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A field test of inducible resistance to specialist and generalist herbivores using the water lily *Nuphar luteum*

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Abstract We tested whether grazing by the specialist beetle *Galerucella nymphaeae* (Coleoptera: Chrysomelidae) induced resistance to herbivory in the water lily *Nuphar luteum macrophyllum* (Nymphaeaceae) using both the specialist beetle and the generalist crayfish *Procambarus clarkii* (Decapoda: Cambaridae). For 2 months, we allowed natural densities of beetles to develop on control plants of *Nuphar*, while removing beetles every 2–3 days from adjacent plants that were paired by location within our field site. By the end of the 2-month manipulation, beetle grazing had damaged twice as much leaf surface on control plants as on removal plants (30.6% vs. 14.2%, respectively). We then offered tissues from control and removal plants to adult and larval beetles and to crayfish in laboratory assays. Increased levels of previous attack by the specialist beetle either did not affect or increased water lily attractiveness to beetles, but significantly decreased attractiveness to the generalist crayfish. Beetle larvae did not feed preferentially on control vs. removal *Nuphar* in assays using either immature, undamaged leaves that had not yet reached the pond surface or intermediate aged leaves that had reached the surface and experienced some beetle grazing. Adult beetles consumed significantly more immature leaf tissue from the heavily grazed controls than from the less grazed removal plants but did not discriminate between control and removal leaves of intermediate age in either feeding or oviposition preference. In contrast, generalist crayfish consumed significantly more plant tissue from the less grazed treatment than from the more heavily grazed controls. Crude chemical extracts from *Nuphar* strongly deterred

crayfish feeding, but neither phenolic content, protein content, nor differential effects of crude extracts from control vs. removal plants explained crayfish feeding on control versus removal leaves. Our assays suggest that induced resistance to crayfish may be chemically mediated, but the particular mechanisms producing this response remain unclear. Responses may be due to defensive metabolites that degrade rapidly following extraction.

Key words Chemical ecology · Induced defenses · Plant-herbivore interactions · Specialist vs. generalist herbivores · Water lily

Introduction

Traditionally, herbivory on freshwater macrophytes has been considered to be minimal and to have little effect on the ecology or evolution of the aquatic plants (Shelford 1918; Lamberti and Moore 1984). However, recent reviews of this topic suggest that herbivory on living freshwater macrophytes can be substantial and thus deserves further study (Lodge 1991; Newman 1991; Jacobsen and Sand-Jensen 1992; Lodge et al. 1998). Numerous authors have hypothesized that antiherbivore defenses, particularly chemical defense, may be common in freshwater vascular plants (Otto and Svensson 1981; Ostrofsky and Zettler 1986; Kerfoot 1988; Suren 1989), but few chemical defenses have been documented (Newman et al. 1992, 1996).

Cyr and Pace (1993) suggest that herbivory, including herbivory on aquatic macrophytes, is generally greater in aquatic than in terrestrial ecosystems. Despite such evidence, investigations of interactions between plants and herbivores in freshwater systems are rare compared to studies in terrestrial or marine habitats. In particular, inducible responses to herbivory have been studied extensively in terrestrial plants (reviewed by Karban and Baldwin 1997), with numerous investigations docu-

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menting increased production of defensive secondary metabolites following herbivory (e.g., Tallamy and Raupp 1991; Karban and Baldwin 1997). Induction of chemical defenses in response to herbivory also occurs in marine seaweeds (Van Alstyne 1988; Cronin and Hay 1996a) but may occur less commonly than for terrestrial plants (Hay 1996). In contrast to the many studies from terrestrial communities, and the several from marine communities, very few studies have examined whether freshwater macrophytes, which are descendants of terrestrial plants, respond to herbivore attack by increasing defenses (but see Jeffries 1990).

The water lily genus *Nuphar* is rich in secondary metabolites (Wrobel 1967), and a recent investigation (Cronin 1998), as well as data we present here, indicate that *Nuphar luteum* produces metabolites that deter feeding by generalist consumers such as crayfish. We therefore chose this plant to investigate the possibility of inducible resistance to herbivory in response to attack by the water lily leaf beetle *Galerucella nymphaeae*, which can dramatically reduce lifetimes of individual *Nuphar* leaves (Wallace and O'Hop 1985; Juliano 1988; Kouki 1991a) and change the relative production of different leaf morphologies (Kouki 1993). Wallace and O'Hop (1985) proposed that beetle herbivory caused compensatory growth and increased leaf turnover in *Nuphar*; however, experimental tests have shown no increased leaf production in grazed vs. ungrazed water lilies (Kouki 1991a, 1993). This suggests water lily leaf beetles may negatively impact plant fitness and therefore could select for defensive responses in *Nuphar*.

Several studies of terrestrial plant responses to herbivore attack have included considerations of how induction affects different types of herbivores (e.g., Kogan and Fischer 1991; Rausher et al. 1993). Some authors have argued that induced defenses may be more effective against generalists than against specialists because specialists are often adapted to the defenses of their host plant (Rhoades 1979; Van Dam et al. 1993). In support of this, some studies show that specialist herbivores can be unaffected by, or can even benefit from, increased levels of host plant chemical defenses (e.g., Smiley et al. 1985). However, other specialist herbivores are negatively affected by their host's chemical defenses (Zangerl and Berenbaum 1993; Osier et al. 1996), and herbivore-mediated induction of defenses deters feeding by numerous specialists (Karbon 1993). The effects of plant defenses can thus vary considerably among different herbivores in both marine and terrestrial systems (Hay and Steinberg 1992; Rausher 1992), and a recent synthesis (Karbon and Baldwin 1997) indicates that it is not yet clear that induced defenses will produce consistent or predictable differences in the responses of generalist versus specialist herbivores. In light of this unresolved question, our study examined how differing levels of herbivory by a beetle that specializes on water lilies affected subsequent feeding by both the specialist beetle and a generalist crayfish.

Materials and methods

Rationale

Studies attempting to detect induced responses to herbivory have used a variety of methods, including comparisons of naturally grazed and undamaged plants, simulated herbivory (e.g., clipping), and herbivore caging. These approaches each have limitations. Traits of naturally grazed versus ungrazed plants can be confounded by selective herbivory and environmental factors (Neuvonen and Haukioja 1985; Strauss 1988), plant responses to real vs. simulated herbivory can sometimes differ (Baldwin 1990, 1994; Kouki 1993), and caging can influence plant chemistry through shading or other confounding factors (Stamp and Bowers 1994). Furthermore, many studies report changes in plant chemistry due to herbivory but fail to demonstrate whether the responses actually protect plants from further herbivore damage (Fowler and Lawton 1985; Hay 1996). To minimize these concerns, we chose to manipulate herbivore numbers by manually removing water lily leaf beetles, their eggs, and larvae, from naturally growing *Nuphar*. We then compared subsequent herbivore preferences for plants that differed in their history of herbivore attack.

Study site and organisms

The study site, in a residential area of Morehead City, North Carolina, USA (34°44'N, 76°48'W), is a 1- to 2-ha pond that is 1–3 m deep and has a sand bottom with a flocculent organic covering. The pond is characterized by high biomass and species diversity of both submersed and emergent aquatic macrophytes.

The water lily *N. luteum macrophyllum* (Small) Beal. (Nymphaeaceae) is a rhizomatous, perennial herb with a wide geographic distribution throughout the eastern and central United States (Godfrey and Wooten 1981). At our study site, its growing season begins in March or April and lasts well into November. Long-petioled leaves and flowers reach the water surface, often forming circular to oval clusters of leaves. Shape of individual clusters is obscured where leaves from multiple plants continuously blanket large areas of the pond.

At our study site, *Nuphar* is attacked mainly by the water lily leaf beetle *G.* (= *Pyrrhali*) *nymphaeae* (Coleoptera: Chrysomelidae), which completes all life history stages on floating and aerial leaf surfaces (Scott 1924). Larvae of a species of pyralid moth also attack *Nuphar* by cutting elliptical pieces from the leaves, but this type of damage at our site is minor compared to the damage characteristic of water lily leaf beetles (see Wallace and O'Hop 1985). Laboratory studies indicate that *Galerucella* specializes on species of *Nuphar* and occasionally *Polygonum* (Kerfoot 1988; G. Cronin, T. Schlacher, D.M. Lodge submitted). Larvae live on and consume upper leaf surfaces for roughly 9 days before pupating and metamorphosing into adults that feed and oviposit on leaves (Wallace and O'Hop 1985). The beetles' relatively sedentary nature makes field manipulation possible.

The Louisiana red crayfish *Procambarus clarkii* (Decapoda: Cambaridae) is a commercially exploited species that has become well established far beyond its native range in the southeastern United States and northern Mexico (Hobbs 1989). Crayfish are relatively mobile, generalist omnivores that occur commonly throughout North Carolina (Hobbs 1989) and can have dramatic effects on the distribution and abundance of aquatic macrophytes (Creed 1994; Lodge et al. 1994, 1998). Crayfish occur at our study site but did not appear to be abundant. For this study, crayfish were obtained from a local aquaculture facility and kept at the Institute of Marine Sciences in Morehead City, North Carolina. They were maintained in 34 × 28 × 13 cm tubs with 2 l of dechlorinated tap water that was changed every 4–5 days. The animals were fed daily with commercial fish food and Oceanstars algal pellets.

Beetle removal experiment

We selected and marked ten pairs of *Nuphar* plants (paired by location and approximate size) over a wide area of the experimental pond on 17 April 1996. In an effort to minimize beetle movement among our experimental plants, we selected water lilies that were separated from all others by at least 2 m. These spatially discrete clusters of leaves almost certainly represented entire plants (Luther 1983), but we were unable to check this rigorously because diving and probing the bottom to verify the isolation of each plant's rhizomes resuspended the flocculent bottom material and lowered visibility to zero. The average distance between paired plants was 5 m, similar to other studies in which *Galerucella* densities on *Nuphar* were manipulated (Kouki 1991a, 1993). Each member of a pair was randomly assigned as either a control or a beetle removal treatment. Since the study began early in the growing season, there was little existing damage on *Nuphar* leaves prior to the start of our experiment.

Using a canoe as a working platform, we removed and destroyed adults, larvae, and egg masses from all floating and aerial leaves of removal plants. We also visited and disturbed (to simulate the mechanical effects of beetle removal) leaves of control plants but did not remove any beetles. This was done three times per week or approximately every other day for 2 months. Because of the uneven spatial distribution of beetles in the pond early in the growing season, and because a few of our control replicates remained initially free of herbivores, a one-time addition of 30–50 larvae was made to each control plant 2 weeks after the start of the experiment to ensure that some herbivory did occur. The length of time in which the added larvae grazed before pupating (approx. 9 days or less) was short compared to the total duration of the experiment (over 60 days), and beetle densities on control plants did not appear to differ from natural densities over the length of the experiment. Therefore, we believe that control plants experienced herbivory levels comparable to unmanipulated plants at our study site.

After the initial extermination, very few larvae were found on removal plants. However, some adult beetles recolonized and deposited new egg masses during the few days between removals. Toward the end of the experiment, single, late-instar larvae were occasionally found on a removal replicate. We assume that these were transported on the water surface tension from other water lilies in the pond.

Beetle densities, plant traits, and leaf damage of grazed versus removal water lilies

We recorded beetle densities on control and removal water lilies on 18 June 1996. This occurred 3 days after the last removal, roughly corresponding to the next scheduled removal if the experiment had been continued. Counts of adults and larvae per leaf were made in the field for each of the 20 plants that constituted our experiment.

To estimate the effects of beetle herbivory on these plants, we measured plant size (by assuming each cluster of leaves was an ellipse and measuring the long and short axes of the patch), counted the total number of leaves per plant (including immature leaves not yet at the surface, aerial leaves, and decayed leaves), and counted the number of flowering stems. Aerial leaves were defined as leaves that completely cleared the water surface. Decayed leaves were defined as leaves that were greater than 50% yellowed or brown and disintegrating. *Nuphar* also produces submersed leaves that never reach the surface and have a distinct morphology; in one previous study, herbivory by *Galerucella* resulted in the production of more submersed leaves of this type (Kouki 1993). However, in our study, submersed-type leaves were not quantified due to time constraints, the large size of many of our experimental plants (50–60 leaves per plant), and the difficulty of being certain that all submersed leaves had been collected, given the low underwater visibility in the pond.

After recording the above parameters in situ, three immature leaves from just below the water surface and three intermediate-age

leaves from each control and removal plant were set aside for laboratory feeding and oviposition assays. The intermediate-aged leaves were selected randomly from a subset of leaves on each plant that were of adequate size (> 10 cm wide), had no yellowed or decayed tissue, and had a smooth, unbroken perimeter. After selection of the assay leaves, all remaining leaves were harvested and brought to the laboratory where 20 leaves (10 control and 10 removal) were randomly selected from each replicate and examined to obtain the percentage of leaf area damaged and individual leaf size. All leftover leaves were frozen at -70°C . To measure percent cover damaged, a transparency was marked with 100 points in a stratified random design and placed on the upper surface of the leaf such that a designated point on the transparency overlapped the juncture of petiole and leaf. The leaf condition directly underlying each point was recorded as one of seven categories: (1) green (undamaged), (2) grazed (in a characteristic fashion – the upper leaf surface was partially excavated but not perforated), (3) missing, (4) brown and dry, (5) torn, (6) yellowed, and (7) decayed and disintegrating. Many of the points failed to overlap the smaller leaves, in which case we recorded all points hitting the leaf and then moved the leaf further up the transparency to a second predetermined mark. We repeated this process until we had recorded a total of at least 50 points for each leaf. To assess individual leaf size, we recorded leaf length from the base of the sinus to the apex for each of the ten leaves used in percent cover measurements. These data were used to calculate individual leaf surface area in the following manner: a LI-COR area meter was used to measure the projected surface area of leaves of known lengths; we then calculated the regression equation: leaf surface area (cm^2) = $-14.282 + 8.578 \times$ leaf length (cm). Leaf length accurately predicted leaf surface area ($R^2 = 0.997$), and this equation was used to calculate surface areas of the leaves we had analyzed for percent cover of damage.

Individual leaf surface area and percent of leaf area damaged for each of the seven categories of damage were averaged for the ten leaves subsampled from each plant. These mean values for each of the ten control and ten removal water lilies were then used in our statistical analyses of differences between the two treatments (see below).

Beetle feeding and ovipositing assays

To determine whether the experimental reduction in beetle herbivory affected the palatability or attractiveness of *Nuphar* for ovipositing or feeding beetles, we offered adult and larval *Galerucella* a choice of leaf portions from control and removal plants. Immature leaves and leaves of intermediate age were compared in separate assays. For each of the ten pairs of plants, three immature leaves from control plants were haphazardly paired with three immature leaves from corresponding removal plants, yielding a total of 30 pairs of leaves. Squares were cut from randomly selected areas of leaves (4×4 cm for assays with adults and 3×3 cm for assays with larvae). Control and removal leaf squares were placed side by side on damp paper towels in the bottom of clear plastic, lidded containers. The beetles used in these assays were collected from *Nuphar* scattered over a wide area of the experimental pond (to avoid collecting closely related siblings) on the day that the assays were set up. In larval assays, three larvae were placed on the juncture of the two adjacent squares in each container. In assays using adults, four adults were haphazardly added to each container to enhance the chances of including at least one gravid female. The duration of both larval and adult assays was 24 h. The surface area (mm^2) consumed in each replicate was measured by placing a transparency over each replicate and tracing the damage with a black marker. The transparencies were then run through a LI-COR area meter to quantify the area of leaf surface consumed. Ovipositing was measured as the number of egg masses deposited on the leaf squares; the number of eggs per egg mass was also quantified.

If no feeding took place, we assumed that the leaf tissues were not sampled and that the herbivores had not made a choice, and we excluded that replicate from our analysis of feeding preference. Such cases, in which the difference between control and treatment is

zero, would decrease the chance of detecting a real difference in feeding but would not affect our conclusion if there were, in fact, no difference in herbivore preference for control vs. treatment. The exclusion of replicates in which no feeding occurred was standard practice in the feeding experiments reported in this study; however, only 5 of 28 replicates in the larval assay of immature leaves were omitted in this fashion.

Assays comparing intermediate-aged leaves were performed in the same manner as described for immature leaves, except that existing damage to leaf squares was colored onto transparencies before the assays were started, and the amount consumed was calculated as the final minus the initial grazed surface area. It was not obvious when no feeding had occurred; therefore, we analyzed all replicates. Because we had only ten independent pairs of experimental water lilies, we averaged the feeding of the three groups of beetles given leaves from the same pair of control and removal plants, and we used these ten means as individual replicates in our statistical analyses. Differences in amount eaten (mm^2) between control and removal *Nuphar* were analyzed using paired *t*-tests.

Crayfish feeding assays

To assess the response of a generalist consumer to our experimental *Nuphar* plants, we offered portions of the freshly collected leaves from control and removal treatments to individually housed crayfish. To control for changes in leaf mass due to factors other than crayfish consumption, equivalent leaf portions were also set up in containers with no crayfish. However, very few animals ate measurable amounts of the fresh tissue, resulting in poor replication and low power to detect a difference in palatability of the two choices. This lack of feeding is consistent with observations that *Nuphar* is a low-preference plant for crayfish (Cronin 1998). Additionally, crayfish sampling of leaf edges sometimes caused leaf portions to gain more weight than the control pieces, probably through water uptake of damaged tissue.

Because of these difficulties, we used an alternate method (see Hay et al. 1994) to create *Nuphar* mimics consisting of freeze-dried plant tissue in an alginate matrix. Crayfish consumed this food more readily than fresh *Nuphar*. Frozen leaves remaining from the harvest of control and removal treatments were freeze-dried and ground to a fine powder in a Wiley mill. These leaves represented pooled subsamples from our ten control or ten removal replicates. For both the control and removal treatment, 1.5 g plant powder was mixed with 0.37 g alginic acid and 18.8 ml distilled water. These two mixtures were poured into two slots of a rectangular mold, beneath which lay a fiberglass window screen. Calcium chloride solution (0.25 M) was sprayed on the food, causing it to solidify and adhere to the screen. After the mold was removed, the screen was cut into strips containing equal amounts of the two *Nuphar* diets and offered to individual crayfish ($n = 32$). Because both foods were affixed to the screen, consumption of each food could be measured as the number of squares on the screen that were cleared of food (see Hay et al. 1994). We monitored the animals periodically and attempted to remove strips when roughly half of either choice was consumed. We ended the assay after 17 h. Excluded from statistical analysis were replicates in which the food was not eaten at all, completely eaten, or dislodged from the screen.

The different assay methods for beetles versus crayfish (fresh leaf squares vs. artificial diets) might have confounded our study if cutting leaf squares for the beetle assays caused rapid induction of chemical changes in both control and removal treatments. Thus, crayfish could have been presented with tissue in which initial plant chemistry was "preserved" by freeze-drying, whereas beetles could have been offered uniformly induced tissues. We addressed this problem using preliminary assays to test whether crayfish fed differently on artificial diets made of *Nuphar* that was promptly frozen, freeze-dried, and made into alginate foods (potentially preserving initial chemistry) versus *Nuphar* that was cut into squares that sat at room temperature for 1 h (allowing for rapid induction of defenses) and then frozen, freeze-dried, and made

into alginate foods. Feeding was similar on both treatments ($P = 0.264$, $n = 17$, paired *t*-test), indicating that cutting leaves did not alone affect their palatability to crayfish.

Neither the fresh plant tissue nor the artificial foods appeared to differ in morphology or structural traits between control vs. removal *Nuphar*. Other plant traits that are known to affect herbivore preferences include chemical defenses (Rosenthal and Berenbaum 1992) and nutritional value, particularly nitrogen content (Mattson 1980). To assess whether plant chemistry influenced crayfish feeding in the assay described above, we incorporated crude chemical extracts from control versus removal *Nuphar* into palatable foods and offered the two foods to crayfish. For each of the two treatments, 1.5 g of freeze-dried *Nuphar* powder was extracted with 2:1 dichloromethane (DCM):methanol, filtered, extracted with 7:3 methanol:distilled water, and filtered again. After the solvents had been removed by rotary evaporation, the extract was partitioned between DCM and water. The DCM-soluble (lipophilic) portion was dried, dissolved in anhydrous ethyl ether, and applied to 1.5 g of a palatable mixture of broccoli and iceberg lettuce (fresh-frozen, freeze-dried and ground to a powder). We removed ether from the powder by rotary evaporation, resulting in the extract from 1.5 g of *Nuphar* being coated onto 1.5 g of broccoli-lettuce powder; this was done for both control and removal treatments. The two powders were then used to make experimental food in the manner described above. The distilled water, which was mixed with the plant powder and alginate, contained the appropriate water-soluble extract from either control or removal tissue. Aside from the addition of the extracts and the use of broccoli-lettuce (1:1 by dry mass; 8% soluble protein, 4.1% N, 39.6% C, unpublished data), methods used in this assay were identical to the assay testing entire *Nuphar* tissues.

In addition to offering crayfish a choice between two foods treated with extracts from either our *Nuphar* control or removal treatments, we conducted two separate assays in which a palatable food with no extract was offered in tandem with the extract-treated foods. Thus, we determined the individual effects of total crude extracts from control or removal *Nuphar* on consumption of an otherwise palatable food, in addition to the effects of the extracts relative to one another. These two assays followed the previously described methods. Freeze-dried broccoli-lettuce was treated with either solvent alone or solvent plus extract. Rotary evaporation removed the solvents, yielding a powder treated with extract and a "solvent control" powder. These were used to make our experimental foods.

Analysis of total phenolics and protein content

Polyphenolics are a class of secondary plant metabolites commonly hypothesized to deter herbivores (Feeny 1976; Coley and Aide 1990). Herbivory, as well as environmental factors, can cause changes in concentrations of plant phenolics (Waterman and Mole 1989; Tallamy and Raupp 1991). Therefore, we tested for an effect of differential beetle grazing on phenolic concentration in *Nuphar*, using the Folin-Ciocalteu method for quantification of total phenolics (Folin and Ciocalteu 1927). Waterman and Mole (1994) suggest that this method is less prone to interference by non-phenolic metabolites and is one of the best available for total phenolics. Six replicate samples of the freeze-dried, finely ground leaves from control versus removal treatments (the same tissues used in crayfish assays) were analyzed, and tannic acid standards were used to convert sample absorbency at 760 nm to phenolic concentration in tannic acid equivalents (TAE).

As a measure of plant nutritional value, soluble protein content was quantified in control versus removal *Nuphar* using the method of Bradford (1976), modified for small samples (Cronin and Hay 1996b). Three replicate samples of freeze-dried, finely ground tissue (the same tissues used in crayfish assays) were digested in 1 M sodium hydroxide for 24 h at 1°C. A 50- μl aliquot of the supernatant solution was added to 5 ml Bradford reagent, and absorbance at 595 nm was recorded. The standard curve was calculated using samples of bovine serum albumin (BSA).

Statistical analyses

Overall differences between control and removal water lilies in vegetative traits and percent cover of leaf damage were analyzed by multivariate analysis of variance (MANOVA). Vegetative traits of replicate water lilies were represented by a vector of the following variables: aerial leaves, immature leaves, decayed leaves, flowering stems, total leaves, leaf density, and individual leaf area. The response vector for percent cover of leaf damage was composed of the seven categories of leaf damage listed previously. In both cases, the pairing effect was nonsignificant (vegetative traits: $F = 0.877$, $P = 0.702$; percent cover leaf damage: $F = 1.085$, $P = 0.369$), so we left it out of the analyses and performed one-factor MANOVAs testing whether response vectors differed between control and removal plants (the multivariate equivalent of a t -test, see discussion of Hotelling's T^2 -statistic in Morrison 1976). Following the result of a significant difference between the multivariate responses, univariate unpaired t -tests were performed on each variable to further determine in what manner the control and removal plants differed.

Because the pairing of plants by location was statistically unimportant, we analyzed all other variables, e.g., beetle densities, with unpaired t -tests. Laboratory assays of feeding and ovipositing by beetles and of feeding by crayfish were analyzed with paired t -tests, because individual herbivores were offered control and removal leaf tissue simultaneously in every case.

Results

Effects of beetle removal on beetle densities, leaf damage, and attributes of *Nuphar*

Our removal treatment reduced adult *Galerucella* density by 66% ($P = 0.001$) and larval density by 93% ($P < 0.0001$) on removal relative to control plants (Fig. 1). These densities were recorded 3 days after the last removal, indicating that the effects of our treatment were relatively long-lasting. The success of removal was probably similar or better throughout the experiment because it began when the overall beetle population was much lower, and the longest interval between removals was 3 days.

The reduction of beetle numbers on the removal treatment had statistically significant effects on several of the plant traits we measured (Table 1, Figs. 2, 3). Because plants were assigned randomly as control or removal, we assume that initial plant traits were similar between the two treatments. After 2 months, removal plants had 57% fewer decayed leaves ($P = 0.010$), 23% fewer immature leaves ($P = 0.0006$) that had not yet reached the surface, and 38% fewer flowering stems ($P = 0.044$; Fig. 2A). Removal plants tended to have more total leaves ($P = 0.095$; Fig. 2B) than control

plants. Density of leaves/m² and average area per leaf did not differ between treatments (Fig. 2C, D).

Leaf damage differed substantially between control and removal *Nuphar* (Table 1, Fig. 3). Removal leaves had 38% more green tissue ($P = 0.0002$) and 54% less grazed area ($P = 0.0002$; Fig. 3). Removal leaves were also less decayed ($P = 0.020$) and tended to have less leaf area missing ($P = 0.091$) than control leaves. Tears in the leaves resulted from mechanical damage due to wind, disturbance from our canoe, and a hailstorm (R.C. Bolser, personal observation). The small but significant ($P = 0.002$) difference in percentage of torn leaf area (Fig. 3) is probably due to tears remaining more evident on intact surfaces of removal leaves. This type of damage was always minor ($1.1 \pm 0.2\%$ for removal, $0.5 \pm 0.2\%$ for control), and grazing may have obscured tears on the more heavily grazed, control leaves (Fig. 3).

In an attempt to integrate several of the above parameters, we calculated the total area of green leaf tissue per plant by multiplying the mean percent green area per leaf by the mean individual leaf area, and then multiplying this by the total number of leaves for each of the 20 experimental plants (Fig. 4). The significant effect of beetle removal on the percentage of green leaf tissue at the scale of individual leaves (Fig. 3), together with the

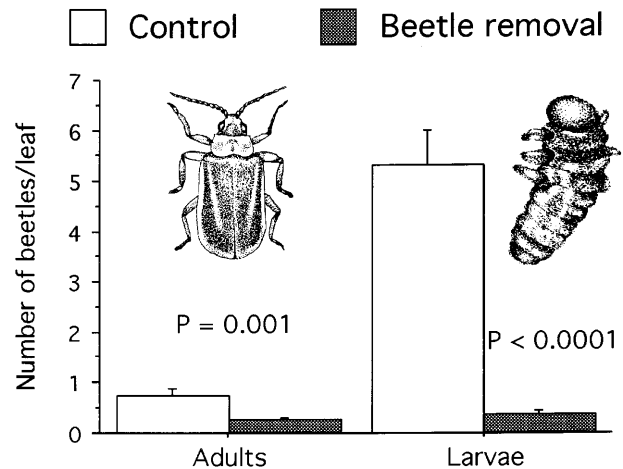


Fig. 1 Mean densities (± 1 SE) of water lily leaf beetles on removal vs. control *Nuphar* plants after 2 months of experimental removal. Two-tailed P -values were generated by unpaired t -tests comparing mean beetle density between removal and control plants ($n = 10$ for each bar)

Table 1 Results of MANOVAs testing for overall difference between control and removal *Nuphar* in vegetative traits and percent cover of leaf damage. Type refers to either control or removal. The effect of pairing plants was statistically nonsignificant and was thus omitted from the analyses

	<i>df</i>	Hotelling-Lawley trace	<i>F</i>	num. <i>df</i>	denom. <i>df</i>	<i>P</i> -value
Vegetative traits						
Type	1	4.815	8.255	7	12	0.0009
Residuals	18					
Leaf damage						
Type	1	3.415	5.854	7	12	0.004
Residuals	18					

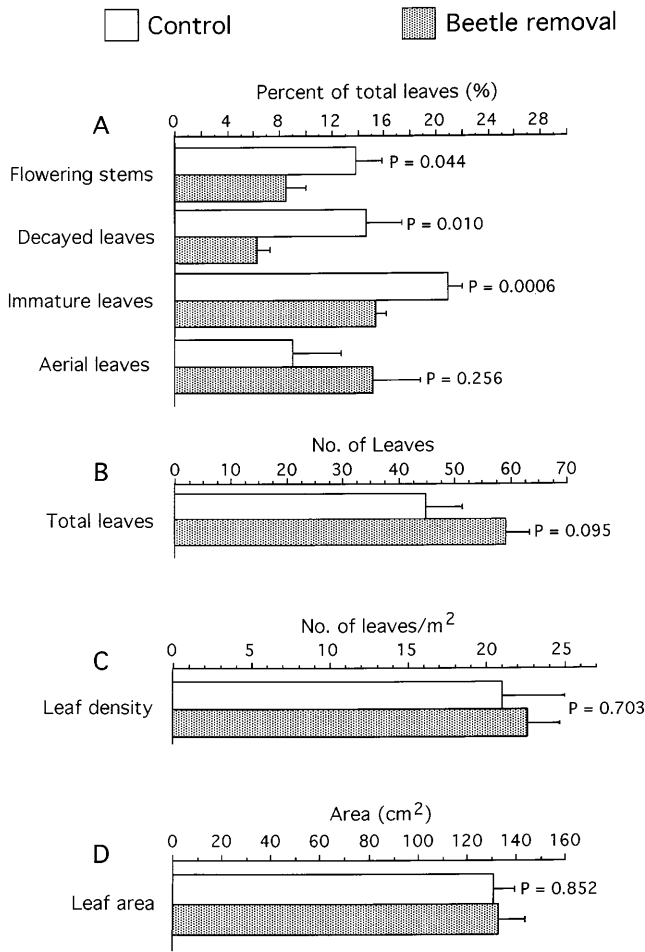


Fig. 2A–D Traits of removal and control *Nuphar* plants after 2 months of experimental manipulation. Two-tailed *P*-values were generated by unpaired *t*-tests. **A** Flowering stems and different categories of leaves (decayed, immature and aerial), expressed as percent of total leaves from a plant. **B** Total number of leaves from a plant. **C** Total leaf density (leaves/m²). **D** Average individual leaf area (cm²)

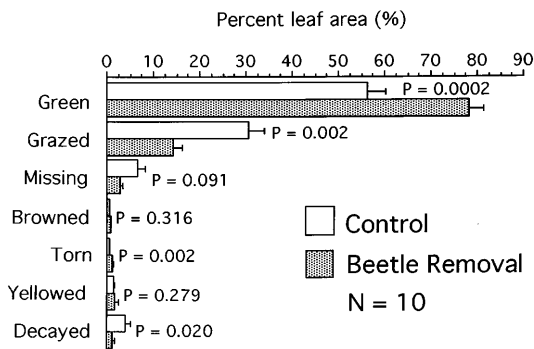


Fig. 3 Differences in damaged leaf area (%) between removal and control *Nuphar* ($n = 10$ plants, with measurements made on ten leaves from each plant). Categories of damage are: *green* (undamaged), *grazed*, *missing*, *browned* (and dry), *torn*, *yellowed*, and *decayed* (and disintegrating). Two-tailed *P*-values were generated by unpaired *t*-tests

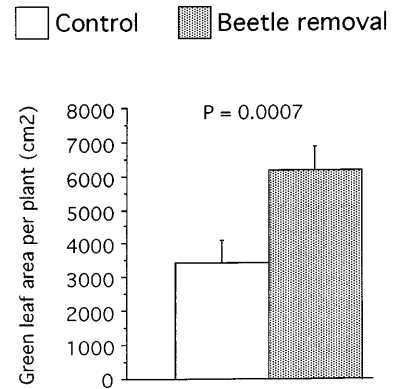


Fig. 4 Total surface area of green leaf tissue per experimental plant, calculated by the equation: mean % green leaf area × mean leaf area (cm²) total number of leaves per plant. Two-tailed *P*-value was generated by an unpaired *t*-test. $n = 10$ for each bar

tendency of removal plants to have more leaves (Fig. 2B), resulted in removal plants having 80% more green, photosynthetic tissue than control plants ($P = 0.0007$, Fig. 4).

Galerucella feeding and ovipositing assays

Galerucella readily ate and oviposited on *Nuphar* in laboratory assays. Of the four assays we performed (immature and intermediate-age leaves offered to both adults and larvae), only the adult assay comparing immature leaves from control and removal plants showed a statistically significant difference in feeding (Table 2). Adult beetles consumed almost twice as much leaf area of immature leaves from control as from removal plants, contrary to what we would expect if *Nuphar* defenses against *Galerucella* were being induced by previous herbivory. Beetles did not feed preferentially in assays using intermediate-age leaves, nor did adults oviposit preferentially on control versus removal treatments in any of the assays (Table 2). The high larval feeding on immature, removal tissue (48.9 ± 32.8 mm²) was influenced by one extreme measurement; without this replicate, the mean consumption of control versus treatment was very similar.

Crayfish feeding assays

When offered a choice between artificial foods made with freeze-dried, powdered leaves from control vs. removal water lilies, crayfish consumed twice as much tissue from the less grazed, removal treatment as from the more heavily grazed control ($P = 0.049$; Fig. 5A). This result is consistent with the hypothesis that *Nuphar* has inducible defenses, but total crude extracts from control versus removal treatments did not differ in their effects on crayfish feeding ($P = 0.478$; Fig. 5A). However, both control and removal *Nuphar* were chemically

Table 2 Feeding and ovipositing assays where adult and larval beetles were offered a choice between *Nuphar* leaves from control plants versus beetle removal plants. Adults were offered 4 × 4 cm,

and larvae 3 × 3 cm squares, of each leaf type. Shown are means ± 1 SE. *P*-values are from paired *t*-tests (except for unpaired *t*-tests for number of eggs/egg mass) and are two-tailed in all cases

Assay	Immature leaves			Intermediate age leaves		
	Grazed	Beetle removal		Grazed	Beetle removal	
Adult feeding (mm ² eaten/beetle)	28.3 ± 3.9	15.2 ± 2.4	<i>P</i> = 0.013 <i>n</i> = 10	20.6 ± 4.8	18.9 ± 4.0	<i>P</i> = 0.828 <i>n</i> = 10
Ovipositing: Number of egg masses/leaf	0.800 ± 0.187	0.900 ± 0.186	<i>P</i> = 0.769 <i>n</i> = 10	0.467 ± 0.099	0.317 ± 0.080	<i>P</i> = 0.244 <i>n</i> = 10
Number of eggs/egg mass	14.3 ± 0.6	14.1 ± 0.6	<i>P</i> = 0.894 <i>n</i> = 40	12.8 ± 0.4	12.9 ± 0.6	<i>P</i> = 0.952 <i>n</i> = 21
Larval feeding (mm ² eaten/larva)	26.9 ± 13.2	48.9 ± 32.8	<i>P</i> = 0.324 <i>n</i> = 9	51.9 ± 13.5	52.5 ± 9.3	<i>P</i> = 0.975 <i>n</i> = 10

defended; extracts from both strongly depressed feeding by crayfish when tested individually against a control lacking *Nuphar* extract (Fig. 5B). Consistent with the previous test (Fig. 5A, right-hand bars), we could detect no significant difference in the magnitude of deterrence exhibited by each of the extracts (*P* = 0.302; unpaired *t*-test on differences in percent eaten between extract foods and solvent controls for control versus removal *Nuphar* extracts; Fig. 5B).

Total phenolic content and plant protein

Leaves from the less grazed, removal treatment had total phenolic concentrations that were 35% higher than concentrations in leaves from the more heavily grazed control (*P* < 0.0001; Table 3). Soluble protein content did not differ between treatments (*P* = 0.934; Table 3).

Discussion

Galerucella had negative effects on water lilies at the scale of whole plants (Table 1, Figs. 2, 4) and individual leaves (Table 1, Fig. 3). Despite these effects, leaves from heavily grazed plants were not less palatable to beetles in any of our feeding assays (Table 2). There is ample evidence that some specialist herbivores, while tolerant of host plant defenses, can still be negatively affected by the secondary metabolites of their hosts (Zangerl and Berenbaum 1993; Osier et al. 1996) and can be deterred by induced increases in host chemical defense following attack (Karban 1993). However, in our study, high levels of attack resulted in plants becoming more palatable, or not changing in palatability, to the beetles (Table 2), while becoming less palatable to the generalist crayfish (Fig. 5A). Thus, beetle grazing may induce increased defenses against crayfish, but we found no evidence that it induces traits that deter beetle feeding.

We infer a chemically mediated response of crayfish because our food preparation procedure removed structural or morphological differences between tissues.

However, the precise mechanisms driving the difference in palatability between more and less grazed *Nuphar* tissue remain unclear. Crude chemical extracts from the two treatments did not differentially affect crayfish feeding (Fig. 5A), although both extracts did deter crayfish feeding relative to foods without extracts (Fig. 5B). No difference in nutritional value, as indicated by protein content, was detected, and total phenolics were higher in removal than in control *Nuphar* (Table 3). Phenolics were, thus, positively related to crayfish consumption, which is inconsistent with their hypothesized role as antiherbivore defenses. Furthermore, extensive efforts to separate, purify, and identify deterrent elements in *Nuphar* extracts have indicated that *Nuphar* possesses chemical feeding deterrents, but that these degrade and lose their deterrence against crayfish following extraction and exposure to various separation techniques (G. Cronin, personal communication). It is possible that subtle, but important, chemical differences between extracts from treatment versus control plants produced the selective feeding seen in Fig. 5A but were removed by our extraction or bioassay procedures.

There are several possible arguments why we would expect water lily leaf beetles to be unaffected or stimulated by inducible defenses of *Nuphar*. First, the fact that beetles specialize on *Nuphar* suggests that they may have circumvented the antiherbivore defenses of their host. Early formalizations of plant defense theory argue that inducible responses that occur within days or weeks of damage should deter generalist consumers only (Rhoades 1979). The evidence for this hypothesis is somewhat mixed; however, in several studies, specialist insect herbivores perform as well or better on regrowth foliage from previously damaged plants as on foliage from undamaged plants (Crawley and Nachapong 1984; Pullin 1987), and there are numerous examples of plants becoming more susceptible to specialist grazers following attack (summarized in Karban and Baldwin 1997). Second, for some specialist herbivores that sequester plant chemical defenses to deter their own enemies, use of host plants may increase if plants increase production of chemical defenses (Smiley et al. 1985). *Galerucella* are reported to be unpalatable to several predators (Juliano

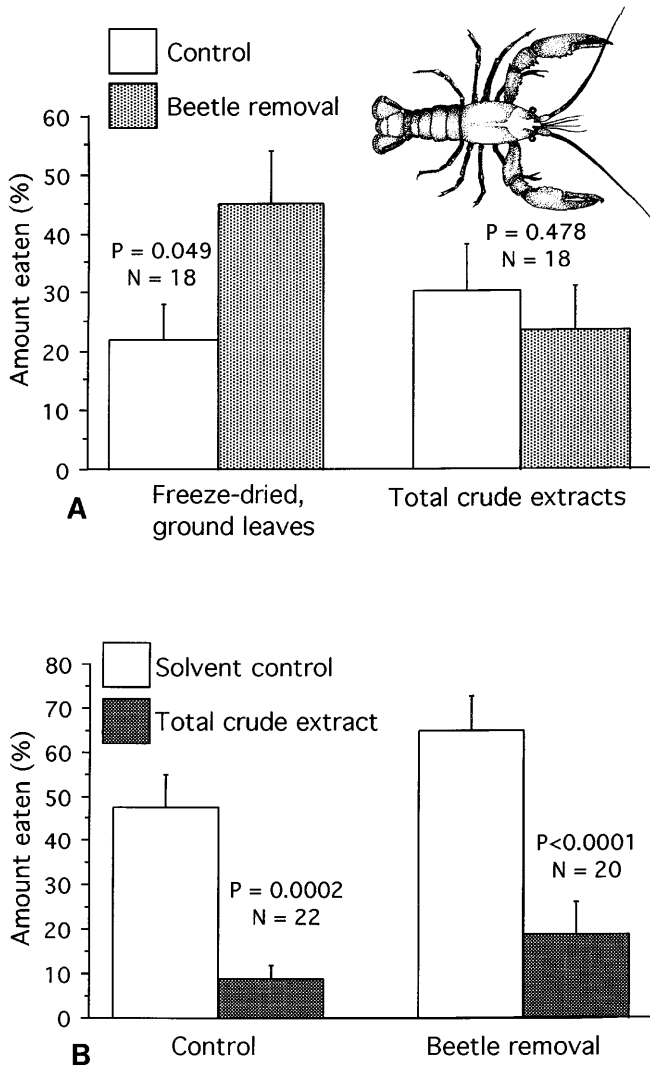


Fig. 5A,B Effects of chemical extracts from experimental *Nuphar* on a generalist crayfish. Two-tailed *P*-values were generated by paired *t*-tests. **A** Feeding by *Procambarus clarkii* on (1) artificial foods (freeze-dried, finely ground plant tissue in an alginate matrix) made with control or removal *Nuphar* leaves, and (2) palatable, artificial foods (1:1 lettuce:broccoli) treated with total crude (lipophilic and water-soluble) extracts from control or removal *Nuphar* leaves. **B** Feeding by *P. clarkii* on (1) artificial foods treated with either total crude extract from control *Nuphar* or with solvent only (= solvent control), and (2) artificial foods treated with either total crude extract from removal *Nuphar* or solvent only

1988; Otto and Wallace 1989), and some authors have suggested that the beetles sequester noxious metabolites from *Nuphar* (Otto and Wallace 1989). This untested hypothesis should be investigated; it is consistent with our finding that beetles were undeterred by the responses of waterlilies to intense herbivory. Third, it is commonly believed that inducible defenses should be much more effective against mobile consumers than sedentary herbivores that have no choice but to remain on their host plant (Karban and Baldwin 1997). Water lily leaf beetles are relatively sedentary herbivores; adults do fly between neighboring plants, but typically only for several seconds at a time (R. Bolser, personal observation). Also, we have visited several locations in the region of our study site where *Nuphar* is common yet *Galerucella* is conspicuously absent, suggesting that the beetles' dispersal ability may be limited at these larger scales.

At the opposite extreme, crayfish are relatively mobile, generalist consumers, and our study suggests *P. clarkii* is more sensitive than *Galerucella* to induced responses in *Nuphar* (Fig. 5A). The high nutritional value of *Nuphar* (10% protein; Table 3) suggests that it might be a preferred food for generalist herbivores if it did not possess strong chemical deterrents to feeding (Fig. 5B). Although these defenses may keep crayfish from being major consumers of water lilies, even minor feeding could negatively impact plants because, through sloppy feeding, crayfish often cause much more plant tissue to be lost than they actually consume (Lodge and Lorman 1987). In mesocosm studies, crayfish have been shown to reduce the biomass of *Nuphar* and other macrophytes (Chambers et al. 1990). It is thus conceivable that chemical deterrents in *Nuphar* are aimed at generalist consumers like crayfish, and that it is more important to deter these types of consumers than to deter specialist water lily leaf beetles. In aquatic systems, it is common for generalist herbivores to drive plants to local extinction; generalists may therefore be strong agents in selecting for plant chemical defenses (Hay and Steinberg 1992).

The pattern of results we found for new and mature leaves is similar to several other studies comparing the responses of both specialist and generalist herbivores to previously damaged plants. Center and Wright (1991) argued that preference of a specialist weevil for young leaves of water hyacinth was stimulated by changes in plant chemistry following damage, but that these changes deterred feeding by a generalist caterpillar. Rausher et al. (1993) found that generalist herbivores and one of

Table 3 Analysis of soluble protein and total phenolic content of freeze-dried, finely ground tissue (pooled subsamples) from control vs. removal *Nuphar* rosettes. Values are means \pm 1 SE. Two-tailed

P-values were generated by unpaired *t*-tests. *BSA* bovine serum albumin, *TA* tannic acid

	Grazed	Beetle removal	
Soluble protein (% dry mass, BSA equivalents)	10.4 \pm 0.5 <i>n</i> = 3	10.3 \pm 1.1 <i>n</i> = 3	<i>P</i> = 0.934
Total phenolics (% dry mass, TA equivalents)	16.0 \pm 0.4 <i>n</i> = 6	21.6 \pm 0.4 <i>n</i> = 6	<i>P</i> < 0.0001

two specialists were deterred by induced responses in older leaves, but stimulated by changes in newly produced leaves. We also showed that a specialist herbivore preferentially consumed more young tissue from plants with heavy prior damage, although mature leaves from the same plants were not grazed selectively (Table 2). Because the crayfish assay used artificial foods made with mostly older leaves, crayfish avoided mature plant tissue from more heavily grazed *Nuphar* (Fig. 5A). A comparable assay using only tissue from immature leaves was not performed with crayfish due to limited availability of this type of leaf; therefore, we cannot assess the response of the generalist to newly produced leaves. Rausher et al. (1993) argued that if more defenses are mobilized to leaves experiencing heavier damage, fewer resources may be left for resistance in subsequently produced leaves of the same plant. A possible explanation of our results is that control plants allocated more defenses to intermediate-age leaves, which affected the feeding choice of the generalist but not the specialist, and that this reallocation caused a concomitant decline in the defenses of newly produced, control leaves compared to new leaves of removal plants.

Our results do not clearly argue that induced defenses against water lily leaf beetle never occur in *Nuphar*. The defensive response could be triggered at lower levels of beetle grazing than occurred in our study. Because some adult beetles recolonized *Nuphar* plants during the 2-day intervals between removals (Fig. 1), our removal plants still experienced herbivory. Beetle grazing on our removal treatment was about 50% of that experienced by controls, but the leaf area grazed was still $14.2 \pm 2.1\%$ (mean \pm 1 SE) of total leaf area; (Fig. 3). This level of damage is comparable to reported natural herbivory on *Nuphar* in Georgia, USA (13.2% leaf area; Wallace and O'Hop 1985) and in Finland (17% leaf area; Kouki 1991b). These levels of beetle herbivory cause leaves to decompose and sink significantly faster than ungrazed leaves, presumably because feeding trenches are quickly colonized by bacteria and disintegrate (Wallace and O'Hop 1985). Thus, both our removal and control plants might have exhibited induced defenses. However, the differences in palatability and chemical composition we noted between the heavily grazed control and less grazed, removal treatment (Tables 2, 3, Fig. 5) indicate that plants in the two treatments did differ in some traits to which beetles or crayfish responded. Furthermore, induced responses in general may not be "on-off" phenomena triggered by some threshold level of damage; evidence from many terrestrial studies suggests that both rapid and longer-term induced resistance become stronger as damage levels are increased (see Table 4.5 in Karban and Baldwin 1997).

In conclusion, we found no evidence that water lilies experiencing higher levels of beetle grazing induced defenses that deterred subsequent grazing by the water lily leaf beetle. However, crayfish did avoid feeding on *Nuphar* tissue that had been previously attacked by high densities of beetles. These patterns suggest that *Nuphar*

may be changing in response to beetle attack; however, feeding by the specialist beetles is unaffected, or stimulated, by the changes while feeding by the generalist crayfish appears to be diminished. Levels of herbivory measured in this study are among the highest reported for the *Nuphar-Galerucella* system, and for freshwater macrophyte-insect interactions in general. Knowledge of the dynamics of such systems is important not only to the scientific ecology community, but also to practitioners of classical biological control of nuisance aquatic plants (Center and Van 1989; Center and Wright 1991). Growing evidence for significant herbivore damage occurring on natural populations of aquatic macrophytes (Lodge 1991; Newman 1991; Jacobsen and Sand-Jensen 1992, Lodge et al. 1998) and for the occurrence of plant chemical defenses in freshwater habitats (Newman et al. 1992, 1996; Cronin 1998, Bolser et al. in press; Fig. 5B) indicates the need for more studies on plant-herbivore interactions in aquatic systems for determining the role that chemical defenses may play in mediating these interactions.

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