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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

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Abstract

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Across many species, an individual's behavior in a social competition can be predicted by factors like size, condition, and previous competitive experience. But how does an individual's behavior in nonsocial contexts influence performance? This study explored how *Betta splendens* performance in social competition correlates to performance across cognitive and behavioral tasks. To investigate this correlation, the question was divided into two experiments, the first consisting of four behavioral tests and the second of five social exposures. The behavioral tests, novel object interaction (NOI), scototaxis, mirror, and detour were conducted to explore how individual subject behavior, stress, and cognition predicted real-life social competition. I found that individual performance in the scototaxis and NOI were not predictive of real-life social competition while the mirror test did predict aggressive behavior. The five days of social exposure testing were organized to also answer the question of how betta behavior differs in competition with a novel versus a familiar opponent. No differences were found in behavior nor neural activity between the two groups suggesting betta behavior does not change depending on familiarity with an opponent. Notably, it was found that a positive correlation existed between the percent time spent in the object zone of the NOI and the standard length of the fish indicating larger fish are less neophobic. Additionally, standard length was also correlated to the principal component analysis for Day 1 social exposure, which suggests that subjects who exhibited more a "preparedness for direct contact" strategy, tended to also be bigger in size.

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Introduction

In all facets of life, the social decisions we make in our relationships can impact our future behaviors and perceptions towards new connections. As the importance of social cognition, defined as "the mechanisms by which animals acquire, process, store, and act on information from other individuals," becomes more and more prevalent in human and animal communities, research in this topic deepens (Bshary et al., 2014). In a recent study about social cognition, rhesus monkeys deviated from human behavior and were found to demonstrate an increase in negativity bias with age, meaning their behaviors were more affected by negative stimuli rather than positive ones of the same intensity (Rosati et al., 2018). This study compared age-dependent social goals between humans and monkeys, shedding light on how social cognition develops in different species. Similarly, research investigating the mechanisms of social cognition in mice has found that the hypothalamic neurons activate both during aggression and when witnessing aggression, helping to develop more accurate social cognition models for us to learn about humans and other species (Yang et al., 2023).

In addition to rodent and primate studies, other taxa, including fish, have been established as model organisms of social cognition, especially as it relates to cooperation and social decision-making (Bshary et al., 2014). Due to the existing homologies between fish and mammals in both brain structures and ability to use learning to solve intricate problems (O'Connell & Hofmann, 2012), findings on fish behavior are more generalizable across species than previously thought. Already studies have found that isotocin may be the fish homolog for oxytocin due to its role in regulating social behaviors in cichlid (Reddon et al., 2012). Despite the observed importance of social cognition in many animals including fish, there is still much to learn about how social cognition develops.

Across species, social cognition can be influenced by many factors, specifically in *Betta splendens,* also known as the Siamese fighting fish, it has been seen that their environment and development can impact their interactions with others. A study by Ichihashi et al. (2004), has demonstrated that bettas who were individually housed and visually isolated during rearing had an increased win rate than conspecifics who were exposed to other betta fish. It has also been documented that the presence of other *betta* in the surroundings depending on if they were conspecific or heterospecific could change the strength of preference of the male *bettas* (Justus & Mendelson, 2018). Similarly previous literature demonstrates that the social surroundings can also determine specific behaviors in *betta* with gill flaring being more expressed in front of male audiences and tail beats in front of female audience (Dzieweczynski & Perazio, 2012).

Due to the large role that the environment plays in a social situation it is important to reflect on *betta* behaviors in non-social situations as well. Prior literature has shown how changes in Siamese fighting fish living conditions such as water temperature (Forsatkar, Nematollahi, Biro, et al., 2016), water disturbances (Hurtado-Parrado et al., 2019), and pollutant levels (Tudor et al., 2019) can influence fish to act bolder, with higher anxiety or even with higher avoidance. A question that remains is can animal behavior in a nonsocial situation reflect their social behavior as well? According to Agues-Barbosa et al. (2022), betta fish who expressed an individual behavior of non-nest builders, also tended to be the most aggressive subjects with highest levels of cortisol. Identifying relationships in behaviors across different tasks, especially those relevant to stress responses, can be key to learning more about how individual behavior influences the outcome of a social competition, which “loosing” for a betta fish can come at a high cost.

Thus, here I explored how an animal's performances in cognitive and behavioral tasks predict response to social novelty in a species that exhibits high levels of aggression in social competitions. Both behavioral experiments and neurobiological analysis were conducted with twenty-four male betta fish subjects. *Betta splendens*, also known as Siamese fighting fish, are a model organism due to their territorial nature and aggressive, combative behavior (Alyan, 2010). The study was divided into two experiments, the first experiment consisting of four behavioral tasks (novel object interaction [NOI], mirror interaction, scototaxis, and detour) to determine how individual *betta* behavior, stress, and cognition predict real-life social competition. For the second experiment, five social exposures were conducted to determine how *Betta splendens* behavior differs in competition with a novel vs familiar opponent.

I hypothesized that individual *betta* behavior would be consistent across behavioral and cognitive tasks, meaning those who spend an increased amount of time in front of the mirror would also be more exploratory in scototaxis and novel object interaction due to previous correlations found between boldness levels in betta fish and aggressive response (Hebert et al., 2014). Furthermore, this consistency would correlate to behavior in a social competition with another live male *betta* and would potentially even predict the winner. Based on previous literature findings that ecological factors influence *Betta splendens* behavior (Brandão et al., 2019), I specifically predicted that male betta who spend a large proportion of time being aggressive towards their mirror image and significantly exhibit aggressive fighting tendencies during the mirror task will also demonstrate significant aggressive behavior during social exposure. I also predict that subjects who exhibit anxiety-like behavior in the scototaxis task or spend less time exploring the novel object will be less aggressive in social exposures, regardless of novelty or familiarity (Maximino et al., 2010). While previous work has shown that bettas exposed to a familiar vs novel opponents do not differ in behavior (Alyan, 2010), given the large amount of research on the neural mechanisms involved in social behavior (Gorlick, 1990), I hypothesized that bettas in social competition that differ in opponent familiarity would show different neural activity in brain regions related to social memory due. As a result of the many different neural mechanisms one *betta* can have, I predicted to see different patterns in the fish homolog of the hippocampus between the fish who were exposed to a novel opponent compared to those who were exposed to a familiar opponent (reflecting memory of the opponent), as well as differences in behavior due. Previous literature has found that Zebrafish can discriminate familiar from nonfamiliar individuals 24 h after the first encounter through visual, olfactory, and acoustic cues (Madeira & Oliveira, 2017).

Methods

Subjects and Housing

Throughout the experiment, the subjects remained the same: 24 adult male *Betta splendens,* ranging in size and color. Although their previous life history remains unknown, they were all purchased from the same local provider, arrived on the same day, and were given one week acclimation before the start of the experiment. All 24-betta fish were individually housed in 1L tanks for a week before commencing any testing to reset their social baseline, and during this time color and standard length were measure for everyone. Subjects were fed commercial pelleted food and were on a 12-hour light: 12-hour dark cycle. The room temperature was set to 80 degrees which also housed a water reservoir filled with reverse osmosis water for use inside of the tanks. Water was fully changed in each tank twice a week. All the subjects were housed according to Emory IACUC regulations (PROTO202200088).

Experimental Design Overview

Two methods of testing, behavioral tasks and repeated social exposures, were conducted, followed by brain tissue analysis. For the behavioral testing, each fish was video recorded as they responded to a novel object interaction (NOI) test, scototaxis test, mirror test, and detour test. All behavioral testing took place within four days, with the NOI and mirror test happening on day 1 and the scototaxis and detour happening on day 4 **(Figure 1)**. Water was replaced between each trial throughout the entire experiment to prevent odor cues.

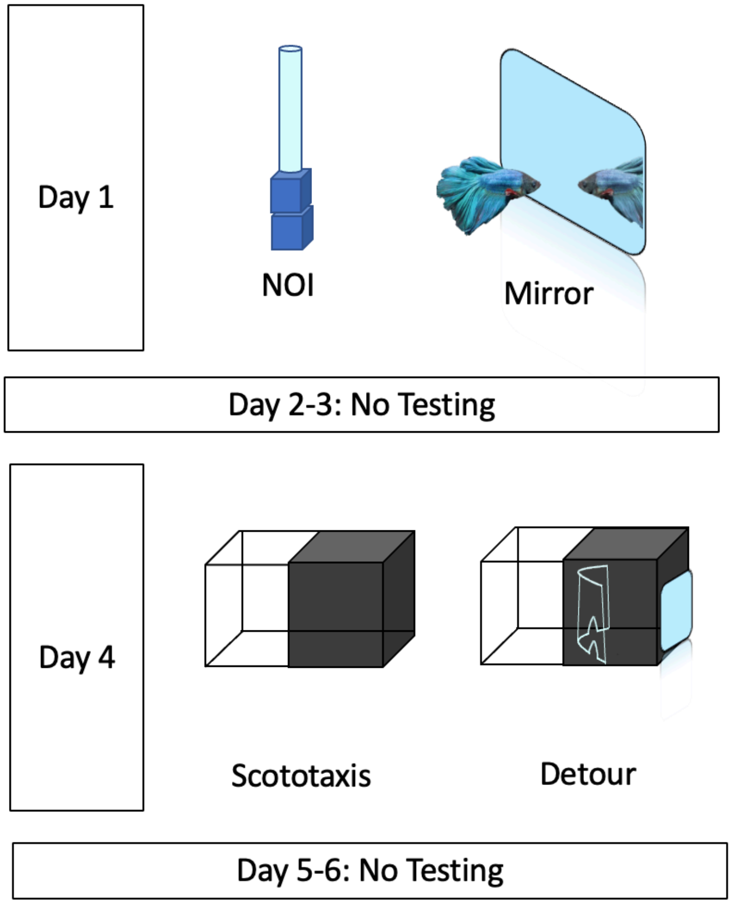

Figure 1. *Behavioral Testing Schematic*. Behavioral testing included a novel object interaction test (day 1), mirror test (day 1), scototaxis (day 4), and detour test (day 4). These tasks all took place during the first week of the study.

Following the behavioral tasks, on day 7, the *betta* fish participated in a social exposure test in which the subjects were evenly assigned to two treatment groups. Half of the *betta* fish interacted with the same opponent for all five consecutive social encounters, one per day, while the other half interacted with the same familiar opponent for days 1 through 4 but found themselves with a novel opponent on day 5 **(Figure 2)**. Brain activity was examined by staining for phosphorylated ribosomal subunit 6 (pS6), a neural activity marker that works similarly to an immediately early gene, staining in response to neural activation (Butler et al., 2019). After immunohistochemical staining, the dorsolateral telencephalon (Dl), the homolog to the mammalian hippocampus (Northcutt, 2005), was imaged count the number of PS6 labelled cells. Specifically, the dorsal division of the Dl was selected for imaging based on ease of consistent identification on stained tissue.

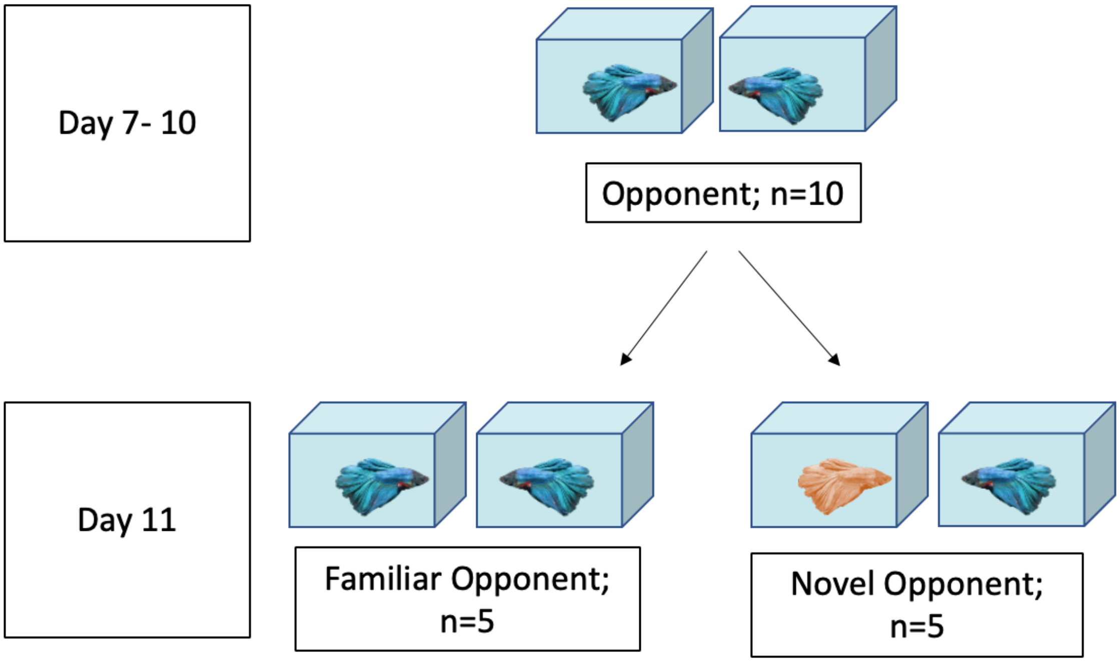

Figure 2. *Social Exposure Testing Schematic*. Social exposure testing was conducted throughout 5 days. On day 5 of the social exposures (day 11 of entire experiment), subjects were split into two experimental groups one receiving a familiar opponent and the other one a novel one.

Behavioral Testing

To observe *betta* fish response to a **novel object,** a novel object interaction test (NOI) was utilized. During NOI tests, the betta fish subjects were each placed inside an experimental tank (33.5 cm x 7.62 cm x 19.5 cm) and given three minutes to habituate to the new environment. For another ten minutes, its behavior was recorded following the insertion of a novel object, a small, blue (4cm x2cm x2cm) plastic rectangle (Lucon-Xiccato & Dadda, 2016). I observed the subject's behavior (neophobia) in response to the novel object by tracking the fish's location, path, and duration of time in each of the following zones: object, object wall, far, far wall. To determine the zones, the experimental tank was split in half, the half that contained the novel object was the object zone while the other half was labeled far object. The walls were calculated as the area 7% from the wall **(Figure 3)**.

To assay anxiety and boldness levels in the *betta* fish, a **scototaxis test** was utilized (Maximino et al., 2010). Here, the subjects were placed in an experimental tank (33.0 cm x 7.62 cm x 19.1 cm) that is divided in half. One half was covered on the sides and bottom in an all-white casing made from laminated construction paper and the other all in an all-black from the same material. In this scenario, the fish get to decide whether to spend their time in the dark or light area, assaying scototaxis. Fish who are more exploratory will investigate the white space, despite it being more exposed, while more anxious individuals will remain in the safer dark area as seen in Zebrafish, goldfish, guppies, and tilapias fish species (Maximino et al., 2010). Additionally, time spent along the wall of the tank were recorded to assay thigmotaxis. At the start of each test each of the fish were positioned in the center and held in place by an opaque plexiglass cylinder (diameter 6.35cm) for a 3-minute habituation period. After, they were released and allowed to roam the entire tank for a duration of 10 minutes. Duration and entry times to each of the following zones are recorded: center black, black wall, center white, and white wall **(Figure 3)**.

A **mirror assay** was conducted to examine the social behavior of *betta* fish. Subjects interacted with a mirror placed on the side of the tank for 10 minutes after a 3-minute habituation period while I record their behavior. The *betta* fish were released in the center of the tank and explore the three zones: mirror, middle, and upper **(Figure 3)**. I recorded the duration of time the *betta* fish spent in each location zone as well as the number of entries into each zone. Additionally, due to the social qualities of the mirror test, I also assessed the aggressive behaviors: ramming, surface breathing, gill-covered display (also referred to as operculum extension), tail beating, lateral swimming, and unengaged. These social interactions, adapted from Dzieweczynski and Perazio (2012); Balzarini et al. (2014); Alton et al. (2013); Vu et al. (2020), were recorded based on proportion of time and number of entries.

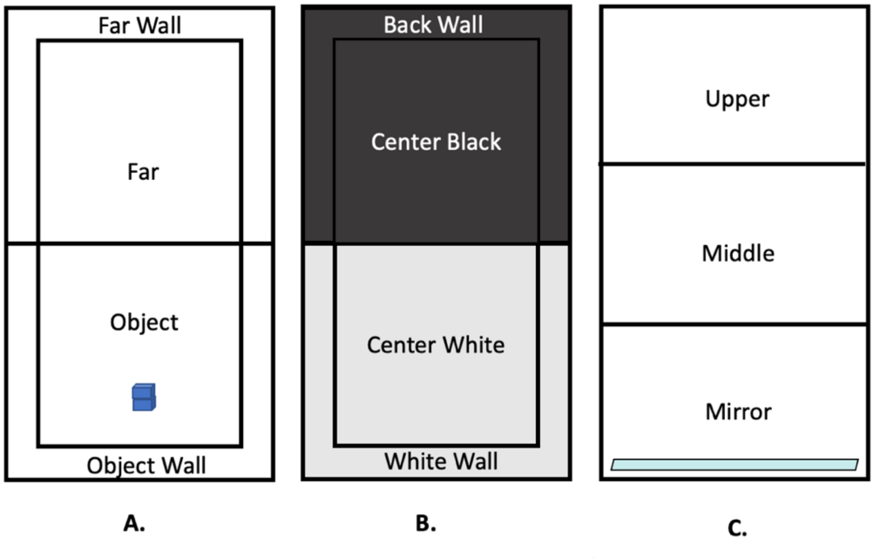

Figure 3. *Video Scoring Zones for Behavioral Tests*. **(A) NOI**: the zones were divided into object vs far (depending on the location of the novel object) and center vs wall. **(B) Scototaxis**: zones were based of the color and whether it was the center or wall. Detour was conducted in this same tank, so it used the same scoring in addition to visual assessment of when an individual crossed the barrier **(C) Mirror:** the zone nearest to the mirror corresponded to the mirror zone, and followed by the middle, and lastly the upper zone was the farthest away.

Ethogram 1. Mirror Behavioral Testing

	Behavior	**Description**
Aggressive	Ramming	Fish making rapid, targeted contact with the mirror using body or mouth.
	Surface breathing	Fish swimming up to the surface of the water to inhale oxygen.
	Gill-covered display	Fish flaring their gills.
	Tail beating	Fish bending body continually to making the motion of an "S" with their tails.
	Lateral swimming	Fish swimming with body parallel to mirror at a proximity.
Nonsocial	Unengaged	Fish that are not engaging in any of the above behaviors.

For the last behavioral test, the **detour task**, I tested the *Betta splendens* ability to employ cognitive flexibility, meaning the ability to recognize that there is a barrier, so they need to change their behavior to achieve a better solution (Brandão et al., 2019). Following a 3-minute habituation period, the fish were released inside the same experimental tank as the scototaxis test (half covered in white and the other in black) but now with the addition of a mirror as a social stimulus. Instead of being able to freely swim up to the mirror, though, and interact with it, there was a transparent plastic barrier made of lamination sheets restricting the path in a semicircle around the barrier. The subject fish needed to identify this obstacle and navigate their way to either of the two holes cut out of the bottom of the barrier which were 10.16 cm away from each other, these would allow them to enter and interact with mirror. I recorded the time it took for each individual fish to successfully reach the stimulus mirror with a max recording time of 10 minutes.

Social Exposure Testing

For the second experiment conducted, social exposure (SE) was examined in addition to behavioral testing. In this experiment, two focal fish in their individually housed 1L tanks were placed side by side. For 3-minutes, an opaque divider was utilized to prevent them from seeing each other. After three minutes, the opaque divider was removed, allowing visual (but not olfactory or tactile) contact. Their interactions were video recorded for 10 minutes (Day 1-4 SE) and 15 minutes (Day 5 SE). Behaviors assessed were ramming, surface breathing, gill-covered display, tail beating, lateral swimming, and unengaged. These social interactions, adapted from Dzieweczynski and Perazio (2012); Balzarini et al. (2014); Alton et al. (2013); Vu et al. (2020), were recorded based on proportion of time and number of entries. The subjects in this task were divided into two separate treatment groups to examine the effects of the “Dear enemy effect” and if the response correlated to the behavioral tasks described previously. From the cohort of 24, 12 fish received a familiar opponent for all five days of behavioral testing while the other 12 fish received the same familiar opponent until day five, when they were introduced to a novel *betta* fish they have never interacted with before. On day 5 SE (day 11 of the entire experiment), the fish had a 15-minute social exposure followed by a 45-minute resting period and then tissue was collected and processed in the methods described below. This resting period was chosen because it provided appropriate time for the P6S neural activity marker to reach its highest activation level to best measure protein expression through immunohistochemistry on sectioned and stained brain tissue.

Ethogram 2. Social Exposure Testing

	Behavior	Description
Aggressive	Ramming	Fish making rapid, targeted contact with the wall of the tank that faces the opponent, using body or mouth.
	Surface breathing	Fish swimming up to the surface of the water to inhale oxygen.
	Gill-covered display	Fish flaring their gills on the half of the tank that faces the opponent.
	Tail beating	Fish bending body continually to making the motion of an "S" with their tails on the half of the tank that faces the opponent.
	Lateral swimming	Fish swimming with body parallel to the wall of the tank that faces opponent at a proximity.
Nonsocial	Unengaged	Fish are on the other half of the tank that does not face the opponent or are not engaging in any of the above behaviors.

<u>Immunohistochemistry</u>

Following the last social exposure test on day 11, brains were harvested from all subjects. After 45 minutes of resting, subjects were euthanized via rapid decapitation following anesthetization in an ice bath, and whole head tissue was stored in 4% paraformaldehyde for four hours. Tissue was then rinsed in 30% sucrose in Phosphate Buffer Saline (PBS) for 30 seconds and the brain was extracted from the skull and stored in 30% sucrose overnight. Tissue was then preserved in OCT until ready for sectioning. Brain tissue was sectioned on a cryostat at 20 uM at -23C and plated on microscope slides. Once ready to begin immunohistochemistry, on day 1, the slides (with the tissue) were rinsed for 5 minutes in 1000uL of 1X Tris Buffer Saline (TBS) 5 times on a shaker set to low speed. Subsequently, they were rinsed for 5 minutes in 1000uL of 4% paraformaldehyde once on a shaker. The final wash was rinsing the tissue for 5 minutes in 1000uL 1X TBS twice on the shaker. Following this, the block (1 X TBS base, Triton 0.3% of base, and Normal Donkey Serum (NDS) 10% of base) was added to the slides and slides were transferred into a humid chamber at room temperature and stored for 1 hour. A hot plate was used throughout as necessary to assist with tissue adhesion to slide. Afterwards, primary antibodies (PS6 0.02% of base and diluent base) were added to the slides and stored in a humid chamber (an IBI Scientific immunohistochemical staining tray with water in the reservoir under the slides) in the fridge for 24 hours.

Day 2 of immunohistochemical staining began with 2, 30-minute rinses in 1X TBS on shaker. Next, secondary antibodies (Donkey anti-Rabbit 594 0.03% of base and diluent base) were added to the slides and slides were transferred into a humid chamber at room temperature and stored for 2 hours. Lastly tissue was rinsed in 1X TBS for 20 minutes once on shaker and DAPI was added before the cover slip. Following a few hours to dry, slides were sealed with clear nail polish until imaging. Slides were then images using a Zeiss Axio Image Microscope set to 10x magnification. I assessed neural activity by counting the number of PS6 expressing cells in the Dld region of the *betta splendens* brain, with quantification methods described below.

Scoring and Data Analysis

All behavioral and social exposure videos were scored using an event logging software called Cowlog. In Cowlog, an association was created between behavior and location cues and keyboard letter presses. When a letter was clicked that corresponds to a certain area of the experimental tank or to a specific behavior, Cowlog recorded the appropriate event and time. In addition to Cowlog, the application Picture in Picture (PiP) was used to create a grid with specific dimensions that could be overlayed onto every video thus removing confounding variable related to differences in zone size (represented in **Figure 3**). Using the R package "Cowlogdata" (Wallace 2020), the logs were converted into summaries that included time durations spent in certain zones of the tank, time of initiation of each behavioral event, and number of events recorded for each behavior type. Furthermore, this analysis allowed data to be gathered on the path the animal takes to get to a certain zone or the behaviors the proceeded it. To collect the neural activity data, the application Image J was used in addition to Cell Profiler. Image J allowed me to choose a region of interest with the same dimensions for each of the tissue images. Cell Profiler was used to count number of cells through the same objective algorithm. All data analysis was conducted in R studio (version 2021.09.0). For behavioral testing I conducted two principal components analyses and linear models. For the social exposure comparisons, Wilcoxon and t-tests were utilized. I choose to look at proportion of time spent expressing gill covered display, lateral swimming, and unengaged and to look at number of tail beats, ramming, and surface breathing based on the characteristics of the behaviors.

Results

Behavioral Testing Overview

The percentage of time all 24-*betta* fish spent in each zone was calculated for the three behavioral tests: mirror, scototaxis, and NOI as seen in **Figure 4**. It was identified that the *betta* fish preferred to spend most of their time in the mirror zone (64.8%) for mirror task, black wall zone (49.0%) for scototaxis, and far wall zone for (40.3%) for the NOI. From this data it can also be seen that on average the *betta* fish spent much of their time in the wall zones for both the NOI (77.9%) and the scototaxis (76.5%) tests. Notably for the NOI, there was less than a 3% difference between the average time spent in the object wall zone (37.6%) vs time spent in far wall zone (40.3%) as well as time spent in object zone (12.5%) vs far zone (9.6%).

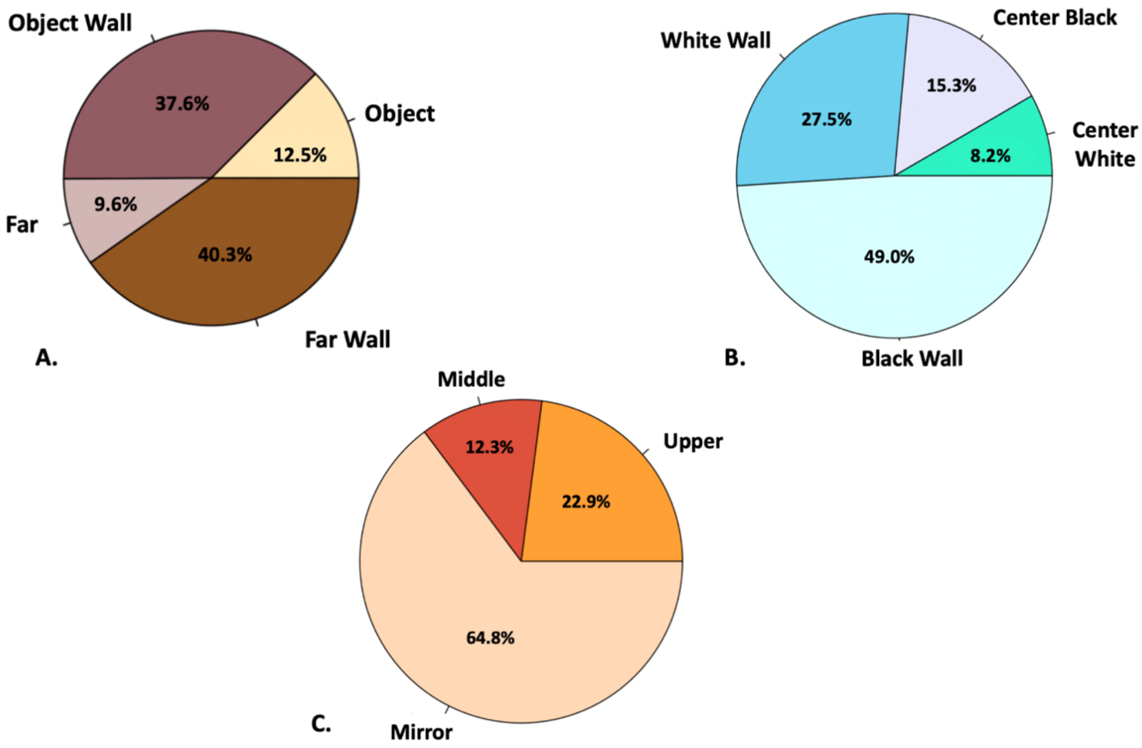

Figure 4. *Percentage of time spent in zones for mirror, scototaxis, and NOI behavioral tests (n=24)* **(A)** Percentage of time *betta* fish spent in the object wall zone (dark purple), object zone (beige), far wall zone (brown), and far zone (light purple) during the novel object interaction test (NOI). **(B)** Percentage of time *betta* fish spent in the white wall zone (dark blue), center black zone (purple), center white zone (green), and black wall (light blue) during the scototaxis test **(C)** Percentage of time *betta* fish spent in the mirror zone (beige), middle zone (orange), and upper zone (red) during the mirror test.

Behavioral Testing and SE Day 1 Correlations

To answer the first question of how individual fish behavior, stress, and cognition predicts real-life social competition, behavioral data from the mirror, scototaxis, and NOI tests were correlated with *betta* behaviors from the social exposure (SE) task. Time spent in the object zone of the NOI was individually compared to all six SE behaviors from the first day of social exposure (i.e., when all animals received a novel opponent for the first time) but no significant correlation was found, suggesting interaction with a novel object does not predict social real-life behaviors (**Table 1**). Time spent in the center white zone of scototaxis task, used to assess stress and boldness in the *betta* fish, was also compared to the six SE behaviors from Day 1 using a linear regression analysis but no significant correlation was found between time spent in center white zone of the scototaxis and the six SE behaviors **(Table 1)**.

The mirror task was conducted to examine the individual social behavior of the *betta* fish. To explore how mirror task compared to SE behaviors, I compared the time spent in the mirror zone to the Day 1 social exposure behaviors. A linear model indicated a significant correlation between time spent in mirror zone and the behaviors, gill-covered display (p=0.0084, n=10, **Figure 5A***)* and unengaged (p=0.016, n=10, **Figure 5B**) (**Table 1**). These results show that there is a positive correlation between the time the *betta* fish spent gill flaring and the time spent in the mirror zone. In contrast, there is a negative correlation between the time spent not engaging with the other *betta* fish and time spent in the mirror zone. The detour test used to determine *betta* fish cognitive flexibility was not conclusive as only 16.7% fish successfully completed the task as it was designed (*n=24*).

Table 1	**Behavioral Zones**		
Social Exposure Behaviors (Day 1)	Object Zone (NOI Task)	Center White Zone (Scototaxis Task)	Mirror Zone (Mirror Task)
Ramming	*p=0.16*	*p=0.76*	*p=0.65*
Surface breathing	*p=0.13*	*p=0.63*	*p=0.99*
Gill-covered display	*p=0.54*	*p=0.56*	***p=0.0084***
Tail beating	*p=0.27*	*p=0.068*	*p=0.40*
Lateral swimming	*p=0.72*	*p=0.84*	*p=0.53*
Unengaged	*p=0.71*	*p=0.71*	***p=0.016***

Table 1. *Social exposure day 1 and NOI, scototaxis, and mirror tasks correlations* (*n=10*). P-values are expressed for each correlation run between the three behavioral zones (object, center white, and mirror) and the six SE behaviors (ramming, surface breathing, gill-covered display, tail beating, lateral swimming, and unengaged).

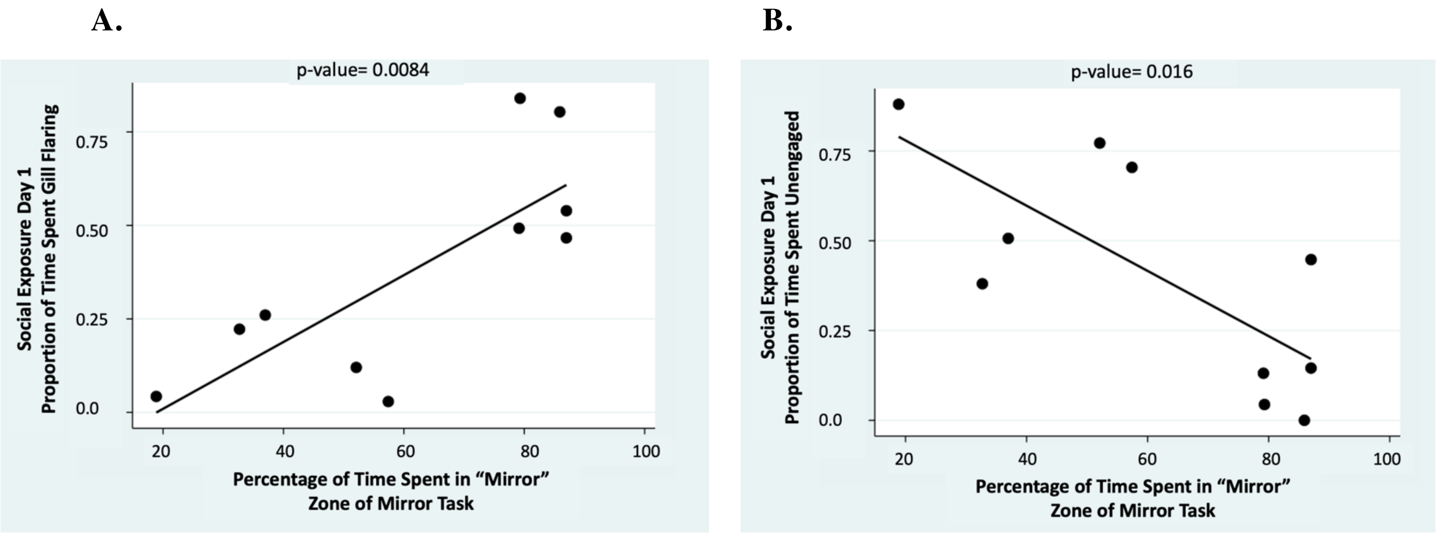

Figure 5. *Significant correlations between social exposure day 1 and mirror task (n=10).* **(A)** Proportion of time *betta* fish spent with gill-covered display is positively correlated with the percentage of time spent in the mirror zone of the mirror task (*p=0.0084)* **(B)** Proportion of time *betta* fish spent unengaged is negatively correlated with the percentage of time spent in the mirror zone of the mirror task (*p=0.016)*.

SE Day 1 Multidimensional Relationships

Beyond looking at individual behaviors in the social competition, I used a multidimensional analysis to explore general patterns of behavior in the day 1 SE task. To examine the relationship between the six SE behaviors for day 1 SE testing a principal components analysis (PCA) was run. Only the first two components were looked as they account for the largest proportion of variance (42.3% and 29%). As seen by **Table 2**, for PC1, the analysis loadings found an inverse relationship between engaged and unengaged behaviors. This shows that fish who were unengaged were participating less in any of the other behaviors. Additionally, the PC2 analysis loadings found an inverse relationship between the more “display-oriented” strategy consisting of gill covered display and tail beating and the more “preparedness for direct contact” strategy of lateral swimming, unengaged, surface breathing, and ramming. SE Day 1 relationship loadings can also be seen in **Figure 6**.

Table 2	**PCA Day 1 Component Loadings**	
Social Exposure Behaviors Day 1	PC1 “Engagement”	PC2 “Strategy”
Ramming	*-0.0791*	***0.449***
Surface breathing	*-0.298*	***0.570***
Gill-covered display	***-0.516***	*-0.274*
Tail beating	*-0.312*	***-0.499***
Lateral swimming	***-0.406***	*0.374*
Unengaged	***0.614***	*0.0972*

Table 2. *Social exposure day 1 PCA loadings* (*n=10*). The day 1 PCA loadings (eigenvalues) reveal the relationships that exist between the unengaged vs engaged behaviors in PC1 and “display-oriented” strategy vs “preparedness for direct contact” strategy in PC2. Red values represent strong relationships (> 0.4, < -0.4).

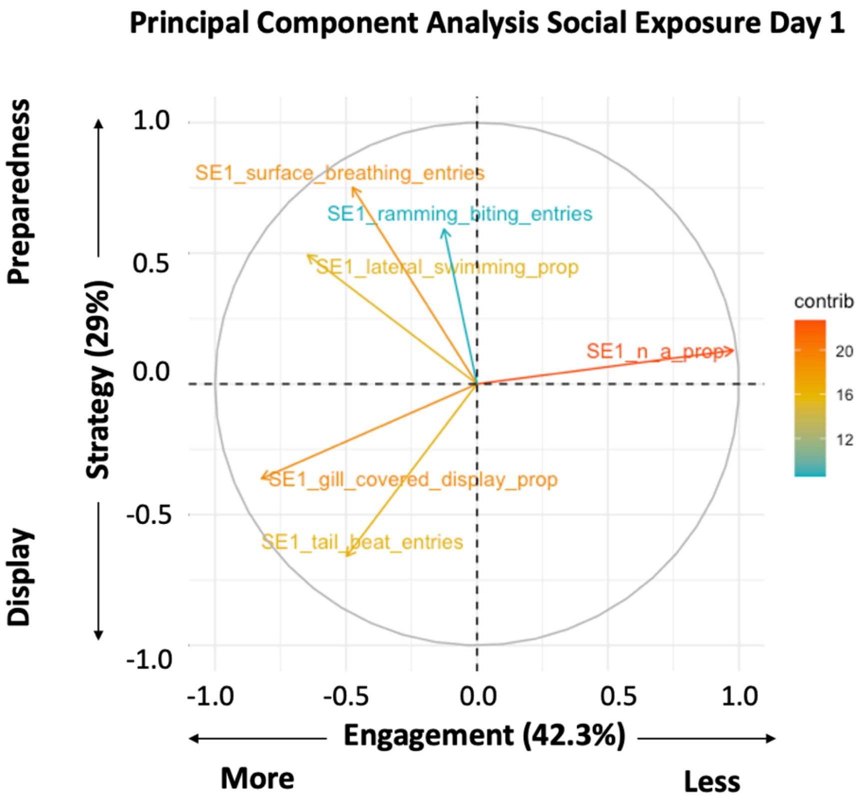

Figure 6. *Social exposure day 1 PCA* (*n=10*). PC1 can be seen along the x-axis representing the first component loading (42.3% percent of variance explained) which highlights the relationship that exists between "engaged" vs "nonengaged" behaviors. PC2 can be seen along the y-axis which represented the second component loading (29% percent of variance explained) and stresses the relationship between "display-oriented strategy" and "preparedness for direct contact strategy".

<u>Behavioral Testing and SE Day 1 PCA Correlations</u>

In addition to individually comparing the six SE Day 1 behaviors to behaviors in the mirror, scototaxis, and NOI tasks, the PCA values were also compared. A linear model was conducted for SE Day 1 PC1 and PC2 compared to object zone in the NOI, but no statistical correlation was identified (**Table 3)**. Furthermore, when PC1 and PC2 were compared to center white zone of the scototaxis task, no significance was established (**Table 3**). The PCA for SE Day 1 was also analyzed in correlation with time spent in mirror zone and a significant correlation was identified with PC1 (*p=0.038,* **Figure 7 and Table 3***)* demonstrating a correlation between fish who were engaged in the fight and time spent in mirror zone, as seen previously in the individual comparisons between the mirror task and the social exposure. In the preliminary analysis of exploring the different zones of the behavioral tasks, it was found that a positive correlation existed between the percent time spent in object zone of the NOI and the standard length of the fish (*p = 0.040*, **Figure 8).** Thus, I compared standard length to the PC1 & PC2 scores of SE day 1 and a significant correlation was found with PC2 (**Figure 9**). Subjects who exhibited more a preparedness for direct contact strategy were bigger in size.

Table 3	**Behavioral Zones**		
PCA Components	Object Zone (NOI Task)	Center White Zone (Scototaxis Task)	Mirror Zone (Mirror Task)
PC1	*p=0.90*	*p=0.89*	***p=0.038***
PC2	*p=0.053*	*p=0.52*	*p=0.56*

Table 3. *PCA components and NOI, scototaxis, and mirror tasks correlations* (*n=10*). P-values are expressed for each correlation run between the three behavioral zones (object, center white, and mirror) and the two PCA components (PC1 and PC2).

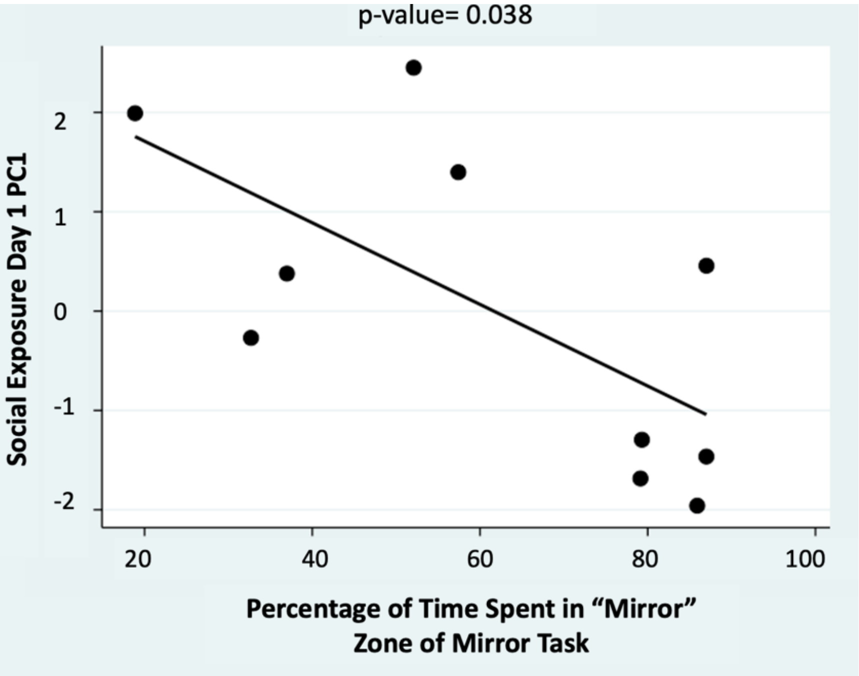

Figure 7. *Significant correlation between PC1 from social exposure day 1 and mirror task (n=10)*. Behaviors from PC1 day 1 are positively correlated with the percentage of time spent in the mirror zone of the mirror task (*p=0.038)*. The subjects who behaved more "engaged", also spent more time in the mirror zone.

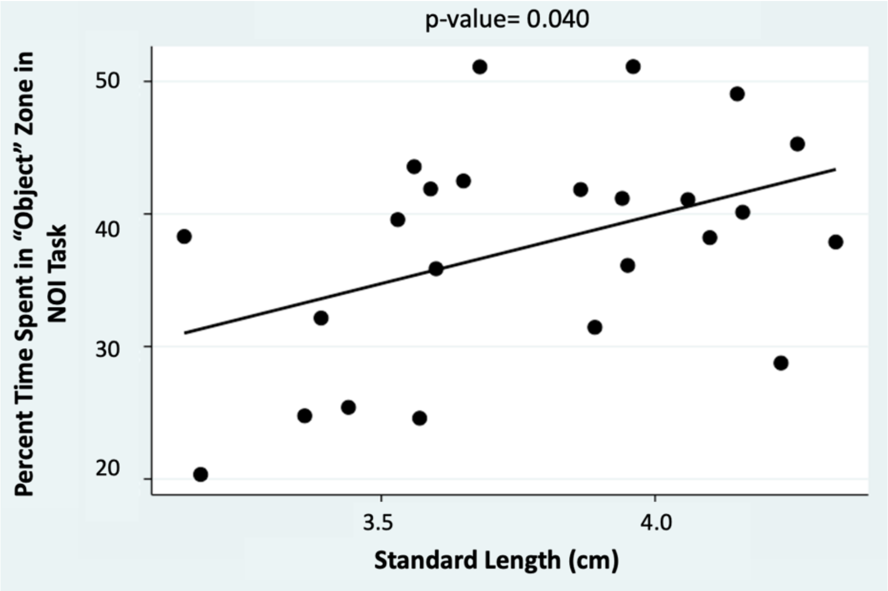

Figure 8. *Significant correlation between percentage of time spent in object zone and standard length (n=10)*. Proportion of time *betta* fish spent in the mirror zone of the mirror task was positively corelated to standard length of fish (*p=0.040)*.

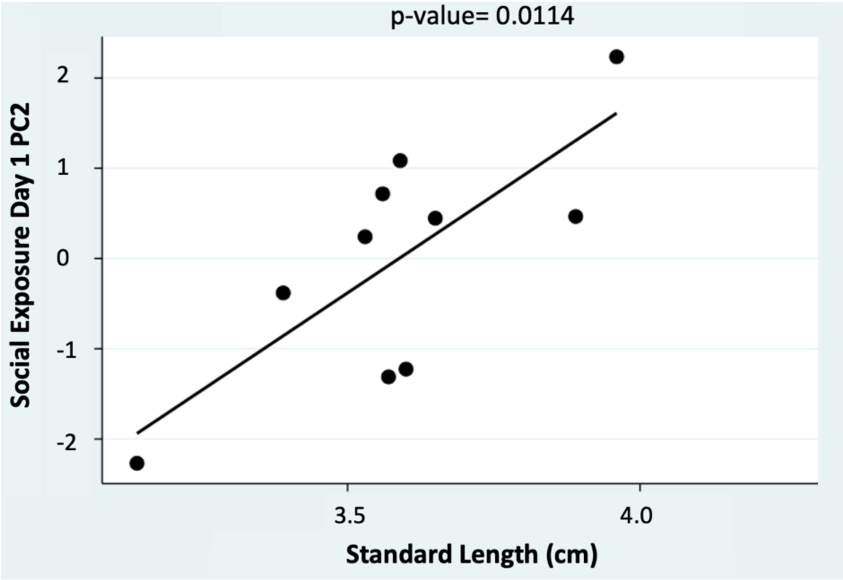

Figure 9. *Significant correlation between PC2 from social exposure day 1 and standard length (n=10).* Behaviors from PC2 day 1 are positively correlated with the fish standard length (*p=0.0114*). The subjects who behaved with more "preparedness for direct contact" strategy also were bigger sized.

SE Day 1 and SE Day 5 Differences

To assess whether individuals that received the novel vs familiar opponent differed in behavior during on the fifth and final day of social exposure, I first compared treatment groups in their scores on the PCA from day 1 (described previously), when there should be no differences because all animals were meeting their opponent for the first time. I then compared treatments in each of the six behaviors recorded on Day 5 and finally conducted a PCA from day 5 (described below) and compared scores between treatments, when the differences between a familiar and novel opponent should appear. As seen by **Table 4 and Figure 10**, when a principal component analysis was conducted on the behaviors observed in day 5, the ramming behavior loaded with the unengaged behavior, which was different from the previous established relationship loadings from the day 1 PCA. Additionally, the ramming behavior also loaded positive in relation to the two display behaviors which suggest that PC2 identified a set of behaviors that are more selfdirected (surface breathing, lateral swimming, and unengaged) and a set of behaviors that are more opponent directed (tail beating, gill-covered display, and ramming).

Table 4	**PCA Day 5 Component Loadings**	
Social Exposure Behaviors Day 5	PC1	PC2
Ramming	*-0.278*	***0.429***
Surface breathing	***0.499***	*-0.153*
Gill-covered display	*0.346*	***0.418***
Tail beating	*0.166*	***0.636***
Lateral swimming	***0.454***	***-0.402***
Unengaged	***-0.566***	*-0.227*

Table 4. *Social exposure day 5 PCA loadings* (*n=10*). The PC1 and PC2 loadings (eigenvalues) for social exposure day 5. Red values represent strong relationships (> 0.4, < -0.4).

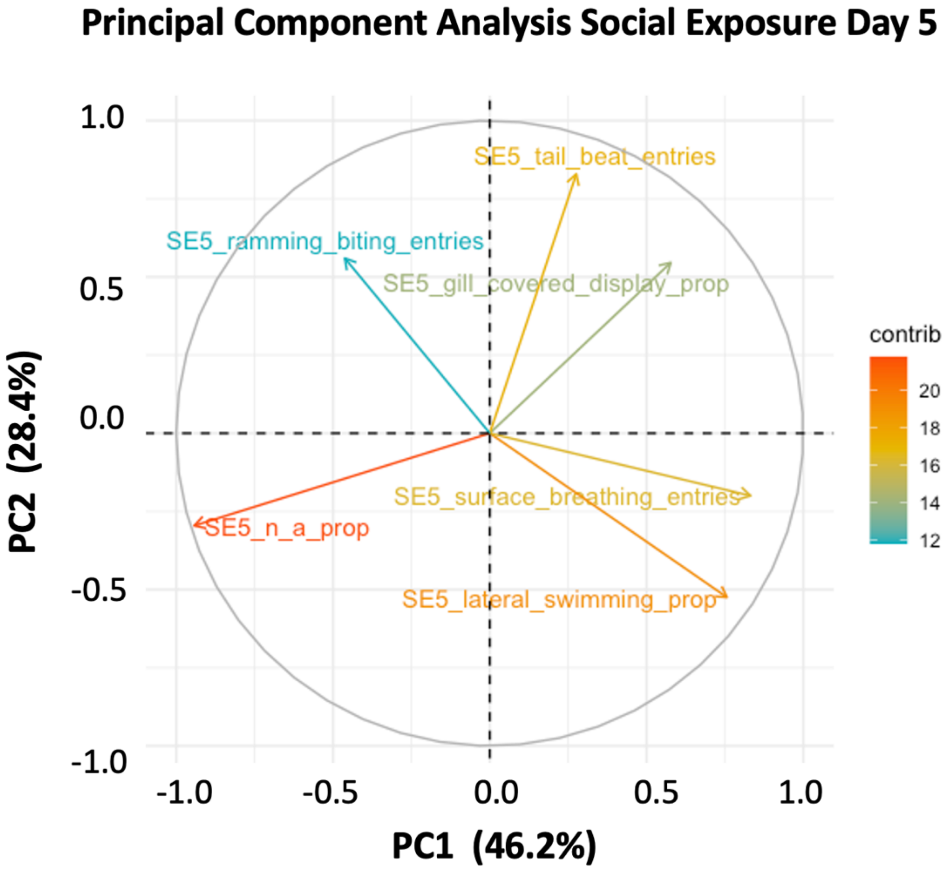

Figure 10. *Social exposure day 5 PCA* (*n=10*). PC1 can be seen along the x-axis representing the first component loading (46.2% percent of variance explained). PC2 can be seen along the y-axis which represented the second component loading (28.4% percent of variance explained).

To then answer if *betta* behavior differed in competition with a novel vs familiar opponent, the behaviors for both day 1 (same treatment) and day 5 (different treatment) social exposures were compared between animals that were in the "familiar" treatment vs the "novel" treatment. Data from Wilcox and T-test indicate that there were no significant differences in any of the six SE behaviors for either day. SE day 1 PC1 and PC2 were also compared between familiar and novel with the expectation of no difference because they all received the same treatment; and indeed, no significant differences were found. Then PC1 and PC2 for Day 5 SE were tested to see if there was any significant difference between the novel vs familiar treatment groups but no significance was found **(Figure 11).**

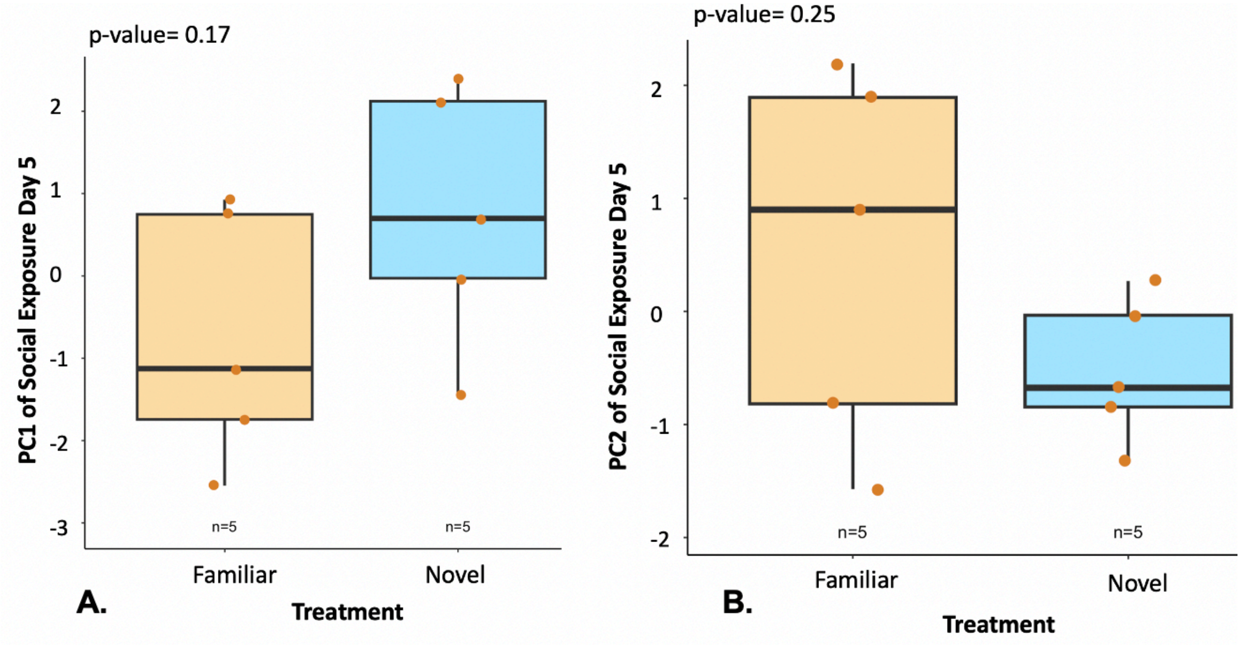

Figure 11. *No significant difference was found for PCA day 5 between treatment groups (n=10).* **(A)** The PC1 social exposure day 5 values were compared to each treatment group, novel vs familiar, but no significant differences were found. **(B)** The PC2 social exposure day 5 values were compared to each treatment group, novel vs familiar, but no significant differences were found.

SE Day 5 and Neural Activity Differences

Neural activity was measured for 10 subjects, 5 from the familiar treatment, and 5 from the novel treatment. Specifically, the Dld was imaged the and number of cells that expressed PS6 were counted for each animal (**Figure 12**). On average 15.6 cells were counted. In comparison we found no significant differences in neural counts between fish in the novel vs the familiar treatment (**Figure 13**).

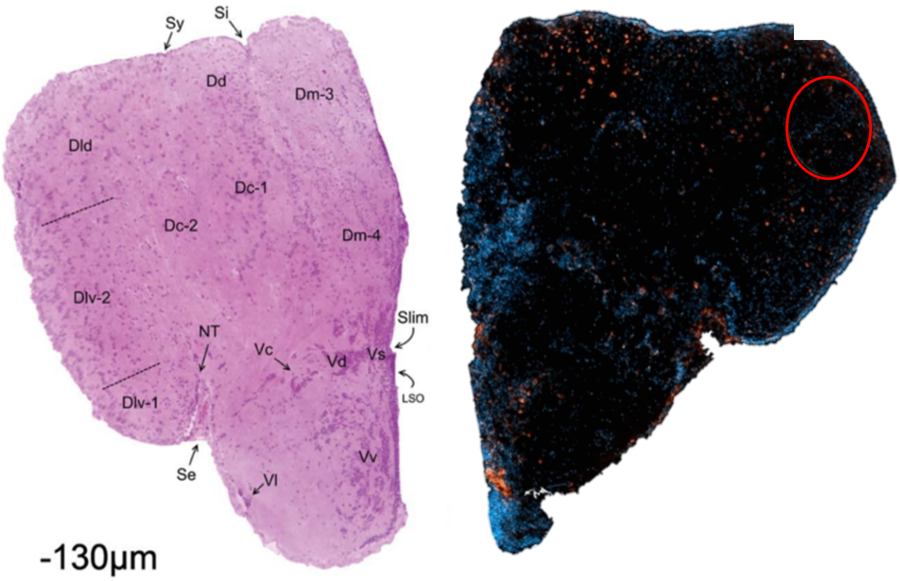

Figure 12. *Betta splendens telencephalon transverse brain slice*. **(Left)** A brain structure labelled image of a *betta* telencephalon (transverse plane) was stained with hematoxylin and eosin. Dld label indicates region of interest. Sourced from Magalhães Horn and Rasia-Filho (2017). **(Right)** The telencephalon (transverse plane) of *betta* subject, Gabriel, stained with PS6 and DAPI. Red circle indicated region of interest.

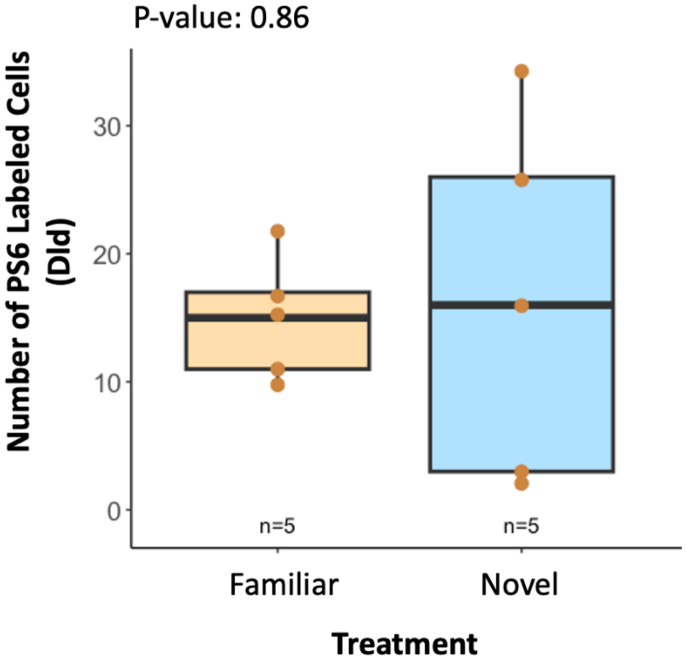

Figure 13. *No significant difference was found for number of PS6 labeled cells between treatment groups (n=10)*. The number of PS6 labeled cells did not significantly differ between the two treatment groups.

Discussion

This study explored how performance in a social competition correlate to *Betta splendens* performance across cognitive and behavioral tasks. To investigate this correlation, the question was divided into two experiments, the first consisting of four behavioral tests and the second of five repeated social exposures. The behavioral tests, NOI, scototaxis, mirror, and detour were conducted to explore how individual subject behavior, stress, and cognition predicted real-life social competition, which was observed in a social contest with another individual. It was found that individual performance in the scototaxis and NOI were not predictive of real-life social competition while the mirror test was. The five days of social exposure testing were organized to answer the question of how *betta* behavior differs in competition with a novel vs a familiar opponent. I found no differences in behavior nor neural activity between the two groups suggesting *betta* behavior does not change depending on familiarity with an opponent. Notably, it was found that a positive correlation existed between the percent time spent in the object zone of the NOI and the standard length of the fish indicating larger fish are more likely to interact with a novel object. Additionally, standard length was also correlated to PC2 from the social exposure, which suggests that subjects who exhibited more a "preparedness for direct contact" strategy, tended to also be bigger in size.

Behavioral Testing

Previous studies have demonstrated the effectiveness of the open field test in assaying boldness levels in fish (Forsatkar et al., 2016; Hebert et al., 2014). In our experiment, I specifically tested to see how *betta* fish interacted with a novel object and if this could predict their aggressiveness later in real-life competition. The results show that, on average, the fish spent 8.2% of time in the object zone (the most unfamiliar zone), which is congruent with findings in previous literature of rainbow trout spending less than 5% of time exploring the novel object, while spending around 25% exploring the familiar object (Sneddon et al., 2003). I predicted that amount of time spent in this zone would be correlated to decreased stress and expected to see correlations between individual *betta* behavior in social exposures but discovered none. This data challenges findings from Hebert et al. 2014, which found positive correlations between time that *betta* fish spent gill flaring and activity level in a boldness assay, although this discrepancy could be due to the differences in measured boldness behaviors. While I measured time spent in the most unfamiliar zone, they measured "time spent actively moving during the trial". As a future direction, I could explore the correlation between time spent moving through NOI zones and individual behaviors during social exposure because increase time entering the center white requires more risk.

Similarly, scototaxis tests have been shown to assess anxiety levels in many fish species, including zebrafish, goldfish, and even *bettas* (Maximino et al., 2010). The construct validity of the scototaxis test is dependent on the innate preference the fish have for the sheltered areas, which is opposite to its simultaneous motivation to explore the unknown exposed environment (Kavaliers & Choleris, 2001). I found that, when color zones are compared, the *betta* fish spent most of their time in the black zone of the scototaxis task on average. This is consistent with previous literature on betta fish, which has found that control *bettas* spent more than 50% of their time in the black area (Tudor et al., 2019). I also hypothesized an association between time spent in the center white zone (the most exploratory zone) to individual aggressive behaviors expressed by the fish during the first social exposure day, but no significant correlations were found. This suggests that anxiety levels do not correlate to *betta* behavior in real-life social competition, which could be due to the powerful role aggression plays in mating for *bettas* and their innate tendency to engage in combative behavior, which trumps their preference for safety (Dzieweczynski et al., 2013).

It is known that *betta* fish are not able to recognize themselves in a mirror, instead perceiving its reflection as an opponent (Oliveira et al., 2022; Ramos et al., 2021; Desjardins & Fernald, 2010). This allows a mirror to be a great tool for assessing aggressive behaviors (Balzarini et al., 2014). I found that the *betta* fish spent most of their time in the mirror zone interacting with the perceived "opponent". I expected to see a positive correlation between time spent in the mirror zone and aggressive behaviors such as gill flaring and tail beating. In support of previous literature, I found significant positive correlation between time spent engaging in gill flaring and time spent interacting with mirror, suggesting that the mirror test can predict real-life *betta* competition (Arnott et al., 2016; Ramos & Gonçalves, 2019). Additionally, I also found a negative correlation between time spent unengaged and time spent in mirror zone further supporting the predictability of the mirror task. An aggressive behavior in *betta* fish is reflective of the effort they are putting forth into guarding their territory to ensure reproductive success (Simpson, 1968; (Jaroensutasinee & Jaroensutasinee, 2003). These innate behaviors can explain the findings, as *betta* fish would treat any perceived opponent in the same aggressive way to better their chances as survival and reproduction. In addition to these findings, Arnott et al. (2016) revealed a significant difference between number of surface breathing in mirror vs real-life competitor, a path that could be further paved in future experiments.

Social Exposure Testing

To better understand the role social competition plays in cognitive & behavioral tasks, I then explored the question of how *betta* behavior differs in competition with a novel vs a familiar opponent through dividing subjects into two groups and conducting five days of social exposures. After comparing the behaviors from day 1, to the behaviors from day 5, I found no significant differences between the two groups suggesting *betta splendens* behavior does not change depending on familiarity with opponent. These results are consistent with findings from Alyan (2010) which also found *betta's* to be exempt from the "dear enemy phenomenon". Because the previous literature was inconclusive as to if the lack of distinction between opponents was due to difficulty in identifying one as novel and the other as familiar, I decided to further explore if neural activity differences could be seen between the two groups in a brain region related to memory. Our research specifically looked at the dorsal division of the lateral zone of the dorsal telencephalon (Dld) – the fish homolog for human hippocampus – and found an average of 15.6 cells with expressed PS6. No significant difference in PS6 labelled cells was found between the novel vs familiar group, suggesting that the explanation for a lack of difference in behavior could be a result of no memory recall of the opponent. For future directions studying the fish homolog of the amygdala, a region involved in emotional learning and aggression across many species would allow me to further distinguish if the lack of difference between novel and familiar opponents stems from recognition or subsequent behavioral strategy.

Standard Length

In the process of exploring the different zones of the behavioral tasks, it was found that a positive correlation existed between the percent time spent in object zone of the NOI and the standard length of the fish. The bigger fish was, the more likely it was to spend time exploring the novel object, which could be a result of higher locomotor activity in older fish since standard length and age are associated (Yue et al., 2022; Lucon-Xiccato et al., 2020). This finding led to the question of does standard length predict social exposure behaviors, and while no significance was found between the SE Day 1 behaviors and standard length, PC2 from the Day 1 PCA was found to be positively correlated to fish size. Thus, the larger the fish the more likely it was to engage in surface breathing, ramming, and lateral swimming. This could be because of a larger fish size, they are less fearful of their smaller opponent, engaging more in "preparedness" strategy instead of display. There is much more to explore regarding the impact of size and its correlates (age, condition, prior experience) to fight outcomes. Furthermore, the principal component analysis identified two "strategies" that have previously not been described in *betta* fighting and are interesting areas of future work.

Conclusion

This comprehensive experiment found consistent individual variation in behavior across contexts in *Betta splendens*, but familiarity to an opponent did not have an impact on behavior or on the brain. While it yielded unexpected results (both significant and nonsignificant), this work highlights how examining the relationship between behavioral testing, neural activity, and performance in social competitions offers unique insight into the effects of social cognition not only in this species but in many other vertebrates including humans.

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