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Application of metamorphosis assay to a native Japanese amphibian species, *Rana rugosa*, for assessing effects of thyroid system affecting chemicals

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ABSTRACT

The aims of this study were to assess the utility of a metamorphosis assay for detecting thyroid hormone-disrupting chemicals using *Rana rugosa*, a domestic frog species in Japan, and to compare species differences in sensitivity to thyroxine (T_4) and propylthiouracil (PTU) among *R. rugosa*, *Xenopus laevis* and *Xenopus* (*Silurana*) *tropicalis*. Tadpoles of *R. rugosa* (TK stages III/IV) were exposed to standard test chemicals for acceleration (T_4) and inhibition (PTU) of metamorphosis for 28 days in semi-static condition and total body length and developmental stage (TK stage) were recorded every week. T_4 (0.61 and 2.24 μg/L in actual concentrations) and PTU (19.73, 76.83, and 155.67 mg/L in actual concentrations) induced significant acceleration and inhibition of metamorphosis, respectively. The present results indicate that the metamorphosis assay is successfully applied to the domestic frog species, *R. rugosa*, suggesting this assay can be used for the assessment of chemicals on ecological impacts in wild frog species.

1. Introduction

A large number of chemicals in the environment have the potential to disrupt endocrine systems in vertebrates (Colborn et al., 1993). Some chemicals have potential to cause adverse effects on reproductive development and function in wildlife by disrupting the endocrine system (Colborn, 1995; Tyler et al., 1998). For the detection and characterization of environmental chemicals with potential thyroid-disrupting activities, the amphibian metamorphosis assay was selected by the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) as an *in vivo* assay (OECD, 2003; Opitz et al., 2005). *Xenopus laevis* has been used as a test animal for the metamorphosis assay for detecting chemicals with agonistic and antagonistic activities of thyroid hormone, since the metamorphosis induced by thyroid hormone has been well characterized in this species (Goleman et al., 2002; Kloas et al., 2002, 2003; Degitz et al., 2005; Opitz et al., 2005; Tata, 2006). A current study demonstrated that the metamorphosis assay was successfully applied to *Xenopus* (*Silurana*) *tropicalis* (Mitsui et al., 2006). *X. tropicalis* offers some advantages over *X. laevis* as a model animal, including diploid vs. tetraploid genome (Amaya et al., 1998; Hirsch et al., 2002; Gilchrist et al., 2004), and its shorter life cycle is likely to be advantageous in chronic ecotoxicity testing, for example, for endocrine disrupting chemicals. Both these species can be used in metamorphosis assays for detecting thyroid hormone disrupters. In addition, the development of a comparable test protocol for domestic species will lend greater support to ecological risk assessment of chemicals.

The wrinkled frog, *Rana rugosa*, is distributed as a domestic species in Japan (Maeda and Matsui, 2003). *R. rugosa* has been frequently used to study sex lampbrush chromosomes, evolution of heterogametic sex, and sex determination (Miura et al., 1996, 1997, 1998; Kato et al., 2004). Effects of thyroid hormone and its antagonist on tail resorption have been reported in *R. rugosa* (Hanada et al., 2003; Kitamura et al., 2005; Goto et al., 2006); however, no common protocol for a metamorphosis assay has been established.

In the present study, *X. laevis* metamorphosis assay (XEMA) protocol (Opitz et al., 2005) was applied to a domestic species in

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Japan, *R. rugosa*, for the first time, to establish detailed protocol of metamorphosis assay for this species.

2. Materials and methods

2.1. Chemicals

Thyroxine (T_4; CAS 51-48-9) and propylthiouracil (PTU; 6-n-propyl-2-thiouracil; CAS 51-52-5) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) at the highest purity available. Stock solutions of T_4 and PTU were prepared in 0.1 M NaOH and 0.7 M NaOH, respectively. All experimental aspects were conducted in compliance with the institutional guidelines for the care and use of animals.

2.2. Preparation of tadpoles

R. rugosa embryos were supplied from the Institute for Amphibian Biology, Hiroshima University. Ovulation was induced by an injection using suspension of crushed pituitaries of *Rana catesbeiana* (bull frog) into the body cavity of *R. rugosa* females. Eggs were collected from females and artificially fertilized with suspension of crushed testes. Embryos were transferred into the test medium consisting of 0.25 g/L commercial salt mixture, Tropic Marin Meersalz (Dr. Biener GmbH, Wartenberg, Germany), pH 7.0 ± 0.5, in deionized aerated water. Embryos were developed at 22, 25, and 28 °C in each experiment.

The normal development of *R. rugosa* tadpoles was staged according to Taylor and Kollros (1946) (TK stage), which is the normal table of *Rana pipiens*. The normal table was compared with the normal table of *X. laevis* established by Nieuwkoop and Faber (1994).

2.3. Experiments

2.3.1. Effect of rearing temperature

Initially, the optimal temperature for rearing tadpoles of *R. rugosa* was investigated. Ten embryos were assigned to each of six test vessels (glass aquaria, $22 \times 25 \times 30$ cm), which were filled with 3.3 L of aerated test medium at three temperatures (22, 25, and 28 °C). Cohorts of tadpoles developed in duplicate tanks per temperature for 7 weeks after fertilization. Tadpoles were fed boiled spinach daily (0.2–0.5 g per tank) and maintained in 12-h light/12-h dark photoperiod. The test medium was changed three times per week (i.e. tadpoles were reared in semi-static conditions). Time to metamorphosis of developing tadpoles was recorded weekly from weeks 3 to 7. Determination of developmental stage was performed under a stereo-microscope according to TK stage.

2.3.2. Metamorphosis assay

A metamorphosis assay using *R. rugosa* was performed based on the *X. laevis* metamorphosis assay (Kloas et al., 2003; Opitz et al., 2005). Tadpoles were reared in test medium at 25 °C during the pre-exposure phase for 3 weeks, and then 10 tadpoles were placed in each test vessel (glass aquaria, $22\,cm \times 25\,cm \times 30\,cm$) containing 3.3 L of test medium at TK stages III/IV, which corresponds to stages 49–51 of *X. laevis* (Nieuwkoop and Faber, 1994). Tadpoles were exposed to 0.25, 1.0, and 4.0 μg/L (0.32, 1.29, and 5.15 nM) T_4 in nominal concentration, as positive controls for thyroid hormone agonistic activity (2 tanks per concentration) and 18.75, 75, and 150 mg/L (0.11, 0.44, and 0.88 mM) PTU in nominal concentration, as positive controls for anti-thyroid hormone activity (2 tanks per concentration), or test medium alone (3 tanks for controls). Tadpoles were exposed for 28 days under semi-static conditions in a 12-h light/12-h dark photoperiod. All tadpoles were fed boiled spinach, and total daily food ratio was increased along with tadpole growth, approximately 0.2–0.5 g/tadpole through the course of the study. Test medium in all tanks was changed completely three times a week. During the test duration, the tadpoles were checked daily for mortality, and developmental (TK) stage and total body length were measured and recorded weekly. A caliper was used to measure the total body length of the tadpole. Determination of developmental stage was performed under a stereo-microscope according to TK stage.

2.4. Histology

Five tadpoles exposed to T_4 and controls were harvested, and were anaesthetized on ice when metamorphosis had been completed (5–7 weeks after exposure). Since PTU-exposed tadpoles did not complete the metamorphosis, tadpoles (five samples) were harvested 12 weeks after exposure. The whole body including thyroid glands was fixed in Neofix (Merck, Darmstadt, Germany), dehydrated and embedded in paraffin. Tadpoles were placed in ventral recumbency and decapitated in a plane perpendicular to the caudal-rostral axis. Five-step sections were taken from each block, and two serial sections of each step are placed. Sections were cut at 6 μm thickness and stained with hematoxylin and eosin. Sections in each sample were acquired from the central portions of the thyroid glands to provide an accurate reflection of thyroid size (OECD, 2007), and thyroid glands were observed pathological changes such as hypertrophy, atrophy, follicular cell hypertrophy, and follicular cell hyperplasia including histological endpoints selected in the OECD amphibian metamorphosis assay (OECD, 2007; Grim et al., 2009). A section of the largest thyroid gland including the middle area of thyroid gland was selected and observed.

2.5. Chemical measurement

Chemical analysis of T_4 and PTU in the test medium was carried out using high-performance liquid chromatography (HPLC)–mass spectrometry (MS). T_4 and PTU concentrations were measured twice. The first sample was taken immediately after chemical exposure to the test medium (fresh water after medium exchange) and the second was taken from the test vessels before test medium exchange to determine the relative quantity of T_4 and PTU remaining (water sample at 2 days after exposure with the test medium containing tadpoles and feed). Water samples were maintained in glass bottles at −30 °C until analysis.

For measurement of actual T_4 concentrations in the test medium, water samples (50–200 mL) were diluted in methanol. Each sample was loaded on PS-2 cartridges (Waters, MA, USA), which were conditioned with methanol and double-distilled water (DDW) in advance. T_4 was extracted from the cartridge with methanol at a flow rate of 10 mL/min, and then the eluted T_4 was reconstituted in 1 mL by methanol purging with N_2. Each aliquot of the sample (5 μL) was injected into HPLC (Agilent 1100, Agilent Technologies, Tokyo, Japan) housing a Mightysil Rp-18 GP (2.0 mm × 150 mm) column (Kanto Chemical Co. Inc., Tokyo, Japan). The oven temperature was 40 °C during the injection. The column was eluted with 0.1% formic acid: methanol gradient programmed from 50% to 70% of methanol for 15 min at a flow rate of 0.2 mL/min, and the post-column reagent is determined at m/z of 778.6 by the API-400 Q-Trap MS system (Applied Biosystems Inc., CA, USA). The recovery ratio of standard T_4 (0.2 μg/L) in this method was 79.5% of nominal concentration.

Water samples (50 mL) from test vessels containing PTU were diluted in methanol. In total 5 μL of each sample was injected into a Develosil C30-UG-5 (2.0 mm × 150 mm) column (Nomura Chemical Co., Ltd., Aichi, Japan). The column was maintained at 40 °C during the injection, and then eluted with 0.1% formic acid: methanol gradient programmed from 0% to 60% of methanol for 12 min at a flow rate of 0.2 mL/min. The post-column reagent was analyzed by the MS system, and then determined at an m/z of 171.1. The recovery ratio of standard PTU (100 μg/L) in this method was 99.7% of nominal concentration.

2.6. Statistical analyses

Statistical analyses were performed according to the XEMA ring-test (Opitz et al., 2005). The normal development of *R. rugosa* tadpoles was staged according to the normal table of *R. pipiens* by Taylor and Kollros (1946) (TK stage), which is indicated by Roman letters. Consequently, these data were converted to Arabic numbers prior to statistical analysis of the developmental-stage parameter. Non-parametric Kruskal–Wallis test followed by Dunn's test was used to determine differences for the developmental stage exhibited between each exposure group and control. Total body length data were analyzed for normal distribution (Kolmogorov and Smirnov test) and homogeneity of variance (Levene test) by each day was recorded. For normally distributed data, Dunnett's test was performed for difference compared from the control group to all exposure groups. For data not meeting criteria of normality and homogeneity of variance, non-parametric Kruskal–Wallis test followed by Dunn's test was used. These statistical analyses were conducted with Stat Light Software (Yukms Co. Ltd., Tokyo, Japan) and SPSS software (SPSS Japan Inc., Tokyo, Japan).

3. Results

3.1. Effects of rearing temperature on R. rugosa development and metamorphosis

The effect of temperature on metamorphosis was assessed in *R. rugosa* to inform our selection of test temperature for the XEMA-type metamorphosis assay. Embryos at all temperatures, 22, 25, and 28 °C, developed successfully for 7 weeks after fertilization. After 3 weeks, tadpoles reared at 25 and 28 °C reached stages III/IV. Between weeks 4 and 7, significant acceleration of development was observed in tadpoles reared at 25 and 28 °C, compared to those at 22 °C (Fig. 1). No tadpole mortalities were observed in the course of the study.