Thyroidal Hormone Disrupting Effects and Histopathological Examination in an Amphibian Metamorphosis Assay

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The interference of a chemical with the thyroidal hormone system, that is the normal function of the hypothalamic-pituitary-thyroid (HPT) axis, is considered as an endocrine disrupting effect. The amphibian metamorphosis assay (AMA), which is an OECD and EPA guideline study, represents a generalized vertebrate model to evaluate such a chemical action not only on wildlife but also mammals. This review deals with the state-of-the-art of frog metamorphosis processes and thyroid hormone regulation, and introduces our attempts, especially the histopathological examination, for more precise evaluation of AMA.

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Introduction

Research on the theme of endocrine disruption by chemical substances is moving forward in a broad range that includes not only the effects on human health but also the effects on wildlife. A large number of chemical substances that possibly act on the thyroid hormonal system (hypothalamus - pituitary - thyroid (HPT) axis) in vertebrates have been reported; therefore, establishing a suitable vertebrate model for carrying out risk assessments is an urgent problem. In addition to exposure to chemical substances via the gills or oral exposure via feed, it is postulated that tadpoles, which are the larvae of frogs (amphibians) living in water, are exposed percutaneously because, unlike adults, their skin is thin and has high permeability. Furthermore, tadpoles become frogs through new formation, retraction and reconstruction of structures and functions from larval tissues to adult tissues through the process of metamorphosis; however, this process is primarily controlled by thyroid hormones, and tadpoles are thought to have extreme hypersensitivity to chemical substances that affect the thyroid hormones. The morphological and functional changes to tadpoles during metamorphosis are dramatic, and abnormalities in the process of metamorphosis yield effective information as parameters that reflect thyroid hormone disruption. From the point of view described above, the Organization for Economic Cooperation and Development (OECD) and the US Environmental Protection Agency (EPA) have focused on thyroid toxicity and have formulated guidelines for detailed investigations of the process of metamorphosis in African clawed frogs (*Xenopus laevis*, **Fig. 1**).[1), 2)] The thyroid gland, which is in the heads of tadpoles and which is the target of histological examinations in toxicity studies, is on a line between the two eyes (**Fig. 2**). Because the tissue is extremely small at the beginning of metamorphosis and because the shape of the head changes as metamorphosis progresses, with the position of the thyroid gland changing with it, there is a difference from normal mammals. It is been found that accumulating know-how for pathological studies is necessary for accurate evaluations of toxicity.

From the point of view described above and in consideration of the most recent trends in regulations in

Fig. 1 A larva and a froglet of African clawed frogs (*Xenopus laevis*)

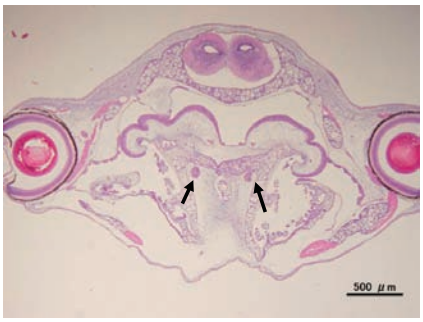


Fig. 2 Arrows indicate thyroid glands in Stage 51

Europe and the United States, the Environmental Health Science Laboratory at Sumitomo Chemical has investigated the metamorphosis and mechanisms of thyroid hormonal control in frogs as a part of exhaustive safety evaluations for endocrine disrupting action by chemical substances set up to include organisms in the environment, and has established exhaustive evaluations of the effects of chemical substances on the thyroid hormonal system using tadpoles.

Thyroid Hormones and Related Factors

The thyroid hormones include thyroxine (T4) and triiodothyronine (T3) (**Fig. 3**). The thyroid gland primarily synthesizes and secretes T4, and T4 is deiodinated and converted into T3 which have a higher level of bioactivity . The thyroid hormones are transported in the blood in a form binding to the transport protein transthyretin (TTR), are conjugated in the liver and excreted into bile. The thyroid hormones, their control and impacts of toxicity are known for mammals.[3] The structure of thyroid hormones in vertebrates is completely preserved, and the modes of synthesis and secretion are basically the same in amphibians and mammals.

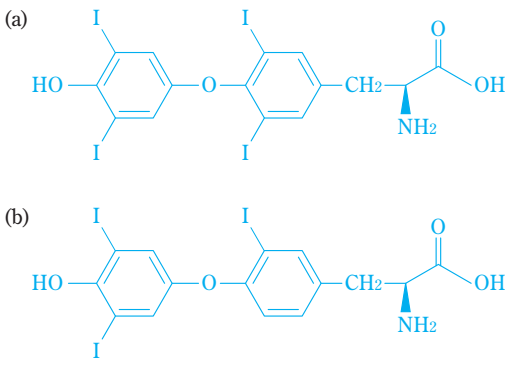


Fig. 3 Thyroxine: T4 (a), and 3,3',5- triiodothyronine: T3 (b)

The thyroid hormones are controlled by the HPT axis, and the mechanisms controlling them and factors affecting them are shown in **Fig. 4**. Inhibiting the uptake of iodine by the thyroid gland and control of thyroid peroxidase disrupts the synthesis of thyroid hormones. In addition, suppression of deiodinase in peripheral tissue suppresses the production of T3 from T4. The derivation of hepatic drug metabolizing enzymes facilitates the bile excretion of T4 and T3. In addition, when a chemical substance binds to the thyroid hormone transport protein and competes antagonistically with a thyroid hormone, the level of the thyroid hormone in the blood is reduced. Thus, each of the points shown in **Fig. 4** can also be a target of contaminating substances in the environment as factors for varying thyroid hormones.

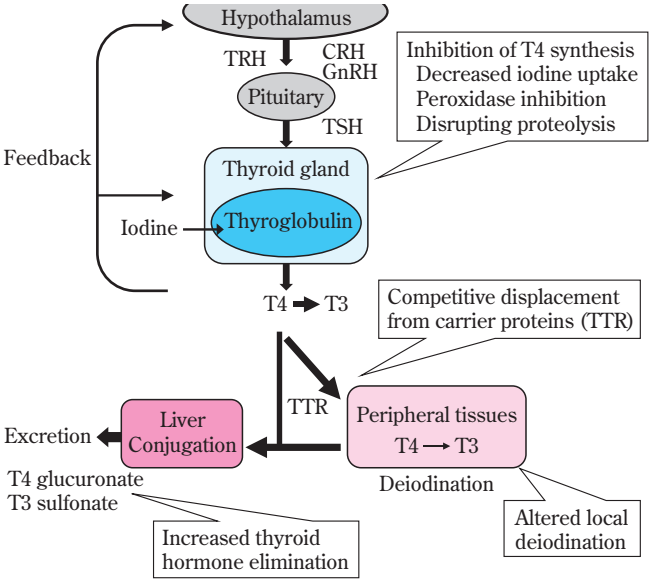


Fig. 4 Overview of the thyroid hormone pathway and regulation

It is known that the ratio of T3 : T4 activity that accompany fluctuations in deiodinase varies the processes of development and metamorphosis.[4), 5)] Thyroid hormones primarily undergo sulfate conjugation and are excreted by the liver, but there are reports of suppression of sulfate conjugation by several chemical compounds.[6)] In addition, there are few reports of glucuronidation with tadpoles, and this is characterized more as an additional metabolic pathway than it is with mammals. However, glucuronidases, which are the principal enzyme group for this pathway, are thought to reduce the thyroid hormones in the circulatory blood through bile excretion as in mammals. Further-

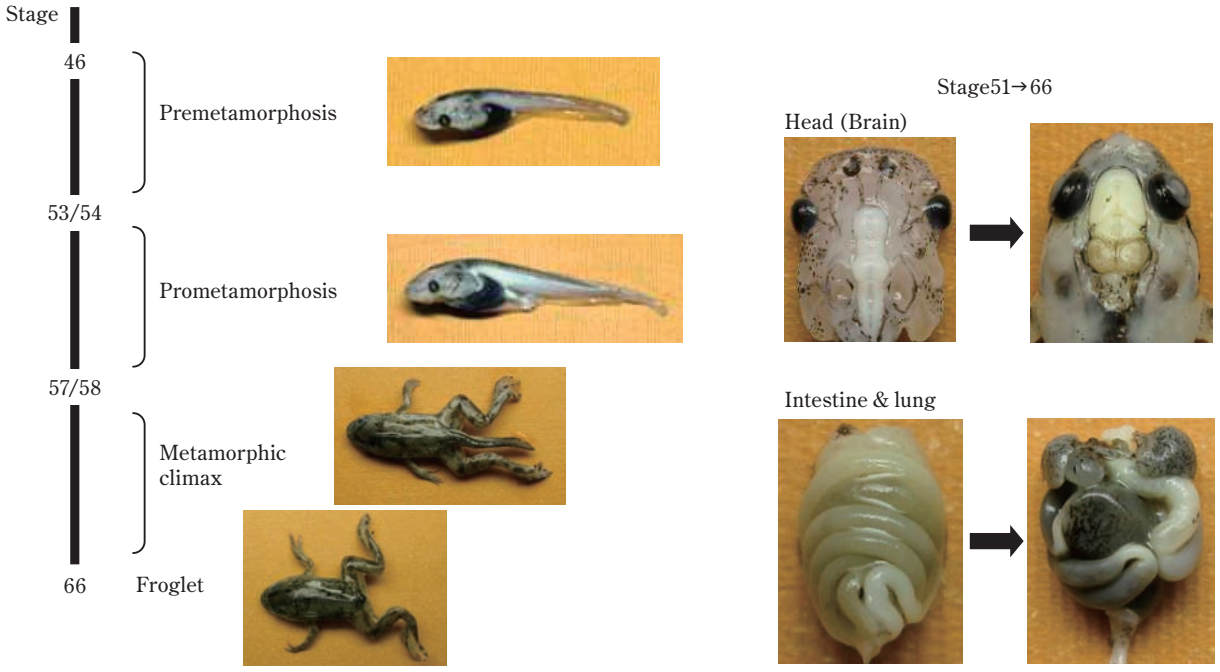
more, the derivation of hepatic microsomal enzyme cytochrome P450 2B1 by pentobarbital in the same manner as in mammals has been reported in some species of frogs.[7)]

A characteristic point in frogs is, unlike mammals, the affinity of TTR is greater for T3 than for T4.[6), 8)] Binding to TTR affects the free hormone concentration in blood serum and affects cell uptake, biological reactivity and maintenance of thyroid hormone levels in the blood. It has been reported that several chemical compounds[9)–12)] have a high affinity for TTR and possibly cause disturbances of the thyroid hormones.

Metamorphosis and Related Hormones

Metamorphosis in frogs can be roughly divided into three stages, and they are called premetamorphosis, prometamorphosis and metamorphic climax (**Fig. 5**). Premetamorphosis is up to the stage that can be reached without thyroid hormones, until the appearance of the hind limbs. After that, the period until the appearance of the anterior limbs is classified as prometamorphosis, and after that, the period until the tail and gills are absorbed and disappear, the lungs developed and the adult formed is classified as the metamorphic climax. In the process of metamorphosis, the feeding habits change from herbivory to carnivory, and along with this, the intestinal tract goes from being long and narrow to short and wide. After prometamorphosis, metamorphosis is controlled systemically by thyroid hormones, and if there is exposure to thyroid hormone receptor antagonists, metamorphosis is inhibited, and the frog is not completely formed.[13)] Conversely, if there is exposure to a substance with thyroid activity, metamorphosis is induced.[14)] Sensitivity to thyroid hormones changes according to the stage of metamorphosis, and the amount of thyroid-stimulating hormone (TSH), which is of a higher order, increases from the latter half of prometamorphosis through the metamorphic climax and then decreases thereafter.[15)]

In frogs, corticotrophin-releasing hormone (CRH) and gonadotropin-releasing hormone (GnRH) are known to promote the release of TSH in the same manner as thyrotropin releasing hormone (TRH).[16)–18)] In addition, mammalian luteinizing hormone-releasing hormone (LHRH), androgen, which is a male hormone, prolactin (PRL) and also corticosteroids and aldosterones, which are adrenocortical hormones, influence the level of thyroid hormones during metamorphosis or influence the process of metamorphosis itself. During the metamorphosis of tadpoles, these hormones have complex effects.[17), 19)–21)]



Developmental stages are according to Nieuwkoop and Faber

Fig. 5 The stage of metamorphosis. The premetamorphosis is the period until the appearance of the hind limbs, and the prometamorphosis is from their appearance to that of the forelimbs. During the period of metamorphic climax, resorption of the tail and gills and development of lungs occur

Effects of Chemical Substances

Investigations of thyroid hormone disrupting action using frogs have been attempted for the past 10 years or so by evaluating the presence or absence of effects by various exogenous substances on metamorphosis.[22)–25)] There have been reports of delays and acceleration of metamorphic stages, effects on hind limb length, morphological changes in thyroid gland tissue and enhancement of the expression of TSH genes in pituitary gland tissue. Among chemical substances that contaminate the environment, there are reports of antithyroid action due to perchlorates,[26), 27)] polychlorinated biphenyls (PCBs),[28)] bisphenol A (BPA),[29)] polybrominated diphenyl ethers (BDE47), pentabromodiphenyl ether mixtures (DE-71)[30)] and methoxychlor.[31), 32)] On the other hand, it has been reported that the herbicide acetochlor acts to accelerate metamorphosis by making the appearance of anterior limbs and the beginning of the metamorphic climax period earlier, in other words, it has an action similar to thyroid hormones.[33)] When substances that contaminate the environment are considered, there are a variety of substances in the wastewater released by municipalities and other entities; therefore, there are indications that effects on metamorphosis in frogs are possible.[34)] In fact, in field tests at the Athabasca oil sands region in the northern part of Alberta in Canada, there have been reports of delays in metamorphosis in indigenous species of frogs.[35)] There have been similar reports of effects on the thyroid hormone system by many substances that contaminate the environment, but these are experimental results with concentrations that are clearly higher than the concentrations to which frogs are exposed in the natural environment. More research and studies are necessary regarding whether these occur in the same manner with concentrations that can be present in the actual environment.

On the other hand, there are also reports of negative data. It has been reported that UV filters such as 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC) do not have thyroid hormone-like action or an antithyroid hormone action in the concentrations that are in environmental exposure,[36)] and it has been reported that atrazine, which is a herbicide, does not affect the process of metamorphosis in frogs.[37)]

The fungicide Triclosan has a structure that is similar to the thyroid hormones; therefore, attention has been given to release into the environment and effects on metamorphosis in amphibians. While Veldhoen et al. have reported the effects on the length of hind limbs,[38)] Fort et al. have counterargued[39)] that there are no effects on metamorphosis at the concentrations in environmental exposure in compliance with GLP guideline tests, and there is still no conclusion in this dispute.[40)–44)]

Amphibian Metamorphosis Assay and Work on Histopathological Investigations in Particular

The various mechanisms and the various compounds described above change the thyroid hormone level in the blood. When there is a decrease in thyroid hormones, HPT axis positive feedback operates, and TSH secretion increases in the pituitary gland. The mechanism mediated via the frog HPT axis is controlled in the same manner as in rodents[45)]; therefore, amphibian metamorphosis assays (AMAs) are evaluation tests based on HPT axis functions in vertebrates, inclusive of humans. The thyroid gland becomes larger because of increases in TSH secretions, and hypertrophy and/or hyperplasia of follicular epithelial cells are induced. O'Connor et al. have reported that thyroid gland histology is the most informative information among the effects of substances with thyroid toxicity in rodents.[46)] Research up to now has also shown that, in the same manner, tests on thyroid tissue are also the most sensitive indices in frogs.[47)]

An overview of AMA testing is shown in **Tables 1** and **2**. The stage classifications of Nieuwkoop and Faber[48)], which are based on morphological changes are used for growth in African clawed frogs. In AMAs, exposure is started at stage 51 where the hind limbs are just in a form like grains of rice. The exposure period is 21 days, and the primary endpoint and timing of observations are daily observations (mortality rate), growth stages at seven and 21 days of exposure, hind limb length, snout-vent length, body weight and thyroid tissue examination at 21 days of exposure. The most distinctive morphological change that can be used as a morphological index of the growth stage is the development of the hind limbs, and it has been reported that there is a positive correlation between the growth stage and (anti-) thyroid hormone-like action.[49), 50)]

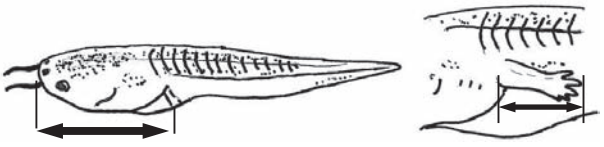
In histological examinations of the thyroid gland, it is necessary to take quantitative criteria into account

Table 1 Protocol of the AMA

Initial larval stage	Nieuwkoop and Faber stage 51
Exposure period	21 days
Larvae selection criteria	Developmental stage and total length (optional)
Test concentrations	Minimum of 3 concentrations spanning approximately one order of magnitude
Exposure regime	Flow-through (preferred) and/ or static-renewal
Test system flow rate	25 mL/min
Larval density	20 larvae/test vessel (5 larvae/L)
Test solution/ test vessel	4–10 L (10–15 cm minimum water)/ glass or stainless steel test vessel (e.g., 22.5 cm × 14 cm × 16.5 cm)
Replication	4 replicate test vessels/ test concentration and control
Acceptable mortality rate in controls	≤ 10% per replicate test vessel
Water temperature	22 ± 1°C
Lighting	12 h Light : 12 h dark, 600 to 2000 lux
Thyroid fixation	Davidson's fixative
pH	6.5 – 8.5
Dissolved oxygen concentration	> 3.5mg/L (> 40% air saturation)

Table 2 Observation time points for primary endpoints in the AMA

Apical Endpoints	Observation time points
Mortality	Daily
Developmental stage	Day 7 and 21, comply with N&F Stage
Hind limb length	Day 7 and 21
Snout-vent length	Day 7 and 21
Wet body weight	Day 7 and 21
Thyroid gland histology	Day 21, comply with guidance for histopathology



Snout-vent length　　Hind limb length

Table 3 Diagnostic criteria, severity and grading for histopathology in AMA

Core criteria (severity graded)
- Thyroid gland hypertrophy/atrophy
- Follicular cell hypertrophy
- Follicular cell hyperplasia

Additional criteria (severity graded and/or qualitatively described)
- Follicular lumen area: reduced or increased
- Colloid quality: homogeneous, heterogeneous, lacy or granular
- Follicular cell height/shape: squamous, cuboidal, low/high columnar

Grading (For multifocal or diffusely-distributed alteration, the percentage of tissue area involved should be considered.)
- Grade 0 (not remarkable to minimal, less than 20%)
- Grade 1 (mild, 30-50%)
- Grade 2 (moderate, 60-80%)
- Grade 3 (severe, over 80%)

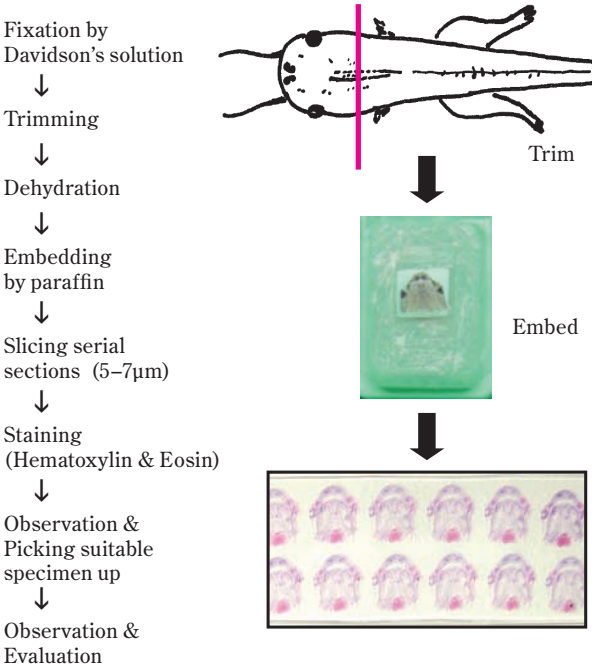


Fig. 6 Histology procedure

in addition to the core criteria given in **Table 3** and to have suitable grading. The thyroid tissue of frogs is extremely small, and cannot be distinguished by the naked eye. In addition, the shape of the head changes with metamorphosis, and the position of the thyroid gland is shifted slightly.

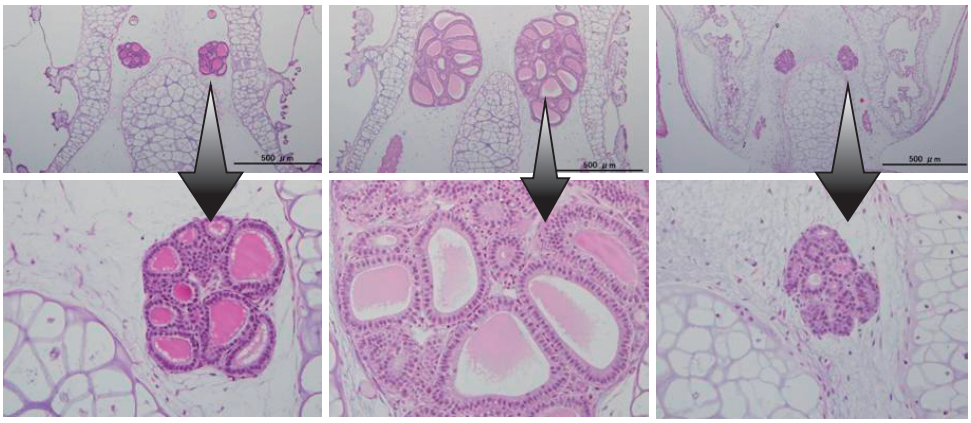
Therefore, before the observation of thyroid tissue, there enters in the time and effort for selecting suitable slice samples for observation from pathology specimens formed from continuous thin slices of the head, which includes the thyroid gland in a number of slices (**Fig. 6**).

In addition, in the process of metamorphosis in tadpoles, the morphology of the thyroid gland differs according to the growth stage. It is extremely small tissue in premetamorphosis, but in prometamorphosis, it gradually becomes larger, and reaches a maximum in the latter half of the metamorphic climax, with the follicular epithelial cells also being at a maximum. Thus, to carry out histological examinations of the thyroid

gland, particular techniques and knowledge are necessary; therefore, guidance on sample preparation techniques and diagnostic methods in AMAs,[51), 52)] and standard documentation on histology and histopathology of the thyroid gland for supporting AMA guidelines have been published along with an atlas of normal tissues during the period that covers the process of metamorphosis.[53)]

At the Sumitomo Chemical Environmental Health Science Laboratory, we have constructed AMA tests in our laboratory using substances with thyroid action and substances with antithyroid action and carried out histopathological examinations for the purpose of establishing AMA test evaluation techniques. **Fig. 7** shows an image of the thyroid tissue in African clawed frogs exposed to the thyroid hormone T4 and propylthiouracil (PTU), which is a typical antithyroid acting substance. PTU not only inhibits thyroid peroxidase, but also causes depletion of T4 and T3 through inhibition of deionation, and TSH secretion from the pituitary gland increases via the feedback mechanism. The thyroid gland receives a continuous stimulus from the level above, and while T4 synthesis and secretion are inhibited, the hormone synthesis is facilitated incompletely. As a result, the thyroid tissue as a whole becomes larger, and hypertrophy and hyperplasia of follicular epithelial cells are exhibited. The amount and concentration of the colloid stored in the area close to the follicular epithelium are reduced, showng pale staining drops, and a heterogenous image is presented. On the other hand, T4 is the thyroid hormone itself, and an excessive amount of the thyroid hormone is present in the body. The negative feedback mechanism operates and reduces TSH. As a result, the activity of the thyroid gland is stopped, and this brings about atrophy of the thyroid tissue and atrophy of the follicular epithelial cells. The necessity for storing the colloid disappears, and the colloid area exhibits a small size. **Fig. 8** shows the results of histopathological examinations that have been scored. Both thyroid hormone-like action and antithyroid hormone-like action exhibit a reaction correlated with the exposure concentration, and it can be seen that it has been grasped well in histopathological terms. The growth stages and growth of the hind limbs are also important end points for the effects of antithyroid acting substances, but secondary effects also influence the delay in growth stages; therefore, standards for judging antithyroid action cannot be obtained from this alone, and histopathological evaluations are a necessary element.

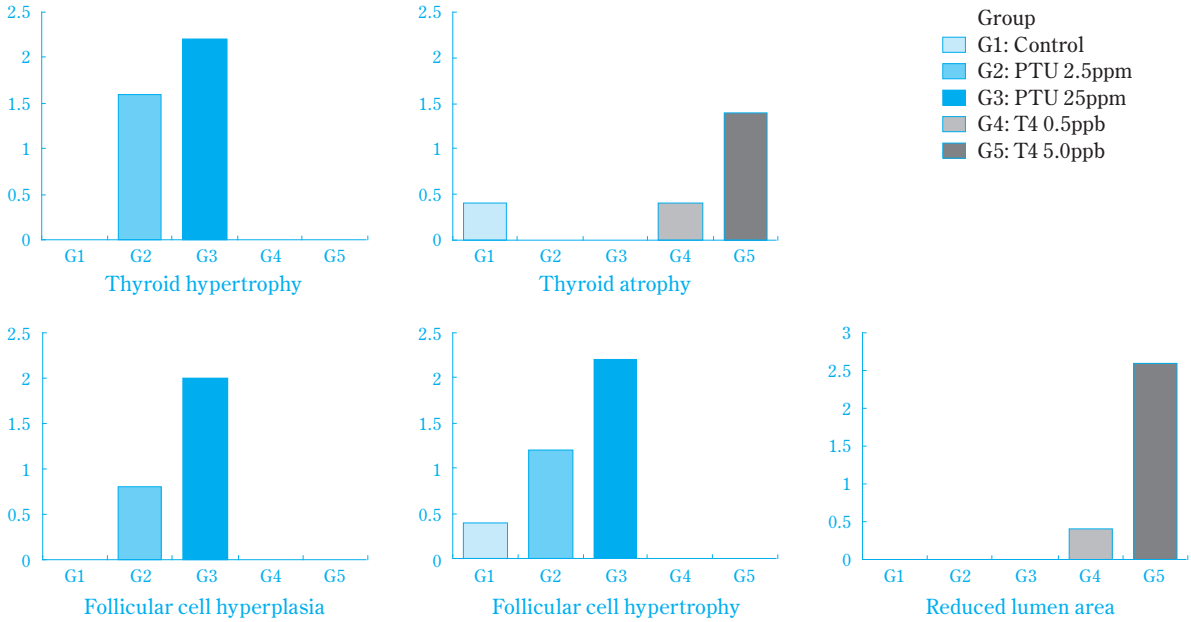
Histopathological examinations of the thyroid gland in the process of metamorphosis in frogs are extremely complicated. During the climax of the normal process of metamorphosis, the synthesis and secretion of TSH by the pituitary gland and also the synthesis and secretion of T4 by the thyroid gland reach a maximum level, and a reduction in the follicular colloid of the thyroid gland and a large increase in the follicular epithelial cell height arise.[53)] Therefore, when such images of thyroid tissue are observed, one is dogged by the difficulty in distinguishing whether there are changes because of the effects on the HPT axis or just a reflection of the growth of the tadpole. To gain knowledge of secondary effects related to stress and reduced weight, we investigated exposure to chemical compounds under constrained food intake. **Fig. 9** (b) shows thyroid tissue when there has been no exposure to a chemical substance but only a restriction of feed to 60%, but the constrained food intake clearly caused atrophy of the thyroid tissue. This change is the same as the change with exposure to thyroid acting substances, and when a reduction in the amount of food consumption is found, we must differentiate whether the histological changes are effects on the thyroid gland by chemical compounds or ones due to secondary effects. Furthermore, the reproducibility of the effects of exposure to chemical compounds has been confirmed, but the differences between individuals and the differences between lots have proven to be large. The image in **Fig. 9** (c) is the thyroid gland of an individual exposed to PTU, but more advanced hypertrophy and hyperplasia have occurred than in the image shown in **Fig. 7**. In the triclosan controversy, interpretation of the presence or absence of mild hypertrophy in the thyroid tissue and changes in the follicular epithelium as well as the effect on the size of the individual body on the thyroid hormones is difficult.[41), 42)] From the standpoint of individual differences, the selection of the individuals to be tested in histopathological examinations of thyroid glands in AMA tests is also extremely important. As is described in the guidelines, comparison with a control group with matched growth stages is vital for accurate evaluation of the effects in exposed groups. Furthermore, we must consider the possibility that chemical exposure affects the growth of tadpoles, and we must also consider individual differences in growth in each exposed group.



(a) Control (b) PTU 25 ppm (c) T4 5.0 ppb

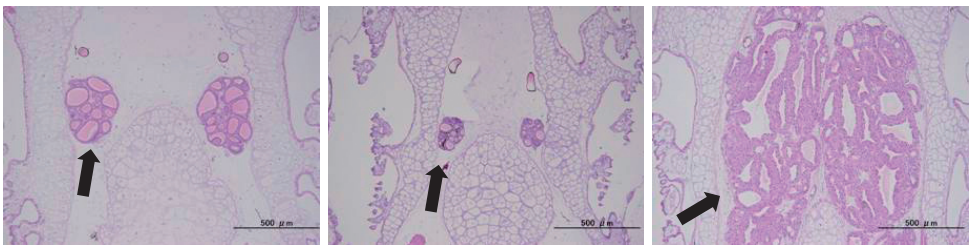
PTU induces thyroid hypertrophy, follicular cell hyperplasia and altered colloid quality. T4 causes follicular atrophy.

Fig. 7 Effect on thyroid histopathology



The scores show the mean of grade. Grade: 0~3

Fig. 8 Result of histopathological examination



(a) Control (b) Restricted feeding 60% (c) PTU 25ppm

Restricted feeding induced thyroid atrophy (b). There is considerable morphological differences among individuals affected (c).

Fig. 9 Effect of food intake and individual difference

Conclusion

As one phenomenon in endocrine disruption, the action of chemical substances on the thyroid hormone system (HPT axis) has been recognized as an important toxicity for human health and organisms in the environment. Amphibian metamorphosis assays (AMAs) are evaluation tests based on HPT axis functions, not only in frogs but also in vertebrates as a whole, inclusive of human beings. At the Sumitomo Chemical Environmental Health Science Laboratory, we have examined the process of metamorphosis and frogs and the control mechanisms for thyroid hormones and have worked toward establishing an evaluation system with high precision, aiming at more exhaustive safety evaluations for chemical substances.

In the process of metamorphosis and frogs, the tissue images of the thyroid gland change incessantly and dramatically; therefore, toxicological pathologists, who observe and evaluate samples, must be familiar with the physiological changes that accompany metamorphosis. Naturally, knowledge of the physiology of the thyroid gland, factors affecting the thyroid gland and reactions with thyroid acting substances is necessary, but familiarity with the range of individual variations that make interpreting evaluations difficult and the changes due to secondary effects such as growth, food and stress is also necessary. We would like to take advantage of the information and techniques acquired through the work above in safety evaluations for chemical substances as we move forward.

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