

# Proximate and ultimate causes for transatlantic variation in seaweed defenses

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**ABSTRACT:** Previous tests of theories predicting tradeoffs between constitutive and inducible defenses are inconclusive. This may result from focusing on individual traits or using cultivated plants subjected to artificial selection. We examined this tradeoff in the seaweed *Ascophyllum nodosum* (hereafter *Ascophyllum*)—one of the best-studied seaweeds with inducible defenses. Although several European populations respond to grazing with induced resistance, a US population failed to express inducible defenses. This variation may be related to corresponding variation in constitutive resistance and consumer pressure. We compared the constitutive resistance of *Ascophyllum* from an inducing and a non-inducing population (Sweden and USA, respectively). We quantified constitutive resistance by conducting choice feeding assays on seaweed from both populations after inducible defenses had relaxed, rather than focusing on single traits. We measured constitutive phlorotannins to confirm that defenses had relaxed and to compare between-population levels. Periwinkles consistently preferred relaxed Swedish *Ascophyllum*, suggesting that US *Ascophyllum* without inducible resistance had higher constitutive resistance. Neither snail source nor experimental arena influenced this result. Phlorotannins were also higher in relaxed US *Ascophyllum*. To test the hypothesis that defense type may be related to current consumer pressure, we compared per capita grazing rates and average snail densities from both locations. US snails grazed more and were more abundant in the USA, suggesting that current consumer pressure may be higher on US *Ascophyllum*. Consistent with theory, constitutive and inducible resistance were negatively related between our sites. Inducible resistance may be absent from the US population because these individuals have high constitutive resistance, perhaps because they encounter greater consumer pressure.

**KEY WORDS:** Plant–herbivore interactions · Constitutive defense · Phlorotannins · Chemical ecology · Spatial variation

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## INTRODUCTION

Plant defense theories (including optimal defense, carbon nutrient balance, and growth rate hypotheses) predict a tradeoff between constitutive and inducible defenses among populations and among species because environmental features differentially select for these defense types (Coley 1988, Mattson et al. 1988, Karban & Baldwin 1997, Stamp 2003). Consistent with theory, constitutive and in-

ducible plant traits were negatively related across plant genotypes in a recent meta-analysis (Koricheva et al. 2004, but see Thaler & Karban 1997 for an exception). However, the interpretation of this correlation probably should be considered tentative, as most existing studies are limited by 2 primary shortcomings. First, most studies (11 of 15 summarized in Koricheva et al. 2004) measured the response of a single morphological plant trait (e.g. trichome density), secondary metabolite (e.g. chlorogenic acid), or

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structural chemical class (e.g. non-indolyl glucosinolates). Given that most plants rely on multiple chemical, nutritional, and morphological traits to deter their consumers (Hay et al. 1994, Agrawal 2007) and that specific defensive traits mediating interactions are often unknown, focusing on a single defensive trait may have little relevance to overall levels of defense (Underwood et al. 2002). A more integrative measure of plant defense is herbivore response (i.e. resistance *sensu* Karban & Baldwin 1997). Interestingly, studies focused on herbivore responses (e.g. habitat or feeding preferences) indicate no significant relationship between inducible and constitutive resistance across genotypes (Brody & Karban 1992, English-Loeb et al. 1998, Underwood et al. 2000) or a positive relationship (Salgado & Pennings 2005, Long et al. 2011). A second limitation is that most studies on tradeoffs between inducible and constitutive defenses used cultivated plants that are inbred and have undergone bouts of selection within agricultural and unnatural environments (Brody & Karban 1992). Thus, apparent tradeoffs in agricultural settings may never arise in more natural settings. Clearly, understanding the relationship between constitutive and inducible defenses of plants requires more attention.

Unlike constitutive defenses, inducible defenses are expressed after grazer attack (Karbon & Baldwin 1997). By exposing some plants to herbivores and keeping other plants grazer-free, several studies have identified and compared the strength of inducible defenses (Brody & Karban 1992, English-Loeb et al. 1998, Underwood et al. 2000). Similarly, constitutive defenses can be compared between populations by growing plants without herbivores and allowing any inducible defenses to relax. For example, Underwood et al. (2002) used this approach to compare the constitutive defense levels of soybean genotypes. Similarly, English-Loeb et al. (1998) compared constitutive defenses between grape plant populations by assaying herbivorous mite response towards ungrazed plants. Thus, constitutive defenses are commonly measured by comparing herbivore response to plants that have grown for some period without herbivores.

One model system to examine tradeoffs in constitutive and inducible plant defenses is the seaweed *Ascophyllum nodosum* (hereafter *Ascophyllum*) interacting with one of its primary grazers, the smooth periwinkle *Littorina obtusata*. These are dominant species in wave-protected areas along rocky shores in the North Atlantic. Swedish and UK populations of *Ascophyllum* consistently respond to grazing or grazer

cues by increasing phlorotannin production and decreasing palatability (Table 1). These changes, in turn, increase *L. obtusata* movement (Coleman et al. 2007) and decrease *L. obtusata* egg hatching success (Toth et al. 2005). In contrast, a US population of *Ascophyllum* did not respond to similar levels of grazing pressure—phlorotannin levels and palatability were equivalent between grazed and ungrazed *Ascophyllum* (Long & Trussell 2007). Thus, *Ascophyllum* displays geographic variation in inducible defenses. The proximate and ultimate causes of this variation remain unknown, but one hypothesis is that *Ascophyllum* displays a corresponding geographic variation in constitutive defenses. For example, US *Ascophyllum* may fail to express inducible responses because it maintains higher levels of constitutive defenses. Such an example of local adaptation is possible given the broad range and limited dispersal of this seaweed.

To test this hypothesis, we conducted a reciprocal, laboratory transplant experiment between locations containing *Ascophyllum* previously known to vary with respect to inducible defenses (inducible: Tjärnö, Sweden; non-inducible: Boston, Massachusetts; Table 1). We compared the constitutive resistance of *Ascophyllum* between these populations by measuring the palatability of non-induced *Ascophyllum* from both locations during choice feeding assays and by measuring total phlorotannin levels. Also, we compared the per capita grazing rate of Swedish and US snails because some evidence suggests that plant defenses are correlated with grazing rate (Pennings & Silliman 2005, Salgado & Pennings 2005). Finally, we compared snail densities between these sites.

## MATERIALS AND METHODS

To compare the relative palatability of *Ascophyllum* with relaxed inducible defenses from Sweden and the USA, we conducted a reciprocal, choice feeding, laboratory experiment with organisms from both locations. In May, we collected *Ascophyllum* and *Littorina obtusata* from Nahant, Massachusetts, USA and Tjärnö, Sweden. At each location, we collected organisms (snails and seaweed) from 4 sites separated by at least 1 km. An equal number of *Ascophyllum* from each site were pooled by location and experimental individuals were randomly selected from this pool. We removed epiphytic organisms from seaweeds manually and via submergence in fresh water for 5 min. Half of the collected *Ascophyllum* and *Littorina* were kept at the local marine labo-

Table 1. *Ascophyllum*. Studies that have tested for inducible defenses in response to grazing or grazer cues. Induction has been observed in populations in Sweden and the UK, but not the USA. Using 2-sample *t*-tests, we compared our relaxed phlorotannin levels ( $3.3 \pm 0.2\%$  dry mass [DM]; mean  $\pm$  SE) to previous levels of induced and non-induced Swedish populations. *p*-values for these tests are shown in parentheses for the Swedish populations

Induction?	<i>Ascophyllum</i> population	Induction effect(s)	Induced phlorotannins (% DM $\pm$ SE)	Non-induced phlorotannins (% DM $\pm$ SE)	Source
Yes	Sweden (Tjärnö)	Total phlorotannins increased	$9.2 \pm 0.4$ ( $p < 0.001$ )	$7.0 \pm 0.2$ ( $p < 0.001$ )	Pavia & Brock (2000)
	Sweden (Tjärnö)	Total phlorotannins increased, consumption decreased	$6.9 \pm 0.4$ ( $p < 0.001$ )	$3.5 \pm 0.1$ ) ( $0.3 < p < 0.4$ )	Pavia & Toth (2000)
	Sweden (Kristineberg)	Total phlorotannins increased, consumption decreased	$3.4 \pm 0.3$ ( $p > 0.05$ )	$2.9 \pm 0.3$ ( $0.3 < p < 0.4$ )	Toth & Pavia (2000)
	Sweden (Tjärnö)	Total phlorotannins increased, egg hatching decreased	$3.8 \pm 0.1$ ( $0.05 < p < 0.10$ )	$3.4 \pm 0.3$ ( $p > 0.5$ )	Toth et al. (2005)
	Sweden (Tjärnö)	Total phlorotannins increased	$4.6 \pm 0.1$ ( $p < 0.001$ )	$3.6 \pm 0.1$ ( $0.3 < p < 0.4$ )	Svensson et al. (2007)
	Sweden (Tjärnö)	Total phlorotannins increased	$5.6 \pm 0.2$ ( $p < 0.001$ )	$4.9 \pm 0.2$ ( $0.005 < p < 0.010$ )	Toth et al. (2007)
	UK (Plymouth)	Total phlorotannins increased, consumption decreased	$6.8 \pm 1.2$	$3.2 \pm 0.4$	Borell et al. (2004)
	UK (Plymouth)	Total phlorotannins increased, consumption decreased, grazing scar sizes decreased, movement increased	$5.6 \pm 0.6$	$0.8 \pm 0.1$	Coleman et al. (2007)
No	USA (Nahant)	No effect on total phlorotannins or consumption	$3.3 \pm 0.2$	$4.2 \pm 0.4$	Long & Trussell (2007)

ratory (Sweden: Tjärnö Marine Biological Laboratory; USA: Northeastern University Marine Science Center) and the other half were translocated to the other marine laboratory. Translocations consisted of placing seaweeds and snails in coolers with damp paper towels and dry ice, carrying them aboard aircraft, and placing in closed aquaria at the translocated site ~22 h later. We did not observe any negative effects of translocations on these organisms.

To create relaxed *Ascophyllum*, we grew local and translocated seaweeds without grazers or grazer cues in the laboratory for 14 d at both locations. The purpose of the relaxation period was to allow any inducible defenses that were being expressed in the field to relax. As such, the starting level of inducible defense in these algae was unimportant for this assay. To confirm that inducible defenses were relaxed, we measured total phlorotannin levels because they are positively correlated with inducible defenses in *Ascophyllum* (Table 1). After 14 d, total phlorotannins in apical tip tissues were measured using the Folin-Ciocalteu method (Van Alstyne 1995) and compared with reported phlorotannin levels of induced and non-induced *Ascophyllum* from Sweden (e.g. Pavia & Toth 2000). For the phlorotannin

analysis, apical tip *Ascophyllum* samples from the USA or Sweden were individually homogenized and extracted in 80% methanol at 4°C for 24 h (N = 28). Fifty microliters of extract was diluted with 1 ml deionized water and 1 ml 40% Folin-Ciocalteu reagent. After 5 min, 1 ml of a saturated sodium carbonate solution was added. Samples were vortexed and then heated in a 50°C water bath for 30 min. Absorbance was read at 760 nm and compared with a standard curve generated with phloroglucinol (Sigma) samples. Phlorotannin concentration was corrected by a seaweed dry/wet mass ratio and reported as % dry mass.

We proposed that low phlorotannin levels at the end of our assay (similar to previously published reports of control *Ascophyllum* not experiencing grazing, but different from previously published reports of grazed *Ascophyllum*) would indicate that we had successfully relaxed the inducible defenses. Furthermore, the limited evidence available suggests that inducible seaweed defenses should relax within this time period in the absence of grazer cues (e.g. <14 d; Ceh et al. 2005). However, relaxation times may be different (either shorter or longer) in *Ascophyllum* as Ceh et al. (2005) examined different

algae genera with different defense types (e.g. non-polar chemical defenses) from a different region (Indian Ocean).

We performed 3 types of analyses of the phlorotannin data. First, we conducted 2-tailed, 2-sample *t*-tests to determine whether our mean phlorotannin concentration was different from previously published values for Swedish *Ascophyllum* grown without grazers (Table 1). Previously published means and standard errors were determined from original values using imageJ. Second, we conducted 2-tailed, 2-sample *t*-tests to determine whether our mean phlorotannin concentration was different from previously published values for induced Swedish *Ascophyllum* grown with grazers (Table 1). Significant *p*-values for this comparison would also suggest that our approach allowed inducible responses to relax. Third, we compared total phlorotannin levels in US and Swedish *Ascophyllum* with a 2-tailed, 2-sample *t*-test.

After 14 d of growth without grazers or grazer cues, we conducted choice feeding assays using relaxed *Ascophyllum* from Sweden and the USA. During each feeding assay, we offered *Littorina obtusata* (3 snails per replicate, *n* = 10) a choice of 1.0 to 1.5 g (dry blotted wet weight) apical tip *Ascophyllum* tissue from the USA and Sweden. The 2 choices were differentiated with labeled binder clips. Grazing was allowed for 3 to 5 d, after which we measured final wet seaweed mass.

Grazing rates were corrected for autogenic growth in equivalent grazer-free controls using the formula  $T_i(C_f/C_i) - T_f$ , where  $T_i$  and  $T_f$  represent the initial and final masses, respectively, of tissue subjected to grazing, and  $C_i$  and  $C_f$  represent the initial and final masses, respectively, of control tissue. To examine the importance of snail source and experimental location, we conducted experiments with both local and translocated snails at both locations. We analyzed grazing choices by conducting 2-tailed, paired *t*-tests because choices were paired during the feeding assays. We applied a Bonferroni adjustment to account for the number of comparisons we conducted ( $k = 4$ ). Additionally, we converted grazing rates in choice assays to the percentage of the total consumption represented by US *Ascophyllum*. After arcsin transformation, we used this percentage as a response variable in a 2-factor ANOVA with Experimental Location (USA, Sweden) and Snail Source (USA, Sweden) as fixed factors.

In the choice feeding assays, snails preferred to feed on relaxed *Ascophyllum* from Sweden, suggesting that US *Ascophyllum* had higher levels of consti-

tutive defenses. To examine the relationship between seaweed constitutive defenses and per capita grazing rates of *Littorina obtusata*, we determined the grazing rates of snails from both populations in no-choice feeding assays. No-choice assays were conducted in Sweden only. Both local and translocated snails were offered apical tip *Ascophyllum* tissue (~1 g) from either the USA or Sweden (*N* = 10). After correcting for growth with an equal number of autogenic controls (see above), we compared grazing rates using ANOVA with Snail Source and Seaweed Source as fixed factors because we were specifically interested in comparing the US and Swedish populations. Snail mass did not differ between US and Swedish populations (0.89 and 0.85 g, respectively;  $p = 0.27$ ).

## RESULTS

After 14 d of growth without grazers, total phlorotannin concentrations in Swedish *Ascophyllum* ( $3.3 \pm 0.2\%$  dry mass; mean  $\pm$  SE) were equivalent to, or lower than, all published reports for non-induced Swedish *Ascophyllum* (Table 1). These phlorotannin levels were below published reports for induced Swedish *Ascophyllum* in 4 studies (Table 1; Pavia & Brock 2000, Pavia & Toth 2000, Svensson et al. 2007, Toth et al. 2007) and marginally below published reports for induced Swedish *Ascophyllum* in another study (Toth et al. 2005). Our phlorotannin levels were not different from published reports of induced Swedish *Ascophyllum* in only 1 of the 6 previous studies (Toth & Pavia 2000). Given that our phlorotannin levels were at or below published values for non-induced *Ascophyllum* in 6 of 6 studies and that our phlorotannin levels were not below published values for induced *Ascophyllum* in only 1 of 6 studies, we propose that we successfully relaxed the inducible defenses in Swedish *Ascophyllum*. Also, total phlorotannin levels were 66% higher in relaxed *Ascophyllum* from the USA than Sweden (Fig. 1;  $t_{1,54} = 8.19$ ,  $p < 0.001$ ).

In 3 of the 4 combinations tested, *Littorina obtusata* strongly preferred relaxed *Ascophyllum* from Sweden rather than US *Ascophyllum* (Fig. 2a,b,d). Grazing rates were 4 to 7 times higher on relaxed Swedish *Ascophyllum* relative to relaxed US *Ascophyllum* in these 3 assays. For the experiment in the USA, snail source did not change this preference (Fig. 2a,b). In the fourth experiment (US snails feeding in Sweden), there was a similar trend for a preference of Swedish *Ascophyllum*, but this difference was not statistically significant—perhaps because of lower grazing rates

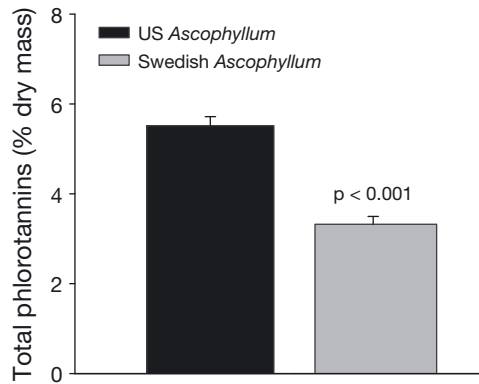


Fig. 1. *Ascophyllum*. Mean (+SE) total phlorotannins in apical tips from either US or Swedish populations. *Ascophyllum* was grown without grazers for 14 d before analysis to allow induced phlorotannin production to relax. N = 14

of these snails (Fig. 2c). In the overall ANOVA analysis, there was not a significant effect of Experimental Location ( $F_{1,36} = 0.000$ ,  $p = 0.999$ ) or Snail Source ( $F_{1,36} = 0.406$ ,  $p = 0.528$ ) on the percentage of total consumption represented by US *Ascophyllum*. There was also no significant interaction of Experimental Location and Snail Source ( $F_{1,36} = 0.735$ ,  $p = 0.397$ ).

In no-choice feeding assays, US snails grazed seaweeds 3.6 times more than Swedish snails (Fig. 3;  $F_{1,36} = 23.19$ ,  $p < 0.001$ ). Unlike the choice assays, there was no significant effect of seaweed source in the no-choice assays ( $F_{1,36} = 0.28$ ,  $p = 0.600$ ). Furthermore, there was no interaction between snail source

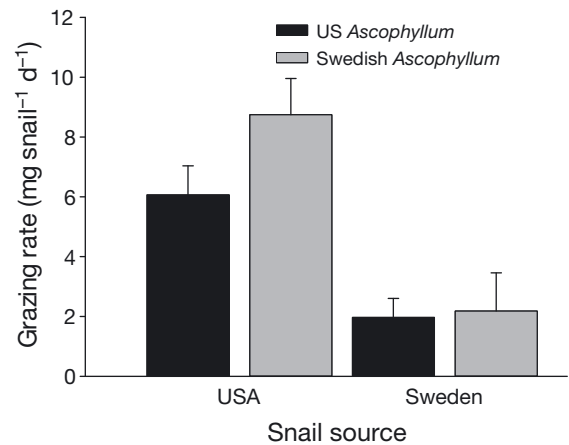


Fig. 3. *Littorina obtusata* grazing *Ascophyllum*. Mean (+SE) grazing rates in no-choice assays when offered apical tips from either the USA or Sweden. Before these assays, *Ascophyllum* was grown without grazers for 14 d. We only conducted these assays in Sweden. N = 10

and seaweed source on grazing rate in no-choice assays ( $F_{1,36} = 0.49$ ,  $p = 0.487$ ).

## DISCUSSION

Inducible and constitutive defenses were inversely related between *Ascophyllum* from our sites on opposing sides of the Atlantic Ocean—US seaweeds that lacked inducible defenses (see Long & Trussell 2007) were less palatable than non-induced Swedish seaweeds, suggesting that US seaweeds had higher constitutive defenses. This result was robust given that it did not depend on snail source (Sweden or USA) or assay location (Swedish or US labs).

It is possible that had we allowed relaxation of inducible defenses for longer than 14 d, the inducible *Ascophyllum* population (i.e. Sweden) would have become more palatable. We think this is unlikely given that (1) other seaweeds, including other brown seaweeds, relax inducible defenses within this time period (Ceh et al. 2005) and (2) phlorotannins, which are positively correlated with inducible defenses that affect *Ascophyllum* palatability (Pavia & Toth 2000, Toth & Pavia 2000, Borell et al. 2004, Coleman et al. 2007), in our relaxed seaweed were lower than published values for induced *Ascophyllum* (in 5 of 6 studies) and not different from non-induced *Ascophyllum*. Regardless, our results are conservative because any additional relaxation in the Swedish population would have made it more likely for us to arrive at our conclusion that constitutive defenses were lower in Swedish *Ascophyllum*.

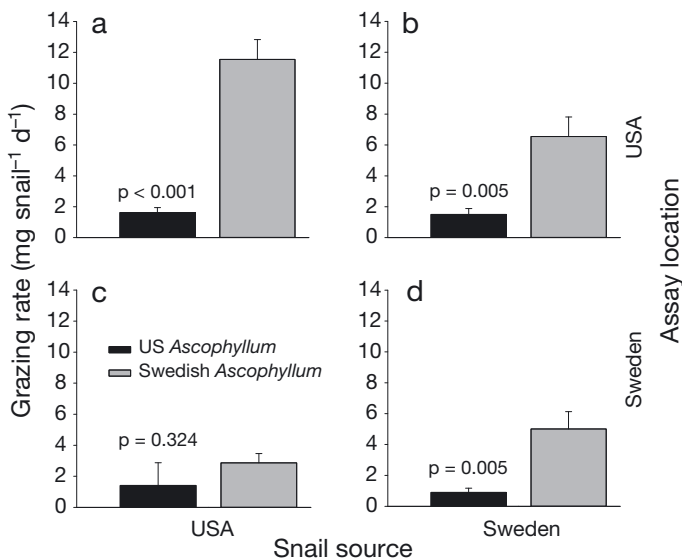


Fig. 2. *Littorina obtusata* grazing *Ascophyllum*. Mean (+SE) grazing rates when offered a choice of apical tips from the USA and Sweden. Before these assays, *Ascophyllum* was grown without grazers for 14 d. We conducted assays in both locations and with snails from both locations. N = 10

A proximate cause of higher constitutive defenses in our population of US *Ascophyllum* is higher concentrations of total phlorotannins—a putative class of chemical defenses. The defensive role of phlorotannins has been debated (Deal et al. 2003, Kubanek et al. 2004), but some of the strongest evidence in support of this claim comes from a study of inducible defenses in Swedish *Ascophyllum* (Pavia & Toth 2000). In that study, phlorotannins incorporated into agar discs at low concentrations (0.5 to 2.0%) reduced *Littorina obtusata* feeding compared with phlorotannin-free foods. However, we can not exclude the hypothesis that other factors, such as nutrients or secondary metabolites, may have contributed to geographic differences in palatability (e.g. galactolipids; Deal et al. 2003).

Multiple environmental factors could ultimately have selected for this transatlantic variation in defense type. We estimated current consumer pressure by measuring per capita grazing rates and surveying the literature for reported *Littorina obtusata* densities at these sites. Per capita grazing rate was measured because a recent study found that lowlatitude populations of salt marsh cordgrass containing higher levels of constitutive defenses (Salgado & Pennings 2005) encounter herbivores with higher per capita grazing rates compared with northern populations (Pennings & Silliman 2005). We observed 3.6 times higher per capita grazing rates by US snails. Similarly, there is a trend for higher densities of *L. obtusata* at our US sites ( $161 \pm 22 \text{ m}^{-2}$ ; J. D. Long pers. obs.) than our Swedish sites ( $113 \pm 35 \text{ m}^{-2}$ ; Toth et al. 2007). However, *L. obtusata* densities can vary dramatically within a region (e.g. mean snail densities at sites within 2 km of our US site vary from 22 to  $555 \text{ m}^{-2}$ ; Lubchenco 1980, 1983). If (1) current conditions are representative of historic conditions across evolutionary time and (2) other factors (e.g. abiotic stress) do not differentially suppress US consumer pressure relative to Sweden, then the combination of more abundant herbivores with higher per capita grazing rates in the USA may have differentially selected for constitutive resistance in our US *Ascophyllum* population. However, there are known abiotic differences between these sites (e.g. tidal regime) that could also be important in the evolution of seaweed defenses.

Two recent studies suggest that seaweed species might be particularly promising for comparing tradeoffs in defense types because of significant intraspecific variation in seaweed palatability. First, Swedish snails non-indigenous to native regions of *Fucus evanescens* (Iceland) strongly preferred *F. evanescens* from Iceland compared with Sweden (Forslund

et al. 2010). Similar to our study, this preference was related to levels of total phlorotannins. Second, the relative palatability of 2 ephemeral seaweeds (*Ulva* and *Porphyra*) towards snails completely switched between 2 populations in the Gulf of Maine (Dolecal & Long 2013). At present, it is unclear if these differences represent variation in constitutive resistance, inducible resistance, or both. Yet this strong geographic variation in seaweed palatability could improve our ability to detect geographic tradeoffs in defense types.

Given the logistical challenges of our reciprocal experiment that crossed seaweed source, snail source, and assay location (2 levels for each factor), we were unable to add an additional factor of Site nested within seaweed and snail source. To prevent confounding variance among sites at a location with variance between continents, we selected plants from 4 sites at each location and pooled these, thus achieving a mean response to plants from multiple sites at each geographic location. After pooling within a region, we still observed strong differences in feeding preferences for Swedish *Ascophyllum* regardless of snail source and assay location. Although this approach limits our ability to predict constitutive defense levels beyond the focal populations, our data clearly indicate spatial variation in constitutive resistance in *Ascophyllum*. Additional studies are needed to determine whether this pattern persists throughout multiple populations within each region.

A major strength of our study was the inclusion of herbivore choice feeding assays as the primary measure of algal defense, rather than focusing exclusively on a subset of algal traits. Using this approach, we observed a tradeoff between defense types (i.e. higher constitutive resistance in a US population that lacked inducible resistance). Surprisingly, this result contrasts with the limited number of studies that use herbivore behavior to compare defense types (e.g. habitat or feeding preferences). Although we observed an inverse relationship between constitutive and inducible resistance across populations, other studies have found either no relationship (English-Loeb et al. 1998, Underwood et al. 2000) or a positive relationship (Brody & Karban 1992, Salgado & Pennings 2005, Long et al. 2011). At present, it is unclear why we observed a tradeoff in defense types when other similar studies did not. However, it seems possible that these systems differed in the strength and predictability of herbivore pressure. To test such a prediction, future studies will need to quantify the intensity and predictability of herbivore pressure *in situ* across systems.

*Acknowledgements.* Discussions with E. Sotka and G. Trussell provided the initial motivation for these experiments. R. Dolecal, E. Jones, C. Kwan, and A. Warneke provided thoughtful comments on early versions of the manuscript. This research was supported by the National Science Foundation (OCE-0825846), the Centre for Marine Chemical Ecology ([www.cemace.science.gu.se](http://www.cemace.science.gu.se)) and the Linnaeus Centre for Marine Evolutionary Biology ([www.cemeb.science.gu.se](http://www.cemeb.science.gu.se)) at the University of Gothenburg, and by the Swedish Research Council (contracts 621-2007-5779 and 621-2011-5630). This is Contribution No. 31 of the Coastal and Marine Institute Laboratory, San Diego State University.

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*Submitted:* June 7, 2013; *Accepted:* August 7, 2013  
*Proofs received from author(s):* November 13, 2013