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The effects of *phloroglucinol* on *Tegula herbivory*

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Abstract

Primary producer's strategies for defending themselves against herbivores has manifested in many ways. Chemical defense is one strategy that many plants have utilized to become less palatable to herbivores. The production of secondary metabolites such as tannins in terrestrial plants and phlorotannins in marine algae are hypothesized to have a deterring effect on herbivore grazing. Phloroglucinol (1,3,5 - trihydroxybenzene) is the monomer of all phlorotannins found in brown algae (Division *Phaeophyceae*). The monomer can be arranged in a variety of different ways for a variety of functions. Research on the function of phloroglucinol as the active deterrent of herbivory has conflicting results. To address this, two snail species from the genus *Tegula* were used to determine if the presence of phloroglucinol reduced herbivory. We exposed *Tegula funebralis*, an intertidal species and *Tegula pulligo*, a subtidal species to experimental seaweed plates with varying concentrations of phloroglucinol. The two *Tegula* species were selected as closely related representatives from different tidal heights to see if habitat played a role in phloroglucinol tolerance. We measured consumption and preference of experimental plates with different concentrations of phloroglucinol. Consumption was measured directly and preference was determined using a Y-maze. Our study shows that there is no deterring effect of phloroglucinol on either of the two *Tegula* species. The function of phloroglucinol may not have any implications in defense against *Tegula* herbivores. The production of phlorotannins in kelp species could be a general stress response without a specific stressor activating the production, and many compounds likely carry out chemical defense.

Keywords — Phlorotannins, Chemical defence, Algae, Preference, Y-maze

1. Introduction

Herbivory is at the core of all ecosystems and is essential to support higher levels of life. In response to herbivory, many prey organisms have evolved defensive strategies to reduce their losses. Defensive strategies can take many forms, including or combining mechanical, chemical, constituted or induced mechanisms. Induced chemical defenses occur when the prey organism responds directly or indirectly to grazing with the increased production of a chemical deterrent [1]. Examples of induced and constituted chemical defenses are well known and described in vascular

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terrestrial plants [2], while examples of chemical defenses in marine primary producers are less understood.

Phlorotannins are a marine example of a chemical defense that is seen only in the division Phaeophyceae or the brown algae [3]. Phlorotannins are polymers of phloroglucinol (1,3,5 - trihydroxybenzene) and can have different structural forms [4]. Phlorotannins are secondary metabolites that have many speculated functions such as, protection from ultraviolet (UV) radiation [5, 6], reduction of localized phytoplankton levels, reduction of fouling organisms [7], increased wound repair [4], and chemical herbivore defense [3, 8, 9]. The simplest form of phlorotannin is the monomer phloroglucinol. The functions of different forms of phlorotannins are not well understood and it is unclear as to whether phloroglucinol is active in minimizing herbivory. It has been disputed whether phlorotannins function as a chemical defense against herbivores [10].

Concentration and allocation of phlorotannins vary in tissues of the same individual [7], between different life stages of conspecifics [9], and throughout populations [11]. Closely related brown algal species from the same geographical area can have varying amounts of phlorotannins [3, 8]. Species from different climates can also have a variety of phlorotannin concentrations. Temperate brown algae tend to have higher amounts of phlorotannins compared to tropical species [3] and kelp of the Pacific Northwest have been found to have less phlorotannins than Australian kelp species [8].

Research of phlorotannins and phloroglucinol as herbivore deterring compounds, similar to phlorotannin, allocation research, yields conflicting results. Steinberg (1988) found that different amounts of phlorotannins and polyphenolics mixed into agar plates had similar herbivore deterrence, while Deal et al. (2003) found that in *Fucus vesiculosus*, galactolipids rather than phlorotannins deterred urchin herbivory. These discrepancies in the literature suggest that there is a wide variety of responses both by brown algae to produce phlorotannins and herbivores being deterred by phlorotannins [4]. Studies that use the same herbivore-seaweed relationships also have different results, as reviewed in [4]. With that, experiments investigating the effects of phlorotannins, polyphenols, and phloroglucinol on herbivory must further investigate and consider what is involved in this complex interaction.

Phloroglucinol has been tested specifically for its involvement in deterring herbivory and has had inconsistent results. Steinberg (1988) found that the monomer did not deter herbivory by the intertidal snail *Tegula funebralis*. In contrast, Pereira et al. (2015) measured the distance *Tegula trindentata* traveled in response to the addition of an agar plate containing phloroglucinol and found that on average, the snails moved farther away from plates containing higher concentrations of phloroglucinol [12].

To contribute to the research into whether phloroglucinol is the active molecule of the herbivore deterring properties of phlorotannins, we exposed two gastropod grazers to varying phloroglucinol levels to determine if there is an effect. Two snails of the genus *Tegula* were selected. *Tegula funebralis*, an intertidal species and *Tegula pulligo*, a subtidal species. These species were selected for their relatedness and differing habitats. Since phlorotannins and phloroglucinol offer protection to seaweeds against UV radiation [5, 6], we suspect that intertidal herbivores such as T. *funebralis*, would be more resilient to phloroglucinol exposure. Subtidal herbivores such as *T. pulligo*

would be less resilient to phloroglucinol as there is less need for protection from UV radiation at greater depths. We sought out to determine if increasing concentrations of phloroglucinol results in deterrence from food choices containing phloroglucinol and if it reduces grazing by *Tegula* species. We hypothesize that both species of *Tegula* will prefer food sources lacking phloroglucinol and that grazing will be reduced in both species at higher concentrations of phloroglucinol. In addition, the intertidal species, *T. funebralis*, may be less affected by the presence of phloroglucinol compared to *T. pulligo*.

2. Materials and Methods

2.1. Animal Collection and Preparation

One hundred *Tegula funebralis* specimens were collected from Aguilar Point in Bamfield, B.C. on November 8th, 2016. Ninety-three *Tegula pulligo* specimens were collected from Bamfield Inlet on November 10th, 2016. Snails were kept in groups of 20 in five liter containers, the same as those used in the consumption experiment.

2.2. Animal Preparation

Seventy-five snails of each species were starved for 90 hours before starting the consumption experiment. Of those not selected for consumption trials, 12 snails of each species were starved for 14 days prior to the preference experiment. The weight of each snail was recorded before beginning the consumption trials and then randomly assigned a treatment container. Snails used for the preference trials were randomly assigned but not weighed.

2.3. Agar Plate Preparation

We prepared agar plates infused with seaweed (*Macrocystis pyrifera*) and isolated phloroglucinol. The phloroglucinol was added to 100 mL of distilled water, five grams of seaweed and 2.5 grams of bacteriological agar. The five grams of seaweed consisted of 2.5 grams of blade tissue, and 2.5 grams of stipe tissue. The use of equal parts of both stipe and blade tissue was to account for any variations in the natural levels of phlorotannins in kelp. The seaweed was blended in water and then heated to a gentle boil. The agar and phloroglucinol were added to the mix and then immediately poured into six 60mm x 15mm sterile polystyrene Petri Dishes. Treatment concentrations of phloroglucinol were as follows, control was 0 mg/mL, low was 8 mg/mL, medium was 12 mg/mL, high was 17 mg/mL, and very high was 22 mg/mL (as per Steinberg, 1988). Each treatment was prepared separately. The agar plates were left to sit for three hours until the agar set and were stored in a fridge at 6 °C until experiments could start ($\sim$48 hours). Plates weighed approximately 18 grams. Plates were then soaked in seawater for 12 hours to allow the plates to absorb water and sink. Plates were weighed again after soaking to be able to determine amount consumed by the snails.

2.4. Experiment 1: Consumption

One agar plate was secured to the bottom of a clear, 5 L testing container with a small amount of hot glue. Each container was randomly assigned three weighed snails and a sea table. Five replicates of each treatment and a control replicate (without snails) were done. This resulted in 25 containers with snails and 5 control containers without snails (a total of 30). This was done for both species for a total of 60 trials. All the snails used were starved for 90 hours before starting the experiment. The snails were left in the containers with the pre-weighed agar plates for five days. When done, each agar plate was removed, reweighed and used to determine the amount of agar consumed by the snails.

$$\text{wet mass before} - \text{wet mass after} = \text{amount consumed}$$

The amount of agar consumed was then divided by the mass of the three snails used in the trial. This determined the consumption to biomass ratio for the trial.

$$\text{amount consumed}/\text{total biomass} = \text{con : biom ratio}$$

2.5. Experiment 2: Preference

A Y-maze made of corrugated plastic cardboard was used. The maze was set up in a sea table with water flow. Each trial had one control plate and one treatment plate. Three concentrations of phloroglucinol were used as treatment plates. A control with 0 mg/mL, a low with 8 mg/mL, and a high with 17 mg/mL of phloroglucinol were used. The seawater flowed over each plate and toward the proximal end of the maze. Three zones were distinguished in the Y-maze. Zone 1 referred to the center, Zone 2 referred to the left arm and Zone 3 referred to the right arm. Before commencing a trial, a control plate and treatment plate (either control, low or high) was randomly assigned to either Zone 2 or Zone 3 and glued in the distal end of the Y-maze. Once water was flowing, a snail was placed at the proximal end of the maze. The 12 *T. funebralis* and 12 *T. pulligo* used for these trials were starved for 14 days. We were limited in the availability of *T. pulligo* so 12 snails was the largest sample size attainable.

We ran four trials of each treatment with each species of *Tegula* for 30 minutes. Time spent in each zone was recorded. The snail's preference (control or treatment) was established by which tray they arrived at or by which zone they spent the most time in. All snails tested made a choice. Preference was recorded at the end of the trial. The maze was rinsed between trials to prevent any influence from mucus trails left by previous snails.

3. Results

3.1. Consumption

Consumption to biomass (con:biom) ratios were calculated and negative ratios, resulting from an increase in plate mass, were disregarded for our analysis. Causes for the negative consumption ratios, which indicate an increase in weight are discussed later in this manuscript. There was a total of 4 negative con:biom values (3 from *T. funebralis*

and 1 from *T. pulligo trials*). Mean con:biom ratios for *T. funebralis* trials were 0.105 for control, 0.069 for low, 0.080 for medium, 0.159 for high, and 0.113 for very high. Standard deviations of these means were 0.089, 0.073, 0.040, 0.108 and 0.113 respectively. The mean consumption ratio for T. pulligo trials were 0.112 for control, 0.163 for low, 0.110 for medium, 0.088 for high, and 0.122 for very high (Figure 1). Standard deviations of these means were 0.049, 0.025, 0.059, 0.044 and 0.083 respectively. These ratios represent the average mass of agar consumed per unit of snail biomass.

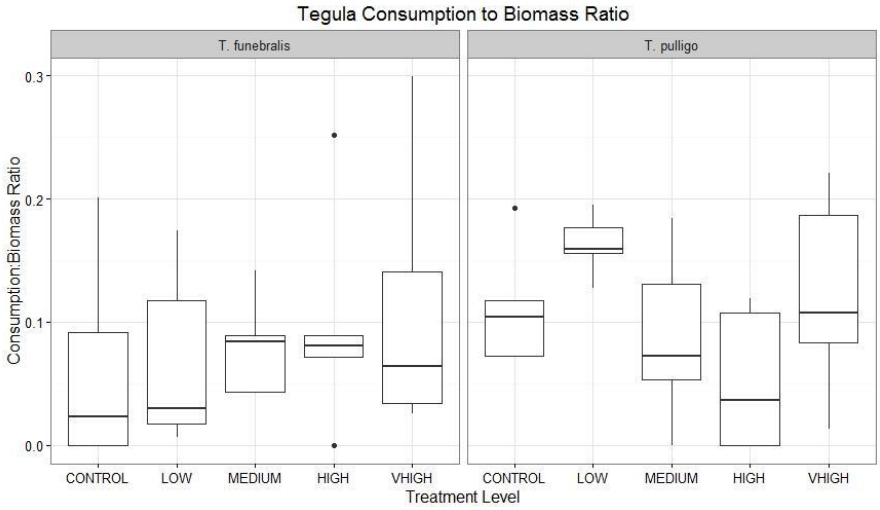

Figure 1: *The consumption:biomass ratio for each treatment level in the consumption experiment. VHIGH = Very High treatment.*

	T. funbralis		*T. Pulligo*	
	treatment	control	treatment	control
Zero	2	2	2	2
Low	2	2	3	1
High	3	1	3	1

Table 1: *The choice made by each snail in each trial run in the Y-maze. Zero = Control treatment (0 mg/mL), Low = Low treatment (8 mg/mL), and High = High treatment (17 mg/mL).*

3.2. Preference

All snails tested made a choice of either the treatment or the control. *Tegula funebralis* chose the treatment 50% of the time in control trials, 50% of the time in low trials, and 75% of the time in high trials. *Tegula pulligo* preferred the treatment 50% of the time in control trials, 75% of the time in low trials, and 75% of the time in high trials (Figure 2).

The time spent in the treatment zone of the Y-maze was averaged for each treatment and species. *Tegula funebralis* spent 49.92% of the time in treatment zone during control trials, 38.54% of the time in the treatment zone during low trials, and 52.43% of the time in the treatment zone during high trials. *Tegula pulligo* spent 40.89% of the time in

treatment zone during control trials, 35.53% of the time in the treatment zone during low trials, and 46.52% of the time in the treatment zone during high trials.

3.3. Analysis

Results of statistical analysis using linear models to fit consumption data returned no effect of concentration, species, or any combination of the two factors on con:biom ratios (p-values$>$ 0.05). A chi-squared test was used to determine the independence of phloroglucinol concentration from preference choice. The results from the test indicate that concentration of phloroglucinol was independent of choice (p-value$=$ 0.586, $\chi^2 = 1.066$).

4. Discussion

Statistical analysis of results from both experiments found no effect of phloroglucinol concentration or species on consumption or choice. In the consumption experiment, con:biom ratios for increasing phloroglucinol concentrations in each species of *Tegula* were not significantly different from one another and showed no trend. There was neither an increase nor decrease in consumption by either species; therefore the phloroglucinol monomer did not affect the amount of *Tegula* grazing. There was no effect of species on the amount of agar consumed or con:biom ratios. These results suggest there is no habituation in higher intertidal snails due to increased phloroglucinol from UV radiation. The amount of the agar consumed was low in all trials. This could be due to the short duration of trials and short starvation time prior to the experiment. The negative con:biom ratios that were disregarded in our analysis suggest that there may have been inaccuracies in the measuring techniques or unforeseen increases in agar mass that were not considered, such as absorption of water by the agar or bacterial growth on the agar.

In the preference trials, choice was independent of concentration, but there was a noted affinity for the phloroglucinol treatment plates over the control plates. More snails of both species chose phloroglucinol plates over control plates in the high concentration treatment (75% for both *T. funebralis* and *T. pulligo*). Both species on average spent a large proportion of time in the treatment zone during both low and high trials. This might suggest that phloroglucinol as a monomer could attract Tegula to the seaweed, influencing food choice. The preference experiment would benefit from more replicates.

Both the consumption and preference experiments had unexpected results. Our result of a slight preference for phloroglucinol may suggest that its presence in seaweed may be an indicator of food to *Tegula* species. The lack of effect of phloroglucinol concentration on consumption suggests that phloroglucinol does not affect the amount consumed by *Tegula* species. Considering both results, the amount of grazing is likely not affected by the presence of phloroglucinol, but in food sources may influence *Tegula* food choice. Seaweed that produces large amounts of phloroglucinol may attract herbivores resulting in greater overall herbivory on that individual. Since phloroglucinol may promote herbivory, it is fair to speculate that it it does not occur in high levels naturally. It is likely that phloroglucinol must be polymerized into phlorotannins or polyphenols for any herbivore deterring properties to arise. There are

multiple configurations of phlorotannins with many functions and benefits [4]. Further research of phlorotannin configurations and their function would aid in determining the influence of phlorotannins on herbivory.

Our results suggest there is more involved in herbivore deterrence than simply the monomer phloroglucinol. There are a number of potential alternatives to phlorotannins and phloroglucinol as herbivore deterrents in marine algae. An alternative is that phlorotannins and phloroglucinol may affect different species differently. Similar to our study, Steinberg (1988) found no effect of phloroglucinol on *T. funebralis* herbivory, but found that phloroglucinol did affect Echinoid herbivores such as *Strongylocentrotus purpuratus*. Another alternative is that there are other compounds responsible for herbivore deterrence. Deal et al. (2003) used bioassays to separate compounds and suggests that galactolipids are responsible for herbivore deterring properties of seaweeds. As phlorotannins are difficult to isolate and quantify [10], they are often tested as an extracted concoction of potentially confounding compounds. Deal et al. (2003) suggests that galactolipids are a confounding chemical that is extracted with phlorotannins and has likely influenced previous research. This raises the notion that other bioactive metabolites may be uninvestigated and have unknown properties within phlorotannin extracts.

Phlorotannin production in kelps is often observed in stressed individuals [5]. The production of these compounds appears to be in direct response to stressors like herbivory, UV radiation, and others. The stress response could be a general one that is initiated as an attempt to lessen the stress, regardless of its source. If phlorotannin production is a general response to stress, it would be difficult to determine which stressor the phlorotannins most effectively reduce.

Chemical defenses are not rare in the marine environment. Additional examples of chemical defenses are demonstrated in the Genus *Desmarestia*. These brown algae are capable of producing sulfuric acid that can contribute up to 18% of the algae's dry mass [13]. The acid produced by these algae can dissolve mouth parts of urchins and deter herbivore grazing [13]. Other brown algae in the Order *Dictyotales*, can produce a diterpenoid alcohol that has shown to greatly decrease herbivore grazing [14]. Understanding the relationship and interactions between primary producers and herbivores is essential to understanding the fundamental ecology of any ecosystem.

5. Conclusion

The function of phloroglucinol as the active molecule in phlorotannin herbivory deterrence is not clear from our experiments. The monomer appears to have no deterring effect on the amount a herbivore will consume. Phloroglucinol may attract herbivores to food sources but more conclusive evidence is required. Extracting phlorotannins that are free of confounding compounds from seaweeds is essential to determining the specific function of each phlorotannin, and determining phloroglucinols role in the seaweed.

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