AQUATIC PLANT-HERBIVORE INTERACTIONS ACROSS
MULTIPLE SPATIAL SCALES

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AQUATIC PLANT-HERBIVORE INTERACTIONS ACROSS MULTIPLE SPATIAL SCALES.

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td></td>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td>SUMMARY</td>
<td>ix</td>
</tr>
<tr>
<td></td>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Induced chemical defenses in a freshwater macrophyte suppress herbivore fitness and the growth of associated microbes.</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.1 Abstract</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.2 Introduction</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.3 Methods</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.4 Results</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1.5 Discussion</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1.6 References</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>Feeding and growth rates among native and non-native apple snails (Ampullaridae) in the U.S.</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>2.1 Abstract</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>2.2 Introduction</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2.3 Methods</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>2.4 Results</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>2.5 Discussion</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>2.6 References</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>Latitudinal patterns in the palatability of freshwater macrophytes.</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>3.1 Abstract</td>
<td>58</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>3.3 Methods</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>3.4 Results</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>3.6 References</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>4. Herbivores prefer plants that are evolutionarily naïve: Herbivore phylogeography and preference for native vs exotic plants.</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>4.1 Abstract</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>4.3 Methods</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>4.4 Results</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>4.5 Discussion</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>4.6 References</td>
<td>102</td>
<td></td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Collection sites for fresh and freeze-dried data-sets</td>
<td>63</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Consumption of $N$ vs $S$ fresh plant species ($N=8$)</td>
<td>69</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Consumption of $N$ vs $S$ ground plants ($N=8$)</td>
<td>72</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>Consumption of $N$ vs $S$ ground plants (larger dataset; $N=22$)</td>
<td>74</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>Grand means comparing consumption of $N$ vs $S$ plants</td>
<td>76</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Plants utilized for comparisons of native and exotic confamilial pairs</td>
<td>91</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Plant preferences in (A) <em>P. canaliculata</em> and (B) <em>P. clarkii</em></td>
<td>14</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Consumption of induced and non-induced <em>Cabomba</em> (reciprocal test)</td>
<td>15</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Consumption of foods treated with extracts from induced versus non-induced plants</td>
<td>16</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Consumption of control and treatment <em>Cabomba</em> (waterborne cues)</td>
<td>17</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Juvenile snail growth when fed induced versus non-induced <em>Cabomba</em></td>
<td>19</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Percent inhibition of microbes grown with extracts from induced and non-induced plants</td>
<td>20</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Mean consumption of plants by snails during the choice assays</td>
<td>39</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Visual estimates of snail consumption across time</td>
<td>41</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Total grams of plant consumed during the choice assays</td>
<td>42</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Consumption during the no-choice assays (comparisons between plants)</td>
<td>43</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>Consumption during the no-choice assays (comparisons between snails)</td>
<td>44</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Percent growth in snail mass (comparisons between plants)</td>
<td>45</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Percent growth in snail mass (comparisons between snails)</td>
<td>47</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>Growth efficiency of snails</td>
<td>48</td>
</tr>
<tr>
<td>Figure 2.9</td>
<td>Snail mortality during the one-month feeding experiment</td>
<td>50</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Consumption of N vs S fresh macrophytes (N=8)</td>
<td>68</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Consumption of N vs S freeze dried and ground macrophytes (N=8)</td>
<td>71</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Consumption of N vs S freeze dried and ground macrophytes (N=22)</td>
<td>73</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Consumption of native vs exotic macrophytes</td>
<td>95</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Correlations in plant preferences between herbivore species</td>
<td>96</td>
</tr>
</tbody>
</table>
### LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_i$</td>
<td>Initial weight of control plant</td>
</tr>
<tr>
<td>$C_f$</td>
<td>Final weight of control plant</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>$T_i$</td>
<td>Initial weight of plant exposed to consumer</td>
</tr>
</tbody>
</table>
SUMMARY

For decades scientists believed that herbivory had minimal impact on freshwater ecosystems. We now know that herbivory in freshwater systems equals or exceeds herbivory in terrestrial and marine systems. In extreme cases, herbivores can change clear, macrophyte dominated ecosystems into turbid plankton dominated ecosystems. Even though research on plant-herbivore interactions in freshwater systems has increased, there is still much that is unknown. This thesis is comprised of four studies investigating freshwater plant-herbivore interactions across multiple spatial scales. The first study investigated how induced chemical defenses in *Cabomba caroliniana* suppress herbivore consumption and growth as well as how this herbivore-generated change in plant chemistry affects the growth of plant associated microbes. At the spatial scale of individual ponds or lakes, consumers that induce their host plants may also be indirectly affecting other consumers and microbial pathogens via changes in this shared resource.

The second study moves to an ecosystem scale and investigates how exotic versus native apple snails may impact Everglades’ habitats. We investigated plant preference, consumption, growth and conversion efficiencies in the singly native apple snail to occur in the U.S. (*Pomacea paludosa*) versus four introduced species (*P. canaliculata, P. insularum, P. haustrum* and *P. diffusa*). We found that even though plant preferences are similar, invasive snails tend to eat more, grow more rapidly, and sometimes more efficiently than natives. This suggests that invasive species could have a large impact on the environment, especially the abundance of submerged plants.
The third study investigated how palatability of freshwater plants varies with latitude (i.e. geographic scale). Increased herbivory at lower latitudes is hypothesized to select for increased plant defenses, which has been shown to be true for tropical forests, salt marshes, and seaweeds. When we contrasted eight confamilial plants collected in Indiana versus Southern Florida, three of four herbivores significantly preferred northern plants. When we evaluated a second set of plants collected from Indiana versus Central Florida, only one of three herbivores preferred the northern plants. Overall, our results suggest a preference for northern plants, but the strength of this relationship was variable. We hypothesize that this variability may be driven by 1) local variance in herbivore pressure that creates variance in plant defenses, and/or 2) the effect of winter length on the survival and feeding rate of herbivores.

The final study expanded to a world scale, and investigated herbivore preference for native vs exotic plants. We found that both N. American crayfish and S. American snails preferred exotic plants over confamilial natives, despite responding to different plant characteristics. The single species of apple snail that occurs in N. American showed no preference for native or exotic plants from a N. American perspective, but instead exhibited preferences that correlated with its history of evolution in S. America. As the N. American species is a sister species of the S. American snails, feeding by the N. American snail appears more affected by its S. American lineage than its recent history in N. America. This suggests that phylogenetic legacy will affect choices of the herbivore as well as resistance or susceptibility of plants.
CHAPTER 1

INDUCED CHEMICAL DEFENSES IN A FRESHWATER MACROPHYTE SUPPRESS HERBIVORE FITNESS AND THE GROWTH OF ASSOCIATED MICROBES

1.1 Abstract

The freshwater macrophyte *Cabomba caroliniana* induces a chemical defense when attacked by either the crayfish *Procambrus clarkii* or the snail *Pomacea canaliculata*. Induction by either consumer lowers the palatability of the plant to both consumers. When offered food *ad libitum*, snails feeding on non-induced *Cabomba* grew 2.6 - 2.7 times more than those feeding on induced *Cabomba*. Because snails fed less on induced plants, this could be a behavioral effect (reduced feeding), a physiological effect of the induced metabolites on the consumer, or both. To assess these possibilities, we made artificial diets with lipid extracts of induced vs non-induced *Cabomba* and restricted control snails to consuming only as much as treatment snails consumed. Growth measured as shell diameter was significantly lower on the diet containing extract from induced, as opposed to non-induced, plants; change in snail mass showed a similar, but non-significant, trend. Thus, snails reduce feeding on induced plants because these plants suppress consumer fitness. The induced defenses also suppressed growth of co-occurring microbes. When two bacteria and three fungi isolated from *Cabomba* surfaces were cultured with the lipid extract from induced and non-induced *Cabomba*, both extracts inhibited the microbes, but the induced extract was more potent against three of the five potential pathogens. Thus, induced plant defenses can act against both
consumers and microbes whose access to the plant may be facilitated by herbivore damage.

1.2 Introduction

For decades there was consensus that herbivory had minimal impact on freshwater macrophytes (Shelford 1918; Hutchinson 1975; Lamberti and Moore 1984). However, levels of herbivory in freshwater systems equal or exceed those in terrestrial and marine systems (Cyr and Pace 1993; Lodge et al. 1998), and numerous recent studies show substantial impacts of herbivores on freshwater macrophyte biomass (Søndergaard et al. 1996; Lodge et al. 1998; Chambers et al. 1990) and species composition (Sheldon 1987; VanDonk 1998; Gross et al. 2001; see also the early reviews of Lodge 1991; Newman 1991). Herbivores have even been implicated in changing clear macrophyte dominated ecosystems into turbid plankton dominated ecosystems (Søndergaard et al. 1996; Carlsson et al. 2004). Recognition of herbivory as a strong selective agent in aquatic systems suggests the need to better understand the dynamics of chemical defenses in aquatic macrophytes and the impacts of these defenses on consumers.

Induced defenses are well studied among terrestrial plants (Karban and Baldwin 1997), have recently been documented for several marine seaweeds and phytoplankton (Long et al. 2007b; Toth and Pavia 2007), but are poorly investigated among freshwater macrophytes and their consumers. To date, 22 species of freshwater macrophytes have been demonstrated to produce chemical defenses against herbivores (Newman et al. 1996; Cronin 1998; Bolser and Hay 1998; Bolser et al. 1998; Kubanek et al. 2001; Cronin et al. 2002; Prusak et al. 2005; Parker et al. 2006, 2007; Miller and Provenza 2007; Erhard 2007), three species have been shown to induce either structural defenses
(Center and Van 1989; Kouki 1993) or an unidentified defense (Jeffries 1990), but only one species is known to induce a chemical defense (Bolser and Hay 1998). The chemicals responsible for this induced defense were not identified and the physiological effects on herbivores were not studied (Bolser and Hay 1998). This contrasts with terrestrial systems where more than 100 species induce defenses, with many of these compounds identified and some effects on herbivores demonstrated (Karban and Baldwin 1997). Because freshwater macrophytes reinvas ed aquatic environments from terrestrial systems (Cook 1990), induced chemical defenses might be expected among aquatic macrophytes; however, neither their occurrence nor consequences are well studied.

Herbivore induction of plant defenses can impact not only plant-herbivore interactions, but also interactions among herbivores via their shared plant resource (Agrawal 2005, Long et al. 2007, Toth and Pavia 2007, Kessler and Halitschke 2007). Not all herbivores induce plant defenses and not all induced defenses are equally effective against different herbivores (Van Zandt and Agrawal 2004); this creates considerable potential for indirect effects among herbivores mediated by induction of their shared plant resources (Long et al. 2007). As possible examples, small herbivores tend to induce defenses in marine algae more commonly than do larger herbivores (Hay 1996, Toth and Pavia 2007), even though the induced defenses may strongly affect some of the large herbivores. Even consumers within the same guild can differentially affect induction: the snail Littorina obtusada induces a defense in the brown seaweed Fucus while Littorina littorea does not and this differentially affects Fucus use by the two herbivores (Long et al 2007). Similarly, induction of milkweed impacts the growth of an herbivorous butterfly but not an herbivorous beetle (Van Zandt and Agrawal 2004).
Alternatively, multiple examples exist for cross-induction - where different herbivores induce similar plant responses and these responses have similar impacts on multiple herbivores (Kessler and Halitschke 2007). It was initially hypothesized that generalist consumers more commonly caused induced plant defenses than did specialists who had co-evolved with the plant, but this pattern does not appear to hold (Agrawal 2000). More research is needed to clarify why plants have evolved such variable responses to different herbivores, and why herbivores are so differentially affected by these induced defenses.

Induced chemical defenses can impact both herbivores and plant pathogens (Krischick et al. 1991), and some cases of induction following herbivore attack may, in fact, be induction in response to microbial pathogens transmitted by herbivores (Raffa and Smalley 1995). The relative roles of direct herbivory vs microbes that invade plants via herbivore bites is unclear; the plant could be responding directly to herbivory (i.e., loss of plant matter), to open wounds caused by herbivory, or to chemicals or pathogens present in the saliva of the herbivore (Raffia and Smalley 1995; Coleman et al. 2007).

Here, we investigated the induced chemical response of the freshwater macrophyte *Cabomba caroliniana* to two generalist consumers and the effects of this induction on one of the herbivores and on microbes associated with the plant being attacked. Specific objectives included:

1. Documenting whether feeding by the snail *Pomacea canaliculata* and the crayfish *Procambarus clarkii* induced chemical defenses in *Cabomba*.

2. Resolving whether induction was specific to the herbivore attacking the plant or whether induction due to attack by one herbivore also affected feeding by the other.
3. Determining whether induction occurred in response to direct grazing or also to water-borne cues from attacks on neighboring conspecifics.
4. Attempting to identify the chemicals induced.
5. Exploring the impact of the induced response on herbivore growth.
6. Investigating whether metabolites induced in response to herbivores might also suppress co-occurring microbes that might function as plant pathogens.

1.3 Methods

1.3.1 Collections

_Cabomba caroliniana_ is a submerged macrophyte native to sub-tropic-temperate regions of eastern North and South America; it occurs in ponds, lakes, and quiet streams (Godfrey and Wooten 1979). It is clonal, with runners producing upright ramets. Growth can be prolific, and reproduction is both asexual via fragmentation and sexual via emergent flowers. _Cabomba_ is a “problem weed” in parts of the USA, Canada, Greece, Japan, China, and Australia due to its fast growth and displacement of native species (Schooler et al. 2006). _Cabomba_ was collected from Sibley Pond, Georgia (N 33.9365 and W -84.4409).

Apple snails (_Pomacea canaliculata_) were collected in Florida during winter 2006 by Neighborhood Fish Farm, Miami, Florida and mailed overnight to the Georgia Institute of Technology. Snails were maintained in 38 L tanks and fed leaf lettuce 2-3 times per week. Adults laid eggs; juveniles from these eggs were grown on lettuce and algal pellets until used in experiments.
Crayfish (*Procambarus clarkii*) were acquired from Carolina Biological Supply in summer 2007 and raised in the lab. Females with zygotes were isolated; when their offspring hatched, the juveniles were raised in tanks on commercial herbivore food until needed for experiments.

### 1.3.2 Herbivore preference:

Crayfish and snails were offered small pieces of ten species of local freshwater macrophytes (*Alternanthera philoxeroides, Cabomba caroliniana, Egeria densa, Hydrocotyle sp., Ludwigia sp., Myriophyllum aquaticum, Myriophyllum sp., Nymphaea odorata, Pontederia cordata, and Sagittaria sp.*) simultaneously in 739 ml containers and allowed to feed for up to four days. All plants were collected from ponds and rivers in Atlanta on the same day. To standardize herbivore encounter across species, we adjusted plant mass to assure approximately equivalent 2-D surface area of each plant species in each container. Assays were divided into 10 blocks, where each block included one replicate with snails, one with crayfish, and one control (no herbivore included) to assess changes in plant mass unrelated to consumption (Roa 1992; Stachowitz and Hay 1996). All pieces within each block were cut from the same plant when possible, and no individual plant was used in more than one block. Grazing in individual replicates was stopped (herbivore removed) when approximately half of the plants had been consumed. At the end of four days, all remaining herbivores were removed from the assays and all plants weighed. Initial plant weights were corrected for autogenic changes using the formula: \( T_i \times (C_f/C_i) \), where \( T_i \) was the initial weight of plants exposed to consumers and \( C_i \) and \( C_f \) were the initial and final weights of the plants from the matching controls (Stachowitz and Hay 1996).
Choice assays were analyzed using a modified Friedman’s Test because consumption of each plant was not independent of consumption of the other plants available in the assay (Conover and Iman 1981, Roa 1992). Friedman’s test requires dependent variables (i.e. consumption) to be ranked within each replicate; however, instead of running the comparison with a chi-square table (the usual Friedman’s statistic, Conover 1999) we instead utilized two-way analysis of variance (with replicate and treatment as the independent variables) as this is more reliable with larger matrixes (Conover and Iman 1981). Paired comparisons were completed on the rank data using Tukey’s pairwise comparisons. Induction: Cabomba plants were grown and induced in laboratory tanks. Thirty-eight L tanks were filled with 4.5 liters of 3:2 mix of commercial topsoil and sand, then topped with 1.5 liters of river pebbles. Filtered tap water was added to the tanks and allowed to age for 4-5 days before the plants were added. Each tank was lighted with daylight deluxe and coral grow fluorescent lights set on a 12:12 light: dark cycle. 34-54 g of Cabomba were patted dry to remove water weight and planted evenly spaced into the tanks. Plants were allowed to adjust to the new conditions for 1-2 months before induction experiments started. Tanks were randomly assigned to treatments, utilizing a modified block across each group of tanks that shared the same shelf and fluorescent lights.

Snails with shell widths of 1-2 cm or crayfish with carapace length of 1.0-2.4 cm were added to one tank in each block to generate snail-grazed and crayfish-grazed plants. Herbivores were allowed to feed for 12 days and were removed when approximately 25 percent of the plant material appeared to have been consumed. Plants were allowed to recover for five to nine days, and then live plants from induced and control tanks were
compared in paired assays to confirm occurrence of induction (see below). Within three
to five days of the induction trials, plants were either utilized in live plant experiments, or
harvested and frozen at -51°C for future analyses.

To determine if plants induced via water-borne cues from attacked conspecifics, 946 ml plastic containers containing three medium sized snails (shell width 1-2 cm) feeding *ad libitum* on fragments of *Cabomba* plants were placed within tanks containing planted *Cabomba*. All containers had 16 holes punched in the bottom and sides to allow water exchange between plants being consumed and those not contacted by consumers. In addition, water was drained from these containers into the tanks twice a week to ensure greater mixing. Ten tanks were randomly assigned to either the treatment (containers with snails and *Cabomba*) or control (equivalent empty containers). After three weeks, plants from the control and treatment tanks were paired and offered to herbivores.

### 1.3.3 Extractions

To test for induction of chemical deterrents, attacked and control *Cabomba* were freeze dried and extracted 3-4 times for 1-2h each time in a 2:1 mixture of dichloromethane: methanol. Solvents were filtered from the plant material and the plant’s extract concentrated in a rotary evaporator. Extracts were stored dry in the freezer until needed.

We conducted bioassay guided fractionation in an attempt to identify the chemical(s) being induced. The crude extract from induced *Cabomba* was divided into 4 partitions using a modified Kupchan scheme of liquid-liquid partitions (Kupchan et al. 1975) producing hexane-, chloroform-, ethyl acetate- and water-soluble fractions. All fractions were tested to determine their influence on herbivore feeding (see below).
Because only the ethyl acetate partition deterred feeding, it was partitioned into 17 fractions via a reverse-phase Supelco C18 column. Thin layer chromatography was used to recombine these into four partitions with minimal metabolite overlap (i.e. fractions with similar characteristics were combined). Effects of these partitions on snail feeding were assessed and only the least polar partition was active. This was further divided using isocratic high performance liquid chromatography (HPLC). The HPLC revealed five distinct peaks plus an irregular baseline. Bioassays showed none of the peaks to be active either individually nor combined, but the baseline was active. Therefore the baseline was collected and partitioned on a gradient HPLC. Approximately ten peaks were collected and combined into 6 fractions, which were tested first individually and then recombined together to evaluate effects on snail feeding.

1.3.4 Bioassays

Live pieces of induced and non-induced *Cabomba* were matched by appearance and weight, attached to plastic clips labeled as either control or induced, and offered to herbivores in either 946 ml (*P. canaliculata*) or 1140 ml (*P. clarkii*) containers for 1-3 days. Assays were ended when approximately 50% of one of the plant pieces was consumed. Autogenic changes in plant mass were measured by placing weighed pieces of control and induced *Cabomba* into containers without herbivores, and the weight of the plants measured at the end of the experiment to correct for changes in weight unrelated to herbivory. Plant start weights were corrected in the same manner as in the preference experiment described above. Paired T-tests evaluated feeding on induced vs non-induced plants. Each source tank was considered a single replicate in these assays; if plants from the same tank were offered to more than one herbivore, then these were
considered sub-samples of the same replicate and were pooled to generate one mean for that tank replicate. This avoids confounding tank-effects with treatment-effects and prevents pseudoreplication (Hurlburt 1984).

Assays with artificial food tested the effects of *Cabomba* extract on feeding. All plants from induced tanks were combined and extracted, as were all plants from control tanks. Extracts were dissolved in methanol, added to freeze dried and ground green leaf lettuce, the solvent removed via rotary evaporation, and this extract-treated food imbedded in an agar gel to make a artificial plants containing the extract from *Cabomba* (see Hay et al. 1994). The agar food was created so as to match the dry mass per volume of the live plant (0.07 ± 0.02 g/ml). The recipe involved mixing 4.7 ml of deionized water with 1 g of ground plant matter, then mixing this with 0.66 g of agar in 9.2 ml of deionized water that had been heated to boiling. Agar and plant mixtures were combined and quickly spread into a fiberglass mold overlaying a base of window screen. This created two parallel, thin strips of artificial food with the same dimensions (see Hay et al. 1998 for an illustration). The mold was removed, and the screen was cut into strips so that each snail could be offered the same amount of control and treatment foods. A small notch was cut at the corner of the screen at one end to mark the food as either treatment or control. Amount eaten was quantified by counting the number of screen squares from which food had been removed. Experiments were conducted until half of either of the foods had been consumed. Control food differed depending on the extract being tested. When testing the whole induced extract compared to non-induced extract, the treatment contained extract from induced plants and the control contained extract from non-induced
plants. When testing extract fractions, the control was ground lettuce treated with only the solvent used as carrier for the test fraction.

1.3.5 Effects on herbivore growth

To determine how induced defenses in *Cabomba* influenced herbivore consumption and growth, we fed 20 pairs of snails *ad-libitum* on either induced or non-induced *Cabomba*. We did not test crayfish in this assay due to their sloppy feeding and the difficulty in determining what had been consumed versus shredded and discarded. Forty small snails were measured (max width of shell) and weighed (after patting dry) and randomly assigned to either treatment or control groups. Snails were kept in 550 ml containers with screening on 3 sides to allow water movement between the containers, which were held in a 0.91x1.83 m flow through water table. Snails were fed *ad-libitum* for 1 month. Tanks with *Cabomba* were kept in an induced state by keeping a low number of snails feeding on the plants at all times. New pieces of *Cabomba* were provided to experimental snails every three days, and harvesting completed on only one to two tanks at a time to limit the influence of harvesting on plant chemistry (i.e. control tanks were each harvested once over the one month period to limit any impact of cutting on induced defenses). Snail growth was measured as both a change in mass and shell width. All measurements were completed blind to prevent bias.

To determine if the induced extract had negative impacts on snail growth when consumption was held constant, we fed snails identical diets treated with extracts from induced and non-induced *Cabomba* and limited control snails to consuming only the amount of food consumed by treatment snails. Forty small snails were measured, weighed and paired according to size, with one member of each pair randomly assigned.
to the control and the other to the treatment group. A known mass of food treated with induced extract was offered to the treatment snails. After 3 days, the food was removed, re-weighed, and the amount eaten calculated; that same mass of food treated with extract from non-induced plants was offered to control snails. This was repeated every 3 days for 30 days with both groups of snails – thus producing pairs of treatment and control snails that had consumed the same mass of agar-based food. At the end of the month, measurements of width and mass were completed blind (i.e. the researcher did not know which were treatment or control snails), and growth was calculated as percent change. Mortality of snails reduced the sample size from 20 to 18. Because our hypothesis was that induced *Cabomba* would reduce snail growth, data were analyzed using a one-tailed T-test.

### 1.3.6 Effects on microbes

Bacteria and fungi naturally associated with *Cabomba* were collected by placing 0.5-1.0 cm long pieces of live *Cabomba* from Sibley Pond onto sterile plates created by mixing YPM media, agar, penicillin, and streptomycin. It was assumed that the bacteria and fungi already growing on *Cabomba* were those that might be most resistant to its defenses and thus potential pathogens. Small pieces of agar were cut from the center of each growing microbe colony after 24 hours and placed onto a new plate to isolate individual species. This was repeated another 2-3 times to acquire mono-specific cultures. Cultures were placed into a YPM, glycerin mixture and frozen at -15°C. Samples were defrosted after nine months, placed into 10 ml YPM liquid media and allowed to grow. Eliza bioassays quantified pathogen growth in the presence of extracts from induced and non-induced *Cabomba*. We completed these assays for 2 bacterial and
3 fungal isolates that grew well under our culture conditions. To run the bioassays, 200 ul of dense pathogen cultures were added to 12 ml of autoclaved YPM media and vortexted for 10 s to ensure proper mixing. 100 ul of this microbe-containing broth was added to each cell on a 96 well plate. Test extracts dissolved in DMSO were added to treatment wells at concentrations of 1x, 0.5x, 0.25x, 0.12x, 0.06x, and 0.03x the natural yield from our extraction. Growth was compared to controls receiving only the DMSO carrier solvent. Turbidity readings were taken at the onset of the experiment and again after 64 h to assess microbe growth. Percent inhibition was calculated by comparing the change in turbidity between the various extract concentrations and the DMSO negative controls. Comparisons between induced and non-induced extracts were completed with t-tests corrected for multiple comparisons (P = 0.008) within each pathogen.

1.4 Results

When offered fresh plants collected from the field, *Pomacea canaliculata* and *Procambarus clarkii* varied considerably in feeding preferences (Figure 1.1), but *Cabomba* ranked among the top food choices for both consumers. However, *Cabomba* became significantly less palatable to both herbivores when either herbivore had previously fed on the plant; feeding on induced plants was reduced by 71-83% compared to feeding on non-induced plants (P≤0.01 for all contrasts; Figure 1.2). The induced defense was chemical; when artificial diets were treated with extracts from induced and non-induced *Cabomba* (i.e. retaining only the chemical traits), feeding on the food treated with extract from induced plants was reduced by 64-83% compared to feeding on the food treated with extract from non-induced plants (P<0.001; Figure 1.3). Induction
Figure 1.1. Plant preferences in (A) the snail *Pomacea canaliculata* and (B) the crayfish *Procambarus clarkii* when simultaneously offered ten species of macrophytes. Error bars represent SE. Letters above bars denote significant groupings by Tukey’s multiple comparison on consumption ranked within each replicate (Friedman’s test). *Procambarus* drawing is from Huxley (1879).
Figure 1.2. Consumption of induced and non-induced *Cabomba* plants eaten in paired assays using live plants. (A) Induced by snails and fed to snails. (B) Induced by crayfish and fed to snails. (C) Induced by snails and fed to crayfish. (D) Induced by crayfish and fed to crayfish. Error bars represent SE. P-values are from paired T-tests.

occurred due to direct feeding, not due to water-borne cues from attacked conspecifics.

Plants grown in tanks receiving water-borne cues from attacked neighbors had similar palatability to those not receiving such cues (N=5, P=0.68, Figure 1.4).
Figure 1.3. Consumption of artificial foods treated with chemical extracts from induced versus non-induced plants. (A) Induced by snails and fed to snails. (B) Induced by snails and fed to crayfish. Error bars represent SE. P-values are from paired T-tests.

The compound responsible for deterring herbivore feeding was found in the ethyl acetate partition (70% suppression, P<0.001, N=12, paired t-test); the hexane-, chloroform-, and water-fractions showing no significant effects on feeding (P= 0.77, N=12; P= 0.69, N=11; and P=0.10, N=11, respectively). We could follow the feeding
deterrent activity from the ethyl acetate partition through two additional separation
procedures (a reverse phase column separation followed by isotonic HPLC), but when
further separated into six fractions via gradient HPLC, none of these fractions
significantly suppressed feeding (P-values ranged from 0.14 to 0.99 for the six assays,
N=10-15, paired t-tests). However, recombined fractions again deterred feeding
(p=0.009, N=14), suggesting an additive or synergistic effect of multiple metabolites.

![Graph](image)

**Pomacea canaliculata**

N = 5, P = 0.68

Figure 1.4. Consumption of control and treatment
*Cabomba*, where treatment *Cabomba* represents plants that were
grown in the presence of waterborne cues of grazers attacking
conspecifics. Error bars represent SE. P-value is from a T-test.

When juveniles of the snail *Pomacea canaliculata* were fed *ad libitum* on induced
and non-induced *Cabomba*, growth of snails on non-induced and induced *Cabomba* was
111% and 30%, respectively for mass (p<0.001) and 25% and 7% respectively for shell
diameter (p<0.001; Figure 1.5A). When mass of food eaten each day was measured over the last 9 days of the experiment, snails with access to non-induced Cabomba consumed 1.9 times more plant mass than those constrained to feeding on induced Cabomba (p<0.001; N=18; t-test). Thus, the lower growth of snails fed induced plants could have been due to a decrease in feeding on induced plants, a physiological effect of the induced chemicals on snail growth, or a combination of both. To assess these possibilities, we created artificial diets containing induced and non-induced extracts and constrained both groups of snails to consuming equal quantities of food. Under these conditions, snails feeding on equal quantities of artificial food prepared with non-induced compared to induced plant extracts grew significantly more in diameter (5% versus 1%, respectively (P=0.01, one-tailed paired T-test) and tended to grow more in mass but not significantly so (13% versus 9 %, respectively; P=0.13, one-tailed paired T-test) (Figure 1.5B). We considered shell growth to be the more rigorous measure of growth here due to snails appearing to vary in how much water they rejected from their shells before closing when we were patting them dry before weighing.

Extracts from both the induced and non-induced Cabomba suppressed growth of all 5 microbes isolated from Cabomba. Antimicrobial activity declined with decreasing concentration of the extract (Figure 1.6), and the extract from induced plants tended to be more potent than the extract from control plants. This difference was significant for one of the two bacteria and for two of the three fungi.
Figure 1.5. Juvenile snail growth (± 1SE) when: (A) fed ad libum on induced versus non-induced *Cabomba* plants for 30 days, and (B) fed on an agar-based diet treated with lipid extracts from induced versus non-induced *Cabomba*. In (B), snails feeding on the non-induced diet were constrained to consuming only as much food as was consumed by snails feeding on the induced diet. P-values are from 1-tailed, paired t-tests.
Figure 1.6. Percent inhibition (compared to controls) of two bacteria and three fungi when grown in broth treated with extracts from induced and non-induced plants. Inhibition was calculated by comparing pathogen growth between cells with and without the added extracts. Test concentrations are shown as % of natural yield in our extractions. Asterisk represent significant differences using paired T-tests corrected for multiple comparisons (p<0.008). Error bars represent standard errors.
1.5 Discussion

Twenty-two aquatic macrophytes have been shown to chemically deter herbivores (Newman et al. 1996; Bolser and Hay 1998; Bolser et al. 1998; Cronin et al. 1998, 2002; Kubanek et al. 2001; Prusak et al. 2005; Parker et al. 2006, 2007; Erhard 2007; Miller and Provenza 2007), with active metabolites identified for five species (Newman et al. 1996; Bolser et al. 1998, Kubanek et al. 2001; Parker et al. 2006, 2007). Induced chemical defenses have been detected in only one other aquatic macrophyte: *Nuphar luteum* (Bolser and Hay 1998), with neither that study nor this one identifying the compounds responsible. Our study indicates that aquatic herbivores have been enough of a force to select for induced defenses in macrophytes, that these defenses suppress herbivore feeding due to negative effects on their fitness, and that the metabolites induced by herbivory might also defend macrophytes from microbes that could attack plants via herbivore generated feeding scars.

Although initially a favored food of both snails and crayfish (Figure 1.1), *Cabomba* induces a chemical defense in response to direct attack, and this induction reduces herbivore feeding by 71-83%. Herbivores are selected to detect and avoid induced plants because consuming induced plants lower herbivore growth. The detrimental impact of induced defenses on herbivores may favor *Cabomba* by: (1) causing herbivores to switch to nearby competing plants, (2) increasing herbivore mortality by slowing their growth and keeping them in smaller size classes that are more susceptible to consumers, and (3) reducing herbivore reproduction and the local numerical response of consumers. In a meta-analysis of terrestrial plants and their insect herbivores, Nykanen and Koricheva (2004) found that induced defenses had a significant
impact on herbivore fitness, but no impact on plant consumption. Such findings may be due to a focus on insects, which tend to specialize on specific host species and to feed primarily as larvae that have a limited ability to move among hosts (Bernays and Graham 1988). The herbivores in our study were more mobile generalists that are likely to switch from induced *Cabomba* to more palatable plants, thus producing a direct fitness benefit to *Cabomba*. *Cabomba* is an introduced pest in many locations, and efforts at biological control are underway (Schooler et al. 2006). Given our results, evaluations of potential bio-control agents (herbivores or microbes) should consider the effects of these enemies on induced as well as non-induced *Cabomba*.

Airborne chemical cues to consumer presence occur in terrestrial plants (Karban and Baldwin 1997) and waterborne cues stimulate induced defenses among marine seaweeds and phytoplankton (Toth and Pavia 2000, Long et al. 2007b). It therefore makes sense that waterborne cues could stimulate induced defenses in freshwater macrophytes, however we detected no evidence of induction due to waterborne cues alone. Lack of such cuing could occur due to *Cabomba* growing in slow moving waters where chemical cues propagate slowly, or due to *Cabomba* being a clonal species that communicates effectively among ramets via direct connections of underground stems, such that plants need not also respond to dissolved cues.

Terrestrial and marine investigations of chemical defenses have emphasized higher order processes such as how induced defenses impact competitive and trophic interactions among multiple community members (Long et al. 2007a; Toth and Pavia 2007). Our research suggests that higher order interactions could occur in this system as well. Because both snails and crayfish induced a chemical defense in *Cabomba* that
impacted not only their feeding, but also that of the other herbivore, induction due to feeding by herbivore 1 could impact the fitness of herbivore 2 (see Long et al. 2007a). Both herbivores could also be impacting the microbial community associated with *Cabomba*. Extracts from induced plants more strongly suppressed microbes; this could change host-pathogen dynamics or rates of microbial remineralization of plant material, as has been suggested for terrestrial leaves subject to insect attack (Fonte and Schowalter 2005; Kay et al. 2008).

We are unsure what triggers the induced chemical response in *Cabomba*. It could be loss of leaves from direct herbivory, increased susceptibility to pathogens due to wounds from the herbivory, or a response to pathogens present within the herbivore’s saliva. Preliminary data indicates that artificial herbivory (i.e. cutting the plant with scissors) does not induce defenses (A. Brown *personal communication*), suggesting that actual herbivory, not just leaf damage is necessary.

In summary, *Cabomba caroliniana* induces a chemical defense in response to two generalist herbivores. The response is reciprocal in that induction by either herbivore impacts the feeding of the other. The defense is chemical and suppresses both consumer feeding and growth efficiency, but we were unable to identify the metabolites involved due to additive or synergistic effects. Induced plants deter not only herbivores, but also tend to suppress microbes associated with *Cabomba*. Our results suggest that consumers that induce defenses in their host plants may also be indirectly affecting other consumers and microbial pathogens via changes in this shared resource.

### 1.6 References


CHAPTER 2

FEEDING AND GROWTH RATES AMONG NATIVE AND NON-NATIVE APPLE SNAILS (AMPULLARIIDAE) IN THE UNITED STATES

2.1 Abstract

The United States hosts one native and five non-native species of aquatic apple snails, with all species currently located in or around the Everglades ecosystem in South Florida. These introduced apple snails have devastated wetlands in Southeast Asia, but little is known about their impact on the Everglades. To evaluate potential impacts of introduced apple snails, we investigated plant preference, consumption rates, growth rates, and growth efficiencies for four introduced and one native species across six to eight aquatic plants native to South Florida. Three of the non-native snails are invasive, one has shown no tendency to expand (innocuous), and one has minimal impact on macrophytes due to its diet. All macrophyte consuming snails exhibited similar feeding preference, with *Utricularia* being the highest preference, *Bacopa, Sagitaria, and Nymphaea* being intermediate preference, and *Eleocharis, Pontederia, Panicum* and *Typha* being avoided. The invasive species *Pomacea insularum* and *P. canaliculata* tended to eat more, grow more, and have higher conversion efficiencies than the native species *P. paludosa* or the non-invasive *P. haustrum*. These contrasts were more often significant for *P. insularum* than for *P. canaliculata*. Consumption and growth were minimal for *P. diffusa* on all macrophytes; this species is mainly an algivore. In general,
invasives showed greater rates of feeding and growth, and sometimes increased efficiency, compared to native and innocuous non-native species - suggesting a mechanism for greater rates of expansion by invasive species.

2.2 Introduction

Some 50,000 non-native species have been introduced into the United States, producing an economic cost estimated at $120 billion per year (Pimentel et al. 2005). In addition to economic costs, introduced species can also disrupt native ecosystems and threaten native species. It is estimated that 50% of presently imperiled species are at risk due to competition with or predation by non-native species (Wilcove et al. 1998). Invasive species impact all habitat types (Parker et al. 1999), but appear to have stronger impacts on freshwater than on terrestrial ecosystems (Sala et al. 2000). Not all introduced species negatively impact native ecosystems, necessitating an improved understanding of which species are most threatening and under what circumstances (Parker et al. 1999).

Apple snails are large omnivorous snails with a voracious appetite for aquatic plants. They are named after the fruit as their sizes are often similar; however, larger species can reach shell heights of over 25 cm (Cowie 2002). Apple snails prefer shallow, turbid sites with minimal water movement (Cowie 2002, Cazzaniga 2006). They are obligate lung breathers and can survive drying of their habitats by aestivating in mud (Cowie 2002). Identification of the different apple snails is difficult as morphological characteristics can vary with environmental conditions (Cazzaniga 2006). Similarly, lifespan and reproduction also vary depending on latitude and climate (Cowie 2002). For
example, in tropical climates, *P. canaliculata* matures in 7 months and lives for one year. In more seasonal areas, the same species matures after two years and lives for four years (Cowie 2002). Apple snails have internal fertilization, and egg laying can occur above water (*Pomacea*) or under water (*Marisa*) depending on the species. Fecundity varies among species (Cowie 2002) but lifetime egg production can exceed 10,000 eggs for an individual (Cazzaniga 2006).

Apple snail introductions have caused negative economic and ecological impacts worldwide (Cowie 2002). Apple snail introduction into Taiwan cost US $30 million in lost rice farming revenue in 1986 alone (Mochida 1991). Their impacts on other commercial crops, such as taro, water spinach, water chestnut, and lotus also are considerable (Cowie 2002; Carlsson 2006). Apple snails not only have negative economic impacts, but can also disrupt natural ecosystems. The introduction of *Pomacea canaliculata* into Thailand changed aquatic ecosystems from clear, macrophyte dominated systems into turbid, plankton dominated ones (Carlsson et al. 2004). Due to this history, *P. canaliculata* is currently listed as “100 of the World’s Worst Invasive Alien Species” (Lowe et al. 2000).

Five species of non-native apple snails have been introduced into the southern United States including California, Texas, Arizona, Georgia, and Florida (Rawlings et al. 2007). All five currently occur in and around the Everglades in South Florida (Rawlings et al. 2007; pers comm. Rawlings). Only one species of apple snail, *Pomacea paludosa*, is native to North America, and it occurs exclusively in South Florida. The native species is an important component of South Florida ecosystems and it serves as a critical, almost exclusive, prey for the endangered Everglades Kite (Bennetts et al. 1994). Of the five
species that have been introduced into the U.S., three (*Pomacea insularum*, *Pomacea canaliculata*, and *Marisa cornuarietis*) are considered “invasive” pests, one (*Pomacea hastrum*) appears “innocuous” because it has been in the US for 30 years without becoming invasive (Rawlings et al. 2007), and the fifth species (*Pomacea diffusa*) consumes primarily algae and has little impact on aquatic macrophytes (Howells 2002). Phylogenetic studies show *P. canaliculata* and *P. insularum* to be sister species closely related to the native *P. paludosa*. *P. haustrum* and *P. diffusa* are sister species more distantly related to the native *P. paludosa* (see Figure 1 from Rawlings et al. 2007).

Due to the potential impact of introduced apple snails on commercial crops and native vegetation, the U. S. Department of Agriculture has banned movement of apple snail species across state lines without a permit and requires all facilities housing the snails to show proper precautions to prevent escape. Given these circumstances, field determination of impacts of the introduced apple snails on native vegetation is difficult. We thus conducted a laboratory study to determine the feeding preferences, feeding rates, growth rates, and growth efficiencies of the one native versus 4-5 non-native apple snails that occur in the U.S.

The impact of the introduced apple snail species on aquatic habitat could differ depending on the introduced species and invaded habitats. Habitats that have evolved with the native apple snails may be impacted differently than habitats lacking native apple snails. The quick spread of *P. insularum* (Rawlings et al. 2007), combined with data showing reduced growth of the native when in the presence of the larger introduced species (Conner et al. 2008), suggest that the invasive species might displace the native *P. paludosa*, but mechanisms underlying this were not investigated.
To assess possible impacts of these non-native species, we quantified differences in food preference, food consumption, growth and efficiencies between the native, innocuous, and invasive species of apple snails. Our goal was to evaluate possible ramifications if the exotics were to replace the native species in and around the Everglades National Park. In addition, quantification of consumption and growth patterns might predict snail impacts if they expanded into other wetland areas (e.g., the Okefenokee Swamp in SE Georgia). Specific objectives included:

1. Compare food preference between the one native and five introduced snails when offered common aquatic macrophytes from the Everglades National Park.
2. Evaluate plant consumption rates among native, introduced, and invasive species of snails.
3. Contrast growth among snail species when offered *ad libitum* one of six species of native plant.
4. Compare growth efficiencies among the different snails.

### 2.3 Methods

#### 2.3.1 Animals

Collection and transport of snails were conducted under Department of Agriculture permit number P526P-07-07248. *Pomacea paludosa* and *P. insularum* were collected as eggs; *P. insularum* from Lake Lure (N 31° 33.210' W 82° 28.947') in Georgia and Lake Tohopekaliga (N 28° 13.033 W 81° 22.533) in Florida, and *P. paludosa* from Lake Tohopekaliga in Florida. Adult *P. canaliculata* were obtained from Neighborhood Fish Farm in Miami Florida; *P. diffusa, P. haustrum, and Marisa*
cornuarietis were obtained from Paradise Aquatics in Winterhaven, Florida. These species produced viable eggs that hatched in the lab; juveniles hatched from eggs were used for all experiments. All species were identified according to characteristics of their eggs (Rawlings et al. 2007), and species were held separately in labeled tanks because juveniles are difficult to identify. An individual snail was used in only one experiment and then euthanized. All snails used in experiments were hatched between 2 June and 29 July 2008.

Because we wanted to estimate how non-native apple snails might affect areas occupied by the native apple snail, plant species were chosen for our experiments based on their importance in the Florida Everglades and our ability to easily obtain the species. Plants were considered important if they were listed as common species by two books describing the Ecosystems of the Everglades (Gunderson 1994, Lodge 2005). The exception was Typha, which appeared only in Lodge (2005), but was included because of its increasing abundance in disturbed aquatic habitats (pers. comm. Cynthia Guerra, Miami Audubon). *Nymphaea odorata, Panicum hemitomon, Typha sp., Pontederia cordata, and Utricularia sp.* were all collected throughout Georgia. *Sagittaria latifolia, Bacopa caroliniana,* and *Eleocharis cellulosa* were ordered from Biosphere Consulting Inc, a plant nursery that guaranteed no pesticides were used on the plants. All plants were planted in 72 L tubs and grown in a greenhouse at the Georgia Institute of Technology so that they could be harvested as needed for experiments.

**2.3.2 Choice Assays**

Snails were simultaneously offered small pieces of eight plant species (*Bacopa, Eleocharis, Nymphaea, Panicum, Pontederia, Sagittaria, Typha,* and *Utricularia*) in
replicate 739 ml containers and allowed to feed for three days. Snails encountered plants by crawling across the bottom of the container or floating across the surface of the water. We adjusted plant mass to assure approximately equivalent 2-D surface area of each plant species in each container to standardize herbivore encounter rate. Since *Utricularia* is so diffuse, in order to have a sufficient mass to allow for measurements, we bundled a larger amount of plant together using a small piece of craft wire. Similarly, small pieces of *Typha* and *Panicum* were also joined together using craft wire. This caused these species to sink while the rest floated. As the *Utricularia* was eaten first and the *Panicum* and *Typha* not eaten at all (see results), the location of the plants on the surface vs. bottom did not appear to impact the results. Assays were spatially grouped into 16 blocks of replicates, where each block included one replicate of each snail species plus one control to monitor autogenic changes in plant mass unrelated to feeding (Roa 1992; Stachowitz and Hay 1996). Within each block, all pieces were cut from the same plant when possible, and no individual plant was used in more than one block. The mass of snails utilized within each block was similar, adjusting the number of snails as needed to match total snail mass. Assays were checked every 4-8 h to visually estimate the percentage of each plant consumed. At the end of three days, all snails were removed from the assays, the remaining plants blotted, and a wet mass determined. Plant starting mass was corrected for autogenic change according to the formula (Stachowitz and Hay 1996): \( T_i \times (C_f/C_i) \), where \( T_i \) are the initial masses of plants available for consumption by the snails and \( C_i \) and \( C_f \) are the initial and final masses of the plants from the matching controls.

2.3.3 Growth
We measured snail growth when fed different plants by placing individual snails (N= 16/snail species) into 296 ml plastic containers and feeding them excess amounts of one of the plant species. To keep replicates to a manageable number, only six plant species and five snail species were utilized. Plant species included most of those utilized in the choice assays (Utricularia, Bacopa, Eleocharis, Nymphaea, Panicum, and Pontederia) except for the exclusion of one low and one high preference species; snails included all species but M. cornuarietis, which had suffered a mortality event in the lab. Each of 16 blocks consisted of 33 cups randomly assigned to a location within the block. 

P. diffusa was offered only four species of plants due to its algal specific diet (Howells 2002). For each snail species, a control snail was included in each block to measure changes in mass when starved. Snail mass was measured blind relative to treatment at the beginning and end of the experiment. Cups were checked every three days to add water and food as needed. Once a week, half of the water and all of the food was replaced. Dead snails were noted. Originally cups were filled to approximately two cm from the top and covered with aluminum foil with holes. The snails experienced some mortality with this methodology, necessitating replacement of the dead snails and modification of the tops from aluminum to screen mesh. All snails that died during the first week of the growth experiment were replaced and the date of replacement noted. After the first week, dead snails were not replaced. Growth was corrected for actual days of growth at the end of the experiment (1 month ± 1 week). Snails ranged between 0.05 and 1.7 grams wet mass at the start of the experiment. Ideally, all snails in the experiment would be the same starting size; however, due to the magnitude of this experiment, we were unable to find 528 snails across 5 species that matched in size.
Therefore, we matched by wet mass all snail sizes within a block. The experiment was initiated with no significant difference in sizes among snail species. However, smaller *P. paludosa* experienced considerable mortality the first week of the experiment and were replaced with slightly larger snails, leading to a significantly larger start size for *P. paludosa* (mean mass = 0.54 g) compared to the rest of the species (mean mass ranged from 0.24 to 0.31 g) (F=12.6, P<0.001). This was incorporated into all subsequent statistical analyses.

### 2.3.4 Growth efficiencies

During the last three days of the growth experiment, mass eaten by each snail was measured. As with the choice assays, for each replicate, food pieces were cut from the same individual plant and a control piece set aside to measure changes in plant mass unrelated to grazing. At the end of three days the leftover plant pieces were weighed and corrected for changes in mass of the control specific for that block. Feeding was assessed as mass of plant consumed per mass of snail. Growth efficiency was calculated as the daily growth of the snail in grams/daily consumption in grams. Since growth was measured over one month, but consumption only quantified for the last three days, we assumed that the consumption in the last three days represented average consumption throughout the month. We removed data points where either snail growth or consumption was less than or equal to zero because calculating efficiencies with these numbers creates biologically unrealistic values.

### 2.3.5 Statistical analyses

Choice assays were analyzed using a modified Friedman’s Test to account for non-independence among the plant species within each replicate (Conover and Iman
Because of non-independence among food choices, the Friedman’s test requires dependent variables (i.e. consumption) to be ranked within each replicate. However, instead of running the comparison with a chi-square table (the usual Friedman’s statistic, Conover 1999) we instead utilized two-way analysis of variance (with replicate and treatment as the independent variables) as this is more reliable with larger matrixes (Conover and Iman 1981). Paired comparisons were completed on the rank data using Tukey’s pairwise comparisons. The no-choice feeding assays, growth assays, and efficiencies were analyzed using the Analysis of Covariance (ANCOVA) on ranked transformations with snail mass as the covariate. Non-parametric rank transformations were utilized because data did not meet the assumptions of homogeneous variance and no other transformations adequately corrected this problem. Tukey’s adjustment was utilized on all pairwise comparisons to keep Type I error to a minimum. When the results of the ANCOVA suggested the covariate (snail mass) was not significant, pairwise comparisons were completed on the results from the one way Analysis of Variance (ANOVA). To determine if individual results were significantly different from zero, t-tests were completed with the significance level (α) corrected by dividing by the number of comparisons completed per graph. Chi-square contingency tables were used to evaluate differences in mortality across snail species and plant diet. Due to small sample sizes and the lack of variance measurements, analyses tested for the occurrence of any differences among snail species or plant species, but no pairwise comparisons could be reliably completed.
2.4 Results

Figure 2.1. Mean consumption (error bars = SE) of plants by snails during the choice assays. P-values are from ANOVA. Different letters represent significant differences using Tukey’s multiple comparison tests on ranked data. Asterisks indicate consumption is significantly different from zero using T-tests with α corrected to 0.006 for multiple comparisons.
All snail species showed crudely similar patterns of preference when offered a choice among the eight macrophytes (Figure 2.1). _Utricularia_ sp. was readily consumed by all species with _Sagittaria_ also being heavily consumed by _Pomacea canaliculata_ and _P. insularum_. _Bacopa_ and _Nymphaea_ were consumed at intermediate rates by most snail species, with _Paniculm, Pontedaria, Eleocharis, and Typha_ being largely avoided. Although _P. diffusa_ consumed so little of all macrophytes that there were no significant differences in its feeding across all plants tested. The response of _P. diffusa_ to _Utricularia_ was intriguing and suggested considerable intraspecific variance in their willingness to feed on this plant; 44% of these snails consumed none of the plant, while 31% consumed >80% of the available mass. Visual estimates of plant consumption over time (Figure 2.2) suggested that all snail species consumed _Utricularia_ first. The consumption of the second, third and fourth choices varied among snail species. _P. paludosa_ consumed _Bacopa_ second, followed by _Sagittaria_ and _Nymphaea_ at similar and low rates. _P. insularum_ consumed _Sagittaria_ and _Bacopa_ at similar rates, followed by _Nymphaea_. _P. canaliculata_ consumed _Sagittaria_ second before switching to _Bacopa_ and later _Nymphaea_. _P. haustrum_ and _P. diffusa_ consumed _Sagittaria, Bacopa, and Nymphaea_ at similar low rates, while _Marisa cornuarietis_ consumed _Bacopa_ as a second choice and other species at minimal rates.

When we summed all feeding on all plant species in each replicate to evaluate how total consumption rates varied among snail species, _P. insularum_ and _P. canaliculata_ were statistically indistinguishable and consumed about 190 mg of plant/ g snail/ day (Figure 2.3). _M. cornuarietis_ and _P. haustrum_ consumed significantly less at about 70-115 mg/ g snail /day. _P. diffusa_ consumed less than all other species, and could
Figure 2.2. Visual estimates of snail consumption (mean ± SE) across time during the choice assays.
not be demonstrated to be feeding because the mean mass eaten did not differ significantly from zero (N = 16, P= 0.88). The native snail *P. paludosa* fed at an intermediate rate of about 140 mg of plant/ g snail/ day; this was significantly more feeding than *P. haustrum* and *P. diffusa*, but did not differ significantly from other species.

![Figure 2.3](image)

Figure 2.3. Total grams of plant consumed across all plant species per gram of snail per day (mean ± SE) during the choice assays. Statistical tests and symbols as in Figure 2.1, except that the data were not ranked.

When confined with a single plant species (rather than having a choice among plants), three of five snail species tested consumed significantly more *Utricularia* and *Bacopa* than any other species (Figure 2.4). *P. haustrum* showed a similar pattern but its consumption of *Utricularia* did not differ significantly from its feeding on *Nymphaea*. As in the choice assays, *P. diffusa* could not be demonstrated to feed on any plant, even when given no other choice. All five of the snail species tested consumed very little (0-45 mg/ g snail/ day) of *Nymphaea, Eleocharis, Pontederia*, and *Panicum*. *M.*
Figure 2.4. Consumption (mean ± SE) during the no-choice assays. Statistical tests and symbols as in Figure 121.

- **P. paludosa**
  - N = 7-12, P < 0.001

- **P. insularum**
  - N = 11-13, P < 0.001

- **P. canaliculata**
  - N = 11-12, P < 0.001

- **P. haustrum**
  - N = 11-12, P < 0.001

- **P. diffusa**
  - N = 9-11, P = 0.7

- No data
cornuarietis was not included in this study due to mortality prior to the experiment. If we compared across snail species for the two plant species that were most readily consumed, *P. insularum* and *P. canaliculata* consumed more *Utricularia* than *P. diffusa*, with other snails consuming intermediate masses that did not differ significantly from other snail species (Figure 2.5). *P. insularum* and *P. canaliculata* consumed more *Bacopa* than either *P. paladosa* or *P. diffusa*, with *P. haustrum* being intermediate.

Diet strongly affected growth (p<0.001) for all snail species (Figure 2.6). All species grew well (by 20 - 266%/month) on *Utricularia*; all grew to a lesser extent on
Figure 2.6. Percent (+SE) growth in snail mass when feeding on different single-species diets. Statistical tests and symbols as in Figure 2.1.
Bacopa (by 6 - 101%). Only P insularum achieved positive growth on Nymphaea. Growth on all other snail-plant combinations did not differ significantly from zero. P. diffusa did not have positive growth on any plant species; P. hastrum had positive growth on only Utricularia; P. paludosa and P. canaliculata had positive growth on Utricularia and Bacopa; and P. insularum had positive growth on Utricularia, Bacopa and Nymphaea.

To facilitate comparisons among snail species, growth of snails was compared for each of the three plant species that provided the best growth (Figure 2.7). When feeding on Utricularia, P. canaliculata and P. insularum increased their mass by more than 200%, followed by P. hastrum and P. paludosa, increasing by 70-90%, with P. diffusa growing by only 20%. Results for Bacopa were lower for all species but followed a similar pattern among snail species; P. insularum grew the most (101%), P. diffusa the least (6%), with the other species falling between these extremes (30-60%). Growth on Nymphaea was limited for all species; P. insularum grew 28%, P. canaliculata by 8%, P. paludosa 6%, and the other two species grew less than 3%. The introduced species P. insularum always grew significantly more than the native P. paludosa, while the introduced species P. canaliculata showed a similar trend but this was significant only for Utricularia. Growth efficiencies ranged between zero and 0.13 across all snail and plant contrasts (Figure 2.8). There were no significant differences in efficiencies on Bacopa or Nymphaea across snail species, however, on Utricularia, P. insularum had significantly higher efficiency compared to the native P. paludosa and the algivore P. diffusa.
Figure 2.7. Percent (± SE) growth in snail mass when feeding on one of the three plant species that supported positive growth over the month long experiment. Statistical tests and symbols as in Figure 2.1.
Figure 2.8. Growth efficiency of snails (±SE) when feeding on each of the three plant species that supported positive growth. Statistical tests and symbols as in Figure 2.1.
When summed across all diets, there were among-snail differences in total mortality ($X^2 = 21.7 \ P = 0.0002$). When all snail species were summed across a specific diet, there were also significant among-plant differences in survivorship of snails confined to those diets ($X^2 = 18.0 \ P = 0.006$). In general, the native snail *P. paludosa* and the non-macrophyte consuming *P. diffusa* were the least hardy snails, experiencing mortality of ~20% across all diets combined (Figure 2.9). For the other three snail species, mortality was only 4-8%. As might be expected from the feeding preference data, snails confined to the preferred diets of *Utricularia* and *Bacopa* survived well (2-3% mortality), while those feeding on other plant diets experienced 10-15% mortality, with starved snails suffering 22% mortality.

### 2.5 Discussion

Invasive species are second only to habitat loss in negatively impacting native species (Wilcove et al. 1998). It has been estimated that 50,000 non-native species have been introduced into the United States (Pimentel et al. 2005), but only a small number of these become invasive. For introduced apple snails in the United States, three species appear invasive; *Pomacea canaliculata*, *P. insularum* and *Marisa cornuarietis* are spreading rapidly (Rawlings et al. 2007). One species (*P. hastrum*) has been in South Florida for years, but has not spread beyond this initial area (Rawlings et al. 2007) and is thus considered innocuous. The final introduced species (*P. diffusa*) appears to consume algae instead of macrophytes and is predicted to have minimal impact on the structure of macrophyte communities (Howells 2002). The ecosystem effects of these species are unknown, but given the large impact of *P. canaliculata* in Southeast Asia (Cowie 2002; Carlsson et al. 2004), impacts on native flora and fauna in the U.S. could become
Figure 2.9. Snail mortality during the one-month feeding experiment.
substantial. Thus, apple snails provide an opportunity to compare species that are native, non-native, invasive, or innocuous.

Food preferences were crudely similar across all species. *Utricularia* was always a preferred food, and *Panicum, Pontederia, Typha*, and *Eleocharis* were always avoided. *Bacopa, Sagittaria* and *Nymphaea* were consumed at intermediate rates by all species with the exception of *P. diffusa*, which fed at low rates on all macrophytes (Figure 2.2). Apple snail food preferences have been assessed previously (Estebenet 1995; Lach et al. 2000; Carlsson et al. 2004, Carlsson and Lacoursiere 2005; Gettys et al. 2008), but only a few macrophytes were rejected in those assays; Estebenet (1995) found rejection of *Elodea canadensis* and Carlsson et al. (2004) found rejection of *Typha sp*. Our results indicate that apple snails are generalist herbivores that show strong preferences for some macrophytes and nearly complete avoidance of others. Plants included in our assays were abundant within the Everglades National Park, which is impacted by the native apple snail *P. paludosa*. Therefore, some plants in our assay may have been selected to deter feeding by the native snail, potentially expressing traits that deter other apple snails as well.

Growth of all apple snails was low on all macrophytes except for *Utricularia* and *Bacopa*, where growth in mass was 20-266% and 6-101% over one month, respectively (Figure 2.6, 2.7). Sharfstein and Steinman (2001) measured mass increases in *P. paludosa* that were an order of magnitude higher than what we measured. They specifically stated that their *Utricularia* and *Eleocharis* included associated periphyton (detritus, algae, and microbes) which could be an additional food source. In contrast, our plants did not contain any obvious periphyton growth. Estebenet (1995) measured
growth in *P. canaliculata* over a period of four months. The growth over the last month of her experiment (when the size of her snails matched those we used) was similar the growth rates we measured. This suggests that our growth is accurate for snails fed individual macrophyte species, but the former studies suggests that a diverse diet that includes detritus and algae allows for higher growth.

Consumption rates from the choice assays indicated that the native apple snail fed at rates indistinguishable from the invasive species *P. insularum* and *P. canaliculata* and the non-invasive *M. cornuarietes*, with *P. haustrum* and *P. diffusa* feeding significantly less than the native species (Figure 2.3). When confined to single plant diets (Figure 2.4, 2.5), the invasive apple snail species consumed *Bacopa* at greater rates than the native species and showed a similar, but non-significant, pattern for *Utricularia*. Mean consumption measured for the choice and no choice assays ranged between 0 – 0.19 and 0 – 0.34 grams of plant per gram of snail per day, respectively. Carlsson and Bronmark (2006) found consumption of 0.25 grams of plant per gram of snail per day for newly hatched *P. canaliculata*. Rigorous comparisons with other studies are not possible due to different snail sizes and macrophyte species being used among studies. Growth efficiency of the invasive and native snails was similar for all contrasts except for the invasive species *P. insularum* having a significantly greater efficiency than the native species when fed *Utricularia* (Figure 2.8). When pooled across all diets, mortality tended to be higher for the native *P. paludosa* than for the non-native species, suggesting it may be less hardy (Figure 2.9).

There are differences in apple snail life history characteristics that are not covered in this paper. Snail densities may vary between the native and exotic species, though data
from the field are lacking. Native snail densities range between 0-3 snails per square meter in South Florida (Darby et al. 2004). No density measurements are available for the invasive species in Florida, but *P. canaliculata* have been found to reach densities of 130 and 150 per square meter in rice patties in Southeast Asia (Cowie 2002). Additionally, sizes of the adult snails also differ and can be important. The invasive *P. insularum* can be up to four times heavier than the native *P. paludosa* (Conner et al. 2008). Batch fecundity is highly variable between species; native *P. paludosa* have on average 30 eggs per batch (maximum 141) while *P. insularum* can have over 1000 eggs per batch (Cowie 2002; Rawlings et al. 2007). If the snails produce the estimated 22 batches per year (Cowie 2002), this translates into a large difference in number of offspring. However, since the eggs of *P. insularum* are smaller (see Figure 2 from Rawlings et al. 2007), information on survival of hatchlings is also needed. We measured differences in juvenile mortality, but matched species across size and not age. Since eggs (and hence hatchlings) are much larger in *P. paludosa* compared to *P. insularum* (Rawlings et al. 2007), initial hatchling mortality may be lower, and we may have missed any increased mortality associated with the smaller sizes of the invasive hatchlings.

Even though we measured similar food preferences across *Pomacea* species, the differences in feeding rates, growth, efficiencies, and mortality when combined with differences in size and fecundity suggest that a change in *Pomacea* species could impact aquatic habitats of South Florida. However, our results suggest that complete denudation of the flora would not occur as all apple snail species rejected *Panicum, Eleocharis*, and *Pontederia*, even when provided no other choice. Our study suggests that *Utricularia*
will be one of the first plants impacted. In Georgia, *P. insularum* is currently located just 18 miles outside the Okefenokee Swamp (pers. comm. Chad Sexton, Georgia DNR).

Introduction of *P. insularum* could impact the Okefenokee Swamp due to the high prevalence of *Utricularia* in this ecosystem (Greening and Gerritsen 1987). Invasive species often have a large impact if they fill a novel function in the new habitat (Parker et al. 1999). Introducing invasive apple snails into areas without native apple snails may allow them to occupy an unfilled niche, which could lead to a larger impact as plant species in this area may not be able to resist or tolerate the voracious appetite of the apple snails.

In summary, the invasive *P. insularum* had increased growth, increased efficiency, and decreased mortality compared to the native apple snail even though its consumption of various plants was similar (Figures 2.3, 2.7, 2.8, 2.9). The invasive *P. canaliculata* had similar characteristics in magnitude to *P. insularum*, but was significantly higher than the native *P. paludosa* for growth on *Utricularia* only. Comparatively, the innocuous *P. haustrum* did not differ from the native *P. paludosa* in growth or efficiency (Figures 2.7 and 2.8) but did have a lower consumption rate (Figure 2.3). This might explain why *P. insularum* is currently expanding its range while *P. haustrum* is not (Rawlings et al. 2007). In fact, *P. insularum* is currently replacing *P. haustrum* in some Florida areas (Pers. Comm. Tim Collins, Florida International University). The similarity in results between *P. insularum* and *P. canaliculata* were not surprising given that these are sister species (Rawlings et al. 2007). However, the fact that *P. insularum* was consistently higher than *P. canaliculata* on all characteristics measured was surprising. *P. canaliculata* is listed as one of the top 100 worst invasive
species (Lowe et al. 2000), but our results suggest that *P. insularum* may be capable of causing equal or greater environmental damage. This is especially true given that fecundity is higher in *P. insularum* than in *P. canaliculata* (Rawlings et al. 2007).

Overall, our results suggest that invasives feed faster and grow more than both the native and the non-native innocuous species, providing a mechanism for the increased expansion in the former and lack of expansion in the later.

### 2.6 References


CHAPTER 3
LATITUDINAL PATTERNS IN THE PALatability OF FAshWATER MACROPHYTES

3.1 Abstract

Increased herbivory at lower latitudes is hypothesized to select for increased plant defenses. Feeding assays with salt marsh plants and seaweeds, as well as leaf damage patterns in forests, have supported this hypothesis, with low latitude plants experiencing greater damage in the field and being less palatable than higher latitude plants when offered to herbivores. We tested this hypothesis for freshwater macrophytes because they offered an independent plant lineage and habitat type in which to test this general hypothesis and because the patchiness of consumer occupancy across small and often isolated water bodies might produce local variance in herbivore pressure that would override geographic variance and produce different results for this habitat type. When we fed 8 congeneric pairs of live plants collected from Indiana vs South Florida (150 and 365 frost-free days respectively) to three species of crayfish and one species of snail, three of the four herbivores significantly preferred northern to southern plants. For two species of crayfish that differed in feeding on live plants (one favoring northern plants one not), we retested feeding using foods composed of freeze-dried and finely ground plant matter, thus removing structural characteristics while retaining chemical/nutritional traits. In this assay, both herbivores strongly preferred northern plants, suggesting that lower latitude plants had been selected for more deterrent chemical traits. When we
collected 22 pairs of congeneric plants from Central Florida (a 350 km reduction in north-south distance, but a decrease from 365 to 270 frost-free days/yr) vs Indiana and tested these in feeding assays with three crayfishes using dried, ground, and reconstituted plant material, we found a significant effect of latitude for only one of three species of herbivore. Overall, our results suggest a preference for northern plants, but the strength of this relationship varied between our two data-sets. We hypothesize that the differential strength of preference for northern vs southern plants in these two assays may be driven by 1) large changes in frost-free days that occur over small spatial scales affecting the consistency of herbivore selection for plant defenses or 2) local variance in herbivore pressure at the level of collection site producing a high variance in plant defenses.

3.2 Introduction

Results comparing plant palatability across latitude from broad leaf forests (Coley and Aide 1991), salt marshes (Pennings et al. 2001, Pennings et al. 2007), and marine algae (Bolser and Hay 1996) suggest that increases in prey defenses at low latitudes may be a general trend. Exceptions appear to occur for phenolics in seaweeds (VanAlstyne and Paul 1990) and in some terrestrial plants from high (≥40°) latitudes (Swihart et al. 1994, Vihera-Aarnio and Heikkila 2006), but the defensive nature of these compounds is variable, not predictable from metabolite class alone, and may sometimes be confounded with effects of other metabolites (Steinberg and Van Altena 1992, Hay 1996, Deal et al. 2003). It is generally assumed that diminished palatability of plants from lower latitudes is driven by the higher diversity and activity of herbivores in tropical locations selecting for increased plant defenses (Bolser and Hay 1996, Kicklighter and Hay 2006, Pennings et al. 2009, Schemske et al. 2009). This is consistent with Coley and Aide (1991) finding
greater herbivore damage to leaves of tropical versus temperate trees, and Pennings et al. (2009) finding that herbivory on saltmarsh plants was two orders of magnitude higher in low vs high latitude salt marshes.

In this study, we addressed whether the palatability of freshwater macrophytes also decreases at lower latitudes. These plants offer a different taxonomic group and environment for testing this hypothesis, and there are several reasons that freshwater systems might differ from forests or marine systems. First, latitudinal gradients in species diversity tend to be weaker and less steep in freshwater than in marine or terrestrial systems (Crow 1993, Hillebrand 2004, Covich 2009), and previous studies have assumed that greater enemy diversity equates with greater attack and greater selection for defense (e.g., Mitchell and Power 2003, Torchin et al. 2003), so one can question whether freshwater systems will follow trends seen in other habitat types. Additionally, the patchiness, variable sizes, and variable degree of isolation of freshwater systems (some colonized by larger consumers, some not) might select for differing prey traits on this patch scale and might obscure or negate latitudinal trends. Given these uncertainties, we tested whether the palatability of freshwater macrophytes declined at lower latitudes, as has been determined for a limited number of other ecosystems.

3.3 Methods

3.3.1 Herbivores

Feeding assays were conducted with the crayfishes *Procambarus spiculifer*, *Procambarus clarkii*, and *Orconectes rusticus* and the snail *Pomacea paludosa*. Crayfishes were chosen to represent species occurring across the ranges of the plants used in the feeding assays. The natural range of *Procambarus spiculifer* is the
southeastern United States including Mississippi, Alabama, Florida, Georgia, and South Carolina (http://iz.carnegiemnh.org/). *Procambarus clarkii* naturally occurs from Illinois south to Florida, not including Indiana (http://nas.er.usgs.gov), and the natural range of *Orconectes rusticus* includes Indiana, western Ohio and northern Kentucky (http://nas.er.usgs.gov). The herbivorous apple snail *Pomacea paludosa* is restricted to South Florida (Rawlings et al. 2007). *P. spiculifer* were collected in the Chattahoochee River, *O. rusticus* were ordered from Connecticut Biological Supply, *P. clarkii* were ordered from Carolina Biological Supply and *P. paludosa* were collected as eggs in Florida and allowed to hatch and grow in the lab, feeding on lettuce and algal pellets until they reached an appropriate size for feeding assays. Most of the *P. clarkii* and *P. spiculifer* utilized in the bioassays were hatched from eggs in the lab and raised on commercial herbivore food until large enough for bioassays. Crayfish were housed in 946 ml individual containers placed in a 0.91x1.83 m flow through water table. Bioassays were completed within the individual containers. Snails were housed in 38 L tanks before assays, but transferred to individual 946 ml containers for bioassays.

### 3.3.2 Plants

Two collections of plants were made for testing latitudinal differences in palatability. For the first assays we collected eight pairs of live, conspecific or congeneric plants, one member of each pair from the Everglades in South Florida (about 25° Latitude) and the other member of the pair from Indiana (between 39 and 41° latitude; thus higher latitude plants were growing about 1,700 Km north of lower latitude plants and experienced an average of 215 vs zero days of frost/yr (Table 3.1). High and low latitude plants were collected simultaneously on June 14, 2009 and either shipped
(FL) or driven (IN) overnight to Georgia where assays were completed. All plants were treated the same from both locations: roots or stems (when roots were impossible to collect) were wrapped in wet paper towels, placed into a plastic bag and then packed into a Styrofoam shipping box with ice. Even though the IN plants were driven and the FL plants shipped, we mimicked the same conditions for both sets. For example, the IN plants were packed in a box and left overnight in a car to mimic the experience of the shipped plants. All assays were completed within one week of collection. Each of the eight plant pairs represented a different plant family and all plant pairs were offered to all four herbivore species. Three of the comparisons represent conspecifics from the different latitudes (see Table 3.1); the rest either being conspecific or congeneric contrasts (some species cannot be identified unambiguously without flowers or seeds, which were not always present, see below).

The requirements of collecting live plants simultaneously at two distant locations and rapidly running feeding assays limits the number of contrasts that can be conducted. In contrast, collecting, immediately freezing, freeze-drying, powdering this material and reconstituting it in a gel-based food for feeding tests facilitates greater numbers of contrasts (Bolser and Hay 1996), but could involve changes in plant traits that may be artifacts of the preservation and presentation methods (Pennings et al 2001, Cronin et al. 2002, Siska et al. 2002). To determine if freeze drying, grinding to remove structural traits (but retain most chemical traits), and presenting plant material to herbivores in an
Table 3.1. Collection sites for fresh and freeze dried data sets. Superscripted numbers match plants into pairs and identify data points on figures 3.1, 3.2.

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<tr>
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<th>Longitude</th>
<th>Frost days</th>
<th>Plants collected</th>
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<td>87 13.140</td>
<td>197</td>
<td>Chara¹, Nuphar luteum³, Sagittaria⁶, Utricularia⁷</td>
</tr>
<tr>
<td>Spear Lake</td>
<td>41 21.429</td>
<td>85 39.874</td>
<td>212</td>
<td>Ludwigia², Pontederia cordata⁴</td>
</tr>
<tr>
<td>Lake James</td>
<td>41 41.272</td>
<td>85 01.960</td>
<td>229</td>
<td>Vallisneria americana⁸, Potamogeton⁵</td>
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<td>85 22.999</td>
<td>229</td>
<td></td>
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<tr>
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<td>80 40.363</td>
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<tr>
<td>Ft Cooper Park</td>
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<td>82 18.321</td>
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<tr>
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<td>28 55.844</td>
<td>81 50.590</td>
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</table>
agar-based matrix produced result paralleling those with live plants, plants from these same eight pairs were freeze dried, ground, reconstituted in an agar gel (methods of Hay et al. 1994) and, then tested for latitudinal patterns in palatability (see below for methods). Due to limited plant material for this assay, only two of the four herbivores (P. clarkii and O. rusticus) were utilized for these assays.

Given the general consistency between assays with live versus freeze-dried and reconstituted plants in the above assay (see results), we conducted another collection to increase sample size and the numbers of plant species included and conducted feeding assays using freeze-dried plant material. These assays using powdered plant material included 22 comparisons of related plants from northern versus southern sites. Northern collections came from 39-41° Latitude and southern plants came from Central Florida at 27-28° Latitude – about 1400 Km apart and experiencing 215 vs 95 days of frost/yr (Table 3.1). To conserve available plant material, we conducted assays with the three crayfish, but not the snail. Plants were collected from fourteen sites in Central Florida and nine sites throughout Indiana and frozen within 24 h to preserve freshness. Each plant collection from a site was utilized in only one assay to prevent pseudoreplication (Hurlburt 1984). However, if a plant species was collected from 2 northern sites and 2 southern sites, then 2 separate comparisons could be completed. The 22 comparisons represented 15 plant families (Alismataceae, Araceae, Ceratophyllaceae, Characeae, Haloragaceae, Hydrocharitaceae, Lemnaceae, Lentibulariaceae, Najadaceae, Nymphaeaceae, Onagraceae, Pontederiaceae, Potamogetonaceae, Saururaceae, Typhaceae); seven contrasts were repeats within families. Ideally, all comparisons would be between conspecifics, however, for many aquatic plants, species identification
depends on flower or seed characteristics (Godfrey and Wooten, 1979) which were often lacking. Therefore, plants that could not be identified to species were paired according to morphology within a genus. We know that at least 7 of the 22 comparisons were between conspecifics plants, with the remaining contrasts being between either conspecifics or congenerics. Plants were collected on July 5-8 for Indiana and July 21-24 for Florida. All plants were freeze-dried and ground using a Wiley- mill with a 60 um sieve.

3.3.3 Assays with live plants

Pieces of related higher and lower latitude plants were matched by surface area and mass and offered to herbivores in 946 ml containers. Assays were grouped into 10 blocks of replicates, where each block included one replicate of each herbivore species plus one control to monitor autogenic changes in plant mass unrelated to feeding (Roa 1992; Stachowitz and Hay 1996). Plant starting masses were corrected for autogenic change according to the formula: \( T_i \times \left( \frac{C_f}{C_i} \right) \) where \( T_i \) is the initial mass of plants available for consumption and \( C_i \) and \( C_f \) are the initial and final masses of the plants from the matching controls (Stachowitz and Hay 1996). All pieces within each block were cut from the same plant when possible, and no individual plant was used in more than one block. After 50% of one of the individual plants was consumed, or after 48 h (whichever happened first), the remaining plants were blotted and a wet mass determined. This produced assay durations of 2-48h depending on the rate at which each replicate fed. Replicates were discarded if no consumption occurred by 48 h or if all of both plants were consumed. Paired t-tests assessed differences in consumption for each contrast. We also conducted a paired t-test using the mean from each paired contrast as a single
replicate to test the overall preference for northern versus southern plants for each consumer. To evaluate across all consumers and contrasts, we scored the number of times a northern collection was significantly preferred to a southern one and the number of times a southern was preferred to a northern one versus and conducted a sign test to determine if the frequency of significant preferences differed between northern versus southern plants. One-tailed sign tests were completed as we expected a preference for northern plants.

3.3.4 Assays with freeze dried plants:

An agar food was created that matched live plant dry mass per volume. Plant densities (dry mass/volume) were calculated by measuring volumetric displacement of live plant tissue and mass of the associated freeze dried material to calculate g/ml (N=5 per plant collection). The agar gel recipe included mixing 3 ml of deionized water with enough ground plant matter to equal 10 ml of live plant. Concurrently, 7 ml of deionized water was also mixed with 0.19 grams of agar and heated until boiling (adapted from Hay et al. 1994). Agar and plant mixtures were combined and quickly spread onto assay “dominoes”. “Dominoes” were 102 by 55 mm pieces of flat PVC with 30 small indentations drilled into each side of the block. The warm agar food is scraped into the indentations where it hardens as it cools. The northern and southern plants were placed into a side of the domino labeled as such; allowing for identification of the plants during the bioassays. Feeding was quantified as the number of indentations from which crayfish removed and consumed the food. Experiments were conducted until half of one of the foods had been consumed. Replicates where either all of both plants or none of either
plant were eaten were discarded. Statistical analyses were as described for the above tests.

### 3.3 Results

When fed live plants, the snail and two of the three crayfish species significantly preferred northern versus southern plants (Figure 3.1, Table 3.2). When averaged across all plant pairs (insets in Figure 3.1, Table 3.5), the snail consumed 286% more of the northern vs southern plants, *Procambarus spiculifer* consumed 121% more of the northern plants, and *P. clarkii* consumed 71% more. Only *Orconectes rusticus* did not significantly prefer northern plants (p=0.16), but it trended in this direction, consuming 40% more northern than southern plants overall and showing significant preference for northern species in 3 pairings and no significant preference for southern species.

The eight plant pairs fed to four herbivores produced 32 feeding assays. In 15 of these contrasts, northern plants were consumed significantly more than southern congeners; no contrasts were significant in the opposite direction. Thus, 47% of all contrasts indicated northern plants were significantly more palatable, while 0% indicated that southern plants were preferred (p<0.001; 1-tailed sign test). All herbivores tested preferred northern over southern *Potamogeton* and *Sagittaria*; 3 of the 4 preferred northern over southern *Pontederia* and 2 of the 4 preferred northern over southern *Ludwigia* or *Nuphar*. When these comparisons were repeated with two of the four herbivore species, but using freeze-dried and ground plants where all structural traits would have been removed (but chemical/nutritional traits retained), both *P. clarkii* and *O. rusticus* significantly preferred northern over southern foods overall (Figure 3.2, Table 3.3). *O. rusticus* consumed 83% more northern than southern plant material, and *P.
Figure 3.1. Consumption (mean ±1SE) of northern vs southern fresh macrophytes (N=8) by four herbivore species when presented as congeneric pairs. Northern plants were collected in Indiana, and southern plants in South Florida. The sloping solid line in each figure represents the 50:50 distribution expected if there is no preference due to latitude. The filled-in symbols indicate contrasts where consumers showed a significant preference for one plant in that pair. P-values are from one-tailed paired T-tests following corrections for autogenic changes unrelated to consumption and are for the overall data set.
Table 3.2. Consumption of northern vs southern fresh plant species (N=8). Northern plants were collected in Indiana, and southern plants in South Florida. All analyses were completed on live plants corrected for autogenic changes. P-values represent two-tailed paired T-tests. Means ( SE) are provided. ID matches plant pairs in Table 3.1, and points on Figure 3.1, 3.2.

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<th>animal species</th>
<th>ID</th>
<th>plant genus</th>
<th>change north mean %</th>
<th>change south mean %</th>
<th>N</th>
<th>P-value</th>
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<td>0.01</td>
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<tr>
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<td>3 ± 3</td>
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<td>&lt;0.001</td>
</tr>
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<td>Potamogeton</td>
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</tr>
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<td>0.02</td>
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<tr>
<td></td>
<td>7</td>
<td>Utricularia</td>
<td>32 ± 12</td>
<td>34 ± 10</td>
<td>9</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Vallisneria</td>
<td>51 ± 10</td>
<td>64 ± 21</td>
<td>7</td>
<td>0.56</td>
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</table>

| O. rutilus      | 1  | Chara       | 30 ± 10             | 43 ± 5              | 7 | 0.40    |
|                 | 2  | Ludwigia    | 31 ± 8              | 30 ± 10             | 10| 0.88    |
|                 | 3  | Nuphar      | 42 ± 7              | 21 ± 8              | 9 | 0.02    |
|                 | 4  | Pontederia  | 41 ± 9              | 0 ± 4               | 10| 0.004   |
|                 | 5  | Potamogeton | 60 ± 9              | 21 ± 8              | 10| 0.01    |
|                 | 6  | Sagittaria  | 47 ± 10             | 13 ± 8              | 10| 0.03    |
|                 | 7  | Utricularia | 25 ± 10             | 41 ± 12             | 9 | 0.27    |
|                 | 8  | Vallisneria | 11 ± 9              | 36 ± 9              | 10| 0.10    |

| P. spiculifer   | 1  | Chara       | 38 ± 7              | 32 ± 7              | 8 | 0.65    |
|                 | 2  | Ludwigia    | 33 ± 13             | 24 ± 12             | 10| 0.60    |
|                 | 3  | Nuphar      | 53 ± 11             | 18 ± 5              | 10| 0.31    |
|                 | 4  | Pontederia  | 57 ± 10             | 6 ± 4               | 9 | <0.001  |
|                 | 5  | Potamogeton | 52 ± 8              | 5 ± 8               | 10| <0.001  |
|                 | 6  | Sagittaria  | 60 ± 7              | 13 ± 7              | 10| 0.002   |
|                 | 7  | Utricularia | 38 ± 9              | 34 ± 9              | 10| 0.72    |
|                 | 8  | Vallisneria | 32 ± 11             | 33 ± 10             | 10| 0.93    |

| P. polydora     | 1  | Chara       | 40 ± 10             | 17 ± 6              | 7 | 0.13    |
|                 | 2  | Ludwigia    | 80 ± 7              | 2 ± 3               | 10| <0.001  |
|                 | 3  | Nuphar      | 26 ± 8              | -3 ± 2              | 8 | 0.01    |
|                 | 4  | Pontederia  | 56 ± 9              | -3 ± 2              | 2 | 0.13    |
|                 | 5  | Potamogeton | 15 ± 6              | 0 ± 4               | 8 | 0.04    |
|                 | 6  | Sagittaria  | 64 ± 9              | 0 ± 3               | 9 | <0.001  |
|                 | 7  | Utricularia | 23 ± 8              | 10 ± 7              | 10| 0.37    |
|                 | 8  | Vallisneria | 42 ± 10             | 68 ± 10             | 6 | 0.15    |
*clarkii* consumed 68% more. For the 16 feeding assays constituting these trials, northern plants were significantly preferred in 10 (63%) of the contrasts and southern plant in none (p=0.004; 1-tailed sign test). For 12 of the 16 feeding assays, results from the assays with powdered plant material paralleled results from experiments using whole plants (compare Figures 3.1, 3.2). There were four exceptions: *Chara* showed no latitudinal pattern in assays with whole plants but northern collections were preferred by both consumers in assays with ground plants; the same pattern occurred for *Ludwigia* when fed to *O. rusticus*; in contrast to the above, the preference of *O. rusticus* for northern *Nuphar* when fed whole plants was lost when the plants were ground to a fine powder. Thus, in assays with ground plants, consumers preferred northern over southern plants as consistently as or more consistently than in assays using whole plants, suggesting that most preferences were driven by plant chemistry rather than plant structure, since preferences were retained following loss of structural traits.

The logistics of collecting and simultaneously feeding consumers live plants from distant areas constrain the number of comparisons that can be completed. To test a larger number of contrasts, we collected 22 pairs of related plants from northern and southern locations, quickly froze and then freeze-dried these, powdered the dried material and fed these pairs of plants to the three species of crayfishes in the lab. However, for these collections, we compared plants from Indiana to plants from Central Florida instead of plants from Southern Florida, while this difference was only about 350 Km further north than the South Florida collections, the number of days of frost/yr increased from zero to 95 when moving from southern to Central Florida. For the 22 comparisons, *P. clarkii* preferred northern plants in 7 assays, southern plants in 7 assays, and neither in 8 assays,
Figure 3.2. Consumption of northern vs southern freeze dried and ground macrophytes (N=8) offered as congeneric pairs of plants. Due to limitations of plant material, we made this contrast for only 2 of the 4 herbivores used in Figure 3.1. Statistical tests and symbols are as in Figure 3.1.
producing an overall lack of preference across latitudes ($p=0.36$; Figure 3.3, Tables 3.4, 3.5). *P. spiculifer* preferred northern plants in 8 assays, southern plants in 3, and neither in 13 assays, again producing no significant overall pattern of preference as a function of latitude. In contrast, *O. rusticus* (that failed to prefer northern plants in whole plant assays; Figure 3.1) significantly preferred northern over southern plants in this assay ($p=0.01$, Figure 3.3, Tables 3.4, 3.5) – showing a significant preference for northern plant pairs in 5 assays and never preferring southern plants. Pooling across all contrasts within a herbivore species, *O. rusticus* consumed 24% more of the northern plants ($p=0.01$), *P. spiculifer* consumed 22% more of the northern plants ($p=0.12$), and *P. clarkii* consumed

<table>
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<th>mean % change south</th>
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Figure 3.3. Consumption of northern vs southern freeze-dried and ground macrophytes (larger dataset; N=22) offered as congeneric pairs of plants. Northern plants were collected in Indiana, and southern plants in central Florida. Statistical tests and symbols are as in Figure 3.1.
Table 3.4. Consumption of northern vs southern ground plants (larger dataset; N=22). Northern plants were collected in Indiana, and southern plants in Central Florida. All analyses were completed on freeze dried and ground plants. P-values represent two-tailed paired T-tests. Means (± SE) are provided.

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<tr>
<td>Sagittaria</td>
<td>19</td>
<td>57 ± 9</td>
<td>27 ± 6</td>
<td>9</td>
<td>0.037</td>
<td>67 ± 7</td>
</tr>
<tr>
<td>Sagittaria</td>
<td>20</td>
<td>63 ± 11</td>
<td>10 ± 6</td>
<td>8</td>
<td>0.009</td>
<td>53 ± 6</td>
</tr>
<tr>
<td>Arum</td>
<td>21</td>
<td>37 ± 6</td>
<td>17 ± 4</td>
<td>15</td>
<td>0.006</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>Saururus</td>
<td>22</td>
<td>50 ± 6</td>
<td>17 ± 5</td>
<td>8</td>
<td>&lt;0.001</td>
<td>57 ± 9</td>
</tr>
</tbody>
</table>
only 6% more of the northern plants (p=0.36). If one compares frequency of significant preference for northern versus southern plants across all 66 assays run for these 3 herbivores, then a consumer preferred the northern plant material in 20 (30%) of 66 contrasts and preferred southern plant material in 10 (15%) contrasts (p=0.05; 1-tailed sign test).

3.5 Discussion

Assays with intact plants and powdered material of these same plants indicated that northern species and populations are more palatable than southern ones. When we offered 3 crayfish and 1 snail species whole plants collected about 1700 Km apart on a north-south axis, 3 of the 4 herbivores strongly preferred the northern plants, consuming 71-286% more of the northern plants on average (Figure 3.1). Even the fourth species consumed a non-significant 40% more of the northern plants. When we removed plant structural traits but retained chemical traits by grindings plants to a fine powder, imbedding this material in agar, and feeding it to two crayfishes, even the species that did not feed selectively in the whole plant assays strongly preferred the northern plant tissues (Figure 3.2), suggesting that the southern plants possess stronger chemical deterrents to feeding. These findings parallel findings of greater palatability and lesser chemical defenses of higher versus lower latitude seaweeds (Bolser and Hay 1996) and saltmarsh plants (Pennings et al. 2001, 2007, 2009, Siska et al. 2002). Overall, it appears that tropical consumers have selected more strongly for prey chemical defenses - resulting in tropical prey being better defended and less palatable than their temperate relatives (Levin 1976, Coley and Aide 1991, Bolser and Hay 1996, Siska et al. 2002, Kicklighter and Hay 2006, Pennings et al. 2009). In contrast to our consistent findings for live plants
Table 3-5. Grand means (± SE) comparing consumption of northern vs. southern plants. P-value represents one-tailed paired T-tests.

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Grand Mean Live Plants (N=8)</th>
<th>Grand Mean Freeze dried &amp; Ground Plants (N=8)</th>
<th>Grand Mean Freeze dried &amp; Ground Plants (N=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>North</td>
<td>South</td>
</tr>
<tr>
<td>P. clarkii</td>
<td>8</td>
<td>55 ± 6</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>O. rusticus</td>
<td>8</td>
<td>36 ± 5</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>P. spiculifer</td>
<td>8</td>
<td>45 ± 4</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>P. paludosla</td>
<td>8</td>
<td>43 ± 8</td>
<td>11 ± 8</td>
</tr>
</tbody>
</table>
and dried tissues from these plants (Figures 3.1, 3.2), our tests using dried and powdered material from a larger number of species collected from Indiana versus Central Florida (350 Km north of the previous Florida collections) were considerably less consistent and effect sizes were smaller (Figure 3.3).

We can envision three hypotheses to explain why the strength and consistency of our results varied between the contrasts using plants from South Florida versus Central Florida. First, freeze drying the plants may have compromised, or eliminated, some plants defenses, and compromised patterns that might have been apparent with live plants (Pennings et al. 01, Cronin et al. 2002, Siska et al. 2002); however, our relatively consistent findings for live plants and freeze-dried material of the same plants (Figures 3.1, 3.2) argues against this hypothesis. Second, the live plants from South Florida were collected 350 kilometers further south than the plants from Central Florida (the Everglades versus Orlando). This is a relatively small decrease in total distance, but it represents a large difference in the frequency of cold days each year; the Everglades experiences zero days/yr of frost while Central Florida experiences 95 days/yr of frost (Commerce Department 2005). By contrast Indiana experiences 215 days/yr of frost. Given that most ectothermic herbivores feed less or become inactive as temperatures decline (Garton and Stickle 1980, Seals et al. 1997) this large difference in frost frequency may result in less selection for herbivore deterrent traits among plants in Central versus Southern Florida. At higher latitudes, the shorter growing season for ectothermic herbivores may reduce their impact on aquatic macrophytes and limit selection for plant defenses; however, more research is needed regarding how herbivore pressure varies as a function of frost-free days/yr. Finally, spatial variance in herbivory
among freshwater habitats could select for among-site variance in plant palatability within each latitudinal area, and that the greater diversity of habitats from which we made our Central Florida collections may have added variance that prevented detection of significant differences between latitudes. Freshwater habitats have been compared to islands, with limited interactions of organisms between neighboring units (Jackson et al. 2001, Hillebrand 2004, Covich 2009). This could lead to a substantial variability in biotic interactions among sites within a latitude or region. For example, Jackson et al (2001) found completely different assemblages of cyprinids in lakes with and without top predators. Freshwater environments can be highly variable with differences in origin (Covich 2009), ph, nutrient availability, oxygen concentrations (Santamaria 2002), temperature, wind, precipitation, geomorphology, light, substrate, and salinity (Lacoul and Friedman 2006). This among site variation in physical and biotic conditions may select for spatial variance in phenotypes or genotypes (Santamaria 2002), possibly overriding latitudinal patterns. Most contrasts of palatability across latitude assume that lower latitude prey have been selected for greater defenses (Coley and Aide 1991, Bolser and Hay 1996, Kicklighter and Hay 2006, Pennings et al. 2001). Salgado and Pennings (2005) used common garden transplantation experiments to demonstrate that the differences they documented were genetic; however, this need not always be the case. The patterns we have documented among freshwater plants could be an evolutionary adaptation to increased herbivore pressure, but they might also result from induced responses in ecological time rather than genetic responses over evolutionary time. Many plants induce defenses in response to recent herbivore attack (Karban and Baldwin 1997), and induced defenses do occur in some aquatic plants (Bolser and Hay 1998, Morrison
and Hay in review), but there are too few studies to assess whether this is common or rare. If herbivores were more active in Florida than in Indiana prior to our plant collections, then it is conceivable that more of our Florida plants were induced and that the lower palatability we documented resulted from differences in induction rather than selection.

In summary, our results show that palatability of freshwater plants to herbivores generally increases with increasing latitude. When we contrasted 8 confamilial plants collected in Indiana (150 frost free days) versus Southern Florida (365 frost free days), three of four herbivores significantly preferred northern plants. This significance was upheld (or increased) when the same plants were freeze-dried, ground and re-offered as agar gels – thus, suggesting greater chemical defenses among the lower latitude plants. When we evaluated palatability of a second set of freeze-dried plants collected from Indiana (150 frost free days) versus Central Florida (270 frost free days), only one of three herbivores preferred the northern plants. Given these results, we suggest that the decrease in palatability at lower latitude is due to either the impact of winter (frost days) on the duration and activity of herbivory or to increased patchiness of herbivore pressure across freshwater habitats and the evolutionary or ecological (induced) response to this variance.

3.6 References


CHAPTER 4
HERBIVORES PREFER PLANTS THAT ARE EVOLUTIONARILY NAÏVE: HERBIVORE PHYLOGEOGRAPHY AND PREFERENCE FOR NATIVE VS EXOTIC PLANTS

4.1 Abstract

The enemy release and biotic resistance hypotheses predict that native herbivores will either facilitate or hinder, respectively, the spread of exotic species. Enemy release predicts that native herbivores will avoid novel, non-native plants, while biotic resistance predicts the opposite – that native herbivores will prefer exotic plants that have not been selected to deter these herbivores. Using five herbivore species and nine congeneric pairs of native and exotic plants, we tested the predictions of these hypotheses. Four of five herbivores preferred exotic over native plants. Three species of South American apple snails (*Pomacea sp.*) preferred North American over South American macrophytes. In contrast, the North American crayfish *Procambarus spiculifer* preferred South American over North American macrophytes. Both patterns suggest that biotic resistance may affect invading plants more than enemy release and that generalist herbivores prefer naïve, non-native plants that have thus not been selected to deter native herbivores. Apple snails have their center of diversity in South America, but a single species (*Pomacea paludosa*) occurs in North America, with its range limited primarily to South Florida. This species with a South American lineage but a North American distribution did not differentiate between South American and North American plants. However, its
preferences among confamilial plant pairs correlated with preferences of its South American relatives rather than with preferences of the North American crayfish, suggesting that its feeding preferences are affected more by its lineage than its present distribution. General tests of the roles of plant structure, chemistry, and protein content in affecting feeding indicated that the crayfish was responding primarily to plant structure while the apple snails were responding primarily to plant chemistry; herbivore preferences were not correlated with plant protein content.

4.2 Introduction

Non-native species are a problem world-wide due to their disruption of native ecosystems and the economic and environmental costs they produce (Pimentel et al. 2005, Byrnes et al. 2007). Approximately 50,000 non-native species have been introduced into the United States; these non-native species incur an economic cost estimated at $120 billion per year (Pimentel et al. 2005). Invasive species occur in and impact all habitat types (Parker et al. 1999), but appear to have greater impacts on freshwater than on terrestrial ecosystems (Sala et al. 2000).

There are many hypotheses regarding the predictors of species invasions, their dynamics of establishment, and their patterns of spread (Kolar and Lodge 2001, Hayes and Barry 2008). However, two prominent and alternative theories address how interactions between herbivores and plants may exacerbate or retard the invasion and spread of non-native plants - the enemy release hypothesis and the biotic resistance hypothesis. The enemy release hypothesis postulates that when non-native plants enter novel environments, their natural enemies are no longer present and this release from co-evolved enemies allows the exotics to increase in abundance and distribution (Maron and
The biotic resistance hypothesis suggests that native species function as natural enemies (consumers, pathogens, competitors) of non-native invaders and suppress their establishment and spread in the new habitat (Maron and Vila 2001, Levine et al. 2004, Parker et al. 2006). These two hypotheses predict opposite responses of native herbivores toward native versus exotic plants. The enemy release hypothesis predicts that native herbivores will prefer native plants, giving exotics a competitive advantage. Conversely, biotic resistance predicts that native herbivores will prefer exotic plants (which are evolutionarily naïve to native herbivores and have not been selected to deter these herbivores), helping to stop their establishment and spread.

Support for enemy release has been documented via preference for native plants over exotic plants. Fields tests and common garden experiments with terrestrial plants have sometimes shown higher insect damage on native vs exotic species (Siemann and Rogers 2003, Agrawal et al. 2005, Carpenter and Cappuccino 2005, Parker and Gilbert 2007, Liu et al. 2007). Only Parker and Gilbert (2007) identified the herbivores as either generalists or specialists, and noted that the differential herbivory was due to generalist insect herbivores. Similarly, a snail and deer also preferred native over exotic plants (Xioang et al. 2008, Eschtruth and Battles 2009). Conversely, Parker and Hay (2005) found that several generalist herbivores (crayfish, slugs, grasshoppers) from a variety of environments selectively consumed exotic in preference to native plants in laboratory feeding assays. Moreover, in a meta-analysis of 68 field experiments, Parker et al. (2006) found that native herbivores generally suppressed non-native plants and that non-native herbivores generally suppressed native plants and facilitated the invasion of non-native plants, especially those from their own native region. Thus, they suggested that
herbivores were selectively feeding on evolutionary naïve plants that had not co-evolved with native herbivores and thus had no opportunity to be selected for defense. It is unclear what drives the variance in the results discussed above, but three hypotheses exist. First, the impact of generalist and specialist herbivores on plants may differ as generalist often have a larger impact on plant population dynamics than specialists (Parker et al. 2006). Additionally, phylogenetic isolation (when native herbivores do not co-occur with a close relative of the exotic plant that may share its defensive traits) (Ricciardi and Ward 2006, Hill and Kotanen 2009, Dawson et al. 2009), and “invasiveness” (i.e. the impact and spread) (Cappuccino and Carpenter 2005) of the non-native plant may be important in determining its interaction with the native community (see discussion).

We focused on testing the response of generalist herbivores to native vs non-native plants using aquatic herbivores and macrophytes from North and South America. We determined herbivore preference for nine pairs of confamilial native and exotic plants using one crayfish and one apple snail native to the United States and three apple snails native to South America. We also conducted analyses of possible plant traits (chemical, structural, nutritional) influencing feeding preference by determining if preference for live plants was correlated with 1) preference for plants that had been dried, ground to a fine powder, and imbedded in a gel-matrix (thus removing structural but retaining chemical and nutritional traits), 2) preference for extracts from plants coated on top of ground lettuce and offered in an artificial agar food (thus retaining only chemical traits), or 3) plant protein concentrations. By using a suite of herbivores (apple snails) whose distribution is primarily South American, but that has one species native to the southern
most region of the United States (South Florida), we were also able to conduct an initial
assessment of the possibility that phylogenetic history of the herbivore (the history of
South American evolution) will override recent ecological and evolutionary history (one
species’ occurrence in only North America) and result in it retaining preferences more
similar to its South American relatives.

4.3 Methods

4.3.1 Collections

Crayfish and apple snails are omnivores capable of drastically impacting
The crayfish, *Procambarus spiculifer*, is native to the southeastern United States
(including Mississippi, Alabama, Florida, Georgia, and South Carolina) (Carnegie
Museum of Natural History; Global Crayfish Resources. http://iz.carnegiemnh.org/).
This crayfish had previously demonstrated preference for exotic over native plants
(Parker and Hay 2005), however, as preferences can change (Agarawal et al. 2005), we
re-evaluated this species. Adult crayfish were collected from the Chattahoochee River in
Atlanta. The offspring from these crayfish were fed commercial herbivore food and
frozen shrimp until large enough to be utilized in bioassays. All apple snail species are
currently present in South Florida, but three are native to the South America (*Pomacea
canaliculata* to Argentina, Bolivia, Paraguay, Uruguay and Brazil; *P. hastrum* to Brazil,
Peru and Bolivia; and *P. insularum* to Argentina, Brazil, Bolivia, Uruguay and Paraguay)
and only one species (*P. paludosa*) is native to North America (Rawlings et al. 2007).
*Pomacea paludosa* and *P. insularum* were collected as eggs; *P. insularum* from Lake
Lure (N 31° 33.210’ W 82° 28.947’) in Georgia and Lake Tohopekaliga (N 28° 13.033 W
81° 22.53) in Florida, and *P. paludosa* from Lake Tohopekaliga in Florida. Adult *P. canaliculata* were obtained from Neighborhood Fish Farm in Miami Florida; *P. haustrum* were obtained from Paradise Aquatics in Winterhaven Florida. These species produced viable eggs that hatched in the lab; juveniles hatched from eggs were used for all experiments. Snails were reared on lettuce until they reached a size where they could be utilized in assay experiments. All species were identified according to characteristics of their eggs (Rawlings et al. 2007). Because juveniles are difficult to identify, species were held separately in labeled tanks. All snails used in experiments were hatched between 2 June and 29 July 2008. Crayfish were housed in 946 ml individual containers placed in a 180x90 cm flow through water table. Snails were housed in 38 L tanks until used in feeding assay; for assays, they were transferred to 946 ml containers. Replicates of all assays were in separate containers to assure independence.

Nine pairs of congeneric native and exotic plants were utilized (Table 4.1). Distributions (native vs exotic) were determined using the USDA Germplasm Resources Information Network (GRIN) as this was the best reference for North and South American plants. There is uncertainty surrounding the native distribution of *Ludwigia hexapetala* and *Pistia stratiotes*. Both species are listed as non-native by the Atlas of Florida Vascular Plants (Wunderlin and Hansen 2008), and were considered exotic by Parker and Hay (2005); however, GRIN lists them as native to S. Florida. Results are thus presented both with and without these comparisons included. Plants were considered native to the South American snails if the native distribution of the snail overlapped with the native distribution of the plant. Two of the plants considered “exotic” to the South American snails were listed as native in either Colombia or
Venezuela. As *Pomacea* are not listed as native in these countries, we assumed there was no historical overlap of *Pomacea* apple snails with these plant species and that they would be “novel” to the snails. We were able to collect nine pairs of related plants where one was native to North America and one was exotic (see Table 4.1). Only four of these nine pairs represented a native and an exotic species pairing from the perspective of the South American herbivores (Table 4.1). When possible, related pairs of plants were collected from the same location to minimize confounding effects due to local conditions (see Table 4.1), however, this was not possible for four of the comparisons. All plants were either used within 24 hours of collection or planted in 72 L tubs and grown in a greenhouse at the Georgia Institute of Technology until needed.

### 4.3.2 Assays

Pieces of confamilial native and exotic plants were matched by surface area and mass and offered to herbivores in 946 ml containers. Assays were grouped into 10 blocks of replicates, where each block included one replicate of each herbivore species plus one control to monitor autogenic changes in plant mass unrelated to feeding (Roa 1992; Stachowitz and Hay 1996). Plant starting masses were corrected for autogenic change according to the formula: \( T_i \times \left( C_f/C_i \right) \), where \( T_i \) is the initial mass of plants available for consumption by the herbivores and \( C_i \) and \( C_f \) are the initial and final masses of the plants from the matching controls (Stachowitz and Hay 1996). All pieces within each block were cut from the same plant when possible, and no individual plant was used in more than one block. After 50% of one of the plant species was consumed or after 5 days (whichever happened first) the assay was stopped for that replicate. Remaining plants were blotted dry at the end of the assay and a wet mass determined. This produced assay
Table 4.1. Plants utilized for comparisons of native and exotic congeneric pairs. Native distributions based on USDA Germplasm Information Network database. Superscript numbers represent collection sites. 1 Clayton County Water Authority, 2 Arizona Aquatic Gardens, 3 Chattahoochee River, 4 Texas, 5 Lake Lanier, 6 Piedmont College.

<table>
<thead>
<tr>
<th>PAIR #</th>
<th>NATIVE PLANT</th>
<th>NATIVE DISTRIBUTION</th>
<th>EXOTIC PLANT</th>
<th>EXOTIC DISTRIBUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td><em>Pontederia cordata</em></td>
<td>US, Brazil, Bolivia, Argentina, Paraguay, Uruguay, Colombia, Equador</td>
<td><em>Eichhornia crassipes</em></td>
<td>Venezuela, Brazil, Guyana, Suriname</td>
</tr>
<tr>
<td>2a</td>
<td><em>Myriophyllum pinnatum</em></td>
<td>US, Canada, Africa, Asia, Europe</td>
<td><em>Myriophyllum simulans</em></td>
<td>Australia</td>
</tr>
<tr>
<td>3a</td>
<td><em>Orotonium aquaticum</em></td>
<td>US</td>
<td><em>Colocasia esculenta</em></td>
<td>Tropical Asia</td>
</tr>
<tr>
<td>4a</td>
<td><em>Peltandra virginica</em></td>
<td>Canada, US</td>
<td><em>Colocasia esculenta</em></td>
<td>Tropical Asia</td>
</tr>
<tr>
<td>5a</td>
<td><em>Vallisneria americana</em></td>
<td>US, Meso America, Venezuela</td>
<td><em>Hydrilla verticillata</em></td>
<td>Asia</td>
</tr>
<tr>
<td>5a</td>
<td><em>Vallisneria americana</em></td>
<td>US, Meso America, Venezuela</td>
<td><em>Egeria densa</em></td>
<td>Brazil, Argentina, Uruguay</td>
</tr>
<tr>
<td>7a</td>
<td><em>Myriophyllum heterophyllum</em></td>
<td>US</td>
<td><em>Myriophyllum aquaticum</em></td>
<td>Brazil, Argentina, Bolivia, Equador, Peru, Chile, Paraguay</td>
</tr>
<tr>
<td>8a</td>
<td><em>Peltandra virginica</em></td>
<td>Canada, US</td>
<td><em>Pistia stratiotes</em></td>
<td>FL, TX, Africa, Brazil, Argentina</td>
</tr>
<tr>
<td>9a</td>
<td><em>Ludwigia palustris</em></td>
<td>US, Mexico, Costa Rica, Guatemala, Colombia</td>
<td><em>Ludwigia hexapetala</em></td>
<td>FL, SC, TX, Guatemala, Brazil, Paraguay, Argentina</td>
</tr>
<tr>
<td>6b</td>
<td><em>Egeria densa</em></td>
<td>Brazil, Argentina, Uruguay</td>
<td><em>Vallisneria americana</em></td>
<td>US, Meso America, Venezuela</td>
</tr>
<tr>
<td>7b</td>
<td><em>Myriophyllum aquaticum</em></td>
<td>Brazil, Argentina, Bolivia, Equador, Peru, Chile, Paraguay</td>
<td><em>Myriophyllum heterophyllum</em></td>
<td>US</td>
</tr>
<tr>
<td>8b</td>
<td><em>Pistia stratiotes</em></td>
<td>FL, TX, Africa, Brazil, Argentina</td>
<td><em>Peltandra virginica</em></td>
<td>Canada, US</td>
</tr>
<tr>
<td>9b</td>
<td><em>Ludwigia hexapetala</em></td>
<td>FL, SC, TX, Guatemala, Brazil, Paraguay, Argentina</td>
<td><em>Ludwigia palustris</em></td>
<td>US, Mexico, Costa Rica, Guatemala, Colombia</td>
</tr>
</tbody>
</table>
durations of 1-5 days for each replicate depending on the rate of feeding. If no consumption occurred by 5 days or if all of both plants were consumed between times checked, that replicate was discarded because it provided no information on the relative preference for the plants in that replicate. Paired T-tests evaluated differences in consumption for each native vs exotic contrast. A second paired t-test using the mean from each paired contrast as a single replicate, evaluated the overall preference of each consumer for native versus exotic plants.

We were also interested in determining if plant palatability was correlated with plant structural, chemical, or nutritional traits. Due to a limited amount of plant matter, we were unable to run these tests with all herbivore species, so included the crayfish species (*P. spiculifer*), the North American snail species (*P. paludosa*) and the fastest eater among the South American snail species (*P. insularum*). To destroy structural traits but retain chemical and nutritional traits, plants were freeze-dried, ground with a Wiley Mill until particles could pass through a 60um mesh, and these ground particles reconstituted into a gel-based food as described in Hay et al. (1998). To assess the effects of chemical traits unrelated to structural and nutritional traits, freeze dried plants were extracted 3-4 times for 1-2h each time in a 2:1 mixture of dichloromethane: methanol and this extract coated onto freeze-dried lettuce to create an artificial food in a gel-matrix (Bolser and Hay 1998). Masses of lettuce and extract were varied so as to match the dry mass per volume of the natural plants being evaluated. Plant densities (dry mass/volume) were calculated by measuring volumetric displacement of live plant tissue and mass of the associated freeze dried material to calculate g/ml (N=5 per plant species). The agar gel recipe included mixing 3 ml of deionized water, enough ground plant matter to equal
10 ml of live plant, and then 0.19 g of agar in 7 ml of boiling deionized water (adapted from Bolser et al. 1998). Agar and plant mixtures were combined and quickly spread into either a fiberglass mold with window screen underneath (see Hay et al. 1998 for illustration) or into assay “dominoes.” Dominoes were 102 by 55 mm pieces of flat PVC with 30 small indentations drilled into opposite halves of each block. The warm agar food was scraped into the indentations where it hardened as it cooled. The native and exotic plants being compared were randomly assigned to opposite ends of a domino and ends labeled to allow identification at the end of each bioassay. Feeding was quantified as the number of indentations from which crayfish removed and consumed the food. Dominos proved to be a good methodology for crayfish, whose sloppy feeding makes measurement of consumption from fiberglass screen gels difficult. The fiberglass mold was appropriate for apple snails because their radulas could more effectively graze from the flat surface of the gel than from the holes in the dominoes.

Preferences were converted to a single number by calculating the proportion consumed that was exotic from a North American perspective (grams of exotic consumed divided by the sum of the grams of exotic and native plants combined). Correlations were completed between the results from the live plants and ground plants or live plants and extracts from the plants to determine the influence of structural and chemical characteristics. Similarly, protein content was measured using a modified Bradford assay (methods in Prusak et al. 2005) and correlated with live plant preferences. This provides a crude measurement of the importance of structural, nutritional (as measured by protein) and chemical characteristics.
4.4 Results

The native crayfish *P. spiculifer* strongly preferred exotic plants when offered confamilial pairs of native and non-native plants (N=9 P=0.006; Figure 4.1a). The preference for exotic plants remained significant when the two plants with questionable native distributions were excluded (N=7 P=0.003; Figure 4.1a). All of the South American snails (*P. canaliculata, P. insularum, P. haustrum*) showed a preference for North American (exotic to them) over South American (native to them) plants (N=4 P=0.003; N=4 P<0.001; N=4 P=0.009; respectively; Figure 4.1b-d). The single apple snail species native to North America (*P. paludosa*) showed no preference for either native or exotic plants (N=9 P=0.28; or N=7 P=0.61, Figure 4.1e) when plants were considered as native or exotic to the Southeastern US (the only area in which this species has occurred). All *Pomacea* snails showed the same general pattern of plant preferences. Of the 36 comparisons (9 plants x 4 snails), snails showed the same significant preference in 92% of the comparisons. Where they did not agree (3 cases or 8% of the contrasts), the difference was due to a lack of a significant choice in one of the South American snails and not a switching of preferences. This occurred when *P. haustrum* showed no preference for either *Eichhornia* or *Hydrilla*, and *P. insularum* showed no preference for *Colocasia* over *Orontium*. Preferences of the native snail, *P. paludosa*, were strongly correlated with preferences of the three South American congenerics (Figure 4.2 b-d) but not correlated with preferences of the North American crayfish *P. spiculifer* (Figure 4.2a).
Figure 4.1. Consumption (mean ± 1SE) of native vs exotic macrophytes by five herbivore species when presented as confamilial pairs. The sloping solid line in each figure represents the 50:50 distribution expected if there is no preference for either native or exotic plants. The filled-in symbols indicate contrasts where consumers showed a significant preference for one plant in that pair. Inset histograms show the mean consumption of the exotic and native species. P-values are for the pooled data shown in the histogram and are from two-tailed paired T-tests. The triangles present in a) and e) represent comparisons including *Ludwigia hexapetala* and *Pistia stratiotes*, plants whose native distribution is in question. P-values for these two graphs are provided with (N=9) and without (N=7) these two data points.
The crayfish *P. spiculifer* showed no correlation between live plant preference and preference toward ground plants or preference toward extracts from these plants (N=8, $r^2 = .16$, P= 0.33; and N=8, $r^2 = 0.00$, P=.94, respectively) suggesting that this species is not strongly affected by plant chemical traits and may be responding to plant structural characteristics. Conversely, snail preference for live plants was correlated with...
preference for ground plants (N=8, r² = 0.96, P<0.001; N=8, r² = 0.83, P= 0.002; for P. paludosa and P. insularum, respectively). Correlations between live plant preference and preference toward extracts from plants were significant for P. insularum (N=8, r² = 0.64, P=.02), and nearly so for P. paludosa (N=8, r² = 0.45,P=.07). These patterns suggest that the snails are more strongly affected by plant chemical traits. None of the tested species showed a correlation between preference and protein concentration of the test plants (N=8, r² = 0.05, P= 0.60; N=8, r² = 0.05, P= 0.59; N=8, r² = 0.24, P= 0.21; for P. paludosa, P. insularum and P. spiculifer, respectively).

4.5 Discussion

Both the crayfish native to North America and the three snails native to South America preferred exotic over native plants (Figure 4.1). However, the lack of a preference by the North American apple snail species (P. paludosa) for either native or non-native species and its preferences correlating closely with those of South American apple snails, suggests that there may be considerable evolutionary inertia and that the preferences of P. paludosa result more from evolutionary lineage than recent ecology. We measured only herbivore feeding preference rather than impact in the field, but previous studies showing a preference of native herbivores for non-native plants (Parker and Hay 2005) have been consistent with impacts occurring in the field (Parker et al. 2006 but see Best and Arcese 2009). Parker et al. (2006) found that native herbivores selectively suppressed non-native plants, and that non-native herbivores selectively impacted native plants and facilitated non-native plants from the herbivore’s native region. Thus, non-native plants appeared to be following their native, generalist herbivores into new regions rather than escaping them. However, competition and other
factors will also affect plant invasions in field settings (Carpenter and Cappuccino 2005, Best and Arcese 2009).

Our results are consistent with those of Maron and Vila (2001), Parker and Hay (2005), and Parker et al. (2006) in demonstrating that native herbivores prefer non-native plants. However, other studies have found herbivores preferring native over exotic plants (Sieman and Rogers 2003, Agrawal et al. 2005, Carpenter and Cappuccino 2005, Liu and Stiling 2006, Parker and Gilbert 2007, Liu et al. 2007, Xiaoang et al. 2008, Eschutrush and Battles 2009). Carpenter and Cappuccino (2005) suggest that this dichotomy could be caused by inclusion of exotic plants that are not highly invasive; they noted a relationship between herbivore damage and invasiveness (Carpenter and Cappuccino 2005, Jogesh et al. 2008). They found that invasive plants were more likely to have unique chemical defenses that deterred native herbivores (Cappuccino and Arnason 2006), and suggested that inclusion of less invasive plants might obscure support for the enemy release hypothesis. Our findings are unlikely to be explained by this hypothesis given that many of the exotic species we utilized are highly invasive. On average, the non-native plants we used are listed as a weed for 6 ± 6 U.S. states (Ranges from 0 for *M. simulans* to 21 for *Hydrilla*), and one plant (*Eichhornia*) is listed as one of the top 100 worst invasive species (Lowe et al. 2000). Additionally, a meta-analysis of field experimental results failed to find a relationship between plant invasiveness and herbivore impact (Parker et al. 2006).

Our results support the biotic resistance hypothesis and not the enemy release hypothesis. These two hypotheses predict opposite responses of native herbivores to native versus exotic plants. On average, biotic resistance suggests that non-native plants
have not been selected to resist native herbivores and will be selectively consumed by
native herbivores (Maron and Vila 2001, Levine et al. 2004, Parker et al. 2006); in
contrast, the enemy release hypothesis predicts that native herbivores will avoid non-
native plants since they have traits that the native herbivores have not evolved to tolerate
(Maron and Vila 2001, Keane and Crawley 2002). Our findings that herbivores
selectively attack evolutionary naïve plants with which they have not previously co-
occurred is consistent with biotic resistance, and counter to predictions of the enemy
release hypothesis.

Investigators documenting support for the enemy release hypothesis via herbivore
preference for native plants note that preference for natives accounts for a very small
percentage of the variance in preferences (Liu et al 2007, Carpenter and Cappuccino
2005), and may not lead to differential mortality (Sieman and Rogers 2003, Parker and
Gilbert 2007). This suggests that while low palatability of exotics may be important in
some cases, it is not the primary mechanism accounting for the spread and survival of
invasive plants. Other characteristics besides, or in conjunction with palatability, have
been found to be important for the establishment and spread of exotics including
tolerance to grazing (Ashton and Lerdau 2008), faster growth or higher fecundity
(Callaway and Maron 2006), a positive response to disturbance (Eschtruth and Battles
2009), and facilitation by non-native herbivores (Parker et al. 2006).

We note that our study tested confamilial pairs of native and exotic plants.
Research suggests that herbivore familiarity with a relative of the invasive species can
impact preference. However, there is conflicting information on the direction of this
relationship. Some studies indicate that herbivores prefer phylogenetically novel plants
(Hokkanen and Pimentel 1989, Ricciardi and Ward 2006) while others indicate an avoidance of phylogenetically novel plants (Hill and Kotanen 2009, Dawson et al. 2009). Hokkanen and Pimentel (1989) found that successful biological control agents were often novel enemies who have no history of co-evolution. Additionally Ricciardi and Ward (2006) show that exotic plants without native congeners have a lower survival when attacked by either vertebrate or invertebrate herbivores when compared to exotic plants with native congeners. Conversely, Hill and Kotanen (2009) and Dawson (2009) looking at leaf damage by herbivores found increased herbivore damage on plants with a higher number of confamilial native species. The former studies show a negative impact (decreased survival) while the later shows a positive impact (decreased herbivory) of being phylogenetically novel. This discrepancy in results could be due to the methodology implemented: both Hill and Kotanen (2009) and Dawson (2009) measure leaf damage by insects, but Ricciardi and Ward examine plant survival in response to vertebrate and invertebrate herbivory. When herbivores affect plant survival by removing entire plants, this does not leave a record of their effect (leaf damage) and may result in a biased estimate of impact when leaf damage alone is assessed.

There was no correlation in plant preference between the one snail native to North America (P. paludosa) and the North American crayfish P. spiculifer; however, there were significant correlations between the preference of P. paludosa and the three South American snails. The strongest correlations were between the snail native to North America (P. paludosa) and its closest relatives in South America: both P. insularum and P. canaliculata are sister species to P. paludosa (Rawlings et al. 2007). This suggests that P. paludosa is responding to preferences formed historically when the genus was
evolving in South America. No estimate exists as to when *P. paludosa* split from the rest of the Pomacea family (T. Collins, FIU pers comm.), but the close genetic relationship between *P. paludosa* and its sister species (Rawlings et al. 2007) suggest the divergence was recent. These results agree with earlier assertions that phylogenetic history can impact herbivore preferences. However, previous papers have concentrated on the phylogenetic history of the exotic prey species (i.e. are close relatives present in the new environment). We expand this idea to include not only the phylogenetic history of the exotic prey species (Cappuccino and Carpenter 2005, Strauss et al 2006, Hill and Kotanen 2009, Dawson et al. 2009) but also the phylogenetic history of the native consumer.

Interestingly, our results show both generalist crayfish and snails preferred exotic over native species, even though they respond to different plant traits, with crayfish most affected by plant structural traits (i.e., preference patterns for live plants changing once the plants are dried and ground –see Cronin et al. 2002) and snails responding more to plant chemical traits (i.e., the consistent preferences across live plants, ground plants, and plant extracts). Neither crayfish nor snails showed a correlation between plant preference and protein content, suggesting that protein (which commonly limits some herbivores – Mattson 1980) had minimal influence on these feeding choices. It would be interesting to test if preferences of a South American crayfish align with the preferences of the South American snails or the North American crayfish to see if phylogeny or geography influences preference in response to structural or chemical traits, respectively.

In summary, our results supported the biotic resistance hypothesis over the enemy release hypothesis. Both North American crayfish and South American snails preferred
exotic plants over confamilial natives, despite responding to different plant
characteristics. The single species of apple snail that occurs in North American showed
no preference for native or exotic plants from a North American perspective, but instead
exhibited preferences that correlated with its history of evolution in South America. As
the North American species is a sister species of the South American snails, feeding by
the North American snail appears more affected by its South American lineage than its
recent history in North America. This suggests that phlogenetic legacy will affect
choices of the herbivore as well as resistance or susceptibility of host plants.

4.6 References


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