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Changes in thyroid histomorphology and thyroglobulin immunostaining upon exposure to thiourea in *Triturus* newts

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ABSTRACT

Amphibians are useful bioindicators for monitoring aquatic health and the influence of xenobiotics such as endocrine disrupting chemicals. Because aquatic ecosystems experience the majority of global pollution, aquatic organisms are most exposed and vulnerable to endocrine disruptors. Furthermore, penetration of endocrine disruptors into aquatic organisms especially in amphibians is even easier because of more permeable skin, resulting in high bioavailability and bioaccumulation of chemicals. One of the most potent endocrine disruptors is thiourea, which chemically blocks the synthesis of thyroid hormones and prevents metamorphosis in amphibians. We investigated the influence of thiourea on histomorphology of the thyroid gland in *Triturus* newts at the metamorphic stage, when thyroid hormone concentrations should reach their maximum level. Chronic exposure to thiourea induced hypertrophy and hyperplasia of follicular cells as well as a significant reduction of interstitial tissue. The intensity of the thyroglobulin immunostaining signal significantly decreases upon chronic exposure to thiourea. Successful cross-reactivity of human primary antibody in immunochemical detection of thyroglobulin in Urodela confirms potential homology in thyroglobulin structure throughout the vertebrates.

1. Introduction

The aquatic ecosystem is one of the most fragile ecosystems because it receives most of the pollutants released into the environment. One of the best explored examples of endocrine disrupting chemicals in wildlife was obtained from fishes (Arcand-Hoy and Benson, 1998; Carnevali et al., 2018). Such information in amphibians is still scarce, despite their worldwide decline (Kloas, 2002; Stuart et al., 2004). Amphibians are characterized by a biphasic life cycle with an aquatic larval stage, followed by metamorphosis into a more or less terrestrial adult. The most notable changes include resorption of the tail fin, loss of the external gills, closure of gill slits, remodeling of the cranial skeleton, formation of eyelids and nasolacrimal duct, as well as loss of Leydig cells in the skin and keratinization of the skin (Lynn, 1961; Gilbert, 2000; Smirnov, 2006; Takamura et al., 2020).

Thyroid hormones (THs; L- thyroxine, T_4 and L- triiodothyronine, T_3) are crucial for larval development, growth and especially metamorphosis in amphibians. Generally, metamorphosis depends on levels of THs in the bloodstream and their production is maintained by a classical negative feedback loop involving the hypothalamic (thyrotropin-releasing hormone) – pituitary (thyrotrophic cells) – thyroid axis (Kim and Mohan, 2013; Bassett and Williams, 2016; Ortiga-Carvalho et al., 2016). During larval development secretion of THs increases towards metamorphosis, reaches a maximum at metamorphosis, and decreases after metamorphosis (White and Nicoll, 1981; Norman et al., 1987). THs are produced by the thyroid gland, which is one of the largest endocrine glands found in all vertebrates. In endothermic vertebrates, the most important function of this gland is to regulate the rate of metabolism. Also, the thyroid gland is possibly the most conservative gland throughout the vertebrates considering anatomy, function and the hormones produced (McNabb and King, 1993; Norris, 1997).

In amphibians, the thyroid gland is a paired structure, unlike in

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sharks, reptiles and mammals, where it is one single mass. The thyroid gland is composed of numerous, more or less spherical follicles consisting of simple epithelium surrounding the central cavity or lumen filled with colloid, which constitutes as a thyroid hormones reservoir (Norris and Lopez, 2005). The follicles are bound together by interfollicular connective tissue or interstitium. One of the major components of colloid is thyroglobulin (Tg), a glycoprotein synthesized by the thyrocytes, secreted apically and sequestered in the follicle lumen. Various vertebrate groups differ in the timing when the thyroid gland becomes active during development. In amphibians, a histologically identifiable thyroid gland was recorded during early larval development just after hatching (Jennings and Hanken, 1998; Kent et al., 2014), but the pituitary-thyroid axis does not begin functioning until the middle/late larval stage (Irikhimovitch, 1948). This late development of the thyroid gland in amphibians relative to mammalian embryos is probably associated with the high levels of maternal thyroid hormones stored in the eggs' yolk, which are sufficient for normal embryonic development but not for metamorphosis (Rupik, 2011).

Endocrine disrupting chemicals, in contrast to thyroidectomy (surgical removal of the thyroid gland), do not fully block THs synthesis and/or secretion, and a small amount of hormones still exist in the blood stream (Brown, 1997). One of the most potent endocrine disrupting chemicals is thiourea, which is a well-established agent in treating hyperthyroidism or Grave's disease in humans. Thiourea causes the pituitary gland to overproduce thyroid-stimulating hormone in response to the drop in the level of THs in the blood that leads to thyroid gland hypertrophy. Beside strong inhibitory effect on THs synthesis in different organisms (fish, amphibians, lizard and mammals) this anti-thyroid agent shows other properties such as a high gastrointestinal absorption (Degitz et al., 2005; van der Ven et al., 2006). The mechanism of action is very similar for all anti-thyroid agents via inhibition of the thyroid peroxidase enzyme, thereby inhibiting iodination of tyrosine residues in thyroglobulin, the oxidative coupling of iodinated tyrosine, and therefore the biosynthesis of the thyroid hormones (Davidson et al., 1979). Thiourea exposure was reported to inhibit metamorphosis in anuran tadpoles (Steinmetz, 1950; Krishnapriya et al., 2014) and cause a delay in cranial osteology of newly metamorphosed newts (Smirnov and Vassilieva, 2003a, 2003b, 2005; Smirnov et al., 2011; Ajduković et al., 2021). In nature, thiourea may be present in surface waters and sediments over prolonged periods (Mutic et al., 2017) because it is added to fertilizers to inhibit the nitrification process (Wang et al., 2017) and thiourea could thus directly threaten amphibians in their natural habitat. Also, thiourea can be found in drinking water and food and humans can be exposed to the harmful effects of this chemical.

The aim of this study is to investigate the effects of thiourea on thyroid histomorphology in *Triturus* newts. Thyroid histological examinations are a valuable and sensitive diagnostic for evaluation of changes in THs production and potential disruption of the hypothalamic – pituitary – thyroid axis by endocrine disrupting chemicals during development and metamorphosis (Degitz et al., 2005; Grim et al., 2009). Moreover, some data indicate the possibility that a substituted derivative of thiourea, ethylene thiourea, may provoke development of thyroid tumors in rodent models (Hurley, 1998). Therefore, we aimed to determine the severity of histopathological changes in the thyroid gland upon chronic exposure to thiourea. Finally, to improve histopathological analysis, we tested mammalian Tg antibodies for immunohistochemical assessment of the thyroid follicular tissue, which, to the best of our knowledge, has never been previously explored in urodeles.

2. Materials and methods

2.1. Animal housing

The genus *Triturus* forms a well-supported monophyletic clade of newts within the family Salamandridae (Wielstra et al., 2019; Rancilhac et al., 2021). According to current taxonomy, the genus *Triturus* consists of nine species: two marbled (*T. marmoratus* and *T. pygmaeus*) and seven crested (*T. anatolicus, T. carnifex, T. cristatus, T. dobrogicus, T. ivanbureschi, T. karelinii* and *T. macedonicus*) newt species (Wielstra and Arntzen, 2016). In this study, we used hybrid individuals obtained from crosses between two crested newt species: *T. ivanbureschi* and *T. macedonicus*. These two species hybridize in nature at their contact zone (Arntzen et al., 2014; Wielstra et al., 2017) and hybrids cannot be distinguished based on their external features from their parental species (Arntzen et al., 2018). Subtle differences in life history and morphological traits between hybrids and their parental species are in the range of differences between the two parental species (Bugarčić et al., 2022; Vučić et al., 2019, 2022).

Matings were performed in March 2019 in common containers (500 L) filled with dechlorinated tap water and covered with a protective net. After mating, gravid females were transferred to separate aquaria filled with dechlorinated tap water and with plastic strips for egg deposition. Eggs were collected daily and kept submerged in dechlorinated tap water in plastic Petri dishes until hatching. Hatchlings were transferred to a small common aquarium, where they developed under a natural day-night light regime at an air temperature of 18° ± 1 °C and a water temperature of 16° ± 1 °C. Under these laboratory conditions, normal larval development of *Triturus* newts, from hatchling to metamorphosis, lasts approximately 120–130 days (Furtula et al., 2009). Larvae were fed *ad libitum* with *Artemia* sp. at earlier developmental stages and with *Tubifex* sp. at later stages.

2.2. Experimental settings

Three experimental groups were defined: 1) control (dechlorinated tap water – C), 2) low thiourea concentration (0.05% solution of thiourea – TU 0.05%) and 3) high thiourea concentration (0.1% solution of thiourea – TU 0.1%). These concentrations of thiourea were established in previous work on salamanders and both induce goitrogenic effects (Wheeler, 1953). Thiourea (p.a. ≥99.0%; Sigma, St. Louis, MO, USA) solutions were prepared by dissolving thiourea crystals in dechlorinated tap water.

Developmental stage 62 is the last larval stage before metamorphosis, characterized by fully developed limbs, well-formed external gills, and yellowish color skin with rounded melanophores (Glücksohn, 1931). When larvae reached this stage, approximately 46 days after hatching, they were randomly transferred from common aquaria to 2-L plastic containers half-filled with different media depending on the experimental group (control and two thiourea concentrations). The medium of the experimental groups was changed three times per week. To avoid potential high larval density caused by the increase in larval body mass and size, which invariably leads to some detrimental effects, such as reduction in larval growth or size at metamorphosis (Altwegg, 2003), larvae were kept at the same density (three larvae per container). Exposure to thiourea lasted roughly three months.

Metamorphic individuals are characterized by the resorption of external gills and the complete closure of gill slits. Also, dramatic changes in the skin are notable, especially in pigmentation and thickness (skin became darker with white dots spread along the midline of the body). Because thiourea inhibits THs synthesis, and therefore inhibits the ability to metamorphose, sampling of larvae was based on normally metamorphosing individuals in the control group. Metamorphosed juveniles and treated larvae were euthanized with ethyl 3-aminobenzoate methanesulfonate, better known as MS 222 (CAS Number: 886–86–2; Sigma, St. Louis, MO, USA), and processed for further histological analysis (see Section 2.3. for detailed explanation). For histological analysis, we used 17 individuals from three experimental groups: C (n = 6), TU 0.05% (n = 6) and TU 0.1% (n = 5). For immunohistochemical staining we used 9 individuals: C (n = 3), TU 0.05% (n = 3) and TU 0.1% (n = 3).

Collection of adults of parental species from natural populations was approved by the Ministry of Energy, Development and Environmental

Protection of the Republic of Serbia (permit no. 353–01–75/2014–08) and the Environmental Protection Agency of Montenegro (permit no. UPI-328/4). The experiment was approved by the Ethical Committee of the "Siniša Stanković" Institute for Biological Research (decision no. 02–07/19). All experimental animals were treated in compliance with European directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes.

2.3. Tissue processing and staining methods

The entire lower jaw was removed from each animal and immediately fixed in 4% neutral buffered formaldehyde (Centrohem, Serbia) for 24 h (Bancroft and Gamble, 2002). The tissue was then dehydrated with increasing grades of ethanol solution (30–100%), cleared in xylene and embedded in Histowax (Histolab Product AB, Göteborg, Sweden). Serial longitudinal tissue Section (5 μm thickness) were obtained on a rotary microtome (RM 2125RT Leica, Wetzlar, Germany). Hematoxylin-eosin histochemical staining (H&E) was performed on deparaffinized and rehydrated tissue sections, using Mayer's haematoxylin (Sigma-Aldrich) for three minutes and counterstained with eosin (Sigma-Aldrich) for five minutes.

Novelli histochemical method was used as previously reported (Šošić-Jurjević et al., 2015). In brief, deparaffinized and rehydrated tissue sections were incubated in hot 1 N HCl (60 °C, three minutes), followed by staining in 1% acid fuchsin (Fluka Chemie AG, Switzerland; 30 s) and 1% light green (Sigma-Aldrich, USA; 30 s). In between, the slides were washed in distilled water. For both histochemical procedures, after the final dehydration of samples through graded series of ethanol solution (70–100%), the slides were cleared with xylene and mounted in DPX (Sigma-Aldrich Co., USA).

Immunohistochemical (IHC) staining was performed as previously described for the Wistar rat thyroid tissue (Šošić-Jurjević et al., 2015), using rabbit antiserum directed against human thyroglobulin (Tg; Dakopatts, Glostrup, Denmark). In brief, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 15 min. Reduction of non-specific background staining was achieved by incubation with normal porcine serum (Dakopatts, Denmark; 1:10) for 45 min. The rabbit antiserum directed against human Tg (1:500) was applied overnight at 4ºC. Swine anti-rabbit IgG- horseradish peroxidase (HRP; Dakopatts, Denmark; 1:200) was applied as a secondary antiserum for 1 h. Visualizations were performed using diaminobenzidine tetrahydrochloride (DAB; Dakopatts, Denmark) at concentrations suggested by the manufacturer. All washes and dilutions were performed using 0.1 M PBS (pH 7.4). The sections were counter-stained with hematoxylin and mounted in DPX medium (Sigma-Aldrich Co., USA). For the control of secondary antibody activity, the primary antibody was substituted with PBS (pH 7.4). Wistar rat thyroid sections served as the positive control to confirm the species cross-reactivity of the antiserum.

Digital images were made on a LEITZ DM RB light microscope (Leica Mikroskopie & Systems GmbH, Wetzlar, Germany), with a LEICA DFC320 CCD camera (Leica Microsystems Ltd., Heerbrugg, Switzerland) and Leica DFC Twain Software (Leica, Germany).

2.4. Morphometric analysis and quantitative image analysis

Morphometric assessment was performed on the H&E-stained thyroid sections according to Šošić-Jurjević et al. (2015). Four to five sections taken from the central part of both glands from all 17 specimens were analyzed. The measurements were carried out using the newCAST stereological software package (VIS – Visiopharm Integrator System, version 3.2.7.0; Visiopharm; Denmark), at an objective magnification of 40x. The counting area was defined using a mask tool; a test grid (5 ×5) with uniformly spaced test points and lines was provided by the new-CAST software. The relative volume densities (V_V) of follicular epithelium, colloid and interstitium were calculated as the ratio of the number of points hitting the respective tissue phase divided by the number of points hitting the analyzed reference space (i.e. the sum of points on the epithelium, colloid and interstitium): V_V (%) = Pp/Pt × 100 (Pp, counted points hitting the tissue phase, Pt, the total of points of the test grid hitting the reference space).

For the quantification of the DAB IHC signal of Tg we used the ImageJ Fiji software (Image J, Version 1.49j) with the open source plugin IHC profiler (Varghese et al., 2014). Six unbiased captured images (2592 ×1944 pixels, 40x objective magnification) per animal were analyzed. The IHC profiler plugin allowed color deconvolution of IHC images (using the set optical density vectors for DAB and hematoxylin) and profiling of the DAB-stained cytoplasmic stained image sample, which was then, using the threshold function of ImageJ, used for the analysis of mean gray intensity. Optical density (OD) was calculated as OD = log (max intensity/mean intensity).

2.5. Statistical analysis

We used a one-way analysis of variance (ANOVA) to test whether there is any statistical difference between the control group and two thiourea treatments in relative volume for all tissue phases (epithelium, colloid and interstitium). To determine which specific groups differed from each other, we used the Tukey HSD post hoc test. A T-test was used to determine if there is a significant difference between the means of the control group and two thiourea treatments in the optical density of Tg immunopositivity. All statistical analyses were done in Statistica 10 (StatSoft Inc. 2011). For graphical presentation the *ggplot2* package (Wickham, 2016) in R version 4.0.5 (R Core Team, 2021) was used.

3. Results

3.1. Histomorphometric analysis of the thyroid gland

All individuals from the control group normally metamorphosed at the end of the experiment, unlike individuals from both thiourea treatments, which retained larval characteristics and failed to metamorphose. Histomorphometric analysis of the thyroid gland revealed some notable changes between the control group and thiourea-treated animals.

The thyroid gland of control newts is present as paired oval structures lying in the lower jaw anterior to the arterial arch and lateral to the geniohyoideus muscles. They are generally composed of a small number of thyroid follicles and predominant interstitial tissue (Fig. 1A, arrows). Thyroid follicles are characterized by a tall, prismatic epithelium and the eosinophilic luminal colloid with clearly visible resorption vacuoles (Fig. 1B). Within the interstitium, large elliptical erythrocytes with the purple nuclei clearly evident on Novelli-stained sections were the most prevalent stromal tissue elements, which indicate prominent vasculature of the thyroid gland (Fig. 1C). Low and high concentrations of thiourea caused hypertrophy and hyperplasia of follicular cells (Fig. 1D, G, arrows). A greater number of thyroid follicles ware noticed in the thyroid gland of both thiourea-treated animals in comparison with the control group, characterized by columnar and taller follicular epithelium (Fig. 1E, H). The luminal colloid in the follicles was greatly reduced, in line with expected thyroid-stimulating hormone, which stimulated endocytosis and intracellular breakdown of the colloid (Fig. 1E, H, asterisks). Chronic exposure to both thiourea concentrations induced significant changes in thyroid tissue, which were primarily reflected by an increase of the follicular and a reduction of the interstitium tissue phase, as well as vascularity, in line with the histological appearance of a parenchymatous goiter in mammals (Fig. 1F, I, arrowheads).

The morphometric analysis demonstrated that in the thyroid tissue of the control group the relative V_V of follicular epithelium represented 28.07 ± 8.8%; V_V of the colloid was 9.33 ± 3.3 while V_V of interstitium represented 62.6 ± 11.5%. The ANOVA results revealed a statistical significance difference ($p < 0.05$) between the control group and the

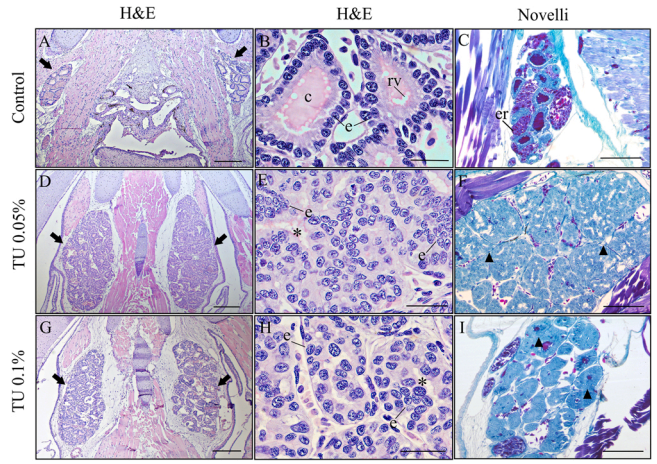

Fig. 1. Representative micrographs of the thyroid gland of *Triturus* newts from metamorphosed control group and newts exposed to two concentrations of thiourea (TU 0.05% and TU 0.1%). Hematoxylin and eosin (H&E) staining, 2.5x objective magnification (bar=500 μm; A, D and G) and 20x objective magnification (bar=100 μm; B, E and H). Novelli staining, 10x magnification (bar=200 μm; C, F and I). Arrows point to the thyroid glands, which were significantly enlarged upon thiourea treatments (D and G) compared to the control group; asterisks indicate follicles devoid of colloid (E and H); and arrowheads point to diminished interstitial vasculature (H-I); c, colloid; e, epithelium; er, erythrocytes; rv, resorption vacuoles.

two thiourea treatments for V_V of follicular epithelium and interstitium (Table 1). A Tukey HSD test for multiple comparisons revealed that mean values of epithelium and interstitium were significantly different ($p < 0.05$) between the control group and the two concentrations of thiourea (TU 0.05% and TU 0.1%), but there was no statistically significant difference between two thiourea concentrations (Table 2). Both thiourea concentrations induced a 60% and 55% increase ($p < 0.05$) of relative V_V of follicular epithelium, while the V_V of the colloid was not significantly altered in comparison to the corresponding control values. On the other hand, V_V of the interstitium was reduced after both thiourea concentrations exposure by 43% and 35% ($p < 0.05$). The obtained data are summarized in Fig. 2.

3.2. Immunohistochemical analysis of Tg immunostaining in the thyroid gland

In control newts, Tg immunostaining was highly specific and diffuse in the cytoplasm of thyroid follicular epithelial cells (Fig. 3A, B), being the strongest at the apical surface of the follicular cells (Fig. 3B, arrows). Tg immunopositivity was also evident in the luminal colloid, but to a smaller extent (Fig. 3B, D, F). Upon exposure to both thiourea concentrations, the pattern of Tg immunostaining remained unchanged – being more intense in the follicular epithelium than in the colloid (Fig. 3C-F). However, the distribution of intense immunopositivity within the thyrocytes changed from the apical to the perinuclear region (Fig. 3D, F, arrows). The optical density (OD) of Tg immunopositivity was 41% ($p = 0.007$) and 39% ($p = 0.013$) lower in TU 0.05% and TU 0.1% treatments, in comparison with OD value of the control group (Fig. 4).

Table 1
Relative volume density for control and two thiourea treatment groups for all tissue phases (epithelium, colloid and interstitium) of the *Triturus* newt thyroid gland. ANOVA test; the statistical significance $P < 0.05$ is indicated by boldface.

	df	F	p
Epithelium	2	21.15	**< 0.0001**
Colloid	2	0.59	0.5683
Interstitium	2	14.63	**0.0004**

Table 2
Summary of the Tukey HSD post hoc test between control (C) and two thiourea treatment groups (TU 0.05% and TU 0.1%) for all tissue phases (epithelium, colloid and interstitium) of the *Triturus* newt thyroid gland. The statistical significance $P < 0.05$ is indicated by boldface.

	Treatments	C	TU 0.05%	TU 0.1%
Epithelium	C		**0.0002**	**0.0008**
	TU 0.05%	**0.0002**		0.6149
	TU 0.1%	**0.0008**	0.6149	
Colloid	C		0.6552	0.6071
	TU 0.05%	0.6552		0.9920
	TU 0.1%	0.6071	0.9920	
Interstitium	C		**0.0006**	**0.0039**
	TU 0.05%	**0.0006**		0.6289
	TU 0.1%	**0.0039**	0.6289	

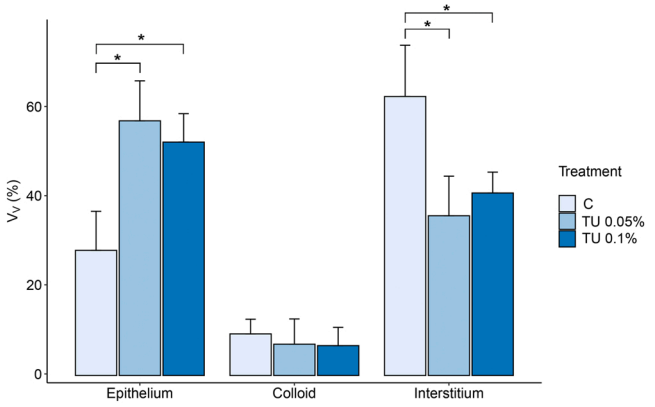

Fig. 2. The relative volume density (V_V;%) of follicular epithelium, colloid and interstitium in the thyroid gland of *Triturus* newts from the metamorphosed control group (C) and newts exposed to two concentrations of thiourea (TU 0.05% and TU 0.1%). The values are mean ± SE; $p < 0.05$ and (*) present a statistical significant difference between the control group and two thiourea concentrations.

Moreover, some follicles were almost completely colloid-depleted. Aside from epithelial hyperplasia of the follicles and development of parenchymatous goiter, epithelial atypia that may eventually progress to carcinoma was not detected, despite chronic exposure to thiourea during the late larval period (almost three months).

4. Discussion

The mechanism of thiourea action, as a potent endocrine disruptor, is chemical blockade of THs synthesis, which leads to a delay in the development and metamorphosis process in amphibians (Gobdon et al., 1943; Degitz et al., 2005; Thambirajah et al., 2019). Even small concentration of this endocrine disruptor can induce delay in metamorphosis in poikilothermic animals (Trijuno et al., 2002).

The histomorphological results of this study revealed that chronic exposure to thiourea induce hypertrophy and hyperplasia of thyroid epithelial cells in *Triturus* newt, without development of thyroid epithelial atypia upon exposure to both low and high concentrations of thiourea. A difference in histomorphology existed between the control group and both thiourea concentrations, but there was no significant difference between the two thiourea concentrations. The thyroid gland of treated animals showed reduced interstitium and vascularity due to hypertrophy of thyroid follicles, which indicates that the thyroid glands were exhausted and THs synthesis was inhibited compared to the control group. The obtained histological changes are in agreement with previously published ones in adult newts from the genus *Desmognathus* and anuran tadpoles from the genus *Lithobates* (Wheeler, 1953; Takamura et al., 2020) and indicate suppression of TH production (Grim et al.,

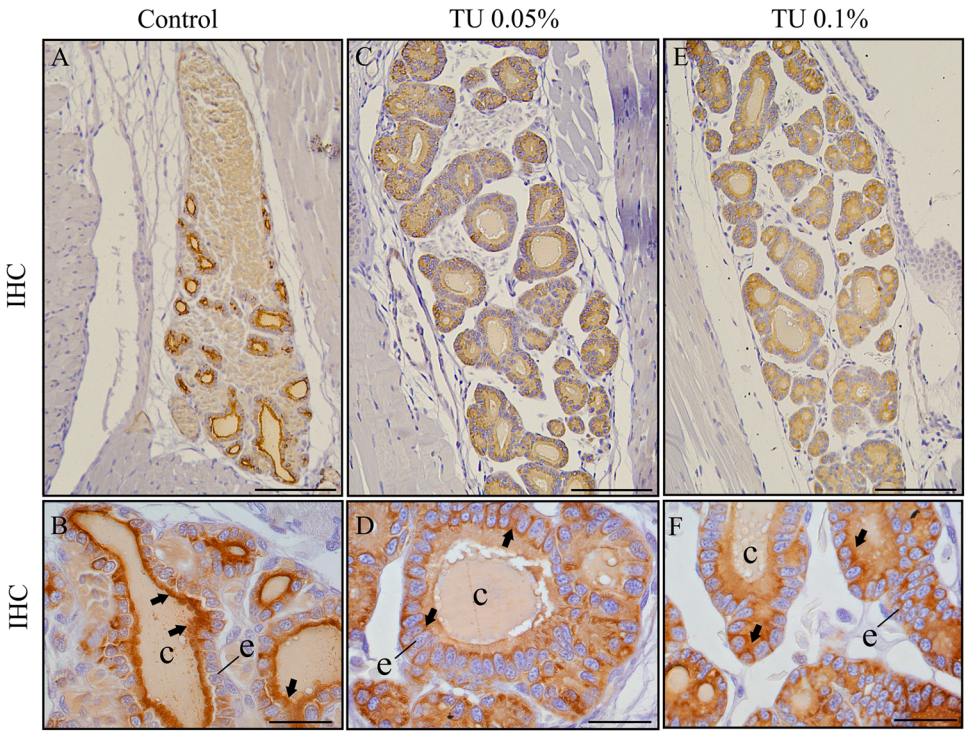

Fig. 3. Representative micrographs of immunohistochemical staining for thyroglobulin (Tg) in the thyroid gland of *Triturus* newts from the metamorphosed control group and newts exposed to two concentrations of thiourea (TU 0.05% and TU 0.1%). For micrographs (A, C and E) at 10x objective magnification, bar= 200 μm, and for micrographs (B, D and F) at 40x magnification, bar= 50 μm. c, colloid; e-epithelium. Arrows point altered distribution of intense Tg immunopositivity within follicular epithelium, from apical to perinuclear region of cytoplasm.

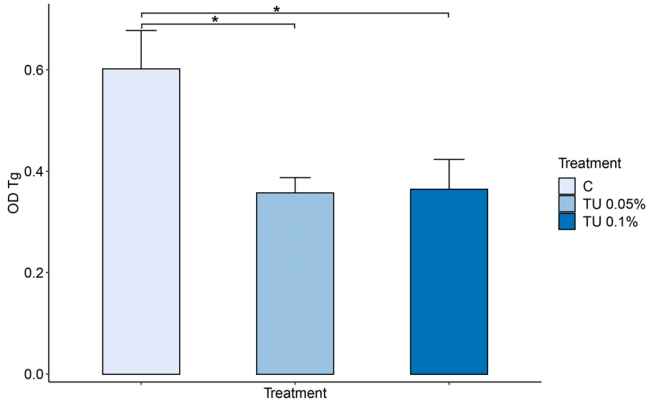

Fig. 4. Optical density (OD) of thyroglobulin (Tg) immunopositivity in the thyroid gland of *Triturus* newts from the metamorphosed control group (C) and newts exposed to two concentrations of thiourea (TU 0.05% and TU 0.1%). The values are mean ± SE; $p < 0.05$ and (*) present a statistical significant difference between control and two thiourea concentrations.

2009).

Decrease in Tg immunostaining intensity correlated with redistribution of apical membrane proteins into perinuclear region of the thyrocytes in thiourea treated animals. The intense perinuclear Tg immunostaining in thiourea treated newts probably corresponds to newly synthesized Tg in dilated cisternae of endoplasmic reticulum and Golgi apparatus, keeping in mind results of Yi et al. (1997). These authors demonstrated by immunogold electron microscopy that administration of propylthiouracil to rat stimulated synthesis and secretion of Tg in the thyroid follicular epithelium, but at the same time inhibited reabsorption and degradation of Tg.

In the case of *Triturus* newts, both concentrations of thiourea (low and high) induced pronounced changes in the thyroid gland and these two concentrations can be optimal for causing histomorphological and physiological changes in thyroid gland. In some other amphibians, an optimal concentration of thiourea (a concentration that induces change but is not lethal) affects metabolic costs and energy allocation. In anurans, treated tadpoles can reach almost twice the length of control tadpoles, but the morphology, internal histology, and biochemistry remain larval (Krishnapriya et al., 2014; Ruthsatz et al., 2019).

Some notable changes in phenotype are evident in *Triturus* larvae treated with thiourea relative to metamorphic individuals from the control group. Larvae treated with thiourea have "arrested metamorphosis", which means they did not metamorphose but continue to growth while retaining larval characteristics (larval cranial morphology with well-developed gills, larval thin skin and pigmentation). Also, results are in agreement with thiourea effects on cranial morphology obtained from the same *Triturus* larvae used in this study (Ajduković et al., 2021) and from independent set of *Triturus* larvae used for oxidative stress status (Gavrić et al., 2021). The deficit in the THs level in the blood in thiourea-treated larvae slowed down cranial remodeling during metamorphosis and they retained late larval cranial morphology (Ajduković et al., 2021). The higher concentration of thiourea affected the antioxidative parameters to the extent that oxidative damage could not be avoided, contrary to the lower concentration which has antioxidative properties due to chronic exposure (Gavrić et al., 2021). Otherwise, exposure to thiourea in *Triturus* newts did not induce thyroid carcinoma development, in contrast to results obtained in rodent models exposed to thyroid inhibitors (Fang et al., 1994).

Some studies report the use of thiourea to obtain so-called synchronized amphibian metamorphosis assay used to obtain tadpoles and larvae available at any time for laboratory metamorphosis experiments (Gutleb et al., 2007; Chiba et al., 2012). The advantage of this protocol is that tadpoles at the same stage of development are available at any time to start the metamorphosis experiment and experimental groups are much more homogenous at the beginning of the experimental procedure. The use of thiourea as a pretreatment to synchronize development in amphibians must be taken with caution and morphological, physiological and histological effects should not be ignored.

To the best of our knowledge, this is the first study that confirms cross-reactivity of human primary antibody to be used for thyroglobulin immunohistochemical detection in Urodela. The observed results support the conservative anatomy of the thyroid gland and homology in

protein structure throughout the vertebrates (Holzer et al., 2016). Future studies of immunohistochemical expression of changes in thyroid receptors alpha and beta, whose expression is dominant in all tissue and which are present even before the organism has a functional thyroid gland, may contribute to a better understanding of the mechanisms of thiourea action.

5. Conclusion

Chronic exposured to two thiourea concentrations induce similar changes in histomorphology of the thyroid gland in *Triturus* newts. This was evident as hyperplasia and hypertrophy of follicular cells, while the amount of interstitial tissue was diminished. The obtained changes are in line with the histological appearance of parenchymatous goiter, with no indices of tumor development, and indicate a deficit in THs production at the metamorphic stage of *Triturus* newts, when THs concentration should reach their maximum. Our study confirms cross-reactivity of human primary antibody in immunochemical detection of thyroglobulin in Urodela for the first time. Various endocrine disrupting chemicals can be a significant threat to aquatic biodiversity, but exposure consequences are little understood. Amphibian larvae can be a useful bioindicator for monitoring aquatic health and the influence of various xenobiotics and endocrine disrupting chemicals on amphibian development and metamorphosis because the larval stages are the most sensitive to environmental pollution. The obtained data may serve as a basis for future endocrine disruptor research in amphibians because thyroid hormone disrupting compounds may contribute to the observed global decline of amphibian species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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