A Thesis Presented to

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Blues in *Betta splendens*

by

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ABSTRACT

Betta splendens are a very popular fish. They are commonly kept as pets, but are also occasionally used as models for research. With such a wide variety of colors possible, and breeders often looking for specific colorations, there should be much information regarding genetic inheritance available. However, the few sources available are less than reputable, and have no research to back up their models. Blue color in *Betta splendens* covers other colors when fully exhibited. I hoped to learn the inheritance pattern of the three blue: royal blue, turquoise, and steel blue. I hypothesized that the popular model of incomplete dominance was correct, but that Steel Blue was the heterozygous genotype, and royal blue and turquoise were the homozygous genotypes. However, our breeding attempts were not successful. In addition to inheritance, I also tested color preference of female *Betta splendens* between the three blues. I hypothesized that females would prefer the richer colors of royal blue and turquoise, over the less visually rich steel blue. I found that turquoise was the most preferred color by females, with the most time spent with the turquoise male overall.

INTRODUCTION

Many people are familiar with "betta fish" found in pet stores across the world. Often called "Siamese fighting fish," these are domestic *Betta splendens* (Figure 1). The *Betta* genus includes seventy-three known species that vary wildly in appearance, temperament, and behavior. Only the *Betta splendens* species has been truly domesticated at this point in time, though there are still wild populations. The domestic varieties have a wide array of colors and tail shapes available, making these fish flashy and eye catching - and very popular.

Figure 1. Male *Betta splendens* obtained from Petco (Low quality steel blue color)

Most breeders are located in Thailand, where these fish are native. However, there is a general lack of genetic knowledge, there are a great number of "culls" produced in each spawn - fish that are undesirable in some way (incorrect color, bad form, deformities, etc) and are often euthanized by various methods. Breeders tend to breed the same color to the same color and expect the same color to result, and when this doesn't happen, they are left with an undesirable spawn of offspring that they have wasted time and money on. There is a singular model of genetics that can be found reiterated on many disreputable sites ("Betta…" 2019, Gianne 2017, van Esch, "The Basics…") but little research to back it up. If the colors and genetics of these fish could be understood more concretely, it could streamline the breeding process and benefit both the breeder and purchasers of *Betta splendens* - both individuals who purchase directly, and large box stores that purchase in bulk. Breeders would be able to produce the desired colors more efficiently, and focus on other traits, such as form or health.

With at least eleven base (Such as the solid blues, blacks, reds, oranges, and yellows) colors, and at least ten modifiers (such as the different spread (coverage) of the colors, the "marble gene" that causes patches of color, and other similar genes) that affect the base colors (van Esch), understanding the genetics of each color and how they may affect each other is a time consuming task. The colors/pigments are located in layers, one color over another. The top layer of color is blue (or iridescent), which is produced by iridophores and the guanine crystals,

which reflect blue light to our eyes (Amiri 2012). Below that is the black layer of pigment, whose color is produced by the melanin, located in melanophores (Usui 2018). The lowest color layer of pigment is the red (and yellow) layer, whose color is determined by erythrophores and xanthophores (Chaplen 2002). Below that is what is often called the cellophane layer - a layer with no pigment. It appears flesh-colored, and the fins are transparent. (Chaplen 2002)

The color layer I chose to begin with is the blue. This is because it is the most external and covers the other colors, making it the most easily visible layer of color. There are three colors of blue: Royal Blue, Turquoise, and Steel Blue (Figure 2). Royal Blue is the richest color, a dark blue. Turquoise is a lighter blue, often with a green sheen visible in the right light. Steel Blue is the lightest of the colors, a grey blue. The Steel Blue is the hardest of the three to find, and can be mistaken for other colors by those not familiar with it. Two other colors may also occur in this blue layer - copper and dragonscale (A thick, white color of the scales). However, these are supposedly bred in from other *Betta* species, and as such were not included yet. (van Esch)

Figure 2. Left to right: Royal Blue male, Turquoise male, Steel Blue male. Actual fish purchased for the study.

The popular model states that these blues are regulated by incomplete dominance, which is when there are two alleles, and the one does not fully mask the other, resulting in an

intermediate color (a common example used in classes is a red and white flower producing pink flowers)("Betta…" 2019, Gianne 2017, van Esch, "The Basics…"). With incomplete dominance, it is impossible to determine which allele is dominant and which is recessive, because neither is dominant or recessive. With this model, the popular consensus is that turquoise and steel slue are the homozygous genotypes, and royal blue is the heterozygous genotype (van Esch). However, consider: steel blue is the rarest of the three, and turquoise and royal blue the most common. With the breeders almost always breeding the same color together, one could infer that the homozygous genotypes would be the most prevalent, as few heterozygotes would be produced this way. In a wild population, the ratio would likely be different, as there are not humans interfering with the breeding and mate choices. However, in this case, the numbers are going to be skewed towards homozygosity. Because of this, I hypothesize Steel Blue is actually the heterozygous phenotype, and Turquoise and Royal Blue the homozygous phenotypes.

There have been studies in the past testing female *Betta splendens* color preference (Brownwell 2015). These found that females tended to prefer red males over blue males, and dark red males over light red males. However, they do not specify which color blue was used, and simply said they were "of varying pigment". This is a common issue seen in other research articles that used iridocytes from blue *Betta splendens*. They did not specify which blue, or investigate if the blues had different effects for their study (Chaplen 2002, Amiri 2012). Brownwell et. al (2015) speculated their females preferred red over blue as a rich red is tied to healthier immune systems in some other fish, and may be indicative of a better forager. However, they did not take into consideration the coloration of wild *Betta splendens,* which features red, but only reduced spread of blue (Figure 3). No studies were located comparing female color preference between blue colors in males. I hypothesized that females would prefer

the richer coloration of blue over less rich, as they preferred the darker reds; preferring Royal Blue the most, and Steel Blue the least. Though it is possible they may prefer Turquoise over Royal Blue as it is the wild type. However, the wild type is a reduced spread - meaning the iridophores are not a solid mass, creating more of an iridescent sheen, and are not always visible. The fish acquired for this project are full spread blues, for easier identification. The full spread means the iridophores completely cover the fish, blocking out any other colors, making the fish solidly blue, hence why it is easier to differentiate between the colors.

Figure 3. A wild caught male *Betta splendens* showing black, reduced red (meaning it is only on the fins), and reduced spread Turquoise blue (meaning it is only iridescence, and only visible when light shines on it). (Sriboribun)

The blue in *Betta splendens*, like in many other species, isn't the result of a pigment. Instead, the iridophores contain guanine crystals that reflect blue light to our eyes, giving the blue appearance. Different orientations of the crystal stacks reflect different wavelengths, producing the different colors. The crystals rest in a chitin framework that can be used to regulate distance and orientation (Kimura 2020) (Figure 4). The crystals' positions can be modified by the fish as well. When stressed, the crystals can shift to reduce their reflection of light, making the color seem to fade visually. This can start to expose the underlying melanin (Amiri 2012). Colloquially, when this happens, it is called "stress stripes" and is often used by

betta keepers to identify when something is wrong - whether it is the result of illness, bullying, fear, water parameters, etc. (Figure 5).

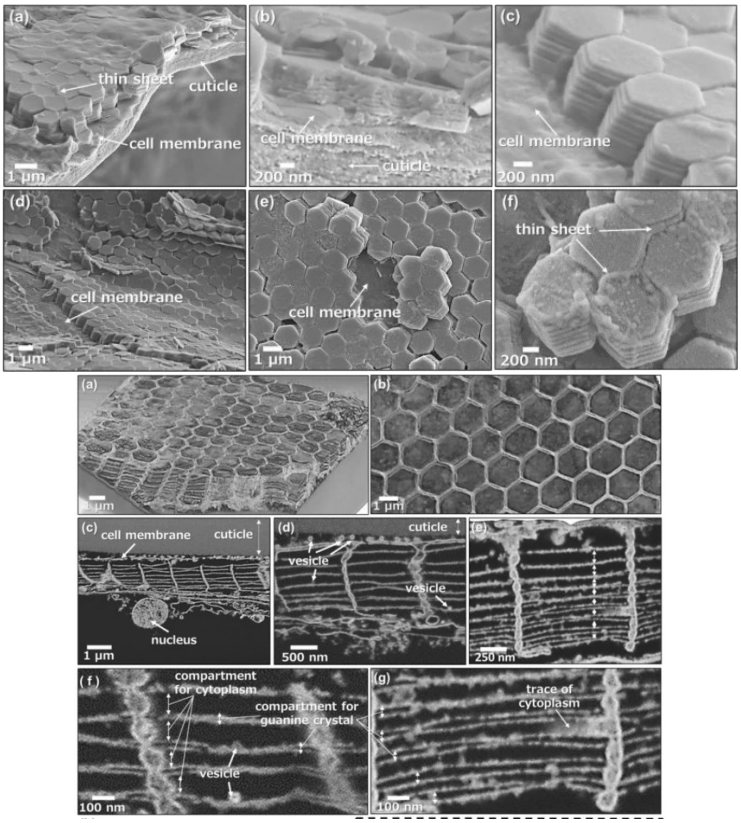

Figure 4. SEM images of guanine crystals (top) and the chitin framework (bottom) from Kimura et al (2020)

Figure 5. Turquoise Female 1 (TF1) exhibiting minor stress stripes due to the stress of being introduced to a new tank and the other females. The stripes are barely visible in the left image, moderate in the center image, and prominent in the right image.

These properties of guanine crystals give them several potential uses for humans, beyond providing color for ornamental fish. Because of their reactions to stressors, they can be used to detect toxins in the environment - both chemical pollutants and bacteria can be detected with this method. Chaplen (2002) created a method for doing this, and engineered a portable device for analysis in the field. Kimura (2020) experimented with using guanine crystals from an unspecified fish to reflect light to create a finely tunable way to direct light when using microscopy. Studying the different blues in bettas may provide important information to others looking to study similar topics as Chaplen et. al (2002) and Kimura et. al (2020) with information to help them refine their methods. I chose to begin the inspection of these blue colorations in *Betta splendens* by looking at inheritance and preference because that information will enable others to more efficiently produce their own fish of these colorations for their own studies.

The guanine crystals and their chitin framework rest on the outside of the cell (Figure 4). Chitin is a polysaccharide made from chains of glucose, which is found in cell walls of fungi, exoskeletons, and certain hard structures in invertebrates and fish (BD Editors 2017). While the chitin in *Betta splendens* has not been specifically studied, there is research regarding it in other species. In fungi, DNA is read and transcribed into RNA, which is translated into a protein. These proteins are combined in the rough endoplasmic reticulum into an inactive form of chitin synthase. This is transported to the golgi apparatus, where it is packaged into a chitosome, which is transported to the cell membrane, and places the chitin synthase onto the interior surface. From here, it produces chitin, which is placed on the outside of the cell membrane (Copen 2001). The chitin on *Betta splendens* cells is likely produced in this same fashion, with the genetics controlling the pattern and size of the framework produced by the chitin synthase. The inter-

guanine plate distance is what regulates the light wavelength reflected, and changing the distance of that space changes the reflection, however, the mechanism for that change has not yet been found (Kimura 2020). However, it was noted by Kimura et al. (2020) that the inter-plate distance did shrink when their framework samples dried out.

The life cycle of a *Betta splendens* can be divided into several steps. Bukhori (2016) choses several stages to divide the life of the fish by, including: egg, fry, free swimming, juvenile, and sexually mature adults. A male looking to mate will build a nest of bubbles (often called a bubble nest). These bubbles are coated in saliva to prevent popping, yet still require still water. Once the male deems his bubble nest sufficient, he will begin showing off for females - flaring his fins and attempting to lead her back to the nest. She will inspect the nest, and if she does not find it adequate, she will destroy it or ignore the male (Robert 2018). If sufficient, they will mate by "wrapping" - twisting their bodies around each other to place the reproductive organs close together. The male will fertilize eggs as the female releases them. The pair will then pick the eggs up off the bottom using their mouths, and place them in the nest. After this, the female is removed (Robert 2018)

The male monitors the eggs in the bubble nest for approximately 36 hours until the eggs hatch. The offspring, called fry, remain in the bubble nest for approximately three days before becoming free swimming. The male should be removed and the fry independent at this point. Around three to six weeks, the fry will begin developing their labyrinth organ, and require humidity for proper development (Adam 2021). At around two and a half months of age, the fry will begin reaching sexual maturity. In a tank, males, and some females will need to be removed to prevent aggression. Females have a visible ovipositor (Colloquially called an "egg spot")

located between their ventral fins and anal fin, however, males, particularly young males, can have a false egg spot, making this only a marginally successful way to determine sex. In domestic *Betta splendens* of the halfmoon tail type, males have significantly longer fins than females. However, this is also not a reliable way to determine sex, as there are shortfinned variants that can occur in males. The best way is to determine sex is, if possible, to look through the fish and observe ovaries. Only females will have them. This is, unfortunately, not always possible. A combination of these methods is most often used. The reproductive prime for these fish is four to 12 months of age, and the average lifespan, in captivity, is three to five years (Robert 2018).

MATERIALS AND METHODS

Fish selection

Using an intermediary (4k Bettas, located in California), an unknown breeder was located, and two pairs of royal blue, two pairs of turquoise, and two steel blue males were purchased. The breeder did not have any Steel Blue females, so a single female was located from an alternative breeder (Ake Bettas in Thailand). Toplines (The shape of their back) and finnage of all fish meet International Betta Congress standards (*). Fish were four months of age, the beginning of the sexually mature period (four to twelve months is optimal). Photos and videos were requested prior to purchasing to ensure proper coloration. Overnight shipping from the intermediary to the lab was used. The breeder provided the following information regarding the pairs and steel blue male: When asked if the fish were related, the breeder claimed they were not, and when asked what colors were the parents, the breeder said “same color”. It is unclear what this latter statement means.

Bettas with full spread blue coloration were used, and the “Halfmoon” tail type. Full spread ensured there was no ambiguity about the color, as there can be with reduced spread (which is more of an iridescence). Halfmoon tail type ensured that any offspring would be easy to sex, due to the males having long fins and the females having short fins, and that there would be a lot of the color easily visible for identification.

Fish care

Males were kept in individual two and a half gallon aquariums with no filters. Paper was placed between the tanks to ensure they could not see each other. Tanks were heated to 79 F using Freesea 50W heaters. They received once weekly 50% water changes. Females were placed in a fifteen gallon aquarium together (which is called a sorority), also heated to seventy-nine F with a Freesea 50W heater, the 15 gallon received 25% weekly water changes. Females were floated in bags next to each other for one hour to reduce fighting during introduction. All tanks had *Terminalia catappa* leaves (Commonly called Indian Almond Leaves, abbreviated IALs) added for tannins, and *Pistia stratiotes* (Commonly called dwarf water lettuce) was added for enrichment. All tanks used tap water that was treated with Seachem Prime.

All fish were fed a diet of Omega One betta buffet pellets once a day, except when otherwise specified in the following procedures.

Breeding procedure

Two attempts at breeding the fish were made - fish were conditioned for one week the first time, and two weeks the second time. This involved feeding three times a day with frozen bloodworms, and placing the female in a clear cup in the males’ tank for 10 to 15 minutes once a day. During the conditioning period, small sponge filters were allowed to acquire nitrifying bacteria in a privately owned and established tank (not laboratory conditions) that contained one

male betta and seven neon tetras. This was done so that living bacteria could be easily transported into the breeding tanks when needed, to facilitate the nitrogen cycle and prevent ammonia or nitrite poisoning. This process is commonly called “Seeding media”.

After this period, males were moved to ten gallon breeding tanks that were filled with six inches of water heated to 83 F. *Terminalia catappa* leaves and *Pistia stratiotes* were added for the male to build a nest under. The seeded sponge filters were added and adjusted to have as little air output as possible to prevent the bubbling of the filter from disturbing the surface, and ruining the males bubble nests. Again, paper was placed between tanks so males could not see each other. Once fish were in the tanks, plastic wrap was used to cover the tank to increase humidity. Females were placed in 64 ounce mason jars in the center of the 10 gallon tanks (Figure 6).

I bread three pairs, one of each color - a Royal Blue male and Royal Blue female, Turquoise male and Turquoise female, Steel Blue male and Steel Blue female. Because I had two fish of each color and two of each color per gender, the male was selected based on which of the two built the better bubble nest during conditioning. The female was selected based on best color or form, or most visibly containing eggs at the end of the conditioning period.

Once males in the breeding tanks had constructed a sufficient bubble nest and were showing appropriate behaviors - including flaring his fins at the female, and attempting to lead her back to his nest - females were released.

After mating, females were removed. Males remained in the tank and received continuous light - about 12 hours of white light during the day, and about 12 hours of blue light at night, using a Nicrew brand light, 18W - while they monitored the nest. The light is provided at all times so that the male is able to find and retrieve any eggs that have fallen from the nest. Eggs hatch after approximately 36 hours. Male continues to monitor the baby fish, called fry,

while they remain in the bubble nest for approximately three days. After this period, the fry become free swimming, and the males are removed.

Babies receive once daily 90% water changes, and three times daily feeding with live foods. Vinegar eels were used for the first two weeks, then a mix with baby brine shrimp. Salt water and vinegar solution were not allowed to enter the tank water, to prevent any potential negative effects on the fry.

Figure 6. Breeding tanks (without sponge filters and saran wrap)

Color preference testing

Ten gallon tanks were filled with six inches of water. The tank was (visually) divided into thirds using paper under the tank. Each thirds' center was marked to ensure equal spacing. The sides of the tank were also covered with paper to eliminate distractions. The tank was heated to 80 F using an adjustable aqueon 100W heater - this is because the females being used came out of a personal sorority that is also at 80 F, this eliminated the need for a period for temperature acclimation. A tank light was placed on top - an AquaNeat 10 gallon light. Males were placed in

64 ounce mason jars - one of each color blue was used. These were placed in the center of their third of the tank (Figure 7)

Females were then released one at a time into the tank. An ipad was placed directly above the tank, looking down. This was used to record a 15 minute period. After the 15 minute period, the video was reviewed, and the female replaced in the sorority. Using time stamps on the video, it was calculated how much time the female spent interacting with each male. Interacting included, but was not limited to: looking at directly, flaring at, swimming into the glass, and purposefully circling the jar. Data was then recorded while the next female was placed in the tank, and the ipad set to record again.

Fifteen females of varying color, age, and tail type were used. The males used were the same ones, from the breeder mentioned above. None of the females were of the same coloration (completely) and included every base color. The females were transported from sorority to test tank using a cup. There were a few *Salvinia minima* present in the tank as they are present in the sorority tank, and were transferred with the water that was in the cup with the female.

Figure 7. The testing tank set up.

RESULTS AND DISCUSSION

Breeding

The first round of breeding attempts did not result in any offspring. The fish had shown good behaviors, discussed earlier,h prior to being released, and continued to do so while released, but none attempted to mate, referred to as wrapping. The Turquoise female (TF1) released her eggs in her container prior to being released. As the fish continued to not wrap, and I knew TF1 may not have many eggs left, females and males were all removed to their original tanks for additional conditioning.

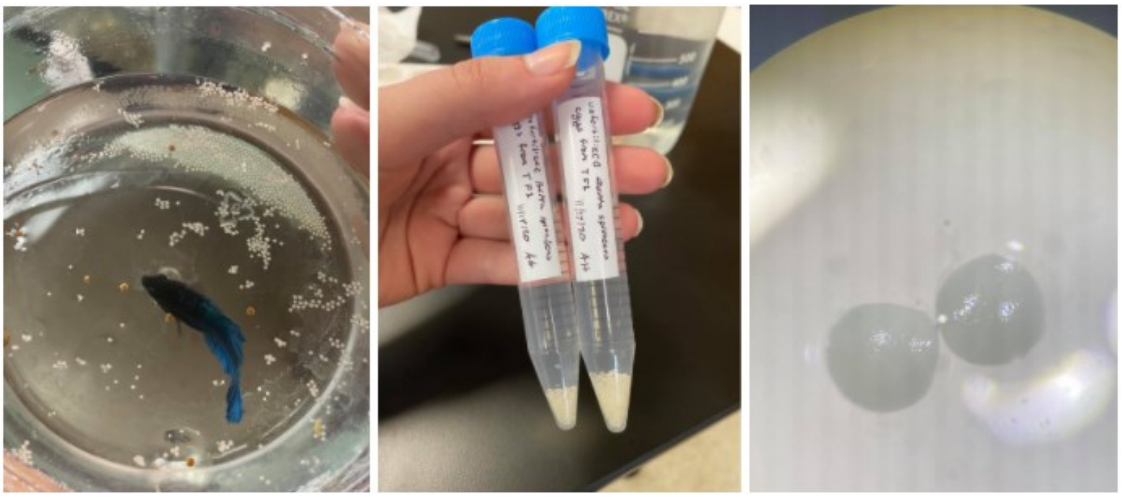

Figure 8. Left to right: TF1 with her eggs in the cup as I found her. Eggs preserved in ethanol. Two eggs under 100x magnification.

TF1's eggs, when found, had about half floating and half residing on the bottom. The eggs were preserved in 80% ethanol with as little water as possible. Eggs were not dried. Eggs were observed under 100x magnification - appeared slightly textured and had a clear coating around them (Figure 8).

The second attempt at breeding resulted in all three pairs wrapping and dropping eggs. The Steel Blue pair was the most violent with each other, and I nearly pulled the female for

worry she would be seriously hurt. They, however, successfully wrapped. The Turquoise pair were the most interested in wrapping, but struggled due to TF1's large amount of eggs (she was too round for the male to position himself correctly). However, they were eventually able to successfully wrap and drop eggs. The Royal Blue were the most dysfunctional pair - the male would send the female mixed signals - he would have her follow him to the nest, then chase her away. Eventually they did wrap and drop eggs.

The Royal Blue male ate his eggs the first night. He was pulled from the breeding tank for reconditioning. The Turquoise and Steel Blue males successfully hatched their babies and kept the babies in the nest until they were free swimming. At two weeks, the Turquoise fry all died at approximately the same time. The Steel Blue fry hardly grew, and slowly died off. The last few died after the tank was moved. I suspect the stress of the move killed the last few, but they may have been sickly as a whole.

I also tried the secondary Royal Blue pair (Royal Blue Male 2 (RBM2) and Royal Blue Female 2 (RBF2), after the first pair failed. They exhibited the appropriate behaviors while the female was contained. After she was released, they continued to show good behaviors. They were left unobserved for a period of two hours, during which the female attacked the male (Figure 9). He was treated to prevent infection and changed to a slightly better diet than the Omega One pellets, as well as received additional vitamin supplements. Ultimately he recovered.

Figure 9. RBM1 immediately after being pulled (left), and RBM1 after one month of treatment and regrowth

The females in the sorority fell ill after their tank was moved as well. They were treated with API General Cure, Furan-2, and KanaPlex. Only RBF1 survived.

Between the loss of the fry and loss of the females, the breeding attempts were halted.

Color Preference

Females' time spent interacting with each male was recorded. The male that the female spent the most time with was deemed to be her "preferred male". The Steel Blue male was the most preferred male, with six females spending the most time with him. The Turquoise male was the least preferred male with only four females preferring him (Figure 10). The data was analyzed with a chi-squared goodness-of-fit test, and was found to be not significant, with a P-value of 0.819.

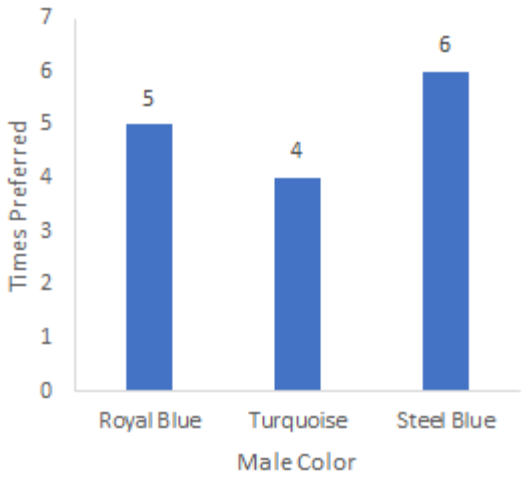

Figure 10. The number of instances where the male of each color was the preferred male by the females

The male the females spent the most time with total, was the Turquoise male, with the females spending 839 total seconds with him. The Steel Blue male, despite being the most often preferred male, had 787 total seconds spent with him. The Royal Blue had the least amount of total time spent with him, at only 559 seconds total (Figure 11). This data was also analyzed with a chi-squared goodness-of-fit test, and was found to be significant, with a P-value of 0.000.

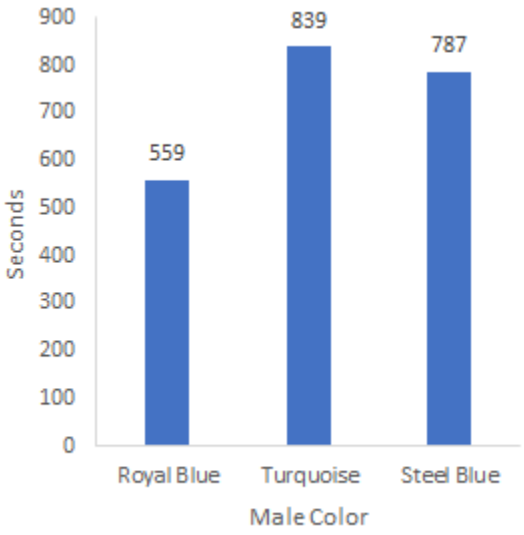

Figure 11. Total number of seconds females spent with each male

Also recorded were the number of individual interactions each female had with each male. The Steel Blue male had the most interactions, followed by the Turquoise male, with the Royal Blue male having the fewest interactions (Figure 12). The females' first interaction with a male was also counted, as most females swam around before interacting with a male. The males were all interacted with first five times, meaning there was no one male that attracted females more significantly at first. This data was again analyzed with a chi-squared goodness-of-fit test, and was found to be not significant, with a P-value of 0.305.

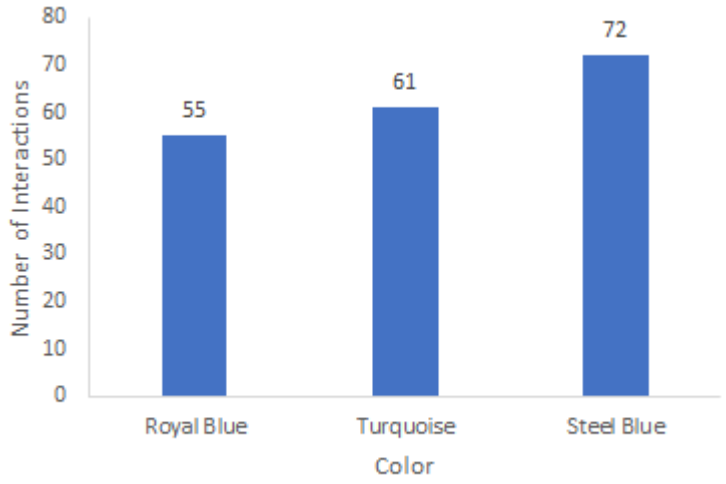

Figure 12. Total number of interactions females had with each male

The steel blue male was the most preferred male and had the highest number of individual interactions, but with this result being not significant, we must turn to the data relating to the total time spent with each male. The turquoise male had the most time spent with him, and it was significant, indicating that the turquoise male is preferred most by the females.

CONCLUSION

I think testing with a larger group of females would be necessary to confirm the color preferences. The turquoise male was the least often preferred male, yet the individual females who spent the most time with him, spent much more time with him than other females did with their preferred male. This could be due to the nature of the females tested - perhaps the ones who preferred the turquoise male had more obsessive tendencies. Or perhaps, something about preferring the turquoise color leads to a higher level of interest, such as temperament.

I hypothesized that royal blue would be preferred due to the richness of its color, and past tests finding dark red was preferred over light red (Brownwell 2015). However, turquoise is also the natural color of blue found in bettas. Perhaps the natural color of the fish, even in altered forms (Such as the full spread instead of the reduced spread of the blues, or the extended spread of the red instead of the reduced spread) is preferred over more recent mutations. This could be studied further, especially if a wild type *Betta splendens* could be obtained for testing.

Further testing regarding color preference with an increased number of females could be beneficial, to see if this preference for turquoise holds true, or if there are any temperaments affecting the results. The royal blue male, for example, was the most aggressive out of the three males used for breeding. Perhaps this aggression stressed the females during the color preference test, leading to them spending less time with him, because of temperament, rather than color. It would also be interesting to test if females of one color (such as all royal blues) would collectively prefer one color of male, this would determine if the color of the female had any impact on the color of male that was preferred.

I hypothesized that the royal blue color, being a richer visual color, would be preferred over turquoise, which, in turn, would be preferred over steel blue. Individually the steel blue was

the most preferred male among the females, but that was found not significant after statistical analysis. However, the turquoise male had the most time spent with him by the females overall, which was significant, meaning the turquoise was preferred over the steel blue and the royal blue. This disproves the hypothesis, with the royal blue actually having the least amount of time spent with him by females.

While no fry were produced and color inheritance was not determined, it was interesting that the Royal Blue pairs were both very aggressive with each other. In the future, it may be interesting to investigate temperament and if there are any differences specifically related to color. I hypothesized that the steel blue is heterozygous, and the royal blue and turquoise colors are homozygous genotypes, but this was neither proven, nor disproven during this study.

In the future, I would like to attempt to breed these fish again to more concretely determine inheritance. I plan to speak to breeders and look for alternative conditioning methods to try, to better prepare the fish for breeding. I will also look into obtaining new females and/or males, in case there were any genetic weaknesses present in the offspring that lead to the deaths. I am excited by the potential to continue these studies, and plan to do so.

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