

Effect of Dietary Carotenoids on Reproducers of
Amazon River Prawn *Macrobrachium amazonicum*.
Part 1: Metabolism, Morphometric / Zootechnical
Indexes, Body Composition and Gametes

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Abstract

The inclusion of natural extracts and solution of synthetic astaxanthin in the *Macrobrachium amazonicum* diet were tested to verify their effect on performance, body indexes and total accumulation of astaxanthin in the reproductive tissues and gametes of this crustacean in comparison with newly captured wild animals. The experiment was randomized in blocks

(five treatments with three replicates). Four groups were submitted, during 20 days, to diets in recirculation tanks: control diet (CONT); diet containing natural extract of “buriti” (CAR); diet with inclusion of natural “urucum” extract (BIXN), diet with synthetic astaxanthin (ASTX) and NATURAL group (not fed with ration). 180 prawns were used, with 60 males (6.08 ± 1.96 g) and 120 females (4.55 ± 1.03 g) distributed in five groups containing four males and eight females each. There were no significant differences in performance and body indexes. The number of released spermatozoids, live spermatozoids, body and egg pigmentation was higher in BIXN and ASTX treatments. The ASTX treatment was superior to the NATURAL group in the body pigmentation of females and eggs and release of spermatozoids by males. These results demonstrate that the use of natural and artificial carotenoid pigments in the diet are beneficial for reproduction of *M. amazonicum*.

Keywords: buriti, urucum, astaxanthin, electroejaculation, reproductive performance, prawn culture

1. Introduction

Carotenoids are important food components for the metabolism of crustaceans (Meyers, 2000; Zhao et al., 2019). These pigments influence the overall development, influencing normal development, productivity, resistance to stress and survival (Niu et al., 2009; Aguirre-Hinojosa et al., 2012). According to Wade et al. (2017), carotenoids are indispensable for the perfect development of the reproductive characteristics of the crustaceans used in aquaculture, besides the improvement of the product, and subsequently quality and price.

Freshwater prawn genus *Macrobrachium* demonstrate differences in reproductive performance in response to the use of carotenoid-rich foods in their diet and improved their performance (Tizkar et al., 2014). Astaxanthin is the main pigment accumulated by crustaceans, it plays key roles in reproductive performance and reproductive precocity (Paibulkichakul et al., 2008) and for the development of embryonic and larval phases of *Macrobrachium* (Ribeiro et al., 2001).

The Amazon river prawn *Macrobrachium amazonicum* is one of the most produced species in the northern region of Brazil by artisanal fishing (Marques et al., 2012; Melo et al., 2020). Its natural occurrence is in the monsoon climate region of the Amazon. However, the species has already been introduced for commercial cultivation in several parts of South America (Silva et al., 2017).

Foods used in the Amazon region such as “buriti” *Mauritia flexuosa* and “urucum” *Bixa orellana* are rich in pigments (Saini et al., 2015). These pigments can promote positive effects on the crustacean species. Plant extracts can provide precursor carotenoids which have the potential to be converted into astaxanthin by crustaceans (Vernon-Carter et al., 1996; Arredondo-Figueiroa et al., 2003; Göcer et al., 2006; Aguirre-Hinojosa et al., 2012).

The objective of this study was to test the inclusion of natural extracts and synthetic astaxanthin in wild Amazon river prawn diet, verify its effect on performance, body indexes, and the total accumulation of astaxanthin in the reproductive tissues and gametes of this

crustacean compared to recently caught animals, and check if these pigments help reproductive efficiency in the period of adaptation to captivity.

2. Method

2.1 Experimental Design and Diets Formulation

The experiment was conducted in a 2 x 5 factorial design (two sexes and 5 treatments). A total of 180 adult prawns were distributed in five groups containing four males (6.08 ± 1.96 g) and eight females (4.55 ± 1.03 g) according to the diet: Group 1 - animals submitted to a control diet without inclusion of pigments (CONT); group 2 - animals submitted to a diet containing natural extract of “buriti” that has beta-carotene as the main pigment (CAR); group 3 - animals submitted to diet with natural “urucum” extract including bixin as the main pigment (BIXN); group 4 - animals submitted to diet with synthetic astaxanthin (ASTX) and group 5 - animals that were not submitted to any diet (NATURAL). Thus, a group representing the wild diet, two groups with low concentration of pigments and two groups with high concentration of pigments as described in Table 1. The pigments were included in the diets by spraying the extracts on the surface of the pellet.

The prawns were obtained from fishermen in January 2018, from the region of Macapá-AP (Lat: 00° 02' 20" N, Long: 51° 03' 59" W), Brazil, and taken to the Aquaculture & Fishery laboratory of the Agroforestry Research Center of Amapá, Embrapa-Amapá. The 36 prawns, divided into 3 repetitions, used to study the wild characteristics were readily analyzed according to the parameters of the study. The other 144 animals (in the treatments with experimental diets) were grown in twelve 60 liter tanks, consisting in four treatments with 3 replicates in each one. The recirculation system has a mechanical/biological filter and a water flow of 120 L per hour per tank.

Feed was provided for 20 days in the amount of 2 g per replica daily (3.3% of the average live weight) divided into two feeds, being 0.5 g in the morning (10:00 am) and 1.5 g in the evening (6:00 pm). The uneaten feed was collected and frozen daily (8:00 am) for subsequent drying in an oven at 60°C and determining the feed conversion and feed efficiency.

Table 1. Concentration of nutrients and pigments in dry matter (DM) of the experimental diets of Amazon river prawn

Composition analyzed	Experimental diets			
	CONT	CAR	BIXN	ASTX
Dry matter (DM) (%)	92.36	89.71	91.49	92.15
Crude protein (% in DM)	37.83	37.19	36.70	37.87
Ethereal extract (% in DM)	7.31	7.98	7.4	7.67
Crude fiber (% in DM)	4.43	3.46	5.74	4.03
Ashes (% in DM)	14.37	14.73	14.46	14.44
Non-nitrogenous extract (% in DM)	32.8	30.21	32.93	32.18
Calcium (% in DM)	1.88	1.41	1.96	1.68
Phosphorus (% in DM)	0.86	0.88	0.92	0.83
Carotenoids and total retinoids				
β-carotene (mg/kg)	15.71	38.09	-	-
Bixin (mg/kg)	-	-	289.71	-
Astaxanthin (mg/kg)	-	-	-	197.45
Retinol (mg/kg)	8.06	8.0	8.01	8.04

Experimental diets contain the following additives: CONT without pigment supplementation, CAR with “buriti” extract supplementation, BIXN with “urucum” extract supplementation and ASTX with synthetic astaxanthin supplementation.

In order to prepare the ethanol extracts, 100 g of each fruit (“buriti” and annatto) were crushed in a blender together with 96°GL ethanol, rested for 24 h at 30°C to extract the pigments. The extracts were separated from the fibrous residue by filter and their volume corrected for 100 mL. In the case of the synthetic astaxanthin, 2 g of the product were diluted in water (10 mL) and the rest of the volume completed with the same ethanol to be equal to the other extracts. Only the ethanol and water solution were reserved for the control group. The extracts were included in the diets by spraying them on the surface of the extruded

commercial feed pellets (38% Crude Protein) and dried for 8 h in an oven at 60°C. The ration was supplied in the amount of 2 g per replica daily (3.3% of live weight) divided into two rations, being 0.5 g in the morning (10:00 a.m.) and 1.5 g in the evening (06:00 p.m.).

For the pigment concentration analysis, one male and one female from each treatment ($n = 2$) were randomly sampled and dissected to separate the organs to be analyzed (stomach, hepatopancreas, and gonads).

The proximal composition of the foods (Table 1) was made based on the following methodologies: Dry matter (DM): drying in an oven at 105°C until constant weight, according to methodology of IAL (2008); Total crude protein (TCP): determined by the Kjeldahl method. The crude protein content was calculated by multiplying the total nitrogen by factor 5.46 ($\% N \times 5.46$) (Nogueira and Souza, 2005; IAL, 2008); Ethereal extract (EE): determined in Soxhlet apparatus with petroleum ether under reflux for 4 hours (Nogueira and Souza, 2005; Silva and Queiroz, 2006); Ashes (ASH): determined gravimetrically in muffle at 105°C/4h (Nogueira and Souza, 2005; IAL, 2008); Crude fiber (FB): Determined gravimetrically through the remaining residue of acid and alkaline digestion (Nogueira and Souza, 2005; Silva and Queiroz, 2006); Phosphorus (P): by complexation of phosphorus with vanadium-ammonium molybdate and determination by spectrophotometry in the visible region and determination of Calcium (Ca): complexometric titration with EDTA, according to IAL methodology (2008); Determination of non-nitrogenous extract (ENN) by difference = $100 - (PB + FB + EE + MM)$. The diets presented similar levels of retinol, which is important to avoid interference in the effects expected by carotenoids in crustaceans as described by Liñán-Cabello et al. (2002).

2.2 Water Quality Parameters

The water quality parameters: Temperature (T°C), hydrogenation potential (pH), dissolved oxygen (DO), electrical conductivity (EC), total dissolved solids (TDS), salinity (SAL) and potential of oxidation (PO) were measured with U-5000 multiparameter probe (HORIBA®, Kyoto, Japan). Nitrogen as total ammonia (TA-N), nitrogen as nitrite (NO₂⁻-N) and alkalinity (ALC) were determined using colorimetric kits and HI 83200 multi-parameter photometer (HANNA INSTRUMENTS®, Woonsocket, Rhode Island, USA).

The physicochemical variables of the water had the following mean values: temperature 27.45°C (± 0.47); pH 8.5 (± 0.42); dissolved oxygen 7.06 (± 1.14); electrical conductivity 0.45 (± 0.04); total dissolved solids 0.30 (± 0.03); salinity 0.2; total ammonia 0.12 (± 0.04); nitrite 0.09 (± 0.02); oxidation potential 205.45 (± 26.96) and alkalinity 43.49 (± 9.59).

2.3 Performance

2.3.1 Zootechnical Indexes

In addition to the percentage of survival (S), the zootechnical indexes were made based on the following equations:

Specific growth rate (SGR) - relative to daily growth of animals, percentage values. For calculation, the expression (1) was used:

$$SGR (\% /day) = \frac{\ln final\ weight\ (g) - \ln initial\ weight\ (g)}{time\ (days)} \times 100 \quad (1)$$

Apparent feed conversion (AFC) - equivalent to the amount of feed needed for the animal to gain 1 kg live weight (2):

$$AFC = \frac{consumed\ feed\ (g)}{final\ weight\ (g) - weight\ initial\ (g)} \quad (2)$$

Weight gain (WG) final average weight of animals in the tank subtracted final average weight (3):

$$GP = final\ weight\ (g) - initial\ weight\ (g) \quad (3)$$

Gain length (GL) final average length of animals in the tank subtracted final average length (4):

$$GL = final\ length\ (mm) - initial\ length\ (mm) \quad (4)$$

Relative condition factor (Kr) - indirectly measures the physiological state of the animal in relation to stored energy reserves, such as hepatic glycogen and body fat. Practically indicating if the animal is fat or lean according to the regression curve of the population mean (5):

$$Kr = observed\ weight\ (g) \div expected\ weight\ (g) \quad (5)$$

According to LE CREN (1951), expected weight, determined through the length weight curve obtained over the entire period, observed weight, obtained from the individual weighing of each organism.

2.3.2 Body Yield Indices

The calculation of body yield indices was made based on the following equations:

Gastrossomatic index (GaSI) equivalent to the weight of the stomach in relation to the prawn body. Described by equation (6):

$$GaSI = \frac{stomach\ weight\ (g)}{total\ body\ weight\ (g)} \times 100 \quad (6)$$

The gonadosomatic index (GSI) represents the proportion of the animal's gonads weight in relation to body weight. It should be measured separately between the sexes, since the gender defines considerable differences in this variable (7):

$$GSI = \frac{\text{weight of gonads (g)}}{\text{total body weight (g)}} \times 100 \quad (7)$$

Hepatosomatic index (HSI), variable that measures the proportion of the liver of the animal in relation to the total weight of the crustacean (8):

$$HSI = \frac{\text{liver weight (g)}}{\text{total body weight (g)}} \times 100 \quad (8)$$

Head off yield (HSY) is the proportion of marketable product as described without the crustacean cephalothorax, i.e., only the abdomen with its attachments. For its calculation it is used the individualized weighing of this part of the animal divided by the total weight (9):

$$HSY = \frac{\text{abdome weight(g)}}{\text{total body weight (g)}} \times 100 \quad (9)$$

Yield of fillet (clean prawn without the shell) (YSF) considered the abdominal part, excluding the carapace and annexes as pleopods (10):

$$YSF = \frac{\text{peeled weight (g)}}{\text{total body weight (g)}} \times 100 \quad (10)$$

2.3.3 Centesimal Composition of Animals

The proximal composition of the animals was obtained based on the following methodologies: Moisture (MOI): Drying in oven at 105°C until constant weight, according to IAL (2008); total lipids (TLP): determined in soxhlet apparatus with petroleum ether under reflux for 4 hours (Nogueira and Souza, 2005; Silva and Queiroz, 2006), Ashes (CNZ), Crude Protein (CP), Phosphorus (P) and Calcium (Ca) were analyzed according to the methodologies described in item 2.1.

2.3.4 Reproductive Performance of the Female

The spawning per female (SgF) were measured by daily observation of all females in each tank and the data were annotated for later statistical comparison by dividing the number of spawning by the initial number of females as in expression (11).

$$SgF = \frac{\text{total number of spawns}}{\text{initial number of females}} \quad (11)$$

In order to count the number of eggs per female weight (EFW), the egg count was carried out after laying the eggs mass, counting three individual plots and determining the average number of eggs per gram, then multiplying the value by the total mass of eggs and dividing by the

weight of the female as in expression (12). With this, we reduced the effect of the female's weight on the total amount of eggs produced to favor the analysis of the treatment effect.

$$EFW = \frac{\text{total number of eggs}}{\text{female weight (g)}} \quad (12)$$

To obtain a visual reference of the egg's color related to the concentration of pigment measured, the eggs were observed macroscopically by three trained observers and their subjective appearances under consensus of the three people annotated on the day of the spawning up to a maximum 12 hours after their occurrence.

2.3.5 Reproductive Performance of the Male

Two males from each repetition were used to collect material. For the spermatophore removals in the males, the electroejaculation was carried out in the prawns immobilized with rubber gums attached to a foam base, using up to three 9V shocks at the base of the fifth pair of pereopods and collecting with sterile forceps. The live sperm counts (LS) were performed according to the Leung-Trujillo and Lawrence (1987), using hematocytometer counts using hematoxylin-eosin as contrasts. Four groups of 100 spermatozooids were selected from each male and the numbers of live cells counted. Then, the individual mean of these four counts represented the sperm viability as stated in expression (13).

$$LS = \text{mean of 4 groups with 100 spermatozooids of each male} \quad (13)$$

In addition to this count, the number of spermatophores collections (SP) was measured in a final period of 24 hours after the experiment in the treatments with food and in the initial 24 hours for the NATURAL group, as in the expression (14).

$$SP = \text{average number of collections per replicate} \quad (14)$$

2.4 Analysis of Pigments and Retinol

The prawn tissues were macerated in 96°GL ethanol and analyzed in a SP220 UV spectrophotometer (Bioespectro®, Curitiba-PR, Brazil), at the wavelength of 478 nm absorbance (ABS) of astaxanthin and absorption coefficient E1% 1cm 2100 for calculating astaxanthin concentration (mg/kg) contained in the tissue (ACT). For the diets (Table 1), the same technique was used as the ASTX diet with the same values for the animals, for CONT and CAR, ABS of 452nm and absorption coefficient of E1% 1cm 2620 and in the diet BIXN, the ABS of 457nm and the absorption coefficient of E1% 1cm 3443. For the determination of retinol, ABS was used at 325 nm and the absorption coefficient E1% 1cm 1780, as in the methodology described by Dias et al. (2010). The calculation was made by the methodology of Rodriguez-Amaya (2001); Rodriguez-Amaya and Kimura (2004), following the equation (15):

$$\left[\text{mg} \frac{\text{pigment}}{\text{kg}} \right] = \frac{\text{ABS} \times \text{DF} \times \text{V}}{\text{CA} \times (\text{g})} \quad (15)$$

At where:

ABS = Absorbance of the pigment in the ethanol solution (nm)

DF = Dilution factor (= 1,000,000)

V = Volume of solution

CA = Coefficient of absorption E1% 1cm of the pigment in ethanol

(g) = grams of sample used

2.5 Statistical Analysis

The data (Mean and standard deviation) were initially submitted to the Shapiro-Wilk normality test. ANOVA Duncan's tests for the parametric variables and Kruskal-Wallis were used for the non-parametric variables, both at a significance level of 5%. The statistical analysis was done in the INFOSTAT program, version 2017 (Casanoves et al., 2012).

3. Results

3.1 Diet

The composition of the experimental diets is described in table 1, where the treatments, pigment additives and concentrations are identified. The other ingredients of the commercial ration were not altered.

3.2 Performance

3.2.1 Zootechnical Indexes

Table 2 shows that there were no significant differences ($p > 0.05$) among treatments for WG, GL, AFC, SGR and S.

Table 2. Zootechnical indexes (mean \pm standard deviation) of Amazon river prawn rations containing different pigment sources

Zootechnical indexes					
Groups	WG (g)	GL (cm)	AFC*	SGR (%)	S (%)
CONT	0.31 \pm 0.05	-0.07 \pm 0.22	8.40 \pm 1.44	1.54 \pm 0.23	86.16 \pm 4.81
CAR	0.37 \pm 0.14	0.06 \pm 0.05	7.66 \pm 2.95	1.87 \pm 0.71	83.33 \pm 14.43
BIXN	0.44 \pm 0.19	-0.01 \pm 0.14	6.44 \pm 2.28	2.19 \pm 0.94	94.44 \pm 4.81
ASTX	0.37 \pm 0.24	0.15 \pm 0.10	10.21 \pm 8.14	1.85 \pm 1.22	83.33 \pm 14.43

WG = weight gain, GL = gain in length, AFC = apparent feed conversion, SGR = specific growth rate, S = survival. There were no significant differences in the Duncan parametric test ($p > 0.05$) or in the variable marked with * by the non-parametric Kruskal-Wallis test ($p < 0.05$). Experimental diets contain the following levels of total carotenoids: CONT without supplementation of pigment (15 mg/kg) of beta-carotene, CAR with supplementation of “buriti” extract (38 mg/kg) of beta-carotene, BIXN with supplementation of “urucum” extract of bixin (298 mg/kg) and ASTX with supplementation of synthetic astaxanthin (197 mg/kg).

3.2.2 Body Yield Indices

Table A1 (in Appendix) shows that the GSI, GNI, HSI, HSY and YSF indices showed no significant differences between treatments ($p < 0.05$). Only a significant difference in GSI between the sexes was observed due to the inherent morphological differences of each sex. In the case of the condition factor (Kr), it was evidenced that the animals (grouped sex) submitted to treatments CONT, CAR, BIXN and ASTX presented higher mean values than the NATURAL diet group ($p < 0.05$). Analyzing each sex, Kr response was higher in females in the experimental groups ($p < 0.05$), which showed a significant difference in the treatments compared to the NATURAL group, while the males mean values did not show the same behavior ($p < 0.05$).

3.3 Centesimal Composition of Prawns

Table 3 shows that the M content in the prawn was higher in the control treatment (CONT) in relation to the other treatments ($p < 0.05$), although the TCP content in this treatment was higher than the treatments CAR and ASTX ($p < 0.05$). The treatment CONT still presented lower ASH and P indices in relation to the other groups ($p < 0.05$). The group of animals fed the NATURAL diet had the lowest EE values in relation to the animals fed with the rations ($p < 0.05$). The analyzed levels of Ca presented numerically superior results in the two treatments with more concentrated amounts of carotenoids (BIXN and ASTX) and in the NATURAL group ($p < 0.05$), in relation to the other treatments.

Table 3. Centesimal composition (mean \pm standard deviation) of the Amazon river prawn cultivated with different sources of pigments in natural matter

Groups	Composition					
	M (%)	TCP (%)	ASH (%)	TLP (%)	Ca (%)	P (%)
NATURAL	71.25 \pm 1.46 ^a	17.66 \pm 0.71 ^{bc}	4.67 \pm 0.08 ^b	2.34 \pm 0.11 ^a	1.39 \pm 0.28 ^c	0.28 \pm 0.04 ^b
CONT	74.38 \pm 0.44 ^b	18.36 \pm 1.27 ^c	2.43 \pm 0.38 ^a	3.51 \pm 0.22 ^b	0.77 \pm 0.24 ^{ab}	0.19 \pm 0.04 ^a
CAR	71.42 \pm 1.5 _a	16.04 \pm 0.09 ^{ab}	4.35 \pm 0.14 ^b	3.82 \pm 0.31 ^b	0.69 \pm 0.05 ^a	0.30 \pm 0.01 ^b
BIXN	72.04 \pm 1.46 ^a	16.83 \pm 0.67 ^{abc}	4.80 \pm 0.40 ^b	3.59 \pm 0.66 ^b	1.11 \pm 0.11 ^{bc}	0.33 \pm 0.05 ^b
ASTX	70.06 \pm 0.64 ^a	15.38 \pm 1.13 ^a	4.96 \pm 0.17 ^b	3.72 \pm 0.11 ^b	1.45 \pm 0.29 ^c	0.34 \pm 0.04 ^b

M = moisture, TCP = total crude protein, ASH = ash, TLP = total lipids, Ca = calcium, P = phosphorus. Different letters in the same column indicate significant differences by the Duncan parametric test ($p < 0.05$). Experimental diets contain the following levels of total carotenoids: CONT without supplementation of pigments (15 mg/kg) of beta-carotene; CAR supplemented with “buriti” extract (38 mg/kg) of beta-carotene; BIXN with supplementation of “urucum” extract (298 mg/kg) of “bixin” and; ASTX with supplementation of synthetic astaxanthin (197 mg/kg). The NATURAL group was evaluated after capture and only received feed from the natural environment.

3.4 Female Reproductive Performance

The female prawns, as described in table 4, did not present significant differences in reproductive performance in the tested SgF and EFW variables ($p > 0.05$). However, they showed visible differences in the external color of the eggs, with the females being submitted to the treatments CONT, CAR of ocher color similar to the natural diet group and, in the other animals, the color of the eggs was darker with brown shade in the treated animals BIXN and black in the ASTX group (Table 4).

Table 4. Reproductive indexes (mean \pm standard deviation) of Amazon river prawn females cultivated with different pigments in the diets

Treatments	SPF	E(g)F	Eggs Color
CONT	1.00 \pm 0.13	454 \pm 222	ocher
CAR	0.875 \pm 0.13	509 \pm 115	ocher
BIXN	1.125 \pm 0.13	530 \pm 125	brown
ASTX	0.875 \pm 0.13	509 \pm 180	black

SPF = spawns per female, E(g)F = eggs per gram of female. There were no significant differences between treatments ($p < 0.05$) by the Duncan test. CONT - without supplementation of pigments (15 mg/kg) of beta-carotene; CAR - supplemented with “buriti” extract (38 mg/kg) of beta-carotene; BIXN with supplementation of “urucum” extract (298 mg/kg) of bixin and ASTX with supplementation of synthetic astaxanthin (197 mg/kg).

3.5 Male Reproductive Performance

In the figure 1., ASTX treatment had a superior performance in the number of SP units in relation to the NATURAL and CONT groups of male prawns, while CAR and BIXN treatments, males presented intermediate results ($p < 0.05$). Figure 2. shows that the percentage of LS was lower in the control treatment (CONT) and intermediate in the CAR treatment in relation to the other groups ($p < 0.05$).

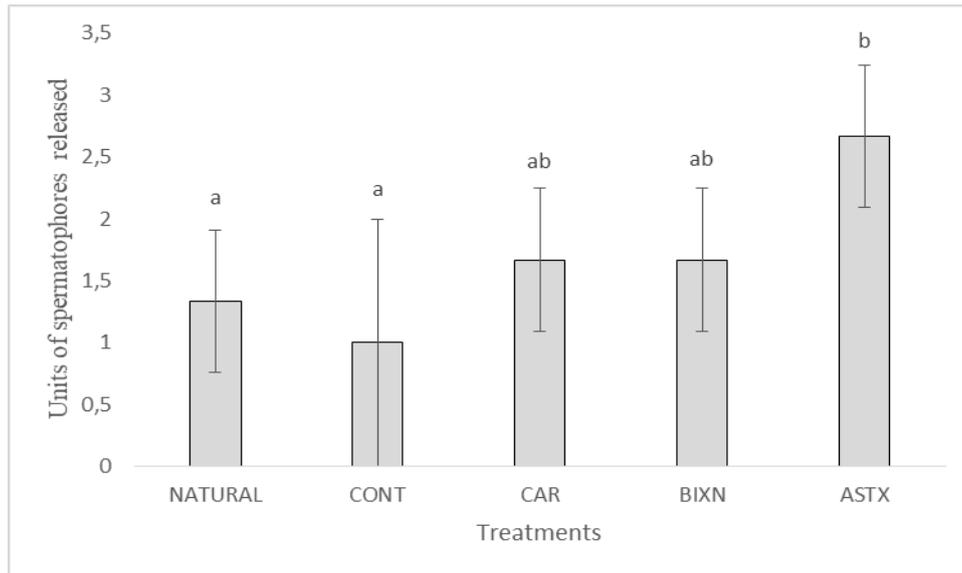


Figure 1. Number of spermatophores (SP) by Amazon river prawn males (n = 2) during 24 h post-capture (NATURAL) and after 20 days of culture in the other groups (mean ± standard deviation)

Different letters at the top of the columns indicate significant differences by the Duncan test (p<0.05). Experimental diets contain the following levels of total carotenoids: CONT without supplementation of pigment (15 mg/kg) of beta-carotene, CAR with supplementation of “buriti” extract (38 mg/kg) of beta-carotene, BIXN with supplementation of “urucum” extract (298 mg/kg) of bixin and ASTX with supplementation of synthetic astaxanthin (197 mg/kg). The NATURAL group was evaluated after capture and only received feed from the natural environment.

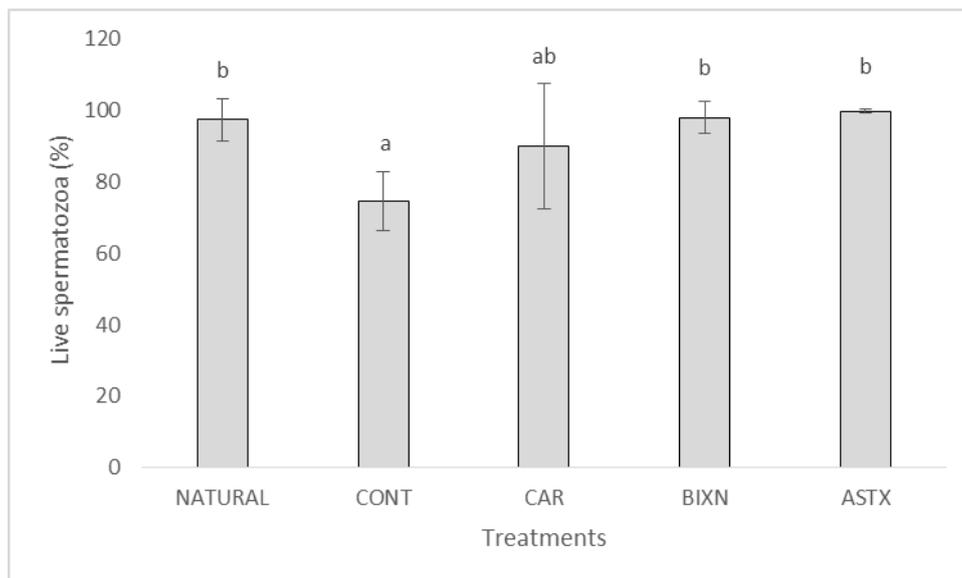


Figure 2. Percentage of live spermatozooids by Amazon river prawn males (n = 2) during 24 h post-capture (NATURAL) and after 20 days of culture in the other groups (mean ± standard deviation)

Different letters at the top of the columns indicate significant differences by the Duncan test ($p < 0.05$). Experimental diets contain the following levels of total carotenoids: CONT without supplementation of pigment (15 mg/kg) of beta-carotene, CAR with supplementation of “buriti” extract (38 mg/kg) of beta-carotene, BIXN with supplementation of “urucum” extract (298 mg/kg) of bixin and ASTX with supplementation of synthetic astaxanthin (197 mg/kg). The NATURAL group was evaluated after capture and received only feed from the wild environment.

3.6 Concentration of Pigments

The ACT in the female (Figure 3.) and males (Figure 4.) prawns varied according to the treatment used ($p < 0.05$). In the control treatment females (CONT), in which the feed was not supplemented with pigment additives. The astaxanthin accumulation was lower than the group of prawn females nourished with the NATURAL diet and the ASTX group, whereas the animals of the groups CAR and BIXN assumed intermediate values. The ASTX treatment alone took the largest ACT in both males and females ($p < 0.05$). The treatments CONT and CAR were shown to have low ACT values, while BIXN assumed an intermediate value. The ASTX treatment obtained the best ACT result among treatments with pigment addition. However, the NATURAL diet group still had a higher level of ACT in relation to CONT, CAR and BIXN9 ($p < 0.05$). The concentration of astaxanthin in the eggs of Amazon river prawn (Figure 5.) was higher in the treatments BIXN and ASTX, intermediate in the CAR treatment and lower in the control treatment CONT ($p < 0.05$).

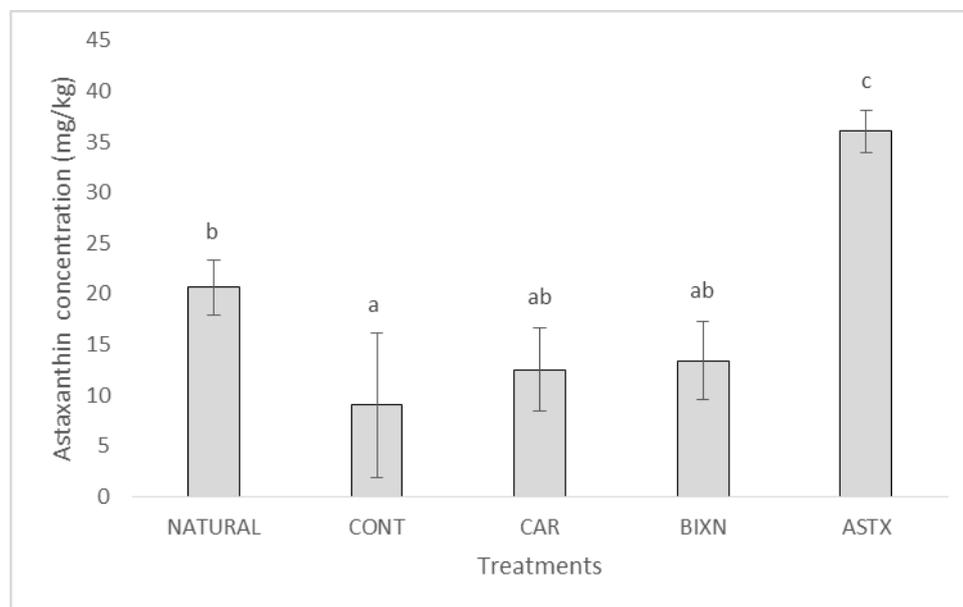


Figure 3. Concentration of astaxanthin in the body of Amazon river prawn females ($n = 1$) post-capture (NATURAL) and after 20 days of culture in the other groups (mean \pm standard deviation)

Different letters at the top of the columns indicate significant differences by the Duncan test ($p < 0.05$). Experimental diets contain the following levels of total carotenoids: CONT without supplementation of pigment (15 mg/kg) of beta-carotene, CAR with supplementation of

“buriti” extract (38 mg/kg) of beta-carotene, BIXN with supplementation of “urucum” extract (298 mg/kg) of bixin and ASTX with supplementation of synthetic astaxanthin (197 mg/kg). The NATURAL group was evaluated after capture and received only feed from the wild environment.

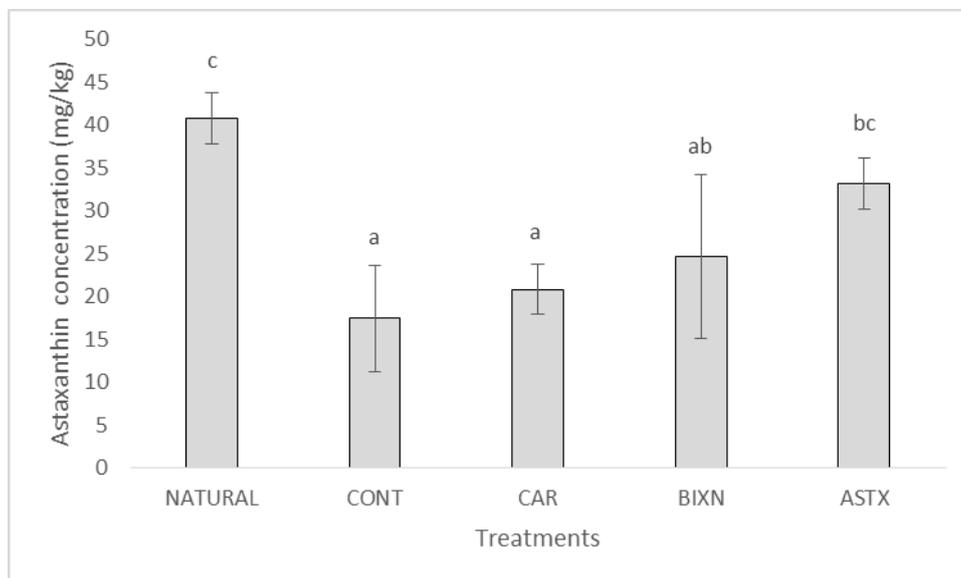


Figure 4. Concentration of astaxanthin in the body of Amazon river prawn males (n = 1) post-capture (NATURAL) and after 20 days of culture in the other groups (mean ± standard deviation)

Different letters at the top of the columns indicate significant differences by the Duncan test ($p < 0.05$). Experimental diets contain the following levels of total carotenoids: CONT without supplementation of pigment (15 mg/kg) of beta-carotene, CAR with supplementation of “buriti” extract (38 mg/kg) of beta-carotene, BIXN with supplementation of “urucum” extract (298 mg/kg) of bixin and ASTX with supplementation of synthetic astaxanthin (197 mg/kg). The NATURAL group was evaluated after capture and received only feed from the wild environment.

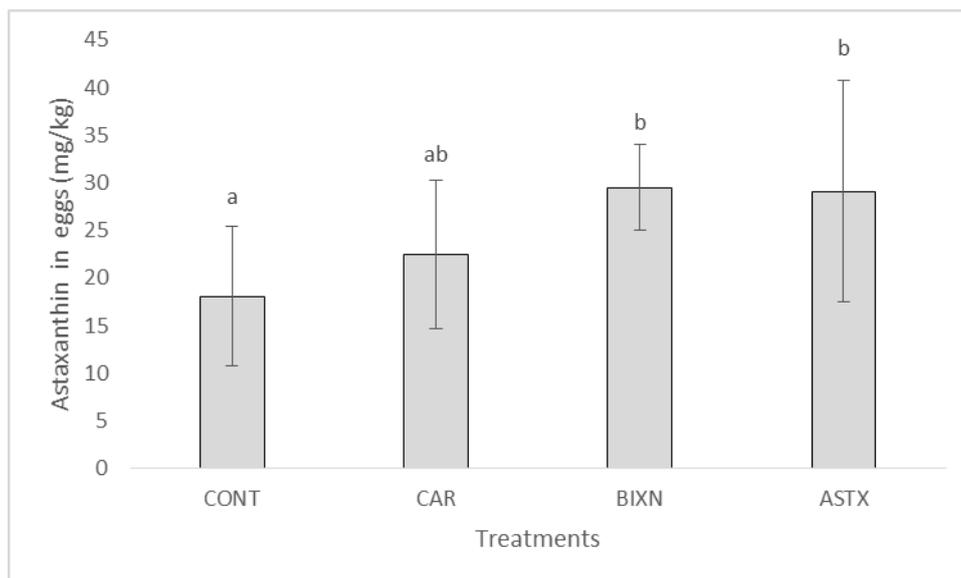


Figure 5. Concentration of astaxanthin in eggs of Amazon river prawn (n = 3) after 20 days of culture (mean \pm standard deviation). Different letters at the top of the columns indicate significant differences by the Duncan test ($p < 0.05$)

Experimental diets contain the following levels of total carotenoids: CONT without supplementation of pigment (15 mg/kg) of beta-carotene, CAR with supplementation of “buriti” extract (38 mg/kg) of beta-carotene, BIXN with supplementation of “urucum” extract (298 mg/kg) of bixin and ASTX with supplementation of synthetic astaxanthin (197 mg/kg).

4. Discussion

The water physicochemical values were in accordance with those found for cultivation of *M. amazonicum* in recirculation systems (Henares et al. 2015) and traditional aquaculture systems (Keppeler et al. 2015).

In this study, the activity of prawn exploring the environment and searching for food was concentrated at night. During the day, the animals took refuge in shelters and had little interest in the food provided, a behavior also reported by Fernandes (2016). Since it was possible in some cases to collect almost all the food at the time of cleaning the tank. For Santos et al. (2017), there are no differences in the development of *Macrobrachium carcinus* fed once or 2.5 to 6x per day. Goda et al. (2010) observed that two feeds per day for *Macrobrachium rosenbergii* provide greater development than 3 and 4x. In this way, feeding at night and collecting food waste in the morning would be efficient and would prevent degradation of water quality.

In early life forms of crustacean, increased levels of carotenoid pigments in the diet lead to greater WG, SGR and better AFC (Zhang et al., 2013), GL (Ghasem et al., 2018) and S (Niu et al., 2009). However, in the present study, the zootechnical indexes and body yield indexes tested did not present significant differences, most probably because the animals were in adulthood and in the reproductive period and by the time of study. According to Pangantihon-Kühlmann (1998), the prawn growth is reduced as these animals reach

reproductive maturity, allocating part of their reserves for reproduction. Regarding the condition factor (Kr), Rocha et al. (2015) suggest that factors such as time of year, food availability and reproductive condition influence the Kr of *M. amazonicum*. Thus, in spite of the time of the year that is the same, the fact of receiving a formulated diet and being in the reproductive period influenced positively the result mainly of the group of females fed with ration in relation to the group of prawn fed with NATURAL diet.

Kim et al. (2013) did not observe significant differences in protein and lipid content of juveniles of *Macrobrachium rosenbergii* raised with different levels of carotenoids. The differences in these levels in the animals may be related to the levels found in the diets that are heterogeneous due to the characteristics of the added extracts and their digestibility and species-specific effectiveness.

Yanar et al. (2011) found higher values of Ca and astaxanthin and lower values of P and EE in wild penaeid *Penaeus semisulcatus* (19.7 ± 0.21 g) than in the cultured animals. According to Milograna et al. (2010), the demand for ionic calcium (Ca^{2+}) in *Macrobrachium olfersii* is more accentuated in the groups with higher concentration of pigment, because it is necessary to use the ion in the process of intercellular transport. Possibly this is the reason for a higher level of this element present in the groups with greater body accumulation of the pigment and a lower level of P, ASH and M in the control group, since the tissue demand of Ca and P in *Macrobrachium* is interrelated and proportional (Wang et al., 2003).

Possibly increased pigment availability causes prawn physiological response by increasing mineral absorption to meet their need for transporters. The lower TLP level in the NATURAL diet group corroborates the Kr result since the two indexes are highly correlated as described by Herbing and Friars (1991), because Kr is a way to evaluate the energy reserves of aquatic animals. The higher level of TCP in the CONTROL treatment contrasts with the low relative levels of ASH, Ca, P and M in treatments with higher pigment values. The low mineralization in this treatment suggests that there may be a relationship between the levels of minerals and the consumption/accumulation of pigments.

Studies point out that, similarly to what happened with *M. amazonicum*, there are no differences in GNI (Liñán-Cabello et al. 2003), HIS (Ahmad et al. 2003) using pigments in the diet of other crustaceans. However, no studies were found that correlated GSI, HSY, YSF, with dietary supplementation of carotenoids for comparison. Thus, as *M. amazonicum* did not show differences in these body indexes, it is believed that if there are changes in these variables, they are related to other factors.

The eggs production of adult females was not influenced by the addition of pigments. It is believed that if feeding with pigments is made from the growth phase and for a longer period, significant effects occur at the precocity of the gonad's maturation and spawning time (Menasveta et al., 1994; Pangantihon-Kühlmann et al., 1998). In the case of males, diet pigments influenced the production of spermatophore and the survival of spermatozoid. According to Perez-Valazquez et al. (2003), the lack of astaxanthin in the diet impairs the production of spermatophores and the sperm quality of the prawn. There may be differences between the time of pigment administration and reproductive efficiency between the sexes.

This is because males produce their genetic material in approximately 24 hours and females in a few weeks (New et al., 2010).

For Braga et al. (2013), the supplementation of some carotenoids (astaxanthin precursors) may be efficient in improving the quality of the prawn spermatophore and spermatozooids. These authors still suggest differences in carotenoid requirements between males and females of the same species. This may therefore explain the different body concentrations of pigment as observed in the present study.

Body accumulation of astaxanthin was increased in treatments using synthetic astaxanthin and “urucum” extract. This is because astaxanthin has a higher pigmentation power, since it does not expend so much energy expenditure and is not required in large quantity for use by prawns (Arredondo-Figueiroa et al., 2003). However, bixin from “urucum” can be better exploited in view of a low-cost frequent feed for prawn farms in the Amazon as it is already used in aquaculture effectively (Fries et al., 2014; Dananjaya et al., 2017). This is due to the fact that bixin functions as a precursor to astaxanthin in the body of this prawn, which, like other organisms, converts this carotenoid to astaxanthin (Costa and Miranda-Filho, 2020).

The group with NATURAL food presented higher pigment reserves compared to CONTROL and CAR, probably due to the availability of pigment in foods consumed in the native environment. According to Quintana-López et al. (2019), shrimps *Litopenaeus vannamei* raised in an intensive system with commercial feed had an accumulation of astaxanthin in the hepatopancreas (the main storage site for this pigment), but lower than those collected in the wild. This is due to the availability of food from primary production origin rich in natural carotenoids. Phytoplankton are responsible for the synthesis of these carotenoids and their dispersion in the aquatic environment related to the level of sunlight that provides photosynthesis and development of pigments as a form of protection (Huang et al., 2017). Thus, in protected intensive systems where there is no direct incidence of light, as in the present study, the development of these organisms is compromised.

The accumulation of astaxanthin in prawn eggs, observed in the present study, by the addition of synthetic astaxanthin and bixin is desirable, since astaxanthin assists in increasing hatching rate (Maulana et al., 2017) and subsequently in increased survival, development and resistance to stress in prawn larvae (Wang et al., 2018). Rashidiyan et al. (2018) also associated the greater presence of astaxanthin in eggs with the larger diameter of them, the greater larval somatic index, the greater larval length in *Macrobrachium rosenbergii*. Although the amount of spawning of *M. amazonicum* was not influenced during the study period (20 days), longer periods of time such as 41 days (Maulana et al., 2017) and 61 days (Pangantihon-Kühlmann et al., 1998) showed better results for the spawning of crustaceans supplemented with pigments, suggesting that frequent use can contribute to their reproductive efficiency.

The color of the eggs shows that they take darker colorations when they receive higher concentrations of pigments and the accumulation of these pigments is more effective. Chang and Shih (1995) reported that the most accentuated coloration in *M. rosenbergii* eggs occurred due to the accumulation of vitellin, which is the main biomolecule of

lipo-glyco-carotenoprotein stored in the eggs of this crustacean in the vitelogenesis process. Being responsible for the embryonic nutrition process during incubation.

In general, the greater amount of body astaxanthin in prawn is indicative of greater potential antioxidant activity and greater accumulation of essential amino acids, such as glutamic acid, aspartic acid, lysine and leucine, which are components of the carotenoproteins, being responsible for the pigment fixation in the shell (Pattanaik et al., 2020). According to Long et al. (2017), the inclusion of pigments improves body composition, antioxidant activity and increases the color of the eggs of chinese mitten crab (*E. sinensis*) females. Ribeiro et al. (2001) observed in *M. olfersii* that the carotenoids contained in eggs are consumed in the catabolic processes during embryonic development, mobilizing nutrients to produce proteins, cellular membranes and by the consumption of the yolk of the vitellin reserves.

Considering the data from this study, it is possible to consider bixin as an interesting precursor to astaxanthin for prawn cultivation, especially for breeding, both in the maintenance of breeders and as a possible aid in gametes development. Annatto source of bixin is a widely used and commercialized agro-industrial product (Rivera-Madrid et al., 2016), which facilitates its potential use by the feed industry. New studies may explore the definition of the ideal doses of bixin in the cultivation of species of *Macrobrachium* and other crustaceans, as well as the effect of its inclusion in the diet of breeders and young forms from these treatments. It is also important to measure the animal's energy expenditure for converting this carotenoid to astaxanthin and the economic comparison of the cost of producing prawn using bixin compared to the synthetic astaxanthin which is traditionally used by the aquatic feed industry.

5. Conclusions

Feeding with commercial feed improves the condition factor of Amazon river prawn breeders, especially in the case of females. The addition of carotenoid pigments in the diet of these crustaceans provides greater production of spermatophores and sperm survival. Diets containing natural bixin and synthetic astaxanthin contribute to the accumulation of body astaxanthin in prawn and eggs. No difference was observed in the performance in tested animals with the different pigments but the use of these supplements contributed to the adaptation and maintenance of the reproductive characteristics of the species in captivity.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

Ethical approval

The study with invertebrate species does not require an ethical approval.

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Appendix

Table A1. Body indexes and relative condition factor (mean \pm standard deviation) of the Amazon river prawn cultivated with different pigment sources

Treatments	Body indexes				Condition factor		
	GSI (%)*	GNI (%)*	HSI (%)*	HSY (%)	YSF (%)	Kr*	
NATURAL	0.91 \pm 0.34	2.19 \pm 2.02	5.14 \pm 2.63	53.28 \pm 2.99	39.92 \pm 4.45	0.98 \pm 0.14 ^a	
CONT	0.79 \pm 0.26	1.67 \pm 2.14	3.63 \pm 1.33	50.65 \pm 5.76	37.71 \pm 7.44	1.03 \pm 0.12 ^b	
CAR	0.70 \pm 0.16	3.03 \pm 3.13	4.03 \pm 1.07	50.50 \pm 4.63	36.56 \pm 3.02	1.03 \pm 0.11 ^b	
Total	BIXN	1.05 \pm 0.59	2.58 \pm 3.57	3.40 \pm 0.90	48.81 \pm 2.47	35.96 \pm 3.51	1.05 \pm 0.11 ^b
	ASTX	1.06 \pm 0.74	2.79 \pm 3.13	3.65 \pm 1.24	51.41 \pm 2.99	37.54 \pm 3.91	1.06 \pm 0.13 ^b
	Males	0.78 \pm 0.18	0.52 \pm 0.21 ^a	4.37 \pm 1.84	51.53 \pm 3.30	37.91 \pm 4.02	1.02 \pm 0.13
	Females	1.24 \pm 0.55	4.39 \pm 2.66 ^b	3.57 \pm 1.20	50.32 \pm 4.31	36.92 \pm 5.01	1.00 \pm 0.14
	NATURAL	0.65 \pm 0.16	0.57 \pm 0.45	6.78 \pm 3.04	55.43 \pm 1.69	38.76 \pm 1.37	1.02 \pm 0.15
	CONT	0.82 \pm 0.29	0.52 \pm 0.28	4.23 \pm 0.94	48.28 \pm 2.10	36.78 \pm 3.99	1.01 \pm 0.10
Males	CAR	0.73 \pm 0.18	0.55 \pm 0.03	3.97 \pm 0.30	52.46 \pm 4.12	36.40 \pm 1.46	0.98 \pm 0.06
	BIXN	0.87 \pm 0.12	0.51 \pm 0.01	3.50 \pm 1.04	50.47 \pm 2.06	37.13 \pm 4.54	1.00 \pm 0.61
	ASTX	0.82 \pm 0.13	0.43 \pm 0.09	3.38 \pm 0.96	50.99 \pm 2.36	36.97 \pm 0.76	1.02 \pm 0.15
	NATURAL	1.16 \pm 0.24	3.80 \pm 1.48	3.50 \pm 0.18	51.05 \pm 2.24	35.38 \pm 5.34	0.95 \pm 0.13 ^a
	CONT	0.77 \pm 0.30	2.83 \pm 2.71	3.05 \pm 1.58	53.02 \pm 7.85	38.77 \pm 10.91	1.04 \pm 0.13 ^b
Females	CAR	0.67 \pm 0.18	5.51 \pm 2.46	4.09 \pm 1.66	48.28 \pm 5.03	36.71 \pm 1.46	1.06 \pm 0.12 ^b
	BIXN	1.22 \pm 0.88	4.65 \pm 4.35	3.30 \pm 0.96	47.15 \pm 1.64	34.79 \pm 5.10	1.07 \pm 0.12 ^b
	ASTX	1.40 \pm 0.76	5.15 \pm 2.79	3.90 \pm 1.64	51.82 \pm 0.56	38.12 \pm 3.02	1.05 \pm 0.15 ^b

GSI = gastsomomatic index, GNI = gonadosomatic index, HSI = hepatosomatic index, HSY = headless yield, YSF = fillet yield. Different letters in the same column in variables marked with * indicate significant differences by non-parametric Kruskal-Wallis test ($p < 0.05$). In the other variables there were no significant differences for the Duncan parametric test ($p > 0.05$). CONT - without supplementation of pigment (15 mg/kg) of beta carotene; CAR - with supplementation of buriti extract (38 mg/kg) of beta carotene; BIXN - with urucum extract supplementation (298 mg/kg) of bixin and; ASTX supplemented with synthetic astaxanthin (197 mg/kg). The NATURAL group was evaluated after capture and only received feed from the natural environment.

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