The impacts of anti-fouling paint on the ecophysiology of *Hemigrapsus oregonensis* determined through stress responses parameters

Introduction:

Along the coast of Washington and within the Puget Sound, shipping activity has increased with the increasing demand for goods. The port of Seattle contains four terminals housing goods from Asia, Europe, and various Latin American countries (Port of Seattle, 2016). Additionally, ferries that operate within the Puget Sound are prominent, running multiple routes daily. These shipping routes and ferries rely on the fact of quick and reliable travel times. However, these ships face one major issue that slows the travel, biofouling. When objects are placed into the ocean, they act as a substrate for organisms to attach to, in a process called biofouling. It happens naturally and quickly, with algae, barnacles, and other microorganisms accumulating over time. The buildup increases drag on the hull, causing ships to slow down (University of Portsmouth, 2021). Not only does this increasing shipping time, but it also raises shipping costs. Therefore, biofouling is avoided by the use of anti-fouling paint. This paint is used to eliminate the chances of organisms and algae from growing and attaching on the hull of the vessel. Furthermore, anti-fouling paints are used on other objects placed in the ocean that would be damaged by the process of biofouling (buoys, mile markers, etc.). Anti-fouling paint works by releasing ions of heavy metals, creating a barrier, to prevent the attachment of organisms to the surface.

While anti-fouling paint is effective, it causes trace heavy metals such as copper, zinc, and lead to be released into the water. The paint contains a multitude of chemicals, the most notable being cuprous thiocyanate biocide. This chemical dissociates when it comes in contact with water and releases copper ions into the ocean. Copper released through anti-fouling paint from shipping and boating is known to be the second largest input of copper into the ocean, accounting for 37% (“Baltic Sea Shipping Should Avoid Copper in Antifouling Paints and Open-Loop Scrubbers to Mitigate Pollution,” 2023). This has quickly become a novel source of pollution.

Organisms along the coasts and within the water column of Puget Sound and Washington are exposed to the heavy metal released by anti-fouling paint. Heavy metal exposure is toxic to organisms, such as crustaceans native to the Puget Sound, creating a multitude of impacts. A study by Persoone & Castritsi-Catharios (1989) found an 80%-95% rate mortality of brine shrimp larvae (*Artemia*) when in high levels of exposure. Programmed cell death (apoptosis) was also observed in Chinese mitten crab (*Eriocheir sinensis*) when exposed to copper (Bu et al., 2022). Lastly, the osmotic and ionic processes within mudflat fiddler crabs (*Minuca rapax*) cease, preventing the organism from being in homeostasis resulting in increased stress (Capparelli et al., 2017). This stress impairs the normal processes that allow for the continued survival of the organism. These effects can be long-lasting as well, due to the process of bioaccumulation. Copper can accumulate in the stomach and outer shell; this was observed in mud crabs (*Scylla paramamosai*) (Luo et al., 2020). The copper released by anti-fouling is harmful to organisms, potentially resulting in death and results in changes to the chemical and ecological composition of the ecosystem.

The hairy shore crab (*Hemigrapsus oregonensis*) is one of the native species along the coast and within the Puget Sound. It will be used as a model organism for our study due to its abundance and its ability to reflect possible stress responses in crustaceans. Righting time and lactate levels will be measured to assess the stress experienced by the crabs. Righting time is an indicator of stress as increases in righting time have been correlated with increased stress and fatigue (Stoner, 2012). Lactate levels indicate the level of anaerobic respiration, a process that increases in response to increased energetic demands in stressful states (Lorenzon et al., 2008).

While the general impacts of anti-fouling paints on organisms are known, the specific impacts to stress physiology in crustaceans native to the Puget Sound, such as the hairy shore crab, is unknown. The overall aim of this study is to characterize the stress response *Hemigrapsus oregonensis* demonstrates in response to being exposed to the antifouling paint. To address this, we test the following three hypotheses: (i) exposure to increasing levels of antifouling pain will increase the righting time of individuals; (ii) exposure to increasing levels of antifouling pain will increase the lactate levels of individuals, (iii) exposure to increasing levels of anti-fouling paint will reduce the alertness of individuals.

Methods:

In order to test the stress response of the hairy shore crab induced by anti-fouling paint, we carried out a week-long experiment using Trilux® 33 Aerosol, an anti-fouling paint containing the active ingredient cuprous thiocyanate biocide. The experiment consisted of three experimental tanks (each labeled with the appropriate size square) and one control tank (shared among all groups). The salinity and temperature were kept constant across all four tanks in all 15 °C and 33 ppt, respectively.

Preparation of the anti-fouling coated samples consisted of using aluminum foil as our substrate to mimic how the paint adheres to an aluminum hull. The aluminum foil was cut into squares measuring 2x2 cm, 4x4 cm, and 8x8 cm. These squares were then coated with Trilux® 33 Aerosol, spraying under a fume hood for safety. The squares were allowed to dry for 24 hours before a second coat of the anti-fouling paint was applied. After six days of drying the paint covered squares were brought to the lab to begin the experiment.

Three experiment tanks were filled with 2 L of 15°C and 33 ppt water. 15 hairy shore crabs were gathered from the larger control tank. The crabs were randomly selected to reduce human error. 5 crabs were placed into each of the three experiment tanks. Then, an anti-fouling paint covered squares was placed in the tank paint side down to ensure the most exposure to the paint. The three squares were placed into their respective tanks with the same labeling. These experiment tanks were then placed in a water bath to ensure temperature was constant and given constant oxygen through a tube. The crabs were exposed to the paint for a course of a week.

At the end of the week, the crabs were removed from the tanks. Righting time and hemolymph extractions were conducted. Righting time was done by flipping the crab on its back and timing, in seconds, how long it takes for them to flip right side up. Hemolymph was extracted by injecting a needle into the most proximal leg joint and drawing up the hemolymph. The hemolymph was then tested for lactate levels using the Cayman Chemical L-Lactate Assay. This procedure was done to all 15 experimental crabs as well as 5 control crabs. Righting times and lactate levels were analyzed using graphs as well as t-tests for statistical results. The levels of exposure are indicated by surface area-to-volume ratios. Qualitative data was also collected on the behavior of the crabs during each test.

Results:

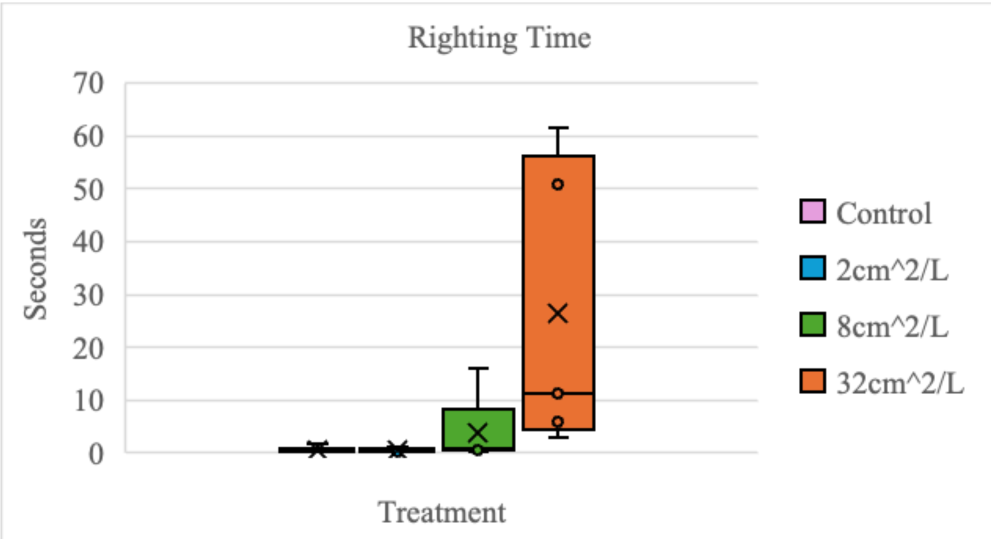

Figure 1: Righting times (s) of individual hairy shore crabs indicated by the circles and ends of range lines. The 'x' represents the average for the group. Results are separated by levels of exposure indicated by surface area-to-volume ratios

Table 1: Results of t-tests comparing the average righting time (s) of each experimental group to the control, as well as to $2cm^2/L$.

t-Test Comparison	Control $2cm^2/L$	Control $8cm^2/L$	Control $32cm^2/L$	$2cm^2/L$ $32cm^2/L$	$2cm^2/L$ $8cm^2/L$	$8cm^2/L$ $32cm^2/L$
p-Value	0.89	0.36	0.10	0.10	0.35	0.15

The righting times of the crabs increased as exposure increased. The average righting times are as followings, starting with the control and increasing in exposure: 0.376s, 0.325s, 3.56s, and 26.304s (Fig 1). The crabs exposed to the $32cm^2/L$ had the longest righting time and was the most significantly different from the control with a p-value of 0.15 (table 1). The crabs exposed to the lowest amount of anti-fouling paint had similar righting times as the control. Fig 1 depicts the average righting times with “x” and bars to indicate the range of values in each treatment. Qualitatively, the crabs were observed to become more lethargic and easier to work with as the level of exposure increased. Crabs in the $32cm^2/L$ required multiple seconds to begin flip over and had failed attempts at flipping over. Contrasted to the control crabs, who flipped almost instantly and remained active after completing the test.

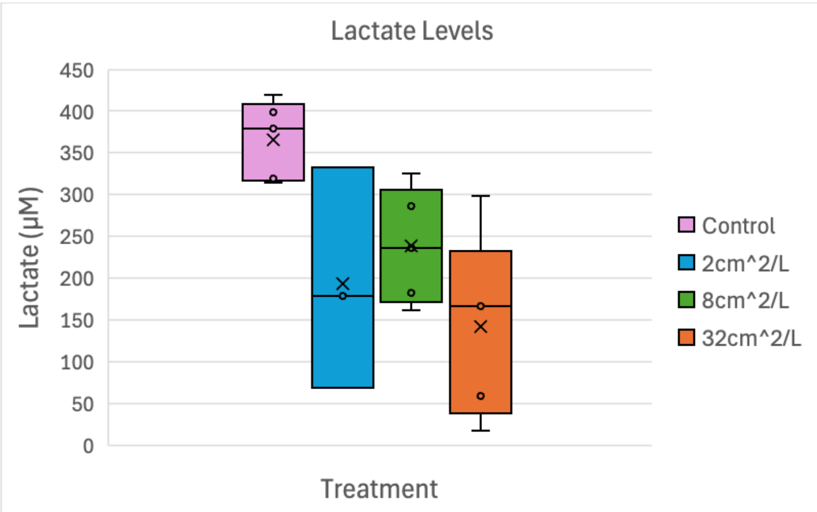

Figure 2: Lactate levels (µM) of individual hairy shore crabs indicated by the circles and ends of range lines. The 'x' represents the average for the group. Results are separated by levels of exposure indicated by surface area-to-volume ratios

Table 2: Results of t-tests comparing the average lactate levels (µM) of each experimental group to the control, as well as to 2cm^2/L.

t-Test Comparison	Control 2cm^2/L	Control 8cm^2/L	Control 32cm^2/L	2cm^2/L 32cm^2/L	2cm^2/L 8cm^2/L	8cm^2/L 32cm^2/L
p-Value	0.16	0.01	0.008	0.60	0.62	0.14

The lactate levels were analyzed using a plot depicted in Fig. 2. The control crabs exhibited an average lactate level of 365.44 µM (Fig 2). The experimental groups experienced decreasing levels of lactate levels with increasing levels of exposure. The average lactate levels for the 2cm^2/L, 8cm^2/L, and 32cm^2/L are 192.59µM, 237.97µM, and 141.32 µM respectively (Fig 2). The p-value for comparing the highest level of exposure to the control is 0.008 (table 2). Qualitatively, the same behaviors were seen with the righting time tests. The crabs in the high level of exposure exhibited little defensive behavior. The crabs did not engage in pinching and offered little opposition to the needle being inserted. The control crabs and 2cm^2/L, however, were aggressive, pinching and squirming.

Lastly, it is important to note there was one mortality in the 2cm^2/L treatment. Additionally, a pregnant female in the 2cm^2/L treatment, preventing hemolymph from being extracted.

Discussion:

The visualization in Fig. 1 and the qualitive data regarding the righting time of the individual crabs, indicate the exposure of anti-fouling paint positively correlates to an increase in righting time. By using the righting time as a proxy for stress, the increasing levels of anti-fouling paint resulted in increased stress levels of the crabs. The average righting time for the crabs in the 32cm^2/ L treatment was longer than all of the other treatments. This indicates a very high stress level among the individuals. Even though, the p-values comparing the righting times do not indicate a significant difference, this can simply be due to the small sample size of 5 individuals per group. The increased righting time supports previous studies that indicate increased righting times due to copper exposure. This increase in stress can occur for many reasons, as copper interacts with many facets of the crab ecophysiology. Increased exposure to copper would increase the level of maladaptive responses the crab is undergoing in order to remove the toxin from its body (Stoner, 2012). Increasing the responses will shunt energy away from being able to recover from additional stressors, such as being placed on the back, increasing the righting time. Inflammatory responses, such as immune responses to remove the copper from the tissues, increase the fatigue experienced by the individual, increasing the righting time (Bu et al., 2022). Furthermore, oxidative stress, the imbalance of free radicals in the hemolymph, will cause damage to multiple tissues and molecular structures, such as lipids, DNA, and proteins (Capparelli et al., 2017). These responses were observed through the qualitative data gathered. The lethargic state of the individuals crabs in the $32cm^2/L$ supports the explanation of increased stressed levels. The individuals were unable to flip themselves back over or engage in defensive behaviors, suggesting the stress had left them incapacitated. Increases in damage to the body, will increase the physiological stress the crab is in. If the stress is too severe, death can occur. This study did conclude in one mortality. It is unknown the reasoning as to why one crab would die and the other would not.

The results from the lactate assays show a significant decrease in lactate levels as the exposure to anti-fouling paint increases. This is opposite from what is expected. Previous studies have shown the increase in copper to cause increases in lactate due to increases in energetic needs and anerobic respiration (Dunbar et al., 2017). Furthermore, lactate is expected to increase because of the reduction of glycogen stores with the presence of copper (Dunbar et al., 2017). However, this is not what we observed. A possible explanation, is the process of anaerobic respiration ceases due to high stress or the interaction between the copper and proteins. If the copper ion being released has the ability to prevent or limit the process of anerobic respiration, organisms would be unable to synthesize energy in low oxygen environments. This has not been observed in other studies and would require further experimentation to determine a relationship.

There are possible errors associated with this experiment that should be taken into account and tested further. The small sample size of only 5 crabs in each treatment and the reduction to only 3 crabs in the $2cm^2/L$ treatment caused a large variation in righting times and could impact the ability to accurately analyze the data. In addition to the small sample size, this study contains pseudoreplication and each sample is not a true replicate. Therefore, if there are any errors in the environment or experimental set up then all 5 individuals in the treatment will experience the error. Limitations in resources prevented us from conducting an experiment with true replication. Further studies should be conducted to test with true replication.

Overall, this study was able to both qualitatively and quantitatively describe the effects of anti-fouling paint on the ecophysiology and stress response of hairy shore crab through observation and the righting times. The larger environmental impact of this study is rooted in the growing concentration of heavy metals, like copper, in the water column due to the use of anti-fouling paint. The increase stress on organisms can reduce the ability to fight infection or diseases, lowering the overall fitness of the population. With increasing impacts from climate change and global warming, organisms will be unable to survive the increase in stressors. The addition of heavy metals via anti-fouling paint can act as a comorbidity for individuals, reducing populations and impacting ecological structuring. Actions on reducing the use of anti-fouling paint needs to be prioritized to reduce the probability of population and subsequently ecological change.

Resources:

Baltic sea shipping should avoid copper in antifouling paints and open-loop scrubbers to mitigate pollution. (2023, May 24). *European Commission*. https://environment.ec.europa.eu/news/baltic-sea-shipping-should-avoid-copper-antifouling-paints-and-open-loop-scrubbers-mitigate-2023-05-24_en

Bu, X., Song, Y., Pan, J., Wang, X., Qin, C., Jia, Y., Du, Z., Qin, J. G., & Chen, L. (2022). Toxicity of chronic copper exposure on Chinese mitten crab (Eriocheir sinensis) and mitigation of its adverse impact by myo-inositol. *Aquaculture*, *547*, 737511. https://doi.org/10.1016/j.aquaculture.2021.737511

Capparelli, M. V., McNamara, J. C., & Grosell, M. (2017). Effects of waterborne copper delivered under two different exposure and salinity regimes on osmotic and ionic regulation in the mudflat fiddler crab, Minuca rapax (Ocypodidae, Brachyura). *Ecotoxicology and Environmental Safety*, *143*, 201–209. https://doi.org/10.1016/j.ecoenv.2017.05.042

Dunbar, S. G., Shives, J., & Boskovic, D. S. (2017). Lactate accumulation in the intertidal hermit crab, Pagurus samuelis, in response to burial-induced hypoxia. *Crustacean Research*, *46*, 121–132. https://doi.org/10.18353/crustacea.46.0_121

Lorenzon, S., Giulianini, P. G., Libralato, S., Martinis, M., & Ferrero, E. (2008). Stress effect of two different transport systems on the physiological profiles of the crab Cancer pagurus. *Aquaculture*, *278*(1–4), 156–163.

Luo, J., Zhu, T., Wang, X., Cheng, X., Yuan, Y., Jin, M., Betancor, M. B., Tocher, D. R., & Zhou, Q. (2020). Toxicological mechanism of excessive copper supplementation: Effects on coloration, copper bioaccumulation and oxidation resistance in mud crab Scylla paramamosain. *Journal of Hazardous Materials*, *395*, 122600.

Persoone, G., & Castritsi-Catharios, J. (1989). A simple bioassay with Artemia larvae to determine the acute toxicity of antifouling paints. *Water Research*, *23*(7), 893–897. https://doi.org/10.1016/0043-1354(89)90014-6

Port of Seattle. (2016, April). *A SELF-GUIDED TOUR OF THE SEATTLE CONTAINER TERMINALS*. https://www.portseattle.org/sites/default/files/2018-03/Terminal_Tour_4-2016.pdf

Stoner, A. W. (2012). Assessing Stress and Predicting Mortality in Economically Significant Crustaceans. *Reviews in Fisheries Science*, *20*(3), 111–135. https://doi.org/10.1080/10641262.2012.689025

University of Portsmouth. (2021, February 24). Hidden pollution from shipping threatens sustainable ocean efforts. *Phys.Org*. Phys.org. https://phys.org/news/2021-02-hidden-pollution-shipping-threatens-sustainable.html