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The Effects of Length and Increasing Nitrate Concentrations on Behavior of Betta Splendens

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THE EFFECTS OF LENGTH AND INCREASING NITRATE CONCENTRATIONS ON BEHAVIOR OF *BETTA SPLENDENS*

by

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ABSTRACT

Nitrates are an important compound in nature. However, through a number of human activities, nitrate concentrations have exponentially increased in nature. Most of the anthropogenic nitrates come from fertilizers, sewage water, and other wastewater. These nitrates contaminate the water which can get into lakes, rivers, ponds, and other natural bodies of water. *Betta splendens* are a well studied species in the animal behavior community. They have defined and quantifiable displays and characteristics during aggressive and mating ritual behavior. *Betta splendens* live in freshwater habitats that have most likely come in contact with the increased levels of nitrates. This study observed anxiety-like behavior of female *B. splendens* when exposed to increasing levels of nitrates (0 ppm, 10 ppm, 100 ppm). We also looked at whether or not there was a relationship between the body size and anxiety-like behavior of the females. We found that the individuals showed anxiety-like behavior when exposed to 10 ppm of nitrate. We also found that there may be a correlation between female body size and anxiety-like behavior, however we cannot be certain due to having a small data set.

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INTRODUCTION

Betta splendens, also known as Siamese Fighting fish, are common pets in many households. *B. splendens* are part of the family *Osphronemidae*. They are native to Southeast Asia and are found in shallow freshwater swamps, rice fields, creeks and rivers (Nur et. al., 2022). Male *B. splendens* are known to have many aggressive behaviors during reproductive and territorial matters that are easily quantifiable (Jaroensutasinee & Jaroensutasinee, 2001). *B. splendens* are farmed commercially for ornamental purposes. They are a well studied species because of their availability and unique behavioral characteristics (Alton et. al., 2013; Clotfelter et. al.,2007; Dupeyron and Wallace, 2023).

Nitrates are a naturally occurring compound that occur in the nitrate cycle. It can begin as ammonia, $NH4^{+}$, which gets converted into nitrites to nitrates by nitrifying bacteria. These nitrates can get used by plants (Canfield et. al., 2010). Apart from the naturally occurring nitrates in the environment, there has been an increase of anthropogenic made nitrates which are commonly found in fertilizers, wastewater and sewer waters, animal manures and waste (Bertram et al., 2019; Canfield et. al., 2010). These nitrates can and have started to increasingly contaminate runoff which can get into groundwater, streams, marshes, and lakes. This can cause eutrophication in the ecosystem (Siarkos, Mallios, and Latinopoulos, 2024; Deegan et al., 2012). In a contained area such as a recirculating aquaculture system, it is possible to have unknowingly high nitrate levels which can harm the species being reared (Morris et. al., 2011). For humans, elevated nitrate levels in drinking water have been known to cause methemoglobinemia in infants and thyroid gland problems (Ward et al., 2018). According to the World Health

Organization, the guideline for maximum contaminant level for nitrate is 50 mg/L as NO_3 (Ward et al., 2018). In fish, high nitrate concentrations have been found to be an endocrine disruptor (Kellock, Moore, and Bringolf, 2017). There have been studies conducted looking at how a diverse range of water contaminants affect aquatic organisms' behavior and physical health such as caffeine on zebrafish (*Danio rerio)* (Alia and Petrunich-Rutherford, 2019), fluoxetine in guppies (*P. reticulata)* (Fursdon et al, 2019), arsenic in *B. splendens* (Tudor, 2018), nitrate in Whitespotted Bamboo Sharks (Morris et.al., 2011).

Though *B. splendens* are a well studied species, most studies focus on males (Tudor, 2018). This study's focal point is on female *B. splendens*. We are observing how the females choose their mates and their levels of anxiety-like behavior. To measure anxiety-like behavior, we are using the scototaxis test. This test takes place in a tank divided in half horizontally and lined with black and white felt (Figure 7). If the subject stays in the black section, it indicates that the fish may be more anxious. In contrast, if the fish is in the white section or actively exploring the tank, it suggests that the fish is more bold and less anxious. A scototaxis test has been tested to be a valid ecological behavioral test (Maximino et al, 2010). It has been used in studies observing anxiety in various fishes such as zebrafish (Maximino et. al, 2011), talitrid amphipods (Cohen and Putts, 2013), *B. splendens* (Dupeyron and Wallace, 2023; Tudor, 2018). In addition to observing how increasing levels of nitrate affect *B. splendens'* anxiety, we are interested in whether the body size of the female has an effect on anxiety. Body size has been found to affect the behavior of organisms in aspects such as reproduction (Clotfelter, Curren, and Murphy, 2006), predator-prey relationship (Nakazawa, Ohba, and Ushio, 2013;

Niiranen et. al., 2019), boldness and exploring environments (Brown and Braithwaite, 2004). It is important to study how these various water contaminants affect the aquatic organisms' ecological behavior. From this perspective, we can understand the organism's reproductive, feeding, environmental, and evolutionary behavior.

METHODS

Subjects

The *B. splendens* came from a commercial fish supplier (Segrest Farms, Gibsonton, FL). There were two shipments over the testing periods. We found no differences of results between the two shipments. Each shipment had an even amount of red and blue males and between 40-70 red females. The females had an ovipositor showing that they are sexually mature.

Husbandry

In the lab, there were tanks filled with water from a reverse osmosis filtration system. Males and females were separated on different shelves. Females had visual contact with other females. White corrugated plastic sheets were placed between each tank in the male section to avoid aggression between fish. Every week, fish were checked between 10-11 AM. Fish were fed two pellets of Hikari Vibra Bites Tropical Aquatic Diet every other day. Full water changes were conducted every week. The date, temperature of water in the tank, fish behavior and health, if the fish got fed, if the water got changed, initials, and other notes were recorded on a log located in the lab once a day. The fish were exposed to a ratio of 12:12 light and dark exposure. The health of the fish was observed everyday. Fish that displayed signs of sick symptoms were noted in the log and moved to a different shelf. During the summer of 2023, temperatures ranged between 67 to 85.6 degrees Fahrenheit. During the summer and fall of 2023, it was suspected that the warm temperatures caused a bacterial infection, killing many of the individuals. Fish that had symptoms of discoloration, clamped fins, poor swimming abilities and staying near the bottom of the tank, and white film on the body were considered to be sick. For

cleaning and sterilizing purposes, ten percent bleach was used as a disinfectant. The tanks were scrubbed, rinsed, sprayed with the bleach solution, and left to dry for 24 hours. The tanks were then rinsed with tap water and left to air dry for at least 24 hours. Nets were disinfected in the bleach solution and rinsed after handling the fish to avoid the spread of bacteria.

Nitrate doses

A stock solution of standard sodium nitrate was mixed with distilled water. The stock solution was then diluted to get the necessary ppm of nitrate. The concentration groups were the control group with 0 ppm, 10 ppm of sodium nitrate, and 100 ppm of sodium nitrate. We used these concentration amounts because 10 ppm is the maximum concentration for the US public drinking water (Ward et al, 2018) and 100 ppm is double the amount (50 mg/L or 50 ppm) that is the guideline according to the World Health Organization ("Guidelines for Drinking Water Quality: Fourth Edition Incorporating the First and Second Addenda, 2022) and is a realistic amount that could be found in the environment.

Scototaxis Testing Procedure

Each dosing week, three to five females were picked at random and dosed with the same concentration amount. The females remained in the dosed water for 96 hours before being tested to ensure the nitrate was in her system. No fish that looked sick were chosen. During the testing day, a 50-minute mate choice test was administered right before the scototaxis test. The scototaxis test was performed in a 37.9 liter tank (50.8 x 25.4 x 30.5 cm, L x W x H). The tank was divided in half with black and white felt lined along the tank (Figure 1.) The felt provided the distinctive areas and created a non-reflective surface. The tank was filled with clean RO water. A light was suspended above

the tank to provide uniform lighting in the tank. The female was placed in a white plastic rectangular tube located in the middle of the tank for acclimatization for 10 minutes. The female was released from the tubing and observed for 10 minutes. Two people observed the fish with one person stating the time. The female crossed to and from the black section while the other person recorded the time. Right after the testing, the female was moved to the housing tanks with fresh, clean water and measured.

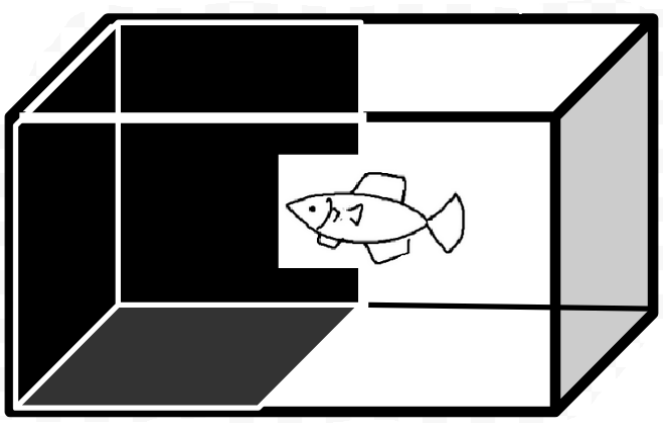

Figure 1. Evenly divided through the middle vertically with black and white felt lining the tank. Tank dimensions (50.8 x 25.4 x 30.5 cm, L x W x H)

Statistical Analysis

All statistical data was processed in the software JMP pro 17. We ran an ANOVA and a Post-hoc Tukey-Kramer test for time spent in the black against nitrate concentration. A Tukey-Kramer test was performed to see how each concentration related with the time spent in the black section. A generalized linear mixed model was conducted with the variables; nitrate concentration and length together and separately against time spent in the black section. This was administered to see how each variable interacted with the dependent variable (time spent in the black section).

Combined data Arsenic data (increase of sample size)

In order to increase the data size, specifically in the control group, we added the control group (0 ppb of arsenic) from Dr. Scarlett Tudor's dissertation on *B. splendens* conducted in 2018 (Tudor, 2018). All of the methods, tests, and dosage amounts were identical to this study. This additional data was added to see if there were stronger trends because the original data set was small.

RESULTS

Concentration Comparisons (Nitrate Lab Data)

The nitrate concentration was found to have an effect on the time spent in the black section from the ANOVA test ($p = 0.0274$). Upon further exploration, through a Post-hoc test, it was found that there was significant difference between the 10 ppm and 100 ppm concentration groups (Tukey-Kramer: 0.0229; Figure 2). The control females did not spend significantly more time in the black sections than the dosed groups. The females dosed with 10 ppm had the highest average of time spent in the black section (460.88 ± SE: 123.96 s). The average time spent in the black section for the control group was 352.72 ± SE: 202.82 s. The combined average time spent in the black section for the nitrate dosed groups was 384.42 ± SE:155.40 s. The 100 ppm had the lowest average time spent in the black section (227.84 ± SE:131.04). There were 29 individuals in this data set. In this data set, 11 female fish did not go through the mate choice test before the scototaxis test.

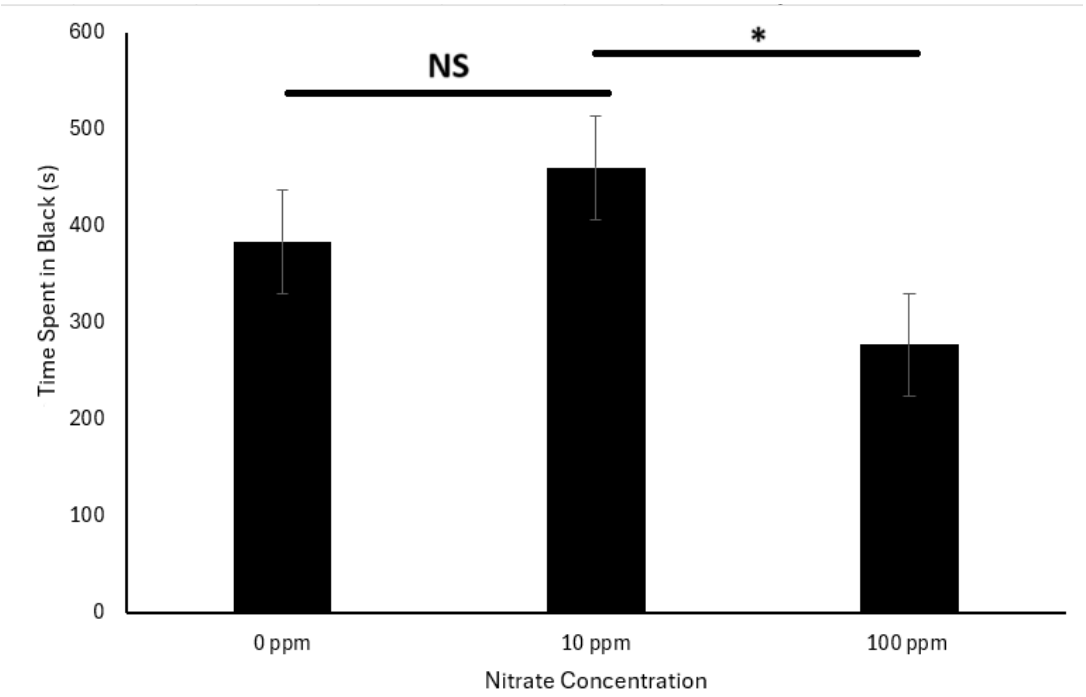

Figure 2. Comparison of the mean time females spent in the black section of the tank among nitrate concentrations. An asterisk represents the significant difference between the 10 ppm and 100 ppm concentration groups (Tukey's post-hoc: 10 ppm: p= 0.0229). Females in the 100 ppm concentration group spent the least amount of time in the black.

Female Body Size Linear Regressions (Nitrate Lab Data)

There was a correlation shown between the female body size and 0 ppm (r^2= 0.6235, N=7, p= 0.0347; Figure 3). The average length within the control group was 35.85 ± SE: 7.75 mm. There was no correlation between female body size and time spent in the black section in either the 10 ppm (linear regression: r^2= 0.1773, N=9, p=0.2590; Figure 4) or 100 ppm (linear regression: r^2= 0.1390, N=13, p=0.2095; Figure 5). The average length within the 10 ppm group was 40.30 ± SE: 9.20 mm. The average length within the 100 ppm group was 42.19 ± SE: 7.21 mm. The average length of females in the whole data set was 40.07 ± SE: 8.12 mm.

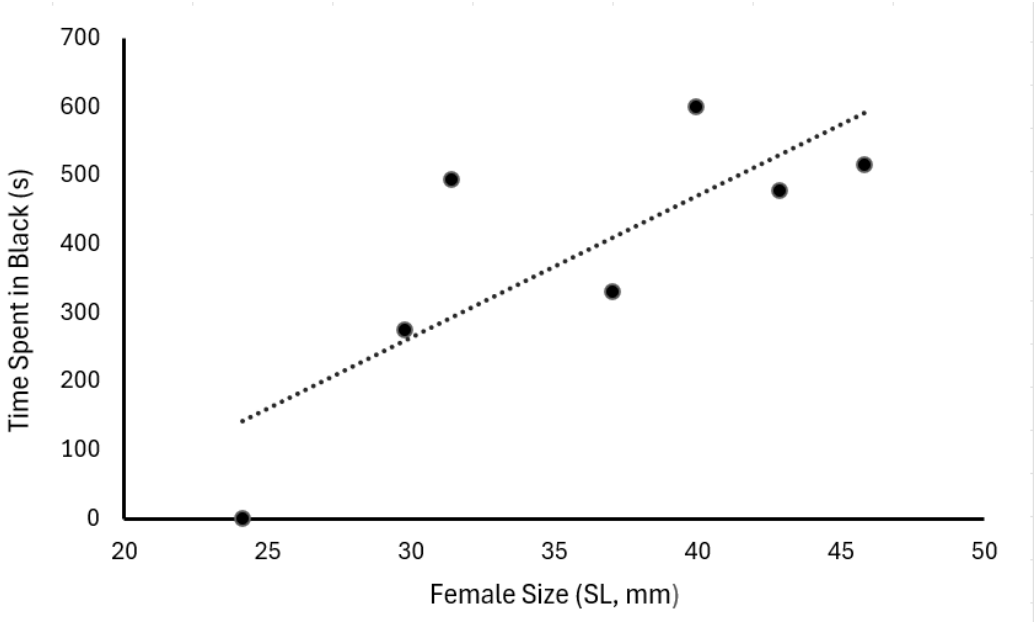

Figure 3. The relationship between female body length and time spent in the black for the control group (0 ppm)(r^2= 0.6235, N=7). There was a significant p value of 0.0347 which suggests a high correlation.

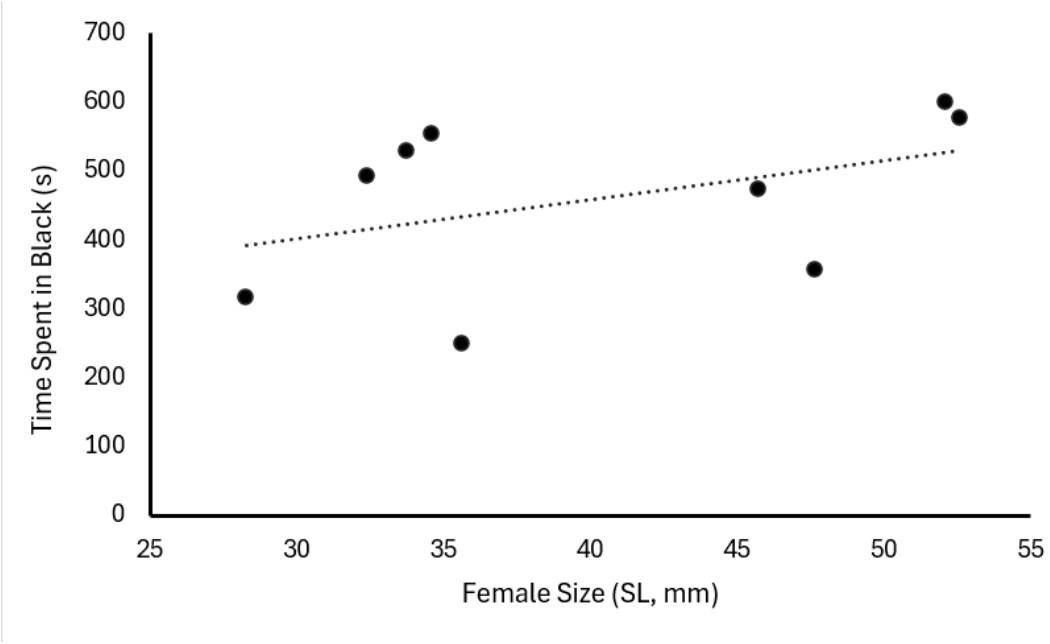

Figure 4. The relationship between female body length and time spent in the black in the 10 ppm concentration group (r^2= 0.1773, N=9, p=0.2590).

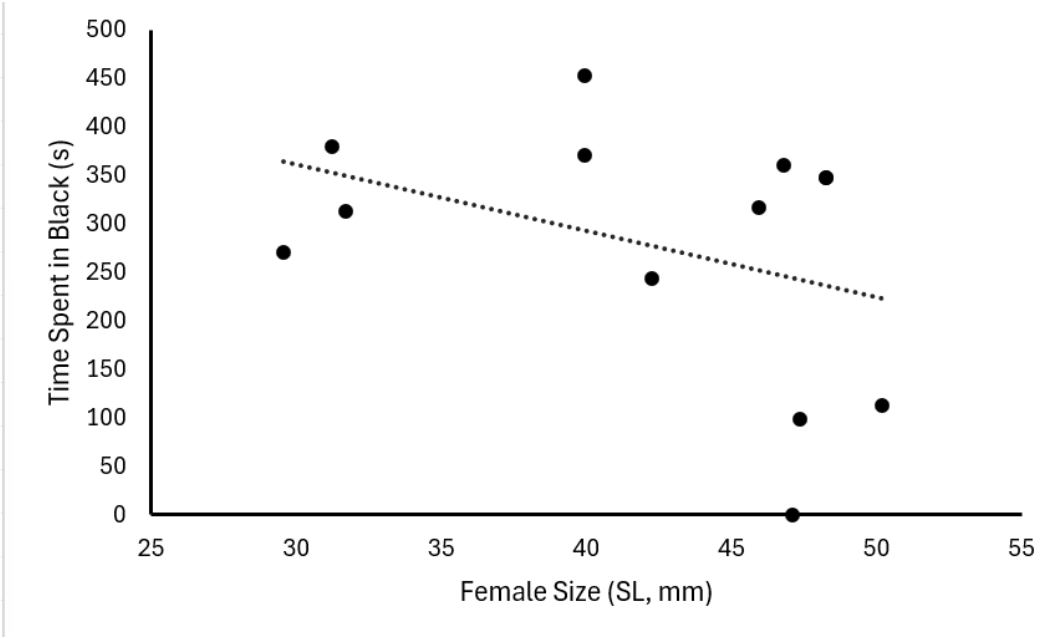

Figure 5. The relationship between female body length and time spent in the black in the 100 ppm concentration group (r^2= 0.1390, N=13, p=0.2095).

General Linear Mixed Model (Nitrate Lab Data)

There were no correlations or significance found between nitrate concentration, length and the combination of the two variables (Table 1).

Source	Chi-Square	p-value
Nitrate Concentration	2.2998	0.3167
Female Size	1.4607	0.2268
Nitrate Concentration * Female Size	1.852	0.3961

Table 1. Linear model examining the influence of nitrate concentration and female body size on time spent in the black section.

Concentration Comparisons (Nitrate Lab and Arsenic Control Data)

The addition of the arsenic control group data was to increase the sample size. The average time spent in the black section for the control group was 354.32 ± SE: 138.86 seconds (Figure 6). The average length of females was 37.14 ± SE: 8.42 mm. There was a significance between the 10 ppm and 100 ppm concentrations. There was no significance between the 0 ppm and 10 ppm or the 100 ppm respectively. There were 41 individuals in this data set. Identical to the nitrate lab-only data set, the same 11 female fish did not go through the mate choice test before the scototaxis test.

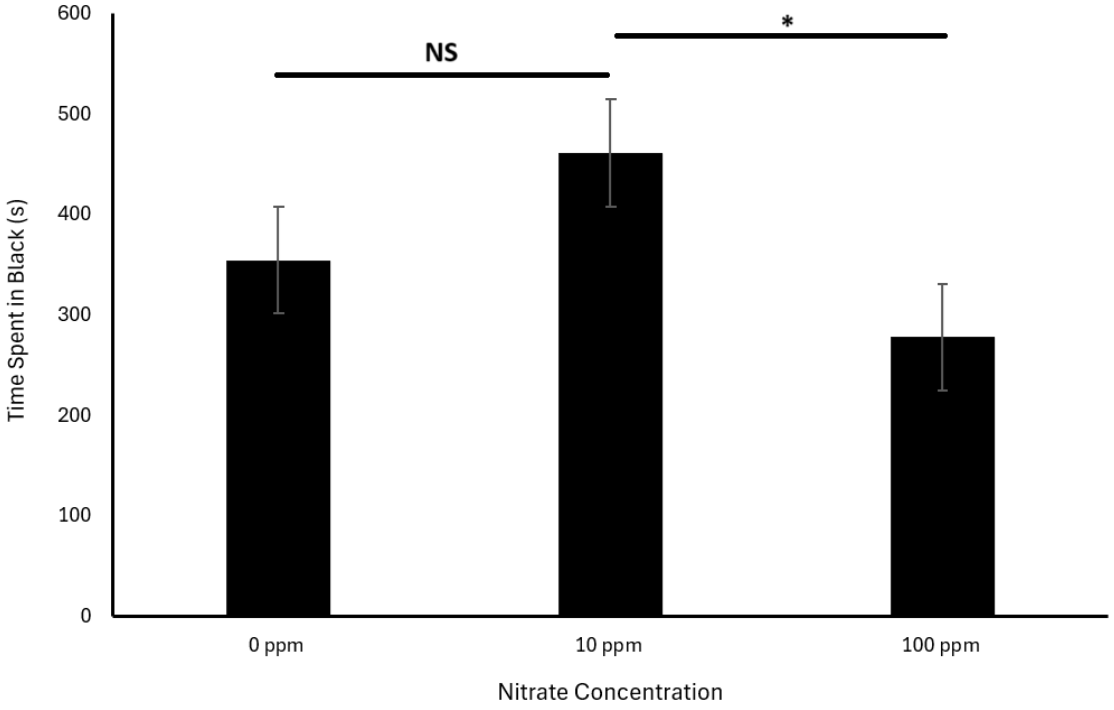

Figure 6. Comparison of the mean time females spent in the black section of the tank among nitrate concentrations. An asterisk represents the significant difference between the 10 ppm and 100 ppm concentration groups (ANOVA: p= 0.0117; Tukey's post-hoc: 10 ppm: p= 0.0084). Females in the 100 ppm concentration group spent the least amount of time in the black.

Female Body Size Linear Regressions (Nitrate Lab and Arsenic Control Data)

There was a significance found between female body size and time spent in the black section for the control group (r^2= 0.2507, N= 19, p=0.0290; Figure 7). The significant p-value= 0.0290 suggests a high correlation between the two variables. The average length in the control group is 32.20 ± SE 6.00 mm.

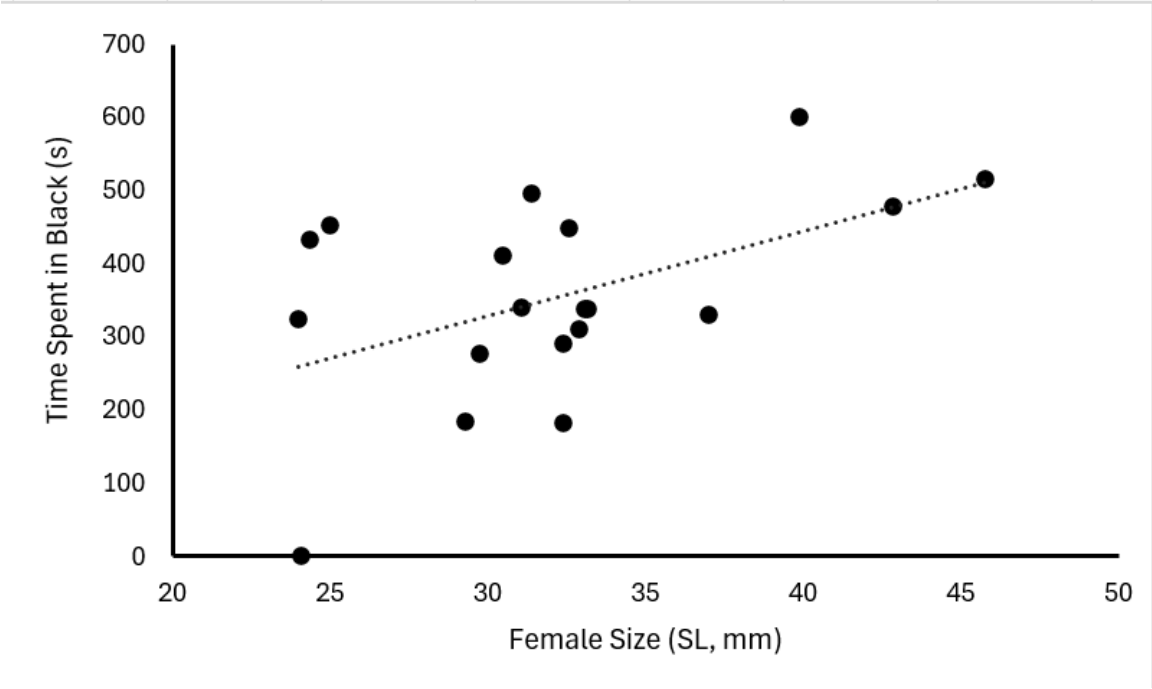

Figure 7. The nitrate lab and arsenic data's relationship between female body length and time spent in the black in the 0 ppm concentration group (r^2= 0.2507, N= 19, p=0.0290).

General Linear Mixed Model (Nitrate Lab and Arsenic Control Data)

There were no significant p-values found in this analysis (Table 2).

Source	Chi-Square	p-Value
Length	0.8815578	0.3478
Nitrate Concentration	1.05655924	0.5896
Nitrate Concentration * Length	1.2142174	0.5449

Table 2. Linear model examining the influence of nitrate concentration and female body size on time spent in the black section using the nitrate lab and arsenic control group data.

DISCUSSION

Nitrate Concentration

There were no major differences in nitrate concentration results between the nitrate lab-only data and the data set with arsenic data added. In both of the data sets, the nitrate concentration 10 ppm had the highest amount of time spent in the black section and with further investigation using Tukey-Kramer's test, there was a significance between 10 ppm and 100 ppm (p= 0.0229; Figure 2 & 6). These results did not follow the trend that was seen in a similar study focusing on arsenic contaminants and *B. splendens.* The arsenic study found that fish spent more time in the dark section when they had higher concentrations of arsenic in their system (Tudor, 2018). Another study observing effects of 17α-ethinylestradiol, an estrogen mimic on *B. splendens*, found that there was a reduction of boldness when exposed to the compound. (Dzieweczynski, Campbell, Marks, Logan, 2014). In contrast, a study observing male *B. splendens*'s behaviors of boldness and decision making when exposed to 17α-ethinylestradiol found that exposure to the contaminant decreased boldness (Hebert et. al., 2014). These differences may be due to the tests being used, testing environment, dosage amount, or multiple of these factors and more.

Female Body Size

In the nitrate lab-only data set, there was a significant p-value in the control group (0 ppm) linear regression (Figure 3). This suggests that there is a high correlation between body size and anxiety behavior. When the female is smaller, she is more anxious. A study with wild populations of *Brachyraphis episcopi* taken from different locations of the same stream found that the larger fish were less bold and slower to

emerge from a shelter (Brown and Braithwaite, 2004). To increase the sample size of the control group, we added control group data from Dr. Tudor's study on *B. splendens* and increasing levels of arsenic (Tudor, 2018). This increased the sample size of the control group from 7 individuals to 19 individuals. When re-analyzing the new data, we still saw significance in the control group (Figure 7). This follows the same trends of the control group in the nitrate lab-only data (Figure 3). The 10 ppm and 100 ppm data was the same for both data sets. Thus, we can conclude that the other concentration groups (10 ppm and 100ppm), have no significant p values (Figure 4 & 5). These trends follow a study conducted with guppies (*Poecilia reticulata)* also found that there was no significance between boldness and body size (Harris, Ramnarine, Smith, Pettersson, 2010). This study had 167 test subjects with mixed sexes. This leads to the conclusion that though we tried to increase the sample size by adding control data from the arsenic study (Tudor, 2018) ultimately, we need a bigger sample size from all of the different concentration groups to gain a better understanding of whether there is a correlation between body size and anxiety. A bigger sample size would also allow us to have more flexibility in the statistical tests that could be used. In addition to a bigger data set, we want an equal sample size within each concentration group.

CONCLUSION

Betta splendens are an important species in this scientific community. They have become a staple for animal behavior, specifically in aggression, but they can provide so much more information if we expand our areas of study. Though there are naturally occurring nitrates in the cycle of life, we humans are elevating the concentrations at an alarming rate. This study has merely suggested that nitrate contaminants and body length have some effect on anxiety-like behavior in *Betta splendens*. We have only looked at a sliver of what we can explore and study. This study could be further developed by increasing its sample size which in return would allow for stronger trends and results. This study and other studies exploring the effects of different water contaminants on aquatic ecosystems are so important to understanding animal behaviors and their evolutionary history.

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APPENDICES

Appendix A: Institutional Animal Care and Use Committee (IACUC) Form & Approval

Office of the Vice President
For Research
Institutional Animal Care
and Use Committee

THE UNIVERSITY OF MAINE

311 Alumni Hall
Orono, Maine 04469-5717
Tel: 207-581-2657

www.umaine.edu

DATE: 8/29/2023

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
Office of the Vice President for Research
311 Alumni Hall

ANNUAL REVIEW OF APPROVED PROTOCOL

In compliance with federal regulation, please complete this form and return it to umric@maine.edu no later than three weeks from the date of this memo.

NAME: Mary Scarlett Tudor

APPROVED PROTOCOL #: A2021-07-01

TITLE: The mechanisms of altered reproductive behavior of Betta splendens due to contaminant exposure.

APPROVAL PERIOD: 10/18/2021 THROUGH 10/17/2024

1. Check correct line below. Additional information required on reverse side.

_____ Project involving the protocol referenced above has been completed and is no longer active.

__X__ Project is active and approved protocol is being followed.

_____ Project is active, but the protocol needs to be modified. (IACUC approval required; please submit an amendment. Modifications must be approved before they are implemented. See IACUC website for amendment instructions.)

_____ Project is active, but approval period will expire before project is completed. (A new protocol must be submitted for review, and project may not continue past the expiration date until a new protocol is approved.)

_____ Other (please explain).

MORE INFORMATION TO COMPLETE ON REVERSE SIDE!!

AUTHOR'S BIOGRAPHY

Tess Hureau was raised in Fort Collins, Colorado. She attended the University of Maine pursuing a degree in marine science with a concentration in aquaculture. During her college career, she participated in the swim club and crew club. She worked as a lifeguard at the Wallace pool for three years. In the fall semester senior year, she did Semester by the Sea at the Darling Marine Center in Walpole, ME.