Activated and Constitutive Chemical Defenses in Freshwater Plants

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Activated and Constitutive Chemical Defenses in Freshwater Plants

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DEDICATION

This thesis is dedicated to my family.
Thank you for teaching me confidence, and giving me unlimited support and love.

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SUMMARY

Although macrophyte-herbivore interactions in freshwater systems were generally disregarded for many years, recent data suggest that herbivory is intense and important in structuring freshwater communities. Therefore, it has been hypothesized that chemical defenses should be common among freshwater plants, but few studies have reported such chemical defenses, and no previous studies have assessed the frequency of chemical defenses among freshwater plants. In a study of 21 freshwater plant species co-occurring in a Southeastern USA wetland environment with the generalist omnivorous crayfish *Procambarus verrucosus*, we found that 52 % of plant species were chemically defended. For these plants crude extracts deterred feeding by *P. verrucosus* when tested in artificial foods at natural concentrations. Of these chemically defended plant species, only one, *Eupatorium capillifolium*, showed consistent changes in extract palatability following mechanical damage to plant tissue, indicative of an activated chemical defense mechanism. Because herbivores select food based on several plant traits, including plant nutritional value, it might be expected that chemical defenses would be especially important for protein-rich plants. However, we found no relationship between soluble protein concentration and deterrence of plant extracts. Overall, it appears that chemical defenses are common among freshwater plants, but that activated defenses are rare.

CHAPTER 1

INTRODUCTION

Community structure and plant abundance are strongly influenced by herbivory in both aquatic and terrestrial ecosystems (McNaughton, 1985; Carpenter, 1986; Carpenter and Kitchell, 1988; Huntley, 1991). Plant-herbivore interactions have been intensely studied, especially in terrestrial and marine ecosystems (Lubchenko and Gaines, 1981; Hay and Fenical, 1988; Howe and Westley, 1988; Bryant et al., 1992; Hay, 1997). It was previously thought that herbivory on freshwater macrophytes was rare and had negligible effects on community structure and plant diversity (Hutchinson, 1975; Wetzel, 1983). However, investigations suggest that macrophyte abundance and diversity can be greatly affected by herbivory in freshwater ecosystems (Lodge and Lorman, 1987; Lodge, 1991; Cyr and Pace, 1993; Lodge et al., 1998).

Considering the detrimental effects of herbivory (Lodge, 1991; Krebs, 2001), plants should be selected to defend themselves in areas of high herbivory. Many plant species use morphological (Hay, 1981; Steneck, 1986; Lewis et al., 1987; Van Alstyne, 1989), associational (Hay, 1986), or chemical defenses (Paul and Van Alstyne, 1992; Hay, 1996; Karban and Baldwin, 1997), as well as combinations of different defense strategies (Hay et al., 1994). Plant chemical defenses have been shown to be important in determining plant species diversity within aquatic and terrestrial ecosystems (Hay and Fenical, 1996; Karban and Baldwin, 1997).

Chemical defenses may be constitutive (always present), induced (production up-regulated gradually), or activated (produced immediately from available precursors) in response to enemy attack (Harvell, 1990; Paul and Van Alstyne, 1992; Karban and Baldwin, 1997). Induced and activated chemical defenses are triggered by plant injury or contact with herbivores, resulting in chemical changes within plants that increase resistance to herbivory. Unlike induced responses that occur over a time scale of hours to months (Karban and Baldwin, 1997), activation can occur within seconds of plant injury (Paul and Van Alstyne, 1992), however, the actual kinetics of activation are unknown.

In some cases, the use of chemical defenses has been shown to be costly to the producer, reducing growth and reproduction (Baldwin, 1998; Pavia et al., 1999). If herbivory is constant and predictable, a plant may benefit from a constitutive defense because costs of defense could be offset by the benefit of continuous protection. In contrast, if herbivory is spatially or temporally variable, costs associated with constitutive defense may exceed the benefits of protection, and so the use of an induced or activated defense may be preferred, if induced and activated defenses incur lower costs, including lower allocation (energetic), opportunity, and ecological costs (Karban and Baldwin, 1997; Koricheva, 2002).

In some cases, constitutively expressed chemical defenses are used by specialist herbivores to locate host species and to stimulate feeding, incurring an ecological cost that could negatively affect the fitness of plants constitutively producing the defense (Alder and Karban, 1994). For example, the green alga *Chlorodesmis fastigiata* produces chlorodesmin, deterring generalist fishes (Hay et al., 1989). However, a specialist crab that exclusively lives and feeds on this alga uses this metabolite as a feeding cue. Similarly, the herbivorous amphipod *Pseudamphithoides incurvaria* avoids predation by reef fishes by wrapping itself in the chemically defended brown alga *Dictyota* *bartayresii.* The amphipod's behavior is cued by the alga's constitutively expressed metabolite pachydictyol A, which deters grazing by reef fishes but not by the amphipod (Hay et al., 1990). The use of an activated defense may carry lower ecological costs than a constitutive defense by enabling a plant to "hide" from specialist herbivores or parasites that may otherwise locate their food or host by using host chemical defenses as cues. Because of their infrequent production, activated defenses may be unreliable cues for adapted consumers. However, when the mountain pine beetle, *Dendroctonus ponderosae*, bores into the lodgepole pine *Pinus contorta*, the pine releases terpenes (that deter other herbivores) which act as precursors to, and synergists of, the beetle's aggregation pheromone, thus attracting more beetles until a threshold is reached, at which point host terpene production is exhausted and beetles begin mating (Raffa and Berryman, 1983). Overall, however, the potentially lower ecological costs for plants with activated chemical defenses relative to constitutive chemical defenses could be a significant benefit that could select for activation as a defensive strategy.

Activated chemical defenses may present many additional benefits to plants relative to constitutive or induced defenses. First, activation increases resistance to herbivores at the onset of an attack, possibly repelling the initial attacker, rather than protecting the plant only from herbivores which feed very slowly or from future attacks, as with induced defenses. Second, if chemical defenses are toxic to plants that produce them, then by producing or up-regulating them only when attacked and only for short periods of time, costs associated with autotoxicity are minimized, relative to constitutive and induced defenses. Third, an unstable compound could be useful as an activated defense, decomposing soon after its exposure to herbivores, but may be less effective as an induced or constitutive defense, for which the defended state lasts for hours to months, or continuously. Fourth, compounds that have alternative functions within a plant, or that serve as precursors to several biosynthetic products, one of which is the activated defense, may also be more efficiently used for an activated than constitutive defense. If activation occurs infrequently, the metabolite may be used elsewhere within the plant, or the precursor may be diverted to synthesize different products, when the plant isn't being attacked.

Activation of chemical defenses may occur by at least three possible mechanisms. First, activation can occur when a plant is physically damaged, by the conversion of a somewhat deterrent compound to a more deterrent compound. In three green algae in the genus *Halimeda,* a less deterrent compound, halimedatetratraacetate, is converted into a more potent compound, halimedatrial, upon plant injury (Paul & Van Alstyne, 1992). Second, activation of a chemical defense may occur by the production of a deterrent compound from non-deterrent precursors. For example, hydrogen cyanide is produced by plants in the families Rosaceae, Leguminosae, Gramineae, Araceae, Compositae, Euphorbiaceae, and Passifloraceae when plant tissue is crushed or cellular structure is disrupted, by releasing enzymes that hydrolyze cyanogenic glycosides (Conn, 1979). Third, activation may occur by increasing the concentration of a deterrent metabolite that is normally expressed at lower levels, as occurs in the freshwater orchid *Habenaria repens* (Bolser et al., 1998; Wilson et al., 1999). Because activated defenses involve metabolic changes occurring within a time frame of seconds (Paul and Van Alstyne, 1992; Cetrulo and Hay, 2000), it is likely that metabolic precursors and enzymes are present prior to the attack, because transcription and translation to yield enzymes can take up to 40 seconds (Mathews et al., 1999), longer than the time it may take some herbivores to critically damage a plant. This requirement, along with the need to either activate plant defenses at the site of damage or to rapidly move defenses from sites of synthesis to damaged sites, may constrain the effectiveness of activated chemical defenses as a widespread strategy among plants.

In a recent investigation of activated chemical defenses in macroalgae, 17 % (7 of 42) of algal species tested underwent changes in palatability upon physical damage that were consistent with activated chemical defenses, although deterrent compounds were not identified (Cetrulo and Hay, 2000). Activation of plant chemical defenses is known to occur in freshwater ecosystems, but the frequency of activation has not been established. Newman et al. (1990, 1992, 1996) found that when watercress (*Nasturtium officinale*) tissue is damaged, it releases 2-phenylethyl isothiocyanate, deterring feeding by freshwater amphipods, caddisflies, and snails. As with terrestrial glucosinolate-containing plants, the deterrent compound is the product of a glucosinolate compound and a myrosinase enzyme, compartmentalized into separate cells until tissue damage. Bolser et al. (1998) measured feeding preferences of the crayfish *Procambarus clarkii* to four species of freshwater macrophytes, and found two plants to be chemically defended, one of which, the freshwater orchid *Habenaria repens*, contained a deterrent compound habenariol (bis-*p*-hydroxybenzyl-2-alkyl-2-hydroxysuccinoate). Crayfish preferred extracts of frozen *H. repens* tissue over extracts of frozen and subsequently thawed *H. repens* tissue, which contained three times the concentration of habenariol found in frozen tissue, suggesting that production of habenariol was activated by cell lysis.

Approximately 22,000 secondary metabolites have been isolated and identified from terrestrial plants (most of unknown ecological function), although only 20-30 % of higher plants have been investigated (Wink, 1999). In contrast, approximately 1,880 secondary metabolites have been isolated and identified from marine plants, mostly from macroalgae (McClintock and Baker, 2001), and some of these have been shown to affect herbivore feeding behavior or fitness (Cruz-Rivera and Hay, 2003). Compared to the thousands of published studies on the identity and effects of terrestrial and marine chemical defenses, only three publications report separate freshwater plant chemical defense systems that have been chemically characterized and their ecological consequences tested (Newman et al., 1992; Bolser et al., 1998; Kubanek et al., 2001). The existence of chemical defenses in general has only been investigated for 14 freshwater macrophytes (11 higher plants, three algae). Of these 14 freshwater species investigated, 57 % (8 of 14) have been documented to have chemical defenses (Newman et al., 1992; Bolser et al., 1998; Bolser and Hay, 1998; Cronin, 1998; Cronin et al., 2002). Of the eight chemically defended species, two have been shown to utilize activated chemical defenses. We hypothesized that because of the advantages activated chemical defenses may bring to plants in areas with intense and unpredictable herbivory, activated chemical defenses could be important to macrophytes in freshwater ecosystems.

Freshwater habitats include ponds, lakes, streams, rivers, and wetlands, and are home to many herbivores, including terrestrial and aquatic herbivores, both migratory and non-migratory. Wetlands are diverse ecosystems with high habitat and species diversity (Brinson et al., 1981). Many wetlands alternate between flooded and dry states, and therefore support plants and herbivores adapted to these conditions. Because wetlands are a transitional zone between water and land, wetland plants may be vulnerable to both aquatic herbivores such as beavers, otters, waterfowl, fish, crayfish, snails, amphipods, isopods, and insect larvae, as well as terrestrial herbivores such as deer, muskrats, raccoons, rabbits, and adult insects. Approximately 4600 wetland plant species have been reported to exist in the United States alone, of which roughly 7 % are non-native species (USDA, 2004). Despite this species and habitat diversity, relatively few studies have investigated how wetland plants persist. One way assess this is to ask whether wetland plants possess defenses, such as chemical defenses, against common herbivores.

In this investigation, we asked what proportion of a group of 21 wetland plant species use chemical defenses, and activated chemical defenses in particular, against a co-occurring omnivorous crayfish, *Procambarus verrucosus*. We also examined the relationship between plant nutritional content, the type of chemical defense (activated or constitutive), and the magnitude of the deterrent effect. We hypothesized that plants of high nutritional value to herbivores (i.e., high protein content) would be under greater threat of herbivory and greater selection to evolve defenses, and therefore may be better protected by chemical defenses, either activated or constitutive. Because of field constraints, we did not test for induced chemical defenses. From these experiments, we expected to better understand plant-herbivore interactions in freshwater ecosystems and to determine what role activated and constitutive chemical defenses may play.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Study Organisms*:

We used crayfish as bioassay organisms for testing constitutive and activated chemical defenses in freshwater macrophytes because crayfish are generalist feeders, have well-developed chemoreceptive organs shown to be important in perceiving food odors (Tierney et al, 1985), behave well in laboratory assays (Bolser et al., 1998), and can have potentially great effects on macrophyte biomass in natural systems (Lodge and Lorman, 1987; Lodge et al., 1994; Lodge et al., 1998). The omnivorous crayfish *Procambarus verrucosus*, commonly found living among macrophytes in our study, is native to and distributed throughout the Chattahoochee River wetlands in Alabama and Georgia (Hobbs, 1972). *P. verrucosus* was collected from wetlands at the Chattahoochee National Recreation Area (Cochran Shoals Unit, near Atlanta, Georgia, USA) from January to September 2003 and were identified using Hobbs (1972). Crayfish were housed in a large water table with continuous flow of dechlorinated tap water, and were separated from each other by plastic dividers creating 125 x 125 cm chambers. On days when the crayfish were not being used for feeding assays they were fed commercial trout pellets.

Multiple sites within the Chattahoochee National Recreation Area wetlands in north Georgia with high seasonal variation in water level, water temperature, and herbivore abundance (A. Prusak, personal observation), were used for plant collections. Twenty-one freshwater macrophyte species were chosen for study, based upon availability from

May to October, 2003 (see Table 1). We focused on abundant plants and avoided very rare plants because of sample size limitations. The macrophyte *Agrostis scabra* was inadvertently collected at two locations, and so results from each collection are included separately. The macrophytes *Eupatorium capillifolium*, *Galium tinctorium*, *Murdannia keisak*, and *Sagittaria latifolia* were collected twice to repeat activation experiments. Macrophytes were identified using Godfrey and Wooten (1979, 1981).

Macrophytes were gently pulled up by the roots, placed in Ziploc bags, and stored on ice for transport to the laboratory at Georgia Institute of Technology. At the lab, plants were gently rinsed, patted dry, weighed, and each collection was sorted into two batches of equal size, as measured by wet mass and volumetric displacement in water. Pressed voucher specimens are stored at Georgia Institute of Technology.

2.2 *Chemical Methods*:

To mimic the shredding action of feeding crayfish and thus simulate herbivore damage, macrophytes were shredded for 45 seconds in water using a blender, to generate the simulated "grazed" treatment. This method has been used successfully by previous researchers examining activated defenses among macroalgae (Paul and Van Alstyne, 1992; Cetrulo and Hay, 2000). To simulate a non-grazed plant (simulated "non-grazed" treatment), plant material was blended in methanol (MeOH), which was expected to inhibit enzymatic activity within the plant, preventing activation of plant chemical defenses. After blending each treatment, plant material and solvent were placed in 1 L flasks, and equal amounts of the opposite solvent were added, resulting in a 1:1 blend of MeOH/H_2O. After extracting for 1 hour at 4 ºC, extracts were vacuum filtered and solvents were removed from extracts by rotary evaporation. To maximize both the yield and polarity range of compounds extracted, plant material was further extracted once each with three additional solvent systems: 100 % MeOH; then 1:1 MeOH/dichloromethane; then 1:2 MeOH/dichloromethane. These extracts were combined with the 1:1 MeOH/H_2O extract, dried by rotary evaporation, and stored at –20 °C until used in bioassays.

2.3 *Bioassay Methods:*

Two types of feeding assays were conducted: (1) tests of extracts from simulated "grazed" treatments compared to extracts from simulated "non-grazed" treatments, and (2) tests of "grazed" or "non-grazed" treatments compared to no-extract controls. Twenty-five to 40 crayfish were used for each bioassay, and one or two assays were run with each group of crayfish each day.

Bioassay foods and experimental design followed procedures of Hay et al. (1994) and Bolser et al. (1998). Bioassay food consisted of freeze-dried, finely ground broccoli and lettuce (1:1) in an agar matrix with the addition of plant extracts (either "grazed" or "non-grazed" extracts) or no extracts (no-extract control). For testing of each plant species, we used a quantity of dried broccoli/lettuce corresponding to the plant's dry mass for a 20 mL volume of fresh plant tissue. An aliquot of extract corresponding to 20 mL of fresh plant material was dissolved in acetone/methanol/ethyl ether (1:1:3) and added to the broccoli/lettuce powder. Solvents were then removed on a rotary evaporator, and the extract-coated broccoli/lettuce mixture was suspended in 7.0 mL distilled water, combined with a boiled solution of 0.37 g agar in 12.0 mL distilled water, and quickly poured into a mold beneath which lay window screen. Once cooled, the food adhered to the window screen and each food patch was cut with a razor blade to measure 9 x 7 window screen squares. No-extract control foods were prepared in the same manner, including the organic solvents described above, but without plant extracts.

To directly contrast the effects of extracts from “grazed” vs. “non-grazed” treatments, each crayfish was independently offered a food strip consisting of one patch of food with “grazed” extract and one patch of food with “non-grazed” extract. For tests of “grazed” or “non-grazed” treatments vs. no-extract controls, each crayfish was offered a food strip consisting of one patch of food with either “grazed” or “non-grazed” extract, and one patch of no-extract control food. Each crayfish was allowed to feed until approximately half of the total amount of food on its strip was eaten, typically taking between one and six hours, but sometimes up to 24 hours. Once removed from crayfish, the number of food squares missing (eaten) from each treatment patch was counted.

2.4 *Statistical Analyses:*

A Shapiro-Wilk test indicated non-normal distribution of feeding assay results. Therefore, the non-parametric Wilcoxon signed-rank test was used to compare consumption of “grazed” treatments vs. “non-grazed” treatments, and to compare consumption of “grazed” or “non-grazed” vs. no-extract controls. The non-parametric Mann-Whitney U statistical test was used to compare the amount consumed of the “grazed” treatment from the “grazed” vs. no-extract control bioassay, to the amount consumed of the “non-grazed” treatment from the “non-grazed” vs. no-extract control bioassay (Sokal and Rohlf, 1994). We call this the indirect comparison of consumption on “grazed” vs. “non-grazed” extracts. A Mann-Whitney U test was also used to compare data from experiments conducted at different times but using the same extracts from the same collection, in order to determine whether the data could be combined. A linear regression t-test was used to test for a relationship between protein content and extract deterrence. P-values for linear regression represent whether the slope of the linear correlation between protein content and extract deterrence was different from a slope of zero. Extract deterrence was calculated for each replicate in a given bioassay by subtracting the amount of "grazed" treatment eaten from the amount of the no-extract control food eaten, and dividing this by the total amount eaten, and then averaging across all replicates of one bioassay (consumption of "grazed" treatment rather than "non-grazed" treatment was chosen for this calculation so that extract deterrence of plants with activated chemical defenses would be evaluated at their activated levels). For plant species for which multiple collections or bioassays were conducted, results from the second bioassay were used to generate a value of extract deterrence. An extract deterrence value of +1 indicates that only no-extract control food was consumed (i.e., strong deterrence); a deterrence value of 0 indicates that there was no preference between no-extract control food and "grazed" extract food (i.e., no chemical defense). Negative deterrence values suggest feeding stimulation by the plant extract. Data in figures are presented as means $\pm$ one standard error. In all analyses, $p < 0.05$ was considered statistically significant.

2.5 *Interpretation of Results:*

Based on bioassay results, macrophytes were categorized as having a constitutive chemical defense, an activated chemical defense, or no observed chemical defense. A macrophyte was considered to have a constitutive chemical defense if the "non-grazed" chemical extract significantly deterred crayfish feeding compared to a no-extract control, and "non-grazed" extracts were not significantly preferred over "grazed" extracts, by either the direct or indirect methods of comparing consumption on these foods (see above). Macrophytes were considered to have an activated chemical defense if: 1) "grazed" extracts significantly deterred feeding relative to no-extract controls, and 2) foods containing "non-grazed" extracts were significantly preferred over foods containing "grazed" extracts, by either the direct or indirect methods of comparing consumption on these foods (see above). A macrophyte showed no evidence for chemical defense if the "non-grazed" extract did not significantly deter crayfish feeding relative to no-extract controls, and if no significant difference was detected between "grazed" and "non-grazed" extracts, by either the indirect or direct methods of comparing consumption of these foods (see above).

2.6 *Plant Nutritional Analyses*:

Volumetric displacement, wet mass, dry mass, and ash-free dry mass were determined for 5 separate plants for each macrophyte species. All samples were patted dry and weighed, dried at 45 °C for 24 hrs and weighed to obtain dry mass, and placed in an ashing oven at 450 °C for 4 hrs and weighed to obtain ash-free dry mass. To determine the approximate nutritional value of each plant species, soluble protein content was measured according to Bradford (1976) for 19 of the 21 plants species (two plant species were unavailable for re-collection at the time of this measurement). Whole plant samples were weighed, freeze-dried, and ground. Three 5 mg samples of each plant were digested in 1.0 mL NaOH (1 mol/L) for 24 hours at 4 °C, and then centrifuged, and 100 μL aliquots of the supernatant were added to 3.0 mL of Bradford Reagent. After 15-20 minutes, absorbance was measured at 595 nm using a Spectronic 21D spectrophotometer against bovine serum albumin (BSA) standards. Five protein standards were used to construct a standard curve (r^2=0.997).

2.6 *Chemical Analyses:*

Thin layer chromatography (TLC) was performed to qualitatively compare the composition of the "grazed" vs. "non-grazed" extracts. Plant extracts were dissolved in acetone/MeOH/ether (1:1:3), and spotted on four silica TLC plates. Different mobile phase systems were used for each plate: 1) 3:1:1 *n*-butanol/acetic acid/H_2O, 2) 100% ethyl ether, 3) 1:1 ethyl ether/petroleum ether, 4) 1:4 ethyl ether/petroleum ether. Compounds were analyzed under natural light, short-wave ultraviolet light (254 nm), and stained with 7 % sulfuric acid in ethanol and heated to reveal oxidized products.

CHAPTER 3

RESULTS

Fifty-two percent (11 of 21) of species tested possessed chemical defenses, since their crude extracts, tested in artificial foods at natural concentrations, significantly depressed crayfish feeding relative to no-extract controls (Figures 1-2). The chemically defended plants include one floating macrophyte, *Utricularia* sp., and 10 emergent macrophytes: *Alisma subcordatum* (Alismataceae), *Aster* sp. (Asteraceae), *Eupatorium capillifolium* (Asteraceae), *Galium tinctorium* (Rubiaceae), *Hydrolea quadrivalvis* (Hydrophyllaceae), *Hypericum mutilum* (Clusiaceae), *Mimulus ringens* (Scrophulariaceae), *Polygonum densiflorum* (Polygonaceae), *Sagittaria latifolia* (Alismataceae), and *Saururus cernuus* (Saururaceae). Collections of one additional emergent species, *Agrostis scabra* (Poaceae), appeared be chemically defended at one site, (Cochran Shoals, Figure 2A), but not at another site (Johnson Ferry, Figure 3A). The remaining nine macrophytes' crude extracts did not significantly deter crayfish feeding when tested at natural concentrations, and therefore these plants appear to not be chemically defended (Figure 3). These plants include one submersed macrophyte *Cabomba pulcherrima* (Cabombaceae), and eight emergent macrophytes: *Alternanthera philoxeroides* (Amaranthaceae), *Cyperus odoratus* (Cyperaceae), *Ludwigia repens* (Onagraceae), *Lycopus virginicus* (Lamiaceae), *Murdannia keisak* (Commelinaceae), *Myriophyllum aquaticum* (Haloragaceae), *Polygonum punctatum* (Polygonaceae), and *Polygonum sagittatum* (Polygonaceae). Table 1 lists plant species studied, some plant traits, and type of chemical defense determined in this study.

When originally tested, three macrophyte species (*Eupatorium capillifolium*, *Galium tinctorium*, and *Murdannia keisak*) appeared to utilize activated chemical defenses, since plant extracts generated by the simulated "grazed" treatment (i.e., physically damaged prior to extraction) significantly deterred crayfish feeding relative to extracts from the simulated "non-grazed" treatment (i.e., physically damaged after treatment with organic solvent had started) (Figure 1A, 1C, 1E). The evidence for activated chemical defenses among these three plants came either from direct comparison of crayfish consumption of foods containing "grazed" vs. "non-grazed" extracts (*E. capillifolium*; p=0.032), or indirectly, by comparing data from bioassays testing consumption of treated foods vs. no-extract controls (*M. keisak*; p=0.001), or by both direct and indirect comparisons (*G. tinctorium*; p=0.026, 0.002, respectively). Additionally, the "grazed" extract of *Sagittaria latifolia* was found to be deterrent relative to no extract controls (p=0.020), whereas its "non-grazed" extract was not (p=0.21), although there was no significant difference in consumption of foods containing these extracts relative to each other (p=0.18 direct comparison; p=0.22 indirect), so this plant did not appear to possess an activated defense (Figure 1G). To re-assess *S. latifolia*'s mechanism of chemical defense and to test whether the activated defense results for the three plant species were reproducible, these four species were re-collected, extracted, and new bioassays performed. Crayfish again strongly preferred "non-grazed" over "grazed" extracts from *E. capillifolium* (p=0.006; Figure 1B), confirming that this plant utilizes a chemical defense activated by physical damage although it also possesses a constitutive defense since "non-grazed" extracts were also deterrent. However, although crayfish were again deterred by extracts of *G. tinctorium*, there was no significant difference in palatability of “grazed” and “non-grazed” extracts (p=0.97 direct comparison; p=0.083 indirect; Figure 1D), suggesting a constitutive rather than activated chemical defense for this species. Bioassays of the second collection of *S. latifolia* also suggested a constitutive chemical defense, since both “grazed” and “non-grazed” extracts were unpalatable to crayfish and their effects were indistinguishable from each other (Figure 1H). Neither “grazed” nor “non-grazed” extracts of *M. keisak* significantly deterred crayfish feeding relative to no-extract controls in the second bioassay (although non-significant trends towards deterrence were observed; p=0.057 and 0.14, respectively, Figure 1F), and the “non-grazed” extract did not deter crayfish feeding in the first bioassay (p=0.87, Figure 1E), suggesting that this plant may contain no chemical defense. Therefore, overall, only one of the 21 macrophytes studied, *E. capillifolium*, showed consistent changes in extract palatability characteristic of a chemical defense activated by physical damage.

In some cases, results of initial bioassays were difficult to interpret. For example, bioassays directly comparing palatability of “grazed” and “non-grazed” extracts of *Saururus cernuus* and *Agrostis scabra* (Cochran Shoals collection) suggested crayfish preference for “grazed” over “non-grazed” extracts (Figure 2A, 2H), whereas comparisons of palatability of each of these extracts with no-extract controls indicated strong chemical defenses. For these two plant species, bioassays using extracts from the initial collections were repeated, and crayfish preference for “grazed” extracts was not observed the second time. Bioassays directly testing “grazed” vs. “non-grazed” extracts for these plants took up to 20 times longer than typical assays, probably because crayfish avoided feeding on both treatment foods offered, which may account for the lack of reproducibility in these assays.

Similar difficulties in interpreting data were encountered for *Polygonum sagittatum*, which appeared to not be chemically defended (Figure 3I). Compared to no-extract controls, neither *P. sagittatum* "grazed" nor "non-grazed" extracts deterred crayfish feeding (p=0.40, p=0.54, respectively), yet in the initial bioassays directly comparing "grazed" vs. "non-grazed" extracts, "grazed" extracts were significantly preferred over "non-grazed" extracts (p=0.048). A bioassay repeating this direct comparison showed an opposite pattern, with "non-grazed" being preferred over "grazed" extracts (p=0.022). *P. sagittatum* appears to use no chemical defenses since both of this plant's extracts were similarly palatable to no-extract controls.

Thin layer chromatography (TLC) analysis of plant extracts revealed approximately 10-15 UV-absorbing and sulfuric acid-staining compounds for each species and exposed some differences in chemical composition of "grazed' vs. "non-grazed" extracts. TLC analysis of *Galium tinctorium*, which appeared to possess an activated chemical defense from the first experiment, but upon re-collection and extraction suggested a constitutive rather than activated defense, was only conducted on extracts from the second collection because there was no remaining extract material from the initial collections. This analysis revealed four polar spots in the "grazed" extract of *G. tinctorium* that were not present in the "non-grazed" extract, including three spots that became visible after staining with sulfuric acid, and one yellow spot visible under natural light. TLC did not reveal obvious differences in extract chemical composition of *Eupatorium capillifolium* (which possessed an activated chemical defense evident from two independent collections).

In addition, TLC differences between "grazed" and "non-grazed" extracts were observed for four species of plants with constitutive chemical defenses: *Aster* sp., *Lycopus virginicus*, *Mimulus ringens*, and *Sagittaria latifolia*. A few polar, UV-active spots were evident by TLC in the "non-grazed" extracts, which were not present in the "grazed" extracts, of both *S. latifolia,* and the *Aster* sp. TLC showed the presence of a mid-polar, yellow spot in the "non-grazed" extract of *L. virginicus*, which was not evident in the "grazed" extract. These differences for *S. latifolia, Aster sp.,* and *L. virginicus* suggest that compounds present in the plant, and observed by TLC in the "non-grazed" extract, may have decomposed due to the grazing simulation treatment and thus were missing from the "grazed" extract. A very prominent polar, UV active spot was evident in the "grazed" extract of *M. ringens* that was not present in the "non-grazed" extract. No TLC differences were observed for other plant species.

For the 19 plant species for which total soluble protein was measured, positive correlations were identified between: 1) dry mass per volume of fresh plant tissue and soluble protein per volume of fresh plant tissue ($p<0.001$, Figure 4A); 2) ash-free plant dry mass per total plant dry mass and total soluble protein per total plant dry mass ($p<0.001$, data not shown); 3) ash-free plant dry mass per total plant dry mass and total soluble protein per volume of fresh plant tissue ($p<0.001$, data now shown). Compared to the 19 plants analyzed, the broccoli/lettuce mixture used as food base for the feeding assays had the highest value of protein per dry mass and the second highest value of protein per volume of fresh plant tissue.

No significant correlation was found between the magnitude of deterrence of plant extracts (calculated from feeding data on "grazed" extracts vs. no-extract controls for each plant species) and plant protein content, neither when protein was expressed as soluble protein per volume of fresh plant tissue ($p=0.95$, Figure 4B), nor when protein was expressed as a percentage of plant dry mass ($p=0.67$, Figure 4C). No significant correlation existed between magnitude of deterrence and protein content both when chemically defended plants were grouped together and when non-chemically defended plant were grouped together ($p> 0.13$, $p>0.29$, respectively, whether calculated using protein as a proportion of plant volume or of dry mass).

CHAPTER 4

DISCUSSION

Despite the high rate of herbivory in freshwater systems (Horne and Goldman, 1994), and the prediction that selection for anti-herbivore defenses, including chemical defenses, should be strong (Lodge et al., 1998), only 14 species of freshwater plants had been previously examined for chemical defenses prior to this study. The current study more than doubles the available data on the frequency of chemical defenses among freshwater plants, providing evidence for 19 previously untested species, and establishes that a majority of freshwater plant species tested (52 % of 21 species in this study; 55 % of 33 species tested overall to date) utilize chemical defenses against generalist herbivores (Newman et al., 1992; Bolser and Hay, 1998; Bolser et al., 1998; Cronin, 1998; Kerfoot et al., 1998; Kubanek et al., 2001; Cronin et al., 2002, this study [Figures 1-3]). Because almost all of the freshwater plant species chosen for this and previous studies were selected based upon availability and not because they were suspected of being chemically defended, it is possible that freshwater plants in general are commonly chemically defended. It is difficult to compare the frequency of chemical defenses in terrestrial, freshwater, and marine ecosystems because very few studies have documented the frequency of chemical defenses from an unbiased sampling of multiple species. Paul and Hay (1986) found that 56 % of 70 seaweed species examined contained unusual secondary metabolites (according to TLC analysis), and that the presence of secondary metabolites in seaweeds correlated with low susceptibility to herbivory. However, bioassays testing the effects seaweed extracts on herbivory were not performed.

Nevertheless, results from Paul and Hay (1986) and from our current investigation suggest that the frequency of chemical defenses among marine and freshwater plants may be similar.

Our finding that 52 % of freshwater plant species examined are chemically defended may be an overestimation, if our choice of abundant plants resulted in selective examination of species that are resistant to herbivores (since plants may be abundant because herbivores avoid them). Additionally, our assessment of plant chemical defenses was based solely on bioassays using crude extracts, rather than on the identification of the compounds responsible for the deterrence of plant extracts. Therefore, we may have generated artifacts during extraction causing deterrence in some cases, or decomposition of deterrent compounds during extraction leading to a lack of observed deterrence in other cases. However, in previous studies of freshwater plant chemical defenses (e.g., Newman et al, 1992; Bolser et al., 1998; Kubanek et al., 2001), fractionation of deterrent crude extracts did eventually lead to the identification of plant natural products as being responsible for the observed feeding deterrence, so it is likely that most species we identified as being chemically defended actually do possess antifeedant natural products.

This is the first study that has measured the frequency of activated chemical defenses among a group of freshwater plants. We found that only one (*Eupatorium capillifolium*) of 21 species had a chemical defense that was reliably activated by mechanical damage, indicated by palatability differences between simulated "grazed" vs. "non-grazed" plant extracts (Figures 1A, 1B). Although three other species (*Galium tinctorium*, *Murdannia keisak*, and *Sagittaria latifolia*) were suspected of possessing activated chemical defenses from initial experiments, repeated experiments failed to confirm this (Figure 1C-H).

Chemical differences between “grazed” and “non-grazed” extracts were not obvious by thin layer chromatography (TLC) for *E. capillifolium*, although in *G. tinctorium* extracts, at least four TLC spots corresponding to polar, organic compounds were observed in “grazed” extracts that were not present in “non-grazed” extracts. The failure to observe TLC differences between these two extracts of *E. capillifolium*, the one plant that consistently showed extract palatability differences indicating an activated defense, does not indicate that chemical differences did not exist. Our TLC method may not have revealed 1) compounds that were present at very low concentration, 2) compounds that had similar TLC retardation factors to that of abundant compounds such that some spots were obscured, 3) compounds that did not absorb visible or short-wave UV light or stain with sulfuric acid, or 4) quantitative changes in compounds between treatments.

The one plant that consistently showed evidence for an activated chemical defense, *Eupatorium capillifolium*, can vary greatly in size, growing up to 3 meters tall. *E. capillifolium* specimens collected in our investigation were slender, delicate, bore no flowers, were no more than 20 cm tall, and were therefore suspected to be young. Young plant tissue may be expected to be more highly defended against herbivory than mature plant tissue because young tissue is often more nutritious, and therefore may be more vulnerable and valuable to the plant (Feeny, 1976; Rhoades, 1979). However, a competing plant-herbivore model has suggested than young tissue may be physiologically constrained from producing defensive metabolites (Herms and Mattson, 1992). One potential problem for immature, growing organisms could be autotoxicity associated with the production or storage of chemical defenses, but the use of an activated defense such as that of *E. capillifolium* could greatly reduce autotoxic effects because defenses would only infrequently be produced and could be short-lived. Based upon superficial examination, our *E. capillifolium* appeared to have no obvious structural defenses, and might therefore rely solely on chemical defense. However, since mature *E. capillifolium* was not included in our study and its chemical defenses have not been previously studied, we cannot make intraspecific comparisons.

Including the one freshwater macrophyte species with an activated chemical defense from this study (*Eupatorium capillifolium*), and the two macrophytes, *Nasturtium officinale* and *Habenaria repens*, found to possess activated chemical defenses in previous investigations (Newman et al., 1992, 1996; Bolser et al., 1998), a total of 9 % (3 of 33) of freshwater plant species tested for chemical defenses, have been found to display consistent changes in palatability characteristic of activated chemical defenses. Compared to the numerous investigations examining chemical defenses in marine ecosystems (reviewed by Paul, 1992; Hay and Fenical, 1996), only two investigations of marine plants (macroalgae) have directly tested for chemical defenses activated by physical damage (Paul and Van Alstyne, 1992; Cetrulo and Hay, 2000). Of the 45 species included in these two investigations, 22 % (10 of 45) had activated chemical defenses, although the deterrent compounds have only been identified for three of these species (Paul and Van Alstyne, 1992). Additionally, some marine algae are known to use activated chemical defenses, whereby dimethylsulphoniopropionate is hydrolyzed to yield dimethyl sulfide and acrylic acid, one or both of which deter grazing by protozoans and sea urchins, when algal cells are lysed (Wolfe et al., 1997; Van Alstyne et al., 2001; Van Alstyne and Houser, 2003). Thus, activated chemical defenses do exist among marine plants, and Cetrulo and Hay (2000) suggest they may be fairly common. In contrast, a relatively smaller proportion of freshwater plants (3 of 33 species tested) have been found to possess activated chemical defenses (Newman et al., 1990; Bolser et al., 1998, this study), although 45 % of species in this group possessed constitutive chemical defenses.

Among some of the 33 freshwater plant species previously studied, but which were not included in our study, cases of activated chemical defenses may have been overlooked due to 1) damaged treatments not being performed; and 2) some plants' defenses becoming activated by standard collection and handling practices, such that these activated defenses would be misinterpreted as constitutive defenses. Therefore, among the 10 freshwater plant species previously tested for chemical defenses but not for activated chemical defenses, we cannot be sure that activated defenses were not present in the four species (*Iris virginica*, *Nuphar advena*, *Nuphar luteum*, *Typha angustifolia*) that were found to possess chemical defenses, nor in the six species (*Brachospermum* sp., *Carex vesicaria*, *Ceratophyllum demersum*, *Chara* sp., *Potamogeton ampliflius*, *Spirogyra* sp.) that were reported to not possess chemical defenses (Newman et al., 1992; Bolser and Hay, 1998; Bolser et al., 1998; Cronin, 1998). Thus, the overall frequency of activated chemical defenses among these 33 plant species could be as high as 39 %, but based upon species which were tested for activated defenses, only 13 % (3 of 23) have been shown to use activated chemical defenses.

In our study, the low frequency of activated chemical defenses (1 of 21 plant species) may also be an underestimate of the actual frequency, due to methodological limitations. First, because our feeding assays focused on only one herbivore, the crayfish *Procambarus verrucosus*, we may have missed chemical defenses, including activated chemical defenses, which deter other herbivores but not crayfish. Second, plants may respond to specific cues or types of damage from herbivores rather than responding simply to physical damage, as has been shown for some induced defenses (Karban and Baldwin, 1997). For example, deterrent phlorotannins were induced in the brown alga *Ascophyllum nodosum* when directly grazed by a snail, *Littorina obtusata*, known to feed on this seaweed, but defenses were not induced by physical damage with a hole-puncher (Pavia and Toth 2000). Turlings et al. (1990) found that when caterpillars grazed corn seedlings for several hours, a mixture of terpenoids (α-*trans*-bergamotene, (*E*)-β-farnesene, and (*E*)-nerolidol) were released by the plant that in turn attracted wasps that parasitize the caterpillars. These compounds were not released by undamaged seedlings nor by plant tissue damaged artificially. However, in some instances mechanical damage has been shown to induce a defensive chemical response. Green and Ryan (1972) established that accumulation of proteinase inhibitor I, suggested to function as protection against herbivores, was induced in potato and tomato plants by insect or mechanical damage. However, not all methods of artificial damage used were equally effective; crushing of tissue was apparently more effective that hole-punching. Yet, no such examples of herbivore specific cues are known for activated chemical defenses. In fact, because activation can occur within seconds of damage (Paul and Van Alstyne, 1992), we hypothesize that cell lysis or tissue rupture, leading to contact and chemical reaction between available precursors, for example as occurs with the glucosinolate-myrosinase system (Van Etten and Tookey, 1979), may be the most reliable trigger for a rapidly activated chemical defense. Previous studies that reported activated chemical defenses in aquatic plants have used physical damage such as grinding in water (Cetrulo and Hay, 2000), or freezing to lyse cells followed by thawing (Bolser et al., 1998; Newman et al., 1990, 1992). We expect that our method of grinding plant tissue in water with a blender should reasonably mimic the natural shredding of crayfish feeding behavior in that both the artificial and natural methods result in rapidly rupturing many individual plant cells in an aqueous medium. However, we did not investigate the possibility that crayfish-specific cues might have led to activation of chemical defenses that we did not observe.

Another methodological limitation that may have affected our results is the use of organic solvents to prevent activation and to extract plants. In our study, simulation of herbivore grazing was achieved by blending fresh plant material in water for 45 seconds, followed by addition of methanol to begin extraction, whereas our "non-grazed" treatment consisted of blending fresh plant material in methanol for 45 seconds, which we expected would prevent enzymatic conversion of precursors to activated defenses or prevent up-regulation of defense biosynthesis, also hypothesized to occur enzymatically. However, if enzymes associated with production of defenses remained active in methanol or aqueous methanol (e.g., Khan et al., 2000), which in our case included approximately 15 % water from the plant itself, then chemical defenses may have been activated even in the "non-grazed" treatments, leading us to conclude that such plants were in fact constitutively chemically defended. This would have led to an over-reporting of constitutive defenses and an under-reporting of activated defenses. Some solvents may also create or destroy active compounds (Puyana et al., 2003), which may have over- or under-exaggerated our findings of chemical defenses in general. Presumably, our extraction method (which included use of water, methanol, and dichloromethane) with multiple solvent evaporation steps selected for stable, non-volatile, organic, non-protein (i.e., non-denaturable) chemical defenses. However, our method should have resulted in the efficient extraction of all known types of plant chemical defenses excluding proteins and hydrogen cyanide, but including alkaloids, saponins, terpenes, tannins, lignoids, acetylinic acids, isothiocyanates, and nitriles, as well as typically non-defensive plant compounds like pigments, steroids, glycosides, and fatty acids.

Our ability to detect potentially small, localized changes in plant defensive chemistry was inhibited by our methodology of grinding up entire plants. Such within-plant variation in constitutive of induced chemical defenses occurs in both terrestrial and aquatic plants (McKey, 1979; Steinberg, 1984; Cronin and Hay, 1996a; Taylor et al., 2002), and may allow a plant to efficiently defend its most valuable or vulnerable tissues (Rhoades, 1979). For example, Taylor et al. (2002) found that the brown alga *Sargassum filipendula* induces chemical resistance to grazing only in the top stipes of the plant, which contain valuable meristematic tissue responsible for new growth. Older stipes which secure the plant to the sea floor were constitutively, morphologically defended by tissue toughness. If plant chemical defenses can be activated locally, which has not yet been assessed, then in our study this occurrence might have been detected either as plant defenses wholly activated (if the activation was strong enough to change the palatability of a whole plant extract), or as a lack of plant defenses (if activated defenses were ineffective once diluted by extracts of whole plants).

Whether activated defenses can occur locally within plant tissues, or whether damage in one part of a plant changes palatability of a whole plant is difficult to test, because dissection of plant tissues for the purpose of analysis of each tissue separately requires causing plant damage that could activate defenses. A possible, yet experimentally demanding option might be to expose plants to herbivore or mechanical damage, then to flash-freeze whole plants in liquid nitrogen (at approximately –200 °C), which would immobilize cell contents. Plant parts could then be dissected in liquid nitrogen, without activating enzymes or allowing chemical reactions to take place. Then, dissected plant parts could be placed in solvent while still frozen, and extracted. Palatability and chemical composition of extracts from plant parts at differing distances from damage could be compared to each other, and to undamaged controls. While the intra-plant variation in activated defenses and the mechanisms by which plants exchange information about damage and coordinate responses to damage are intriguing, we decided to focus the current study on the broader question of the frequency of these defenses because so little is known about this in freshwater systems (Horne and Goldman, 1994).

Based on results from previous investigations (Feeny, 1976; Cronin and Hay, 1996b; Puglisi and Paul, 2000) and from our investigation, chemical defenses may vary by location, time, and/or environmental conditions. Extracts of *Agrostis scabra* collected from two different sites and in different months had different effects on crayfish feeding behavior, with a collection from the Cochran Shoals site suggesting a constitutive chemical defense (Fig 2A) and a collection from the Johnson Ferry site suggesting no chemical defense (Fig 3A). Similarly, extracts of *Alternanthera philoxeroides* from our investigation were palatable (Fig 3B), however, Cronin et al. (2002) reported a constitutive chemical defense for *A. philoxeroides* collected in North Carolina and assayed using a crayfish congeneric to ours. Four other macrophyte species (*Eupatorium capillifolium*, *Galium tinctorium*, *Murdannia keisak*, and *Sagittaria latifolia*) for which our initial experiments suggested the possibility of activated chemical defenses (Figure

1A, 1C, 1E, 1G) were each re-collected (during a different month than the initial collection but at the same sites), extracted, and assayed, with the result that an activated chemical defense could only be confirmed for one of these (*E. capillifolium*, Figure 1B). Because the results of bioassays with *E. capillifoium* were reproducible, we are confident in our assessment that this plant uses an activated chemical defense and that the results were not the cause of a statistical artifact. Feeding assay results of the second experiments for *G. tinctorium*, and *S. latifolia* indicated constitutive chemical defenses (Figure 1D, 1H), and suggested no chemical defense for *M. keisak* (1F). This raises four possibilities: 1) results from the first bioassays for *G. tinctorium*, *M. keisak*, and *S. latifolia* produced a Type II experimental error; 2) results from the second bioassay for *G. tinctorium*, *M. keisak*, and *S. latifolia* produced a Type I experimental error; 3) these plants used activated chemical defenses in early summer (when initial collections were made) and constitutive or no chemical defenses in fall (when the second collections were made); 4) at the time of the second collection, physical stress or herbivore attack had resulted in defenses already being activated in *G. tinctorium* and *S. latifolia*, such that even the "non-grazed" extracts had elevated levels of deterrent compounds. At this time, we cannot distinguish between these possibilities, but because extracts of *G. tinctorium* and *S. latifolia* were generally unpalatable to crayfish relative to no-extract controls (Figure 1C, 1D, 1G, 1H), we are confident in attributing constitutive chemical defenses to these plants. Most *M. keisak* extracts, in contrast, did not deter crayfish feeding relative to no-extract controls (Figure 1E, 1F).

Some other variations in bioassay results, for example direct comparisons of feeding on simulated "grazed" and "non-grazed" extracts of *Agrostis scabra* (Cohcran Shoals collection), *Saururus cernuus,* and *Polygonum sagittatum* (Figure 2A, 2H, 3I) may be explained by one of the following reasons: 1) crayfish disliked both "grazed" and "non-grazed" extracts, such that when "grazed" and "non-grazed" extracts were directly tested in feeding assays, crayfish avoided both treated foods, and took up to 20 times longer to feed adequately, making actual preferences hard to detect (*S. cernuus*, *A. scabra*); 2) results from one or both of these bioassays led to a Type II error; and 3) by the time of the second bioassay using extracts stored frozen since shortly after collection, degradation of some compounds may have occurred. Because of the uncertainty of results from these direct comparisons of palatability of "grazed" vs. "non-grazed" extracts, we used data from bioassays involving no-extract controls and statistical analysis from indirect comparisons to establish that *S. cernuus* and *A. scabra* are constitutively defended and that extracts of *P. sagittatum* are palatable.

The mechanism of chemical defense that a plant evolves (e.g., constitutive, induced, or activated) may depend on the type of herbivores selecting for plant defenses. Large wetland herbivores such as moose, deer, beavers, rabbits, and fowl may remove an entire plant in a few bites. Facing these herbivores, plant defenses that 1) prevent an herbivore from taking the first bite (e.g., a morphological defense such as tissue toughness), or 2) deter herbivore interest prior to the first bite (e.g., a deterrent odor) should be most effective. Alternatively, an herbivore could learn to avoid certain plants following an early experience with a constitutive of activated chemical defense that left it sick (Provenza, 1995), or an herbivore could learn to avoid a plant species after finding it distasteful (Kimball et al., 2002). These learning experiences could be coupled with plant odor or visual cues to remind experienced herbivores to avoid the defended plant.

Natural selection for chemical defenses based on learning by large herbivores would require that either individual plants survive an initial attack, or that kin or clones of a consumed plant benefit from herbivores' learning experiences; however there is no experimental evidence that the latter occurs. In the case of mid-sized to large herbivores that could destroy a plant in one meal consisting of several bites over a period of seconds to minutes, an activated or constitutive chemical defense could be successful at repelling herbivores after an initial unpleasant bite, whereas defenses induced over periods of hours to months may be ineffective. Activated and constitutive chemical defenses may also be used by plants co-occurring primarily with generalist herbivores, since generalists may feed from a variety of plant species, and if food is not limited, generalists are known to select plants with high nutritional value, low defenses, or other desirable traits, avoiding unpalatable plants (Cronin, 1998). Our collection sites included several such mid-sized to large generalist herbivores, for example crayfish (*Procambarus verrucosus* used as a model herbivore in this study), as well as fishes, fowl, beavers, rabbits, and deer (A. Prusak, personal observation).

If allocation, opportunity, or ecological costs of plant chemical defenses are substantial (Baldwin, 1998; Koricheva, 2002; Zavala et al., 2004), and if herbivory is unpredictable in space or time, then activated chemical defenses may be more efficient than constitutive defenses. In the current study, we found that constitutive chemical defenses were common among freshwater plants (52 % of species tested), but activated chemical defenses were rare (5 % of species tested). Since we did not test for induction, we cannot assess its frequency, but it is possible that some of the plants that either appeared to be constitutively defended or undefended may have possessed induced chemical defenses that were not observed. Nevertheless, the high frequency of constitutive chemical defenses found in this study suggests that: 1) herbivory is or has been predictably high in wetland ecosystems, possibly because plants are vulnerable to both aquatic and terrestrial herbivores, diminishing the benefit of activated defenses over constitutive chemical defenses; and/or 2) that costs of resistance are not limiting for wetland plant species.

Since herbivores are often nitrogen-limited and not carbon-limited (Mattson, 1980), protein content should be a good proxy of plant nutritional quality. Thus, plants with high protein levels may be more desirable to herbivores, possibly selecting for plant defensive traits including chemical defenses. However, our initial hypothesis that plants with greater nutritional value are more strongly chemically defended was not supported by our data, which suggested no correlation between deterrence of plant extracts and protein content (whether protein was expressed as a proportion of plant volume or dry mass) (Figure 4B, 4C). Whether plant species were grouped all together (Figure 4B, 4C), or chemically defended plants grouped separate from plants with no chemical defenses, no correlation was observed ($p > 0.13$ for chemically defended plants, $p > 0.29$ for plants with no chemical defenses). This lack of correlation also causes us to reject an alternative hypothesis that protein-rich plants may be physiologically constrained from devoting resources to the production of chemical defenses (whose biosynthesis require the use of enzymes). However, because we measured deterrence of extracts rather than whole plant palatability, a correlation between protein content and resistance to herbivory in general may exist that we were not able to detect. Our investigation did not assess morphological or associational defenses, nor did it assess additive or synergistic effects of multiple defenses.

Previous investigations have indicated that prey nutritional quality and prey chemical and morphological defenses work in concert to affect consumer food choices. For example, calcium carbonate and deterrent terpenes appear to act synergistically to defend the green alga *Halimeda goreauii* from herbivory by a sea urchin (Hay et al., 1994). Additionally, some herbivores are more willing to consume deterrent or toxic compounds if found in a nutritionally-rich food than if found in a poor food (e.g., Bernays, 1981; Duffy and Paul, 1992; Hay et al., 1994). In our investigation, a direct positive correlation was found between plant dry mass/volume and protein content/volume (Figure 4A), which may have affected feeding assay results, because our feeding assays exposed crayfish to natural concentrations of plant extracts incorporated into artificial foods with the appropriate dry mass of plant food (broccoli and lettuce) for each plant species. Thus, extracts from high dry mass plants were tested in artificial foods with high protein content, and because of this high protein content, may have been perceived as less deterrent than extracts from low dry mass plants. Therefore, our ability to directly compare the deterrence of extracts from different plants is confounded by potential differences in plant nutritional quality. If, instead, we had compared the deterrence of all extracts in artificial foods with equal, average protein content, then we might have found that protein-rich plants had more potent defenses. However, this would have been a flawed approach, because to be effective in the field, chemical defenses must operate to deter consumption of plant tissue containing that plant's own nutritional value, so by testing plant extracts at natural concentrations in foods with appropriate dry mass, we reasonably assessed chemical defenses for each plant. In fact, because the broccoli and lettuce mixture we used as experimental food had a high concentration of protein relative to its dry mass, our assays were conservative in testing plant extracts against a background of nutritionally-rich food.

Although based on little experimental evidence, it was previously thought that macrophyte-herbivore interactions in freshwater ecosystems were negligible and did not have significant effects on community structure (Hutchinson, 1975; Wetzel, 1983). Recent studies of macrophyte-herbivore interactions have demonstrated that herbivory is as high in freshwater ecosystems as it is in terrestrial and marine ecosystems (Cyr and Pace, 1993), and that these interactions can have greater effects on community structure than previously assumed (Lodge et al., 1998). Grazing by generalist herbivores can either increase or decrease plant diversity and is thought to be dependent on consumer preference and prey defenses (Lubchenco, 1978). High herbivory and herbivore diversity in freshwater ecosystems may be expected to select for diversity of defensive mechanisms, including chemical defenses.

It has been demonstrated that crayfish have an important role in structuring macrophyte communities, and can significantly reduce plant species richness (Lodge and Lorman, 1987, Lodge et al., 1994). Generalist herbivores have been shown to base their feeding preference on morphology, nutritional value, and chemical defenses (Lodge, 1991; Cronin, 1998; Cronin et al., 2002). Our finding that 11 of 21 wetland macrophyte species investigated have chemical defenses that significantly deterred feeding by the generalist crayfish *Procambarus verrucosus* provides evidence that chemical defenses in freshwater ecosystems are common, although chemical defenses activated by physical damage were observed for only one of these plant species. When combined with previous studies on freshwater plant chemical defenses, it is clear that many freshwater macrophytes use chemical defenses, particularly constitutive chemical defenses, to defend themselves against generalist herbivores.

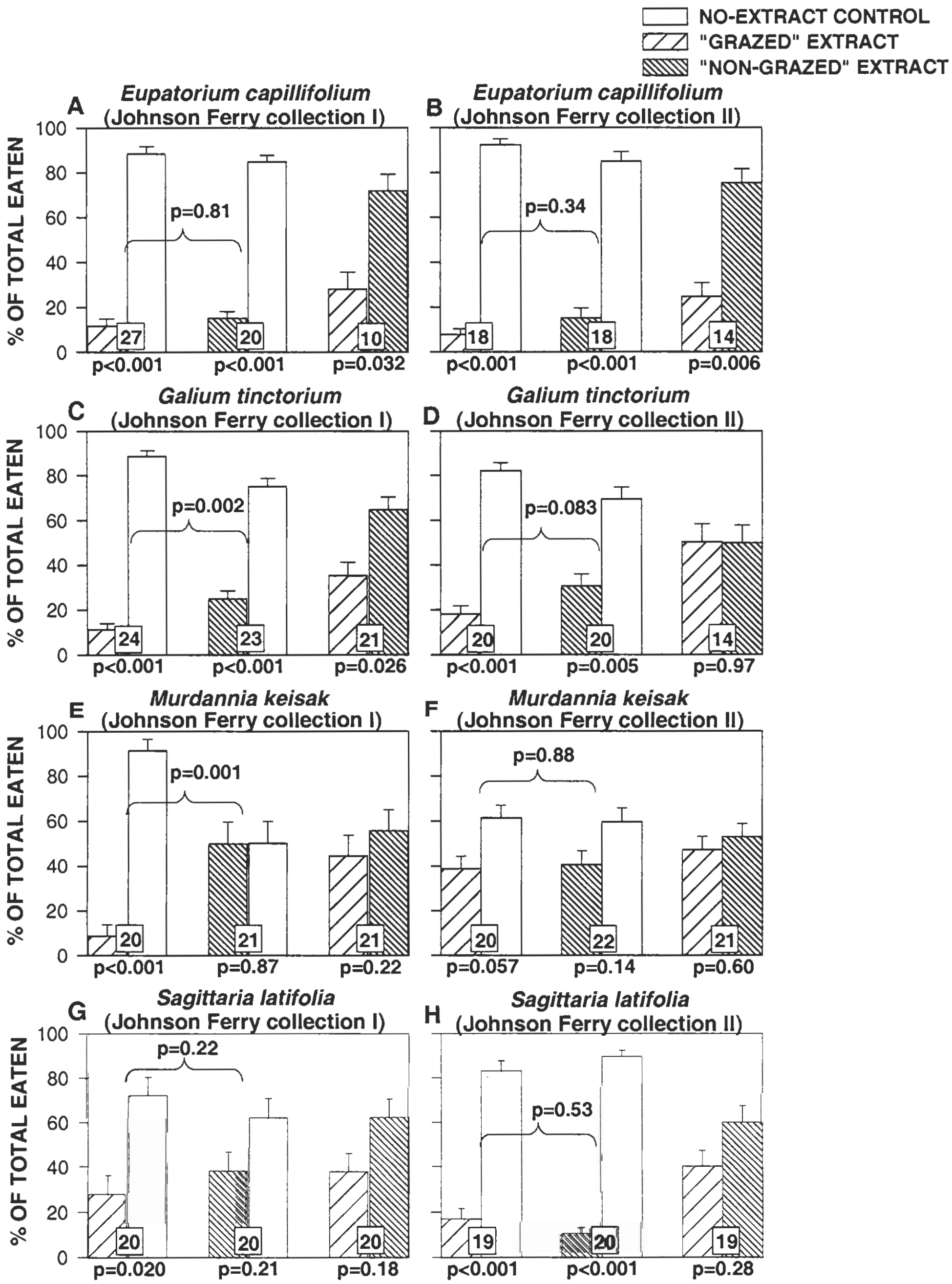

Figure 1. Activated Chemical Defenses. Effects of simulated "grazed" and "non-grazed" freshwater plant extracts on crayfish feeding behavior: macrophytes found to have activated chemical defenses (*Eupatorium capillifolium*) and macrophytes with initial data suggesting activated defenses, but upon repeated experiments, found to have constitutive chemical defenses (*Galium tinctorium* and *Sagittaria latifolia*) or no chemical defenses (*Murdannia keisak*). Histogram pairs represent paired experiments. Sample size is noted in a box at the base of each histogram.

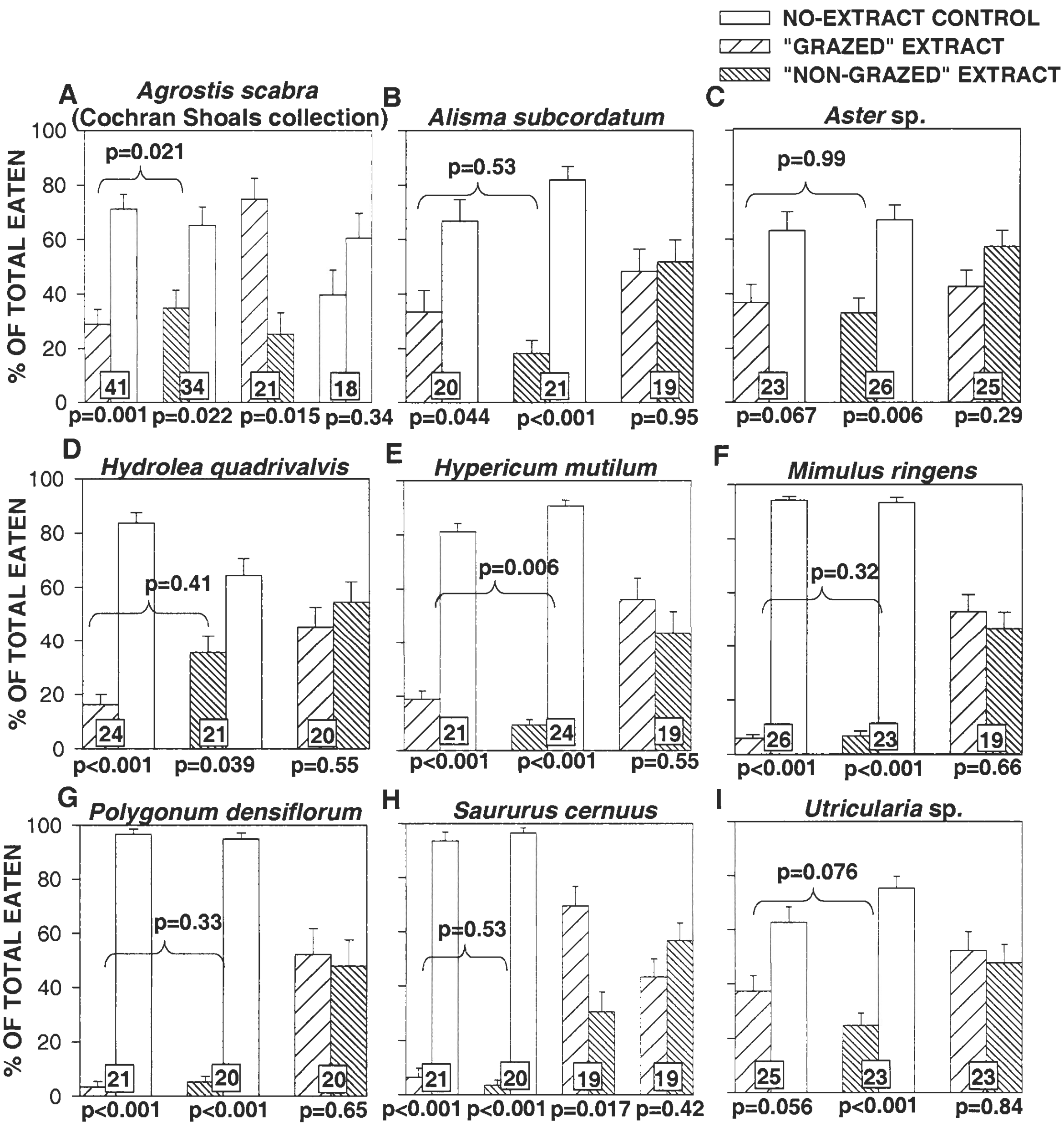

Figure 2. Constitutive Chemical Defenses. Effects of simulated "grazed" and "non-grazed" freshwater plants extracts on crayfish feeding behavior: macrophytes found to have constitutive chemical defenses. Analysis and symbols as in Figure 1.

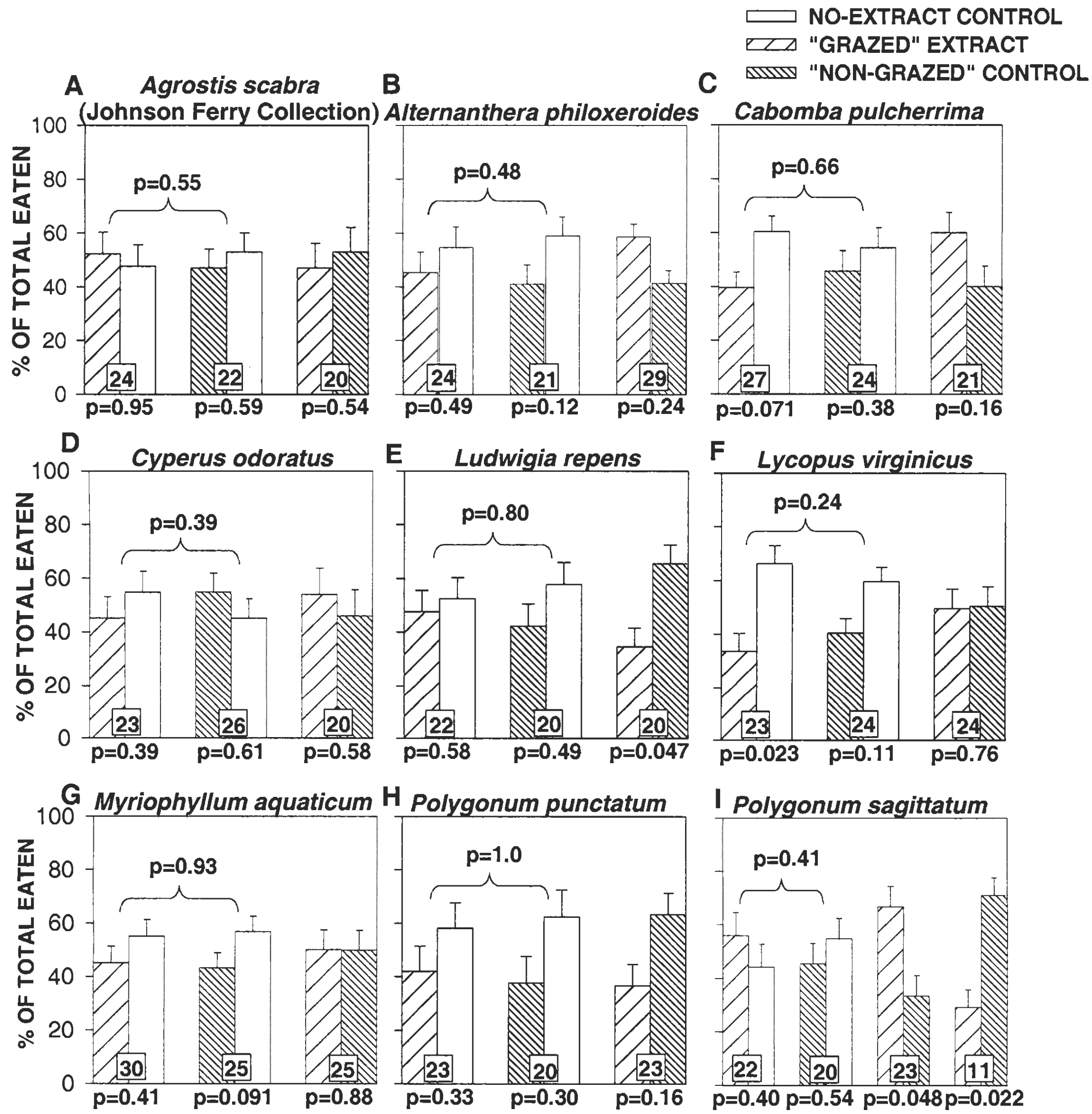

Figure 3. No Chemical Defenses. Effects of simulated "grazed" and "non-grazed" freshwater plant extracts on crayfish feeding behavior: macrophytes found to have no chemical defenses. Analysis and symbols as in Figure 1.

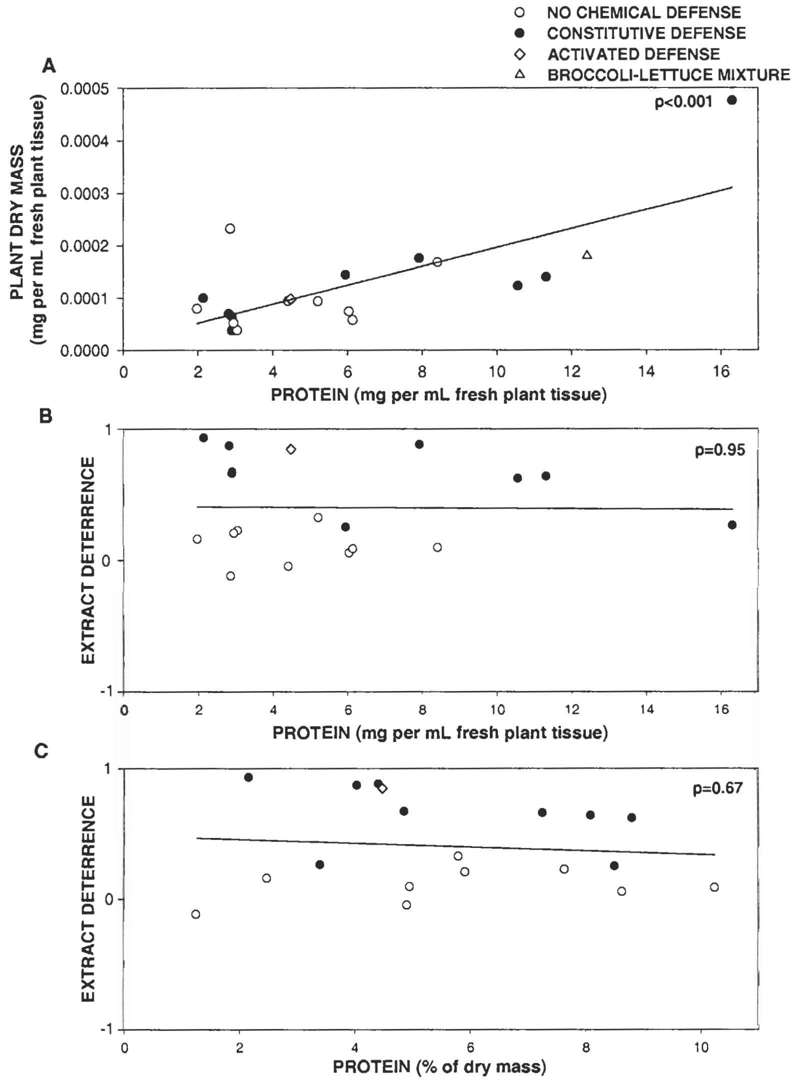

Figure 4. Protein and Extract Deterrence. A. Relationship between soluble protein concentration and plant dry mass for 19 freshwater plant species in this study and broccoli/lettuce mixture used as experimental food. r^2=0.534. B. Relationship between soluble protein concentration per volume of fresh plant tissue and deterrence of plant extracts for 19 freshwater plant species. A positive extract deterrence value indicates strong deterrence, zero indicates no deterrence, and a negative value indicates feeding stimulation by the extract. C. Relationship between soluble protein concentration as a proportion of plant dry mass and deterrence of plant extracts. Symbols and analysis as Figure 4B.

Table 1. Freshwater Plant Traits. Freshwater plant species used in this study, some plant traits, and assessment of chemical defenses. Protein content data are also provided for the broccoli and lettuce mixture that was used as the experimental food. Protein content is reported as mean ± 1 SE. [1] USDA (2004) [2] Godfrey and Wooten (1979) [3] (1981).

Plant Species	Common Name	Duration [1]	Origin [1, 2, 3]	Chemical Defense	Protein (% of dry mass)	Protein (mg/mL fresh plant tissue)
Agrostis scabra	rough bentgrass	perennial	US Native	none	4.90 ± 0.08	4.41 ± 0.08
Alisma subcordatum	American water plantain	perennial	US Native	constitutive	not measured	
Alternanthera philoxeroides	alligatorweed	perennial	S. America Native	none	not measured	
Aster sp.		perennial		constitutive	3.40 ± 0.13	16.3 ± 0.6
Cabomba pulcherrima	fanwort	perennial	US Native	none	5.91 ± 0.50	2.95 ± 0.25
Cyperus odoratus	fragrant flatsedge	annual/perennial	US Native	none	4.95 ±0.07	8.41 ± 0.12
Eupatorium capillifoium	dogfennel	perennial	US Native	activated	4.48 ± 0.29	4.48 ± 0.29
Galium tinctorium	stiff marsh bedstraw	perennial	US Native	constitutive	8.08 ± 0.17	11.3 ± 0.2
Hydrolea quadrivalvis	waterpod	perennial	US Native	constitutive	4.85 ± 0.18	2.91 ± 0.11
Hypericum mutilum	dwarf St. Johnswort	annual/perennial	US Native	constitutive	8.80 ± 0.49	10.6 ± 0.6
Ludwigia repens	creeping primrose-willow	perennial	US Native	none	8.63 ± 0.26	6.04 ± 0.18
Lycopus virginicus	Virginia water horehound	perennial	US Native	none	5.79 ± 0.22	5.21 ± 0.20
Mimulus ringens	allegheny monkeyflower	perennial	US Native	constitutive	4.41 ± 0.39	7.93 ± 0.70
Murdannia keisak	marsh flower	perennial	Asia Native	none	7.63 ± 0.44	2.90 ± 0.17
Myriophyllum aquaticum	parrot-feather	perennial	S. America Native	none	10.2 ± 0.7	6.14 ± 0.42
Polygodum densiflorum	denseflower knotweed	annual/perennial	US Native	constitutive	2.15 ± 0.11	2.15 ± 0.11
Polygodum punctatum	dotted smartweed	annual/perennial	US Native	none	2.47 ± 0.15	1.98 ± 0.12
Polygodum sagittatum	arrowleaf tearthumb	annual/perennial	US Native	none	1.24 ± 0.05	2.87 ± 0.16
Sagittaria latifolia	broadleaf arrowhead	perennial	US Native	constitutive	7.25 ± 0.63	3.05 ± 0.27
Saururus cernuus	lizard's tail	perennial	US Native	constitutive	4.04 ± 0.16	2.83 ± 0.11
Utricularia sp.	bladderwort		US Native	constitutive	8.50 ± 0.54	5.95 ± 0.38
broccoli/lettuce control					12.4 ± 0.58	12.4 ± 0.58

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