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Ph.D. thesis

Ecological aspects on induced defenses in macroalgae by mesoherbivore attacks

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CONTENTS

SUMMARY

Plants have a diverse arsenal to cope with herbivore attacks. For example, plants can utilize a constitutive and induced form of the anti-herbivory defenses. Induced responses are different from constitutive defenses because their initiation is regulated by cues, which are triggered by feeding attacks. Induced defenses are generated when the cues are present or induced defenses are potentially reset if the cues are absent. As induced defenses are well-matched to the risk of attack from consumers, plants can reduce the cost for unnecessary defenses, and alternatively allocate their resources to growth and reproduction. Moreover, the dynamic aspects of induced defenses can provide benefits for the responding plant to prevent the chance of chronic tolerance of herbivores to defensive compounds. Thus, plants could adopt induced defenses preferably over constitutive responses when their consumer pressures are variable and predictable.

Current studies have identified induced anti-herbivory defenses in macroalgae in responses to single herbivore species. However, the induced response of a plant can modify reactions in diverse interacting species as well as a single herbivore species. Thus, it is necessary to include diverse interacting species in order to generalize the aspect on the induced responses. Here, I confirmed which grazing cues (Publication 1) and specific herbivore (Publication 2) induced antifeeding defenses in macroalgae. Comparing responsive patterns between a non-native and a native macroalgae in terms of herbivore specificity and its temporal variation, I found that herbivore-induced defense in macroalgae is a coevolved product that matches the risk of the coexistent herbivore (Publication 2). Additionally, I tested whether direct effects of induced

defense in a plant on an herbivore could be diversified by adding other interacting players such as plants (Publication 3) or herbivore species in simple trophic interactions (Publication 4).

Overall, *Fucus vesiculosus* became less palatable in response to direct grazing, showing that antifeeding defenses can be induced in this macroalga. Moreover, *F. vesiculosus* deterred feeding in response to herbivory-related cues, which are released from a grazed neighbor, *F. vesiculosus,* as well as in the presence of a non-feeding grazer. Thus, macroalgae may have a strong ability to trigger anti-herbivore defenses with consumer-related cues.

The other studied species*, Mastocarpus stellatus*, which was newly introduced into Helgoland, has increased anti-herbivory defenses toward *Idotea granulosa*, an isopod that has coevolved with its original populations. The anti-isopod defenses in *M. stellatus* were active even after grazing ceased, suggesting unmatched responses to the temporal variation of herbivores in new habitat. However, the non-native species did not induce defenses to a non-coevolved herbivore, *Littorina littorea*, which is absent from its original habitat. In contrast to *M. stellatus*, *Chondrus crispus*, as a native counterpart at Helgoland, was resistant to *L. littorea*. Accordingly, missed responses to the non-coevolved herbivores (i.e. periwinkle snail) or overcharged response to the coevolved herbivores (i.e. isopod), can support that defense inductions and their temporal variability in macroalgae may result from the adaptive process to herbivores through co-evolved history.

Similar to direct feeding, waterborne cues from a grazer-attacked macroalga can induce anti-herbivore responses in the neighbor macroalgal species. Except for *Fucus vesiculosus* and *F. spiralis*, both *Cystoseira humilis* and *Halidrys siliquosa* increased their antifeeding resistances responding to the cues derived from *Sargassum muticum*. This indicates that the cues from *S. muticum* can communicate with other macroalgal species, which would experience the feeding damage later. In an opposite direction, *S. muticum* did not increase their resistance when exposed to waterborne cues from grazed heterospecific macroalgae, showing that *S. muticum* does not respond to the heterospecific cues. Thus, the grazed cues may be utilized as preventive agents for the specific responding plants to encounter consumer attacks in the future.

Moreover, induced responses of the grazed macroalgae affected amounts of the consumption of other herbivore species to interact with these two species. This means that the efficiency of antifeeding traits in induced responses was differentiated by specific herbivores. The previous feeding of *L. littorea* on *F. vesiculosus* deterred feeding by the other consumers (i.e. *I. baltica*), rather than that for itself. Yet, previous grazing by *I. baltica* affected the consumption amount of *I. baltica*, not that of *L. littorea*. This shows that the effects of feeding-induced defenses were only effective to *I. baltica* regardless of inducer identity. This herbivore-dependent sensitivity suggests that trait modifications may reframe the interactions between grazed plants and other herbivore species, and thereby mediate asymmetric interactions among multiple herbivores.

In conclusion, induced defenses in macroalgae are specialized by herbivory-relevant cues, direct feeding loss, feeder cues, herbivore specificity, temporal dynamics, and co-evolution period. Moreover, the efficiency of induced responses can be shifted by the presence of other herbivores or macroalgal species. Ultimately, trait plasticity may trigger direct and indirect interactions between herbivores and macroalgae, consequently enhancing complexities in the food webs. The obtained results give important insights in the functioning of macroalgae-herbivore communities.

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1. INTRODUCTION

1.1. Macroalgae and their essential roles

Benthic primary producers such as macroalgae in freshwater and marine ecosystems play an important role in the annual carbon cycle on the planet. Especially marine macroalgae contribute to 3 % of the primary production of global carbon (Mann 1973) and its amount per area exceeds those of plants in terrestrial systems (Smith 1981). Further, macroalgae occupy from the littoral to the sublittoral zone (down to 20 to 300 m), and macroalgae form the major ecological components in marine communities. For instance, macroalgae serve significant roles by providing food, nurseries, and habitats for diverse animals such as fishes, crustaceans, and mollusks (Lubchenco & Gaines 1981, Hay 1997). Currently, their economic values has become more important as substitute resources such as curative biomedicines (reviewed in Paul & Williams 2008) and biofuels (Aresta et al. 2005).

1.2. Macroalgae and herbivory

Macroalgae are frequently exposed to threats from herbivores in littoral zones (Duffy & Hay 2000, Jonathan et al. 2002). The consumption amount by herbivores is close to 60-100 % of macroalgal growth per day, which exceeds the consumption by the most intensive herbivores in terrestrial communities (McNaughton 1985). Moreover, feeding-attacked parts in macroalgal shoots become weaker and break down easily, consequently it intensifies the biomass loss of the attacked individual during foraging activity of herbivores (Viejo & Åberg 2003, Toth & Pavia 2006). In addition to the feeding-related loss, gradients of feeding-preference by herbivores on specific species in macroalgal assemblages or particular stage of plants in life-cycle can determine the macroalgal community structure (Lotze et al. 2001, Wikström et al. 2006).

Macroalgal communities in temperate regions are typically exposed to diverse groups of mesoherbivores (length < 2.5cm), such as amphipods, isopods and gastropods (Brawley 1992). The mesoherbivores consume a relatively small portion of macroalgal production, compared to large herbivores like fishes and sea urchins (Hay & Steinberg 1992). Some mesoherbivore species known as generalist feeders can be mostly abundant in a specific season since their life cycles and living styles are regulated by seasonality. Further, their mobility could cause temporally variable pressures on host plants in the course of hours or days. Despite the variation of feeding pressures by the mesoherbivores, the herbivores utilize macroalgae as for habitat (Brawley 1992, Hay & Steinberg 1992), by residing among individual thalli for long periods. Thus, mesoherbivores can become a threat to putative feeding damage for host macroalgae (Pavia et al. 1999, Duffy & Hay 2000).

1.2.1. Macroalgal strategies against herbivory

Macroalgae are known to protect themselves from their consumer attacks with different strategies: avoidance, tolerance, resistance, and the combination of more than two factors mentioned above (Lubchenco & Gaines 1981, Cronin 2001). For instance, some macroalgae live in a close association with less tasty species to reduce grazing (Wahl & Hay 1995, Poore & Hill 2005) or to defeat their grazers by attracting enemies of the grazers (Coleman et al. 2007a). Further, some plants can tolerate herbivory through consistent growth in order to compensate feeding loss from actual grazing (Karban & Baldwin 1997, Nykänen & Koricheva 2004). The compensative growth in the attacked plants can be equivalent to that of non-attacked conspecifics. Plants that are able to compensate growth can invest the saved energy into gamete production, unlike their conspecifics that do not have the ability to compensate growth.

Moreover, macroalgae can become resistant through special mechanical are permanently present in plants. Using these two manners properly, macroalga properties (i.e. tissue calcifications, Cronin 2001) and/or chemical compounds (i.e. feeding deterrents and assimilation reducers, Sotka & Whalen 2008), consequently lowering feeding efficiencies of the herbivores. These antifeeding resistances in macroalgae are subdivided in constitutive and induced forms (reviewed in Karban & Baldwin 1997, Haavisto et al. 2010). Induced responses to herbivory are different from constitutive forms in that they are generated in a reaction to feeding attacks or their subsequent cues, while constitutive defenses can increase their resistances to herbivore risks effectively (Taylor et al. 2002). The variation of defense levels of plants is referred to optimal defense hypothesis (reviewed in Cronin 2001, Stamp 2003).

1.2.2. Induced defenses in macroalgae to consumers

Induced defenses in plants need a so-called 'lag time' until they are fully active (reviewed in Karban & Baldwin 1997, Metlen et al. 2009, Fig. 1). In this regard, feeding attacks in the middle of induction process may be predicted to cause irreversible impacts in the responding plants (Zangerl 2003). For example, if the grazed plants have large amounts of feeding damage within a short time, the plants cannot induce defenses due to low amounts of remained tissues. Thus, induced defenses may not be a proper strategy to resist against intense herbivores.

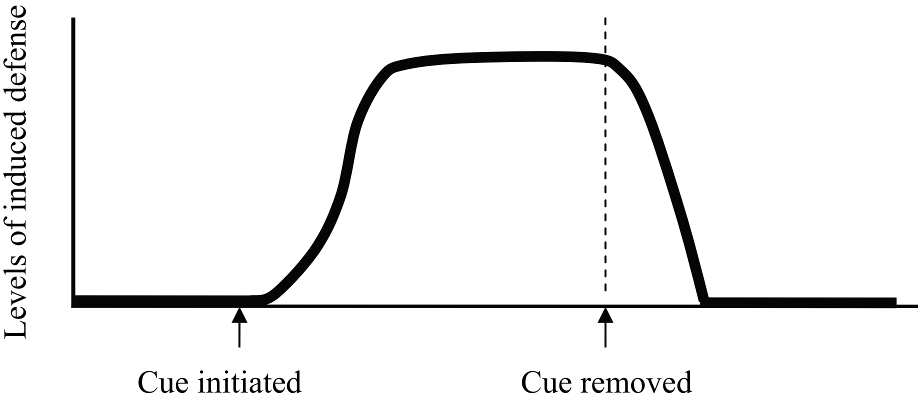


Fig. 1. A model illustrating the defense induction and reduction in plants. Induced defenses appear in response to the onset and the removal of herbivore-associated cues such as direct grazing or herbivore appearance.

Nonetheless, defense inductions in macroalgae are highly correspondent to the variation of herbivory pressure (Fig. 1.). For example, if the risk of herbivory is present, defense inductions in plants can be triggered. However, if the risk is absent, the induced responses in plants could be reduced to the original state (reviewed in Metlen et al. 2009). Concurrently, instead of investing resources to the unnecessary defenses, plants would reallocate the resources into growth and reproduction (Zangerl 2003, Agrawal 2005). Therefore, induced defenses are considered cost-saving strategies by on-demand response (Karban & Baldwin 1997, Karban et al. 1999).

Induced anti-consumer defenses can lead to reduced grazing pressures in various ways. For instance, induced responses would (1) decrease the palatability of a host plant, (2) slow down or avoid counteradapting of herbivores to defensive compounds derived from attacked plants (Karban & Baldwin 1997), (3) attract the natural enemy of herbivores, and thereby reduce the risk of herbivore attacks indirectly (Coleman et al.

2007a, Heil 2008, Allison & Hare 2009), (4) increase resistance of the non-grazed plants via cues emitted from either grazer itself or grazed neighbor plants (Coleman et al. 2007b, Howe & Jander 2008), and (5) require the negligible expense which does not reduce the fitness in the damaged plants (i.e. Rohde et al. 2004).

Moreover, induced responses to herbivory provide many benefits to plants. For instance, induced defense can reduce the risk of self-intoxication from the production and storage of defensive metabolites (Agrawal & Karban 1999) or it can increase the allelopathic compounds known to have deleterious effects on other counterpart competitors (Thelen et al. 2005).

1.3. Commonness of induced defensese in macroalgae

Induced antiherbivore resistances in macroalgae are reported in approximately 33 species in 23 studies, published between 1986 and 2010 (Van Alstyne 1989, Cronin & Hay 1996, Pavia & Toth 2000, Toth & Pavia 2000, Sotka et al. 2002, Taylor et al. 2002, Borell et al. 2004, Hemmi et al. 2004, Rohde et al. 2004, Weidner et al. 2004, Ceh et al. 2005, Macaya et al. 2005, Rothausler et al. 2005, Diaz et al. 2006, Molis et al. 2006, Coleman et al. 2007b, Long et al. 2007, Long & Trussell 2007, Toth 2007, Yun et al. 2007, Molis et al. 2008, Rohde & Wahl 2008a, 2008b): 14 red macroalgae (*Ahnfeltia plicata, Ceramium virgarum, Chondrus crispus, Chondracanthus chamissoi, Delesseria sanguinea, Furcellaria lumbricalis, Galaxaura diessingiana, Gracilaria capensis, Hypnea spicifera, H. pannosa, Osmundea ramosissima, Phyllophora pseudoceranoides, Polyides rotundus,* and *Pterocladiella capillace*), 15 brown macroalgae (*Ascophyllum nodosum, Chordaria flagelliformis, Cystoseira nyrica, Dictyota mestrualis, Ecklonia* *cava, Fucus evanescens, F. serratus, F. vesiculosus, Glossophora kunthii, Halidrys siliquosa, Lessonia nigrescense, Lobophora variegata, Sargassum asperifolium, S. muticum,* and *S. filipendula*) and 4 green macroalgae (*Cladophora rupestris*, *Codium platylobium*, *Enteromorpha intestinalis*, and *Ulva lactuca*)..

In these studies mentioned above, macroalgae were tested to trigger the induced responses after exposure to herbivory-associated cues: direct grazing, simulated grazing (clipping), herbivore presence, and feeding-related chemicals (digestive enzyme). Although some studies reported that even simulated feeding can change the algal traits (e.g. Hemmi et al. 2004), others found no such effect (e.g. Rohde et al. 2004). This discrepancy suggests that induced response may be generated by combining more than three factors, such as herbivore foraging behavior, physical attributes by actual grazing, and defensive chemicals in plants (e.g. Coleman et al. 2007b).

1.4. Comparison of induced response in native to non-native species

Induced defenses towards putative or actual grazing have been investigated within native macroalga-herbivore pairs (Toth & Pavia 2007 and herein references), showing various responses to herbivore specificity and/or temporal variation of the feeding pressure (Pavia & Toth 2000, Molis et al. 2008). For example, plants have developed to resist against specific herbivores to offer intensive impacts (Karban & Baldwin 1997). Further, the levels of induced antifeeding traits are changed in concert with the dynamics of feeding loss in hour or day or season through a coevolved history with consumers (reviewed in Metlen et al. 2009). However, it is not well studied how plants respond to specific herbivores which have no coadaptive period with the plants (Orians

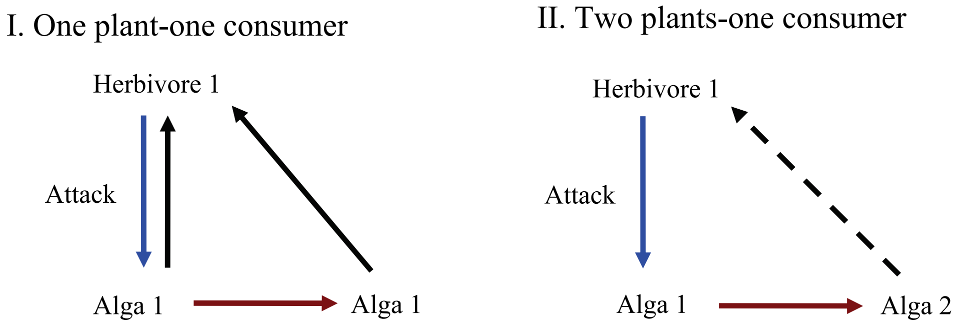
& Ward 2010).

Plants in a new habitat (i.e. non-native plants) can encounter attacks from non-coevolved herbivores, which are missing in their home range. In this case, due to lack of coadaptive periods to the novel herbivores, the non-native species would induce improper responses (Verhoeven et al. 2009), e.g. unmatched responses to the variation of feeding pressures. In contrast, if herbivores coexist with their prey in the old range, herbivore-specific feeding cues might trigger induced defenses even in a new habitat (Smith 2009). Thus, because of differences in the composition and temporal variability of herbivore species between new and old habitats, the non-native species in the new habitat would experience different feeding pressures compared to its original habitat (Agrawal 2001).

To estimate the antifeeding capacity of the non-native species, recent studies have measured the quantity of feeding deterrent chemicals (e.g. total concentration of phlorotannins in marine macroalgae or pyrrolizidine alkaloids in terrestrial plants, Joshi & Vrieling 2005, Wikström et al. 2006, Eigenbrode et al. 2008, Caño et al. 2009). However, these studies are not enough to evaluate the availability of antifeeding properties with variable consumers due to simple measurement of a single chemical. Studies on a single factor in induced defenses are not suitable for estimating capacity of the induced antifeeding properties in native plants which can vary on herbivore specificity and temporal variation (e.g. Pavia & Toth 2000, Molis et al. 2006). Thus, it is needed to examine how non-native species respond to coevolved and non-coevolved herbivores, and it is needed to test how non-native species respond to temporal variation of herbivores

1.5. Indirect species interactions via induced defenses

Most research has focused on simple herbivore-macroalga pairs (Toth & Pavia 2007 and herein references, Fig. 2-I.), showing that plants have a capacity to change their traits in response to herbivory-associated cues. Further, some studies have shown that effects from induced responses in the attacked plants can be extended to other species, so called trait mediated indirect interactions (TMIIs, Ohgushi 2005, Utsumi et al. 2009, van Dam 2009). This means that interactions of the modified plant with other plants or herbivores may be changed, if one plant modifies its antifeeding properties in response to one herbivore. Thus, a species can have an indirect effect on the others through changes in its traits (Fig. 2-II. & 2-III.).



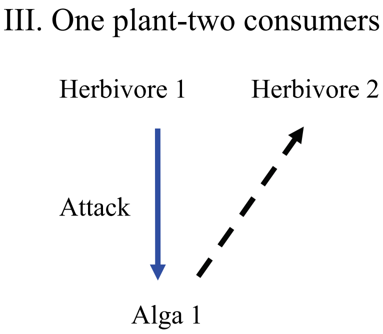


Fig. 2. The schematic overview of interactions between plants and herbivores presented in this thesis. Direction of herbivore attacks and attack-relevant signals is arrowed inblue and red lines, respectively. I) shows direct effect (by black solid lines) of herbivore-induced responses in the grazed or nongrazed conspecific plants. II) and III) can show indirect effect (by dotted lines) of directly grazed plants via induced response on the heterospecific plants and herbivores, respectively.

TMIIs are well documented in both aquatic and terrestrial communities (Werner & Peacor 2003, Molis & da Gama 2009). Recent works suggest that trait modifications as mediator of TMIIs can show immediate effects on the entire populations of preys when consumers are present. Further, altered traits of the prey individuals after consumer attacks have broad effects in the level of population and community (reviewed in Miner et al. 2005, Berg & Ellers 2010). For example, induced response of the attacked prey can prevent from increasing the population growth rate of its consumer (Ramos-Jiliberto 2003, Abrams & Matsuda 2004, Haavisto et al. 2010), allowing the same prey to have a coexistence with the consumer within the community (reviewed in Miner et al. 2005). However, TMIIs in marine communities have not been well-studied in the context of macroalgae-herbivore trophic interactions (but see Long et al. 2007, Molis et al. 2010).

1.5.1. Macroalgal interactions mediated by induced defense

One plant growing close to another plant affected by simulated grazing or actual grazing may become more resistant to herbivores in terrestrial systems (reviewed in Heil & Karban 2010). This phenomenon indicates that plants can emit the cues in response to herbivore attacks and the cues can act as a warning about risk of herbivore attacks to other plants ('talking tree', Karban & Baldwin 1997, Dicke 2009). Besides terrestrial plants, there have been several studies on ‘talking macroalgae’ (i.e. brown macroalgae *Ascophyllum nodosum* and *Fucus vesiculosus*). These studies about talking in macroalgae showed that defense in the brown algae can be induced by waterborne cues derived from grazed conspecifics (Toth & Pavia 2000, Rohde et al. 2004, Toth 2007).

The feeding-cues from attacked plants can be a preventive agent to tailor the defensive traits in heterospecific plants (reviewed in Karban 2008, Heil & Karban 2010, Fig. 2-II.). The cues can be accessible to heterospecific plants, which share similarity of signaling systems (e.g. Karban et al. 2004). Moreover, the availability of heterospecific cues among preys, which are exposed to common consumer attacks, depends on a relatedness among plant community members, e.g. phylogenetic distance (Chivers & Smith 1998, Mirza & Chivers 2003, Schoeppner & Relyea 2005). So, it is likely that cue chemicals are similar among closely related preys, and that cues induce similar antiherbivore responses accordingly.

Despite lack of evidence for interspecific signaling in macroalgae, the cues from grazed macroalgae should be also important for defensive decisions of the heterospecific macroalgae. In ecological aspects, diverse macroalgal species in dense stands encounter the threat of generalist mesoherbivores. These herbivores are closely associated with their host plants, but are sensitive to the induced responses (Hay 1996). The commonness of the herbivores in marine communities may imply that grazing pressure on one algal species becomes a big threat even to different neighbor macroalgae. Indeed, the induced defenses in the grazed algal specimen can increase the mobility of the herbivores (Borell et al. 2004), probably allowing the herbivores to switch host plants. Thus, it is assumed that attacks of the mesoherbivores can be shared information to indicate feeding loss among macroalgae.

1.5.2. Herbivore interactions mediated by induced defenses

Several herbivore species with diverse dietary preferences share host plants (e.g.

Buschbaum et al. 2006), providing an arena for multiple grazer interactions. Recent studies on plant-herbivore interactions suggest that interspecific herbivore competitions are mediated by induced changes in host plant traits (reviewed in Denno & Kaplan 2007, Fig. 2-III.). Such interactions have not been well-studied in marine systems (but see Long et al. 2007, Molis et al. 2010, Yun et al. 2010), even though there is accumulating evidence about herbivory-induced trait modifications in macroalgae (Toth & Pavia 2007).

In trophic interactions, macroalgae can mediate interactions of multiple herbivores by indirect effects of their induced plant defenses. The induction of defensive traits in macroalgae varies on specificity of herbivore species because of differences in risk presented by each herbivore (Pavia & Toth 2000, Molis et al. 2006, Molis et al. 2008). Moreover, it is suggested that each herbivore has a phylogenetically different tolerance or avoidance to the chemical defenses of macroalgae (Poore et al. 2007). These consumer-specific patterns in defenses of macroalgae could ultimately impact the efficiency of the induced defenses. Thus, specificity of defense induction in host plants can regulate interspecific herbivore interactions and thereby determine superior competitors among herbivores (reviewed in Ohgushi 2005).

1.6. Aims

The herbivore-induced defenses in macroalgae may decrease their own palatability in response to direct feeding attacks, consequently becoming an undesirable food to consumers. Despite the prevalence of induced antifeeding responses in macroalgae, it is less known how herbivore-induced responses of one plant can influence other interacting herbivore and macroalgal species. This study aims to confirm the direct effects of herbivore- induced responses in terms of herbivore-associated cues, herbivore specificity, and temporal variation of herbivores. Furthermore, this study examines its indirect effects on other species using trophic interactions between herbivores and macroalgae in marine ecosystems by addressing the following questions:

In trait-mediated direct effects,

1. Similar to direct grazing, will grazer-associated cues with grazer presence or grazing activity induce the response in *Fucus vesiculosus*? (Publication 1)
2. Will the herbivore-induced response be based on changes in chemical traits of macroalgae? (Publication 1, 2, 3 & 4)
3. Will two macroalgae, native *Chondrus crispus* and non-native *Mastocarpus stellatus*, induce changes to their palatability in response to direct grazing of specific herbivores? (Publication 2) The specific herbivores included a coevolved species, *Idotea granulosa*, and a non-coevolved species, *Littorina littorea*, after comparing the herbivore composition between original habitat and introduced habitat of *M. stellatus*.
4. Will induced responses be recovered after removal of feeding attacks? (Publication 2)

In trait-mediated indirect effects,

5. Will induced responses of macroalgae increase resistances in the other macroalgae? (Publication 3)
6. Will induced responses of macroalgae affect feeding preferences of different grazers later? (Publication 4)

2. MATERIAL AND METHODS

2.1. Sampling sites and organisms

In two regions along the NE Atlantic shore (Helgoland in Germany and Faro in Portugal), several macroalgal species with diverse herbivore assemblages were selected as targeted organisms.

At Helgoland (54° 11' N, 7° 52' E), a brown macroalga (*Fucus vesiculosus*) and two red macroalgae (*Chondrus crispus* and *Mastocarpus stellatus*) were collected in ‘Bunker’ and ‘Westwatt’ of the intertidal shore. All three species occur frequently in all seasons. Contrarily, two additional browns (*Halidrys siliquosa* and *Sargassum muticum*) were sampled since they are the most abundant species in the shallow subtidal (Bartsch & Kuhlenkamp 2000). *Mastocarpus stellatus* and *S. muticum* are the non-native species, which were introduced into Helgoland less than 30 years ago, and have dominated in the intertidal and subtidal region, respectively (Bartsch & Kuhlenkamp 2000). In these sites, dominant herbivore groups were composed of isopods, amphipods, and gastropods, which are closely associated with macroalgal assemblages (Reichert & Buchholz 2006). Among mesoherbivores, I used two isopod species (*Idotea baltica* and *I. granulosa*) and one gastropod (*Littorina littorea*) for the further experiments.

At Faro in Portugal, four brown macroalgae species (*Cystoseira humilis, F. spiralis, F. vesiculosus* and *S. muticum*) were abundant. These macroalgae were collected at two sites: intertidal rocky shores of São Rafael (37°05´N, 8°15´W), and intertidal pools of Praia de Queimado (37° 49' N, 8° 47' W). As herbivore groups, either an assemblage of amphipod species (*Gammarus insensibilis*, *Gamarella fucicola* and

Cymadusa filosa) or an isopod species (*Stenosoma nadejda*) was chosen since they are associated with feeding on the macroalgae selected in this work.

2.2. General experimental set-ups

Under indoor conditions, incubation experiments were conducted with herbivores and macroalgae. At the beginning of experiments, targeted species were collected and transferred to the laboratory within 3 hours. After removal of visible epibionts, macroalgal individuals were cut into pieces (Fig. 3-I.) from the entire individual. In *C. crispus* and *M. stellatus,* whole individuals were used. Then, macroalgal pieces (or whole individuals, if applicable) were distributed to aquaria (8 l and 2.9 l at Helgoland and Faro, separately), and the algal pieces were randomly allocated either to aquaria with herbivory-related treatments or without treatments (see the detailed treatments in each of publications). In the aquaria, the algal pieces were allowed to acclimatize to the indoor set-up conditions and to recover from potential grazing damage in the field. Indeed, macroalgal relaxation from *in situ* grazing effects is known to occur within 4 days (Rohde & Wahl 2008b). Every aquarium was continuously supplied with filtered ambient seawater from near shore (flow rate: 0.25 l h^{-1} in Helgoland, 0.75 l h^{-1} in Faro). The aquaria were lit for 12 hours daily in a total irradiance of 65.5 ± 2 µmol m^{-2} s^{-1} in Portugal and 34.4 ± 2.5 µmol m^{-2} s^{-1} (LI-COR, UWQ 6534) in Helgoland.

In the beginning of induction phase, herbivores were added into the aquaria for herbivory-related treatments, while simultaneously no herbivores were put into the control aquaria (Fig. 3-II.). After induction period, the grazers were removed from every treated aquarium. Then, the macroalgal pieces were withdrawn from each of treated and control aquaria, transferred to a feeding arena and allocated for bioassays.

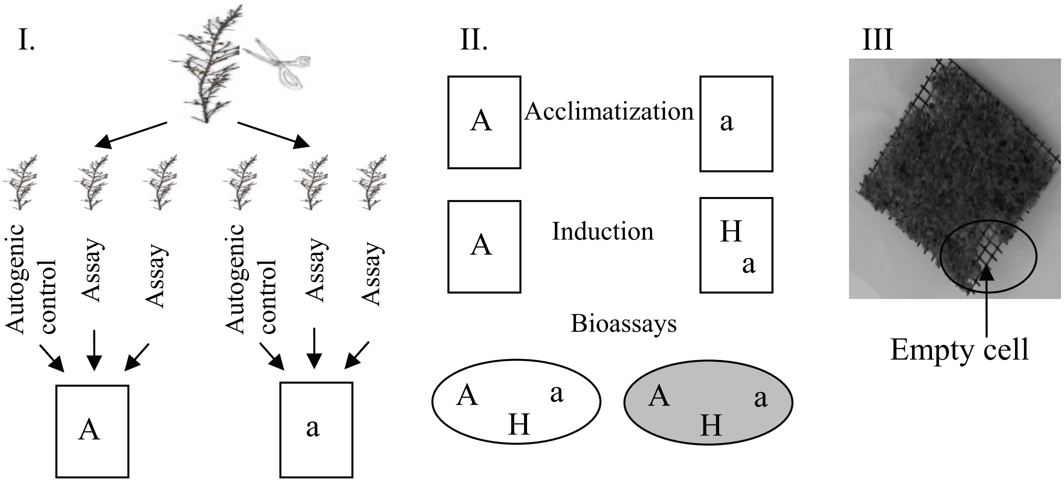


Fig. 3. Overview of experimental setups (only shown for single replicate). I) Sample preparation steps for autogenic control and assaying. The fresh samples were allocated to aquaria (large rectangles), including either the algal samplesnamed 'A' for grazing-free control or 'a' for grazing-related treatments. II) Experimental sequences. After acclimatization phase, herbivores (H) were added. After induction phase, algae from control aquaria and treated aquariawere transferred to feeding arenas (ovals) for fresh (white oval) and reconstituted food assays (grey oval). III) Photo showsa reconstituted food pellet and empty cell by grazing in bioassays.

To detect induced trait changes by herbivory, treated and control pieces were offered to herbivores and consumption amounts of each algal piece were measured. A significant preference of control over treated pieces in bioassays was considered as an induction of antiherbivore defenses. These choice experiments were performed in two types of bioassays with either fresh macroalgal pieces or reconstituted food pellets.

Firstly, fresh bioassay was used to assess defense ability (morphological and chemical traits). One pair of a treated piece and a control piece was transferred into a feeding arena (glass Petri dish, 25 cm Ø, experimental unit = EU), where herbivores could choose between one pair of treated and control food. The other pair of pieces was also transferred to an arena, accounting for non-feeding related changes in macroalgae (i.e. autogenic changes) during bioassays. After macroalgal pieces were blotted with paper towels for 15 seconds and spun 10 times in a salad spinner, their weights were measured to the nearest 0.001 g on a balance (Sartorius LE323S). In 3 days of incubation period of macroalgae with herbivores, all pieces were reweighed. Then, consumption amounts of treated and control pieces were calculated with a formula (equations from Cronin & Hay 1996): A_{start} x $(B_{end} / B_{start}) - A_{end}$, where A_{start} and A_{end} represent initial and final wet mass of an assayed piece, respectively, and B_{start} and B_{end} represent initial and final mass of the autogenic control piece, respectively.

To detect chemical trait modifications, morphological properties were destroyed by either pulverizing the pieces or extracting the feeding-deterrent chemicals from the pieces into DCM solvent or freeze-drying (Study question #2). The powder (or extractant coated with powder of generally palatable species, *Ulva* spp.) was mixed with molten agar. Then, the agar-powder mixtures were poured over a mosquito net and flattened between two glass plates (methods adapted from Hay et al. 1994). After solidification, macroalgae-agar mixtures were cut into food pellets of 15 x 15 mm^2. Reconstituted foods made of the algal pieces from treated aquarium and control aquarium were transferred into a feeding arena with herbivores for 36 hours. Feeding rates were determined by measuring the weight change or counting the empty cells (see Fig. 3-III.) in the food pellets between the start and the end of two-choice feeding assays.

2.3. Specific experimental set-ups

2.3.1. Experiment design 1: cues to induce antifeeding defenses in a macroalga

This experiment aimed to identify which grazing-related cues released from feeding activity of herbivores, can induce defenses in macroalga (Study question #1, Publication 1). Using model species of *F. vesiculosus*, multiple treatment experiments were conducted: 1) actual grazing on macroalgal pieces, 2) neighbor grazing, 3) only existence of herbivore and 4) control (Fig. 4a-I.).

To control the effects of neighbor grazing and only existence of herbivore effectively, aquaria (12 x 18 x 11 cm^3, 2 l) were divided by a plastic net (pore size: 1 mm^2) into the upstream (near the inflow of seawater) and the downstream (near the outlet of seawater) compartment. Water flow direction (1.8 l h^{-1}) was controlled from the upstream to the downstream compartment, allowing delivery of the reactant metabolites upstream to algal pieces downstream.

I.

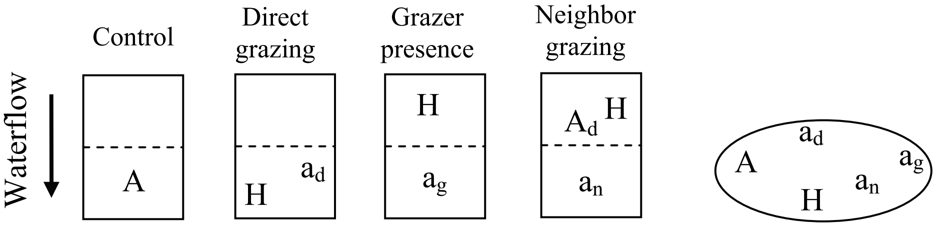


Fig. 4a. I) Experimental setup for identifying which herbivore-related cues can induce responses in macroalgae with 4 cue types: direct grazing (a_d), grazer presence (a_g), neighbor grazing (a_n) and control (A). H inidcates herbivore. Each aquarium was divided into two parts (dashed lines). After induction phase, 4 algal pieces were transferred to a feeding arena (oval) for bioassays.

After an induction period of 14 days, the pieces were withdrawn from 4 treated aquaria in the downstream compartment and were transferred to a feeding arena. Subsequently, feeding preference tests were conducted with 4 foods in bioassays.

2.3.2. Experiment design 2: comparing induced defenses in native and non-native macroalgae

To test how dynamic herbivore-induced defenses are, the responding patterns between two red macroalgae (i.e. native *C. crispus* and non-native *M. stellatus*) were compared in terms of herbivore specificity and temporal variation (Study question #3& #4, Publication 2). Species groups were compared between introduced and native habitats of *M. stellatus* and *Idotea granulosa* and *Littorina littorea* were chosen because *I. granulosa* is a coevolved herbivore and *L. littorea* is a non-coevolved herbivore of *M. stellatus*, respectively.

Since macroalgae are expected to increase their defenses strongly in response to direct grazing loss, the grazing-related treatments here consisted of direct grazing and its control. After 6 days of induction period, the macroalgal pieces were withdrawn from both the treated and control aquaria and were transferred to a feeding arena. Then, grazers were removed from macroalgae in all treatment aquaria. Subsequently, the grazed macroalgae were incubated without grazers during 6 days (reduction phase). By doing so, I examined whether the induced state was relaxed (Study question #4, Publication 2). For detecting the reduced state, bioassay tests were conducted between control and treated specimens.

2.3.3. Experiment design 3: effect of induced response on macroalgal interactions

I examined macroalgal specific ability to change the antifeeding traits in response to the waterborne signals from grazed plants (Study question #5, Publication 3). For this investigation, herbivores were added to algal pieces only in the upstream compartment of treated aquaria during 12 days of induction phase, and the grazing signals from upstream were conveyed to macroalgae in the downstream compartments via controlling the direction of waterflow (e.g. Bradbury & Vehrencamp 1998, Fig. 4b-II.). Simultaneously, herbivores were not added to the control aquaria. For testing perspective of interspecific signaling, the macroalgal species that had experienced the herbivory attack upstream were different from those downstream. Further, for detecting the possibility of intraspecific signaling, the macroalgal species exposed to the herbivory attack upstream were the same as downstream. This experiment was conducted similarly in two sites, Faro and Helgoland, using local algae and herbivore species.

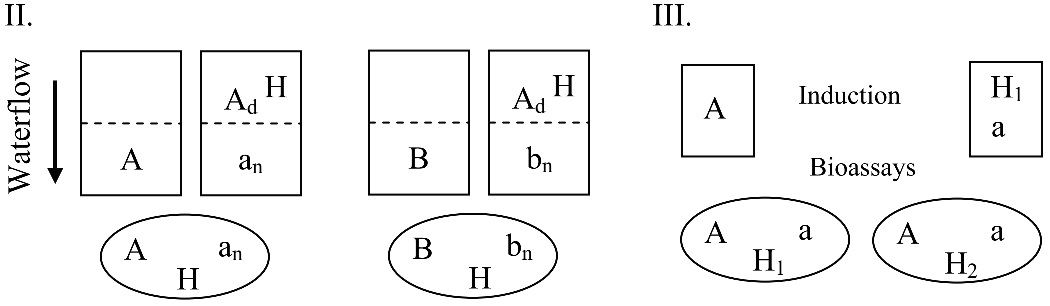


Fig. 4b. Both II) and III) test TMIIs via herbivory-induced responses in simple trophic interactions. Symbols as in Fig. 4a. II) Plant-plant-herbivore relation via the waterborne signals. B and b_n indicates different algal species from producer species (A_d) of waterborne signals exposed to herbivores. III) Plant-herbivore-herbivore relation. H_1 is one herbivore species different from H_2, which were used in inductions and bioassays.

2.3.4. Experiment design 4: effect of induced response on herbivore interactions

For investigating the efficiency of induced responses, either the same or different herbivores were involved in feeding arenas, compared to the herbivore species used for induction phase (see Fig. 4b-III.). For example, when *I. baltica* was used for inducing macroalgal responses, either the identical or different species (i.e. *L. littorea*) was used for subsequent feeding assays (Study question #6, Publication 4). Additionally, if *L. littorea* was applied in induction phase, *L. littorea* as well as *I. baltica* was offered in bioassays.

2.4. Statistics

A normal distribution in consumption rates was confirmed with the Kolmogorov-Smirnov test. Data from 4-food choice experiments (see the Fig. 4a-I.), where more than two-alternative foods were presented to consumers, were analyzed by resampling without replacement, using a Monte Carlo analysis with 10000 permutations. As post-hoc tests, paired *t*-tests were used (Publication 1).

A normal distribution in consumption rates was confirmed with the Kolmogorov-Smirnov test. Consumption rates by herbivores on two food types, i.e. directly grazed food and non-grazed food, were compared by paired *t*-tests, separately with algal species and herbivores, type of bioassay, and experimental phase (Publication 2). Despite the large number of *t*-tests in total 12 bioassays shown in this thesis, the signicifant level was not adujsted for multiple statistical tests, i.e. sequential Bonferroni adjustments.That is, the expected probability for signfiicant assays in total 12 bioassays is 1 (i.e. $12 \times 0.05 = 0.6$). Compared to the expected number for the significant assay, there were 5 significant assays observed. The number of the oberserved significant assays was statistically greater than what could be expected by chance alone, based on chi-square tests ($\chi^2 < 6.63$, $p < 0.0001$).

A normal distribution in consumption rates was confirmed with the Kolmogorov-Smirnov test. Consumption rates of macroalgae located in downstream compartments were also measured using 36 bioassays to detect the effects of intraspecific grazing *vs.* control and interspecific grazing *vs.* control, independently of two sites and two food types. The feeding amounts between treated and control food were analyzed by paired *t*-tests (Publication 3). The number of the oberserved significant assays was 11 in total (36 assays). This is statistically greater than what could be expected by chance alone (i.e. 36 x 0.05 = 1.8), based on chi-square tests ($\chi^2 < 6.63$, $p < 0.0001$). Thus, the signicifant level of sequential Bonferroni adjustments was not adujsted for multiple statistical tests.

A normal distribution in consumption rates was confirmed with the Kolmogorov-Smirnov test. To detect the herbivore sensitivity to induced defenses, consumption rates of fresh and reconstituted algae were analyzed using three-factorial repeated-measures ANOVAs. The consumption rates from one feeding assay were repeatedly measured (fixed, two levels: grazed and control), while identity of the inducer (fixed, two levels: *L. littorea* and *I. baltica*) and type of consumer (fixed, two levels: conspecifics and heterospecific) was represented by orthogonal grouping factors (Publication 4). For post-hoc tests, paired *t*-tests were used.

3. PUBLICATIONS

Publication 1. Testing for the induction of anti-herbivory defenses in four Portuguese macroalgae by direct and water-borne cues of grazing amphipods

Yun HY, Cruz J, Treitschke M, Wahl M & Molis M. (2007) Helgoland Marine Research 61:203-209

Publication 2. Comparing the ability of a native and a non-indigenous seaweed to induce anti-herbivory defenses

Yun HY & Molis M (In preparation)

Publication 3. Waterborne cues from grazed algae can induce chemical anti-herbivory defenses in conspecific and heterospecific neighbors

Yun HY, Engelen AH, Santos RO & Molis M (Submitted to Oecologia)

Publication 4. Seaweed-mediated indirect interaction between two species of meso-herbivores

Yun HY, Rohde S, Linnane K, Wahl M, Molis M (2010) Marine Ecology Progress Series 408:47-53

Helgol Mar Res (2007) 61:203–209
DOI 10.1007/s10152-007-0067-6

ORIGINAL ARTICLE

Testing for the induction of anti-herbivory defences in four Portuguese macroalgae by direct and water-borne cues of grazing amphipods

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Abstract Herbivory is a key factor in regulating plant biomass, thereby driving ecosystem performance. Algae have developed multiple adaptations to cope with grazers, including morphological and chemical defences. In a series of experiments we investigated whether several species of macroalgae possess anti-herbivore defences and whether these could be regulated to demand, i.e. grazing events. The potential of direct grazing on defence induction was assessed for two brown (*Dictyopteris membranacea, Fucus vesiculosus*) and two red seaweeds (*Gelidium sesquipedale, Sphaerococcus coronopifolius*) from São Rafael and Ria Formosa, Portugal. Bioassays conducted with live algal pieces and agar-based food containing lipophilic algal extracts were used to detect changes in palatability after exposure to amphipod attacks (=treatment phase). *Fucus vesiculosus* was the only species significantly reducing palatability in response to direct amphipod-attacks. This pattern was observed in live *F. vesiculosus* pieces and agar-based food containing a lipophilic extract, suggesting that lipophilic compounds produced during the treatment phase were responsible for the repulsion of grazers. Water-borne cues of grazed *F. vesiculosus* as well as non-grazing amphipods also reduced palatability of neighbouring conspecifics. However, this effect was only observed in live tissues of *F. vesiculosus*. This study is the first to show that amphipods, like isopods, are capable to induce anti-herbivory defences in *F. vesiculosus* and that a seasonally variable effectiveness of chemical defences might serve as a dynamic control in alga–herbivore interactions.

Keywords Amphipods · Anti-herbivory response · Induction · Macroalga-grazer interaction · Portugal · Seaweeds

Introduction

Herbivory is a key factor for controlling biomass accrual and community structure of macroalgae (Hay and Fenical 1988; Hay 1991; Cyr and Pace 1993) and, thus, affecting a central ecosystem service. Depending on the food web structure of a system, meso-herbivores (Brawley 1992) may benefit from the presently observed overexploitation of predatory fish (Myers and Worm 2003), which would enhance their impact on macroalgae. Despite their low mobility and consumption rates relative to macro-herbivores (e.g. fishes), meso-herbivores (e.g. amphipods) due to their elevated numbers and more site-bound life histories may shape species composition of macroalgal communities (Brawley and Adey 1981; Duffy and Hay 2000).

In contrast to macro-herbivores, meso-herbivores may use algae as both food and habitat because they consume only part of an algal individual (Hay et al. 1987). This more lasting but less destructive association should influence algal responses to meso-herbivory. Algae can actively deter herbivores morphologically and/or chemi-

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cally (Hay 1996; Cronin 2001). To cope with a typically more sudden, more intense but less permanent attack by macro-herbivores such as fishes, a constitutive defence may be optimal. In contrast, meso-herbivores consume smaller portions of their food over longer time spans, which could favour the selection for defences that are adjusted to grazing pressure (inducible defence). In an evolutionary context, macroalgae displaying inducible anti-herbivory defences may have a selective advantage over constitutively defended algae for several reasons. First, the risk of self-intoxication is reduced because the production and storage of potentially harmful chemicals is minimised (Agrawal and Karban 1999). Second, algae with inducible defences represent a temporally and spatially variable (''moving'') target to herbivores, exacerbating herbivore adaptations to defensive algal traits (sensu Karban et al. 1999). Third, meso-herbivores move from induced to non-induced algae, which in turn exposes them more to predators (Borell et al. 2004).

An induction of anti-herbivory defences is well documented for vascular plants (Karban and Baldwin 1997), but only limited evidence—essentially restricted to brown algae—exists for seaweeds. Present knowledge suggests that anti-herbivory defences in algae are a quite differential response. For instance, Pavia and Toth (2000) demonstrated that grazer identity matters as to whether defences were induced or not. Moreover, algal responses may be tissue-specific, when valuable algal parts, e.g. holdfasts, display constitutive and other tissues inducible defences (Taylor et al. 2002). Seasonal patterns of induction add further complexity to alga-grazer interactions (Molis et al. 2006). Induced defences were shown to be temporally and spatially variable. For example, the defence levels in *F. vesiculosus* were lowered to pre-grazed levels within 10 days (Hemmi et al. 2004). In addition, the repulsive response was not spreading to adjacent tissue (Hemmi et al. 2004). However, Toth and Pavia (2001) suggested that the information to defend may be propagated by water-borne cues within and between individual algae. To date only few studies have demonstrated the effects of water-borne cues in the context of inducing anti-herbivory defences in macroalgae (Toth and Pavia 2000; Rohde et al. 2004; Macaya et al. 2005). Knowledge on whether effective water-borne cues stem from the grazer or the alga is extremely scarce. Furthermore, little is known about the identity of chemical compounds that are induced during anti-herbivory responses.

The aim of this study was to test whether (1) direct grazer attacks induce defences in selected red and brown seaweeds, (2) water-borne cues from either conspecifics and/or grazers could induce anti-herbivory defences, and (3) the effects of the water borne cues might decrease, after grazing stopped.

Materials and methods

Collection sites and organisms

All algae and herbivores were collected at the intertidal rocky shores of São Rafael, Albufeira (37°05′N, 8°15′W) and the lagoon-system Ria Formosa (37°1′N, 7°55′W), Portugal. To ensure the ecological relevance of the research, study algae were selected by regional abundance and their supposed role as habitat engineers (large, bushy, perennial), and relevant herbivores were selected after assessing feeding preferences in pilot studies (data not shown). As a result, we chose two species each of red (*Gelidium sesquipedale* and *Sphaerococcus coronopifolius*) and brown seaweeds (*Dictyopteris membranacea* and *Fucus vesiculosus*). As herbivores, we selected an assemblage of three amphipod species (*Gammarus insensibilis, Gamarella fucicola*, and *Cymadusa filosa*) naturally associated with and feeding on the selected macroalgae at both collection sites. Hereafter algae are referred to by their generic names and herbivores as amphipods.

Experimental set-up and design

We conducted two induction experiments, one in July and one in September 2003. These were divided into either two (acclimation and treatment, July experiment) or three (additional recovery, September experiment) 2-week phases. First, the effects of herbivore attack on the induction of anti-herbivory defences in live algal pieces were assessed for all four selected species of macroalgae. Treatment effects, i.e. change in palatability, were identified by feeding assays, where after the treatment phase the consumers could choose between a grazed and a non-grazed algal piece. To test whether an observed induction was based on non-polar secondary metabolites, we conducted feeding assays with artificial food containing broad-spectrum DCM extracts. The effects of water-borne cues were assayed in the September experiment with only *Fucus.*

For the July experiment, five specimens of each algal species ($n = 5$) were collected and transferred in a cooler to the Marine Laboratory of Ramalhete, Faro, Portugal, within 2 h after collection on 18 July 2003. On the same day, all macroscopic epibionts were gently removed from all algae with a sponge. Six pieces (ca. 3 g per piece) of comparable tissue type were cut from each specimen. These six pieces were distributed at random between one control and one treatment aquarium (experimental units EUs), resulting in a total of 40 EUs (4 species × 5 individuals × 2 treatment levels) containing three genetically identical thallus pieces each. Each transparent plastic aquarium (2 l volume) was individually supplied with a continuous, unidirectional flow of seawater from a reservoir directly connected to the coastal lagoon next to the marine station.

At the start of the July experiment, algal pieces remained for 14 days without consumers (acclimation phase) to adjust to cultivation conditions and to equalise possible differences in defensive traits, which may had been acquired by unknown grazing histories in the field. After acclimation, one (for *Fucus* and *Dictyopteris*), two (for *Sphaerococcus*), or three (for *Gelidium*) amphipod individuals were randomly drawn from the three-species mixture and added to each treatment EU and allowed to consume the macroalgae for 14 days, while no grazers were added to the control EUs (treatment phase). Thus individual study algae were exposed to one to three grazer species, but averaged over all replicates for a given algal species, the consumption quantified was due to the naturally occurring mixture of the three-amphipod species. Working with different consumer species within one treatment increases the scatter of results but reflects what is happening in the field. Grazer densities varied among algal species according to observed densities in the field in order to keep grazing pressure in our experiment comparable to natural levels of herbivory. At the end of the treatment phase, grazers were removed. The palatability of live algal pieces, and, when appropriate, artificial food was assessed in choice feeding assays (see below).

For the September experiment, seven *Fucus* specimens (n = 7) were collected in the Ria Formosa lagoon on 13 September 2003. All EUs were vertically divided by a mesh (1 mm mesh size) into equally sized up- and downstream compartments. Twenty pieces of comparable tissue-type were cut from single plants and five pieces each randomly allocated to the downstream compartments of four EUs at the beginning of the acclimation phase (7 plants × 4 treatments = 28 EUs total). Four different treatment levels were allocated to the EUs: (1) addition of amphipods to the downstream compartment (test for direct grazer attack), (2) addition of amphipods and *Fucus* pieces to the upstream compartment (test for water-borne cues from nearby grazed conspecific algae and/or grazing amphipods), (3) addition of amphipods to the upstream compartment (test for water-borne cues from non-grazing amphipods), and (4) no addition of amphipods or algae to the upstream compartment (control). At the end of the treatment phase, all herbivores were removed from the EUs as well as all *Fucus* pieces from the upstream compartments. Moreover, 2 algal pieces were randomly withdrawn from the downstream compartment of each EU and used for either multi-choice feeding assays of live pieces or agar-based food containing lipophilic *Fucus* extracts, respectively. Two of the other three algal pieces remained in the EUs for another 14 days (recovery phase). The third piece was transferred during the assay period into a separate, amphipod-free Petri dish (see next paragraph) to measure autogenic changes in wet mass and returned to EUs after assays were terminated. At the end of the recovery phase, the same algal piece was used again to measure autogenic changes in wet mass during assays. The remaining two algal pieces were withdrawn from EUs to assess their palatability levels in multi-choice feeding assays of either live pieces or agar-based food (testing for reduction of the defence after treatment).

Feeding preference assays

Two-way choice (July experiment) or multiple-choice (September experiment) feeding assays compared the relative palatability of differently treated algal pieces. In the July experiment, one live piece of a control and a grazed alga were offered to amphipods simultaneously in a Petri dish (15 cm Ø) for 4 days. The water in Petri dishes was exchanged every 12 h. In the September experiment, 4 differently treated algal pieces each (see above) was offered in the same Petri dish. For distinction during the feeding assays, algal pieces were coded by coloured threads. In the multiple-choice assay with artificial food, the pellets were individualized by different incision patterns. Different amphipod individuals were used in the treatment phase and in subsequent feeding assays to avoid consumer adaptations. Wet mass of assayed algal pieces was measured to the nearest 0.001 g at the beginning and end of feeding assays. Moreover, the wet mass change of a control algal piece from the same treatment as the assayed alga was used to correct consumption rates for non-feeding-related autogenic changes in wet mass, according to the Peterson and Renaud (1989) formula:

$$\text{consumption} = T_{\text{start}} \times (C_{\text{end}}/C_{\text{start}}) - T_{\text{end}}$$

where T_{start} and T_{end} represent pre- and post-assay wet mass of pieces of an assayed alga, respectively, and C_{start} and C_{end} represent pre- and post-assay wet mass of autogenic control pieces, respectively.

Preparation of artificial food

To determine whether the induced defences were due to secondary metabolites (activated or induced only for defensive purposes), feeding assays with lipophilic algal-extracts were performed for those algae that showed significant responses in assays using live algal pieces. Algal pieces were submerged in 10 ml of dichloromethane for 2 day at room temperature. Subsequently, the dichloromethane supernatant was poured onto *Ulva lactuca* powder and allowed to evaporate. In this manner, the *Ulva* powder was coated by the non-polar algal compounds. *Ulva* has been used previously as a standard food, palatable to most herbivores, in assays using algal extracts (e.g. Deal et al. 2003). A mixture of 0.72 g Agar and 10 ml distilled water was boiled, allowed to cool to ca. 40°C, mixed with the algal compound-coated *Ulva* powder, and poured over a mosquito mesh flattened between two fibreglass panels (method adapted from Hay et al. 1994). After cooling, a 15×15 mm^2 section was cut from each pellet and used in feeding assays.

Statistical analysis

Consumption rates from choice feeding assays (July experiment) were analysed by two-tailed paired *t*-tests. Prior to statistical analyses, the normal distribution of differences between control and grazed alga pieces was confirmed with the Kolmogorov–Smirnov test. Consumption rates from multiple-choice feeding assays (September experiment only) were analysed by resampling without replacement, using a Monte Carlo analysis with 10,000 permutations (Bärlocher 1999). Pairwise *t*-tests were used as post-hoc tests, for which no Bonferroni correction was calculated, if the probability of finding that the number of significant *t*-tests by chance was below 5% (Moran 2003).

Results

July experiment

At the end of the treatment phase, live specimens of *Gelidium*, *Sphaerococcus*, and *Dictyopteris* showed a non-significant trend of higher palatability in control pieces than in amphipod-attacked pieces (all paired *t*-test: $t_4 \leq 1.85$, $P > 0.05$). Only for live *Fucus* pieces, amphipod consumption of control pieces was significantly higher (by 60% on average) than consumption of amphipod-attacked pieces (paired *t*-test: $t_4 = 4.83$, $P = 0.008$, Fig. 1). This pattern was also detected when agar-based food containing lipophilic *Fucus*-extracts was used. Control pieces were significantly preferred by 31% over agar-based food containing lipophilic extracts from amphipod-attacked conspecifics (paired *t*-test: $t_4 = 4.67$, $P = 0.01$, Fig. 2).

September experiment

The experiment was performed with *Fucus*, the only species that had shown a significant reduction in palatability in response to amphipod attacks.

1. Live *Fucus*: At the end of the 2-week treatment phase, amphipod consumption was significantly different between differently treated *Fucus* pieces (resampling, $P = 0.019$). The palatability of individuals exposed to water-borne cues from nearby amphipod-grazed conspecifics and/or grazing amphipods, as well as to non-grazing amphipods, was significantly lower than the palatability of control pieces, while the palatability of directly grazed *Fucus* pieces was non-significantly decreased (Fig. 3a). Control pieces were on average 48% more palatable than grazed pieces. At the end of recovery phase, the palatability of *Fucus* controls increased even more. Control pieces were on average a significant 91% more consumed than *Fucus* pieces exposed to the other three treatments (resampling $P = 0.050$).

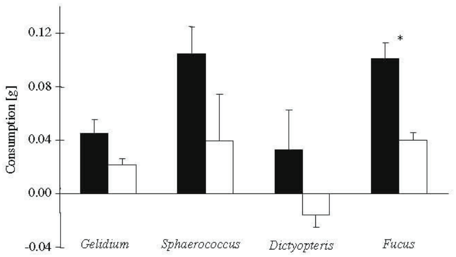

Fig. 1 Mean (+SE) amphipod-consumption on live pieces of different alga species during 4-day long feeding assays ($n = 5$) at the end of the treatment phase (see "Materials and methods" for details). *Black bars* controls, *open bars* directly amphipod attacked, * significantly different

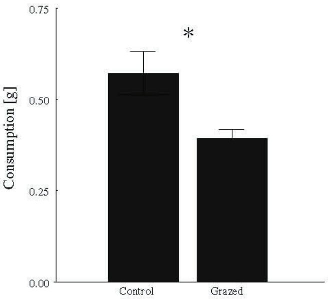

Fig. 2 Mean (+SE) amphipod-consumption of artificial *Fucus vesiculosus* food during 4-day long feeding assays ($n = 5$) at the end of the treatment phase (see "Materials and methods" for details). * Significantly different

2. Agar based food containing lipophilic *Fucus*-extracts. At the end of both treatment and recovery phase, amphipod consumption was not significantly affected by grazing treatments (resampling: treatment phase $P = 0.137$, recovery phase $P = 0.561$; Fig. 3b).

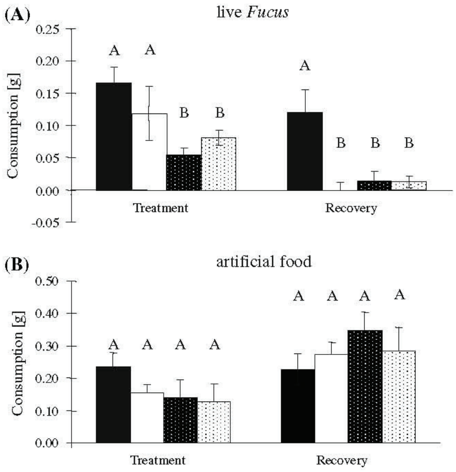

Fig. 3 Mean (+SE) amphipod-consumption on *Fucus vesiculosus* during 4-day long feeding assays ($n = 7$) at the end of the treatment and recovery phase (see "Materials and methods" for details) for **a** live algae and **b** agar-based food containing lipophilic *Fucus*-extracts. Treatments sharing the *same letter* are not significantly different. *Black bars* control, *open bars* directly amphipod attacked, *white dotted bars* waterborne cues from nearby-grazed conspecifics, *black dotted bars* waterborne cues from non-grazing amphipods

Discussion

In all algae species tested in the July experiment, control pieces were preferred over grazed pieces, but this was statistically significant only for *Fucus*. Also in the case of assays using artificial food containing lipophilic *Fucus*-extracts, control pieces were preferred over directly grazer-exposed pieces. As shown in the September experiment, exposure of *Fucus* to water-borne cues from nearby compartments containing either amphipods grazing on *Fucus* or amphipods alone, also reduced the palatability of live alga pieces but not that of artificial food containing a respective alga extract. A change in palatability of grazed *Fucus* may theoretically also occur if the consumers removed the more palatable parts of the alga, leaving less palatable portions untouched. However, this seems unlikely because amphipods consumed only a small fraction, i.e. <5%, of *Fucus* wet mass during the treatment phase. In addition, control pieces of *Fucus* continued to be more palatable than grazed pieces even after grazing had ceased for 14 days and new tissues had been formed. Alternatively, this change in palatability suggests that direct amphipod grazing induced an anti-herbivore response of morphological and/or chemical origin. In addition, the significant reduction in consumption of artificial food containing extracts of amphipod-attacked *Fucus*, hints at an induced production or mobilization of lipophilic, i.e. non-phlorotannin, compounds with anti-herbivory effects. An induction of chemical anti-herbivory compounds in brown seaweeds has been reported by several studies (e.g. Rohde et al. 2004). The role of phlorotannins has been well studied in this context, but results are controversial. Algal palatability after induction of phlorotannins stimulated (Pavia et al. 1997), decreased, or did not affect (Pavia and Toth 2000) seaweed consumption by the mesograzers *Littorina obtusata* or *Idotea granulosa*. Also, even at elevated concentrations, phlorotannins lack repulsive effects against at least some mesograzers, including amphipod species (Kubanek et al. 2004). The rigorous bioassay guided study of Deal et al. (2003) revealed that *F. vesiculosus* compounds, which were repulsive to urchins, were neither within the phlorotannin- nor the DCM-soluble fraction. According to the present study, and contrary to Deal et al. (2003), an amphipod repulsive compound seems to occur primarily in the lipophilic fraction of the *Fucus*-chemistry. In addition to chemical defences, amphipods may induce morphological anti-herbivory defences in *Fucus*. An induction of morphological defences has been described for other *Fucus* species. For example, *F. distichus* generates adventitious branches in response to the attack of littorine snails (van Alstyne 1989). We did not observe the production of new tissues, but cannot rule out that *Fucus* induced less obvious morphological changes, e.g. tissue toughness or blade shape (Lowell et al. 1991; Ruuskanen and Back 1999), for which no measurements were taken. But morphological defences—if induced—were in any case not the only ones, since a (slightly smaller) anti-herbivory activity was also found in the extracts.

Inductive patterns were altered in two ways when experiments were repeated later in the year. On the one hand, anti-herbivory defences were detected with some delay in live *Fucus*, i.e. only at the end of the recovery phase, and on the other hand effects were not detected with artificial food containing lipophilic *Fucus*-extracts. It could be speculated that the delayed inductive response may result from a seasonally variable herbivore activity. For example, *Fucus* sensitivity to amphipod grazing may change seasonally, due to differential allocation of defensive compounds in response to seasonal performance for growth and reproduction. Second, seasonal differences in amphipod abundance are known to affect grazing pressure on algae (e.g. Worm 2000), but these data are missing for our study site. Finally, amphipod activities are temperature-dependent. Decreasing water temperature lowers grazing rates in molluscs, isopods, or urchins (Jonne et al. 2006). In the present study, temperature dropped an almost negligible 2°C between the July and the September experiments, making temperature-dependent effects on amphipod grazing unlikely. Seasonal differences in the inducibility of anti-herbivory defences were rarely investigated, but reported for the kelp *Ecklonia cava* (Molis et al. 2006). Here water temperature had been identical across seasons, suggesting that factors other than temperature drove changes in repulsive responses in *Ecklonia cava*. The second deviation from the July experiment, i.e. lacking effects in assays using artificial food containing lipophilic *Fucus*-extracts, is interesting. This may be due to changes in the identity of effective algal anti-herbivory compounds across seasons. Phenotypic plasticity is expected to lower the risk of consumer co-evolution (Agrawal and Karban 1999). If algae vary the kind of anti-herbivore compounds in combination with plastic responses, the risk of co-evolving consumers should be further lowered compared to inducing always the same defensive compound, as consumers need to adapt (1) to different defence molecules that are (2) not constantly present in the food.

Amphipods consumed significantly less tissue from *Fucus*-specimens located down-stream of nearby grazed conspecifics than from control algae. This suggests an induction of anti-herbivory defences by water-borne cues, which has been already shown for three species of macroalgae, including *F. vesiculosus* (Pavia and Toth 2000; Rohde et al. 2004; Macaya et al. 2005). At the collection site, *Fucus* grows in dense stands. This facilitates the action of water-borne cues, which confer to *Fucus* conspecifics information about impending grazing (Rohde et al. 2004). Our study extents existing evidence about water-borne induction responses in *Fucus* driven by isopod grazers to amphipods. In contrast to the study by Rohde et al. (2004) dealing with isopod grazers, the present study indicates that water-borne info-chemicals released by the amphipod grazer alone, induced anti-herbivory defences in *Fucus*. Water-borne cues from mere grazer presence were generally considered as a deficient signal to induce stronger response to grazer attacks (van Alstyne 1988; Toth and Pavia 2000; Rohde et al. 2004) rather than direct and nearby grazing. However, our study reveals that *Fucus* may be sensitive to even non-grazing amphipods. This algal susceptibility to amphipods alone is surprising because amphipods use *Fucus* also as a habitat. Thus, *Fucus*-specimens should permanently receive the inducing cue, which should lead to a permanent induction, i.e. a de facto constitutive defence. According to Karban et al. (1999), this quasi-constitutive response should minimise for the algae the selective advantage of using water-borne cues from non-grazing herbivores to induce anti-herbivory defences. Dissipation and dilution of cues emitted by the amphipods were possible much lower in our experimental units than in the natural environment. Possibly, this leads to cue concentrations, which in the field, would signal particularly high amphipod densities. Alternatively, *Fucus* may benefit from the ability to initiate defences as soon as the first signals of a strong grazer density are perceived, if amphipod abundances fluctuate sharply, resulting in sudden changes of grazing pressure. Testing this hypothesis requires identification of signal thresholds as well as seasonal monitoring of amphipod abundance. Finally, widespread induction response by *Fucus* should increase the number of distasteful conspecifics, which in turn may increase amphipod movements among algae, increasing amphipod visibility to their predators (Borell et al. 2004).

In conclusion, the effectiveness of lipophilic compounds as well as speed and strength of inducible anti-herbivory defences were variable in time. This variation adds complexity to algae-mesoherbivore interactions, because inducible algae remain at least to a certain extend part of herbivore's diet rather than constitutively defended algae. Therefore, inducible defence could possibly advance stability in benthic systems similar to what is known from planktonic systems (Verschoor et al. 2004).

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Publication 2

Comparing the ability of a native and a non-indigenous seaweed to induce anti-herbivory defenses

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ABSTRACT

Phenotypic plasticity frequently is considered to be an important mechanism of successful species invasion. Few studies, however, have evaluated whether non-indigenous and native species differ in their ability to use plastic antifeeding resistance when introducing new habitats. Here, we investigated whether grazing by the periwinkle *Littorina littorea* and the isopod *Idotea granulosa* induced anti-herbivory defenses in one non-indigenous (*Mastocarpus stellatus* Stackhouse Guiry) and one native (*Chondrus crispus* Stackhouse) species from a site in Helgoland, Germany. In Iceland, as donor region of Helgoland *M. stellatus* population, periwinkles are missing whereas isopods are present. At our study site, algal growth was measured to assess whether induced defenses incurred metabolic costs. Furthermore, feeding preferences of each grazer species were determined. *L. littorea* preferred *C. crispus* over *M. stellatus*, whereas *I. granulosa* showed no preference for either algal species. Moreover, grazing by *I. granulosa* but not *L. littorea* induced defenses in *M. stellatus* that were functional even after grazing ceased. Induced responses did not affect growth of *M. stellatus*.

Grazing of *C. crispus* by *L. littorea* induced resistance in both seasons, whereas grazing by *I. granulosa* triggered season-specific responses. Furthermore, inducible defenses decreased the growth rates of *C. crispus*. It indicates that the non-indigenous seaweed exhibited permanent defenses against periwinkle, whereas isopod induced defense responses in both algal species. Thus, success of the non-indigenous *M. stellatus* in the recipient region appeared to be linked to the use of constitutive defenses against an unfamiliar grazer species and cost-saving induced defenses against a familiar grazer species.

Key index words: consumer, gastropod, inducible defenses, mesoherbivores, non-indigenous species, phenotypic plasticity, rocky shore, trophic interaction

INTRODUCTION

Herbivory strongly affects biomass accumulation of plants (Elton 1958, Cyr & Pace 1993, Keane & Crawley 2002), which may ultimately influence the species composition of plant communities (e.g. Duffy & Hay 2000). Herbivory also can promote phenotypically plastic responses in plants and seaweeds, such as the induction of anti-herbivory defenses (Karban & Baldwin 1997, Fordyce 2006, Toth & Pavia 2007). In other words, plants utilize morphological and/or chemical modifications to reduce their vulnerability to the impact of herbivory. Induced anti-herbivory responses should be favored over constitutive defenses when the risk of consumption is relatively high but variable in space and/or time (Karban & Nagasaka 2004), which is generally the case in temperate habitats where herbivores are more abundant and active in summer than in winter.

Induced plant responses to herbivory are considered to be an adaptive strategy because they save resources that can be allocated to other metabolic processes, like growth or reproduction (Karban & Baldwin 1997, Agrawal 2005). However, little is known about the costs associated with the expression of induced defenses in seaweeds. If the defenses require negligible costs, they can be expressed all the time without reduction in fitness and therefore would not be tied directly to the risk of attack. In contrast, defenses with high fitness costs should be optimized to the actual risk of herbivory, which would prevent the allocation of resources to unnecessary defenses until they are required (reviewed in Strauss *et al.* 2002).

Inducible defenses vary among herbivores. The identity of grazers, which cause severe damage to their prey, affects the appearance of herbivory-induced responses (Pavia & Toth 2000, Toth & Pavia 2007). Seasonal variations in herbivory, which mainly are caused by seasonal increases in herbivore abundance (Molis et al. 2006), also influence the levels of induction of anti-herbivory responses. The efficacy of induced responses relies on temporal dynamics of herbivory, resulting in reversibility of the responses. For example, induced levels can recover to their original state after impacts from grazers disappear (Karban & Agrawal 2002, Rohde & Wahl 2008b).

Although the induction of anti-herbivory responses by native grazers feeding on seaweeds is well documented (Toth & Pavia 2007), less is known about the effects of grazers on non-indigenous seaweeds in their new range. When seaweeds are introduced to new habitats, they may encounter grazers that do not exist in their home range. In this case, the non-indigenous alga will be exposed to novel herbivore cues, which should lower the likelihood that the seaweed will induce the proper responses (Verhoeven et al. 2009). In contrast, if herbivores coexist with that algal species in its old range, feeding cues from herbivores should be able to trigger inducible defenses in the new habitat (Smith 2009). Recent studies demonstrated the existence of non-grazer specific chemical traits in non-indigenous plants that can deter herbivores (Joshi & Vrieling 2005, Wikström et al. 2006, Eigenbrode et al. 2008, Cańo et al. 2009).

In this study, we examined how two species of seaweed, *Mastocarpus stellatus* (non-indigenous to Helgoland) and *Chondrus crispus* (native to Helgoland), responded to grazing by the periwinkle *Littorina littorea* and the isopod *Idotea granulosa*. In Iceland, which is the donor region of Helgoland *M. stellatus* population, *L. littorea* does not exist (Ingolfsson 2006); thus, the *M. stellatus* population from Iceland had not been exposed to *L. littorea* grazing. We measured the relative palatability of the native red alga *C. crispus* and the non-indigenous seaweed *M. stellatus* (both collected from Helgoland), as well as their ability to induce and reduce defenses against local herbivores. We also investigated different feeding preference patterns of herbivores and induced defenses of *M. stellatus* from Helgoland (introduced range) and from Iceland (old range). The goal of the comparison between the sites was to determine whether feeding preferences and patterns of induced defenses in *M. stellatus* were consistent in the donor and recipient regions. To evaluate defense dynamics in light of seasonal variation in trophic interactions between seaweeds and mesograzers, experiments were conducted in spring and fall.

Using two-choice feeding assays in the laboratory, we tested whether 1) grazers showed different patterns in feeding preferences between the native *C. crispus* and the non-indigenous *M. stellatus* and between *M. stellatus* from the donor and recipient regions, 2) the ability to induce anti-herbivory defenses against both grazer species differed between the native and indigenous seaweed, and 3) putative costs in anti-herbivory responses differed between the seaweed species.

MATERIALS AND METHODS

Study site and organisms

Our target algal species was *M. stellatus*, which was accidentally introduced from Iceland to Helgoland about 25 years ago (Kornmann & Sahling 1994). Since then, it has dominated the upper intertidal region and is present throughout the intertidal zone (Bartsch & Kuhlenkamp 2000). The native seaweed *C. crispus*, which coexists with and appears ecologically and morphologically similar to *M. stellatus* (Lüning 1990), was selected for study. These two red seaweeds co-occur on the rocky intertidal platform of Westwatt, Helgoland, NE Atlantic (53°11´N 7°53´E). Several species of mesoherbivores (size < 2.5 cm), mainly gastropods and isopods, also inhabit the algae-dominated study area (Reichert & Buchholz 2006). Among the grazers, two generalist feeders, the periwinkle *L. littorea* and the isopod *I. granulosa,* were selected for study because they are very abundant at the study site (e.g. Eschweiler et al. 2009), consume considerable amounts of *M. stellatus* (unpublished data HY Yun), and differ in distribution: in Iceland, which is the donor region for Helgoland population of *M. stellatus*, *L. littorea* does not occur, whereas *I. granulosa* inhabits both regions (Ingolfsson 2006, Reichert & Buchholz 2006).

Experimental design and set-up

Feeding preference experiments. Choice feeding experiments were conducted to evaluate whether *L. littorea* and *I. granulosa* exhibit a different feeding preference for *C. crispus* or *M. stellatus* (inter-specific preference: 1 to 10 May 2006) and for *M. stellatus* from Helgoland or Iceland (population-specific preference: 1 to 10 November 2006).

All grazers and macroalgae except *M. stellatus* from Iceland were collected at Westwatt and transported within 1 hour to the laboratory at the Biologische Anstalt Helgoland, Germany. Specimens of *M. stellatus* were also collected at Hafnarfjörður, southwestern Iceland, NE Atlantic (N: 64° 04′, W: 21°60′). All specimens of *I. granulosa* and *L. littorea* used in feeding preference experiments were kept until the start of experiments on a mixed algal diet in an aerated container (50 L) with water flow-through. On the day of algae collection, all visible epibionts were removed with a soft sponge without damaging the algae. Subsequently, each algal specimen was spun separately 15 times in a salad spinner and blotted between paper towels to remove additional water before being weighed with a laboratory scale (Sartorius 1602 MP) to the nearest 0.0001 g. Next, one specimen of each algal species (mean ± SE initial wet mass *C. crispus* 0.67 ± 0.04 g and *M. stellatus* 0.77 ± 0.06 g) was placed in one transparent plastic aquarium (8 L) to test for inter-specific feeding preference. To explore population-specific preference for *M. stellatus*, one specimen from each *M. stellatus* population (initial wet mass Helgoland 0.33 ± 0.03 g and Iceland 0.25 ± 0.02 g) was placed in one aquarium. A total of 40 (inter-specific preference) and 28 (population-specific preference) aquaria were used in the experiments. Aquaria were supplied with a unidirectional flow-through (25 ml min^{-1}

flow rate) of ambient, cotton-filtered seawater. Fluorescent lamps illuminated the aquaria at a mean irradiance of 34.4 ± 2.5 µmol $s^{-1}m^{-2}$ (LI-COR, UWQ 6534) with a light-dark cycle of 12:12 hours. This light regime corresponded to ambient PAR levels at 1 m water depth during the time when the experiments were conducted (unpublished data HY Yun).

Experiments were conducted with 10 replicates (inter-specific preference) and 7 replicates (population-specific preference) respectively. To assess herbivore selectivity, each replicate was setup with 4 aquaria by adding either five *I. granulosa* or five *L. littorea*, and each control. After a 6 day grazing period, grazers were removed from the set up and all algae were weighed. To account for non-feeding related changes in wet mass (i.e., autogenic changes) during bioassays, actual consumption (AC) of both grazer species was corrected using the formula: $H_b \times (C_e/C_b) - H_e$, where H and C represent the wet weight of an algal specimen used in a feeding assay and as an autogenic control, respectively, and the subscripts b and e indicate the beginning and end of feeding assays, respectively (equation adopted from Cronin & Hay 1996). Autogenic controls had the same treatment history (grazer present or absent) as the algal pieces used in the assays and the same sample size to minimize type 1 errors due to low variability in the assessment of non-feeding related change in wet mass (Roa 1992).

Induction experiment. To assess whether an herbivore-specific ability to induce anti-herbivory defenses exists in native *C. crispus* and non-indigenous *M. stellatus*, induction experiments with *L. littorea* and *I. granulosa* were conducted in spring (20 March to 10 April 2006) and fall (10 October to 1 November 2006) (Fig. 1). The day on which the experiments were started, 200 specimens of *C. crispus* (initial wet mass spring: 0.69 ± 0.05 g, fall: 0.26 ± 0.01 g), *M. stellatus* from Helgoland (spring: 0.46 ± 0.02 g, fall: 0.56 ± 0.15 g), and *M. stellatus* from Iceland (only fall: 0.28 ± 0.02 g) without severe grazing marks were collected in the field. All macroscopic epibionts were removed with a soft sponge without damaging the algae. For each type of algae, there were 40 aquaria for the 200 *C. crispus* samples, 40 aquaria for the 200 *M. stellatus* Helgoland samples, and 40 aquaria for the *M. stellatus* Iceland samples. Within aquarium, five randomly selected specimens were included (for specifications see above) after marking the five group members individually with colored threads. Subsequently, algae were kept for 4 days without grazers in the set-up for acclimatization of algae to laboratory conditions and to allow for a reduction of putative *in situ* grazing effects; such effects are known to occur in the brown seaweed *F. vesiculosus* within 4 days (Rohde & Wahl 2008b).

At the beginning of induction phase, 10 aquaria containing *C. crispus* and *M. stellatus* were left without grazers (control), whereas 10 aquaria received 10 *I. granulosa* individuals and 10 aquaria received 10 *L. littorea* individuals (grazed). All aquaria were covered with sheets of Plexiglas to hinder grazer escape. At the end of the induction phase, all herbivores were removed from the set-up along with three algal pieces from each aquarium (Fig. 1). The remaining two algal pieces in each aquarium were used to test whether putative induced anti-herbivory defenses in algae disappeared during the next 6-day-long grazer-free period (reduction phase).

Bioassays. Two types of bioassays were used for each species of seaweed. First, bioassays using fresh algae were used to assess whether grazing induced chemical and/or morphological anti-herbivory defenses in the seaweeds at the end of the induction and reduction phases. Prior to the 3-day-long bioassays, the wet mass of specimens with and without exposure to grazers in the induction phase was measured to the nearest 0.0001 g after the algae was blotted dry with paper tissue for 10 seconds and spun 15 times in a salad spinner. One previously grazed and one non-grazed specimen were transferred to a feeding arena (glass Petri dish, experimental unit = EU) containing 1.5 L of seawater. Feeding arenas (n = 10) contained either five specimens of *I. granulosa* or five *L. littorea*. To avoid confounding effects of grazer adaptations to food quality, naïve grazer individuals were used in these feeding assays. Seawater was exchanged every 12 hours and algal specimens were re-weighed as described above at the end of bioassays. Actual consumption of algae was determined by correcting for autogenic changes (see description in the feeding preference experiments section). The autogenic change in wet mass was measured from one algal specimen in the aquarium during the reduction phase (Fig. 1).

Second, reconstituted food assays were conducted after the induction phase to assess whether induced anti-herbivory defenses were chemical in nature. Due to the small size of the algae, two specimens were jointly used in the preparation of food pellets (Fig. 1). To reconstitute algal structure, algae were freeze-dried after the induction phase and ground to a homogenously fine powder with a mortar and pestle. Next, 0.2 g of algal powder was suspended in 0.8 ml of distilled water. Subsequently, this algal suspension was mixed with melted agar (0.036 g in 1 ml distilled water), the mixture was allowed to cool to 55 °C before being poured over a mosquito net, and then it was flattened between two glass plates (methods adopted from Hay et al. 1994). After solidification, food pellets of 15 x 15 mm² were cut from agar-algae mixtures and marked by different incision patterns to distinguish pellets originating from different treatments. One pellet of each non-grazed and grazed alga was transferred into a feeding arena (EU) with either five isopods or five snails. Each pair of foods was replicated 10 times. Consumption was calculated as the change in wet mass of the food pellets prior to and after 36-hour-long assays.

Net Growth. Growth rates of non-grazed and previously grazed specimens of each *C. crispus* and *M. stellatus* were compared to evaluate effects of herbivory-induced responses on algal performance and to assess putative costs of this induction. In order to estimate wet mass changes at the end of the induction phase and 3 days later (Fig. 1), we randomly selected a specimen from both treatments and measured their wet mass to the nearest 0.0001 g. The net growth rate was expressed as the percent change in wet mass after dividing final by initial wet mass.

Statistical analysis

Data from feeding preference experiments were analyzed using two-tailed paired t-tests for each type of herbivore. Consumption rates between ungrazed and previously grazed algal specimens from the induction experiments were analyzed using two-tailed paired t-tests, separately with algal species and herbivores, type of bioassay, season, and experimental phase. Prior to the analysis, normality of differences was tested using the Kolmogorov-Smirnov test. Differences in the change in wet mass between previously grazed and ungrazed specimens were compared using Student's t-tests. Data not meeting the assumption of normality after log transformation were analyzed using the

Mann-Whitney U-test.

We did not consider a multiple-comparison procedure (such as Bonferroni), although we conducted in total 30 bioassays in the experiments. With the large number of feeding assays at $\alpha \leq 0.05$, about two assays should yield significant results by chance alone (i.e. Moran 2003). To test this, a chi-square test was used to assess whether the number of observed significant assays was greater than what could be predicted by chance alone.

RESULTS

Feeding preference

Inter-specific preferences. I. granulosa showed no significant feeding preference between *C. crispus* and *M. stellatus* (Fig. 2a, $t_9 = 0.934$, $p = 0.375$). However, *L. littorea* preferred *C. crispus*, on average, 2.3 times more than *M. stellatus* (Fig. 2a, $t_9 = 2.677$, $p = 0.025$).

Inter-population preferences. I. granulosa showed no significant feeding preference between *M. stellatus* originating from Helgoland and Iceland (Fig. 2b, $t_6 = 2.25$, $p = 0.066$), whereas *L. littorea* significantly preferred *M. stellatus* from Helgoland 12 times more than conspecifics from Iceland (Fig. 2b, $t_6 = 2.65$, $p = 0.038$).

Induction experiment

Fourteen of the 30 feeding assays conducted showed significant differences between grazed and ungrazed algae. This proportion is significantly greater than the predicted two assays that would have occurred by chance alone (chi-square: $\chi^2_1 = 10.31$, p =

0.001).

Spring

C. crispus. At the end of the induction phase, grazing by *I. granulosa* and *L. littorea* significantly decreased the palatability of *C. crispus* by 50% and 80%, respectively, compared to that of ungrazed conspecifics (Table 1a, Fig. 3). Furthermore, net growth of *C. crispus* being grazed by *I. granulosa* and *L. littorea* was significantly lower (5% and 3%, respectively) than that of non-grazed conspecifics (Table 2a, Fig. 4). After the reduction phase, *I. granulosa* showed no significant preference in the consumption of control and grazed algae (Table 1a, Fig. 3a). However, previous grazing by *L. littorea* significantly reduced the palatability of *C. crispus* by 50% compared to that of ungrazed conspecifics (Table 1a, Fig. 3b).

At the end of the induction phase, both species of grazer preferred reconstituted food made of non-grazed *C. crispus* 29% (*I. granulosa*) and 36% (*L. littorea*) more than reconstituted food made of grazed conspecifics (Table 2a, Fig. 5).

M. stellatus. At the end of the induction phase, the palatability of fresh *M. stellatus* grazed by *I. granulosa* was not significantly different from that of non-grazed algae (Table 1a, Fig. 3a). Similarly, grazing by *L. littorea* did not significantly affect the palatability of fresh algae when compared to the ungrazed control (Table 1a, Fig. 3b). Furthermore, net growth between grazed and ungrazed *M. stellatus* was not significantly different, regardless which of the two species of grazers consumed *M. stellatus* during the induction phase (Table 2a, Fig. 4). At the end of the reduction phase, previous grazing by *I. granulosa* had significantly decreased the palatability of fresh *M. stellatus* by 35% compared to ungrazed algae, whereas grazing by *L. littorea* did not significantly alter the palatability relative to that of ungrazed *M. stellatus* (Table 1a, Fig. 3).

At the end of the induction phase, *I. granulosa* preferred reconstituted food made of non-grazed *M. stellatus* significantly (1.3 times) more than reconstituted food of grazed conspecifics (Table 2a, Fig. 5). *L. littorea* did not show a preference for reconstituted grazed *M. stellatus* or the non-grazed control.

Fall

*C. crispus***.** After the induction phase, the palatability of *C. crispus* was not significantly affected by grazing by *I. granulosa*, whereas *L. littorea* preferred ungrazed over grazed algae by 50% (Table 1b, Fig. 3). Net growth of the ungrazed algae was a significant 41% higher than that of *I. granulosa* grazed conspecifics, whereas grazing by *L. littorea* did not have a significant effect on net growth (Table 2b, Fig. 4). After the reduction phase, both herbivores showed no significant preference for ungrazed or grazed fresh algae (Table 1b, Fig. 3).

At the end of the induction phase, *I. granulosa* consumption of reconstituted grazed and ungrazed *C. crispus* did not differ significantly (Table 2b, Fig. 5a). *L. littorea* consumed 57% less reconstituted grazed *C. crispus* compared to reconstituted ungrazed *C. crispus* (Table 2b, Fig. 5b).

M. stellatus from Helgoland. After the induction phase, the consumption of grazed fresh *M. stellatus* by *I. granulosa* was 52% lower than the consumption of controls (Table 1b, Fig. 3a). However, *L. littorea* consumed ungrazed and grazed algae equally (Table 1b, Fig. 3b). Moreover, net growth of algae grazed by each species of herbivore was not significantly different from net growth of ungrazed conspecifics (Table 2b, Fig. 4). After the reduction phase, *I. granulosa* still preferred ungrazed fresh *M. stellatus* two times more than grazed conspecifics, whereas *L. littorea* showed no preference in the consumption of ungrazed or grazed algae (Table 1b, Fig. 3).

At the end of the induction phase, *I. granulosa* consumed 26% more reconstituted ungrazed algae than reconstituted grazed algae, whereas consumption by *L. littorea* did not significantly affected the palatability of *M. stellatus* (Table 2b, Fig. 5).

M. stellatus from Iceland. At the end of the induction phase, *I. granulosa* preferred fresh ungrazed algae twice as much as grazed conspecifics (Table 1b, Fig. 3a). However, *L. littorea* showed no significant preference for either food (Table 1b, Fig. 3b). Moreover, net growth was not significantly different between grazed and ungrazed algae, regardless of grazer species (Table 2b, Fig. 4). After the reduction phase, neither consumption by *I. granulosa* nor by *L. littorea* was significantly different between grazed and ungrazed algae (Table 1b, Fig. 3).

At the end of the induction phase, *I. granulosa* consumed 25% less reconstituted grazed algae than reconstituted ungrazed conspecifics, whereas *L. littorea* showed no such significant difference (Table 2b, Fig. 5).

DISCUSSION

Patterns of inducible anti-herbivore responses in the non-indigenous seaweed *M. stellatus* differed from those of the native seaweed *C. crispus*. In contrast to the isopod *I. granulosa*, the periwinkle *L. littorea* was unable to induce anti-herbivory defenses in *M. stellatus*. Both species of herbivores, however, induced anti-herbivory defenses in *C.*

crispus. Grazing had detrimental effects on the growth of *C. crispus*, whereas growth of *M. stellatus* was not affected by grazing. Furthermore, *M. stellatus* was consumed less than the native alga by the periwinkle *L. littorea*, whereas both algal species were equally consumed by the isopod *I. granulosa*. These results suggest that *M. stellatus* may use a different strategy than *C. crispus* to cope with the grazing impact of local herbivores.

Induced defenses in native *C. crispus*

In spring, specimens of *C. crispus* exposed to *I. granulosa* and *L. littorea* were less palatable than ungrazed conspecifics, showing that grazing by both species induced an anti-herbivory response in this seaweed. This pattern was apparent in assays using fresh algae and those using reconstituted food, suggesting a chemically mediated interaction. In contrast, Toth (2007) did not demonstrate an induction of anti-herbivory defenses in fresh and reconstituted *C. crispus* in response to grazing by *I. granulosa*. This suggests that the ability of seaweeds to induce anti-herbivory defenses against the same grazer species may vary within the NE Atlantic region. In fall, however, grazing by *I. granulosa* did not affect the palatability of *C. crispus*, indicating seasonal variation in the ability of isopods to induce anti-herbivory defenses in *C. crispus*. Similarly, Molis et al. (2006) reported that the induction of defenses in the alga *Ecklonia cava* was seasonally variable. A season-specific ability to induce defenses in seaweeds of the temperate zone may be due to strong seasonal differences in grazing pressure; it may be more effective to tolerate low grazing pressure and defend only above some fitness-related threshold (Karban et al. 1999). Patterns in the density of *I. granulosa* at the study site seem to agree with this interpretation, as this isopod was 10-fold more abundant in summer than in fall (personal observation HY Yun). This interpretation is further corroborated when abundance patterns of *L. littorea* are considered. The density of this species showed no seasonal variation on a nearby intertidal Helgoland shore (unpubl. data M Molis), which may explain why the palatability of periwinkle-grazed *C. crispus* continued to be lower than that of ungrazed conspecifics (i.e., defenses were also induced in fall).

Defenses in *C. crispus* were reduced after grazing by *I. granulosa* ceased, indicating that induced responses may be tailored to the actual grazing threat and perhaps to demand for metabolic costs. Grazing pressure by the fast swimming isopods may vary greatly over relatively short periods of time (i.e., hours) compared to grazing by the slow-moving periwinkles. Consequently, the reduced defenses in *C. crispus* might be related to feeding behavior of *I. granulosa*. A fast relaxation of induced defenses has been shown for other seaweed-isopod interactions (Rohde & Wahl 2008b). Activity patterns of an herbivore may influence the evolution of plasticity in defensive traits (Karban & Nagasaka 2004). A timely reduction in defenses may be a way to save costs. The slower growth of grazed compared to ungrazed algae suggests that the observed induction of defenses may incur some metabolic cost in *C. crispus*, which will redirect resources from growth processes to the production of anti-herbivory defenses. Detrimental effects on growth of *C. crispus* were also detected with grazing by *L. littorea*, suggesting that periwinkle grazing also generates costly defenses. Furthermore, *C. crispus* sustained defenses after *L. littorea* was removed, which indicates that the expense of defenses induced by periwinkles can be even more costly than those induced by isopods. Consequently, grazing by local herbivores seemed to detrimentally affect the fitness of *C. crispus*, which may be critical in the population dynamics of other competing seaweed species, such as the non-indigenous *M. stellatus*.

Induced defenses in non-indigenous *M. stellatus*

The non-indigenous *M. stellatus* displayed different responses to local grazers from Helgoland compared to the native *C. crispus*. First, *M. stellatus* did not induce defenses against grazing periwinkles. Second, *M. stellatus* decreased its palatability in response to grazing isopods in both seasons. Third, isopod grazing induced changes in the palatability of *M. stellatus* that continued even after the herbivores were removed; in contrast, defenses in *C. crispus* by identical herbivore species relaxed. This suggests that the introduced *M. stellatus*, although principally able to induce anti-herbivory defenses, could not tailor its responses to the grazing regimes of the herbivores from the recipient region in as timely a period as the native seaweed. Previous studies corroborate that seaweeds in a new habitat rarely exhibit accurate responses to local herbivores. For example, introduced *Fucus evanescens* contains excessively abundant anti-herbivore chemicals to reduce the risk from generalist herbivores compared to its competing native counterparts (Wikström et al. 2006).

Inducible defenses bear a selective advantage if they increase prey fitness relative to the undefended or constitutively defended condition (Karban et al. 1999). Therefore, induced anti-herbivory responses have the potential to promote successful introductions of non-indigenous species to new ranges. The relatively limited expression of induced anti-herbivory responses and the moderate match to the grazing regime of important grazer species were shown in *M. stellatus* in the new habitat. It would imply that there is a limitation in successful establishment of *M. stellatus* in Helgoland. Yet, in reality *M. stellatus* has established itself successfully in Helgoland. Part of our experiments can explain this discrepancy. First, growth data for *C. crispus* suggest that the induction of anti-herbivory defenses is associated with the cost of producing chemical feeding deterrents. In contrast, grazing did not affect growth of *M. stellatus*, despite the excessive induction of chemical anti-isopod defenses. Thus, the non-indigenous seaweed seemed to repel grazers at relatively little fitness-associated cost compared to the native seaweed. Second, periwinkles strongly preferred *C. crispus* over the non-indigenous seaweed, despite the lack of induced defenses. Moreover, growth in the latter was unaffected by grazing damage by the periwinkles. Thus, *M. stellatus* seems to be liberated from feeding by one important grazer in the recipient range.

In contrast to *L. littorea*, *I. granulosa* consumed comparable amounts of both algae. This grazer-specific feeding preference can be explained by the different time spans of shared history between grazer and seaweed. The periwinkle does not occur in Iceland and thus had only a limited time to induce anti-herbivory traits in *M. stellatus*. In contrast, *I. granulosa* coexists in Iceland with *M. stellatus* and therefore the seaweed had time to adapt to this grazer by developing inducible anti-herbivory defenses. Similar to the observed feeding preferences between algal species, differences in palatability between *M. stellatus* specimens from the two populations showed that *L. littorea* preferred *M. stellatus* specimens from the recipient population. In contrast, isopods, which coexist with *M. stellatus* in both the donor and the recipient regions, consumed algal specimens from both regions equally well. Other studies have shown that herbivores that lack a history of interaction with non-indigenous plants can fail to recognize them as suitable food sources (Siemann & Rogers 2003, Lankau et al. 2004).

Our results show that the population of *M. stellatus* on Helgoland can reduce the potential damage from herbivores in the recipient region with relatively little or no additional defensive cost. It is possible that *M. stellatus* in the presence of herbivores contains chemical compounds that provide not only anti-grazing responses but also other functions. For instance, phlorotannins, which are known to be feeding deterrence chemicals in brown algae, can play additional roles, such as wound healing (Hemmi et al. 2004) or UV screens (Henry & Van Alstyne 2004). With these low-budget responses to grazers, *M. stellatus* may allocate more resources than *C. crispus* to enhance its performance in establishing a sustainable population in the new habitat.

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Table1. Results from paired t-tests comparing consumption of previously grazed and non-grazed fresh algae (*Chondrus crispus* or *Mastocarpus stellatus*) in 3 day-long bioassays performed at the end of a 6 day-long induction and reduction phase in (a) spring and (b) fall. Herbivores were either the isopod *Idotea granulosa* or the periwinkle *Littorina littorea*. Bold font indicates statistically significant differences at $\alpha \leq 0.05$.

(a) Spring experiment

Algae	Grazer	Induction		Reduction	
		t-value	p-value	t-value	p-value
C. crispus	*I. granulosa*	4.566	**.001**	0.886	.409
	L. littorea	3.447	**.007**	2.721	**.024**
M. stellatus	*I. granulosa*	1.052	.320	2.375	**.042**
	L. littorea	1.760	.112	1.362	.206

(b) Fall experiment

Algae	Grazer	Induction		Reduction	
		t-value	p-value	t-value	p-value
C. crispus	*I. granulosa*	0.819	.434	1.946	.084
	L. littorea	2.856	**.019**	0.927	.378
Helgoland	*I. granulosa*	2.687	**.025**	2.793	**.021**
M. stellatus	*L. littorea*	0.034	.974	1.519	.163
Iceland	*I. granulosa*	2.485	**.035**	1.115	.294
M. stellatus	*L. littorea*	1.115	.294	2.226	.053

Table 2. Results from paired t-tests comparing consumption of reconstituted food made of previously grazed and non-grazed algae (*Chondrus crispus* or *Mastocarpus stellatus*) in spring (a) and fall (b) in 1.5 day-long assays at the end of the induction phase (n=10). Growth rates of previously grazed and non-grazed algae were compared with Student´s t-test or Mann-Whitney U-test (italics). Bold font indicates statistically significant differences at α ≤ 0.05.

(a) Spring experiment

Algae	Grazer	Net growth		Reconstituted food	
		t-value	p-value	t-value	p-value
C. crispus	*I. granulosa*	4.563	**.001**	2.290	**.048**
	L. littorea	5.994	**1x10^{-5}**	2.470	**.036**
M. stellatus	*I. granulosa*	1.503	.150	4.121	**.003**
	L. littorea	0.839	.413	1.002	.343

(b) Fall experiment

Algae	Grazer	Net growth		Reconstituted food	
		t-value	p-value	t-value	p-value
C. crispus	*I. granulosa*	2.891	**.010**	0.563	.587
	L. littorea	0.508	.683	2.400	**.040**
Helgoland	*I. granulosa*	1.511	.152	2.989	**.015**
M. stellatus	*L. littorea*	0.151	.880	1.428	.187
Iceland	*I. granulosa*	0.302	.766	2.473	**.035**
M. stellatus	*L. littorea*	1.172	.257	1.198	.261

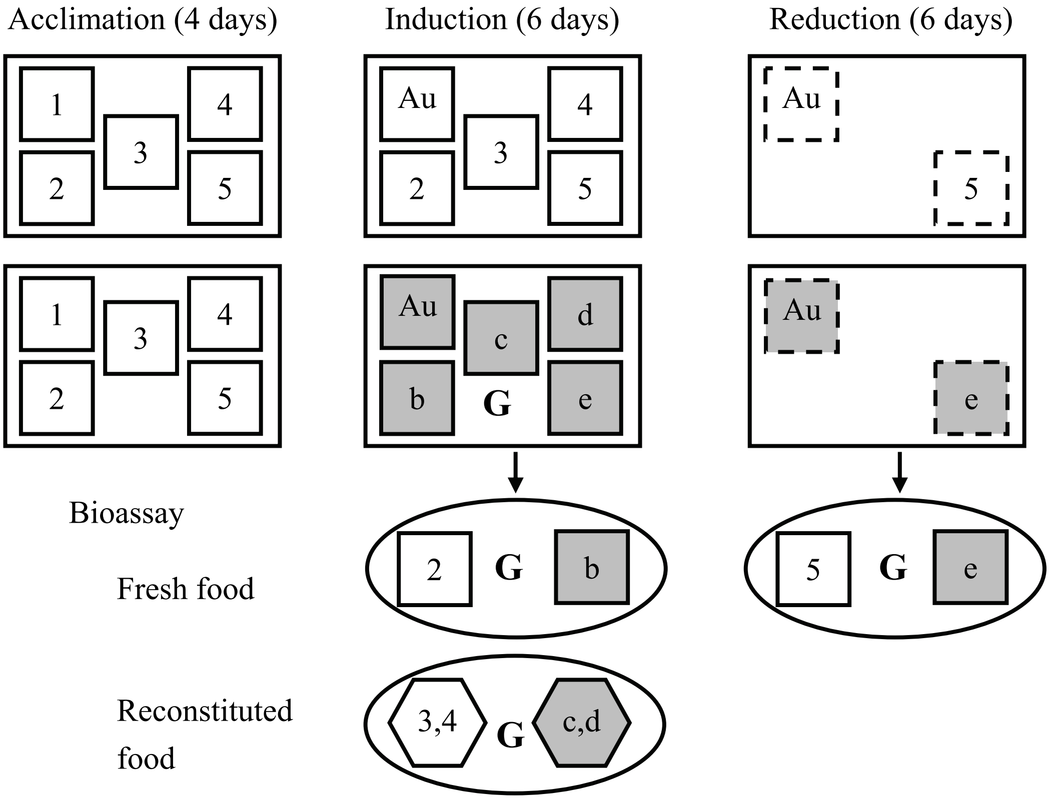


Fig. 1. Experimental set up (displayed for one replicate). Large rectangles indicate aquaria, each containing five algal individuals (squares). After 4 days of acclimation, grazers (G) were added to half of the aquaria for 6 days (induction phase). Filled and open squares indicate algal individuals exposed to grazers and algae left without grazers, respectively. At the end of the induction phase, grazers were removed and two algae incubated for a further 6 days (reduction phase). At the end of the induction and reduction phase, algal individuals were transferred to feeding arenas (ovals) for two choice bioassays (Numbers: non-grazed algal individuals, Letters: grazed algal individuals, Au: autogenic control, Hexagon: reconstituted food made of two algal individuals).

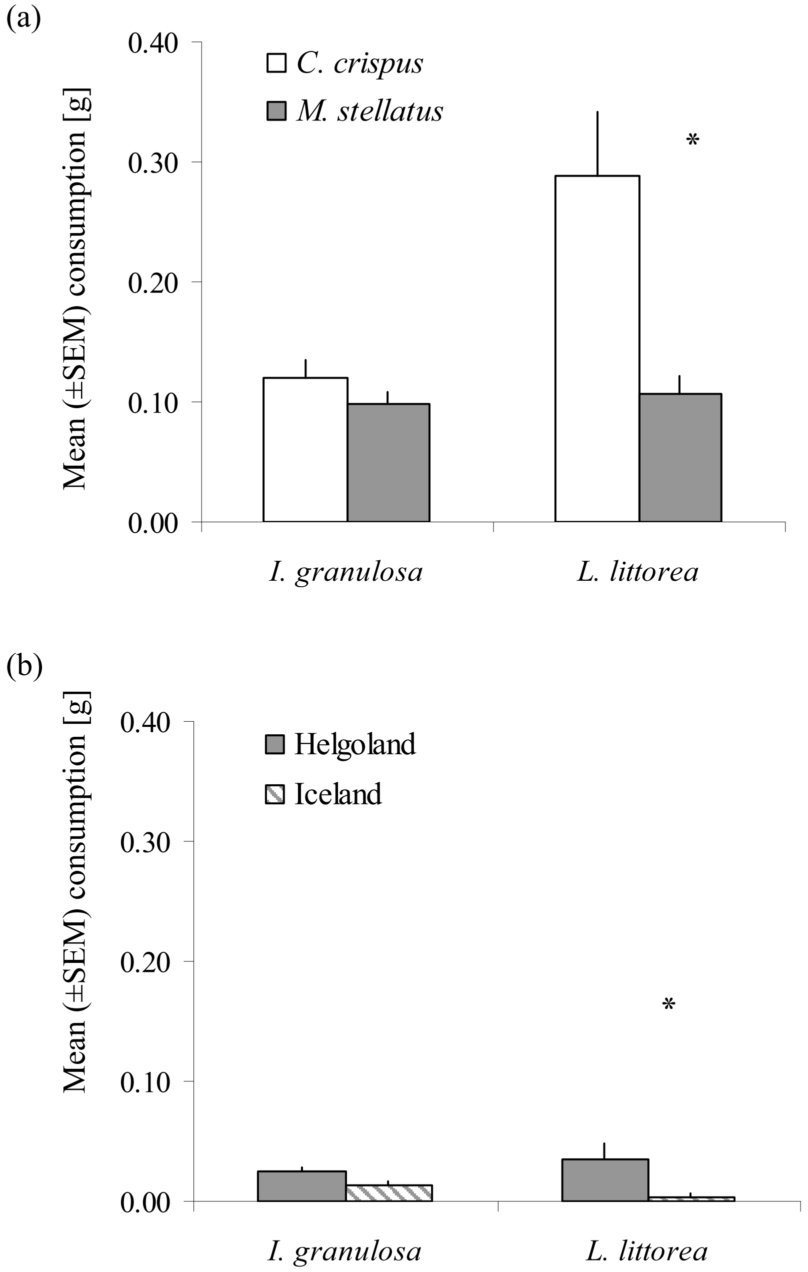
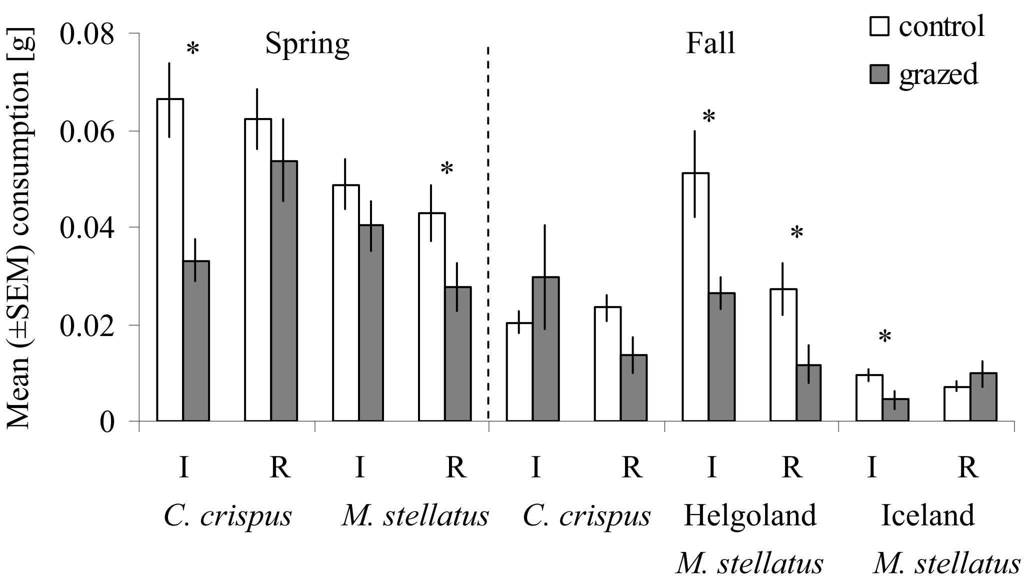


Fig. 2. Consumption by *Idotea granulosa* and *Littorina littorea* during 6 day-long choice feeding assays (n = 10), in which (a) one specimen of *Chondrus crispus* and *Mastocarpus stellatus* or (b) one specimen of *M. stellatus* originating from Helgoland (recipient region) and one specimen from Iceland (donor region) were simultaneously offered to grazers. Asterisks indicate statistically significant differences at $\alpha \leq 0.05$.

(a) Induced by *I. granulosa*



(b) Induced by *L. littorea*

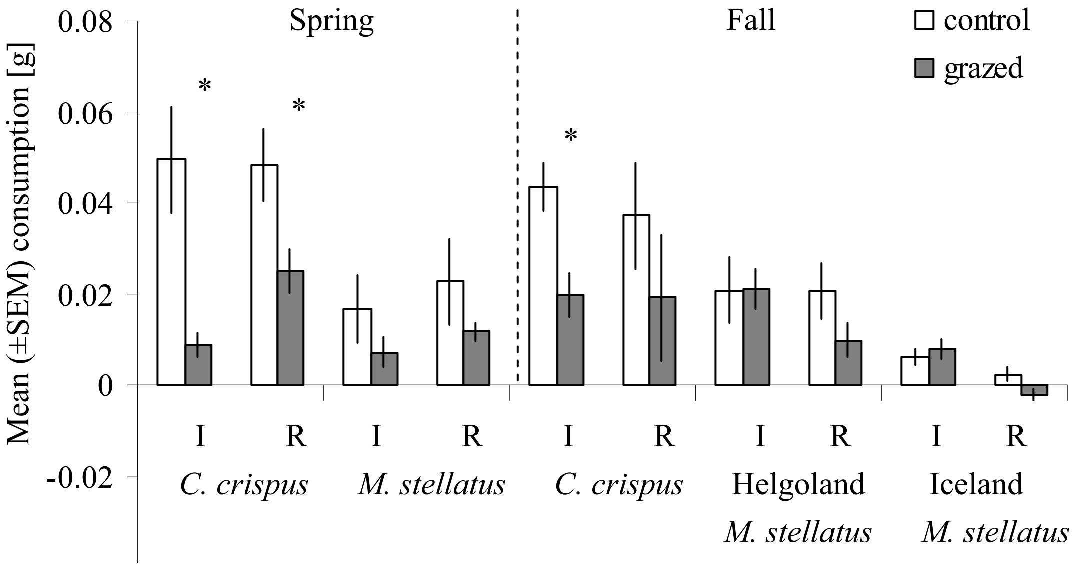
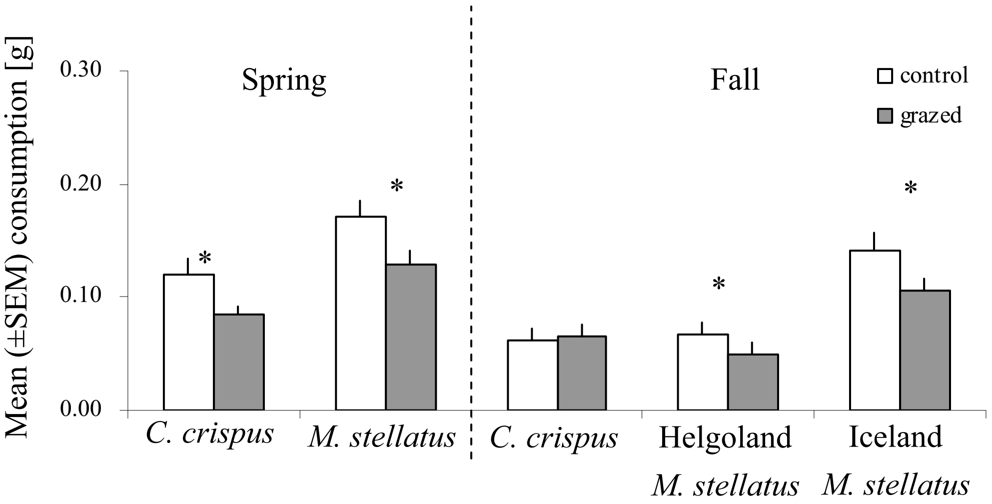


Fig. 3. Fresh algal assays in spring and fall. Consumption of previously grazed and non-grazed *Chondrus crispus* and *Mastocarpus stellatus* by (a) the isopod *Idotea granulosa* and (b) the periwinkle *Littorina littorea* during 3 day-long feeding assays at the end of the induction (I) and reduction phase (R) (n = 10). Asterisks indicate statistically significant differences at $\alpha \leq 0.05$.

(a) *I. granulosa*



(b) *L. littorea*

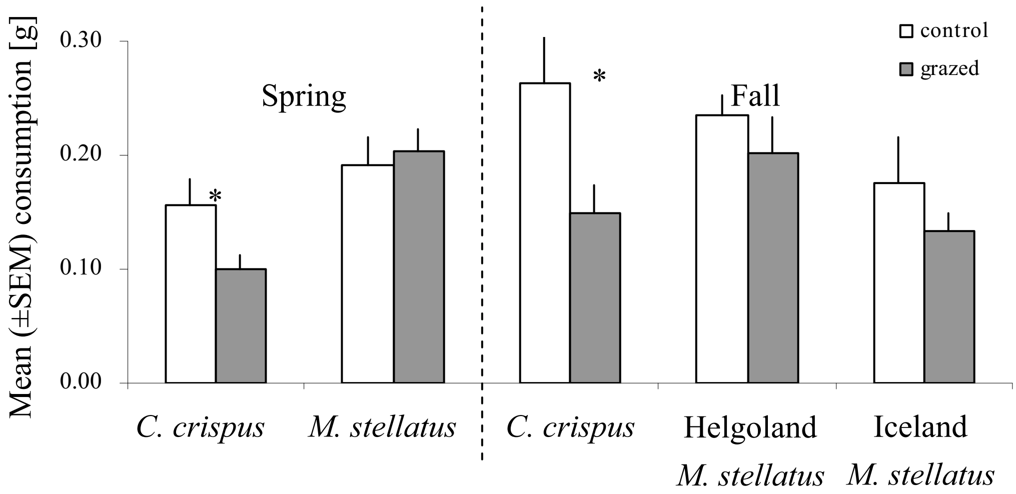
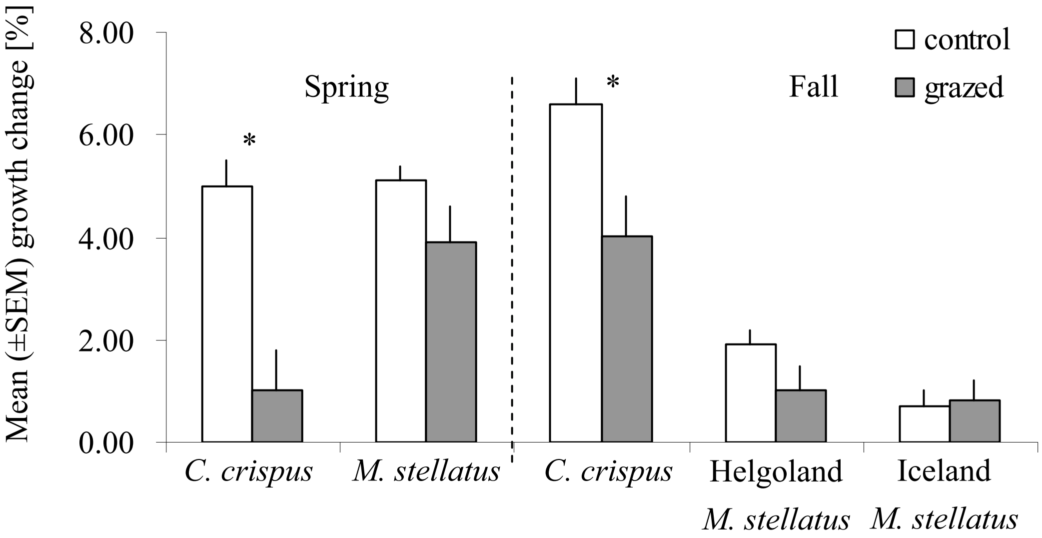


Fig. 4. Net growth of previously grazed and non-grazed *Chondrus crispus* and *Mastocarpus stellatus* during 3 days in spring and fall using (a) the isopod *Idotea granulosa* and (b) the periwinkle *Littorina littorea* as grazers (n = 10). Asterisks indicate statistically significant differences at α ≤ 0.05.

(a) *I. granulosa*



(b) *L. littorea*

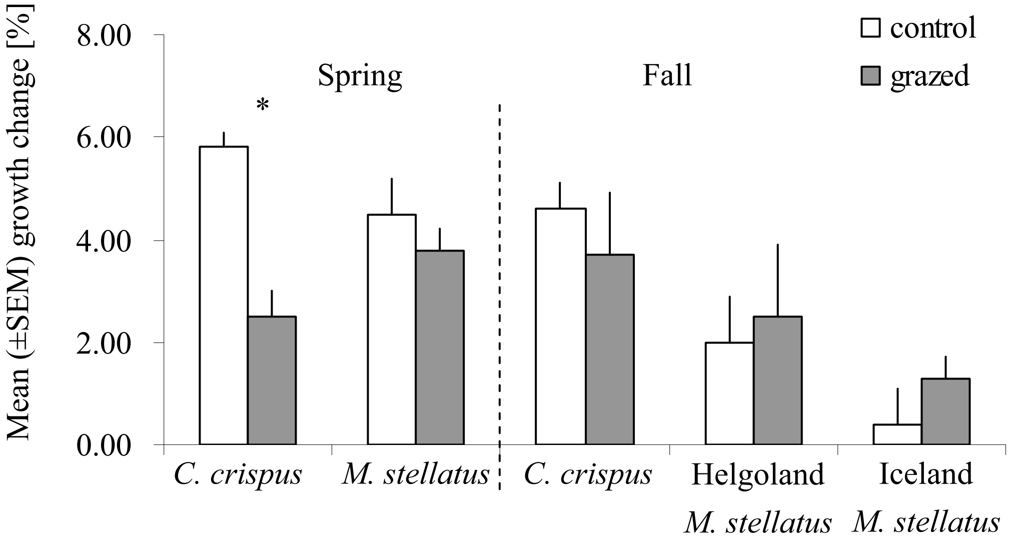


Fig. 5. Reconstituted food assay in spring and fall. Consumption of reconstituted food made of previously grazed and non-grazed *Chondrus crispus* and *Mastocarpus stellatus* by (a) the isopod *Idotea granulosa* and (b) the periwinkle *Littorina littorea* during 1.5 day feeding assays at the end of the induction phase (n = 10). Asterisks indicate statistically significant differences at $\alpha \leq 0.05$.

Publication 3

Waterborne cues from grazed algae can induce chemical anti-herbivory defenses in conspecific and heterospecific neighbors

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ABSTRACT

Terrestrial plants become more resistant in responses to cues released from other plants damaged by herbivores. Here, we examined whether macroalgae trigger such anti-herbivory responses to direct grazing or waterborne cues from either conspecific or heterospecific neighbours attacked by grazers. Experiments were conducted in two NE Atlantic sites using the non-native brown macroalga *Sargassum muticum* and two native browns of each region with a local isopod species as generalist herbivore. In fresh and reconstituted bioassays, algal trait modfications were detected by feeding preference tests between grazer-treated and non-treated conspecifics. Directly grazed native macroalgae in a type of either fresh food or reconstituted pellet were less consumed than ungrazed conspecifics, but not the non-native *S. muticum*. Exposure to waterborne cues from grazed conspecifics decreased the palatability for reconstituted food made of *Fucus spiralis* (Portugal) and *F. vesiculosus* (Germany), respectively. The palatability of *S. muticum* was not affected by waterborne cues from grazed heterospecifics, while exposure to cues from grazed *S. muticum* decreased the palatability of *Cystoseira humilis* (Portugal) and *Halidrys siliquosa* (Germany) but did not affect the palatability of either *Fucus* species. Moreover, cues emitted from grazed *F. spiralis* lowered the tastefulness of *C. humilis* and vice versa. Waterborne cues derived from grazed neighbour macroalgae have shifted anti-herbivory traits in some con- and heterospecifics. The cues from even non-indigenous *S. muticum* grazed by local herbivores can induce anti-feeding traits of its confamilar, native macroalgae, although *S. muticum* appeared insensitive to direct grazing and waterborne cues. Therefore, we can suggest that 'eavesdropping' occurs in macroalgal species and that specific native species would benefit restrictedly from a non-native macroalga via changes of anti-herbivory trait to encounter their future consumer's attack.

Key-words: eavesdropping, herbivore-induced trait changes, macroalgal specificity, non-native species, *Sargassum muticum*

INTRODUCTION

Impacts of herbivory can lead to changes in morphological and/or chemical traits of plants and macroalgae (Karban & Baldwin 1997, Fordyce 2006, Toth & Pavia 2007). Some induced modifications by direct grazing may result in reduced susceptibility or palatability to herbivores (Karban & Baldwin 1997). These induced responses ultimately can prevent further vulnerability of plants from grazers (Borell et al. 2004, Sotka & Whalen 2008), e.g. by an induction of anti-herbivory defences (i.e. Karban et al. 1999). Moreover, plants attacked by herbivores may release some chemicals that inform conspecifics about the potential risk of grazing damage, which refers to ‘talking tree’ phenomenon (Karban and Baldwin 1997). The ungrazed conspecifics that received risk cues have induced anti-herbivory defences to lower their susceptibility to future grazing. This phenomenon has been shown for both vascular plants (Karban et al. 2003, Baldwin et al. 2006, Heil & Karban 2010) and the macroalgae *Ascophyllum nodosum* and *Fucus vesiculosus* (Toth & Pavia 2000, Rohde et al. 2004).

Volatile cues of grazed plants may also offer valuable information about consumer attacks to undamaged specimens of different plant species. For example, non-grazed tobacco plants (*Nicotiana attenuata*) decreased their palatability to herbivores after exposure to cues released from grazed sagebrush (*Artemisia tridentate*) (Karban et al. 2004). Similarly, non-grazed barley (*Hordeum* spp.) decreased its attractiveness to herbivores when they had encountered cues released by grazed thistles (*Cirsium* spp.) (Glinwood et al. 2004). Thus, plants may use warning cues originated from the grazed and heterospecific neighbour plants in order to protect themselves against nearby active grazers (Callaway et al. 2003, Heil & Karban 2010).

Plants can respond selectively to the alarm cues released from the specific plant among diverse neighbour plants (Karban et al. 2004). Yet, it is not clear how strongly the cues from damaged plants are effective among nearby heterospecific plants. Among multiple preys to encounter a shared consumer, it is suggested that the cue effects on cue-receiving preys depend on their phylogenetic distances to the cue-emitting species (e.g. Schoeppner & Relyea 2005). Based on this work, we could expect that even non-native species would provide volatile cues to only phylogenetically related native species, but not to distantly related species, and vice versa (e.g. Smith 2009). In contrast, the non-native species could not receive cues from distantly related species and vice versa.

Alarm cues in intertidal communities exist as either waterborne (Tollrian & Harvell 1999) or airborne chemicals (i.e. jasmonates, Arnold et al. 2001). In contrast to terrestrial habitats, the role of alarm cues for plant-herbivore interactions has been studied only among conspecific macroalgae (Toth & Pavia 2007, Rohde & Wahl 2008a). Yet, in marine community, generalist consumers as common herbivores can be a reliable threat to coevolved (native plant) as well as non-coevolved members (newly introduced plant) of multi-species assemblages (Duffy & Hay 2000, Van Zandt & Agrawal 2004, Parker & Hay 2005, Pearse & Hipp 2009), and consumption on one plant by a generalist would also increase the possibility of feeding attack in an adjacent other plant species. Thus, the commonness of generalist consumers in marine habitats may suggest that algae could also use eavesdropping, which occur in mixed species stands (Brawley 1992).

In this study, we examined whether the brown non-native *Sargassum muticum* (Japanese wireweed) and two native algal species induce anti-herbivory resistances in responses to either direct grazing by isopods or waterborne cues derived from grazed conspecific or heterospecific macroalgae. To generalize patterns of induced responses, similar laboratory experiments were conducted in two NE Atlantic regions, i.e. Portugal and Germany. Here we asked whether 1) direct grazing will decrease the palatability in native but not in non-native species of macroalgae; 2) waterborne cues released from grazed native but not from non-native macroalgae will lower the palatability of undamaged conspecifics; 3) cues emitted from grazed non-native *S. muticum* induce anti-herbivory responses in phylogenetically close but not in phylogenetically distant native algal species; and 4) waterborne cues released by closely but not by more distantly related native algal species induce anti-herbivory responses in the non-native *S. muticum*.

MATERIALS AND METHODS

Collection sites and organisms

Experiments were conducted with macroalgae and herbivores collected at two NE Atlantic shores that are ≥1400 km apart: (i) Praia de Queimado, SW coast of Portugal (37° 49' N, 8° 47' W, Southern Europe) and (ii) Nordstwatt, Helgoland, Germany (54° 11' N, 7° 52' E, Northern Europe). The non-native brown macroalga *Sargassum muticum* (Yendo) Fensholt was recorded to arrive in the end of 1980s on the southern and northern European shores (Kornmann & Sahling 1994, Lluch et al. 1994). At the southern shore, both *S. muticum* and native species *Cystoseira humilis* belong to Sargassaceae family and co-exist in dense stands in sheltered rock pools. Moreover, the native *F. spiralis*, which belongs to Fucaceae family, exists in Portugal within a range of 1 m in the intertidal zone around the rockpools. At the northern shores, *S. muticum* and native species *Halidrys siliquosa* belonging to the Sargassaceae family coexist in the shallow subtidal, while the native *F. vesiculosus* (Fucaceae family) is restricted to the mid to upper intertidal, i.e. in the range of tens of metres away from the subtidal sites inhabited by *S. muticum* and *H. siliquosa*. As herbivores we used either the isopod *Stenosoma nadejda* (Portugal) or *Idotea baltica* (Germany), which are common to abundant at the respective shores (Reichert & Buchholz 2006, Xavier et al. 2009).

Induction experiment

To generalize algal trait changes in responses to (i) direct grazing, (ii) waterborne signals from grazed conspecifics, or (iii) heterospecifics, similar experiments were conducted from March to May in 2007 at Marine Laboratory of Ramalhete (Southern European shore) and from June to August in 2007 at Biologische Anstalt Helgoland (Northern European shore), respectively. At each study site, three 19 d long induction experiments were conducted. The day induction experiments started, grazers and 16 specimens of each algal species were collected and macroscopic epibionts were gently removed from the algae with a sponge. Then, 6, 12, or 24 apical pieces (1.5-2g wet mass) were cut from each specimen and marked individually by coloured threads. Then, the algal pieces per specimen were allocated to transparent plastic experimental aquaria (Portugal: 2L, Germany: 8L) to test for effects of (i) direct grazing; (ii) waterborne cues released by grazed conspecifics; or (iii) grazed heterospecifics, respectively (Fig. 1). However, due to the small size of *F. spiralis*, only two pieces (0.8-1.0g wet mass) were cut and marked by coloured threads per specimen so that 24, 48 and 96 individuals of *F. spiralis* had to be collected for the three induction experiments. During the subsequent 4 d long acclimation phase, algal pieces remained without grazes in the aquaria. This allows for algae to reduce putative in situ grazing effects, which is known to occur within 4 d (Rohde & Wahl 2008b), and for their acclimatization to laboratory conditions. Moreover, all grazers used in the experiments were kept throughout the acclimation phase on a mixed algal diet (but target algal species were excluded) in a separate aerated container (50L) with water flow-through. Each experimental aquarium was supplied by a uni-directional flow of cotton-filtered seawater from the nearby sea at an average rate of 300 (Portugal) or 120 ml min^{-1} (Germany). Fluorescent lamps (58 Watt Osram in Portugal, 36 W Philips in Germany) irradiated aquaria in a 12 hour light: 12 hour dark cycle with, on average (±SD), 65.5 (±2.0) and 34.4 (±2.5) $\mu molm^{-2}s^{-1}$ PAR in Portugal and Germany, respectively (LI-COR, UWQ 6534). This irradiance simulated ambient PAR levels at 1 m water depth during the time when the experiments were conducted.

Direct grazing: The first induction experiment tested whether the palatability in macroalgae is changed by direct grazing (Fig. 1A). This experiment started on 15-03-2007 (Portugal) and 06-06-2007 (Germany). At the end of the acclimatization phase, 4 isopods were added to a randomly selected half of the 16 aquaria containing the same algal species, while no grazers were added to the remaining 8 aquaria (induction phase, n = 8). At the end of the 12 day long induction phase, grazers were discarded. One algal piece from each aquarium was used for conducting bioassays with fresh pieces, the second piece was used to correct consumption rates in bio-assays as a result of non-feeding related (autogenic) changes in algal biomass, and the final piece was used for bio-assays with reconstituted food (see bioassays below).

Waterborne induction: The second induction experiment examined whether the palatability in undamaged macroalgae changes in response to receiving waterborne cues released by nearby grazed conspecifics. This experiment started in 07-04-2007 (Portugal) and 30-06-2007 (Germany). Prior to the experiment, a plastic net (1mm mesh size) was vertically inserted in each aquarium to divide aquaria into equally sized up- and downstream compartments. Six pieces from the same specimen were added evenly to the up- and the downstream compartment of an aquarium (except for *F. spiralis*) (Fig. 1 B). The experiment started by adding 4 isopods to the upstream compartment of a randomly selected half of the 16 aquaria used for each species, while the remaining 8 aquaria were kept grazer-free controls (n = 8, total of 48 aquaria per site). At the end of the experiment, grazers were discarded, and algal pieces from downstream compartments allocated to bioassays as described for the first induction experiment.

Eavesdropping: The third induction experiment tested whether the palatability in undamaged macroalgae changes in response to receiving waterborne cues released by nearby grazed heterospecific species. This experiment started on 01-05-2007 (Portugal) and 02-07-2007 (Germany). The principle experimental set-up was identical to that of the second induction experiment, however, there were 6 different sets of combinations with 3-different algal species positioned in the up- and the downstream compartments of each aquarium (see Fig. 1C for general set-up and Fig. 2C and 3C for detailed species combination in Portugal and Germany, respectively).

Bioassays

At the end of the induction phase, a change in algal palatability was assessed by measuring consumption rates in treated and control pieces of either fresh or reconstituted food (Fig. 1). Feeding assays using fresh pieces tested for a chemical and/or morphological modification of grazed algae. Prior to the assay, algal pieces were blotted with paper towels for 15 seconds, were spun 10 times in a salad spinner, and were weighed on a balance (Sartorius LE323S) to the nearest 0.001g. Separated by species, one treated and one control piece were randomly selected and transferred into a feeding arena (200ml glass Petri dish, experimental unit = EU) together with 2 naïve isopods. To account for non-feeding related (autogenic) changes in wet mass in assayed algae for fresh bioassays, genetically identical pieces were paired in a separate Petri dish after preparing them in the same way like assays pieces (Peterson & Renaud 1989). Seawater in feeding arenas was exchanged twice daily to reduce artefacts on grazer consumption by e.g. waste products accumulating in feeding arenas. At the end of 3 d long feeding assays, each food was reweighed. Actual consumption rates in each replicate bioassay were calculated as: B_{start} x (A_{end} / A_{start}) – B_{end}, where B_{start} and B_{end} represent initial and final wet mass of an assayed piece, respectively, and A_{start} and A_{end} represent initial and final mass of the autogenic control piece, respectively (equations from Cronin & Hay 1996). A significant preference of control over treated algal pieces was interpreted as an induction of anti-herbivory defences.

Feeding assays using reconstituted food tested for changes in only chemical resistance traits. After freeze drying, algal pieces were ground to a homogenous fine powder and 0.2g of this powder were suspended in 1ml of distilled water. This algal suspension was mixed with molten agar (0.043g in 1.2ml distilled water) after the agar had cooled to 55°C, poured over a mosquito net (1 mm^2 mesh size), and flattened between two glass plates (methods adapted from Hay et al. 1994). After solidification, food pellets of 15 x 15mm^2 were cut from algae-agar mixtures and marked by different incision patterns to distinguish between control and treated pellets. One control and one treated pellet were exposed to feeding by 2 isopods for 36 hours under identical conditions as in assays using fresh algal pieces. Feeding rates were determined by mass changes in food pellets between start and end of two-choice feeding assays, using the same laboratory balance as for assays with fresh algae.

Statistical analysis

A normal distribution of differences in consumption rates of control and treated food pieces originating from the same feeding arena was confirmed with the Kolmogorov-Smirnov test. Differences in the consumption of treated and control food pieces were compared with paired t-tests. Due to the large number of tests (i.e. 24), we used the χ^2 test statistic to calculate the probability of finding 8 and 9 significant fresh and reconstituted food bioassays (see results), respectively, by chance alone (i.e. 24 x 0.05 = 1.2) to be very unlikely (fresh food: $\chi^2 = 19.64$, $p < 0.0001$, reconstituted food: $\chi^2 = 26.73$ $p < 0.0001$). Consequently, we did not use the sequential Bonferroni adjustment for multiple tests.

RESULTS

Southern Europe experiments (Portugal)

Direct grazing. Stenosoma nadejda significantly preferred ungrazed pieces of *C. humilis* to previously grazed pieces by, on average, 30 and 3 times in bioassays using fresh and reconstituted feed, respectively (fresh $t_7 = 3.40$, $p = 0.011$; reconstituted $t_7 = 3.54$, $p = 0.009$, Fig. 2A). Although previous grazing reduced the palatability of fresh *F. spiralis* pieces by 72% compared to that of control pieces, this difference was non-significant ($t_7 = 1.82$, $p = 0.111$, Fig. 2A). In contrast, reconstituted food made of previously ungrazed *F. spiralis* was significantly preferred over reconstituted food made of previously grazed *F. spiralis* ($t_7 = 2.68$, $p = 0.032$). Previously grazed *S. muticum* pieces were not significantly preferred by *S. nadejda* over ungrazed pieces in both fresh and reconstituted food assays (fresh $t_7 = 0.84$, $p = .429$; reconstituted $t_7 = -0.11$, $p = .913$, Fig. 2A).

Waterborne cues. The consumption by *S. nadejda* was not significantly different between pieces of *C. humilis* positioned downstream of grazed and ungrazed conspecifics in both bioassays using fresh or reconstituted pieces of *C. humilis* (fresh $t_7 = 1.405$, $p = 0.203$; reconstituted $t_7 = 0.566$, $p = 0.589$, Fig. 2B). In contrast, pieces of *F. spiralis* located downstream of grazed conspecifics were less palatable, on average, by a significant 52% to *S. nadejda* than *F. spiralis* pieces positioned downstream of ungrazed conspecifics (Fig. 2B). This pattern was apparent in both, assays using fresh or reconstituted pieces of *F. spiralis* (fresh $t_7 = 2.98$, $p = 0.021$; reconstituted $t_7 = 3.46$, $p = 0.011$). The palatability of *S. muticum* downstream of grazed conspecifics was not significantly different from that of *S. muticum* pieces located downstream of ungrazed alga in both fresh and reconstituted assays (fresh $t_7 = 1.40$, $p = 0.21$; reconstituted $t_7 = 0.47$, $p = 0.653$, Fig. 2B).

Eavesdropping. In assays with fresh and reconstituted food, *S. nadejda* significantly preferred *C. humilis* food that were positioned downstream of ungrazed *F. spiralis*, on average, 2 and 3.4 times more than *C. humilis* food that were positioned downstream of grazed *F. spiralis*, respectively (fresh $t_7 = 2.56$, $p = 0.037$; reconstituted $t_7 = 3.65$, $p = 0.008$, Fig. 2C). Similarly, fresh and reconstituted pieces of *C. humilis* that were positioned downstream of ungrazed *S. muticum* were significantly more palatable to *S. nadejda* than *C. humilis* pieces downstream of grazed *S. muticum* (fresh $t_7 = 3.40$, $p = 0.011$; reconstituted $t_7 = 2.99$, $p = 0.020$, Fig. 2C). Isopods significantly preferred fresh pieces and reconstituted food made of *F. spiralis* pieces that were positioned downstream of ungrazed *C. humilis* pieces on average 2.4- and 2.5-fold, respectively, compared to *F. spiralis* food that was positioned downstream of grazed *C. humilis* pieces (fresh $t_7 = 2.73$, $p = 0.029$; reconstituted $t_7 = 3.14$, $p = 0.016$, Fig. 2C). Yet, isopods showed no preference between pieces of *F. spiralis* that were located downstream of grazed and ungrazed pieces of *S. muticum* (fresh $t_7 = -1.82$, $p = 0.112$; reconstituted $t_7 = -0.55$, $p = 0.602$, Fig. 2C). The consumption of *S. muticum* pieces kept downstream of grazed and ungrazed pieces of either *C. humilis* or *F. spiralis* was not significantly affected (all $t_7 < 0.55$, $p > 0.05$, Fig. 2C).

Northern Europe experiments (Germany)

Direct grazing. Idotea baltica significantly consumed ungrazed fresh pieces of *H. siliquosa*, on average, 2-fold more than previously grazed pieces ($t_7 = 4.585$, $p = 0.003$, Fig. 3A). Yet, corresponding assays with reconstituted *H. siliquosa* as food revealed that isopods did not significant differentiate in the consumption of ungrazed and previously grazed pieces of *H. siliquosa* ($t_7 = -1.59$, $p = 0.157$). In assays using fresh and reconstituted pieces of *F. vesiculosus*, *I. baltica* significantly preferred ungrazed food, 2.5 and 1.9 times more than previously grazed food, respectively (fresh $t_7 = 2.89$, $p = 0.023$; reconstituted $t_7 = 3.38$, $p = 0.012$, Fig. 3A). Previous grazing by *I. baltica* did neither affect the palatability of fresh nor reconstituted food of *S. muticum* significantly (fresh $t_7 = 1.92$, $p = 0.097$; reconstituted $t_7 = 1.31$, $p = 0.231$, Fig. 3A).

Waterborne cues. I. baltica did neither significantly prefer fresh pieces nor reconstituted food made of *H. siliquosa* that were located downstream of ungrazed and grazed conspecifics (fresh $t_7 = 1.96$, $p = 0.091$; reconstituted $t_7 = 0.30$, $p = 0.977$, Fig. 3B). Fresh pieces made of *F. vesiculosus* positioned downstream of ungrazed conspecifics were not significantly preferred by *I. baltica* to pieces located downstream of grazed conspecifics ($t_7 = 1.00$, $p = 0.349$, Fig. 3B). However, there was significant preference by 47% in the corresponding reconstituted food assays ($t_7 = 3.54$, $p = 0.009$). The consumption of *S. muticum* downstream of grazed conspecifics was not significantly different to that of *S. muticum* pieces located downstream of ungrazed ones in both fresh and reconstituted assays (fresh $t_7 = -2.18$, $p = 0.066$; reconstituted $t_7 = 0.61$, $p = 0.563$,

Fig. 3B).

Eavesdropping. I. baltica did neither prefer fresh pieces nor reconstituted food made of *H. siliquosa* pieces that were located downstream of ungrazed *F. vesiculosus* significantly to those located downstream of grazed *F. vesiculosus* (fresh $t_7 = -0.40$, $p = 0.699$; reconstituted $t_7 = .403$, $p = 0.699$, Fig. 3C). Yet, isopods consumed, on average, 2.5 (fresh pieces) and 1.8 times (reconstituted food) more of *H. siliquosa* pieces that were positioned downstream of ungrazed than of previously grazed pieces of *S. muticum* (fresh $t_7 = 2.918$, $p = 0.023$; reconstituted $t_7 = 3.13$, $p = 0.017$, Fig. 3C). In all other assays, *I. baltica* showed no feeding preferences (all $t_7 < 0.91$, $p > 0.05$, Fig. 3C).

DISCUSSION

Our results revealed that macroalgae have an ability to modify anti-feeding traits in responses to direct grazing and/or waterborne cues from grazed neighbours in both Portuguese and German shores studied. Further, the cues (i.e. originated from the non-native *S. muticum*) were efficient to generate the anti-feeding features of some confamiliar native species, i.e. *C. humilis* and *H. siliquosa* in two experimental sites, respectively. Algal capacity to tailor their anti-feeding defences was detected restrictedly to the native species, but not to the non-native species *S. muticum*, which was consistently insensitive to direct grazing or its waterborne cues.

Effects of direct grazing on macroalgal palatability

Either fresh and/or reconstituted pieces of ungrazed native species from both studied shores (*C. humilis*, *H. siliquosa*, *F. spiralis*, and *F. vesiculosus*) were preferred over grazed pieces. Although patterns in fresh assays were not fully matched by assays using reconstituted food, a strong tendency that isopods preferred ungrazed over grazed pieces of native algae was apparent in all assays. This indicates that grazing by local isopods can induce anti-herbivory defences in the native algal species used in this study. In all but one native species, i.e. *H. siliquosa*, results from assays using reconstituted food showed significant preferences for ungrazed over grazed pieces, which suggests an induction of feeding deterrent chemicals. While this is the first report about an induction of anti-herbivory defences in *C. humilis*, *H. siliquosa*, and *F. spiralis*, several studies across a wide geographical range (including both studied shores) already demonstrated the ability of *F. vesiculosus* to induce chemical defences against grazers (Hemmi et al. 2004, Rohde et al. 2004, Long et al. 2007, Yun et al. 2007, Yun et al. 2010).

Lacking preferences in isopod consumption between ungrazed and previously grazed pieces of *S. muticum* indicate that the non-native species was unable to induce anti-herbivory defences against both species of isopods. In contrast, Toth (2007) reported that there was an induced anti-isopod responses of *S. muticum* from a Swedish shore. Several factors could explain this contradicting pattern. First, the herbivore species used in our study were different from that used in the Toth (2007) study. Such grazer-specificity of inducible anti-herbivory responses has also been demonstrated in other species of macroalgae (Molis et al. 2006, Molis et al. 2008) as well as vascular plants (Agrawal 2000). Secondly, differences in the ability of *S. muticum* to induce anti-herbivory defences between geographic regions may stem from the relatively high phenotypic variability, which has been reported to occur in non-native plants in their new habitats (reviewed in Cox 2004, Ghalambor et al. 2007, Thompson 2009).

Instead of herbivore-induced responses, *S. muticum* may deter isopods constitutively. However, this seems unlikely as the consumption level of control and treated pieces of *S. muticum* was at both sites of comparable magnitude to the level of consumption of ungrazed pieces of the other species used in this study. This suggests that there is no constitutive anti-herbivore resistance in *S. muticum*, rather *S. muticum* seems to tolerate grazing, e.g. by compensating grazing-induced losses of biomass with consistent growth regardless of grazing damage (e.g. Rohde et al. 2004, Toth et al. 2007). Indeed, previously grazed *S. muticum* shows comparable growth rates to ungrazed *S. muticum* in its new habitats (Monteiro et al. 2009). We also observed that consistent growth of *S. muticum* pieces at both shores of our study, regardless of feeding loss (unpublished data).

Effects of conspecific waterborne cues on induced defences

Assays using reconstituted food showed that both *F. spiralis* and *F. vesiculosus* were less preferred when positioned downstream of grazed than of ungrazed conspecifics. This suggests that both species induced some chemical anti-herbivory defence in response to exposure to waterborne cues that were released from grazed conspecifics. This corroborates finds of previous studies using same or different populations of *F. vesiculosus* from the NE Atlantic (Rohde et al. 2004, Yun et al. 2007) as well as the fucoid seaweed *Ascophyllum nodosum* (Toth & Pavia 2002). However, not all three species (*C. humilis, H. siliquosa* and *S. muticum*) reduced their palatability in response to waterborne cues released from grazed conspecifics. Similarly, a lack of response to the neighbouring grazing was reported in other brown macroalgae (Sotka et al. 2002, Weidner et al. 2004, Toth 2007, Rohde & Wahl 2008a). This suggests that the ability to respond to waterborne cues from grazed conspecifics with an induction of anti-herbivory defences may be species specific. One reason for such species-specificity could be the distribution of algae in the field (e.g. Rohde & Wahl 2008a). For example, in dense stands, the distance between emitter and receiver conspecifics is short which could minimize the dilution of the signals and thus optimize the transfer of signals. Thus, we can expect that species in dense stands (e.g. *F. spiralis* and *F. vesiculosus*) may be more likely to respond to waterborne cues from adjacently grazed conspecifics than seaweed species with a scattered distribution (e.g. *H. siliquosa* and *S. muticum*).

Eavesdropping - effects of heterospecific cues

The palatability of *C. humilis* in downstream of grazed *F. spiralis* was lower for isopods from the Portuguese shore than in pieces of *C. humilis* in downstream of ungrazed *F. spiralis* and vice versa. These feeding patterns were apparent in assays using fresh and reconstituted pieces of *C. humilis* and *F. spiralis*, strongly suggesting that both species induced a chemical defence against isopods in response to waterborne cues released by isopod-grazed heterospecific species. This is to our knowledge the first report on eavesdropping in marine macroalgae, although it is known to be common in terrestrial plants (Karban et al. 2003, Glinwood et al. 2004, Karban et al. 2004). It provides an example of a trait-mediated indirect interaction among different macroalgae in front of a consumer attack.

The ability to eavesdrop on different plants may be relevant to the ecological distribution of macroalgal assemblages. For example, *C. humilis* and *F. spiralis* coexist in the intertidal zone with a close distance < 1 m. Such close distance among algal habitats can determine the extent of distance over which the trans-specific signals travel in field conditions (e.g. Heil & Karban 2010). In fact, it is reported that the effectiveness of volatile cues to mediate plant signalling in terrestrial systems was limited to plants in a close distance, i.e. within ca. 60 cm (Karban et al. 2006). In this manner, it is assumed that the effects of the cues might be absent if there was a long distance between algal species. Our results between *H. siliquosa* and *F. vesiculosus* are consistent with distribution pattern of macroalgae. *H. siliquosa* occur in shallow subtidal in Germany, in which they are separated by tens of metres from *F. vesiculosus* habitat. However, it is inconsistent between *H. siliquosa* and *S. muticum*. The cues between the two marcroalgae were efficient, even though they distributed in long distances. Collectively, the inconsistent trend indicates that habitat closeness between macroalgae could not fully explain mechanisms of the cue availability to mediate inter-specific plant signalling.

Similar to the observed changes of tastefulness in *H. siliquosa* in Germany, palatability of fresh and reconstituted pieces of *C. humilis* was strongly reduced when positioned downstream of grazed *S. muticum* compared to ungrazed *S. muticum* in Portugal. It implies that grazed *S. muticum* can release the alarm cues utilized by *C. humilis* and *H. siliquosa*. In contrast to *C. humilis* and *H. siliquosa* as cue-receiving species, neither *F. spiralis* nor *F. vesiculosus* lowered its palatability when positioned downstream of grazed than of ungrazed *S. muticum*. The contrasting responses imply that the ability to eavesdrop on the signals from *S. muticum* is restricted on its confamiliar species at both shores, while the phylogenetically further distant *Fucus* species (i.e. different family) were lacking this ability, although it is largely unknown how non-native species or native species respond to the signals from each other in their new habitats (e.g. Smith 2009). Previous studies corroborate with this pattern, as they showed that phylogenetic distance between cue-receiver and cue-emitter can affect the efficacy of inter-specific signals on grazer attacks of a shared consumer (Mirza & Chivers 2003, Schoeppner & Relyea 2005). Interestingly, there was no change in the level of palatability between *S. muticum* positioned downstream of grazed and ungrazed native macroalgae from both shores. Thus, this lack in eavesdropping by *S. muticum* represents an asymmetric interaction in which native species may benefit from nearby grazed *S. muticum*, while the latter has no advantage when neighbouring native macroalgae are grazed.

Conclusion

Similar to signalling among terrestrial plants, macroalgae in marine environments can inform a potential load of herbivory attacks to neighbouring algal species. Consequently, their alarm cues are used to cope with the grazing impacts in advance and to induce defences in some conspecific as well as heterospecific algae. Furthermore, the capacity to recognize cues from other algae can be different depending on species-specificity, although its underlying mechanisms remain unclear. Such cue availability among macroalgae may reframe ultimately macroalgae-herbivore interactions in a way that could not be predicted from the cue effectiveness within species.

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Yun HY, Rohde S, Linnane K, Wahl M, Molis M (2010) Seaweed-mediated indirect interaction between two species of meso-herbivores. Marine Ecology Progress Series 408:47-53

Figure captions

Fig. 1 Experimental set-up (displayed for one replicate), testing for induced defenses in response to (A) direct grazing, (B) neighbour grazing by conspecific, and (C) by heterospecific species. For neighbour grazing effects, aquaria (big rectangles) were divided by a net into two compartments and arrow means direction of water flow. Each aquarium contains three or six macroalgal pieces). One macroalgal pieces was used in each fresh or reconstituted food assays or as autogenic controls. After 4 d of acclimatization, grazers were added to half of aquaria during subsequent 12-day induction phase. Macroalgal pieces were transferred to feeding arenas (ovals) for conducting two choice feeding assays. Solid and stippled lines represent control and treated algal pieces, respectively, in fresh and reconstituted feeding assays. For clarity, autogenic controls of fresh food were omitted.

Fig. 2 Portugal (Southern European site). Mean consumption (±SE) of treated (open bar) and control food (black bars) from fresh and reconstituted algal pieces i.e. direct grazed *vs.* control (A), macroalgae affected by grazing on neighbouring conspecifics *vs.* control (B), and macroalgae exposed to grazed on heterospecific algae *vs.* control (C). UP = macroalgal species located in the upstream compartment and DOWN macroalgal species in the downstream compartment. * = significantly different in paired t-test (n =8).

Fig. 3 Germany (Northern European site). Mean consumption (±SE) of fresh and reconstituted algal pieces. Symbols and their interpretation as in Fig. 2.

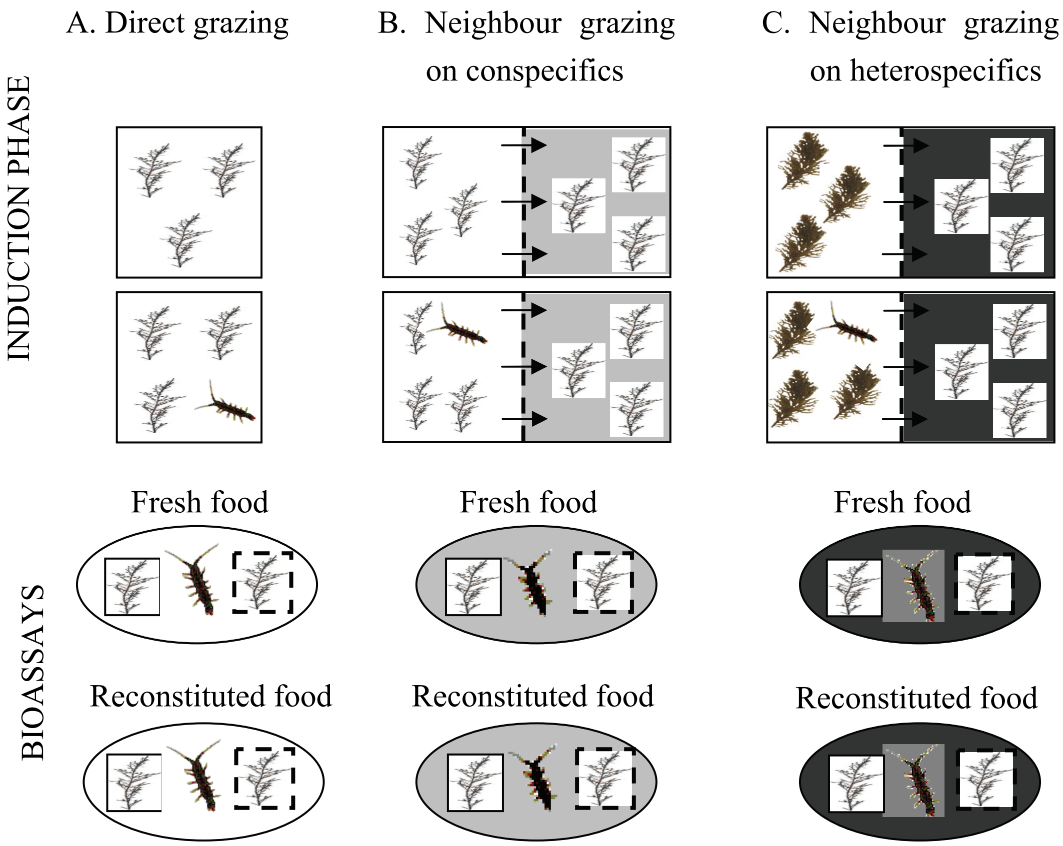

Fig. 1 Yun et al.

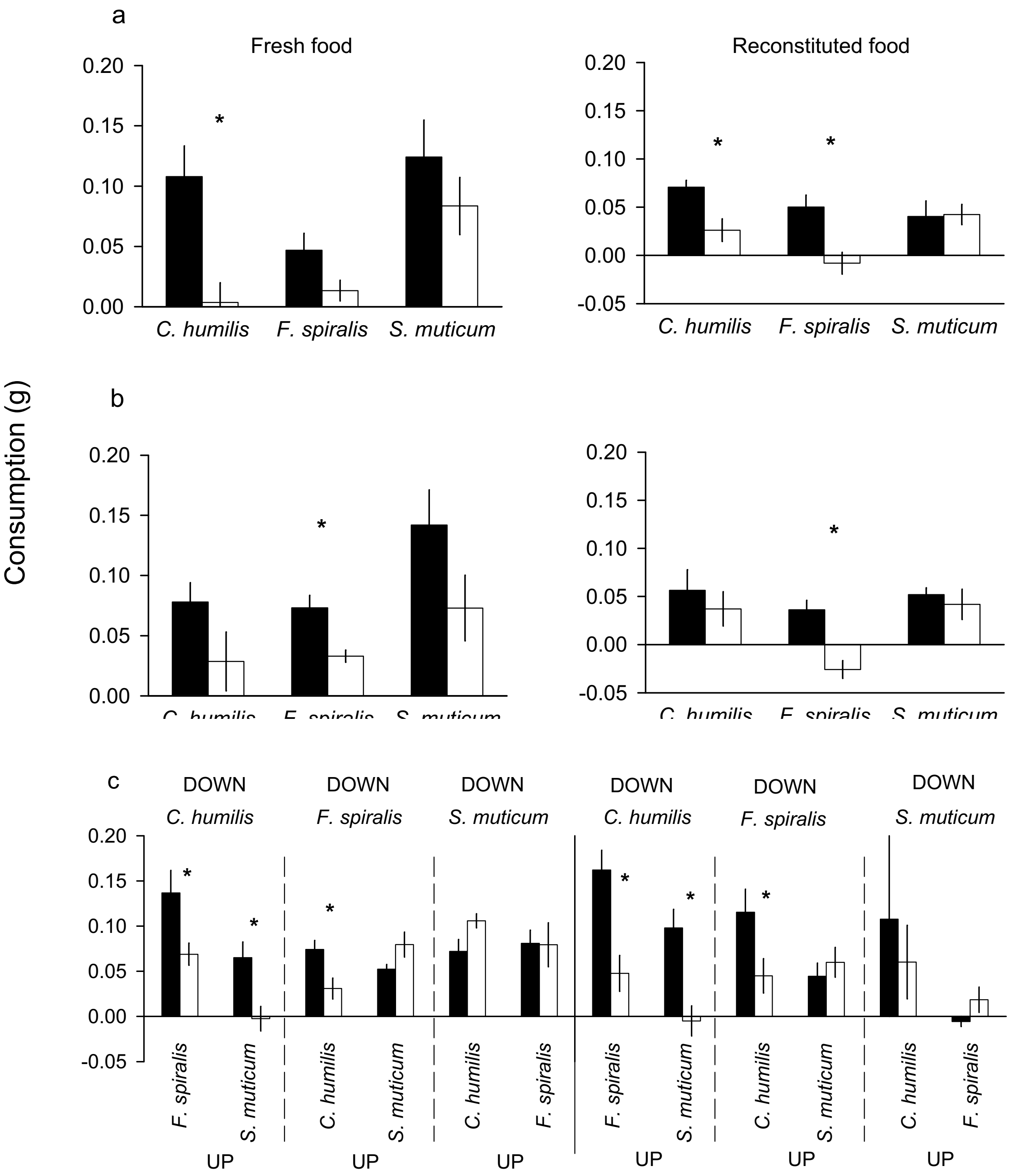

Fig. 2 Yun et al.

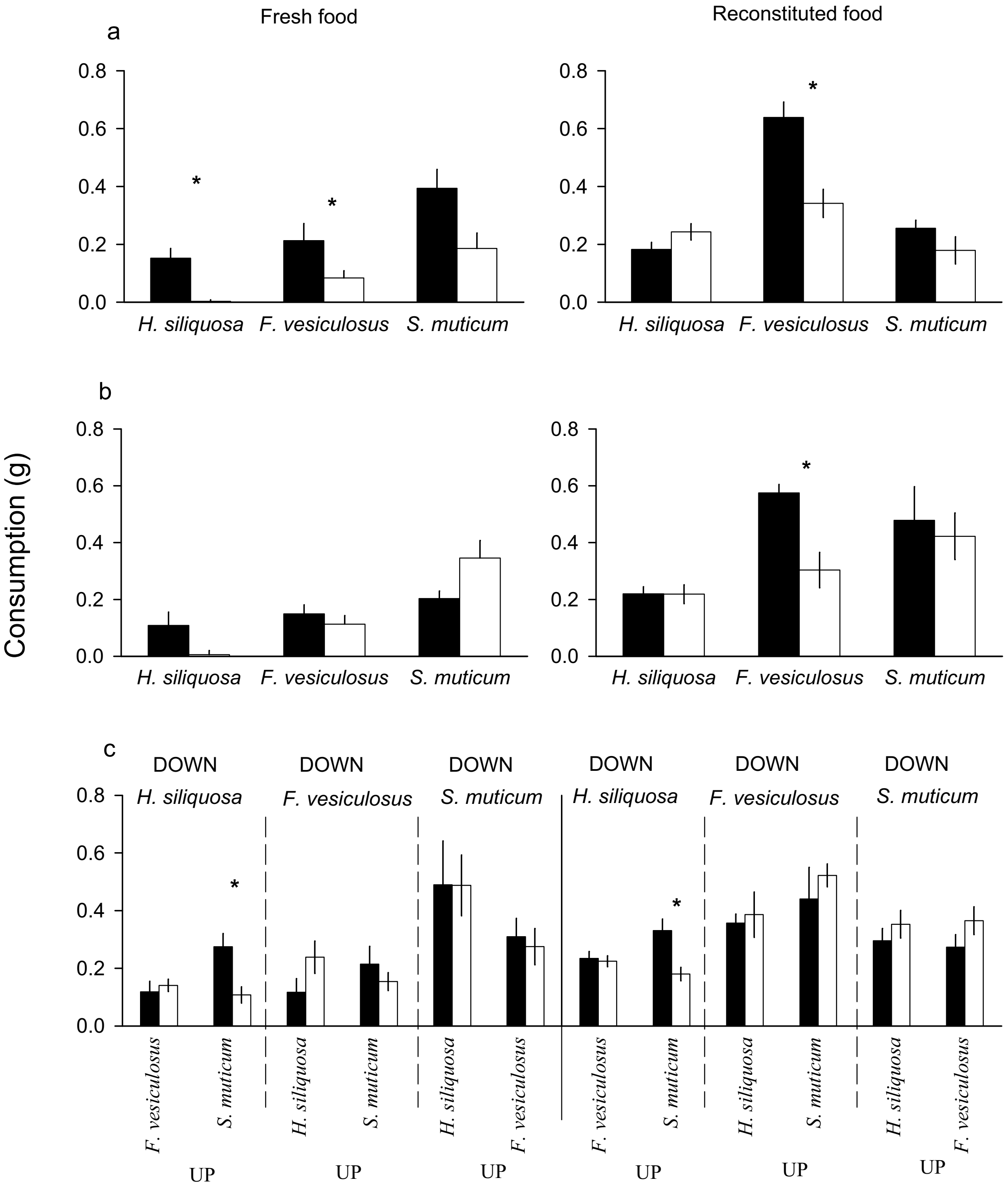

Fig. 3 Yun et al.

Seaweed-mediated indirect interaction between two species of meso-herbivores

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ABSTRACT

Previous studies on trait-mediated trophic interactions in marine ecosystems were restricted to pair-wise interactions between one species of meso-herbivore and plant, though multi-grazer interactions are more common in nature. We investigated whether the feeding of one consumer, either the periwinkle *Littorina littorea* or the isopod *Idotea baltica*, affected consumption by the other consumer via anti-herbivory defence induction in the brown seaweed *Fucus vesiculosus*. To test the generality of our findings, we ran similar experiments with seaweed/grazer populations in the North and Baltic Seas (NE Atlantic). Grazer-specificity in induction strength was assessed by using the same species of grazer for induction and consumption. 'Indirect' induction effects were assessed by using different species of grazers for induction and consumption. Palatability assays were run with live algae and with reconstituted food to distinguish between different mechanisms of resistance. Grazing by herbivores induced a chemical defence in *F. vesiculosus*. In the North Sea population, the induced defences were only effective against *I. baltica*, regardless of inducer identity. The sensitive responses of *I. baltica* to the induced defences were also detected in the reconstituted food assays using Baltic Sea organisms. Thus, marine meso-grazers may be affected by previous feeding through the same or a different species of consumer by modified prey traits, such as induced chemical defences. Furthermore, the magnitude of the effect in the induced defences can be determined by species-specific sensitivity.

KEY WORDS: Multi-species interactions, Trait-mediated indirect interaction, *Fucus vesiculosus*, *Idotea baltica*, *Littorina littorea*, Herbivore–seaweed interactions

INTRODUCTION

Herbivory can strongly alter the structure and diversity of terrestrial and marine communities (reviewed by Duffy & Hay 2000, Van Zandt & Agrawal 2004). Herbivory may be a driver for the evolution of phenotypic plastic responses in plants (Karban & Baldwin 1997, Toth & Pavia 2007). Inducible anti-herbivore responses in plants could represent a more favourable strategy than constitutive defences where the risks from consumers are relatively high, but variable in space and time (Karban & Nagasaka 2004). Herbivore-induced responses at the same time reduce the vulnerability of plants to consumers (reviewed by Karban & Baldwin 1997) and modify the feeding behaviour of herbivores (Borell et al. 2004). Typically, the induced defence persists for some time, then is reduced again, either after grazing pressure ceases (Rohde & Wahl 2008) or even before (Weidner et al. 2004). Induced chemical defences may contribute to intra-specific signalling in plants (reviewed by Baldwin et al. 2006) and seaweeds (Toth & Pavia 2000) and affect inter-specific interactions with other community members (Ohgushi 2005, Coleman et al. 2007, Denno & Kaplan 2007).

Several herbivore species with diverse dietary guilds in marine systems may share a single seaweed host (e.g. Buschbaum et al. 2006), providing arenas for multiple grazer interactions. Despite this, previous studies on marine communities have mainly focused on the effects of single herbivore species on trait changes in their single host alga, because competition among herbivores is usually not considered due to the supposed ubiquity of food (Strong et al. 1984). However, besides this direct consumer–consumer interaction, a consumption-induced modification of food quality may constitute trait-mediated indirect interactions among consumers (TMIIs) (reviewed by Schmitz et al. 2004, Fordyce 2006, Long et al. 2007).

While TMIIs may be powerful, the mediating trait, inducible defence, within a prey species may vary regionally (i.e. Long & Trussell 2007). For instance, previous research on a congeneric species to that used in the present study, *Fucus radicans*, has shown that it exhibits different adaptive traits regionally, due to restricted gene flow (Bergström et al. 2005). Indeed, induction of anti-herbivore resistance as a plant property to mediate herbivore interactions can be geographically variable within a specific population (Long & Trussell 2007). Also, herbivores may adapt to the local particularities of their hosts (Sotka & Hay 2002, Jormalainen et al. 2008). Grazer tolerance to the chemical defences of algae may be broad but differ phylogenetically

(Poore et al. 2007). Therefore, herbivore–herbivore interactions mediated by algal hosts may not only vary regionally but also with regard to the consumer group considered.

TMIIs are potentially of high ecological relevance, due to their immediate effects on the entire prey population when consumers are present (Peacor & Werner 2001). To better understand whether TMIIs are generally important drivers in species interactions, 2 aspects of TMIIs need to be experimentally considered. First, it should be clarified whether the very few examples of TMIIs in marine systems depict a bias in research effort or a real pattern (Schmitz et al. 2004). Second, to assess regional variability, TMIIs of identical interaction webs should be compared between locations with different environmental conditions. In a recent study from North America, Long et al. (2007) suggested that an asymmetry in the competition for food between the periwinkles *Littorina obtusata* and *L. littorea* and the isopod *Idotea baltica* was mediated by trait changes in their shared food, the brown seaweed *Fucus vesiculosus*. In the present study, since a similar food web also exists along NE Atlantic shores, we examined the role of TMIIs in the food web with seaweed *F. vesiculosus* and the meso-grazers *I. baltica* and *L. littorea*, at 2 different locations in the brackish Baltic Sea and the fully marine North Sea, respectively. First, we hypothesised that grazing decreases the palatability in *F. vesiculosus*. Second, we tested whether the grazing-induced response may be derived from a chemical cue. Third, we investigated whether grazing by a given herbivore species decreased the palatability of seaweed pieces for naïve conspecific or heterospecific consumers. To generalize induced response patterns, experiments were conducted with 2 regionally distinct (North and Baltic Seas) food webs of *F. vesiculosus* and its grazers.

MATERIALS AND METHODS

Sampling sites and organisms. Laboratory experiments were conducted with the gastropod *Littorina littorea*, the isopod *Idotea baltica*, and the brown seaweed *Fucus vesiculosus*, which were collected at 2 NE Atlantic sites: (1) at Bülk (54°26 N, 10°11 E) in the Kiel Fjord (western Baltic Sea) and (2) at Bunker (54°11'N, 7°52'E) in the Nordwatt of Helgoland (southern North Sea). These sites are ca. 900 km apart by seaways and differ with respect to their abiotic conditions. The Baltic Sea is an almost atidal (tidal range <10 cm), semi-enclosed, brackish sea (salinity of 15 at the collection site) of relatively young geological age. In contrast, the North Sea has a tidal range of >2.35 m at the collection site, is fully marine (salinity of 33 at the collection site) and represents a geologically older marine environment than the Baltic Sea. The distribution of the fauna and flora in the Baltic Sea reflects a depleted sub-set of the North Sea biota, indicating similarity in biotic conditions at both sites to a certain extent.

Experimental set-up and design. ***Induction of anti-herbivore defences:*** To test the causes and effects of herbivory-induced defence at the regional scale, identical 21 d laboratory experiments were conducted at the IFM-Geomar (Baltic Sea) and Biologische Anstalt Helgoland (North Sea).

Twenty individuals of *Fucus vesiculosus* without visible grazing marks were collected from the shore at each region and immediately transported to the laboratory. Ten apical pieces of similar length of each *F. vesiculosus* specimen were cut, and all visible epibionts were removed with a soft sponge without damaging the alga. Each *F. vesiculosus* piece was then marked using coloured threads. The 10 pieces of each *F.*

vesiculosus specimen were evenly divided between 2 transparent plastic aquaria, each thus containing 5 genetically identical *F. vesiculosus* pieces. Subsequently, the *F. vesiculosus* pieces were kept for 4 d without treatment to allow for the reduction of putative *in situ* grazing effects, which are known to occur within 4 d (Rohde & Wahl 2008), and acclimatisation of alga to laboratory conditions (Fig. 1). At the same time, *Idotea baltica* and *Littorina littorea* specimens for use in the experiment were incubated in an aerated container (50 l) with water flow-through and fed on a mixed algal diet.

In both regions, the aquaria were supplied with local seawater. Off Kiel (Baltic Sea), seawater from the nearby Kiel Fjord was UV-sterilized (hw-Wiegand water sterilizer 500), filtered (1.2 µm), and stored in a tank (150 l) before supplying the individual aquaria (2.9 l) at a flow rate of 0.25 l h–1. Off Helgoland (North Sea), seawater from the North Sea was filtered by synthetic cotton fibre mesh and stored in a tank (200 l) from which each aquarium (8 l) was supplied at a flow rate of 1.2 ± 0.3 l h–1. In both regions, fluorescent tubes illuminated each aquaria at a total irradiance of 65.5 ± 2 µmol m–2 s–1 (LI-COR, UWQ 6534) off Kiel (Baltic Sea) and of 34.4 ± 2.5 µmol m–2 s–1 (LI-COR, UWQ 6534) off Helgoland (North Sea) with a 12 h light:12 h dark cycle. These light regimes corresponded to photosynthetic active radiation at 1 m water depth in ambient levels during the time when the experiments were conducted.

During 14 d of induction, algal pieces either were left without grazers (‘control’) or were exposed to grazing (‘grazed’). Potential defences in the seaweed were induced by direct grazing consisting of adding either 15 *Idotea baltica* or 15 *Littorina littorea* (hereafter ‘inducer’) in each of 10 aquaria (Fig. 1). Aquaria were covered with a wire mesh (Kiel) or Perspex plates (Helgoland) to prevent *L. littorea* emigration from the aquaria. Throughout the induction phase, pieces of *Fucus* *vesiculosus* were visually checked for the occurrence of feeding damage to confirm that all algal pieces were consumed by grazers. In addition, measuring the consumption rates of 3 *F. vesiculosus* pieces per aquarium (n = 10) during the induction phase (only North Sea) revealed no significant differences in grazing impact within aquaria (see 'Results'). At the end of the induction phase, all inducers were removed from the set-up and returned to the sea.

Bioassays: After the induction phase, herbivory-induced changes in algal palatability were tested by 2 types of choice feeding assays comparing the consumption of grazed and non-grazed algal pieces (Fig. 1).

In the first type of assay, the palatability of previously grazed versus un-grazed fresh algal pieces was tested to detect induced responses of a chemical and/or morphological nature. After algal pieces were blotted with paper towels for 15 s and spun 10 times in a salad spinner, then they were weighed to the nearest 0.001 g on a balance (Sartorius LE323S). One previously grazed and 1 non-grazed piece of *Fucus vesiculosus* were transferred into a feeding arena (glass Petri dish, 25 cm diameter, termed experimental unit [EU]) containing 1.5 l of seawater. Each feeding arena contained either 6 *Littorina littorea* or 6 *Idotea baltica*, which either same or different specimens were used for induction and consumption (hereafter ‘consumer’) (Fig. 1; total 40 EUs, n =10). To avoid confounding effects of grazer familiarity with previously grazed food, only naïve individuals without prior contact with the test algae were used as consumers. The EUs during the feeding assays were maintained under the same light regime as that used during the induction phase. Seawater was changed every 12 h. Algal pieces were reweighed after 3 d. To account for non-feeding-related changes in wet mass (i.e. autogenic changes) during bioassays, feeding rates were corrected using the formula (equation from Cronin & Hay 1996): *T*start × (*C*end / *C*start) – *T*end, where *T*start and *T*end represent initial and final wet mass of an assayed *F. vesiculosus* piece, respectively, and *C*start and *C*end represent initial and final mass of the autogenic control piece, respectively. A significant preference of non-grazed over previously grazed *F. vesiculosus* pieces was interpreted as an induction of anti-herbivory defences. Autogenic changes in seaweed wet mass yielded in some cases negative consumption rates, i.e. growth in the presence of grazers more than compensated feeding loss.

The second type of feeding assay used reconstituted *Fucus vesiculosus*, in which any morphological defence was destroyed in the alga, but its chemistry preserved, to assess whether induced anti-herbivory defences were of chemical in nature. To reconstitute *F. vesiculosus*, algal pieces were freeze-dried after the induction phase, ground to a homogenous fine powder with a mortar and pestle, and 0.5 g of this powder was suspended in 2 ml distilled water. The molten agar (0.09 g in 2.5 ml distilled water) was mixed, after cooling to 55°C, with the *F. vesiculosus* suspension, poured over a mosquito net (1 mm2 mesh size) and flattened between 2 glass plates (methods adapted from Hay et al. 1994). After solidification, food pellets of 15 × 15 mm^2 were cut from *F. vesiculosus*–agar mixtures and marked by different incision patterns to distinguish pellets originating from control and grazed treatments. One control and 1 grazed pellet were transferred into a feeding arena (EU) with isopods or snails for 36 h (Fig. 1; total 40 EUs, n = 10) under conditions similar to those in the fresh bioassay. Feeding rates were determined by counting the number of empty mosquito net cells in the food pellets at the end of 2-choice feeding assays.

Statistical analysis. Since the 2 regions differ in a number of environmental factors, we did not attempt to interpret the absolute differences in consumption rates between 2 sites, but restricted our analysis to within-region comparisons. In both Baltic and North Sea data, consumption rates of fresh and reconstituted alga were analyzed using 3-factorial repeated-measures ANOVAs. Consumption rates from 1 feeding assay were the repeated measure (fixed, 2 levels: grazed and control), while the identity of the inducer (fixed, 2 levels: *Littorina littorea* and *Idotea baltica*) and type of consumer (fixed, 2 levels: *L. littorea* and *I. baltica*) represented orthogonal grouping factors. Testing for sphericity was not relevant because the repeated measure had only 2 treatments (Quinn & Keough 2002). Due to ambiguous selection of an appropriate error term for post hoc analysis involving within-subject by between-group interactions (Winer et al. 1991), 1-tailed paired *t*-tests were performed as alternative post hoc tests with sequential Bonferroni adjustment to account for the number of comparisons made during analysis (i.e. $k = 2$, where k refers to the number of levels of the between-group factor; Rice 1989).

Results

Induction phase

Idotea baltica and *Littorina littorea* consumed on average 14 and 15% of the *Fucus vesiculosus* pieces during the induction phase, respectively. Direct measurements of consumption (only North Sea) and visual inspections of algae for grazing marks (both study sites) confirmed that all *F. vesiculosus* pieces in each aquarium were equally consumed by both grazer species.

Fresh food assays

Baltic Sea

Consumption was significantly lower in grazer-treated algal pieces than in the non-grazed pieces (Table 1, Fig. 2a). The absence of significant interactions suggests that this effect was independent of inducer identity and consumer identity (Table 1).

North Sea

Herbivores significantly preferred non-grazed pieces by 51% over previously grazed *Fucus vesiculosus* pieces (Table 1, Fig. 2b). In contrast to the lack of interaction with inducer identity, previous grazing had a significant interaction with consumer identity; this indicates that the efficacy of anti-herbivory defences depends on the consumer (Table 1). When *Idotea baltica* was the consumer, the non-grazed algae were significantly preferred, by 29%, over the grazed *F. vesiculosus* pieces (paired *t*-test: $t_{19} = 6.31$, $p_{corrected} < 0.025$), while *Littorina littorea* showed no significant preference (paired *t*-test: $t_{19} = 0.26$, $p_{corrected} > 0.025$).

Reconstituted food assays

Baltic Sea

Control pieces of non-induced *Fucus vesiculosus* were preferred 2.3 times over induced pieces (Table 1, Fig. 3a). The previous grazing-effect varied on consumer identity, regardless of inducer identity (Table 1). *Idotea baltica* as the consumer significantly preferred non-grazed pellets rather than previously grazed pellets (paired *t*-test: $t_{19} = 3.91$, $p_{corrected} < 0.025$), whereas the consumer *Littorina littorea* did not show a significant preference between the 2 types of food (paired *t*-test: $t_{19} = 0.26$ $p_{corrected} > 0.025$).

North Sea

Similar to assays using fresh *Fucus vesiculosus* pieces, overall consumption of control pellets was significantly higher than that of previously grazed *F. vesiculosus* pellets (Table 1, Fig. 3b). The effect of previous grazing was not significantly different among inducer species, but it was among consumers (Table 1). The consumer *Idotea baltica* significantly preferred ungrazed over grazed *F. vesiculosus* pellets; in contrast, *Littorina littorea* showed no preference (paired *t*-test: *I. baltica* $t_{19} = 3.46$, $p_{corrected} < 0.025$; *L. littorea* $t_{19} = 1.11$, $p_{corrected} > 0.025$).

Discussion

Meso-grazers induce chemical defence in *Fucus vesiculosus* in both regions studied. The amount of feeding reduced by these defences varied with consumer identity in 2 regions. *Idotea baltica* might be a more sensitive responder to grazing-induced modification in algal palatability than *Littorina littorea*.

The observed consistent preference of previously non-grazed over grazed pieces of *Fucus vesiculosus* using fresh or reconstituted food indicates that grazing can change some chemical seaweed trait(s). Chemical anti-herbivore defences have been described in *F. vesiculosus* (Rohde et al. 2004, Rohde & Wahl 2008) and in other species of fucoids (Toth & Pavia 2000, Taylor et al. 2002, Koivikko et al. 2005).

Quality and quantity of induced defences seems to be independent of inducer identity, at least for the 2 herbivore species tested, and the resulting patterns appeared to be surprisingly consistent. Regardless of whether the inducer was a con- or heterospecific species, *Idotea baltica* preferred non-grazed pellets over grazed ones in the North and Baltic Seas. Similarly, the preference pattern displayed by *Littorina littorea* did not depend on the inducer species. Tendencies similar to those shown in the reconstituted food assays were found in the other assays using live algal pieces. While the inducer species and region did not change the pattern, the sensitivity of the consumer species did. In all instances, *L. littorea* was more tolerant towards the chemical defences of *Fucus vesiculosus* than was *I. baltica*.

To our knowledge, only Long et al. (2007) have previously investigated the species-specific effects of induced seaweed responses using diverse herbivore species. In their study, grazing by the periwinkle *Littorina obtusata* reduced consumption rates of *L. littorea*, *L. obtusata* and *Idotea baltica* on *Fucus vesiculosus*, while previous grazing by *L. littorea* did not affect the palatability of *F. vesiculosus* for any of the 3 herbivores. While they suggested that inducer identity matters for the efficacy of the induced defence, we additionally show here that sensitivity to the induced defence may vary substantially among consumer species. Notably, *I. baltica*, but not *L. littorea*, were efficiently deterred by induced algae. Thus, all 3 components, the capacity of herbivores to trigger defence induction, the ability of the algal prey to react to grazing by inducing a defence, and the sensitivity of local herbivore species to this induced defence (i.e.

herbivore offence; Sotka & Whalen 2008) will determine the outcome of this multilateral interaction.

Differences in the responses of herbivores to inducible defences can cause plant-mediated interactions to appear asymmetric (Kaplan & Denno 2007). An induction of anti-herbivory defences has been shown to increase the dispersal of feeding damage, as well as the frequency of grazer movements (Borell et al. 2004). More mobile grazers like *Idotea baltica* may increase foraging activity in response to induced chemical seaweed defences and migrate to less well-defended prey, while slow moving grazers like *Littorina littorea* have to cope with the defense longer. In combination with the possibility to gain protection from defended prey, this may have selected for greater tolerance in slow moving meso-grazers (i.e. Sotka & Whalen 2008), which has also been demonstrated in terrestrial systems (reviewed by Karban & Agrawal 2002).

Studies on multiple herbivore interactions mediated by the traits of a host alga are just emerging for the marine community. The presence of multiple herbivore species that differ in their degrees of sensitivity towards defences will put some selective pressure on the defence traits of the host plant. This knowledge regarding consumer–prey interactions is highly desirable, because the intensity and directions in consumer–consumer interactions can be determined by the trait-mediated effects of prey (Trussell et al. 2003, Creel & Christianson 2008). Assessing the contribution of indirect trait-mediated effects in species interactions will help to refine existing knowledge on species interactions towards a more realistic perspective of the interactions occurring in the overall food web.

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Table 1. Results of 3-factorial repeated measures ANOVA from a) Baltic Sea and b) North Sea, comparing feeding rates affected by previous grazing (2 levels, non-grazing and grazing), the identity of the inducer (2 levels, *Idotea baltica* and *Littorina littorea*), and the identity of the consumer (2 levels, *I. baltica* and *L. littorea*). Consumption was assessed in two choice bioassays using either fresh or reconstituted pieces of *Fucus vesiculosus* (n = 10). df = degrees of freedom.

a) Baltic Sea

	Fresh food				Reconstituted food			
	df	MS	F	p	df	MS	F	p
G	1	0.52	16.17	< 0.001	1	2387.11	17.60	< 0.001
G x I	1	0.04	1.12	0.298	1	189.11	1.39	0.245
G x C	1	0.13 x 10^{-2}	0.04	0.842	1	1911.01	14.09	0.001
G x I x C	1	0.76 x 10^{-3}	0.02	0.877	1	340.31	2.51	0.122
Error	36	0.03			36	135.64		

b) North Sea

	Fresh food				Reconstituted food			
	df	MS	F	p	df	MS	F	p
G	1	0.08	19.66	< 0.001	1	11834.11	12.14	0.001
G x I	1	0.27 x 10^{-2}	0.66	0.421	1	32.51	0.03	0.856
G x C	1	0.04	10.60	0.002	1	5628.01	5.77	0.022
G x I x C	1	0.07 x 10^{-4}	0.18 x 10^{-2}	0.967	1	400.51	0.41	0.526
Error	36	0.15			36	974.68		

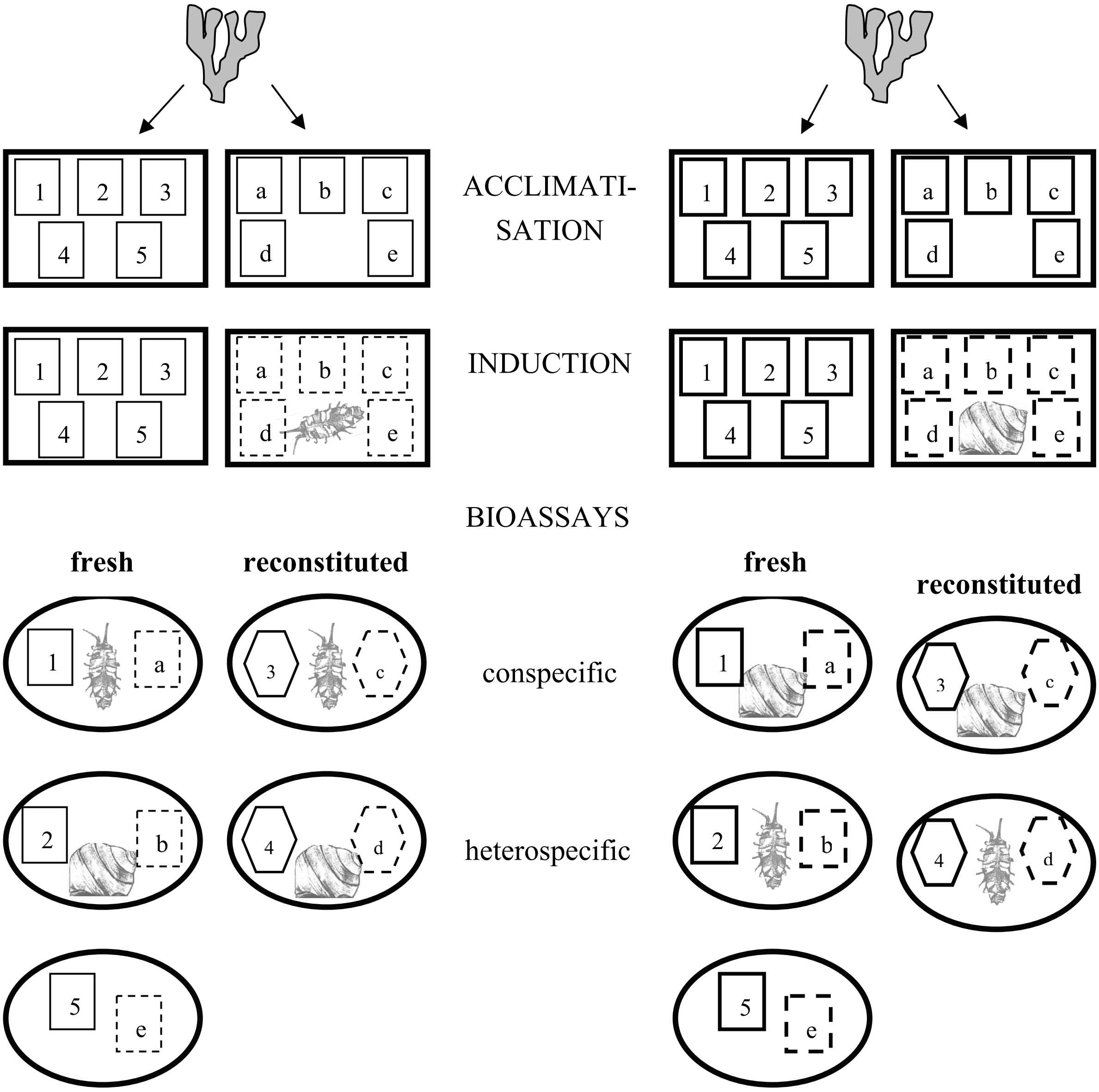


Fig. 1. Experimental design (only shown for a single replicate, requiring two algal specimens), testing for effects of trophic interactions between the brown seaweed *Fucus vesiculosus* and the two meso-herbivores *Idotea baltica* (isopod) and *Littorina littorea* (gastropod). Large rectangles indicate aquaria, each containing five *F. vesiculosus* pieces (small rectangles). After a 4 d acclimatisation phase, grazers were added to half of the aquaria for 14 d. Pieces of *F. vesiculosus* grazed by *I. baltica* and *L. littorea* (thin and thick stippled rectangles, respectively) were transferred to feeding arenas (ovals) for two choice feeding assays. Numbers and letters label control and grazed pieces of *F. vesiculosus*, respectively, while rectangles and hexagons indicate fresh and reconstituted pieces of *F. vesiculosus* in feeding assays, respectively. For clarity, autogenic controls of reconstituted food were omitted.

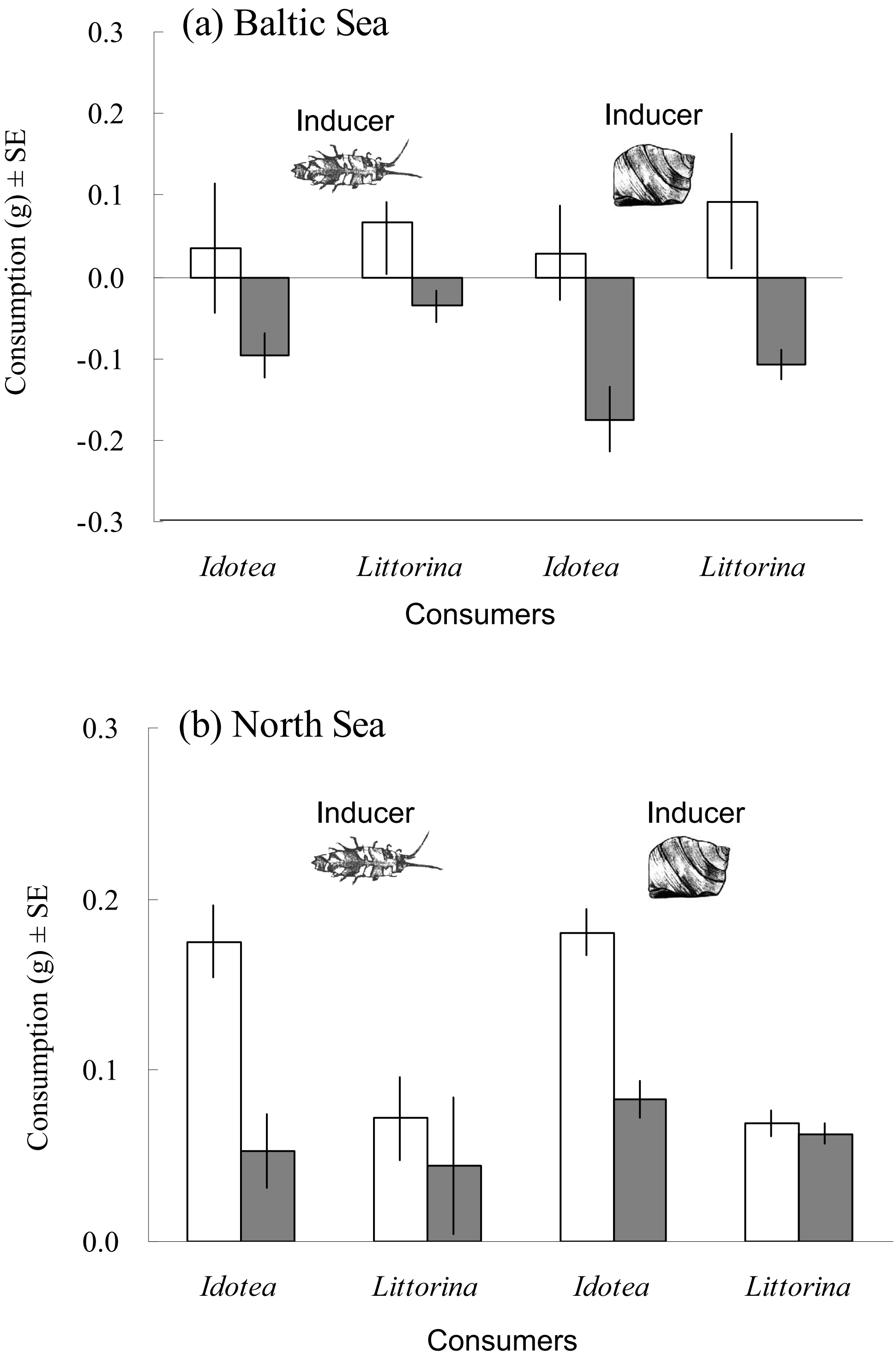


Fig. 2. Mean (± SEM) consumption rates of control (white bars) and grazed (black bars) pieces of fresh *Fucus vesiculosus* from (a) Baltic and (b) North Sea (n = 10). Pictures of grazers above the graphs indicate grazer species used during the induction phase (inducer), while the names below the graphs indicate the species of grazer used in the feeding assays (consumer).

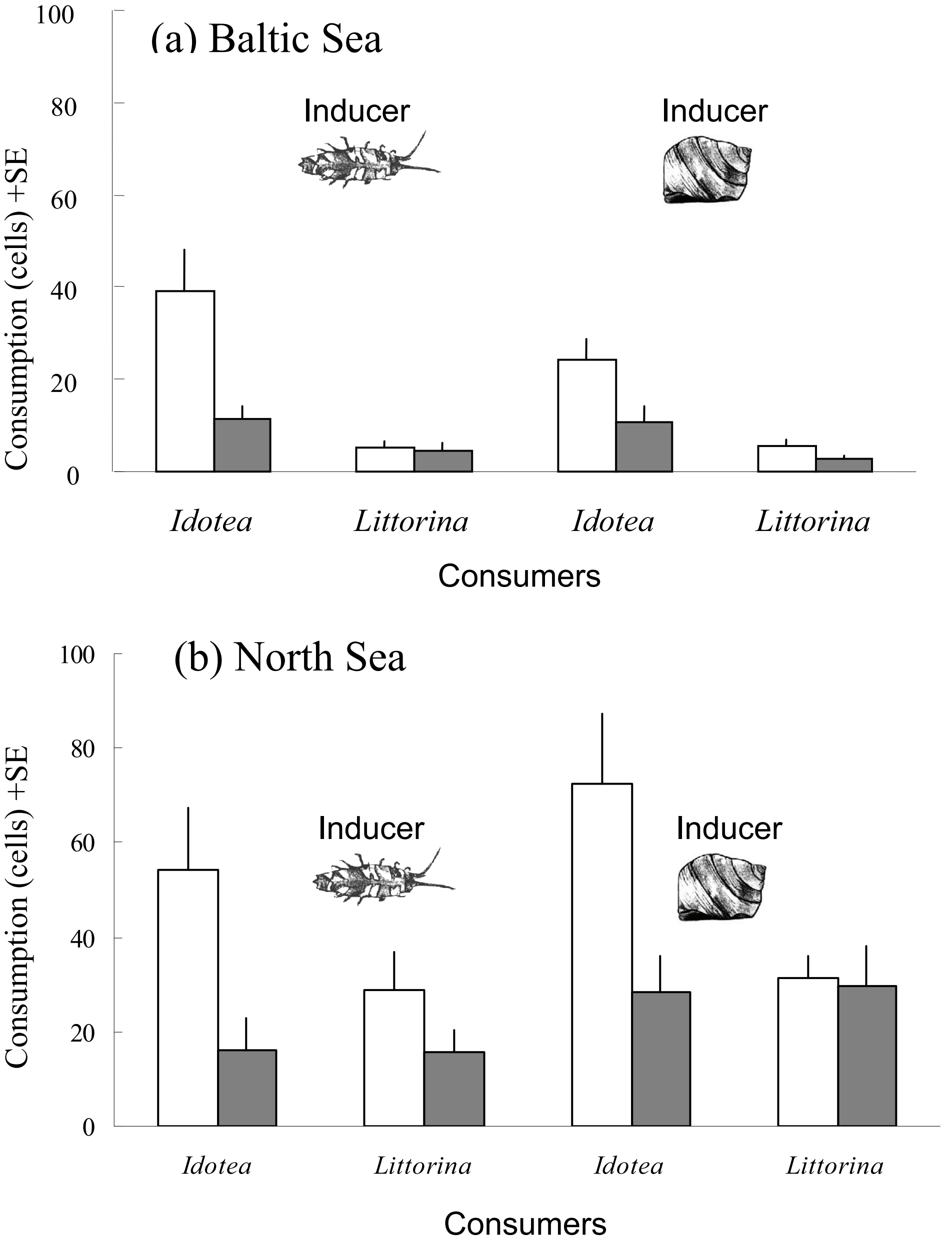


Fig. 3. Mean (+ SEM) consumption rates of grazed and control pieces of reconstituted *Fucus vesiculosus* food from (a) Baltic and (b) North Seas (n =10). Symbols as in Fig. 2.

4. SUMMARY OF RESULTS

4.1. Cues to induce defenses in *Fucus vesiculosus*

Amphipod consumption was significantly lower in *F. vesiculosus* pieces exposed to nearby amphipod-grazed conspecifics, as well as to non-grazing amphipods in comparison with direct grazed pieces and control pieces (resampling, $p = 0.019$, Fig. 5.). However, amphipod consumption to food pellets in DCM extract was not significantly different from 3 grazing treatments with control (resampling, $p = 0.137$, Fig. 5.).

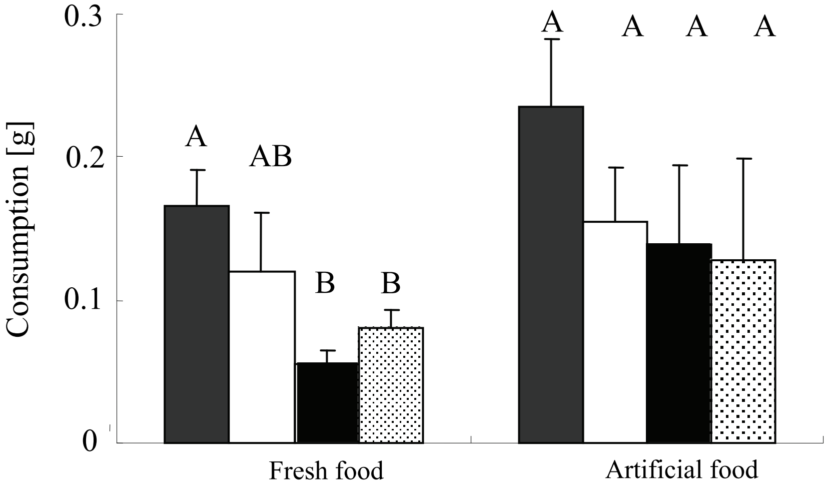


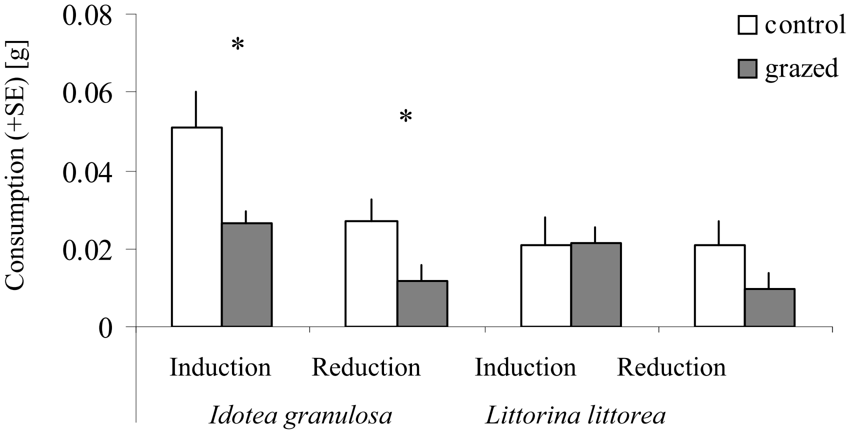
Fig. 5. Mean (+SE) amphipod-consumption on *Fucus vesiculosus* during feeding assays (n = 7) at the end of the induction phase (see material and methods for details) for (A) live algae and (B) agar-based food containing lipophilic *Fucus*-extracts. Black bars = control, open bars = directly amphipod attacked, white dotted bars = waterborne cues from nearby-grazed conspecifics, black dotted bars = waterborne cues from non-grazing amphipod. Treatments sharing the same letter are not significantly different after post-hoc test. Figures are redrawn from Publication 1.

4.2. Induced defenses in non-native *Mastocarpus stellatus vs.* native *Chondrus crispus*

4.2.1. Herbivore specificity

Anti-isopod response. After induction phase, the palatability of fresh *M. stellatus* grazed by coevolved *Idotea granulosa* was significantly different from that of non-grazed algae (Fig. 6-I.). However, grazing by this isopod species did not decrease the palatability of *C. crispus* compared to that of ungrazed conspecifics (Fig. 6-II.).

I. *Mastocarpus stellatus*



II. *Chondrus crispus*

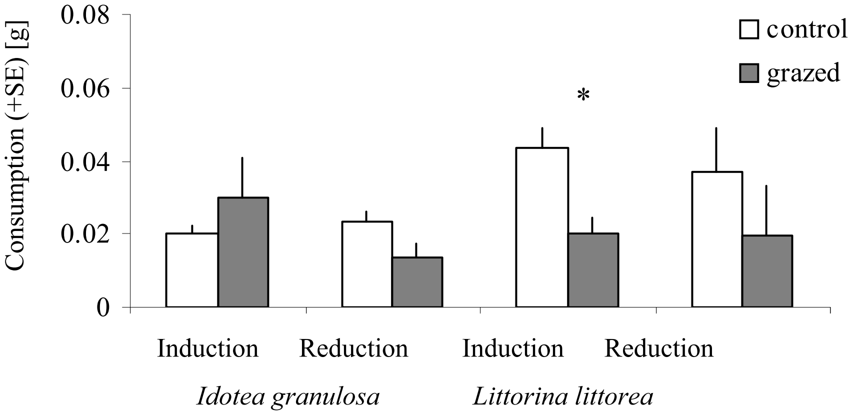


Fig. 6. Mean (+ SE) consumption of (I) *Mastocarpus stellatus*, and (II) *Chondrus crispus* in fall experiment grazing by *Idotea granulosa* and *Littorina littorea* for 3 day feeding assays for fresh algae at the end of induction and reduction phase (n =10). Asterisks indicate a significant difference ($p < .05$) between the palatability of grazed and control tissues by paried *t*-tests (redrawn from Publication 2).

Anti-gastropod response. After induction phase, there was no significant preference between fresh grazed and control *M. stellatus* by non-coevolved *Littorina littorea* (Fig. 6-I.). In contrast to *M. stellatus*, the consumption of fresh *C. crispus* control was significantly 5.6 times higher than that of previously grazed conspecifics (Fig. 6-II.).

The herbivore specific patterns in fresh food assays were similarly found in the reconstituted food assays (detailed result in Publication 3).

4.2.2. Reversibility of induced defenses

Anti-isopod response. After reduction phase, grazed fresh *M. stellatus* was significantly less palatable to *I. granulosa* by 35 % compared to ungrazed alga, even after reduction phase (Fig. 6-I.). Contrarily, there was no significant difference in the consumption of control and grazed *C. crispus* (Fig. 6-II.).

Anti-gastropod response. After reduction phase, *L. littorea* did show feeding preference for controls compared to grazed *M. stellatus* (Fig. 6-I.). Similarly, the palatability of grazed *C. crispus* was not different from that of ungrazed conspecifics (Fig. 6-II.).

4.3. Macroalgal interaction via induced defenses

4.3.1. Effects of grazing signals on conspecific macroalgae

Faro. The consumption by *Stenosoma nadejda* was decreased in fresh and reconstituted *F. spiralis* when it was exposed to cues from a grazed conspecific rather than that from an ungrazed one (fresh $t_7 = 2.29$, $p = 0.02$; reconstituted $t_7 = 3.46$, $p = 0.01$). However, this pattern was not found in *Cystoseira humilis* nor in *S. muticum* (fresh $t_7 < 1.41$, $p >$

0.05; reconstituted $t_7 < 0.57$, $p > 0.05$). It implied that released signals from grazed conspecifics can induce antifeeding defenses only in *F. spiralis*.

Helgoland. The feeding of *I. baltica* on fresh foods made of *F. vesiculosus* was not significantly higher downstream of controls than that of grazed conspecifics, while there was significant preference in its corresponding reconstituted foods (fresh $t_7 = 1.00$, $p = 0.35$; reconstituted $t_7 = 3.54$, $p = 0.01$). Yet, signals released from *I. baltica*-attacked macroalgae did not change palatability of conspecific *H. siliquosa* and *S. muticum* (fresh $t_7 < 1.96$, $p > 0.05$; reconstituted $t_7 < 0.61$, $p > 0.05$).

4.3.2. Effects of grazing signals on heterospecific macroalgae

Faro. *S. nadejda* preferred *C. humilis* when it was exposed to ungrazed *F. spiralis* rather than *C. humilis* affected by grazed *F. spiralis* (Fig. 7-I.), implying that *C. humilis* changed its palatability in responses to feeding signals released from attacked *F. spiralis*. Similarly, *C. humilis* shifted its tastefulness in responses to signals from grazed *S. muticum*. Moreover, *F. spiralis* changed its palatability when it encountered grazed *C. humilis*, rather than grazed *S. muticum*. However, *S. muticum* did not modify its traits depending on grazing signals from *C. humilis* and *F. spiralis*, respectively. The overall patterns in fresh food assays were similarly found in reconstituted food assays (detailed result in Publication 3).

Helgoland. *I. baltica* did not significantly prefer *H. siliquosa* pieces downstream of ungrazed *F. vesiculosus* to those in *H. siliquosa* pieces downstream of grazed *F. vesiculosus* (Fig. 7-II.). Yet, the isopods increased the consumption of *H. siliquosa* subjected to ungrazed *S. muticum* over grazed *S. muticum*. These two results suggest that *H. siliquosa* decreased their palatability in responses to feeding signals from *S. muticum*, not from *F. vesiculosus*. Contrarily, the palatability of *F. vesiculosus* was not suppressed by signals from either *S. muticum* or *H. siliquosa*. Moreover, *S. muticum* was not affected by heterospecific signals. Patterns in fresh food assays were comparably found in reconstituted food assays (detailed result in Publication 3).

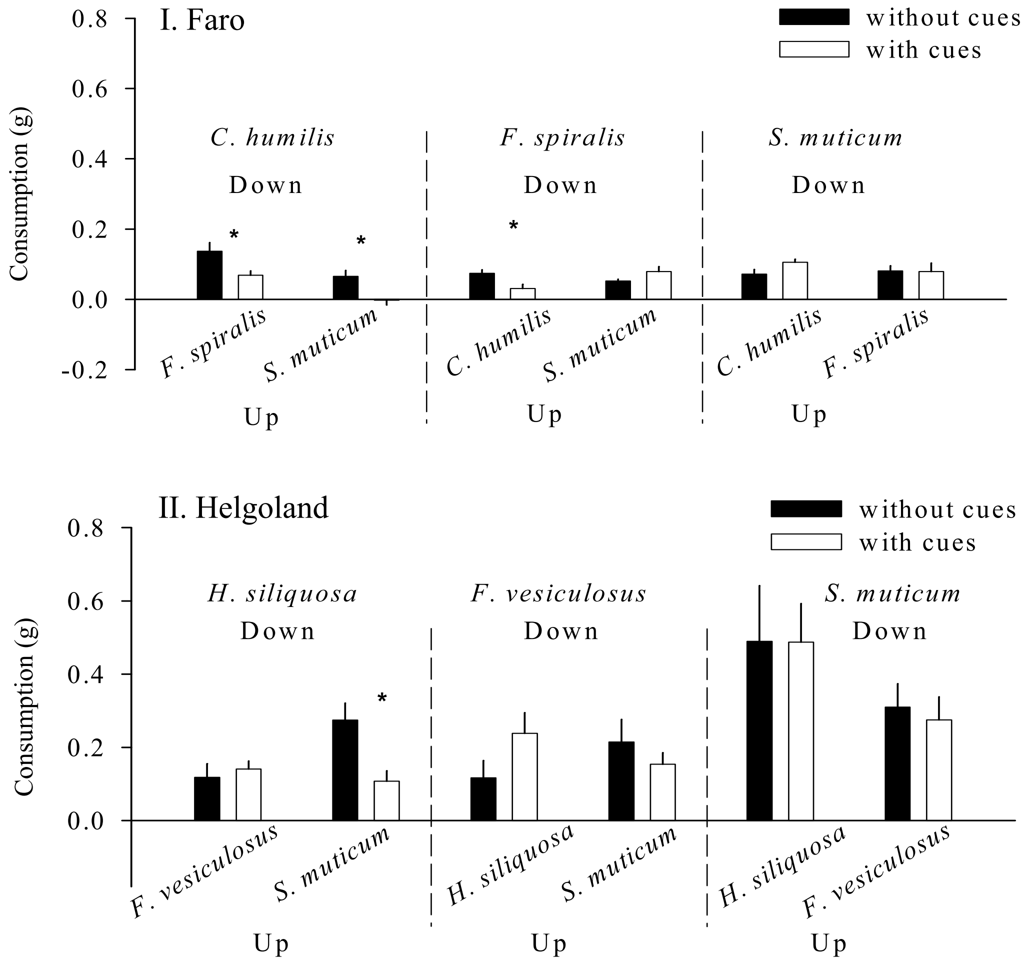


Fig. 7. Mean cconsumption rates of three algae exposed to waterborne cues released from grazing on heterospecific algae (open bar) and those exposed to non-grazing (black bar) through fresh bioassays, in (I) Faro and in (II) Helgoland. Bars indicate mean + standard error of the averaged consumption rates measured in each assay (n =8). * = significantly different in paired *t*-test. This figure is redrawn from Publication 3.

4.4. Herbivore interaction mediated by antiherbivore defense

A significant grazing x inducer x consumer interaction was detected ($F_{1,36}$ = 10.60 p = 0.002). When *I. baltica* was the inducer, the consumption of non-grazed algae was significantly higher to conspecific consumer than the grazed *F. vesiculosus* pieces (t_9 = 4.21, p = 0.001), while there was no preference pattern to heterospecific consumer (i.e. *L. littorea,* t_9 = 0.64, p = 0.284, Fig. 8.). When *L. littorea* was the inducer, the consumption of ungrazed macroalgae was significantly increased to heterospecific consumer (*I. baltica*, t_9 = 4.45, p = 0.001), but not conspecific consumer (t_9 = 0.88, p = 0.202), over grazed algal pieces. The patterns of fresh food assays were similarly found in those of reconstituted assays (detailed information in Publication 4).

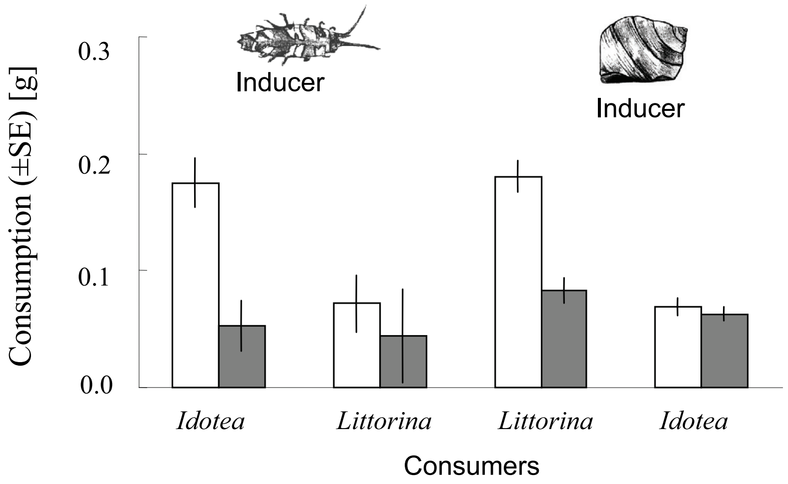


Fig. 8. Mean consumption rates of grazed (black bars) and control pieces (white bars) of *Fucus vesiculosus* from fresh food assays in Helgoland (n = 10). Pictures of grazers above the graphs indicate grazer species used during the induction phase (inducer), while the names below the graphs indicate the species of grazer used in the feeding assays (consumer). Bars indicate mean ± standard error of the averaged consumption rates measured in each assay. This figure is rredrawn from Publication 4.

5. SUMMARY OF GENERAL DISCUSSION

This dissertation highlights the ability of macroalgae to protect themselves by inducing their palatability changes in the face of consumer attacks. In particular, I examined how macroalgae change their chemical traits in response to herbivore properties classified by herbivore specificity, temporal variation of the herbivores, and feeding cues. Further, I tested macroalgae can predict the herbivory and induce defenses by the cues from an attacked conspecific and a heterospecific species. Finally, I investigated the effective magnitude of the induced responses using non-native macroalgae as well as species-specificity of herbivores. These approaches support that herbivore-induced defenses are not accidental reactions but adaptive options of plants through coevolved history with specific herbivores.

5.1. Diverse patterns of induced anti-herbivore defenses

5.1.1 Induced defenses by direct grazing

Herbivores significantly preferred ungrazed macroalgae to directly grazed ones, which were shown in fresh assays (Publication 1, 2, 3 & 4). Consistently, 3 brown macroalgae (*Fucus vesiculosus*, *F. spiralis* and *Cystoseira humilis*) and 2 red macroalgae (*Chondrus crispus* and *Mastocarpus stellatus*) showed significant preferences of control foods in reconstituted food assays. These similar results between two types of feeding assays indicate that macroalgae can modify chemical traits to increase feeding deterrence after direct grazing. In several brown macroalgae (such as fucoids including the browns studied here), there are the variable changes of concentration in phlorotannins (polyphenolic compounds), which can be triggered by direct grazing (e.g. Pavia & Toth 2000, Toth & Pavia 2000). Furthermore, chemical compounds against herbivory have been identified in red macroalgae (reviewed in Potin 2008). However, the induced chemicals themselves do not prevent feeding damages in non-grazed macroalgae completely (Pavia & Toth 2000). Thus, it is needed to identify the active compounds which can provide antiherbivore defenses in responses to direct grazing.

Moreover, patterns of induced responses are differentiated by the herbivore species. Namely, *F. vesiculosus* changed its palatability in responses to grazing by *Idotea baltica*, not to *Littorina littorea* in this study (Publication 4). However, such herbivore specific responses in *F. vesiculosus* were not detected in other studies. For example, Rohde et al. (2004) demonstrated an induction of anti-herbivory defenses in response to grazing by *L. littorea*. The contrasting pattern by *L. littorea* in *F. vesiculosus* from Rohde et al. (2004) can be due to interactive effects of local environmental conditions (e.g. consumer abundance) on algal chemical defense (i.e. Sotka & Hay 2002, Sotka et al. 2002, Long & Trussell 2007, Jormalainen et al. 2008). A similar reaction has been shown by comparative studies by Pavia & Toth (2000) and Long & Trussell (2007), showing that induction of anti-herbivore resistance of *Ascophyllum nodosum* is geographically variable in response to the other herbivore periwinkle *L. obtusata*. The inconsistent pattern of Long & Trussell (2007) to Pavia & Toth (2000) is explained by variable levels of consumer pressure across sites. For understanding the associated role of consumer variability to algal plasticity at population levels, it is necessary to compare the herbivore community between Baltic and North Seas.

The induced responses to herbivores are considered as a significant strategy of cost savings for plants in plant-herbivore interactions (reviewed in Fordyce 2006, Metlen et al. 2009). It means plants only invest in defense when necessary and allocate resources to growth and reproduction when not under consumer attack (Zangerl 2003). Therefore, regulation of defensive traits is affected by dynamics of herbivory pressures. This study shows that previously grazed *C. crispus* was less palatable to *L. littorea* than ungrazed individuals in 6 days of herbivore incubation, while the difference in palatability disappeared after 6 days of herbivore exclusion (Publication 2). This suggests that the induced state of antifeeding traits in *C. crispus* was reset to the original state before the attacks occurred. Consistently, the reversibility of induced responses has been reported in other macroalgal species, e.g. *F. vesiculosus* and *Ecklonia cava* (Molis et al. 2006, Rohde & Wahl 2008b), in terms of induced defenses regulated by herbivory dynamics (reviewed in Metlen et al. 2009). Thus, the reversed way of induced defenses reemphasizes the adaptive ability of macroalgae to trigger on-demand responses to a changing herbivore environment.

5.1.2. Induced defenses by herbivore cues

Interestingly, *F. vesiculosus* reduced its palatability to amphipods in response to waterborne cues released from the mere presence of grazers (Publication 1). It implies that cues originated from chemicals from herbivores can trigger induced anti-amphipod response in *F. vesiculosus*. Indeed, one study identified that the chemical compounds derived from herbivore foraging activity, i.e. secretion of digestive enzymes during feeding, can increase resistance traits in brown macroalgae (Coleman et al. 2007b). The herbivore cue might especially be recognized by the macroalgae if it indicates strong impacts to the host species. Considering a living style of herbivores, amphipods make a house by rolling the thallus and staying on the host macroalgae for relatively long periods. Due to the manner of residence, the presence of grazers would be expected as a prolonged feeding damage by the plants (e.g. Karban et al. 1999). Thus, only the presence of the herbivore can be a comparatively strong agent to increase macroalgal resistances similar to direct grazing.

Moreover, signals from grazed macroalgae can be used for alarming the herbivore attack to the ungrazed conspecifics (Publication 1 & 3). Exposure to the signals from grazed *F. spiralis* and *F. vesiculosus* reduced the palatability of each conspecific macroalga, shown in reconstituted food assays. Similarly, other studies have shown that macroalgal palatability was decreased by conspecific cues of *Ascophyllum nodosum* (Toth & Pavia 2000) and *F. vesiculosus* (Rohde & Wahl 2008a). Thus, even nonattacked algae can expect herbivore attacks from the attacked algae and defend themselves via the signals derived from the attacked conspecifics.

In contrast to two *Fucus* spp., three other species: *C. humilis*, *Halidrys siliquosa* and *Sargassum muticum* did not reduce their palatability when they were exposed to cues from their respective conspecific grazing. Such algal specific responses to conspecific signals can be associated with the distribution patterns of macroalgal populations. For example, when the macroalgae grow in dense stands (showed in two *Fucus* spp. distribution pattern), distance between the emitter and receiver plant should be shorter (i.e. Rohde & Wahl 2008a). Thus, the consumption of one individual by mobile mesoherbivores can increase the chance of feeding loss to other individuals, implying putative feeding damage at the level of macroalgal populations. Additionally, if the distance between cue-emitter and cue-receivers is closer, it might reduce the possibility for dilution of the signals, consequently providing correct information about consumer attack to conspecific neighbors. Thus, the signals can be available for these *Fucus* individuals, which grow much closer together compared to *H. siliquosa* and *S. muticum*.

5.1.3. Indirect induced defenses via signaling

Macroalgae are capable of recognizing the signals derived from the attacked heterospecifics as a preventive agent to alarm consumer attacks. Indeed, I found that *C. humilis* and *H. siliquosa* changed their palatability after receiving cues from grazed heterospecifics (i.e. *S. muticum*), respectively. This demonstrates macroalgal capacity to induce antiherbivore responses to the cues released even from different macroalgal species. Plants are known to respond to information provided by damaged neighbors, if the information content is reliable to indicate herbivory risks (Karban et al. 1999). Due to dominance of mesoherbivores as generalist feeders in the sample collection site (Buschbaum et al. 2006, Molis et al. 2010), the herbivore attacks could be a common impact to macroalgal community members. Consequently, the commonness of the mesoherbivores can affect the availability of heterospecific cues to mediate interspecific signaling in macroalgae.

In contrast to *C. humilis and H. siliquosa,* both *F. spiralis* and *F. vesiculosus* near grazed *S. muticum* did not suppress further consumption by grazers. This suggests that cues emitted from *S. muticum* cannot be received by *Fucus* spp., but by other species.

Ranges of interspecific cues about consumer attacks can be related to the plant identity which share phylogenetic relatedness with other plants (e.g. among preys about shared consumer attack in Schoeppner and Relyea 2005). Based on this aspect, grazing cues from *S. muticum* influenced effectively to induce responses in the same familiar species, Sargassaceae *C. humilis* and *H. siliquosa*, which coexist in submerged conditions in tide pool and shallow subtidal area, respectively. In contrast, *S. muticum* did not induce the response in different species of the same family, Fucaceae, i.e. *Fucus* spp., which occur in intertidal zone beyond the distribution range of *S. muticum*. To generalize the commonness of herbivore cues across macroalgae, it is necessary to conduct experiments across numerous phylogenetic groups (Schoeppner & Relyea 2005, Schoeppner & Relyea 2009).

5.2. Magnitude of induced defenses by properties of herbivores

5.2.1. Coevolved background between plants and herbivores

In plant-herbivore interactions, plants have regulated their defensive traits through an adaptive period with herbivores. However, it is questioned how plants respond to native herbivores in newly introduced region. My results show that non-native *M. stellatus* did not change its palatability after grazing by *L. littorea*, indicating that *M. stellatus* fails to induce responses to *L. littorea* grazing (Publication 2). However, *L. littorea* is a strong agent to shift the antifeeding traits in other species, i.e. native *C. crispus*. The contrasting pattern suggests that there might not be enough time for *M. stellatus* to evolve induced defenses against *L. littorea* grazing. Comparing the herbivore composition between old and new ranges of the non-native species, *L. littorea* is a novel herbivore to *M. stellatus* since the snail does not exist in its old habitat

(Ingolfsson 2006, Reichert & Buchholz 2006). According to Colautti et al. (2004), non-coevolved herbivores cannot be recognized as feeding damage agents. Moreover, non-native plants show improper responses in newly introduced ranges in terms of higher variability of induced anti-grazing compounds (e.g. Cipollini et al. 2005, Eigenbrode et al. 2008), which is not tested in this study. In this regard, the non-native *M. stellatus* could not display a proper response to grazing by novel herbivores. This is supported by contrasting response of *M. stellatus* to a co-evolved herbivore, i.e. *I. granulosa*. The isopod species imposes feeding pressures commonly on native and introduced *M. stellatus* populations (Ingolfsson 2006, Reichert & Buchholz 2006). The co-evolved grazers may still consume the non-native species even in new habitat (i.e. Helgoland). Therefore, the different response in *M. stellatus* between two herbivores indicates that efficiency of defenses is an outcome of plants via coevolved time with herbivores.

5.2.2. Species specificity of herbivores

Effects of the plant-induced responses can vary depending on species identity of herbivores (Agrawal 2001). Consistently, in macroalgal herbivore interactions, grazing decreased the algal palatability overall, but effects of the grazing-induced responses vary depending on species identity of herbivores (Publication 4). This implies that multiple herbivore coexistence can affect the efficiency of the induced defenses to decrease the consumption loss in the attacked macroalgae. Similarly, Long et al. (2007) demonstrated that the previous attack by periwinkle, *L. obtusata,* reduced the algal palatability for other species, i.e. *L. littorea* and *I. baltica,* and their research argued that these interactions may lead to the grazer migration away from the attacked macroalgae in intertidal community. Consequently, interactions between one plant and one herbivore could influence the food preference of other herbivore populations in marine communities. In other words, grazing-induced changes in host plants can mediate interspecific interactions between herbivores (i.e. Ohgushi 2005, Molis & da Gama 2009, Utsumi et al. 2010).

The induced defenses were effective restricted to *I. baltica* over *L. littorea*. It shows that the previously grazed *F. vesiculosus* by *I. baltica* was relatively distasteful to *I. baltica* itself, but not to *L. littorea*, compared to a non-grazed individual. Again, macroalga grazed by *L. littorea* was less preferred food only for *I. baltica*, but not to *L. littorea*. Such individual capacity of grazers to deal with defense induction of plants is known to affect the direction of the herbivore interaction accordingly, which determines superior competitor among herbivores (Denno & Kaplan 2007). Differences in life-history traits, e.g. dispersal, promote the competitive superiority in herbivores (Karban & Agrawal 2002). Considering the mobility of herbivores, the active isopod might have different strategies to handle the induced host plant relative to the less-active gastropod. It means that the mobility of herbivores might drive to reduce a time to stay in the low-quality food derived from the induced feeding deterrence in the attacked plant and then to move to a better un-induced food easily. The counteradaptive strategy of marine herbivores to the algal induced defenses has recently been explained in a way that herbivores can tolerate resistant chemicals in the induced host algae through detoxification enzymes (e.g. Sotka & Whalen 2008). However, it is unknown how the activity of the physiological neutralizer to detoxify the feeding deterrence from the host in isopod is different from that in gastropod.

5.3. Conclusions and future study

The present study has emphasized that macroalgae have sophisticated mechanisms to deal with interacting herbivore species through modifying their antifeeding traits. In detail, defense inductions of macroalgae are specialized by herbivore-relevant cues and herbivore-species. Further, reversibility of the responses in macroalgae is responsible for counterbalancing the temporal variation of herbivores. Moreover, the induced responses to one herbivore in one alga are affected indirectly by additional algal species. Indirect induced defenses of macroalgae, which respond to herbivory risks from heterospecific neighbors, would mediate macroalgal communication about consumer attacks and contribute to improve their immunity against feeding damages later. Thus, the macroalgae can have fine-tuned ability to tailor their induced defense to risk of herbivory. However, in order to deter the herbivory effectively, induced responses require a coevolved time for macroalgae to encounter to attacks from specific consumer attacks. Even though defense is induced in some algae, the effectiveness of induced changes in grazed macroalgae could be diversified among diverse herbivore species, due to ranges of counteradaptive ability of herbivores to deal with algal induced response. Consequently, herbivore-induced defenses in macroalgae cannot complete solution to resist to all herbivore attacks. Rather, herbivore-induced defenses can contribute to modify interactions among multiple species, enhancing complexities in the trophic interactions between macroalgae and herbivores.

Direct grazing effects by one herbivore on one macroalga have been extensively studied in the last 20 years (reviewed in Jormalainen & Honkanen 2008, Paul & Williams 2008, Pereira & da Gama 2008). However, the aspects of defense inductions in complex environmental conditions have received relatively little attention (Kant et al. 2009, Dicke et al. 2009, Snoeren et al. 2010). The chemical complexity of the real environments is one of the most important aspects to be considered in future work. Macroalgae live in marine systems where are chemically rich environments. Some chemical cues are intentionally released to inform the feeding risk or others are simply by-products of metabolism for diverse ecological functions (Hay 2009). From these chemical mixtures, it is questioned whether macroalgae can selectively respond to valuable information to indicate the feeding attack. However, when we evaluate the effects of informative cues on cue-receiver species in experiments, it is typically done under relatively controlled conditions consisting of cues from only one herbivore and one plant. In other words, these methods cannot totally represent chemical noise in natural conditions, but can overestimate the capacity of macroalgae to detect and respond to consumer cues. To understand selective capacity of plants to respond to cues, it is necessary to conduct experiments that compare defense inductions across a diverse range of chemicals released from grazed macroalgae.

Extensive attention has been focused on determining defense inductions in macroalgal individuals to consumer attacks. That is, current results were obtained over relatively short-term periods (i.e. a few days or weeks), shorter than the life spans of herbivores. Such short period experiments using a few species may have limited our insight of herbivore-induced responses and have difficulties to estimate realistic values of induced responses in whole marine systems. For enhancing our understanding about how induced responses play a role in structuring communities broadly, we should consider two aspects in future studies. First, we should measure the population parameters (i.e. demographics) of the interacting species within the communities using long-term experiments (see to Hammill & Beckerman 2010). Over long-term period through multiple generations, we should include diverse species which occur in realistic ecosystems in future experiments.

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