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Regulation of anti-herbivore defence by *Fucus vesiculosus* in response to various cues

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Summary

1 We examined whether the marine macroalga *Fucus vesiculosus* induces defences against herbivory and, if so, which factors trigger this induction. In addition, we assessed whether induced defences are reduced after consumption stops.
2 Induced effects were measured as changes in palatability rather than changes in the chemistry of the algae. We also tested for reductions in growth rate to determine whether induced defence incurs metabolic costs.
3 We tested whether direct grazing, feeding on neighbouring plants, clipping and presence of a non-grazing herbivore could trigger induction. The isopod *Idotea baltica* and the gastropod *Littorina littorea* were used as herbivores.
4 Both direct feeding of *I. baltica* and feeding on neighbouring plants induced chemical defence in *F. vesiculosus*, whereas the snail *L. littorea* only induced defence by direct grazing. Simulated herbivory (clipping), or the presence of herbivores without grazing, did not lead to defence induction. All induced defences were reversed within 2 weeks of consumption ending.
5 Thus, *F. vesiculosus* differentiates between physical damage and natural herbivory. Furthermore, feeding by *I. baltica* on *F. vesiculosus* releases signals that trigger neighbouring *Fucus* individuals to induce defence.
6 We found no evidence that metabolic costs incurred as a consequence of induced defence were sufficient to lead to growth reduction.
7 This algal species demonstrates defence plasticity (i.e. induction and reduction of anti-herbivore defences 'on demand'), with the response depending on both grazer identity and grazing pattern matter.

Key-words: Baltic Sea, chemical defence, defence induction, *Fucus vesiculosus*, herbivory, *Idotea baltica*, *Littorina littorea*, Macroalgae, plant–herbivore interactions, water-borne cues

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Introduction

Fucus vesiculosus L. (hereafter *Fucus*) is an extensively studied alga and the main constituent of the littoral biome in the western Baltic (Kangas *et al*. 1982). Several studies have detected a decline of up to 95% in biomass in *Fucus* populations during the last three decades (Kautsky *et al*. 1986; Vogt & Schramm 1991; Schramm & Nienhuis 1996), possibly due to increasing grazing pressure, especially by the isopod *Idotea baltica* Pallas (Kangas *et al*. 1982; Salemaa 1987; Schaffelke *et al*. 1995). Algae have evolved several strategies to deal with herbivory: many ephemeral algae compensate for consumption by high growth and reproduction rates, whilst others rely on morphological defences, low nutritional value or chemical defences (Lubchenco & Gaines 1981). These adaptations may be persistently expressed (constitutive defence) (Baldwin 1998) or generated 'on demand' (inductive defence) (Karban & Baldwin 1997; Harvell & Tollrian 1999). Although inductive defence is likely to allow allocation of resources to defence to be reduced when not required (Agrawal & Karban 1999), the resulting benefits are not well understood. Besides saving resources, the possible advantages of defence induction include: (i) a higher variability of algal chemical composition may prevent herbivores becoming adapted to defensive mechanisms over ecological or evolutionary time-scales (Whitham 1983); (ii) attack by specialist herbivores, epibionts or

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parasites that are attracted by specific defence-related metabolites is avoided when the plant is not in the induced state (Giamoustaris & Mithen 1995); and (iii) the risk of autotoxicity is reduced if toxic defensive metabolites do not need to be permanently stored (Baldwin & Callahan 1993). The induction of chemical defence against herbivory has been demonstrated for many vascular plant species (see review by Karban & Myers 1989), but few examples have been reported in marine algae (Van Alstyne 1988; Yates & Peckol 1993; Cronin & Hay 1996a; Pavia & Toth 2000). This may result from a research bias (Cronin 2001), or because, unlike vascular plants, algae tend to be relatively incapable of transporting the induction stimulus from the site of grazing damage to other parts of the plant (Cronin & Hay 1996b). Also, many previous studies of algae measured changes in chemistry rather than in palatability, or used inappropriate induction cues and may therefore have drawn invalid conclusions (see Baldwin 1990; Hay 1996).

Nearly all studies on induction of chemical defence in marine algae have involved brown algae (e.g. Van Alstyne 1988; Yates & Peckol 1993; Cronin & Hay 1996a; Hammerstrom *et al.* 1998), and chemical ecologists have focused mainly on phlorotannins as feeding deterrents (reviewed by Ragan & Glombitza 1986; Steinberg & van Altena 1992). However, although some studies reported that phlorotannins decrease feeding by herbivores (e.g. Geiselman & McConnell 1981; Steinberg 1988; Boettcher & Targett 1993; Pavia & Toth 2000), others found no such effect (e.g. Pavia *et al.* 1997; Steinberg & van Altena 1992; Van Alstyne *et al.* 2001). *F. vesiculosus* has been shown to possess natural defensive properties and its chemical composition is sufficient to explain repulsion of the grazer *Littorina littorea* L. (Geiselman & McConnell 1981). Bioassays showed that polyphenols (phlorotannins) acted as feeding deterrents, with *Fucus* tissue with higher concentrations of polyphenols being significantly less palatable. However, it is unclear whether *Fucus* is capable of regulating its defences, and, if so, which cues trigger induction, or how responses vary with different types of natural and artificial damage. In addition to direct grazing (Pavia & Toth 2000), mechanical simulation of herbivory (clipping) (Paul & Van Alstyne 1992; Steinberg 1995a; Pavia *et al.* 1997) has been used to induce resistance against herbivores. However, mechanical simulations of herbivory often differ from actual grazing in some physical attributes (see Baldwin 1990), or may lack some chemical or biological cues that are necessary to induce chemical defence (e.g. Raffa & Smalley 1995). In aquatic systems, water-borne cues could originate from neighbouring grazed plants, although only one example of such transmission is known in marine algae (Toth & Pavia 2000). In the case of natural herbivory, plants may exhibit different chemical responses to different species of herbivore (Stout *et al.* 1998). In this study we examined whether defence is inducible in *F. vesiculosus*. Furthermore, we tried to identify the cues that can induce chemical resistance against the herbivores *I. baltica* and *L. littorea* by applying different grazer treatments: (i) direct grazing, (ii) grazing of neighbouring plants, (iii) clipping, and (iv) mere presence of the grazer. We tried to differentiate between chemical and structural defence by using different types of feeding assays. Finally, we assessed whether defences were reduced again after grazing had ceased.

Materials and methods

STUDY SITE AND ORGANISMS

All organisms were collected from a rocky shore in the Kiel Fjord, western Baltic (54°26′ N, 10°11′ E), where the perennial seaweed *F. vesiculosus* (bladder wrack) forms dense, almost monospecific stands. *Fucus* plants were transferred in coolers from the subtidal zone (0.2–0.7 m) to the laboratory and maintained in aerated seawater until required (within 24 hours). We chose the isopod *I. baltica* and the snail *L. littorea* as herbivores for our experiments because both use *Fucus* and other macroalgae as both habitat and food source, and can significantly reduce the biomass of these large seaweeds (Nicotri 1980; Watson & Norton 1985; Salemaa 1987).

INDUCTION EXPERIMENTS

Two induction experiments were run in a constant temperature chamber (15 °C) from August to December 2002 at the Leibniz-Institute of Marine Sciences (IfM-GEOMAR) in Kiel, Germany. The experimental set-up consisted of a flow-through system of 42 transparent plastic aquaria (2.9 L), each of which was divided into an upstream and a downstream compartment by plastic mesh (1 mm). The algal pieces that were tested for defence induction were always kept in the downstream compartment. Ambient water was obtained from the nearby Kiel Fjord, filtered (1.2 µm) and stored in a tank (150 L) before supply to the aquaria, which were individually regulated with roller clamps to give a flow rate of 0.25 L h^{-1}. Light was provided by fluorescent tubes (OSRAM FLUORA L 36 W/77 25X1), which were mounted in parallel above the aquaria, so that total irradiance was 14.24 ± 0.04 Wm^{-2} with a light/dark period of 12/12 hours. *I. baltica* served as the herbivore in the first experiment and *L. littorea* in the second.

Both experiments were divided into three phases (acclimatization, treatment and recovery), each of 2 weeks duration (Fig. 1). All algal pieces were first acclimatized in the absence of grazers to reduce potential grazing effects carried over from the field, as well as effects caused by cutting the algae. Algae were then exposed to different treatments (see below) to induce defences. The recovery phase was used to test whether induced resistance disappeared after the treatments (i.e. grazing or threat of grazing) had been removed. At the end of each phase, feeding choice assays were run to

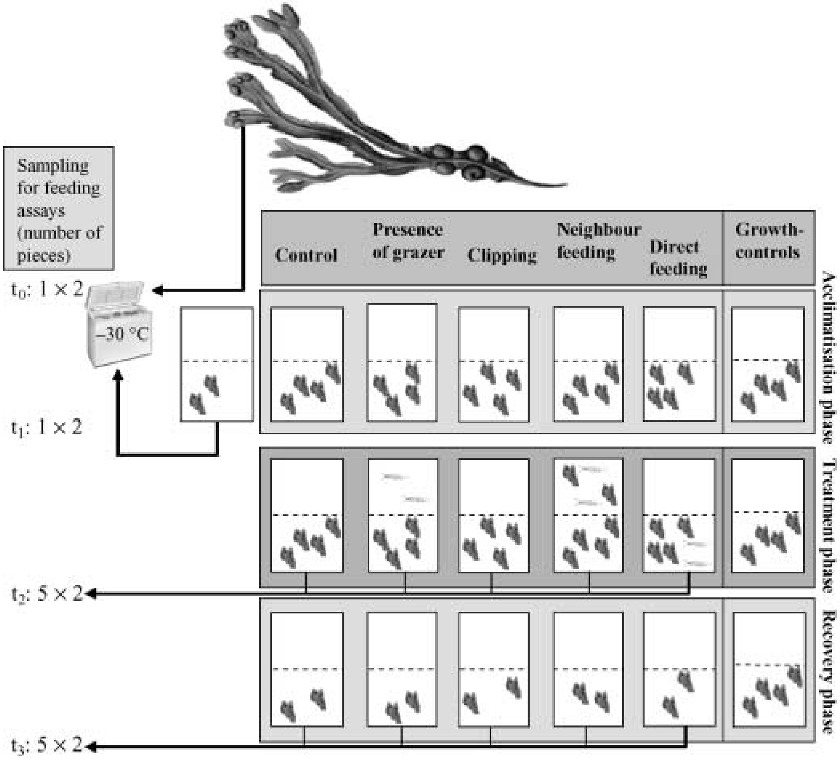

Fig. 1 Experimental design showing three sequential phases and sampling for one replicate. An algal individual was cut into 28 comparable pieces and randomly distributed as shown. Two pieces were deep frozen immediately, and a further two after being kept without grazers during the acclimatization phase. Four pieces were left as controls for growth measurements, while for the five treatments, two algal pieces from each aquarium were used for feeding assays at the end of the treatment phase (t_2) and the remaining two after the recovery phase (t_3).

test for differences in palatability (see below). Before and after every phase, all algal pieces were carefully blotted dry and weighed to the nearest 0.001 g to determine whether the metabolic costs of induction of defence were sufficient to reduce growth.

Six *Fucus* individuals, of equal size and without severe grazing damage, were collected on the starting day of each experiment (an algal individual is comprised of the tissue descending from a single holdfast). To control for genetic variation in phlorotannin concentration, which may greatly exceed induced responses (Jormalainen *et al*. 2003), each of the six replicate individuals was divided into comparable pieces and distributed randomly between treatments. Each individual contributed 28 similar apical pieces (1.5–2 g wet weight), which were cleaned of all visible epiphytes. Two pieces were frozen for later measurement of the *in situ* level of defence (Fig. 1, t_0) and the remainder distributed between seven aquaria (two in one, four each in the remaining six). At the end of the acclimatization phase (t_1), the two pieces were frozen to identify changes in palatability compared with the initial sample. Algal pieces in each of the remaining six aquaria were marked at the base by clipping a small, distinctively shaped piece out of the algae so that each sample could be identified in the multiple choice feeding assays. One aquarium per replicate provided tissue for measuring for autogenic changes in mass over the time period of feeding assays (growth control, Fig. 1), while the remainder were subjected to one of five treatments. Controls contained algae without grazer, while 'presence of grazer' had four individuals of the chosen grazer in the upstream compartment and the target algae in the downstream compartment. 'Clipping' had grazing simulated by cutting small pieces of tissue from the lower lateral parts of the algae, without damaging the apical meristem, with scissors every second day. The cumulative amount of tissue removed during the treatment phase was less than 10% of total wet weight. The clipping treatment proved to be without effect in the first experiment and was not therefore included in the second. 'Neighbour grazing' was as in 'presence of grazer', except that the grazers were allowed to feed on additional *Fucus* pieces of the same genotype as the target algae. For 'direct feeding' three individuals of *Idotea* or four individuals of *Littorina* were allowed to feed on the target algae in the downstream compartment. In the recovery phase, all grazers were removed from the aquaria and clipping was stopped.

PREFERENCE FEEDING ASSAYS

After the acclimatization phase (Fig. 1, t_1) two paired choice feeding assays were conducted for each replicate (comparing t_0 and t_1 samples), while after treatment and recovery phases (t_2 and t_3) two multiple choice feeding assays were used (comparing treatments and control for that time point).

The first assay in each case was conducted with intact algal pieces to assess chemical and/or structural defences. Algal pieces were carefully blotted dry, weighed, and transferred to a 2.9-L aquarium containing seawater, to which either four *I. baltica* or six *L. littorea* were added. Algal pieces were reweighed after 3 days and the biomass consumed in each replicate was calculated as $H_0 \times (C_f/C_0)-H_f$, where H_0 and H_f were pre-assay and post-assay wet weights of the algae in the feeding trials and C_0 and C_f were the weights of the growth controls, i.e. C_f/C_0 represents autogenic changes in mass (Sotka *et al.* 2002).

The second assay used reconstituted food to assess resistance based on chemical or nutritional differences. Algal pieces were freeze-dried and ground to a fine powder with a mortar and pestle and then reconstituted in agar. Agar was heated to boiling point in a microwave oven (0.18 g Agar + 5 mL distilled water), allowed to cool to *c*. 40 °C and mixed with the moistened algal powder (0.25 g of powdered algae + 1 mL distilled water). The mixture was poured over a mosquito net (mesh size 1 mm) and flattened between two layers of wax paper (Hay *et al.* 1994). The net was cut into squares of 16 × 16 cells and treatments were identified by clipping the corners in different shapes. The reconstituted tissues were offered to three isopods or snails for 24 h. We then counted the number of net cells consumed from each treatment.

STATISTICAL ANALYSIS

As consumption values within an aquarium cannot be assumed to be independent, classical ANOVA is inappropriate (Roa 1992). Permutation tests were used instead, performed with Resampling Stats 5.0 for Windows (Resampling Stats Inc., Arlington, VA, USA) (Bärlocher 1999; Good 1999). Differences in multiple choice feeding assays were analysed by a Monte Carlo analysis with 10 000 permutations. As post hoc tests, four (first induction experiment) or three (second induction experiment) pairwise Monte Carlo tests were conducted between control and treatments. Significance levels of post hoc tests were Bonferroni corrected to α = 0.0125 and 0.0166, respectively.

Fucus growth rates were calculated as percentage biomass change per day. Prior to a one-way ANOVA, data were arcsin-transformed to obtain normality (Kolmogorov-Smirnov) and homogeneity of variance (Cochran).

Results

ASSAYS WITH *I. BALTICA*

After acclimatization (i.e. 2 weeks without grazing) palatability of *Fucus* did not differ from that of *Fucus* that was immediately frozen after collection, when tested either as intact algae or as reconstituted food (resampling statistics, $P > 0.05$, Fig. 2).

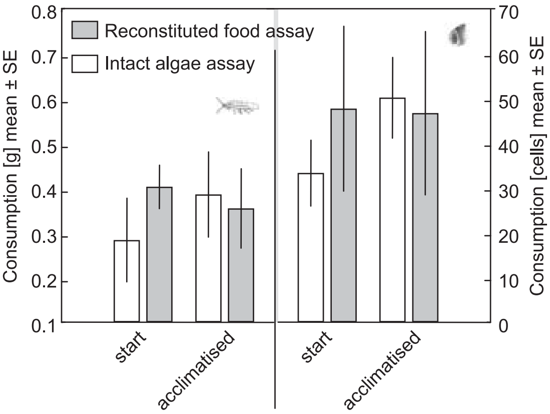

Fig. 2 The amount of *Fucus vesiculosus* tissue (intact algae, white bars) and reconstituted food (grey bars) consumed by *Idotea baltica* and *Littorina littorea* in paired choice feeding assays after acclimatization phase ($n = 6$). Consumption of intact algae included a correction for autogenic changes (see text for details). For assays with artificial food, *Fucus* pieces were freeze-dried, ground and built into an agar matrix with an embedded mosquito mesh and consumption recorded as the number of cells completely eaten.

At the end of the treatment phase, algae exposed to direct *Idotea* grazing and algae exposed to neighbour feeding were significantly less palatable than control algae (both $P < 0.001$, Fig. 3). In contrast, neither the mere presence of non-grazing *Idotea* nor clipping affected *Fucus* palatability.

After the recovery phase, no significant differences in palatability persisted between treatments and controls, either in intact algae assays, or in reconstituted food assays ($P > 0.05$, Fig. 3).

ASSAYS WITH *L. LITTOREA*

L. littorea did not distinguish between acclimatized vs. non-acclimatized plants offered as intact algae or reconstituted food ($P > 0.05$, Fig. 2). At the end of the treatment phase, directly grazed *Fucus* was significantly less palatable than controls both for intact algae and reconstituted food ($P < 0.001$, Fig. 3), but, in contrast to the assays with *Idotea*, neighbour feeding by *Littorina* did not reduce palatability compared with controls ($P > 0.05$). As in the *Idotea* assays, the mere presence of *Littorina* did not affect *Fucus* palatability ($P > 0.05$). At the end of the recovery phase, differences in palatability between directly grazed *Fucus* plants and controls diminished for both intact algae and reconstituted food ($P > 0.05$, Fig. 3), so that no treatment differed significantly from the control.

GROWTH RATES DURING THE INDUCTION EXPERIMENTS

We found no significant differences in *Fucus* growth rates between different grazing treatments within any phase in the first experiment (Fig. 4, ANOVA, Table 1). Due to tissue loss during the treatment phase, growth

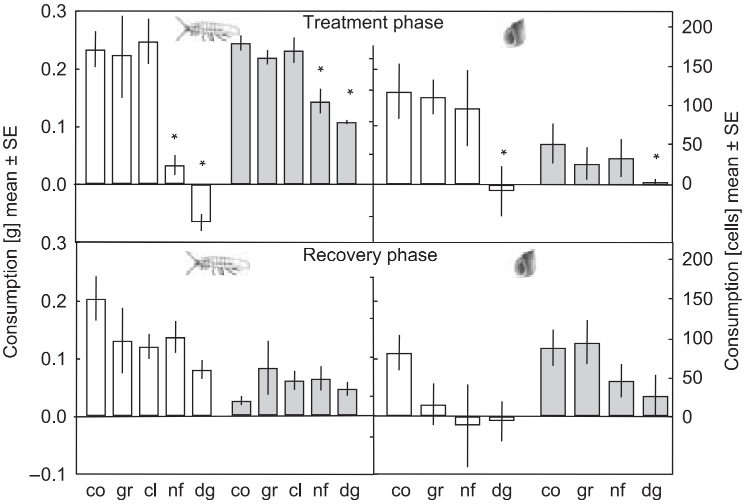

Fig. 3 The amount of *Fucus vesiculosus* tissue (intact algae, white bars) and reconstituted food (grey bars) consumed by *Idotea baltica* and *Littorina littorea* in multiple choice feeding assays after treatment and recovery phase ($n = 6$). Negative consumption was caused by autogenic weight change correction (see text for formula). co = controls; gr = presence of grazer; cl = clipping; nf = neighbour feeding; dg = direct grazing. Treatments that are significantly different to controls are marked with *. For further explanations see Fig. 2.

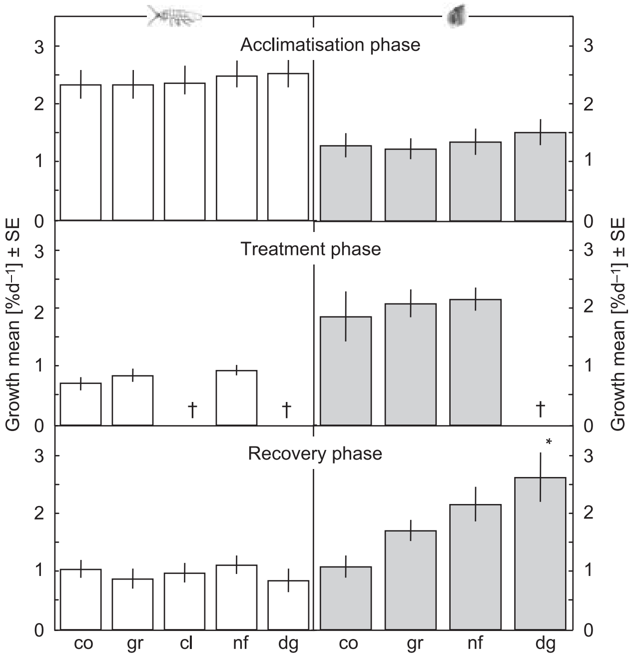

Fig. 4 Growth rates of *Fucus vesiculosus*: white bars represent the growth rates of the first experiment; grey bars represent growth rates of the second experiment ($n = 6$); † = bars are absent, because these values were not included in the analyses.

rates for clipped and directly grazed algal pieces had to be excluded from the analyses. There was a high variation in growth rates between the experimental phases: growth rates were two to three times higher in the acclimatization phase than in the following two phases.

Similar results were observed within acclimatization and treatment phases of the second experiment. In the recovery phase, however, *Fucus* samples that had previously been exposed to direct grazing by *L. littorea* grew significantly faster than the control plants (Fig. 4, ANOVA, Table 1).

Table 1 ANOVA for the growth rates of *Fucus vesiculosus* during the induction experiments. Significant differences ($P < 0.05$) are indicated by bold numbers

	1st induction experiment			2nd induction experiment		
	d.f.	*F*	*P*	d.f.	*F*	*P*
Acclimatization phase	4 (25)	0.228	0.919	3 (20)	0.343	0.793
Treatment phase	2 (15)	0.873	0.437	2 (15)	0.256	0.777
Recovery phase	4 (25)	0.432	0.784	3 (20)	5.075	**0.008**

Discussion

Both induction experiments showed clearly that palatability of *F. vesiculosus* can be reduced by herbivory, providing evidence for induced defences, and that the differences are reduced when grazing stops. Return to pre-induction levels after the end of stimulation should be an integral characteristic of inducible defence, but this has not been demonstrated before for marine algae. Different grazer species appear to trigger the anti-herbivore defences of *Fucus* via different cues. Defence induction did not reduce growth rates of *Fucus*, but, interestingly, algae that had been grazed by *Littorina* showed increased growth during the recovery phase.

The finding that reconstituted food and fresh algae produced very similar results, suggests that chemical, rather than structural, mechanisms are involved. An induction of chemical defence as a response to herbivory has only been demonstrated in the three brown algae *Sargassum filipendula* (Sotka *et al*. 2002; Taylor *et al*. 2002), *Dictyota menstrualis* (Cronin & Hay 1996a) and *Ascophyllum nodosum* (Pavia & Toth 2000; Toth & Pavia 2000). Consequently, *F. vesiculosus* is the fourth algal species for which induced resistance has been demonstrated. Except for *D. menstrualis*, all are members of the phlorotannin-producing Fucales (Cronin 2001). Some phlorotannins are feeding deterrents (Targett & Arnold 1998). For instance, Van Alstyne (1988) induced higher phlorotannin concentrations by clipping in *Fucus distichus*, and this decreased feeding by *Littorina sitkana*, and Pavia & Toth (2000) found that phlorotannins from *A. nodosum* reduced its palatability. However, high phlorotannin concentrations do not always provide a high level of defence. Feeding preference assays with *I. baltica* showed that although *F. vesiculosus* had the highest phlorotannin concentrations of all the algal species offered, it was also the most preferred (Jormalainen *et al*. 2001). Recently, Deal *et al*. (2003) found that galactolipids rather than phlorotannins were herbivore deterrents in *F. vesiculosus*.

The various treatments used to try to induce resistance gave different results. Earlier studies used clipping as a method to simulate herbivory (e.g. Pfister 1992; Pavia & Toth 2000). However, earlier reviews have already cautioned that clipping does not simulate herbivory adequately and it should not be assumed to do so without proper testing (Karban & Myers 1989; Baldwin 1990). In the present study, clipping, in contrast to natural grazing, never induced a higher resistance. In its natural habitat (the high sublittoral), *Fucus* may often be subject to physical damage due to water turbulence. If this type of damage was sufficient to induce defence, it is probable that *Fucus* would be in an induced state for most of its lifetime. Plants that react to 'wrong' signals by producing potentially costly defensive metabolites may be at a selective disadvantage (Karban & Baldwin 1997). Although it is possible that cutting the algae to divide them between the different treatments may have caused physiological changes, including changes in chemistry, these effects would have been similar across all treatments.

With *I. baltica*, both direct feeding and feeding on neighbours induced chemical defence in *Fucus*. Effects of neighbour feeding suggest that it is not physical damage alone that triggers defence induction; water-borne cues are also emitted by the grazer or the consumed conspecific. In terrestrial plants, several studies have found defence to be induced by herbivore grazing on neighbouring plants (Bruin *et al*. 1995a, 1995b). Hartley & Lawton (1991) showed that a signal from herbivore saliva or symbiotic fungi can trigger defensive reactions in vascular plants, but no such signals have been identified for algae (see review by Karban & Baldwin 1997). There are only two studies that have tested whether chemical compounds released by algae or feeding herbivores could induce a defensive response in neighbouring algae (Toth & Pavia 2000; Sotka *et al*. 2002), but only Toth & Pavia (2000) demonstrated such an effect (in *Ascophyllum nodosum*). However, their study could not determine whether the signals were released by the alga or by the grazers. The mere presence of grazers was not sufficient to induce a defensive reaction in *F. vesiculosus*, indicating that neither *Idotea* nor *Littorina* release defence-inducing signals when they are not feeding. Only *Idotea* induced chemical defence in ungrazed neighbours, although direct grazing by either species was effective. Assuming a consumer-specific evolution of defensive strategies (Hay & Steinberg 1992), ignoring neighbour feeding of *Littorina* may not be disadvantageous for *Fucus.* A single *Fucus* individual can provide abundant food and habitat for the slow moving *Littorina* (Gendon 1977), so the risk of a nearby individual being attacked is relatively small. In contrast, *Idotea* is a fast swimming isopod that often switches between *Fucus* individuals (Jormalainen & Tuomi 1989; Jormalainen *et al*. 2001), and defence triggered by nearby feeding of *Idotea* might therefore have a selective value. Sotka *et al*. (2002) suggest that induction by direct grazing is a more important defensive strategy than induction by water-borne cues, because it tends to induce a stronger response (Van Alstyne 1988; Cronin & Hay 1996a; Toth & Pavia 2000), but the importance of induction by water-borne chemical cues must not be underestimated.

Neither *Idotea* nor *Littorina* differentiated between freshly collected algae and acclimatized samples, suggesting that no chemical or structural changes took place in the algae during acclimatization. There may have been no grazers at the field collection site, but feeding assays at the end of the recovery phase suggest that this cannot be due to defences present in the field persisting during the 2-week acclimatization, as all experimentally induced defences returned to pre-treatment levels within 2 weeks. As this is the first study we know to have tested the reversal of herbivore-induced defences in algae, there are no data about the speed of defence responses. Induction did not appear to take place over the course of the feeding assays, implying that it needs more than 3 days of grazing. Earlier studies, however, did induce defence in *F. vesiculosus* within 3 days (Peckol *et al*. 1996) and in *F. distichus* within 2 weeks (Van Alstyne 1988), but they did not test how long the defence was upheld. However, Hammerstrom *et al*. (1998) reported that in some kelp species increased phlorotannin concentrations, which had been induced by clipping, decreased to normal values within 7 days of the treatment ending. However, this study did not investigate changes in palatability, and it is therefore possible that phlorotannin levels did not relate to defence.

Defences induced by *Idotea* had no effect on the growth of *Fucus*, but direct *Littorina* grazing enhanced growth of *Fucus*, an effect that persisted throughout the recovery phase. The resource allocation model predicts that the production of defensive metabolites may incur substantial metabolic costs that can lead to trade-offs (see Discussion in Cronin 2001). Thus, if growth is energy limited, allocation to defence would result in reduced growth rates in defence-induced algae. Three explanations could account for this unexpected pattern: the defence induced in *Fucus* did not require substantial metabolic costs; the defence was induced at the expense of metabolic processes other than growth; or resources were not limiting in our experiment. Almost all studies that have tried to demonstrate that chemical defence is costly have involved terrestrial plants and have reported equivocal results (Bazzaz *et al*. 1987; Simms 1992). Investigations on brown algae showed either no growth reduction following defence induction (Pfister 1992), or a negative correlation between phlorotannin concentration and growth rates (Yates & Peckol 1993; Steinberg 1995b; Pavia *et al*. 1999). To our knowledge, there is no study on marine algae that has rigorously demonstrated that herbivore-induced defence has resulted in growth reduction.

One possible explanation for the enhanced growth rate of *Fucus* following grazing of *Littorina* could be compensatory growth to redeem the tissue loss (Strauss & Agrawal 1999; Toth & Pavia 2002). However, it remains unclear why this response is not shown after *Idotea* grazing.

In conclusion, *F. vesiculosus* can reversibly regulate its level of chemical defence against herbivores, without negative effects on its growth rate. The plasticity of the defensive response appears to reflect several different underlying mechanisms, and experimental investigation of these may elucidate more general patterns of plant–herbivore interactions.

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