

## CHEMICAL DEFENSE IN A MARINE ALGA: HERITABILITY AND THE POTENTIAL FOR SELECTION BY HERBIVORES

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**Abstract.** Herbivores have major impacts on many marine algae and are assumed to have been important forces driving the evolution of algal chemical defenses. However, an evolutionary change in chemical defenses in response to herbivory requires that there is both heritable variation for that trait and a response by herbivores to such variation. The subtidal red alga *Delisea pulchra* produces four main secondary metabolites (furanones) that vary in concentration at a variety of spatial and temporal scales. Here we determined the heritability of furanone concentrations and then, with a series of feeding experiments, determined how feeding by herbivores associated with *D. pulchra* varied as a function of nonpolar extract and furanone concentration. Total furanone concentration showed significant broad-sense heritability ( $h^2 = 0.234$ ), but broad-sense heritability of the four individual furanones varied in magnitude, ranging from 0.058 to 0.321. Compound 3, which is often the most abundant furanone, was the only one of the four furanones to show significant genetic variation. However, all four furanones were strongly genetically correlated, indicating that selection acting on any one of them will cause a corresponding change in the others. With the exception of the mesograzer gastropod *Phasianotrochus eximius*, herbivores generally consumed *D. pulchra* at lower rates compared to other common macroalgae. Macrograzers (sea urchins and large gastropods) were mostly deterred by nonpolar extract that contained levels of furanones that spanned the range of furanone variation in the field. The amphipod *Ampithoe ngana* was not deterred at the lowest extract concentration, and *P. eximius* was not deterred by any naturally occurring extract concentration. Compound 3 was deterrent to macrograzers at 3 and 5 mg/g dry mass, but was not deterrent at 1 mg/g dry mass. Compound 3 was also not deterrent to *A. ngana* at any of those concentrations. For the most part *D. pulchra* in the field contains concentrations of furanones that are strongly deterrent to most herbivores. However, at some places and times, furanones do occur at lower concentrations that are not deterrent to herbivores. Given the heritable variation for furanones, when this overlap occurs there is the potential for selection by herbivores for higher concentrations of furanones in *D. pulchra*.

**Key words:** chemical defense; *Delisea pulchra*; feeding deterrence; furanones; herbivores; heritability; marine algae; marine plant–herbivore interactions; natural selection; quantitative genetics.

### INTRODUCTION

The idea that herbivores exert selective pressure on plants is pervasive and underlies many of the theories that attempt to explain plant–herbivore interactions. This assumption is not surprising given the major impact herbivores have in reducing the growth, reproduction, and survivorship of terrestrial and marine plants (Hay and Steinberg 1992, Marquis 1992). Herbivory in marine systems in particular, can be extreme. Tropical marine herbivores, principally fishes, can remove as much as 100% of daily algal production (Car-

penter 1986). In temperate regions where sea urchins and gastropods are generally the dominant herbivores, grazing is not quite as intense (Hay and Steinberg 1992). Nonetheless, temperate sea urchins can remove all fleshy and foliose algae where they graze and create patches of “barrens” or “deforested” areas (Dean et al. 1984, Fletcher 1987, Andrew 1993). Temperate gastropods (Hawkins and Hartnoll 1983), fishes (Andrew and Jones 1990), and amphipods (Tegner and Dayton 1987, Duffy and Hay 2000) can also have significant impacts on temperate marine plants.

Many terrestrial and marine plants produce secondary metabolites that deter feeding by herbivores (Fritz and Simms 1992, Hay and Steinberg 1992, Rosenthal and Berenbaum 1992, Paul et al. 2001). Generally, it is assumed that these compounds have evolved in response to natural selection by enemies such as herbivores (Erlich and Raven 1964, Futuyma 1983). However, such selection has rarely been demonstrated (see

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Marquis 1992, Rausher 1996). Few studies of terrestrial plants have clearly demonstrated a selective impact by herbivores for different concentrations of secondary metabolites (Mauricio and Rausher 1997, Shonle and Bergelson 2000). Mauricio and Rausher (1997) also demonstrated selection by herbivores for a physical defense (trichome density) and Simms and Rausher (1989) demonstrated selection by herbivores for resistance itself.

For an evolutionary change to occur in response to natural enemies, genetic variation for a resistance trait is required (Simms and Rausher 1992). In many terrestrial plants, quantitative variation in resistance (e.g., Berenbaum et al. 1986, Fritz and Price 1988, Simms and Rausher 1989) and traits that confer resistance (e.g., secondary metabolites and trichomes) have a significant genetic basis (Berenbaum and Zangerl 1992, Mauricio 1998, Hamilton et al. 2001). The degree of genetic control over variation in the concentrations of secondary metabolites differs among species, but heritabilities are generally in the order of 0.5 or greater (Berenbaum and Zangerl 1992, Hamilton et al. 2001). Thus, quantitative variation of many terrestrial plant secondary metabolites is under substantial genetic influence and has the potential to respond to selection by natural enemies.

Marine algae produce secondary metabolites similar to those found in terrestrial plants such as terpenes, acetogenins, and polyphenolics, but they also produce other active secondary metabolites not found in terrestrial plants, such as those that contain halogens (Hay and Steinberg 1992, Paul et al. 2001). Algal secondary metabolites deter a range of natural enemies including herbivores (see Hay and Steinberg 1992, Paul et al. 2001), epiphytes (de Nys et al. 1995, Schmitt et al. 1995), bacteria (Maximilien et al. 1998), and competitors (de Nys et al. 1991). Quantitative variation in the concentrations of algal secondary metabolites is widespread (Van Alstyne et al. 2001) and the variation within populations can be considerable (Wright et al. 2000b). Importantly, the deterrence of natural enemies of marine algae by secondary metabolites is often concentration dependent (e.g., Hay et al. 1987, Steinberg 1988, de Nys et al. 1995, Schmitt et al. 1995). Of these natural enemies, herbivores are usually considered to have the greatest impact on marine algae, and models for the evolution of algal secondary metabolites generally invoke an important role for resistance to herbivory (Hay and Steinberg 1992, Steinberg 1992).

To date, research examining the factors responsible for quantitative variation in algal secondary metabolites has focused on phenotypic change in response to environmental factors such as nutrients, light, desiccation, or induction due to herbivory (Yates and Peckol 1993, Arnold et al. 1995, Cronin and Hay 1996a, b, Puglisi and Paul 1997). There are no studies describing the extent to which quantitative variation in algal secondary metabolites is heritable and only one study

demonstrating qualitative inheritance of these compounds (Masuda et al. 1997). Indeed, only one study has examined the heritability of a quantitative defensive trait in a marine organism. Harvell (1998) found significant genetic variation and heritability  $>0.64$  for spine length in the bryozoan *Membranipora membranacea*. The absence of data describing the heritability of algal secondary metabolites represents a major gap in understanding the evolution of these compounds.

In this paper we investigated the potential for the secondary metabolites of a common subtidal red alga to respond to selection (i.e., their heritability) and then, with a series of feeding experiments, the potential for herbivores to impose such selection. Initially, we documented patterns of variation in the secondary metabolites (furanones) from the red alga *Delisea pulchra* (Greville) Montagne (Bonnemaisoniales, Rhodophyta) and herbivore abundance (macrograzers and mesograzers) at two locations. We then asked the following specific questions (1) Is quantitative variation in furanones from *D. pulchra* heritable? (2) Are the different furanones genetically correlated (i.e., will selection on one furanone cause a parallel change in the others)? (3) What are the feeding rates of herbivores on *D. pulchra* vs. other common macroalgae? (4) Are non-polar extracts and the furanone from *D. pulchra* that is often most abundant deterrent to herbivores? (5) Does feeding by herbivores vary with the concentration of nonpolar extract and the furanone from *D. pulchra* that is often most abundant? If quantitative variation in furanones is heritable, and feeding by herbivores increases with decreasing furanone concentration, then there is the potential for herbivores to impose selection for higher concentrations of these compounds.

## METHODS

### *Study sites and organisms*

Algae and herbivores used in this study were collected primarily from Bare Island, near Sydney, Australia (33°59'32" S, 151°13'50" E). Sampling of variation in furanones and herbivores associated with *Delisea pulchra* was also conducted at nearby Cape Banks (34°00' S, 151°14'33" E). Some individuals of the sea urchin *Holopneustes purpurascens* were collected from Long Bay (33°57'38" S, 151°15'56" E), and some individuals of the amphipod *Ampithoe ngana* were collected from Shark Bay (33°51'9" S, 151°16'0" E) when there were insufficient number of these herbivores at Bare Island. The macroalgal and herbivore communities on subtidal rocky reefs at the depths we worked (1–3 m) at these locations are broadly similar. The common macroalgae at these locations are *D. pulchra* (except for Shark Bay), the brown algae *Ecklonia radiata*, *Sargassum vestitum*, *Sargassum linearifolium*, *Zonaria diesingiana*, and *Dilophus marginatus*, and coralline turfing red algae *Corallina officinalis* and *Amphiroa anceps*. The common herbivores are the sea urchins

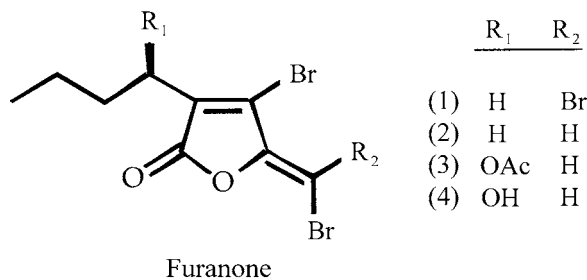


FIG. 1. Structure of the major secondary metabolites of *Delisea pulchra* (compounds 1–4).

*Heliocidaris erythrogramma*, *Heliocidaris tuberculata*, *Centrostephanus rodgersii*, and *H. purpurascens*; the large gastropods *Turbo torquatus*, *Turbo undulatus*, and *Australium tentiforme*; and a variety of mesograzers. Mesograzers common in this region are juvenile *H. purpurascens*, a number of gastropods including *Aplysia parvula* and *Phasianotrochus eximius*, Nereid polychaetes and amphipods from the families Ampithoidae, Hyalidae, and Aoridae (Rogers et al. 1998, Poore et al. 2000, Wright et al. 2000b). For further description of these sites see Fletcher (1987), Poore and Steinberg (1999), and Wright and Steinberg (2001).

*Delisea pulchra* is a common shallow subtidal foliose red alga occurring throughout southern Australia, the Antarctic Peninsula, and various Subantarctic Islands (Ricker 1987, Bonin and Hawkes 1988). Although it is described in the literature as having a triphasic life history (Bonin and Hawkes 1988, Womersley 1996), populations of *D. pulchra* around Sydney are maintained through asexual reproduction of tetrasporophytes. Random amplified polymorphic DNA (RAPDs) indicated the presence of clones in populations of *D. pulchra* (Wright et al. 2000a), and an 18-month study at Bare Island showed that all reproductive plants over that time were tetrasporophytes (Wright and Steinberg 2001). Furthermore, *D. pulchra* at Bare Island recycle tetrasporophytes from tetraspores (J. T. Wright, unpublished data), and this appears to be widespread as haploid plants were found in only one out of 15 populations over a 650-km range (Wright et al. 2000b).

*Delisea pulchra* produces a range of nonpolar halogenated furanones of which four are consistently present in plants and make up ~95% of total secondary metabolites (Fig. 1; de Nys et al. 1992, 1993). Furanones are contained within specialized gland cells both within and at the surface of the alga (Dworjanyn et al. 1999) and they deter settlement of both eukaryotic and prokaryotic epiphytes (de Nys et al. 1995, Maximilien et al. 1998). Furanones further affect a range of bacterial phenotypes via interference with specific signal-based bacterial regulatory systems (Manefield et al. 1999, Hentzer et al. 2002). *D. pulchra* is a low preference food for the sea urchin *Tripneustes gratilla*, the gastropod *T. undulatus* (Steinberg and Van Altna

1992), and the sea hare *A. parvula* (Rogers et al. 1995, 2000) and furanones deter feeding by *H. purpurascens* (Williamson et al. 2004). There is considerable variation in the concentrations of furanones in *D. pulchra* both within plants (de Nys et al. 1996, Dworjanyn et al. 1999, Rogers et al. 2000) and among plants on geographic (between locations) and small (within locations) spatial scales, between different times of the year, and between different life-history stages (Wright et al. 2000b).

#### Small-scale variation in furanones and herbivores

Patterns of variation in furanones and herbivore abundance were examined in October 1997 at Cape Banks and Bare Island using the plants and sites described previously to examine genetic structure in *D. pulchra* (see Wright et al. 2000a for a full description of sites and sampling design). Briefly, four 3 × 3 m sites were set up at the two locations, which are ~2 km apart. Within each of these sites, three 0.5 × 0.5 m quadrats were placed and within each quadrat five *D. pulchra* plants were randomly collected. At Bare Island, two additional sites were also set up adjacent to the other four sites (Wright et al. 2000a). These two sites had been invaded by high densities of *H. erythrogramma* and had 92.5% fewer plants than the adjacent uninvaded sites. Plants were collected haphazardly from within these two sites and not from within nested quadrats.

Plants were removed from the substratum, placed into clip seal plastic bags underwater, and taken back to the laboratory. Once there, mesograzers were removed (see next paragraph) and plants weighed. Approximately 1.5 g of tissue were removed for DNA extraction (Wright et al. 2000a), 20% of the remaining plant tissue was taken to measure fecundity (Wright 2000), and the remainder was frozen at -20°C until extraction and quantitative analysis of furanones. Furanones were extracted and quantified using gas chromatography-mass spectrometry (GC-MS) following the methods of de Nys et al. (1996) and Wright et al. (2000b). Spatial variation in the concentration of total furanones was analyzed using a nested ANOVA with the levels location, sites within location, and quadrats within sites. Variation in the concentrations of individual furanones was examined with a nested analysis containing the same three spatial levels (location, site, and quadrat) and the additional factors of compound and plant (blocked factor).

As we were interested in examining the potential for selection, we only counted herbivores known to consume *D. pulchra*. All macroinvertebrates that co-occur with *D. pulchra* consume it to some extent (Steinberg and Van Altna 1992; J. T. Wright, unpublished gut content analysis). Macroinvertebrates were counted in each quadrat at the same time as the *D. pulchra* was collected. In the two sites invaded by *H. erythrogramma*, macrograzers were counted within 10 haphazardly

placed quadrats. Gut content analyses or feeding experiments also revealed that four mesograzers: *H. purpurascens*, *A. parvula* (Rogers et al. 1995, 2000), *P. eximius*, and the nereid polychaete *Platynereis dumerilii* consumed *D. pulchra*. The abundance of these four mesograzers on *D. pulchra* was counted by shaking each plant vigorously in freshwater for 20–30 s, which removed all individuals of these species (J. T. Wright, unpublished data). Variation in the abundance of total macrograzers (all species pooled, data  $\log x + 1$  transformed) was examined between locations and among sites within locations. Variation in the abundance of total mesograzers (all four species pooled, data  $\log x + 1$  transformed) was examined between locations, among sites within locations, and among quadrats within sites. The two sites invaded by *H. erythrograma* were not included in any of these hierarchical analyses as *D. pulchra* and herbivores were not sampled from within nested quadrats in those sites.

#### *Heritability of furanones and gland cells*

The heritability of furanone concentrations and gland cell densities in *D. pulchra* was examined on plants grown from tetraspores released by 20 tetrasporophytes from Bare Island. As *D. pulchra* recycles diploid tetrasporophytes from asexual tetraspores, the offspring derived from each parent represent clones. Fertile plants were collected and ~5 g of tissue with tetrasporangia was removed. This tissue was soaked in 1% betadine solution (1:10 dilution of provadine–iodine solution, Foulding Pharmaceuticals, Salisbury, South Australia) for 3 min, rinsed three times in clean sea water, and then put into a 2-L beaker containing filtered sea water and ceramic settlement tiles (4 × 4 cm). Plant tissue was left in these beakers with aeration for 48 h to release tetraspores onto the unglazed side of the settlement tiles. After 48 h, the tiles were checked for attached tetraspores. Most plants had released sufficient numbers of tetraspores in that time, although four plants were left for an additional 24 h to release more tetraspores. Individual tiles ( $n = 4$  per parent) were then placed into 200-mL culture jars containing half strength Provasoli's enriched seawater (PES; Provasoli 1968), 0.01 mg/mL of Penicillin G, and 0.25 mg/mL germanium dioxide.

The tetraspores were kept at 20°C on a 16:8, light:dark cycle with light at ~50 mmol·m<sup>-2</sup>·s<sup>-1</sup>. After 10 d, the tiles were transported back to Bare Island and attached to the substratum in a cage (mesh size 10 mm). The tiles were caged as a pilot study showed that uncaged tiles containing sporelings were completely grazed by gastropods and sea urchins (J. T. Wright, unpublished data). Six tiles that lacked sporelings but had been kept in the same culture conditions were also attached to examine natural recruitment of *D. pulchra* during the experiment. No *D. pulchra* recruited naturally onto the tiles during the experiment. After 71 d two plants (1.89 ± 0.24 mg dry mass, mean ± 1 SE)

were collected from each tile (plants were all nonreproductive) to determine the concentrations of furanones and the number of surface gland cells. All plants were frozen at -20°C until required for extraction or microscopy.

One plant from each tile was freeze-dried, weighed to four decimal places, and then extracted exhaustively with dichloromethane. Methods for the preparation of samples for GC-MS were as described in Wright et al. (2000b) with the following changes: only three extractions were done with volumes of 800 and 500 μL (twice) of dichloromethane; the extract was not filtered; 50 or 25 μL (for very small samples) of the internal standard was added to the extracts; and samples were pipetted into inserts that were then placed into GC-MS tubes. GC-MS was then performed as described in de Nys et al. (1996) and Wright et al. (2000b).

Gland cell densities were determined on the second plant following the methods of Dworjanyn et al. (1999). Although gland cells occur both internally and on the surface of *D. pulchra* they are more abundant in the top third of the plant compared to the middle and base (Dworjanyn et al. 1999). We examined genetic variation for the number of surface gland cells 5 mm down from the mounted apical tip on each plant, in the center of the thallus using a 100 × 100 μm grid.

Differences among clones in the total and individual concentrations of secondary metabolites were analyzed with a one-way analysis of covariance (ANCOVA) with plant mass as the covariate. Mass was not included as a covariate in the analysis of the number of surface gland cells as it was not measured. Differences among clones for the number of surface gland cells were determined using one-way ANOVAs. For all of these analyses, a significant clone effect indicates heritable variation for that trait.

The heritability of these traits was determined as broad-sense (or clonal) heritability (Falconer 1989). Broad-sense heritability ( $h_C^2$ ) estimates the proportion of the phenotypic variance due to genotypic variance and was calculated as

$$h_C^2 = s_G^2 / (s_G^2 + s_E^2)$$

where  $s_G^2$  and  $s_E^2$  are the variance components due to clone and error, respectively, calculated from the ANOVAs (Falconer 1989, Lynch and Walsh 1998). In non-clonal species, broad-sense heritability overestimates actual heritability as, in addition to additive genetic variance, it includes dominance and interaction effects (Falconer 1989). However, for asexual species such as *D. pulchra*, it provides an evolutionarily relevant measure of heritability (Lynch and Walsh 1998).

#### *Genetic correlations between furanones and gland cells*

Genetic correlations between furanones and gland cells were estimated and represent the correlations due only to genetic factors. Precise estimates of genetic

correlations are very difficult to obtain (Cheverud 1988, Falconer 1989, Lynch and Walsh 1998). There are three main methods used to estimate genetic correlations between traits: (1) a pairwise comparison of relatives based on a parent-offspring regression, (2) a nested sib analysis partitioning the covariance into genetic vs. environmental effects, and (3) a correlation between traits using family means (Lynch and Walsh 1998). Method three is considered less rigorous than methods one and two, and at best provides an approximation of the genetic correlation. However, even knowledge of the sign of a genetic correlation is revealing (Lynch and Walsh 1998), and thus genetic correlations based on family means are a useful step in understanding genetic correlations among traits. We estimated genetic correlations among furanones and gland cells for the plants from the heritability experiment by performing correlations on the clone means. Given that *D. pulchra* reproduces asexually, this was the only one of the three methods that could be applied to examine genetic correlations between traits here.

#### General methods for feeding experiments

To investigate the response of herbivores to variation in furanones, we examined the feeding rates on different algae and the effects of nonpolar extract and furanones from *D. pulchra* on feeding by six of the most common herbivores in this system: *H. erythrogramma*, *H. purpurascens*, *T. torquatus*, *T. undulatus*, *P. eximius*, and *A. ngana* (Rogers et al. 1998, Poore et al. 2000, Wright et al. 2000b). All feeding experiments were done in the School of Biological, Earth, and Environmental Sciences, University of New South Wales 30-kL recirculating seawater system. Experiments for *H. erythrogramma*, *T. torquatus*, and *T. undulatus* were conducted in 7-L perspex tanks divided in half with a piece of plastic mesh (1-cm mesh size). Experiments for the canopy dwelling *H. purpurascens* were conducted in individual 130 × 200 cm plastic cages (mesh size ranges from 0.5 to 1 cm<sup>2</sup>) placed in 20-L plastic bins. Six of these cages were placed in each bin with a hose providing flowing seawater directly into each cage. The feeding rate of *A. ngana* was examined in 50-mL perspex jars, while the experiments examining the effects of nonpolar extract and pure compound 3 on this species were done in 20-L plastic bins divided into 20 rectangular compartments (8 cm long, 8 cm wide, 11 cm high) separated by 300- $\mu$ m mesh supplied with flowing sea water (see Poore and Steinberg 1999). Experiments for *P. eximius* were done in the 20-L plastic bins divided into 20 compartments as described for *A. ngana*.

*Heliocidaris erythrogramma*, *H. purpurascens*, *T. torquatus*, and *T. undulatus* were collected, placed into the experimental tanks, and allowed to feed on a mixture of fresh algae for 2–3 d, after which the experiments were set up. *A. ngana* and *P. eximius* were collected and held in cultures in 7-L perspex tanks on a

mixture of fresh algae for 1–2 wk until the start of the experiments. Animals were not starved prior to any of the feeding experiments.

#### Feeding rates of herbivores

The feeding rates of *H. erythrogramma*, *H. purpurascens*, *T. torquatus*, *A. ngana*, and *P. eximius* on *D. pulchra* were contrasted to four other algal species (*C. officianalis*, *E. radiata*, *S. vestitum*, and *Z. diesingiana*) in no-choice feeding assays. The feeding rate of *T. undulatus* on *D. pulchra*, *E. radiata*, *S. vestitum*, and *Z. diesingiana* has been described previously and indicates that *D. pulchra* is rarely consumed (Steinberg and Van Altena 1992). In our experiments, pieces of algae visually free of epiphytes were cut from freshly collected plants, blotted to a constant wet mass, and weighed. *H. erythrogramma*, *H. purpurascens*, and *T. torquatus* were presented with ~5-g pieces, *A. ngana* with ~25-mg pieces, and *P. eximius* with ~1-g pieces of a given alga. In each experiment there were between 8 and 12 replicates of each algal species with herbivores (one animal per tank) and the same number of replicates without herbivores to control for autogenic changes. Herbivore plus and herbivore minus treatments were randomly assigned to a given tank. Experiments ran for 48 h after which each piece of alga was blotted to a constant wet mass, reweighed, and the differences in feeding rates analyzed with a two-way ANOVA (algal species × herbivore presence/absence). All replicates were included in the analyses except for several *A. ngana* individuals that died during the experiment. Tukey's post hoc test within the herbivore treatment was then used to examine for differences among the means of algal species after a correction for autogenic change, using the error mean square from the two-way ANOVA in the Tukey's post hoc tests (Peterson and Renaud 1989).

#### Effect of nonpolar extract on feeding

Because *D. pulchra* was rarely consumed by herbivores here (see *Results*) and handling of plants for the duration of a feeding experiment causes an unpredictable induced response in furanone concentrations (R. de Nys, O. Ison, A. G. B. Poore, J. T. Wright, and P. D. Steinberg, *unpublished manuscript*), we were unable to compare consumption of *D. pulchra* tissue with different furanone levels. Therefore, we proceeded to directly test the effects of variation in concentrations of nonpolar extract and compound 3 on feeding.

The effect of nonpolar crude extract from *D. pulchra* on feeding by all six herbivores was examined at three concentrations. Initially we tested the nonpolar extract at an average "natural concentration." This was determined by taking a bulk collection of ~175–200 *D. pulchra* plants from Bare Island (695.6 g dry mass), extracting it sequentially with dichloromethane and then methanol (three times each), and then pooling these extracts. This extract was reduced in vacuo and

the resultant extract partitioned between dichloromethane and water. The dichloromethane fraction was subsequently reduced in vacuo to yield 41.54 g dry mass of nonpolar extract that represented a mean concentration of 5.97% of nonpolar extract. As we wanted to examine the effects of variable concentrations of nonpolar extract on feeding by herbivores, the extract was tested at 5.97%, one-third of this concentration (1.98%), and one-sixth of this concentration (0.99%). The concentrations of the four main furanones in this bulk nonpolar extract were determined with GC-MS as described previously. These concentrations (per dry mass of plant) were: compound 1 (2.93 mg/g), compound 2 (4.15 mg/g), compound 3 (9.02 mg/g), and compound 4 (5.17 mg/g). Therefore concentrations (per dry mass of plant) of total furanones (compounds 1–4) in the nonpolar extract used in these experiments were 21.26 mg/g (for 5.97%), 7.087 mg/g (for 1.98%), and 3.544 mg/g (for 0.99%).

Experiments to test the deterrent effects of nonpolar extract on feeding by *H. erythrogramma*, *T. torquatus*, *T. undulatus*, and *A. ngana* were done using an artificial diet made up of agar and freeze-dried *Ulva* sp. ground to a powder. *H. purpurascens* and *P. eximius* would not consume any artificial diet and experiments for these two species were done by coating extract onto palatable algae: *E. radiata* (for *H. purpurascens*) and *Ulva* sp. for (*P. eximius*).

To make the artificial diet, freeze-dried *Ulva* was coated with nonpolar extract at the appropriate concentration (milligrams per gram dry mass) by dissolving the extract in diethyl ether, mixing in the *Ulva*, and allowing the diethylether to evaporate. For the control food, freeze-dried *Ulva* was coated with diethylether only. Agar (4% m/v) was boiled in a microwave and allowed to cool to ~50°C before the precoated *Ulva* was mixed into the agar. The dry:wet mass ratio of the artificial diet (1:8) approximated that of *D. pulchra* (mean dry:wet mass ratio 1:7.5,  $N = 48$  plants). For *H. erythrogramma*, *T. torquatus*, and *T. undulatus* disks were made by pouring the mix into 7.5-mL petri dishes with a numbered washer added to identify the different treatments and to ensure the disk sank. For *A. ngana* the agar mix was poured between two sheets of perspex and flattened to ~1 mm thick (see Hay et al. 1994). Pieces ~25 mg (wet mass) were cut from this sheet and a small piece of fishing line was pushed through each piece to identify the different treatments.

For *H. purpurascens*, ~2.5-g (wet mass) pieces of *E. radiata* were coated on both sides at the appropriate concentration by pipetting the required amount of nonpolar extract dissolved in diethylether. For *P. eximius* 200-mg (wet mass) pieces of *Ulva* were coated on both sides at the appropriate concentration with the nonpolar extract dissolved in diethylether. For both assays, control pieces of algae were coated with diethylether only.

The two types of food, one with nonpolar extract and one without extract, were presented to single animals

in choice experiments. A similar number of tanks were set up with no herbivores to control for autogenic changes during each experiment. Experiments were run until most animals had consumed ~50% of one food (24–48 h) when the entire experiment was stopped, and analyzed by calculating the difference in mass between the food with and without nonpolar extract for each replicate and performing a *t* test on these differences between the herbivore plus and herbivore minus treatments (Peterson and Renaud 1989). Herbivore replicates where no consumption occurred were removed from the analyses. This number varied among herbivores: most gastropods and *A. ngana* ate during these experiments but between 13% and 30% of *H. purpurascens* and 15% and 47% of *H. erythrogramma* did not eat during the experiments. Final sample sizes for these experiments ranged from 10 to 15 replicates. Following this, we then examined whether there were differences in the feeding of each herbivore among the three extract concentrations using a one-factor ANOVA. To do this, for each plus-herbivore replicate we calculated consumption of the plus-extract food scaled to the average consumption of the no-extract food for that experiment (after adjustments for autogenic change), log-transformed the data, and then performed the ANOVA among the three concentrations. Significant variation across the three concentrations indicates concentration-dependent feeding by that herbivore in response to nonpolar extract.

#### *Effect of compound 3 on feeding*

Compound 3 is typically the most abundant furanone in *D. pulchra* (de Nys et al. 1996, Wright et al. 2000b). It was isolated and purified from the dichloromethane extract using vacuum liquid chromatography followed by high performance liquid chromatography (de Nys et al. 1992). The structure of compound 3 was confirmed by comparison of <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance with reference data (de Nys et al. 1992).

We examined how variation in compound 3 affected feeding by *H. erythrogramma*, *T. torquatus*, *T. undulatus*, and *A. ngana* by incorporating it into the artificial diet (see *Methods: Effects of nonpolar extract on feeding*) at 1, 3, and 5 mg/g dry mass. These concentrations for compound 3 represent relatively low, mean, and high concentrations based on some previously published values (de Nys et al. 1996, Dworjanyn et al. 1999, Rogers et al. 2000, Wright et al. 2000b), although they are low relative to the concentration in the nonpolar extracts used in our experiments. *H. purpurascens* is strongly deterred by compound 3 at 1 mg/g dry mass (Williamson et al. 2004) and was not tested here. The design and analyses of the experiments with compound 3 were the same as for the experiments with nonpolar extract.

## RESULTS

### *Small-scale variation in furanones and herbivores*

Concentrations of total and individual furanones did not differ between Bare Island and Cape Banks, or

TABLE 1. Nested ANOVAs for the effect of location, sites within locations, and quadrats within sites on total and individual furanones in *Delisea pulchra*.

Source	df	MS	F	P
Total furanones				
Location	1	50.115	0.745	0.421
Site (location)	6	67.233	2.143	0.105
Quadrat (site)	16	31.378	1.825	0.038
Error	96	17.19		
Individual furanones				
Location	1	0.251	0.295	0.607
Site (location)	6	0.852	2.023	0.122
Quadrat (site)	16	0.421	6.923	<0.005
Plant (block)	4	0.395	6.492	<0.005
Compound	3	33.051	68.858	0.003
Location × compound	3	0.480	3.919	0.026
Site (location) × compound	18	0.122	1.964	0.032
Quadrat (site) × compound	48	0.062	1.025	0.433
Error	380	0.061		

among sites within those locations (Table 1). Concentrations of total furanones at Bare Island were  $12.25 \pm 0.49$  mg/g dry mass ( $N = 90$ ) and at Cape Banks were  $13.35 \pm 0.60$  mg/g dry mass (mean  $\pm 1$  SE,  $N = 60$ ). However, there was significant variation among quadrats for both total and individual furanones indicating that most of the variation in concentrations of these compounds lies at small spatial scales (Table 1). Compounds 3 and 4 occurred at higher concentrations than compounds 1 and 2. The concentrations (milligrams per gram dry mass) of these four compounds (mean  $\pm 1$  SE for  $N = 150$  plants across locations) were:  $1.55 \pm 0.05$  (compound 1);  $1.17 \pm 0.04$  (compound 2);  $4.91 \pm 0.16$  (compound 3); and  $5.06 \pm 0.19$  (compound 4).

Three macrograzers were recorded in quadrats: the sea urchins *Heliocidaris erythrogramma* and *Holopneustes purpurascens*, and the gastropod *Turbo torquatus*. In the two sites where a wave of high densities of *H. erythrogramma* had entered, there were an average of 82.8 and 61.2 *H. erythrogramma*/m<sup>2</sup>. *H. purpurascens* and *T. torquatus* were much less abundant, rarely being more than one animal/m<sup>2</sup>. There were significantly fewer macroinvertebrates at Cape Banks compared to Bare Island ( $F_{1,6} = 11.644$ ,  $P = 0.014$ ): only one *H. erythrogramma* and two *T. torquatus* were recorded at Cape Banks. There were also significant differences in the abundance of total macroinvertebrates among sites within locations ( $F_{6,16} = 3.062$ ,  $P = 0.034$ ). The most abundant mesograzers was the gastropod *Phasianotrochus eximius*, which occurred at all sites and reached maximum site densities (mean  $\pm 1$  SE per gram wet mass *D. pulchra*) of  $0.53 \pm 0.12$  at Bare Island and  $0.57 \pm 0.14$  at Cape Banks. Overall, there was no difference in total mesograzers per gram *D. pulchra* (wet mass) between Bare Island and Cape Banks ( $F_{1,6} = 0.114$ ,  $P = 0.747$ ), there were significant differences in total mesograzers per gram among sites within locations ( $F_{6,16} = 3.963$ ,  $P = 0.013$ ), but not among quadrats within sites ( $F_{16,96} = 1.567$ ,  $P = 0.093$ ).

#### Heritability of furanones and gland cells

The total concentration of furanones was extremely variable among clones, ranging from  $3.8 \pm 0.5$  to  $33.9 \pm 2.5$  mg/g dry mass (mean  $\pm 1$  SE). Concentrations of the four individual furanones were also highly variable among clones. For all furanones however, there were significant positive phenotypic correlations between concentration and plant mass ( $r$  ranged from 0.473 to 0.752;  $P < 0.001$ ). Therefore mass was included as a covariate in the analyses of genetic variation in concentrations of furanones. There was significant genetic variation in the concentrations of compound 3 and total furanones (ANCOVA; Table 2). Compounds 1, 2, and 4 showed no significant genetic variation (although note  $P$  values for compounds 1 and 2; Table 2). Broad-sense heritabilities for the concentrations of total and individual furanones were relatively low, ranging from 0.058 for compound 4 to 0.321 for compound 3. The number of surface gland cells also showed significant genetic variation but as was the case for furanones, broad-sense heritability for gland cells was relatively low: 0.240 (Table 2).

Based on the clone means, there were significant positive genetic correlations among all four compounds (Table 3). The number of surface gland cells was not genetically correlated with any of the other traits.

#### Feeding rates of herbivores

All herbivores showed differences in feeding rates among algal species (Fig. 2). Qualitatively, *D. pulchra* was consumed at the lowest rate for all three macrograzers (Fig. 2). Quantitatively, compared to the consumption of *D. pulchra*, *H. purpurascens* consumed significantly more *Ecklonia radiata*, *Sargassum vestitum*, and *Zonaria diesingiana*; *H. erythrogramma* and *T. torquatus* consumed significantly more *Corallina officinalis* and *E. radiata*; *Ampithoe ngana* consumed significantly more *Z. diesingiana* and *S. vestitum*; and *P. eximius* consumed significantly more *Z. diesingiana*.

TABLE 2. Summary of ANCOVAs testing for differences among clones in furanone concentrations and gland cell densities.

Trait	Mass (covariate)		Clone		$h_c^2$
	$F_{1,54}$	$P$	$F_{19,54}$	$P$	
Total furanones	32.162	<0.001	2.224	0.011	0.234
Compound 1	34.954	<0.001	1.750	0.056	0.158
Compound 2	29.861	<0.001	1.710	0.063	0.151
Compound 3	9.974	0.003	2.895	0.001	0.321
Compound 4	50.665	<0.001	1.246	0.258	0.058
Gland cells	...	...	2.266†	0.009	0.240

Notes: Mass was included as a covariate in all analyses for furanones. Broad-sense heritabilities ( $h_c^2$ ) were calculated using the variance components from the ANCOVA models (see Methods).

†  $F_{19,60}$ .

Compared to the other four herbivores, *P. eximius* consumed high amounts of *D. pulchra* relative to preferred algae. To further examine the feeding of *P. eximius* on *D. pulchra*, we tested whether *P. eximius* fed preferentially on the tops vs. bottom of *D. pulchra* in a choice assay. The tops of *D. pulchra* contain higher concentrations of furanones than the bottoms (de Nys et al. 1996, Dworjanyan et al. 1999), and *P. eximius* consumed significantly more of the tops ( $32.1 \pm 3.7$  g) of *D. pulchra* compared to the bottom ( $11.8 \pm 3.0$  g;  $t = -4.93$ ,  $df = 18$ ,  $P < 0.001$ ).

#### Effect of nonpolar extract on feeding

Nonpolar extract of *D. pulchra* at 5.97% dry mass was highly deterrent to feeding by all herbivores except *P. eximius* (Fig. 3). At 1.98%, nonpolar extract was significantly deterrent to *T. torquatus*, *T. undulatus*, and *A. ngana* and there was no significant reduction in feeding by *H. purpurascens* ( $P = 0.085$ ; Fig. 3). Feeding by *H. erythrogramma* was not deterred by nonpolar extract at this concentration. At 0.99%, nonpolar extract significantly deterred feeding by *H. purpurascens* and *T. undulatus*, but did not deter feeding by *T. torquatus* and *A. ngana*. There was no significant reduction in feeding by *H. erythrogramma* at this concentration ( $P = 0.085$ ). As *P. eximius* was not deterred at 5.97%, we increased the concentration of nonpolar extract by three and then six times instead of decreasing it. At a concentration of 17.91%, nonpolar extract still did not reduce feeding by *P. eximius*. However, at 35.82%, feeding by *P. eximius* was significantly deterred by nonpolar extract (Fig. 3). ANOVAs performed across the three extract concentrations on scaled consumption of plus-extract food relative to consumption of no-extract food revealed significant dif-

ferences in feeding by *H. erythrogramma*, *T. undulatus*, *A. ngana*, and *P. eximius* in response to differences in extract concentration. For *T. undulatus*, *A. ngana*, and *P. eximius* there was a decrease in feeding with an increase in extract concentration, but for *H. erythrogramma* there was lower feeding when the extract was at 0.99% and 5.95% compared to 1.98% (Fig. 3). Importantly, with the exception of *H. erythrogramma* and *P. eximius*, an increase in extract concentration from 1.98% to 5.99% did not result in significantly greater feeding deterrence.

#### Effect of compound 3 on feeding

Compound 3 did not deter feeding by any of the four herbivores at 1 mg/g dry mass but was deterrent to *H. erythrogramma*, *T. torquatus*, and *T. undulatus* at 3 and 5 mg/g dry mass (Fig. 4). Compound 3 did not deter feeding by the amphipod *A. ngana* at any of the three concentrations. ANOVAs performed across the three concentrations on scaled consumption of plus-compound 3 food relative to consumption of no-compound 3 food revealed a significant difference in feeding by *T. torquatus* with concentration, but no significant differences for *H. erythrogramma*, *T. undulatus*, or *A. ngana* (Fig. 4). Similar to the response of most herbivores to the nonpolar extract, an increase in the concentration of compound 3 from 3 to 5 mg/g did not result in significantly greater feeding deterrence for any herbivores tested.

## DISCUSSION

### Heritability of chemical defense

Although it is well established that quantitative variation in many secondary metabolites from terrestrial

TABLE 3. Genetic correlations ( $r$ ) among furanone concentrations and gland cell densities based on the clone means ( $n = 20$  clones).

	Compound 1	Compound 2	Compound 3	Compound 4	Gland cells
Compound 1		0.970**	0.937**	0.837**	0.143
Compound 2			0.904**	0.897**	0.169
Compound 3				0.680*	0.142
Compound 4					0.143

\*  $P < 0.05$ ; \*\*  $P < 0.001$ .



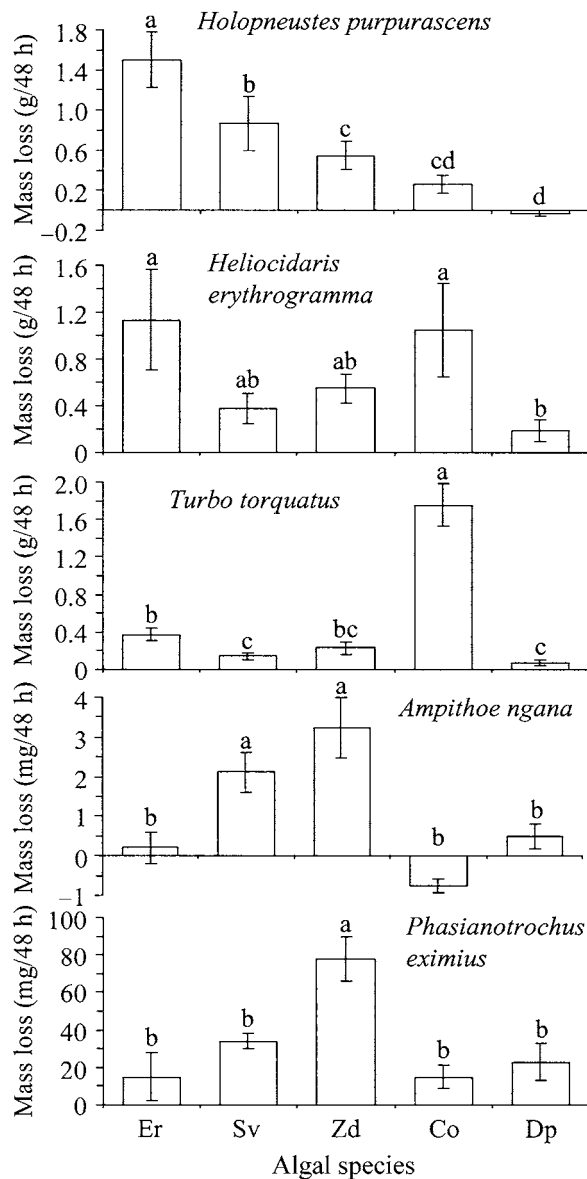


FIG. 2. Feeding rates of herbivores on five algae. The data show the mean ( $\pm 1$  SE) of wet mass loss ( $n = 10$  for *Holopneustes purpurascens*, *Turbo torquatus*, and *Phasianotrochus eximius*;  $n = 8$  for *Heliocidaris erythrogramma*;  $n = 7-11$  for *Ampithoe ngana*). Mass loss has been adjusted for autogenic changes in algae in treatments without herbivores. Bars labeled with the same letter do not differ significantly ( $P < 0.05$ ) in Tukey's post hoc analyses. Key to algal species abbreviations: Er, *Ecklonia radiata*; Sv, *Sargassum vestitum*; Zd, *Zonaria diesingiana*; Co, *Corallina officinalis*; Dp, *Delisea pulchra*.

plants is heritable (Berenbaum and Zangerl 1992, Hamilton et al. 2001), there have been no previous examinations of the genetic basis to quantitative variation in the secondary metabolites of marine algae. In *Delisea pulchra*, variation in total furanone concentration had a significant genetic basis but heritability of the four individual furanones varied. Similar differences

in the heritability of secondary metabolites occur within species of terrestrial plants (Zangerl et al. 1989, Zangerl and Berenbaum 1990). Interestingly, in *D. pulchra* the heritabilities of both total and individual furanones were generally lower than those described for secondary metabolites from terrestrial plants (Berenbaum and Zangerl 1992, Hamilton et al. 2001) suggesting strong environmental effects on their variation. A range of environmental factors influence quantitative variation in algal secondary metabolites (Yates and Peckol 1993, Arnold et al. 1995, Cronin and Hay 1996a, b, Puglisi and Paul 1997) but their relative effects on furanones are unclear. As furanones are restricted to gland cells in *D. pulchra* (Dworjanyn et al. 1999), gland cell density may have important implications and possibly place constraints on furanone concentrations (e.g., Zangerl et al. 1989). However, despite the significant genetic basis to surface gland cell density, the absence of significant correlations between surface gland cell abundance and furanones suggests no constraints on furanone concentrations as a function of gland cell density, although the density of internal gland cells and the size of both surface and internal gland cells were not measured here.

Maternal effects on offspring (Lynch and Walsh 1998) may have inflated our estimates of broad-sense heritability for furanones and gland cells. The maternal effect of tetrasporophytes on tetraspores is unknown. However, red algal spores are small (*D. pulchra* tetraspores are  $\sim 20$   $\mu\text{m}$  diameter), contain very limited amounts of nutritional material (Santelices 1990), and mechanisms for maternal effects in red algae are unknown. Hence, we have assumed that maternal effects on variation in furanone concentration and gland cell density were small.

#### Feeding by herbivores and the potential for them to impose selection

The finding of genetic variation for concentrations of furanones in *D. pulchra* indicates that there is the potential for furanones to evolve in response to selection by natural enemies, although the relatively low heritability for furanones suggests that the response would be slow. In terrestrial plants, insect herbivores can select for both increased concentrations of secondary metabolites (Mauricio and Rausher 1997) or to maintain intermediate concentrations (Shonle and Bergelson 2000). In contrast to terrestrial plants where specialized feeding by insect herbivores is common (Bernays and Chapman 1994) and a specific defensive response to individual herbivore species may be expected, marine algae are predicted to evolve defenses that are deterrent to a diverse range of generalist herbivores because specialist marine herbivores are rare (Hay and Steinberg 1992). The low consumption of *D. pulchra* by most herbivores and the strong deterrence by naturally occurring levels of nonpolar extract (Table 4) indicate the chemical defenses of *D. pulchra* fit that

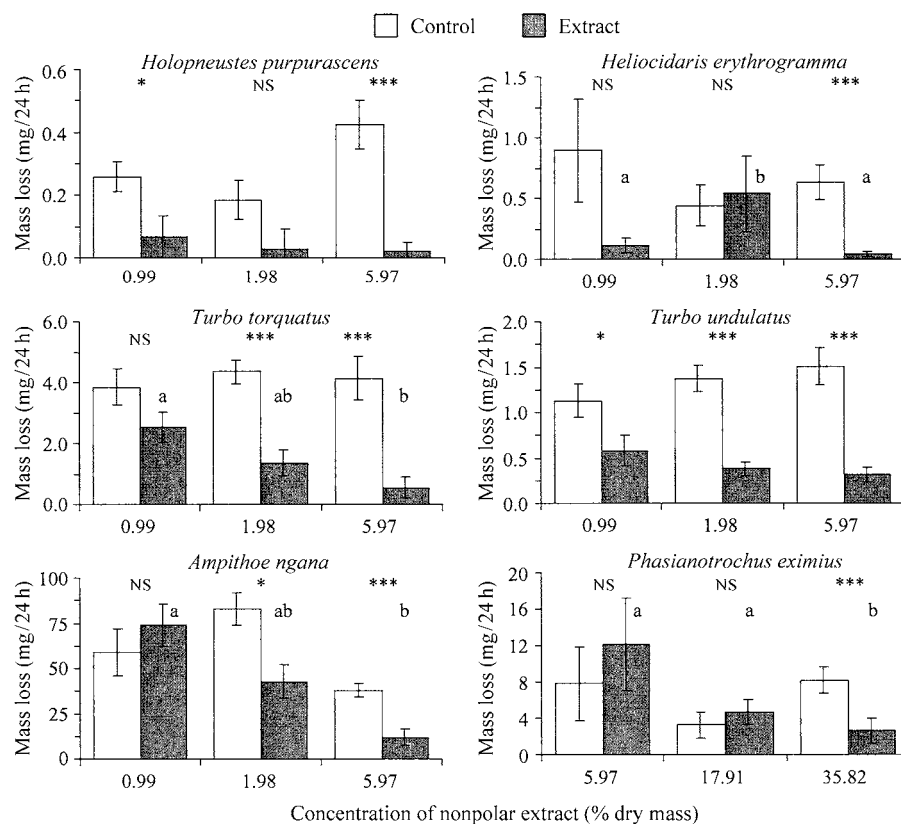


FIG. 3. The effect of three concentrations of nonpolar extract from *Delisea pulchra* on feeding by six herbivores. Bars show the mean ( $\pm 1$  SE) amount of food consumed (wet mass) per replicate over 24 h after adjustment for autogenic change ( $n$  ranged from 10 to 15). Asterisks indicate  $P$  values from  $t$  tests following Peterson and Renaud (1989): \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; NS, not significant. The results of ANOVAs testing for differences in deterrence among the three concentrations were: *H. purpurascens* ( $F_{2,33} = 1.129$ ,  $P = 0.336$ ); *H. erythrogramma* ( $F_{2,31} = 5.338$ ,  $P = 0.010$ ); *T. torquatus* ( $F_{2,35} = 5.052$ ,  $P = 0.012$ ); *T. undulatus* ( $F_{2,40} = 0.398$ ,  $P = 0.674$ ); *A. ngana* ( $F_{2,27} = 10.184$ ,  $P = 0.001$ ); *P. eximius* ( $F_{2,38} = 4.282$ ,  $P = 0.021$ ). For the herbivores for which the three extract concentrations differed in deterrence, shared letters above the plus extract bars indicate those concentrations that did not differ ( $P > 0.05$ , Tukey's test).

prediction. The herbivores associated with *D. pulchra* appear to fall into two broad groups: species that consume very little *D. pulchra* and are deterred by naturally occurring concentrations of furanones except when they occur at very low levels (most herbivores), and species that appear adapted to consuming *D. pulchra* and are not deterred by any naturally occurring concentration of furanones (e.g., *Phasianotrochus eximius*: Table 4). The finding that macrograzers were not deterred by compound 3 at 1 mg/g dry mass but were deterred at 3 mg/g dry mass indicates the potential for selection by those herbivores for concentrations of compound 3  $> 1$  mg/g dry mass. Compound 3 occurred at concentrations  $> 3$  mg/g dry mass in 79% of plants at Bare Island and 88% of plants at Cape Banks. Thus, most plants in those populations were chemically defended against those herbivores and overall, deterrence occurred at the low end of the range of concentrations. Studies examining the effects of quantitative variation in other algal secondary metabolites on feeding by herbivores are uncommon, but they suggest deterrence of

ten occurs at the high end of the concentration range at which those compounds occur (Cronin and Hay 1996b, c). Lower concentrations of furanones in *D. pulchra* have been recorded in plants at other places and times (de Nys et al. 1996, Wright et al. 2000b), and they may represent crucial windows for herbivores to act as selective agents on furanone concentrations.

Of the herbivores tested in this system, the two mesograzers were less deterred by nonpolar extract and furanones than macrograzers. *P. eximius* also preferentially consumed furanone rich tops of plants to the bottoms, as does the sea hare *Aplysia parvula* (Rogers 2000). Mesograzers are often less deterred by concentrations of algal secondary metabolites that are deterrent to macrograzers and herbivorous fish (Hay et al. 1987, Duffy and Hay 1994). By feeding on chemically defended plants, some mesograzers experience lower predation by fish than they would on more palatable species (Hay et al. 1989, 1990, Duffy and Hay 1994). The mechanism by which *P. eximius* is able to tolerate feeding on *D. pulchra* is unclear. It is not a specialist

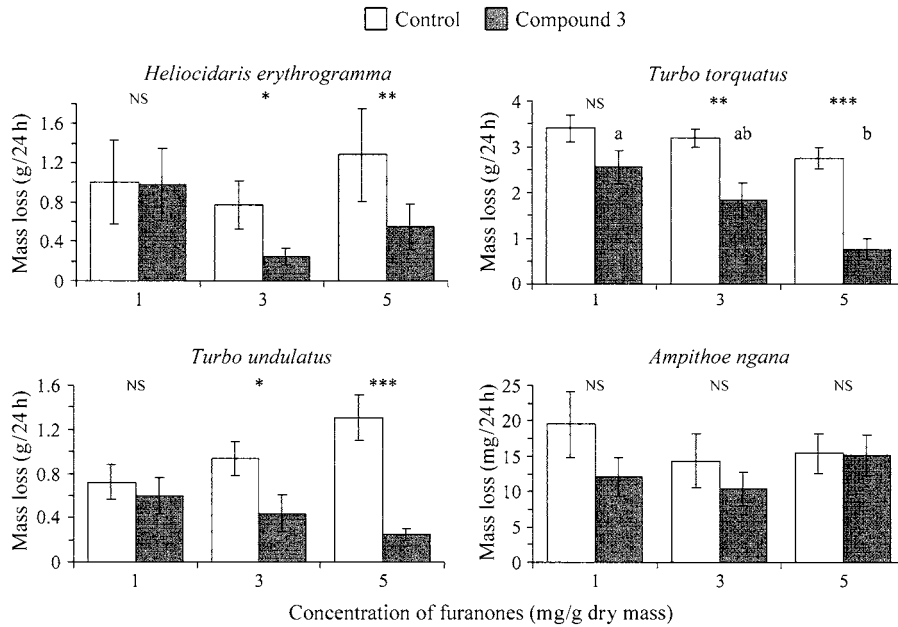


FIG. 4. The effect of quantitative variation in compound 3 on feeding by four herbivores. Bars show the mean ( $\pm 1$  SE) amount of food consumed (wet mass) per replicate over 24 h after adjustment for autogenic change ( $n$  ranged from 6 to 13). Asterisks indicate  $P$  values from  $t$  tests following Peterson and Renaud (1989): \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; NS, not significant. The results of ANOVAs testing for differences in deterrence among the three concentrations were as follows: *H. erythrogramma* ( $F_{2,25} = 1.607$ ,  $P = 0.220$ ); *T. torquatus* ( $F_{2,32} = 4.311$ ,  $P = 0.022$ ); *T. undulatus* ( $F_{2,31} = 0.309$ ,  $P = 0.069$ ); *A. ngana* ( $F_{2,30} = 1.196$ ,  $P = 0.316$ ). For *T. torquatus*, shared letters above the plus compound-3 bars indicate those concentrations that did not differ ( $P > 0.05$ , Tukey's test).

feeder and does not sequester or store these compounds (R. de Nys, unpublished data). The furanone-containing gland cells, which occur at higher densities in the tops of the alga, are not limited to the apical meristems (Dworjanwn et al. 1999), and *P. eximius* does not selectively graze around small regions of tissue with low concentrations of secondary metabolites as has been documented for herbivorous amphipods on *Zonaria augustata* (Poore 1994).

The potential selective impact of any herbivore species is likely to be important if its impact is large relative to that of all other species. Although mesograzers can have important effects on temperate macroalgae (Duffy and Hay 2000), the sea urchin *H. erythrogramma* is very abundant, consumes more mass of *D. pulchra* per capita than other herbivores, and has a major impact on *D. pulchra* recruitment and survivorship when it reaches high densities (Wright and Steinberg

TABLE 4. Summary table describing the feeding by herbivores on *D. pulchra* vs. other macroalgae, and the effects of three concentrations of nonpolar extract and three concentrations of compound 3.

Herbivore	<i>D. pulchra</i> vs. other algae	Nonpolar extract (% dry mass)			Compound 3 (mg/g dry mass)		
		0.99	1.98	5.97	1	3	5
Sea urchins							
<i>Heliocidaris erythrogramma</i>	low	(D)	N	D	N	D	D
<i>Holopneustes purpurascens</i>	very low	D	(D)	D	D	...	...
Gastropods							
<i>Turbo torquatus</i>	low	N	D	D	N	D	D
<i>Turbo undulatus</i>	low†	D	D	D	N	D	D
Mesograzers							
<i>Phasianotrochus eximius</i>	mid	...	...	N	...	...	...
<i>Ampithoe ngana</i>	low/mid	N	D	D	(D)	N	N

Notes: Key to symbols: D, deterrent at  $P < 0.05$ ; (D), deterrent at  $P < 0.1$ ; N, not deterrent. Ellipses (...) indicate "not tested."

Sources: Steinberg and Van Altena (1992), Williamson et al. (2004), and present study.

† Choice and no-choice experiments done.

2001). Despite this abundance and impact, *H. erythrogramma* does not appear to select *D. pulchra* individuals on either a chemical or genetic basis. At Bare Island, *D. pulchra* from the sites where >90% of plants had been removed by grazing by high densities of *H. erythrogramma* did not differ in concentrations of total furanones ( $F_{1,58} = 0.961$ ,  $P > 0.5$ ) or random amplified polymorphic DNAs (RAPD) banding patterns (Wright et al. 2000a) compared to adjacent ungrazed sites. All *D. pulchra* were eventually removed at these high urchin densities, and the longer survivorship of some plants may simply be determined by the distribution of *H. erythrogramma* in space.

There are three constraints regarding our conclusions that herbivores have the potential to act as selective agents for concentrations of furanones above very low levels. First, the presentation of furanones in the artificial diets may not accurately reflect how they are presented to herbivores in the plants. This issue is important because we were unable to provide any data on the feeding rate by herbivores on *D. pulchra* tissue that differed in concentrations of furanones, and we used different artificial diets for different herbivores. However, in general both the qualitative and quantitative responses of all herbivores to nonpolar extract and compound 3 reflected their feeding rates on *D. pulchra* tissue (Table 4). Thus we are confident our experiments provide meaningful data. Second, feeding in laboratory experiments may not reflect field consumption. Because we examined the feeding response of individual species, controlling for other herbivores and ensuring autogenic controls lacked herbivores were most easily done in the laboratory. Experiments to examine feeding deterrence of marine algal secondary metabolites are routinely performed in laboratory experiments (see references in Hay and Steinberg 1992, Paul et al. 2001). Third, we have not examined feeding by herbivorous fishes. Although several herbivorous fish occur in this region, their diet and habitat preferences suggest that with the exception of *Girella tricuspidata*, most are unlikely to consume *D. pulchra* (Andrew and Jones 1990, Clements and Choat 1997, Curley et al. 2002) and none have ever been observed grazing it.

There are also two important evolutionary constraints. First, because furanones also deter the settlement of epiphytes (de Nys et al. 1995) and inhibit colonization, movement, and biofilm development by bacteria (Maximilien et al. 1998, Hentzer et al. 2002), there are many natural enemies that may impose selective pressure on different furanones and/or furanone concentrations. However, relative to the combined (diffuse) selective pressures by natural enemies, any selective pressure imposed by individual species will be weak. Consequently, a specific pairwise coevolutionary response between furanones and single enemies is unlikely, because selection should favor generalized defenses (Fox 1981), which appears to be the case for *D. pulchra*. In addition, because all four furanones are

genetically correlated, selection on individual furanones will cause a correlated change in the others. Genetic correlations arise because of pleiotropy, which is likely if structurally similar compounds such as the four furanones are produced via the same biochemical pathways, or through linkage disequilibrium (Falconer 1989, Lynch and Walsh 1998).

Finally, any cost of furanones to *D. pulchra* may represent a further constraint on their evolution. Most models for the evolution of resistance in plants assume that as well as having a benefit, resistance traits also impose a cost by directing resources away from growth and reproduction (Strauss et al. 2002) and stress tolerance (Cronin 2001). Furanones clearly provide a benefit to *D. pulchra* by deterring natural enemies but they usually occur at higher concentrations than needed to achieve this, suggesting they impose little cost. A companion study to this paper indicates the cost of furanones to *D. pulchra* is complex and depends on the life-cycle stage (sporelings, juveniles, and adults) being examined (S. A. Dworjanyn, J. T. Wright, N. Paul, R. de Nys, and P. D. Steinberg, *unpublished manuscript*).

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