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Influence of Annatto (*Bixa orellana*) Extract and Different Shading Levels on *Litopenaeus vannamei* Color Reared Inland Using Biofloc Technology

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Abstract

Shrimp color is important when choosing the crustacean by the consumer. Pacific white shrimp *Litopenaeus vannamei* can convert various types of carotenoids into astaxanthin and accumulate them in its body, giving the desired reddish color. In this study, the shrimps were kept in a biofloc aquaculture system away from the coastal zone, fed with commercial feed supplemented with annatto extract (*Bixa* sp.). The experiment was carried out with a 2 x 4 factorial design with shad levels (50% and 80%) and four feeding regimes (0, 7, 14, and 21 days) to evaluate the most efficient model to promote the increase in the surface color of the shrimp. Leaching in artificial brackish water and the influence of biofloc pigments on shrimp color. According to the results, bixin sprinkled on the feed surface undergoes leaching in brackish water the measure of the time it is immersed. Carotenoids present in the biofloc showed a correlation with chlorophyll content by the *R* test. Raceway with 80% water shad and feed containing 1,235 mg/Kg of bixin showed the most significant results in the shell color of shrimps (fresh and cooked) and lower ammonia levels.

Keywords: color enhancer, carotenoid, Penaeidae shrimp, pigment leaching, shade

1. Introduction

Shrimp pigmentation is variable in nature, but after cooking, it takes on a reddish coloration. The red color determines the consumer choice of fish and crustaceans (Daniel et al., 2017). This color is provided primarily by astaxanthin (3,3'-dihydroxy-β-carotene-4,4dione) $C_{40}H_{52}O_4$, a carotenoid not synthesized by these aquatic animals that should be included in their diets (Kamata et al., 1990).

Astaxanthin has an important role in the physiology of crustaceans, being fundamental in the processes of growth, reproduction, health, anti-oxidative processes, protection against radiation, and animal mimicry according to Costa & Miranda-Filho (2020). This pigment is released from the protein molecule and becomes visible in the red spectrum (Howell & Matthews, 1991).

The Penaeidae shrimp *Litopenaeus vannamei* can assimilate and convert carotenoids present in food into astaxanthin and accumulate them in the body (Baron et al., 2008; Kop et al., 2010). However, to have efficient pigmentation after cooking, it is necessary to have enough portions of carotenoids in the shrimp feed. If this ratio is low, the crustacean will have a pale color when cooked (Göçer et al., 2006; Aguirre-Hinojosa et al., 2012).

Annatto is known as the “urucuzeiro” (*Bixa* sp.) red color seed dye, bixin (methyl hydrogen 9′-cis-6,6′-diapocaroteno-6,6′-dioate ester) $C_{25}H_{30}O_4$, the main pigment, responsible for about 80% of the carotenoid content fat soluble in natural form (Rivera-Madrid et al., 2016). In aquaculture, annatto was also used, obtaining positive results in skin pigmentation in kinguio *Carassius auratus* (Fries et al., 2014; Dananjaya et al., 2017), and Chinese mitten crab (Wang et al., 2018). Likewise, for muscle pigmentation in rainbow trout *Oncorhynchus mykiss* (Safari & Atash, 2015).

Aquaculture production using the Biofloc Technology (BFT) system has outstanding biological diversity, such as algae, cyanobacteria, yeasts (producing carotenoids), zooplankton (eating these living beings), protozoa, bacteria, etc. The consumption of these food items by shrimp, in addition to the feed provided, can give the necessary carotenoids for their metabolism and, if not assimilated, are liable to accumulate in the body. However, the composition of the biofloc is quite heterogeneous and varies according to the location, climatic conditions, feeding, water shading or quality, and composition of the water (Avnimelech et al., 2015; Samocha et al., 2017). In Brazil, cultivation in BFT far from the coast in brackishwater conditions has gained relevance due to the demand for fresh shrimp close to consumer markets, since this system sustains high densities, which makes it viable for the continent, and for that, the use of artificial salt, which increases production costs, must be reduced (Spelta et al., 2021).

In shrimp farming using the BFT system in the greenhouse, water shading can also determine the difference in the color of crustaceans (You et al., 2006). The use of shad nets is frequent in closed aquaculture systems to contain the development of autotrophic microorganisms and to provide the development of heterotrophic microorganisms, which must be dominant in this system (Suantika et al., 2018).

According to Khoa et al. (2020), in the cultivation of *L. vannamei* in biofloc, the use of a 50% shade net, similar to the control treatment without shad, provides better survival, final biomass, reduces the amount of *Vibrio* spp. and nitrogen compounds. Also, a higher proportion of protein and lipids in biofloc concerning treatments with two and three layers of this shade net suggest that this is due to a greater balance in the ratio between the autotrophic and heterotrophic biota in the cultivation tank. However, external coloring influences the shrimp market, and the reddish ones have greater acceptance by the consumer and better sales value in the trade, this condition being stimulated by cultivation in darker environments (Tume et al., 2009; Parisenti et al., 2011; Tomas et al., 2019).

Commercial shrimp feeds are generally not supplemented with pigmentation additives. What can affect the quality of the final product in intensive systems, where the use of natural food is limited? This makes farmers need alternatives for using concentrated pigments at the end of the growing period. In this way, shrimp culture was carried out in a BFT system, using greenhouse raceways with different feeding regimes with a commercial ration containing annatto extract and employing 50 and 80% water shades to test the response time for pigment inclusion during the *L. vannamei* high-density cultivation.

2. Method

The study with invertebrate species does not require ethical approval.

2.1 Structure and Location

The experiment was conducted in sixteen raceways lined with a non-toxic PVC blanket with an internal weft of 100 m^3, with central partition and rounded ends, measuring 5 m wide and 20 m long. These tanks were arranged side by side in a greenhouse. The aeration of the system was "nozzle" type injectors connected to centrifugal pumps, creating a water circulation. The production unit was installed in the city of Sete Lagoas - MG, Brazil, 19°28′4″ S, 44°14′52″ W, 766 meters of altitude, and 600 km from the Atlantic Ocean.

2.2 Preparation and Experimental Conditions

The raceways were filled with freshwater, pumped from surface wells, artificially salinized to 24 g/L, and covered with shad nets (50 and 80%) with VeroMix sea salt premix (Veromar®, Campinas, SP, Brazil) and sodium chloride 2:1. Freshwater was replenished in the system to compensate for the effects of evaporation. They matured with 50% biofloc inoculum for 4 days and received 70,000 juvenile shrimp with 5.0 g (± 0.5 g). The density in each tank was 700 shrimps/m^3.

During the experimental period, the animals were fed 4 times a day (8:00 am, 12:00 a.m., 4:00 pm, and 8:00 p.m.), with a commercial extruded carotenoid level of 8.4 mg/kg (± 0.3) (Poti Guaçu AD, 35% CP, Guabi®, Guabi Animal Nutrition, Campinas, SP, Brazil) with the inclusion of 1,235 mg/kg bixin (value measured after processing and drying). Bixin was obtained in the form of urucum extract in ethanol (24.1 g/L bixin in the solution), made from annatto seed, and commercially available. In this way, we intend to test the influence of feed administration with a high carotenoid concentration in a short period during *L. vannamei* cultivation.

Addition of sugar cane (when total ammonia ≥ 1 mg/L) in the proportion of 20:1 of the measured total ammonia (TA-N), as recommended by Avnimelech et al. (2015). This management was done in a fractionated way at the times of 10:00 a.m., 2:00 p.m., 6:00 p.m., and 10:00 p.m. Decanter was always carried out when the value of the sedimentable solids (SS) exceeded 15 mL/L until the minimum value of 8-10 mL/L.

2.3 Experimental Model

A completely randomized design in a 2 x 4 factorial design (two shad levels and four sampling times) was used for this study. Four shrimp 6.5 g (± 1.39) from each tank were collected in four different opportunities. Six repetitions for each of the two shad levels and collection times. In the first sampling, there was no addition of pigment in the feed. The other samples were taken after 7, 14, and 21 days of feeding with inert feed containing anatto. Thus, considering the two conditions of shading (50 and 80%), eight treatment combinations were tested, divided into two shades, and the four administration times of pigmented ration of 0, 7, 14, and 21 days. The treatments tested were 50-0, 50-7, 50-14, 50-21, 80-0, 80-7, 80-14, and 80-21.

2.4 Water

Temperature, dissolved oxygen (DO), pH, and salinity were measured with an AK88 multiparameter probe (AKSO®, Akso Produtos Eletrônicos Limitada, São Leopoldo, RS, Brazil). Total ammonia (TA-N), nitrite (NO_2^--N), nitrate (NO_3^--N), and phosphate (PO_4^{-3}-P) were determined according to the methodology described by UNESCO (1983). For alkalinity, the APHA (1998) method was used, and for the sedimentable solids, direct reading in the Imhoff sedimentation cone was employed.

2.5 Analyzes of Pigments

Quantitative carotenoid analyzes were performed by adapting the methodology proposed by Rodriguez-Amaya (2001). Four feed samples were macerated in porcelain grains to form a thin paste. A one-gram weight portion was separated in a test tube to extract the carotenoids in anhydrous ethyl alcohol and then filtered on quantitative filter paper and allowed to stand protected from light until measurement. The extracts were corrected in their volume until the solution obtained a 5-mL sample for analysis. The amount of bixin was determined by the UV spectrophotometer Libra S22 (Bichrom®, Cambridge, UK) at 457 nm (ABS 457) using the specific extinction coefficient in the cuvette of 1 cm optical path E1%, 1 cm = 3443 nm, according to the calculation described by Weber (1990).

Equation 1:

$$\mu g\ bixin\ /g = \frac{ABS\ 457\ x\ DF\ x\ V}{3443\ x\ g}$$

where: ABS 457 = Absorbance of bixin in ethanol solution at 457 nm; DF = Dilution factor; V = Sample volume; 3443 = Absorption coefficient of bixin; g = grams of sample.

Biofloc samples were prepared by filtering the solids in a 20-µm mesh. Samples were cooled at 5°C for up to 4 h and dehydrated in a forced ventilation oven at 55°C for 24 h, for total moisture elimination. Pigment concentration was based on the dry matter (DM) of the biofloc.

Equation 2, adapted from Kuroda et al. (2005), was used to determine the total chlorophyll based on the amount of chlorophyll-a:

Equation 2:

$$\mu g\ chlorophyll - a/g = 27{,}9\ x\ (ABS\ 665 - ABS\ 750)\ x\ \frac{1.000\ x\ DF}{V\ x\ 1}$$

where: 27,9 = Coefficient of absorption of beta carotene in ethanol; ABS 665 = Absorbance of chlorophyll in ethanol solution at 665 nm; ABS 750 = Absorbance other pigments (no chlorophyll) in ethanol solution at 750 nm; DF = Dilution factor; V = Sample volume; 1 = optical length of the cuvette.

Total carotenoid concentrations in the dry biofloc and feed samples (Equation 3) were determined spectrophotometrically at 452 nm (ABS 452) using the specific extinction coefficient of E1%, 1 cm = 2100 based on the amount of beta-carotene according to the same equation of astaxanthin, but subtracting the chlorophyll value to avoid interference. Conversely, the chlorophyll determination equation proposed by Kuroda et al. (2005).

Equation 3:

$$\mu g\ total\ carotenoids/g = \frac{(ABS\ 452 - ABS\ 665)\ x\ DF\ x\ V}{2100\ x\ g}$$

where: ABS 452 = Absorbance of total carotenoid at 452 nm; ABS 665 = Absorbance of chlorophyll in ethanol solution at 665 nm; DF = Dilution factor; V = Sample volume.

2.6 Bixin Leaching Test

Homogenized samples of the same experimental feed supplied to shrimps were collected, crushed in porcelain grains, weighed (1.0 g), and analyzed in quadruplicate by the determination methodology of bixin described in item 2.5. For the control condition, the analyses were performed without immersion of the feed in an aqueous medium. Other feed samples were collected and immersed in test tubes with 10 mL of distilled water solution with salinity 25 g/L, pH 6.0, and 30ºC for 15, 30, 60, and 120 minutes in a thermostatic bath under shaking.

2.7 Color Analysis

The colorimetric analysis of the shrimp body surface was performed by reflective spectroscopy using a digital colorimeter (Minolta Color Reader CR-400), according to the CIELAB three-dimensional color space system. Defined by the International Lighting Commission (CIE 1976), by the coordinates L * for lightness from black to white, a * from green to red, and b * from blue to yellow (Skrede, 1987). For standardization purposes, readings were performed in the shell abdominal region of the pleura between the first and second somites in each shrimp sampled. After cooking (1 minute) at the approximate temperature of 100°C, the same animals were evaluated again by the same method.

2.8 Statistical Analysis

For data analysis, the Shapiro-Wilk normality test was initially performed. For parametric data, ANOVA and Tukey's means comparison test was used (Temperature, DO, TA-N, SS, PO_4^{-3}-P), and the Kruskal-Wallis test in non-parametric data (pH, salinity, NO_2^--N, NO_3^--N, alkalinity and coloration). Significant differences were compared at the significance level of 5% probability. Regressions among the variables were made to evaluate the relationship among the parameters (carotenoids, chlorophyll, and bixin). The Spearman R test was used for the correlation tests. The analyses were performed by the INFOSTAT software version 2017 according to the manual (Casanoves et al., 2012).

3. Results

3.1 Water Parameters

Table 1 shows the water quality parameters evaluated in the two raceways throughout the experiment (21 days). Salinity, total ammonia, nitrite, nitrate, and alkalinity parameters showed significant differences between the two levels of shad ($P<0.05$). In figure 1, it is observed the difference in the color of the shrimp farming water in BFT. Since 50% of shad predominates the autotrophic composition evidenced by the green coloration that chlorophyll from photosynthetic microorganisms. In the shading of 80%, the predominance of heterotrophic organisms leaves the water with a brownish appearance.

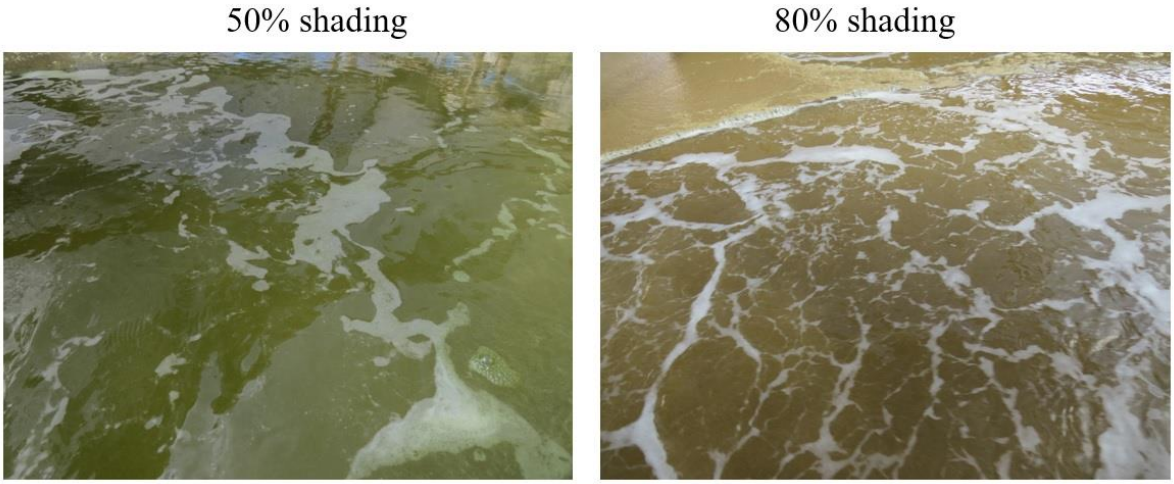

Figure 1. The visual appearance of shrimp cultivation raceways in BFT.

Table 1. Water quality parameters during the test in two different shade raceways (%)

Parameter	50% shade	80% shade
Temperature (°C)*	28.65 ± 1.88	29.43 ± 1.86
pH	7.70 ± 0.11^{b}	7.60 ± 0.16^{a}
DO (mg/L)*	5.60 ± 2.35	5.62 ± 0.93
Salinity (‰)	22.09 ± 8.99^{a}	25.93 ± 0.55^{b}
TA-N (mg/L)*	0.69 ± 0.80^{b}	0.26 ± 0.34^{a}
NO_2^--N (mg/L)	0.68 ± 2.34^{a}	2.58 ± 0.39^{b}
NO_3^--N (mg/L)	16.33 ± 1.10^{a}	60 ± 21.38^{b}
Alkalinity (mg $CaCO_3$/L)	299.18 ± 0.91^{b}	294 ± 3.63^{a}
SS (mL/L)*	10.32 ± 5.38	12.59 ± 3.23
PO_4^{-3}-P (mg/L)*	2.38 ± 0.26	2.26 ± 0.32

Different letters represent significant differences (P<0.05) by the non-parametric Kruskal-Wallis paired test. Parameters marked with * were evaluated by the Tukey parametric test, different letters represent significant differences (P<0.05).

3.2 Pigment Analysis

The total carotenoid concentration in the biofloc, as shown in figure 2A, was higher (P<0.05) in the 50% shad raceway compared to 80% shad (day 0). However, a contrasting response began to occur, resulting in equal total carotenoid content (in both raceways) by the end of the monitoring period (day 21st). The concentration of chlorophyll-*a*, as seen in figure 2B, was lower in the 80% shad raceway than 50% shad, which was higher throughout the experiment (P<0.05).

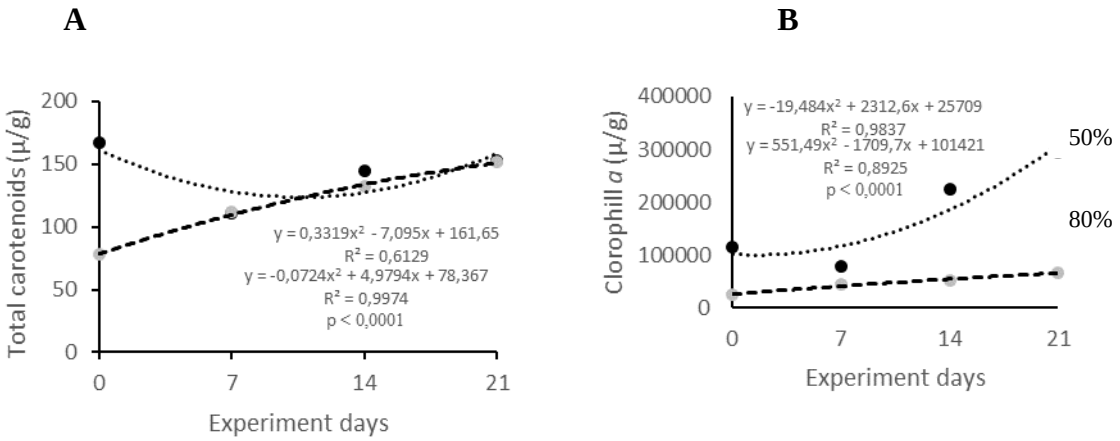

Figure 2. Relationship between pigments of the biofloc based on the dry matter (DM) and the time of the experiment in days being (A) total carotenoids and (B) chlorophyll a. Line (.....) represents shad 50%, and line (----) represents shad 80%

Table 2 shows the correlations (Spearman's R coefficient) between the total carotenoids of the biofloc, chlorophyll-*a*, and L * a * b * coordinates measured in the shrimps (fresh and cooked). The color coordinate evaluations measured in the shrimps are described in figure 5.

Table 2. Correlation (R Spearman) between the total carotenoids contained in the bioflocs and the chlorophyll levels of the color of the *in natura* and cooked prawns

Correlation between	R	valor de P
Biofloc carotenoids versus biofloc chlorophyll	0.71	<0.0001
Biofloc carotenoids versus L* *in natura* shrimp	0.11	0.2118
Biofloc carotenoids versus L* *in natura* shrimp	-0.29	0.0010
Biofloc carotenoids versus b* *in natura* shrimp	-0.06	0.4865
Biofloc carotenoids versus L* cooked shrimp	0.08	0.3879
Biofloc carotenoids versus a* cooked shrimp	0.08	0.3879

Biofloc carotenoids versus b* cooked shrimp	0.17	0.0491

The values of L * (luminosity), a * (Red), and b * yellow correspond to the CIELAB system of color coordinates.

3.3 Bixin Leaching Test

The leaching test (Fig. 3) showed that the feed immersion in brackish water changed the concentration of bixin pigment present in the feed. After two hours of immersion, the amount of carotenoid was lost to the water concerning the non-immersed ratio (control) (P<0.05).

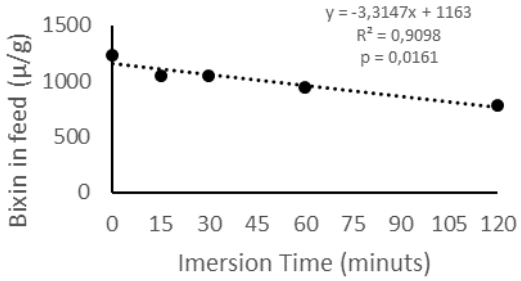

Figure 3. Relationship between the immersion time of pigmented feed in NaCl distilled water solution (25 g/L, pH 6.0 and 30°C) and the level of bixin measured

3.4 Color Analysis

In figure 4A, it is possible to observe that lightness of fresh shrimp (L *) was higher (P<0.05) in animals fed with feed containing bixin independent of shad. The green color of fresh shrimp (a *) was more pronounced (P<0.05) in the treatments 50-14, 50-21, 80-21, and 80-7 (Fig. 4B). Figure 4C shows that treatment 80-14 (fresh shrimp) presented a more pronounced (P<0.05) yellowish coloration (b *) than the other treatments. Figure 4D represents the lightness (L *) of the cooked shrimp. The lightness of the treatment 80-14 was lower (P<0.05) than the other conditions, which did not differ among them (P>0.05). The red color (a *) of the cooked shrimp was more pronounced (P<0.05) in the 80-14 treatment (Fig. 4E). The other treatments did not differ from each other (P>0.05). The yellow coloration (b *) of cooked shrimp was more pronounced (P<0.05) in the 50-21, 80-0, 80-14, and 80-21 than in 50-7 and 50-14 treatments (Fig. 4F). The other conditions did not differ significantly from each other (P>0.05).

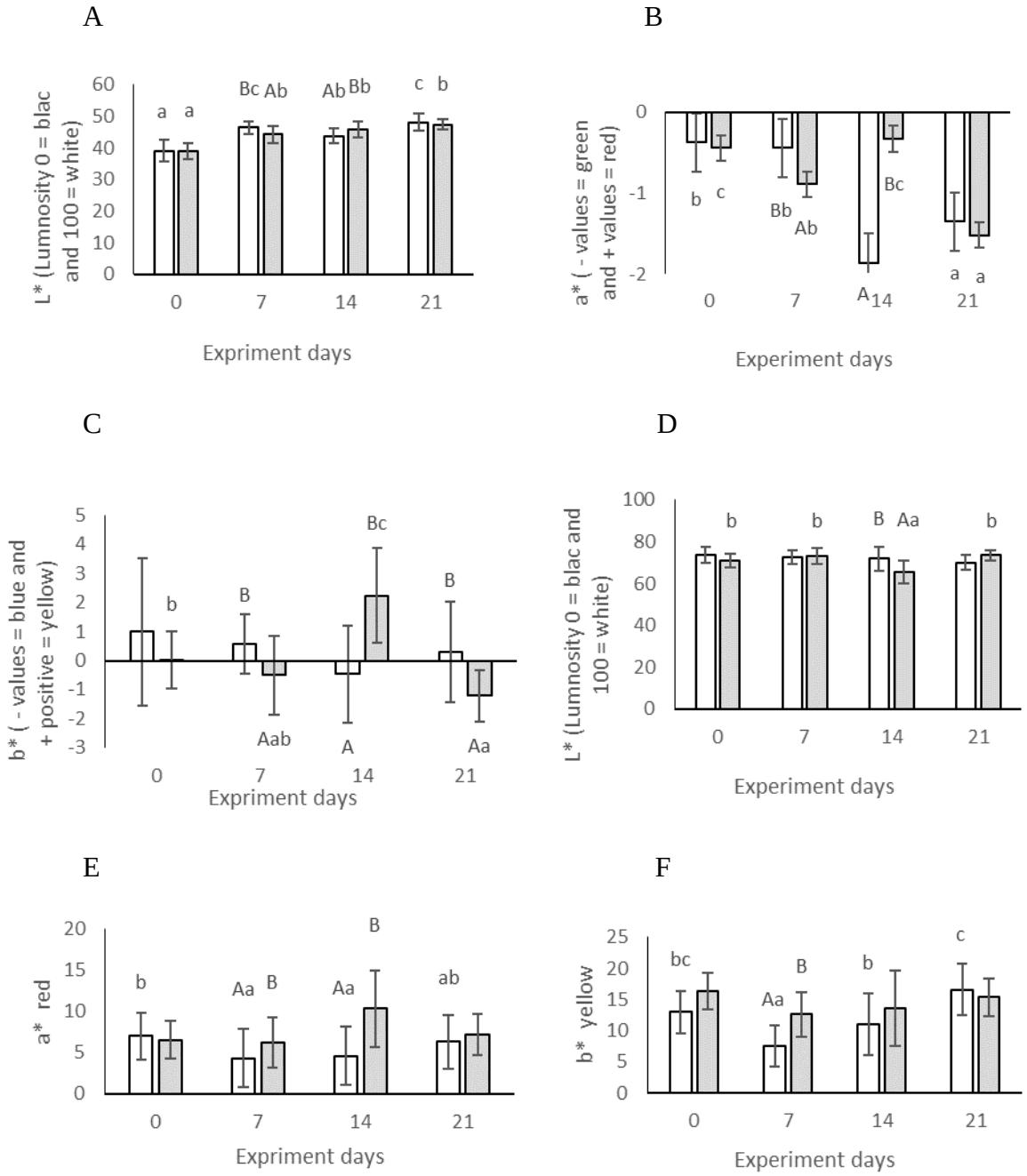

Figure 4. Mean (SD ±) of the coloration measured in shrimp *L. vannamei in Natura* L* (A) a* (B) b* (C) and cooked L* (D) a* (E) b* (F) with the inclusion of annatto in the ration in two shad levels. White columns represent treatments with 50% shading, and gray columns represent treatments with 80% shading. Different CAPITAL letters represent significant differences (P<0.05) by the non-parametric Kruskal-Wallis pairs test for shad and minuscule letters for cultivation time in the same shad

4. Discussion

Increasingly common in Brazil, *L. vannamei* cultivation far from the coast is characterized by

using brackish water in the BFT system. However, this system ensures productive viability without influencing the shrimp's nutritional quality (Pinto et al., 2020). But the supplied diets generally do not contain carotenoids, and the shad systems are not well-defined in the literature.

Heterogeneous cultivation conditions such as those described in the present study are decisive for the manifestation of different color characteristics for each cultivation tank, and it is necessary to assess which one meets the ideal recommended by the consumer market. The color of a crustacean´s shell is influenced by diet, habitat, life story, water quality, and the interaction among binding proteins and pigment (Wade et al., 2017; Pan et al., 2019; Pattanaik et al., 2020).

The measured water quality parameters are those commonly found in biofloc aquaculture systems (Krummenauer et al., 2014) and heterogeneity between tanks. The observed differences possibly occurred because of the microbiota in each tank and were probably influenced by the different shade conditions in the present study (Avnimelech et al., 2015; Samocha et al., 2017).

Considering the need for a balanced and favorable environment for heterotrophic bacteria development (Avnimelech et al., 2015) and that autotrophic activity did not increase the pigment effectiveness in shrimp pigmentation in the present study, it was preferable to use a shade of 80% instead of 50% in the *L. vannamei* farming (BFT system).

The concentration of chlorophyll *a* in the biofloc system showed the intensity of the primary production as observed by Arst et al. (2008). In the present study, chlorophyll *a* showed a positive correlation with carotenoid concentration present in the biofloc (dry matter) ($P<0.05$). Shade (80%) may have caused a reduction in photosynthetic rate, as suggested by León & Galván (1999), reducing the amount of chlorophyll present weekly throughout the experiment in comparison to 50% water shading ($P<0.05$), but did not determine the reduction of carotenoids proportionally to chlorophyll.

The probable reason for this response is that the sources of carotenoids in this study are diverse and not only from photosynthetic organisms. Phytoplankton is responsible for the biosynthesis of these carotenoids and their dispersion in the aquatic environment related to the level of sunlight that provides photosynthesis and the development of pigments as a form of protection (Huang et al., 2017). In Quintana-López et al. (2019) study, *L. vannamei* grown with commercial feed without the addition of pigments in an extensive system had a higher concentration of astaxanthin in the shell, than those cultivated in an intensive system or captured in a natural environment due to the consumption of microbiota present in greater quantity in this system.

Carotenoids may also be bioaccumulated from zooplankton (Yagi et al., 2001; Esatbeyoglu & Rimbach, 2017), be derived from yeasts and other non-chlorophyllized microorganisms (Andrews & Starr, 1976; Johnson et al., 1980), or have leached from the experimental feed itself, bixin from 2 h immersed in salt water ($P<0.05$). Water shade can be a form of control for the desirable microbiota in the biofloc media, since the largest shade may favor

heterotrophic organisms in comparison to autotrophs as discussed by Samocha et al. (2017).

However, there is a low or nonexistent correlation of the color in shrimp (L * a * b *) with the level of total carotenoids found in the biofloc, and these carotenoids may be insufficient to meet the shrimp needs or only partially available because they are protected by the cell wall of the consumed food which has not undergone a process that would release them to the extracellular medium to be absorbed as reported by Chien & Shiau (2005). Another possibility is related to the consumption of the biofloc by the shrimp, since it may not have been ingested in significant quantities to supply their needs for carotenoids. In addition, the difference in species-specific efficiency of the ability to conversion of precursor carotenoids to astaxanthin and their capacity for body accumulation by shrimps must be considered (Latscha, 1991; Schiedt et al., 1993; Gouveia et al., 2003; Güroy et al., 2012) and this can influence its color as this is the main pigment of shrimp accumulation.

Regarding the coloration of fresh shrimp, the treatments with the addition of annatto extract showed higher lightness (L* higher values close to 100 are more whitish, lower values close to 0 are more blackened) in comparison to the two treatments without pigments. According to Parisenti et al. (2011), the consumer prefers whiter fresh crustaceans, and the orange-red color is considered ideal when cooking shrimp. This characteristic can be evidenced in the a* (red) and b* (yellow) coordinates with positive values, in which treatment 80-7 is among the best results, and treatment 80-14 was greater than 50-14 in red color, while in treatment 50-21 was higher than 50-7 and 50-14, and isolated as the best result on the L* characteristics, cooked with darker staining, and b* fresh with more yellowish color ($P < 0.05$).

The use of carotenoid precursors of astaxanthin for shrimp from plants is efficient, and annatto has been successfully tested for pigmentation of other aquatic organisms (Costa & Miranda-Filho, 2020). Diet and environment-related color variations may be due to differences in the profile and amount of accumulated carotenoid pigments such as astaxanthin, beta-carotene, and others (Liñán-Cabello et al., 2003). Astaxanthin is responsible for the red color of the shrimps and is the main carotenoid present in the body of *L. vannamei* participating among other processes as a strong antioxidant in its body and making this shrimp a functional food for humans (Silva et al., 2015).

It was observed that some treatments with 80% shading did not show higher pigmentation after cooking, although having a greater or similar amount of shrimp bixin. Other studies have also found that higher pigment accumulation in tissues does not always determine the external coloration of aquatic organisms (Choubert, 2010; Safari & Atash, 2015). In some cases, the supplementation of carotenoids may not be as efficient due to the metabolic cost of the necessary molecular transformation of the precursor pigment into astaxanthin and accumulation in the shrimp body (Latscha, 1991).

The difference in shrimp color during the same experimental period and different shading can be explained by the influence of the lightness on the astaxanthin accumulation in *L. vannamei* as demonstrated by You et al. (2006) and the natural mimicry of crustaceans in different environmental conditions (Bedini, 2002; Bedini, 2006). These aspects make the shrimp present different colors to imitate the environment around them, reducing possible predation

when in captivity.

Darker cultivation environments have contributed to the improvement of the body color of shrimp by increasing the size of the chromatophores and more accumulation of pigments in them. The total amount of carotenoids in the body does not necessarily indicate higher carapace coloration since for this color to be externally visible, it must be within the chromatophores (Tume et al., 2009; Tomas et al., 2019).

As already observed with other plants that have a high concentration of carotenoids in their composition (Vernon-Carter et al., 1996; Arredondo-Figueroa et al., 2000), and according to the present study, annatto may be a potential food supplement to improve *L. vannamei* pigmentation from the seventh day of bixin supplementation. Bixin can be used in the concentration of 1,235 mg/Kg in the ration in short periods in the final days of cultivation. However, it was not possible to affirm that it increased the red/yellow pigmentation on the first day (0), a contribution of the biofloc carotenoid, and only its use was efficient after reducing this natural quantity, observed a difference of coloring first in the tank of 80% shading and at the end of the experiment in the 50% shading.

Annatto seed of the selected strain produces 3.5 to 6% by weight of carotenoids and has wide availability around the world representing about 70% of all pigments marketed (Fabri & Teramoto, 2015). Considering an economic study by Giuliani (2017) to estimate the fair market price of annatto seed at 5.7% bixin and the dollar price in Brazil (the world's largest annatto producer) in the year of the study, we observed that the value of pure bixin contained in the wholesale seed is $ 42.58 / kg. In the present study, the preparation of 1 kg of bixin, in the form of annatto extract, costs approximately $ 140.00. In contrast, astaxanthin, which is the main pigment marketed as a supplement for animal feed, has prices ranging from $ 2,500.00 to $ 7,000.00/kg depending on the degree of purity (Shah et al., 2016). Thus, even if high amounts of annatto are used (at least ten times higher) to replace astaxanthin sources, the bixin diet can be more affordable than those using astaxanthin as a carotenoid supplement.

Future studies test the pigmentary efficiency of annatto in different concentrations for *L. vannamei*, and it is important to verify the effect of different shading and concentrations of microbiota on the shell color of this Peneidae.

5. Conclusion

The inclusion of pigment in the diet was more efficient in coloring the shrimp than the primary production (algae) present in the biofloc. And higher shading apparently favored production conditions. In the conditions that simulate the immersion of the feed in the water, there were no significant losses of bixin before two hours of time. Being an interesting alternative to be used as an additive in shrimp feed. Considering the characteristics observed in this study, it is recommended the use of 80% shade in the cultivation of *Litopenaeus vannamei* in BFT and supplementation with annatto extract, 1,235mg/kg of bixin in the feed, during the last 14 days of cultivation, to obtain better results in the coloring of the final product with a positive effect on the reduction of ammonia in the culture tanks.

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