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Article

Determining the impact of caffeine on aggression in *Betta splendens*

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SUMMARY

Caffeine is a naturally occurring chemical stimulant found in various products that is becoming more widespread. Around 90% of the world's population consumes caffeine daily, which is absorbed and released into the body in the form of fluids. Consequently, caffeine has appeared in aquatic environments around the world as wastewater contains human fluids that contain caffeine. However, its effects and safety for the health of humans and wildlife remain inconclusive. Thus, in our study, we investigated the impact of varying caffeine concentrations on the aggressive behavior of the Siamese fighting fish *Betta splendens* territory holders after seeing an intruder. We compared aggression among four experimental groups, including the control group without any caffeine added, low dose caffeine (120 µg/L), medium dose caffeine (200 µg/L), and high dose caffeine (280 µg/L). We predicted that if there was an increase in the caffeine concentration, then the *B. splendens* would exhibit more aggressive behaviors because of the natural tendency of caffeine to stimulate movement. We found that caffeine exposure did not significantly alter male territory-holding behavior in *B. splendens.* A better understanding of the relationship between the biological systems of *B. splendens* responsible for aggressive behavior and caffeine is needed to clarify the urgency of the issue of increasing caffeine concentrations found in bodies of water worldwide.

INTRODUCTION

Caffeine is a natural stimulant most commonly found in tea, coffee, and cacao plants. Approximately 90 percent of all people in the world consume caffeine daily (1). Caffeine's main effect on the human body is stimulating the brain to boost energy and alertness (1). Adenosine is a neurotransmitter that builds up as energy and is used throughout the day, leading to elevated levels of tiredness (2). Caffeine interrupts the effects of adenosine, making one feel more energized by preventing adenosine molecules from binding to adenosine receptors in the brain, preventing a slowdown in nerve cell activity, which results in prolonged energy levels (2). However, the human body does not metabolize and absorb all the caffeine ingested as the maximum amount of caffeine that is able to be absorbed is around 400 mg/L (3). Consequently, the caffeine is excreted from the body through bodily fluids and is subsequently flushed into the wastewater, which is filtered and recycled back into the environment (4,5).

As a result, caffeine has been found in waterways worldwide, especially in the United States of America and Switzerland. The maximum acceptable average concentrations of caffeine in raw wastewater, treated wastewater, river, drinking water, groundwater, lake, catchment, reservoir, and rainwater samples are 3.60 mg/L, 55.5, 19.3, 3.39, 0.683, 174, 44.6, 4.87, and 0.0054, respectively (6). Previous studies have shown that the caffeine in the waterways had negative impacts on marine life (algae, clams, and mussels), including reduced reproduction and growth rates (6). In particular, eutrophication occurs when the environment becomes enriched with nutrients and leads to increasing levels of plant and algae growth in estuaries and coastal waters (7). Eutrophication may be triggered when caffeine ends up in waterways, causing oxygen to decrease, excessive plant production, blooms of harmful algae, increased frequency of anoxic events, and the dying of marine life (7).

In our experiment, the subjects are *Betta splendens* Siamese fighting fish. *B. splendens* are freshwater fish often found in the marshes, ponds, or slow-moving streams of Southeast Asia and are available in pet stores worldwide. Male *B. splendens* have a tendency to defend their territory from other fish (8,9). They are carnivorous animals that, in nature, primarily consume insects and insect larvae near their surroundings. Male *B. splendens* build bubble nests for their babies with their mouths and attract female *B. splendens* to them. When the female begins to lay eggs, the male retrieves them and deposits them in the nest (9). The males then chase the female away due to their inherent aggressive behaviors and guard the nest against intruders and predators until the eggs hatch. Male *B. splendens* will exhibit very aggressive behavior against other fish, such as anal fin spreading or attacking the opponent fish, as they act as territory-holders once they are used to their habitat due to their natural tendencies (9). If two male *B. splendens* can see each other without an escape route, they will fight and, in some cases, die due to extreme fighting (10). According to previous research, caffeine has been shown to increase energy levels across various organisms, such as *B. splendens* (11). The fish will exhibit more aggressive behaviors by either flaring their fins or increasing their movements; this is why we decided to measure ventral fin expansions, caudal fin expansions, rams against the tank wall, and the number of gill cover expansions as indicators of aggression. Since these are characteristics common to *B. splendens,* we can use these features as indicators for changes in behavior due to the consumption of caffeine (11, 12).

In this study, we explored the effects of different caffeine concentration levels on aggressive behavior in *B. splendens*. We expected that when the amount of caffeine increased, the *B. splendens* fish would become more aggressive due to caffeine's natural tendency to increase movement among

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organisms. However, no difference in the number of ventral fin expansions, caudal fin expansions, rams against the tank wall, and the number of gill cover expansions was observed among fish exposed to no caffeine, low caffeine, medium caffeine, or high caffeine. More research is needed to solidify these findings and find a convincing correlation between caffeine and aggression in *B. splendens*.

RESULTS

We sought to compare aggressive behaviors in *B. splendens* in response to an intruder after exposure to varying caffeine concentrations. We added caffeine concentrations (dissolved from powder into the fish tanks) of 0 µg/L dose (control), 120 µg/L (low dose), 200 µg/L (medium dose), and 280 µg (high dose) into each tank for 24 hours prior to experimentation; when the experiment started, we introduced an intruder fish next to the tank of the caffeinated fish and then measured aggressive behaviors over a period of 5 minutes (**Figure 1**).

In general, all *B. splendens* with doses of caffeine displayed visually aggressive and hyper behavior compared to their non-caffeinated counterparts (**Figure 2**). However, the differences in aggression were not statistically significant. For every behavior (ventral fin expansion, caudal fin expansion, gill flairs, and rams against the tank) we calculated an Analysis of Variance (ANOVA) test for baseline vs. control and baseline vs. low dose intruder, baseline vs. medium dose intruder, and baseline vs. high dose intruder. The p-value for the behaviors mentioned above in the baseline vs. control comparison group, in order, was 0.0812, 0.2893, 0.3921, 0.0225 respectively. For the baseline vs. low dose intruder, it was 0.6649, 0.8904, 0.0666, and 0.2095 respectively. For the baseline vs. medium dose intruder, it was 0.2788, 0.6393, 0.6621, and 0.1577 respectively. For the baseline vs. high dose intruder, it was 0.6495, 0.0407, 0.0642, and 0.3545 respectively.

Most of the ANOVA test values across all the behaviors exceeded the significance threshold of our study (0.05) (**Figure 3**). In our experiment we chose a significance level of 0.05, therefore, if the ANOVA p-value is greater than 0.05, there is no significant difference in the behaviors of the compared trials. For a p-value less than 0.05, we can then say our study showed a significant difference in the compared trials. Only the ram baseline vs. intruder (0.0225) and caudal fin baseline vs. intruder (0.0407) had p-values less than 0.05. These outcomes suggest a lack of substantial

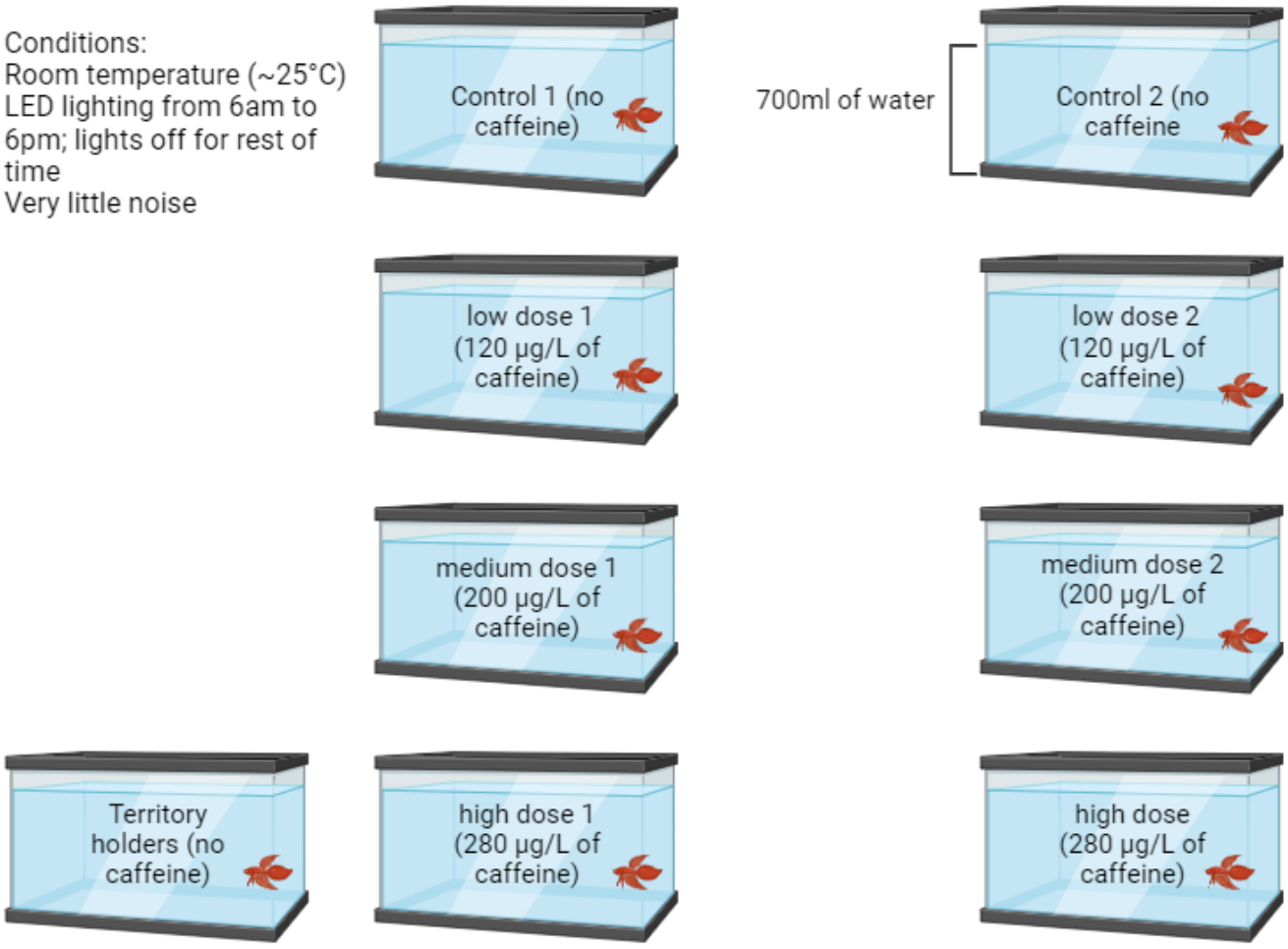

Figure 1: *B. splendens* samples and conditions used in this experiment. Each container (filled with 700 mL tap water) was stored in a room temperature environment (around 25°C) with LED lighting and very little noise. There is one *B. splendens* per container, all of which are territory holders, while the intruders are stored separately in their own individual containers in other laboratories. During experimental trials, the intruders are put into a new container containing the territory holder via a net.

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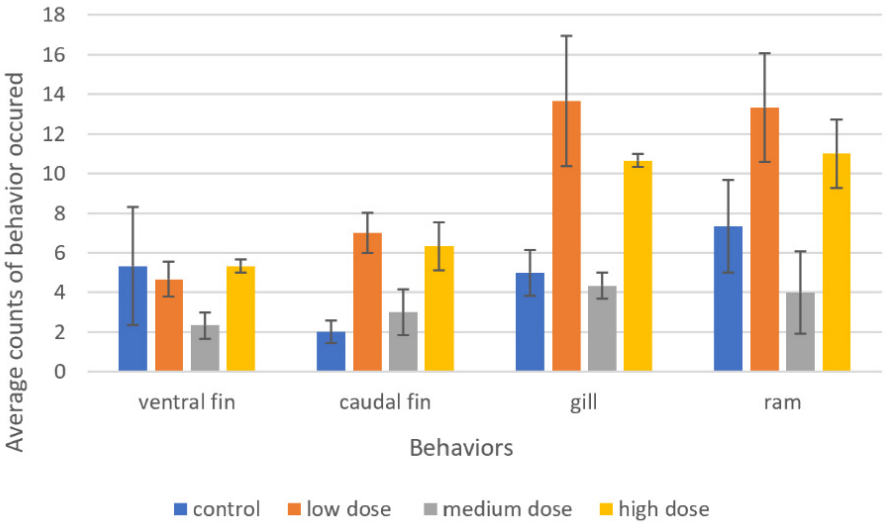

Figure 2: Average counts of aggressive behaviors displayed in all four trials for varying caffeine concentrations. Error bars represent standard error. We measured and noted aggressive behaviors in fish with varying caffeine concentrations. Bars represent the mean results for three trials in each category (n=3) to test the number of times a specific behavior (4 in total) occurred during the experiment when shown an intruder. The legend represents the different caffeine concentrations the fish received in the experiment: the control with 0 µg/L dose, the low dose with 120 µg/L, the medium dose with 200 µg/L, and the high dose with 280 µg. Prior treatment of unimpeded habitat acclimatization lasted 48 hours.

variance between the diverse trial conditions. Specifically, *B. splendens* exposed to varying caffeine doses displayed analogous behavioral patterns regardless of the presence of an intruding fish. When faced with an intruder, the response of the sampled fish did not consistently align with the level of caffeine in their environment. These findings collectively suggest that caffeine consumption among *B. splendens* does not exert a significant influence on their aggression.

DISCUSSION

In our experiment, we aimed to determine whether there was a correlation between caffeine concentrations and aggressive behaviors in *B. splendens* using visual analysis of aggressive behaviors in fish before and after receiving varying caffeine concentrations. Our experiment did not draw a concrete relationship between caffeine and aggression in *B. splendens.* As seen in the results, the higher the caffeine concentration, the *B. splendens* did not display more frequent aggressive behavior (**Figure 2**). There is no clear upward trend as we predicted in the hypothesis. Additionally, only two out of the sixteen ANOVA test values throughout the experiment were below the significance level of 0.05 (**Figure 3**). Thus, the only significant behavior change that was observed in our study is in **Figure 3**. The *B. splendens* with varying doses of caffeine exhibited similar behavior despite seeing another *B. splendens* fish around its territory. When the caffeine-dosed *B. splendens* fish saw an intruder *B. splendens,* levels of aggression were not different among groups exposed to no, low, medium, or high caffeine concentrations. This indicates that across all experimental groups, caffeine consumption by the *B. splendens* does not influence their behavior under stress.

Nevertheless, one potential source of error in this experiment was the inconsistency in rest periods for intruder fish when transitioning between different trials. The *B. splendens* intruder fish did not rest for 24 hours as they were just used in the previous trials. As a result, the state of the *B. splendens* intruders was not specific, and the fish possibly behaved more aggressively after seeing other *B. splendens* fish. In future experiments, the intruder fish should rest for 24 hours after every trial so that they can be in a calm state. In addition, a possible source of error was the sounds of moving in a chair or banging the table with the legs, which could impact the state of the fish. The sampled fish could panic and display abnormal behavior during the trials, which would decrease the reliability of the data. This issue could be solved by being gentler and more careful when sitting on chairs and tables when recording the data. Lastly, the caffeine concentration needed to be more consistent. After the first day of the experiment, some of the caffeinated water evaporated from the tank, leading to changes in caffeine concentrations. In future investigations with more time to conduct trials, experimenters should make new concentrations every trial

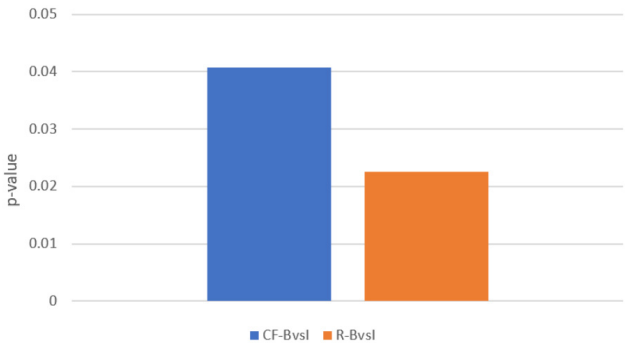

Figure 3: ANOVA test p-values for ram baseline vs. intruder (R-BvsI; blue bar) and caudal fin baseline vs. intruder (CF-BvsI; orange bar). Our significance level for the ANOVA Test is 0.05. R-BvsI and CF-BvsI were the only two out of the sixteen ANOVA Test trials where the p-values were less than 0.05 (p-value < 0.05; significant difference was shown in behaviors).

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to ensure the consistency of the water of each experimental group.

In summary, our results show that currently there is not much evidence to support the theory that caffeine consumption in male territory-holding *B. splendens* will make them exhibit more aggressive behavior when an intruder is visible compared to when it is in a calm state. Nonetheless, other caffeine-related studies have demonstrated mixed findings. For example, a study investigating the effect of caffeine on aggression in mice found that acute caffeine administration increased aggression (13). However, another study found no significant effect of caffeine on aggression in mice (14). These conflicting results suggest that the relationship between caffeine and aggression may be complex and dependent on various factors, including dosage, species, and experimental conditions (such as time of caffeine contact). In addition, numerous studies have been conducted on the effects of caffeine on behavior in other animals and humans. In rodents, caffeine has been shown to influence locomotor activity, anxiety-related behavior, and social interactions (15). In humans, caffeine has been shown to increase aggression, including both perpetration and victimization (16).

To better understand the implications of our findings in the larger context, further research is necessary. Future studies could investigate the anatomical explanations of aggressive behavior in *B. splendens* and explore how caffeine may interact with these factors. Additionally, conducting comparative studies with other animal species, including mice or other fish species, could help elucidate the generalizability of our findings. Potential avenues of research are to better understand the anatomy of *B. splendens* and how it correlates with aggressive behavior when responding to caffeine. A possible implication of the current conclusion is that it will shed light on issues with water quality in waterways and allow the government and organizations to focus their efforts on removing other water contaminants, like caffeine. This way, efforts can focus on eliminating more harmful contaminants along with less harmful ones like caffeine from water, such as nitrogen, metals, and pesticides, to clean our waterways.

MATERIALS AND METHODS

B. splendens housing

Sixteen *B. splendens* were obtained from a local fish shop. Eight were assigned as territory holders and the other eight as intruders. All of the *B. splendens* were isolated in their own individual transparent 1.5 L water tanks filled with 700 mL of water surrounded by opaque covers to prevent them from being agitated due to the sight of other *B. splendens*. The territory holders were split into four separate groups, with two fish in each group. One group was the control group without any caffeine added, the second was the low dose (120 µg/L of caffeine), the third was the medium dose (200 µg/L of caffeine), and the fourth was the high dose (280 µg/L of caffeine). All eight intruders were placed in water tanks without any added caffeine. All fish were fed regularly in intervals of 8 hours with fish food.

Adding Caffeine to Experimental Groups

In this experiment, we used a Sigma-Aldrich caffeine powder with a molecular weight of 194.19 g/mol to make the caffeine concentrations. We made a 2.5 L stock solution for caffeine of 0.02 g/L, then used proportions to find the amount of caffeine concentration per 0.700 L.

For the low dose group, we added 12 mL of caffeine stock solution into the 700 mL water; for the medium dose group, we added 20 mL of caffeine stock solution into the 700 mL water; for the high dose group, we added 28 mL of caffeine stock solution into the 700 mL water.

Conducting the Experiment

Before experimenting, we allowed the territory holder fish to rest (meaning they did not have any contact with other fish) for 24 hours in the water with each respective caffeine concentration. The intruders also rested for 24 hours with regular tap water (without added caffeine). After the resting period of 24 hours, we obtained a territory holder and an intruder fish and placed their fish tanks side by side so they could see each other. We observed the behaviors of the territory holders for a total of 5 minutes with 10 seconds in between recording the aggressive behaviors of the fish with a check mark, each indicating that the territory fish has exhibited a specific aggressive behavior. We focused on observing four established aggressive behaviors in *B. splendens*, which were Operculum (Gill Cover) flair, Ventral (Pelvic) Fin flair, Caudal (Tail) Fin flair, and rams against the fish tank. Each experimental group was repeated for three trials, noting only the behavior of the intruder fish. After repeating this process for all four experimental groups, we used a t-test to see the differences in behavior between the control fishes and the fishes with varying caffeine doses.

Statistical analysis

After gathering the data after each trial, we compared the data of each behavior of the territory holder in each caffeine dose to the control group territory holder using an ANOVA: Single Factor test in Microsoft Excel. We used a significance level of 0.05, meaning that we are confident that 95% of our results are accurate, but there is still a 5% chance of a Type I error (false positive). A calculated p-value in our study below 0.05 therefore means a significant difference in comparisons. A p-value greater than 0.05 in our study means there is no significant difference in comparisons.

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