Ecotoxicology and Environmental Safety 86 (2012) 86–92

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Examination of an amphibian metamorphosis assay under an individual-separated exposure system using *Silurana tropicalis* tadpoles

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ARTICLE INFO

Article history:
Received 21 June 2012
Received in revised form
27 August 2012
Accepted 28 August 2012
Available online 12 October 2012

Keywords:
Amphibian
Metamorphosis assay
Silurana tropicalis
Thyroxine
Propylthiouracil
Exposure system

ABSTRACT

We examined the validity of an amphibian (*Silurana tropicalis*) metamorphosis assay (a 28-day semistatic test) under an individual-separated exposure system, where tadpoles were individually held in small glass beakers. We first conducted a comparative rearing experiment for 28 days between this exposure system and the traditional individual-grouped exposure system, both of which held 30 tadpoles (stages 49 and 50) in dechlorinated tap water (a control solution). The former system served to reduce interindividual variability in regard to three morphological measures (developmental stage, hind limb length, and total body length). Under this system, we tested thyroxine (T4, 1 μg/L) and propylthiouracil (PTU, 75 mg/L) for 28 days of exposure. The morphological data collected at 7-day intervals indicated that significant metamorphic acceleration and retardation were consistently induced in the tadpoles exposed to T4 and PTU, respectively. In addition, the thyroid glands of the tadpoles exposed to T4 and PTU clearly exhibited atrophy and hypertrophy accompanied with severe follicular cell hyperplasia, respectively. Our results are in agreement with the historical data generated from previous studies employing the traditional exposure system, thus indicating the validity of our alternative testing protocol.

1. Introduction

Amphibian metamorphosis, which is induced and regulated by thyroid hormones, accompanies drastic transformations in essentially every organ and tissue. Particularly in anurans, the process of morphological alteration caused by metamorphosis (tadpole-to-frog transformation) can be easily monitored by visual inspection. In addition, postembryonic development has been thoroughly studied as a biological model to elucidate thyroid functions (Shi, 2000). By exploiting these advantages, an in vivo assay to detect chemicals disrupting thyroid functions has been designed using *Xenopus laevis*, a worldwide experimental anuran species (Kloas, 2002; Kloas et al., 2003; OECD, 2004; Opitz et al., 2005). The experimental protocol of this assay has been standardized by OECD (2009). Meanwhile, similar metamorphosis assays have also been successfully developed using other anuran species, such as *Silurana tropicalis* (formerly called *Xenopus tropicalis*) (Mitsui et al., 2006) and *Rana rugosa*, an endemic species in Japan (Oka et al., 2009). In particular, *S. tropicalis*, which is phylogenetically related to *X. laevis*, has attracted attention as a new model species, due to its experimental superiority over *X. laevis*: a shorter life cycle and a diploid genome that increase the practicability of use for multi-generational tests and genetic analyses (Song et al., 2003; Fort et al., 2004a, 2004b; Kashiwagi et al., 2010).

However, traditional assays have practical difficulties when conducted by one or two individual researchers due to the large number of tadpoles required for the examination of morphological endpoints. As demonstrated by historical studies (Kloas et al., 2003; Opitz et al., 2005; Mitsui et al., 2006; Oka et al., 2009), tadpoles, even within a control, tend to develop enhancing individual differences in growth and development during exposure periods. This tendency would become more conspicuous under individual-grouped exposure systems where 20–30 tadpoles are held together in a single test tank, because the tadpoles can easily interfere with each other during feeding. An evaluation of results yielded using amphibian metamorphosis assays is based largely on statistical comparisons of morphological data concerning tadpole development. Accordingly, such large within-group variability necessitates the use of a large number of tadpoles for control and chemical treatment groups. In addition, when including replication, the number of tadpoles increases two- to four-fold. Therefore, an exposure system to exclude interactions among tadpoles can serve to minimize interindividual variability of morphological data and thereby allow the number of tadpoles to be reduced. The simplest design fit for this purpose is

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0147-6513/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2012.08.034

an individual-separated exposure system in which each tadpole is held in a single test vessel. This system also enables individual identification without marking of the tadpoles, which is technically difficult (Rice et al., 1998). However, in the individual-separated exposure system, which requires as many test vessels as the total number of test tadpoles, the test vessel must be reduced in size due to the limited volume of the temperature-constant chamber. This exposure system inevitably reduces the amount of swimming space available for each tadpole. For certain anuran species, it has been suggested that tadpole development can be accelerated by gradually reducing aqueous space that may stimulate the release of thyrotropin, a thyroid-stimulating hormone (Denver, 1998; Denver et al., 1998). The anuran metamorphosis assay under the individual-separated exposure system may therefore not truly detect thyroid-disrupting chemicals, unless sufficient volume of test solution is provided for each tadpole during the test.

The current work aimed to establish an amphibian metamorphosis assay practicable by one or two individual researchers, using *S. tropicalis* tadpoles as a test species. We first conducted a comparative rearing experiment between the individual-separated exposure system and the traditional individual-grouped exposure system in order to confirm that the former served to reduce the interindividual variability of the morphological without inducing developmental acceleration that would affect the test results. Subsequently, under the individual-separated exposure system, we tested thyroxine (T4, one of thyroid hormones) and propylthiouracil (PTU, an antithyroid chemical), both of which have been used as test chemicals in traditional metamorphosis assays. By comparing the results with the traditional data, we verified the validity of the amphibian metamorphosis assay under the individual-separated exposure system.

2. Materials and Methods

2.1. Animal husbandry

Adult pairs of *S. tropicalis* were obtained from the Institute of Amphibian Biology of Hiroshima University. The adult frogs were kept in polypropylene aquaria filled with dechlorinated tap water under the following conditions: water depth, 9 cm; water temperature, 25 ± 1 °C; photoperiod, 12-h light/12-h dark; frog density, six frogs per 1800 cm^2 of water surface area; feeding, thrice per week with a commercial diet for aquatic frogs (XL-2, Oriental Yeast, Tokyo, Japan).

After an acclimatization period of 4–6 weeks, breeding was induced by injecting the frogs into the dorsal lymph sac with human chorionic gonadotropin (Wako Pure Chemical Industries, Osaka, Japan): each male received a small primer dose of 20 IU (7 days prior to mating) followed by a final dose of 200 IU (several hours prior to mating), and each female received a single dose of 300 IU (several hours prior to mating). Spawned eggs and developing tadpoles were reared in polypropylene aquaria filled with dechlorinated tap water under the following conditions: water depth, 7 cm; water temperature, 25 ± 1 °C; photoperiod, 12-h light/12-h dark; tadpole density, approximately 200 individuals per 1800 cm^2 of water surface area; feeding, daily with a commercial tadpole food (Sera Micron®, Sera GmbH, Heinsberg, Germany) started on day 5 postfertilization. The amount of daily food was adjusted according to tadpole size so as to avoid water deterioration from excess food. The water was gently aerated during the rearing period. Two weeks after fertilization, tadpoles at stages 49 and 50 (Nieuwkoop and Faber, 1994) with a total body length of approximately 20 mm were used to start each experiment, which employed tadpoles derived from three different frog pairs.

The care and treatment of all frogs and tadpoles, including the survivors after the completion of the experiments, complied with the current laws of Japan and the guidelines presented by the American Society of Ichthyologists and Herpeto logists (ASIH, 2004).

2.2. Test chemicals and preparation of test solutions

Since both T4 and PTU (the highest grade available, Sigma-Aldrich, St. Louis, MO., USA) were hardly soluble in neutral water, these chemicals were first dissolved in 0.7 M NaOH and 1 M NaOH, respectively. These solutions (T4, 3000 mg/L; PTU, 90 g/L) were used as stock solutions. Test solutions (T4, 1 μg/L; PTU, 75 mg/L) were prepared by diluting (3×10^6-fold for T4 and 1200-fold for PTU) the stock solutions with a diluent, as described in Table 1. The pH of the test solution for PTU was adjusted to approximately 7.0 by adding a small amount of 1 M HCl. Adjustment of pH was not done for the test solution of T4 because its pH dropped to nearly 7 due to 3×10^6-fold dilution.

Table 1
The experimental protocol of the metamorphosis assay using *Silurana tropicalis* tadpoles.

Duration	28 days
Temperature	25 °C (with a deviation of ± 1 °C)
Light	12-h light/12-h dark photoperiod using white fluorescent lamps
Diluent	Dechlorinated tap water (hardness, approximately 40 mg/L as $CaCO_3$; pH, 6.5–7.5; dissolved oxygen, 95%–105% saturation at 25 °C; iodine concentration, approximately 4 μg/L)
Test procedure	Semistatic test with solution renewal three times a week (Monday, Wednesday, and Friday)
Group size	Thirty individuals for each group (a diluent control and chemical treatment groups)
Feeding	Daily with 1% (w/v) Sera Micron® solution: 0.5 mL (days 0–6) and 1 mL (days 7–28) per individual
Aeration	None

2.3. Experiments

2.3.1. Assay protocol

The current work consisted of two experiments that were conducted following the protocol shown in Table 1. Although OECD (2009) presented a standard protocol for conducting an amphibian metamorphosis assay under the traditional individual-grouped exposure system, the current experiments employing an individual-separated exposure system involved two major alterations: (1) the prolonged test duration from 21 to 28 days and (2) the use of total body length instead of snout–vent length as one of morphological endpoints. Most of historical data of amphibian metamorphosis assays have been yielded by employing the 28-day exposure duration and measuring total body length to monitor growth of tadpoles (Kloas et al., 2003; Opitz et al., 2005; Mitsui et al., 2006; Oka et al., 2009). Since the validation of an individual-separated exposure system would be based largely on comparisons between the above historical data and those obtained from the current work, the two experiments were also performed during 28 days of exposure, using total body length as an indicator of tadpole growth.

2.3.2. Experiment 1: A comparative rearing experiment between two different exposure systems

A rearing experiment involving no chemical treatment groups was conducted to compare tadpole development between an individual-separated exposure system and an individual-grouped exposure system. In the former system, each of 30 tadpoles was held in a 500-mL (53 $cm^2 \times 11$ cm) glass beaker containing 330 mL of the diluent (i.e., control solution). In the latter system, 30 tadpoles were held together in a 45-L glass aquarium (1600 $cm^2 \times 28$ cm) containing 10 L of the diluent. The two exposure systems were therefore designed to provide the same rearing conditions of tadpole loading (one individual per 330 mL) and water depth (6 cm). On days 0 and 28, each tadpole was inspected alive for developmental stage, hind limb length, and total body length on its ventral image captured using a digital microscope (CCD camera and controller, VH-6300; zoom lens, VH-Z05; Keyence, Osaka, Japan). The developmental stage was determined consulting the normal table of *X. laevis* (Nieuwkoop and Faber, 1994). The hind limb length (from the root of the thigh to the tip of the fourth toe) and total body length (from the tip of the snout to the end of the tail fin) were measured to the nearest 0.1 mm. The length measurement was made as if the objective was stretched straightforward.

2.3.3. Experiment 2: A trial experiment of the metamorphosis assay using T4 and PTU

The metamorphosis assay was conducted under the individual-separated exposure system that held tadpoles individually in a 500-mL glass beaker containing 330 mL of the test solution. In this experiment, T4 (a thyroid system agonist; 1 μg/L) and PTU (an antithyroid substance; 75 mg/L) were tested. Each tadpole was inspected at 7-day intervals for developmental stage, hind limb length, and total body length using the same method as that described in experiment 1. In addition, observations without a microscope were made daily regarding mortality, abnormal behavior, and grossly visible malformations. At the termination of the test, all tadpoles were fixed (see below) and then (2 days after the fixation) weighed to the nearest 1 mg after removing adherent water with a dry paper towel.

2.4. Histological examinations of the thyroid glands

At the termination of experiment 2, all tadpoles were inactivated by gradually cooling down the water temperature to approximately 15 °C, in order to suppress