The Phenology of Turion Formation in *Spirodela polyrhiza* as an Adaptation to
Unpredictable Environments: Implications for the Evolution of Bet Hedging and
Phenotypic Plasticity

by

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ABSTRACT

Reliable environmental cues may lead to the evolution of adaptive phenotypic plasticity. However, when cues are unreliable and the environment is thus unpredictable, “bet hedging” is expected to evolve. Here, the phenology of turion formation in (*Spirodela polyrhiza* (L.)) is used to study bet hedging and phenotypic plasticity in the field. Mean days to turion germination and formation were found to differ among pond environments- fronds from ponds of greater unpredictability had greater variability in the timing of turion formation. In a transplant study fronds behaved differently from when in their home environments, suggesting phenotypic plasticity in turion formation. In a common garden experiment, comparison of trait expression under homogenous conditions provided evidence for population differentiation. This study provides a test of putative diversification bet hedging, phenotypic plasticity and population differentiation in *S. polyrhiza*.

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CHAPTER 1

**TESTS OF PUTATIVE BET HEDGING IN TURION PHENOLOGY OF *SPIRODELA
POLYRHIZA* (L.) IN REPSONSE TO ENVIRONMENTAL UNPREDICTABILITY**

Introduction

1.1.1 Implications of Environmental Fluctuations

Life-history traits such as juvenile development, age at sexual maturity, germination and even death are known to be under strong natural selection (Roff 1992). However, there is uncertainty in the association between the phenology of these life-history traits and fitness because of underlying trade-offs between possible fitness gains and risks involved with unpredictability of temporal environmental patterns and disturbances (Stearns 1989; Varpe 2012; Varpe *et al.* 2007; Iwasa and Levin 1995; Landa 1992; Boyce 1978; Giesel 1976). Such disturbances are further complicated by climate change (most notably temperature), which is occurring more rapidly than previously observed (Walther *et al.* 2002) and is expected to continue on this trajectory (Stenseth and Mysterud 2002) due to anthropogenic activities (Rosenzweig *et al.* 2008; Chevin *et al.* 2010). Therefore, the predictability of important environmental cues signaling the need to move from one life-history stage to another, for example, may be compromised. Possible direct responses to temporal variability include changes in behaviour, range shifts and migration. Evolutionary responses include, but are not limited to, the evolution of locally well-adapted ecotypes, phenotypic plasticity and, less commonly studied, bet hedging (Slatkin 1974; Seger and Brockman 1987; Simons 2011).

Bet hedging trades off the arithmetic (immediate/expected) fitness with the geometric mean (long-term) fitness across generations, and is thus characterized by low variance in fitness (Gillespie 1974; Slatkin 1974; Seger and Brockman 1987; Simons 2011). Often described as risk spreading, bet hedging is expected to evolve under environmental uncertainty and reduces the risk of complete failure, thus reducing the

variance in long-term fitness (Seeger and Brockman 1987). Under normal conditions, bet-hedging traits seem maladaptive since the immediate fitness is sacrificed to inhibit the possibility of complete failure. Bet hedging may include the phenological variability of important life-history strategies that include migration (Lundberg 1988), germination (Simons and Johnston 2006) and dormancy (Dumont and Ali 2004).

There are two kinds of bet hedging: diversification and conservative (Seeger and Brockman 1987; Philippi and Seeger 1989). When faced with environmental unpredictability, an individual may express an array of different phenotypes that are suited to different environments, thus reducing the likelihood of failure at the cost of expected fitness (Seeger and Brockman 1987; Philippi and Seeger 1989; Bulmer 1984; Simovich and Hathaway 1997; Rajon *et al* 2009; Simons 2007; Simons and Johnston 2006). For example, the amount of diversification in the timing of seed germination in *Lobelia inflata* matches predictions of bet-hedging theory since fitness functions for survival were found to vary within and among generations under fluctuating natural selection acting on seed germination in the field (Simons 2009). In addition to phenotypic variation, variance in fitness may be reduced by a single phenotype that avoids risk (Slatkin 1974; Seeger and Brockman 1987; Philippi and Seeger 1989). This is conservative bet hedging and may be referred to as risk-aversion. For example, the initiation of reproduction (“bolting”) appears to be a conservative bet-hedging trait in that the timing of this event is restricted to safe, early dates even though bolting at later dates would be optimal under normal circumstances (Simons and Johnston 2003). Both of the above bet-hedging strategies reduce the variation in fitness by a reduction in the arithmetic mean fitness.

1.1.2 Dormancy as Bet Hedging

Dormancy is interpreted as an adaptation allowing plants to withstand unfavourable conditions in which survival under normal metabolic activity would be unlikely (Vegis 1964). Environmental cues regulating dormancy will vary across time and space, and dormancy phenologies are adapted to prevailing conditions (Bewley 1997; Finch-Savage and Leubner-Metzger 2006; Baskin and Baskin 2004). Temperature, photoperiod, nutrient deficiency and water stress are environmental signals that control how plant hormones that regulate dormancy are produced and perceived (Horvath *et al.* 2003). When environmental cues reliably predict environments, phenotypic plasticity may be the mechanism by which dormancy is regulated. For example, dormancy duration in various species is a plastic trait expressed in response to drought stress (Sawhney and Naylor 1982). Alternatively, when environmental variability is unpredictable in that cues are unreliable, bet hedging is expected to evolve (Seger and Brockman 1987; Simons 2002).

The classic example of bet hedging is the dormancy strategy of desert annual plants under unpredictable precipitation (Phillipi 1993; Evans *et al.* 2007; Cohen 1966; Pake and Venable 1996; Adondakis and Venable 2004). The timing of conditions suitable for seed germination (sufficient precipitation and temperatures) in desert climates may be inconsistent year-to-year (good year versus bad year). Adaptive bet hedging as a response to desert unpredictability allows for one individual to produce several seeds that remain dormant for different periods of time (Clauss and Venable 2000) resulting in overlapping generations (Cáceres and Tessier 2003) and reduced fitness variance (Venable and Lawlor 1980). Bet hedging has been assessed in a very broad range of taxa that include

birds, plants, insects and microbes (Simons 2011). Exploring the occurrence of bet hedging in microbes, the simplest life forms, suggests the importance of risk spreading—it may have been one of the earliest of evolutionary responses to fluctuating environments (Beaumont *et al.* 2009).

Observing and quantifying bet hedging traits is difficult since they are only adaptive over multiple generations of fluctuating selection, they are not easily identified and they may be blended with other evolved responses (Simons 2011; Simons and Johnston 2003). Conservative bet-hedging traits are especially difficult to discern since there are no obvious clues (Simons 2011) (phenotypic variation suggests diversification bet hedging) and it is difficult to choose a non-risk averting strategy for comparison (Hopper 1999). Demonstrating bet hedging requires empirical evidence that the geometric-mean fitness differs from the arithmetic mean fitness. For these reasons, evidence for bet hedging is weak (Simons and Johnston 1997; Simons 2011). Since bet hedging is expected to evolve under low predictability (Stearns 1992; Roff 2002), one approach to study of bet hedging is to compare phenotypes that have evolved in habitats that differ in the degree of unpredictable environmental variability (Hairston and Olds 1984; Philippi 1993a; Philippi 1993b; Khatchikian *et al.* 2010; Philippi *et al.* 2001; Hopper 1999; Simons 2011). In this study, it is proposed that the phenology of formation of turions, small seed-like vegetative organs, has evolved as a bet-hedging phenotype in response to environmental unpredictability in *Spirodela polyrhiza* (L.). This is the first study to propose bet hedging in *S. polyrhiza*.

1.1.3 The Model System: *Spirodela polyrhiza*

S. polyrhiza is a free-floating hydrophytic perennial of the subfamily lemnoideae. It provides an ideal model system for this study since it may be found in a highly diverse range of environments; from inlets of large lakes to ditches and wetland swamps (Dudley 1987; Jacobs 1947; Hillman 1961). Lemnoideae produce “daughter” fronds from two pockets at the proximal end (Figure 1.1) which, in turn, become mothers. Often, daughters may remain attached to the mother while making daughters of their own; this is known as a colony. Fronds of *S. polyrhiza* are proposed to be a highly reduced type of rosette (Jacobs 1947) and a modified leaf-like appendage (Hillman 1961).

Figure 1.1 Life cycle of *Spirodela polyrhiza* (Diagram M. Compton)

Individuals survive drought and low temperatures through a dormancy organ called a turion. Turions are vegetative organs (Jacobs 1946) formed in the same budding pouch as regular fronds (Dudley 1987). The budding pouch is composed of pocket sheaths that enclose meristematic cells (Smart and Trewavas 1983). Budding pouches are found on both regular fronds and turions; fronds have two and turions have one. Formation of turions is important because regular fronds cannot tolerate low winter temperatures of temperate climates (Appenroth 2003). Turions are smaller than regular fronds and are filled with starch grains with few or no aerenchyma. High density thus causes them to sink to the sediment where they remain until the onset of favourable conditions in the spring (Hillman 1961; Jacobs 1947) when “germination” commences (Figure 1.2) through a phytochrome-mediated response (Appenroth 2003). The turions of *S. polyrhiza* are considered true turions because they are highly modified morphologically from their leafy counterpart (Hillman 1961), and they do not grow any further once produced (Appenroth *et al.* 1996).

S. polyrhiza fronds reproduce almost exclusively clonally but flowering in this species is an alternative pathway (Figure 1.1) that is very rare (Crawford and Landolt 1993). Seed production in all species of Lemnoideae is considered negligible as a mode of dispersal due to the success of vegetative propagation (Jacobs 1947). Contact with neighbour fronds (crowding) seems to be important in flowering by increased ethylene formation causing reduced growth rate and physiological switch from vegetative growth to flowering or to turion formation (Scharfetter *et al.* 1987; Appenroth 1990a). When flowering does occur, seeds are non-dormant and germination occurs almost immediately after abscission from the frond.

The life span of *S. polyrhiza* is cited to be 33 days with a reproductive rate of 0.08 fronds per day (Lemon *et al.* 2001). The release of the daughter frond from the mother may have an effect on subsequent production of daughter fronds through apical dominance. Every subsequent daughter will be smaller than the preceding daughter (due to fewer cells) and produce fewer daughters themselves (Hillman 1961). In the laboratory, individual fronds may produce two daughter fronds simultaneously, two turions simultaneously and may even produce one daughter and one turion simultaneously within one colony (personal observation; Figure 1.3).

1.1.4 Turions of *S. polyrhiza*

The “decision” to form turions is incompletely understood in the field, but is induced by fluctuating environmental cues or stress factors signaling the onset of unfavourable conditions (Appenroth and Nickel 2010). These signals include low temperature (Appenroth 2002a) and a shortage of nutrients (Dudley 1987) such as phosphorus (Appenroth 2002a), nitrate, sulphate (Malek and Cossins 1983; Appenroth *et al.* 1989) and abscisic acid (Flores and Smart 2000; Chaloupková and Smart 1994; Smart and Fleming 1993; Perry and Byrne 1969). Short photoperiod is an environmental signal important for anticipating the end of a growing season for many perennial plants, although it does not seem relevant to turion formation in *S. polyrhiza* (Appenroth 2003). Low nighttime temperatures and fluctuations in nutrients are essential in the developmental pathway leading to turion formation (Appenroth 2003; Appenroth 2002a).

The starch stored by the turion is degraded for sucrose synthesis slowly over time, ensuring survival during unfavourable conditions, and is then degraded rapidly as needed for frond growth upon germination when conditions are again suitable (Ley *et al.* 1997).

After-ripening, the slow degradation of germination inhibitors (Finch-Savage and Leubner-Metzger 2007) occurs over the winter at the sediment (Appenroth and Gabrys 2001), and causes responsiveness to phytochrome (Appenroth *et al.* 1990a) in the spring. Phytochrome mediates both germination and starch degradation (Appenroth 2011; Appenroth and Gabrys 2001; Dölger *et al.* 1997). Germination is induced by light (Appenroth *et al.* 1992; Perry 1968), by temperature (Appenroth *et al.* 1996) and is dependent on the availability of starch. Rapid growth provides a competitive advantage over other surface-dwelling organisms. This requires the carbohydrate supply from the starch reserve that is dependent on the presence of nitrate (Appenroth and Zeigler 2008) provided by mineralizing bacteria in the water. When the signal (temperature of 15 C° and higher) to begin germination is received, the respiration pathway in the turion is altered and a CO₂ gas bubble is produced within its tissues, and the turion buoys to the surface (Newton *et al.* 1978; Hillman 1961). At the surface, vegetative proliferation continues very rapidly whilst temperatures remain favourable (15-35 C°) (Jacobs 1947).

Figure 1.2 a. Upper surface of germinating *Spirodela polyrhiza* turions. b. Underside of germinating turions. The new frond originates from the budding pouch at proximal end of the turion (Photo M. Compton).

Figure 1.3 *Spirodela polyrhiza* fronds producing daughter fronds and turions a. Upper surface of colony; mother (top right) and daughters (I,I) (top left and bottom middle). One daughter is producing its own daughter (II) (bottom right). b. Underside of colony. Arrows indicate turions (Photo M. Compton).

With respect to formation, dormancy and germination, turions are analogous to seeds yet they are different because turions are vegetative organs (Appenroth *et al.* 1996). Like seeds, the effect of light on germination of turions in the spring is mediated by photoreceptor phytochrome (Appenroth *et al.* 1990a) through biosynthesis of gibberellins (Appenroth and Augsten 1991). In temperate climates, turions remain dormant under favourable conditions to avoid germination before winter (Appenroth 2002b), common with dormancy in seeds (Bewley 1997). There is also a trade-off between ensuring turions are produced before the onset of fatal winter conditions and continuing successful vegetative propagation (synthesizing enough starch to fill turions is a taxing process on the mothers) (Jacobs 1946).

Because of temporal and spatial variance in temperature and nutrient conditions, the onset of winter is unpredictable among years and variable among ponds that duckweed populations inhabit. More specifically, smaller ponds will gain heat (Collinson *et al.* 1995) and lose heat (Duguay *et al.* 2003) more quickly than ponds of larger size. Small ponds also experience higher nutrient concentration fluctuations (Bennion and Smith 2000). Environmental variability may be predictable, however it is expected that the variability observed in smaller ponds is unpredictable in that it does not serve as a reliable indication of the length of time remaining in the growing season. Larger water systems have been found to be more stable and predictable than smaller water systems (Koops *et al.* 2003; Gregersen *et al.* 2009) since intuitively, a higher volume of water is a better buffer against fluctuations than smaller volumes of water. Therefore, ponds of varying sizes are suitable environments to observe phenotypes in environments that differ in the degree of environmental predictability. Since bet hedging is expected to evolve

under environmental unpredictability, the timing of turion formation of *S. polyrhiza* that have evolved under unpredictable conditions, may be a bet-hedging phenotype (diversification or conservative).

A bet-hedging prediction is that mean days to turion formation will be earlier (more conservative) as unpredictability increases (ex. with decreasing pond size). Producing turions earlier than necessary will ensure survival through winter even if the onset of winter occurs earlier than normal, but is not an optimal strategy under normal conditions. In preliminary laboratory observations, vegetative propagation of individual fronds decreases, and completely ceases once turion formation is initiated. An important part of the ecology of *S. polyrhiza* is the advantage it gains by spreading across the pond surface and out-competing other aquatic species. Therefore, producing turions earlier would be a trade-off between its competitive advantage and ensuring survival.

A second bet-hedging prediction is that the variance (potential diversification) in turion formation will not be equal among ponds differing in environmental uncertainty and this variance will be greater (more diversified) in less predictable environments (e.g. with decreasing pond size). Diversifying the timing of turion production ensures continued regular propagation and an appropriate response to the variable onset of unsuitable conditions. Both strategies inhibit the possibility of complete failure.

The goals of this study are twofold: to contribute to the development of *S. polyrhiza* as a model system for evolutionary ecology by gaining basic knowledge of turion formation and germination in the field, and to assess whether turion formation has the characteristics of a bet-hedging strategy. Empirical evidence for bet hedging is scant and there is yet to be a study that tests the existence of bet hedging in a hydrophyte

subject to a temperate climate. Strong tests of bet hedging require long-term study (Phillipi *et al.* 2001) and quantitative assessment of optimality (quantifying maximization of the geometric mean) (Simons 2011); our study aims to provide evidence on potential bet hedging by asking whether population-level characteristics of turion formation across environments that differ in unpredictability are qualitatively consistent with predictions of bet hedging.

Ponds of varying attributes containing duckweed were chosen and pond properties were measured over the course of a growing season. In Chapter 1, important pond parameters for duckweed overwintering phenology were monitored, and unpredictability from the perspective of duckweed was inferred from variability in these pond parameters. Because phenology may reflect population differentiation and/or a plastic response to pond environment, in Chapter 2 population differentiation is assessed and phenotypic plasticity is proposed as an alternative hypothesis to bet hedging through a transplant study in the field and a common garden experiment in the laboratory.

1.2 Methods

1.2.1 Environmental Unpredictability Within and Among Ponds

At the beginning of April 2011, eight ponds that varied in size (large to ephemeral) on Queens University Biological Station (QUBS) land holdings at Chaffey's Locks, Ontario (44°34'01.19" N and 76°19'26.09" W) were selected as focal environments for study. However, only four of the original ponds were found to contain populations of *S. polyrhiza*: Cow Island Marsh (CI), Leroi Swamp (LS), Barb's Marsh (BM) and Blackberry Pond (BB). Therefore, the other four ponds were eliminated from the study. Ponds were monitored throughout the spring, summer and fall (April 29-

November 14). To observe differences in environmental variation, change in pond depth, total phosphorus concentration, overall size and depth, water surface temperature and sediment temperature were measured over the course of this study. Surface area of each pond was measured in the field and by using Google Earth (© 2013 DigitalGlobe).

Pond depth was measured using duplicate 1"x5' PVC posts set as deep as possible into the sediment using a post driver and a rubber mallet 4-5 m away from shoreline and 4-8 m away from one another. The distance from the sediment to the top of the water surface was measured, recorded and averaged between the two pipes to determine initial depth. Weekly, the distance from the top of the pipe to the surface of the water was measured, recorded and averaged to obtain the weekly change in depth.

Triplicate samples of water from all focal ponds were taken at each sampling (six times) to determine total phosphorus concentration (TP). Using unfiltered samples, TP concentration was analyzed with ascorbic acid modification of the molybdenum technique followed by potassium persulphate digestion under pressure, as outlined by Bourassa and Cattaneo (2000).

Water temperatures at the pond surface and at the sediment interface were monitored using iButtons (Maxim Integration © 2012). These monitors were programmed to record temperature hourly. For waterproofing, they were placed in small plastic Petri dishes with lids and filled with silicone. An elastic band was set in the silicone as a means of attachment. For iButtons allocated to measure the sediment temperature, a weight was affixed to one side of the Petri dish. In the field, the section of the rubber band remaining outside of the Petri dish was placed around one of the PVC

pipes used for measuring pond depth. The weighted Petri dishes sank to the bottom of the pipe and the non-weighted Petri dishes floated on the water's surface.

1.2.2 Pond Description

All ponds were within QUBS land holdings (Figure 1.4) and spanned a distance of approximately 6 km with an average of 2.8 km between sites. Lake Opinicon is a major contributor to the waterway system. Cow Island Marsh is a small inlet off the man-made Lake Opinicon ($17.4 \times 10^3 \text{ m}^2$) and is furthest from all other sites (Figure 1.4). This lake is surrounded by both coniferous and deciduous trees and hydrophytic plants including sedges (Cyperaceae) and rushes (Juncaceae), water lilies (Nymphaeaceae), and duckweed (*Lemna trisulca*, *Wolffia* spp., *Spirodela polyrhiza*). The average depth of the test area was 37 cm. Leroi Swamp ($7\,000 \text{ m}^2$) is a marshy swamp off the Skycroft Trail, part of both the Rideau and Cataraqui Trail systems. The swamp is surrounded mainly by deciduous species with sedges (Cyperaceae) and rushes (Juncaceae) around the perimeter. Here, the shoreline receded more than any other focal pond (approximately 5 m). The average depth throughout observation was 55 cm. Barb's Marsh ($4\,200 \text{ m}^2$) was surrounded by tall grass to a distance of approximately 50m. Grasses (Poaceae), sedges (Cyperaceae) and rushes (Juncaceae) surrounded the shoreline with some duckweed present (*Lemna minor*, *Spirodela polyrhiza*). The average depth was 29 cm. Blackberry Pond (655 m^2) was a small pond along the border of the QUBS land holdings property. It was found within coniferous and deciduous woods with decaying matter throughout the pond and surrounding area. The sediment was quite saturated and water very muddy. The pond was frequented by several Anuran species. *Wolffia* spp. was extremely abundant

and covered the entire surface area of the pond with *Spirodela polyrhiza* dispersed throughout. The average depth was 28 cm.

Figure 1.4 A Google Satellite (© 2013 DigitalGlobe) image of Queen's University Biological Station (QUBS) land holdings and test sites at Chaffey's Locks, Ontario, Canada.

1.2.3 Turion Phenology

To catch germinating fronds, *up traps* (Figures 1.5a and 1.7) were constructed using 4" PVC pipes and elbows. Four pieces (18" length) were cut and cemented to the elbows to make a frame. The seams of the frame were sealed with silicone. To ensure germinated fronds were contained (Figure 1.6) aluminum mesh screening was affixed, extending 6" above and below the frame (Figure 1.7). Two traps at each pond were tethered into place 4-6 m away from the shoreline and 2-8 m away from one another. Weekly, germinated turions and fronds were recorded and removed from the trap. The average from the two traps was taken to determine the total number of re-germinated turions per unit area. Mean days to frond germination was used for analysis with May 1 as day 0.

To catch turions formed by fronds at the surface in the late summer, frames described above were modified. Aluminum mesh folded into baskets that fit the inside of the frame replaced the mesh fence described above (Figure 1.5b). These traps are referred to as *down traps* (Figure 1.8). Four *down traps* with baskets were tethered at each pond 4-10 m away from shoreline and 2-6 m away from one another. Fronds collected from within each pond (n=50) were placed within the respective down traps on July 12. Each week thereafter, total fronds and total turions were counted and a frond population of n=50 was maintained by removing excess fronds or adding from the surrounding population.

Figure 1.5 Schematic of *traps* made to contain *Spirodela polyrhiza* fronds and turions from PVC pipe and aluminum mesh. a. *Up trap* - collects germinating turions b. *Down trap* - contains fronds and abscised turions.

Figure 1.6 *Spirodela polyrhiza* in an *up trap* at Queen's University Biological Station (QUBS), Chaffey's Locks, Ontario, Canada (Photo M. Compton).

Figure 1.7 *Up trap* deployed in the field and containing *Spirodela polyrhiza* fronds at Queen's University Biological Station (QUBS), Chaffey's Locks, Ontario, Canada (Photo M. Compton).

Figure 1.8 Example of *down trap* with aluminum mesh basket to contain *Spirodela polyrhiza* fronds and their turions (Photo M. Compton).

1.2.4 Statistical Analysis

To compare the sensitivity of pond water to ambient temperature for the focal ponds, cross-spectral analysis (Sousa *et al.* 2009) using Matlab (©1994-2013 The MathWorks) was conducted. Cross-spectral analysis allows for characteristics of stationary series data to be examined as they vary across space by breaking down the series into cyclical components of different frequencies (Bassett and Tinline 1970). The results of the cross-spectral analysis gives a time lag between both the surface and ambient, and sediment and ambient temperatures.

The daily mean, daily minimum and daily maximum temperatures were calculated from the hourly iButton recordings for the surface and sediment temperatures. Principal components analyses (PCA) were used to reduce these variables to a set of uncorrelated (orthogonal) variables for further analysis. Three PCAs were performed; one each for the three surface, three sediment, and for all six temperature variables. The first principal component (PC1) accounts for 93.9% of the variance in surface temperatures, 93.8% for sediment, and 91.2% of the variance for all six temperature measures. Since replicate iButton recordings were not used, daily average temperatures were separated into eight independent temporal replicates (detailed in Results) and the standard deviations of each replicate were calculated to quantify and compare environmental variance for each pond. A Levene's test was used to assess inequality of variance among ponds. Plots of TP and depth were plotted through time to observe fluctuations.

Turion yield was calculated as the total number of turions/(total number of fronds)/unit area. Mean days to turion germination and formation were used for analysis with May 1 as day 0. A one-way ANOVA was used to determine if mean days to turion

formation differed among ponds. Homoscedasticity was determined through a Levene's test. Environmental variances determined (above) were correlated with the variance in the phenology of turion formation. Insufficient data was collected from Barb's Marsh and therefore it was eliminated from this section of the analysis.

1.3 Results

1.3.1 Depth and Phosphorus

The depth changed the most at Leroi Swamp (Figure 1.9), consistent with the observation of greatest shoreline recession described earlier. The means of pond depth differed significantly ($P < 0.0001$), with Cow Island Marsh and Leroi Swamp differing from one another and both differing from Barb's Marsh and Blackberry Pond (Figure 1.10). Blackberry Pond and Barb's Marsh are shallower than Leroi Swamp and Cow Island Marsh. Pond depths were not consistent within focal ponds. Leroi Swamp had the greatest overall change in depth which coincides with dramatic observed changes to shoreline throughout the season. Between June and July, the depth at Blackberry Pond decreased significantly, coinciding with temperatures of 35°C and greater at the surface of the pond, possibly indicating higher evaporation since evaporation is a function of temperature in small ponds (Stewart and Rouse 1976).

Total phosphorus concentrations showed consistent trends through time (Figure 1.11). TP means were found to differ among ponds ($P < 0.05$). Blackberry Pond had the highest mean TP concentration (134 µg/L) (Figure 1.12). From a Tukey-Kramer HSD test, TP in Blackberry Pond and Barb's Marsh differed from Cow Island Marsh and Leroi Swamp.

Figure 1.9 Depth of focal ponds: Leroi Swamp (LS), Cow Island Marsh (CI), Barb's Marsh (BM), and Blackberry Pond (BB) from April 29-November 7, 2011 at QUBS, Chaffey's Locks, Ontario, Canada. Error bars represent ± 1 SE.

Figure 1.10 Mean depth in four focal ponds: Cow Island Marsh (CI), Leroi Swamp (LS), Barb's Marsh (BM), and Blackberry Pond (BB). Box plot is showing the median (midline), interquartile range (box), and 1.5 times the interquartile range (bars).

Figure 1.11 Total phosphorus in ponds: Leroi Swamp (LS), Cow Island Marsh (CI), Barb's Marsh (BM), Blackberry Pond (BB) from May to October 2011 at the QUBS, Chaffey's Locks, Ontario, Canada. Error bars represent $\pm 1SE$.

Figure 1.12 Mean total phosphorus concentrations of focal ponds: Cow Island Marsh (CI), Leroi Swamp (LS), Barb's Marsh (BM) and Blackberry Pond (BB). Box plot is showing the median (midline), interquartile range (box) and 1.5 times the interquartile range (bars).

1.3.2 Surface and Sediment Temperatures

Mean, minimum and maximum daily temperatures were calculated for surface and sediment water temperatures (Table 1). Time series plots for surface and sediment temperatures for all ponds may be found in Appendix A. Because temperature data were missing from CI surface from some time periods, and because replicate iButtons were not used, daily temperature measurements were divided into 8 independent replicate time periods. Replicates 1-7 consisted of 25 consecutive days of daily temperature measurements (April 29-May 23, May 24-June 17, June 18-July 12, July 13-August 6, August 7-August 31, September 1-25, September 26-October 20, respectively) and repetition 8 consisted of 15 days (October 21-November 5).

Table 1.1 Surface and sediment water temperature overall means, actual minimum, actual maximums and coefficient of variation (CV) of four focal ponds at the QUBS, Chaffey's Locks, Ontario (April 29-November 14, 2011). Temperature range is given in parentheses.

	<i>Daily surface temp (°C)</i>				<i>Daily sediment temp (°C)</i>			
	Overall mean	Min	Max	CV	Overall mean	Min	Max	CV
Cow Island Marsh	16 ±0.09 (24-7= 17)	13 (26- 4=22)	20 (31- 9=22)	0.37	15 ±0.10 (26- 4=22)	14 (24- 3=21)	19 (31- 5=26)	0.36
Leroi Swamp	17 ±0.12 (27- 3=24)	12 (23- 2=21)	23 (31- 6=26)	0.46	15 ±0.10 (25- 5=20)	13 (23- 2=21)	16 (31- 6=25)	0.39
Barb's Marsh	20 ±0.04 (27- 5=22)	13 (24- 1=23)	22 (40- 19=21)	0.45	-	-	-	-
Blackberry Pond	16 ±0.11 (28- 0=28)	13 (25- 0=25)	22 (41- 5=36)	0.47	15 ±0.10 (29- 5=24)	12 (24- 5=19)	18 (37- 1=36)	0.37

^a Temperature based on hourly readings

Due to iButton loss/failure, surface temperature data from June 11 to September 24 for BM was missing and sediment temperatures for BM were missing for the entire study and therefore BM was eliminated from the temperature portion of the analysis. Surface temperature data was also missing for CI from June 11 to September 24. Missing sections of temperature data would bias the comparison among ponds because of changes in mean temperature through time. Therefore, to measure among pond variability, surface and sediment temperature measurements for all ponds from June 11 to September 24 (repetitions 3-6) were eliminated.

Variability is the greatest at BB in surface minimum and maximum temperature (Figure 1.13), sediment maximum (Figure 1.14) and PC1 of sediment and of surface and sediment temperature together (Figure 1.15). There were no significant differences in temperatures among ponds (Surface: $P=0.6903$, Sediment: $P=0.2229$, Surface + Sediment: $P=0.8940$). A Levene's test rejects equal variances among ponds from PC1 for surface ($P<0.0001$) sediment ($P<0.0004$) and surface and sediment together ($P<0.0001$).

Cross-spectral analysis of hourly ambient outdoor and hourly water surface temperature found a three-fold disparity among lag times, with Cow Island Marsh showing the longest lag time (3 hours) followed by Barb's Marsh and Blackberry Pond (both 2 hours) and Leroi Swamp (1 hour).

Figure 1.13 Standard deviation of surface temperature from the average, minimum and maximum daily temperatures (April 29-June 11, September 24- November 5) of focal ponds: CI (Cow Island Marsh), LS (Leroi Swamp) and Blackberry Pond (BB). Error bars represent ± 1 SE.

Figure 1.14 Standard deviation of surface temperature from the average, minimum and maximum daily temperatures (April 29-June 11, September 24-November 5) of focal ponds: CI (Cow Island Marsh), LS (Leroi Swamp) and BB (Blackberry Pond). Error bars represent ± 1 SE

Figure 1.15 Standard deviation of temperature based on PC1 of the surface, sediment temperatures (April 29-June 11, September 24-November 5) of focal ponds: CI (Cow Island Marsh), LS (Leroi Swamp) and Blackberry Pond (BB). Error bars represent ± 1 SE.

1.3.3 Turion Phenology; Germination

Initial germination of turions in the spring was the greatest and earliest at Blackberry Pond (Mean days=40) (Figure 1.16). Cow Island Marsh followed Blackberry Pond (Mean days=73) and turions at Leroi Swamp germinated the latest (Mean days=91). A one-way ANOVA found mean days to turion germination differed among ponds (DF=2, F=1385.279, $P<0.0001$) (Figure 1.17) and from a post-hoc Tukey-Kramer HSD, all ponds differed significantly from one another (all $P<0.0001$). Box plot (Figure 1.17) shows the median (midline), interquartile range (box), and 1.5 times the interquartile range (bars).

Figure 1.16 Turion germination of *Spirodela polyrhiza* at Cow Island Marsh (CI), Leroi Swamp (LS) and Blackberry Pond (BB) (May 18-November 7, 2011). Additional y-axis added to adjust for scale issues for the first month of data collection. Error bars represent $\pm 1SE$.

Figure 1.17 Mean days to turion germination of *Spirodela polyrhiza* at Cow Island Marsh (CI), Leroi Swamp (LS) and Blackberry Pond (BB) (May 18-November 7, 2011). Box plot is showing the median (midline), interquartile range (box), and 1.5 times the interquartile range (bars).

1.3.4 Turion Phenology; Formation

Blackberry Pond was the first population observed to form turions (Figure 1.18). However, mean days was found to be significantly earlier at Cow Island Marsh with mean days to turion formation being 123 days at Cow Island Marsh, 131 days at Leroi Swamp and finally, 135 days at Blackberry Pond (Figure 1.19). Box plot (Figure 1.19) shows the median (midline), interquartile range (box), and 1.5 times the interquartile range (bars). From a one-way ANOVA, mean days to turion formation was found to differ among ponds ($DF=2$, $F= 9.4387$, $P<0.0001$). A post-hoc Tukey-Kramer HSD test found that Leroi Swamp and Blackberry Pond differed from Cow Island Marsh ($P=0.0016$ and $P=0.0003$, respectively).

The standard deviation of the days to turion formation was the greatest at Blackberry Pond ($SD=29$) and lower at Leroi Swamp ($SD=20$) and Cow Island Marsh ($SD=24$) (Figure 1.19). A Levene's test found that variances in days to turion formation were not equal ($P<0.0003$). Mean days to turion formation was not consistently related to environmental variance (Figure 1.20). From the variance and the standard deviation (Figure 1.21), variability in turion phenology is the greatest at Blackberry Pond.

Figure 1.18 Turion yield of *Spirodela polyrhiza* at focal ponds: CI (Cow Island Marsh), LS (Leroi Swamp) and Blackberry Pond (BB) at the QUBS, Chaffey's Locks, Ontario, Canada (July 26-November 7, 2011). Error bars represent $\pm 1SE$.

Figure 1.19 Mean days to turion formation of *Spirodela polyrhiza* in focal ponds: CI (Cow Island Marsh), LS (Leroi Swamp) and Blackberry Pond (BB). Box plot is showing the median (midline), interquartile range (box), 1.5 times the interquartile range and standard deviation (blue).

Figure 1.20 Mean days to turion formation of *Spirodela polyrhiza* and the standard deviation of PC1 temperature (see text) of surface and sediment temperatures of focal ponds: CI (Cow Island Marsh), LS (Leroi Swamp) and Blackberry Pond (BB). Error bars represent ± 1 SE.

Figure 1.21 Standard deviation of mean days to turion formation of *Spirodela polyrhiza* and the standard deviation of PC1 temperature (see text) of surface and sediment temperatures of focal ponds: CI (Cow Island Marsh), LS (Leroi Swamp) and Blackberry Pond (BB). Error bars represent ± 1 SE.

1.4. Discussion

Environmental variance is predictable to the extent that cues are available and reliable. In the face of environmental uncertainty, plants may reduce risk of failure through conservative behaviour, through suboptimal timing of flowering (Simons and Johnston 2003), or through diversification in important life-history traits, such as in the timing of seed germination (Simons 2009). In this chapter, I studied putative conservative and diversification bet hedging in turion phenology in three populations of *Spirodela polyrhiza* (L.) inhabiting different ponds that differ in unpredictability.

To assess the predictability of environmental variance, ponds varying in morphological attributes were compared. Pond depths infer water volume (with overall surface area), which would affect the thermal capacity and therefore temperature and may have consequences for nutrient concentrations. Blackberry Pond was the smallest and shallowest of all ponds (Figures 1.9 and 1.10) and would therefore be the least buffered against temperature and nutrient concentrations fluctuations. This conjecture is supported by the greater sensitivity to ambient temperature from the cross-spectral analysis and high variance in surface and sediment water temperatures (Figures 1.14 and 1.15). These findings are consistent with the assumption that smaller ponds are more unpredictable (Koops *et al.* 2003; Gregersen 2009).

The density of duckweed species themselves may further complicate variability at Blackberry Pond. The entire surface area of Blackberry Pond was saturated with a *Wolffia* spp. and *S. polyrhiza*. Cow Island Marsh also contained a *Wolffia* species, *S. polyrhiza* and *Lemna trisulca*, yet the larger surface area resulted in lower densities. Dale and Gillespie (1976) found that the temperature within floating mats of *Lemnoideae* (formerly *Lemnaceae*) covering the surface, like at Blackberry Pond resulted in larger

and more variable energy exchanges at the surface. Whereas in open water systems, like Cow Island Marsh (low density of vascular plant coverage), where radiant energy may easily penetrate and wind may mix the water, surface temperatures are more homogenous. Therefore, the presence and abundance of duckweed at Blackberry Pond may indirectly contribute to the variability of their own environment.

Evidence for substantial variability and differences among populations in the phenology of both turion germination and formation was found (Figures 1.17 and 1.19, respectively). Although observations of turion germination in the field provide novel life-history information for this species, the history of deposition of turions to the sediment is unknown; thus, observed germination behaviour is an indirect measure of phenology. Prior to this study, knowledge of variability in turion germination and formation in nature was lacking. Evidence of phenological variability in both turion germination and formation behaviour renders *S. polyrhiza* as a suitable model organism to study putative bet-hedging phenotypes.

A safe phenotype that appears to be suboptimal ensures future success and reduces the possibility of failure in unpredictable environments. If turion formation was observed to be significantly earlier in the more unpredictable environment compared to the more predictable environments, then a conservative bet hedging would be implied. However, since mean days to turion formation was not found to be significantly earlier in the more unpredictable pond (Figure 1.20), a conservative bet hedging trait cannot be concluded.

The lag in water temperature behind ambient air temperature in the small pond was less than the lag time in the larger pond; thus, smaller ponds will freeze up faster at

the onset of winter. Therefore, the onset of winter would be less certain in ponds that are more vulnerable to freezing and a safe phenotype would ensure that turions were produced prior to the expected optimal date. Rapid clonal propagation over the surface of the pond surface offers a competitive advantage to *S. polyrhiza* populations. In this system, continued population growth may confer greater fitness benefits.

Conservative bet-hedging traits are difficult to recognize since there are no obvious ‘clues’ of their existence (Hopper 1999; Simons 2011) and this study does not test for optimality in fitness traits over a long-time scale required to properly infer bet hedging.

In the face of environmental uncertainty, high phenotypic variance diversifies response such that there is a phenotype for an array of environmental outcomes (Seger and Brockman 1987). Thus, the population inhabiting the most unpredictably variable pond was expected to have the highest variance in mean days to turion formation. The observation of the highest variation in turion formation in Blackberry Pond—the pond with the highest measures of environmental variance (Figure 1.21)—is consistent with a diversification bet-hedging strategy. However, it should be noted that the probability of obtaining this concordance by chance alone is high, given that only four ponds were compared.

The variance in water temperature was found to be highest in Blackberry Pond. Temperature is an important cue for the induction of turion formation (Appenroth and Nickel 2010, Appenroth 2002a). Unpredictability of this cue may have led to the evolution of diversification bet hedging in *S. polyrhiza* fronds. However, phenotypic plasticity may account for observed phenotypic variability and this is tested in Chapter 2.

Measuring trait variance is insufficient to confidently conclude a diversification bet-hedging trait. Fitness consequences of environmental variance must be assessed (Simons 2009). Nevertheless, the observation of high trait variance in turion formation, and of differences in trait variance among ponds is suggestive of diversification bet hedging, and demands further evaluation.

Our initial design included replicate ponds of similar morphometrics, namely size. However, several ponds were dropped from the study because of the absence of *S. polyrhiza*. Replicate ponds would have allowed direct inferences of effects of pond size on turion phenology in addition to increasing power of statistical analyses. All ponds are connected to the Opinicon Lake system, which may facilitate gene flow; however, genetic population differentiation in plants has been found at spatial scales as small as a few millimeters (Linhart and Grant 1996; Waser and Price 1985; Cole and Voskuil 1996).

Tests over several generations that measure optimality and demonstrate maximization of the geometric mean fitness would be required to conclude bet hedging as an explanation for the occurrence of apparently suboptimal but risk-averting “safe” phenotypes (conservative bet hedging) or for phenotypic variance (diversification bet hedging) (Gillespie 1974; Seger and Brockman 1987; Phillipi and Seger 1989; Simons and Johnston 2003).

Phenotypic divergence among populations in timing of turion formation may be attributed to bet hedging; however, other mechanisms may be responsible. Diversification bet hedging is expected to evolve when environmental variability is unpredictable; but when cues may be relied upon to predict future environments, adaptive phenotypic plasticity is expected to evolve (Sultan and Spencer 2002). Furthermore, a species may

consist of several ecotypes locally adapted to their specific environments (Bradshaw 1984; Schlichting and Piglucci 1993). For locally adapted genotypes to evolve there must be limited gene flow and little temporal variation (Kawecki and Ebert 2004). These alternative hypotheses are addressed in Chapter 2.

This study was motivated both by the lack of basic natural history knowledge of turion phenology, and by the possibility that turions act, in a manner similar to seeds, to reduce risk under environmental unpredictability. Here, we have found evidence consistent with bet hedging—that *S. polyrhiza* populations differ in turion phenology in nature. Although the observation that phenotypic variance increases with unpredictability is suggestive, it does not constitute strong evidence for bet hedging (Simons 2011) however, further evaluation of bet hedging is warranted for both turion germination and formation from observed variance in phenology.

Since there is insufficient replication in this study, it is difficult to conclude confidently that turion phenology is diversified in all ponds that are more unpredictable, or more specifically, in smaller ponds. Replicate ponds would allow for the inference that turion phenology may be diversified based on size, or high variance in temperature and not by chance alone if consistent results among similar environments were found.

This is the first study to directly measure turion germination and formation in the field, and it demonstrates that *S. polyrhiza* has the potential to be a useful model for asking important questions in evolutionary ecology.

CHAPTER 2

PHENOTYPIC PLASTICITY AND POPULATION DIFFERENTIATION IN *SPIRODELA POLYRHIZA*

2.1 Introduction

2.1.1 Climate Change and Phenology

Recent findings suggest that climate change (Rozenweig *et al.* 2008; Jones *et al.* 2001) has notable ecological consequences (Visser 2008; Gienapp *et al.* 2008; Bradshaw and Holzapfel 2006) beyond those of naturally occurring environmental fluctuations, particularly on phenologies (Walther *et al.* 2002). Spring phenologies have been found to have advanced 2-5 days per decade (Menzel *et al.* 2006; Walther *et al.* 2002; Root *et al.* 2003; Parmesan and Yohe 2003) and plant development through life stages has been found to be occurring at faster rates (Saxe *et al.* 2002) since temperature directly affects plant metabolic processes. More specifically, examples of earlier flowering (Fitter and Fitter 2002; Primack *et al.* 2004; Parmesan and Yohe 2003) at a rate of 0.94 days earlier per decade (Panchen *et al.* 2012) in addition to examples of earlier spring green up (Menzel and Fabian 1999; Cleland *et al.* 2007; Wolfe *et al.* 2005; Chmielewski and Rötzer 2001) have been documented. However, a phenological shift is not necessarily an adaptation or strategy to cope with environmental variability. Rather, it may be a direct response to the environment.

2.1.2 Phenotypic Plasticity, Bet Hedging and Population Differentiation

Environmental cues may be used as information to (imperfectly) predict future conditions to persist under fluctuating environments (Tufto 2000). A cue may be used by an individual to assume a phenotype appropriate for future conditions through phenotypic plasticity. Environmental predictability may be thought of as a high correlation between a cue and the fitness outcome of a particular phenotype (Phillipi 1993b; Cohen 1967). Environmental cues should not be confused with physiological triggers that result in

direct responses; cues are indicative of conditions relevant to future success, to which a response may evolve (Visser 2008). For example, the environment may select for the timing of life stages (e.g. reproduction) based on required resource availability. Therefore, the existence and reliability of environmental cues are crucial for selection of adaptive phenotypic plasticity.

By definition, adaptive phenotypic plasticity is the facultative expression of phenotypes (West-Eberhard 1989) that maximize fitness under a range of conditions (Sultan 1996). However, plasticity may be non-adaptive (Langerhans and DeWitt 2002) and, for example, lead to phenological mismatches (Nussey *et al.* 2005). Plasticity is adaptive if the perceived cue is a reliable indication of future conditions, and the resulting phenotype is appropriate (Schlichting 1986) in that survival and reproduction are enhanced (Griffith and Sultan 2004; Richards *et al.* 2006). Plastic phenotypes must have a high global average of fitness as opposed to fitness measured in each environment separately (van Kleunen and Fischer 2004; Releya 2002).

In addition to constraints on plasticity due to the availability and dependability of environmental cues, costs of processing environmental signals and limitations of genetic variance constrain the effectiveness of phenotypic plasticity (DeWitt *et al.* 1998). Another limit imposed on the evolution of phenotypic plasticity is the time lag between the recognition of the relevant cue and the realization of the new phenotype (Padilla and Adolf 1996), referred to as developmental sensitivity (Moran 1992). Furthermore, climate change may disrupt temporal patterns of environmental cues rendering them less reliable (Visser 2008).

“Bet-hedging” is expected to evolve when cues are unreliable (Stearns 1992; Roff 2002; Simons 2002) and, more generally, under circumstances that limit the evolution of phenotypic plasticity and adaptive tracking (Simons 2011). Diversification bet hedging is defined as the evolution of high phenotypic variance and maximization of the geometric mean fitness over time (Seger and Brockman 1987; Simons 2009). In diversification bet hedging, risk is spread among two or more phenotypes, thereby reducing fitness variance among generations (Simons and Johnston 2006). It is possible for a blend of both diversification and phenotypic plasticity to evolve in one trait with the expression depending on the degree of environmental predictability (Simons 2011).

The possible set of plastic responses to a given set of environments is known as the norm of reaction (Schlichting 1986). Understanding the form of the norm of reaction is important since it is a direct indication of the capacity of phenotypic response (Simons and Wagner 2007). Although it has been suggested that plasticity is a by-product of selection acting upon phenotypic values for given sets of environments (Via 1993), plasticity itself is a character that is genetically variable and subject to natural selection (Schlichting and Pigliucci 1998). It has been suggested that if populations do not have the capacity to exhibit adaptive plasticity across potentially encountered environments and show a significant genotype-environment interaction, ecotypic variation may result (Via 1991; Sultan and Spencer 2003). Adaptive phenotypic plasticity evolves under heterogeneous conditions and some degree of gene flow (Sultan and Spencer 2002), but under low gene flow and low temporal variability, divergent selection will lead to local adaptation (Kawecki and Ebert 2004). Since plasticity is a trait that is genetically

variable, it is possible to encounter population differentiation of plastic (and bet-hedging) phenotypes.

Genetic differentiation distinguishes local adaptation from phenotypic plasticity (Kawecki and Ebert 2004) and occurs when there is sufficient genetic variation within populations and variation in selection among populations (Ronce and Olivieri 1997). Reciprocal transplants and common garden experiments are used to detect local adaptation by comparing mean fitness in genotypes between environments, disentangling the contributions of environment, population differentiation and phenotypic variation.

2.1.3 Reciprocal Transplants

Selection may differ consistently across environments, resulting in population differentiation (Linhart and Grant 1996). If so, a prediction of reciprocal transplant manipulations is that a given population will behave similarly in its “home” and “away” environments, and that it should perform better at “home” (Joshi *et al.* 2001; Macel *et al.*, 2007; Kawecki and Ebert 2004). However, when a population performs differently from in its environment of origin, phenotypic plasticity is implicated (Lortie and Aarssen 1996). Differences among populations established through a transplant study may be further examined in a common garden experiment to confirm population differences and to allow for trait comparisons.

2.1.4 Common Gardens

Common garden experiments allow genetic and environmental sources of variation of natural populations to be determined (Sears and Angilletta 2003; Nuismer and Gandon 2008). This approach may test hypotheses about specific environmental factors inducing divergent selection by explicitly testing the role of such contributions on

trait expression in a controlled environment (Kawecki and Ebert 2004). Reciprocal transplant and common garden experiments may impose logistical and practical challenges in execution. Replication of identical genotypes is difficult with sexually reproducing plants and life cycles may be long and result in overlapping generations complicating stage-dependent studies. An ideal study system with a life-history trait with obvious fitness consequences in temperate environments may be found in the overwintering strategy of *Spirodela polyrhiza* (L.), the free-floating hydrophyte commonly known as Greater Duckweed. These hydrophytes are non-sessile, rarely reproduce sexually (Hillman 1961; Crawford and Landolt 1993) and propagate clonally very rapidly.

2.1.5 *Spirodela polyrhiza*, Plasticity and Population Differentiation

In seeds, germination timing is a critical stage in a plant's life history and plasticity in its timing has been found (Donohue *et al.* 2005a; Donohue *et al.* 2005; Donohue 2002; Sultan 1996; Wagner and Simons 2009a, Wagner and Simons 2009b). *S. polyrhiza* produce a modified vegetative over-wintering organ (Hillman 1961), a turion that remains dormant through unfavourable conditions (Appenroth *et al.* 1996). Regular *S. polyrhiza* fronds float at the water's surface until specific cues are given that indicate the onset of unfavourable conditions (Appenroth and Nickel 2010). When this cue is perceived, regular frond propagation switches to turion formation. The turion is dense (no aerenchyma and filled with starch) and settles at the sediment (Jacobs 1947) where it undergoes after-ripening during the winter (Appenroth *et al.* 1996), slowly degrading its stored starch until the cue for germination is given when conditions are favourable again in the spring (Newton *et al.* 1978; Hillman 1961; Appenroth *et al.* 1992; Perry 1968;

Appenroth *et al.* 1996). Temperature and nutrient concentrations are the most important cues for turion germination and formation (Appenroth and Nickel 2010; Appenroth *et al.* 1989; Dudley 1987; Malek and Cossins 1983).

Vegetative fronds cannot survive winter conditions (Appenroth 2003) and therefore turion phenology is crucial for survival in temperate and subarctic climates. In this study, we use the phenology of turions of *S. polyrhiza* to test the hypothesis that phenotypic plasticity evolves when environmental cues are reliable and cues are expected to differ among environments duckweed inhabit.

S. polyrhiza are robust and can be found growing in almost any body of freshwater (Dudley 1987) in temperate and subarctic climates. Any still or slow-moving body of water and even muddy patches of swamps provide suitable habitat for duckweed. Pond environments vary based on their size, variability in depth, total phosphorus concentrations, temperatures (Bennion and Smith 2000) and in their sensitivity to ambient temperatures (Sousa *et al.* 2009). Ponds of larger size are less variable, thermally more stable, and therefore more predictable, as established in Chapter 1. Turion formation may be a plastic trait that responds to environmental cues. This hypothesis was tested in a transplant study in the field. In addition, population differentiation may govern differences in phenotypes among populations. A common garden experiment tested this hypothesis.

If turion formation in *S. polyrhiza* is a phenotypically plastic trait, then when transplanted, turion formation phenology will be different from the home population and like the host population, responding to altered environment and new environmental cues therein. To test whether differences observed in the reciprocal transplant study are due to

local population differentiation, and to allow comparison of trait expression under homogeneous conditions, a common garden experiment was conducted in the laboratory. If genetic differences among populations exist, the timing and the frequency of turion production will differ among ponds when populations used for the transplant study are subjected to identical, controlled conditions. Studying phenotypic plasticity in plants is of particular interest. The rates of human-induced environmental changes mentioned earlier occur at rates that may be too rapid for evolutionary tracking (elimination of suboptimal forms by natural selection) and therefore plasticity may allow for sessile and non-sessile organisms alike to persist despite these changes (Sultan 2000; Jump and Peñuelas 2005).

2.2 Methods

2.2.1 Field Sites and Apparatus

This study was conducted at the Queens University Biological Stations (QUBS) at Chaffey's Locks, Ontario (44°34'01.19" N and 76°19'26.09" W) during the summer and fall of 2011 (July 26-November 14). Four focal ponds found to vary in predictability (see Chapter 1) and contain populations of *S. polyrhiza* were selected: Cow Island Marsh (CI), Leroi Swamp (LS), Barb's Marsh (BM) and Blackberry Pond (BB).

To contain fronds and capture turions for the transplant study, frames identical to the *Down* traps from Chapter 1 were constructed using 4" PVC pipes and elbows. Four pieces (18" length) were cut and cemented to the elbows to make a frame. The seams of the frame were sealed with silicone. Aluminum mesh baskets were folded to fit inside the frame and affixed to the inside perimeter. See Chapter 1 (Figure 1.5) for schematics.

2.2.2 Experimental Design

A transplant study was conducted where fronds from the medium ponds (LS and BM) were transplanted to both the large (CI) and small pond (BB). Fronds from the large pond (CI) were transplanted to the small (BB) pond and fronds from the small (BB) were transplanted to the large pond (CI) (Figure 2.1). Insufficient data was collected from BM and was therefore data from this site was eliminated from statistical analysis. Due to practical and logistic constraints, a complete reciprocal transplant was not implemented in this experiment.

There were four traps dedicated to each transplanted population at both transplant locations. Weekly, total fronds and total turions were counted and a frond population of $n=50$ was maintained by removing excess fronds or adding to from the respective home population. Frond and turion counts from all frames were averaged to determine turions per frond produced. Mean days to turion formation were used for analysis.

Figure 2.1 Schematic of experimental design for transplant study. Sizes of circles represent relative sizes of Cow Island Marsh, Leroi Swamp and Blackberry Pond.

Because turion formation is highly dependent on temperature, phenotypic plasticity in the phenology of turion formation may also be highly dependent on temperature. A common-garden experiment was conducted in the laboratory at three different temperatures (15°C, 24°C and 30°C) in separate growth chambers using frond populations collected during the field component, including a population from BM (excluded from transplant experiment). Turion production in each population was induced in the lab, turions were collected, after-ripened and allowed to germinate in Appenroth's (1996) medium three weeks before manipulation in the winter of 2012. Only the second and third generations (daughters II and III, see Chapter 1) were used for the experiment to eliminate both non-genetic maternal and carry-over effects from confounding results.

The location of each population's replicate flask was randomized to eliminate any possible positional effects on subsequent manipulation during the initial germination stage and during temperature manipulations. Media was replenished only once before replicate flasks (n=4 for each population) containing 25-90 fronds were transplanted to the growth chambers to signal turion formation by nutrient depletion. Flasks were checked each day and new fronds and turions were counted. The production of a turion was recorded when a mature turion was observed detached from the mother frond.

2.2.3 Statistical Analysis

For the reciprocal transplant study, turions per frond was calculated and averaged for each treatment. Using the mean days to turion formation, a two-way analysis of variance (ANOVA) was used to observe the effect of home and away and the interaction

of home*away. Since LS was not reciprocally transplanted, it was eliminated from the two-way ANOVA.

Frond and turion counts for each treatment were converted into a frequency value that was then transformed using an arcsin-squareroot transformation to observe the frequency of turion production through time. A logistic regression was performed using all effects and their interactions. Dummy variables '1' and '0' were assigned to a new turion and a new frond, respectively.

2.3 Results

2.3.1 Transplant Experiment

In Chapter 1, the mean days to turion formation was found to differ among ponds. To test for phenotypic plasticity, fronds from Cow Island Marsh, Leroi Swamp and Blackberry Pond were transplanted to Cow Island Marsh (predictable environment) and to Blackberry Pond (unpredictable environment) to test for adjusted days to turion formation in response to a new environment.

Fronds transplanted to Cow Island Marsh began producing turions a few weeks after the transplant and produced a higher yield towards the end of the season (Figure 2.2). Turions were produced by fronds transplanted to Blackberry Pond almost immediately after the transplant (Figure 2.2). A two-way ANOVA tests for main effects of home environment, away environment, and the interaction between home and away environments. Because Leroi Swamp was not reciprocally transplanted, it was not included in this analysis. An effect of home environment and an interaction between home and away were found to have a significant effect on mean days to turion formation (Table 2.1). Underlying the ANOVA results are the following trends: mean days to turion formation was later in the more predictable environment (Cow Island Marsh) for fronds from Cow Island Marsh (mean days=133) and from Leroi Swamp (mean days=139) populations and earlier when transplanted to the unpredictable environment (Blackberry Pond) (mean days=123 and 113, respectively). Fronds transplanted from Blackberry Pond however, produced turions earlier when in the more predictable environment (Cow Island Marsh, mean days=133) than when in its home environment (mean days=137)

The mean days to turion formation for fronds in the predictable environment fell in a range between 132 and 139, whereas in the more unpredictable environment (Blackberry Pond), mean days to turion formation was spread between 113 and 137 days (Figure 2.3). Therefore, fronds seemed to behave quite similarly to each other in the predictable pond, and like the 'host' population but different from each other in the unpredictable environment, and unlike the 'host' population.

Figure 2.2 Turion yield (turions per frond) of *Spirodela polyrhiza* fronds when transplanted from each of three home ponds to Cow Island Marsh (a) and Blackberry Pond (b), QUBS, Ontario, Canada. Error bars represent ± 1 SE.

Figure 2.3 Phenology of turion formation of *Spirodela polyrhiza* when fronds transplanted from home populations Cow Island Marsh (CI), Leroi Swamp (LS) and Blackberry Pond (BB) are transplanted to host ponds (abscissa) Cow Island Marsh and Blackberry Pond. Error bars represent ± 1 SE.

Table 2.1 Results of two-way ANOVA of mean days to turion formation of *Spirodela polyrhiza* in a transplant study at QUBS in two populations: Cow Island Marsh and Blackberry Pond (July 26-November 14, 2011).

Effect	<i>df</i>	T Ratio	P
Home	2	3.10	0.0021
Host	2	1.50	0.1352
Home*Host	4	-2.27	0.0237

2.3.2 Common Garden Experiment

To test for population differentiation, fronds were collected from the field and were brought back to the laboratory. Fronds from Barb's Marsh (eliminated from all field studies due to insufficient results) were included in this portion of the study. Second and third generation fronds were subjected to standardized conditions in a common garden experiment across three different temperatures (15°C, 23.5°C and 30°C). New turions and new fronds were monitored.

The cold temperature induced the earliest (Figure 2.4) and the highest frequency (0.60) of turions within all ponds and was significantly different from frequencies at the room (0.34) and the high (0.32) temperature treatments (Figure 2.5).

A logistic regression, using dummy response variables '1' for the formation of a turion and '0' for the formation of a new frond, found that temperature, population home (pond) and day all had significant effects on turion formation; day having the most significant effect followed by temperature ($R^2=0.12$) (Table 2.2). The cold temperature induced the earliest switch from producing fronds to turions compared to the room and hot temperatures from trends observed in Figure 2.6. Cow Island Marsh appears to have spent the most time producing new fronds before turions when compared to the other ponds (Figure 2.6). In this model, all paired interactions (Day*Temp, Day*Pond, Pond*Temp) are significant but no three-way interaction was found (Day*Temp*Pond, $P=0.1168$) (Table 2.2).

Table 2.2 Logistic regression of the effects of time (day), pond (Cow Island Marsh, Leroi Swamp, Barb's Marsh and Blackberry Pond), temperature (15°C, 23.5°C and 30°C) and their interactions on turion formation of *Spirodela polyrhiza* in a common garden experiment

Effect	<i>df</i>	Chi Square	P
Day	1	6840.59	<0.0001
Temperature	2	609.68	<0.0001
Pond	3	98.16	<0.0001
Day*Temperature	2	29.34	<0.0001
Day*Pond	3	13.91	0.00030
Temperature*Pond	6	117.05	<0.0001
Day*Temperature*Pond	6	10.19	0.1168

Temperature*Day- Chi Square=609.68, $P<0.0001$

Figure 2.4 Turion formation frequencies of four populations of *Spirodela polyrhiza* in the common garden experiment at cold (a), room (b) and hot (c) temperatures.

Figure 2.5 The effect of cold, room and hot temperatures (15°C, 23.5°C and 30°C, respectively) on frond and turion formation frequencies of *Spirodela polyrhiza* from four different populations (Cow Island Marsh, Leroi Swamp, Barb's Marsh and Blackberry Pond) in a common garden experiment. Box plot is showing the median (midline), interquartile range (box), and 1.5 times the interquartile range (bars).

Figure 2.6 Logistic regression analyses of *Spirodela polyrhiza* frond and turion formation from focal ponds (Cow Island Marsh, Leroi Swamp, Barb's Marsh and Blackberry Pond) at the cold temperature, 15°C, room temperature 23.5°C and the hot temperature, 30°C.

2.4 Discussion

2.4.1 Reciprocal transplant

Spirodela polyrhiza populations have a vast geographic range (Dudley 1987) and thrive under diverse environmental conditions that include large lakes and rivers to small ponds to puddles of water formed in ditches. As established in Chapter 1, factors including temperature and nutrient concentrations will be highly variable in smaller bodies of water. Persistence through such variable conditions may be attributed to adaptive phenotypic plasticity (Sultan and Spencer 2003) or to the evolution of bet-hedging strategies (Seeger & Brockman 1987). Furthermore, when environments are more stable and gene flow is low, differences among populations may be attributed to local adaptation (Kawecki and Ebert 2004) governing trait differences in phenotypic plasticity (and/or bet hedging).

In the first experiment, we tested turion formation of fronds from populations that vary in the degree of environmental variance. Phenotypically plastic fronds were expected to behave differently from when in their ‘home’ and like the ‘host’ population and to produce a significant home*host interaction (Table 2.1). Fronds were found to alter their behaviour when transplanted and a significant interaction was confirmed by a two-way ANOVA. These differences are suggestive of plasticity in turion formation of *S. polyrhiza*.

All populations behaved similarly when in Cow Island Marsh but quite differently when in Blackberry Pond. Fronds from Blackberry Pond satisfy both requirements in our prediction regarding phenotypic plasticity in that they behaved differently from their ‘home’ and like their ‘host’. For populations from Cow Island Marsh and Leroi Swamp,

plasticity is also inferred; however, this plasticity may not be adaptive. If we assume that the home population phenotype is best suited to the prevailing conditions (greatest phenotype-fitness association), then we would expect an adaptive plastic strategy to assume the same phenotype as the home population. The association between a cue and fitness may differ among ponds; thus, cues in a host environment may not be properly “interpreted” by a transplanted frond. There is also the possibility of non-genetic maternal and carry-over effects where physiological or developmental processes of the home environment continue to effect response, post-transplant (Bakken 1995).

Further tests with higher statistical power might provide more conclusive results. However, we may conclude that phenotypic plasticity in turion formation exists in *S. polyrhiza*. Adaptive phenotypic plasticity is expected to evolve under conditions in which environmental variance has predictable fitness outcomes (DeWitt *et al.* 1998) and in Chapter 1, the timing of turion formation in Blackberry Pond was the most variable in the most unpredictable environment. This is also suggestive of a putative diversification bet hedging strategy. It is possible for a blend of both diversification bet hedging and phenotypic plasticity to evolve in one trait and the expression depends on the degree of unpredictability (Simons 2011).

Furthermore, differences observed in mean days to turion formation may be due to population differentiation. A common garden experiment helps to disentangle population differences as well as emphasize important environmental factors controlling phenotypic response (Kawecki and Ebert 2004).

2.4.2 Common Garden

Local adaptation is expected to evolve when there is low gene flow and low temporal variability. To test for population differentiation, fronds from all of the four original test populations (Barb's Marsh, included here but excluded from all other analyses) were subjected to common garden conditions at three different temperatures to observe differences in turion production. If turion production is found to differ among ponds of origin, population differentiation may be inferred.

Consistent with the literature, temperature is an important effect inducing turion formation (Appenroth and Nickel 2010; Appenroth 2002; Newton *et al.* 1978). Temperatures of 15 °C or lower will induce turion production earlier than at warmer temperatures, regardless of population origin. Colder temperatures potentially indicate the end of favourable conditions and therefore induce a plastic response to switch to turion production sooner than at warmer temperatures. The frequency of turions was also the greatest at the cold temperature (mean frequency=0.60) and differed among ponds with Cow Island Marsh producing the most (mean frequency=0.53), and Leroi Swamp producing the lowest frequency of turions overall (mean frequency=0.37) (Figure 2.4). Perhaps cooler temperatures indicate a shorter growing season and may require a strategy that produces many turions that will germinate in the spring (synchronously or not) allowing for a greater number of germinating fronds over the surface. Differences in strategies may be a result of population differentiation.

A logistic regression that finds day, pond, temperature and their paired interactions to be significant indicates that there is local population differentiation in phenology of turion production. However, local population differentiation does not

imply local adaptation: an assessment of fitness of the phenotypes expressed in each environment would be necessary to draw conclusions about adaptation. From observation of fronds under common garden conditions at all temperatures, it was evident that once the ‘decision’ to make turions was made, individuals from all populations continued to produce turions and very few new fronds. This demonstrates an important observation of the life history at *S. polyrhiza*. It appears that once the physiological switch has been made for an individual to produce turions, new fronds are no longer produced which may have fitness implications. This requires further tests that follow *S. polyrhiza* at the individual-level.

This study provides evidence that populations differ in frequency and phenology of turion formation and of differentiation in the effects of temperature on turion formation based on the logistic regression. Populations are in close proximity (See Chapter 1, Figure 1.4) and sexual reproduction is a rare occurrence (Crawford and Landolt 1993; Jacobs 1946; Saeger 1929). Duckweeds are non-sessile and are easily moved by water currents through continuous water systems or across the surface of water by wind. Birds and mammals may also serve as dispersal agents since fronds, turions and the rare occurrence of seeds of *S. polyrhiza* are easily entangled in feathers and fur (Jacobs 1946). The aforementioned factors would facilitate gene flow and thereby hamper local adaptation. However, genetic divergence of *L. minor* (flowering also very rare) has been found in populations only 0.8 km apart (Cole and Voskuil 1996) and therefore it is not unreasonable to find population differentiation among the focal ponds in this study.

Genetic variation is not an alternative to phenotypic plasticity or bet hedging since these traits are simply properties of a genotype that are subject to evolution by natural selection (Richards *et al.* 2006). In other words, a phenotypically plastic or bet-hedging trait allows for the response of a genotype to different environments. Further tests of phenotypic plasticity in nature at higher statistical power and testing for genetic differences would allow for results that are conclusive. It is hoped that this work will lead to future studies that more fully exploit the large geographical range of *S. polyrhiza* to test for population differentiation, plasticity and bet hedging.

CONCLUSIONS

Tests of bet hedging require long-term studies across several generations that can demonstrate a maximization of the geometric mean fitness and optimality. In addition to the difficulties of testing for bet hedging, putative bet-hedging traits are difficult to recognize. Therefore, empirical evidence supporting bet hedging is lacking.

In this study, in the field, we sought to 1) identify possible hedging traits, 2) quantify whether or not apparent sub optimality and/or variation in phenotype is due to a conservative diversification or bet-hedging strategy, 3) test for phenotypic plasticity as an alternative hypothesis, and in the laboratory, 4) test for local population differentiation. In the process, important information on the life history of *S. polyrhiza* was obtained.

In Chapter 1, evidence supporting phenotypic variation among populations was found for both germination and turion formation, and therefore, possible bet-hedging traits. Variance in days to turion formation was correlated with a high degree of environment variance, inferring putative diversification bet hedging. No evidence was found to support conservative bet hedging.

In Chapter 2, a transplant study in the field was used to assess phenotypic plasticity in turion formation. When transplanted, fronds behaved differently from when in their home environments, suggesting that the timing of turion formation is a phenotypically plastic trait. To test for potential for population differentiation governing phenotypic traits, a common garden experiment in the laboratory tested for differences in trait expression by subjecting fronds to standardized, homogenous conditions. Evidence of population differentiation was found based on a logistic regression.

Bet hedging and phenotypic plasticity are traits of a genotype and thus may evolve through natural selection, given genetic variation for these traits. Assessment of the adaptive significance of plasticity and bet hedging, however, would require estimation of fitness over multiple environments.

This was the first study to assess turion germination and formation directly in the field and this study has established methods and collected empirical data regarding both. Information regarding growth patterns and turion formation is difficult to find in the literature. I observed that not only can a frond produce a daughter while producing a turion, it can also produce two turions simultaneously and can continue to produce subsequent turions. There is also reason to believe that once the switch to produce turions is made, the frequency of new fronds decreases rapidly, or ceases.

In addition, temperature as an important factor controlling the induction of turion formation was revealed by the common garden experiment. A very high yield of turions was produced after a short period and therefore an efficient method established for any future studies requiring a high yield of turions.

Finally, *Spirodela polyrhiza* has been established as a suitable model organism to be used to ask important questions in the field of evolutionary ecology.

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APPENDIX A
SUPPLEMENTARY TEMPERATURE PLOTS

Figure A.1 Cow Island Marsh (CI) average, minimum and maximum surface temperatures from hourly iButton recordings (April 29-November 7, 2011).

Figure A.2 Cow Island Marsh (CI) average, minimum and maximum sediment temperatures from hourly iButton recordings (April 29-November 7, 2011).

Figure A.3 Leroi Swamp (LS) average, minimum and maximum surface temperatures from hourly iButton recordings (April 29-November 7, 2011).

Figure A.4 Leroi Swamp (LS) average, minimum and maximum sediment temperatures from hourly iButton recordings (April 29-November 7, 2011).

Figure A.5 Barb's Marsh (BM) average, minimum and maximum surface temperatures from hourly iButton recordings (April 29-November 7, 2011).

Figure A.6 Blackberry Pond (BB) average, minimum and maximum surface temperatures from hourly iButton recordings (April 29-November 7, 2011).

Figure A.7 Blackberry Pond (BB) average, minimum and maximum sediment temperatures from hourly iButton recordings (April 29-November 7, 2011).