Marine and terrestrial herbivores display convergent chemical ecology despite 400 million years of independent evolution

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Chemical cues regulate key ecological interactions in marine and terrestrial ecosystems. They are particularly important in terrestrial plant–herbivore interactions, where they mediate both herbivore foraging and plant defense. Although well described for terrestrial interactions, the identity and ecological importance of herbivore foraging cues in marine ecosystems remain unknown. Here we show that the specialist gastropod *Elysia tuca* hunts its seaweed prey, *Halimeda incrassata*, by tracking 4-hydroxybenzoic acid to find vegetative prey and the defensive metabolite halimedatetraacetate to find reproductive prey. Foraging cues were predicted to be polar compounds but instead were nonpolar secondary metabolites similar to those used by specialist terrestrial insects. Tracking halimedatetraacetate enables *Elysia* to increase in abundance by 12- to 18-fold on reproductive *Halimeda*, despite reproduction in *Halimeda* being rare and lasting for only ∼36 h. *Elysia* swarm to reproductive *Halimeda* where they consume the alga's gametes, which are resource rich but are chemically defended from most consumers. *Elysia* sequester functional chloroplasts and halimedatetraacetate from *Halimeda* to become photosynthetic and chemically defended. Feeding by *Elysia* suppresses the growth of vegetative *Halimeda* by ∼50%. *Halimeda* responds by dropping branches occupied by *Elysia*, apparently to prevent fungal infection associated with *Elysia* feeding. *Elysia* is remarkably similar to some terrestrial insects, not only in its hunting strategy, but also its feeding method, defense tactics, and effects on prey behavior and performance. Such striking parallels indicate that specialist herbivores in marine and terrestrial systems can evolve convergent ecological strategies despite 400 million years of independent evolution in vastly different habitats.

chemical cue | defense | eavesdropping | herbivory | prey tracking

Chemical cues and signals govern the processes that shape species demography, community structure, and ecosystem function in both terrestrial and marine ecosystems. However, the chemicals regulating these processes are better known for terrestrial systems, especially with regard to plant–herbivore interactions (1, 2). Terrestrial insect herbivores, most of which are trophic specialists (3), commonly locate prey by tracking plant volatiles (4). In response, plants induce chemical defenses both to reduce herbivory and to limit the spread of pathogens introduced by herbivore feeding (5); some also release volatile compounds that attract predators of their herbivores (6, 7). These volatile compounds involved in insect foraging and plant defense are well described; generally, they are nonpolar secondary compounds (or blends of compounds) that are plant-specific. In contrast, few herbivores in marine ecosystems are trophic specialists (8), and whether they use chemical cues to locate their prey remains poorly understood (9, 10). The identities of such foraging cues are unknown but are predicted to be polar compounds that diffuse readily through water (9, 10).

Halimeda incrassata is the dominant seaweed within Caribbean seagrass ecosystems (11, 12). It facilitates seagrass bed formation (13) and generates the majority of carbonate sediments within the ecosystem (12). Organisms affecting *H. incrassata* performance therefore may impact ecosystem-level ecology and biogeochemistry. The potent chemical defenses (14) and calcified thallus (85% $CaCO_3$, by dry mass) (12) of *H. incrassata* deter most herbivores (15). However, the sea slug *Elysia tuca* (hereafter *Elysia*) tolerates these defenses and selectively associates with and feeds on chemically rich species of green seaweed including *H. incrassata* (16). *Elysia* pierces the calcified thallus of its prey with a modified radula (17) and then feeds suctorially (18). It performs pharyngeal regurgitation while feeding to reduce the viscosity and facilitate withdrawal of cytoplasm (18). By sequestering its prey's chemical defenses (19) and chloroplasts (20), *Elysia* becomes chemically defended and cryptic; acquired chloroplasts continue photosynthesis within *Elysia*, transferring up to 60% of fixed carbon to the herbivore (20). Although *Elysia* has been found associated with other seaweeds, it is most common on *Halimeda* species (16), and its larvae settle and metamorphose only on *Halimeda* species, including *H. incrassata* (21). As one of the few herbivores that preferentially consume *Halimeda* species, *Elysia* may locate its prey by tracking *Halimeda*-derived chemical cues. However, the identities of distance foraging cues are unknown for this, or any other, marine herbivore.

Significance

We report, for the first time to our knowledge, compounds that specialist marine herbivores use to find their prey. The seaweed *Halimeda incrassata* produces metabolites that deter feeding by generalist herbivores. However, a specialist sea slug, *Elysia tuca*, follows these defensive compounds and not only attacks the seaweed but does so preferentially while the seaweed is reproducing. *Elysia* sequester *Halimeda's* chemical defenses (to deter predators) and chloroplasts (becoming photosynthetic). *Elysia* feeding reduces *Halimeda* growth by ∼50%, but the alga drops branches occupied by *Elysia*, possibly to avoid fungal infection associated with herbivory and to rid itself of *Elysia*. These interactions parallel many involving terrestrial insects and plants, even though marine and terrestrial herbivores have evolved independently for 400 million years.

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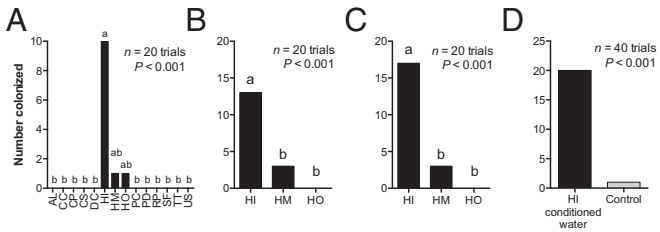

Fig. 1. *Elysia* host preference. Number of trials in which an *Elysia* colonized one of 14 common seaweeds and seagrasses ($n = 20$) (*A*), three co-occurring seaweeds in the genus *Halimeda* ($n = 20$) (*B* and *C*), or a cotton ball laced with *H. incrassata*-conditioned seawater vs. seawater only ($n = 40$) (*D*), when offered in a still water arena (*A*, *B*, and *D*) or in the field (*C*). Choice was assessed after 2 h (*A*–*C*) or within a 5-min period (*D*). Results were analyzed by a Cochran's Q (*A*–*C*) or Fisher's exact (*D*) test. In *A*–*C*, different letters above bars indicate significant differences among seaweeds in terms of *Elysia* colonization frequency, as determined by Wilcoxon sign tests (corrected for multiple comparisons). AL, *A. longicaulis*; CC, *Caulerpa cupressoides*; CP, *Caulerpa prolifera*; CS, *Caulerpa sertularioides*; DC, *Dictyosphaeria cavernosa*; HI, *H. incrassata*; HM, *H. monile*; HO, *H. opuntia*; PC, *Penicillus capitatus*; PD, *Penicillus dumetosus*; RP, *Rhipocephalus phoenix*; SF, *S. filiforme*; TT, *T. testudinum*; US, *Udotea* sp.

Here we used a series of field and laboratory experiments to determine (*i*) whether *Elysia* is a specialist; (*ii*) whether it tracks its prey using chemical cues; (*iii*) the identities of such foraging cues; (*iv*) the ecological effects of *Elysia* on *Halimeda*; and (*v*) whether *Halimeda* employs counterdefenses that limit grazing effects, including the spread of an *Elysia*-associated fungus.

Results and Discussion

At our field sites, 96% of *Elysia* occupied *H. incrassata* (mean density: ~1–4 per seaweed across sites and times; Table S1). In laboratory experiments in which we simultaneously offered *Elysia* 14 species of co-occurring seaweeds and seagrasses, *Elysia* colonized only *Halimeda* species, with 83% choosing *H. incrassata* (Fig. 1*A*). When offered only three species of *Halimeda* in laboratory and field experiments, 81–85% of *Elysia* selected *H. incrassata* over *Halimeda monile* and *Halimeda opuntia* (Fig. 1 *B* and *C*). Thus, *Elysia* preferentially tracks to and associates with *H. incrassata*.

Prey tracking was chemically mediated; in laboratory experiments *Elysia* colonized cotton balls laced with *H. incrassata*-conditioned water at 20 times greater frequency than unconditioned controls (Fig. 1*D*). Bioassay-guided fractionation of *H. incrassata* extracts (Fig. S1) resulted in the identification of a single attractant compound, 4-hydroxybenzoic acid (4-HBA) (Fig. 2*A*). This molecule occurs on the surface of *H. incrassata* (22) and was detected in two of 15 water samples drawn from enclosures placed around *H. incrassata* for 30 min in the field. When we coated 4-HBA (at natural concentration) or solvent only (control) onto 6 × 6 cm cloth squares and staked these squares adjacent to *H. incrassata* in the field for 24 h, seaweeds enriched with 4-HBA were colonized by 110% more *Elysia* than were control seaweeds (Fig. 2*B*), confirming that *Elysia* tracks to 4-HBA under natural field conditions.

In nature, *Elysia* were 12–18 times more abundant on reproductive *H. incrassata* than on vegetative individuals (Table S2), even though reproductive individuals constitute less than 5% of the population and persist for only 36 h (23). It is unlikely that *Elysia* uses 4-HBA to distinguish reproductive seaweeds rapidly, because the concentration of 4-HBA (the percent of the total dry mass, mean ± SE) within vegetative and reproductive thalli does not differ (reproductive: 0.03 ± 0.01%, vegetative: 0.09 ± 0.03%; $n = 4$; Wilcoxon signed rank test, $P = 0.125$; *SI Materials and Methods*). We therefore hypothesized that reproductive seaweeds produce an additional and more preferred attractant. In support of this hypothesis, when we coated a natural concentration of hydrophobic extracts from reproductive or vegetative *H. incrassata* onto 6 × 6 cm cloth squares and staked these squares adjacent to vegetative *H. incrassata* in the field for 24 h, seaweeds enriched with reproductive extract were colonized by 152% more *Elysia* than seaweeds enriched with vegetative extract (Fig. 2*C*). Bioassay-guided fractionation of reproductive *H. incrassata* extracts (Fig. S2) identified halimedatetraacetate (HTA) (Fig. 2*A*) as the primary cue that *Elysia* uses to locate reproductive *H. incrassata*. Although HTA deters large generalist herbivores from feeding on *Halimeda* species (24), *Elysia* sequesters HTA from *Halimeda* (19) and in doing so becomes unpalatable to fish predators (Fig. S3). Before spawning, *H. incrassata* forms uncalcified reproductive structures (gametangia) on its exterior. They contain high levels of HTA and chloroplasts (14), both of which are sequestered by *Elysia*. These concentrated resources may explain *Elysia's* preference for and aggregation on reproductive prey.

Increasing evidence suggests that small specialist herbivores that are scarce, have low metabolic rates, and remove little biomass while feeding are nonetheless capable of strongly impacting seaweed performance, in part because they can tolerate host defenses and preferentially target important structures such as gametes that otherwise are chemically defended (16). Our study supports and expands on this view. High abundances of *Elysia* on reproductive *H. incrassata* (Fig. S2) and observations of *Elysia* feeding on gametes (25) together suggest that *Elysia* likely reduces the fecundity of *H. incrassata*. In field experiments, when we manipulated *Elysia* densities on vegetative *H. incrassata*, a natural density of *Elysia* reduced seaweed growth by 54% and increased branch loss by 118% after 3 d, relative to seaweeds without *Elysia* (Fig. 3 *A* and *B*). Field experiments involving herbivore enclosures further demonstrated that *H. incrassata* selectively loses branches inhabited by *Elysia* (Fig. 3*C*). These findings suggest that the impacts of *Elysia* grazing are ecologically important and may scale up to have ecosystem-level consequences, because *H. incrassata* facilitates the establishment of seagrass (the ecosystem's foundation species) and generates a majority of the carbonate sediments within Caribbean seagrass habitats (12, 13).

Elysia feeding might weaken branches and cause their detachment (26). Alternatively, *H. incrassata* may amputate branches

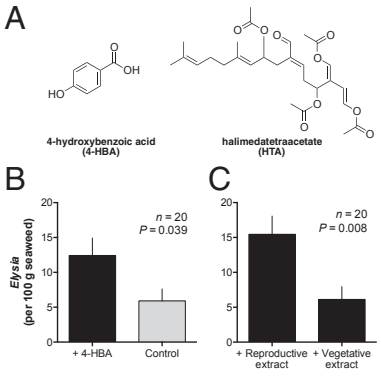

Fig. 2. Chemical attractants produced by *H. incrassata*. (*A*) *Elysia* hunts its seaweed prey by tracking *H. incrassata* cues. Shown are the structures of 4-HBA, which *Elysia* uses to locate vegetative *H. incrassata*, and HTA, which *Elysia* uses to locate reproductive *H. incrassata* during rare and ephemeral spawning events. Isolation of 4-HBA and HTA are reported in Figs. S1 and S2, respectively. (*B* and *C*) Mean (+ SE) number of *Elysia*/100 g seaweed that colonized *H. incrassata* in nature 24 h after seaweeds were enriched with 4-HBA or with a solvent control ($n = 20$) (*B*) or with extracts from vegetative or reproductive *H. incrassata* ($n = 20$) (*C*). Each result was analyzed by a Mann–Whitney test.

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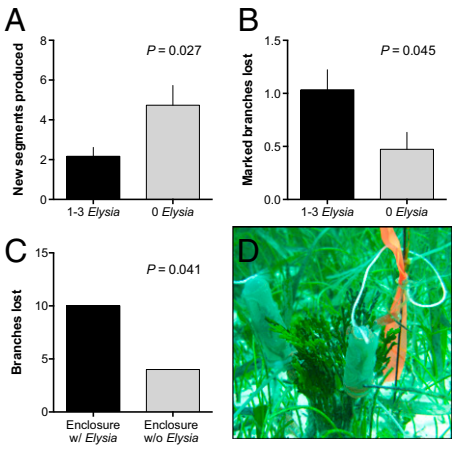

Fig. 3. Effects of *Elysia* grazing on *H. incrassata*. Mean (+ SE) number of new segments produced (per eight marked branches) (*A*) and marked branches lost (per individual seaweed) (*B*) by *H. incrassata* after 3 d, when occupied by a natural density of *Elysia* (black bars, $n = 29$) or no *Elysia* (gray bars, $n = 19$). (*C*) Number of *H. incrassata* that lost branches that were occupied (black bar) or unoccupied (gray bar) by *Elysia* for 5 d ($n = 15$). Seaweeds that lost their empty enclosure also lost their occupied enclosure. (*D*) Experimental setup. Results were analyzed by a Welch's *t* test (*A*), a Mann–Whitney test (*B*), or McNemar's test (*C*).

to rid itself of *Elysia*; a related seaweed (*Avrainvillea longicaulis*) amputates blades that are fouled (27), and numerous terrestrial plants drop leaves to rid themselves of herbivores (28). Amputation would be considered an extreme response to *Elysia's* suctorial feeding (which removes little biomass) unless *Elysia*, like many sap-sucking insects (29), vectors pathogens while injecting anticoagulants to feed (18). To investigate this possibility, we isolated and cultured fungi from *Elysia* radulae (the feeding apparatus) and assessed *H. incrassata* tissue loss following injection of one of the isolated fungal strains, Et-2. The elongation factor-1 alpha (*EF1α*) gene of the Et-2 isolate exhibited 93% nucleotide sequence similarity to that of *Lulworthia grandispora*, a fungus commonly found on decaying mangrove wood, leaves, fruits, and seedlings (Table S3). When injected, the fungus caused *H. incrassata* to drop segments above the inoculation site (Fig. 4*A*). Segment loss did not occur after injection of media alone or after injection of the marine fungus *Lindra thalassiae*, a pathogen of the seagrass *Thalassia testudinum* and several seaweeds (Fig. 4*B*). Regardless of mechanism, branch loss appears adaptive in that it removes *Elysia* and *Elysia*-vectored fungi that are potential pathogens.

It is hypothesized that trophic specialists in marine ecosystems should be selected to use prey-specific secondary metabolites as foraging cues, whereas generalists should track to mixtures of primary metabolites indicative of a variety of prey (9). This hypothesis was untested, because foraging cues have been identified for only a few marine consumers, none of which are specialist herbivores (10). We identified 4-HBA as the chemical cue that *Elysia* tracks to locate vegetative *H. incrassata* and HTA as the primary cue that it uses to locate the few reproductive *H. incrassata* that occur during each spawning event (23). *Elysia* may use HTA to track reproductive *H. incrassata*, because it is produced by *Halimeda* in species-specific amounts and is concentrated externally in the alga's gametangia during reproduction (14, 24). Our discovery of *Elysia* foraging cues reveals that *Elysia* uses prey secondary metabolites as foraging cues, a strategy common among terrestrial insects but previously unknown for marine herbivores (4).

Elysia displays remarkable similarities to some terrestrial insect herbivores in both its hunting strategy and in its feeding method, defense tactics, and effects on prey behavior and performance. Marine herbivores capable of consuming seaweeds, including sacoglossans, did not evolve until the late Mesozoic Era (30, 31); tight associations between specialist herbivores and such seaweeds arose more recently and often did so suddenly rather than through long histories of reciprocal coevolution (32). This timing is synchronous with the proliferation of modern insects and flowering plants and the beginning of their interaction (33). However, today's terrestrial insects and marine mollusks evolved independently throughout the Paleozoic Era. Thus, our results suggest that some specialist herbivores in marine and terrestrial ecosystems have evolved convergent hunting, feeding, and defense strategies despite some 400 million years of independent evolution in vastly different physical environments. Studies of other sacoglossans and insect-like marine herbivores should reveal whether this evolutionary convergence is common or unique to these species.

Materials and Methods

Study Sites and Organisms. Field surveys and experiments were conducted from 2007–2010 in shallow seagrass beds near Pickles Reef (24.9896 N, 80.4172 W) and Rodriguez Key (25.0478 N, 80.4526 W) in the northern section of the Florida Keys. These sites, 7.4 km apart, are both dominated by the seagrasses *T. testudinum* and *Syringodium filiforme* but differ in depth, exposure, adjacent habitat, and seaweed composition. Pickles Reef is 3–6 m deep, exposed, and adjacent to a reef flat; Rodriguez Key is 1–2 m deep, sheltered, and adjacent to a mangrove island. For seaweed community composition, see Table S1.

Interspersed among seagrasses at these sites are a variety of rhizophytic green seaweeds (Order: Bryopsidales). We selected one such seaweed, *H. incrassata,* to study, because it is (*i*) colonized by the sacoglossan sea slug *Elysia* (16); (*ii*) well described in terms of its chemical defenses (14); (*iii*) the dominant seaweed within seagrass habitats throughout both the Florida Keys reef tract (11) and the wider Caribbean (12); and (*iv*) critical to the ecology of (13) and carbonate cycling within (12) Caribbean seagrass ecosystems. We studied *Elysia* because it (*i*) is thought to be a specialist, presumably associating with and feeding on *Halimeda* species (16, 17, 19, 21, 25); (*ii*) has been studied previously with regard to its sequestration of seaweed chloroplasts (34) and chemical defenses (19); and (*iii*) is the most abundant and widespread sacoglossan in the Florida Keys, which harbors the highest densities of sacoglossans in the Caribbean (35). Organisms were collected in the immediate vicinity of Pickles Reef and Rodriguez Key. Unless otherwise noted, collections for chemical extraction were frozen (*H. incrassata*), or chemical extraction was performed immediately (*Elysia*) upon return from the field.

Laboratory *Elysia* colonization experiments (see below) were conducted in 2007 and 2009 on shaded tables in Key Largo, FL. Similar experiments were conducted in 2007 and 2010 in a temperature-controlled chamber at the Georgia Institute of Technology, Atlanta, GA. Feeding assays to test the deterrence of *Elysia* chemical extracts were conducted at 17-m depth on Conch Reef (24.9524 N, 80.4525 W), because it harbors an abundance of

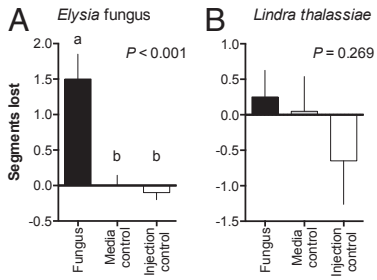

Fig. 4. Effect of fungal injection on *H. incrassata* tissue loss. Mean (+ SE) number of segments lost from *H. incrassata* above the site of inoculation when injected with a fungus isolated from the radula of *Elysia* (*A*) or with the common marine fungal pathogen *L. thalassiae* (*B*). Fungal effects (black bars) were assessed after 8 d, relative to media (gray bars) or needle-puncture (white bars) controls that were applied to other branches of the same seaweed ($n = 20$). Data from each experiment were analyzed by a Friedman's test. Letters above bars indicate significant differences among treatments within an experiment, via Friedman's post hoc tests.

predatory fishes (e.g., *Thalassoma bifasciatum*) that are commonly used in tests of feeding deterrence (36).

Field Surveys. Once in July and once in August 2007, we assessed *Elysia* host-use patterns by evaluating their abundance on the most common seagrasses and seaweeds at both Pickles Reef and Rodriguez Key. At each site, we carefully slipped a plastic zip-lock bag (Ziploc, SC Johnson) over each seaweed or bundle of seagrass, sealed the bag around the holdfast, severed the thallus at the seaweed–substrate interface, and closed the bag ($n = 10$ collections per species per site). Collections were made at least 5 m apart. In the laboratory, each sample was rubbed in a shallow pan of water to remove *Elysia*. After all *Elysia* were counted, each individual seaweed was blotted with paper towels and weighed. *Elysia* abundances then were scaled by seaweed biomass (number of *Elysia*/100 g seaweed). Data from each sampling period violated, and could not be transformed to meet, key assumptions of parametric analysis of variance (i.e., normality and homoscedasticity). Therefore, we used its nonparametric analog, the rank-based Kruskal–Wallis test, to test for differences in *Elysia* abundance among seaweed species. *Elysia* were largely absent from all seaweeds except *Halimeda*, creating asymmetry in the dispersion of the data, which violates an assumption of the Kruskal–Wallis test (37, 38). Notwithstanding, sample sizes were equal, and the abundance patterns observed in our surveys were clear and consistent across sites and sampling dates; therefore it is unlikely the tests generated spurious conclusions. As a second evaluation, we tested the null hypothesis that *Elysia* are distributed equally across all seaweed species, using χ^2 tests (37, 38). When comparing the observed *Elysia* densities/100 g seaweed on each seaweed species with the density that would be expected if all species were used equally, the null hypothesis of equivalent host use was rejected ($P < 0.001$) for each site and sampling period.

In July–August 2007 and May–June 2009, we also surveyed *Elysia* abundances on reproductive *H. incrassata* (i.e., seaweeds with gametangia on their exterior) to assess whether *Elysia* preferentially occupy reproductive individuals when such are present. Because spawning events are infrequent and each involves less than 5% of the population (12), and because reproductive thalli persist for only 36 h and then die (12, 23), we opportunistically collected reproductive seaweeds whenever found at Pickles Reef (as described above). With each, we also collected the nearest (within 2 m) vegetative *H. incrassata* of similar size to assess whether *Elysia* densities on reproductive seaweeds differed from the local baseline. Paired samples were returned to the laboratory where *Elysia* were enumerated and seaweeds were weighed as described above. *Elysia* densities then were scaled by seaweed biomass (*Elysia*/100 g seaweed). Paired replicates were pooled by collection year ($n = 12$ for 2007, $n = 12$ for 2009). Differences in the number of *Elysia* occupying vegetative or reproductive seaweeds in 2009 were assessed with a paired t test. The 2007 data violated an assumption of the paired t test (normality) but met the assumptions of its nonparametric analog, the Wilcoxon signed rank test (37, 38), so we analyzed the 2007 data using the latter test.

Colonization Experiments. In nature, patterns of *Elysia* distribution among seaweed species could reflect host specialization but also could be generated by ecological constraints such as predation. To quantify host preference per se, we conducted colonization experiments in small aquaria, using the same 14 seaweeds and seagrasses that we surveyed in the field (Table S1). We cut all species into pieces of similar projected surface area and randomly interspersed single pieces of each species across a 20-cm-diameter glass dish ($n = 20$), the bottom of which was covered with a thin layer of sand and filled with seawater to a depth of 4 cm. A single *Elysia* was deployed in the center of each dish. After 2 h, we scored which seaweed the slug colonized (or no choice). Based on the results of the first experiment, a second was conducted as above but with only the three species of *Halimeda* found locally (*H. incrassata*, *H. monile*, and *H. opuntia*). For that experiment, two pieces of each species were deployed in each dish. To verify that host preferences were similar in situ, where hydrodynamics may affect *Elysia* sensory function, we also conducted a colonization experiment at Pickles Reef. Within a large sand patch we deployed *H. incrassata*, *H. monile*, and *H. opuntia*, with an individual of each species serving as a point of a 10-cm equilateral triangle ($n = 20$). We randomized the location of each species within each triangle and spaced replicate triangles ~1 m apart. A single *Elysia* was placed in the center of each triangle; its choice of host (or no choice) was assessed after 2 h. Because these experiments were designed to determine relative host preference, trials in which *Elysia* selected no host were excluded from analysis. For these laboratory and field experiments, the data from each trial were neither continuous in scale nor independent (i.e., *Elysia*'s host selection may depend on the other seaweeds present in a trial), thus precluding the use of ANOVA (37). Hence the differences in the frequency with which *Elysia* colonized each seaweed species were assessed for each experiment using a Cochran's Q test (38). In all three cases, results were significant; to assess *Elysia* preference among hosts, we used pair-wise Wilcoxon signed rank tests, corrected for multiple comparisons (Benjamini–Hochberg procedure).

Colonization experiments indicated that *Elysia* is a specialist on *H. incrassata* and may locate the seaweed using chemical cues (i.e., in experiments *Elysia* commonly raised the front portion of its body from the substrate, spread its rhinophores (chemosensory organs), waved them back and forth as if testing for a chemical cue, and then proceeded to *H. incrassata*). To assess the role of chemical cues in *Elysia* foraging rigorously, we conducted a colonization experiment similar to those described above but used a mimic laced with *H. incrassata*-conditioned water instead of the seaweed. To condition seawater, we soaked 45 g of *H. incrassata* in 1 L of seawater (Instant Ocean, Spectrum Brands) for 3 h; we then removed the seaweed and soaked a cotton ball in the conditioned water for 2 min. Control cotton balls were soaked for 2 min in the same volume of unconditioned seawater, which originated from the same batch of Instant Ocean. Next, one treated and one control cotton ball were placed in opposite corners of a rectangular, 740-mL plastic container filled with 400–450 mL of seawater ($n = 40$). Treated and control balls were always in opposite corners of the arena, but their locations were randomized. After 2 min, a single *Elysia* was placed in the center of each arena. We scored whether it first colonized the treated or control ball within a 5-min period. *Elysia* that climbed the arena wall and became caught on the surface tension of the water were placed back in the center. A score of "no choice" was given if *Elysia* did not colonize either ball or climbed the wall and became caught in the surface tension twice within the test period. This experiment produced binary response data (colonized vs. not colonized). Consequently, differences in the frequency with which treated vs. control cotton balls were colonized was assessed using a Fisher's exact test (37, 38).

Isolation of Attractant Cues Produced by *Halimeda*. To isolate the chemical cue(s) that *Elysia* uses to locate *H. incrassata*, we collected a bulk sample of *H. incrassata*, measured its volume, extracted it exhaustively in methanol, fractionated the resulting organic extract, and tested the attractant qualities of each fraction via colonization experiments. Subsequently, each active fraction was separated further, and its constituents were tested, a process that was repeated until individual attractant molecules were isolated and purified. Purification methods are described in detail in *SI Materials and Methods*. To prepare a bioassay, we coated a natural volumetric concentration of an extract onto a small piece of cotton ball (one/sixth of a cotton ball) using diethyl ether and allowed the solvent to evaporate. Controls coated only with diethyl ether were created using the same procedures. Colonization experiments ($n = 10$–20 per fraction) were conducted and analyzed as described above for the conditioned water experiment. These assays isolated the *Halimeda*-derived cues that *Elysia* uses to locate vegetative and reproductive *H. incrassata* (4-HBA and HTA, respectively).

Structural Elucidation of Attractant Cues. 4-HBA isolated from *H. incrassata* was characterized by NMR spectroscopy and mass spectrometry. Liquid chromatography/electron spray ionization mass spectrometry (LC/ESI-MS) indicated the presence of a nonhalogenated small molecule (ESI-MS $C_7H_6O_3$ $[M - H]^-$ m/z 136.88). The ^{1}H NMR spectrum of the isolated compound showed two chemical shifts at δ_H 7.85 (d, 6.8 Hz) and 6.78 (d, 6.8 Hz) ppm (CD_3OD, 500 MHz). Collectively, these data indicated that the structure of the molecule was 4-HBA. These results were confirmed by comparison of spectroscopic data with an authentic sample of 4-HBA purchased from Alfa Aesar (catalog no. A13700).

HTA isolated from *H. incrassata* was identified by a combination of 1D and 2D NMR spectroscopy and mass spectrometry. LC/ESI-MS indicated the presence of a molecule (ESI-MS $C_{28}H_{38}O_9$ $[M - H]^-$ m/z 517.58, $[M + Na]^+$ m/z 541.63). The ^{1}H and ^{13}C NMR spectral data of the isolated compound ($CDCl_3$, 500 MHz) were identical to those previously reported for HTA (39, 40): ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.33 (s, 1H), 7.62 (d, $J = 12.6$ Hz, 1H), 7.26 (s, 1H), 6.43 (t, $J = 7.6$ Hz, 1H), 5.97 (t, $J = 8.1$ Hz, 1H), 5.82 (d, $J = 11.2$ Hz, 1H), 5.51 (dt, $J = 8.3$, 8.1 Hz, 1H), 5.08 (d, $J = 8.1$ Hz, 1H), 5.02 (m, 1H), 3.03 (dt, $J = 14$, 8.0 Hz, 1H), 2.72 (dt, $J = 14$, 7.9 Hz, 1H), 2.55 (d, $J = 7.8$ Hz, 1H), 2.17 (s, 3H), 2.14 (s, 3H), 2.06 (s, 3H), 2.02 (m, 1H), 1.97 (d, $J = 8.0$ Hz, 1H), 1.94 (s, 3H), 1.65 (s, 6H), 1.57 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 193.8, 170.0, 169.8, 167.7, 166.8, 149.4, 141.2, 141.2, 137.2, 134.5, 131.7, 123.7, 122.5, 118.1, 108.8, 70.0, 67.9, 39.4, 32.5, 29.5, 26.2, 25.6, 21.2, 20.9, 20.6, 20.6, 17.7, 16.8 ppm.

All LC/MS work was performed with a reversed-phase column (Waters Symmetry C_{18}, 4.6 × 150 mm, 5 μm) using a Waters 2695 Separations Module coupled to a Waters 2996 Photodiode Array Detector and Waters ZQ2000 ESI mass spectrometer. NMR spectra were recorded on a Bruker DRX-500

instrument, using a 5-mm broadband probe for ^{1}H and ^{13}C experiments and were referenced to residual CD_3OD (3.31 and 49.0 ppm, for ^{1}H and ^{13}C, respectively) or $CDCl_3$ (7.24 and 77.0 ppm, for ^{1}H and ^{13}C, respectively).

Field Assessments of Attractant Cues. To verify that 4-HBA operates as an *Elysia* foraging cue under natural hydrodynamic conditions, we conducted experiments at Pickles Reef in which we enriched *H. incrassata* with either 4-HBA or solvent only (control) and assessed the abundance of *Elysia* that colonized the seaweeds after 24 h. To prepare the experiment, we dissolved 4-HBA (Alfa Aesar) in diethyl ether, coated 6 cm × 6 cm cotton cloth squares with a natural volumetric concentration of the compound (treatment) or an equivalent amount of diethyl ether (control), allowed the solvent to evaporate, and speared each square with a bamboo stake ($n = 20$). In the field, we staked each square within 1–2 cm of a defaunated, vegetative *H. incrassata*, with treatment and control seaweeds interspersed and replicates separated by at least 2 m. After 24 h, we carefully bagged the seaweeds, returned them to the laboratory, counted the *Elysia* on each seaweed, blotted each seaweed dry, and weighed each seaweed. *Elysia* densities then were scaled by seaweed biomass (*Elysia*/100 g seaweed). The data violated an assumption of the *t* test (normality) but met the assumptions of the nonparametric Mann–Whitney test (37, 38), so we used the latter to test for differences in the abundance of *Elysia* occupying treatment vs. control seaweeds.

Elysia were more abundant on reproductive *H. incrassata* than on nearby vegetative individuals (Table S2), despite the rare and ephemeral nature of reproductive seaweeds (23). To determine if swarming to reproductive *H. incrassata* is mediated by a greater chemical attraction to them, we conducted an experiment at Pickles Reef similar to that described in the previous paragraph, but instead we enriched *H. incrassata* with extracts of reproductive or vegetative *H. incrassata*. To prepare the experiment, we exhaustively extracted reproductive and vegetative seaweeds (each 200 mL volumetric displacement) in methanol, filtered the extracts, removed the methanol under vacuum, partitioned each extract between water and ethyl acetate (4×), retained only the lipophilic (ethyl acetate) fraction of each extract (water fractions were not active; Figs. S1 and S2), and removed solvents from each extract by rotary evaporation. We then dissolved each extract in diethyl ether, coated each at a natural volumetric concentration onto 6 cm × 6 cm cotton cloth squares, allowed the solvent to evaporate, speared each square with a bamboo stake ($n = 20$ per extract), and placed each stake within 1–2 cm of a defaunated, vegetative *H. incrassata* in the field. Treatments were interspersed, with replicates separated by at least 2 m. After 24 h, we collected the samples and processed them as described in the previous paragraph. Differences in the abundance of *Elysia* occupying seaweeds treated with extract from reproductive or vegetative *Halimeda* were assessed with a Mann–Whitney test, for the reasons described in the previous paragraph.

Isolation of *Elysia* Antipredator Chemical Defenses. In addition to using experiments to guide our isolation of attractant cues produced by *H. incrassata*, we also conducted feeding experiments to determine whether *Elysia* is chemically defended from predators, and, if so, whether protection is caused by a sequestration of chemicals from *H. incrassata* (19). We extracted *Elysia* and partitioned the extract based on polarity (detailed methods are described in *SI Materials and Methods*) and then tested whether a predatory reef fish (*T. bifasciatum*) rejected food pellets containing each fraction or purified compound. To prepare feeding assays, we dissolved each fraction or compound in solvent and incorporated it at a natural volumetric concentration into a food gel composed of 5% dried squid and 5% sodium alginate. This gel was formed into a noodle-like strand, firmed with 0.25 mol/L aqueous $CaCl_2$, and cut into individual pellets (for methods see ref. 36). Control pellets were prepared in the same manner, except they lacked slug extract. In the field, we first fed an individual *T. bifasciatum* a control pellet to confirm it was feeding and then fed it a treatment pellet and assessed whether the pellet was consumed or rejected ($n = 20$ per fraction). If the treatment was rejected, another control pellet was offered to assure rejection was not caused by satiation. However, no fish were found to be satiated following rejection of the treatment pellet. Differences in the frequency with which treated vs. initial control pellets were rejected were assessed for each fraction using a Fisher's exact test. These assays identified HTA as the compound that *Elysia* sequesters from *Halimeda* (19) to deter fish predators.

Grazing Experiments. In August 2007, the effects of *Elysia* feeding on *H. incrassata* growth and branch loss were assessed with a manipulative field experiment. At Pickles Reef we selected and flagged 20 groups of *H. incrassata* that met the following criteria: (*i*) four individuals were present within an area of 1–2 m, and their positions approximated a square; (*ii*) they were similarly sized; and (*iii*) they showed evidence of recent growth (i.e., new growth tips). After resident *Elysia* were manually removed, dental floss was tied around eight branches on each individual seaweed, with the floss positioned just below the second segment from the apex; these markers served as a baseline to gauge new growth (which is apical) or the loss of marked branches. We then applied no, one, two, or three *Elysia* onto one of the four seaweeds in each block. After 3 d, seaweeds were carefully bagged and returned to the laboratory. We then counted the *Elysia* occupying each individual seaweed and the number of new segments produced by each remaining marked branch. Fewer than eight remaining marked branches was interpreted as branch loss.

Few replicates gained *Elysia*, but many lost *Elysia* during the experiment. Hence in many cases treatments were not fully retained. Replicates were excluded from analysis if they did not initially receive *Elysia* but were colonized during the experiment. Similarly, replicates were excluded if they initially received *Elysia* but were not inhabited by any *Elysia* at the end of the experiment. Seaweeds that retained some *Elysia* were pooled as one-to-three *Elysia* ($n = 29$) and were compared with seaweeds that remained without *Elysia* throughout the experiment (0 *Elysia*; $n = 19$). Such an approach is conservative, given that 23 of the 29 replicates in the one to-three *Elysia* group originally received two or three *Elysia* but harbored only one at the end of the experiment. Likewise, our calculation of growth (new segments produced per eight marked branches) also was conservative: Our analysis assumed zero growth on lost branches but did not penalize for the loss of biomass from lost branches. Data regarding branch loss violated an assumption of the *t* test (normality) but met the assumptions of its nonparametric analog, the Mann–Whitney test. Data regarding growth were neither normally distributed nor homoscedastic. Differences in branch loss and growth between treatment ("one-to-three *Elysia*) and control (no *Elysia*) seaweeds therefore were evaluated with a Mann–Whitney test and Welch's *t* test, respectively (37).

In June 2009, we conducted a manipulative experiment to determine whether branch loss was associated with *Elysia* grazing. To do so, we constructed slug enclosures and assessed whether a branch encased by an enclosure containing a slug was lost from *H. incrassata* more frequently than a paired branch encased by an empty enclosure (Fig. 3*D*). Each enclosure was constructed from the end of plastic test tube (5 cm length × 1.5 cm diameter), which could be slipped over the tip of a branch. To construct each enclosure, we (*i*) bored four holes (1-cm diameter) through the tube and glued nylon mesh from L'eggs stockings (Hanesbrands Inc.) over each hole to create four windows that allowed water to pass in and out of the enclosure; (*ii*) glued and cable-tied a nylon mesh skirt to the bottom opening of the tube to create a complete seal around the branch; and (*iii*) attached a small weight to the tube to make it neutrally buoyant.

At Pickles Reef, we selected 30 similarly sized *H. incrassata* spaced ~2 m apart and deployed enclosures, one empty and one containing a single *Elysia*, on opposite sides of each seaweed. We closed each by sealing the skirt shut with a small cable tie. Paired enclosures were leashed by nylon thread to a wire flag so they could be retrieved if dropped and to verify that treatments were retained during the experiment (Fig. 3*D*). After 5 d, we evaluated branch loss as a function of treatment. In eight instances, *Elysia* escaped from treatment enclosures. In addition, seven seaweeds underwent sexual reproduction during the experiment. Those 15 replicates were excluded. Because the response data were nominal, treatments and controls were paired, and multiple outcomes (both enclosures lost, neither lost, enclosure with slug lost, enclosure without slug lost) were equally possible, the data were evaluated with a McNemar's test (41). A robust sample size ($n > 10$) allowed approximation of the χ^2 distribution (41). With one degree of freedom, Yates correction for continuity was applied (41). This approach allowed us to test for differences in the frequency with which treatment and control branches were dropped from experimental seaweeds while controlling for the instances in which neither or both were dropped.

Isolation and Identification of Fungi Associated with *Elysia* Radulae. In August 2007, we cultured fungi from the radulae of 20 randomly selected *Elysia* collected from Pickles Reef. Each radula was removed by dissection with sterile needlepoint tweezers and was plated on a separate culture plate containing yeast extract/peptone/mannitol penicillin/streptomycin (YPM P/S) media (2 g/L yeast extract, 2 g/L peptone, 4 g/L D-mannitol, 16 g/L agar, and 250 mg/L L-penicillin G/streptomycin sulfate in artificial seawater). Isolation plates were sealed with Parafilm (Bemis NA) and incubated at 27 °C. Culture plates were monitored daily, and emerging fungi were replated until a pure culture was obtained. In total, we successfully cultured four fungi (one from each of four radulae).

Genomic DNA was extracted from the four freshly collected fungal isolates (which we designated "Et-1," "Et-2," "Et-3," and "Et-4") using the DNeasy Tissue Extraction Kit (Qiagen). *EF1*α and 28S large subunit ribosomal RNA (*28SrRNA*) genes then were amplified and sequenced (see *SI Materials and Methods* for details), and sequences were deposited in GenBank with accessions nos. KT246475–KT246481. Sequences were edited manually in BioEdit version 7.0.5.3 (42) and aligned using ClustalW (43). Sequence similarity of the target genes to other fungal taxa was determined for each isolate in a BLAST search (44). *28SrRNA* from isolate Et-2 failed to amplify using the primers Lul28Sfor and Lul28Srev. E-values and maximum scores from the BLAST queries revealed moderate to high similarity of each isolate to several fungal genera (Table S3). After identification, small sections of mycelium from each fungal isolate were frozen in 1 mL liquid YPM media (containing 10% glycerol) in cryo-vials at −80 °C for use in future experiments.

Fungal Inoculation Experiments. In September 2010, we thawed and plated the four *Elysia*-associated fungal isolates. Isolate Et-2 resumed growth after 48 h, but the others did not after more than 21 d; thus Et-2 was the sole isolate used in the inoculation experiment. At Pickles Reef, we flagged 40 *H. incrassata* of similar size and spaced 1–2 m apart ($n = 20$ for Et-2 experiments; $n = 20$ for *L. thalassiae* experiments). To each seaweed, a piece of red, white, or black thread was tied on each of three branches, just below the fifth segment from the apex of the branch. Then, under the tissue layer of the fifth segment from the apex of the three marked branches, we injected 0.05 mL of inoculum from the fungus Et-2 or from the common marine fungal pathogen *L. thalassiae*, YPM media as a control, or a blank injection (i.e., only a needle puncture) as a control. The preparation of fungal suspensions is described in *SI Materials and Methods*. After 8 d, we scored the number of segments gained or lost above the inoculation site and whether the branch was missing. Because branch loss was limited to only 10% (12 of 120) of experimental seaweeds and showed no clear pattern, we focused on segment loss as a response metric. Both treatments and controls were deployed on each individual seaweed and therefore were not independent; as such, differences among treatments and controls with respect to segment loss or gain were evaluated for each experiment using a Friedman's test and post hoc comparison (37, 38).

Statistical Analysis Software. We performed Cochran's Q and Friedman's tests in R version 2.15.0 (45) using the package RVAideMemoire version 0.9-27 (46) and agricolae version 1.1–8 (47), respectively. Welch's *t* test also was performed in R. All other statistical analyses were performed in SigmaStat version 3.5 (Systat Inc.).

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