LIFE ON THE EDGE: A STUDY OF CRYOBIOSIS IN THE TARDIGRADA

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OF

CRYOBIOSIS IN THE TARDIGRADA

By

ALEXANDRA PONTEFRACT, B.Sc.

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AUTHOR: Alexandra Pontefract, B.Sc. (McMaster University)

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Abstract

The search for life on other planets has brought with it a renewed interest in the study of extremophiles as it relates to cold-tolerance. The means for the elucidation of these studies has been through the use of analog-sites in the polar regions of Earth as approximations of extra-planetary environments. These extreme environments are typified by low annual temperatures, low levels of available liquid water, food and light. Despite these conditions, however, life prevails. Perhaps one of the most "extreme" organisms residing in these environments are tardigrades. These micrometazoans are capable of withstanding temperature extremes from 150°C to -276°C, pressure, X-ray radiation, dessication and salinity. Of relevance to this thesis is the ability of the tardigrade to withstand extreme low temperature, which they do by entering a cryobiotic, or latent state. Cryobiosis is seen as an extreme form of cold-tolerance, but apparently lacking a lower lethal temperature (LLT). Despite the incredible advantages that this strategy confers, cryobiosis remains poorly understood. This study provides a review of the literature on freeze tolerance and cryobiosis to bridge the two spheres of research, as well as clarifying the nomenclature used in these papers. Particular attention is paid to the terms of cryoprotective dehydration and cryobiosis, proposing that cryoprotective dehydration be thought of instead as a process leading to the latent state. Experiments were conducted to explore the relationship that time and temperature have on cryobiotic capability in the tardigrade *Macrobiotus harmsworthii*. Results showed that both time and temperature played a significant role above -80°C, with poor survivability at -20°C. At -80°C, however, time no longer appeared to play a role in viability.

Preface

Three years ago, I traveled to Ward Hunt Island, Nunavut. This 5 km long landmass is situated off the northern coast of Ellesmere Island, and is the northern-most point of land in Canada. It was here that an intense interest in psychrophiles and polar environments was catalyzed, including an interest in analog environments. It was then that I witnessed first-hand the rapid changes that are occurring in the high-Arctic, and had my first view of the giant crack in the Ward Hunt Ice Shelf that would later detach in July of this year. With the calving of the Ward Hunt Ice Shelf, and most recently, the complete detachment of the Markham Ice Shelf from Ellesmere Island, field sites visited in 2005 and again in 2007, are now gone. March of 2009 will mark the end of the International Polar Year, a scientific program that has brought unprecedented focus to the Polar Regions, providing funding for researchers around the globe. It is hoped that the end of this program will not be an end to polar research, and that this thesis will help to encourage further research in a place we are only just beginning to understand, before it is no longer possible.

I would like to thank the Origins Institute, Warwick Vincent, Ralph Pudritz, Ben Evans, Grant McClelland, Pat Chow-Fraser, Ralph Schill, Brent Sinclair, Andrea Morash, Maria Abou-Chakra and Dave Anderson for all of their help and support. I would also like to thank my Biology ladies, as well as Lindsay Baxter, Hannah Elias, Mikhail Inozemtsev and my parents, who kept me sane and kept me on track.

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Table of Contents

Abstract.........iii

Preface.........iv

Table of Contents.........v

List of Figures.........vi

List of Tables.........vii

Introduction.........1

Glossary of Terms.........4

1. Frozen Worlds: A review of the physiological mechanisms of freezing in invertebrates.........8

The Physics of Freezing.........10

Freeze Avoidance Strategies.........13

Freeze Avoidance.........15

Freeze Tolerance.........18

Cryobiosis.........24

Re-evaluation of Terms.........30

2. Some like it cold: a study of cryobiosis in the eutardigrade *Macrobiotus harmsworthii* (Preceded by preface).........36

Introduction.........36

Materials and Methods.........39

Results.........41

Discussion.........44

Conclusion.........51

Appendix.........54

List of Figures

Chapter 1

Figure 1: Diagrammatic representation detailing the strategies and processes of employed by organisms when encountering freezing conditions........................11

Figure 2: Relationship between the supercooling point and the lower lethal limit....16

Chapter 2

Figure 1: Ventral and side views of *Macrobiotus harmsworthii*..........................39

Figure 2a: Diagrammatic representation of the statistical 3-way Anova log-linear model..44

Figure 2b: Diagrammatic representation of the statistical 3-way Anova log-linear model with the above-zero temperature set removed......................................45

List of Tables

Chapter 2

Table 1: Data from freeze/thaw study showing survivorship at four different temperatures with three different time periods..........42

Introduction

The question 'does life exist on other planets?' has plagued humans for millennia, but it is only in recent decades that technology has provided a biological perspective for the planets in our solar system. With each investigation, it becomes clearer that if life does exist elsewhere, then it does so at very low temperatures. On Mars, for example, the atmospheric pressure is so low that liquid water is unstable on the surface. Nevertheless, hydro-geological evidence from the Mars Global Surveyor indicates that water may still flow beneath the Martian surface and in possible brine veins below the polar ice caps (Rothschild & Mancinelli 2001). Europa is thought to harbour a subsurface water ocean beneath a layer of ice that is too thick to allow photosynthesis to occur (Rothschild & Mancinelli 2001). However, some researchers hypothesize that disequilibrium chemistry occurring in the ice, "driven by charged particles accelerated in Jupiter's magnetosphere, could produce sufficient organic and oxidant molecules for an Europan biosphere" (Rothschild & Mancinelli 2001). Given this knowledge, it is of paramount importance to begin to understand the limits at which life exists and the strategies that life employs to reside therein – with a specific emphasis on cold adaptation.

Tardigrades may be considered as a prime example of an organism adapted to withstanding freezing conditions. Sister taxa to the Onychophora (Gabriel *et al.*.. 2007), the Tardigrada are multi-cellular, micro-invertebrates, typically 500μm in size (Kinchin 1997), which possess no form of internal

thermoregulation (Lozina-Lozinskii 1974). As these organisms enjoy a global distribution, there are several species that are endemic to the Polar Regions, frequently residing directly on the ice in the cryoconite holes of ice-shelves. These holes are open for only a short period each year and can be subject to diurnal freezing. As a result, tardigrades find themselves in a rather tenuous position. The first and most immediate problem is that of injuries sustained as a result of freezing. As ice-crystals form externally and internally, sharp edges pierce cuticle and membrane alike, resulting in death (Cavicchioli *et al.*. 2000). At the molecular level, the helical and super coiled strands of DNA become too stable for RNA polymerase to attach to, thus inhibiting transcription. Furthermore, low temperatures support unfavourable secondary RNA structures, which interfere with the processes of translation. At the cellular level, membrane fluidity decreases at lower temperatures, affecting membrane-associated cellular functions (Phadtare *et al.* 1999). Accompanying these is a lowering of enzymatic activity as well as transport processes (Cavicchioli *et al.* 2000).

Despite such adverse conditions, these obligate thermoconformers have developed several strategies for dealing with the problems associated with freezing (Wright 2001). This is accomplished either by avoiding extreme conditions altogether or, when avoidance is not a viable option, through the evolution of specific adaptations that control metabolic activity and freezing within the body (Danks 2006, Elster & Benson 2004, Leather *et al.* 1993, Lozina-Lozinskii 1974, Sinclair *et al.* 2003, Sømme 1999). It is these adaptations that are

the focus of the first chapter of this thesis. In answering the question: 'what are the mechanisms available to organisms facing freezing conditions?' the first chapter provides a review of freeze avoidance and freeze tolerance as well as an in-depth discussion of the metabolic state known as cryobiosis and how it relates to tardigrades. A form of cryptobiosis (cryptic life), cryobiosis is defined as a complete cessation of metabolic activity brought on by sufficiently low temperatures (Crowe 1974, Kinchin 1997, Rebecchi *et al.* 2006). In this state, tardigrades are capable of remaining dormant for an indefinite period of time at temperatures down to absolute zero (Ramløv & Westh 1992, Rothschild & Mancinelli 2001), and it is this incredible ability to survive such extreme conditions that is the focus of this thesis. Cryobiosis is continually under-represented and misrepresented in the literature, and this chapter serves to provide an accurate representation of the process as well as a thorough explanation of freeze tolerance and freeze avoidance mechanisms, which is essential to the understanding of cryobiosis.

After establishing the mechanisms involved in freeze adaptations as well as the nuances in terminology found throughout the related literature in the first chapter, the discussion of cryobiosis shifts from the theoretical to the experimental. Very little is understood about the evolution of this ametabolic state, the mechanisms by which it occurs, as well as the strength of adaptation between species inhabiting different climates (Wright 2001). In the second chapter, these topics are explored through an experiment examining the ability of

local temperate tardigrades to enter cryobiosis, withstanding varying sub-zero temperatures for different lengths of time in an effort to answer the question: what are the boundaries of cryobiosis? If time plays no role in survival through cryobiosis, then the implications may be far-reaching. On our own planet, the capabilities afforded by cryobiosis may serve as proof that life could have survived through extended periods of glaciation reaching millions of years, such as during the posited "snowball earth" events (Vincent *et al.* 2004). In addition to implications for our own planet, however, the ability of organisms to survive for prolonged periods of time in freezing conditions provides further argument for the presence of life elsewhere in our solar system.

Following the dictum that "basic biological principles are most easily elucidated by examining living systems found on the edges of adaptive ranges" (Crowe 1974), it is the intention of this study to convey the ability of life to survive in the most extreme conditions and the idea that cryobiosis may serve as a model for the survival of life on other planets.

Glossary of Terms

Cold hardening: *collectively, the strategies of freeze-tolerance and freeze-avoidance.*

Cryptobiosis: *a reversible suspension of metabolism resulting from adverse changes in one or more environmental factors, resulting in a loss of the liquid water state (Kinchin 1997, Wright 2001).*

Cryobiosis: *cold-induced cryptobiosis, viewed as an extreme form of freeze-tolerance characterized by the lack of a lower lethal limit (Wright 2001).*

Cryoprotective dehydration: *the process wherein organisms possessing low dessication resistance will dehydrate when exposed to frozen material (ie. soil), due to the lower vapour pressure of ice (Sinclair et al. 2003).*

Cryoprotectants: *low molecular weight alcohols and sugars, which act to lower the melting point of a given substance as a function of increased osmolarity (Storey & Storey 1992).*

Gel-phase transitions: *transitions which may occur in lipid bilayerst, where changes in osmolarity and temperature can result in a phase change of the bilayer from a liquid phase to a gel phase (Wright 2001).*

Diapause: *a hormonal, predictive, lowering of metabolism brought on by changing photoperiod and/or temperature (Leather et al. 1993).*

Freeze dehydration: *see cryoprotective dehydration.*

Freeze avoidance: *strategy employed by organisms incapable of withstanding internal freezing; thermal hysteresis proteins as well as polyols and sugars lower the melting point of cellular fluid, allowing the organism to supercool (Storey & Storey 1992).*

Freeze tolerance: *organisms employing this strategy are capable of surviving the formation of ice in their bodily tissues and employ ice nucleating proteins in to promote controlled ice crystal growth (Storey & Storey 1992).*

Heterogeneous nucleation: *ice formation caused by introduction by a foreign particle, which will disrupt the water molecules and result in crystallization at temperatures below freezing (Smith 1961).*

Homogeneous nucleation: *ice formation in a pure substance: ice nucleation may occur spontaneously in a supercooled liquid because water molecules have a tendency to arrange themselves in tetrahedral formations which provide a surface for further nucleation (Smith 1961).*

Ice nucleating proteins: *proteins that provide a surface for ice nucleation by lowering the energy required for the formation of a crystal (Crowe et al. 2004).*

Latent states: *a state characterized by "reductions in metabolic activities, temporary cessations of growth and reproduction, and often by much enhanced resistance to environmental extremes" (Crowe 1974).*

Lower lethal limit: *defined as the observed limits of cold-tolerance (Sinclair 1999).*

Membrane phase-transitions: *lipid membranes are usually quite mobile in their liquid crystal state, with disordered hydrocarbon chains; at lower temperatures, however, membranes undergo a phase change into a crystalline state, where lipid tails are highly ordered (Diwan 2007).*

Quiescence: *the response of an organism to "a sudden unanticipated, non-cyclic and usually short duration deviation of normal weather conditions" (Leather et al. 1993).*

Supercooling: *a condition wherein a liquid undergoes thermal hysteresis, yeilding a freezing point that differs from its melting point (Leather et al. 1993).*

Thermal hysteresis proteins: *proteins that are capable of lowering the freezing point of the liquid in which they are in, creating a hysteresis and effectively supercooling the liquid; also known as antifreeze proteins (Storey & Storey 1992).*

Vitrification: *the process of conversion of a liquid to a glass; cooling causes the molecular motions of a liquid to slow and eventually stop, resulting in extreme viscosity, characterized as a solidified, amorphous liquid state (Taylor et al. 2004).*

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Frozen Worlds: A review of the physiological mechanisms of freezing in invertebrates

Alexandra Pontefract

Department of Biology, McMaster University, Hamilton, Ontario, L8S 3M1 Canada
Email: pontefaj@mcmaster.ca

Abstract: The search for life on other planets has brought with it a renewed interest in the study of 'psychrophily' and cold-hardy organisms. The literature available on this subject is quite extensive, though the focus has centered mainly on freeze-avoidance and freeze-tolerance in insects. Existing independently from this is a body of work devoted to the study of cryobiosis as it pertains to tardigrades. Tardigrades, microscopic invertebrates related to the Nematoda, are only now beginning to enter the spotlight as ideal organisms for space research, due to their unique ability to withstand extreme radiation, dessication, temperature and the vacuum of space. Though extensive studies are available detailing cold hardening and cryobiosis, scant literature is available describing the processes in relation to each other. As a result, a discussion of the cryobiotic state is generally absent in reviews describing freeze-avoidance and freeze-tolerance strategies and processes such as cryoprotective dehydration. This paper seeks to provide a thorough review of these strategies and processes in relation to cryobiosis, in an attempt to amalgamate these research areas, as well as serving to eliminate inconsistencies in the usage of relevant nomenclature.

Introduction

As scientists expand the search for life in the solar system, they have also begun to take a closer look at our own planet and have come to realize over the last decade that temperature is not as significant a boundary to the existence of life as once thought (Rothschild & Mancinelli 2001). Expeditions to Mars and the moons of Jupiter have brought renewed interest to the polar regions of Earth, in an attempt to describe the strategies that life employs to survive such extreme cold.

To withstand such adverse conditions, organisms living at the poles must tolerate prolonged periods of sub-zero temperatures along with low levels of available liquid water, nutrients and even light (Cavicchioli *et al.* 2000, Coulson & Birkemoe 2000, Elinitsky *et al.* 2007, Elster & Benson 2004, Mueller *et al.* 2005). Dropping temperatures cause intra- and extracellular fluids to freeze and the dry winter air frequently attracts moisture through the cuticles of micro-invertebrates (Danks 2005, Wright 2001). These organisms, however, have acquired strategies to combat these conditions – employing a wide array of biomolecules to help control internal freezing or avoid it altogether (Coulson & Birkemoe 2000, Crowe 1974, Crowe *et al.* 2004, Danks 2005, Danks 2006, Elster & Benson 2004, Leather *et al.* 1993, Lee & Costanza 1998, Lozina-Lozinskii 1974, Sinclair 1999, Sinclair *et al.* 2003, Sømme 1999, Storey 1999, Storey & Storey 1990, Wharton & Ferns 1995, Wright 2001). Figure 1 reveals the range of strategies available to organisms residing in areas affected by low temperature, as well as the relationships among them. The pinnacle of these strategies, however, may be found in the nematodes, rotifers, and of most relevance to this paper, tardigrades residing in the cryoconite holes of ice-shelves. These organisms are unique in their ability to tolerate internal freezing while simultaneously entering an ametabolic state known as cryobiosis (Crowe 1974, Hengherr *et al.* 2007, Jönsson & Bertolani 2001, Jönsson & Jaremo 2003, Kinchin 1997, Nelson 1991, Nelson 2002, Ramløv & Westh 1992, Rebecchi *et al.* 2007, Rebecchi *et al.* 2006, Rothschild & Mancinelli 2001, Sømme & Meier 1995, Wright 2001). Despite the

damage posed to cellular membranes, proteins and DNA or RNA strands (Cavicchioli *et al.* 2000, Phadtare *et al.* 1999), tardigrades can enter this latent state for years at a time, surviving temperatures approaching absolute zero, with no ill effect (Crowe 1974, Kinchin 1997, Wright 2001).

Surprisingly, very little is known about cryobiosis, a sub-category of the overall metabolic state known as cryptobiosis, and the strategy is quite frequently absent in the literature pertaining to cold-hardiness. The following report will serve as a review of the processes and states associated with cold-hardiness, with a specific focus on the mechanisms of freeze-tolerance and cryobiosis as they pertain to tardigrades and other micro-invertebrates.

The Physics of Freezing

To understand the strategies employed by organisms enduring freezing conditions, it is first necessary to understand the mechanism of freezing at the macro and micro levels. From the perspective of thermodynamics, freezing is generally termed as being a first-order phase change, with the melting point being the only instance where the solid and liquid may coexist at standard pressure in equilibrium (Franks 1981). Liquid water is metastable below 0°C and, as such, will freeze spontaneously under the proper conditions (Franks 1981), which is of considerable concern for any living organism. However, water does not necessarily freeze at the freezing point, and it, as well as other bodily fluids of many living organisms, are capable of entering a supercooled state, where fluids

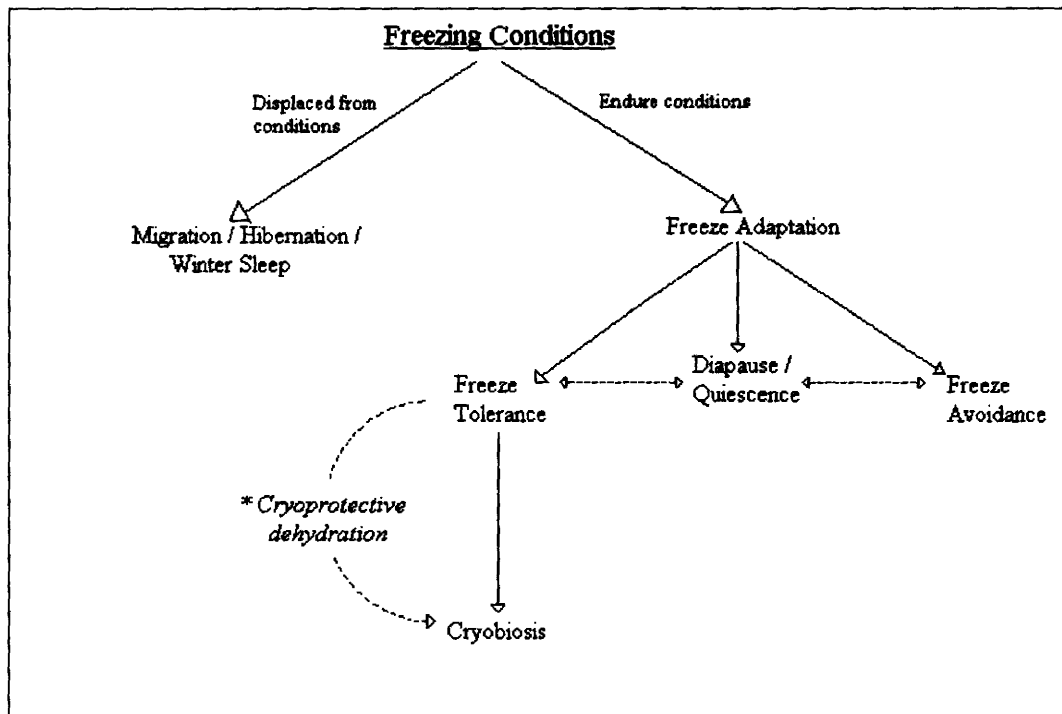

Figure 1. Mechanisms for surviving freezing conditions and the relationships among them. Dotted lines indicate a situation where the relationship is not obligatory. For example, some organisms amass cryoprotectants associated with cold-hardening strategies before, or without, entering a latent state. Alternatively, some organisms must first be in diapause or quiescence before making use of freeze-tolerance or avoidance strategies. Moreover, it should be recognized that cryoprotective dehydration (italicized term) is considered herein as a process leading to the state of cryobiosis, and that not all organisms entering cryobiosis must undergo cryoprotective dehydration*.

undercool but do not freeze (Farrant & Woolgar 1970, Franks 1981, Luyet 1969). The reason that liquids do not necessarily freeze is that, for crystallization to occur, there must be a point of nucleation wherein a group of water molecules of a critical size assumes a configuration that is thermodynamically favourable (Lee & Costanza 1998, Muldrew *et al.* 2004). This configuration may be recognized by other water molecules as an "ice-embryo" to which they will then attach themselves, resulting in a reduction in entropy and chemical potential (Franks 1981). From here, an ice crystal may grow, adopting many different crystal

morphologies depending on the temperature, cooling rate, and the nature and concentration of any solutes present (Luyet 1969).

Ice Formation and the Implications for Biological Systems

Ice crystal formation may occur by means of homogeneous nucleation, heterogeneous nucleation, or seeding (Pitt 1992). Within a biological system such as a cell, ice formation generally occurs through heterogeneous nucleation, where nucleation occurs not only on the surface of the liquid but on suspended particles and minute bubbles present on that surface. Energetically speaking, this type of ice formation is much more favourable than is homogeneous nucleation, as some energy is provided by the destruction of solute-liquid interface by the ice crystal – allowing further nucleation to occur. The ice-crystals that do form are metastable, however, and, unless cooling is slow enough to allow for supercooling, they may frequently recrystallize into a more stable morphology, causing serious injuries both from outside and within a cell (Franks 1981).

Aside from the physical injury received from ice crystal formation, ice nucleation also introduces secondary concerns in maintaining osmotic equilibrium between the intra- and extra-cellular environments and, on a larger scale, between and organism and its environment. Depending on the rate of freezing, a cell may not be able to maintain osmotic equilibrium with the external environment (Farrant & Woolgar 1970). When freezing occurs, solutes are excluded from ice, and thus, the osmotic gradient increases. When freezing is slow in the

extracellular fluid (ECF), cells are able to maintain osmotic equilibrium through dehydration to the ECF and, thus, avoid freezing (Muldrew *et al.* 2004). However, when the cooling rate is too great, freezing occurs too rapidly within the extracellular solution for the cell to undergo compensatory dehydration. The cytoplasm becomes supercooled, and the cell is then in great danger of intracellular ice formation occurring (Muldrew *et al.* 2004, Pitt 1992). A secondary consideration of injury due to loss of osmotic equilibrium is also in the injury imposed on cells due to shrinkage through dehydration. The shrinkage of the cell causes the phospholipid bilayers to undergo harmful gel-phase transitions (Crowe *et al.* 2004, Phadtare *et al.* 1999, Wright 2001). This leads to a displacement of integral membrane proteins, also causing some of the membrane to be internalized as vesicles within the cell (Wright 2001).

Freeze Avoidance Strategies

The destructive forces of ice crystals experienced by organisms has, expectedly, lead to a range of physical and biochemical mechanisms directed towards either avoiding or tolerating freezing conditions. There are two options when dealing with freezing conditions, which separate into the categories of those animals that are capable of escaping the conditions entirely – either through migration, hibernation or winter-sleeping – and those that must endure the conditions.

For those animals that must endure such adverse conditions, two main strategies have evolved, that of freeze-avoidance and freeze-tolerance. A third strategy, which can be considered in addition to the aforementioned tactics, is that of quiescence and diapause, which both result in the slowing of metabolism found commonly within arthropods, primarily insects. Though diapause could be thought of as a form of hibernation, it is considered to be separate, as diapausing insects also make use of freeze avoidance and freeze tolerant strategies in addition to a lowering of metabolic rate (Leather *et al.* 1993). As the majority of literature on cold-hardiness focuses on insects, a brief discussion of the relevant material will provide a good beginning for further discussion on the biochemical adaptations associated with freeze-avoidance and freeze-tolerance in relation to tardigrades. This will lead to a discussion of cryobiosis (freeze-induced cryptobiosis) and cryoprotective dehydration – protective dehydration related to the strategies of freeze tolerance and cryobiosis.

Quiescence and Diapause

Quiescence, as defined by Leather *et al.,* 1993 is "the response of individual [organisms] to a sudden unanticipated, non-cyclic and usually short-duration deviation of normal weather conditions". It is likely that this strategy occurs in early winter or is related to winter-active insects, resulting only in retardation of growth. Diapause, on the other hand, is considered to be the most prevalent form of dormancy in insects (Leather *et al.* 1993) and is typified by

entry into complete arrested development. This type of dormancy is predictive, and the organism must go through significant preparation to enter this overwintering state, usually brought on by a change in daily temperature and photoperiod (Lozina-Lozinskii 1974). Though organisms in both temperate and polar regions must overwinter, there are several advantages and disadvantages inherent in such a phenomenon. The first and most-obvious advantage is that overwintering allows an organism to survive inconstant and harmful conditions that would be unfavourable for growth and reproduction. These life-forms are also generally protected from predation and do not have to deal with the dangers of starvation inherent in winter environments (Storey & Storey 1992). It should also be noted that, in general, diapausing organisms have a higher tolerance to freezing. As seasonal synchronisation is found to be associated with diapause, it has been hypothesised that freeze adaptation (or cold hardiness) in an organism may actually have evolved in diapausing individuals (Sømme 1999). In some species (insects for example), an individual cannot become cold hardy unless it is in diapause, and it has been found that juvenile hormone appears to be involved in the regulation of both processes (Danks 2005).

Freeze Avoidance

Cold hardiness is generally broken down into two categories, those that avoid freezing and those that tolerate it; however, it should be noted that within the two categories, there is a varying level of tolerance to cold temperatures

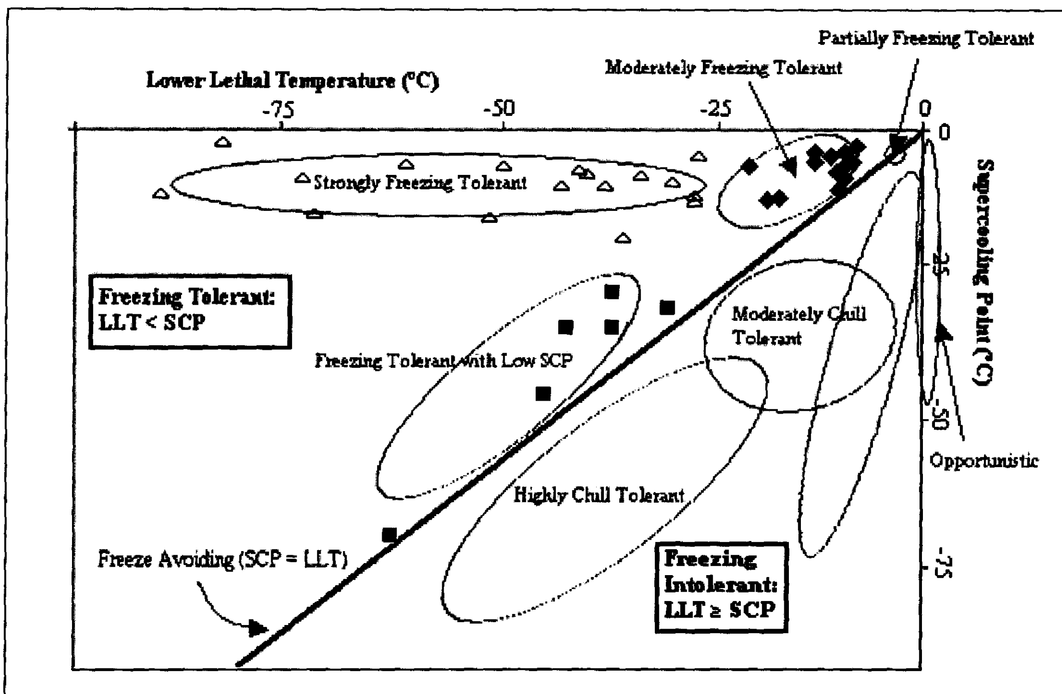

Figure 2. The relationship between the supercooling point and the lower lethal temperature for four freeze tolerant and five freezing intolerant groups of insects adapted from Sinclair (1999).

(Figure 2)(Sinclair 1999). Freeze avoidance, thought to have evolved as a means of cold hardiness prior to the evolution of freeze tolerance (Sinclair *et al.*. 2003), is practiced by organisms that cannot tolerate any form of intracellular freezing. Instead, they take advantage of the ability of their bodily fluids to supercool, further utilizing thermal hysteresis (or antifreeze) proteins along with increases in one or more polyols or sugars (Leather *et al.* 1993). For example, many beetles have been reported to have supercooling points (SCPs) of approximately -7°C to -12°C without the addition of antifreeze proteins (Storey & Storey 1992). As this range is within the environmental conditions likely to be encountered, their task is to lower the supercooling point well below these anticipated conditions. To do so, they must first increase the synthesis of antifreeze proteins in the body fluids, along with the removal of any sites of potential ice nucleation (Block 2002, Danks

2005, Leather *et al.* 1993, Lozina-Lozinskii 1974, Sømme 1999, Storey & Storey 1990, Storey & Storey 1992). Secondly, they must increase the concentration of low molecular weight solutes or decrease body water content so as to lower the crystallization temperature and stabilize the membranes at such low temperatures (Leather *et al.* 1993, Lozina-Lozinskii 1974, Sinclair *et al.* 2003, Sømme 1999, Storey & Storey 1992). This allows them to supercool their body to temperatures as low as -50°C or -60°C (Leather *et al.* 1993) (See Lee and Costanza, 1998 for a detailed review).

Antifreeze Proteins

Antifreeze proteins (AFPs), also referred to as thermal hysteresis proteins (THPs), act in much the same way as the antifreeze in a car does. These proteins are associated with the post-modification of ice-crystal growth and structures, adsorbing to the growing surfaces of minute ice crystals in the intra- and extracellular spaces (Elster & Benson 2004, Storey & Storey 1992). Aside from modifying crystal growth at very high concentrations, AFPs cause a thermal change in the solution in which they are contained, resulting in a difference between the melting and freezing points for that solution (Elster & Benson 2004). Antifreeze proteins achieve this by restricting the growth of crystals to many small fronts between the molecules, creating a high surface-to-volume ratio. This creates a surface energy too high for ice to continue to form, and so ice can only continue to accumulate once the temperature is lowered (Storey & Storey 1992).

AFPs do not act colligatively; however, their ability to lower the freezing temperature is so great that they are as much as 500 times more effective than are other solutes (Danks 2006, Elster & Benson 2004). Furthermore, AFPs act to inhibit recrystallization, which can occur if the supercooling point is breached or external temperature warms and then cools again unexpectedly (Danks 2006, Wharton *et al.* 2005a). Antifreeze proteins are prevalent in many species, found in plants, insects and various fish, and their regulation is precisely controlled, incorporating photoperiod, temperature, circadian rhythms and hormone action to match protein expression with particular environments (Storey & Storey 1992).

Freeze Tolerance

As freeze avoidance is thought to have evolved first as a reliable strategy for dealing with freezing conditions, it is surprising that freeze tolerance would have evolved at all (Sinclair *et al.* 2003, Sømme 1999). Within invertebrates, however, freeze tolerance occurs in many species of insects, marine intertidal organisms and meiofauna (Storey 1999). This is because supercooling is not without its own dangers. Supercooled liquid is metastable (see section on thermodynamics) and so, as the cooling period lengthens and temperature continues to decrease, the probability of spontaneous ice formation increases. If the temperature exceeds the supercooled point or the cooled liquid encounters nucleators, freezing will occur instantaneously and result in death (Elster & Benson 2004, Storey & Storey 1990). Freeze tolerant species, then, adopt an

almost opposite strategy when dealing with freezing conditions relative to their freeze avoiding counterparts. Instead of removing sites of nucleation, these organisms add ice-nucleating proteins (INPs) to their extracellular fluids, along with THPs and low molecular weight cryoprotectants, creating the conditions for slow, controlled ice crystal growth in non-critical areas of the organism down to a lower lethal temperature, the value of which varies from species to species(Elster & Benson 2004, Leather *et al.* 1993, Lozina-Lozinskii 1974, Sinclair *et al.* 2003, Smith 1961, Sømme 1999, Storey & Storey 1990, Storey & Storey 1992). For example, the goldenrod gallfly (*Eurostata solidiganis*) is capable of surviving to -50°C, whereas larvae of the fruit fly, *Chymomyza costata*, are capable of freezing to -80°C without fatality (Sinclair *et al.*. 2003).

Ice Nucleating Proteins

In contrast to the role of antifreeze proteins, ice nucleating proteins (INPs), also known as ice nucleating agents (INAs) and protein ice nucleators (PINs), help to induce and control the formation of extracellular ice, raising the supercooling point of the fluid they are in to as little as 2°C below the freezing point of the ECF (Duman 2001, Leather *et al.* 1993, Sinclair *et al.* 2003, Sømme 1999, Storey & Storey 1992, Wilson & Ramløv 1995). Ice nucleators are any particles that are large enough to provide a surface on which an ice embryo can grow to reach critical size (Leather *et al.* 1993). In the absence of ice nucleators, which control the formation of ice, freezing can occur intracellularly, causing an

influx of water from the ECF, resulting in cell death through lysing. With controlled extracellular freezing, however, the rates of ice formation are slow, enabling cells to maintain osmotic balance and vapour pressure with the ECF, letting the cytoplasm cool without freezing (Duman 2001). Ice nucleating proteins may already be present in the body, in the form of food particles or grains of mineral dust (Storey & Storey 1992), but the primary nucleators are generally proteins added to the ECF (Storey 1999). One of the first INPs to be studied came from *Vespula maculate* (Bald-faced hornet, Hymenoptera) and was found to be 74 kDa in size, containing strongly hydrophilic amino acids such as glutamate, glutamine, serine and threonine (Duman 2001, Sømme 1999). The second set of INPs to be purified came from *Tipula trivitatta* (Crane fly, Diptera), which contained proteins and lipoproteins, the latter being 800 kDa in size and composed of 11% phosphotidylinositol (PI) – a property unique to these ice nucleating protein (Duman 2001, Sømme 1999). Thirty years later, the body of knowledge on these proteins has not grown, but the presence of hydrophilic amino acids, along with the increase in composition of PI, suggests that nucleating agents are capable of organizing water to form ice embryos on nucleator surfaces (Duman 2001). In freeze-tolerant species, therefore, the role of INPs is to ensure that freezing occurs extracellularly in a controlled fashion before nucleation can occur in areas where freezing would be lethal. The sites of nucleation can differ from species to species, ranging from inoculative freezing through the cuticle, to nucleators in the gut and haemolymph (Sinclair 1999,

Wilson & Ramløv 1995). It should be further noted that the rates of freezing and the temperatures at which freezing can occur may also vary within the different compartments of the given organism, thus altering the amount of injury that will result (Leather *et al.* 1993). For a more-detailed discussion of ice nucleating proteins see Duman (2001).

Cryoprotectants

As mentioned earlier, both freeze avoiding and freeze tolerant organisms synthesize low molecular weight cryoprotectants to protect their cells from the dangers associated with freezing. The predominant cryoprotectants (CPTs) used are glycerol, sorbitol, sucrose, glucose and trehalose; however, mannitol, threitol, erythritol, myo-inositol, ribitol, ethylene glycol and fructose are also used (Danks 2005, Danks 2006, Leather *et al.* 1993, Lee & Costanza 1998, Lozina-Lozinskii 1974, Montiel 2000, Sinclair *et al.* 2003, Sømme 1999, Storey & Storey 1990, Storey & Storey 1992). The choice of cryoprotectants used for the cell is very specific, and the molecules used must be highly soluble, present at low concentrations, be chemically inert (*eg.* Browning reactions), and act to stabilize protein conformation (Storey & Storey 1992). The above molecules can be placed into two categories, those that are colligative (polyols) and those that are membrane stabilizers (glucose, sucrose, trehalose and proline) (Storey & Storey 1990). In freeze avoiding species, colligative CPTs at concentrations anywhere from 8%-30% of the fresh body weight (Sømme 1999, Storey & Storey 1992)

help to lower the supercooling point; however, in freeze-tolerant species, they limit the amount of ice that is capable of forming and help to maintain osmotic equilibrium during dehydration (Danks 2006, Leather *et al.* 1993, Muldrew *et al.* 2004, Sinclair 1999, Sømme 1999, Storey & Storey 1990, Storey & Storey 1992). The reason there are so many different polyols present is due to the fact that, if any one CPT was present at the levels mentioned above, the results could be highly toxic for the cell (Leather *et al.* 1993). By having many different types, no one CPT need reach dangerous levels while providing protection to the cell. It has also been hypothesized that mixtures of polyols and sugars at high concentrations are actually capable of undergoing a glassy transition at -25°C, a process known as vitrification, which would be less damaging for cells (Storey & Storey 1992). In insects, the accumulation of cryoprotectants is a seasonal event, with the organism building up carbohydrate stores near the end of summer for winter usage (Montiel 2000) and then breaking down the synthesized molecules during spring. This is especially important for insects which overwinter in their larval stages, as the broken down cryoprotectants can then be used to fuel larval development into the pupal and adult stages in the spring (Storey & Storey 1990).

Freezing and Dehydration

The main distinction between freeze avoiding and freeze tolerant organisms is that the latter can tolerate the formation of ice in the tissues, where the former cannot. There is considerable overlap in the level of cold tolerance

that both strategies confer, but the strategies must be seen as separate from each other (Danks 2005, Sinclair 1999). Freeze tolerance is manifested along a spectrum, where some organisms are weakly freeze tolerant and some are strongly so. For example, in the insect *Tipula paludosa*, internal freezing can be tolerated above the SCP (which is relatively high), but individuals will die before their body temperatures reach equilibrium with the environment (Sinclair 1999). The strongest freeze tolerant species are those that freeze at relatively high temperatures but are capable of surviving to very low temperatures. A well-known example of this latter category is seen in the larvae of the golden rod gall fly, which freezes at -6°C, but can survive temperatures as low as -50°C (Sinclair 1999, Storey & Storey 1990)

Another factor that must be considered is the degree of dehydration exhibited by freeze tolerant species. As described earlier, during ice formation in the extracellular fluid, freeze tolerant species experience an osmotic gradient as well as a gradient due to the vapour pressure of ice, drawing water out of the cell to maintain equilibrium with the ECF. It is because of this that the ability to tolerate freezing is also related to the ability to tolerate dehydration (Sømme 1999). There are many similarities between the mechanisms involved in freeze tolerance and dessication, as both rely on the synthesis of polyols and sugars (especially trehalose) to maintain osmotic balance and protein conformation, while also requiring the organism to be capable of making adjustments to cellular water content (Danks 2005). Cells can prevent dehydration to a certain extent by

the use of cryoprotectants, which, aside from lowering the temperature of freezing within the cell, are capable of binding large amounts of water (Muldrew *et al.* 2004). This organic water, or "bound" water, is not capable of interacting with forming ice crystals due to the energy required to break the bonds (Franks 1981). In this way, cells can help to limit the amount of freezing that can occur. Though dehydration occurs naturally in cells as a result of freezing, some organisms actually employ the strategy of dehydrating before freezing, thus limiting the amount of freezing that could conceivably occur. Indeed, it was found that many insects try to evacuate as much water from their bodies as possible, either through the evacuation of the gut (Franks 1981) or through the increase in cellularly bound water (Danks 2005). Finally, it should be noted that many micro-invertebrates use dehydration as the mechanism for surviving freezing (Danks 2005, Montiel 2000).

Cryobiosis

A form of cold-induced cryptobiosis, cryobiosis may be seen as an extreme form of freeze tolerance. Cryptobiosis, itself, (first coined by Keilin in 1959) is a reversible cessation of metabolic activity in response to environmental stressors (Crowe 1974, Dewel *et al.* 1993, Jönsson & Bertolani 2001, Jönsson & Jaremo 2003, Kinchin 1997, Nelson 1991, Nelson 2002, Neuman 2006, Ramløv & Westh 1992, Rebecchi *et al.* 2007, Rebecchi *et al.* 2006, Sømme & Meier 1995, Watanabe 2006, Watanabe *et al.* 2004, Wright 1989, Wright 2001), which in the

tardigrade is generally accompanied by the formation of a "tun" state (The tun, or *Tönnchenform* state, in terms of morphology, results in a lower relative surface area for the animal and is achieved by the tardigrade through an invagination of the limbs and head, accompanied by an overlapping of the cuticular plates, thus creating an impenetrable surface. It should be realized also, that formation of a tun is not strictly necessary for entry into cryobiosis) (Kinchin 1997, Wright 2001). True cryptobiosis should also be further defined as a loss of the liquid water phase either through dessication (anhydrobiosis) or freezing (cryobiosis), where the latter case also involves a loss of water due to the vapour pressure of ice (Wright 2001). It should be noted that this description of cryptobiosis is a deviation from the definition most commonly used in the literature. It is, however, more accurate in that it employs a common mechanism (loss of the water state) uniting the varying forms of cryptobiosis. This process also should not be confused with the latent states of quiescence and diapause. The former, though a response to environmental stress, is a slowing down of metabolism rather than a complete cessation of metabolic activity. With diapause, the main differences separating it from cryptobiosis lie in the fact that diapause is 1) predictive – in that that organism is able to anticipate the yearly event and prepare accordingly prior to the initiation of adverse conditions – and 2) regulated by hormonal factors in response to photoperiod. It is very likely that cryptobiosis is regulated by changes in internal osmolarity as a result of freezing or dehydration (Watanabe *et al.* 2003).

Cryptobiotic ability is widely dispersed among taxa, ranging from unicellular organisms to invertebrates, with the primary literature focusing on the anhydrobiotic (dessication-induced) response in tardigrades, nematodes, bdelloid rotifers, artemia shrimp and chironimid species *Polypedilum vanderplanki*. Though *P. vanderplanki* is the largest organism (7-8mm) to undergo cryptobiosis (Watanabe 2006), it does so in the larval stage and, so, is protected in comparison to the adult stage. Tardigrades, alternatively, are capable of undergoing cryptobiosis during any stage of development (Kinchin 1997, Rebecchi *et al.* 2006) and of surviving in that state for an indefinite amount of time depending on external environmental conditions, averaging 7 years (Jönsson & Bertolani 2001, Newsham *et al.* 2006).

Many of the studies of the cold-adaptive abilities of cryobionts have been performed with the use of anhydrobiotic organisms (Watanabe *et al.* 2003), so the distinction between the two processes is lost. In cryobiosis among tardigrades, dessication and the formation of a tun are not required for the survival of freezing conditions. It is here as well that cryobiosis deviates from the strategy of freeze tolerance, by apparently lacking a lower lethal limit and surviving to indefinitely low temperatures (Ramløv & Westh 1992, Wright 2001). The tardigrade, therefore, must be either capable of rendering its bodily fluids "unfreezable" or be able to withstand intracellular freezing. (Wharton & Ferns 1995, Wright 2001) The types of cryoprotectants, potential ice nucleators and THP's have not been studied in tardigrades to this date and currently remain unknown, along with the

overall mechanisms of the adaptation, with only five recent bodies of work published in the last sixteen years on the subject (Coulson & Birkemoe 2000, Newsham *et al.* 2006, Ramløv & Westh 1992, Sømme & Meier 1995, Wharton & Ferns 1995). In the 1992 and 1995 studies, the researchers took tardigrades and studied the effects of cooling rate, trehalose level and short-term acclimation on hydrated and dehydrated specimens (Ramløv & Westh 1992, Sømme & Meier 1995). They found that dehydrated organisms had higher survival rates than did hydrated animals but that both groups had good survivorship when pre-acclimated to low temperatures and exposed to freezing temperatures over a short period of time (Ramløv & Westh 1992, Sømme & Meier 1995).

Trehalose

The differences in survivorship found in the previous studies between hydrated and dehydrated specimens likely resulted from the fact that anhydrobiotic organisms, in general, are capable of undergoing dessication to such an extent that up to 98% of all water, both bound and unbound, is lost (Watanabe 2006). Thus, one could argue that an anhydrobiont is incapable of freezing. Though it is not known exactly what triggers the anhydrobiotic response, or how the organisms deal with such severe dessication, it is thought that the sugar trehalose plays a very large role in survivorship (Crowe 1974, Crowe *et al.* 2004, Hengherr *et al.* 2007, Montiel 2000, Ramløv & Westh 1992, Rebecchi *et al.* 2007, Rebecchi *et al.* 2006, Watanabe 2006, Watanabe *et al.* 2003,

Wright 1989). Ramløv and Westh (1992) showed that, when tardigrades were cooled at rates comparable to those found in their environment, animals possessing higher concentrations of trehalose had higher survivorships (Ramløv & Westh 1992). The role of trehalose in anhydrobionts has been fairly well explored, with studies on *Artemia* occurring as early as the 1960's, noting concentrations that ranged as high as 13-18% of the dry weight of the cyst (Hengherr *et al.* 2007). All studies exploring the efficacy of sugars in preserving different tissues in freeze-dried conditions have revealed that trehalose appears to be the most successful. It is not specifically known why this is so, but it has been suggested that trehalose, unlike other sugars at similar concentrations, has access to the interiors of vesicles, which may give it superior abilities in preserving these types of membranes (Crowe *et al.* 2004).

Cryoprotective Dehydration

The term "cryoprotective dehydration" appeared first in a paper by Holmstrup and Wharton in 1994 (Wharton *et al.* 2005b). Essentially, the authors described three mechanisms of dealing with freezing conditions; those of freeze avoidance, freeze tolerance and cryprotective dehydration. The authors state that cryprotective dehydration occurs in small invertebrates with a permeable epidermis, inhabiting soil or bryosystems. These organisms do not supercool to any significant extent and do not experience the formation of ice within the tissues but, instead, are found to lose water to the environment (Elinitsky *et al.*

2007, Holmstrup *et al.* 2002, Pederson & Holmstrup 2003, Sinclair 1999, Wharton *et al.* 2005b). The mechanism behind this process lies in the organisms' abilities to dehydrate, rapidly equilibrating their liquid melting points with those in the external environment. All fluids are capable of supercooling to some extent (Muldrew *et al.* 2004); however, the vapour pressure of supercooled water is higher than the vapour pressure of ice. Thus, when ice forms in the environment of a soil dwelling organism at a constant temperature above the SCP, the organism dehydrates but maintains vapour equilibrium and, so, freezing does not occur (Holmstrup *et al.* 2002). A study performed by Wharton *et al.*. (2005) on the Antarctic nematode *Panagrolaimus davidi* found that ice did not form in 25% of the specimens, which instead dehydrated. This evidence was used to further support the strategy of cryoprotective dehydration.

Cryobiosis as extreme freeze-tolerance accompanied by a cessation of metabolic activity, is a situation where organisms are exposed to slow dehydration as a consequence of the low vapour pressure of ice (Wright 2001). Cryobionts do not possess an extensive capacity to supercool, possessing high SCPs of approximately -2°C. It is at this stage that cryobionts may experience loss of water to the surrounding freezing medium, before succumbing to extracellular freezing as the temperature drops. In the experiment by Wharton *et al.*. (2005), the nematodes that experienced dehydration were kept at -1°C, which is above the SCP for this cryptobiotic organism. It is not surprising, then, that the organism did not undergo freezing and, instead, dehydrated (Wharton *et al.* 2005b). Many

of the organisms used in the studies of cryoprotective dehydration, such as arctic midges, nematodes and some collembolans, are well known cryptobiotic organisms (Block 2002, Watanabe 2006). It is surprising then, that none of the associated literature mentions the strategy of cryptobiosis, nor entertains a discussion of the differences between the two processes (Coulson & Birkemoe 2000, Elinitsky *et al.* 2007, Holmstrup *et al.* 2002, Pederson & Holmstrup 2003, Sinclair 1999, Wharton *et al.* 2005b).

The exclusion of cryobiosis from the discussion of cold-adaptation in the literature is not a recent event, and the metabolic states have generally been treated as an extension of freeze tolerance in most papers (Wright 2001). It is possible that, in the literature pertaining to cryoprotective dehydration, this oversight has again occurred; however this omission allows for speculation on whether or not there are actually differences between the two strategies. The described state of cryobiosis and the role that dehydration plays therein suggests that cryoprotective dehydration exists more as a mechanism resulting in the cryobiotic state – where the two terms have existed in separate spheres due to a lack of the appreciation of the differences between cryobiosis and freeze tolerance.

Re-evaluation of Terms

The body of literature present on cold-adaptation is quite broad, spanning from cold avoidance and cold hardiness, to freeze tolerance, cryptobiosis and

finally cryoprotective dehydration. This review, however, has revealed areas where research is thin and the processes are not well understood. From the forgoing paragraphs, I propose to reclassify cryoprotective dehydration (italicized term in Figure 1) as a process leading to the metabolic state of cryobiosis, rather than an alternate strategy of cryoprotection unto itself. This review has shown, that the differences between cold-hardening strategies are not easily delineated, where varying environmental stressors have blurred the lines between avoidance and tolerance, freezing and dehydration. It is important then that the processes of cryptobiosis and cryoprotective dehydration are further explored in the interests of resolving confusion in the freeze-tolerance literature.

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Preface

The following study was conducted along with my supervisor, Jonathon Stone. All of the fieldwork was completed by myself, with logistical support provided by J. Stone and W. Vincent and team. I conducted the in-lab studies, which were a collaboration with J. Stone, who made suggestions on the experimental set-up, and aided in the statistical analysis.

Some like it cold: a study of cryobiosis in the eutardigrade *Macrobiotus harmsworthii*

Alexandra Pontefract and Jonathon Stone

Department of Biology, McMaster University, Hamilton, Ontario, L8S 4M6, Canada.
E-mail: pontefaj@mcmaster.ca

Abstract: With recent missions to Mars and the moons of Jupiter, research into psychrophiles and the psychrophilic lifestyle has become paramount to the astrobiological community. The study of analog sites and the organisms that reside therein have led to an essential understanding of the limits at which life can survive. In the Canadian high-Arctic, several Mars-analog sites exist on Devon Island and Ward Hunt Island, Nunavut. These sites typify an Arctic desert, with mean summer high temperatures of 4°C, increased saline conditions and low levels of available liquid water. Despite these environmental challenges, life remains abundant – characterized by extensive microbial communities on the surface and within the topsoil. Among the organisms in these communities, tardigrades (water-bears) are perhaps the most 'extreme' life form present. Studies have shown that Arctic tardigrades are capable of withstanding freezing temperatures, down to -80°C for several years, but it is not known whether this ability is inherent to Arctic species or is widespread throughout tardigrades. The purpose of this study was to elucidate the relationship between temperature, duration and survival on a species of tardigrade found in Cootes Paradise, Hamilton: *Macrobiotus harmsworthii*. Specimens were frozen at -20°C, -40°C and -80°C for different lengths of time to assess their cryptobiotic abilities. The length of time spent in a frozen state had no apparent effect on the tardigrades' abilities to survive, but the temperature at which they were frozen played a crucial role, with survival at -80°C being achieved with greatest frequency.

Key words: Psychrophiles, Cryptobiosis, Tardigrades, Extremophiles, Analog research

Introduction

One of the main foci of astrobiology is the study of life in extreme environments, and, subsequently, determining life's extremes. From the deepest parts of the ocean, where light cannot reach, to supercooled water droplets in the Earth's atmosphere (Sattler *et al.* 2001), life endures, evolving and adapting

mechanisms we are only beginning to understand. Possibly one of the most "extreme" life forms, and the focus of this study, are tardigrades. These tiny (200-1000 μm in length) micro-invertebrates (Kinchin 1997) share with rotifers, nematodes, bdelloid rotifers, artemia shrimp and chironomid larvae the ability to survive a wide array of environmental extremes (Rebecchi *et al.* 2006). Studies have shown that these organisms can withstand temperatures from -253°C to 151°C, X-ray radiation, dessication, vacuum conditions, and pressures up to 600Mpa, by retreating into a dormant state known as cryptobiosis (Rothschild & Mancinelli 2001).

Coined by Keilin in 1959, cryptobiosis in tardigrades is defined as a complete cessation of metabolic activity, frequently accompanied by entrance into a "tun" state (Crowe 1974, Dewel *et al.* 1993, Jönsson & Bertolani 2001, Jönsson & Jaremo 2003, Kinchin 1997, Nelson 1991, Neuman 2006, Ramløv & Westh 1992, Rebecchi *et al.* 2007, Rebecchi *et al.* 2006, Sømme & Meier 1995, Watanabe 2006, Watanabe *et al.* 2004, Wright 1989, Wright 2001). Cryptobiosis can occur as the result of several different environmental stressors, such as dehydration (anhydrobiosis), salinity (osmobiosis) and extreme cold (cryobiosis), which is the main focus of this study (Crowe 1974, Kinchin 1997, Ramløv & Westh 1992, Wright 2001). Cryobiosis is generally seen as an extreme form of cold tolerance, differing only in the fact that the former process apparently lacks a lower lethal limit, with the organism capable of withstanding temperatures approaching absolute zero (Sinclair 1999). The ability of tardigrades to tolerate

such low temperatures is attributed generally to the anhydrobiotic state, a condition that dominates cryptobiotic literature (Ramløv & Westh 1992). This is an inaccurate portrayal, however, and it should be noted that tardigrades are capable of maintaining viability at low temperatures in a hydrated and dehydrated state, though the latter does afford increased survivability. Cryobiotic tardigrades do undergo some dehydration during the freezing process due to the lower vapour pressure of ice – the amount of which is dependent on the rate of freezing.

Previous experiments have shown that tardigrades are capable of remaining in a frozen state for up to several years (A. Pontefract, pers. obs., Coulson & Birkemoe 2000, Newsham *et al.* 2006, Ramløv & Westh 1992, Sømme & Meier 1995, Wharton & Ferns 1995); this capability, however, has not been well documented. The most-complete study of cryobiosis was an experiment that analyzed the effects of cooling rate on the eutardigrade *Adorybiotus coronifer*, down to -196°C (Ramløv and Westh 1992). In the current study, we present data detailing the effects of temperature and time on survival for the tardigrade species *Macrobiotus harmsworthii* (Figure 1) while in a hydrated state and discuss the implications of these results in relation to cryobiosis and astrobiological research.

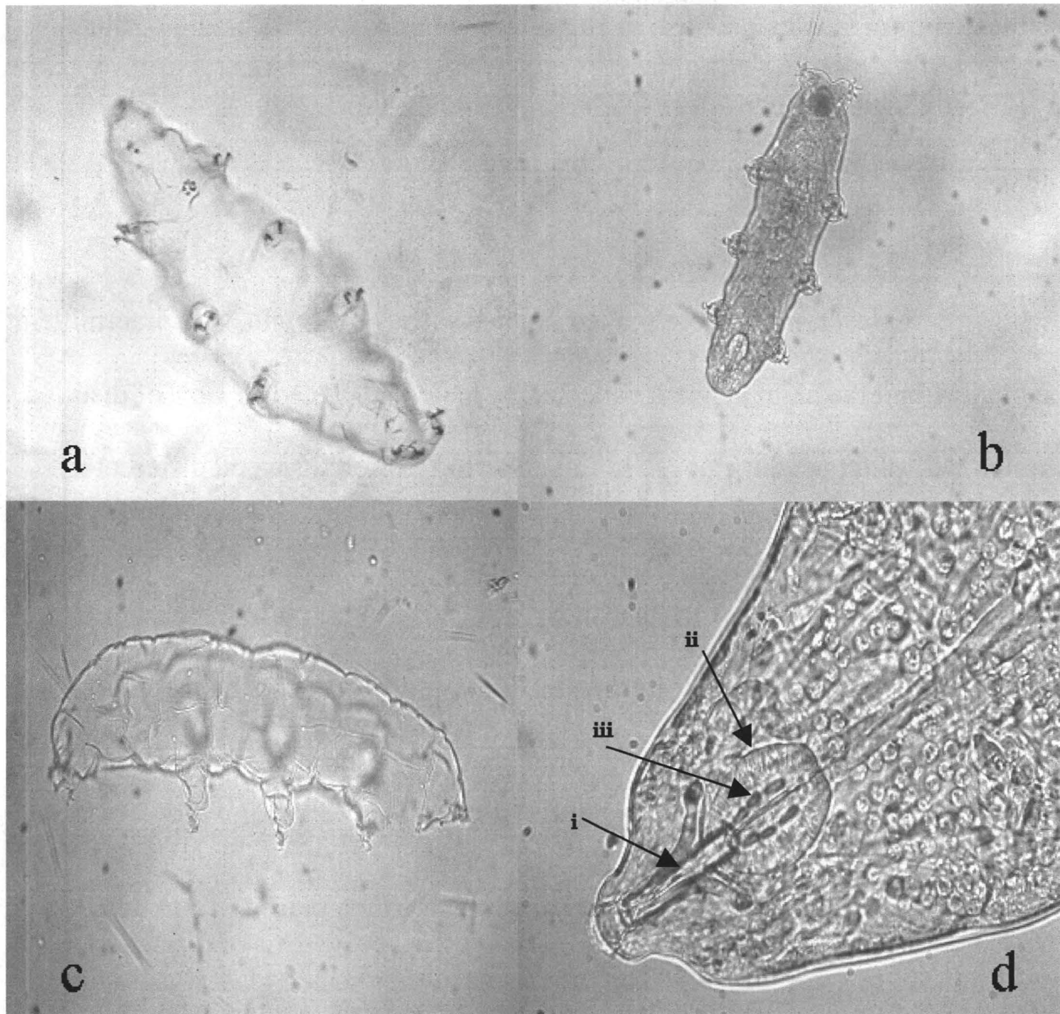

Figure 1. Ventral views of adult *M. harmsworthii* (a) and a juvenile (b). Approximate size of juvenile is 100um. Side view of tardigrade (c). Close up of buccal apparatus (d) clearly showing pharyngeal apparatus (i), pharyngeal bulb (ii) and macroplacoids (iii). Photos by A. Pontefract.

Materials and Methods

Organisms

The experimental animal species used in this study, *M. harmsworthii*, belongs to the Eutardigrada and is classified as a limno-terrestrial organism, as individuals require the presence of a water film to remain active even though they live 'on land.' Samples of moss taken from the bark of decomposing trees were

collected from Cootes Paradise in Hamilton, Ontario, Canada in 2007-2008. After collection, the moss was stored in containers at 4°C until use, with no mortality.

Culturing

Samples of moss were soaked in glass dishes overnight, with intermittent agitation, and the animals were collected by hand and placed in 1mL of distilled water in 2 mL eppendorf tubes. Initial culturing explored several different methods (see discussion); using milled sphagnum, glass vials in co-culture with algae and agar with algae. In this study, tardigrades were cultured at room temperature with a species of freshwater algae, along with the inclusion of moss debris for an additional walking substrate that had been rinsed clean of protists and other microinvertebrates. Moss leaves were added to the tubes to improve the level of dissolved oxygen in the water. Tubes were then placed in a holder, which was situated in approximately 5 cm of water within a sealed glass dish to limit evaporation. Half of the water in each tube was replaced every two days, and counts were made every four days. Identification of the species was performed with a species key found in Kinchin (1994), using egg dentition.

Freezing

Tardigrades, $a=9$ groups and $n=37$ individuals, were placed in 0.25 mL of distilled water in 2 mL eppendorf tubes, to which 0.25 ml of algal solution were added. Each tube was kept at 4°C for 24 hours to allow the animals to acclimate before freezing, approximating an actual environmental scenario. Finally, each

tube was placed at -20°C, -40°C, or -80°C, for 30, 60, or 90 days. Tubes placed at the -40°C and -80°C temperatures were placed at -20°C for 24 hours before being moved to their respective temperatures, to maintain a freezing rate that remained below 5°C min^{-1}. In previous studies, cooling rates above this value were shown to result in decreased survival rates for the specimens (Ramløv & Westh 1992). As a control, 9 eppendorf tubes, each containing 37 tardigrades suspended in 0.25 mL of ATW and 0.25 mL of algal solution, were kept at room temperature for 30, 60 and 90 days. Due to the duration of the experiment, water for the control tubes was changed every 2 days to maintain quality, and tardigrade eggs were removed prior to hatching.

Thawing

Upon termination of the experiment, tubes were placed at 4°C for several hours, until thawed, and then maintained at temperatures less than 15°C for counting. The samples were then placed at 4°C for the next 24 hours and counted again to ensure viability.

Results

Several methods were attempted to create a monoxenic culture for the species. It was found that using eppendorf tubes with algal infused water and moss debris yielded the best results for this species, and specimens could be kept alive for up to 70 days before cultures began to deteriorate. Eggs were laid every15-20 days, ranging in size from 1 to 9 eggs per individual. Hatching time

Temperature ($a=4$) A	Time (days) ($b=3$) B	Mortality ($c=2$) C		
		Healthy	Dead	Totals
23°C - control*	30	35	2	37
	60	3	34	37
	90	0	37	37
		38	73	111
-20°C	30	2	35	37
	60	0	37	37
	90	0	37	37
		2	109	111
-40°C	30	31	6	37
	60	24	13	37
	90	15	22	37
		70	41	111
-80°C	30	36	1	37
	60	27	10	37
	90	35	2	37
		98	13	111
Totals		208	236	444

Table 1. Results of freeze thaw experiment over 30, 60 and 90 days. The data points for each time point at the control temperature represent an average of three trials, where position 111 *(abc)* was rounded up and 112 was rounded down. Position 121 is an average of only two trials as the third was lost due to human error.

varied from 14 to 30 days. Initial clutches maintained a moderate hatching rate, with 22 of 34 (64%) eggs resulting in viable offspring. However, this diminished over the course of two months with ultimately only 48 of 102 eggs hatching, at a rate of 47%. It was not possible to discern the sex of the tardigrades used; however, it was observed that the species is either parthenogenic or a self-fertilizing hermaphroditic species or both. Further life history traits have not been included, as studies are still ongoing.

Freeze/Thaw Study

Upon thawing, tubes were counted in the first five minutes, after 24 hours and then after a week to ensure viability. Tardigrades were scored as having survived once normal leg and torso movement was observed (Table 1). Statistical analysis of the data was performed using a 3-way anova log-linear model (Sokal & Rohlf 1995), calculated using BIOMstat (Rohlf & Slice 2002) (Figure 2a). The log-linear model used was:

$$\ln \hat{f}_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} \qquad (\alpha\beta\gamma = 0)$$

where μ is the mean of the logarithm of the expected frequencies. α_i, β_j, and γ_k are the effects of categories *i, j* and *k* of factors A (temperature), B (time) and C (mortality) respectively. The term $\alpha\beta_{ij}$ term of interaction expresses the dependence of category *i* of factor A on category *j* of factor B, and so on. The expected frequencies for this three-way model cannot be calculated directly and an iterative model must be used to estimate the $\hat{f}_{ijk}$, which are then compared to actual f_{ijk} using the G-test (Sokal & Rohlf 1995). In the model in Figure 1a, the red circle indicates that the three-way interaction term is significant. In this case, the model cannot be simplified and the $\alpha\beta\gamma_{ijk}$ term cannot be dropped. It should be noted that all of the cases in this figure are significant, but that this is irrelevant due to the presence of a complete interaction and so, significance for the other terms has not been indicated. In Figure 2b, the data pertaining to the non-freezing temperature have been excluded from the calculations in order to tease out more subtle associations between the trials experiencing freezing conditions.

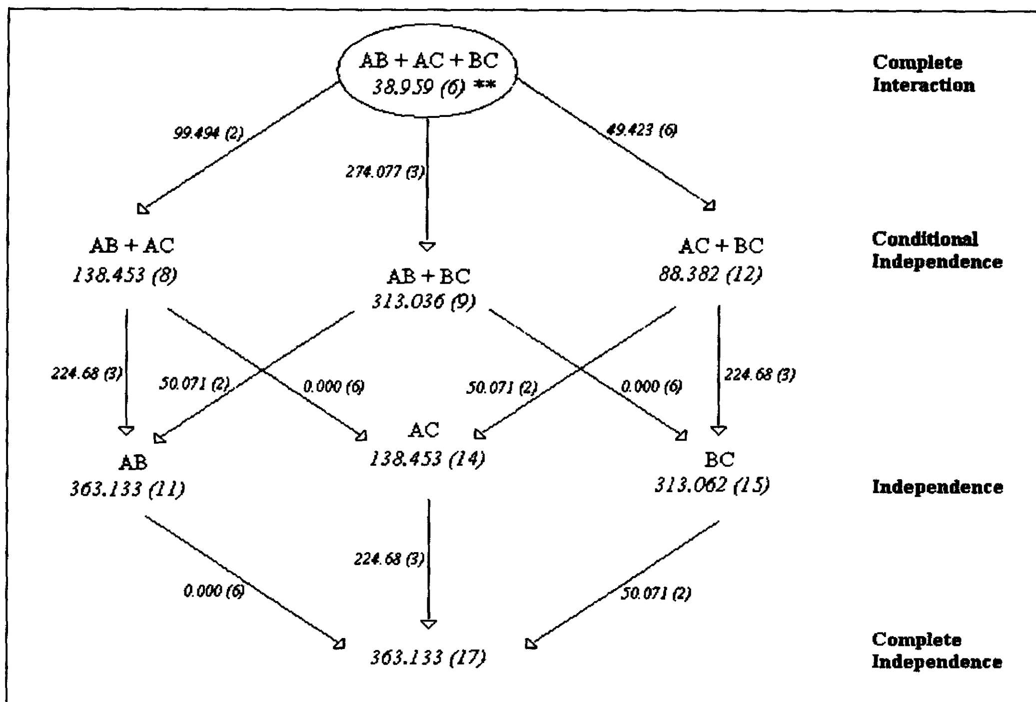

Figure 2a. Representation of the tests using the log-linear model. The starred value indicates a significant G-value at the 0.001 level. The case at the top of the figure indicates that the G-test for three-way interaction term was significant, so it cannot be omitted and no other terms need to be tested, but results from those G-tests have been shown for completeness. The values along the arrows show the differences in G-values between steps in the model. The values in parentheses indicate the degrees of freedom. Figure adapted from Sokal and Rohlf (1995).

Discussion

Culturing

Studies as far back as 1914 have reported the maintenance of tardigrades in the laboratory (Pennak 1953). Despite this, tardigrades have proven notoriously difficult to culture, with maintenance of axenic cultures being virtually impossible (Kinchin 1997). Previous studies have used saturated milled sphagnum in co-culture with nematodes in the rearing of predaceous tardigrades, with good results (Sayre 1969). As well, cultures have been maintained with stream water and algae in glass vials (Dougherty 1964) or agar as a walking

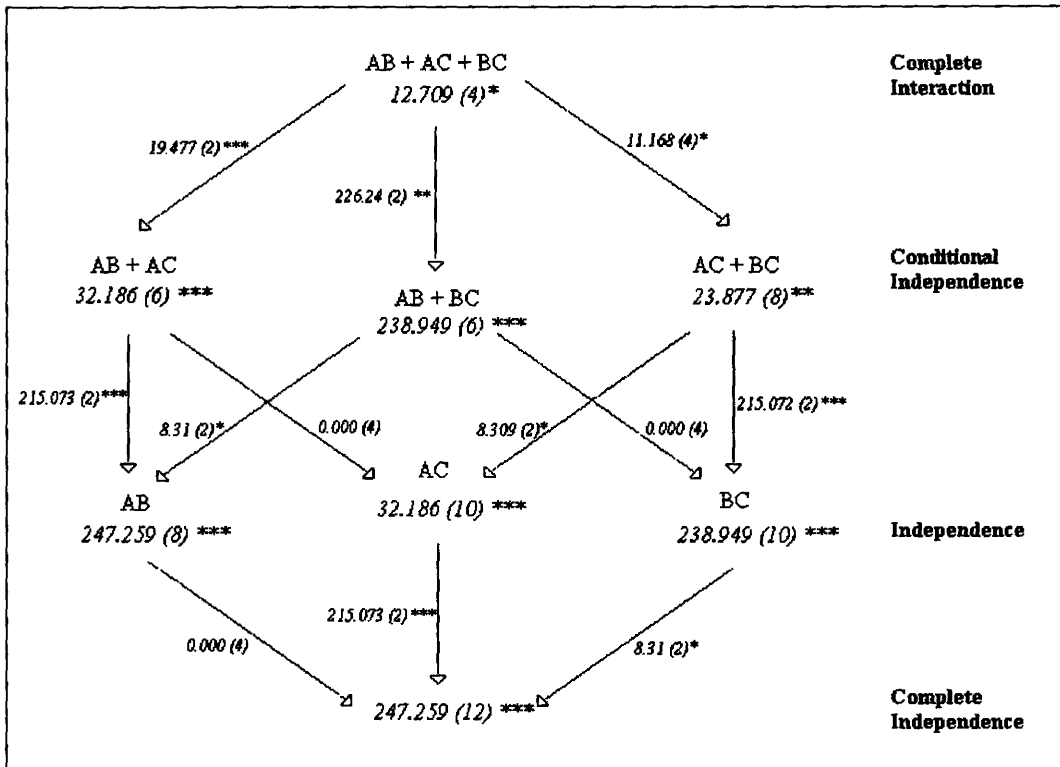

Figure 2b. Representation of the three-way model at the 0.001 significance level with the above-zero temperature set removed from the data set. * Indicates 0.05 significance, **0.01, and ***0.001. At the 0.01 level, the case AC + BC is significant. Figure adapted from Sokal and Rohlf (1995).

substrate (Schill *et al.* 2004). All of the aforementioned culturing methods were attempted with the specimens collected, with poor results. Animals were eventually moved into the 2 mL eppendorf tubes when the sphagnum and agar substrates proved to be unsuccessful. Additionally, the glass vials were found to be an ineffective substrate, as the tardigrades had a difficult time maintaining their purchase on the surface. This study provides a culturing method for *M. harmsworthii*, for which none had been published. The method created in this paper was not ideal, but did suffice in the maintenance of cultures for a couple of months, though viability was low beyond the P_1 generation. It is possible that the

media used to maintain the alga was not sufficiently high enough in nutrients to maintain the tardigrades. Further experimentation should be conducted using an algal medium such as BG-11, which may provide sufficient nutrients.

Freeze/Thaw Study

Though a few studies explore the survivability of tardigrades in relation to cold (Coulson & Birkemoe 2000, Newsham *et al.* 2006, Ramløv & Westh 1992, Sømme & Meier 1995, Wharton & Ferns 1995), this is the first study to investigate the relationship between temperature and time duration as a means to understanding the capabilities of cryobiotic tardigrades. This experiment found that temperature does, indeed, play an important role in the rate of survival and, furthermore, that time spent at a given temperature will also have measurable effects. It was discovered that viability increases with decreasing temperature, and decreases with increased time at temperatures above -80°C. At -80°C, time no longer appears to play a role in survivorship, and it is possible that the tardigrades could remain indefinitely at this temperature. At -20°C, viability was lowest, at 2%, whereas -40°C and –80°C were characterized by 63% and 88% survivability respectively.

When data from all four temperatures (24°C, -20°C, -40°C and -80°C) were included in the statistical analysis, it was clear that all of the variables of time, temperature and mortality interacted to a certain extent, though it was not possible to elucidate the depth of the interaction. Once data obtained from the non-freezing temperature were removed, however, it was then possible to see the

pairwise interactions between the different variables. The term testing the independence of time and temperature from mortality was not significant, and the terms testing the independence of time from temperature and mortality, as well as time and mortality from temperature had a lower G-value in comparison with the other terms (eg. $G = 32.186$ vs. $G = 247.259$). From this, it is clear that though time does play a role in the survival of tardigrades, it is weak in comparison to the effect of temperature on survivability. Temperatures as high as -20°C, likely still allow for chemical reactions to occur within the tardigrade, the temperature possibly not being low enough to induce a cryobiotic state. As these organisms were frozen in the hydrated state, trehalose replacement likely did not play a major role in the maintenance of membrane and protein integrity (Ramløv & Westh 1992). Therefore, as the duration of time spent at a temperature increased, the tardigrades suffered fatal damage as a result of low oxygen levels, high osmolarity and a toxic build-up of chemicals. At -80°C, and even at -40°C, however, temperatures were cold enough to slow metabolic activity within the tardigrade to acceptable levels, inducing a cryobiotic state and limiting damage to key biological systems.

It is remarkable to note that, although *M. harmsworthii* was collected in a temperate habitat, it still possessed a level of viability equal to, and in some cases, surpassing, Arctic and Antarctic tardigrades also frozen at -80°C. Sømme and Meier (1995) found that, in hydrated specimens *Echinscus jenningsi*, mortality increased with duration of exposure from 7 to 150 days at -80°C, while mortalities

of the other two species studied (*Macrobiotus furciger* and *Diphascon chilenense*) did not change. This evidence suggests that the cryobiotic capability is not prevalent at the same level across different tardigrades species. It is of interest then to conduct such freeze/thaw experiments across all tardigrade taxa capable of cryobiosis in an attempt to elucidate the evolutionary origins and patterns of cryobiosis, as well as discerning the differences between taxa resulting in a higher or lower cryobiotic response. Though this study is the first to look at the interaction of temperature and time with respect to cryobiotic capability, it has only scratched the surface in terms of the mechanisms governing cryobiosis and the ability of tardigrades to employ this strategy.

Acknowledgements

This study was originally meant to serve as a comparison of cryobiotic capability between Arctic and temperate tardigrades. The Arctic samples were collected on and around Ward Hunt Island in August of 2007, and were unfortunately lost due to circumstances beyond the authors' control. Despite this, we thank the Northern Scientific Training Program, NSERC, and the Origins Institute for funding travel, as well as W. Vincent and his team from Université Laval (D. Antonaides, J. Veillette, J. Pouliot and D. Sarrazin) for allowing A. Pontefract to accompany them. We thank the Polar Continental Shelf Project (PCSP) for logistical support and Quttinirpaq National Park for allowing the use of their facilities. Thank you to R. Pudritz, B. Evans, R. Schill, P. Chow-Fraser,

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Conclusion

The research in this thesis focused on answering two main questions: 1) what are the strategies available to organisms facing freezing conditions? and 2) what are the temperature-temporal boundaries of cryobiosis? Though the above questions involve disparate bodies of information, answering the first question leads to an important revelation about the relationship between cryoprotective dehydration and cryobiosis, which helps in understanding the limits of cryobiosis.

In Chapter 1, a tardigrade on ice was presented as a way to understand the hazards faced by organisms in such freezing conditions. This was followed by a discussion of the strategies adopted in cold-hardiness, with a detailed review of freeze-avoidance and freeze-tolerance. These considerations led to a discussion of cryobiosis, calling attention to the paucity of information present on this subject as well as the lack of attention the strategy receives in other freeze-tolerance literature, most notably on how it pertains to cryoprotective dehydration. This recently conceptualized term has been studied mainly in well-known cryptobiotic organisms, though there is no mention of cryobiosis in these studies. In a comparison of the mechanisms leading to each "state" (Figure 1), it becomes clear that cryoprotective dehydration is the process leading to the cryobiotic state. That this connection has not been previously made is a testament to the lack of representation that cryobiosis receives in cold adaptation literature. This review, therefore, provides a necessary bridge in the literature between the cold-hardiness

and cryobiotic "camps", to strive for a more unified understanding of cold tolerance.

Chapter 2 presented an experimental analysis of cryobiosis, in an effort to determine what, if any, impact time and temperature have on the viability of cryobiotic tardigrades. Their cryobiotic capabilities were limited at temperatures above -80°C, where time was a key factor – with longer durations resulting in lower survival rates. At -80°C, time no longer constituted a significant factor. When these results are combined with those from previous studies detailing the survival of tardigrades after years spent at -80°C, it becomes evident that this metabolic capacity may be unconstrained under suitable environmental conditions. Indeed, the report for the longest survival of a tardigrade may be found in a paper by Franchesci (1948), in which leg movement in an anhydrobiotic tardigrade, which was hydrated after 120 years of dormancy, was reported (Jönsson & Bertolani 2001). Though that tardigrade did not revive successfully, the report is nonetheless important in displaying the incredible capacity of tardigrades to remain in a dormant state without damage to critical systems. It has been noted that the survival of tardigrades in a dormant state tends to decline in the presence of normal oxygen conditions; however, this phenomenon is not observed in the presence of a vacuum (Jönsson & Bertolani 2001). The implications of this observation are far-reaching. Aside from making tardigrades excellent candidates for space research (Jönsson 2007), this increased

viability in low oxygen environments strengthens the case for the presence of life on other planets.

Author's Note

After the completion of this thesis, Ingemar Jönsson of Kristianstad University, Sweden, successfully revived tardigrades that had been kept in a low Earth orbit for 10 days while being exposed to vaccum and full solar radiation (Jönsson *et al.*. 2008). They are now the first animal to have survived open space.

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Appendix

The strategy of cryobiosis is poorly represented in the literature and very little is known about the mechanisms governing entrance into this latent state, the maintenance of biological systems in cryobiotic organisms and a return to an active state. In an attempt to an answer to these questions, I attempted the first analysis of gene expression during entry into cryobiosis in tardigrades, using *454* pyrosequencing. This form of sequencing allows for an analysis of expression without the use of whole genome sequencing and microarrays.

Methods

RNA Extraction and cDNA Synthesis

Tardigrades were kept at 23°C, 0°C and -20°C for 24 hours in 50 µL of distilled water in 2 mL Eppendorf tubes, with 100 tardigrades per tube. At the time of sampling, tubes were plunged into liquid nitrogen to preserve relative gene expression at that time point. RNA extraction was performed using the TRIzol method outlined by Schill *et al..*. (2004). 200 µL of TRIzol were added to the samples and incubated at room temperature for 5 minutes. 40 µL of chloroform were then added, tubes were shaken rapidly for 15 seconds and then allowed to incubate at room temperature for an additional 5 minutes. Tubes were then centrifuged for 15 min at 12000 g at 4°C. The aqueous supernatant was removed, placed in new tubes, precipitated with 100 µL of isopropyl alcohol and vortexed. Samples were incubated for 10 minutes at room temperature and then centrifuged

for 20 minutes at 12000 g at 4°C. The supernatant was decanted and the pellet rinsed in 500 μL of ice-cold 75% ethanol and vortexed. Samples were then centrigued for 5 min at 7500 g at 4°C. Supernatant was again decanted and the previous step repeated. After a pulse-spin for 10 s, any remaining alcohol was allowed to evaporate in a fume hood. The pellet was then reconstituted in 10 μL of PCR quality water and quantified using a NanoDrop.

For first-strand cDNA a working RNA dilution was made at 5 μg/ μL using PCR quality water. A master mix was made containing 1 μL of 10X DNase I reaction buffer (Sigma #R6273), 1 μL DNase I, Amplification Grade (Sigma #D5307), and 6 μL of PCR quality water. This mix was added to 2 μL of diluted RNA and incubated at room temperature for 15 minutes. The sample was then inactivated at 65°C for 10 minutes and then chilled on ice for 2-3 minutes. 1 μL of AP primer (oligo DT) was added along with 1 μL of 10 mM dNTP mix. Sample was incubated at 65°C for 5 minutes and then chilled on ice for 10 minutes. Then a mix of 4 μL of 5X 1st strand buffer, 2 μL 0.1 M DTT, 1 μL RNase out (Invitrogen; 40 unit/ μL; #10777-019), and 0.3 μL of SuperscriptII RNase H- Reverse Transcriptase (Invitrogen; 200 U/ μL; # 18064-014), were added. Tubes were then incubated at 42°C for 50 minutes, and then inactivated at 70°C for 5 minutes.

454 Pyrosequencing

Unfortunately, pyrosequencing could not be completed due to issues with back-orders for the materials needed to perform the sequencing, as well as delays with equipment. It is hoped that this project will be able to be completed in the near future.

Schill RO, Steinbruck GHB, Kohler HR. 2004. Stress gene (*hsp70*) sequences and quantitative expression in *Milnesium tardigradum* (Tardigrada) during active and cryptobiotic stages. *The Journal of Experimental Biology* 207: 1607-13

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