The following supplements accompany the article

Defensive role of macroalgal phlorotannins: benefits and trade-offs under natural herbivory

Fiia Haavisto*, Riitta Koivikko, Veijo Jormalainen

*Corresponding author: fiia.haavisto@utu.fi

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SUPPLEMENT 1

Table S1. Test of difference between the two treatment levels allowing herbivore access on algae (fish exclosure, open) on grazing damage from high autumn herbivory. Grazing damage was measured as lost thallus area. Initial size of the algae and its interaction with genotype did not contribute to variation in the model and were not included in the final model.

	Thallus area		
Fixed factors	ndf, ddf	F	P
Treatment	1, 10	0.19	0.67
Random factors	$s^2 \pm SE$	χ^2	P
Cage (Trm)	0.026 ± 0.012	218	<0.0001
Genotype	0.022 ± 0.0064	166	<0.0001
Residual	0.043 ± 0.0026		

Table S2. Test of difference between the three treatments in the phlorotannin content of the algae under high herbivory of autumn and under low herbivory of spring. In treatments algae were either protected from herbivores (No herbivory), exposed to herbivores, and fish predators were excluded (Fish-exclosure), or algae were exposed to natural herbivory together with fish predators (Open). Manipulation was conducted in natural marine sublittoral and lasted for three weeks.

	AUTUMN			SPRING		
Source of variation						
Fixed factors	ndf, ddf	F	P	ndf, ddf	F	P
Treatment, Trm	2, 11.4	22.1	<.0001	2, 8	4.10	0.060
Random factors	$s^2 \pm SE$	χ^2	P	$s^2 \pm SE$	χ^2	P
Genotype	35.8± 13.9	31.5	<.0001	37.8 ± 14.0	12.1	0.0005
Cage (Trm)	8.75 ± 6.08	6.01	0.007	0	0	
Residual	65.1			112		

SUPPLEMENT 2

To study whether herbivory triggers qualitative differences in individual phlorotannins, we conducted an HPLC analysis on a small set of algae that were manipulated in the laboratory.

METHODS

MANIPULATION OF ALGAE

We manipulated vegetative tips from 10 genotypes of *F. vesiculosus* in three different groups. In the grazed group, *I. balthica* males grazed directly on the algal pieces (IG). The second group of algal pieces was exposed to water-borne cues from grazing (NG), but not direct herbivory. To do this, we placed one *I. balthica* male into a small plastic mesh-cage to feed on a piece of *F. vesiculosus*; this cage was placed in a glass container together with a focal algal piece. Algae in the control group were reared in pure seawater (Control). Manipulations lasted for five days in 1-L glass containers under four 400-W sodium vapor lamps (Sylvania), with a light rhythm of 14 hours light and 10 hours dark, at 15°C.

SAMPLE EXTRACTION

After the manipulation, algal pieces were freeze-dried, ground, and held at -20ºC until further analysis. A 200-mg aliquot was prepared from each piece, from which lipids were removed by multiple solid-liquid extractions with hexane (Koivikko et al. 2007). Following the removal of hexane, phlorotannin extraction was conducted four times with a preparation of 7:3 acetone:water (10 mL) that included ascorbic acid (0.3 % m/v) to prevent oxidation. The extraction aliquots were combined and acetone was removed in a fume hood (room temperature). The water-based residue was centrifuged and the liquid portion was frozen and freeze-dried. The dried sample was dissolved twice with purified water (0.5 mL) and filtered on an 0.45-µm PTFE filter, at which point an aliquot was taken for HPLC analyses.

HPLC ANALYSES

The HPLC analyses were conducted by normal-phase (NP) chromatography, in which chromatographic separation is based on the increasing polarity of the analytes. With this method, individual compounds or groups of similar-sized compounds of lower polarity appear in the beginning of the chromatograph, whereas higher-polarity compounds have longer retention times. In general, polarity increases with the size of the phenolic compound (Koivikko et al. 2007).

The NP-HPLC system consisted of an HP 1090 liquid chromatography apparatus (Hewlett-Packard, Palo Alto, CA, USA) with build-in DAD and HP Chemstation software for LC 3D systems. The column was a LiChrospher Si 60 (250 × 4 mm i.d., 5 µm; Merck) that was equipped with a guard cartridge (LiChrospher Si 60; 4 × 4 mm i.d.; 5 µm; Merck). The binary mobile phase consisted of (A) dichloromethane, methanol, water, and acetic acid (82:14:2:2, v/v) and (B) methanol, water, and acetic acid (96:2:2, v/v). The elution gradient was: 0 min, 100% A (isocratic); 0-30 min 0-17.6% B in A (linear), 30-45 min 17.6-30.7% B in A (linear); 45-50 min 30.7-87.8% B in A (linear); 50-60 min 87.8% B in A (isocratic); 60-80 min 87.7-0% B in A (linear); 80-105 min 100% A (isocratic). The detection wavelength was 280 nm, the flow rate was 1.0 mL/min, and the injection volume was 1 µL (Koivikko et al. 2007).

Due to the lack of commercial standards for individual phlorotannins, the traces of the HPLC chromatograms were integrated and the integrated areas (hereafter 'size') were used for statistical analyses.

RESULTS

Altogether, HPLC analyses revealed 29 separate traces. To test statistically the difference in the sizes of traces between control algae and those having direct (IG) or indirect grazing (NG) cues, we pooled the results from IG and NG groups (n=4) and conducted t-tests (Table S3). Significant elevations in IG and NG algae were tracked in traces #10, #20, #21, #23, of which trace #10 had been recognized as a phlorotannin tetramer in an earlier study (Koivikko et al. 2007). In addition, traces #22 and #24 were marginally higher in the treatment groups, while traces #8, #16, and #29 were less abundant. Trace #27 was also interesting, but varied among measurements, which brought into question the repeatability of the trace (Fig. S1).

Table S3. Results from t-tests that compared the sizes of eight traces in the HPLC chromatograms between algae that were exposed to herbivore grazing (IG and NG) and those kept in pure seawater (Control).

Trace #	df	t	P
8	4	2.3	0.084
10	4	3.5	**0.024**
16	4	2.3	0.086
20	4	2.8	**0.049**
21	4	3.5	**0.024**
22	4	2.2	0.093
23	4	3.1	**0.036**
24	4	2.4	0.074
29	4	2.7	0.055

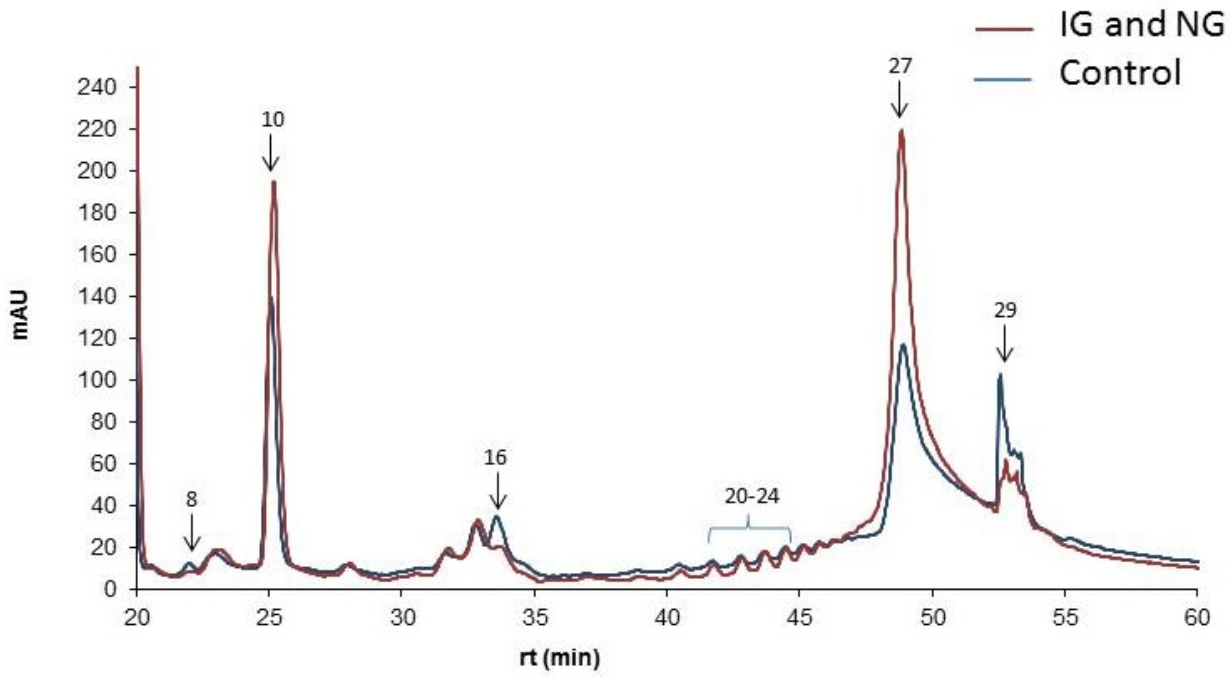

Fig. S1. NP-HPLC chromatograph of the traces of soluble phenolics in *F. vesiculosus* after five-day exposure to direct grazing (IG) or to water-borne cues from grazing of conspecific algae (NG) versus manipulation in pure seawater (Control).

REFERENCES

Koivikko R, Loponen J, Pihlaja K, Jormalainen V (2007) High-performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus vesiculosus*. Phytochem Anal. 18: 326-332 doi:10. 1002/pea.986.