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EVOLUTION OF INVASIVE SPECIES IN RESPONSE TO MANAGEMENT: POTENTIAL
FOR HERBICIDE RESISTANCE IN WATERMILFOIL

Lindsey-Ann Louise Schulte

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

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For the Degree of

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Robert B. Annis Water Resources Institute

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Abstract

Herbicides are commonly used to control invasive plants. While agricultural weed scientists have focused on herbicide resistance, little is known about its importance in non-agricultural settings. Hybrid Eurasian watermilfoil (*Myriophyllum spicatum* x *M. sibiricum*) is an invasive aquatic plant that is frequently managed with herbicides throughout the United States. In this thesis, I consider the potential for natural populations of this taxon evolving resistance to 2,4-dichlorophenoxyacetic acid (2,4-D), the most commonly used systemic herbicide.

Evolutionary change in a trait between generations as a result of selection requires both genetically based variation of the trait within a generation (heritable variation) and differential survival and/or reproduction among members of that generation with different values of the trait (selection differential). Using a laboratory herbicide assay, I tested for differences in vegetative growth and response to 2,4-D among distinct genotypes of hybrid Eurasian watermilfoil collected from natural populations. I found heritable variation in growth and response to 2,4-D, indicating that heritable variation required for 2,4-D resistance evolution is present in natural populations of hybrid Eurasian watermilfoil. I also considered whether herbicide application in a natural lake creates a selection differential. Using a point-intercept survey, I collected plants before and after the lake was treated with herbicides. I then conducted a laboratory 2,4-D assay on the watermilfoil collected during the two time periods. I found increased growth rates and reduced sensitivity to 2,4-D among plants collected post-treatment compared to the pre-treatment plants (all from the same generation), indicating that herbicide exposure creates a selection differential among plants with different growth rates and 2,4-D sensitivities. Taken together, these results indicate that the components necessary for 2,4-D resistance evolution (i.e. heritable variation and a selection differential) are present in natural populations subject to management.

These results illustrate the importance of considering evolutionary potential when managing invasive species.

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General Introduction:

It is well known that evolution can be important in the establishment of invasive species (Lee 2002; Maron et al 2004; Bossdorf et al. 2005; Facon et al. 2006; Richardson and Pysek 2006; Lavergne and Molofsky 2007; Novak 2007; Keller and Taylor 2008; Prentis et al. 2008). Management programs are designed to limit the distribution and abundance of these species and thus are expected to impose selection pressures on them. But outside of artificial agricultural systems, little is currently known about the role evolution plays in determining the response of invasive species to management programs (but see Michel et al. 2004; Thum et al. 2012).

The breeder's equation provides a conceptual framework for what components are necessary for adaptive evolution to occur. It has the simple form,

$$R = h^2s.$$

Here, s is the selection differential (or strength of selection) for the trait of interest in the one generation, h^2 is the heritability of that trait, and R is the evolutionary response of that trait (across generations) to selection imposed on the previous generations (Falconer 1960; Lynch and Walsh 1998). For evolution of the trait to occur ($R > 0$ or $R < 0$), there must be phenotypic variation in the trait on which selection can act, this variation must be at least partially heritable ($h^2 > 0$), and selection must be applied ($s > 0$ or $s < 0$). Thus, if herbicide application alters important properties such as survival, growth, and/or reproduction in an invasive plant population, if there is variation among members of the population in the magnitude of these effects, and if at least part of this variation is heritable, then there is the potential for herbicide resistance evolution.

Herbicide resistance has evolved in many weed species subject to intense control efforts in agricultural systems (Holt et al. 1993; Powles and Holtum 1994; Powles et al. 1998; Powles and Yu 2010; Délye 2013; Délye et al. 2013; The International Survey of Herbicide Resistant

Weeds). Since resistance is known to evolve in these highly managed systems, the question becomes, does evolution of resistance to management efforts also occur in natural populations of invasive plants in non-agricultural systems, such as aquatic or wildland ecosystems? With increasing invasive aquatic plant populations in freshwater lakes, herbicide use for control has likewise increased, creating the potential to see herbicide resistance evolve.

Eurasian watermilfoil (*Myriophyllum spicatum* L.; hereafter ‘EWM’) is an invasive aquatic plant found throughout North America (Aiken et al. 1979). This species is commonly found with its native sister species, northern watermilfoil (*M. sibiricum* Komarov; hereafter ‘NWM’). The two species have hybridized resulting in hybrid Eurasian watermilfoil (*M. spicatum* x *M. sibiricum*; hereafter ‘hybrid watermilfoil’; Moody and Les 2002; Moody and Les 2007; Sturtevant et al. 2009; Zuellig and Thum 2012). EWM appears to have been introduced to North America at least two times, and appears to have frequently hybridized with NWM, creating numerous distinct lineages of hybrid watermilfoil (Moody and Les 2007; Zuellig and Thum 2012).

The hybrid watermilfoils are themselves sexually viable, which means they also have the potential to create novel genotypes and increase genetic variation through recombination (LaRue et al. 2013b). Hybrid watermilfoils on average grow faster and exhibit reduced sensitivity to commonly used rates of the herbicide 2,4-D when compared to their invasive parental species, EWM (LaRue et al. 2013a). These hybrid genotypes may have heritable variation for traits important to management, which would lead to their potential evolutionary response to selection pressures such as herbicides (Délye et al. 2013; Délye 2013).

What is not understood is whether there is heritable variation in growth and herbicide response—the h^2 part of the breeder’s equation—and if the herbicides used exert selection within

lake populations of watermilfoil—the s part of the breeder's equation. Variation in growth and herbicide response of watermilfoil populations and the heritability of this variation are discussed in detail in Chapter 1, while herbicide-induced selection is discussed in detail in Chapter 2. Demonstrating both of these components is necessary to establish the potential for herbicide resistance evolution in invasive watermilfoil populations. Demonstrating that resistance evolution actually occurs—the R part of the breeder's equation—requires comparisons across generations in successive years and is not addressed here. In this thesis, I show the raw components necessary for evolution are present among natural populations of invasive watermilfoil and that based on this, I can predicted that there is the potential for the herbicide resistance evolution among invasive watermilfoil populations, and more broadly, the potential for evolution in response to management programs.

**Chapter 1: Heritable Variation for Growth and Response in the Presence and Absence of
2,4-D Among Hybrid Watermilfoil Genotypes**

Running header: Potential for resistance evolution in watermilfoil

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Data Archival Statement: Data for this study are available at: (to be completed after the manuscript is accepted for publication).

Abstract:

There are numerous examples of invasive species adapting to new environments, but few studies have examined the potential for invasive species to adapt to management programs implemented to limit their distribution and abundance. Herbicides are commonly used to control invasive plants, and while agricultural weed scientists focus heavily on herbicide resistance, little is known about its importance in non-agricultural settings. Hybrid Eurasian watermilfoil (*Myriophyllum spicatum* x *M. sibiricum*) is an invasive aquatic weed that is frequently managed with herbicides throughout the United States to mitigate negative environmental and economic impacts. Aquatic plant managers are increasingly concerned about the potential for herbicide resistance evolution in hybrid watermilfoils. Here, I consider the potential for evolution of resistance to 2,4-dichlorophenoxyacetic acid (2,4-D), the most commonly used systemic herbicide for controlling hybrid watermilfoil. Using a laboratory assay, I tested for differences in vegetative growth in the presence and absence of 2,4-D among distinct genotypes collected from natural populations. I found heritable variation in growth in both the presence and absence of 2,4-D and in the degree of reduction of growth in response to 2,4-D exposure. These results indicate that genetic variation required for 2,4-D resistance evolution is present in natural populations. More broadly, our results illustrate the importance of considering the evolutionary potential of invasive species when designing, implementing, and evaluating control programs in natural systems.

Key Words: *Myriophyllum spicatum*, hybrid Eurasian watermilfoil, evolutionary potential

Introduction:

It is widely recognized that rapid evolution is one of the mechanisms by which introduced species may become invasive (Lee 2002; Maron et al. 2004; Bossdorf et al. 2005; Facon et al. 2006; Richardson and Pysek 2006; Lavergne and Molofsky 2007; Novak 2007; Keller and Taylor 2008; Prentis et al. 2008). However, the potential for established invaders to rapidly evolve in response to human efforts to control them has been little studied in nonagricultural systems. Efforts to control invasive species by chemical or other means clearly can create strong selection pressures. But in order for an evolutionary response to occur, there must be heritable variation in relevant phenotypic traits. Studies of heritable variation in traits that confer or reflect resistance are therefore important in assessing the potential for evolutionary response to management.

Herbicide resistance is an evolutionary adaptation to selection imposed by herbicide exposure (Powles and Yu 2010; Délye 2013; Délye et al. 2013). It has been documented in many weed species in heavily managed crop systems (Holt et al. 1993; Powles and Holtum 1994; Powles et al. 1998; The International Survey of Herbicide Resistant Weeds) but has rarely been studied in nonagricultural settings (but see Michel et al. 2004). However, because herbicides are widely used to control invasive plants in natural environments, it is logical to think that herbicide resistance may sometimes evolve in these systems.

Eurasian watermilfoil (*Myriophyllum spicatum*, L.; hereafter EWM) is a widespread aquatic invasive weed that is frequently managed with herbicides. EWM sensu lato can be split into two distinct forms: pure EWM (sensu stricto) and hybrid watermilfoil (*Myriophyllum spicatum* x *Myriophyllum sibiricum* Komarov; hereafter hybrid), consisting of interspecific crosses between EWM and native northern watermilfoil (hereafter NWM). There is particular

concern about invasiveness and control of hybrid watermilfoils among aquatic plant managers because hybrids on average grow faster and exhibit decreased sensitivity to the commonly used herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (LaRue et al. 2013a).

Molecular genetic studies have demonstrated a surprising amount of genotypic diversity in hybrid watermilfoils, suggesting that sexual reproduction is more common than previously expected for a taxon capable of vegetative propagation (Zuellig and Thum 2012; LaRue et al. 2013a and 2013b). However, it is not known whether increased growth rate and decreased sensitivity to 2,4-D are common to all hybrid watermilfoils or whether there is significant variation among hybrid genotypes. Understanding patterns of genetic variation in these traits is important for understanding the evolutionary potential of hybrid watermilfoils to evolve invasiveness which, in turn, has strong implications for management decisions such as prioritization of populations and the development of management prescriptions.

The purpose of this study was to test for heritable variation in growth rate and 2,4-D sensitivity in hybrid watermilfoil. I hypothesized that hybrids exhibit quantitative genetic variation for these traits and therefore have the potential to evolve in response to control efforts. In particular, I was interested in whether hybrid watermilfoils have the potential to evolve resistance in response to selection imposed by the herbicide 2,4-D.

Materials and Methods:

Plant Populations and Growth Conditions

I isolated one plant stem from each of 12 lake populations scattered across the northern tier of the U.S. (ten hybrid genotypes and two EWM genotypes, which I used as reference genotypes that were known to be sensitive to 2,4-D). Previous studies demonstrated genetic differentiation among water bodies (Zuellig and Thum 2012; LaRue et al. 2013a). I sampled a

single stem from each lake to increase the probability that each stem was a unique genotype.

This study design therefore evaluates the potential for herbicide resistance evolution at a broad scale, and further work should consider evolutionary potential at local scales.

Individual stems were planted in separate 18.9-L buckets containing potting soil supplemented with 2.2 mL kg⁻¹ Osmocote (19:6:12, nitrogen/phosphorus/potassium) and capped with sand to prevent soil dispersing into the water column. As plants grew, I propagated each genotype vegetatively by removing branches and replanted them to produce genetically identical ramets for each genotype in our herbicide assay. Members of each genotype were planted in the same bucket, and all buckets were randomly placed in 1136-L mesocosm tanks at the Robert B. Annis Water Resources Institute. Cultures were checked daily to ensure there was no cross-contamination among genotypes within the same tank. All tanks were filled with filtered water from Muskegon Lake and lit with a full-spectrum sodium lamp (Sylvania M1000/U M47/S Metalarc) on a 14:10 hour light/dark cycle with water temperature ranging between 21-24°C throughout all studies. Cultures were maintained in this manner until each genotype had a sufficient number of stems for the herbicide assay (see below).

2,4-D Assay

Herbicide assays were conducted using a block design, both to increase sample size (since a sufficient number of replicates of each genotype could not all be grown and exposed at once) and to combat potential pseudo-replication. To minimize random environmental variation among genotypes, all ramets of different genets for each block were randomly assigned to squares in a single mesocosm tank divided into a grid. Each ramet was replanted at least once to minimize maternal effects before the assay.

For each assay, apical stem segments 11.8 cm long were collected for each genotype, with three ramets per genet per exposure level. Initial wet weight was recorded for each plant. Each meristem was labeled, wrapped individually in a permeable netting, and randomly placed in one of two treatments (500 $\mu\text{g L}^{-1}$ and 1000 $\mu\text{g L}^{-1}$ 2,4-D) or a control (filtered Muskegon Lake water). The number of treatment levels I could investigate was limited by space; these two levels were chosen because they represent the normal to high range of exposure. Target field concentrations for 2,4-D are usually 1-2 mg L^{-1} , but 2,4-D is typically used as a ‘spot treatment’ in nuisance areas within a lake and the applied solution rapidly dilutes as it disperses. Thus, many plants inside and out of treated areas experience a lower rate of 2,4-D (Bugbee et al. 2003; WIDNR and USACE of ERDC 2011). An independent assay was done for each block to avoid pseudo-replication.

Each exposure lasted 2 days. At the end of exposure, each meristem was planted in an individual 115-mL pot with soil supplemented with 2.2 mL kg^{-1} Osmocote, capped with sand and placed in a mesocosm tank (also filled with soil supplemented with the fertilizer and capped with sand) to grow for ~3 weeks. At the end of the growth period, length gained (final length minus 11.8 cm) and wet weight gained (final total weight minus initial weight) were recorded, following LaRue et al. (2013a).

Statistical Analysis

I tested for differences in growth among hybrid genotypes using a two-way ANOVA with interaction. Separate analyses were run with length gained and wet weight gained as the response variable; I present only the results for length gained, because the two growth measures were strongly correlated (Spearman’s $\rho=0.67$, $p<0.001$) and qualitatively the same. Genotype, treatment, genotype x treatment, and block were treated as fixed effects. Data were transformed

using the Box-Cox method to satisfy the ANOVA assumptions of approximately Gaussian residuals with homogenous variance.

I also tested for differences among hybrid genotypes in sensitivity of growth to 2,4-D using a one-way ANOVA. Sensitivity was operationally defined as the reduction in growth at 1000 $\mu\text{g L}^{-1}$ 2,4-D compared to the control. Genotype and block were treated as fixed effects. Data were transformed by adding 23.7 to each value, so that there were no negative or zero values, and then using a Box-Cox transformation to satisfy the ANOVA assumptions. All statistical analyses were performed with R (R Development Core Team 2014).

The genetic contribution to observed phenotypic variation in growth among hybrids was assessed both by pairwise comparisons of genotypes and by estimating broad-sense heritability, which was calculated as the ratio of among-genotype variance to total variance across blocks, within each treatment level and the control (Falconer and Mackay 1996).

Results:

Growth (length gained) varied significantly among genotypes and treatment levels (Table 1, Figure 1), but there was no significant interaction between genotype and treatment. In each treatment level, at least some genotypes differed significantly from others (Figure 1, Supplementary Table 1). Sensitivity varied significantly among genotypes (Table 2, Supplementary Table 2). Broad sense heritabilities for growth were estimated at 0.34, 0.22, and 0.27 for the control, 500 $\mu\text{g L}^{-1}$, and 1000 $\mu\text{g L}^{-1}$ treatments, respectively. Within genotypes, growth decreased with increasing 2,4-D concentration.

Discussion:

The evolutionary potential for invasive aquatic plants to adapt to control measures such as herbicides is of clear importance to aquatic plant management, yet few relevant empirical

studies have been conducted to date (but see Michel et al. 2004; Thum et al. 2012). Our results contribute meaningfully to current understanding of the potential for 2,4-D resistance evolution in hybrid watermilfoil.

Resistance evolution requires heritable variation for growth in the presence of an herbicide over the range of concentrations and exposure times that populations experience in the field, such that genotypes that are less affected by the herbicide replace genotypes that are more strongly inhibited by it with repeated treatment. Our study provides compelling evidence that hybrid watermilfoils have the potential to evolve 2,4-D resistance by demonstrating that variation in growth rate and sensitivity to 2,4-D has a genetic basis (i.e., is heritable). This heritable variation is evidenced by significant variance among genotypes in growth and in degree of reduction in growth following 2,4-D exposure (Table 1 and 2, Figure 1). For example, the Lake2 genotype grew better than the Lake3 genotype in the control and after exposure to both levels of 2,4-D, indicating faster growth in the former compared to the latter (Supplementary Table 1). Thus, from these laboratory data I can predict that naturally-occurring hybrid genotypes that are less affected by 2,4-D could replace genotypes that are more affected within and among lakes following treatment with 2,4-D, which results in an evolutionary trajectory selecting for faster/less affected growers.

It is important to note that while our study demonstrates the *potential* for 2,4-D resistance evolution, it does not prove that the less sensitive genotypes collected from some populations occur as the result of resistance evolution. Weed scientists distinguish between tolerance and resistance. Briefly, resistance is the result of evolutionary response to prior selection from herbicide exposure on an ancestral population, whereas tolerance is inherent without prior exposure to and selection by the herbicide. It is possible our genotypes that are less affected by

2,4-D do not have any history of herbicide exposure to their ancestral population, and simply exhibit a higher tolerance to 2,4-D than other genotypes. However, based on our data, if two or more genotypes with different sensitivity growth rates and responses occurred in the same lake before the application of 2,4-D, I predict that the less affected genotypes would have higher survivorship and reproduction compared to more affected genotypes. This would lead to a shift in the relative proportions of genotypes present before versus after treatment, and would constitute resistance evolution.

Although I found significant differences for growth in the presence and absence of 2,4-D among our genotypes, our results corroborate the findings of LaRue et al.'s (2013a) that hybrid watermilfoils are on average less sensitive to 2,4-D than parental Eurasian watermilfoil. This result was true even at the 1000 $\mu\text{g L}^{-1}$ 2,4-D treatment, two-fold higher than LaRue et al. (2013a) used in their study, and encompasses a recommended exposure for Eurasian watermilfoil (Green and Westerdahl 1990). In fact, growth of some hybrid genotypes in 1000 $\mu\text{g L}^{-1}$ 2,4-D was similar to that of Eurasian watermilfoil in the control. Thus, this study lends further credence to the notion that hybrid watermilfoils in natural populations are more likely to exhibit a muted response to operational 2,4-D applications, which in turn is consistent with the hypothesis that hybridization can be a mechanism for stimulating adaptive evolution (Anderson and Stebbins 1954; Arnold 2004; Ellstrand and Schierenbeck 2000). Given the general pattern that hybrids are less sensitive to 2,4-D compared to pure Eurasian individuals, I recommend that aquatic plant managers distinguish between parental and hybrid Eurasian watermilfoil within a lake to carefully consider management strategies. However, there remains much to be learned regarding the genetic basis of the observed variation for growth in and response to 2,4-D in order to better predict when and where populations will be or become resistant (monogenic versus polygenic

basis, target site versus non-target site evolution, influence of parental background; see Powles and Yu 2010; Délye 2013; Délye et al. 2013).

From a management perspective, it is also important to note that while there is a clear genetic component to variation in growth with/without the presence of 2,4-D, a number of environmental factors are also likely to influence 2,4-D response. Our heritability estimates (the proportion of phenotypic differences that can be attributed to genetic differences) for watermilfoil growth were 34%, 22%, and 27% in the control, 500 $\mu\text{g L}^{-1}$, and 1000 $\mu\text{g L}^{-1}$ treatments, respectively, showing that phenotypic variation also includes a substantial environmental component. This shows genotypes can respond differently to 2,4-D based on a number of environmental factors. I therefore think it is important that aquatic plant managers recognize that while our results provide strong evidence for herbicide resistance potential, they do not demonstrate that any genotypes that are resistant under laboratory conditions will necessarily be so under more complex natural conditions. Future work should examine herbicide response in field settings, which may be most easily conducted with careful pre- and post-treatment monitoring.

Our study illustrates the potential for invasive species to evolve resistance to management efforts, which could exacerbate invasive species problems environmentally and economically. I recommend that natural resource managers consider the potential for evolution in designing, implementing, and evaluating the effectiveness of invasive species management programs.

Table 1. Two-way ANOVA with interaction results for hybrid genotypes. Data were transformed using the Box-Cox method. Interaction term indicated by x.

Factor	df	SS	MS	F-Value	P-Value
Treatment	2	69.72	34.86	59.74	<0.001
Genotype	9	53.40	5.93	10.17	<0.001
Genotype x Treatment	18	11.17	0.62	1.06	0.39
Block	2	7.01	3.50	6.00	
Residuals	238	138.87	0.58		

df, degrees of freedom; SS, sum of squares; MS, mean square

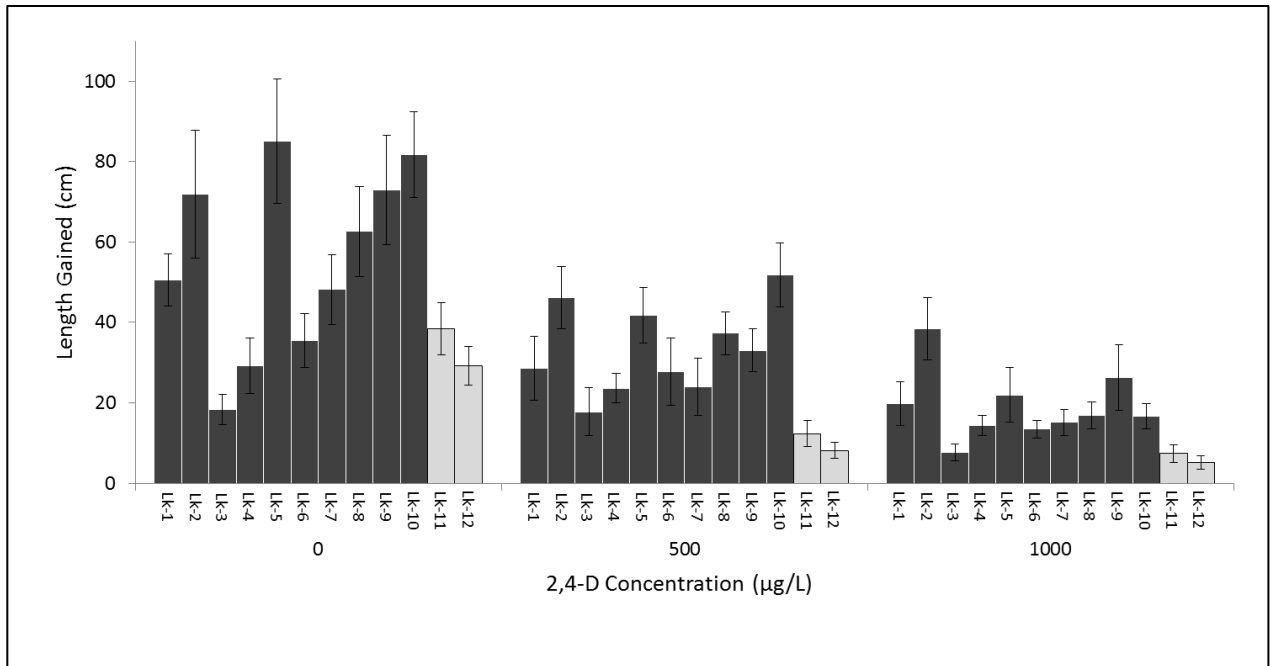
Table 2. One-way ANOVA results for response among the hybrid genotypes. Data were transformed using the Box-Cox method.

Factor	df	SS	MS	F-Value	P-Value
Genotypes	9	17.23	1.92	2.81	0.007
Block	2	10.45	5.23	7.67	
Residuals	78	53.19	0.682		

df, degrees of freedom; SS, sum of squares; MS, mean square

Figure 1. Growth of hybrid watermilfoil genotypes (dark grey bars) and Eurasian watermilfoil reference genotypes (light grey bars) in two concentrations of 2,4-D and a control after 3 weeks of growth post-treatment (\pm standard error). The same genotype from each lake is present in both treatments and the control. The mean across blocks within treatment for each genotype is shown.

Figure 1. Heritable variation for growth in the presence and absence of 2,4-D among watermilfoil genotypes.



Supplementary Materials:

Supplementary Table 1. Pairwise comparisons between hybrid genotypes within treatments showed statistically significant differences with Tukey's test.

Comparison	Treatment ($\mu\text{g L}^{-1}$ 2,4-D)	p-value
Lk1-Lk3	0	0.05
Lk2-Lk3	0	0.003
Lk5-Lk3	0	<0.001
Lk8-Lk3	0	0.007
Lk9-Lk3	0	0.001
Lk10-Lk3	0	<0.001
Lk5-Lk4	0	0.006
Lk9-Lk4	0	0.04
Lk10-Lk4	0	0.005
Lk2-Lk3	500	0.03
Lk10-Lk3	500	0.01
Lk2-Lk3	1000	<0.001

Supplementary Table 2. Pairwise comparisons for sensitivity between hybrid genotypes that showed statistically significant differences with Tukey's test. Sensitivity was operationally defined as the reduction in growth at $1000 \mu\text{g L}^{-1}$ 2,4-D compared to the control.

Comparison	p-value
Lk10-Lk3	0.02
Lk10-Lk4	0.04

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Chapter 2: Monitoring Selection on a Watermilfoil Population in Michigan

Running Footer: Monitoring Selection on Watermilfoil

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There is widespread evidence that adaptive evolution can play an important role in the successful establishment and spread of introduced species, but few studies have examined the potential for these species to adapt in response to management programs used to control them in natural habitats. Herbicides are commonly used to control invasive weeds, and the evolution of herbicide resistance is a major focus in agricultural settings. Little is known, however, about the potential for resistance evolution outside of agricultural settings. Eurasian watermilfoil (*Myriophyllum spicatum*) is an invasive aquatic plant that hybridizes with native northern watermilfoil (*Myriophyllum sibiricum*). Hybrid watermilfoils exhibit faster vegetative growth and reduced response to the commonly used herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) compared to Eurasian watermilfoil, both of which are commonly controlled with herbicides. There is increasing concern for the potential of herbicide resistance evolution in watermilfoil populations because hybrid watermilfoils exhibit heritable variation for growth and sensitivity to 2,4-D. Here, I consider whether herbicide treatment of watermilfoil in a large Michigan lake creates a selection differential favoring less sensitive phenotypes. I tested for differences in

vegetative growth in the presence of 500 $\mu\text{g L}^{-1}$ 2,4-D by collecting plants before and after the lake was treated with auxin herbicides (2,4-D and triclopyr) using a point-intercept survey and conducting a laboratory 2,4-D assay on the watermilfoil collected during the two time periods. I found increased growth by the plants collected post-treatment compared to the pre-treatment plants, suggesting the herbicide acts as a selection agent resulting in a shift towards phenotypes that grow better when exposed to 2,4-D. Our results, paired with previous research on watermilfoil, indicate that the components necessary for 2,4-D resistance evolution (i.e. heritable variation and a selection differential) are present in natural populations treated with 2,4-D. These results illustrate the importance of considering the evolutionary potential of invasive species when designing, implementing, and evaluating management programs natural systems.

Nomenclature: 2,4-D; Eurasian watermilfoil, *Myriophyllum spicatum* L.; northern watermilfoil, *Myriophyllum sibiricum* Komarov; hybrid watermilfoil, *Myriophyllum spicatum* x *Myriophyllum sibiricum*

Key Words: Herbicide resistance, invasive species management, evolutionary potential

There is evidence that adaptive evolution can play an important role in the successful establishment and spread of invasive species (Lee 2002; Maron et al. 2004; Bossdorf et al. 2005; Facon et al. 2006; Richardson and Pysek 2006; Lavergne and Molofsky 2007; Novak 2007; Keller and Taylor 2008; Prentis et al. 2008). Introduced species are frequently managed to control their distribution, abundance, and spread; however, invasive species evolution in response to management actions has not been carefully evaluated in natural systems. For adaptive evolution to occur, there must be heritable variation in relevant phenotypic traits, as well as selection acting on those traits. Therefore, it is important to understand both of these components when determining the potential for evolution in response to management.

Herbicide resistance is the evolutionary adaptation to herbicide applications (Powles and Yu 2010; Délye 2013; Délye et al. 2013). Resistance has been documented in a variety of weed species in heavily managed crop systems (Holt et al. 1993; Powles et al. 1998; The International Survey of Herbicide Resistant Weeds), but has rarely been studied in nonagricultural systems (but see Michel et al. 2004; Thum et al. 2012). However, given that herbicides are commonly used to control invasive plant populations in natural environments, there clearly is the potential for resistance evolution if there is heritable variation in response to exposures and the herbicides act as selection agents.

Hybrid Eurasian watermilfoil (hereafter hybrid) consists of the interspecific crosses between invasive Eurasian watermilfoil (*Myriophyllum spicatum*, L.; hereafter EWM) and native northern watermilfoil (*Myriophyllum sibiricum* Komarov; hereafter NWM). Hybrids on average grow faster and exhibit a reduced response to the commonly used herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (LaRue et al. 2013a; Chapter 1). Molecular studies of hybrids have shown a large amount of genetic diversity within and among watermilfoil

populations, which suggests multiple hybridization events and sexual reproduction for a plant that is more prominently known for its vegetative propagation (Zuellig and Thum 2012; LaRue et al. 2013a, 2013b). Furthermore, growth rate and response to 2,4-D vary significantly among hybrid genotypes, providing evidence of heritable phenotypic variation in traits relevant to management among hybrid watermilfoil collected from natural populations (Chapter 1). Since heritable variation may be present in hybrid populations, it is important to determine if herbicides used to control these populations are acting as selection agents, shifting the mean among surviving plants towards increased growth and muted herbicide response. Understanding if herbicide treatments select for faster growing or less sensitive genotypes is important for understanding the evolutionary potential of hybrid watermilfoil to evolve invasiveness, which has strong implications for management decisions.

The purpose of this study was to conduct a temporal change experiment within one lake to test for selection acting within a lake population of watermilfoil through herbicide application. I hypothesized that plants collected after a prescribed lake herbicide treatment would be better or faster growers in the presence of 2,4-D than plants collected before treatment. In particular, I was interested to see if the lake herbicide treatment acted as a selection agent, resulting in a phenotypic shift among surviving plants towards better growth when exposed to 2,4-D.

Materials and Methods

Study Site. I sampled plants from Houghton Lake, Roscommon County, Michigan. This lake has a surface area of 8,111.52 hectares, a maximum depth of 6.71 meters, and a mean depth of 2.29 meters. Sampling sites occurred at depths ≤ 3.96 meters using a point-intercept survey of 996

total sampling sites (Figure 1). Houghton Lake has been heavily managed in previous years with fluridone, 2,4-D amine, and triclopyr (Progressive AE, personal communication).

Plant Material. I collected plant material through rake tosses from 20 out of 428 total sites with plants present near the beginning of the growing season in June 2014 before the prescribed herbicide treatment of the lake. The lake was then spot-treated with 2,4-D amine (Sculpin G, SePro Corporation) and triclopyr (Renovate OTF, SePro Corporation). Near the end of the growing season in September 2014, I collected plant material from 14 out of 298 total sites with plants present. Pre- and post-treatment sites were chosen independently and opportunistically based on the presence and abundance of plant material (i.e. greater than 15-20 individual stems). Among the plants collected post-treatment, there was likely differential exposure due to proximity relative to the herbicide treatment, meaning the plants collected likely varied in their exposure to herbicide.

Plant material from each of the 20 pre-treatment collection sites and 14 post-treatment collection sites was planted in 18.9-L buckets containing potting soil supplemented with 2.2 mL kg⁻¹ Osmocote (19:6:12, nitrogen/phosphorus/potassium) and capped with sand to prevent soil dispersing into the water column. At the Robert B. Annis Water Resources Institute, buckets with pre-treatment material were distributed between two 1136-L mesocosm tanks, each containing 10 buckets, while buckets containing post-treatment material were distributed between two additional mesocosm tanks, each containing 7 buckets. Cultures were checked daily to ensure there was no cross-contamination among sites within the same tank. All tanks were filled with filtered water from Muskegon Lake and illuminated with a full-spectrum sodium lamp (Sylvania M1000/U M47/S Metalarc) on a 14:10 hour light:dark cycle with water temperature ranging between 21-24°C throughout the study. Plant cultures were grown and replanted several

times (when plants reached the surface) for approximately 2 months. Through the replantings, I vegetatively propagated stem by stem, but also removed and replanted fragments from each stem, reducing the potential for maternal effects and limiting differences in the health of the plants due to pre-treatment plants being cultured in laboratory conditions longer than post-treatment plants.

2,4-D Assay. Herbicide assays were conducted using a randomized complete block design. To minimize micro-environmental variation among blocks, plants from each site (20 pre-treatment plants and 14 post-treatment plants), were randomly assigned a square in a mesocosm divided into a grid. Each stem was replanted at least once in addition to the replantings described above to decrease any lingering micro-environmental effects from other mesocosms.

For each assay, one 11.8-cm apical stem was collected for each site. Initial wet weight was recorded. Each stem was labeled and wrapped individually in a permeable netting and placed into one treatment of $500 \mu\text{g L}^{-1}$ 2,4-D. Target field concentrations for 2,4-D are usually $1\text{-}2 \text{ mg L}^{-1}$ (applied at a rate of $160 \text{ lbs acre}^{-1}$ on Houghton Lake), but 2,4-D is typically used as a ‘spot treatment’ in nuisance areas within a lake and the applied herbicide rapidly dilutes as it disperses. Thus, many plants inside and out of treated areas experience a lower rate of 2,4-D (Bugbee et al. 2003; WIDNR and USACE of ERDC 2011). An independent assay was done for each block to avoid pseudo-replication.

I chose to use only 2,4-D since it is the most commonly used herbicide and was used more often for treatment in Houghton Lake than triclopyr. I did not expose Houghton Lake plants to a control because the objective was not to determine growth differences between control and treated plants; however, to determine our $500 \mu\text{g L}^{-1}$ 2,4-D treatment caused an effect

on growth, I exposed a reference genotype used in Chapter 1, and by using a t-test, I determined the plants were being affected by the herbicide treatment ($p=0.002$).

The plants were exposed to 2,4-D for 48 hours and then each meristem was planted in an individual 115-mL pot with soil supplemented with 2.2 mL kg^{-1} Osmocote, capped with sand and placed back in the mesocosm it came from (also filled with soil supplemented with the fertilizer and capped with sand) to grow for 3 weeks. At the end of the growth period, length gained (final length minus 11.8-cm) and wet weight gained (final total weight minus initial weight) were recorded, following LaRue et al. (2013a) and methods in Chapter 1.

Statistical Analysis. I tested for differences in growth in the presence of $500 \mu\text{g L}^{-1}$ 2,4-D using a mixed-effects analysis of variance with length gained as the response variable. Separate analyses were run with length gained and wet weight gained as the response variable; I present only the results for length gained, because the two growth measures were strongly correlated (Pearson's $r=0.95$, $p<0.001$) and qualitatively the same. Time of collection (i.e. pre- versus post-treatment plant collection) was treated as a fixed effect, while site was a random effect nested within time of collection since the same sites were not always collected post-treatment as were collected pre-treatment. Although difference in growth among sites is not our primary interest, site still needs to be represented within the model to account for variation among sites within time of collection. Block was treated as a fixed effect. Data were square root transformed to satisfy the ANOVA assumptions of approximately Gaussian residuals with homogeneous variance. All statistical analyses were performed with R (R Development Core Team 2015).

Results and Discussion

The potential for evolution in response to management efforts, such as herbicides, is increasingly important to aquatic plant managers. For such evolution to occur, both heritable variation in relevant traits, such as growth and response to herbicides, as well as selection on those traits are necessary. Chapter 1 showed hybrid watermilfoils have ecologically relevant heritable variation. The present results show that herbicide treatment in Houghton Lake created a selection differential in the mean growth rate when exposed to 2,4-D.

Our study provides compelling evidence that operational herbicide applications could act as selection agents resulting in a shift towards relatively better or faster growing plants following lake treatment. This presence of selection is supported by significantly higher growth in 2,4-D among post-treatment plants from Houghton Lake than among pre-treatment plants ($p=0.030$; Table 1, Figure 2). Furthermore, our study shows some level of phenotypic diversity within the lake by showing variation for growth in $500 \mu\text{g L}^{-1}$ 2,4-D among the different sites within time of collection ($p=0.013$; Table 1). Together with previous research showing variation in growth and response to herbicides (Chapter 1), I can predict a change in the lake composition of watermilfoil following management, with the potential for watermilfoils that are less affected by 2,4-D replacing watermilfoils that are more affected, resulting in the evolutionary potential for herbicide resistance.

It is important to note that while our study provides evidence for the selection component necessary for the evolution of herbicide resistance, I did not show that an evolutionary response to selection occurred. This additional step will require monitoring over two or more growing seasons. Hendry et al. (2008) notes that phenotypic traits can change more quickly in populations subject to anthropogenic forces. Many organisms can respond to rapid changes in their

environment via phenotypic plasticity, but plasticity can also serve as an initial rapid response to changes in the environment that, in time, could facilitate genetic changes (Lande 2009; Hansen et al. 2012). Ultimately, I have shown a within-generation snapshot of the watermilfoil population's response to management in Houghton Lake. Further detailed monitoring, both phenotypic and genotypic, on Houghton Lake, as well as other lakes, needs to be conducted to examine whether the evolutionary response I predict actually occurs.

An inherent issue with laboratory assessment of plant performance in most temporal monitoring studies such as this one is the amount of time the organisms are cultured under laboratory conditions. The pre-treatment plants were cultured approximately 3 months longer than the post-treatment plants, suggesting the post-treatment plants could have been healthier from not being subject to laboratory conditions as long as the pre-treatment plants or vice versa. One way to combat this issue is to culture all plants for a long enough time to effectively remove potential variation in plant health as a result of the length of time in laboratory conditions. I was unable to do this, since I did not know how long it would take. Instead, I replanted all plant material repeatedly before exposure to 2,4-D to increase the likelihood that all plant material used in the assay was of similar health before treatment.

Plants were collected at the beginning and end of the growing season. I have no data on the response of the plants immediately following the herbicide application, so I do not know the immediate effects of the herbicide treatment. It is possible that the treatment was not efficacious, thus producing low die back and rapid regrowth of the plants able to withstand the treatment. Alternatively, the treatment could have been effective for a time, but plants grew back from root crowns, the seed bank, or untreated areas. Again, continued monitoring on lakes being managed

with herbicides is needed to determine where the regrowth comes from and to effectively manage future watermilfoil populations.

In summary, our study shows that herbicide treatment in Houghton Lake created a selection differential within the watermilfoil population. Paired with previous research on watermilfoil demonstrating extensive genetic variation as well as heritable variation in growth and sensitivity to 2,4-D (Zuellig and Thum 2012; LaRue et al. 2013a; Chapter 1), this finding shows the potential for this invasive species to evolve resistance to management. While herbicide resistance evolution is common in agricultural settings (for review see Powles and Yu 2010), there are few studies that consider the potential for resistance evolution in non-agriculture systems (again, see Michel et al. 2004; Thum et al. 2012). Invasive watermilfoil is highly managed, and by monitoring for selection on these plants, it will be easier to predict whether or not management programs are effective. Herbicide resistance evolution is something that managers should be proactively considering. Management programs should consider ways of reducing genetic variation and/or selection pressures within lakes without having negative side-effects. Our study demonstrates the importance for natural resource managers to consider the potential for evolution when designing, implementing, and especially when monitoring invasive species management actions.

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Table 1. Mixed-effects one-way ANOVA results comparing watermilfoil growth following exposure to 500 $\mu\text{g L}^{-1}$ 2,4-D for plants collected pre- and post-treatment from Houghton Lake, Michigan.

Factor	df	SS	MS	F-value	P-value
Time of collection	1	14.88	14.877	4.967	0.030
Site	32	186.53	5.829	1.946	0.013
Block	2	34.25	17.125	5.718	
Residual	61	182.71	2.995		

df, degrees of freedom; SS, sum of squares; MS, mean sum of squares

Figure 1. Sampled sites from the Houghton Lake point-intercept survey. Plants were collected from 20 sites before Houghton Lake was treated with 2,4-D amine and triclopyr (yellow and orange points) and 14 sites were collected after the herbicide treatment (red and orange points).

Figure 2. Growth of plants collected from Houghton Lake, Michigan pre-herbicide treatment (white bars) and post- herbicide treatment (grey bars) 3 weeks following the laboratory assay of $500 \mu\text{g L}^{-1}$ 2,4-D (\pm standard error). The mean length gained (cm) was calculated for each time of collection period using the untransformed length gained data.

Figure 1. Pre- and post-treatment sampling site locations across Houghton Lake.

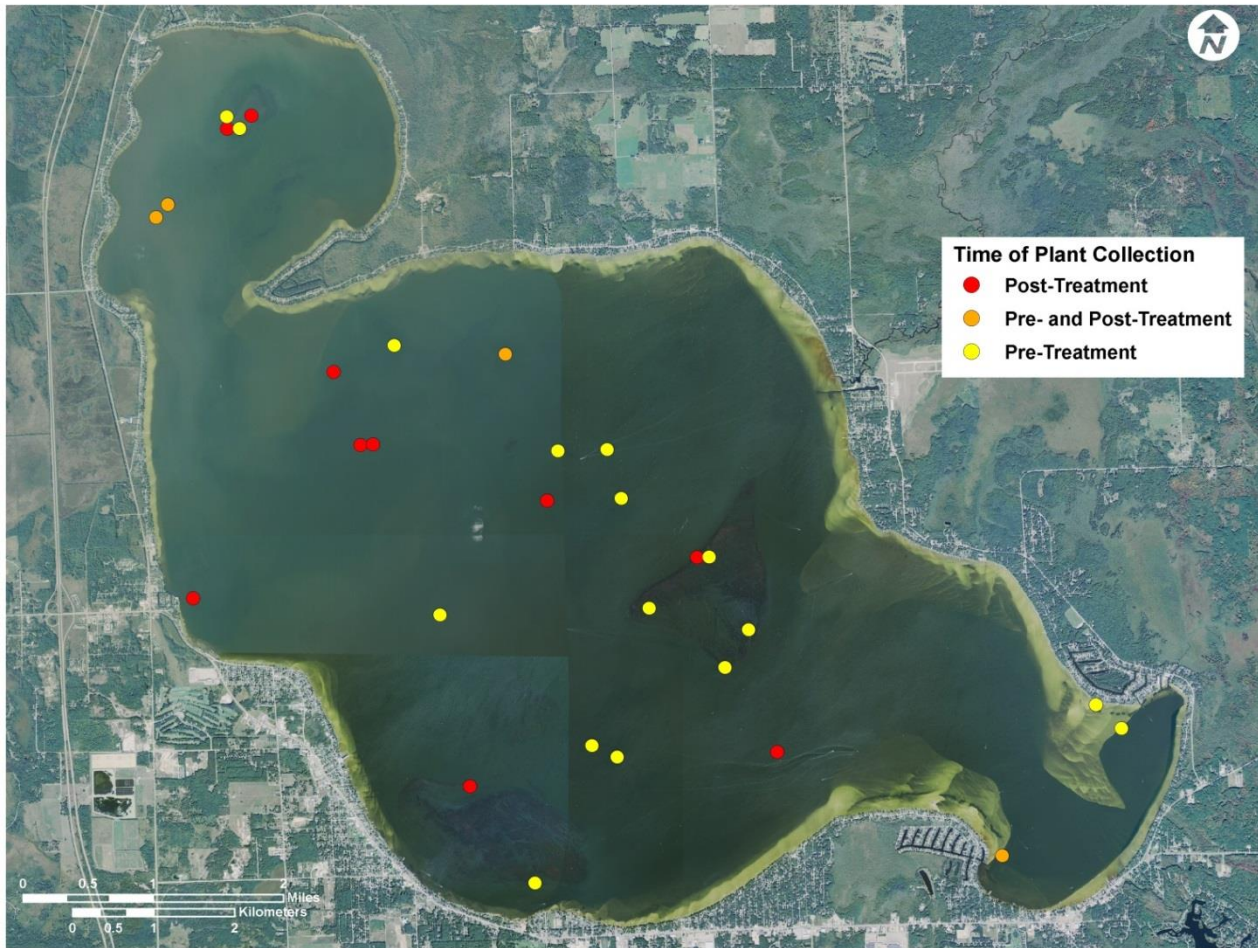
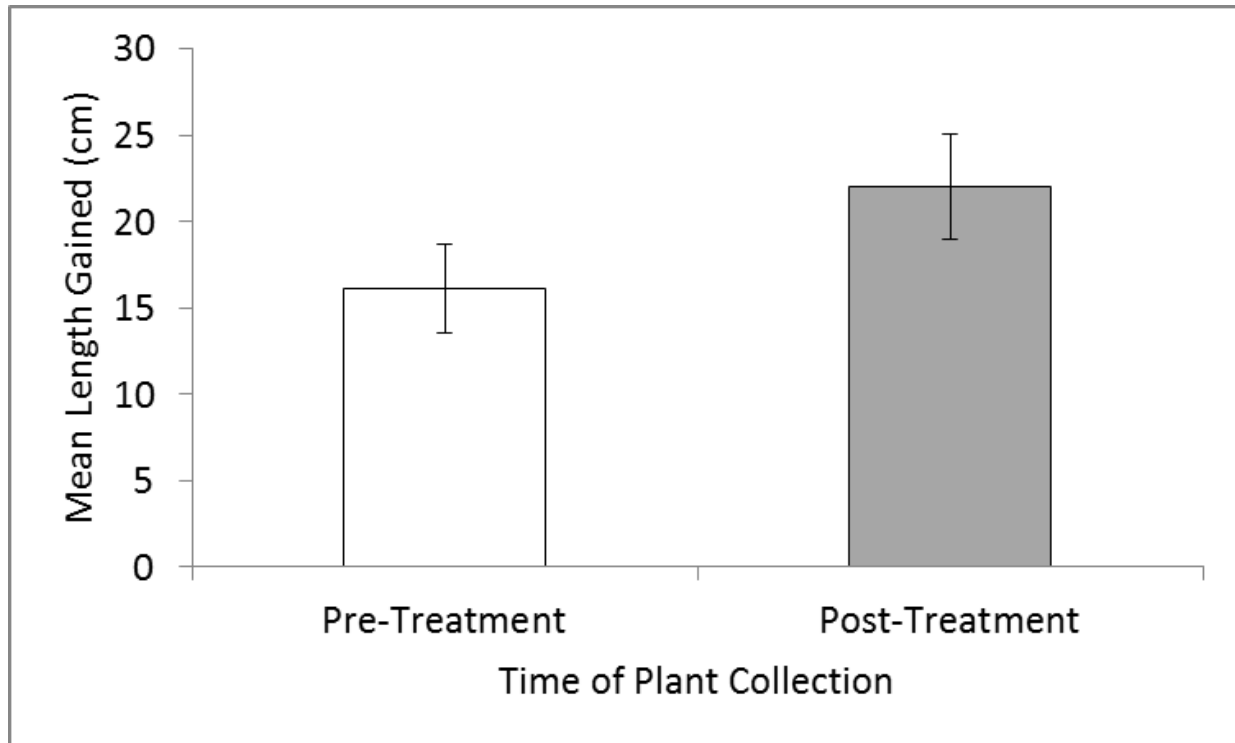


Figure 2. Mean length gained (cm) following the laboratory 2,4-D exposure among plants collected pre- and post-treatment from Houghton Lake.



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General Conclusions:

These studies examine the status of the two primary components necessary for herbicide resistance evolution in watermilfoil. Chapter 1 demonstrates that hybrid watermilfoils collected from natural populations exhibit heritable variation for the traits of growth and sensitivity to 2,4-D, evidence for the heritable variation in adaptive traits part of the breeder's equation, " h^2 ". This means that there is the potential for better growing genotypes to replace genotypes with lower growth rates when 2,4-D is applied. Chapter 2 provides a case study of selection acting on a lake population of watermilfoil as a result of herbicide application, providing evidence for the selection component of the breeder's equation, " s ". It is not surprising that I have found heritable variation for growth and 2,4-D sensitivity, at a broad scale, since watermilfoil exhibits a substantial amount of molecular variation present within and among lakes (Zuellig and Thum 2012), but there have been no previous studies suggesting variation in phenotype is related to genotype until the hybrid study in Chapter 1. Also, it is not surprising to see that herbicide application can act as a selection agent since this is well known in agriculture; however, up to this point, there have been no studies suggesting that the same can occur in a lake system, until the case study in Chapter 2. Although these were two separate studies, viewing them holistically provides a compelling argument the raw material is available in natural populations for herbicide resistance evolution.

Given that these studies suggest resistance evolution can occur among populations of invasive watermilfoil, aquatic plant managers should consider the possibility of evolutionary impacts when developing and implementing control programs. Controlling these invasive plant populations may not be as straightforward as increasing herbicide rates. We, as a scientific community, need to continue to learn more about the evolutionary effect of treatment on these

populations, which means studies conducting pre- and post-treatment monitoring for selection and a more thorough understanding of the heritable variation for growth and herbicide sensitivity present in natural populations are needed. The next step would then be to determine which genes lead to specific phenotypes. These two studies are just the beginning for providing evidence of the potential for watermilfoil evolution in response to management programs, and there is still much to learn about how best to address control issues.

More broadly, the two studies reported here show the potential for invasive species evolution in response to management in a non-agricultural, wild lands system, which has not been thoroughly studied in aquatic organisms (see Michel et al. 2004; Thum et al. 2012). These studies provide compelling evidence that invasive species, writ large, have the potential to evolve in response to the management efforts used to control them. We now need to learn more and adjust our own management practices to lessen this problem.

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