EFFECTS OF PARENTAL AGE AND STRESS ON STRESS TOLERANCE OF OFFSPRING IN DUCKWEED

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Abstract

Demographic senescence is the population-level declines in rates of survival and reproduction with increasing age. Although offspring are often assumed to be of equal quality, offspring fitness can change with parental age. Many factors contribute to offspring fitness, including the ability to tolerate stress. Parental and ancestral environments also have the ability to affect offspring stress tolerance. The effects of parental age and ancestral salt stress on offspring ability to tolerate salt stress were examined in an asexual, aquatic plant. It was found that parental age affected the response, but not the overall fitness of offspring exposed to salt stress. Ancestral stress prepared offspring for stress through a reduced time to produce a first offspring, although these offspring may have been of lower quality. The studies done show the importance of stress history on future fitness.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Thesis overview

Increasing age often comes along with declines in survival and reproduction, known as demographic senescence. While offspring are often assumed to be of equal quality, it has been shown that offspring traits such as lifespan or fitness can also change with increasing parental age. This is a phenomenon known as parental age effects, and can have impacts on the age-specific force of natural selection. Offspring fitness, survival, and reproduction have been investigated in relation to parental age effects, but less is known about the effects of parental age on offspring ability to tolerate stress, which can also impact fitness. Parental and ancestral environment can affect offspring stress tolerance both adaptively, and non-adaptively. In an asexually-reproducing population, for which genetic variation is expected to be low, inherited responses to a stressful environment are likely to be non-genetic in mechanism. In this thesis, I examine the effects of history on the stress tolerance and fitness of offspring, using an aquatic plant species. I investigate whether parental age changes the ability of offspring to tolerate stress. I also examine if ancestral exposure to stress allows future generations to have a higher fitness when exposed to the same stress, indicating greater stress tolerance.

In this chapter, I provide background information on senescence, including evolutionary and mechanistic theories of senescence, followed by further consideration of how senescence occurs in plants. I review the concept of fitness, how components of fitness can be traded-off, and parental age effects. As I use the salt sodium chloride (NaCl) as a stressor, I also review the effects of this salt, salt tolerance strategies of plants, and how stress and senescence can interact. Finally, I introduce the study species I use in my analysis.

In Chapter 2, I investigate the effects of parental age on offspring stress tolerance. I use two birth orders to examine offspring with different-aged parents, and place these offspring in multiple intensities of stress to examine if fitness differs between treatments.

Chapter 3 examines the effect of ancestral environment on offspring fitness. I expose ancestral plants to a multigenerational stress, followed by a gap of 0-3 generations in non-stressful conditions, before exposing focal plants to an immediate stress. I examined these to see if fitness in this immediate stress is altered by ancestral exposure to stress, as compared to a control group, in which ancestors never experienced the stressor.

In the final chapter I provide a general discussion and synthesis of the results found in Chapters 2 and 3. I note some limitations of the studies done, and suggest future directions.

1.2 Theories of senescence

1.2.1 Evolutionary theories of senescence

Humans are concerned with aging, as individuals experience progressive degradation of bodily functions, structures, and abilities, and an increased chance of injury. At the population level, increasing age can result in declines in the rates of survival and reproduction, a phenomenon known as demographic senescence. From a human perspective, senescence seems inevitable, and was once thought to be ubiquitous and common to all organisms (Hamilton, 1966). How did something so seemingly maladaptive evolve, and across such a wide range of species that it seems universal? Organisms that are best able to survive and reproduce are favoured by natural selection; so, shouldn't an organism that never ages or loses its ability to reproduce be favoured over an organism that senesces?

The three major theories for the evolution of senescence are the mutation accumulation theory, the antagonistic pleiotropy theory, and the disposable soma theory. These share a recognition that with increasing age, comes a weakening of the force of natural selection (Hamilton, 1966), although they each have subtly different emphases. Reproductive value is an age-specific estimation of mean future reproductive success, calculated from demographic values (Fisher, 1930). Since survival and reproduction in the future are not guaranteed, reproductive value tends to decline with age, and this also reflects the weakening of natural selection with age. In addition to this, compared to offspring which are produced later, early production of offspring also allows grand-offspring to be produced earlier, further decreasing the force of selection with age.

The mutation accumulation theory states that because of the weaker force of natural selection at later ages, detrimental mutations affecting an organism later in life are not selected against as strongly as mutations that show effects earlier in life. Delaying the onset of these negative effects is also beneficial, since they affect reproduction less when they occur later in life. Thus, according to the mutation accumulation theory, senescence occurs because of the resulting accumulation of detrimental mutations affecting older, and not younger age classes (Medawar, 1946, 1952).

Antagonistic pleiotropy theory is similar to mutation accumulation theory, but notes that wholly detrimental mutations should still be selected against, however slightly. Pleiotropic mutations that are beneficial at young ages, and detrimental at older ages, would be selected for by natural selection, as early effects are more important drivers of fitness than later ones. Under antagonistic pleiotropy theory, the resulting negative effects which predominantly appear later in life, result in senescence (Williams, 1957).

Finally, the disposable soma theory suggests that senescence is due to a breakdown of somatic maintenance, which is less important than the maintenance of germline tissue and production of offspring. Under this framework, resources are preferentially directed towards reproduction, at the cost of the eventual deterioration of the soma, which allows the accumulation of random defects in cells and results in senescence (Kirkwood & Holliday, 1979; Kirkwood & Rose, 1991).

1.2.2 Mechanistic theories of senescence

Apart from the evolutionary theories of senescence, which seek to understand *why* senescence has evolved, mechanistic theories have been developed to explain *how* senescence occurs. Many theories of senescence have been proposed, with an estimated 300 existing as of 1990 (Medvedev, 1990). As more evidence is collected, some theories are supported and connected, with an ultimate goal of developing a general, unified theory of aging (Rattan, 2006; Chmielewski, 2017). While mechanistic theories are not the focus of this thesis, I briefly review several influential mechanistic theories.

In rate-of-living theory, observations of the connection between metabolism and longevity are addressed. Rate-of-living theory suggests that all organisms are born with a certain metabolic potential, determined by genotype. The rate at which this metabolic potential is used up determines the organism's longevity, with exhaustion of resources as the cause of death once this potential is gone. Under the rate-of living theory, organisms with a lower metabolic rate are predicted to live longer (Pearl, 1928; Sohal, 1986). The death-by-starvation hypothesis proposed by Molisch (1938) also suggests that the cause of senescence and death in annual plants is resource exhaustion resulting from the high demands of seed production. Although some recent studies have found use

in resource exhaustion theories to interpret results (Thomas, 2002; Brys et al., 2007; Rosbakh & Poschlod, 2018), these are not currently in favour, as other theories have greater support (Chmielewski, 2017).

Following from rate-of-living theory, and its connection of metabolism with longevity, Parsons (1995, 2002b) notes that these traits are also linked with stress and stress resistance. Stressful conditions often increase metabolism and energy usage, and the ability to better tolerate the stress mitigates this. Taking this into account, the rate-of-living theory should predict an increase in longevity when stress resistance is increased. Selection for greater stress resistance can result in increased longevity and decreased metabolism, and vice versa - selection for increased longevity can result in greater stress resistance. The reformulation of the rate-of-living theory to the stress theory of senescence shifts focus from metabolism to the more relevant trait of stress resistance (Parsons, 1995, 2002b).

The stress theory of senescence is in agreement with some other theories of senescence, including the network theory of aging (Kowald & Kirkwood, 1994; Parsons, 1995). The network theory of aging suggests that senescence is the result of the breakdown of the various maintenance processes which protect homeostasis. This theory combines protein error theory and free radical theory to begin to describe the network of maintenance processes that maintain homeostasis, and show that these can break down over time (Kowald & Kirkwood, 1994). Protein error theory suggests that proteins responsible for protein translation can themselves be translated with errors. Translation errors would accumulate in a feedback loop, resulting in an error catastrophe, and causing deterioration of cells with aging (Orgel, 1963, 1973). Free radical theory focuses on the role of free radicals and reactive oxygen species (ROS) as a cause of senescence, as they cause

damage within cells that accumulates over time (Harman, 1956, 1992). ROS are produced endogenously within organisms, with damaging effects protected against by antioxidants.

As a greater number of studies examining ROS and oxidative damage are done, the free radical theory has since been updated, incorporated by oxidative damage theory, and damage theory (Lin & Beal, 2003; Gems & Doonan, 2009). With newer information available, it has been noted that oxidative damage theory is compatible with rate-of-living theory, mentioned previously (Brys et al., 2007). Sohal (1986) notes that besides resource exhaustion, rate-of-living theory could occur if an unidentified substance accumulated in relation to metabolism. As ROS are produced as a natural product in the pathways of metabolism (Gill & Tuteja, 2010), ROS could be the identity of the hypothesized substance. The combined theories would predict that as metabolism increases, the amount of ROS produced would also increase, leading to a greater amount of oxidative damage, and resulting in aging.

While free radical theory has considerable evidence supporting it, some studies have found results which do not follow its predictions. Pomatto and Davies (2018) argue that free radical theory is overly simplistic, considering increases in free radicals and ROS as 'bad', and greater antioxidants as 'good', without taking into account potential complexities often seen in biological systems – such as compensatory mechanisms or redundancies. This could explain the ambiguous and sometimes contradictory results seen in some studies. The adaptive homeostasis theory of aging proposes that senescence occurs when the ability to adaptively change the homeostatic range of stress-protective systems declines with age, resulting in an increasing accumulation of damage. Older organisms are less able to adapt to stress and adjust protective pathways to environmental conditions. This theory allows a clearer understanding of the free radical theory, which better fits the observations made since free radical theory was originally proposed (Pomatto & Davies, 2018).

1.3 Senescence in plants

Senescence has most often been studied in animal species, as these give more relevant information to understanding human aging, which is often the main point of interest. To better understand why senescence has evolved, it is important to look both at species very similar, and very different from each other (Jones et al., 2014).

Senescence was previously thought to be ubiquitous among all living organisms (Hamilton, 1966). However, this is not the case, with negligible and negative senescence occurring in various organisms, including plants (Vaupel et al., 2004; Jones & Vaupel, 2017). While senescence is characterized by decreasing rates of survival and reproduction with age, negligible senescence shows rates that do not change with age, and negative senescence shows increases in these rates. Indeed, negligible or negative senescence seems to be quite common in plants; a study examining senescence in angiosperms found that 93% showed no senescence, a surprising change from the commonality of senescence in animals (Baudisch et al., 2013).

Negligible and negative senescence can occur in organisms for which size, rather than age, predicts survival and reproduction. Indeterminate growth allows increasingly greater sizes to be achieved, and often accompanies cases of negative or negligible senescence (Jones & Vaupel, 2017). While plants are often able to grow indeterminately from meristems, animals more often exhibit determinate growth, in which individuals grow to a maximum size, and then stop. Plant size can also be affected by shrinkage, which changes a population's structure independently of age or stage through survival, post-disturbance speed of recovery, and trade-offs with sexual reproduction and dormancy (Salguero-Gómez & Casper, 2010). When plants senesce, it is often associated with reproduction. In monocarpic plants such as annuals and biennials, seeds are produced in a single reproductive event near the end of life, followed by rapid degeneration of the

plant organs and tissues (Noodén et al., 2004; Davies & Gan, 2012). Polycarpic or perennial plants live longer, and have the possibility of multiple reproductive events (Thomas, 2013). In many perennial species, senescence and its associated declines and degradation do not occur (Munné-Bosch, 2008, 2015).

Unlike many animals, plant forms are often modular, consisting of repeating units or modules which have their own distinct lifespans (Harper, 1980). Modules can remain attached, or can separate from each other to form populations of genetically identical, but physiologically separate ramets. All of the clonally produced ramets that share a genetic source make up a genet. Ramets that are independent of each other benefit from the containment of risk and damage to individual parts, allowing unaffected parts to continue to survive and reproduce, potentially in more favourable environments (Hutchings & Bradbury, 1986; Price et al., 1996; Salguero-Gómez, 2018). This clonal reproduction via ramets can be considered continuous growth of the genet (the genetic individual), which can allow an escape from senescence (Vaupel et al., 2004). Ramets, however, can exhibit the expected declines in rates of survival and reproduction that accompany senescence, despite the potential 'immortality' of the genet of which they are a part. Functionally, it is at the level of ramets that the probabilities of survival and reproduction differ, and groups of ramets act as a population (Orive, 1995). In this document, 'individuals' indicates single ramets.

Plant biology also differs from animals in the lack of distinction between somatic and germline tissues. In plants, the germline cells are thought to separate from the soma very late in development, or not at all (Walbot, 1985; Sutherland & Watkinson, 1986), as both reproductive organs and clonally-produced offspring seem to come from somatic cells of the plant. More recent studies have questioned this assumption, showing evidence of a 'functional germline' in which an undifferentiated, slowly-dividing cell lineage is responsible for eventually producing reproductive

tissues (Watson et al., 2016; Lanfear, 2018). This would give similar benefits to an early-segregating germline, such as a lower accumulation of deleterious mutations in offspring, and prevention of the inheritance of somatic mutations.

In some theories of senescence, a clear distinction of germline and soma is necessary. The antagonistic pleiotropy theory states that a distinct soma that is not passed on to offspring is a necessary condition for senescence (Williams, 1957). This assumption cannot be made in the case of asexual reproduction, with Williams (1957) noting that when identical asexual clones are produced, senescence should not occur. However, organisms with asexual reproduction (differentiated from asexual clones in that there is a distinct 'parent' and 'offspring' produced, rather than two identical clones) can be considered to have a soma, and therefore can exhibit senesce. Plants are directly addressed, with the conclusion that while ramets do senesce, the genets they comprise should not.

Disposable soma theory also requires a distinct germline, recognizing that maintenance and repair in germ cells are very important for the survival of genes, but maintenance and repair of the soma is less so, and may be more costly than it is worth (Kirkwood, 1977; Kirkwood & Holliday, 1979). However, it is also noted when the assumption of a distinct and irreversibly differentiated germline is not met, such as in plants, modification to the theory is necessary (Kirkwood & Holliday, 1979). While this suggests that plants would need to spend more energy to decrease mutations in somatic cells, this does not seem to be the case – high somatic mutation rates in plants have been argued to be beneficial (Whitham & Slobodchikoff, 1981; Otto & Hastings, 1998). Intra-individual somatic selection of cell lineages with lower mutation rates, or with beneficial mutations, has been suggested as a method to avoid the typically detrimental effects of heritable somatic mutations (Whitham & Slobodchikoff, 1981; Antolin & Strobeck, 1985; Gill et al., 1995;

Otto & Hastings, 1998). Further investigation is needed to clarify this topic, but the basic concept of the disposable soma theory – an energetic trade-off between reproduction and maintenance of the rest of the body – remains plausible. The ability to maintain a low mutation rate in the genetic information passed to offspring is not the only measure of successful reproduction, and energy could be invested in other aspects of reproduction instead. Indeed, the presence of a segregated germline may be less important than the change in force of natural selection with increasing age (Orive, 1995; Baudisch et al., 2013). Other theories, including the mutation accumulation theory (Medawar, 1946, 1952), and the ideas proposed by Hamilton (1966), make no assumptions regarding the presence of a segregated germline.

1.4 Fitness

The concept of fitness is very important, yet has had considerable disagreement over its definition. The idea originates with Darwin (1859), who noted that amongst the variation of traits in a population, certain traits are more advantageous compared to others. Individuals with these advantageous traits are better adapted to their environment, and so are more likely to survive and reproduce – they have greater fitness. Fitness can be understood as the probability of contributing genes to future generations, the predicted number of offspring an individual will produce over its lifetime (Hamilton, 1966; Winkler & Fischer, 1999). Fitness is used as a measurement of performance or success in an environment, and is used as a tool to examine the workings of natural selection (Stearns, 1989; Charlesworth, 1994; Benton & Grant, 2000). Fitness is often examined by 'type' of individual, referring either to individuals of the same genotype or phenotype, and can be understood in the context of other 'types', which may be more or less fit, comparatively (McGraw & Caswell, 1996). Fitness is also highly dependent on the environment, as the traits that

give a high fitness in one environment do not necessarily give the same advantage in a different environment (Stearns, 1992; Winkler & Fischer, 1999).

Fitness is made up of multiple components, which can make it difficult to choose a component to measure it. No single quality can be the most advantageous in all situations, so measurement of a single component does not always accurately reflect fitness. Various types of measures have been used to quantify fitness, with common ones often based on measures of reproductive success, population growth, or population size, among others (Benton & Grant, 2000). Measures of reproductive success include net reproduction – the total number of offspring produced throughout the lifespan of an organism (Clutton-Brock, 1988; Newton, 1989; Partridge, 1989; Benton & Grant, 2000). This takes both reproduction and longevity into account, although not the timing of when offspring are produced. The timing of reproduction is important as reproductive value decreases with increasing age (Fisher, 1930). Earlier-produced offspring have a greater value than later-produced offspring, since offspring produced earlier are themselves able to produce offspring earlier, and so spread their genes faster (Stearns, 1976). Another common measure of fitness is the intrinsic rate of increase (r – also known as the Malthusian parameter), which is a per-capita measure of population growth (Fisher, 1930; Hamilton, 1966; Benton & Grant, 2000). This takes into account the lifespan, reproduction, and timing of reproduction of an organism, and is often considered a good measure of fitness (Hamilton, 1966; Murray Jr, 1992; Benton & Grant, 2000). Using r as a measure of fitness requires an assumption of a constant environment and stable population, and is applicable only to the environment in which it was measured (Metz et al., 1992; Metcalf & Pavard, 2007). While a measure of population growth seems to imply the necessity of a population of individuals, which gives a single value, it is

possible to determine the intrinsic rate of increase for each individual, as described by McGraw and Caswell (1996), giving a greater sample size.

These measures of fitness take into account some of its most important aspects, although individual measures often do not capture the entirety of the concept. The quality of offspring produced is another component of fitness that is overlooked when using *r* or net reproduction. The ability of an organism to reproduce is certainly important, but it is also important for the offspring produced to survive and produce their own offspring. The use of the term 'quality' here is similar to fitness – high-quality offspring are very fit, and low quality offspring are not – but is used to describe the offspring of the individual of interest, whereas fitness is used to describe the individual itself. Depending on the life history of the organism in question, it may be more or less beneficial to produce high-quality offspring. For example, in the case of fish when replacement of parents is important, it may be beneficial to produce a few high-quality offspring, rather than many low-quality offspring (Svardson, 1949; Williams, 1966a; Stearns, 1976). Size of offspring is a trait that can contribute to quality, with larger sizes often providing benefits such as an increased chance of survival, greater competitive ability, and better resistance to environmental risks (Stanton, 1984; Hutchings, 1991; Vasseur et al., 1995; Einum & Fleming, 2000; Krist, 2011; Rollinson & Hutchings, 2013). A common trade-off between the number of offspring and their size was originally studied in birds (Lack, 1947, 1948), but is applicable to other organisms, and has been found to occur in other taxa including plants (Smith & Fretwell, 1974; Vasseur et al., 1995; Sadras, 2007).

The fitness of an organism is highly dependent on the environment in which it is measured (Stearns, 1992; Winkler & Fischer, 1999). Some environments are highly variable, while others are more constant, with natural environments exhibiting at least some level of change. If it is

assumed that organisms living in an environment are adapted to some extent to the average conditions of that environment, then fluctuations should expose these organisms to suboptimal, and potentially stressful conditions, in which the organism's fitness is detrimentally affected. The ability to respond to these fluctuations, through adaptation or plasticity, allows organisms to maintain their fitness under these suboptimal conditions (Bradshaw, 1965; DeWitt et al., 1998). In environments where fluctuations are common, adaptation and plasticity are the most advantageous, however, in constant environments, it is possible for these abilities to be disadvantageous if there is a cost of plasticity (Alpert & Simms, 2002; Hallsson & Björklund, 2012; Lande, 2014). As anthropogenic activities change natural environments through climate change and salinization, however, adaptation and plasticity may be increasingly important (Kefford et al., 2016; Donelson et al., 2018). The ability to respond to and tolerate suboptimal and stressful conditions generally increases fitness and quality, although it is important to recognize how the environment affects this component of fitness, and the specific conditions in which it may not increase fitness.

1.5 Trade-offs

Trade-offs have been mentioned previously in this chapter, although they have not yet been directly addressed. The concept of trade-offs stems from the recognition that all organisms are limited in the amount of resources they are able to obtain, and so cannot maximize all components of fitness simultaneously (Williams, 1966b; Law, 1979). Instead, they must divide the resources available to them amongst these components. The cost of allocating resources towards one component comes at the cost of one or more others (Stearns, 1976; Pease & Bull, 1988). Like fitness, trade-offs can be complicated and multi-faceted, although they are often addressed in a

binary view, and they can be misinterpreted if some of the components involved are not measured (Pease & Bull, 1988).

Many trade-offs exist; however, only trade-offs considered relevant to the evolution of senescence will be discussed here. The disposable soma theory is based on a trade-off between maintenance of the soma and reproduction, in which the optimal balance of resource allocation typically favours reproduction (Kirkwood & Holliday, 1979; Kirkwood & Rose, 1991). The lack of maintenance of the soma is what results in senescence, so this could also be considered a trade-off between reproduction and longevity. A trade-off of current reproduction versus survival, or future reproduction, occurs in the sense that expending resources to reproduce comes with costs to the parent, decreasing the chances of survival and reproduction in the future (Williams, 1966b). Trade-offs within reproduction exist, including offspring number versus quality trade-offs, which can be seen when the number of offspring is negatively correlated with components of quality, such as size (Lack, 1947; Smith & Fretwell, 1974). The ability to reproduce faster can also come at the cost of offspring quality (Gibson & Mace, 2006; Thompson et al., 2016).

1.6 Parental age effects

While offspring quality is often considered in the context of trade-offs, the same is not true in the theories of the evolution of senescence. In many cases, it is assumed that all offspring are of equal quality, and only the rates of survival and reproduction decline with increasing age (Hamilton, 1966; Kirkwood & Rose, 1991; Vaupel et al., 2004). This assumption is not always met, as indicated by phenomena such as the Lansing effect, in which offspring of younger parents have a longer lifespan than offspring of older parents (Lansing, 1947, 1948). Parental age can also affect the quality of offspring, through decreases in survival, reproduction, or intrinsic rate of

increase of offspring (Descamps et al., 2008; Bouwhuis et al., 2010; Gillespie et al., 2013a; Barks & Laird, 2015). Besides these direct indicators of offspring quality, offspring size and development time can also be affected by parental age (Ashby et al., 1949; Berkeley et al., 2004; Barks & Laird, 2015).

If offspring quality varies with parental age, it will affect the force of natural selection on different age categories, similarly to the age-related declines in survival and reproduction found in senescence (Kern et al., 2001; Priest et al., 2002). If older individuals have a low chance of surviving and reproducing, as well as producing offspring which are themselves less likely to survive or reproduce, mutations which occur at older ages will experience only weak selection. There is a risk of underestimating age-related declines in the force of natural selection, if declines in offspring quality with increasing parental age are ignored (Pavard et al., 2007; Gillespie et al., 2013b; Barks & Laird, 2015, 2020). As such, offspring quality is important, and should be taken into account in studies of senescence (Kern et al., 2001; Barks & Laird, 2015).

1.7 Salt stress and tolerance

1.7.1 Salinization

Salinization is a complex process in which water-soluble salts accumulate above natural concentrations, with noticeably detrimental effects (Rengasamy, 2006; Herbert et al., 2015). It occurs in both soils and water, affecting human agriculture and economy as well as natural systems. While the salt NaCl (and the Na^+ and Cl^- ions it forms) is often the dominant salt, salinization can more broadly include other major ions such as Ca^{2+}, K^+, Mg^{2+}, HCO_3^-, CO_3^{2-}, and SO_4^{2-} (Williams, 1987; Rengasamy, 2006; Cañedo-Argüelles et al., 2013). These salts are found

at different concentrations in many ecosystems, and their natural accumulation, called primary salinization, occurs over long time periods.

Secondary salinization, the accumulation of salts caused by anthropogenic activities, occurs much more rapidly (Herbert et al., 2015). Human activities such as irrigation, mining, and de-icing, among others, can cause considerable increases in salt concentrations in freshwater and soils, and these effects are predicted to increase with global climate change (Neubauer & Craft, 2009; Cañedo-Argüelles et al., 2013; Herbert et al., 2015).

1.7.2 Salt stress in plants

High concentrations of NaCl result in detrimental effects for organisms that live in environments with normally low levels. In plants, salt stress occurs in two phases – osmotic stress occurs quickly at exposure, followed by the slower effects of ionic stress. Osmotic stress is caused by high salt concentrations outside the roots drawing water out of the plant, and is very similar to stress caused by drought. Ionic stress occurs relatively slowly, as it requires a build-up of ionic concentrations inside plant tissues. The accumulation of either Na^+ or Cl^- ions can be toxic. In many plant species, Na^+ accumulates more rapidly than Cl^-, and is responsible for the negative effects observed. The shoot systems of plants are most sensitive to Na^+ accumulation, as ions tend to be transported within the plant to the shoots, allowing roots to maintain lower concentrations (Munns & Tester, 2008). The ability to prevent Na^+ from reaching the shoots can result in cases of Cl^- accumulating to toxic levels before Na^+, such as in citrus trees (Storey & Walker, 1998; Prior et al., 2007; Munns & Tester, 2008). Movement of Na^+ into the root vascular tissue occurs primarily through symplastic pathways, as the apoplastic movement in the root is prevented by the casparian strip and suberin lamellae, which act as barriers (Chen et al., 2011; Foster & Miklavcic,

2017; Basu et al., 2021). The toxic effects of salt are caused by a combination of the water-related effects of osmotic stress, and the toxicity of ions in the cytoplasm that result in ionic stress. High concentrations of salt outside of the plant cells allow passive entry of Na^+ into root cells through non-selective cation channels (Amtmann & Sanders, 1998; Tester & Davenport, 2003; Munns & Tester, 2008). Influx of Na^+ ions can also occur through high-affinity K^+ transporters, high-affinity K^+-uptake transporters, and low-affinity cation transporters (Schachtman et al., 1997; Plett & Møller, 2010; Assaha et al., 2017; Basu et al., 2021).

Salt stress has a major effect on the growth and yield of plants. The immediate physiological drought caused by osmotic stress inhibits growth and cell elongation. After a period of adjustment, plants are able to continue growth, although at a slower rate (Munns, 2002; Munns & Tester, 2008). Decreased yields are common, which is important for agricultural species and practices (Francois et al., 1994; Pitman & Läuchli, 2002; Munns et al., 2006; Bybordi, 2010). Salt stress negatively affects photosynthesis. Stomata close in response to the reduced water in the plant, in order to preserve water. This affects the availability of CO_2 for photosynthesis, as conductance is reduced both through stomata and the mesophyll (Flexas et al., 2004; Galmés et al., 2007; Chaves et al., 2009). The decreases in photosynthetic rate may also be attributed to inhibition of other biochemical processes. The quantum yield of photosystem II is decreased under salt stress, as the efficiency of photosystem II and the electron transport chain is altered (Chen et al., 1999; Stępień & Kłobus, 2006; Khan et al., 2009; Mehta et al., 2010; Oukarroum et al., 2015). In the cytosol, high concentrations of Na^+ inhibit enzyme activity (Greenway & Osmond, 1972), and protein synthesis (Hall & Flowers, 1973). Salt stress increases the amount of reactive oxygen species (ROS) produced, in part due to decreased photosynthesis, resulting in increased oxidative stress and damage (Munns & Tester, 2008).

1.7.3 Salt tolerance in plants

Salt tolerance is typically achieved through preventing the accumulation of Na^+ in tissues, which can occur through various mechanisms. Preventing influx of Na^+ into cells can be done through the downregulation of some of the transporters that allow Na^+ into cells, but may also prevent the uptake of other important elements such as K^+ (Munns & Tester, 2008; Assaha et al., 2017). At a cellular level, the mechanisms of extrusion and compartmentation are important in keeping cytosolic concentrations low, although they are energetically costly. Na^+ ions can be extruded from the cytosol back into the apoplastic space or the environment via Na^+/H^+ antiporters, which require energy to form the H^+ gradient that drives the movement. One well-characterized antiporter is the membrane-bound SOS1 (salt overly sensitive 1). SOS1 extrudes Na^+ from cells at the plasma membrane, and is found at high levels in cells which are highly dependent on apoplastic efflux of Na^+ from the cytosol, such as in root tips where a lack of vacuoles prevents compartmentation (Maathuis et al., 2014; Basu et al., 2021). SOS1 also plays a role in the regulation of long-distance Na^+ transport, through loading Na^+ into the xylem and transporting it to the shoots (Shi et al., 2002; El Mahi et al., 2019; Basu et al., 2021). High affinity K^+ transporters (HKTs) are involved in long-distance transport of Na^+, although with an opposite effect to SOS1. HKTs move Na^+ from the xylem and shoots to the phloem, allowing it to move back towards the roots, and preventing accumulation of Na^+ in the shoots (Almeida et al., 2017; Basu et al., 2021). Within cells, compartmentation allows the regulation of cytosolic concentrations through the sequestration of Na^+ ions in vacuoles. Na^+/H^+ exchangers (NHX) are antiporters found on vacuolar membranes, which use an H^+ concentration gradient to move Na^+ into vacuoles (Hamaji et al., 2009; Yamaguchi et al., 2013; Basu et al., 2021). Compartmentation can occur in both root and shoot parts of the plant. In the shoots, Na^+ ions accumulate in older leaves, and increase the rate of leaf senescence. Accumulation of Na^+ in vacuoles may have a benefit of acting to balance

osmotic pressures, allowing water to enter cells and maintaining turgor, and helping to counteract the effects of osmotic stress (Shabala & Mackay, 2011; Shabala & Pottosin, 2014). Concentrations must be carefully maintained to prevent toxicity, but the easily-accessible Na^+ attenuates the need to synthesize other, more costly organic solutes to balance the osmotic forces (Munns & Tester, 2008; Maathuis et al., 2014).

Salt tolerance is also related to the maintenance of the cellular Na^+/K^+ ratio, typically through the regulation of cytosolic K^+ concentrations. The physiochemical properties of Na^+ and K^+ ions are very similar (Maathuis & Amtmann, 1999), but while K^+ is an essential nutrient, Na^+ is not essential for most plant species (Maathuis, 2014). K^+ has a role in many physiological processes, including enzyme function, photosynthesis, protein synthesis, transport of photosynthate, osmoregulation including the maintenance of turgor and movement of other ions, and resistance to stresses (Cakmak, 2005; Wang et al., 2013). Small amounts of Na^+ can have a beneficial effect on growth, particularly when K^+ concentrations are low (Maathuis, 2014). However, Na^+ ions at high concentrations can compete with K^+ in both uptake from the environment, and for binding sites in various important physiological processes. In the cytosol, high concentrations of Na^+ depolarize the membrane, opening outward-rectifying K^+ channels, which results in the efflux of K^+ from cells and further K^+ deficiency (Wang et al., 2013). The ability of a plant to maintain high K^+ concentrations during salt stress is associated with better salt tolerance (Maathuis & Amtmann, 1999; Shabala & Pottosin, 2014).

Oxidative stress occurs when the ROS produced in a cell increases beyond the protective capabilities of antioxidant reactions, resulting in damage (Bartosz, 1997). ROS consists of both molecules and free radicals, including H_2O_2 (hydrogen peroxide), O_2^- (superoxide ion), $^1O_2$ (singlet oxygen), O_2^{2-} (peroxide), HO_2 (perhydroxyl radical), OH^- (hydroxyl radical), and RO^- (alkoxy

radicals) (Ahanger et al., 2017). ROS are produced in plant cells under normal conditions, with most produced by chloroplasts and peroxisomes in light conditions, and mitochondria in dark conditions (Foyer & Noctor, 2003; Gill & Tuteja, 2010). This normal production is balanced by antioxidant systems which react with and degrade ROS, preventing them from causing damage. Antioxidant systems are composed of enzymatic parts (such as superoxide dismutases [SOD], catalases [CAT], ascorbate peroxidase [APX], and glutathione reductase [GR]), and non-enzymatic parts (such as ascorbic acid [AsA], glutathione [GSH], and proline [Pro]) (Gill & Tuteja, 2010). Various external factors can cause an increase in the production of ROS, including high light intensity, water deficit, and salt (Bartosz, 1997; Miller et al., 2010; Choudhury et al., 2017). In mitochondria, aerobic metabolism produces ROS. Stresses can result in over-reduction of electron carriers, and the subsequent electron leakage has enough energy to reduce O_2, forming more ROS (Keunen et al., 2011). In chloroplasts, $^1O_2$ is a normal by-product of photosynthesis. When the electron transport chain is overloaded, O_2 is reduced instead of CO_2, and electron leakage can also occur (Gill & Tuteja, 2010). Higher production of ROS can be balanced by increasing antioxidant defense systems. The upregulation of antioxidants is seen in response to abiotic stresses, such as salt, and overexpression of antioxidants gives a greater stress tolerance (Gill & Tuteja, 2010; Chang et al., 2012; Hasanuzzaman et al., 2012). However, this tolerance is a result of the balance between ROS and antioxidants, and so can be limited in its capacity to tolerate stress (Arora et al., 2002).

1.8 Expected effects of stress on longevity and senescence

What effects should increased stress have on longevity and senescence? Often, organisms exposed to stress exhibit a biphasic dose response, known as hormesis (as well as other names,

such as a U-shaped dose-response curve). Used commonly in toxicology, hormesis is a response to a chemical or environmental condition in which a low dose has a beneficial or stimulatory effect, and a high dose has a detrimental, toxic, or inhibitory effect (Calabrese & Baldwin, 2002; Mattson, 2008; Calabrese et al., 2015). In the field of aging and senescence, longevity hormesis is of interest. This refers to the beneficial effects such as longevity, which occur in response to an organism's cellular responses to a mild stress (Rattan, 2008). The hormetic stimulatory/beneficial effect can be caused by a direct stimulatory effect, or by an overcompensation reaction when homeostasis is disrupted, both of which result in similar effects. This overcompensation is thought to be an adaptive response, as it uses a small amount of extra resources to repair damage, and leads to larger beneficial effects including stress tolerance and protection against future damage (Rattan, 2008; Calabrese et al., 2015). These upregulated maintenance and repair mechanisms should also counteract the effects of aging and senescence, whether it occurs through direct repair of damage, or through maintenance of the ability to maintain homeostasis (Rattan, 2008; Milisav et al., 2012). These ideas are similar to those of Pomatto and Davies (2018), and their adaptive homeostasis theory of aging.

Many types of stresses have been shown to result in longevity hormesis (Rattan, 2004), supporting the idea that stress response pathways are connected with longevity pathways (Hunt et al., 2011; Calabrese et al., 2015). Longevity-promoting stresses include the 'classical' examples of radiation (Davey, 1917; Cork, 1957), and caloric restriction (McCay et al., 1935; Masoro, 1998). Caloric restriction – a treatment in which the amount of food or calories that an organism consumes is reduced – can result in malnutrition or starvation when restriction is too severe, but increases longevity at milder restrictions. It occurs in a large variety of heterotrophic organisms including yeast, nematodes, flies, rodents, and monkeys (Taormina & Mirisola, 2014), and has also been

shown to occur in the plant *Arabidopsis thaliana*, although this is less well studied (Minina et al., 2013). Exhibiting a hormetic response, caloric restriction can be considered a nutritional stress, and has been shown to result in stress tolerance and increased protective mechanisms, including increased autophagy, and protection against oxidative stress (Yu & Chung, 2001; Szafranski & Mekhail, 2014). However, the increased longevity resulting from caloric restriction also often results in decreases in reproduction (Holehan & Merry, 1985; Partridge et al., 2005; Mair & Dillin, 2008). This follows the predictions made by the disposable soma theory, that when resources are allocated towards maintenance and repair processes, there is less available to be allocated towards reproduction.

1.9 Study species

The group commonly referred to as duckweeds consists of 36 related plant species in the Lemniodeae subfamily (Family Araceae). As aquatic angiosperms, they can be found floating in slow-moving and still bodies of freshwater, at or near the surface (Landolt, 1986; Lemon & Posluszny, 2000). They are a diverse and cosmopolitan group, found on almost every continent, with the exception of arctic and Antarctic regions (Landolt, 1986). Individual ramets of the duckweed species *Lemna minor* consist of a frond (a leaf and stem structure), with a single root protruding from the bottom (Lemon & Posluszny, 2000). Although flowering is possible, the production of offspring is done almost exclusively asexually, with vegetative propagules forming in the two meristematic pockets present on fronds (Lemon & Posluszny, 2000). *L. minor* exhibits 'handedness', in that clones from the same strain consistently produce the first offspring from the meristematic pocket on the same side (left or right) (Hillman, 1961; Barks & Laird, 2016). Subsequent offspring are produced from alternating meristematic pockets. Offspring remain

attached to their parents via a stipe until they are full grown, after which the stipe breaks, releasing the offspring (Lemon & Posluszny, 2000). This allows *L. minor* to reproduce quickly, to the point at which it can cover the surface of the body of water.

Duckweeds are used in a variety of fields, including ecotoxicology and bioremediation (Wang, 1990; Megateli et al., 2009; Ziegler et al., 2016), plant development (Hillman, 1976), production of feedstock and biofuel (Cheng & Stomp, 2009; Sree et al., 2015), and even as food for humans (Appenroth et al., 2017; Sree et al., 2019; McNamara, 2020), and can be considered a model system (Lam et al., 2014; Laird & Barks, 2018). Of relevance here, demographic senescence has been studied in duckweeds for over 70 years (Ashby & Wangermann, 1949, 1951; Claus, 1972). This research has focused primarily on the duckweed species *L. minor* (Ashby & Wangermann, 1949, 1951, 1954; Barks & Laird, 2015, 2020), and *Lemna turionifera* (Barks & Laird, 2016; Ankutowicz & Laird, 2018; Barks et al., 2018; Barks & Laird, 2018), although *Lemna gibba* exhibits senescence as well (Chmilar & Laird, 2019). For these reasons, I chose to use the species *L. minor* here.

CHAPTER 2: EFFECTS OF PARENTAL AGE ON THE SALT TOLERANCE OF OFFSPRING IN AN AQUATIC PLANT

2.1 Abstract

Parental age affects offspring fitness. The ability to tolerate stress also affects fitness, but less is known regarding changes in offspring stress tolerance with increasing parental age. I examined first-born and fifth-born offspring (using birth order as a proxy for parental age), and compared their fitness in several sub-lethal concentrations of salt (NaCl), to investigate the interactive effects of birth order and salt stress on the offspring of the aquatic plant *Lemna minor*. I found that increasing salt concentration detrimentally affected fitness, primarily due to reduced reproduction at early ages. Fifth offspring had greater fitness than first offspring. I attributed this result to a possible hump-shaped change in offspring fitness with increasing birth order, with the fifth-born treatment falling near the peak of the hump. I found no interactive effect of birth order and salt concentration on offspring fitness; however, there was an interactive effect on the time to first reproduction and size of fronds. Specifically, first-born offspring showed that increasing salt concentrations slowed time to first reproduction and allowed growth to a greater size, while fifth-born offspring showed little change in either variable with increasing salt. Thus, variation in birth order affected offspring response to salt stress, although not in terms of fitness.

2.2 Introduction

Demographic senescence is characterized by declines in rates of survival and reproduction with increasing age (Hamilton, 1966; Kirkwood & Rose, 1991; Vaupel et al., 2004). Offspring quality can also vary with parental age. Older parents sometimes produce offspring with decreased lifespans (Lansing, 1947, 1948; Bell, 1984), survival (Tarín et al., 2005; Descamps et al., 2008;

Reichert et al., 2019), reproduction (Tarín et al., 2001; Smits et al., 2002), and fitness as determined by a measure of population growth (Gillespie et al., 2013a; Barks & Laird, 2015).

Parental age effects are important to examine because, like the declines in survival and reproduction with increasing age, changes in offspring quality with increasing parental age may affect the force of natural selection (Kern et al., 2001; Priest et al., 2002). Declines in offspring quality could affect the evolution of senescence, with the age-specific force of natural selection decreasing faster than expected when only survival and reproductive age-trajectories are taken into account (Pavard et al., 2007; Gillespie et al., 2013b; Barks & Laird, 2015, 2020).

Fitness, the ability to contribute genes to future generations, is a concept that cannot be accurately reflected by a single component in all environments. The intrinsic rate of natural increase (r) is considered a widely applicable measure of fitness, as it takes into account an organism's lifespan, reproduction, and timing of reproduction (Fisher, 1930; Hamilton, 1966; Murray Jr, 1992; Stearns, 1992; Benton & Grant, 2000). Fitness is highly dependent on the environment of the organism (Hamilton, 1966; Stearns, 1992; Winkler & Fischer, 1999). The ability to respond to changes in environment allows organisms to maintain their fitness in suboptimal or stressful environments (Bradshaw, 1965; DeWitt et al., 1998).

Stressful environments confer detrimental effects, decreasing survival and reproduction, resulting in a decrease of fitness (Parsons, 2002a, 2004). Tolerance of stressful conditions requires an expenditure of resources to protect against and repair damage. In plants, stress caused by the salt sodium chloride (NaCl), is well characterized. Salt stress causes osmotic stress through reduced uptake of water, and ionic stress, caused by an accumulation of ions (often Na^+) inside the cytosol, causing damage to intracellular molecules (Munns & Tester, 2008). Osmotic stress can be somewhat mitigated through the production of organic molecules that adjust the osmotic potential

to allow for greater water uptake, which requires an expenditure of resources (Munns & Tester, 2008; Acosta-Motos et al., 2017). Tolerance to ionic stress often occurs by preventing accumulation of ions, such as Na^+, in the cytosol, where they have damaging effects. Mechanisms to do this include preventing the uptake of Na^+, as well as actively extruding Na^+ out of cells, and moving Na^+ into vacuoles, where these ions cause less damage (Munns & Tester, 2008).

Stress tolerance is also important to examine in this context due to its connection with longevity (Parsons, 1995, 2002b, 2003). Adaptive responses to stress include the upregulation of various protective and repair mechanisms (Milisav et al., 2012). Some mechanistic theories of senescence attribute it to an accumulation of damage over time (Harman, 1992; Kowald & Kirkwood, 1994; Lin & Beal, 2003; Gems & Doonan, 2009). The upregulation of mechanisms caused by stress can repair the damage that would normally result in senescence, thus taking a longer time to accumulate damage, and extending longevity (Milisav et al., 2012). There is overlap of some pathways involved in stress tolerance and longevity with a positive relationship, such that they are simultaneously affected (e.g., both increased) by manipulation of one pathway (Broughton et al., 2005; Vermeulen & Loeschcke, 2007; Prigione et al., 2010; Zuin et al., 2010; Shaposhnikov et al., 2015). This can result in a hormetic effect, in which a small stress results in an increase in longevity, while a larger stress results in a decrease (Mattson, 2008; Calabrese et al., 2015). The initiation of stress response mechanisms overcompensates for the small stress, and provides the benefit of protection against future damage (Rattan, 2008; Calabrese et al., 2015).

Some work has been done on the effect of parental age on stress tolerance, with varying results. A study in the red flour beetle (*Tribolium castaneum*) found that increasing parental age decreased offspring starvation tolerance, but had no effect on cold tolerance (Halle et al., 2015). Maternal age in a spider (*Argiope radon*) showed no effect on starvation tolerance, although it

increased with increasing egg sac order (Ameri et al., 2019). In *Drosophila melanogaster*, the effects of increasing parental age on offspring lifespan are strain-dependent (Priest et al., 2002), and examination of a strain exhibiting increasing offspring lifespan with older parents also showed that offspring tolerance to oxidative stress and heat shock stress increased with increasing parental age (Lee et al., 2019).

To examine the effects of parental age on offspring stress tolerance, I used the predominantly asexual, aquatic plant *Lemna minor* (common duckweed), which produce offspring at relatively regular intervals, allowing birth order to stand in as a proxy for parental age (Barks & Laird, 2015, 2016, 2018). Duckweed has exhibited senescence and changes in offspring fitness with increasing birth order and parental age in previous studies, typically as a hump-shaped pattern of offspring fitness, with an initial increase at early parental ages followed by a decline (Barks & Laird, 2016, 2018). It is important to note that the initial increase can be extremely subtle, especially when compared to the larger and more steady decline in older ages (Barks & Laird 2015). I hypothesized that offspring of differing birth orders, and therefore of differing parental age, would show different abilities to tolerate salt stress. While offspring of very high birth orders were predicted to have decreased fitness and stress tolerance, the possibility of a hump-shaped pattern at low to moderate birth orders prevented a hypothesis regarding the direction of the change in fitness and stress tolerance.

2.3 Materials and methods

2.3.1 Study species and growth conditions

Duckweeds are a group of closely related aquatic angiosperms within the Lemnoideae subfamily (Family Araceae), consisting of multiple species including *Lemna minor*, and are found

floating in slow-moving bodies of water (Landolt, 1986; Bog et al., 2010). Individual ramets consist of a leaf and stem structure called a frond, which has two meristematic pockets from which offspring are produced asexually. Offspring are attached to a parent via a stipe which breaks at maturity (Lemon & Posluszny, 2000), allowing a frond to be 'born'. A small amount of stipe tissue remains in the meristematic pocket, off of which the subsequent offspring is produced, resulting in a slow accumulation of stipe tissue (Lemon & Posluszny, 2000). Fronds produce offspring quickly and at relatively regular intervals, allowing birth order to stand in as an estimate of the age of the parent (Barks & Laird, 2015, 2016, 2018). A frond's birth order is a count of offspring produced by a parent up to the date of its detachment, and not pocket-specific, so a frond that is the N^{th} total offspring produced by its parent would have a birth order of N.

In this study, I used *L. minor* fronds obtained from the Canadian Phycological Culture Centre (CPCC 492; collected originally from Elk Lake, British Columbia, Canada, 48° 31′30″ N, 123° 23′18″ W; GenBank accession no. MG000373; MG000447). Fronds were grown individually in 60 x 15 mm (diameter x height) petri dishes containing 10 mL Schenk and Hildebrandt growth medium (S6765, Sigma-Aldrich, St. Louis, MO, USA), at one-eighth strength (0.4 g L^{-1}). Fronds were cultured axenically, and to maintain a constant environment, I transferred fronds to fresh growth medium weekly. Unlike some previous studies that supplemented the growth medium with sucrose, tryptone, and yeast extract to more easily detect microorganism contamination, I did not include additions to the medium. Nevertheless, I detected contamination by microorganisms in a number of petri dishes over the course of the study; I discarded these dishes. I kept fronds in growth chambers at 24˚C, under a 15:9 (light:dark) photoperiod with an average photosynthetic photon flux density of 15.20 μmol m^{-2} s^{-1} at plant height. Fronds were arranged in a randomized design in a Conviron E15 growth chamber (Controlled Environments Limited, Winnipeg, MB).

2.3.2 Treatments and sample sizes

I began with a total of 300 fronds, which were split among eight treatments. Treatments consisted of a combination of one of four salt (NaCl; S7653, Sigma-Aldrich, St. Louis, MO, USA) concentrations, crossed with one of two parental ages. I chose a range of concentrations – a control ("C": 0 g L^{-1}), low ("L": 1 g L^{-1}), medium ("M": 2 g L^{-1}), and high ("H": 4 g L^{-1}) concentration, adding salt to the growth medium before autoclaving. These concentrations were based on preliminary experiments, in which these concentrations affected frond fitness, lifespan and offspring production, without the lethality observed at higher concentrations. Birth order was used as a proxy for parental age, with first-born offspring considered to be born from young parents, and fifth-born offspring considered to come from older parents.

The fronds I took directly from stock culture had unknown ages, birth orders, and previous numbers of offspring produced, and were used as progenitor (P) fronds, which were marked with a speck of diluted, autoclaved India ink. The first offspring produced by a P frond had never produced any offspring, but was of an unknown birth order, and so was considered an unknown (P-U) frond. Upon detachment from its parent (considered its birth), I kept and marked the P-U fronds, and discarded the parents. Since I had observed them since their birth, I knew the first offspring produced by a P-U frond was the first offspring that frond had produced, with a birth order of 1 – this offspring was considered a first offspring (P-U-1), born to a young parent. By counting the number of offspring produced by a P-U frond, the offspring produced fifth (that is, having a birth order of 5) could be identified – this offspring was considered a fifth offspring (P-U-5), born to an older parent. To produce the first-born lineage, I grew P-U-1 fronds, and took the first offspring for 2 more generations, producing P-U-1-1-1 (birth order 1: "1") focal fronds. Three generations were used to ensure a consistent recent genealogy, as grandparental effects occur in

duckweeds (Barks & Laird, 2016, 2018). I produced the fifth-born lineage in a similar way, but using fifth offspring instead – I grew P-U-5 fronds, and took the fifth offspring for two more generations, producing P-U-5-5-5 (birth order 5: "5") focal fronds.

Each of the P-U-1-1-1 and P-U-5-5-5 fronds were placed into one of the four salt concentrations on the day they were born, giving the eight treatments – Control-First (C1), Control-Fifth (C5), Low-First (L1), Low-Fifth (L5), Medium-First (M1), Medium-Fifth (M5), High-First (H1), and High-Fifth (H5). Fronds remained in the appropriate salt concentration until they were determined to be dead, which was defined as the date their last offspring detached. Throughout their entire lifespan, I observed the fronds daily to record reproduction. I began with 35 fronds for each of the four first-born treatments, and 40 fronds for each of the four fifth-born treatments, anticipating greater sample loss from the longer time needed to produce fifth offspring. The number of focal fronds produced was less than the original starting number for all treatments with a mean final sample size of 27.75 fronds (ranging from 23-33; Table 2.1), owing to both contamination by microorganisms, and parents producing fewer than five total offspring during the production of focal fronds.

2.3.3 Frond size

I photographed each focal frond after it had detached its last offspring, using a microscope-mounted digital camera. I placed fronds between a microscope slide and cover and applied gentle pressure to flatten them before they were photographed. The images were taken under conditions in which frond edges were in focus, and there was a high contrast between the frond and the background, to optimize the shape analysis. Damaged fronds were excluded from analysis (n = 17). I determined frond area and perimeter from these images using code developed by

Ankutowicz and Laird (2018). All image analysis was performed in MATLAB (version R2016a, The MathWorks Inc., Natick, USA, 2016).

2.3.4 Sample preparation and analysis by flame atomic absorption spectroscopy

After their last offspring had detached, fronds were individually rinsed with deionized water. I placed rinsed fronds in foil containers to dry at 60 ˚C until a constant weight was achieved, signifying fronds were completely dry. To achieve a minimum dry weight, I pooled six individual whole plants from the same treatment together to form one sample. Fronds comprising a pool were randomly selected from within each treatment. I used an analytical balance accurate to the hundredth of a milligram to record the weight of each sample. I digested samples in 2 mL high-density polyethylene conical vials, using 12 N trace-metal grade nitric acid at a ratio of 1 mg of dried tissue to 10 µL of acid. Samples were digested over 3 hours at 80 ˚C. Following digestion, I allowed samples to cool to room temperature before diluting them at a ratio of 1 mL acid to 10 mL water (utilizing UltraPure Millipore water). Samples were then stored at 4 ˚C until analysis.

I quantified the amount of sodium within samples by flame atomic absorption spectroscopy utilizing an Agilent 240FS (Agilent Technologies, USA). A standard curve was established by the dilution of a certified sodium standard (SCP SCIENCE, Product #140-001-111) to 200 µg L^{-1}, 400 µg L^{-1}, and 800 µg L^{-1} sodium. The standard curve was re-sloped after every ten samples, and recalibrated every twenty samples. I ran the flame atomic absorption spectroscopy at SpectraAA (Agilent software) factory settings with the minor adjustments of setting the lamp current to 8.0 mA, and limiting the slit width to 0.5 nm. I read samples at a wavelength of 589.0 nm. To verify the standard curve, SLRS-6 (River Water Certified Reference Material for Trace Metals and other

Constituents, National Research Council Canada) was tested, and sodium concentrations read within 10% of accepted values for the reference material.

2.3.5 Data analysis

I performed a two-way ANOVA to compare the effects of salt concentration, birth order, and their interaction on the intrinsic rate of natural increase (*r*) measured at the level of focal fronds (McGraw & Caswell, 1996) as a primary measure of fitness. To identify the sources of differences in fitness, I performed two-way ANOVAs on the time to first reproduction, total number of offspring, and lifespan. I also used two-way ANOVAs to measure tissue concentration of Na^+, dry mass, area, and perimeter. I assessed normality and homoscedasticity with residual-by-predicted plots and normal quantile-quantile plots, and found that some variables exhibited skew and/or heteroscedasticity. I performed transformations on these variables, so data would meet the assumptions of ANOVA. The latency data were ln(x+1) transformed due to the presence of '0' values in the data, tissue concentration of Na^+ was square-root transformed ($\sqrt{x}$), area data were squared (x^2), and perimeter data were ln(x) transformed. I was unable to transform the *r* data to satisfactorily meet the assumptions of an ANOVA. Instead, I performed a permutation test, which was a non-parametric alternative, and so did not require assumptions of normality and homoscedasticity.

To examine differences among salt concentrations within each birth order treatment, I performed post-hoc contrasts. I used pairwise t-tests corrected for multiple comparisons using the Benjamini and Hochberg method within each birth order treatment. This test was not appropriate for the intrinsic rate of increase data, which did not meet the assumptions of normality and

homoscedasticity. Instead, I used non-parametric pairwise Wilcoxon rank sum tests, corrected for multiple comparisons using the Benjamini and Hochberg method.

I calculated lifespan from the date a frond detached from its parent, to the date of detachment of its last offspring. Some fronds had offspring that did not detach at the end of their life, although they sometimes produced grand-offspring. I excluded these non-detaching offspring when determining reproductive lifespan, as alternative methods of determining death were not possible, but included them in the total offspring number, as they still contributed to future generations. To facilitate statistical analysis, reproduction data were analysed in a binary manner, in which reproduction either did or did not occur on a particular date, as per Barks and Laird (2015). Multiple reproduction events were uncommon (0.8% of all reproductive events), and were not further examined as a separate phenomenon.

To examine senescence, I fit the daily proportion of surviving fronds to exponential, Weibull, Gompertz, and logistic models for each treatment (Pletcher et al., 2000; Ricklefs & Scheuerlein, 2002; Barks & Laird, 2015). Unlike the Weibull, Gompertz, and logistic models, the exponential model has a constant survival with age, which implies no senescence. I used the Akaike Information Criterion corrected for small sample sizes (AICc) to select the best model, with $\Delta AICc < 2$ indicating a fit indistinguishable from the 'best' model. I analysed the probability of reproduction using a generalized estimating equation (GEE) approach coupled with a Wald's test. This approach accounted for the possibility that individual fronds were unlikely to reproduce on consecutive days, potentially resulting in autocorrelation in the data. I tested three correlation structures for each treatment: an 'independent' correlation structure, a first-order autoregressive (AR1) correlation structure, and an exchangeable correlation structure. In cases where the definite covariation matrix was not positive, this correlation structure was ruled out, as suggested in Shults

et al. (2009). The correlation structures were compared using the Rotnitzky-Jewel (RJ) criteria (Rotnitzky & Jewell, 1990).

All analyses were done in R v. 3.6.3 (R Core Team, 2020).

2.4 Results

2.4.1 Life history and physiological traits

Increasing salt concentration significantly decreased the intrinsic rate of increase of fronds (Figure 2.1a; permutation test, $F_{3, 206} = 20.93$, $p = 2.00 \times 10^{-4}$). Post-hoc tests showed that all salt concentrations within the first-born treatment had significantly different intrinsic rates of increase from each other. All salt concentrations within the fifth-born treatment also had significantly different intrinsic rates of increase from each other, with the exception of the control and low concentrations (Figure 2.1a). The intrinsic rate of increase was significantly increased with the fifth-born fronds (Figure 2.1a; permutation test, $F_{1, 206} = 42.71$, $p = 2.00 \times 10^{-4}$), and there was no significant interaction effect (Figure 2.1a; permutation test, $F_{3, 206} = 1.01$, $p = 0.40$).

Fronds from the first-born treatment had a significantly longer time to first reproduction than fifth-born fronds (Figure 2.1b; two-way ANOVA, $F_{1, 203} = 104.55$, $p < 2.00 \times 10^{-16}$). There was also a significant effect of salt concentration (Figure 2.1b; two-way ANOVA, $F_{3, 203} = 11.15$, $p = 8.23 \times 10^{-7}$), and an interaction effect on time to first reproduction (Figure 2.1b; two-way ANOVA, $F_{3, 203} = 7.90$, $p = 5.22 \times 10^{-5}$). First-born fronds showed an increasing time to first reproduction with increasing salt concentration. Post-hoc tests found the medium salt concentration had a significantly longer time to first reproduction than the control and low concentrations, and the high concentration had a significantly longer time than all other concentrations. In the fifth-born birth order treatment, the time to first reproduction remained

similar among all salt concentrations, which showed no significant differences in post-hoc tests (Figure 2.1b).

Offspring number was significantly lower in fronds exposed to high salt concentrations, compared to the other concentrations (Figure 2.1c; two-way ANOVA, $F_{3, 203} = 114.13$, $p < 2.00 \times 10^{-16}$). Birth order did not have a significant effect (Figure 2.1c; two-way ANOVA, $F_{1, 203} = 0.042$, $p = 0.84$), but there was a significant interaction effect (Figure 2.1c; two-way ANOVA, $F_{3, 203} = 3.09$, $p = 0.028$). Within the first-born fronds, post-hoc tests showed the low salt concentration had a significantly higher number of offspring than the control and medium salt concentrations, and a significantly lower number of offspring in the high salt concentration. Within the fifth-born birth order treatment, there was no significant difference in offspring number for the control, low, and medium salt concentrations, but the high salt concentration had a significantly lower number (Figure 2.1c).

Lifespan was significantly affected by salt concentration, with low and medium concentrations increasing lifespan, and the high concentration decreasing lifespan, compared to the control (Figure 2.1d; two-way ANOVA, $F_{3, 203} = 53.92$, $p < 2.00 \times 10^{-16}$). There was no significant effect of birth order (Figure 2.1d; two-way ANOVA, $F_{1, 203} = 0.11$, $p = 0.75$), or interaction effect of salt treatment and birth order (Figure 2.1d; two-way ANOVA, $F_{3, 203} = 1.47$, $p = 0.22$) on lifespan. Post-hoc tests found no significant difference between the low and medium salt concentrations, but a significant difference between all other concentrations, in both the first- and fifth-born birth order treatments (Figure 2.1d).

The tissue concentration of Na^+ significantly increased with salt concentration (Figure 2.2a; two-way ANOVA, $F_{3, 25} = 27.38$, $p = 4.57 \times 10^{-8}$). Post-hoc tests found a significant increase in tissue concentration from the control to the low treatment, and no significant difference between

the low and medium treatments. There was a significant increase in tissue concentrations from the medium to high treatments. In the first-born birth order treatment, the low and high salt concentrations showed no significant difference (Figure 2.2a). There was no significant effect of birth order (Figure 2.2a; two-way ANOVA, $F_{1, 25} = 0.87$, $p = 0.36$), nor a significant interaction effect (Figure 2.2a; two-way ANOVA, $F_{3, 25} = 0.70$, $p = 0.56$).

The dry mass of pooled fronds showed a significant effect of salt concentration (Figure 2.2b; two-way ANOVA, $F_{3, 25} = 25.28$, $p = 9.69 \times 10^{-8}$), birth order (Figure 2.2b; two-way ANOVA, $F_{1, 25} = 5.67$, $p = 0.025$), and an interaction effect (Figure 2.2b; two-way ANOVA, $F_{3, 25} = 8.51$, $p = 4.56 \times 10^{-4}$). Within the first birth order treatment, dry mass increased with increasing salt concentration. Fifth-born fronds showed no significant differences among salt treatments with the exception of the high concentration, which was significantly higher than the dry masses of the low and medium concentrations, although not the control. First-born fronds had a greater dry mass than fronds from the fifth birth order when exposed to any salt, though this trend was reversed in the control treatment (Figure 2.2b).

First-born fronds were significantly larger than fifth-born, both in surface area (Figure 2.2c; two-way ANOVA, $F_{1, 189} = 165.91$, $p < 2.00 \times 10^{-16}$), and perimeter (Figure 2.2d; two-way ANOVA, $F_{1, 189} = 22.85$, $p = 3.51 \times 10^{-6}$). Surface area showed a significant effect of salt concentration (Figure 2.2c; two-way ANOVA, $F_{3, 189} = 4.11$, $p = 7.52 \times 10^{-3}$), and interaction effect (Figure 2.2c; two-way ANOVA, $F_{3, 189} = 4.03$, $p = 8.25 \times 10^{-3}$). First-born fronds were larger with increasing salt concentration, with a significant increase from the control to low concentration, no difference between the low and medium salt concentrations, and a significant increase from the medium to high concentrations. Fifth-born fronds showed no significant differences in surface area, regardless of salt concentrations (Figure 2.2c). There was a significant effect of salt

concentration on perimeter (Figure 2.2d; two-way ANOVA, $F_{3, 189} = 5.91$, $p = 7.07 \times 10^{-3}$), but no significant interaction effect (Figure 2.2d; two-way ANOVA, $F_{3, 189} = 0.17$, $p = 0.92$). Perimeter increased with increasing salt concentration from the control to medium concentrations, followed by a decrease in the high salt concentration. Post-hoc tests found a significant difference in perimeter between the control and medium concentrations within the first-born birth order treatment, but no significant differences within the fifth-born treatment (Figure 2.2d).

2.4.2 Senescence

Almost all treatments showed declines in daily survival with increasing age (Figure 2.3), with the exception of the H5 treatment. The exponential model, which indicates no senescence, was one of three co-best fitting models for this group ($\Delta AICc < 2$; Table 2.2). All other groups had weaker support for the exponential model ($\Delta AICc = 7$ for H1, and $\Delta AICc > 15$ for all other treatments). The Gompertz model was found to have the best fit (lowest $\Delta AICc$) for the M5 group, but in all other cases, the Weibull model was found to have a co-best fit ($\Delta AICc < 2$) with either the Gompertz or the logistic model fitting equally well ($\Delta AICc < 2$; Table 2.2).

The working correlation structures for models of the probability of reproduction were not unique in half of the treatments, when using the RJ criteria. The AR-1 model was found to be best, or co-best in all treatments except the high salt (H) treatments, for which the independent working correlation structure was the best. Nevertheless, the best-fit lines for all working correlation structures overlapped in each treatment indicating little difference between models, with the exception of L1. The RJ criteria found that the AR-1 and exchangeable working correlations structures could not be separated in the L1 treatment, and the lines for these models were visibly different (Figure 2.4b). As models were indistinguishable for most treatments, the AR-1 model

was selected based on biological relevance (i.e., negative temporal autocorrelations between successive reproduction events), although the exchangeable model is also reported for the L1 treatment. The probability of reproduction showed a decline with increasing age in all treatments except H1 (Figure 2.4) when examined with the AR-1 model. Declines in probability of reproduction according to the AR-1 model were significant for all treatments that exhibited them (Table 2.3). The change in probability of reproduction of the L1 treatment was significant for both the AR-1 and exchangeable models (Table 2.3), although the exchangeable model shows an increase in probability of reproduction with increasing birth order (Figure 2.4b).

2.5 Discussion

2.5.1 Effects of salt concentration on fitness

As expected, higher salt concentrations resulted in decreased fitness of fronds, as measured by the intrinsic rate of natural increase (Figure 2.1a). The highest salt treatment was the most detrimental, significantly reducing the total offspring number and lifespan (Figure 2.1), which were the components responsible for the decrease in fitness of the fronds in the high salt concentration. In addition to a shorter lifespan, the high salt treatment affected the survival of fronds (Figure 2.3d, h), and the timing and trajectory of reproduction. Detrimental effects of initial reproduction on fitness were reflected in the slower time to first reproduction of the H1 treatment (Figure 2.1b), and the low proportions of fronds reproducing at age 1 in both high salt treatments (Table 2.3).

As fitness decreased with increasing salt concentration, fronds in the low and medium concentrations of salt showed fitnesses between those of fronds in the control and high salt treatments (Figure 2.1a). Unlike fronds in the high salt treatment, the components responsible for

the decrease in fitness were less obvious. The offspring number and lifespans of fronds in the low / medium treatments were the same or greater than those observed for the control (Figure 2.1c, d), and the trajectories of survival indicate fronds in these treatments survived for longer than those in the control (Figure 2.3). The time to first reproduction was significantly slower only for fronds in the M1 treatment (Figure 2.1b), and would not have decreased fitness for any other group. The components responsible for the decreases in fitness of fronds in the low and medium salt treatments were the proportion of fronds reproducing at age 1, which decreased with increasing salt concentration, and the smaller declines in probability of reproduction over the maximum lifespan, compared to the control (Table 2.3, Figure 2.4).

The higher fitness of fronds in the control treatments compared to those in the low, medium, and high treatments indicates that the schedule of reproduction was highly important. The low and medium salt treatments showed an increase in offspring number, lifespan, and a high probability of survival with age compared to the control, which all seem beneficial, yet they had a decreased fitness. The time to first reproduction does not fully explain this decrease. It is the initial proportion of fronds reproducing at age 1 that is responsible for the decreases in fitness seen for fronds in the low and medium salt treatments, as well as in the high, although other components also contribute in the latter case. In this study, the production of offspring early in life is more important to fitness than the number of offspring produced, longevity, or a high probability of survival with age – a sensible conclusion given the benefits of early reproduction (Stearns, 1992).

The steady decline of fitness with increasing concentration of salt is a pattern that is expected with toxic substances – effects are stronger with greater amounts. Declines are observed in all components of fitness in the high salt treatment, but surprisingly, the mean lifespans of the low and medium concentrations show an increase (Figure 2.1c, d). This is a phenomenon known

as longevity hormesis (Rattan, 2004; Mattson, 2008; Rattan, 2008; Calabrese et al., 2015). The ability to tolerate stress is connected with longevity and decreased senescence (Parsons, 1995; Hunt et al., 2011). As some mechanistic theories attribute senescence to an accumulation of damage over time, an increase in protective maintenance and repair mechanisms caused by a stress could also repair the normal damage that would eventually result in senescence (Parsons, 1995; Rattan, 2008; Milisav et al., 2012). Once the stress becomes too large, the compensatory actions of these stress response mechanisms are overwhelmed, resulting in a drop in survival and reproduction. However, investigation of specific mechanisms in the *L. minor* stress response that could cause such a response are beyond the scope of this study.

2.5.2 Salt stress

Salt stress is composed of two parts - osmotic stress, in which a physiological water deficit is caused by a high concentration of ions outside of the plant, and ionic stress, in which high ion concentrations inside cells cause damage. Osmotic stress occurs more immediately, as ionic stress requires time for ions to accumulate within cells, and osmotic stress is often considered responsible for decreases in growth and reproduction, similar to drought stress (Munns & Tester, 2008). However, the osmotic and ionic effects of salt stress can occur simultaneously, making them difficult to separate. Due to the immediate effect, it is likely that the lower probability of reproduction observed in salt treatments (Figure 2.4) was caused by osmotic stress, and decreased availability of water within cells. Salt is known to affect plant sexual reproduction, although the direction of the effect depends on species and concentration of salt (Pitman & Läuchli, 2002; Van Zandt et al., 2003; Bybordi, 2010). Asexual reproduction is also affected by salt, with an examination in hygrophilous plants done by Cheng et al. (2018). They found that low to moderate

concentrations increased the number of reproductive events in several species, while higher concentrations decreased them, which was attributed to osmotic stress and disruption of ion homeostasis.

As the salt concentrations of treatments increased, there were increases in whole-plant tissue concentrations of Na^+ (Figure 2.2c). Salt tolerance mechanisms of plants commonly prevent the accumulation of Na^+ ions in the cytosol where they can damage important molecules. These mechanisms include preventing the uptake of Na^+ into cells, active extrusion of Na^+ from plant cells, and compartmentation of Na^+ into vacuoles (Munns & Tester, 2008). Fronds in the low and medium salt concentrations were able to prevent more accumulation of Na^+ within their cells, as compared to the high salt treatment. The high whole-plant tissue concentrations with increasing salt treatments indicates that the exclusion or extrusion of Na^+ ions from cells was unable to prevent accumulation within cells. It is likely that some Na^+ was moved into vacuoles, although the data collected cannot show where in the cell that Na^+ is found. It is reasonable to surmise that there was at least some accumulation of Na^+ within the cytosol, based on the high concentrations in the treatment and tissue, along with the strong detrimental effects observed.

2.5.3 Effects of birth order on fitness

Fifth-born offspring had a higher fitness than first-born offspring, as measured by the intrinsic rate of increase (Figure 2.1a). This difference in fitness is attributable to the difference in time to first reproduction, which was slower for first-born fronds (Figure 2.1b). A previous study in the same strain showed a very slight increase in offspring fitness at a young parental age, followed by a decline with increasing parental age (Barks & Laird, 2015). Similar results were also reported in closely related *L. turionifera*, in which the fitness of offspring initially increased

to a maximum at the fifth total offspring produced (reported there as a pocket-specific birth order of 3), before declining with increasing birth order thereafter (Barks and Laird 2016, 2018). Whether and why there is an initial increase in offspring fitness with increasing birth order / parental age, followed by a long decline, is worthy of further study. In particular, it would be instructive to examine the effects of stress on offspring of a greater birth order than tested in the current study.

The declines in survival and reproduction with increasing age that characterize demographic senescence are due to the weakening in the force of natural selection with age (Hamilton, 1966). Offspring quality may show similar declines with age, that further weaken the force of natural selection at greater ages (Kern et al., 2001; Priest et al., 2002; Pavard et al., 2007; Gillespie et al., 2013b; Barks & Laird, 2020). Why, then, would we see an increase in fitness with increasing parental age, in a species such as duckweed that exhibits senescence? It seems reasonable to assume that the increase in fitness from the first-born treatment to the fifth-born is due to a hump-shaped pattern with increasing parental age, similar to that found by Barks and Laird (2016), with maximal fitness found at the fifth total offspring produced. This shape is reminiscent of the hump-shaped fertility trajectories commonly found in mammals, in which fertility increases as individuals reach maturity, before the senescent decline (Gage, 2001). Older parents can be larger than younger parents, and often produce higher-quality offspring due to greater ability to provision offspring (Marshall et al., 2010). In *L. minor*, the declines in offspring quality with increasing birth order were suggested to be caused by the accumulation of stipe tissue in meristematic pockets after offspring detach (Lemon & Posluszny, 2000), which would increase after each detachment occurred, and could result in structural changes in the developmental environment of offspring (Barks & Laird, 2015, 2016). However, this did not explain the initial

increase in fitness observed by Barks and Laird (2016), who noted that the developmental environment of the first offspring also differs from that of later-born offspring, in that they develop while their parent remains attached to its own parent. This differing developmental environment should only be applicable for the first two offspring produced. Fronds typically do not detach offspring until they themselves have detached, and produce only one offspring per meristematic pocket at a time (Lemon & Posluszny, 2000), meaning only two offspring will experience this different developmental environment. Offspring fitness increased up to the fifth offspring born, leaving the increased fitness of the third to fifth-born offspring unaccounted for. Fronds are considered to be full-grown and mature upon detachment from their parent (Landolt, 1986). However, *L. minor* has a very simple structure, with little visible difference between a mature and immature frond besides size, which is an ambiguous indicator. Perhaps this assumption is incorrect, and the initial increase in offspring fitness is related to the full maturation of the parent. If different conditions affect the speed at which the fronds mature, this could also explain why there is a difference in the results of this study, and that by Barks and Laird (2015).

Alternatively, perhaps birth order of offspring is used to generate variation in unpredictable environments. Birth order in the duckweed species *Spirodela polyrhiza* was found to generate differences in turion phenology as a bet-hedging strategy, in order to ensure that some turions are produced at the optimal time for maximal fitness, given the unpredictable timing of freezing in the winter (Mejbel & Simons, 2018), as well as for turion reactivation in the spring (Morris et al., 2020). Bet-hedging is a way of reducing risk and increasing long-term fitness in unpredictable environmental conditions through diversification, or the expression of conservative 'safe' traits, which can decrease fitness in the short-term (Philippi & Seger, 1989; Simon & Meyers, 2011). If offspring of differing birth orders have a greater fitness under different conditions, this variation

would allow at least some of these offspring to survive no matter what conditions are present, thus reducing the risk to the genet. While salt tolerance does not change with differing birth orders, offspring fitness appears to, although the specific pattern is still unclear, given the differences between this study and Barks and Laird (2015). Fitness is highly dependent on environment (Stearns, 1992; Winkler & Fischer, 1999), and perhaps the conditions of this study allowed the fifth-born offspring to maximize their fitness, whereas the conditions of Barks and Laird (2015) allowed offspring of an earlier birth order to maximize fitness. Differences from this study include the light intensity, photoperiod, and medium used. Further studies should be done to explore these possibilities.

2.5.4 Interactive effects of birth order and salt concentration

There was no significant interaction effect of salt concentration and birth order on fitness, as measured by the intrinsic rate of increase (Figure 2.1a). Post-hoc tests showed a significant decrease in fitness from the C1 to L1 groups, but no significant difference between the C5 and L5 groups (Figure 2.1a), suggesting that a low concentration of salt did not affect the fitness of fifth-born fronds. In the high salt concentration, the H5 group had a large variability in fitness, showing that there is variability in the tolerance of a strong stress in this group. While the variability of fronds in the H5 group was the greatest, all of the groups within the fifth-born birth order treatment showed a noticeably greater variability in fitness than first-born fronds.

Despite this lack of interactive effect on fitness, first-born offspring were more sensitive to higher salt concentration in some of the traits measured. First-born offspring required a significantly longer time to produce their own first offspring with medium or high concentrations of salt, while fifth-born offspring showed no significant differences with increasing salt (Figure

2.1b). The ability to reproduce early in life is advantageous, with offspring produced earlier being more contributory towards fitness than those produced later, as they are able to begin their own reproduction earlier and contribute more strongly to the growth of the population (Fisher, 1930; Stearns, 1976). This slowing of reproduction at higher salt concentrations allowed fronds to grow to a greater size, as both dry mass and frond area showed increases within the first-born treatment (Figure 2.2b, c). A greater surface area in first-born offspring is consistent with Barks and Laird (2015), although Barks and Laird (2016) found that fronds increased in size up to the fifth total offspring produced before declining. As fronds in this study were from the same strain as the 2015 study, perhaps birth order-related declines in size are consistent in this strain, rather than being affected by environment. Dry mass increased with increasing salt concentration in the first-born treatment, but within the fifth-born treatment, only the H5 group showed a significant increase (Figure 2.2c).

Size is often used as an indicator of fitness and reproductive ability in plants (Younginger et al., 2017), but it is unclear if size plays a role in the fitness of *L. minor*. It has been shown that larger frond sizes gives a greater competitive ability in resource-limited conditions than increased reproduction, although this was unexpected, since there is no obvious benefit for a larger size in duckweed (Vasseur et al., 1995). Fronds in this study were grown individually, without any competition from others. The increase in size with increasing salt concentration for first-born fronds is likely due to an accumulation of starch. Salt is known to result in the accumulation of starch in duckweed, including *L. minor* (Sree & Appenroth, 2014; Sree et al., 2015). Starch may act as an osmoregulator, counteracting the effects of osmotic stress caused by the salt (Adem et al., 2014; de Morais et al., 2018). In this study, however, the increased size did not have an observable benefit on fitness.

2.6 Conclusions

Increasing salt concentrations detrimentally affect fitness in *L. minor*, with no apparent interaction of birth order. Low and medium concentrations exhibit hormesis, showing an increase in lifespan and offspring number. The early production of offspring was the most important component of fitness. Birth order affected response to stress, with first-born offspring producing their first offspring more slowly, in order to increase their size. Fifth-born offspring were less sensitive to increasing concentrations of salt, with no change in time taken to produce a first offspring, and almost no effect on size.

2.7 Tables

Table 2.1: Sample sizes of each treatment before birth order selection, the number of focal fronds produced, and final sample sizes.

Treatment	Initial n	Total focal fronds	Final sample size
C1	35	32	30
L1	35	31	29
M1	35	32	27
H1	35	34	30
C5	40	26	25
L5	40	33	33
M5	40	28	25
H5	40	27	23
	Mean:	30.375	27.75

Table 2.2: Comparison of four survival models for each treatment. The model considered best has the lowest AICc and ΔAICc values, although models with ΔAICc < 2 are considered to fit equally well. The best model(s) for each treatment are bolded.

Treatment	Model	No. parameters	Deviance	AICc	ΔAICc	AICc weight
C1	Exponential	1	317	319	23.95	4.22×10^{-6}
	Weibull	**2**	**291**	**295**	**0.00**	**0.670**
	Gompertz	2	296	300	4.68	6.46×10^{-2}
	Logistic	**3**	**290**	**297**	**1.85**	**0.266**
L1	Exponential	1	332	334	58.44	1.20×10^{-13}
	Weibull	**2**	**271**	**275**	**0.00**	**0.585**
	Gompertz	**2**	**272**	**277**	**1.34**	**0.300**
	Logistic	3	272	279	3.26	0.115
M1	Exponential	1	306	308	47.846	1.84×10^{-11}
	Weibull	**2**	**256**	**261**	**0.297**	**0.390**
	Gompertz	**2**	**256**	**261**	**0.00**	**0.452**
	Logistic	3	256	263	2.095	0.159
H1	Exponential	1	221	223	7.040	1.48×10^{-2}
	Weibull	**2**	**212**	**216**	**0.00**	**0.500**
	Gompertz	**2**	**212**	**217**	**0.608**	**0.369**
	Logistic	3	212	219	2.918	0.1162
C5	Exponential	1	267	269	15.62	2.20×10^{-4}
	Weibull	**2**	**249**	**253**	**0.00**	**0.54288**
	Gompertz	2	252	257	3.53	9.297×10^{-2}
	Logistic	**3**	**247**	**254**	**0.80**	**0.36392**
L5	Exponential	1	368	370	52.53	2.67×10^{-12}
	Weibull	**2**	**313**	**317**	**0.00**	**0.680**
	Gompertz	2	318	323	5.27	4.87×10^{-2}
	Logistic	**3**	**312**	**319**	**1.84**	**0.271**
M5	Exponential	1	285	287	24.43	4.74×10^{-6}
	Weibull	2	267	272	9.73	7.35×10^{-3}
	Gompertz	**2**	**258**	**262**	**0.00**	**0.954**
	Logistic	3	262	269	6.42	3.85×10^{-2}
H5	**Exponential**	**1**	**192**	**194**	**0.00**	**0.409**
	Weibull	2	192	196	2.029	0.148
	Gompertz	**2**	**191**	**195**	**0.992**	**0.249**
	Logistic	**3**	**188**	**196**	**1.499**	**0.193**

Table 2.3: Results of the Wald tests done for probability of reproduction for each treatment, probability of reproduction at age 1, and the change in probability over the maximum age for the treatment. The AR-1 working correlation structure was favoured based on its biological relevance, and lack of visible distinction from other models in all treatments except L1. Bolded p-values indicate significance ($p < 0.05$).

Treatment	Working correlation structure	Probability of reproduction at age 1	Change in probability of reproduction between age 1 and maximum age of treatment	χ^2	df	p
C1	AR-1	0.16	-0.11	108.0	1	$\mathbf{2.0 \times 10^{-16}}$
L1	AR-1	0.12	-0.074	74.2	1	$\mathbf{2.0 \times 10^{-16}}$
	Exchangeable	0.084	0.078	6.2	1	**0.013**
M1	AR-1	0.10	-0.060	61.8	1	$\mathbf{3.8 \times 10^{-15}}$
H1	AR-1	0.029	0.085	6.9	1	$\mathbf{8.5 \times 10^{-3}}$
C5	AR-1	0.16	-0.11	25.9	1	$\mathbf{3.6 \times 10^{-7}}$
L5	AR-1	0.13	-0.092	36.9	1	$\mathbf{1.3 \times 10^{-9}}$
M5	AR-1	0.099	-0.061	16.5	1	$\mathbf{4.8 \times 10^{-5}}$
H5	AR-1	0.082	-0.044	1.6	1	0.21

2.8 Figures

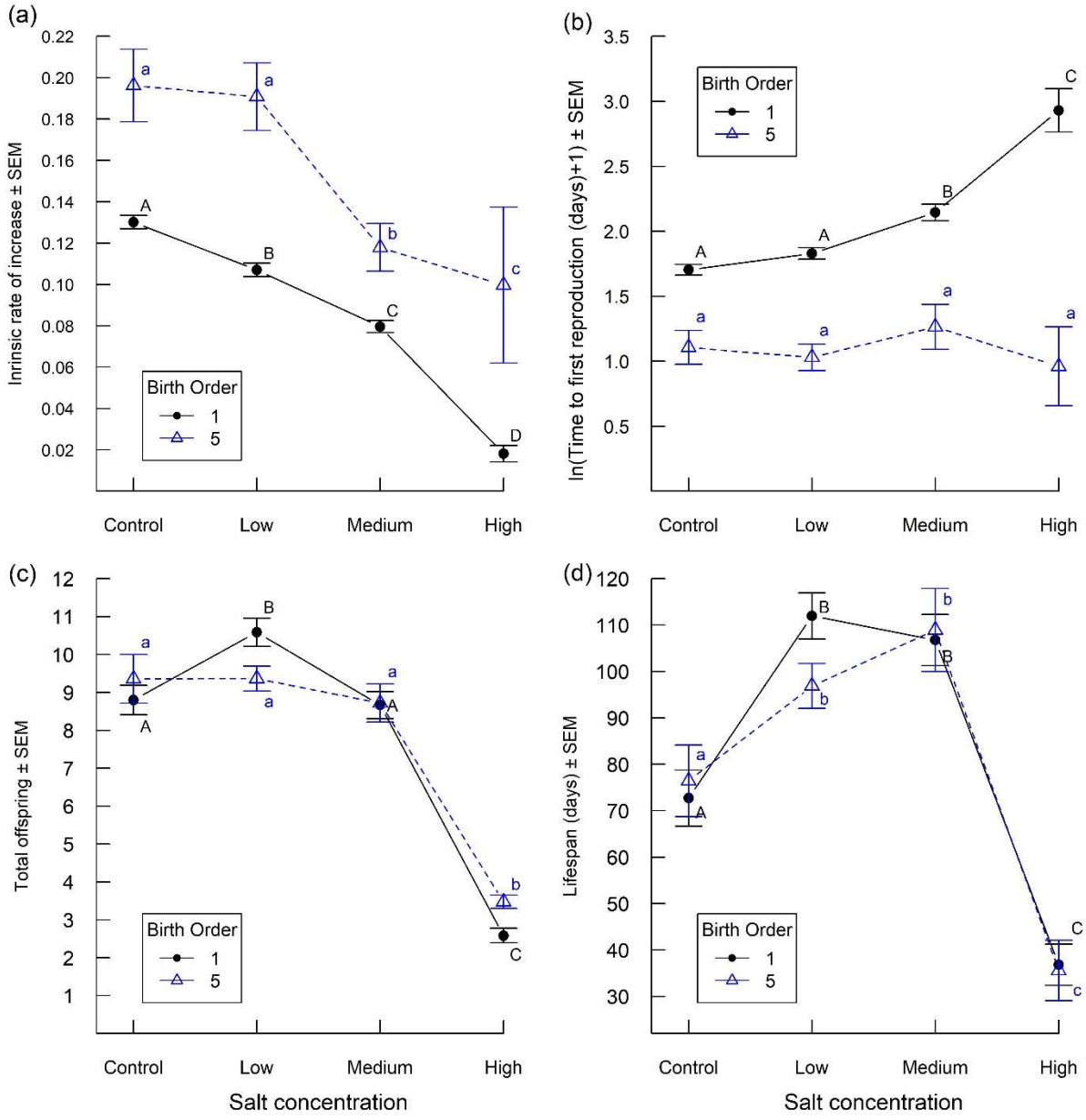

Figure 2.1: Interactive effects of salt concentration (horizontal axis; Control = 0 g L^{-1}, Low = 1 g L^{-1}, Medium = 2 g L^{-1}, and High = 4 g L^{-1}) and birth order (black circles = birth order 1-1-1; blue triangles = birth order 5-5-5) on (a) mean intrinsic rate of natural increase (r), (b) ln-transformed mean time to first reproduction, (c) mean total offspring number, and (d) mean lifespan. Error bars indicate one standard error of the mean. Means within each birth order treatment sharing the same letter are not significantly different ($p < 0.05$) according to post-hoc tests. Pairwise t-tests corrected for multiple comparisons were performed for (b) - (d). Pairwise Wilcoxon tests corrected for multiple comparisons were performed for (a), due to the non-normal distribution of data.

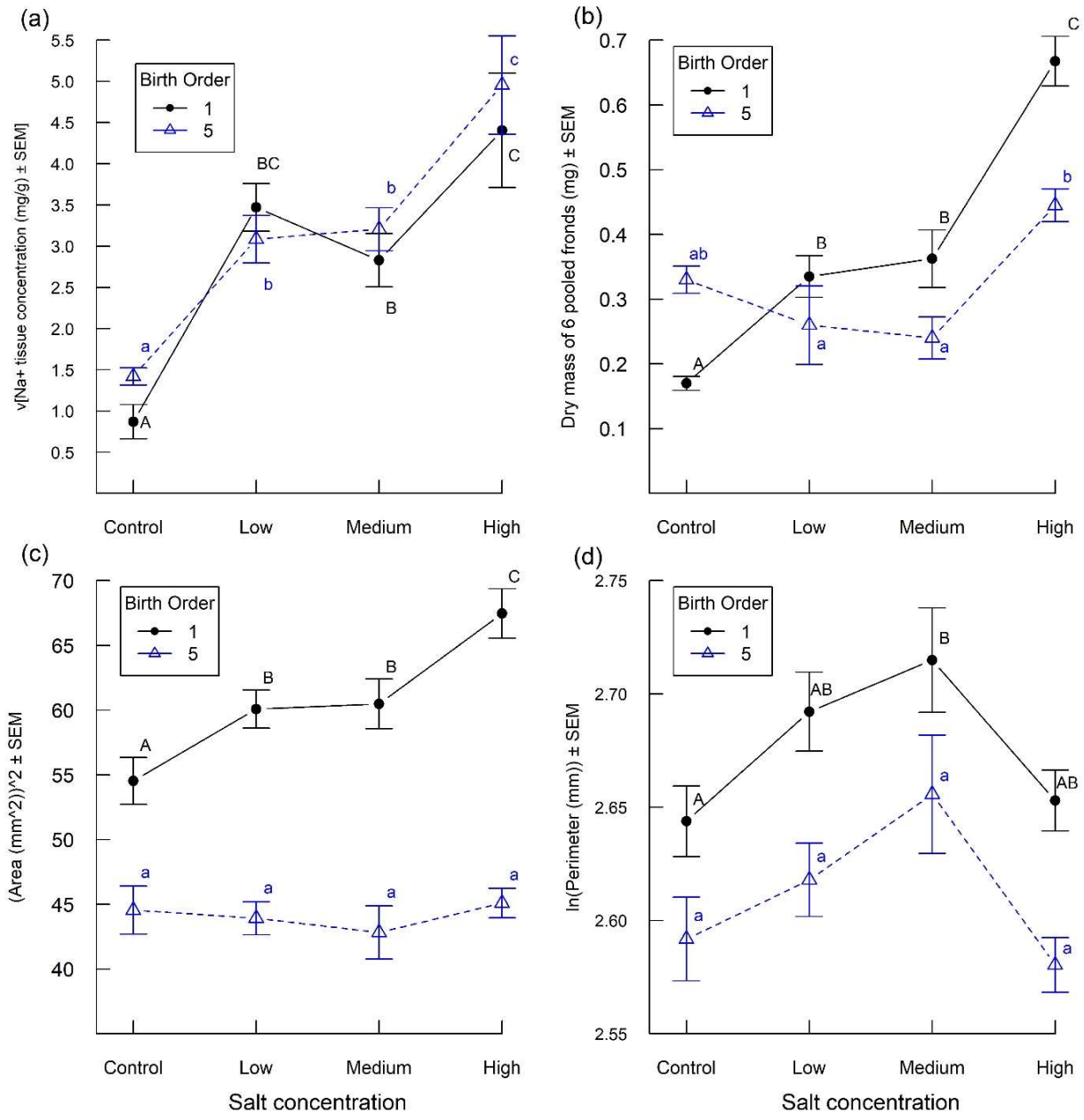

Figure 2.2: Interactive effects of salt concentration (horizontal axis; Control = 0 g L^{-1}, Low = 1 g L^{-1}, Medium = 2 g L^{-1}, and High = 4 g L^{-1}) and birth order (black circles = birth order 1-1-1; blue triangles = birth order 5-5-5) on (a) whole-plant tissue concentration of Na+, (b) dry mass of 6 pooled fronds, (c) frond surface area, and (d) frond perimeter. Error bars indicate one standard error of the mean. Means within each birth order treatment sharing the same letter are not significantly different ($p < 0.05$) according to post-hoc pairwise t-tests corrected for multiple comparisons. Fronds for (a) and (c) were pooled into samples consisting of six fronds, giving n = 4 for each combined treatment, with the exception of L5 (Low-Fifth), for which n = 5.

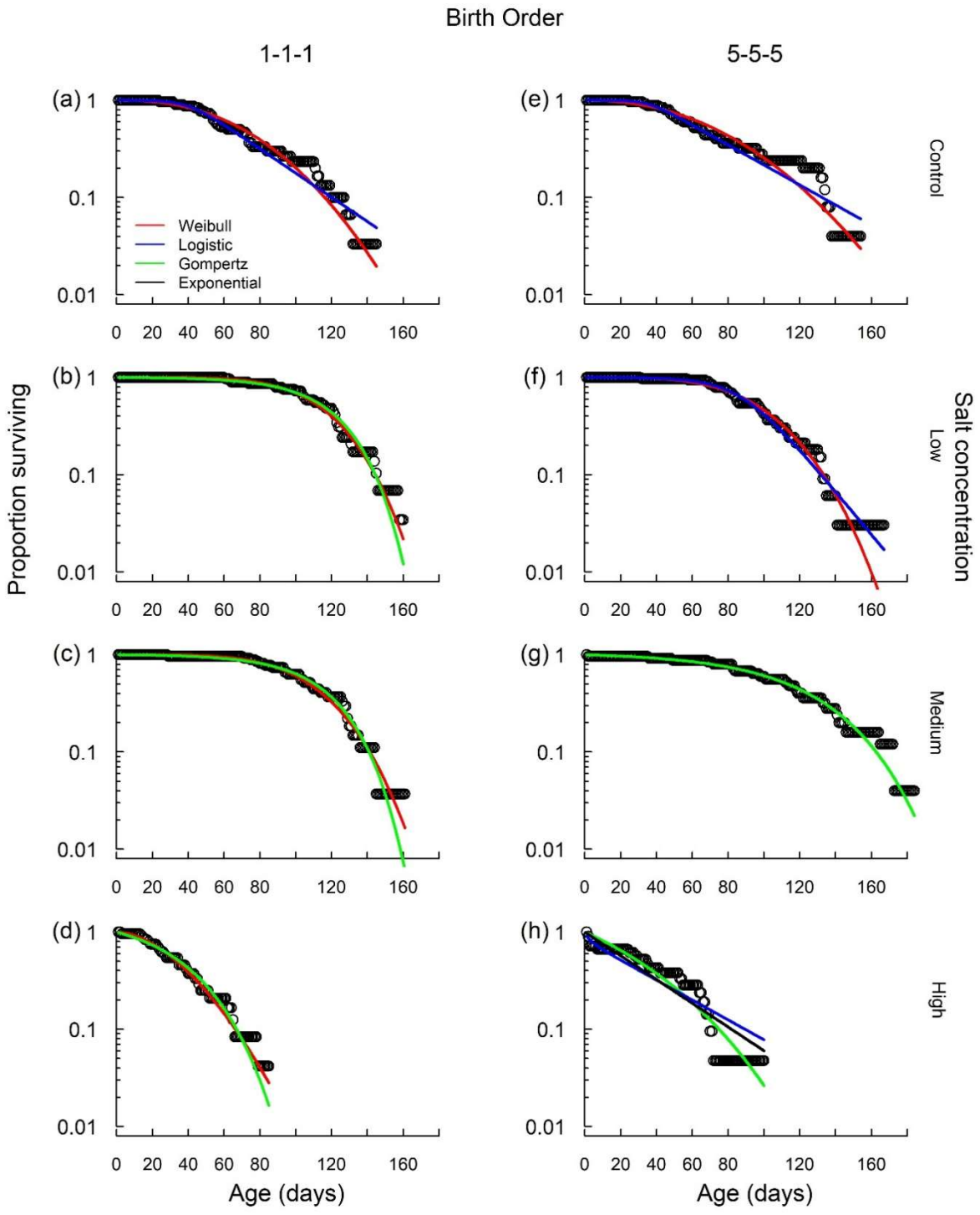

Figure 2.3: Declines in survival with increasing age for each treatment. Lines indicate model(s) with the best fit (ΔAICc < 2; red = Weibull, blue = logistic, green = Gompertz, black = exponential). (a) – (d) have a birth order of 1-1-1; salt concentrations of control (0 g L^{-1}), low (1 g L^{-1}), medium (2 g L^{-1}), and high (4 g L^{-1}) respectively. (e) – (h) have a birth order of 5-5-5; salt concentrations of control (0 g L^{-1}), low (1 g L^{-1}), medium (2 g L^{-1}), and high (4 g L^{-1}) respectively.

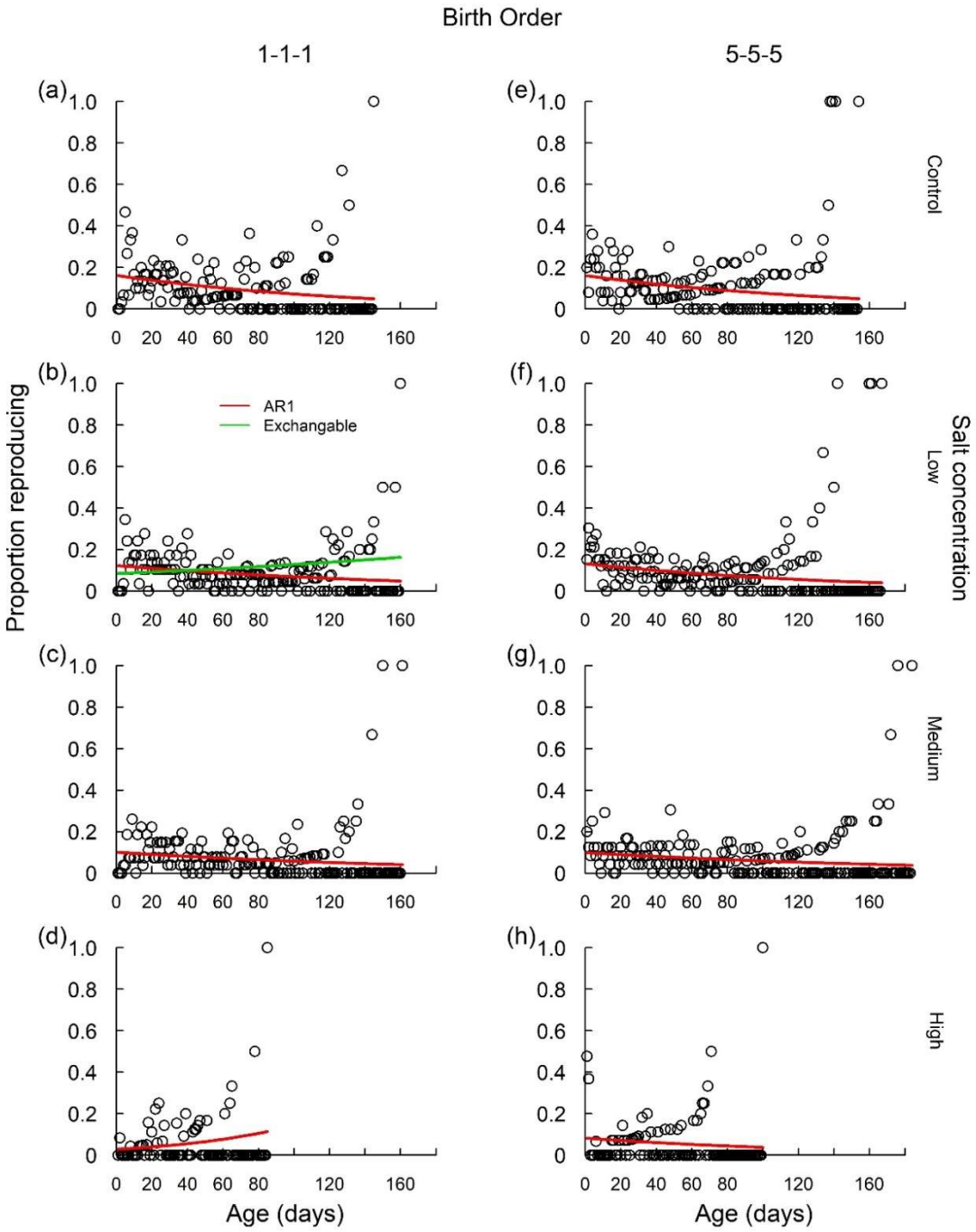

Figure 2.4: Changes in reproduction with increasing age for each treatment. Red lines indicate the AR-1 model, as other models have overlapping lines. The exception to this is (b) which shows a green line indicating the exchangeable model. (a) – (d) have a birth order of 1-1-1; salt concentrations of control (0 g L^{-1}), low (1 g L^{-1}), medium (2 g L^{-1}), and high (4 g L^{-1}) respectively. (e) – (h) have a birth order of 5-5-5; salt concentrations of control (0 g L^{-1}), low (1 g L^{-1}), medium (2 g L^{-1}), and high (4 g L^{-1}) respectively. As death is defined as the date of last reproduction, there is always an instance of a 1.0 proportion of reproduction at the maximum age, as the last frond produces its final offspring before death.

CHAPTER 3: INTERACTIVE EFFECTS OF IMMEDIATE AND ANCESTRAL STRESS ON FITNESS IN DUCKWEED

3.1 Abstract

Organisms that reproduce asexually must respond to abiotic stresses in their environment with the disadvantage of the reduced ability for genetic variation through recombination. Common duckweed (*Lemna minor*) is an aquatic plant that reproduces asexually, through the budding of ramets. As a freshwater plant, duckweed experiences stress from salt (e.g., NaCl), which detrimentally affects growth, photosynthesis, and cellular processes. I took a demographic approach to examine whether ancestral exposure to salt stress affects the ability of duckweed offspring to tolerate immediate exposure to the same stress. To do this, I varied schedules of ancestral stress by placing three consecutive generations of duckweed in an environment with 2 g L^{-1} NaCl, followed by 0-3 generations in a control (no stress) environment. After these treatments, the offspring produced were used as the focal plants for the experiment. Half of these were placed back into an environment containing salt, and half remained in control conditions. Immediate stress decreased fitness, due to a slowing of reproduction, and suggesting a cost of stress tolerance. The effects of ancestral stress, and the interaction of immediate and ancestral stress were more complex. Ancestral stress prepared offspring for immediate stress, by producing offspring more quickly, but potentially of lower quality.

3.2 Introduction

Species that reproduce asexually have the disadvantage of reduced variation in offspring, as except for rare mutations, all offspring produced contain the same genetic information as their parent (Crow & Kimura, 1965). This should make it difficult to respond to environmental changes and stresses, yet organisms are able to adapt to changes through mechanisms such as epigenetics,

and phenotypic plasticity (Bradshaw, 1965; Castonguay & Angers, 2012; Verhoeven & Preite, 2014). Parental and ancestral environments can have the ability to affect the traits and fitness of offspring and future generations (Agrawal et al., 1999; Rassoulzadegan et al., 2006; Dunn & Bale, 2011; Burgess & Marshall, 2014). In an asexual species, does ancestral stress affect future generations' ability to tolerate the same stress?

Fitness is the capacity of an organism to contribute genes to future generations, with greater representation indicating a greater fitness (Hamilton, 1966; Winkler & Fischer, 1999). As such, survival and reproduction are very important components of fitness. A measure of these, the intrinsic rate of increase, is a commonly used measure of fitness, although it assumes a constant environment (Hamilton, 1966; Benton & Grant, 2000; Metcalf & Pavard, 2007). Fitness is highly dependent on the environment in which an organism lives (Stearns, 1992; Winkler & Fischer, 1999). As resources are limited, trade-offs occur among components of fitness, to optimize species' life history characteristics. For example, a trade-off between offspring number versus offspring quality is commonly seen (Lack, 1947; Smith & Fretwell, 1974). Offspring quality can be considered as the fitness of offspring – their own ability to survive and reproduce, although it is often measured through other characteristics, such as offspring size.

The force of natural selection is age-specific, acting more strongly on younger individuals (Hamilton, 1966). This is used in various theories to explain the observations of demographic senescence - the population level declines in rates of survival and reproduction with increasing age. Disposable soma is one such theory, which suggests that senescence is a result of a trade-off between reproduction and somatic maintenance. Allocation of resources to reproduction is favoured, resulting in an accumulation of damage to somatic tissue over time, and deterioration with age (Kirkwood & Holliday, 1979; Kirkwood & Rose, 1991).

Stress tends to decrease fitness, as the detrimental effects of stress decrease chances of survival and reproduction. Tolerance of stress is costly, and requires the expenditure of resources that could be used for other activities. Mild doses of a stress, however, can sometimes have the opposite effect, in a biphasic dose-response curve known sometimes as hormesis (Calabrese & Baldwin, 2002; Mattson, 2008; Calabrese et al., 2015). One example of this is longevity hormesis, in which a mild stress results in an increased lifespan, often attributed to an increase in maintenance and repair mechanisms. While a large stress shortens lifespan through detrimental effects, upregulation of pathways by mild stress is considered adaptive as a method of damage repair and stress tolerance, and also counteracts the accumulation of damage caused by aging. Damage accumulates more slowly, and senescence is delayed, resulting in a longer lifespan (Rattan, 2008; Calabrese et al., 2015). Stress tolerance is linked with lifespan, as many stressors have been shown to result in longevity hormesis, suggesting that stress response and longevity pathways overlap (Davey, 1917; McCay et al., 1935; Rattan, 2004; Hunt et al., 2011). Selection for stress resistance can result in an increased longevity, consistent with the stress theory of senescence (Parsons, 1995)

Sodium chloride salt (NaCl) is a well-known stressor of plants, with both Na^+ and Cl^- ions exhibiting detrimental effects, although Na^+ tends to accumulate in cells faster. Salt negatively affects growth, photosynthesis, and cellular processes through osmotic and ionic stress. Plants are often able to tolerate moderate salt concentrations through the energetically-expensive processes of extruding Na^+ from cells, excluding it from uptake, and compartmentation in vacuoles, away from sensitive cytosolic components (Munns & Tester, 2008). Salt stress is important to investigate, as anthropogenic activities are causing rapid salinization in freshwater ecosystems, which is predicted to continue to increase with climate change (Cañedo-Argüelles et al., 2013; Herbert et al., 2015). Increases of salt concentrations in natural environments can affect multiple

generations of organisms, particularly in organisms which reproduce quickly, since rapid reproduction means multiple generations can be exposed to the same immediate conditions.

Previous studies examining the effects of ancestral stress on future generations' stress tolerance have been done in the plant species *Arabidopsis thaliana*. Exposure of ancestral generations to a mild heat stress resulted in a higher fitness of future generations exposed to the same stress, as compared to those without the ancestral stress exposure, with changes attributed to epigenetic memory (Whittle et al., 2009). Exposure of *A. thaliana* to ancestral salt stress also improved the salt tolerance of future generations exposed to the same stressor (Boyko et al., 2010; Suter & Widmer, 2013a). Another study examining multigenerational heat and salt stress found that the changes observed depended on the strain of *A. thaliana* (Suter & Widmer, 2013b).

In this study, I used an aquatic, asexual plant to investigate the effects of ancestral salt stress on the fitness of offspring, and their ability to tolerate the same stress in their immediate environment. I expected that the combined effects of the immediate and ancestral stress would be less than additive, with offspring being prepared for the salt stress they were exposed to. However, it was also a possibility that the combined effects of immediate and ancestral salt stress would be more than additive, with multigenerational stress resulting in the production of lower quality offspring.

3.3 Materials and methods

3.3.1 Study species, growth conditions, and experimental procedures

Duckweeds are small, free-floating aquatic angiosperms, found in slow-moving freshwater bodies on nearly every continent (Landolt, 1986). Part of the Lemnoideae subfamily (Family Araceae), duckweeds include the species *Lemna minor,* also known as common duckweed or lesser

duckweed. *L. minor* ramets consist of a frond (a leaf and stem structure) and a single root, and reproduce almost exclusively asexually through the budding of offspring from two meristematic pockets. Offspring are attached to their parent during development via a stipe, which breaks after they are mature, releasing the offspring from their parent (Lemon & Posluszny, 2000). Duckweeds reproduce quickly, and may have visible grandoffspring developing before the offspring have detached from their parent themselves. The small size, short lifespan, and previous work on senescence in *L. minor* makes it a suitable study species for demographic analyses and other types of studies (Lam et al., 2014; Laird & Barks, 2018).

The plants used in this study were derived from a culture obtained from the Canadian Phycological Culture Centre (CPCC 492 *L. minor*; originally collected from Elk Lake, British Columbia, Canada, 48° 31′30″ N, 123° 23′18″ W). I grew fronds under axenic conditions in individual 60 x 15 mm (diameter x height) petri dishes. These contained 10 mL of Schenk and Hildebrandt growth medium (S6765, Sigma-Aldrich, St. Louis, MO, USA), at one-eighth strength (0.4 g L^{-1}). Every seven days, I transferred fronds to fresh medium to maintain a constant environment. Previous studies supplemented this medium to more easily detect contamination by microorganisms through visual inspection; however, I chose not to include supplements in this study. A small number of petri dishes experienced contamination during the study; these fronds were discarded. Fronds were kept in a growth chamber at 24°C, with a photoperiod of 15:9 (light:dark hours), and an average photosynthetic photon flux density at plant height of 15.20 μmol m^{-2} s^{-1}.

3.3.2 Ancestral and immediate salt exposure treatments

Experimental preparations and treatments are summarized in Figure 3.1. To avoid parental age effects in *L. minor* (Barks and Laird 2015), and thereby reduce noise in the data, I ensured recent genealogical homogeneity of experimental fronds through selection of first offspring over several generations. Each 'progenitor' frond was initially selected from a stock culture, and the first offspring to detach from it, post-selection, was of unknown birth order. I marked this 'unknown' offspring and discarded the progenitor. The first offspring to detach from the unknown frond had a birth order of one; I marked it, and discarded its parent (i.e., the 'unknown' frond). Subsequent selection of first offspring over two additional generations led to the fronds I used in the next phase of the application of experimental treatments (Figure 3.1).

These fronds and/or some of their descendants were subjected to one of five levels of the 'ancestral' salt exposure treatment (Figure 3.1). Throughout the application of the ancestral treatment, I continued to select and retain the first offspring produced for all fronds, thus maintaining genealogical homogeneity across treatment levels. During the ancestral treatments, I exposed fronds to a salt stress by moving the fronds to medium with 2 g L^{-1} of salt (NaCl; S7653, Sigma-Aldrich, St. Louis, MO, USA) added. This concentration was chosen as an 'intermediate' stress, following Hayden (2018) and after pilot experiments showed it to negatively affect *L. minor* life-history trajectories without being immediately lethal. I applied the ancestral treatment over six generations for each frond lineage. It typically consisted of zero to three generations placed in the control (c) medium, followed by three generations placed in the stress (S) medium. After that, I placed fronds back in the control medium for the balance of the six generations. Frond lineages in the ancestral control treatment were never exposed to salt through the six generations – resulting in a total of five ancestral treatments: cccccc, SSSccc, cSSScc, ccSSSc, and cccSSS (Figure 3.1).

I designated the first offspring produced by the last generation of the fronds in the ancestral treatment as the 'focal' frond. By way of example, a focal frond in the cSSScc ancestral treatment had its great^4-grandparent grown in control growth medium, followed by its great^3-, great^2-, and great-grandparent grown under stressful conditions, followed by its grandparent and parent grown in control growth medium.

I exposed the focal fronds to one of two levels of the 'immediate' salt exposure treatment. Half of the focal fronds from each ancestral treatment were placed in the control medium, and the other half were placed in the stress medium (Figure 3.1). I followed these fronds for their entire lifespan, and observed them daily to record offspring production. For each of these offspring fronds, the date of detachment from its parent was recorded as its birth date, and death date was defined as the date its last offspring detached from it, accounting for the entire reproductive lifespan of the frond. In some of the focal fronds, the last offspring produced did not detach from its parent, remaining attached until both were dead. In these cases the date of death used was that of the last detaching offspring. Total offspring number was also calculated excluding any non-detaching offspring, as reproduction was considered to occur when an offspring detached. While I began with 560 fronds divided evenly among 10 treatments, and distributed randomly between two growth chambers, the malfunction of one chamber resulted in the death of half of the fronds. The remaining fronds (from the functioning growth chamber) had similar sample sizes among treatments, which were still large enough for statistical analyses. The sample size of each treatment was also decreased by contamination of fronds, and death of fronds during ancestral treatment (before focal fronds were produced), resulting in a mean final sample size of 21 fronds per treatment (range: 18 to 25; Table 3.1).

While offspring traits were not measured directly, a benefit of the design of ancestral treatments is that focal fronds from one ancestral treatment can have the same genealogical exposures as the *offspring* of the focal fronds from another ancestral treatment. For example, the offspring of the ccSSSc-C group's focal fronds have two generations between themselves and the ancestral stress – the focal frond (the parent), and the parent of the focal frond (the grandparent). Likewise, focal fronds stemming from the cSSScc ancestral treatment have two generations between the application of ancestral stress and the focal frond. Therefore, focal fronds in the combined distant ancestral stress and immediate control treatments (e.g., cSSScc-C) should have similar traits to the offspring of focal fronds in more recent ancestral treatments (e.g., ccSSSc-C), an attribute of this experimental design that can be used to infer offspring characteristics.

3.3.3 Sample preparation and analysis by flame atomic absorption spectroscopy

I used flame atomic absorption spectroscopy to assess the realized salt concentration within tissue of the different treatments. To ensure enough material was available for analysis, I pooled whole plants from each treatment into one sample per treatment, as single fronds did not provide enough dry mass to run individually. I rinsed fronds with deionized water, placed them into foil dishes, and dried them at 60 °C until a constant weight was achieved, signifying that the samples were completely dry. I weighed each sample on an analytical balance, and recorded the weight on a scale accurate to the hundredth of a milligram. I placed samples in 2 mL high-density polyethylene conical vials and digested them using 12 N trace-metal grade nitric acid at a ratio of 1 mg of dried tissue to 10 µL of acid. Samples were digested over 3 hours at 80 °C. Following digestion, I allowed samples to cool to room temperature before diluting them at a ratio of 1 mL

acid to 10 mL water (utilizing UltraPure Millipore water), then stored samples at 4 °C until analysis.

I quantified the amount of sodium within samples by flame atomic absorption spectroscopy utilizing an Agilent 240FS (Agilent Technologies, USA). I diluted a certified sodium standard (SCP SCIENCE, Product #140-001-111) to 200 µg L^{-1}, 400 µg L^{-1}, and 800 µg L^{-1} sodium to establish a standard curve. The standard curve was re-sloped after every ten samples, and recalibrated after every twenty samples. I ran the flame atomic absorption spectroscopy at SpectraAA (Agilent software) factory settings with minor adjustments: the lamp current was set to 5.0 mA, and slit width limited to 0.5 nm. I read samples at a wavelength of 589.0 nm. To verify the standard curve, SLRS-6 (River Water Certified Reference Material for Trace Metals and other Constituents, National Research Council Canada) was tested and sodium concentrations read within 10% of accepted values for the reference material.

3.3.4 Data Analysis

I used a two-way ANOVA to compare the effect of the ancestral treatment, immediate treatment, and their interaction on the intrinsic rate of natural increase (r) measured at the level of focal fronds (McGraw & Caswell, 1996), which was the primary measure of fitness. The effects on the time to first reproduction, lifespan, and total number of offspring produced were also examined with a two-way ANOVA, to identify the source of differences in fitness across treatments. Normality and homoscedasticity of data were assessed with residual-by-predicted plots and normal quantile-quantile plots, to determine whether the assumptions of ANOVA were met. Post-hoc pairwise t-tests corrected for multiple comparisons using the Benjamini and Hochberg

method were performed within each immediate treatment to examine differences among ancestral treatments.

Senescence data were also examined. I fit the daily proportion of fronds surviving with exponential, Weibull, Gompertz, and logistic models for each treatment (Pletcher et al., 2000; Ricklefs & Scheuerlein, 2002; Barks & Laird, 2015). The exponential model is the only one of these four models that has constant survival with age, implying no senescence. I used the Akaike Information Criterion corrected for small sample sizes (AICc) to select the best model, with a ΔAICc lower than two taken to indicate a fit effectively indistinguishable from the 'best' model.

I analysed reproduction data in a binary manner, such that fronds either reproduced, or did not reproduce on a particular date. This was done following the methods of Barks and Laird (2015), in order to facilitate statistical analyses. I did not further examine multiple reproduction events, as the number of these was very small (1.2% of all reproduction events, across all treatments). Thus, I used a generalized estimating equation (GEE) approach coupled with a Wald's test to analyse the probability of reproduction. This approach accounted for potential autocorrelation in the data stemming from the possibility that individual fronds were unlikely to reproduce on consecutive days. Three correlation structures were tested for each treatment: an 'independent' correlation structure, a first-order autoregressive correlation structure (AR-1), and an exchangeable correlation structure. I chose to use the AR-1 correlation structure as it was the most biologically relevant, accounting for the temporal autocorrelation from the decreased chance of reproducing on consecutive days. Other working correlation structures were either rejected for not meeting the positive definite criterion of Shults et al. (2009), or an ambiguous determination of the best working correlation structure using the Rotnitzky-Jewel criteria (Rotnitzky & Jewell, 1990), and all structures produced qualitatively indistinguishable fits.

All analyses were done in R v. 3.6.3 (R Core Team, 2020).

3.4 Results

3.4.1 Life history traits

The intrinsic rate of natural increase (*r*) was significantly greater for fronds in the immediate control treatments than the immediate stress treatments (Figure 3.2a; two-way ANOVA, $F_{1, 204} = 234.26$, $p < 2.0 \times 10^{-16}$). The effect of ancestral stress was also significant, with *r* values increasing as stress became more recent (Figure 3.2a; 2-way ANOVA, $F_{4, 204} = 2.56$, $p = 0.04$). However, despite the significant main effect of ancestral stress, post-hoc contrasts showed no significant differences among ancestral treatments within either immediate treatment (Figure 3.2a). There was no significant interaction effect (Figure 3.2a: 2-way ANOVA, $F_{4, 204} = 0.84$, $p = 0.50$).

The time to first reproduction was significantly longer for the immediate stress group than the immediate control group (Figure 3.2b; two-way ANOVA, $F_{1, 204} = 76.21$, $p = 9.2 \times 10^{-16}$). The ancestral treatment was also significant, with time to first reproduction typically decreasing as ancestral stress became more recent (Figure 3.2b; two-way ANOVA, $F_{4, 204} = 5.27$, $p = 4.7 \times 10^{-4}$). There was a significant interaction effect between the immediate and ancestral treatments (Figure 3.2b; two-way ANOVA, $F_{4, 204} = 2.79$, $p = 0.028$). Within the immediate stress treatment, recency of ancestral stress tended to decrease the time to first reproduction, with post-hoc tests finding the ccSSSc-S treatment significantly lower than when ancestral stress was absent or very distant (cccccc-S and SSSccc-S, respectively). Time to first reproduction also declined with recency of ancestral stress within the immediate control treatments, for which post-hoc tests found

a significant difference between the most recent ancestral stress (cccSSS-C), and absent ancestral stress (cccccc-C; Figure 3.2b).

Fronds exposed to the immediate salt treatment had a longer lifespan than those in the immediate control treatment (Figure 3.2c; two-way ANOVA, $F_{1, 204} = 140.42$, $p < 2.0 \times 10^{-16}$). The ancestral treatment alone did not have a significant effect on lifespan (Figure 3.2c; two-way ANOVA, $F_{4, 204} = 1.70$, $p = 0.152$), but there was a significant interaction effect of the immediate and ancestral treatments on lifespan (Figure 3.2c; 2-way ANOVA, $F_{4, 204} = 3.69$, $p = 6.4 \times 10^{-3}$). Contrasts within the immediate control treatment showed the distant ancestral stress groups (SSSccc-C and cSSScc-C) had significantly shorter lifespans than the other ancestral treatments. There were no significant differences found between groups in the immediate stress treatment (Figure 3.2c).

The immediate treatment did not have a significant effect on offspring number (Figure 3.2d; two-way ANOVA, $F_{1, 204} = 0.381$, $p = 0.538$), but there was a significant effect for ancestral treatment (Figure 3.2d; 2-way ANOVA, $F_{4, 204} = 3.10$, $p = 0.017$). There was also a significant interaction effect of the immediate and ancestral treatments on offspring number (Figure 3.2d; two-way ANOVA, $F_{4, 204} = 3.847$, $p = 4.9 \times 10^{-3}$). Within the immediate stress treatment, offspring number tended to decrease with recency of ancestral stress, but the post-hoc contrasts did not detect any differences. In the immediate control treatment, distant ancestral stress (i.e., SSSccc, cSSScc) had a lower number of offspring, while recent ancestral stress (i.e., ccSSSc, cccSSS) showed similar offspring numbers, relative to when ancestral stress was absent (i.e., cccccc).

3.4.2 Tissue concentrations of Na^+

Due to the necessity of pooling individuals to obtain a sample with sufficient biomass for flame atomic absorption spectroscopy, only one sample was obtained and measured for each combination of treatments. With this limitation, it was difficult to use conventional hypothesis tests. A two-way ANOVA could be done if the interaction term was omitted. However, visual inspection of the data shows that an interaction was very likely, making this statistical approach inappropriate. As such, significant differences between groups could not be determined. With this limitation in mind, there appeared to be an effect of immediate stress, with stress treatments having a higher tissue concentration of Na^+ (Figure 3.3). This is consistent with previous literature. In salt stress conditions, plants are able to sequester Na^+ within vacuoles, and can extrude Na^+ from cells depending on the concentration gradient between the cells and the environment (reviewed by Munns & Tester, 2008). Ancestral treatment did not appear to affect tissue concentration within the immediate control treatment, but the two most recent ancestral stress treatments had very high concentrations of Na^+, when combined with immediate stress. These high concentrations imply that Na^+ may be accumulated multigenerationally, although returning to control conditions for more than two generations reduced the tissue concentrations back to normal levels. Naturally, further studies with greater replication will need to be done in order to confirm these results, and support this interpretation.

3.4.3 Senescence

All treatments showed decreases in daily survival rate with increasing age (i.e., concave-down survivorship curves; Figure 3.4). Moreover, in no cases was the exponential model (indicating 'no senescence') selected as the best-fit model, and in every case the exponential model

had very poor support (ΔAICc > 16 for all; Table 3.2). The Weibull model was found to have the best fit (lowest ΔAICc), or had a co-best fit (ΔAICc < 2) in all cases. When there were models with co-best fits, either the Gompertz or logistic model was deemed to fit equally well (ΔAICc < 2; Table 3.2).

All treatments showed a decline in probability of reproduction with increasing age (Figure 3.5). The decline in probability of reproduction with increasing age was significant for most treatments, with the exceptions being SSSccc-C, cSSScc-C, and cccccc-S (Table 3.3), when examined with a GEE model with an AR-1 correlation structure.

3.5 Discussion

3.5.1 Effects of immediate stress

Immediate salt stress had a strong negative effect on the fitness of fronds, as seen in the considerably lower intrinsic rate of natural increase of the immediate stress treatments (Figure 3.2a). This is expected due to the detrimental effects of salt stress, which should decrease fitness when there are increased costs of stress resistance. Since intrinsic rate of natural increase is a measure of the capacity for population growth, it can be affected by variation in survival, number of offspring, and the schedule of reproduction. The observed lowered fitness is attributable to the longer time to produce first offspring when exposed to the immediate stress (Figure 3.2b), and that fact that offspring number was not affected by immediate stress (Figure 3.2d), while lifespan increased (Figure 3.2c). The measure of lifespan is defined in such a way that it encompasses the reproductive lifespan of fronds, and the increase in the immediate stress indicates that fronds also take a longer time to finish reproduction. While fronds exposed to the immediate stress were able to produce a similar number of offspring, they were only able to do so over a significantly longer

time – often with an additional 20 days or longer (Figure 3.2). As early-produced offspring contribute more to population growth and fitness than late-produced offspring (Fisher, 1930; Stearns, 1976), the extended reproductive schedule in fronds experiencing immediate stress led them to have a lower intrinsic rate of increase. Combined, this evidence suggests that immediate salt stress slows reproduction, and 'stretches' the life of fronds, resulting in decreased fitness due to requiring a longer time for similar total reproduction. This interpretation is also supported by the shape of the declines in survival with increasing age, as almost all fronds exposed to immediate stress (Figure 3.4f – j) survive for much longer than those in immediate control conditions (Figure 3.4a – e). The proportion of fronds reproducing at age 1 is lower in the immediate stress treatments than the immediate control treatments (Table 3.3). The declines in reproduction with increasing age also appear stretched in the immediate stress treatments (Figure 3.5f – j), compared to the immediate control treatments (Figure 3.5a – e), which show similar declines over different lengths of life (Table 3.3).

If the results observed in this study follow the predictions made by the disposable soma theory, resources should be traded off between reproduction and somatic maintenance and repair (Kirkwood & Holliday, 1979; Kirkwood & Rose, 1991). In light of this, the longer time to produce offspring in the immediate stress treatment could indicate that those fronds had fewer resources available for reproduction, having shifted these resources towards the upregulation of tolerance or repair processes instead, with increased lifespan being an incidental by-product of slowed reproduction. This is supported by the concept of longevity hormesis, and the connection of stress resistance and longevity. Salt is detrimental, negatively affecting growth, reproduction, photosynthesis and increasing oxidative damage (Munns & Tester, 2008), but moderate, non-lethal concentrations of salt such as the 2 g L^{-1} used in this study, induce an adaptively plastic response

in *L. minor* that allow survival through stress resistance (Tkalec et al., 2001; Panda & Upadhyay, 2004; Sikorski et al., 2013). The increased longevity observed in the immediate salt treatments (Figure 3.2c) suggests that maintenance and repair upregulation was an adaptively plastic response. Maintenance and repair pathways increased in response to damage from stress can also protect against and repair the damage done by aging, thus decreasing damage, and slowing the aging process (Parsons, 1995; Rattan, 2008; Milisav et al., 2012). This would result in a greater lifespan, although it is important to remember that it comes at the cost of overall fitness. More generally, the ability to maintain homeostasis with increasing age, including the ability to respond to changing or stressful environments, can reduce senescence and the effects of aging (Rattan, 2008; Pomatto & Davies, 2018). Investigating the mechanisms involved is beyond the scope of this demographically oriented study, although mechanisms involved with oxidative stress and reactive oxygen species have been suggested (Harman, 1956, 1992; Lin & Beal, 2003; Gems & Doonan, 2009; Pomatto & Davies, 2018).

3.5.2 Effects of ancestral stress

The subtle increase in intrinsic rate of increase seen with recent ancestral stress (e.g., cccSSS; Figure 3.2a) is predominantly due to the shorter time to first reproduction (Figure 3.2b), as offspring produced early are themselves able to reproduce sooner, which will impact how quickly a population can grow (Stearns, 1976). When ancestral stress is distant (e.g., SSSccc), the time to first reproduction is slower, and the intrinsic rate of increase is lower, becoming more similar to the average values of the control treatment (cccccc). The higher intrinsic rate of increase observed with recent ancestral stress suggests that a recently-stressed population should grow at a faster rate than a distantly-stressed population. While it is common to use intrinsic rate of increase

to measure fitness, this comes with the assumption that there is a constant environment (Benton & Grant, 2000). In this study, the changes in environment required for the ancestral treatments (with the exception of cccccc) mean this assumption cannot be met, so it is important to note that intrinsic rate of increase must be interpreted with caution with respect to ancestral stress.

3.5.3 Patterns within the immediate stress treatment

Within the immediate salt treatment, ancestral stress may prepare offspring for stressful conditions. Fronds with ancestral exposure to stress respond to immediate stress differently than fronds with no ancestral exposure to stress (Figure 3.2). Previous studies have reported that salt stress results in initial mortality and decrease in growth, followed by recovery (O'Brien et al., 2020), suggesting that responses during the multigenerational stress are adaptively plastic, to resist the stress and maximize fitness under suboptimal conditions.

Trends in the time to first reproduction and offspring number of the immediate stress treatments suggest a trade-off. When ancestral stress is more recent, the time to first reproduction is faster, and slightly fewer offspring are produced, although there were no significant differences detected among the immediate stress treatment offspring numbers. The values for the reproductive traits of the distant ancestral stress groups (i.e., SSSccc-S) are also more similar to the control group with no ancestral stress exposure (cccccc-S). These differences among ancestral treatments suggest that the reproductive strategy of duckweeds in stress conditions favours speed of reproduction at the cost of offspring number, while control conditions balance speed of reproduction with a slightly higher offspring number. When there is recent ancestral stress, fronds are better able to achieve faster reproduction in immediate stress.

It is interesting to note that the recent ancestral stress combined with immediate stress treatments (ccSSSc-S and cccSSS-S) have very high Na^+ tissue concentrations compared to all other treatments (Figure 3.3). This suggests that fronds with multigenerational salt stress may acquire Na^+ from parental or grandparental fronds, or may have an impaired ability to extrude Na^+ from tissues when in stressful conditions themselves, although further studies with replication need to be done to confirm these results. These high tissue concentrations could be responsible for some of the differences seen in the life history traits of the same groups (Figure 3.2). While there are large differences in the tissue concentrations of the recent ancestral stress groups, life history traits exhibit smaller and more subtle differences. There are also slight differences in the life history traits of the distant ancestral stress groups (SSSccc-S and cSSScc-S) compared to the control, although their tissue concentrations appear very similar.

3.5.4 Patterns within the immediate control treatment

The groups with combined immediate control and recent ancestral stress (e.g., cccSSS-C) show a faster time to first reproduction, and an offspring number and longevity not significantly different from that of the control (cccccc-C; Figure 3.2). This suggests that even with recent ancestral stress, a return to immediate control conditions returns resource allocation to a state similar to when no ancestral stress has occurred, although faster time to first reproduction is still observed. The most important change that salt stress causes is the slower time to first reproduction, which is slowly lost as the stress becomes more distant.

While the post-hoc tests detected no significant differences between the control (cccccc-C) and either of the recent ancestral stress groups (cccSSS-C, ccSSSc-C) for offspring number, lifespan, or intrinsic rate of increase, it is interesting to note that these appear to be slightly higher

with recent ancestral stress than in the control, and perhaps a study with greater statistical power would detect differences in these. A moderately higher longevity in the recent ancestral stress combined with immediate control groups suggests a slower re-allocation of resources. Early-life damage could be repaired, delaying senescence, before resources are shifted back towards reproduction. Under this explanation, a greater offspring number would indicate a shift of resources back towards reproduction. This supports the interpretation that recent, multigenerational ancestral stress prepares offspring for stress through optimal allocation of resources for that environment. When the environment no longer contains the stressor, preparation for a stressful environment is no longer beneficial, and so these changes are lost. While the lack of significant differences between the control and recent ancestral stress groups indicate that this occurs quickly, studies with greater power may detect a lag in the return to normal.

The total resources available to all fronds across the immediate control treatment should be the same, due to their identical immediate environments. However, the recent ancestral stress groups have a faster time to first reproduction, and a similar offspring number and lifespan compared to the control. This should require more resources, yet there seem to be no traits indicating a decrease in resource allocation. This suggests that trade-offs are occurring in some trait that has not been measured. In many taxa, offspring number versus offspring quality is a common trade-off (Lack, 1947; Svardson, 1949; Williams, 1966a; Smith & Fretwell, 1974; Stearns, 1976; Vasseur et al., 1995; Sadras, 2007), and could be the unmeasured trait in question here. If the faster reproduction seen with recent ancestral stress comes at the cost of offspring quality, these offspring may not be able to survive or reproduce well themselves. While offspring traits were not measured directly, the study design allows an estimation of offspring traits from equivalent genealogical stress exposures. In particular, the focal fronds in the cSSScc ancestral

treatment should have a similar genealogical exposure to salt stress as the offspring of the ccSSSc-C treatment (i.e., two generations in control media), and should show similar reactions. The significantly shorter lifespan and low offspring number, as well as the slightly longer (although not significant) time to first reproduction in the combined distant ancestral stress and immediate control treatment (e.g., cSSScc-C), as compared to the control, supports the hypothesis that these are low-quality fronds, which may have been the price their parent paid to produce many offspring quickly. Alternatively, perhaps these low values are due trade-offs these fronds are performing themselves – sacrificing lifespan, offspring number, and reproduction speed, to produce a few high-quality offspring, and indicating a recovery from the ancestral stress. Further studies that include observation of generations more distant from the stress, or measurement of a greater number of fitness indicators could indicate which, if either, of these occurs.

3.5.5 Adaptive responses in an asexual species

All plants in this study reproduced asexually, which means that every frond should be genetically identical to its parent (i.e., aside from rare mutants). Yet responses to stress were different between the ancestral treatments, with ancestral stress preparing focal fronds for immediate stress, and implying that stress resistance strategies were inherited from previous stressed generations. While the possibility of a direct transfer of salt from parent to offspring cannot be dismissed, due to the high tissue concentrations seen in the ccSSSc-S and cccSSS-S groups (Figure 3.3), it would not explain the differences among the immediate control treatments, which show similar tissue concentrations. An alternative explanation is that *L. minor* may pass information to offspring through non-genetic means, such as epigenetics. Epigenetic changes in DNA methylation have been shown to occur in *L. minor* in response to thermal stress, some of

which can be inherited through several generations of asexually-produced offspring (Prelovšek, 2018). It has been suggested that epigenetic variation provides adaptive phenotypic plasticity in response to changing environments, which may be important for short-term adaptation and non-genetic evolution of asexual species (Latzel & Klimešová, 2010; Verhoeven & Preite, 2014; Dodd & Douhovnikoff, 2016). As asexual reproduction cannot produce variation through recombination, and genetic adaptation is relatively slow, epigenetic changes could provide variation in response to changing conditions instead. Asexual reproduction may mean epigenetic markers are more easily inherited, as the epigenetic resetting associated with meiosis is avoided (Feng et al., 2010; Verhoeven & Preite, 2014; Wilschut et al., 2016). Epigenetic factors may also affect changes in genome stability, which has been shown to be affected by salt stress in plants (Boyko et al., 2010; Boyko & Kovalchuk, 2011). While genetic similarity of fronds was assumed due to the mode of reproduction in this study, it is possible that the salt stress affected genome stability, and induced genetic changes. Further studies should be done, both to examine if salt stress induces epigenetic or genetic changes in duckweeds, and if these changes are inherited by offspring.

In particular, genetic studies using organisms with mutations that alter the expression of genes can be useful in determining molecular mechanisms. Identification of stress tolerance genes and their specific functions and mechanisms can be done through loss-of-function and overexpression studies (Cushman & Bohnert, 2000; Alberts et al., 2002). Studies in duckweed have been done, including the insertion and overexpression of transgenes, resulting in an increased tolerance to salt stress (Yang et al., 2013; Yang et al., 2017).

A limitation of the present study, related to *Lemna* biology, is the lack of clear distinction between the environments of different generations. Attachment to parents and grandparents can result in brief, but direct exposure to the parental and grandparental environments during development and the pre-birth period. As such, the ancestral stress treatments for which it can be said confidently that focal fronds were not themselves directly exposed to ancestral stress is the cSSScc treatment, or those with more distant ancestral stress. The cause of the results seen for the recent ancestral stress treatments could be due to non-genetic inheritance from stressed ancestors, or simply due to the brief stress they experienced early in life. For example, the ccSSSc-S group could be interpreted as exhibiting preconditioning, a type of hormesis in which a brief, mild exposure to a stressor allows an individual to better tolerate a larger stress later (Calabrese et al., 2007). Genealogically, in the earliest stages of their development, the focal fronds of the ccSSSc-S group may have briefly experienced the stressful grandparental environment, followed by a short period in the control conditions while attached to their parent, then after detaching, were returned to stress conditions for the immediate treatment. A brief exposure to the grandparental environment may have allowed a stronger adaptively plastic response to the immediate stress in the very fast time to reproduction of the ccSSSc-S group, compared to both immediate exposure alone (cccccc-S), or continuous multigenerational and immediate exposure to stress (cccSSS-S).

3.6 Conclusion

Immediate salt stress results in decreased fitness, attributable to the slowed speed of reproduction. Fronds produce both their first and last offspring more slowly when in immediate stress, and are only able to produce a similar number of offspring over the extended lifespan they exhibited. The longer lifespan is likely a result of an increase in maintenance and repair, as a

protective mechanism against salt stress, which also repairs the damage that comes with increasing age. The effects of ancestral stress were more complex than those of immediate stress. However, the general effect of ancestral stress was to prepare fronds for stress by causing them to produce offspring more quickly, albeit of potentially lower quality. Overall, these results emphasize the multigenerational nature of environmental stress.

3.7 Tables

Table 3.1: Initial and final sample sizes of each treatment.

Treatment	Initial n	n remaining after growth chamber malfunction	Final n
cccccc-C	56	24	22
SSSccc-C	56	27	25
cSSScc-C	56	23	20
ccSSSc-C	56	33	24
cccSSS-C	56	35	24
cccccc-S	56	30	23
SSSccc-S	56	27	18
cSSScc-S	56	23	21
ccSSSc-S	56	31	18
cccSSS-S	56	27	19
Mean:	**56**	**28**	**21**

Table 3.2: Comparison of four survival models for each treatment. The model with the lowest AICc and ΔAICc values are considered the best. Models with ΔAICc < 2 are considered equally well fitting as the model with the lowest AICc. The best model(s) for each treatment are bolded.

Treatment	Model	No. parameters	Deviance	AICc	ΔAICc	AICc weight
cccccc-C	Exponential	1	218.24	220.43	23.72	5.10×10^{-6}
	Weibull	**2**	**192.09**	**196.72**	**0.00**	**0.72**
	Gompertz	2	196.92	201.55	4.83	0.064
	Logistic	3	191.81	199.14	2.42	0.21
SSScccC	Exponential	1	229.23	231.41	36.15	9.05×10^{-9}
	Weibull	**2**	**190.71**	**195.26**	**0.00**	**0.64**
	Gompertz	2	194.08	198.63	3.37	0.12
	Logistic	**3**	**190.06**	**197.21**	**1.95**	**0.24**
cSSScc-C	Exponential	1	188.60	190.81	30.55	1.13×10^{-7}
	Weibull	**2**	**155.60**	**160.31**	**0.041**	**0.48**
	Gompertz	2	160.79	165.50	5.23	0.036
	Logistic	**3**	**152.77**	**160.27**	**0.00**	**0.49**
ccSSSc-C	Exponential	1	244.56	246.74	41.42	7.03×10^{-10}
	Weibull	**2**	**200.75**	**205.32**	**0.00**	**0.70**
	Gompertz	2	203.89	208.47	3.14	0.14
	Logistic	3	201.05	208.25	2.93	0.16
cccSSS-C	Exponential	1	241.50	243.68	30.31	2.56×10^{-7}
	Weibull	**2**	**208.81**	**213.38**	**0.00**	**0.97**
	Gompertz	2	215.98	220.55	7.17	0.027
	Logistic	3	273.23	280.43	67.05	2.68×10^{-15}
cccccc-S	Exponential	1	248.83	251.02	31.11	1.36×10^{-7}
	Weibull	**2**	**215.31**	**219.91**	**0.00**	**0.78**
	Gompertz	2	218.55	223.15	3.25	0.15
	Logistic	3	217.42	224.68	4.77	0.071
SSScccS	Exponential	1	198.94	201.19	23.18	4.24×10^{-6}
	Weibull	**2**	**173.36**	**178.16**	**0.15**	**0.43**
	Gompertz	**2**	**173.21**	**178.01**	**0.00**	**0.46**
	Logistic	3	173.03	180.74	2.73	0.12
cSSScc-S	Exponential	1	228.31	230.52	38.34	2.93×10^{-9}
	Weibull	**2**	**187.51**	**192.18**	**0.00**	**0.62**
	Gompertz	2	189.64	194.31	2.13	0.21
	Logistic	3	187.39	194.80	2.62	0.17
ccSSSc-S	Exponential	1	195.34	197.59	30.45	1.63×10^{-7}
	Weibull	**2**	**162.34**	**167.14**	**0.00**	**0.67**
	Gompertz	2	164.80	169.60	2.46	0.20
	Logistic	3	162.57	170.29	3.15	0.14
cccSSS-S	Exponential	1	207.20	209.43	16.94	1.30×10^{-4}
	Weibull	**2**	**188.79**	**193.54**	**1.06**	**0.37**
	Gompertz	**2**	**187.74**	**192.49**	**0.00**	**0.62**
	Logistic	3	192.80	200.40	7.91	0.012

Table 3.3: The results of Wald tests, and the change in the probability of reproduction between age 1 and the maximum age for each treatment using the AR-1 working correlation structure. Bolded p-values indicate significance ($p < 0.05$).

Group	Probability of reproduction at age 1	Change of probability of reproduction between age 1 and maximum age of treatment	χ^2	df	p
cccccc-C	0.22	-0.14	15.80	1	$\mathbf{7.00 \times 10^{-5}}$
SSSccc-C	0.20	-0.025	1.10	1	0.29
cSSScc-C	0.20	-0.054	2.11	1	0.15
ccSSSc-C	0.19	-0.087	15.90	1	$\mathbf{6.80 \times 10^{-5}}$
cccSSS-C	0.21	-0.15	33.40	1	$\mathbf{7.70 \times 10^{-9}}$
cccccc-S	0.13	-0.046	2.98	1	0.084
SSSccc-S	0.12	-0.058	7.56	1	$\mathbf{6.00 \times 10^{-3}}$
cSSScc-S	0.12	-0.058	5.02	1	**0.025**
ccSSSc-S	0.13	-0.082	22.60	1	$\mathbf{2.00 \times 10^{-6}}$
cccSSS-S	0.13	-0.075	18.60	1	$\mathbf{1.60 \times 10^{-5}}$

3.8 Figures

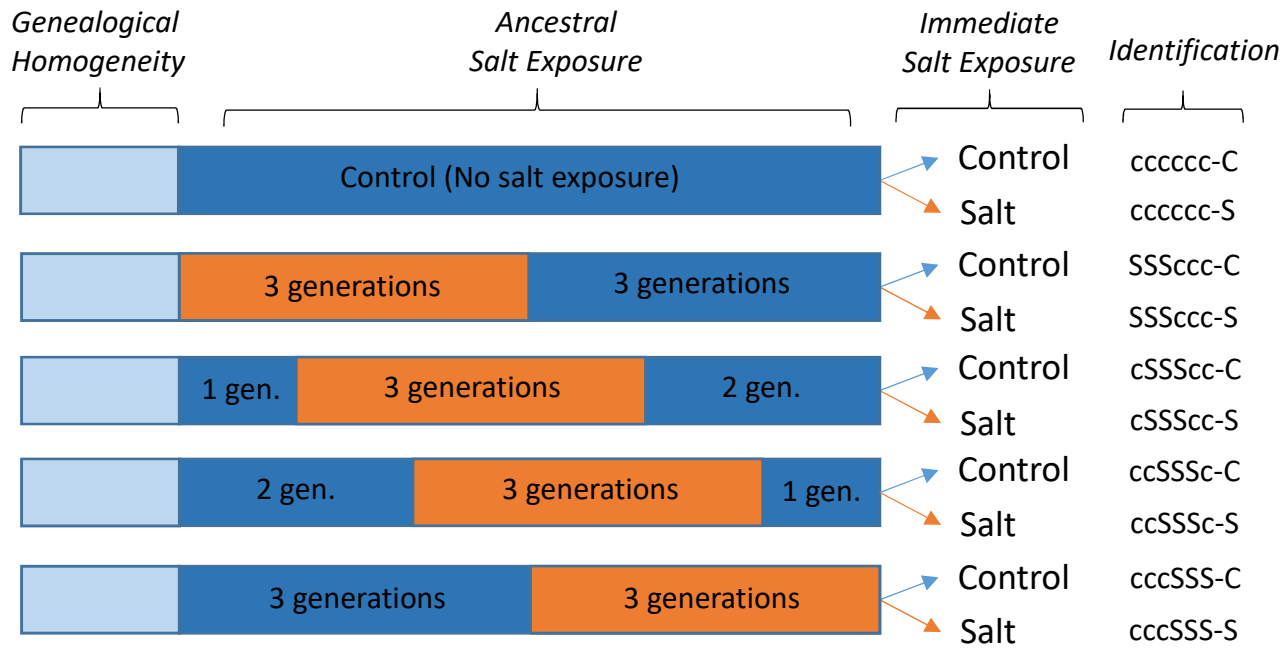

Figure 3.1: Diagram of the experimental procedure used. 'Genealogical Homogeneity' refers to the process of selecting first offspring over several clonal generations to ensure genealogical homogeneity. Ancestral salt exposure shows the six consecutive generations of fronds (going from left to right) which are exposed to either salt (orange) or control (blue) growth medium. Each row shows a different ancestral treatment, for a total of five. Immediate salt exposure shows that the fronds from each ancestral treatment are split into two groups, which are exposed to the immediate treatment. Identification gives the name of each treatment combination.

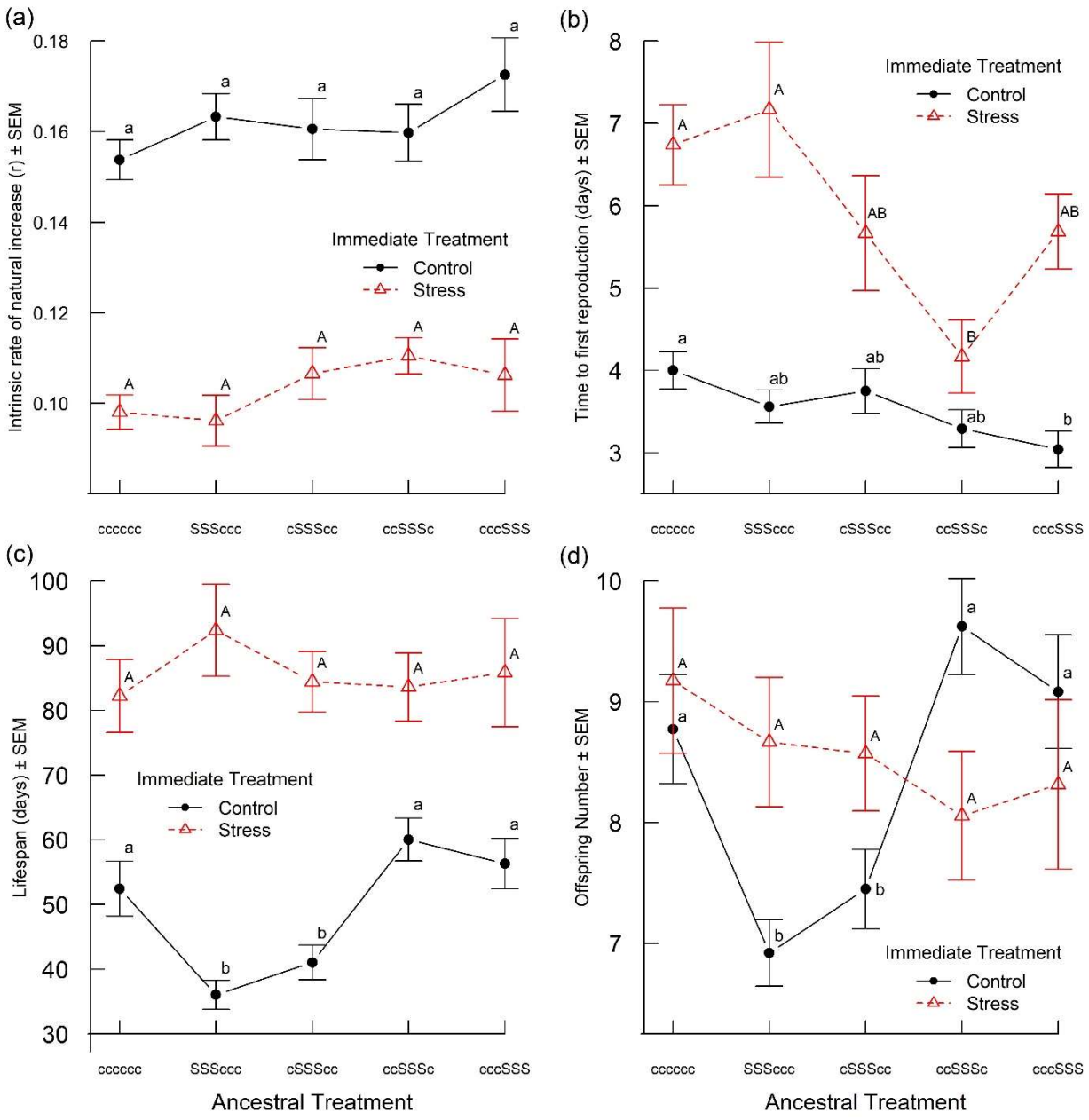

Figure 3.2: Interactive effects of ancestral treatment (horizontal axis) and immediate treatment (black circles = control; red triangles = stress) on (a) mean time to first reproduction, (b) lifespan, (c) mean total offspring number, and (d) mean intrinsic rate of natural increase (*r*). Error bars indicate one standard error of the mean. Means within each immediate treatment sharing the same letter are not significantly different ($p < 0.05$) according to post-hoc pairwise t-tests corrected for multiple comparisons.

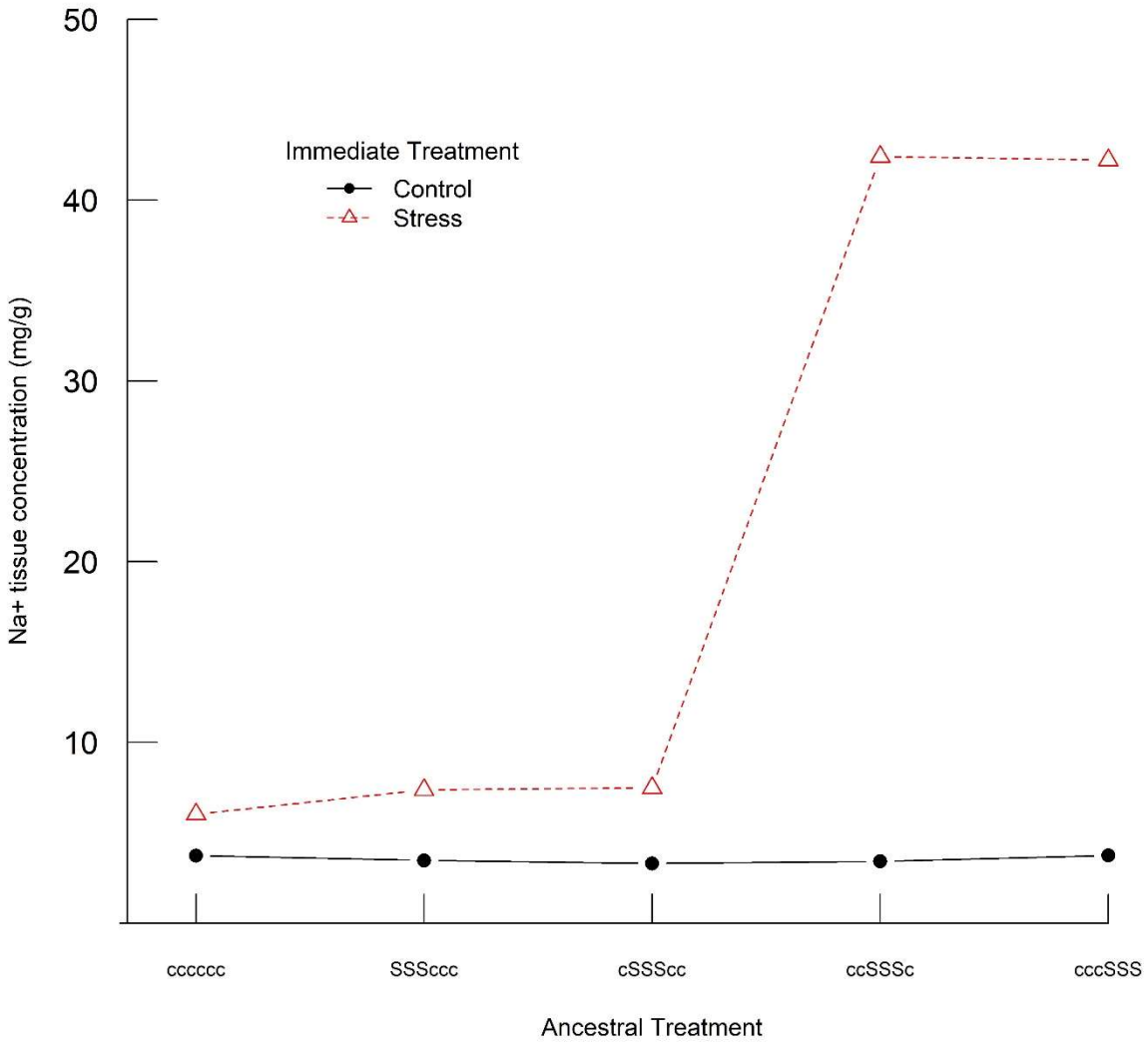

Figure 3.3: Interactive effects of ancestral treatment (horizontal axis) and immediate treatment (black circles = control; red triangles = stress) on whole-plant Na^+ tissue concentrations, as measured by flame atomic absorption spectroscopy. Variance and statistics were unable to be measured due to lack of replication (n = 1 per treatment combination), and likely interaction effect.

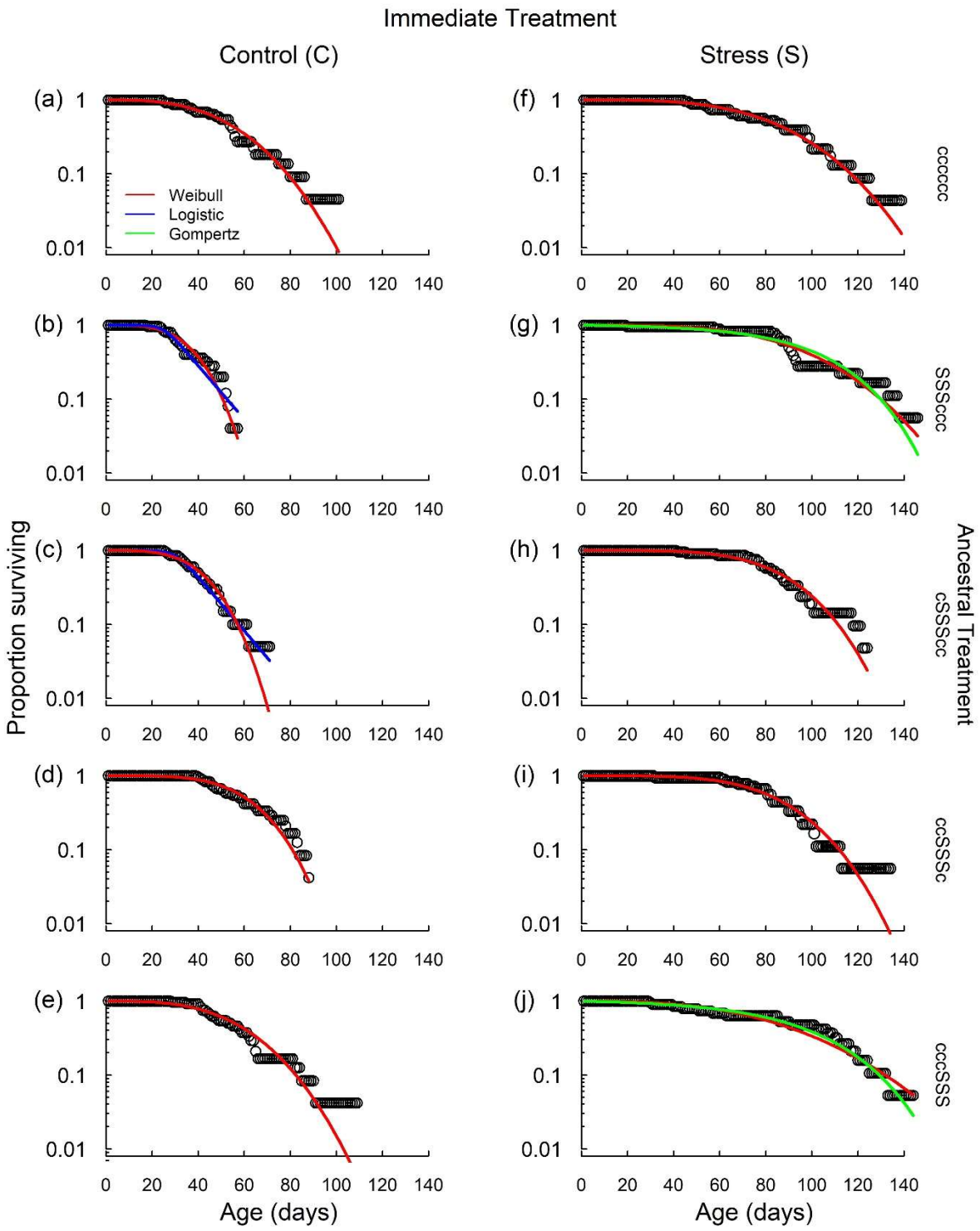

Figure 3.4: Declines in survival with increasing age for each treatment. Lines indicate model(s) with the best fit (ΔAICc < 2; red = Weibull, blue = logistic, green = Gompertz). (a) – (e) Immediate treatment C (control); ancestral treatments cccccc, SSSccc, cSSScc, ccSSSc, and cccSSS, respectively. (f) – (j) Immediate treatment S (stress); ancestral treatments cccccc, SSSccc, cSSScc, ccSSSc, and cccSSS, respectively.

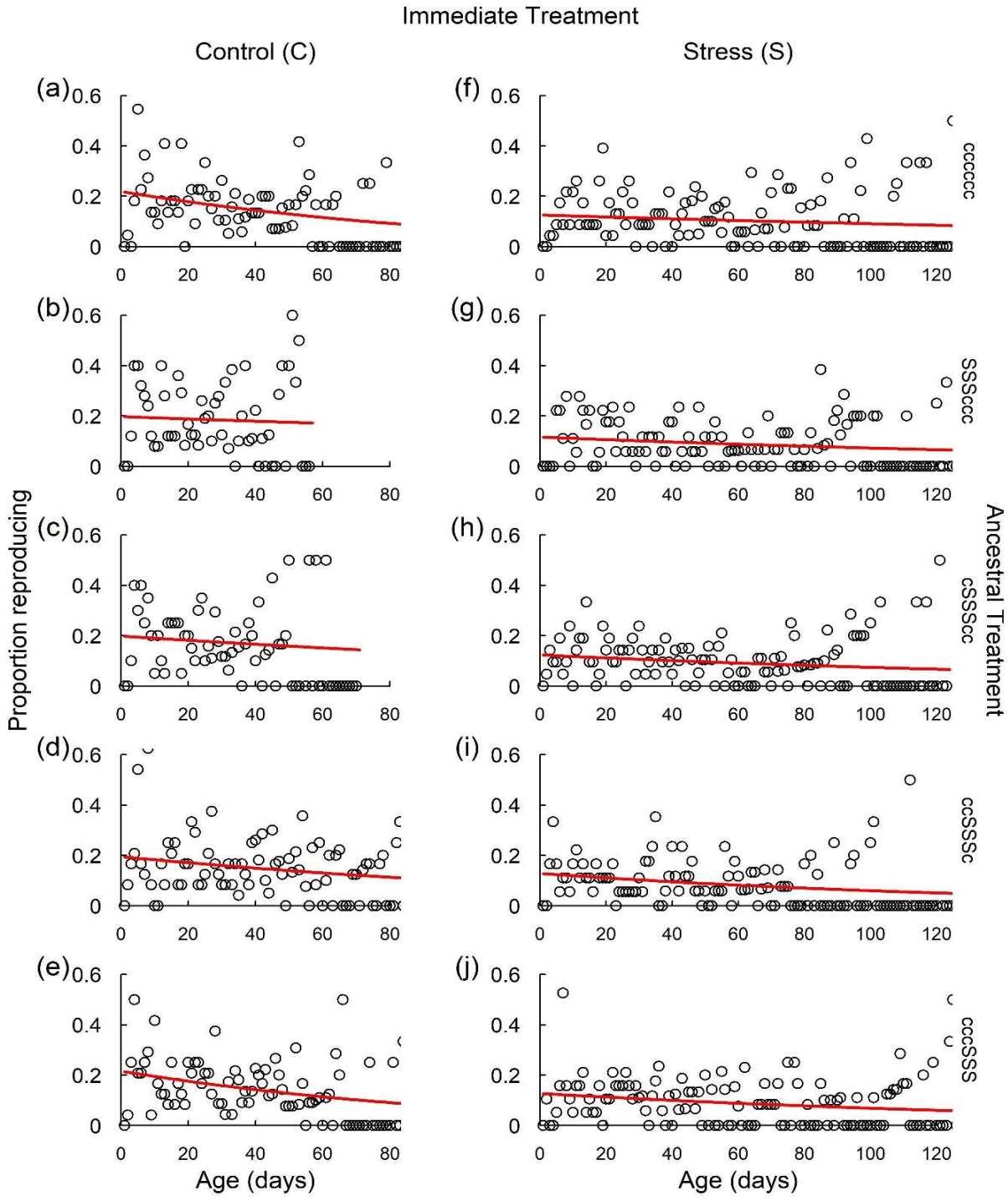

Figure 3.5: Declines in reproduction with increasing age for each treatment. For better visibility, graphs have been truncated at 0.6 proportion reproducing, and at 90 and 130 days for the immediate control and stress treatments respectively. The full data can be seen in Supplementary Figure 2.1. Lines indicate the AR1 model. (a) – (e) Immediate treatment C (control); ancestral treatments cccccc, SSSccc, cSSScc, ccSSSc, and cccSSS, respectively. (f) – (j) Immediate treatment S (stress); ancestral treatments cccccc, SSSccc, cSSScc, ccSSSc, and cccSSS, respectively.

CHAPTER 4: GENERAL DISCUSSION

4.1 Research summary

The ability to tolerate stress is important in environments with variable conditions, as higher stress tolerance is related to higher fitness (Bradshaw, 1965; DeWitt et al., 1998). While many factors can contribute to a stress tolerance ability, I aimed to examine how history affects offspring stress tolerance and fitness in this thesis. All offspring produced by a parent are not of the same quality, since offspring fitness varies with parental age, and this can have impacts on the age-specific force of natural selection and how it is estimated (Kern et al., 2001; Priest et al., 2002; Pavard et al., 2007; Gillespie et al., 2013b; Barks & Laird, 2015, 2020). Parental and ancestral environments can affect the fitness of offspring, and provide greater ability to tolerate stress in some, but not all cases (Boyko et al., 2010; Suter & Widmer, 2013b).

In Chapter 2, I showed that offspring fitness changes with birth order, and decreases with increasing salt concentration. Birth order affected the way in which offspring responded to stress, although their overall tolerance to the stress in terms of fitness was not affected by these changes. First-born fronds grew larger with increasing salt concentrations at the cost of producing their first offspring more slowly, compared to fifth-born offspring, which showed little change in size or time to first reproduction with increasing salt concentrations. The component of offspring fitness that was most affected by the salt stress was the early production of offspring, although other components were also affected.

Chapter 3 showed that immediate salt stress conditions resulted in a decreased fitness of fronds, due to slowed reproduction. The effects of ancestral stress were more complex. Fronds were prepared for stress and able to produce their first offspring more quickly, particularly when the ancestral stress was recent. However, these offspring could be of lower quality, suggested by

the decreased offspring number and lifespans of the fronds in immediate control conditions combined with distant ancestral stress.

The timing of reproduction is highly important to the fitness of *L. minor*. Generally, the timing of reproduction is known to be important, as early-produced offspring contribute more to population growth than those produced later in life (Stearns, 1992), and reproductive values decline with age (Fisher, 1930). This is illustrated particularly well in the low and medium salt treatments of Chapter 2, showing a similar number of offspring, time to first reproduction, and increased lifespan compared to the control, which are all changes that should increase fitness. Yet the fitness of fronds in these treatments is decreased, simply due to the low proportion of fronds reproducing at young ages. In Chapter 3, ancestral stress prioritizes a faster time to first reproduction, with a greater proportion of fronds reproducing early in life. Stress conditions slowed reproduction, and fronds had greater fitness when they were able to reproduce more quickly.

Chapter 2 showed longevity hormesis with increasing salt concentration, in which the salt concentrations of the low and medium treatments increased the lifespan of fronds, but had a strong detrimental effect at the high concentration. Exposure to a mild stress disrupts homeostasis, and can result in an overcompensation of maintenance, repair, or protection mechanisms, providing a greater ability to tolerate stress in the future (Calabrese & Baldwin, 2002; Rattan, 2008; Calabrese et al., 2015), and repairing or slowing damage accumulation that can result in senescence (Rattan, 2008; Milisav et al., 2012). While examination of mechanisms of senescence is beyond the scope of this thesis, an example of a potential mechanism responsible for the changes observed could be reactive oxygen species (ROS), and the oxidative damage they cause. ROS are produced by normal cellular functions (Foyer & Noctor, 2003; Gill & Tuteja, 2010), and the accumulation of oxidative damage over time can result in senescence (Harman, 1992; Lin & Beal, 2003; Gems & Doonan,

2009; Barja, 2019). Stress can increase the production of ROS, and has been shown to increase in duckweed exposed to salt stress (Bartosz, 1997; Oukarroum et al., 2015). Antioxidants counteract ROS, so increasing the production of antioxidants can decrease the oxidative damage caused by ROS (Panda & Upadhyay, 2004; Gill & Tuteja, 2010; Chang et al., 2012; Hasanuzzaman et al., 2012). As ROS play a role in both stress-induced damage, and age-induced damage, the benefits obtained from upregulated antioxidant production in the stress response will also act on the damage that causes senescence. This could delay senescence, and increase longevity. The hormesis effect is also visible in the extended lifespans of the immediate treatment of Chapter 3.

While all ramets in this study were asexually produced and genetically identical to each other (i.e., aside from rare mutations), they exhibited differing responses in different treatments, indicating the ability to produce plastic responses. Previous studies have examined phenotypic plasticity in *L. minor*, with fitness being the least plastic trait measured (Vasseur & Aarssen, 1992). Plastic responses can be adaptive, or non-adaptive, best differentiated by the effects on fitness (Merilä & Hendry, 2014). The fronds of birth order 1 in the increasing salt concentrations of Chapter 2 show a non-adaptive plastic response, considered non-adaptive since the observed larger frond size and slower time to first reproduction show corresponding decreases in fitness. Chapter 3 however, suggests an adaptive plastic response, which may be inherited by offspring. Recent multigenerational ancestral stress results in a faster time to first reproduction, which increases fitness. This is also supported by previous studies, which have found that in duckweed exposed to salt stress, following the initial period of mortality and decreased growth, population growth exhibits a recovery (O'Brien et al., 2020). Since variation in genetic inheritance is not expected due to the asexual nature of reproduction, this suggests *L. minor* is capable of a non-genetic method of inheritance.

Plastic responses can allow asexual organisms to respond quickly to changes in their environment (Latzel & Klimešová, 2010; Verhoeven & Preite, 2014; Dodd & Douhovnikoff, 2016). However, the plastic responses can depend on reliable environmental cues, and may have associated costs of expression (Van Kleunen & Fischer, 2005; Auld et al., 2010; Murren et al., 2015). When cues are unreliable, or plasticity is constrained, bet-hedging can evolve (Gillespie, 1974), and it is possible for plasticity and bet-hedging to occur simultaneously (Donaldson-Matasci et al., 2013; Simons, 2014; Gremer et al., 2016). Bet-hedging can be diversifying, in which multiple phenotypes are produced with the expectation that at least one will survive, a strategy which increases long-term fitness at the cost of immediate fitness of some individuals (Simons, 2011). This has been seen previously in duckweed (Mejbel & Simons, 2018), and could be a reason for the change in fitness between the first- and fifth-born offspring.

4.2 Study limitations

While the intrinsic rate of increase is a generally good indicator of fitness, it assumes a constant environment and an unchanging population growth rate and age composition (Hamilton, 1966; Murray Jr, 1992; Benton & Grant, 2000; Metcalf & Pavard, 2007). The nature of the treatments in Chapter 3 result in this assumption not being met, which means this fitness measure must be interpreted with caution in that instance. Additionally, the intrinsic rate of increase was calculated at the level of individual fronds (McGraw & Caswell, 1996), but future studies involving non-constant environments would benefit from directly tracking realized population growth as a means of measuring fitness.

Another limitation based on the physiology of *L. minor* is that offspring remain attached to parents, and sometimes grandparents for a short duration before their birth (Lemon & Posluszny,

2000). In Chapter 3, this called into question if changes observed were due to inheritance of characters from a parent, or due to a short, direct exposure of the recent ancestral stress treatment. While the results of Chapter 3 suggest a non-genetic transfer of information, further evidence must be collected. Tissue concentrations of Na^+ suggest a direct transfer of salt from parent to offspring, which could explain some, but not all changes. Epigenetic changes are a plausible cause, given the benefits of adaptive phenotypic plasticity in a changing environment for an asexual species (Latzel & Klimešová, 2010; Verhoeven & Preite, 2014), as well as the finding that epigenetic changes occur with thermal stress in *L. minor* (Prelovšek, 2018).

The studies in Chapters 2 and 3 use a single strain of *L. minor*, from which all ramets are asexually produced. As such, all individuals are from the same genet. While groups of ramets function as a population (Orive, 1995), and are an appropriate level to examine here, only a single genet was investigated. The conclusions of these studies are applicable to this strain of *L. minor*, but further investigation in other genets should be done to see if similar results emerge, before drawing conclusions about the species as a whole.

4.3 Future directions

From these studies come questions which require further investigation. In chapter 2, a higher fitness in the fifth birth order than the first was observed, suggested to be due to a hump-shaped curve in offspring fitness with increasing parental age, similar to that observed in *L. turionifera* (Barks & Laird, 2016). A hump-shaped pattern was found in the same strain of *L. minor*, although the peak of offspring fitness occurred much earlier in this study, and appeared as a near-monotonic decline with increasing parental age (Barks & Laird, 2015). Investigation of older birth orders should be done to observe changes in offspring quality with older parental age,

followed by investigation into the causes of these changes. Is the change in offspring quality with increasing birth order caused by differing developmental environments, dependent on parental size or maturity, followed by declines due to stipe accumulation? Or are offspring of differing birth orders optimized for different environments, following a diversifying bet-hedging strategy, and would show different fitnesses in differing environmental conditions?

Following from chapter 3, the values of the intrinsic rate of increase calculated from population projection matrices should be compared to values obtained by observing population growth, in order to determine how closely the former reflects the latter under changing environmental conditions such as temporary salt stress exposure. It would also be beneficial to determine if multigenerational stress increases fitness compared to a single stressed generation, indicating an adaptively plastic response to salt stress. Further, a greater number of ancestral generations should be examined, to see if the declines in offspring quality suggested with distant ancestral stress continue, or if they show recovery. The evidence of non-genetic inheritance in response to salt stress should also be examined further. This could include investigating epigenetic changes caused by salt stress in *L. minor*, examination of whether there is a direct transfer of Na^+ from parent to offspring, or if some other type of non-genetic inheritance occurs.

In both chapters, hormesis was observed, suggesting that salt stress upregulates repair pathways that also affect longevity. Perhaps cross-tolerance of different stressors may also occur. Investigation into these mechanisms and pathways could provide insight into the connection of stress and longevity, and allow comparison of the pathways present in plants, as compared to animals.

4.4 Conclusion

The chapters of this thesis show the importance of ancestral history on the fitness of *L. minor*. Parental age and birth order changes the fitness of offspring, and their strategy of stress tolerance, although it does not increase their overall stress tolerance. Ancestral exposure to salt stress affects how future generations respond to the same stressor, potentially indicating an adaptively plastic response. Both parental characteristics and ancestral environments affect offspring in *L. minor*.

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Appendix 1: Supplementary Tables for Chapter 2

Supplementary Table 1.1: The parameters of the best-fit models of survival (ΔAICc < 2) for each treatment. The lines for the Weibull models are given by $[proportion\ surviving] = \exp(-(a[age])^b)$ when a and b are as specified in the appropriate treatment row. The lines for the logistic models are given by $[proportion\ surviving] = (1 + \frac{ac(\exp(b[age])-1}{b})^{-1/c}$, when a, b, and c are as specified in the appropriate treatment row. The lines for the Gompertz models are given by $[proportion\ surviving] = \exp(-\left(\frac{a}{b}\right)(\exp(b[age]) - 1)$ when a and b are as specified in the appropriate treatment row. Lines for the exponential models are given by $[proportion\ surviving = \exp(-a[age])$ when a is as specified in the appropriate treatment row.

Treatment	Best fit model(s)	*a*	*b*	*c*
C1	Weibull	0.012	2.42	-
L1	Weibull	8.19×10^{-3}	4.97	-
	Gompertz	2.31×10^{-4}	0.042	-
L1	Weibull	8.55×10^{-3}	4.42	-
	Gompertz	3.53×10^{-4}	3.94	-
H1	Weibull	0.024	1.76	-
	Gompertz	0.028	0.011	-
C5	Weibull	0.012	2.19	-
	Logistic	4.94×10^{-5}	0.17	7.39
L5	Weibull	9.34×10^{-3}	3.80	-
	Logistic	7.40×10^{-5}	0.075	1.47
M5	Gompertz	3.53×10^{-4}	0.039	-
H5	Exponential	0.028	-	-
	Weibull	0.030	0.89	-
	Gompertz	0.011	0.028	-

Supplementary Table 1.2: Parameters of the generalized estimating equations used fit to reproduction data for each treatment. The AR1 correlation structure was used for all, based on the biological relevance of the model. The lines are given for each group by $[proportion\ reproducing] = \frac{\exp(a+b[age])}{1+\exp(a+b[age])}$, where *a* and *b* are as specified in the appropriate treatment row. The exchangeable correlation structure is also provided for the L1 treatment, as it provided an equally good fit, with a visually different line, and is given by $[proportion\ reproducing] = \frac{\exp(a+b[age])}{1+\exp(a+b[age])}$.

Group	Correlation structure	*a*	*b*
C1	AR-1	-1.65	-9.10×10^{-3}
L1	AR-1	-1.96	6.41×10^{-3}
	Exchangeable	-2.39	4.70×10^{-3}
M1	AR-1	-2.19	-6.08×10^{-3}
H1	AR-1	-3.53	0.017
C5	AR-1	-1.65	-8.45×10^{-3}
L5	AR-1	-1.89	7.87×10^{-3}
M5	AR-1	-2.21	-5.65×10^{-3}
H5	AR-1	-2.41	-8.24×10^{-3}

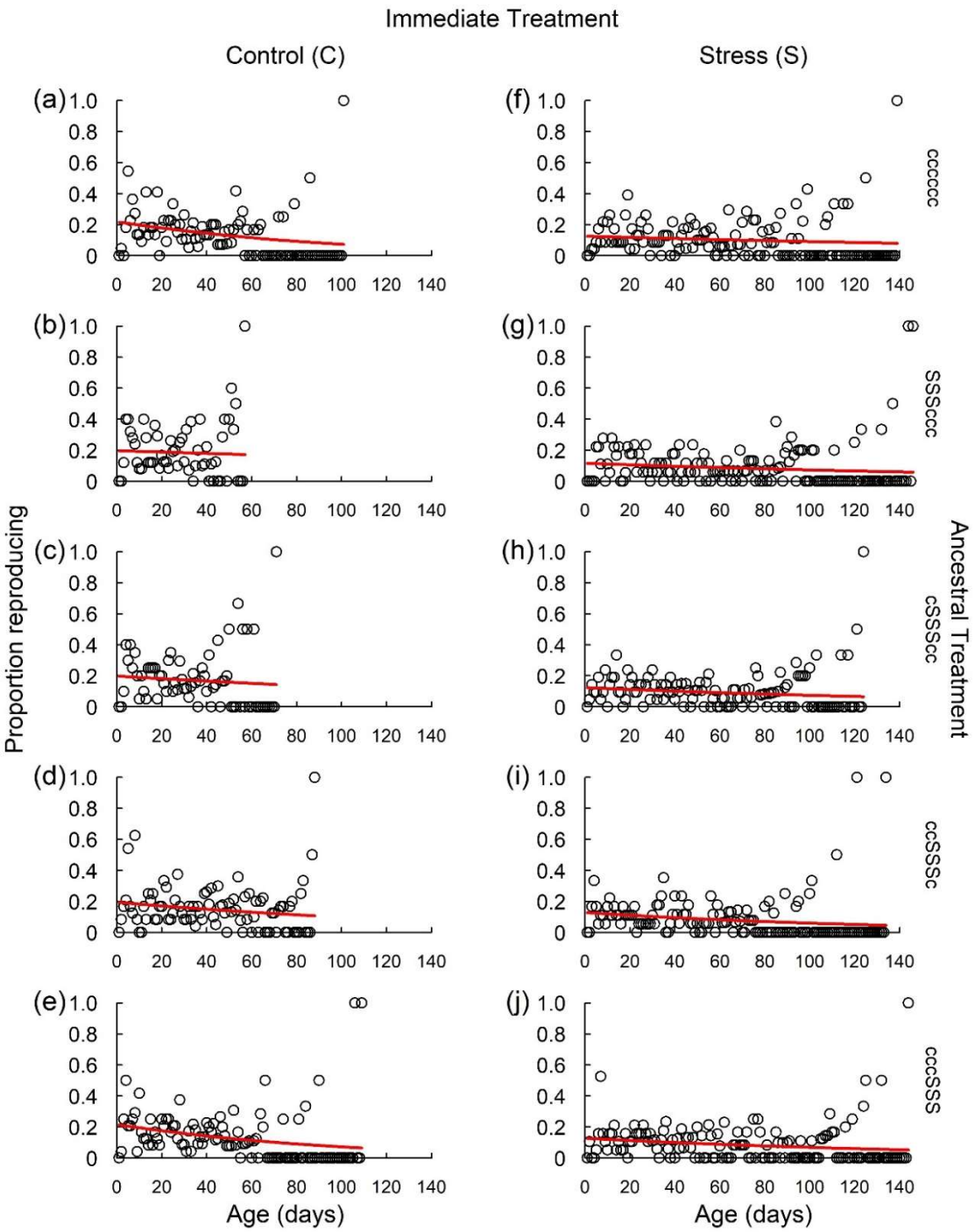
Appendix 2: Supplementary Tables and Figures for Chapter 3

Supplementary Table 2.1: The parameters of the best-fit models of survival (ΔAICc < 2) for each treatment. The lines for the Weibull models are given by $[proportion\ surviving] = \exp(-(a[age])^b)$ when a and b are as specified in the appropriate treatment row. The lines for the logistic models are given by $[proportion\ surviving] = (1 + \frac{ac(\exp(b[age])-1}{b})^{-1/c}$, when a, b, and c are as specified in the appropriate treatment row. The lines for the Gompertz models are given by $[proportion\ surviving] = \exp(-\left(\frac{a}{b}\right)(\exp(b[age]) - 1)$ when a and b are as specified in the appropriate treatment row.

Treatment	Best fit model(s)	*a*	*b*	*c*
cccccc-C	Weibull	0.017	2.88	-
SSSccc-C	Weibull	0.025	3.58	-
	Logistic	7.88×10^{-6}	0.37	4.44
cSSScc-C	Logistic	4.61×10^{-6}	0.33	3.86
	Weibull	0.022	3.66	-
ccSSSc-C	Weibull	0.015	4.17	-
cccSSS-C	Weibull	0.016	3.10	-
cccccc-S	Weibull	0.011	3.43	-
SSSccc-S	Gompertz	9.28×10^{-4}	0.034	-
	Weibull	9.78×10^{-3}	3.48	-
cSSScc-S	Weibull	0.011	4.49	-
ccSSSc-S	Weibull	0.011	4.20	-
cccSSS-S	Gompertz	1.61×10^{-3}	0.029	-
	Weibull	0.010	2.71	-

Supplementary Table 2.2: Parameters of the generalized estimating equations used fit to reproduction data for each treatment. The AR1 correlation structure was used for all, based on the biological relevance of the model. The lines are given for each group by $[proportion\ reproducing] = \frac{\exp(a+b[age])}{1+\exp(a+b[age])}$, where a and b are as specified in the appropriate treatment row.

Group	*a*	*b*
cccccc-C	-1.27	-0.013
SSSccc-C	-1.40	-2.95×10^{-3}
cSSScc-C	-1.40	-5.46×10^{-3}
ccSSSc-C	-1.42	-7.99×10^{-3}
cccSSS-C	-1.29	-0.013
cccccc-S	-1.94	-3.64×10^{-3}
SSSccc-S	-2.03	-5.18×10^{-3}
cSSScc-S	-1.96	-5.64×10^{-3}
ccSSSc-S	-1.91	-8.40×10^{-3}
cccSSS-S	-1.92	-6.82×10^{-3}



Supplementary Figure 2.1: Declines in reproduction with increasing age for each treatment. Full data shown, truncated version can be seen in Figure 3.5. Lines indicate the AR1 model. (a) – (e) Immediate treatment C (control); ancestral treatments cccccc, SSSccc, cSSScc, ccSSSc, and cccSSS, respectively. (f) – (j) Immediate treatment S (stress); ancestral treatments cccccc, SSSccc, cSSScc, ccSSSc, and cccSSS, respectively. At the maximal lifespan of each treatment, reproduction always has a proportion of 1.0, as death is defined as the date of last reproduction, and very few fronds (often n = 1) reach the maximal lifespan.