Effects of temperature on the immune response of *H. oregonensis*

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Introduction

The Environmental Protection Agency (EPA) recorded 2023 as the warmest year on record for sea surface temperatures as anthropogenic climate change continues to drive up global warming (U.S. Environmental Protection Agency [EPA], 2023). This warming trend is accompanied by an increase in the frequency and intensity of climate extremes, including marine heatwaves (Oliver et al, 2018). Heatwaves can dramatically alter marine ecosystems since many marine organisms are sensitive to changes in temperature (Oliver et al, 2018). Among the most visible impacts are disruptions to species distributions, reproductive success, metabolic rates, and susceptibility to pathogens. These impacts can especially be seen in ectotherms like crabs, whose physiological functioning is dependent on external temperatures (Chisholm & Smith, 1994).

These climate change-driven impacts are not only ecological but also economic. In 2018–2019, the Bering Sea snow crab (*Chionoecetes opilio*) population declined by 90% (Litzow et al, 2024). Before the collapse, the fishery had an ex-vessel value of approximately $227 million annually (Litzow et al, 2024). Scientists still debate the exact cause of the snow crab population collapse, but it was likely caused by the abnormal amounts of marine heatwaves that occurred in the Bering Sea that year (Litzow et al, 2024). Increased temperatures from heat waves could have led to starvation due to an increased metabolism, or a lack of suitable habitat could have caused the crabs to die off (Szuwalski et al, 2023). Notably, warmer ocean conditions may weaken crabs immune responses, leaving them vulnerable to pathogens and diseases such as the bitter crab disease (BCB) (Litzow et al, 2024). Invertebrate immune systems, which rely on hemocytes for pathogen defense, are at risk of thermal stress, with extreme temperatures shown to suppress immune function and increase disease susceptibility (Adamo 2012; Shields 2019; Truscott and White, 1990).

Research suggests that temperature-related stress can lower the threshold for infection and intensify host-pathogen interactions (Truscott & White, 1990). Decreases in host immunity and range/more individuals in closer quarters combined with an increase in pathogen susceptibility all warn of a potential disease (Litzow et al, 2024). These changes in host immunity, pathogen transmission, and habitat ranges caused by heat waves are cause for concern regarding commercially important species like the Dungeness crab (*Metacarcinus magister*). It's possible that if a marine heatwave were to occur in Washington waters the Dungeness crab could face a similar collapse. There are currently no studies investigating the coupling effects of rising temperatures and disease susceptibility in Washington's crabs.

To address this gap, this study uses the hairy shore crab (*Hemigrapsus oregonensis)* as a proxy to investigate immune responses under thermal stress. By subjecting *H. oregonensis* to pathogens and higher temperatures, immune response and stress can be measured using hemocyte counts and a resazurin assay. This species inhabits overlapping habitats with Dungeness crabs, and they are physiologically similar. Understanding how rising temperatures affect *H. oregonensis*' hemocyte concentrations and stress levels when exposed to pathogens will provide insight into how disease susceptibility in related species may change under future ocean conditions.

Methods

3 10x20x15cm tanks were set up each with 6 crabs in them. Two of the tanks were kept at 27 °C and the other was kept at 13°C. These temperatures were chosen as 13°C is the average temperature for shallow waters in Puget Sound and 27°C is near the thermal extreme that *H.*

oregonensis can tolerate (Dehnel, 1960). Mud/sand was collected from Golden Gardens Park in Seattle Washington on April 25th, 2025 around 1 pm and put in one 27°C tank and one of the 13°C tanks, the mud-covered the bottom of the tank and rose 1.5 inches off the bottom. Mud was used to introduce pathogens to the crabs. The mud collected from their natural environment is almost certain to contain naturally occurring pathogens for these shore crabs, which also live in Golden Gardens (Karbasdehi et al, 2017). 1.5 liters of 33 ppm saltwater was added to each tank. There was also a control tank with no mud at 13°C that had control crabs for various experiments. Shells were also placed in the tanks to provide shelter and minimize stress.

After one week of exposure to the pathogens 2 crabs from each treatment were subjected to a resazurin-based metabolic assay. The resazurin solution was prepared by combining 0.5 g resazurin salt, 10 mL deionized water, and 10 µL dimethyl sulfoxide (DMSO). For the resazurin working solution, 148 mL of DI water mixed with Instant Ocean to 23–25 ppt salinity was combined with 333 µL of resazurin stock solution, 150 µL DMSO, and 1.5 mL of an antibiotic solution (Penicillin/Streptomycin and Amphotericin B Fungizone). Each crab beaker required 35 mL of working solution.

The metabolic activity of shore crabs (*Hemigrapsus oregonensis*) was assessed using a resazurin assay. Each crab was dried with paper towels and weighed to the nearest 0.01 g using a milligram-scale balance to allow for the normalization of metabolic activity by mass. Individual crabs were placed in beakers containing 35 mL of the resazurin working solution and a timer was started. Every 30 minutes, 200 µL of the solution was pipetted and transferred to the chambers of a 96-well culture plate. This process was repeated 3 times for each crab, once at 30 minutes, 60, and 90 minutes. Following the final sampling time point, crabs were removed using gloved hands, rinsed with 33–35 ppt saltwater, and returned to holding tanks. Crabs that completed the assay were separated from those that had not by a mesh fence placed in the middle of the tank. Fluorescent values were obtained by running the plate at Excitation 590 and Emission 590. Fluorescent values were then normalized by crab weight. This process was repeated a week later with different crabs.

Hemolymph was extracted from the joint connecting the legs to the carapace. After hemolymph was extracted crabs were separated by a mesh fence to keep track of those who had been tested. Hemolymph extraction sometimes leads to mortalities. Hemocyte concentrations were counted using a hemocytometer. .1µL was placed in the hemocytometer and the count was multiplied by 10 to get the concentration per µL.

Results

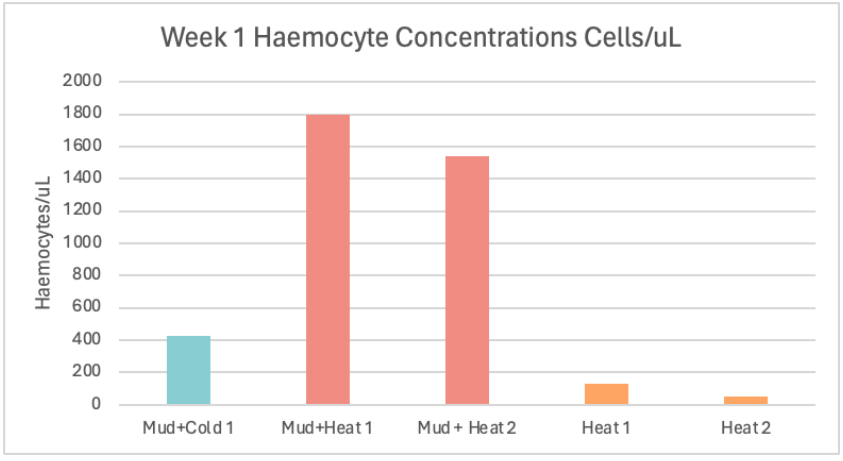

Fig. 1 Hemocyte counts per µL in different treatment groups after 1 week of exposure.

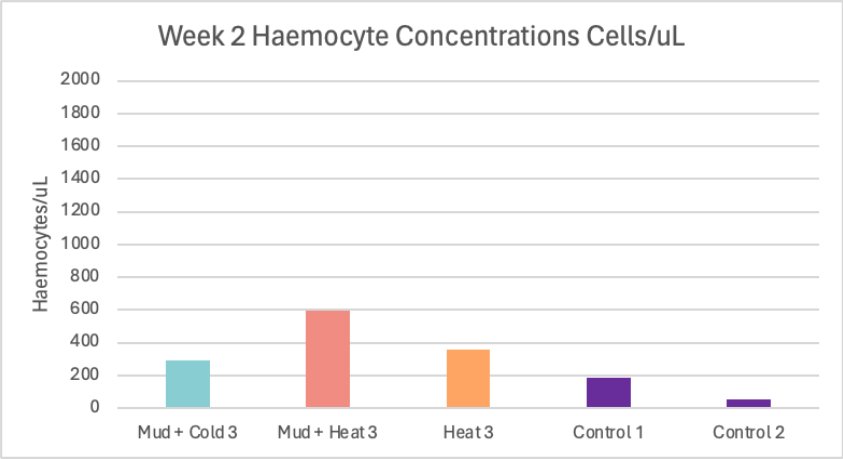

Fig. 2 Hemocyte counts per µL in different treatment groups after 2 weeks of exposure.

In Week 1, hemocyte concentrations varied visibly across treatment groups. The Mud + heat 1 and mud + Heat 2 groups exhibited the highest hemocyte counts, approximately 1800 and 1500 cells/µL respectively, indicating a strong immune response to combined thermal and pathogenic stress (Fig. 1). In contrast, the mud + cold treatment group showed a much lower hemocyte concentration, around 400 cells/µL, and the groups exposed only to heat had significantly smaller counts ranging from ~150 to ~50 cells/µL suggesting that heat stress alone did not trigger a substantial immune activation and that mud without elevated heat did cause an immune response of a smaller magnitude compared to mud and heat.

Hemocyte concentrations declined across all groups by week 2. The mud + heat 3 group still had the highest concentrations but had dropped to approximately 600 cells/µL. The mud + cold group also declined to ~ 300 cells/µL. Average hemocyte counts rose for the crabs in the 27°C tank to approximately 350 cells/µL. Control groups had hemocyte counts below 200, suggesting that to be a baseline for crabs not undergoing thermal stress or immune response (Fig. 3). Overall, these results suggest that combined mud and heat exposure initially induce the strongest immune response, which subsides by Week 2, potentially suggesting an immune suppression with temperature.

The resazurin assay showed that the crabs treated with pathogens in the 13°C water had the highest relative fluorescent unit (RFU) values (Fig. 3). A higher RFU means that more oxygen is being consumed. Higher oxygen consumption would imply a higher metabolic rate, which would make fighting an infection more difficult (Szuwalski et al, 2023). It should also be noted that the error bars for this treatment were the highest, and with a small sample size of 3 crabs, an anomaly is possible. There was no statistically significant difference between the other groups, however, the mud/pathogen+heat group is seen to have the slowest metabolism(Fig. 3).

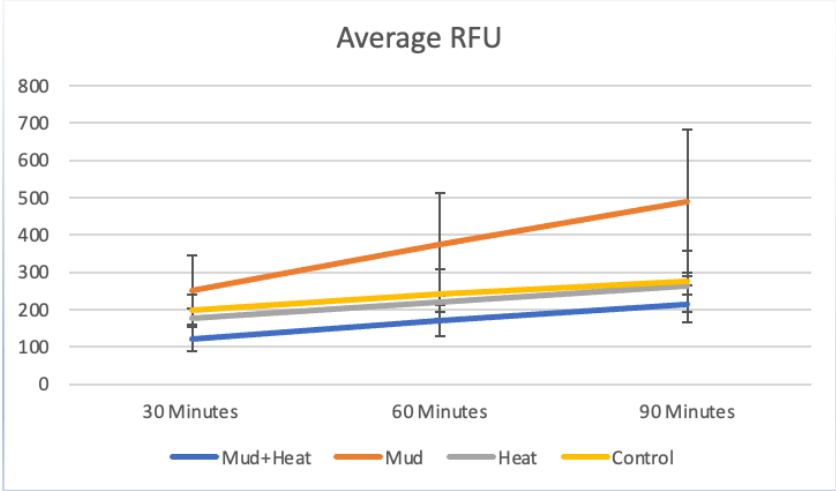

Fig. 3 Relative fluorescent units (RFU) of the different groups over time normalized by weight—average RFU values of 3 crabs per group, measured at 30, 60, and 90 minutes.

Discussion

The results showed that increased temperature combined with the introduction of pathogens significantly increased the hemocyte counts in *H. oregonensis*. The hemocyte counts in the mud + cold treatment were double that of hemocyte counts in treatments not containing mud. This immune response in mud groups suggests that there were pathogens present in the mud that infected the crabs. The heightened response in the mud + heat group suggests that the increase in heat leads to a more severe infection (Qin et al, 2012). This supports the hypothesis that elevated temperatures, such as marine heatwaves, will lead to more infections in crabs, and potential die-offs. By Week 2, the hemocyte counts in both mud treatment groups had gone down. This could suggest that the pathogens were being successfully fought, or that immune suppression was beginning to onset. In the future, a longer study should be done to determine the cause of the hemocyte decline over time. These findings align with existing literature which shows that invertebrate immune response can be suppressed by heat, and that heat leads to higher disease susceptibility (Adamo 2012; Shields 2019).

The resazurin assay results were less clear than the hemocyte counts. The mud + cold group showed the highest metabolic rate, potentially reflecting increased energy expenditure for immune activation at tolerable temperatures. In contrast, the mud + heat group showed the lowest metabolic rate, which could point toward metabolic suppression (Shields, 2019). This could be due to thermal stress overwhelming the crab's physiological capacity, as previously documented in crustaceans at thermal extremes (Dehnel, 1960). Unfortunately, high error bars and limited replicates (n=3) weaken the statistical power of these findings, and future studies should incorporate larger sample sizes to reduce the effects of individual variation.

Along with larger sample sizes, future studies should use replicate tanks to be sure that treatment effects could be confounded by tank-specific conditions. Future studies could also benefit from using confirmed samples of pathogens instead of natural mud/sand. In this study, the actual pathogen content was not quantified which led to some uncertainty in the results that would not occur if specific known pathogens were used to isolate immune responses better. Despite these limitations, this study offers preliminary insight into how heat waves could negatively impact immune function in Washington crab species. Given that *H. oregonensis* shares habitat and physiological traits with *M. magister*, these findings raise concerns about the

vulnerability of Dungeness crabs under further warming scenarios. If rising ocean temperatures reduce immune function and increase stress, commercial crab populations could face similar risks to those experienced by the Bering Sea snow crab (Litzow et al, 2024). Even if the pathogen is not deadly, infected crabs often have meat that tastes bitter, resulting in economic losses (Litzow et al, 2024).

This study provides evidence that thermal stress in combination with natural pathogens can elevate immune activity in *H. oregonensis*, and that after longer exposure to heat *H. oregonensis* may undergo immune suppression. The uncertainty in the results calls for future studies. As global oceans warm it is becoming increasingly important to understand environmental stressors and disease dynamics when assessing the future resilience of economically important marine species in a warming world.

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