HERBIVORE RESISTANCE IN INVASIVE AND NATIVE

MYRIOPHYLLUM SPICATUM AND *MYRIOPHYLLUM HETEROPHYLLUM*

A Thesis

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ABSTRACT

Invasive plant species are increasing worldwide and often have significant impacts on local ecosystems. Many attempts have been made to use augmented herbivore populations as a biological control of nuisance populations, however, the success of these efforts has been variable. One character which can affect the success of these biological controls is the expression of plant herbivore resistance. In this study I examined inducible and constitutive herbivore resistance in native and invasive samples of the aquatic angiosperms, *Myriophyllum spicatum* and *Myriophyllum heterophyllum*.

M. spicatum is native in Europe, Asia and north Africa and invasive in much of North America. When fed on by the ecologically significant herbivore, *Acentria ephemerella, M. spicatum* plant tips changed from green to red with a simultaneous structural change from closed upward pointing leaves to open drooping leaves. A similar response was observed in this study when native and invasive *M. spicatum* plant tips were exposed to jasmonic acid, a plant hormone known to stimulate the expression of plant defensive genes. *A. ephemerella* fed plant tips exposed to jasmonic acid had reduced growth compared to individuals fed unexposed plant tips indicating the induction of herbivore resistance. Not all of the herbivore resistant traits we examined, however, were induced by jasmonic acid. Total phenolics and tellimagrandin II concentrations were not affected by jasmonic acid and changes in nitrogen concentrations were inconsistent. Tissue reddening, in part caused by decreased chlorophyll and increased anthocyanin concentrations, was consistently induced and may affect the visibility of herbivores in the field. However, this trait can not explain the reduced herbivore growth so it is likely that other herbivore-resistant traits, not examined in this study, were induced in *M. spicatum*.

M. heterophyllum is native to North America (except New England) and has invaded many New England and European lake ecosystems. When exposed to jasmonic acid in this study, concentrations of total phenolics, and an unknown phenolic compound were increased and nitrogen concentrations were decreased. These induced plant tips reduced the growth of feeding *A. ephemerella*. This response, however, was only observed in German (invasive) *M. heterophyllum* and not in samples from Maine (invasive) or New York (native). The constitutive expression of traits presumed to confer herbivore resistance was also significantly different among invasive and native populations. Maine had significantly higher concentrations of total phenolics and the unknown phenolic was only present in German *M. heterophyllum*. These differences in constitutive and inducible herbivore resistance among native and invasive *M. heterophyllum* populations may be a result of rapid evolution of invasive populations or their hybridization with native *Myriophyllum* species.

The constitutive and inducible herbivore resistance of *M. spicatum* and *M. heterophyllum* shown in this study would have a negative impact on biological control species which has not previously been considered by lake managers. Furthermore, differences in the expression of herbivore resistance among invasive and native populations of *M. heterophyllum* may partially explain the variable success of this invasive species.

BIOGRAPHICAL SKETCH

Alene Onion was raised on the shores of Flying Pond in Vienna, ME. Here she developed her interests in aquatic ecosystems, viola and cross-country skiing with her parents, brother and sisters. She carried these interests through her years of undergraduate study and, in 2002, she received her Bachelor's of Science with honors in Biology - Ecology and Evolution from Cornell University. Advised by Paul Feeny and Nelson Hairston Jr., her honors thesis examined the inducibility of micrcystin in the cyanobacteria species, *Microcystis aeuriginosa*. Thanks to the advice of Nelson Hairston Jr. and funding from the German government, she then began her Master's research in Konstanz, Germany with Elisabeth Gross. While in Konstanz she learned to respect both Eurasian watermilfoil and the Swiss Alps. Although she enjoyed this experience, she gratefully returned to her friends and colleagues at Cornell University for her second and final year. After graduation she will sorely miss Ithaca's gorges and the friends she leaves behind.

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Chapter I

HERBIVORE RESISTANCE INDUCED BY JASMONIC ACID IN

Myriophyllum spicatum

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Abstract—Few studies have investigated induced responses to herbivory in aquatic angiosperms despite the fact that herbivore damage and the expression of plant defenses are comparable to terrestrial systems. *Myriophyllum spicatum* is a submersed aquatic angiosperm native to Europe, Asia and north Africa and invasive in many North American lakes. When fed upon by aquatic larvae of the lepidopteran moth, *Acentria ephemerella,* plant tips frequently change from green to red with a simultaneous structural change from closed, upward pointing leaves to open, drooping leaves. In this study, these changes were induced in native (German) and invasive (New York) populations of *M. spicatum* by exposure to jasmonic acid, a hormone known to stimulate the production of herbivore defenses in terrestrial plant and macroalgae species. *A. ephemerella* growth rates were significantly reduced by this induced response indicating the induction of herbivore resistance. Not all of the potential resistant traits we examined, however, were induced. Total phenolics and the specific polyphenol, tellimagrandin II, were unaffected by jasmonic acid. Nitrogen concentrations were reduced in *M. spicatum* from native but not invasive cultures. Tissue reddening, attributable to higher anthocyanin and lower chlorophyll concentrations, was induced and would make herbivores more visible to fish predators. This trait, however, would not affect herbivore growth so it is likely that other resistant traits are affected by jasmonic acid that we have not examined. Comparison of red and green plant tips collected from a pond in which *A. ephemerella* was present as a major herbivore indicated that exposure to high irradiance may, apart from herbivory by Acentria or jasmonic acid, induce red tips. Finally, observations from our growth studies indicated that *M. spicatum* may respond specifically to younger *A. ephemerella* larvae.

INTRODUCTION

Many plants are able to identify herbivory and respond to it by producing appropriate resistant or defensive traits. Such induced responses are thought to be beneficial because plants avoid production costs when no herbivore is present (for a review, see Karban and Baldwin, 1997 or Tollrian and Harvell, 1999). Over 100 examples of herbivore-induced responses have been observed in terrestrial vascular plants (Karban and Baldwin, 1997). In aquatic ecosystems, investigations have focused primarily on phytoplankton species (Rengefors et al., 1998; Van Donk et al., 1999; Luerling and Van Donk, 2000) and marine macroalgae (Peckol et al., 1996; Pavia and Toth, 2000; Amsler, 2001; Arnold et al., 2001). Fewer studies have examined aquatic angiosperms (Jeffries, 1990; Bolser and Hay, 1998) and none of these have identified the specific defenses induced. Aquatic angiosperms suffer herbivore attacks comparable to terrestrial systems (Cyr and Pace, 1993) and express a number of resistant and defensive characters (Newman et al., 1996; Bolser et al., 1998; Wilson et al., 1999; Kubanek et al., 2001; Choi et al., 2002) any of which is potentially inducible.

Myriophyllum spicatum (Halorgaceae)*,* commonly known as Eurasian watermilfoil, is a submersed aquatic angiosperm. It is a ubiquitous species native to Europe, Asia, and north Africa and invasive in 45 U.S. states and three Canadian provinces (Newman, 2004). In its invaded habitat, it often forms dense mats that both inhibit native macrophyte growth and interfere with the recreational use of lakes (Johnson and Blossey, 2002). Because of this, *M. spicatum* is considered a nuisance invasive species.

When fed on by aquatic larvae of the lepidopteran moth, *Acentria ephemerella* [formerly *A. nivea* Olivier, see Passoa (1988) or *Acentropus niveus* (Berg, 1942)]*, M. spicatum* plant tips sometimes change from green to red with a simultaneous structural

change from closed, upward pointing leaves to open, drooping leaves (Figure 1.1A; R.L. Johnson, personal communication). This induced response may reflect an induction of resistant or defensive characters. *A. ephemerella* is a generalist herbivore native to Europe and invasive in North America (Sheppard, 1945; Johnson and Blossey, 2002). It is found feeding on *M. spicatum* in both its native and invaded habitats (Gross et al., 2002; Johnson and Blossey, 2002) and has had a significant impact on invasive *M. spicatum* populations (Painter and McCabe, 1988; Johnson et al., 1998; Johnson et al., 2000; Gross et al., 2001). Studies are now examining augmented *A. ephemerella* populations as a potential biocontrol of nuisance *M. spicatum* beds (R.L. Johnson, personal communication). Since induced resistant or defensive characters would affect the success of this biological control, it is important that we understand this plant's induced response.

A number of traits are potentially inducible in *M. spicatum* which may confer herbivore resistance. The leaves and meristems of *M. spicatum* contain high concentrations of total phenolics, the most abundant of which is the hydrolysable polyphenol, tellimagrandin II (1,2,3-tri-*O*-galloyl-6 (S)-hexahydroxydiphenoyl-*ß*-D-glucose; Gross et al., 1996). Previous investigations have shown that these compounds inhibit gut bacteria isolated from *A. ephemerella* (Walenciak et al., 2002), and that *A. ephemerella* fed *M. spicatum* had reduced growth compared with individuals fed *Potamogeton perfoliatus* which contains no hydrolysable tannins and only 10% of Folin-sensitive phenolics compared to *M. spicatum* (Choi et al., 2002). Nutritive quality, specifically nitrogen availability, may also be affected by the induced response. Reduced nitrogen has been shown to have a negative impact on the growth (e.g., Feeny, 1970; Moran and Hamilton, 1980; Schroeder, 1986) and feeding preference (e.g., McNeill and Southwood, 1978; Schroeder, 1986; Athey and Connor, 1989) of terrestrial herbivores. Finally, the color change itself, quantified as changes in

chlorophyll and the red pigment, anthocyanin, may confer herbivore resistance in *M. spicatum* since red plant tips would make feeding herbivores more visible to predators (Archetti, 2000).

The purpose of this study was to determine whether herbivore resistance is induced in *M. spicatum* and to identify which traits are included in this response. To do this we exposed plant tips from both Germany and New York to jasmonic acid (JA), a plant-specific hormone known to stimulate the expression of a number of plant defensive genes (Howe, 1996; Devoto and Turner, 2003). We used JA instead of feeding *A. ephemerella* because we found that the plant response to herbivory was inconsistent. To test the effect this induced response has on feeding herbivores, we fed *A. ephemerella* larvae JA-induced and non-induced plant tips and observing their growth response. We next examined the effect JA had on traits presumed to confer herbivore resistance: total phenolics, tellimagrandin II, nitrogen, and tissue reddening. Finally, in a third experiment, we examined these same characters in red and green tips collected from the field in order to compare the laboratory response to JA with potential induction by herbivory in the natural habitat.

METHODS AND MATERIALS

Plant Cultures Used in Induction Experiments. For the German *M. spicatum* culture, we used an established axenic culture based on plants that had been collected in the Schöhsee, an 82 ha mesotrophic lake in northern Germany. Shoots had been surface-sterilized and cultured using the method developed by Kane et al. (1991) and modified by Gross et al. (1996). Cultures were maintained at 22 ± 1 °C and 36.5 µmol photons $s^{-1}m^{-2}$ light at L14:D10 photoperiod using OSRAM L36W/72 Biolux lamps. For the New York *M. spicatum* culture, root stalks were collected from Marsh 100, Cornell University Experimental Ponds Facility, Ithaca, NY, in the summer of 2003.

After being cleaned of epiphytes and invertebrates, stalks were planted in soil and immersed in 60 cm of lake water. Cultures were kept in a greenhouse at 20 ± 7 °C and with natural light supplemented with greenhouse lamps L14:D10 photoperiod. Although axenic plant samples were preferred because parasite or pathogen attacks could affect the response to JA (Thaler et al., 2002), we did not sterilize New York cultures due to time constraints.

   Inducing the Response with Jasmonic Acid. Plant tips were exposed to JA for four days to induce the anti-herbivore response. To do this, we added JA dissolved in 100% ethanol to empty flasks or jars and left the containers standing for 10 minutes while the ethanol evaporated. After this time, we filled the containers with medium and placed one plant tip in each. As a control, plant tips were held for the same duration of time in clean containers. In experiments examining the inducibility of specific traits, plant tips were also held in containers from which the same volume of ethanol (without JA) had evaporated. This treatment was needed to control for the effect of the solvent. German *M. spicatum* plant tips were three cm long, treated under axenic conditions, and were exposed to 20 µM JA. We used this concentration because it produced the most dramatic healthy response in preliminary experiments (higher concentrations produced an unhealthy response). A standard lake water equivalent medium was used with 2 mM calciumchloride dehydrate, 0.5 mM magnesium heptahydrate, 0.77 mM sodium bicarbonate, and 0.000077 M potassium monochloride in ultrapure water (German industrial norm: DIN EN ISO 7346-3). New York *M. spicatum* plant tips were five cm long, treated under non-axenic conditions, and were exposed to 10 µM JA (chosen for similar reasons as in experiments with German *M. spicatum*). Filtered water (GF/C Whatman 1.2 µm) from the Cornell University experimental ponds was used.

Larval Growth Experiments. To test the effect the induced response had on *A. ephemerella* growth, larvae were fed induced or control plant tips every five days for at least 15 days. To monitor larval growth, head capsule width was measured roughly every five days using digital imaging at a mean precision of 1.5%. For the larval growth experiment using German *M. spicatum* (16-31 May, 2003), we used 18 larvae recently forced out of diapause. Diapausing larvae of *A. ephemerella* were collected from Lake Constance, Germany in September 2002 by snorkeling. To maintain this resting state, we held larvae in the dark at 4°C. Water was refreshed regularly with cooled tap water (piped from Lake Constance with only trace chlorine levels). Immediately before the experiment, we forced the larvae out of diapause by bringing them to 20°C. During the experiment, animals were maintained at 22 ± 1 °C at L14:D10 photoperiod using OSRAM L36W/72 Biolux lamps. For the larval growth experiment using New York *M. spicatum* (November 18 - December 4, 2003), we used 40 freshly hatched larvae from a continuous culture. This culture had been initiated with individuals collected from Marsh 100 at the Cornell University Experimental Ponds Facility, Ithaca, NY in the early summer of 2003 and was maintained in a greenhouse on a mixture of *Ceratophyllum demersum* and *M. spicatum*. During the experiment, animals were held in a greenhouse under a L14:D10 photoperiod (36.5 µmol photons $s^{-1}m^{-2}$) with temperatures varying between 18 °C at night and 27 °C on sunny days.

Testing the Inducibility of Herbivore Resistant Traits. Concentrations of total phenolics, tellimagrandin II, anthocyanin and chlorophyll were compared among induced, control, and solvent control plant tips from both German and New York cultures. Nitrogen concentrations were only compared between induced and control treatments. The experiment using German *M. spicatum* was repeated three times (23-27 March, 2003; 3-7 August, 2003; 12-16 January, 2004). In the first experiment, we

had five replicates (with two plant tips per replicate), nitrogen was not measured, and chlorophyll measurements were only taken from four of the five replicates; in the second, we used four replicates (with five plant tips per replicate); in the last, we had ten replicates (two plant tips per replicate) of induced and control treatments to measure nitrogen concentrations only. The experiment using New York *M. spicatum* was performed once (10-14 December, 2003) with ten replicates (one plant tip per replicate).

Comparison of Red and Green Field Samples. To compare our results using jasmonic acid with what may be induced by herbivory, red and green *M. spicatum* plant tips were collected throughout Deisendorfer pond, Germany (47°42'N, 9°10'E) on June 18, 2003. Plant tips were three cm long and five replicates of each color (two plant tips per replicate) were chemically analyzed.

Chemical Analyses. Immediately at the end of each induction experiment and immediately after field samples were brought back to the laboratory, plant tips were washed with tap water and blotted dry before being shock frozen with liquid nitrogen, freeze dried and finely ground. Each sample was then divided into triplicate subsamples for phenolic, anthocyanin, and chlorophyll analyses. For the control and JA-exposed treatments, a fourth subsample was analyzed for nitrogen concentrations. We decided to analyze these compounds separately and not simultaneously because the small size of our samples required accurate measurements.

We measured concentrations of total phenolics using the Folin-Ciocalteau assay with tannic acid (Sigma) as the standard (Gross et al., 1996). Tellimagrandin II was measured with high pressure liquid chromatography using a method modified from that developed by Gross et al. (1996). Our only modification was to lengthen the first leg of the elution profile by 20 minutes to improve separation of the compounds. Nitrogen concentrations were determined by an Elementar Analysator NCS 2500 (CE

Instruments / Thermoquest) with atropine sulfate as a standard (Choi et al., 2002). To quantify the anthocyanin found in *M. spicatum,* cyanidin glycoside (Hegnauer, 1966), we used a method described by Murray and Hackett (1991), modified for small samples. Samples were extracted with aqueous methanol-HCl (0.1% HCl, v/v) and absorbance was measured at 532 nm and 653 nm using a Beckman Cary 50 spectrophotometer. To quantify chlorophyll *a* and *b* concentrations, we extracted samples using an 80% acetone solution and read absorbance at 663.6 nm and 646.6 nm (Porra, 2002). In the following, results for chl *a* and *b* are expressed as chlorophyll.

Statistical Analyses. Initial head capsule sizes and growth rates of *A. ephemerella* fed induced or control plant tips were compared using one-way ANOVA. Total phenolic, tellimagrandin II, nitrogen, anthocyanin and chlorophyll concentrations were compared among treatments using one-way ANOVA with Tukey's multiple comparison of means ($\alpha_{over\ all} = 0.05$). Because the interaction between experiment date and treatment was not significant for these concentrations (total phenolics: two-way ANOVA, $F_{2,\ 24}$=0.22, p=0.80; anthocyanin: one-way ANOVA, $F_{2,\ 24}$=2.49, p=0.10; nitrogen: $F_{1,\ 30}$=2.33, p=0.14), replicate experiments with German *M. spicatum* were also combined in a two-way ANOVA with Tukey's multiple comparison of means ($\alpha_{over\ all} = 0.05$) to compare concentrations of total phenolics, anthocyanin, and nitrogen among treatments. All statistical analyses were carried out using SAS software (SAS Institute, 1999).

RESULTS

Larvae Growth Experiments. Growth rates were significantly reduced in larvae fed induced *M. spicatum* from either Germany or New York (Figure 1.2; one-way ANOVA; larvae fed German *M. spicatum*: $F_{1,\ 19}$=7.94, p=0.01; larvae fed New York *M. spicatum*: $F_{1,\ 27}$=8.19, p=0.008). This was not a consequence of initial size

differences since head capsule widths were not significantly different at the start of each experiment (Figure 1.2; one-way ANOVA; larvae fed German *M. spicatum*: $F_{1, 19}=1.79$, $p=0.20$; larvae fed New York *M. spicatum*: $F_{1, 27}=0.34$, $p=0.57$). In experiments with New York *M. spicatum*, many larvae fed untreated plants pupated before day 16 which may explain why the slope of the line decreases between the final two dates (Figure 1.2). These high pupation rates were probably due to occasionally high (up to 27 °C) greenhouse temperatures. Also during this experiment, herbivore damaged plant tips visibly changed from green to red while the larvae were less than ten days old. After *A. ephemerella* were older than ten days, no color change was observed in the milfoil. This effect was not observed in feeding experiments with German *M. spicatum,* perhaps because larvae were older than ten days at the start of the experiment.

Inducibility of Herbivore Resistant Traits. With one exception, JA exposure had no effect on concentrations of total phenolics or tellimagrandin II in either German or New York *M. spicatum* (Figure 1.3; total phenolics in German *M. spicatum*, March and August experiments combined: two-way ANOVA, $F_{2, 26}=0.30$, $p=0.74$; total phenolics in German *M. spicatum,* March: one-way ANOVA, $F_{2, 12}=0.13$, $p=0.88$; total phenolics in German *M. spicatum,* August: one-way ANOVA, $F_{2, 12}=0.48$, $p=0.63$; total phenolics in New York *M. spicatum*: one-way ANOVA, $F_{2, 27}=0.78$, $p=0.47$; tellimagrandin II in German *M. spicatum,* March: one-way ANOVA, $F_{2, 12}=0.11$, $p=0.90$; tellimagrandin II in New York *M. spicatum*: one-way ANOVA, $F_{2, 27}=2.96$, $p=0.07$). The one exception was in the August experiment with German *M. spicatum* where significantly lower concentrations of tellimagrandin II were observed in JA-exposed plant tips (Figure 1.3: one-way ANOVA, $F_{2, 12}=9.52$, $p=0.003$).

Nitrogen concentrations (% dry mass) were lower in induced German *M. spicatum*. This difference was highly significant in the December experiment,

marginally significant in the August experiment, and highly significant when the two experiments were combined (Figure 1.3; German *M. spicatum,* August: one-way ANOVA, $F_{1, 13}$=3.32, p=0.09; German *M. spicatum,* December: one-way ANOVA, $F_{1, 17}$=18.64, $p<0.001$; German *M. spicatum,* August and December experiments combined: two-way ANOVA, $F_{1, 31}$=18.64, $p<0.001$). JA exposure had no effect, however, on nitrogen concentrations in New York *M. spicatum* (one-way ANOVA, $F_{1, 16}$=1.28, p=0.27).

Plant tips of both German and New York *M. spicatum* exposed to JA appeared red compared with unexposed plant tips or plant tips exposed to ethanol alone (Figure 1.1B). With one exception, a significant treatment effect was observed in both measurements of anthocyanin and chlorophyll a+b (Figure 1.3; one-way ANOVA ; anthocyanin in German *M. spicatum,* March: $F_{2, 12}$=9.84, p=0.003; anthocyanin in New York *M. spicatum*: $F_{2, 27}$=225.58, $p<0.001$; chlorophyll a+b in German *M. spicatum,* March: $F_{2, 11}$=37.54, $p<0.001$; chlorophyll a+b in German *M. spicatum,* August: $F_{2, 12}$=75.84, $p<0.001$; chlorophyll a+b in New York *M. spicatum*: $F_{2, 27}$=6.9, p=0.004). The one exception was in the anthocyanin concentrations of the August German *M. spicatum* experiment but this result was significant when combined with the other replicate German *M. spicatum* experiment (anthocyanin in German *M. spicatum,* August: one-way ANOVA, $F_{2, 12}$=2.27, p=0.15; anthocyanin German *M. spicatum,* March and August experiments combined: two-way ANOVA, $F_{2, 26}$=8.31, $p<0.001$). A Tukey's multiple comparison of means revealed that these significant differences were attributable to higher anthocyanin concentrations and lower chlorophyll a+b concentrations in JA-exposed plant tips and that the solvent did not have a significant effect (Figure 1.3; $\alpha_{over\ all} = 0.05$). The structure of the apical meristem also changed from closed, upward pointing leaves to open, drooping leaves (Figure 1.1B,C).

Comparison of Red and Green Field Samples. Compared with green tipped *M. spicatum* collected from the field (Figure 1.1C)*,* red plant tips had significantly higher concentrations of total phenolics, tellimagrandin II, and anthocyanin and significantly lower concentrations of chlorophyll a+b and nitrogen than green plant tips (Figure 1.3; one-way ANOVA; total phenolics: $F_{1, 8}$=147.37, $p<0.001$; tellimagrandin II: $F_{1, 8}$=111.11, $p<0.001$; anthocyanin: $F_{1, 8}$=518.12, $p<0.001$; chlorophyll a+b: $F_{1, 8}$=283.83, $p<0.001$; nitrogen: $F_{1, 8}$=18.03, $p=0.001$).

Figure 1.1 A. *M. spicatum* plant tip before (left) and after (right) being fed on by an *Acentria ephemerella* larvae. **B.** German (a) and New York (b) *M. spicatum* plant tips exposed to jasmonic acid (right) and unexposed (left). **C.** Green (left) versus red (right) plant tips collected from *Deisendorfer* pond in southern Germany.

A

Ba

Bb

C
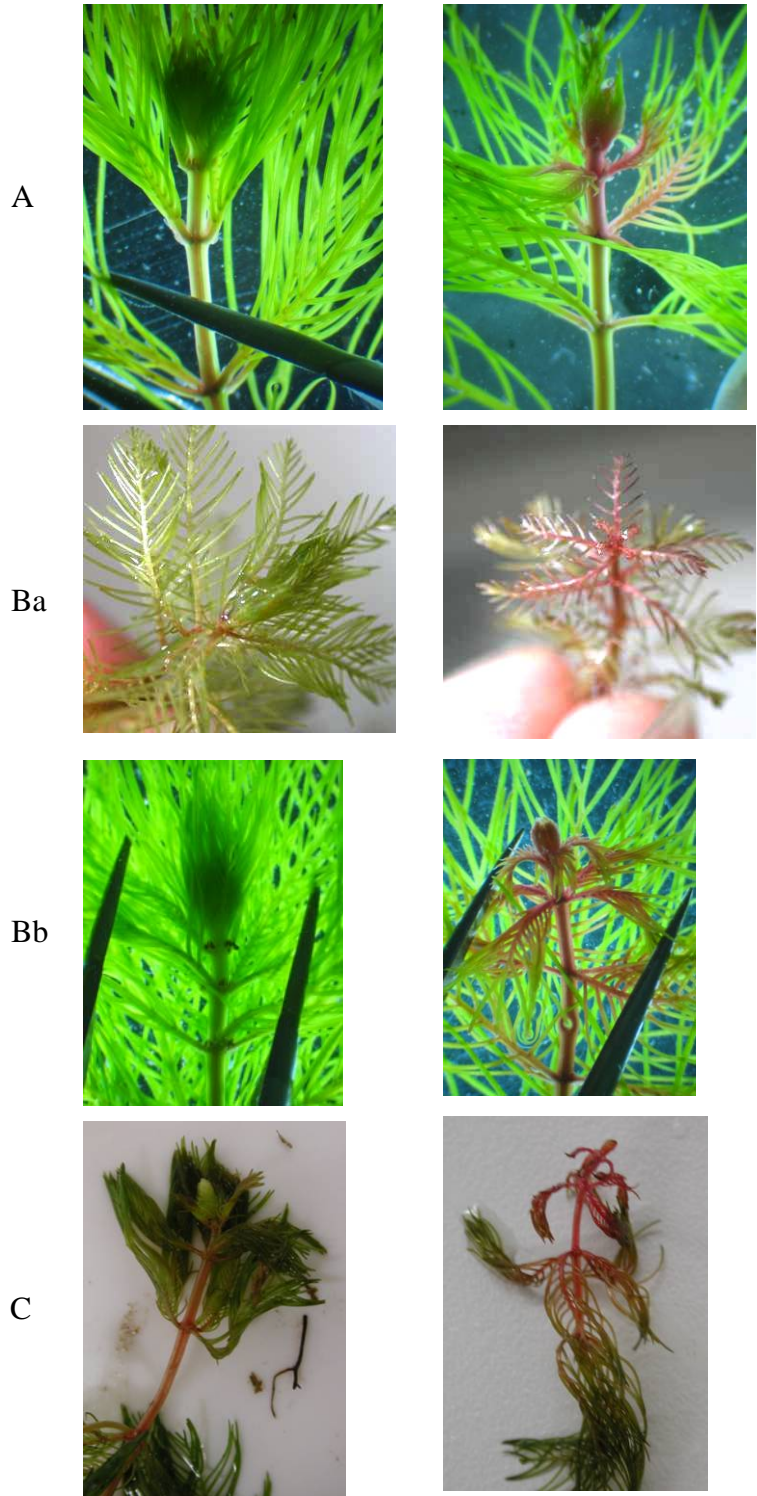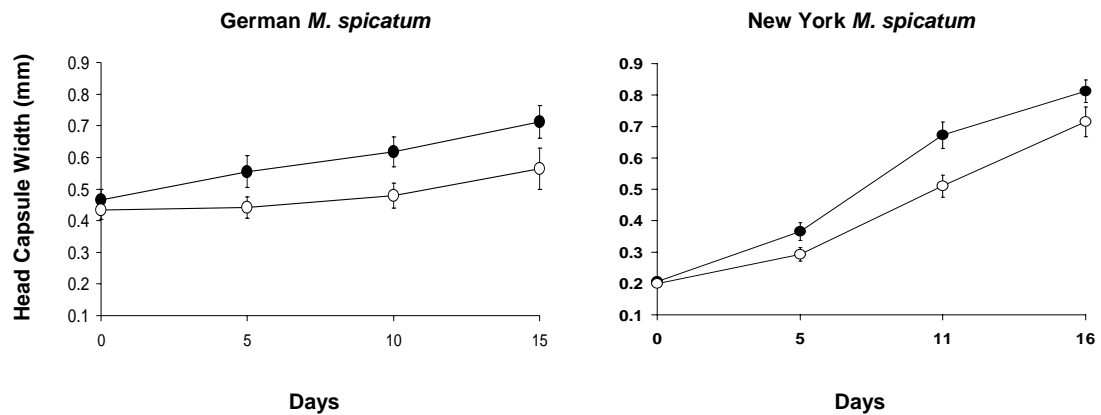
Figure 1.2 Head capsule width (± 95% C.I.) of *Acentria ephemerella* larvae fed German (A) or New York (B) *M. spicatum* plant tips exposed to jasmonic acid (white circles) or unexposed (black circles). Growth rates were significantly different in both experiments (one-way ANOVA, larvae fed German *M. spicatum*: $F_{1, 19}$=7.94, *p*=0.01; larvae fed New York *M. spicatum*: $F_{1, 27}$=8.19, *p*=0.008).

Figure 1.3 Concentrations of anthocyanin, chlorophyll, total phenolics, tellimagrandin II, and nitrogen (± 95% C.I.) in German and New York *M. spicatum* plant tips subjected to 1 of 3 treatments: unmanipulated (black bars), exposed to ethanol alone (grey bars), or exposed to jasmonic acid dissolved in ethanol (hatched bars). Also given are concentrations found in green (black bars) and red (striped bars) plant tips collected from a southern German pond. Letters above the bars indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison of means; $p<0.05$). Letters above the German *M. spicatum* experiments indicate significant differences when these experiments were combined (two-way ANOVA followed by Tukey's multiple comparison; $p<0.05$).
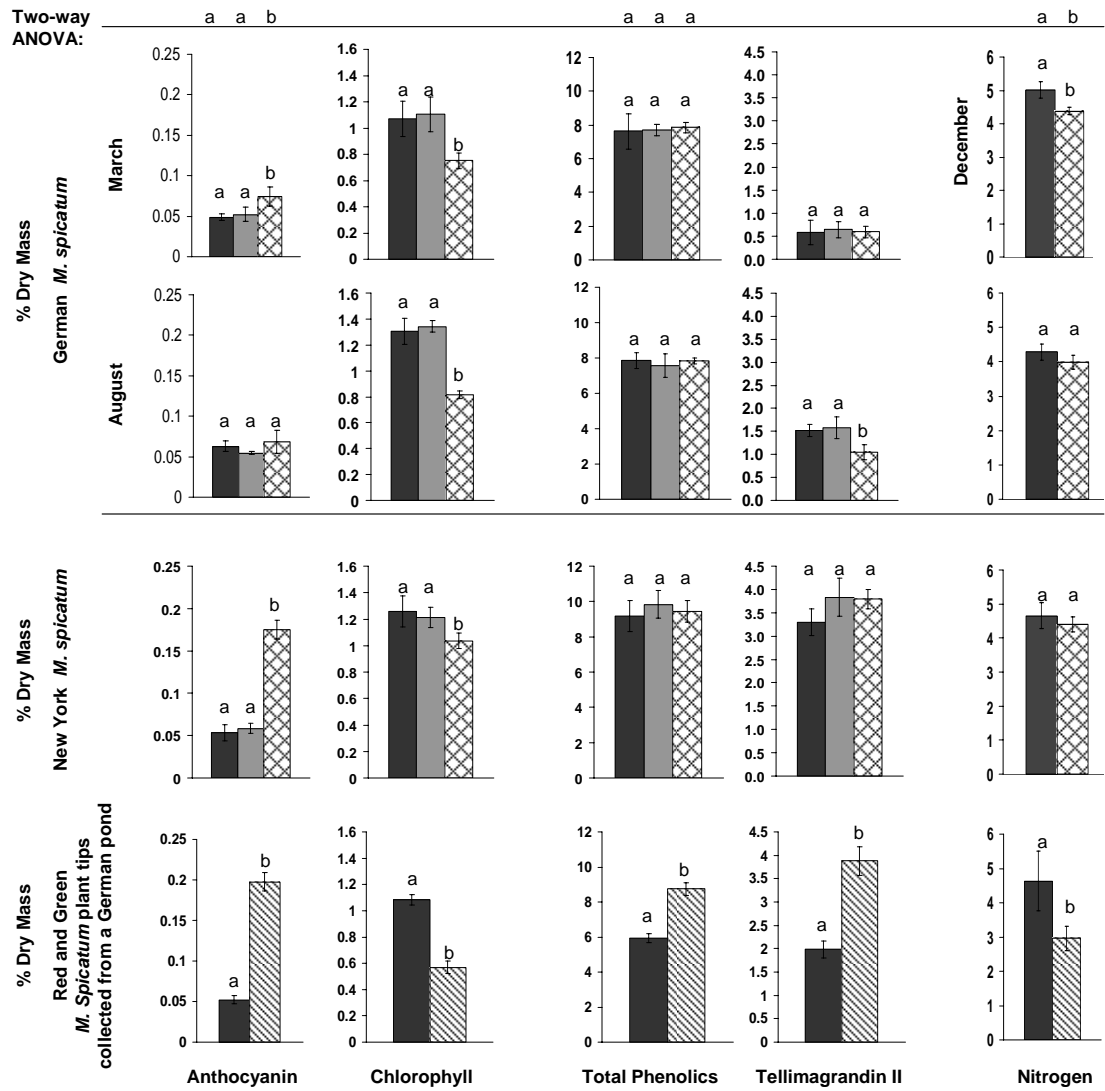
DISCUSSION

Herbivore resistance was induced by JA in *M. spicatum* from both Germany and New York. *A. ephemerella* larvae fed JA-induced plant tips from either source population had reduced growth compared with individuals fed untreated plant tips (Figure 1.2). This may serve as a plant defense because larvae with slower growth rates would be exposed to parasites and predators over a longer period of time and would suffer from increased mortality (Moran and Hamilton, 1980). Also, larvae may be able to detect the induced response and move to neighboring, undefended plants (Feeny, 1970; Moran and Hamilton, 1980; Karban and Baldwin, 1997). Since *M. spicatum* often forms dense interwoven canopies at the water's surface, the herbivore could easily transfer among plant tips.

Not all of the specific traits presumed to confer herbivore resistance were induced by JA in *M. spicatum.* Concentrations of total phenolics and of the specific phenolic, tellimagrandin II were not elevated in JA-exposed plant tips from either Germany or New York (Figure 1.3). This is interesting considering the uncertain effect these compounds have on feeding herbivores. Total phenolics in *M. spicatum* reduce *A. ephemerella* growth (Choi et al., 2002) and both total phenolics and the specific phenolic, tellimagrandin II, inhibit the gut bacteria of this herbivore (Walenciak et al., 2002). However, the anti-microbial activity *M. spicatum* phenolics (Walenciak et al., 2002) would protect herbivores against bacterial or fungal pathogens (Choi et al., 2002). The net impact may be insignificant which would explain why these compounds are not induced in response to JA exposure. A second explanation for the lack of response is that the production of phenolic compounds in *M. spicatum* may not be induced by JA. JA stimulates production of the enzyme phenylalanine ammonia-lyase (PAL), which is the first regulatory enzyme of the phenylpropaoid metabolic pathway (Gundlach, 1991). Gross (1992) hypothesized that

phenolic compounds are produced either by this pathway or by a pathway derived from dishikimic acid. If a product of the latter, phenolic concentrations would not be affected by JA. Finally, it is important to note that concentrations of total phenolics and tellimagrandin II were substantially lower in German *M. spicatum* compared with plants from the New York culture. This was not surprising considering the fact that axenic cultures are known to express significantly lower concentrations of these compounds (Gross, unpubl. results).

Nitrogen availability was inconsistently reduced by JA. A significant reduction was observed in experiments with German *M. spicatum* but not in experiments with New York *M. spicatum* (Figure 1.3). This is surprising considering the fact that chlorophyll, which accounts for much of the nitrogen present in photosynthetic tissues (Lee et al., 2003), was significantly lowered in both experiments (Figure 1.3). It is unlikely that this difference is due to the fact that lower JA concentrations were used with New York *M. spicatum,* since the induction of anthocyanin was more significant in these experiments (Figure 1.3). However, because of the inconsistent response, it is uncertain whether JA induces changes in nitrogen availability. It is also important to note that JA is known to induce tissue senescence as well as defensive traits (He, 2002). Since senescence is correlated with reduced nitrogen concentrations (Chalker-Scott, 1999; Lee et al., 2003), changes in nitrogen concentrations observed in this study may be a result of the senescing property of JA and not an anti-herbivore induced response. Experiments using herbivore inducers are needed to determine the inducibility of this trait.

The color change from green to red of plant tips exposed to JA was consistently observed in experiments with both German and New York *M. spicatum* (Figure 1.1Ba,Bb) and was similar to that we observed in plant tips damaged by young *A. ephemerella* larvae (Figure 1.1A). This color change was caused at least in part by

elevated anthocyanin and lower chlorophyll a+b concentrations (Figure 1.3). The response was visibly and chemically more dramatic in plant tips from New York compared with those from Germany which is most likely due to the fact that German samples were held under lower light levels (Figures 1.1, 1.3).

It may be that feeding herbivores are more visible to fish predators on these induced red plant tips. Predation experiments are needed, however, to confirm that herbivore resistance is conferred by this induced response. Furthermore, it is possible that the induced color acts as a visible signal, attracting visual predators such as bluegill sunfish (P.P. Feeny, personal communication). Bluegills can see in the green - red color range (Hawryshyn et al., 1988) and are known to feed on *A. ephemerella* larvae (R.L. Johnson, personal communication).

It is likely that other herbivore resistant characters, not examined in this study, were induced by JA exposure. This is especially true considering the fact that *A. ephemerella* growth was reduced by German *M. spicatum* exposed to JA whereas traits presumed to affect herbivores directly (total phenolics, tellimagrandin II, and nitrogen) were not consistently affected in these plant tips. A specific phenolic other than tellimagrandin II may have been induced in this experiment; such changes do not always affect total phenolic concentrations (Gross, 2003). Also, leaf tissue sometimes appeared thicker in induced plant tips (Figure 1.1) which may have indicated a change in tissue toughness. Finally, concentrations of protease inhibitors and oxidative enzymes are induced in terrestrial plants (for a review see Constable, 1999) and may also be effected in *M. spicatum.*

The response induced by feeding *A. ephemerella* larvae may differ from that induced by JA. Concentrations of total phenolics and tellimagrandin II were all significantly different between red and green tips collected from the field, but were not consistently induced by JA in the laboratory (Figure 1.3). While collecting *M.*

spicatum in the field, we found fewer red tips on plants growing in the shade of over-hanging trees than in those exposed to full sun. UV radiation has been shown to induce tannin and anthocyanin production (Pavia et al., 1997; Lee et al., 2003) and these compounds are thought to provide photoprotection (Swanson, 2002; Lee et al., 2003). Thus, it may be that sun exposure alone, or sunlight interacting with herbivory, is important in color variation observed in the field.

Herbivore inducers were not used in this study because the plant response to feeding *A. ephemerella* was inconsistent. An effect of larval age may account for some of this variation. During *A. ephemerella* growth experiments with New York cultures, a visible response was induced by herbivores feeding in control plant tips when larvae were zero and five days old but not when they were ten days old. According to the head capsule measurements of Gross et al. (2002), these larvae were respectively in I, III, and V instar size classes. Plants are known to distinguish among herbivore species (Pavia and Toth, 2000; Voeckel and Baldwin, 2004), and, although no one has yet shown that they are able to discriminate among herbivore instars (Van Dam et al., 2001), it is possible that *M. spicatum* induces defenses specifically in response to young larvae. In natural populations, *M. spicatum* suffers continuous attacks from feeding *A. ephemerella* larvae, however hatching egg clutches result in sudden bursts of attacking herbivores. If a plant is able to augment defenses when young larvae become abundant, it would significantly reduce future herbivore damage.

The induced response to herbivory in *M. spicatum* may affect control efforts of nuisance populations. Augmentations of *A. ephemerella* numbers in lakes in New York State have had varying success as a biological control (R.L. Johnson, personal communication). Induced herbivore resistance would negatively impact herbivore growth in a way that has not previously been considered by lake managers.

Furthermore, if *M. spicatum* induces resistant traits specifically against young *A. ephemerella* larvae, augmentations using older insect larvae might be more successful.

In summary, we have demonstrated that herbivore resistance is induced by JA in *M. spicatum* from both Germany and New York. Tissue reddening was induced and may affect herbivore predation, however, the specific traits responsible for reducing herbivore growth remain to be elucidated. Experiments testing other herbivore resistant traits such as polyphenol oxidase and protease inhibitors are needed. It would also be interesting to examine further the effects of herbivore age and solar radiation. These interactions merit further investigation particularly since an understanding of this plant's induced response to herbivory has the potential to improve biological control efforts on nuisance *M. spicatum* populations.

Chapter II

A COMPARISON OF CONSTITUTIVE AND JASMONIC ACID INDUCIBLE

HERBIVORE RESISTANCE AMONG NATIVE AND INVASIVE

POPULATIONS OF THE AQUATIC ANGIOSPERM, *Myriophyllum*

heterophyllum

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Abstract – Many studies have attempted to identify the characters which contribute to the variable success of invasive plant populations. One such character is the differential expression of herbivore resistant traits. *Myriophyllum heterophyllum* is a submersed aquatic angiosperm native to eastern North America (except New England) and has successfully invaded many New England and European lake ecosystems. In this study we compared constitutive and inducible traits presumed to confer herbivore resistance among Maine, German, and New York *M. heterophyllum* populations. Constitutive concentrations of total phenolics were significantly higher in Maine *M. heterophyllum* compared with German or New York samples. Concentrations of an unknown phenolic compound were only detected in German *M. heterophyllum.* Exposing plant tips to jasmonic acid, a plant hormone known to stimulate the expression of herbivore resistant genes, caused reductions in tissue nitrogen concentration and increases in phenolic concentrations (both total phenolics and the above mentioned unknown phenolic compound) only in German *M. heterophyllum.* Decreases in chlorophyll and increases in the red pigment, anthocyanin, were also observed in these plant samples but did not produce a visible color change as has been observed in *M. spicatum.* This response induced by JA in German *M. heterophyllum* was effective at reducing growth of the ecologically significant herbivore, *Acentria ephemerella*. The differential expression of constitutive and inducible herbivore resistant traits observed in this study may be the result of rapid evolution of invasive populations or their hybridization with native *Myriophyllum* species. Regardless of their origin, anti-herbivore traits probably contribute to the differential success of invasive *M. heterophyllum* populations and are significant when considering prevention and control strategies.

INTRODUCTION

The spread of exotic plant species is increasing worldwide and is a primary cause of current biodiversity loss (Vitousek et al., 1997). The success of an invading species, however, is not consistent among populations (Buchan and Padilla, 2000; Huston, 2004; Russel and Lauda, 2004). This is a consequence of both genetic and ecosystem variation between populations (Siemann and Rogers, 2001; Huston, 2004). A comparison of such systems has the potential to reveal the mechanisms underlying the success of an invasive species.

One mechanism for successful establishment that may vary among invasive plant populations is the differential expression of anti-herbivore defenses. Such variation has been documented among native plant populations (Strauss and Agrawal, 1999). Selection pressures on invasive species should differ from those they experience in their native habitats and may result in high variability of anti-herbivore defense expression (Vermeij, 1996; Garcia-Rossi et al., 2003). Examples include invasive populations of salt marsh cordgrass (Garcia-Rossi, 2003) and of rush skeleton weed (Burdon et al., 1981).

In this study we compare constitutive and inducible herbivore resistant traits among a native (New York) and two invasive (German and Maine) populations of the aquatic angiosperm, *Myriophyllum heterophyllum* (variable watermilfoil; Halorgaceae). *M. heterophyllum* is a submersed aquatic plant native to all of the eastern United States except for New England (Les and Mehrhoff, 1999). Within the last 70 years, it has invaded lakes in Connecticut, Rhode Island, Massachusetts, Vermont, New Hampshire and Maine (Les and Mehrhoff, 1999). It has also invaded lakes in Germany, Austria, Switzerland, and Great Britain within the last 60 years (Wimmer, 1997). In New England invaded habitats, *M. heterophyllum* forms dense mats that frustrate boaters and swimmers and are associated with declines in native

macrophyte populations (Sheldon, 1994; Halstead et al., 2003). Interestingly, such problems have not occurred European lakes where populations have become established.

To compare herbivore resistance among these populations, we examined traits presumed to have a negative impact on plant attackers. *Myriophyllum* species with high concentrations of total phenolics have been shown to inhibit herbivore growth (Choi et al., 2002). Reduced nitrogen concentrations in terrestrial plants have a negative impact on herbivore growth (e.g., Feeny, 1970; Moran and Hamilton, 1980; Schroeder, 1986) and feeding preference (e.g., McNeill and Southwood, 1978; Schroeder, 1986; Athey and Connor, 1989). Finally, an inducible green to red color change quantified as reduced chlorophyll and augmented anthocyanin concentrations, has been observed in the congeneric and sometimes sympatric milfoil species, *M. spicatum,* and is thought to make herbivores more vulnerable to visible predators (Chapter 1).

In this study, we compared constitutive concentrations of total phenolics and inducible concentrations of total phenolics, nitrogen, anthocyanin and chlorophyll among New York, Maine and German *M. heterophyllum.* To induce *M. heterophyllum*, we exposed plant tips to jasmonic acid (JA), a plant specific hormone known to stimulate the transcription of a wide range of plant defensive genes (Devoto and Turner, 2003; Howe, 1996). When an induced response was observed, we tested the effect these changes had on a feeding herbivore.

METHODS

Plant Cultures. For German *M. heterophyllum*, we used an established axenic culture based on plants that had been collected from a pond on the University of Constance campus in Constance, Germany. Shoots had been surface-sterilized and

cultured using the method developed by Kane et al. (1991) and modified by Gross et al. (1996). Cultures were maintained at 22 ± 1 °C and 36.5 µmol photons $s^{-1}m^{-2}$ light at L14:D10 photoperiod using OSRAM L36W/72 Biolux lamps. For the New York cultures, root stalks were collected from both Jenkins Bay in Tupper Lake, Franklin County in the Adirondack Mountains and from Canaan Lake, Suffolk County near the Long Island Atlantic coast. For the Maine culture, root stalks were collected from Lake Auburn, Auburn. After being cleaned of epiphytes and invertebrates, Maine and New York samples were planted in soil and immersed in 60 cm of lake water. Cultures were kept in a greenhouse at 20 ± 7 °C and with natural light supplemented with greenhouse lamps at L14:D10 photoperiod. Although axenic plant samples were preferred because parasite or pathogen attacks could affect the response to JA (Thaler et al., 2002), we did not sterilize New York or Maine cultures due to time constraints.

 Jasmonic Acid Exposure. Plant tips were exposed to JA for four days to induce the anti-herbivore response. To do this, we added JA dissolved in 100% ethanol to empty flasks or jars and left the containers standing for 10 minutes while the ethanol evaporated. After this time, we filled the containers with medium and placed one plant tip in each. As a control, plant tips were held for the same duration of time in clean containers. In experiments examining the inducibility of specific traits, plant tips were also held in containers from which the same volume of ethanol (without JA) had evaporated. This treatment was needed to rule out the effect of the solvent. The German *M. heterophyllum* plant tips were three cm long, treated under axenic conditions, and were exposed to 20 µM JA. We used this concentration because it produced the most dramatic healthy response in preliminary experiments (higher concentrations produced an unhealthy response). A standard lake water equivalent medium was used with 2 mM calciumchloride dehydrate, 0.5 mM magnesium heptahydrate, 0.77 mM sodium bicarbonate, and 77 µM potassium monochloride in

ultrapure water (German industrial norm: DIN EN ISO 7346-3). The New York and Maine *M. heterophyllum* plant tips were five cm long, treated under non-axenic conditions, and were exposed to 10 µM JA (chosen for similar reasons as in experiments with German *M. spicatum*). Filtered water (GF/C Whatman 1.2 µm) from the Cornell University experimental ponds was used as medium.

   Comparing Constitutive and Inducible Traits. To examine the inducibility of traits presumed to confer herbivore resistance in German, Maine, New York-Adirondack, and New York-Long Island *M. heterophyllum,* plant tips were exposed to jasmonic acid using the methods described above; the constitutive expression of total phenolics was determined by examining concentrations in untreated-control plant tips. German *M. heterophyllum* was exposed to JA in three successive experiments on 21-25 June, 2003, 3-7 August, 2003, and 12-16 January, 2004. In the first experiment, we had five, four, and three replicates (with two plant tips per replicate) for the JA, ethanol-control and no-solvent-control treatments respectively. In the second experiment, we used four replicates (with five plant tips per replicate). In the last, we had ten replicates (with two plant tips per replicate) of only JA and control treatments. In this last experiment we measured only nitrogen concentrations. New York-Adirondack, New York-Long Island, and Maine *M. heterophyllum* were exposed to JA once (10-14 December, 2003) with five replicates (one plant tip per replicate).

   Chemical Analyses. Immediately at the end of each JA-exposure experiment, plant tips were washed with tap water and blotted dry before being shock frozen with liquid nitrogen, freeze dried and finely ground. Each sample was then divided into triplicate subsamples for phenolic, anthocyanin, and chlorophyll analyses. For the control and JA-exposed treatments a fourth subsample was analyzed for nitrogen concentrations. We analyzed these compounds separately and not simultaneously because the small size of our samples required accurate measurements.

To quantify the anthocyanin found in *Myriophyllum* sp.*,* cyanidin glycoside (Hegnauer, 1966), we used a method described by Murray and Hackett (1991), modified for small samples. Samples were extracted with aqueous methanol-HCl (0.1% HCl, v/v) and absorbance was measured at 532 nm and 653 nm. To quantify chlorophyll *a* and *b* concentrations, we extracted samples using an 80% acetone solution and read absorbance at 663.6 nm and 646.6 nm (Porra, 2002). The content of chlorophyll a and *b* is in the following expressed as 'chlorophyll'. All absorbances were read using a Beckman Cary 50 spectrophotometer. Total phenolics were measured using the Folin-Ciocalteau assay with tannic acid (Sigma) as the standard (Gross et al., 1996) and nitrogen concentrations were determined using an Elementar Analysator NCS 2500 (CE Instruments / Thermoquest) with atropine sulfate as a standard (Choi et al., 2002).

In a qualitative analysis of German *M. heterophyllum,* we also identified an unknown phenolic compound whose concentrations were affected by JA exposure. This unknown phenolic was resolved at 15.8 minutes using a high pressure liquid chromatography method modified from that developed by Gross et al. (1996) for the analysis of *M. spicatum* phenolic compounds. Our only modification was to lengthen the first leg of the elution profile by 20 minutes to improve separation of the compounds.

was quantified as peak area per gram dried sample. Interestingly, no such peak was observed in analyses of Maine or New York *M. heterophyllum.*

Larval Growth Experiment. To test the effect the induced response in German *M. heterophyllum* had on herbivore growth, JA treated and control plant tips were fed to aquatic larvae of the lepidopteran moth, *Acentria ephemerella* [formerly *A. nivea* Olivier, see Passoa (1988) or *Acentropus niveus* (Berg, 1942)]. This generalist herbivore is native to Europe and invasive in many lakes of northeastern North

America (Sheppard, 1945; Johnson, 2000). In lakes where they co-occur, *A. ephemerella* has been observed feeding on *M. heterophyllum* (R.L. Johnson, personal communications). In this experiment, we used *A. ephemerella* recently forced out of diapause. Diapausing larvae were collected from Lake Constance, Germany in September 2002 by snorkeling. To maintain this resting state, we held the insects in the dark at 4°C. Water was refreshed regularly with cooled tap water (piped from Lake Constance with only trace chlorine levels). Immediately before the experiment, larvae were brought to room temperature to force them out of diapause. 26 individuals were then fed induced or control plant tips every five days for 15 days. To monitor larval growth, head capsule width was measured every five days using digital imaging at a mean precision of 1.5%. During the experiment, animals were maintained at 22 ± 1 °C at L14:D10 photoperiod using OSRAM L36W/72 Biolux lamps.

Statistical Analyses. Constitutive concentrations of total phenolics were compared among source populations using one-way ANOVA with Tukey's multiple comparison of means ($\alpha_{over\ all} = 0.05$). To examine the inducibility of total phenolics, the unknown phenolic, nitrogen, anthocyanin and chlorophyll, we compared concentrations among JA, solvent control, and control treatments using one-way ANOVA with Tukey's multiple comparison of means ($\alpha_{over\ all} = 0.05$). Initial head capsule sizes and growth rates of *A. ephemerella* fed induced or control plant tips were compared using one-way ANOVA. All statistical analyses were carried out using SAS software (SAS Institute, 1999).

RESULTS

Comparison of Constitutive Total Phenolics. Total phenolic concentrations in untreated-control *M. heterophyllum* plant tips were significantly different among source populations (Figure 2.1; one-way ANOVA, $F_{4,\ 17} = 75.2$, $p<0.001$). A Tukey's

multiple comparison of means ($\alpha_{\text{over all}}$=0.05) revealed that concentrations were highest in Maine *M. heterophyllum,* lowest in German *M. heterophyllum,* and not significantly different between New York populations. Replicate measurements from German *M. heterophyllum* were also not significantly different (Tukey's; $\alpha_{\text{over all}}$=0.05).

Comparison of Inducible Traits. No visible response was induced by JA in *M. heterophyllum* from any source population (Figure 2.2). In experiments with German invasive *M. heterophyllum,* however, a significant treatment effect was observed in concentrations of total phenolics, the unknown phenolic compound, anthocyanin, chlorophyll, and nitrogen (Figure 2.1; one-way ANOVA; June total phenolics: $F_{2, 9}$=31, p<0.001; August total phenolics: $F_{2, 9}$=66, p<0.001; June unknown phenolic: $F_{2, 9}$=300, p<0.001; August unknown phenolic: $F_{2, 9}$=305, p<0.001; June anthocyanin: $F_{2, 9}$=170, p<0.001; August anthocyanin: $F_{2, 9}$=15, p=0.003; June chlorophyll: $F_{2, 9}$=144 p<0.001; August chlorophyll: $F_{2, 9}$=25, p<0.001; August nitrogen: $F_{1, 13}$=6.4, p=0.025; December nitrogen: $F_{1, 17}$=26, p<0.001). A Tukey's multiple comparison of means revealed that these differences were attributable to higher anthocyanin, lower chlorophyll, lower nitrogen, higher total phenolic, and higher unknown phenolic concentrations in the JA-exposed plant tips (Figure 2.1; Tukey's $\alpha_{\text{over all}}$=0.05). With two exceptions, the solvent did not have a significant effect (Figure 2.1; Tukey's $\alpha_{\text{over all}}$=0.05). The first exception was a significant increase in total phenolic concentrations in the June experiment. However, these concentrations were still significantly lower than those found in JA-exposed plant tips. The second exception was a significant increase in anthocyanin concentrations in the August experiment (Figure 2.1; Tukey's $\alpha_{\text{over all}}$=0.05).

In experiments with Maine and New York *M. heterophyllum,* treatments did not have a significant effect on anthocyanin concentrations (Figure 2.1; one-way

ANOVA; Maine: $F_{2, 12}$=0.1, p=0.9; New York-Adirondack: $F_{2, 12}$=0.44, p=0.66; New York-Long Island: $F_{2, 12}$=2.6, p=0.12). Although significant treatment effects were observed in chlorophyll and total phenolic concentrations, with one exception, none of these treatment effects were attributable to differences between the control and JA-exposed plant tips (Figure 2.1; one-way ANOVA with Tukey's multiple comparison of means $\alpha_{\text{over all}}$=0.05; Maine chlorophyll: $F_{2, 12}$=8.8, p=0.005; New York-Adirondack chlorophyll: $F_{2, 12}$=18, p<0.001; New York-Long Island chlorophyll: $F_{2, 12}$=1.6, p=0.24; Maine total phenolics: $F_{2, 12}$=2.1, p=0.16; New York-Adirondack total phenolics: $F_{2, 12}$=5.6, p=0.02; New York-Long Island total phenolics: $F_{2, 12}$=2.7, p=0.11). The one exception was that chlorophyll concentrations were significantly lower in JA-exposed plant tips of the experiment with New York-Adirondack *M. heterophyllum*. Finally, nitrogen concentrations were significantly higher in JA-exposed New York-Adirondack *M. heterophyllum* (Figure 2.1; one-way ANOVA; $F_{1, 8}$=12, p=0.008).

Larval Growth Experiment. Growth rates were significantly reduced in larvae fed induced German *M. heterophyllum* (Figure 2.3; one-way ANOVA; $F_{1, 23}$=16.1, p<0.001). This was not a consequence of initial size differences since head capsule widths were not significantly different at the start of each experiment (Figure 2.3; one-way ANOVA; $F_{1, 24}$=0.06, p=0.81).

Figure 2.1 Concentrations of anthocyanin, chlorophyll, total phenolics, tellimagrandin II, and nitrogen (± 95% C.I.) in German, Maine, and New York *Myriophyllum heterophyllum* plant tips subjected to 1 of 3 treatments: unmanipulated (black bars), exposed to ethanol alone (grey bars), or exposed to jasmonic acid dissolved in ethanol (hatched bars). Letters above the bars indicate significant differences (one-way ANOVA followed by Tukey's multiple comparison of means; $p<0.05$).
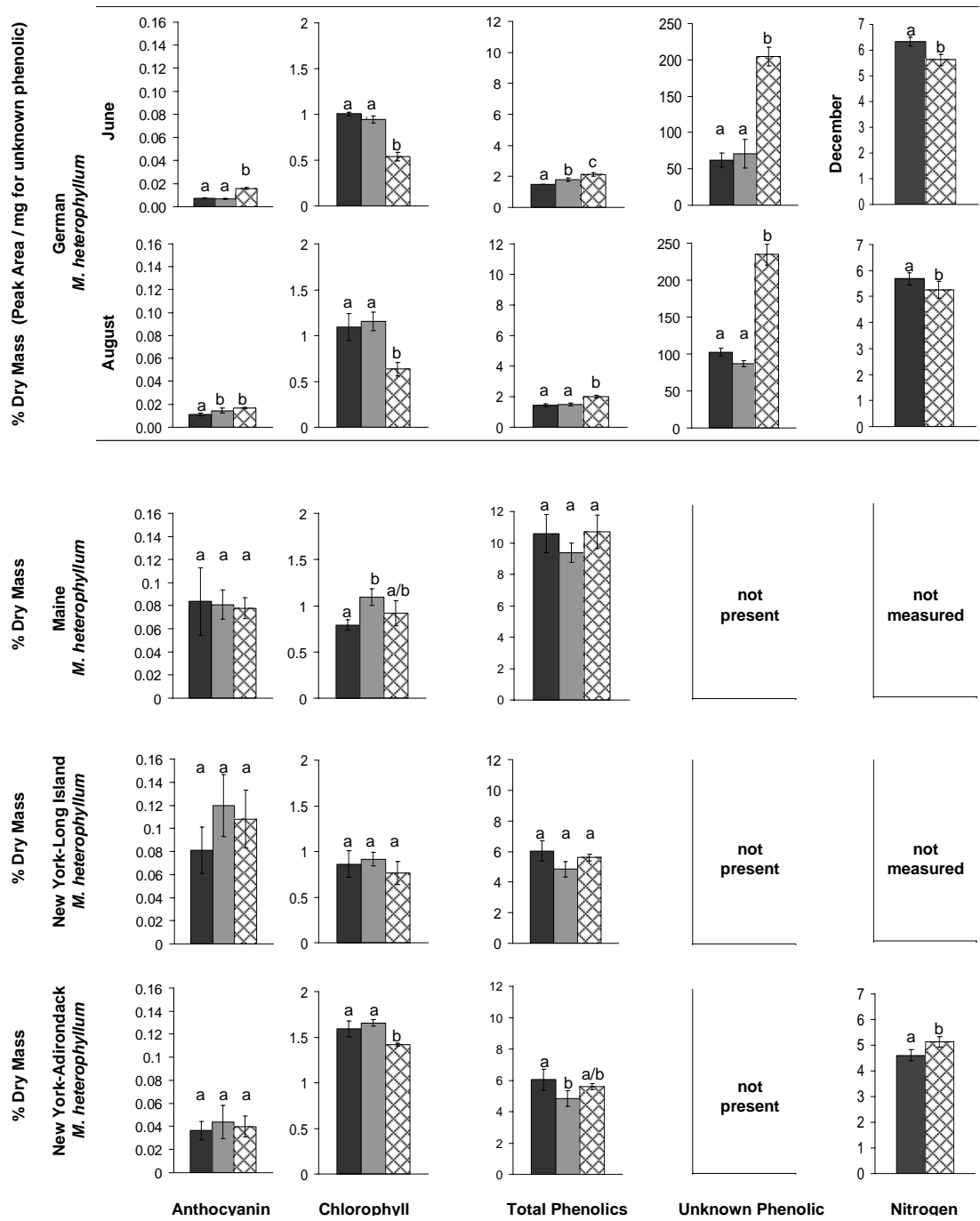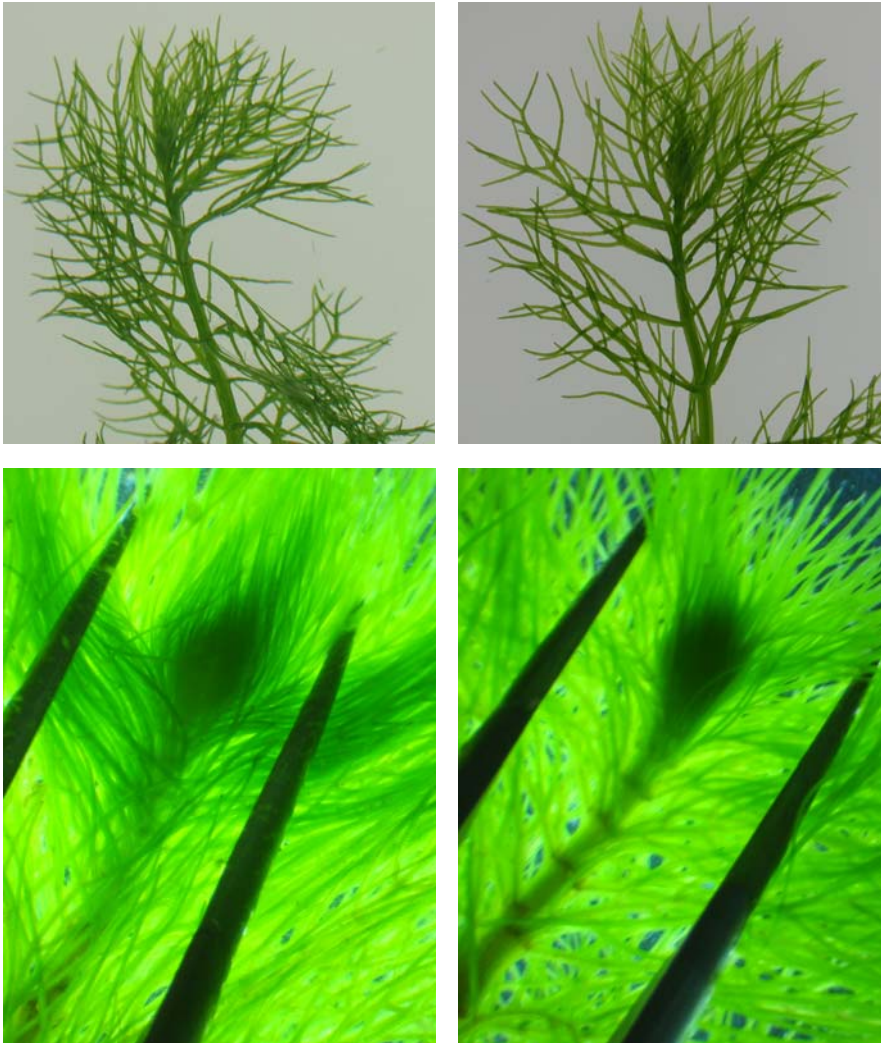
Figure 2.2 German (top) and New York-Adirondack (bottom) *Myriophyllum heterophyllum* plant tips exposed to jasmonic acid (right) and not exposed (left). The forceps seen in some photos were used to spread the leaves.
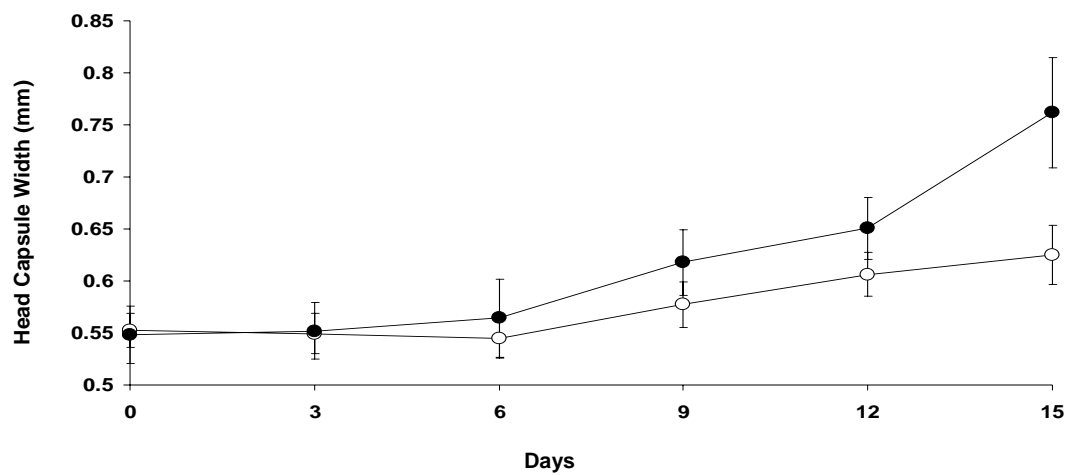
Figure 2.3 Head capsule width ($\pm$ 95% C.I.) of *Acentria ephemerella* larvae fed German *M. heterophyllum* plant tips exposed to jasmonic acid (white circles) or unexposed (black circles). Growth rates were significantly different in both experiments (one-way ANOVA; $F_{1,23}$=16.1, p<0.001).

DISCUSSION

Constitutive and inducible traits presumed to confer herbivore resistance were significantly different among New York, Maine, and German *M. heterophyllum* populations. Constitutive concentrations of total phenolic compounds were highest in the Maine *M. heterophyllum* population, lowest in the German population and not significantly different between the two New York populations (Figure 2.1). Total phenolics may not, however, actually differ between German and New York populations in nature since axenic *Myriophyllum* cultures generally have lower total phenolic concentrations than non-axenic samples (Gross, unpubl. results). Also, previously published concentrations measured in non-axenic samples from our German source population are within the range found in New York *M. heterophyllum* (Choi et al., 2002). Constitutive expression of the unknown phenolic was also significantly different among source populations because we were only able to detect concentrations of this compound using high pressure liquid chromatography in German *M. heterophyllum* and not in samples from New York or Maine. Since *Myriophyllum* species with higher phenolic concentrations have been shown to reduce the growth of feeding *A. ephemerella* (Choi et al., 2002), variation in constitutive phenolic concentrations may affect herbivore resistances in these different source populations

A JA-induced response was observed in German *M. heterophyllum* for which concentrations of anthocyanin, chlorophyll, nitrogen, total phenolics, and of the unknown phenolic were significantly different between JA-exposed and unexposed plant tips (Figure 2.1). This response was unlikely a result of exposure to the solvent of JA since concentrations of chlorophyll and of the unknown phenolic compound were unaffected by exposure to the solvent alone and concentrations of anthocyanin and total phenolics were unaffected by solvent exposure in one of two replicate

experiments (Figure 2.1). Despite the increases in anthocyanin and decreases chlorophyll concentrations that we documented, we could not detect any visible color change (Figure 2.2) of the type that is striking in *M. spicatum* (Chapter 1). This response in pigment content may reflect the changes we observed in nitrogen concentrations since chlorophyll accounts for much of the nitrogen present in photosynthetic tissues (Lee et al., 2003). The lower nitrogen concentrations we observed in JA-exposed tissue would probably reduce herbivore growth as has been shown in terrestrial ecosystems (e.g., Feeny, 1970; Moran and Hamilton, 1980; Schroeder, 1986). The increased concentrations of total phenolics and of the specific unknown phenolic compound could also have a negative impact on feeding herbivores (Choi et al., 2002). Because of this, it was not surprising that *A. ephemerella* larvae fed *M. heterophyllum* exposed to JA had slower growth rates compared with those fed unexposed plant tips (Figure 2.3). Traits resulting in lower herbivore growth could potentially serve as a plant defense because larvae with slower growth rates would be exposed to parasites and predators over a longer period of time and would suffer from increased mortality (Moran and Hamilton, 1980). Also, larvae may be able to detect the induced response and move to neighboring, undefended plants (Moran and Hamilton, 1980; Karban and Baldwin, 1997).

Herbivore resistant traits were not induced in New York or Maine *M. heterophyllum.* Concentrations of total phenolics, anthocyanin, and chlorophyll were unaffected by JA exposure in Maine and New York-Long Island *M. heterophyllum* (Figure 2.1). Chlorophyll concentrations were significantly lower in New York-Adirondack *M. heterophyllum* exposed to JA (Figure 2.1), whereas anthocyanin and total phenolic concentrations were unaffected and nitrogen concentrations were actually higher in these JA-exposed plant tips (Figure 2.1). Because of this, we

conclude that it is unlikely that herbivore resistance was induced by JA in Maine or New York *M. heterophyllum.*

It is surprising that an induced response was only observed in German *M. heterophyllum* and completely absent in samples from North America. We used a higher concentration of JA in experiments with German as opposed to North American *M. heterophyllum* samples because axenic samples were less sensitive to JA exposure in complementary experiments with *M. spicatum* (Chapter 1). Although it is possible that inducible herbivore resistance was only observed in German *M. heterophyllum* because higher JA concentrations were used, A. Jörg (personal communications) observed a similar response in German *M. heterophyllum* exposed to JA at 20% the concentration we used for the Maine and New York *M. heterophyllum.* We conclude that it is unlikely that the differing JA concentrations had a significant effect in this study. A second explanation for why German *M. heterophyllum* was the only sample to respond to JA is that these plants may have been misidentified. *Myriophyllum verticillatum* commonly co-occurs with *M. heterophyllum* and the two species are often confused by experts (Les and Mehrhoff, 1999). Although the identification of our Maine and New York *M. heterophyllum* samples was confirmed (R.L. Johnson, personal communications), we have not confirmed the identification of our German *M. heterophyllum* sample.

It is not surprising that constitutive and inducible herbivore resistance should vary among invasive and native *M. heteorphyllum* populations. Invasive populations presumably face many new selection pressures in newly invaded habitats. This argument has been suggested by several authors as the reason for rapid evolution observed in other invasive plant species (e.g., Burdon et al., 1981; Willis et al., 1999; Siemann and Rogers, 2001; Garcia-Rossi et al., 2003; Siemann and Rogers, 2003). A second explanation is that invasive populations may be composed of hybrid species. A

recent molecular study done by Moody and Les (2002) determined that many invasive populations in New England are actually composed of *M. heterophyllum* × *M. pinnatum* hybrids misidentified because they are morphologically very similar to *M. heterophyllum sensu stricto*. Because of this, it is possible that our *M. heterophyllum* samples were actually hybrid species.

Differences in constitutive and inducible herbivore resistance likely contribute to the variable success of invasive *M. heteorphyllum* populations. Higher constitutive total phenolic concentrations were observed in Maine *M. heterophyllum* whereas inducible herbivore resistance and concentrations of the unknown phenolic were only observed in *M. heterophyllum* sampled from Germany. These differences are significant when considering efforts to prevent or control nuisance populations. Herbivorous insect species are presently being considered for biological control of nuisance *M. heterophyllum* populations (Halstead et al., 2003), and their success may be affected by the herbivore resistance expressed by the plants. Furthermore, accidental introductions of plant material from well defended *M. heterophyllum* may be more likely to become nuisance population than those with lower herbivore defenses.

In summary, we have shown that significant differences in constitutive and inducible anti-herbivore defenses exist among German, Maine, and New York *M. heterophyllum* populations. These differences may be a result of rapid evolution, hybridization, or misidentification. Regardless of its origin, this variation probably contributes to the success of invasive populations. It is also possible that the differences we observed between German and Maine populations also exist among populations within Germany or among populations within Maine. Comparison studies at finer geographic scales are needed particularly since this variation may affect prevention and control strategies of nuisance populations.

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