Caffeine Concentrations on the Stress Response of *Hemigrapsus Oregonensis*

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Introduction

Caffeine is among the most widely consumed central nervous system stimulants with an estimated ~80% of the global population having at least one caffeinated beverage daily (Heckman et. al., 2010). However, the prevalence of this stimulant means that caffeine is emerging as a contaminant of concern in the aquatic environment. Scientists have identified caffeine as one of the most ubiquitous pharmaceutically active compound pollutants in the environment, meaning that very little oceanic life avoids its toxicologic impact (Li et. al., 2019). Caffeine primarily finds its way into water bodies via wastewater, especially from human excretion. Wastewater treatment facilities can successfully and efficiently remove caffeine from wastewater, but current facilities have been outpaced by production (Mustard, 2013) (Li et. al., 2019). Due to caffeine's utilization by humans, it is used as a marker of anthropogenic activities and pollution in a given water body (Hillebrand et. al., 2012). Long-term exposure to caffeine results in accumulation within marine life tissues, with relevant amounts reported in coral, fish, mussels, and algae (Vieira et. al., 2022).

Caffeine has significant detrimental effects on marine species including genotoxicity, oxidative stress, lipid peroxidation, neurotoxicity, mortality, and reproductive and growth inhibition (Vieira et. al., 2022). The presence of caffeine in the water negatively influences species' abundance, biomass, and fitness. This not only impacts individual species, but the integrity of an ecosystem as a whole. Food webs can be thrown into disarray when caffeine is introduced, as species will have reproduction complications, increased stress levels, and requiring more food consumption and oxygen (Vieira et. al., 2022). Caffeine has a detriment to countless marine species in many different ways and the stress experienced by an organism leads to negative shifts in ecosystem dynamics, as all these species are intertwined.

Crabs are essential to ecosystem dynamics, playing an integral role in the intertidal as both mobile predators and abundant prey. Crustaceans are important to scientists as well, since their abundance and role as bioindicators allows for ease in testing whether environmental changes alter biological functions and stress responses. Caffeine has been found to primarily alter crabs' digestive tract, muscle functions, and gill membrane. It depolarizes membrane potential, blocking delayed K+ conductance within the neuronal membrane (Hermann, 1981). In the hepatopancreas, the digestive gland of arthropods, caffeine exposure led to damage to DNA, higher antioxidative responses, lipid peroxidation, and inhibition in the termination of nerve responses (Baracchini et. al., 2024). Within the muscles, caffeine has been found to induce tension in depolarized muscle and cause contractions. This is due to caffeine accelerating the release of Ca2+ and suppressing the active binding of mycoplasma Ca2+ (Huddart, 1969). The presence of caffeine also causes a release in calcium from the endoplasmic reticulum, resulting in a flux of intracellular cadmium and copper within gill cells, affecting its sodium-potassium pump (Ortega et. al., 2014; Sa and Zanotto, 2013).

In Washington state, a prolific crab within the rocky intertidal is the shore crab *Hemigrapsus oregonensis*. Our study aims to assess how different environmental concentrations of caffeine facilitated physiological stress responses within *H. oregonensis.* By answering this question, we will be able to determine the limit of caffeine that these crabs can endure and what responses they have to the foreign pollutant. Using the data we gather, we will be able to monitor *H. oregonensis* within the intertidal and recognize when individuals or ecosystems may be exposed to debilitating or even fatal concentrations of caffeine within the water.

We hypothesized that higher environmental concentrations of caffeine will have an increased effect on the stress response in *Hemigrapsus oregonensis.* To test this hypothesis, we

designed an experiment in which multiple *H. oregonensis* crabs will be exposed to low, medium, and high concentrations of caffeine dissolved into their water. We will collect data regarding their righting time, dissolved oxygen consumption, and lactate concentration within crab hemolymph, as well as record behavioral observations and mortalities, to determine whether higher doses of caffeine result in increased stress responses.

Methods

This experiment was conducted over the course of 14 days, in three 2.5 L tanks. The temperature and salinity of the tank water was kept at constant, controlled values. 100mg caffeine capsules (Nutricost brand) were emptied to obtain caffeine powder. Caffeine powder was added to a beaker of water and stirred until dissolved. One tank was treated with 5 μg/L of caffeine, amounting to an addition of 1.5 mL of the dissolved caffeine solution. Next, a second tank was treated with 15 μg/L of caffeine, resulting in an addition of 4.5 mL dissolved caffeine solution. Lastly, 6 mL of the caffeine solution was added to the third tank, about 20 μg/L of caffeine. These tanks represented our low, medium, and high dose treatments, respectively. After 7 days of exposure, the water and caffeine treatments within each tank were replaced.

Hemigrapsus oregonensis crabs were supplied by the University of Washington and obtained by cages in the intertidal. Crabs selected for this experiment were of similar size and length. 6 crabs were added to each of the three test tanks. A control tank was set up as well, though this tank was communal for all studies participating in this labspace. These crabs were maintained at the same water temperature and salinity as well.

Tests for righting time were conducted to observe stress and the effect of caffeine on locomotion. Each individual crab was placed on their back within their test tank. The time it took each crab to flip over was measured and recorded to the hundredth of a second. Righting time tests were conducted for all three crab populations after 7 and 14 days of caffeine exposure.

Resazurin tests were conducted to determine the oxygen consumption and metabolic rate of the crabs. A crab was selected from each caffeine treatment for testing. First, individual crab chambers were prepared by loading 35 mL of resazurin working solution into each. Crabs were patted dry and weighed to the nearest hundredth of a gram. Once crabs were placed within their respective chambers, a timer was set. Every 30 minutes, 200 μL were withdrawn from each chamber and placed within the wells of a 96 well plate. After an hour and thirty minutes, crabs were withdrawn from their chambers and washed thoroughly with 33-35 ppt seawater. The 96 well plate was run in an Agilent BioTek Synergy HTX Multi-Mode Microplate Reader at Excitation 530; Emission 590 to obtain fluorescence values. All fluorescent values were divided by individual crab weight to normalize. Resazurin tests were performed after 7 and 14 days of caffeine exposure.

Hemolymph extraction was performed by inserting a needle into the base of their back walking legs. Extracted hemolymph was deposited in a 6 microtube strip. Each treatment had hemolymph extracted from two crabs. Once all hemolymph was extracted, the microtube strip was placed in the freezer to be kept until testing. Hemolymph was extracted from three control crabs and deposited in a separate microtube strip. Tests were run on extracted hemolymph to determine the lactate levels present within each sample. Lactate was selected to assess increases in aerobic respiration associated with an increased stress response. Each hemolymph sample was run twice, regardless of the amount present. Hemolymph extractions were only conducted after 14 days of caffeine exposure, due to the risk of mortality and limb loss associated with extractions.

Behavioral observations were recorded to assess how exposure to caffeine affects crabs' individual responses and community dynamics. Behavior was recorded both in notes and with videos. Mortality throughout the experiment was documented to assess the possible fatality of caffeine exposure to crabs.

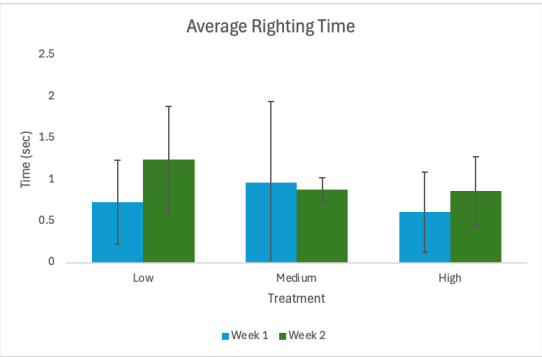
Results

The effects of environmental caffeine exposure on the physiological stress responses of crabs were assessed through measurements of righting time, dissolved oxygen consumption, and lactate concentration, as well as observations of behavior and mortality across different caffeine concentrations. Crabs exposed to different doses of caffeine had different average righting times (Fig 1.). Between the first and second weeks of the experiment, the average righting time of the low and high doses decreased. In contrast, the average righting time of the medium dose increased between the first and second weeks of the experiment. During week 1 of testing, the shortest righting time was observed in the high dose (0.18 secs) and the longest was observed in the medium dose (2.4 secs). During the second week of testing, both the shortest and longest righting times were observed in the low dose (0.54 and 2.26 secs, respectively).

Overall, the fluorescence of resazurin increased as time went on during the experiment (Fig 2). During week 1 of testing, the highest fluorescence was observed in the high dose with 707 RFU after an hour and a half. During the second week of testing, the low dose had the highest overall fluorescence of 840 RFU after an hour and a half. The lowest fluorescence observed was found in the control crabs across both weeks.

Lactate concentrations were taken from all four testing groups' hemolymph (Fig 3.). The control group had the highest lactate concentrations, averaging around 257 µM. Between the three dose groups, crabs within the high dose had the highest overall lactate concentrations (167 µM).

Mortality was the highest among the high dose caffeine group (Fig 4.). Within the high dose, 3 crabs died across 14 days of testing. The medium dose group had 2 mortalities in the first 7 days of testing and the low dose group had 1 mortality on the 14th day of testing. Notable behavioral observations include escaping, cannibalism, and muscle constrictions. On the 7th day of testing, we observed crabs attempting to escape their tanks by climbing oxygen tubes. Our 2 mortalities in week 2 were a result of low and high dose crabs escaping. In the high dose tank, a crab had died and appeared to be cannibalized by its tankmates. Finally, we observed several crabs in the medium and high doses with tensed exterior walking legs during day 14. Crabs in the high dose appeared to be more lethargic as the experiment went on, as they didn't struggle or fight back as hemolymph was extracted, opposed to the low and medium dose crabs.



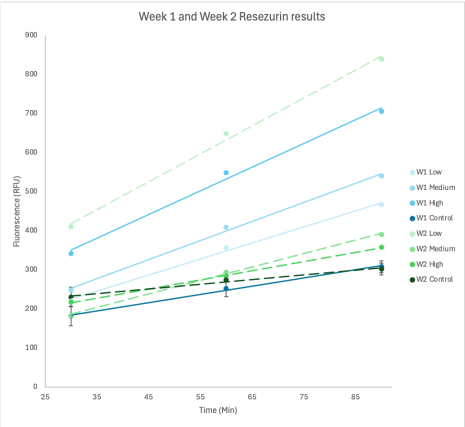


Fig 1. (left) Average righting time of three test groups between week 1 and week 2, measured in seconds. **Fig 2. (right)** Resazurin results for four test groups over 2 weeks. Week 1 values are solid blue lines, week 2 values are dashed green lines.

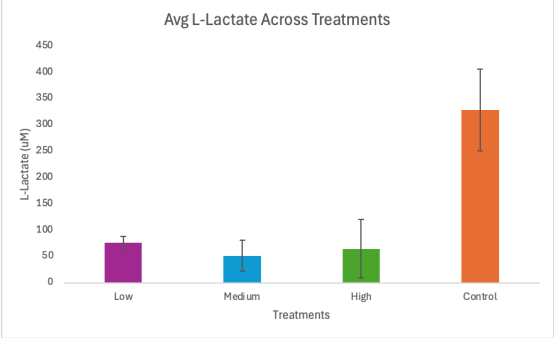


Fig 3. (left) Average concentration of lactate (μM) over four test groups. **Fig 4. (right)** Mortality in three test groups over the duration of two weeks.

Discussion

In this experiment, the aim was to answer whether environmental concentrations of caffeine facilitate physiological stress responses in *H. oregonensis*. Results indicate that there is indeed a stress response triggered by the presence of caffeine. For the low and high dose groups, we found that righting times decreased between the first and second week. This indicates that the longer these crabs were exposed to caffeine, the harder it became to flip themselves back over. This could be due to a lethargic state brought about by constant energy usage in a high stress state or muscle constrictions making it difficult for these crabs to maneuver their limbs. The medium dose test group had longer righting times in the first week compared to the second, although this is likely due to an individual in this group having the highest righting time overall. This individual could have been an outlier, but since we didn't measure righting time more than once a day, we had a small dataset to pull from. For future experiments, taking righting time more often may lead to more accurate, decisive results.

Resazurin results indicated a reverse trend in week 2 compared to week 1. In week 1, the highest rate of O2 consumption was done by the high dose crab, but in week 2 the highest rate was done by the low dose crab. This could be due to the lethargic state taken on by the high dose crabs on the 14th day of the experiment, indicating that these crabs may have been close to the end of their lives. Low dose crabs weren't exposed to as much caffeine, so their high O2 consumption could be due to panic brought on by the resazurin testing procedure.

Lactate concentrations were highest in the control group; however, these crabs were continuously exposed to stressors. Unlike the test tanks prepared for this experiment, the control tanks were communal for all testing groups within the facility. This means that there were lots of hands reaching in the tank and pulling crabs out, which could induce a high stress state. Among our three testing groups, the high caffeine dose crabs had the highest lactate concentration, indicating that there was a high metabolic stress response to this dose.

Mortality was highest in the high dose group, due to the high stress response that this dose brings about. Overall, 4 crabs died during the first week of exposure, one of which being cannibalized by the remaining 4 crabs in the high dose. This cannibalization could be a result of a requirement for energy intake, as all crabs in this experiment were starved. Higher metabolic rates induced by caffeine exposure could result in crabs requiring more food to sustain their needs. On the 14th day of testing, 2 crabs died by escaping their tanks and falling into waste

collection bins beneath the testing site. We observed crabs climbing the oxygen tube before, which is likely how these 2 escaped. This is reason to believe that caffeine concentrations within the water induced such a high stress response that the crabs escaped their tanks to be free from it, even if this resulted in a swift death shortly after. Crabs within the medium and high doses were also observed having constricted back legs and struggling to walk. This suggests that high levels of caffeine exposure causes uncontrollable muscle contractions, constricting limbs to the point that they're immovable.

While this test is ultimately limited due to the small sample sizes of each group, we have found that the presence of environmental caffeine in *H. oregonensis* crabs' waters may induce major behavioral, metabolic, and physiological stress effects. Furthermore, the higher the concentration of caffeine, the higher the stress response and mortality rate. As mortality and stress responses are found across all three treatments, this suggests that even small concentrations of caffeine can cause adverse health problems in crabs. This may have significant implications for crabs in the wild. Studies have found that the rate of caffeine that is being added to water systems is higher than the rate of removal via wastewater treatment (Li et. al., 2019). Accumulation of caffeine within bodies of water is a result of this unsteady state. Caffeine also accumulates within marine tissues. Along with this accumulation, crabs that live close to urban infrastructure are likely to experience higher levels of caffeine within the water. Runoff and sewage spills are major ways in which the wastewater containing caffeine reaches these water systems (Vieira et. al., 2022). With constant additions of caffeine from human pollution and accumulation within their own tissues, we will see compromised crab health over time, worsening the longer these crabs are exposed.

Along with this, caffeine doesn't only affect crabs. Fish, mussels, algae, and coral have all been found with relevant amounts of caffeine accumulation within their tissues (Vieira et. al., 2022). These animals are all at the very core of their ecosystems. Crabs, fish, and mussels are all important consumers, but are also food for secondary predators and humans. Algae are primary producers, laying the very foundation for food webs. Coral populations provide habitats for entire ecosystems. If these integral pieces of ecosystems suffer health complications and increased mortality rates because of the overabundance of caffeine within the water system, we will see entire ecosystems and habitats collapse. This study doesn't just show that caffeine facilitates stress responses, it shows that actions must be taken to minimize the damage that caffeine concentrations cause to ecosystems. By using crabs as a bioindicator, we can catch when caffeine has begun to seep into habitats before it begins a cascading effect. Caffeine is far too prevalent in our bodies of water for us to proceed further without putting a plan into action to reduce runoff into water systems and monitor habitats for caffeine exposure.

Citations

Baracchini, C., Messager, L., Stocker, P., & Leignel, V. (2024). The Impacts of the Multispecies Approach to Caffeine on Marine Invertebrates. *Toxics*, *12*(1), 29. https://doi.org/10.3390/toxics12010029

Heckman, M. A., Weil, J., & de Mejia, E. G. (2010). Caffeine (1, 3, 7-trimethylxanthine) in Foods: A Comprehensive Review on Consumption, Functionality, Safety, and Regulatory Matters. Journal of Food Science, 75(3), R77–R87. https://doi.org/10.1111/j.1750-3841.2010.01561.x

Hermann, A. (1981). Action of caffeine on pyloric motorneurons in the crustacean stomatogastric ganglion. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 69(2), 191–197. https://doi.org/10.1016/0306-4492(81)90128-3

Li, S., Wen, J., He, B., Wang, J., Hu, X., & Liu, J. (2020). Occurrence of caffeine in the freshwater environment: Implications for ecopharmacovigilance. Environmental Pollution, 263, 114371. https://doi.org/10.1016/j.envpol.2020.114371

Mustard, J. A. (2013). The buzz on caffeine in invertebrates: effects on behavior and molecular mechanisms. Cellular and Molecular Life Sciences, 71(8), 1375–1382. https://doi.org/10.1007/s00018-013-1497-8

Ortega, P., Custódio, M. R., & Zanotto, F. P. (2014). Characterization of cadmium plasma membrane transport in gills of a mangrove crab Ucides cordatus. Aquatic Toxicology, 157, 21–29. https://doi.org/10.1016/j.aquatox.2014.09.006

Sá, M. G., & Zanotto, F. P. (2013). Characterization of copper transport in gill cells of a mangrove crab Ucides cordatus. *Aquatic Toxicology*, *144-145*, 275–283. https://doi.org/10.1016/j.aquatox.2013.10.018

Vieira, L. R., Soares, A. M. V. M., & Freitas, R. (2022). Caffeine as a contaminant of concern: A review on concentrations and impacts in marine coastal systems. Chemosphere, 286(2), 131675. https://doi.org/10.1016/j.chemosphere.2021.131675