Review

Thyroid Hormone-disrupting Effects and the Amphibian Metamorphosis Assay

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Abstract: There are continued concerns about endocrine-disrupting chemical effects, and appropriate vertebrate models for assessment of risk are a high priority. Frog tadpoles are very sensitive to environmental substances because of their habitat and the complex processes of metamorphosis regulated by the endocrine system, mainly thyroid hormones. During metamorphosis, marked alteration in hormonal factors occurs, as well as dramatic structural and functional changes in larval tissues. There are a variety of mechanisms determining thyroid hormone balance or disruption directly or indirectly. Direct-acting agents can cause changes in thyroxine synthesis and/or secretion in thyroid through effects on peroxidases, thyroidal iodide uptake, deiodinase, and proteolysis. At the same time, indirect action may result from biochemical processes such as sulfation, deiodination and glucuronidation. Because their potential to disrupt thyroid hormones has been identified as an important consideration for the regulation of chemicals, the OECD and the EPA have each established guidelines that make use of larval African clawed frogs (Xenopus laevis) and frog metamorphosis for screening and testing of potential endocrine disrupters. The guidelines are based on evaluation of alteration in the hypothalamic-pituitary-thyroid axis. One of the primary endpoints is thyroid gland histopathology. Others are mortality, developmental stage, hind limb length, snout-vent length and wet body weight. Regarding histopathological features, the guidelines include core criteria and additional qualitative parameters along with grading. Taking into account the difficulties in evaluating amphibian thyroid glands, which change continuously throughout metamorphosis, histopathological examination has been shown to be a very sensitive approach. (DOI: 10.1293/tox.25.1; J Toxicol Pathol 2012; 25: 1–9)

Key words: frog, amphibian, metamorphosis, test guideline, thyroid, histopathology

Introduction

There are many concerns about endocrine-disrupting chemical effects on wild animals as well as humans. Considerable numbers of chemicals have been shown to alter the thyroid system in vertebrates, and disruption of the thyroid axis has been identified as an important consideration for the regulation of chemicals. Thus, focusing on thyroid toxicity, the OECD and EPA established guidelines for investigation of frog metamorphosis1-2. Because amphibian tadpoles have a thin, permeable skin and inhabit aquatic environments, they are exposed to toxicants through both dermal and dietary routes and appear to be particularly susceptible to a number of contaminants in wastewater discharged from agricultural fields and from industrial and household areas. In addition, the fact that frog metamorphosis is regulated by thyroid hormones (THs), promoting the remodeling of the aquatic larvae into an adult tetrapod, means that the dramatic structural and functional changes of larval tissues can be readily applied as parameters reflecting endocrine disruption.

The frog thyroid glands, located between the eyes (Fig. 1), show various morphological changes during metamorphosis and under conditions of exposure to certain compounds. Here, the regulation by THs, focusing on affecting factors in addition to the metamorphosis assay, is reviewed.

Thyroid Hormones and Their Related Effectors

The thyroid gland secretes thyroxine (T4), which is converted to a more biologically active form, 3,3',5-triiodothyronine (T3), mainly in the peripheral target tissues. Transport of THs in the blood is accomplished through binding to transport proteins, predominantly transthyretin (TTR), with conjugation and degradation processes occurring in the liver and subsequent secretion in bile. Some THs are coupled with bacterial deconjugation and undergo reabsorption in the intestine. TH structures are conserved...
between vertebrates, and their production and secretion are essentially the same in amphibians and mammals.

There are several processes that influence the thyroid hormone balance, such as sulfation, deiodination and glucuronidation (see Fig. 2). Both inhibition of thyroidal iodide uptake and suppression of thyroidal peroxidase activity can disrupt TH synthesis and secretion. In addition, inhibition of 5-deiodinase in the peripheral tissue is related to reduction of conversion from T4 to T3, while induction of hepatic microsomal enzymes such as phenol sulfotransferase and UDP-glucuronyl transferase leads to increase in excretion of both T4 and T3 into bile. Furthermore, competitive binding to thyroid transport proteins results in reduced levels of total and free THs in serum. Therefore, there are many potential targets of environmental contaminants that could be involved in disruption of TH metabolism.

Rahman and Yamauchi found TH sulfating activity to be present in the liver cytosol in frog tadpoles and that the T3:T4 sulfating activity ratio varies during developmental stages, sulfation being inhibited by chemical compounds such as halogenated phenol and phenolic compounds, p-nitrophenol, dopamine, 17beta-estradiol (E2) and dihydroxyandrostosterone. Compared with sulfation, glucuronidation has been poorly studied in amphibians, in line with its character as a relatively minor pathway. However, a subset of the responsible enzymes, glucuronidases (UGTs), can reduce circulating levels of THs through biliary elimination as in mammals. Regarding phase I species, hepatic microsomal cytochrome P450 2B1 was found to be induced by pentobarbital in adults of the semiaquatic frog, Rana pipiens, but not in frog tadpoles and adults of the aquatic frog Xenopus laevis. Deiodinase enzymes, type 2 and 3 iodothyronine deiodinase, are found in several peripheral target tissues in amphibians, and the level of expression is closely connected with metamorphosis.

The most characteristic feature of TTR from nonmammalian vertebrates, such as amphibians, is their higher affinity for T3 than for T4. Binding of chemicals to TTR decreases their effective free concentrations in plasma and changes the apparent affinity for THs, which can diminish cellular uptake and biological responses and would alter plasma TH homeostasis. Therefore, chemicals interfering with T3 binding to TTR may directly affect the free concentration of plasma T3 and its plasma clearance rate. Chemical compounds including diethylstilbestrol (DES),

![Fig. 1. Arrows indicate thyroid glands in Stage 51.](image1)

![Fig. 2. Overview of the thyroid hormone pathway.](image2)
phenolic and phenol compounds\textsuperscript{10,11} and pentachlorophenol and oxyinil\textsuperscript{8,9} have the potential to bind to TTR, and Yamauchi \textit{et al.} reported that with amphibian TTR, DES possesses similar affinity to T3, which is the natural ligand\textsuperscript{8}.

\section*{Metamorphosis and Related Hormones}

Amphibian metamorphosis is divided roughly into 3 stages, premetamorphosis, prometamorphosis and climax (see Fig. 3). The premetamorphic stage is the period until the appearance of the hind limbs, and the prometamorphic period 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.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{stage_of_metamorphosis.png}
\caption{The stage of metamorphosis is divided into 3 stages. The premetamorphic stage is the period until the appearance of the hind limbs, and the prometamorphic period 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.}
\end{figure}

Metamorphic retardation or even complete blockade of thyroid hormone receptor antagonists (e.g., NH-3) leads to extremely high levels evident at the metamorphic climax\textsuperscript{19}. Globulin are found in tadpoles at the early stages, but expecially but not larval cells\textsuperscript{24}. Kaneko \textit{et al.} demonstrated that TH suppressed the CRH-induced release of TSH, but not the basal release, from larval, juvenile and adult bullfrog pituitaries in \textit{vitro}\textsuperscript{18}. In addition, Jacobs \textit{et al.} reported that intravenously injected mammalian luteinizing hormone-releasing hormone (LHRH) was able to raise circulating levels of T4 as well as testosterone (T) in three frog species, \textit{Rana ridibunda}, \textit{Rana temporaria} and \textit{Rana esculenta}\textsuperscript{21}. Regarding prolactin (PRL), inhibitory effects may be exerted on metamorphosis, but this is controversial. Huang and Brown concluded that PRL does not play a role as a juvenile hormone in \textit{X. laevis}, but overexpression of PRL does specifically inhibit some but not all programs of tail resorption\textsuperscript{22}.

Research has indicated concomitant elevation of THs and corticoids as metamorphosis progresses\textsuperscript{26–28}. According to Kikuyama \textit{et al.}, the aldosterone plasma level is low prior to the onset of climax, but then there is a sharp rise, and combined adrenocorticotropic (ACTH) and T treatment causes a marked increase in concentration\textsuperscript{28}. Gray and Janssens observed that corticosterone stimulated T3-induced metamorphosis in \textit{X. laevis} tadpoles\textsuperscript{29}.

Steroid sex hormones also serve to modulate thyroid system functions. Gray and Janssens observed that testosterone and E2 inhibited T3-induced metamorphosis in \textit{X. laevis} tadpoles\textsuperscript{29}, while Hogan \textit{et al.} demonstrated a delay in the time for \textit{Rana pipiens} tadpoles to undergo metamorphosis when exposed to ethinylestradiol during either mid-metamorphosis or throughout the entire larval period\textsuperscript{30}.

\section*{Effects of Chemical Compounds on Amphibian Metamorphosis}

In the past few decades, several thyroid disrupting substances have been tested for toxicity using amphibians. Xenopus and other anurans have been generally applied to assess the developmental effects of a variety of xenobiotics. There have been a considerable number of reports regarding inhibitors of T4 synthesis that inactivate peroxidases. After exposure to propylthiouracil (PTU), also known to inhibit deiodinase, \textit{X. tropicalis} tadpoles showed no signs of decline in body length or body weight but a considerable reduction in the developmental stage and hind limb length\textsuperscript{31}. Opitz \textit{et al.} reported effects of exposure to PTU and another peroxidase inhibitor ethylthioureia (ETU), in \textit{X. laevis}\textsuperscript{32,33}. Metamorphic retardation caused by ETU was associated with concentration-dependent histological changes in the thyroid gland and increased mRNA expression of TSH-beta in the pituitary\textsuperscript{33}. Deitz \textit{et al.} exposed pre- and pro-metamorphic larvae to methimazole, PTU and T4\textsuperscript{34} and induced changes in a concentration-dependent manner. Methimazole and PTU caused a delay in larval development and morphological changes in the thyroid gland, which were characterized as reduced colloid, glandular hypertrophy and cellular hyperplasia and hypertrophy. On the other hand, T4 treatment resulted in a concentration-dependent increase in
the developmental rate. A single other potent inhibitor of T4 synthesis, perchlorate, disturbs iodide uptake by the follicular cells of the thyroid gland. Ammonium perchlorate was found to inhibit forelimb emergence, hindlimb development and tail resorption, linked to significant hypertrophy of the thyroid follicular epithelium at concentrations below those reported in contaminated surface waters. Tietge et al. showed that sodium perchlorate inhibits TH synthesis via effects on the sodium-iodide symporter, resulting in retarded metamorphosis and histological effects on thyroid as the most sensitive endpoint. Hu et al. reported the results of thyroid immunohistochemistry for T4 in X. laevis exposed to perchlorate; T4 immunoreactivity was concentrated in a ring of colloid adjacent to follicle cells, independent of the developmental stage. Theirs is the only report regarding this point of view, but they suggested the utility of this immunohistochemical biomarker, because the intensity of the colloidal T4 ring is more sensitive than any other morphological changes (such as hind limb length, forelimb emergence, tail resorption, thyrococyte hypertrophy or colloid depletion).

There are limited reports concerning environmental contaminant chemicals. Gutleb et al. found that time until metamorphic transformation was prolonged, the body weights of frogs were increased after exposure to polychlorinated biphenyls (PCB) and T4 levels were lowered, although not significantly. Iwamuro et al. established that bisphenol A (BPA) induced deceleration of both spontaneous and T4-induced metamorphic changes, with suppression of thyroid hormone receptor (TR) beta gene expression. Polybrominated diphenyl ether mixture (DE-71) caused delay in tail regression in X. laevis. According to Carlsson et al., autoradiograms revealed high concentrations and long-term retention of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) in adipose tissue and melanin in frogs exposed as both tadpoles and juveniles, with lower developmental stages suggesting possible thyroid hormone disruption. Fort et al. recorded delayed metamorphosis and enlarged thyroid glands with follicular hyperplasia in X. tropicalis and X. Laevis tadpoles exposed to the insecticide methoxychlor. Using a GLP study in compliance with the guidelines mentioned below, and indicated that environmentally relevant concentrations do not alter the normal course of thyroid-mediated metamorphosis in this standard anuran model. The effect of triclosan has been controversial.

Guideline Establishment

The amphibian hypothalamic-pituitary-thyroid (HPT) axis controls TH levels in the same way as it does in mammals, although CRH also plays a role. Therefore, the amphibian metamorphosis assay (AMA) represents a generalized vertebrate model to the extent that it is based on the functions of the HPT axis.

On September 7, 2009, the Organization for Economic Co-operation and Development (OECD) adopted the AMA using X. laevis as a guideline for testing of chemicals. The US Environmental Protection Agency (EPA) Office of Prevention, Pesticides, and Toxic Substances (OPPTS) also includes the AMA for detection of thyroid-active chemicals in Tier 1 testing of their endocrine screening program. The EPA developed the OECD test guidelines through a process of harmonization in October 2009. Previously, in 1998, the OECD initiated a high-priority activity to revise existing guidelines and develop new guidelines for the screening and testing of potential endocrine disrupters. One element was a test guideline for the screening of substances active on the thyroid system of vertebrate species. The 1st OECT Expert Consultation on Endocrine Disrupter Testing in Amphibians was held in 2001. Subsequently, the AMA underwent extensive validation programs consisting of 3 phases, which included intra- and interlaboratory studies demonstrating the relevance and reliability of the assay. Mainly the U.S., Germany and Japan took part in these trials.

For validation phase 1, T4 and PTU were chosen as test chemicals. The primary objective was a comparative
evaluation of the utility and sensitivity of two proposed exposure scenarios for detection of stimulating and inhibiting effects of thyroid system-disrupting substances.

Phase 2 was interlaboratory multichemical testing with a harmonized protocol. The United Kingdom and Switzerland also participated in this phase in addition to Germany, Japan and the United States. Three model substances representing different modes of action on the thyroid system were used: sodium perchlorate, T4 and iopanoic acid (IOP). IOP is an inhibitor of iodothyronine deiodinases.

Phase 3 studies were conducted to assess the utility of the AMA protocol to detect weakly active thyroid system-disrupting substances and to distinguish thyroid system-related changes from activity resulting from mechanisms not directly related to thyroid system function. Benzophenone-2 (BP-2) and E2 were selected as weakly active and non- or indirectly related substances. As a result, the effect of a weakly antithyroid substance, BP-2, was detected, while the endpoints with exposure to E2 did not indicate any effect on the thyroid system. Subsequently, validation of the assay was subjected to peer review by a panel of independent experts.

The procedure in the guidelines is shown simply in Table 1 and Table 2. Exposure should be initiated at developmental stage 51, according to Nieuwkoop and Faber. Hind limb development is used qualitatively for determination of the developmental stage. The exposure duration is 21 days. The primary endpoints and observation time points are mortality daily, developmental stage, hind limb length, snout-vent length and wet body weight on days 7 and 21 and thyroid gland histology on day 21. The most prominent morphological staging landmark is hind limb morphology, which is positively associated with agonistic effects.

Regarding the histopathology of the thyroid gland, the guidelines mention core criteria and additional qualitative criteria along with grading, as shown in Table 3.

For a more detailed understanding, information is available in "Amphibian Metamorphosis Assay: Part 1 – Technical guidance for morphologic sampling and histolog-

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### Table 1. Protocol of the AMA

| Component | Specification |
|-----------|---------------|
| **Test animal** | *Xenopus laevis* larvae |
| **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** | > 3.5 mg/L (> 40% air saturation) |

### Table 2. Observation Time Points for Primary Endpoints in the AMA

| Observation time points | **Apical endpoints** |
|-------------------------|----------------------|
| **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 |

### Table 3. Diagnostic Criteria, Severity and Grading for Histopathology in AMA

| Criteria | Description |
|----------|-------------|
| **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%) |
ical preparation” and “Amphibian Metamorphosis Assay: Part 2 – Approach to reading studies, diagnostic criteria, severity grading and atlas”⁶¹,⁶². In addition, in support of the OECD AMA test guideline, a document was developed that provides a standardized approach for evaluating the histology/histopathology of thyroid glands⁶³, including an atlas of the normal architecture of amphibian thyroid glands over the course of metamorphosis.

One article regarding an examination conducted in compliance with these guidelines supported the use of the AMA as a Tier 1 endocrine screen for detection of potential thyroid pathway activity, but the lack of a true negative response (no effect) during the validation process prevents full evaluation of this assay’s specificity at this time⁶⁴.

The Importance and Difficulty of Histopathological Examination

Each of the mechanisms mentioned above can alter concentrations of circulating THs. Under conditions of TH decrease, a result of feedback of circulating THs on the HPT axis, there is increased secretion of TSH from the pituitary gland. Such excessive stimulation would be expected to result in an increased thyroid gland size, increased degree of follicular cell hyperplasia and/or increased degree of follicular cell hypertrophy, as is well documented for the rodent thyroid gland⁶⁵. O’Connor et al. stated that histopathological change of the thyroid gland is the most valuable information regarding thyroid toxicants⁶⁶, and histological examination of the amphibian thyroid gland has been shown to be a very sensitive approach in several studies. Figure 4 illustrates thyroid glands exposed to T4 or PTU along with controls.

Although the developmental stage and hind limb length are important endpoints of antithyroid activity, developmental delay cannot, by itself, be considered a diagnostic indicator of antithyroidal activity. Therefore, conducting histopathological analyses of the thyroid glands is an essential requirement.

A series of validation assays verified that thyroid histopathology is sensitive and reliable for antithyroidal activity resulting from either inhibition of thyroidal iodide uptake or iodide organification. On the other hand, according to the phase 1 validation study, histopathological analyses of thyroid glands of T4-treated tadpoles were less consistent between the laboratories and more difficult to interpret compared with the effects seen in the PTU studies⁵⁷. Furthermore, the phase 2 validation report mentioned that while thyroid histopathology was sensitive for antithyroidal activities, weak agonistic activity could not be reliably detected⁵⁸.

Thyroid histopathology is complex. Depletion of colloid stores and increases in epithelial cell height are known to occur at climax stages during normal development when TSH synthesis and release by the pituitary and T4 synthesis and secretion by the thyroid gland reach maximum levels⁶³. Therefore, there may be difficulty in distinguishing whether histological changes occur in response to alterations of the HPT axis or alternatively merely reflect advanced stages in tadpoles. In fact, the guidelines insist that the most appropriate sampling approach for histological analyses is to use stage-matched individuals whenever possible. In order to select stage-matched individuals, all larvae should first be staged prior to selection and subsequent processing for data collection and preservation. This is necessary because normal divergence in development will result in differential stage distributions within each replicate tank.

There is one example showing that amphibian thyroid histopathology is extremely complex⁵³, and when misinterpretation occurs, it leads to the opposite conclusion. In a controversial issue regarding the effect of triclosan, Fort
and Pawlowski reported in regard to the histopathological changes that an increase in the occurrence of minimal thyroid gland hypertrophy was not accompanied by follicular hypertrophy or hyperplasia. The lack of follicular hypertrophy or hyperplasia was suggested to be the result of both the minimal nature of the response and the increased body size of the treated specimens.

**Conclusion**

Because disruption of the thyroid axis has been identified as an important consideration for regulation of chemicals, the OECD and EPA have established guidelines that make for use of larval African clawed frogs (X. laevis) and frog metamorphosis for screening and testing of potential endocrine disrupters. In the test guidelines, thyroid gland histology is one of the primary endpoints, along with mortality, developmental stage, hind limb length, snout-vent length and wet body weight. A series of validation studies and many reports have revealed that the histopathology of the thyroid gland is very sensitive and reliable for detection of antithyroidal activity but, on the other hand, is less useful for weak agonistic activity. Since the morphology of the amphibian thyroid gland changes continuously throughout metamorphosis with the fluctuation in TH levels, the guidelines stress the need for well experienced toxicologic pathologists who are familiar with normal X. laevis thyroid histology, thyroid gland physiology and general responses of the thyroid gland to agonists or antagonists.

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