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A Study of Defensive Mechanisms Employed by Two Species of Nudibranchs using Toxicity and Unpalatability Analyses

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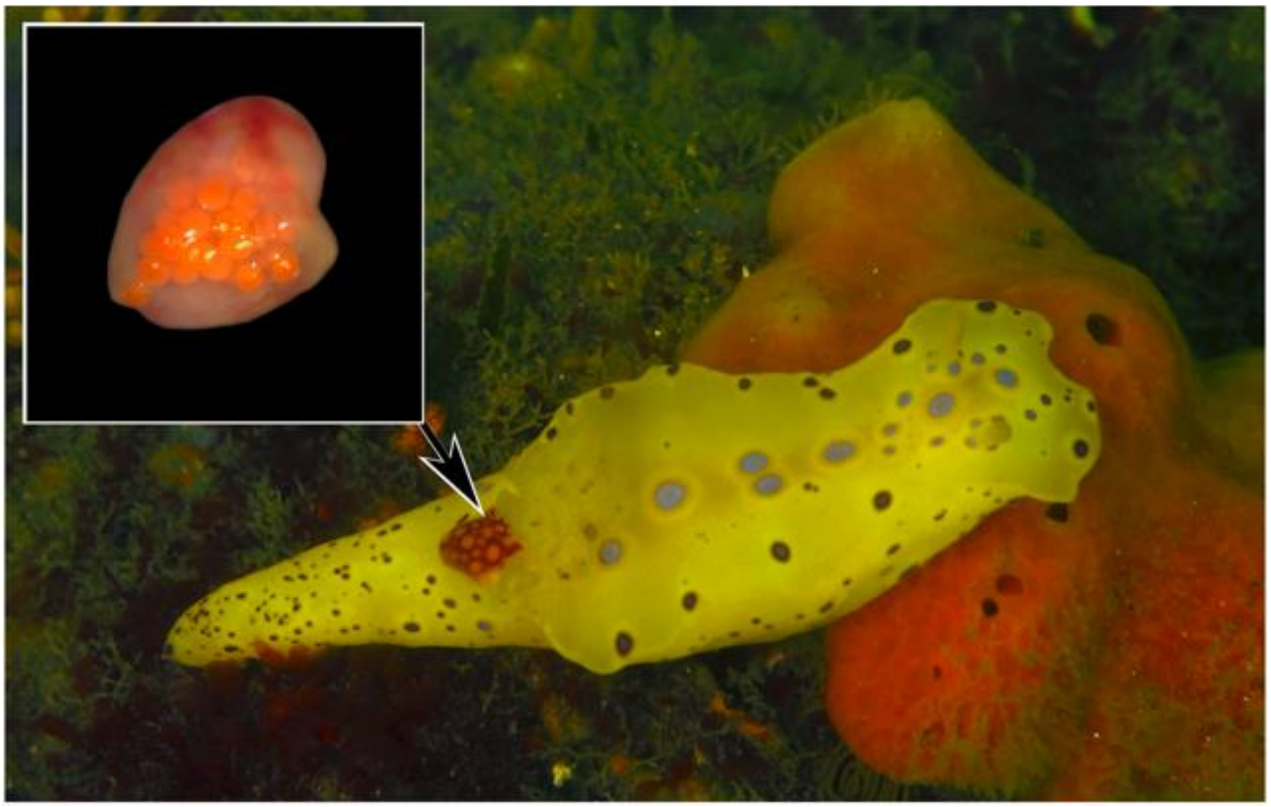

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Abstract

Nudibranchs are marine invertebrates that have developed an intriguing defense mechanism, including warning coloration and the use of chemicals accumulated through their sponge diet. The goal of this study was to determine whether the strength of chemical defenses differs between dietary and accumulated secondary metabolites for two species: *Glossodoris vespa* and *Ceratosoma brevicaudatum*. First, NMR spectroscopy was used to not only identify specific compounds in the mantle (outer covering) and the viscera (gut) but also to analyze the possibility of nudibranch species transporting more toxic compounds for defensive purposes. Next, toxicity (brine shrimp) and palatability (*Palaemon* shrimp) assays were used to examine whether accumulated compounds differ in anti-predator activity. The results of this study show increased toxicity in the mantle compared to the viscera for both species. and while both species exhibited the possibility of selective sequestration, *Glossodoris vespa* hinted that nudibranchs may have other methods of chemical sequestration including chemical modification that would explain why more toxic and unpalatable compounds are found in the mantle. However, there was no significant change in unpalatability between the mantle and the viscera. Finally, comparisons between genera that have mantle dermal formations along the mantle rim (*Glossodoris*) and those that have mantle dermal formations concentrated in the mantle horn (*Ceratosoma*) show that despite varying classes of dietary chemicals and selectivity of sequestration, both species exhibited a chemical arsenal in the mantle that was more toxic than dietary metabolites, suggesting that toxicity is an important part of their defensive strategy.

Keywords: nudibranch, toxicity, unpalatability, mantle dermal formations, selective sequestration

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Acknowledgements

First and foremost, I would like to thank Dr. Karen Cheney for allowing me to conduct such an exciting project in her lab with her equipment. Everyday I came in, she made sure that I had a plan for my experiments and assays while ensuring that I had all the resources necessary to be successful during the entire duration of my stay. This was the first time I have conducted research in the marine biology field, and I couldn't ask for a more supportive advisor to show me the ropes.

I honestly could not have done this without the help of Anne Winters. Not only did she teach me about nudibranchs and the specific species I was studying during the month, but she also was willing to meticulously review each step of the chemical purification process and the two assays we performed. She came on the weekends to help set up experiments and talked with me for hours regarding my research paper and the results of our experiments in general. From the moment I stepped into the lab, she was very courteous to me and always asked if I needed any clarification on what we were doing. I, therefore, want to sincerely thank her for all of her help and expertise that she bestowed upon me during my time in Brisbane.

I finally want to take time to thank the rest of the Cheney Lab, including Cedric and Emily for being so accommodating from the first day. Lost looks on my face were aplenty in and around the lab, and they were always happy to help me with whatever I needed.

Last but not least, I want to thank Tony Cummings for being the helpful and sarcastically funny teacher he was over the course of the semester. Thank you for dealing with 19 college students and ensuring our safety wherever we were. These last three months will always be remembered.

List of Figures

Introduction

Chemical defenses are prevalent in the animal kingdom, but organisms vary in how they accrue these chemicals as a source of protection. Some animals such as the cane toad (*Rhinella marina*) are able to synthesize their own toxins using basic chemical building blocks and accumulate them on their skin as a deterrent to predators such as the Slaty-grey snake (Phillips et al., 2003). However, other organisms have evolved to be able to sequester toxins from their diet such as poison dart frogs that accumulate the toxins found in millipedes and ants (Daly et al., 1994). While most organisms utilize one form of chemical accretion, the marine gastropods known as nudibranchs (Gastropoda, Mollusca, Animalia) have been observed to engage in both selective sequestration, the ability to only use certain chemicals from their diet, and chemical modification of dietary metabolites (Kubanek et al., 2000).

The clade of Nudibranchia

There are over 3,000 species of nudibranchs around the world from the warm Caribbean Sea to the frigid Antarctic waters. The term 'nudibranch' means 'naked gills' which aptly describes their appearance: unlike many of their snail-like relatives, these marine invertebrates have evolved to shed their hard outside shell following their larval stage. Energy economy is a possible reason for this transformation, as the nudibranchs would save the cost of using and transporting a shell if they could develop a new form of protection (Faulkner & Ghiselin, 1983). Instead, nudibranchs have evolved to use chemical compounds as a deterrent. There are two primary methods of accruing these toxins. The first is *de novo synthesis*, which is the act of creating toxin molecules from more simple biomolecules such as sugars and amino acids. Through this process, species such as the *Cadlina luteomarginata* and the *Melibe leonina* are

able to synthesize defensive chemicals only when threatened (Kubanek et al. 2000). The second and most common method is the ability of these invertebrates to 'steal' secondary metabolites from sponges that they feed on. Unlike primary metabolites, secondary metabolites, such as terpenes and alkaloids, are organic compounds that are not directly related to growth and development. Nudibranchs are able to bioaccumulate these compounds in various parts of their body, namely the mantle (outer covering) and the viscera (gut).

Nudibranch Anatomy and Chemical Storage

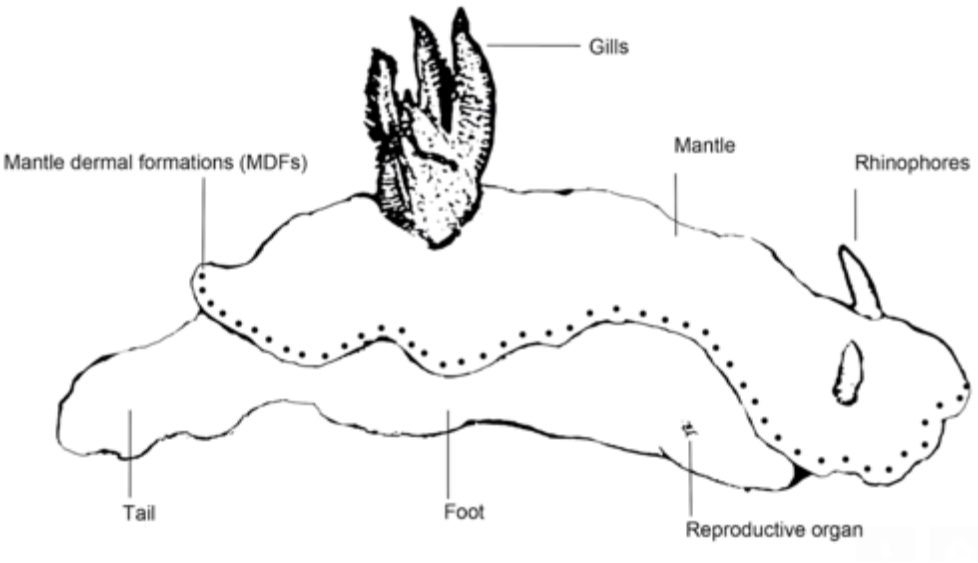

Figure I. Anatomical Representation of a Generic Nudibranch

Nudibranchs have a simple external anatomy, including gills, a mantle, and rhinophores. Rhinophores are external appendages that are used for odor detection, the mantle acts as an outer covering, and the gills are used for respiration. In response to a predator attack, most nudibranchs are able to invert themselves, retracting vital parts such as the gills and rhinophores towards the inside of its body while flaring the mantle outwards (Pawlik et al., 1988). Scientists observing

this behavior hypothesized that chemical toxins that were found in the viscera of various families of nudibranchs may be present in the mantle to deter predators from eating the nudibranch (Avila & Paul, 1997). Further research showed that there were records of toxins in the mantle that were especially concentrated in small dots on the edge of the mantle rim called mantle dermal formations (MDFs) while also uniformly distributed across the mantle at lower concentrations (Carbone et al., 2013). MDFs are globular masses that measure approximately 250 micrometers in diameter and are composed of multiple cells, each with a large vacuole that holds the toxins (Fontana et al., 1994). Across nudibranch families, MDFs contain a wide range of chemicals: some are identical to the toxins found in the viscera, but others are different, which raises a few hypotheses. First, nudibranchs may sequester more toxic sponge compounds in the mantle and less toxic compounds in the viscera to reduce the risk of autotoxicity, which is inadvertent self-poisoning due to the presence of toxic compounds in the body. Certain species in the *Doriprimatica* and *Chromodoris* genera are able to selectively sequester more toxic compounds in the mantle and allow less toxic compounds to accumulate in the viscera (Cheney et al., 2016). Second, nudibranchs may ingest more benign sponge compounds and later chemically modify those compounds to be more active and then transport them to the mantle and MDFs. NMR spectroscopy with certain *Glossodoris* individuals have shown that the 12-keto scalarane compounds such as heteronemin that are present in the nudibranch cannot, themselves, be found in their sponge diet but are presumed to be derivatives of compounds that can be found in the sponge (Manzo et al., 2007). Although there are examples that support both hypotheses, in most cases though, nudibranchs seem to have varying chemistries between their mantle and viscera.

Unpalatability vs. Toxicity

In terms of feeding, nudibranch families are usually associated with a certain family of sponges whether due to dietary preference and/or geographical location, and there are also many that are even species-specific (Rudman & Berquist, 2010). However, the variability of nudibranch chemical defenses relies mainly on the availability of secondary metabolites in their dietary sponges (Wägele et al., 2006). The primary way of characterizing these toxins that are applicable to the nudibranchs' anti-predator defenses is to compare the toxicity levels and unpalatability levels. Toxicity is the measure of a metabolite that causes physiological harm to the predator that ingests it while unpalatability refers to food containing the metabolite that are quickly rejected by predators without any subsequent damage (Pawlik, 2012). In terms of unpalatability, the secondary metabolites are insoluble in water, and many species of nudibranchs accumulate terpenoids, which are hydrocarbons that come from their sponge diet. Terpenoids are widely distributed in plant families and because they are volatile in nature, other terrestrial organisms sense them through odorant receptors (Tholl, 2015). However, in a marine context, organisms are able to distinguish these hydrophobic molecules by taste receptors, and therefore, unpalatability is a more appropriate description (Atema, 2012). Nudibranchs release these chemicals in high localized amounts through sacrificial body parts such as the mantle dermal formations, which are predominantly located along the mantle rim (Carbone et al., 2013). In addition to the variety of compound classes including alkaloids, terpenes, and macrolides, there are also many combinations of unpalatability and toxicity found in nudibranchs. For example, some chemicals are toxic and palatable; this would eventually severely harm or kill the predator but would not prevent the nudibranch from being eaten. Although this method seems to have limited effectiveness, fish assays using *Chasmodes bosquianus* showed that nudibranchs

with unpalatable or toxic compounds that caused physiological damage to the fish when ingested led to both immediate rejection through vomiting and a learned behavior by the fish to avoid any nudibranchs with similar chemical compounds (Long & Hay, 2006). However other chemicals are non-toxic but unpalatable, which would serve the nudibranch well not only because they act as antifeedant molecules but also because they would decrease the risk of autotoxicity while the compounds are stored in the nudibranch. Naturally, the most effective combination would be unpalatable and toxic. Therefore, this is an interesting topic of discussion from an evolutionary perspective because there are nudibranch species exhibiting each of these combinations: *N. gardineri* is toxic and palatable, *D. tuberculosa* is not toxic and palatable, *C. elisabethina* is toxic and unpalatable, and several *Goniobranchus* species showed low to no toxicity and high unpalatability (Winters, *unpublished*).

Study Species

A) B)

Figure II. Images of Study Species. (A) *Glossodoris vespa* (B) *Ceratosoma brevicaudatum*

Two genera of nudibranchs were analyzed in this study. *Glossodoris* nudibranchs feed exclusively on *Thorectidae* sponges, which possess sesterterpenes that are complex biomolecules with five isoprene branches (Manzo et al., 2007). The two most common sesterterpenes are

scalaradials and heteronemin, which can be found in *Glossodoris* species such as the *Glossodoris pallida* (Manzo et al., 2007). These nudibranchs rely primarily on mantle dermal formations to store their toxins, with scalaradials consisting of approximately 15% of the dry mass of the MDFs (Manzo et al., 2007). One unique characteristic of *Glossodoris* nudibranchs is that some species are able to transform selected dietary scalaranes into compounds that act as detoxifiers but also on some occasions, increase the toxicity of raw sponge chemicals to improve the effectiveness of predator deterrence (Rogers & Paul, 1991). The second genus comprises of the *Ceratosoma* nudibranchs that feed on *Dysideidae* sponges, which possess sesquiterpenes including furanosesquiterpenes (Rudman, 1984). Unlike any other nudibranch genera, *Ceratosoma* individuals not only have MDFs around the mantle rim, but they also have MDFs near the rhinophores and inside a mantle horn that is present near the gills and possesses a much higher concentration of mantle dermal formations and toxins than the rest of the mantle. Its purpose is most likely to be a primary target for potential predators and distract them away from essential appendages such as the gills and the rhinophores using contrasting coloration from the rest of the mantle (Mollo et al., 2005).

This study will explore two nudibranch species (*Glossodoris vespa*, *Ceratosoma brevicaudatum*) and analyze the chemical compositions in the mantle and the viscera in each. Comparing the unpalatability and toxicity levels of these chemicals will not only shed light on whether these two species, similar to others, sequester different toxins from the sponge tissues in their gut and their outer mantle rim but also allow us to test the hypothesis that they detoxify raw sponge compounds to avoid autotoxicity. Furthermore, I hypothesize that nudibranchs sequester the more toxic and more unpalatable toxins to the mantle and mantle dermal formations to deter

predators more effectively while allowing the less active compounds to pass through the viscera to decrease the threat of autotoxicity.

Materials and Methods

Animals

Specimens of the nudibranch *Glossodoris vespa* (18 individuals) were collected in Currimundi reef, Gneerings reef, and Mudjimba Island off the coast of Mooloolaba, Sunshine Coast, Queensland between the months of May and October 2016. *Ceratosoma brevicaudatum* (3 individuals) were collected at Nelson Bay Pipeline in March 2016. Collections were done on SCUBA, and specimens were stored at -20 degrees Celsius until chemical extract analysis was carried out. The *Palaemon* shrimp were collected at Moffatt beach, King beach, and Shelley beach by hand-netting in the intertidal zone on October 30th, 2016 and stored in aquaria at UQ until use.

Dissection and Extraction

Nudibranch individuals from each species were dissected and separated into viscera masses and mantle masses and put into separate 20 mL vials along with 10 mL of acetone. Using a scale and the previously recorded mass value of a dry vial, the wet weight of the mantles and viscera were measured and recorded. In addition, the volumes were recorded using acetone displacement in a graduated cylinder. The mantles and viscera were then put in separate beakers and sonicated for 4-5 minutes to break up cellular membranes, allowing the chemical toxins to seep into the surrounding acetone solution. The contents were allowed to settle and were subsequently filtered using cotton wool. The sonication-filtration procedure was repeated three

times or until the liquid in the respective beakers were clear, which signaled that most of all the chemicals from the nudibranch tissues were removed from the tissue. The filtered concentrate was put in a rotary evaporator until dry. The solution was then partitioned with diethyl ether, placed under a separator funnel and added to 5-7 mL of diethyl ether and MQ-H2O. After three inversions of the separator funnel, the clear organic solution was pipetted into a separate beaker along with sodium sulfate that acts as a drying agent to remove any traces of water. The darker solution consisting of lipids and tissue was poured back into the funnel, and the same procedure was repeated until there were no more traces of the organic liquid. The resulting clear liquid was subsequently pipetted into a final vial, leaving the sodium sulfate particles. The final vial was then placed under a nitrogen-releasing machine which removes all of the diethyl ether. The final result was crude nudibranch toxin extract. This entire process was completed for both the mantle and the viscera chemicals. Lastly, NMR spectroscopy was performed on the final extracts to ascertain what chemical compounds were present in both.

Brine Shrimp Toxicity Assays

The brine shrimp (*Artemia sp.*) eggs were put in a beaker filled with saltwater and aerated for about 30 hours, allowing them to hatch. The crude extract was diluted with dichloromethane (DCM) to create a stock solution at natural concentration and 3 replicates were used for each treatment (0.5 mL of stock solution, 0.25 mL, 0.025 mL, 0.0025 mL) for both the mantle crude extract and the viscera crude extract. There were also 3 control vials that did not have any toxins. Circular pieces of glass microfiber filter paper were fit at the bottom of each vial with the specific amount of toxins soaked and dried into it. Only DCM was added to the control vials. Then, 10 brine shrimp along with 2.5 mL of seawater were put in each vial and set with caps not

fully screwed on to allow the brine shrimp to respire but also to limit the amount of water that evaporates out of the vial. These vials were put under a light source and untouched for exactly 24 hours. The following day, brine shrimp were pipetted into a petri dish one vial at a time. To ensure that all the brine shrimp were transferred, the filter paper was washed with saltwater and poured into the petri dish as well. Under a microscope, the number of living, dead, and slow-moving brine shrimp were counted and recorded. Slow-moving shrimp were characterized as those that beat their swimming appendages at a rate of less than 60 beats per minute with location static, have reduced range of motion of appendages, or exhibit erratic beating at a rate of less than 70 beats per minute.

White-Gloved Shrimp (*Palaemon* sp.) Unpalatability Assays

Assay Preparation

Two plastic eight-compartment boxes (33 cm x 27 cm) were placed in each of the four tanks that were filled with saltwater with a salinity in the range of 1.020 and 1.023 PSU and aerated. Small holes were bore in all of the outer walls and in each of the dividers inside the box to allow water flow through the compartments. The water level was approximately half an inch below the top of the boxes. One white-gloved shrimp (*Palaemon serrifer*) was placed in each compartment that was labeled with a number from the range of 1 to 64. For a period of 2-3 days, the shrimp were fed with standard fish food that was colored green and then starved for 1 day. Using a random number generator, each shrimp was randomly assigned one of the nine conditions that corresponded to the four concentrations that were tested for both the mantle and viscera (0.25 mL, 0.125 mL, 0.0625 mL, 0.03125 mL) and a control. Each chemical dosage was 0.25 mL in total. Therefore, the 0.25 mL of mantle stock solution represented the natural

concentration, and the 0.125 mL of mantle stock solution with 0.125 mL of dichloromethane solvent to reach the 0.25 mL mark represented half of the natural concentration. Overall, 63 shrimp were used. In addition, the mantle toxin concentration and viscera toxin concentration were constant to ensure that the types of chemicals were the focus rather than the amount of chemicals: both stock solutions for the *Glossodoris vespa* were at a concentration of 31.03 mg/mL while those for the *Ceratosoma brevicaudatum* were at a concentration of 11 mg/mL.

Pellet Preparation

The following dry contents were measured to two decimal places using a scale: 25 mg freeze dry squid, 15 mg alginic acid, and 15 mg sand. Then 0.25 mL of DCM/stock solution combination (9 total combinations) were added to the dry contents and mixed. The mixture was then allowed to settle for 20 minutes to allow the DCM to evaporate, and then 0.25 mL of distilled water and 1 drop of red food coloring were added. The combination of wet and dry contents was mixed until gelatinous to ensure toxins were evenly spread within the mixture and using a scraper, the red mass was put into the back of the front end of a 10-mL syringe. The back end of the syringe was then used to push the mixture to the tip of the syringe. With the front end of the syringe in a petri dish of calcium chloride, the red mass was exuded slowly out of the syringe, where it solidified in the $CaCl_2$ solution. Finally, the long resulting tube was picked up with tweezers, dipped into distilled water to ensure all the $CaCl_2$ was removed from the pellets, and placed in a cleaned petri dish. This exact process was performed for each condition, except for the control condition. Because the control condition was used for both the seven shrimp in its condition group in the beginning and also as a method of ensuring that lack of hunger was not the cause of pellet rejection by the shrimp, all of the contents were quadrupled.

Assay Completion

After creating the red food pellets, each shrimp was fed its designated toxin concentration food pellet with tweezers and whether it accepted the pellet at the time of feeding or not was recorded. After fifteen minutes from the first feeding, the shrimps were checked for red spots in their transparent bodies, which determined whether they ingested the pellet or not. Small red spots, large red spots, and no red spots were recorded in a table. The same was done for all shrimps at the thirty-minute mark and the one-hour mark. If any shrimp were not observed to eat the pellet during the entire experiment, they were fed a control pellet (no toxins) to ensure that their rejection of the pellet was due to the toxins rather than a lack of hunger. If the shrimp rejected the control pellet, it was omitted from the study.

Data analysis procedure

Data was recorded in Excel spreadsheets, and graphs were created in the GraphPad 7 Prism program. In addition, LD_{50} values were calculated for each brine shrimp condition using Abbott's formula on Excel. This was done to account for natural mortality of the brine shrimp (observed in the control samples); after the results were altered, the LD_{50} was calculated. The LD_{50} is the amount of toxins required for a 50% mortality rate of the brine shrimp. This value was calculated using the data and graphs via interpolation of sigmoidal curves. Also, ED_{50} values were calculated for each *Palaemon* shrimp condition, which represented the amount of toxins required for a 50% food pellet rejection by the shrimp. Chemical analysis of the mantle and viscera extracts were performed by NMR spectroscopy machines provided by the University of Queensland before the assays were started.

Results

Compounds detected by the NMR

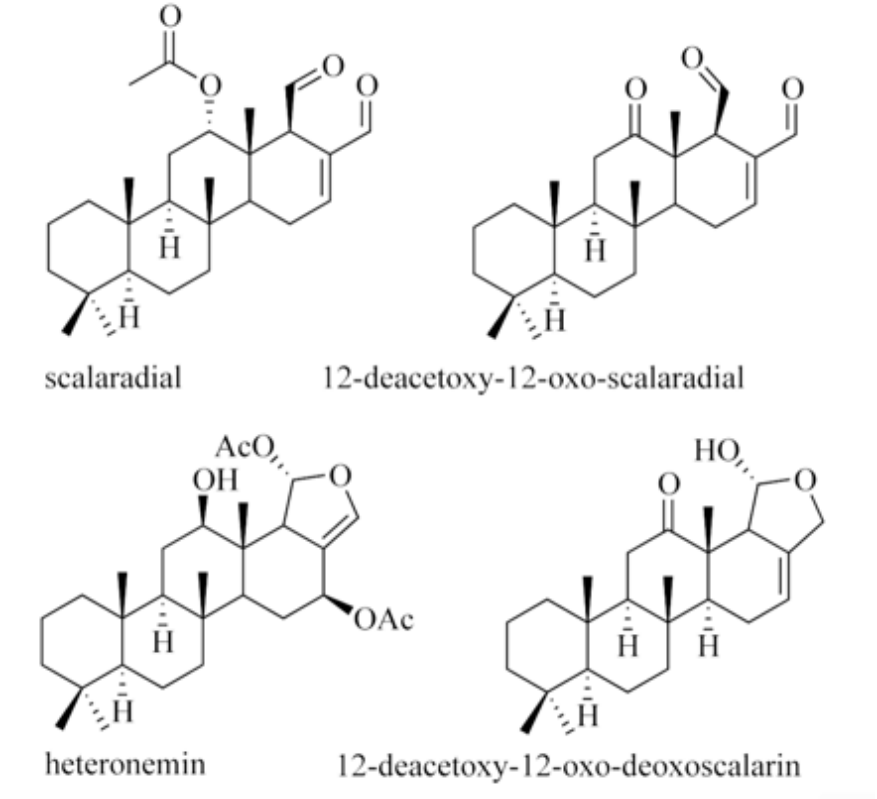

Figure III. Chemical structures of sesterterpene molecules in the mantle and viscera of *Glossodoris vespa*. The locations of these compounds are shown in **Table I**.

Table I. Identification and location of chemical compounds in the mantle and viscera of the *Glossodoris vespa*. "P" signifies the presence of the compound and "NP" signifies non-presence.

Species	Location	Scalaradial	12-deacetoxy-12-oxo-scalaradial	12-deacetoxy-12-oxo-deoxoscalarin	Heteronemin
G. vespa	Mantle	P	P	P	NP
	Viscera	NP	NP	NP	P

The NMR spectroscopy image showed that the mantle and the viscera of the *Glossodoris vespa* consisted of different chemical compounds. Through organic analysis, three distinct scalaradial compounds were found in the mantle: scalaradial, 12-deacetoxy-12 oxo-scalardaial, 12-deacetoxy-12-oxo-deoxoscalarin. However, only one distinct compound was found in the viscera and identified as heteronemin.

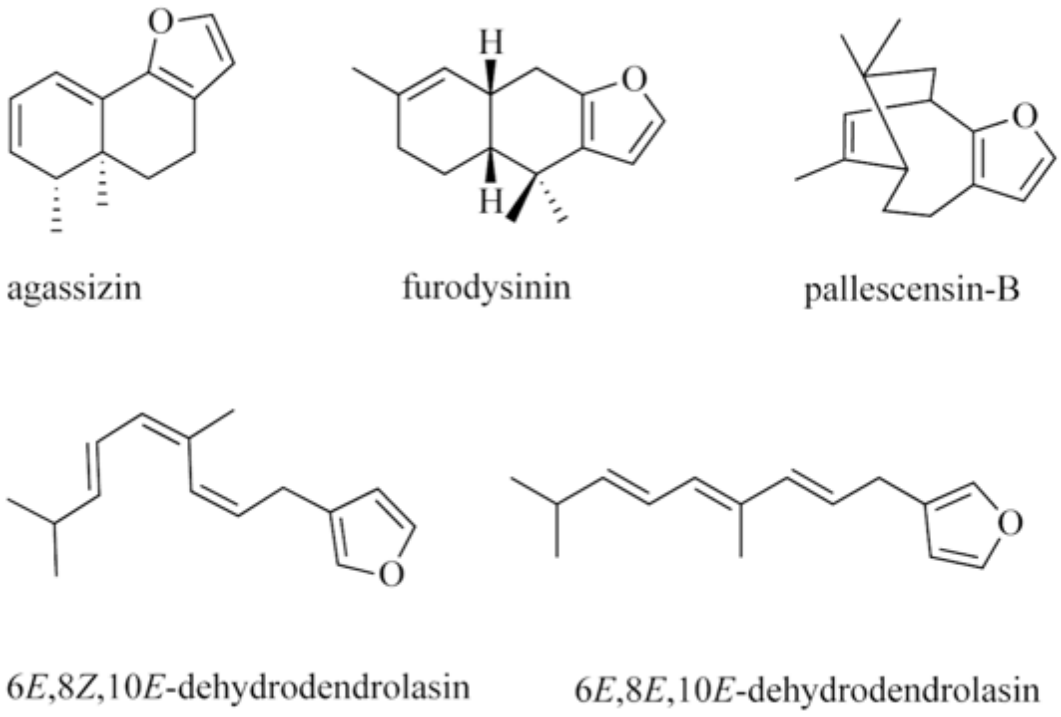

Figure IV. Chemical structures of sesquiterpene molecules in *Ceratosoma brevicaudatum*. The locations of these compounds are shown in **Table II**.

Table II. Identification and location of chemical compounds in the mantle and viscera of the *Ceratosoma brevicaudatum*. "P" signifies the presence of the compound and "NP" signifies non-presence.

Species	Location	Agassizin	6E,8E,10E-dehydro-dendrolasin	6E,8Z,10E-dehydro-dendrolasin	Pallescensin B	Furodysinin
C. Brevi-	Mantle	P	P	P	P	P
	Viscera	P	P	NP	P	NP
	Horn	P	P	P	P	P

The NMR spectroscopy image showed that the compounds in the viscera were also present in the mantle and the mantle horn, but the mantle held one additional sesquiterpene while the mantle horn held two. Agassizin, 6E, 8E, 10E – dehydrodendrolasin, and Pallescensin B were found in the mantle, the mantle horn and the viscera. However, furodysinin was found to be a major compound only in the mantle and mantle horn while 6E, 8Z, 10E – dehydrodendrolasin was only found in the horn.

Palatability Results

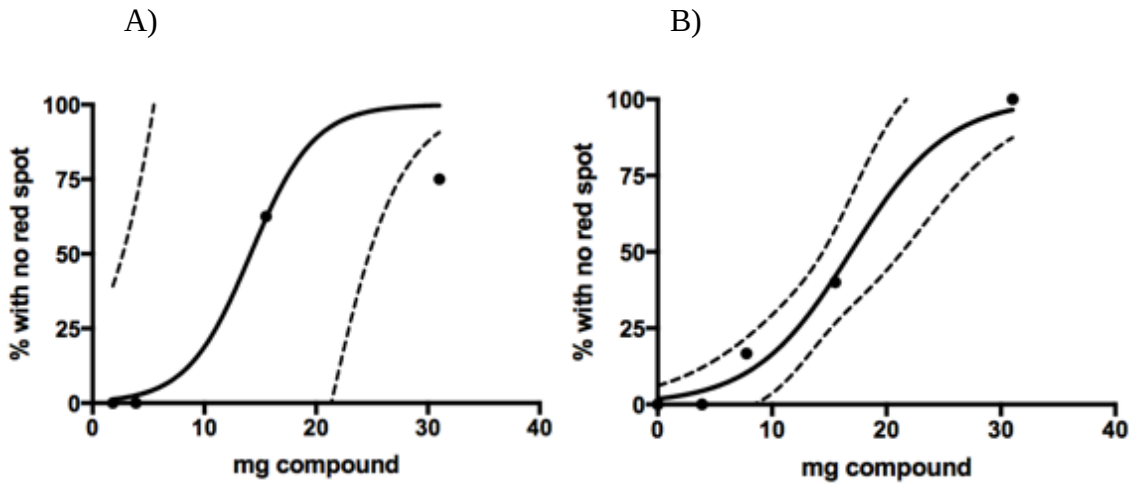

Figure V. Unpalatability results from *Palaemon* shrimp assays using A) *G. vespa* mantle extract and B) *G. vespa* viscera extract. The x-axis represents the amount of extract used and the y-axis represents the corresponding percentage of food rejection by the shrimp. % with no red spot (y-axis) is synonymous with % of pellets rejected because of toxins present in pellets.

Palatability assays were run for the mantle and viscera extract for the *Glossodoris vespa* using *Palaemon* shrimp, and the results are shown in **Figure V.** For the assays using viscera toxins, the corresponding ED_{50} value was 14.14 mg with the upper limit as 24.75 mg and the lower limit as 2.71 mg using 95% confidence intervals. For the assays using mantle toxins, the corresponding ED_{50} value was 16.89 mg with the upper limit as 21.38 mg and the lower limit as 14.4 mg while also using 95% confidence intervals.

A) B)

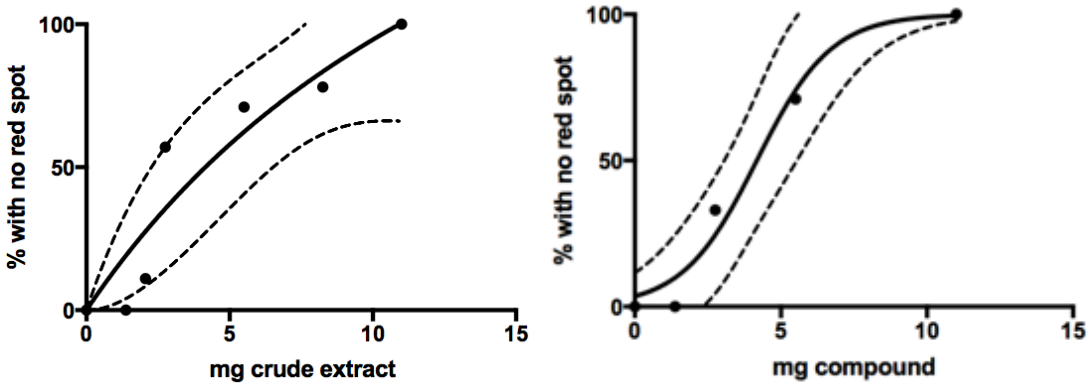

Figure VI. Unpalatability results from *Palaemon* shrimp assays using A) *C. brevicaudatum* mantle extract and B) *C. brevicaudatum* viscera extract. The x-axis represents the amount of extract used and the y-axis represents the corresponding percentage of food rejection by the shrimp. % with no red spot (y-axis) is synonymous with % of pellets rejected because of toxins present in pellets.

The mantle and viscera palatability results for *Ceratosoma brevicaudatum* are shown in **Figure VI**. For the assays using viscera toxins, the ED_{50} value was 4.18 mg with the upper limit as 5.55 mg and the lower limit as 3.06 using 95% confidence intervals. For the assays using mantle toxins, the ED_{50} value was 2.67 mg with the upper limit as 3.31 mg and the lower limit as 2.04 mg using 95% confidence intervals.

Toxicity Results

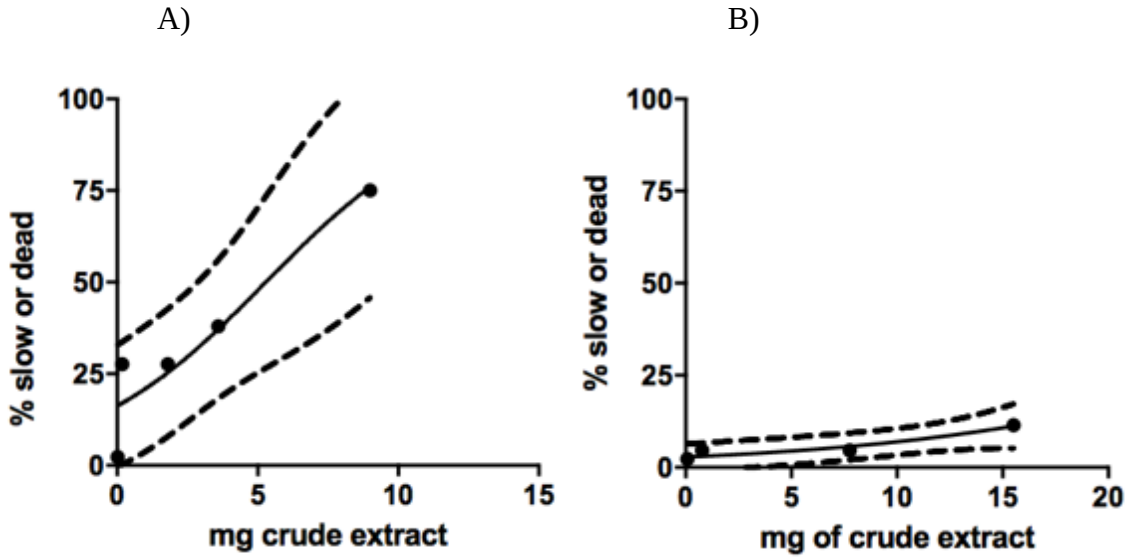

Figure VII. Brine shrimp toxicity assays for the *Glossodoris vespa* using A) mantle extract and B) viscera extract. 95% confidence intervals are shown as the dotted lines. The x-axis shows the amount of toxins used while the y-axis shows the corresponding percentages of brine shrimp that were slow-moving or dead after a period of 24 hours of exposure to the nudibranch toxins.

The brine shrimp toxicity results (dose responses) for the mantle and viscera of *Glossodoris vespa* are shown in **Figure VII.** For the assays using mantle toxins, the LD_{50} value was 5.29 mg of the compounds with no calculated upper limit and the lower limit as 2.86 mg using 95% confidence intervals. The LD_{50} value for the assays using viscera toxins was not able to be calculated because no experimental condition resulted in at least 50% brine shrimp mortality after 24 hours of exposure to the toxins.

A) B)

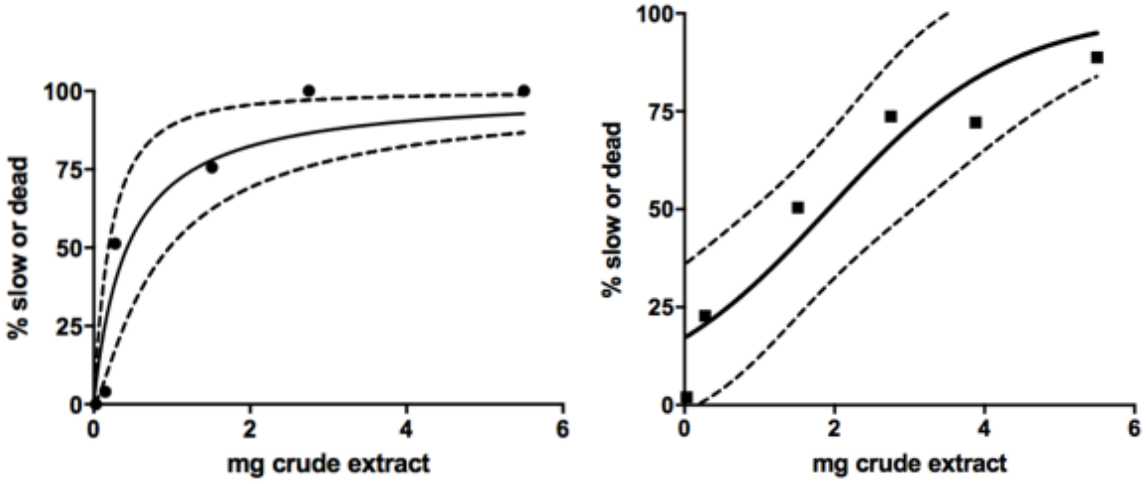

Figure VIII. Brine shrimp toxicity assays for the *Ceratosoma brevicaudatum* using A) mantle extract and B) viscera extract. 95% confidence intervals are shown as the dotted lines. The x-axis shows the amount of toxins used while the y-axis shows the corresponding percentages of brine shrimp that were slow-moving or dead after a period of 24 hours of exposure to the nudibranch toxins.

The toxicity results (dose responses) for the mantle and viscera of *Ceratosoma brevicaudatum* are shown in **Figure VIII**. For the assays using mantle toxins, the LD_{50} value was 0.84 mg of the compounds with the upper limit as 1.81 mg and the lower limit as 0.20 mg using 95% confidence intervals. The LD_{50} value for the assays using viscera toxins was 1.92 mg of the compounds with the upper limit as 3.04 mg and the lower limit as 0.90 mg using 95% confidence intervals.

Discussion

1. Hypothesis that nudibranchs detoxify raw chemical compounds from sponges is refuted while the hypothesis that they are still eventually able to accumulate toxic compounds in the mantle is supported

One of the primary goals of this study was to assess the manner in which certain species of nudibranchs store toxic compounds from their sponge diets. As stated previously, the first hypothesis involved nudibranchs detoxifying raw sponge toxins to avoid the possibility of autotoxication of the nudibranch as the compounds pass through and are stored in the viscera and mantle. Data from the brine shrimp and *Palaemon* shrimp assays revealed the opposite: there was, in fact, an *increase* in toxicity between compounds in the viscera and the mantle of the same species. For the *Glossodoris vespa,* there was a 62% increase in mortality at natural concentrations from the viscera and the mantle and a 27% increase for the *Ceratosoma brevicaudatum.* In addition, the LD_{50} value for the *Glossodoris vespa* mantle was 5.29 mg while the LD_{50} for its viscera was much higher than 15 mg, which shows that a much smaller amount of mantle toxins is required for a 50% mortality rate when compared to viscera toxins (**Fig VII**); the *Ceratosoma brevicaudatum* also showed the same result with an increase of 1.08 in LD_{50} value from mantle to viscera (**Fig VIII**). This trend was also found in various *Chromodoris* species including *C. elisabethina* and *C. magnifica;* the mantles contained Latrunculin A, a potent chemical that at natural concentration, led to 100% mortality in brine shrimp assays while the viscera possessed two more benign compounds that exhibited less than 25% mortality (Cheney et al., 2016). The second hypothesis stated that to maximize predator deterrence, nudibranchs have evolved a complex mechanism to store more toxic compounds in the mantle than in the viscera. Olfaction and taste of chemicals in the water are believed to have evolved to allow marine organisms to not only

find nutrient sources but also to avoid toxins (Lunceford & Kubanek, 2015). Therefore, more toxic compounds released by the nudibranch would lead to greater chemical deterrence. The similarity of unpalatability levels between the mantle and viscera for both nudibranch species proved to be an interesting finding (**Fig V, Fig VI**); although we expect a strong correlation between unpalatability and toxicity, there are no clear associations between the two amongst various species (Glendinning, 1994). Some were unpalatable yet non-toxic such as the *Goniobranchus* nudibranchs while others were toxic yet palatable such as the *Mexichromis* (Wägele et al., 2006). Overall, though, these results support the hypothesis in which nudibranchs prioritize predator deterrence over the threat of autotoxication. Further research should be focused on how nudibranchs are able to withstand the deleterious effects of these toxins and especially in the mantle, store them in such high concentrations. Even in the viscera, the *Ceratosoma brevicaudatum* exhibited a 73% mortality at natural concentrations with an LD_{50} value of 1.92 mg (**Fig VIII**). Therefore, even in the dietary tract near vital organs, the toxicity levels of these chemical compounds are still quite high. One possible explanation may involve the development of enlarged vacuoles inside the cells of digestive tract tissue that allows separation between organelles and the toxins. Similar to those in mantle dermal formations, these vacuoles would essentially allow the nudibranch to not only ingest these compounds but also to store them in large quantities in the viscera (Wägele & Klussmann-Kolb, 2005).

2. There is evidence of both selective sequestration and chemical modification of raw sponge chemicals

There are two methods of creating a more toxic and/or more unpalatable mantle covering, and NMR spectroscopy of the chemical compounds in each species shows that both may

possibly be used. For the *Glossodoris vespa*, there were records of sesterterpenes, which are compounds commonly found in *Thorectidae* sponges. In the mantle, the predominant compound was scalaradial along with two other sesterterpenes while the viscera had high concentrations of heteronemin (**Table I**). Although this has not been proven, *Glossodoris vespa* most likely accrues its mantle toxins through selective sequestration. *Thorectidae* sponges that are the primary feeding target for *Glossodoris vespa* individuals are known to contain heteronemin and scalaradial derivatives, which leads to the conclusion that scalaradial and scalaradial derivatives are selectively sequestered by the nudibranch to its mantle for increased predator deterrence. However, the fact that not all *Thorectidae* sponges contain scalaradials and the structural similarity between the mantle and viscera toxins introduces the possibility of these nudibranchs chemically transforming less toxic compounds such as heteronemin into more toxic compounds such as 12-deacetoxy-12 oxo-scalaradial and moving them to the mantle dermal formations. The difference in structures lies in the absence of one closed ring structure (in contrast to the open ring structure of the three mantle compounds) and a dialdehyde functional group. This behavior was also observed in the species *Cadlina luteomarginata*, where compounds such as pallescensin A and furodysinin were not found in the sponge they feed on, *Leosella idia* (Pawlik, 1993). Therefore, those two compounds must have been biosynthesized by the nudibranch itself. In terms of the *Glossodoris vespa,* future studies may involve using radioactive carbons to label heteronemin compounds found in *Thorectidae* sponges and tracking those carbons to decipher whether the heteronemin was transformed into another compound or not in the viscera. Studies with *Dendrodoris limbata* involved using carbon isotope markers to label mevalonic acid to prove that these nudibranchs are able to biosynthesize their own compounds without using sponge chemicals; I believe that this mechanism could be used to ascertain whether *Glossodoris*

nudibranchs are able to chemically modify sponge chemicals into ones that are more toxic and therefore more effective against predators (Fontana et al., 2000). On the other hand, the *Ceratosoma brevicaudatum* exhibits the possibility of selective sequestration that may contribute to the higher toxicity in this species. All of the chemical compounds found in both the mantle and viscera of the nudibranch can also be found in the *Dysideidae* sponges that they feed on, where the more toxic and more unpalatable compounds are in the mantle. This species stored all compounds in the mantle that were also found in the viscera. This contrasts with *Glossodoris vespa*, which only had one compound in the viscera and did not store that compound in the mantle. Additional evidence for this comes from the fact that the mantle is the only part of the nudibranch that shows furodysinin and 6E,8Z,10E- dehydrodendrolasin in the NMR spectroscopy results, which leads us to believe that both compounds may have been selectively concentrated from previous sponge meals and cause the increase in toxicity from the viscera to the mantle (**Table II**). Furthermore, both compounds can be found in the *Dysideidae* sponges; these points lead to the probable selective sequestration mechanism that the *Ceratosoma brevicaudatum* employs. Finally, only *C. luteomarginata* and *Dendrodoris grandiflora* are known to manifest both selective sequestration and chemical modification, so further research must be devoted to finding if there are more nudibranchs that exhibit this dual mechanism of sequestration (Kubanek et al., 2000).

3. Toxin concentration plays an important role with the development of mantle dermal formations and the mantle horn of *Ceratosoma brevicaudatum*

Not only are the levels of unpalatability and toxicity important for predator deterrence but concentration of the chemical compounds in the mantle are vital as well. As mentioned earlier, most nudibranch species carry mantle dermal formations on the mantle rim that consist of cells

carrying relatively high volumes of sponge toxins. These are positioned in such a way around the mantle rim and also colored differently than the rest of the mantle to maximize the probability of predator exposure to the toxins when an organism attempts to bite the nudibranch (Marin et al., 1997). In an experiment with the species *Glossodoris pallida*, experimental removal of MDFs along the outside of the mantle corresponded with increased rates of predation towards the nudibranch, which highlights the importance of these specialized cells (Avila & Paul, 1997). While this is the case for the *Glossodoris vespa*, the *Ceratosoma brevicaudatum* not only has MDFs along the mantle rim but also has MDFs at a higher concentration in the mantle horn that is colored differently than the mantle. The mantle horn has been observed as a sacrificial appendage which was frequently damaged by predators but decreased the risk towards vital parts of the nudibranch; this was seen in experiments with *C. trilobatum* and *C. gracillimum* (Mollo et al., 2005). In the *Ceratosoma brevicaudatum* extracts, the toxin concentration in the mantle horn was 90 mg/mL while those of the mantle and the viscera were 5.2 mg/mL and 11 mg/mL, respectively; the mantle toxin concentration was lower than that of the viscera because the toxins are mainly found in the mantle dermal formations, which comprise a small proportion of the overall surface area of the mantle. However, the fact that the mantle horn is known to hold a higher concentration of toxins and MDFs than the mantle is a possible reason why the *Ceratosoma brevicaudatum* mantle is more toxic than the *Glossodoris vespa* mantle with the two highest concentrations (based on natural *viscera* concentrations) at 100% rate of mortality in the brine shrimp assays for the *Ceratosoma brevicaudatum* while the corresponding rates for the *Glossodoris vespa* were 75% and 30% respectively. Further research can be focused on finding additional roles of the MDFs such as possibly allowing nudibranchs to transform sponge toxins in the MDFs rather than the viscera and transporting them to the MDFs; this would decrease the

risk of autotoxicity. In addition, how do nudibranchs transport the toxins from the viscera to the mantle? Do the MDFs have certain chemoreceptors that aid in selective sequestration: accumulating more toxic and more unpalatable compounds in the mantle? In addition, because it is proven that *Ceratosoma brevicaudatum* individuals accumulate more toxic compounds in the mantle, future studies should address how the nudibranchs are able to gauge the relative toxicity of multiple compounds to perform selective sequestration of sponge chemicals.

4. Proof of nudibranchs' multimodal defense mechanism

Finally, this data supports the notion that nudibranchs' aposematic characteristics are multimodal in nature. These organisms have evolved to not only possess bright coloration as a warning signal to potential predators but to also be unpalatable and even toxic by using sponge toxins from their diet. Whether it be selectively sequestering and increasing the local concentration of more toxic compounds in the mantle or possibly transforming less toxic sponge compounds into more toxic compounds, nudibranchs have utilized a complex defense mechanism that presents scientists around the world exciting questions to answer.

Conclusion

This study showed how complex nudibranch defense mechanisms are, and how much there is still to learn about these incredible invertebrates. The two species that were examined exhibited a variety of different kinds of toxins, different ways of using them when threatened by a potential predator, and two possible ways of accumulating the most toxic and unpalatable compounds in the mantle. Another primary finding of this study is that nudibranchs are capable of modifying the chemical arsenal of secondary metabolites from their sponge diet to increase

the effectiveness of predator deterrence. Both species showed differences in the toxin composition of the mantle and viscera that were either present or not present in the sponges that they feed on. Finally, the ability to accrue a multitude of toxins both in their viscera and mantle and the way they are able to withstand toxicity in their bodies are topics of prime importance in future research. Overall, understanding transport mechanisms, selective sequestration, and how they are able to chemically modify sponge toxins will help us piece together the evolutionary history of what are seemingly primitive organisms are actually an intriguing model system to study chemical and anti-predator defenses.

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Image Citations

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