FRESHWATER RED ALGAE USE ACTIVATED CHEMICAL DEFENSES AGAINST HERBIVORES

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FRESHWATER RED ALGAE USE ACTIVATED CHEMICAL DEFENSES AGAINST HERBIVORES

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Dedicated to my parents, Douglas A. and Jean M. Goodman,
who have taught me (by example) to make the most of my opportunities.

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SUMMARY

Chemically mediated interactions have important ecological and evolutionary effects on populations and communities. Despite recognition that herbivory can significantly affect the biomass and composition of freshwater macrophyte communities, there are few investigations of chemical defenses among freshwater vascular plants and mosses and none of freshwater red algae. This study compares the palatability of five species of freshwater red algae (*Batrachospermum helminthosum*, *Boldia erythrosiphon*, *Kumanoa sp.*, *Paralemanea annulata*, and *Tuomeya americana*) that occur in the southeastern United States relative to two co-occurring macrophytes (the chemically defended aquatic moss *Fontinalis novae-angliae* and the broadly palatable green alga *Cladophora glomerata*). We assessed the potential role of structural, nutritional, and chemical traits in reducing macrophyte susceptibility to generalist crayfish grazers. Both native and non-native crayfish significantly preferred the green alga *C. glomerata* over four of the five species of red algae. *B. erythrosiphon* was palatable, while the cartilaginous structure of *P. annulata* reduced its susceptibility to grazing, and chemical defenses of *B. helminthosum*, *Kumanoa sp.,* and *T. americana* rendered these species as unpalatable as the moss *F. novae-angliae*. Extracts from these latter species reduced feeding by ~30-60% relative to solvent controls if tissues were crushed (simulating herbivore damage) prior to extraction in organic solvents. However, if algae were first soaked in organic solvents that inhibit enzymatic activity and then crushed, crude extracts stimulated or had no effect on herbivory. *B. helminthosum*, *Kumanoa sp., and T. americana* all exhibited "activated" chemical defenses in which anti-herbivore

compounds are produced rapidly upon herbivore attack via enzymatic processes. In an additional accept/reject behavioral assay, *B. helminthosum* extracts reduced the number of crayfish willing to feed by >90%.

Given that three of the five red algal taxa examined in this study yielded deterrent crude extracts, selection for defensive chemistry in freshwater rhodophytes appears to be substantial. Activated chemical defenses are thought to be an adaptation to reduce the resource allocation and ecological costs of defense. As such, activated chemical defenses may be favored in freshwater red algae, whose short-lived gametophytes must grow and reproduce rapidly. Roughly 20% of the known chemical defenses produced by marine algae are activated; further examination is needed to determine whether the frequency of activated chemistry is higher in freshwater red algae compared to their marine counterparts. Continued investigation of chemical defenses in freshwater red algae will contribute to among-system comparisons, providing new insights in the generality of plant-herbivore interactions and their evolution.

CHAPTER I

INTRODUCTION

Herbivory was once believed to be unimportant in freshwater systems (Shelford 1918, Hutchinson 1975), however contemporary experiments and meta-analyses revealed that rates of herbivory in freshwater systems are as high as those in marine and terrestrial systems (Cyr and Pace 1993, Feminella and Hawkins 1995, Hillebrand 2009). Herbivory can significantly affect the biomass (Lodge et al. 1998, Hillebrand 2008), species composition (Lamberti and Resh 1983, Power et al. 1985) and dominant morphological form (Hill et al. 1992, Holomuzki et al. 2006) of freshwater macrophyte and periphyton communities.

Despite the recognition that herbivory may be a strong force selecting for antiherbivore defenses (Lodge et al. 1998), relatively few studies have examined chemical defenses in freshwater systems. To date, 23 freshwater vascular plants and one aquatic moss have been demonstrated to produce chemical defenses against herbivores (reviewed in Morrison and Hay 2010). Hundreds of marine macroalgae are known to be chemically defended against grazers (McClintock and Baker 2001), and marine red algae are arguably the most chemically rich group in terms of both number and type of defensive compounds isolated (Blunt et al. 2009, Stout and Kubanek 2010). Freshwater macroalgae and cyanobacteria produce toxins that are allelopathic, bactericidal, and insecticidal (see Gross 2003, Camacho 2008 for a discussion), yet it remains unknown whether freshwater macroalgae use secondary metabolites to deter grazers. Therefore, we investigated the possibility of chemical defenses in stream-dwelling red macroalgae.

The relatively short life spans of freshwater algae have been predicted to reduce the evolutionary pressure for these algae to synthesize defensive chemistry (Steinman 1996). The fast growth of aquatic algae often allows them to out-run grazers, and grazers may only be able to regulate primary production when nutrient limitation or seasonal flooding reduce algal biomass (Pringle and Hamazaki 1997). However, the relative strength of biotic and abiotic selection pressures continues to be debated in lentic (streams and rivers) systems (Power et al. 1998, Power et al. 2008, Sponseller et al. 2010, Holomuski et al 2010). Investigating whether stream-dwelling red algae produce defensive chemistry will contribute to among-system comparisons, providing new insights into the generality of plant-herbivore interactions and their evolution (Hay 1991, Lodge et al. 1998).

Freshwater red algae (Rhodophyta) are potentially a valuable food source for grazers in cold-water streams and rivers. These algae are distributed worldwide and have been found in 51% of North American stream reaches surveyed (Sheath and Hambrook 1990, Sheath and Cole 1992). Rhodophyte abundance in stream segments varies considerably, however red algae can account for 30-60% of seasonal primary production in some locations (Muller 1978, Stock et al. 1987). The majority of freshwater rhodophyte taxa exist perennially as prostrate, microscopic forms that produce upright, macroscopic reproductive stages in the spring (Sheath and Hambrook 1990).

Considerable attention has been focused on springtime diet shifts by invertebrate grazers from terrestrial detritus to aquatic primary production (e.g. algae and byrophytes; Mulholland et al. 2000, Finlay 2004, Thorp and Delong 2002, McNeely et al. 2007, Lau et al. 2009). Algal food sources are often more nutritious than detritus and vascular plants

due to higher protein and lipid content as well as lower C:N ratios in algal tissue (Lamberti 1996, Frost et al. 2002), and selective feeding by minnows, crayfish, snails, and caddisfly larvae often eliminates filamentous chlorophytes from benthic communities (Lamberti and Resh 1983, Power et al. 1985, Creed 1994, Gelwick and Matthews 1997). Despite the fact that red algae can be abundant in springtime and may represent a valuable food source for grazers (Sheath and Hambrook 1990), trophic interactions between freshwater red algae and stream herbivores have rarely been examined.

The general palatability of freshwater red algae remains unclear, though pieces of red algae have been reported in the guts of 39 stream-dwelling animals including 26 aquatic insects, 9 amphipods, 2 snails, and 2 Asian carp (Sheath and Hambrook 1990, Sheath 2003). Presence/absence data may not reflect feeding preferences and the *relative* frequency that red algae are consumed; in fact, diatoms were the most frequently ingested item by the majority of these animals (Shih 1941, Jones 1949, 1950, Mecom and Cummins 1964, Hambrook and Sheath 1987). Hambrook and Sheath (1987) examined how grazing rates relate to freshwater rhodophyte nutritional traits, but the palatability of rhodophytes was not tested against co-occurring food sources. Two studies report that grazers avoided consuming red algae while reducing the biomass of diatoms and chlorophytes in streams (Stewart 1987, Steinman et al. 1991), however mechanisms underlying grazer selectivity were not examined.

Seaweeds and freshwater macrophytes reduce grazer damage through structural traits, low nutritive value, secondary metabolites, or a combination of these mechanisms (Duffy and Hay 1990, Hay et al. 1994, Hay 1996, 2009, Bolser et al. 1998, Cronin et al. 2002). Here, we test the palatability of five freshwater red algae, spanning five genera

and four morphological forms, relative to a generally palatable green alga and a common aquatic moss known to be chemically defended. Our objective was to (1) determine the palatability of freshwater red algae relative to other commonly co-occurring macrophytes and (2) identify traits of freshwater red algae affecting their susceptibility to grazing.

CHAPTER II

METHODS

2.1 Study Organisms

Five species of freshwater red macroalgae (*Batrachospermum helminthosum*, *Boldia erythrosiphon*, *Kumanoa sp.*, *Paralemanea annulata,* and *Tuomeya americana*) were collected from rivers and spring-fed streams throughout Georgia and Alabama (Table 1). We frequently found these red algae co-occurring with aquatic mosses and the green alga *Cladophora glomerata*, as previously documented (Jones 1949, Thirb and Benson-Evans 1985, Everitt and Burkholder 1991, Filkin and Vis 2004). Therefore, we assessed the palatability of these red algae in relation to *C. glomerata* as well as the moss *Fontinalis novae-angliae*, known to be chemically defended against crayfish (Parker et al. 2007). Macrophytes were collected 24-48 h prior to feeding assays, transferred on ice to the laboratory at Georgia Institute of Technology, and maintained in aerated spring water at 14°C until used.

We used crayfish as bioassay organisms to assess macrophyte palatability because crayfish (1) are generalist feeders known to consume a wide variety of macrophytes (Gaeveskaya 1969, Hobbs 1993) and (2) can regulate the biomass and distribution of algae and macrophytes in freshwater systems (Lodge and Lorman 1987, Creed 1994, Lodge et al. 1994). The crayfish *Cambarus latimanus* is endemic to streams throughout the eastern Piedmont Plateau and Coastal Plain (Hobbs 1989). We found *C. latimanus* co-occurring with rhodophytes at several locations in Georgia and collected this species from the Georgia State Botanical Gardens in Spring 2009. Feeding assays were also conducted with the non-endemic Louisiana red crayfish, *Procambarus clarkii,* because it

is a generalist feeder, performs well in laboratory assays (Bolser et al. 1998, Cronin et al. 2002), and it is the most widely distributed crayfish, having spread worldwide from its native range (Gherardi 2006). *P. clarkii* were obtained from Carolina Biological Supply. We housed crayfish in individual containers (15x15 cm; water depth=14 cm) with perforated sides to allow passage of re-circulating, filtered water in flow-through tables, and fed them Bio-Blend herbivore pellets 3-4 times per week.

2.2 Feeding assays with living tissues

We conducted two crayfish feedings assays with live macrophytes. In May 2009, we performed a multiple-choice assay comparing the red algae *Batrachospermum helminthosum*, *Boldia erythrosiphon*, *Kumanoa sp.* and *Paralemanea annulata* with the aquatic moss *Fontinalis novae-angliae* and the green algae *Cladophora glomerata.* This allowed direct comparison of palatability rankings for each macrophyte across both crayfish species. Crayfish in this initial experiment readily consumed *Cladophora* while *Fontinalis* was low preference, as expected due to its chemical defenses (Parker et al. 2007). Thus, these two macrophytes served as known palatable and unpalatable comparatives in this and subsequent assays. In May 2011, paired-choice assays were conducted to gain greater statistical resolution between the consumption of each macrophyte and the palatable *Cladophora* "control". We used only *P. clarkii* in these paired-choice assays because the two crayfish species displayed equivalent feeding preferences in the initial multiple-choice assay (see Results). One day prior to feeding assays with live macrophytes, crayfish were transferred to individual containers with aerated, dechlorinated tap water in a controlled environmental chamber set at 14°C.

For the multiple-choice assay that used both crayfish species, we arranged 72 containers (each 8 cm in diameter and holding 120 ml dechlorinated tap water) in 24 replicated blocks of three randomly assigned treatments: one *P. clarkii* (30-50 mm in carapace length), one *C. latimanus* (equal in size to *P. clarkii*), and one no-crayfish control to account for potential autogenic changes in prey mass unrelated to consumption. All six macrophyte species were prepared in the following manner: three pieces of tissue were taken from the same individual macrophyte, spun in a salad spinner to remove excess water, and adjusted in size so that the mass of each piece was 50 mg ± 10%. We used 24 separate individuals (one per block) of each macrophyte species to maintain independence among replicates. One section of each species was placed into each container, resulting in all containers holding equivalent masses of all prey species. Crayfish were allowed to feed until ~ 50% of the total macrophyte tissue in each replicate had been consumed or for a maximum of 12 h, whichever occurred first. After feeding, all identifiable pieces of macrophyte were collected, spun in a salad spinner, and reweighed. Percent mass change due to consumption was calculated using the formula: $(T_i \text{ x } C_f/C_i) - T_f/(T_i \text{ x } C_f/C_i)$, where T_i and T_f were initial and final wet masses of tissue exposed to crayfish grazing and C_i and C_f were initial and final wet masses of control tissue (formula modified from Stochowicz and Hay 1996).

For paired-choice assays, *Cladophora glomerata*, *Fontinalis novae-angliae, Batrachospermum helminthosum,* and *Paralemanea annulata* were collected from the same populations used in the multiple-choice assay. *Boldia erythrosiphon* was not available at the previous location and was therefore collected from a new population (Table 1). We also added a fifth species of red algae, *Tuomeya americana*, which was

not abundant enough to collect in 2009. Feeding assays comparing each of the six macrophytes to *Cladophora* were conducted simultaneously. We interspersed treatments by grouping 96 individual containers (15x15 cm; water depth = 14 cm) into eight replicated blocks of 12 containers each; within each block of 12 containers, treatments were haphazardly assigned so that one *Procambarus clarkii* crayfish (60-80 mm in carapace length) container and one autogenic control container was created for each macrophyte comparison (n = 8). As before, pieces of macrophyte tissue were spun in a salad spinner to remove excess water and weighed. Each container received equivalent masses of *Cladophora* and the paired macrophyte (50 mg ± 10% for *Tuomeya* pairings due to its rarity; 100 mg ± 10% for all other pairings). This time, autogenic control pieces could not be directly matched to treatment pieces due to tissue pooling; within a replicate, it was often necessary to pool tissue from 2-3 individual macrophytes to attain 100 mg. Crayfish were allowed to feed until ~ 50% of the total macrophyte tissue in each replicate had been consumed or for a maximum of 12 h, whichever occurred first. Two crayfish did not eat and these replicates were omitted from analysis resulting in n = 7 for two comparisons. After feeding, all identifiable pieces of macrophyte were collected, spun, and reweighed. We calculated the percent mass change due to consumption using the formula: $(T_i - T_f)/T_i - C$ where T_i and T_f were initial and final wet masses of tissue exposed to crayfish grazing and the control factor C was the mean percent mass change of the eight control replicates.

2.3 Feeding assays with ground tissues

Herbivore preferences are influenced by the combined roles of macrophyte structure, nutritional value, and secondary chemistry (e.g. Hay et al. 1994, Cronin et al. 2002). To evaluate potential effects of structural traits, we removed structural differences by freeze-drying and grinding macrophytes to a fine powder using a high-energy ball mill (SPEX SamplePrep 8000D Mixer/Mill®). When reconstituted into agar-based foods, powdered tissue should retain species differences in secondary chemistry and nutritional value but lose species-specific structural traits (see Hay et al. 1994 for an overview on this method). Natural plant densities (dry mass/volume) were calculated by measuring volumetric displacement of live macrophyte tissue and mass of the associated freeze-dried material to calculate mg dry mass/ml (n = 10 per species). To make agar-based foods, 2 ml of deionized water was mixed with enough macrophyte powder to equal 10 ml of live macrophyte, this was then quickly mixed and stirred with 8 ml of deionized water and 0.25 grams of agar that had been heated until boiling. The resulting gel was quickly spread onto "dominoes" consisting of 102 by 55 mm pieces of flat PVC with 30 indentations (3 mm wide x 1mm deep) drilled into opposite halves of each block. The gelled food was pressed into indentations so that the control food containing powered *Cladophora* at its natural density was on one half of the domino and a treatment food containing one other powered macrophyte at its natural dry mass/volume was on the second half of the domino. This recipe yielded 25-30 replicate dominos. The number of food dots removed by crayfish during feeding was tallied separately for the control and treatment sides and divided by 30 dots to calculate the percent of each food type consumed. Plates were counted when approximately 50% of the entire 60 dots were

consumed; replicates were omitted from analysis if crayfish consumed less than 10% or greater than 90% of the 60 dots, as these replicates experienced too little or too much feeding to give a reliable indication of relative preference.

If powdered macrophytes reconstituted at natural densities were unpalatable to crayfish, it suggested that low-preference macrophytes were chemically defended or that they offered inadequate reward in terms of dry mass/volume. Because the density of *Cladophora* was up to 10 fold higher than some of the red algal species (Table 1), we conducted a second assay in which the dry mass/volume of *Cladophora* was matched to the natural density of the other macrophyte with which it was paired. This allowed the assessment of deterrent properties unconfounded by differences in dry mass/vol.

2.4 Feeding assays with crude extracts

We investigated the defensive role of chemical extracts for all five red algal species. Because some chemical defenses are only produced (or made more deterrent) when herbivores damage algal cells and allow enzymatic reactions to "activate" defenses (Paul and Van Alstyne 1992, Cetrulo and Hay 2000), we tested both activated and non-activated crude extract from frozen tissue of each species collected on the same day. Activated tissue was allowed to thaw in deionized water for several minutes before extraction in methanol (freezing lyses cells, as does herbivore feeding, and thawing allows enzymes to function prior to extraction). Non-activated tissue was thawed in 100% methanol, which inhibits enzymatic activity, preventing activation of deterrent chemistry. Once the tissue was nearly thawed, each treatment was ground separately for 120 sec using a mortar and pestle to assure efficient extraction. The tissue was

transferred back to its original flask and the reciprocal solvent was added to each treatment to create a 1:1 methanol/water solution that totaled three times the volume of the tissue extracted. After 2 h, solvents were vacuum filtered through cheesecloth and filter paper and all algal tissue was extracted 4 additional times in 100% methanol for 2 h each. Successive extractions were pooled and the entire crude extract was filtered a final time through diatomaceous earth (Fisher Scientific Celite 545®) to remove larger polysaccharides. Solvents were removed via rotary evaporation and crude extracts were stored at -30°C, and tested in bioassays within 72 h.

We re-suspended crude extracts in 70:30 methanol/water and coated *Cladophora* with natural concentrations (by algal dry mass) of extracts in solution, i.e. the extract obtained from 210 mg of dry *Batrachospermum* (the amount of dry mass in a 10 ml equivalent of *Batrachospermum*) was transferred to 210 mg of dry *Cladophora* (see Hay et al. 1998 for an overview of this method). Powdered treatment foods coated with extract were dried under vacuum to remove solvents before use in domino assays. Solvent control foods were made by coating the same amount dry *Cladophora* with the volume of 70:30 methanol/water solution used to transfer the crude extract onto treatment foods. This controlled for the possibility of trace amounts of solvents, or impurities from the solvents, remaining on the artificial foods. Extract-coated treatment foods and matching solvent control foods were gelled as described for ground tissue assays and spread onto domino plates.

One additional assay was conducted for *Batrachospermum* extracts made from frozen tissue collected in 2010. This tissue was thawed and extracted 4 times in 1:1 methanol/water for 2 h each with a final overnight extraction in 100% methanol. The

amount of crude extract in a 5 ml equivalent of *Batrachospermum* was dried onto powdered *Cladophora* at natural concentration. A matching solvent control was prepared and treatment and control powders were added to a solution of 30% sodium alginate by dry mass in 5 ml of deionized water. Food pastes were drawn into 100-μL glass capillary tubes and ejected into a hardening solution of 0.25 M calcium chloride. The resultant algal noodles were rinsed in deionized water and cut to yield feeding pellets 6-8 mm long and 1-2 mm in diameter. Twenty individual *P. clarkii* were first offered a control food pellet; if they accepted, they were offered a treatment pellet. If the treatment food was rejected, crayfish were offered a second control pellet to ensure the rejection response was not due to satiation. Crayfish that rejected either control pellet were eliminated from analysis. This assay allowed a more rapid assessment of chemical deterrence compared with the domino assay, in which crayfish feeding times ranged from 10-80 min, which could have allowed for leaching or degradation of water-soluble or unstable feeding deterrents.

2.5 Macrophyte nutritional analysis

We measured several macrophyte traits as potential indicators of nutritional value including: dry mass/volume, AFDM/volume, soluble protein/volume, and soluble protein/dry mass for 5-10 replicates of each macrophyte species for each collection period and location. The amount of macrophyte tissue that displaced 2-5 ml of deionized water was weighed to calculate wet mass/volume, freeze-dried to a constant mass to calculate dry mass/volume, and finally combusted at 500°C for 4 h to calculate ash free dry mass (AFDM)/volume. Soluble protein content was measured using the method of

Bradford (1976). Five to ten replicates of each species were freeze-dried and ground to a powder using a high-energy ball mill (SPEX SamplePrep 8000D Mixer/Mill®). Then a 5 mg powdered aliquot was digested in 1.0 ml 1M sodium hydroxide for 24 h at 4°C, centrifuged, and 10 μl aliquots of the supernatant were added to 200 μl of Bradford reagent in a 96-microwell plate. After 15 min, absorbance was measured at 595 nm using a BioTek multi-mode plate reader against bovine serum albumin (BSA) standards.

2.6 Statistical analysis

We analyzed the multiple-choice assay involving live macrophyte tissues using non-parametric rankings as recommended for assays with non-independence among treatments (macrophyte species) within each replicate (Roa 1992). Percent consumption was ranked within replicates (adjusting for ties) and a two-way ANOVA, with the fixed factors macrophyte *species* and *crayfish species*, was used to analyze ranked data (Conover and Inman1981). Differences among macrophyte species were evaluated via Tukey multiple comparison tests. The mass consumed (mg individual $^{-1}$) of each macrophyte was compared between the two crayfish to evaluate feeding patterns between crayfish species for each macrophyte species; matched samples (tissue taken from the same macrophyte individual) were analyzed with two-tailed, paired sample *t*-tests. All feeding assays pairing either live or powdered *Cladophora* controls to treatment foods were also analyzed with 2-tailed, paired sample *t*-tests. The non-parametric Mann-Whitney U test was used to compare the effect sizes of activated versus non-activated extract-coated treatment foods for each species (Zar 2010). The number of individual crayfish consuming extract-coated pellets compared to solvent-control pellets was

analyzed with Fisher's exact test. We analyzed differences among macrophyte traits with Kruskal-Wallis tests in lieu of one-way ANOVAs, because data transformations could not control for non-normality. Dunn's pairwise comparisons were run to elucidate differences between macrophyte traits.

CHAPTER III

RESULTS

3.1 Feeding on living tissues

When offered four red algae, one green alga, and an aquatic moss, the crayfish *Cambarus latimanus* and *Procambarus clarkii* displayed similar feeding preferences (Figure 1; $P = 0.958$, two-way ANOVA on ranked data). There was also no interaction between the fixed factors *crayfish species* and *macrophyte species* ($P = 0.784$). Comparisons of *C. latimanus* and *P. clarkii* consumption indicated no significant differences between crayfish species in the amount of each macrophyte eaten ($P > 0.05$

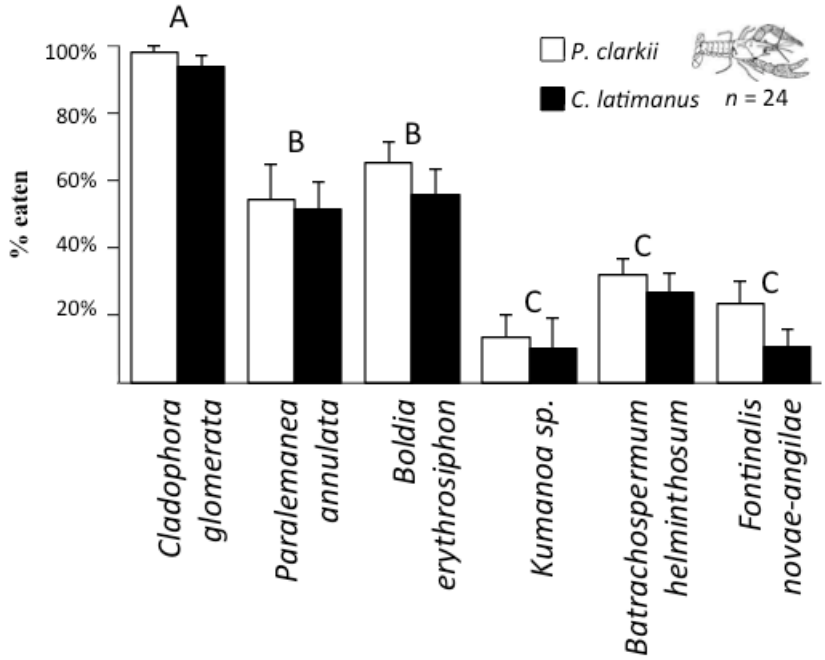

Figure 1. Percent consumption (mean + 1 SE) of living macrophytes offered in multiple-choice feeding assays to the crayfishes *Procambarus clarkii* and *Cambarus latimanus* ($n = 24$ for both crayfishes). Letters above bars denote significant groupings by Tukey's multiple comparisons ($P < 0.05$) on mean consumption of each macrophyte ranked within each replicate following a two-way ANOVA on ranks ($P < 0.001$). Ranked feeding preferences of the two crayfishes were similar ($P = 0.958$, two-way ANOVA).

and $n = 24$ for all contrasts, two-tailed paired *t*-tests). Macrophytes fell into three general groups based on crayfish feeding; the filamentous green alga *Cladophora glomerata* was highly preferred, the red algae *Boldia erythrosiphon* and *Paralemanea annulata* were of intermediate preference, while the red algae *Batrachospermum helminthosum* and *Kumanoa sp.* were least preferred, along with the moss *Fontinalis novae-angliae* (Figure 1). In paired-choice assays (Figure 2), *P. clarkii* crayfish grazed 62-76% more *Cladophora* compared to *Batrachospermum*, *Kumanoa*, *Paralemanea*, and *Tuomeya americana* ($P < 0.001$ for all contrasts, two-tailed paired *t*-tests). Crayfish consumed similar amounts of *Cladophora* and *Boldia* (Figure 2A).

3.2 Feeding on artificial foods made with ground tissues

When macrophyte tissues were ground to a fine powder (destroying structural traits) and reconstituted into agar-based foods at natural density, *Procambarus clarkii* fed 56-98% less on the aquatic moss *Fontinalis novae-angliae* the red algae *Batrachospermum helminthosum*, *Kumanoa sp.*, and *Tuomeya americana* compared to *Cladophora glomerata* (Figure 2B). However, *P. clarkii* preference in live-tissue assays was reversed when the cartilaginous structure of *Paralemanea annulata* was destroyed; consumption of ground *Paralemanea* was 112% greater than ground *Cladophora* at natural densities. In the above assays, differential feeding could have been based on different dry mass/volume of reconstituted foods or on chemical traits of the foods. To separate these effects, we ran a second set of ground tissue assays in which the dry mass/volume of *Cladophora* was matched to the dry mass/volume of contrasting macrophytes to eliminate difference in reward/bite in terms of dry mass (Figure 2C). At

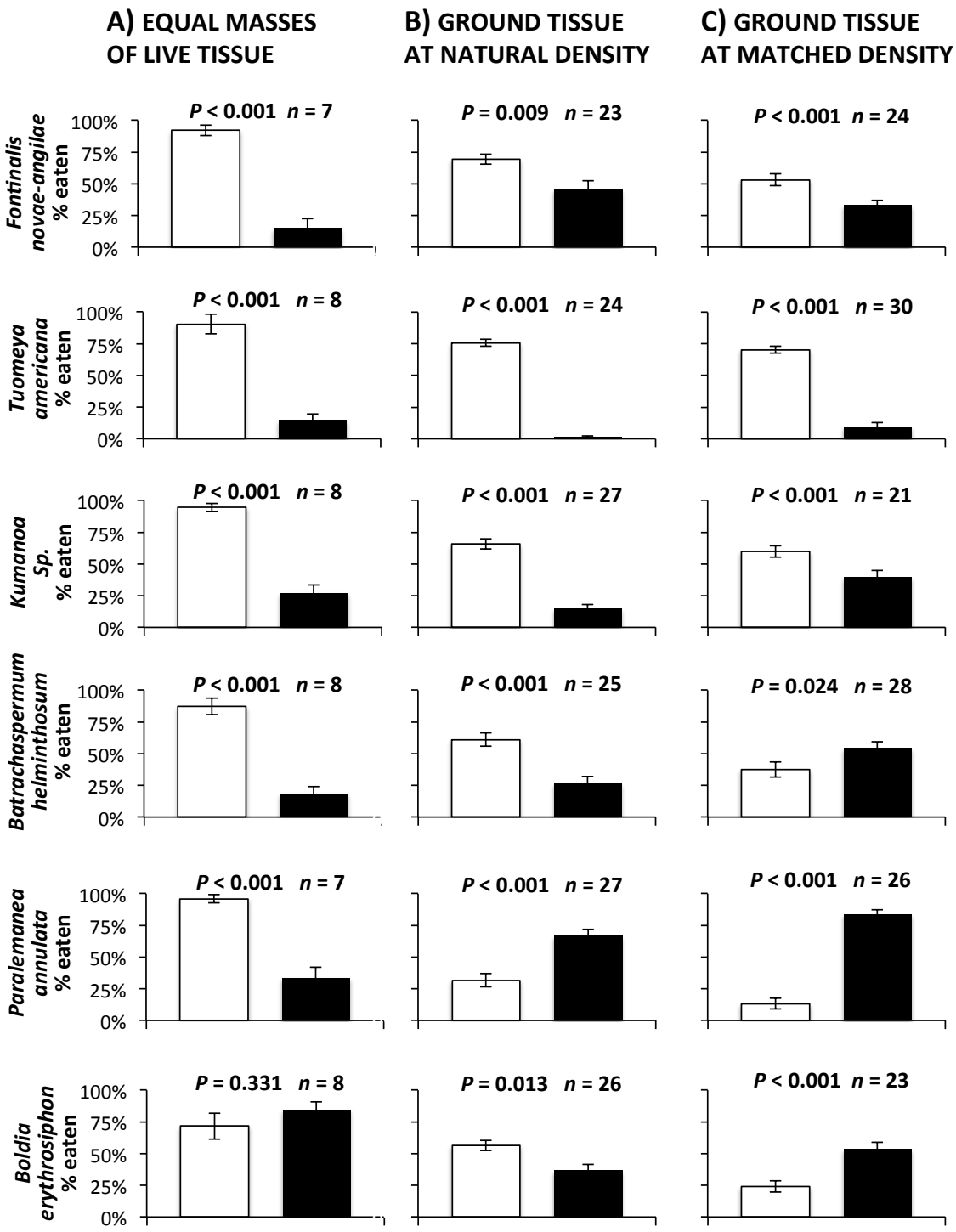

Figure 2. Consumption by the crayfish *Procambarus clarkii* in paired-choice assays when given (A) equal masses of living tissue *Cladophora glomerata* (white bars) and the aquatic moss *Fontinalis novae-angilae or* five red algal species (black bars), (B) ground tissues of *Cladophora* (white bars) and paired macrophytes (black bars) each reconstituted at their natural densities, and (C) ground *Cladophora* (white bars) matched to the natural density of each paired macrophyte (black bars). Error bars represent ± 1 SE. Sample size and *P*-values from a 2-tailed, paired-sample *t*-test are given above each comparison.

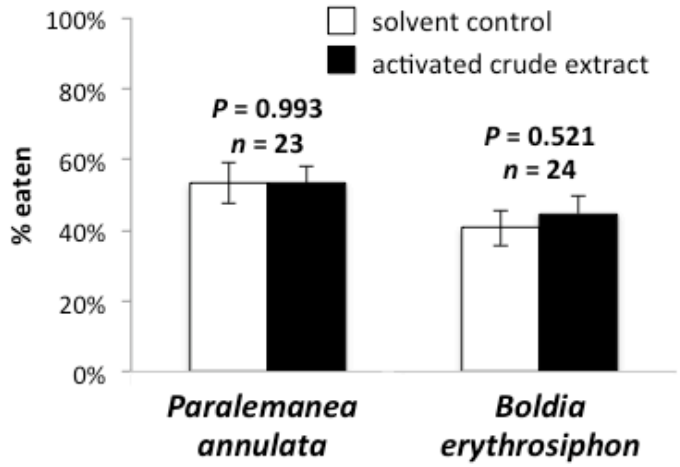

Figure 3. Assays with extracts from freshwater red alga showing no chemical defense. *Procambarus clarkii* crayfish were given a choice between solvent control foods (white bars) and food treated with activated extracts from crushed algal tissue (black bars). Error bars represent ± 1 SE. Sample size and *P*-values from a 2-tailed, paired-sample *t*-test are given above each comparison.

these matched food densities, crayfish feeding was still suppressed by 38% for the chemically defended moss *Fontinalis novae-angliae*, by 89% for *Tuomeya*, and by 33% for *Kumanoa.* These data suggest low feeding levels on *Kumanoa* and *Tuomeya* in live tissue assays were due to factors other than morphology and tissue density. Increased feeding on ground tissues of *Boldia erythrosiphon, Batrachospermum*, and *Paralemanea* when matched by dry mass/volume suggests that the patterns of feeding on live algae and ground algae at natural densities were due to 1) value differences in natural dry mass/volume, 2) structural traits destroyed in grinding, or 3) loss of chemistry in the freeze-drying and grinding process.

3.3 Feeding on artificial foods with crude algal extracts

Extracts of *Paralemanea annulata* and *Boldia erythrosiphon* had no effect on crayfish feeding (Figure 3), but crude extracts of *Batrachospermum helminthosum*,

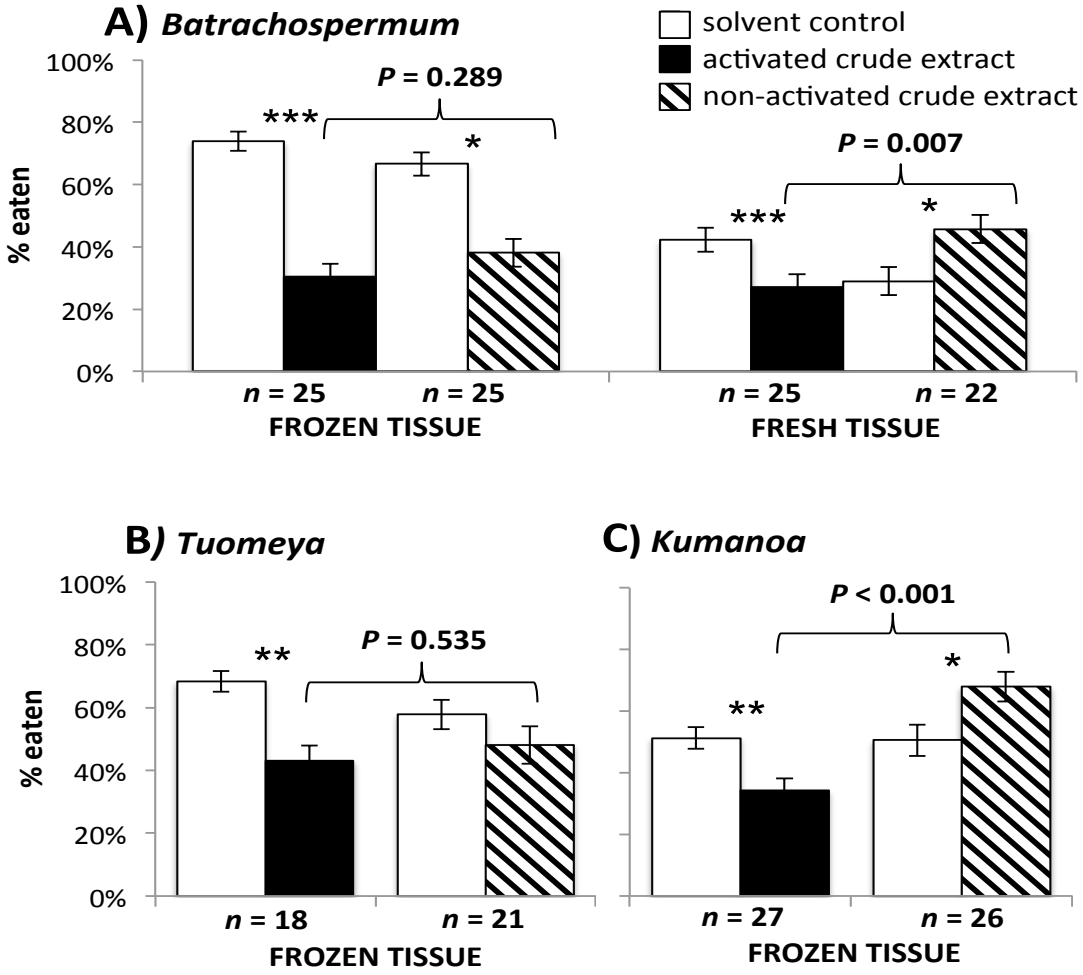

Figure 4. Assays with crude extracts from freshwater red algae. *Procambarus clarkii* crayfish were given a choice between solvent control food (white bars) and either food treated with activated extracts from tissue damaged in water (black bars) or food treated with non-activated extracts from frozen tissue damaged in methanol (hatched bars). Percent consumption of food when treated with (A) extracts from frozen and fresh tissue of *Batrachospermum helminthosum,* (B) extracts from frozen tissue of *Tuomeya americana*, and (C) extracts from frozen tissue of *Kumanoa sp.* Sample size is given at the base of each extract-solvent control pairing. Error bars represent ± 1 SE. Asterisk represent *P*-values from a 2-tailed, paired-sample *t*-test comparing consumption of extracts and solvent controls: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Bracketed *P*-values are from Mann-Whitney rank sum tests comparing consumption of activated and non-activated extracts.

Kumanoa sp., and *Tuomeya americana* all significantly suppressed crayfish feeding relative to solvent controls (Figure 4). All three chemically defended species (*Batrachospermum*, *Kumanoa*, and *Tuomeya*) show evidence of activated chemical defenses. Activation is supported by 1) tests in which activated and non-activated extracts were each compared to solvent controls and 2) post-hoc statistical tests directly comparing the consumption of activated and non-activated food in the two separate feeding assays. Comparisons of activated vs. non-activated extracts from frozen *Batrachospermum* were not significantly different from each other, but it is possible that the "non-activated" extract was activated in the process of freezing and thawing due to the unusually high water content of this species. Therefore, *Batrachospermum* was re-collected and activated and non-activated extracts were made from live tissue. Crayfish ate significantly less food containing activated extracts made from fresh *Batrachospermum*; this was also the case for frozen *Kumanoa* compared to non-activated extracts ($P = 0.007$ and $P < 0.001$, Mann-Whitney Rank Sum Tests). Relative to solvent controls, activated extracts of *Batrachospermum* and *Kumanoa* suppressed feeding while non-activated extracts stimulated feeding. Activated *Tuomeya* extracts significantly suppressed crayfish feeding relative to solvent controls ($P = 0.002$, paired *t*-test), while non-activated extracts had no affect on crayfish feeding ($P = 0.256$, paired *t*-test). There was no statistical difference between consumption of treatment foods coated with either activated or non-activated extracts in post-hoc comparisons ($P = 0.535$, Mann-Whitney Rank Sum Test).

We ran one additional accept/reject behavioral assay with crude extracts from *Batrachospermum* (Figure 5). Only 1 of 18 crayfish accepted the extract-coated

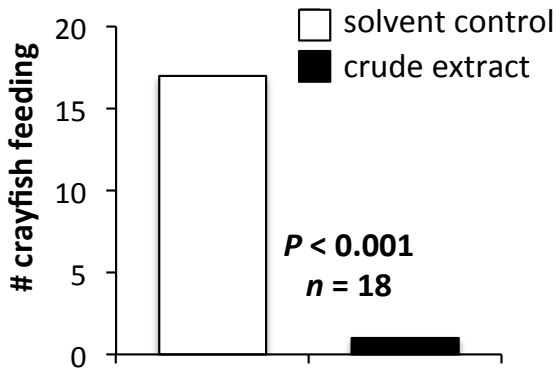

Figure 5. *Procambarus clarkii* crayfish acceptance of control food pellets versus those treated with the crude extract from the red alga *Batrachospermum helminthosum*. Sample size and *P*-values from a Fisher's exact test are denoted.

treatment pellet, while no crayfish rejected the first solvent control pellet ($P < 0.001$, Fisher's exact test). Two additional individuals were eliminated from analysis because they refused the second control pellet after rejecting the treatment and we could not confirm that rejections were not due to satiation. Crayfish took an average of three bites, which were spit out, before discarding the treatment pellets while control pellets were consumed entirely.

3.4 Macrophyte nutritional analysis

For the six macrophytes we measured soluble protein content (Table 1) and found it was positively correlated with total dry mass when these traits were expressed per volume of fresh macrophyte tissue ($r^2 = 0.951$, $P < 0.001$, Figure 6A). No relationship was found between rank-order palatability (derived from the 2009 live-tissue multiple-choice assay) and dry mass per volume, protein per volume, nor percent protein by dry mass ($P > 0.67$

in all cases, all three trends were similar so only palatability verses protein per volume is depicted).

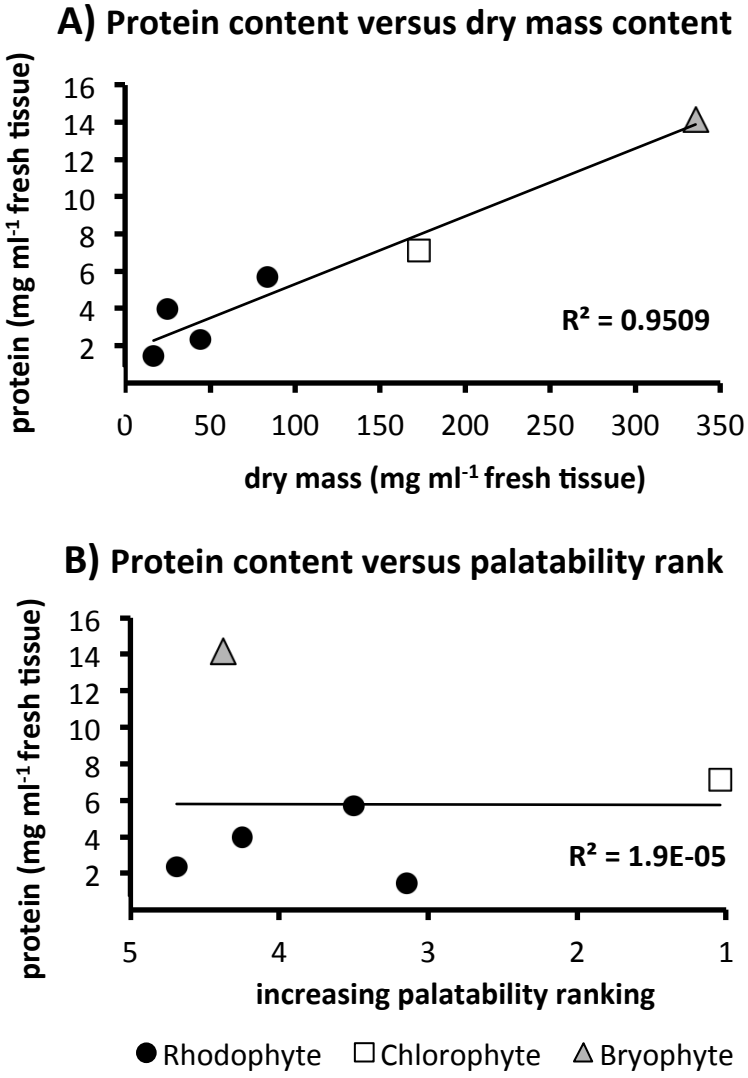

Figure 6. The relationship between nutritive value and palatability ranks for six stream macrophytes. (A) Soluble protein concentration verses macrophyte density (B) Soluble protein concentration versus palatability rankings (accounting for ties) of macrophytes from assays with living tissue.

Table 1. Macrophyte collection sites and nutritional traits. Mean ± 1 SE and sample sizes (in parentheses) are given. Different bolded letters (following sample sizes) denote statistical differences between species collected in the same year (Dunn's multiple comparisons tests following Kruskal-Wallas ANOVAs on ranks.).

Macrophyte	Morphological Form	Gametangia duration	Collection Date	Dry mass/vol (mg/ml)	AFDM/vol (mg/ml)	AFDM (% dry mass)	Soluble protein (mg/ml)	Soluble protein (% dry mass)	Collection Sites
Rhodophyta									
Boldia erythrosiphon	fleshy, cylindrical tubes	Apr-Jun	5-May-09	16.5 ± 1.1 (n=5) **A**	----------	----------	1.3 ± 0.2 (n=5) **A**	7.5 ± 0.5 (n=5) **AB**	Little Schultz Creek, Bibb County, Alabama (33.02'11.83N, 87.07'15.88W) in 2009; North River, Tuscaloosa County, Alabama (33.33'42.63N, 87.37'48.86W) in 2011
			12-May-11	25.8 ± 0.6 (n=10) **A**	22.4 ± 0.6 (n=9) **A**	87.2 ± 0.7 (n=9) **AB**	0.7 ± 0.3 (n=10) **AB**	2.7 ± 0.1 (n=10) **AB**	
Batrachospermum helminthosum	mucilaginous filaments with a beaded appearance	Apr-Jun	6-May-09	22.3 ± 2.5 (n=10) **AB**	----------	----------	4.1 ± 0.7 (n=10) **BC**	16.5 ± 0.8 (n=10) **A**	Chattahoochee River, Gwinnett County, Georgia (Jones Bridge Park Unit, 34' 00.053N, 84' 14.220W)
			13-May-11	20.6 ± 1.3 (n=10) **A**	18.6 ± 1.0 (n=10) **A**	90.6 ± 0.9 (n=10) **A**	1.8 ± 0.2 (n=10) **A**	8.57 ± 0.9 (n=10) **AB**	
Kumanoa sp.	mucilaginous filaments with a beaded appearance	Apr-Jun	5-May-09	43.9 ± 1.6 (n=10) **ABC**	----------	----------	2.6 ± 1.9 (n=9) **AB**	5.8 ± 1.3 (n=9) **BC**	Cripple Creek, Tuscaloosa County, Alabama (33.29'33.59N 87.33'45.27W)
			12-May-11	49.8 ± 1.2 (n=10) **AB**	34.9 ± 0.6 (n=10) **AB**	70.3 ± 0.9 (n=10) **BC**	0.6 ± 0.1 (n=10) **A**	1.3 ± 0.1 (n=10) **BCD**	
Tuomeya americana	densly branched cartilaginous filaments	May-Jun	6-May-09	----------	----------	----------	----------	----------	Chattahoochee River, Gwinnett County, Georgia (Jones Bridge Park Unit, 34' 00.053N, 84' 14.220W)
			13-May-11	168.7 ± 9.7 (n=10) **ABC**	37.2. ± 0.2 (n=10) **AB**	77.9 ± 1.7 (n=10) **AB**	4.3 ± 0.4 (n=10) **BC**	2.6 ± 0.2 (n=10) **ABC**	
Paralemanea annulata	cartilaginous unbranched thali arranged in tufts	Mar-May	6-May-09	83.7 ± 8.1 (n=5) **ABC**	----------	----------	2.9 ± 0.4 (n=5) **AB**	3.5 ± 0.1 (n=5) **C**	Big Cloud Creek, Watson Mill Bridge State Park, Madison County, Georgia (34.01'43.55N, 83.04'41.39W)
			13-May-11	211.9 ± 8.0 (n=10) **BC**	194.3 ± 7.7 (n=10) **C**	91.6 ± 0.3 (n=10) **A**	2.7 ± 0.3 (n=10) **BC**	1.3 ± 0.5 (n=10) **CD**	
Chlorophyta									
Cladophora glomerata	filamentous mats	---------	6-May-09	173.1 ± 13.9 (n=10) **BC**	----------	----------	9.4 ± 2.3 (n=10) **C**	5.4 ± 0.4 (n=10) **ABC**	Georgia State Botanical Gardens, Clark County, Georgia (33.54'10.40N, 83.22'53.91W); Chattahoochee River (Jones Bridge Park Unit)
			13-May-11	383.6 ± 25.9 (n=10) **C**	81.9 ± 4.3 (n=10) **BC**	31.0 ± 2.0 (n=10) **C**	4.4 ± 0.7 (n=10) **BC**	1.1 ± 0.2 (n=10) **D**	
Bryophyta									
Fontinalis novae-angliae	long, rigid phyllids (leaves)	---------	6-May-09	227.1 ± 11.6 (n=9) **C**	----------	----------	8.1 ± 1.7 (n=10) **C**	3.6 ± 0.2 (n=10) **C**	Chattahoochee River (Jones Bridge Park Unit)
			13-May-11	186.0 ± 4.5 (n=10) **BC**	88.2 ± 2.9 (n=10) **BC**	47.5 ± 1.4 (n=10) **CB**	2.14 ± 0.2 (n=10) **ABC**	1.15 ± 0.1 (n=10) **CD**	
H-value, DF, *P*-value			May 2009	46.7, 5, <0.001	----------	----------	45.0, 5, <0.001	40.0, 5, <0.001	
			May 2011	64.2, 6, <0.001	63.7, 6, <0.001	63.3, 6, <0.001	53.7, 6, <0.001	51.1, 6, <0.001	

CHAPTER IV

DISCUSSION

4.1 Antigrazer traits of freshwater red algae

Contrary to once widely held views (Hutchinson 1975), herbivory often strongly regulates macrophyte abundance and community composition in freshwater systems (Lamerti and Resh 1983, Feminella and Hawkins 1995, Steinman 1996, Lodge et al. 1998). Algal morphology (i.e. prostrate or upright growth form) influences macrophyte susceptibility to herbivory (Hill et al. 1992, Holomuski and Biggs 2006), but the role of chemical defenses in protecting stream algae has rarely been studied (Camacho 2008). Our data indicate that secondary chemistry can strongly reduce risk of consumption by herbivores; it may also interact in important ways with morphology and nutritional value to affect palatability (Figure 2, Table 1), as occurs for seaweeds in marine systems (Hay et al. 1994, Hay 1996). We detected chemical defenses in 3 of the 5 red algae investigated. Crude chemical extracts of *Batrachospermum helminthosum, Kumanoa sp.,* and *Tuomeya americana* reduced crayfish grazing by ~33-60% relative to solvent controls (Figure 4); extracts of *Paralemanea annulata* and *Boldia erythrosiphon* had no effect on herbivory (Figure 3). The mucilage of some red algae has been suggested to mechanically deter invertebrate grazers in streams (Hambrook and Sheath 1987, Steinman et al. 1992); but removing the mucus layer of *Batrachospermum* and *Kumanoa* (by freeze-drying and grinding their tissues) did not increase their palatability, suggesting a primacy of chemical deterrents in these species. The cartilaginous structure of *Paralemanea* appeared to reduce crayfish feeding on this alga; when intact, it was

consumed 65% less than *Cladophora*, but when its structure was destroyed and its powdered tissue was reconstituted at natural density, *Paralemanea* was consumed 112% more than *Cladophora* (Figure 2). *Boldia* showed no evidence of structural or chemical defenses, but its low dry mass/volume (Table 1) may make it a less valuable food; in paired-choice assays crayfish preference switched from *Cladophora* to *Boldia* when the density of *Cladophora* was reduced to match the natural density of *Boldia* (Figure 2B,C). In addition to its chemical defenses, *Batrachospermum* also showed evidence of low palatability due to nutritional inadequacy. Thus structural, chemical, and nutritive traits of freshwater red algae appear to suppress feeding by generalist herbivores, analogous to well-described antiherbivore adaptations possessed by marine macroalgae (Lubchenco and Gaines 1981, Duffy and Hay 1990, Hay et al. 1994, Cruz-Rivera and Hay 2003, Hay 1996, 2009).

We did not find a positive correlation between grazer preference and protein content of red algae (Figure 6), in contrast to Hambrook and Sheath (1987), the single other study to examine grazing on freshwater red algae. We found that the presence of deterrent chemistry better explained the relative palatability of red algal taxa. Differences in secondary chemistry between the algal species and populations collected by Hambrook and Sheath, *Audouinella violacea*, *Batrachospermum virgatum*, and *Tuomeya americana*, compared to those we used may account for the incongruous results. Alternatively, food selection by grazing caddisfly and mayfly larvae used by Hambrook and Sheath (1987) may be constrained by factors different from those affecting crayfishes. Aquatic insect larvae and amphipods that undergo rapid growth, metamorphosis, and reproduction may select foods primarily based on dietary essentials,

such as lipids and fatty acids, and nutritional value (Cummins and Klug 1979, Lamberti and Moore 1984, Cargill et al. 1985, Torres-Ruiz et al. 2009) while tolerating consumption of deterrent compounds, especially if this tolerance enhances escape from their own consumers via association with an unpalatable host (e.g. Parker et al. 2007), as also occurs for small invertebrate grazers in marine systems (Hay 1996, 2009).

4.2 Activated defenses in freshwater red algae

Chemical defenses may be constitutive (always present), induced (production upregulated in response to herbivory and other environmental cues), or activated (produced enzymatically from available precursors within seconds of macrophyte injury) (Harvell 1990, Paul and Van Alstyne 1992, Karban and Baldwin 1997). Feeding on foods treated with extracts of *Batrachospermum helminthosum*, *Kumanoa sp.*, and *Tuomeya americana* is indicative of activated chemical defenses. The "activated" extracts, produced by crushing tissue prior to extraction with organic solvents were significantly deterrent for these three red algae. However, when we inhibited enzymatic activity by soaking algal tissue in organic solvents prior to crushing, the "non-activated" crude extracts stimulated or had no effect on herbivory.

At least three types of activated defenses have been described, though for most cases the mechanism of activation remains unknown. First, a deterrent compound may be made from non-deterrent precursors; examples include hydrogen cyanide (HCN), which is produced by enzymatic hydrolysis of non-toxic glycosides upon injury of many vascular plants and dimethylsulfoniopropionate (DMSP), found in many marine chlorophytes and microalgae, which is hydrolyzed into the deterrent products dimethyl

sulfide (DMS) and acrylic acid (Conn 1980, Van Alstyne et al. 2001, Van Alstyne and Houser 2003). Secondly, a mildly deterrent compound may be biotransformed into a more deterrent compound like the conversion of halimedatetraacetate to the more potent compound halimedatrial by marine green algae in the genus *Halimeda* (Paul & Van Alsyten 1992). Finally, activation may increase the concentration of an already deterrent compound normally expressed at lower levels as seen in the freshwater orchid *Habenaria repens* (Bolser et al. 1998). The later two modes of activation could explain why grazing patterns on activated and non-activated extracts of *Tuomeya* showed similar trends though activated extracts were significantly deterrent, while non-activated extracts were not.

Chemical defenses may be costly to the producer, reducing fitness and growth when they are not needed for defense (Baldwin 1998, Pavia et al. 1999). It is possible that activated defenses reduce opportunity costs when herbivory is unpredictable in space and time (Harvell 1990, Karbon et al. 1999). If precursors to deterrent compounds are used in several biosynthesis pathways, they can be diverted to the production of other metabolites when defense is not needed. In addition, autotoxicity may be minimized if toxic compounds (or their precursors) are confined in gland cells and released for only a short time after herbivore exposure before decomposing rapidly. Activated defenses may also carry lower ecological costs. Activated compounds that are released unreliably in space and time (i.e. a moving target) may enable macrophytes to escape detection by specialist herbivores that use deterrent compounds as cues to find their hosts (Alder and Karban 1994). Small herbivores often live and feed on chemically defended macrophytes, representing enemy-free space, to avoid incidental consumption by larger

consumers (Jeffries and Lawton 1984, Hay 1996, Lankau 2007). Mayflies, midge larvae, amphipods, and isopods were more abundant on the chemically defended moss *Fontinalis novae-angliae* compared to the palatable (to geese and crayfish) riverweed *Podostemum ceratophyllum* growing in the Chattahoochee River (Parker et al. 2007). We found these invertebrates to be absent from *Batrachospermum helminthosum* and *Tuomeya americana* collected from the same location in the Chattahoochee River. Thus, activated chemistry may reduce allocational, opportunity, and ecological costs of defense in freshwater red macroalgae that have a relatively short season in which gametophytes must grow and reproduce rapidly. Given that the three species in which we detected chemical defenses, all had activated defenses, a more comprehensive investigation is warranted to determine whether freshwater macroalgae in general, or freshwater red algae in particular, have a higher frequency of activated defenses compared to their marine counterparts (Cetrulo and Hay 2000) and other freshwater macrophytes (Prusak et al. 2005). To date, only three previous cases of activated defenses have been detected in freshwater primary producers, and these were all in vascular plants (Newman et al. 1992, Bolser et al. 1998, Prusak et al. 2005). Additionally, only ~ 20% of chemically defended seaweeds utilize activated defenses (Paul and Van Alstyne 1992, Cetrulo and Hay 2000).

4.3 Intraspecific variation in susceptibility to grazers

Intraspecific variation in nutritional, chemical, and structural resistance mechanisms may be of importance for freshwater red algae. Freshwater red algae, like their marine counterparts, have complex life cycles and alternation of generations. Heteromorphic life history strategies in marine algae have been proposed as "bet

hedging" in that different sources of mortality are spread over two stages that can differ in their susceptibility to different biotic and abiotic disturbances (Littler and Littler 1980, Lubchenco and Cubit 1980). Differential rates of herbivory have been documented within marine algae that have heteromorphic life cycles in which the morphologically distinct gametophyte (sexual) and sporophyte (asexual) occupy different ecological niches (Lubchenco and Cubit 1980, Slocum 1980, Vergés et al. 2008).

Ninety-nine percent of the sexually reproducing freshwater red algae display a heteromorphic life pattern (Sheath and Hambrook 1990). A perennial, prostrate "chantransia" stage produces macroscopic gametophytes in late winter to early spring. The gametophyte stages of freshwater red algae may be more prone to macrograzers, while the inconspicuous chantransia may escape consumption. For example, grazing crayfish and minnows can eliminate upright chlorophytes from algal communities (Power et al. 1985, Creed 1994, Gelwick and Matthews 1997). However, the prostrate form of chantransia makes them less susceptible to larger consumers like crayfish and minnows, but probably still prone to grazing by small invertebrates with rasping and scraping feeding apparatuses (Steinman 1996, Kohler and Wiley 1997, Rosemond et al. 2000, Holomuzki et al. 2006). Chantransia stages of the genus *Batrachospermum sp.* were noted as heavily grazed by *Goniobasis* snails (Minckley and Tindall 1963) and were detected with regular frequency in the guts of *Baetis* mayfly nymphs (Jones 1950). Thus, macro and micro stages of freshwater red algae may be experiencing differing selection regimes influenced by grazer feeding modalities.

Optimal defense theory predicts that tissues, which contribute more to plant fitness or are more susceptible to consumers, should be more heavily defended (McKey et

al. 1979, Rhoades et al. 1979). Organs for sexual reproduction, including the carposporophyte, grow attached to the gametophyte of many freshwater red algae. As seen in other algae and plants, special reproductive structures can be more chemically defended (Strauss et al. 2002). For example, the female gametophytes of the marine red alga *Asparagopsis armata* contain the highest concentration of defensive compounds in attached carposporophytes and are grazed less compared to male gametophytes and the vegetative stage (Vergés et al. 2008). Here we show that the gametophyte stage of *Batrachospermum helminthosum* is chemically defended against crayfish, however it remains unknown whether the chantransia stage is also defended. Steinman et al. (1991) found that the addition of grazing snails to laboratory streams increased the relative percent cover of *Batrachospermum moniliforme* chantransia to greater than 50% and decreased the relative percent cover of diatoms to less than 10%. It is unknown why snails avoided consuming *B. moniliforme*, however chantransia may be chemically defended, nutritionally inadequate, or less susceptible due to morphological or structural traits. Considering the higher proportion of heteromorphy in stream red algae compared to their marine counterparts (Sheath and Hambrook 1990), freshwater red algae may be ideal taxa for optimal defenses studies examining intraspecific variation.

4.4 Implications for macrophyte community structure

Numerous studies have demonstrated that herbivores can exert strong control over the distribution and abundance of stream macrophytes and periphyton (Feminella and Hawkins 1995, Steinman 1996, Holomuzki et al. 2010). Grazers often structure benthic assemblages in terms of dominant taxa (Lamberti and Resh 1983, Power et al. 1985,

Creed 1994) and morphology (Hill et al. 1992, Steinman et al. 1992, Holomuzki et al. 2006). For example, stalked, erect and filamentous growth forms are vulnerable to most grazers and their consumption can shift benthic assemblages toward prostrate, understory growth forms (Hill et al. 1992, Creed 1994, Holomuzki et al. 2006). In marine systems, palatable species are often restricted to growing in areas where grazers are not active, while defended species are able to persist in areas where grazing is strong (Lubchenco and Gaines 1981, Hay 1984, Menge et al. 1985, Duffy and Hay 1990, Hay 1996). Palatable stream-dwelling macrophytes may spatially "escape" herbivory by growing in shallow depths and/or high currents where grazers are more prone to predation (Creed 1994), less efficient at grazing (Poff and Ward 1992, 1995, Opsahl et al. 2003), or incapable of colonizing or feeding due to rapid flow (Poff et al. 1991, Hart 1992, Swan and Palmer 2000).

Chemical defenses may allow some freshwater red algae to exist where palatable algae cannot. Stewart (1987) noted that when algae were transferred to deeper pools containing the herbivorous minnow *Campostoma anomalum*, four species of green algae were completely consumed within one hour while *Batrachospermum sp.* remained uneaten after four days. Likewise, we collected *Batrachospermum* from deeper pools of a stream within the Georgia State Botanical Garden, while *Cladophora glomerata* was restricted to rocky shoals where swiftly moving water was < 5 cm deep. Herbivore exclosure studies have shown that crayfish exclude *C. glomerata* from deeper stream pools while the relative cover this green algae can exceed 75% in shallow regions (Creed 1994).

Alternatively, *Paralemanea* and *Lemanea* may not need chemical defenses as these genera specialize in high-flow regions with a mean velocity of 49 cm s^{-1} (Sheath and Hambook 1988) and have been found in velocities greater than 1 m s^{-1} (Sirjola 1969, Everitt and Burkholder 1991, Vis et al. 1991). Hart (1992) reported that crayfish did not graze stream substrata in areas where current velocities exceeded 50 cm s^{-1} and Minckley (1962) noted that *Lemanea* was restricted to the highest-velocity regions of a Kentucky stream that were inaccessible to grazing invertebrates. Thus, antiherbivore mechanisms, including defensive chemistry, may play an important role in the distribution and abundance of macrophytes within slower-flowing stream reaches.

4.5 Conclusions

The relative strength of biotic and abiotic selection pressures continues to be debated in streams and rivers systems (Holomuski et al 2010), and it has been predicted that selection pressure for the production of antigrazer compounds in freshwater algae is low (Steinman 1996). Given that three of the five red algal taxa examined in this study yielded deterrent crude extracts, the potential for defensive chemistry in freshwater rhodophytes appears high. Considering all three of the chemical defenses we detected appear to be activated by enzymatic processes upon tissue lyses, it is possible that previous studies missed such defensive chemistry by not evaluating the potential for activated metabolites. Further investigation of chemical defenses in freshwater red algae will contribute to among-system comparisons (Hay 1991, Hay and Steinberg 1991, Lodge et al. 1998), providing new insights into the generality of plant-herbivore interactions and their evolution.

REFERENCES

Adler, F. R. and R. Karban. 1994. Defended fortresses or moving targets? Another model of inducible defenses inspired by military metaphors. American Naturalist 144:813-832.

Baldwin, I. T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proceedings of the National Academy of Sciences 95:8113-8118.

Blunt, J. W., B. R. Copp, W.-P. Hu, M. H. G. Munro, P. T. Northcote, and M. R. Prinsep. 2009. Marine natural products. Natural Products Reports 26:170-244.

Bolser, R. and M. E. Hay. 1998. A field test of inducible resistance to specialist and generalist herbivores using the water lily *Nuphar luteum*. Oecologia 116:143-153.

Bolser, R. C., M. E. Hay, N. Lindquist, W. Fenical, and D. Wilson. 1998. Chemical defenses of freshwater macrophytes against crayfish herbivory. Journal of Chemical Ecology 24:1639-1658.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72:248-254.

Camacho, F. A. 2008. Macroalgal and cyanobacterial chemical defenses in freshwater communities. Pages 105-119 *in* C. D. Amsler, editor. Algal Chemical Ecology. Springer-Verlag Berlin.

Cargill, I. I., S. Austen, K. W. Cummins, B. J. Hanson, and R. R. Lowry. 1985. The role of lipids as feeding stimulants for shredding aquatic insects. Freshwater Biology 15:455-464.

Cetrulo, G. L. and M. E. Hay. 2000. Activated chemical defenses in tropical versus temperate seaweeds. Marine Ecology Progress Series 207:243-253.

Conn, E. E. 1980. Cyanogenic compounds. Annual Review of Plant Physiology 31:433-451.

Conover, W. J. and R. L. Iman. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. American Statistician 35:124-129.

Creed, R. P. 1994. Direct and indirect effects of crayfish grazing in a stream community. Ecology 75:2091-2103.

Cronin, G., D. M. Lodge, M. E. Hay, M. Miller, A. M. Hill, T. Horvath, R. C. Bolser, N. Lindquist, and M. Wahl. 2002. Crayfish feeding preferences for freshwater macrophytes: the influence of plant structure and chemistry. Journal of Crustacean Biology 22:708-718.

Cruz-Rivera, E. and M. E. Hay. 2003. Prey nutritional quality interacts with chemical defenses to affect consumer feeding and fitness. Ecological Monographs 73:483-506.

Cummins, K. W. and M. J. Klug. 1979. Feeding ecology of stream invertebrates. Annual Review of Ecology and Systematics 10:147-172.

Cyr, H. and M. L. Pace. 1993. Magnitude and patterns of herbivory in aquatic and terrestrial ecosystems. Nature 361:148-150.

Duffy, E. D. and M. E. Hay. 1990. Seaweed adaptations to herbivory Bioscience 40:368-375.

Everitt, D. T. and J. A. M. Burkholder. 1991. Seasonal dynamics of macrophyte communities from a stream flowing over granite flatrock in North Carolina, USA. Hydrobiologia 222:159-172.

Feminella, J. W. and C. P. Hawkins. 1995. Interactions between stream herbivores and periphyton: a quantitative analysis of past experiments. Journal of the North American Benthological Society 14:465-509.

Filkin, N. and M. L. Vis. 2004. Phenology of *Paralemanea annulata* (*Lemaneaceae*, Rhodophyta) in an Ohio woodland stream. Hydrobiologia 518:159-168.

Finlay, J. 2004. Patterns and controls of lotic algal stable carbon isotope ratios. Limnology and Oceanography 49:850-861.

Frost, P. C., R. S. Stelzer, G. A. Lamberti, and J. J. Elser. 2002. Ecological stoichiometry of trophic interactions in the benthos: understanding the role of C: N: P ratios in lentic and lotic habitats. Journal of the North American Benthological Society **21**:515-528.

Gaeveskaya, N. S. 1969. The role of higher plants in the nutrition of the animals of freshwater basins. National Lending Library for Science and Technology, Yorkshire, England.

Gelwick, F. P. and W. J. Matthews. 1997. Effects of algivorous minnows (Campostoma) on spatial and temporal heterogeneity of stream periphyton. Oecologia 112:386-392.

Gherardi, F. 2006. Crayfish invading Europe: the case study of *Procambarus clarkii*. Marine and Freshwater Behaviour and Physiology 39:175-191.

Gross, E. M. 2003. Allelopathy of aquatic autotrophs. Critical Reviews in Plant Sciences 22:313-339.

Hambrook, J. A. and R. G. Sheath. 1987. Grazing of freshwater rhodophyta. Journal of Phycology 23:656-662.

Hart, D. D. 1992. Community organization in streams: the importance of species interactions, physical factors, and chance. Oecologia 91:220-228.

Harvell, C. D. 1990. The ecology and evolution of inducible defenses. The Quarterly Review of Biology **65**:323-340.

Hay, M. E. 1984. Patterns of fish and urchin grazing on Caribbean coral reefs: are previous results typical? Ecology 65:446-454.

Hay, M. 1991. Marine-terrestrial contrasts in the ecology of plant chemical defenses against herbivores. Trends in Ecology & Evolution **6**:362-365.

Hay, M. E. 1996. Marine chemical ecology: What's known and what's next? Journal of experimental marine biology and ecology 200:103-134.

Hay, M. E. 2009. Marine chemical ecology: Chemical signals and cues structure marine populations, communities, and ecosystems. Annual Review of Marine Science 1:193-212.

Hay, M. E., J. E. Duffy, C. A. Pfister, and W. Fenical. 1987. Chemical defense against different marine herbivores: are amphipods insect equivalents? Ecology 68:1567-1580.

Hay, M. E., Q. Kappel, and W. Fennical. 1994. Synergisms in plant defenses against herbvores: interactions of chemistry, calcification, and plant-quality. Ecology 75:1714-1726.

Hay, M. E., J. J. Stachowicz, E. Cruz-Rivera, S. Bullard, M. S. Deal, N. Lindquist, K. F. Haynes, and J. G. Millar. 1998. Bioassays with marine and freshwater organsisms. Pages 39-141 *in* F. Haynes and J. G. Millar, editors. Methods in Chemical Ecology. Chapman and Hall, New York.

Hill, W. R., H. L. Boston, and A. D. Steinman. 1992. Grazers and nutrients simultaneously limit lotic primary productivity. Canadian Journal of Fisheries and Aquatic Sciences 49:504-512.

Hillebrand, H. 2008. Grazing regulates the spatial variability of periphyton biomass. Ecology 89:165-173.

Hillebrand, H. 2009. Meta-anlysis of grazer control of periphyton biomass across aquatic ecosystems. Journal of Phycology 45:798-806.

Hobbs, H. H. 1989. An Illustrated Checklist of the American Crayfishes (Decapoda, Astacidae, Cambaridae, Parastacidae). Smithsonian Contributions to Zoology 480:1-236.

Hobbs, H. H. 1993. Trophic relationships of North American freshwater crayfishes and shrimps. Milwaukee Public Museum Contributions in Biology and Geology 85:1-110.

Holomuzki, J. H., R. L. Lowe, and J. A. Ress. 2006. Comparing herbivory effects of stream macroinvertebrates on microalgal patch structure and recovery. New Zealand Journal of Marine and Freshwater Research 40:357-367.

Holomuzki, J. R., J. W. Feminella, and M. E. Power. 2010. Biotic interactions in freshwater benthic habitats. Journal of the North American Benthological Society 29:220-224.

Hutchinson, G. 1975. A Treatise on Limnology. Wiley & Sons, New York.

Jeffries, M. J. and J. H. Lawton. 1984. Enemy free space and the structure of ecological communities. Biological Journal of the Linnean Society 23:269-286.

Jones, J. 1949. An ecological study of the River Rheidol, North-Cardiganshire, Wales. Journal of Animal Ecology 18:67-88.

Jones, J. 1950. A further ecological study of the River Rheidol: the food of the common insects of the main-stream. The Journal of Animal Ecology 19:159-174.

Karban, R., A. A. Agrawal, J. S. Thaler, and L. S. Adler. 1999. Induced plant responses and information content about risk of herbivory. Trends in Ecology & Evolution 14:443-447.

Kohler, S. and M. Wiley. 1997. Pathogen outbreaks reveal large-scale effects of competition in stream communities. Ecology 78:2164-2176.

Lamberti, G. 1996. The role of periphyton in benthic food webs. Algal ecology: freshwater benthic ecosystems. Academic Press, San Diego:533–572.

Lamberti, G. and J. Moore. 1984. Aquatic insects as primary consumers. Pages 164-195 *in* V. Resh and D. Rosenberg, editors. The ecology of aquatic insects. Praeger, New York.

Lamberti, G. and V. Resh. 1983. Stream periphyton and insect herbivores: an experimental study of grazing by a caddisfly population. Ecology 64:1124-1135.

Lankau, R. A. 2007. Specialist and generalist herbivores exert opposing selection on a chemical defense. New Phytologist 175:176-184.

Lau, D., K. Leung, and D. Dudgeon. 2009. Are autochthonous foods more important than allochthonous resources to benthic consumers in tropical headwater streams? Journal of the North American Benthological Society 28:426-439.

Littler, M. and D. Littler. 1980. The evolution of thallus form and survival strategies in benthic marine macroalgae: field and laboratory tests of a functional form model. American Naturalist 116:25-44.

Lodge, D., G. Cronin, E. van Donk, and A. Froelich. 1998. Impact of herbivory on plant standing crop: comparisons among biomes, between vascular and nonvascular plants, and among freshwater herbivore taxa. Pages 149–174 *in* E. Jeppeson, M. Sondergaard, and K. Christoffersen, editors. The structuring role of submerged macrophytes in lakes. Springer-Verlag New York, Inc., New York.

Lodge, D., M. Kershner, J. Aloi, and A. Covich. 1994. Effects of an omnivorous crayfish (Orconectes rusticus) on a freshwater littoral food web. Ecology 75:1265-1281.

Lodge, D. and J. Lorman. 1987. Reductions in submersed macrophyte biomass and species richness by the crayfish *Orconectes rusticus*. Canadian Journal of Fisheries and Aquatic Sciences 44:591-597.

Lubchenco, J. and J. Cubit. 1980. Heteromorphic life histories of certain marine algae as adaptations to variations in herbivory. Ecology 61:676-687.

Lubchenco, J. and S. D. Gaines. 1981. A unified approach to marine plant-herbivore interactions. I. Populations and communities. Annual Review of Ecology and Systematics 12:405-437.

McClintock, J. B. and B. J. Baker. 2001. Marine chemical ecology. CRC Press, Boca Raton, Florida.

McKey, D. 1979. Distribution of secondary compounds within plants. Pages 55-134 *in* G. Rosenthal and D. Janzen, editors. Herbivores: their interaction with secondary plant metabolites Academic Press, Inc., New York.

McNeely, C., J. C. Finlay, and M. E. Power. 2007. Grazer traits, competition, and carbon sources to a headwater-stream food web. Ecology 88:391-401.

Mecom, J. O. and K. W. Cummins. 1964. A preliminary study of the trophic relationships of the larvae of *Brachycentrus americanus* (Banks)(Trichoptera: Brachycentridae). Transactions of the American Microscopical Society 83:233-243.

Menge, B. A., J. Lubchenco, and L. R. Ashkenas. 1985. Diversity, heterogeneity and consumer pressure in a tropical rocky intertidal community. Oecologia 65:394-405.

Minckley, W. L. 1962. Studies of the ecology of a spring stream: Doe Run, Meade County, Kentucky. Wildlife Monographs 11:3-124.

Minckley, W. L. and D. R. Tindall. 1963. Ecology of *Batrachospermum sp.* (Rhodophyta) in Doe Run, Meade County, Kentucky. Bulletin of the Torrey Botanical Club 90:391-400.

Morrison, W. and M. Hay. 2010. Induced chemical defenses in a freshwater macrophyte suppress herbivore fitness and the growth of associated microbes. Oecologia 165:427-436.

Mulholland, P., J. Tank, D. Sanzone, W. Wollheim, B. Peterson, J. Webster, and J. Meyer. 2000. Food resources of stream macroinvertebrates determined by natural-abundance stable C and N isotopes and a 15 N tracer addition. Journal of the North American Benthological Society 19:145-157.

Newman, R. M., Z. Hanscom, and W. C. Kerfoot. 1992. The watercress glucosinolate-myrosinase system: a feeding deterrent to caddisflies, snails and amphipods. Oecologia 92:1-7.

Opsahl, R. W., T. Wellnitz, and N. LeRoy Poff. 2003. Current velocity and invertebrate grazing regulate stream algae: results of an in situ electrical exclusion. Hydrobiologia 499:135-145.

Parker, J. D., D. Burkepile, D. O. Collins, J. Kubanek, and M. E. Hay. 2007. Stream mosses as chemically-defended refugia for freshwater macroinvertebrates. Oikos 116:302-312.

Paul, V. J. and K. L. Van Alstyne. 1992. Activation of chemical defenses in the tropical green algae Halimeda spp.* 1. Journal of experimental marine biology and ecology 160:191-203.

Pavia, H., G. Toth, and P. Åberg. 1999. Trade offs between phlorotannin production and annual growth in natural populations of the brown seaweed Ascophyllum nodosum. Journal of Ecology **87**:761-771.

Poff, N. L., R. D. DeCino, and J. V. Ward. 1991. Size-dependent drift responses of mayflies to experimental hydrologic variation: active predator avoidance or passive hydrodynamic displacement? Oecologia 88:577-586.

Poff, N. L. and J. V. Ward. 1992. Heterogeneous currents and algal resources mediate in situ foraging activity of a mobile stream grazer. Oikos 65:465-478.

Poff, N. L. and J. V. Ward. 1995. Herbivory under different flow regimes: a field experiment and test of a model with a benthic stream insect. Oikos 72:179-188.

Power, M. E., W. J. Matthews, and A. J. Stewart. 1985. Grazing minnows, piscivorous bass, and stream algae: dynamics of a strong interaction. Ecology 66:1448-1456.

Power, M. E., M. S. Parker, and W. E. Dietrich. 2008. Seasonal reassembly of a river food web: floods, droughts, and impacts of fish. Ecological Monographs 78:263-282.

Power, M. E., R. J. Stout, C. E. Cushing, P. P. Harper, F. R. Hauer, W. J. Matthews, P. B. Moyle, B. Statzner, R. Irene, and W. De Badgen. 1988. Biotic and abiotic controls in river and stream communities. Journal of the North American Benthological Society 7:456-479.

Pringle, C. M. and T. Hamazaki. 1997. Effects of fishes on agal response to storms in a tropical stream . Ecology 78:2432-2442.

Prusak, A. C., J. O'Neal, and J. Kubanek. 2005. Prevalence of chemical defenses among freshwater plants. Journal of Chemical Ecology 31:1145-1160.

Rhoades, D. F., G. A. Rosenthal, and D. H. Janzen. 1979. Evolution of plant chemical defense against herbivores. Pages 4-53 *in* G. A. Rosenthal and D. H. Janzen, editors. Herbivores: their interaction with secondary plant metabolites. Academic Press, Inc., New York.

Roa, R. 1992. Design and analysis of multiple-choice feeding-preference experiments. Oecologia **89**:509-515.

Rosemond, A. D., P. J. Mulholland, and S. H. Brawley. 2000. Seasonally shifting limitation of stream periphyton: response of algal populations and assemblage biomass and productivity to variation in light, nutrients, and herbivores. Canadian Journal of Fisheries and Aquatic Sciences 57:66-75.

Sheath, R.G. 2003. Red Algae. Pages 197-224 *in* J. Wehr and R. Sheath, editors. Freshwater algae of North America: ecology and classification. Academic Press, San Diego.

Sheath, R. and B. Cole. 1992. Biogeography of stream macroalgae in North America. Journal of Phycology **28**:448-460.

Sheath, R. G. and J. A. Hambrook. 1990. Freshwater ecology. Pages 423-453 *in* K. M. Cole and R. G. Sheath, editors. Biology of the red algae. Cambridge University Press, New York.

Sheath, R. G. and J. A. Hambook. 1988. Mechanical adaptations to flow in freshwater red algae. Journal of Phycology 24:107-111.

Shelford, V. E. 1918. Conditions of existence. Pages 21–60 *in* H. B. Ward and G. C. Whipple, editors. Freshwater Biology. Wiley, New York.

Shih, H. J. 1941. On the foods of some cyprinoic fishes. Sinesia 12:235-237.

Sirjola, E. 1969. Aquatic vegetation of the river Teuronjoki, south Finland, and its relation to water velocity. Annales Botanici Fennici 6:68-75.

Slocum, C. J. 1980. Differential susceptibility to grazers in two phases of an intertidal alga: advantages of heteromorphic generations. Journal of experimental marine biology and ecology 46:99-110.

Sponseller, R. A., N. B. Grimm, A. J. Boulton, and J. L. Sabo. 2010. Responses of macroinvertebrate communities to long-term flow variability in a Sonoran Desert stream. Global Change Biology 16:2891-2900.

Stachowicz, J. and M. E. Hay. 1996. Facultative mutualism between an herbivorous crab and a coralline alga: advantages of eating noxious seaweeds. Oecologia 105:377-387.

Steinman, A. D. 1996. Effects of grazers on freshwater benthic algae. Pages 341-373 *in* R. J. Stevenson, M. L. Bothwell, and R. L. Lowe, editors. Algal Ecology: Freshwater Benthic Ecosystems. Academic Press, San Diego.

Steinman, A. D., P. J. Mulholland, and W. R. Hill. 1992. Functional responses associated with growth form in stream algae. Journal of the North American Benthological Society 11:229-243.

Steinman, A. D., P. J. Mulholland, and D. B. Kirschtel. 1991. Interactive effects of nutrient reduction and herbivory on biomass, taxonomic structure, and P uptake in lotic periphyton communities. Canadian Journal of Fisheries and Aquatic Sciences 48:1951-1959.

Stewart, A. J. 1987. Responses of stream algae to grazing minnows and nutrients: a field test for interactions. Oecologia 72:1-7.

Stock, M. S., T. D. Richardson, and A. K. Ward. 1987. Distribution and primary productivity of the epizoic macroalga *Boldia erythrosiphon* (Rhodophyta) in a small Alabama stream. Journal of the North American Benthological Society 6:168-174.

Stout, E. P. and J. Kubanek. 2010. Marine macroalgal natural products. Pages 41-65 *in* L. Mander and H. W. Lui, editors. Comprehensive Natural Products II: Chemistry and Biology. Eslevier, Oxford.

Strauss, S. Y., J. A. Rudgers, J. A. Lau, and R. E. Irwin. 2002. Direct and ecological costs of resistance to herbivory. Trends in Ecology & Evolution 17:278-285.

Swan, C. M. and M. A. Palmer. 2000. What drives small scale spatial patterns in lotic meiofauna communities? Freshwater Biology 44:109-121.

Thirb, H. H. and K. Benson-Evans. 1985. The effect of water temperature, current velocity and suspended solids on the distribution, growth and seasonality of *Lemanea fluviatilis* (C. Ag.), Rhodophyta, in the River Usk and other South Wales rivers. Hydrobiologia 127:63-78.

Thorp, J. H. and M. D. Delong. 2002. Dominance of autochthonous autotrophic carbon in food webs of heterotrophic rivers. Oikos 96:543-550.

Torres-Ruiz, M., J. D. Wehr, and A. A. Perrone. 2007. Trophic relations in a stream food web: importance of fatty acids for macroinvertebrate consumers. Journal of the North American Benthological Society **26**:509-522.

Van Alstyne, K. L. and L. T. Houser. 2003. Dimethylsulfide release during macroinvertebrate grazing and its role as an activated chemical defense. Marine Ecology Progress Series 250:175-181.

Van Alstyne, K. L., G. V. Wolfe, T. L. Freidenburg, A. Neill, and C. Hicken. 2001. Activated defense systems in marine macroalgae: evidence for an ecological role for DMSP cleavage. Marine Ecology Progress Series 213:53-65.

Vergés, A., N. A. Paul, and P. Steinberg. 2008. Sex and life-history stage alter herbivore responses to a chemically defended red alga. Ecology 89:1334-1343.

Zar, J. H. 2010. Biostatistical Analysis. Fifth edition. Pearson Prentice Hall, Upper Saddle River, New Jersey.