Effects of starvation and refeeding cycles on spermatogenesis and sex steroids in the Nile tilapia *Oreochromis niloticus*

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ABSTRACT

Food restriction is part of the life cycle of many fish species; however, nutritional deficiency may negatively influence gametogenesis and gonadal maturation. The aim of this study was to evaluate the effects of food restriction on the spermatogenesis of Nile tilapia. For this, adult males were submitted to starvation and re-feeding cycles (alternating periods of starvation and feeding) for 7, 14, 21, and 28 days. After 7 days of starvation, glycaemic and lipid levels were significantly reduced, followed by reduction of plasma testosterone (T) and 11-ketotestosterone (11-KT). In addition, reduced proliferation of spermatogonia and increased apoptosis of spermatocytes, spermatids, and spermatozoa was observed in starvation groups. In the refeeding groups, the sex steroids and the proportion of germ cells had no significant alterations compared to the control group, except for spermatozoa. In this sense, the present study suggests that starvation after 7 days progressively reduces T and 11-TK, resulting in damage to the production of spermatogenic cells, while refeeding may delay spermatogenesis but does not lead to testicular impairment.

1. Introduction

Nutritional deficiency is associated with metabolic changes that may be reflected in the circulating concentrations of hormones such as testosterone and gonadotrophins, which regulate spermatogenesis and testicular maturation (Cheah and Yang, 2011; Churchill et al., 2019; Gilad et al., 2018; Grizard et al., 1997). In addition to hormonal impairment, nutrient deficiencies interfere with spermatogenesis, especially deficiency of ions and vitamins that act protecting the testis from oxidative damage and improve sperm quality, as well as are related with the differentiation of spermatogonia (Alonge et al., 2019; Chung et al., 2009). In fish, long fasting periods can also negatively influence the reproductive potential depending on the species, sex, age of the animal, and its capacity to mobilise the energy reserves (Jobling, 2016; Luquet and Watanabe, 1986). However, the mechanisms by which spermatogenesis is influenced by starvation remain unknown.

Fish spermatogenesis is a cyclic process finely regulated by endocrine, paracrine, and autocrine factors involving complex interactions between somatic and germ cells (Batlouni et al., 2009; Schulz et al., 2010). The germ cells development can be divided into three main phases: spermatogonial or proliferative, when undifferentiated spermatogonia originate type A and type B spermatogonia; spermatocytary or meiotic phase, when meiotic division originates primary and secondary spermatocytes; and spermiogenesis, when a series of morphological changes leads to the differentiation of spermatids into spermatozoa (Schulz et al., 2010). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) regulate the production of sex steroids, testosterone, and 11-ketotestosterone by Leydig cells, and these hormones mainly act on the mitotic proliferation and differentiation of spermatogonia and spermiation (de Waal et al., 2009; Haider, 2007).

In testes, a complex and well organised balance between proliferation and cell death maintains tissue homeostasis (Ribeiro et al., 2017; Russell et al., 2002; Wang et al., 2012). During spermatogenesis, apoptosis is responsible for maintaining the appropriate number of germ cells supported by the Sertoli cells and for elimination of defective germ cells (Barnes et al., 1998; Richburg, 2000; Shaha et al., 2010).

Stressors such as thermal shock, exposure to toxic substances, hormonal changes, and starvation increase germ cells apoptosis, impairing sperm production (Cheah and Yang, 2011; Lascarez-Lagunas et al., 2014; Shaha et al., 2010; Wang et al., 2012, 2017).

Food deprivation and fasting are common in the life cycle of many fish species in response to environmental fluctuations under both natural and farming conditions. The physiological and metabolic response to fasting is variable among species, with carnivorous fish being better adapted to periods of food restriction than herbivores and omnivores (Gadomski and Petersen, 1988). Nile tilapia *Oreochromis niloticus* is a omnivorous fish that is very attractive for aquaculture due to its large size, rapid body growth, flesh palatability, ease of reproduction, adaptability to a wide range of environmental conditions, resistance to diseases and infections, and stress tolerance (Little and Hulata, 2000). In addition, males have favourable features to study the structure and function of the testes since they present cystic organisation of germ cells that allows a reliable analysis of spermatogenesis (Melo et al., 2016; Vilela et al., 2003). Although studies have associated food deprivation with reduced reproductive potential in fish species (Pérez-Jiménez et al., 2007; Pikle et al., 2017), knowledge about the consequences of food restriction at metabolic, hormonal, and morphological levels is still incipient. In this sense, the goal of this study was to evaluate the effects of refeeding cycles (alternating periods of starvation and feeding) and starvation on blood biochemical parameters, androgens concentration, and spermatogenesis of Nile tilapia.

2. Material and methods

2.1. Experimental design and fish sampling

The experiment was conducted at the Laboratory of Aquaculture (LAQUA) of the Universidade Federal de Minas Gerais (UFMG), and the study was approved by the Ethics Committee on Animal Use (CEUA, UFMG 67/2017). For acclimation, a breeding stock of 94 adult males of *O. niloticus* (GIFT lineage, age between 6 and 8 months, 24.31 ± 0.31 cm total length, 268.37 ± 11.21 g body weight) were equally distributed in six 1 m^3 culture tanks and kept for 30 days with a mechanical and biological filtration system under controlled conditions. The culture conditions of the experiment were maintained by heaters with thermostat for stabilization of temperature, continuous supplementary aeration by an air diffuser and the photoperiod was kept at 12 h light to 12 h dark. Throughout the experiment period, the water parameters of the tanks were monitored once a week using a Horiba U51 multi-parameter probe and the average values were obtained: temperature 29.06 ± 0.04 °C, dissolved oxygen 7.06 ± 0.02 mg/l, pH 7.31 ± 0.04, conductivity 0.87 ± 0.11 mS/cm, total dissolved solids 0.51 ± 0.02 g/l, and salinity 0.36 ± 0.02 (values represent mean $\pm$ SEM).

During the experiment, the fish were divided into three groups (control $n = 30$ fish, refeeding $n = 32$, and starvation $n = 32$) with a duplicate tank for each group. In the control group, fish were fed *ad libitum* with commercial feed containing 32% crude protein, and three/four animals per tank were collected at the following sampling times (7, 14, 21 and 28 days). In the refeeding group, four animals per tank were collected at the following sampling times: refeeding 1 (day 14, after a week of starvation, followed by a week of *ad libitum* feeding), refeeding 2 (day 21, after a week of starvation, followed by a week of feeding, and another week of starvation), refeeding 3 (day 28, after alternating two weeks of starvation and feeding). In the starvation group, fish were submitted to total food restriction, and four animals per tank were collected with 7, 14, 21 and 28 days (starvation 7D, 14D, 21D and 28D). The fish were euthanized with 285 mg/l eugenol solution following the ethical principles established by the National Council for Animal Experimentation Control (CONCEA). From these animals, the body weight (BW), total length (TL), and gonad weight (GW) were obtained and the gonadosomatic index (GSI = 100GW/BW) and Fulton

condition factor (K = 100BW/TL^3) were calculated. Blood plasma and testis samples were obtained for analyses using different techniques.

2.2. Light and electron microscopy

For histology, the middle section of the testis of each specimen were fixed in Bouin's fluid for 24 h, embedded in paraffin, sectioned at 5 μm thickness, and stained with haematoxylin-eosin. For electron microscopy, testis samples were fixed in Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1 M sodium phosphate buffer pH 7.3 for 24 h at 4 °C and post-fixed in 1% osmium tetroxide with 1.5% potassium ferrocyanide for 2 h and then embedded in Epon/Araldite plastic resin. The ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a Tecnai G2-135 12 Spirit 120 kV transmission electron microscope (FEI Company, Hillsboro, OR, USA).

2.3. Blood biochemical parameters

For biochemical analyses, blood samples of each fish were collected in a heparinized syringe and transferred to Eppendorf tubes at 4 °C, also heparinized. The Accu-Chek kit with a minimum detection limit of 10 mg/dl and satisfactory accuracy according to European standard EN ISO 15197 was used for determination of glucose. Then, 2 ml blood samples were centrifuged for 4 min at 3000 rpm and stored in a freezer at −80 °C. Concentrations of total cholesterol and triglycerides were determined using BioTechnique kits following the manufacturer's recommendations.

2.4. TUNEL in situ assay

In order to detect the apoptotic DNA fragmentation, testis samples of each fish were fixed in 4% paraformaldehyde solution for 24 h at 4 °C, embedded in paraffin, and sectioned at 5 μm thickness. The sections were subjected to the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay using the FragEL DNA fragmentation detection kit QIA 33 (Calbiochem, San Diego, CA, USA), following the manufacturer's protocol. For this, the sections were washed in phosphate buffered saline (PBS) pH 7.4, treated with 20 μg/ml proteinase K in PBS for 15 min, and then treated with and 3% hydrogen peroxide (H2O2) in PBS for 15 min to inactivate the endogenous peroxidase. Next, the sections were incubated with terminal deoxynucleotidyl transferase (TdT) and biotinylated deoxynucleotides for 90 min at 37 °C. After this, the sections were incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min at room temperature and revealed with diaminobenzidine (DAB, Sigma Aldrich's Corp., St. Louis, MO, USA) in PBS for 2 min and counterstained with haematoxylin. In the negative control, treatment with TdT/deoxynucleotides was omitted.

2.5. Immunohistochemistry

Testis samples were submitted to immunohistochemistry reaction for detection of the antibody Ki67 (Sigma), a marker of cell proliferation/mitosis. For this, samples were fixed in 4% paraformaldehyde solution for 24 h at 4 °C, embedded in paraffin, and sectioned at 5 μm thickness. For endogenous peroxidase blocking, the sections were incubated with 3% H_2O_2 in PBS for 30 min at room temperature. Subsequently, the sections were subjected to antigen recovery with 10 mM sodium citrate buffer pH 6.0 for 20 min at 96 °C. Non-specific binding was blocked with 2% bovine albumin in PBS buffer for 30 min at room temperature. Then, the sections were incubated with the primary antibody Ki67 in a humid chamber at 4 °C overnight at 1:100 dilution. Following, the sections were submitted to the Dako EnVision™ + Dual Link System-HRP (kit K4063 Dako), revealed with DAB, and counterstained with haematoxylin. For the negative control, one of the sections did not receive the primary antibody.

2.6. Plasma sex steroids

For the determination of testosterone, 11-ketotestosterone and cortisol, plasma samples of each fish were stored at −80 °C. The samples were submitted in duplicate to ELISA assay using 11-ketotestosterone kit (Cayman Chemical, Michigan, USA), testosterone kit (Cayman, Instruments GmbH, Marburg, German), and cortisol kit (DRG Instruments GmbH, Marburg, German) following the manufacturers' protocols, as described previously (Weber et al., 2019). The sensitivity of the assays was 3.9 pg/ml (testosterone), 0.8 pg/ml (11-ketotestosterone), and 2.5 ng/ml (cortisol).

2.7. Morphometry

For morphometry of spermatogenesis, four animals per sampling/ group were randomly chosen and digital histological images were obtained using an image analysis system with Zeiss Axiovision software coupled to an Axioplan 2 Zeiss Microscope. To evaluate the proportion of the testicular components and positive cells for Ki67 and TUNEL, 30 randomly chosen fields per animal were photographed at 400× magnification. Spermatogenic cells were identified according to features previously established (Schulz et al., 2010). The images were analysed in ImageJ software using a grid of 540 points. The proportion of somatic and germ cells was determined based on the number of points on the testicular cells in relation to the total points analysed in each field (Melo et al., 2016; Ribeiro et al., 2017).

2.8. Statistical analyses

Data were statistically analysed using Minitab 16.1 and GraphPad Prism 6.03 software. The data did not present normal distribution, so Kruskal-Wallis test followed by Dunn's *post-hoc* test were used. The results were considered significant at 95% confidence interval and values were expressed as mean ± S.E.M.

3. Results

3.1. Testicular alterations and biological indices

During the experiment, animals from the control and refeeding groups did not present relevant histological alterations in the testes (Fig. 1A). However, after 7 days of starvation, germ cells at different stages of development had highly compacted chromatin (Fig. 1B), and type A undifferentiated spermatogonia (A_{und}) with cytoplasmic vacuolization were observed (Fig. 1C). In the fish collected with 14, 21, and 28 days of starvation, inflammatory infiltrate, spermatogonia and spermatocytes in the tubular lumen, and hyperplasia of Leydig cells was also detected (Fig. 1D and E). Furthermore, the animals submitted to 28 days of starvation had testicular regions with disorganisation and degeneration of the seminiferous tubules (Fig. 1F). In the ultrastructure, the sampling times starvation 7D, 14D, 21D, and 28D showed spermatocytes with vacuolated cytoplasm and degenerate mitochondria being released into the lumen together with cellular debris and apoptotic bodies (Fig. 1G–I). At this time, the spermatids presented abnormalities in the chromatin compaction, degeneration of the midpiece with inefficient elimination of cytoplasm material (Fig. 1J-L). In addition to the histological and ultrastructural changes described above, the animals submitted to starvation and refeeding cycles presented testes with a significantly lower GSI when compared to the control group (p < 0.05), although the Fulton condition factor did not show any variation among groups (Fig. 1M and N).

3.2. Morphometry of the germ and somatic cells

In the spermatogonial phase, the proportion (%) of type A undifferentiated and differentiated spermatogonia (A_{und} and A_{diff}) was

reduced in the starvation 21D and 28D when compared to the other sampling times. In contrast, the proportion of type B spermatogonia did not show a significant difference between the treatments (Fig. 2A). Starvation for 7, 14, 21 and 28 days affected the spermatocytary phase, leading to a significant reduction (p < 0.05) in the proportion of spermatocytes (Fig. 2B), but no significant variation was detected in the sampling times refeeding 1, 2 and 3 compared to the control. During spermiogenesis, no significant variation was found in the proportion of spermatids, but a significant reduction in spermatozoa ratio (p < 0.01) was observed in all treatments when compared to the control (Fig. 2B).

The proportion of Leydig cells was significantly higher at all the sampling times in animals submitted to starvation when compared to the control group (p < 0.01) (Fig. 2C). In the animals collected in the sampling times refeeding 1, 2 and 3, an increase in the Leydig cells ratio was also observed, but without statistical difference when compared to the control group. In addition, there was a significant reduction in the proportion of Sertoli cells in the starvation 21D (Fig. 2C). Interstitial tissue did not show significant changes in the treated groups compared to the control, except on 14 days of total food restriction (starvation 14D) when there was a significant increase (p < 0.05) in relation to the other groups (Fig. 2C).

3.3. Blood biochemical parameters

In the sampling times starvation 7D, 14D, 21D, 28D and refeeding 2 the blood glucose was significantly lower than that observed in the control group (p < 0.05) (Table 1). Plasma levels of total cholesterol and triglycerides were higher in the control group when compared to the experimental groups, except for refeeding 1 (p < 0.05).

3.4. Plasma concentrations of androgens and cortisol

The animals submitted to 7, 14, 21, and 28 days of starvation presented a significant reduction (p < 0.05) in plasma concentrations of 11-ketotestosterone and testosterone compared to the control group, with a drastic reduction of these hormones at 28 days of total food restriction (Fig. 3A and B). The sampling times refeeding 1 and 3 did not show significant variations for both androgens when compared to the control, while the sampling time refeeding 2 presented a significant reduction for both androgens. Plasma cortisol levels were significantly higher in the starvation 7D, 14D and refeeding 2 when compared to the control (p < 0.05) but no significant variation was found between the starvation 28D and control group. During starvation, a cortisol peak was found at 7 days after starting the treatment, and it was gradually reducing until reaching values close to the control group after 21 days of total food restriction (Fig. 3C).

3.5. Cell proliferation and apoptosis

Since the refeeding group showed few alterations in the morphological analyses compared to the control, cell proliferation and apoptosis were only evaluated in the control and starvation groups. The proportion of A_{und}, A_{diff}, and Sertoli cells positive for Ki67 showed a significant decrease after 21 and 28 days of total food restriction when compared to the control group (Fig. 4 A, B and E). In addition, after 7 days of starvation, a significant increase of Leydig cells labelled by Ki67 was observed. In the sampling times starvation 14D, 21D, and 28D, the proportion of TUNEL-positive spermatocytes, spermatids, and spermatozoa increased significantly and progressively over the course of total food restriction (Fig. 4 C, D, and F).

4. Discussion

Response to nutritional stress encompasses metabolic and hormonal changes, which negatively reflect on fish spermatogenesis, as also reported in mammals (Grizard et al., 1997; Yu et al., 2016). In general,
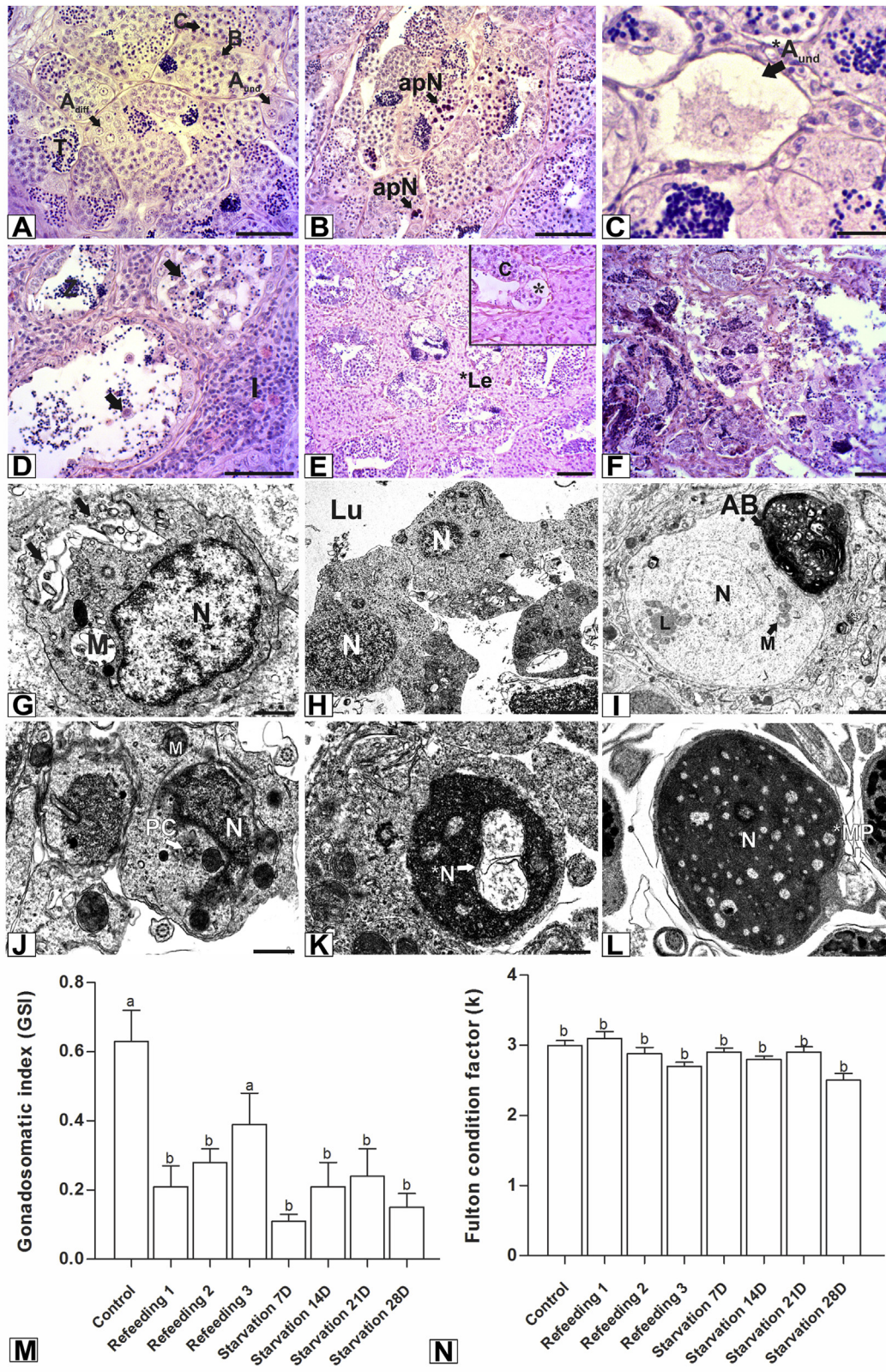
Fig. 1. Histological sections stained with haematoxylin-eosin (A–F), ultrastructural sections (G–L), and biological indices (M, N) of testes of Nile tilapia. A: Control group containing type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{dif}), type B spermatogonia (B), spermatocytes (C) and spermatids (T) without morphological alterations. B: Cysts containing cell clusters with apoptotic nucleus (apN). C: A_{und} with dilatation and cytoplasmic vacuolization. D: Spermatogonia inside the lumen of the seminiferous tubule (black arrow) and inflammatory infiltrate with acidophilic granulocytes in the interstitial tissue (I). E: Hypertrophy and hyperplasia of Leydig cells (*Le) and empty cysts (*). F: Seminiferous tubules in degeneration. G–H: Spermatocytes in degeneration with vacuolization in the cytoplasm (black arrow) being released in the tubular lumen together with cellular debris. I: Apoptotic body (AB) next to a spermatogonia. J–L: Anomalies in the compaction of chromatin and degeneration of the midpiece (*MP) in spermatids. Nucleus (N); mitochondria (M); proximal centriole (PC); lumen (Lu); lisossome (L). Different letters indicate significant difference among sampling times. Scale bars (A, B, D-F) 50 μm, (C) 20 μm, (G, J-L) 500 nm, (H) 1 μm and (I) 2 μm.
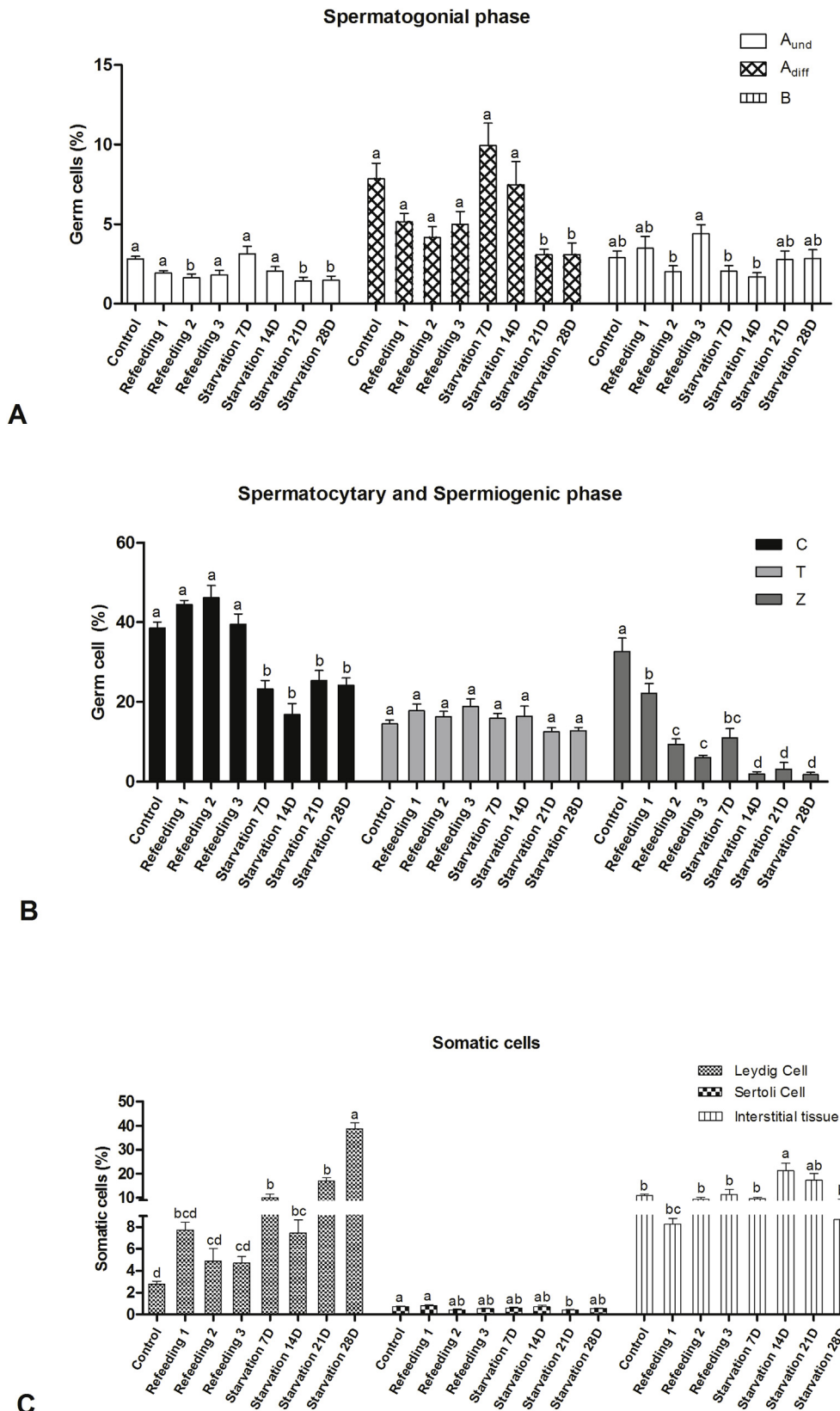
Fig. 2. Proportion of germ and somatic cells during spermatogenesis of Nile tilapia submitted to refeeding cycles and starvation (A–C). Type A undifferentiated spermatogonia (A$_{und}$), type A differentiated spermatogonia (A$_{dif}$), type B spermatogonia (B), spermatocytes (C), spermatids (T) and spermatozoa (Z). In each spermatogenic germ cell, different letters indicate significant difference among sampling times (p < 0.05).

Table 1
Blood biochemical parameters (mg/dL) in Nile tilapia submitted to refeeding and total food restriction.

Groups	Glucose	Total cholesterol	Triglycerides
Control	56.7 ± 5.10[a]	238.1 ± 33.23[a]	228.05 ± 22.39[a]
Refeeding 1	68.0 ± 2.90[a]	175.0 ± 9.45[b]	141.55 ± 26.39[b]
Refeeding 2	20.6 ± 1.70[b]	107.5 ± 10.15[bc]	88.59 ± 6.18[b]
Refeeding 3	34.7 ± 3.00[a]	102.3 ± 11.45[bc]	112.52 ± 11.67[b]
Starvation 7D	18.5 ± 0.80[b]	83.1 ± 4.30[c]	95.05 ± 12.39[b]
Starvation 14D	27.3 ± 2.90[b]	81.7 ± 6.90[c]	93.33 ± 6.39[b]
Starvation 21D	20.6 ± 1.60[b]	84.10 ± 13.23[c]	131.51 ± 1.79[b]
Starvation 28D	20.6 ± 1.60[b]	73.41 ± 9.70[c]	93.42 ± 5.39[b]

Different letters in the same columns represent significant difference between the sampling groups for each biochemical parameter ($p < 0.05$).

females are more sensitive than males to the effects of food restriction (Grone et al., 2012; Ridelman et al., 1984), but few studies have been reported the effects of fasting on fish spermatogenesis (Pikle et al., 2017). In the Nile tilapia, severe morphological changes were observed in the germ cells development during starvation, and there was a decrease in the percentage of spermatogonia, spermatocytes, and spermatozoa that may be related to increased apoptosis and reduced cell proliferation.

The gonadosomatic index (GSI) is an indicator of the gonadal development widely used to evaluate the effects of different environmental stressors on fish reproduction (Collins and Anderson, 2002; Duston and Saunders, 1999; Frantzen et al., 2004; Grone et al., 2012). Studies have shown that GSI is negatively affected by nutrient deprivation in both sexes as observed in the European seabass *Dicentrarchus labrax,* amago salmon *Oncorhynchus masou ishikawae,* and catfish *Clarias gariepinus* (Chatzifotis et al., 2011; Silverstein and Shimma, 1994; Suchiang and Gupta, 2011). In addition, findings of the present study show that GSI reduction during food restriction is associated with changes in testicular morphology. Although the tissue response to nutritional deficiency varies among fish species depending on the exposure period and their capacity to mobilise energy reserves, severe tissue changes such as cell degeneration and structural disorganisation have been reported after long periods of food restriction (Pikle et al., 2017; Suchiang and Gupta, 2011). These changes may be related to the poor nutritional status, culminating in the reduction of androgens and elevation of cortisol, as detected in the Nile tilapia of this study.

Short periods of food restriction cause nutritional deficiencies that increase the plasma lipid levels. However, after long periods of food restriction, in addition to lipid deficiency, lipogenesis is depressed while lipolysis is accelerated (Yu et al., 2016). The reduction of lipid levels, mainly cholesterol during food restriction, has been observed in several fish species (Pérez-Jiménez et al., 2007; Prasad, 2015; Rossi et al., 2015). Cholesterol is required for testosterone synthesis in the Leydig cells (Wayne Hou et al., 1990). In this sense, the reduction of testosterone levels as well as 11-ketotestosterone after 7 days of starvation found in this study may be due to the reduction of plasma total cholesterol levels, which has been well documented in humans and other mammalian species (Eacker et al., 2008; Morrison et al., 2002; Velasco-Santamaría et al., 2011).

In addition to sex steroids, glucocorticoids, especially cortisol, may act negatively at all stages of spermatogenesis in both fish and mammals (Dey et al., 2010; Milla et al., 2009; Weber et al., 2002). High plasma levels of cortisol are related to the reduction of the steroidogenic potential of Leydig cells by reducing their sensitivity to the luteinizing hormone (LH) (Orr and Mann, 1992; Whirledge and Cidlowski, 2010). In this study, high levels of cortisol are apparently related to decreased 11-ketotestosterone, increased apoptosis, and decreased of germ cell proliferation.

Proliferation of Leydig cells along with a higher proportion of these cells after 7 days of starvation may be a compensatory mechanism to
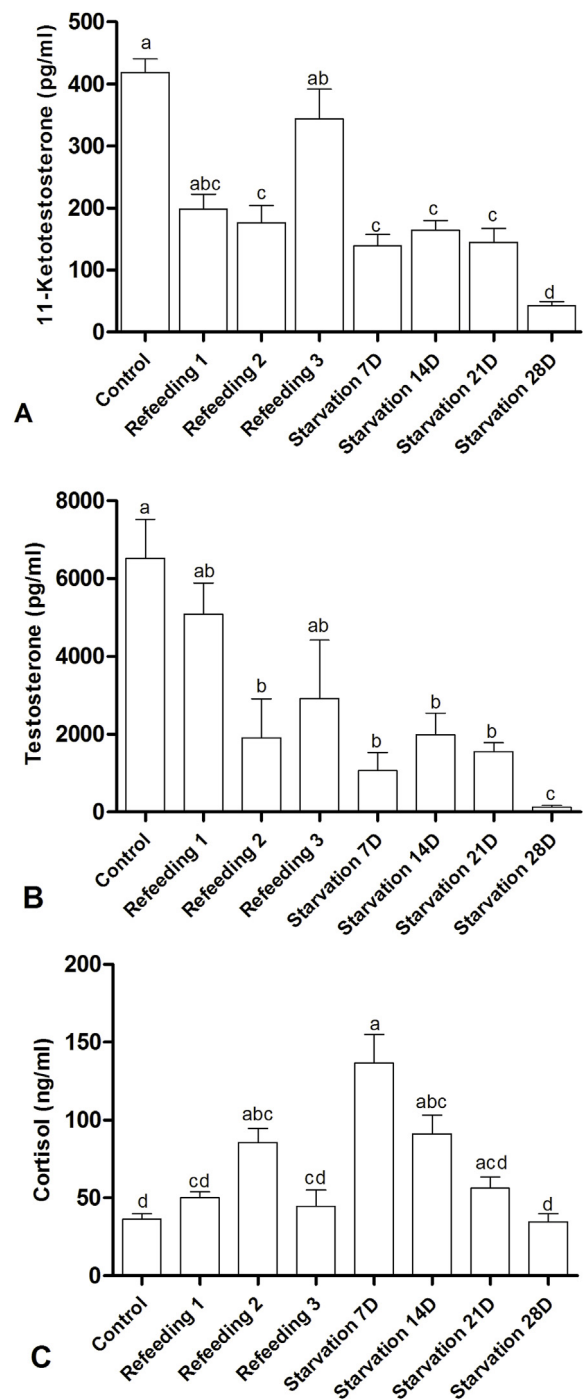
Fig. 3. Effect of refeeding cycles and starvation on androgen and cortisol concentrations in Nile tilapia. Plasma levels of 11-ketotestosterone (A), testosterone (B) and cortisol (C). Different letters indicate statistical difference among sampling times ($n = 8$ per sampling, $p < 0.05$).

increase steroidogenesis and androgen production in order to support the spermatogenesis and sperm production in the Nile tilapia under starvation conditions. Leydig cells proliferation in response to low testosterone concentration was observed in rats during the fetal period (Mylchreest et al., 2002). In fish testes, testosterone is converted to 11-ketotestosterone, the main androgen during fish spermatogenesis (Gazola and Borella, 1997; Ohta et al., 2007). The 11-ketotestosterone acts by suppressing the release of anti-Müllerian hormone (AMH) and stimulating the production of activin B by the Sertoli cells, and these molecules act antagonistically, as activin B being a stimulator and AMH
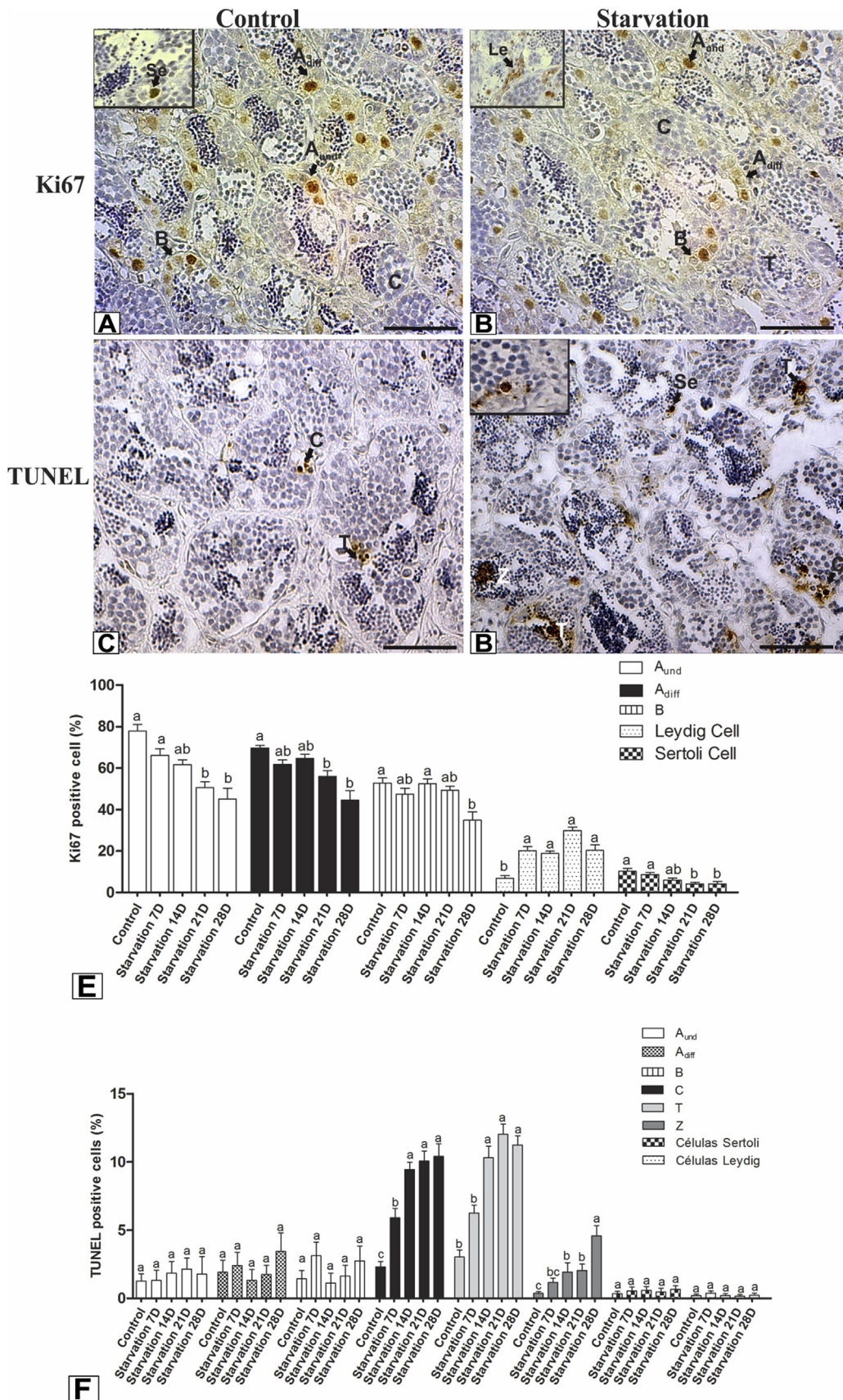
Fig. 4. Immunohistochemistry for Ki67 (A–B) and TUNEL assay (C–D) in testes of Nile tilapia from control and starvation groups. Type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{dif}), type B spermatogonia (B), spermatocytes (C), spermatids (T), spermatozoa (Z), Leydig cells (Le) and Sertoli cells (Se). Proportion of Ki67 and TUNEL positive cells in control and starvation groups (E–F). Different letters indicate statistical differences among sampling times ($p < 0.05$). Scale bars (A–D) 50 μm.

Fig. 5. Effects of refeeding cycles and starvation in Nile tilapia spermatogenesis and its relationship with sexual hormones and biochemical parameters. 11-ketotestosterone (11 KT); Testosterone (T); Cortisol (C); Cholesterol (CH) Triglycerides (TG), Type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{dif}), type B spermatogonia (B), spermatocytes (C), spermatids (T) and spermatozoa (Z).

an inhibitor of spermatogonial proliferation (Ohta et al., 2007; Schulz et al., 2010; Skaar et al., 2011). Moreover, 11-ketotestosterone induces the synthesis of 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) that is required for early meiosis and spermiation (Milla et al., 2009; Ozaki et al., 2006). In this sense, the low concentration of testosterone and 11-ketotestosterone observed in this study may be associated with a reduction in the spermatogonial proliferation (Ki67-positive cells) and a reduction in the proportion of spermatogonia, spermatocytes, and spermatozoa after 7 days of starvation. In addition, the survival and development of germ cells depend on their association with Sertoli cells, which showed a significant reduction in proliferation at 21 days of starvation.

Due to reproductive strategies or low food availability, some animals experience lifelong feeding and fasting periods (Pérez-Jiménez et al., 2007). In addition, alternating fasting and feeding periods may induce compensatory growth and decrease the loss of water quality and fish farming costs (Jobling, 2016; Morshedi et al., 2017). Our data suggest that refeeding changes the metabolic levels of carbohydrates and lipids as also reported by Pérez-Jiménez et al. (2007); however, the animals submitted to refeeding 1 3 did not present a marked reduction in the analysed androgen levels and kept their testicular architecture and germ cell morphology. The high proportion of spermatocytes observed in the refeeding group suggests a delay in germ cells recruitment to spermatogenesis progression. Indeed, changes in temperature, fasting, or exposure to xenobiotics may induce breakdown of DNA in primary spermatocytes and delay the progression of the meiotic phase of spermatogenesis (Alvarenga and França, 2009; Liu et al., 2013; Peñaranda et al., 2016).

During spermatogenesis, apoptosis maintains tissue homeostasis, regulating the production of spermatozoa and preventing the formation of abnormal gametes (Baum et al., 2005; Kaptaner and Kankaya, 2013; Ribeiro et al., 2017). However, external stimuli, such as nutrient deprivation, are associated with increased apoptosis during spermatogenesis in amphibians and mammals (González et al., 2018; Wang et al., 2012). In fish, the relationship between nutrient deprivation and its influence on apoptosis during fish spermatogenesis is poorly studied. In Nile tilapia, our findings show that starvation significantly increased the proportion of TUNEL-positive germ cells in the testes. In addition, the ultrastructural changes found in these cells may indicate that nutritional deficiency negatively affects spermatogenesis, culminating in abnormal cell elimination by apoptosis. These findings may justify the reduction in the proportion of spermatocytes and spermatozoa in animals submitted to starvation. Corroborating our results, Escobar et al. (2014) also observed a strong relationship between food restriction and increased apoptosis of spermatogenic cells in *Dicentrarchus labrax*.

Taken together, our results suggest that starvation can progressively

reduce testosterone and 11-ketotestosterone levels, with consequent morphological changes in the Nile tilapia spermatogenesis. In addition, prolonged total food restriction is associated with severe damage to testicular function and germ cell death (Fig. 5). Although refeeding cycles may delay spermatogenesis, they do not result in permanent damage to the testicular function and can be recommended for maintenance of breeding stock in the fish farming.

Declaration of competing interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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