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Effect of dietary omega-3 polyunsaturated fatty acids supplementation of *Astyanax lacustris* males on semen quality

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This study aimed to verify whether the inclusion of omega-3 polyunsaturated fatty acids (PUFAs-ô3) in the diet of *Astyanax lacustris* males improves seminal quality. Four hundred fish were arranged in 20 boxes of 180 L in the recirculation system. These were fed with isoprotein diets (32% CP), until apparent satiation, with four levels of marine fish oil rich in PUFAs-ô3 inclusion (In% = 0, 3, 6, and 9), for 105 days. After, males were hormonally induced to spermiation, and semen was collected after 226 ATUs. Parameters evaluated were osmolality, seminal volume and color, spermatic concentration, sperm morphology, membrane integrity and, sperm motility (total and progressive motility, velocity curvilinear, straight line and average path, linearity and rectilinearity coefficients, trajectory oscillation, head lateral displacement amplitude, and cross-beat frequency). Seminal volume was greater in the In-0 and In-3 groups. Inclusion of PUFAs-ô3 positively influenced the kinetic parameters, as treatment with 6% and 9% of inclusion fish oil resulted in higher values for most of these parameters and did not differ statistically from each other. Thus, it is concluded that the addition of PUFAs-ô3 to the feed of breeders significantly improved the seminal quality of *A. lacustris* males.

Keywords: Fish sperm, Marine fish oil, Reproduction, Yellowtail lambari.

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Este estudo teve como objetivo verificar se a inclusão de ácidos graxos poliinsaturados ômega-3 (PUFAs-ô3) na dieta de machos de *Astyanax lacustris* melhora a qualidade seminal. Quatrocentos peixes foram dispostos em 20 caixas de 180 L no sistema de recirculação. Estes foram alimentados com dietas isoproteicas (32% PB) até aparente saciedade, com quatro níveis de óleo de peixe marinho rico em inclusão de PUFAs-ô3 (In% = 0, 3, 6 e 9), durante 105 dias. Após, os machos foram induzidos hormonalmente à espermiação e o sêmen foi coletado após 226 UTAs. Os parâmetros avaliados foram osmolalidade volume e cor seminal concentração espermática morfologia espermática integridade de membrana e motilidade espermática (motilidade total e progressiva velocidade curvilínea linha reta e trajeto médio coeficientes de linearidade e retilinearidade, oscilação da trajetória, amplitude de deslocamento lateral da cabeça e frequência de batida cruzada). O volume seminal foi maior nos grupos In-0 e In-3. A inclusão de PUFAs-ô3 influenciou positivamente os parâmetros cinéticos, pois o tratamento com 6% e 9% de inclusão de óleo de peixe resultou em valores mais elevados para a maioria desses parâmetros e não diferiram estatisticamente entre si. Assim, conclui-se que a adição de PUFAs-ô3 na ração dos criadores melhorou significativamente a qualidade seminal dos machos de *A. lacustris*.

Palavras-chave: Lambari-do-rabo-amarelo, Óleo de peixe marinho, Reprodução, Sêmen de peixe.

INTRODUCTION

The success of fish reproduction in captivity depends on the quality of the gametes. High-quality gametes are structurally well-formed, possess fertilization capacity, and generate living descendants (Valdebenito *et al*., 2015). In captivity, the production of these gametes can be controlled by environmental factors, such as photoperiod, water temperature, or spawning substrate; in addition, the nutritional and physiological conditions of the breeders, in particular, have a direct effect on the quality of the gametes and consequently on the performance of the fish's reproductive system (Bobe, Labbé, 2010; Mylonas *et al*., 2010, 2017). Studies carried out by Izquierdo *et al*. (2001), Watanabe, Vassallo-Agius (2003), Ling *et al*. (2006), Hachero-Cruzado *et al*. (2009), and Norambuena *et al*. (2012) demonstrated that nutrition influences reproductive parameters such as gonadal development, the quantity and quality of oocytes and sperm, and the quality of larvae produced, as the availability of essential biochemical components for gametogenesis and reproduction control can be affected by the nutritional status of the reproductives (Izquierdo *et al*., 2001; Norambuena *et al*., 2012).

One of the two main nutritional factors that significantly affect reproductive performance in fish is the content of essential fatty acids (EFA) in the diet (Watanabe *et al*., 1984). Lipids, mainly long-chain polyunsaturated fatty acids (LC-PUFAs) that include arachidonic acid (20:4 n-6, ARA), eicosapentaenoic acid (20:5 n-3, EPA), and docosahexaenoic acid (22:6 n-3, DHA), are required in the diet because they are eicosanoid precursors that are involved in various physiological processes, including

steroid production, gonadal development, and maintenance of membrane integrity (Jaya-Ram *et al.*, 2008).

LC-PUFAs are also important for other functions, such as controlling the reproduction and development of fish embryos and/or larvae, as well as improving sperm quality (Izquierdo *et al.*, 2001; Norambuena *et al.*, 2012). LC-PUFAs can also be synthesized from two polyunsaturated fatty acids (PUFAs), linolenic acid (18:3 n3, ALA) and linoleic acid (18:2 n-6, LA), also known as omega-3 and omega-6, respectively. These fatty acids are considered EFA because of the inability of fish to synthesize them in the body and must be provided in the diet (Izquierdo *et al.*, 2001; Norambuena *et al.*, 2012).

The positive effects of the addition of lipids to the diet on aspects of fish reproduction, either in improving the quality of semen and oocytes or in increasing the fecundity rate, hatching rate, and larval survival, have already been reported for several species, such as *Danio rerio* (Hamilton, 1822) (Jaya-Ram *et al.*, 2008), *Oreochromis niloticus* (Linnaeus, 1758) (Ng, Wang, 2011), *Colisa fasciatus* (=*Trichogaster fasciata* Bloch & Schneider, 1801) (Hossen *et al.*, 2014), *Oncorhynchus mykiss* (Walbaum, 1792) (Hajiahmadian *et al.*, 2016), *Acipenser baerii* Brandt, 1869 (Luo *et al.*, 2017), *Sparus aurata* Linnaeus, 1758 (Ferosekhan *et al.*, 2021), and *Cyprinus carpio* var. *koi* (Harshavardhan *et al.*, 2021). In the evaluation of seminal characteristics, the following parameters must be analyzed: seminal color and volume, rate and duration of sperm motility, sperm morphology and sperm concentration (Solis-Murgas *et al.*, 2011; Zhang *et al.*, 2017). Knowledge of the physiology of reproduction, together with the numerous studies of fish biology, has allowed the determination of management procedures that allow the induction of gonadal maturation of fish in captivity and, consequently, the artificial fertilization of oocytes, enabling the production of fish on a large scale, thus allowing the growth of the fish farming industry (Zaniboni-Filho, Weingartner, 2007).

The yellowtail lambari, *Astyanax lacustris* (Lütken, 1875) (Garutti, Britski, 2000), is a rustic species of small size, with a fast life cycle and high productivity in intensive cultivation because of its ease of handling, artificial feeding, and high prolificity. It is considered a model species, in addition to presenting great economic importance in the market of live fish and for human consumption (Sabbag *et al.*, 2011; Fonseca *et al.*, 2017; Brambila-Souza *et al.*, 2021). Therefore, this study aimed to evaluate whether the inclusion of omega-3 polyunsaturated fatty acids (PUFAs-ô3) in the diet of *Astyanax lacustris* would influence the seminal quality of breeders of the species.

MATERIAL AND METHODS

Experimental diets. An isoprotein diet (32% CP) was formulated (Tab. 1) and divided into four treatments with three levels of inclusion of purified marine fish oil (Campestre) containing high levels of PUFAs-ô3 (3, 6, and 9% in the diet) and a control diet without oil inclusion. To prepare the diets, the ingredients were ground, mixed, moistened and processed in an Exteec extruder, model Ex Micro, with a 3 mm die. The granules were dehydrated in a forced ventilation oven at 55 °C for 24 h. Afterward, the different amounts of marine fish oil were mixed in the feed. Before processing the feed, a bromatological analysis of all its ingredients was carried out (Laboratório de Bromatologia, UNESP/FEIS). The percentages of 1st and 2nd dry matter, percentage of crude protein by the

Kjeldahl method, percentage of fat by the Soxhlet method and percentage of ash, in addition to the gross energy determined in a Parr calorimetric bomb, were determined according to the AOAC (2000). The fatty acid profile of marine fish oil (Tab. 2) and the four diets was carried out at the Laboratório de Estudos de Fisiologia Animal (LEFISA), UNESP/FEIS; Laboratório de Metabolismo e Reprodução de Organismos Aquáticos (LAMEROA-USP) (Tab. 3). Feed processing was carried out at the Instituto de Pesca, located in the administrative region of São José do Rio Preto, São Paulo, Brazil.

TABLE 1 | Composition of experimental feed formulated for *Astyanax lacustris* breeders. (1) Mineral mix (Premix Raguife; Santa Fé do Sul, SP, Brazil) [Fe 20 g/kg; Cu 3,500 mg/kg; Zn 24 g/kg; I 160 mg/kg; Mn 10 mg/kg; If 100 mg/kg; Co 80 mg/kg; vitamin A 2,400,000 IU/kg; vitamin D3 600,000 IU/kg; vitamin E 30,000 IU/kg; vitamin K3 3,000 mg/kg; vitamin C 60 g/kg; vitamin B1 4,000 mg/kg; vitamin B2 4,000 mg/kg; vitamin B6 3,500 mg/kg; vitamin B12 8,000 mcg/kg; inositol 25 g/kg; choline 100 g/kg; Pantothenic Ac 10 g/kg; biotin 200 mg/kg; B.C. Folic 1,200 mg/kg; niacin 20 g/kg; antioxidant *etc 5,000 mg/kg]. (2) Based on the analysis of ingredient composition. (3) Nitrogen-free extract (NFE) = dry matter – (crude protein + lipid + mineral matter + crude fiber). In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3.

	Experimental diets			
Ingredients (%)	**In-0**	**In-3**	**In-6**	**In-9**
Soybean-45% PB. bran	31	32	32	32.5
Corn	22.27	24.37	17.97	14
Fish (tilapia). flour	15	15	15.3	15
Rice. grits	16	10.9	14	12.7
Corn. gluten	7	7	7	7
Wheat. bran	6	5	5	7.22
Marine fish oil	0	3	6	9
Dicalcium phosphate	1.5	1.5	1.45	1.4
Limestone	0.40	0.4	0.45	0.35
Premix[1]	0.50	0.5	0.5	0.5
BHT. antioxidant	0.30	0.3	0.3	0.3
Antifungal (Filax)	0.03	0.03	0.03	0.03
TOTAL	**100**	**100**	**100**	**100**
Composition[2]				
Dry Matter (%)	91.54	91.88	92.06	92.29
Crude Protein (%)	32	32	32	32
Ethereal Extract (%)	3.11	6.11	8.96	11.84
Mineral Matter (%)	7.12	7.15	7.15	7.17
Gross Fiber (%)	2.84	2.77	2.69	2.9
Non-Nitrogen Extractive[3] (%)	44.09	41.47	38.89	36.15
Gross Energy (cal/g)	3965.4	4135.77	4292.94	4463.51
Calcium	1.5	1.5	1.53	1.46
Phosphor	0.75	0.75	0.75	0.73
Lysine	1.9	1.91	1.92	1.92
Methionine	0.67	0.66	0.66	0.65

TABLE 2 | Fatty acid profile (% of total detected) of marine fish oil used in the composition of experimental diets for *Astyanax lacustris* breeders.

Fatty acids	Fish oil
C18:0	0.78
C18:1	1.12
C18:2n6	4.41
C18:3n3	31.92
C20:4n3	7.95
C20:5n3	8.98
C22:5n3	35.29
C22:6n3	9.54

TABLE 3 | Fatty acid profile (%) detected in the four diets fed to *Astyanax lacustris* breeders. In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3.

	Experimental diets			
Fatty acids	In-0	In-3	In-6	In-9
C14:0	1.65	1.54	1.67	1.94
C16:0	20.00	16.95	8.87	9.41
C16:1	3.04	2.92	4.50	3.34
C18:0	3.85	4.25	5.02	4.43
C18:1	31.42	6.32	6.68	5.74
C18:2n6 (LA)	34.36	16.38	7.82	7.10
C18:3n3 (ALA)	0.28	2.15	3.17	2.30
C18:3n6	1.82	7.41	4.50	1.16
C18:4n3	0.72	4.28	6.09	4.50
C20:2n6	0.00	1.56	0.35	0.96
C20:3n6	0.30	4.14	3.74	4.51
C20:4n6	0.66	8.14	7.66	5.07
C20:5n3 (EPA)	0.72	8.96	11.40	18.80
C22:5n3	0.14	1.71	1.91	2.39
C22:6n3 (DHA)	1.20	13.25	27.24	27.37
SAT	25.50	22.74	15.56	15.78
MUFA	34.47	9.24	11.18	9.08
PUFA	40.18	67.99	73.88	74.16
PUFAn6	37.13	37.64	24.07	18.80
PUFAn3	3.05	30.35	49.81	55.36

Experimental design. We used 400 males of *A. lacustris* two months old, with an average weight of 2.08 ± 0.44 g and average total length of 5.4 ± 0.39 cm. These were distributed in 20 polyethylene boxes of 180 L arranged in a recirculation system, with a density of 20 fish per box. The experiment was conducted in a completely randomized design with four treatments and five replications, totaling 20 experimental units. The treatments consisted of four levels of inclusion of PUFAs-ô3: In-0 (GC) = 0%, In-3 = 3%, In-6 = 6% and In-9 = 9%. Fish were fed twice a day (9 am and 5 pm) for 105 days until apparent satiation. During the entire experimental period, the following physicochemical variables of the water of the four treatments were monitored: pH, temperature, dissolved oxygen, and electrical conductivity measured every day in two periods in the morning and afternoon using a multiparameter sensor (ASKO - AK88v2). Total ammonia and nitrite levels were measured three days per week (Alcon Labcon Test Kit) (Tab. 4).

Semen collection. After 105 days, *A. lacustris* males were hormonally induced using Ovopel® (20 µg of GnRHa + 10 mg of metoclopramide [dopamine antagonist]) in a single dose (3 mg/kg of live fish) (Yasui *et al*., 2015). After 226 accumulated thermal units (226 ATUs) (Carneiro-Leite *et al*., 2020), the males were anesthetized with 1% benzocaine solution (Sigma - Aldrich E1501), and the semen was extruded using abdominal massage in the anteroposterior direction of the body and collected with the aid of micropipettes of 10–100 µL (Kasvi-K1-100B). Contamination of the semen with blood, feces and urine was carefully avoided through visual observation, for which a pre-assessment of the semen under microscopy was carried out to also verify if there was spermatic activation.

Semen analysis. To verify the influence of the inclusion of marine fish oil on the quality of *A. lacustris* semen, the following parameters were analyzed: duration of motility, considering the activation of spermatozoa to the observation of 10% motile spermatozoa; seminal volume, considering until the moment of ejaculation stop or observation of contamination with blood; and sperm concentration (spermatozoa/mL), measured using a Neubauer-type Hematimetric Chamber, for which the semen was diluted in formalin-saline solution in a proportion of 1:1000, respectively (Ninhaus-

TABLE 4 | Water quality variables recorded during the 105-day experimental period. In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3.

	Treatments			
Variable	In-0	In-3	In-6	In-9
Temperature (°C)	28.5±2.0	28.4±1.9	28.6±1.9	28.5±1.9
pH	7.9±0.3	7.8±0.3	7.7±0.3	7.8±0.2
Dissolved oxygen (mg L^{-1})	4.9±2.5	5.2±2.4	5.6±1.1	4.9±2.3
Total ammonia (ppm)	0	0	0	0
Electric conductivity (µS cm^{-1})	334±61.3	321±27.5	328±17.0	336±31.7
Nitrite (µg L^{-1})	0	0	0	0

Silveira *et al*., 2006). The osmolality of the seminal plasma was also determined, for which the semen was centrifuged at 3,000 rpm for 15 min, and the supernatant was collected and analyzed in an osmometer (OSMOMAT model 030, Berlin, Germany). The semen was characterized in terms of transparency, viscosity and color, compared to clean water and color patterns, but these parameters were not used for qualitative comparison between treatments Motility parameters were evaluated using a CASA system (ISAS® Integrated Semen Analysis System, Proiser, Valencia, Spain) coupled to a UB200i phase contrast microscope (UOP/Proiser) with an objective of 10x negative phase contrast. The images were captured with an ISAS 782C camera (Proiser, Spain) and processed with CASA software using 50 frames per second (fps). Semen was activated by adding 30 µl of distilled water to 0.5 µl of semen in a Makler™ camera (Sefi Medical Instruments Ltd, Israel), and an analysis was performed after 10 s of activation. Total motility (MOT, %), Progressive motility (PRG, %), rapid sperm (SptzFast, %), curvilinear velocity (VCL, µm/s), straightlinear velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness coefficient (STR, %), mean oscillation of the spatial trajectory (WOB, %), amplitude of lateral displacement of the head (ALH, µm), crossbeat frequency (BCF, Hz). Sperm with VAP < 10 µm/s were considered immobile, with velocity > 25 µm/s = medium sperm and velocity > 50 µm/s fast sperm. All analyses were performed for four treatments: In-0 (GC), In-3, In-6 and In-9.

The integrity of the sperm membrane was measured using the eosin-nigrosin staining method, for this analysis the spermatozoa were stained in the proportion 1:10:10 (semen:eosin:nigrosin). Using a light microscope, 200 spermatozoa per slide were considered alive when they remained colorless, indicating an intact membrane, or dead when stained pink, indicating a ruptured membrane, according to Lopes *et al*. (2018). Five animals were used per box per treatment.

In addition to the analyses mentioned above, the morphological normality of the spermatozoa was also evaluated. For this, the semen of five animals per treatment was fixed in formalin-saline solution at a proportion of 1:1000 (semen: fixative), stained with rose bengala, in the proportion of 1:10 (dye: semen), and 10 µL was deposited on a glass slide and covered with a coverslip (Streit-Junior *et al*., 2008). On each slide, 100 spermatozoa were analyzed, and the analysis was performed under optical microscopy (Zeiss/AXIOCAM-MRc5), at 1000X magnification. Spermatozoa were classified as normal or damaged, with damage classified as primary and secondary alterations, according to Miliorini *et al*. (2011).

Statistical analysis. Analysis of variance ($p<0.05$) was applied to the data, the Tukey test was applied for parametric data, and the Kruskal-Wallis test was applied for non-parametric data. The statistical program R Studio was used for data analysis. To the boxes were considered as an experimental unit.

RESULTS

The semen had a whitish to yellowish color and a slightly viscous appearance, regardless of the treatment considered. Regarding the seminal volume, the treatments with 6% and 9% of oil inclusion provided the smallest semen volumes, with a decrease of 40 and 45% respectively in relation to the control treatment (In-0). Sperm motility time for the In-9 treatment showed significantly lower values between treatments (Tab. 5). For seminal osmolarity, sperm concentration, and membrane integrity, no statistical differences were observed between the treatments (Tab. 5). No significant difference was found for the percentage of primary and secondary morphological alterations, but the In-0 (GC) treatment was the one that presented the highest values for both, in addition to having a smaller amount of normal spermatozoa, with the treatment In-6 being what showed a higher percentage of normal spermatozoa (Tab. 6).

TABLE 5 | Seminal parameters of lambaris (*Astyanax lacustris*) fed with the inclusion of omega-3 polyunsaturated fatty acids (PUFAs-ô3) in their diet, for 105 days. Different letters indicate statistical difference between treatments (Kurskal-Wallis, Tukey, $p<0.05$). In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3.

Parameters	Treatments			
	In-0	In-3	In-6	In-9
Seminal Volume (µl)	42.5±32.1 A	40.2±18.3 A	25.5±14.0 AB	23±14.8 B
Motility Duration (s)	40.3±7.1 AB	41.3±6.0 AB	45.1±7.4 A	36.4±4.0 B
Osmolality (mOsm)	300±0.0 A	286±0.0 A	277±0.0 A	290±0.0 A
Sperm Concentration (sptz/µl)	$11.5\pm5.3x10^{9}$ A	$10.3\pm3.9x10^{9}$ A	$10.7\pm3.1x10^{9}$ A	$11.4\pm4.5x10^{9}$ A
Membrane Integrity (%)	94.6±11.1 A	98.2±1.9 A	99±2.2 A	99.2±1.4 A

TABLE 6 | Analysis of the morphological normality of spermatozoa from fresh semen of *Astyanax lacustris* fed with the inclusion of omega-3 polyunsaturated fatty acids (PUFAs-ô3) in their diet, for 105 days. In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3.

	Treatments			
	In-0	In-3	In-6	In-9
Primary damages	44.6±20.5	32±6.7	32.6±9.3	38.6±10.7
Secondary damages	19.4±3.8	14.4±6.8	10.6±4.6	13.4±12.7
Total Defects (%)	64±11.6	47.8±8.7	43.2±7.3	52±6.8
Normal (%)	36±12.7	52.2±9.2	56.8±8.7	48±15.8

For the MOT and PRG, a significant increase was observed with the inclusion of PUFAs-ô3, with an emphasis on 9% of oil inclusion, which presented higher values that differed significantly from these two parameters of In-0 (Fig. 1; Tab. 7). For the parameters VCL, VSL, VAP, number of fast sperm, LIN, STR, and WOB, treatments In-6 and In-9 provided significantly higher values than In-0 and In-3 (Figs. 2–3; Tab. 7). The treatments with greater amounts of oil inclusion (In-6 and In-9) resulted in higher values for the ALH and BCF, which statistically differed from the In-0 and In-3 treatments. (Figs. 4A–B).

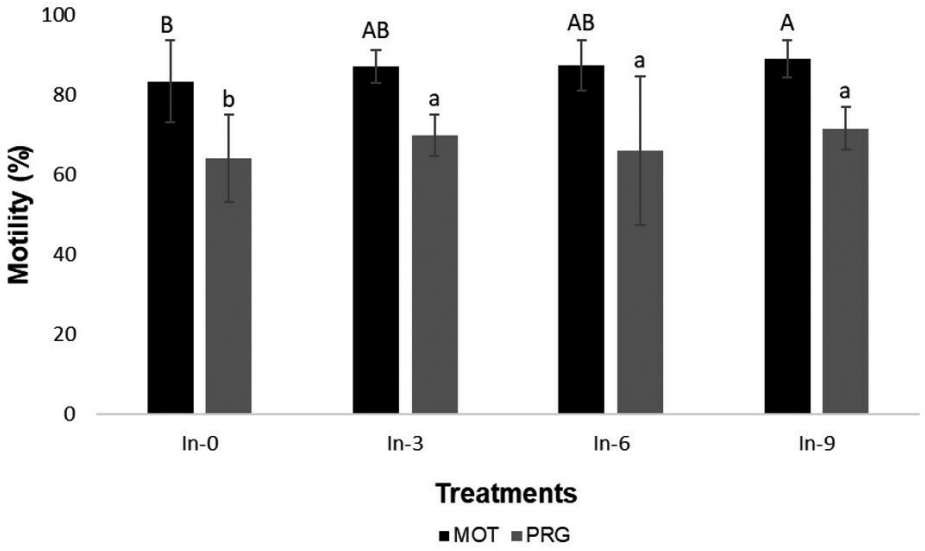

FIGURE 1 | Results obtained for total (MOT, %) and progressive (PRG, %) sperm motility after feeding with the inclusion of PUFAs-ô3 in the diet of *Astyanax lacustris* for a period of 105 days. In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3. Different capital letters indicate statistical difference between treatments for MOT. Different lowercase letters indicate statistical difference between treatments for PRG (Kruskal-Wallis, $p<0.05$).

TABLE 7 | Variation of sperm kinetic parameters of broodstock treated with marine fish oil in relation to control. Total motility – MOT; Progressive motility – PRG; Rapid sperm – SptzFast; Curvilinear velocity – VCL; Linear velocity – VSL; Average velocity – VAP. In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3; I – percentage increase compared to control.

Treatments	In-0	In-3		In-6		In-9	
Cinetic parameters	Total	Total	I (%)	Total	I (%)	Total	I (%)
MOT. (%)	83.4	87.2	4.5	87.5	4.8	89.1	6.8
PRG. (%)	64.2	69.9	8.8	66.0	2.7	71.6	11.5
SptzFast. (%)	15.9	19.2	20.5	77.9	389.2	80.2	403.5
VCL. (µm/s)	46.4	48.2	3.8	79.5	71.2	79.6	71.4
VSL. (µm/s)	35.2	36.9	4.5	79.5	125.5	68.2	93.4
VAP. (µm/s)	34.9	42.6	21.8	68.6	96.2	75.8	116.7

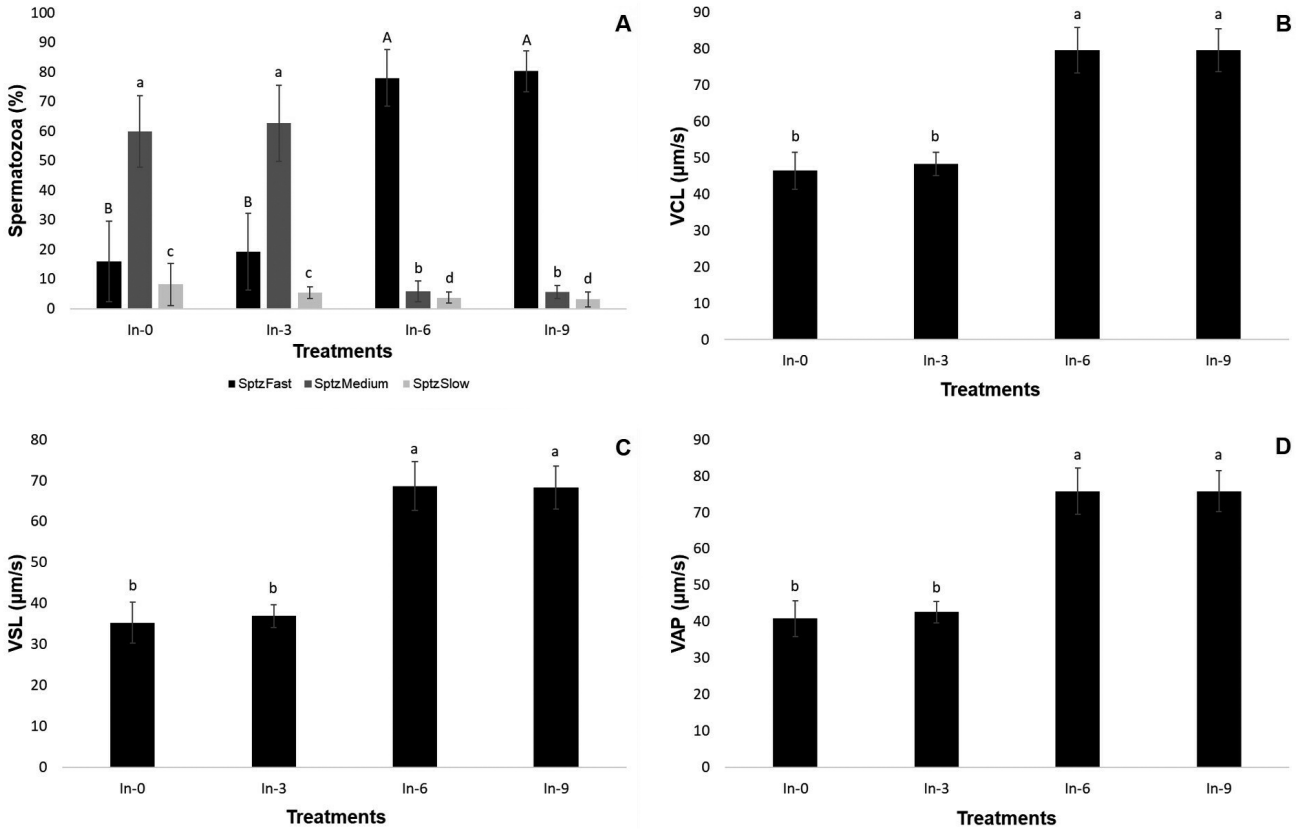

FIGURE 2 | Results obtained for **A.** Percentage of fast (SptzRápido), medium (SptzMedium) and slow (SptzSlow), **B.** Curvilinear velocity (VCL, µm/s), **C.** Linear velocity (VSL, µm/ s), **D.** Mean velocity (VAP, µm/s%) after feeding with the inclusion of PUFAs-ô3 in the diet of *Astyanax lacustris* for a period of 105 days. In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3. Different capital letters indicate statistical difference between treatments for SptzFast. Different lowercase letters indicate statistical difference between treatments for SptzMedium, SptzSlow percentage, VCL, VSL and VAP (Kruskal-Wallis, $p<0.05$).

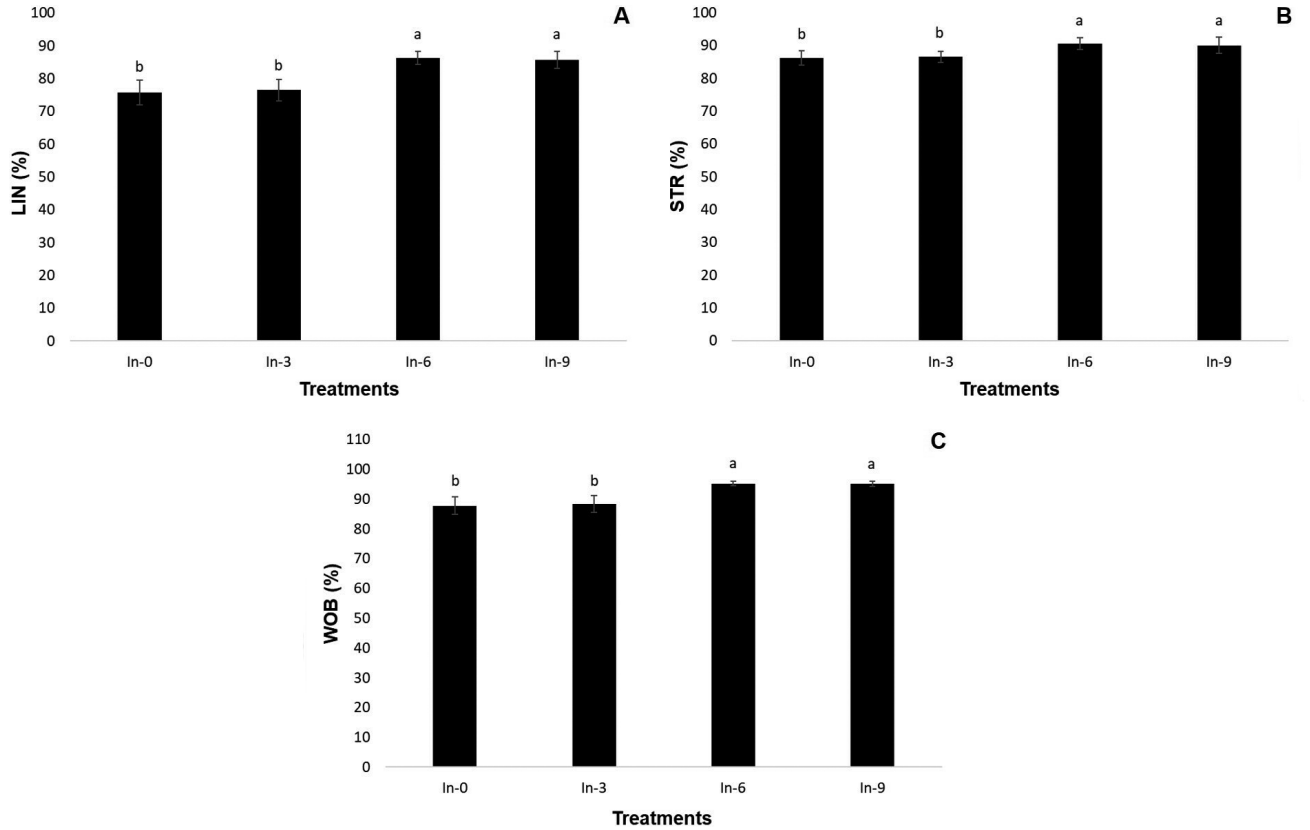

FIGURE 3 | Results obtained for **A.** Linearity (LIN, %), **B.** Straightness coefficient (STR, %) and **C.** Mean oscillation of the spatial trajectory (WOB, %), after feeding with the inclusion of PUFAs-ô3 in the diet of *Astyanax lacustris* for a period of 105 days. In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3. Different lowercase letters indicate statistical difference between treatments for LIN, STR and WOB (Kruskal-Wallis, $p<0.05$).

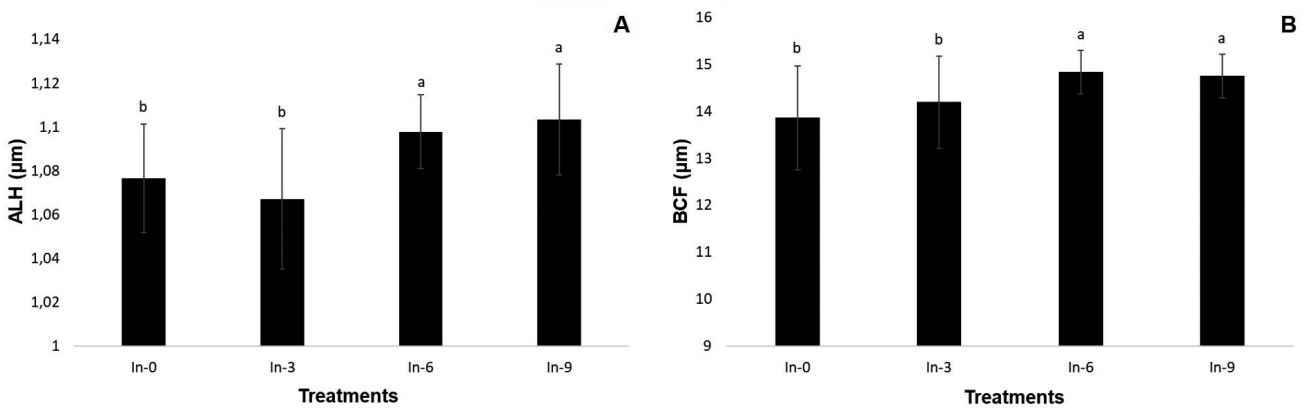

FIGURE 4 | Results obtained for **A.** Head lateral displacement amplitude (ALH, µm) and **B.** Crossbeat frequency (BCF, Hz), after feeding with the inclusion of PUFAs-ô3 in the diet of *Astyanax lacustris* for a period of 105 days. In-0 – diet without inclusion of PUFAs-ô3; In-3 – feed with inclusion of 3% PUFAs-ô3; In-6 – feed with inclusion of 6% of PUFAs-ô3; In-9 – feed with inclusion of 9% PUFAs-ô3. Different lowercase letters indicate statistical difference between treatments for ALH and BCF (Kruskal-Wallis, $p<0.05$).

DISCUSSION

The quality of gametes is essential for obtaining high rates of fertilization and hatching in the reproductive process and is considered a limiting factor for reproductive success (Gallego *et al*., 2013; Köprücü *et al*., 2015). Sperm motility can be evaluated and measured using parameters related to the fertilizing capacity of sperm, including total and progressive sperm motility, speed of sperm movement, sperm concentration, presence of abnormalities, morphological changes in gametes, and integrity of the plasma membrane (Fauvel *et al*., 2010; Gallego *et al*., 2013).

Studies have shown that introducing higher amounts of PUFAs into fish diets improves semen quality (Köprücü *et al*., 2015; Yonar *et al*., 2020). According to Lahnsteiner *et al*. (2009), lipids are the main energy resources of the sperm in salmonids and are important for maintaining sperm viability. This is noticeable in our work with *Astyanax lacustris*, as many of the parameters used to evaluate seminal quality showed a positive increase with the introduction of marine fish oil into the diet of breeders.

In the present study, treatments with higher oil inclusion (In-6 and In-9) had lower seminal volume when compared to the control group (In–0) and In-3; however, for the species *Dicentrarchus labrax* (Linnaeus, 1758) (Asturiano *et al*., 2001) and *Oncorhynchus mykiss* (Köprücü *et al*., 2015), the supplementation with PUFAs-ô3 resulted in greater seminal volume when compared to the control, which can be considered a result of the difference in the physiology of the referred species; these have different reproductive cycles and live in colder environments, as well as the need for a refinement of studies to determine the most adequate nutritional conditions for the inclusion of PUFAs-ô3.

As observed in *Oncorhynchus mykiss* (Köprücü *et al*., 2015) and *Sparus aurata* (Ferosekhan *et al*., 2021), the duration of sperm motility was increased by the inclusion of PUFAs-ô3 in the diet of breeders of these species, corroborating the data obtained in our experiment with *A. lacustris*. However, in the present study, the results showed that for *A. lacustris*, the inclusion of limit oil was 6% because, with the highest inclusion (9%), the duration of motility was significantly reduced. Regarding the parameters of sperm concentration and integrity of the sperm plasma membrane, the inclusion of marine fish oil had no positive or negative influence, since the number of spermatozoa per volume of semen is a species-specific characteristic not connected to nutritional supplementation and, in this case, the high level of sperm membrane integrity must be related to the good genetics of the specimens used.

As for the presence of gametes without formation deformities, there was no significant change considering the inclusion or absence of PUFAs-ô3 in the diet. This result was also observed for *Rhamdia quelen* (Quoy & Gaimard, 1824) (Rodrigues *et al*., 2022) with dietary supplementation with different sources of PUFAs. What can be highlighted is that, although no statistical difference was observed, GC (0%) provided a lower percentage of normal spermatozoa, which can be conjectured that despite not having a significant improvement, PUFAs-ô3 influenced cell formation.

The effect of PUFAs on motility was also observed in *Oncorhynchus mykiss* (Köprücü *et al*., 2015), where the use of a diet rich in n-3 polyunsaturated fatty acids increased motility compared to controls (no addition of PUFAs-ô3). Butts *et al*. (2015) found a similar effect for *Anguilla anguilla* (Linnaeus, 1758) semen; the addition of polyunsaturated fatty acids (EPA, DHA, and ARA) in the feed of breeders resulted in a significant increase in sperm motility. With a more sensitive motility analysis methodology, we corroborated

the above reports with other fish species in which the inclusion of PUFAs-ô3 provided an increase in sperm motility and, more specifically, in our experiment with *A. lacustris* MOT and PRG.

The sperm kinetic parameters VCL, VSL, and VAP were also positively affected by the diet with 6% marine fish oil, corroborated by Luo *et al*. (2017), who found that diets containing higher amounts of PUFAs resulted in increased sperm kinetics in *Acipenser baerii*. This is an important effect because these kinetic parameters have been used as indicators of sperm quality and are highly correlated with fertilization capacity because they allow sperm to find and penetrate the micropyle faster (Figueroa *et al*., 2016; Gallego *et al*., 2017; Leite *et al*., 2018). In addition, spermatozoa need energy to move, and the improvement of these parameters in treatments with greater oil inclusion may be related to greater mitochondrial β-oxidation for energy generation, due to the greater availability of PUFAs in these treatments, which can be used for the production of ATP through oxidative phosphorylation (Mansour *et al*., 2003; Díaz *et al*., 2021).

According to Beirão *et al*. (2011), spermatozoa from fish with a more linear trajectory had the highest correlation with fertilization rate, demonstrating that this characteristic is one of the important parameters to be taken into account in the determination of seminal quality in fish. In our experiment, treatments with higher oil inclusion (In-6 and In-9) increased the percentage of fast sperm and the kinetic parameters LIN, STR, WOB, ALH, and BCF, further corroborating the idea that PUFAs are beneficial for the improvement of *A. lacustris* sperm quality.

Another interesting kinetic parameter to consider is ALH, considered a good indicator of sperm maturation (Kowalski *et al*., 2006; Król *et al*., 2009). In our experiment, treatments In-6 and In-9 provided significantly higher values for ALH, which may indicate that the sperm of these treatments will provide greater seminal quality and, consequently, are more suitable for fertilization.

Thus, we can conclude that the inclusion of purified marine fish oil containing high levels of PUFAs-ô3 brought benefits to the seminal quality of *A. lacustris*. The In-6 and In-9 treatments improved the evaluated seminal parameters most efficiently. However, considering that the differences between the evaluated parameters are subtle between the two treatments, as well as the volume of oil to be used, we consider that In-6 is the best treatment for improving the seminal quality of *A. lacustris*.

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AUTHORS' CONTRIBUTION

Laicia Carneiro-Leite: Conceptualization, Data curation, Investigation, Methodology, Writing-original draft, Writing-review and editing.

Lorena Pacheco da Silva: Formal analysis, Methodology.

Hellen Buzollo Pazzini: Conceptualization, Formal analysis, Methodology.

Stella Indira Rocha Lobato: Investigation, Methodology

Laís Pedroso Borges: Investigation, Methodology

Yasmin dos Santos Reis: Investigation, Methodology.

Luciane Gomes-Silva: Investigation, Methodology

Cristiéle da Silva Ribeiro: Data curation, Investigation, Formal analysis, Methodology.

Rosicleire Veríssimo-Silveira: Methodology, Resources, Visualization.

Alexandre Ninhaus-Silveira: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Writing-review and editing.

ETHICAL STATEMENT

The experiment was carried out at Universidade Estadual Paulista "Júlio de Mesquita Filho," Campus de Ilha Solteira, at the Laboratório de Ictiologia Neotropical (LINEO). All technical procedures used in this study were approved by the Ethics Committee on the Use of Animals (CEUA) of the Faculdade de Engenharia – Campus de Ilha Solteira in the process CEUA - 12/2019 FEIS/UNESP, the animals used in this project were not collected from the wild, so there was no need to obtain a license from SISBIO.

COMPETING INTERESTS

The author declares no competing interests.

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