

EFFECTS OF THYROXINE ON LIVER STRUCTURE OF
XENOPUS LAEVIS

A Report of a Senior Study

by

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ABSTRACT

Anurans such as *Xenopus laevis* undergo metamorphosis to transition from a larval to an adult form. During metamorphosis thyroxine induces changes in body structures and tissues. In this study stage 64 juvenile *Xenopus laevis* were treated with 1mg of L-thyroxine, or NaOH as control. After treatment, livers and gall bladders were removed and histological slides were made to examine what, if any, structural changes were made to the liver. The slides were analyzed to determine if there was a significant difference between the treated and control groups in body weight, liver to body ratio, hepatocyte width, hepatocyte density, and Von Kupffer cell density. No significant difference ($p > .05$) was found for body weight, liver to body ratio, hepatocyte width, or hepatocyte density. However, a significant difference was found for the density of Von Kupffer cells ($p = 1.45 \times 10^{-17}$). Von Kupffer cells are phagocytes in the liver and act to remove waste, toxins, or old cells. Thus, it is possible that the thyroxine caused hepatotoxicity explaining the increase in Von Kupffer cells.

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CHAPTER I

INTRODUCTION

Amphibian Metamorphosis

Amphibian metamorphosis is a complex process regulated by a number of external (environmental) and internal (hormonal) processes. Environmental factors (see figure 1) initiate a sequence of biochemical events that cause the metamorphosis of amphibians from the larval to the adult morphology. Some of the environmental factors regulating metamorphic rate include temperature (Hayes et al., 1993), food levels (Kupferberg et al., 1994), predator presence (Kupferberg et al., 1994), tadpole densities (Semlitsch and Caldwell, 1982), and pond evaporation rates (Denver et al., 1998; Figure 2). The rate of metamorphosis of the frog is accelerated by increased temperature, decreased food, overcrowding, pond evaporation, and increased predation (Hayes, 1997).

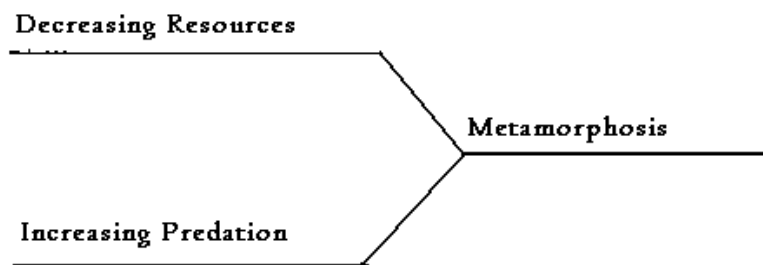


Figure 1. As food levels, habitat, and other resources diminish and predation levels and risk of

not being capable to successfully compete increase, the rate of metamorphosis is accelerated.

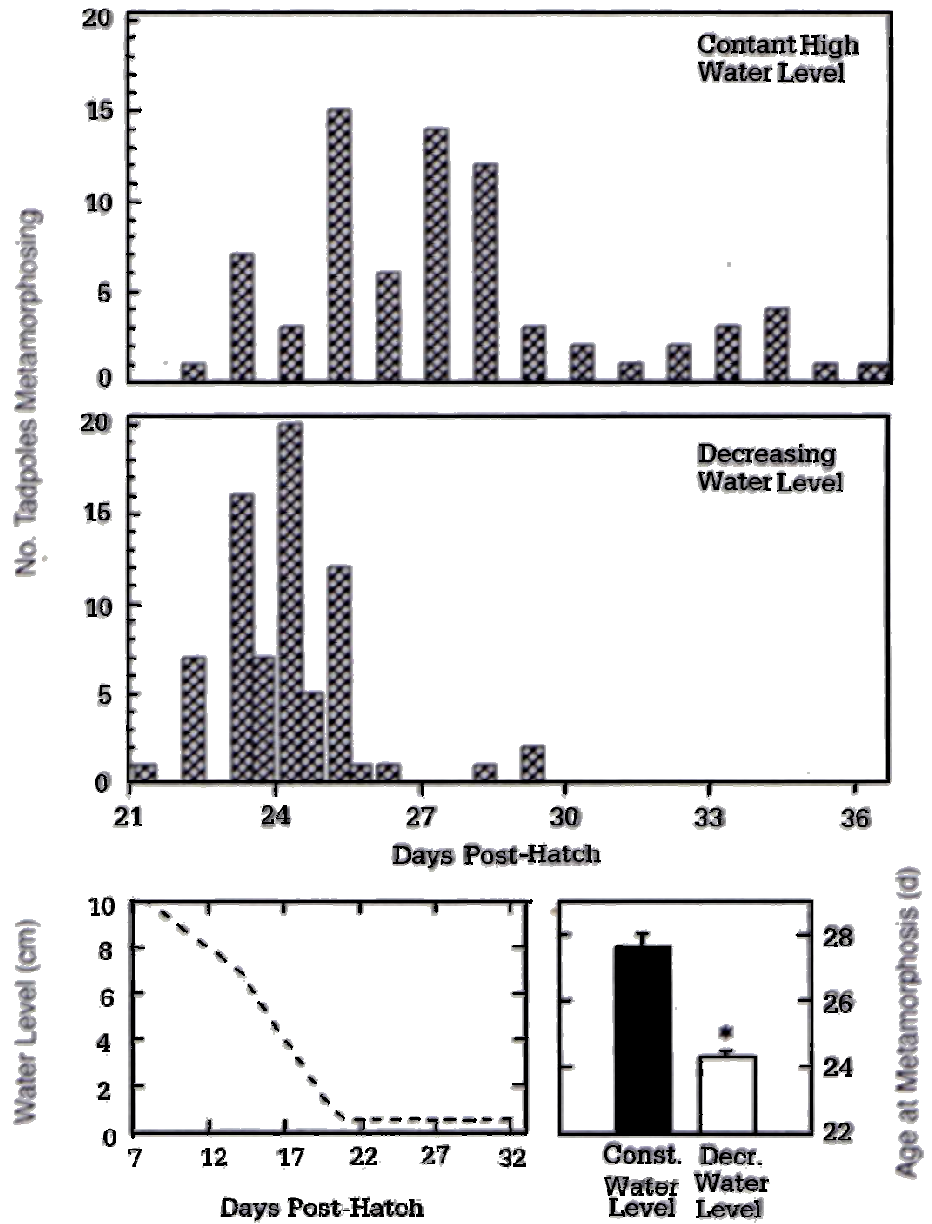


Figure 2. Denver et al. kept tadpoles in either a constant volume of water or decreasing amount of water. The tadpoles in the decreasing water were found to undergo metamorphosis at a

younger age than those in a constant volume of water.

The timing of metamorphosis greatly impacts the fitness of the adult amphibian. Frogs that metamorphose at a larger size are more likely to survive and, therefore, reproduce. However, to metamorphose at a larger size, amphibians must remain in the larval stage longer and risk the resources diminishing further (Altwegg and Reyer 2003). Frogs that undergo metamorphosis at a smaller size have the benefit of attaining a new food source and resources first, but those that undergo metamorphosis at a larger size are more fit and able to out compete the smaller amphibians (Denver et al., 1998). Thus, the time at which an amphibian undergoes metamorphosis is critical. If metamorphosis occurs too early the amphibians will be small and unable to compete in the adult form; however, if metamorphosis is too late, the amphibian will risk the resources in the larval stage running out before it is able to leave.

Hormonal Control of Metamorphosis

The thyroid hormones are responsible for bringing about the organ transformations that occur during metamorphosis of amphibians. Thyroxine, T_4 , and triiodothyronine, T_3 , are the two hormones secreted by the thyroid, both iodinated forms of the amino acid tyrosine. Iodine is absorbed into the blood from food in the gastrointestinal tract. The thyroid removes iodine from the blood to produce the thyroid hormones. Thyroglobulin, a glycoprotein produced by the thyroid combines with iodine to form each of the thyroid hormones. The hormones are stored in this state in the thyroid for periods up to three months. When released into circulating blood, T_4 and T_3 must be cleaved from thyroglobulin. In the blood the thyroid hormones bind to plasma proteins. The majority of thyroid hormone released into the blood is in the form of T_4 , however,

once in the blood most thyroxine is deiodinized to form T_3 . The thyroid hormones are introduced into cells where they bind to intracellular proteins. Once inside the cell, the thyroid hormones bind to receptors on DNA and initiate transcription inside of cells (Guyton and Hall, 2006). In amphibians, thyroid hormone levels are low during early larval development, and peak at metamorphic climax (Mondou and Kaltenbach, 1979).

Organ changes in amphibian metamorphosis

The elevation in T_4 concentration induces metamorphic changes in numerous body organs (see Table 1). Thyroid hormones stimulate jaw and head transformations during metamorphosis (Hanken and Summers, 1989). During metamorphosis, the skull undergoes ossification and calcification to form bone of the adult form (Hanken and Hall, 1988). In addition to altering the skeletal structure of the head, the facial features are also changed to be more advantageous to the adult. In *Xenopus laevis* tadpoles, the eyes are located laterally on the head while in adults the eyes are located dorsally. The shift in eye position is due to the changing of the skull structure during metamorphosis. According to Armstrong et al. (1999), thyroxine induces ventral ciliary marginal zone cells to increase in proliferation and produce projecting ganglion cells accounting for the asymmetric growth of the retinas. The repositioning of the eyes enables the frogs to transition into new role in environment.

Table 1: Metamorphic changes and timing in *Xenopus laevis* (Nieuwkoop and Faber, 1994)

Stage	External Development	Internal Development
46-52	-all four limbs bud and develop	-thyroid function initiates -degradation of external gills -lung development begins -intestinal changes occur -ribs and cartilaginous bones develop -sexual differentiation of gonads
53-54	-fore and hind limbs in paddle stage -fingers and toes indicated	-chondrification of femur -histogenesis of muscle fibers in thigh and leg
55-58	-elbow and wrist defined -angle of elbows greater than 90°	-limb musculature develops -rows of tooth germs evident -ossification of bones in limbs
59-60	-darkening of pigment on back -transition to adult skin	-lymph sacs develop
61	-head becomes narrowed -openings of gills smaller	-hypertrophy of skin at end of tail
62	-ventral fin disappears	-lower eyelid formation
63	-head narrower than body -corner of mouth level with eye	-notochord of tail shrivels -degeneration of muscle in tail -gill slits close
64	-corner of mouth behind eye -length of tail diminished	-nostrils develop -notochord of tail degenerated
65	-almost entirely adult skin -tail 1/10 size of body	-tongue developed -epithelium of colon differentiation
66	-tail no longer visible -completely adult skin	-dorsal horns of lungs reduced

The structure of the jaw changes as the structure of the skull is changed, but muscle attachments to the jaw to control movement must also develop (Denver 1998.) The diet of the adult amphibian is often different than the diet of the larval amphibian; this explains the need for the jaw to change in structure. Due to this change in diet, the gastrointestinal tract of the

amphibian must also be altered to enable the amphibian to receive the proper nutrients from its new food source.

The role of thyroxine in gastrointestinal metamorphosis was tested by treating amphibians with thyroxine to induce metamorphosis and examining the hormones and types of cells present in the gastrointestinal tract (Maake et al. 1999). In the subjects treated with thyroxine, it was found that the presence of the neurohormones neurotensin, substance P, calcitonin gene-related peptide, gastrin/cholecystokinin, somatostatin, and serotonin were increased and that, in the gastrointestinal system, the number of nerve fibers and endocrine cells increased (Maake et al. 1999). Histochemical patterns of alkaline phosphatase activity in the digestive tract of frogs treated with thyroxine were examined by Kaltenbach et al. (1977). The activity of phosphates would indicate that enzymes were being activated or deactivated. It was found that before thyroxine induced metamorphosis, the activity was focused in the striated border of the duodenum, after metamorphosis activity in the connective tissue of the entire digestive tract increased and new mucosal epithelium developed. The new mucosal border to the digestive tract would regulate the substances crossing the lining and accessing the blood stream. In order for the digestive tract to be transformed into that of the adult form, the larval form must be slowly destroyed and the adult built. During metamorphosis, the number of macrophages and aggregates of fibroblast cells in the connective tissue surrounding the intestinal tract increased. This indicates that the thyroxine induced the macrophages to breakdown the larval intestinal tract while the fibroblast developed the adult intestinal tract, thus connective tissue cells affect epithelial transformation from larval to adult stage (Ishizya-Oka and Shimoszawa 1994).

During metamorphosis, the number of peroxisomes progressively increases, and these organelles function to remove waste from cells, in the liver, kidneys, and intestinal tract. The waste products from larval systems are broken down and converted to adult systems (Dauca et al.1983). Thyroxine also affects the way that proteins are synthesized, as different amounts and types of proteins are needed in the adult that were not in the larva. One protein that thyroxine accelerates the synthesis of is carbamyl phosphate, a molecule that is involved in ridding the body of excess nitrogen in the urea cycle, in the liver of tadpoles (Paik and Cohen, 1960). The differentiation in protein content also indicates that the adult makes different use of its environment than the larva did.

One of the final stages in metamorphosis for many amphibians is the loss of the tail. Thyroxine induces tail loss through apoptosis, or self-destruction of cells in the tail by promoting DNA fragmentation and inhibiting catalase activity by enhancing NO generation (Kashiwagi et al.1999). Induction of ubiquitin, a biochemical marker for apoptosis, was found to be caused by treatment with thyroxine on tadpole tails. Atrophying tadpole tails also develop a mechanism for hydrogen peroxide production, which may contribute to cell death in this organ (Kashiwagi et al. 1999).

For many amphibians, the adult form is able to leave the water and so the gills of the larval form must be transformed into lungs. Gill absorption is one of the final events of metamorphosis, occurring during the climax is induced by thyroxine. The gills are made up of epithelial cells of several types, and depending on the type are either remaining viable cells or undergoing apoptosis (Hackford et al. 1977).

Metamorphosis of the Liver

The liver is of particular interest to this study for both functional and physical changes that may occur during metamorphosis. One functional change that occurs is increased protein production. When *Rana catesbeiana* tadpoles are induced to metamorphose by injection of 0.5-1.0 μ g thyroxine and maintained in water containing a dose of 5-10 μ g thyroxine, the total number of proteins in the frog's liver increases by double in some instances (Tata, 1967). After two days the rate of ribosomal RNA synthesis in the liver is increased. New ribosomes were formed that seemed more firmly attached to the endoplasmic reticulum than in pre-metamorphic frogs. As the new ribosomes formed, the number of phospholipids produced increased.

Another functional change associated with hepatic metamorphosis is the action of the RNA polymerase activity in frog's livers. The DNA dependent RNA polymerases form RNA using DNA as a template. The effects of thyroxine on the stimulation of RNA polymerases I and II were monitored and found that they were equally effected (Griswold and Cohen, 1971).

In tadpoles before and during metamorphosis, the liver is the site of erythropoiesis, the formation of red blood cells, but in adult frogs the bone marrow is site of erythropoiesis. The liver contains a higher percent of immature red blood cells than can be found in the blood of premetamorphic tadpoles. During the climax of metamorphosis, mature erythroid cells are present in large numbers in the liver and the number of immature erythroid cells in the blood increases (Maniatis and Ingram, 1971). One would expect that the transition of erythropoiesis from the liver to the bone marrow to take place during metamorphosis as erythropoiesis occurs only in bone marrow in adult frogs. The finding acknowledges that erythropoiesis occurs in the

bone marrow after the larval stages yet it is unclear at what time this transition from the liver to the bone marrow occurs.

The effects of thyroxine on the liver of *Xenopus laevis* during metamorphosis are apparent in the synthesis of proteins and the composition of proteins in the liver. The identity of the proteins present in the adult that were absent in the larva could give indication to function. No indication has been made as to whether the changes that occur in the liver would be visible in histological slides. The purposes of this study are to examine the structure of the liver after treatment with a physiological dose of thyroxine and determine if any hepatic histological changes are induced by the treatment.

CHAPTER II

MATERIALS AND METHODS

Animal Husbandry

Xenopus Laevis tadpoles in stages 62-64 were obtained from Nasco (Fort Atkinson, WI). The forty-three tadpoles were randomly divided into groups of five or six and placed into plastic containers filled with three liters of tap water treated with Kent Fresh/Marine Detox (1ml/5gal.) to dechlorinate the water. Twenty-one tadpoles were used in the control group and twenty-two tadpoles were used in the treated group. The water in the tanks was changed every Monday and Thursday during the experiment. Crushed Nasco frog brittle was feed to all groups every weekday of study period. Frogs were on a 14:10 photoperiod with both room and water temperatures maintained at 20° C.

Treatment

1 mg of L-thyroxine (Sigma, lot 111 H02 11) was added to the experimental group per liter of water. The L-thyroxine was made by adding 0.010g of L-thyroxine (Mettler Toledo PB303-D Delta Range) to 5mL of 1% NaOH (Fisher Scientific, lot 896729) to create a 2mg/mL solution. The control frogs were treated by adding 1.5mL of 1% NaOH to each tank. The treatments occurred at each water change. The frogs were treated for 21 days, and then underwent 21 days without treatment before finally being treated for another 21 days.

Sample Preparation

After the treatment period ended the frogs were anesthetized in a 0.05% MS222 solution. The anesthesia was made by combining 1.0 L H₂O, 0.50g MS222, and 0.42g of NaHCO₃ to make a 0.05% MS222 solution. The frogs were submerged in the solution in the groups that they had been divided into of five. The frogs did not exhibit any eye reflexes after 2 minutes in the solution and were allowed to remain in the solution for 4 minutes. Each frog was massed; the liver and gallbladder of each frog were removed using micodissection and massed. After the livers and gallbladders were removed, the frogs were euthanized.

Livers were fixed in Bouins solution, which was replaced with 75% ethanol after a period of a week. After the samples were cleared of excess Bouins, the tissues were embedded in paraffin wax, oriented so the gallbladder was dorsal, and sliced at 12 μm using a Shandon Finesse 325 microtome. The ribbons were floated in a bath of water and glycerol, mounted onto slides and labeled in sequential order. Slides were stained using Hematoxylin and Eosin after which a cover slip was applied with Permount and examined using an Accu-scope and pictures of the tissues were obtained using a Sony DSC-F707 digital camera.

Measurements

To quantitatively determine the effects of the thyroxin treatment, the hepatocyte density, hepatocyte width, body weight ratio, liver to body ratio, and Von Kupffer cell density were measured. The hepatocyte density was obtained by counting the number of hepatocyte cells present in a 40x magnification field of view. To find the density of Von Kupffer cells, a random selection of liver samples were examined and the number of Von Kupffer cells in the field of view, 100x magnification, were counted. To determine the hepatocyte width, only hepatocyte

cells with the entire diameter visible and undisrupted were measured using a 400x magnification. Ten random samples were obtained from each individual and hepatocyte width measured.

The rulers of the microscopes used were calibrated using a micrometer and the measured widths of the hepatocytes were converted to μm . The mean values of hepatocyte width, hepatocyte density, Von Kupffer cell density, body ratio, and liver to body ratio were each calculated and compared using two-tailed t-test assuming equal variances. Graphs were constructed in order to provide visual representation of the results.

CHAPTER III

RESULTS

Effects of thyroxine exposure to liver tissue of *Xenopus laevis* were examined by comparing body weight, liver to body ratio, hepatocyte width, hepatocyte density, and Von Kupffer cell density.

Body Weight and Liver Size Analysis

The livers of each individual were massed; a comparison found no significant difference in the body weight of the treated and the non treated frogs ($p=0.481$). The livers were also massed then divided by the body weight of that individual. From both the control and experimental group 8 frogs were analyzed. Thyroxine had no significant effect on the size of the frog's livers respective to each frog's body weight ($p= 0.213$)

Tissue Analysis

Histological analysis was performed on the liver and gallbladder of 8 *Xenopus laevis*. The mean diameter of the two groups showed no significant difference ($p =0.0918$) in hepatocyte width (Figure 3) due to treatment with thyroxine.

The livers of the *Xenopus laevis* treated with thyroxine had an increased amount of Von Kupffer cells ($p =1.45 \times 10^{-17}$). Figure 4 shows the difference in Von Kupffer cell density. A comparison of the number of Von Kupffer cells between the treated and control frogs is provided

in figure 5.

The density of hepatocytes was measured by counting the number in a 100x field of view from a random sampling of individuals. Thyroxine treatment had no significant effect on the density of hepatocytes ($p = 0.470$).

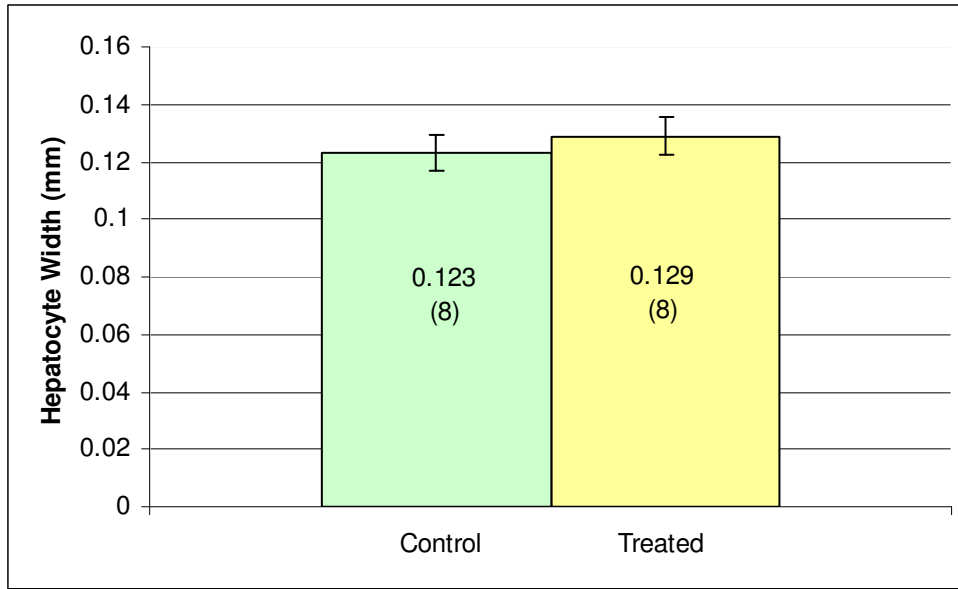


Figure 3: Average hepatocyte width (± 1 SE) for control and thyroxine treated *Xenopus laevis* at 400x magnification.

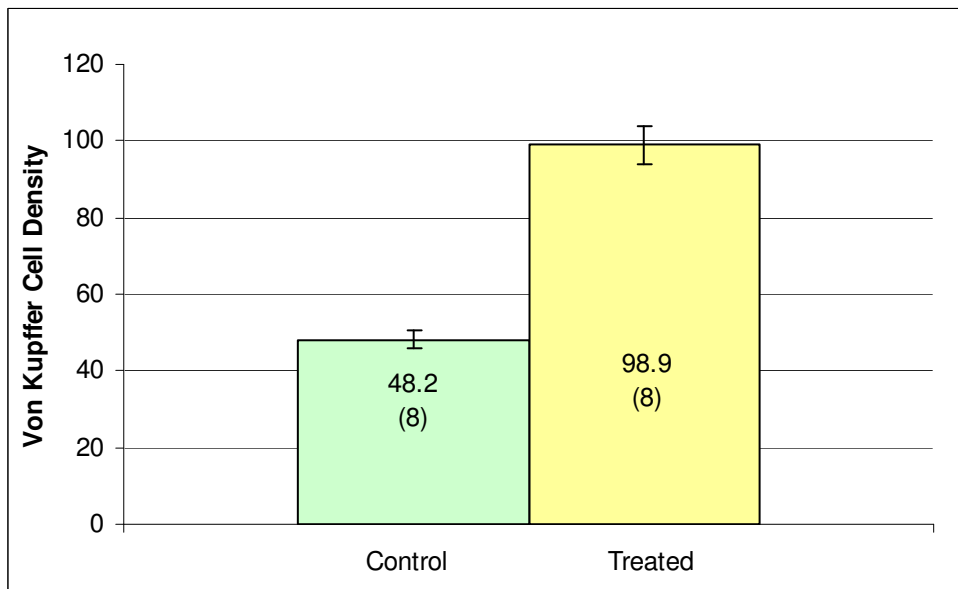


Figure 4: Von Kupffer Cell density (± 1 SE) for control and thyroxin treated *Xenopus laevis* at a

magnification of 100x.

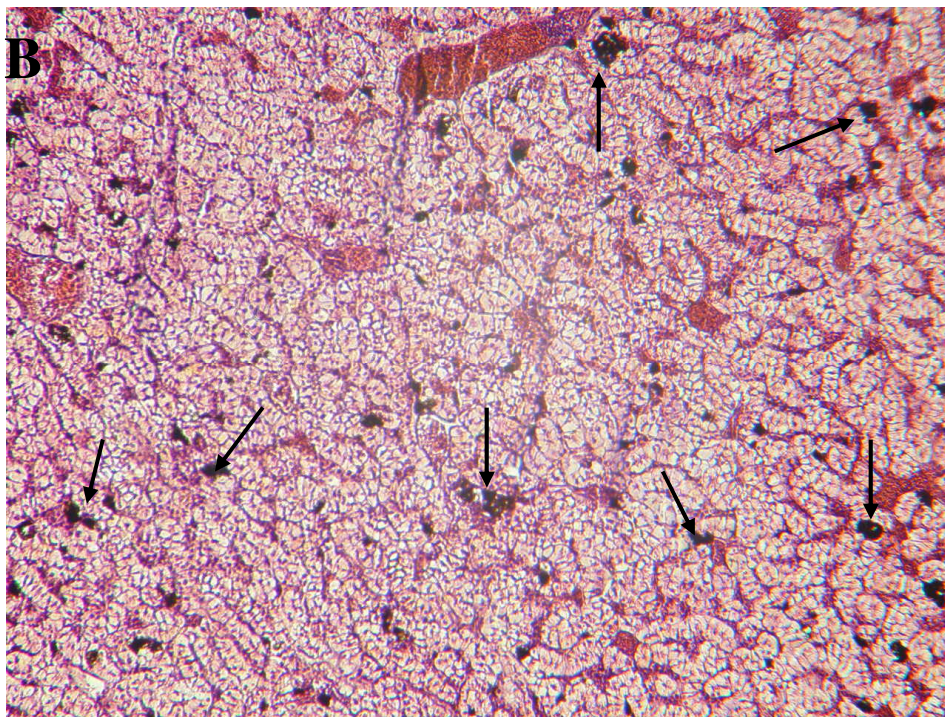
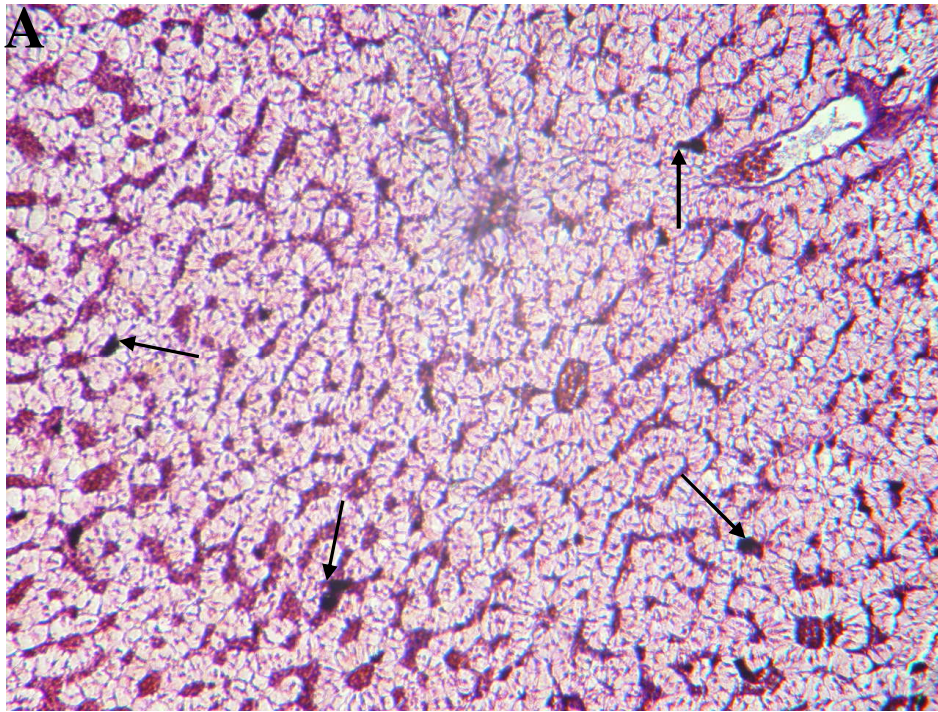


Figure 5: Control livers (A) and thyroxine treated livers (B), demonstrating the greater density of Von Kupffer cells (arrows) in thyroxine treated individuals, magnification of 100x.

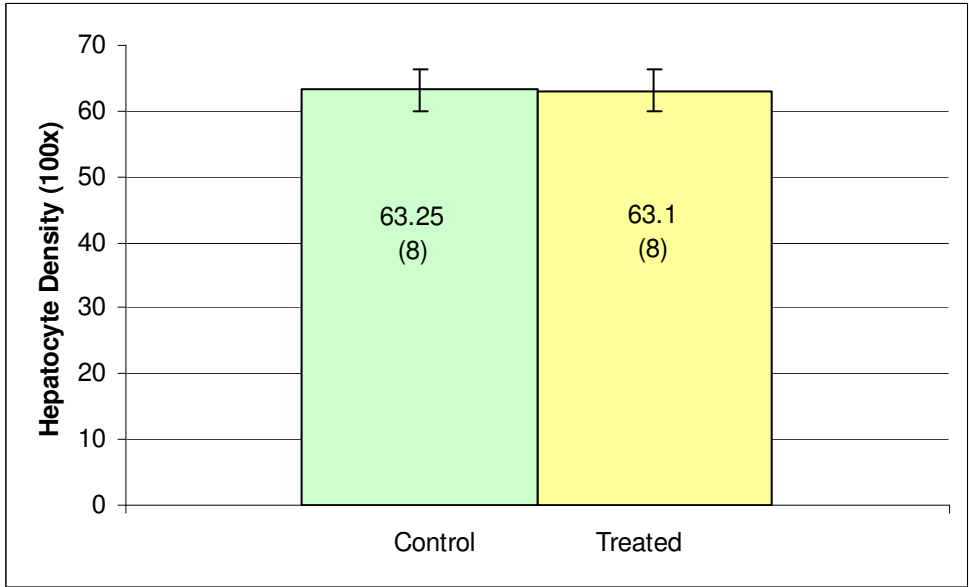


Figure 6: Hepatocyte density (\pm SE) for control and thyroxine treated *Xenopus laevis* at a magnification of 100x.

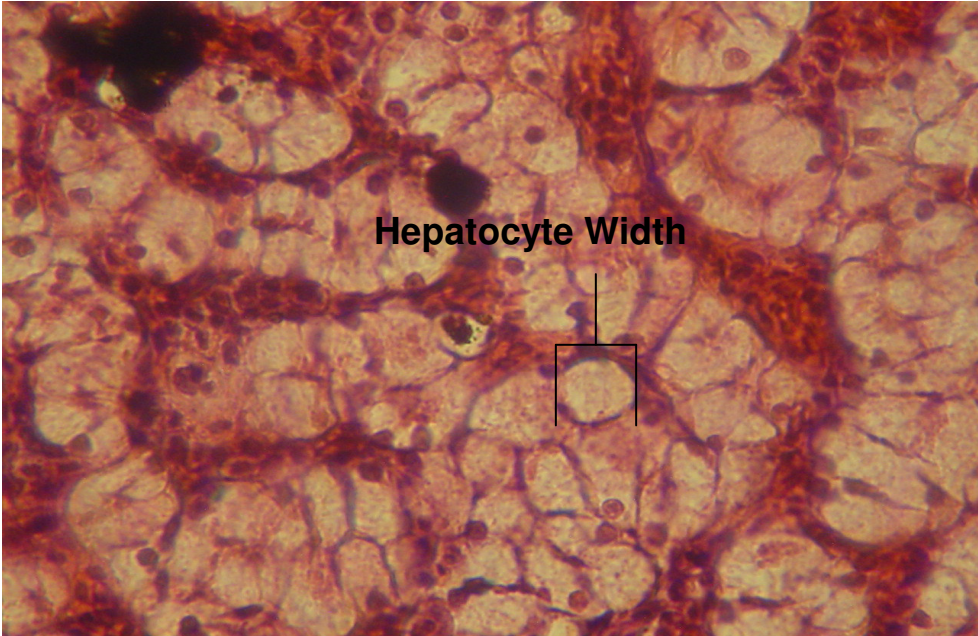


Figure 7: Measurement of Hepatocyte Width at a magnification of 400x.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Because thyroxine is known to increase metabolism of organisms, it was expected that the overall body size and the liver size of the thyroxine treated individuals would be reduced. No significance was found for either body weight or liver/body ratio. However, the results of this study suggest that two 21-day exposures to L-thyroxine on post metamorphic tadpoles had an effect on the density of Von Kupffer cells present in the liver. No significant effect was found on either the density of hepatocytes or the width of hepatocytes.

The lack of thyroxine to alter the density of hepatocytes, the width of hepatocytes, and the liver to body-size ratio was unexpected because thyroxine has been found to increase metabolism (Moreno et al., 2002). Other studies involving thyroxine treatment on livers found that thyroxine blocked the accumulation of free sphingosine, which is phosphorylated into a signaling lipid, in the liver (Babenko and Natarova, 1999). A study exposing rats to long-term thyroxine treatment did not find any significant morphology alterations in liver parenchyma (Croce et al., 2007). However, similar to this present study, a study of the effects of atrazine, an herbicide, on several organs of *Xenopus laevis* also found a significant increase in the density of Von Kupffer cells (Hedden et al., 2007). It was theorized that the increase in Von Kupffer cells was an effort of the liver to remove the atrazine from the liver.

The treatment series on these tadpoles may not have been long enough or at the correct

stage of development to have effects on the mass of the liver. Further study could treat during metamorphosis as opposed to immediately following to ensure that the increase in thyroxine was present while the body structures were still changing and forming.

Thyroxine was shown to have an effect on the phagocytic cells of the liver, the von Kupffer cells. Individuals treated with thyroxine had a marked increase in numbers of such cells. No difference was observed in the number of hepatocytes. Von Kupffer cells remove waste, old cells, and are able to produce melanins (Sichel). Because the Von Kupffer cells are phagocytic this would suggest that the hepatocytes are being degraded at a higher rate in the thyroxine treated livers than in the control group. Further research would be needed to find out what is causing the hepatocytes to be degraded at an accelerated rate.

Embryonic exposure to iron and copper alter the ability of von Kupffer cells to function by “enhancing their respiratory burst activity without modifying particle phagocytosis” (Videla et al., 2003, pp108). Croager et al. treated young rats with a liquid ethanol diet and found ethanol consumption also increases permeability of the small intestine, resulting in Kupffer cell activation, this would also possibly increase the number of von Kupffer cells although this study did not measure von Kupffer density as part of analysis.

From this study and others, it is evident that the von Kupffer cells of the liver are activated or their density is increased by both foreign and natural substances. This is likely due to the fact that the liver functions in part to remove toxins and foreign substances. The von Kupffer cells located in the liver act as phagocytes to remove these toxins, therefore, it is understandable that an increase in toxins to an organism would increase the number of von Kupffer cells.

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