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PLANT ANIMAL INTERACTIONS

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Dopamine functions as an antiherbivore defense in the temperate green alga *Ulvaria obscura*

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Abstract On northeastern Pacific coasts, *Ulvaria obscura* is a dominant component of subtidal "green tide" blooms, which can be harmful to marine communities, fisheries, and aquaculture facilities. *U. obscura* is avoided by herbivores relative to many other locally common macrophytes, which may contribute to its ability to form persistent blooms. We used a bioassay-guided fractionation method to experimentally determine the cause of reduced feeding on *Ulvaria* by echinoderms, molluscs, and arthropods. Our results indicated that dopamine, which constituted an average of 4.4% of the alga's dry mass, was responsible for decreased feeding by sea urchins (*Strongylocentrotus droebachiensis*). Subsequent experiments demonstrated that dopamine also reduced the feeding rates of snails (*Littorina sitkana*) and isopods (*Idotea wosnesenskii*). Dopamine is a catecholamine that is a common neurotransmitter in animals. The catecholamines dopamine, epinephrine (adrenaline), and norepinephrine also occur in at least 44 families of higher plants. The functions of catecholamines in plants are less well known than in animals but are likely to be diverse and include both physiological and ecological roles. Our results are the first experimental demonstration of a plant or algal catecholamine functioning as a feeding deterrent. This novel use of dopamine by *Ulvaria* may contribute to the formation and persistence of harmful *Ulvaria* blooms in northeastern Pacific coastal waters.

Keywords Algae · Chemical defense · Dopamine · Herbivory · Plant–herbivore interactions

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Introduction

Many marine seaweeds produce novel natural products that provide protection from grazing by invertebrate and vertebrate herbivores (McClintock and Baker 2001; Paul 1992). Because the intensity of herbivory is thought to be higher at lower latitudes, tropical species are thought to produce more effective chemical defenses than temperate species (Bolser and Hay 1996; Cronin et al. 1997). As a result, chemical defenses are better documented for tropical species. Studies of temperate algal defenses have primarily focused on the production of brown algal phlorotannins (Amsler and Fairhead 2006); however, recent studies have found that many temperate green algae also contain herbivore-deterrent natural products (Van Alstyne and Houser 2003; Van Alstyne et al. 2001). The production of chemical defenses by temperate green macroalgae is ecologically relevant because many of these species are capable of rapid population growth and can form large blooms that have a variety of detrimental effects on the structure and diversity of local communities (Fletcher 1996; Raffaelli et al. 1998).

Increasing evidence suggests that the production of toxic or deterrent natural products by ulvoid green macroalgae (phylum Chlorophyta, order Ulvales) may be an important but overlooked aspect of their ecology. Chemicals produced by ulvoid macroalgae can be responsible for inhibiting invertebrate settlement (Magre 1974) and herbivore feeding (Van Alstyne and Houser 2003; Van Alstyne et al. 2001), causing larval mortality (Johnson and Welsh 1985), and slowing phytoplankton growth (Jin and Dong 2003). Blooms of ulvoid green algae occur worldwide (Fletcher 1996). They are often

associated with increases in seawater nutrient concentrations (Schramm and Nienhuis 1996; Valiela et al. 1997), although the abundance and productivity of blooms can also be limited by a variety of potentially interacting abiotic and biotic factors, including competitors (Lotze et al. 1999), light (Henley and Ramus 1989; Peckol and Rivers 1995), and herbivores (Aronson and Precht 2000; Lotze and Worm 2000; Rowcliffe et al. 2001). Therefore, macroalgal natural products that limit consumption by herbivores or that slow the growth of competitors could potentially affect the formation and persistence of blooms.

In this study, we explored the production of chemical feeding deterrents by the ulvoid green alga *Ulvaria obscura* var. *blytii*. *U. obscura* is a low intertidal to subtidal alga that occurs along the North Pacific and North Atlantic coasts (O'Clair and Lindstrom 2000). In Washington coastal waters, it can be the dominant component of subtidal ulvoid blooms (Nelson et al. 2003a), which are commonly known as "green tides". Initial evidence suggests that *U. obscura* produces at least one bioactive natural product. Water-soluble exudates from *U. obscura* inhibit brown algal zygote germination, oyster larval development, and macroalgal growth (Nelson et al. 2003b). Green sea urchins also avoid consuming *U. obscura* in the laboratory, even though they will congregate around it in the field (Himmelman and Nedelec 1990). The purpose of this study was to assess whether *U. obscura* is a low-preference food for multiple types of grazers, relative to other co-occurring macrophytes, and to determine why grazers avoid it. We found that dopamine, a ubiquitous neurotransmitter in animals, was responsible for grazers avoiding *U. obscura*.

Materials and methods

Multiple-choice feeding-preference experiments

To assess feeding preferences of herbivores for *U. obscura* relative to other sympatric macrophytes, we conducted multiple-choice feeding-preference assays with three phylogenetically diverse, locally common herbivores. Green sea urchins *Strongylocentrotus droebachiensis* (one per container) were simultaneously offered ~0.5 g pieces of *Alaria marginata, Laminaria saccharina, Mazzaella splendens*, *Nereocystis luetkeana*, *Ulva linza*, *Ulva lactuca*, *U. obscura*, and *Zostera marina* in 20 cm-diameter Rubbermaid containers that had four sets of ten holes drilled on the sides with 1-mm-mesh fiberglass screens placed over them to allow for water flow. The algae were collected from the beach in front of the Shannon Point Marine Center (hereafter referred to as the Shannon Point Beach). Isopods *Idotea wosnesenskii* (five per container) and snails *Littorina sitkana* (ten per container) were offered ~0.1 g pieces of *Fucus gardneri, M. splendens, Mastocarpus papillatus, Microcladia borealis, U. linza*, *U. lactuca*, and *U. obscura* in 20 cm-diameter Rubbermaid containers. To measure autogenic algal mass changes, each experimental container was paired with a control container that held algae but no herbivores. Algal pieces were weighed prior to the start of the experiments. Containers were checked periodically and herbivores were removed when approximately half the algae in the container had been consumed or after 2 days. At the end of the experiment, experimental and control algae were reweighed to determine mass changes. A Yao's *R*-test (Manly 1993) was used to determine if there were significant differences in the consumption rates of the algae.

Isolation of the active metabolite in *U. obscura*

A bioassay-guided fractionation method was used to isolate feeding-deterrent compounds from *U. obscura* (Fig. 1). Algae were collected from a seagrass bed on the Shannon Point Beach on 7 July 2001 from a mixed stand of *U. obscura*, *U. fenestrata,* and *U. linza*. At the time of collection, ulvoid green algae were the dominant algae in the subtidal and intertidal zones at this site, but the populations were not sufficiently large to be considered a bloom. To obtain crude polar and non-polar extracts, algae (~320 g) were blotted dry, weighed, and extracted in 2:1 dichloromethane (DCM):methanol (MeOH) in darkness at −10°C for 2 days. Preliminary experiments indicated that the active compounds could be affected by the preparation and extraction method (Nelson 2003); therefore, we chose to extract the algae for a longer period of time at low temperature rather than conducting more rapid extracts at room temperature. The extracts were filtered, separated into polar (water-soluble) and non-polar (DCM-soluble) fractions with a separatory funnel, dried on a rotary evaporator, and dissolved in either MeOH (polar) or DCM (non-polar). To prepare the algal base for the agar-based foods used in feeding experiments with *S. droebachiensis*, either extracts (experimental foods) or solvents (control foods) were mixed with 5 g freeze-dried ground *L. saccharina* and dried with a rotary evaporator. To make the agar-based foods, 1.5 g agar and 43.5 ml distilled water were heated in a microwave. The extract or solvent-coated *L. saccharina* was added immediately before the agar solidified at approximately 40°C to avoid thermal decomposition of the extracts. Each mixture was then poured onto a glass plate over sand (3 g) that was spread on the bottom of the plate to keep the food from floating in the containers. After it cooled, the agar was cut into approximately 1 g pieces and weighed.

Each urchin ($n = 15$) was offered an experimental food piece containing an extract and a control food piece that lacked the extract. In order to control for autogenic mass changes, each experimental container was paired with an identical control container that lacked herbivores. Animals were allowed to feed until approximately half the food in the container was re-

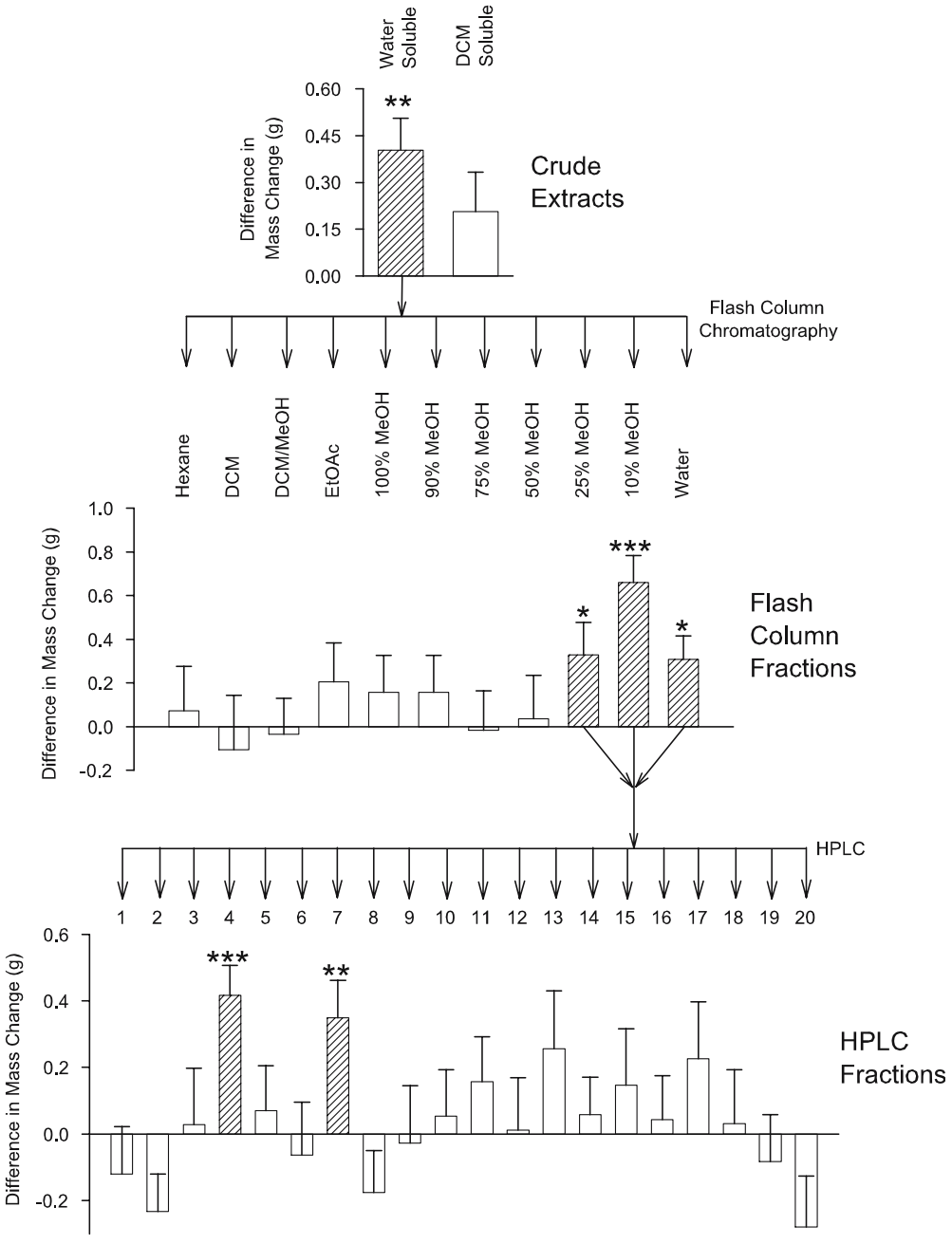

Fig. 1 Bioassay-guided fractionation method used to isolate bioactive compounds in *Ulvaria obscura*. Differences in mass change are the mass losses of the control foods (corrected for losses in containers lacking urchins) minus mass losses of food with extracts or fractions (corrected for losses in containers lacking urchins) in urchin bioassays. *Error bars* are ± 1 SE ($n = 15$–30). Flash column fraction types and high performance liquid chromatograph (*HPLC*) fraction numbers (corresponding to minutes on the HPLC) are given *above* the corresponding data for feeding experiments conducted with those fractions. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *EtOAc* Ethyl acetate, *DCM* dichloromethane, *MeOH* methanol

moved, but for no more than 4 h. All replicates in which $< 25\%$ or $> 75\%$ of the food was eaten were excluded from further analyses. The change in mass of the food with extracts was subtracted from the change in mass of the food with no extracts for each experimental and control replicate. Then the difference in the control container (no herbivores) was compared to the difference in the experimental container with a paired *t*-test ($\alpha = 0.05$). Wilk–Shapiro/Rankit plots were calculated with Statistix (version 2) to determine normality.

To further isolate the activity in the polar extract, additional *U. obscura* was collected from the Shannon Point Beach on 2–4 August 2001 and was extracted twice in MeOH, concentrated with a rotary evaporator, and then dried on a Savant Speed Vac Plus SC210A. The extract was then partitioned with reverse-phase vacuum flash chromatography through Hyperprep C18 silica (Supelco 13325). Extract (20 g) was loaded onto a 60 ml column and 50 ml of each of the following solvents was pulled through the column with a vacuum: water, 10% MeOH, 25% MeOH, 50% MeOH, 75% MeOH, 90% MeOH, 100% MeOH, 1:1 DCM:MeOH, ethyl acetate (EtOAc), DCM, and hexane. Each fraction was collected, dried with a Savant Speed Vac Plus, weighed, and re-dissolved in the extraction solvent. To make the experimental foods, the equivalent amount of fraction from 50 g fresh mass (FM) of *U. obscura* was added to 4 g freeze-dried *L. saccharina* and dried with a rotary

evaporator. To make the control foods, an equal volume of the corresponding solvent was added to 4 g *L. saccharina* and dried. Agar foods were made as described above and feeding preference experiments with the fractions were conducted in which urchins ($n = 15$) were given a choice between experimental food containing an extract fraction and an identical control food that lacked the extract.

To further isolate the activity within the active fractions (Fig. 1), the water, 10% MeOH, and 25% MeOH fractions were combined and separated with a Varian ProStar high performance liquid chromatograph (HPLC). Approximately 30 sets of HPLC fractions were each collected over 1-min intervals for 20 min (Varian ProStar UV detector: 225 and 375 nm; Varian C-18 column (25 cm×4.6 mm×5 μm); 1.0 ml/min, linear gradient of 90–10% aqueous acetonitrile). No peaks were detected after 12 min with the detector set at 225 nm. Fractions from individual runs were combined to yield 20 HPLC fractions. Each fraction was then dried with a rotary evaporator, weighed, and dissolved in MeOH. Bioassays were conducted as described above with the 20 HPLC fractions using the equivalent material from 50 g FM *U. obscura* for the experimental foods.

Gas chromatography/mass spectroscopy (GC/MS) and nuclear magnetic resonance (NMR) spectroscopy were then used to examine the two deterrent HPLC fractions (Fig. 1). GC/MS analyses were conducted with a Varian CP-3800 GC/Saturn 2000 ion trap detector [scan range of 40–650 atomic mass units (AMU)] with a J&W DB5-MS column (30 m×0.25 mm, 0.25 μ) using a split injection of 10:1 at 250°C with helium as a carrier at 1.2 ml/min and an oven temperature of 50°C ramping to 275°C at 10°C/min. Data were compared to spectra from a National Institute of Standards and Technology database and data from commercially obtained pure compounds. NMR analyses were conducted on a Mercury-300BB NMR spectrometer in deuterated MeOH.

Measurements of tissue dopamine concentrations

To quantify tissue dopamine concentrations with HPLC in *U. obscura, U. lactuca*, and *U. linza*, approximately 0.1 g pieces of algae ($n = 10$ per species) were extracted by soaking them in 10 ml of 80% aqueous MeOH for 1 week at −70°C. The extracts were filtered through GF/A glass fiber filters then though a 0.22 μm filter. Concentrations were determined by injecting 10 μl extract on a Waters HPLC running an isocratic elution of 0.1 M phosphate buffer (pH 4.0) containing 1.0 mM heptane sulfonic acid and 10% MeOH (Shah et al. 2003) at 1.0 ml/min on a Supelcosil LC-ABZ column (5 cm×4.6 mm×5 μm). Peak areas (at 1.6 min) were determined using electrochemical detection (+600 mV) and compound identifications were verified with a photodiode array detector. Commercially obtained dopamine hydrochloride (Sigma H-8502) was used as a standard (standard range: 0.2–1.2 μg, $R^2 = 0.995$, $P < 0.001$).

Agar-based feeding experiments with dopamine

To assess the impact of dopamine on feeding by a more diverse group of herbivores, *I. wosnesenskii, L. sitkana*, and *S. droebachiensis* were given choices of agar-based diets containing dopamine (added as the hydrochloride salt; Sigma H-8502) and control diets lacking it. These experiments were conducted at several concentrations of dopamine that were less than or equal to mean naturally occurring concentrations of dopamine in *U. obscura* (on a FM basis). Dopamine was initially offered to each herbivore at 0.34% FM, or at about one-third of the natural concentration. In subsequent experiments, the concentration was either decreased by a factor of 2 if foods containing the compound were significantly avoided ($\alpha = 0.05$) or increased by about 0.3% FM if the addition of the compound had no significant effect on feeding.

The experiments were conducted as described above for *U. obscura* fractions with the following exceptions. In the *L. sitkana* experiments, no sand was used, five *L. sitkana* were in each of the experimental containers, the experiments were conducted in 10 cm-diameter plastic Petri dishes, foods were embedded in a 1 mm^2 fiberglass mesh and the animals were offered 10 mm×10 mm pieces of foods, and the amount of surface area removed from the mesh was measured, rather than the loss of mass. For *I. wosnesenskii* experiments, no sand was used, the animals were offered 10 mm×10 mm pieces of foods embedded in fiberglass mesh, the amount of surface area removed from the mesh was measured, and there were two individuals in each of the experimental containers.

Results and discussion

U. obscura was a low preference food relative to other morphologically similar green algae for three locally common herbivores that are sympatric with it. For some of these herbivores, *U. obscura* was even a low preference food relative to other algae that produce chemical defenses or are structurally more complex. In multiple-choice feeding-preference experiments, *I. wosnesenskii*, *L. sitkana*, and *S. droebachiensis* had significant (Yao's *R*-test, $P < 0.05$) but distinct preferences for the foods offered (Fig. 2). *I. wosnesenskii* and *L. sitkana* showed strong preferences for the chlorophytes *U. lactuca* and *U. linza* (Fig. 2a, b); however, *U. obscura* was eaten in much lower amounts by both grazers. *I. wosnesenskii* ate about one-third as much *U. obscura* as *U. lactuca* and *U. linza*, whereas the consumption rate of *U. obscura* by *L. sitkana* was about one-fifth as much as on the other green algae. *S. droebachiensis* preferred kelps, especially *N. luetkeana* and *L. saccharina* (Fig. 2c). *U. linza* was the only green alga the urchins ate readily. *U. lactuca* was eaten in moderate amounts by *S. droebachiensis*; *U. obscura* and the eelgrass *Z. marina* were eaten least.

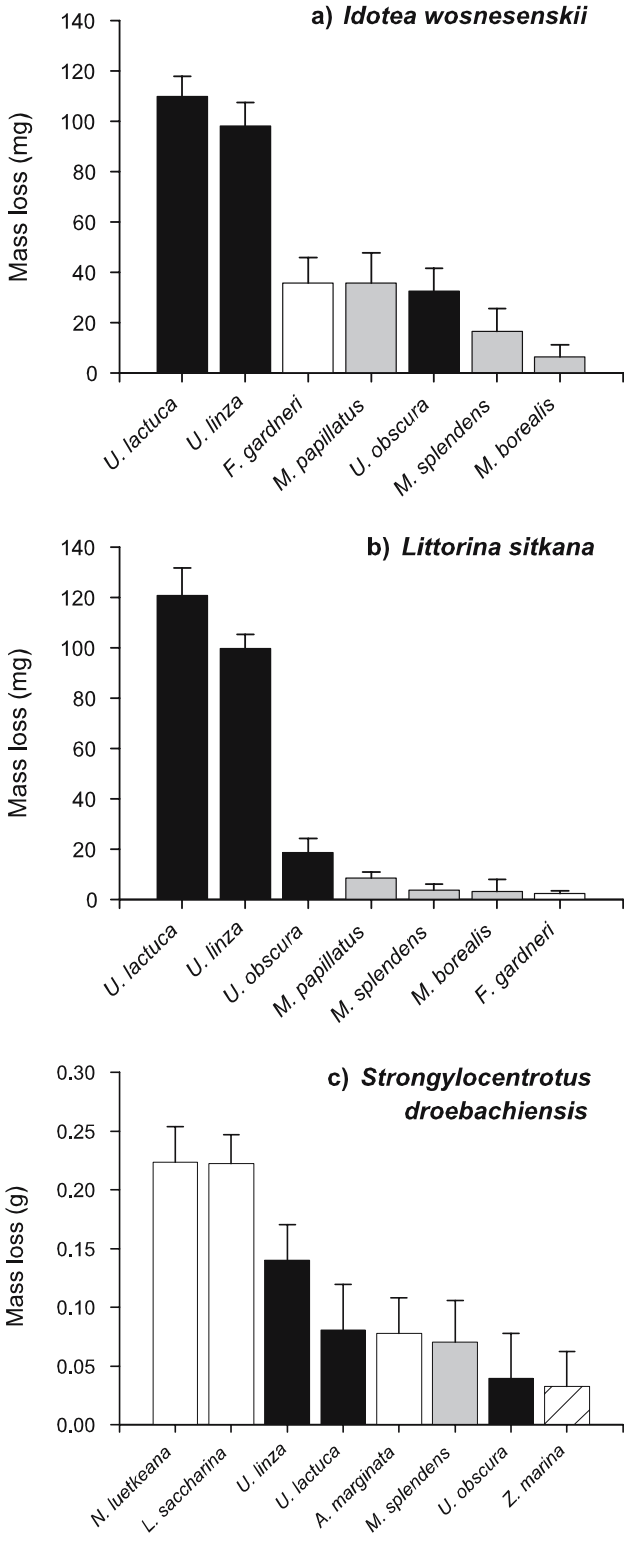

Fig. 2 Food preferences of **a** the isopod *Idotea wosnesenskii*, **b** the snail *Littorina sitkana,* and **c** the urchin *Strongylocentrotus droebachiensis* for common macroalgae and a seagrass in multiple-choice feeding-preference assays. Data are given as means ± 1 SE. *Black bars* Green algae, *grey bars* red algae, *white bars* brown algae, *hatched bars* eelgrasses. Feeding rates among species are significantly different (*I. wosnesenskii*, Yao's $R = 614.08$, $P = 0.006$, $n = 9$; *L. sitkana* Yao's $R = 2305.4$, $P = 0.001$, $n = 9$; *S. droebachiensis*, Yao's $R = 50533$, $P = 0.024$, $n = 8$)

It is unlikely that structural properties of *U. obscura* are responsible for its avoidance by herbivores because the alga is a thin, monostromatic flat blade that is structurally less complex than all of the other species used in the feeding-preference assays. Nutrient concentrations also do not explain the herbivore preferences because *U. obscura* has relatively high tissue nitrogen concentrations that range from about 4 to 6% of the alga's dry mass (K. L. Van Alstyne, unpublished data). These values are comparable to concentrations in *U. lactuca* and *U. linza* and are higher than those found in several of the red and brown algae used in this study (K. L. Van Alstyne et al., unpublished data). Therefore, we focused subsequent investigations on isolating natural products that deterred feeding by *S. droebachiensis* (Fig. 1). Only the addition of polar extracts significantly reduced urchin feeding (Fig. 1; paired *t*-test: $t = 3.90$, $P = 0.002$) when the MeOH:DCM extracts from *U. obscura* were separated into polar and non-polar fractions and incorporated into agar-based foods. When polar extracts were separated with vacuum flash column chromatography and the resulting fractions were offered to *S. droebachiensis* in agar-based foods, only the water (paired *t*-test: $t = 2.82$, $P = 0.014$), 10% MeOH (paired *t*-test: $t = 5.43$, $P < 0.001$), and 25% MeOH (paired *t*-test: $t = 2.25$, $P = 0.041$) fractions significantly reduced feeding, relative to controls (Fig. 1). When the three deterrent flash column fractions were combined and separated into 20 HPLC fractions, feeding was only reduced by HPLC fractions 4 and 7 (Fig. 1; paired *t*-test: $t = 3.13$, $P = 0.008$ and $t = 6.16$, $P < 0.001$, respectively). No other fractions had significant effects on urchin feeding rates (paired *t*-tests: $P > 0.05$).

Examination of the two deterrent HPLC fractions with GC/MS showed that the same two major compounds occurred in both the fourth and seventh HPLC fractions. The fragmentation pattern for the first compound showed a base peak at an *m*/*e* of 124 AMU and matched the fragmentation pattern of commercially obtained dopamine hydrochloride (Sigma H-8502). The identification of this compound was further confirmed by comparing the ^{1}H NMR spectra for the fourth fraction and dopamine.

The second compound had a base peak at an *m*/*e* of 120 AMU, and showed fragmentation patterns that were consistent with patterns from commercially obtained dimethylsulfoniopropionate (DMSP; University of Groningen). DMSP is a component of an activated antiherbivore defense (Van Alstyne et al. 2001) that is present in *U. obscura* at concentrations of about 4–6% of the alga's dry mass (K. L. Van Alstyne, unpublished data); however, it needs to be enzymatically cleaved to dimethylsulfide (DMS) and acrylic acid to be deterrent. The solvents used in our isolation process would have deactivated the lyase enzyme and prevented DMSP cleavage. Furthermore, DMSP alone has been demonstrated to be a feeding attractant to *S. droebachiensis*, not a deterrent (Van Alstyne et al. 2001). Therefore, we

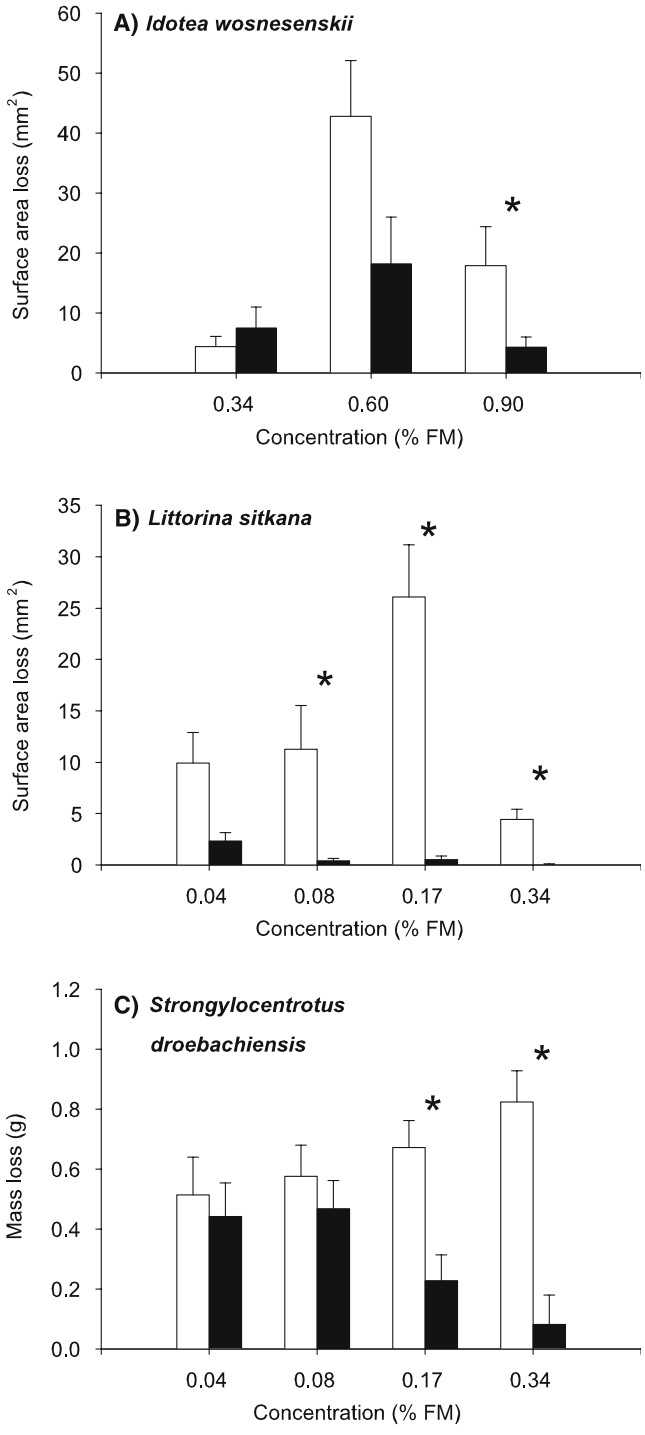

Fig. 3 Effects of dopamine at various concentrations on feeding by **a** isopods *I. wosnesenskii*, **b** snails *L. sitkana*, and **c** sea urchins *S. droebachiensis*. Data are means ± 1 SE. *Asterisks* indicate experiments in which control foods were eaten significantly ($P < 0.05$) more than foods containing dopamine (paired *t*-test). *Black bars* Foods with dopamine, *white bars* foods lacking dopamine

ruled it out as the deterrent compound in the *U. obscura* extracts.

Tissue dopamine concentrations from freshly extracted *U. obscura* were 4.42 ± 0.82% by dry mass (mean ± 1 SD; $n = 10$) or 0.94 ± 0.18% FM ($n = 10$) when measured with HPLC. Dopamine was not detected in *U. lactuca* and *U. linza*.

Commercially obtained dopamine that was incorporated into agar-based foods deterred feeding by *I. wosnesenskii*, *L. sitkana*, and *S. droebachiensis*, even at concentrations that were much lower than those occurring in the alga (Fig. 3). *S. droebachiensis* were deterred at concentrations as low as 0.17% FM or approximately 20% of naturally occurring concentrations (Fig. 3c). *L. sitkana* were deterred at about 10% of natural dopamine concentrations (Fig. 3b). *I. wosnesenskii*, which was the least affected of the three herbivores, was deterred at slightly less than natural concentrations (Fig. 3a).

The fifth and sixth HPLC fractions were not examined with GC/MS or NMR spectroscopy so we did not determine if DMSP and dopamine were present in these fractions. Because DMSP is a feeding stimulant and the relative amounts of dopamine and DMSP may have differed in the fourth up to an including the seventh fractions, it is possible that the stimulatory effects of DMSP could have countered the inhibitory effects of dopamine in the fifth and sixth fractions. It is also possible that other unidentified compounds that were not present in the fifth or sixth fraction could have been acting additively or synergistically with the dopamine in the fourth and seventh fractions to inhibit urchin feeding.

The catecholamines dopamine, epinephrine (adrenaline), and norepinephrine are aromatic nitrogenous compounds that are common in animals. In vertebrates, catecholamines function primarily as neurotransmitters and hormones (Kandel et al. 2000). In animals, dopamine modulates both excitatory and inhibitory transmissions across synapses. Catecholamines also occur in at least 44 families of higher plants (Kuklin and Conger 1995). Their functions in plants are less clear but are likely to be as diverse as those in animals and include both physiological and ecological roles. For example, dopamine is involved in photophosphorylation, the regulation of ion permeability, the synthesis of alkaloids, and in responses to wounding (Roshchina 2001). Catecholamines, including dopamine, increase in concentration when potato leaves are injured (Szopa et al. 2001) and dopamine production increases in healthy tissue surrounding a wound in giant cactus (Steelink et al. 1967).

To our knowledge, *U. obscura* is the only alga that produces a catecholamine. Dopamine (Tocher and Craigie 1966) and a phenolase enzyme, which could use dopamine as a substrate for oxidation (Tocher and Meeuse 1966), have been previously isolated from *U. obscura*. The phenolase enzyme (Tocher and Meeuse 1966) may allow ingested dopamine to be oxidized to quinones (Mason 1955), which are highly reactive and have a number of deleterious biological effects (Appel 1993). This mechanism would be similar to activated defenses reported from other green algae (Paul and Van Alstyne 1992; Van Alstyne et al. 2001). When tissues of *U. obscura* are damaged by high temperatures or desic-

cation, they darken as dopamine is presumably converted to quinones by phenolase (Tocher and Meeuse 1966). Damaged algae also release a water-soluble reddish-black substance that inhibits the development of brown algal embryos, reduces macroalgal and epiphyte growth rates, and causes increased mortality in oyster larvae (Nelson et al. 2003b).

In temperate ulvoid green algae, herbivore-deterrent natural products such as dopamine, DMS and acrylic acid (Van Alstyne and Houser 2003; Van Alstyne et al. 2001) may slow grazing sufficiently that macroalgal populations can rapidly increase when environmental conditions are favorable. *U. obscura* contains both dopamine and the DMSP activated defense, a combination that is likely to make it unpalatable to a broad range of herbivores. *U. obscura* exudates have toxic effects on seagrass epiphytes, macroalgae, and invertebrate larvae (Nelson et al. 2003b) and dopamine in seawater can inhibit the germination of marine macroalgae (M. Cataldo and K. L. Van Alstyne, unpublished data). Thus, toxic or deterrent natural products may play an important role in the ecology of temperate ulvoid macroalgae by reducing grazing or by impacting sympatric species.

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