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The effect of polyunsaturated aldehydes on zebrafish larvae heart rate and mortality

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ABSTRACT

This research provides insight on the effects that polyunsaturated aldehydes (PUAs) have on model species zebrafish heart rate and mortality. Zebrafish were used as an analog for forage fish, which are of both ecological and economic importance. Forage fish often interact with diatoms, some species of which have been known to emit allelopathic chemicals, PUAs. These chemicals can have a deleterious effect on invertebrates that cohabitate with or consume diatoms, but their effects on vertebrates was unknown. In the lab, zebrafish eggs were exposed to three different concentrations of PUAs plus two controls and observed to determine any effects on their heart rate and ability to survive. A non-parametric ANOVA was used to compare the average heart rates of the larvae from each treatment and a Kaplan Meier survival analysis was used to determine the percent chance of larval survival for a given time for each treatment. The data showed that under high concentrations, PUAs decrease larval heart rate and decrease their chance of survival.

INTRODUCTION

Diatoms, a type of phytoplankton, are a vital member of aquatic ecosystems. Their cell walls, called frustules, are made of transparent silica creating striking patterns. They are unicellular and can exist as a single cell or in colonies. Diatoms carry out about one-fifth of the earth's photosynthesis, producing more oxygen than all the rainforests combined (Armbrust 2009). They are the basis of the marine food web and play an important role in the biological carbon pump; the diatoms themselves are directly or indirectly consumed by larger organisms, supporting both natural populations and fisheries alike while their detritus sinks to the ocean depths and is eaten by deep-water dwellers or remineralized into CO2 (Armbrust 2009).

Polyunsaturated aldehydes (PUAs) are allelopathic chemicals produced by diatoms (Romano et al. 2010). They are considered to be oxylipins and are formed when diatoms are faced with environmental stressor. Studies have shown that diatoms have an inhibitory effect on the reproduction of the copepods that graze on them, reducing both hatching success and larval development (Ianora & Miralto, 2010). PUAs can be detected for up to 14 days in seawater, and although it is of low concentration, it is still harmful to the benthic copepods that consume it (Romano, 2010). Although most PUA-producing diatoms are pelagic species, there is some evidence that some benthic species can produce PUAs (Pezzolesi et al. 2017). Many of the studies done on diatom-derived PUAs show a teratogenic effect on invertebrates (Romano et al. 2010), but there have not been any studies on PUAs effects on vertebrates that share a habitat with benthic diatoms.

Forage fish often coexist with diatoms. Forage fish are of both ecological and economical importance; they are a food source for many other organisms and a key part of the marine food chain. Some of the benthic diatoms that exist in the Salish Sea have recently been shown to emit

PUAs (Johnson, unpublished data) and many of the forage fish that live there, like the Pacific Herring, lay their eggs on aquatic vegetation that also harbor benthic diatoms (Prazukin et al. 2022). It is very likely that the eggs and larvae of Salish Sea forage fish develop in the vicinity of the PUAs and eat the substrate on which the benthic diatoms reside. However, it is unknown if PUAs affect forage fish development, hatching success, or chance of survival.

The purpose of this study is to determine if PUAs emitted by benthic diatoms may have significant effects on the forage fish who consume the diatoms or whose eggs develop in the same vicinity. Any extreme negative effects on forage fish would directly impact marine ecosystems and indirectly impact the economy. To test this hypothesis I used a model organism, the zebrafish. My null hypothesis was that zebrafish heart rates and mortality will be unaffected by the additions of PUAs. My alternative hypothesis was that zebrafish heart rates and mortality will increase due to an addition of PUAs. If my null hypothesis is rejected, this study would be a springboard to examining the effects of PUAs in other vertebrates.

METHODS

For these experiments, zebra fish embryos were exposed to a range of PUA concentrations and assessed for differences in heart rate and time to mortality. Heart rate is an easily measurable indicator of stress and time to morality will show the larvae's ability to resist starvation.

Chemicals

The three polyunsaturated aldehydes (trans,trans-2,4-Decadienal ($C_{10}H_{16}O$), (2E,4E)-Octa-2,4-dienal ($C_8H_{12}O$), trans,trans-2,4-Heptandienal ($C_7H_{10}O$)) were purchased from Thermo Scientific, Molport, and Sigma-Aldrich, respectively. Methanol was used to dissolve the PUA lipid chains to ensure homogeneity within the water solution. Dimethyl sulfoxide is a solvent frequently used to deliver toxins in zebrafish bioassays, but results from initial experiments showed that the embryos were acutely sensitive to the concentration of DMSO needed to dissolve PUAs, so methanol was used instead.

Zebrafish

Zebrafish were bred in Dr. James Cooper's lab at Western Washington University and embryos delivered to Dr. Brady Olson's lab one day post fertilization (dpf). AB wildtype zebrafish were used for breeding to allow for a broad genetic pool and to mimic a natural population. While it is recognized that zebrafish are tropical and not representative of Salish Sea forage fish, they are a model organism and much is known about their toxicological responses. Therefore, they serve as a model for initial tests on the toxicity of diatom-derived PUAs.

Experimental Design and Set Up

Table 1. PUA Concentrations (heart rate experiment):

Units	**PUA**	**Low**	**Mid**	**High**

ug/ml	Octa	1.12	5.59	11.18
	Hepta	0.66	3.30	6.61
	Deca	0.38	1.90	3.81

Table 2. PUA Concentrations (mortality experiment):

Units	**PUA**	**Low**	**Mid-Low**	**Mid-High**
ug/ml	Octa	1.12	2.24	3.36
	Hepta	0.66	1.32	1.98
	Deca	0.38	0.76	1.14

Heart rate

For the heart rate experiments, three PUA concentrations were used (Table 1), low, mid, and high, and two controls. One control was 1% methanol and the other was plain embryo water. Controls were used to determine if any effects on heart rate were due to the methanol instead of the PUAs. Each treatment had 3 replicates and the PUAs were introduced to petri dishes that contained 25 embryos, 24 hours post fertilization. To negate any detrimental effects to the embryo from fungus or bacteria, new chemical solutions were mixed and added to treatments during daily water changes. Any dead or decaying eggs were removed each day before water changes. The petri dishes with the embryos were stored in an incubator at 28 degrees Celsius to mimic the water temperature at which natural populations of zebrafish occur in. Three dpf, zebrafish embryos are developed enough for an observer to see their hearts. At 3 dpf, a camera attachment was connected to a microscope and 10 randomly-selected embryos from each treatment replicate were recorded for 10 seconds. ImageJ was used to open the videos and the number of heartbeats per 10 seconds were manually counted and then multiplied by six to determine beats per minute (bpm).

The fish in the high treatment replicates as well as one replicate in the medium treatment died before filming day, so analyses were done on the remaining 11 plates. Statistical analyses of data were run using RStudio. The data from the replicate plates were aggregated to determine the average bpm for each treatment. Ggplot was used to create a bar plot and boxplot to visualize the means, spread, and outliers. Before running an ANOVA, a Shapiro-Wilk normality test and Levene's Test for Homogeneity of Variance were run. A non-parametric ANOVA, Kruskal-Wallis rank sum test, was used to determine if there were significant differences between treatments and the Wilcoxon rank sum test with continuity correction was used to determine which treatments were significantly different.

Mortality

For mortality experiment three PUA concentrations (Table 2.) and two controls, 1% methanol and plain embryo water, were used. The middle and high concentrations had to be lowered to Mid-Low and Mid-High after all fishes in each of the replicate dishes for those treatments died within 24 hours of fertilization. The treatments for this experiment were introduced 2 hours post fertilization as opposed to the 1 day done in the heart rate experiment. Zebrafish embryos naturally hatch 3 dpf, but the treated embryos were not hatched at that time so they were manually hatched at the same time as the control larvae were naturally emerging from their eggs. New PUA solutions were added during daily water changes for each replicate plate. The petri dishes with the embryos were stored in an incubator at 28 degrees Celsius to mimic the water temperature at which natural populations of zebrafish occur in The number of dead larvae were recorded every day until all fish died. Dead fish were removed prior to water change to prevent decay in treatment.

Statistical analyses of collected data were run using RStudio. A Kaplan-Meier survival analysis was run to provide a visual of the survival probability at a given time. To determine if there were any significant differences in survival between treatments, a log rank analysis was ran and then a pairwise comparison was done to determine between which treatments were significantly different.

RESULTS

Heart rate

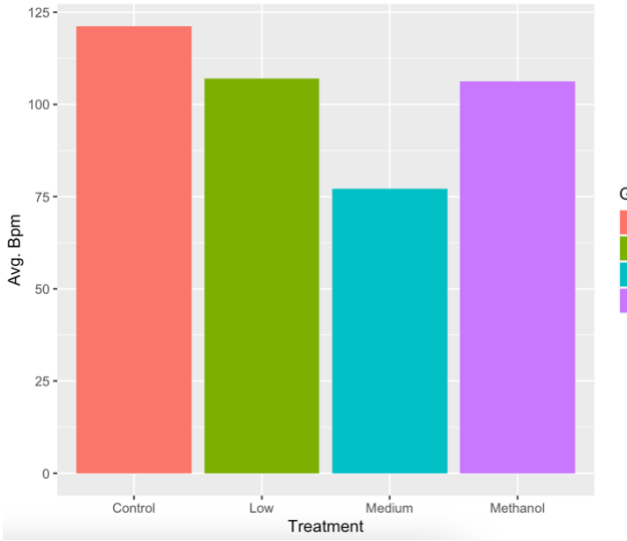

Figure 1. Bar plot of the average beats per minute of the control, low, medium, and methanol treatments

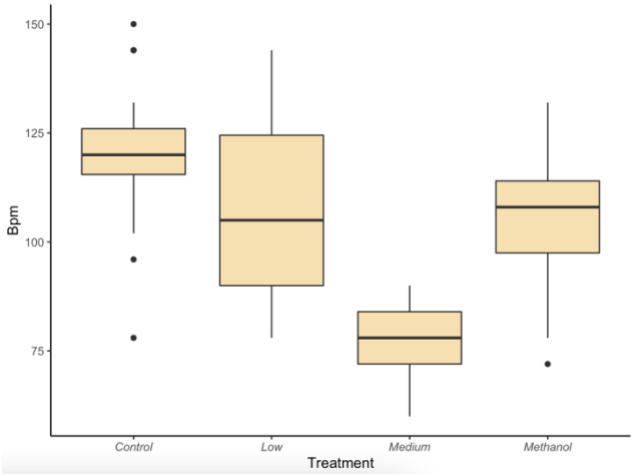

Figure 2. Box plot of beats per minute of the control, low, medium, and methanol treatments. Black points indicate outliers.

The control treatment heart rate was the highest at an average of 121.2 bpm. The low and methanol treatments were similar with an average of 107 bpm and 106.2 bpm respectively. The medium treatment heart rate was the lowest at 77.1 bpm (Figure 1.) Figure 2 shows the data distribution of the four treatments. The control had four outliers. The results from the Shapiro-Wilk normality test showed that the control and medium plates are distributed normally with p-values of 0.0508 and 0.1466, but the low and methanol treatments had abnormal distributions with p-values of 0.02897 and 0.03429. The results from the Levene's Test for Homogeneity of

Variance had a p-value of 0.0001294, meaning the variance between treatments were very different. Because the assumptions for a parametric analysis of variance were not met, the Kruskal-Wallis rank sum test, a non-parametric ANOVA, was used. Its p-value of 3.26e-11 showed that there were significant differences between treatments but doesn't indicate which treatments. Results from the Pairwise Wilcoxon rank sum test determine that the low and methanol treatments were the only two treatments not significantly different from each other (p-value = 0.92292).

Mortality

The graph from the Kaplan-Meier analysis (Figure 3) shows the probability of survival on a certain day for each treatment. There were visible differences between treatments, but to determine if they were significant differences, a log-rank analysis was done. Its p-value of less than 2e-16 indicates that there were significant differences. Pairwise comparisons indicated which treatments were significantly different from each other. The comparison between control and methanol treatment had a p-value of 0.59, meaning that they are fairly similar. Low and medium-low were also similar to each other, with a p-value of 0.1742. They were, however, significantly different to the controls and towards the high treatment. The medium-high treatment was significantly different to all other treatments.

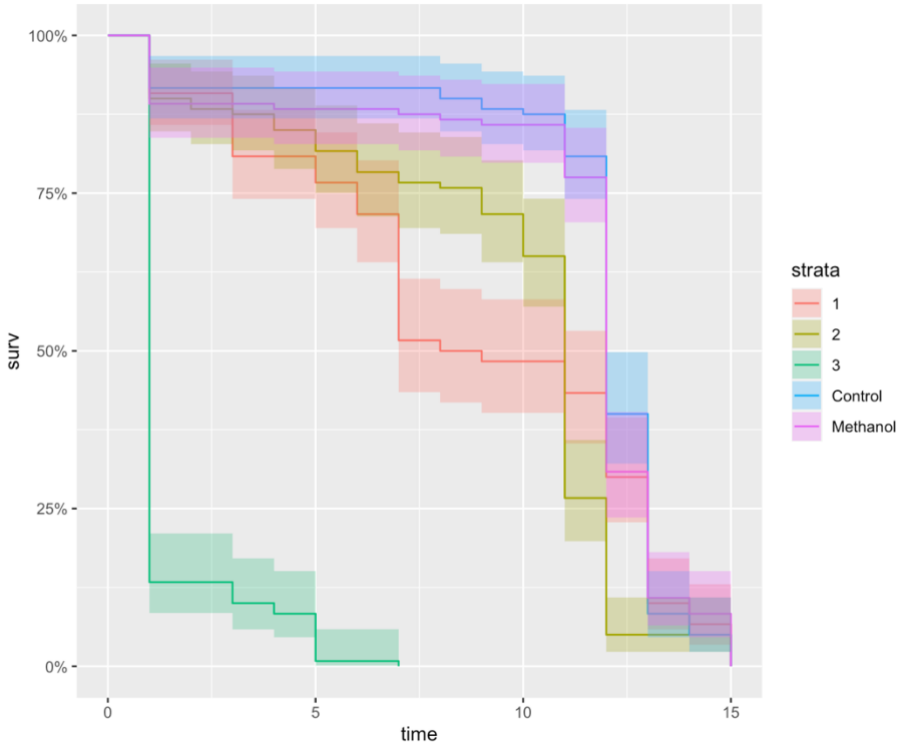

Figure 3. Kaplan-Meier survival analysis. Treatment 1 = low, treatment 2 = mid low, treatment 3 = mid-high. Shaded area around solid lines is the 95% confidence interval

DISCUSSION

My research showed that in high concentrations, PUAs had a significant effect on zebrafish embryo heart rate. Studies have demonstrated that when under stress, fish exhibit a change in heart rate (Wang et al. 2020). My results from the heart rate experiment support that finding. The fish exposed to the highest concentration of PUAs had the lowest heart rate. The low and methanol treatments' average heart rate were quite similar to each other. They were not significantly different from each other, indicating that at that a low concentration, PUAs have the same level of toxicity for zebrafish as methanol alone does. The low and methanol control hear rates were, however, significantly lower than the control treatment. When more aldehyde was added, the average heart rate of the affected

embryos were significantly lower than the three other treatments. It is known that most species exhibit a jump in heart rate when stressed, so it was surprising that the embryo bpm decreased when put under stress. Some studies have shown that exposure to polycyclic aromatic hydrocarbons, derived from crude oil spills, and other small molecules can cause zebrafish to experience reduced ventricular contractility and block key voltage-gated potassium and calcium ion channels controlling excitation-contraction coupling (Incardona et al 2014, Milan et al 2003). This could suggest that exposure to PUAs could block an ion channel controlling a similar physiological process.

The results here also show that at high concentrations, PUAs had a significant effect on zebrafish larvae ability to survive. The fact that untreated embryo water control and the methanol control were similar to each other suggests that methanol does not have an effect on the larvae and can be removed as a mortality factor. In other words, any significant differences in survival can be attributed to the addition of the PUAs. The low and medium-low treatments were also similar to each other. While the increase in PUAs from the low to the medium-low treatment did not have a significant effect on the larvae, the two treatments are significantly different from the control treatments. This suggests that the two lower treatments did affect mortality but in similar ways. The highest PUA concentration had a significant effect on larvae mortality, and the results from that treatment were significantly higher than the low, medium-low, and the control treatment results.

The results from my research indicate that there is significant cause to believe polyunsaturated aldehydes emitted by benthic diatoms in the Salish Sea could have detrimental effects on the forage fish that live there. One future study would be to repeat the heart rate experiment with the high treatment. Because the high treatment replicates died before they were able to be measured, it would be interesting to determine if the average bpm continues to decrease with the increase in PUAs or if it would plateau at the same average as the mid treatments. Another study would be to replicate both experiments with Salish Sea forage fish in their natural habitat. If the PUAs were to affect these fish, the ecological and economic impact could be devastating.

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