

DESCRIPTION AND INITIAL EVALUATION OF A *XENOPUS* METAMORPHOSIS ASSAY FOR DETECTION OF THYROID SYSTEM–DISRUPTING ACTIVITIES OF ENVIRONMENTAL COMPOUNDS

ROBERT OPITZ,[†] THOMAS BRAUNBECK,[‡] CHRISTIAN BÖGI,[§] DANIEL B. PICKFORD,^{||} GERRIT NENTWIG,[#]

JÖRG OEHLMANN,[#] OSAMU TOOI,^{††} ILKA LUTZ[†] and WERNER KLOAS^{*†‡‡}

[†]Department of Inland Fisheries, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin D-12587, Germany

[‡]Department of Zoology, University of Heidelberg, Heidelberg D-69120, Germany

[§]BASF Aktiengesellschaft, Product Safety—Regulations, Toxicology, and Ecology, D-67056 Ludwigshafen, Germany

^{||}AstraZeneca Global Safety, Health, and Environment, Brixham Environmental Laboratory,

Freshwater Quarry, Brixham, Devon TQ5 8BA, United Kingdom

[#]Department of Ecology and Evolution, University of Frankfurt, Frankfurt am Main D-60054, Germany

^{††}Towa Kagaku, Hiroshima 730-0841, Japan

^{‡‡}Department of Endocrinology, Institute of Biology, Humboldt University Berlin, D-10115 Berlin, Germany

(Received 26 April 2004; Accepted 1 September 2004)

Abstract—A need is recognized for the development and evaluation of bioassays for detection of thyroid system–disrupting compounds. The issue of testing for thyroid disruption can be addressed by exploiting amphibian metamorphosis as a biological model. In the present study, a test protocol for a *Xenopus* metamorphosis assay (XEMA) was developed and its interlaboratory transferability was evaluated in an informal ring test with six laboratories participating. In the XEMA test, exposure of *Xenopus laevis* tadpoles was initiated at stages 48 to 50 and continued for 28 d. Development and growth of tadpoles were assessed by means of developmental stage and whole body length determinations, respectively. For initial test protocol evaluation, thyroxine (T4), and propylthiouracil (PTU) were used as positive controls for thyroid system–modulating activity, and ethylenethiourea (ETU) was used as a test compound. Exposure of tadpoles to 1 µg/L T4 produced a significant acceleration of metamorphosis whereas PTU concentrations of 75 and 100 mg/L completely inhibited metamorphosis. Five different ETU concentrations (5, 10, 25, 50, and 100 mg/L) were tested and a concentration-dependent inhibition of metamorphosis was observed. None of the compounds affected tadpole survival, and only PTU caused a slight retardation in tadpole growth. This study demonstrates that the XEMA test provides a sensitive, robust, and practical testing approach for detection of compounds with both agonistic and antagonistic effects on the thyroid system in *Xenopus* tadpoles.

Keywords—*Xenopus* Metamorphosis Thyroid system Endocrine disruption Ring-test study

INTRODUCTION

In the past several years, the issue of endocrine-disrupting activities of environmental compounds has received a great deal of attention in toxicological, ecotoxicological, and epidemiological studies [1,2]. With respect to the increasing number of compounds that are suspected to act as potential endocrine disruptors (ED), a tiered testing strategy recently has been proposed for the evaluation of the many existing and newly produced chemicals for endocrine-disrupting activity [3–5]. In this regard, effects of ED on reproductive biology by far have been attracting the most attention [6,7]. However, recent reviews on possible thyroid system–disrupting effects of environmental chemicals strongly support the hypothesis that the thyroid system represents another important target of ED action [8–10]. The current bias of research activities on ED with (anti-)estrogenic and (anti-)androgenic properties appears to be, at least in part, a result of the fact that more and better validated bioassays are available to evaluate responses associated with chemical disruption of sex steroid function [7,11,12] compared to disruption of thyroid system function [6,13,14]. By focusing attention on thyroid toxicants disrupting thyroid hormone (TH) homeostasis, current mammalian testing

strategies mainly rely on measurements of plasma concentrations of thyroxine (T4), triiodothyronine (T3), and thyroid-stimulating hormone as well as on histopathological analysis of the thyroid gland [15,16]. These testing strategies pay only little attention to the possible effects of ED on TH signaling in peripheral tissues [10]. However, there is growing evidence for agonistic and antagonistic action of ED on cellular TH signaling [17–20]. Accordingly, disruption of TH action in target tissues should deserve adequate consideration as a possible mode of action when developing a comprehensive testing strategy for thyroid system disruption.

Postembryonic development in anuran amphibians (metamorphosis) is one of the best-studied biological models for TH function and, therefore, anuran metamorphosis represents a valuable biological model to investigate the effects of ED on various aspects of thyroid system functioning [21]. Furthermore, the development of a functional amphibian metamorphosis assay for detection of ED affecting thyroid system function has been recognized as an important research need toward the establishment of a comprehensive screening and testing strategy for endocrine disruption [6,22]. The regulatory role of THs during anuran metamorphosis has been studied at all levels of biological organization [23]. Anuran metamorphosis is dependent obligatorily on THs, best exemplified by the fact that surgical removal of the thyroid gland or chemical

* To whom correspondence may be addressed
 (werner.kloas@igb-berlin.de).

blockage of TH synthesis leads to complete blockade of metamorphic development [23]. The thyroid system of anuran tadpoles responds to treatment with classical antithyroidal agents (e.g., perchlorate, propylthiouracil, methimazole) in a fashion similar to that seen in mammals. Exposure of tadpoles to such goitrogens not only has been shown to retard metamorphic development but also to cause reductions in plasma TH levels accompanied by hypertrophic and hyperplastic changes in the thyroid and pituitary glands [24–26]. On the other hand, addition of minute amounts of THs to the rearing water of tadpoles during premetamorphosis leads to a precocious induction of metamorphosis [27]. The highly reproducible and stereotypic response pattern shown by premetamorphic tadpoles following TH treatment offers considerable potential for identification of thyromimetic activities of ED under in vivo conditions. Although the circulating levels of TH provide a major factor in the regulation of amphibian metamorphosis, the highly coordinated remodeling of diverse tadpole tissues also is critically dependent upon various cellular factors controlling T3 action in target cells [23,27]. It is particularly the latter aspect that makes the process of amphibian metamorphosis an attractive and unique biological model for testing of thyroid system–disrupting compounds. Accordingly, an amphibian metamorphosis assay may provide a simple means for detection of both antithyroidal as well as thyromimetic activities of environmental compounds. Previous attempts to establish an amphibian-based assay for thyroid system disruption used *X. laevis* tadpoles at late developmental stages [28], but results were unsatisfactory, particularly with regard to the sensitivity of the assay to detect stimulating and inhibiting effects of known thyroid system agonists and antagonists [6,29].

In this study, an alternative testing protocol for a *Xenopus* metamorphosis assay (XEMA) was developed specifically to address ED effects on the thyroid system. During test development, special emphasis was placed on the ability of the test protocol to detect sensitively both antithyroidal and thyromimetic activities of test compounds. Furthermore, attention was given to the practicability of the test system in order to facilitate its widespread use. Therefore, the core elements of the testing approach taken were initiation of exposure at premetamorphic stages and determination of developmental stages as an easily measurable endpoint to monitor tadpole development throughout the exposure period. The utility of this testing approach was evaluated in experimental studies using known thyroid system agonists and antagonists such as T4 and propylthiouracil (PTU) [30] and an informal ring-test study was performed to characterize the general interlaboratory transferability of the testing protocol. Ethylenethiourea (ETU) was selected as a test compound for the initial evaluation of the XEMA test because of its well-documented ability to disrupt thyroid system function in mammalian species. Studies have shown that ETU inhibits TH synthesis in mammals by inhibition of thyroid peroxidase activity [31] and treatment of rats with ETU caused large reductions in circulating TH levels accompanied by increased plasma levels of thyroid-stimulating hormone, increases in thyroid gland weight and thyroid gland hyperplasia [32–34]. To the best of our knowledge, data on thyroid system–disrupting effects of ETU in anuran tadpoles were not available. However, information available suggested moderate to low toxicity of ETU to *X. laevis* tadpoles [35]. The results presented in this paper provide evidence that known mammalian thyroid system–disrupting compounds are capable of affecting metamorphosis in *X. laevis* tadpoles and

show that a morphological metamorphosis assay could provide a simple means to detect antithyroidal as well as TH-mimicking activities of ED.

METHODS

Description

Test species. The South African clawed frog *X. laevis* was selected as a test species because the biological processes and their regulation by TH during postembryonic development are particularly well explored. Extensive investigations on metamorphic development have made this species one of the leading resources for understanding TH function during vertebrate development [23], and revealed a great amount of baseline information on various morphological, histological, biochemical, and molecular biological aspects of metamorphic transformations [23,36,37]. Embryonic and larval development in *X. laevis* has been divided into 66 stages [36], and the relationship between the functional state of the thyroid system and the progress of metamorphic development is well-documented in this species. The premetamorphic period (stage 37 to stages 53/54) is characterized by rapid growth of tadpoles with only minor morphological changes occurring during this period [36]. Thyroid follicular formation starts around stages 46/47 in *X. laevis* tadpoles [38], and the thyroid gland develops into a functional gland until stage 53 [36]. Accordingly, TH levels are very low during premetamorphosis [39] and premetamorphic development (e.g., forming of hind limb buds) appears to be independent of TH. Hind limb growth and differentiation are the earliest TH-induced modifications marking the onset of the prometamorphic period at stages 54/55. Levels of circulating TH steadily increase in tadpoles during prometamorphosis [39] stimulating further morphogenesis and differentiation of the limbs. Fore limbs emerge at stage 58, which marks the beginning of the climax phase of metamorphosis, a period during which rapid and dramatic changes occur in morphology (e.g., gill and tail resorption) under the influence of peaking TH levels [39]. At the end of metamorphosis (stage 66), TH levels decline to lower levels.

The ease of culture and reproduction makes this anuran species the preferable test species for routine testing [40,41]. Large numbers of embryos can be obtained easily on demand in the laboratory, because breeding can be induced by injection with human chorionic gonadotropin throughout the year. Optimal rearing conditions for *X. laevis* larvae are easy to achieve in ecotoxicological laboratories [40,41] and tadpoles can be reared in great numbers to specific developmental stages. Compared to many other anuran species, metamorphic development in *X. laevis* proceeds relatively rapid, thus allowing for shorter test periods. From a toxicological perspective, it is important to note that *X. laevis* tadpoles have been used in numerous studies investigating the effects of environmental chemicals on metamorphosis providing a considerable database on lethal and sublethal effect concentrations for many substances [42]. In the majority of published studies, static or semistatic exposure systems have been employed for chemical treatment of *X. laevis* tadpoles. However, for compounds displaying rapid degradation, flow-through techniques may be required in order to meet more closely the demands of aquatic toxicology testing. Although recent studies indicate that the use of flow-through systems is not detrimental to *X. laevis* tadpole development [43], a comparative analysis of tadpole development under flow-through and static-renewal exposure condi-

tions is required to obtain more information on the advantages and disadvantages of each of these exposure techniques.

Animals and husbandry. During this evaluation study, all tests were performed with animals bred in the laboratory. Spawning of adult *X. laevis* was induced by injection of human chorionic gonadotropin (Sigma, Deisenhofen, Germany) into the dorsal lymph sac as described by Kloas et al. [44]. Breeding of adult *Xenopus*, induction of spawning, and initial rearing conditions for spawned eggs and newly hatched larvae are described in greater detail elsewhere [44]. Briefly, eggs and larvae were maintained at $22 \pm 1^\circ\text{C}$ and $\text{pH } 7.0 \pm 0.5$ during all phases of development. The light:dark cycle was 12:12-h. All tanks used for rearing of tadpoles were aerated continuously by airstones. The rearing medium was formulated by adding 2.5 g of the commercial salt mixture Tropic Marine Meersalz (Tagis, Dreieich, Germany) to 10 L of deionized water. This medium was used for rearing of eggs and tadpoles during the pre-exposure period and provided the dilution water for formulation of test solutions during the actual exposure phase. Spawned eggs and developing larvae initially were reared in large holding tanks (e.g., 50-L tanks) filled with 40 L of rearing medium. Upon reaching stages 47/48, tadpoles were transferred from the holding tanks to smaller test tanks (glass aquaria, 11 L) containing 10 L of rearing medium. Larval density was reduced to 40 to 45 tadpoles per 10 L at this time. Tadpoles were reared under these conditions for 2 to 3 d to allow for a first acclimation to subsequent test conditions (e.g., water level, tank water volume, light intensity). During all phases of development, tadpoles were fed daily a commercial tadpole food, Sera Micron® (Sera, Heinsberg, Germany). Feeding of tadpoles started at day 5 postfertilization. The amount of food was adjusted as needed so that the water became clear within 24 h. During the pre-exposure phase, the total daily food ration was increased to accommodate tadpole growth.

General protocol. The XEMA test involved aqueous exposure of premetamorphic tadpoles to five different concentrations of the test compound and a solvent control (SC) for 28 d in a static replacement exposure system. The exposure design consisted of at least two replicate tanks for each treatment group. The test was initiated with tadpoles at developmental stages 48 to 50 according to the staging criteria described by Nieuwkoop and Faber [36]. At stages 48 to 50, tadpoles should have reached a whole body length (WBL) of 16 to 25 mm and only tadpoles at developmental stages 48 to 50 with a WBL > 16 mm were considered for the exposure phase. Tadpoles that meet these criteria were assigned randomly to the test tanks. Test tanks were filled with 10 L of test solution and 30 tadpoles were placed in each test tank resulting in a larval density of three tadpoles per liter. Test solutions were replaced completely three times a week (Monday, Wednesday, Friday), at which time tadpoles were removed from the test tanks by gentle scooping using a small strainer in order to avoid any injury of the test organisms. Toxic effects arising from the use of latex gloves [45] have been reported, and so we recommend using vinyl or nitril gloves during exchange of test solutions. Before refilling the test tanks with fresh test solution, the inner tank sides, the bottom, and the glass covers were cleaned with warm tap water, followed by thorough rinsing with deionized water.

During the test, tadpoles were fed daily a specified amount of Sera Micron. The food was added to the test tanks as dry food according to the following schedule: 200 mg/day from

exposure days 0 to 5 and 300 mg/day from exposure days 6 to 28. In conjunction with the renewal schedule of the test solutions, this feeding regime was sufficient to maintain adequate water quality and has been found to ensure proper growth and development of *X. laevis* tadpoles [44].

Biological endpoints. The primary endpoint for the XEMA test is the developmental stage [36] to which the tadpoles develop during 28 d of exposure. Antithyroidal compounds (e.g., PTU) are expected to retard metamorphic development, causing lower developmental stages compared to untreated controls. On the other hand, compounds that mimic or enhance TH action (e.g., T4) would cause accelerated metamorphic development leading to more advanced developmental stages compared to untreated controls. Measurements of WBL were performed in order to determine possible effects of test substances on the growth rate of tadpoles. Although measurements of tadpole weight might provide a more precise estimation of tadpole growth, weight determinations during the course of the experiments technically are more demanding and would cause greater handling stress to the test organisms.

For determinations of developmental stage and WBL, tadpoles were removed from the test tanks by scooping with a small strainer and transferred to a transparent plastic chamber approximately $80 \times 30 \times 15$ mm containing a few ml of test solution. It was not necessary to anaesthetize the tadpoles for this procedure. Experimental animals were handled carefully during this transfer in order to minimize stress and avoid any injury to the tadpoles. The developmental stage of all test animals was determined according to the staging criteria of Nieuwkoop and Faber [36] by using a binocular dissection microscope. For length measurements, the transparent measurement chamber was placed on a length scale that allows length measurements to the nearest mm. Alternatively, length measurements can be performed using an image analyzing system. Developmental stage and WBL were determined for each tadpole at the beginning of the exposure (day 0) and again on days 7, 14, 21, and 28 of exposure.

In addition, all test tanks were checked daily for dead tadpoles, and the numbers were recorded. Dead tadpoles were removed from the test tanks as soon as observed. Further measurements of nonspecific toxicity could be incorporated into the XEMA test protocol, including the monitoring of tadpoles for abnormal behavior (e.g., uncoordinated swimming, hyperventilation, atypical quiescence) or malformations (e.g., limb deformities, kinked tails). Such effects, although difficult to quantify, can aid in the interpretation of test results by providing some information on additional toxic side effects of the test substances. However, at this stage of the XEMA evaluation process, no distinct criteria for a qualitative and quantitative assessment of possible morphological or behavioral abnormalities are provided.

Evaluation

Organization of the ring test. The utility of the XEMA testing protocol to detect thyroid system-disrupting effects was evaluated in a series of experiments performed in six laboratories during an informal international ring-test study [44]. The ring-test study was organized and coordinated by the Department of Inland Fisheries at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries in Berlin, Germany. For standardization of test performance between laboratories, a standard operation procedure [44] was developed by the organizing laboratory in Berlin and provided to all participants.

Table 1. List of participating institutions in the ring-test study. Throughout this report, each laboratory is referred to only by a randomly allocated code number, not representing the order of listing shown below

Country	Research institution	Replication of tests
Germany	Department of Inland Fisheries, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin D-12587	2
	BASF Aktiengesellschaft, Product Safety—Regulations, Toxicology, and Ecology, D-67056 Ludwigshafen	2
	Department of Zoology, University of Heidelberg, Heidelberg D-69120	2
	Department of Ecology and Evolution, University of Frankfurt, Frankfurt am Main D-60054	2
Japan	Towa Kagaku, Hiroshima 730-0841	1
United Kingdom	AstraZeneca Global Safety, Health, and Environment, Brixham Environmental Laboratory, Freshwater Quarry, Brixham, Devon TQ5 8BA	1

Initially, all participating institutions (Table 1) were asked to perform the XEMA test only with animals bred in their own laboratories. However, one laboratory (laboratory 3) did not maintain a breeding stock of adult *X. laevis* and, in order to keep within the time schedule for the ring test, it was decided to transport newly hatched larvae a short distance from laboratory 2 to laboratory 3 on demand.

The salt mixture Tropic Marine Meersalz and the tadpole food Sera Micron were purchased in bulk quantities from the same lot and were then shipped to each laboratory. The test chemicals T4, PTU, and ETU were purchased from Sigma at the highest purity available. Unfortunately, it was not possible to obtain bulk quantities of the test chemicals (ETU, PTU, and T4) from the same lot. Hence, different lots of the test chemicals had to be used during this study. Stock solutions of T4 were prepared in 0.1 M NaOH (Roth, Karlsruhe, Germany). Stock solutions of PTU and ETU were made in 0.7 M NaOH and 1% dimethylsulfoxide ([DMSO], Sigma), respectively.

Experimental design. In all experiments conducted during the ring-test study, tadpoles were exposed to nominal concentrations of 5, 10, 25, 50, and 100 mg/L ETU ($n = 2$ tanks for each concentration) and 0.01% DMSO as a SC ($n = 3$ tanks). Further treatments with 75 or 100 mg/L PTU ($n = 3$ tanks) and 1 $\mu\text{g/L}$ T4 ($n = 3$ tanks) represented positive control groups for antithyroidal and thyroid system agonistic activities, respectively. In preliminary experiments, DMSO did not affect developmental rates, growth, or survival of tadpoles at concentrations up to 0.1% (data not shown). Therefore, it was decided to omit the use of a dilution water control during this initial evaluation study in order to reduce the number of test animals. Notably, no effects of DMSO on the apical endpoints used in this study were detected in XEMA test trials performed in laboratory 5 where the experimental setting included both SC and dilution water control treatment groups [44]. The concentrations of T4, PTU, and ETU used in this study were selected on the basis of results from preliminary range-finding experiments that were performed in the Berlin laboratory. Two concentrations of PTU were used during this study. In a first series of four tests, a concentration of 100 mg/L PTU was employed, but this concentration was reduced to 75 mg/L PTU during a later phase of the ring test. Chemical analyses of actual water concentrations of T4, PTU, and ETU were performed only in laboratory 5 in order to confirm the stability of all compounds during the static-renewal exposure regime used in this study [44].

In the tail-resorption assay described by Fort et al. [28], exposure had been initiated with tadpoles at stage 60 and tail-length measurements provided the main endpoint in their as-

say. In order to assess the utility of this parameter in the XEMA test, the tail length additionally was determined for each tadpole at days 0, 7, 14, 21, and 28 of exposure.

Statistical analysis. Standardized data reporting forms were prepared and sent to all participating laboratories. Raw data were sent electronically by the participating laboratories to the Berlin laboratory. Statistical analyses of test data were performed using the software package Sigma Stat[®] 2.0 (SPSS-Jandel Scientific, Erkrath, Germany). Tail length and WBL data were first analyzed for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). For normally distributed data, Dunnett's test was used to compare length data from the SC group to all other treatment groups. In cases where length data were not distributed normally, data were analyzed using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test to compare length data from the SC group to all other treatment groups. Developmental stage data usually were not distributed normally and the nature of these data suggests that calculation of mean values is not a meaningful method. Therefore, only nonparametric methods were considered for statistical analysis of developmental stage data. The Kruskal-Wallis test was used to determine whether significant differences existed between the developmental stage composition of treatment groups and, if indicated, Dunn's multiple comparison test was used for comparisons with the SC group. Differences in growth and developmental indices between treatments were considered significant at the level of $p < 0.05$. Percent mortality was calculated at the end of each test trial by dividing the total number of tadpoles that died in all tanks of a given treatment group by the number stocked in this treatment group.

RESULTS

Participation

A total of 10 XEMA test trials using ETU as a test substance were conducted in six laboratories (Table 1). Four laboratories performed two test trials with ETU and two laboratories conducted only a single test with ETU.

Solvent control

In the SC group (0.01% DMSO), mortality during the exposure period of 28 d was very low (Table 2). No mortality was detectable in the SC group in seven out of a total of 10 test trials. In this study, growth of tadpoles was assessed by means of WBL measurements. Body weight of tadpoles was not determined. At test initiation, mean WBL of tadpoles in the SC groups ranged between 16.5 and 24.5 mm (Fig. 1). In

Table 2. Mortality following 28-d exposure of tadpoles to the indicated nominal concentrations of dimethylsulfoxide (DMSO), thyroxine (T4), propylthiouracil (PTU), and ethylenethiourea (ETU) as observed in different test trials during the ring-test study. Data are presented as % mortality in a given treatment

Lab	Replicate test	DMSO	T4	PTU	ETU (mg/L)				
		0.01%	1.0 µg/L	75 mg/L	5.0	10	25	50	100
1	R1 ^a	0	2.2	2.2	0	0	0	0	0
	R2	0	1.1	0	0	0	0	0	3.3
2	R1 ^a	0	0	1.1	0	0	0	1.7	0
	R2 ^a	0	0	1.1	0	1.7	0	0	0
3	R1	0	0	1.1	0	0	1.7	0	1.7
	R2	0	0	0	1.7	0	1.7	0	0
4	R1 ^a	2.2	5.6	3.3	3.3	5.0	5.0	3.3	3.3
	R2	2.2	3.3	6.6	1.7	3.3	3.3	1.7	0
5	R1	0	5.0	1.7	0	1.7	3.3	1.7	0
6	R1	2.2	2.2	2.2	1.7	1.7	0	1.7	3.3

^a Nominal PTU concentration was 100 mg/L in this test trial.

all test trials, mean WBL increased until day 21 of exposure. In five tests, mean WBL increased until test termination at day 28 of exposure. The reduction in mean WBL between exposure days 21 and 28 as observed in the other five test trials was due to an increased number of tadpoles in the SC group displaying tail resorption at exposure day 28 (Fig. 1C). Moreover, inspection of raw data revealed that the suitability of WBL as a parameter to assess tadpole growth should be limited to the initial 14 d of exposure, because several tadpoles already showed development beyond stage 61 by exposure day 21 in some test trials. Therefore, our analysis of possible effects of chemicals on tadpole growth focused on changes in WBL during the initial 14 d of exposure. The term initial growth rates is used throughout this paper to describe this approach of data interpretation. Comparison of results from measurements taken at exposure day 14 in different tests revealed that mean values of WBL spread over a large range from a minimum of 33.1 ± 6.1 mm to a maximum of 51.7 ± 6.2 mm (Table 3).

The developmental rate of tadpoles was assessed by means of stage determination according to Nieuwkoop and Faber [36]. During the exposure period of 28 d, tadpoles in the SC group displayed development to prometamorphic and early climax stages (Fig. 1A, Table 3). It was evident that, despite the use of a standardized protocol, differences in baseline developmental rates still existed across the participating laboratories (Table 3). Median values of the developmental stage reached by tadpoles in the SC group at test termination ranged from 55 to 62 between laboratories (Fig. 1A). In the tests performed in laboratories 1, 3, and 6, tadpoles showed somewhat higher basal developmental rates compared to tests in laboratories 2, 4, and 5. The results summarized in Table 3 further indicate a considerable interindividual variability in tadpole development as became evident from the range of stages to which the tadpoles developed during 28 d.

Thyroxine

A low concentration of T4 (1 µg/L) was used as a positive control for TH agonist activity during the initial evaluation of the XEMA test protocol. This T4 concentration was nontoxic to premetamorphic tadpoles. Mortality was not observed in four tests and ranged between 1.1% and 5.6% in the other six tests (Table 2). Higher T4 concentrations (≥ 5 µg/L), as tested in preliminary range-finding experiments, caused rapid developmental changes but inhibited tadpole growth and resulted in much higher mortality within 28 d (data not shown). Results

from WBL measurements at day 14 did not indicate differences in initial growth rates between the SC group and the 1 µg/L T4 treatment group (Table 3). At day 14, a significant reduction in WBL of T4-treated tadpoles compared to the SC group was detected in only one test in laboratory 2. Based on the analysis of developmental stage data, 1 µg/L T4 caused a statistically significant acceleration of metamorphic development compared to the SC group in all 10 test trials (Table 3). The enhanced development in the T4 treatment group was significant from exposure day 7 on throughout the entire testing period of 28 d. The effects of 1 µg/L T4 on tail length were less consistent, if compared to the results from developmental stage determinations (Table 4). In two test trials, T4 treatment did not cause any effects on tail length compared to the SC group. In the other eight test trials, mean tail length at day 28 was significantly lower in the T4 treatment group compared to the SC group.

Propylthiouracil

As a positive control for antithyroidal activity, PTU was selected for the initial evaluation of the XEMA test protocol. Two concentrations of PTU were used during this study. In a first series of four tests, a concentration of 100 mg/L PTU was employed. Because growth-retarding effects were noticed at 100 mg/L PTU, the PTU concentration was reduced to 75 mg/L PTU in a later phase of the ring test. Exposure to either PTU concentration did not result in increased mortality compared to the SC group (Table 2). However, in comparison to the SC group, a significant reduction in initial growth rates of tadpoles was observed for both PTU concentrations (Table 3). Both concentrations caused a complete inhibition of metamorphosis, which was evident from the observation that tadpoles did not develop beyond stage 54 within 28 d of exposure (Table 3). During all test trials, retardation of metamorphic development by either 100 or 75 mg/L PTU was significant from exposure day 14 on throughout the entire testing period of 28 d. The growth retardation caused by PTU, as observed in nine tests, complicated the assessment of possible effects of PTU on tail length. Compared to the SC group, mean tail length in PTU treatment groups was significantly reduced at days 7 and 14 in nine out of 10 tests. At day 21, mean tail length in PTU treatment groups still was reduced significantly in five out of 10 test runs. At test termination (day 28), a significantly greater tail length in the PTU treatment group

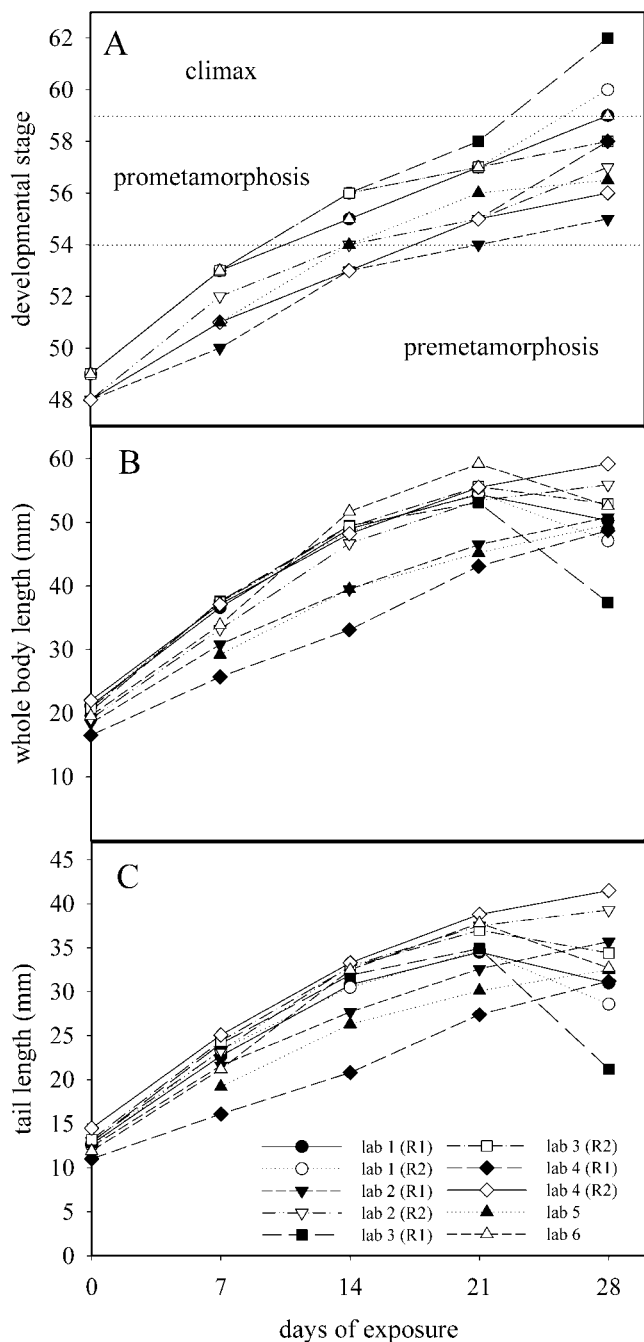


Fig. 1. Temporal changes in developmental stage (A), whole body length (B), and tail length (C) in the solvent control group (0.01% dimethylsulfoxide) during 28 d of exposure. Data from 10 independent test trials performed in six laboratories are shown. Replicate tests (R1, R2) performed in laboratories 1, 2, 3, and 4 are shown separately. Median (A) and mean values (B, C) are shown; standard deviation bars are omitted to reduce clutter.

compared to the SC group was observed in only four test trials (Table 4).

Ethylenethiourea

At the concentrations tested, ETU apparently was nontoxic to premetamorphic tadpoles. Exposure to ETU did not affect survival of tadpoles (Table 2) and, in most test runs, no effects of ETU on initial growth rates were detectable (Table 3). In all 10 XEMA test trials performed with ETU, results from developmental stage determinations revealed a concentration-

dependent inhibition of metamorphic development (Table 3). Figure 2 shows a typical box plot of developmental stages determined at day 28 in laboratory 1 during the present study. The two highest ETU concentrations (50 and 100 mg/L) caused a complete inhibition of metamorphic development in all test trials as became evident from the observation that tadpoles did not develop beyond stage 54 within 28 d of exposure. Partial inhibitory effects were observed at lower ETU concentrations. In seven test trials, 25 mg/L ETU caused a strong retardation of tadpole development leading to significantly lower developmental stages compared to SC tadpoles by day 28. The inhibitory effect of 25 mg/L ETU did not reach statistical significance during two tests performed in laboratory 2 and during one test in laboratory 4. In contrast to the two highest ETU concentrations, 25 mg/L ETU did not completely inhibit metamorphic development but metamorphosis proceeded much slower compared to the SC group. In eight test trials, median values for the developmental stage of tadpoles in the 25 mg/L ETU treatment group ranged between 53 and 55 at test termination (Table 3). The low median values indicate that a large number of tadpoles exposed to 25 mg/L ETU did not develop to prometamorphic stages within 28 d. However, it was observed that a certain number of tadpoles in this treatment group still were capable of developing to late prometamorphosis and even early climax stages, thus indicating a considerable interindividual variability in developmental responses of tadpoles to this partially inhibiting ETU concentration. Ethylenethiourea at 10 mg/L produced weak inhibitory effects, reaching a statistically significant level in only one test in laboratory 4 (Table 3). Lowest-observed-effect concentration (LOEC) values of inhibitory effects of ETU on metamorphic development were calculated from developmental stage determination data at day 28 (Table 3). Within this ring-test study, LOECs ranged between 10 and 50 mg/L ETU, thus minimum and maximum LOECs differed by a factor of five. In six out of 10 tests, a LOEC of 25 mg/L ETU was determined. Intralaboratory comparison of LOECs for inhibitory effects of ETU on metamorphic development showed that replicate tests performed in laboratory 1 (LOEC: 25 mg/L ETU), laboratory 2 (LOEC: 50 mg/L ETU), and laboratory 3 (LOEC: 25 mg/L) produced comparable results. A higher intralaboratory variability was observed for the replicate tests performed in laboratory 4 (LOECs: 10 and 50 mg/L ETU). In contrast to developmental stage determinations, tail-length measurements proved to be a far less sensitive parameter to detect inhibitory activities of ETU on metamorphic development during XEMA (Table 4). Inhibitory effects of ETU (25, 50, 100 mg/L) on tail resorption, as indicated by a higher mean tail length compared to the SC group at exposure day 28, were detected only in five test runs (Table 4).

DISCUSSION

The general aim of the present study was to develop the conceptual and methodological framework of a standardized amphibian testing approach for detection of thyroid system-disrupting effects by environmental compounds. This issue was addressed by exploiting anuran postembryonic development as a biological model. The underlying idea of this amphibian metamorphosis assay is that potential thyroid system-disrupting effects become evident in *X. laevis* tadpoles as morphological alterations during metamorphosis due to the obligatory dependence of normal metamorphic development on undisturbed function of the thyroid system [23]. Within this

Table 3. Summary of results from measurements of developmental stage (exposure day 28) and whole body length (WBL), exposure day 14) in the interlaboratory study. DMSO = dimethylsulfoxide; T4 = thyroxine; PTU = propylthiouracil; ETU = ethylenethiourea

Endpoint	Lab	Replicate test	DMSO		T4 ($\mu\text{g/L}$)		PTU (mg/L)		ETU (mg/L)		LOEC ^a (mg/L)	
			0.01%		1.0	75	5.0	10	25	50	100	
Stage (day 28)	1	R1 ^b	59 (55-65) ^e	62 (57-66) ^d	52 (48-54) ^e	59 (54-65)	58 (54-63)	54.5 (51-59) ^e	52 (50-54) ^e	52 (50-54) ^e	25	
		R2	60 (53-65)	63 (59-66) ^d	53 (51-54) ^e	59 (56-64)	58 (53-65)	57 (49-62) ^e	53 (52-54) ^e	53 (52-54) ^e	25	
	2	R1 ^b	55 (51-64)	59 (58-62) ^d	51 (50-54) ^e	55 (54-62)	55 (52-60)	55 (50-59)	51 (50-55) ^e	54 (50-54) ^e	50	
		R2 ^b	57 (55-64)	59 (59-65) ^d	53 (51-54) ^e	56 (54-64)	57 (54-62)	56 (52-59)	56 (52-59)	53 (51-54) ^e	50	
	3	R1	62 (57-66)	64 (59-66) ^d	53 (52-54) ^e	62 (57-65)	62 (54-65)	54 (52-63) ^e	53 (52-53) ^e	53 (50-53) ^e	25	
		R2	58 (54-66)	61 (57-66) ^d	53 (52-54) ^e	57 (56-64)	57 (54-66)	53 (52-58) ^e	53 (52-54) ^e	53 (52-54) ^e	25	
4	R1 ^b	58 (55-61)	60 (58-61) ^d	52 (50-53) ^e	57 (53-59)	55 (51-57) ^e	54 (50-58) ^e	52 (51-54) ^e	52 (51-54) ^e	10		
	R2	56 (51-61)	57 (57-62) ^d	53 (51-54) ^e	56 (52-59)	56 (52-59)	55 (51-58)	51 (50-54) ^e	52 (51-54) ^e	25		
5	R1	56.5 (53-60)	57 (56-63) ^d	52 (49-53) ^e	57 (54-62)	57 (55-61)	53 (51-56) ^e	51 (50-53) ^e	52 (50-53) ^e	25		
	R2	60 (53-65)	63 (58-66) ^d	53 (51-55) ^e	61 (54-66)	62 (51-65)	54 (51-58) ^e	53 (51-54) ^e	53 (51-55) ^e	25		
WBL (day 14)	1	R1 ^b	49.0 \pm 4.9 ^f	46.8 \pm 5.6	35.9 \pm 6.3 [*]	48.3 \pm 5.0	48.2 \pm 4.9	49.0 \pm 4.2	48.1 \pm 3.6	47.2 \pm 4.4	47.1 \pm 3.4	
		R2	48.6 \pm 4.9	46.7 \pm 4.6	45.8 \pm 5.1 [*]	48.1 \pm 3.5	47.3 \pm 3.9	48.2 \pm 4.3	48.2 \pm 4.3	47.2 \pm 4.4	47.1 \pm 3.4	
	2	R1 ^b	39.5 \pm 5.5	35.2 \pm 4.8 [*]	33.6 \pm 5.2 [*]	39.6 \pm 3.9	39.0 \pm 4.9	42.4 \pm 5.9 [*]	39.9 \pm 6.6	35.1 \pm 4.9 [*]	35.1 \pm 4.9 [*]	
		R2 ^b	46.7 \pm 4.1	45.0 \pm 3.8	41.2 \pm 5.7 [*]	44.9 \pm 4.7	45.9 \pm 4.6	46.0 \pm 3.8	45.1 \pm 3.8	45.2 \pm 4.5	45.2 \pm 4.5	
	3	R1	49.5 \pm 3.6	48.0 \pm 4.4	45.1 \pm 4.8 [*]	48.8 \pm 4.5	49.2 \pm 4.5	49.5 \pm 4.5	48.2 \pm 3.8	48.0 \pm 4.5	48.0 \pm 4.5	
		R2	49.3 \pm 4.2	47.5 \pm 4.0	46.2 \pm 4.3 [*]	48.4 \pm 4.0	49.0 \pm 3.6	49.2 \pm 4.2	48.2 \pm 4.4	48.3 \pm 3.3	48.3 \pm 3.3	
4	R1 ^b	33.1 \pm 6.1	33.1 \pm 7.5	29.6 \pm 6.1 [*]	33.1 \pm 7.6	35.1 \pm 7.6	36.4 \pm 8.6	36.4 \pm 6.0	33.3 \pm 6.2	33.3 \pm 6.2		
	T2	48.2 \pm 5.6	48.8 \pm 5.3	47.4 \pm 4.5	47.5 \pm 4.2	48.8 \pm 4.6	46.7 \pm 5.2	46.3 \pm 4.7	47.7 \pm 4.4	47.7 \pm 4.4		
5	R1	39.5 \pm 5.4	40.7 \pm 5.1	36.3 \pm 4.9 [*]	40.7 \pm 4.2	40.2 \pm 5.1	40.9 \pm 4.6	40.7 \pm 5.4	40.0 \pm 4.7	40.0 \pm 4.7		
	R1	51.7 \pm 6.2	49.2 \pm 6.7	41.6 \pm 8.1	52.5 \pm 6.4	53.9 \pm 6.3	55.2 \pm 6.9	55.2 \pm 6.9	52.4 \pm 8.9	52.4 \pm 8.9		

^a Lowest-observed-effect concentration (LOEC) values for inhibitory effects of ETU on development were calculated based on developmental stage data at exposure day 28.

^b A PTU concentration of 100 mg/L was used in this test trial.

^c Values shown are the median stage with the total range of stages in parentheses.

^d Acceleration of development (Dunn's test, $p < 0.05$).

^e Retardation of development (Dunn's test, $p < 0.05$).

^f Values shown are means and standard deviations.

^{*} Significantly different from the DMSO control (Dunnnett's test, $p < 0.05$).

Table 4. Summary of results from tail length measurements (exposure day 28) in the interlaboratory study. DMSO = dimethylsulfoxide; T4 = thyroxine; PTU = propylthiouracil; ETU = ethylenethiourea

Endpoint	Lab	DMSO		T4 ($\mu\text{g/L}$)		PTU (mg/L)		ETU (mg/L)			
		0.01%		1.0	1.0	75	75	5.0	10	25	50
Tail length (day 28)	1	31.0 \pm 10.9 ^b	21.0 \pm 14.5*	34.6 \pm 6.7	32.4 \pm 10.4	33.5 \pm 7.0	38.3 \pm 4.3*	38.1 \pm 3.8*	37.9 \pm 5.1*		
	2	28.6 \pm 12.8	17.4 \pm 15.7*	35.3 \pm 4.3*	32.9 \pm 8.2	32.8 \pm 8.6	38.0 \pm 5.2*	37.9 \pm 4.5*	37.8 \pm 3.7*		
	2	35.7 \pm 5.3	32.8 \pm 4.8*	34.6 \pm 5.2	36.7 \pm 3.7	36.8 \pm 4.5	37.9 \pm 4.3	36.9 \pm 5.3	35.6 \pm 4.9		
	3	39.3 \pm 4.4	33.4 \pm 9.5*	38.6 \pm 4.6	39.4 \pm 4.8	40.3 \pm 3.3	41.7 \pm 2.8*	40.0 \pm 4.0	40.8 \pm 4.1		
	3	21.2 \pm 14.7	14.3 \pm 14.5*	36.6 \pm 3.3*	21.7 \pm 14.3	27.1 \pm 12.6	39.4 \pm 3.5*	38.2 \pm 3.5*	38.1 \pm 3.4*		
	4	34.4 \pm 11.6	27.4 \pm 12.9*	37.1 \pm 3.1	37.1 \pm 6.0	36.8 \pm 7.4	39.8 \pm 3.3*	38.9 \pm 4.1	39.2 \pm 3.6*		
	4	31.2 \pm 4.8	28.7 \pm 5.8*	26.7 \pm 5.9*	31.9 \pm 5.5	31.1 \pm 6.0	31.1 \pm 6.6	31.8 \pm 7.5	30.5 \pm 6.2		
	5	41.5 \pm 4.8	39.8 \pm 3.8	41.0 \pm 3.7	41.4 \pm 3.8	41.6 \pm 3.9	41.0 \pm 4.3	40.6 \pm 4.5	40.4 \pm 3.9		
	5	32.5 \pm 4.1	31.8 \pm 4.3	32.8 \pm 5.9	33.0 \pm 3.7	32.4 \pm 4.3	34.0 \pm 3.6	32.9 \pm 5.4	32.7 \pm 5.2		
	6	32.7 \pm 13.1	17.3 \pm 12.8*	38.7 \pm 7.7	29.6 \pm 14.6	32.4 \pm 13.1	44.0 \pm 7.0*	44.7 \pm 6.9*	43.7 \pm 7.6*		

^a PTU concentration of 100 mg/L was used in this test trial.

^b Values shown are means and standard deviations.

* Significantly different from the DMSO control (Dunn's test, $p < 0.05$).

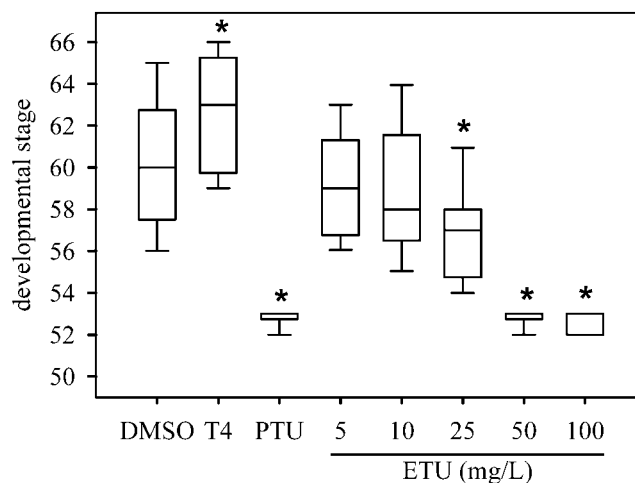


Fig. 2. Distribution of developmental stages in different treatment groups after 28 d of exposure to the solvent control (0.01% dimethylsulfoxide [DMSO]), 1 $\mu\text{g/L}$ thyroxine (T4), 75 mg/L propylthiouracil (PTU), and the indicated nominal concentrations of ethylenethiourea (ETU). Results from a test trial conducted in laboratory 1 (replicate test R2) are shown. Bars indicate median values, box demarcates 25 and 75%iles, whiskers indicate 5 and 95%iles. Statistically significant differences from the solvent control (0.01% DMSO) are marked by asterisks ($*p < 0.05$, Dunn's multiple comparison test on ranks).

conceptual framework, it generally can be assumed that compounds that inhibit thyroid function or antagonize TH action cause reductions in developmental rates of tadpoles [29,46] whereas substances that mimic or enhance TH activity accelerate metamorphosis [23]. Determination of developmental stages of *X. laevis* tadpoles by using the staging criteria of Nieuwkoop and Faber [36] was selected as the primary endpoint of this test protocol because stage determination provides an integrative and convenient means to assess treatment-related deviations from normal morphological development. In addition, growth and mortality of tadpoles were monitored because these endpoints should provide first indications of modes of action unrelated to the thyroid system.

When developing the protocol for the XEMA test, special attention was given to a sensitive detection of both agonistic and antagonistic activities on TH-regulated metamorphosis. From an endocrinological perspective, the period from premetamorphosis throughout prometamorphosis was considered to be the most relevant developmental phase to be covered by the XEMA testing protocol. Although endogenous concentrations of TH are very low in premetamorphic tadpoles [39], they display the competence to respond to exogenously administered TH by precocious induction of metamorphosis-associated biochemical and morphological changes [27]. Accordingly, initiation of exposure at premetamorphic stages (stages 48 to 50 in XEMA) was considered to be a prerequisite for a sensitive detection of the potential stimulating effects of compounds with thyromimetic activities. So far, only few studies investigated the potential of chemicals to exert thyromimetic activity by directly interfering with thyroid hormone receptor-mediated TH action in target tissues [17,19,47]. Recently, Kitamura et al. [17] reported that brominated and chlorinated bisphenol A derivatives compete with T3 for binding to the mammalian thyroid hormone receptor and display TH-mimicking activity toward TH-responsive gene expression under in vitro conditions. Furthermore, some hydroxylated metabolites of PCBs and bromodiphenyl ethers have been re-

ported to bind with weak affinity to human thyroid hormone receptors [47], and there is also some evidence of direct effects of PCBs on TH-responsive gene expression in fetal rat brain [10]. It should be noted, however, that the hypothesis of xenobiotic interference with TH action in peripheral target tissues has so far not been investigated adequately in experimental studies. Nevertheless, by assembling all the pieces of evidence obtained in various studies, we think that disruption of TH signaling should find adequate consideration as a possible mode of action when developing a testing strategy for thyroid system disruption.

It has been known for long that the precocious metamorphosis model offers considerable potential to reveal possible thyromimetic activities of compounds [48]. In order to assess the utility of the XEMA assay to detect compounds sensitively with thyromimetic activities, a low concentration of thyroxine (T₄; 1 µg/L), serving as a positive control for a TH agonist activity, was included in the exposure scheme of all XEMA tests performed during this ring-test study. The selected T₄ concentration was remarkably nontoxic to tadpoles but always produced a significant acceleration of metamorphosis. In this respect, it should be noted that alternatively proposed amphibian testing protocols using tadpoles at later developmental stages (stage 60) such as the tail-resorption assay [28] were limited in their ability to detect stimulating effects even at higher concentrations of T₄ or T₃ [6]. The failure of TH treatment to enhance development relative to the untreated controls in assays using tadpoles at late developmental stages most likely was due to the high endogenous level of TH already present in these tadpoles and, thus, additional exogenous TH could not significantly affect the system. Regarding the detection of TH agonistic activities in *X. laevis* tadpoles, the superior sensitivity of premetamorphic stages, compared to early and late prometamorphic stages, also has been shown for the up-regulation of specific TH-responsive genes following short-term TH treatment [49].

For the initial evaluation of the XEMA testing protocol, PTU was used as a positive control for antithyroidal activities. Propylthiouracil inhibits the synthesis of T₄ in the thyroid gland by inactivating thyroid peroxidase [8]. In all tests performed during this ring-test study, PTU caused a pronounced inhibitory effect on metamorphosis. The observed inhibition of metamorphosis by PTU is consistent with the reported antithyroidal activity of PTU in mammals [8] and in *X. laevis* tadpoles [30]. By determination of developmental stages, inhibition of metamorphic development by PTU rapidly was detectable from exposure day 7 throughout the entire exposure period. The observation that the antithyroidal activity of PTU caused a pronounced and rapidly detectable inhibition of metamorphosis in *X. laevis* tadpoles, when exposure is initiated at early developmental stages, is in agreement with earlier studies using various goitrogens to inhibit metamorphosis [29,50]. Together with the low mortality rates, the results from the positive control groups (T₄, PTU) clearly demonstrate the practicability and general ability of a testing protocol covering premetamorphic and prometamorphic developmental phases to detect both stimulating and inhibiting effects on the thyroid system by means of monitoring metamorphic development in *X. laevis* tadpoles.

At the time of initiation of the XEMA evaluation study, the tail-resorption assay [28] was recommended by the Endocrine Disruptor Screening and Testing Committee as a Tier I frog metamorphosis assay [4]. Therefore, measurements of

tail length were included as an additional endpoint in this study because of its proposed utility to detect changes in thyroid system function. Compared to determination of developmental stages, tail-length measurements provided a much less-sensitive and less-reliable parameter to assess changes in thyroid function in our study. This was not unexpected given that the XEMA testing protocol covers premetamorphic and prometamorphic developmental phases. Although one might argue that tail length measurements are of limited value to detect changes in thyroid function, there is at least one mode of action, namely the inhibition of type III monodeiodinase (D3) activity, where tail-length measurements may provide a valuable means of detection. During prometamorphosis, increased D3 expression in tadpole tail protects this tissue from precocious transformation in response to rising T₃ plasma levels [51]. By using iopanoic acid, an inhibitor of all iodothyronine deiodinases [52], the protective effect of D3 can be overcome, thus leading to precocious induction of tail resorption in tadpoles during late prometamorphosis (R. Opitz, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany, unpublished data), and increased rates of T₃-induced tail resorption in tail-culture experiments [53]. Accordingly, before tail-length measurement is rejected completely as an endpoint, its possible diagnostic value to indicate changes in D3 activity should be explored.

Ethylenethiourea was used as a test substance during the initial evaluation of the XEMA test because of its reported antithyroidal activity in mammals [8]. To the best of our knowledge, this is the first study reporting antithyroidal activity of ETU in amphibian tadpoles. Ethylenethiourea is a common metabolite, breakdown product, and contaminant of ethylenebisdithiocarbamate fungicides [54]. It has been classified as a goitrogenic, carcinogenic, and teratogenic environmental contaminant [55]. A main target organ of ETU is the thyroid gland [34]. Mechanistic studies have shown that ETU is a potent inhibitor of T₄ synthesis and that the inhibitory activity results from an inhibition of thyroid peroxidase activity [31]. In the XEMA test, a concentration-dependent inhibition of metamorphic development was observed for ETU. The highest test concentrations of ETU (50 and 100 mg/L) completely inhibited development at stages 53/54, indicating that these concentrations were sufficient to cause an almost complete inhibition of TH synthesis in *X. laevis* tadpoles. Lower concentrations of ETU (10 and 25 mg/L) also showed partial inhibitory effects on development. Although it is difficult to relate observations made in this study with results from mammalian exposure studies, our data indicate elevated sensitivity of the testing approach used in XEMA to detect the antithyroidal activity of ETU. Exposure of male Wistar rats for 28 d to 100 to 300 mg/L ETU in drinking water caused significant reductions in serum T₄ and T₃ concentrations that were accompanied by elevated plasma thyroid-stimulating hormone levels, but these changes were not reflected in thyroid gland morphology when assessed by light microscopy [33]. Preliminary results from histological analyses of thyroid glands dissected from *X. laevis* tadpoles after ETU exposure during XEMA revealed morphological alterations at ETU concentrations in the lower mg/L range (R. Opitz, unpublished data).

The distinction between thyroid system-related and unrelated mechanisms altering metamorphic development is an important problem that needs to be investigated thoroughly in order to ensure the specificity of any metamorphosis assay for thyroid system-related effects. In this study, a first step was

undertaken to address this issue by assessing the utility of growth parameters to serve as indicators of nonspecific mechanisms affecting tadpole development. Measurements of WBL were used to describe tadpole growth but the utility of this parameter was limited to the initial 14 d of exposure, because at exposure day 21, some tadpoles already showed signs of tail resorption with a resultant reduction in WBL. At the low concentration used in this study, T4 did not alter tadpole growth rates within the initial 14 d of exposure. It should be noted, however, that higher concentrations of T4 as used in preliminary range-finding experiments greatly reduced tadpole growth and survival while promoting faster development. For PTU, the effect pattern concerning tadpole development and growth was more difficult to interpret. Though strongly inhibiting metamorphic development, PTU treatment also reduced tadpole growth rates. The observed growth retardation in PTU-treated tadpoles was unexpected because results from several other studies suggested that even complete blockade of TH synthesis and thus, complete inhibition of metamorphosis does not necessarily inhibit growth of tadpoles [29,44,56]. Consistent with these observations, the antithyroidal activity of ETU was not associated with altered growth rates in *X. laevis* tadpoles in our studies. One possible explanation for the growth-retarding effects by PTU may be toxic side effects arising from extrathyroidal modes of action [57]. Therefore, it is concluded that chemically induced alterations in thyroid system function do not necessarily affect tadpole growth during pre- and prometamorphic development and altered growth rates may indicate the presence of additional toxic side effects that are not related to the thyroid system. In turn, the results from the PTU treatment indicate that alterations in tadpole growth cannot be regarded as a sufficient criterion to reject the hypothesis of thyroid system disruption as being responsible for changes in metamorphic development.

The inherent rate at which tadpole development proceeds is considered a key factor that may influence the results of any amphibian metamorphosis assay. Accordingly, establishing comparable rates of tadpole development across different laboratories is a prerequisite to generating reproducible data on effects of environmental chemicals on the developing amphibian thyroid system. Apart from the interlaboratory validation studies conducted for the Frog Embryo Teratogenesis Assay—*Xenopus* [58], we could not identify published studies investigating the interlaboratory variability of growth and developmental parameters during *X. laevis* development. A review of the published literature revealed a considerable variability in inherent rates of growth and development of *X. laevis* tadpoles in different laboratories [29,36,59,60]. Main sources of variability may be differences in animal husbandry techniques as well as genetic factors. In this study, the use of a testing protocol standardizing numerous abiotic and biotic factors determining tadpole growth and development (temperature, light:dark cycle, feeding regime, larval density, culture medium) provided the basis for an initial assessment of the inherent variability associated with tadpole development in the laboratory setting. A comparison of development and growth data obtained from the ring-test study showed that the use of a standardized testing protocol clearly could reduce the inherent variability of rates of tadpole growth and development between laboratories. Importantly, despite the fact that moderate differences in basal developmental rates still existed, all laboratories were able to detect the stimulating and inhibiting effects on metamorphosis produced by the positive controls

T4 and PTU, respectively. Moreover, qualitatively and quantitatively similar results also were obtained for the test substance ETU, demonstrating the robustness of the XEMA test to detect both stimulating and inhibiting effects on metamorphic development across different laboratories. Nevertheless, a more robust exposure verification by chemical analysis is warranted in future studies to examine whether the remaining variability was due to biological factors or differences in actual water concentrations of the test compounds.

Determination of the developmental stage of tadpoles provides an integrated measure of chemical effects on metamorphic development that can be determined easily. However, apical morphological endpoints generally are considered to suffer from low sensitivity and specificity for detection of effects on thyroid axis functioning. Despite these limitations, the measurement of apical endpoints is a valuable and practicable approach given the specific purposes of this first evaluation study of the XEMA test. Nevertheless, we are aware that complementary analyses of histological, biochemical, and molecular endpoints are required to improve the assay regarding sensitivity, specificity for effects on the thyroid system, and diagnostic value for specific modes of action. Preliminary work showed that analyses of mRNA expression levels of thyroid hormone receptor β in various tadpole tissues and thyroid-stimulating hormone β -subunit in the pituitary offer considerable potential to increase the sensitivity and diagnostic value of the XEMA test [49]. Another classical approach to detect antithyroidal activities of chemicals is histological analysis of the thyroid gland [24,26,30]. However, little is known about concentration-dependent changes of thyroidal histological markers in anuran larvae following exposure to thyroid-disrupting compounds. The analysis of the various histological criteria that may bear a potential to indicate changes in the functional state of the thyroid gland in *X. laevis* tadpoles currently is in progress.

CONCLUSION

In summary, a testing protocol was developed for a *Xenopus* metamorphosis assay, using *X. laevis* larvae as test organisms. A core element of the testing approach used in XEMA was that exposure of tadpoles is initiated at premetamorphic stages 48 to 50 and continued for a total of 28 d to allow development of tadpoles throughout prometamorphosis. This testing approach proved functional to detect the stimulating effect of a low concentration of T4 and the inhibitory effects of the known mammalian antithyroidal chemicals PTU and ETU. Determination of the developmental stages of the test organisms provided an endpoint to reveal the alterations in the thyroid system caused by these substances. The results presented provide evidence that chemicals known to inhibit thyroid function in mammalian animal systems are capable of affecting metamorphosis in *X. laevis* tadpoles, and that these effects clearly were detectable using simple and practical morphological endpoints in the XEMA test. The general interlaboratory transferability of the test protocol was confirmed in an international ring-test study. Critical parameters influencing the test results could be identified and a further refinement and optimization of the test protocol is proposed. Based on the data presented, the XEMA testing protocol is regarded a sensitive and easy-to-handle testing approach for the detection of thyroid system-disrupting activities of environmental compounds. Further studies are in progress to identify additional histological, biochemical, and molecular endpoint measure-

ments that may help to refine the testing protocol with respect to its sensitivity and diagnostic value for thyroid system-related effects.

Acknowledgement—The cooperation and enthusiasm of all laboratories and their associated technical staff gratefully are acknowledged. The authors gratefully acknowledge the dedicated assistance of W. Schumacher, A. Tillack, A. Schulz, O. Jagnytsch, S. Hartmann, K. Weber, G. Elter, C. Sagoe, N. Santo, T. Fujii, M. Miyahara, T. Oka, N. Mitsui, L. Bickley, N. Pounds, C. Brown, and M. Hetheridge. This work was supported by the Federal Environmental Agency Germany under Contract 200-67-409 and 203-67-450 and by the fund for endocrine disruptors from the Japanese Ministry of Environment.

REFERENCES

- Crisp TM, Clegg ED, Cooper RL, Wood WP, Anderson DG, Baetcke KP, Hoffmann JL, Morrow MS, Rodier DJ, Schaeffer JE, Touart LW, Zeeman MG, Patel YM. 1998. Environmental endocrine disruption: An effects assessment and analysis. *Environ Health Perspect* 106(Suppl 1):11–56.
- Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV, Brandt I, Vethaak AD. 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *Crit Rev Toxicol* 30:71–133.
- Ankley GT, Johnson RD, Toth G, Folmar L, Detenbeck NE, Bradbury SP. 1997. Development of a research strategy for assessing the ecological risk of endocrine disruptors. *Rev Toxicol* 1:231–267.
- U.S. Environmental Protection Agency. 1998. Endocrine-disruptor screening and testing advisory committee (EDTAC) final report. Washington, DC.
- Huet M-C. 2000. OECD activity on endocrine disruptors test guidelines development. *Ecotoxicology* 9:77–84.
- Gray LE, Ostby J, Wilson V, Lambright C, Bobseine K, Hartig P, Hotchkiss A, Wolf C, Furr J, Price M, Parks L, Cooper RL, Stoker TE, Laws SC, Degitz SJ, Jensen KM, Kahl MD, Korte JJ, Makynen EA, Tietge JE, Ankley GT. 2002. Xenoendocrine disruptors—tiered screening and testing: Filling key data gaps. *Toxicology* 181–182:371–382.
- Jobling S. 1998. Review of suggested testing methods for endocrine-disrupting chemicals. *Pure and Applied Chemistry* 70: 1805–1827.
- Brucker-Davis F. 1998. Effects of environmental synthetic chemicals on thyroid function. *Thyroid* 8:827–856.
- Rolland RM. 2000. A review of chemically induced alterations in thyroid and vitamin A status from field studies of wildlife and fish. *J Wildl Dis* 36:615–635.
- Zoeller RT. 2003. Challenges confronting risk analysis of potential thyroid toxicants. *Risk Analysis* 23:143–162.
- Yamasaki K, Sawaki M, Ohta R, Okuda H, Katayama S, Yamada T, Ohta T, Kosaka T, Owens W. 2003. OECD validation of the Hershberger assay in Japan: Phase 2 dose response of methyltestosterone, vinclozolin, and *p,p'*-DDE. *Environ Health Perspect* 111:1912–1919.
- Owens W, Koeter HB. 2003. The OECD program to validate the rat uterotrophic bioassay: An overview. *Environ Health Perspect* 111:1527–1529.
- DeVito M, Biegel L, Brouwer A, Brown S, Brucker-Davis F, Cheek AO, Christensen R, Colborn T, Cooke P, Crissman J, Crofton K, Doerge D, Gray E, Hauser P, Hurley P, Kohn M, Lazar J, McMaster S, McClain M, McConnell E, Meier C, Miller R, Tietge J, Tyl R. 1999. Screening methods for thyroid hormone disruptors. *Environ Health Perspect* 107:407–415.
- Opitz R, Levy G, Bogi C, Lutz I, Kloas W. 2002. Endocrine disruption in fish and amphibians. *Recent Research and Development in Endocrinology* 3:127–170.
- Marty MS, Crissman JW, Carney EW. 2001. Evaluation of the male pubertal assay's ability to detect thyroid inhibitors and dopaminergic agents. *Toxicol Sci* 60:63–76.
- O'Connor JC, Frame SR, Davis LG, Cook JC. 1999. Detection of thyroid toxicants in a tier I screening battery and alterations in thyroid endpoints over 28 days of exposure. *Toxicol Sci* 51: 54–70.
- Kitamura S, Jinno N, Ohta S, Kuroki H, Fujimoto N. 2002. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. *Biochem Biophys Res Commun* 293:554–559.
- Crump D, Werry K, Veldhoen N, van Aggelen G, Helbing CC. 2002. Exposure to the herbicide acetochlor alters thyroid hormone-dependent gene expression and metamorphosis in *Xenopus laevis*. *Environ Health Perspect* 110:1199–1205.
- Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, Hataya Y, Shimatsu A, Kuzuya H, Nakao K. 2002. Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J Clin Endocrinol Metab* 87:5185–5190.
- Yamada-Okabe T, Aono T, Sakai H, Kashima Y, Yamada-Okabe H. 2004. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin augments the modulation of gene expression mediated by the thyroid hormone receptor. *Toxicol Appl Pharmacol* 194:201–210.
- Kloas W. 2002. Amphibians as model for the study of endocrine disruptors. *Int Rev Cytol* 216:1–57.
- Hutchinson TH, Pickford DB. 2002. Ecological risk assessment and testing for endocrine disruption in the aquatic environment. *Toxicology* 181–182:383–387.
- Shi Y-B. 1999. *Amphibian Metamorphosis. From Morphology to Molecular Biology*. Wiley-Liss, New York, NY, USA.
- Goleman WL, Carr JA, Anderson TA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. *Environ Toxicol Chem* 21:590–597.
- Miranda LA, Paz DA, Dezi RE, Pisanó A. 1995. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in *Bufo arenarum* larvae with inhibited thyroid function. *Gen Comp Endocrinol* 98:166–176.
- Miranda LA, Pisanó A, Casco V. 1996. Ultrastructural study of thyroid glands of *Bufo arenarum* kept in potassium perchlorate solution. *Biocell* 20:147–153.
- Tata JR. 1998. *Hormonal Signaling and Postembryonic Development*. Springer, Heidelberg, Germany.
- Fort DJ, Rogers RL, Morgan LA, Miller MF, Clark PA, White JA, Paul RR, Stover EL. 2000. Preliminary validation of a short-term morphological assay to evaluate adverse effects on amphibian metamorphosis and thyroid function using *Xenopus laevis*. *J Appl Toxicol* 20:419–425.
- Goleman WL, Urquidí LJ, Anderson TA, Smith EE, Kendall RJ, Carr JA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. *Environ Toxicol Chem* 21:424–430.
- Goos HJ, Zwanenbeek HC, van Oordt PG. 1968. Hypothalamic neurosecretion and metamorphosis in *Xenopus laevis*. II. The effect of thyroxine following treatment with propylthiouracil. *Arch Anat Histol Embryol* 51:267–274.
- Doerge DR, Takazawa RS. 1990. Mechanism of thyroid peroxidase inhibition by ethylenethiourea. *Chem Res Toxicol* 3:98–101.
- Graham SL, Hansen WH. 1972. Effects of short-term administration of ethylenethiourea upon thyroid function of the rat. *Bull Environ Contam Toxicol* 7:19–25.
- Kurtio P, Savolainen K, Tuominen R, Kosma V-M, Naukkarinen A, Männistö P, Collan Y. 1986. Ethylenethiourea and nabam induced alterations of function and morphology of thyroid gland in rats. *Arch Toxicol Suppl* 9:339S–344S.
- Tsuda H. 1983. Goiter, adenoma, and carcinoma of the thyroid induced by amitrole and ethylenethiourea, rat. In Jones TC, Mohr U, Hunt RD, eds, *Monographs on Pathology of Laboratory Animals, Endocrine System*. Springer, Heidelberg, Germany, pp 204–211.
- Birch WX, Prahlad KV. 1986. Effects of nabam on developing *Xenopus laevis* embryos: Minimum concentration, biological stability, and degradative products. *Arch Environ Contam Toxicol* 15:637–645.
- Nieuwkoop PD, Faber J. 1994. *Normal Table of Xenopus laevis (Daudin)*, 3rd ed. Garland, New York, NY, USA.
- Deuchar EM. 1975. *Xenopus: The South African Clawed Frog*. John Wiley, New York, NY, USA.
- Jayatilaka AD. 1978. An ultrastructural study of the thyroid gland in the premetamorphic *Xenopus laevis* (Daudin) tadpole. *J Anat* 125:579–591.
- Leloup J, Buscaglia M. 1977. La triiodothyronine, hormone de la métamorphose des amphibiens. *CR Acad Sci Hebd Seances Acad Sci D* 284:2261–2263.
- Bantle JA. 1995. FETAX—a developmental toxicity assay using

- frog embryos. In Rand GM ed, *Fundamentals of Aquatic Toxicology. Effects, Environmental Fate, and Risk Assessment*. Taylor & Francis, Washington, DC, USA, pp 207–230.
41. Wu M, Gerhart J. 1991. Raising *Xenopus* in the laboratory. *Methods Cell Biol* 36:3–18.
 42. Pauli BD, Perrault JA, Money SL. 2000. RATL: A database of reptile and amphibian toxicology literature. Technical Report Series 357. National Wildlife Research Centre, Canadian Wildlife Service, Environmental Conservation Branch, Ottawa, ON.
 43. Pickford DB, Hetheridge MJ, Caunter JE, Hall AT, Hutchinson TH. 2003. Assessing chronic toxicity of bisphenol A to larvae of the African clawed frog (*Xenopus laevis*) in a flow-through exposure system. *Chemosphere* 53:223–235.
 44. Kloas W, Opitz R, Lutz I. 2003. Ring test: Effects of pesticides and other chemicals on thyroid system in the amphibian *Xenopus laevis*. Research Report 20067409. Federal Environmental Agency, Berlin, Germany.
 45. Gutleb AC, Bronkhorst M, van den Berg JHJ, Murk AJ. 2001. Latex laboratory gloves: An unexpected pitfall in amphibian toxicity assays with tadpoles. *Environ Toxicol Pharmacol* 10:119–121.
 46. Lim W, Nguyen N-H, Yang HY, Scanlan TS, Furlow JD. 2002. A thyroid hormone antagonist that inhibits thyroid hormone action in vivo. *J Biol Chem* 277:35664–35670.
 47. Cheek AO, Kow K, Chen J, McLachlan JA. 1999. Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. *Environ Health Perspect* 107:273–278.
 48. Selenkow HA, Asper SP. 1955. Biological activity of compounds structurally related to thyroxine. *Physiol Rev* 35:426–474.
 49. Opitz R, Levy G, Lutz I, Kloas W. 2002. Development of molecular biomarkers to detect thyroid-disrupting activities of environmental chemicals in *Xenopus laevis* tadpoles. *Proceedings, 21st Conference of European Comparative Endocrinologists*, Bonn, Germany, August, 26–30, pp 99–102.
 50. Henriques U. 1962. Bioassay of thyroid hormone using hypothyroid tadpoles. *Acta Endocrinol* 41:143–153.
 51. Kawahara A, Gohda Y, Hikosaka A. 1999. Role of type III iodothyronine 5-deiodinase gene expression in temporal regulation of *Xenopus* metamorphosis. *Dev Growth Diff* 41:365–373.
 52. Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR. 2002. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* 23:38–89.
 53. Shintani N, Nohira T, Hikosaka A, Kawahara A. 2002. Tissue-specific regulation of type III iodothyronine 5-deiodinase gene expression mediates the effects of prolactin and growth hormone in *Xenopus* metamorphosis. *Dev Growth Diff* 44:327–335.
 54. Lentza-Rizos C. 1990. Ethylenethiourea (ETU) in relation to use of ethylenebisdithiocarbamate (EBDC) fungicides. *Rev Environ Contam Toxicol* 115:1–37.
 55. Vettorazzi G, Almeida WF, Burin GJ, Jaeger RB, Puga FR, Rahde AF, Reyes FG, Schwartsman S. 1995. International safety assessment of pesticides: Dithiocarbamate pesticides, ETU, and PTU—a review and update. *Teratogen Carcinog Mutagen* 15:313–337.
 56. Denver RJ. 1997. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. *Am Zool* 37:172–184.
 57. Bandyopadhyay U, Biswas K, Banerjee RK. 2002. Extrathyroidal actions of antithyroid thionamides. *Toxicol Lett* 128:117–127.
 58. Bantle JA, Burton DT, Dawson DA, Dumont JN, Finch RA, Fort DJ, Linder G, Rayburn JA, Buchwalter D, Maurice MA, Turley SD. 1994. Initial interlaboratory validation of FETAX: Phase I testing. *J Appl Toxicol* 14:213–233.
 59. Carr JA, Gentles A, Smith EE, Goleman WL, Urquidi LJ, Thuett K, Kendall RJ, Giesy JP, Gross TS, Solomon KR, Van Der Kraak G. 2003. Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environ Toxicol Chem* 22:396–405.
 60. Delgado MJ, Gutiérrez P, Alonso-Bedate M. 1987. Melatonin and photoperiod alter growth and larval development in *Xenopus laevis* tadpoles. *Comp Biochem Physiol A* 86:417–421.