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Effects of Ecologically Relevant Concentrations of Nitrate on Behavior in Betta Splendens

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EFFECTS OF ECOLOGICALLY RELEVANT CONCENTRATIONS OF NITRATE ON BEHAVIOR IN *BETTA SPLENDENS*

by

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ABSTRACT

Nitrate is a ubiquitous pollutant that is becoming more prevalent in both fresh and saltwater environments. Short term effects are often studied, but an organism's response to stress can change due to whatever the stressor is. In order to research this, a study was run using environmentally relevant nitrate treatments (0 mg/L, 10 mg/L NO3-N, and 100 mg/L NO3-N) to see the effect of nitrate on scototaxis behaviors, a measure of anxiety. This study used *Betta splendens* because they are easy to rear and have very well documented behaviors. In addition to the scototaxis, the effect of temperature on mortality was also analyzed. It was found that mortality had a positive correlation with temperature and females spent more time on the black side in the 10 mg/L NO3-N treatment than the 100 mg/L NO3-N treatment.

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INTRODUCTION

Betta splendens are native to Southeast Asia, and are usually found in shallow and still waters, such as rice paddies (Lichak et al. 2022). They are often selectively bred for aggression, but even without selective breeding, male bettas are known for their aggressive behaviors (Lichak et al. 2022, Forsatkar et al. 2016, Tudor et al. 2019, Vu et al. 2020). They frequently show stereotypical displays of dominance (Vu et al. 2020) which include flaring their gills and doing performance-like tail movements (Lichak et al. 2022). These responses are common due to the territoriality of the males (Lichak et al. 2022, Forsatkar et al. 2016), which can even happen when they are faced with their own reflection (Tudor et al. 2019). *B. splendens*, also known as bettas, have been studied for years so they have very well documented behaviors and are easy to rear which makes them a very good species for lab experiments (Tudor et al. 2019).

Behavior can be impacted by many different things, but temperature is one of the most important things that can have major effects on both behavior and physiology. There have been studies done that have found that temperature changes of just 2º, 2.5º, or 3ºC can change behavior. Not only can behavior be impacted, but temperature can also affect growth rates, respiration, locomotion, and energy budgets of different ectotherms. Temperature has a specific impact on growth and reproduction because the temperature range for these to occur is much smaller than the normal temperature range a species can experience daily (Forsatkar et al. 2016).

In addition to temperature, contaminants are another big problem both in the wild as well as in aquaculture. Examples of contaminants include suspended solids, organic and inorganic materials, toxic metal compounds (Paul et al. 2019), fossil fuel combustion, runoff, agricultural fertilizers (Gomez et al. 2021), pesticides (Paul et al. 2019, Kellock et al. 2018), and endocrine disrupting chemicals (EDCs) which include steroid hormones and pharmaceuticals (Kellock et al. 2018). EDCs can impact reproduction, metabolism, growth, and development (Kellock et al. 2018), and unregulated release of chemicals from agriculture has caused problems in the past (Paul et al. 2019). Contaminants can enter aquatic systems from both direct and indirect sources (Paul et al. 2019)

Nitrogen is an important part of our atmosphere and ecosystems, so it is important to study the effects it can have. The air we breathe is made up of almost 80% nitrogen. Nitrogen can be found in many different forms, including but not limited to N_2, N_2O (nitrous oxide), NO (nitric oxide), NO_2 (nitrogen dioxide), and NH_3 (ammonia) (Alahi and Mukhopadhyay 2018). The most common forms found are nitrate, nitrite, and ammonium because of the nitrogen cycle (Camargo et al. 2005, Davidson et al. 2017). The nitrogen cycle will take ammonium and turn it to nitrite, which is then turned into nitrate. Due to this, nitrate is often found in higher concentrations than ammonium or nitrite (Camargo et al. 2005). Nitrate is known to be a very common pollutant worldwide, and it is one of the most ubiquitous pollutants that exists (Kellock et al. 2018).

Nitrate can occur both naturally and unnaturally. Naturally, it can occur from atmospheric deposition, runoff from ground and surface water, geological deposits, organic matter degradation, and the nitrogen cycle (Camargo et al. 2005). It can also enter the environment from non-natural sources, including agricultural runoff, industrial wastes, and sewage effluents (Camargo et al. 2005). Nitrate occurs naturally at very low concentrations (less than 2 mg/L NO3-N) but due to human activity those levels are rising and can be found at concentrations higher than 100 mg/L NO3-N (Gomez et al. 2021).

In an aquatic system, too much nitrate is detrimental. When there is too much nitrate, it can stimulate eutrophication (Gomez et al. 2021, Alahi and Mukhopadhyay 2018) and become toxic to various species. Nitrate is able to enter the body of a fish through the gills, which has an impact on toxicity, behavior, and physiology (Gomez et al 2021). Toxicity has been known to increase with increased exposure time and increasing concentrations, but it can decrease with increasing body sizes, adaptation, and increases in salinity. Due to this, it seems that marine species are less sensitive to nitrate than freshwater species (Camargo et al. 2005). Previous studies have found the extended exposure to nitrate can cause side swimming, gill hyperplasia, decreased feed efficiency (Davidson et al. 2014), decreased growth rates, general physiological changes, and reproductive physiological changes (Kellock et al. 2018, Davidson et al. 2014).

In the wild, bettas live in temperatures ranging from 25º to 31.5ºC (77º-88.7ºF). In lab settings, bettas have been kept in ranges from 21º-30ºC (69.8º-86ºF) (Forsatkar et al. 2016). Every species has an ideal temperature range, and falling outside of this range can have effects on feeding activity, digestion, and behavior (Sirakov and Velichkova 2023).

Scototaxis is a test that looks at preference of light vs. dark. It has been established as a measure of anxiety in both rodents (Maximino et al. 2010) and fish (Tudor et al. 2019). In a study done with zebrafish, the test was run after the fish were exposed to a pharmaceutical that was known to cause changes in anxiety in humans. The results found that more anxious fish spent more time in the black (Tudor et al. 2019). In previous scototaxis studies, both the number of entries into each side as well as time spent on each side was recorded. These studies also noted that the black and white materials should be as non-reflective as possible (Maximino et al. 2010).

The goals of this study were to identify the impacts of varying levels of nitrate on scototaxis behaviors. Based on previous research findings, the original hypothesis was that *B. splendens* would become more stressed and spend more time in the black side of the tank with increasing nitrate concentrations. As the study progressed, an additional goal of determining whether mortality was correlated with high temperatures was included. It was hypothesized that the increase in temperature led to higher mortality.

METHODS

Study Species and Housing

B. splendens were initially obtained from Segrest inc, a commercial fish supplier. During the testing period, there were two different shipments of fish that were both obtained from the same supplier, but there were also four sets of male pairs (eight total fish) purchased from a local pet store. The first shipment came in during early summer (May), but due to high mortality there was another shipment ordered during the fall. Male pairs from the pet store were purchased prior to the second shipment arriving. All female fish that were purchased were red, half of the males were blue, and half of the males were red. All fish were acclimated to the tanks for at least seven days prior to the start of the experiments.

All fish were fed to satiation 5-6 times a week with a commercial pellet diet. The room was kept on a 12:12 hour light:dark photoperiod to keep the fish on a consistent schedule. Once a week, each tank had a 100% water change using reverse osmosis (RO) water that was supplemented with RO Right solution. Water temperature was tracked by a sensor that stayed in a tank with only water 24/7. No other water quality parameters needed to be checked consistently since the RO water was used for every water change. All fish were held in individual clear plastic 1.4L tanks. Each tank and fish had an associated number that was randomly assigned so that we could track which females were dosed and which fish had been used in all previous tests. Males had white corrugated plastic dividers in between them to prevent male to male interactions, but females had no dividers to allow social interaction.

Every day, there was a visual inspection done of every fish to check for discoloration or odd behaviors. There was a husbandry sheet that kept track of the date, daily temperature, if fish were fed, if water was changed and if so which tanks, and any important notes about behaviors. If any fish had died, they were immediately removed from the tank, wrapped in paper towels, and disposed of in the trash. All tanks that held dead fish were sprayed with a 10% bleach solution, left for 24 hours, and rinsed to disinfect.

This project was approved by and followed the guidelines of the University of Maine's Animal Care and Use Committee (protocol number A2021-07-01).

Nitrate Exposure

Nitrate concentrations were made by using sodium nitrate salt and calculating the necessary amount to mix with water in order to make 10 mg/L NO3-N and 100 mg/L NO3-N concentrations. These treatment concentrations were chosen because 10 mg/L NO3-N is the current limit for nitrate concentrations in drinking water that is still safe to drink (Kellock et al. 2018, Camargo et al. 2005, US EPA 2013), and 100 mg/L NO3-N can still be found naturally so it is an ecologically relevant concentration (Camargo et al. 2005). In addition to these two treatments, there were fish tested in a control group that were not exposed to any nitrate.

Females were dosed 96 hours prior to being tested as this period has been shown to cause behavioral changes for acute exposure studies (Paul et al. 2019). On the scheduled date that the exposure started, four females were taken and had water changes done with the treatment water instead of RO water. Those fish were then clearly marked so that they did not get water changes again for four days but they were still fed regularly.

If the testing day was for the control group, there was nothing to be done four days prior unless it was a regularly scheduled water change for those fish, but the females being tested were recorded so they were not used again. Males were never exposed to the nitrate treatments.

On the day of testing, females start with a different test that looks at mate choice behaviors. There was one pair of males used with each female. Immediately after the conclusion of the mate choice test, the females began the scototaxis test. Once that test finished, the females were measured before being put in a fresh tank with fresh RO water. Mate choice data was not analyzed in this study, but going through this test first may have impacted the scototaxis results that were examined.

Scototaxis Procedure

Scototaxis is a test that has been shown to determine anxiety in fish (Tudor et al. 2019). For this study, scototaxis was measured immediately after running a test for mate preference. The test tank was 37.9-liters (50.8x25.4x30.5cm, L x W x H), and the entire inside was lined with half black and half white felt (Fig. 1). Bettas have been known to show aggressive behaviors at their own reflections (Tudor et al. 2019). Although it is mostly males that display this behavior, it is still important to use a non-reflective surface to avoid all possible variables that could cause misleading data. For this reason, felt was chosen as the material to cover the tank, and it had to be placed on the inside so that there would be no glass interference. The felt was glued to the tank in order for it to stay in place.

To begin the test, the tank was filled to the top with fresh RO water in order to see the effects of the nitrate from only the 96-hour exposure. The female that had just been through the mate preference test would be taken and put in a white corrugated plastic tube in the middle of the tank for 10 minutes to allow her to acclimate to the water. After the 10 minutes was up, the tube was removed and the female was allowed to go anywhere in the tank for another 10 minutes. During that period, the time spent on each side and the number of times the female crossed the middle line was recorded. The time spent was recorded by using the lap function on a cellphone, recording which lap number correlated to which side of the tank, and adding up all of the lap times for each side at the end. A cross of the centerline was counted as when her eye fully crossed from one felt side to the other. These were tallied under sections labeled “black to white” or “white to black” to note which direction the female was moving.

After that 10 minute period was over, the female was removed, put in a fresh tank with fresh RO water, measured, and returned to the shelf. This process was repeated for each female being tested on that day.

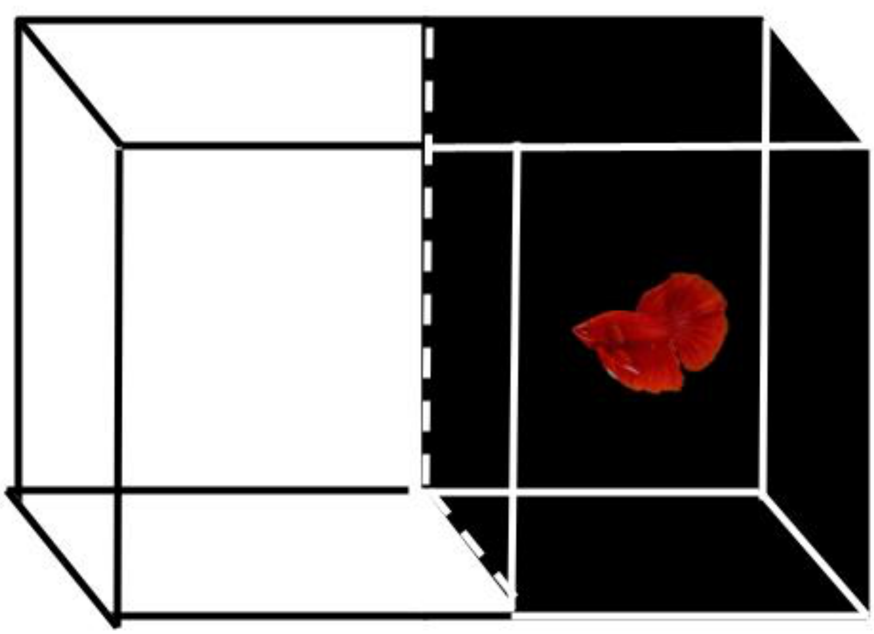

Figure 1. Schematic drawing of scototaxis tank used. A 37.9 liter tank (50.825.430.5cm, L W H) was divided in half by felt to make one black section and one white section. Betta fish image taken from online.

Statistical Analysis

A normal distribution test was run on all data to see what data was normally distributed and what was not. To determine if increases in temperature influenced mortality, a non-parametric linear regression was run. A Kruskal-Wallis test was run to determine if there were any significant differences between shipments and number of crosses. A t-test was run to determine what the relationship was between the shipments and time spent in black. A Kruskal-Wallis test was also run to determine if there were any significant differences between the nitrate concentrations and the number of crosses. The relationships between nitrate concentrations and the time spent in black were determined with an ANOVA and post-hoc test with a threshold of significance at 0.05.

RESULTS

Husbandry and Mortality

Temperature was positively correlated with mortality (non-parametric linear regression: p=0.0358; Fig. 2). The lowest recorded temperature was 67ºF, and the highest temperature recorded was 85.6ºF. Days that had 3 mortalities or more all happened at a temperature of at least 77ºF. The highest number of mortalities did not happen on the highest temperature day, but the days that had higher mortalities only happened at higher temperatures. No mortalities occurred while the fish were exposed to nitrate, they only occurred during the holding periods.

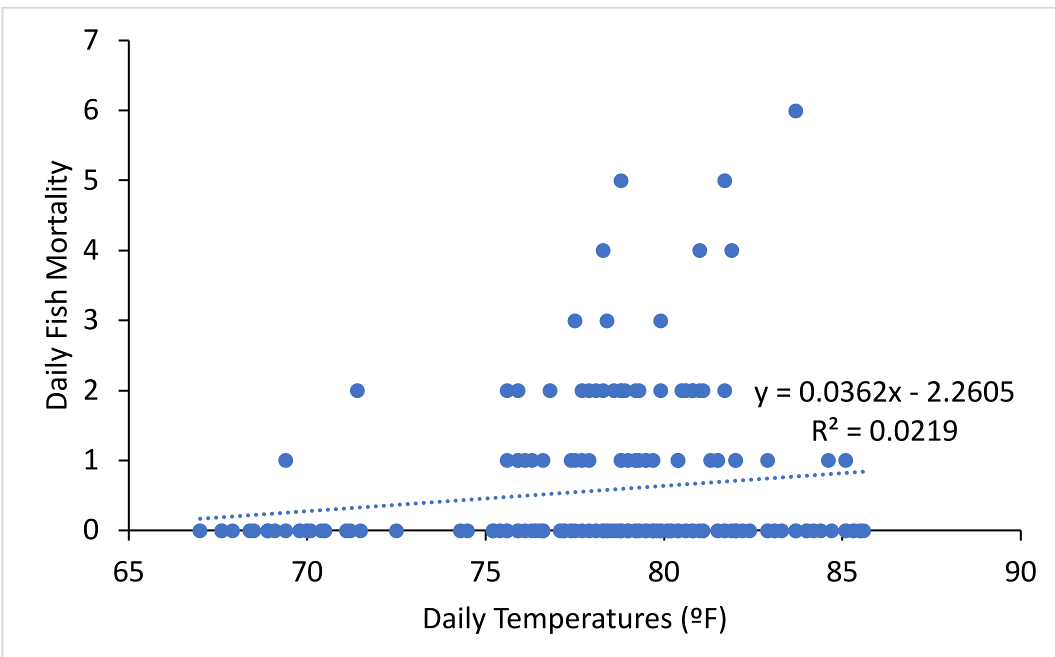

Figure 2. The number of fish that died daily compared with the temperature on that day. There is a positive correlation of temperature with mortality (non-parametric linear regression: p=0.0358). The equation given is the equation of the regression line shown. The R^2 value is how correlated the data are. No mortalities occurred while exposed to nitrate.

Scototaxis

Distributions of normality were determined using parametric tests for all data. The proportion of time in both black and white was normally distributed. The number of crosses was not normally distributed. After determining that, it was important to determine whether the shipment had any effects on behavior. There was no significant difference between the shipments and the time spent on the black side (proportion of mean time in black ± SD: shipment 1=0.575±0.387; shipment 2=0.587±0.252; ANOVA, t-test: p=0.5267; Fig. 3). There was a significant difference between the shipments and the average number of crosses (mean number of crosses ± SD: shipment 1=2.33±2.71; shipment 2=9.08±8.84; Kruskal-Wallis: chi-squared=5.5717; DF=1; p=0.0183; Fig. 4).

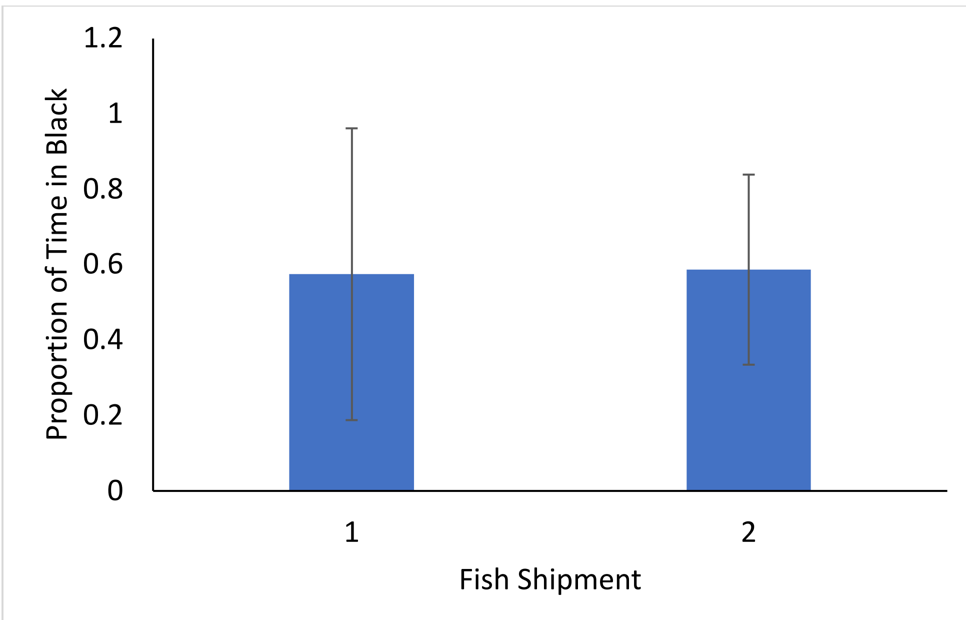

Figure 3. The average amount of time spent in black for all treatments in shipment one compared to shipment two. There was no significant difference between shipments (t-test: p=0.5267). The error bars represent the standard deviation for each data set.

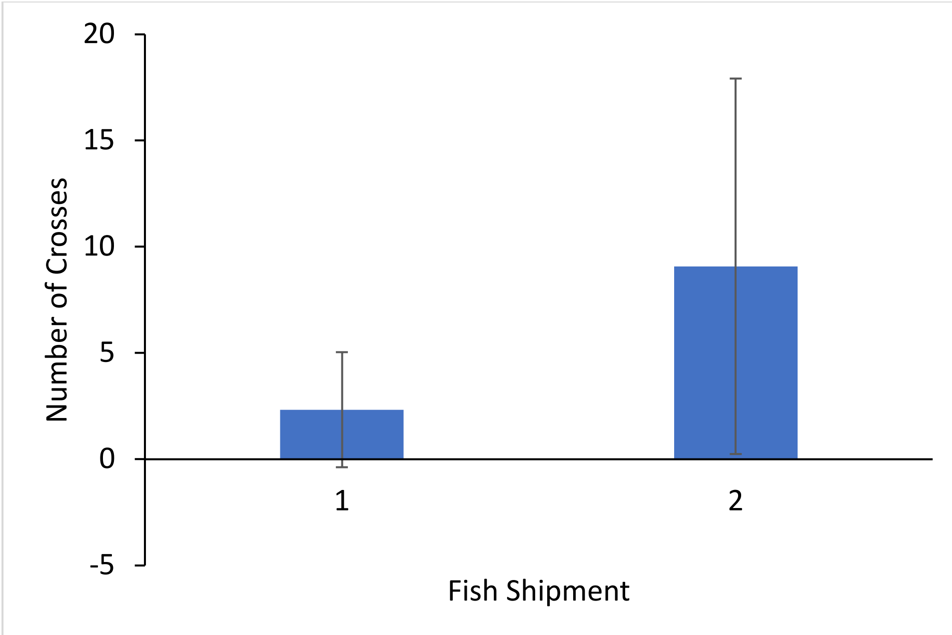

Figure 4. The average number of times females crossed the centerline for all treatments in shipment one compared to shipment two. There is a significant difference between the two shipments (Kruskal-Wallis: chi-squared=5.5717; DF=1; p=0.0183). The error bars represent the standard deviation for each data set.

Since the data for the time in black was not significantly different between shipments, both shipments were combined to analyze the concentration data. There was a significant difference between the 10 mg/L NO3-N treatment and the 100 mg/L NO3-N treatment, but neither treatment was significantly different from the control (0 mg/L) treatment (proportion of mean time in black ± SD: 0 mg/L=0.605±0.357; 10 mg/L=0.851±0.136; 100 mg/L=0.396±0.266; ANOVA: p=0.0067; Student's t-test: 10-100 p=0.0018; 10-0 p=0.1072; 0-100 p=0.1334; Fig. 5). There was not a significant difference between any of the treatments and the average number of crosses (mean number of crosses ± SD: 0 mg/L=6.17±7.05; 10 mg/L=2.57±2.57; 100 mg/L=7.45±9.06; Kruskal-Wallis: chi-squared=1.0891; DF=2; p=0.5801; Fig. 6).

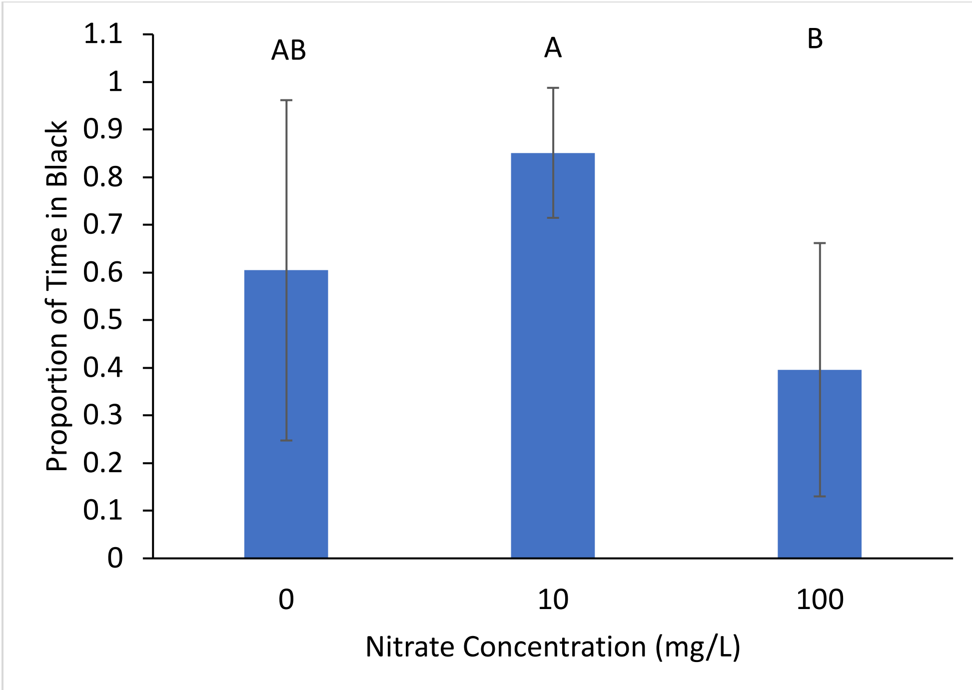

Figure 5. The average amount of time spent in black for each concentration treatment. There is a significant difference between the average time from 10 mg/L to 100 mg/L, but no significant difference between the control group to either treatment (ANOVA: p=0.0067; post-hoc test: 10-100 p=0.0018; 10-0 p=0.1072; 0-100 p=0.1334). The error bars represent the standard deviation for each data set. Different letters represent significant differences between those data.

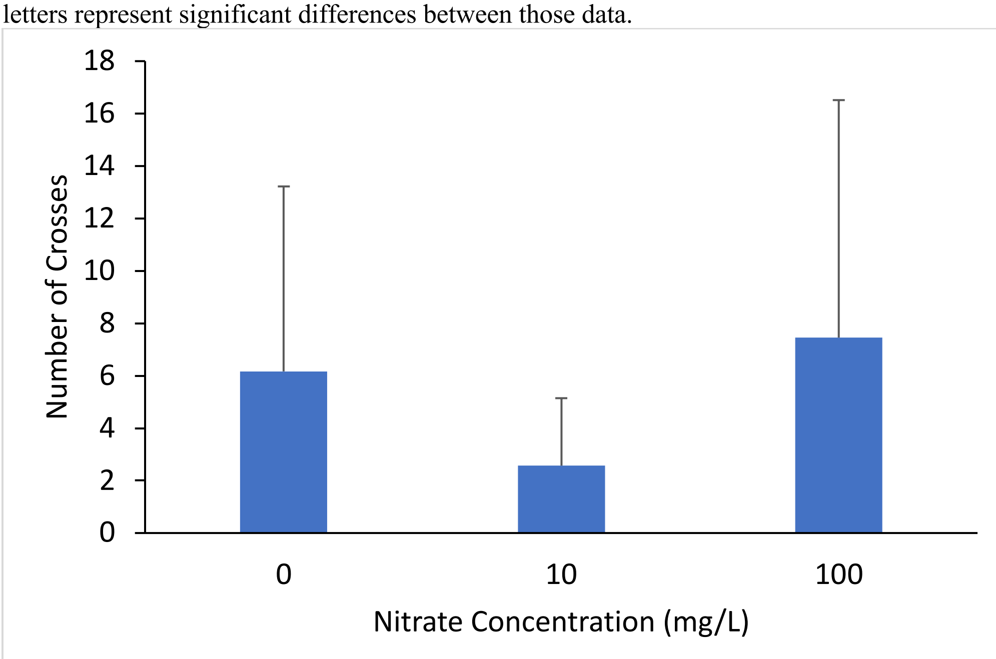

Figure 6. The average number of crosses by females in each nitrate concentration. There is no significant difference between any of the concentrations (Kruskal-Wallis: chi-squared=1.0891; DF=2; p=0.5801). Error bars represent the standard deviation for each data set.

DISCUSSION AND CONCLUSION

Mortality

Mortality was found to be positively correlated with temperature. Most of the days during the experiment duration had temperatures within the 70-80ºF range, which is within the normal temperature range of *B. splendens*. Fully aquacultured fish tend to be cultured in slightly lower temperatures (low 70s and 80s) whereas wild fish can be found living in places where the temperature reaches high 80s (Forsatkar et al. 2016). This study found that all days that had three or more mortalities were on days where the temperature was above 77ºF. Another study done with bettas kept the rearing system at 28ºC, which is 82.4ºF. That study mentioned that temperatures this high can reduce the viability of some pathogens so it helps to reduce illness (Lichak et al. 2022). This was not the case for this study. The bettas used for this experiment tended to show more sick behaviors and higher mortalities at high temperatures.

When bettas are sick, they can present with clamped fins, lethargy, disinterest in food, or buoyancy disorders. They will either float towards the surface of the water or their tail will sink so they are vertical in the tank. Sinking is more common and is often linked to excessive feeding. Floating fish often have a bloated abdomen. About 50% of bloating cases will resolve themselves within 24-48 hours, but if they don't that can be a sign of bacterial infection (Lichak et al. 2022).

Bettas are more susceptible to disease when water quality in general is poor. Although they are freshwater fish, small amounts of saltwater can be added into a system in order to reduce diseases and improve growth (Lichak et al. 2022). Water quality is not the only factor that can cause negative behaviors in fish. It has been found that female bettas are negatively affected by social isolation (Tudor et al. 2019), so they should be able to see each other at all times. Since this is only true for females and males have more aggressive tendencies, male bettas have to have dividers in between them (Lichak et al. 2022).

Shipment

There was no significant difference between the shipments and the overall time that the females spent in the black side of the tank, but there was a significant difference between the shipments and the total number of times the females crossed from one section to the other. A potential reason for this significant difference is increased activity levels at higher temperatures. A study done with bettas looked at how small temperature differences affect activity levels of the fish (Forsatkar et al. 2016). They studied boldness, which was done by tracking movement and latency times in various treatments of fish. They used 26ºC as the control temperature and 30ºC as the elevated temperature. They found that the fish in the elevated temperature treatment had lower latency times, used the area furthest from the dropping point more than the control fish, and were overall more active than the control fish (Forsatkar et al. 2016). Due to the timing of the arrival of the second shipment of fish for this experiment, those fish were exposed to the higher temperatures for longer. The increased exposure to high temperatures could be a reason that the crosses differed while the overall time spent did not. For this reason, both shipments were combined for all subsequent analyses.

Nitrate Concentrations

There was no significant difference between treatments for the number of crosses, but there was a significant difference between the 10 mg/L NO3-N treatment and the 100 mg/L NO3-N treatment for the amount of time spent on the black side. Fish can start to exhibit sluggish behavior if exposed to temperatures at the high end of their ideal range for too long (Sirakov and Velichkova 2023). Depending on when the fish were tested and which fish were tested, there could have been other factors that impacted these results. If they had survived through the high temperatures, if they had shown signs of illness but recovered, or if they had been exposed to other kinds of stress, they might have acted due to more than just nitrate exposure. This could be why the control treatment had a higher average time in black than the 100 mg/L NO3-N treatment and why the 10 mg/L NO3-N treatment had the highest average time in black out of all of the treatments. The fish in this experiment were held for multiple months before testing began, so they had to survive a long time before ever being tested.

Future Studies

If this study were to be done again, there are some things to keep in mind. At least for the fish acquired from Segrest inc, the temperature needs to be kept in the lower 70ºF range for increased health. The testing also should ideally start sooner after receiving them to try to get ahead of any disease outbreaks. The fish should have enough time to acclimate to the new conditions, but not be held for an extended amount of time past that. The fish used in this experiment were held for a few months before testing began, but they were fully acclimated after 2-4 weeks. After the second shipment was received, there was a protocol change for disinfecting tanks that allowed bleach soaks that should have been implemented sooner to avoid disease spread. Along with this, nets used for water changes should be bleached consistently to avoid spreading of bacteria.

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AUTHOR'S BIOGRAPHY

Sarah Janson is a fourth year student at the University of Maine who will graduate with a bachelor's of science in Marine Science with a double concentration in Marine Biology and Aquaculture in May 2024. She had betta fish growing up, so when the opportunity came around to work in this lab starting in the summer of 2023, it seemed like a perfect fit. She hopes to work in the field of conservation science after graduation to be able to continue to work hands-on with various species.