

## Induction of phlorotannin production in a brown alga: defense or resource dynamics?

Veijo Jormalainen, Tuija Honkanen, Riitta Koivikko and Janne Eränen

Jormalainen, V., Honkanen, T., Koivikko, R. and Eränen, J. 2003. Induction of phlorotannin production in a brown alga: defense or resource dynamics? – *Oikos* 103: 640–650.

Increase of phenolic secondary metabolites, phlorotannins, in brown algae due to gastropod grazing has been interpreted as an anti-herbivore adaptation. Here we tested whether such a response could be due to changes in truly available resources for the alga, not by the grazing activity of snails as such. We allowed two species of snails, *Theodoxus fluviatilis* and *Physa fontinalis* to graze on *Fucus vesiculosus*. These species feed on epibiota and particulate matter on the thallus but do not eat the thallus of *F. vesiculosus*. We further simulated snail grazing by nutrient enhancement, removal of epibiota and by a combination of the two. Manipulations of nutrient and light availability revealed the crucial role of epibiota in mediating resource availability for *F. vesiculosus*. Nutrient enhancement alone increased epibiota and decreased phlorotannins. Cleaning the thallus resulted in increased growth, and together with nutrient enhancement also in a trade-off with phlorotannins. Presence of *T. fluviatilis* on the thallus induced phlorotannin production, a response differing from the simulations of snail grazing. However, we suggest that the increase in phlorotannins may not be an induced defense but rather a consequence of a specific way of resource manipulation by this snail species. *T. fluviatilis* removes hyaline hairs that facilitate nutrient uptake. *P. fontinalis* did not remove hyaline hairs and the response of the alga to its grazing was similar to the treatment where we mechanically removed epibiota suggesting that cleaning of the thallus is the major mechanism how this snail species affects *F. vesiculosus*. Genetic variation in phlorotannin concentrations highly exceeded the induced responses of simulated or real snail grazing. This casts doubt for the efficiency of induced phlorotannin production to act as a defense, but is not contradictory with the interpretation of phlorotannins responding to variation in resource availability.

V. Jormalainen, T. Honkanen, R. Koivikko and J. Eränen, Dept of Biology, Univ. of Turku, FIN-20014 Turku, Finland (veijo.jormalainen@utu.fi).

Brown algal phlorotannins are phenolic secondary metabolites, with a putative adaptive role in defense against herbivory (Targett and Arnold 1998). A characteristic of phlorotannins is their plasticity to a variety of environmental factors. Phlorotannin production has been found to respond plastically, for example, to changes in nutrient environment (Yates and Peckol 1993, Van Alstyne and Pelletreau 2000), light (Cronin and Hay 1996, Pavia and Toth 2000b), depth (Peckol et al. 1996), salinity (Jormalainen and Honkanen 2001) and grazing (Pavia and Brock 2000, Pavia and Toth

2000a) or other mechanical wounding (Van Alstyne 1988, Hammerström et al. 1998). To what extent such plasticity may represent inducible defense against herbivory or other adaptive or non-adaptive responses has been controversial. Suggestions for other adaptive roles for phlorotannins include protection against ultraviolet radiation (Pavia et al. 1997) or function as anti-fouling substances (Lau-Stanley and Qian 1997). Furthermore, phlorotannins may play a role in the primary chemistry of algae, e.g. as storage compounds (Ragan and Glombitza 1986) and act as structural compounds in cell-

Accepted 7 May 2003

Copyright © OIKOS 2003  
ISSN 0030-1299

walls (Schoenwaelder and Clayton 1998, Schoenwaelder 2002).

The suggested defensive role of phlorotannins is based on three kinds of evidence: (1) Several case studies have shown that phlorotannins deter feeding by herbivores (Steinberg 1985, Van Alstyne and Paul 1990, Steinberg et al. 1995, Pereira and Yoneshigue-Valentin 1999, Pavia and Toth 2000a). (2) Phenolic compounds may decrease the performance of herbivores by binding with proteins in the gut thereby decreasing their assimilation efficiency (Stern et al. 1996) and there is some direct evidence for reduced assimilation efficiency of herbivores due to phlorotannins (Ireland and Horn 1991, Boettcher and Targett 1993). However, these functions of phlorotannin are by no means unanimous. Several cases exist where deterrence (Steinberg and Van Altena 1992, Steinberg et al. 1995, Pavia et al. 1999a, Van Alstyne et al. 2001, Jormalainen et al. 2001) or negative effects on performance (Targett et al. 1995, Jormalainen et al. 2001) have not been verified, and sometimes even a preference for phlorotannin rich algae has been found (Pavia et al. 1997, Jormalainen et al. 2001). This suggests that even if the original adaptive function of phlorotannins were the defense against herbivory, a number of herbivore species have adapted to utilize phlorotannin rich algae. (3) Grazing by herbivores or simulated grazing has been found to produce very variable responses in experiments where the existence of inducible responses have been specifically tested. Induced increases in phlorotannin production have been found in some cases (Van Alstyne 1988, Yates and Peckol 1993, Peckol et al. 1996, Hammerström et al. 1998, Pavia and Toth 2000a, Toth and Pavia 2000, Arnold et al. 2001), but there are several cases where no responses were found (Yates and Peckol 1993, Steinberg 1994, Martinez 1996, Peckol et al. 1996, Pavia et al. 1997, Toth and Pavia 2002). Thus, the existence of induced defensive response of phlorotannins to grazing and biomass loss cannot be unanimously assumed. Furthermore, whether an induced response, when there is one, can be interpreted as adaptive plasticity, evolved through selection imposed by herbivores, or as a non-adaptive consequence of resource manipulation due to removal of biomass or other influences of the presence of grazers, has remained even more unclear.

The resource-based hypotheses on plant secondary chemistry, carbon-nutrient balance hypothesis (Bryant et al. 1983) and growth-differentiation balance hypothesis (Herms and Mattson 1992), assume the production of carbon based secondary metabolites, such as phenolics, to vary largely according to the resource availability for the plant (Herms and Mattson 1992). Although the value of these hypotheses, especially CNB, as an explanatory paradigm has been doubted (Hamilton et al. 2001, Close and McArthur 2002, Koricheva 2002, Nitao et al. 2002) they can be useful in offering proximate mechanisms for allocation of carbon between

growth and secondary metabolism (Karban and Baldwin 1997, Koricheva et al. 1998, Lerdau and Coley 2002) and in providing an alternative to overly straightforward adaptive explanations. These hypotheses predict that when nutrients are readily available for growth, the allocation of photosynthetic carbon is primarily directed to demands of growth and, consequently, the production of secondary metabolites decreases. On the other hand, when nutrients limit growth but photosynthesis is not limited by light availability, secondary metabolites can be produced with low or no costs for the growth of the plant (Tuomi et al. 1988). Therefore, the availability of both light and nutrients should generate variability in phlorotannin production, and the magnitude of the effect of one resource depends on the level of the other. For example, increased light availability is expected to increase production of secondary metabolites when nutrients are limiting, but not when nutrients are abundant. Covariation of phlorotannin production with resource availability has been shown in several studies (Ilvessalo and Tuomi 1989, Yates and Peckol 1993, Arnold et al. 1995, Steinberg 1995, Pavia et al. 1999b, Pavia and Toth 2000b, Van Alstyne and Pelletreau 2000).

Induced responses to natural herbivory in phlorotannin production have been found only when using snails as herbivores (Van Alstyne 1988, Pavia and Toth 2000a, Toth and Pavia 2000, Amsler 2001), and such responses are often interpreted as specific anti-herbivore adaptations, induced defenses. Snails typically graze on the surface of the thallus feeding on periphyton and epiphytic algae (Skoog 1978, Blanchard et al. 2000), on deposited matter and sometimes on the focal thallus as well (Hyman 1967). Hence, such a feeding pattern may modify the resource pool for the alga in several ways: First, removal of epibiota eliminates shading and thereby affects the light quantity and the amount of UV irradiation on the thallus. Second, nutrient resources for the alga improve because nutrient absorbency by epiphytes decreases and especially because snails produce organic nutrients through their digestion. Third, as brown algae can store resources in their thallus (Carlson 1991, Lobban and Harrison 1994), biomass losses due to consumption of the thallus therefore reduce the amount of stored resources. Thus, it is possible that the induced response of phlorotannins to snail grazing follows from the changes in resource levels rather than representing a specific anti-herbivore adaptation.

Here we test whether snail grazing induces changes in phlorotannin concentration and growth of the brown alga *Fucus vesiculosus* (L.) and to what extent these changes are attributable to changes in resource availability. As grazers we use two species of snails that feed on epibiota on the thallus but do not eat the thallus of adult *F. vesiculosus*. Because we use species that are not herbivores on *Fucus*, we do not directly test

the inducible defense model. Instead, we test whether a mere occurrence of non-herbivorous snails on algae can induce responses that would easily be interpreted as induced defenses in a case of herbivorous snails. We compare the effects of snail grazing to simulations of snail presence, removal of epibiota and nutrient enhancement, on growth and phlorotannin production of the alga. We predicted that if resource availability explains the plasticity of these traits, the presence of snails should generate responses similar to the ones in treatments with removal of epibiota, nutrient enhancement, or both, depending on the relative effect of snails on availability of light and nutrients. We further explore the amount of genetic variation in phlorotannin production. To be effective defenses, we expect the magnitude of inducible responses to exceed the amount of genetic variation.

## Material and methods

*Fucus vesiculosus* is a perennial, belt-forming brown alga that dominates littoral rocky bottom vegetation especially in the nutrient poor areas in the northern Baltic Sea. The growth of *F. vesiculosus* occurs by means of apical meristems (Van den Hoek et al. 1995). Nutrients are absorbed both through the thallus and through phaeophycean hairs, so called hyaline-hairs, that are multi-cellular, uniseriate, unbranched colourless filaments erecting in tufts from cryptostomata in apical parts of the thallus (Hurd et al. 1993, Van den Hoek et al. 1995). In *F. vesiculosus* in our study area, there are one to seven hair-tufts of dozens of hairs per cm<sup>2</sup> of thallus (pers. obs. by authors). Translocation of resources occurs from older parts of thallus to growing apices (Honkanen and Jormalainen 2002).

The snails *Theodoxus fluviatilis* and *Physa fontinalis* occur on *Fucus* feeding on epibiota and particulate matter on thallus (Skoog 1978, Jones et al. 1999, Malm et al. 1999), but they do not feed on mature thallus of *F. vesiculosus* (pers. obs. by authors, verified by scanning electron microscopy). *Theodoxus fluviatilis* is very abundant in our study area reaching densities of hundreds of individuals per individual alga. In a sample distribution of 120 *F. vesiculosus* collected throughout the summer, 25%, 50% and 75% quartiles were 19, 52 and 109 individuals of *T. fluviatilis* per alga, respectively (unpubl. data). *Physa fontinalis* is clearly less abundant occurring in densities of at most a few individuals per alga (Pettay 2001).

We conducted the experiment in outdoor through-flow aquaria, covered with UV-permeable plastic. We used a total of 28 plastic aquaria, with a volume of 15 l and seawater through-flow of six l h<sup>-1</sup>. The algae were collected from the Archipelago Sea, northern Baltic Sea (22°18.65'E, 60°06.50'N), randomly along a

dense *Fucus* belt from the depth of one meter. The algal material consisted of 15 large individuals, an individual being defined as the entity growing from a single stem on a holdfast (which commonly contains several stems) and representing a unique genotype. Each genotype was split to 28 apical pieces, each about five cm in length and carrying two to four apical meristems. One piece of each genotype was anchored in the bottom of each of the aquaria by a clothes-peg. Thus, each aquarium had the same genotypic composition of algae, 15 genotypes in each aquarium.

We cloned the genotypes in order to both control and evaluate the amount of genotypic variation in growth and phlorotannins as well as to explore genetic correlation between these traits. Clonal material is commonly used to evaluate genetic effects (Schwaegerle et al. 2000). However, conclusions from clonal material concern total genetic variation, not just the additive genetic variance, and hold under assumption of minimal maternal, or "carry-over" effects from the clonal parent.

The experiment started by randomly assigning the aquaria to seven treatments, four replicate aquaria to each treatment. The treatment (1) acted as a control having no manipulations. The following three treatments acted as simulations of possible snail effects on resources available to algae. The treatment (2) got nutrient enhancement and in the treatment (3) we removed periphyton, filamentous algae and deposited material from the surface of the thallus. The treatment (4) experienced both the nutrient enhancement and the removal of epibiota. In the treatment (5) we had *T. fluviatilis* grazing on algae and in the treatment (6) we had *T. fluviatilis*, but they were inhibited from grazing on the algae. The last treatment (7) had *P. fontinalis* snails that were allowed to graze on the algae.

Nutrient enhancement (treatments 2 and 4) was done by adding inorganic nutrients (NO<sub>3</sub>, NH<sub>4</sub>, PO<sub>4</sub>) into the aquaria in a continuous flow of droplets. The attempted concentrations for these nutrients in the nutrient enhancement aquaria were 70 µg/l, 30 µg/l and 20 µg/l, respectively. In the beginning of the experiment, even distribution of nutrients in the aquaria was confirmed by using colored solution. Epibiota and deposited material was removed (treatments 3 and 4) by brushing the thallus with a smooth brush every second day. In the treatments including *T. fluviatilis* we placed initially (12th May) 100 individuals into each aquaria either freely (treatment 5) or enclosed into a mesh-net bag to keep the snails away from the thallus (treatment 6), and later we (4th June) added specimens so that their total number in each aquarium was 150 individuals. In the mesh-net bags we had plastic plants in order to provide substratum for periphyton on which the snails graze. In the treatment with *P. fontinalis* (treatment 7) we had a total of 52 free ranging individuals. A smaller number than in the *T. fluviatilis* treatment was used due to the larger size and higher mobility of this species.

Nutrient manipulation in aquaria was monitored by analyses of water samples. The low number of replicates, two per treatment, does not allow rigorous statistical testing, but the data documents the efficiency of nutrient manipulation: actual nutrient concentrations remained slightly lower than the attempted values, most likely due to fast biological fixation (Fig. 1). Furthermore, the snails increased ammonium concentration in the water as we expected, but such an effect was evident only in the treatments 5 and 7 where snails were feeding freely on algae (Fig. 1).

In the beginning (12th May) and at the end (4th July) of the experiment we measured the weight, maximum length and the number of apical parts of the algae.

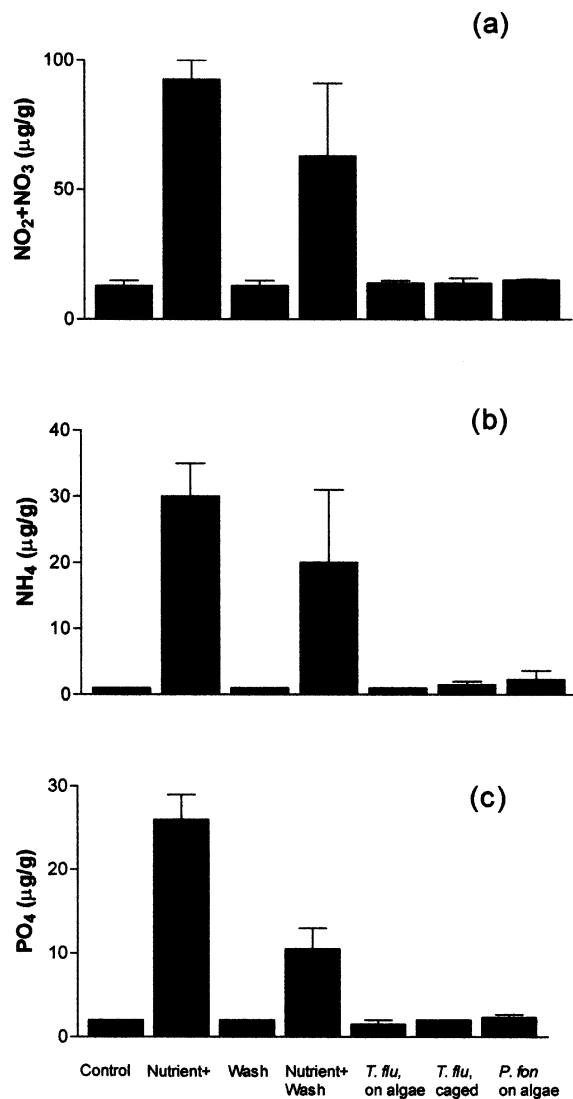


Fig. 1. Mean (+SE) nitrate and nitrite nitrogen (a), ammonium nitrogen (b) and phosphate phosphorus (c) concentrations (µg/l) in experimental aquaria. Means are based on two replicate samples.

Growth in terms of weight and length increment and production of apical tips was calculated as the difference between the final and initial measurements. At the end of the experiment, we also measured the amount of periphyton growing on algae by washing the algae individually distilled water and filtering the periphyton on pre-weighed glass-fiber filters, which were dried and weighed. This was done two days after the last removal of epibiota (treatments 3 and 4), thus, the estimates represent minimum effects of these treatments on epibiota.

We quantified the total content of phenolic compounds at the end of the experiment by a modification of the Folin–Ciocalteu method (Nurmi et al. 1996) by using phloroglucinol as a standard agent. The Folin–Ciocalteu assay also quantifies non-phenolic hydroxylated aromatic compounds, but these make up < 5% of the total Folin–Ciocalteu-reactive compounds (Van Alstyne 1995). In the following, we use the term ‘phlorotannins’ for these total phenolics, since brown algae are not known to contain other polyphenols (Targett and Arnold 1998). In the phlorotannin analyses, we used only apical thallus grown during the experiment. In order to ensure enough biomass for the analyses, two replicate pieces belonging to the same genotype and treatment, but originating from separate aquaria, were combined for each determination.

In order to explore the grazing effects of the two snails and the effect of the washing treatment on the thallus of *F. vesiculosus* in detail, we reared pieces of algae under four treatments: control, presence of *T. fluviatilis*, presence of *P. fontinalis* and the washing treatment. We used ten different algal genotypes, which were each cloned into four pieces. One piece of each genotype was assigned to every treatment, singly in one-liter containers. Washing was conducted as above every second day when also water was changed in all containers. In the treatments with snails, we had 20 *T. fluviatilis* individuals or eight *P. fontinalis* individuals grazing freely in each container. The rearing lasted for 15 days, under natural light and water temperature of 15°C, after which we freeze-dried the algae for microscopic examination. The length of the tufts of hyaline hairs on thallus was evaluated under a dissecting microscope by measuring the length of ten randomly chosen hair-tufts from each alga. In addition, we analyzed 12 algae, three from each treatment, by scanning electron microscopy in order to visualize the consequences of snail grazing on the surface of the thallus.

For statistical analyses we used mixed model ANOVAs (PROCEDURE MIXED in SAS 6.12, Littell et al. 1996), where the genotype and aquarium (when applicable) were treated as random factors and the treatment as a fixed effect. Initial size was used as a covariate in the analyses of growth. A priori contrasts were performed between the control and the other treatments, and between the two treatments with *T.*

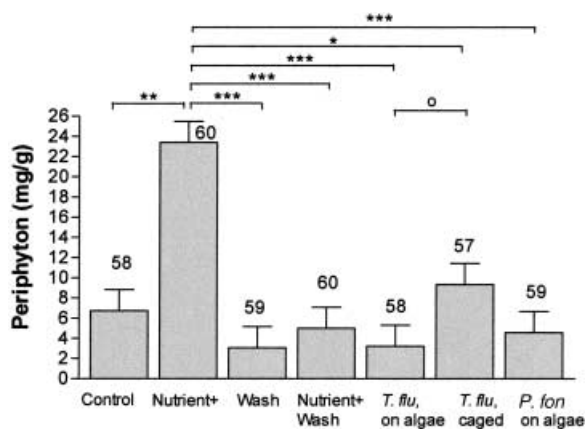


Fig. 2. Mean ( $\pm$ SE) epibiont (mainly periphyton and filamentous algae) load on algae (mg dry weight epibiota/g fresh weight algal tissue) in each experimental treatment. N-values are given on the top of each bar. Horizontal hair-lines connect the treatments differing significantly according to the Tukey's a posteriori comparison of all pair-wise means.  $^{\circ}$  =  $P < 0.10$ , \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , and \*\*\* =  $P < 0.001$ .

*fluviatilis*. Because not all of these comparisons are orthogonal, the experiment-wise error rate will be somewhat higher than 0.05. Therefore, we also conducted conservative a posteriori testing of differences between all pair-wise means using Tukey's test statistics. In the chemical analysis of phlorotannins, we combined algal pieces (see above) and, therefore, used genotypic mean values in the statistical analysis with the consequence of being unable to test the genotype-by-treatment interaction. To compare the amount of variance in phlorotannins due to the treatments and genotype, we calculated the respective variance components (PROCEDURE VARCOMP in SAS 6.12, Littell et al. 1996). Correlations between growth and phloro-

tannin concentration were calculated for each treatment separately using the genotypic mean values of the traits. Thus, correlations represent genotypic correlations, under general limitations of interpreting genetic effects from clonal material.

## Results

Nutrient increment effectively increased the periphyton biomass growing on *F. vesiculosus* (Fig. 2, Table 1). Furthermore, the washing treatment removed periphyton from the algae, which was seen especially clearly in the comparison between the nutrient enhanced and nutrient enhanced plus washed algae (Fig. 2). *Theodoxus fluviatilis*, although they increased the nutrient concentration in the water, effectively removed periphyton when grazing on algae (Fig. 2, Table 1).

The treatments significantly affected growth of *F. vesiculosus*, measured either in terms of increment in weight or length (Table 2). The average growth during the 53-day duration of the experiment in the control group was  $0.496 \pm 0.03$  g ( $n = 59$ ) in weight or  $15.4 \pm 1.0$  mm ( $n = 59$ ) in length. Nutrient increment alone tended to decrease growth (Fig. 3, Table 2), most likely due to increased load of periphyton (Fig. 2) and consequent decrease in truly available light and nutrients for *F. vesiculosus*. The crucial role of epibiota is further emphasized by the result that nutrient increment with the simultaneous removal of epibiota by washing significantly increased growth (Fig. 3, Table 2). Washing alone increased growth (Fig. 3, Table 2) implying that in control environment algal growth was depressed due to the presence of epibiota on algae. Accordingly, the number of apical parts that arose during the experiment was significantly higher in the group of washed algae

Table 1. Mixed model ANOVA tests of the effects of genotype and treatments on the amount of periphyton on algae. A priori contrasts between the control and each of the treatments are shown. Data were log transformed for the analysis and are shown in Fig. 2.

Source of variation	Variance estimates			
Random effects	$s^2 \times 10^3$	$SE \times 10^3$	Z	P
Genotype	8.98	4.1	2.19	<0.05
Aquarium(Trm)	29.5	10.2	2.90	<0.01
Genotype $\times$ Trm	0			
residual	51.8	3.8		
Fixed effects	Tests of fixed effects			
	ndf, ddf	F	P	
Trm	6, 21	11.6	<0.001	
Ctrl vs Nutrient+	1, 21	24.1	<0.001	
Ctrl vs Wash	1, 21	3.49	0.07	
Ctrl vs Nutrient+, Wash	1, 21	0.55	NS	
Ctrl vs <i>T. fluviatilis</i> on algae	1, 21.1	4.40	<0.05	
Ctrl vs <i>T. fluviatilis</i> caged	1, 21.1	0.89	NS	
Ctrl vs <i>P. fontinalis</i> on algae	1, 21	1.26	NS	
<i>T. flu</i> on algae vs caged	1, 21.1	9.26	<0.01	

Table 2. The statistical analyses of the effects of genotype and treatments on growth and phlorotannin concentration. Genotype and its interactions with the treatments and the aquaria are treated as random effects and the manipulation as a fixed effect. In the analysis of growth, the initial size (either in weight or length, respectively) is used as a covariate. Data are shown in Fig. 3.

Source of variation	Variance estimates Growth, in weight			Variance estimates Growth, in length			Variance estimates Phlorotannin concentration					
	s <sup>2</sup> × 10 <sup>4</sup>	SE × 10 <sup>4</sup>	Z	P	s <sup>2</sup>	SE	Z	P	s <sup>2</sup>	SE	Z	P
Random effects												
Genotype	90	37	2.46	<0.01	9.03	3.61	2.50	<0.01	0.41	0.16	2.49	<0.05
Aquarium(Trm)	11	6.7	1.61	0.05	0.63	0.49	1.28	NS	not applicable			
Genotype × Trm residual	0	—	—	—	0	—	—	—	not applicable			
	160	12	—	—	13.8	1.02	—	—	0.18	0.03	—	—
Fixed effects												
Treatments												
Contrasts												
ctrl vs nutrient+	6, 21	6.55	6.55	<0.001	6, 20.7	6, 20.7	5.85	<0.01	6, 84	6, 84	4.48	<0.001
ctrl vs wash	1, 21	3.37	3.37	0.08	1, 20.6	1, 20.6	1.66	NS	1, 84	1, 84	4.07	<0.05
ctrl vs nutrient+, wash	1, 21.2	2.24	2.24	NS	1, 20.9	1, 20.9	12.4	<0.01	1, 84	1, 84	0.08	NS
ctrl vs <i>T. fluviatilis</i> on algae	1, 20.8	8.30	8.30	<0.01	1, 20.5	1, 20.5	7.69	<0.05	1, 84	1, 84	5.18	<0.05
ctrl vs <i>T. fluviatilis</i> caged	1, 20.9	1.65	1.65	NS	1, 20.5	1, 20.5	0.05	NS	1, 84	1, 84	4.43	<0.05
ctrl vs <i>P. fontinalis</i>	1, 21.2	0.55	0.55	NS	1, 21.2	1, 21.2	1.19	NS	1, 84	1, 84	0.24	NS
<i>T. fluviatilis</i> on algae vs caged	1, 20.8	4.51	4.51	<0.05	1, 20.7	1, 20.7	5.08	<0.05	1, 84	1, 84	2.32	NS
Covariate	1, 21	0.29	0.29	NS	1, 21	1, 21	0.74	NS	1, 84	1, 84	6.75	<0.05
	1, 404	287	287	<0.001	1, 398	1, 398	7.50	<0.01				

than in the control (data and full ANOVA not shown; Control vs wash contrast  $F_{6, 21} = 4.24$ ,  $P = 0.05$ ).

The presence of *T. fluviatilis* in aquaria, either grazing on algae or caged, had no effect on growth of *F. vesiculosus* when compared to the control treatment (Fig. 3, Table 2). However, growth in the treatment with *T. fluviatilis* grazing on algae was lower than in the treatments with wash or with wash and nutrient enhancement (Fig. 3). On the contrary, when *P. fontinalis* was allowed to graze on the thallus, the growth of *F. vesiculosus* was significantly better than that of the control algae. It is also worth to note that a significant amount of variation in both the amount of epibiota (Table 1) and growth (Table 2) was due to the genotype, and that there was no significant genotype by treatment interaction in growth.

The average phlorotannin concentration of the genotypes in the control treatment was  $4.6 \pm 0.2\%$  ( $n = 15$ ) of the dry-weight of algae. Our treatments significantly affected phlorotannin concentration (Fig. 3, Table 2). Nutrient increment, either alone or with washing away epibiota, significantly decreased phlorotannin concentration (Fig. 3, Table 2). The presence of *T. fluviatilis* on algae had an opposite effect as they significantly increased phlorotannins. However, the sole presence of these snails in aquaria without a physical contact with the algae had no effect on phlorotannins (Fig. 3, Table 2)[EL1]. The presence of *P. fontinalis* on algae had no effect on phlorotannins. Although phlorotannins showed plastic responses to our manipulations, the among-genotype variation was much higher than the variation due to the manipulations: the variance component due to the treatments was only 6.6% of the total variance, whereas the variance component for among genotype variation was 64.8% (for visualization of among-genotype variation, see Fig. 4).

Although some treatments, especially the one including nutrient enrichment and removal of epibiota, had opposite effects on growth and phlorotannins, genotypic correlation between these traits in the pooled data over all the treatments was positive ( $r = 0.52$ ,  $P < 0.05$ ,  $n = 15$ ) and tended to be positive also within the individual treatments (Fig. 4).

The microscopic examination of the algae revealed that *T. fluviatilis* did not make any visible injury on cells in the surface of the thallus, but they removed hyaline hairs that erect from the thallus (Fig. 5). This removal action of the hairs also showed up as the shorter length of the hair-tufts in the treatment with *T. fluviatilis* ( $1.20 \pm 0.15$  mm,  $n = 10$ ) as compared to control ( $2.32 \pm 0.15$  mm,  $n = 10$ ), washing ( $2.30 \pm 0.15$  mm,  $n = 10$ ) and *P. fontinalis* ( $1.96 \pm 0.15$  mm,  $n = 10$ ) treatments. The mean length of the hair-tufts differed significantly between the treatments (ANOVA:  $F_{3,27} = 13.5$ ,  $P < 0.0001$ ), and the contrast between the control and *T. fluviatilis* treatments was statistically significant ( $F_{1,27} = 31.0$ ,  $< .0001$ ; control vs washing -contrast:

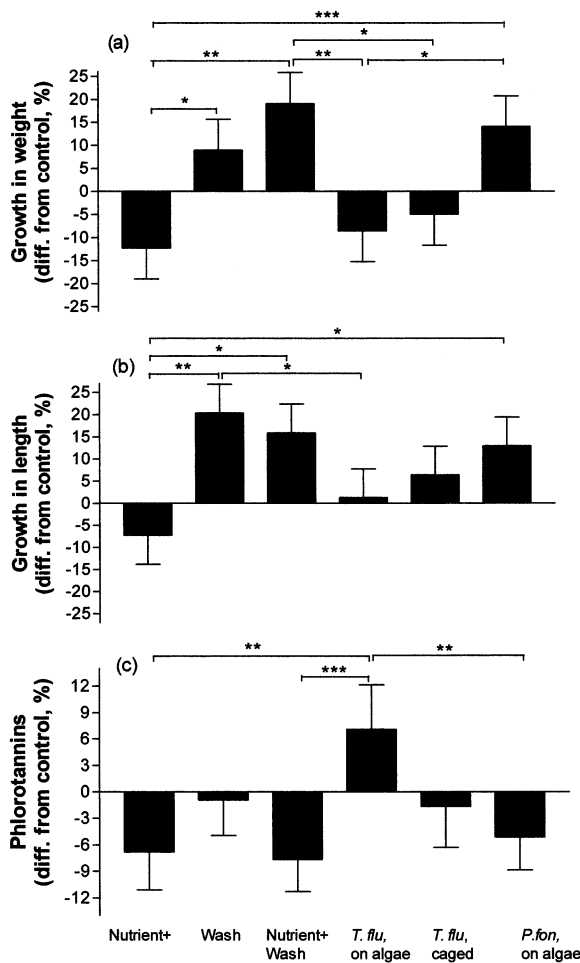


Fig. 3. Growth, in terms of weight (a) and length (b) increment, and phlorotannin concentration (c) in different treatments as a percentile difference (mean + SE) from the mean value of the control treatment. Horizontal hair-lines connect the treatments differing significantly according to the Tukey's a posteriori comparison of all pair-wise means. ° =  $p < 0.10$ , \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , and \*\*\* =  $P < 0.001$ .

$F_{1,27} = 0.01$ , NS; control vs *P. fontinalis* -contrast:  $F_{1,27} = 3.17$ ,  $P = 0.09$ ). The hair-tufts or the surface of the thallus in control, washing and *P. fontinalis* treatments did not differ from each other in our visual inspection by scanning electron microscopy (Fig. 5a).

## Discussion

Simulations of snail grazing activity by nutrient addition and removal of epibiota and particulate matter from the thallus, both alone and in combination, effectively induced responses in growth and/or phlorotannin production of *F. vesiculosus*. This clearly shows that manipulations of resource availability alone are capable of generating significant changes in growth and production of secondary metabolites, and highlights the im-

portance to separate these effects from adaptive induced defenses against herbivory.

The grazing action of *T. fluviatilis* on the thallus induced an increase in phlorotannin production. This response clearly differed from the effects of all the other treatments which either did not affect or decreased phlorotannin production. The response presupposed physical contact of *T. fluviatilis* with the thallus as the sole presence of snails in aquarium had no effect on either growth or phlorotannins. Can this response be interpreted as an induced defense by *F. vesiculosus* against snail grazing or could it rather be explained as a consequence of resource manipulation by the snail?

Manipulation of resources is involved in the interaction as *T. fluviatilis* efficiently removes epibiota from the thallus. However, although the availability of carbon increased, growth was not consequently stimulated as in treatments including cleaning the thallus. Instead, this could be interpreted as a cost of carbon allocation to production of secondary metabolites. This may seem to fulfill two of the prerequisites for a trait to represent an induced defense: a specific and costly response to the presence of a herbivore (Rhoades 1979). That *T. fluviatilis* does not feed on, or damage the surface of the photosynthetic thallus of *F. vesiculosus*, contradicts such interpretation because the presence of this species on thalli does not indicate increased risk of becoming grazed. Furthermore, *T. fluviatilis* is very abundant in our study area being practically always present on *Fucus* during the active growing period. High abundance and predictability of the herbivore species should, in general favor rather constitutive than induced defenses (Rhoades 1979, Steinberg 1994). Thus, we find it difficult to interpret the increased phlorotannin production of *F. vesiculosus* as a defense against *T. fluviatilis*.

The very specific way of resource manipulation by *T. fluviatilis* provides an alternative explanation. The grazing action of this snail may simultaneously both increase light availability by removal of epibiota and decrease nutrient uptake through removal of the hyaline hairs that has been shown to function in phosphate uptake (Hurd et al. 1993). Because hyaline hairs occur throughout Phaeophyceae (Van den Hoek et al. 1995), we suspect that the importance of such hairs could possibly be generalized to several other alga-herbivore interactions. The length of the tufts of hyaline hairs in the treatment with *T. fluviatilis* was only about half of that of the control, which may indicate a consecutive decrease in nutrient uptake. Then, nutrients may limit growth and the increase of secondary metabolites could be interpreted as a consequence of increased photosynthesis. Phlorotannins are located in sub-cellular bodies, physodes, that are especially abundant in epidermal, outer cortical and meristematic cells; they are not known to exist in hyaline hairs (Ragan and Glombitza 1986). Therefore, the observed increase of phloro-

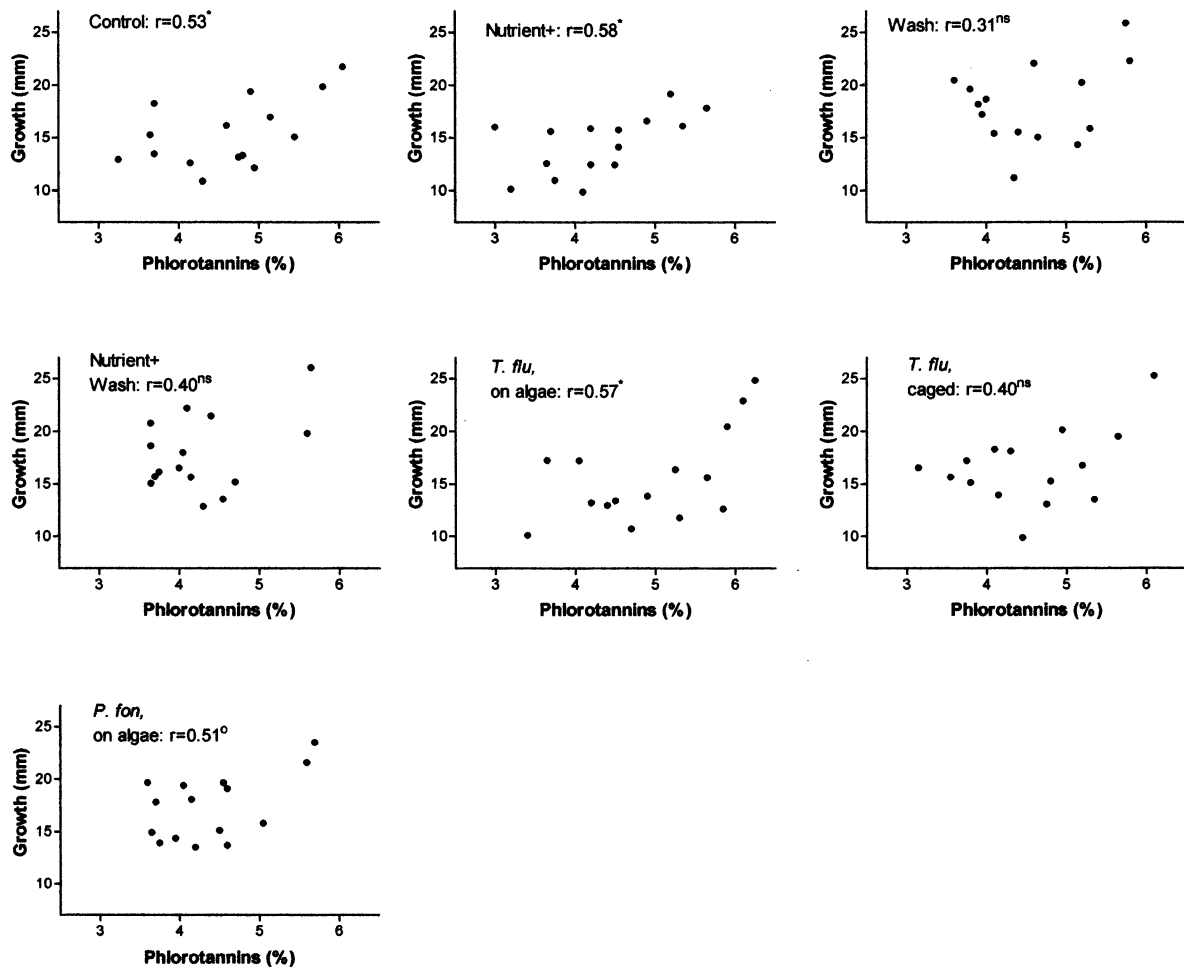


Fig. 4. Genotypic correlations between phlorotannin concentration and growth (in terms of length increment), separately for individual treatments.

tannins will probably not concern hyaline hairs, hence hardly being capable of functioning as a defense against hair removal by *T. fluviatilis*. Washing the thallus by a brush removed epibiota but did not harm the hyaline hairs and, consequently, led to increased growth. Increased growth, in turn, by forming a sink of carbon led to a trade-off with the amount of secondary metabolites.

Interestingly, the effect of *P. fontinalis* on *F. vesiculosus* differed from that of *T. fluviatilis*. Growth increased but there was no effect on phlorotannins. As such, the consequences of *P. fontinalis* grazing on the thallus were similar to the washing treatment, implying that cleaning the thallus from epibiota and particulate matter is the major mechanism for how this snail species affects *F. vesiculosus*. As a consequence of increased light availability and, in addition, possibly enhanced nutrient conditions there is a positive effect on the growth of *F. vesiculosus*. This growth, however, neither affected induction of phlorotannin production nor cor-

relative changes in phlorotannin production. Also, the different response of *F. vesiculosus* to *P. fontinalis* and *T. fluviatilis* shows that there is no such general cue as snail grazing triggering phlorotannin production, which could easily be interpreted as an anti-herbivore adaptation.

Nutrient addition decreased phlorotannin production, which is consistent with the predictions from the resource-based CNB and GDB -hypotheses on allocation to products of secondary metabolism. However, these hypotheses assume that the allocation of carbon shifts from the production of secondary metabolites to growth in conditions of good nutrient availability (reviewed by Herms and Mattson 1992). This was not true in our case where growth was deprived rather than increased. Instead, epibiota played a crucial role here: nutrient enhancement tripled the amount of epibiota on thallus. Thus, nutrient enhancement led to decreased carbon availability due to shading by epibiota, which in turn both slowed growth rate and decreased production

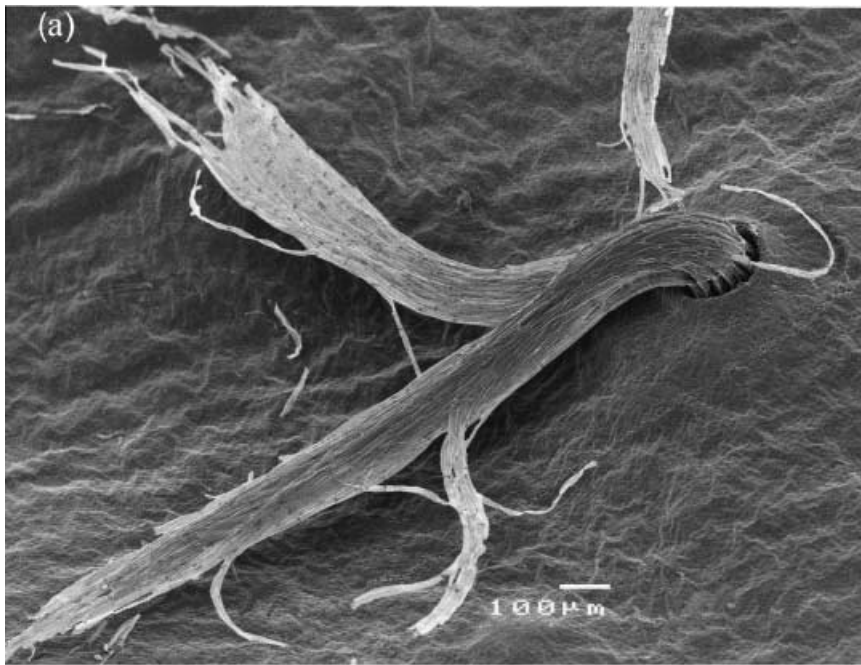
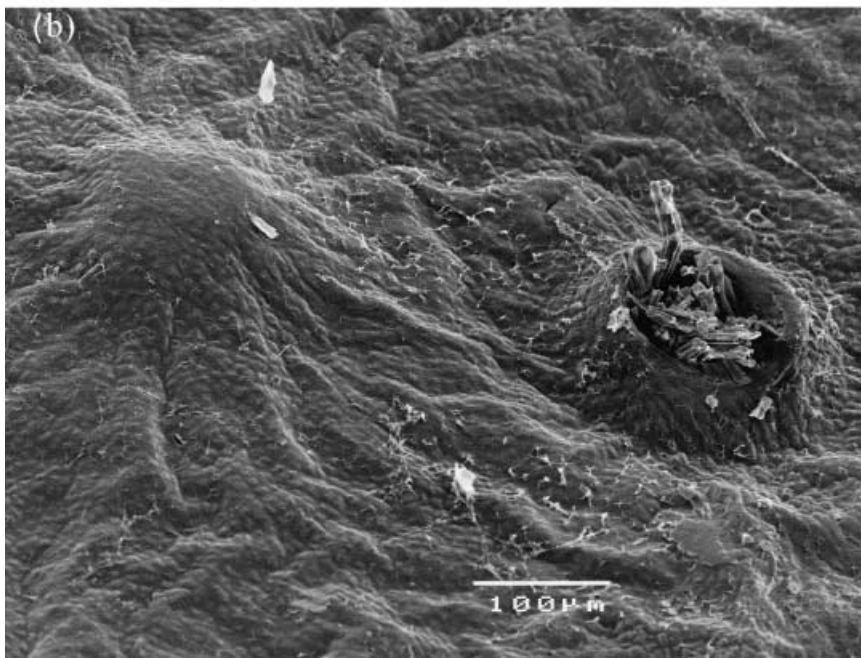


Fig. 5. Typical tufts of hyaline hairs arising from cryptostomata on apical parts of thallus of *F. vesiculosus* as seen by scanning electron microscopy in the control treatment (a) and in the treatment with *T. fluviatilis* (b).



of secondary metabolites. Removal of epibiota by washing the thallus increased growth. Washing the thallus together with nutrient enhancement both increased growth and decreased phlorotannin production, which is consistent with the predictions from the resource-based hypotheses, which assume prioritization of growth when nutrient conditions are not limiting.

Phenotypic trade-off in allocation of carbon between growth and secondary metabolism was thus evident when neither nutrients nor light were limiting. How-

ever, all genotypes changed their allocation pattern in a similar way as a response to the manipulations. Among the genotypes there was no trade-off between growth and phlorotannins; instead there was a positive relationship indicating that some genotypes were better able to both grow and produce phlorotannins. This may be related, for example, to genetic differences in photosynthetic efficiency or to genotypic variation in the amount of epibiota on algae. We are not aware of any earlier data on genetic trade-offs between growth

and secondary metabolism in brown algae, but phenotypic trade-offs have been found to be variable between populations (Yates and Peckol 1993, Pavia et al. 1999b) and in time (Steinberg 1995). The variability in the phenotypic trade-off implies that the costs of producing secondary metabolites vary according to the resource availability.

The variation of phlorotannins was mainly due to the genotype of algae. Excluding the fixed effects, genotypic variation comprised almost 70% of the variation. But most interestingly, genotypic variation (~65% of total variation) also highly exceeded the variance due to the manipulations (~7% of total variation). This clearly emphasizes the importance of genetic variation as compared to phenotypic plasticity of secondary metabolism. When genetic variation between individual algae in the production of secondary metabolites largely exceeds the range of induced changes, these should be inefficient in directing herbivory. The large genetic component of variation suggests that induced changes are likely to be poor defenses and therefore less likely to evolve in the context of plant-herbivore interaction. The high genetic variation of phlorotannins in *F. vesiculosus* does not preclude the possibility of induced defenses but it makes them less efficient because food choices are likely to be based on genotypic levels of defenses. The interpretation of plasticity of phlorotannins due to snail grazing being a consequence of variation in resource availability is by no means contradictory with the observed high level of genetic variation. Furthermore, this does not exclude the possibility that the plasticity of phlorotannins under varying resource availability represents adaptations to other selective factors (Close and McArthur 2002, Nitao et al. 2002).

*Acknowledgements* – We thank the Archipelago Research Institute of the Univ. of Turku for providing facilities and the working environment, and Outi Vesakoski and Jenni Pettay for help with running the experiment. The study was financed by the Academy of Finland (project 44086) and the Wihuri foundation (to T. Honkanen).

## References

Amsler, C. D. 2001. Induced defenses in macroalgae: the herbivore makes a difference. – *J. Phycol.* 37: 353–356.

Arnold, T. M., Tanner, C. E. and Hatch, W. I. 1995. Phenotypic variation in polyphenolic content of the tropical brown alga *Lobophora variegata* as a function of nitrogen availability. – *Mar. Ecol. Prog. Ser.* 123: 177–183.

Arnold, T. M., Targett, N. M., Tanner, C. E. et al. 2001. Evidence for methyl jasmonate-induced phlorotannin production in *Fucus vesiculosus* (Phaeophyceae). – *J. Phycol.* 37: 1026–1029.

Blanchard, G. F., Guarini, J. M., Provot, L. et al. 2000. Measurement of ingestion rate of *Hydrobia ulvae* (Pennant) on intertidal epipellic microalgae: the effect of mud snail density. – *J. Exp. Mar. Biol. Ecol.* 255: 247–260.

Boettcher, A. A. and Targett, N. M. 1993. Role of polyphenolic molecular size in reduction of assimilation efficiency in *Xiphister mucosus*. – *Ecology* 74: 891–903.

Bryant, J. P., Chapin III, F. S. and Klein, D. R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. – *Oikos* 40: 357–368.

Carlson, L. 1991. Seasonal variation in growth, reproduction and nitrogen content of *Fucus vesiculosus* L. in the Øresund, southern Sweden. – *Bot. Mar.* 34: 447–453.

Close, C. D. and McArthur, C. 2002. Rethinking the role of many plant phenolics – protection from photodamage not herbivores? – *Oikos* 99: 166–172.

Cronin, G. and Hay, M. E. 1996. Effects of light and nutrient availability on the growth, secondary chemistry, and resistance to herbivory of two brown seaweeds. – *Oikos* 77: 93–106.

Hamilton, J. G., Zangerl, A.R., DeLucia, E. H. et al. 2001. The carbon-nutrient balance hypothesis: its rise and fall. – *Ecol. Lett.* 4: 86–95.

Hammerström, K., Dethier, M. N. and Duggins, D. O. 1998. Rapid phlorotannin induction and relaxation in five Washington kelps. – *Mar. Ecol. Prog. Ser.* 165: 293–305.

Hermes, D. A. and Mattson, W. J. 1992. The dilemma of plants: to grow or defend? – *Q. Rev. Biol.* 67: 283–335.

Honkanen, T. and Jormalainen, V. 2002. Within-plant integration and compensation: effects of simulated herbivory on growth and reproduction of the brown alga, *Fucus vesiculosus*. – *Int. J. Plant Sci.* 163: 815–823.

Hurd, C. L., Galvin, R. S., Norton, T. A. et al. 1993. Production of hyaline hairs by intertidal species of *Fucus* (Fucales) and their role in phosphate uptake. – *J. Phycol.* 29: 160–165.

Hyman, L. H. 1967. The invertebrates: Mollusca I. – McGraw-Hill.

Iivessalo, H. and Tuomi, J. 1989. Nutrient availability and accumulation of phenolic compounds in the brown alga *Fucus vesiculosus*. – *Mar. Biol.* 101: 115–119.

Irelan, C. D. and Horn, M. H. 1991. Effects of macrophyte secondary chemicals on food choice and digestive efficiency of *Cebidichthys violaceus* (Girard), an herbivorous fish of temperate marine waters. – *J. Exp. Mar. Biol. Ecol.* 153: 179–194.

Jones, J. I., Young, J. O., Haynes, G. M. et al. 1999. Do submerged aquatic plants influence their periphyton mutualists? – *Oecologia* 120: 463–474.

Jormalainen, V. and Honkanen, T. 2001. Multiple cues for phenotypic plasticity in phlorotannin production of the bladder wrack *Fucus vesiculosus*. – *Phycologia* 40: 59–60.

Jormalainen, V., Honkanen, T. and Heikkilä, N. 2001. Feeding preferences and performance of a marine isopod on seaweed hosts – cost of habitat specialization. – *Mar. Ecol. Prog. Ser.* 220: 219–230.

Karban, R. and Baldwin, I. T. 1997. Induced responses to herbivory. – Univ. of Chicago Press.

Koricheva, J. 2002. The carbon-nutrient balance hypothesis is dead; long live the carbon-nutrient balance hypothesis? – *Oikos* 98: 537–539.

Koricheva, J., Larsson, S., Haukioja, E. et al. 1998. Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. – *Oikos* 83: 212–226.

Lau-Stanley, C. K. and Qian, P. Y. 1997. Phlorotannins and related compounds as larval settlement inhibitors of the tube-building polychaete *Hydroides elegans*. – *Mar. Ecol. Prog. Ser.* 159: 219–227.

Lerdau, M. and Coley, P. D. 2002. Benefits of the carbon-nutrient balance hypothesis. – *Oikos* 98: 534–536.

Littell, R. C., Milliken, G. A., Stroup, W. W. et al. 1996. SAS® System for Mixed Models. – SAS Institute Inc.

Lobban, C. S. and Harrison, P. J. 1994. Seaweed ecology and physiology. – Cambridge Univ. Press.

Malm, T., Engkvist, R. and Kautsky, L. 1999. Grazing effects of two freshwater snails on juvenile *Fucus vesiculosus* in the Baltic Sea. – *Mar. Ecol. Prog. Ser.* 188: 63–71.

- Martinez, E. A. 1996. Micropopulation differentiation in phenol content and susceptibility to herbivory in the Chilean kelp *Lessonia nigrescens* (Phaeophyta, Laminariales). – *Hydrobiologia* 327: 205–211.
- Nitao, J. K., Zangerl, A. R., Berenbaum, M. R. et al. 2002. CNB: requiescat in pace? – *Oikos* 98: 540–546.
- Nurmi, K., Ossipov, V., Haukioja, E. et al. 1996. Variation of the total phenolic content and individual low-molecular-weight phenolics in foliage of mountain birch trees (*Betula pubescens* ssp. *tortuosa*). – *J. Chem. Ecol.* 22: 2023–2040.
- Pavia, H. and Brock, E. 2000. Extrinsic factors influencing phlorotannin production in the brown alga *Ascophyllum nodosum*. – *Mar. Ecol. Prog. Ser.* 193: 285–294.
- Pavia, H. and Toth, G. 2000a. Inducible chemical resistance to herbivory in the brown seaweed *Ascophyllum nodosum*. – *Ecology* 81: 3212–3225.
- Pavia, H. and Toth, G. B. 2000b. Influence of light and nitrogen on the phlorotannin content of the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus*. – *Hydrobiologia* 440: 299–305.
- Pavia, H., Cervin, G., Lindgren, A. et al. 1997. Effects of UV-B radiation and simulated herbivory on phlorotannins in the brown alga *Ascophyllum nodosum*. – *Mar. Ecol. Prog. Ser.* 157: 139–146.
- Pavia, H., Carr, H. and Åberg, P. 1999a. Habitat and feeding preferences of crustacean mesoherbivores inhabiting the brown seaweed *Ascophyllum nodosum* (L.) Le Jol. and its epiphytic macroalgae. – *J. Exp. Mar. Biol. Ecol.* 236: 15–32.
- Pavia, H., Toth, G. and Åberg, P. 1999b. Trade-offs between phlorotannin production and annual growth in natural populations of the brown seaweed *Ascophyllum nodosum*. – *J. Ecol.* 87: 761–771.
- Peckol, P., Krane, J. M. and Yates, J. L. 1996. Interactive effects of inducible defence and resource availability on phlorotannins in the north Atlantic brown alga *Fucus vesiculosus*. – *Mar. Ecol. Prog. Ser.* 138: 209–217.
- Pereira, R. C. and Yoneshigue-Valentin, Y. 1999. The role of polyphenols from the tropical brown alga *Sargassum furcatum* on the feeding by amphipod herbivores. – *Bot. Mar.* 42: 441–448.
- Pettay, E. 2001. The responses of *Fucus*-inhabiting animal species to nutrient enrichment caused by fish farming. 2001. M. Sc.-thesis, Univ. of Turku, Dept of Biology, Turku.
- Ragan, M. A. and Glombitza, K. W. 1986. Phlorotannins, brown algal polyphenols. – In: Round, F. E. and Chapman, D. J. (eds), *Progr. Phycol. Res.* Vol. 4. Biopress, pp. 129–241.
- Rhoades, D. F. 1979. Evolution of plant chemical defense against herbivores. – In: Rosenthal, D. A. and Janzen, D. H. (eds), *Herbivores: their interaction with secondary plant metabolites*. Academic Press, pp. 3–54.
- Schoenwaelder, M. E. A. 2002. The occurrence and cellular significance of physodes in brown algae. – *Phycologia* 41: 125–139.
- Schoenwaelder, M. E. A. and Clayton, M. N. 1998. Secretion of phenolic substances into the zygote wall and cell plate in embryos of *Hormosira* and *Acrocarpis* (Fucales, Phaeophyceae). – *J. Phycol.* 34: 969–980.
- Schwaegerle, K.E., McIntyre, H. and Swingley, C. 2000. Quantitative genetics and the persistence of environmental effects in clonally propagated organisms. – *Evolution* 54: 452–461.
- Skoog, G. 1978. Influence of natural food items on growth and egg production in brackish water populations of *Lymnea peregra* and *Theodoxus fluviatilis* (Mollusca). – *Oikos* 31: 340–348.
- Steinberg, P. D. 1985. Feeding preferences of *Tegula funebralis* and chemical defenses of marine brown algae. – *Ecol. Monogr.* 55: 333–349.
- Steinberg, P. D. 1994. Lack of short-term induction of phlorotannins in the Australasian brown algae *Ecklonia radiata* and *Sargassum vestitum*. – *Mar. Ecol. Prog. Ser.* 112: 129–133.
- Steinberg, P. D. 1995. Seasonal variation in the relationship between growth rate and phlorotannin production in the kelp *Ecklonia radiata*. – *Oecologia* 102: 169–173.
- Steinberg, P. D. and Van Alstene, I. A. 1992. Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. – *Ecol. Monogr.* 62: 189–222.
- Steinberg, P. D., Estes, J. A. and Winter, F. C. 1995. Evolutionary consequences of food chain length in kelp forest communities. – *Proc. Natl Acad. Sci. USA* 92: 8145–8148.
- Stern, J. L., Hagerman, A. E., Steinberg, P. D. and Mason, P. K. 1996. Phlorotannin-protein interactions. – *J. Chem. Ecol.* 22: 1877–1899.
- Targett, N. M. and Arnold, T. M. 1998. Predicting the effects of brown algal phlorotannins on marine herbivores in tropical and temperate oceans. – *J. Phycol.* 34: 195–205.
- Targett, N. M., Boettcher, A. A., Targett, T. E. and Vrolijk, N. H. 1995. Tropical marine herbivore assimilation of phenolic rich plants. – *Oecologia* 103: 170–179.
- Toth, G. B. and Pavia, H. 2000. Water-borne cues induce chemical defense in a marine alga (*Ascophyllum nodosum*). – *Proc. Natl Acad. Sci. USA* 97: 14418–14420.
- Toth, G. B. and Pavia, H. 2002. Lack of phlorotannin induction in the kelp *Laminaria hyperborea* in response to grazing by two gastropod herbivores. – *Mar. Biol.* 140: 403–409.
- Tuomi, J., Niemelä, P., Chapin III, F. S. et al. 1988. Defensive responses of trees in relation to their carbon/nutrient balance. – In: Mattson, W. J., Levieux, J. and Bernard-Dagan, C. (eds), *Mechanisms of woody plant defenses against insects: search for pattern*. Springer, pp. 57–72.
- Van Alstene, K. L. 1988. Herbivory grazing increases polyphenolic defenses in the intertidal brown alga *Fucus distichus*. – *Ecology* 69: 655–663.
- Van Alstene, K. L. 1995. The comparison of three methods for quantifying brown algal polyphenolic compounds. – *J. Chem. Ecol.* 21: 45–58.
- Van Alstene, K. L. and Paul, V. J. 1990. The biogeography of polyphenolic compounds in marine macroalgae: temperate brown algal defenses deter feeding by tropical herbivorous fishes. – *Oecologia* 84: 158–163.
- Van Alstene, K. L. and Pelletreau, K. N. 2000. Effects of nutrient enrichment on growth and phlorotannin production in *Fucus gardneri* embryos. – *Mar. Ecol. Prog. Ser.* 206: 33–43.
- Van Alstene, K. L., Whitman, S. L. and Ehlig, J. M. 2001. Differences in herbivore preferences, phlorotannin production, and nutritional quality between juvenile and adult tissues from marine brown algae. – *Mar. Biol.* 139: 201–210.
- Van den Hoek, C., Mann, D. G. and Jahns, H. M. 1995. *Algae – an introduction to phycology*. – Cambridge Univ. Press.
- Yates, J. C. and Peckol, P. 1993. Effects of nutrient availability and herbivory on polyphenolics in the seaweed *Fucus vesiculosus*. – *Ecology* 74: 1757–1766.