

Induced defense mechanisms in an aquatic angiosperm to insect herbivory

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Abstract In terrestrial angiosperms, defense and resistance mechanisms against herbivores have been studied extensively; yet this topic is poorly understood in aquatic angiosperms. We investigated induced response mechanisms in *Myriophyllum spicatum* to the generalist insect herbivore *Acentria ephemerella* in three independent experiments. Various morphological and chemical response variables were examined in grazed apical shoots and compared to undamaged controls. We further estimated plant palatability of induced and non-induced apices in choice assays, and assessed the growth response of *Acentria* larvae in no-choice feeding assays. Leaves of induced apices were splayed out horizontally and changed in color from green to red. The dry matter content and thus plant toughness increased by up to 19 %, but silica levels stayed constant.

Induced apices exhibited a decline in chlorophyll content of up to 34 %, reflected also by a 10 % decrease in nitrogen levels, while nitrogen increased by 14 % in lower parts of grazed shoots. Also, herbivore-deterrent total phenolic compounds increased by up to 20 % in apices. In choice trials, *Acentria* larvae strongly avoided grazed tips, and growth was reduced by 25 % on induced apices. In total, we observed five different induced resistance and defensive traits in grazed apices: changes in appearance, increased plant toughness, delocalization of N-containing metabolites, increased polyphenols, and reduced nutritional value. The observed changes prevent herbivore damage and loss of apical tissue, which are most valuable for plant fitness. Our study presents the first evidence of multiple, parallel defense strategies including constitutive and induced defense mechanisms in a freshwater angiosperm.

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Introduction

The complex and multifaceted interactions between plants and their herbivores have gained much attention, both in terrestrial and aquatic systems. Plants need to counterbalance growth and competitive ability with either constitutive or inducible defense mechanisms (Ito and Sakai 2009). Consequently, most plant responses towards herbivores rely on more than one defensive trait. The combination of these different traits results in specific plant defense syndromes, such as the balance between high nutritional quality and physical or chemical constitutive defenses (Agrawal and Fishbein 2006), or the alternate or concomitant expression of several inducible defensive traits, depending on the plant

species (Karban 2011; Orians et al. 2011). Competitive species might be faced with competition-defense tradeoffs affecting the investment in either constitutive or induced defenses. A recent study suggests that this dilemma holds for constitutive resistance and is complemented by a positive relationship of competitive ability with induced resistance (Kempel et al. 2011). Induced responses can be divided into induced resistance describing a response of the attacked plant resulting in a decreased fitness of the attacking herbivore and induced defense describing a response of the attacked plant resulting in an increased fitness of the attacked plant (Karban and Baldwin 1997). We will use these terms accordingly and refer to both as defense mechanisms.

Even though all aquatic (marine and freshwater) angiosperms are secondarily aquatic (Cook 1999), little is known about whether they respond to herbivore damage in the same way; i.e., whether they exhibit comparable constitutive or induced defense mechanisms. While terrestrial plants may respond with a variety of constitutive and induced responses (Karban and Baldwin 1997), much less is known about allelochemical interactions between freshwater vascular plants and herbivores (Forbey et al. 2013; Gross and Bakker 2012; Morrison and Hay 2011). Some studies indicated that herbivory on primary producers, including angiosperms, in freshwater systems may even exceed that in terrestrial systems (Cyr and Pace 1993; Lodge et al. 1998), and this would suggest that herbivore defenses in aquatic plants are less widespread. Indeed, fully aquatic leaves of amphibious species exhibited higher grazing rates than aerial leaves, possibly due to a lower structural defense (Cronin et al. 1998).

Constitutive chemical resistance in freshwater plants against herbivores has been well described (Bolser et al. 1998; Erhard et al. 2007; Kubanek et al. 2000; Prusak et al. 2005). Such constitutive defense mechanisms are presumed to prevail in these freshwater macrophytes because of a high and lengthy exposure to mostly generalist herbivores (Ostrofsky and Zettler 1986; Otto and Svensson 1981). The fact that most freshwater herbivores are generalists might explain why induced resistance mechanisms in freshwater plants attained less attention (Morrison and Hay 2011).

While several studies have shown induced responses in marine macroalgae (e.g., Toth et al. 2005; Toth and Pavia 2000; Van Alstyne and Pelletreau 2000), the situation seems more complex in seagrasses (Darnell and Heck 2013; Steele and Valentine 2012). The impact of macrograzers on the production of phenolic acids and condensed tannins in the seagrasses *Thalassia testudinum* and *Halodule wrightii* varied somewhat idiosyncratically with season, urchin density, and distance from urchin damage (Steele and Valentine 2012).

In terrestrial systems, mechanisms of induced defense and resistance against herbivores are favored over constitutive defenses under conditions when the kind of herbivore and the severity of damage vary across space and/or time (Karban 2011). This might also explain the variable results found in seagrasses (Darnell and Heck 2013; Steele and Valentine 2012), and might also be applicable to submersed freshwater angiosperms. Most freshwater vascular plants are able to distribute via fragmentation, resulting in genetically identical plants which are exposed to variable habitats, including differences in herbivore abundance and composition. Therefore, mechanisms of induced resistance in aquatic vascular plants can be predicted by theory (Karban 2011) and are expected to be prevalent. In view of this, and with respect to the extensive research in terrestrial systems, it is surprising that herbivore-induced responses in aquatic vascular plants so far have received considerably less attention. Until now, only four studies have reported induced responses to herbivory in freshwater angiosperms (Bolser et al. 1998; Jeffries 1990; Lemoine et al. 2009; Morrison and Hay 2011), and a few others recently focused on marine angiosperms (Darnell and Heck 2013; Steele and Valentine 2012; Valentine et al. 2004). None of them identified an induced allelochemical, and the small number of studies does not allow any general conclusions on the prevalence of this defense mechanism (Morrison and Hay 2011).

An increase in plant toughness is an efficient structural defense against herbivores with limited biting force. It can be measured using the dry matter content (DMC), a plant trait reflecting tissue toughness both in terrestrial and aquatic plants (Cornelissen et al. 2003; Elger and Lemoine 2005). The pond snail *Lymnaea stagnalis* L. preferred plant tissue with lower DMC values (Elger and Lemoine 2005). Silica content may affect plant toughness and DMC, and may enable plants to respond adaptively to environmental stresses, including herbivory (Cooke and Leishman 2011).

Induced resource sequestration is an alternative or complementary response strategy in terrestrial plants (Meldau et al. 2012; Orians et al. 2011). Induced resource reallocation to belowground storage organs (rhizomes) has been found in the submerged macrophyte *Potamogeton perfoliatus* L. after damage to shoots by the herbivorous larvae of *Acentria ephemerella* Denis and Schiffermüller (Crambidae, Lepidoptera) (Miler and Straile 2010). Simulated herbivory led to changed nitrogen enzyme levels, assimilation and allocation in roots and non-clipped outer leaves of the marine angiosperm *Thalassia testudinum*, which suggests induced changes in resource allocation (Valentine et al. 2004).

Phenolic compounds are among the most widespread defensive compounds in higher plants and macroalgae (Dixon and Pavia 1995; Toth and Pavia 2000). While

phlorotannins are specific for phaeophytes (Toth et al. 2005; Toth and Pavia 2000), hydrolysable and condensed tannins, flavonoids and phenolic acids have all been associated with a defensive function against herbivores in terrestrial and marine higher plants (e.g., Salminen and Karonen 2011; Steele and Valentine 2012). In freshwater systems, most submerged living higher plants are monocotyledons, containing rather low concentrations of phenolic compounds (Gross and Bakker 2012).

Members of the dicotyledonous *Myriophyllum* (Haloragales), however, are different. *Myriophyllum spicatum* L. is widely distributed in northern Eurasia and North Africa and invasive to North America. It is generally competitive and fast growing, with its distribution and propagation relying mostly on vegetative fragmentation but not on seed dispersal. Apical meristems are most valuable for dispersal, and their presence relates to plant fitness (Riis et al. 2009). This species has one of the highest contents of hydrolysable polyphenols known in submerged macrophytes, ranging from 50 to 138 mg g⁻¹ dry mass in leaves to up to 250 mg g⁻¹ in apices (Hempel et al. 2009). The ellagitannin tellimagrandin II is the main hydrolysable polyphenol in *M. spicatum* (Gross et al. 1996). The shoots of *M. spicatum* exhibit a strong vertical gradient of nutrients and polyphenols (including tellimagrandin II), which declines with increasing distance from the apex (Hempel et al. 2009). The decline in the polyphenol concentration in lower shoots might represent a dilution of defensive compounds initially formed during plant growth (Koricheva 1999). However, the highly pro-oxidant ellagitannins should be specifically deleterious for herbivores (Salminen and Karonen 2011), and thus apical meristems in *M. spicatum* might be well defended against grazers. Indeed, the polyphenols in *M. spicatum* have multiple allelochemical actions and already present an efficient constitutive defense against multiple stressors: they are allelopathically active against algae and cyanobacteria (Gross et al. 1996), reduce the growth of *A. ephemera* larvae (hereafter “*Acentria*”, since there is only one species in the genus) (Choi et al. 2002), possibly by negatively affecting gut bacteria (Walenciak et al. 2002). Nevertheless, *Acentria* can completely develop on *M. spicatum* (Gross et al. 2001). So far, our observations in the field and laboratory indicate a complex interaction between *M. spicatum* and the larvae of *Acentria*.

Despite this high constitutive defense specifically in the apices, *M. spicatum* is subjected to severe grazing pressure on these plant parts by several invertebrate grazers, among them both specialists and generalists (Gross et al. 2002; Newman 2004). Considering that specialist herbivores are often well adapted to induced resistance mechanisms and generalist herbivores are frequently deterred by induced responses (Karban 2011), we would expect that generalist herbivores such as *Acentria* are able to induce changes

in grazed plants. *Acentria* larvae cause very noticeable changes in *M. spicatum* apices, similar to those seen when subjecting these plants to jasmonic acid (Onion 2004), suggesting that herbivore-induced signal cascades have been triggered. These prior observations indicated a localized and specific response of *M. spicatum* apices towards herbivory by *Acentria*.

The aim of our study was thus to elucidate the multiple possible induced plant responses, i.e. all morphological, structural and chemical changes involved in the defense and resistance of *M. spicatum* against this insect herbivore. We specifically asked: does *Acentria* herbivory (1) change structural properties of the apices, (2) cause changes in nutrient allocation between grazed and non-grazed tissues, (3) affect the content of defensive phenolic compounds, and (4) result in a lower palatability and food quality of the grazed tissue for larvae? These questions were addressed in three independent experiments, using different plant source material and optimized micro-extraction methods to account for highly localized effects.

Materials and methods

Experiments

Experiment I: response in the field

We investigated the response of *M. spicatum* to *Acentria* grazing under semi-natural conditions by sampling a mesocosm at the Limnological Institute, Constance, Germany (47°41'42"N, 9°11'37"E). A self-sustaining stand of *M. spicatum* has been growing for 5 years in this outdoor 5 × 10 × 1-m (width × length × depth) large concrete basin, filled with natural Lake Constance sediment (10 cm) and circulating lake water (70 cm). The plants harbor a self-sustaining population of *Acentria*.

We sampled 300 fragments of *M. spicatum* by gently whipping a fishnet through the stand at a depth of 20–50 cm when the first *Acentria* larvae broke hibernation and started feeding on 8 April 2011. Fragments were transported to the laboratory within 30 min. Each shoot was examined for the presence and position of *Acentria* larvae or other herbivores. Visual inspections showed no damage caused by other herbivores, such as *Eubrychius velutus* and *Lymnaea stagnalis*.

Forty-eight *Acentria*-damaged apical shoots were found and 40 control shoots were randomly selected from the undamaged fragments. The relation between the fragments was not identified, thus multiple fragments could have originated from the same plant individual. This factor was taken into account in the following experiments, but not considered here. To exclude the problem of dilution

of defensive compounds (Koricheva 1999), we used treatment and fragment length as predictor variables. Shoots were cut below the feeding scar and the length of this apical shoot was measured. For comparison, size classes were assigned to the nearest full centimeter, e.g., 0.51–1.49 cm was assigned to 1 cm. Control shoots were cut to lengths of 1, 2 and 3 cm, reflecting the size distribution of the treatment (grazed) shoots.

All shoots were photographed to document changes in leaf position and color of the apex. Cross sections (20 μm) of a subsample of stems with feeding scars ($n = 10$) were prepared using a hand-microtome and examined with a dissecting microscope for the depth of feeding scars (2–10 \times magnification).

All plant samples were carefully blotted dry using paper tissue, and weighed to the nearest 0.1 mg to determine the fresh weight. Care was taken to avoid excessive drying and thus damage to the shoots. Each shoot was then individually shock frozen in liquid nitrogen and freeze dried. We determined the dry weight of shoots before grinding them to a fine powder. The powder was stored in amber glass vials.

DMC was determined by dividing the dry weight by the fresh weight of the sample. We used micro-analytical methods to analyze plant fragments as small as ca. 1 cm (0.5–1 mg dry weight), providing a very high resolution of changes in the immediate microenvironment of the damaged tissue. Silica was analyzed after alkaline digestion (0.1 mg in 0.5 ml of 0.2 M sodium hydroxide; 90 min; 99 °C) as a silico–molybdate complex at 810 nm (Krause et al. 1983). Nitrogen and carbon were analyzed with an Elementary Analyzer NCS 2500 (Thermoquest–CE Instruments) using atropine sulfate as a standard. Chlorophyll (chl) was extracted [0.1 mg sample in 200 μl acetone/TRIS–hydrochloric acid–buffer, 8/2 (v/v); pH 7.8; 2 h at 16 °C (Hempel et al. 2009)]. The absorbance at 664 nm and 647 nm was used to calculate the chl $a + b$ content. Anthocyanin was extracted with acidified methanol (methanol/water/hydrochloric acid 37 %, 80/20/1; 0.1 mg in 200 μl solvent) for 2 h at 16 °C. The absorbance at 532 and 653 nm was used to calculate the anthocyanin content (Hempel et al. 2009).

Total phenolic compounds (TPC) were extracted in aqueous acetone [50/50 (v/v); 0.1 mg sample in 200 μl solvent] followed by the Folin–Ciocalteu assay with tannic acid (no. 403040, batch 06817CJ; Sigma–Aldrich) as standard, modifying the protocol of Choi et al. (2002). We also determined the proportion of pro-oxidant polyphenols of the TPC according to the protocol of Salminen and Karonen (2011). Specifically ellagitannins are prone to oxidation. First, the TPC was determined, and then an aliquot of each sample was oxidized for 90 min under alkaline conditions. After neutralization, and taking into account the

dilution of the sample, the total phenolic content (representing the non-oxidized phenolic compounds) was quantified again using a modified Folin assay and gallic acid as standard.

Experiment II: response under controlled conditions in the field followed by choice and no-choice feeding assays

To verify if the induced responses observed in experiment I can be reproduced under more controlled conditions, and if the resulting induced changes affect herbivore performance, we set up smaller enclosures in June 2011. Fresh, non-grazed plant material was obtained from one existing stand of *M. spicatum* in a small concrete mesocosm (2 \times 2 \times 1 m; width \times length \times depth). We separated the plant stand into two neighboring populations (distance 15 cm) using two polypropylene foil tubes each of 1 m². The tubes were fixed in the sediment by a metal frame, and reaching above the surface fixed to a floating Styrofoam frame. Plants in the tubes were cut to a length of 15 cm, and all cut-off material with the attached herbivores was removed carefully to guarantee for similar age, depth, and light conditions of shoots, and the exclusion of herbivores. The few remaining herbivores, mainly *E. velutus*, were removed manually directly after plant cut-back. Within 2–3 weeks, new shoots had grown to the surface.

Plants were then exposed to *Acentria* larvae of the same instar stage for a defined time. The larvae were derived from a sampling made in December 2010 by snorkeling at 1- to 2-m depth near the southwest tip of the Isle of Reichenau (47°42'0"N, 9°2'31"E). Larvae hibernating within basal stems of *P. perfoliatus* were stored in water-filled buckets with aeration at 4 °C in the dark. Hibernation was broken by exposing stem sections to 22 \pm 1 °C for 1–3 days in a climate chamber and offering emerging larvae fresh *M. spicatum* as food. Instar classes were defined according to the classification given in Choi et al. (2002).

We selected pairs of neighboring apical shoots of different plants reaching the same depth and with similar visual appearance, within each enclosure. One larva of instar III was placed on one tip of each pair and labeled as treatment. Sampling of treatment and the respective control plants, always within the same enclosure, took place 24 h later. Treatment plants were cut off below the internodes where the larvae were located and a feeding scar was visible. Fragment lengths were 0.6–1.5 cm, with a mean of 0.9 cm (\pm 0.3 cm) for both treatment and control groups. The respective control tips were cut to a similar fragment size. Samples were individually placed in 15-ml Falcon tubes and transported immediately to the laboratory. Each of the 31 paired replicates for treatment and control were individually chemically analyzed, using the same methods as described in experiment I.

Another set of these unconnected shoots with and without feeding marks was used in the choice and no-choice feeding assays. For the choice assays, small glass jars ($n = 19$; 5 cm diameter, 4 cm height) filled to 3 cm depth with lake water were each equipped with a pair of control and treatment apical shoots. Jars were randomly positioned in a growth chamber at a light:dark regime of 14:10 h and 22 °C, corresponding to summer conditions in Lake Constance. Only the light intensity was kept lower than summer conditions at $150 \mu\text{E m}^{-2} \text{s}^{-1}$ in order to prevent further induction of polyphenols by high irradiance (Gross 2003). Each jar received one larva of instar II, which was placed between both apices in the middle of the jar. After 24 h, the positions of the larvae, indicated by the construction of leaf shelters and fecal pellets, were located.

For the no-choice feeding assays, we used instar II larvae weighed to the nearest 0.001 mg (SC2 ultramicro scale; Sartorius, Göttingen, Germany) and placed each with either a control or a grazed plant apex in a glass jar. Larvae were allowed to feed for 6 days ($n = 16$ each). The relative growth rate (RGR) was calculated as

$$\text{RGR} = (\ln W_2 - \ln W_1) / (t_2 - t_1);$$

with W_1 and W_2 representing larval weights at times t_1 and t_2 .

Experiment III: response under controlled conditions and resource allocation to lower shoots

To examine changes of plant parameters similar to those observed in the previous two experiments and to track the changes of nutrients observed in attacked tips along a vertical gradient, we collected *M. spicatum* plants at the end of April 2011 in the small stream Markelfinger Aach close to where it flows into Lake Constance (N47°44'16.422" E9°0'7.384"). Plants were washed and freed from invertebrates. We used 36 pots (6 cm diameter) filled with lake sediment; each pot was planted with four apical plant shoots of 12 cm length. Pots were placed in clear polyvinylchloride tubes (50 cm height, 10 cm diameter), which were placed in one concrete mesocosm filled to 40 cm depth with lake water. Tubes were randomly allocated to 18 treatment and 18 control tubes. Treatment plants were exposed to *Acentria*, with four larvae per tube.

Here, samples for the assessment of induced responses were taken after 5 days. As before, treatment apices were cut off at the height where the feeding damage was located and control plants were cut to similar size (1.0 ± 0.5 cm). Larvae found within treatment samples were placed back into the tubes to maintain approximately the same herbivore density, since plants grew fast and developed side tips. Herbivores were allowed to feed for 2 additional weeks. Then the lowest 10-cm stem section of each plant was cut

off and freed from roots. The apical shoots were analyzed as described above, and the lower stems were analyzed for DMC and nitrogen content.

Statistical analysis

Data were analyzed using R 2.15.1 (R Development Core Team 2011). Differences between groups of experiment II were identified using a paired *t*-test (but see supplementary Appendix A1 for details on the negligible effect of enclosures), and in experiment III using Student's *t*-test. The Shapiro test was used to verify normal distribution of the data or residuals for the applied linear regressions in experiment I. In the case of a lognormal distribution, data were log transformed prior to statistical tests. We fitted linear regression models to test effects of fragment length and treatment on chlorophyll, DMC, carbon, nitrogen and TPC levels including both predictors and their interaction. Non-significant interaction terms were dropped from the models prior to final statistical effect evaluation. Data of the choice feeding assay were evaluated using the χ^2 -test. Effect size is given as Cohen's *d*-value.

Results

Plant appearance

Acentria herbivory caused visible color and morphological changes in *M. spicatum* apices (Fig. 1a–f). We observed in all experiments that *Acentria* (Fig. 1h) constructed a shelter with leaves of two consecutive nodes, and at the enclosed internode we observed feeding scars. Cross-sections of ten treatment stems at the feeding scars showed that caterpillars always stopped their feeding activity before or at the aerenchyme and that damage was exclusively next to the leaf shelter (Fig. 1g). We observed distinct changes in appearance above the feeding scars, specifically a color change from green to red. Also, leaves above the feeding scar were splayed out so that all leaves, including the ones next to the apex, stood crippled, almost perpendicular to the stem while they were arranged almost parallel along the apex in controls.

Structural response

In experiment I, the variance in DMC is only explained by treatment as a significant explanatory variable, but not by length, or the interaction between these variables (Fig. 2a). In contrast, the DMC in lower stems of experiment III was not affected by the presence of herbivores. Accordingly, the DMC was always higher in apices of the treatment groups in all experiments (Fig. 3). The silica content of shoots did not differ between treatment and control plants (Table 3).

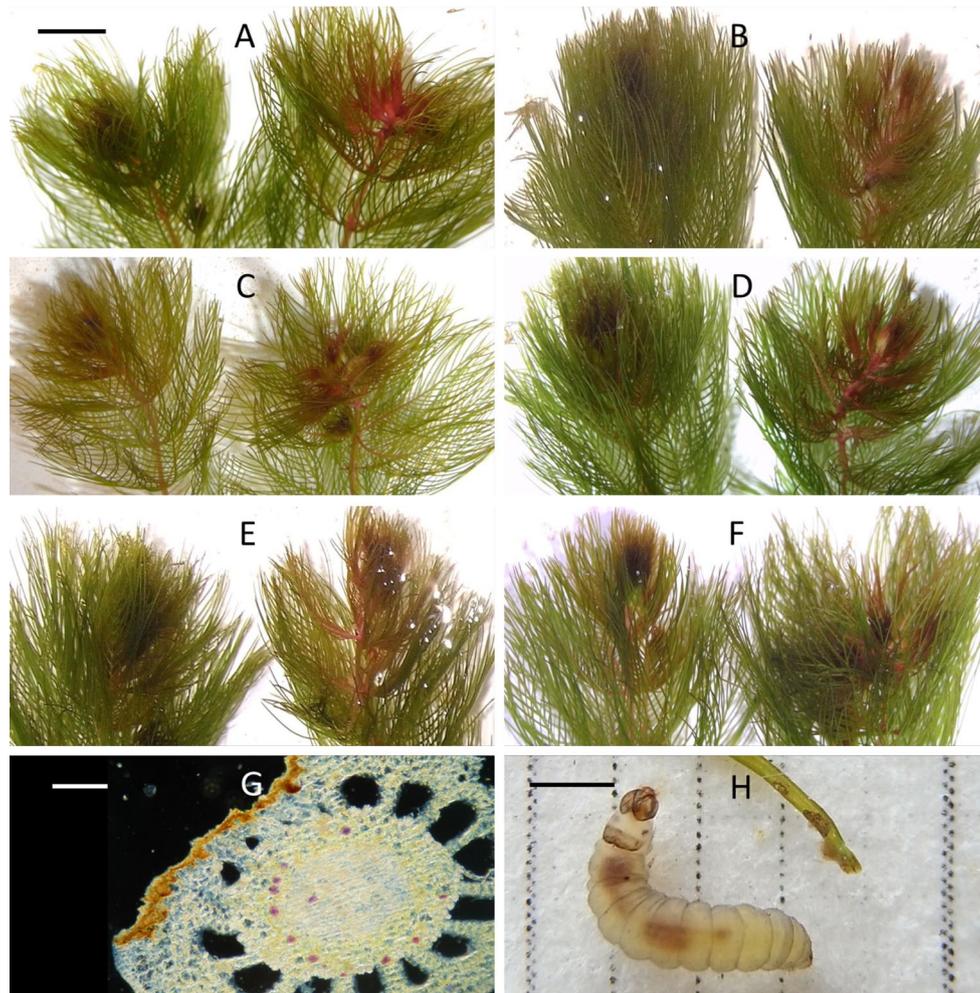


Fig. 1 Induced morphological changes in *Myriophyllum spicatum* after 24 h of *Acentria ephemerella* feeding. **a–f** Six sample pairs of apices taken from the outdoor mesocosm in experiment II. In each picture, the *left side* shows the control plant with normal appearance, and the *right side* shows the treatment plant, exhibiting a paler green but more red appearance due to a significant reduction in chlorophyll content, and enhanced visibility of anthocyanin pigmentation, of leaves and stalks. Leaves of the attacked plant tips are partly crippled

and oriented at a wider angle along the stem, exposing the apex and causing a splayed out appearance. The *scale bar* in **a** represents 1 cm, and applies to **a–f**. **g** Cross-section of *M. spicatum* at the feeding scar. *Lower right* Normal aerenchyme surrounded by undamaged tissue. *Upper left* feeding scar damaging the aerenchyme. The *scale bar* represents 1 μm . **h** Picture of a 2nd instar larva of *A. ephemerella*. The *scale bar* represents 1 mm

Chlorophyll, carbon and nitrogen content

The chl *a+b* of treatment apices was significantly reduced in all experiments (Fig. 3). In experiment I, shoot length was positively correlated with chlorophyll level in the treatment group but had no effect on the control group (Fig. 2b). The identified interaction between length and treatment shows that treatment effects decreased with increasing fragment length.

Carbon and nitrogen levels were only analyzed in apical shoots of experiment I and lower stems of experiment III. Herbivory significantly reduced carbon and nitrogen levels in apical shoots, but only resulted in a marginally higher carbon/nitrogen molar ratio, with a significant interaction

term for treatment \times length (Tables 1, 3). We observed an inverse effect in basal stem sections (Tables 2, 3), suggesting different resource sequestration in young and old tissues.

Phenolic compounds

The anthocyanin levels of apices in experiment I were neither affected by treatment nor by fragment length. In experiment II, we observed a slight increase in anthocyanin in grazed apices, but the effect size was low with Cohen's *d*-value inferior to 0.4.

Both treatment and control apices show a significant negative correlation of TPC with length, but treatment

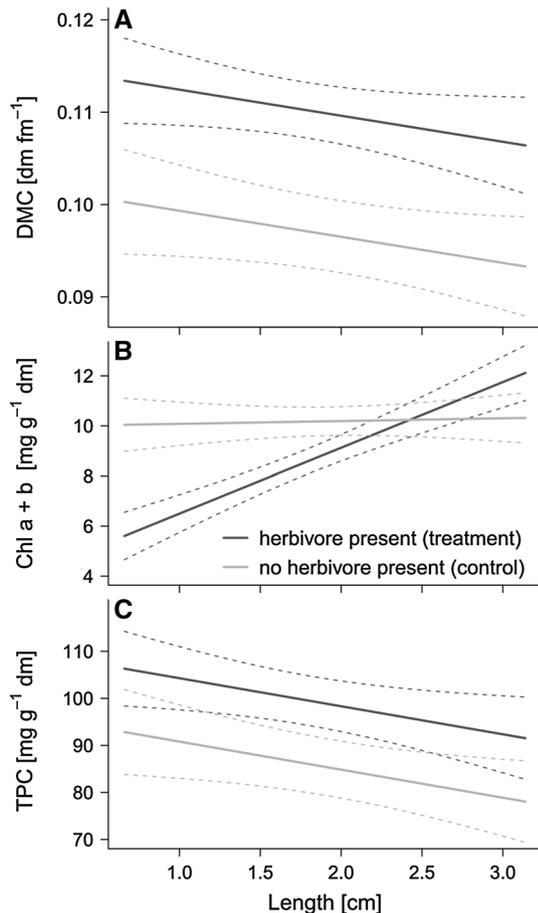


Fig. 2 Main responses of *M. spicatum* to *A. ephemera* herbivory depending on fragment length. Shown are the regression lines of the linear models (solid lines) of experiment I; the full statistical results are presented in Table 1. Dashed lines represent 95 % confidence intervals. **a** Dry matter content (DMC): $df = 3,74$, $R^2 = 0.31$. **b** Total phenolic content (TPC): $df = 3,84$, $R^2 = 0.15$. **c** Chlorophyll *a+b* (Chl *a+b*): $df = 3,83$, $R^2 = 0.40$

apices always contained higher TPC levels (Fig. 2c). TPC levels of the treatment groups were significantly increased in all experiments (Fig. 3). Recently, the pro-oxidant activity of polyphenols was highlighted as a specific mode of action in plant-herbivore interactions (Salminen and Karonen 2011). However, when applying this new method to a subset of samples from experiment I, we found no increase in the ratio of pro-oxidant polyphenols in herbivore-exposed shoots (Tables 2, 3).

Feeding trials

In all feeding assays within experiment II, a maximum of 50 % of the plant material offered was consumed, thus ad libitum food supply was guaranteed. In the choice feeding assays, 17 out of 19 *Acentria* larvae preferred control plants over shoots with prior feeding experience (χ^2_1 ,

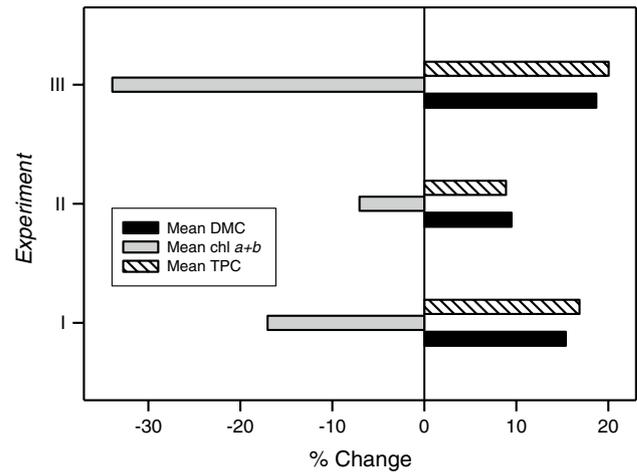


Fig. 3 Effect sizes for major structural and chemical changes across all three experiments. Cross-experiment comparison of treatment means of the DMC, chl *a+b* and TPC as percentage change of the corresponding control means. For experiment I, all data irrespective of fragment length were pooled. For abbreviations, see Fig. 2

$_{19} = 11.84$, $P = 0.00057$, $d = 2.5$). In the no-choice feeding assays, growth rates of *Acentria* larvae reared on treatment plants were reduced compared to larvae reared on control plants (RGR 0.143 ± 0.06 mg day $^{-1}$ compared to 0.193 ± 0.07 mg day $^{-1}$, $t_{31} = 2.30$, $P = 0.027$, $d = 0.79$).

Discussion

Our study shows induced responses of the freshwater angiosperm *Myriophyllum spicatum* to herbivory by aquatic moth larvae of *Acentria ephemera*. We demonstrate herbivore-induced changes in plant appearance and changes in the amount of defensive chemical compounds in plant shoots; additionally our data indicate induced nutrient reallocation. The induced defense mechanisms reduced herbivore preference and performance and can therefore be regarded as induced resistance.

Due to the optimized analytical procedures, we were able to detect changes in *M. spicatum* shoots in apical fragments as small as 0.5-cm length. In experiment I, we studied fragments of 1- to 3-cm length to see if the vertical gradient for polyphenols and nutrients typically found in this plant (Hempel et al. 2009) is also visible in attacked shoots with different distances of the feeding scars to the apex. Depending on the response variable regarded, we found different effects of length of the apices and treatment, e.g. Fig. 2; Table 3.

We offer two explanations for these observations. First, the intensity of the induced response is altered by the distance of the feeding scar to the apex. This may be the case, if protection of the apex, which has the highest

Table 1 Effect of treatment (*Acentria ephemerella* grazing, no herbivore grazing) on the plant chemistry of apices of different length in *Myriophyllum spicatum*

Response variable	ndf, rdf	adj. R^2	Source of variation					
			Treatment		Length		Treatment \times length	
			<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
DMC	2, 75	0.31	33.2	<0.0001	3.81	0.054	–	–
Chl <i>a+b</i>	3, 83	0.40	18.59	<0.0001	27.54	<0.0001	25.02	<0.0001
Carbon	2, 51	0.12	7.48	0.0085	2.09	0.15	–	–
Nitrogen	2, 53	0.16	10.64	0.0019	2.1	0.15	–	–
Carbon/nitrogen	3, 51	0.17	3.35	0.073	3.22	0.078	4.11	0.047
Anthocyanin	2, 83	0.013	3.12	0.08	0.012	0.91	–	–
TPC	2, 85	0.15	12.64	0.0006	5.17	0.025	–	–

Results of two-way ANOVA analyses for experiment I. Non-significant interaction terms were removed from the analysis

ndf Numerator df, rdf residual df, adj. adjusted, DMC dry matter content, Chl *a+b* chlorophyll *a* and *b*, carbon/nitrogen carbon/nitrogen molar ratio, TPC total phenolic content

P < 0.05 in *italic*

Table 2 Effect of treatment (*A. ephemerella* grazing, no herbivore grazing) on the plant chemistry of apices of *M. spicatum*

Experiment	Response variable	df	<i>t</i>	<i>d</i>	<i>P</i>
Experiment I					
Apices	Silica	22	0.10	0.042	0.91
	TPC-ox.	10	0.46	0.26	0.65
Experiment II					
Apices	DMC	29	4.87	0.86	<0.0001
	Chl <i>a+b</i>	30	–2.32	0.43	0.026
	Anthocyanin	30	2.25	0.38	0.031
	TPC	30	3.03	0.54	0.0048
Experiment III					
Apices	DMC	43	4.55	1.35	<0.0001
	Chl <i>a+b</i>	37	–5.46	1.76	<0.0001
	TPC	40	4.53	1.4	<0.0001
Lower stems	DMC	36	–1.41	0.45	0.16
	Carbon	33	4.26	1.44	0.00015
	Nitrogen	31	4.09	1.42	0.00028
	Carbon/nitrogen	31	3.36	1.14	0.002

Results based on Student's *t*-tests for experiments I and III, and on a paired *t*-test for experiment II. For the analyses of silica content and proportion of pro-oxidant phenolic compounds of TPC (TPC-ox.) within experiment I, all fragment lengths were pooled (all results of experiment I accounting for length are presented in Table 1)

d Cohen's *d*-value—effect size; for other abbreviations, see Table 1

value in terms of growth and dispersal for *M. spicatum* and thus is most important for plant fitness, has the highest priority for the plant. Second, the response is induced exclusively in the apex and lower induction levels in longer fragments are a result of dilution effects caused by cell elongation and division of the growing shoot (Koricheva 1999).

Plant responses

The appearance of apical shoots in *M. spicatum* changed dramatically upon herbivory by *Acentria*. Potentially triggered by feeding scars on the stem of *M. spicatum* reaching the aerenchyme two apparent modifications of grazed apices were documented. Both changes in color and leaf shape or orientation might represent adaptations to counteract feeding or shelter preference by the attacking larvae, by other larvae and/or even a “cry for help,” e.g., by attracting predators such as sticklebacks (Miler et al. 2008). *Acentria* larvae usually build shelters from whorls close to the apex, but larvae might have difficulties bending the modified leaves into a shelter, and then would move to non-grazed shoots. The color change of the apex from green to red is a result of the decrease in chlorophyll, but not an increase in anthocyanin in induced plants. These changes in appearance might affect inter- and intraspecific interactions such as attraction of predators or deterrence of egg-laying females, although we have no experimental evidence for such indirect effects so far (Chen and Huang 2013).

Structural responses to herbivory, documented as an increase in DMC have been observed in freshwater macrophytes (Elger and Willby 2003), and terrestrial plants (Cornelissen et al. 2003). Plant toughness, considered a major mechanical defense mechanism against herbivores (Lucas et al. 2000), is difficult to measure in the feathery leaves of *M. spicatum*. We consider that DMC represents a good measure of plant toughness. In all our experiments, induced plant apices showed increased levels of DMC. In contrast, the observed decline in DMC in *M. spicatum* due to grazing by *L. stagnalis* (Lemoine et al. 2009) could be an artifact since the authors used complete shoots and observed massive regrowth of side shoots, which likely accounted

Table 3 Detailed summary of induced changes in *M. spicatum* apical shoots and lower stems after herbivory by *A. ephemerella*

Experiment	Response variable	Length (cm)	Control Mean \pm SE	Treatment Mean \pm SE	
Experiment I					
Apices	DMC (g d.m. g ⁻¹ f.m.)	1	<i>0.10 \pm 0.003</i>	<i>0.11 \pm 0.002</i>	
		2	<i>0.10 \pm 0.005</i>	<i>0.11 \pm 0.002</i>	
		3	<i>0.09 \pm 0.002</i>	<i>0.12 \pm 0.004</i>	
	Silica (mg g ⁻¹ d.m.)	1–3	0.77 \pm 0.15	0.80 \pm 0.19	
		Chl <i>a+b</i> (mg g ⁻¹ d.m.)	1	<i>9.65 \pm 0.63</i>	<i>6.44 \pm 0.27</i>
			2	<i>11.19 \pm 0.44</i>	<i>9.59 \pm 0.48</i>
	Carbon (mg g ⁻¹ d.m.)	3	9.80 \pm 0.42	10.20 \pm 0.73	
		1	44.41 \pm 0.41	43.48 \pm 0.41	
		2	43.99 \pm 0.43	43.86 \pm 0.32	
	Nitrogen (mg g ⁻¹ d.m.)	3	44.26 \pm 0.32	42.99 \pm 0.33	
		1	6.09 \pm 0.28	5.38 \pm 0.19	
		2	5.62 \pm 0.19	5.49 \pm 0.15	
	Carbon/nitrogen (mol mol ⁻¹)	3	5.88 \pm 0.19	4.99 \pm 0.14	
		1	8.77 \pm 0.32	9.52 \pm 0.22	
		2	9.22 \pm 0.22	9.43 \pm 0.15	
	Anthocyanin (mg g ⁻¹ d.m.)	3	8.83 \pm 0.26	10.21 \pm 0.19	
		1	1.62 \pm 0.08	1.68 \pm 0.08	
		2	1.68 \pm 0.11	1.64 \pm 0.1	
	TPC (mg g ⁻¹ d.m.)	3	<i>1.82 \pm 0.08</i>	<i>1.41 \pm 0.13</i>	
		1	87.57 \pm 5.24	108.80 \pm 5.41	
2		86.00 \pm 4.49	96.45 \pm 3.33		
TPC-ox. (%)	3	81.50 \pm 4.94	87.10 \pm 4.51		
	1–3	15.38 \pm 0.98	16.08 \pm 1.09		
Experiment II					
Apices	DMC (g d.m. g ⁻¹ f.m.)	1	<i>0.11 \pm 0.002</i>	<i>0.12 \pm 0.002</i>	
	Chl <i>a+b</i> (mg g ⁻¹ d.m.)	1	7.21 \pm 0.25	6.70 \pm 0.22	
	Anthocyanin (mg g ⁻¹ d.m.)	1	1.41 \pm 0.08	1.56 \pm 0.08	
	TPC (mg g ⁻¹ d.m.)	1	130.36 \pm 3.49	141.93 \pm 2.63	
Experiment III					
Apices	DMC (g d.m. g ⁻¹ f.m.)	1	<i>0.12 \pm 0.0029</i>	<i>0.14 \pm 0.0039</i>	
	Chl <i>a+b</i> (mg g ⁻¹ d.m.)	1	6.56 \pm 0.36	4.33 \pm 0.19	
	TPC (mg g ⁻¹ d.m.)	1	128.31 \pm 4.08	154.01 \pm 3.89	
Lower stems	DMC (g d.m. g ⁻¹ f.m.)	10	0.15 \pm 0.004	0.15 \pm 0.003	
	Nitrogen (mg g ⁻¹ d.m.)	10	1.51 \pm 0.04	1.78 \pm 0.05	
	Carbon (mg g ⁻¹ d.m.)	10	39.51 \pm 0.15	40.46 \pm 0.18	
	Carbon/nitrogen (mol mol ⁻¹)	10	30.86 \pm 0.85	26.85 \pm 0.83	

Results are based on three independent experiments; for details, see Tables 1 and 2

d.m. Dry matter, *f.m.* fresh matter; for other abbreviations, see Tables 1 and 2

Statistically significant changes are in *italic*

for the lower DMC in grazed versus control plants. Our approach in detecting changes in the direct vicinity of damage determines more precisely the effect of herbivory on DMC, unveiling induced structural defense. The increased DMC might deter young *Acentria* larvae more strongly as they may have difficulty handling the increased toughness of the induced apices (Müller and Dearing 1994). We do not yet know which factor is responsible for the increase in DMC. Silica levels did not differ in shoots from controls and treatments, although increased silica levels may play a

role in plant-herbivore interactions (Cooke and Leishman 2011). Another possibility might be an increase in (structural) polyphenols bound to the cell walls.

Herbivores prefer plants and plant parts with a high nutritional value, and chlorophyll and nitrogen levels are indicators of the nutritional value (Cornelissen et al. 2003). A low nutritional value negatively affects freshwater herbivores such as *Acentria* (Miler and Straile 2010). We observed a decreased chlorophyll and nitrogen content of 20 and 11 % in treatment apices, respectively. Thus, the

nutritional value was reduced and possibly contributed to the decreased herbivore preference for induced plants.

Plant polyphenols are well-known defensive metabolites against generalist herbivores, leading to higher mortality and prolonged development times in terrestrial and aquatic lepidopteran larvae (e.g. Choi et al. 2002; Lindroth and Hemming 1990). The deterrent effect of polyphenols was long believed to be a result of protein precipitation. Recently, Salminen and Karonen (2011) proposed more focus on deleterious pro-oxidant tannins, e.g. ellagitannins. In our study, the level of TPC increased in attacked apical shoots on average by 15.3 %, supporting the defensive role of polyphenols. However, the proportion of pro-oxidant tannins did not increase. Since there are yet only a few studies on pro-oxidant polyphenols, it is unclear whether an increase can be expected upon induction by herbivores. However, since the level of TPC increased, the absolute amount of pro-oxidant tannins also increased.

Resource reallocation

Herbivore-induced resource reallocation from attacked tissues to storage organs, also termed “induced resource sequestration,” was recently reviewed by Orians et al. (2011), and shown in another submerged macrophyte, *Potamogeton perfoliatus* by Miler and Straile (2010). The latter argued that resource reallocation to storage organs might be a common strategy in macrophytes against the multivoltine *Acentria* and suggested that the stored resources may fuel regrowth of new shoots in the following growth season. Our experiments show altered contents of carbon and chlorophyll or nitrogen in treatment apices and lower stems compared to the controls. Grazed shoots exhibited lower chlorophyll content, an indirect measure of nitrogen allocation, in all three experiments and this even as early as after 24 h, indicating active transport rather than dilution or bleaching. Nitrogen declined in grazed shoots in experiment I, and both carbon and nitrogen levels were increased in the lower stems of grazed plants in experiment III compared to the control. A potential caveat of the results from lower stems is that the sampling had been performed 2 weeks after sampling of apical fragments, and with a longer exposure to herbivores, to give effects occurring at the tips of the plants time to potentially reach the lower plant parts. Certainly future experiments are needed to verify our findings, e.g., using stable isotopes to monitor resource reallocation more closely. Nevertheless, we assume that induced resource sequestration from grazed tissue to storage organs in *M. spicatum* should benefit the whole plant, by facilitating the development of new shoots and side shoots during the ongoing and/or next year’s growth period.

A change in allocation of carbon or nitrogen to defensive compounds upon herbivore attack has been described for

terrestrial systems (e.g., Meldau et al. 2012). In our study, the combined effect of increased defense and changed nutrient content of valuable apices is evident from the changes in chlorophyll, carbon and nitrogen and the resulting carbon/nitrogen molar ratio, as well as in the increased TPC content. Whether more nitrogen was allocated to lower shoots, and more carbon was allocated to defensive compounds, is the subject of ongoing investigations.

Induced defense reactions

An induced defense reaction is defined as an increase in fitness of attacked plants after herbivory compared to fitness levels of undefended plants (Karban and Baldwin 1997). Plant fitness was not directly addressed in this study because our primary aim was the localized response in apical shoots, but we consider that induced changes present in apical shoots, which increase the resistance towards herbivores, are highly related to an increased fitness of induced plants. The reproduction of *M. spicatum* relies strongly on the number of fragments released, and fragments with apical tips have been shown to have the highest relative growth rates (Riis et al. 2009). As shown in experiment I (Table 1), the observed changes were most predominant in the apical tips of the sampled fragments. Even though the interactions of treatment and length were not significant for TPC and DMC, we could show that the most valuable parts for plant fitness showed the strongest response.

Despite this induced defense, herbivory by *Acentria* can result in severe damage of *M. spicatum*. In lower Lake Constance up to 82 % of the apical meristems were found damaged or missing, showing clear signs of *Acentria* damage [remaining leaf shelters, bite marks (Gross et al. 2002)]. Fragmentation is very common in *M. spicatum* and even increased under nutrient limitation (Smith et al. 2002). Our hypothesis is that at least some missing apices have not been fully consumed but fragmented. Hence negative effects on plant fitness might have been compensated for by induced defense. Another result of the observed induced defense reactions in *M. spicatum* could be that herbivory damage is spread more evenly over the whole plant or plant population, avoiding too severe damage at one site, and thus potentially allowing plants to maintain canopy-forming growth under low to moderate herbivore pressure. In Gross et al. (2001), we had observed a decline in biomass and main shoot length with increasing density of *Acentria* larvae, but the number and length of side shoots did not change. These observations are in agreement with a modeling study that predicted that plant defenses and compensatory growth can work together to increase plant fitness under herbivory (Lehtila 1996).

Induced plant resistance

Induced plant resistance is represented by any trait that reduces the preference or performance of herbivores. It is often confused with plant defense but contrary to this mechanism does not have any implications on the resulting plant fitness (Karban and Baldwin 1997). We verified induced resistance of grazed apices using choice and no-choice feeding trials. We demonstrated a decreased preference and an inferior performance of *Acentria* when offered induced plant fragments. The reduced growth rate of *Acentria* on induced plant fragments might be a result of the lower nutritional value in terms of nitrogen content and/or a decreased palatability through increased TPC and DMC levels, or a combination of all these factors. High TPC levels can impair larval growth and may affect population development. *Acentria* larval size at pupation was negatively correlated with the amount of TPC found in the food plant (Choi et al. 2002), and this should result in a lower fecundity of adult females.

The induced defense traits (see section “[Induced defense reactions](#)” above) force *Acentria* larvae to leave their shelters and move to less-defended side shoots, enhancing the larvae’s risk of being preyed on (Miler et al. 2008). Regardless whether DMC, nutritional value or TPC, or a combination of all factors reduced larval growth rate, the final impact on these herbivores is severe. Consequently, larvae of *Acentria* avoided feeding on induced plant parts when given a choice. They can easily move to lower plant parts or neighboring apices (F. F., personal observation), but will experience deleterious effects such as a reduced growth rate if only induced plant material is available, as seen in the no-choice assays.

Outlook

We demonstrate a change in appearance of apical shoots coupled with morphological, structural and chemical induced defense and resistance mechanisms in the already constitutively well-defended aquatic angiosperm *M. spicatum* against larvae of *A. ephemerella*, together with indications of resource sequestration of major elements to less herbivore-susceptible plant parts and/or storage organs.

Many recent reviews evaluated different plant strategies against herbivores. Different analyses of general patterns in plant responses towards herbivory resulted in descriptions of plant defense syndromes (Agrawal and Fishbein 2006), trade-offs between constitutive and induced resistance (Kempel et al. 2011), conditions selecting for induced defense, constitutive defense, and no defense (Ito and Sakai 2009), or a fulcrum model selecting between “growth and/or defend favored vs. induced sequestration favored” (Orians et al. 2011). None of these match the strategies present

in *M. spicatum*. We propose two approaches to explain the observations of this study. First, previous studies mainly dealt with experimental studies in terrestrial systems. Freshwater vascular plants are known to differ from terrestrial plants in terms of, e.g., resource acquisition, dispersal strategies, structural carbon and herbivore consumption rates (Cyr and Pace 1993; Lodge et al. 1998). The little cited study by Lehtila (1996) supports our findings and shows that induced responses and herbivore tolerance effects can co-occur. In respect to this, and based on the currently poor knowledge on defense mechanisms in freshwater macrophytes, we request more studies questioning if models for terrestrial systems can generally be applied to interactions between herbivores and freshwater vascular plants. Second, changes in the concentration of various compounds can be very local, as shown here in experiment I, and discussed by Koricheva (1999). Taking this into account, many possibly induced allelochemicals might have been overlooked in studies of terrestrial plants but especially in low-DMC plants, like aquatic macrophytes. Therefore, more studies like the one we present here, determining changes in the closest proximity to the attack, could significantly improve our understanding of combined defense and resistance traits in plants.

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