

Appendix 3: Thyroid Histology Report (Germany)

Methods

Lower jaw tissue including the thyroid gland was taken from 10 randomly selected tadpoles per treatment group at test termination of each of the IGB studies. The methods used for histological analysis are described in detail in the corresponding SOP. Slides were prepared at the Department of Zoology at the University of Heidelberg (Germany). Tissues were sectioned (5 μm thick) in a transverse plane from dorsal to ventral and stained with hematoxylin and eosin. For each tadpole, 5 sections of the middle part of the right thyroid lobe were analyzed for exposure-related changes in the following qualitative parameters:

- Overall size of the thyroid gland (reduction, increase)
- Follicle size (reduction, increase)
- Follicle shape (regular, irregular, uniform)
- Colloid content (increase or reduction in colloid area; absence of colloid)
- Colloid density (homogeneous or heterogeneous tinctoral quality; pale, lacy, or granular colloid; peripheral vacuolation of colloid)
- Follicular cell shape (cuboidal, columnar, tall)
- Follicular cell height (increase, reduction)
- Structure of the epithelium (single cell layer or stratification resulting in multiple layers, papillary infoldings of the epithelial cell layer into the lumen)

In addition to a qualitative description, attempts were made to quantify selected changes in thyroid gland morphology by means of image analyzing techniques. The right lobe of 10 tadpoles per treatment group was analyzed regarding epithelial cell height and total cross section area. Epithelial cell height was measured for 3 randomly selected follicles per section. The height of 4 follicular cells per follicle was measured and a total of 5 different sections of the right lobe were analyzed for each animal ($n=60$ measurements per lobe). The maximum cross section area of the right lobe was determined by analyzing the total cross section area for at least 5 sections of the middle part of the right lobe.

Effects of PTU Exposure on Thyroid Histology

In the stage 51 and the stage 54 exposure studies with PTU, the light histological appearance of thyroid glands from the 2.5 mg/L PTU treatment group did not differ from the control group (Figure 1). A single layer of cuboidal follicular epithelial cells was present in glands from these treatment groups. The colloid showed a homogeneous tinctoral quality and peripheral vacuolation of the colloid was minimal.

At PTU concentrations of 5, 10 and 20 mg/L, concentration-dependent increases in thyroid gland size, follicle size and the degree of colloid resorption were observed in both exposure studies with PTU. The prominent changes seen in the 5 mg/L PTU group included a minimal increase in follicle size and a resulting diffuse enlargement of the thyroid gland (Figures 2A, 5A). The prevalence of these changes was greater in tadpoles from the stage 51 exposure study compared to the stage 54 study. At 5 mg/L PTU, the follicular epithelium still consisted of a single layer of cuboidal follicular cells and as judged from the homogeneous tinctoral quality of the colloid, no changes were detectable for the density of the colloid. The selected changes observed in the 5 mg/L PTU group were also seen in tadpoles from the 10 mg/L PTU treatment group but increased in prevalence and severity (Figures 2B, 5B). In addition, the marked peripheral vacuolation of the colloid as observed in some glands of the 10 mg/L PTU group indicated a partial depletion of colloid content. Furthermore, a number of follicles in the glands from the 10 mg/L PTU group showed a change in shape of epithelial cells from cuboidal to columnar. The thyroid glands of tadpoles from the 20 mg/L PTU group were characterized by marked increases in follicle size and a pronounced enlargement of the gland (Figures 3, 6). A high prevalence of follicular cell hypertrophy and hyperplasia was observed together with markedly enhanced colloid depletion. Further, collapsed follicles devoid of colloid were noted in few glands from this treatment group.

Comparing the results from the qualitative analyses of thyroid gland histology between the two exposure studies, it was found that particularly at the lower PTU

concentrations (5 and 10 mg/L), prevalence and severity of the aforementioned changes in thyroid gland histology were higher in tadpoles exposed from stage 51 for 21 days than in tadpoles exposed from stage 54 for 14 days.

Results from the quantitative measurements of epithelial cell heights are shown in Figure 7. In the stage 51 exposure study, increases in epithelial cell height were observed at the two highest PTU concentration (10 and 20 mg/L), but only the effect at 20 mg/L PTU was statistically significant. In the stage 54 exposure study, the epithelial cell height was significantly increased at 10 and 20 mg/L PTU. Thus, the results of these quantitative measurements confirm the qualitative description of the thyroid glands regarding the presence of follicular cell hypertrophy in tadpoles exposed to 10 and 20 mg/L PTU.

The maximum cross section area of the right thyroid lobe was determined in order to quantify the apparent enlargement of the thyroid glands in PTU-exposed tadpoles. In the stage 51 exposure study, enlargement of the thyroid glands following exposure to 5, 10 and 20 mg/L PTU could be confirmed by detection of increases in the maximum cross section area of the right lobe (Figure 8). This effect was statistically significant at PTU concentrations of 10 and 20 mg/L. The mean values of the maximum cross section area were approximately 2 and 3.5 times greater at these PTU concentrations than in untreated control. Statistically significant increases in the maximum cross section area of the right thyroid lobe were also observed in tadpoles exposed to 10 and 20 mg/L PTU during the stage 54 exposure study (Figure 8). In this study, the effect was less pronounced as evident from 1.7- and 2.3-fold increases in mean values of the maximum cross section area.



(A) control

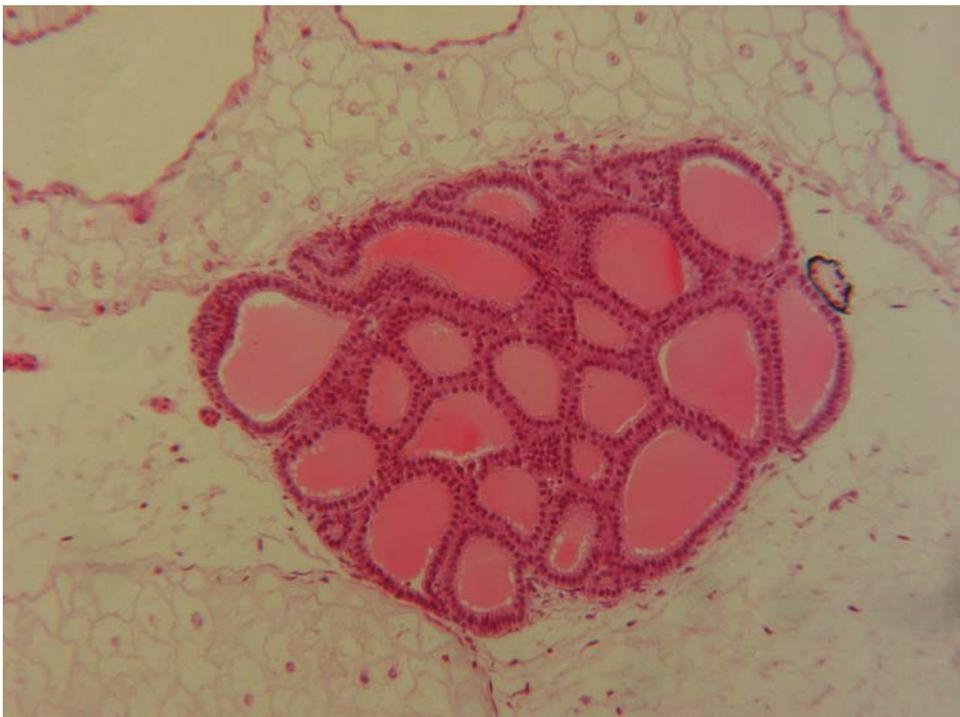


(B) 2.5 mg/L PTU

Figure 1. Light micrographs (10 x magnification) of histological sections of thyroid glands from *X. laevis* tadpoles after 21 days of exposure to PTU during the stage 51 exposure study. See text for details.

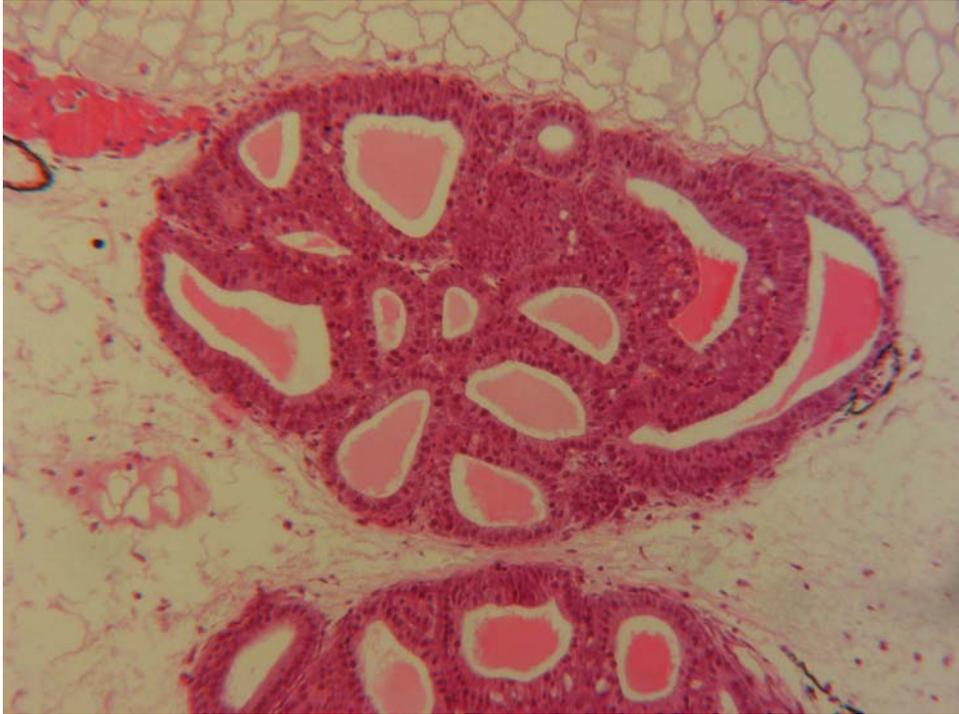


(A) 5.0 mg/L PTU



(B) 10 mg/L PTU

Figure 2. Light micrographs (10 x magnification) of histological sections of thyroid glands from *X. laevis* tadpoles after 21 days of exposure to PTU during the stage 51 exposure study. See text for details.



20 mg/L PTU

Figure 3. Light micrograph (10 x magnification) of a histological section of thyroid gland from *X. laevis* tadpole after 21 days of exposure to PTU during the stage 51 exposure study. See text for details.



(A) control



(B) 2.5 mg/L PTU

Figure 4. Light micrographs (10 x magnification) of histological sections of thyroid glands from *X. laevis* tadpoles after 14 days of exposure to PTU during the stage 54 exposure study. See text for details.

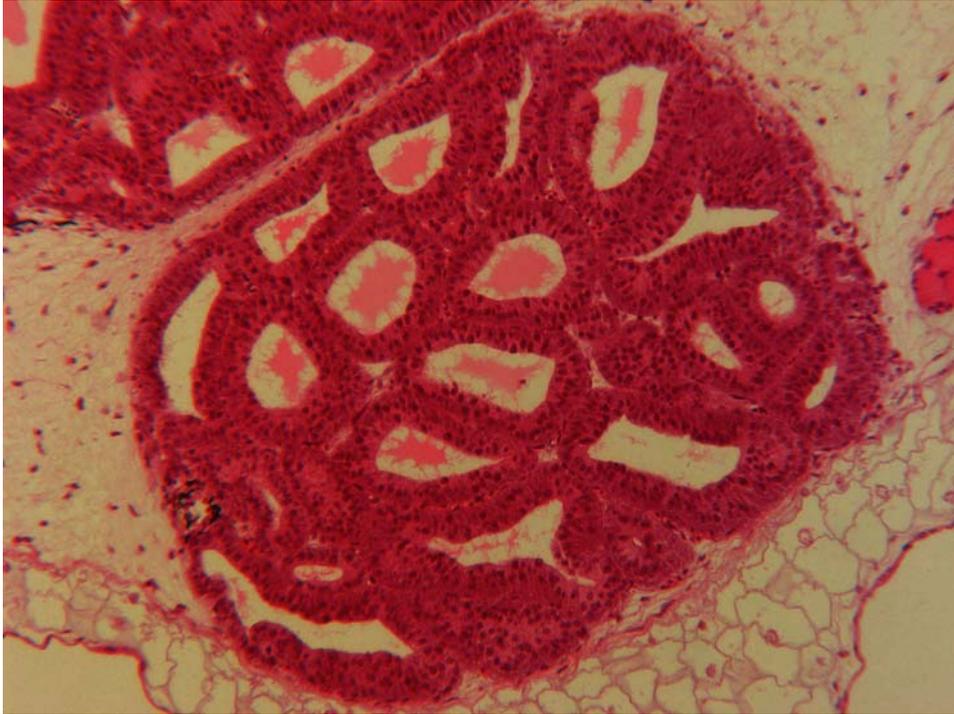


(A) 5.0 mg/L PTU



(B) 10 mg/L PTU

Figure 5. Light micrographs (10 x magnification) of histological sections of thyroid glands from *X. laevis* tadpoles after 14 days of exposure to PTU during the stage 54 exposure study. See text for details.



10 mg/L PTU

Figure 6. Light micrograph (10 x magnification) of a histological section of thyroid gland from *X. laevis* tadpole after 14 days of exposure to PTU during the stage 54 exposure study. See text for details.

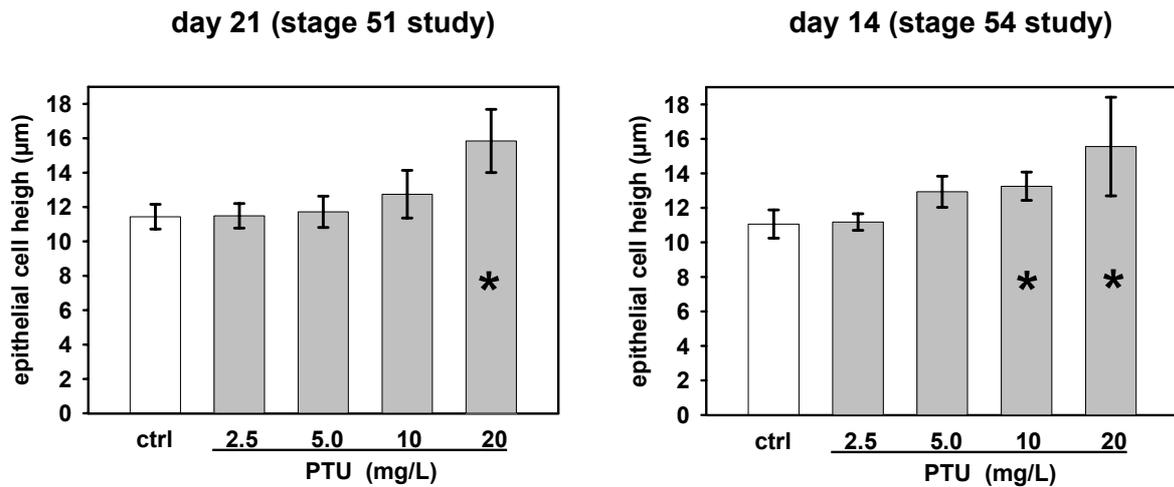


Figure 7. Effects of PTU exposure on epithelial cell height in thyroid glands of *X. laevis* tadpoles. Columns and bars represent mean values \pm SD of 10 animals per treatment group. Significant differences from the control group (ctrl) are marked by asterisks (* $p < 0.05$; Dunnett's test)

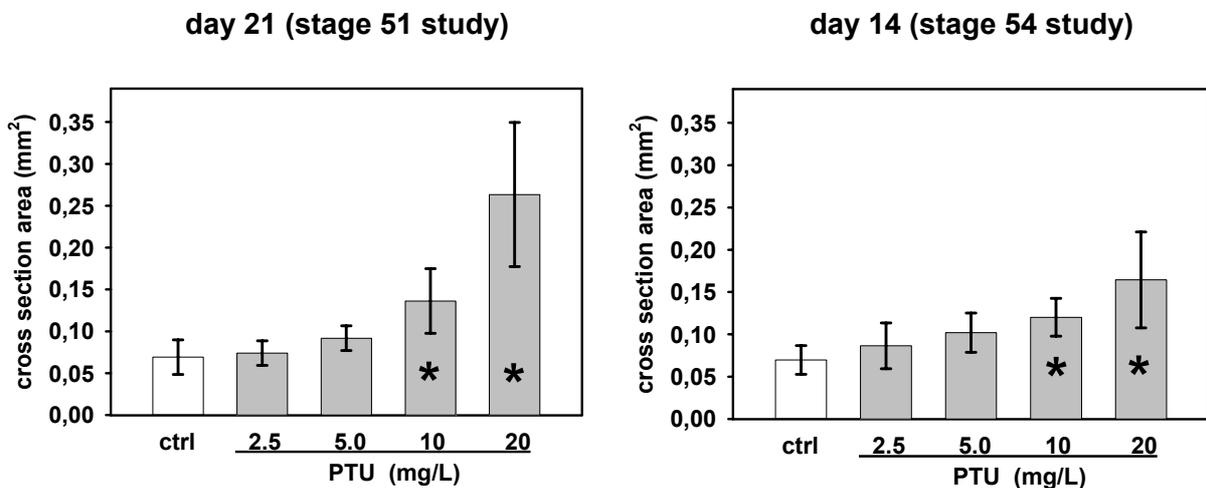
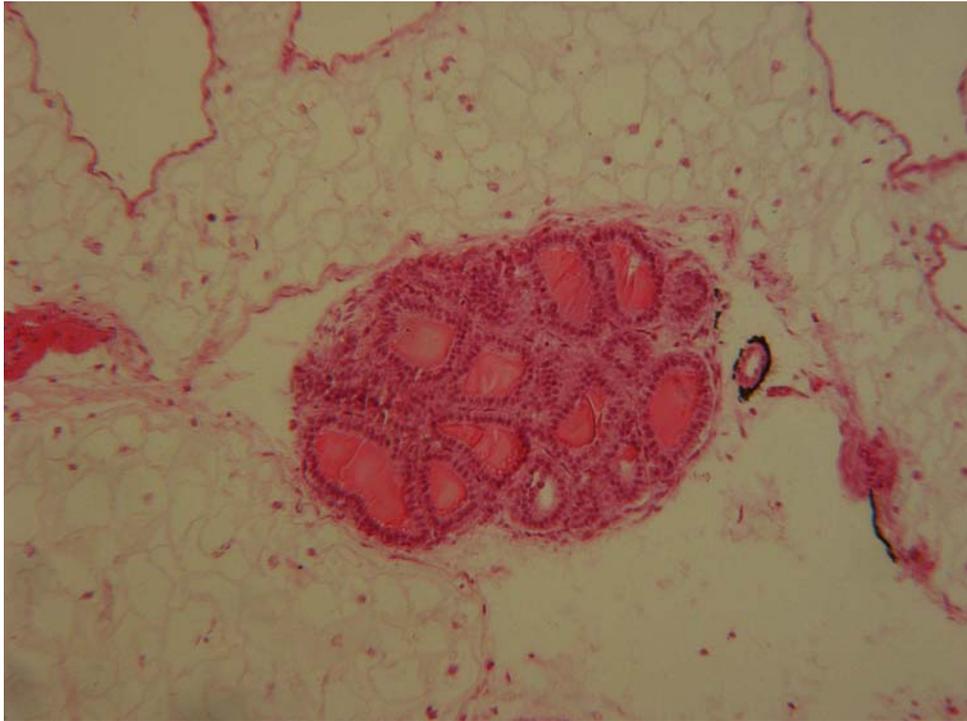


Figure 8. Effects of PTU exposure on maximum cross section area of thyroid glands of *X. laevis* tadpoles. Columns and bars represent mean values \pm SD of 10 animals per treatment group. Significant differences from the control group (ctrl) are marked by asterisks (* $p < 0.05$; Dunnett's test)

Effects of T4 Exposure on Thyroid Histology

In the stage 51 and stage 54 exposure studies with T4, the light histological appearance of thyroid glands from tadpoles exposed to T4 concentrations of 0.25, 0.5 and 1.0 µg/L did not differ from the control group (Figures 9A, 9B, 10A and 10B). A single layer of cuboidal follicular epithelial cells was present in glands from these treatment groups. The colloid showed a homogeneous tinctoral quality and peripheral vacuolation of the colloid was minimal. In tadpoles exposed to the highest T4 concentration (2.0 µg/L), a slight increase in the degree of peripheral vacuolation of the colloid was observed (Figure 11). Furthermore, an increased prevalence of follicles lined by columnar epithelial cells was noticed. These changes were observed in both exposure studies with T4.

A quantitative analysis of epithelial cell heights and maximum cross section area of the right thyroid lobe is currently in progress for tissue samples from both T4 exposure studies. Results from measurements of epithelial cell heights are shown in Figure 15. Statistically significant increases in epithelial cell height were detected at 2.0 µg/L (stage 51 exposure study) and 1.0 and 2.0 µg/L T4 (stage 54 exposure study).

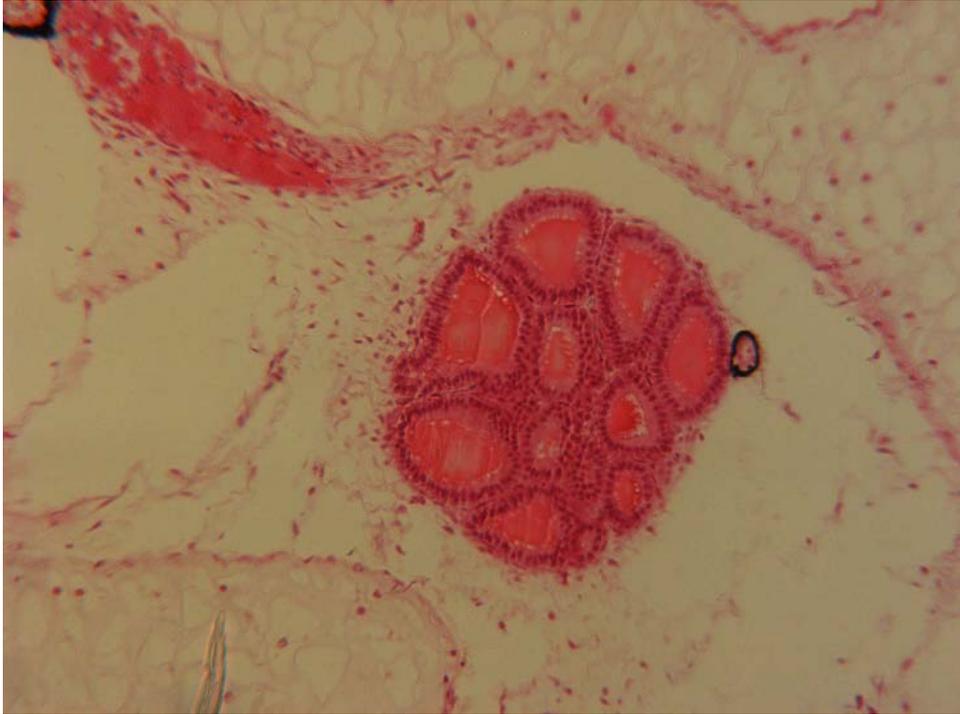


(A) control

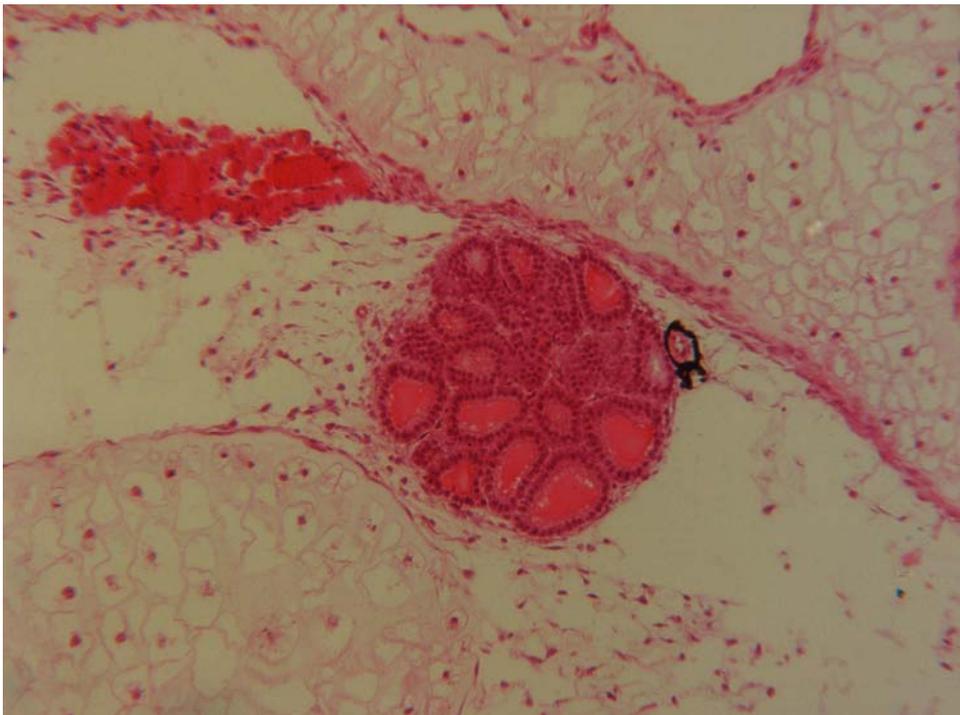


(B) 0.25 µg/L T4

Figure 9. Light micrographs (10 x magnification) of histological sections of thyroid glands from *X. laevis* tadpoles after 21 days of exposure to T4 during the stage 51 exposure study. See text for details.

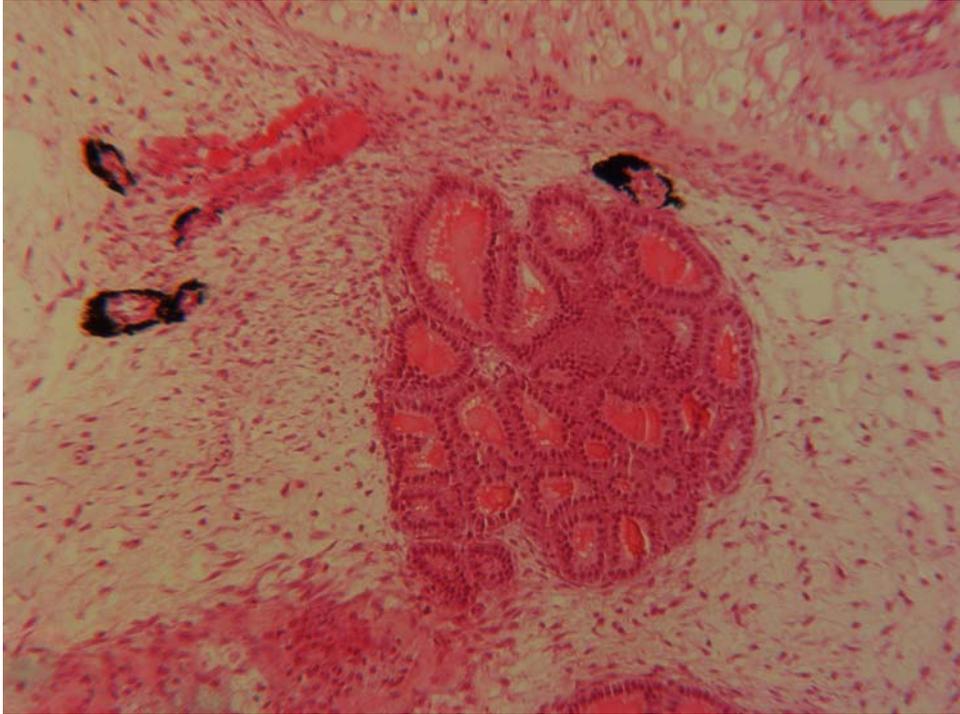


(A) 0.5 µg/L T4



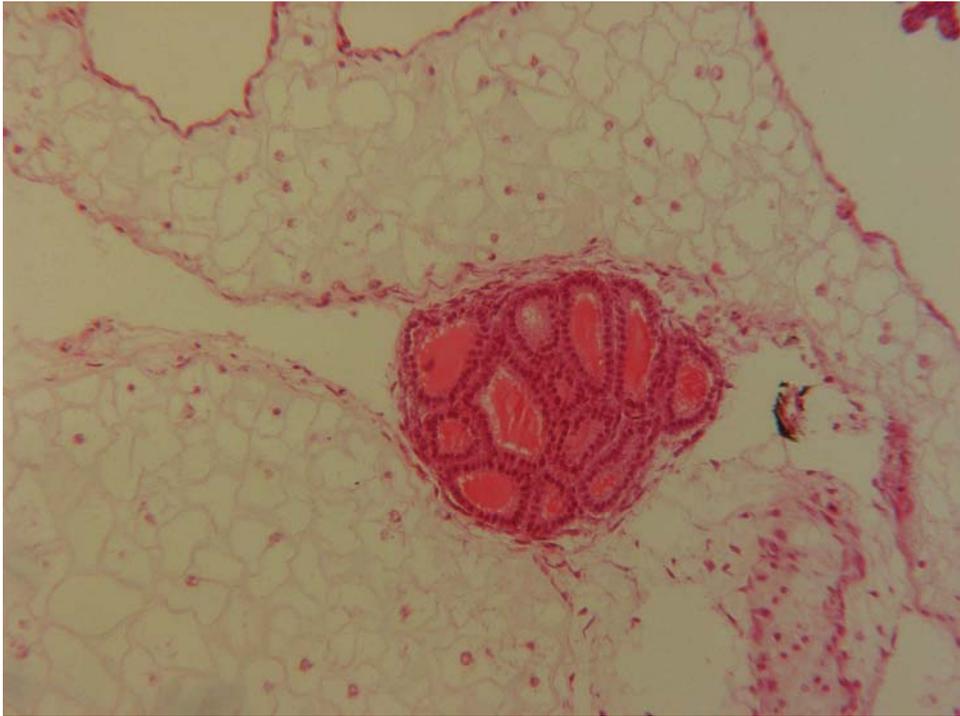
(B) 1.0 µg/L T4

Figure 10. Light micrographs (10 x magnification) of histological sections of thyroid glands from *X. laevis* tadpoles after 21 days of exposure to T4 during the stage 51 exposure study. See text for details.

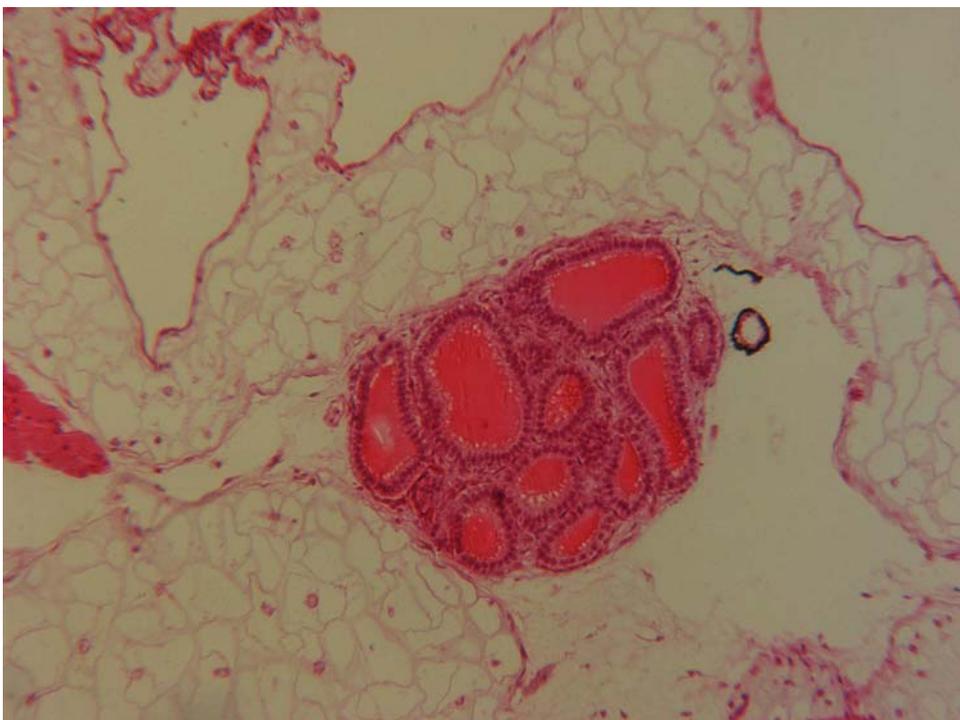


2.0 µg/L T4

Figure 11. Light micrograph (10 x magnification) of a histological section of thyroid gland from *X. laevis* tadpole after 21 days of exposure to T4 during the stage 51 exposure study. See text for details.

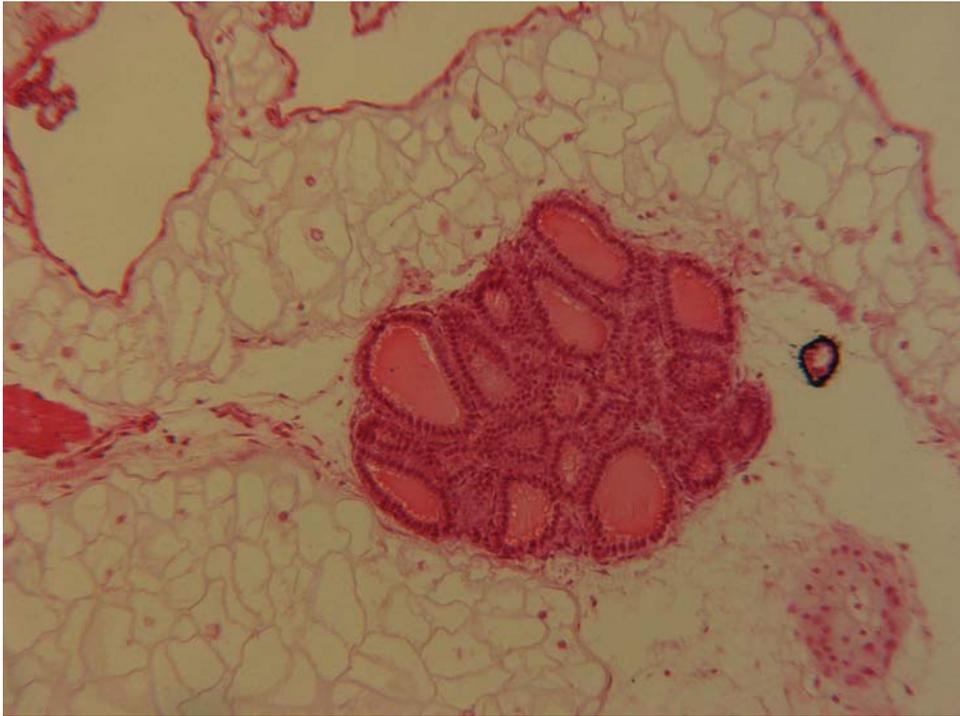


(A) control



(B) 0.25 µg/L T4

Figure 12. Light micrographs (10 x magnification) of histological sections of thyroid glands from *X. laevis* tadpoles after 14 days of exposure to T4 during the stage 54 exposure study. See text for details.

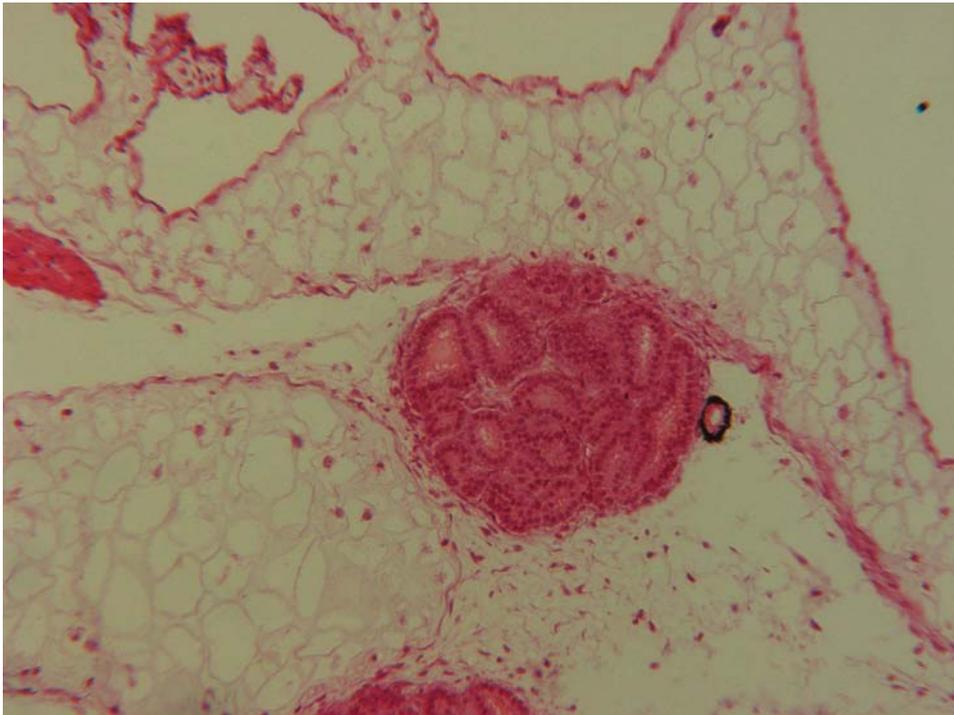


(A) 0.5 µg/L T4



(B) 1.0 µg/L T4

Figure 13. Light micrographs (10 x magnification) of histological sections of thyroid glands from *X. laevis* tadpoles after 14 days of exposure to T4 during the stage 54 exposure study. See text for details.



2.0 µg/L T4

Figure 14. Light micrograph (10 x magnification) of a histological section of thyroid gland from *X. laevis* tadpole after 14 days of exposure to T4 during the stage 54 exposure study. See text for details.

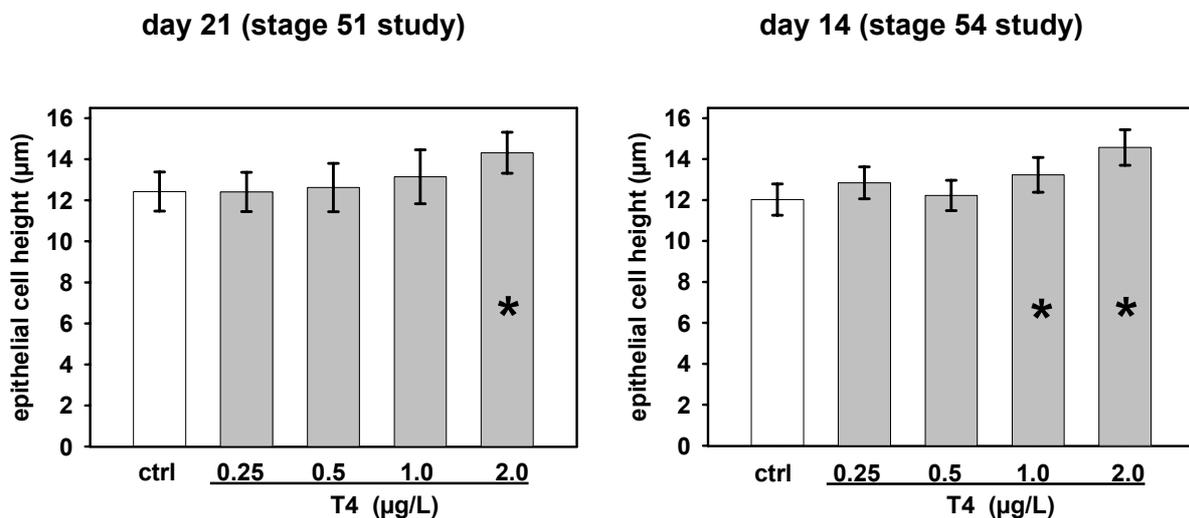


Figure 15. Effects of T4 exposure on epithelial cell height in thyroid glands of *X. laevis* tadpoles. Columns and bars represent mean values \pm SD of 10 animals per treatment group. Significant differences from the control group (ctrl) are marked by asterisks (* $p < 0.05$; Dunnett's test)