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PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Fouling mediates grazing: intertwining of resistances to multiple enemies in the brown alga *Fucus vesiculosus*

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Abstract Macroalgae have to cope with multiple natural enemies, such as herbivores and epibionts. As these are harmful for the host, the host is expected to show resistance to them. Evolution of resistance is complicated by the interactions among the enemies and the genetic correlations among resistances to different enemies. Here, we explored genetic variation in resistance to epibiosis and herbivory in the brown alga *Fucus vesiculosus*, both under conditions where the enemies coexisted and where they were isolated. *F. vesiculosus* showed substantial genetic variation in the resistance to both epibiosis and grazing. Grazing pressure on the alga was generally lower in the presence than in the absence of epibiota. Furthermore, epibiosis modified the susceptibility of different algal genotypes to grazing. Resistances to epibiosis and grazing were independent when measured separately for both enemies but positively correlated when both these enemies coexisted. Thus, when the enemies coexisted, the fate of genotypes with respect to these enemies was intertwined. Genotypic correlation between phlorotannins, brown-algal phenolic secondary metabolites, and the amount of epibiota was negative, indicating that these compounds contribute to resistance to epibiosis. In addition, phlorotannins correlated also with the resistance to grazing, but this correlation disappeared when grazing occurred in the absence of epibiota. This indicates that the patterns of selection for the type of the resistance as well as for the resistance traits vary with the occurrence patterns of the enemies.

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Introduction

Plants have to cope with multiple natural enemies, such as herbivores, pathogens and, especially in aquatic communities, epibionts. To accomplish this, plants may evolve either a generalized defense, meaning that the same defensive trait, often a chemical one, is efficient against different enemies, or a specialized defense for each enemy (Krischik et al. 1991; Biere et al. 2004). Depending on the type of enemies and the interactions among them, selection for resistance is expected to lead to varying patterns of genetic correlations among resistances to different enemies; these often show positive associations, although may show no association at all or be negative (Leimu and Koricheva 2006). Positive genetic correlations between resistances to different enemies are particularly expected when the enemies harm the host in a similar manner—for example, between resistances to herbivores having similar phenology and feeding mechanism (Fritz 1992). Weaker correlations are expected when the enemies are very different, e.g., pathogens and herbivores, and negative correlations indicate a trade-off in resistances to different enemies (Leimu and Koricheva 2006).

Macroalgae are susceptible to multiple natural enemies, including herbivores and epibionts (organisms living on the algal surface). Herbivory in macroalgal communities is

generally intense (Cyr and Pace 1993) and its roles both in community regulation (Hay 1997; Duffy and Hay 2000; Lotze et al. 2001; reviewed by Shurin et al. 2002) and as an evolutionary agent in selection for plant defenses (Hay and Fenical 1988; Cronin 2001) are well known. The ecological and evolutionary consequences of epibiosis for macroalgae are far less studied, despite epibiosis being a ubiquitous phenomenon in aquatic communities (Davis et al. 1989; Steinberg and de Nys 2002).

Epibiosis is considered mainly harmful for the host alga (the basibiont). Epibiota compete with the basibiont for light and nutrients, thereby decreasing growth and reproduction (D'Antonio 1985; Cebrian et al. 1999); raise mortality by increasing drag (D'Antonio 1985); and may attract grazers, thereby increasing tissue loss (Bernstein and Jung 1979; Wahl and Hay 1995; Wahl et al. 1997). Such negative effects on basibiont fitness can be expected to select for efficient anti-fouling defenses. However, we still lack a comprehensive view of the strength of selection for anti-fouling defenses (Steinberg et al. 2001). Demonstrations of the effects of epibiota on the host fitness are still rare and there is very little data on intraspecific variation of fouling resistance or on selection for resistance (Honkanen and Jormalainen 2005), although these form the basis for evolutionary responses of the anti-fouling traits.

Ecological interactions among natural enemies affect whether the selective impacts of each enemy are independent or correlated with each other (Rausher 1996; Strauss et al. 2005). A plant is expected to respond to selection by an enemy in a pairwise, straightforward manner if the pattern of selection imposed by one enemy is independent of the presence of the other and if the resistances to enemies are not genetically correlated; otherwise, the evolutionary response of the plant is diffuse depending on the abundance of the enemies and the genetic association of the resistances (Rausher 1996). When enemies coexist on the same hosts, generalized defenses may be selected for. In the case of epibionts and herbivores, epibiosis has been found to encourage herbivory on the basibiont—so called "shared doom" effect (Wahl and Hay 1995). This could be expected to generate positive genetic correlations between the resistance to epibiosis and the resistance to herbivory. Although both the "shared doom" and its opposite "associational resistance" have been documented in host–epibiota–enemy interactions (Bernstein and Jung 1979; Wahl and Hay 1995; Littler et al. 1995; Wahl et al. 1997; Karez et al. 2000), we are not aware of any study that has explored the relationship of the host resistance to epibiosis with that to another enemy.

Resistance is commonly measured as the reversal of the damage due to an enemy or as the reversal of the abundance of the enemy (Leimu and Koricheva 2006). Genetic correlations among resistances to different enemies, measured in this way, are commonly positive (Rausher 1996; Leimu and Koricheva 2006). However, such measures do not tell on which traits the resistance is based or whether the resistance traits are the same for the different enemies. Therefore, in order to understand the evolution of defenses against multiple enemies, there is a need for a more trait-oriented approach (Strauss et al. 2005; Leimu and Koricheva 2006).

Phlorotannins are brown-algal phenolic secondary metabolites, which probably have multiple ecological functions (Schoenwaelder 2002; Arnold and Targett 2003; reviewed by Amsler and Fairhead 2006). Most often phlorotannins have been associated with resistance to herbivory (reviewed by Targett and Arnold 1998; Pavia and Toth 2000a; Jormalainen et al. 2005). In addition, phlorotannins have been suggested to act as anti-foulants because they have been found to inhibit settlement of bacteria (Sieburt and Conover 1965; Nagayama et al. 2002), algal spores (Jennings and Steinberg 1997) and references therein (Nagayama et al. 2003), and protists (Langlois 1976). Recent studies have found that even small concentrations of phlorotannin can deter settlement of invertebrate larvae (Lau-Stanley and Qian 1997; Lau and Qian 2000; Wikström and Pavia 2004). However, the capability of phlorotannins to deter epibionts has been questioned because, as highly polar compounds, they are unlikely to adhere to the surface of the plant (Jennings and Steinberg 1997). However, phlorotannins are exuded from the thallus (Koivikko et al. 2005) and these exudates may have deterrent properties.

In this study, we explored the genetic variation in resistance of the brown alga *Fucus vesiculosus* L. to herbivory and epibiosis. We hypothesized that resistances to these two enemies are independent or negatively correlated when only one enemy is present but, owing to the interaction of the enemies, positively correlated when the enemies coexist. We manipulated experimentally both the amount of epibiota and grazing on algae, and, using clonal material, determined the genetic correlations between the resistances to fouling and herbivory. Resistances were defined as the inverse of the amount or probability of grazing and as the reverse of the biomass of epibiota on algae. Because phlorotannins have been suggested to have a role in both of these interactions, we further quantified the genotypic variation in phlorotannins and explored their relationship with the resistance to grazing and epibiosis.

Materials and methods

Experimental design

We sampled 30 *F. vesiculosus* genotypes from a large population covering several islands and skerries (60°08′, 22°17′) on 11 April 2005, and transferred them to the

Archipelago Research Institute (University of Turku) on Seili Island, where we conducted the experiment. Genotypes were defined as entities growing from a single branch of thallus on a holdfast. We divided each genotype into 12 apical pieces; each part was about 4–6 cm in length and consisted of one or two dichotomous branching points. We conducted the experiment in 12 through-flow (12 l h^{-1}) mesocosms (60 l volume) that were located outdoors under a natural-light and sea-water temperature (1–15°C) regime, with a water pump to provide water movement. Mesocosms were protected from rainfall by a thin plastic cover. We randomized the algal pieces among the mesocosms; one piece of each genotype was anchored to the bottom of each mesocosm. We transferred all pieces into the mesocosms for 3 weeks before the start of the experiment. The rationale for this pre-experiment period in mesocosms was to acclimatize the genotypes to this common environment, thereby diminishing the possible carry-over environmental effects of the clonal "mother". Such carry-over effects may for instance include variable nutrient resources in the thallus (Honkanen and Jormalainen 2002; Jormalainen and Honkanen 2004), which we expected to even out when reared in a common environment. When the analysis of genetic effects is based on cloned genotypes, conclusions concern total genetic variation, not just additive genetic variance, and hold under the assumption of minimal maternal or "carry-over" effects from the clonal parent (Schwaegerle et al. 2000).

In the experiment, we manipulated the amount of epibiota and the occurrence of herbivory. For the first 3 weeks of the experiment, we slightly enriched the nutrient concentration of the water by adding 50 g controlled release N–P–K fertilizer (Osmocote 3–4 M) to every mesocosm. This promoted quick development of fouling epibiota. Epibiota consisted mainly of periphyton, dominated by diatoms, but also included some filamentous algae (mainly *Pilayella littoralis*, L.). These groups usually dominate the epibiotic community on *F. vesiculosus* in the natural environment. The manipulation of epibiota included two levels: (1) natural fouling load accumulating on algae in the mesocosms (hereafter "Fouling") and (2) removal of fouling load by washing the algae (hereafter "Clean-up"). The washing was conducted by gently brushing each piece with a soft brush, underwater, at 2- to 3-day intervals (three times a week). The manipulation of epibiota lasted for the 6-week duration of the experiment. Manipulation of herbivory included two levels: (1) no herbivores present and (2) *Idotea baltica* (Pallas) present for the last 10 days of the experiment. The epibiosis treatment was started prior to the start of the herbivory treatment, because the development of the epibiotic community takes time and we wanted to measure resistance to grazing under both conditions—with and without epibiota. We initiated the herbivory treatment by adding 50 *I. baltica* to each mesocosm, but gradually increased their amount to 85 in order to ensure a measurable amount of grazing. Thus, the experimental design was a two-by-two factorial, with epibiosis and herbivory as fixed factors. In addition, we had the genotype as a random factor (as described above). We had a total of three replicate mesocosms in each fouling-by-herbivory treatment combination.

At the beginning of the experiment, we measured the weight [wet weight (WW) after drying between a tissue] and length (as an average of all straight lines from the midrib of the basal intersection to each apex) of the pieces, and counted the number of apical meristems. At the end of the experiment, we performed the same measurements and, in addition, measured the fouling load of the pieces and analyzed the amount of grazing from the treatment group with isopods present. The pieces of algae were frozen prior to quantification of phlorotannins. We measured the fouling load by washing each piece in filtered seawater, scraping off all periphyton and filamentous algae carefully from the thallus, filtering the water on pre-weighed micro-filters (Millipore) and dry-weighing the filter. We calculated fouling load as the dry-biomass of epibiota per unit dry-biomass of *F. vesiculosus*. We calculated the final dry-weight (DW) from the final WW using the regression equation DW = 0.026 + 0.180FW ($df = 150$, $R^2 = 92\%$) derived from the sub-sample of the algal pieces that were dry-weighed for the chemical analyses. We determined the amount of grazing from digital photographs, taken at the beginning of the herbivory treatment and at the end of the experiment. Grazing was measured as the total area of all grazing marks, using an image-analysis program. Phlorotannins were quantified (in % of algal DW) from each whole piece of lyophilized and powdered algae by means of the Folin–Ciocalteau technique (described in detail in Koivikko et al. 2005), using phloroglucinol as the standard agent.

Statistical analysis

We analyzed fouling load, growth (in terms of a change both in biomass and length), and phlorotannin concentration using mixed-model ANOVAs (run by SAS, Procedure Mixed; SAS Institute 1999), where fouling and herbivory treatments were treated as fixed factors, and the genotype and mesocosm as random factors. The procedure uses restricted maximum likelihood estimation for the random factors. All possible interactions between genotype and fixed factors were initially included in the model, but the model was simplified on the basis of the Akaike's Information Criterion (AIC) values (Littell et al. 2006), i.e., the interaction was removed if it was non-significant and its inclusion did not improve the model fit. The significance testing of the fixed factors was based on *F* statistics and Kenward–Roger adjusted degrees of freedom. The signifi-

cance of random factors was tested using the deviance of the two models with that factor included versus that excluded from the model, and comparing that to one-tailed χ^2-tables (Littell et al. 2006).

The design of the experiment did not allow the estimation of the interaction between the two random factors, genotype and mesocosm. At that level, the design was analogous to an "unreplicated randomized block" design, in which the tests of treatment and block effects are justified only under assumption of no interaction of these. Although we have no reason to expect the genotype-by-mesocosm interaction, we cannot rule out such a possibility. If such interaction exists, it is included in the residual variation and may bias the estimation of the genotypic variance component.

Assuming no genotype-by-mesocosm interaction, the genetic variances in fouling load, growth, and phlorotannin concentration were highly significant. We estimated the genotypic values by calculating the "best linear unbiased predictors" and their standard errors; these are considered as the best estimates for the random effects (Littell et al. 2006). For fouling load, these estimates were calculated for the Fouling group with no herbivory, because herbivores affected the amount of epibiota. For phlorotannins, estimates were calculated for the Clean-up group, either over both the herbivory treatment levels or for the No-herbivory group only, depending on the context (see "Results"). When measuring growth (in terms of length), we only included non-grazed apices in the length measures; therefore, growth measures are not directly affected by grazing and we estimated the genotypic values for growth over both the herbivory levels. Again, depending on the context, we used estimates calculated for either the Clean-up or Fouling treatment levels. Genotypic estimates were used to explore the genetic covariation among the traits above by Pearson correlations. For illustrative purposes in the bivariate plots, we calculated the best fitting line describing the genetic correlations between fouling, growth and phlorotannins using the Deming model II regression that takes into account the different error variance of the two variables (implemented by the Graph Pad Prism software, Motulsky and Christopoulos 2003).

The actual amounts of loss by grazing were relatively small, and many pieces not grazed at all, which resulted in non-normal distribution of the grazing loss. Therefore, we analyzed grazing as a binomially distributed response (grazed, not grazed), fouling and genotype as grouping factors, using a generalized linear model (run by SAS, Procedure Genmod). This procedure estimates the probability of grazing using maximum likelihood estimation and logit-link function, and tests the significance of factors by the difference in deviance of the two models, one including and the other excluding the factor. The model fit was assessed by the close to one ratio of deviance to the degrees of freedom (Dobson 2003). To visualize the genetic variation in grazing resistance, we calculated the estimates for grazing loss (in terms of area loss) for each fouling treatment-by-genotype combination. The genotypic estimates for grazing loss correlated well with the probability of grazing (cleaning group: $r = 0.70$, $P < 0.0001$, $n = 30$), so these two different methods for estimating grazing gave parallel results.

One potential problem in the analysis of grazing is that its measurement is not completely independent across genotypes, since grazing on one genotype may affect that on other genotypes within a mesocosm. This problem is inherent to the method, since feeding preferences (in our case, the probability of grazing) cannot be measured without offering alternatives. Consequently, there might be a negative correlation between genotypes, which generally will lead to an increased probability of type-I error (Underwood 1997, p. 179). We do not, however, consider this a serious problem here because the grazing situation has been replicated in mesocosms and the type-I error level of the genetic difference in the probability of grazing is lower than 0.001 (in the Clean-up group, see "Results").

We used logistic regression to explore how the probability of grazing was affected by fouling load and phlorotannin concentrations (run by SAS, Procedure Logistic). In these analyses, we used genotypic estimates for phlorotannins and fouling load, and thus the logistic regressions represent relationships at the genetic level. We checked the fit of the model by the Hosmer and Lemeshow test (Quinn and Keough 2002).

Results

The fouling load was minimal in the Clean-up group and large in the Fouling group (Fig. 1a, Table 1). Similarly, herbivores removed most fouling organisms, decreasing the difference between the fouling treatment levels, as indicated by the significant fouling-by-herbivory interaction (Fig. 1a, Table 1). The genotype-by-fouling interaction was significant, arising mainly from the difference in genotypic variance between the levels of the fouling treatment (Fig. 1b, Table 1). However, when tested separately within the fouling treatment levels, variance among genotypes in fouling load was statistically significant in both the Clean-up [$s^2 = 0.09 \pm 0.04$ (estimate $\pm$ SE), $\chi^2 = 12.4$, $P < 0.001$, 23% of phenotypic variation] and Fouling ($s^2 = 14.5 \pm 6.65$, $\chi^2 = 11$, $P < 0.001$, 19% of phenotypic variation) groups. The genotypic fouling loads in the Clean-up and Fouling groups correlated positively ($r = 0.94$, $P < 0.0001$, $n = 30$), implying that the rank order of genotypes with respect to the fouling load remained similar under both conditions.

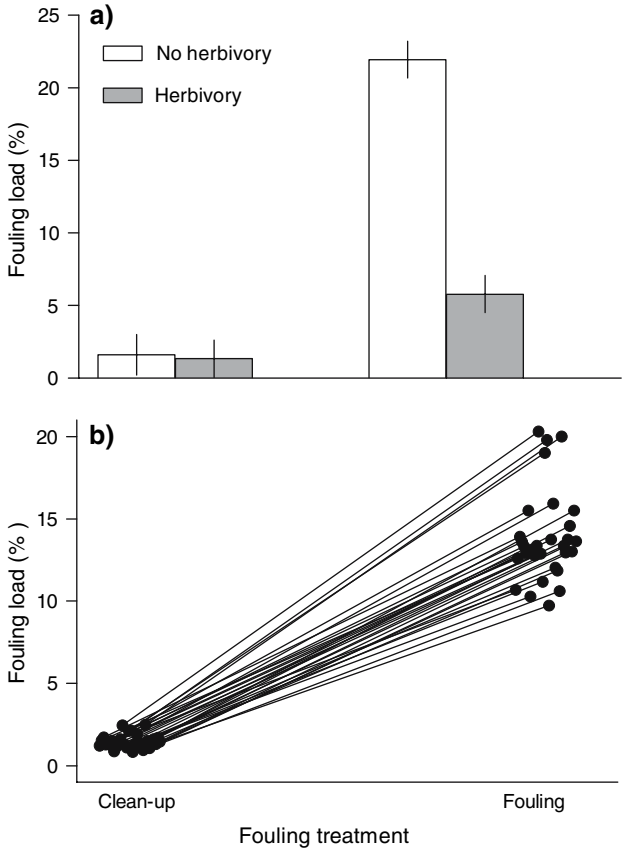

Fig. 1 Fouling load (dry-mass of epibiota as a percentage of the dry-mass of the basibiont) on *F. vesiculosus* with different fouling and herbivory treatments (**a**) and the genetic variation in fouling load in Clean-up and Fouling groups (**b**). *Lines* combine estimates for each genotype in the two environments, calculated for both herbivory treatments

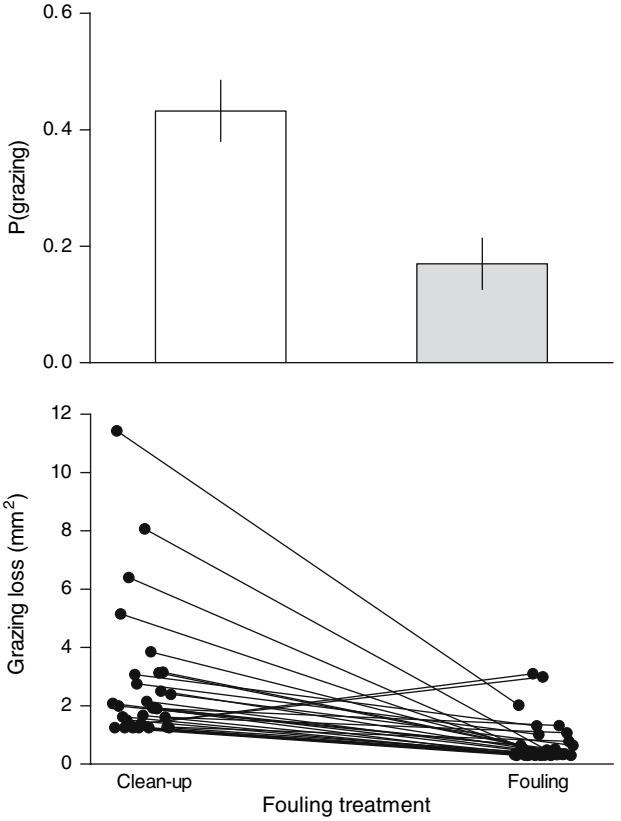

Fig. 2 The probability of grazing (grazed or not grazed; for calculation, see text) and the genetic variation in grazing loss (measured as the area of grazing marks) in *F. vesiculosus* separately for the two fouling treatments. *Lines* combine estimates for each genotype in the two environments, calculated for the Herbivory group only

Table 1 Mixed-model ANOVA tests of the effects of fouling manipulation and presence of herbivores on fouling load on algae, and of the variation among genotypes in fouling load. Data are summarized in Fig. 1

Source of variation	Variance estimates			
Random effects	s^2	SE	χ^2	*P*
Mesocosm (fouling × herbivory)	3.00	2.09	9.6	<0.01
Genotype	1.38	2.70	0.3	NS
Genotype × fouling	5.97	3.31	6.8	<0.01
Residual	34.3	3.00		
	Tests of fixed effects			
Fixed effects	*ndf*, *ddf*	*F*	*P*	
Fouling	1, 12.6	82.9	<0.0001	
Herbivory	1, 8.54	46.5	<0.0001	
Fouling × herbivory	1, 8.54	43.4	<0.001	

The probability of grazing was higher in the group of Clean-up algae than in those with fouling cover (Fig. 2a; Fouling treatment, $df = 1$, $\chi^2 = 14.6$, $P < 0.001$). The genotype-by-fouling interaction was significant ($\chi^2 = 47.1$, $df = 29$, $P < 0.05$), indicating both that the genotypic variance in the probability of grazing depended on the fouling load and that the ranks of the genotypes with respect to resistance to grazing changed between the fouling treatment levels (Fig. 2b). Genotypic variance in the probability of grazing was high and statistically significant in the Clean-up group ($\chi^2 = 60.3$, $df = 29$, $P < 0.001$) and lower, just above the significance level, in the fouling group ($\chi^2 = 42.2$, $df = 29$, $P = 0.054$). The same pattern was found when we analyzed grazing in terms of area loss: the genetic variance component was about 31% in the absence of epibiota and only about 7% in the presence of epibiota (Fig. 2b). The genotypic grazing loss in the Clean-up and Fouling groups was not correlated ($r = 0.14$, ns, $n = 30$), implying that the rank order of genotypes with respect to grazing loss changed between the environments. Thus, there was genetic variation in the resistance to grazing, the rank of the genotypes depended on the fouling load, and under fouled conditions the genetic variation evened out to a large extent.

Both the growth and phlorotannin concentration responded to the fouling treatment by decreasing under the fouling load (Fig. 3, Table 2). The herbivory treatment had

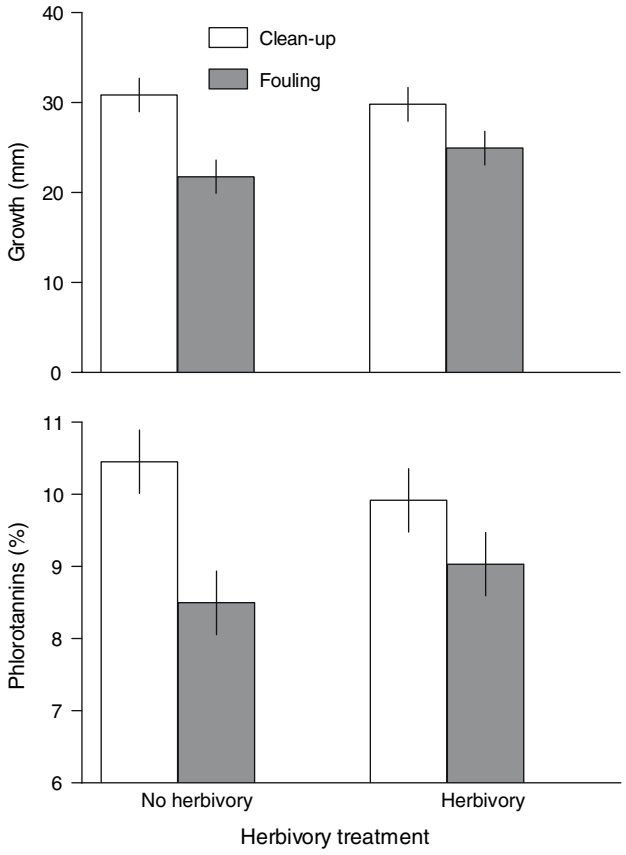

Fig. 3 Growth rate (in terms of length increment during the experiment) and phlorotannin concentration (in percentages of dry-weight) of *F. vesiculosus* in different fouling and herbivory treatment levels. Growth is adjusted for the covariate initial length. For statistical analyses, see Table 2

no effect on growth or phlorotannins (Fig. 3, Table 2). We measured growth in terms of both a change in biomass and in length; as both measurements showed very similar patterns with respect to the treatments, we give the summary statistics only for growth in length (Fig. 3a). Genetic variation in both growth and phlorotannin concentration was statistically highly significant (Table 2). In addition, there was a significant genotype-by-fouling treatment interaction in the weight increase, implying some genetic variance in the tolerance to fouling.

Genotypic estimates of fouling load and growth correlated negatively (Fig. 4). The same negative genetic correlation between growth in length and fouling load remains regardless of whether the growth is measured under fouling load (as in the Fig. 4) or Clean-up conditions, because there is no genotype-by-fouling treatment interaction (Table 2) and the ranks of the genotypic estimates therefore remain the same in both groups. Also growth in weight (estimates calculated for the no-herbivory group only, because herbivory decreases weight) was negatively correlated with fouling load (Clean-up: $r = -0.38$, $P < 0.05$; Fouling: $r = -0.35$, $P = 0.06$). Growth rate and phlorotannin concentration covaried positively at the genotypic level (Fig. 5), suggesting that there was no cost of phlorotannin production in terms of growth.

The genetic correlation between phlorotannin concentration and fouling load was negative (Fig. 6a). The relationship between the probability of grazing and the genotypic phlorotannin concentration depended on whether grazing took place on fouled or on clean algae (Fig. 6b; logit-model of the probability of grazing: fouling treatment-by-phlorotannin concentration interaction: $\chi^2 = 10.1$, $df = 1$, $P < 0.01$, model $R^2 = 20.7\%$). In the Clean-up group, the probability of grazing increased with the increasing phlorotannin concentration (Fig. 6b, $\beta = 0.60 \pm 0.21$, $\chi^2 = 8.3$, $df = 1$, $P < 0.01$, $R^2 = 14\%$). In the Fouling group, there was instead a tendency for the probability of grazing to decrease

Table 2 Mixed-model ANOVA tests of the effects of fouling manipulation and presence of herbivores on growth and phlorotannin concentration of algae, and of the variation among genotypes. When the interactions of genotype with the fixed factors were non-significant, they were excluded from the model. In the analysis of growth, the initial size (either in weight or length) was used as a covariate. Data for growth in weight, however, is not shown; for other variables is summarized in Fig. 2

Source of variation	Variance estimates growth (in weight)				Variance estimates growth (in length)				Variance estimates phlorotannin concentration			
Random effects	$s^2 \times 10^2$	SE $\times 10^2$	χ^2	P	S^2	SE	χ^2	P	s^2	SE	χ^2	P
Mesocosm (fouling × herbivory)	5.52	2.96	71.8	<0.0001	7.44	4.11	49.6	<0.0001	0.359	0.227	16.5	<0.0001
Genotype	6.23	2.25	12.8	<0.001	21.8	6.19	165	<0.0001	1.26	0.394	75.3	<0.0001
Genotype × fouling	2.20	1.12	8.7	<0.01	–	–			–	–		
Residual	11.8	0.993			20.1	1.66			2.77	0.222		
Fixed effects	*ndf*, *ddf*	F	P		*ndf*, *ddf*	F	P		*ndf*, *ddf*	F	P	
Fouling	1, 9.19	13.4	<0.01		1, 7.93	17.8	<0.01		1, 8.01	13.3	<0.01	
Herbivory	1, 8.01	0.03	NS		1, 7.92	0.43	NS		1, 8.01	0.00	NS	
Fouling × herbivory	1, 8.02	1.27	NS		1, 7.92	1.63	NS		1, 8.01	1.89	NS	
Covariate	1, 328	120	<0.0001		1, 317	3.25	0.07		–	–		

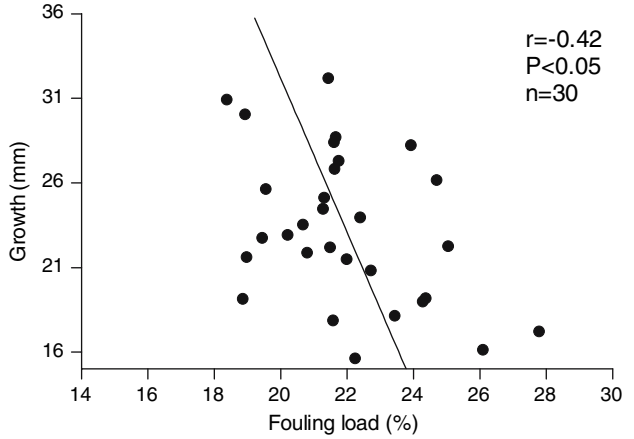

Fig. 4 Genetic correlation between fouling load (dry-mass of epibiota as a percentage of the dry mass of the basibiont) and growth rate (in terms of length increment during the experiment). Genotypic estimates are calculated for the treatment level having fouling (Fouling group). For growth, both herbivory levels are included and the estimates are adjusted for the average initial length; for the fouling load, the estimates are for the No-herbivory treatment level alone. The *line* is a model-II Deming regression adjusted for the standard errors of the variables

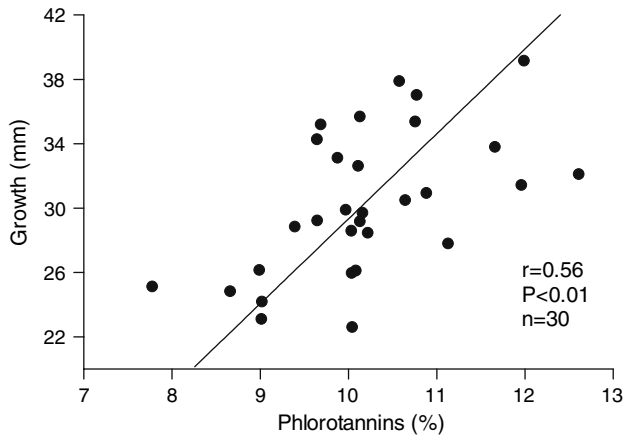

Fig. 5 Genetic correlation between phlorotannin concentration (as percentages of dry weight) and growth rate (in terms of length increment during the experiment). Genotypic estimates are calculated for both the herbivory treatments, for the Clean-up fouling treatment level. The *line* is a model-II Deming regression adjusted for the standard errors of the variables

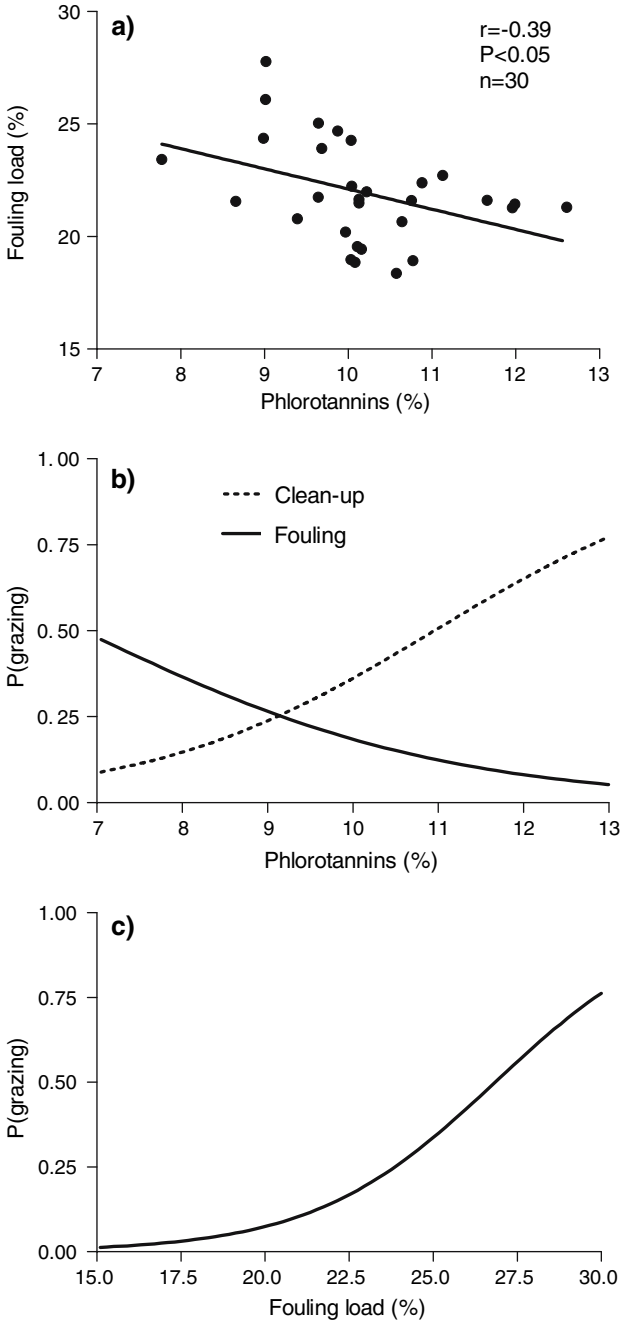

Fig. 6 Genetic correlations between phlorotannin concentration, fouling load, and the probability of grazing. Genotypic estimates for phlorotannins are from the Clean-up treatment level for both herbivory treatment levels (**a**), or from the Clean-up treatment level with no herbivory (**b**). Fouling load is estimated for the Fouling group with no herbivory (**a**, **c**). The probability of grazing is estimated separately for the Clean-up and Fouling groups in **b** and only for the Fouling group in **c**. **a** The model-II Deming regression line. **b, c** The *lines* represent logistic regression models

with increasing phlorotannin concentration, although this was not significant at $\alpha = 0.05$ (Fig. 6b, $\beta = -0.47 \pm 0.26$, $\chi^2 = 3.16$, $df = 1$, $P = 0.07$, $R^2 = 6.5\%$). When the logistic regression was performed using phenotypic phlorotannin values, i.e., values from each algal piece instead of genotypic means, the overall pattern was similar, but only the negative relationship in the Fouling group ($\beta = -0.48 \pm 0.17$, $\chi^2 = 7.4$, $df = 1$, $P < 0.01$, $R^2 = 16\%$) was statistically significant. Probability of grazing did not depend on phlorotannins in the Clean-up group ($\beta = 0.10 \pm 0.10$, $\chi^2 = 0.94$, $df = 1$, ns, $R^2 = 1.4\%$). Similarly, the relationship between the probability of grazing and the fouling load of the genotype depended on whether grazing took place in the presence or absence of epibiota (fouling treatment-by-fouling load interaction: $\chi^2 = 8.9$,

$df = 1$, $P < 0.01$, model $R^2 = 18.6\%$). When herbivores grazed on algae cleaned of epibiota (Clean-up group), there was no significant relationship between the probability of grazing and the genotypic fouling load (measured in Fouling group without herbivores; $\beta = -0.13 \pm 0.10$, $\chi^2 = 1.7$, $df = 1$, ns, $R^2 = 2.7\%$). When herbivores grazed on fouled algae, the probability of grazing increased with the fouling load (Fig. 6c; $\beta = 0.37 \pm 0.13$, $\chi^2 = 7.6$, $df = 1$, $P < 0.01$, $R^2 = 15.1\%$).

Discussion

Epibiosis was costly to *F. vesiculosus* as growth, in terms of length, decreased about 27% and the concentration of phlorotannins decreased about 20%. This indicates that epibiota shaded the basibiont, thereby decreasing photosynthesis. Genotypes varied in their resistance to fouling, the variance component due to genotype being roughly 20% of the phenotypic variation. Genotypic variation in resistance to fouling has been found repeatedly in different local populations of *F. vesiculosus*, both in a mesocosm environment (Jormalainen et al. 2003) and in the field (Honkanen and Jormalainen 2005), suggesting that it prevails among populations and is expressed in different environments.

In the absence of epibiota, genetic differences in resistance to grazing were relatively high, the proportion of among-genotype variation being about 30% of the phenotypic variation. The resistance to grazing in isolation from epibiosis was not provided by phlorotannins, rather the contrary; the genotypes characterized by high phlorotannin concentrations were grazed more often. However, in the analysis conducted at the phenotypic level, phlorotannins did not explain the probability of grazing. These results imply that it was not phlorotannins as such, but some other trait, e.g., growth rate or some resistance mechanism (Deal et al. 2003), genetically correlating with phlorotannins, that generated the differences in susceptibility to herbivory in the absence of epibiota.

Grazing effects on the macroalgal host have previously been shown to depend on the amount or identity of epibionts (Bernstein and Jung 1979; Wahl and Hay 1995; Karez et al. 2000). In the current study, herbivory and epibiosis were closely intertwined as grazing pressure on *F. vesiculosus* depended on the occurrence of epibiota. *I. baltica* readily fed on epibiota and, therefore, herbivory directed less at the *F. vesiculosus* thallus under fouled conditions. Such a shift in feeding is possible when herbivores are generalists, or at least have some degree of diet mixing, which is the case in *I. baltica* (Jormalainen et al. 2001) and is typical of aquatic mesograzers in general (Cruz-Rivera and Hay 2000; Sotka and Hay 2002). Thus, grazing pressure on *F. vesiculosus* by this key herbivore in the Baltic Sea (Engkvist et al. 2000) may vary depending on the amount of the epibiotic community.

While the presence of fouling organisms generally decreased grazing pressure on the basibiont, the genotype-by-fouling interaction in the probability of grazing implied that the resource use of *I. baltica* among the genotypes changed in the presence of epibiota; it was the most fouled genotypes that faced the largest grazing loss. Two mechanisms may explain why the basibiont was grazed more the more there were epibiota: either the high fouling load attracted numerous grazers or those algal genotypes that suffered high fouling loads were consumed more readily by individual *I. baltica*. We cannot, unfortunately, separate these possibilities. The first explanation suggests that isopods used the amount of epibiota instead of the characteristics of *F. vesiculosus* as their food choice criteria and that the basibiont faced a shared doom (Wahl and Hay 1995) with the epibionts. However, the availability of alternative food and the consequent option to feed on a mixed diet that provides better growth (Hemmi and Jormalainen 2004) may have made it possible for individual *I. baltica* to adjust their consumption of the basibiont. For example, diet mixing may effectively dilute defensive metabolites, thereby allowing shifting of resource use among the *F. vesiculosus* genotypes, i.e., altering food choice criteria. This indicates also that the grazing strategy of the herbivore may be sensitive to the composition of the host assemblage and therefore different traits may provide resistance in distinctive situations, even against the same enemy.

Our study showed that epibiosis can change the rank of the genotypes in their susceptibility to grazing, suppress genetic variation in resistance to grazing and modify the patterns of selection for different types of resistances. While selection by herbivory for the traits responsible for the grazing resistance may be strong under conditions with low epibiosis, such as in environments where wave motion keeps algae clean, under fouled conditions this selection relaxes and the influences of epibiosis and grazing on the fate of the basibiont become intertwined. When there is only one enemy, the lack of the genetic correlation between resistances to fouling and grazing indicates that there may be selection for specialized defenses. The very dissimilar types of attacks of these two enemies may favor specialized defenses and consequent genetic decoupling of resistances. However, when the enemies coexist the positive genetic correlation between resistances to epibiosis and grazing indicates that a generalized defense may be selected for. Variation in epibiosis is thus likely to contribute to the highly variable patterns in selection found in *F. vesiculosus* (Jormalainen and Honkanen 2004). Variable selection may be important for the maintenance of genetic variation in resistances to grazing and fouling.

We found a positive genetic correlation between the phlorotannin concentration and fouling resistance. We interpret this to indicate that phlorotannins influence fouling resistance. If the causality were reversed, we would expect to find the relationship only under fouled conditions; it however remained also when phlorotannins were measured from the cleaned algae. There was also a positive genetic correlation between growth and fouling resistance, even in the absence of fouling. An alternative interpretation could then be that slowly growing genotypes get more fouling and the correlation with phlorotannins arises as an indirect consequence of the positive genetic correlation between growth and phlorotannins. We do not, however, consider this to be the explanation: genotypic variation in resistance to fouling was detected also in the Clean-up group and the genotypic fouling loads correlated positively between the Clean-up and Fouling groups, indicating that genetic differences show up within a few days of cleaning the thallus, already at the early stage of colonization by microbial epibiota. Thus, genotypic differences in growth cannot explain the fouling load at the end of the experiment. Phlorotannins decreased in the presence of epibiota and, hence, do not function as an inducible resistance to fouling, but they may provide constitutive resistance.

Extracts containing phlorotannins have previously been documented to inhibit settlement of epibiotic organisms (Jennings and Steinberg 1997; Wikström and Pavia 2004). The weakness of these studies is that it is not clear how much of these polar compounds the epibionts actually encounter under field conditions. To be an efficient antifouling compound, the metabolite must either adhere onto the surface of the thallus or be exuded into the boundary layer of the thallus in such an amount that it can deter propagules of the fouling organisms (Jennings and Steinberg 1997). We know that *F. vesiculosus* exudes phlorotannins (Koivikko et al. 2005) and that some of the phlorotannins, presumably the large-sized polymers, remain in agar matrix without dissolving into water for substantial periods (Jormalainen et al. 2005). The significant genotypic correlation between phlorotannin concentration and resistance to fouling in our study provides further evidence that phlorotannins may have an ecologically significant role as antifouling defense.

Jennings and Steinberg (1997) reported a slight positive phenotypic correlation in field data in the kelp *Ecklonia radiata*, but since the phlorotannin concentration explained only a very small amount of variation in the coverage of epiphytes (less than 2%), they considered the relationship biologically insignificant. In our experiment, phlorotannins explained about 15% of the variation in resistance to fouling. Thus, much unexplained variation still remained despite the controlled conditions. One explanation for this could be that the resistance to fouling is provided by certain phlorotannin(s), while we have measured the pooled concentration of soluble phlorotannins. The phlorotannins include an array of different polymers, which may have different properties and different activities (Ragan and Glombitza 1986; Nagayama et al. 2003). Explicit studies of different polymers, as well as the connection between tissue concentrations and the concentrations encountered by epibionts at the thallus surface, are needed to fully understand phlorotannins' role as an anti-fouling defense in brown algae.

To be regarded as a defense trait, resistance is expected to be costly in terms of decreased growth and/or reproduction in the absence of the enemy. Expression of costs of resistance or of certain chemical defense traits have turned out to be very variable, suggesting that realization of the costs depends on environmental variation, especially on resource availability (reviewed by Bergelson and Purrington 1996; Strauss et al. 2002; Koricheva 2002). We did not find any cost of resistance to fouling. On the contrary, the genetic correlation between resistance to fouling (reverse of the fouling load) and growth was positive and, thus, the genotypes that grew fast also had the best resistance. Accordingly, the relationship between growth and phlorotannin concentration was positive, showing that there was no genetic trade-off in the allocation of carbon to growth and phenolic secondary metabolites. Production of quantitative secondary metabolites is usually highly dependent on light availability (reviewed by Koricheva et al. 1998), and this has been found to be the case also with phlorotannins (Pavia and Toth 2000b). We suggest that the potential to photosynthesize carbon is crucial to the expression of the allocation costs of quantitative secondary metabolites such as phlorotannins. In through-flow mesocosm environments, algae grow close to the surface and are relatively unshaded, have abundant light and, thus, carbon does not become limiting. Genotypes that are efficient in photosynthesizing under high-light conditions are capable to both grow and produce phlorotannins, as has also been found previously (Jormalainen et al. 2003). Under field conditions, the expression of allocation costs becomes more variable; either no correlation or negative correlations have been observed (Jormalainen and Honkanen 2004).

In conclusion, the genetic correlation between resistance to epibiosis and resistance to grazing was positive when these enemies coexisted. When resistances for both enemies were measured independently, the positive relationship disappeared, suggesting that the selection for resistance depends on the context, e.g., selection by herbivory alone will vary depending on the occurrence of epibiota. This suggests that the pattern of selection for resistance traits depends on the presence of multiple enemies. When enemies do not coexist, specialized defenses may be selected for by each enemy, but when they do coexist, the

patterns of selection exerted by the different enemies are interdependent and selection may favor generalized defenses. Phlorotannins provide resistance to fouling but do not deter grazers in the absence of epibiota. However, in the presence of epibiota, a positive relationship between the grazing resistance and phlorotannins arises as phlorotannins increase resistance to fouling and resistances to fouling and grazing are positively correlated.

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