GAME VI: Flexibility in macroalgal response to herbivory: the influence of grazing pressure on the induction of chemical defences in seaweeds

Study Question

Grazer-algae interactions are vitally important in structuring coastal ecosystems. Herbivores can control the vertical and horizontal distribution of seaweed populations, which in turn are very important habitat engineers in the sub- and intertidal (Valiela 1995, Duffy & Hay 2000). It is long known that macroalgae have several defence mechanisms to reduce grazing pressure by a multitude of herbivores such as snails, amphipods, isopods, urchins, and fish (Duffy & Hay 1990, Hay 1996, Amsler et al. 2005). A number of recent studies showed that macroalgae can induce chemical defences as a response to the presence of grazers (Pavia & Toth 2000, Ceh et al. 2005, Molis et al. 2006, Toth & Pavia 2007). Little is known so far about the flexibility in this response. Experimental studies on the brown seaweed *Fucus vesiculosus* revealed that the time needed by the algae to induce a chemical defence against isopod grazing is about two weeks (Hemmi et al. 2004, Rohde et al. 2004) – a time span that seems too long to ensure efficient protection in the case of a massive herbivore attack. In this study, we want to investigate whether the length of the period until a defence is induced is a function of grazing pressure. This will be done with several species of macroalgae from different costal habitats in different climate zones.

Inducible chemical defences in macroalgae

Several studies showed that brown and red, and to a lesser extent, also green seaweeds can regulate their chemical defence: it is induced in the presence of herbivores and reduced again in their absence. It is widely assumed that there is selective pressure for flexible responses to grazer attack in marine macrophytes, since the maintenance of a constitutive defence is presumably costly in terms of metabolic energy, bears the risk of self-intoxication, and allows a faster adaptation of herbivores to plant chemical defences (Hay & Fenical 1988, Harvell 1990, Rohde et al. 2005, Dworjanyn et al. 2006).

Though inducible chemical defences have been described for numerous species worldwide, little is known about the dynamics of induction processes. We do not know whether algae, for which the presence of an inducible defence was already

shown, exhibit this trait at all times and in all habitats. Intraspecific and even intra-individual variation in space and time is known for many ecological and physiological traits of algae (e.g. Cronin & Hay 1996, Cronin 2001, Sotka et al. 2001) and may exist for the induction of defences as well.

Experimental set-up and test organisms

All experiments should be conducted in tank systems/aquaria, since it is problematic to control grazer densities in the field. Small plastic container will do; we normally use aquaria that have a volume of 6-10 l.

Red and brown algae, like the Fucales (Fig. 2), are the most promising groups of seaweeds for this experiment. We know from the first GAME project that chemical defence is not very wide spread among green algae, so they should not be the first choice. Since it is known that different parts of algae are defended to different degrees (Toth et al. 2005), we should agree on a certain part of the thallus to be used in the experiments. From our experience, intertidal algae are easier to keep since they are more stress tolerant than subtidal species, but we do not have intertidal zones at all the stations.

Grazers can be amphipods, isopods, decapods (shrimps, shore crabs, hermit crabs, spider crabs etc.), gastropods (periwinkles, sea slugs, limpets, abalones etc.), urchins or fish. In previous GAME projects we most often worked with mesograzers (amphipods, isopods) because they are easy to collect in large numbers and relatively easy to keep in tanks. They feed selectively and consume measurable amounts in short times. Fish are more difficult to catch and keep, while urchins often show a rather unselective feeding behaviour.

The aquaria can be placed outdoor, where they experience natural light regimes. A good place would be on a pier or a jetty, where a flow-through system can easily be installed by the use of a pump and a larger water container from which the incoming seawater is then distributed to the single aquaria. With a flow-through system we hopefully do not have to take care of cooling or additional oxygen supply. If there is no flow-through system installed, we could use refillable storage tanks (let's say 250-1000L) that are connected to the small, single aquaria so that the water volume in the aquaria can be exchanged every 1-2 days. A third option would be a closed water cycle with a filter system. Each of these systems should be equipped with air supply.

If you have lamps that allow the cultivation of macroalgae, you could run the experiments in constant temperature rooms what would also solve the problem of overheating. If you work under natural light conditions, it may be necessary to cover the aquaria with some kind of gauze or net material to reduce the amount of incoming radiation. Here, I would like to ask the incoming students and their supervisors to discuss the facilities at their institutes before the course in Kiel starts, so that we can try to keep the methods as comparable as possible.

Figure 1: The brown seaweed *Fucus serratus* from the Baltic Sea. Photo: Martin Wahl

Pilot study I: Determining grazer densities

In this first approach, we will gain knowledge about average grazer consumption and algal growth rates. After choosing a promising alga-grazer combination (see below), a set-up with 12 aquaria will allow to investigate the impact of 3 different grazer densities on the seaweed of interest. A control group will provide information about the growth of algae in the absence of herbivores. Grazer densities should be chosen on the base of field observations, while each of the levels has 3 replicates (i.e. aquaria). This study will be run for three days. After this, we should be able to determine a density of herbivores that leads to a loss in plant biomass of 5-10 % during the given time span, what, if algal growth and grazer consumption rates are constant, should result in a total loss of 25-50 % within 15 days.

Pilot study II: Test for inducible defences

The second step in the experiment is to test for the presence of an inducible defence in the chosen seaweed. The most promising candidates are macroalgae that were already tested in GAME I, GAME V, or in other studies. Macroalgae and grazers will be collected in the field and then, like in the first GAME project, kept in small aquaria (Ceh et al. 2005, Molis et al. 2006, Weidner et al. 2004, Yun et al. 2007). One group of algae will be exposed to grazing, while a control group remains ungrazed throughout this pilot trial. There should be 5 replicates in each of the groups. Information about an appropriate grazer density result from pilot study I. Agal material will be taken from both groups at intervals of 2-3 days and will be offered to herbivores of the same species as used for the induction in two-way choice feeding assays. A significant difference in the consumption of algal tissue between the grazed and ungrazed group indicates the presence of a defence (Fig. 2). The absence of a difference may indicate a constitutive defence or the absence of any resistance towards grazing. A third control group in which green seaweeds of the genus *Ulva*, which normally do not show any chemical defence, are offered to the grazers can help to test for the general presence of any defensive trait. This pilot study should be run for 14-15 days.

Speed of induction and reduction (in *Fucus vesiculosus*)

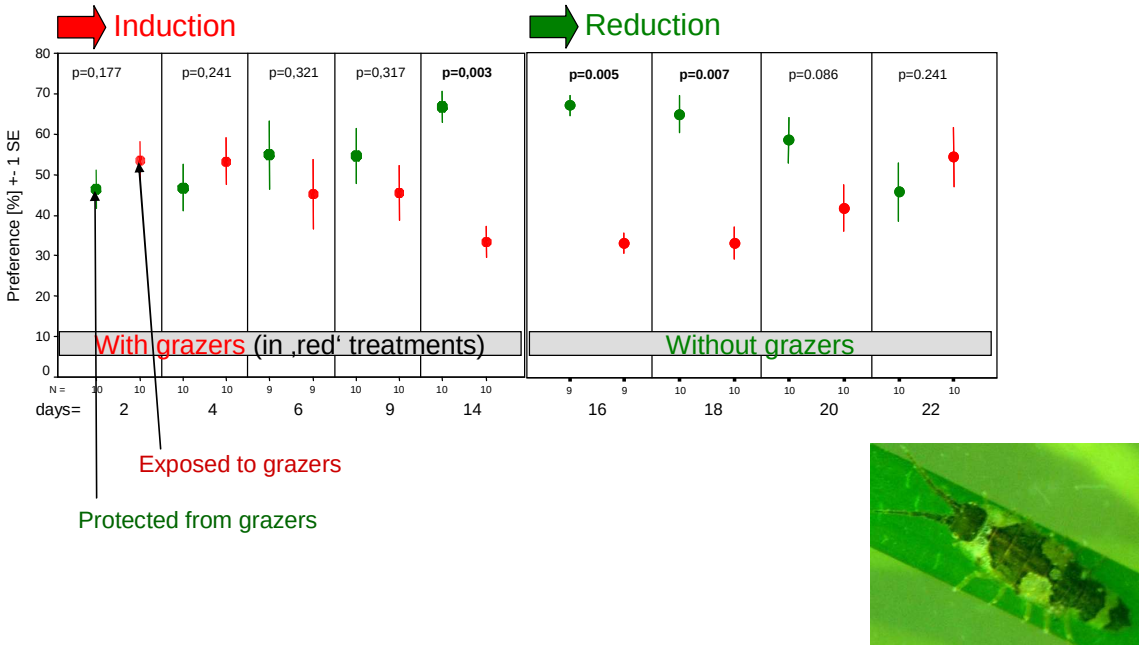

Figure 2: Induction and reduction of a chemical defence in *Fucus vesiculosus*. Source: Sven Rohde

Main experiment: Influence of grazer density on defence induction time

Once an alga exhibiting an inducible defence has been identified, it will be exposed to different levels of grazing pressure. The minimum number of treatment levels is two: one grazer density above the point when grazer consumption exceeds algal growth and one below it. If this study shows a significant difference in induction times between levels, a subsequent experiment with the same seaweed species and two intermediate grazer densities could round off the picture. In both cases, an ungrazed group of algae with 20 replicates will serve as a reference that allows to identify when the defense sets in. The number of replicates should be 10 per treatment level. With a replication of 10 experimental units per treatment level and 20 control units we would end up with 40 aquaria. At the moment, for GAME V, we use a maximum number of 96 aquaria. Small plastic tanks are not too expensive, so that space might rather be the limiting resource here.

Samples of algal tissue should be taken subsequently from the different replicates in short time intervals (2-3 days) to ensure a high temporal resolution in this study. These samples will then be offered to the grazers and consumption rates will be compared between each of the treatment levels and the controls. To make sure that

all the subsequent samples experienced the same grazing pressure, algae or algal fragments should be kept in single compartments within the tanks. Each compartment at a given grazer density level contains the same number of herbivores. With a maximum experimental duration of 18 days, 6 compartments will be enough. Cylinders made from a gauze material are a cheap and fast way of installing compartments.

Feeding assays

We suggest the use of two-way choice feeding assays. There are three options to prepare the algal material for these assays; the first would be to work with living algal material. In this case, we need to control for growth that occurs during the feeding experiments to disentangle biomass accrual and consumption. The maximum time of exposing the algal material to herbivores should be 72 h. If you use amphipods or isopods, Petri dishes are sufficient as containers for the feeding assays. The number of herbivores per assay will depend on their consumption rates. Please make sure that you use the same species of grazers (but not the same individuals!) that you used for the induction of the defence. It would also be interesting to run tests with other herbivores to answer the question whether the induced defence is grazer specific. This would be an interesting topic for additional studies.

Working with living algae has several disadvantages: a) we can not store the material, b) we have to control for growth during the experiment, what means that we will need additional algal material for the controls, and c) we can not control for non-chemical defence mechanisms. Another option would therefore be to work with freeze-dried material. The resulting powder is storable (in a normal freezer) what makes time management easier and we exclude mechanical defence mechanisms. For the feeding assays the powder will be embedded in agar and the powder-agar mixture will then be poured onto a piece of gauze that functions as a matrix (Fig. 3). These procedures will be practised here in Kiel during the introductory course. We will determine consumption by counting the consumed quadrates. We have to make sure that we standardize the consumed material for the body size of the grazers.

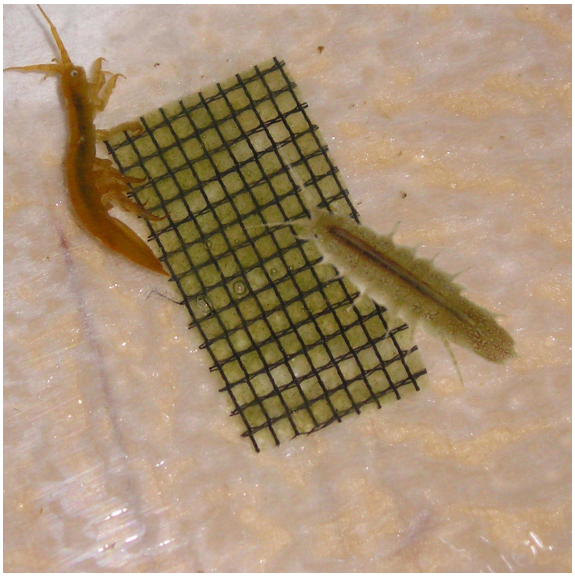

Figure 3: Mesograzers feeding on algal material embedded in agar and fixed in a gauze matrix. Photo: Sven Rohde.

If freeze-drying is not possible, it could be an option to air-dry the algal material and grind it afterwards using a mortar or an electric coffee mill. A third option would be to extract the fresh material using a solvent covering a broad polarity range (e.g. a mix of methanol and hexane). These extracts are storable and can also be incorporated in an agar matrix. In this case, Ulva powder should be added to make the food pellets more attractive for the grazers. We will discuss the pros and cons of the different methods during the course in Kiel and we will practise the different techniques in practicals. Again, the incoming students and their supervisors should discuss on beforehand which approach could be the best with regard to their facilities and resources. Do you have access to freeze-driers, rotatory evaporators, etc?

Extra experiment: Is clipping inducing a defence?

In case you can not use intact algal individuals, because they are too big or can not be detached from their substratum, you have to take single blades or fragments of the thallus what would mean you have to cut it. To make sure that this treatment is not inducing a defense that will later confuse our study; you should test for the effects of clipping. For this one group of algae (10 replicates) will be kept over a period of 14 days and will be clipped every second day. A control group will be clipped in the beginning and not again thereafter. After two weeks, feeding assays will test for the presence of a defense in the clipped individuals. If a defense is present the

experiment should be prolonged while the clipping will be stopped to see how fast the defense will be reduced again (Fig. 2)

Data analysis

To check for the presence of an inducible defence and for the effects of clipping the paired t-test or its non-parametric equivalent, i.e. the Wilcoxon-signed-rank test, will be used to test for significant differences in the consumption of previously stimulated and non-stimulated seaweed material. In the main experiment, our response variable is the time until an inducible defence becomes detectable in the feeding assays. In the case of only two treatment levels, a simple independent t-test will suffice to test for differences in the length of mean induction times, while in the case of more than 2 levels we could employ either analysis of variance to compare group means or we could model induction time as a function of grazing pressure using regression analysis. Our design, in principal, allows both approaches (replicated regression, *sensu* Cottingham et al. 2005). To identify the induction time we will calculate the size of the repulsion effect, i.e. the difference in consumption (in percent) on induced and non-induced seaweeds, for all sampling events. These values will be plotted over time for each single replicate and a straight line will be fitted to the data. The point in time when repulsion exceeds 30 % will be recorded as the respective induction time (Fig. 4).

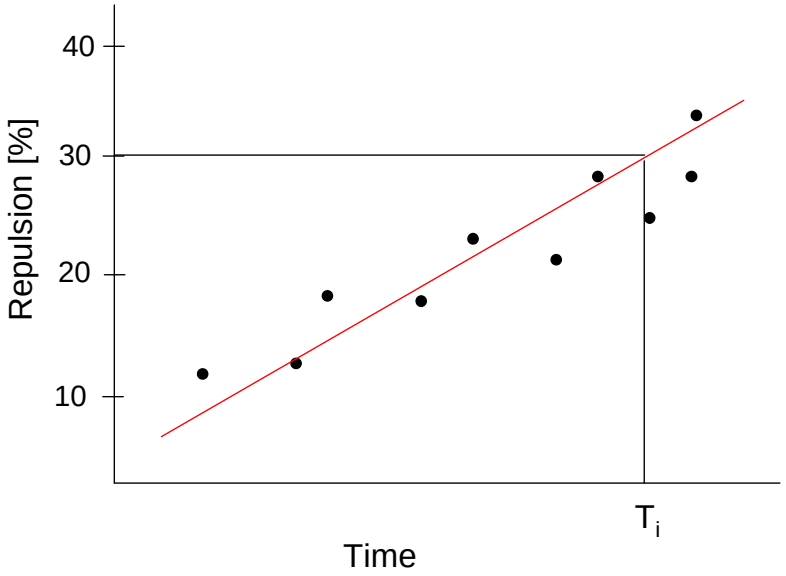

Figure 4: We assume a defence to be induced when the repulsion, i.e. difference in the consumption on grazed and ungrazed seaweed material, reaches 30 %. This indicates the induction time T_i.

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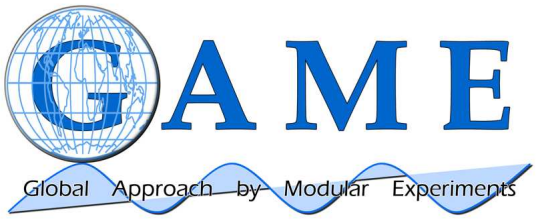
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