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BEHAVIORAL ASSESSMENT OF TAMBAQUI JUVENILES (*Colossoma macropomum*) EXPOSED TO DIFFERENT AMBIENT COLORS AND SOCIAL STRESS

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Abstract
Animal welfare regards the quality of life and the environment in which animals live or are exposed. Hence the importance of studies assessing the environmental influence on the biology and behavior of fishes from the *Colossoma macropomum* species, considering their higher potential for fish-farming and as a test organism in scientific research. This study aimed to assess, in a controlled environment, the behavioral changes expressed by juveniles from the *Colossoma macropomum* species exposed to different ambient colors or social situations. The results did not show an influence from the different ambient colors or social situations on fish distribution in the water column. The color pattern showed dark tones in black- and blue-colored environments, and light tones in white-colored environments or with the presence of a mirror. The time of locomotor activity/frequency did not change in any of the treatments of exposure to environments with colors and a mirror. Moreover, the fishes remained with folded fins and a straight posture, maintaining a pattern of rhythmic operculum beating in a normal frequency range described for the species. This suggests that animal welfare did not change in any of the situations tested and that there was a pattern of adaptive response to the type of environment.

Keywords: Animal behavior. Animal welfare. Color patterns in fish.

1. Introduction

Animal welfare is a current topic extensively addressed in recent decades and, despite the lack of a globally accepted definition for this term, it regards the quality of life, physical health, proper biological function, and emotional and behavioral health that could be applied equally to wild animals, animals raised in captivity or zoos, experimental animals, or pets. More recently, these precepts expanded to fish (Alonso et al. 2020; Kristiansen and Bracke 2020).

An increased interest in fish welfare has emerged mainly because of the growth in fish-farming activities, increased consumer awareness, the need for quality production (Dawkins 2017; Hvas et al. 2021), and the increasingly expressive use of these animals as test-organisms in scientific research, in which the health and welfare are important to produce valid experimental results (Utne-Palm and Smith 2020).

Several studies define fish as sentient beings, meaning they can be aware of sensations and subjective feelings such as pain and fear. To ensure the presence of this condition, the authors based it on anatomical and physiological characteristics of teleost fish, such as the presence of a nociceptive system similar to that of mammals. However, there are still opposing opinions to this affirmative, which became the target of countless discussions; therefore, the question of sentience in fish is not a consensus in the scientific community (Oliveira and Galhardo 2007; Browman et al. 2019; Sloman et al. 2019; Kristiansen and Bracke 2020; Snedon 2020).

Considering the difficulties of producing studies involving a methodical collection of physiological, biochemical, and behavioral data, the Fisheries Society of the British Isles suggested using the following practical welfare indicators: changes in the color pattern of the body and eyes, changes in opercular movement rates, changes in swimming activity and other behaviors, anorexia, reduction of growth rate, morphological anomalies, scars, diseases, and reproductive inhibition, among others (FSBI 2002).

Currently, several studies have been developed to understand the effects of the environment, background color or luminosity, and social stress on fish health and welfare. Marandi et al. (2018) evaluated the combined effects of three stocking densities and two aquarium colors (black and white) on the growth, body composition, and skin color of the common carp (*Cyprinus carpio*) and found that the tank color and stocking density significantly affected the growth and feeding performance of this fish, while there was no combined interaction between the two factors examined.

According to McLean (2021), the tank color affects the larval and post-larval fish culture. In the larvae, this variable affects the survival, growth, and feed conversion but in post-larval fish, the tank color can cause high levels of stress and affect the health, growth, body color, behavior, and reproductive performance. For Navarro and Navarro (2017), the color effect on productive and reproductive performances of fish also depends on a few factors such as the species, sex, and development stage evaluated. Such an interaction must be understood to allow developing efficient techniques and practices to promote fish health and welfare when maintained and cultivated in a controlled environment.

The *Colossoma macropomum* species is popularly known in Brazil as tambaqui and was described by George Cuvier, in 1818. It is a native fish from the Amazon River, Orinoco River, and their tributaries, and belongs to the Actinopterygii, Characiformes order, Serrasalmidae family (Goulding and Carvalho 1982; Hilsdorf and Hallerman 2017). The tambaqui is a teleost neotropical model of great economic and cultural importance for artisanal fishing and commercial fish farming (Morais and O´Sullivan 2017; Wood et al. 2017). Thus, potentially stressful environments for the species must be understood to aid fish-farming or maintenance studies with experimental animals (Santos et al. 2018a).

Hence, this study aimed to assess, in a controlled environment, the behavioral changes expressed by juveniles from the *Colossoma macropomum* species exposed to different ambient colors and social situations.

2. Material and Methods

This study was approved by the Ethics Committee for Animal Use of the Federal University of Western Pará (CEUA/UFOPA - Protocol Nº 0520190072). All the methods and experiences were performed according to the approved guidelines.

The experiments were performed at the Laboratory of Chemistry Applied to Toxicology, Environmental Sanitation, and Water Resources of the Federal University of Western Pará – Tapajós Campus, Santarém, PA, Brazil. Tambaqui juveniles (*Colossoma macropomum*) were obtained from the Santa Rosa fish-farming station (PA370, KM 34, Santarém, Pará, Brazil), acclimated for 90 days in a PVC box with a capacity of 0.1 m^3, and maintained in a natural periodic cycle, continuous forced aeration, room temperature of 27°C±0.5, pH of 7±0.2, and daily feeding of 3% of body weight in the form of commercial extrusion. The tanks were also siphoned daily until the animals reached the average size of 7.7 cm to start the experiments.

During acclimatization, observation aquariums were prepared by producing styrofoam plates in the aquarium sizes, which were later covered with colored EVA, composing four treatments: black, white, brown, and blue (Figure 1). One color was used per week and these covered plates were placed on the sides of the aquarium, including the bottom, to eliminate the reflexes for both the fish and the footage. A ceramic tile with the same width and length was placed next to the bottom background styrofoam plate to prevent it from floating in the water during the experiments, and both were covered with EVA.

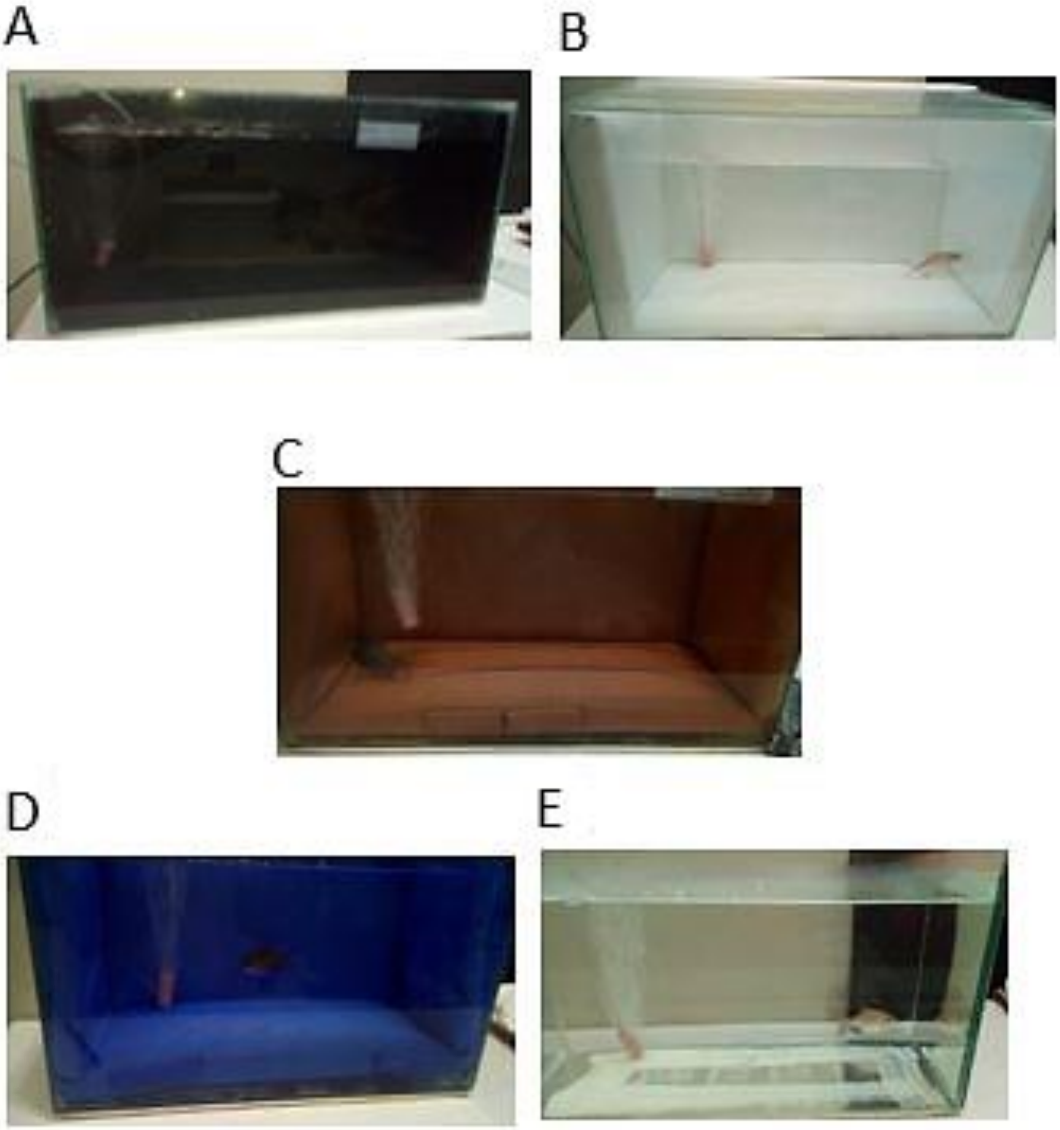

Figure 1. Aquariums characterized in the five different treatments that composed the experiments. A – First week: black color; B – Second week: white color; C – Third week: brown color; D – Fourth week: blue color; E – Fifth week: social situation (with a mirror).

After acclimatization, the animals were divided and selected from a total of 25 subjects of similar lengths and weights. Each fish, with an average length of 7.7 cm and an average weight of 6.1 g, was isolated in a glass aquarium with a capacity of 0.024 m³, equipped with continuous forced aeration, at a temperature of 27°C±0.5, for a 12-hour photoperiod, and fed once a day. They were placed between 16 and 24 hours in advance in the prepared aquarium for an adaptation period. It should be noted that only one aquarium was used at a time, remaining for an entire week with the same color, and changed only the following week.

The experiments started on Monday when a fish was placed in the aquarium, and the footage occurred only on Tuesday. Then, the fish was euthanized, the aquarium was cleaned, the water was changed, and a new fish was placed for acclimatization, which was only filmed the next day, and so on throughout the week. At the end of each week, there was a total of five different fishes for each treatment.

Daily filming sessions were performed for five weeks (from Tuesday to Saturday), with the duration of 16 minutes, and, in the first four weeks, the data were registered with the animals (total of 20 animals, five for each color) exposed to the presence and the absence of different colors to compare fish behavior in both situations.

In the last week, five fishes were exposed individually to a social situation by placing a mirror inside the side of the aquarium to simulate the presence of another fish (virtual intruder). At this stage, there were also daily filming sessions in both situations: with the presence and the absence of a mirror.

For the filming sessions, video cameras coupled to a monitor were placed one meter away from the test aquarium to ensure adequate video recording and monitor the location and swimming activity of the fish. After each session, the animals were anesthetized (eugenol solution, via bath) for euthanasia.

To evaluate the observed behavior when exposed to different ambient colors or social situations by simulating the presence of a virtual intruder (image reflected on the mirror), adjustments were made for the tambaqui and the ethogram elaborated by Merighe et al. (2004) was used, with patterns detailed below:

a) Distribution in the water column: based on the three predetermined regions of the water column - lower, middle, and upper regions -, the spatial distribution of each individual was registered by the minute. The observation of this behavioral pattern aimed to study the occurrence of territoriality and its spatial position over time;

b) External animal color: there were changes in animal colors, determining whether the animal remained predominantly lighter or darker. This behavior pattern was used as an indicator of animal welfare;

c) Locomotor activity: the time each subject spent in locomotion was recorded during the 16 minutes of each filming session;

d) Dorsal fin position: the dorsal fin position was registered, considering it retracted when its rays were longitudinally arranged relative to the back of the animals; or bristly, when they were approximately perpendicular to the back;

e) Animal posture: characterization of the position in which the fish was represented, either straight or slanted. This pattern also indicated animal welfare.

These variables were statistically analyzed with the GraphPad Prism 6.0 software, and the results were expressed as the mean ± standard error of the mean for the experiments. The appropriate statistical tests were used: Shapiro-Wilk normality test (applied to all data), analysis of variance – one-way ANOVA (to analyze locomotor activity) and two-way ANOVA (to analyze the fish distribution in the water column) -, multiple comparisons test - Tukey's test and Fisher's exact test (to analyze the variable of the external color of the animal) -, considering a p-value ≤ 0.05 as the level of statistical significance.

3. Results

Fish distribution in the water column

To verify the influence of colors and social situations on fish distribution in the different regions of the water column, the two-way ANOVA statistical test was performed. The results showed that, for this parameter, there was no influence of any of the test situations ($p=0.9999$). Figure 2 shows the preference of fish positioning in the lower region of the water column, in the presence of both colors and a mirror.

External Animal Color

For this parameter, Tukey's test and Fisher's exact test were performed to assess whether there were differences or changes in the color pattern of the fish in the different treatments, considering the fish light or dark according to the tone expressed when exposed to colors or a mirror. Thus, the level of significance was verified by comparing the fish in a black environment versus the fish in a white environment ($p=0.0476$), a brown environment versus a white environment ($p=0.1667$), a blue environment versus a white environment ($p=0.0079$), and a brown environment versus a blue environment ($p=04444$). It is noteworthy that in the white environment and the presence of a mirror, the animals presented the same tone (Figure 3).

The results show a significant p-value when comparing the color pattern of the fish in black and blue environments with the pattern observed in the white environment. In other words, there were more fishes in dark tones in the presence of black and blue colors (Figure 4) in contrast to the animals in light tones in a white environment and exposed to a mirror (Figure 5).

The boxes in which the fishes were maintained during acclimatization were blue, which justifies selecting this color to compare the behavior and color of fishes when they were in blue and other colors.

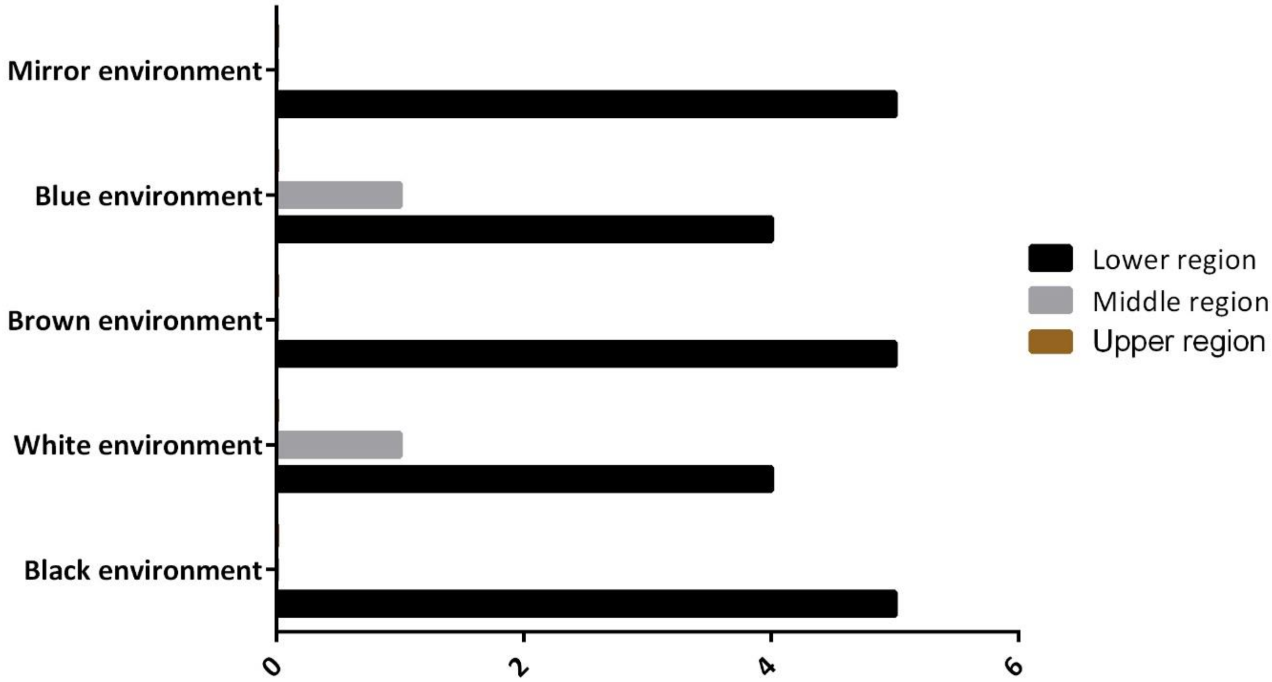

Figure 2. Fish distribution in the water column in different treatments.

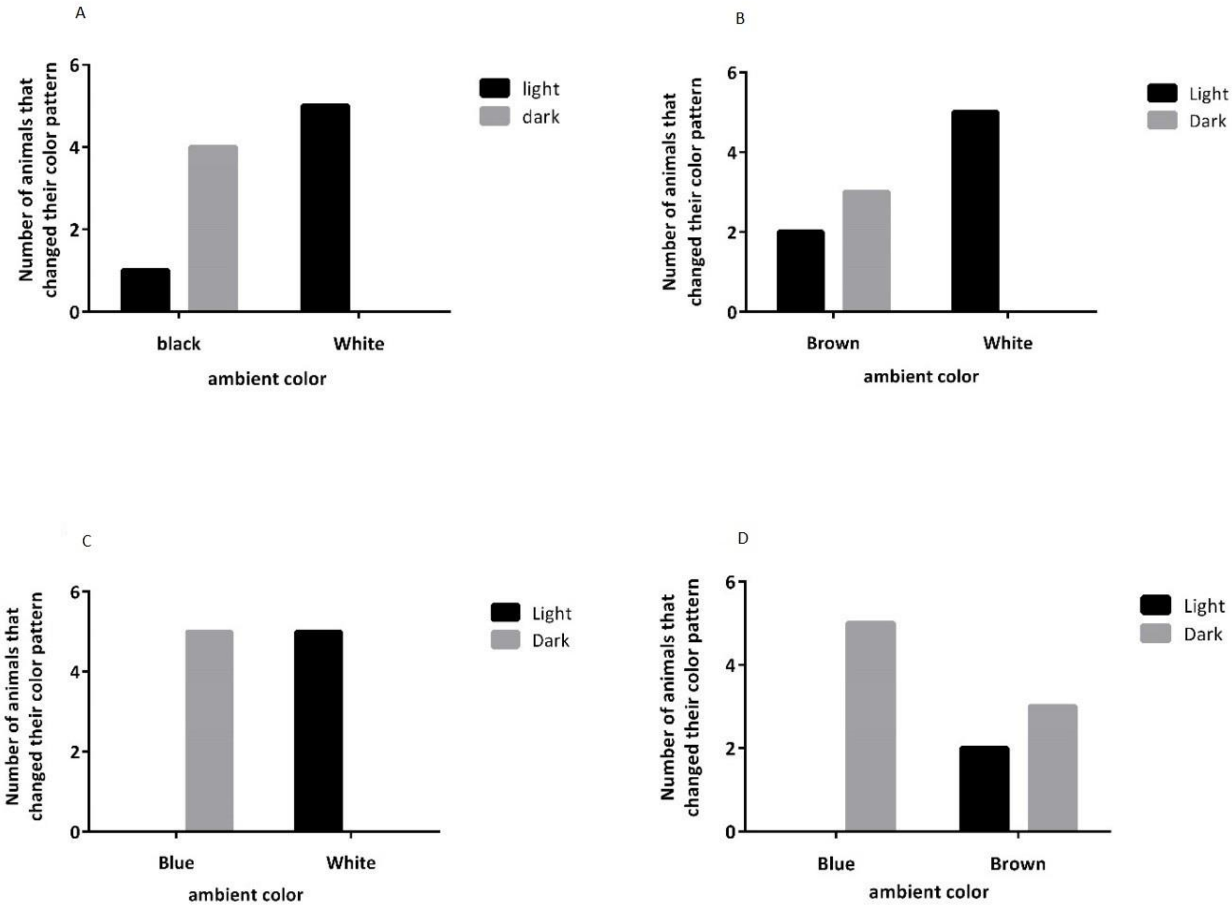

Figure 3. Animal color pattern, comparing the different environments. A – black vs. white; B – brown vs. white; C – blue vs. white; D – brown vs. blue.

Figure 4. Fishes in dark color. The dark spots spread on the body while in the environments: A – black; B – blue; C – brown.

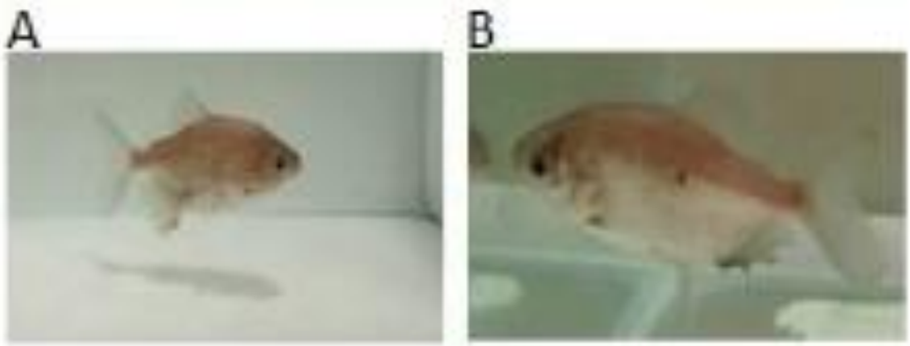

Figure 5. Fishes in light color: white color on the lower part and a rosier tone on the upper part while in the A – white environment and B – in the presence of a mirror.

Locomotor activity

The analyses of variance (one-way ANOVA) showed that, for this pattern, there was no significant difference (p=0.4429) in the different treatments (Table 1). The time of locomotor activity/frequency did not change in the presence of colors or a mirror. There was also no aggressive or agonistic behavior related to the image of the virtual intruder.

Table 1. Mean and standard error of the time of locomotor activity of tambaqui juveniles (*C. macropomum*) maintained isolated in different ambient colors and submitted to the reflection of their image in the mirror, for 16 minutes of observation.

	Locomotor activity (seconds)	
	Arithmetic mean	Standard error
BLACK	16.00	7.95
WHITE	33.40	20.66
BROWN	89.60	40.17
BLUE	48.60	32.52
MIRROR	100.60	62.26

4. Discussion

Regarding the distribution in the water column, Merigue et al. (2004) highlight in their study on the effect of ambient color on social stress in Nile Tilapia (*Oreochromis niloticus*) the preference of fishes for positioning in the lower region of the water column during the trial period, being significantly higher (p=0.0079) than middle and upper regions.

Regarding the color pattern of the animals in the different treatments tested, a higher number of fish remained dark in the blue, black, and brown environments, whereas in the white environment and the presence of a mirror, they presented lighter colors. This suggests there was a process of acclimatization/adaptation to lighter environments. According to Salis et al. (2019), the color pattern has a clear ecological and behavioral meaning, with a wide range of functions in animals, particularly teleosts.

In a similar experiment, Ninwichian et al. (2018) evaluated the effect of five colors of experimental tanks (white, red, green, blue, and black) on growth, stress response, and skin color in the *Trichogaster pectoralis* species. The result showed that fish skin color varied from very pale (high skin clarity) in the white tanks to very dark (low skin clarity) in the black tanks, and 80% of the variation in skin clarity was explained by tank clarity, as the use of a tank resulted in normal skin color, thereby revealing that blue is the most appropriate tank color for the *T. pectoralis* culture.

According to Shawkey and D'Alba (2017), many animals, including fish, can adapt their skin pigmentation in response to color fluctuations and environment luminosity, as animal integumentary coloration plays a crucial role in the protection, visual communication, and camouflage dynamic. It is also an example of phenotypic plasticity with significant importance for adapting and surviving in new environments, varying between species and populations (Sköld et al. 2016), as the effect of tank color on foraging capacity, growth, metabolism, physiology, survival, and skin pigmentation of larvae from the *Chanos chanos* species (Bera et al. 2019) and juveniles from the *Lophiosilurus alexandri* species (Costa et al. 2016).

It is noteworthy that the skin pigmentation pattern is a specific characteristic of each species and depends on the number, shape, and spatial combination of several types of chromatophores, which through different cellular mechanisms, lead to changes in the color of animals, which may occur in minutes, hours, or more slowly in weeks (Sköld et al. 2016; Delgadin et al. 2020).

Changes in pigmentation/skin color are influenced by several aspects (such as stage of development and reproductive cycle, or environment aspects such as biotic and abiotic factors). Fish pigmentation is one of the most important quality criteria because it defines the market value of species in fish-farming and ornamental species, serving as an externally visible sign to infer the welfare and cultivation conditions used (Linhares et al. 2018; Vissio et al. 2020).

The results show that the time of locomotor activity/frequency did not change in the different colors tested or in the presence of a mirror. They also did not show observable aggressive and agonistic behaviors related to the image of the virtual intruder. These findings may also be associated with the gregarious nature of social species that live in flock/groups from the species used (Barbosa et al. 2009).

According to Gonçalves-de-Freitas et al. (2019), the social behavior of fish may be affected by artificial environments, particularly in species that show aggressive behavior to define the social rank hierarchy, although aggressive interactions are part of the natural behavior of fishes. If constant and intense, it may cause serious bodily injuries, increase energy expenditure, and lead animals to social stress, negatively affecting the welfare of the species.

Merigue et al. (2004), when evaluating the effect of ambient color on social stress in Nile Tilapia (*Oreochromis niloticus*), verified that, in isolation, there was no significant difference ($p>0.05$) of this pattern for the different colors tested. Santos et al. (2018b) found significant differences ($p\leq0.05$) in the social behavior of males from the *Betta splendens* species under the effect of different ambient colors and with a simulated presence of a co-specific with a mirror. According to Santos et al. (2019) and Ruchin (2021), this difference in behavior can be explained by the fact that the receptivity of fish to ambient light/color changes profoundly according to the species and development status evaluated.

Conversely, many behaviors are based on visual information. Tatemoto and Serra (2021) observed the behavior of Nile Tilapia juveniles under two light extremes: high light and low light, and verified that the low light condition decreases but does not eliminate confrontations. However, the high light simulation in artificial environments potentially compromised the welfare of the species by increasing the number of agonistic interactions.

In the study by Silveira et al. (2020), the behavior of damselfish (*Stegastes fuscus*) was evaluated in natural and controlled conditions to verify how territory and familiarity affect the aggressive behavior of the species. Field and laboratory data suggested that territoriality and damselfish aggression are related to the size of the area defended and the identity of the competitor, as reduced areas can result in increased aggression and the presence of heterospecifics.

In turn, Otsuka et al. (2020) conducted a light-dark preference test using medaka fish (*Oryzias latipes*) with or without a neighboring co-specific. The fish, when isolated, preferred the light environment and avoided the dark one. When presenting another fish of the same species (co-specific), it increased the time the male fish spent in the dark environment, showing that this condition encouraged the development of risky behavior.

Studies of this nature are important because investigating the behavior of a species allows understanding the laws and principles that explain the activities of fishes and their reasons, based on the understanding of motor patterns; motivations; and evolutionary, genetic, and environmental influences (Volpato et al. 2020).

It is noteworthy that in all the treatments proposed, the dorsal fin position and posture of *C. macropomum* juveniles did not show detectable changes, as the fins remained retracted, the posture straight, and the opercular beat rhythmic, thus suggesting that fish welfare did not change in any of the experiments performed.

5. Conclusions

Overall, the results of this study did not show visible behavioral changes in juveniles from the *Colossoma macropomum* species in the different ambient colors and social situations evaluated. This further suggests the versatility of this species, which could adapt to several ambient colors by changing the coloring pattern. However, further studies are required for this species, mainly for knowing more about its behavior and developing tools to assess welfare and effective ways of cultivation that can be applied to both fish-farming activities and scientific experimentation areas.

Authors' Contributions: AGUIAR, O.F.: conception and design, acquisition of data, analysis, interpretation of data and drafting the article; LISBOA, J.D.B.: conception and design, acquisition of data, analysis and interpretation of data; DE SANTANA, M.B.: conception and design, acquisition of data, analysis and interpretation of data and critical review of important intellectual content; LOPES, R.B.: critical review of important intellectual content; DE MOURA, L.S.: critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Animal Use Ethics Committee (CEUA) of the Federal University of Western Pará (Protocol No. 0520190072).

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