Adrian Shaw

Introduction

Pollution from human activities is resulting in increasing levels of heavy metal contamination in marine environments. Wastewater from sewers and industrial facilities are major sources of heavy metal pollution in coastal and marine environments (Naser, 2013). Antifouling paints used on the hulls of boats and ships are another source of heavy metal pollution, primarily in the form of copper and zinc, and many heavy metals can also be found in runoff from roads and stormwater (Adeleye et al., 2016; Gao et al., 2022; Liu et al., 2018).

In Washington, the Puget Sound and its watersheds are among the areas impacted by anthropogenic pollution, with a number of heavy metals counted as chemicals of concern by the Puget Sound Assessment and Monitoring Program (Essington et al., 2025). Marinas within the Puget Sound have been found to have high levels of copper contamination, both bound to sediment suspended in the water and deposited on the substrate, likely from antifouling paints (Hobbs et al., 2022).

Because of the increasing presence of heavy metals in our waters, it is important to understand the potential impacts of this pollution. One major way that heavy metals are taken up by marine organisms is through the gills absorbing heavy metals in the water, due to their large surface area and ion-exchange processes. These heavy metals then bind to lipids and proteins and accumulate in tissues (Oros, 2025).

In crabs, copper exposure has been linked to both physiological and behavioral impairments to reproduction. Multiple studies have linked copper exposure to negative effects to ovarian function in European green crabs and Pacific rock crabs (Elumalai et al., 2005; Medesani et al., 2004). Additionally, copper exposure in European green crabs has been shown to impair the ability of male crabs to both find mates and successfully mate with them (Krång et al., 2006).

This study aims to use yellow shore crabs (*Hemigrapsus oregonensis*) as a model organism to better understand the behavioral effects of copper exposure on the various crab species living in the Puget Sound, and to hopefully gain insight into how those effects might affect their ability to avoid threats and survive in the wild.

Methods

Experimental Setup

210 yellow shore crabs (*Hemigrapsus oregonensis*) were collected from Lion's Park boat launch between 11:30 AM and 1:34 PM in Bremerton, Washington during a low tide. The shore crabs were moved to a large seawater tank held at 13°C and 33ppt, with a number of rocks and shells scattered around as hides. Of those crabs, 18 were selected at random and divided into two groups of nine crabs each. Each group was placed into a smaller tank with the same temperature and salinity and given a few hides. All crabs were fasted for the duration of the experiment.

Copper sulfate pentahydrate was dissolved in the water of the two smaller tanks. One tank – designated the "low copper" treatment – had two liters of seawater with 25 mg/L concentration of copper sulfate pentahydrate, and the other tank – designated the "high copper" treatment – had three liters of seawater with 167 mg/L concentration of copper sulfate pentahydrate. These concentrations are higher than the LD_{50} values found for copper sulfate for *Cancer antennarius* by Lara Jacobo et al. (2016), but were chosen due to the different method of administering the copper sulfate – dissolved in the water rather than through injections.

Control crabs were selected at random from the crabs remaining in the larger tank that were not exposed to any copper.
Testing was done after one week, and then again at the end of the second week.

Righting Time
Crabs were placed one at a time into a wide tray of seawater approximately three inches deep and flipped onto their backs by hand. A timer was started when letting go of the crab and stopped when the crab had flipped itself over and was standing normally. After week one, four crabs from each group were tested. After week two, four low copper crabs and one high copper crab was tested due to mortality among the high copper crabs.

Resazurin
A stock resazurin solution was created by mixing 0.5 g resazurin salt, 10 mL deionized water, and 10 µL DMSO. This was used to create a working resazurin solution consisting of 148 mL seawater, 333 µL resazurin stock solution, 150 µL DMSO, and 1.5 mL antibiotic solution 100x Penn/Strep & 100x Fungizone. The seawater was created using deionized water and Instant Ocean and adjusted to 23-25 ppt.
Crabs were dried and weighed before being placed individually into beakers containing 35 mL of working resazurin solution. The tops of the beakers were covered with tinfoil to form an approximate seal. 200 µL samples were taken at 30, 60, and 90 minutes and placed in the wells of a microplate. Samples were run in a plate reader at Excitation 530; Emission 590 to obtain fluorescence values, which were normalized using the weights of the crabs as measured. Three low copper and three high copper crabs were tested after week one, and four low copper crabs and one high copper crab was tested after week two. Several control crabs were tested each week as well.

Threat Response
Crabs were placed one at a time into a wide tray of seawater approximately three inches deep with a couple of hides available. A trifold was set up as a barrier so that the crabs would react less to observers. After a crab was placed in the tray, it was given a period of time – roughly thirty seconds – to adjust to its environment. Once the crab had hidden or settled near one of the hides, the hide was removed and an image of a predator (a seagull) was presented to simulate an encounter with a threat in the wild. If the crab moved to the other hide, this was considered a flight response and the time taken to react was noted. If the crab remained stationary for thirty seconds, this was considered a freeze response.

Hemolymph
Hemolymph was extracted from several crabs with a syringe after week one and week two, but due to outside complications no data from that is being reported in this study.

Results
Mortalities
Both groups had mortalities over the course of the two weeks. After the first week one crab in the low copper group and five in the high copper group had died, and after the second week four additional crabs in the low copper group and four additional crabs in the high copper group had died as well. Additionally, one low copper crab in week one and two low copper crabs in week two died during hemolymph extractions. There isn't exact data on mortalities among the control group, but there were no mass mortalities among the control crabs akin to what the two copper treatment groups experienced.

Righting Time

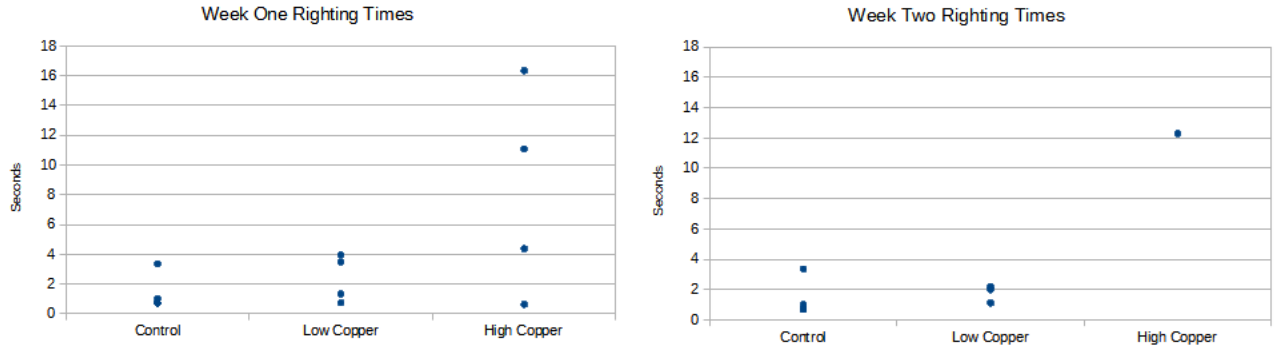

Fig. 1
Righting times in seconds after one and two weeks of exposure. Righting times for crabs from the control group were only measured once, and those values are shown for both weeks. One crab from the low copper group in week two was unable to right itself and is not shown.

The control crabs had righting times of 0.71, 0.71, 1.01, and 3.36 seconds. In week one, the low copper crabs had a similar range of 0.74, 1.33, 3.48, and 3.96 seconds while the high copper crabs had much more variation with righting times of 0.63, 4.38, 11.08, and 16.33 seconds. In week two, the low copper crabs had righting times of 1.11, 2.00, and 2.20 seconds with the fourth crab unable to right itself, and the high copper crab took 12.3 seconds to right itself.

Resazurin

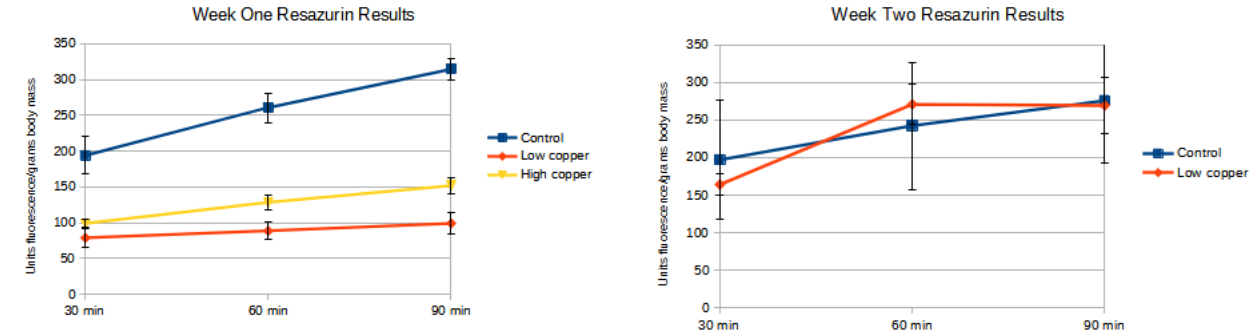

Fig. 2
Resazurin results after one and two weeks of exposure. One high copper crab was removed from the week one data along with two low copper crabs and the only high copper crab from the week two data due to the deaths of those crabs during the assay. Two different sets of control data are shown as several control crabs were tested after each week – both control groups consist of three crabs.

Although the sample sizes are too small to achieve statistical significance, there does seem to be a difference in week one between the control crabs and the crabs in the two copper treatments, with the control crabs seeming to have higher respiration. In week two, there does not seem to be any difference in respiration between the control crabs and the low copper crabs.
Notably, though two low copper crabs were still moving after being removed from the resazurin solution in week two, only one crab had any increase in fluorescence at all between the sample at 60 minutes and the sample at 90 minutes (going from 289.8 units fluorescence/gram to 295.4 units fluorescence/gram). All of the other three low copper crabs had slightly lower fluorescence in the 90 minute sample than in the 60 minute sample. This pattern – an increase in fluorescence between the 30

and 60 minute samples but minimal change in the 90 minute sample – was not observed in any of the other surviving crabs, which all had fairly steady increases between all samples, but was observed in all of the dead crabs.

Threat Response

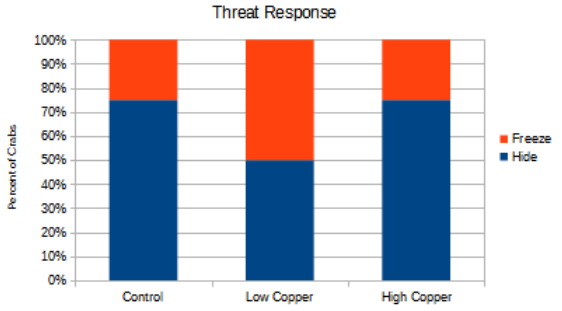

Fig. 3
Threat responses among the treatment groups in the study – in the control and high copper groups, three crabs fled and one froze, and in the low copper group, two crabs fled and two froze.

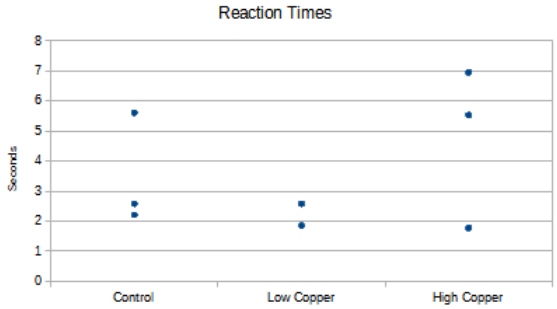

Fig. 4
Reaction times among the crabs that chose to flee in each group.

There was not any notable variation between the groups in terms of response chosen or time taken to react. The low copper group did not have as slow of a slowest reaction time as either the control or high copper crabs, but the group sizes are too small to see any significant differences.

Discussion

Unfortunately, due to both the low initial sample sizes and the high rate of mortalities, the data collected is very limited in scope and lacks statistical significance. Because of this, it is difficult to draw any concrete conclusions, although the results do suggest possible effects from the copper exposure. However, the aforementioned high mortalities do show some effect, and although the sample size was not large enough for statistical significance, the higher maximum righting times in week one among the high copper crabs and in week two among both the low and high copper crabs suggest some form of impairment.

This is in line with previous studies, which have shown copper exposure to result in male European green crabs taking much longer to react to pheromones and to perform specific mating behaviors, as well as performing non-mating behaviors more frequently (Krång et al., 2006). Toxic copper concentrations have also been linked to neurobehavioral effects in other animals, along with dysfunction of other organs (Kumar et al., 2015). While it is not possible to detect the precise nature of the potential impairment from this study, it is likely to be the result of similar impacts to those observed in other species.

There were a number of difficulties with testing the crabs' responses to a threat. The primary difficulty was in the environment, as these tests were taking place in a loud environment where other people were working. Additionally, we needed to be able to observe the crabs at least somewhat, to determine if they had settled down. Because of those factors, the use of a barrier to try and prevent the crabs from being distracted by the outside environment was less effective than ideal, and there was never really a point where the crabs truly calmed down. Instead, the crabs would frequently react to occurrences and

people outside of the testing environment of the tray, and were frequently already somewhat agitated before any predator related disturbance could be simulated.

Along with that, there was some difficulty with adequately measuring the response of the crabs once the disturbance was presented. It is difficult to tell the difference between a crab that freezes initially before deciding to flee and a crab that decides to flee but reacts slowly. We also had no way to measure the speed at which the crabs moved when they did flee, which could potentially have shown a difference between a normal and an impaired reaction.

The resazurin assays also presented challenges as several crabs died during the assays, resulting in less complete data. It is possible that the resazurin assay itself had an adverse effect on the crabs, but with the lack of mortalities among the control crabs it is likely that any effect it has is minimal. The increased stress and physical effects of the copper exposure could potentially have exacerbated ordinarily minor effects of the resazurin assay, but even accounting for that, the primary cause of death in those cases would still be the copper exposure, so I feel confident counting those mortalities among the mortalities caused by the treatment.

While the data in this study is very inconclusive, the impairments that were observed could signal potential difficulty escaping predators and other threats in crabs exposed to copper contamination in the wild. Further work with larger sample sizes and a more effective methodology focusing on the ability of the crabs to evade threats could shed more light on how copper contamination affects the ability of these crabs to respond to and escape from danger.

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