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Chemical Defenses Promote Persistence of the Aquatic Plant *Micranthemum umbrosum*

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Abstract Five of the most common macrophytes from an aquaculture facility with high densities of the herbivorous Asian grass carp (*Ctenopharyngodon idella*) were commonly unpalatable to three generalist consumers—grass carp and the native North American crayfishes *Procambarus spiculifer* and *P. acutus*. The rooted vascular plant *Micranthemum umbrosum* comprised 89% of the total aboveground plant biomass and was unpalatable to all three consumers as fresh tissues, as homogenized pellets, and as crude extracts. Bioassay-guided fractionation of the crude extract from *M. umbrosum* led to four previously known compounds that each deterred feeding by at least one consumer: 3,4,5-trimethoxyallylbenzene (**1**) and three lignoids: β-apopicropodophyllin (**2**); (−)-(3*S*,4*R*,6*S*)-3-(3′,4′-methylenedioxy-α-hydroxybenzyl)-4-(3″,4″-dimethoxybenzyl)butyrolactone (**3**); and (−)-hibalactone (**4**). None of the remaining four macrophytes produced a chemically deterrent extract. A 16-mo manipulative experiment showed that the aboveground biomass of *M. umbrosum* was unchanged when consumers were absent, but the biomass of *Ludwigia repens*, a plant that grass carp preferentially consumed over *M. umbrosum*, increased over 300-fold. Thus, selective feeding by grass carp effectively eliminates most palatable plants from this community and promotes the persistence of the chemically defended *M. umbrosum*, suggesting that plant defenses play critical yet understudied roles in the structure of freshwater plant communities.

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Introduction

Herbivores were historically thought to have little impact on the ecology and evolution of freshwater plant communities (e.g., Shelford, 1918; Hutchinson, 1975). However, quantitative reviews show that herbivore impacts in freshwater systems rival those of marine and terrestrial systems, and aquatic herbivores often reduce the standing stock and alter the species composition of freshwater plant communities (Lodge, 1991; Newman, 1991; Cyr and Pace, 1993; Lodge et al., 1998). Additionally, aquatic herbivores exhibit selective avoidance of chemically or structurally defended plants (Newman et al., 1996; Bolser et al., 1998; Cronin, 1998; Cronin et al., 2002); yet there is surprisingly little direct evidence linking consumer feeding preferences to particular plant traits, or ultimately to shifts in plant community structure. For example, the introduced crayfish *Orconectes virilis* selectively consumes the filamentous green alga *Cladophora* over the blue-green alga *Gleotrichia*, and instigates a shift from green to blue-green algae in experimental ponds (Dorn and Wojdak, 2004), but the mechanisms conferring resistance to herbivores in *Gleotrichia* were not investigated.

A number of studies show that freshwater macrophytes are frequently unpalatable and contain a variety of secondary metabolites that could function as herbivore deterrents (Ostrofsky and Zettler, 1986; Cronin et al., 2002). More than one half of the crude extracts from 21 species of aquatic macrophytes that Prusak et al. (2005) surveyed, for example, deterred feeding by an omnivorous crayfish, although they did not identify the metabolites responsible for feeding deterrence. In fact, we know of only three freshwater plants with identified compounds that deter herbivores—watercress, *Rorippa nasturtium-aquaticum* (L.) Hayek (Newman et al., 1996), the waterspider bog orchid, *Habenaria repens* Nutt. (Bolser et al., 1998; Wilson et al., 1999), and lizard's tail, *Saururus cernuus* L. (Kubanek et al., 2000, 2001)—with a total of nine described secondary metabolites that influence herbivore feeding. In contrast, hundreds to thousands of secondary metabolites that deter consumers have been described from marine and terrestrial primary producers (Seigler, 1998; Faulkner, 2002 and references therein). These molecules can have strong cascading impacts on the ecology and evolution of plant–herbivore interactions in these systems (Hay and Fenical, 1988, 1996; Hay, 1996), suggesting that plant chemical defenses may play similar, but relatively uninvestigated, roles in freshwater systems.

Here, we examined the feeding preferences of three generalist consumers among five species of macrophytes collected from an aquaculture facility stocked with high densities of the herbivorous Asian grass carp *Ctenopharyngodon idella*. To determine the traits promoting macrophyte persistence under intense herbivory, we assessed the palatability of (1) whole plants, (2) plant tissues ground and imbedded in a gel-like matrix to retain most of the chemical and nutritional traits but with normal morphological traits removed, (3) plant crude extracts, and (4) specific metabolites isolated using bioassay-guided fractionation. We also conducted

a manipulative field experiment excluding herbivorous fishes. We then assessed the changes in the littoral plant community after 16 mo to determine whether well-defended species were disadvantaged in the absence of herbivores.

Methods and Materials

Study Organisms

We collected macrophytes from two 91 × 61 m wide, 1.3 m deep earthen ponds at the Owens and Williams fish hatchery in Hawkinsville, GA, USA. Each pond was stocked with >100,000 juvenile, triploid Asian grass carp, *C. idella*, an exotic herbivorous fish introduced throughout the United States to reduce aquatic plant abundance (USGS, 2005). On one occasion, we observed one turtle (pond slider—*Trachemys scripta* Wied) and evidence of crayfish (i.e., a single crayfish moult) in one of the ponds; these omnivores also consume macrophytes and, if common, could have further enhanced herbivore impact (Lodge et al., 1998). However, their effects were likely small relative to the large numbers of grass carp in each pond. The rooted, vascular plant *Micranthemum umbrosum* appeared to be the predominant plant species in one pond, while the floating green alga *Spirogyra* sp. appeared to be the predominant plant species in the other. The hatchery owner informed us that grass carp would frequently bite *M. umbrosum* but then forcibly reject it, and that *Spirogyra* sp. often persisted until all other macrophyte species had been consumed (P. Williams, personal communication). Based on these observations, the high density of herbivores in these ponds, and the acrid taste of *M. umbrosum* (J.P., personal observation), we hypothesized that these macrophytes possessed defensive traits promoting their persistence under intense herbivory.

On April 26, 2004, we determined the abundance of macrophytes in each pond by randomly locating five 0.25-m^2 quadrats on the littoral fringe (<1 m depth) of one side of each pond and determining the identity of macrophytes located beneath 36 points in each quadrat (we did not sample the remaining sides because they were disturbed by seine netting to capture fish). The five most common macrophytes (the green alga *Spirogyra* sp., and the vascular plants *M. umbrosum, Ludwigia repens*, *Juncus repens* Michx., and *J. effusus* L.)—were collected, transported to the laboratory in a chilled cooler, and fed to three consumer species within 24 hr of collection. We fed macrophytes to juvenile grass carp and to the native North American crayfishes *Procambarus spiculifer* and *P. acutus*. Both crayfishes have ranges across the southeastern United States (Hobbs, 1981). We used crayfish as a bioassay organism because they can have strong impacts on aquatic macrophyte communities (Lodge and Lorman, 1987; Creed, 1994; Lodge et al., 1994; Dorn and Wojdak, 2004), they are diverse and abundant foragers in aquatic habitats throughout North America (Lodge et al., 2000), they feed well in the laboratory (Bolser et al., 1998; Parker and Hay, 2005), and we observed evidence of crayfish in one of the ponds.

P. spiculifer were collected from the Chattahoochee River, Atlanta, GA (33°54′N, 84°27′W); *P. acutus* were collected from an adjacent wetland. We housed each crayfish in a separate 12 × 12 × 10 cm cubicle with perforated walls that received recirculating, filtered water. All animals were fed a maintenance diet of Bio-Blend Herbivore food (Marineland Labs) 3–4 times wk. Grass carp would not

feed when kept individually, so we housed them in small groups of 3–6 animals in 3.5-l buckets with recirculating water.

Feeding Assays

We determined the relative palatability of all five macrophyte species by offering 12–15 individuals of each consumer species a bite-sized portion of each macrophyte and recording whether each portion was eaten or rejected. If rejected, we fed consumers a piece of palatable aquatic macrophyte (*Egeria densa* Planch.) to ensure that they were not satiated. If the palatable macrophyte was rejected, that replicate animal was not included in the assay because it appeared satiated. Because grass carp were kept in small groups, we report results from the first fish that fed in each bucket as a replicate (N = 12–15 separate buckets). Order of macrophyte presentation was randomized separately for each replicate consumer. We then analyzed (using a Fisher's exact test) the proportion of animals that were willing to feed on each individual macrophyte species relative to the palatable control.

Low palatability of fresh macrophyte tissues could result from structural, morphological, nutritional, chemical, or other characteristics. To determine whether macrophyte morphology could account for feeding preferences, we destroyed morphological traits by incorporating freeze-dried and finely ground macrophyte tissues into gel-based foods constructed with 30% sodium alginate by dry mass (Hay et al., 1998). We added enough macrophyte powder to the paste to approximate the same dry mass per volume of macrophyte found in tissues from each species of macrophyte being assayed (see Macrophyte Traits). The gel was then coated onto the interior wall of a glass Petri dish and immersed in a hardening solution of 0.25 M calcium chloride. After approximately 1 min, the gel was removed, rinsed in water, and cut into bite-sized portions. This method resulted in reconstituted macrophytes with similar morphologies and a soft, fleshy texture not unlike cooked pasta. Nutritional values and chemical defenses should have remained similar to those of intact macrophytes (however, freeze-drying can alter the structure and the activity of some metabolites; Cronin et al., 1995). These artificially softened macrophytes were then assayed against a palatable control food—a 1:1 mixture of freeze-dried and powdered broccoli and lettuce ("broc-let") that herbivores readily accept as food (Bolser et al., 1998). Broc-let content matched the dry mass per volume of each macrophyte being assayed. Feeding on pellets was recorded as the frequency of acceptance or rejection of treatment or control pellets, with these pellets being offered alternately. We analyzed (via Fisher's exact test) the proportion of animals feeding on each individual reconstituted macrophyte species relative to the palatable control.

If gel-based treatments were unpalatable, this suggested a chemical basis for feeding rejection. We tested for chemical defenses by conducting feeding assays with crude extracts from each macrophyte incorporated into broc-let based sodium-alginate pellets as above (see above and Hay et al., 1998 for a general review). Extracts were acquired by macerating fresh macrophyte tissues in a 1:1 mixture of water and methanol overnight, then successively extracting the macrophyte material for at least 2 hr in 1:1 and 1:2 methanol/dichloromethane. The extracts were combined, and solvents were removed under vacuum to yield a crude extract. For food preparation, each crude extract was dissolved in acetone, incorporated into broc-let powder and sodium alginate, and the solvent was evaporated by vigorous stirring in a fume hood. Control foods were treated identically (including addition of

acetone) but without the addition of crude extracts. The dry mass content of treatment and control pellets matched the dry mass per volume content of each macrophyte being assayed. Pellets were fed to animals and the results were statistically analyzed as described above.

Macrophyte Traits

We measured macrophyte traits that are thought to be indicative of macrophyte nutritional quality or availability as a food, including: toughness, dry mass/volume, ash-free dry mass/volume, soluble protein/volume, and soluble protein/dry mass. Dry mass/volume was determined by drying 3–8 replicate samples of known volume at 60°C for at least 2 d; ash-free dry mass/volume was determined by combusting these same samples at 450°C for at least 6 hr. Toughness was estimated by using a penetrometer (see Duffy and Hay, 1991) to determine the mass required to pierce a leaf with a needle. Two of the five macrophytes could not be adequately tested with this approach. The rush *J. effusus* was too tough to pierce with our penetrometer; the strands of the green alga *Spirogyra* were too thin to accept the needle.

Soluble protein content was estimated with the Bradford (1976) method. Triplicate composite samples of ground macrophyte material from each species (~5 mg) were digested in 1 ml sodium hydroxide (1 mol/l) for 24 hr at 2.5°C, centrifuged, and 100-μl aliquots of the supernatant were added to 5-ml samples of Bradford reagent. After 10–15 min, absorbance of each sample at 595 nm was measured using a Spectronic 21D spectrophotometer against bovine serum albumin (BSA) standards.

Results were analyzed with ANOVA followed by Tukey multiple comparison tests, with transformations (log + 1) to correct heteroscedastic variances when necessary. Protein analyses were conducted on pooled samples of tissues from many individual plants. These data were not statistically analyzed because variances associated with the means were methodological rather than associated with differences among individual, replicate plants.

Bioassay-Guided Fractionation

M. umbrosum was the only macrophyte species with consistent evidence for a strong chemical defense. To separate and identify the defensive compounds, we used bioassay-guided fractionation of the total crude extract by assessing the feeding response of the crayfish *P. spiculifer*. We used crayfish rather than grass carp for these assays because grass carp had not yet acclimated to feeding in the laboratory when we began this fractionation. We did, however, use all three consumer species to test the deterrence of each isolated metabolite and also of the remaining crude extract minus these compounds. Extracts were initially tested at twice their extracted concentrations (by volume) to offset loss due to inefficient extractions and/or compound decomposition. Chromatographic fractions and pure compounds were tested by offering crayfish broc-let based pellets incorporated with fractions or compounds vs. control foods; results were statistically analyzed with Fisher's exact tests.

Fresh tissues of *M. umbrosum* were extracted successively with dichloromethane, acetone, and methanol, and these extracts were combined to produce a crude extract. The deterrent crude extract was fractionated by using silica gel flash chromatography (40–63 μm Aldrich silica gel eluting with increasing concentration

of ethyl acetate in petroleum ether). The resulting 36 fractions were grouped by similar thin layer chromatography (TLC) properties into seven fractions, of which two were deterrent. The deterrent component within the less polar deterrent fraction was purified by repeated flash chromatography, as described above, followed by silica gel high-performance liquid chromatography (HPLC) with hexane/ethyl acetate as the mobile phase. The more polar deterrent fraction yielded two deterrent pure compounds via: (1) repeated silica gel flash chromatography eluting with toluene/ethyl acetate or toluene/petroleum ether/ethyl acetate; (2) recrystallization from hexanes/methanol/toluene (3:2:1); and (3) silica gel HPLC eluted with hexane/ethyl acetate, using a Zorbax RX-SIL HPLC column (9.4 × 250 mm; 5 μm) attached to a Waters Breeze HPLC system consisting of Waters 515 pump and Waters 2487 UV detector recording at 210 and 254 nm. A third fraction from the initial flash column separation did not initially deter crayfish feeding, but unusual ^{1}H NMR signals motivated the purification of a fourth compound by flash column chromatography and HPLC as described above.

Pure compounds from each fraction were identified on the basis of ^{1}H, ^{13}C, and 2D NMR spectroscopy, and comparisons of NMR, IR, and mass spectral data with literature data. Optical rotations were obtained using a Jasco P-1010 polarimeter. IR data were acquired on a Nicolet 520 FTIR spectrophotometer with thin films on NaCl plates. ^{1}H, ^{13}C, and 2D NMR spectral data were obtained on a Bruker Avance DRX 500 MHz spectrometer using $CDCl_3$ referenced to residual $CHCl_3$ (δ 7.28).

Quantification of Isolated Compounds

To determine whether our isolated yields were comparable to the natural concentrations in plant tissues, we quantified the concentrations of each of the four compounds from five separate individuals of *M. umbrosum* collected at the same time and under the same conditions as the bulk material used in this study. Frozen plants were individually extracted with a 1:1 mixture of water and methanol, then successively for at least 2 hr in 1:1 and 1:2 methanol/dichloromethane.

Quantification of natural products was achieved by LC-MS/MS using a Micromass Quattro triple quadrupole mass spectrometer in conjunction with an Agilent 1100 HPLC. A reversed-phase Zorbax eclipse XDB-C8 column (1.0 × 150 mm, 3.5 μm) was used to separate the natural products with a gradient system of water/acetonitrile (0.1% formic acid) 95:5 (v:v) to 5:95 (v:v) over 31 min. Three or four standard solutions (0.0001–0.10 mg/ml) of each of the four pure compounds were used to measure sample concentration by integration of the peaks areas for monitored transitions arising from dissociation of $[M + H]^+$ precursor ions to a structure-specific fragment ion for each compound. These data were used to establish standard curves ($R^2 > 0.99$ for each compound) for the quantification of compounds in crude extracts of the five macrophyte samples. Once natural concentrations were known, we tested the effects of each compound at its natural concentration and at its isolated yield with each of our three consumers species.

Experimental Exclusion of Herbivores and Tests of Herbivore Preference among Plants

To determine whether the chemically defended *M. umbrosum* was disadvantaged relative to less-defended plants in the absence of herbivory, we excluded grass carp

and other potential consumers from caged areas of the pond for 16 mo and measured the abundance of plants in caged vs. control areas. On April 26, 2004, we established five blocks in the pond with three treatments in each block: (1) an uncaged treatment allowing full herbivore access, (2) a three-sided cage control allowing herbivore access but controlling for cage artifacts, and (3) 2 four-sided cages excluding herbivores. Each block had 2 four-sided cages because we had originally intended to establish another treatment in one of the cages. We never imposed this treatment, thus, both cages were considered replicates in the same block to calculate the cage effect. Each treatment area was $0.9 \times 0.9 \times 0.9$ m, with the cage control and cage areas marked by 1.0-m-tall steel rebar posts. Cages were constructed of 3.0-mm plastic mesh affixed to the rebar posts with cable ties. A 10- to 15-cm skirt was anchored around each cage to prevent consumers from burrowing under the mesh walls. On only one occasion did we encounter grass carp in the cages; both fishes were removed and were likely too small (< 2 cm in length) to have begun feeding on macrophytes given that grass carp typically do not become herbivorous on macrophytes until they reach approximately 3 cm in length (Hickling, 1966). Treatment blocks were established in linear arrays separated from each other by at least 4.0 m along the shoreline that was not used to seine fish. Treatments were randomly assigned to each position in the block, with the restriction that the open treatment was on either end of the block. Watermarks on the cage walls suggested that the average treatment depth was approximately 15 cm, but we observed that cages were occasionally dry or up to 30 cm deep, consistent with the variability of water depth that we observed in natural ponds in the area. Poor water clarity, however, prevented monitoring of plant cover when water depth exceeded approximately 10 cm.

We estimated initial plant cover in the treatments by determining the identity of macrophytes located beneath 36 points in a 0.25-m^2 quadrat placed directly in the center of each cage or open treatment. We analyzed the initial total plant cover and the initial cover of the two species (*M. umbrosum* and *L. repens*) that were most abundant at the end of the experiment with a blocked one-way ANOVA, transforming (log + 1) to correct heteroscedastic variances (determined via Cochran's tests) when necessary.

On August 29, 2005, we harvested all of the aboveground plant material from each cage and weighed it to the nearest gram. We analyzed the total aboveground biomass and the biomass of the two most common plant species in our treatments with a blocked one-way ANOVA, transforming (log + 1) to correct heteroscedastic variances (determined via Cochran's tests) when necessary. Significant ANOVA results were followed by multiple comparisons (Tukey tests) among treatment means.

Although our earlier laboratory feeding assays allowed us to determine which plant species were unpalatable, they were unsuitable for determining preference hierarchies among plant species. Thus, we conducted choice-feeding assays comparing grass carp preference for *M. umbrosum*, the plant that dominated cover in one of the grass carp ponds, with *L. repens*, a formerly rare plant that heavily recruited into our exclusion cage treatments (see Results). We also compared grass carp preference for *M. umbrosum* vs. *Najas guadalupensis*, and *L. repens* vs. *N. guadalupensis*; we chose *N. guadalupensis* because it dominated (>80% cover, $N = 20$ quadrats) the cover of a nearby (~300 m distant) pond of natural origin that did not have grass carp. We hypothesized that the dominant plants from the grass

carp pond (*M. umbrosum* and *L. repens*) would be of lower preference than the dominant plant (*N. guadalupensis*) from a habitat that lacked grass carp, and that *M. umbrosum* would be of lower preference than a plant that recruited only to cages where we excluded grass carp.

Each replicate assay consisted of placing a binder clip with a 2.0-cm portion of each of two plant species into 18 buckets containing 2–5 grass carp. Each replicate was periodically checked to determine which plant had been eaten first, with all treatments harvested the following morning. We did not retain the replicates where both plants had been eaten, as we could not determine which plant had been eaten first. We analyzed the number of occasions in which each species was eaten first with Fisher's exact tests.

Results

Macrophyte Abundance

In late April 2004, the littoral fringe of both grass carp ponds was largely unvegetated (mean ±1 SE of bare space; Pond 1 = 76.7 ± 10.6%, Pond 2 = 80.6 ± 10.3%, both $N = 5$), but macrophyte cover in each of the ponds was dominated by a single species (Pond 1: *M. umbrosum* = 86.5 ± 6.8% of total plant cover; Pond 2: *Spirogyra* = 94.4 ± 5.6% of total plant cover). Of the remaining four macrophyte species, *J. effusus* represented 9.8 ± 5.3% of total plant cover in Pond 1 and 2.8 ± 2.8% in Pond 2, *J. repens* comprised 2.8 ± 2.8% in Pond 2, and there were trace amounts of *L. repens* in Pond 1. A single individual of the sedge *Carex* sp. occurred in Pond 1; because this was only one individual, we did not include this species in our feeding assays. No other aquatic macrophytes were observed in the ponds.

Feeding Assays

When offered as fresh macrophyte tissues, each of the five macrophyte species assayed was unpalatable relative to a control food to at least two of the three consumer species tested (Fig. 1A). Of the two most common macrophytes, *M. umbrosum* was significantly less palatable than control food (*E. densa*) to all three consumers, whereas *Spirogyra* was significantly less palatable to *Procambarus acutus* and *C. idella*, but not to *P. spiculifer*. Although relatively uncommon in the ponds, *L. repens* also was significantly less palatable to all three consumers than was the control food. After we destroyed plant morphological traits, palatability increased for some macrophytes, but feeding on *M. umbrosum*, *Spirogyra*, and the rush *J. repens* remained similar to that on intact plants (Fig. 1B). When the crude extracts from macrophytes were incorporated into a palatable control food, only *M. umbrosum* remained unpalatable—suggesting a strong chemical deterrent to feeding by all three consumers (Fig. 1C).

Macrophyte Traits

Table 1 shows toughness, dry mass, ash-free dry mass, and protein content of the macrophytes examined. *M. umbrosum* was the softest macrophyte that we tested with the penetrometer, was of intermediate rank in dry mass and in soluble protein

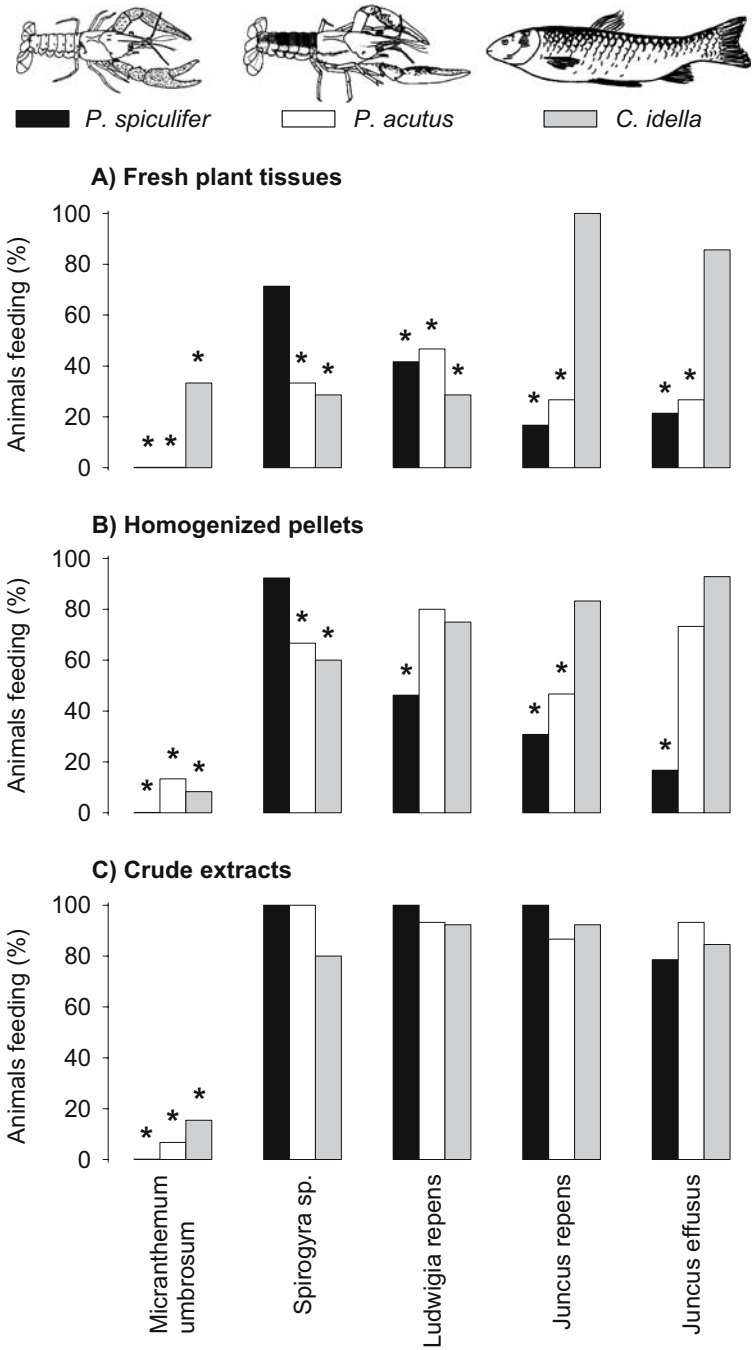

Fig. 1 Percentage of 12–15 individual *Procambarus spiculifer* (filled bars), *P. acutus* (open bars), and *Ctenopharyngodon idella* (gray bars) feeding on (A) fresh macrophyte tissues, (B) homogenized macrophyte pellets at natural dry mass content, and (C) crude extracts from five aquatic macrophyte species. Asterisks denote statistically significant ($P < 0.05$) reductions in feeding relative to a palatable control (*Egeria densa*) for each consumer species (Fisher's exact tests)

Table 1 Mean (± SE) and sample sizes (in parentheses) for each analysis of selected macrophyte traits*

Macrophyte	Mass to pierce (mg)	Dry mass/ vol. (mg/ml)	Ash-free dry mass/vol. (mg/ml)	Soluble protein (mg/ml)	Soluble protein (% dry mass)
Micranthemum umbrosum	5.40 ± 0.768 (5)a	88.0 ± 7.57 (8)b	25.6 ± 5.30 (8)b	4.83	5.49
Spirogyra sp.	Too thin to test	45.2 ± 4.90 (4)a	7.26 ± 1.95 (4)a	3.06	6.77
Ludwigia repens	9.19 ± 1.31 (5)a	73.3 ± 10.1 (5)ab	13.7 ± 2.44 (5)ab	4.77	6.51
Juncus repens	13.3 ± 1.04 (5)b	142 ± 14.3 (5)c	24.4 ± 4.46 (5)b	6.96	4.90
Juncus effusus	Too hard to test	80.3 ± 9.97 (5)b	10.5 ± 2.54 (5)ab	4.81	5.99
Broc-let control	N/A	55.2 ± 1.41 (3)	6.27 ± 0.475 (3)	4.04	7.32
ANOVA *P* values	0.001	0.001	0.004	N/A (Composite samples)	N/A (Composite samples)

*Species that share a letter within a column are not significantly different from one another in unplanned comparisons following ANOVA; broc-let not included in analyses.

per volume of plant, but had the highest ash-free dry mass per volume of macrophyte. *Spirogyra* could not be tested with the penetrometer because of its filamentous morphology, but it has no obvious structural barriers to grazing. *Spirogyra* was generally nutritionally poor relative to the other plants; it ranked lowest in dry mass, ash-free dry mass, and protein content per volume of plant (Table 1). *L. repens* was relatively soft and of intermediate to low rankings in mass and protein content. The prostrate rush *J. repens* was the toughest macrophyte that we could test, and it had the highest dry mass, second highest ash-free dry mass, and protein content when measured volumetrically, but the lowest protein content when expressed as a % of dry mass. The emergent rush *J. effusus* was too tough to test with the penetrometer, and had intermediate dry mass per volume, relatively low ash-free dry mass per volume, and moderately low protein content. Our palatable control food, a 1:1 mixture of powdered broccoli and lettuce (broc-let), had relatively low dry mass, ash-free dry mass, and protein content per volume of plant, but it had the highest protein content of all the foods when expressed as a % of dry mass (Table 1).

Bioassay-Guided Fractionation

The crude extract of *M. umbrosum* strongly deterred feeding by *P. spiculifer* (Fig. 2). Two of the initial seven fractions from this extract strongly reduced crayfish feeding (fractions B and E, Fig. 2). Purification of the active component in fraction B via three silica gel chromatographic columns revealed 3,4,5-trimethoxyallylbenzene (elemicin) (**1**) as the bioactive metabolite (Fig. 2). Similar bioassay-guided separation of fraction E, followed by HPLC purification, led to identification of the deterrent compounds β-apopicropodophyllin (**2**) and (−)-(3*S*,4*R*,6*S*)-3-(3′,4′-meth-

Fig. 2 Bioassay-guided fractionation of crude extracts from *Micranthemum umbrosum*. Each graph shows the percentage of 12–15 individual *P. spiculifer* feeding on a solvent-only control food (open bar) vs. control food containing macrophyte extracts. Shaded graph panels denote statistically significant ($P < 0.05$) feeding reductions relative to the palatable control (Fisher's exact tests). See Methods and Materials for mobile phases and chromatographic details

ylenedioxy-α-hydroxybenzyl)-4-(3′,4′-dimethoxybenzyl)butyrolactone (**3**) (Fig. 2). Although fraction C did not initially deter crayfish feeding (Fig. 2), unusual ^{1}H NMR signals motivated the purification of (−)-hibalactone (**4**), also known as (−)-savinin, from this fraction (Fig. 3).

Quantification of Compounds **1–4**

We initially tested the deterrent fractions at twice their yield (by volume) to make up for assumed losses during purification, but compound quantification by LC-MS/MS showed that even doubling the presumed natural concentration did not approach the actual concentration occurring in the crude extract for each of the four compounds assayed (Table 2). The isolated yields of compounds **1** and **2** were 19% and 16% of their natural concentrations, respectively, while compounds **3** and **4** were isolated at only 1% and 8% of their natural concentrations, respectively.

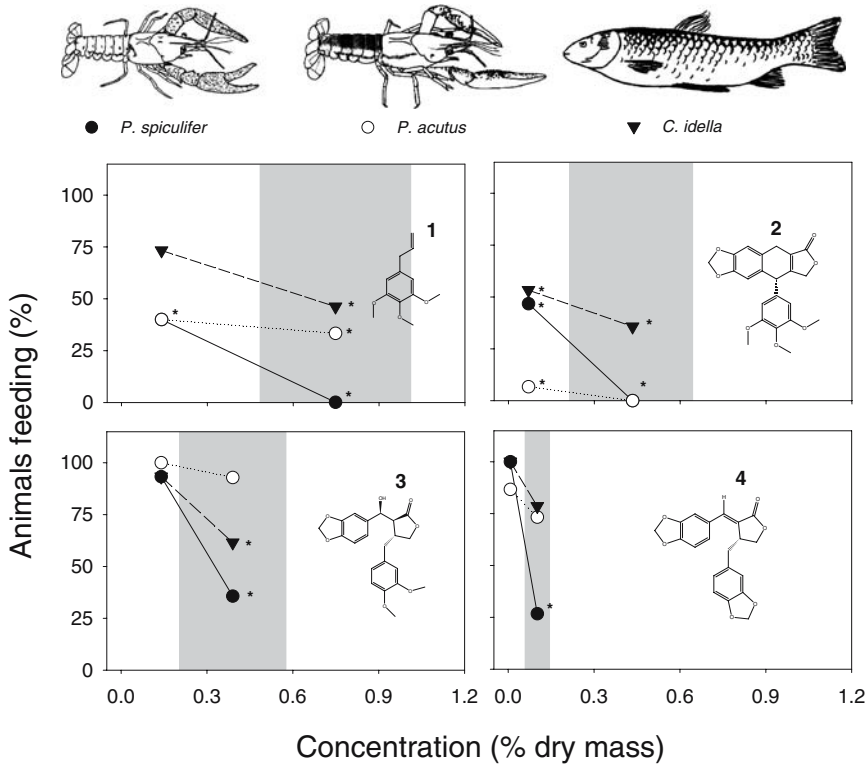

Fig. 3 Percentage of 12–15 individual *P. spiculifer* (filled circles), *P. acutus* (open circles), and *Ctenopharyngodon idella* (filled triangles) feeding on pellets containing (A) 3,4,5-trimethoxyallylbenzene (**1**); (B) β-apopicropodophyllin (**2**); (C) (−)-(3*S*,4*R*,6*S*)-3-(3′,4′-methylenedioxy-α-hydroxybenzyl)-4-(3″,4″-dimethoxybenzyl)butyrolactone (**3**); and (D) (−)-hibalactone (**4**). Asterisks denote statistically significant reductions in feeding relative to a palatable control for each consumer species ($P < 0.05$; Fisher's exact tests). The shaded area is the quantified natural concentration (by dry mass) ± 1 standard deviation (see Table 2); feeding assays to the left of this shaded area were conducted at the isolated yield of each metabolite

When elevated to their natural concentrations, compounds **1** and **2** were both deterrent to all three consumers (Fig. 3). In contrast, when we elevated compounds **3** and **4** to their natural concentrations, compound **3** was deterrent to *P. spiculifer* and to grass carp, but not to *P. acutus*, whereas compound **4** was deterrent to *P. spiculifer* but not to the other consumers (Fig. 3). For three of the four

Table 2 Isolated yield and quantitatively determined dry mass concentrations (% of dry mass ± SD) of four deterrent compounds isolated from the crude extract of *Micranthemum umbrosum*

Quantification method	Concentration of compound (% of macrophyte dry mass)			
	1	2	3	4
Isolated yield ($N = 1$)	0.14	0.070	0.0045	0.0078
LC-MS/MS ($N = 5$) quantification	0.75 ± 0.26	0.43 ± 0.22	0.39 ± 0.19	0.10 ± 0.04

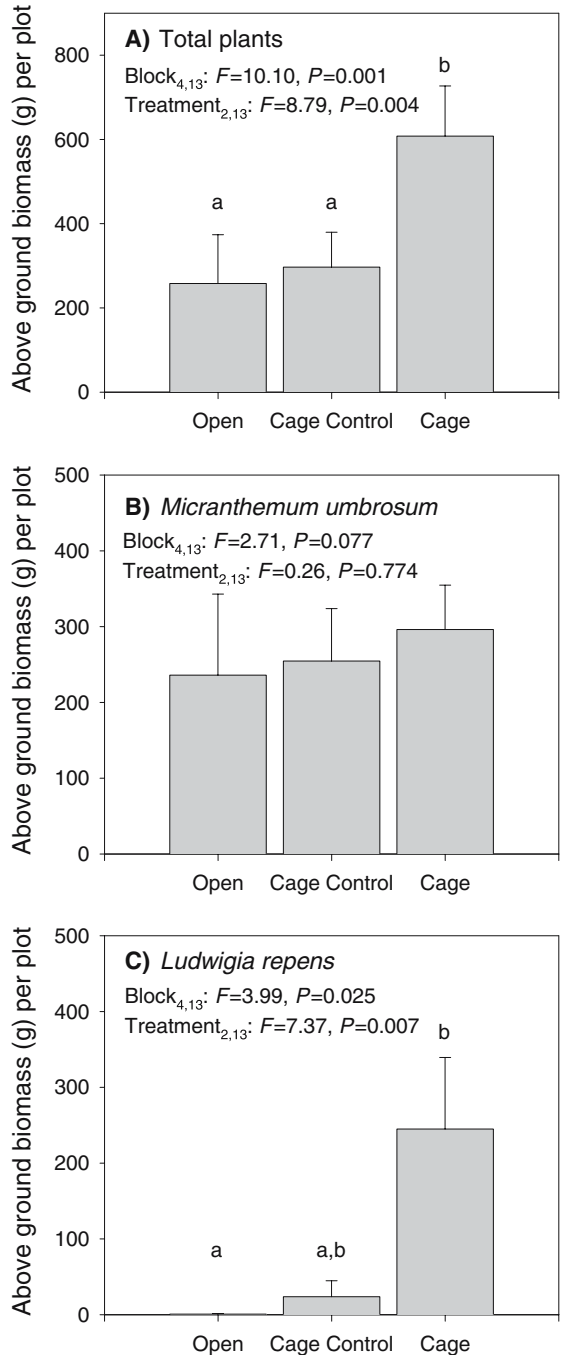

Fig. 4 Final aboveground biomass per plot of (A) all plants, (B) *M. umbrosum*, and (C) *Ludwigia repens* in open, cage control, and cage treatments after 16 months. Statistical results are from one-way blocked ANOVAs. Bars that share the same letter were not statistically different ($P < 0.05$) from one another in Tukey's tests

compounds tested, the magnitude of feeding depression appeared stronger for *P. spiculifer* than for the other consumers (Fig. 3).

We also tested whether we had isolated all of the strongly deterrent compounds by assaying the crude extract minus the fractions containing the four isolated compounds (i.e., we used TLC to group fractions from the first silica gel column that lacked compounds **1**–**4**). None of our three test consumers were significantly deterred by this "crude minus deterrent fractions" extract (N = 13–15 for each consumer species, % acceptance $\geq$ 86.7%, $P \geq 0.50$). However, given the significant compound degradation and/or inefficient yields that we observed (Table 2), it is possible that unknown, but potentially deterrent, compounds within this crude extract were tested at concentrations significantly lower than their natural levels.

Experimental Exclusion of Herbivores

At the initiation of the experiment, there was no difference in total plant cover (P = 0.833), the cover of *M. umbrosum* (P = 0.089), or the cover of *L. repens* (P = 0.641) among the open, cage control, and cage treatments (data not shown). After 16 mo of excluding grass carp and other potential herbivores (e.g., crayfish, turtles), there was 2.4-fold more total plant biomass (P = 0.004, Fig. 4A) and over 300-fold more *L. repens* (P = 0.007, Fig. 4C) in the cage vs. open treatments. Biomass of the unpalatable macrophyte *M. umbrosum* was unaffected (P = 0.774, Fig. 4B). Thus, herbivore exclusion allowed other species to increase in abundance but did not alter the abundance of the chemically defended *M. umbrosum*.

When offered a choice between two plant species, grass carp preferred *L. repens* over *M. umbrosum* by 14 to zero ($P < 0.001$), *N. guadalupensis* over *M. umbrosum* by 11 to zero ($P < 0.001$), and *N. guadalupensis* over *L. repens* by 14 to zero ($P <$ 0.001). The striking differences in preference for all contrasts clearly establishes a preference hierarchy of *N. guadalupensis* > *L. repens* > *M. umbrosum*.

Discussion

It is a common pattern in marine and terrestrial habitats for selective feeding by herbivores to shift plant species composition toward chemically or structurally defended plants (Hay and Fenical, 1988; Rosenthal and Berenbaum, 1992; Hay, 1997). In contrast, although aquatic herbivores commonly reduce plant standing stock and alter species composition (Lodge, 1991; Newman, 1991; Cyr and Pace, 1993; Lodge et al., 1998), experimental investigations linking herbivore feeding preferences to particular plant traits and ultimately to shifts in plant community structure are rare. Here, we show that five of the most common macrophytes collected from an aquaculture facility for herbivorous Asian grass carp, *C. idella,* were commonly unpalatable to three generalist consumers—nonnative grass carp and the native North American crayfishes *P. spiculifer* and *P. acutus*. The most common macrophytes—*M. umbrosum* and *Spirogyra* sp.—comprised 87% and 94%, respectively, of the total macrophyte cover in two grass carp ponds, and both were unpalatable to grass carp (Fig. 1). *Spirogyra* appeared nutritionally inadequate to these consumers, and *M. umbrosum* was chemically defended by at least four secondary metabolites (Figs. 2 and 3). When we excluded grass carp and other potential herbivores from experimental portions of one of the ponds, a plant that

was preferred over *M. umbrosum*—*L. repens*—increased over 300-fold in the herbivore exclusion treatment. A nearby natural pond that lacked grass carp was dominated by *N. guadalupensis,* a plant that grass carp preferentially consumed over both *M. umbrosum* and *L. repens*. Thus, selective feeding by grass carp effectively eliminates most palatable plants from this community and promotes the persistence of less palatable, chemically defended or nutritionally inadequate plants.

Grass carp and most crayfish species are generalist consumers that will eat a variety of plants (Parker and Hay, 2005) but still selectively feed among species based on their structural, nutritional, and chemical traits (Cronin et al., 2002). However, knowledge of traits alone may not be predictive of feeding preferences among different consumer species (e.g., Hay et al., 1987; Hay and Fenical, 1996). For example, both the grass carp and crayfish *Procambarus acutus* rejected the filamentous green alga *Spirogyra* (Fig. 1). The crayfish *P. spiculifer*, however, readily consumed *Spirogyra* (Fig. 1), and in another study the crayfish *Pacifastacus leniusculus* preferred it over other aquatic plants (Warner and Green, 1995). Among the five plant species that we tested, *Spirogyra* had the lowest protein content per volume of plant (Table 1), suggesting that nutritional inadequacy may explain its low palatability to *P. acutus* and to grass carp, but the variation among consumers (Fig. 1; Warner and Green, 1995) suggests that palatability depends on the palate of the consumer, or on considerable intraspecific variance in the defensive traits of the plants studied (e.g., Taylor et al., 2003). Moreover, *Spirogyra* and other filamentous algae reportedly persist in these and other ponds only until submersed macrophytes have been selectively removed by grass carp (P. Williams, personal communication; Van Dyke et al., 1984). This suggests that plants can delay or reduce herbivory by being nutritionally poor, but they may be unlikely to escape consumption once higher preference plants have been removed.

It is uncertain why *L. repens* was rejected in the fresh tissue assays (Fig. 1). It was readily eaten over *M. umbrosum* in choice assays with fresh tissue, was readily eaten by all but one consumer as homogenized pellets (Fig. 1B), and was eaten by all consumers when extracts were incorporated into a palatable control food (Fig. 1C). It could be that our methodology of offering bite-sized pieces rather than whole plants altered the acceptability of this or other plant species. The prostrate rush *J. repens* and the emergent rush *J. effusus* were both tough plants that may have been structurally defended from consumption by crayfishes (Table 1, Fig. 1). However, at least one crayfish species rejected the softened, homogenized pellets of each species (Fig. 1B), although no consumers rejected the chemical extracts (Fig. 1C). Thus, it appears that both rushes could be structurally defended, but we cannot exclude the possibility that deterrent compounds in the softened foods were lost during the extraction process. Alternatively, the higher protein content of broc-let powder (Table 1) may have provided extra feeding incentives that counterbalanced deterrent chemistry. Other investigations have shown that consumers are more likely to feed on chemically defended foods if they are nutritionally rich (Duffy and Paul, 1992; Cruz-Rivera and Hay, 2003).

Despite these ambiguities for some consumer and macrophyte species, *M. umbrosum* was clearly chemically distasteful to all three consumers (Fig. 1), and we isolated four natural products that serve as chemical defenses against herbivory (Figs. 2 and 3). Each of the four compounds has previously been described, but this is the first study to report on their ecological function as defenses against herbivory. Compound **1** is an essential oil commonly found in aromatic plants including nutmeg

and parsley (De Vincenzi et al., 2004). It has been implicated as an antimicrobial compound (Marston et al., 1995), a growth inhibitor of green algae (Della-Greca et al., 1992), and as an insecticide (Miyazawa et al., 1992). β-Apopicropodophyllin (**2**) has previously been isolated from the Mexican medicinal plant *Hyptis verticillata* "bushmint," and is from a class of lignoids active against several cancer cell lines (e.g., Novelo et al., 1993). Compound **3** has been synthesized but was not previously known as a natural product (Pelter et al., 1988). Lignan **4** occurs in juniper and several woody plant species (e.g., Hartwell et al., 1953); it inhibits prostaglandin E_2 production (Ban et al., 2002), tumor necrosis factor-α production and T-cell proliferation (Cho et al., 2001), and is a synergist for insecticides (Matsubara, 1972). We lost from 81% to 99% of the natural concentrations of these four molecules during isolation procedures (Table 2). Given this poor yield, it is possible that additional deterrents were present but recovered at concentrations too low to be biologically active.

Prior to this study, there were only three freshwater plants with described herbivore feeding deterrents—watercress, *R. nasturtium-aquaticum* (L.) Hayek (Newman et al., 1990, 1996); the waterspider bog orchid, *H. repens* Nutt. (Bolser et al., 1998; Wilson et al., 1999); and lizard's tail, *S. cernuus* L. (Kubanek et al., 2000, 2001)—with a total of nine described secondary metabolites demonstrated to influence herbivore feeding. Our study brings the new total of described herbivore antifeedants in freshwater plants to 13. Of these 13, 10 are lignoids, including three in this study and seven compounds isolated from *S. cernuus* (Kubanek et al., 2001). This general, though still preliminary, pattern suggests that lignoids—of which several thousand have been described from numerous plant taxa (Seigler, 1998; Ward, 1999)—are common, but often overlooked, defensive compounds warranting additional study.

Plant defense theory predicts that chemically defended plants have fewer resources for growth and will be competitively displaced by less defended plants when herbivore pressure is lessened (Herms and Mattson, 1992). To test this, we excluded grass carp for 16 mo and documented a 300-fold increase in the abundance of *L. repens* (Fig. 4), a plant that was preferred over *M. umbrosum* in a choice feeding assay. However, we did not observe a decrease in the abundance of *M. umbrosum* in the cage treatments (Fig. 4). Thus, although chemical defenses in *M. umbrosum* appear to promote its persistence in the face of intense herbivory, we saw little evidence to suggest competitive displacement of *M. umbrosum* by *L. repens* in the absence of herbivores. There are several potential explanations. Our experiments ran through two growing seasons, but the long history (>20 yr) of grass carp herbivory in this habitat may have consistently excluded other species and reduced the potential pool of new colonists exhibiting high-growth, low-defense strategies. In support of this hypothesis, *M. umbrosum* typically takes at least 4 yr to recruit into new ponds in this system, after which it persists indefinitely (P. Williams, personal communication). Additionally, the only species that showed a large increase in abundance—*L. repens*—is also relatively unpalatable (Fig. 1), and may not be a much better competitor than *M. umbrosum*. Moreover, despite the long-standing view that constructing and storing defensive compounds is physiologically costly and detracts from growth and reproduction, empirical evidence is conflicting (Koricheva, 2002), suggesting that investment in chemical defense need not necessarily restrain growth and competitive ability (Cronin, 2001). Finally, grass carp will repeatedly sample foods even if they do not ingest the plants (P. Williams,

personal communication); this chronic sampling may have depressed *M. umbrosum* abundance in the open and cage control treatments and obscured competitive effects in the cage treatments. Nevertheless, on a percentage basis, excluding herbivores led to dramatic increases in *L. repens* that reduced the relative abundance of *M. umbrosum* from 89% to 54% of the total plant community, indicative of chemical defenses promoting the relative dominance of *M. umbrosum* in this community.

Herbivory in freshwater systems is more important than previously thought (Lodge and Lorman, 1987; Newman, 1991; Cyr and Pace, 1993; Lodge et al., 1994, 1998; McKnight and Hepp, 1995), and freshwater plants are frequently chemically or structurally defended from consumers (Newman et al., 1996; Bolser et al., 1998; Cronin, 1998; Kubanek et al., 2001; Cronin et al., 2002; Prusak et al., 2005). Rarely, however, have the mechanisms of deterrence (e.g., structural or chemical defenses) been linked to the broader context of community structure. Here, we show that selective herbivory by grass carp shifts the species composition of freshwater plant communities toward plants that are distasteful, structurally defended, or nutritionally inadequate (Figs. 1 and 4), suggesting that plant defenses can play critical yet understudied roles in the structure of freshwater plant communities.

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