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Stream mosses as chemically-defended refugia for freshwater macroinvertebrates

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Marine and terrestrial studies show that small, sedentary herbivores that utilize plants as both food and habitat can gain enemy-free space by living on hosts that are chemically defended from larger, generalist consumers. Although large herbivores are increasingly recognized as important consumers of macrophytes in freshwater communities, the potential indirect effects of herbivory on plant-associated macroinvertebrates have rarely been studied. Here, we show that the large, generalist consumers in a riverine system, Canada geese, *Branta canadensis*, and crayfish, *Procambarus spiculifer*, both selectively consumed riverweed, *Podostemum ceratophyllum*, over an aquatic moss, *Fontinalis novae-angliae,* even though moss comprised 89% of the total plant biomass on riverine rocky shoals. Moss supported twice as many plant-associated macroinvertebrates as riverweed, suggesting that it might provide a spatial refuge from consumption by these larger consumers. Bioassay-guided fractionation of moss extracts led to the isolation of a C_{18} acetylenic acid, octadeca-9,12-dien-6-ynoic acid, that deterred crayfish feeding. In contrast to results with Canada geese and crayfish, both the amphipod *Crangonyx gracilis* and the isopod *Asellus aquaticus* consumed significant amounts of moss but rejected riverweed in laboratory feeding assays. Moreover, neither amphipod nor isopod feeding was deterred by the crude organic extract of *Fontinalis*, suggesting that these mesograzers tolerate or circumvent the chemical defenses that deterred larger consumers. Thus, herbivory by large, generalist herbivores may drive freshwater plant community structure towards chemically defended plants and favor the ecological specialization of smaller, less mobile herbivores on unpalatable hosts that represent enemy-free space.

Large, generalist herbivores can profoundly alter plant communities by selectively consuming palatable species and avoiding chemically or structurally defended plants (Hay and Fenical 1988, Crawley 1989, Rosenthal and Berenbaum 1992, Lodge et al. 1998). In many cases, however, even the most unpalatable plants are still colonized and consumed by a diverse suite of smaller, often more specialized herbivores that use plants as both habitat and food (e.g. insects) (Strong et al. 1984, Hay 1992). Although the distribution of specialized insects on their hosts can be related to intrinsic differences in plant quality (Ehrlich and Raven 1964), extrinsic factors may also affect host-preference (Jeffries and Lawton 1984, Murphy 2004). In systems with large herbivores, for example, small grazers are at risk of being eaten when living on plants that are palatable to these larger consumers; thus, living on host plants that are well defended from large herbivores can allow smaller grazers to escape incidental predation (Duffy and Hay 1994, Hay 1996). This provision of enemy-free space is hypothesized to have played a large role in the evolutionary radiation of specialist herbivores onto chemically-defended marine and terrestrial plants (Hay et al. 1987, Bernays and Graham 1988, Hay 1992, Singer and Stireman 2005).

Herbivores often remove as much or more plant standing stock from freshwater systems as from marine and terrestrial systems (Cyr and Pace 1993, Lodge et al. 1998). Selective feeding by large, generalist herbivores, including waterfowl (Sondergaard et al. 1996, Van Donk and Otte 1996, Weisner et al. 1997, Santamaria 2002), crayfish (Lodge and Lorman 1987,

Lodge 1991, Lodge et al. 1994, Dorn and Wojdak 2004), mammals (Qvarnemark and Sheldon 2004), and fish (Van Donk and Otte 1996), can alter the abundance and species composition of freshwater plant communities and drive plant assemblages towards dominance by chemically defended plants (Parker et al. 2006). Although macroinvertebrates are often abundant on submersed aquatic plants (Brusven et al. 1990, Bowden 1999, Hutchens et al. 2004), and suffer intense predation from omnivorous crayfish (Lodge et al. 1994), fish (Sheldon 1987, Johansson 1991, Flecker and Townsend 1994), and waterfowl (Marklund et al. 2002), the feeding and host-plant preferences of small, plant-associated grazers relative to the feeding preferences of larger consumers in the same freshwater system have rarely been considered.

Here, we addressed whether small, sedentary herbivores in a riverine environment preferentially resided and fed on plants that were chemically repugnant to large herbivores. We asked the following questions: 1) how does plant abundance on riverine rocky shoals correlate with palatability to Canada geese and crayfish? 2) Are small, plant-associated macroinvertebrates more abundant and more likely to feed on a plant that is unpalatable to geese and crayfish? 3) Does plant secondary chemistry differentially affect feeding preferences of crayfish vs macroinvertebrates?

Methods

Study area and sampling of vegetation and macroinvertebrates

Investigations were conducted in the Chattahoochee River National Recreation Area near Atlanta, Georgia, USA. The rocky shoals of the Chattahoochee are covered by dense stands of plants, including riverweed, *Podostemum ceratophyllum*, a vascular plant that attaches directly to the rocky substratum via holdfasts, and *Fontinalis novae-angliae*, an aquatic moss that also attaches directly to the rock surface. Other plants observed during our study were Brazilian waterweed *Egeria densa*, the emergent bur-reed *Sparganium americanum*, the submersed milfoil *Myriophyllum pinnatum* and the green alga *Nitella flexilis*, each referred to hereafter by genus.

In the Chattahoochee River, herbivory on macrophytes appears strong. We commonly observed Canada geese (*Branta canadensis*) and crayfish (*Procambarus spiculifer*) grazing along the shoals and riverbanks, with some groups of geese surpassing 100 individuals. There was also conspicuous evidence of grazing by beaver (*Castor canadensis*) and muskrats (*Ondatra zibethicus*).

We measured the percent cover and biomass of the common plants located on rocky shoals in an area where we frequently observed geese feeding (the Jones Bridge Park Unit, 34' 00.053N, 84' 14.220W). To determine percent cover, we haphazardly placed 11 1.0 m^2 quadrats on the substratum and recorded the identity of plants located beneath 25 points within each quadrat. We then collected all of the plant biomass from three randomly selected 0.04 m^2 areas within each quadrat (i.e. a total of 0.12 m^2 within each quadrat). In the laboratory, these samples were sorted to species, spun in a salad spinner to remove excess water, and weighed to the nearest mg. The mean of these three sub-replicates was computed for each quadrat and the means for the eleven separate quadrats used to estimate mean plant biomass per m^2. Percent cover and biomass data were analyzed with one-way analysis of variance (ANOVA) following log transformation to correct heteroscedastic variances as detected with Cochran's tests.

We also characterized the abundance and species composition of plant-associated macroinvertebrates on the two most abundant macrophytes (*Podostemum* and *Fontinalis*) in our study area. We collected 17 samples of each plant by detaching macrophytes from the substratum and quickly placing them into downstream plastic bags while still underwater. A previous study using similar methodologies resulted in less than 1% loss of moss-associated insects (Glime and Clemons 1972). In areas where we observed Canada geese feeding, most of the *Podostemum* was grazed down to a turf of only about 2 cm in height. We avoided these areas by collecting similarly-sized *Podostemum* and *Fontinalis* plants from nearby, deeper (>0.75 m) areas of rapid flow. Canada geese had difficulty feeding in these areas and were often swept downstream while attempting to feed. In the laboratory, we rinsed each macrophyte over a 0.5 mm sieve and enumerated all animals retained on the sieve. Crustaceans were identified to species; insects were identified to order or family. Plants were then spun in a salad spinner to remove excess water and weighed to the nearest mg. Macroinvertebrate densities were expressed as a function of macrophyte wet mass; t-tests were used to evaluate differences in faunal abundance between host plants.

Feeding by Canada geese, crayfish, and macroinvertebrates

To determine the feeding preferences of the common herbivores in this system, we fed six species of aquatic macrophytes (*Podostemum, Egeria, Sparganium, Myriophyllum*, *Nitella* and *Fontinalis*) to Canada geese and crayfish. We also fed the most common plant species at our study area, *Podostemum* and *Fontinalis*, to two species of relatively small consumers, the amphipod

Crangonyx gracilis and the isopod *Asellus aquaticus*, that we found on submersed vegetation.

To feed Canada geese, we collected plants and transported them in a chilled cooler to a nearby riverine location with large numbers of geese. Working from a canoe, we used bread to attract ~75 geese and to initiate goose feeding. After geese were sufficiently acclimated and feeding (generally within 10 min), we picked a random goose and pitched a small handful of foliage from a randomly-chosen species of macrophyte directly in front of it. Geese that picked up and ate the plant were recorded as accepting the plant as food. Geese that picked up the plant and then rejected it were subsequently thrown a piece of bread to determine if they were satiated. Geese that rejected the plant and ate the bread were considered to have rejected the plant; geese that rejected both the plant and piece of bread were not counted in the feeding assay. We used a Fisher's exact test to analyze the number of animals that fed on each macrophyte species relative to the control level of 100% acceptance of bread by non-satiated geese.

We conducted similar feeding assays with crayfish in the laboratory. We collected over 50 *Procambarus spiculifer* from the Chattahoochee River and housed each crayfish in a separate $12 \times 12 \times 10$ cm cubicle with perforated walls receiving recirculating, filtered water. Crayfish were fed a maintenance diet of Bio-Blend Herbivore food 3–4 times per week. We determined the relative palatability of plants by offering 15–17 of these crayfish a 0.5–1.0 cm^2 portion of each macrophyte species that we had fed to geese and recorded whether each portion of food was eaten or rejected. If rejected, we fed crayfish a piece of palatable aquatic macrophyte (*Ludwigia palustris*) to assure they were not satiated. If the palatable macrophyte was rejected, the replicate was not included as the animal appeared satiated. Order of macrophyte presentation was randomized separately for each replicate consumer. We then used Fisher's exact test to analyze the number of animals that fed on each macrophyte species relative to the palatable control.

For assays with amphipods and isopods, we placed three amphipods or two isopods into one compartment of an ice cube tray containing 18–22 mg portions of either plant species and approximately 25 ml of water. Each plant portion was sonicated for 5–15 s before use to remove particulate organic matter that could confound estimates of plant tissue loss. Sonicating did not rupture macrophyte tissues. Controls for changes in plant mass unrelated to herbivory consisted of identical portions from the same individual plants placed into the same ice cube tray but without herbivores. After three days we calculated the mass change of plants exposed to herbivores using the formula: $T_f\text{-}(T_i \times C_f/C_i)$, where T_f and T_i were final and initial wet masses of tissue exposed to herbivores, and C_i and C_f were initial and final wet masses of controls (modified from Cronin and Hay 1996). Results were analyzed with t-tests.

Plant traits

We measured several macrophyte traits as potential indicators of nutritional quality. These included: dry mass/volume, ash-free dry mass/volume, soluble protein/volume, and soluble protein/dry mass for all six macrophyte species. Soluble protein was estimated with the Bradford method (Bradford 1976, as described in Parker et al. 2006). Results were analyzed with ANOVA followed by Tukey multiple comparison tests; log transformations corrected heteroscedastic variances when necessary.

We also conducted feeding assays testing whether plant morphology or secondary chemistry could account for differences in plant palatability between *Podostemum* and *Fontinalis*. We destroyed differences in plant morphology by freeze-drying and grinding tissues of *Podostemum* and *Fontinalis* to a fine powder and imbedding these powders into alginate-based foods that had similar morphologies and textures but retained the chemical and nutritional differences among macrophyte species (see Hay et al. 1998 for a general review, and Parker et al. 2006 for detailed methodologies). Alginate-based foods approximated the natural dry mass per volume of each macrophyte species and were assayed against a palatable control food (1:1 mixture of freeze-dried, powdered broccoli and lettuce 'broclet') that herbivores readily consumed (Bolser et al. 1998). Feeding on pellets was recorded as the frequency of acceptance or rejection of treatment or control pellets, with treatment pellets always offered first. We then analyzed the number of animals feeding on each individual reconstituted macrophyte species relative to the palatable control using Fisher's exact test.

If gel-based treatments were unpalatable to herbivores, it suggested that the plant was chemically defended or nutritionally inadequate. We tested for chemical defenses by incorporating crude extracts from *Podostemum* or *Fontinalis* into broc-let based alginate foods and then feeding pellets of these foods to crayfish. Extracts of each plant were acquired by macerating macrophyte tissues in a mixture of water and methanol (1:1 v:v) overnight, then successively extracting the macrophyte material for at least two hours in methanol:dichloromethane (1:1 v:v and 1:2 v:v). The solvents were then removed under vacuum. For food preparation, the 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 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 results were statistically analyzed as above.

We offered amphipods and isopods similar artificial foods treated with the crude extracts from *Podostemum* or *Fontinalis*, but we used a different method to quantify feeding for these much smaller herbivores. Foods were constructed as above, but we spread control (broc-let with solvent only) and treatment (broc-let treated with either *Podostemum* or *Fontinalis* extract in solvent) paste over window-screen mesh prior to hardening it in the calcium chloride solution (Hay et al. 1998). Either three amphipods or two isopods were then offered a choice between a control and treatment food held in 5.5 cm diameter petri dishes with 15 ml of water. Feeding was quantified as the number of mesh squares cleared from the 25 available for each food type. Feeding was measured and that replicate harvested when half of either food had been eaten (up to 8 days). The proportion of control and treatment squares eaten relative to the total squares eaten was analyzed with paired t-tests. To evaluate potential changes in *Fontinalis* extract over the duration of these assays, we incorporated extract into broc-let as above and qualitatively analyzed TLC plates for compound degradation after 8 days. We also fed crayfish control and treatment foods that had soaked in water for 8 days to determine whether *Fontinalis* extracts were still deterrent after this time period.

Bioassay-guided fractionation

The crude extract of *Fontinalis* deterred crayfish feeding so we used bioassay-guided fractionation of this extract to isolate and identify the compounds responsible for deterrence. Wet moss (200 ml) was blended in 250 ml of water and then allowed to soak for 5–10 min to trigger any enzyme-activated reactions (Newman et al. 1996, Bolser et al. 1998) before adding an equal volume of methanol. After three hours, this extract was decanted and solvents were removed via rotary evaporation. The remaining plant material was successively extracted in 400 ml of methanol:dichloromethane (1:1 v:v) for 12 h and 400 ml of methanol:dichloromethane (1:2 v:v) for three hours, removing solvents by rotary evaporation after each step. These extracts were then combined and tested as before by offering crayfish broc-let based pellets incorporated with chemical extracts vs control foods that were treated identically but without the addition of extracts. Extracts were tested at their isolated concentrations by volume (i.e. the extract from 1 ml of plant was incorporated into 1 ml of treatment food), but at an elevated concentration by dry mass to attempt to offset loss due to inefficient extractions and/or compound decomposition (i.e. we incorporated this extract into 0.07 g of broc-let whereas 1 ml of *Fontinalis* was equivalent to 0.31 g of dry plant mass, Table 1).

The deterrent crude extract was then partitioned into five fractions of increasing polarity using a modified Kupchan liquid-liquid partition (Kupchan et al. 1975). These fractions were assayed for effects on crayfish feeding and deterrent fractions were further fractionated using silica gel flash column chromatography (40–63 μm Aldrich silica eluted with a gradient of hexane and ethyl acetate). Chromatographic fractions were grouped by common thin layer chromatographic (TLC) properties and tested for deterrence of crayfish feeding. Deterrent fractions were subjected to flash chromatography as before, and this was repeated as necessary until achieving a deterrent fraction that consisted of one major TLC spot. This fraction was further separated using high performance liquid chromatography (HPLC-Zorbax RX-SIL silica column, 9.94×250 mm, 5-micron, attached to a Waters Breeze HPLC system, 515 pump, with a Waters 2487 UV detector at 210 and 254 nm, eluted with a gradient of hexane and acetone), resulting in one deterrent compound. At this step we attempted to obtain a structure by NMR spectroscopy, but there was insufficient quantity of material for analysis. Thus, we obtained and extracted new plant material as before and used

Table 1. Mean (± SE) and sample sizes (in parentheses) for each analysis of selected macrophyte traits. Species that share a letter within a column are not significantly different from one another in unplanned comparisons following ANOVA (broc-let not analyzed).

Macrophyte	Dry mass/vol. (mg ml^{-1})	AFDM/vol. (mg ml^{-1})	Soluble protein (mg ml^{-1})	Soluble protein (% dry mass)
Podostemum ceratophyllum	175 ± 22.5 (10)c	132 ± 17.4 (10)c	5.53	9.68
Egeria densa	78.9 ± 5.74 (10)b	66.0 ± 3.29 (10)b	4.23	3.34
Sparganium americanum	19.0 ± 3.09 (10)a	12.63 ± 0.74 (10)a	2.71	0.515
Nitella flexilis	109 ± 7.66 (10)b	80.5 ± 5.29 (10)b	6.38	6.95
Myriophyllum pinnatum	65.1 ± 2.44 (10)b	51.6 ± 1.81 (10)b	7.74	5.03
Fontinalis novae-angliae	314 ± 30.7 (10)d	274 ± 27.9 (10)d	3.22	10.1
Broc-let control	55 ± 1.41 (3)	6.27 ± 0.47 (3)	4.03	7.34
ANOVA p-values	$p < 0.0001$	$p < 0.0001$	N/A (composite samples)	N/A (composite samples)

repeated flash chromatography to obtain a fraction that displayed similar TLC characteristics to the deterrent fraction from the previous extraction. Fractions from the final separation were pooled according to common NMR spectral traits and tested for deterrence of crayfish feeding. The major compound from the fraction that deterred crayfish feeding was then identified on the basis of ^{1}H, ^{13}C, and ^{2}D NMR spectroscopy. All NMR spectral data were obtained on a Bruker Avance DRX 500 MHz spectrometer using $CDCl_3$ as solvent with TMS as internal standard referenced to residual $CHCl_3$ (δ 7.28).

Results

Plant abundance

Two macrophytes, *Podostemum* and the aquatic moss *Fontinalis*, covered 84% of the available area and comprised 98% of the total plant cover on rocky shoals at our study site. The remaining plant cover was predominantly the benthic green alga *Vaucheria* sp. The cover of *Podostemum* ($49.5 \pm 6.08\%$, mean $\pm$ SE) and *Fontinalis* ($34.9 \pm 7.57\%$) did not differ significantly ($p = 0.151$, t-test), but the wet plant biomass per area of *Fontinalis* was $\sim 10\times$ greater than that of *Podostemum* (331 ± 109 g m^{-2} vs 35.7 ± 13.6 g m^{-2}, respectively, $p = 0.023$, t-test). *Fontinalis* commonly grew as large (~ 30 cm long), bush-like macrophytes while *Podostemum* occurred primarily as a short (~ 2 cm), turf-like layer in most places. However, we observed large clumps of *Podostemum* (~ 30 cm long) growing in deeper areas of rapid flow. In these areas, when geese 'tipped up' to feed, they were rapidly swept downstream.

Feeding and/or colonization of plants by Canada geese, crayfish and macroinvertebrates

Of the six macrophyte species we fed to consumers, *Podostemum* was among the most palatable whereas *Fontinalis* was among the least palatable to both Canada geese and crayfish (Fig. 1A, 1B). Both geese and crayfish readily fed on *Podostemum* and the exotic plant *Egeria*, while feeding by both consumers was significantly lower on the native plants *Sparganium*, *Nitella*, and *Myriophyllum* (Fig. 1A, 1B).

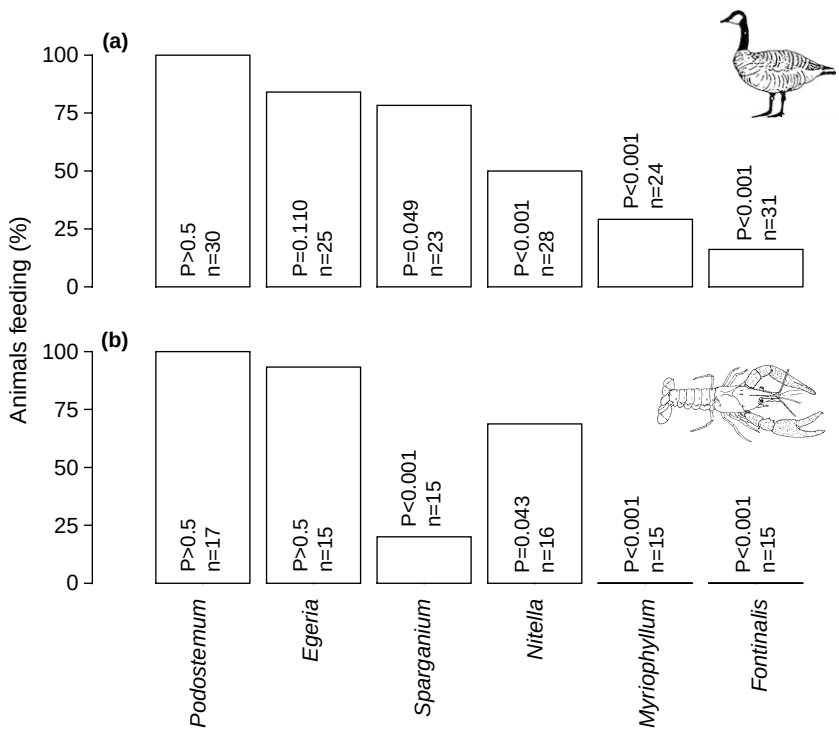

Fig. 1. Percentage of (A) Canada geese, *Branta canadensis,* and (B) crayfish, *Procambarus spiculifer,* feeding on fresh tissues from six aquatic macrophyte species collected from the Chattahoochee River. Statistics are from Fisher's exact tests assessing feeding on each plant relative to a palatable control food that was always consumed (bread for geese, *Ludwigia palustris* for crayfish) for each consumer species.

Plant-associated fauna were approximately twice as abundant on *Fontinalis* versus *Podostemum* ($p = 0.002$, Fig. 2). Mayflies (Ephemeroptera), midge larvae (Chironomidae: Diptera), the amphipod *Crangonyx gracilis* (Crustacea), and the isopod *Asellus aquaticus* (Crustacea) were all more abundant on *Fontinalis* than on *Podostemum* (Fig. 2). In contrast, blackflies (Simuliidae: Diptera) were significantly more abundant on *Podostemum*, and caddisflies (Hydropsychidae: Trichoptera) trended in the same direction (Fig. 2).

In contrast to Canada geese and crayfish, the amphipod *Crangonyx gracilis* and the isopod *Asellus aquaticus* both consumed *Fontinalis* but not *Podostemum* (Fig. 3). Amphipods and isopods ate the green leaves from *Fontinalis*, leaving behind only the fibrous stems (Parker and Burkepile, pers. obs.).

Plant traits relative to consumer feeding

The least nutritious plant we assayed was *Sparganium*; it had the lowest dry mass, ash-free dry mass, and protein content (by % dry mass) of the macrophytes we analyzed (Table 1). *Egeria*, *Nitella*, and *Myriophyllum* were of moderate values for these traits. *Podostemum*, which was readily consumed by geese and crayfish, had the second highest dry mass, ash-free dry mass, and protein while *Fontinalis*, which was rejected by geese and crayfish, had the highest values for each of these traits (Table 1). Thus, the two most abundant plants at our study site, *Podostemum* and *Fontinalis*, were also potentially the most nutritious, but were the most and least preferred food, respectively, for Canada geese and crayfish (Fig. 1A, 1B).

When plant morphology was destroyed by freeze drying, grinding, and incorporating plant tissues into homogeneous pellets, crayfish consumed pellets of *Podostemum* as readily as they consumed pellets of broc-let (both 100% feeding, $p > 0.5$, $n = 16$), but feeding on pellets of *Fontinalis* was depressed by 56% relative to feeding on broc-let pellets (only 43.8% of animals consumed *Fontinalis* pellets; 100% consumed broc-let pellets, $p < 0.001$, $n = 16$). When crude extracts from each plant were incorporated into broc-let, crayfish readily consumed foods treated with *Podostemum* extract (100% fed on control and 90% on *Podostemum* extracts, $p = 0.487$, $n = 20$), but feeding was suppressed 94% by incorporation of *Fontinalis* extract (Fig. 4). Thus, crayfish rejected *Fontinalis* because it was chemically distasteful, not because it was morphologically tough.

In contrast to crayfish, neither amphipods nor isopods were deterred by the crude extract from *Fontinalis* (Fig. 4). Amphipod feeding was stimulated, however, by *Podostemum* extract ($65.3 \pm 6.25\%$ of the total squares eaten were of broc-let incorporated with *Podostemum* extract, $p = 0.023$, $n = 23$), whereas isopods were not affected ($42.3 \pm 4.59\%$ of the total squares eaten were of broc-let incorporated with *Podostemum* extract, $p = 0.339$, $n = 20$). Although feeding assays with amphipods and isopods took up to eight days to complete, there were no qualitative changes in the *Fontinalis* extract over eight days according to TLC, suggesting that the majority of secondary metabolites were stable over this time period. Further, deterrence of the *Fontinalis* extract did not degrade over the 8-day period; 10 crayfish fed on the control food while only one fed on the treatment food containing 8-day old *Fontinalis* extract ($p = 0.001$, Fisher's exact test).

Bioassay guided fractionation

The crude extract of *Fontinalis* was deterrent to crayfish (1 of 17 animals ate the treatment food, $p < 0.001$, Fisher's exact test), as were the hexane and chloroform soluble portions of this extract (11 of 17 animals

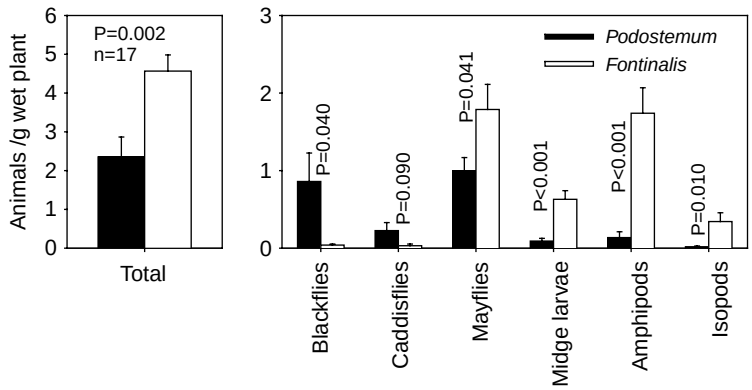

Fig. 2. Abundance of plant-associated macroinvertebrates per g of wet *Podostemum ceratophyllum* and *Fontinalis novae-angliae* collected from the Chattahoochee River. Statistics are from t-tests.

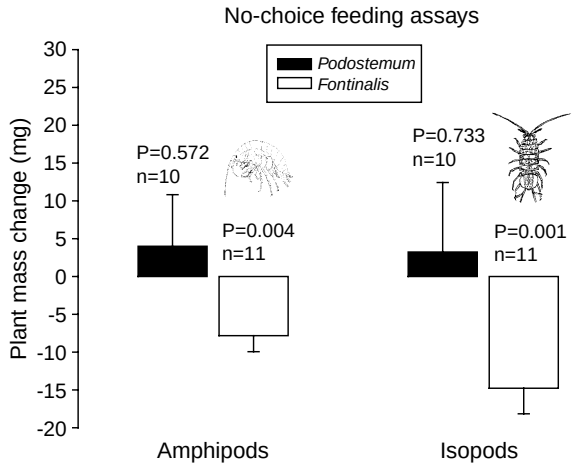

Fig. 3. Plant mass change (mean + SE) due to consumption by the amphipod *Crangonyx gracilis* and the isopod *Asellus aquaticus* when offered fresh tissues of either *Podostemum ceratophyllum* or *Fontinalis novae-angliae*. Statistics above each bar are from t-tests and tested whether mass change for each plant species was significantly different from zero in the presence of herbivores.

feeding, $p = 0.018$, and 4 of 17 animals feeding on fractions, $p < 0.001$, respectively). The remaining fractions (ethyl acetate, butanol, and water soluble portions) from this first partition were not deterrent ($p > 0.5$, $n = 20$). Silica gel flash column chromatography of the combined hexane- and chloroform-soluble fractions resulted in one fraction that was deterrent to crayfish (0 of 20 animals feeding, $p < 0.001$). Further separation of this fraction via flash chromatography and HPLC resulted in one fraction that deterred crayfish feeding (12 of 19 animals feeding, $p = 0.008$); this appeared to be a single metabolite. However, once purified, the compound decomposed before we could obtain a structure by NMR spectroscopy. A second extraction was performed and, using repetitive flash chromatography and HPLC as before, we obtained a fraction that had TLC characteristics similar to the previous deterrent fraction; this new fraction also deterred crayfish feeding (12 of 19 animals feeding, $p = 0.008$). NMR spectroscopy followed by comparison of spectral data with previous accounts (Jamieson and Reid 1976) revealed the major component in this fraction to be a C_{18} acetylenic fatty acid, octadeca-9,12-dien-6-ynoic acid (Fig. 4). The isolated yield of this compound was 0.0036% of moss dry mass.

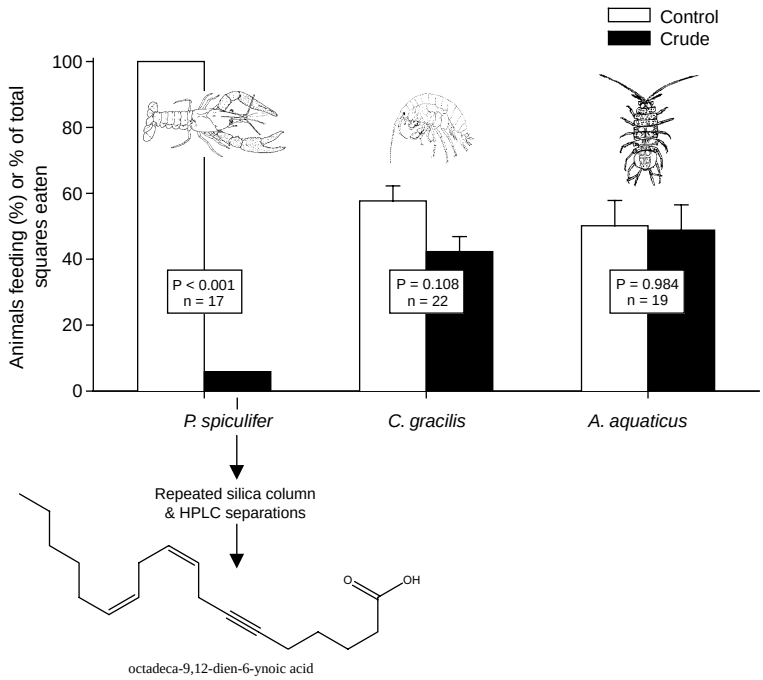

Fig. 4. Consumption by the crayfish, *Procambarus spiculifer,* amphipod, *Crangonyx gracilis*, and isopod, *Asellus aquaticus* when feeding on foods treated with the crude extract from *Fontinalis novae-angliae.* Control and treatment diets were freeze-dried, powdered broccoli and lettuce with and without the addition of extract (at natural dry mass concentrations). Crayfish were tested by offering individual pellets of each diet in succession; the number of animals feeding on each diet was analyzed with a Fisher's exact test. Amphipods and isopods were tested by offering each diet simultaneously on window screen squares; the relative proportion of each diet eaten was tested with paired t-tests. Bioassay-guided fractionation (see Methods and Results for details) of crude extract led to the isolation of a C_{18} acetylenic fatty acid that deterred feeding by crayfish.

Discussion

Our data provide initial evidence that small, sedentary herbivores in freshwater systems can gain enemy-free space by feeding on plants that are chemically defended from larger consumers, corroborating similar patterns from marine and terrestrial systems (Hay et al. 1987, Bernays and Graham 1988, Hay 1992, Murphy 2004, Singer and Stireman 2005). The most abundant plant along the rocky shoals of the Chattahoochee River, the aquatic moss *Fontinalis novae-angliae*, was unpalatable to both Canada geese and crayfish (Fig. 1), but was colonized by twice as many invertebrates as was the most palatable plant to these herbivores, *Podostemum ceratophyllum* (Fig. 2). *Fontinalis* was chemically defended from crayfish consumption by a C_{18} acetylenic acid, octadeca-9,12-dien-6-ynoic acid, but both the amphipod *Crangonyx gracilis* and the isopod *Asellus aquaticus* consumed the fresh tissues and crude extracts of *Fontinalis* in laboratory feeding assays (Figs. 3, 4). This differential host-use and plant consumption by large and small grazers is similar to patterns found in other systems (Hay 1992, Hay and Fenical 1996), and suggests that these mesograzers may have been selected to tolerate or circumvent the chemical defenses that deter larger consumers as a way of gaining enemy-free space.

Other studies of small herbivores in freshwater systems support our general conclusion that these herbivores should select habitat (and often food) that reduces their encounter rate with large consumers. Aquatic caterpillars of the pyralid moth *Munroessa gyralis* (which have limited mobility among plants) preferentially feed on two water lilies, *Nymphaea odorata* and *Brasenia shreberi* (Dorn et al. 2001), that are unpalatable to omnivorous crayfish (Cronin et al. 2002, Parker and Hay 2005). Similarly, the beetle *Galerucella nymphaeae* specializes on and is even stimulated by secondary metabolites in a water lily (*Nuphar luteum macrophyllum*) that is chemically repugnant to crayfish (Bolser and Hay 1998). Insects also avoid associating with a palatable green alga, *Cladophora glomerata,* that crayfish exclude from streams (Creed 1994). Thus, small invertebrates in freshwater systems often shun plants that are acceptable to larger consumers, or are attracted to plants that larger consumers avoid, paralleling patterns found with marine crustaceans and some terrestrial insects. Mosses are widespread in freshwater habitats (Everitt and Burkholder 1991, Baattrup-Pedersen et al. 2006, this study), and many of these mosses are heavily colonized by macroinvertebrates (Suren 1991, Chantha et al. 2000, Linhart et al. 2002, this study) and possess novel secondary metabolites (Dembitsky and Rezanka 1995, Liao and Glime 1996, this study). These patterns suggest that this phenomenon may be particularly prevalent among freshwater mosses and invertebrates.

Host-plant selection by small invertebrates in freshwater systems has traditionally been assigned to differences among macrophytes in the provision of habitable living spaces, entrainment of particulate organic matter, surfaces for epiphytic algal growth, and shelter from turbulent flow (Lodge 1985, Brusven et al. 1990, Suren 1991, Linhart et al. 2002). Our data do not allow us to control for these potentially covarying factors, but some of the variation in plant traits and herbivore use in our study are inconsistent with these viable alternative hypotheses. For example, *Podostemum* appeared relatively clean and free of debris, whereas *Fontinalis* contained more particulates, suggesting that mesograzers could have been attracted to *Fontinalis* because of these alternative food sources. However, both amphipods and isopods ate significant quantities of moss tissue even after we removed this layer of particulate organic matter (Fig. 3). Aquatic mosses also have been found inside the guts of several aquatic insects (Jones 1950, Pritchard and Berte 1987, Suren and Winterbourn 1991, Bowden 1999), isopods (LaCroix 1996), and amphipods (Minckley and Cole 1963), confirming that aquatic mosses are not merely a habitat, but also a food, for numerous small invertebrates. Finally, protein is often the limiting macronutrient for herbivores (Mattson 1980), yet protein content did not differ between *Podostemum* and *Fontinalis* (Table 1), further suggesting that this aspect of plant nutritional quality was not driving use of these plants by small invertebrates. Thus, although host-plant quality may explain some differences in host-plant preference, the importance of enemy-free space may still be a driving force maintaining the ecological specialization of macroinvertebrates on this chemically noxious bryophyte.

Although herbivory is not often considered a strong ecological process in riverine environments, grazing by Canada geese and crayfish appeared to drive the plant community on these shallow rocky shoals towards species that can either tolerate or resist herbivory. We commonly saw >100 Canada geese foraging on *Podostemum ceratophyllum*, a plant that was highly palatable to both geese and crayfish in feeding assays (Fig. 1A, 1B). However, grazing removed only the upright foliage, not the rock-adhering basal roots. In addition to anchoring this plant against rapidly flowing water, these rock-adhering roots may allow riverweed to tolerate chronic herbivory by retaining both the basal meristems and a starchy root that persists even when the upright foliage has been removed, much like rhizomatous terrestrial grasses (McNaughton 1983). In contrast, *Fontinalis novae-angliae* comprised nearly 90% of the total plant standing stock, was rarely eaten by geese or crayfish (Fig. 1A, 1B), and was chemically defended

against crayfish in laboratory feeding assays (Fig. 4). Although we did not develop methodologies to test whether Canada geese were also deterred by the same chemical defenses that deterred crayfish, these patterns suggest that *Fontinalis* may be the most abundant plant on these rocky shoals because it is chemically defended and rarely eaten by the dominant consumers in this system. The strong impacts of herbivory on this system were further suggested by the increased abundance of palatable species like *Podostemum* in deeper, rapidly flowing areas where geese feeding appeared to be inhibited by flow.

Evidence for chemical defenses in freshwater macrophytes has lagged behind that of terrestrial plants and marine macroalgae. Thousands of secondary metabolites in marine and terrestrial plants are known to influence herbivory (Seigler 1998, Faulkner 2002), but herbivore feeding deterrents have only been described for five freshwater macrophytes – watercress, *Rorippa nasturtium-aquaticum* (Newman et al. 1996), the waterspider bog orchid, *Habenaria repens* (Bolser et al. 1998), lizard's tail, *Saururus cernuus* (Kubanek et al. 2001), baby's tears, *Micranthemum umbrosum* (Parker et al. 2006) and *Fontinalis novae-angliae* (this study). The acetylenic acid that we isolated from *Fontinalis* as well as other acetylenic fatty acids are common to aquatic and terrestrial mosses (Anderson et al. 1975, Jamieson and Reid 1976, Kohn et al. 1987, Zinsmeister et al. 1991) but are uncommon in vascular plants (Seigler 1998). Some acetylenic fatty acids have antimicrobial and antifungal properties (Borel et al. 1993, Li et al. 1994), suggesting other potential defensive roles for this compound. Additionally, acetylenic compounds are inherently unstable when exposed to light, heat, and oxygen (Seigler 1998), perhaps explaining why our isolated yield was only 0.0036% of dry mass (by contrast, the same compound was found at 0.29% in another aquatic moss, *F. antipyretica* (Jamieson and Reid 1976).

Although terrestrial and marine studies have commonly shown that plant chemical defenses can have cascading impacts on food webs (reviewed by Hay 1997, Ohgushi 2005), similar multi-trophic level perspectives have rarely been examined in freshwater systems. However, increasing evidence suggests that aquatic macrophytes are often chemically defended against aquatic consumers (Bolser et al. 1998, Prusak et al. 2005), ultimately influencing plant community structure (Parker et al. 2006) and host-plant use by plant-associated invertebrates (this study). Thus, although historically underappreciated, plant chemical defenses appear to have strong impacts on freshwater systems that may extend beyond their direct impacts on consumer feeding behavior.

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