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Effect of dietary crude glycerin on the productive performance of Nile tilapia fingerlings

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Abstract: The aim of this study was to evaluate the effect of different crude glycerin levels in the diet of Nile tilapia fingerlings (mean initial weight 0.32 ± 0.06 g, n = 450) on growth performance parameters, whole-body composition, blood glucose and liver morphology. Crude glycerin was tested at six different levels (0, 4, 8, 12, 16, and 20%) in diets containing 30% digestible protein and 3,000 kcal kg^{-1} digestible energy. After 37 days of feeding, the inclusion of crude glycerin resulted in positive effects on final weight, visceral fat, weight gain, feed conversion, specific growth rate and feed intake. The different treatments did not influence fillet yield, glycemia, survival and hepatosomatic index, but intermediate levels of inclusion decreased the area of hepatocytes. Regarding fish body composition, significant differences were found in moisture and ash contents, with no changes in crude protein and total lipid. The inclusion of crude glycerin in the Nile tilapia diet improves growth performance without negatively affecting survival rate and glycemia of fingerlings.

Key words: by-product, GIFT lineage, glycerol, histology, *Oreochromis niloticus*.

INTRODUCTION

The increase in world biodiesel production has led to an increase in crude glycerin stocks, the most important by-product obtained during oil transesterification (Stelmachowski 2011). The main glycerin component is glycerol, and both terms are treated as synonyms in several scientific studies (Balen et al. 2017).

Glycerol (propane-1,2,3-triol) is a small organic molecule rapidly absorbed by the animal gastrointestinal tract (Herting et al. 1956, Lin 1977). It is used as an important gluconeogenic substrate in the form of glyceraldehyde-3-phosphate in the liver and kidney, whereas its entry is in the form of dihydroxyacetone phosphate in the glycolytic pathway (Hagopian et al. 2008). Glycerol also participates in the lipogenesis metabolic pathway, in which it is esterified with three fatty acids and gives rise to triacylglycerol (Wang et al. 2013).

The energy values of maize and glycerol are similar (Zijlstra et al. 2009), and facilitate their exchange in animal feed composition. In addition, possible increased maize prices, or even those of other energy ingredients, may stimulate the use of crude glycerin. The apparent digestibility coefficient of energy from dietary glycerol is 0.89 for Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) (Meurer et al. 2012).

The inclusion of crude glycerin in Nile tilapia diets was recommended in up to 11% during the sexual reversal phase (Meurer et al. 2016). Although up to 10% glycerol in the diet did not impair the productive performance and health of Nile tilapia juveniles (Neu et al. 2013), a significant weight gain was achieved when included at 5.9% (Gonçalves et al. 2015).

Taking into account the first sexual maturation, its administration did not influence growth, but impaired the male spermatogenesis process (Mewes et al. 2016).

Dietary glycerol is metabolized to lipids or carbohydrates and used as an energy source by tilapia *Oreochromis mossambicus* (Peters, 1852) juveniles (Costa et al. 2017). In *O. niloticus*, dietary crude glycerol completely replaced maize for individuals ranging from 10 to 30 g (Moesch et al. 2016) and from 190 to 355 g (Santos et al. 2019). In individuals weighing over 35 g, inclusion levels greater than 10% characterize it as a lipogenic nutrient (Costa et al. 2015).

Due to the different results reported for the early life stages of this species, the aim of the present study was to evaluate the effect of dietary crude glycerin on the growth performance, survival, glycemia and liver histomorphology of Nile tilapia (*O. niloticus*) fingerlings.

MATERIALS AND METHODS

Experimental design and diets

The procedures adopted in this trial followed the Ethical Principles in Animal Experimentation and were approved by the Comissão de Ética no Uso de Animais (CEUA/Palotina-UFPR, protocol no. 11/2009).

A total of 450 sexually reversed GIFT Nile tilapia fingerlings displaying 0.32 ± 0.06 g initial weight were distributed in thirty 60 L polyethylene tanks, in a completely randomized design consisting of six treatments and five repetitions, with each experimental unit consisting of a tank containing 15 fingerlings.

The treatments consisted of practical isoproteic, isoenergetic and isophosphoric diets (Table I), divided into six levels of crude glycerin inclusion (0, 4, 8, 12, 16, and 20%). The inclusion of glycerin was performed to replace maize in the diet and considered its digestible energy values, according to Meurer et al. (2012) and Boscolo et al. (2002).

The crude glycerin was produced from soybean oil and beef tallow by BSBIOS Energia Renovável, Marialva plant, Brazil. According to the manufacturer, the product is 82-85% pure, containing 5.5% chlorides, 5.0-5.5% ash and 10-13% moisture.

Defatted soybean meal and whole maize were ground using a 0.5 mm sieve, mixed with the other ingredients and then processed into feed. The pelletizing process was carried out using a dough extruder machine (Gastromaq, model ME-20, Brazil), preceded by wetting the mixture with water at 52 °C. After pelleting, the feeds were dried in a forced ventilation oven at 55 °C for 24 h. The pellets were broken to fit the size of fish mouths, discarding particles smaller than 1.0 mm. The diets were provided to the fish four times a day (7h00, 11h00, 15h00 and 19h00) for 37 days.

Recirculation system and water quality

The water used in this experiment was obtained from an artesian well. All tanks were connected by a mechanical and biological filtration (two 500 L biofilters) water recirculation system. The daily water renewal rate was about five times the total water volume (3,000 L) and the temperature was maintained by a 3000 W heater and controlled by a thermostat. The aeration system consisted of a 58 W electromagnetic air pump connected to PVC pipes, which delivered oxygen through silicone hoses with a microporous air stone at the end, one for each experimental unit.

Each experimental unit was cleaned by siphoning twice a day to remove feces and uneaten food. pH was determined using a digital bench pH meter (MS TECNOPON mPA 210, Brazil), water temperature and dissolved oxygen were measured using a portable oximeter (ALFAKIT AT 315, Brazil). Total ammonia was determined

Table I. Formulation and proximate composition of the experimental diets for Nile tilapia fingerlings.

Ingredients (%)	Inclusion levels (%)					
	0	4	8	12	16	20
Soybean meal	69.47	70.33	71.19	72.05	72.91	73.78
Ground maize	24.41	19.54	14.67	9.80	4.93	0.00
Crude glycerin	0.00	4.00	8.00	12.00	16.00	20.00
Soy oil	2.69	2.67	2.64	2.62	2.59	2.58
Dicalcium phosphate	2.12	2.15	2.19	2.22	2.26	2.30
Vitamin-mineral premix *	1.00	1.00	1.00	1.00	1.00	1.00
Salt (NaCl)	0.30	0.30	0.30	0.30	0.30	0.30
BHT#	0.01	0.01	0.01	0.01	0.01	0.01
Calcitic limestone	0.00	0.00	0.00	0.00	0.00	0.03
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated composition						
Linoleic acid (%)	2.42	2.32	2.22	2.12	2.02	1.92
Starch (%)	24.58	21.67	18.75	15.83	12.91	9.96
Calcium (%)	0.76	0.76	0.78	0.78	0.80	0.82
Ashes (%)	7.29	7.32	7.35	7.38	7.41	7.47
Gross energy (kcal kg^{-1})	4,065.90	4,046.13	4,026.37	4,006.60	3,986.83	3,966.80
Digestible energy (kcal kg^{-1})	3,000.00	3,000.00	3,000.00	3,000.00	3,000.00	3,000.00
Crude fiber (%)	4.59	4.54	4.50	4.46	4.41	4.37
Total phosporous (%)	0.86	0.86	0.86	0.86	0.86	0.86
Fat (%)	4.67	4.45	4.22	4.00	3.78	3.57
Total lysine (%)	1.99	2.00	2.02	2.03	2.04	2.05
Dry matter (%)	89.29	85.66	82.02	78.39	74.76	71.13
Methionine + total cystine (%)	0.97	0.97	0.96	0.95	0.94	0.94
Crude protein (%)	33.52	33.53	33.55	33.57	33.58	33.60
Digestible protein (%)	30.00	30.00	30.00	30.00	30.00	30.00

***Levels per kg of diet: folic acid, 2 mg; pantothenic acid, 40 mg; biotin, 0,4 mg; copper, 20 mg; iron, 125 mg; iodine, 2 mg; manganese, 75 mg; niacin, 50 mg; selenium, 0,7 mg; vitamin A, 10,000 IU; vitamin B1, 19 mg; vitamin B12, 35 mg; vitamin B2, 20 mg; vitamin B6, 24 mg; ascorbic acid, 500 mg; vitamin D3, 5,000 IU; vitamin E, 200 IU; vitamin K3, 5 mg; zinc, 250 mg.**
#Butylated Hydroxy Toluene (antioxidant).

according to Koroleff (1976) and nitrite was determined according to Baumgarter et al. (1996). Total alkalinity and hardness were determined by the titrimetric method (Macêdo 2003).

Water chemical variables such as pH (7.77 ± 0.39), total ammonia (0.05 ± 0.02 mg L^{-1}), nitrite (0.02 ± 0.01 mg $N\text{-}NH_4$ L^{-1}), total alkalinity (88.33 ± 13.62 mg $CaCO_3$ L^{-1}) and hardness (48.63 ± 9.42 mg $CaCO_3$ L^{-1}) were monitored once a week, while temperature (29.24 ± 1.30 °C) and dissolved oxygen (6.26 ± 0.61 mg L^{-1}) were determined twice a day. According to Suresh & Bhujel (2012), the water quality parameters remained adequate for *O. niloticus* development throughout the experiment.

Growth performance parameters

At the end of the experimental period and 24 h of fasting, all fish were anesthetized with 10% benzocaine (Merck, Darmstadt, Germany) and slaughtered for total weight measurements. In addition, three specimens from each experimental unit were used to remove visceral fat and liver.

Subsequently, fillet yield (FY), visceral fat rate (VFR), weight gain (WG), feed conversion rate (FCR), specific growth rate (SGR), feed intake (FI), survival rate (SUR) and hepatosomatic index (HSI) were calculated as follows:

FY = (weight of the skinless fillet, g/body weight, g) × 100

VFR = (visceral fat weight, g/body weight, g) × 100

WG = final body weight (g) - initial body weight (g)

FCR = feed consumed (g, dry weight)/weight gain (g)

SGR = [(ln final body weight - ln initial body weight)/days] × 100

FI = 100 × total amount of the feed consumed/ [days × (initial body weight + final body weight)/2]

SUR = (final fish count /initial fish count) × 100.

HSI = (liver weight, g/body weight, g) × 100.

Determination of blood glucose level and body proximate composition

Due to the small amount of blood present in fingerlings, plasma glucose concentrations were determined using the SD CodeFree™ self-test device (SD Biosensor, Inc., Suwon, Korea), with collection performed by cutting the caudal peduncle of three individuals from each experimental unit.

Whole-body chemical composition was determined by grinding three fish from each experimental unit using a meat grinder. Moisture (Method n° 950.46), crude protein (Method n° 981.10), ether extract (Method n° 960.39) and ash (Method n° 920.153) values were obtained according to the AOAC (2005) methodology.

Histological evaluation

The collected liver fragments (three fish per tank) were fixed in a 10% formaldehyde solution for 12 hours and then stored in 70% alcohol. They were then dehydrated in an ascending series of alcohol, diaphanized in xylol, and included in paraffin, to obtain semi-partial histological sections. Microtomies were performed and histological 5 µm sections were obtained with the aid of a disposable Leica RM 2155 rotary microtome knife (Leica Microsystems GmbH, Nussloch, Germany). Histological sections were stained by the hematoxylin-eosin (HE) method. Subsequently, the areas of 30 hepatocytes per repetition were measured, totaling 150 cells per treatment. Image capture was performed using a Zeiss AxioCam ERc 5s photomicroscope (Carl

Zeiss Microscopy GmbH, Jena, Germany) under a 40x objective using the Image-Pro® Plus - Version 4.5 Computer Imaging System for Windows (Media Cybernetics, Inc., Rockville, USA).

Statistical analysis

Statistical analysis was performed using the STATISTICA software version 7.0 (StatSoft, Inc., Tulsa, USA). All data were submitted to a one-way ANOVA to compare significant differences among treatments, while Tukey's test was used to compare the means. Before performing the ANOVA, the data were checked for normality using the Shapiro-Wilk test and the data expressed as percentages were transformed into a sine-arc, however, the untransformed data are presented. Significance was set at $p < 0.05$.

RESULTS

The growth performance and survival rate of Nile tilapia fed diets containing different levels of crude glycerin inclusion are presented in Table II.

The inclusion of crude glycerin in the diet of fingerlings improved the mean values of final weight, weight gain, feed conversion rate and specific growth rate. The best results for these variables were obtained in fish fed diet containing 20% crude glycerin. The use of dietary glycerol led to decreased visceral fat percentages. In addition, a decrease in the daily feed intake rate was observed from the 8% inclusion level. On the other hand, the different diets did not influence ($p > 0.05$) fillet yield and fish survival rate.

Blood glucose levels and the hepatosomatic index were not influenced by the assessed diets ($p > 0.05$) (Table III). Conversely, hepatocyte areas were higher for fish fed the 0, 4 and 20% glycerin inclusion diets, and smaller for those fed 12% inclusion diets.

Liver cells exhibited an arrangement in endothelial cell-lined cords and sinusoids

Table II. Production responses of Nile tilapia *(Oreochromis niloticus)* fingerlings fed diets containing different levels of crude glycerin inclusion.

Variables	Inclusion level (%)					
	0	4	8	12	16	20
IBW (g)	0.33±0.01	0.32±0.00	0.33±0.00	0.32±0.01	0.33±0.01	0.33±0.01
FBW (g)	$4.97±0.23^{c}$	$5.27±0.14^{bc}$	$5.60±0.19^{ab}$	$5.58±0.13^{ab}$	$5.35±0.23^{bc}$	$5.87±0.20^{a}$
FY (%)	24.28±1.94	23.97±0.35	25.81±1.18	25.20±1.01	26.03±1.36	26.56±1.13
VFR (%)	$0.35±0.07^{b}$	$0.23±0.11^{ab}$	$0.29±0.11^{ab}$	$0.15±0.09^{ab}$	$0.15±0.12^{ab}$	$0.10±0.10^{a}$
WG (g per fish)	$4.64±0.23^{c}$	$4.95±0.13^{bc}$	$5.28±0.19^{ab}$	$5.26±0.13^{ab}$	$5.03±0.24^{b}$	$5.55±0.20^{a}$
FCR	$1.25±0.03^{d}$	$1.23±0.03^{cd}$	$1.13±0.03^{ab}$	$1.11±0.03^{ab}$	$1.16±0.02^{bc}$	$1.09±0.03^{a}$
SGR (% day^{-1})	$7.27±0.06^{d}$	$7.44±0.13^{cd}$	$7.54±0.06^{abc}$	$7.68±0.10^{ab}$	$7.51±0.09^{bc}$	$7.70±0.03^{a}$
FI (% day^{-1})	$6.43±0.16^{b}$	$6.34±0.14^{b}$	$5.81±0.23^{a}$	$5.79±0.14^{a}$	$5.98±0.13^{a}$	$5.71±0.18^{a}$
SUR (%)	97.33±3.65	97.33±3.65	97.33±3.65	100.00±0.00	100.00±0.00	96.00±3.65

Data are expressed as the means ± SD.
The values in the same row with different superscripts are significantly different ($p < 0.05$).
IBW, initial body weight; FBW, final body weight; FY, fillet yield; VFR, visceral fat rate; WG, weight gain; FCR, feed conversion ratio; SGR, specific growth rate; FI, feed intake; SUR, survival rate.

with absence of cytoplasmic vacuolizations, while most nuclei displayed a rounded shape and central position, a normal organization for this type of tissue (Figure 1a). The 12% glycerin inclusion diet resulted in a decrease in hepatocytes area (Figure 1b).

Results for the whole-body chemical composition are displayed in Table IV. The mean values of moisture and ash increased with the inclusion of crude glycerin ($p < 0.05$), and no statistical differences were observed for crude protein and ether extract contents in the whole-body.

DISCUSSION

The increase in dietary levels of crude glycerin improved the growth performance of fingerlings and glycerin may be included in up to 20% of the Nile tilapia diet. At this level, it promotes the total replacement of maize.

Crude glycerol has already been evaluated as an alternative dietary energy source for poultry, pigs and cattle, and recommended dietary levels range from 10 to 28%, without impairing zootechnical performance (Lin et al. 1976, Lammers et al. 2007, San Vito et al. 2015). Concerning fish, Li et al. (2010) found that the channel catfish, *Ictalurus punctatus* (Rafinesque, 1818), can utilize about 10% dietary glycerol without adverse effects on feed consumption, weight gain, and feed efficiency ratio. Higher levels reduced the weight gain, feed efficiency and fillet yield of this species.

No significant effects were observed for FBW, WG and FCR in Nile tilapia juveniles fed diets containing up to 15% purified glycerol (Costa et al. 2015). On the other hand, Gonçalves et al. (2015) observed no negative effects up to 12% dietary inclusion, whereas an increase in glycerol inclusion to 16% worsened WG, FCR, protein retention efficiency and SGR.

In the present study, the decreases noted for mean FCR and the increased FBW due to the use of dietary glycerol indicate that fish energy requirements were met. Inclusion of non-protein energy has been shown to spare dietary protein from catabolism to provide energy and enhance its utilization for growth (Ghanawi et al. 2011). These results are similar to those reported by Moesch et al. (2016), who observed the lowest FCR in fingerlings from 10 to 30 g fed total maize substitution by crude glycerol diets. On the other hand, Gonçalves et al. (2015) observed an increase in FCR of Nile tilapia fed dietary 16% glycerol. This may be related to the way the food was supplied, as glycerol leaching to the water may occur before animal consumption. Glycerol displays a hygroscopic nature and significant

Table III. Blood glucose and hepatic parameters of Nile tilapia *(Oreochromis niloticus)* fingerlings fed diets containing different levels of crude glycerin inclusion.

Variables	Inclusion level (%)					
	0	4	8	12	16	20
Blood glucose (mg dL^{-1})	42.92±2.23	47.67±9.19	49.53±7.16	43.42±1.40	56.73±5.07	51.33±11.89
HSI (%)*	1.15±0.36	1.17±0.14	1.13±0.16	1.16±0.13	1.28±0.16	1.03±0.09
Hepatocyte area (μm^2)	202.96±5.43^{a}	202.72±7.92^{a}	187.22±18.63^{ab}	169.67±6.41^{b}	189.51±11.11^{ab}	195.61±5.51^{a}

Data are expressed as the means ± SD of three replicates.
Values in the same row with different superscripts are significantly different ($p < 0.05$).
***HSI, hepatosomatic index.**

water solubility, due to the presence of three hydroxyl groups in its structure (Beatriz et al. 2011).

The mean value of 25.31% for FY were lower than those described by Moesch et al. (2016) for Nile tilapia fingerlings, of 32.97%. This difference can be attributed to the size difference of the fish used in each of these studies. In the present study, besides the fact that the different treatments did not affect FY, the observed means were close to those reported by Silva et al. (2016) for this species in the harvesting and industrial processing phase (350-1,000 g).

The inclusion of crude glycerin did not affect the survival of the Nile tilapia fingerlings. The high survival rate observed herein may have been influenced by non-variations in initial mean fish weight and appropriate stocking density. In cichlids, larger males are more aggressive and tend to become the dominant fish (Beechine 1992), and unevenness of initial stocks favor increased mortality.

Crude glycerol may contain some impurities resulting from the biodiesel production process, such as methanol, inorganic salts and even trace amounts of heavy metals (Pyle et al. 2008, Jun et al. 2010, Pagliaro & Rossi 2010) and can cause fish metabolism disturbances even at low concentrations (Öner et al. 2008). The liver performs numerous functions vital to the vertebrate metabolism (Grisham 2009) and hepatocytes are considered the primary toxicity target for several compounds (Zelikoff 1998). However, no negative interference on growth, histological analysis and health was observed throughout the experiment.

The histological analysis of the liver revealed the absence of vacuolization, which can be explained by glycogen deposition in the cell cytoplasm to the detriment of lipids, since the liver cell cytoplasm is influenced by the nutritional status of the animal and greater glycogen deposition is observed when an adequate diet is provided (Rigolin-Sá 1998).

Hepatocyte area was lower in treatments where glycerin inclusion was intermediate, indicating a dose-dependent response. The efficiency of the gluconeogenic role of glycerol was also dose-dependent in juvenile tilapia livers, with absorbed ^{14}C-glycerol found deposited mainly as a carbohydrate (Costa et al. 2017). In rainbow trout liver, the glycerol incorporation into glycogen was higher than into lipids (Lech 1970). Retained glycerol also seems to primarily

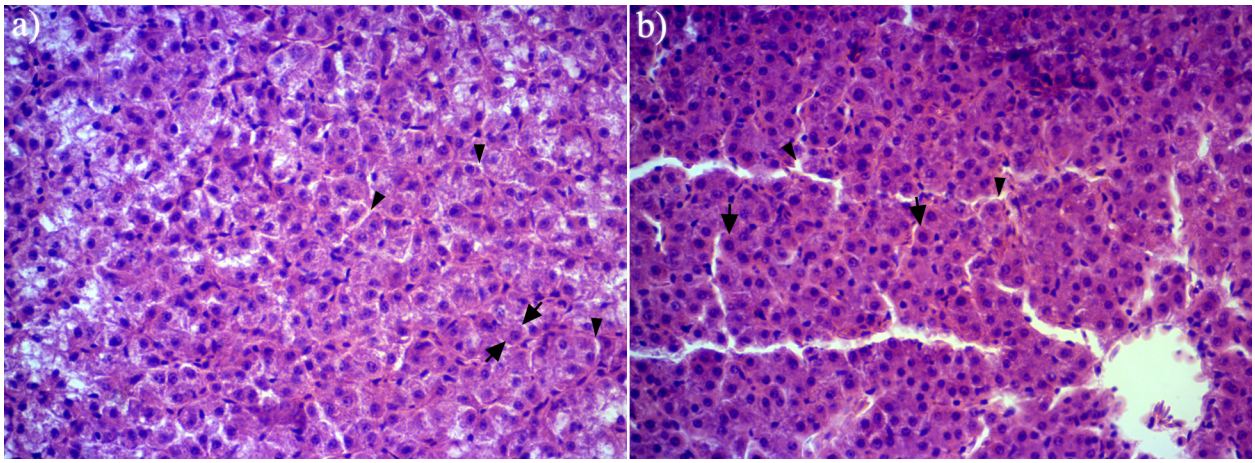

Figure 1. Photomicrography of the histological aspects of Nile tilapia hepatic tissue fed diets containing different crude glycerin levels: a) Hepatocyte arrangement surrounded by sinusoidal capillaries (control treatment); b) Hepatocytes presenting decreased area. The arrows indicate hepatocytes and the arrowheads indicate sinusoidal capillaries. HE staining. 40× objective lens.

have followed gluconeogenesis, rather than the lipogenesis pathway in *O. mossambicus* tilapia (Costa et al. 2017).

The hepatosomatic index was not influenced by the different diets, despite the decrease in the area of hepatocytes observed in some treatments. In contrast, a lower HSI at 16% inclusion was observed in fingerlings between 10 and 30 g, without significant differences between hepatocyte areas (Moesch et al. 2016). On the other hand, increased dietary glycerol inclusion did not affect the HSI of juveniles, despite increasing triglyceride content in liver (Costa et al. 2015). In channel catfish, dietary glycerol levels above 10% caused increased HSI and decreased liver fat content (Li et al. 2010).

Although the amount of starch decreased as maize was replaced by glycerin, fish glycemia was not influenced by the assessed diets. The values obtained for blood glucose remained as described as standard for *O. niloticus* in intensive cultivation systems (Tavares-Dias 2015), lower than those observed for Nile tilapia juveniles (Neu et al. 2013, Costa et al. 2015). Conversely, the addition of glycerol caused increased blood glucose in channel catfish (Li et al. 2010) and rainbow trout, resulting in hyperglycemia, indicating that glycerol was converted to glucose which is not an efficient energy source for carnivorous species (Menton et al. 1986).

In the present study, the inclusion of dietary glycerin caused a significant increase in ash and moisture contents of the whole-body, while protein and lipid deposition were not affected. In larger Nile tilapia, the proximate composition of the whole-body was not affected by diets containing up to 16% glycerol (Gonçalves et al. 2015). In addition, the ash content was slightly lower than that reported by Neu et al. (2013) and Gonçalves et al. (2015) for Nile tilapia juveniles (initial weight of 29.15 g and 7.73 g, respectively).

The amount of water, proteins, carbohydrates, fats, and minerals deposited in living tissues is not constant, but rather changes with fish size (Bureau et al. 2000). A strong relation between ash and water mass is observed, which reflects a strong relation between ash and protein mass, where the ash:water ratio increases slightly with body size (Breck 2014). In addition, lipid deposition reduces body water content and tends to increase with fish size (Bureau et al. 2000, Santos et al. 2012). When lipids are deposited in tissues, they generally substitute water and, consequently, protein gains generally result in significant live weight gain, whereas lipid gains generally result in little or no weight gain (Bureau et al. 2000).

Table IV. Whole-body composition of Nile tilapia *(Oreochromis niloticus)* fingerlings fed diets containing different levels of crude glycerin inclusion.

Variables (%)	Inclusion level (%)					
	0	4	8	12	16	20
Crude protein	14.64±0.59	14.67±0.58	14.59±1.53	14.16±1.37	14.23±0.87	15.17±1.16
Ether extract	3.23±1.47	2.14±0.60	3.13±0.74	2.79±0.37	3.03±0.68	2.68±0.53
Moisture	74.54±2.31^{b}	76.87±0.51^{ab}	76.83±0.45^{ab}	76.95±1.73^{ab}	77.38±0.29^{a}	77.49±1.02^{a}
Total ash	2.45±0.20^{b}	2.53±0.16^{ab}	2.76±0.12^{ab}	2.72±0.33^{ab}	2.77±0.11^{ab}	3.20±0.79^{a}

Data are expressed as the means ± SD of three replicates.
Values in the same row with different superscripts are significantly different ($p < 0.05$).

The results indicate that the use of crude glycerin as a dietary energy source was efficient for *O. niloticus* fingerlings between 0.3 and 6 g, and can totally replace dietary maize. In conclusion, the inclusion of crude glycerin in the diet of Nile tilapia improves growth performance of fingerlings and decreases the area of hepatocytes at intermediate levels with no negative effect on survival rate and plasma glucose concentration.

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