

**Clonal variation: birth order produces life-history diversity in the Greater
Duckweed, *Spirodela polyrhiza***

by

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Abstract

Evolutionary response to environmental variation is required for survival and may occur through adaptive tracking, phenotypic plasticity or bet hedging. Variable dormancy is a classical bet-hedging trait in literature, and enables survival through harsh conditions. Greater Duckweed, *Spirodela polyrhiza*, forms overwintering propagules called “turions”. However, *S. polyrhiza* propagates clonally, and it is unknown how diversified dormancy behaviour is generated among genetically identical offspring. Here, I investigate sources of variation in turion formation in *Spirodela polyrhiza*. I tested whether turion production is stimulated by birth order, size and temperature under controlled conditions of a growth chamber and a thermogradient incubator. Parental birth order was found to have a significant effect on turion formation; furthermore, this effect is temperature-dependent. This study addresses fundamental questions on mechanisms generating diversity in dormancy behavior, and provides insight into the natural history of *Spirodela polyrhiza*, an emerging model organism for ecological studies.

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Glossary

Adaptive phenotypic plasticity: the modification of trait expression in response to changes in environmental conditions such that reproductive success is increased. If an organism normally faces certain environmental conditions (ex. seasonality) or a range of environments and the phenotype expressed is appropriate and reproductive success is maintained or enhanced, adaptive plasticity has evolved. In response to environmental variability, adaptive phenotypic plasticity is ideal, however its effectiveness is limited. This is due to the dependence on reliable cues as well as the constraints of limited heritable genetic variance.

Adaptive tracking: The most general form of microevolution where the selection of different phenotypes is favoured and results in genetic changes in populations over time. In response to environmental change, suboptimal traits are continuously removed while changes in optimal trait values lead to the expression of phenotypes that have increased allele frequency of those optimal traits. The effectiveness of tracking thus depends on the genetic variance availability and rate of environmental change.

Bet hedging: an alternate form of adaptation as a response to unpredictable environmental conditions not normally encountered by an organism. It is the “sacrifice” of arithmetic mean fitness that decreases the variation in fitness and leads to the maximization of long-term geometric fitness over generations. Bet hedging may seem maladaptive under average conditions, as the immediate fitness within generations is not maximized. There are two forms of bet hedging. Conservative bet hedging, or playing it safe, avoids risk by expressing a single safe phenotype rather than the optimal phenotype for current conditions, and reduces the among-generation variance in fitness. Diversified bet hedging is an alternative form that expresses several phenotypes to spread the risk instead of a single “optimal” phenotype and thus avoids complete failure. This reduces between-individual correlations in fitness. Thus far, very little research has been conducted on this mode of response.

Environmental cue: information that reliably predicts future conditions. This could potentially lead to the evolution of a response. Environmental cues can be seasonally characteristic such as temperature and length of photoperiod, and can trigger the onset or termination of a particular behaviour (e.g. dormancy) at the most appropriate time. This could impact organismal growth and development. An environmental cue has to be reliable for the evolution of phenotypic plasticity but if the cue is unreliable, bet hedging may evolve.

Environmental unpredictability: spatiotemporal variation in the conditions faced by an organism including physical, chemical and/or biological factors, such as temperature, light, and resource availability for which no reliable cues exist. Organisms are unable to predict the future state accurately in these conditions. If faced with unpredictable environmental change, bet-hedging traits (see **Bet hedging** above) are expected to evolve.

Evolution: while different definitions of evolution exist, it could be described as a heritable changes in a population over successive generations. Different sources can cause evolutionary change in a population. The four main forces of evolution are mutation, gene flow, genetic drift, and natural selection. A **mutation** is a random change to an organisms DNA sequence resulting from errors during replication. **Gene flow** (e.g. migration) occurs when genetic material is exchanged between separate populations and works to reduce the variation between the populations. **Genetic drift** is the random change in allele frequency in a population caused by random sampling of gametes or a bottleneck event. Genetic drift has the strongest effects on small populations and effects genes already existing in the population. **Selection** is the variation in reproductive success that is associated with variation in that particular trait. Variation in the trait has to have a genetic basis for the trait to evolve through natural selection. The rate at which the trait evolves depends on how much variation exists and its interaction with the environment. Negative selection removes suboptimal alleles while positive selection favours optimal alleles associated with phenotypes that are more likely to increase the number of offspring. These causes may influence populations over many generations and can have beneficial, harmful or neutral effects.

Fitness: A measure of the ability to survive to reproductive age and produce offspring. Fitness can depend on several factors, such as mutation and the environmental conditions faced by the organism. Relative fitness is measured by an organism's reproductive success compared to that of the fittest individual in the population, whereas absolute fitness is measured as the organism's total reproductive success.

Genetic variance: variation in genetic composition of individuals within populations, often measured as "heritability." Variation could have a negative or neutral impact on a population's ability to survive or could enhance a population's ability to adapt to changing environmental conditions and persist.

Life-history trait: Traits that affect an organism's life table, including lifespan, number and size of offspring, and age at reproduction. Life history traits vary depending on the environmental conditions encountered and evolve through natural selection. Variation in one trait may be correlated with the variation of another (characterized as tradeoffs between fecundity, growth, and survival).

Norm of reaction: The pattern of phenotypes expressed of a given genotype for a single trait at specified levels of particular environmental factors. Norms of reaction can thus depict phenotypic plasticity graphically, as a function of the phenotypic distribution for a given genotype and its interaction with changing environmental factors. Variance in norms of reactions can be used to assess genetic differences among individuals or populations to respond to change, as well as the evolution of phenotypic plasticity. Norms of reaction are not expected to be adaptive to novel environmental variation. If cues are available and allow the prediction of future environments, norms of reaction may evolve such that appropriate phenotypic modifications can be made to adjust the phenotype to the environment.

Phenotypic plasticity: The variable expression of phenotypes across different environmental conditions. Organisms may respond either directly to environmental changes, or to environmental cues, such as temperature and light. Plasticity can be expressed at the morphological (changes in leaf size, for example), behavioural (timing of germination), or physiological level (temperature acclimation) and can evolve if there is adequate genetic variation in norms of reaction. The response may be adaptive (see **Adaptive phenotypic plasticity**) or non-adaptive.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Implications of environmental variation

Assessing the effects environmental variability has on fitness is necessary for predicting organismal survival in changing environments. Every organism experiences environmental variation to some degree and because this variation may affect reproductive success, appropriate response to these changes is required. Organisms may respond directly to environmental change through physiological or behavior alterations (Chown *et al.*, 2010, Cleland *et al.*, 2007), indirectly through evolutionary change (Bell *et al.*, 2009, Charmantier and Garant 2005), or possibly a combination of direct and indirect responses (Simons 2011, Travis *et al.*, 2013). For instance, direct physiological or behavioral responses are often temporary and ultimately molded by evolution (Chown *et al.*, 2010). Due to the potential detrimental effects of climate and other environmental changes on biodiversity and ecosystem services, an increasing amount of research is committed to elucidating evolutionary response to environmental variation (Bell *et al.*, 2009, Bellard *et al.*, 2012, Chown *et al.*, 2010).

Changing environments may cause variable selection, where traits that are favorable at one time (generation or season, for example) may be unfavourable later on (Ellner *et al.*, 1999, Simons 2009). Adaptive tracking and phenotypic plasticity are two potential evolutionary responses to environmental change that have been extensively studied (Botero *et al.*, 2014, Cleland *et al.*, 2007, Simons 2011, Tufto 2015). With adaptive tracking, natural selection favors different phenotypes, resulting in genetic

change in populations over time (Cleland *et al.*, 2007, Bell and Gonzalez 2009, Gonzalez *et al.*, 2012). The effectiveness of tracking is limited by the availability of genetic variance, and by the rate of environmental change (Auld *et al.*, 2009, Charmantier and Garant 2005, Wilson *et al.*, 2006). Alternatively (or concurrently), organisms may survive predictable environmental change (such as seasonality) through expression of adaptive phenotypic plasticity, where organisms adapt to change through an adjustment of phenotypic expression during their lifetime either through development, morphology or physiology (Levins 1968, Thomas 1991). Phenotypic plasticity may be a non-adaptive result of a changing environment, or an adaptive response (Ghalambor *et al.*, 2007, Nussey *et al.*, 2005). The alteration of trait expression such that reproductive success is increased—adaptive phenotypic plasticity—may only occur when the organism faces predictable change in environmental conditions (Levins 1968). For example, due to drier spring weather in Yukon, white spruce (*Picea glauca*) cone production has increased, allowing female North American red squirrels (*Tamiasciurus hudsonicus*) to advance their parturition by 18 days over a ten year period (Réale *et al.*, 2003). This adjustment in breeding dates could have resulted from phenotypic plasticity as a direct response to environmental conditions (warmer temperatures, increased food abundance) or from the microevolution towards earlier breeding times. The reproductive success of the squirrels was maintained following the climate change, suggesting that earlier breeding dates is appropriate and that the squirrels have adapted to those environmental conditions (Charmantier *et al.*, 2008). However, as the evolution of adaptive plasticity requires a predictable relationship between environments and fitness associated with a certain trait, norms of reaction are not expected to be adaptive to novel environmental variation.

Studies that assessed the effectiveness of plasticity in changing environments found certain norms of reaction to be maladaptive and that the efficiency of phenotypic plasticity depended on the temporal stability of the processes essential to reproductive trade-offs (Charmantier *et al.*, 2008, Nussey *et al.*, 2005, Simons 2011).

By expressing the appropriate phenotype, adaptive phenotypic plasticity in response to changing environments is ideal; however, the evolution of plasticity is seriously limited (van Kleunen and Fischer 2005). The effectiveness of adaptive phenotypic plasticity is limited due to the dependability of environmental conditions as well as the constraints of limited genetic variance (Auld *et al.*, 2009, Murren *et al.*, 2015, van Kleunen and Fischer 2005). In addition, time delays occur between receiving relevant environmental cues and processing novel phenotypes, further limiting adaptive plasticity effectiveness (Moran 1992, Snell-Rood 2012). Although research interprets adaptive phenotypic plasticity as an evolutionary response independent of adaptive tracking, it itself evolves through tracking over longer time scales (Simons 2011). Thus, in conditions under which adaptive tracking and phenotypic plasticity are constrained, survival must depend on a different form of response.

Although adaptive tracking and phenotypic plasticity are both evolutionary responses to environmental change, they are not effective responses to unpredictability. Organisms may respond to unpredictable environmental change through an alternate form of adaptation, bet hedging (Slatkin 1974). A bet-hedging strategy is a trait that maximizes long-term fitness in varying and unpredictable environments by sacrificing mean fitness to decrease variation in fitness (Philippi and Seger 1989, Seger and Brockmann 1987). Technically, bet-hedging traits maximize geometric mean fitness over generations

(Philippi and Seger 1989). Because maximization of geometric mean fitness occurs across generations and thus does not maximize immediate or “expected” fitness within generations, bet-hedging adaptations appear to be maladaptive under average conditions (Gillespie 1974, Seger and Brockmann 1987, Simons 2009).

Bet-hedging traits are expressed as either being conservative or diversified. Conservative bet hedging, or “playing it safe”, is risk avoidance through the expression of a phenotype that reduces fitness variation (Philippi and Seger 1989, Seger and Brockmann 1987). For example, conservative bet hedging is expressed when reproduction of semelparous plants is assured by being restricted to a safe period earlier in the season, even though later dates would be optimal during “normal” seasons (Hughes and Simons 2014, Simons and Johnston 2003). This is because initiating reproduction late in the season may lead to complete reproductive failure if the season is unexpectedly truncated. Alternatively, diversified bet hedging occurs when an organism expresses several phenotypes to “spread the risk”, rather than a single phenotype that is expected to be optimal, thus reducing fitness variation and preventing complete failure (Philippi and Seger 1989). For example, although seeds produced by a parental *Lobelia inflata* plant are genetically identical, they show a high degree of variation in the timing of germination within seasons that appears to be optimal for the extent of environmental unpredictability affecting seedling success (Simons 2009).

Environmental variation is both predictable and unpredictable, and a combination of evolutionary responses to variation may be expressed (Simons 2014). Under fluctuating environmental conditions, phenotypic plasticity and bet hedging may jointly evolve to impact fitness. For example, both plasticity and bet hedging can be co-

expressed in seed germination (Cohen 1967, Simons 2009). While plasticity evolves under reliable environmental cues, the cues received by an organism may be imperfect, and may not allow optimal expression in this new environment. As the elucidation of the interaction between plasticity and bet hedging is lacking in the literature, more research is needed to better analyze behaviours that jointly express these two modes of responses.

1.2 Dormancy as phenotypic plasticity and bet hedging response

Dormancy is commonly cited as an adaptation to environmental change as both a bet-hedging strategy and as adaptive phenotypic plasticity (Gremer *et al.*, 2016, Gremer and Venable 2014, Fernandez-Pascual and Jimenez-Alfaro 2014, Nilsson *et al.*, 1996, Simons 2014). Many animal and plant species exhibit dormancy and evolve life-history stages to mitigate survival through harsh environments (e.g., winter) by depressing metabolic functions and stopping development (Silsbury 1961). Furthermore, norms of reaction, or the pattern of phenotypic expression across environments, frequently evolve as a response to local heterogeneous environments (Reed *et al.*, 2011, Via and Lande 1985). Dormancy periods differ in time and in level of metabolic reduction, ranging from slightly slowing metabolism to more intense reductions and of prolonged length. Certain cues that may trigger the onset of dormancy include temperature (Baskin and Baskin 1998, Martin 1946), photoperiod (Vleeshouwers *et al.*, 1995), nutrient availability (Baskin and Baskin 1998, Martin 1946) and moisture (Probert 2000, Steadman *et al.*, 2003). If the environmental cues are reliable, dormancy may be regulated through phenotypic plasticity. When environmental conditions are unpredictable and cues are not 100% reliable, bet hedging may evolve.

Dormancy stages in some organisms range from short quiescence periods induced by sudden adverse environmental fluctuations, to longer diapause periods of metabolic suppression (Belozerov 2008). Diapause is usually cited as a plastic response to seasonal changes, combined with a bet-hedging response where diapausing eggs are produced early in development as a response to unreliable environmental cues that would otherwise be devastating to non-dormant eggs (Andrewartha 2008, Furness *et al.*, 2015). A recent study that analyzed the egg hatching of an annual killifish in relation to predictable and unpredictable environmental cues indicated that diapause period lengths respond to predation, early drying and temperature, suggesting adaptive phenotypic plasticity (Furness *et al.*, 2015). However, killifish eggs exhibited diversified bet hedging by ensuring variation at multiple levels, including length of diapause periods and timing of hatching, even after controlling both genetic and environmental sources of variation. Plasticity was also present as the eggs that entered diapause were influenced by environmental factors such as temperature and light (Furness *et al.*, 2015).

Different plants demonstrate different dormancy responses. Some plants exhibit vegetative dormancy where the plant lives through more than one season but delays the transition to a reproductive state when conditions are deteriorating (Bentsink and Koornneef 2008, Shefferson 2009). Seed dormancy is believed to be both a phenotypically plastic and a bet-hedging strategy where seeds will not germinate if conditions are unfavourable, or will show partial germination under environmental uncertainty (Shefferson 2009, Simons 2014). Once conditions are favorable, viable seeds germinate and plant development commences (Shefferson 2009). The timing of seed germination is an example of a life-history trait that expresses both phenotypic plasticity

and bet hedging. In a study that assessed desert annual plants along a precipitation gradient, germination fractions displayed phenotypic plasticity to water availability within the season and bet hedging was observed as a response to environmental unpredictability by producing multiple dormant seeds for varying periods of time to reduce fitness variance (Clauss and Venable 2000). Cohen's classic bet-hedging model (Cohen 1966, 1967), in which the evolution of dormancy fraction depends on the encountering of 'bad years', allowed for the direct assessment of the relationship between adaptive phenotypic plasticity and bet hedging (Simons 2014). The germination behaviour of seeds from *Lobelia inflata* (L.) was found to show a negative correlation between plasticity and potential diversification bet-hedging and a positive correlation between plasticity and germination fraction, both indicative of a trade off between plasticity and bet hedging (Simons 2014).

Assessing the joint expression in phenotypic plasticity and bet hedging continues to be a challenging task due to the difficulty of perceiving reliable and unreliable environmental cues from an organism's standpoint and because the adaptive significance of bet hedging traits must be assessed over multiple generations of fluctuating selection. The current understanding of the joint expression between these two modes of responses is underdeveloped and more research is necessary. While vegetative rosette and seed stages continue to be used as models for plasticity and bet hedging, several aquatic plant species are characterized by an alternate dormant stage that may help further elucidate dormancy as a joint plasticity and bet hedging response.

1.3 *Spirodela polyrhiza* and turion formation

Some species of the subfamily Lemnoideae (Araceae) produce turions— seed-like overwintering dormant propagules (Appenroth 1989, Jacobs 1947, Wang *et al.*, 2014). These specialized vegetative organs survive harsh environmental conditions by sinking to the sediment surface and lying dormant in the mud until conditions are favourable again (Perry 1968). *Spirodela polyrhiza*, or Greater Duckweed, is an aquatic perennial plant that can be found nearly worldwide in many types of freshwater habitats (Jacobs 1947). When water temperature drops below 10°C, cell growth, synthesis and absorption abilities decrease enough to severely damage duckweed fronds (Kuehdorf *et al.*, 2014, Song *et al.*, 2006). As the environmental conditions deteriorate during the fall and winter, and as nutrient availability decreases, *S. polyrhiza* develops turions in the place of normal fronds. Turions lack aerenchyma (Appenroth 2002), and contain high levels of starch, making them denser than the pond water (Newton *et al.*, 1978). Turions thus sink to the sediment and remain there during winter (Appenroth 2002). The “decision” to form turions is not completely understood; however, external stress signals are essential in switching to the pathway that leads to turion formation (Appenroth 2002). The signals that induce turion formation include low temperatures (Appenroth and Nickel 2010), nutrient deficiency (Appenroth *et al.*, 1989; Gerard and Triest 2014) such as phosphate (Appenroth and Adamec 2014), nitrate (Malek and Cossins 1982), and sulphate (Malek and Cossins 1982), increasing amounts of abscisic acid (Smart and Trewavas 2006) and metal stressors (Olah *et al.*, 2014; Olah *et al.*, 2015). The formation of turions may also depend on the length of the growth season (Kuehdorf *et al.*, 2013), where longer growth periods and warmer temperatures result in enhanced fitness (Appenroth 2002, Kuehdorf

et al., 2014). During the winter, or while conditions are unfavourable, the starch stored in the turions at the sediment surface is slowly degraded to sucrose until conditions are favorable again (Ley *et al.*, 1997). Turion germination is induced by warmer spring weather and longer photoperiods (Perry 1968). After the starch conversion, carbon dioxide bubbles are released and trapped under the turion, causing the turion to float to the surface (Perry 1968, Beer 1985). This allows duckweed to quickly reproduce and grow rapidly while other plant species slowly begin development.

If turion formation has evolved jointly as adaptive plasticity and a bet-hedging strategy, response to reliable external cues as well as diversification in phenology of turion production are both expected. The mechanisms underlying the switch to turion production however, have not been completely elucidated. Timing of turion formation varies between individual fronds of duckweed (Appenroth and Nickel 2010). *Spirodela polyrhiza* reproduces asexually by budding and produces a frond from one of two meristematic pockets (turions have one pouch) that eventually matures within a few days (Jacobs 1947). Fronds made by *S. polyrhiza* are commonly referred to as mother fronds, daughter fronds, or granddaughter fronds, which follows the norm for naming connected duckweed fronds (Figure 1.1) (Landolt 1986). A daughter that buds off a mother frond is genetically identical to the mother (Hillman 1961). Therefore, if diversification in turion production is favoured, the “problem” for *S. polyrhiza* is that variation must be generated among genetically identical offspring.

The release of the daughter frond from the mother may have impacted the offspring’s growth, as each subsequent offspring will be smaller than the preceding daughter (Hillman 1961). This reduction in daughter frond size is not due to smaller cell

size but due to the presence of fewer cells found in late daughters (Hillman 1961). This may be a result of stipe tissue buildup at the meristematic pouch of the mature parent frond (Barks and Laird 2015). There appears to be a rejuvenescence cycle where fronds that are small and produced later in the birth order produce first offspring that are larger than themselves due to higher number of cells (Ashby and Wangermann 1949). It might be concluded from this that variation of turion timing may be due to the size of the frond. However, variation in size is generated through birth order. It is not known whether size, birth order, or both play a role in the variation of turion production.

Temperature is an important cue to form turions in duckweed. *S. polyrhiza* will make only vegetative fronds at temperatures higher than 18°C and anything colder than 10°C will likely induce only turions (Appenroth 2002; Kuehdorf *et al.*, 2014) Between these extreme temperatures, however, variation in turion production may result from variation in temperature, size and/or birth order.

Environmental cues may be reliable, allowing for the expression of adaptive plasticity; however, due to temporal and spatial variance in temperature and nutrient levels, the onset of winter conditions may be unpredictable between years and among bodies of water and bet hedging may have evolved. Turions remain dormant under favorable conditions in temperate climates to inhibit winter germination (Appenroth 2002). This is believed to have evolved as a risk avoidance strategy that ensures survival and allows for future successful vegetative propagation.

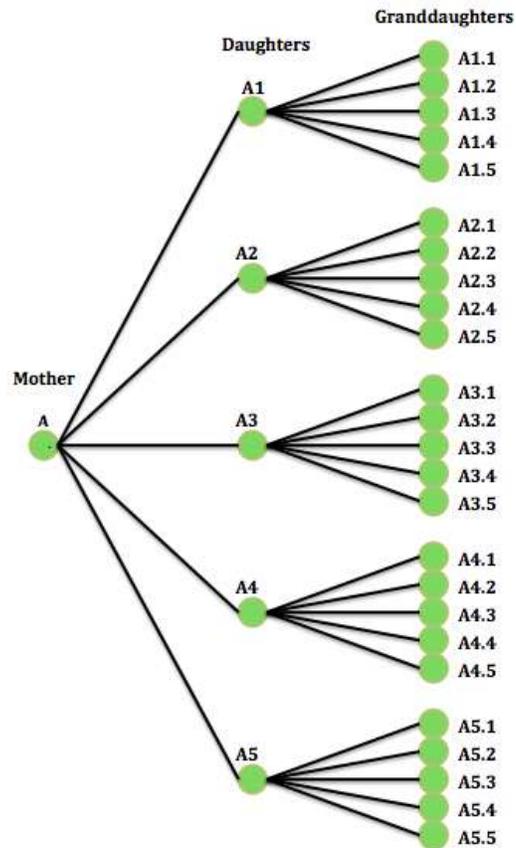


Figure 1.1 Birth order and naming system of *Spirodela polyrhiza* fronds. The first letter A, indicates the progenitor mother, AX as the daughter and AX.Y as the granddaughter.

The purpose of this study is to identify the mechanisms that generate diversification in turion formation of *Spirodela polyrhiza*. Duckweed provides an ideal model system for this study due to its small size, its rate of reproduction and the use of turions as a dormancy strategy. Because *S. polyrhiza* daughter fronds are genetically identical to the mothers, the analysis of variation in turion formation allows for further understanding of the evolutionary responses expressed in dormancy. In chapter 2, I hypothesized that turion production is stimulated by birth order and size and that they are potential sources of dormancy variation as a candidate diversification bet-hedging strategy. I tested this by looking at a set of parents and allowing them to grow for three generations under constant environmental conditions, all the while noting birth order, size and daughter type. In chapter 3, I hypothesized that the effect of birth order on turion formation depends on temperature and that these effects are potential sources of diversification bet hedging and plasticity in *S. polyrhiza*. With the use of a thermogradient incubator, each birth order is replicated across a range of water temperatures, and first turion birth order, days to first turion and turion frequency are recorded. The results will provide insight into how potential diversification in phenology—known to be an important strategy expressed in propagules of other organisms—is generated among genetically identical individuals.

CHAPTER 2

SOURCES OF DIVERSIFICATION IN *SPIRODELA POLYRHIZA* TURION FORMATION: AN EMPIRICAL TEST OF BIRTH ORDER AS A PUTATIVE BET HEDGING MECHANISM

2.1 Introduction

2.1.1 Environmental effects on life-history traits

In a variable environment, the evolutionary success of an organism depends on its capability to respond to environmental change, whether directly (Chown *et al.*, 2010, Cleland *et al.*, 2007), or indirectly (Bell *et al.*, 2009, Charmantier and Garant 2005). The evolution and expression of life history traits, such as age at first reproduction, reproductive lifespan, and germination, can depend on the extent of environmental variation perceived by the organism (Levins 1968). Environmental variation is a characteristic of all environments, and may be increasing with the rapid and extensive nature of anthropogenic changes (Chevin *et al.*, 2010, Wong 2015). Evolutionary responses that may be expressed in response to environmental change include adaptive tracking (Cleland *et al.*, 2007, Bell and Gonzalez 2009, Gonzalez *et al.*, 2012), phenotypic plasticity if cues are predictable (Bradshaw 1965, Levins 1968, Stearns 1989, Via and Lande 1985), and bet hedging if cues are unpredictable (Gillespie 1974, Seger and Brockmann 1987, Slatkin 1974).

While there has been a recent surge of interest in bet hedging (Beaumont *et al.*, 2009, Furness *et al.*, 2015, Graham *et al.*, 2014, Gremer and Venable 2014, Gremer and Venable 2016, Simons 2014), very little research has been conducted thus far on this mode of response (Simons 2011). Bet hedging is characterized by low fitness variance, as immediate arithmetic fitness is traded off for long-term geometric fitness across generations (Philippi and Seger 1989, Seger and Brockmann 1987). If fitness variance is reduced by the expression of a single phenotype and avoids risk, conservative bet hedging has evolved (Philippi and Seger 1989). Diversification bet hedging is expressed

when several phenotypes are exhibited in response to unpredictable cues and the risk is spread (Philippi and Seger 1989). Sometimes, combinations of evolutionary responses are co-expressed in a life-history trait (Simons 2014). For example, timing of flowering can mediate both conservative and diversified bet hedging (Rees *et al.*, 2004). However, most research in the context of bet hedging has been conducted on diversification bet hedging and seed dormancy (Clauss and Venable 2000, Simons 2009).

2.1.2 Sources of dormancy variation in plants

Dormancy is a state in which an organism ceases growth and metabolic activity in response to adverse environmental conditions (Silsbury 1961). Different dormancy behaviours are observed across kingdoms (Belozarov 2008, Shefferson 2009, Sussman and Douthit 1973). In plants, dormancy is often expressed as either vegetative in which transition to the reproduction stage is delayed (Bentsink and Koornneef 2008, Shefferson 2009) or as seed dormancy where germination is delayed until conditions are favourable (Shefferson 2009, Simons 2014). While cues that trigger the onset or termination of dormancy are well studied (Baskin and Baskin 1998, Martin 1946, Vleeshouwers *et al.*, 1995), sources that cause dormancy variation within seasons have not been completely elucidated. If several phenotypes are exhibited and variation in dormancy behaviour is observed, response to environmental unpredictability may occur through diversification bet hedging. Assessing sources of dormancy behaviours may further elucidate putative bet hedging strategies.

Several different sources of variation in dormancy behaviour exist within species. In plants, sources of variation in vegetative dormancy or seed germination may include size (Halpern 2004), genetics, environmental cues (Simons and Johnston 2006) or a

combination of sources. With size, organisms are theoretically expected to produce offspring of a consistent size that depends on the tradeoff between offspring size and number (Smith and Fratwell 1974). However, within-species and within-individual variation in offspring size is well studied. For example, seed sizes vary fivefold among individual *Lupinus perennis* plants (Halpern 2004). Natural selection may favor parents that produce diverse seed sizes. For example, differences in size can affect fitness through timing of germination (Simons and Johnston 2000) or dispersal (Westoby *et al.*, 1996). If the environment the offspring faces is unpredictable, diversification may enhance long-term fitness as a bet-hedging strategy. However, size variation may be the result of non-adaptive evolution if selection for same-sized offspring size is weak or if the underlying genetic basis prevents it (Byers *et al.*, 1997, Mojoinnier 1998). *Spirodela polyrhiza* produces genetically identical offspring through clonal reproduction, yet diversity is observed in dormancy behaviour (Appenroth and Nickel 2010, Sultan 2000). The generation of variation among genetically identical individuals in a trait potentially associated with unpredictable survival makes *S. polyrhiza* a suitable species for study of diversification bet hedging.

2.1.3 *Spirodela polyrhiza* as the model system

Spirodela polyrhiza is a small free-floating aquatic plant that can be found in a range of different fresh-water habitats (Jacobs 1947). When environmental conditions begin to deteriorate and water temperature drops, metabolic activity begins to slow down (Kuehdorf *et al.*, 2014, Song *et al.*, 2006). To survive the harsh conditions during the fall and winter, *S. polyrhiza*, develops turions in place of vegetative fronds (Appenroth and Nickel 2010, Wang *et al.*, 2014). Turions are overwintering dormant propagules that sink

to the sediment surface and lay dormant during the winter until conditions are favourable again (Perry 1968). The formation of turions may enhance long-term fitness depending on the growth period and temperature (Appenroth 2002, Kuehdorf *et al.*, 2014). If a duckweed frond produces turions prior to encountering unfavourable winter conditions, immediate fitness is reduced relative to other individuals, and interspecific competitive ability is also reduced because other aquatic plant species continue to proliferate (Leng 1999). However, when conditions are favourable again, turions float to the surface and vegetative proliferation occurs rapidly (Perry 1968, Beer 1985).

Research shows that external stress signals are essential in the formation of turions (Appenroth 2002), yet the phenology of turion formation, or the switch from frond to turion production, is not understood. Based on observations, timing of turion formation varies between individual duckweed fronds within a season (Appenroth and Nickel 2010). Ponds that contain *S. polyrhiza* exhibit variation in turion timing, in that some fronds produce turions earlier in the season than other individual fronds (Appenroth and Nickel 2010). By forming turions at various times throughout the season, complete failure may be prevented, in that some fronds will not form a turion and continue to reproduce while other fronds form turions early to survive the winter.

Duckweed fronds of the subfamily Lemnoideae asexually produce daughters through budding from two meristematic pouches (Jacobs 1947). Under optimal conditions, *Spirodela polyrhiza* fronds live for about 30 days, have a reproductive rate of 0.08 fronds per day and produce up to 15 daughters (Barks and Laird 2015, Lemon *et al.*, 2001). These daughter fronds later become mothers as they produce daughters themselves. Daughter fronds remain attached to their mother through stipe tissue, which

then severs after daughter maturation (Landolt 1986). Daughter fronds are genetically identical to the mother (Hillman 1961). However, diversification has been shown to be adaptive in other organisms, seed germination for example (Cohen 1966, Gremer and Venable 2014, Simons 2009) and generating variation in turion formation is thus a problem for a plant that makes genetically identical offspring. The formation of a daughter may have an effect on succeeding daughter formation, as each subsequent daughter is smaller than preceding daughters (Hillman 1961). This is due to fewer cells within the daughter frond (Hillman 1961) but may also be a result of a small amount of stipe tissue buildup left behind in the mother's meristematic pouch (Lemon and Posluszny 2000). If daughter size depends on when the mother released it, size variation is generated through birth order. Variation in dormancy through turion formation may then result from size and/or birth order.

While other studies have shown the maternal effects on seed size and seed germination (Barks and Laird 2016, Galloway 2001, Halpern 2004, Lu *et al.*, 2016, Mercer *et al.*, 2011), no previous work has been conducted on maternal birth order effects on dormancy behavior. The goals of my study are threefold; first, I aim to identify possible sources of variation in turion formation in genetically identical fronds of *Spirodela polyrhiza* by assessing birth order and size through three generations (mother, daughter, and granddaughter). It is hypothesized that the type of daughter produced is dependent on the birth order and size of the mother; specifically, that daughters produced later in the mother's lifespan are more likely to produce fewer vegetative fronds with an earlier switch to turion formation. Second, I assess if a single genotype exhibits a potential diversification bet-hedging strategy. This will be the first study to assess

maternal effects as a potential source of turion variation and the expression of putative bet hedging in turion formation. If birth order and size do generate diversification in dormancy phenology, then further work would be merited to assess the joint effects of diversification and plasticity. Third, I aim at acquiring additional knowledge of *Spirodela polyrhiza* fronds and their development of turion formation as a model system for future evolutionary ecology studies.

2.2 Materials and Methods

2.2.1 Pre-experiment set up

Duckweed fronds were obtained from various locations in Barb's Marsh at Queen's University Biological Station. Turions made by these fronds were preserved in a refrigerator set at 4°C before being used. To begin this study, I placed a single turion in the seed germinator (Biochambers model SG-30), set to 15 hour days and 9 hour nights at 21°C. Vegetative growth continued until the surface of a 1200mL Pyrex crystallizing dish was almost completely covered. The dish was filled with 1L of Appenroth's growth medium (Appenroth *et al.*, 1996) and changed every four days to replenish nutrients and prevent algae growth.

Twenty six fronds were taken out of the duckweed pool from the crystallizing dish, separated carefully with tweezers, put in separate 15mL tubular glass vials and labeled A to Z. These were the "mothers". Vials were filled with 10mL of Appenroth's growth medium. In order to remove any environmental effects, and to standardize effects of birth order, each parent frond used in the study was a first daughter of a first daughter (etc.) going back at least three generations.

2.2.2 Experimental design

The purpose of this part of the study was to test whether turion production is correlated with birth order and size. All 26 vials, labeled A to Z were kept in a SG-30 seed germinator. Temperature was set at 18°C with 15 hour days and 9 hour nights. Light intensity was kept at 40 $\mu\text{mol}/\text{m}^2/\text{s}$. Each daughter produced was gently separated from the mother and placed in a 12-well plate and moved to another SG-30 at 17°C with 14 hour days and 10 hour nights to record frond and turion formation (Figure 2.1). A daughter was only separated from the mother when it became a mature adult frond. A frond is considered to be mature when it begins making a frond of its own (Lemon *et al.*, 2001). 17°C was chosen as vegetative fronds and turions are expected to be produced in equal proportions at this temperature and 18°C was too warm for turion formation. The daughters became the focal generation to determine whether their birth order affects the type of daughter (here, “granddaughter”) produced. The daughters inside the 12-well plates were labeled with the letter of the mother they originated from and the birth order in which they were produced. Once the daughters produced granddaughters (whether a vegetative frond or a turion), they were separated carefully with tweezers and photographed. The granddaughters were then placed in a new set of 12-well plates within the SG-30 carrying the daughters and labeled with the mother letter, daughter birth order, and granddaughter birth order (Ex. A1.1). This was done to keep track of the total number of offspring made by the daughters. The positions of the 12-well plates were randomized regularly within the SG-30. Nutrient medium in all plates was replaced every four days to replenish nutrient levels, ensuring all fronds experienced similar conditions, and to limit algae growth. Two mother fronds died after making only three daughters and

were removed from the study. The rest of the mothers made at least eight daughters in their cycle, while a few mothers made more than eight daughters. To analyze the effect of daughter birth order on granddaughter turion formation, only the first eight daughters made by each mother were assessed. To limit the number of 12-well plates used in the seed germinator, after a granddaughter had been photographed with the microscope, the next granddaughter from the same daughter replaced it in the plate (i.e. A1.2 would replace A1.1).

2.2.3 Frond and turion measurements

After separating a frond, the frond or turion produced was removed from the medium and photographed using an Olympus SZX12 microscope with a Pixelink Camera attachment using a 0.5X lens at 10X magnification. The digital photographs were calibrated, and frond length (apex to opposite edge) measurements were taken using NIH ImageJ version 1.49v. A micrometer scale was measured (in pixels) to obtain a known distance of 10 mm. Each frond measurement was repeated at least five times to assess precision and the final result was taken as the average value. Calibration was performed each time ImageJ was opened.

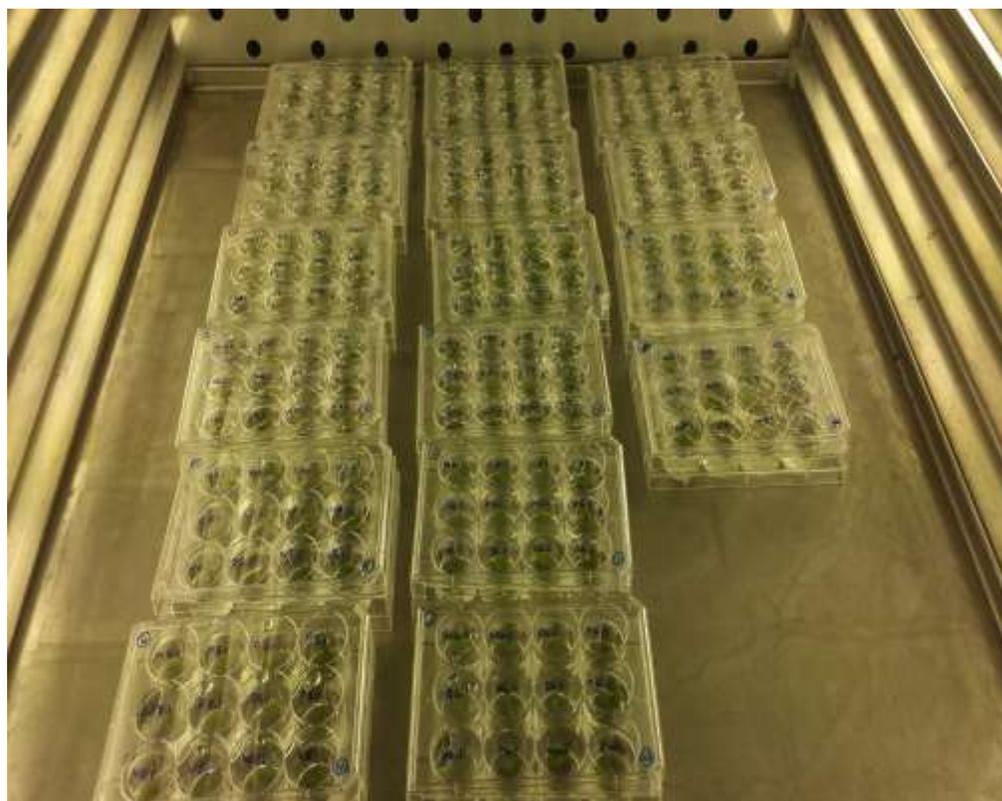


Figure 2.1 Set up of 12-well plates containing *S. polyrhiza* fronds in SG-30 seed germinator. Fronds were labeled according to the frond they originated from (Ex. A) and the birth order they were produced in (Ex. A.1, A.2...).

2.2.4 Statistical analysis

All statistical analyses were conducted using SAS JMP version 13.0. Analyses of the effect of birth order on first turion birth order would be biased if the daughters that never produced turions were eliminated from statistical models. The exclusion of this large amount of data (especially at warmer temperatures where turion formation is reduced) could potentially skew the results of the effects of birth order on turion formation. Because I am mainly identifying sources of turion formation, rather than testing if bet hedging is expressed, a hypothetical, additional late turion—as a granddaughter following the final true granddaughter—was added to the dataset for daughter fronds that did not produce any turions. In this case, if a daughter frond produced ten granddaughter fronds during its life cycle and died without producing any turions, a hypothetical eleventh granddaughter was added as a potential turion. This follows the norm of slight data alterations in other studies that assess seed formation and germination. A single granddaughter turion was added the dataset only to daughter fronds that never made a turion and only for the measured response of first turion birth order (not turion frequency).

In order to first assess the effect of size on turion formation, a multiple regression analysis was conducted on mother size and daughter size on first turion birth order (i.e. first turion granddaughter), which infers to the number of vegetative fronds produced before a turion formed. Another multiple regression was conducted on the effects of daughter birth order and daughter size on first turion birth order. A post-hoc simple regression of daughter birth order on first turion birth order was conducted. Mean first turion birth order for each daughter birth order was calculated.

To assess the impact of birth order and size on turion formation, turion frequency was also analyzed as an alternate measure of phenology. Turion frequency was calculated as the total number of turions made by a daughter divided by the total number of granddaughters made (fronds and turions). A multiple regression analysis was conducted on the effect of daughter birth order and size on turion frequency. A post-hoc simple regression of daughter birth order on turion frequency was also conducted.

2.3 Results

2.3.1 First turion birth order

Mother duckweed fronds produce sequentially smaller daughters, and size may thus be a source of variation in turion behaviour. To study the expression of putative diversification bet hedging in genetically identical fronds, the effect of mother and daughter size on first turion birth order was analyzed. A multiple regression analysis that included the effect of mother size, daughter size and their interaction finds that only daughter size has a strong effect on first turion birth order (Table 2.1). Larger daughter fronds make a first turion on average later than do smaller daughter fronds. The slope of the regression line confirms a weak positive relationship (Figure 2.2).

Table 2.1 Multiple regression of the effect of mother and daughter size on first granddaughter turion birth order.

Effect	df	Sum of squares	F-ratio	Probability
Mother size	1	6.50	0.98	0.3245
Daughter size	1	97.25	14.60	0.0002*
Mother size*daughter size	1	3.96	0.59	0.4418

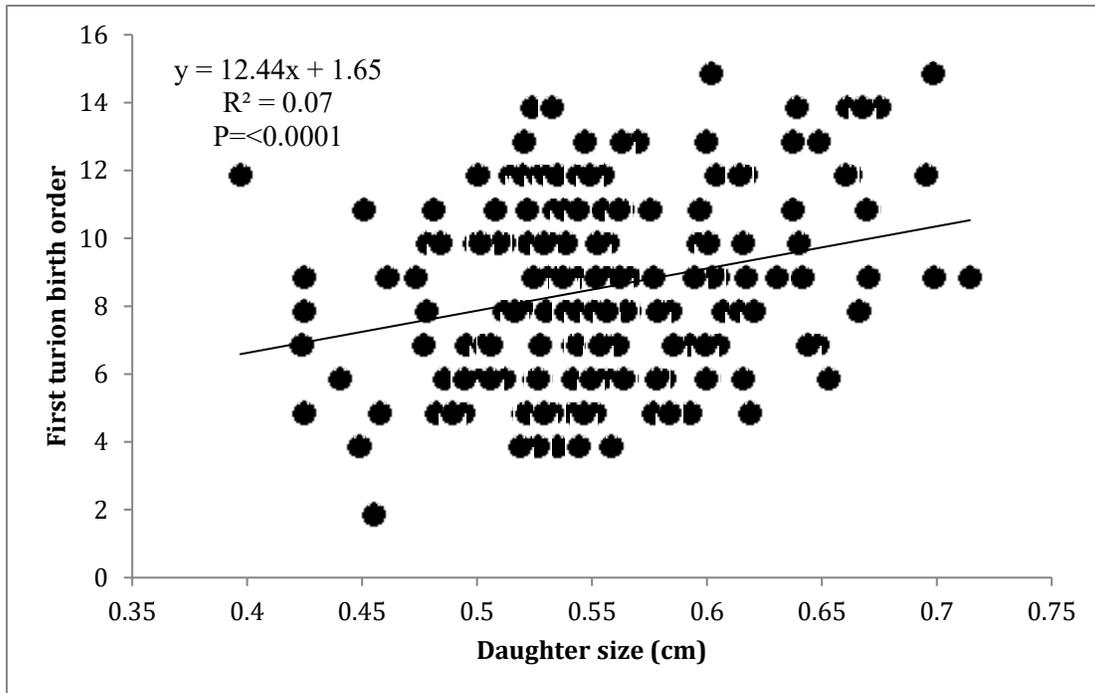


Figure 2.2 Effect of daughter size on first turion formation. Daughters make a first turion sooner than larger daughter fronds. A simple regression confirms the significant effect.

2.3.2 The effect of size and birth order on first turion birth order

The decrease in frond size is generated through birth order; therefore, the effect of daughter birth order on turion formation was also assessed as a potential source of variation in turion phenology among genetically identical fronds. Because the effect of mother size was nonsignificant (Table 2.1), only daughter size was assessed for subsequent analyses. All mother fronds were first daughters of first daughters, thus only daughter birth order and daughter size were included in the multiple regression on the effects of first turion birth order (as a granddaughter). The multiple regression model shows that after correcting for size, daughter birth order and the interaction between daughter birth order and size, have strong effects on first turion birth order (Table 2.2). A post-hoc simple regression confirms the significant effect ($F=19.75$, $P=<0.0001$, $R^2=0.09$). The negative slope indicates that the later the daughter birth order, the earlier the first turion is produced (Figure 2.3). On average, birth order 2 daughters produce the turions latest while birth order 7 mothers make the turions earliest.

Table 2.2 Multiple regression of the effect of daughter size and birth order on first granddaughter turion birth order.

Effect	df	Sum of squares	F-ratio	Probability
Daughter size	1	2.51	0.40	0.5293
Daughter birth order	1	42.06	6.67	0.0106*
Daughter size*daughter birth order	1	42.61	6.75	0.0101*

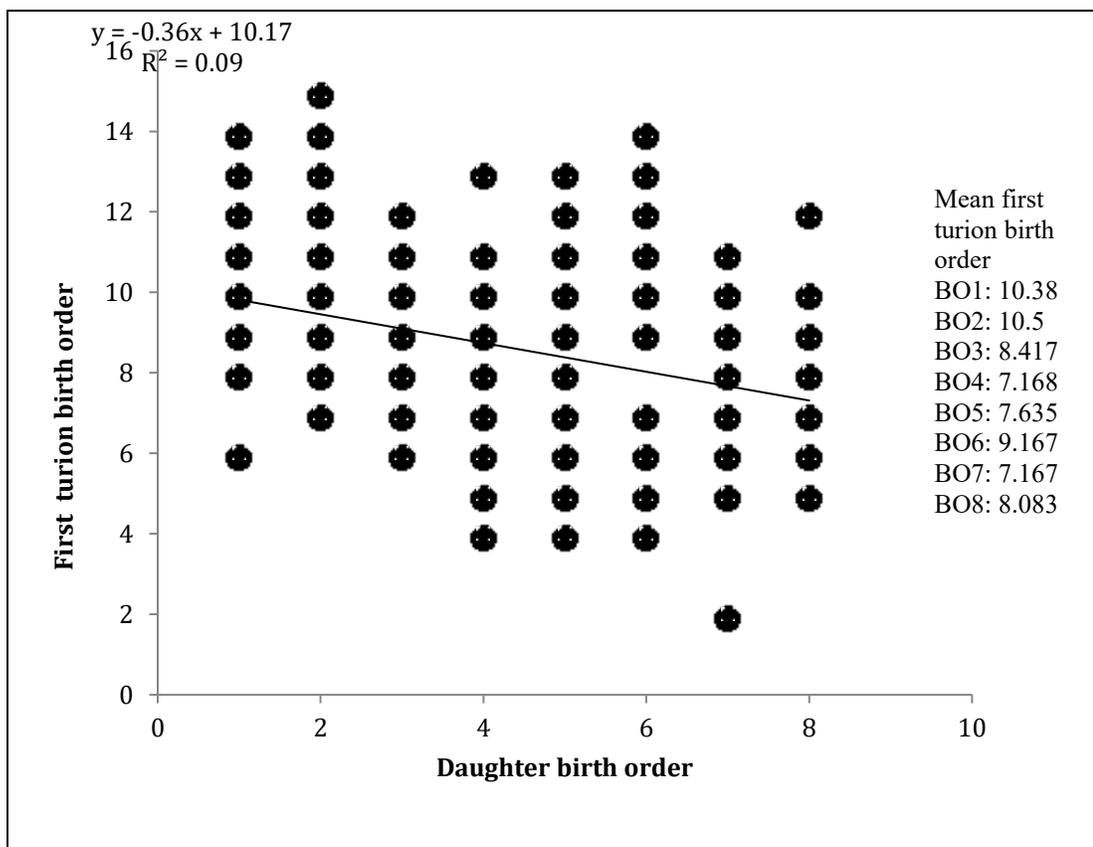


Figure 2.3 Effect of daughter birth order on first turion birth order. The dots reflect the first turion birth order for each of the 24 daughter fronds (A to X) per daughter birth order.

2.3.3 Turion frequency

The second measure of turion phenology assessed was turion frequency. Mother birth order and size were not found to be significant (Table 2.1), and were thus not included in the regression. A multiple regression analysis including the effects of daughter birth order, daughter size and their interaction on the total number of granddaughter turions formed shows a significant effect of daughter birth order and the interaction, but not daughter size (Table 2.3). A post-hoc simple regression of daughter birth order confirms the significant effect ($F=11.93$, $P=0.0007$, $R^2=0.06$).

Table 2.3 Multiple regression of the effect of daughter size and birth order on turion frequency.

Effect	<i>df</i>	Sum of squares	F-ratio	Probability
Daughter size	1	0.02	0.95	0.3317
Daughter birth order	1	0.26	9.11	0.0029*
Daughter size*daughter birth order	1	0.27	9.62	0.0022*

2.4 Discussion

Response to change is necessary depending on the temporal and spatial scale of the variation. Under unpredictable environmental conditions, “bet hedging” (Slatkin 1974) is expected to evolve. In average conditions, variability in life history traits may seem maladaptive and immediate arithmetic mean fitness is sacrificed (Gillespie 1974, Seger and Brockmann 1987, Simons 2009). However, this may be beneficial because variance in fitness is reduced and geometric mean, or long-term fitness is maximized (Philippi and Seger 1989). Empirical evidence that bet hedging occurs naturally in stochastic environments is rare (Simons 2011) and more work is needed to analyze bet-hedging strategies in the wild. Here, I asked if diversification bet hedging is expressed by assessing variation in dormancy behaviour among genetically identical offspring. Specifically, this study aimed at identifying the maternal effects of both birth order and size as potential sources of variation in turion production. Daughter fronds were exposed to a homogeneous temperature environment, and turion formation was assessed, all the while measuring first turion birth order and turion frequency.

Results showed that birth order but not size affects first turion birth order and turion frequency (Table 2.2, 2.3). My results are therefore consistent with my hypothesis, in that birth order has a direct effect on turion formation and a daughter with a later birth order produces a turion sooner than a daughter with an early birth order (Figure 2.3). The consistent differences in turion formation between birth orders found here cannot be a result of environmental factors because fronds were exposed to constant environmental conditions in the seed germinator.

Diversification bet hedging is favoured in other species that also exhibit variation in dormancy behavior (Clauss and Venable 2000, Furness *et al.*, 2015, Simons 2009, 2014). For example, Cohen (1996) suggested that in order to survive unreliable cues in variable environments, seed germination in annual plants is spread among different years as a diversification strategy. In semelparous perennial plants, diversification could be expressed through the development of a “rosette bank”, where offspring of a single genotype flower at different ages so that seed dispersal is spread through time (Rees *et al.*, 2006). Diversification is often favoured for this type of trait (i.e. dormancy). If diversification in turion formation is favoured in the long term, then birth order is a mechanism whereby it may be generated among genetically identical individuals. Specifically, timing variation “spreads the risk” and that late birth order mothers produce turions earlier than other duckweed fronds thereby preventing complete failure in the season and that at least some fronds will survive.

Previous studies have reported the effects of mother age and environment on offspring quality (Barks and Laird 2015, Fox 1993, Mousseau and Fox 1998), however very few studies have assessed the effect of maternal birth order on offspring. Barks and Laird (2016) demonstrated that *Lemna minor* (common duckweed) exhibit deterioration in offspring fitness with increasing mother birth order. This decline could be a result of stipe tissue buildup near the meristematic pouches of the mother (Lemon and Posluszny 2000), or a result of the decline in environmental quality in which offspring develop (Fox 1993). Furthermore, birth order 1 daughters are produced while the mother is still developing whereas subsequent daughters are produced from a mature mother. Thus, in the context of my study, late birth order daughters could also exhibit a decline in fitness,

resulting in the production of fewer vegetative fronds and ensuring survival in subsequent seasons by forming a turion early.

This was the first study to assess and identify sources of variation in dormancy behaviour of clonal offspring. If a diversification bet hedging strategy is favoured in turion formation of genetically identical offspring, a long-term study should be conducted to evaluate if the variation in turion phenology demonstrates the maximization of geometric mean fitness (Simons 2011). In this study, all the mother fronds were birth order one, because they were all first daughters of first daughters, and only daughter fronds exhibited different birth orders. To measure the continuing consequences of variation in dormancy behaviour, a multigenerational study including ancestral birth orders (i.e. mother birth order, daughter birth order, and granddaughter birth order) is needed to assess variation in timing of turion formation and their effects on future progeny. In addition, molecular genetic analysis could ask whether more than one genotype exists at a location and whether there is genetic variation with respect to timing of turion formation.

This study provides further support of maternal influences on the phenology and frequency of turion formation in *Spirodela polyrhiza* fronds. However, all fronds were exposed to constant environmental conditions. Natural environments are variable and the effects environmental cues have on dormancy behaviour must also be analyzed. After assessing the effects of mother birth order and size on timing of turion formation, work is now needed to assess both maternal and environmental effects on turion phenology. If faced with predictable environmental conditions, dormancy behaviour is expected to adapt through phenotypic plasticity (Levins 1968, Thomas 1991). Thus, if maternal and

environmental effects are potential sources of variation in turion formation, integrated strategies combining diversification bet hedging and adaptive phenotypic plasticity can occur. Studies often assess these two modes of evolutionary response in isolation and only rarely on the integration of phenotypic plasticity and bet hedging (Gremer *et al.*, 2016, Gremer and Venable 2014, Simons 2014).

Evidence of observed phenological variability in dormancy behaviour renders *Spirodela polyrhiza* an appropriate model organism for future ecological studies. Additionally, *S. polyrhiza* is easy to maintain, reproduces quickly, and offspring are genetically identical, allowing for further elucidation of evolutionary responses expressed in dormancy. Here, I demonstrated the potential for maternal effects to generate variation in life history traits as diversification; that birth order leads to turion formation diversity in clonal offspring. The next step is to assess the integrated effects of potential bet hedging and plasticity through birth order and environmental effects on the phenology of turion formation.

CHAPTER 3

MATERNAL AND ENVIRONMENTAL EFFECTS AS SOURCES OF DIVERSIFICATION BET HEDGING AND PHENOTYPIC PLASTICITY IN *SPIRODELA POLYRHIZA*

3.1 Introduction

3.1.1 Adaptation to environmental change

Understanding how organisms cope with and adapt to environmental change is required to elucidate species persistence and survival in variable environments. In changing environmental conditions, different modes of evolutionary responses are likely to be favoured (Bell *et al.*, 2009, Charmantier and Garant 2005, Simons 2011, Travis *et al.*, 2013). Two evolutionary modes of response in which organisms respond to variance include phenotypic plasticity and bet hedging (Levins 1968, Philippi and Seger 1989, Seger and Brockmann 1987, Slatkin 1974, Thomas 1991, Via and Lande 1985). Under predictable environmental change, organisms may express adaptive phenotypic plasticity, where adaptation to change occurs through the adjustment of phenotypic expression (Bradshaw 1965, Levins 1968, Stearns 1989, Via and Lande 1985). Plasticity is adaptive when observed cues are reliable indicators of future conditions, and the phenotype is appropriate such that reproductive success is increased (Lloyd 1984, Stearns 1989). However, the effectiveness of plasticity is limited due to the dependability of environmental cues (van Kleunen and Fischer 2005). Additionally, limitations of genetic variance and the cost of processing cues perceived by the environment also limit plasticity efficiency (Auld *et al.*, 2009, Murren *et al.*, 2015, van Kleunen and Fischer 2005). In conditions where cues are not reliable indicators of future conditions—the remainder of a growing season for an annual plant, for example—or phenotypic plasticity is constrained, an alternate mode of evolutionary response is necessary.

When environmental conditions are unpredictable or plasticity is constrained, bet hedging is expected to evolve (Gillespie 1974, Seger and Brockmann 1987). In

unpredictable variable environments, organisms may produce several offspring phenotypes, increasing the probability that at least some will survive future conditions (Cohen 1966). This bet-hedging or risk-spreading strategy leads to a decrease in arithmetic mean fitness and increased geometric-mean fitness of populations over time and across environments (Dempster 1955, Philippi and Seger 1989, Seger and Brockmann 1987). Bet-hedging traits are either conservative or diversified. Conservative bet hedging minimizes fitness variance across environmental conditions by “playing it safe” and avoiding risk (Philippi and Seger 1989, Seger and Brockmann 1987). Diversified bet hedging is defined as spreading the risk by expressing several phenotypes and maximizing geometric-mean fitness through the reduction of among-generation fitness variance (Philippi and Seger 1989).

Because natural environmental variation has both predictable and unpredictable components, the joint evolution of phenotypic plasticity and bet hedging is expected. Although adaptive phenotypic plasticity continues to be extensively researched and interest in bet-hedging strategies is rising (Beaumont *et al.*, 2009, Graham *et al.*, 2014, Gremer and Venable 2014, Simons 2009), the interaction and joint expression between these two modes of responses is underdeveloped in literature.

3.1.2 Dormancy as plasticity and diversification

Adaptive plasticity and bet hedging may be jointly expressed in dormancy (Gremer *et al.*, 2016, Gremer and Venable 2014, Fernandez-Pascual and Jimenez-Alfaro 2014, Nilsson *et al.*, 1996, Simons 2014). Dormancy is a mechanism allowing escape from detrimental conditions used by a wide range of taxa, including microorganisms, plants, and animals (Belozarov 2008, Shefferson 2009, Sussman and Douthit 1973).

When faced with unfavourable environmental conditions, organisms that enter dormancy reduce metabolic activity and conserve energy, thus increasing the likelihood of survival through those harsh environments (Silsbury 1961). Organisms demonstrate different dormancy behaviours in response to environmental change. For instance, some animals delay egg development until conditions are optimal again (Furness *et al.*, 2015), while certain plants use the time for the dispersal of seeds (Lu *et al.*, 2013). However, some organisms rely on environmental cues to trigger the onset or termination of dormancy. Cues that can influence dormancy behaviour include photoperiod (Vleeshouwers *et al.*, 1995), nutrient and moisture availability (Baskin and Baskin 1998, Martin 1946, Probert 2000), and temperature (Baskin and Baskin 1998, Martin 1946). Species respond differently to environmental cues. For instance, shorter photoperiods may trigger the onset of dormancy in some species while other species are unaffected by the photoperiod (Hilton and Bitterli 1983, Simpson 1990). Organisms that rely on light availability for survival may enter the dormancy stage early if the photoperiod is short (Masuda *et al.*, 2006), while other species that although still suggest an operative phytochrome system, lack photoperiod sensitivity for dormancy regulation (Heide 2011).

If cues are reliable indicators of future environments, dormancy may be adjusted through phenotypic plasticity. If, however, cues are not completely reliable, dormancy may be regulated through bet hedging. Joint expression may evolve between these two modes of response as the two strategies are not mutually exclusive and cues to predict future environments are weak and unreliable (Simons 2014). A classic example of the possible joint evolution of these two modes of responses is found in seed germination (Clauss and Venable 2000, Simons 2014). If cues are available and allow the prediction

of future environments, norms of reaction may evolve such that appropriate phenotypic modifications can be made to adjust the phenotype to the environment (Lloyd 1984). This adjustment may occur through early development or parents transmit it to offspring, ensuring their survival in those forecasted environments (Stearns 1989). However, bet hedging may concurrently evolve where seed germination of an annual plant is spread over several years, in case some years are unfavorable for reproduction and survival (Philippi 1993).

Dormancy can occur at different stages in different plant species. For example, vegetative dormancy occurs over a prolonged period where herbaceous perennial plants persist below ground, but delay sprouting above ground for one or more seasons (Bentsink and Koornneef 2008, Shefferson 2009). Seed dormancy is a mechanism in which viable seeds do not germinate—even under suitable environments—until dormancy requirements are satisfied (Shefferson 2009, Simons 2014). This allows seeds to survive unsuitable environmental conditions by temporarily arresting or slowing metabolism and development. Vegetative rosette and seed dormancy have been used as models to assess the joint expression of adaptive plasticity and bet hedging in plant species (Clauss and Venable 2000, Simons 2014). However, tests of bet hedging are difficult, as fitness must be evaluated over multiple generations of fluctuating selection. A dormant vegetative stage found in some short-lived aquatic species provides an alternative for study of the joint evolution of these two modes of response.

Spirodela polyrhiza, or Greater duckweed, is an aquatic perennial plant of the subfamily Lemnoideae (Araceae) found in a highly diverse range of environments (Appenroth 2002, Appenroth and Nickel 2009, Jacobs 1947, Wang *et al.*, 2014). It

provides an ideal model system for study of plasticity and potential diversification because of its small size, rapid rate of reproduction, and especially because of the formation of turions: seed like over-wintering dormant vegetative propagules (Appenroth 2002). Turions are produced in place of normal fronds when environmental conditions deteriorate (Appenroth 2002).

3.1.3 Clonal variation in *Spirodela polyrhiza* turions

As *Spirodela polyrhiza* fronds inhabit variable environments, the phenology of turion dormancy is expected to evolve as phenotypic plasticity and bet hedging. Cues that are known to trigger the formation of turions in *S. polyrhiza* include low temperatures (Appenroth and Nickel 2010), nutrient deficiency (Appenroth *et al.*, 1989; Gerard and Triest 2014) and plant hormone levels (Smart and Trewavas 2006). As temperatures drop in autumn and winter, cell growth slows down, initiating turion formation. When water temperature drops below 10°C, duckweed fronds produce mainly turions (Kuehdorf *et al.*, 2014, Song *et al.*, 2006). Limited levels of phosphate, nitrate and sulfate concentrations in water can also induce turion formation (Appenroth and Adamec 2014, Malek and Cossins 1982). Additionally, studies have found that higher levels of abscisic acid inhibit *S. polyrhiza* growth and induce the production of turions (Smart and Trewavas 2006). When these environmental conditions occur in a pond containing *S. polyrhiza*, variation in the timing of turion production is observed (Appenroth and Nickel 2010).

3.1.4 Questions and hypotheses

Under variable conditions in the field, the timing of turion formation that maximizes fitness is unpredictable. Early turion formation is maladaptive under expected

conditions because immediate fitness is reduced by lost reproductive potential. However, late turion formation risks lineage extinction under early onset of lethal conditions; therefore, the evolution of diversification bet hedging is expected. However, daughter fronds are asexually produced, and are genetically identical to the mother (Hillman 1961, Jacobs 1947). Therefore, if diversification in turion production is favoured, *S.polyrhiza* faces the problem of how to generate variation among genetically identical offspring. This problem is the focus of this chapter. Mother fronds release up to fifteen daughters sequentially through budding from two meristematic pouches, and offspring development may be affected. Each subsequent daughter will be smaller than preceding daughters, a result of fewer cells in each offspring (Hillman 1961). Variation among genetically identical offspring is thus generated through birth order, but it is unknown whether size, birth order, and/or environmental cues result in variation in turion phenology.

Results from chapter 2 indicate that birth order influences the phenology of turion formation. To understand how variation in turion phenology might be generated by single genotypes under variable environments, here I study the effect of birth order on turion phenology across a range of thermal environments.

My goal is to assess the joint effect of plasticity to temperature and potential diversification generated by birth order on inducing turion formation. Specifically, I hypothesize that temperature and mother birth order play important roles in inducing turion formation, and that the effect of birth order differs across temperature environments. Thus, this study aims to provide insight into sources of dormancy variation among genetically homogeneous individuals, and if plasticity and potential diversification bet hedging can be co-expressed under variable environments. In

addition, it will provide needed information on the natural history of *Spirodela polyrhiza*, an emerging model organism, and suggest what may influence phenology in the field.

3.2 Materials and Methods

3.2.1 Experimental design and thermogradient setup

In chapter 2, all birth orders were exposed to the same environmental conditions (temperature, lighting schedule and media changes). There, I was focused on the direct relationship between birth order and phenology of turion formation. Here, I ask about the relationship between this potential diversification and phenotypic plasticity to temperature through the use of a thermogradient incubator.

Fronde of *Spirodela polyrhiza* are found in a diversity of environments, and a thermogradient incubator may be used to ask about norms of reaction in turion formation over a range of temperatures simultaneously. Thermogradient incubators have been used to investigate seed germination in response to temperature in several species (Medany and Hegazy 2007, Orozco-Segovia *et al.*, 2010, Simons 2014, Thomson and Whatley 1984), and are used here to assess plasticity to temperature in turion formation for different birth orders. The thermogradient incubator is constructed from an aluminum block measuring $126 \times 46 \times 11$ cm. Through the use of a water chiller, cold water flows in counter-current through two closely spaced holes drilled through one end, while heated water flows in counter-current from a water heater through two holes through the opposite end (Figure 3.1). An aquatic chamber is installed on the surface of the aluminum block, and is split by a waterproof barrier into two replicate lanes, allowing for simultaneous runs of a study. The aquatic chamber was filled with Appenroth's growth nutrient medium (Appenroth *et al.*, 1996), and topped up as necessary. Two Symban

fluorescent lamps (model F32T8/841/ECO) above the thermogradient provided a 14-hour day and ten-hour night photoperiod. White perforated barriers were placed in the aquatic chamber to reduce eddy flow and stabilize the temperature gradient, effectively dividing the aquatic chamber into seven sections. The continuous range of temperatures from 12 to 18°C was monitored daily using temperature probes. This range was chosen because, under homogenous nutrient conditions, *S. polyrhiza* produces only vegetative fronds at water temperatures above 18°C and anything colder than 10°C will likely induce only turions (Kuehdorf *et al.*, 2014, Song *et al.*, 2006). The goal was thus to determine plasticity to a temperature range that induces the production of both fronds and turions.

Prior to beginning the study, the thermogradient was left on for one week with eight liters of Appenroth's nutrient medium in each replicate lane to stabilize temperatures. The thermogradient was covered to control evaporation of the nutrient medium. Fronds were contained within 1-inch diameter strainer "traps"; these traps were supported at the water surface by cutout Styrofoam floats (Figure 3.2). Five frond traps were positioned at each temperature per replicate lane, resulting in a total of 70 traps per thermogradient run (35 per replicate). The birth order traps were randomly positioned in each Styrofoam float to ensure that there were no spatial effects within the thermogradient impacting turion formation. Because results in chapter 2 demonstrate that turion timing variation is induced by birth order, here, sample size was increased by including only birth orders 1, 3, 5, 7, and 9 replicated and placed at positions differing by 1°C along the thermogradient. Furthermore, mother fronds produce daughters from two pouches almost simultaneously (Jacobs 1947), and these paired daughters behave similarly. Each trap was colour coded with sticker labels to identify the birth order.

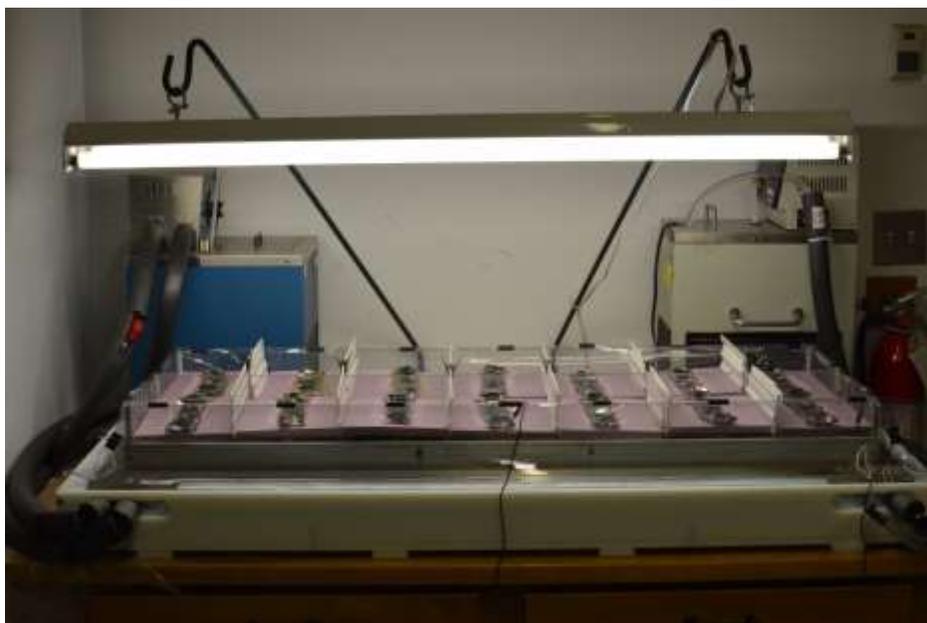


Figure 3.1 Thermogradient incubator setup with water chiller on left and heater on right. 50 mL of plumbing antifreeze was added to cooler to prevent freezing. Duckweed fronds were placed in strainers to separate birth orders. Two replicates of five birth orders were represented at each temperature position.



Figure 3.2 Set up of frond traps showing two replicates of all birth orders at one example temperature location along the thermogradient. White perforated barriers separated neighbouring temperatures. Each trap was colour coded to indicate the mother birth order.

3.2.2 Frond preparation

Preparation of fronds for the thermogradient study required elimination of environmental effects and standardizing effects of birth order by producing first daughters of first daughters (etc.) going back at least five generations. Preserved turions that were stored in a refrigerator were placed in a 1200mL crystallization dish with 1 liter of Appenroth's growth medium (Appenroth *et al.*, 1996). The dish was then placed in a seed germinator (Biochamber SG-30), where turions germinated to produce a source pool of *S. polyrhiza* fronds. After germination, 84 individual fronds were carefully separated with tweezers and placed in 12-well plates (7 plates in total). Sample size was 4 fronds per each birth-order trap at each replicated temperature (56 fronds of each birth order= 280 fronds); however, extra fronds were maintained in the 12-well plates in case of death. These plates were maintained at room temperature on a light shelf with 14-hour days and 10-hour nights.

The thermogradient study was performed twice; as a "forward run" and "backward run" of sequential birth orders. For the forward run, as the fronds in the plates made a first daughter (birth order 1), they were placed into the appropriate trap. Four fronds of birth order 1 were placed in replicate at each temperature. Even numbered birth orders were teased apart from the mother at a standard stage and discarded. Because not all fronds in the plates reproduced simultaneously, the lids of each 12-well plate was labeled with a number indicating how many daughters each frond produced. Once the fronds in the plates produced subsequent daughters, they were separated from the mother and placed in the corresponding birth order traps in the thermogradient. Growth medium was replaced each time a new birth order was placed in the thermogradient to ensure all

birth orders were exposed to the same environment, and was replaced weekly for the remainder of the run.

To verify that there were no temporal effects driving observed results of birth order, an “extension” run was performed immediately following the forward run (prior to the backward run) and without altering the conditions. In this extension run, only birth order 1 mothers were placed in the thermogradient so that their behaviour could be compared to the original birth-order one mothers.

For the backward run, birth order 9 was placed in the thermogradient first, with birth order 1 placed in last. To prepare birth order 9 mothers with the 12-well plates, the first fronds made (birth order 1) were discarded. This continued until birth order 8. Birth order 9 was then separated from the mother and placed in the birth order 9-designated traps inside the thermogradient. All birth orders were similarly prepared, and were transferred to the thermogradient with the same temporal separation as the forward run, but in reverse order (7, 5, 3, 1). To ensure that birth orders experienced the same medium changes, the medium was replenished once a week and the placement of a new birth order inside the incubator was scheduled so that it would fall on the same day as the medium change. In chapter 2, measured phenological responses only included first turion birth order and turion frequency. Here, measured phenological responses include birth order of first turion, days to first turion and turion frequency. Length and width of all the mother and daughter fronds were measured to assess the effect of size on turion phenology.

3.2.3 Frond and turion measurements

Once a mother frond produced a daughter, whether a frond or turion, it was separated carefully from its mother using tweezers. A daughter was only separated from the mother when it became a mature adult frond. A frond is considered to be mature when it begins making a frond of its own (Lemon *et al.*, 2001). Each mother and daughter produced was photographed using an Olympus SZX12 microscope with an Infinity 3 Lumenera camera using a 0.5X lens at 10X magnification. The digital photographs were calibrated, and frond length (apex to opposite edge) and width (perpendicular to length at widest point) measurements were taken using NIH ImageJ version 1.49v. A known distance of 10 mm from a glass micrometer scale was used to calibrate the photos. Each frond measurement was repeated at least five times to assess precision and the final result was taken as the average value. Calibration was performed each time ImageJ was opened for measurements.

3.2.4 Statistical analysis

The effects of mother birth order, mother size, and temperature on first turion birth order were analyzed using SAS JPM 13 software. Data for forward and backward thermogradient runs were combined into one dataset to include the effect of thermogradient run. Analyses of turion phenology would be biased if mothers producing zero turions were simply eliminated from statistical analyses. Because I am assessing the effects of birth order and temperature as sources of turion diversification and analyzing potential overall trends, a hypothetical turion is added to mothers that did not produce any turions. Studies that assess seed germination and formation also follow this norm. Therefore, for these analyses, a, late turion—as a daughter following the final true

daughter—was added to the dataset for mother fronds that did not produce a turion during their lifecycle. For example, if a mother frond produced ten daughter fronds during its life cycle and died without producing any turions, a hypothetical eleventh daughter was added as a potential turion. A single daughter turion was added the dataset only to mother fronds that never made a turion and only for the measured response of first turion birth order and days to first turion (not turion frequency).

A multiple regression analysis that combined both the forward and backward thermogradient run was performed to determine the effect of mother birth order, temperature, thermogradient run and their interactions on first turion birth order. Separate post-hoc multiple regression analyses were conducted for the forward and backward runs to further examine the significance of the effect of temperature and mother birth order in each independent run in the event of interactions with thermogradient run. The effects of mother birth order and temperature on first turion birth order were further assessed independently through post-hoc simple regressions combining both runs. A regression analysis was also conducted to compare birth order 1 mothers in the forward run and extension run where only birth order 1 mothers were placed in the thermogradient. This was performed to confirm no temporal effects were driving the birth order results. The effect of temperature on first turion birth order was compared between the two runs.

Turion phenology may be a result of mother size rather than, or in addition to mother birth order. Thus, after determining whether mother length or width is a more appropriate size measure, separate multiple regression analyses tested for effects of mother size, mother birth order, and temperature on first turion birth order in each run.

Days to first turion and turion frequency are alternate measures of turion phenology. These were similarly assessed by conducting multiple regression analyses of the effect of temperature, mother birth order, thermogradient run, and their paired interactions. Turion frequency was calculated as the total number of turions divided by the total number of daughters produced by the mother. The thermogradient run (forward and backward) was included to account for differences between runs. Separate post-hoc multiple regression analyses were conducted for the forward and backward runs to further analyze the data. A logistic regression was conducted to predict the effect of temperature, mother birth order and daughter birth order on daughter state being a frond or turion.

3.3 Results

3.3.1 First turion birth order

When all mothers were exposed to the same temperature (chapter 2), mother birth order was found to have a significant effect on first turion birth order. Here, to study the joint expression of phenotypic plasticity and putative bet hedging, mothers of birth orders 1, 3, 5, 7, and 9 were exposed to a range of temperatures, aiming to test the effect of birth order, temperature and their interaction on turion formation.

A multiple regression analysis that combined forward and backward thermogradient runs to assess the main effects of temperature, mother birth order and thermogradient run (TGR) on first turion birth order found that all three factors were significant, along with several interaction effects involving TGR (Table 3.1).

Given the finding of interactions with TGR, including a three-way interaction between TGR, temperature and mother birth order, post-hoc analyses were performed to examine the two individual runs in more detail. In separate multiple regression analyses (Table 3.2), the effects of mother birth order and temperature were confirmed to have a significant effect on first turion birth order. However, the interaction between mother birth order and temperature —found to be nonsignificant in the whole model— is significant in both the forward and backward run.

Table 3.1 Multiple regression of the effects of temperature and mother birth order on first turion birth order in a single model including forward and backward thermogradient runs (Thermogradient run = TGR).

Effect	<i>df</i>	Sum of squares	F-Ratio	Probability
TGR	1	86.43	57.91	<0.0001*
Temperature	1	432.26	289.61	<0.0001*
Mother birth order	1	163.56	109.58	<0.0001*
TGR*Temperature	1	70.01	46.90	<0.0001*
TGR*Mother birth order	1	4.63	3.10	0.0806
Temperature*Mother birth order	1	0.43	0.29	0.5914
TGR*Temperature* Mother birth order	1	19.56	13.10	0.0004*

Table 3.2 Multiple regression of the effects of temperature and mother birth order on first turion birth order shown separately for the forward thermogradient run and backward thermogradient run.

Thermogradient run	Effect	<i>df</i>	Sum of squares	F-Ratio	Probability
Forward	Temperature	1	425.09	197.55	<0.0001*
	Mother birth order	1	56.58	26.29	<0.0001*
	Temperature*Mother birth order	1	12.90	5.99	0.0170*
Backward	Temperature	1	77.18	92.61	<0.0001*
	Mother birth order	1	111.61	133.92	<0.0001*
	Temperature* Mother birth order	1	7.09	8.50	<0.0048*

Because the interpretation of main effects is controversial in the presence of an interaction effect (Gotelli and Ellison 2013), the effects of mother birth order and temperature on first turion birth order were further assessed independently through post-hoc simple regressions combining thermogradient runs (mother birth order: $F=27.85$, $P=0.0001$, $R^2=0.17$; temperature: $F=110.13$, $P=0.0001$, $R^2=0.44$). Thus, as indicated by the trends in figure 3.3, daughters made later in the mothers' life cycle, i.e. higher birth orders 5 to 9, produce turions on average sooner than daughters made earlier in the mothers' life cycle. For example, in both runs, birth order 9 consistently produced a first turion early across all temperatures, followed by birth order 7, 5 and 3, while birth order 1 mothers made a first turion the latest on average (Figure 3.3).

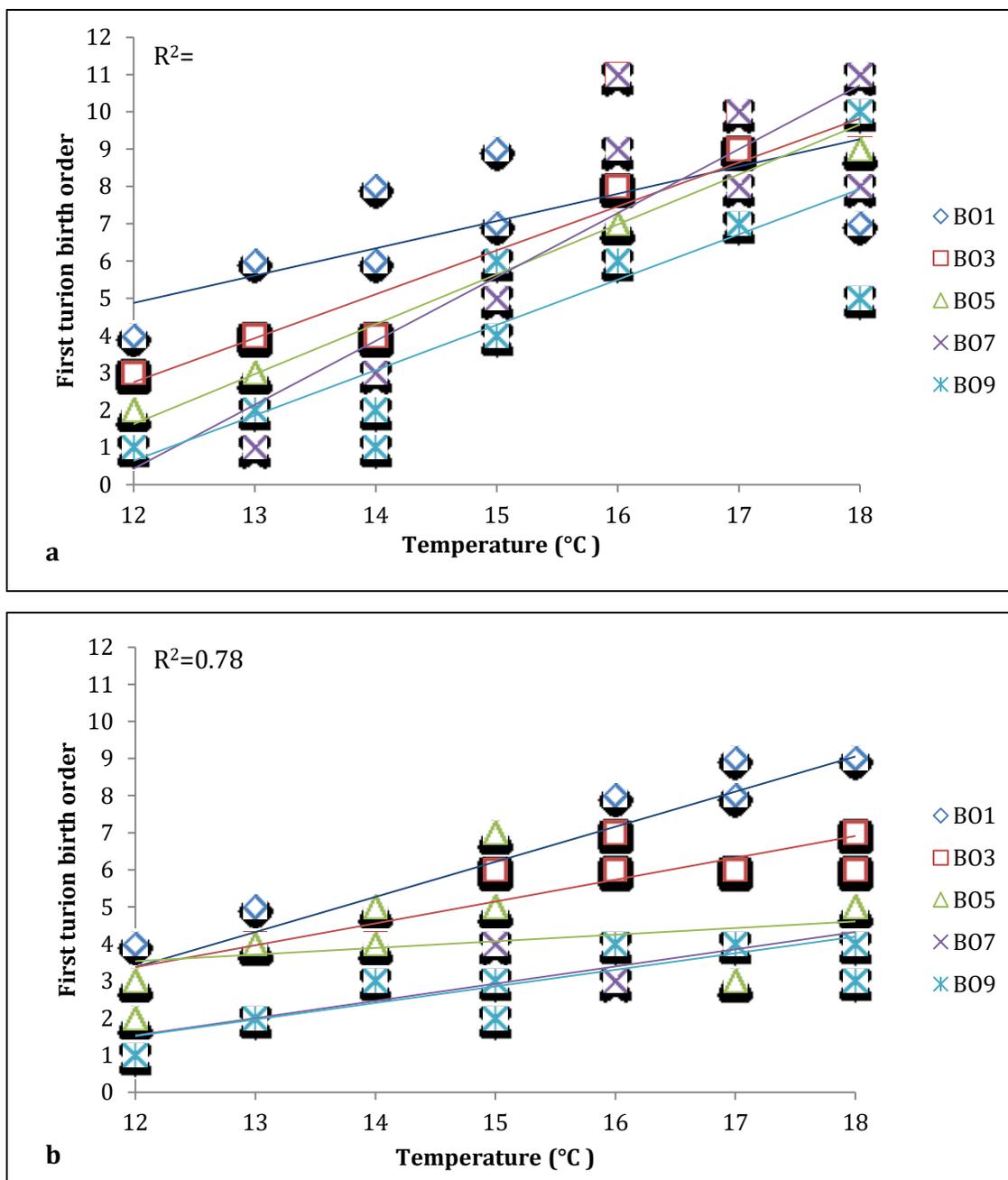


Figure 3.3 The effects of mother birth order and temperature on first turion birth order from the forward (a) and backward (b) thermogradients runs. The thermogradients incubator is split into two replicates, reflected by the two points for each birth order at each temperature.

Immediately following the forward thermogradient run, and without alteration to the thermogradient setup, an additional “extension” run was conducted using only birth order 1 mothers to confirm that no unintended temporal effects were driving birth order results. This allowed a comparison of turion production of birth order 1 mothers during the extension phase to their behaviour at the beginning of the forward run. Separate analyses were conducted on the effect of temperature on first turion birth order in the forward run and extension run. Birth order 1 mothers have similar results across thermogradient runs (Forward: $F=19.05$, $P=0.0009$, $R^2=0.61$; Extension: $F=23.90$, $P=0.0004$; $R^2=0.67$). A comparison of the results of birth order 1 mothers in the extension run to birth order 1 mothers in the forward run, confirms that temperature had a significant effect on first turion birth order, with a similar increase in first turion birth order in both (Figure 3.4).

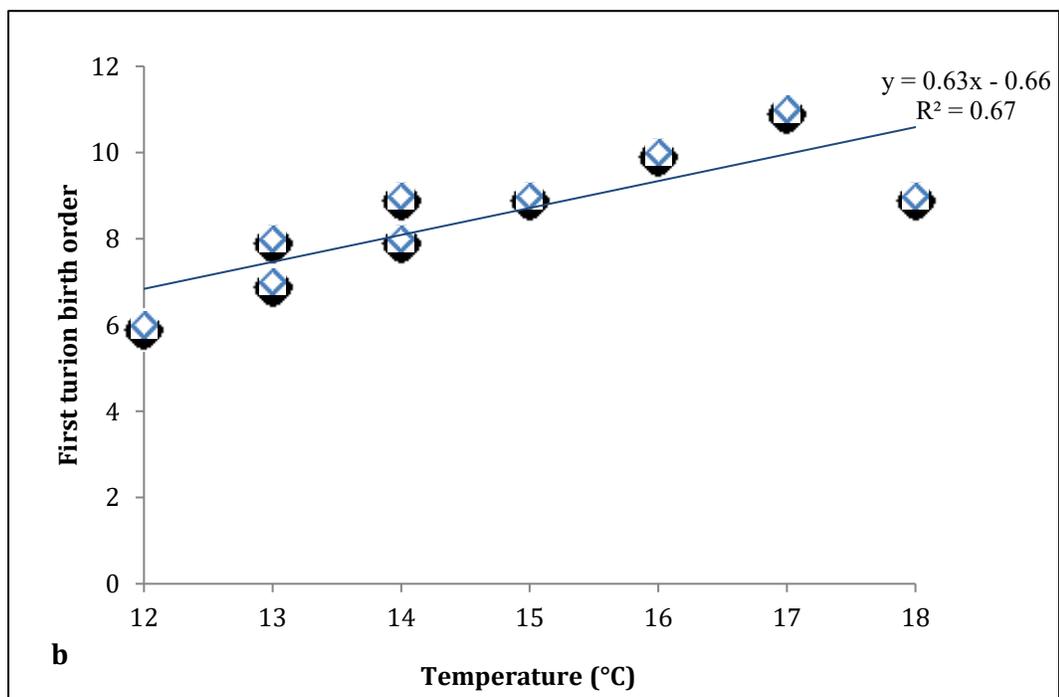
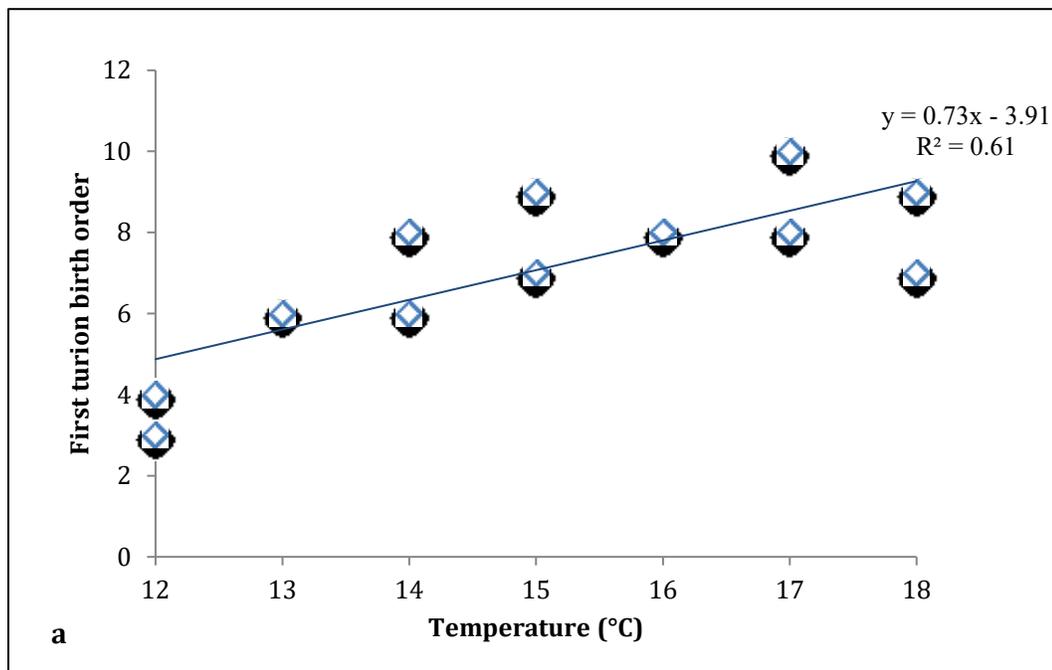


Figure 3.4 The effects of temperature and birth order 1 mothers on first turion birth order in the forward (a) and extension (b) run where only birth order 1 mothers were placed in the thermogradient.

Results from chapter 2 showed that frond size plays an important role in turion formation. Here, I ask whether mother size explains variation in turion phenology beyond that explained by birth order across thermal environments. First, to determine whether length or width is a better indicator of mother size the variance explained by length and width was compared in a multiple regression that also included temperature and interaction effects on first turion birth order. Mother width has a significant effect on first turion birth order, whereas mother length does not, regardless of whether only main effects (width: $F=45.8$, $P<0.0001$; length: $F=0.98$, $P=0.32$) or interaction effects (width: $F=23.9$, $P<0.0001$; length: $F=0.24$, $P=0.62$) are included in the model. Therefore, further analyses use mother width to represent size.

To ask whether turion phenology is a result of mother birth order beyond the effect of size, separate multiple regression analyses were conducted for each thermogradient run that included the effects of temperature, mother birth order, mother size (width), and their interactions. Both runs show strong effects of mother birth order and temperature, but only the backward run shows a strong effect of width (Table 3.3). There is clearly an independent effect of mother birth order beyond the effect of mother size. For this reason, and because models including all variables and their interaction effects are large and difficult to interpret, mother birth order but not size are used for further analyses of turion phenology.

Table 3.3 Multiple regression of the effects of temperature, mother birth order and mother width on first turion birth order shown separately for the forward thermogradient run and backward thermogradient run.

Thermogradient run	Effect	<i>df</i>	Sum of squares	F-ratio	Probability
Forward	Temperature	1	1478.58	725.79	<0.0001*
	Mother birth order	1	129.47	63.55	<0.0001*
	Mother width	1	4.14	2.03	0.155
	Temperature*mother birth order	1	41.26	20.25	<0.0001*
	Temperature*mother width	1	20.65	10.14	0.0016*
	Mother birth order*mother width	1	0.98	0.48	0.4883
	Temperature*mother birth order*mother width	1	3.95	1.94	0.1649
Backward	Temperature	1	47.96	81.25	<0.0001*
	Mother birth order	1	44.78	75.87	<0.0001*
	Mother width	1	12.50	21.19	<0.0001*
	Temperature*mother birth order	1	11.07	18.76	<0.0001*
	Temperature*mother width	1	0.89	1.51	0.2198
	Mother birth order*mother width	1	8.41	14.26	0.0002*
	Temperature*mother birth order*mother width	1	29.55	50.07	<0.0001*

3.3.2 Time to first turion

A second measure of turion phenology is the amount of time taken to produce a first turion. A multiple regression analysis to assess the effects of temperature, mother birth order, TGR, and their interactions finds that the independent effect of mother birth order and temperature are significant (Table 3.4).

Like in the analysis of first turion birth order, a three-way interaction was found here; therefore, post-hoc multiple regression analyses were conducted to assess thermogradient runs separately. Results differ between the forward and backward run (Table 3.5), with the forward run showing stronger effects of temperature and the backward run showing stronger effects of mother birth order (Figure 3.5)

Table 3.4 Multiple regression of the effects of mother birth order and temperature on days to first turion for both forward and backward thermogradient runs (Thermogradient run = TGR).

Effect	<i>df</i>	Sum of squares	F-Ratio	Probability
TGR	1	249.78	3.56	0.0615
Temperature	1	3182.54	45.32	<0.0001*
Mother birth order	1	3708.43	52.80	<0.0001*
TGR*Temperature	1	2102.19	29.93	<0.0001*
TGR*Mother birth order	1	1180.80	16.81	<0.0001*
Temperature*Mother birth order	1	83.06	1.18	0.2788
TGR*Temperature* Mother birth order	1	503.57	7.17	0.0084*

Table 3.5 Multiple regression of the effects of temperature and mother birth order on days to first turion shown separately for the forward thermogradient run and backward thermogradient run.

Thermogradient run	Effect	<i>df</i>	Sum of squares	F-Ratio	Probability
Forward	Temperature	1	5228.93	62.05	<0.0001*
	Mother birth order	1	352.03	4.18	0.0450*
	Temperature*Mother birth order	1	497.83	5.91	0.0178*
Backward	Temperature	1	55.80	0.99	0.3226
	Mother birth order	1	4537.21	80.74	<0.0001*
	Temperature*Mother birth order	1	88.80	1.58	0.2132

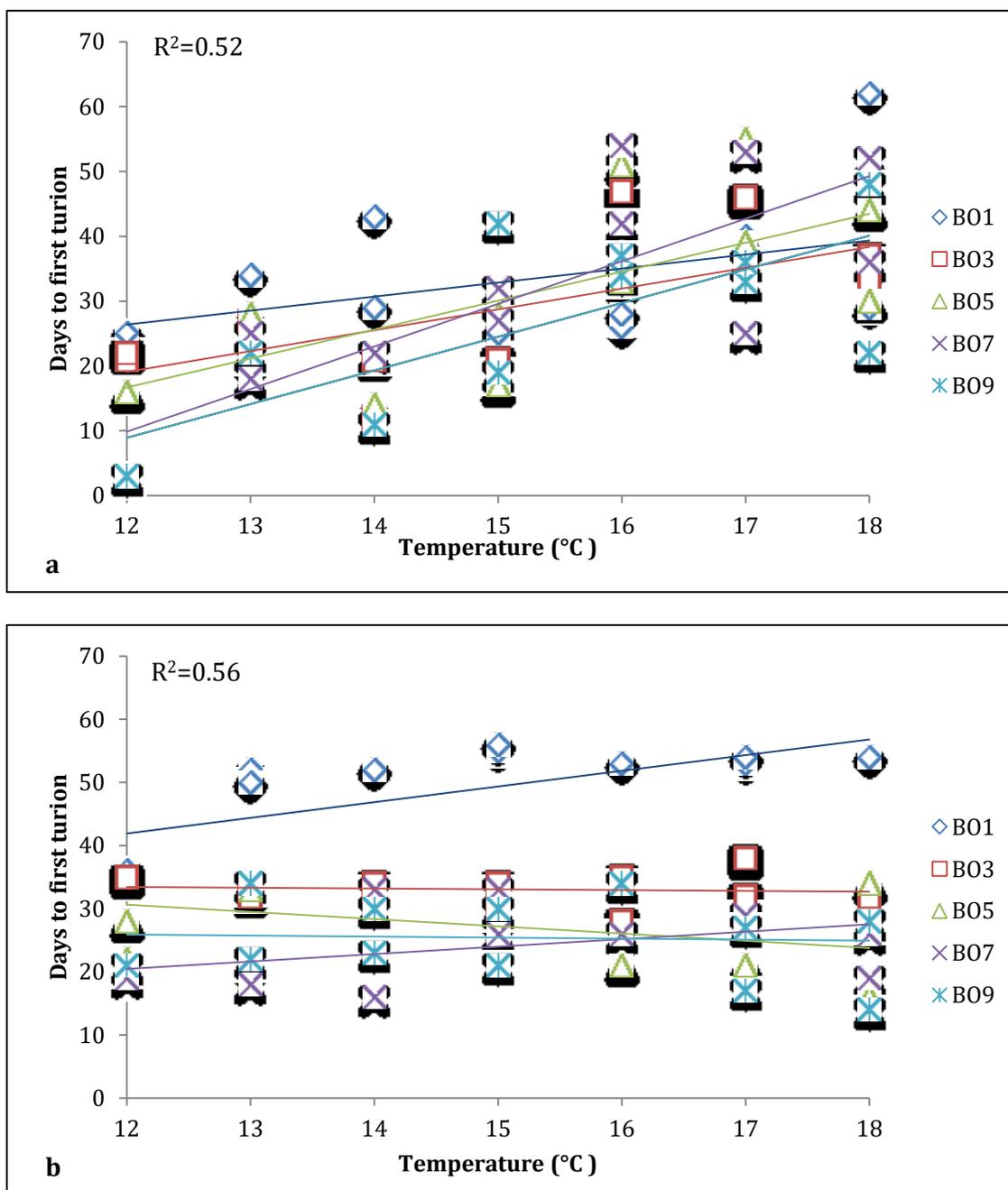


Figure 3.5 The effects of mother birth order and temperature on days to first turion from the forward (a) and backward (b) thermogradient run. The thermogradient incubator is split into two replicates, reflected by the two points for each birth order at each temperature.

3.3.3 Turion frequency

The phenology of turion formation may also be measured as the frequency of turion production, or the proportion of daughters that are turions. A multiple regression to analyze the effect of temperature, mother birth order and thermogradient run on turion frequency found all three main effects to be significant (Table 3.6). Again, the three-way interaction is significant, and separate post-hoc multiple regression analyses were performed for both thermogradient runs independently (Table 3.7). All birth orders showed a decrease in turion frequency at increasing temperatures, and an increasing frequency of turion formation with increasing birth order (Figure 3.6).

Table 3.6 Multiple regression of the effects of temperature and mother birth order on turion frequency for both forward and backward thermogradient runs (Thermogradient run = TGR).

Effect	<i>df</i>	Sum of squares	F-Ratio	Probability
TGR	1	0.31	19.70	<0.0001*
Temperature	1	2.96	186.15	<0.0001*
Mother birth order	1	2.10	131.84	<0.0001*
TGR*Temperature	1	0.19	11.97	0.0007*
TGR*Mother birth order	1	0.10	6.51	0.0119*
Temperature*Mother birth order	1	0.56	35.03	<0.0001*
TGR*Temperature* Mother birth order	1	0.13	8.32	0.0046*

Table 3.7 Multiple regression of the effects of mother temperature and mother birth order on turion frequency shown separately for the forward thermogradient run and backward thermogradient run.

Thermogradient run	Effect	<i>df</i>	Sum of squares	F-Ratio	Probability
Forward	Temperature	1	2.32	133.43	<0.0001*
	Mother birth order	1	0.63	36.39	<0.0001*
	Temperature*Mother birth order	1	0.62	35.35	<0.0001*
Backward	Temperature	1	0.82	57.38	<0.0001*
	Mother birth order	1	1.56	108.94	<0.0001*
	Temperature*Mother birth order	1	0.07	5.09	0.0273*

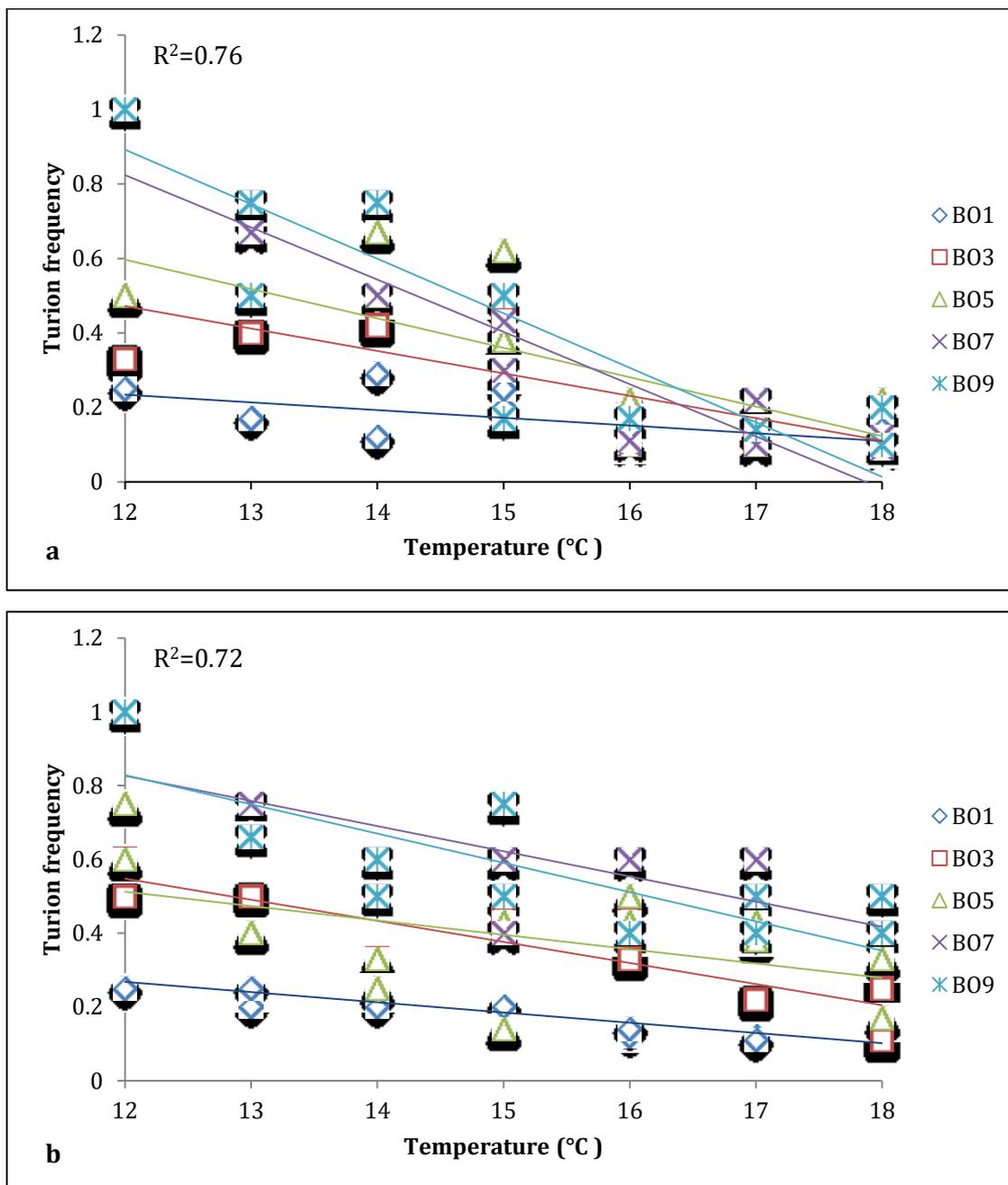


Figure 3.6 The effects of mother birth order and temperature effects on turion frequency from the forward (a) and backward (b) thermogradient run. The thermogradient incubator is split into two replicates, reflected by the two points for each birth order at each temperature.

As a final analysis of turion phenology, a logistic regression was used to model the equation predicting the type of daughter (vegetative frond or turion) produced by a mother. The effects of temperature, mother birth order and daughter birth order are strong predictors in the formation of turions ($R^2=0.48$) (Table 3.8).

Table 3.8 Logistic regression of the effects of temperature, mother birth order and daughter birth order on predicting turion formation.

Effect	Parameter estimate	Wald chi-square	<i>df</i>	Probability
Intercept	16.31	68.33	-	<0.0001*
Temperature	-1.64	84.69	1	<0.0001*
Mother birth order	0.41	35.77	1	<0.0001*
Daughter birth order	1.12	84.90	1	<0.0001*

3.4 Discussion

Phenotypic plasticity and bet hedging are two evolutionary modes of response to varying environmental conditions (Philippi and Seger 1989, Seger and Brockmann 1987, Slatkin 1974, Via and Lande 1985). However, very little is understood about the relationship between these two strategies. *Spirodela polyrhiza* inhabits variable environments in which the timing of turion formation has direct fitness consequences: early turion formation reduces potential fitness through reduced reproduction, but if turion formation is delayed, overwinter survival may be zero. Turions exhibit putative bet hedging through diversification of turion phenology among offspring, and plasticity to environmental conditions such as temperature (Hillman 1961, Jacobs 1947). Furthermore, *S. polyrhiza* provides a convenient model for this study because all offspring fronds of a single mother are genetically identical yet show diversity in phenology of turion formation— a trait critical for overwinter survival (Hillman 1961, Jacobs 1947). It is known that variation in propagule dormancy behaviour is adaptive in a wide range of organisms (Philippi 1993, Venable 2007). For example, Clauss and Venebale (2000) showed that variation in seed germination occur in varying environmental conditions and that germination fractions exhibit adaptive phenotypic plasticity in response to water availability. Delayed germination expressed a bet hedging strategy in response to the distribution of environmental conditions. Here, I asked how genetically identical individual duckweed fronds produce turions at varying times and whether plasticity and diversification bet hedging can be co-expressed.

Maternal effects on offspring traits may be a direct consequence of intrinsic variation in maternal condition in space (architecture) or time (development), or may be

driven by trade-offs (Braun *et al.*, 2013, Smith and Fretwell 1974); offspring phenotype may also be molded directly by the environmental variability mothers face. This study aimed at identifying possible sources of turion variation by analyzing the effects of mother birth order and size across a range of temperatures through the use of a thermogradient incubator. Behavioural diversity in genetically identical individuals may be adaptive or non-adaptive (Ghalambor *et al.*, 2007, Nussey *et al.*, 2005). I found that the mother's birth order, both through its direct effect and its interaction with temperature, can be an important source of potential bet-hedging diversification. The main effect of temperature suggests that if environmental temperature is a reliable cue predicting future conditions, mothers can express phenotypic plasticity to adjust turion timing. Thus, my results suggest that diversification bet hedging and plasticity in the timing of turion formation can be co-expressed under variable environmental change.

Mother size was also assessed as a potential source of variation in turion formation in addition to birth order. Unlike in chapter 2, where size was nonsignificant after controlling for birth order, size had a significant effect on turion phenology, along with the effects of birth order and temperature (Table 3.3). This suggests that when mothers face environmental unpredictability in the wild, variation in mother size and birth order increase daughter turion timing variation, possibly as a bet-hedging strategy. However, the effect of mother birth order was consistently significant and stronger than the effect of size; therefore, mother birth order, temperature and daughter birth order (as this indicates to the type of daughter produced), were used in a logistic regression model (Table 3.8). Each variable contributes significantly to predicting the type of daughter produced by a mother; whether a frond or turion. This furthers our understanding of the

possible causal relationship between these predictor variables and turion phenology. Future studies could be conducted to test this model in mesocosms or in the field.

These findings on turion phenology further our understanding of how the interplay between maternal traits and environmental conditions may affect the expression of phenotypic variance. Effects of maternal environment on timing of turion formation were clearly apparent as turions formed more rapidly at cooler temperatures, regardless of birth order (Figure 3.3, 3.4, 3.5a, 3.6). Water temperature and nutrient availability may vary in time and on a fine spatial scale in natural populations (Schlosser 1990). This suggests that patterns of plasticity and norms of reaction in daughters could be selected in response to environmental variability faced by the mother. Relationships between maternal effects and offspring life-history behaviours have been found in prior research (Dudley 1991, Mousseau and Fox 1998, Roach 1984). Maternal effects, including age, environment and their interaction, have been shown to generate variation in seed emergence timing (Galloway 2001, Lu *et al.*, 2016, Mercer *et al.*, 2011). This thermogradient study provides further support of maternal influences on life-history traits; variation in turion formation is impacted by the birth order of the mother and the temperature they were exposed to in the thermogradient incubator.

The interpretation of results here is intended to identify potential sources of dormancy variation and to assess the relationship between the expression of plasticity and diversification. The phenotypic variation an organism expresses is diversified bet hedging only if the variation is adaptive (Philippi and Seger 1989). For example, seeds of *Lobelia inflata* are genetically identical, yet a high degree of variation in germination timing is found within seasons (Simons and Johnston 2006), and is found to be optimal for the

environmental unpredictability affecting seedling success (Simons 2009). However, environmental variation is both predictable and unpredictable, and the joint expression of plasticity and bet hedging is expected (Simons 2014). In a recent study, divergent germination strategies of several winter annual plant species were found to be influenced by both environmental variation and competition. Strong fitness benefits were observed to varying germination timing, but predicting time of germination was not perfect, suggesting that the evolution of germination timing requires integrating plasticity and bet-hedging strategies (Gremer *et al.*, 2016).

Although I now know that maternal effects (birth order and size) and environmental effects play important roles in determining turion phenology, a fuller understanding of turion variation in genetically identical individuals will require inferences about adaptation to environmental variance. This is difficult because predictable and unpredictable components would need to be assessed, which depends on the way an organism perceives them. To identify a diversification strategy, a long-term study of its fitness effects would have to be conducted. In addition, plasticity is assumed to be adaptive if the perceived cues are reliable indicators of future conditions (Levins 1968, Thomas 1991), which would require determining whether the appropriate phenotypic adjustments are made across environments.

This norms-of-reaction study in turion formation isolates effects of temperature, but is not meant to imply that no other cues exist. Studies of cues, including nutrient levels in water and duration of growth season, can further add to our understanding of the relationship between plasticity and diversification. Although the balance between plasticity and bet hedging cannot be measured, I can conclude that the effects of birth

order and temperature play substantive roles in turion formation, indicating the importance of the joint expression of these two modes of response in the field.

This study was motivated by the lack of understanding of sources that generate diverse dormancy behaviour in clonal offspring and to improve the understanding of the joint expression of phenotypic plasticity and bet hedging. Previous research studies have analyzed the effect of metal stressors on *S. polyrhiza* growth (Olah *et al.*, 2014; Olah *et al.*, 2015), uptake capabilities (Zhang *et al.*, 2009), and cues that induce turion formation including nutrient levels and temperature (Appenroth *et al.*, 1989, Appenroth and Nickel 2010, Gerard and Triest 2014), but not what causes behavioral variation within genetically identical fronds. Although this study's focus was on turion production, and thus environmental variation in the current season, the full consequences of variation in turion production include also turion germination variance the following season. Future work to build on evidence provided here should include a test of bet hedging; a test of the quantitative fit of a candidate bet-hedging trait—both the timing of turion formation and germination—to the degree of fluctuating selection (Simons 2011). This study is the first to assess sources of variation in turion formation of *Spirodela polyrhiza* fronds and provides insight on the natural history of *Spirodela*, an emerging model organism, suggesting what mechanisms may influence phenology and generate diversity in dormancy behaviour in the field. In addition, it demonstrates that *S. polyrhiza* is an appropriate model for future ecological studies because, among other reasons, potential diversification and plasticity are generated among genetically identical individuals.

References

- Andrewartha, H.G. 2008. Diapause in relation to the ecology of insects. *Biological Reviews*, **27**: 50-107.
doi:10.1111/j.1469-185x.1952.tb01363.x
- Appenroth, K.J. 2002. Co-action of temperature and phosphate in inducing turion formation in *Spirodela polyrhiza* (Great duckweed). *Plant, Cell and Environment*, **25**:1079-1085.
doi:10.1046/j.1365-3040.2002.00885.x
- Appenroth, K.-J. and Adamec, L. 2014. Specific turion yields of different clones of *Spirodela polyrhiza* depend on external phosphate thresholds. *Plant Biology*, **17**:125-129.
doi:10.1111/plb.12154
- Appenroth, K.J., and Nickel, G. 2010. Turion formation in *Spirodela polyrhiza*: The environmental signals that induce developmental process in nature. *Physiologia Plantarum*, **138**:312-320.
doi: 10.1111/j.1399-3054.2009.01319.x.
- Appenroth, K., Hertel, W., Jungnickel, F. and Augstenn, H. 1989. Influence of nutrient deficiency and light on turion formation in *Spirodela polyrhiza* (L.) Schleiden. *Biochemie und Physiologie der Pflanzen*, **184**:395-303.
doi:10.1016/S0015-3796(89)80036-8
- Appenroth, K., Teller, S., and Horn, M. 1996. Photophysiology of turion formation and germination in *Spirodela polyrhiza*. *Biologia Plantarum*, **38**: 95.
doi: 10.1007/BF02879642
- Ashby, E. and Wangermann, E. 1949. Senescence and rejuvenation in *Lemna minor*. *Nature*, **164**:187.
doi:10.1038/164187a0
- Auld, J.R., Agrawal, A.A. and Relyea, R.A. 2009. Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proceedings of the Royal Society B.*, **277**: 503-511.
- Barks, P.M and Laird, R.A. 2015. Senescence in duckweed: age-related declines in survival, reproduction and offspring quality. *Functional Ecology*, **29**:540-548.
doi:10.1111/1365-2435.12359
- Barks, P.M., and Laird, R.A. 2016. A multigenerational effect of parental age on offspring size but not fitness in common duckweed (*Lemna minor*). *Journal of Evolutionary Biology*, **29(2016)**: 748-756.
doi: 10.1111/jeb.12823

- Baskin, C.C., Baskin, J.M. 1998. Seeds— ecology, biogeography, and evolution of dormancy and germination. San Diego, CA, USA: Academic Press.
- Beaumont, H.J.E., Gallie, J., Kost, C., Ferguson, G.C. and Rainey, P.B. 2009. Experimental evolution of bet hedging. *Nature*, **462**: 90-97.
- Beer, S. 1985. Effects of CO₂ and O₂ on the photosynthetic O₂ evolution of *Spirodela polyrhiza* turions. *Plant Physiology*, **79**:199-201.
- Bell, G. and Gonzalez, A. 2009. Evolutionary rescue can prevent extinction following environmental change. *Ecology Letters*, **12**: 942-948
doi:10.1111/j.1461-0248.2009.01350.x
- Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W. and Courchamp, F. 2012. Impacts of climate change on the future of biodiversity. *Ecology Letters*, **15(4)**: 365-377.
doi: 10.1111/j.1461-0248.2011.01736.x
- Belozеров, V.N. 2008. Diapuse and quiescence as two main kinds of dormancy and their significance in life cycles of mites and ticks (Chelicerata: Arachnida:Acari) Part 1. Acariformes. *Acarina*, **16**: 79-130.
- Bentsink, L. and Koornneef, M. 2008. Seed Dormancy and Germination. *Arabidopsis Book*, **6**: 1-18.
doi: 10.1199/tab.0119
- Botero, C.A., Weissing, F.J., Wright, J. and Rubenstein, D.R. 2014. Evolutionary tipping points in the capacity to adapt to environmental change. *Proceedings of the National Academy of Sciences*, **112**:184-189.
- Bradshaw, A.D. 1965. Evolutionary significance of phenotypic plasticity in plants. *Advances in Genetics*, **13**: 115-155.
- Braun, D.C., Patterson, D.A. and Reynolds, J.D. 2013. Maternal and environmental influences on egg size and juvenile life-history traits in Pacific salmon. *Ecology and Evolution*, **3(6)**: 1727-1740.
- Byers D. L., Platenkamp, G.A.J., and Shaw, R.G. 1997. Variation in seed characters in *Nemophila menziesii*: evidence of a genetic basis for maternal effect. *Evolution*, **51**: 1445-1456.
- Charmantier, A., McCleery, R.H., Cole, L.R., Perrins, C., Kruuk, L.E. and Sheldon, B.C. 2008. Adaptive phenotypic plasticity in response to climate change in a wild bird population. *Science*, **9(320)**: 800-803.
doi: 10.1126/science.1157174.

- Charmantier, A., Garant, D. 2005 Environmental quality and evolutionary potential: lessons from wild populations. *Proceedings of the Royal Society B*, **272**: 1415–1425.
doi:10.1098/rspb.2005.3117
- Chevin, L.-M., Lande, R. and Mace, G.M. 2010. Adaptation, Plasticity, and Extinction in a Changing Environment: Towards a Predictive Theory. *Public Library of Science Biology*, **8**: 1-7.
doi: 10.1371 /journal, pbio. 1000357.
- Chown, S.L., Hoffman, A.A., Kristensen, T.N., Angilletta, M.J., Stenseth, N.C., and Pertoldi, C. 2010. Adapting to climate change: a perspective from evolutionary physiology. *Climate Research*, **43**: 3-15.
doi: 10.3354/cr00879
- Clauss, M.J. and Venable, D.L. 2000. Seed germination in desert annuals: An empirical test of adaptive bet hedging. *American Naturalist*, **155**: 168-186.
- Cleland, E.E., Chuine, I., Menzel, A., Mooney, H.A. and Schwartz, M.D. 2007. Shifting plant phenology in response to global change. *TRENDS in Ecology and Evolution*, **22**: 357-365.
doi:10.1016/j.tree.2007.04.003
- Cohen, D. 1966. Optimizing reproduction in a randomly varying environment. *Journal of Theoretical Biology*, **12**: 119–129.
- Cohen, D. 1967. Optimizing reproduction in a randomly varying environment when a correlation may exist between conditions at time a choice has to be made and subsequent outcome. *Journal of Theoretical Biology*, **16**: 1–14.
- Dempster, E.R. 1955. Maintenance of genetic heterogeneity. *Cold Spring Harbor Symposia on Quantitative biology*, **20**: 25-32.
- Dudley, E.C., eds. 1991. Maternal effects in evolution, in *The Unity of Evolutionary Biology. Proceedings of the Fourth Annual Meeting of the Fourth International Congress of Systematic and Evolutionary Biology*, pp. 719–799, Dioscorides Press.
- Ellner, S. P., Hairston, N. G., Kearns, C. M., and Babai, D. 1999. The roles of fluctuating selection and long-term diapause in microevolution of diapause timing in a freshwater copepod. *Evolution*, **53**: 111–122.
doi:10.2307/2640924

- Fernandez-Pascual, E. and Jiminez-Alfaro, B. 2014. Phenotypic plasticity in seed germination relates differentially to overwintering and flowering temperatures. *Seed Science Research*, **24**: 273-280.
doi:10.1017/S0960258514000269
- Fox, C.W. 1993. The influence of maternal age and mating frequency on eff size and offspring performance in *Callosobruchus maculatus* (Cleopatra: Bruchidae). *Oecologia*, **96**: 139-146.
- Furness, A.I., Lee, K. and Reznick, D.N. 2015. Adaptation in a variable environment: Phenotypic plasticity and bet-hedging during egg diapause and hatching in an annual killifish. *Evolution*, **69**: 1461-1475.
doi:10.1111/evo.12669
- Galloway, L.F. 2001. The effect of maternal and paternal environments on seed characters in the herbaceous plant *Campanula americana* (Campanulaceae). *American Journal of Botany*, **88(5)**: 832-840.
- Gerard, J. and Triest, L. 2014. The effect of phosphorus reduction and competition on invasive Lemnids: Life traits and nutrient uptake. *International Scholarly Research Network Botany*, **2014**: 1-9.
doi:10.1155/2014/514294
- Ghalambor, C.K., McKay, J.K., Carroll, S.P. and Reznick, D.N. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, **21(3)**: 394-407.
doi: 10.1111/j.1365- 2435.2007.01283.x
- Gillespie, J. 1974. Natural selection for within-generation variance in offspring number. *Genetics*, **76**: 601–606.
- Graham, J.K., Smith, M.L., Simons, A.M. 2014. Experimental evolution of bet hedging under manipulated environmental uncertainty in *Neurospora crassa*. *Proceedings of the Royal Society B.*, **281**: 20140706.
- Gonzalez, A., Ronce, O., Ferriere, R. and Hochberg, M.E. 2012. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **368**: 20120404.
doi:10.1098/rstb.2012.0404
- Gotelli, N.J., and Ellison, A.M. 2013. *A Primer of Ecological Statistics*. University of Vermont and Harvard Forest: Sinauer Associates, Inc.

- Gremer, J.R., Kimball, S. and Venable, D.L. 2016. Within-and among-year germination in Sonoran Desert winter annuals: bet hedging and predictive germination in a variable environment. *Ecology Letters*, **19**: 1209-1218.
doi: 10.1111/ele.12655
- Gremer, J.R. and Venable, D.L. 2014. Bet hedging in desert winter annual plants: optimal germination strategies in a variable environment. *Ecology Letters*, **17**: 380–387.
- Halpern, S.L. 2004. Sources and consequences of seed size variation in *Lupinus perennis* (Fabaceae): adaptive and non-adaptive hypotheses. *American Journal of Botany*, **92(2)**: 205-213.
- Heide, O.M. 2011. Temperature rather than photoperiod controls growth cessation and dormancy in *Sorbus* species. *Journal of Experimental Biology*, **62(15)**: 5397-5404.
- Hillman, W.S. 1961. The Lemnaceae, or duckweeds. A review of the descriptive and experimental literature. *The Botanical Review*, **27**: 221-287.
- Hilton, J.R. and Bitterli, C.J. 1983. The influence of light on the germination of *Avena fatua* L. (wild oat) seed and its ecological significance. *New Phytologist*, **95**: 325-333.
- Hughes, P.W and Simons, A.M. 2014. Secondary reproduction in the herbaceous monocarp *Lobelia inflata*: time-constrained primary reproduction does not result in increased deferral of reproductive effort. *BMC Ecology*, **14**:15.
doi: 10.1186/1472-6785-14-15
- Jacobs, D.L. 1947. An ecological life-history of *Spirodela polyrhiza* (Greater Duckweed) with emphasis on the turion phase. *Ecological Monographs*, **17**: 437-469.
- Kuehdorf, K., Jetschke, G., Ballani, L. and Appenroth, K-J. 2014. The clonal dependence of turion formation in the duckweed *Spirodela polyrhiza*-an ecogeographical approach. *Plant Physiology*; **150**: 46-54.
doi:10.1111/ppl.12065
- Landolt, E. 1986. The family Lemnaceae—monographic study. 1. Ver-offentlichungen des Geobotanischen Institutes der ETH, Stiftung Rubel, Zurich. Heft 71.
- Lemon, G. D., Posluszny U. and Husband, B. C. 2001. Potential and realized rates of vegetative reproduction in *Spirodela polyrhiza*, *Lemna minor*, and *Wolffia borealis*. *Aquatic Botany*, **70**: 79- 87.
doi: 10.1016/S0304-3770(00)00131-5
- Lemon, G.D. and Posluszny, U. 2000. Comparative shoot development and evolution in the Lemnaceae. *International Journal of Plant Sciences*, **161**: 733–748.

- Leng, R.A. 1999. Duckweed: A tiny aquatic plant with enormous potential for agriculture and environment. FAO Publications, Rome, Italy.
- Levins, R. 1968. Evolution in Changing Environments. Princeton University Press: Princeton.
- Ley, S. Dolger, K. and Appenroth, K-J. 1997. Carbohydrate metabolism as a possible physiological modulator of dormancy in turions in *Spirodela polyrhiza* (L.). *Plant Science*, **129**: 1-7.
- Lloyd, D.G. 1984. Variation strategies of plants in heterogeneous environments. *Biological Journal of the Linnaean society*, **21**: 357-385.
- Lu, J.J., Tan, D.Y., Baskin, J.M., and Baskin, C.C. 2013. Trade-offs between seed dispersal and dormancy in an amphi-biscarpic cold desert annual. *Annals of Botany*, **112(9)**: 1815-1827.
- Lu, J.J., Tan, D.Y., Baskin, J.M., and Baskin, C.C. 2016. Effects of germination season on life history traits and on transgenerational plasticity in seed dormancy in a cold desert annual. *Scientific Reports*, **6**: 25076.
- Malek, L., and Cossins, E.A. 1982. Senescence, turion development, and turion germination in nitrate- and sulfate-deficient *Spirodela polyrhiza*. Relationships between nutrient availability and exogenous cytokinins. *Canadian Journal of Botany*, **61**:1887-1897.
- Martin, A.C. 1946. The comparative internal morphology of seeds. *American Midland Naturalist* **36**: 513–660.
- Masuda, J-I., Urakawa, T., Ozaki, Y., and Okubo, H. 2006. Short photoperiod induces dormancy in lotus (*Nelumbo nucifera*). *Annals of Botany*, **97(1)**: 39-45.
- Medany, M. A., and Hegazy, A. K. 2007. Prediction of seed germination and seedling growth of four crop plants as affected by root zone temperature. *World Journal of Agricultural Sciences*, **3(6)**: 714-720.
- Mercer, K.L., Alexandr, H.M., and Snow, A.A. 2011. Selection on seedling emergence timing and size in an annual plant, *Helianthus annuus*, (Common sunflower, Asteraceae). *American Journal of Botany*, **98**: 975-985.
- Mojonnier, L. E. 1998. Natural selection on two seed-size traits in the common morning glory *Ipomea purpurea* (Convolvulaceae): patterns and evolutionary consequences. *American Naturalist*, **152**: 188-203

- Moran, N.A. 1992. The Evolutionary maintenance of alternative phenotypes. *The American Naturalist*, **139**: 971-989.
- Mousseau, T.A. and Fox, C.W., eds. 1998. Maternal Effects as Adaptations (A Symposium organized for the 1996 Annual Meetings of the Society for the Study of Evolution, Washington University, St. Louis MI, USA), Oxford University Press.
- Murren, C.J., Auld, J.R., Callahan, H., Ghalambor, C.K., Handelsman, C.A., Heskell, M.A., Kingsolver, J.G., Maclean, H.J., Masel, J., Maughan, H., Pfenning, D.W., Relyea, A., Seiter, S., Snell-Rood, E., Steiner, U.K. and Schlichting, C.D. 2015. Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. *Heredity*, **115**: 293-301.
doi:10.1038/hdy.2015.8
- Newton, R.J., Shelton, D.R., Disharoon, S. and Duffey, J.E. 1978. Turion Formation and Germination in *Spirodela polyrhiza*. *American Journal of Botany*, **65**: 421-428.
- Nilsson, P., Tuomi, J. and Astrom, M. 1996. Bud Dormancy as a bet-hedging strategy. *The American Naturalist*, **147**: 269-281.
- Nussey, D.H., Postma, E., Giennap, P. and Visser, M.E. 2005. Selection on heritable phenotypic plasticity in a wild bird population. *Science*, **310**: 304-306.
doi: 10.1126/science.1117004
- Olah, V., Hepp, A. and Meszaros, I. 2015. Comparative study on the sensitivity of turions an active fronds of giant duckweed (*Spirodela polyrhiza* (L.) Schleiden) to heavy metal treatments. *Chemosphere*, **132**:40-46.
- Olah, V., Hepp, A., Lakatos, G. and Meszaros, I. 2014. Cadmium-induced turion formation of *Spirodela polyrhiza* (L.) Schleiden. *Acta Biologica Szegediensis*, **58**:103-108.
- Orozco-Segovia, A., Gonzalez-Zertuche, L., and Mendoza, A., Orozco, S. 1996. A mathematical model that uses Gaussian distribution to analyze the germination of *Manfreda brachystachya* (Agavaceae) in a thermogradient. *Physiologia Plantarum*, **98**: 431-438.
- Perry, T.O. 1968. Dormancy, Turion formation and germination by different clones of *Spirodela polyrhiza*. *Plant Physiology*, **43**:1866-1869.
- Philippi, T. 1993. Bet-hedging germination of desert annuals: beyond the first year. *American Naturalist*, **142**(3): 474-487.
- Philippi, T. and Seger, J. 1989. Hedging ones evolutionary bets, revisited. *Trends in Ecology and Evolution*, **4**: 41-44.

- Probert, R.J. 2000. The role of temperature in the regulation of seed dormancy and germination. In: Fenner M, ed. *Seeds: the ecology of regeneration in plant communities*. Wallingford, UK: CAB International, 261–292.
- Réale, D., McAdam, A.G., Boutin, S. and Berteaux, D. 2003. Genetic and plastic responses of a northern mammal to climate change. *Proceedings of the Royal Society B*, **270(1515)**: 591-596.
doi: 10.1098/rspb.2002.2224
- Rees, M., Childs, D. Z., Rose, K. E. and Grubb, P. J. 2004. Evolution of size dependent flowering in a variable environment: partitioning the effects of fluctuating selection. *Proceedings of the Royal Society of London B*, **271**: 471–475.
- Rees, M., Childs, D.Z., Metcalf, J.C., Rose, K.E., Sheppard, A.W., Grubb, P.J. 2006. Seed dormancy and delayed flowering in monocarpic plants: selective interactions in a stochastic environment. *The American Naturalist*, **168(2)**: E53-E71.
doi: 10.1086/505762
- Reed, T.E., Schindler, D.E. and Waples, R.S. 2011. Interacting Effects of Phenotypic Plasticity and Evolution on Population Persistence in a Changing Climate. *Conservation Biology*, **25(1)**: 56-63.
- Roach, D. A. 1984. Ecological Genetics of Life-History Characteristics in *Geranium carolinianum*. Ph.D. Dissertation, Duke University.
- SAS Institute. 2016. SAS/STAT User's guide, version 13.0. Cary, North Carolina, USA: SAS Institute.
- Schlosser, I.J. 1990. Environmental variation, life history attributes, and community structure in stream fishes: implications for environmental management and assessment. *Journal of Environmental Management*, **14**: 621–628.
- Seger, J. and Brockmann, H.J. 1987. What is bet-hedging? *Oxford Surveys in Evolutionary Biology*, **4**: 182–211.
- Shefferson, R.P. 2009. The evolutionary ecology of vegetative dormancy in mature herbaceous perennial plants, *Journal of Ecology*, **97**: 1000-1009.
doi:10.1111/j.1365-2745.2009.01525.x
- Silsbury, J.H. 1961. A study of dormancy, survival and other characteristics in *Lolium perenne* L. at Adelaide, S.A. *Australian Journal of Agriculture Research*, **12**: 1-9.
- Simons, A. M., and Johnston, M.O. 2000. Variation in seed traits of *Lobelia inflata* (Campanulaceae): sources and fitness consequences. *American Journal of Botany*, **87**: 124-132.

- Simons, A. M. and Johnston, M. O. 2003. Suboptimal timing of reproduction in *Lobelia inflata* may be a conservative bet-hedging strategy. *Journal of Evolutionary Biology*, **16**, 233–243.
doi:10.1046/j.1420-9101.2003.00530.x
- Simons, A. M. and Johnston, M. O. 2006. Environmental and genetic sources of diversification in the timing of seed germination: implications for the evolution of bet hedging. *Evolution*, **60(11)**: 2280-2292.
- Simons, A.M. 2009. Fluctuating natural selection accounts for the evolution of diversification bet hedging. *Proceedings of the Royal Society of London B: Biological Sciences*, **276**: 1987-1992.
- Simons, A.M. 2011. Modes of response to environmental change and the elusive empirical evidence for bet hedging. *Proceedings of the Royal Society B*, **278**:1601-1609.
doi: 10.1098/rspb.2011.0176
- Simons, A.M. 2014. Playing smart vs. playing safe: the joint expression of phenotypic plasticity and potential bet hedging across and within thermal environments. *Journal of Evolutionary Biology*, **27**:1047-1056.
doi: 10.1111/jeb.12378
- Simpson, G.M. 1990. Seed dormancy in grasses. Cambridge, UK: Cambridge University Press.
- Slatkin, M. 1974. Hedging ones evolutionary bets. *Nature*, **250**: 704–705.
- Smart, C.C. and Trewavas, A.J. 2006. Abscisic-acid-induced turion formation in *Spirodela polyrrhiza* L.II. Ultrastructure of the turion; a stereological analysis. *Plant, Cell and Environment*, **6**:515-522.
doi:10.1111/1356-3040.ep11588143
- Smith, C. C. and Fretwell, S.D. 1974. The optimal balance between size and number of offspring. *American Naturalist*, **108**: 499-506.
- Snell-Rood, E. C. 2012. Selective processes in development: implications for the costs and benefits of phenotypic plasticity. *Integrative and Comparative Biology*, **52(1)**: 31-42.
- Song, G., Hou, W., Wang, Q., Wang, J. and Jin, X. 2006. Effect of low temperature on eutrophicated waterbody restoration by *Spirodela polyrrhiza*. *Bioresource Technology*, **97**:1865-1869.

- Steadman, K.J., Crawford, A.D., Gallagher, R.S. 2003. Dormancy release in *Lolium rigidum* seeds is a function of thermal after-ripening time and seed water content. *Functional Plant Biology*, **30**: 345–352.
- Stearns, S.C. 1989. The evolutionary significance of phenotypic plasticity. *Bioscience*, **39**: 436-445.
- Sultan, S.E. 2000. Phenotypic plasticity for plant development, function and life history. *Trends in Plant Science*, **5(12)**: 537-542.
- Sussman, A.S. and Douthitt, H.A. 1973. Dormancy in microbial species. *Annual Review Plant Physiology*, **24**: 311-352.
- Thomas, J.D. 1991. Phenotypic plasticity as a component of evolutionary change. *Trends in Ecology and Evolution*, **6**:246-249.
doi:10.1016/0169-5347(91)90070-E
- Thompson, K., and Whatley, J.C. 1984. A thermogradient bar apparatus for the study of the germination requirements of buried seeds *in situ*. *New Phytologist*, **96(3)**: 459-471.
- Travis, J.M.J., Delgado, M., Bocedi, G., Baguette, M., Barton, K., Bonte, D., Boulaenger, I., Hodgson, J.A., Kubisch, A., Penteriani, V., Saastamoinen, M., Stevens, V.M. and Bullock, J.M. 2013. Dispersal and species' responses to climate change. *Oikos*, **122**:1532-1540.
- Tufto, J. 2015. Genetic evolution, plasticity and bet-hedging as adaptive responses to temporally autocorrelated fluctuating selection: A quantitative genetic model. *Evolution*, **69**: 2034-2049.
doi:10.1111/evo.12716
- van Kleunen, M. and Fischer, M. 2005. Constraints on the evolution of adaptive phenotypic plasticity in plants. *New Phytologist*, **166(1)**: 49-60.
- Venable, D.L. 2007. Bet hedging in a guild of desert annuals. *Ecology*, **88(5)**: 1086-1090.
doi: 10.1890/06-1495
- Via, S. and Lande, R. 1985. Genotype-Environment Interaction and the Evolution of Phenotypic Plasticity. *Society for the Study of Evolution*, **39**: 505-522.
- Vleeshouwers, L.M., Bouwmeester, H.J., and Karssen, C.M. 1995. Redefining seed dormancy: an attempt to integrate physiology and ecology. *Journal of Ecology*, **83**: 1031–1037.

- Wang, W., Haberer, G., Gundlach, H., Glaber, C., Nussbaumer, T., Luo, M.C., Lomsadze, A., Borodovsky, M., Kerstetter, R.A., Shanklin, J., Byrant, D.W., Mockler, T.C., Appenroth, K.J., Grimwood, J., Jenkins, J., Chow, Choi, C., Adam, C., Cao, X-H., Fuchs, J., Schubert, I., Rokhsar, D., Schmutz, J., Michael, T.P., Mayer, K.F.X. and Messing, J. 2014. The *Spirodela polyrhiza* genome reveals insights into its neotenus reduction fast growth and aquatic lifestyle. *Nature Communications*, **5**: 3311.
doi:10.1038/ncomms4311
- Westoby M., Leishman, M. and Lord, J. 1996. Comparative ecology of seed size and dispersal. *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences*, **351**: 1309-1318
- Wilson, A. J., Pemberton, J. M., Pilkington, J. G., Coltman, D. W., Mifsud, D. V., Clutton-Brock, T. H., Kruuk, L. E. B. 2006. Environmental coupling of selection and heritability limits evolution. *Public Library of Science Biology*, **4**:1270-1275.
doi:10.1371/journal.pbio.0040216
- Wong, B.B.M., and Candolin, U. 2014. Behavioural responses to changing environments. *Behavioural Ecology*, **26(3)**: 665-673.
- Zhang, X., Hu, Y., Liu, Y., and Chen, B. 2009. Arsenic uptake, accumulation and phytoremediation of duckweed (*Spirodela polyrhiza* L.). *Journal of Environmental Sciences*, **23(4)**: 601-606.