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Thiourea effects on the metamorphosis and development of bullfrog tadpole (*Lithobatescatesbeianus*)

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Abstract: *During the metamorphosis climax, a series of structural, physiological, biochemical and behavioral changes are produced due to the increase of thyroid hormone levels. Thiourea is a potent endocrine disruptor able the circulating thyroid hormones levels. This potent goitrogenic compound can be administered on tadpoles at low doses to deaccelerate the metamorphosis climax, and to obtain bigger tadpoles which will produce larger froglets, reducing the mortality and morbidity rates. Additionally, the time required for the animals to reach the slaughter weight or for reproductive purposes can been reduced. In this study, we evaluated the effect of thiourea on the development of bullfrog's tadpoles fed with diets containing different thiourea concentrations. At the end of experimental period, the tadpoles treated with 7 mg/kg of thiourea showed higher weight and superior total and partial lengths. On the other hand, no significant differences for body composition were observed between treatments. The body nutrients deposition obtained during the trial were in accordance with the normal growth of bullfrog's tadpoles. Thioureacan be used in bullfrog tadpoles to increase growth, without changing the body's nutrient deposition.*

Keywords:*Aquatic toxicity; froglets; metamorphosis; thyroid gland; weight gain.*

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I. INTRODUCTION

Early development of vertebrates is under the control of hormones, among which thyroid hormones (THs) play an important role during different processes throughout the life of these animals [1, 2, 3]. Thyroid hormones perform an essential role on metabolic regulation, normal development, growth, thermogenesis and energy balance in mammals [4, 5, 6]. In the same way, THs in fish are involved in morphogenesis, skin pigmentation, osmoregulation, thermoregulation, muscle function and reproduction [7, 8, 9], and in amphibians they have a critical role during metamorphosis [1, 10, 11].

Synthesis and release of THs are regulated by the thyroid gland. Thyroid gland release thyroxine (T4) is the main thyroid secretion product. T4 is deiodinated to 3,3′,5-triiodothyronine (T3), which is the biologically active form and the principal ligand of thyroid hormone receptors (TRs). T3 production and secretion is essential during the amphibian's metamorphosis [4, 6, 11,12]. Thus, the switch that induces the developmental changes is the T4 to T3 conversion. T3 action on tissues is regulated and mediated through binding to the TRs [2, 13, 14]. In amphibians, TRs are differentially expressed between males and females [15]. However, the mechanisms through which THs could affect amphibian development are currently unknown.

In amphibians, tadpoles and adults differ in several morphophysiological aspects like respiration, locomotion, diet and feeding[16,17, 18]. In addition, the synthesis and release of THs is different in tadpoles when compared to adult frogs. Indeed, these hormones directly affect the metamorphosis climax in tadpoles[4, 11, 19, 20, 21, 22] and consequently these differences are reflected in diverse behaviors and response to environmental factors.

The metamorphosis process in amphibians involves significant morphological changes such as keratinization of the skin, changes in feeding habits (passage from herbivores to carnivores), tail regression and the appearance of the extremities. Most of the changes in amphibian life are produced by the action of the

thyroid hormones [23]. During the metamorphosis T4 levels increase and a series of radical changes occurs that culminate in the modification of larval features, such as: hepatic differentiation [24], remodeling of intestines [25], respiratory organs [26], immune system [27], eyes [28], pituitary gland [29] and the majority of bone formation [30].

Chemicals that disrupt thyroid gland cause blocking of the synthesis and release of thyroid hormones and affect their transport and catabolism. Thus, potential THs disrupting effect was found for several environmental pollutants [31, 32, 33, 34]. Besides this, several researches have been shown that steroids (corticosterone and estradiol) perform a role as modulators of thyroid hormones [35, 36]. For example, amphibian prolactin can be used to inhibit the effect of thyroid hormones during metamorphosis [1, 10]. However, these compounds are not currently used due to their deleterious effects on reproductive and productive parameters, sexual reversion, gonadal alterations [37, 38], hermaphroditism, testicular atrophy, loss of sperm quality [39], changes in larval gonadal differentiation [40] and decrease in the growth rate [41]. In this context, several goitrogenic agents can be used to treat these effects. An example is thiourea which belongs to a class of drugs commonly used. Thiourea causes hypertrophy and hyperplasia of the thyroid gland follicular cells and suppress the thyroid hormones' production [14, 42].

The use of thiourea in different concentrations has demonstrated its goitrogenic efficacy in the development of poikilothermic animals. In addition, this compound shows other properties such as high gastrointestinal absorption, rapid elimination by tissues and urine, and a strong inhibitory effect on thyroid hormone synthesis [43, 44]. Therefore, thiourea administration can be a viable alternative for the study of new techniques to delay the amphibian metamorphosis process in order to obtain larger, active and healthy froglets. Thus, the aim of this work was to evaluate the thiourea administration during the development of bullfrog's tadpoles (*Lithobatescatesbeianus*) and their possible effect on post-metamorphic animals.

II. MATERIALS AND METHODS

The experiment was carried out at the Aquaculture Center of the São Paulo State University (UNESP) and was divided into two phases. Phase one (pre metamorphic phase): consisted of a period of 90 days, which began in January and ended in April, this phase used bullfrog tadpoles. Phase two (post-metamorphic phase): which began in April and ended in August, during this phase a random sample of froglets from the first phase was used. All procedures were approved by the Ethics Committee on Animal Use (CEUA) of the Faculty of Agricultural and Veterinary Sciences, UNESP (Protocol No. 019715/14), and were conducted in accordance with ethical principles in animal experimentation adopted by Brazilian College of Animal Experimentation (COBEA).

2.1.Experimental design and sampling

Bullfrog tadpoles from the same spawning were used (Gosner's stage 26), obtained from a commercial frog farm located in Matão, São Paulo, Brazil. The tadpoles went through a seven-day adaptation period (adaptation to the facilities, daily management and feeding), before the start of the experiment. The mean values and standard deviations for weight, total length and partial length of tadpoles were 0.019 ± 0.004 g; 10.76 ± 0.72 mm; 4.38 ± 0.35 mm, respectively.

2.2. Pre-metamorphic phase

Tadpoles during pre-metamorphic phase were housed in 12 groups with an initial density of 1.2 animals per liter (L), in cement boxes of 500 L, with in-line continuous water flow (obtained from an artesian well source) and individual water intake. Tadpoles were distributed in a completely randomized design with four treatments (0, 3, 5 and 7 mg of thiourea per kg of feed) and three replications. The cement boxes were siphoned weekly to remove the feces and feed leftovers. Water quality was constantly monitored and the following parameters were evaluated: dissolved oxygen (O_2D), pH and electrical conductivity (EC). The water temperature in the boxes was checked daily, twice a day, in the early morning and late afternoon. The mean values observed during the experimental period for the water physical and chemical parameters were: O_2D 5.5 ± 0.78 mg L-1; pH 7.43 ± 0.08; EC 184.15 ± 9.21 μS cm-1; and temperature 29.7 ± 3.26°C (at morning) and 31.9 ± 2.78 °C (at afternoon). The pre-metamorphic animals were fed with a powder diet containing 27% digestible protein and 3676 kcal kg-1 of digestible energy three times a day, avoiding leftovers, thus, the quantity offered was considered consumed [45].

Thiourea (TU) was mixed into the feed and offered to tadpoles for 30 days according to each treatment T0 (control group, 0 mg TU per kg feed), T1 (3 mg TU per kg feed), T2 (5 mg TU per kg feed) and T3 (7 mg TU per kg feed). Subsequently, all groups of tadpoles were fed with a diet without TU (the same consumed by the control group) until the occurrence of the metamorphosis climax. Monthly and until the metamorphosis climax, 10% of the tadpoles from each experimental box were weighed and measured individually to obtain

body weight (BWT), partial length (snout to tail insertion, PL) and total length (snout to tail end, TL), in total three measurements were performed at 30 (DT30), 60 (DT60) and 90 days (DT90).

Another random sample was taken on day 15 (DT15), 30 (DT30) and 45 (DT45), in which some tadpoles of each box (approximately 35 g of live weight) were sacrificed to estimate their body composition (crude protein (CP), ether extract (EE), dry matter and ashes (DM). The tadpoles sampled were placed in containers with ice for desensitization and later euthanized (immersion in 10% benzocaine solution). The celomatic-visceral cavity was opened to remove the gastrointestinal tract and then the animals were packed in plastic bags and frozen for further laboratorial processing for determination of the body composition. Additionally, 5 animals per treatment were collected at DT15, DT30 and DT45 for morphological analysis of thyroid gland.

For body composition analysis, frozen tadpole samples were triturated in a waste grinder and immediately lyophilized at -50 °C to obtain the pre-dried matter. Later, the samples were milled in a ball mill and sent to the laboratory for protein analysis by the Dumas method in Leco 528 LC equipment (Etheridge et al., 1998); quantification of ether extract by the Ankom XT-15 extractor (Ankon Technology, Macedon, NY - https://www.ankom.com); ash and dry matter measurement through incineration in muffle at 550 °C and oven drying at 105 °C for 12 hours, respectively.

For histological and morphological analysis of thyroid gland, (n=30) tadpoles were sacrificed and fixed in Karnovsky's solution, decalcified in 0.25M EDTA and included in historesin. Serial longitudinal sections of 5µm thickness were performed, from the dorsal to ventral portion. The sections were stained with toluidine blue solution. For each tadpole, five sections of the medial portion of the thyroid gland were evaluated by treatment considering the following parameters: general thyroid gland size (enlargement or reduction area), follicular size (perimeter), follicular shape (regular, irregular, uniform or not), follicular cell structure (cuboidal, columnar or flat), epithelial structure (single cell layer or stratified with multiple layers) and follicular cell height. The perimeters, areas, and heights were obtained by Image J 1.49 software (National Institute of Health, Bethesda, Maryland, USA - http//:imagej.nih.gov/ij).

2.3.Post-metamorphic phase

In this stage 696 froglets were used (2.52 ± 0.27 g) from the previous phase. The frogletswere kept in three m2 stalls with cement floor and masonry walls, maintaining an initial maximum stocking density of 50 animals per m2. The animals were placed randomly into the stalls, taking to account the treatment and the replicate received during the pre-metamorphic phase. Each stall had a shelter, water channel and food vibrating trays arranged linearly. The water used was from artesian well, with continuous flow. Daily, the stalls and the water channels were cleaned and the food replaced on food vibrating tray.

The feed consisted of an extruded commercial diet containing 40% crude protein and 4366 kcal kg-1 of crude energy, which was offered “ad libitum”. The food pellet size was 2 to 4 mm in the first 45 days and 6 to 8 mm after this period. Room temperature and minimum was measured daily with a thermometer placed 30 cm from the floor. The mean maximum and minimum room temperatures observed were 33.1 ± 3.2 °C and 26.2 ± 2.6 °C, respectively.

Biometric measures (weight and length) were performed every 30 days. The frogs were individually weighed (BWF) with an electronic scale and measured (snout-vent length, SVL) with a digital caliper. In total, four biometric measurements were performed for each treatment at 30 (DF30), 60 (DF60), 90 (DF90) and 120 days (DF120). Then, internal organs as liver, kidney, spleen, gonads and adipose tissue were removed to determine the hepatosomatic (HIS % = liver weight/body weight x 100), gonadosomatic (IGS % = gonads weight/body weight x 100) and liposomatic index (ILS % = weight of body fat/body weight x 100).

Samples of liver, kidney and spleen and were fixed in Karnovsky's solution and included in historesin. The samples were sectioned in cross sections of 5µm thickness which were stained by means of the Hematoxylin-Eosin staining technique.

2.4.Statistical analyses

All data were analyzed for normality and analysis of variance and average comparisons (parametric and non-parametric) were performed according to the results. The results presented are expressed as mean ± SEM (standard error). Comparisons between means (between and within the different groups) were performed using two-way ANOVA or one-way ANOVA followed by the Bonferroni test ($p < 0.05$) for parametric data and Kruskal-Wallis test was used for non-parametric data followed by the Dunn test. All statistical analyzes were performed using SAS V9.0 and Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA, USA - http://www.graphpad.com) software.

III. RESULT AND DISCUSSION

3.1. Pre-metamorphic phase

Tadpoles treated with 7 mg kg-1 of TU exhibited the highest partial and total lengths. A detailed analysis indicates that at DT30 the tadpoles of T0 displayed the lowest PL, followed by the tadpoles of T2, with differences of 19 % and 17 % with respect to those of T3, respectively (Figure 1).

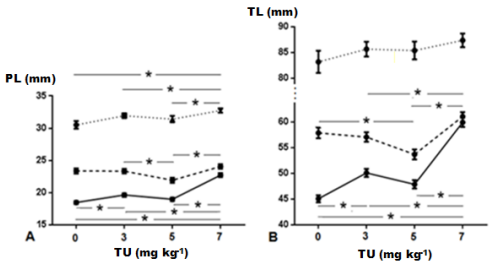

Figure 1:Partial and total length of bullfrog tadpole treated with TU. (A): partial length (mm); (B): total length (mm). 30 days (DT30) (continuous line); 60 days (DT60) (dashed line) and 90 days (DT90) (dotted line). Points represent the mean ± SEM. The stars indicate significant differences ($p < 0.05$) between the treatments.

At DT60, although no significant differences were observed in PL between the treatments T3/T0 and T3/T1, the treatments T0 and T1 presented a PL 3% lower than that obtained by T3. At DT90, significant differences were observed between T3 and the other treatments evaluated. Significant differences were observed between the treatments at DT30 and DT60. At DT30 the treatment T3 presented a significant highest TL when compared with the other treatments (Figure 1B). This condition was maintained during DT60 between treatments T3 "versus" T2, T3 "versus" T1, and T2 "versus" T0. For weight, the animals from DT30 of the T3 treatment showed significantly superior weight than the other treatments (2.18 g), with a difference of 93% in relation to the weight of tadpoles from control (1.13 g). At DT60 the control group which at the firstly measurement showed the lowest weight, obtained an increase in weight of 90 % on average, however did not present differences with respect the average weight of T3 group (2.31 g). Furthermore, T2 group presented low weight and was less than that observed in T0. In DT90, T0 showed significant differences from the other three treatments (Figure 2).

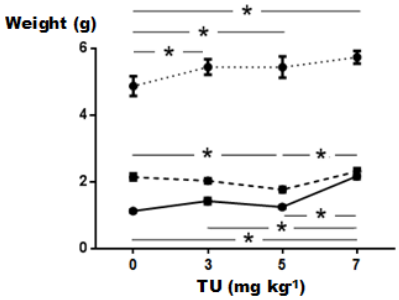

Figure2:Weight (g) of bullfrog tadpole treated with TU during the pre-metamorphic phase. 30 days (DT30) (continuous line); 60 days (DT60) (dashed line) and 90 days (DT90) (dotted line). Points represent the mean ± SEM. The stars indicate significant differences ($p < 0.05$) between the treatments.

The second parameter analyzed during the pre-metamorphic phase was body composition. During the evaluated period there was an increase in the body nutrient deposition of tadpoles. The CP mean value was 53.10% at DT15, and fifteen days later (DT30) CP increased to 56.20% which represents a raise of 5.84% on CP body composition. In the DT45, a minimal difference was observed in the corporal deposition of CP, approximately 0.07%. Major differences were observed for body fat deposition. Between DT15 and DT30 there was an increase on EE of 12.11%, and between DT30 and DT45 there was a noted increase of 26.17%. The same trend of CP was observed for ash and DM, there was an increase of both between DT15 and DT30 (4.53% of ash and 5% of DM) and the lowest values were observed between DT30 and DT45 (1.76% of ash, 1.60% DM).

At the end of the evaluated period, no significant differences were observed between treatments in total length, but partial length was different. Partial length represents only the size of the tadpole body without considering the tail and is an indicator of the size that the animal will reach after the metamorphosis [46]. During the transition from aquatic to terrestrial life, tadpoles may lose up to 70% of their total weight [4]. Tail

absorption and morphophysiological changes that occur in this period (e.g., alteration of the jaws and digestive system) can lead to animals ceasing food intake. Thus, during the final phase of the metamorphosis, which precedes the climax, it is important to obtain larger animals, especially in relation to body size and weight. In this work, animals treated with 7 mg/kg TU showed higher partial length and weight at the end of the experiment.

The metamorphosis climax was initially observed 45 days after the start of phase 1. After 60 and 90 days, most of the animals reached the metamorphosis climax, 30% to 40% of the tadpoles in the first period and 50% to 75% of the animals during the second period. We also observed that animals of the T3 showed a higher number of post metamorphic animals, 32% in relation with other treatments. This effect may be due to lower TH levels and higher levels of prolactin, since prolactin inhibits metamorphosis by blocking the effects of TH effects [47, 48]. Another explanation is that during the feed with TU, low levels of TH are maintained for a short time and, subsequently, the production of TSH increases, gradually stimulating the synthesis and release of TH, promoting rapid differentiation and climax.

Several studies demonstrate the goitrogenic efficacy of TU on amphibian metamorphosis through the analysis of the hormonal mediators involved in the metamorphosis process [35, 49, 50, 51]. However, no studies were found that evaluate the use of TU and that support the objectives proposed in this study. To date, this is the first study using thiourea in order to prolong the pre metamorphosis stage, and obtain larger tadpoles, resistant to stress and with better health conditions.

As a consequence of analyzing the second parameter, we observed an increase in tadpole body nutrient deposition. The increase of corporal nutrients such as proteins, lipids and minerals determined the animal growth and can be influenced by endogenous factors (species, genetics and/or life stage) and exogenous factors (diet composition, breeding environment, etc.) [52]. Throughout the period evaluated, there was a huge increase in protein deposition at 15 - 30 days and subsequently decrease from 45 days. [53] reported similar results, where they described an increase in body protein deposition (until 36 days) and subsequently a remarkable decrease. The deposition and accumulation of proteins (mainly in tadpole tail), are essential for survival during the metamorphosis climax [54].

Lipid deposition showed a gradual increase and was observed throughout the evaluated period, which suggests the formation of an energy reserve for the metamorphosis climax. In bullfrog tadpoles, body lipids gradually increases with increasing the body mass during the pre-metamorphosis, reaching maximum deposition at the beginning of the metamorphosis climax [55]. [56]evaluated the emergence of fat accumulation during the larval phase and the metamorphosis of five species of Anura: *Rana curtipes*, *R. cyanophlyctis*, *R. tigerina*, *Polypedatusmaculatus* and *Bufomelanosticus*. The authors reported that body fat varies by species, larval period, and is higher before the onset of the metamorphosis climax. Similar results were reported by [53], where by evaluating the body nutrient deposition of bullfrog tadpoles they found that the lipid body deposition gradually increased throughout the experiment, reaching its peak at 41 days, composing an energy reserve necessary for the beginning of the metamorphosis process.

In the evaluation of the possible effects of the TU on the thyroid gland, the size of the gland (ThA) was affected, thus, during the DT15 the control group presented the smallest area (23.69 mm2) while the T3 group showed the largest area (37.93 mm2). However, despite the difference observed between the two groups (63%), the treatments showed no significant differences (Figure 3).

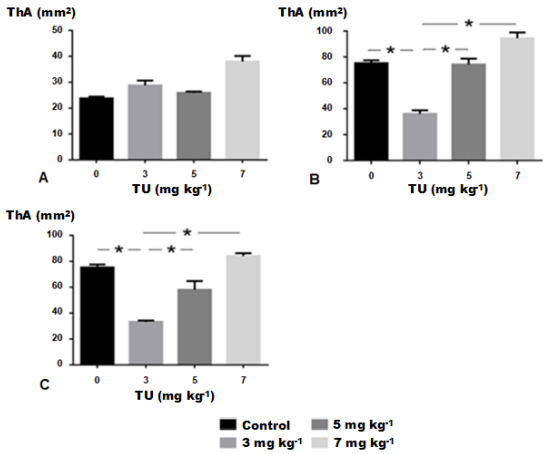

Figure 3: Thyroid gland size of bullfrog tadpole treated with TU during the pre-metamorphic phase. (A): 15 days (DT15); (B): 30 days (DT30); (C): 45 days (DT45). Bars represent the mean ± SEM and are expressed in square millimeters. The asterisks indicate significant differences ($p < 0.05$) between the treatments.

At DT30, it was noted that a ThA increase occurred in all evaluated treatments, showing values of 318% in T0, 126% in T1, 224% in T2 and 250% in T3. The T1 group that received 3 mg of TU presented the smallest ThA, differing statistically from the other treatments (Figure 3B). At DT45 the ThA in T0 showed similar values to that of the previous period and in the other treatments the ThA dropped by 8%, 22% and 11% for T1, T2 and T3, respectively (Figure 3C). Regarding to the follicles size (FolS), in T1 FolS remained practically unchanged. The other treatments showed a significant increase on FolS (72% in T0,25 % in T2 and 86% in T3) at DT15 and DT30. Conversely, at DT45 it was noted that follicle atrophy occurred, with a FolS decrease of approximately 31% in T0 and 5 – 18% in T3 (Figure 4).

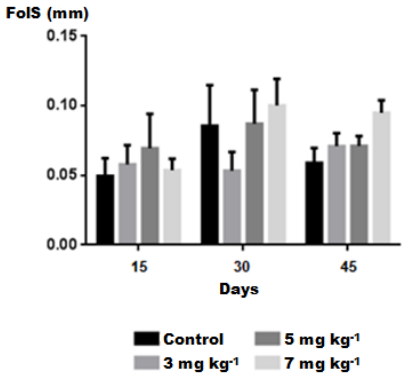

Figure 4: Mean follicle size (FolS) of bullfrog tadpole thyroid gland by treatments and days evaluated. Bars represent the mean ± SEM ($p<0.05$) and the measurements are expressed in millimeters.

In relation to the follicular shape (regular or irregular and uniform or not uniform) at DT15, all treatments showed few follicles with irregular shape and different sizes. Histologically it was observed that cuboidal and columnar follicular cells were present in both the in control group and in TU treated groups. In general, the most observed follicular structure was the cuboidal shape. No stratified epithelium or presence of papillary invaginations in the epithelial cell layer of the lumen were observed (Figure 5). At DT30 in all treatments the follicles showed irregular shape with different follicle structures, at the same time, follicle with cuboidal structures and uniform shapes were also detected. In all treatments an increase in the follicles number was observed, particularly in T2 and T3.The morphology of the thyroid gland is an important aspect in the diagnosis of several histomorphological characteristics mediated by regulatory elements of hypothalamic-pituitary-thyroid axis (HPT), particularly TSH, a hormone that regulates the synthesis and release of thyroid hormone [59]. During the metamorphosis, the morphology of the thyroid gland changes gradually to reach the physiological requirements necessary for the transition from aquatic to terrestrial life [26]. The thyroid gland increases in size and volume, due to the follicular proliferation that increases in size and number of follicular cells [57]. This fact was confirmed by the data obtained in the control group, where the tadpoles fed with food without thiourea presented hypertrophy of the thyroid gland, in this case due to the normal development of the animals during the metamorphosis.

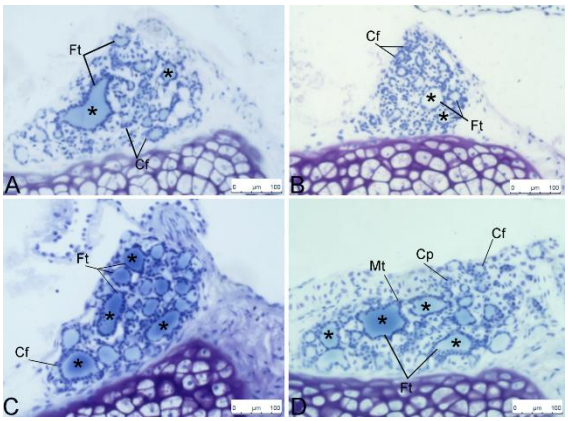

Figure 5: Transversal sections of the thyroid gland of bullfrog tadpole after 30 days of thiourea (TU) treatment. (A): T0 control group; (B): T1 3 mg kg-1 TU; (C): T2 5 mg kg-1 TU; (D): T3 7 mg kg-1 TU. Thyroid follicle (Ft); Parafollicular cells (Cp).

Tadpoles that received 3 mg TU/kg presented hypertrophy of the thyroid gland only during the initial phase and subsequently gland atrophy. This suggests that the low TU dosage used was not able to keep low TH levels in plasma to stimulate the thyroid hypertrophy. Animals in T2 showed hypertrophy of the thyroid gland up to 30 days. This event was characterized by follicular cell hypertrophy, and increase of number, size and luminal area of the follicles. After this period, the size of the thyroid gland matches to T0 tadpoles. In this context, the subsequent atrophy observed at the end of the period could be due to the low concentration of TU used. This suggests that the TU concentration was not able to maintain the low TH levels after cessation of the inhibitor administration, leading to regression of follicular cells and follicle size.

On the other hand, the tadpoles that received 7 mg TU/kg showed hypertrophy of the thyroid gland throughout the period as a consequence of follicular cell hypertrophy, hyperplasia and increase of the luminal area of the follicle. The exposure of the thyroid gland to inhibitors of TH synthesis (such as thiourea), induces follicular cell hyperplasia and hypertrophy, changes in the follicular luminal area and glandular growth, due to alterations associated with compensatory mechanisms modulated by the increase in circulating TSH concentrations in response to the decrease of blood TH [43, 50].

3.2. Post-metamorphic phase

At the beginning of the experiment, the mean weights of control and treated animals did not differ significantly (2.52 ± 0.27 g), however, after 30 days, pronounced differences were observed between treatments. At DT45 control and treated groups presented a significant weight increase (153% T0, 229% T1, 96% T2 and 257% T3) when compared to the previous measurement (Figure 6).

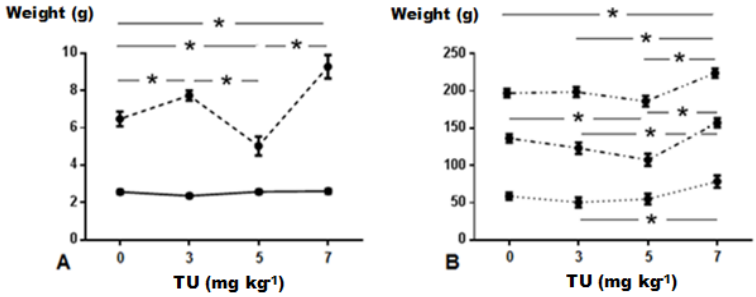

Figure 6: Mean of the weight of bullfrogs treated with TU during the pre-metamorphic phase. (A): beginning of the experiment (solid line), 30 days (DT30) (dashed line); (B): 60 days (DT60) (dotted line); 90 days (DT90) (dash-dotted line); 120 days (DT120) (dash-dot-dot line). Points represent the mean ± SEM. The asterisks indicate significant differences ($p < 0.05$) between the treatments.

Significant differences were observed between the T0, T1 and T3. T2 showed a 22% and 54% lower weight than T0 and T3, respectively. At DT60, significant weight differences were observed only between treatments T1 and T3 (Figure 6B). In this period, there was an increase in the mean weight of the animals compared to the previous period in all treatments, mainly in T2 (998%) and T0 (811%). In the posterior periods, the weight gain was 130% (T0), 141% (T1), 94% (T2) and 99% (T3) for DT90, and 45% (T0), 62% (T1), 74% (T2) and 43% (T3) for DT120. In both periods, T3 animals presented the highest weights, 156 g (DT90) and 224 g (DT120), when compared to the other treatments (Figure 7). On the other hand, T0, T1 and T2 presented similar values of final weight. The snout-vent length (SVL) exhibited the same trend of weight gain with a significant increase in the first 60 days and then moderate gains at about 120 days.

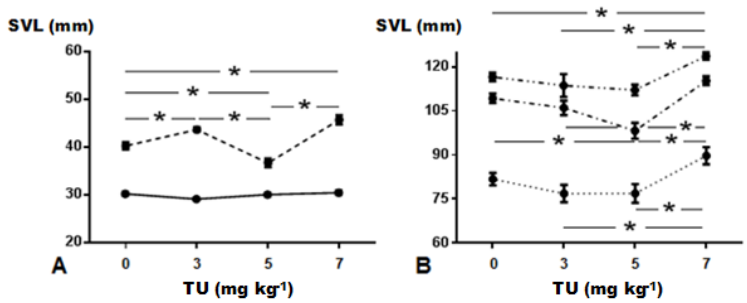

Figure 7: Mean of the snout-vent length (mm) of bullfrogs treated with TU during the pre-metamorphic phase. (A): beginning of the experiment (solid line), 30 days (DT30) (dashed line); (B): 60 days (DT60) (dotted

line); 90 days (DT90) (dash-dotted line); 120 days (DT120) (dash-dot-dot line). Points represent the mean ± SEM. The asterisks indicate significant differences ($p < 0.05$) between the treatments.

At the beginning of the experiment and DT30 there were observed differences between the treatments (Figure 7A), and in DT60, DT90 and DT120 there was a significant length increase (Figure 7B). At DT60, there was a notable increase in the average weight of the animals on treatments T2 (998%) and T0 (811%) compared to the previous period. This significant increase is similar to the gains obtained with the compensatory gain. Weight gains followed by compensatory growth were reported for Nile tilapia after a short fasting period and subsequent feedback [58]. Similar results were described for the Genetically Improved Farmed Tilapia (GIFT) lineage [59] and for *Piaractusmesopotamicus* [60]. In subsequent periods, weight gains begin to decrease progressively, probably as a function of the energy redistribution from growth to reproduction.

To date, it is still difficult to establish comparisons of the zootechnical bullfrog performance due to the limited information available and the environmental climatic differences in the facilities [61]. [62]reported a bullfrog performance during the fattening phase with initial mean weight of 52 g and fed with different commercial diets, treated frogs obtained mean daily weight gains approximately 2.71 g day-1 after 60 days. The present study obtained similar weight gains of 2.29 (T0), 2.46 (T1), 2.18 (T2) and 2.42 g day-1 (T3).

Analyzing the effects of TU in the organs, we found in all treatments the presence of cytoplasmic rarefaction associated with dissociation of hepatic trabeculae presence of numerous mast cells and melanomagrophages in the liver (Figure 8).

In the kidney of animals treated with 3 mg TU showed an increase of the interstitial space in the renal parenchyma, dystrophic calcifications, dilated tubules with thinning of the tubular epithelium, hypertrophic glomeruli which are usually associated with hemodynamic and hypotrophic changes that lead to increase of Bowman space, loses of interstitial tissue associated with increased interstitial space, tubulonephrosis and presence of mast cells (Figure 9).

Kidney analysis of animals treated with 5 mg TU also presented an increase of interstitial tissue, dilated tubules with thinning of the tubular epithelium, mast cells, melanomacrophagous and the presence of necrotic granulomas. Animals treated with 7 mg TU showed the absence of tubular lumen, increased interstitial space, glomerular degeneration, presence of mast cells and melanomacrophages. Transversal spleen sections showed normal cells and structures such as melanocytes, arteries and veins, red pulp and white pulp. Regardless of the administration TU and the amount used, the examined structures presented lymphoid follicles, innumerable mast cells, melanomacrophages, eosinophils and tissue damage (Figure 10).

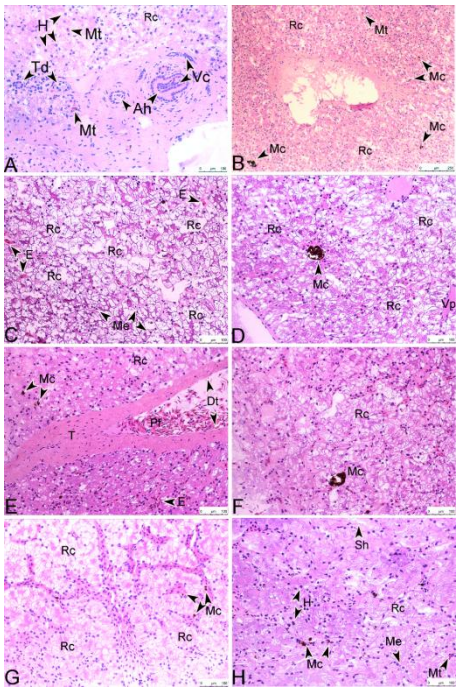

Figure 8:Histological sections of bullfrog liver. (A and B): Control animals; (C and D): animals treated with 3 mg TU; (E and F): 5 mg TU; (G and H): 7 mg TU. Hepatocytes (H); Distal tubules (Td); Hepatic artery (Ah); Congestive vessels (Vc); Mast cells (Mt); Melanomacrophages (Mc); Cytoplasmic rarefication (Rc);

Eosinophils (E); Melanocytes (Me); Portal vein (Vp); Splenic trabecula (T); Proliferation of fibroblasts (Pf); Trabecular dissociation (Dt).

Thiourea addition to the tadpoles during the pre-metamorphic phase did not affect the organs weight. The liposomatic, hepatosomatic and gonadosomatic index obtained in this work are in agreement with the expected values cited for this species in literature [63, 63, 64]. Analysis of the tissue morphology of liver, kidney and spleen revealed morphological alterations that caused concern and will likely require further investigation. Cellular and morphological alterations in these organs can be caused by failures in sanitary and zootechnical management, presence of infectious agents, lesion (cell swelling, steatosis, necrosis and apoptosis) and cell death [65, 66, 67, 68, 69].

The analysis of liver morphology of treated animals and control group, showed the presence of melanomacrophages, mast cells and cytoplasmic rarefaction. This suggests a toxic effect caused by the addition of TU. However, the analysis of liver sections of T0 showed the presence of the same structures as melanomacrophages, mast cells and cytoplasmic rarefaction associated with dissociation of hepatic trabeculae. The presence of these signs may indicate that the quality of the commercial diets are inappropriate for these animals [67, 68, 70]. Even if the animals were consuming the expected ammount or the normal quantity of feed they are not getting the total benefits of the protein contained in the feed. This could potentially be because the proteins are of deficient quality or are under the quantity required.

Studies evaluating the performance of bullfrogs fed with commercial diets based on nutritional information for carnivorous fish and with crude protein content over 40 % show satisfactory zootechnical results [62, 71]. However, the use of these commercial diets have caused damages in some organs such as liver, kidney, intestine and pancreas [68, 70, 72]. Kidney and spleen morphological analysis further increases the suspicions of inadequacy of the commercial diet.

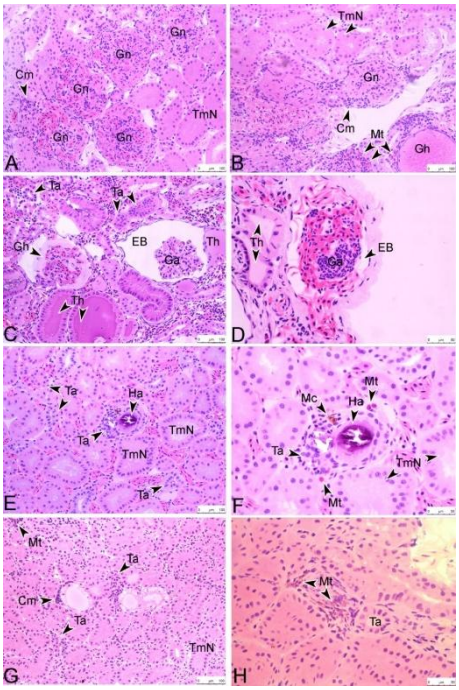

Figure 9:Histological sections of the bullfrog kidney. (A and B): Control animals; (C and D): animals treated with 3 mg TU; (E and F): 5 mg TU; (G and H): 7 mg TU. Glomeruli (Gn); Mesonephric tubules (TmN); Mesenchymal cells (Cm); Mast cells (Mt); Atrophic tubules (Ta), Hypertrophic tubules (Th); Spacing of Bowman's capsule (EB); Atrophic glomeruli (Ga); Melanomacrophages (Mc); Hyalinized glomerulus (Gh); Necrotic granuloma (Ha).

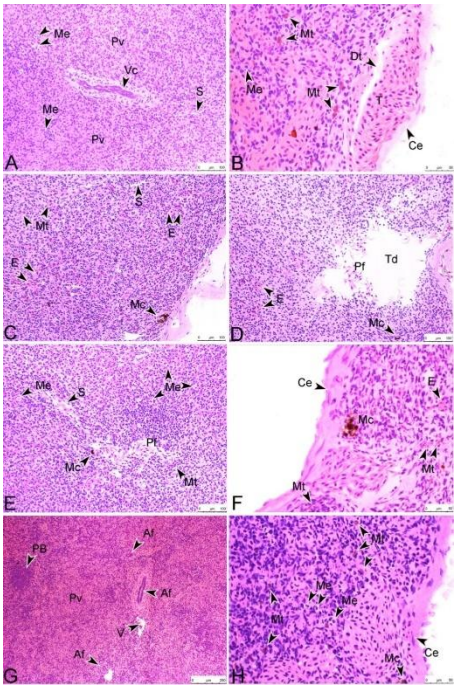

Figure 10:Histological sections of the bullfrog spleen. (A and B): Control animals; (C and D): animals treated with 3 mg TU; (E and F): 5 mg TU; (G and H): 7 mg TU. Red pulp (Pv); White pulp (PB); Congestive vessels (Vc); Splenic breasts (S); Melanocytes (Me); Mast cells (Mt); Dissociation of splenic trabecula (Dt); Splenic capsule (Ce); Eosinophils (E); Melanomacrophages (Mc); Proliferation of fibroblasts (Pf); Degenerated tissue (Td); Follicular arteries (Af); Vessels and veins (V).

IV. CONCLUSION

In conclusion, our findings suggest that the administration of thiourea in the bullfrog tadpoles feed resulted in larger post-metamorphic animals for rearing, and at the end of the fattening period. Use of thiourea during the initial stage of development, and at the dosages used in this work, did not cause toxicity or any gonadal alteration in adult animals. These results encourage new research surrounding this theme, aiming to improve this new technique, and making it more suited to the reality of the frog breeders.

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CONFLICTS OF INTERESTS

All authors declare no competing interests.

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