EXTREME TOLERANCE IN THE EUTARDIGRADE SPECIES *H. DUJARDINI*

EXTREME TOLERANCE IN THE EUTARDIGRADE SPECIES *HYPSIBIUS DUJARDINI*

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TITLE: Examining the Upper and Lower Limits of Extreme Tolerance in the Eutardigrade Species *Hypsibius dujardini*

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LAY ABSTRACT

While interest in tardigrade extreme tolerance research has increased over the last decade, many research areas continue to be underrepresented or non-existent. And, while recognized tardigrade species have been increasing steadily in number, fundamental biological details, like individual life history traits, remain unknown for most. The main objectives in this thesis therefore were to survey the life history traits for the freshwater tardigrade species *Hypsibius dujardini*, increase knowledge about its extreme-tolerance abilities and describe its utility in astrobiological and biological studies. Research involved tardigrade tolerance to hypergravity, pH levels and radiation exposure (and associated radiation-induced bystander effects) as well as responses to temperature changes during development. Findings reported in this dissertation provide new data about *H. dujardini*, thereby narrowing the information gap that currently exists in the literature for this species.

ABSTRACT

Tardigrades are microscopic animals that can survive exposure to multiple extreme conditions. This remarkable ability makes them suitable laboratory model organisms for conducting biological to astrobiological research. Whereas tardigrade extreme-tolerance research has been focused predominantly on their ability to endure extreme desiccation, responses to other extraordinary conditions (i.e. hypergravity, pH, radiation and low temperature) remain un-described. These extreme tolerance research areas, in addition to life history traits, were documented in our studies on the eutardigrade species *Hypsibius dujardini*.

We found that specimens tolerated extreme g-equivalent forces (i.e., 16060*g*) and radiation levels (i.e. 5 kGy), with decreased survivorship at increased accelerations and radiation doses. Radiation induced bystander effects (RIBEs) manifested as a threshold response, with the threshold value between 3 and 5 kGy. Extreme acidic (pH 1 and 2) and alkaline (pH 11 to 14) conditions caused death instantaneously, while exposures to pH 4, 6, 8, 9, and 10 were tolerated. Tardigrade eggs reared at 0 °C for 4 days developed relatively slowly, diminishing their biological age relative to their chronological age. Extending cold exposure (0 °C) time (days = 10, 20 and 40) decreased incubation time (days = 3, 2 and 1, respectively) at 22 °C; lengthening cold exposure time led to decreased growth in juveniles and lowered survivorship in adults, suggesting that costs are associated with increasing incubation time at 0 °C.

Tolerance to hypergravity, large radiation doses and a wide-range in pH conditions support the notion that tardigrades are suitable organisms for astrobiological research, particularly in exploring parameters associated with potential transfer and habitability in extreme environments. RIBEs in adult tardigrades and the ability for tardigrade embryos to alter their 'biological clocks' based on exposed cold temperature duration have applications in biological research. Characterizing molecules involved in bystander signaling and response and biological clock adjustments during development could have important implications for improving biological practices such as radiotherapy and cryopreservation.

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LIST OF ABBREVIATIONS

^{60}Co	Cobalt 60
BYST3	Bystander (unirradiated) animals exposed to a single individual irradiated at 3 kGy level
BYST5	Bystander (unirradiated) animals exposed to a single individual irradiated at 5 kGy level
CO_2	Carbon dioxide
CON	Control
Dsup	Damage suppressor
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
EP	Egg production
GPa	Gigapascals
Gy	Grays
H. dujardini	*Hypsibius dujardini*
H_2S	Hydrogen sulfide
H_2SO_4	Sulfuric acid
HSP(s)	Heat shock protein(s)
kGy	kilograys
KOH	Potassium Hydroxide
LA_{50}	Lethal acceleration dose at which 50% of the population dies
LD_{50}	Lethal dose at which 50% of the population dies
LEA proteins	Late embryogenesis abundant proteins
M. tardigradum	*Milnesium tardigradum*
MPa	Megapascals
pH	Power of hydrogen
R. coronifer	*Richtersius coronifer*
RAD3	Populations irradiated at the 3 kGy level
RAD5	Populations irradiated at the 5 kGy level
RIBEs	Radiation induced bystander effects
RoTaRad	Rotifers, tardigrades and radiation
TARSE	Tardigrade resistance to space effects
TDP	Tardigrade-specific intrinsically disordered proteins
UV	Ultraviolet
UVC	Ultraviolet C

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

First discovered in 1773 by German zoologist Johann Goeze as “kleiner Wasserbär”, meaning ‘little water bears’, and named in 1776 by Italian biologist Lazzaro Spallanzani as “Tardigrada”, which translates to ‘slow stepper’, tardigrades are microscopic multicellular organisms renowned for their abilities to survive extreme environmental conditions.

1.1 Tardigrade Habitats

Tardigrades are invertebrates that often are described as cosmopolitan in their distribution; some species exhibit limited tolerance and inhabit specific environments (Nelson et al. 2015). Tardigrades have been documented as inhabiting marine, brackish water, freshwater, limnoterrestrial and terrestrial environments (Nelson et al., 2015). Most species are found in terrestrial environments, and true limnic species are few in number; some limnoterresterial species can inhabit terrestrial and freshwater environments (Nelson et al., 2015). Although typically found in moist environments like dampened mosses, liverworts and lichens, tardigrade populations also have been documented as living along shorelines and in lakebeds, forest canopies, sand dunes, subterranean caves and cryoconite holes (Chang et al., 2015; Nelson et al., 2015; Devasurmutt & Arpitha, 2016).

1.2 Tardigrade Phylogeny

Closely related to Onychophora and Arthropoda and, as an ecdysozoan, sharing some derived traits with Nematoda, water bears are classified formally in the phylum Tardigrada, which contains more than 1200 known species (Degma et al., 2015). The Tardigrada can be subdivided into two groups: Eutardigrada and Heterotardigrada (Nelson et al., 2015). The Class Heterotardigrada includes marine and terrestrial species; some marine and all terrestrial species are characterized by thickened, segmented dorsal (i.e. armored) cuticles, with limbs that terminate in claws or discs and digestive tracts that terminate in an anus (Nelson et al., 2015). The Class Eutardigada includes species characterized by dorsal cuticles that are thin and undivided (i.e. unarmored), live in freshwater and terrestrial environments and have legs that terminate in claws and digestive tracts that end in a cloaca (Nelson et al., 2015). The phylogenetic relationships between the two classes (Heterotardigrade; Eutardigrada) and their orders (Arthrotardigrada and Echiniscoidea; Apochela and Parachela) are supported by morphological (e.g. Ramazzotti and Maucci, 1983; Nichols et al., 2006) and molecular data (e.g. Garey et al., 1999; Nichols et al., 2006; Bertolani et al., 2001). A third class, Mesotardigrada was established based on the discovery of a single species (*Thermozodium esakii*) reported from a hot spring in Japan by Gilbert Rahm in 1937. Its only known habitat later was lost to an earthquake and, since, no other species belonging to the class has been recorded. As such, while

member species might exist, the class Mesotardigrada often is considered as 'of dubious status' (Grothman et al., 2017).

1.3 Tardigrade Morphology

All tardigrades share a distinctive body plan comprising 5 segments. The head, located anteriorly, can contain two eyespots (in most eutardigrades and some heterotardigrades; Nelson et al., 2015), the 'brain' and the mouth (Chang et al., 2015). During feeding, tardigrades use their stylets – two needle-like mouth apparatus – to pierce objects (Ruppert and Barnes, 1994). Whereas most tardigrade species are herbivorous, some species are carnivorous (e.g. feed on nematodes and rotifers; Schill et. al., 2011) or detritivorous (i.e. feed on debris and dead material; Devasurmutt & Arpitha, 2016). Connected to the mouth is the buccal tube, which can vary in size depending on the diet, with carnivorous tardigrades having a broader buccal tube than do herbivorous tardigrades (Nelson et al., 2015). Each among the remaining 4 body segments contains paired stubby legs that terminate in claws or adhesive discs. The body is covered by cuticle, which sits atop epidermis and comprises chitin, mucopolysaccharides, proteins and lipids (Ruppert and Barnes, 1994; Somme, 1996). The cuticle, stylets, claws, discs as well as the lining in the foregut and hindgut are shed periodically throughout life (Ruppert and Barnes, 1994). Each tardigrade also has a complete digestive system (i.e. a foregut, a midgut and a hindgut; Dewel et al., 2003) but lacks respiratory and circulatory systems. Respiration instead is accomplished via gas exchange while circulation occurs via a haemocoel – an

open system that allows fluids to circulate throughout the body cavity (Ruppert and Barnes, 1994). Excretion occurs through a variety of modes, at the buccal glands during molting and at the lining of the midgut or through excretory glands (i.e. eutardigrades have three Malpighian glands that function in excretion; Nelson et al., 2015). Tardigades also have a simple nervous system, wherein three nerve tissue bands connect the dorsal tripartite brain to the subpharyngeal ganglion and nerve tracts connect four ventral ganglia (Persson et al., 2012).

1.4 Tardigrade Reproduction

Although sexual dimorphism is unpronounced among tardigrade species, males generally are considered to be smaller than females (Nelson & Marley, 2002). Depending on the species and the colonized environment, tardigrades can undergo sexual or asexual reproduction modes (Bertolani, 2001). Most tardigrade species are dioecious – each individual contains a male or female reproductive system (Ruppert and Barnes, 1994). Such gonochorism is the condition for most marine tardigrade species, with hermaphroditism cited only once and parthenogenesis thought to be absent (Bertolani, 2001; Nelson et al., 2015). Limnoterrestrial tardigrade species, however, are thought to have adopted several reproductive modes (i.e. dioecy, hermaphroditism and parthenogenesis; Bertolani, 2001). When males are present in a species, tardigrades can reproduce sexually. Males either fertilize eggs, which are laid inside (i.e. *Milnesium tardigradum)* or outside (i.e. *Macrobiotus hufelandi)* female molted cuticles (external fertilization), or deposit sperm into female reproductive tracts

before molting is complete (internal fertilization) (Bertolani, 2001; Glime, 2013; Ruppert and Barnes, 1994). Some tardigrades species reproduce via self-fertilization or simultaneous hermaphroditism – individuals possess male and female reproductive organs but outcross (Bertolani, 2001; Nelson & Marley, 2002). In some species, where simultaneous hermaphrodites are absent and males are unknown or uncommon (e.g. *Hypsibius dujardini*), tardigrades reproduce asexually via parthenogenesis – offspring development from unfertilized eggs (Glime, 2013). Among known parthenogenetic modes, tardigrades have been documented to reproduce only via thelytokous parthenogenesis (Bertolani, 2001). Depending on the species, as many as 30 eggs can be laid at one time, taking typically 2 weeks to hatch (Ruppert & Barnes, 1994).

2. CRYPTOBIOSIS

Given that the term ‘anabiosis’ – exhibiting no signs for life, yet being viable under the right conditions – could be misinterpreted easily with ‘abiotic’ and ‘abiogenesis’ (i.e. death), Keilin (1958) proposed the term ‘cryptobiosis’ to describe the period in which an organism enters a latent state where any sign for life become undetectable (e.g. an ametabolic state). Tardigrades can become cryptobiotic when surrounding conditions become suboptimal for active survival (Bertolani et al., 2004; Nelson, 2002). Tardigrades have been recognized formally as being able to engage in at least 4 cryptobiotic processes: anhydrobiosis

(response to desiccation), cryobiosis (response to low temperatures), osmobiosis (response to increased solute concentrations) and anoxybiosis (response to low oxygen levels) (Bertolani et al., 2004; Somme, 1996). These 4 types also are considered formally in this introductory section, although other processes in tolerating extreme conditions are possible and considered subsequently.

2.1 Anhydrobiosis

Metazoans like rotifers, nematodes and tardigrades can tolerate desiccation for prolonged periods (Schill et al., 2009). Anhydrobiosis is the term used to describe the process by which an organism enters an ametabolic state when surrounding conditions become too arid (Hengherr 2009; Horikawa 2012). Anhydrobiotic tardigrade bodies adopt barrel-like appearances, which therefore are referred to as ‘tuns’ (Horikawa et al., 2007). During tun formation, tardigrades undergo morphological contortions including contracting the cuticle layer as well as invaginating the anterior segments and lobopodous legs to reduce surface area (Somme, 1996). Tardigrades also promote wax extrusions to the cuticle surface to minimize water transpiration (Crowe, 1972). These morphological modifications combined with molecular changes enable anhydrobiotic tardigrades to reduce their total body water content to 1%-3%, protecting themselves against environmental extremes (Crowe, 1972; Horikawa et al., 2008). Following rehydration, normal metabolic function resumes – a response that can take anywhere from 4 minutes to a few hours to manifest (Schill et al., 2009; Horikawa, 2012; Glime, 2013).

Successful revival following desiccation depends on slow dehydration (Somme, 1996; Crowe, 1972). In 1972, Crowe showed increased survivorship among active tardigrades that were desiccated at relative humidities greater than 70% while anaesthetized tardigrades desiccated under identical conditions died. Anaesthetized tardigrades were unable to form tuns during desiccation, indicating that active metabolism is necessary for successful tun formation. While several studies have started to elucidate the biochemical mechanisms associated with anhydrobisis, it has yet to be elucidated completely. Up-regulated cell protectants like trehalose, glycerol, heat shock proteins (HSPs) and late embryogenesis abundant (LEA) proteins have been suggested to aid cellular stabilization during desiccation (e.g. Crowe, 1972; Glime, 2013; Schill et al., 2009; Boothby et al., 2017). Tardigrade-specific intrinsically disordered proteins (TDP) also recently have been linked to anhydrobiosis, where high expression levels for TDP genes were documented in multiple tardigrade species that underwent desiccation (Boothby et al., 2017).

Given that water helps in maintaining integrity in cellular structures and membranes, two hypotheses have been proposed to explain macromolecular stabilization during desiccation (Hengherr et al., 2009). The water replacement hypothesis states that hydrophilic molecules, such as non-reducing sugars (i.e. trehalose), accumulate during desiccation and interact with existing

macromolecules (via hydrogen bond interactions) to replace water (Hengherr et al., 2009; Schill et al., 2009). The vitrification hypothesis states that hydrophilic molecules enter a glassy state, thereby preventing denaturation, aggregation and disintegration through immbolization (Hengherr et al., 2009; Schill et al., 2009). The hypotheses are non-mutually-exclusive, and, therefore, some overlap between the two explanations ultimately might provide the most appropriate description.

2.2 Cryobiosis

When surrounding conditions become too cold for active survival, tardigrades undergo another cryptobiotic mode, known as cryobiosis (Nelson & Marley, 2000; Wright et al., 1992). Entering a cryobiotic state allows an individual to survive freezing and thawing (Nelson et al., 2015). Given these properties, tardigrades are found commonly in polar regions, at altitude on mountains and in glacial cryoconite holes (Nelson, 2002; Nelson et al 2015).

For decades, researchers have tried to define the upper limit for tardigrade cold-tolerance. In the early 1920s, Rahm reported that the tardigrade species *Ramazzottius oberhaeuseri, Milnesium tardigradum* and *Macrobiotus* sp. survived temperatures as low as -253°C in a hydrated state (Somme & Meier, 1995). This suggests that the ability to tolerate extremely cold temperatures is independent from the mechanisms associated with anhydrobiosis, despite the common association (e.g. Ramlov & Westh, 1992). In 1950, Becquerel

demonstrated survival by desiccated specimens from two eutardigrade species following exposure to temperatures near absolute zero (-273 °C). Tun formation is unnecessary for cryobioisis, in distinction to the situation with anhydrobiosis (Crowe & Madin, 1974; Crowe, 1975; Sømme, 1996; Halberg et al., 2013). In 1992, Ramlov and Westh showed that hydrated specimens from *Adorybiotus coronifer* survived cooling to -196 °C over a broad range in cooling rates, with increased survivorship at lower cooling rates (i.e. < 5-10 °C/minute). More-recent publications, however, have reported that desiccated tardigrades have high recovery rates compared to their hydrated counterparts at low temperatures. Somme and Meier reported in 1995 that, whereas the species *Echiniscus jenningsi, Macrobiotus furciger* and *Diphascon chilenense* exhibited high survival rates following exposure to -22 °C in hydrated and dehydrated states, only dehydrated specimens from all three species survived exposure to -180 °C. Horikawa also showed that dehydrated tardigrades survived with greater frequency at -196 °C than did hydrated individuals (2008). These recent findings suggest that, although tardigrades are freeze-tolerant, they are more tolerant to cold temperatures when desiccated (Horikawa, 2013).

2.3 Osmobiosis

Osmobiosis is induced by increased osmotic pressure and the least-studied cryptobisis in tardigrades (Nelson, 2002; Wright et al., 1992). Like cryobiosis, tun formation is unnecessary for osmobiosis; however, entering a tun state does provide increased survival (Wright et al., 1992; Nelson, 2002). Active and

asphyxiated tardigrades have shown tolerance to high salinity water, however, prolonged immersion in moderate to high salinity water has been shown to impart detrimental effects (Wright et al., 1992). The biochemical processes involved in osmobiosis have yet to be characterized.

2.1 Anoxybiosis

Anoxybiosis has been documented in marine, limnic and terrestrial tardigrades (Nelson et al., 2015). Most researchers consider anoxybiosis as a non-cryptobiosis type, as it involves water uptake rather than loss (Wright et al., 1992; Glime, 2013). Anoxybiosis is induced by low oxygen or increased aqueous CO_2 and H_2S (Wright et al., 1992). Osmoregulation is lost when oxygen levels are low, causing water to enter cells; as a result, anoxybiotic tardigrades become turgid and stationary (Wright et al., 1992; Glime, 2013). Tolerance to anoxybiotic conditions is species dependent – some species survive for only a few hours, whereas others can survive for up to 6 months in sealed vials (Nelson 2002; Wright et al., 1992; Glime, 2013). Long-term tolerance to anoxybiosis has yet to be resolved.

3. TARDIGRADE EXTREME-TOLERANCE RESEARCH

Renowned for the ability to engage in the 4 cryptobiosis modes among species in the phylum, tardigrades also have been recognized to tolerate other extreme environmental conditions, such as high ionizing radiation doses, hydrostatic pressures and temperatures. Tardigrades additionally have become the first

animals to have survived simultaneous exposure to the vacuum and solar radiation in outer space (Jönsson et al., 2008). Unlike other animals that have been proposed for space exploration, tardigrades are small in size (ranging 0.1 to 1mm in size) and therefore require only simple facilities for space experimentation (Guidetti et al., 2012). Moreover, given the short life cycles characterizing many tardigrade species, researchers are able to examine effects from space environments over many generations. For these reasons, tardigrades make suitable laboratory models for biological and astrobiological research.

3.1 Tolerance to High Temperatures

As early as 1842, Doyère reported that anyhdrobiotic specimens from the tardigrade species *Macrobiotus hufelandi* survived exposure to temperatures as high as 125 °C (Ramlov & Westh, 2001). A few decades later, Rahm reported that dehydrated specimens from *Milnesium tardigradum* and *Ramazzotius oberhaeuseri* survived exposure to temperatures as high as 151 °C (Horikawa, 2013). In 2001, Ramlov and Westh showed that specimens from *Adorybiotus (Richtersius) coronifer* possessed relatively moderate tolerance to high temperatures, with decreased survivorship beyond 70 °C. Why temperatures at which different tardigrade species can be resurrected successfully varies is unknown. One obvious possible explanation for the discrepancy is that different species invoke different mechanisms. Alternatively, the duration at which tardigrades were exposed to high temperatures (Doyère: a few minutes; Rahm: 35 minutes; Ramlov & Westh: 60 minutes) might have been a factor in the

different responses. Anhydrobiosis also might play a role in enabling tardigrades to endure high temperatures, as desiccated specimens from *Ramazzottius varieornatus* showed 90% recovery following post-heating treatment (90 °C for 1 hour) while hydrated individuals died (Horikawa et al., 2008). Tolerance to high temperatures is a research area requiring attention, especially for astrobiological applications, such as lithotranspermia scenarios (e.g. Vasanthan et al. 2017).

3.2 Tolerance to Radiation

Tardigrades are renowned for the ability to tolerate ionizing radiation in high doses. In astrobiology, researchers are interested in radiation-tolerant animals, as they are more likely to survive exposure to the UV radiation and cosmic radiation that would be encountered in outer space and, so, transpermia scenarios. In radiation biology, the mechanisms that enable tardigrades to tolerate ionizing radiation could have application in preventative measures with practitioners and treatment measures with patients. Radiation induces double strand breaks in DNA, thereby leading to cell damage and ultimately cell death (Jonsson et al., 2005). Understanding DNA repair mechanisms in radiation-tolerant animals, like tardigrades, could prove to be useful in enhancing current radiation therapies and technologies against diseases like cancer and aging (Nilsson et al., 2010).

Tardigrades have shown tolerance to many radiation types including gamma, UV, cosmic, heavy ion, and proton radiation (Jonsson et al., 2005; Altiero et al., 2011;

Persson et al., 2011; Horikawa et al., 2008; Nilsson et al., 2010). Tardigrade tolerance to radiation has been documented to be dose dependent, decreased survivorship with increased radiation (Jonsson et al., 2005; Horikawa et al., 2006). The means by which high radiation levels are tolerated are known incompletely; efficient DNA repair systems are believed to be involved, including the genes *RAD51* (induced by gamma radiation; Beltran-Pardo et al., 2013), *phrA* (induced by UVC radiation; Horikawa et al., 2013) and the tardigrade-specific damage suppressor gene *Dsup* (induced by X-ray radiation; Hashimoto et al., 2016). The LD_{50} – the lethal dose at which 50% of individuals in an irradiated population die – varies among species. The variations are attributed differences in radiation sources, tardigrade species tested and whether specimens were hydrated or desiccated during irradiation (Nilsson et al., 2010). Researchers have documented that hydrated specimens in some species are more tolerant to radiation than are desiccated specimens (e.g. Horikawa et al., 2006) while other species return the opposite result (e.g. Horikawa et al., 2013). Still other researchers have demonstrated that an anhydrobiotic state during irradiation confers no particular advantage to survival post-irradiation (e.g. Horikawa et al., 2008). Studying the mechanisms that allow tardigrades to endure high radiation doses may be useful in understanding why some species are more tolerant to radiation in the hydrated state than the desiccated state and vice versa.

3.3 Tolerance to High Hydrostatic Pressure

Tardigrades also have been shown to tolerate exposure to high pressures. This is remarkable considering that high pressure has damaging effects on biological material, including DNA, proteins and cellular membranes (Seki & Toyoshimo, 1998). Most organisms, including bacteria, cannot survive pressures beyond 300 MPa (Seki & Toyoshimo, 1998). The terrestrial tardigrade species *Macrobiotus occidentalis* and *Echiniscus japonicas* survived exposure to 600 MPa – equivalent to 6000 times atmospheric pressure at sea level (Seki & Toyoshimo, 1998). Additional experiments have shown that tardigrades can survive exposure to pressures as high as 1.2 GPa and 7.5 GPa, the latter equivalent to pressures 180 km below Earth surface (Horikawa et al., 2007; Ono et al., 2008).

4. PROJECT INTENT

The objective of the thesis was to elucidate tardigrade extreme-tolerance by characterizing the upper and lower limits for survivability to extreme conditions and describe relevant applications to astrobiology and biology. To characterize tardigrade extreme-tolerance, laboratory experiments were performed using the parthenogenetic eutardigrade species *Hypsibius dujardini*. Tardigrade extreme-tolerance experiments were focused primarily on factors that are underrepresented and in one case nonexistent in literature. Areas of research included tardigrade tolerance to hypergravity mimicked through exposure to extreme g-equivalent accelerations (Chapter 3); acidic and alkaline pH levels

(Chapter 4); radiation (and radiation-induced bystander effects) (Chapter 5) and cold tolerance during early development (Chapter 6). In addition to extreme-tolerance research, a life history project for *H. dujardini*, chronicling its lifecycle and reproductive activity, also was undertaken (Chapter 2). With its genome recently sequenced, increased interest has emerged in the scientific community to better understand *H. dujardini* as a model organism for research inquiry.

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CHAPTER 2: LIFE HISTORY TRAITS OF THE EUTARDIGRADE SPECIES *HYPSIBIUS DUJARDINI* UNDER LABORATORY CONDITIONS

2.1 PREFACE

This chapter contains the manuscript in preparation titled “Life History Traits of the Eutardigrade Species *Hypsibius dujardini* Under Laboratory conditions*”* authored by Vasanthan T. and Stone J.

Whereas recognized tardigrade species has increased in number over the years, individual life history traits for most are unknown. The present study investigated the life history traits ffor the parthenogenetic eutardigrade species *Hypsibius dujardini*, chronicling its growth, reproductive activity and lifespan under laboratory conditions. While eggs exhibited 100% hatching success, approximately 10% among individuals failed to reach sexual maturity and 20% among individuals experienced growth impediments, suggesting that vulnerability to growth defects and fatality occur soon after birth. Maximum age achieved in adults was 75 days, with individuals laying on average 42 eggs. Egg production increased with age, with eggs laid per exuvium maximizing on days 18, 21 and 24, post hatching.

2.2 CONTRIBUTIONS

Tarushika Vasanthan performed the experiments, wrote the manuscript and generated Tables 1 and 2 and Figures 1 through 3. Jonathon Stone provided a great deal of direction and feedback to enhance the quality of the paper.

Title Page

Life History Traits of the Eutardigrade Species *Hypsibius dujardini* under

Laboratory Conditions

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1. Introduction

The phylum Tardigrada contains over 1200 known species (Degma et al, 2016). Cosmopolitan in distribution, tardigrades can be found in marine, freshwater and terrestrial environments (Devasurmutt & Arpitha, 2016). While recognized species have been increasing in numbers over the years, scant data are available for individual life history traits. And while tardigrade tolerance has been investigated for a variety of factors, fundamental biological phenomena like embryonic development, reproduction and lifespan have been described in relatively few species (i.e. < 5%; Schill et. al., 2013).

We examined life history traits for the freshwater eutardigrade species *Hypsibius dujardini* (Doyère, 1840). This species was chosen because it is a suitable model organism for evolutionary-developmental studies (Gabriel et al., 2007), characterized by a short generation times, stereotypical embryonic stages and easy continuous culturing under laboratory conditions. Discrepancies among

results in recent *H. dujardini* genome sequencing projects (the first tardigrade genome to have been published) have received much attention in the scientific community (e.g. Boothby et. al., 2015; Koutsovoulos et al., 2016; Yoshida et al., 2017). Researchers additionally have made great advances in exploring the effects from exposure to environmental extremes such as ionizing radiation, radiation-induced bystander effects, desiccation, temperature and hypergravity on *H. dujardini* (Beltrán-Pardo et. al., 2015; Fernandez et. al., 2016; Boothby et al., 2017; Guidetti et al., 2011; Vasanthan et al., 2017). Whereas *H. dujardini* research has been concentrated on extreme tolerance, life history traits like hatching success, fecundity rates and maximum lifespan are unavailable (Schill, 2013). The present study was implemented to provide those data.

2. Materials and Methods

2.1 Tardigrade Sampling

To characterize life history traits in *Hypsibius dujardini* (Z151, Sciento, Whitefield, Manchester), approximately 100 egg-laying mothers were isolated. Individuals were monitored under light microscopy (Nikon SMZ1000) until eggs were deposited and mothers had exited their exuvia. Eggs were isolated and housed in 1.5 mL microtubes containing 15 µL of spring water (Aberfoyle, Puslinch Ontario; Nestlé Pure Life). Exuvia typically contained a single embryo, but some containing 2 and 3 embryos also were used. To maintain synchronous development among individuals, only eggs collected from mothers within 1 hour post laying were used. Seventy eggs were sampled. Eggs were monitored daily

under light microscopy until hatching. Hatchlings were stored individually in 1.5 mL microtubes and monitored daily until day 6, when individuals began ovipositing. Adults were monitored every three days until death. To score growth, egg production and survivorship, individuals were transferred from microtubes onto a 35-mm Petri dish (Falcon) and observed by light microscopy. Survivorship data were recorded every three days, when water (100 µL) and algal food (10 µL; *Chlorococcum* sp.*;* A68, Sciento, Whitefield, Manchester) were replenished and used microtubes were replaced with new microtubes. Eggs produced by individuals were removed.

2.3 Body Length Measurements

An additional 12 eggs were collected to measure individual body lengths for hatchlings (before first feeding), juveniles (day 3 post hatching) and adults (days 6 and 8 post hatching). Measurements were taken via stereomicroscope eyepiece reticule (Wild Heerburg, M3C) and calibrated with a stage micrometer. Egg measurements were taken after mothers had exited exuvia. Body lengths were measured during walking when specimens were extended maximally. As described by Suzuki (2003), body length measurements were taken as the distance from head to juncture on the posterior-most segment with legs.

2.4 Tardigrade Life History Traits

The following life history traits were documented in 70 individuals: gestational period (days), individual growth, age at fist oviposition (days), ovipositions per

lifespan, eggs laid per molt, maximum clutch size, eggs laid per lifetime, average lifespan (days) and maximum lifespan (days). Where applicable, values were reported with means and ranges.

3. Results and Discussion

3.1 Hatching Success and Growth

All eggs (N=70) hatched on day 4 post laying, confirming that *H. dujardini* embryos take 4 days to complete embryogenesis (Gabriel et al. 2007). Analogous to embryonic development in *Milnesium tardigradum* where eggs become more transparent with development, *H. dujardini* embryos transitioned from dark brown to a more-transparent hue as development ensued (Suzuki, 2003). While 100% hatching success was observed in this study, previous pilot projects indicated that successful hatching rates typically are lower (approximately 90%). Whereas hatching was synchronous, hatchlings were asynchronous in growth, with 21.43% (N=15/70) and 11.43% (N=8/70) among specimens exhibiting delayed or no growth by day 6, respectively (Figure 1). The impaired or lacking growth might be explained by malnutrition. Given their semi-transparent bodies, guts in *H. dujardini* are visible when magnified. Unfilled guts observed among individuals that exhibited delayed or no growth suggests that they either began feeding at later stages or had failed to feed entirely. Similar observations have been documented for the tardigrade species *Hypisibius convergens* (Bauman, 1961) and *Paramacrobiotus tonollii* (Lemloh et al., 2011), where survival in hatchlings was attributed to adequate food intake during the first day (i.e. 24 hours). Given

the absence in locomotion, individuals that failed to grow were removed from the study.

3.2 Body Measurements

Day 1 hatchling (unfed; N=12 individuals) body lengths ranged from 119.0 µm (minimum) to 142.8 µm (maximum), averaging 138.0 µm. Day 6 and day 8 post hatching adult (fed; N=12) body lengths measured on average 220.15 µm (minimum = 190.4 µm; maximum = 238.0 µm) and 263.8 µm (minimum = 238.0; maximum = 285.6), respectively. These measurements are comparable to those described previously (McInnes, 2007), ranging from 145 µm to 280 µm. While correlation between growth and molting was evident initially (i.e. days 1 through 8 post hatching) – adults were approximately double hatchling size, no correlation was observed once individuals began ovipositing. Gravid females were observed to be slightly longer than were non-egg-carrying females; this difference, however, was only transitory, as pregnant mothers returned to their original sizes once eggs had been deposited. *H. dujardini* adults are small relative to adults in most tardigrade species (Table 1).

3.3 Oviposition, Clutch Size and Molting

By day 6, almost two-thirds (65.7%) among the specimens had deposited eggs into shed exuvia for the first time. While sexual maturity in tardigrades often is described as the time point at which first eggs are laid (i.e. oviposition), this constitutes a slight overestimation (Schill, 2013). *H. dujardini* typically can be

observed carrying eggs approximately one day before eggs are released and deposited into exuvia; thus, a more accurate designation for achieving sexual maturity in *H. dujardini* would be between days 5 and 6 post hatching. First egg production generally yielded between one and three eggs; one individual produced 6 eggs in its first round. Whereas clutch size in *H. dujardini* has been reported previously to range between 1 (minimum) and 10 (maximum) eggs (Gabriel et al., 2007), the maximum output observed in a single molt by any individual in the present study was 8 eggs (Table 2). This number is small compared to values associated with other tardigrades species, which can lay 30 to 40 eggs; thus, exuvia size may dictate egg production in *H. dujardini* (Glime, 2013).

On average, individuals molted approximately 11 times throughout life (Table 2). While egg laying and molting almost always occurred synchronously, molting also occurred without egg production during the earlier and later stages. Thus, the average molts per lifecycle is estimated to be slightly higher than 11. Molting without eggs during early stages may be attributed to growth. Like many tardigrade species, *H. dujardini* is eutelic (Bertolani, 1970). Growth occurs through cell enlargement, where individuals increase body size by increasing cell sizes. Molting without eggs during later stages may result from senescence, where the ability to generate eggs decreases with increased biological age. While lifetime ova production ranged from 15 to 59 eggs, *H. dujardini* specimens laid, on

average, 42 eggs. Eggs laid per exuvium steadily increased with time, with egg production peaking on days 18, 21 and 24, after which it declined gradually (Figure 2).

3.4 Longevity

The maximum age achieved, attained by 5 individuals, was 75 days (Table 1; Figure 3). Although a slight decrease in survivorship was observed immediately after hatching, survival rates were high during early and middle life history stages. Invertebrates typically follow survivorship curves with increased egg production and mortality during early life history stages. The survivorship curve for *H. dujardini*, however, followed age-specific survival, where survival rates were higher during early and middle stages with a steep decrease at later stages. Explanations for this pattern include species-specific characteristics like egg provisioning to enable rapid development and population-specific characteristics like maintenance under constant laboratory conditions. The protection provided by exuvia particularly might explain high hatching rates (i.e. 90-100 %). High survival rates in freely laid eggs in other tardigrade species (e.g. *Paramacrobiotus richtersi)* can be attributed to ornamentation, which includes pores and reticulation patterns (Schill, 2013; Thorp and Covich, 2009).

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7. Figures and Tables

Figure 1

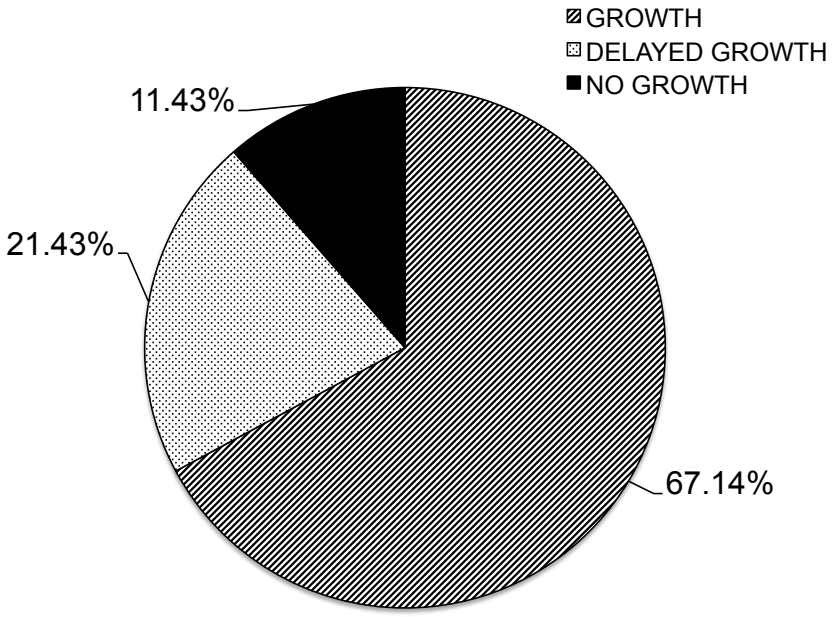

Figure 2

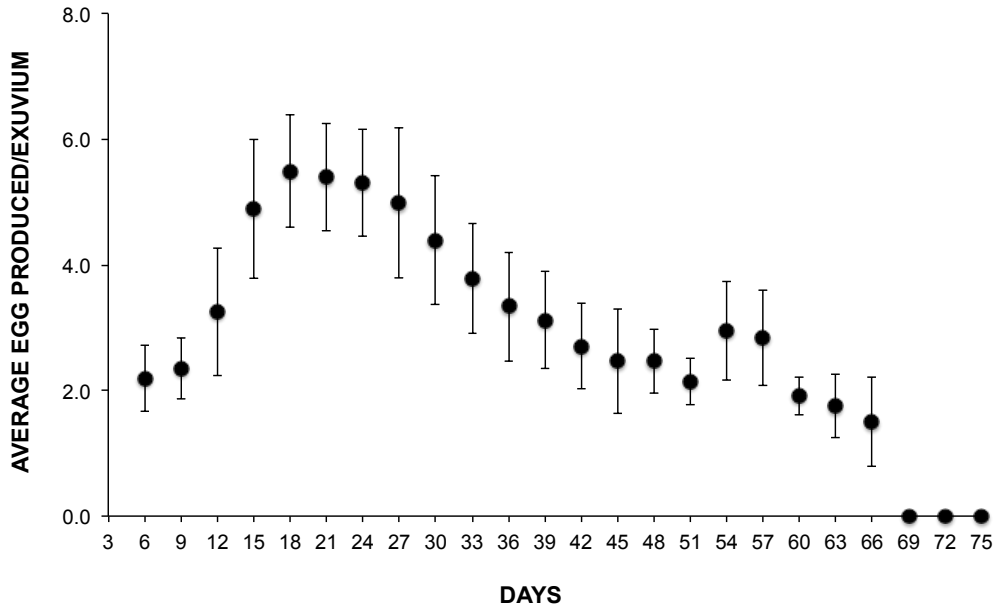

Figure 3

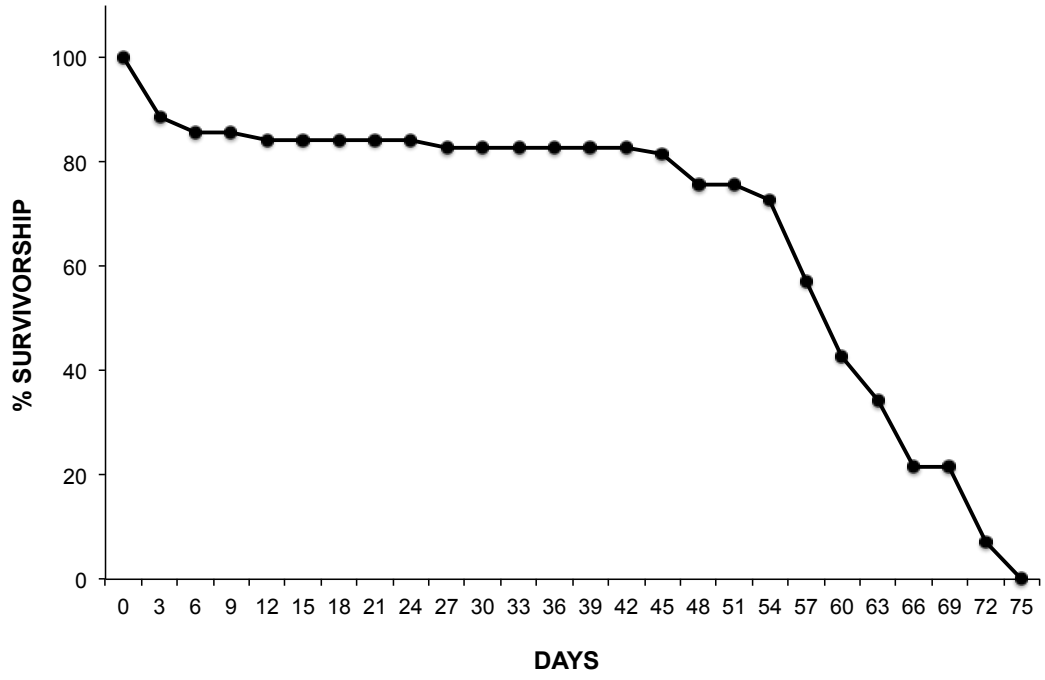

Table 1

Species	Habitation	Mean (µm)	Range (µm)	Gender	Reference
Hypsibius dujardini	Freshwater	263.8	238 – 285.6	Females	Present Study
Dactylobiotus vulcanus [a]	Freshwater	343	235 – 450	n.d.	Kaczmarek et. al. 2012
Parastygarctus renaudue [a]	Marine	153	112 – 168	Females	Grimaldi de Zio et. al. 2009
Stygarctus lambertii [a]	Marine	93	69 – 106	Females	Grimaldi de Zio et. al. 2009
Stygarctus lambertii [a]	Marine	93	76 – 112	Males	Grimaldi de Zio et. al. 2009
Ramazzottius oberhaeuseri [a]	Limno-terrestrial	228.7	107.3 – 375.4	Females	Rebecchi et. al. 2006
Paramacrobiotus tonollii	Limno-terrestrial	654 [b]	n.d.	Females and Males	Lemloh et. al. 2011
Macrobiotus sapiens	Limno-terrestrial	518 [b]	n.d.	Females and Males	Lemloh et. al. 2011

[a] Values obtained from mounted species
[b] Maximum body size

Table 2

Species	Reproduction	Lifespan (days)	Max Lifespan (days)	Age at Oviposition (days)	Hatching Success	Max Eggs/ clutch	Oviposition /Lifespan	Reference
Hypsibius dujardini	Parthenogenesis	61.9 ± 9.9	75	8.0 ± 3.1	90-100 %	8	10.5 ± 2.2	Present Study
Acutuncus antarticus	Parthenogenesis	69.2 ± 36.4	162	9.3 ± 1.1	97.6 %	10	7.5	Tsujmoto et. al. 2015
Paramacrobiotus kenianus (I)	Parthenogenesis	125 ± 35	204	10 [a]	33 %	n.d.	n.d.	Schill 2013
*Paramacrobiotus kenianus (*II)	Parthenogenesis	141 ± 54	212	10 [a]	51 %	n.d.	n.d.	Schill 2013
Paramacrobiotus palaui	Parthenogenesis	97 ± 31	187	10 [a]	54 %	n.d.	n.d.	Schill 2013
Paramacrobiotus tonollii	Bisexual	69.0 ± 45.1	237	24.4 ± 4.4 [b]	82.2 %	19	n.d.	Lemloh et. al. 2011
Macrobiotus sapiens	Bisexual	83.0 ± 33.5	145	16.5 ± 3.8 [b]	78 %	16	n.d.	Lemloh et. al. 2011
Macrobiotus richtersi (I)	Parthenogenesis	194.9 ± 164.4	518	76.9 ± 16.4	83.1 ± 12.7	39	3.4 ± 2.1	Altiero et. al. 2006
Macrobiotus richtersi (II)	Parthenogenesis	137.3 ± 136.4	457	70.7 ± 19.4	51.0 ± 36.0	30	2.0 ± 1.2	Altiero et. al. 2006
Milnesium tardigradum	Parthenogenesis	42.7 ± 11.8	58	15.3 ± 1.6 [c]	77.2 %	12	3.7 ± 1.3	Suzuki et. al. 2003

[a] Median
[b] Eggs observed in ovary
[c] As reported by Tsujmoto et. al. 2015

8. Figures Legends and Table Caption

Figure 1: Proportion among individuals (N=70) that exhibited growth (increase in body length by day 3 of life; 67.1%), delayed growth (increase in body size by day 9 of life; 21.4%) or no growth (11.4%; individuals that did not grow by day 9 were removed from the study).

Figure 2: Number of eggs laid per exuvium (Mean± SD) every three days in *H. dujardini* (N=59).

Figure 3: Survivorship curve of *H. dujardini* (N=59) reared and maintained under laboratory conditions (22 °C).

Table 1: Body measurements for adult tardigrade species, including data from the present study

Table 2: Life history traits for tardigrade species, including data from the present study

CHAPTER 3: G-EQUIVALENT ACCELERATION TOLERANCE IN THE EUTARDIGRADE SPECIES *HYPSIBIUS DUJARDINI*

3.1 PREFACE

This chapter contains the published article "G-Equivalent Acceleration Tolerance in the Eutardigrade Species *Hypsibius dujardini"* authored by Vasanthan T., Alejaldre L., Hider J., Patel S., Husain N., Umapathisivam B. and Stone J. (Astrobiology, 2017, 17: 55-60). The manuscript is reproduced in its published format.

The study describes tolerance to hypergravity in the eutardigrade species *Hypsibius dujardini*. Adult tardigrades were exposed to short-term g-equivalent accelerations via centrifugation (1 min at 3421*g*, 6082*g*, 9503*g*, 13684*g* or 16060g), mimicking effects from hypergravity on survivorship and reproduction. We found that tolerance to hypergravity is acceleration-dependent with reduced egg production and lowered survivorship at higher accelerations. Macroscopic organisms accelerated to levels above 1*g* (9.8 m/s^2) tend to either collapse or suffer from mechanical deformation due to sedimentation of organelles. Microscopic organisms, however, can tolerate high acceleration levels due to their small size. Our findings show that tardigrades can tolerate short-term g-equivalent accelerations at high levels. This is the first study to establish tardigrade tolerance to hypergravity and describe its effects on survivorship and reproduction. These findings provide further indication that tardigrades make suitable organisms for astrobioloigcal inquiry, particularly in the field of lithopanspermia – viable dissemination of life forms via meteoroid material.

3.1 CONTRIBUTIONS

Tarushika Vasanthan conducted the experiments, wrote the manuscript and generated Figure 2, Figure 3 and Table 1 of the published article. Jonathon Stone provided substantial direction and input throughout the research, including providing assistance with computing the LA_{50} values found on Table 1 and significantly improved the writing of the manuscript. The remaining coauthors conducted pilot projects that helped to improve the study design and execution.

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G-Equivalent Acceleration Tolerance in the Eutardigrade Species *Hypsibius dujardini*

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Abstract

Tardigrades are microscopic organisms renowned for their ability to survive extreme environmental conditions. Tardigrade extreme-tolerance research has centered on the ability to withstand desiccation, low and high temperatures, and high hydrostatic pressure and radiation levels. Tardigrade tolerance to hypergravity, however, has yet to be described. We used the eutardigrade species *Hypsibius dujardini* to investigate short-term tolerance to g-equivalent accelerations (*i.e.*, mimicking g-forces). Data obtained from specimens centrifuged between 3421*g* and 16,060*g* for 1 min inclusively reveal tolerance in an acceleration-dependent relation, with lower survivorship and egg production at higher accelerations. This is the first study to demonstrate tardigrade potential for tolerance to hypergravity and describe expected effects on tardigrade survival and reproduction. These findings will prove to be useful in lithopanspermia research (*i.e.*, viable spread in meteoritic rocks). Key Words: Astrobiology—Extreme tolerance—Hypergravity—Tardigrade. Astrobiology 17, 55–60.

1. Introduction

Tardigrades, commonly known as water bears, are classified in the phylum Tardigrada, which contains over 1000 species distributed ubiquitously in marine, freshwater, and semi-terrestrial environments (Nelson and Marley, 2000; Guidetti and Bertolani, 2005; Nelson and Bartels, 2007). In recent years, some tardigrade species have become recognized as organisms that are well suited for astrobiological research, given their tolerance to a wide range of extreme environmental conditions (Horikawa, 2012). Herein, we briefly summarize tardigrade morphology, reproduction, and extreme-tolerance research and show that tardigrades can survive hyperacceleration up to 16,060*g*—values that have been described previously only in bacterial and fungal species (Deguchi *et al.*, 2011). These results distinguish tardigrades as the first multicellular organisms to have survived exposure at such high-magnitude forces and have implications for our understanding of these organisms in astrobiological research.

1.1. Tardigrade morphology

Tardigrades are classified taxonomically into two major classes that are consistent with morphological traits: the Eutardigrada (unarmored) and Heterotardigrada (armored) (Nelson, 2002; Glime, 2013a). Tardigrades are characterized by bilaterally symmetric bodies (approximately 0.1 to 1 mm long), which individually comprise a head and four segments, each bearing paired lobopod legs that terminate in digits or claws (Fig. 1; Nelson, 2002; Glime, 2013a); the first three pairs function in forward locomotion, whereas the last pair is used for receding or arresting movement (Ruppert and Barnes, 1994; Miller, 2004). Tardigrades contain complete digestive systems but lack respiratory and circulatory systems; respiration occurs primarily through body walls, and circulation is accomplished within hemocoels, both diffusion-based (Nelson, 2002; Miller, 2004). Bodies are covered by cuticle layers, which can be smooth or ornamented depending on the class and are shed periodically throughout life (Ruppert and Barnes, 1994).

1.2. Tardigrade reproduction

Tardigrades engage in different reproduction modes. Most tardigrade species are dioecious, each individual containing a single male or female reproductive system (Ruppert and Barnes, 1994). In species wherein males are present, tardigrades can reproduce sexually. Mating almost always occurs during molting. Males deposit sperm into female reproductive tracts (internal fertilization) or directly fertilize eggs (external fertilization), which are laid by females inside exuvia (*i.e.*, molted exoskeletons; *e.g.*, *Milnesium tardigradum*) or in surrounding environments (*e.g.*,

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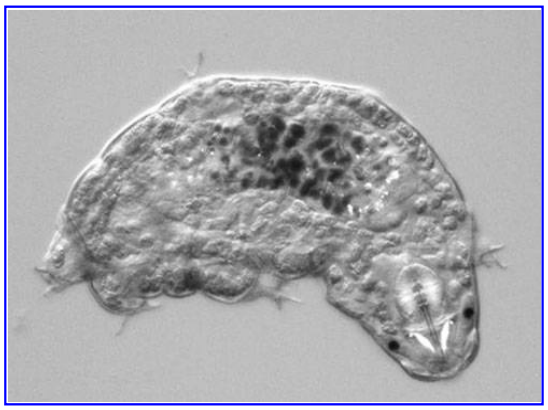

FIG. 1. Eutardigrade species *Hypsibius dujardini* specimen molting and laying two eggs in her exuvium. Adult body length typically ranges from approximately 0.25 mm to approximately 0.5 mm. Photo by Willow Gabriel through EOL Creative Commons.

Macrobiotus hufelandi) (Ruppert and Barnes, 1994; Bertolani, 2001; Glime, 2013b). In species wherein males are absent or uncommon (*e.g.*, in the genus *Ramazzottius*), tardigrades reproduce asexually via parthenogenesis—development from unfertilized eggs (Glime, 2013b). Other species are hermaphroditic (*e.g.*, *Orzeliscus belopus*). Depending on the species, as many as 30 eggs can be laid at one time, typically requiring approximately 2 weeks to hatch (Ruppert and Barnes, 1994).

1.3. Tardigrade extreme tolerance

Like rotifers and nematodes, tardigrades can become cryptobiotic, that is, enter an ametabolic state, when conditions become unfavorable for living (Wright *et al.*, 1992; Nelson, 2002; Bertolani *et al.*, 2004). Tardigrades have been recognized formally as having the capacity to adopt four modes for becoming cryptobiotic: anhydrobiosis (complete desiccation, reportedly up to 100 years; Franceschi, 1948; see Jönsson and Bertolani, 2001, for a review), cryobiosis (freezing temperatures, as low as −273°C; reviewed in Sømme and Meier, 1995), anoxybiosis (low oxygen levels, viable up to 4 days; Crowe, 1975), and osmobiosis (increased solute concentration; Collin and May, 1950). Tardigrades also have survived extremely high temperatures (151°C, for 35 min; Horikawa, 2012), hydrostatic pressures equivalent to those that would be encountered 180 km below Earth's surface (7.5 GPa, for up to 12 h; Ono *et al.*, 2008), and ionizing radiation including gamma (LD_{50}/18 h = 4.7 kGy; Jönsson *et al.*, 2005), heavy ion (LD_{50}/24 h = 6.3 kGy; Horikawa *et al.*, 2006), proton (LD_{50}/48 h = 4.6 kGy; Nilsson *et al.*, 2010), and cosmic (maximum = 4 Gy; Persson *et al.*, 2011). Tardigrades additionally are the first animals known to have survived exposure to the vacuum and ionizing solar and cosmic radiation in outer space (Jönsson *et al.*, 2008; Persson *et al.*, 2011). For these reasons, tardigrades are organisms suited ideally for conducting astrobiological research, particularly concerning habitability in extraterrestrial environments. Tardigrades have been used in exposure experiments on robotic spacecraft: the LIFE TArdigrade Resistance to Space Effects (TARSE) mission (species *Milnesium tardigradum* and *Richtersius coronifer* in the work of Jönsson *et al.*, 2008; species *Macrobiotus richtersi* in the work of Rebecchi *et al.*, 2009) and the Rotifers, Tardigrades, and Radiation (RoTaRad) BIOPAN 6 mission (*Echiniscus testudo*, *M. tardigradum*, *R. coronifer*, and *Richtersius oberhauseri* in the work of Persson *et al.*, 2011). The species *H. dujardini* is becoming appreciated as an animal group suited for experimental investigation (Gabriel *et al.*, 2007), including astrobiological studies (Horikawa, 2012), though it tolerates desiccation poorly (Wright, 1989, 2001; Horikawa *et al.*, 2013).

1.4. G-equivalence tolerance in tardigrades

Despite the aforementioned research, tolerance to hypergravity has yet to be explored in tardigrades. The ability to withstand exposure to extreme forces is requisite to supra-earthly transport and existence, and documenting effects imparted to evolutionarily relevant metrics such as survival and reproduction therefore is required for comprehensive astrobiological studies. In the present study, we investigated tardigrade short-term tolerance to g-equivalent accelerations (*i.e.*, mimicking gravitational forces, or ''g-forces'') and subsequent survivorship and egg production.

2. Material and Methods

To test tardigrade short-term tolerance to g-equivalent accelerations, we used members in the parthenogenetic eutardigrade species *Hypsibius dujardini* (Z151, Sciento, Whitefield, Manchester). We chose this species because it can be cultured easily, is characterized by a short generation time, has been promoted as a model organism for evolutionary and developmental research (Gabriel *et al.*, 2007), and recently has had its genome sequence published (Boothby *et al.*, 2015; Koutsovoulos *et al.*, 2015). Only adult specimens (>0.5 mm) that exhibited coordinated leg movement were used for experimentation. Tardigrades were assigned pseudo-randomly to control (individual number, $n = 10$) or one among five treatment ($n = 10$) groups; two replicates were established for each group (because exact specimen age was unknown, replicates provided a statistical means for testing whether each group constituted a homogeneous population; body sizes were similar between replicates and among groups).

To implement accelerations, specimens (contained in 100 μL springwater; Nestle Pure Life, Ontario, Canada) were centrifuged (Sorvall Pico; maximum radius 8.5 cm) in polypropylene vials (1.5 mL) at 3421*g*, 6082*g*, 9503*g*, 13,684*g*, or 16,060*g* for 1 min (in total, including acceleration and deceleration intervals; for reference, the 9503*g* treatment included 10 s acceleration and deceleration intervals; lower and higher treatments included shorter and longer acceleration and deceleration intervals). Post-centrifugation, tardigrades were housed in polypropylene vials (1.5 mL) containing 75 μL springwater and 25 μL *Chlorococcum* sp. algae as food (Sciento, Whitefield, Manchester; catalogue code A68; culture added directly). To prevent water transpiration, vials were sealed with parafilm, leaving only a small opening to allow oxygen passage. Tardigrades were monitored every 2 days with light microscopy (Nikon SMZ1000) for a 29-day period, and survivorship (% surviving group members,

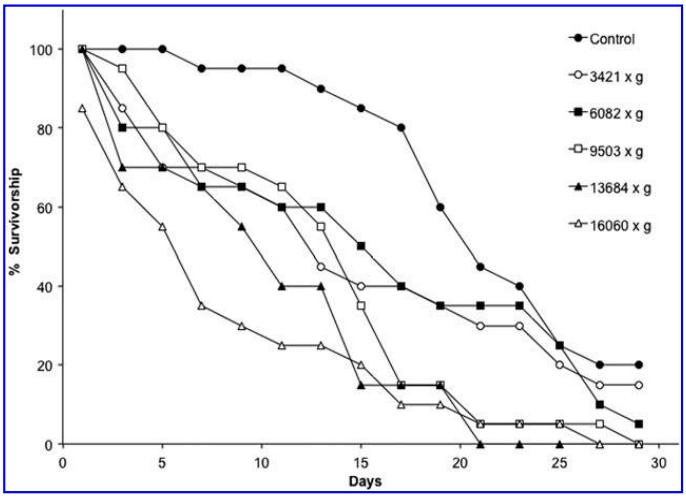

FIG. 2. Acceleration effects on survival in tardigrades post-centrifugation (% Survivorship) in control ($n=20$) and experimental ($n=20$) groups over time (days).

documented on the basis of coordinated leg movements) and egg production were recorded. Eggs were collected and stored in different polypropylene vials (1.5 mL) from those housing adults, in 100 μL springwater. Springwater and algal food were replenished every 2 days. Springwater, pipette tips, and tubes were unautoclaved; all materials and solutions, however, were stored away from possible contamination sources. Pipette tips were changed between replicates and among groups when manipulating and transferring specimens. All groups were maintained at room temperature (22–23°C). Survivorship data comparisons were performed by using logrank tests (Bland and Altman, 2004).

3. Results

No significant differences in survivorship between ($n=10$) replicates within groups were observed, so replicates were combined for subsequent analyses among ($n=20$) control and treatment groups. Specimens withstood short-term exposure to extreme g-equivalent forces (>80% survival among individuals across all treatment groups). Survivorship was greater in control than in treatment groups (degrees of freedom, df = 5, p-value, $p=0.00026$; the survivorship curve for the control group was typical for this laboratory population) and decreased with increased accelerations, especially at 13,684g (df = 1; $p=0.00039$) and, more gradually, 16,060g (df = 1; $p=0.0006$; Fig. 2). Analogously to the median lethal dose (LD_{50}) used in toxicology research (*i.e.*, to quantify the dose required to kill half the members in a population subjected to a treatment for a specified duration), we report the median lethal acceleration (LA_{50}) as the post-centrifugation time at which half the members in a group died (Table 1). No conspicuous differences in external morphology (*e.g.*, mechanical deformations) were observed among specimens across groups. Specimens in control and treatment groups periodically molted and laid eggs. No conspicuous differences in external morphology (*e.g.*, mechanical deformations) were observed among eggs across groups. Egg production (EP) varied among groups, with controls producing the greatest quantities (EP = 18; Fig. 3). Single count-interval egg production interestingly was greatest between days 11 and 13 (EP = 6) for control groups but on day 1 for all treatment groups. Control groups produced more viable eggs than did treatment groups 3421g and 6082g; no eggs hatched in treatment groups 9503g, 13,684g, and 16,060g. Eggs typically hatch within 5 days (*i.e.*, gestation period is 4 days), so we could assess viability with confidence.

TABLE 1. MEDIAN LETHAL ACCELERATION (LA_{50}) VALUES FOR *HYPSIBIUS DUJARDINI*

Acceleration values (g)	*LA_{50} (days)*
3,421	17.6
6,082	18.2
9,503	15.3
13,684	12.0
16,060	10.8

4. Discussion

Individuals in the eutardigrade species *Hypsibius dujardini* can tolerate short-term exposure to extreme g-equivalent forces with >80% survival up to 16,060g. Subsequent survivorship generally decreased with increased accelerations. Because no external mechanical deformations were observed, decreased survivorship with increased acceleration presumably resulted from internal effects.

We were unable to measure egg production per individual, so we recorded (and report herein) total cumulative egg production. Eggs are laid during molting, with embryos deposited into shed exoskeletons, or exuvia, and embryos develop protected inside exuvia for 4–5 days until they hatch (Gabriel *et al.*, 2007). Because tardigrades were monitored every 2 days, we could assume reasonably that eggs that were collected and stored separately had been produced in single egg-laying bouts. Observed egg production rates (*i.e.*, changes in time; typically three eggs per

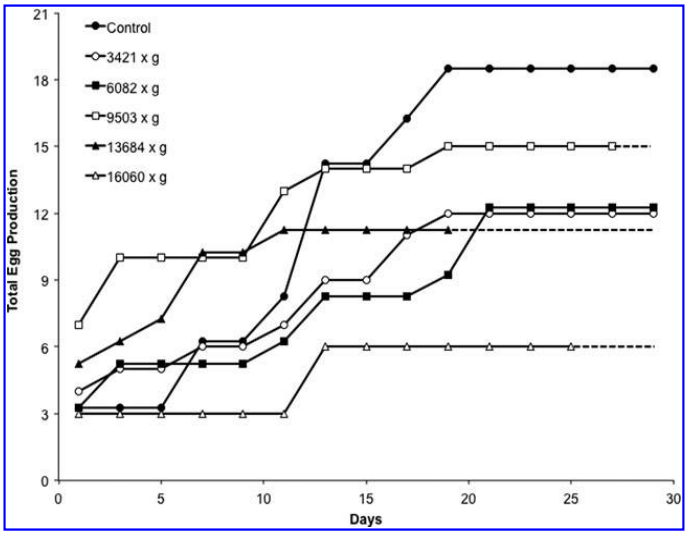

FIG. 3. Acceleration effects on egg production by tardigrades post-centrifugation in control ($n = 20$) and experimental ($n = 20$) groups over time (days); dashed lines indicate no egg production due to 100% mortality.

monitored period, Fig. 3) are consistent with typical values reported previously for *H. dujardini* (mean = 3.4 ± 1.9 SD, range 1–10, $n = 1411$ embryos; Gabriel *et al.*, 2007) and suggest that usually only single individuals laid eggs every other day. Total cumulative egg production was diminished in, and varied among, groups exposed to g-equivalent forces. Egg production was greatest in the first batch produced after acceleration in groups exposed to g-equivalent forces, and viability was maintained only in groups exposed to 3421*g* and 6082*g*. The internal effects that we speculated to have decreased survivorship probably also are responsible for these more-pronounced deleterious effects on fecundity.

Macroscopic organisms that are accelerated to levels slightly greater than that associated with Earth gravity (9.8 m/s^2) tend to suffer severe mechanical deformation or collapse, whereas microscopic organisms can withstand mechanical deformation even under hyperacceleration that is ten-thousand-fold greater than that associated with Earth gravity (Nicholson *et al.*, 2000; Deguchi *et al.*, 2011). The different responses are attributable partially to scale because gravitational potential is size-dependent: organelles like nuclei and mitochondria are at greater risk for sedimentation than are their smaller, constituent proteins and ribosomes (Deguchi *et al.*, 2011). Therefore, organisms that are prokaryotic—each lacking a membrane-bound nucleus—outperform organisms that are eukaryotic under ''hyperaccelerative'' conditions, even at microscopic scales (Deguchi *et al.*, 2011). The prokaryotic, bacteria species *Escherichia coli* and *Paracoccus denitrificans* proliferated when cultured under hyperaccelerative conditions (*e.g.*, 403,627*g;* Deguchi *et al.*, 2011), whereas the eukaryotic, fungus species *Saccharomyces cerevisiae* showed significant impairment in growth with increased acceleration (*e.g.*, 44,893*g* and 52,375*g*) for instance. Bacterial cells consequently have been proposed as model systems for research on extraterrestrial viability—the ability to survive transport to, and in, the troposphere or beyond, ultimately to be transported elsewhere on Earth or a celestial body (Deguchi *et al.*, 2011). Based on our findings, tardigrades—multicellular eukaryotic organisms—can survive short-term high g-equivalent accelerations and thus should be used for research on extraterrestrial viability.

Planets abundantly exchange rocky material. More than 53,000 meteorites have been documented as having impacted Earth, among which between 100 and 200 have been identified as martian (*e.g.*, 105 documented in Worth *et al.*, 2013). If organisms can survive on ejected material from planetary bodies, then Earth-based life could have been seeded from, or transported to, other planets within our solar system (Horneck *et al.*, 2008; Worth *et al.*, 2013). For interplanetary lithopanspermia (*i.e.*, viable spread in meteoritic rocks) to transpire, organisms must be able to (1) survive ejection from a planetary body, (2) withstand conditions encountered during interplanetary travel, and (3) successfully enter the atmosphere surrounding another planet (Mastrapa *et al.*, 2001). To survive steps 2 and 3, organisms must be tolerant to complete desiccation, extremely low temperatures, high ionizing radiation levels, and near-vacuum pressures—conditions to which tardigrades already have demonstrated high tolerance (Ramløv and Westh, 2001; Jørgensen *et al.*, 2007; Horikawa *et al.*, 2008; Jönsson *et al.*, 2008). To survive the initial planetary ejection, tardigrades also would have to be tolerant to short-term extreme g-forces. We found that members in the eutardigrade species *Hypsibius dujardini* can tolerate short-term extreme acceleration, up to 16,060*g* (with a moderate decrease in survivorship over time), and note that the minimum shock pressures required for martian ejecta are estimated to be 5–10 GPa (Fritz *et al.*, 2005), a range that includes the observed static pressure tolerance for tardigrades (7.5 GPa, for up to 12 h; Ono *et al.*, 2008). This constitutes the first study to demonstrate tardigrade tolerance to hyperacceleration and describe its effects on subsequent survival and reproduction. Our findings reveal that hypergravity is another environmental parameter to which tardigrades display extreme tolerance, further broadening their title as poly-extreme-tolerant organisms. We consider the documentation that tardigrades can tolerate hyperacceleration (in addition to

other extreme conditions as yet another instantiation among an increasing number) to suggest that some organisms can survive parameters associated with lithopanspermia rather than demonstrate specifically that tardigrades have been, or will be, candidates for ''lithotranspermia'' (*i.e.*, viable transfer in meteoritic rocks; *e.g.*, between Earth and Mars or *vice versa*).

Tardigrades exhibited reduced survivorship and decreased egg production with increased g-equivalent accelerations. This raises issues about costs and mechanism (or mechanisms) associated with hypergravity tolerance in tardigrades—topics that warrant additional investigation. Furthermore, to determine whether tardigrades can survive accelerations analogous to those that would be experienced if ejected into extraterrestrial conditions (*i.e.*, approximately $10^5 g$), future research should be conducted to define the upper limits for hypergravity tolerance among tardigrade species.

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CHAPTER 4: pH TOLERANCEI N THE FRESHWATER EUTARDIGRADE SPECIES *HYPSIBIUS DUJARDINI*

4.1 PREFACE

This chapter contains the submitted paper "pH Tolerance in the Freshwater Eutardigrade Species *Hypsibius dujardini"* authored by Vasanthan T., Cummins J. and Stone J. (Journal of Limnology, submitted July 12th 2017, paper no. 1690). The manuscript is reproduced in its submitted format.

The study investigated pH tolerance in the eutardigrade species *Hypsibius dujardini* by exposing adult specimens to long-term acidic (pH 1 to 6) and alkaline (pH 8 to 14) environments. We found that while extreme acidic (pH 1 and 2) and alkaline (pH 11 to 14) conditions caused immediate mortality, exposures to pH 4, 6, 8, 9, and 10 were tolerated, with reduced locomotion and fecundity at pH 9 and 10. Individuals exposed to pH 9 and 10 displayed tun-like morphologies with reduced movement and contracted limbs, suggesting that tardigrades can elicit a cryptobiotic response to extreme pH levels. Given that many organisms can survive in only a narrow pH range, tardigrades tolerancee to a wide range of pH levels adds to their repertoire as poly-extreme-tolerant organisms. To date, studies on pH tolerance in tardigrades has consisted of only short-term exposure experiments in unpublished works. This constitutes the first study to examine long-term effects on tardigrade survivorship and reproduction at different pH levels.

4.2 CONTRIBUTIONS

Tarushika Vasanthan conducted the experiments, wrote the manuscript and generated Figures 1 to 3 and Table 1. Julisa Cummins assisted with the pilot project that helped to execute the final study design for the experiment; she also measured, recorded and adjusted solutions to the reference pH for each treatment during the final experiment. Jonathon Stone provided guidance throughout the project and refined writing the manuscript.

TITLE PAGE

pH Tolerance in the Freshwater Eutardigrade Species *Hypsibius dujardini*

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Running Head Line: pH Tolerance in Tardigrades

Key words: Acidic; alkaline; extreme-tolerance; tardigrade.

ABSTRACT

Tardigrades are microscopic panarthropods, remarkable for the ability by some species to tolerate exposure to extreme environmental stress. Under desiccative conditions, tardigrades become anhydrobiotic – an ametabolic, inactive state. Access to a vivifying water supply is required to drive metabolic processes and permit activities. The few investigations conducted on tolerance to pH levels have involved effects for only short-term exposures (<5 days) on survival. Here we report for the first time long-term effects from tardigrade exposure to acidic and alkaline conditions. We used the freshwater eutardigrade species *Hypsibius dujardini* to investigate effects imparted by exposure to acidic (pH 1 to 6) and alkaline (pH 8 to 14) conditions on survival and reproduction. While exposure to extreme acidic (pH 1 and 2) and alkaline (pH 11 to 14) conditions caused immediate mortality (<24 hours), specimens showed remarkable tolerance to exposure to pH 4, 6, 8, 9 and 10, with reduced activity at pH 9 and 10. Specimens responded favorably to exposure to pH 4, laying twice as many eggs as did specimens in a pH 7 control group, while exposure to pH 6, 8 and 9

decreased fecundity. Our findings show that, unlike many aquatic organisms that have a well-defined, narrow pH range within which life can be supported, tardigrades can tolerate a wide range of pH levels outside the normative (pH 7) condition.

1. INTRODUCTION

Tardigrades, commonly known as water bears, are microscopic invertebrates classified in the phylum Tardigrada (superphylum Ecdysozoa). Tardigrades are grouped taxonomically into two classes: the Eutardigrada and Heterotardigrada (Nelson *et al.,* 2015). Renowned for the ability by some species to survive extreme conditions, tardigrades have shown remarkable tolerance to extreme temperatures (ranging from -272 °C to 151 °C: e.g., Rahm, 1921), exposure to outer space conditions (including near absolute-zero temperatures and vacuum pressures on the FOTON-M3 mission: Jönsson *et al*., 2008; cosmic radiation on the RoTaRad project aboard the BIOPAN 6 mission: Persson *et al.,* 2011), high hydrostatic pressures (7.5 GPa: Ono *et al.,* 2008), high ionizing radiation doses (over 4000 Gy: Beltrán-Pardo *et al*., 2015) and chemical treatments (10 min in ethanol: e.g., Ramlov & Westh, 2001; for a comprehensive review on tardigrade survivability to extreme conditions see Guidetti *et al*., 2012). Tardigrade resiliency to extreme conditions has been attributed predominantly to their ability to become anhydrobiotic (e.g., Rebecchi *et al*., 2009), a cryptobiotic state in which individuals become completely desiccated, although the association is nonuniversal (e.g., surviving high radiation doses equally effectively in a dehydrated or hydrated state; Altiero *et al*., 2011; surviving high radiation doses more effectively in a hydrated than in a dehydrated state; Horikawa *et al*., 2006). Whereas most organisms escape ‘in space’ to avoid extreme conditions, cryptobiotic tardigrades escape ‘in time,’ halting development, growth and reproduction until more-favorable conditions prevail. In a cryptobiotic state, tardigrades become ametabolic and immobile, their bodies often adopting barrel-

like shapes, often referred to as being in a 'tun,' state (Glime, 2013). Tardigrades can remain in such a latent state for decades, possibly even longer (120 years in an anhydrobiotic (dried) state: Franceschi, 1948; 30 years in a cryobiotic (frozen) state: Tsujimoto *et al.,* 2016), until surrounding conditions become favorable (Jørgensen *et al*., 2007). The ability to stabilize cellular structures during cryptobiosis, particularly in anhydrobiosis, has been linked to synthesized cell protectants like trehalose, heat shock proteins and intrinsically disordered proteins (Crowe, 1972; Schill *et al*., 2009; Boothby *et al*. 2017). To resume metabolism and locomotion, anhydrobiotic tardigrades require a humid environment. Water, thus, is essential to tardigrade persistence.

Whereas tardigrades have been found worldwide, only some species are cosmopolitan in their distribution, with individuals having a defined tolerance to specific environments (Nelson *et al.,* 2015). Typically tardigrades inhabit marine, brackish water, freshwater, limnoterrestrial and terrestrial environments, with most species residing in terrestrial habitats (Nelson *et al.,* 2015). Tardigrades generally colonize moist environments, like dampened mosses and lichens, but populations also have been observed in lesser known environments, like cryoconite holes on glaciers (Zawierucha *et al*., 2015), hot springs in Greenland (Kristensen, 1982) and the Arctic tundra (Maucci, 1996). Tardigrades also have been observed in wastewater treatment plants: researchers isolated specimens in the tardigrade species *Thulinius ruffoi* from a Polish sewage treatment plant and subsequently used them to investigate effects from ammonia toxicity; Sobczyk *et al.,* 2015). While tardigrades can dwell in extreme habitats where parameters like pH are highly variable (i.e. Arctic tundra tends to be acidic; sewage wastewater tends to be basic), the upper and lower limits for pH tolerance in tardigrades have yet to be characterized completely.

Only three investigations have documented tardigrade tolerance to different pH levels. Individuals in the eutardigrade species *Hypsibius dujardini* (Doyère, 1840) exhibited reduced activity after a 30-hour exposure to pH 4 and a 5-minute exposure to pH 3 but died from a 5-minute exposure to pH 2.8 (Bartels, 2005). Individuals in a eutardigrade species in the genus *Thulinius stephaniae* (Pilato, 1974) exhibited decreased activity following 5-day exposures to pH 3, 4 and 5 (S. Thompson, 2008 unpubl. data). Individuals in the eutardigrade species *Milnesium tardigradum* (Doyère, 1840) tolerated 1-90 minute exposures to pH 1.54 to 12.5, with activities and post exposure survival times increasing with conditions approaching pH 7 (Acevedo, 2008 unpubl. data). These three investigations on tardigrade tolerance to pH involved only short-term effects from exposure. We sought to determine long-term effects from exposure to acidic and alkaline conditions on tardigrade survival and reproduction.

2. METHODS

2.1 Experimental Design

To test tardigrade tolerance to different pH levels, we used the freshwater parthenogenetic eutardigrade species *Hypsibius cf. dujardini* (Z151, Sciento, Whitefield, Manchester). Adult specimens exhibiting coordinated leg movements were chosen for experimentation. To establish acidic (pH 1 to pH 6) and alkaline (pH 8 to pH 14) test solutions, H_2SO_4 and KOH were diluted in spring water. Once the desired pH level was achieved, test solutions were stored and labeled in 250 mL bottles. pH-treated groups (N = 10) were exposed to 100 uL acidic (pH 1 to 6) or alkaline (pH 8 to 14) test solutions; control groups were maintained in spring water (N = 10; pH 7; 100 uL). To test whether deaths resulted from pH treatments rather than poor nutrition (i.e., alga death), a ‘control-starved’ group was established, wherein specimens were maintained in spring water (N = 10; pH 7; 100 uL) without food provisions. Two replicates were established for each treatment (N = 10 x 2 = 20). All animals were maintained under ambient temperature and housed in 1.5 mL microtubes (Figure 1A).

2.2 Survivorship

To record survivorship and egg production, animals were transferred from their respective microtubes, placed on a Petri dish (35 mm) and observed through a stereo microscope (Nikon SMZ1000). Once counted, animals were returned to their microtubes containing 10 uL day-old pH medium topped up with 90 uL pH test solution. Prior to the top up, pH for each test solution was measured and adjusted with reference to a pH meter (Fischer Scientific™ accumet™; Figure 1B). Survivorship and egg production were recorded every other day. Only animals exhibiting coordinated leg movement were scored as viable. Algal food (15uL; *Chlorococcum* sp.*;* A68, Sciento, Whitefield, Manchester) was replenished every 2 days for all groups except the 'control-starved' group. Survivorship comparisons were performed using logrank tests (Bland & Altman, 2004).

3. RESULTS

Mean pH values for acidic (pH 1-6), alkaline (pH 8-14) and control (pH 7) conditions closely approximated prescribed values (Table 1). Exposure to extreme alkaline (pH 11, 12, 13 and 14) and acidic (pH 1 and 2) conditions resulted in complete mortality in less than 24 hours, whereas treatment groups exposed to pH 3, 4, 5, 6, 8, 9 and 10 were characterized by significantly decreased survivorship relative to control groups ($p<0.05$; Figures 2 and 3). Activity was reduced substantially at pH 9 and 10, with specimens displaying tun-like morphologies – retracted limbs and contracted bodies. Whether mortality observed with exposure to pH 3, 5 and 10 resulted from acidic and alkaline conditions alone or accompanied by poor nutrition (e.g., caused directly by acid or alkaline conditions or indirectly through pH level effects on algae) is uncertain, as survivorship curves for these groups were similar to that for the control-starved group. Exposure to acidic and alkaline conditions also affected egg production (EP; Figure 4). Specimens responded favorably to exposure to pH 4, laying approximately twice as many eggs (EP = 106) as did control specimens (EP =

54), whereas egg production decreased considerably with exposure to pH 6, 8 and 9 (EP = 27, 8, 3, respectively); no eggs were laid by specimens exposed to pH 3, 5 and 10. A single egg was produced in the control-starved (pH 7) group.

4. CONCLUSIONS

Freshwater ecosystems often are characterized by seasonal and daily pH fluctuations (Tucker and D'Ambro, 2008). Changes in pH can be attributed to a variety of factors, including mineral acids, like sulfuric acid, which decreases pH, or excessive photosynthesis by underwater plants, which increases pH (Tucker and D'Ambro, 2008; Boyd and Tucker, 2012). Animals also can influence ecosystem pH. In the Arctic, seabirds are instrumental in linking marine ecology (i.e. where they feed) to terrestrial ecology (i.e. where they breed; Zawierucha et al., 2016). Researchers found that soil samples taken from seabird populated areas were more acidic (due to their guano) and on average had a higher tardigrade population density than did soils sampled from areas where seabirds were absent (Zawierucha et al., 2016). Some freshwater organisms consequently have evolved the ability to tolerate a wide range in or even changing pH levels (Tucker and D'Ambro, 2008). Changes in pH, even if within the normal tolerance range for organisms, cannot be too extreme and must be manifested gradually, as exposure to extreme or sudden changes in pH can be fatal (Tucker and D'Ambro, 2008).

Members in the freshwater eutardigrade species *H. dujardini* can tolerate exposure to pH 3, 4, 5, 6, 8, 9 and 10 – levels that are outside normative (pH 7) conditions. This wide range is noteworthy considering that most aquatic organisms are characterized by a well-defined, narrow pH range beyond which they cannot survive. Whereas the molecular mechanisms involved in pH tolerance have yet to be elucidated in tardigrades, mechanisms similar to those found in bacteria might be used. The ability for nonextremophilic bacteria to grow in environments ranging from pH 5.5 to 9.0 has been attributed to their ability to

maintain neutral conditions in their cytoplasm, between pH 7.4 and 7.8, acidifying or alkalinizing internal environments to cope with pH changes in external environments (Padan *et al*., 2005).

Tardigrade tolerance to pH also might be facilitated by the ability to undergo cryptobiosis (with associated trehalose, heat shock protein and intrinsically disordered protein expression). In the extreme alkaline conditions tested herein, specifically pH 9 and 10, specimens entered a cryptobiotic, tun-like state, wherein activity was reduced substantially. By contracting their bodies and assuming tun-like morphologies, specimens reduced their body surface areas, thereby minimizing exposure to the basic environment and increasing their chances for survival. This response is similar to a previously reported cryptobiotic response to survive changes in salinity (Raz, 2005). Exposure to different pH levels also resulted in changes in fecundity. Specimens exposed to pH 6, 8 and 9 laid fewer eggs than did specimens in control groups, while specimens exposed to pH 3, 5 and 10 laid no eggs. As observed with survivorship, decreased egg production in response to exposure to different pH levels suggests that a 'cost' is associated with pH tolerance. Increased egg production in response to exposure to pH 4 is puzzling and warrants additional investigation.

Tardigrades, unlike many aquatic organisms, can tolerate a wide range of pH levels. This constitutes the first study to report long-term effects from exposure to acidic and alkaline conditions on tardigrade survival and reproduction. Given that tardigrades are meiofaunal community members (defined practically as fauna that could pass through a 500 μm sieve) that require specific environmental conditions to remain active and are distributed ubiquitously in marine, freshwater and terrestrial environments, their ability to tolerate acidic and alkaline conditions and potentially respond to changes in pH is quite remarkable. Documenting non-neutral pH tolerance in tardigrades adds to their repertoire in being among the

most extreme-tolerant organisms on the planet and also suggests the possibility that invertebrates like tardigrades could inhabit extreme environments where pH is variable.

5. ACKNOWLEDGEMENT

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7. TABLES AND FIGURES

Table 1

	pH	Mean	Standard Deviation
Acidic Environments	3	2.99	0.03
	4	4.02	0.03
	5	5.02	0.05
	6	5.99	0.03
Control	7	7.00	0.02
Alkaline Environments	8	8.02	0.04
	9	9.00	0.02
	10	10.02	0.04

Figure 1

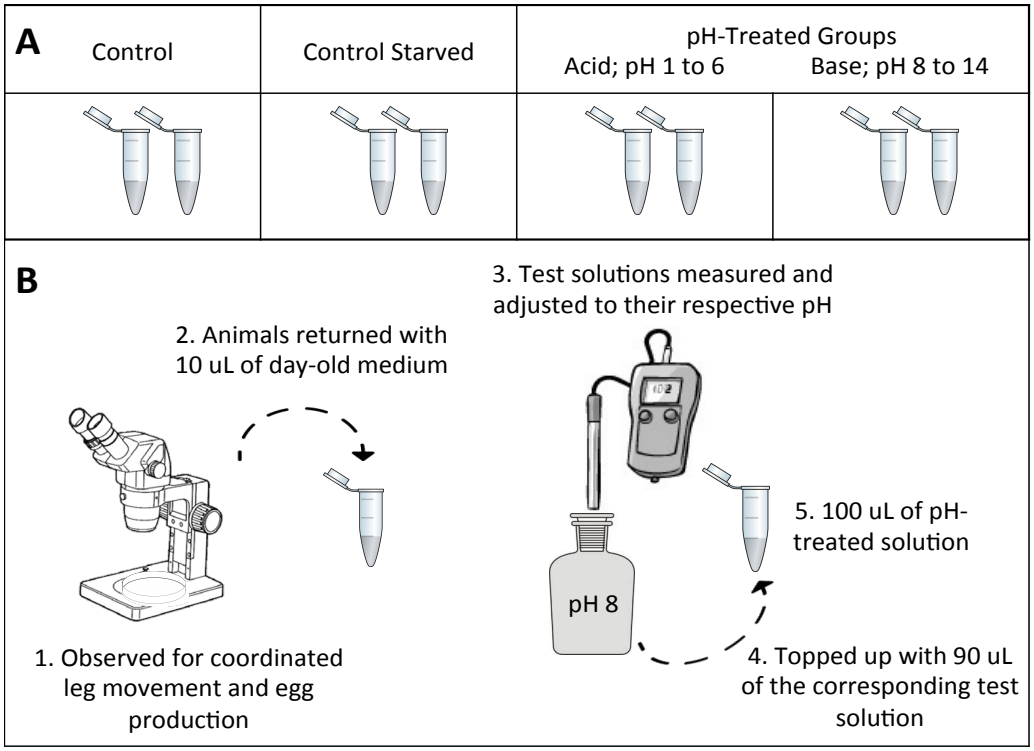


Figure 2

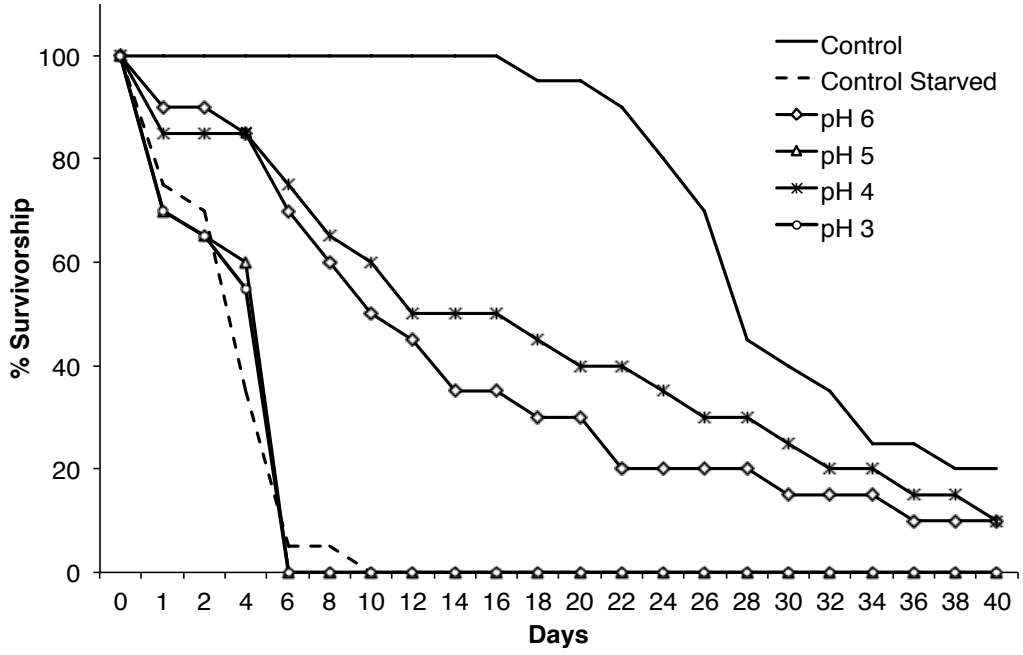


Figure 3

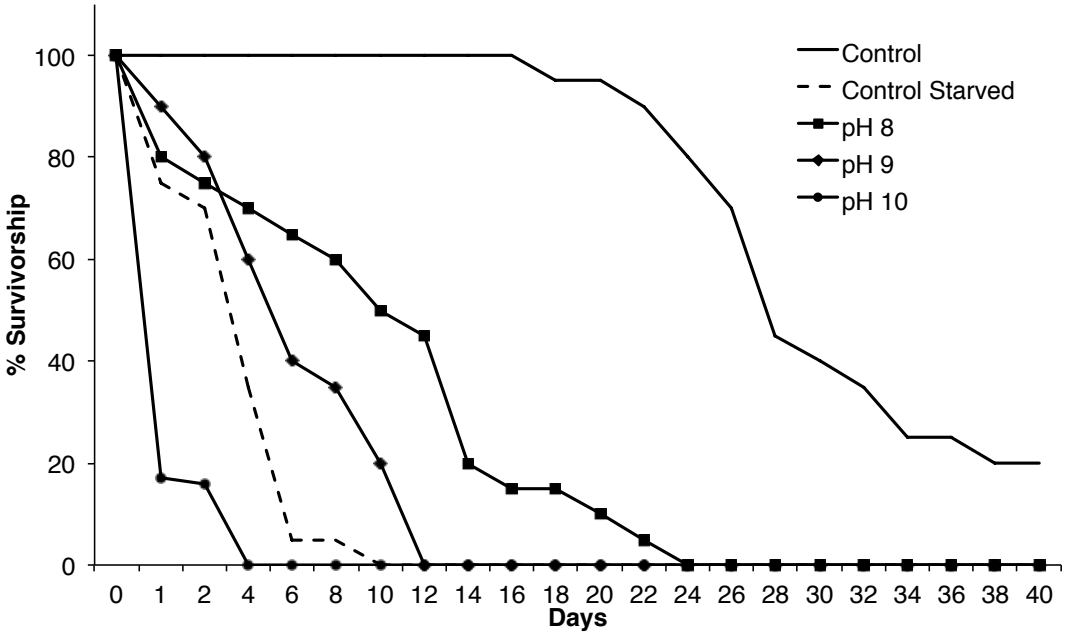
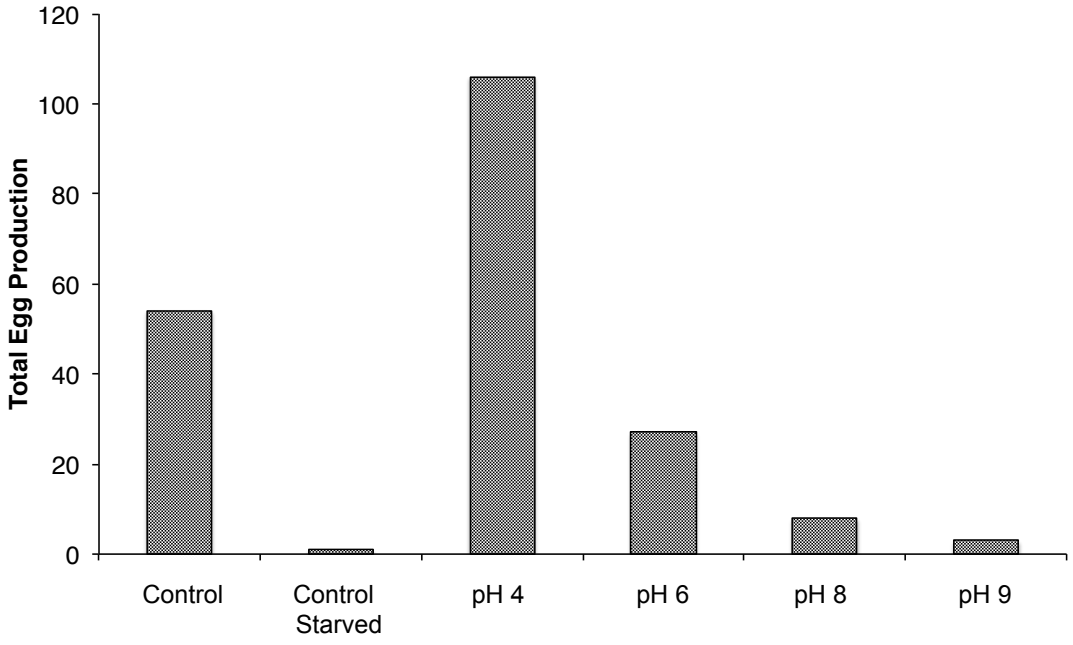


Figure 4



8. FIGURE LEGENDS AND TABLE CAPTION

Table 1. Measured pH values for control and treatment groups

Figure 1. (A) Experimental set up for testing pH tolerance in the eutardigrade species *Hypsibius dujardini*: control (pH 7), control starved (pH 7 and unfed), acidic (pH 1 to 6) and alkaline (pH 8 to 14) groups; N = 20/pH group **(B)** Observation post-pH exposure.

Figure 2. Survivorship curves for tardigrade specimens (N = 20) exposed to acidic (H_2SO_4) conditions, pH 3, 4, 5 and 6. Control specimens (N = 20), fed and starved (N = 20), were maintained in spring water (pH 7).

Figure 3. Survivorship curves for tardigrade specimens (N = 20) exposed to alkaline (KOH) conditions, pH 8, 9 and 10. Control specimens (N = 20), fed and starved (N = 20), were maintained in spring water (pH 7).

Figure 4. Total egg production by specimens in control, fed and starved groups maintained in spring water (pH 7) and treatment groups exposed to acidic (H_2SO_4; pH 4, 6) and alkaline (KOH; pH 8 and 9) conditions.

CHAPTER 5: RADIATION TOLERANCE AND BYSTANDER EFFECTS IN THE EUTARDIGRADE SPECIES *HYPSIBIUS DUJARDINI* (PARACHAELA: HYPSIBIIDAE)

5.1 PREFACE

This chapter contains the published article “Radiation tolerance and bystander effects in the eutardigrade species *Hypsibius dujardini* (Parachaela: Hypsibiidae)*”* authored by Fernandez C., Vasanthan T., Kissoon N., Karam G., Duquette N., Seymour C. and Stone J. (Zoological Journal of the Linnean Society, 2016, 178: 919-923). The manuscript is reproduced in its published format.

The study examined radiation tolerance and radiation-induced bystander effects (RIBEs) in the eutardigrade species *Hypsibius dujardini*. Adult specimens were directly irradiated (3 or 5 kGy) or indirectly exposed to gamma radiation by introducing to unirradiated specimens a single irradiated (3 or 5 kGy) individual. We found that radiation tolerance was dose-dependent in directly irradiated groups, survivorship reduced with increased doses. Tardigrades that received no direct radiation but were exposed to tardigrades that had been irradiated (i.e., bystander animals) exhibited decreased survivorship relative to controls. Interestingly, bystander groups differed significantly in survivorship from groups that were directly irradiated only at the 5 kGy level, suggesting that a threshold for indirect exposure to gamma radiation exists between 3 and 5 kGy. While RIBEs have been reported in other organisms, this is the first study to show that a similar response can be elicited in tardigrades. Our findings add support to the notion that indirect exposure to radiation can be damaging at the organism level. Characterizing the molecules involved in bystander signaling and response could

have important implications for radiotherapy and risk assessment.

5.2 CONTRIBUTIONS

Celia Fernandez conducted the experiments for the study and generated Figure 1. Tarushika Vasanthan trained Celia Fernandez on tardigrade handling, sampling and counting. Tarushika Vasanthan also wrote the manuscript and generated Figure 2. Jonathon Stone provided intellectual guidance and feedback throughout the research and considerably enhanced writing the manuscript. Dr. Colin Seymour provided the necessary background knowledge that inspired the research and provided a working relationship with Robert Pasuta, Supervisor, McMaster Nuclear Reactor, who facilitated sample irradiation. The remaining coauthors performed preliminary experiments that helped to improve the study design, including dose range selection.

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Zoological Journal of the Linnean Society, 2016, **178**, 919–923. With 2 figures

Radiation tolerance and bystander effects in the eutardigrade species *Hypsibius dujardini* (Parachaela: Hypsibiidae)

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Some tardigrade species can tolerate high radiation doses. Radiation-induced bystander effects – damage in unirradiated cells associated with irradiated cells – have yet to be reported in tardigrades. We investigated radiation tolerance and radiation-induced bystander effects on long-term survival in the eutardigrade species *Hypsibius dujardini*. To study direct radiation effects, groups were irradiated with gamma radiation at levels of 3 and 5 kGy. To study radiation-induced bystander effects, unirradiated groups were exposed to a single irradiated (3 or 5 kGy) individual. Survivorship was monitored every 2 days until all individuals had died. Direct radiation decreased survivorship in a dose-dependent manner. Survivorship in bystander groups differed significantly from survivorship in groups irradiated directly with 5 kGy but insignificantly from survivorship in groups irradiated directly with 3 kGy, suggesting that radiation-induced bystander effects had saturated. The eutardigrade species *H. dujardini* can tolerate high radiation doses, with radiation-induced bystander effects that manifest as a threshold response.

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ADDITIONAL KEYWORDS: direct radiation – radiation-induced bystander effects – survivorship – tardigrade.

INTRODUCTION

Tardigrades, known commonly as water bears, are microscopic invertebrate animals renowned for their abilities to survive extreme conditions (Horikawa, 2012). They are classified in the phylum Tardigrada, which contains more than 1200 species distributed ubiquitously in marine, freshwater and semi-terrestrial environments (Nelson, Guidetti & Rebecchi, 2015; Degma, Bertolani & Guidetti, 2016). Tardigrades are among the most radiation-tolerant organisms, having been observed to have survived exposure to protons (Nilsson, Jönsson & Pallon, 2010), ions (Horikawa *et al.*, 2008), UV light (Altiero *et al.*, 2011; Horikawa *et al.*, 2013), gamma rays (Jönsson, Harms-Ringdahl & Torudd, 2005; Horikawa *et al.*, 2006; Beltrán-Pardo *et al.*, 2015), X-rays (May, Maria & Guimard, 1964) and cosmic radiation (Jönsson *et al.*, 2008; Persson *et al.*, 2011). Whereas radiation tolerance and effects have been documented among a variety of tardigrade species, radiation-induced bystander effects have yet to be reported.

Radiation-induced bystander effects occur when unirradiated cells (or organisms) are exposed to irradiated cells (or organisms) and exhibit similar detrimental effects, such as genetic instability or

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decreased survivorship – a phenomenon that was described as early as the 1950s by Parsons *et al.* (1954; Blyth & Sykes, 2011; Seymour & Mothersill, 2004). Radiation-induced bystander effects have been studied with mammalian cells and rodent and fish populations. Mothersill *et al.* (2006) showed that tissue explants from unirradiated fish displayed evidence for radiation damage when paired one-to-one with tissue explants from irradiated individuals. Unirradiated fish that were introduced to water that previously had housed irradiated fish also exhibited evidence for radiation damage (Mothersill *et al.*, 2006). Radiation damage therefore can be imparted from an irradiated to unirradiated individual without direct contact; the effect is hypothesized to be transmitted via stable water-soluble signalling molecules (Mothersill *et al.*, 2006; Blyth & Sykes, 2011). Hu *et al.* (2006) observed that mammalian cells exposed to alpha-particle radiation at low dosage induced double strand breaks in DNA in unirradiated bystander cells situated as far away as 7.5 mm. The likelihood of a neighbouring cell responding to bystander signals released by an irradiated cell therefore depends weakly on distance from the irradiated region (Hu *et al.*, 2006). Whereas radiation-induced bystander effects have been studied predominantly in vertebrates, few studies have been conducted on invertebrates (Mothersill *et al.*, 2006). In this paper, we report direct radiation and radiation-induced bystander effects on survival in the eutardigrade species *Hypsibius dujardini* (Doyère, 1840).

MATERIAL AND METHODS

Study animals

Tardigrade cultures (Sciento Z151; Whitefield) were stored under ambient laboratory conditions (20–23°C). Cultures were maintained in spring water and fed alga (*Chlorococcum* sp.; Sciento A68; Sciento).

Experimental set-up

To study direct radiation effects in adult specimens, groups ($N = 16$) were suspended in 100 μL of spring water in 1.5-mL microtubes. Specimens were exposed to gamma radiation from a ^{60}Co source at levels of 3 and 5 kGy. Irradiation was conducted at the McMaster University Nuclear Reactor Facility (Hamilton, Ontario, Canada). To account for instantaneous deaths, specimens were observed prior to, immediately after and 2 h after irradiation. To study radiation-induced bystander effects, single individuals irradiated at 3 or 5 kGy were selected and transferred to unirradiated groups ($N = 14$), resulting in equal-sized ($N = 15$) irradiated and bystander groups (Fig. 1). Two replicates were established for control (i.e. sham irradiated) and treatment (irradiated and bystander) groups. Two trials were conducted (Trial 1: 2.43 Gy s^{-1}; Trial 2: 2.26 Gy s^{-1}) with two replicates in each group, yielding $2 \times 15 \times 2 = 60$ individuals per treatment.

Observation post-irradiation exposure

Survivorship was recorded on alternate days. Specimens were removed from their respective microtubes, placed on a 35-mm Petri dish (Falcon) and observed by light microscopy (Nikon SMZ1000) for coordinated leg movement. On other days, specimens were left suspended in their respective microtubes with 75 μL of spring water supplied with 25 μL alga. To minimize water evaporation, microtubes were sealed with Parafilm and punctured to create small holes to allow oxygen passage. Water and algae were replenished approximately every 4 days. Eggs laid by individuals were removed and therefore excluded from any statistical analysis. Survivorship data were compared with logrank tests (Bland & Altman 2004).

RESULTS

Survivorship curves in Trials 1 and 2 were indistinguishable statistically ($P > 0.05$). Survivorship curves for replicates within groups were also indistinguishable statistically ($P > 0.05$). Replicates from both trials were therefore combined, and survivorship was reported for the overall population in each group ($N = 60$). Ten pairwise comparisons can be performed for five groups (i.e. one control and four treatment), so $P' = 0.005$ ($= 0.05/10$) was implemented as a Bonferroni correction in performing *post hoc* statistical tests (Sokal & Rohlf, 1995).

Immediately following radiation exposure (i.e. $t = 0$ h), tardigrades were immobile and displayed tun-like morphology (i.e. retracted limbs and contracted bodies). Post-irradiation (i.e. $t = 2$ h), no observable difference in locomotion was observed among control and treatment groups. Control specimens were characterized by an average survival time of 13.2 days; specimens directly exposed to 3 and 5 kGy gamma radiation (RAD3 and RAD5) were characterized respectively by an average survival time of 7.8 and 7.5 days; specimens indirectly exposed as bystanders at 3 or 5 kGy gamma radiation levels (BYST3 and BYST5) were characterized respectively by an average survival time of 9.5 and 10.9 days.

Direct and indirect exposure to 3 and 5 kGy gamma radiation significantly decreased tardigrade survivorship relative to survivorship in unirradiated

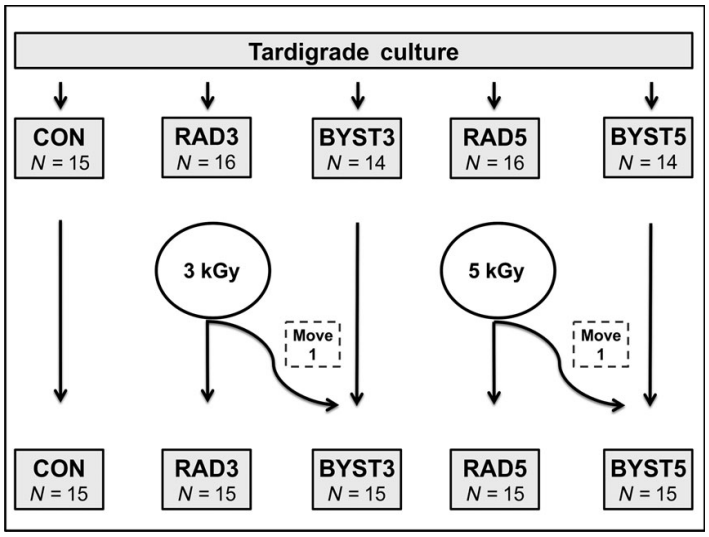

Figure 1. Experimental set-up for testing radiation tolerance and bystander effects in the eutardigrade species *Hypsibius dujardini*: control (CON; 0 kGy), irradiated [received 3 kGy (RAD3) and 5 kGy (RAD5) gamma radiation] and bystander [exposed to an individual irradiated at the 3 kGy (BYST3) or 5 kGy (BYST5) level] groups.

controls (Fig. 2; RAD3, $P < 0.0001$; RAD5, $P < 0.0001$; BYST3, $P = 0.0030$; BYST5, $P = 0.0016$). A significant dose-dependent decrease in survivorship was observed in the directly exposed groups, RAD3 and RAD5 ($P = 0.0025$), with greater survivorship at the lower radiation dose. No significant difference in survivorship was observed between the indirectly exposed groups (BYST3 and BYST5; $P = 0.21$). Groups irradiated directly at the 5 kGy level differed significantly in survivorship from groups irradiated indirectly (BYST3 and BYST5; $P < 0.0001$).

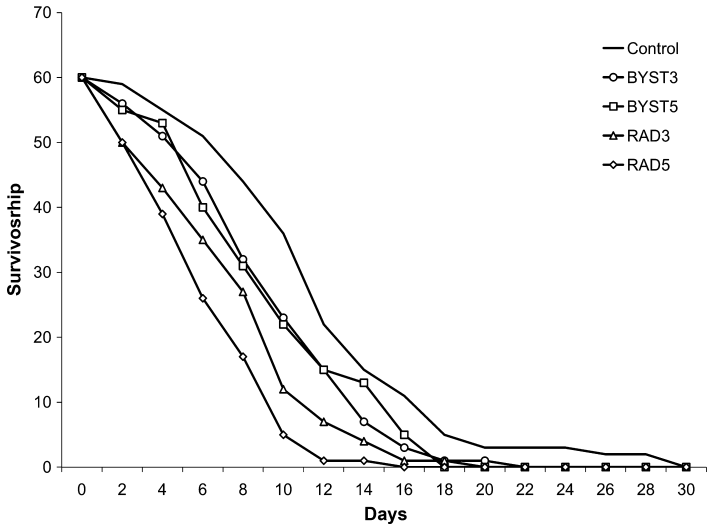

Figure 2. Radiation and radiation-induced bystander effects on the eutardigrade species *Hypsibius dujardini*. Survivorship curves following direct (RAD) and indirect (BYST) exposure to 3 and 5 kGy gamma radiation.

DISCUSSION

Previous studies reported that tardigrade radiation tolerance is impressive and dose-dependent, survivorship decreasing with increasing radiation doses (Jönsson *et al.*, 2005; Horikawa *et al.*, 2006; Beltrán-Pardo *et al.*, 2015) – findings that are consistent with the findings reported herein. We found that *H. dujardini* can tolerate high gamma radiation doses, with significantly decreased, dose-dependent survivorship at 3 and 5 kGy. The value of 5 kGy is identical to the median lethal dose reported previously for *Milnesium tardigradum* (Doyère, 1840; Horikawa *et al.*, 2006).

Bystander groups also exhibited lower survivorship, demonstrating that indirect exposure to radiation imparts damaging effects, the first bystander effect reported in tardigrades. The bystander effect exhibited no dose dependence. This finding is similar to previous cellular studies, revealing no relationship between bystander effects and increasing radiation doses (Blyth & Sykes, 2011). One possible explanation for a lack of dose–response in bystander groups is a constraint on signal release by irradiated cells or organisms (Blyth & Sykes, 2011).

Bystander group specimens in the present study were exposed to only one irradiated individual. Mothersill *et al.* (2006) reported that secondary bystander fish swimming in water that had contained previously irradiated and bystander partner fish showed more potent effects from radiation damage than did the bystander partner fish themselves; thus, an increase in bystander signalling – produced by irradiated and bystander partner fish – might have led to the observed increased secondary bystander effects. We therefore speculate that exposure to more than one irradiated individual at each radiation dose could induce a dose-dependent effect in bystander tardigrades.

Radiation-induced bystander effects in tardigrades might manifest as a threshold response, wherein decreased survivorship is elicited once a minimum dose is reached, rendering animals unable to respond to increased signals produced by irradiated animals (Blyth & Sykes, 2011). This might explain why bystander groups in the present study differed significantly in survivorship from directly irradiated groups only at the 5 kGy level, which suggests that a threshold for indirect exposure to gamma radiation in *H. dujardini* resides between 3 and 5 kGy.

Whether the observed dose independence is attributable to limited signal production by irradiated individuals or a threshold response receptor system in bystander specimens, the mechanism for the bystander effect remains unknown. Molecules that might be involved in bystander signalling and the associated induction modes have yet to be characterized. The data presented in this study provide supporting evidence that radiation-induced bystander effects can elicit detrimental consequences at the organism level. Future research, in addition to the aforementioned exposure to 'multiple-specimen-produced' signals to test the threshold response hypothesis, should be directed at refining the threshold value and determining whether the damaging effects seen in bystander specimens can manifest across generations.

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CHAPTER 6: QUANTUM-LIKE INCREASED INCUBATION PERIOD FOR EMBRYOS WITH INCREASED COLD EXPOSURE

6.1 PREFACE

This chapter contains the submitted paper "Quantum-like increased incubation period for embryos with increased cold exposure*"* authored by Vasanthan T., Nederveen J.P. and Stone J. (Nature Communications, submitted July 26th 2017, NCOMMS-17-18776). The manuscript is reproduced in its submitted format.

Three hypotheses have been proposed to explain effects from desiccation on micrometazoan lifespan: animals stop aging (Sleeping Beauty Hypothesis), continue to age (Rip van Winkle Hypothesis) or age at a decreased rate. Although lifespan extension resulting from desiccation has been described, whether the same premises hold true for cold exposure remains untested. Here we describe effects from exposure to low temperature on embryonic development in the eutardigrade species *Hypsibius dujardini*. Eggs incubated at 0 °C for 4 days did not hatch while controls reared at 22 °C did (4 days = typical gestational period for this species). Cold-exposed eggs hatched 3 days after having been returned to 22 °C, thereby extending their gestational period from 4 to 7 days. Juveniles that hatched from cold-exposed eggs continued to experience a 3-day lag in development compared to controls, indicating that their biological age was less than their chronological age. Extending cold-exposure (0 °C) time to 10, 20 and 40 days yielded incrementally decreased incubation time, 3, 2 and 1, respectively at 22 °C. Lifespan extension by increasing embryological development in response to cold exposure supports the third hypothesis, where

aging continues at a diminished rate. Tardigrade development at low temperature seems to be governed by quantum-like processes, where an energetic-based system is used to control biological aging.

6.2 CONTRIBUTIONS

Tarushika Vasanthan performed the experiments, wrote the manuscript and generated Figure 3. Josh P. Nederveen performed live imaging via DIC microscopy. Josh P. Nederveen and Tarushika Vasanthan collectively generated the composites for Figures 1 and 2. Jonathon Stone provided a great deal of direction and input to enrich the manuscript.

Title: Quantum-like increased incubation period for embryos reared with increased cold exposure time

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Keywords: Aging Rate, Cold Tolerance, Embryogenesis, Tardigrades

ABSTRACT

Three theoretical models have been proposed to explain lifespan extension resulting from exposure to extreme conditions in microscopic animals: individuals become completely dormant and stop aging, continue to age or age but at a diminished rate. Here we show that embryonic cell division in the eutardigrade species *Hypsibius dujardini* is retarded when eggs are reared at 0 °C. Compared to controls, juveniles that hatched from eggs exposed to 0 °C for 4 days experienced a three-day lag throughout life history, indicating that their biological age was less than their chronological age. As cold exposure duration increased (days = 10, 20, 40), incubation time decreased incrementally (days = 3, 2, 1), suggesting that tardigrades involve a quantum-like, energetic-based system for controlling biological aging.

1. INTRODUCTION

An anhydrobiotic state is a condition characterised by reduced, possibly lacking, metabolic activity induced by desiccation at any stage in life history. Anhydrobiosis enables micrometazoans like rotifers, nematodes and tardigrades to tolerate extremely arid conditions that otherwise would be lethal (1). Each ahydrobiotic individual can remain dormant in a desiccated state for weeks, months or even years, until surrounding conditions become favorable for re-animation (2). During desiccation onset, cell protectants like trehalose, heat

shock proteins and intrinsically disordered proteins are synthesized to assist in molecular and cellular stabilization (3-5). Successful resurrection following desiccation is favored by gradual dehydration (1, 6).

Desiccation tolerance effects have been documented at the molecular level (7), but effects on life history have received less attention. Three explanations have been proposed (8). The 'Sleeping Beauty' hypothesis states that metabolic and physiological processes halt in desiccated organisms; no aging occurs, in analogy with the famous fairy tale, wherein a princess sleeps for 100 years and awakens at the same point in life history as when she entered her century-long slumber (8, 9). The 'Rip van Winkle' hypothesis states that metabolic and physiological processes continue in desiccated organisms; aging continues, in analogy with the famous short story, wherein a villager sleeps for 20 years and awakens accordingly advanced in age relative to the point in life history in which he entered his two-decade repose (8, 9). The third hypothesis involves the suggestion that metabolic and physiological processes continue at a lower rate in desiccated organisms; aging continues but in a diminished manner (8, 9).

The Sleeping Beauty hypothesis has been supported by studies on two rotifer species (*Macrotrachela quadricornifera* and *Adineta ricciae*) and one tardigrade species (*Milnesium tardigradum*), wherein desiccated then rehydrated specimens lived for as many active days as did control specimens (9-11). The Rip van Winkle and partial aging hypotheses have been supported by studies on one nematode species (*Panagrolaimus rigidus;* 8, 12). Effects from desiccation on lifespan thus have been documented with adult micrometazoans, but whether cold temperatures could produce similar effects at earlier life history stages remains unknown. We therefore used the tardigrade species *Hypsibius dujardini* to test whether embryonic cell division is retarded when eggs are reared at 0 °C.

2. RESULTS AND DISCUSSION

Cell division in *H. dujardini* embryos is asymmetrical and rapid; nuclear migrations at the 1, 2 4 and 8 cell stage (Figure 1) occur within the first 4 hours post laying (13). Embryos typically change from an opaque to a translucent brown color as they develop. This transition in color was observed in all control embryos (22 °C). Control juveniles hatched 4 days post laying and reached sexual maturity approximately 6 days post hatching (N=30, three trials) – stereotypical development for *H*. *dujardini*. Treatment embryos (0 °C), however, exhibited no colour change during the 4-day cold exposure. Once returned to 22 °C, the stereotypical change in color was observed and juveniles hatched 3 days later (Figure 2). Those juveniles continued to experience this 3-day lag relative to control juveniles throughout ontogeny. The average egg-to-hatchling period for *H. dujardini* is approximately 4 days (13); cold-exposed embryos, however, remained at the egg stage for 7 days (4 days at 0 °C + 3 days at 22 °C) before hatching. Thus, while the chronological age for control and cold-treated eggs was equivalent, the biological age for cold-treated eggs was less (i.e., cold treated eggs appeared younger than their control counterparts).

This finding prompted us to consider three hypotheses, with associated lifespan-altering modes, for subsequent testing: frozen eggs had either retarded, ceased or accelerated development. In the first case, exposure to 0 °C had reduced cell division rate – in the extreme, 4 days at 0 °C had been equivalent to one day at room temperature. In the second case, exposure to 0 °C had induced a developmental checkpoint pathway – in the extreme, cell division halted upon exposure to 0 °C, whereupon a genuine suspended animation ensued. In the third case, re-introduction to 22 °C had increased cell division rate – in the extreme, 3 days at 22 °C after maintenance in suspended animation at 0 °C were equivalent to 4 days at 22 °C. These modes would enable eggs and embryos to tolerate low temperatures for extended time periods and resume cell division and

embryogenesis once conditions suitable for development and subsequent growth and reproduction had resumed.

To try to distinguish among the hypotheses and their associated modes, we incubated eggs at 0°C for longer durations. We found that the lag in development decreased in a quantum-like manner: eggs subjected to 0 °C for 10-, 20- and 40-day exposure periods and then returned to 22 °C were characterized respectively by 13-, 22- and 41-day incubation periods. Thus, as cold-exposure time (in days) doubled from 10 to 20 to 40, embryogenesis time (in days) at ambient laboratory temperature diminished from 3 to 2 to 1 (Figure 3). The most parsimonious interpretation for results obtained with short- and long-term exposure to 0 °C is that tardigrade embryos involve a quantum-like system to control aging, slowing incrementally with decreased 'action' (i.e., energy x time) units.

Lifespan extension resulting from desiccation is a gradual process, as slow drying is required for organisms like rotifers, nematodes and tardigrades to synthesize necessary protectant molecules (1). Similar to anhydrobiosis, cryobiosis requires gradual cooling rates to allow organisms to up-regulate protectant molecules (14). We found contrastingly that tardigrade embryos respond in a cryobiotic-like state without the need for gradual cooling, suggesting the action-dependent incremental developmental response (Figure 3). Tardigrades thus tolerate different extreme conditions through different modes at different stages in life history.

We found support for the partial aging hypothesis. Given that embryos in the tardigrade species *H. dujardini* are neither Sleeping Beauties nor Rip van Winkles and to distinguish the cryobiotic responses reported herein from anhydrobiotic responses (and cryobiotic responses to extreme-cold exposures) reported previously, we propose the 'Snow White' hypothesis: lifespan extension in chilled

organisms resulting from a quantized decrease in cell division rate. The related hypotheses that embryogenesis is affected by a cessation at a developmental checkpoint during cold-exposure (as with Dauer larvae in nematodes (15)) or an increase in embryogenesis after (or both) remain for future definitive testing.

The processes involved in embryogenesis retardation might provide an opportunity for innovative practical application in cryopreserving eggs from other organisms, most-obviously for human *in vitro* fertilisation. Elucidating the associated mechanisms constitutes another exciting challenge for future research.

3. MATERIALS AND METHODS

To examine effects from exposure to low temperature on tardigrade embryonic development, 20 eggs were collected from 15 egg-laying adults in the parthenogenetic eutardigrade species *Hypsibius dujardini* (Sciento Z151). Eggs were stored in 1.5 mL polypropylene microtubes containing 10 µL spring water and maintained at either 22 °C (controls, N=10) or 0 °C (treatment, N=10) in a temperature controlled incubator for 4 days (average gestational period). Eggs and embryos maintained at 22 °C were observed daily with light microscopy (Nikon SMZ1000) to assess normal embryonic development. Hatchlings were stored individually in 1.5 mL polypropylene microtubes containing 50 µL spring water and 15 µL algal food (*Chlorococcum* sp.; Sciento A68) and monitored every three days with light microscopy until all had reached sexual maturity (i.e., first embryogenesis). Three trials were conducted. To test long-term cold exposure on embryonic development, additional eggs were maintained either at 22 °C (controls, N=12) or in a temperature-controlled incubator set at 0 °C for 10 (N=12), 20 (N=12) and 40 days (N=12).

For live imaging, embryos were mounted with spring water on uncoated glass microscope 24x50 mm No.1 slide covers with thickness 0.13 to 0.17 mm (VWR

International). To capture early developmental events, images were captured at the one, two, four and eight-cell stages (approximately 0 to 4 hours post laying; Figure 1). Embryos maintained at 22 °C were imaged at 24, 48, 72 and 96 hours post laying; embryos maintained at 0 °C were imaged at 24, 48 and 72 hours post 4-day cold exposure (Figure 2). Specimens otherwise were maintained on the slide covers and placed in a dark, humid box at 22 °C. Development was observed via differential interference contrast (DIC) microscopy with a Plan Apochromat 60x/1.4 objective lens (Nikon Eclipse Ti), equipped with a high-resolution camera (Photometrics CoolSNAP HQ2 Nikon). Images were viewed and captured with the software NIS Elements AR 4.4 (Nikon Instruments).

4. ACKNOWLEDGEMENT

This research was developed with funds from the Joseph and Joanne Lee Ontario Graduate Scholarship, cultured with specimens from R. McNuff and illuminated with a microscopy suite provided by G. Parise.

5. CONTRIBUTIONS

Under the supervision of and guidance of J.S., T.V. performed the experiments, wrote the manuscript with contributions made from all authors and generated Figure 3. J.P.N. carried out live imaging of the embryos via DIC microscopy. J.P.N. and T.V. collectively generated the composites for Figure 1 and 2.

6. COMPETING FINANCIAL INTERESTS

No competing financial interests to declare.

7. FIGURES

Figure 1

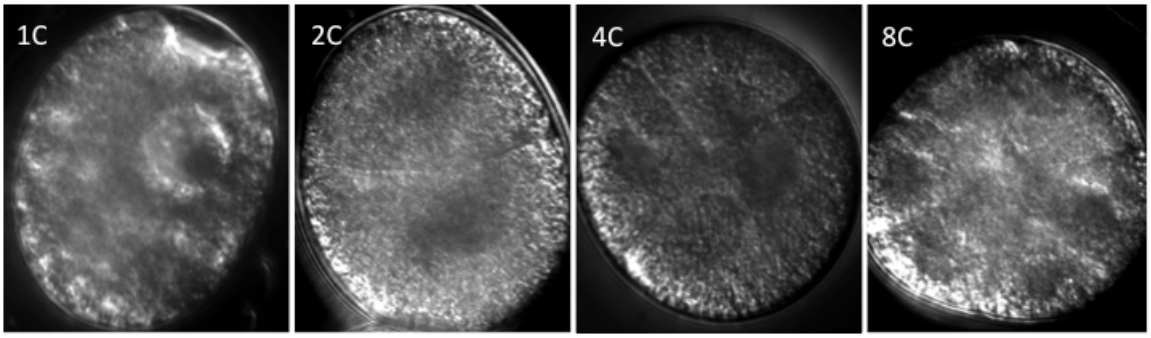


Figure 2

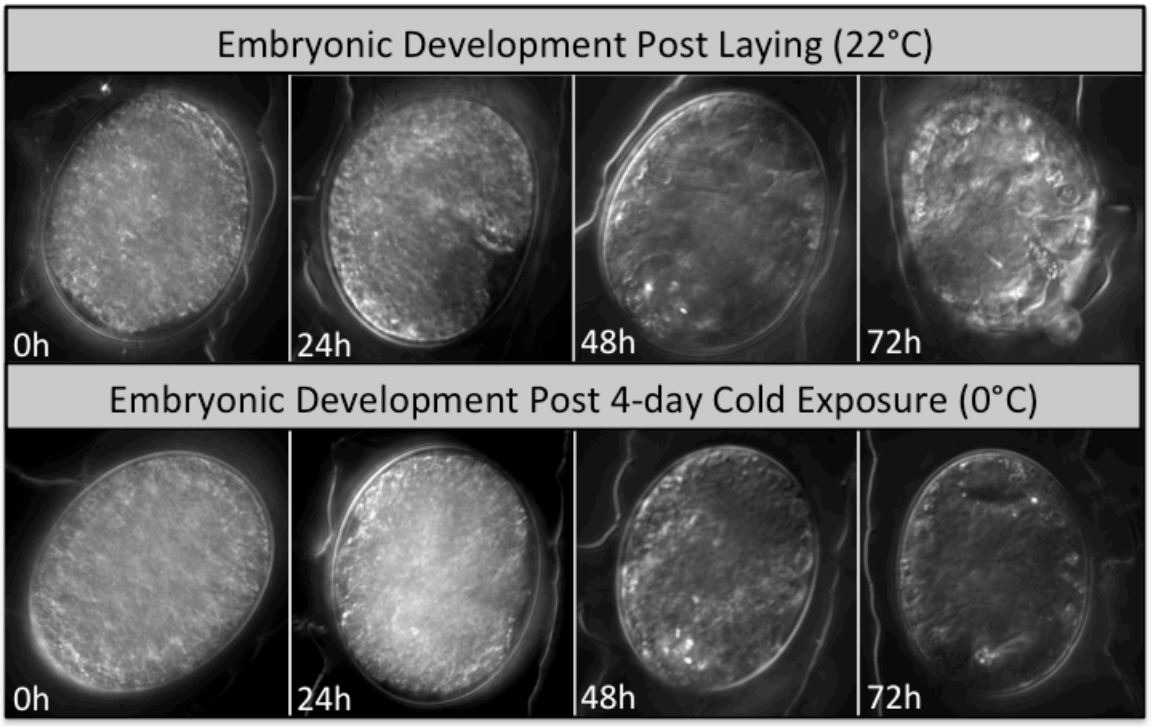
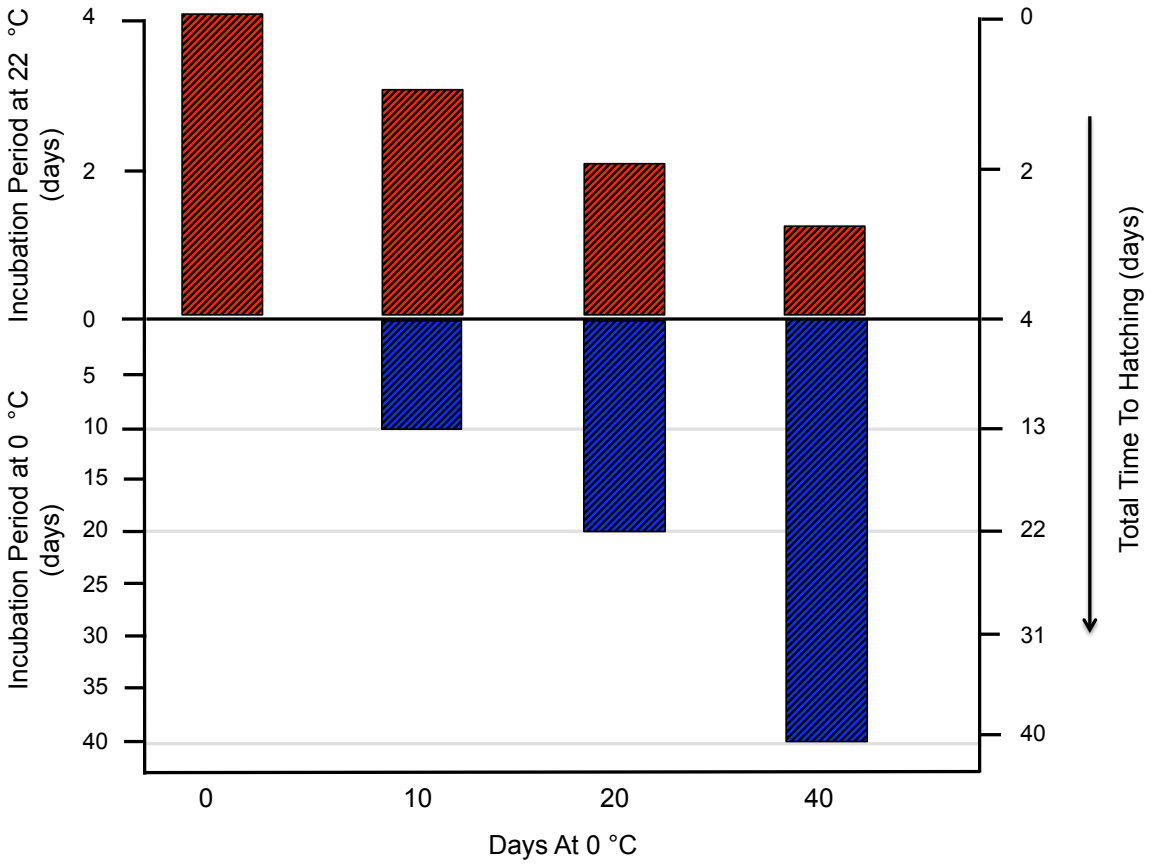


Figure 3



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9. FIGURE LEGENDS

Figure 1: Early cell division (0-4 hours) in *Hypsibius dujardini* embryos maintained at 22 °C, showing nuclear migrations across the one (1C), two (2C), four (4C) and eight (8C) cell stages.

Figure 2: Embryonic development in *Hypsibius dujardini* eggs at 24-hour intervals post laying (22 °C) and post 4-day cold exposure (0 °C).

Figure 3: Incubation periods and total time to hatching for *H. dujardini* eggs (N=12) exposed to 0 °C for 0, 10, 20 and 40 days.

CHAPTER 7: LONG TERM COLD EXPOSURE EFFECTS ON EMBRYONIC DEVELOPMENT IN THE EUTARDIGRADE SPECIES *HYPSIBIUS DUJARDINI*

7.1 PREFACE

This chapter contains the manuscript in preparation titled “Long term cold exposure effects on embryonic development in the eutardigrade species *Hypsibius dujardini ”* authored by Vasanthan T. and Stone J.

For metazoans like tardigrades, successful entry into and out from anhydrobiotic and cryobiotic states requires gradual changes in humidities and temperatures, respectively. The present study examined the effects from exposure to low temperature on tardigrade embryonic development, without inducing a cryobiotic state, through gradual cooling. To examine long term effects, eggs from *H. dujardini* were reared at 0 °C for 10, 20 and 40 days. To examine the effects from varying low temperature during gestational period (i.e. days = 4), eggs were reared in temperature-controlled groups from -2 °C to 20 °C at 2 °C increments. We found that long term cold exposure to 0 °C and varying incubation temperatures extended total time to hatching relative to controls. Lengthening cold exposure intervals impeded growth in juveniles and decreased survivorship in adults, suggesting that costs are associated with increasing incubation time at 0 °C. Given that gradual cooling is un-required to slow down development, characterizing the molecular processes involved in slowed embryogenesis maybe useful for improving biological techniques like cryopreservation.

7.2 CONTRIBUTIONS

Tarushika Vasanthan performed the experiments, analyzed the data, wrote the

manuscript and generated Figures 1 through 3. Jonathon Stone provided direction during the course of the experiment, guiding data analyses and enriching content.

Title Page

Long term cold exposure effects on embryonic development in the eutardigrade species *Hypsibius dujardini*

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Abstract

Lifespan extension generally is associated with the ability to enter a cryptobiotic state. Revival from some cryptobioses, like anhydrobiosis and cryobiosis, requires gradual environmental change, for organisms to upregulate cell protectants. We show that embryonic development in the eutardigrade species *Hypsibius dujardini* can be extended for at least 40 days at 0 °C without undergoing cryptobiosis or gradual cooling. Whether exposure time was doubled sequentially (i.e. 10, 20 and 40 days) at constant incubation temperature (i.e. 0 °C) or incubation temperature was varied over more than a 10-fold range (-2 to 20 °C) at constant incubation period (i.e. 4 days), embryogenesis slowed. Compared to controls, doubling cold exposure time led to impeded growth in juveniles and lowered survival rates in adults, suggesting that costs are associated with prolonging hatching time. Elucidating the molecular processes involved in biological clock adjustments during development could have important implications for improving biological practices, such cryopreservation techniques, and thus warrants further attention

1. Introduction

Lifespan extension through anhydrobiosis and cryobiosis respectively are responses elicited by rotifers, nematodes and tardigrades when exposed to xeric and freezing environments that otherwise would be detrimental to survival. Franceschi (1948) reported on recovering a single tardigrade specimen from a 120-year old dried moss sample. While that observation has been criticized (i.e. whether movement was in fact detected; Jönsson and Bertolani, 2001), the potential for century-long dormancy seems plausible when considering the literature on long-term anhydrobiotic and cryobiotic survival.

The longest recorded anhydrobiotic survival in rotifers (individuals from the genus *Mniobia*) was observed from a 9-year old moss sample collected from Sweden (Guidetti and Jönssen, 2002). The longest recorded anhydrobiotic survival in nematodes (in the plant pathogenic species *Tylenchus polyhypnus*) was observed from a rye seedling leaf, whereon two females and three larvae were revived successfully following a 39-year dormancy (Steiner and Albin, 1946). Reports on long-term cryobiotic survival are less common. Specimens in the nematode species *Plectus murrayi* were revived successfully from frozen (-20 °C) moss samples that had been stored for 25.5 years (Kagoshima et al., 2012). Specimens in the Antarctic tardigrade species *Acutuncus antarcticus* were revived from 30.5 years in cryobiotic (-20 °C) dormancy and even reproduced (Tsujimoto et al., 2015).

Anhydrobiosis and cryobiosis are hypothesized to manifest most effectively when environments change (i.e. dry and cool) gradually, to allow individuals to synthesize protectant molecules (e.g. trehalose, glycerol, intrinsically disordered proteins) used to replace water molecules and minimize cell damage sustained from water loss and crystallization (e.g. Crowe, 1972; Wright, 1989; Boothby et al., 2017). Lifespan extension in desiccated or frozen adult micrometazoans has

been attributed to the ability to successfully enter these cryptobiotic states. Here we show that embryonic development in tardigrade eggs can be slowed down for at least 40 days at 0°C, thereby extending gestation period approximately 10-fold without undergoing cryptobiosis.

2. Materials and methods

2.1 Long term cold exposure

To test lifespan extension resulting from cold exposure, we used the parthenogenetic eutardigrade species *Hypsibius dujardini* (Sciento Z151). Forty eight newly laid eggs were collected from ovipositing mothers at 22 °C. Eggs collected 1 hour post-laying were maintained at 0°C in a temperature controlled incubator for 10 (N=12), 20 (N=12) or 40 days (N=12); control eggs were maintained at 22 °C (N=12). Following cold exposure treatments, eggs were returned to 22 °C and observed daily under light microscopy (Nikon SMZ1000) until hatching. Hatchlings were stored individually in 1.5 mL polypropylene microtubes containing 100 µL spring water (Aberfoyle, Puslinch Ontario; Nestlé Pure Life) and 10 µL algal food (*Chlorococcum* sp.; Sciento A68). Individuals were monitored every two days under light microscopy. Growth, survivorship and egg production were recorded every two days (eggs produced by individuals were removed from microtubes). Used microtubes were replaced with new microtubes and water and algae were replenished every other day. Survivorship curves were analyzed using logrank tests (Bland & Altman 2004).

2.2 Effects from low temperature exposure on total hatching time

To determine the effects from low temperature exposure on gestational period, eggs (N=12) laid by *H. dujardini* at 22 °C were stored at -2 through 20 °C, in 2 °C increments, for 4 days – typical gestational period for this species – in a temperature controlled incubator. A control group (20 °C: N=12) was established for each independently run temperature treatment. Following the 4-day cold

exposure, eggs were returned to 22 °C and monitored daily under light microscopy until hatching. Hatching success and gestational period were recorded.

3. Results

3.1 Long Term Cold Exposure

While survivorship did not differ significantly between controls and eggs exposed to 10- and 20-day cold treatments ($p > 0.05$), a significant decrease in survivorship was observed between controls and 40-day cold-treated eggs ($p < 0.05$; Figure 1). A significant decrease in survivorship also was observed between 40-day cold-treated eggs and 10- and 20-day cold treated eggs ($p < 0.05$). Treatment effects were time-dependent, with increased exposure time resulting in delayed or halted growth. Specimens in control groups followed typical growth schedules (i.e. increased growth on day 3 and sexually maturity achieved by day 6, post-hatching), whereas slowed development was observed among specimens subjected to 10-day cold treatments (18%) and 20- and 40-day cold treatments (8%). Even more juveniles that hatched from 20- and 40-day cold treated eggs failed to grow (75% and 67%, respectively) and died before reaching sexual maturity.

The maximum age was achieved by a single individual in the control group, which lived to 80 days. Maximum age declined with increased cold exposure time; maximum age achieved in 10-, 20- and 40-day cold-treated groups were 76, 64 and 50 days, respectively. Mean lifespan for controls was 57.3 days; mean lifespan for cohorts exposed to 10-, 20- and 40-day cold treatments were 48.9, 62.7 and 41.3 days, respectively. Relative to controls, egg production also declined with increased cold exposure time, with adults laying 21 ± 1.0 and 17 ± 1.4 eggs, in the 10- and 40-day cold-treated groups, respectively; mean egg production in control groups was 25 ± 0.9 (Figure 2). Interestingly, individuals

from the 20 day cold treated cohort produced approximately 1.6 times more eggs than did individuals in control groups (41 ± 1.4; Figure 2).

Effects from low temperature exposure on total hatching time

Exposing tardigrade eggs to declining cold temperatures during the 4-day gestational period increased overall hatching time (Figure 3). While tardigrade eggs reared at room temperature (22 °C) hatched 4 days post-laying (following the typical gestation period for this species), eggs incubated at chilled temperatures (-2 °C, 0 °C, 2 °C, 4 °C, 6 °C and 8 °C) extended hatching time by an additional 3 days; eggs reared at low (10 °C and 12 °C) temperatures extended gestational period by 2 days; eggs reared at moderate (14 °C and 16 °C) temperatures increased gestational period by a single day. Gestational period in eggs incubated at 18 °C and 20 °C was the same as in control groups (i.e. total hatching time = 4 days).

4. Discussion

Lifespan extension commonly is attributed to cryptobiosis, in particular anhydrobiosis, in tardigrades. We found that the gestational period for *H. dujardini* eggs can be extended via cryobiosis, for at least 40 days at 0 °C, increasing hatching time approximately 10-fold. The ability to survive extended periods at low-temperature exposure is remarkable given that embryonic cells typically are more sensitive to stressors (Beltran-Pardo et al., 2015). Increasing incubation time while maintaining a constant temperature (i.e. 0 °C) or varying temperature and maintaining a constant incubation time (i.e. days = 4) altered total time to hatching relative to total time to hatching for control specimens. Specifically and remarkably, doubling incubation time at 0 °C from 10 to 20 to 40 days decreased hatching time at 22 °C from 3, to 2 to 1 days; and increasing temperature from -2-8 to 10-12 to 14-16 °C decreased incubation time at 22 °C from 3 to 2 to 1 days; Figure 3). These findings suggest that tardigrade eggs respond to temperature changes by slowing down embryogenesis independently

from entering a cryptobiotic state. Given that eggs were incubated at low temperatures during early embryonic development (defined as 1-6 hours post-laying; Gabriel et al., 2007), the ability to undergo cryptobiosis may have been unachievable.

While exposure to low temperatures resulted in lifespan extension early in life history (i.e. day 1 for 40-day cold treated eggs was equivalent to day 37 in controls), costs incurred during embryogenesis led to decreased survival rates in adults (Figure 1). Moreover, while hatching success was approximately 100% in all cohorts, juveniles that hatched from eggs reared at 0 °C for 10, 20 and 40 days experienced delayed or stunted growth, compared to controls. Similar growth impediments have been documented in irradiated tardigrade eggs (Beltrán-Pardo et al., 2013; Beltran-Pardo et al., 2015). Juveniles of *M. tardigradum* and *H. dujardini* that hatched from gamma irradiated eggs experienced a lag in development and adults were characterized by lower survivorship, compared to controls (Beltrán-Pardo et al., 2013; Beltrán-Pardo et al., 2015). Embryogenesis in most tardigrade species is regulated tightly (Hejnol and Schnabel, 2005). Irradiation can result in cell loss, with the remaining cells continuing to divide (Beltrán-Pardo et al., 2015) Cell loss experienced during early development is thought to cause growth impediments in juveniles. This might partially explain why eggs reared at 0 °C for 10, 20 and 40 days had growth defects as juveniles and diminished survival rates as adults.

Our findings show *H. dujardini* eggs tolerate long-term cold exposure and varying temperatures by slowing down embryogenesis. Given that gradual cooling is not required to slow down development, identifying the molecular processes involved can have applications for improving biological techniques like cryopreservation and thus requires further attention. Furthermore, while hatching time did not increase among eggs incubated above 18 °C, whether a similar threshold

response to exposure time exists is not yet known. Future research should therefore also aim to define the threshold for long-term cold exposure.

5. Acknowledgements

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7. Figures

Figure 1

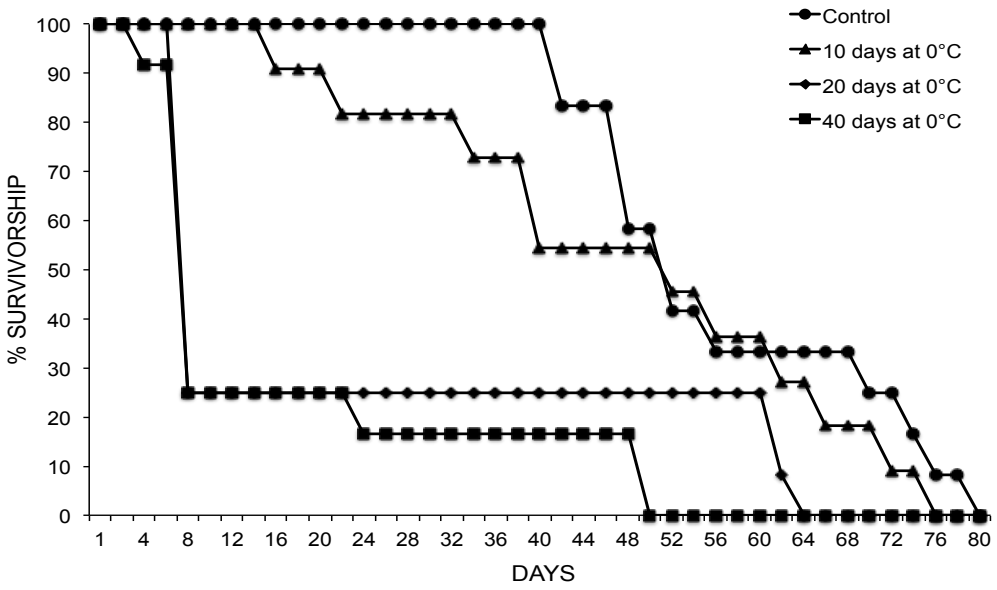


Figure 2

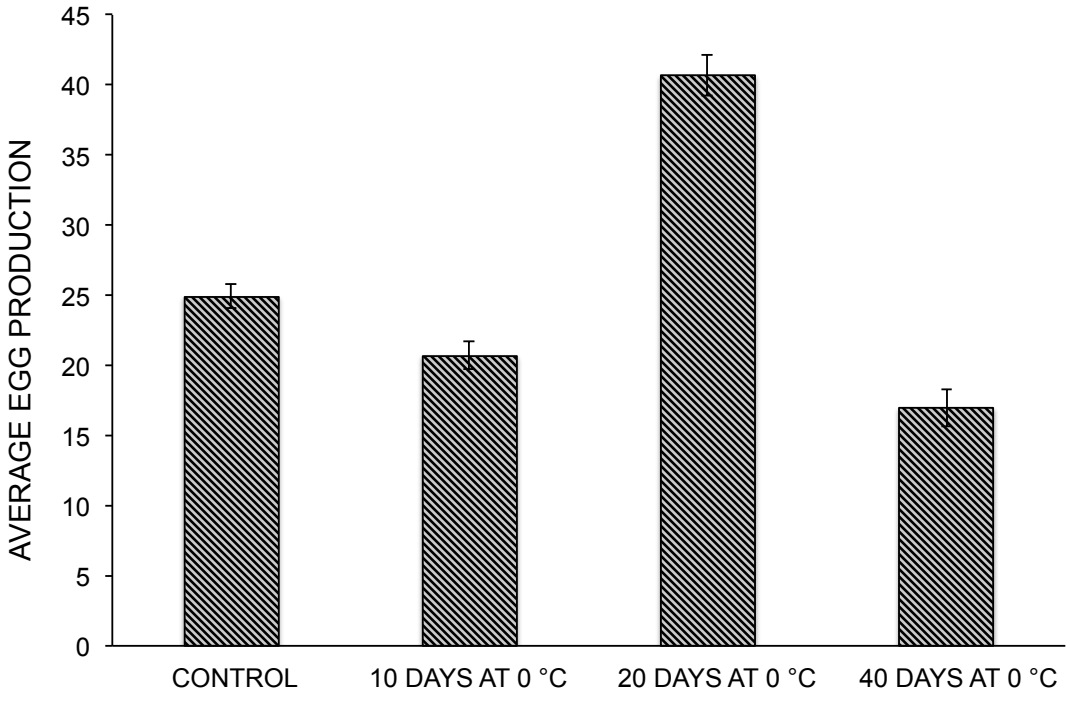
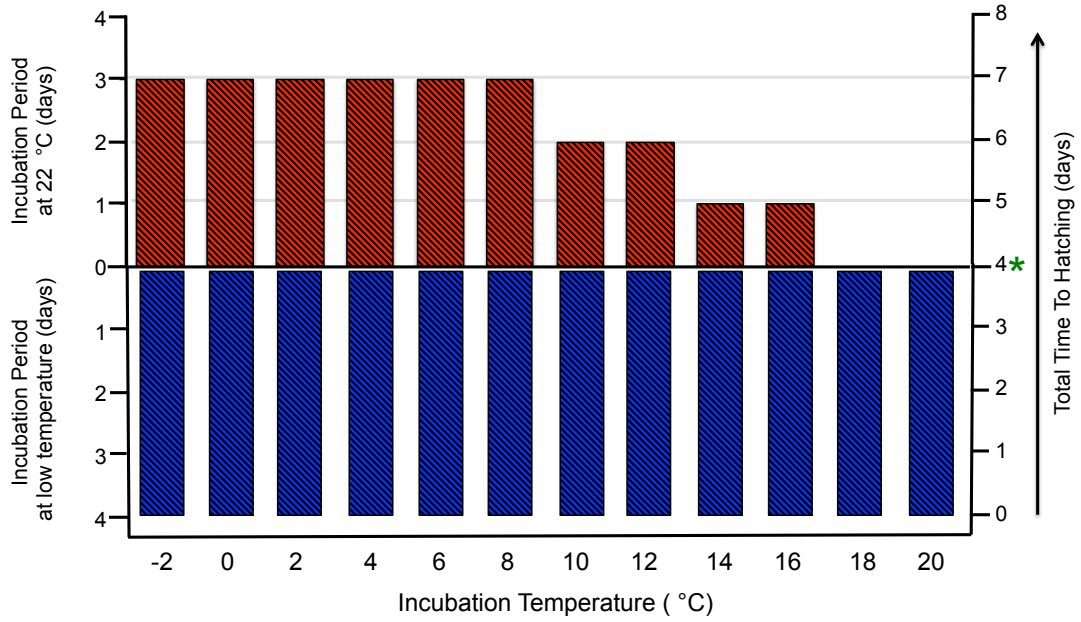


Figure 3



8. Figure Legends

Figure 1: Biological survivorship curves for individual that hatched from eggs reared at 0 °C for 10 (N=12), 20 (N=12) or 40 (N=12) days; controls (N=12) were reared at room temperature (22 °C).

Figure 2: Average (Mean± SD) egg production in adults that hatched from eggs reared at 0 °C for 10 (N=12), 20 (N=12), and 40 (N=12) days; controls (N=12) were reared at room temperature (22 °C).

Figure 3: Incubation period and total time to hatching for eggs (N=12) reared at -2, 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 °C for 4 days; * represents the typical gestation period for *H. dujardini* eggs.

CHAPTER 8: SUMMARY OF FINDINGS

Findings from this dissertation provide a more-complete assessment on *H. dujardini*, its life history and extreme-tolerant abilities. The subsequent text summarizes the research findings and describes their applications to biology and astrobiology.

8.1 Life History of Hypsibius dujardini

The life history traits for *H. dujardini* described in Chapter 2 provide a more comprehensive understanding about its growth, reproduction and lifespan, currently lacking in literature (Schill, 2013). We have shown that the initial stages in life history can be vulnerable for *H. dujardini* hatchlings with one-tenth among individuals failing to reach sexual maturity and one- fifth experiencing delayed growth. Although reduced survivorship was observed soon after hatching, survival rates generally were high during the early and middle stages in life history.

Although *H. dujardini* has a shorter lifespan than do most tardigrade species (e.g. *Paramacrobiotus palaui,* maximum age = 97 ± 31 days; Schill, 2013; Table 1), its life history makes *H. dujardini* ideal for biological investigation wherein multigenerational effects can be observed in a short time period, including damaging effects from exposures to extreme conditions. With a sequenced genome (Boothby et. al., 2015; Koutsovoulos et al., 2016), emerging experimental toolkit (including RNA interference and *in situ* hybridization; e.g. Tenlen et al., 2013), short generation time (approximately 15 to 16 days), easy maintenance under laboratory conditions and, now, having a well-documented life

history, *H. dujardini* is a bona fide candidate for biological inquiry.

While recognized tardigrade species have increased in number during my studies, fundamental biology for most species has yet to be characterized. Future research, therefore, should focus on delineating life history traits for other tardigarde species to provide a more comprehensive understanding about the phylum Tardigrada.

8.2 Tolerance to Hypergravity

For an organism to be considered a candidate for interplanetary travel, it must tolerate three phases: ejection from a planetary body, interplanetary space conditions and, upon arrival, successfully entry of a planet's atmosphere (Mastrapa et. al., 2001). Tardigrade tolerance to extreme desiccation (Ramlov and Westh, 2001), ionizing radiation (Horikawa et. al., 2008), low temperatures (Jorgensen et. al., 2007) and near-vacuum pressures (Jonssen et. al., 2008) would allow it to overcome conditions associated with phases two and three. Whether tardigrades could survive short-term g-forces associated with planetary ejection was an unexplored research area. Chapter 3 describes tardigrade tolerance to hypergravity and their potential to survive planetary departure. We found that g-force tolerance in *H. dujardini* was acceleration-dependent with lowered survivorship at higher accelerations (i.e. LA_{50} (medial lethal acceleration) at 3, 421*g* = 17.6 days vs. LA_{50} at 16,060*g* = 10.8 days; Vasanthan et al., 2017). Higher accelerations also led to decreased egg production. Whereas costs

associated with survivorship and fecundity were apparent, the ability to tolerate high accelerations is quite remarkable in tardigrades. Prokaryotic organisms often are regarded as being more tolerant to hypergravity levels because the likelihood for organelle sedimentation increases with organelle size (Deguchi et. al., 2011). Here we show that tardigrades – eukaryotic organisms – can survive high acceleration rates and therefore can be used for astrobiological investigation.

Analogous to our study on tardigrade tolerance to hypergravity, tardigrade extreme-tolerance research has been reported predominantly on short-term exposure to extreme conditions (Horikawa et. al., 2006; Horikawa, 2012; Jonssen et. al., 2008). To realize tardigrade potential for astrobiological research, future research should examine the effects imparted by long term exposure to extreme conditions, such as prolonged exposure to the space vacuum, ionizing radiation and hypergravity levels. Defining the upper limits for extreme-tolerance in tarigrades may provide new possibilities for interplanetary travel research on multicellular organisms.

8.3 Tolerance to pH Environments

Tardigrade tolerance to pH has only been described only over short time periods and then in unpublished research. Chapter 4 describes effects from long term exposure to acidic and alkaline conditions in *H. dujardini* adults. Whereas extreme alkaline (pH 11 – pH 14) and acidic (pH 1 and pH 2) environments caused immediate death (i.e. within 24 hours), pH 3, 4, 5, 6, 7 and 8 were well

tolerated; exposure to pH 9 and pH 10 resulted in decreased mobilization. Based on our findings, pH tolerance in *H. dujardini* might be enabled by the ability to undergo cryptobiosis. Tardigrades exhibited tun-like morphologies (i.e. retraction of limbs and barrel-like bodies) when pH conditions became extreme (i.e. pH 9 and 10). The observed response to changes in salinity has been documented previously (Raz, 2005). Thus, members in *H. dujardini* seem to tolerate saline conditions better than acidic conditions. The corrosive properties associated with sulfuric acid may explain the lacking tun-like morphologies at lower pH levels.

Whereas most organisms have a well-defined pH range beyond which individuals cannot survive (Tucker and D'Ambro, 2008), tardigrades seem to tolerate a wide range of pH conditions outside their normative range (i.e. pH 7). These findings open up new possibilities in tardigrade extreme-tolerance research and provide support for future astrobiological investigation using tardigrades, particularly parameters concerning habitability in extreme environments. For example, the Phoenix Mars Rover that landed on Mars in 2007 determined that Martian soil is slightly more alkaline than is Earth soil (Kounaves et al., 2010). Moreover, the atmosphere surrounding Venus has been reported to comprise mainly gaseous and liquid sulfuric acid (Oschlisniok et al., 2016). Given that habitability requirements within our solar system involve extreme pH conditions, examining pH tolerance in tardigrades will prove to be useful in the quest for organism habitability on other worlds. Future pH research should focus on identifying the upper and lower limits for pH tolerance in tardigrades. Comparing pH tolerance in

marine, freshwater and terrestrial tardigrade species might be lucrative in determining 'the' ideal tardigrade species for astrobiological exploration.

8.4 Radiation and Radiation-Induced Bystander Effects

According to traditional dogma, effects from ionizing radiation – radiation that carries enough energy to dislodge an electron from an atom or molecule – is restricted strictly to cells that have been targeted directly (Little, 2003). Over the past 50 years, however, evidence has been accumulating to challenge this assumption (Ugwu, 2009). Studies have shown that cells directly exposed to radiation can impart biological changes in naïve, unirradiated neighbouring cells (Little, 1999). The phenomenon where an unirradiated cell (or organism) exhibits similar detrimental effects, such as genetic instability or decreased survivorship, when exposed to an irradiated cell (or organism) became known as the 'bystander effect', or more specifically 'radiation-induced bystander effect' (Little, 1999). Whereas tolerance to radiation has been documented among a variety of tardigrade species, radiation-induced bystander effects (RIBEs) – when an unirradiated organism is exposed to an irradiated organism and exhibits radiation damage effects – had been undocumented. We found that *H. dujardini* can tolerate high radiation doses with decreased survivorship at higher radiation levels and that RIBEs occur in tardigrades and are manifested as a threshold response (Chapter 4; Fernandez et. al., 2016).

Characterizing the molecules involved in bystander signaling and response will

have important implications for radiotherapy and risk assessment. For radiotherapy, studying abscopal effects – radiation responses induced by the irradiated area to an organ or tissue outside the radiation field – is paramount (Snyder, 2004). For example, researchers found that, when lungs in rats were irradiated at the base, out-of-field effects were observed in the apex (Khan *et al*., 1998). Abscopal effects also have been observed in patients diagnosed with chronic leukemias and documented in invertebrates like earthworms (Snyder, 2004; Mothersill and Seymour, 2001). Abscopal effects, however, unlike RIBEs, are systemic effects from radiation damage within an individual rather than a result from a bystander signaling response between cells or organisms (Blyth and Sykes, 2011). Understanding the molecular mechanisms in RIBEs may help explain how abscopal effects occur – findings that have great implications for improving radiation therapy (Blyth and Sykes, 2011; Mothersill and Seymour, 2001). For risk assessment, RIBEs raise cause for concern, as they demonstrate that an unirradiated animal (or cell) experiences indirect and delayed effects from radiation damage when exposed to an irradiated animal (or cell). Thus, 'targeted' objects like tumour cells that undergo radiation therapy can induce genomic instability and chromosomal aberration in unirradiated, normal cells. Characterizing the molecules involved in radiation-induced bystander signalling and response can provide the foundation that is needed to prevent either irradiated cells from producing bystander signals or bystander signals from reaching neighbouring cells outside the targeted field, thereby preventing

bystander response induction in naive cells.

8.5 Effects of Low Temperature on Tardigrade Embryonic Development

In metazoans like nematodes, rotifers and tardigrades, lifespan extension is associated with the ability to successfully enter a cryptobiotic state. To allow organisms to synthesize cellular protectants, cryptobiosis generally requires gradual changes in the surrounding environment. Studies have shown that increasing cooling and drying rates leads to increased mortality in cryobiotic and anhydrobiotic tardigrades, respectively (Ramlov and Westh, 1992; Crowe, 1972). Without inducing a cryobiotic state through gradual cooling, we showed that total hatching time in *H. dujardini* eggs could be extended when reared at low temperatures (Chapter 6 and Chapter 7). Whether eggs were incubated short term (i.e. days = 4), for extended periods (i.e. days = 10, 20 and 40 days) or incubated at a variety of temperatures (i.e. -2 to 20 °C), embryogenesis slowed. Impeded growth in juveniles and decreased survivorship in adults suggests that costs are associated with prolonging hatching time. Despite the initial decline in survivorship following birth, individuals that hatched from eggs reared at 0 °C for extended periods (i.e. days = 10, 20 and 40) went on to reproduce and live relatively long lives.

Three hypotheses have been proposed to explain effects from anhdyrobiosis on lifespan extension: animals stop, continue or slow biological aging (Ricci and Pagani, 1997). While these models have been used to describe anhydrobiosis,

our findings show that the same models can be applied to describe the effects from low temperature (i.e. cryobiotic-like conditions) on tardigrade embryonic development. Whether the same adaptation is employed by other tardigrade species is unknown and warrants further investigation. Characterizing the potential pathways involved in slowed embryogenesis will prove to be useful for improving cryopreservation techniques.

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