Impacts of Antifouling paint on *Hemigrapsus oregonensis* and their stress physiology response

By Cali Weber

INTRODUCTION

Since the 20th century, recreational boating and commercial shipping have expanded significantly (Hall, 2001; Kaplan and Solomon, 2016). To reduce marine biofouling (the accumulation of organisms like algae, barnacles, and mussels on human-made surfaces) both recreational and commercial boats commonly use antifouling coatings on their hulls and propellers (Tian et al., 2021; Venettacci et al., 2023). These coatings can decrease drag by reducing the number of organisms that settle on ships, and therefore also decrease fuel consumption and costs (Tian et al., 2021). To be effective, the chemicals in these paints gradually leach into the water column and thus settle in sediment (Soroldoni et al., 2020). Due to the higher concentration of boats, marinas in both Puget Sound, Washington, and near San Deigo, California have statistically higher levels of these chemicals compared to water outside the marinas (Schiff et al., 2007; Hobbs, McCall, and Lanksbury, 2018). Unfortunately, many of these coatings contain chemicals that are toxic to a wide range of marine organisms, not just the intended target organisms (Soroldoni et al., 2018).

One commonly used ingredient in antifouling paints is copper, including cuprous thiocyanate and cuprous oxide (Cima and Varello, 2023). While copper is an important micronutrient used in metabolism, high concentrations can be toxic when it accumulates in tissues and exceeds what organisms need (Xie et al., 2005). One study by Persoone and Castritsi-Catharios, found *Artemia* larvae to have 100% mortality after being exposed to $112.5 cm^2/L$ of the antifouling paint Trans Long Life antifouling (1989). Another study by Cima and Varello found that exposure to low levels of Cu(I) ions caused cell death and disrupted immune responses in both *Mytilus galloprovincialis*, a mussel often found on the hulls of ships, and *Ruditapes philippinarum*, a soft sediment clam (2023). Despite these findings, less is known about the effects of copper on benthic crustaceans, many of which play key ecological and economic roles in coastal ecosystems.

In Puget Sound, Washington, one ecologically and economically important species is *Metacarcinus magister* (Dungeness Crab). In 2011, it made up just 15% of the total biomass harvested yet generated 44% of the Washington's fishing revenue (Rasmuson, 2013). Ecologically, *M. magister* plays an important role in coastal food webs as an opportunistic predator of bivalves, fish, and shrimp, while also serving as prey for fish, octopuses, and sea otters. To assess the potential effects of copper biocides on Dungeness crabs, researchers used *Hemigrapsus oregonensis* (Hairy Shore Crab) as a model organism due to its high abundance, ease of collection and care, and anatomical and physiological similarities to Dungeness crabs. This study aims to understand how antifouling paint impacts crab ecophysiology. Trilux® 33 Aerosol an ablative antifouling paint with an active ingredient of cuprous thiocyanate was the antifouling paint used in this study. The surface area to volume ratio in experimental tanks was used as a proxy for environmental exposure. Specifically, this study measures righting time, a behavioral indicator of impairment, and hemolymph lactate levels, a physiological stress marker, after one week of exposure. The null hypothesis for this study is that there will be no changes to crab physiology in response to Trilux® 33 Aerosol. The alternative hypothesis is that there will be an increase in the righting time and lactate levels in crabs exposed to higher amounts of antifouling paint.

METHODS

Experimental Design

This study was designed to test the toxicity of antifouling paints on *Hemigrapsus oregonensis*, following the protocol with Artemia larvae by Persoone et al. that used the surface area to volume ratio as a metric for how to increase the paint concentration to test the toxicity of paints used on submerged surfaces. *H. oregonensis* were collected from Lion's Park Boat Launch/Trestle (47°35′07″N, 122°38′42″W) during low tide (-1.68 feet) between 11:30 am and 1:34 pm. Crabs were gathered from a mixed substrate of shell, sand, and pebble.

Three 2-liter experimental tanks were prepared, at 33 ppt salinity and maintained at 13°C. Each tank contained 5 crabs, and an air stone was used to ensure proper oxygenation. The control tank had around 30 crabs and was at the same temperature and salinity conditions. When it came time to test the crabs, only five were randomly selected from the control tank to be tested. Aluminum foil squares (4 cm^2, 16 cm^2, and 64 cm^2) were coated with two even coats of Trilux® 33 Aerosol antifouling paint (active ingredient cuprous thiocyanate) under a fume hood. Each coat was allowed to dry for two days. Once dry, one square was placed in each of the three experimental tanks, with the painted side facing down to mimic the underside of a boat. Over time the squares did eventually sink to the bottom of each tank. The crabs were exposed to these conditions for one week.

Testing Procedures

To evaluate the effects of Trilux® 33 Aerosol on *H. oregonensis* the righting time and the lactate levels were tested for each crab. For the righting time test, each crab was placed on its back, and the time taken to right itself was recorded. A timer was started as soon as the crabs were placed on their backs and stopped once the crab had successfully righted itself. A crab that was 'righted' had all of its walking legs in contact with the table (Day et al., 2019). Any crabs that were able to immediately flip over had righting times recorded as 0.1 seconds to account for the delayed human reaction time in stopping the timer.

Hemolymph was extracted from the joint between the coxa and the body of each crab using a 26-gauge 3/8-inch intradermal bevel needle and disposable syringe for lactate analysis. L-lactate concentrations in crab hemolymph were calculated using the Cayman Chemical L-Lactate Assay Kit, with minor adaptations for non-human samples. Hemolymph was mixed with an equal volume of cold 0.5 M metaphosphoric acid in 1.5 mL microcentrifuge tubes to deproteinate the samples. Tubes were vortexed and incubated on ice for 5 minutes. Samples were centrifuged twice at 10,000 × *g* for 5 minutes at 4°C first to pellet proteins, and again after the addition of 50 µL potassium carbonate to neutralize the acid and remove precipitated salts. Supernatants were collected and assayed using each sample, 100 µL of Assay Buffer, 20 µL of Cofactor Mixture, and 20 µL of Lactate Substrate. Reactions were initiated by the addition of 40 µL of Enzyme Mixture. Plates were incubated at room temperature for 20 minutes, protected from light, and fluorescence was measured with an excitation wavelength of 530–540 nm and an emission wavelength of 585–595 nm. L-lactate concentrations were calculated using Equation 1.

$$\text{L-Lactate } (\mu M) = \left[\frac{\text{CS - (y-intercept)}}{\text{Slope}}\right] \times 2^{*} \times \text{Sample dilution}$$

Equation 1.

Data Analysis

To compare the righting times and lactate levels between the control and each group as well as between groups, two-sample t-tests assuming unequal variance were performed in Microsoft® Excel for Mac (version 16.97).

RESULTS

Righting Time

After one week of treatment conditions, one crab in the $2cm^2/L$ treatment died. From this same treatment, a crab was found with eggs underneath her carapace, so hemolymph was not extracted, however, righting time was still performed. The $32cm^2/L$ treatment had the highest average righting time at 26.30 seconds followed by the $8cm^2/L$ treatment with an average of 3.56 seconds (Figure 1). The control and $2cm^2/L$ treatment had very similar righting times at 0.38 and 0.32 seconds respectively (Figure 1). After performing t-tests, none of the treatments could be considered statistically different from each other using the conventional 0.05 p-value. However, the more concentrated the treatment was, the closer it was to 0.05, with the smallest p-value being between the control and $32cm^2/L$ treatment at 0.10 (Table 1). This lack of significance is likely due to the small sample size, as there is still a clear trend in increasing righting time with higher concentrations of Trilux® 33 Aerosol. Additionally, the fastest righting time of the $32cm^2/L$ treatment was 2.81 seconds, still slower than the slowest righting time of the control and $2cm^2/L$ treatment at 1.48 and 1 second respectively (Figure 1).

Table 1. Results of two sample t-tests assuming unequal variances for righting time (RT) and for lactate levels (LL).

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 (RT)	0.89	0.36	0.10	0.10	0.35	0.15
p-Value (LL)	0.16	0.01	0.008	0.60	0.62	0.14

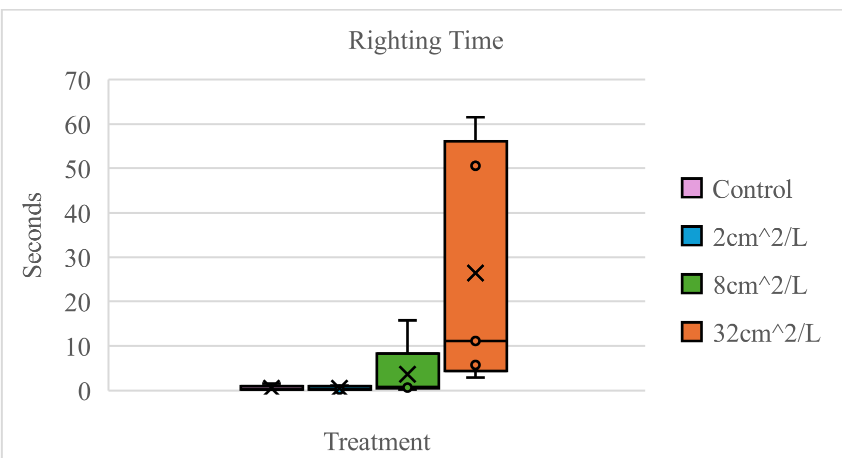

Figure 1. Box and whisker plot of the righting time of H. oregonensis within each treatment group. Average times are denoted with an X and each data point is denoted with an o as well as the whiskers being points.

Lactate Levels

After one week of treatment conditions, the control group had the highest lactate levels with an average of 365.44µM. This was followed by the $8cm^2/L$ treatment with an average of 237.96µM, and then the $2cm^2/L$ and $32cm^2/L$ with average levels being 192.59µM and 141.32µM respectively (Figure 2). After performing t-tests, the control and the $8cm^2/L$ treatment can be considered significantly different from each other with a p-value of 0.01 (Table 1). The control and $32cm^2/L$ treatment can also be considered significantly different from each other with a p-value of 0.008 (Table 1). None of the other t-test comparisons can be considered statistically different from each other.

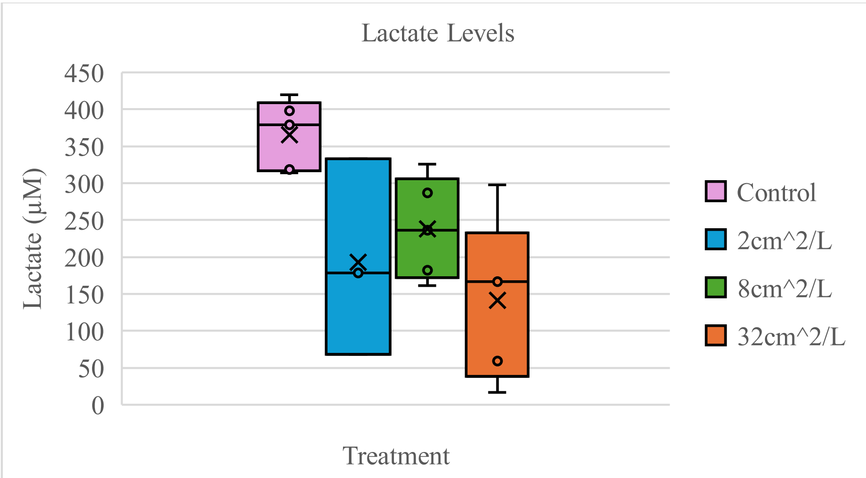

Figure 2. Box and whisker plot of the lactate levels µM of H. oregonensis within each treatment group. Average times are denoted with an X and each data point is denoted with an o as well as the whiskers being points. The $2cm^2/L$ treatment had one crab die and another had eggs underneath her carapace, so hemolymph was not extracted, thus a smaller sample size.

DISCUSSION

The objectives of this study were to assess the impacts of copper-based antifouling paint (Trilux® 33 Aerosol) on the stress physiology and behavior of *Hemigrapsus oregonensis* as a model for Dungeness crab ecophysiology. As the number of boats and thus the levels of antifouling paint increases, it will be important to know how economically and ecologically important species such as the Dungeness crab will react.

After performing the behavioral test for righting time, researchers found that higher paint concentrations caused righting time to increase, indicating a possible dose-dependent behavioral impairment. However, this was not statistically significant at conventional thresholds (<0.05). Conversely, lactate levels decreased with increasing paint concentration, with significant differences between control and higher exposure groups (notably $8cm^2/L$ and $32cm^2/L$ treatments). Both findings lead to the rejection of the null hypothesis, and that *H. oregonensis* is exhibiting a stress physiology response to the increasing concentrations of Trilux® 33 Aerosol.

In crustaceans, righting behavior is considered a reflex that relies on both muscle coordination and neurological control, making it a common indicator of health (Wilson et al., 2022). Increased righting times can signal worsening health, as a crab positioned with its abdomen facing upward is more vulnerable, both due to limited mobility and the exposure of its softer, less protected underside. In this study, while the precise mechanism by which cuprous thiocyanate increases righting time in *Hemigrapsus oregonensis* is not clear, several plausible explanations exist based on related research.

One potential explanation is damage to the statocysts, sensory organs that help crustaceans detect their orientation relative to gravity. A study examining the effects of seismic air guns on rock lobsters found that exposure to intense sound waves physically damaged the lobsters' statocysts, impairing their righting ability (Day et al., 2019). Similarly, it is possible that cuprous thiocyanate affected the statocysts in *H. oregonensis*, disrupting their ability to sense their spatial orientation.

In addition to sensory impairment, copper exposure has been shown to induce a range of physiological stress responses in crustaceans. A study on the Chinese mitten crab (*Eriocheir sinensis*) found that copper can trigger oxidative stress, apoptosis, and endoplasmic reticulum (ER) stress (Feng et al., 2022). Oxidative stress occurs when reactive oxygen species (ROS) overwhelm the cell's antioxidant defenses, leading to damage to DNA, proteins, and lipids. This cellular damage could have contributed to the delayed righting response observed in *H. oregonensis* exposed to Trilux® 33. Feng et al. (2022) also reported that Cu^{2+} exposure upregulated genes involved in apoptosis within the hepatopancreas, gills, and muscles of *E. sinensis*. If a similar response occurred in *H. oregonensis*, cell death in muscle tissue may have impaired motor function, further delaying righting behavior. Additionally, the same study found that copper exposure activated the unfolded protein response, a cellular process aimed at restoring ER homeostasis. Prolonged ER stress can disrupt protein folding, assembly, and transport, potentially interfering with cellular functions critical for coordination and movement (Feng et al., 2022). To better understand which of these mechanisms may be responsible for the observed effects in *H. oregonensis*, future research should include additional molecular analyses, to assess whether their stress response pathways mirror those documented in *E. sinensis*.

While righting behavior provided insight into the behavioral impacts of cuprous thiocyanate, changes in lactate levels can offer further evidence of metabolic stress in *H. oregonensis*. Lactate is a byproduct of anaerobic respiration; it gets formed when cells convert glucose into energy without using oxygen. Cells switch to anaerobic metabolism, producing lactate from pyruvate to regenerate NAD^{+}, which allows for continued ATP production with a lack of oxygen. Copper can damage the gills of crustaceans, potentially impeding their ability to perform aerobic respiration. A study on the freshwater prawn, *Macrobrachium rosenbergii*, copper exposure caused lamellar swelling, hyperplasia, and necrosis, resulting in abnormal gill structures (Asih et al., 2013). These structural changes not only disrupted osmoregulation but may also have compromised respiratory efficiency, possibly leading to increased reliance on anaerobic pathways. *H. oregonensis* however exhibited the opposite pattern. When exposed to higher concentrations of cuprous thiocyanate they appeared more stressed, as indicated by increased righting times, yet exhibited lower lactate levels (Figure 2). This could be because despite being stressed they still had enough oxygen available and did not need to switch to anaerobic respiration. This trend does not appear in any literature and is likely that the observed results were influenced by the small sample size, and additional replicates may yield different outcomes. More molecular analyses should be performed in the future to understand why this trend appeared.

These findings contribute to evidence that copper-based antifouling paints, such as Trilux® 33 Aerosol, can adversely affect non-target marine organisms like *Hemigrapsus oregonensis*, and other benthic crustaceans such as the Dungeness crab. Given the use of antifouling paints in regions like Puget Sound, the potential for impacts on crustacean populations needs attention. While this study highlights behavioral and physiological signs of stress, further research is needed to clarify the underlying mechanisms, especially at the molecular level. Future work could incorporate gene expression analyses to investigate pathways related to oxidative stress, apoptosis, and respiration, as well as longer-term, higher replication, exposure studies to assess potential impacts on survival and reproduction. Understanding how antifouling paints affect species is important for developing more sustainable management practices and for protecting the health of marine food webs.

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