Effects of Abiotic and Biotic Stress on Antimicrobial and Cytotoxic Properties of *Haliclona sp.*

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ABSTRACT

Haliclona sp. is a marine sponge known for its ability to harbor a diverse set of secondary metabolites applicable to antimicrobial and anticancer pharmaceutical development. However, this essential Porifera species may face future issues as a result of biotic and abiotic changes within their environment due to climate change. This study aimed to understand how predation stress and rising temperatures affect the regulation of secondary metabolites by measuring the sponges' cytotoxic and antimicrobial properties. Research in the equatorial region of Mo'orea, French Polynesia, revealed that manipulating temperature and predation stress did not ultimately cause differences between the antimicrobial or cytotoxic properties of different treatments. From this, it is assumed that neither an increase nor decrease in the production of secondary metabolites of *Haliclona sp.* occurred. Comprehensive antimicrobial and cytotoxic properties were observed from extracts of all treatments, and thus, the potential for *Haliclona sp.* to be used as a basis for medicinal use is not dependent on temperature and predation variables. Though methodologies of past research guided this study, uncontrolled variables and experimental limitations, like sponge life stage and definite metabolite concentrations, could have affected the results. Additionally, due to equipment limitations, molecular changes in secondary metabolite expression within the sponges from before and after treatment went unmeasured. This research indicates that the tropical marine sponge *Haliclona sp.* maintains its potential to serve as a pharmacological asset despite the impacts of climate change.

Key words: Porifera; marine sponges; Haliclona sp.*; antimicrobial properties; cytotoxicity; secondary metabolites; pharmacology; predation pressure; temperature stress; chemical defense*

Introduction

Porifera, or sponges, are sessile organisms found worldwide - from polar regions to the tropics, freshwater to salt water. They have evolved over 750 million years and diversified into about 9,490 species (Yang *et al.* 2022). Because sponges spend most of their life stationary, they have evolved to use secondary metabolites as their primary defense mechanism against predation and infection (Radjasa *et al.* 2007, Pawlik *et al.* 2013). Understanding metabolite diffusion patterns of sponges exposed to varying biotic and abiotic factors in their natural environment could be crucial for their conservation as climate change increasingly impacts the marine ecosystem (Luter and Webster 2017). Unfortunately, their ability to adapt to climate change is largely unknown and may vary highly among species (Stevenson *et al.* 2020). Past research on global sponge distribution noted that only about seven percent of all sponge research has focused on environmental impacts and conservation efforts, emphasizing our need to explore sponge acclimation dynamics as they relate to widescale environmental changes (Bell *et al.* 2015). Moreover, preserving these ancient metazoans is critical for society as their naturally derived chemical excretions have led to the synthesis of numerous pharmaceutical products. These derived chemicals have properties that range from antitumor and antimicrobial to antifouling and anti-inflammatory (Sipkema *et al.* 2005).

Latitudinal variations of sponge metabolite studies have shown tropical sponges present a much more extensive array of chemical defenses than their temperate counterparts (Ruzicka and Gleason 2008). This allomone diversity is primarily believed to occur because of higher predation pressures in equatorial regions (Bakus and Green 1974). Additionally, rising temperatures have been shown to increase sponge filtration (Leveque-Eichhorn 2020) and regeneration rates (Runzel 2016), which may influence secondary metabolite excretion.

The combination of an exponentially growing global population and the overuse of antibiotics leading to the emergence of numerous multidrug resistant (MDR) pathogens means the development of new drugs is essential to the future health of our society. In 2019, 7.7 million people died from bacterial pathogens, and in 2022, the use of antimicrobial drugs had significantly increased compared to 2019 and 2020; such shocking statistics are a quantifiable measure of our need for novel medicines (Ikuta *et al.* 2022, "Antimicrobial consumption in the EU/EEA (ESAC-Net) - Annual Epidemiological Report for 2022" 2023). With the ever-increasing demand for innovative drugs, the marine habitat holds latent potential for pharmaceutical exploration (Malve 2016). Because sponges rely heavily on their secondary metabolites for survival, these organisms have extensive chemodiversity. Marine sponges produce antimicrobial peptides anthropogenically used as a source for antibiotics, glycolipids capable of inhibiting viruses such as HIV, and a plethora of other bioactive compounds that have proven effective against MDR pathogens (Varijakzhan et al. 2021). *Haliclona sp.* specifically has been studied as an antifungal treatment for phytopathogenic fungi, as well as common pathogenic yeasts like *Candida albicans* and the filamentous fungus *Aspergillus* (Wattanadilok *et al.* 2007, El-Amraoui *et al.* 2013, El Amraoui *et al.* 2014). Extracts from individual *Haliclona* sponges (Porifera) have been utilized as in-vitro antimicrobial agents against strains such as *Staphylococcus aureus, Vibrio* *parahaemolyticus* - bacteria associated with gastrointestinal illness, and *Aeromonas hydrophila* - a water contaminate bacterium, which generates confidence for the evolution of secondary metabolites as an unorthodox antibacterial agent (Radjasa *et al.* 2007). Additionally, employing the antimicrobial properties of sponges may indirectly lower rates of certain cancers as some bacterium and fungi can cause "persistent infection, evasion of the immune response, chronic inflammation leading to continued cell proliferation, and an increased risk of oncogenic transformation" (Yusuf *et al.* 2023).

Though these antimicrobial properties can prevent infections that induce cancer, sponges also maintain immediate anticancer properties. Cancer treatments generally fall into three categories: cytotoxic drugs aiming to kill dividing cells, targeted therapeutics using enzyme inhibitors to disrupt cell processes, or immunotherapy (Bailly et al. 2020). Around 60% of all anticancer drugs are from natural products, and Porifera contributes to about 30% of all the natural marine compounds identified. Sponge-derived Food and Drug Administration-approved drugs "have been shown to reduce metastatic breast cancer, malignant lymphoma, and Hodgkin's disease." (Calcabrini et al., 2017). Peloruside A, sorbicillactone A, sorbicillactone-B, Cytarabine, Vidarabine, Fascaplysin, and Cladribine are all examples of cancer drugs originating from tropical marine sponges (Malve 2016, Calcabrini *et al.* 2017). Abiotic and biotic stress on this reservoir of bioactive chemicals essential for drug development is largely understudied.

Understanding how temperature and predation affect metabolically rich Porifera provides imperative knowledge for conservation measures with concern for pharmaceutical development. This holds especially true in equatorial regions known for metabolite diversity. *Haliclona sp.*, one of the most abundant species on Mo'orea, an Eastern Pacific island in Polynesia, belongs to an order of sponges "known as the most prolific source of secondary metabolites among sponges" (Lee *et al.* 2021). Preserving the profuse diversity of *Haliclona sp.* (order Haplosclerida) metabolites in equatorial regions will allow research into novel antimicrobial and anticancer drugs to persist through time. Like other tropical sponges, *Haliclona* "synthesize a wide range of metabolites ranging from class terpenes, such as sterols, sesquiterpenoid quinols, glycosphingolipids, and alkaloid bioactive secondary metabolites" (Varijakzhan *et al.*, 2021) which all hold promising therapeutic potential.

This study's objective is to evaluate how the cytotoxic and antimicrobial properties of the marine sponge *Haliclona sp.* are affected when abiotic and biotic factors are manipulated. I exposed sponges to temperature and predation stress treatments, followed by a brine shrimp toxicity test to unveil potential differences in the cytotoxic properties and an antimicrobial zone of inhibition test to understand the variances of antimicrobial properties between sponges of different treatments. These tests are relevant as temperature and predation are common environmental stressors likely to influence metabolite secretion and, thus, increase prophylactic properties. I predict that *Haliclona sp.* exposed to higher temperatures will exhibit higher antimicrobial and cytotoxic properties than those exposed to lower temperatures. Additionally, I predict that *Haliclona sp.* exposed to predation will exhibit higher antimicrobial and cytotoxic properties than individuals not exposed.

Methods

This research sought to investigate how variation in biotic and abiotic factors in the marine environment influence the expression of secondary metabolites in *Haliclona sp.* sponges. The study took place on the island of Moorea, French Polynesia between 22 September and 15 November, 2023. Thirty sponges were collected from three sites in Cook's Bay (Fig. 1). The water temperature, depth, and time of obtainment were recorded at the time of specimen collection. The substrate to which the sponges were attached was also noted. After taking environmental data, the sponges were carefully placed into plastic bags filled with saltwater and closed before being taken from the ocean, as sponges are extremely sensitive to air exposure. The sponges were kept alive in a two foot long, one foot wide, and one foot deep tank within the UC Berkeley Gump Station water tables before experimentation proceeded. Holes on either end of the tank allowed for water to consistently flow through as the ocean water was pumped into the water table.

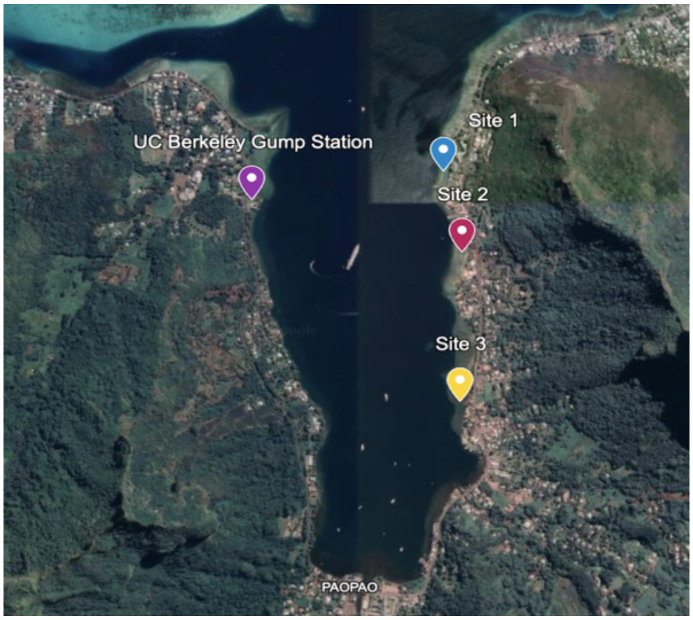

Figure 1: Google Earth (2019) image detailing the location of the three study sites located on Cook's Bay, Mo'orea.

Predation Experiment

After the sponges were left to acclimate to the water table for twenty-four hours, two sponges were moved into two separate tanks for the predation experiment. Both tanks were submerged in the water table and allowed water to circulate through. One sponge was placed in a 6 inch by 1 foot tank with ten nudibranchs of the species *Chromodoris lochi*. Nudibranchs are natural predators of sponges, and since the species *Chromodoris lochi* is abundant in Mo'orea, it is an appropriate predator. The other sponge was kept in a tank of the same dimensions without any nudibranchs. The sponges were left in their respective tanks for 48 hours to acclimate to the new environment, providing time to potentially upregulate their secondary metabolite expression in response to the stressor. After 48 hours, the sponges were removed from the tank, and the extraction of secondary metabolites was performed. This experiment was repeated six times and used twelve sponges in total.

Temperature Experiment

Three sponges were used in each temperature experiment. The sponges were taken from the water tables after one day and moved into three separate temperature regulated tanks. Each sponge was kept in an aerated 2-foot by 1-foot by 2-foot tank with heaters that kept constant saltwater temperatures of 26°C, 29°C, and 32°C, respectively. Any variance in temperature within each of the tanks over the entire 10-week study period was recorded and analyzed in Excel using three ElitechLog Temperature Data Loggers. After 48 hours, the sponges were removed from the tanks for secondary metabolite extraction. This experiment was repeated six times and used a total of eighteen *Haliclona sp.* individuals.

Extraction of Secondary Metabolites

After the experimental treatments, sponges were dried with blotting paper and dropped into 20 mL of 70% ethanol for two minutes. Upon removal, they were patted dry with blotting paper and then macerated using a mortar and pestle. The residual thick purple crude extract was poured over a petri dish lined with Whatman Grade 1 filter paper and left for 24 hours to evaporate the residual seawater and ethanol. Next, the extracts were scraped off the filter paper and moved into 15 mL Falcon tubes, weighed, and diluted in a ratio of 0.1 g crude extract to 1 mL distilled water. These extracts were kept in a -18°C freezer until used in the toxicity and antimicrobial tests.

Antimicrobial Test

Antimicrobial tests were performed by measuring the diameter of a zone of inhibition (ZOI) from sponge extracts on an agar plate covered in a fungal lawn. To culture the fungus, agar plates were made by mixing 300 mL demineralized water with 7.5 g of agar powder. This mixture was microwaved for three minutes, and 20 mL was poured into each petri dish with 1.25 g of plant material. Twelve petri dishes were utilized for testing – six for the temperature treatments and six for the predation treatments. These plates were left to grow mold for 14 days in the Gump Station Dirt Lab. To standardize the antimicrobial test, circular 7 mm filter paper disks soaked in sponge extracts were added to each agar plate an inch away from each other. This filter paper was left on the fungal lawn for 24-hours. Filter paper soaked in distilled water and ethanol were used as negative and positive controls, respectively. After 24-hours, the diameter, in millimeters, of mold killed around the filter paper was recorded. The antimicrobial test was conducted, with two controls, for each of the six temperature and six predation treatments, totaling twelve antimicrobial tests.

To visually analyze the antimicrobial data between secondary metabolites extracted from sponges of different treatments, a box plot utilizing data from the six replicate treatments was produced. This was done in R Studio using the tidyverse and ggplot packages. From there, a one-way ANOVA followed by a Tukey HSD test was used to determine whether there were any significant differences between their mean ZOIs.

Toxicity Test

Toxicity tests were performed using brine shrimp. Brine shrimp were hatched 48 hours prior to the experiment by adding 1 teaspoon of brine shrimp eggs into 1L of 20°C aerated seawater. From the hatched brine shrimp, 100 uL were placed into 15 mL Falcon tubes. Saltwater was then added until there was 5 mL of liquid in the 15 mL Falcon tube. The toxicity test was conducted for replicates, six with three temperature treatments, six with two predation types, and all replicates of both treatment types had a negative control of distilled water. The shrimp were counted by holding the falcon tubes up to the light and using the naked eye to identify individuals suspended in the water column. There was an initial count at T0 of brine shrimp before 100 uL of sponge extract (which contains the secondary metabolites) were added to each tube with a p1000 micropipette. Previous studies have shown that 100 uL kills brine shrimp fast enough to measure the survival curve over a 24-hour period (Perrin 2021). After the extract was added, the living shrimp were then counted twelve times over 24-hours.

For data analysis, I constructed a non-linear regression curve mapping the means of the six replicates for each treatment type to visualize the survival of brine shrimp over the 24-hour period. This non-linear regression curve took time into account by using an asterisks between time and treatment indicating an interaction between the two variables. Analysis was done using tidyverse and sjplot in R Studio. A one-way ANOVA followed by a Tukey HSD test was done at the 12-hour and 24- hour time points to see if any significant differences between treatments had emerged. A logistic regression model was also produced to compare the interactions solely between the three temperature experimental treatments and the two predation experimental treatments without the controls.

Results

The collection of thirty *Haliclona sp.* was taken from an average depth of 1.1 meters and an average ocean temperature of 28.4°C. *Haliclona sp.* sponges were then put into predation tanks and temperature tanks. For the temperature experiment, an ElitechLog Temperature Data Logger instrument, was able to take the temperature of the water baths every five minutes from October 12th to November 1st, 2023. The average water bath temperatures were 26.9°C, 28.9°C, and 31.6°C. The subsequent analysis (i.e. graph labels and statistical tests) references 26°C, 29°C, and 32°C data, as those were the intended temperature treatments, but are correlated to the temperatures mentioned previously. Additionally, it should be noted that after two temperature replicate experiments, the *Haliclona sp.* immersed in the 31.6°C tank became discolored and malodorous.

Temperature Toxicity Test

The percentage of brine shrimp that were alive throughout the entire 24-hour period initially looked to vary as a function of the treatment assigned. The *Haliclona sp.* sponges treated with the three temperature conditions seemed to deviate from the negative control at a similar rate after about 12 and 24 hours. However, an ANOVA and Tukey HSD test indicated that two of the three temperature treatments did not significantly differ from the negative control (F-value: 3.26, $p > 0.05$) at the 12- hour point. The 26°C sponge treatment, however, was statistically different from the negative control curve at the 12-hour mark ($p=0.047$).

The statistical tests replicated at the 24-hour threshold indicated that the three temperature curves, 26°C, 29°C, and 32°C, all significantly differed from the negative control (F-value: 30.35, $p < 0.05$, Fig. 2). Thus, the rate at which the brine shrimp were dying between the control and treatments only significantly differed after the 12-hour mark and not throughout the entirety of the experiment.

A logistic regression model on the experimental data, with time considered, determined that there was no statistically significant difference between the means of the three temperatures (Fig. 3). Setting the temperature treatment of 26°C as a baseline, the logistic regression indicated that the mean temperature treatment of 29°C and mean treatment of 32°C provided insignificant p-values of 0.81 and 0.75, respectively.

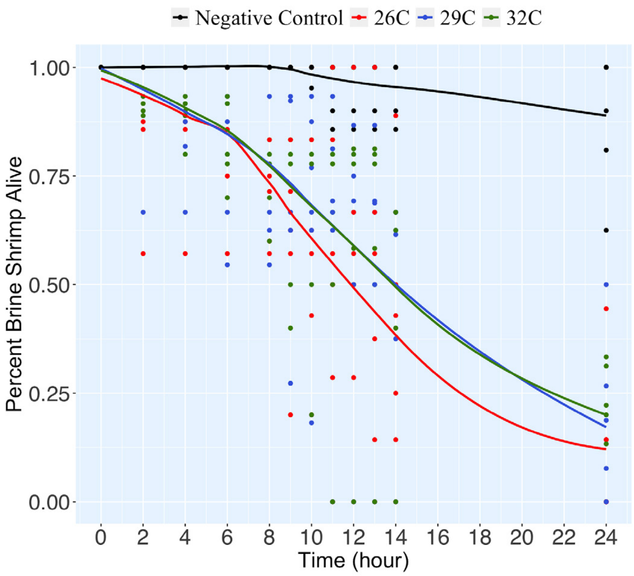

Figure 2: Non-linear Regression Curve of percent survival of brine shrimp over 24-hour periods after being exposed to sponge extract derived from temperature-treated sponges. Brine shrimp trials with *Haliclona sp.* were replicated 6 times (N=6 for each treatment, N=18 total sponges used for data).

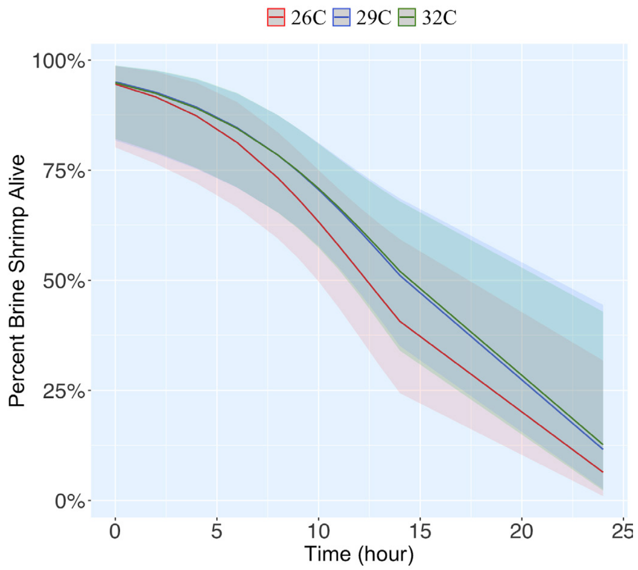

Figure 3: Temperature Logistic Regression Model from extracts taken from temperature-treated sponges (N=18) produced similar brine shrimp survival curves ($p > 0.05$).

Temperature Antimicrobial Test

The temperature treatment did not affect the diameter of the zone of inhibition (ZOI), in millimeters, of fungus killed on the petri dish. As the temperature treatment increased, the ZOI slightly decreased. As expected, the 90% ethanol-positive control had the largest ZOI, whereas the negative control, distilled water, had the smallest average ZOI (Fig. 4).

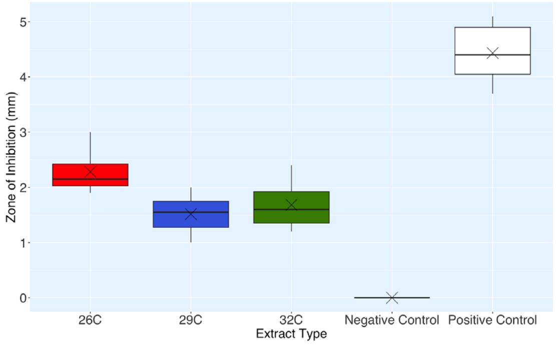

Figure 4: Box plot illustrating antimicrobial properties of temperature-treated sponge extracts. The boxes use data from the six replicates of the zone of inhibition, in millimeters, for the five treatments. The black "X" indicates the mean of the six replicates.

A pairwise comparison (Tukey HSD test) succeeding an ANOVA test (df= 4, SS = 62), revealed that all treatments significantly differed from both controls. As expected, the positive and negative controls were the most different, whereas the temperature treatments were more similar to each other in difference (Table 1). Between temperature treatments, 29°C and 26°C were found to be statistically different from each other, whereas all comparisons made with 32°C were statistically insignificant.

Predation Toxicity Test

Based on Figure 5, predated-on and non predated *Haliclona sp.* looked to differ mainly from the negative control. After utilizing an ANOVA and Tukey HSD test, on data points at the 12-hour (F value: 19.83, $p<0.05$) and 24-hour (F-value: 65.46, $p<0.05$) thresholds, it was found that at both time points, the predation treatments did in fact significantly differ from the control.

In order to compare if the two experimental treatments - predation and non-predation – had statistically different means, I performed a logistic regression statistical analysis that included time as a factor in the data analysis. It was found that there was no significant difference between the means over the 24-hour period between the two treatments (p=0.80, Fig. 6).

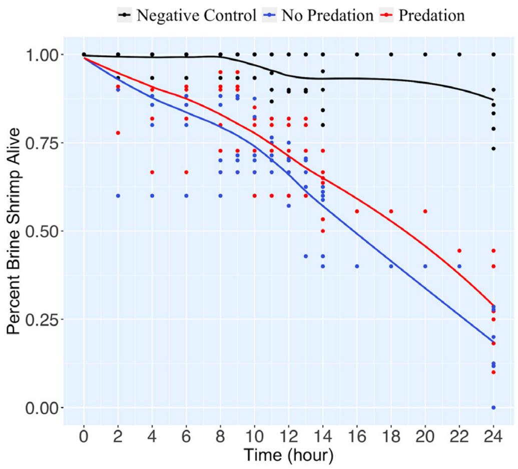

Figure 5: Non-linear Regression Curve of percent survival of brine shrimp over 24-hour periods after being exposed to sponge extract derived from predation-treated sponges. Brine shrimp trials with *Haliclona sp.* were replicated 6 times (N=12).

Pairwise Comparison	Difference	Lower Estimate	Upper Estimate	P-value
Pos. Control - Neg. Control	4.433	3.745	5.122	0.000
Temp 26°C - Neg. Control	2.283	1.595	2.972	0.000
Temp 29°C - Neg. Control	1.517	0.828	2.205	0.000
Temp 32°C - Neg. Control	1.683	0.995	2.371	0.000
Temp 26°C - Pos. Control	-2.150	-2.838	-1.462	0.000
Temp 29°C - Pos. Control	-2.917	-3.605	-2.228	0.000
Temp 32°C - Pos. Control	-2.750	-3.438	-2.062	0.000
Temp 29°C - Temp 26°C	-0.767	-1.455	-0.078	0.024
Temp 32°C - Temp 26°C	-0.600	-1.288	0.088	0.109
Temp 32°C - Temp 29°C	0.167	-0.522	0.855	0.952

Table 1: Tukey HSD pairwise comparisons for the temperature-treated extract antibacterial assay. A 95% confidence interval also provides lower and upper estimates for the difference between the pairs.

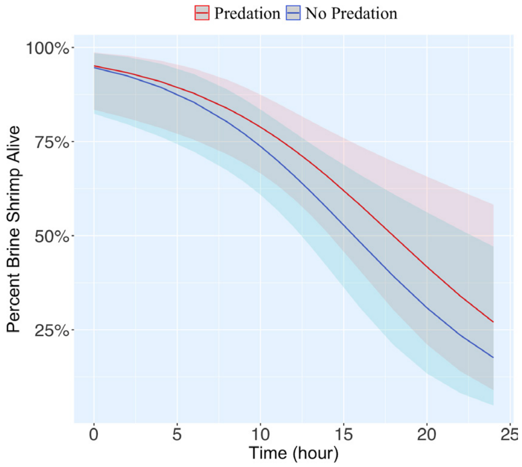

Figure 6: Predation Logistic Regression. Extracts taken from predated sponges (N=6) versus non-predated sponges (N=6) produced similar brine shrimp survival curves (p=0.80).

Predation Antimicrobial Test

Sponge extract exposed to the predation treatment had a slightly larger zone of inhibition than its experimental counterpart. As expected, the negative and positive control made up the smallest and largest average ZOIs, and the two experimental values fell between (Fig. 7).

An ANOVA (df= 3, SS = 51.85) and Tukey HSD pairwise comparison revealed the predated and non-predated sponge extracts did not have significantly different average ZOIs (p= 1.00, Table 2). When compared to the negative control, the two experimental treatments are shown to have the same difference, p-values, and lower and upper estimates.

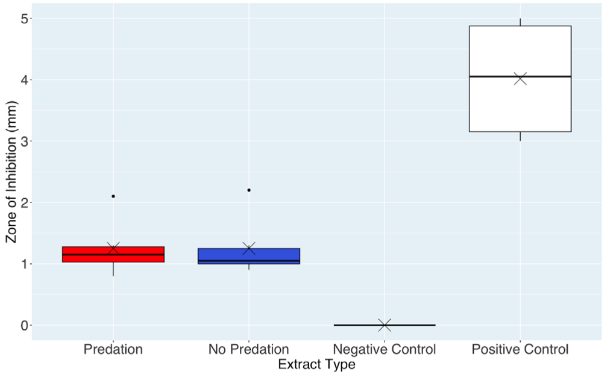

Figure 7: Box plot illustrating the antimicrobial test with predation treated sponge. Presents the mean of six replicates of the zone of inhibition, in millimeters, for the four antifungal treatments. The black "X" identifies the mean.

Discussion

Temperature Toxicity Test & Antimicrobial Test

When examining the toxicity test experimental data versus the negative control, all *Haliclona sp.* extracts clearly killed the brine shrimp at a faster rate than the distilled water. This can likely be attributed to cytotoxic compounds found within *Haliclona sp.* Though *Haliclona sp.* displayed cytotoxic properties in totality, the abiotic factor of temperature did not have a statistically significant effect on toxicity. In other words, the three temperatures did not primarily affect the regulation of secondary metabolites present in the extracts, which would have, in turn, killed brine shrimp at three statistically distinct rates.

All sponge extracts presented antimicrobial properties. Like toxicity, these properties did not differ significantly between the temperature treatments Additionally, the *Haliclona sp.* derivatives

Pairwise Comparison	Difference	Lower Estimate	Upper Estimate	P-value
Pos. Control - Neg. Control	4.017	3.088	4.945	0.000
Predation - Neg. Control	1.250	0.322	2.178	0.006
No Predation - Neg. Control	1.250	0.322	2.178	0.006
Predation - Pos. Control	-2.767	-3.695	-1.838	0.000
Pos. Control- No Predation	2.767	1.838	3.695	0.000
Predation - No Predation	4.441	-0.928	0.928	1.000

Table 2: Tukey HSD pairwise comparisons for the predation-treated extract antibacterial assay. A 95% confidence interval also provides lower and upper estimates for the difference between the pairs.

exhibited smaller zones of inhibition than shown in past studies (Perrin 2021). These results were surprising as a plethora of research has been conducted on the antibacterial and antifungal properties of tropical marine sponge extracts.

The minor differences seen between temperature-treated extracts in the toxicity and antimicrobial tests may be due to overarching differences between the individuals' age, lifecycle, or other confounding variables in each experiment. This finding, though unexpected, may be instrumental to the future of *Haliclona* sponges, as warming water temperatures do not seem to damage their ability to produce chemical defenses. However, the change in temperature also failed to cause upregulation, as hypothesized.

The outcome of this study stands specific to the *Haliclona sp.*, as past research has indicated that warming waters and ocean acidification have varying effects dependent on species, some being unaffected and some made highly impaired (Bell *et al.* 2015). Initially, the hypothesis was that warmer water would cause the upregulation of secondary metabolites due to past research on *Haliclona sp.* illustrating the increase in the antitumor compound salicylihalamide A as temperature increased (Abdo *et al.* 2007). This increase in secondary metabolites when faced with abiotic stressors was presumed to serve as a survival mechanism. However, this was not the case. In fact, two sponges kept at 32°C during the experiment met their upper limits of survival and became discolored and fetid. The remaining majority of *Haliclona sp.* may have been able to maintain their ability to produce secondary metabolites at these higher temperatures as a result of their ability to hold onto endosymbionts(Tianero McIntosh *et al.* 2019). Currently, the global abundance of sponges - due to a worldwide decrease in competition with corals - is skewed towards heterotrophic sponges with stronger connections to their photosynthetic endosymbionts. These endosymbiont-rich sponges, like *Haliclona*, have been found to outcompete their counterparts that lack intracellular microbiota (Bennett *et al.* 2017, Bachtiar *et al.* 2023). Thus, *Haliclona* may be better adapted to survive as climate change continues to warm their environment due to tighter mutualistic interactions rather than increased production of secondary metabolites.

Previous *Haliclona* research has noted that temperature may influence other processes like sponge reproduction cycles, which do have direct implications on secondary metabolite levels (Ternon *et al.* 2017, Reverter *et al.* 2018). Therefore, temperature may not play a direct role in the regulation of metabolites, explaining why this study found no statistical difference in the toxicity or antimicrobial properties between sponges in 26°C to 32°C saltwater. With that being said, temperature might still be influential in larger biological processes like reproduction and development (Ivanisevic *et al.* 2011).

This study's conclusions, though statistically insignificant, have added to an understudied niche of sponge research. Because of the limited body of research currently available on metabolite variance in terms of temperature manipulation, there was little prior knowledge as to whether the *Haliclona sp.* would react to temperature treatments within 48 hours and, if so, whether the methodology of crude extract would be enough to capture the differences. Luckily, the data was able to capture the sponge's acute cytotoxic and antimicrobial properties; however, due to an inability to understand the secondary metabolites present on the molecular level, the experiment could not assess discrete differences between temperature-treated extracts.

Molecular instruments that can take concentration measurements, identify specific secondary metabolites, and refine extractions would be a significant addition to the research. Understanding which exact compounds are changing over the course of the three temperature treatments would further our ability to understand what specific metabolites and resulting molecular defenses may be affected by abiotic stressors. Additionally, as climate change continues to impact our planet, further research into the effects of sudden temperature changes is necessary as each species of sponge inhabiting tropical, temperate, polar, benthic, or shallow water environments presents a unique set of secondary metabolites. Deciphering metabolite regulation is crucial for preserving naturally occurring chemical compounds, which harbor invaluable insights essential for advancing life-saving pharmaceutical development.

Predation Toxicity Test & Antimicrobial Test

Both sponge extracts derived from the predation treatment demonstrated their ability to be cytotoxic within the toxicity trials. However, these *Haliclona sp.* extracts, originating from sponges exposed and unexposed to predation pressure, killed brine shrimp in a manner that was not statistically different from each other. This finding was surprising as past studies indicate that when mechanical stress is present, as in the case of sponges treated with *Chromodoris lochi* nudibranch, they upregulate their metabolites (Proksch 1994). Artificial predation has demonstrated that certain types of sponges exhibit both slow-induced chemical defense reactions, where secondary metabolites are created for the future protection of the sponge, and activated chemical defense mechanisms, where compounds readily available are immediately converted into secondary metabolites (Thoms *et al.* 2006). In the trials, the latter defense mechanism would have been able to produce effective metabolites after 48 hours. For this reason, it was hypothesized that secondary metabolites would upregulate in the presence of physical stressors and, in turn, present more potent cytotoxic and antimicrobial properties.

This study's results were surprising as they contradict a plethora of prior research conducted on the upregulation of metabolites as a response to predation pressure. *Haliclona*, in particular, is quoted to produce "powerful anti-inflammatory and anti-cancer" bioactive compounds increased by predation pressure (Becerro *et al.* 2003, Varijakzhan *et al.* 2021). Additionally, when certain marine sponges are taken from their original habitat, the change in environmental stressors like predation or competition can cause variation in presented metabolites via enzymatic bioconversion (Pohnert 2004). In fact, in a study done to understand sponge aquaculture in New Zealand, genetically identical sponges placed in two different sites retained different chemical proportions of the defense metabolites mycalamide A, pateamine and peloruside A, highlighting the importance of the sponges' ecological niche in its chemical framework (Page *et al.* 2005). For all of these reasons, the results from this study, which showed no statistical difference between toxicity and antimicrobial properties of sponges of varying predation exposure, could have been impacted by experimental limitations.

During these predation experiments, containment of the *Chromodoris lochi*, a dorid nudibranch, was found to be quite difficult. These small animals are able to climb, as well as squeeze through small crevasses, sometimes cutting themselves in half. Nudibranchs are also known to exhibit cannibalistic behavior, so continuous monitoring of their behavior, as well as the quantity of nudibranchs present within the predation tanks, was necessary. Testing these extracts on fungal plates was also found to be a challenging task. After attempting to plate bacteria and fungi from numerous sources, obtaining fungal plates from a peer's experiment allowed the research to proceed. Though this was not ideal, we were eventually able to run the test and better understand the antifungal properties of *Haliclona sp.*

In the future, researchers will be able to understand better the effect predation has on Mo'orean sponges if this experiment is replicated with other natural predators like reef fish. Additionally, predation experiments in the tropical region may affect the secondary metabolites of Demospongia in ways that are unlike Hexactinellida (glass sponges) and Calcarea (calcareous sponges) (Van Soest *et al.* 2012). Despite these limitations and future research possibilities, this investigation into the effects of biotic stress in the form of predation made an undistinguishable difference in *Haliclona*'s cytotoxicity and antimicrobial properties.

This research aimed to understand the intricate connections between abiotic and biotic stressors, such as temperature and predation, and the biochemical mechanisms operating within marine organisms.Overall, this study found no significant difference in cytotoxic and antimicrobial properties between sponges of different treatments. This indicates that *Haliclona sp.* may be resilient to the effects of increased temperature and the presence of predation. It also indirectly indicates that the secondary metabolites may have not increased as a result of exposed stress. However, due to the limitations of this study, more research needs to be done on *Haliclona sp.* to gain a more holistic view of these conclusions, as well as explore the effects of abiotic and biotic stress on alternative sponge species. By exploring the natural defense systems of *Haliclona sp.* in response to realistic environmental stressors, a greater understanding of secondary metabolite regulation and production is attained, effectively providing insight crucial for guiding future conservation and pharmaceutical efforts in marine ecosystems.

ACKNOWLEDGEMENTS

I would like to thank my professors—Rauri Bowie, Seth Finnegan, Stephanie Carlson, Paolo D'Odorico, and Ben Becker—who guided my learning. I would also like to thank the graduate students—Kenzo Esquivel, Rachael Ryan, and Jaemin Lee—and all of my classmates who contributed to this project.

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