same age were fed a bacterial RNAi regimen for 10 days. After ten days, the *Daphnia* were sacrificed for further analysis via Reverse Transcriptase PCR.

4.3.10 Daphnia viability assay following Heat Shock

To determine the ability of *Daphnia* to cope with proteotoxic stress (specifically heat shock), we standardized the temperature and duration of heat shock such that it elicited death after 24 hours of the heat shock. Ten *Daphnia* were placed per microcentrifuge tube in 1 ml of water. The microcentrifuge tube was incubated in a heating block at the designated temperatures for the duration of the heat shock (20 or 30 minutes). Following the heat shock, the *Daphnia* were placed in 100 ml of filtered lake water and allowed to recover for 24 hours before analyzing percentage death. Death was determined by visually inspecting the *Daphnia* under a light microscope to ensure that there was no heartbeat (they have a transparent carapace that makes visual analysis of the heartbeat simple).

4.3.11 Statistics

We performed several statistical tests in order to analyze our results. Either Student T Tests, Analysis of Variance (ANOVA) followed by post hoc Tukey Tests or nonparametric log rank tests (for survivorship data) were performed depending upon the data (Note: figure captions list which statistical test was performed for each data set). Each figure caption denotes p values as set forth by brackets and special characters. Note that our alpha level was $p=0.05$.

4.3.12 *Daphnia* Cultures

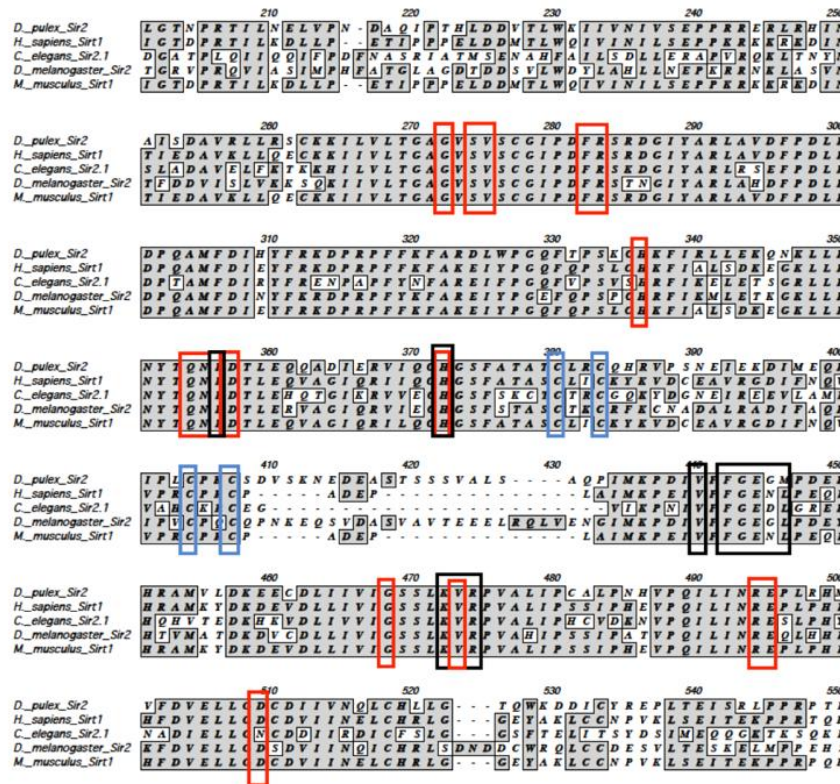
Daphnia pulicaria used in this study were isolated from ponds in Martin, Michigan, USA in 2008 (Lake Sixteen 42.564 N, 85.615 W) and have since been cultured in the lab. No explicit permission is required to collect *Daphnia* from public access lakes in the state of Michigan. *Daphnia* are neither endangered nor protected. More details on the source population of *D. pulicaria* are available in Dudycha 2004 (Dudycha 2004). *Daphnia* were maintained at a temperature of 20⁰ C with a photoperiod of 12:12 L:D (12 hours of light followed by 12 hours of dark) within a Percival growth chamber. *Daphnia* were maintained at a concentration of 3 to 5 organisms per 250 mL beaker in 100 mL of filtered lake water except during RNAi feeding experiments (see below for specific details). *Daphnia* were cleared of young and transferred to a new beaker with fresh water on every alternate day and were fed every day with vitamin supplemented algae *Ankistrodesmus falcatus* at a concentration of 20,000 cells/mL. To generate experimental groups, even-aged cohorts were created by placing neonates individually in 100 ml of COMBO medium, which is an artificial lakewater. Experimental animals were otherwise maintained as in the source cultures.

4.4 Results

4.4.1 *Daphnia* Sir2 protein shows sequence conservation of residues essential for its enzyme activity

The *D. pulex* genome contains a homolog of Sir2 and we wanted to assess sequence conservation in the essential catalytic domain of Sir2, as such conservation would be indicative of similar catalytic protein deacetylase activity of the *Daphnia*

A.



B.

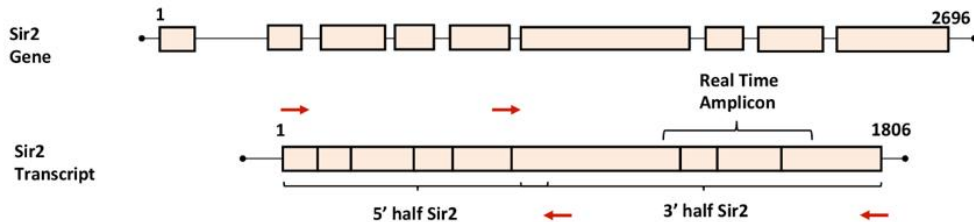


Figure 4.1. Sequence alignment of the Sir2 homologs from invertebrates and vertebrates. A) Sir2 related proteins from 5 model organisms are aligned with *Daphnia* Sir2 on the top. Red boxes depict residues involved in NAD⁺ binding, black boxes depict substrate-binding residues, and blue boxes depict zinc-binding residues. Alignments made using ClustalW in MacVector. B) Schematic representation of *Daphnia sir2* gene and sir2 transcript. PCR primers (red arrows) were designed such that the open reading frame (ORF) was PCR amplified in two parts, a 5' half and a 3' half as indicated. Real-time amplicon is the region of the transcript amplified via real time PCR for expression analysis.

protein. Thus, we aligned the Sir2 protein sequence from the published *D. pulex* genome with previously studied Sir2/Sirt1 sequences from other organisms. As is shown in figure 1A, there is a high degree of sequence similarity between the catalytic domain of *Daphnia* Sir2 and the sequences from *Drosophila*, human, mouse, and worms. Of particular note, the residues that exhibit the highest conservation are from the domains of the protein essential for the deacetylase enzyme functions such as NAD⁺ and substrate binding. The alignment in figure 1A shows the part of *D. pulex* Sir2 that exhibits the highest homology with the corresponding regions from other organisms known to be required for the catalytic function of the Sir2 enzyme.

4.4.2 Characterization of *Daphnia* Sir2

Using PCR to amplify the open reading frame (ORF) in two overlapping pieces as outlined in Figure 1 B, we first obtained the 5' and 3' halves of *D. pulicaria* Sir2 ORF. After verification of the sequence of the PCR products, using the internal restriction enzyme MscI, we joined the two halves as outlined in methods section to create a complete ORF that would be capable of coding for the entire Sir2 protein. We sub-cloned the Sir2 ORF in a mammalian expression vector pcDNA3.1- (Invitrogen) with a Flag tag on its N-terminus so we could check its expression in cell culture as well as in an *in vitro* transcription–translation system to obtain a full-length Sir2 protein for measuring its catalytic activity. Thus, in order to determine if the cloned *Daphnia* ORF would produce a functional enzyme, we performed two experiments, one to determine the expression of Sir2 in mammalian cells and another to ensure enzymatic activity of the Sir2 produced by *in vitro* translation of the ORF. Currently there are no *Daphnia* or

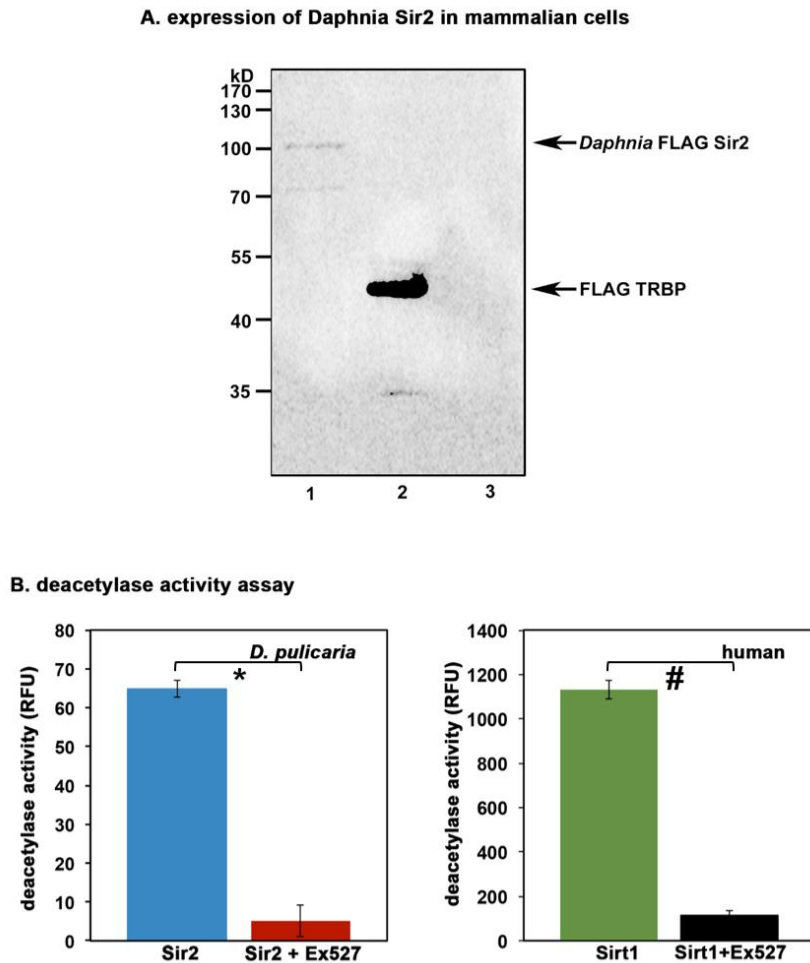


Figure 4.2. *Daphnia* Sir2 ORF produces a functional protein with catalytic activity. A) Expression of *Daphnia* Sir2 in Cos-1 cells. 100 ng each of *Daphnia* FLAG-Sir2/pcDNA3.1-, FLAG-TRBP/pcDNA3.1-, or empty vector pcDNA3.1- were transfected in Cos-1 cells using Effectene (Qiagen) and total protein extract was prepared 24 h after transfection. Western blot analysis was performed with anti-Flag antibody (M2, Sigma). Arrows indicate Flag-Sir2 and Flag-TRBP positions. B) Sirt1 Activity Assay. Activity assay was performed using 5 μ l of *in vitro* translated *Daphnia* Sir2. Human recombinant Sirt1 was used as a positive control. Ex527, a Sirt1/Sir2 specific inhibitor was used to ensure specificity that the observed deacetylase activity was that of *Daphnia* Sir2. Note that *Daphnia* Sir2 data represented is after subtracting the background activity obtained with unprimed (no plasmid DNA added) reticulocyte lysate. Error bars indicate standard deviations. Student T tests were performed to determine statistical significance. The p values are as follows: *: 5.2×10^{-7} (t stat: 22.2, df: 3); #: 4.8×10^{-7} (t stat: 22.6, df: 3).

other crustacean cell lines available, thus we used mammalian cells to express *Daphnia* Sir2. Figure 2A depicts a western blot analysis of the protein extract from Cos-1 cells transfected with *Daphnia* FLAG-Sir2/pcDNA3.1- expression construct or with a positive control FLAG-TRBP/pcDNA3.1-. The FLAG-TRBP/pcDNA3.1- serves as a positive control for the transfection, expression, and western blot as we have used this plasmid for many studies previously (Singh et al 2011, Vaughn et al 2015). As seen in lane 1, the band just above the 100 kD molecular weight marker corresponds to *Daphnia* FLAG-Sir2. The calculated molecular weight of *Daphnia* Sir2 based on its primary sequence is 67kD, however, Sir2 proteins as well as mammalian Sirt1 proteins are known to run heavier on the SDS-PAGE gels than their calculated molecular weight based on the primary sequence, which results from heavy post translational modifications (Vaquero et al 2004). Lane 2 indicates a strong expression of FLAG-TRBP, which is known to express extremely strongly based on our previous work (Singh et al 2011, Vaughn et al 2015). As expected, there is no band present in lane 3 as it contains protein extract from Cos-1 cells transfected with empty vector pcDNA3.1-. Thus, the *Daphnia* Sir2 is expressed in Cos-1 cells and produces a full-length protein.

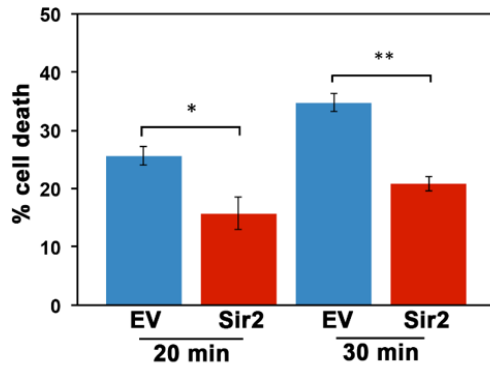
We next wanted to determine if the protein produced from the *Daphnia* Sir2 ORF has deacetylase activity similar to Sir2 from other organisms. We used the FLAG-Sir2/pcDNA3.1- construct to produce an *in vitro* translated *Daphnia* Sir2 protein using the TNT T7 coupled reticulocyte system (Promega). The protein was synthesized using ³⁵S labeled methionine and its production and integrity was checked by SDS-PAGE followed by a phosphorimager scan (data not shown). The deacetylase activity present

in the reticulocyte lysate was measured using a Sirt1 Activity Assay kit (Abcam). Shown in Figure 2B, the reticulocyte lysate containing *in vitro* translated *Daphnia* Sir2 exhibits deacetylase activity (blue bar). A human recombinant Sirt1 sample that was supplied with the kit was used as a positive control (green bar). Note that a reticulocyte lysate only control was performed as a negative control with no Sir2/pcDNA3.1- DNA added as this represents the activity present in reticulocyte lysate. The activity obtained from this negative control is subtracted from the values displayed in figure 2B. The red and the black bars represent the enzyme activity in presence of a Sir2 specific inhibitor Ex527 and establish that both the human recombinant Sirt1 as well as *Daphnia* Sir2 activity is significantly diminished by the inhibitor. These results further confirm that the cloned ORF encodes a functional Sir2 protein.

4.4.3 *Daphnia* Sir2 protects against proteotoxic stress similar to mammalian Sirt1

In order to verify the functional activity of *Daphnia* Sir2, we tested if *Daphnia* Sir2 would offer protection against heat shock in a mammalian cell culture system. Previous work on mammalian Sirt1 in 293T cells established that Sirt1 overexpression provided protection to the cells during severe heat shock (Westerheide et al 2009). To assess if *Daphnia* Sir2 overexpression would offer similar protection, we transfected Cos-1 cells with the *Daphnia* Sir2 expression construct and measured the cell viability following a severe heat shock. Shown in Figure 3A, *Daphnia* Sir2 overexpression in Cos-1 cells resulted in lower percentage cell death following a heat shock. With 20 minutes of heat shock, there was 25.67 +/- 1.62% cell death in cells transfected with empty vector without any Sir2 ORF insert (blue bar). In contrast to this, only 15.74 +/- 2.74% cell death

A.



B.

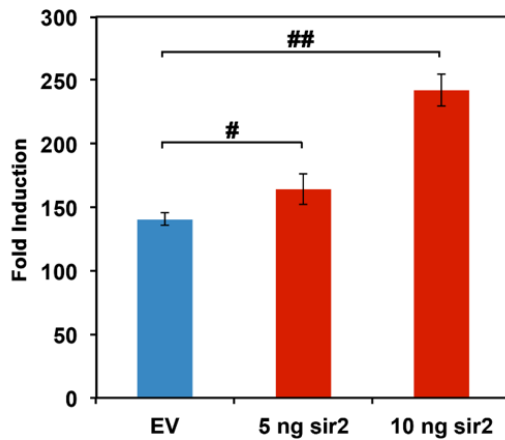


Figure 4.3. *Daphnia* Sir2 exhibits functional activity similar to mammalian Sirt1. A) *Daphnia* Sir2 overexpression confers cytoprotection to heat shock. Cos-1 cells were transfected with *Daphnia* Sir2 expression construct and heat shocked at 42°C for 20 and 30 minutes as indicated. Blue bars depict empty vector control and red bars represent the *Daphnia* Sir2 transfections. Percent cell death was measured using Trypan Blue viability assay 24 hours following heat shock. Error bars represent standard deviations. B) *Daphnia* Sir2 enhances the transcriptional induction of genes regulated by a heat shock. Cos-1 cells were co-transfected either with the empty vector (blue bars) or *Daphnia* Sir2 expression construct (red bars), pGL4.41 (luciferase reporter plasmid, Promega), and pRL-Null (for normalization of transfection efficiency, Promega) and treated with CdCl₂ at 24 hours following transfection. Luciferase assay was performed on cell extracts 6 hours following CdCl₂ treatment. Error bars represent standard deviations. A Student T test of arcsin transformed proportions was used to determine statistical significance in A (* t stat: 3.98, df: 3, p: 0.007; ** t stat: 7.37, df: 3, p: 0.0003). In B an ANOVA was performed (F: 309, df: 2,6, p: 9.2x10⁻⁵) followed by a post hoc Tukey test, both # and ## indicate differences between the means at p < 0.5.

was observed with Sir2 overexpression construct (red bar). When duration of heat shock was 30 minutes, the differences in percentage was greater with control being at 34.79 +/- 1.63% cell death (blue bar) and Sir2 overexpression sample showing 20.90 +/- 1.27% cell death (red bar). Thus, *Daphnia* Sir2 overexpression is clearly protective to mammalian cells following heat shock, potentially acting via the deacetylation of the transcription factor HSF1. The results also indicate that *Daphnia* Sir2 has an enzymatic activity similar to *Drosophila* Sir2 and mammalian Sirt1. In order to further analyze *Daphnia* Sir2's potential in regulating response to proteotoxic stress, we performed co-transfection experiments in Cos-1 cells using *Daphnia* Sir2 expression construct and a luciferase reporter pGL4.41 (Promega), which is regulated transcriptionally by proteotoxic stress. pGL4.41 contains four mammalian heat shock elements (HSEs) that serve as binding sites for HSF1 (Anckar and Sistonen 2012) to induce the transcription of the downstream reporter firefly luciferase ORF only when proteotoxic stress (heat shock or heavy metals) is present. Thus, under proteotoxic conditions, there is an induction of luciferase activity via the activation and binding of HSF1 to the HSEs in pGL4.41. We co-transfected the *Daphnia* Sir2 expression construct and pGL4.41 and treated cells with cadmium chloride (CdCl₂) to induce proteotoxic stress. As shown in Figure 3B, overexpression of *Daphnia* Sir2 caused a dose dependent increase in luciferase activity after CdCl₂ treatment as compared to empty vector control where no *Daphnia* Sir2 was present. The empty vector control (blue bar) showed a 140.50 +/- 4.95 fold induction in response to CdCl₂. Co-transfection of 5 ng and 10 ng of *Daphnia* Sir2 expression construct resulted in 164.16 +/- 11.9, and 242.18 +/- 12.72 -fold induction respectively

(red bars). Thus, *Daphnia* Sir2 overexpression in a mammalian cell culture system results in a more robust cellular response to proteotoxic stress, potentially via the deacetylation of HSF1.

4.4.4 *Daphnia* Sir2 mRNA levels increase with age in *D. pulicaria*

The expression of Hsp70 in response to a heat shock decreases with increasing age in *D. pulicaria* (Schumpert et al 2014), which may be due to a decreased expression of Sir2. In order to analyze the expression of Sir2 during the life span of *D. pulicaria*, we examined the Sir2 transcript levels at ages 1, 4, and 8 weeks. Total RNA was isolated, converted to cDNA, and real time PCR was performed to examine the relative amount of Sir2 mRNA. As represented in Figure 4, *D. pulicaria* steady state Sir2 mRNA levels increased with age. These results indicate that decreased expression of Sir2 mRNA is not a likely cause of decreased HSR and reduced Hsp70 expression in response to proteotoxic stress in old organisms.

4.4.5 Sir2 activity decreases with age in *D. pulicaria*.

We next examined if Sir2 enzyme activity declines with age in *D. pulicaria*. Sir2 activity assays were performed using a commercially available kit (Abcam). As represented in Figure 5, Sir2 activity of *D. pulicaria* declines with age, young organisms displayed the highest activity of Sir2 (410.0 +/- 12.02 RFUs, first blue bar from the left), followed by middle aged *D. pulicaria* (248.6 +/- 4.36 RFUs, second blue bar from the left) and old organisms had the lowest amount of Sir2 activity (178.2 +/- 5.44 RFUs, third blue bar from the left). To ascertain that we were measuring Sir2 activity, we tested the enzyme activity in presence of Ex-527, a known specific inhibitor for Sirt1. As seen in

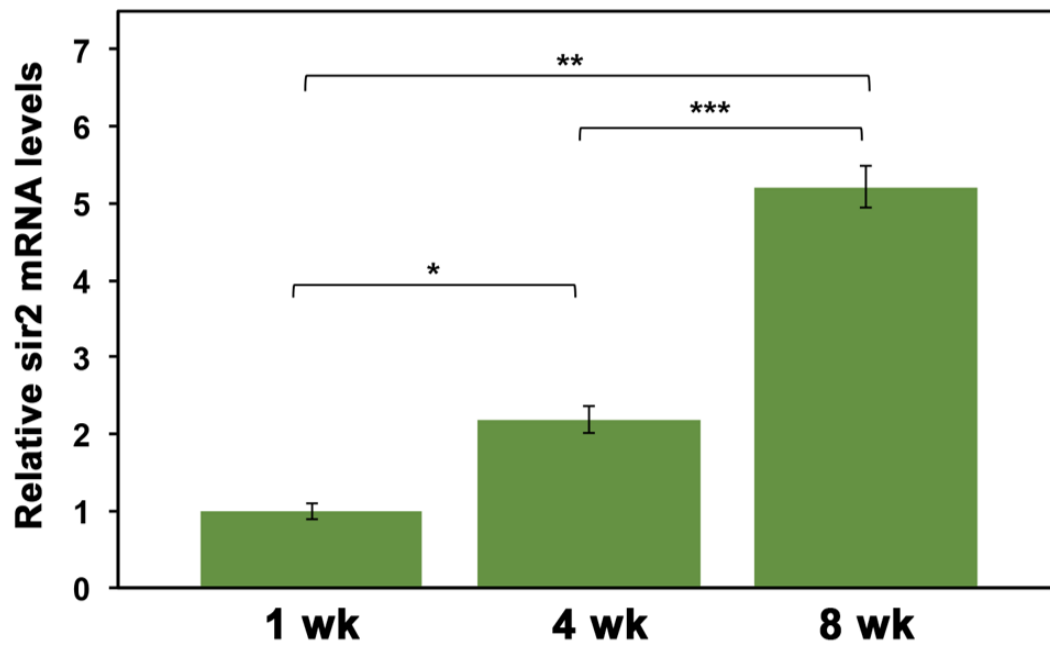


Figure 4.4. Sir2 mRNA levels increase with *D. pulicaria* age. Total RNA was harvested from groups of 15 *D. pulicaria* at each age. Real-time PCR was performed using a BioRad CFX 96 using Sir2-specific primers and GAPDH primers as a normalization control. Data was analyzed using the $2^{\Delta\Delta CT}$ method and are represented as relative sir2 mRNA levels (green bars). Error bars represent standard deviations. The ages are as indicated, wk: weeks. To analyze statistical significance, an ANOVA was performed (F: 145.95, df: 2,9, $p:1.38 \times 10^{-7}$) followed by a post hoc Tukey test. The post hoc analysis revealed that all three means were statistically different from one another and for all *, **, and *** indicate $p < 0.05$.

figure 5 (1 wk + Ex527; red bar and human Sirt1 + Ex527; black bar), the deacetylase activity drops to background levels (grey bar labeled C) in the presence of 800 nM Ex527. Thus, it can be concluded that the commercially available assay system works efficiently to assay *Daphnia* Sir2 activity and that Sir2 activity declines with age in *D. pulicaria*.

4.4.6 Targeted knockdown of Sir2 expression via RNAi shortens *D. pulicaria* lifespan and enhances death following proteotoxic stress.

A decline in Sir2 activity has been shown to be responsible for aging phenotypes of various model organisms (Sinclair and Guarente 2014). In addition, evidence exists for extension of life span by enhancing Sir2 activity with nutraceuticals or by overexpression in model organisms (Imai and Guarente 2014, Sinclair and Guarente 2014). Thus, we next examined if a targeted knockdown of Sir2 activity in *D. pulicaria* would shorten the life span. We recently developed an effective RNAi method for adult *Daphnia* via feeding. In order to achieve RNAi for the gene of interest, a double-stranded RNA (dsRNA) corresponding to a selected region in the target mRNA is expressed in *E.coli* using the L4440 vector (Fire et al 1998, Schumpert et al 2015). *Daphnia* are fed on these bacteria to deliver the dsRNA systemically via the gut. Using this method we have previously achieved effective RNAi of phenoloxidase expression in *D. melanica* and eyeless expression in *D. melanica* and *D. pulicaria* (Schumpert et al, 2015, unpublished). Figure 6A shows the map of the targeting construct for *Daphnia* sir2 using the first 863 bp of the sir2 transcript. We used ten *D. pulicaria* per beaker in 100 ml of filtered lake water for feeding them the bacteria that expressed sir2 or GFP dsRNA for 10 days, after

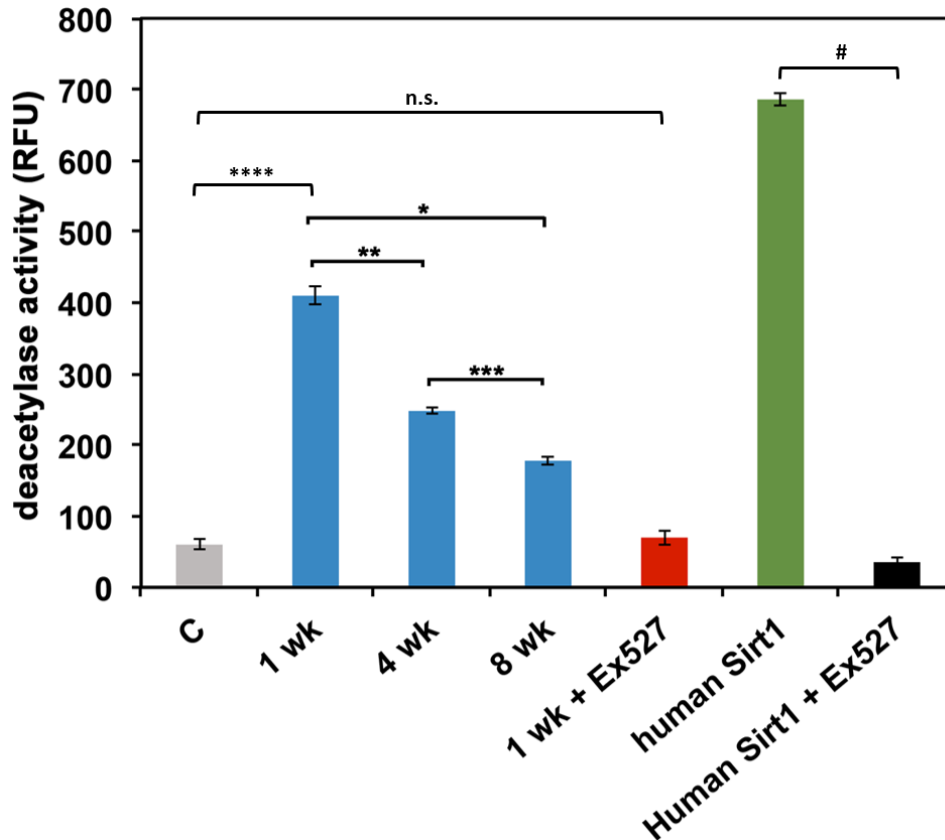


Figure 4.5. *D. pulicaria* Sir2 activity declines with age. The deacetylase activity present in total protein extracts was assayed using a Sirt1 activity assay kit (Abcam). *Daphnia* extracts (1 μ g total protein) prepared from the indicated ages (wk: weeks) were used (blue bars). NAD⁺ was added in excess (non-limiting amounts) to the assay mixtures. Recombinant human Sirt1 (Abcam) was used as a positive control (green bar; 30 ng protein). Ex527, a Sirt1/Sir2 specific inhibitor (800 nM), was utilized to ascertain that the deacetylase activity was from Sir2 and not another sirtuin (red bar for *Daphnia* Sir2, aged 1 week; black bar for human recombinant Sirt1, both 1 μ g protein). A negative control with no extract added is represented as a gray bar. Each age set had 3 separate biological replicates and the data depicted are averages. Error bars represent standard deviations. A Student T-test was performed to examine statistical significance between human Sirt1 and human Sirt1 plus Ex527: # t stat: 563.4, df: 5, p: 3.3×10^{-13} . An ANOVA was performed (F: 911, df: 4,10, p: 9.2×10^{-13}) followed by a post hoc Tukey test. The post hoc analysis revealed that all means were statistically different from one another for all *, **, ***, **** indicate p<0.05, except between control and 1 wk plus Ex527, N.S.: not significant.

which we checked if gene-specific RNAi was achieved. To ensure that the sir2 specific RNAi was achieved, we analyzed the steady state sir2 mRNA levels after the RNAi feeding regimen. Figure 6B displays data from a representative reverse transcriptase PCR demonstrating a knockdown in sir2 mRNA only in RNA isolated from *D. pulicaria* fed on Sir2 dsRNA expressing bacteria (lane 3). RNA isolated from *D. pulicaria* fed on bacteria expressing GFP dsRNA (lane 2) and wild-type *D. pulicaria* controls not fed on bacteria (lane 1) both have constitutive levels of sir2 mRNA expression with little variation in sir2 transcript levels. Thus, our targeting sir2 dsRNA construct achieved very effective and specific sir2 knockdown. In order to measure the effect of sir2 knockdown on viability of *Daphnia* after a heat shock, we first determined the optimal heat shock conditions. We subjected *D. pulicaria* to a range of elevated temperatures as indicated in figure 6C and measured percentage death 24h after heat shock. Based on these results we selected 34⁰ C as the heat shock temperature, as it would allow us to measure an increase or a decrease in lethality after sir2 knockdown. To analyze the effect of Sir2 knockdown on the HSR, *Daphnia* that were fed no bacteria (blue bar labeled “–”, figure 6D), bacteria with a control construct (encoding GFP dsRNA- green bar, figure 6D), or bacteria with the Sir2 targeting construct (encoding Sir2 dsRNA- red bar, figure 6D) for ten days were heat shocked at 34⁰C for 30 minutes. As seen in Figure 6D, the *Daphnia* fed on bacteria with sir2 dsRNA had a significantly higher percentage of death following heat shock (red bar: 90 +/- 4.3%), compared to the negative control GFP (green bar: 67 +/- 5.0%), and wild type *D. pulicaria* (blue bar: 59 +/- 3%). Thus, when Sir2

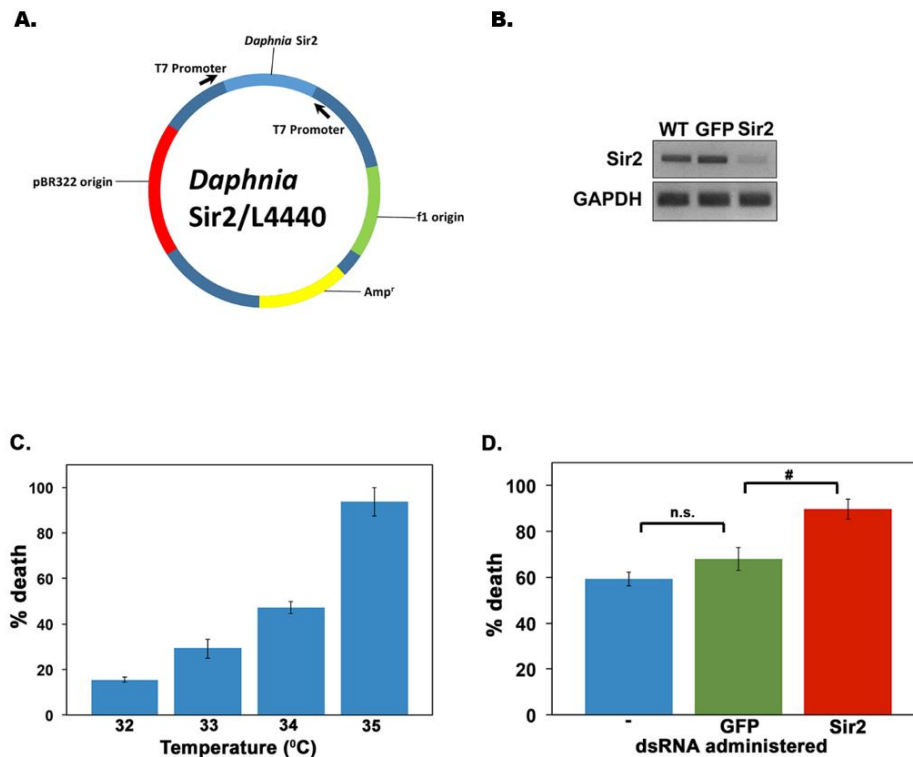


Figure 4.6. Targeted RNAi knockdown of *D. pulicaria* Sir2 severely impairs HSR and survival. A) Diagram of Sir2 targeting construct. B) Sir2 transcript levels are diminished following an RNAi feeding regimen of bacteria expressing sir2 dsRNA. Total RNA was isolated from untreated, GFP dsRNA treated, or Sir2 dsRNA-treated *Daphnia* after the RNAi feeding regimen for ten days. Reverse transcriptase-PCR was performed for 27 cycles with Sir2 or GAPDH specific primers and the product was analyzed on a 1% agarose gel. C) Percentage death following a heat shock in *D. pulicaria*. *D. pulicaria* were heat shocked at various temperatures as indicated for 30 minutes, allowed to recover for 24 hours, at which point the percentage death was measured. Error bars represent standard deviations. D) Percentage death in response to heat shock following Sir2 knockdown. After ten days on an RNAi feeding regimen, untreated *D. pulicaria* (labeled -; blue bar), GFP dsRNA treated *D. pulicaria* (green bar), and Sir2 dsRNA treated *D. pulicaria* (red bar) were subjected to heat shock at 34°C for 30 minutes, allowed to recover for 24 hours, and percentage death was measured. RNAi was performed in a total of 3 different biological replicates. Error bars represent standard deviations. A Student T test of arcsin transformed proportions was used to determine statistical significance in D- # t stat: 5.67, df: 3, p: 4x10⁻³; n.s.: not significant.

is knocked down in *D. pulicaria*, the organism no longer responds appropriately to proteotoxic stress leading to death following a heat shock.

In addition to the deleterious effect of Sir2 knockdown on HSR, we further analyzed the effect of Sir2 knock down on the lifespan of *D. pulicaria*. Sir2 knockdown or mutation has been shown to cause a decline in lifespan for numerous organisms, including yeast, *C. elegans*, and *D. melanogaster* (Kaeberlein et al 1999, Rogina and Helfand 2004, Wang and Tissenbaum 2006). We used ten *Daphnia* neonates per beaker in 100 ml of filtered lake water and subjected them to an RNAi feeding regimen as described in methods. We followed these *Daphnia* throughout life, feeding them daily with either no bacteria (WT, control), bacteria expressing GFP dsRNA, or bacteria expressing sir2 dsRNA. The water was changed every other day and reproduction and death was noted for each treatment group. In Figure 7A, the survivorship curves of *Daphnia* on the RNAi feeding regimens are displayed. WT *Daphnia* fed just algae throughout the course of the experiment lived the longest with the median life span being 31 +/- 2 days (blue line). The *Daphnia* fed on bacteria expressing GFP dsRNA had a median lifespan of 29 +/- 1.8 days (green line) and the *Daphnia* fed on bacteria expressing Sir2 dsRNA had a median lifespan of only 11 +/- 2.5 days (red line). Note that juvenile mortality was removed from this experiment to examine the overall lifespan effects of Sir2 knockdown on adult *Daphnia*. Overall, a knockdown of sir2 by RNAi resulted in a 64 +/- 2% reduction in lifespan of *D. pulicaria* compared to WT *D. pulicaria*. These results are statistically significant as analyzed by a nonparametric log rank test ($X^2=19.1$, p-value= 1.2×10^{-5} comparing WT to Sir2 dsRNA fed sample, $X^2=18.098$, p-

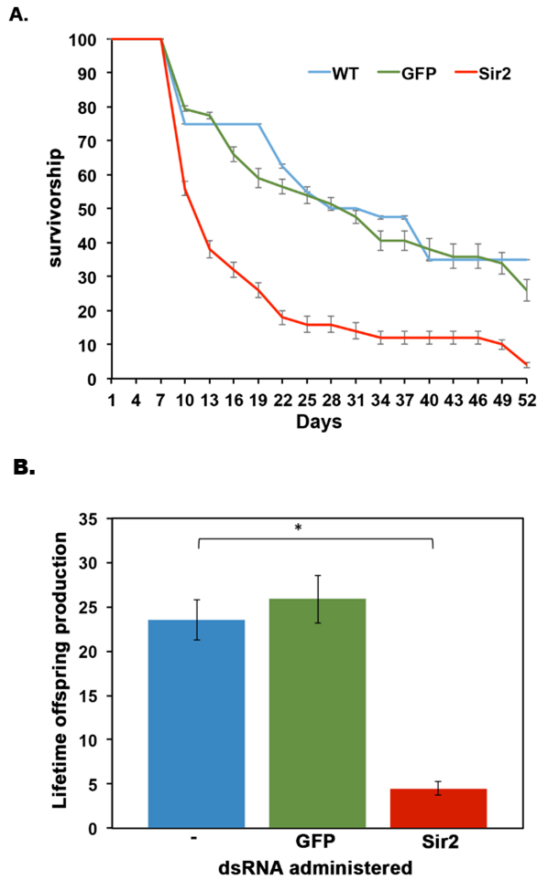


Figure 4.7. Sir2 knockdown in *D. pulicaria* results in reduced lifespan and lifetime offspring production. A) Survivorship curves. The survivorship curves of untreated (blue line), GFP dsRNA-treated (green line), and Sir2 dsRNA-treated (red line) *Daphnia* are represented. Ten *Daphnia* placed in 100 ml of filtered lake water were fed daily on bacteria containing the knockdown constructs. RNAi was performed in a total of 4 different biological replicates. Each plotted point is average survivorship on that day (collectively from our 4 biological replicates) and error bars depict standard error of the mean. No bacteria (untreated) (n=40), GFP (dsRNA control) (n=44), Sir2 (n=50). To determine statistical significance, a nonparametric log rank test was performed and the results are as follows: $X^2=19.1$, p-value= 1.2×10^{-5} comparing Sir2 to WT, $X^2=18.098$, p-value= 2.1×10^{-5} comparing Sir2 to GFP. B) Average number of offspring per *Daphnia* mother. The average number of progeny per mother over the course of its lifespan was calculated by dividing the total number of offspring produced in an experimental set by the total number of adult *Daphnia* mothers in that set. Blue bar is WT control, green bar is GFP dsRNA-treated, and red bar is Sir2 dsRNA-treated. An ANOVA was performed (F: 93, df: 2,6, p: 3.02×10^{-5}) followed by a post hoc Tukey test. The post hoc analysis revealed that – and Sir2 means were statistically different from one another as are GFP to Sir2, * indicate p<0.05. Means – to GFP were not statistically significant in their difference.

value= 2.1×10^{-5} comparing GFP dsRNA fed sample to Sir2 dsRNA fed sample). The lifetime offspring production was also drastically different for the three groups (Figure 7B) with WT producing an average 20.05 ± 2.26 offspring per individual (blue bar), GFP dsRNA treated with 25.85 ± 2.73 offspring per individual (green bar) and Sir2 dsRNA treated *Daphnia* only producing 4.5 ± 0.75 offspring per individual (red bar). These results establish that Sir2 activity is required for a normal life span and reproduction in addition to being required for an efficient protective response to proteotoxicity (Figure 6). This is a first report that uses RNAi to examine the effects of the knockdown of a longevity gene in the emerging model system of *Daphnia*.

4.5 Discussion

The longevity factor and deacetylase Sir2/Sirt1 plays a central role in coordinating cellular stress response and thus affects an organism's ability to respond effectively and appropriately to proteotoxic stress. Various metabolic, genotoxic, and proteotoxic stressors regulate Sir2/Sirt1 expression levels as well as activity and numerous studies have demonstrated Sir2/Sirt1's essential role in cytoprotection, especially in the context of aging (Raynes et al 2012, 2013). Westerheide *et al* demonstrated that one of the deacetylation targets for human Sirt1 is HSF1, the transcription factor responsible for inducing the HSR (Westerheide et al 2009). The acetylated form of HSF1 is inactive for its function as a transcription factor since only the deacetylated form is capable of binding to DNA. The HSR of an organism declines with age and previous studies have implicated the inability of HSF1 to bind to DNA as being the mechanism for this age dependent decline (Fawcett et al 1994, Jurivich et al 1997,

Westerheide et al 2009). This decline in HSR in older organisms is one of the factors thought to be responsible for cellular and organismal aging as it contributes to an increased accumulation of misfolded or damaged proteins (Morimoto and Cuervo 2009, Powers et al 2009). Correspondingly, Sirt1 enzyme activity also declines with age in mammals and it is established to be the causative mechanism for the consequent decline in the HSR via inactivation of HSF1 (Westerheide et al 2009, Raynes et al 2013).

We have previously established that similar to mammals the HSR declines with age in *Daphnia* and this decline is due to the lack of HSF's ability to bind to DNA (Schumpert et al 2014). We hypothesized that *Daphnia* Sir2 may regulate HSF's DNA-binding by deacetylation. Although *Daphnia* is an emerging model for research on aging mechanisms, there are very few molecular studies performed with *Daphnia* and aging (Schumpert et al 2014, Schumpert et al 2015). In order to provide an initial characterization of the molecular mechanisms known to be operative during the aging process in other model organisms, we tested if *Daphnia* Sir2 has deacetylase activity and if it functions to enhance HSR and life span. We cloned Sir2 ORF, tested the activity of encoded protein, and determined the expression levels of Sir2 during the *Daphnia* life span. More importantly, we examined the effects of Sir2 knockdown using our newly developed method for RNAi on the lifespan as well as on HSR. Based on our data, *Daphnia* Sir2 offers cellular protection to proteotoxicity by regulating HSR and also functions in regulation of life span and reproduction. These results establish the involvement of *Daphnia* Sir2 in HSR, lifespan regulation, and reproduction for the first time.

Regulation of Sir2/Sirt1 activity is complex and is known to occur at multiple levels in mammals and invertebrates (Raynes et al 2013, Rovollo and Li 2013). Previous studies have demonstrated that Sirt1 in mammalian cells is regulated by numerous factors including association with activators and inhibitors of catalytic activity, post translational modifications, as well as transcriptional regulation via transcription factors p53 and FOXO (Raynes et al 2013, Revollo and Li 2013). While the steady state levels of Sir2 mRNA showed an increase during *Daphnia* life span, the Sir2 deacetylase activity declined with age. These results suggest a potential regulation at the post-transcriptional or post-translational levels. Although the post transcriptional regulation of Sirt1 (or Sir2) expression has not been studied extensively, some evidence of regulation by the mRNA-binding protein HuR in mammalian cells (Abdelmohsen et al 2007), and modulation of its enzymatic activity by protein phosphorylation and methylation is reported (Raynes et al 2013, Rovollo and Li 2013). Any contribution of such mechanisms in *Daphnia* remains to be explored in the future. In our studies, we could not investigate the relative protein levels of Sir2 during life span in *Daphnia* due to the lack of an antibody that can detect *Daphnia* Sir2 protein and thus such potential modes of regulation may be may only be investigated in future studies once an antibody is available. In addition, cellular NAD⁺ levels are known to regulate Sir2 activity during aging (Imai 2009, Imai and Guarente 2014) and may play a role in the decline of Sir2 activity with age in *D. pulicaria*.

To further investigate the functional role of Sir2 in HSR and life span regulation in *Daphnia*, we investigated the consequence of Sir2 knockdown by RNAi in adult *Daphnia*.

Our results demonstrate a clear increase in mortality following heat shock in *D. pulicaria* with sir2 knockdown by RNAi (Figure 6). In addition, the median life span was significantly shortened in response to Sir2 RNAi (Figure 7). The GFP dsRNA or potentially the presence of bacteria in food alone seem to cause a less significant marginal effect on *Daphnia* life span underscoring the importance of such negative controls. The effect of Sir2 knockdown on *Daphnia* reproduction was striking, as the overall lifetime fecundity was severely diminished in Sir2 knockdown adults (Figure 7). Sir2 is considered to be a metabolic sensor, thus, a potential mechanism for this drastic decrease in fecundity may be due to the organism not being able to effectively gauge the nutrient status of the environment (Raynes et al 2013, Imai and Guarente 2014). Our results not only demonstrate the role of Sir2 in HSR, life span regulation, and offspring production for the first time but also establish that the newly developed RNAi method can be used in adult *Daphnia* to analyze gene function.

The effect of various small molecule activators of Sir2 on the overall lifespan of *D. pulicaria* remains to be explored in future. Numerous natural and synthetic sirtuin activating compounds that work by allosteric mechanisms to stimulate sirtuin activity, are shown to offer health benefits in rodents, primates, and thought to work the same way in humans (Sinclair and Guarente 2014). Resveratrol, a polyphenol found in red grapes, first characterized for its antioxidant properties and subsequently for the activation of sirtuins has been reported in multiple studies to extend lifespan in model organisms including *D. melanogaster* and *C. elegans* (Howitz et al 2003, Wood et al 2004). Although generally resveratrol is thought to have life span extending effects,

some studies in recent years have also claimed that resveratrol showed no life span expansion effects in the same models (Bass et al 2007). In a previous study, no effect of resveratrol on *Daphnia* life span was observed using the short-lived ecotype (*D. pulex* TCO clone), but the overall fecundity was reduced (Kim et al 2014). The effect of resveratrol on Sir2 activity in treated *D. pulex* was not tested and it remains a possibility that the concentrations of resveratrol used in the study may not have affected Sir2 activity levels significantly. Although we have not examined the effects of Sir2 knockdown in *D. pulex*, our results in *D. pulicaria* suggest that a possible variation in the genotypes of the two isolates may reveal important and interesting modifiers of Sir2 dependent regulation.

Another documented and endogenously produced small molecule regulator of sirtuins is melatonin, a free radical scavenger (Jung-Hynes et al 2009, Jung-Hynes et al 2011, Ramis et al 2015). Melatonin levels decline with age and it was reported recently that melatonin activates sirtuins, in addition to regulating circadian rhythms and acting as an anti-inflammatory agent (Jung-Hynes et al 2009, Jung-Hynes et al 2011, Ramis et al 2015). It was reported that melatonin had no effect on *Daphnia magna* life span but attenuated the neck teeth formation and stress response in presence of fish kairomones (Schwarzenberger et al 2014). Although no correlation of the observed effects on stress response or lack of effect on life span with Sir2 activity was tested in this study, it is certainly possible that the melatonin concentrations used may not have affected Sir2 activity.

Caloric restriction extends lifespan of several species and improves healthspan. Many of these benefits have been attributed mainly to the enhanced activity of Sir2 in yeast and invertebrates and Sirt1 in mammals (Wood et al 2004, Rahat et al 2011, Wang et al 2014). This view was challenged by a study describing the failure to observe extension of life span in worms and flies transgenic for the corresponding Sir2 orthologs in a uniform genetic background (Burnett et al 2011). Nevertheless, considering the conflicting evidence, the physiological roles of Sir2 in caloric restriction may be more complex than expected initially. Caloric restriction was not seen to extend life span in *D. pulex*, however, Sir2 expression or activity was not studied in this context (Kim et al 2014). The same study also noted that caloric restriction did extend the life span of *D. pulex* and that genetic polymorphism in organismal response to caloric restriction may be present naturally and contribute to such variation (Kim et al 2014). It could be interesting to study activity of Sir2 under various food levels in different isolates and species of *Daphnia* in future studies.

The decline in Sir2 activity in older organisms is often attributed to a decrease in the essential cofactor NAD⁺ (Braidy et al 2011, Gomes et al 2013, Imai and Guarente 2014). It is possible that the decline we observe in older *Daphnia* is also due to a decline in NAD⁺ levels and this remains to be determined. Another interesting aspect of Sir2 activity regulation is the effect of NAM amidases (Frankel et al 2011, Imai and Guarente 2014). NAM is a byproduct of NAD⁺ conversion during Sirt1 catalysis and is also a noncompetitive inhibitor of Sirt1 (Frankel et al 2011). Multiple studies have shown that

overexpression of a NAM scavenger enzyme, NAM amidase, extends lifespan by released inhibition of Sirt1 (Frankel et al 2011).

The techniques for genome modification to create knockout animals or perform homologous gene replacement are available for *Daphnia* (Hiruta et al 2014, Nakanishi et al 2014). Similarly, techniques to create transgenic *Daphnia* have been established (Kato et al, 2012). It would be interesting to study the effects of Sir2 overexpression on their response to proteotoxicity as well as longevity. Studies using *C. elegans* and *D. melanogaster* indicate an extension of lifespan by as much as 30% when Sir2 is overexpressed (Kaeberlein et al 1999, Frankel et al 2011). Another interesting possibility is to create transgenic *Daphnia* that harbor the sir2 gene from the opposing ecotype, for example, replace the endogenous *D. pulicaria* sir2 with *D. pulex* sir2 locus and vice versa to examine effects on aging and potentially determine the contributions of genetic backgrounds on Sir2 expression, and activity. As *Daphnia* becomes an established model organism for research on aging and longevity, the analysis of Sir2 will be at the forefront of research on aging

Chapter 5:
Development of an efficient RNA interference method by feeding for the
microcrustacean *Daphnia*⁵

⁵ Schumpert C, Dudycha J, Patel R.2015.Development of an efficient RNA interference method by feeding for the microcrustacean *Daphnia*. Submitted to BMC Biotechnology

5.1 Abstract

5.1.1 Background:

RNA interference (RNAi) is an important molecular tool for analysis of gene function *in vivo*. *Daphnia*, a freshwater microcrustacean, is an emerging model organism for studying cellular and molecular processes involved in aging, development, and ecotoxicology especially in the context of environmental variation. However, in spite of the availability of a fully sequenced genome of *Daphnia pulex*, meaningful mechanistic studies have been hampered by a lack of molecular techniques to alter gene expression. A microinjection method for gene knock down by RNAi has been described but the need for highly specialized equipment as well as technical expertise limits the wider application of this technique. In addition to being expensive and technically challenging, microinjections can only target genes expressed during embryonic stages, thus making it difficult to achieve effective RNAi in adult organisms.

5.1.2 Results:

In our present study we present a bacterial feeding method for RNAi in *Daphnia*. We used a melanic *Daphnia* species (*Daphnia melanica*) that exhibits dark pigmentation to target phenoloxidase, a key enzyme in the biosynthesis of melanin. We demonstrate that our RNAi method results in a striking phenotype and that the *phenoloxidase* mRNA expression and melanin content, as well as survival following UV insults, are diminished as a result of RNAi. This method can also achieve gene knockdown in developing embryos and thus may have a broader applicability beyond adult organisms. RNAi mediated targeting of the developmental gene *eyeless*, which is responsible for

development of the compound eye in *Daphnia*, was tested in two species of *Daphnia* (*D. melanica* and *D. pulicaria*) and we observed a deformed eye phenotype in offspring of mothers fed bacteria expressing the *eyeless* targeting dsRNA.

5.1.3 Conclusions:

Overall, our results establish a new method for RNAi in *Daphnia* that significantly advances further use of *Daphnia* as a model organism for functional genomics studies. The method we describe is relatively simple and widely applicable for knockdown of a variety of genes in adult organisms and possibly in developing embryos.

5.2 Introduction

In order to study gene function in intact organisms, effective techniques to manipulate gene expression to achieve an over-expression or knockdown are essential. RNA interference (RNAi) has revolutionized several fields of biology by making it possible to study loss-of-function effects in various organisms without the time-consuming, laborious genetic manipulations. RNAi is a mechanism in which dsRNA molecules trigger gene silencing in a sequence-specific manner, usually resulting in degradation of the transcript complementary to one strand of the dsRNA (Ipsaro and Joshua-Tor 2015 and the references within). The RNAi pathway is conserved throughout eukaryotes with examples of the RNAi mechanism being used to silence gene expression in numerous model organisms including (but not limiting to) *S. pombe*, *Tetrahymena*, *D. melanogaster*, *C. elegans*, *D. rerio*, *Xenopus*, and *M. musculus* (Ketting 2011). This conserved mechanism of gene silencing has led to exceptional use of reverse genetics methods and has led to a better understanding of molecular pathways at mechanistic

levels (Perrimon et al 2010). Beyond enabling the study of diminished expression of a particular gene leading to advancements in understanding molecular pathways, RNAi has demonstrated a potential for being used in therapeutics for treating human diseases (Rettig and Behlke 2012, Deng et al 2014).

Daphnia are freshwater microcrustaceans that inhabit inland waters around the world and often are the critical herbivore in aquatic food webs (Peters and Bernardi 1987, Benzie 2005). They have been a major model system in ecology, population genetics, and ecotoxicology for decades due to the ease with which field- and laboratory-based experiments can be conducted. Furthermore, they are cyclic parthenogens, a life cycle that permits genetically diverse natural populations and allows replication of genetically identical individuals through clonal reproduction in the lab (Zaffagnini 1987). With a fully sequenced genome (*D. pulex*, (Colbourne et al 2011)), *Daphnia* has the potential to be a key model organism in molecular ecology and evolution, and is rapidly emerging as a model organism in non-ecological fields including biology of aging (Pietrzak et al 2008, Dudycha and Hassel 2013, Lohr et al 2014, Murthy and Ram 2014, Schumpert et al 2014, Schumpert et al 2015), and neurobiology (Ungerer et al 2011, Christie and McCool 2012, McCool et al 2012, Weiss et al 2012, Toyota et al 2015). The U.S. National Institutes of Health list *Daphnia* as a model organism for biomedical research (<http://www.nih.gov/science/models/>) citing their extreme phenotypic responses to environmental changes, clonal reproduction, and ecological diversity as advantages in comparison to established biomedical models.

In order to fully realize *Daphnia* for molecular studies, techniques of experimental genetic manipulation are essential. Currently there is only a single technique described for RNAi in *Daphnia* (Kato et al 2011, Hiruta et al 2013) which involves microinjecting small dsRNA molecules into the embryos of *D. pulex* and *D. magna*. Although this system has allowed for the study of some genes regulating embryonic development, there are several drawbacks and limitations to the microinjection method. The microinjection process is technically challenging, involves specialized equipment, thereby making the protocol expensive, tedious and unlikely to be broadly adopted by researchers interested in *Daphnia* (Perrimon et al 2010, Yu et al 2013). Currently, microinjection has been performed successfully only in *Daphnia* embryos. Therefore, this method limits the number of genes one can target as it is mainly applicable to genes expressed during embryonic development. Thus, there is no reliable RNAi method for studying genes expressed later in *Daphnia* life span, or for achieving knock-down for a specific duration during the life span.

In *C. elegans*, the problems associated with microinjection for RNAi were mitigated with the introduction of a new technique that involved feeding the worms with bacteria expressing specific dsRNAs (Timmons and Fire 1998, Timmons et al 2001, Wang and Barr 2005). This method for systemic RNAi via feeding has been adapted for multiple organisms including, but not limited to, the house cricket (*Acheta domesticus*), the lepidopteran pest *Spodoptera exigua*, the brown apple moth (*Epiphyas postvittana*), the termite *Reticulitermes flavipes*, and planarians (Newmark et al 2003, Turner et al 2006, Zhou et al 2008, Tian et al 2009, La Fauce and Owens 2013). Since the

initial report of the systemic RNAi via feeding method in *C. elegans*, several different genes have been identified as being essential for systemic RNAi via feeding. One of these essential genes is Sid-1 (systemic RNAi defective), which encodes a transmembrane protein that forms a dsRNA gated channel (Winston et al 2002, Feinberg and Hunter 2003, Shih and Hunter 2011). We searched the recently published *Daphnia* genome (Colbourne et al 2011) and found that it contains a Daphnid homologue of Sid-1. We also analyzed the *D. pulex* genome for the various proteins known to be involved in RNAi (Ipsarro and Joshua-Tor 2015) using the online tool PANTHER (Protein Analysis Through Evolutionary Relationships) that allows for identification of various protein homologs based on domain structures and evolution of protein function in various organisms (Mi et al 2013). We determined that the *D. pulex* genome contains three homologs of Dicer, two homologs of Argonaute, and two homologs of TRBP (Colbourne et al 2011). Thus, we reasoned that if systemic RNAi via feeding could be achieved for *Daphnia*, transient gene knockdown experiments would rapidly advance development of this organism as a model system. In our present study, we present a method for efficient RNAi mediated gene knockdown in *Daphnia* via feeding.

Our study used three different species, *D. melanica*, *D. pulex*, and *D. pulicaria*. Although these taxa have different names, they are part of the same species-complex (Colbourne et al 1998, Colbourne and Hebert 1996, Cristescu et al 2012, Hebert and Finston 1996, Pfrender et al 2000, Vergilino et al 2011, Miner et al 2013), hybridization among them is frequent in the wild (Hebert et al 1988, Hebert et al 1993, Hebert and

Finston 2001), and experimental crosses do not exhibit reproductive isolation (Heier and Dudycha 2009). Therefore, they have limited divergence of their genomes, and can be used in complementary assays of genetic function. They were labeled as distinct species by molecular taxonomists based on mitochondrial divergence (Colbourne and Hebert 1996, Colbourne et al 1998), though they also have separate ecological niches, with *D. melanica* specialized for habitats with high UV radiation, and *D. pulex* and *D. pulicaria* specialized to small ponds and large lakes with low UV respectively (Benzie 2005, Dudycha 2004, Pfrender et al 2000, Miner et al 2013). *D. melanica* produces melanin as a protective pigment for the high amounts of UV-radiation in its natural habitat (Miner and Kerr 2011, Miner et al 2013). We used the melanin synthesis pathway of *D. melanica* to develop our RNAi technique, as it provides us with an easily measurable visible phenotype (loss of pigmentation) to assess the effectiveness of gene knock down. We used *D. pulex* as a comparison because it is the closest relative to *D. melanica* that does not exhibit pigmentation (Miner and Kerr 2011). In an additional test to determine the general applicability of our technique, we used *D. pulicaria* and examined the process of eye development.

We selected to target *phenoloxidase* gene, which encodes an enzyme essential for melanin synthesis in crustaceans, as a target for RNAi (Adachi et al 2005, Labbe and Little 2009). We also selected *eyeless*, which is a gene essential for normal eye development in numerous organisms including *Daphnia* (Nakanishi et al 2014, Naitou et al 2015) as it can also provide an easily identifiable phenotype if RNAi is successful. *Eyeless* encodes a homeobox transcription factor that is essential for normal eye

development in many organisms including *Drosophila*, mouse, and humans (Ton et al 1991, Quinn et al 1996, Clements et al 2009). *Eyeless* targeted by RNAi via microinjection of *Daphnia* embryos produced an eye deformity phenotype (Nakanishi et al 2014, Naitou et al 2015). Overall, we selected these two target genes based on the easily identifiable phenotypes they would produce if RNAi was successful.

We provide evidence for establishment of an easy, feeding-based RNAi method in *Daphnia*. We demonstrate reduced *phenoloxidase* mRNA levels, diminished melanin levels resulting in a dramatic phenotype, and reduced survival in response to UV radiation in *Daphnia* that are fed on bacteria that express a dsRNA specific for *phenoloxidase*. We also show that RNAi via feeding works systemically to target developing embryos in the bacteria fed mothers. This is the first demonstration that systemic RNAi is possible in *Daphnia*.

5.3 Methods

5.3.1 Daphnia Cultures.

Daphnia pulex and *Daphnia pulicaria* were isolated from waterbodies in southwest Michigan in 2008 and have since been cultured in the lab. *Daphnia melanica* were isolated from high altitude alpine lakes in the Sierra Nevada region in eastern California. The isolate used was known as “Sierra”, and ND5 mitochondrial gene sequencing confirmed the *D. melanica* species identity (Scoville and Pfrender 2010, Miner and Kerr 2011). *D. pulex* and *D. pulicaria* were maintained at a temperature of 20° C with a photoperiod of 12:12 light:dark in a Percival growth chamber. *D. melanica* were maintained at a temperature of 15° C with a photoperiod of 16:8 light:dark. All

Daphnia were maintained at a concentration of 3 to 5 animals per 250 ml beaker in 100 ml of filtered (1µm) lake water until experimentation. Young newborn *Daphnia* were transferred to a new beaker with fresh water on alternate days. *D. pulex* and *D. pulicaria* cultures were fed every day with vitamin-supplemented algae *Ankistrodesmus falcatus* at a concentration of 20,000 cells/ml. *D. melanica* cultures were fed 20,000 cells/ml of *Ankistrodesmus falcatus* on alternate days.

5.3.2. Vectors and Feeding System.

We selected the L4440 plasmid vector for generating dsRNAs in an inducible manner in *E. coli*. L4440 plasmid vector (Fig. 5.1B) was designed by Fire *et al.* (Fire et al 1998). This vector allows cloning of PCR products between two T7 promoters in opposite orientations. The following two bacterial strains harboring λDE3 lysogen (a source of T7 RNA polymerase) were used for generating dsRNA from the recombinant plasmids:

(1) BL21(DE3): ompT hsdS_B (r_B-m_B-) gal dcm (Novagen) - a strain deficient in lon and ompT proteases and used for efficient production of recombinant proteins.

(2) HT115 (DE3) (W3110, rnc14::DTn10 (Addgene, (Dasgupta et al 1998))- a strain deficient in RNase III and used for efficient production of dsRNAs.

E. coli cells that are (DE3) contain an integrated T7 RNA polymerase ORF under the control of a LacUV5 promoter the cultures can be induced with 2mM IPTG to produce T7 RNA polymerase. This leads to the production of dsRNA from the PCR product cloned between the two T7 promoters. We also used the plasmid construct L4417, which contains the 5' half (750 bp) of the GFP ORF cloned between the two T7

promoters (to be used as a negative control in the experiments as it does not have a natural target RNA in *Daphnia*). All the recombinant plasmids that we generated would produce a dsRNA product of about 800 bp in the bacterial host that expresses T7 RNA polymerase. Both L4440 and L4417 were obtained from Addgene (L4440 Plasmid #: 1654, L4417 Plasmid #1649- both gifts from Dr. A. Fire to Addgene).

5.3.3 Target genes and plasmid constructs.

In our current study, we targeted *phenoloxidase* and *eyeless* transcripts for degradation by RNAi. We generated several constructs using L4440 plasmid vector to generate dsRNA corresponding to different regions of our target transcripts (Fig. 5.1C and 5.1D). Note for each construct the PCR product was cloned into pGEMT Easy (Promega) and the sequence was confirmed. The following primers were used to generate the PCR products using either the genomic DNA or cDNA as a template. Appropriate restriction enzyme sites were engineered into the 5'ends of both PCR primers for sub-cloning from pGEMT Easy to L4440. Primers PO245 and GAPDH were used only in qPCR tests of gene expression levels.

P1: (gDNA template) Forward: CACCATGTCAGATTTGCAGC

Reverse: CGCAACATTTGCCTCTTACC

P2: (gDNA template) Forward: AATTCTTGCCGATCAAGGTG

Reverse: GCGAAATACGAACGAGGAAA

P3: (cDNA template) Forward: GCGTGGCAGGTTATTTTCAT

Reverse: CTTTAGAACGAGCCCAGACG

PO245 (qPCR): Forward: CCATTCAGTCCTAAACCGGA

	Reverse: ACCGTCGGAGCATTCTTAAA
<i>eyeless</i> :	Forward: AATTCGATGACGGGATTTCAG
(gDNA + cDNA templates)	Reverse: CTGGAACAGAACTCCAGGC
GFP:	Forward: GCCCGAAGGTTATGTACAGG
	Reverse: AAAGGGCAGATTGTGTGGAC
GAPDH:	Forward: TTATCACCTCCTCAACTTC
	Reverse: CTTCTTCCTTCACTTCTCC

The following abbreviations denote which targeting vector was in the bacteria administered as feed to *Daphnia*: EV: Empty Vector L4440, GFP: GFP Control (L4417), P1: 5' half of *phenoloxidase* gene amplified from genomic DNA, P2: 3' half of *phenoloxidase* gene amplified from genomic DNA, P3: internal region of *phenoloxidase* mRNA amplified from cDNA, NM: Nonmelanic *D. pulex* (Clone: RW20), served as a control.

5.3.4 RNAi feeding protocol.

Ten *Daphnia* selected for experimentation were placed in 100 ml of filtered lakewater in a 250 ml beaker. *E. coli* strain BL21(DE3) bacteria transformed with the plasmid of choice (L4440 with one of the inserts indicated in Fig. 5.1) were grown overnight in Luria Broth (LB) with 2 mM IPTG to induce the expression of T7 RNA polymerase and the dsRNA corresponding to the cloned PCR product. The OD₆₀₀ of the overnight cultures was measured, and bacteria from 2.8 OD₆₀₀ units of overnight culture were pelleted (usually about 1 ml). The pelleted bacteria were resuspended in 1 ml of filtered lake water and dispensed directly into the beakers, which contained *Daphnia*, thereby diluting 1 ml of resuspended bacteria in 100 ml of filtered lake water. This

corresponds to a final OD₆₀₀ of 0.028 or about 2.4×10^7 *E. coli* cells in 100 ml. This same procedure was repeated for ten days, with the *Daphnia* also being fed algae, *Ankistrodesmus falcatus*, at a concentration of 20,000 cells/ml each day for *D. pulex* and *D. pulicaria* and on alternate days for *D. melanica*. The water being changed every other day for *D. melanica* or every day for *D. pulex* and *D. pulicaria*. New bacterial culture in fresh LB was prepared for feeding on each feeding day, and fresh algae added after addition of bacteria to water, thus *Daphnia* were always fed with a mixture of algae and bacteria. During RNAi feeding regimen, the same photoperiods as stated under *Daphnia* cultures section were maintained for each species.

5.3.5 UV treatment of *D. melanica*.

For the experiments involving the knockdown of *phenoloxidase* in *D. melanica*, on the tenth and eleventh days of the bacterial feeding regimen, *Daphnia* were exposed to UV radiation by using a transilluminator with 312 nm UVB emission. *Daphnia* in 250 ml beakers (in 100 ml of water) were placed on the transilluminator. Beakers were arranged such that individual *Daphnia* were an average 10 cm from the UV source. *Daphnia* were exposed to UV for 5 minutes, and then returned to the Percival chamber. On the day 12 after beginning of bacterial feeding, the *Daphnia* were sacrificed and assayed for visual phenotypes and harvested to assay melanin content or isolate total RNA. This UV treatment allowed for a rigorous assessment of the RNAi targeting *phenoloxidase* via feeding method. *D. melanica*, if stressed, can stop production of melanin synthesis and this may result in false positive scoring of the loss of pigmentation phenotype since the presence of bacteria in water may induce some level

of stress. By exposing *Daphnia* to UV radiation, the synthesis of melanin can be induced, over-riding any probable down regulation due to stress.

5.3.6 *Eyeless RNAi Assessment.*

For experiments involving knockdown of the *eyeless* gene, *Daphnia* were fed as per the detailed RNAi feeding protocol above. Offspring of the *Daphnia* being fed bacteria were collected throughout the course of the experiment (RNAi feeding regimen that lasted 15 days) and analyzed, looking for any malformation of the eye associated with a knockdown of *eyeless*.

5.3.7 *Melanin Assay.*

The assay was performed as per Hebert and Emery (Hebert and Emery 1990). The body length of *Daphnia* to be analyzed via the Melanin Assay for their melanin content was measured and then *Daphnia* were placed in 50 µl of 5 M NaOH and incubated at 40°C for 4 days. The melanin content of the resulting solution was determined by measuring optical density at 420 nm with a plate reader. We performed a melanin standard curve using commercially available bovine melanin. By generating a standard curve, we were able to convert our OD₄₂₀ values into micrograms of melanin per millimeter of *Daphnia* (Scoville and Pfrender 2010, Hebert and Emery 1990). We also measured the melanin content of a nonmelanic *Daphnia* species as a negative control.

5.3.8 *UV sensitivity and survival assay.*

We tested *Daphnia*'s ability to survive UV exposure following the loss of pigmentation in response to feeding on bacteria expressing the *phenoloxidase* dsRNA.

Daphnia in control and treatment groups were fed bacteria as described above for 10 days. On the eleventh day, *Daphnia* were subjected to UV radiation (10 minutes of UV radiation on the transilluminator). We subjected the *Daphnia* a second time to this UV dosage on day 12 and on the thirteenth day examined the viability in control and treatment groups.

5.3.9 Reverse Transcriptase (RT)-PCR.

Total RNA was isolated using RNAzol B reagent (TelTest) from 6-10 *Daphnia* following a bacterial RNAi feeding regimen for 10 days. Prior to RNA isolation, for the *Daphnia* fed on bacteria expressing any of the described dsRNAs, the entire gut was removed from each individual to avoid contamination from the bacteria in the gut that contain the dsRNA. *Daphnia* were collected in a microcentrifuge tube, rinsed once with 1 ml of PBS, and were homogenized in 0.8 ml of RNAzol B. Total RNA was isolated as per the supplied protocol. cDNA was synthesized using random hexamer primers, 1 µg total RNA, 10-20 units M-MuLV reverse transcriptase, 500 µM dNTPs, 40 units RNase Inhibitor RNasin (Promega) in appropriate reaction buffer. For each PCR reaction, 2 µl (1/10th of total) cDNA was used with 50 pmoles each of the forward and reverse primers designed to amplify either *phenoloxidase* or *eyeless* PCR product using the Promega GoTaq PCR kit. The following conditions were used for PCR: 95^o C for 5 minutes (initial denaturation), denaturation at 95^o C for 30 seconds, annealing at 58^o C with *phenoloxidase*, 54^o C with *eyeless* products for 30 seconds, extension at 72^o C for 30 seconds for 27 cycles in order to stay within linear range of amplification. The linear

range was determined by varying cycle numbers and performing a densitometric analysis of the amplified product. PCR products were separated on a 1 % agarose gel.

5.3.10 Real Time PCR.

We first determined the efficiency of the real time PCR reactions with serial dilutions of all cDNAs. Every reaction was performed in triplicate in a total volume of 20 μ l. This included 4 μ l cDNA, 250nM *phenoloxidase* or GAPDH primers, and SensiFast Supermix (BioLine). GAPDH was used for normalization. *Phenoloxidase* and GFP primers were validated by running serial dilutions with a template of known quality and the efficiency of the reactions were determined to be greater than 98%. GAPDH primers were previously validated (Scoville and Pfreder 2010). All reactions were run on a BioRad CFX96 Real Time System C1000 Thermal cycler machine with the following conditions: 95⁰ C for 30 seconds, 95⁰ C for 5 seconds, 58⁰ C for 5 seconds for *phenoloxidase* and 54 C for 5 seconds for GFP (the last three steps repeated for 50 cycles), 65⁰ C for 5 seconds, and then 95⁰ C for 5 seconds. We analyzed our data using the Bio-Rad CFX Manager Software with the $2^{-\Delta\Delta C_t}$ method. Note that 3 independent RNA isolations were used from 3 independent groups of *Daphnia* to serve as biological replicates.

5.3.11 Statistics.

To determine statistical significance, a two tailed Student's T-test assuming equal variance or chi square analysis was performed. Each figure legend denotes p values as set forth by brackets and special characters. Note that our alpha level was $p=0.05$.

5.4 Results and Discussion

5.4.1 Selection of target.

In order to establish an RNAi method for *Daphnia* via feeding, we selected target genes that would result in easily identifiable visible phenotypes. A deficiency of phenoloxidase enzyme would result in a reduction of melanin pigment, thus producing a visible loss of pigmentation in *D. melanica*. As shown in figure 5.1A, we selected to target *phenoloxidase* gene based on its involvement in several essential steps in the melanin synthesis pathway (Battistella et al 2009, Labbe and Little 2009). In order to test dsRNAs corresponding to three different regions of *phenoloxidase* gene for their effectiveness, we used three PCR primer pairs to amplify the indicated regions for sub-cloning into the plasmid vector L4440. As is shown in figure 5.1B, two regions from the *phenoloxidase* gene (with primers binding in introns of the gene) corresponding to the 5' region (P1) and 3' region (P2) were selected for PCR amplification. Another primer pair was used with cDNA as a template (therefore no intronic regions would be present), which corresponds to a central region of the *phenoloxidase* transcript (named P3, Figure 5.1C). A different primer pair was used for real time PCR in order to measure changes in *phenoloxidase* transcript levels (termed PO245, Figure 5.1C) after RNAi feeding regimen. The 3' end of the developmental gene *eyeless* was also selected as a target region for producing dsRNA from plasmid vector L4440 as indicated in Figure 5.1D, denoted as E1 (containing intron) and E2 (3' region of *eyeless* transcript).

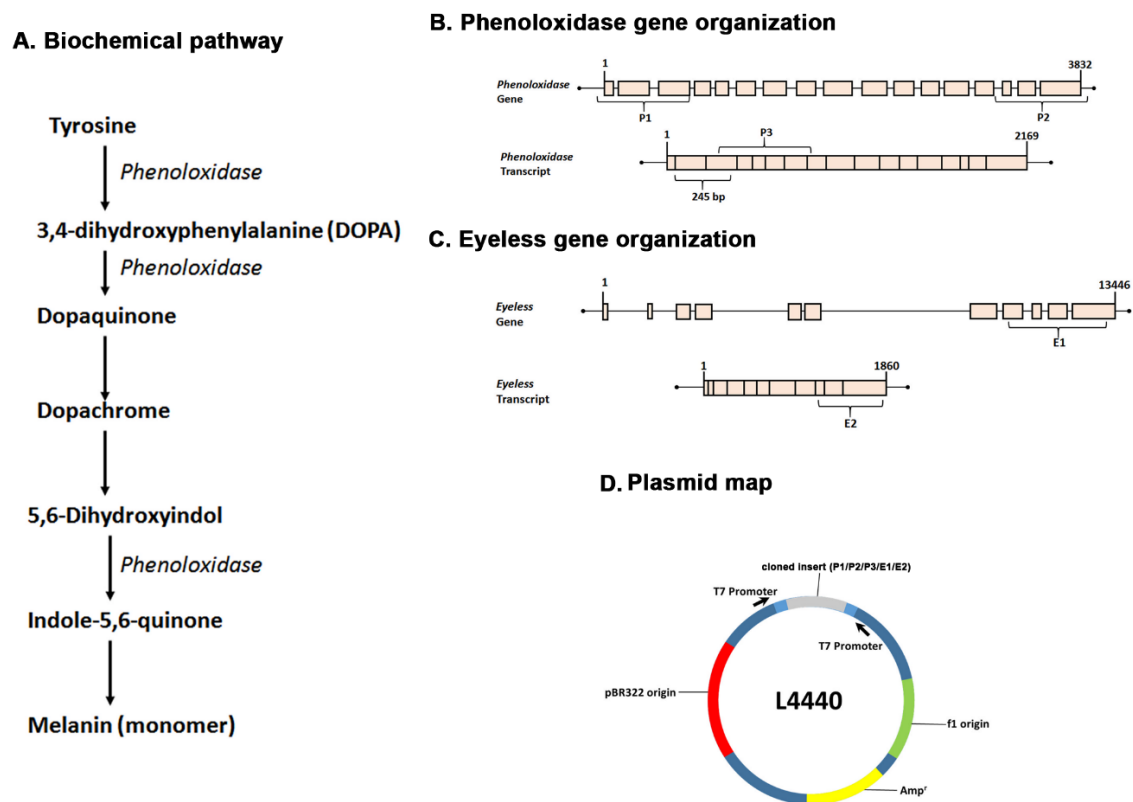
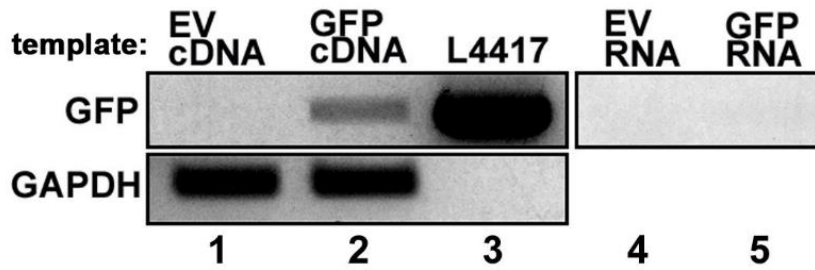


Figure 5.1. Arthropod Melanin Synthesis pathway and schematic map of regions selected for dsRNA expression constructs in *E. coli* plasmid L4440. A) Arthropod melanin synthesis pathway. B) *Daphnia phenoloxidase* gene and transcript. Marked on each diagram are the regions amplified using PCR and cloned in plasmid L4440. C) *Daphnia eyeless* gene and transcript. Marked on each diagram are the regions amplified using PCR and cloned in plasmid L4440. D) Schematic diagram of the L4440 plasmid vector. L4440 is designed to produce double stranded RNA of the cloned insert between the two T7 promoters.

5.4.2 GFP dsRNA can be detected in *Daphnia* after being fed on bacteria expressing GFP dsRNA.

First, we wanted to determine if the dsRNA expressed in bacteria was being effectively delivered to *Daphnia* after being fed on the bacterial suspension. To analyze this, we fed *D. melanica* bacteria expressing GFP dsRNA. The plasmid L4417 (GFP insert cloned in L4440) produces GFP dsRNA from two complementary RNA strands generated from two T7 promoters. Since GFP is not an endogenous *Daphnia* gene, the presence of GFP dsRNA in *Daphnia* would indicate that the dsRNA was delivered from bacteria to *Daphnia* gut and then to the rest of the body. To test this, RNA isolated from *Daphnia* fed on L4417 containing bacteria was subjected to reverse transcriptase PCR analysis. Before isolating total RNA for analysis, the guts of all *Daphnia* were removed carefully to ensure that RNA samples were not contaminated with bacteria from the feed. As is shown in Figure 5.2A, GFP dsRNA was detected in *Daphnia* fed on bacteria with L4417 plasmid (lane 2) but not in *Daphnia* fed on L4440 (EV, lane 1). L4417 plasmid DNA was used as a template positive control for PCR and generated the expected PCR product (lane 3). In order to ensure that there was no plasmid DNA contamination in the RNA preparation originating from bacteria in *Daphnia* guts, we performed PCR using the isolated RNA (and not cDNA) without the reverse transcriptase step as a template. As seen in lanes 4 and 5, no PCR product was generated in the absence of reverse transcriptase reaction thereby confirming that the PCR product was being generated only from GFP dsRNA (Note: all RNA was treated with DNase before being analyzed). Figure 5.2B shows a quantitative reverse transcriptase PCR (qRT-PCR) confirming our

A.



B.

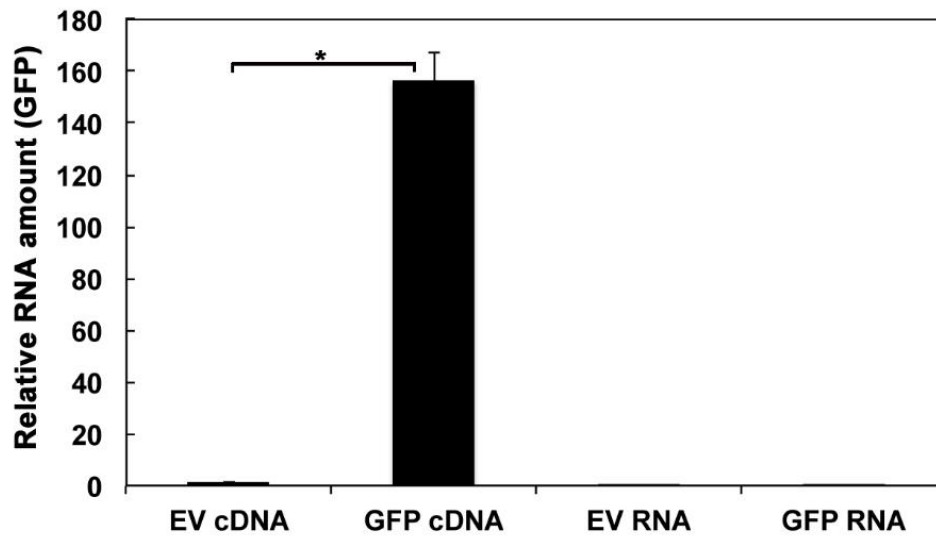


Figure 5.2. dsRNA generated in *E. coli* can be detected in *Daphnia*. A) Reverse transcriptase-PCR data. Lane 1: RNA isolated from *Daphnia* fed on bacteria containing L4440 without any insert (empty vector-EV), Lane 2: RNA isolated from *Daphnia* fed on bacteria containing GFP/L4440, Lane 3: GFP/L4440 plasmid DNA (template positive control), Lanes 4 and 5: reverse transcriptase negative control (the negative control without reverse transcriptase ensures lack of plasmid DNA contamination). GAPDH was used as an internal control for ascertaining that equal amounts of *Daphnia* mRNA were analyzed in lanes 1 and 2. B) Quantitative real time PCR data. Real Time PCR was performed with the same samples as in panel A. *P*-value: $\ast=5 \times 10^{-5}$.

results from Figure 5.2A. The L4417 template positive control was excluded from Fig. 5.2B because as expected it showed an extremely high level of amplification and PCR product. Thus we were able to detect non-endogenous GFP dsRNA in *Daphnia* fed on bacteria containing the plasmid L4417, demonstrating an effective delivery of dsRNA to *Daphnia* tissues other than gut. The results conclusively prove that GFP dsRNA is detected in *Daphnia* tissues other than the gut and indicates effective delivery of dsRNA.

5.4.3 Phenotypic change in *D. melanica* fed on bacteria expressing phenoloxidase dsRNA.

To investigate the effectiveness of RNAi via feeding method, we fed *D. melanica* on bacteria expressing the *phenoloxidase* dsRNA to target this essential enzyme in melanin biosynthesis pathway. After the RNAi feeding regimen, *Daphnia* were observed for loss of pigmentation and the percentages of *Daphnia* exhibiting loss of pigmentation were calculated. Demonstrated in Fig. 5.3D. *melanica* fed on bacteria expressing *phenoloxidase* dsRNA displayed a remarkable loss of pigmentation (Fig. 5.3A and B). This phenotype was not seen in *D. melanica* that were fed L4440 plasmid vector containing no inserts (EV) or a plasmid vector expressing GFP dsRNA (L4417). No visible effect of the *phenoloxidase* dsRNA produced in bacteria was observed in nonmelanic *Daphnia* (*Daphnia pulex*, Clone: RW20) when fed on dsRNA producing bacteria. As is shown in Fig. 5.3A and B, *D. melanica* with no treatments showed a slight change in phenotype (Black bars, Fig. 5.3A and B, 6.0 +/- 5.2%), which can be thought as natural variation in pigmentation.

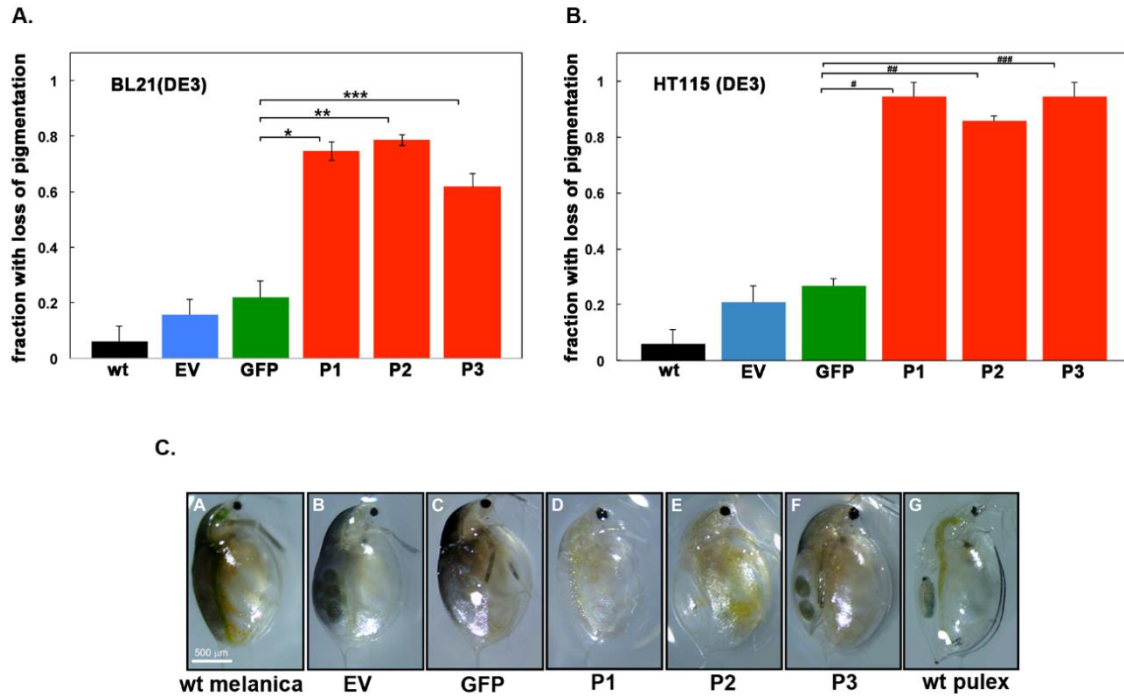


Figure 5.3. *Daphnia* fed on bacteria expressing the *phenoloxidase* dsRNA demonstrate a loss in melanin pigmentation. A and B) Proportion of *Daphnia* demonstrating pigmentation loss phenotype after 10 days on an RNAi feeding regimen with BL21(DE3) and HT115(DE3) strain respectively. Following the feeding regimen, *Daphnia* were UV treated to induce melanin production and then observed under a dissecting scope for pigmentation phenotype. wt (*melanica*): $n = 50$, EV (L4440) $n = 51$, GFP (L44417): $n = 50$, P1 (P1/L4440): $n = 55$, P2 (P2/L4440): $n = 56$, P3 (P3/L4440): $n = 57$. These abbreviations denote which plasmid was transformed into the bacteria before administering as feed to *Daphnia*. The data represent 5 replicate experiments. P -values are as follows: $*$ = 0.0005, $**$ = 0.0032, $***$ = 0.0002, $\#$ = 1.2×10^{-7} , $\##$ = 2.0×10^{-7} , and $\###$ = 0.00016. C) Photographic representation of *Daphnia* from each group as indicated below each panel. The non-melanic species *D. pulex* is shown for comparison.

D. melanica fed on bacteria with EV (blue bars) and GFP control constructs (green bars) showed a pigmentation loss phenotype in some individuals (15.6 +/- 5.9% and 22 +/- 5.4% respectively) in comparison to the group with no treatment. *D. melanica* can show reduced melanin synthesis if they become stressed (Tollrian and Heibl 2004). It is possible that the presence of any dsRNA (a short dsRNA may be produced from the polylinker in EV) could trigger stress signaling and elicit down regulation of melanin production and a minor but noticeable phenotype in a small percentage of individuals. However, *D. melanica* fed on bacteria expressing *phenoloxidase* dsRNA displayed a markedly high percentage of individuals with the pigmentation loss phenotype (Red bars, Fig. 5.3A and B) that was 4-6x greater than the non-target controls. In case of the BL21(DE3) bacterial strain, P1 displayed about 75% (n=55), P2 displayed 78% (n=56) and P3 was slightly more variable with about 62% (n=47) of *Daphnia* displaying the pigmentation loss phenotype (Fig. 5.3A). The bacterial strain HT115(DE3) that lacks the RNase III activity exhibited more efficient RNAi (Fig. 5.3B) as compared to BL21(DE3) strain (Fig. 5.3A) with P1 displaying about 94% (n=16), P2 about 86% (n=14) and P3 about 94% (n=16) of *Daphnia* with loss of pigmentation. Figure 5.3C shows a representative individual from each group of *Daphnia*; either untreated wild type, controls (fed on bacteria harboring EV L4440 or L4417) or fed on bacteria expressing *phenoloxidase* dsRNA (P1, P2, and P3). The dramatic loss of pigmentation is clearly evident in P1, P2, and P3 groups (panels D, E, and F) as compared to untreated and control samples (panels A, B, and C). Panel G shows a non-melanistic *D. pulex* that was fed only on algae for comparison. These results indicate that RNAi method is successful and

it is possible to achieve systemic RNAi via feeding in *Daphnia*. The bacterial strain HT115(DE3) was more effective in generating the RNAi phenotypes as expected since it is deficient in RNase III and is known to accumulate dsRNA at high levels compared to BL21(DE3) strain.

5.4.4 Phenoloxidase transcript levels are diminished in *Daphnia* fed on bacteria expressing Phenoloxidase dsRNA.

Since there was an obvious phenotypic change in *D. melanica* fed on bacteria expressing *phenoloxidase* dsRNA, we analyzed the *phenoloxidase* mRNA levels by reverse transcriptase (RT)-PCR (Fig. 5.4A) as well as quantitative Real Time (qRT)-PCR (Fig. 5.4B). As is shown in Fig. 5.4A, *phenoloxidase* mRNA levels are markedly diminished in *Daphnia* fed on bacteria expressing *phenoloxidase* dsRNA (lanes 3-5) as compared to controls (lanes 1-2). Comparing lanes 1 and 2, it appears that there is more *phenoloxidase* mRNA in the GFP fed *Daphnia*. The presence of dsRNA may elicit an immune response and phenoloxidase is also a key enzyme in responding to immune insults in *Daphnia* (Labbe and Little 2009). Nevertheless, all the *phenoloxidase* targeting constructs (including those with regions corresponding to genomic DNA) resulted in diminished levels of *phenoloxidase* mRNA (lanes 3-5). We also used untreated, non-melanic *D. pulex* (RW20) as a negative control for PCR (lane 6, Fig. 5.4A). Shown in Fig. 5.4A and B, *D. melanica* fed on bacteria expressing *phenoloxidase* dsRNA displayed levels of *phenoloxidase* mRNA similar to non-melanic *Daphnia* (RW20). Thus, when *Daphnia* are fed on bacteria expressing *phenoloxidase* dsRNA, the *phenoloxidase* mRNA levels decline significantly, thereby demonstrating achievement of very effective RNAi.

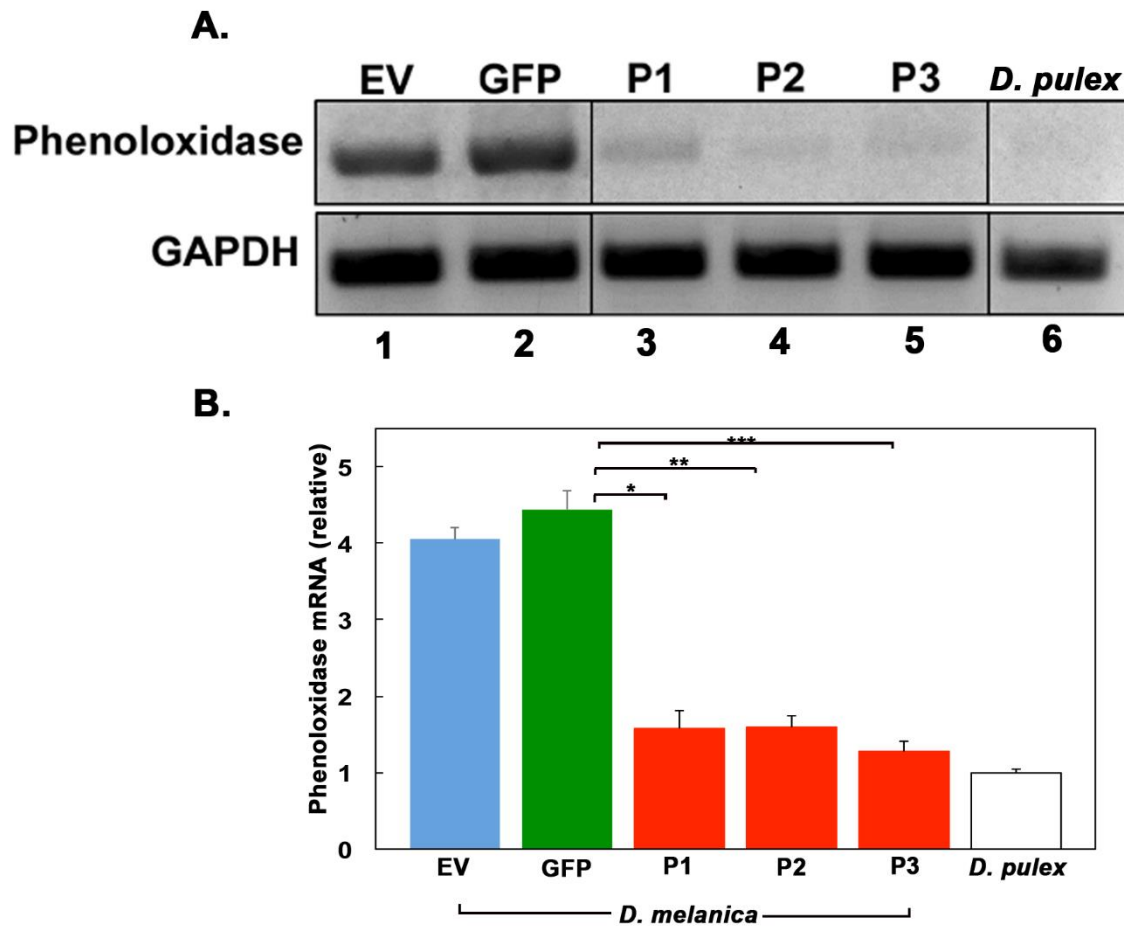
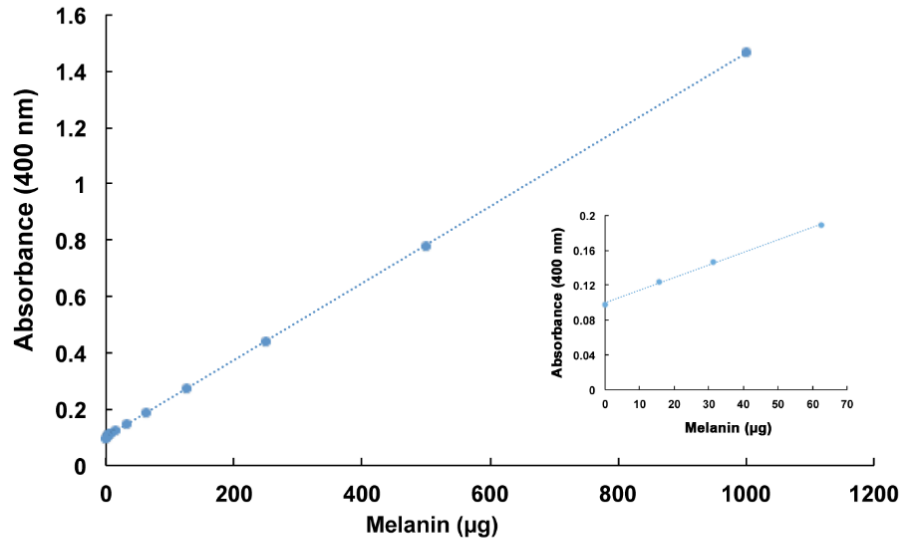


Figure 5.4. *Phenoloxidase* transcript levels are diminished in *Daphnia* fed on bacteria that express *phenoloxidase* dsRNA. A) Reverse Transcriptase PCR examining the expression of *phenoloxidase* mRNA. RNA samples are as follows- Lane 1: EV (L4440 without any insert), Lane 2: GFP (GFP/L4440), Lane 3: P1 (P1/L4440), Lanes 4: P2 (P2/L4440), Lane 5: P3 (P3/L4440), and Lane 6: non-melanic *D. pulex* (isolate RW20) control. GAPDH was used as an internal control for ascertaining that equal amounts of *Daphnia* mRNA were analyzed in each lane. B) Quantitative real time PCR examining the expression of *phenoloxidase* mRNA. The abbreviations under each bar denote which plasmid vector was transformed into the bacteria before administering as feed to *Daphnia*. *P*-values are as follows: $\ast = 1 \times 10^{-7}$, $\ast\ast = 7.2 \times 10^{-8}$, and $\ast\ast\ast = 9.8 \times 10^{-8}$. Blue bar: EV, Green bar: GFP, red bars: P1, P2, and P3, white bar: non-melanic *D. pulex* control.

5.4.5 Melanin levels are diminished in *Daphnia* fed on bacteria expressing phenoloxidase dsRNA.

We further validated that RNAi was achieved by measuring melanin content of *Daphnia* to ensure that the observed phenotypic changes were due to a decline in melanin. The melanin content was determined in *Daphnia* fed on control bacteria (Fig. 5.5B, EV and GFP) and *Daphnia* fed on bacteria expressing the *phenoloxidase* dsRNA (Fig. 5.5B, P1, P2, and P3). First we established a standard absorbance curve for various melanin concentrations using commercially available melanin (Fig. 5.5A). Using the standard curve, the melanin content of *Daphnia* in experimental groups was determined and is shown in Fig. 5.5B. Wild type *D. melanica* (black bar), and the control organisms (blue bar: EV, and green bar: GFP) contained significantly higher amounts of melanin ($32.49 \pm 1.27 \mu\text{g/mm}$, $29 \pm 2.5 \mu\text{g/mm}$, and $35.8 \pm 1.06 \mu\text{g/mm}$ respectively) than *Daphnia* fed on bacteria expressing *phenoloxidase* dsRNA (red bars: P1: $7.2 \pm 1.48 \mu\text{g/mm}$, P2: $5.81 \pm 1.5 \mu\text{g/mm}$, and P3: $8.21 \pm 2.16 \mu\text{g/mm}$ of melanin). We included nonmelanic *D. pulex* as a negative control (white bar: $7.147 \pm 0.75 \mu\text{g/mm}$ of melanin). Comparing P1, P2, and P3 to the non-melanic *Daphnia*, the amount of melanin present is very similar, showing that feeding on bacteria expressing *phenoloxidase* dsRNA resulted in a marked reduction in melanin levels to bring them to the levels in non-melanic *D. pulex* (Fig. 5.5B). (Note that there is some melanin in *D. pulex* because the *Daphnia* eye contains melanin). Thus, the diminished levels of *phenoloxidase* mRNA results in a corresponding decrease in melanin content in *Daphnia*.

A. Melanin Standard Curve



B. Melanin content of *Daphnia*

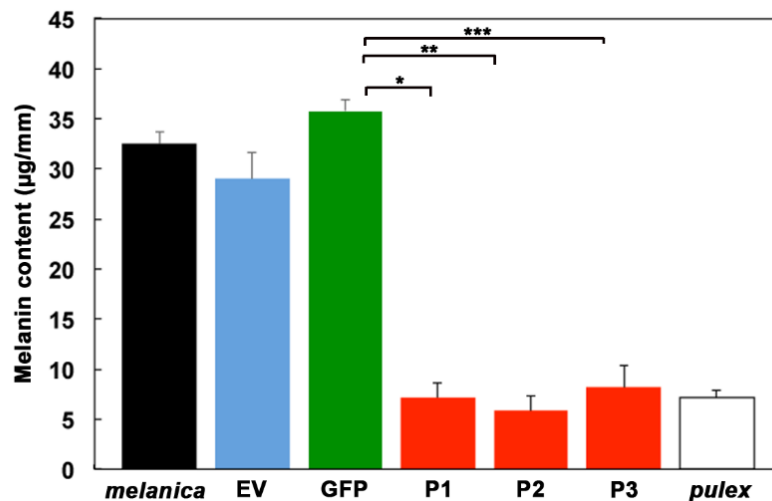


Figure 5.5. Melanin content is diminished in *D. melanica* fed on bacteria expressing *phenoloxidase* dsRNA. A) Melanin standard curve using purified melanin (Sigma). Melanin was dissolved in 5 M NaOH before making serial dilutions to establish a standard curve. B) Melanin Assay performed using extract from various treated *Daphnia*. wt *D. melanica* (*melanica*): $n = 14$, EV: $n = 15$, GFP (GFP/L4440): $n = 14$, P1 (P1/L4440): $n = 11$, P2 (P2/L4440): $n = 12$, P3 (P3/L4440): $n = 13$, *D. pulex* RW20 (*pulex*): $n = 20$. Data represents 2 replicate experiments. The abbreviations indicated below the bars denote the plasmid vector that was transformed into the bacteria before administering as feed to *Daphnia*, except for *pulex*, which indicates non-melanic control without bacterial feed. P - values are as follows: $*$ =0.001, $**$ =0.0002, and $***$ =0.0016.

5.4.6 *Daphnia* fed on bacteria expressing phenoloxidase dsRNA are sensitized to UV radiation.

To investigate the functional effect of melanin loss, *Daphnia* were exposed to UV radiation following the loss of pigmentation after an RNAi feeding regimen. Since melanin protects the DNA damage in response to UV radiation (Brenner and Hearing 2008), we predicted that the *Daphnia* exhibiting loss of pigmentation in response to RNAi knockdown of *phenoloxidase* would be more sensitive to UV. For *D. melanica*, melanin pigmentation in the wild helps them cope with large amounts of UV radiation they encounter daily (Miner and Kerr 2011, Miner et al 2013). Thus, *Daphnia* were treated with UV radiation and their viability was measured following UV exposure. As shown in Fig. 5.6, *Daphnia* that were fed on bacteria expressing *phenoloxidase* dsRNA displayed a dramatically increased sensitivity to UV with about 50% lethality (red bars: P1: 45.8 +/- 2.9 %, P2: 57.7 +/- 3.2%, and P3: 46.7 +/- 3.5% death). In contrast to this, wt *D. melanica* (black bar) and those fed on bacteria with EV (blue bar) or L4417 GFP control (green bar) showed markedly less lethality (EV: 18.1 +/- 1.4%, GFP: 16.7 +/- 3.92%). The non-melanic *D. pulex* was used as a negative control (white bar) and following UV radiation all *D. pulex* individuals died within 2 hours (n=43). These results further confirm that RNAi targeting of *phenoloxidase* worked efficiently in *Daphnia* and exhibited markedly reduced *phenoloxidase* mRNA, melanin content, and markedly increased UV sensitivity. Thus, the knockdown of *phenoloxidase* transcript in *D. melanica* results in a significant reduction in the ability to survive after UV exposure, further validating our RNAi method.

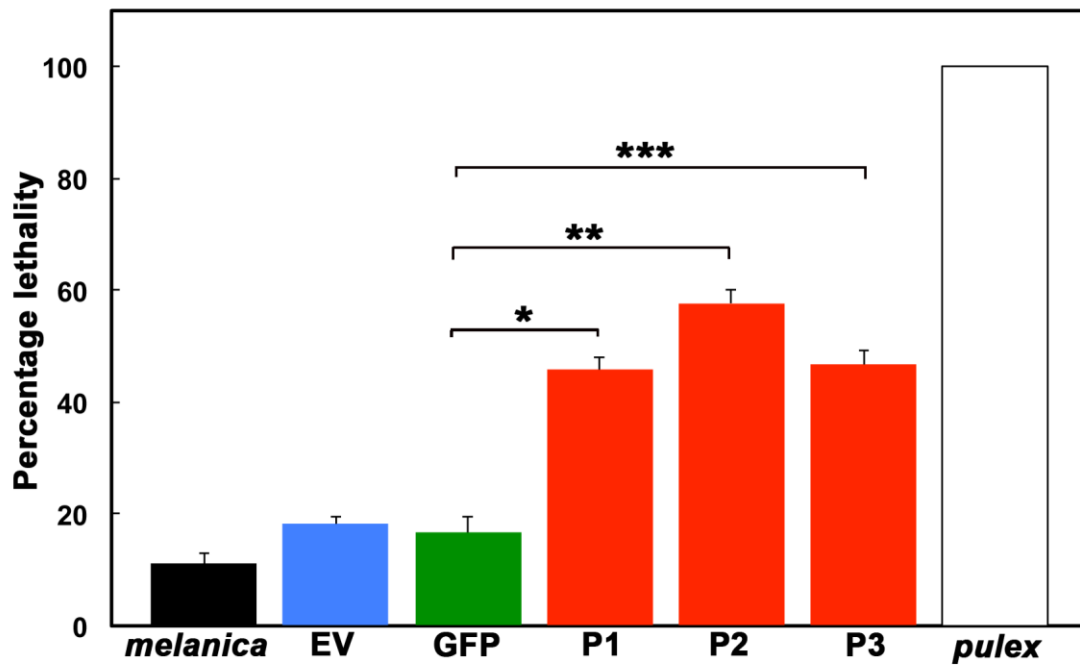


Figure 5.6. Enhanced UV sensitivity of *D. melanica* fed on bacteria expressing phenoloxidase dsRNA. *D. melanica* were subjected to the RNAi feeding regimen for 10 days and then exposed to UV each day for 2 consecutive days. The percent viability of *Daphnia* was assessed 24h after the second exposure. *D. pulex* was used as a control and all individuals died within 2 hours of the initial UV insult. wt *melanica* (*melanica*): $n = 18$, EV: $n = 22$, GFP (GFP/L4440): $n = 24$, P1 (P1/L4440): $n = 24$, P2 (P2/L4440): $n = 26$, P3 (P3/L4440): $n = 30$, *D. pulex* RW20 (*pulex*): $n = 43$. These abbreviations denote the plasmid that was transformed into the bacteria before administering them as feed to *Daphnia*. Data represent 3 replicate experiments. P -values are as follows: $\ast=0.0293$, $\ast\ast=0.0028$, and $\ast\ast\ast=0.0201$.

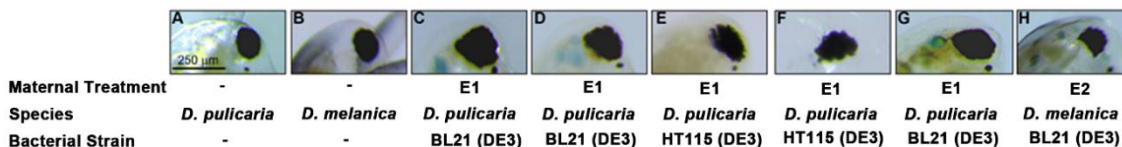
5.4.7 Progeny of Daphnia mothers fed on bacteria expressing eyeless dsRNA exhibit deformed eye phenotype.

To assess the general applicability of the RNAi method, we further tested the method by targeting the developmental gene *eyeless* (Ton et al 1991, Quinn et al 1996, Clements et al 2009, Nakaniski et al 2014). We fed adult *D. melanica* and *D. pulicaria* mothers bacteria expressing the *eyeless* dsRNA and analyzed the offspring of these adults for any eye deformity. As is shown in Fig. 5.7A, deformed eye phenotypes were displayed in offspring of *Daphnia* fed on bacteria expressing *eyeless* dsRNA with eyes that were misshapen or not fully formed. Among the total offspring analyzed, about 23-25% displayed a deformed eye phenotype (Fig. 5.7B) with the BL21(DE3) bacterial strain and about 66% showed deformed eyes with the HT115(DE3) bacterial strain, which indicates a much more effective method for RNAi than previously reported embryo microinjections. The progeny that did not exhibit eye deformity were normal and lacked of any observable phenotypic change. Although both BL21(DE3) and HT115(DE3) strains work in *Daphnia* to produce RNAi phenotypes, the HT115(DE3) strain is more effective and we recommend using this strain for future experiments.

5.4.8 Delivery of dsRNA by feeding is a convenient and reliable RNAi method.

In order to knock down specific gene expression, several methods for the delivery of dsRNA or siRNA have been used in recent years. Among these, RNAi via direct injection of a dsRNA solution is a simple method that works in larger insects due to the easy protocol. It is effective in knocking down expression of target genes in invertebrates such as the cricket *G. bimaculatus*, the mosquito *Aedes aegypti* (Drake et

A. Deformed eye phenotypes



B. Percentages of affected Daphnia

Phenotype	<i>D. pulicaria</i> Fed E1	<i>D. melanica</i> Fed E1	<i>D. melanica</i> Fed E2	<i>D. pulicaria</i> Fed E1
Deformed Eye	25% +/- 7%	23% +/- 6%	25% +/- 4%	66% +/- 11%
Bacterial Strain	BL21 (DE3)	BL21 (DE3)	BL21 (DE3)	HT115 (DE3)

Figure 5.7. RNAi is effective in developing embryos in mothers fed on bacteria expressing *eyeless* dsRNA. *Daphnia* were fed bacteria expressing *eyeless* dsRNA. A) Photographic representation of the eye deformities. B) Percentage of offspring with normal or deformed eye phenotypes. Offspring of 20 *Daphnia* mothers fed on bacteria (*D. pulicaria* E1 (E1/L4440), *D. melanica* E1 (E1/L4440), and *D. melanica* E2 (E2/L4440) expressing *eyeless* dsRNA. Total *D. pulicaria* offspring analyzed: $n = 90$ (E1), Total *D. melanica* offspring analyzed: E1: $n = 27$; E2: $n = 16$. For HT115(DE3), experiments contained 50 adults ($n = 50$) and total 280 offspring were assayed. Data represented are from 2 replicate experiments.

al 2012), the German cockroach *Blattella germanica* (Martin et al 2006), and the silkworm larvae *Bombyx mori* (Liu et al 2013). For a small freshwater microcrustacean such as *Daphnia*, it is difficult to achieve RNAi via injection in adult organisms as injection may result in high mortality since *Daphnia* will lose viability rapidly if not kept immersed in cool water. Thus, delivering dsRNA by feeding offers several advantages over direct injection of dsRNA method, as it is less labor-intensive, less expensive, and is also applicable for screening a large number of essential *Daphnia* genes because of its simplicity. Our study also suggests for the first time that RNAi in *Daphnia* is systemic, and thus has great potential for using this approach for genes involved in early embryonic development by feeding the mothers. Targeting the *eyeless* gene by RNAi via feeding produced more efficient knockdown than the previously reported microinjection method, as well as demonstrating that RNAi in embryos can be achieved by feeding appropriate bacteria to mothers. This is the first report of *phenoloxidase* knock down using RNAi and it not only produced significant reduction in mRNA and melanin levels but it also resulted in a marked increase in lethality in response to UV exposure.

It is worth noting that melanin synthesis was restored in *D. melanica* at about 7 days after bacterial feeding was discontinued, thereby demonstrating that the RNAi was transient and thus this method holds a potential to test the effect of gene knockdown at selected time points during the life span. This may be a very useful feature, especially for aging and longevity research to study the contribution of specific genes in an age-dependent manner. As there was no apparent effect of RNAi bacterial feeding regimen

on reproduction in any species we examined, the technique would be widely applicable to most genes. In this regard, it is worth noting that we attempted a knock down of *distal-less* by the maternal feeding method (similar to *eyeless*). This produced no viable progeny, which could be a sign of embryonic lethality specifically due to achieving efficient RNAi for *distal-less*. *Drosophila distal-less* null mutants die as embryos due to defects in development of sensory organs (Panganiban 2000). Thus, we present a fast and effective method to achieve gene-specific knockdown in adult organisms as well as developing *Daphnia* embryos that holds a tremendous potential to become a mainstream method in various types of biological studies that use *Daphnia* as a model organism.

5.4.9 Conclusions.

We describe a new method to achieve gene specific knockdown by RNAi in *Daphnia* via feeding. By using *E. coli* cells that express gene-specific dsRNAs as a food additive for adult *Daphnia*, we can achieve an efficient RNAi for genes that are expressed in adult tissues as well as in developing embryos. This method provides a powerful tool for genetic manipulation of this important model organism for environmental, evolutionary, as well as developmental genomics.

Chapter 6:

Conclusion

6.1 Conclusion

Aging is a convoluted molecular process that is influenced by numerous molecular mechanisms. In this dissertation, we have presented work establishing *Daphnia* as a potential model organism in the field of research on aging. In **Chapter 2**, we examined the telomere length and telomerase activity in the two ecotypes *Daphnia pulex* and *Daphnia pulicaria* (Schumpert et al 2015). Our findings, that *D. pulex* maintains telomere length throughout lifespan as well as high telomerase activity and processivity while *D. pulicaria* displays a constant decline in telomere length, telomerase activity, and processivity with age suggests that mechanisms other than telomere erosion are affecting the extremely short lifespan of *D. pulex*. In **Chapter 3**, we examined the heat shock response of several different clones of *D. pulex* and *D. pulicaria*, mainly by examination of heat shock protein 70 (Hsp70) protein levels in response to heat shock at multiple ages of the short and long lived ecotypes (Schumpert et al 2014). We found that the short lived *D. pulex* stops responding to heat shock by the mid-point of their life while *D. pulicaria* can still respond effectively by inducing the expression of Hsp70 in response to heat shock. We further investigated the potential cause of this decline in the heat shock response and found the transcription factor HSF loses its ability to bind DNA in old *D. pulicaria*. Thus, in **Chapter 4**, we investigated the role of NAD⁺-dependent deacetylase Sir2 which targets HSF for deacetylation. We found that in *D. pulicaria*, Sir2 activity levels decline with age which is a potential mechanism for the decline in HSF's ability to bind DNA in aged organisms. We also presented a new method in **Chapter 5** for an effective RNAi via feeding method in which transient gene

knockdown can be easily achieved in adult *Daphnia* by feeding them bacteria expressing dsRNA against a gene of interest. In **Chapter 4** we successfully utilized our newly developed method for RNAi via feeding to knock down the expression of Sir2 in *D. pulicaria* to demonstrate a significant decrease in lifespan, and an increase in mortality following heat shock.

We have provided several connected studies examining the longevity of *D. pulex* and *D. pulicaria* and their use as a model system in aging research (Schumpert et al 2014, Murthy and Ram 2014, Schumpert et al 2015). *Daphnia* are currently extensively used in various fields including population genetics, ecology, and ecotoxicology (Benzie 2005); however, *Daphnia* are poised to make an impact on the aging field particularly with an emphasis on natural genetic determinants of the aging process. In humans, although environmental factors are the main determinants of variations in life span, it's estimated that polygenic traits contribute up to 25-30% (Deleen et al 2013). Due to *Daphnia's* unique reproductive strategy of cyclic parthenogenesis, we are able to establish large populations of isogenic individuals (Benzie 2005). From the field, different *Daphnia* isolates from unique geographic locations are thus established as clones in the lab and propagated clonally for each ecotype that we utilized in our studies. Therefore, we are able to compare the genetic differences between the two ecotypes as well as among the various clones within each ecotype with respect to the variation in their median life span. Each clone exhibits a characteristic lifespan and within each ecotype, there are relatively short and long lived clones (Dudycha and Tessier 1999, Dudycha 2001, 2004). It's also important to note that these clones are

naturally occurring variants with differences in lifespan, not artificial clones developed in a laboratory via mutagenesis. The natural history and ecology of *Daphnia* is also well known and studied extensively; hence, a connection between the environmental and genetic aspects of aging could be studied effectively using *Daphnia* (Dudycha and Tessier 1999, Dudycha 2001, 2004). Genetic studies using *Daphnia* may lead to the discovery of specific genetic differences between long lived and short lived clones within each ecotype and between ecotypes in general that could then potentially be expanded to other model organisms and even humans.

The availability of a new RNA interference system for *Daphnia*, that allows for the knock down of a selected gene at specific stages of development (from embryo to juvenile and adult *Daphnia*) also allows for a more comprehensive study of longevity genes in *Daphnia*. This method allows for the knockdown of specific genes whenever the bacterial RNAi feed is administered, allowing for analysis of the effect of gene knockdown at different developmental times or ages during the life span (For example, if we wanted to examine the effects of diminished Sir2 only during the juvenile stages of *Daphnia*, we could easily perform that experiment using our RNAi via feeding system). The RNAi method that we have developed for gene knockdown will also be extremely useful for *Daphnia* biologists all over the world with interests ranging from populations genetics to developmental processes of vision and beyond. Coupling our new RNAi method with recent advances in creating transgenic *Daphnia* using TALEN and CRISPR/Cas9 technologies, *Daphnia* are set to emerge as a model organism into not only

the biology of aging field, but also for other fields invested in mechanistic research (Hiruta et al 2014, Nakanishi et al 2014).

One of the original strengths of *Daphnia* as a model system was in ecotoxicology due to their sensitivity to various compounds (Benzie 2005). Due to this characteristic, *Daphnia* would also be an ideal model to use for the effects of novel compounds on lifespan. High throughput screenings methods can be designed relatively easily to determine the effects of novel compounds and could be administered at different specific ages or over the entire course of their lifespan. The short life span of *D. pulex* makes this organism best suited to be the initial test subject for such potential lifespan extending compounds. The two ecotypes could also be utilized to determine any differences in effect of the identified compounds on the short- versus long-lived ecotypes, which can potentially be investigated further at mechanistic levels to understand the effects of genetic variations on life span extension by such compounds. Following identification of potential compounds that extend lifespan of *Daphnia* offers a possibility for a therapeutic intervention for healthy aging in humans.

Overall, aging is a complicated process that will take a lot more research to fully understand the interconnected pathways and mechanisms underlying the collective biological decline known as aging. With a molecular toolbox that is being further developed at present and isogenic populations that will help pinpoint genetic aspects of the aging process, *Daphnia* are anticipated to make a big impact in the research on aging. This small freshwater crustacean provides a valuable model system, with the most homology to the human genome among all other short-lived invertebrates. In the

end, aging is a process we have all become too familiar with and through continued study, we may one day be able to significantly extend the healthy lifespan of humans.

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