# SENESCENCE IN DUCKWEED: AN INTERSPECIFIC COMPARISON AND THE INFLUENCE OF TEMPERATURE

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## Abstract

The focus of this thesis was to compare the life history traits of three species of duckweed and investigate the impact of temperature on duckweed lifespan. A generalized pace and shape framework was employed to measure six life history traits. Results revealed pace and shape of survivorship to be conserved, while fecundity and frond size were more variable. Two temperatures were then used to determine whether duckweed lifespan was directly impacted by temperature. Results demonstrated that lower temperatures extended lifespan while higher temperatures reduced lifespan, and temporal scaling of survivorship trajectories was observed. A linear model was able to predict the average change in lifespan associated with spending a certain number of days at a high temperature and switching to a low temperature. This study provides an example of how duckweed species remain a useful model system at the forefront of evolutionary and ecological studies focusing on plant senescence.

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# CHAPTER ONE: GENERAL INTRODUCTION

## 1.1 Prologue

Many organisms exhibit a continuous loss of function as they age, typically associated with a decrease in survivorship and reproductive output (Partridge and Barton 1993, Kirkwood and Austad 2000, Hughes and Reynolds 2005, Jones et al. 2014). Termed senescence, this process permeates biology and receives considerable attention within many disciplines. For example, senescence has been shown to influence asymmetrically dividing bacteria (Ackermann et al. 2003), mitochondrial DNA instability in a filamentous fungi (Lorin et al. 2006), the budding potential of mother yeast cells (Fehrmann et al. 2013), the lifespan of honey bee workers (Remolina et al. 2007), sex-specific estrogen levels and telomere length in Japanese medaka (Gopalakrishnan et al. 2013), replicative DNA damage and telomere shortening in human cells (Kaul et al. 2011), and decreases in photosynthesis and stomatal conductance in woody plants (Bond 2000).

This trend towards incorporating senescence research across multiple taxa and disciplines has also brought to light novel ways of looking at the evolution of senescence. For example, recent studies have found that not all organisms display decreases in survivorship and reproductive output with increasing age, but instead seem to exhibit negligible or negative senescence (Vaupel et al. 2004). Defined as an increase in fecundity and survivorship with increasing age (generally associated with indeterminate growth), negative senescence has been shown to exist in many different taxa, including algae, vascular plants, reptiles, and invertebrates (Jones et al. 2014). Given the non-universality of senescence, it is clear that senescence is not inevitable in all

circumstances. Moreover, we might expect there to be strong selection against a process that potentially leads to the death of an organism. Why then does a process that appears to prevent individual organisms from reaching their maximum survival potential persist widely across the tree of life?

## 1.2 Objectives

My thesis has two major objectives, both centered around senescence in duckweeds (floating, aquatic monocots in the genus *Lemna*): (1) Monitor and characterize similarities and differences of survivorship and fecundity trajectories across three species of duckweed, and (2) investigate whether experimental manipulation of growth temperature leads to predictable variation in duckweed lifespan and survival trajectories.

In the following sections, I review theories associated with the evolution of senescence and discuss the variety of senescence patterns observed in the natural world. My focus then narrows to discuss senescence in both clonal and sexually reproducing plants, and whether manipulation of senescence is a phenomenon commonly found in the literature. I also review two important concepts that aid in understanding the evolution of senescence and provide the necessary background for my objectives: the 'pace-shape' framework (associated with Objective 1), and 'temporal scaling' (associated with Objective 2). Once the appropriate background is explained, I then return to my objectives, discussing them in greater detail and providing details about my study species.

### 1.3 Why did senescence evolve?

An early explanation of senescence hypothesized that programmed timing of death would allow for a quicker generation turnover and increase the overall adaptability of a species (reviewed in Kirkwood and Austad 2000). However, like most group-selectionist arguments predicated on selection for the 'good of the species,' this theory has fallen from favour. Most modern theories rely on the observation that extrinsic factors, such as predation, competition, disease, and starvation, are common sources of death in nature (Charlesworth 1994). Given non-zero extrinsic mortality, survivorship declines with age even in the absence of senescence. This decline in survivorship results in a relatively small percentage of a population reaching old age, thus reducing the force of natural selection in older age classes (Hamilton 1966). Based on this reasoning, three prominent, interrelated theories have been devised to explain the evolution of senescence: mutation accumulation (Medawar 1946, 1952), antagonistic pleiotropy (Williams 1957), and disposable soma (Kirkwood 1977).

First of all, mutation accumulation theory predicts that survivorship will be too low at later age classes for natural selection to prevent late-acting deleterious mutations from accumulating in the germ line of long-living organisms (Medawar 1952). This causes a 'selection shadow' to take hold, where mutations that have negative effects later in life are allowed to accumulate over generations with minimal selective pressure against them (Turbill and Ruf 2010). Also, since these genes are only weakly selected against, there is a good chance that they will be evenly distributed throughout the population and thus will impact most if not all individuals that survive to the latest age classes (Kirkwood and Austad 2000).

Second, antagonistic pleiotropy theory elaborates on mutation accumulation theory and predicts that accumulated harmful alleles present within the selection shadow may provide a selective advantage to young individuals and only become deleterious later in life (Williams 1957). Since a selective advantage in early life would disproportionally affect more individuals than a negative effect later in life, natural selection could favour such a trade-off even if the eventual result for the organism is aging and/or death. This is amplified by the importance of early-produced offspring in determining which individuals' descendants will comprise subsequent generations.

Finally, the disposable soma theory predicts that natural selection will favour the optimal allocation of finite resources, involving a trade-off between two critical processes: somatic repair and reproduction (Kirkwood 1977). For example, researchers recently tested this hypothesis by removing the germline from zebrafish and comparing how germline-free fish react to somatic damage when compared to controls with intact germlines. The results showed that germline-free fish were able to recover from fin ablation significantly faster than controls (Chen et al. 2020). Therefore, the cost of maintaining a germline compromises a zebrafish's ability to repair itself, with accelerated accumulation of somatic damage as a consequence.

The above three theories provide complementary, albeit partially overlapping, explanations for why aging is so widely distributed in nature. If the main source of variation in organismal longevity is predicted to be the relative amount of experienced extrinsic mortality, then high levels will lead to quicker attenuation of the force of selection, shorter average life expectancy in the wild, deleterious genes accumulating in early age classes, and there is therefore little selective advantage to maintenance of the

soma. Contrarily, low levels of extrinsic mortality should allow an organism to devote more energy towards improving somatic repair and delaying the negative impacts of deleterious mutations (Kirkwood and Austad 2000). Also, though the disposable soma and antagonistic pleiotropy theories may seem to be arguing two sides of the same coin, the former tends to focus on specific mechanisms, such as somatic maintenance and repair, while the latter predicts the impact of specific genes that may provide pleiotropic effects on a general phenotypic response. Taken as a whole, the above theories provide excellent justification for why senescence would exist, but they fail to address why so many different patterns would be selected for.

### 1.4 How can the diversity of senescence patterns be compared?

There is a wide variety of patterns of senescence in the tree of life (Shefferson et al. 2017). For example, naked mole rats, the longest lived species of rodents, have been shown to negligibly senesce over their 30 year lifespans (Buffenstein 2008), whereas humans can live well over 100 years, but begin to senesce extremely quickly once their average life expectancy (about 78 years) is reached (Bongaarts 2009). Plants show even more striking diversity, with short-lived species such as duckweed (*Lemna* spp.) living a month on average under optimal lab conditions (Barks et al. 2018), to long-lived species such as the bristlecone pine (*Pinus longaeva*) with living specimens that are well over 4000 years old (Lanner and Connor 2001). With such diversity present in nature, how do researchers go about comparing such diverse patterns of senescence?

Baudisch (2011) developed a generalized framework to help alleviate this issue. Instead of relying on an organism's absolute lifespan for comparative purposes, Baudisch

developed a way to decouple general patterns of mortality curves (termed the ‘shape’ of senescence) from the absolute time frame on which these curves occur (termed the ‘pace’ of senescence). Specifically, this approach uses average life expectancies as a unit of relative time, thereby allowing mortality curves to be compared across organisms with very different lifespans (Baudisch 2011). This allows researchers to assess not only how long an organism lives, but how much and how fast each organism ages within their own relative lifespan. For example, a recent study examined 46 disparate species, from humans to green algae, and used the above framework to compare all species relative to their average life expectancies (Jones et al. 2014). They found humans to be an example of extreme relative mortality, demonstrating a flat mortality trajectory post-sexual maturity, followed by a precipitous increase in mortality when approaching one average life expectancy. The unusual shape of human mortality can most likely be attributed to behavioural changes (e.g. health care, agriculture) and not genetic changes, which drive most natural evolution. So, putting humans aside as an example of the extremes that aging patterns can be pushed to, where does the natural variation in patterns come from?

The main focus of recent research has been on the antagonistic pleiotropy theory, specifically on the central prediction that there is a positive correlation between adult extrinsic mortality rates (e.g. starvation, predation, etc.) and the rate of senescence (Williams 1957, Gaillard and Lemaitre 2017). However, a large volume of research has been unable to come to a definitive conclusion, with some studies coming out in favour of this prediction (Stearns et al. 2000, Reznick et al. 2002, Austad and Hoffman 2018), and others criticizing its widespread acceptance (Abrams 1993, Parsons 2007, Wensink et al. 2017). The main argument against the above prediction is that age-independent

mortality cannot change an age-dependent force of selection, therefore any extrinsic cause of death that is age-independent will not give rise to the evolution of senescence (Caswell 2007). While this argument makes intuitive sense and is backed by mathematical theory, it still does not explain why there is so much variability in the patterns of senescence that exist in nature.

### 1.5 Do plants senesce?

When discussing senescence in plants, it is important at the outset to distinguish between the two dominant definitions of plant senescence present in the literature. First, plants may undergo 'physiological' senescence, which refers to intrinsic processes that cause individual plants or modules to age (Dahlgren and Roach 2017). Examples of physiological senescence at the level of plant modules include seasonal abscission (e.g., leaf fall in autumn) and ethylene production, which causes senescence of specific plant organs by limiting auxin transport (Burg 1968). Second, plants may also undergo 'demographic' senescence, which refers to population level changes and is mostly concerned with changes in patterns of mortality and fecundity among individuals within populations and species (Dahlgren and Roach 2017). For example, demographic senescence was observed in *Plantago lanceolata* after researchers found age-dependent changes in mortality patterns between four cohorts grown in a natural environment (Roach et al. 2009). Short-lived plants, such as *Plantago* and *Lemna*, provide an opportunity to observe aging in natural populations as individuals of a known age can easily be marked and followed longitudinally over their entire lifespan (Roach 2004). Long-lived plants, on the other hand, can be more difficult to analyze longitudinally due

to many species living much longer than humans. Therefore, short-lived species will be the focus of the following discussion. Of the two definitions of plant senescence, this thesis will focus on the latter, specifically measuring and comparing demographic senescence in three species of duckweed and determining whether temperature can cause variation in duckweed demography.

Senescence in vascular plants has long been a topic of discussion (Leopold et al. 1959, Leopold 1961, Lockhart and Gottschall 1961) and has recently received significant attention, with researchers providing unique examples of senescence not commonly found in other taxa (Baudisch et al. 2013, Salguero-Gomez et al. 2017, Woo et al. 2018). For example, senescence has traditionally been viewed as something universally negative, which may culminate in the eventual death of an organism. However, many plant species have been shown to have evolved "negative senescence," where survival and fecundity increase with age after reproductive maturity (Vaupel et al. 2004). These developments in aging theory can be attributed to the unique growth form and life history of some plants. For example, most plants exhibit indeterminate growth thanks to the presence of totipotent apical meristems. Indeterminate growth refers to organisms which continue to grow and have a greater reproductive capacity as they age (Thomas et al. 2009). Determinate growth, on the other hand, refers to organisms that reach a maximum size and their reproductive potential stalls as a result. The impact of indeterminate growth on the evolution of senescence has been empirically investigated, with multiple perennial plant species exhibiting negative senescence at the level of the whole plant (Silvertown et al. 2001, Penuelas and Munne-Bosch 2010, Baudisch et al. 2013). However, some plant species are not exempt from senescence, such as duckweed demonstrating senescence at

the ramet level (Barks and Laird 2015), and semelparous plant species (e.g., annuals) that undergo abrupt senescence after a single, exhaustive, terminal reproductive event (Young and Augspurger 1991, Watkinson 1992). These species exhibit determinate growth as defined above.

With such diversity of aging patterns present among plant species, it is apt that some researchers believe plant species are not receiving enough attention in the literature (Monaghan et al. 2008, Salguero-Gomez et al. 2013), especially when compared to the attention mammalian species have received. The relatively limited focus on plant senescence may stem from the driving focus associated with gerontology (i.e., aging in humans), but may also be a result of some unique life history traits of plants. For example, most senescence research requires observing an organism from birth until death, with data collection being conducted over the entire lifespan of the organism of interest. When it comes to species such as the quaking aspen (Barnes 1966, Mitton and Grant 1996) or the bristle cone pine (Lanner and Connor 2001), which have both been shown to grow and reproduce over millennia, researcher longevity becomes the limiting factor with regards to longitudinal data collection. There are still plenty of plant species that live and die well within the average human life expectancy, making certain species easier to analyze than others (Dahlgren and Roach 2017). Another issue that arises from plant life histories is the fact that many plants are able to reproduce both sexually and asexually, confounding the ability of researchers to determine which unit natural selection will target and what constitutes a genetic individual (Tuomi and Vuorisalo 1989, Van Groenendael et al. 1996, Salguero-Gómez 2018). Natural selection may target the ramet, the individual clonal unit, or the genet, a group of ramets all derived from the

same zygote, or both ramets and genets simultaneously. Along with selection pressure, the same can be said for patterns of senescence (Pedersen 1995, Gardner and Mangel 1997). Even though studying senescence patterns in certain plant species may be innately difficult and complex, plants still represent an incredibly diverse group of organisms that can provide novel insight into the evolution of senescence.

### 1.6 Do individual ramets senesce?

The inherent complexity of plant life histories, specifically relating to the ability of certain plants to reproduce both sexually and clonally, has led to most studies investigating senescence in clonal plants to focus on senescence of the genet (Pedersen 1995, Gardner and Mangel 1997, Ally et al. 2010). Since ramets are genetically identical to their parent (Gardner and Mangel 1997), it has been argued that clonality may be a way for genets to postpone (Orive 1995) or completely escape (Pedersen 1999, Vaupel et al. 2004, Shefferson et al. 2017) senescence through a spatially distributed form of indeterminate growth. This is believed to occur through a constant recycling of modules, where ramet birth and death rates reach an equilibrium, providing support for a stable genet (Roach 2004). There are examples of plant genets being able to escape senescence, such as crops of bananas, oranges, and grapes that have avoided genetic decline despite global ramet propagation using very low genetic diversity (Ganapathi et al. 1992, Heloire et al. 1997). Also, the perennial plant *Borderea pyrenaica* shows negative senescence attributed to a highly modular shoot apical meristem arrangement (Garcia et al. 2011, Morales et al. 2013), and Scots pine (*Pinus sylvestris*) demonstrates a lack of ramet senescence when modules are grafted onto older genets (Mencuccini et al. 2014).

However, a recent study examining demographic models from 181 plant species found that 21 out of 22 species employing both sexual and asexual reproduction did in fact senesce at the level of ramet, and were more likely to senesce when compared to exclusively sexually reproducing species (Salguero-Gómez 2018). Therefore, the evidence already present in the literature strongly supports the existence of ramet-level senescence, whereas genet level senescence is far less common.

Ramet senescence has long been a topic of interest, and *Lemna* has been a genus of particular interest in this regard. First, evidence has been provided showing that *Lemna minor* fronds grown in consistent environments show a decrease in survivorship with increasing frond age, demonstrating demographic senescence at the level of ramet (Ashby et al. 1949). Though reproduction was observed in light of impact of nitrogen availability present in different growth media treatments, it is difficult to definitively say that increasing frond age related to a direct reduction in rate of fecundity (Wangermann and Lacey 1955). Temperature has been shown to influence the pace at which *L. minor* senesces, with an increase from 20 °C to 30 °C resulting in a halving of average life expectancy (Wangermann and Ashby 1951). The main focus of the above studies was determining the impact of frond age on the diminution of frond size for successive daughter fronds, for which clear evidence was provided (Wangermann 1952, Ashby and Wangermann 1954). Taken as a whole, the above research provides clear evidence that demographic senescence is an integral part of duckweed life history at the ramet level, and that duckweed lifespan can be manipulated by varying temperatures.

Recently published studies also provide a relevant demonstration of senescence in duckweed and specifically in the three species of interest for this thesis. *Lemna gibba* was

observed to fit a logistic model of frond survival, indicating age-related declines in survival, and had a reduction in the daily probability of reproduction from 0.61 at age 3 days to 0.23 at age 52 days (Chmilar and Laird 2019). *Lemna turionifera* was shown to exhibit highly conserved patterns of senescence, both in pace and shape of survival and fecundity trajectories, when 27 strains were directly compared (Barks et al. 2018). *Lemna minor* was shown to demonstrate demographic senescence, with older plants showing lower levels of survival and reproduction, as well as producing lower-quality offspring, when compared to younger plants (Barks and Laird 2015). Ramet senescence in duckweed is therefore well characterized and understood within individual species, but how demographic traits of species compare to each other has yet to be investigated.

### 1.7 Can senescence be manipulated?

Researchers have been finding ways to manipulate rates of senescence for decades, generally through the use of interventions such as temperature (Thompson and Holliday 1973, Miquel et al. 1976, Partridge et al. 1995, Hsu and Chiu 2009), caloric restriction (Barrows and Kokkonen 1978, Kirkwood and Shanley 2005, Greer and Brunet 2009, Minina et al. 2013), artificial selection (Rose 1984, Reznick et al. 1990, Kawecki et al. 2012) and direct genetic manipulation (Johnson and Wood 1982, Kenyon 2005, Conti et al. 2006, Palmer et al. 2019). It was recently discovered that when the above interventions are used on the nematode worm *Caenorhabditis elegans*, lifespan is manipulated in a way that causes temporal scaling of mortality curves (Stroustrup et al. 2016). Temporal scaling refers to a situation in which the shape of a mortality curve remains the same across manipulations, even as the pace of the curve changes. Different

interventions simply shrink or stretch the curves when plotted against absolute time. One proposed explanation for this phenomenon is that intrinsic biological properties may be responsible for governing the complex metabolic and molecular processes involved in *C. elegans* aging (Pincus 2016). This process involves no singular pathway, enzyme or substrate, but instead reacts to changes in environment, selection, diet, and genome to produce a consistent shape of aging across different treatments. Whether average lifespan increases or decreases, nematodes still undergo the same intrinsic molecular changes consistent with the aging process, but the rate at which these changes occur is either sped up or slowed down depending on the intervention used.

An early theory supported by the above phenomenon, originally coined the 'threshold theory of aging' (Maynard Smith 1963), predicts that individuals that live within different environmental conditions (e.g., high vs. low temperature) will reach a threshold of aging at different rates and begin to die once each relative threshold is crossed. Essentially, aging organisms have a finite number of resources available to them that slowly diminishes over time and once the relative threshold of that organism's specific pool of resources is crossed, the organism will begin to die. A similar theory, the life history theory of senescence, relates this phenomenon to trade-offs organisms must make throughout their lives, generally between reproduction and survival/maintenance (Stearns 1977, 1992). Though the life history theory of senescence has a plethora of research supporting its claims about life history trade-offs (Tuomi et al. 2013, Healy et al. 2019, Roach et al. 2019), the threshold theory of aging has not received as much attention in the literature.

One potential reason for the limited attention is that threshold theory may have evolved into another well researched theory: the free radical theory of aging. Free radical theory posits that an accumulation of intracellular reactive oxygen species (ROS) may ultimately result in the onset of senescence at the cellular level (Wickens 2001, Lu and Finkel 2008, Cui et al. 2012, Gladyshev 2013, Ziegler et al. 2015a). Maynard Smith (1963) supported the threshold theory of aging by monitoring the aging patterns of *Drosophila subobscura* when grown in different ambient temperatures. He was able to demonstrate that flies grown at a higher temperature (25.5 °C) and then switched to a lower temperature (20 °C) died at relatively the same chronological age as control individuals kept exclusively at lower temperatures (Maynard Smith 1963). Relating his results to free radical theory, the flies kept in the higher ambient growth temperature may have undergone cellular respiration at an accelerated rate, potentially resulting in a much faster accumulation of ROS. However, once flies were moved to a lower temperature environment, their internal cellular processes may be slowed down, and ROS accumulation would potentially be slowed down as well. This reduced rate of oxidative stress accumulation may result in the flies exhibiting a phenotypic response akin to never experiencing the increase in ROS production at all. It could therefore be argued that ROS accumulation contributes to the onset of senescence, but did not cause irreversible damage. Could there be similar processes governing plant senescence?

Many plant species exhibit a semelparous mode of growth (Young and Augspurger 1991), which provides support for the threshold theory of aging. Annual plants make an extreme trade-off between survival and reproduction once their environment-specific threshold is crossed (e.g. end of a growth season, see Burghardt and

Metcalf 2017) to give the next generation the greatest likelihood of survival by producing as many offspring as possible, ultimately resulting in the death of the parent plant. Autophagy, or the automatic recycling of internal cellular components, has been hypothesized to be a potential regulator for intrinsic metabolic and molecular processes in plants, specifically through recycling of stored nutrients (Avila-Ospina et al. 2014, Li et al. 2015, Masclaux-Daubresse et al. 2017). For example, autophagy has been shown to negatively impact longevity and fitness in *Arabidopsis* mutants deficient in autophagy-related genes (Minina et al. 2018), and has been shown to promote lifespan extension of *Arabidopsis* plants undergoing caloric restriction through light deprivation (Minina et al. 2013). In a semelparous plant, autophagy may be applied at an extreme to mobilize all nutrients stored throughout the plant's life and repurposing them towards reproduction. While it is interesting that autophagy has been shown to directly influence lifespan in *Arabidopsis*, other plant species have received relatively limited attention when it comes to manipulation of senescence patterns.

### 1.8 Thesis overview

My thesis comprises four chapters, this introduction being the first. My second chapter will focus primarily on my first objective, specifically characterizing similarities and differences of survivorship and fecundity trajectories across three species of duckweed, which are discussed in more detail below. My third chapter will then focus on my second objective; i.e., whether or not temporal scaling is inducible in duckweed, specifically *L. minor*, by manipulating growth temperature. Finally, my fourth chapter will be a general discussion and synthesis of my results, how these results complement

the existing literature and future directions that can be taken to further develop our understanding of the evolution of senescence.

*Objective 1: Comparison of pace and shape of senescence in three species of duckweed*

The first objective of this thesis is to determine whether three species of duckweed exhibit consistent or diverse patterns of survival and fecundity through a comprehensive pace and shape analysis (Baudisch 2011). The three species of interest for this study are *Lemna gibba* L., *L. minor* L. and *L. turionifera* Landolt. Other studies have been conducted looking at the aforementioned species individually (*L. gibba*: Chmilar and Laird, 2019; *L. minor*: Barks and Laird, 2015; *L. turionifera*: Barks et al., 2018), but this is the first study to compare senescence patterns of these three species directly. Also, recent data collection has found that *L. minor* and *L. turionifera* commonly coexist at the pond level within Alberta, Canada (Senevirathna 2021). All three species have a history as model organisms: for example, *L. gibba* has been shown to be an excellent species for bioremediation efforts by effectively removing heavy metal waste from water polluted through mining practices (Sasmaz et al. 2016); *L. minor* has been used as an indicator species for studying eutrophication of polluted water bodies (Khan et al. 2014); and *L. turionifera* has been used to further our understanding of how parental age can affect the fitness of offspring (Ankutowicz and Laird 2018). This study also follows closely with another recently published study, which found overwhelming consistency in the pace and shape of senescence of 27 strains of *L. turionifera* (Barks et al. 2018). Given that such consistency was present within a species of duckweed, in Chapter Two I investigate whether or not the same consistency is present across three species.

*Objective 2: Temporal scaling and predictable change of L. minor aging trajectories*

The second objective of this thesis is to determine whether or not temperature associated temporal scaling (Stroustrup et al. 2016) occurs in *L. minor*. Specifically, I used growth temperature to investigate whether or not *L. minor* exhibits temporal scaling of survivorship trajectories when fronds are grown at two different ambient temperatures. Unpublished data from the lab was used to determine the two temperatures to be used, as temperatures that are too high can accelerate senescence through denaturation of cellular components (Rosenberg et al. 1971), temperatures that are too low can slow senescence to a rate that would make time the limiting factor, and when extremely low can trigger a stress response in some plants (Berberich et al. 1999, Medina et al. 2011). As mentioned previously, manipulation of senescence patterns has been shown to exist in certain plant species, specifically *Arabidopsis,* through caloric restriction (Minina et al. 2013). Therefore, this study will look to employ temperature instead of caloric restriction as a longevity-related intervention in duckweed to test for the presence of temporal scaling. Furthermore, a linear model will be employed to determine if switching fronds from one ambient growth temperature to another can result in predictable changes in average frond lifespan. These will be the main topics of Chapter Three.

## 1.9 Study species

*Lemna* spp. are small aquatic plants belonging to the family Araceae, subfamily Lemnoideae (the duckweeds) and are among the smallest and simplest angiosperms (Hillman and Culley 1978). Commonly found floating on still to slow-moving fresh-

water bodies, these tiny monocots have a cosmopolitan distribution, being found on all continents except Antarctica (Landolt 1986). Though *Lemna* is able to reproduce sexually through flowering (Cleland and Briggs 1967), fronds predominantly reproduce clonally through asexual budding, especially under laboratory conditions. Having one of the fastest relative growth rates documented among vascular plants (Ziegler et al. 2015b), duckweeds can often be mistaken for algal blooms on slow-moving waterbodies. This combination of life history traits along with the ability to reproduce at an exponential rate make duckweeds well suited for certain ecological applications. For example, duckweed has been used as an indicator species for water quality of aquatic ecosystems (Khan et al. 2014), water remediation and toxicity testing (Ziegler et al. 2016), nutrient removal from eutrophic water sources (Cheng et al. 2002, Chen et al. 2018), and as a procedural bioassay for testing the impact of allelopathic compounds (Einhellig et al. 1985).

Each individual *Lemna* (i.e., ramet), commonly referred to as a 'frond,' has a flattened leaf-like structure with a single root protruding from the bottom. The term frond is used to describe the overall make-up of each plant as it is believed that the structure is derived from both leaf and stem tissue (Lemon and Posluszny 2000). Of all the duckweeds, *Lemna* is not the smallest (*Wolffia*, 0.5-1.5mm in diameter, holds this distinction; Lemon et al. 2001), but with an average frond diameter of 2-9 mm, their tiny size makes them ideal for lab-based studies by allowing for large sample sizes in a relatively small area (Laird and Barks 2018).

The asexual offspring of *Lemna* fronds, here termed daughters, develop alternatingly from two meristematic pockets located laterally on both sides of a mother frond (Lemon and Posluszny 2000). Which side the first daughter propagates from is

highly conserved within *Lemna* genets, relating specifically to genotype. Initiation of reproduction happens early in *Lemna* fronds' lives, with fronds being considered 'mature' once their first daughter detaches from the connective tissue present between parent and daughter, termed the 'stipe' (Landolt 1986). Sometimes grand-offspring can begin developing before the stipe of the daughter frond detaches, potentially resulting in multiple generations of fronds growing together in a clumped fashion. Due to this mode of reproduction, it can be potentially difficult to differentiate parent fronds from daughters during data collection. A simple method to resolve this issue is discussed in the subsequent chapters.

# CHAPTER TWO: COMPARISON OF PACE AND SHAPE OF SENESCENCE IN THREE SPECIES OF DUCKWEED: *Lemna minor, Lemna gibba,* and *Lemna turionifera*

## 2.1 Abstract

Traditionally, senescence has been defined as a progressive deterioration over time associated with age-related declines in survival and fecundity. Recent studies have found great diversity in the patterns of aging and theories have been proposed to explain why different patterns of senescence exist, but there is a lack of evidence surrounding the evolutionary sources of this variation. One potential reason for this disparity is the inability to compare the absolute time scales in which organisms exist, as some organisms can live and die in the matter of days, while others live for millennia. To alleviate this issue, a generalized framework was developed to decouple absolute time from analysis and instead standardize life history traits in terms of a species specific average life expectancy, facilitating cross-species and cross-taxa comparisons. This generalized framework was employed to examine the pace (absolute lifespan) and shape (time-standardized measure of the magnitude of change in survival) of aging in three species of duckweed: *Lemna minor* L., *L. gibba* L., and *L. turionifera* Landolt. All three species exhibited declines in survivorship and reproductive output over time, consistent with previous studies looking at these species individually. Interestingly, *L. gibba* exhibited the most drastic decrease in time-standardized survivorship and fecundity when compared to the other two species. *L. gibba* also had fewer total offspring on average and a two-fold larger average surface area and perimeter compared to the other two species. Differences were found between *L. minor* and *L. turionifera* as well, such as *L. turionifera* exhibiting larger average values for measures of survivorship and fecundity

trajectory shape, but these two species were much more similar to each other than to *L. gibba*. This study provides novel insight into the diversity of aging patterns found within the plant kingdom and within the same genus, and also provides further justification for future studies to continue applying the pace and shape framework for cross-species comparisons.

## 2.2 Introduction

Senescence, or the progressive deterioration generally associated with a decrease in survival and reproductive output (Kirkwood and Austad 2000, Monaghan et al. 2008, Nussey et al. 2013, Munne-Bosch 2015), has been well documented in many species. For example, Jones et al. (2014) compared taxa from across the tree of life and found humans to be the greatest outliers among natural populations, exhibiting a 22-fold increase in relative mortality (mortality measured using average life expectancy, rather than absolute time) when approaching one average life expectancy. Mice (Wang et al. 2009, Monteforte et al. 2016), fish (Reznick et al. 2002, Hsu and Chiu 2009), fruit flies (Grotewiel et al. 2005, Archer et al. 2018), honey bees (Remolina et al. 2007), nematodes (Lund et al. 2002, Chen et al. 2007), plants (Roach et al. 2009, Barks and Laird 2015, Edelfeldt et al. 2019), and even bacteria (Ackermann et al. 2003) have shown evidence of senescence. However, certain species have been able to escape the universality of senescence through implementation of an indeterminate form of growth and instead demonstrate negligible or negative senescence (Vaupel et al. 2004, Baudisch et al. 2013). With so many patterns of senescence acting throughout experimental studies and the natural world, understanding variation in patterns of senescence has become a topic of

interest across several disciplines, including bio-gerontology, evolutionary biology, and even climate science (Nussey et al. 2008, Moorad and Promislow 2009, Nussey et al. 2013, Hassall et al. 2017).

The diversity of aging patterns has led researchers to develop general frameworks that facilitate comparison of aging trajectories both within and among species (Bowler and Terblanche 2008, Monaghan et al. 2008, Jones et al. 2014, Barks et al. 2018). One such framework (Baudisch 2011) aims to measure aging trajectories (i.e., population-level patterns of survival and reproduction over time) by investigating two separate measures of mortality and fecundity, known as pace and shape. Pace is the most common measure of senescence in the literature, looking generally at the reproductive lifespan of an organism. For example, many aging studies look exclusively at lifespan in absolute time (Roach 2004, Kaplan and Gurven 2007, Gribble et al. 2014). Shape then quantifies how trends in survival and fecundity change over time by using pace as a unit of time standardization, such that time is relative to an organism's average lifespan rather than using an absolute measure of time (i.e., minutes, days, years, etc.) (Wrycza et al. 2015). This approach facilitates comparisons of organisms and species that age on entirely different time scales, which provides novel insights into how and why differing patterns of senescence have evolved.

When discussing plant senescence, it is first important to distinguish between two common uses of the word senescence in research pertaining to plant-based patterns of aging. Senescence in the literature can either refer to physiological (e.g., seasonal leaf abscission) or demographic senescence (Leopold 1961, Burg 1968, Gan and Amasino 1997, Roach 2004), with the latter being the focus of this study. Demographic senescence

relates to changes in life-history traits associated with increasing age, and not simply organs and tissues that are shed seasonally (Thomas 2013). Plant senescence has received relatively limited attention when compared to mammalian senescence (Roach 2004, Baudisch et al. 2013, Salguero-Gomez et al. 2013). This may be due to certain plant life-history traits such as totipotent apical meristems, which allow many plant species to escape senescence through indeterminate growth (Baudisch et al. 2013). For this reason, plant species that exhibit determinate growth may make better model organisms when studying demographic senescence. Duckweeds, the subject of the current study, are a predominantly clonal plant species exhibiting determinate growth, thus fitting the above criterion. Demographic senescence can be further subdivided into either ramet senescence (i.e. aging of individual clonal units) or genet senescence (i.e. aging of the entire collective group of ramets) (Cook 1983, Gardner and Mangel 1997). This study focuses on ramet senescence.

This study implements the aforementioned pace-shape framework to compare aging across three species of duckweed: *Lemna minor*, *L. turionifera*, and *L. gibba*. An intraspecific comparison of duckweed has already been conducted, looking at the patterns of aging of 27 strains of *L. turionifera* (Barks et al. 2018). This previous work demonstrated that intraspecific survival and fecundity trajectories were highly conserved. Whether or not such trends continue at the species level remains unknown. Duckweeds comprise a diverse and complex taxonomic group (Lemnoideae) with 36 species being identified to date (Bog et al. 2020), and there is a lack of comparative studies looking at how these species relate to each other in terms of patterns of senescence. The focus of this study was to measure the pace and shape of aging in three species of duckweed to

find any potential similarities or differences in life-history traits that may arise when these species are grown under identical conditions.

## 2.3 Methods

### 2.3.1 STUDY SPECIES

Three duckweed species were used in this study: *L. minor* Landolt, *L. turionifera* L., and *L. gibba* L. (family Araceae, subfamily Lemnoideae). Duckweeds are the smallest angiosperms, found free-floating on still or slow-moving fresh water bodies and can be found almost worldwide (Landolt 1986). Duckweeds have one of the highest relative growth rates of all vascular plants (Ziegler et al. 2015b) and can often times be mistaken for algal blooms. Also, *Lemna* has been used as an indicator species for water quality of aquatic ecosystems (Khan et al. 2014) and for treatment of wastewater through micronutrient removal (Cheng et al. 2002). Combining such life-history traits as a predominantly asexual form of reproduction, a relatively small size, and a determinate growth form, *Lemna* remains a model organism for research relating to demographic senescence (Laird and Barks 2018).

The plants being used in this study were originally obtained from a pond outside the University of Lethbridge's Alberta Water and Environmental Sciences Building (strain Wat A, *L. turionifera*; GenBank accession number: MG000496), and from the Canadian Phycological Culture Centre (strains CPCC 492, *L. minor*; GenBank accession number: MG000447; CPCC 310, *L. gibba*; GenBank accession number: MG000445). Each *Lemna* ramet consists of a flattened 'frond' interpreted as a combination of leaf and stem tissue, with a single root protruding from the bottom surface (Lemon and Posluszny

2000). The vegetative budding process through which duckweeds mainly reproduce occurs through two lateral meristematic pockets where daughter fronds alternate developing from the left and right sides. To precisely document reproduction events, data collection must be conducted daily as the life history characteristics of *Lemna* spp. allow them to proliferate very quickly.

### 2.3.2 GROWTH CONDITIONS AND EXPERIMENTAL DESIGN

Each frond was grown under axenic conditions in 60 x 15 mm petri dishes containing 10 mL of half-strength Schenk-Hildebrandt growth medium (Sigma Aldrich S6765) supplemented with sucrose (6.7 g/L), yeast extract (0.067 g/L), and tryptone (0.34 g/L). These supplements were used to facilitate detection of any potential microbial contamination of growth medium. The photoperiod used was 15:9 light-dark. To ensure that growth conditions were consistent throughout the experiment, each frond was transferred to fresh growth medium once per week.

Focal individuals were grown on one of four shelves with each shelf being a random spatial arrangement of 37 individuals from each species (initial N = 444). Each shelf had its own light fixture (AgroBrite FLT46), with six 122 cm high-output fluorescent grow bulbs (T5, 54W, 6400K) positioned 23.5 cm above the plants. During the light cycle, the average photosynthetic photon flux density at plant height was approximately 410 μmol $m^{-2}s^{-1}$ and was measured using a HOBO Micro Station data logger and PAR sensor (Hoskin Scientific, Edmonton, AB). Each shelf was treated as a separate spatiotemporal block to allow for easier division of workload and to account for differences in environment during data analysis. The average temperature for the light

phase of each shelf was measured as: Block 1 (top shelf) = 31.2 °C, Block 2 = 29.7 °C, Block 3 = 27.4 °C, Block 4 (bottom shelf) = 23.6 °C. The average temperature for the dark phase of the cycle ranged between 20 °C and 22 °C across all blocks.

To keep track of focal fronds and to differentiate parental fronds from their daughters, a speck of diluted (1:2, ink to water) and autoclaved India ink was applied to each focal frond. To reduce the possibility of parental age effects, wherein birth order affects offspring quality (Barks and Laird, 2015, 2016), each focal frond was an ancestor of a progenitor frond that had been taken from the relevant species' stock culture. Specifically, each focal individual arose from the same immediate and ancestral birth order (i.e., successive first daughters) over four generations. Each focal frond began being observed once it was 'born', defined as the day it detached from its parent, and observations ended on the day the frond was considered 'dead,' defined as the day the frond's final daughter detached. Thus 'death' in this study is tantamount to the cessation of ramet production, as physiological death in duckweeds is difficult to pinpoint with any precision (Barks and Laird 2015). All plants were observed daily and the number of daughters detached since the previous day was recorded (typically zero or one, and more rarely two). Recorded daughters were discarded.

### 2.3.3 SAMPLE LOSS

Fourteen fronds were lost during the course of the experiment due to microbial contamination or fronds growing in a clumped manner (i.e., multiple generations remaining attached and never separating, making it impossible to distinguish parents from daughters, thus rendering the definition of birth inapplicable). These fronds were

excluded from analysis. The final sample size was N = 430 (*L. gibba*; N = 142, *L. minor*; N = 147, and *L. turionifera*; N = 141).

### 2.3.4 SURVIVAL AND FECUNDITY TRAJECTORIES

Survival and reproduction trajectories were analyzed separately for each of the three species (following Barks and Laird 2015). Four parametric mortality models (exponential, Weibull, Gompertz, and logistic) were fit to the survivorship data. Log-likelihood functions were optimized by using the *optim* function in R and the best-fitting model was found by calculating the Akaike Information Criterion corrected for small sample sizes (AICc). This measure minimizes the mean square error of predictions for each model, with the best-fitting model represented by the lowest AICc value (Burnham and Anderson 2002). The exponential model was the only model that implied no senescence, with the rate of mortality remaining unchanged with increasing age.

A generalized estimating equation (GEE) model was used to fit the proportion of individuals reproducing at a given age. Due to a lag in reproduction experienced by all three species (low reproductive output for first two days), the first two days of reproduction were omitted. Also, due to the decreased probability of a frond reproducing on consecutive days, a first order autoregressive correlation structure was used on all three species to account for temporal autocorrelation (i.e., individuals may be less likely to reproduce the day after reproducing). Fits were found for two other commonly used correlation structures (‘exchangeable’ and ‘independence’), but due to a very similar fit, these correlation structures are not included in the results.

### 2.3.5 COMPARING PACE AND SHAPE OF SENESCENCE

The demographic traits compared across *Lemna* species were lifespan, $shape_{mortality}$, total reproductive output, and $shape_{fecundity}$. Lifespan (our pace measure) was measured as the reproductive lifespan of individual fronds, defined as the time (in days) between the day a frond detached from its parent (birth) and the day of final reproduction (death) (Barks et al. 2018). To quantify $shape_{mortality}$, a previously established framework was used (Wrycza et al. 2015). Specifically, $shape_{mortality}$ was measured as one minus the coefficient of variation in lifespan ($1 - CV_{lifespan}$), where $CV_{lifespan}$ was calculated as the lifespan standard deviation divided by the mean lifespan. Quantifying $shape_{mortality}$ in this way allowed for easy categorization of ageing trajectories: if mortality remained constant with age, $shape_{mortality}$ would equal zero; if mortality increased with age, $shape_{mortality}$ would be between zero and one, approaching one in the limiting case where all individuals died at the same age; and if mortality decreased with age, $shape_{mortality}$ would be negative. One caveat to this framework, however, is that it cannot be applied to individual plants, as each plant only dies once (rendering the standard deviation of lifespan meaningless at the individual level). Therefore, fronds were analyzed at the cohort level to produce 12 $shape_{mortality}$ values, one per block per species.

$Shape_{fecundity}$ was measured as the slope of the relationship between pace-standardized age (independent variable) and mean-standardized fecundity (dependent variable) and was based on linear regressions applied to each plant individually (Barks et al., 2018). Pace-standardized age was calculated as the age of each plant divided by the mean life expectancy of that plant's species, while mean-standardized fecundity was calculated as each plant's cumulative fecundity divided by that plant's reproductive life

span. Unlike $shape_{mortality}$, $shape_{fecundity}$ can be applied to fronds at the individual level, and a value for $shape_{fecundity}$ was therefore calculated for each frond.

In addition to the demographic traits, two plant size traits were measured: frond surface area and perimeter. Photographs of each frond were taken after their final reproduction event had been recorded ('death') using a microscope-mounted digital camera. Image analysis was conducted in MATLAB (version R2018b) using code developed by Ankutowicz and Laird (2018).

To test for among-species differences in demographic and size traits, two-way ANOVA tests coupled with Tukey-Kramer post-hoc tests were used. In addition to the main effect of 'Species,' 'Block' was also included in the model statement to account for differences in traits across the four separate spatiotemporal blocks used in the experiment. Assumptions of two-way ANOVA were assessed visually with residual-versus-fit plots and quantile-quantile plots. Log-transformations were applied to remedy any heteroscedasticity or non-normality observed in lifespan, $shape_{mortality}$, and frond surface area and perimeter. All data were analyzed in R v. 3.6.0 (R Core Team, 2019).

## 2.4 Results

### 2.4.1. SURVIVORSHIP AND FECUNDITY TRAJECTORIES

Age-related declines in survivorship were observed for all three species of duckweed (Figure 2-1). In each case, only about 20% of fronds lived beyond age 30 days. However, the shape of each species' survivorship trajectory was distinct with *L. gibba* showing the most abrupt drop in survivorship (Figure 2-1a), a trend that was apparent when comparing pace-standardized results (Figure 2-1b). *L. minor* and *L. turionifera* had

more gradual decreases in survivorship and followed very similar survivorship trajectories when this measure was pace-standardized – at least until a relative age of 1.5 times the mean life expectancy (Figure 2-1b). Figure 2-2 shows the best fitting parametric mortality model for each species, determined as the model with the lowest AICc value (Table 2-1). The logistic model was the best-fitting parametric mortality model for all three species (Figure 2-2).

In terms of fecundity, age-related declines were also observed for all three species (Figure 2-3). *L. gibba* exhibited a more abrupt drop in absolute fecundity, while the other two species exhibited a smoother decrease. According to the fitted GEE models, the predicted decrease in probability of reproduction for *L. gibba* was from 0.557 at age 3 days, to 0.067 at age 46 days (max lifespan of longest lived individual). For *L. minor*, the predicted decrease was from 0.579 at age 3 days, to 0.095 at age 62 days, and for *L. turionifera* was from 0.562 at age 3 days, to 0.187 at age 55 days.

### 2.4.2 COMPARING PACE AND SHAPE OF SENESCENCE

Even though *L. gibba* had the shortest maximum lifespan, it was on average the longest-lived species with an average lifespan of 26.8 days, followed by *L. minor* with an average lifespan of 26.6 days, and finally *L. turionifera* being the shortest-lived species with an average lifespan of 24.8 days. There was a significant difference between the lifespans of *L. gibba* and *L. turionifera*, but not between any other pairs of species (Figure 2-4a, Table 2-2). Reflecting their respective survivorship curves (Figure 2-1), *L. minor* had the lowest log-transformed $\text{shape}_{\text{mortality}}$ value, followed by *L. turionifera* and *L. gibba*; however, while *L. minor* was significantly different from the other two species,

*L. turionifera* and *L. gibba* were not significantly different from each other with regards to $shape_{mortality}$ (Figure 2-4b, Table 2-2).

*Lemna gibba* produced significantly fewer total offspring, on average, compared to the similar offspring production of *L. minor* and *L. turionifera* (Figure 2-4c, Table 2-2). $Shape_{fecundity}$ values were significantly different among all three species (Figure 2-4d, Table 2-2), reflecting their respective fecundity trajectories, with *L. turionifera* exhibiting the largest average value for $shape_{fecundity}$, followed by *L. minor*, and finally *L. gibba* exhibiting the lowest average value for $shape_{fecundity}$ (Figure 2-3).

The three species showed substantial differences in size (*L. turionifera* < *L. minor* < *L. gibba*), with significant differences in both log-transformed frond surface area and log-transformed frond perimeter (Figure 2-4e, f, Table 2-2).

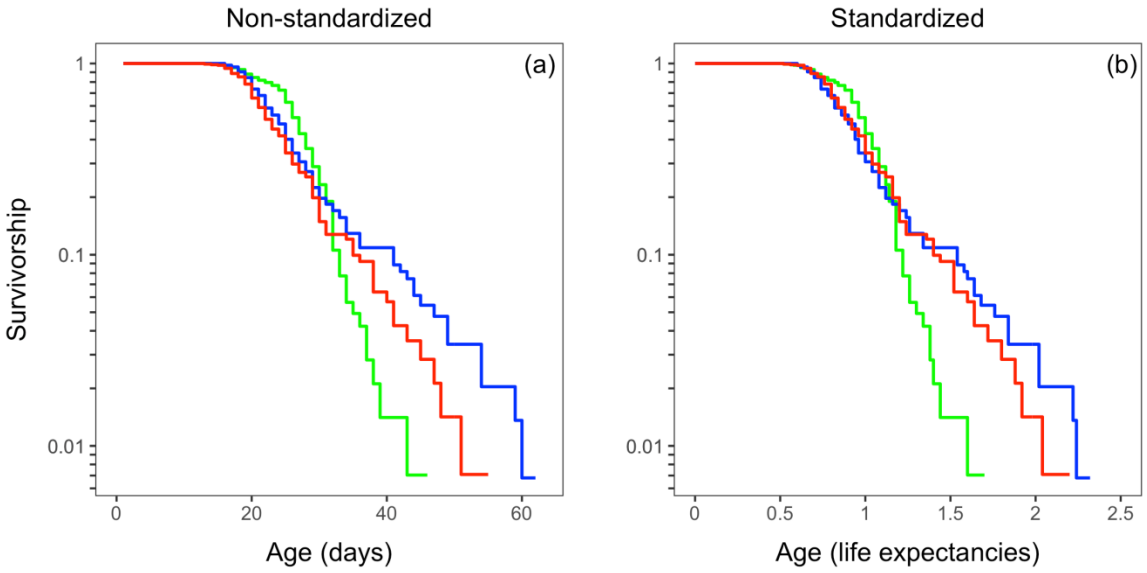


Figure 2-1: Non-standardized (a) and pace-standardized (b) survivorship for three species of duckweed. Colours represent species: *Lemna gibba* = green, *L. minor* = blue, *L. turionifera* = red.

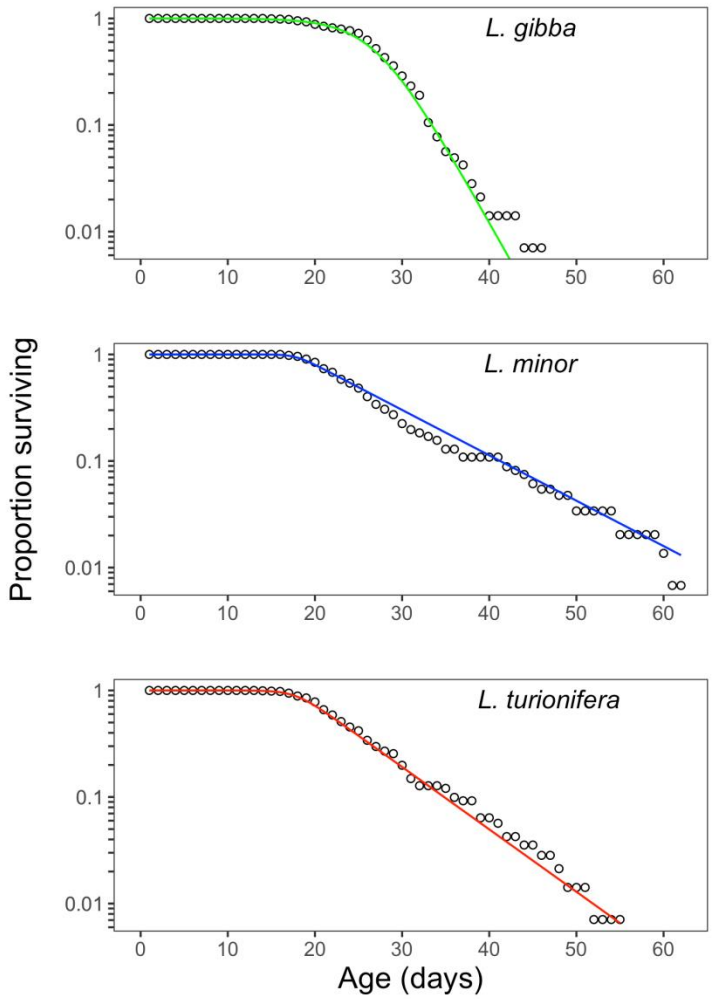


Figure 2-2: Predicted values for the best-fitting parametric mortality model for each species (lines), and empirical values of age-related declines in survivorship (symbols) for three species of duckweed. The best-fitting parametric mortality model for all three species was the logistic model (see Table 2-1).

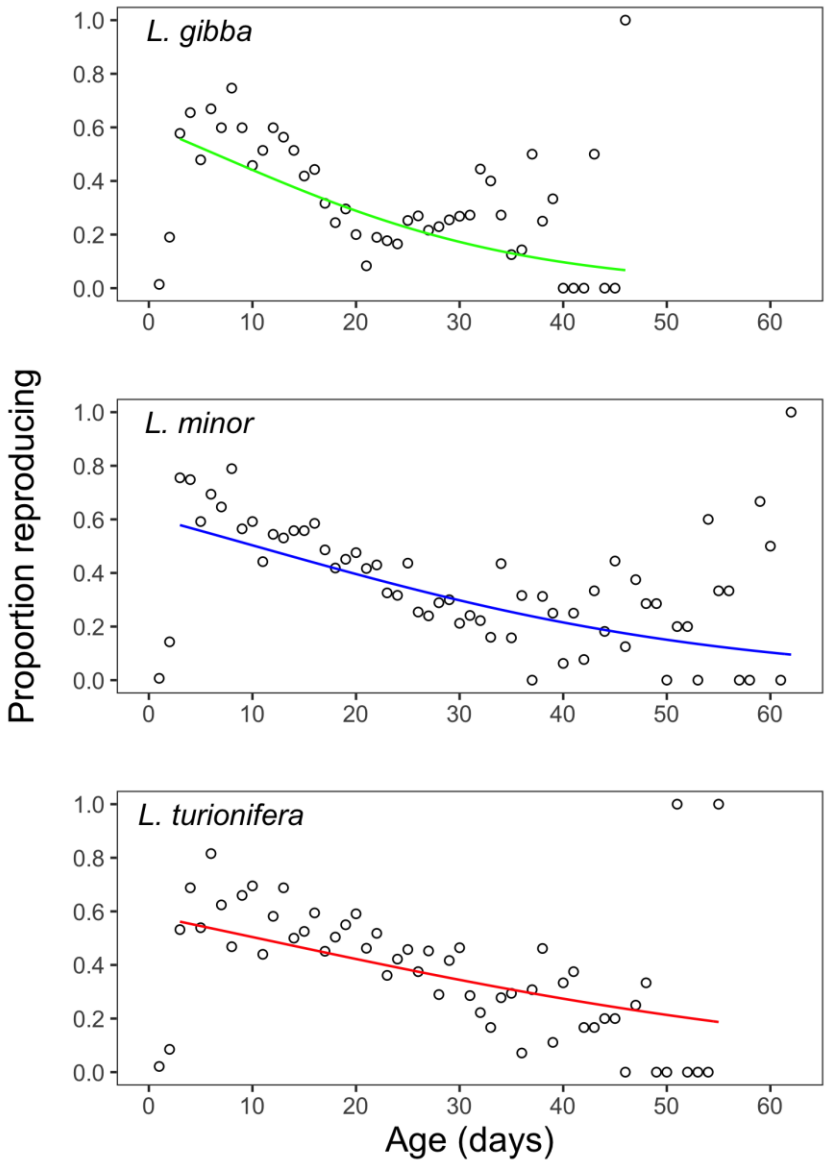


Figure 2-3: Age-related declines in the proportion of individuals reproducing up until the maximum lifespan of each species. The best-fit curve was fitted using a GEE model fit to each species separately. The first two days of reproduction were omitted from the GEE model as a lag in reproduction was experienced by each species.

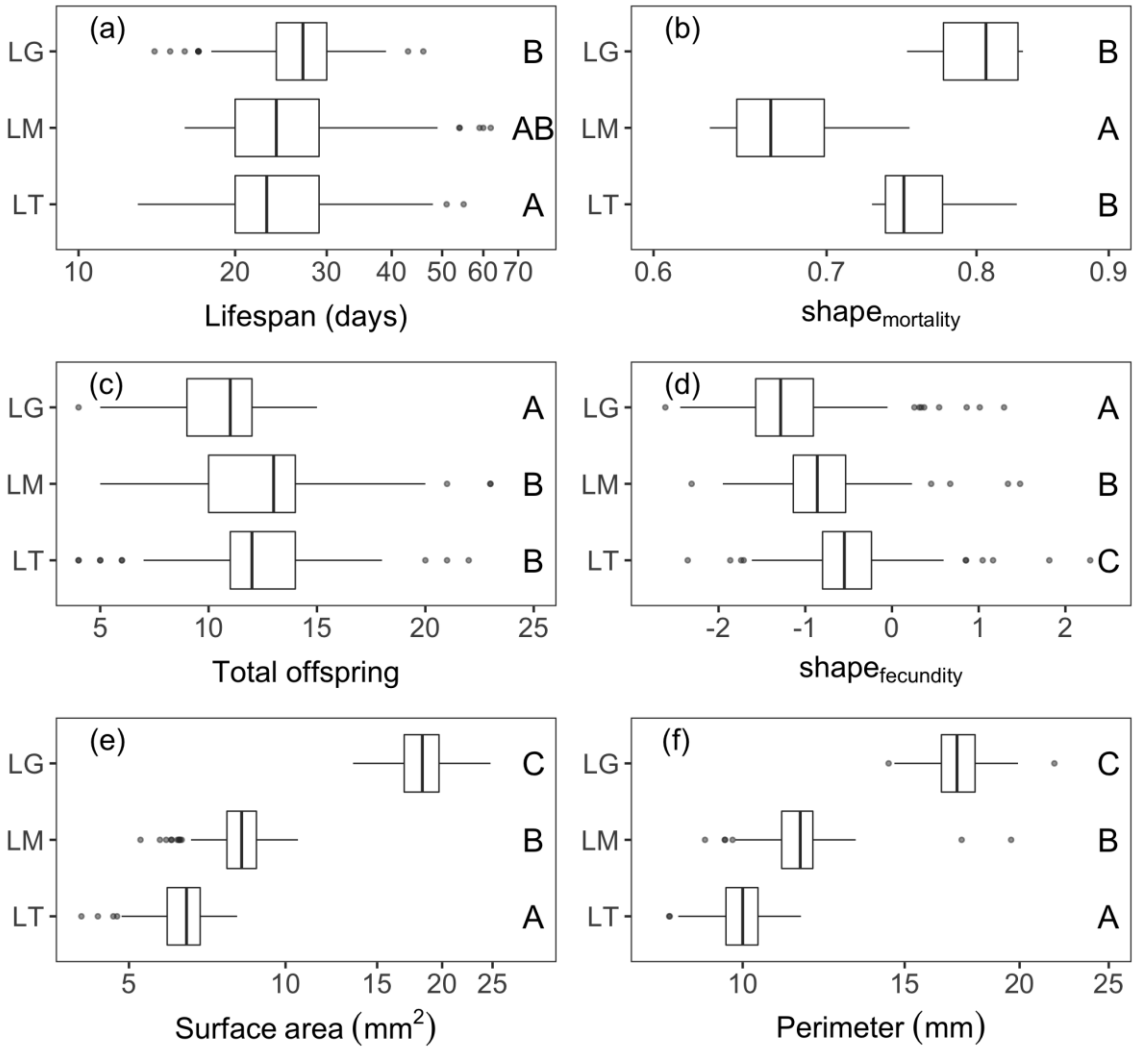


Figure 2-4: Variation in life history traits between three duckweed species (LG = *L. gibba*, LM = *L. minor*, LT = *L. turionifera*). Boxes show the first, second (median), and third quartiles, while the whiskers extend to the minimum and maximum values within 1.5 times the interquartile-range of the first and third quartile, respectively. Letters on the right side of each panel depict the results of a Tukey-Kramer post-hoc test; species with the same letter were not significantly different for the trait in question. Note the logarithmic horizontal axes in panels (a), (b), (e), and (f), reflecting log-transformations necessary to reduce heteroscedasticity or non-normality in the untransformed data.

Table 2-1: AICc values for four parametric mortality models used to model changes in survival over the reproductive lifespan of three species of duckweed. The logistic model (bold) showed the best fit for all three species, as shown by the lowest AICc values.

| **Species** | Model | Parameters | Deviance | AICc | ΔAICc |
|---|---|---|---|---|---|
| ***L. gibba*** | **Logistic** | **3** | **882** | **888** | **0.00** |
| | Weibull | 2 | 894 | 898 | 9.48 |
| | Gompertz | 2 | 924 | 928 | 39.85 |
| | Exponential | 1 | 1218 | 1220 | 331.45 |
| ***L. minor*** | **Logistic** | **3** | **976** | **982** | **0.0** |
| | Weibull | 2 | 1068 | 1072 | 89.7 |
| | Gompertz | 2 | 1129 | 1133 | 151.3 |
| | Exponential | 1 | 1258 | 1260 | 278.2 |
| ***L. turionifera*** | **Logistic** | **3** | **918** | **924** | **0.0** |
| | Weibull | 2 | 980 | 984 | 60.1 |
| | Gompertz | 2 | 1034 | 1038 | 114.1 |
| | Exponential | 1 | 1188 | 1190 | 266.1 |

Table 2-2: Results from two-way ANOVAs comparing traits among three duckweed species distributed among four spatiotemporal blocks (except for $shape_{mortality}$, which was measured at the block level, see Methods). Lifespan, $shape_{mortality}$, frond surface area, and perimeter were log transformed, as the untransformed data violated the assumptions of an ANOVA test.

| log(Lifespan) | **df** | **SS** | **MS** | **F** | **P** |
|---|---|---|---|---|---|
| Species | 2 | 0.12 | 0.0611 | 5.18 | 0.006 |
| Block | 3 | 0.58 | 0.1940 | 16.46 | <0.001 |
| Residuals | 424 | 5.00 | 0.0118 | | |
| Total | 429 | 5.70 | | | |
| log($shape_{mortality}$) | | | | | |
| Species | 2 | 0.0110 | 0.0055 | 48.3 | <0.001 |
| Block | 3 | 0.0059 | 0.0020 | 17.4 | 0.002 |
| Residuals | 6 | 0.0068 | 0.0001 | | |
| Total | 11 | 0.0237 | | | |
| Total offspring | | | | | |
| Species | 2 | 353 | 176.3 | 26.0 | <0.001 |
| Block | 3 | 715 | 238.3 | 35.1 | <0.001 |
| Residuals | 424 | 2879 | 6.8 | | |
| Total | 429 | 3947 | | | |
| $shape_{fecundity}$ | | | | | |
| Species | 2 | 36.25 | 18.127 | 49.343 | <0.001 |
| Block | 3 | 0.88 | 0.294 | 0.799 | 0.495 |
| Residuals | 424 | 155.76 | 0.367 | | |
| Total | 429 | 192.89 | | | |
| log(Surface area) | | | | | |
| Species | 2 | 16.40 | 8.20 | 3238.2 | <0.001 |
| Block | 3 | 0.05 | 0.02 | 6.9 | <0.001 |
| Residuals | 424 | 1.07 | 0.00 | | |
| Total | 429 | 17.52 | | | |
| log(Perimeter) | | | | | |
| Species | 2 | 4.17 | 2.086 | 2172.6 | <0.001 |
| Block | 3 | 0.02 | 0.005 | 5.7 | <0.001 |
| Residuals | 424 | 0.41 | 0.001 | | |
| Total | 429 | 4.60 | | | |

## 2.5 Discussion

The three species of duckweed studied all showed age-related declines in fecundity and survival, as was hypothesized based on previous research (Barks and Laird 2015, Barks et al. 2018, Chmilar and Laird 2019). Survival continually decreased for each species after about half of each cohort's average lifespan, while fecundity consistently decreased after peaking around age eight days. All species exhibited significantly distinct measures of frond size and $shape_{fecundity}$, while other life-history traits remained consistent.

### 2.5.1 PLANT SENESCENCE

There are examples of plant species that demonstrate demographic patterns of senescence (Chapman and Goudey 1982, Downey and Smith 2000, Dahlgren and Roach 2017). However, the large majority of angiosperms exhibit either negligible or negative senescence, generally associated with an indeterminate growth form (Silvertown et al. 2001, Vaupel et al. 2004, Baudisch et al. 2013). Therefore, the relatively unique opportunity that duckweed provides as a study species makes them amenable to studies focusing on the evolutionary foundations of senescence in the plant kingdom. *Lemna* also has the fastest relative growth rate of all angiosperm species (Ziegler et al. 2015b) and the presence of a determinate growth form prevents fronds from escaping demographic senescence, at least at the ramet level. With regards to the genet level, it could be argued that *Lemna* genets use a spatially distributed form of indeterminate growth maintained by constant ramet turnover. Future studies should look to determine whether or not

demographic senescence is present in *Lemna* at the genet level, but such speculation is beyond the scope of this study.

### 2.5.2 PACE OF SENESCENCE

Out of four commonly used models of senescence (see Methods), the logistic model demonstrated the best fit for all species tested. This model of senescence incorporates a deceleration factor and allows for a plateau to be reached, causing mortality to level off at later ages (Pletcher et al. 2000). Neither *L. gibba* nor *L. turionifera* had a significantly different average lifespan than *L. minor*; however *L. gibba* on average lived significantly longer than *L. turionifera* (Figure 2-4a). Based on previously published research (Chmilar and Laird 2019), it was hypothesized that *L. gibba* would be the longest-lived species, on average. This turned out to be the case when all species were grown in a consistent environment, though only by 0.2 days when compared to *L. minor*. Previous research determined the average lifespan of *L. minor* to be about 26 days (Barks and Laird 2015), with *L. turionifera* being about the same (Barks et al. 2018). The longest-lived individuals for each species were 62 days, 55 days, and 46 days for *L. minor*, *L. turionifera* and *L. gibba*, respectively. These fronds, along with other long-lived individuals (>2x average lifespan) were all present within the fourth block, which also had the lowest average temperature across all blocks (see Methods).

It has been shown that *Lemna* fronds may have their lifespans lengthened by lowering the ambient temperature of their growth environment. Therefore, it can be hypothesized that having large differences in average temperature between blocks (about 7.5 °C between block 1 and 4) would have a significant impact on how individual fronds

senesce. Since the longest-lived fronds were present in the block with the lowest average temperature, future studies should ensure that ambient growth temperature across blocks remains more consistent. This may be achieved by using a growth chamber, by not having each block of the experiment physically stacked together, or by exchanging fluorescent bulbs for cooler LED bulbs.

### 2.5.3 SHAPE OF SENESCENCE

Looking at the shape of aging trajectories, *L. gibba* shows a more extreme decrease in standardized survivorship than the other two species (Figure 2-1b). This makes sense given that *L. gibba* also had the largest average value for $shape_{mortality}$ (Figure 2-4b), which was used as an empirical measurement to determine the magnitude of change in mortality experienced by each species (Wrycza et al. 2015). The $shape_{mortality}$ values for *L. minor* and *L. turionifera* were on average smaller, demonstrating that their mortality trajectories show a more gradual decrease over time when being compared to *L. gibba*. Given that *L. minor* and *L. gibba* are taxonomically more closely related to each other than to *L. turionifera* (Wang et al. 2010, Bog et al. 2019), it would be expected that this taxonomic relationship would be reflected in a direct comparison of life history traits. An intraspecific comparison of *L. turionifera* found broad consistency between strains of the same species, so it is interesting that this consistency is not conserved at the species level. Future studies should look to develop a more complete understanding of the different life history traits present within the Lemnoideae subfamily to potentially provide more insight on whether and to what extent taxonomic relationships are reflected in the evolution of differing life history traits.

### 2.5.4 FECUNDITY TRAJECTORIES

Consistent with what was observed with survivorship, *L. gibba* was the species with the most dissimilar fecundity trajectories, showing the lowest average total offspring produced and the lowest average value for $shape_{fecundity}$. Though *L. turionifera* and *L. minor* produced on average very similar numbers of total offspring, *L. turionifera* had the highest average value for $shape_{fecundity}$. These results held true even when the first two days of each focal frond's life were omitted from the fecundity analysis, as individual fronds were less likely to reproduce on the day they were born and the day following. It is interesting that the largest of the three species exhibited not only a sharper relative decline in survivorship, but also the lowest average values for $shape_{fecundity}$ and lowest average number of total ramets produced. A potential explanation for these results may be the phenomenon commonly referred to as allometry, or the relationship between organism size and other traits (Smith and Fretwell 1974, Nielsen and Sand-Jensen 1990, West et al. 1997). Allometric trends in plant species have been shown to exist, demonstrating that metabolic rate scales positively with plant size (Enquist et al. 1998, Gillooly et al. 2001). If the same phenomenon holds true for *Lemna*, it can be argued that *L. gibba* might have the highest average metabolic rate among the three observed species. This increased metabolic rate should in theory result in an increased accumulation of oxidative damage due to an increased rate of metabolic reactions, thus triggering an earlier onset of senescence (shown by Figure 2-1b). However, since metabolic data collection was beyond the scope of this experiment and only three species were examined

in this study, future studies should look to see if this allometric trend continues throughout the Lemnoideae family.

As the fastest reproducing angiosperm (Ziegler et al. 2015b), duckweeds are able to cover large expanses of slow-moving water bodies in a relatively short period of time, being referred to by some as the hypothetical 'Darwinian Demon' (Kutschera and Niklas 2015). Relating this to wild populations, one of the limiting factors on *Lemna* growth rates is frond density (Kufel et al. 2018). It can therefore be assumed that a duckweed species that produces on average larger ramets will have to produce fewer ramets to cover the same surface area of aquatic habitat. Therefore, it would intuitively make sense that the largest of the three species tested would produce the fewest ramets. Since the clonality of duckweed reproduction would be counter-productive if allowed to continue indefinitely, as fronds would eventually shade over each other, a shorter lifespan would allow for older fronds to die off quicker and improve the frond turnover for the entire genet. However, a lack of research on wild populations of *Lemna* makes these claims speculative in nature, and future studies should focus more on the *in vivo* processes surrounding duckweed life history traits.

### 2.5.5 FUTURE DIRECTIONS

Due to how death is defined in this study, post reproductive trajectories are omitted from the analysis. Even though it is likely that fronds continue to use up resources in their immediate area before physiological death, frond discoloration and cessation of reproduction are far too inconsistent to be used as markers of death in an ecological study of this type. Therefore, if reproductive lifespan is used as the sole

measure, it is possible that a model-based approach may overestimate the pace at which *Lemna* ramets senesce. Also, this study looks at the overall senescence patterns in duckweed ramets. Future research should look to develop methods for similar studies to be conducted at the genet level. *Lemna* is a unique example of a plant whose genet would be made up of individual physiological units. So even though individual ramets may develop patterns of senescence, as long as new fronds are constantly being generated, the force of selection would remain independent of genet age, and senescence would not evolve (Silvertown et al. 2001). Finally, given that Lemnoideae is a tremendously diverse taxonomic group (Les et al. 2002), whether or not other species of duckweed share similar life history trait distributions and how populations compare to each other when grown in the wild should also be investigated.

## 2.6 Conclusions

Cross-species comparisons have shed some light on the many different patterns of senescence present in nature (Dudycha 2003, Sherratt et al. 2011) and new generalized frameworks have made it much easier for researchers to compare across taxonomic lines that previously were limited by differences in time scale (Baudisch et al. 2013, Jones et al. 2014). The realization that senescence is not a universal phenomenon has shifted the focus from developing theories that explain why senescence occurs towards theories explaining why there is such diversity in terms of natural patterns of senescence (Kirkwood and Austad 2000, Wensink et al. 2017). Plants, as a specific example, have traditionally been underrepresented when it comes to research examining demographic trajectories of aging (Salguero-Gomez et al. 2013). The above study is an example of

how this disparity can potentially be remedied and at the same time provides evidence that even closely related species can show great diversity in patterns of aging. The pace and shape framework continues to be a useful tool in the characterization of aging trajectories and should be employed in the future to allow for further comparative studies to be conducted.

# CHAPTER THREE: EXPERIMENTAL MANIPULATION OF GROWTH TEMPERATURE LEADS TO PREDICTABLE VARIATION IN DUCKWEED SURVIVAL TRAJECTORIES

## 3.1 Abstract

The manipulation of senescence has long been a topic of interest and recently has shown potential as a means to investigate specific environmental factors and biological mechanisms that govern the physiological deterioration commonly associated with aging. By employing certain interventions, such as direct genetic manipulation, caloric restriction, artificial selection, and altering ambient growth temperature, researchers have shown that the rate of aging can be altered in model organisms. Here, different ambient growth temperatures were used to alter the pace of aging in fronds of a small aquatic monocot, *Lemna minor*. Growth chambers were used to ensure growth temperature remained as consistent as possible throughout the experiment. A difference of 6 °C resulted in a 47.9% increase in average lifespan of fronds grown at a lower temperature (22 °C) when compared to fronds grown at a higher temperature (28 °C). Interestingly, while the pace of frond aging was accelerated by increased ambient temperature, the shape of frond survival curves remained the same when time was standardized and plotted against the average life expectancy of each cohort. Furthermore, it was possible to employ a linear equation to predict the average lifespan of fronds who were initially grown in the two different temperature environments and switched to the opposite environment at scheduled intervals. A linear model was able to accurately predict changes to average *L. minor* lifespan when fronds were moved from a high to low temperature environment, but was unable to accurately predict the alternate treatment

(i.e., low to high). This study provides further evidence that senescence can be manipulated through simple alterations to organismal growth conditions and that temporal scaling can be induced in duckweed aging trajectories. Plant model organisms remain useful in determining what kinds of environmental and biological processes direct the progressive deterioration associated with aging in organisms that employ a determinate growth form.

### 3.2 Introduction

For the better part of a century researchers have been using different interventions to manipulate the natural aging process of organisms to try to understand the evolutionary foundation of this seemingly maladaptive process (McCay et al. 1935, Maynard Smith 1962). Commonly used interventions include genetic manipulation of specific loci (Johnson and Wood 1982, Kenyon 2005, Conti et al. 2006, Palmer et al. 2019), caloric restriction (Barrows and Kokkonen 1978, Kirkwood and Shanley 2005, Greer and Brunet 2009, Minina et al. 2013), artificial selection (Rose 1984, Reznick et al. 1990, Kawecki et al. 2012), and manipulation of ambient growth temperature (Thompson and Holliday 1973, Miquel et al. 1976, Partridge et al. 1995, Hsu and Chiu 2009). The mystery of senescence stems from an inability to pinpoint what biological components determine the progressive deterioration generally associated with increasing age. Recent studies further complicate our understanding by demonstrating that certain organisms can actually improve their survival and reproductive output over time (Vaupel et al. 2004, Jones et al. 2014). Many hypotheses have been proposed and explored, such as the free radical theory centering on damage caused by reactive oxygen species (Ziegler et al. 2015a), and the life

history theory centering on trade-offs between reproduction and somatic maintenance (Harshman and Zera 2007, Flatt et al. 2013, Roach et al. 2019) as well as sex-differences in reproductive investment and immune response (Lawson et al. 2012, Klein and Flanagan 2016, Metcalf et al. 2020). However, without experimental manipulation of environmental and biological factors that directly impact the progression of senescence, it becomes difficult to pinpoint which factors are most important with regards to the evolution of senescence.

A recently explored phenomenon relating to the aforementioned experimental interventions results in either a shrinking or stretching of *Caenorhabditis elegans* ageing trajectories (Stroustrup et al. 2016). Referred to as temporal scaling, these findings demonstrate that *C. elegans* ageing is governed by a single effective rate constant that can alter mortality by determining the rate at which mechanisms related to aging function. Essentially, the pace of aging (absolute time frame in which individuals live and die) in *C. elegans* can be manipulated using different experimental alterations, but the shape of aging (time-standardized measure of overall trends in survivorship) remains consistent across manipulations (Pincus 2016). Interestingly, the threshold theory of aging (Maynard Smith 1963) proposed more than a half century ago is strongly supported by the aforementioned results. Threshold theory posits that an organism continues to age at a consistent rate associated with increasing age until a relative age threshold is attained, after which the organism begins to die. A common example of this phenomenon is observed in survival curves and semelparous plant species (e.g., annuals), where a cohort of organisms all survive for a period of time after reaching sexual maturity and then reach a point when survival begins to fall off, generally associated with the average life

expectancy of the species of interest (Fries 1980, Chen et al. 2007, Bronikowski and Flatt 2010, Gaillard and Lemaitre 2017). It is the objective of this study to investigate how threshold theory impacts the aging trajectories of plants.

Most research on the manipulation of patterns of senescence has centered on model organisms, specifically in invertebrates and certain mammalian species (McCay et al. 1935, Partridge et al. 1995, Sprott 1997, Stroustrup et al. 2016). However, if the goal of gerontological research is to garner a complete understanding of the evolutionary foundations of aging, organisms other than mammals could potentially provide an advantage through study of conserved mechanisms. Plants are no exception when it comes to useful model organisms, with species such as *Arabidopsis thaliana* being used to investigate research questions ranging from how transcriptional elements govern multicellular development (Schmid et al. 2005) to how genetic plasticity can confer adaptive advantages across multiple environments (Cao et al. 2011). Therefore, it can be argued that plant model systems would be just as, if not more, useful than mammalian or invertebrate model systems in investigating the evolutionary mechanisms associated with senescence. Though not investigated directly, temporal scaling has already been shown to exist in *Arabidopsis* through manipulation of specific genetic loci and caloric restriction by limiting light intensity (Minina et al. 2013). Minina et al. (2013) demonstrated that caloric restriction based lifespan extension in *Arabidopsis* is mediated by autophagy-related mechanisms. Duckweeds have also shown promise as plant model organisms (Laird and Barks 2018), demonstrating usefulness in not only evolutionary and ecological analysis (Van Der Heide et al. 2006, Barks and Laird 2016, Barks et al. 2018, Gilbert et al. 2018), but also within disciplines such as bioremediation (Cheng et al. 2002, Sasmaz

et al. 2016), ecotoxicology (Wang 1990, Ziegler et al. 2016), and biofuel production (Su et al. 2014, Xu et al. 2014). With regards to senescence and temporal scaling, duckweed has yet to be studied as an organism of interest. The above developments bring to light how useful plant model organisms can be and demonstrate the need to expand the limited number of studies investigating the phenomenon of temporal scaling in nature by including *Lemna* as a novel study species.

This study uses duckweed, a small, aquatic monocot, as a model organism for the investigation of temporal scaling and manipulation of senescence patterns present in nature, specifically in plant species. The species of interest for this study is *Lemna minor*, as focusing on a single species that is locally present in southern Alberta can provide useful insights on local aquatic ecosystems. An earlier study provided evidence that *L. minor* fronds have differing lifespans when subject to different ambient growth temperatures, demonstrating a two-fold increase in lifespan of fronds grown at 20 °C versus fronds grown at 30 °C (Wangermann and Ashby 1951). This, along with unpublished research from our lab, raises the hypothesis that differing growth temperatures significantly alter frond lifespans. More specifically, higher temperatures will lead to a reduction of frond average lifespan, while lower temperatures will lead to an extension of frond average lifespan.

Extension or reduction of duckweed lifespan has only been indirectly investigated (the aforementioned study investigated the impact of mother frond lifespan on the size of offspring; see Wangermann and Ashby 1951) and whether temporal scaling exists in duckweed aging trajectories remains unknown. The objectives of this study are therefore to implement differing ambient growth temperatures to test manipulation of duckweed

lifespan, determine whether temporal scaling is present, and attempt to predict average changes in duckweed aging when switched from one ambient temperature to another after specific intervals. Switching treatments will involve fronds being grown at an initial temperature (either high or low) and being switched to the alternate temperature once a scheduled number of days at the initial temperature have passed. The hypothesis for this portion of the experiment is that a linear model proposed by Maynard Smith (1963) can be used in conjunction with control frond data to predict the impact that switching from one temperature to another will have on frond average lifespan.

## 3.3 Methods

### 3.3.1 STUDY SPECIES

One species of duckweed was used in this study: *Lemna minor* L., (family Araceae, subfamily Lemnoideae). Duckweeds are small, aquatic monocots found free-floating on still or slow-moving fresh water bodies and have a cosmopolitan distribution, living on all continents except Antarctica (Landolt 1986). Duckweeds have one of the highest relative growth rates of all angiosperms (Ziegler et al. 2015b) allowing new generations of fronds to proliferate very quickly, with most fronds living for about a month under ideal laboratory conditions. Also, *Lemna* shows promise as a useful species in water remediation and toxicity testing (Ziegler et al. 2016), and has been used as an indicator species for water quality of aquatic ecosystems (Khan et al. 2014) and for treatment of wastewater through micronutrient removal (Cheng et al. 2002). With an average lifespan of several weeks, a predominantly asexual form of reproduction, a relatively small size, and a determinate growth form, *Lemna* is a model organism for lab

experiments in ecology and evolution and on plant senescence patterns in particular (Laird and Barks 2018).

The species used in this study was originally obtained from the Canadian Phycological Culture Centre (strain CPCC 492, *L. minor*; GenBank accession number: MG000447). Each *L. minor* ramet consists of a flattened 'frond' that has been interpreted as a combination of leaf and stem tissue, with a single root protruding from the bottom surface (Lemon and Posluszny 2000). The vegetative budding process through which duckweeds predominately reproduce occurs through two lateral meristematic pockets where daughter plants alternate developing from the left and right sides. Data collection must be conducted daily to accurately document reproductive output as the life history characteristics of *Lemna* spp. allow them to proliferate very quickly.

### 3.3.2 GROWTH CONDITIONS AND EXPERIMENTAL DESIGN

Two Conviron E15 growth chambers (Controlled Environments Limited, Winnipeg, MB) were used, one set to 28 °C and one set to 22 °C, while humidity, light intensity, and other potential confounding variables remained constant. Each growth chamber used a combination of incandescent (120V, 60A/99/XL) and fluorescent (F72T12/CW/VHO) lighting with an average photosynthetic photon flux density at plant height of about 400 μmol $m^{-2}s^{-1}$, which was measured using a HOBO Micro Station data logger and PAR sensor (Hoskin Scientific, Edmonton, AB). Each frond was grown under axenic conditions in 60 x 15 mm petri dishes containing 10 mL of half-strength Schenk-Hildebrandt growth medium (Sigma Aldrich S6765) supplemented with sucrose (6.7 g/L), yeast extract (0.067 g/L), and tryptone (0.34 g/L). These supplements were used to

ease detection of microbial contamination within samples, allowing for quick intervention in an effort to prevent growth media contamination from affecting final sample sizes. The photoperiod used was 15:9 light-dark. To ensure consistent growth throughout the experiment, each frond was transferred to fresh growth media and petri dishes once per week. Cultures were reared in a similar fashion to previous studies (single ramet from relevant stock culture), with each focal frond being a fourth generation first daughter to reduce the impact of parental age effects (Barks and Laird 2016). Growth shelves were used to propagate focal individuals. Each shelf had its own light fixture (AgroBrite FLT46), with six 122 cm high-output fluorescent grow bulbs (T5, 54W, 6400K) positioned 23.5 cm above the plants. During the light phase of the cycle, the average photosynthetic photon flux density at plant height was approximately 410 μmol $m^{-2}s^{-1}$. Once focal fronds were acquired, they were labelled and immediately transferred to their respective growth chambers on the day they detached from their mother frond (i.e., date of birth). Each growth chamber contained 11 trays, with 224 fronds being grown in each chamber (total initial N = 448).

To test the impacts that ambient growth temperature has on *L. minor* growth, a subset of each sample was moved from one ambient temperature to the other at scheduled intervals. For the chamber at 22 °C, 16 different treatments were conducted. The first treatment was a control treatment which included 59 individual fronds that were grown at 22 °C for their entire lifespan. The other 15 treatments involved 11 individual fronds for each treatment being switched from an initial growth temperature of 22 °C to a final growth temperature of 28 °C within scheduled intervals. These intervals are represented by the specific treatment number of each frond; treatment one was switched to the final

growth temperature after remaining in the initial growth temperature for one day; treatment two switched after two days, and so on up to a maximum of 15 days at the initial temperature for fronds in treatment 15. For the chamber at 28 °C, a very similar treatment schedule was employed. A control treatment of 59 fronds was allowed to grow at 28 °C for their entire lifespan. The elevated growth temperature was hypothesized to lower the average lifespan of fronds, so 11 treatments were conducted with 15 fronds per treatment. In the same manner as mentioned earlier, fronds in treatment one were grown at the initial ambient temperature of 28 °C and switched to the final ambient temperature of 22 °C after one day; fronds in treatment two were switched after two days, and so on up to a maximum of 11 days at the initial temperature for fronds in treatment 11. Once a frond was switched to its final ambient growth temperature, it remained at that ambient temperature until its date of death, defined as the date its final daughter detached.

To ensure that the growth chambers were not directly impacting the results of the experiment (i.e., confounding temperature treatment and chamber), the temperature that each growth chamber was kept at was randomized throughout the experiment. To accomplish this, every other day during data collection a coin was flipped. If the coin landed tails, the chambers would remain at the same temperatures. If the coin landed heads, the temperature of the growth chambers would be swapped, as would the fronds within the chambers. To limit the time that treatment fronds were exposed to temperatures other than their specific ambient growth temperatures, the chamber set to 22 °C would have its fronds removed during their light cycle. Since average room temperature is closer to 22 °C than to 28 °C, these fronds would be less likely to be affected by a difference in the ambient temperature of their environment while the

chambers were being switched. Once the 22 °C was emptied, the temperature of that chamber was changed to 28 °C and allowed to acclimate. Once acclimatized, the fronds from the originally 28 °C would be moved directly from their original growth chamber to the newly acclimatized chamber to limit exposure to an ambient temperature other than 28 °C. Once the original chamber was empty, the temperature was switched to 22 °C and allowed to acclimatize before the 22 °C treatment fronds were returned to the growth chamber. This process on average took about 30 minutes to complete.

With regards to randomization of frond location within growth chambers, three independently chosen locations were assigned to each frond before the experiment began. Control fronds only required two locations, one during initial propagation and one during focal growth, as they were not switched throughout the experiment. The treatment fronds required three locations, one during propagation, one pre-switch, and one post-switch; therefore the remaining 165 locations were randomly assigned to each treatment frond. Since specific maturation times could not be accurately predicted, if a frond were to be switched to a location that already contained another treatment frond, the nearest unused location would be used instead and documented appropriately. To more easily differentiate mother fronds from daughter fronds, a small dot of autoclaved half-strength India ink was applied to the parental frond to simplify observations. Daughter fronds were recorded on the day they detached to monitor reproductive output and define date of death. Once recorded, daughter fronds were no longer of interest to the objectives of the experiment and discarded.

### 3.3.3 SAMPLE LOSS

A total of ten fronds were lost during the data collection period of this experiment, resulting in a final sample size of 438. All of these fronds were lost due to contamination of the growth media, which resulted in the frond itself becoming contaminated. Appropriate measures were taken to try to alleviate the issue of contaminated media (e.g., immediately switching contaminated fronds to fresh media/petri dish), but to no avail. Three fronds were lost from the 22 °C initial temperature treatments and seven were lost from the 28 °C initial temperature treatments. One specific treatment group was not impacted more than others (each contaminated frond came from a different treatment group) therefore the sample sizes of affected treatment groups were not substantially impacted.

### 3.3.4 SURVIVORSHIP AND TEMPORAL SCALING

To investigate whether temporal scaling of aging trajectories is possible in *L. minor*, survivorship data were analyzed by plotting the proportion of fronds surviving over time. The proportion of fronds surviving to a given age was initially plotted against absolute time to visualize any differences in the pace of aging associated with high and low ambient growth temperatures. The same data were then time-standardized by taking each frond's absolute lifespan and dividing it by the average lifespan of each frond's temperature-specific cohort. These time-standardized data points were then plotted against average life expectancies instead of absolute lifespan, decoupling the rate of aging from the overall analysis to facilitate visualization of temporal scaling. As explained in the introduction, temporal scaling would be present if the two survivorship

curves are not overlapping when plotted against absolute time, but do overlap once time is standardized and represented by average life expectancy.

### 3.3.5 PREDICTING EFFECTS OF SWITCHING TREATMENTS

A previous study has been able to accurately predict the impact that temperature will have on aging trajectories when experimental individuals are switched from an initial growth temperature to a different final temperature (Maynard Smith 1963). Equation 1 represents a linear equation that was employed to analyze the results of switching treatment fronds starting in a low temperature environment:

$$L_3 = L_2 + x\left(1 - \frac{L_2}{L_1}\right) \quad (1)$$

where $L_3$ represents an individual's predicted age at death, $L_2$ represents the average lifespan at the elevated temperature, $L_1$ represents the average lifespan at the lower temperature, and $x$ represents the time spent at the lower temperature before being transferred to the higher temperature. This equation can also be used for high- to low-temperature switching treatments by simply exchanging both instances of $L_2$ with $L_1$ and the single instance of $L_1$ with $L_2$, represented below by equation 2:

$$L_3 = L_1 + x\left(1 - \frac{L_1}{L_2}\right) \quad (2)$$

Employing these equations to try to accurately predict the impact of time spent at each initial temperature hinges on a few inherent assumptions. First, the individuals kept at each initial temperature undergo a similar series of temporal states of aging, $S_0$, $S_1$ … $S_r$ … $S_n$, until reaching the final stage $S_n$ where individuals begin to die; and second, the time it takes to pass from one stage to another, $S_r$ … $S_{r+1}$, at the initial higher temperature abides a consistent ratio to the equivalent time spent at the final lower temperature

(Maynard Smith 1963). It is hypothesized that Equation 1 will result in a positive predicted slope when $L_3$ is plotted against $x$, as spending more time at a lower initial temperature should cause an extension of average lifespan. Conversely, Equation 2 should result in a negative predicted slope, as spending more time at a higher initial temperature should cause a reduction of average lifespan.

### 3.3.6 DATA ANALYSIS

The purpose of Equations 1 and 2 was to provide a theoretical basis that will be used to compare the observed results of each treatment to the predicted values based on the average lifespan of each control treatment. In terms of a null hypothesis for the data analysis, it was hypothesized that the observed intercept and slope extrapolated from the raw data would be the same as the theoretical intercept and slope that resulted from the above equations. To test the validity of this hypothesis, linear regression was employed separately on both the slope and intercept of both the high to low and low to high switching treatments. The *lm* function and a general t-test were employed to test the above hypotheses. All statistical computing was conducted using R v. 4.0.3 (R Core Team, 2020).

## 3.4 Results

### 3.4.1 SURVIVORSHIP AND TEMPORAL SCALING

Using two different ambient growth temperatures, *L. minor* aging trajectories were successfully manipulated with a low (22 °C) and high (28 °C) ambient temperature

difference of 6 °C. Fronds kept in the low temperature environment for their entire lifespan demonstrated a 47.9% increase in mean lifespan when compared to fronds kept in the high temperature environment (Figure 3-1). The average lifespan of control fronds kept in the low temperature environment was $L_1$ = 35.75 days, and the average lifespan of control fronds kept in the high temperature environment was $L_2$ = 24.17 days. When plotted using absolute time, fronds in the low temperature environment began dying after reaching age 22 days, while fronds kept in the high temperature environment began dying after reaching age 15 days.

When plotted against standardized time, temporal scaling in duckweed survivorship trajectories becomes apparent (Figure 3-2). As fronds approach about 60% of their cohort's mean lifespan, fronds in both control treatments begin to die. Less than 10% of individuals were able to live to 1.5 times the average lifespan of each relative cohort, and the relative survivorship trajectory overlapped as age increased relative to the average lifespan of each cohort.

### 3.4.2 PREDICTING EFFECTS OF SWITCHING TREATMENTS

Figure 3-3 represents the empirical results of having treatment fronds switched from one ambient growth temperature to another at scheduled intervals to determine if these switches would result in a predictable change in average lifespan. Firstly, the relation of the empirical data for fronds switched from low to high ambient temperature environments was compared to the theoretical prediction proposed by Equation 1 (Figure 3-3, blue line). With regards to intercept ($L_2$), it was determined that the empirical and theoretical results were significantly different (df = 162, t = -2.6365; P = 0.0092). With

regards to slope $\left(1-\frac{L_2}{L_1}\right)$, it was determined that the empirical and theoretical results were also significantly different (df = 162, t = -2.2024, P = 0.0291). Therefore, in both cases (slope and intercept) the null hypothesis was rejected, however the least squares regression line did move in the same direction as the theoretical prediction line (i.e., with a positive slope). Secondly, the relation of the empirical data for fronds switched from the high to low ambient temperature environments was compared to the theoretical prediction proposed by Equation 2 (Figure 3-3, red line). With regards to intercept ($L_1$), it was determined that the empirical and theoretical results were not significantly different (df = 157, t = 0.2485; P = 0.804). With regards to the slope $\left(1-\frac{L_1}{L_2}\right)$, it was determined that the empirical and theoretical results were also not significantly different (df = 157, t = 0.6633; P = 0.5081). Therefore, in both cases (intercept and slope) the null hypothesis was not rejected. In conclusion, Equation 1 was not able to quantitatively predict mean changes to frond lifespan for fronds moved from a low to high temperature environment, but Equation 2 was able to accurately and quantitatively predict mean changes to frond lifespan for fronds moved from a high to low temperature environment.

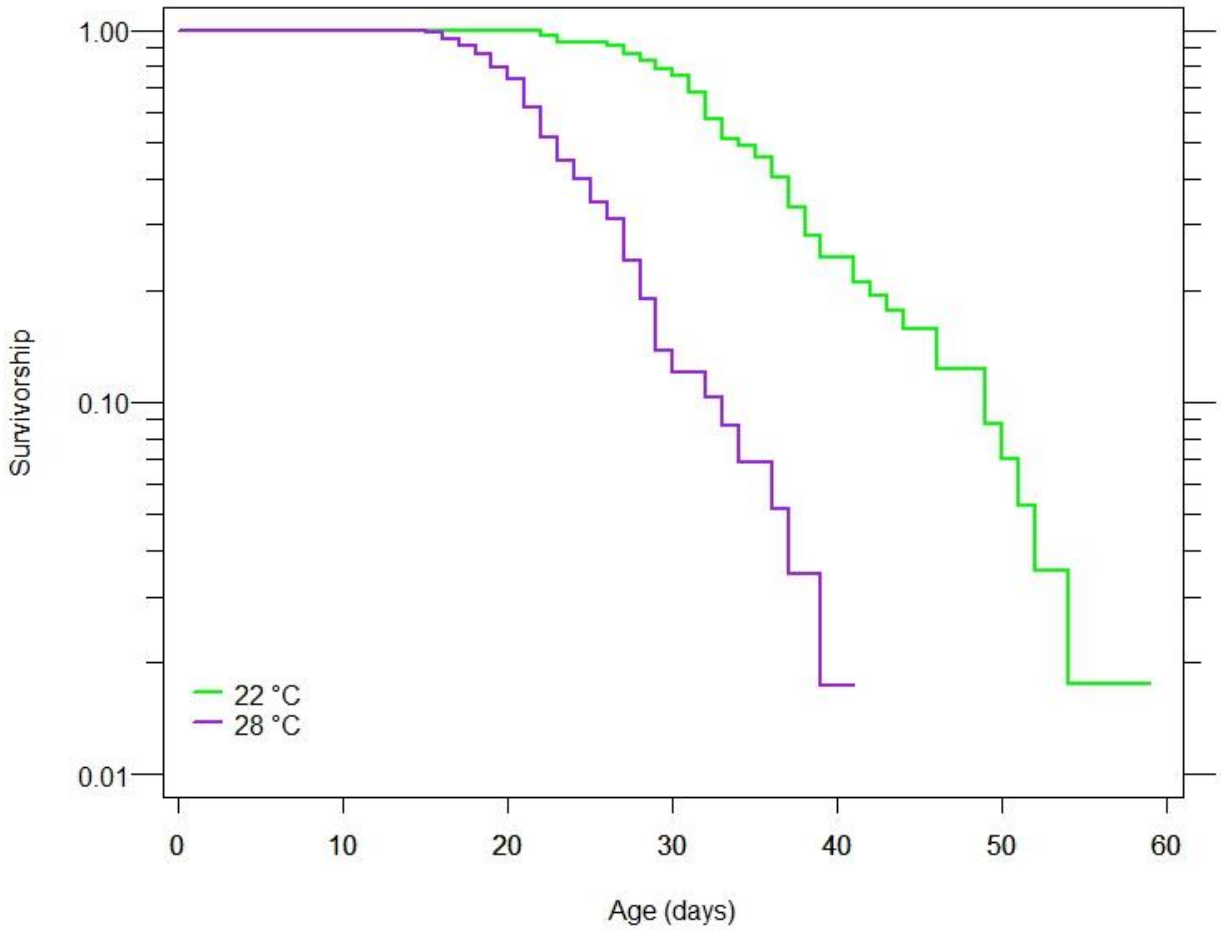


Figure 3-1: Survivorship plotted against absolute time for two different control treatments of *Lemna minor* fronds grown in low (22 °C) and high (28 °C) ambient temperature environments. The low temperature environment resulted in a 47.9% increase in average frond lifespan when compared to the high temperature environment.

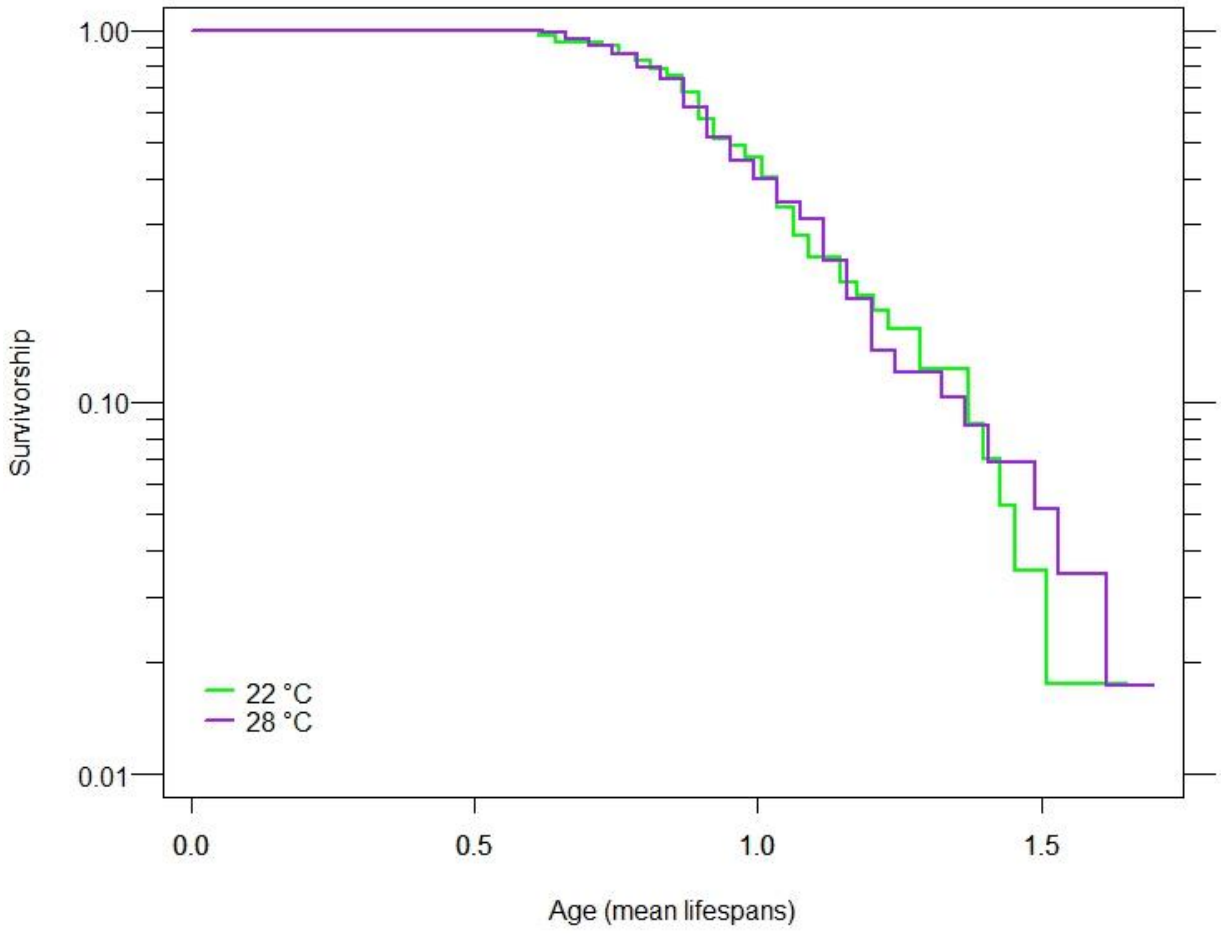


Figure 3-2: Survivorship plotted against standardized time, calculated by dividing each fronds lifespan by the average lifespan of its temperature-specific cohort. The overlap of the survival curves demonstrates temporal scaling of *Lemna minor* survivorship trajectories.

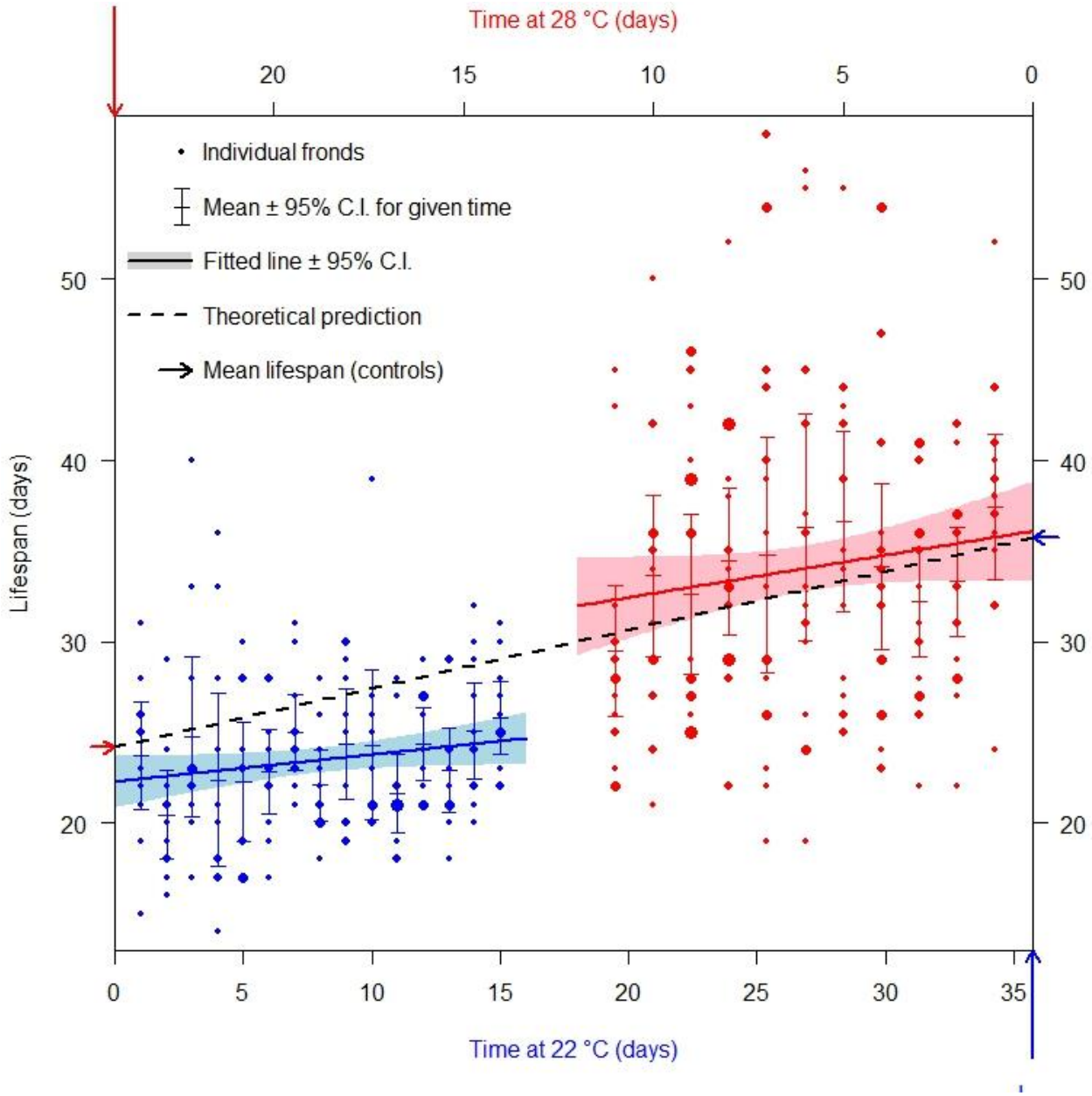


Figure 3-3: Scatter plots representing average lifespans of fronds initially kept at 22 °C and switched to 28 °C (blue) and fronds initially kept at 28 °C and switched to 22 °C (red). Symbol area is proportional to the number of overlapping data points. Theoretical prediction lines follow Equation 1 for fronds moving from a low to high temperature environment, and follow Equation 2 for fronds moving from a high to low temperature environment (lines overlap precisely due to manipulation of the extent and direction of the horizontal axes according to the mean lifespans of controls [indicated by arrows]). Blue and red bands along the least squares regression line of each treatment represent 95% confidence intervals.

## 3.5 Discussion

Through manipulation of ambient growth temperature, this study was able to induce temporal scaling in *L. minor* aging trajectories, demonstrated by time-standardizing and comparing the average lifespans of low and high temperature control treatments. Furthermore, this study was able to employ a theoretical linear model to accurately predict the average change in lifespan caused by spending a scheduled time interval within a high temperature environment and then switching to a low temperature environment. This study represents the first attempt to directly investigate temporal scaling in duckweeds, and even in plants.

### 3.5.1 SURVIVORSHIP AND TEMPORAL SCALING

Previous studies and unpublished lab data had confirmed that temperature has a pronounced impact on the average lifespan of duckweed fronds (Wangermann and Ashby 1951). This study expanded on these findings by employing temperature as an intervention to directly monitor to what extent temperature influences duckweed aging. Through direct manipulation of ambient growth temperature, a 47.9% increase in average lifespan was observed in control fronds grown in a low temperature environment (22 ℃) when compared to control fronds grown in a high temperature environment (28 ℃). Also, when these results were time-standardized, a phenomenon known as temporal scaling was observed where duckweed survivorship trajectories overlapped (Figure 3-2) even though the trajectories followed independent paths through absolute time (Figure 3-1). Temporal scaling has been shown to exist in nematodes (Stroustrup et al. 2016), bacteria (Yang et al. 2019), and now plants. It is interesting that this phenomenon is evolutionarily

conserved across multiple taxonomic clades. Published evidence already supports a theory that has been in circulation for almost six decades; the threshold theory of aging (Maynard Smith 1963). This theory, much like our understanding of senescence patterns, has been evolving over time and most likely has developed into a more specific explanation of how temporal scaling can result from the manipulation of senescence patterns. I discuss a potential theoretical alternative below that relates more closely to recently acquired empirical evidence with regards to theories of senescence.

There is one theory that pertains to temporal scaling that will be discussed hereafter: the free radical theory of aging. Free radical theory posits that the accumulation of reactive oxygen species (ROS) and other reactive molecules over time induces a state in cells reflective of the progressive deterioration associated with aging (Lu and Finkel 2008). Mitochondria, the main energy-producing organelles in eukaryotic cells, have been shown to be a common source of a large percentage of these ROS and other reactive molecules (Abate et al. 2020). Relating back to the threshold theory and temporal scaling, if energy production organelles are exposed to an increased average cytoplasmic temperature, the activation energy required to undergo cellular respiration would be reduced. Therefore, if chemical processes that produce ROS are being completed at faster rates, the accumulation of these reactive molecules will progress much faster, resulting in an earlier onset of cellular senescence. This would cause organisms in an elevated-temperature environment to reach their final aging threshold and begin to die sooner than counterparts kept in a lower temperature environment. It could therefore be hypothesized that ROS accumulation could speed up but not alter intrinsic aging processes, potentially leading to results similar to those shown in Figure 3-2. However, the hypothesis that ROS

or other reactive molecules are the underlying cause of accelerated senescence in duckweed when grown in different ambient temperatures remains to be investigated in more depth.

### 3.5.2 PREDICTING EFFECTS OF SWITCHING TREATMENTS

The objective of the switching treatments was to use a linear model proposed by Maynard Smith (1963) to predict what impact switching from one ambient temperature to another at scheduled intervals would have on the average lifespan of *L. minor*. Interestingly, the model was able to predict with greater accuracy the impact of time spent at a higher temperature then switched to a lower temperature than for the alternate treatment. One potential reason for this could be that fronds initially acclimated to a lower temperature environment may undergo a heat-shock response when moved into a higher temperature environment. Previous studies have shown that small changes in temperature can result in the reorganization of actin filaments into stress fibers (Toivola et al. 2010), fragmentation of organelles such as the Golgi apparatus or endoplasmic reticulum (Welch and Suhan 1986), a reduction in number and integrity of mitochondria and lysosomes (Patriarca and Maresca 1990), and swelling of nucleoli that results in aggregation of incorrectly assembled ribosomes (Boulon et al. 2010, Richter et al. 2010). Since the theoretical prediction line was based on control fronds that remain at the same temperature for their entire lifespan, a heat-shock response in treatment fronds would likely result in a reduction in average lifespan and demonstrate why the least-squares regression line for the low- to high-temperature treatment was significantly different than the theoretical prediction line. Conversely, it would make sense that fronds switched from

a high to low temperature environment were able to have their average lifespans extended to levels reflected by the control treatments. As these treatment fronds were taken from a potentially heat-shock response inducing environment to a lower temperature environment, the accumulation of cellular damage would have slowed down, potentially reversing the accelerated aging caused by acclimating to a high temperature environment.

### 3.5.3 FUTURE DIRECTIONS

Since the control treatments had enough individual fronds to accurately estimate means for $L_1$ and $L_2$ for Equations 1 and 2, improving the switching treatments should be the focus. One potential improvement for the switching treatments would be altering the relative sample sizes of each treatment group. Especially in the low to high treatment groups, spacing out the treatments to switching every other day instead of sequential days would allow for more fronds per treatment group, more precision for each value of $x$ (days spent at each initial temperature), and an improved range of values for $x$. As control fronds kept at 22 ℃ did not begin dying until after age 20 days, reducing the number of treatment groups would allow for the range of $x$ values to be extended and would increase precision of each measurement of $x$ by increasing each treatment group's sample size.

The results of this study develop new insights with regards to temporal scaling in plant aging trajectories as well as how different interventions can cause temporal scaling to occur. As temperature has been shown to manipulate duckweed survivorship trajectories and induce temporal scaling, future studies should employ other interventions, such as genetic manipulation of specific loci and caloric restriction through light intensity manipulation, to see if they impact duckweed aging. Recent studies have

found that autophagy, the catabolic process in which cellular components are transported from the cytoplasm of eukaryotic cells into lysosomes for deconstruction, is an important mechanism with regards to cellular senescence (Perez-Perez et al. 2012, Minina et al. 2014, Michaeli et al. 2016, Abate et al. 2020). Autophagy has been shown to mediate lifespan in *Arabidopsis* when plants were exposed to low-light conditions to promote lifespan extension through autotrophic caloric restriction (Minina et al. 2013). Removal of autophagy-related gene products resulted in an arrest of the lifespan extension phenotype, leading to the conclusion that autophagy plays an important role in *Arabidopsis* lifespan mediation. Future studies employing duckweed as a model organism for investigating the impact of interventions other than temperature on frond life history traits should look towards autophagy-related gene products to determine whether autophagy plays a prominent role in duckweed senescence.

### 3.6 Conclusions

It was determined that ambient growth temperature plays an important role in the mediation of lifespan in *L. minor*. Also, after standardization of the time scale in which two control frond treatments were grown, temporal scaling was observed in duckweed aging trajectories. The phenomenon of temporal scaling has been shown to exist in model invertebrate species as well as bacteria, and now for the first time in plant species. With temporal scaling being conserved across multiple taxa, future research should continue to incorporate lifespan manipulation through means of temperature, caloric restriction, and artificial selection as a way of pinpointing what specific environmental and biological processes are involved in senescence. Switching fronds from one ambient growth

temperature to another at scheduled intervals also demonstrated a relatively accurate predictability associated with the impact of temperature on duckweed aging trajectories, specifically when moving from a high to low temperature environment. This study demonstrates the usefulness of even simple linear models in attempting to predict ecologically significant changes associated with ambient temperatures.

# CHAPTER FOUR: GENERAL DISCUSSION

## 4.1 Pace and shape of survivorship trajectories

The diversity of senescence patterns present in nature has led researchers to look for intuitive explanations for why such patterns are selected for and what mechanisms underlie the evolutionary conservation of senescence (Medawar 1952, Williams 1957, Kirkwood 1977, Stearns 1992, Kirkwood and Austad 2000, Monaghan et al. 2008, Jones et al. 2014, Lemaitre et al. 2015). While many theories have been proposed to explain why the force of selection is reduced at later age-classes, there still remains a lack of understanding with regards to why these patterns are so diverse. In an attempt to alleviate the above issue, recent studies have begun employing a generalized framework to decouple the absolute pace of aging from comparative analysis (Baudisch 2011, Baudisch et al. 2013, Wrycza et al. 2015, Archer et al. 2018, Baudisch et al. 2019). The above framework allows researchers to analyze both the absolute time frame in which an organism lives and dies (the pace of aging) and the more general patterns present in time-standardized mortality curves (the shape of aging). By dividing each experimental lifespan by the average lifespan of the experimental cohort, the survivorship of each individual is time-standardized with respect to the average life expectancy of each cohort. This decouples absolute time from comparative analyses and can provide novel insights with regards to the resulting standardized survivorship curves (Jones et al. 2014).

In Chapter Two, I employed the above generalized framework to observe similarities and differences present in the pace and shape of aging in three species of duckweed: *Lemna gibba* L., *L. minor* L., and *L. turionifera* Landolt. As observed in previous studies (Barks and Laird 2015, Barks et al. 2018, Chmilar and Laird 2019), all

three species have exhibited age-related declines in survivorship and fecundity. While some life history traits, such as average lifespan, $shape_{mortality}$, and total reproductive output, remained relatively consistent among all species, other life history traits, such as $shape_{fecundity}$, frond surface area and perimeter, showed significant differences among species. It is interesting that certain traits are evolutionarily conserved among species of duckweed, while others are not.

In terms of similarities, average lifespan was conserved among species, with the mean lifespan of *L. gibba* being significantly longer than that of *L. turionifera*, but neither being significantly different than *L. minor*. When absolute time is decoupled from the analysis, the $shape_{mortality}$ measure demonstrates that *L. minor* shows significantly more variability in lifespan than the other two species, with the shortest-lived frond surviving to age 16 days and the longest-lived frond surviving to age 62 days. Without time standardization of each cohort's survivorship trajectory, it would seem that each species shows negligible variation in average lifespan. However, through a time-standardized approach, fewer than 1% of *L. gibba* fronds were able to survive past 1.5 times the average life expectancy of their cohort, while over 10% of both *L. minor* and *L. turionifera* fronds were able to survive past 1.5 times their relative cohorts' average lifespans. Even though *L. turionifera* does not demonstrate the same significant variability in average lifespan as *L. minor* (demonstrated by the $shape_{mortality}$ measure), *L. turionifera* fronds were able to live to a higher maximum lifespan on average than *L. gibba* fronds, with certain fronds reaching two times the cohort's average lifespan (age < 50 days).

Some potential explanations for the discrepancy in time-standardized lifespans may relate to the significant differences observed in frond size and average ramet production. In the wild, an evolutionary trade-off could occur between larger and smaller duckweed species, as larger fronds would enjoy a higher photosynthetic potential due to increased chloroplast density, but would require higher metabolic rates to maintain somatic repair mechanisms. This allometric trade-off would ultimately result in a faster relative rate of senescence for larger fronds as the increase in metabolic rate would cause a subsequent increase in the accumulation of oxidative damage. It could also be hypothesized that a larger frond size would be selected for only to a point where the increase in size balances the energy required for somatic development/repair and ramet production. *Lemna gibba* demonstrated the lowest average total offspring production as well as the lowest average $shape_{fecundity}$ value, while maintaining the largest average frond surface area and perimeter. In light of these results, *L. gibba* would require fewer offspring to cover the same amount of aquatic habitat when compared to the much smaller *L. minor* and *L. turionifera*, assuming habitat coverage is a trait under selection in wild duckweed populations. While the Chapter Two results seem to support the above hypothesized trade-off between frond size and ramet production, future studies should look to investigate the 36 species of duckweed identified in nature to achieve a more in-depth analysis of evolutionarily conserved life-history traits. Also, researchers should continue applying the pace and shape framework to analyze duckweed life history traits using longitudinal studies in natural environments.

### 4.2 Predictability of variation in lifespan due to temperature

Senescence patterns in absolute time have shown malleability through the implementation of different types of interventions, such as temperature, artificial selection, caloric restriction, and direct genetic manipulation (Hsu and Chiu 2009, Kawecki et al. 2012, Minina et al. 2013, Palmer et al. 2019, respectively). However, the relative impact of these types of manipulations on time-standardized senescence trajectories has only been investigated recently. Studies have shown that when absolute time is decoupled from the analysis of manipulated lifespan measures, survivorship is either shrunken or extended (depending on the intervention used) along the standardized time axis (Stroustrup et al. 2016, Yang et al. 2019). The resulting phenomenon is known as temporal scaling, demonstrated by the fact that interventions tend to alter the pace but not the overall shape of aging trajectories. This phenomenon interestingly aligns with an already well established theory on the evolution of senescence: the free radical theory of aging. Free radical theory posits that the accumulation of reactive molecular species within the cytoplasm of a cell induces a state in cells that reflects the progressive deterioration associated with senescence (Lu and Finkel 2008). Since mitochondria are the organelles most involved in cellular respiration, it has been hypothesized that biological processes that go on within mitochondria are the main source of these reactive molecules associated with induction of cellular senescence (Abate et al. 2020). Therefore, it can also be hypothesized that any experimental intervention that artificially accelerates the rate of these internal processes will also increase the rate of aging observed in an organism.

In Chapter Three, through manipulation of ambient growth temperature, it was determined that *L. minor* aging trajectories could be manipulated to induce temporal

scaling, and that scheduled switching from one ambient temperature to another led to predictable changes in frond average lifespans. The average lifespan of frond control treatments remaining at the same ambient temperature for their entire lifespan was increased by 47.9% when comparing low temperature control fronds to high temperature control fronds. However, when the results were time-standardized and reported in terms of average life expectancy, the survivorship trajectories overlapped, demonstrating that the rate of aging and not the overall shape of aging was altered. This phenomenon seems to align with the free radical theory, as an increase in the ambient growth temperature would result in an increase in the relative rate of cellular respiration within duckweed mitochondria. This increase in the rate of reaction would directly result in a faster accumulation of reactive molecules, and thus induce aging at a faster rate in cells grown in a higher temperature environment, resulting in an attenuation of average frond lifespan.

Similar results have been observed in other plant species, specifically *Arabidopsis*, where a combination of caloric restriction through light intensity manipulation and genetic knockout of autophagy related gene products resulted in either an extension or reduction of individual plant lifespan (Minina et al. 2013). More specifically, low light conditions resulted in lifespan extension, but when autophagy-related genes were knocked out, the lifespan-extension phenotype was arrested. This led to the conclusion that autophagy, not just light intensity, plays an important role in mediating *Arabidopsis* lifespan (Minina et al. 2014). Though temporal scaling was not analyzed directly in *Arabidopsis*, I hypothesize that a time-standardized comparison of wild-type and mutant low light plants would demonstrate this phenomenon. Future

studies should look to implement caloric restriction through low light intensity treatments and attempt to identify autophagy related genes within duckweed to determine whether these types of interventions play a similar role in duckweed aging.

Using a linear model proposed by Maynard Smith (1963), I also attempted to predict the quantitative change in frond lifespan associated with spending a scheduled amount of time at two different initial temperatures before being switched to the other temperature. The model showed mixed results, being more accurate with regards to fronds initially present in a high temperature environment and switched to a low temperature environment versus the alternate direction. One potential reason for the mixed results of the aforementioned linear model could be attributed to a heat shock response in fronds that were initially acclimated to a lower temperature environment. Even small changes in temperature (two or three degrees) have been shown to alter the structure and function of important internal cellular components and organelles (Welch and Suhan 1986, Boulon et al. 2010, Richter et al. 2010, Toivola et al. 2010). Since fronds that moved from a high to low temperature environment had their average lifespans more accurately predicted, this would make sense as these treatment fronds would not undergo any heat shock response. They would instead potentially experience a lower rate of free radical accumulation and their average lifespan would be more reflective of the control treatment averages used in the linear model. Future studies should continue to implement temperature as an intervention for developing our understanding of the evolutionary conservation of phenomena such as temporal scaling, and to potentially provide novel insights on the evolutionary foundation of diverse patterns of senescence.

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