

Geographic variation in feeding preference of a generalist herbivore: the importance of seaweed chemical defenses

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Abstract The ecological impacts of generalist herbivores depend on feeding preferences, which can vary across and within herbivore species. Among mesoherbivores, geographic variation in host use can occur because host plants have a more restricted geographic distribution than does the herbivore, or there is local evolution in host preference, or both. We tested the role of local evolution using the marine amphipod *Ampithoe longimana* by rearing multiple amphipod populations from three regions (subtropical Florida, warm-temperate North Carolina and cold-temperate New England) and assaying their feeding preferences toward ten seaweeds that occur in some but not all regions. Six of the ten seaweeds produce anti-herbivore secondary metabolites, and we detected geographic variation in feeding preference toward five (*Dictyota menstrualis*, *Dictyota ciliolata*, *Fucus distichus*, *Chondrus crispus* and *Padina gymnospora*, but not *Caulerpa sertularioides*). Amphipod populations that co-occur with a chemically-rich seaweed tended to have stronger feeding preferences for that seaweed, relative to populations that do not co-occur with the seaweed. A direct test indicated that

geographic variation in feeding preference toward one seaweed (*D. ciliolata*) is mediated by feeding tolerance for lipophilic secondary metabolites. Among the four seaweeds that produce no known secondary metabolites (*Acanthophora*, *Ectocarpus*, *Gracilaria* and *Hincksia* *Feldmannia* spp.), we detected no geographic variation in feeding preference. Thus, populations are more likely to evolve greater feeding preferences for local hosts when those hosts produce secondary metabolites. Microevolution of feeding behaviors of generalist marine consumers likely depends on the availability and identity of local hosts and the strength of their chemical defenses.

Keywords Local adaptation · Feeding preference · Plant chemical defenses · Herbivore offense · Ampithoidae · Phylogeography

Introduction

The ecological impact of herbivores on plant communities depends in part on their feeding preferences, which vary strongly among and within herbivore species (Stephens et al. 2007). A powerful approach to understanding the ecological and evolutionary forces that maintain herbivore feeding preferences is to focus on within-species variation (Thompson 2005), and, in particular, on the process of local adaptation (Kawecki and Ebert 2004; Sotka 2005). As examples, widely distributed herbivorous insects often have geographic ranges extending beyond the geographic ranges of their host plants such that individual populations are exposed to locally distinct plant communities throughout the entirety of the species' distribution (Strong et al. 1984). Insect populations commonly evolve a higher affinity for and greater fitness on local plants relative to

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plants from other regions (Fox and Morrow 1981; Thompson 2005; e.g., Rausher 1982; Nitao et al. 1991; Traxler and Joern 1999; Singer and McBride 2009). In fact, it is relatively rare to find generalist terrestrial insects with uniform feeding preferences and performance on host plants across their geographic range (but see Thompson 1993; Wehling and Thompson 1997).

As with terrestrial herbivores, marine herbivores play key roles in the ecology and evolution of nearshore ecosystems. They alter the distribution and abundance of seaweeds (Lubchenco and Gaines 1981; Hay 1997), affect seaweed fitness (Steinberg 1984), and influence recruitment of other species to the ecosystem (Levin and Hay 2002). Cumulatively, these effects alter ecosystem structure and function (Stachowicz et al. 2007). Herbivore feeding preferences vary profoundly across species (Steneck and Watling 1982; Paul et al. 2001; Poore et al. 2008), but much of this variation remains unexplained (Choat and Clements 1998; Sotka et al. 2009), and, consequently, our ability to predict the effects of particular herbivores on local communities remains limited (Burkepile and Hay 2008; Stachowicz et al. 2007).

Given the high frequency by which terrestrial insects show local adaptation in feeding preference, you should expect that small marine herbivores (microgastropods, amphipods, isopods) should regularly evolve local host preferences. However, there are relatively fewer studies that attempt to document local adaptation in feeding preferences among marine herbivores (Poore and Steinberg 2001; Vesakoski et al. 2009). One example is the herbivorous amphipod *Ampithoe longimana*, an abundant member of estuarine communities along the northeastern Gulf of Mexico and the U.S. Atlantic shorelines (Bousfield 1973; Nelson 1980). In North Carolina, *A. longimana* preferentially lives and feeds on chemically defended brown seaweeds in the genus *Dictyota*, which provides a refuge from consumption by large omnivorous fishes (Duffy and Hay 1991, 1994). *Dictyota* is a tropical genus, which is unavailable to cold-temperate populations of *A. longimana* (Richardson 1979). Previous work showed that *A. longimana* populations from warm-temperate North Carolina had stronger feeding preferences for two species of *Dictyota* (*D. menstrualis* and *D. ciliolata*) than did cold-temperate populations from New England, which were allopatric with *Dictyota* (Sotka and Hay 2002; Sotka et al. 2003). Other seaweeds from North Carolina were offered to these same populations, and geographic variation in feeding preference for these other seaweeds was weak (Sotka and Hay 2002). The exception was for a North Carolina population of the red seaweed *Hypnea musciformis*, for which New England populations had a greater feeding preference relative to North Carolina populations (Sotka et al. 2003).

These previous studies were largely focused on the interaction between *A. longimana* and *Dictyota*, and, as such, did not answer the broader question of whether this generalist herbivore consistently responds to biogeographic variation in seaweed availability by evolving feeding preferences for local hosts. That is, does the *A. longimana*–*Dictyota* interaction represent an exceptional case of local evolution in feeding preference, or does *A. longimana* respond evolutionarily to local availability of other hosts (i.e., is local evolution a ‘rule’)? We tested whether populations of *A. longimana* consistently show greater preferences for local seaweed communities by comparing the feeding behaviors of populations from a broader geographic range than was previously studied [cold-temperate New England (41°N), warm-temperate North Carolina (34°N), and subtropical Florida (27°N)]. We combine field surveys, common-garden cultures, feeding preference assays and genetic analysis to determine (1) which seaweeds serve as important hosts for *A. longimana* throughout its distribution, and (2) whether patterns in local host use predict the evolution of local feeding preferences.

Materials and methods

Specimen collection and storage

Populations of *A. longimana* were collected by hand from subtidal seaweeds at less than 1 m depth between May 2006 and August 2007. Multiple populations were collected in each of three regions (each region separated by approximately 7° latitude) along the Atlantic coast of the United States (Table 1). All amphipods were transported to the College of Charleston’s Grice Marine Laboratory where they were cultured in two or more containers (per population) with approximately 4 L of filtered and aerated seawater (~30 ppt) siphoned from Charleston Harbor. Water was changed every 5–7 days. Cultures were maintained within incubators at a temperature of 20 °C, which amphipods in all estuaries likely experience (Sotka and Giddens 2009). Amphipods were fed several seaweeds from among multiple genera (*Ulva*, *Ectocarpus*, *Sargassum*, *Bryopsis*, *Gracilaria*, and *Hypnea*), depending on availability in the field. All seaweeds were rinsed at least twice in freshwater for 30–45 s to remove local epibiota. Amphipods were raised in these ‘common garden’ conditions for at least one generation prior to being used in any feeding assay (between 1–6 months). This minimized the effects of recent history, and as such, geographic variation in feeding response is likely genetically mediated. Specimens for population genetic analysis were removed from cultures.

Table 1 Locations of amphipod populations and identity of local hosts tested

Region	Site	Abbr	Lat; Long	Field survey	<i>Chondrus crispus</i>	<i>Fucus distichus</i>	<i>Ectocarpus siliculosus</i>	<i>Dictyota</i> spp.	<i>Padina gymnospora</i>	<i>Caulerpa sertularoides</i>	<i>Hincksia/Feldmannia</i> spp.	<i>Acanthophora spicifera</i>	<i>Hypnea/Gracilaria</i> spp.
New England	Westport, MA	MA	41.3°N, 71.0°W		X	X	X						
	Jamestown, RI	RI	41.2°N, 71.2°W	Y	X	X	X						
	Niantic, CT	CT	41.2°N, 72.0°W		X	X	X						
North Carolina	Harker's Island	HI	34.4°N, 76.3°W				X	X	X				
	Morehead City	MH	34.4°N, 76.4°W	Y			X	X	X				
	Wrightsville Beach	WB	34.1°N, 77.4°W				X	X	X				
Florida	Fort Pierce	FP	27.3°N, 80.2°W	Y						X	X	X	X
	Tampa Bay	TB	27.5°N, 82.4°W							X	X	X	X

X indicates seaweeds that serve as local hosts from field surveys or personal observations, Y indicates sites where field surveys of amphipods were conducted. Note that *Hypnea* and *Gracilaria* and *Hincksia* and *Feldmannia* are pooled because they were tangled in the field

Fresh *D. ciliolata* and *D. menstrualis* were collected from less than 1 m depth in Morehead City, North Carolina, transported to Grice Marine Laboratory, and aerated in 30 ppt filtered seawater from Charleston Harbor for 24 h before feeding assays began. Fresh *Ulva lactuca* and *Ulva intestinalis* were collected from Charleston Harbor. Seaweeds for lyophilized assays were collected from less than 1 m depth at Jamestown, Rhode Island (*Chondrus crispus*, *Fucus distichus*, and *Ectocarpus siliculosus*); Morehead City, North Carolina (*D. ciliolata*, *D. menstrualis*, and *Padina gymnospora*); and Fort Pierce, Florida (*Acanthophora spicifera*, *Hincksia/Feldmannia* spp., and *Caulerpa sertularoides*; see Supplemental Material Table 5 for biogeographic distributions of seaweeds). *Gracilaria tikvahiae* was obtained from aquacultures at the Harbor Branch Oceanographic Institute (Dr. Dennis Hanisak). All seaweeds for freeze-dried assays were rinsed in freshwater twice for 1 min to remove epibiota, frozen for transport, lyophilized at the Hollings Marine Laboratory in Charleston, SC, and stored at -20°C .

DNA sequence analysis

We analyzed sequences at the mitochondrial locus Cytochrome *c* oxidase I (COI) to determine the degree of genetic differentiation among *A. longimana* populations and to assess whether our morphological identification of *A. longimana* was robust. DNA was extracted from $\sim 1\text{-mm}^3$ tissue samples using a Qiagen DNA Mini Kit following the manufacturer's instructions. The extracted DNA was amplified via polymerase chain reaction (40 cycles; 30 s at 94°C , 30 s at 45°C , and 1 min at 72°C) using genus-specific primers (AmpithoeF 5'-ATC ATA ATT

GGT GGT TTC GGA A-3' and AmpithoeR 5'-CCC TAA CGG GTC AAA AAA AGA A-3'). PCR products were visualized using gel electrophoresis (1.5 % agarose) stained in ethidium bromide. PCR products were cleaned using Shrimp Alkaline Phosphatase and Exonuclease I before they were sequenced in both forward and reverse directions with half reactions of Beckman Coulter CEQ Quick Start chemistry using a Beckman Coulter 8000 sequencer at the Grice Marine Laboratory.

Forward and reverse sequences were aligned using Sequencher 4.7 (GeneCodes), and a consensus sequence for each individual was generated. We also aligned homologous sequences from three confamilial amphipods (*A. lacertosa*, GenBank Accession # GU048163; *A. rubricata*, FJ581493; *A. valida*, GU048170) using CLUSTAL (Larkin et al. 2007) and calculated pair-wise differences between and within species using MEGA 5.0 and an appropriate evolutionary model (HKY+I, by AIC criteria of ModelTest; Posada and Crandall 1998). A distance matrix was visualized using a neighbor-joining algorithm, and statistically evaluated via bootstrapping (2000 reps) using PAUP 4.0. A maximum likelihood search using PhyML 3.0 (Guindon et al. 2010) started with a neighbor-joining tree, followed by the NNI search strategy with the HKY+I model of evolution. The best ML tree was then analyzed with 1,000 bootstrap replicates. The tree was manipulated with the RETREE program in PHYLIP (Felsenstein 1989), and annotated using the APE package in R (Paradis et al. 2004).

Field survey

Because *A. longimana* is relatively sessile, and host use and feeding preference tends to be strongly correlated for this

amphipod, amphipod abundance on a particular seaweed species indicates the importance of that seaweed as a host and food source (Duffy and Hay 1991). We surveyed the dominant macroalgal species at a single location within each region (Jamestown, Rhode Island in New England; Morehead City, North Carolina; and Fort Pierce, Florida; Table 1). We did not quantify seaweed availability within these estuaries, and, as such, these surveys reflect the relative, but not absolute, importance of the seaweeds as host to the amphipods. Five haphazardly chosen individuals of the most abundant seaweed species (5–13 species/region) were placed individually within 4-L plastic bags from the shallow subtidal (≥ 1.0 m MLLW). In order to assess seasonal patterns of abundance, we surveyed each location during early summer (May–June-2006), late summer (August–September-2006), and winter (January–March-2007). In May 2006, we also surveyed amphipods on several dominant seaweed species (*Sargassum hystrix*, *Dictyota crenulata*, and *Dictyota pulchella*) at offshore reefs in the Florida Keys (Pickles Reef, 24.6°N, 80.2°W, and Grassy Key, 24.5°N, 80.6°W).

Each seaweed collected was soaked in freshwater for two 1-min periods, and shaken under running water for at least 30 s to dislodge epifauna. All water was filtered through a 300- μ m filter to collect organisms and then transferred to 20-mL scintillation vials filled with 2–3 % formalin. After a minimum of 1 month, epifauna were transferred to 70 % ethanol for long-term storage. Samples were sorted manually using a dissecting microscope. All *A. longimana* were identified using a dissecting microscope at $\times 10$ – 20 magnification (Bousfield 1973; LeCroy 2002). One-way ANOVAs were performed to determine if *A. longimana* density (number of amphipods per g wet mass of seaweed) differed across seaweed species or across seaweed divisions (i.e., red, brown, and green seaweeds). These ANOVAs were followed by Tukey–Kramer post hoc tests when appropriate ($\alpha = 0.05$).

Feeding assays: fresh tissue

Across its geographic range, *A. longimana* occurs on at least 22 seaweed genera from all divisions of seaweeds (see “Results”). We tested whether amphipods consistently evolve feeding preferences for ten seaweeds that serve as important hosts to some, but not all populations. For all seaweeds tested, we performed feeding choice assays using lyophilized tissue. For the two *Dictyota* species, we also performed feeding choice assays using fresh seaweed tissue and chemical extracts. All populations were assayed at the same time, with the exception of two assays (lyophilized *Dictyota menstrualis* and *D. ciliolata*) in which Tampa Bay populations were assayed 5 months after other populations.

In fresh-tissue assays, amphipods were offered a choice between freshly collected tissue from *U. lactuca* and a single *Dictyota* species (*D. menstrualis* or *D. ciliolata*). *U. lactuca* was chosen as a control because *Ulva* is available in all three regions and previous work indicated no population-level variation in feeding behavior and fitness when isolated with *Ulva* tissue (Sotka and Hay 2002). Moreover, its thin, flattened morphology mirrors that of *Dictyota* when cut into small pieces. One fresh *Dictyota* individual was blotted dry, weighed, and cut into pieces of approximately equal mass (21–24 mg). Pieces of fresh *U. lactuca* with a similar surface area to that of the *Dictyota* pieces were cut from one individual of fresh *U. lactuca*, blotted dry, and weighed (14–18 mg). One piece of *U. lactuca*, one piece of *D. menstrualis* or *D. ciliolata*, and one adult or two juvenile amphipod(s) from each population were placed into a 200-mL plastic cup with ~ 75 mL of seawater. A second cup with tissue from the same seaweed individuals but without amphipods served as a control for autogenic growth. Replicate cups ($n = \sim 20$ per population) were enclosed within plastic containers, and containers were placed haphazardly within a 20 °C incubator in complete darkness. Amphipods were removed when 96 h had lapsed, or when the amphipods had consumed at least half of either seaweed, whichever was first. All seaweed pieces were then removed, blotted dry, and reweighed. We adjusted wet mass consumed while accounting for autogenic growth (cf. Sotka and Hay 2002) and estimated the proportion *Dictyota* consumption by dividing the amount of *Dictyota* consumed by the total amount of *Ulva* and *Dictyota* consumed.

Feeding assays: freeze-dried tissue

We pursued freeze-dried tissue assays because lyophilization can maintain biochemical traits (e.g., nutrients, secondary metabolites) that mediate feeding choices (Cronin et al. 1995) and has the advantage of allowing experiments to be carried out when amphipod populations are available, which did not always coincide with when we were able to collect the seaweeds. While the process of lyophilization reduces the quantity of some secondary metabolites, it is unlikely this difference biases our conclusions because this work primarily focused on the relative differences in feeding behavior between populations exposed to the same foods.

Frozen, lyophilized algae were ground to a fine powder using a Wiley mini-mill with a 180 μ m mesh sieve. Foods were prepared by mixing 0.5 g seaweed with 2 mL water and adding a molten mixture of 0.9 g of agar and 2.5 mL water. This procedure yields foods with 10 % organic content, which is typical of many seaweeds. The seaweed mixture was applied to fiberglass window screen, pressed

between two sheets of wax paper to allow the mixture to solidify and cut into feeding grids measuring 5×6 squares apiece.

The freeze-dried tissue assay was conducted similarly to fresh-tissue assays, with some exceptions. Amphipods were offered a control food of *Ulva* (syn. *Enteromorpha intestinalis*). *U. intestinalis* is available in all three regions, is readily consumed by *A. longimana*, and there are no differences in fitness on *U. intestinalis* among *A. longimana* populations (Sotka and Hay 2002). Replicates were stopped when 96 h had elapsed or when the amphipod consumed 9+ squares from one feeding grid or 10+ squares in total. Replicates where amphipods ate fewer than 9 squares or more than 35 squares across two grids were excluded from analysis because such replicates provide information on feeding rate but bias our ability to detect feeding choices (approximately 16 % of the original replicates). We started each assay with 40 replicate cups per population–seaweed combination. The assay was performed twice for lyophilized *P. gymnospora*, and all replicates were combined ($n = 80$ total) when no difference in feeding behavior was detected between assays.

Feeding assays: seaweed metabolites

After detecting regional differences in feeding preferences for *Dictyota*, we assessed whether this variation in feeding preference was mediated by secondary metabolites. The lipophilic metabolites (and some weakly polar metabolites) were extracted using 2:1 mixture of dichloromethane (DCM): methanol (MeOH) that was 20 times the wet volume of the seaweed. Ground and lyophilized *Dictyota* tissue was immersed in one-third of the DCM:MeOH mixture, allowed to sit for 15 min and then vacuum-filtered through Whatman filters (Grade 3 Qualitative Filter Paper). This extraction was repeated twice and extracts were combined and then dried using rotary evaporation and stored at -20 °C until use. Extracts were then dissolved in ethyl ether and combined with lyophilized *U. intestinalis* at natural concentrations (by dry mass), and then ethyl ether was removed by rotary evaporation. This extraction procedure recovers 88–90 % of the diterpene alcohols of *Dictyota* seaweeds (Cronin et al. 1995). The control, *U. intestinalis*, was treated similarly. Assays were performed as described for freeze-dried assays.

Feeding assays: analysis

Statistical tests for feeding assays focused on two questions. First, paired *t* tests determined whether a single population consumed significantly different amounts of the control and treatment foods. Populations that consumed significantly more (or less) of the treatment than the control

were classified as preferentially consuming (or avoiding) the treatment using two-tailed paired *t* tests. The *p* values of the *t* tests were Bonferroni-corrected by $\alpha = 0.05/k$, where $k =$ number of populations assayed per seaweed.

Second, we tested for regional variation in feeding preference using ANOVAs that nested amphipod populations within one of two regions: Local or Foreign (cf. Kawecki and Ebert 2004). For each seaweed assay, amphipod populations were designated as Local for the treatment seaweed if at least 5 % of amphipods in our field survey were collected on that particular seaweed (i.e., *C. crispus*, *F. distichus*, *D. ciliolata*, *D. menstrualis*, *P. gymnospora*, *G. tikvahiae*, *A. spicifera*, and *Hincksia/Feldmannia*), or, alternatively, if the amphipod population was within the biogeographic range of the seaweed and personal observations indicated that it served as a host for *A. longimana* (i.e., *E. siliculosus* and *C. sertularoides*). All other populations were considered Foreign for that particular seaweed. A signal of regional differences in feeding preferences was inferred when Local populations consumed significantly more of a particular seaweed than did Foreign populations. Because some datasets were non-normal, heteroscedastic, or both, we evaluated statistical significance by comparing *F* ratios with an expected distribution generated from 2,000 permutations of the dataset. When a nested ANOVA indicated a non-significant region effect but significant population effect, we employed a series of Tukey–Kramer post hoc tests on log-transformed or untransformed data. When only two populations were assayed for a given seaweed, data were analyzed using a one-way parametric ANOVA. We also pursued a nested 3-way permuted ANOVA on the freeze-dried tissue data that incorporated region (Local vs. Foreign), population nested within region, and seaweed identity. We excluded *Gracilaria* and *Acanthophora* assays from this analysis because only two populations were used. All analyses had $\alpha < 0.05$.

Results

mtDNA phylogeography of *Ampithoe longimana*

A phylogenetic analysis of 468 bp of COI reveals three well-supported haplotype groups that correspond to biogeographic regions of Florida, North Carolina, and New England (Fig. 1; GenBank Accession numbers KC217656–KC217720). This split between North Carolina and New England populations was documented previously (Sotka et al. 2003), but Florida samples were not described. Average pair-wise differences were 2.9 % between Florida and New England amphipods, 1.5 % between Florida and North Carolina amphipods, and 1.9 % between North

Carolina and New England amphipods. Each of the Florida, North Carolina, and New England individuals were between 19.7 and 26 % divergent from individuals within three confamilial species *Ampithoe lacertosa*, *A. rubricata*, and *A. valida*. Data confirm the identity of *A. longimana* from each region and indicate that regional adaptation can proceed within each region without the homogenizing effect of gene flow from other regions.

Field surveys

In order to identify important seaweed hosts for *A. longimana*, the most abundant species of seaweed from each location were collected during early summer (May–June 2006), late summer (August–September 2006), and winter (January–March 2007). Between 90 and 99 % of local *A. longimana* individuals were represented within the early summer collections in Florida and North Carolina and the late summer collection in Rhode Island (Supplemental Material Table 1). We focused our analysis on these timepoints.

In Florida, we did not find any *A. longimana* on offshore reefs in the Florida Keys. Among the estuarine samples, there was a significant difference in *A. longimana* density across seaweeds ($F_{6,14} = 6.244$, $p = 0.002$). This was driven by the significantly higher abundance on *H. musciformis* and *G. tikvahiae*, which were consistently found tangled together, when compared to all other seaweeds.

There was no significant difference in abundance across red, brown and green seaweeds ($F_{2,18} = 1.930$, $p = 0.174$). Despite the fact that *Dictyota* is tropical in distribution, we found only a small number of *Dictyota* individuals within southern Florida estuaries where we collected *A. longimana*.

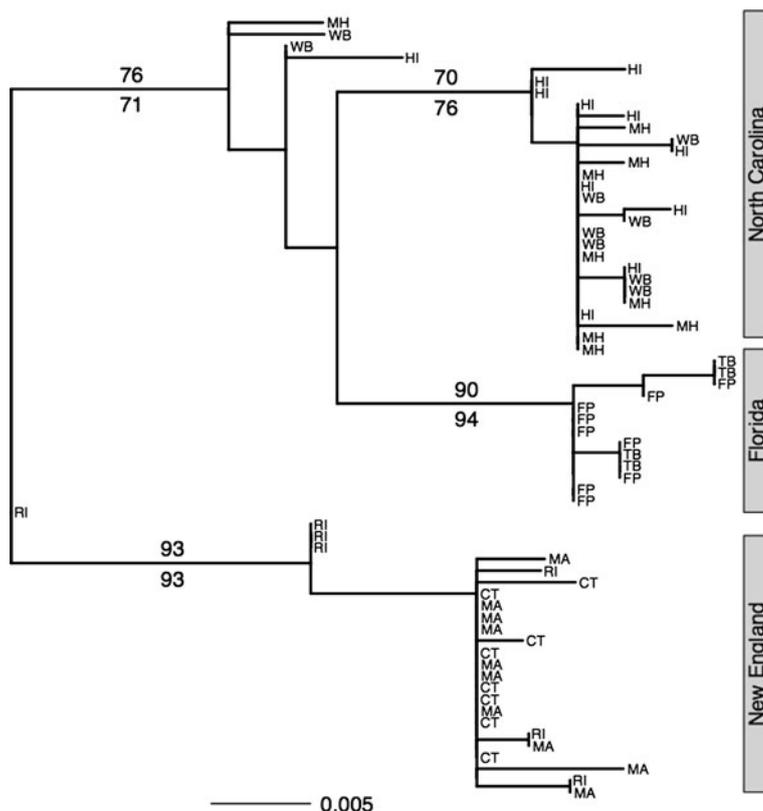
In North Carolina, *A. longimana* was found on every seaweed species collected. However, there were significant differences ($F_{12,26} = 5.319$, $p < 0.001$) in *A. longimana* abundance on North Carolina seaweeds, with a significantly greater number of *A. longimana* found on *D. menstrualis* than all seaweeds except *Sargassum filipendula* and *U. lactuca*. Brown seaweeds were significantly more important as a host than red or green algae ($F_{2,36} = 10.810$, $p < 0.001$); 71, 15, and 14 % of amphipods were collected from brown, red, and green seaweeds, respectively.

In Rhode Island (New England), *A. longimana* was found on every seaweed species collected in the August 2006 collection. There was neither a significant difference in *A. longimana* abundance on Rhode Island seaweed by species ($F_{7,16} = 1.500$, $p = 0.237$), nor when red, brown, and green seaweeds were compared ($F_{2,21} = 0.086$, $p = 0.918$).

Feeding assays: *Dictyota*

Consistent with previous studies (Duffy and Hay 1994; Cronin and Hay 1996), the genus *Dictyota* was an

Fig. 1 Maximum likelihood phylogeny of *Ampithoe longimana* from three regions (New England, North Carolina, Florida) at the mitochondrial locus Cytochrome *c* oxidase I. Nodes at which there was significant bootstrap support using maximum likelihood and neighboring-joining strategies (above and below branches, respectively). Refer to Table 1 for site abbreviations



important host for *A. longimana* in North Carolina with nearly half (44 %) of North Carolina *A. longimana* collected from *D. menstrualis* and *D. ciliolata*. In contrast, *Dictyota* is unavailable to New England amphipods and not regularly available to Florida amphipods. This regional difference in amphipod host usage and seaweed availability predicts the regional difference in feeding preferences. Specifically, North Carolina amphipods more readily consumed *Dictyota* species than New England or Florida amphipods (Fig. 2).

When fresh tissues from *D. ciliolata* and *U. lactuca* were offered in feeding assays, we detected regional differences in feeding preferences using a nested ANOVA in which Local populations (i.e., from North Carolina) consumed relatively more fresh *D. ciliolata* than did Foreign populations (Fig. 2a; see nested ANOVA in Supplemental Material Table 3). A series of paired *t* tests indicated that amphipods from one North Carolina population (NC-HI) consumed significantly more *D. ciliolata* than *U. lactuca* while a 2nd North Carolina population (NC-WB) and all amphipods from New England and Florida fed at equal rates on fresh *D. ciliolata* and *U. lactuca* (NE-MA) (Supplemental Material Table 2).

When assays were repeated using ground, lyophilized tissue from *U. intestinalis* and *D. ciliolata*, Local populations consumed significantly more lyophilized *D. ciliolata* than did Foreign populations (Fig. 2c; Supplemental Material Table 3). Paired *t* tests indicate that two Florida populations (FL-FP and FL-TB) consumed significantly more *U. intestinalis* than *D. ciliolata*, while all other populations consumed statistically indistinguishable amounts of *D. ciliolata* and the control (Supplemental Material Table 2).

When lipophilic secondary metabolites of *D. ciliolata* were offered at natural concentrations, Local populations consumed significantly more of *D. ciliolata* extracts than did Foreign populations (Fig. 2e; Supplemental Material Table 3). Paired *t* tests indicated that populations from New England and Florida were deterred by *D. ciliolata* extracts, while North Carolina populations fed equally on *D. ciliolata* extracts and the control (Supplemental Material Table 2), which was confirmed by a nested ANOVA.

A similar pattern of regional variation in feeding preference emerged when assessing feeding preferences for *D. menstrualis*. During fresh-tissue assays, Local (i.e., North Carolina) populations consumed relatively more fresh *D. menstrualis* than did Foreign populations (Fig. 2b; Supplemental Material Table 3). Paired *t* tests indicate that all NC and FL populations consumed significantly more *D. menstrualis* than *U. lactuca*, while all NE populations fed equally on fresh *D. menstrualis* and the control (Supplemental Material-Table 2). When offered freeze-dried *U. intestinalis* and *D. menstrualis*, there was no significant

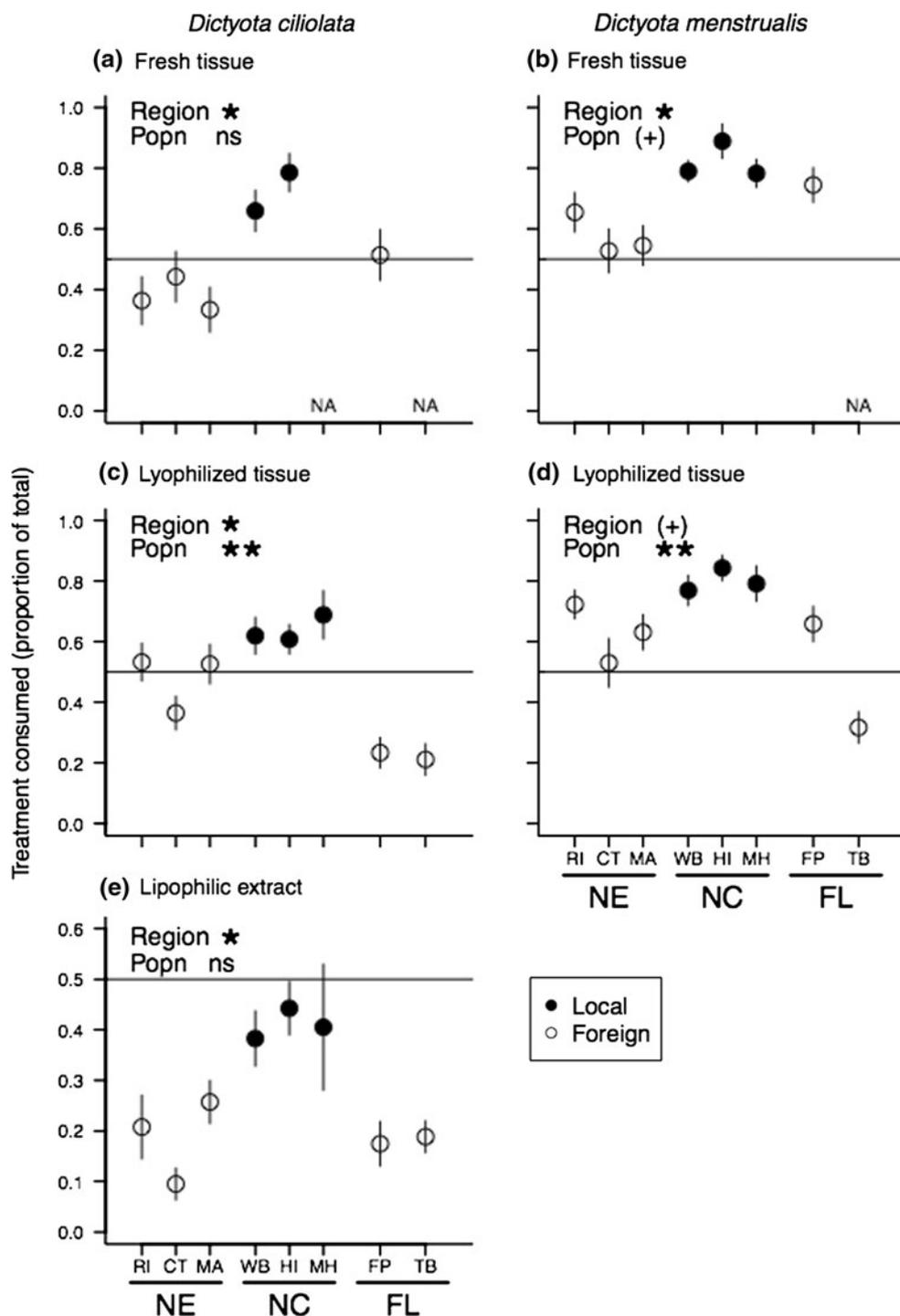
variation in feeding preferences for lyophilized *D. menstrualis* between Local and Foreign populations (Fig. 2d; Supplemental Material Table 3). Populations differed significantly in feeding preference, as amphipods from FL-TB consumed significantly less *D. menstrualis* than amphipods from FL-FP, NC-WB, NC-MH, NC-HI, NE-RI, and NE-MA, and amphipods from NE-CT consumed significantly less *D. menstrualis* than amphipods from NC-HI. Paired *t* tests indicated that NC populations and NE-RI consumed significantly more *D. menstrualis*, FL-TB consumed significantly more *U. intestinalis* and NE-CT, NE-MA, and FL-FP did not distinguish between the two seaweeds (Supplemental Material Table 2). Repeated attempts to extract the secondary metabolites of *D. menstrualis* and assess whether they mediate geographic differences in feeding preference yielded inconsistent results, perhaps because the compounds that mediate deterrence are unstable or are water-soluble and thus not efficiently extracted using our techniques.

Feeding assays: other seaweeds

Analysis of the feeding responses of Local versus Foreign populations was extended to include eight other seaweeds that served as locally important hosts. In contrast with the results seen for *D. ciliolata* and *D. menstrualis* (Fig. 2), regional variation in *A. longimana* feeding preferences was not observed for the eight other seaweeds tested (Fig. 3).

There were three seaweeds for which significant population-level variation in feeding response was found. The red seaweed *C. crispus* hosted *A. longimana* in the greatest abundance in our New England field survey, but is not available to amphipods in North Carolina or Florida. Populations (but not regions) significantly differed in feeding preference (Fig. 3a; Supplemental Material Table 3), as amphipods from NE-CT consumed significantly more *C. crispus* than amphipods from NC-HI, NC-MH, and NC-WB, and amphipods from NE-MA consumed significantly more *C. crispus* than NC-MH amphipods. All amphipods fed equally on *C. crispus* and the control, with the exception of NC-MH amphipods, which consumed significantly less *C. crispus* than *U. intestinalis* and NE-CT amphipods, which ate significantly more *C. crispus* than *U. intestinalis* (Supplemental Material Table 2). Similarly, populations (but not regions) differed in their relative feeding preferences for the brown seaweed *F. distichus* (Fig. 3b; Supplemental Material Table 3), which hosted *A. longimana* in New England but not in North Carolina or Florida. FL-TB amphipods consumed less *F. distichus* than did amphipods from FL-FP, NE-CT, and NE-RI. All populations consumed more *F. distichus* than *U. intestinalis*, with the exceptions of NC-HI and FL-TB, which fed equally on both (Supplemental Material Table 2). Finally,

Fig. 2 Feeding preferences of *Amphithoe longimana* from New England (NE), North Carolina (NC) and Florida (FL) when offered two seaweeds, *Dictyota ciliolata* and *D. menstrualis*. *Dictyota* species occur within Local (filled circles) or Foreign (open circles) habitats. The response variable is the mean (\pm SE) proportion of total consumption that was *Dictyota* when offered a, b fresh tissue, c, d lyophilized tissue, and e lipophilic extract. *P* values for population (Popn) and Region (Local vs. Foreign populations) come from nested ANOVAs (** $p < 0.01$; * $p < 0.05$; (+) $0.05 < p < 0.1$; ns $p > 0.1$). Post hoc tests from lyophilized *D. menstrualis*: RI (a, b); CT (b, c); MA (a, b); WB (a, b); HI (a); MH (a, b); FP (a, b); TB (c), where letters indicate statistically indistinguishable populations. NA indicates a population that was not assayed



populations (but not regions) differed in feeding preference toward *P. gymnospora*, an important host for North Carolina amphipods. All populations consumed significantly more *P. gymnospora* than *U. intestinalis* except for NE-RI, which consumed statistically equivalent amounts (Supplemental Material Table 2).

When we simultaneously analyzed feeding preference data from all lyophilized tissues, the effects of region (i.e., Local vs. Foreign populations), seaweed identity, and population source (nested within host use) were highly significant (all $p < 0.001$; Table 2). The interaction between population source and seaweed identity was

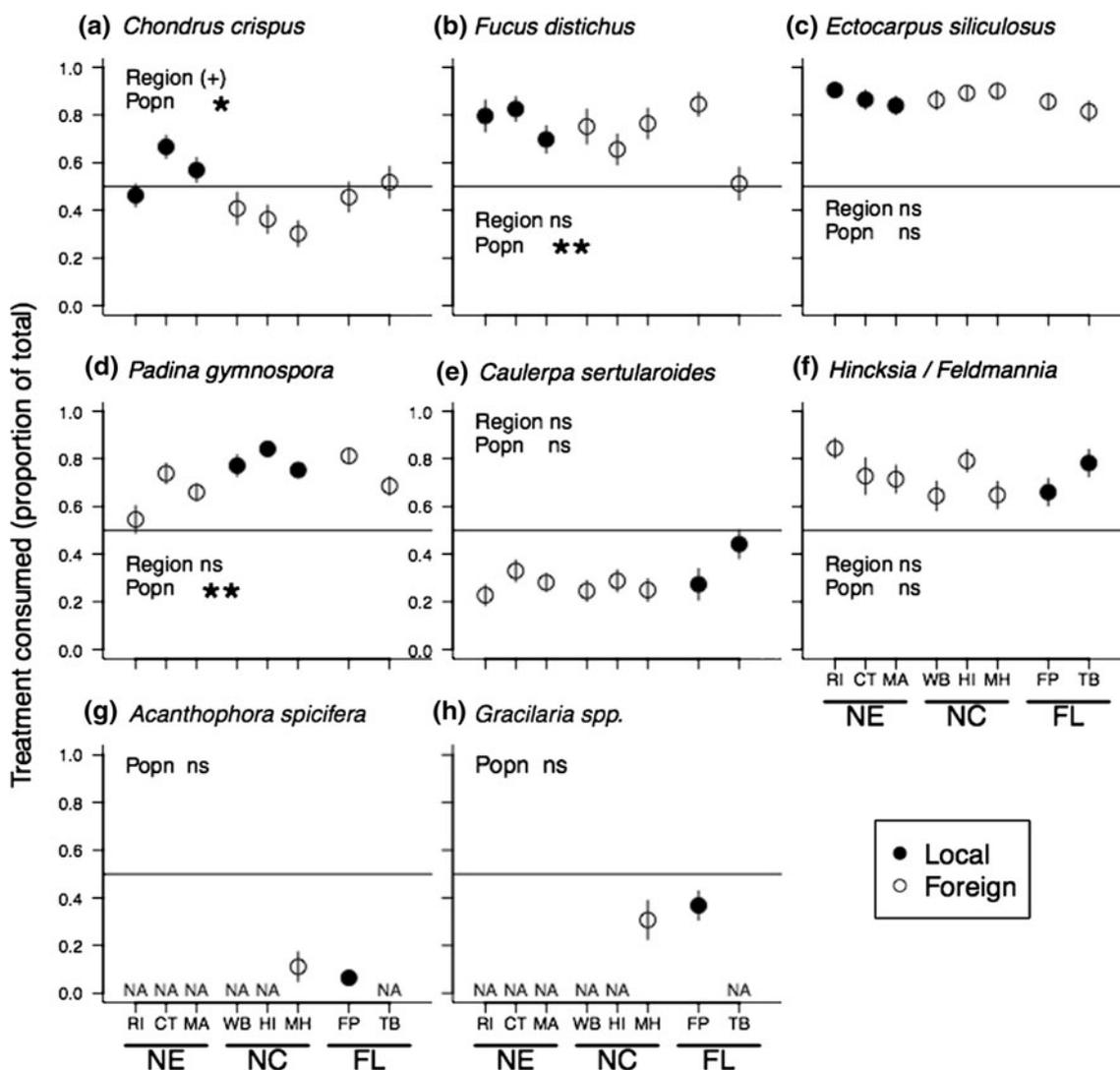


Fig. 3 Feeding preferences of *Amphithoe longimana* from New England (NE), North Carolina (NC), and Florida (FL) for lyophilized seaweeds from a–c NE, d NC, and e–h FL. The response variable is the mean (±SE) proportion of total consumption that was the target seaweed. *P* values for population (Popn) and Region (Local vs. Foreign populations) come from nested ANOVAs (see Fig. 2 legend for abbreviations). *C. crispus* posthoc

tests: RI (a, b, c); CT (a); MA (a, b); WB (b, c); HI (c); MH (c); FP (a, b, c); TB (a, b, c). *Fucus distichus* posthoc tests: RI (a); CT (a); MA (a, b); WB (a, b); HI (a, b); MH (a, b); FP (a); TB (b). *Padina gymnospora* posthoc tests: RI (c); CT (a, b); MA (b, c); WB (a, b); HI (a); MH (a, b); FP (a, b); TB (b, c), where letters indicate statistically indistinguishable populations. NA indicates a population that was not assayed

highly significant ($p < 0.001$), while the interaction between region and seaweed identity was not.

Discussion

The composition of seaweed communities varies considerably throughout the range of *Amphithoe longimana*, which spans from subtropical Florida to cold-temperate New England (Stephenson and Stephenson 1952; Searles 1984; Humm 1969). Seaweed availability thus drives much of the

Table 2 Nested ANOVA testing the effect of region (Local vs. Foreign populations), population source (nested within region), and seaweed identity on feeding preference for lyophilized foods

	df	MS	F	p
Region	1	17.24	54.9	<0.001
Seaweed	7	8.15	28.6	<0.001
Popn{region}	14	0.31	97.7	<0.001
Region × seaweed	6	0.40	1.4	0.251
Popn{region} × seaweed	35	0.29	3.4	<0.001
Residuals	2,095	0.08		

variation in host use among *A. longimana* populations (Table 1). We tested whether regional variation in host use has yielded local evolution in feeding preference and found that the evolutionary response of marine herbivore populations is dependent upon the identity of the seaweed involved (Figs. 2, 3; Table 2). Significant regional variation in feeding preferences were found in response to two seaweeds (*D. menstrualis* and *D. ciliolata*), significant population-level variation was found in response to three other seaweeds (*F. distichus*, *C. crispus* and *P. gymnospora*), while feeding responses of *A. longimana* populations toward the five other seaweeds were statistically indistinguishable. These patterns suggest that, while evolution for greater feeding preference of locally important hosts can occur, such evolutionary responses are not uniform across seaweed hosts, and instead seem to depend on whether the seaweed produces secondary metabolites. We describe these results in more detail below.

Evolution of *Dictyota*–*Ampithoe* interaction

Dictyota is among the most important hosts to North Carolina *A. longimana* (Table 1) and provides *A. longimana* an important refuge from omnivores by producing chemical defenses that are avoided by the local omnivorous fish species (e.g., the pinfish *Lagodon rhomboides*), which consume amphipods (Nelson 1979; Duffy and Hay 2000). The consistently higher feeding preferences for *Dictyota* among North Carolina populations relative to New England populations (Fig. 2) confirm previous findings and suggest that predation maintains strong host use preferences for *Dictyota* in North Carolina (Sotka and Hay 2002; Sotka et al. 2003). However, the fact that North Carolina populations have greater feeding preferences than Florida populations is a novel finding. Because the biogeographic ranges of *Dictyota* and *A. longimana* overlap in both Florida and North Carolina, we hypothesized a priori that Florida and North Carolina populations of *A. longimana* would have equally high feeding preference for *Dictyota*. Instead, surprisingly, the feeding preferences of Florida amphipods for *Dictyota* more closely resemble those of cold-temperate New England populations than of North Carolina.

We hypothesize that the low preference for *Dictyota* among Florida amphipods reflects a historical decline in *Dictyota* in Florida estuaries where *A. longimana* is found. Field surveys from the 1970s and 1980s document abundant populations of *Dictyota* in Florida estuaries before profound anthropogenic changes in salinity and habitat quality (e.g., Dawes 1974; Benz et al. 1979; Supplemental Material Table 6). Surveys undertaken since the 1980s (including our own) indicate that seagrass beds in the Indian River Lagoon and Tampa Bay are generally

dominated by seaweeds that lack lipophilic defenses (e.g., *Hypnea* and *Gracilaria*), and demonstrate that the presence of *Dictyota* is irregular and rare overall. The recent loss of substantial *Dictyota* populations within Florida estuaries suggests that Florida *A. longimana* feeding preferences for *Dictyota* may have degraded via evolution only relatively recently. Such degradation is especially likely given the fitness costs that are incurred in maintaining tolerance for *Dictyota* secondary metabolites. Indeed, we have previously shown via a controlled-natural selection experiment that *Dictyota* preference evolved within five generations (~4 months), and evolution of preference for and fitness on *Dictyota* came at a fitness cost when utilizing other seaweeds (Sotka and Reynolds 2011).

Evolution of feeding response to other seaweeds

Regional correlations among *A. longimana* host use and feeding preference were weak to absent for other seaweeds. None of the New England seaweeds offered (*F. distichus*, *E. siliculosus*, *C. crispus*) were preferentially consumed at greater rates by amphipods from New England (clustered by region) relative to amphipods from southern estuaries (Fig. 3; Supplemental Material Table 3). Of these seaweeds, only *Ectocarpus* occurs in North Carolina, and none occur in Florida (Supplemental Material Table 5). A related species, *Fucus vesiculosus*, occurs in North Carolina, but is only rarely used as a host by *A. longimana* (Sotka, personal observation). There is a suggestion of a marginally significant regional effect ($p = 0.054$) for *C. crispus*, which could indicate either no regional effect or an experimental design that suffered from low statistical power.

While the regional effects toward these eight seaweeds are weak to absent, differences among amphipod populations within regions are detectable in three seaweeds (*C. crispus*, *F. distichus*, and *P. gymnospora*). The mechanisms by which such population-level but not regional variation in feeding response can evolve are uncertain. An intriguing example is *C. crispus*, which could play a role in New England analogous to that for *Dictyota* in North Carolina. *C. crispus* has a more complex morphology than most other seaweeds tested, and may provide increased protection from predators relative to other seaweeds (Hacker and Steneck 1990; Chavanich and Wilson 2000). Additionally, *C. crispus* produces volatile halocarbons and oxygenated fatty acid derivatives, which may be involved in chemical defense (Geiselman 1980; Bouarab et al. 2004) and help shield the mesograzer from consumption by larger omnivores. If such selection advantages are strong at sites that are particularly dense in *C. crispus* but weak when *C. crispus* is rare or absent, then this could yield a pattern of population-level, but not regional, adaptation to

C. crispus. This scenario could also help explain population-level variation in response to *F. distichus*. It is possible that our host-use surveys failed to detect within-region variation in host use, as they were conducted at a single site within each region. Therefore, additional field surveys could help explain the observed population-level variation. Alternatively, it remains possible that neutral evolution (i.e., genetic drift) at loci that control traits related to feeding preferences may be leading to the patchy variation.

Amphipod feeding preferences toward Florida seaweeds were statistically indistinguishable (i.e., *Caulerpa*, *Hinckia*/*Feldmannia*, *Acanthophora*, *Gracilaria*; Fig. 3; Supplemental Material Table 3). Based on other studies, it is possible that seagrasses and their associated epiphytes, rather than macroalgae, serve as important hosts for *A. longimana* in Florida (Stoner 1980; Virnstein et al. 1984; Bell 1991; Brooks and Bell 2001), and there remains the untested possibility that Florida populations have a greater feeding preference for these seagrasses and epiphytes relative to other populations. The only non-*Dictyota* seaweed from North Carolina that was used was *P. gymnospora*, and there was a significant between-population effect on feeding preference.

The role of seaweed secondary metabolites

The unique evolutionary response of *A. longimana* populations toward *Dictyota* reflects adaptation to its deterrent lipophilic metabolites (Sotka and Hay 2002). Tolerating lipophilic metabolites likely requires physiological, behavioral, and/or morphological adaptations to avoid and overcome these defenses (Steneck and Watling 1982; Choat and Clements 1998; Targett and Arnold 2001; Sotka et al. 2009). Similarly, *Caulerpa*, *Padina*, *Chondrus* and *Fucus* all have known water-soluble or lipophilic defenses (Geiselman 1980; Dayton 1985; Bouarab et al. 2004; Kubanek et al. 2004; Cruz-Rivera and Villareal 2006; Bolser and Hay 1996), and population-level variation in feeding preference was detected in response to all of these except *Caulerpa* (Fig. 3). In contrast, there was homogeneity in feeding preference for seaweeds that generally do not produce anti-herbivore metabolites (*Acanthophora*, *Ectocarpus*, *Gracilaria*, and *Hinckia*/*Feldmannia*). Chemically depauperate seaweeds such as these are unlikely to require novel modifications of herbivore physiology. Overall, we find a significantly greater frequency of local evolution in feeding response toward seaweeds with feeding defenses (five of six seaweeds) relative to seaweeds without such defenses (zero of four seaweeds; contingency table *G* test; $G = 2.7$, $p < 0.01$).

Caveats

There are several caveats that temper these conclusions. First, we did not assay all of the seaweeds that are locally

available to *A. longimana* in each region. Within each region, we assayed at least two of the five most important seaweed hosts (as measured by our field surveys) plus one additional seaweed, but it is entirely possible that geographic variation in feeding preference occurs in response to seaweeds we did not assay. However, the addition of more feeding preference assays would not fundamentally alter our primary conclusion that local adaptation occurs for only a subset of locally important seaweeds.

Second, we cannot be certain that our procedures did not remove feeding cues that affect feeding behaviors. While some lipophilic compounds in *Dictyota* are known to withstand our lyophilization, extraction, and storage procedures (Cronin et al. 1995), the responses of most algal metabolites to these procedures are unknown. For example, lyophilization and storage are known to reduce the concentration or activity of secondary metabolites in *F. vesiculosus*, a closely related species to *F. distichus* (Kubanek et al. 2004). Moreover, because we used lyophilized seaweeds for the majority of our assays, we are unable to directly assess whether characteristics such as tissue toughness and morphology play a role in host selection.

Finally, we did not directly assess whether these feeding preferences have consequences for the fitness of the amphipods. However, previous work has demonstrated that feeding preference and performance are linked for herbivorous amphipods (Poore and Steinberg 2001; Taylor and Brown 2006), including *A. longimana* (Cruz-Rivera and Hay 2001). We minimized the possible influence of environmental or maternal differences by raising amphipods for one to several generation(s) in common garden cultures in the laboratory before assays began. Therefore, it is likely that any observed variation in feeding preference is genetically mediated and correlates with variation in fitness traits such as growth, survivorship, and reproduction.

Summary

There remain a modest number of marine studies that display local adaptation in consumer feeding preferences similar to that documented for *A. longimana* (Sotka 2005; Vesakoski et al. 2009; Sanford and Worth 2009) especially when compared with the large number of examples among terrestrial insects (Tilmon 2008). It is possible that the relative lack of marine examples reflects a true and systemic difference among biomes, and is related to biome-specific differences in host specialization. Terrestrial insects are far more specialized than marine amphipods (Poore et al. 2008), and Hay and Steinberg (1992) postulated that marine polyphagy may be evolutionary favored because spawned marine offspring are subject to the vagaries of oceanographic dispersal, seaweed communities are strongly seasonal, marine herbivores rarely undergo

diapause, and herbivores and their hosts have a generation time that is roughly comparable. Specialists are favored when there are strong fitness costs in adapting to alternative hosts, and such costs have been detected in marine consumers (e.g., Sotka and Reynolds 2011; Vesakoski et al. 2009). Overall, though, it appears that fitness costs do not seem strong enough to favor specialization among amphipods. In fact, amphipod amphipods that use a chemically rich seaweed as a host have a larger, rather than smaller, overall host range (Poore et al. 2008). In contrast, terrestrial insects on chemically defended host plants tend to have an even more restricted host range than average (Berenbaum et al. 1996), a pattern that would be predicted by strong fitness costs. Note that sea slugs are an exceptional group of mesoherbivores whose restricted host range mirrors that of most terrestrial insects (Poore et al. 2008).

Thus, using chemically-rich seaweeds may not favor the evolution of specialization in marine mesoherbivores, but the present results indicate that it does predict local strengthening of feeding preferences. Given that such local evolution among terrestrial generalists is not unknown (Thompson 2005; Tilmon 2008), we suggest that the relative lack of examples in marine systems may reflect the fact that biologists rarely test for local adaptation in marine relative to terrestrial systems (Sotka 2005, 2012). To distinguish between these alternatives, we should pay greater attention to patterns of local adaptation in marine consumers, and especially those consumers that utilize chemically-rich prey.

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