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Effect Crude Protein Levels on the Broodstock Spermatic Quality of Nile tilapia (*Oreochromis niloticus*)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MMO, MRF and PVR initiated, designed and wrote the protocol, managed the literature searches, wrote and edited the final manuscript. Authors MBG, ESA, VOF and GCV collected data on fish. Author DVC undertook laboratory analyses while author IBA undertook statistical analyses. He and author LDSM also collaborated with the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Objective: The purpose of this work was to evaluate the reproductive performance and the toxicity of DMSO cryoprotectants and methanol toward semen of Nile tilapia (*Oreochromis niloticus*), fed using diets containing different Crude Protein (CP) levels.
Study Design: completely randomized design.
Place and Duration: The experiment was performed for 90 days, at the Hydrobiology and Fish

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Culture Station of Eletrobrás Furnas, in São José da Barra, State of Minas Gerais, Brazil.
Methodology: 15 broodstock males with around 30 months old were used. They were stocked in ten reservoirs of storage capacity around 8 cubic meters, in which treatments consisted of five diets containing different levels of crude protein (32, 34, 36, 38 and 40%) and 9.5kcal/kg of digestible energy per gram of protein. For the *in-natura* semen, the statistical analyses were carried out using data obtained from a completely randomized design, while for diluted semen, the completely randomized design was also used but, in 5×2 factorial scheme, representing 5 levels of protein and 2 cryoprotectants.
Results: The hepatosomatic index (HSI), gonadosomatic index (GSI), rate and duration of Nile tilapia males *in-natura* semen motility were not influenced ($p>0.05$) by the crude protein levels in the diets. After dilution, significant differences were observed for motility ($p=0.053$) and motility duration ($p=0.021$). The effects of third and fourth degree occurred for the rate ($p=0.0349$) and duration ($p=0.0220$). As these adjustments do not allow a biological interpretation, the Tukey multiple comparison test was used. The diet containing 36% of crude protein level showed mean motility rate similar ($p>0.05$) to those of treatments containing 32, 34 and 40% of crude protein and mean motility rate inferior ($p<0.05$) to treatment containing 38% of crude protein. For motility duration, fish fed using diet containing 38% and 40% of crude protein showed significant differences ($p<0.05$) to only the group of fish fed using diet containing 34% of crude protein.
Conclusion: Diet containing 32, 38 and 40% of crude protein level does not deteriorate the semen quality, so that may be used for feeding tilapia broodstocks. As protein is an expensive input, we can infer that 32% of crude protein is sufficient to determine good quality gametes.

Keywords: Tilapia; reproduction; semen; cryopreservation; fish-male.

1. INTRODUCTION

Tilapias are the second most important group of fish in global aquaculture. They stand out by being fast growing fish, rustic, and by having excellent flesh quality, with good market acceptance and early sexual maturity. While several natural fishing stocks are in their maximum exploration limit, the aquaculture fish production has been increasing in the last few years, for attending the market [1-2]. Besides, fish is one of the most important foods for healthy diet, because is rich in protein and has a great nutritional value [3]. However, it is suggested that fish and seafood shall be preferred in the diet by preventing cardiovascular and other diseases [4]. Then, the improvement of culturing techniques associated to technological advances in nutrition and feeding are necessary, not only for productive performance, but also in reproductive development of fish, in order to secure greater gamete production and greater larvae and fingerling development, which is one of the major difficulties for aquaculture development.

Studies in broodstock nutrition are still limited and relatively expensive, due to the need of large dimension facilities to maintain large groups of adult fish, and high production costs to perform prolonged feeding experiments. Protein is one of the important nutrients in the diet, and determines the increase in reproductive performance of broodstock [5]. Many studies have been performed focused in the improvement of the potential of fish culturing, but few of them have approached nutrition for studying reproductive parameters [6-7]. However, this is an expensive component in the feed manufacturing process, because increases production costs, making necessary studies of protein requirement for each fish species [8-9].

Gonadal development, especially the quality of gametes, depends on the quality of the diet supplied to broodstock [10]. Proteins are found in fish gametes as lipoproteins, hormones and enzymes, determining the quality of the egg and large-scale fish farm production [11].

Semen cryopreservation is a biotechnical method used for the maintenance of viable spermatozoa, minimizing problems, such as synchronization between fish males and females during breeding season, consanguinity and genetic selection [12]. There are three factors which influence the maintenance of spermatic quality during cryopreservation, namely, freezing and thawing methodology, composition of internal and external cryoprotectants and diluter composition [13]. Then, before the freezing process, is important assessing the toxicity of solutions, which are going to be used, considering that, if the cryoprotectant solution is toxic, it will make unfeasible the gametes [7].

Therefore, in this work, we aimed to evaluate the effect of different crude protein levels in the diet of *Oreochromis niloticus* male broodstock, on spermatic quality parameters, as well as evaluating the toxicity of the cryoprotectants, methanol and dimethylsulfoxide (DMSO), toward semen of this fish specie.

2. MATERIALS AND METHODS

2.1 Location

The present study was performed for 90 days at the Hydrobiology and Fish Culture Station of Eletrobrás Furnas, in São José da Barra, State of Minas Gerais, Brazil. Is the result of a partnership between Federal Univesity of Lavras-MG and Eletrobrás-Furnas.

2.2 Animals and Diets

Fifteen Nile tilapia (*Oreochromis niloticus*) broodstock males, Chitralada strain, with around 30 months old were used. Three fish were bred in each one of five reservoirs of storage capacity around 8 cubic meters under continuous water flow. Treatments consisted of feeds of five different crude protein levels, namely, 32, 34, 36, 38 and 40%, and 9.5kcal/kg of digestible energy per gram of protein.

Five experimental diets were made (Table 1) with different crude protein (CP) levels, formulated according to the table of ingredient chemical composition, as determined by [14-15]. All ingredients were ground to a diameter equal or less than to 0.04 mm. The ingredients were weighed, homogenized and water was added, at a proportion of 20% of the total ration weight. The diets were pelletized in an electrical pelletizing machine and dried in a forced air flow oven (50℃) for 24 hours.

The adaptation period to the ration and the environment were 10 day, and during this period, the animals received an experimental ration of 32% CP (Table 1). The fish were fed twice a day (at 09 h and at 15 h) at a daily feeding rate of 2% of the biomass. During the experimental period of 90 days, the broodstock were fed the experimental rations.

2.3 Limnological Parameters

The limnological variables of the tank water were measured daily in the morning (conductivity, pH, dissolved oxygen and temperature) with a double-channel, multi-parameter HQ40D probe, following the Fish Culture Station Eletrobrás Furnas protocol.

2.4 Experimental Methodology

The procedures in this experiment were approved by the Ethics Committee on Animal Use of the Federal Univesity of Lavras-MG, Brazil, certified by the Protocol No. 008/12.

Three fish-males were selected from each reservoir, weighed, measured and, then transferred to reproduction reservoirs around 200 liters of water storage capacity. For semen extrusion, broodstock were taken from the reservoirs, involved in dry towels, and dried their urogenital papillae using napkins, to avoid semen activation during the contact with water, feces or urine. Massage on the coelomic cavity, in the cranium-caudal direction, was done to allow semen samples collection using sterile microtubes (eppendorf).

Subsequently, analyses of motility rate (%) and duration (seconds) were carried out on optic microscope, by means of distilled water activation in 1:4 dilution (semen: water). Motility duration was calculated from the time of activation to the time in which 10% of the spermatozoa were mobile, as outlined in [16].

Semen that showed motility rate above 90% was used for performing toxicity tests. Two cryoprotectant solutions were used: Solution A: 10% dimethylsulfoxide (DMSO) + 5% BTS (Beltsville Thawing Solution®); Solution B: 10% methanol + 5% BTS. The *in-natura* semen was diluted in 1:4 proportion (semen: cryoprotectant solution). For the toxicity evaluation of the cryoprotectant solutions, were verified spermatic motility rate and duration, according to methodology used for *in-natura* semen, right after dilution.

Then fish were euthanized using a 2-phenoxyethanol solution (0.06%) for desiccation to weigh the liver and gonads. From this data, the following indexes were calculated: Gonadosomatic index (GSI) = [(weight of the gonads*100) / weight of the fish]; Hepatosomatic index (HSI) = [(weight of the liver*100) / weight of the fish].

Table 1. Ingredients and calculated composition of the experimental diets supplied to Nile tilápia (*Oreochromis niloticus*) males (based on dry matter)

Ingredients (%)	Levels of protein in the diet (%) 9.5 DE/g of CP				
	32	34	36	38	40
Soybean meal	48.00	53.80	57.50	64.00	68.65
Fishmeal (64% CP)	12.00	12.00	12.00	12.00	12.00
Corn	20.90	16.00	15.90	13.04	5.50
Wheat bran	5.00	5.00	5.00	1.00	0.60
Alginate	0.20	0.20	0.20	0.20	0.20
Soybean oil	0.20	2.00	2.00	4.00	7.80
Dicalcium phosphate	4.95	3.99	1.50	0.34	0.45
Vitamin C	0.06	0.06	0.06	0.06	0.06
Salt	0.20	0.20	0.20	0.20	0.20
Vitamin/Mineral Premix[a]	0.50	0.50	0.50	0.50	0.50
Kaolin	3.65	1.61	0.50	0.10	0.10
BHT [b]	0.02	0.02	0.02	0.02	0.02
Total	100.00	100.00	100.00	100.00	100.00
Calculated composition					
Digestible energy (kcal/kg)	3.040	3.230	3.420	3.610	3.800
Crude protein (%)	32.04	34.24	36.25	38.19	40.05
Ethereal extract	3.87	5.56	5.73	7.64	10.22
Crude fiber (%)	3.74	3.99	4.39	4.30	4.42
Lysine	2.34	2.55	2.54	2.53	2.73
Methionine	0.53	0.55	0.58	0.55	0.58
Threonine	1.44	1.52	1.50	1.46	1.38

[a] *Vitamin/Mineral Premix: Composition/ kg of the product: vit. A = 900.000 UI; vit. D3 = 50.000 UI; vit. E=6.000 mg; vit. K3 = 1200 mg; vit. B1 = 2400 mg; vit. B2 = 2400 mg; vit. B6 = 2000 mg; vit.B12 = 4800 mg; folic acid = 1200 mg; calcium pantothenate = 12.000 mg; vit. C = 24.000 mg; biotin = 6,0 mg; colin = 65.000 mg; nicotinic acid = 24.000 mg; Fe = 10.000 mg; Cu = 600 mg; Mn = 4000 mg; Zn = 6000 mg; I = 20 mg; Co = 2,0 mg e Se = 25mg.* [b] *Butylhydroxytoluene (antioxidant)*

2.5 Statistical Analysis

The experiment using *in-natura* semen was performed in a completely randomized design with three replicates, on which five crude protein levels, namely, 32, 34, 36, 38 and 40% were assessed. The statistical model was as follows:

$$y_{ik} = \mu + \tau_i + \varepsilon_{ik} \quad (2.1)$$

where:

y_{ik} is the observation value regarding to i level of τ factor, and to j level of β factor in the replicate k;
μ is the constant associated to all observations;
τ_i is the i level effect of τ factor, with i=1, 2, 3, 4 and 5;
ε_{ik} is the experimental error regarding to factor i of replicates k, on which $\varepsilon_{ik} \sim N(0, \sigma^2)$.

For the experiment using diluted semen, the completely randomized design (CRD) was also used, but in 5×2 factorial scheme, ie, 5 protein levels and 2 cryoprotectants, totalizing 10 treatments with three replicates, each by using the following statistical model:

$$y_{ijk} = \mu + \tau_i + \beta_j + \delta_{ij} + \varepsilon_{ijk} \quad (2.2)$$

where:

y_{ijk} is the observation value regarding to i level of τ factor and the j level of β factor in the k replicate;
μ is a constant associated to all observations;
τ_i is the effect of i level of τ factor, with i=1, 2, 3, 4 and 5;
β_j is the effect of j level of β factor, with j=1, 2, 3, 4 and 5;
ε_{ijk} is the experimental error regarding all observations, where $\varepsilon_{ijk} \sim N(0, \sigma^2)$.

The assumptions of analysis of variance (ANOVA) were checked using R statistical packages, namely, “stats” version 2.15.1 [17] for normality shapiro test, and “car” version 2.10-12 [18] for Levene homoscedasticity test. After finding statistical differences among means in the ANOVA, the sum of squares was analyzed until

the polynomial of the fourth degree. The "TukeyC" R statistical package version 1.0-7 [19] was also used, but for means comparison of variables with polynomial degree greater than two. [17].

3. RESULTS AND DISCUSSION

3.1 Water Quality

The water quality in the reservoirs during experimental period was found to be within the limit of parameters recommended for tilapia reproduction [20], since means of temperature (ºC), conductivity, pH and dissolved Oxygen ($mg.l^{-1}$) were respectivelly around 22.47, 36.45, 7.43 and 7.02, and are considered appropriate for tilapia initial embryonic development [21].

3.2 Reproductive Index

The reproductive parameters, such as hepatosomatic index (HSI), gonadosomatic index (GSI), spermatic motility rate and duration for *in-natura* semen of Nile tilapia males, were not influenced ($p > 0.05$) by crude protein levels (Table 2).

The HSI may increase depending on the quantity of nutrients in the diet. In this case, the fish convert the excess dietary energy to visceral fat [22]. Therefore, results found in this study suggest that protein in the diet did not influence HSI. Similar results were reported by [23] which, working with 28, 34 and 40% crude protein levels in diets for *Rhamdia quelen* female broodstock, did not find statistical differences for liver weight.
The GSI is used for monitoring gametogenesis progression in teleost fish [24]. In this study, the GSI was not affected by the increase in diet crude protein levels (32, 34, 36, 38 and 40%). These results are similar to those reported in [25], where protein levels of 10, 20 and 35% were evaluated for tilapia.

After semen dilution, there was no significant interaction ($p > .05$) between crude protein levels and cryoprotectants for all variables, except between crude protein levels for motility ($p = .05$) and motility duration ($p = .02$). A significant fourth degree polynomial adjustment ($p < .05$) was found in crude protein levels for motility and, a significant third degree polynomial adjustment ($p < .05$) on crude protein levels for motility duration. The Tukey test was carried out since this adjustment did not refer to a biological interpretation. For motility, 32, 34, 38 and 40% protein levels caused similar effect ($p > .05$), however, the fish fed using diet containing 38% of crude protein showed greater motility than that containing 36% of CP. For motility duration, the fish fed using diet containing 38% of crude protein showed significant difference ($p < .05$) only to group of fish fed using diet containing 34% of crude protein (Table 3).

Some papers referring to tilapia broodstock nutrition showed that spermatic quality is influenced by the diet supplied to the broodstock [26]. In the present study, results showed that crude protein levels in the feed did not change motility parameters and motility duration of fresh semen. The semen quality is measured by seminal composition and motility [27], because, after spermatic activation, the spermatozoa must be able to reach the oocyte surface and penetrate the micropyle [13].

Studies performed aiming to assess the effect of feeding of tilápias [7], showed that dilution of different types of feed oil affected the semen quality. Besides, in the process of assessing toxicity of DMSO cryoprotectants and methanol toward semen, were found better results using both DMSO and fish oil, with motility rate around 90.0±2.00% and duration around 218±9 seconds. This paper describes the importance of assessing diet used to feed broodstock in relation to reproductive parameters of *Oreochromis niloticus*.

Table 2. Means (± standard deviation) of gonadosomatic index (gsi), hepatosomatic index (hsi), spermatic motility rate (mot) and spermatic motility duration (dur) of *in-natura* semen of tilapia broodstock under feeding using different crude protein (cp) levels

Treatment (% of CP)	GSI (%)	HSI (%)	MOT (%)	DUR (s)
32	0.70±0.37	1.43±0.34	98.33±2.89	450.00±132.07
34	0.42±0.73	0.64±1.10	96.67±5.77	1051.67±574.65
36	0.93±0.07	1.54±0.18	98.33±2.89	780.67±370.30
38	0.54±0.55	0.98±0.85	100.00±0.00	1078.67±241.87
40	0.87±0.90	0.91±0.80	98.33±2.89	726.00±344.71

Table 3. Means of motility (%) and motility duration (seconds) after dilution of semen from tilapias fed using diet containing different protein levels and different cryoprotectants

Protein levels (%)	Motility			Motility duration		
	Cryoprotectants					
	DMSO	Methanol	General Mean*	DMSO	Methanol	General Mean*
32	96.7±5.8	90.0±10.0	93.3±8.2 ab	811.7±295.4	514.7±289.0	663.1±125.6 ab
34	97.5±3.5	81.7±12.6	88.0±12.5 ab	367.0±86.3	256.3±72.7	300.6±40.4 b
36	78.3±24.7	73.3±37.9	75.8±28.7 b	807.0±518.8	463.3±453.6	635.1±193.8 ab
38	100.0±0.0	100.0±0.0	100.0±0.0 a	1103.7±125.4	956.7±163.6	1030.1±62.5 a
40	95.0±5.0	88.3±7.6	91.7±6.8 ab	927.7±462.6	971.3±503.9	949.5±176.9 a
General Mean	93.2±13.1	86.7±18.3	89.8±16.1	834.6±378.7	632.5±410.8	730±401.8

** Means followed by the same letter in the column, do not differ one another by Tukey test (p < .05)*

Then, as greater is the seminal quality, greater is the probability of obtaining high fertilization rate, such that the mean motility of fresh semen for all treatments was greater than 96%. By means of semen dilution, was found that the motility rates remained above 70% when tested for toxicity of cryoprotectant solution toward semen. The spermatic motility assessment after freezing is necessary for verifying the efficiency of cryoprotectant in maintaining seminal viability post-freezing. The cryopreservation process, however, may cause injuries to spermatic cells, which results in decreasing of spermatic quality [16]. But, by comparing the crude protein levels of cryoprotectants were found similar results to those reported in [28], on which there was no significant difference in using methanol or DMSO for cryopreservation of tilapia semen. Motility and motility duration pattern of the semen after freezing under different cryoprotectants may vary depending on the species. Therefore, one has to bear in mind that DMSO is the most used cryoprotectant in the majority of the cryopreservation protocols for semen, in concentrations that range from 5 to 15% [29]. Meanwhile, methanol was found to be the most effective for species such as *P. corruscans* [30].

4. CONCLUSION

According to results, we may infer that crude protein levels in the diet for broodstock of Nile tilapia (*Oreochromis niloticus*) affected the spermatic quality pattern, such that is sufficient to determine an effective semen cryopreservation. Because diet containing 32, 38 and 40% of crude protein did not deteriorate semen quality, it may be used for feeding tilapia broodstock. Therefore, because protein is an expensive input in the diet, broodstock may be fed using diet containing 32% of crude protein with no losses in spermatic quality.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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