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## **Influence of *Theobroma cacao* on the Sexual Aggression of Fighting Fish (*Betta splendens*)**

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The study was conducted to determine the influence of exposure to different concentrations of *Theobroma cacao* plant extract on the sexual aggression of *Betta splendens*. Fifteen (15) male Betta fishes were exposed to different concentrations (10%, 20% and 30%) of *T. cacao* and behavioural aggression Betta fishes towards their mirror images were assessed after 12 hours of exposure. Behaviors observed were time it took for each fish to be within 8 cm distance from the mirror, the number of bubbles or surface gulping, number of serpentine movements, duration of shaking movements and the number of times the male *Betta splendens* fishes contact their mirror images.

Results of sexual aggression of male Betta fishes increased in those exposed to 20% concentrations comparable to the positive control (Sildenafil citrate). Courtship behavioural displays such as movement of fishes at 8 cm distance, bubble formation/surface gulping, mirror-contact, and serpentine movements were evidences of sexual receptivity and relative signs of sexual aggressiveness. However, exposure to higher concentrations of *T.cacao* reduced sexual drive.

**Keywords:** *Betta splendens*, fighting fish, *Theobroma cacao*, sexual aggression

### **Introduction**

In fishes, the role of sex steroids especially androgens is very clear (Forsatkar *et al.*, 2014). They are involved in gonadal development, defense of spawning site, mating behaviour performance and in some cases parental care (Dey *et al.*, 2010). These hormonal actions are vital especially for sexually reproducing organisms.

In determining behavioural effects of androgens, the Siamese fighting fish (*Betta splendens*) is a good model organism. Male Bettas have been the focus of behavioral studies of aggression because they are easy to maintain in the laboratory and they exhibit easily identifiable behaviours (Todd *et al.*, 2008).

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Sexual encounters between the female and male *B. splendens* are necessary for the release of gametes that would allow fertilization and subsequent reproduction to occur. Prior to mating though, courtship is an important event in which the males display sexual behaviours to the female.

In *B. splendens*, the females are the one who choose while the males compete with other males for territory and mates (Brownell, 2014). Since the female must ensure that the male can ensure his territory and his bubble nest is suitable for the offspring (Brownell, 2014), she opts to mate with the males displaying behavioural aggression. In cases where there are two or more male, the female chooses the most aggressive one.

Seeing a rival male causes a male Betta to exhibit several types of genetically determined aggressive movements such as spreading his fins, shaking his body, extending his gill opercula and membranes, and generally appearing much larger than his resting size. Since Bettas do not recognize themselves in a mirror, they display to their reflections the same aggression as they might to another male.

Male Bettas construct bubble nests by taking a bubble of air into their mouths, enclosing it in mucus, and depositing it on the surface of the water (Brownell, 2014). The bubble nest is important because it serves as the nest for the fertilized eggs and recently hatched offspring (Braddock & Braddock 1955). Hence, females will mate only in the presence of a nest, which they inspect beforehand.

However, with the pollution and contamination of water systems, the natural habitat of such aquatic organisms is affected. Fluoxetine, for instance, is a selective serotonin reuptake inhibitor (SSRI) drug with 11% of it excreted from body as parent compound (de Vane, 1999) and in natural system is biologically active because of its resistance to hydrolysis and photolysis (Kwon and Armbrust, 2006). Exposure to fluoxetine disrupt reproductive axis of goldfish, *Carassius auratus* (Mennigen *et al.*, 2010). Fluoxetine at a concentration of 54 µg/L reduced significantly serum testosterone levels after 7 days of exposure.

Changes in androgen levels of the male Bettas, caused by the water system contamination and other factors may influence behaviours and the consistency of aggression and courtship in *B. splendens* (Forsatkar *et al.*, 2014).

*Theobroma cacao* is a commercially valued as the source of cocoa and chocolate. Theobromine, a bioactive compound of this, is a stereo-isomer of caffeine which exists as a white powdered alkaloid. Alkaloids have decisive role in sexual selection and aggressiveness which is an advantage for survivorship of its offsprings (Wyat, 2003).

The objective of the study was to determine the behavioural changes in male *B. splendens* after 12 hours of exposure to different concentrations of *T. cacao* aqueous plant extract (10%, 20% and 30%).

## **Materials and Methods**

### ***Plant Materials***

*Theobroma cacao* plants were obtained from the College of Agriculture, Central Luzon State University, Science City of Muñoz, Nueva Ecija.

### ***Test organisms***

Fifteen (15) male *B. splendens* measuring 4.0-4.2cm and weighing 1.5-2.0g were obtained from a local supplier. Each fish was placed in a transparent container with 500mL dechlorinated tap water. They were fed regularly with fish pellets and were allowed to acclimatize for three (3) days.

### ***Preparation of plant extract***

Air dried *T. cacao* leaves were cut into pieces and powdered using a blender. The functional components of *T. cacao* were obtained through distilled water extraction following the procedure of Eguchi *et al.* (1999).

Twenty grams of powdered leaves was added to 300mL of distilled water and stirred in a 1000mL-capacity flask. The mixture was water bathed for two hours at 80-90 °C. The extract was filtered using filter paper and refrigerated prior to assay.

### ***Range Test***

To determine the plant extract concentrations to be used for the study, fishes were initially submerged to a range of concentrations at 10%, 20%, 30%, 40% and 50%. After 12 hours, the fishes were observed for signs of stress including sluggishness or lethargy, loss of appetite, red or swollen gills and cloudy, protruding or sunken eye.

### ***Treatment Preparation***

Three concentrations of *T. cacao* extract (10%, 20% and 30%) were obtained by diluting 50mL, 100mL and 150mL of the extract to 450mL, 400mL and 350mL of dechlorinated tap water, respectively. The total volume for each

treatment was 500mL. The negative control was 500mL of dechlorinated tap water while the positive control was 500 mL of Sildenafil citrate with 54 µg/L concentration.

### ***Assay***

The Betta fishes were exposed to the different treatments for 12 hours (10:00pm-10:00am). Two sets of controls were used to verify that the fishes had heightened sexual behaviours. The positive control used was 500mL of 54 µg/L Sildenafil citrate and the negative control was 500mL of 100% dechlorinated water. Three replicates were performed.

### ***Behavioural Testing***

After 12 hours of exposure to different treatment concentrations, the fishes were transferred to a separate container with dechlorinated tap water. The rectangular container measures 14.5cm x 14.5cm x 8cm (length x width x depth).

A mirror was then attached to the rear side of the container and the behaviours were assessed for 5 minutes following the procedures by Clotfelter and Rodriguez (2006) with some modifications.

The time it took for each fish to be within 8 cm distance from the mirror were initially noted. Other behaviours that were observed include the number of bubbles or surface gulping, number of serpentine movements, duration of shaking movements and the number of times the male *B. splendens* fishes contact their mirror images.

## **Results**

### ***Range Test***

After 12 hours of exposure to different concentrations of *T. cacao*, the Betta fishes exposed to 40% and 50% concentrations exhibited lethargy and loss of appetite. According to Verbeek *et al* (2008), prolonged immobility in Betta fishes indicates stress. *T. cacao* extract at 40%, 50% and increased concentrations were thus excluded from the range of test concentrations. The final treatment concentrations were 10%, 20% and 30%.

### ***Sexual Aggression Based on Behavioural Testing***

The Betta fishes were observed for behaviours indicating sexual aggression after 12 hours of exposure to the different concentrations of the plant extract and both positive and negative control. Table 1 shows the time it took for the fish to be within 8cm distance after exposure to different treatments. Betta fishes exposed to positive control moved perpendicularly towards the mirror at 8 cm distance after 5.33 seconds, 4.67 seconds in 10% concentrations of extract, 5 seconds in 20% concentrations, and 6.33 seconds in 30% concentrations. However, bettas exposed in dechlorinated tap water took a longer time in approaching their mirror images.

**Table 1.** Length of time for the male *B. splendens* fishes to be within 8 cm distance from the mirror at different concentrations of extract.

Concentration (%)	Time (sec) to be within 8cm from mirror
T1 (Sildenafil citrate=54 µL)	5.33 <sup>a</sup>
T2 (0%)	10 <sup>b</sup>
T3 (10%)	4.67 <sup>a</sup>
T4 (20%)	5 <sup>a</sup>
T5 (30%)	6.33 <sup>a</sup>

Means having the same letter of superscript are not significantly different from each other at 5% probability level by DMRT.

Table 2 shows the number of bubbles/surface gulping exhibited by male *B. splendens* fishes at different concentrations of extract. *B. splendens* exposed in positive control (30.33%) and 20% concentrations (34%) of extract showed high number of bubbles. Higher number of bubbles were observed in 10% (45.67%) and 30% (46.67%), but lower number were observed in fishes exposed in dechlorinated tap water.

**Table 2.** Number of bubbles or surface gulping exhibited by male *B. splendens* fishes at different concentrations of extract.

Concentration (%)	Number of Bubbles
T1 (Sildenafil citrate=54 µL)	30.33 <sup>b</sup>
T2 (0%)	22 <sup>a</sup>
T3 (10%)	45.67 <sup>c</sup>
T4 (20%)	34 <sup>b</sup>
T5 (30%)	46.67 <sup>c</sup>

Means having the same letter of superscript are not significantly different from each other at 5% probability level by DMRT

The number of times the male Betta fishes contact their mirror images after exposure to different treatments were also counted and shown in Table 3. Bettas exposed in the positive control and high concentrations of extract have high number of movements in contact with the mirror, which is observed at (46.67%-control, 46.67%-20% concentrations, and 42%-30%). However, Bettas in 10% concentrations had the highest mirror contact (59%) and lowest in dechlorinated tap water.

**Table 3.** Number of times the male *B. splendens* fishes contact their mirror images at different concentrations of extract.

Concentration (%)	Number of Mirror Contact
T1 (Sildenafil citrate=54 µL)	46.67 <sup>b</sup>
T2 (0%)	32.33 <sup>a</sup>
T3 (10%)	59 <sup>c</sup>
T4 (20%)	46.67 <sup>b</sup>
T5 (30%)	42 <sup>b</sup>

Means having the same letter of superscript are not significantly different from each other at 5% probability level by DMRT

Table 4 shows the number of serpentine movements exhibited by the male *B. splendens* fishes. The number of serpentine movements exhibited by Betta fishes exposed to 0% and 10% concentrations are 6.33 and 7.67 respectively. These are significantly lower than those exposed to Sildenafil citrate and 20% concentrations with 13.33 and 13 respectively. Betta fishes exposed to T5 (30%), on the other hand was significantly higher with 26.33 serpentine movements.

**Table 4.** Number of serpentine movements exhibited by the male *B. splendens* fishes.

Concentration (%)	Number of Serpentine Movement
T1 (Sildenafil citrate=54 µL)	13.33 <sup>b</sup>
T2 (0%)	6.33 <sup>a</sup>
T3 (10%)	7.67 <sup>a</sup>
T4 (20%)	13 <sup>b</sup>
T5 (30%)	26.33 <sup>c</sup>

Means having the same letter of superscript are not significantly different from each other at 5% probability level by DMRT

The duration of shaking movements exhibited by the Betta fishes were determined and presented in Table 5. The duration of shaking movements exhibited by fishes exposed to T5 which is 11.62 seconds, is significantly shorter than T3 (10%) which is 21.70 seconds and T2 (0%) which is 29.04

seconds. The shaking movements observed in Betta fishes exposed to T1 (Sildenafil citrate) and T4 (20%) were significantly higher with 47.39 and 49.03 seconds respectively.

**Table 5.** Duration of shaking movements exhibited by the male *B. splendens* fishes at different concentrations of extract.

Concentration (%)	Duration of Fin Shaking Movement (sec)
T1 (Sildenafil citrate=54 µL)	47.39 <sup>d</sup>
T2 (0%)	29.04 <sup>c</sup>
T3 (10%)	21.70 <sup>b</sup>
T4 (20%)	49.03 <sup>d</sup>
T5 (30%)	11.62 <sup>a</sup>

Means having the same letter of superscript are not significantly different from each other at 5% probability level by DMRT

## Discussion

Perpendicular movement in betta fishes is a sign of courtship behavior since it is simultaneous with lateral displays. In a study made by Cantalupo *et al.* (1996), bettas faced with their mirror image, betta fishes showed consistency in their right and left eye use during threat and sexual displays.

There is a significant result regarding the number of bubbles observed in male betta fishes in 20% concentration of extract which is comparable to the positive control.

The males become temporarily attached to their territory and are unlikely to leave once they begin constructing bubble nests (Bronstein 1981a). Nevertheless, upon completion of a bubble nest the male will be ready to court a receptive female. Male Bettas construct bubble nests by taking a bubble of air into their mouths, enclosing it in mucus, and depositing it on the surface of the water (Brownell, 2014). The bubble nest is important because it serves as the nest for the fertilized eggs and recently hatched offspring (Braddock and Braddock 1955).

The increase in surface gulping or bubble forming is an indication of the subject's willingness to engage in a fight (Oliveira *et al.*, 1998). Since there is higher surface gulping or bubble forming in high concentration of extract (30%), it is an indication that high concentrations of *T. cacao* may also lead to a fighting behavior rather than sexual aggression. However, 20% concentrations of extract is just right amount to stimulate sexual aggression since it is comparable to the control.

Male *B. splendens* monitor aggressive interactions between neighbouring conspecifics and use the information on relative fighting ability in subsequent aggressive interactions with the males they have observed (Oliveira *et al.*,

1998). According to Todd *et al.* (2008), the perpendicular confrontation involves the aggressor approaching another fish while swimming very close to its side. This usually results in the second fish swimming away. In this study, gill spreading of the betta fishes is simultaneously done with contact with the mirror. It is usually combined with another behavior like the two broadside displays, perpendicular, head-to-head or chasing. Gill spreading or gill flare is a courtship display which appears to be identical to the aggressive display, (Bronstein, 1981b).

Alkaloids present in theobromine are known to increase testosterone level. Pretreatment with the alkaloid extract increased the serum level of FSH in males compared to control (Udoh *et al.*, 2009). Same effects were also seen in betta fishes in this study in which there is a higher number of mirror contact observed in 10% concentrations than in the positive control and higher concentrations of extract. This means that an insufficient amount of alkaloid alters sexual aggression in Betta fish.

Serpentine movement is a type of head-to-head interaction since it involves movement of the head. The head-to-head interaction is similar to the perpendicular display because the aggressor swims directly towards the other fish's head, causing the less aggressive fish to turn or swim away (Todd *et al.*, 2008).

Fictive air-breathing frequency was increased when isolated brainstem preparations *B. splendens* were superfused due to serpentine proteins (Nguyen *et al.*, 2007). Serpentine is a terpene indole alkaloid produced by several plant extracts.

Sexual behavior in male included more fin spreads and fewer gill flares and color flashes (Todd *et al.*, 2008). Fin spreading is comparable to fin shaking and since gill flaring is simultaneously done with contact with the mirror, there is a significant duration of fin shaking in 20% concentrations of extract (49.03 seconds) which is comparable to the positive control. There is a decreased fin shaking in high concentrations of extract which was observed in 11.62 seconds. Shaking is an indication of sexual receptivity in betta fishes (Castro *et al.*, 2006). This means that high concentrations of *T. cacao* extract decreased sexual aggressiveness in males. This reduced aggression could be used to reduce the reproductive output of male *Betta splendens*.

In some studies, a high display of sexual aggressiveness drove the female betta fish away, yet too passive of a display might cause her to lose interest (Clotfelter *et al.*, 2007). It is known, for example, that female *B. splendens* prefer males who are aggressive to an extent, however, after a certain point, extreme aggression is no longer desirable (Lynn *et al.*, 2007). High concentrations of theobromine reduce sexual drive (Maxwell, 2013). This is



supported in this study since high concentrations of extract reduced sexual aggression like the ones observed in duration of fin shaking.

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