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Effect of dietary protein:lipid ratio on growth and body composition in bullfrog (*Lithobates catesbeianus*)

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ABSTRACT - A feeding trial was performed to assess dietary protein:lipid ratios for the grow-out phase of the bullfrog (*Lithobates catesbeianus*). Nine balanced isoenergetic diets were formulated, combining three different protein levels (300, 400, and 500 g kg^{-1}) with three different lipid levels (50, 100, and 200 g kg^{-1}), designated as P300/50L, P300/100L, P300/200L, P400/50L, P400/100L, P400/200L, P500/50L, P500/100L, and P500/200L. Additionally, a commercial fish feed, commonly used in Mexico to feed bullfrogs, was also tested during the experiment. Growth performance, animal performance parameters, carcass composition, and fatty acid profiles in muscle and liver were evaluated. The feeding trial results showed that all the experimental diets enhanced growth, feed conversion ratio, and frog-leg weight compared with the commercial diet. Bullfrogs had higher growth with 400 and 500 g kg^{-1} of dietary protein regardless of dietary lipid content. It was also notable that with the P500/200L diet, frogs doubled the weight of those fed the commercial diet. DHA, EPA, and total omega-3 fatty acids were double in muscle and two to eight times higher in the liver compared with the commercial diet. In all cases, the final proximal composition of carcass reflected the diet composition. It is suggested that a diet containing 400 g kg^{-1} of protein and 50 g kg^{-1} lipids (protein/lipid ratio: 7.4; gross energy: 18.2 MJ kg^{-1}) is adequate for bullfrog performance during the grow-out phase to achieve market size in a shorter period, thus, reducing farming risks and production costs.

Keywords: carcass composition, culture performance, fatty acid profile, frog grow-out

1. Introduction

Bullfrog (*Lithobates catesbeianus*) has been introduced in different countries for aquaculture purposes due to its easy adaptation to farming conditions and human handling. Although the bullfrog is the most exploited frog species worldwide (Tokur et al., 2008; Huang et al., 2014), the farming technology, including a specific feed formulation, is not currently available, and therefore, fish feeds are commonly used to feed them on farms during the grow-out stage (Casali et al., 2005; Fenerick Jr and

De Stéfani, 2005; FAO, 2022; Zhang et al., 2015). The information that currently exists is the dietary protein requirement for bullfrog (446 g kg^{-1} for tadpoles and 400 g kg^{-1} for juveniles and young adults), using different ingredients and fatty acid sources (Carmona-Osalde et al., 1996; Secco et al., 2005; Olvera-Novoa et al., 2007; Vaz, 2007; Huang et al., 2014; Pereira et al., 2014).

From economic and environmental perspectives, numerous studies have demonstrated the potential of protein sparing when using non-protein energy sources (lipids and carbohydrates) in aquafeeds (Watanabe, 2002; Wu et al., 2015). Minimizing the utilization of protein as energy is the mechanism of saving valuable resources in aquaculture. The supply of an optimum balance of energy and protein in the diet is essential because a deficiency or an excess of nutrients results in suboptimal growth. If the diet is deficient in energy, protein will not be used for tissue synthesis; instead, it will be used as an energy source. Animals meet their energy needs first, so ignoring the protein:energy relationship can lead to adverse effects on the culture performance of a species (Cowey et al., 1975). On the other hand, if the diet contains an excess of energy, the animal will satisfy its appetite before ingesting a sufficient amount of protein to fulfill the needs derived from maximum rates of protein synthesis and growth (Cho, 1987).

This work aimed to determine the best dietary protein:lipid ratio during the bullfrog grow-out phase and its effect on growth, body composition, and fatty acid profile to contribute to the knowledge of adequate dietary requirements and nutrient balance.

2. Material and Methods

The Aquatic Animal Health Code (OIE, 2019) standards were followed during the handling and euthanasia procedure of animals. A 10-week feeding experiment was performed to evaluate the effect of dietary protein:lipid ratio on bullfrog performance in the grow-out phase. The investigation was carried out in a greenhouse located in Morelia, Michoacán, México (19.70° N, 101.18° W, 1,920 m asl.).

The experimental system consisted of thirty 100-L plastic trays (73 × 40 × 33 cm with 15° inclination) with a continuous open water supply of 1.8 L/h and was previously filtered and sterilized with a UV lamp. Frogs (10.78±1 g and 3.73±0.46 cm) were randomly distributed in trays partially flooded (40%) and covered with a mesh (4.5 mm). Each treatment consisted of three replicates (n = 30 frogs/treatment). Ambient and water temperature (26.9±3.5 and 32.9±4.4 °C, respectively) were recorded daily during the experimental period with a natural photoperiod of 14L:10D. At the beginning of the experiment, 30 frogs were sacrificed to obtain their initial proximal composition.

Nine experimental diets with three levels of protein (300, 400, and 500 g kg^{-1}) and three levels of lipids (50, 100, and 200 g kg^{-1}) were evaluated for the bullfrog grow-out phase. These diets were designated as P300/50L, P300/100L, P300/200L, P400/50L, P400/100L, P400/200L, P500/50L, P500/100L, and P500/200L. All experimental diets were formulated using fishmeal, soy protein isolate, cooked and defatted soybean meal, fish oil, and soybean oil as protein and lipid sources; diet composition and proximate analysis were performed (Table 1). A commercial fish diet (432 g kg^{-1} protein, 103 g kg^{-1} lipids) commonly used for bullfrog grow-out phase was also evaluated for practical comparisons. Experimental diets were provided twice a day (10:00 and 18:00 h) in the dry side of the tray, adjusting the feeding ratio to the average weight of the frogs every 14 days, starting with a rate of 12% of body weight on the first days to 5% by the end of the trial. Unconsumed feed was dried and weighed daily to estimate feed intake.

At the end of the experiment, all frogs were fasted for 24 h and then weighed. Fifteen individuals from each treatment were sacrificed to evaluate their growth performance and survival. Proximate composition (n = 6), liver and muscle protein, and lipid and fatty acid composition (n = 3/treatment) were also evaluated. To determine the effect of the treatments, final frog-leg weight (FLW; g) and initial and final body weights (g) were measured. Survival (S), daily weight gain (DWG), weight gain (WG), feed conversion ratio (FCR), protein efficiency ratio (PER), viscerosomatic index (VSI), and hepatosomatic index (HSI) were evaluated using the following equations:

$$S\ (\%) = 100 * \left(\frac{\text{Final number of living frogs}}{\text{Initial number of frogs}} \right)$$

$$DWG \left(\frac{g}{day} \right) = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Time (days)}}$$

$$WG\ (g) = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$WG\ (\%) = 100 * \left(\frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \right)$$

$$FCR = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

$$PER = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

$$VSI = 100 * \left(\frac{\text{Viscera weight}}{\text{Total body weight}} \right)$$

$$HSI = 100 * \left(\frac{\text{Liver weight}}{\text{Total body weight}} \right)$$

Proximal composition analyses were performed on carcasses and diets by triplicate, using the Weende method for ash content, moisture, crude protein, ether extract, crude fiber, and nitrogen-free extract. Additionally, protein and lipids of muscle and liver were also analyzed in triplicate (AOAC, 2000). Fatty acid analyses of liver and muscle were performed only for treatments P300/50L, P300/200L, P400/50L, P500/50L, P500/200L, and the commercial diet, as they reflected the most contrasting and representative results associated with the growth performance of this study. Fat extraction (50 mg of each tissue) (Christie and Han, 2010) and subsequent determination of the fatty acid content followed by a "total lipid derivatization" technique were performed. Fatty acids were analyzed by gas chromatography (Agilent 6850) with a capillary column of fused silica 30 m long by 0.25 µm (film thickness) × 0.25 mm (internal diameter), a polyethylene glycol phase with helium as carrier gas at a flow of 0.7 mL/min and temperature ramp of 110-220 °C. Identification of fatty acids was carried out by comparing the retention time of the sample with commercial standards (FAME Mix from Supelco®).

Survival results, VSI, HSI, FCR, PER, FLW, final weight (FW), WG, DWG, proximal carcass composition, and fatty acid content were evaluated using an analysis of variance (one-way ANOVA). When observing significant differences between the variables ($P<0.05$), Tukey's *post hoc* tests were performed. Data were analyzed using GraphPad Prism 6 software.

Table 1 - Dietary formulation and chemical composition (g kg^{-1}) of experimental and fish commercial diets

Ingredient (g kg^{-1})	Experimental diets[1]									
	P300/50L	P300/100L	P300/200L	P400/50L	P400/100L	P400/200L	P500/50L	P500/100L	P500/200L	Commercial
Proteinic ingredients[2]	450	450	450	590	590	590	740	740	740	NA
Cod liver oil	0	7.8	30	0	2	20	0	0	20	NA
Soybean oil	20	50	100	10	50	100	4.2	40	100	NA
Diatomaceous earth	0	0	0	10	10	10	20	20	20	NA
Corn starch	480	440	340	340	290	190	190	150	50	NA
Butyl hydroxy toluene	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	NA
Mineral premix[3]	30	30	30	30	30	30	30	30	30	NA
Vitamin C[4]	3	3	3	3	3	3	3	3	3	NA
Choline chloride[5]	2	2	2	2	2	2	2	2	2	NA
Vitamin premix[6]	10	10	10	10	10	10	10	10	10	NA
Sodium alginate	2	2	2	2	2	2	2	2	2	NA
Chemical composition (g kg^{-1})[7]										
Crude protein	312	315	311	424	414	410	502	520	506	432
Crude lipids	56	101	198	57	100	194	56	103	198	103
Ash	126	122	121	149	161	153	196	188	157	71
Moisture	22	27	17	18	03	19	50	18	48	40
Protein:lipid ratio	5.6	3.1	1.6	7.4	4.1	2.1	9.0	5.1	3.2	4.2
Gross energy (MJ kg^{-1})	18.0	19.1	21.3	18.2	18.9	21.1	18.4	18.9	21.1	23.0

NA - no information of ingredients composition available from the manufacturer.
[1] P300/50L: 300 g kg^{-1} protein and 50 g kg^{-1} lipid; P300/100L: 300 g kg^{-1} protein and 100 g kg^{-1} lipid; P300/200L: 300 g kg^{-1} protein and 200 g kg^{-1} lipid; P400/50L: 400 g kg^{-1} protein and 50 g kg^{-1} lipid; P400/100L: 400 g kg^{-1} protein and 100 g kg^{-1} lipid; P400/200L: 400 g kg^{-1} protein and 200 g kg^{-1} lipid; P500/50L: 500 g kg^{-1} protein and 50 g kg^{-1} lipid; P500/100L: 500 g kg^{-1} protein and 100 g kg^{-1} lipid; P500/200L: 500 g kg^{-1} protein and 200 g kg^{-1} lipid.
[2] Proteinic ingredients mix: fishmeal (80%), soy protein isolate (11%), cooked and defatted soybean meal (9%).
[3] Mineral premix: macro-elements and trace elements (DSM Nutritional products).
[4] L-Ascorbyl-2-Poliphosphate (AsPP), Rovimix®Stay C®35 (DSM Nutritional products).
[5] Choline chloride (DSM Nutritional products).
[6] Vitamin premix for carnivorous fish (DSM Nutritional products).
[7] Chemical composition of experimental and commercial diets was analyzed in LAMNDA, Universidad Michoacana de San Nicolás de Hidalgo.

3. Results

Survival was higher than 93% in all cases, with no significant differences. Growth, VSI, and HSI results were calculated (Table 2). Feed conversion ratio was similar in all treatments except for P300/50L, P300/200L, and commercial diet, showing the highest FCR. No significant differences were observed between the treatments regarding PER. Frog-leg weights were similar in all treatments except for P300/100L, P300/200L, and the commercial diet, which presented the lowest values (Table 2). Frogs fed P500/200L diet reached a similar average FW (g) than the animals fed diets containing 500 and 400 g kg^{-1} protein regardless of the lipid content. The lowest FW were found in bullfrogs fed P300/200L and the commercial diet. Accordingly, similar results were observed for WG (%) and DWG (g day^{-1}; Table 2).

Despite the level of dietary protein in the diets, treatments with the highest carcass lipid content were those with the highest dietary lipid content (200 g kg^{-1}); on the contrary, P500/50L was the treatment with less carcass lipid content. The body protein content was higher in the P500/50L and P500/100L treatments, and the lowest levels were observed in the P300/200L treatment (Table 3). In all cases, the final proximate composition of the carcass reflected the diet composition.

No significant differences were observed in frog muscle protein content, except between those fed the commercial and the P500/200L diets (Table 4). The lipid content in muscle tissue was lower in all treatments containing 50 g kg^{-1} dietary lipids than those with 100 and 200 g kg^{-1} lipids. Significant changes were observed with the dietary lipid increase from 100 to 200 g kg^{-1} in diets with 300 g kg^{-1} protein but not in 400 and 500 g kg^{-1} protein diets. A similar result was observed in the liver; treatments with 50 g kg^{-1} of fat showed the lowest tissue lipid levels. It can also be observed that in

the treatments with the lowest lipid level (50 g kg^{-1}), but with dietary protein of 400 and 500 g kg^{-1}, lipid deposition in the liver increased; the opposite occurred in treatments with higher fat content (200 g kg^{-1}). Finally, the highest lipid contents were observed in frogs fed the P300/200L and the commercial diets (Table 4).

Regarding polyunsaturated fatty acids (PUFA), there were only significant differences in linoleic acid (18:2n-6), in which the highest content was found in the P300/200L and commercial diets; on the other hand, P500/50L and P400/50L presented the lowest levels. Concerning omega-3 (n-3) PUFA, including eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids, the lowest levels were observed in frogs fed the commercial and P300/200L diets. The sum of DHA and EPA was similar in all treatments, except in the case of the commercial diet, which presented the lowest values of all treatments (Table 5).

The α-linolenic acid (18:3n-3) levels were higher in the two treatments with more dietary lipids (P300/200L and P500/200L), and lower contents were found in the other treatments (Table 6).

Table 2 - Growth performance and feeding efficiency of bullfrog fed experimental and commercial diets during fattening (data are presented means (n = 3) ± standard deviation)

Diet	S (%)	VSI	HSI	FCR	PER	FLW (g)	FW (g)	WG (%)	DWG (g day^{-1})
P300/50L	96.7	17.3±1.5cd	5.0±0.5b	1.7±0.8a	2.3±0.8	31.9±10.2ab	77.0±27.4b	599±263bc	1.0±0.4b
P300/100L	100.0	18.2±1.8abc	4.4±0.4bc	1.5±0.7b	2.5±0.6	29.4±7.9b	77.9±22.3b	609±218bc	1.0±0.4b
P300/200L	96.7	20.3±2.8a	4.1±0.7cd	1.9±0.6a	1.9±0.8	20.3±5.9c	59.7±20.1bc	441±197cd	0.7±0.3bc
P400/50L	100.0	17.2±2cd	4.3±0.7bd	1.2±0.5b	2.1±0.7	36.1±9.8ab	91.2±24.1ab	726±247ab	1.2±0.4ab
P400/100L	93.3	18±2.2abc	4.1±0.6cd	1.1±0.1b	2.2±0.4	33.4±8.2ab	89.0±22.2ab	712±215ab	1.1±0.3ab
P400/200L	96.7	18.4±1.6abc	3.3±0.3ef	1.2±0.3b	2.0±0.4	31.8±6.8ab	86.9±19.8ab	692±196ab	1.1±0.3ab
P500/50L	96.7	15.3±2.4d	3.6±0.6de	1.2±0.3b	1.7±0.5	33.6±10.8ab	95.2±31.1ab	766±300ab	1.2±0.5a
P500/100L	100.0	15.2±2.3d	3.2±0.7ef	1.0±0.3b	1.7±0.3	36.9±7.1ab	94.1±22.8ab	755±232ab	1.2±0.4ab
P500/200L	93.3	17.6±2.6bcd	2.8±0.5f	0.9±0.1b	2.1±0.3	39.3±6a	100.9±19.5a	824±188a	1.3±0.3a
Commercial	93.3	20.1±1.7ab	6.0±0.5a	2.4±0.2a	0.9±0.2	18.5±4.9c	50.6±15.2C	253±141d	0.4±0.2c

S - survival; VSI - viscerosomatic index; HSI - hepatosomatic index; FCR - feed conversion ratio; PER - protein efficiency ratio; FLW - frog-leg weight; FW - final weight; WG - weight gain; DWG - daily weight gain.
P300/50L: 300 g kg^{-1} protein and 50 g kg^{-1} lipid; P300/100L: 300 g kg^{-1} protein and 100 g kg^{-1} lipid; P300/200L: 300 g kg^{-1} protein and 200 g kg^{-1} lipid; P400/50L: 400 g kg^{-1} protein and 50 g kg^{-1} lipid; P400/100L: 400 g kg^{-1} protein and 100 g kg^{-1} lipid; P400/200L: 400 g kg^{-1} protein and 200 g kg^{-1} lipid; P500/50L: 500 g kg^{-1} protein and 50 g kg^{-1} lipid; P500/100L: 500 g kg^{-1} protein and 100 g kg^{-1} lipid; P500/200L: 500 g kg^{-1} protein and 200 g kg^{-1} lipid.
a-f - Values with the same letter in the same column are not significantly different (P≥0.05).

Table 3 - Proximal body composition of bullfrog at the beginning (initial) and at the end of the experiment

Diet	Protein (%)	Lipids (%)	Ash (%)	Moisture (%)
Initial	67.40±0.2ab	14.5±0.05e	14.4±0.04a	81.5±0.1a
P300/50L	60.39±3.5deg	15.8±0.2de	14.2±0.2ab	77.6±1cde
P300/100L	60.04±0.3deg	20.7±0.3c	13.3±0.3abc	76.8±0.5bcdf
P300/200L	53.35±0.5h	27.5±1a	12.8±0.4bc	74.5±3.5fh
P400/50L	63.74±1.6bde	16.2±0.6d	14±0.3ab	78.4±0.8ac
P400/100L	65.67±0.5bcf	16.3±0.3d	13.4±0.7abc	77.8±0.7ad
P400/200L	58.74±2.2g	24.2±0.1b	12±0.7c	76.7±1.2cdgh
P500/50L	70.23±1.4a	12.3±0.2f	14.5±0.4a	79.9±1ae
P500/100L	68.19±1.2ab	14.8±0.2de	13.7±0.6ab	79.4±1.3abeg
P500/200L	61.81±1.2cefg	22.8±0.9b	13.4±0.4abc	76.4±0.6cdfh
Commercial	60.66±1.1eg	19.6±0.9c	10.5±0.3d	76.6±1.5bcdh

P300/50L: 300 g kg^{-1} protein and 50 g kg^{-1} lipid; P300/100L: 300 g kg^{-1} protein and 100 g kg^{-1} lipid; P300/200L: 300 g kg^{-1} protein and 200 g kg^{-1} lipid; P400/50L: 400 g kg^{-1} protein and 50 g kg^{-1} lipid; P400/100L: 400 g kg^{-1} protein and 100 g kg^{-1} lipid; P400/200L: 400 g kg^{-1} protein and 200 g kg^{-1} lipid; P500/50L: 500 g kg^{-1} protein and 50 g kg^{-1} lipid; P500/100L: 500 g kg^{-1} protein and 100 g kg^{-1} lipid; P500/200L: 500 g kg^{-1} protein and 200 g kg^{-1} lipid.
a-h - Values with the same letter in the same column are not significantly different (P≥0.05).

Table 4 - Protein and lipid contents in frog muscle and liver at the beginning and the end of the experiment

Diet	Protein (%)	Lipid content (%)	
	Muscle	Muscle	Liver
Initial	91.06±0.27ab	3.67±0.29a	7.06±0.82f
P300/50L	91.99±0.62ab	1.50±0.05de	5.04±0.36g
P300/100L	91.63±0.31ab	2.28±0.16b	10.61±0.45c
P300/200L	91.96±0.40ab	3.54±0.05a	18.23±0.04a
P400/50L	94.65±0.62ab	1.41±0.18de	7.88±0.04ef
P400/100L	93.16±1.08ab	1.88±0.11bc	9.86±0.31c
P400/200L	94.63±1.02ab	1.89±0.11bc	15.66±0.54b
P500/50L	94.44±0.11ab	1.21±0.07e	8.54±0.23de
P500/100L	96.56±2.98ab	1.58±0.06cd	10.16±0.02c
P500/200L	96±0.33a	1.55±0.12cd	9.45±0.59cd
Commercial	90.58±0.14b	2.12±0.17b	17.64±0.15a

P300/50L: 300 g kg^{-1} protein and 50 g kg^{-1} lipid; P300/100L: 300 g kg^{-1} protein and 100 g kg^{-1} lipid; P300/200L: 300 g kg^{-1} protein and 200 g kg^{-1} lipid; P400/50L: 400 g kg^{-1} protein and 50 g kg^{-1} lipid; P400/100L: 400 g kg^{-1} protein and 100 g kg^{-1} lipid; P400/200L: 400 g kg^{-1} protein and 200 g kg^{-1} lipid; P500/50L: 500 g kg^{-1} protein and 50 g kg^{-1} lipid; P500/100L: 500 g kg^{-1} protein and 100 g kg^{-1} lipid; P500/200L: 500 g kg^{-1} protein and 200 g kg^{-1} lipid.
a-f - Values with the same letter in the same column are not significantly different (P≥0.05).

Table 5 - Fatty acid content (%) in the muscle of bullfrogs fed different diets

Fatty acid	P300/50L	P300/200L	P400/50L	P500/50L	P500/200L	Commercial
14:0	1.0	0.7	0.8	0.7	0.2	0.5
15:0	0.3	0.3	0.2	0.2	0.2	0.2
16:0	22.6a	14.2c	20.2ab	18.5abc	17.7bc	20.1ab
18:0	9.2	9.3	10.9	11.0	9.2	9.8
20:0	0.4	0.5	0.3	0.6	0.3	0.5
16:1n-7	5.2a	1.3ab	3.0ab	1.5ab	0.7b	2.8ab
18:1n-9	19.5abc	26.9a	16.5bc	12.4c	19.5abc	20.5ab
18:1n-7	5.3ab	3.0b	4.6ab	4.2ab	3.8ab	5.9a
20:1n-9 n-11	0.9	1.8	1.4	2.2	1.7	0.9
22:1n-9 n-11	3.7b	11.9ab	12.3ab	19.0a	13.7ab	9.3ab
18:2n-6	7.8bc	10.2ab	6.0cd	3.4d	8.1bc	14.0a
18:3n-6	0.7	0.5	0.3	0.4	0.2	0.2
20:2n-6	0.8	0.6	0.4	0.5	0.2	0.7
20:3n-6	0.7	0.5	0.4	0.5	0.3	1.2
20:4n-6	2.5	1.7	2.6	2.5	2.4	2.0
18:3n-3	1.2	1.6	0.9	0.9	1.3	0.6
18:4n-3	0.6	0.5	0.3	0.5	0.2	0.2
20:4n-3	0.6a	0.4ab	0.5ab	0.5ab	0.2b	0.2ab
20:5n-3	5.3a	4.5ab	5.8a	6.2a	5.7a	2.5b
22:5n-3	2.7	2.2	2.8	3.1	2.5	2.0
22:6n-3	9.0abc	7.3bc	9.8abc	11.0ab	11.9a	5.9c
SFA	33.5a	25.1b	32.4a	31.0ab	27.6ab	31.1ab
MUFA	34.6	44.9	37.8	39.4	39.3	39.3
PUFA	31.9	30.1	29.8	29.6	33.0	29.6
PUFA n-6	12.6abcd	13.5ab	9.6bd	7.4d	11.2bcd	18.1a
PUFA n-3	19.3a	16.6ab	20.2a	22.2a	21.8a	11.5b
DHA:EPA	1.7ab	1.6b	1.7ab	1.8ab	2.1ab	2.3a
DHA + EPA	14.2ab	11.8ab	15.6a	17.2a	17.7a	8.4b

SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; n-3 - omega-3; n-6 - omega-6.
P300/50L: 300 g kg^{-1} protein and 50 g kg^{-1} lipid; P300/200L: 300 g kg^{-1} protein and 200 g kg^{-1} lipid; P400/50L: 400 g kg^{-1} protein and 50 g kg^{-1} lipid; P500/50L: 500 g kg^{-1} protein and 50 g kg^{-1} lipid; P500/200L: 500 g kg^{-1} protein and 200 g kg^{-1} lipid.
a-d - Values with the same letter in the same row are not significantly different (P≥0.05).

Regarding EPA, the commercial treatment presented the lowest level; in contrast, P500/200L, P500/50L, and P400/50L presented the highest results. Concerning DHA, the only different treatments were P300/50L, P300/200L, and the commercial diet, with the lowest values. The DHA:EPA ratio did not show significant differences between treatments (Table 6). Treatments P400/50L, P500/50L, and P500/200L had the highest PUFA levels. There were also notable differences between PUFA n-3 and omega-6 (n-6) content. The highest PUFA n-3 levels were found in P500/200L diet, while the commercial diet presented the lowest values; PUFA n-6 were present in higher concentrations in the P500/200L treatment, then in P300/200L, and finally in the commercial treatment. Treatments with the lowest PUFA n-6 levels contained 50 g kg^{-1} lipids in the diet (Table 6).

Table 6 - Fatty acid content (%) in the liver of bullfrogs fed different diets

Fatty acid	P300/50L	P300/200L	P400/50L	P500/50L	P500/200L	Commercial
14:0	1.2	1.6	1.8	1.7	0.8	1.2
15:0	0.1	0.4	0.3	0.5	0.2	0.1
16:0	20.7ab	15.0b	24.7a	24.0a	20.8ab	21.1ab
18:0	7.1	5.6	7.4	8.5	9.0	7.2
20:0	0.4	0.6	0.6	0.5	0.6	0.6
16:1n-7	13.4a	5.1ab	11.6ab	8.2ab	3.2b	14.8a
18:1n-9	30.8ab	42.5a	24.8bc	17.9c	21.6bc	33.3ab
18:1n-7	4.0	3.0	3.2	4.0	3.5	3.9
20:1n-9 n-11	1.4	2.1	0.8	1.3	1.8	1.1
22:1n-9n-11	7.1a	4.3ab	1.5b	9.5ab	1.6ab	2.9ab
18:2n-6	3.7b	9.7a	3.6b	2.9b	11.1a	8.5a
18:3n-6	0.3	0.2	0.2	0.4	0.4	0.5
20:2n-6	0.2	0.4	0.2	0.2	0.4	0.3
20:3n-6	0.1	0.1	0.2	0.2	0.2	0.1
20:4n-6	1.4bcd	0.7d	2.7ab	2.3abc	3.6a	1.0cd
18:3n-3	0.9b	2.4a	0.7b	0.8b	2.5a	0.8b
18:4n-3	0.2	0.2	0.5	0.2	0.2	0.2
20:4n-3	0.1	0.1	0.2	0.1	0.3	0.4
20:5n-3	2.0b	1.9b	4.2ab	5.1a	6.9a	0.3c
22:5n-3	0.9ab	1.0ab	1.8a	2.9a	2.3a	0.2b
22:6n-3	4.0ab	3.1b	9.2a	8.9a	8.9a	1.6b
SFA	29.6ab	23.2b	34.8a	35.2a	31.4a	30.2a
MUFA	56.7a	57.0a	41.8b	40.8b	31.8b	56.0a
PUFA	13.8b	19.8b	23.4ab	24.0ab	36.9a	13.8b
PUFA n-6	5.7c	11.2b	6.9c	6.0c	15.7a	10.3b
PUFA n-3	8.1cd	8.6bcd	16.5abc	18.0ab	21.1a	3.5d
DHA:EPA	2.0	1.6	2.2	1.7	1.3	5.4
DHA + EPA	6.0b	4.9b	13.4a	13.9a	15.8a	1.9b

SFA - saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; DHA - docosahexaenoic acid; EPA - eicosapentaenoic acid; n-3 - omega-3; n-6 - omega-6.
P300/50L: 300 g kg^{-1} protein and 50 g kg^{-1} lipid; P300/200L: 300 g kg^{-1} protein and 200 g kg^{-1} lipid; P400/50L: 400 g kg^{-1} protein and 50 g kg^{-1} lipid; P500/50L: 500 g kg^{-1} protein and 50 g kg^{-1} lipid; P500/200L: 500 g kg^{-1} protein and 200 g kg^{-1} lipid.
a-d - Values with the same letter in the same row are not significantly different (P≥0.05).

4. Discussion

The bullfrog industry is growing worldwide despite lacking a specific feed formulation for these anurans. The present work evaluated the dietary protein:lipid ratio in grow-out phase performance in bullfrogs. The best growth was observed in diets containing 400 and 500 g kg^{-1} dietary protein regardless of the lipid content. These results agree with a previous report on juvenile bullfrogs, in which the best performance was observed with 420, 500, and 550 g kg^{-1} dietary protein (Olvera-Novoa

et al., 2007). When analyzing the FCR, experimental diet values ranged from 0.9 to 1.9 (P500/200L and P300/200L, respectively) and were 2.4 in the commercial diet. Similar results were found by Olvera-Novoa et al. (2007) and Zhang et al. (2016a), reporting lower FCR values (<0.99) in diets with ≥400 g kg^{-1} protein. In this study, VSI was higher in frogs fed diets with low protein levels (300 and 400 g kg^{-1}) and high dietary lipids (100 and 200 g kg^{-1}). The opposite occurs in diets containing more protein (500 g kg^{-1}), regardless of the lipid content (Table 2). The HSI values reveal excess lipids in the liver (Table 2). In this study, no positive effects were observed on growth with the addition of higher dietary lipid levels. The docosahexaenoic acid, EPA, and total omega-3 fatty acids were double in muscle and two to eight times higher in the liver than in the commercial diet, most likely due to the quality of the lipid ingredients used in the experimental diets. The latter implies that different sources of dietary lipids can be used to increase the final omega-3 fatty acid profile in frog meat production, which is a desirable characteristic for healthier aquaculture products (Xu et al., 2020).

Performance parameters evaluated in this study, such as FLW, FW, WG, DWG, and FCR, presented similar results between treatments with 400 and 500 g kg^{-1} protein. It is worth mentioning that the least favorable results were obtained with the commercial diet, which is currently used as a staple diet in commercial frog farming in Mexico and other parts of Latin America (Casali et al., 2005; Fenerick Jr and De Stéfani, 2005; FAO, 2022). In this study, a diet containing 400 g kg^{-1} of protein (of at least 80% animal origin) and 50 g kg^{-1} lipids (protein:lipid ratio: 7.4; gross energy: 18.2 MJ kg^{-1}) is suitable for bullfrogs ranging from 10 to 100 g body weight. A similar study using bullfrogs of a higher size class (90-300 g body weight) showed that a diet with 400 g kg^{-1} protein and 70 g kg^{-1} lipids is optimal at this stage (Huang et al., 2014). Thus, both studies suggest that bullfrog protein and lipid requirements are similar across the entire grow-out phase. Meanwhile, Carmona-Osalde (1996) states that tadpoles grow better with higher dietary protein (446 g kg^{-1}) and lipid levels (120-140 g kg^{-1}) (Table 7).

Table 7 - Dietary protein and lipid requirements for bullfrogs at different developmental stages

Stage	Protein (g kg^{-1})	Lipids (g kg^{-1})	Reference
Tadpoles	446	120-140	Carmona-Osalde et al. (1996)
Early juveniles (8-58 g)	400	32-51	Olvera-Novoa et al. (2007)
Juveniles (10-100 g)	400	50	Present study
Young adults (100-300 g)	400	70	Huang et al. (2014)

Frogs fed a diet containing 500 g kg^{-1} of protein and 200 g kg^{-1} lipids (protein:lipid ratio: 3.2; gross energy: 21.1 MJ kg^{-1}) grew 308% more than those fed the commercial diet, suggesting that this protein:lipid ratio is more appropriate for the grow-out phase of bullfrogs. On the other hand, because animal protein content in experimental diets was high (80% of total protein) as compared with the commercial diet, and growth and feed utilization in bullfrogs can be affected by different lipid sources (Zhang et al., 2016b), further work is required to assess whether partial or complete substitution with alternative protein sources is possible, because the plant-based commercial diet had the poorest performance despite having an adequate protein:lipid ratio and higher gross energy content. These results have implications for developing a specific diet for the grow-out phase of bullfrogs.

5. Conclusions

Under the experimental conditions, there are no positive effects on bullfrog growth with the addition of dietary lipid levels higher than 50 g kg^{-1}. Since bullfrogs grew better with 400 and 500 g kg^{-1} of dietary protein, regardless of dietary lipid content, a diet containing 400 g kg^{-1} of protein and 50 g kg^{-1} lipids (protein:lipid ratio: 7.4; gross energy: 18.2 MJ kg^{-1}) is adequate for the bullfrog grow-out phase. Finally, due to the metabolic ability of frogs to reflect the dietary lipid composition, it is possible to raise the omega-3 fatty acid profile in frogs rendering them and their subproducts the status of nutraceutical products, increasing their demand and market price.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

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