Sophia Eckhart
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Physiological stress responses in shore crabs (*H. Oregonensis)* from prolonged caffeine exposure

Introduction:

Crabs serve an important role within the intertidal through predator-prey interactions, effect on fisheries and bioindicators (Lorda et al., 2016; Aguirre-Martínez et al., 2012). With anthropogenic influence, many different pollutants enter the watershed through waste water, including pharmaceuticals like NSAIDs, antidepressants and caffeine (Aguirre-Martínez et al., 2012). Caffeine is incredibly abundant within the environment and has been shown to have many negative impacts within different marine organisms, including crabs (Li et al., 2020). *Hemigrapsus oregonensis*, or the yellow hairy shore crab, is a small shore crab found within the intertidal, ranging from Alaska to Baja California and often used as a study organism for laboratory experiments as a proxy for fishery important crabs like *Carcinus magister* or invasive crabs like *Carcinus maenas* (Wicksten 2011; Rodrigues & Pardal, 2014).

Caffeine has been shown to affect crabs in several different ways across different cells within the body. Caffeine changes the permeability and polarity of membranes and decreases the energy needed for the activation for a variety of different cellular processes. Exposure to high concentrations of caffeine leads to significant decreases in the lysosomal membrane stability (Aguirre-Martínez et al., 2012). In the hepatopancreas, a digestive gland within crustaceans, exposure led to damage to the DNA, increases in antioxidative responses, lipid peroxidation which damages lipids in cell membranes, and inhibition of acetylcholinesterase activity which is suppose to terminate nerve impulses. (Baracchini et al., 2024).

Caffeine induces tension development within depolarized muscle, generating contractures as the muscle mechanical threshold is lowered. This is because caffeine accelerates the release of Ca2+ from the sarcoplasmic reticulum in the muscles and suppresses the active binding of mycoplasma Ca2+ (Huddart, 1969). Similar to the muscles, caffeine affects the endoplasmic reticulum which leads to an increased flux of intracellular heavy metals such as Cd and Cu in gill cells through the Na+/K+-ATPAse transporter in the cell (Ortega et al., 2014; Sá & Zanotto, 2013).

This study will look at how different environmental concentrations of caffeine will affect the physiological stress response in *H. oregonensis* by exposing crabs to three different doses of caffeine over the course of a 14-day experiment. To test how chronic stress from exposure will impact *H. oregonensis*, oxygen consumption will be evaluated, behavioral data like righting time will be recorded and hemolymph assay for concentrations of L-Lactate will be performed. Oxygen consumption will reflect the cellular respiration and energy needs of the crabs while L-Laccate will represent the anaerobic respiration that occurs within the crab. The hypothesis is that with an increase in caffeine concentrations, there will be higher rates of oxygen uptake, an increase in the concentration of lactate in the hemolymph and longer righting times from the increase in energy demands.

Methods:

H. oregonensis individuals were collected from the Puget Sound and briefly accumulated to a control environment. Three 2.5 L tanks were filled with a lab-prepared seawater with a salinity of 30 ppt. Six crabs were placed in each tank with a group of control crabs that were kept separately. To dose the three treatments with caffeine, 200 mg caffeine capsules were dissolved into a concentrated solution of water and that was then diluted by adding to the 2.5 L tanks to

produce concentrations of 5 ug/L, 15 ug/L and 20 ug/L. The water in the tanks was replaced and re-dosed with the same concentrations of caffeine half way through the experiment. All crabs were fasted through the entirety of the experiment.

After a week under environmental concentrations, the righting time and respiration of the crabs were recorded. To assess the ability for a crab to right itself, or the righting time, a crab is picked up and placed on its back under the water and timed to see how long the crab took to flip on its front. This was done for each crab across all three treatments. Respiration was found using a resazurin assay which works through the consumption of oxygen which reduces resazurin to resorufin, a fluorescent dye. A working resazurin solution was prepared from a stock solution and 35 mL was transferred into crab chambers. One crab from each treatment was patted dry and weighed to normalize results later, Crabs were then placed in chambers that were then covered. Over the course of 90 mins, a sample of the resazurin from each chamber was taken every 30 minutes and placed into the wells of a labeled well plate. Crabs were then removed from the chambers and rinsed off with saltwater and placed back into their previous tanks. The plate was then read by an Agilent BioTek Synergy HTX Multi-Mode Microplate Reader at Excitation 530; Emission 590 to measure fluorescence values. A linear regression of the fluorescence values for each sample was performed to quantitatively compare the rate of change in oxygen consumption over the 90 minutes.

Hemolymph for the biological assasses were collected on the last day of the experiment, along with righting time and respiration rate again. Two crabs from each experiment were sampled. Hemolymph was extracted through the use of a syringe through the second leg joint of the crab. Hemolymph was injected into a vial and centrifuged to collect at the bottom in preparation for testing. The hemolymph was then used to run assay to determine L-Lactate concentration. After the samples were prepped, 20 ul of each sample was added to the wells of a well plate with 100 ul of Assay Buffer (1X), 20 ul of Cofactor Mixture and 20 ul of lactate substrate. The reactions were catalyzed by 40 ul of Enzyme mixture being added to all wells. The plate was then covered and run to get fluorescence values of the samples which were corrected and then used to determine the L-Lactate (uM) of the samples. To compare L-Lactate concentration across treatment groups, a single-factor ANOVA test was used.

Results:

Over the course of the experiment, there were several mortalities. In the first week, two crabs treated with a medium dose of caffeine and two crabs treated with a high dose died, one of which was cannibalized following their death. In the second week, a crab from the high dose and one from the low dose died through escaping the tank into the water table and water filtration system which had freshwater.

The hemolymph extracted on day 14 was only enough to run one L-Lactate assay. The crabs in the high dose and low dose treatments had slightly more elevated levels of L-Lactate, while the control group had a significantly greater elevation in lactate (Fig.1q). When a single-factor ANOVA test was run on the concentrations of L-Lactate (uM) in the control groups and treatments which found a significant difference between groups ($p>0.05$).

Righting time across the three treatments varied, with several outliers that took longer to right themselves than others (Fig. 1b). After one week, it took crabs with the medium dose longer than the other treatments to right themselves. For the low dose and high dose crabs, they had a longer righting time after the second week compared to the first week, but for the medium dose crabs the righting time was very similar to the first week, if not the same. It took the low

dose crabs the longest to right themselves after two weeks under experimental conditions. However, when observing the behavior of the crabs, at least one of the high dose crabs had muscle contractures of their swimmerrates, or rear, non-walking legs, observed through the non-natural positioning of the legs.

A linear regression was used to assess the different rates of oxygen consumption found from the resazurin assay value. After one week, it showed that the crab under the high dose treatment had the highest rate consumption of oxygen while the control crab had the lowest rate consumption (Fig.1c). This trend was reversed after the second week, with the low dose crab consuming the highest amount of oxygen and the high dose treated crab consuming the least amount (Fig. 1c).

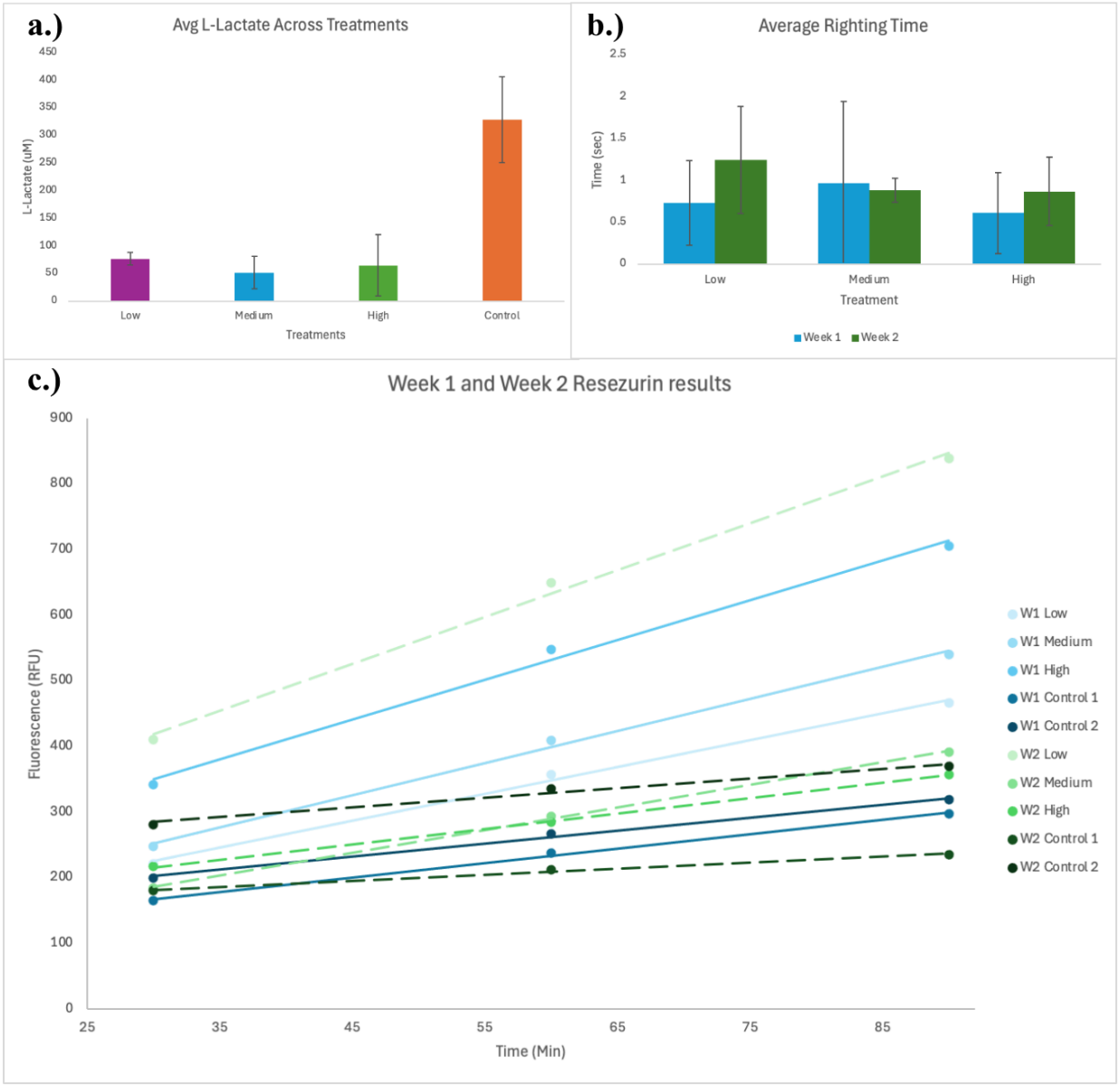

Figure 1. Physiological and behavioral assessments of *H. oregonensis* after one to two weeks under varying concentrations of caffeine. **a.)** The average concentration of L-Lactate (uM) on the

hemolymph after two weeks under treatment conditions. **b.)** The average with standard deviations for righting time across treatments for week 1 and 2. **c.)** Linear regressions of the resazurin results after a week and two weeks. W2 Low has the highest rate of consumption with a slope of 7.14.

Discussion:

This paper aimed to evaluate how varying levels of environmental caffeine concentrations affected the stress response of *H. oregonensis* over a 14-day experiment. To do this, crabs were treated with different concentrations of caffeine and stress response were tested through hemolymph assays, respirometry and behavioral observations like righting time.

After two weeks under stressful conditions, the crabs treated with caffeine would have an increase in metabolic rate to compensate for decreased lysosomal stability, changes in enzyme and nerve activity, and osmoregulation (Aguirre-Martínez et al., 2012; Baracchini et al., 2024; Sá & Zanotto, 2013). At the week one mark, this was seen through the elevated levels of oxygen consumption, indicating an increase in aerobic respiration especially seen under high concentrations of caffeine, but also across all treated groups compared to the control. However, this trend was reversed after two weeks as both the medium and high dose crabs had very similar rates of oxygen consumption compared to the control crabs. This may indicate that at a certain point, the medium and high dose crabs could not maintain the increase in respiration and no longer had an elevated uptake in oxygen, while the low dose crabs had yet to reach that point.

A trend of increased energy needs could be seen in the hemolymph assay as the low, medium and high dose crabs had low concentrations of lactate compared to the control crabs. Given that fermentation is significantly less energetically efficient compared to aerobic respiration, if the crabs were no longer able to maintain increased oxygen consumption, they likely would have also exhausted the use of fermentation as a way to meet their additional needs (Hill et al., 1991). The control crabs were not chronically stressed and could compensate for short-term increased energy needs through the use of lactic acid and lactate.

There were some indications of chronic stress and caffeine on the crabs through their behavior, such as the contractures of the muscles within the swimerrates of the high dose crabs which is likely from changes in activation potential of muscle cells with caffeine (Huddart 1969). Other behaviors included crabs clinging to the tubing of the aeration device within the tanks and two crabs escaping through the tank lid which likely is from chronic stress.

One of the main limitations of the study was the sample size used, there were only six crabs for each treatment group and as mortalities happened over the course of the experiment the number of data points for analysis also decreased. Because of this, hemolymph was only extracted after week 2, to prevent any mortalities related to the process. There was also only enough hemolymph extracted for one biological assay to be performed which led to a limited scope for the understanding of the physiological processes that were occurring throughout the experiment.

Building off of these studies and previous studies on caffeine concentrations and crabs, a critical tissue level threshold for caffeine concentrations can be evaluated to identify the concentration of caffeine where a crab will no longer survive at. Also, a longer, longitudinal study looking at caffeine exposure over 30-days or more could allow for a better understanding on how long term exposure to lower doses of caffeine impacts crabs like the concentrations seen from wastewater run-off.

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