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Re-evaluation of thyroid hormone signaling antagonism of tetrabromobisphenol A for validating the T3-induced *Xenopus* metamorphosis assay

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ABSTRACT

We developed the T3-induced *Xenopus* metamorphosis assay, which is supposed to be able to sensitively detect thyroid hormone (TH) signaling disruption of chemicals. The present study aimed to validate the T3-induced *Xenopus* metamorphosis assay by re-evaluating the TH signaling antagonism of tetrabromobisphenol A (TBBPA), a known TH signaling disruptor. According to the assay we developed, *Xenopus* tadpoles at stage 52 were exposed to 10–500 nmol/L TBBPA in the presence of 1 nmol/L T3. After 96 hr of exposure, TBBPA in the range of 10–500 nmol/L was found to significantly inhibit T3-induced morphological changes of *Xenopus* tadpoles in a concentration-dependent manner in term of body weight and four morphological endpoints including head area (HA), mouth width (MW), unilateral brain width/brain length (ULBW/BL), and hind-limb length/snout-vent length (HLL/SVL). The results show that these endpoints we developed are sensitive for characterizing the antagonistic effects of TBBPA on T3-induced metamorphosis. Following a 24-hr exposure, we found that TBBPA antagonized expression of T3-induced TH-response genes in the tail, which is consistent with previous findings in the intestine. We propose that the tail can be used as an alternative tissue to the intestine for examining molecular endpoints for evaluating TH signaling disruption. In conclusion, our results demonstrate that the T3-induced *Xenopus* metamorphosis assay we developed is an ideal *in vivo* assay for detecting TH signaling disruption.

Introduction

Thyroid hormones (THs, mainly T3 and T4) have been well-known to play crucial roles in vertebrate growth, development, metabolism, *etc.* (Franco et al., 2013; Kim et al., 2016; Murk et al., 2013). The biologically active T3 principally regulates target gene transcription *via* its nuclear receptors, thyroid hormone receptors (TRs), bound to specific DNA sequences called TH-response elements (TREs) in the absence of ligands (Senese et al., 2014). Some of environmental chemicals such as bisphenol A (BPA), polychlorinated biphenyls (PCBs), and tetrabromobisphenol A (TBBPA), can bind to TRs and disrupt the TH signaling pathway because of their similarity in chemical structures to TH (Porterfield, 2000; Zhang et al., 2014;

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Zoeller, 2007). Based on TR binding and transcriptional activities of chemicals, some *in vitro* assays for screening TH signaling agonists or antagonists have been developed and used (Murk et al., 2013). These *in vitro* assays have advantages including speediness, simplicity, and the ability to demonstrate a direct interaction of a chemical with TR. However, they cannot completely evaluate TH signaling disruption of chemicals *in vivo* and consequential effects on vertebrate development. Therefore, *in vivo* bioassays are in need for assessing TH-signaling disruption of chemicals.

Amphibian metamorphosis, controlled by THs, which are regulated by the hypothalamic–pituitary-thyroid (HPT) axis, is believed to be a good model for studying thyroid disruption of chemicals (Degitz et al., 2005). Based on spontaneous metamorphosis of *Xenopus laevis*, OECD (2009) has established the Amphibian Metamorphosis Assay (AMA), which intended to empirically identify substances that may interfere with the normal function of the HPT axis. In addition, it is well-known that premetamorphic tadpoles can be induced by TH to metamorphose precociously, which is mainly mediated by the TH signaling pathway (Tata, 2006). Therefore, several laboratories have begun to use T3-induced amphibian metamorphosis as a model to study TH signaling disruption (Fini et al., 2012; Kitamura et al., 2005; Zhang et al., 2014). Based on this model, we recently developed an assay called the T3-induced *Xenopus* metamorphosis to detect TH signaling disruption of chemicals by choosing appropriate concentrations and exposure durations for T3 induction and establishing quantitatively morphological endpoints (Yao et al., accepted for publication). In the assay, we can determine whether a chemical has potential to disrupt TH signaling based on its effects on T3-induced *Xenopus* metamorphosis. In detail, *Xenopus* tadpoles at stage 52 are exposed to a test chemical in the presence and absence of approximately 1.25 nmol/L T3, which effectively induces metamorphosis. After 48 hr of exposure, the intestine is sampled for examining its morphology and TH-response gene transcription, and the latter is believed to be not only necessary but also sufficient for metamorphosis. After 96 hr of exposure, body weight and morphological endpoints including head area (HA), mouth width (MW), unilateral brain width/brain length (ULBW/BL), and hind-limb length/snout-vent length (HLL/SVL) are measured. Based on all the endpoints, if the chemical inhibits T3-induced metamorphic changes at the both transcriptional and morphological levels, the chemical will be proposed as a TH signaling antagonist. Conversely, it will be proposed as a TH signaling agonist. This simple assay is supposed to be able to effectively detect TH signaling disruption of chemicals. The present study aimed to validate the T3-induced *Xenopus* metamorphosis assay we developed using a known TH signaling disruptor.

Tetrabromobisphenol A (TBBPA) is one of the most widely used brominated flame retardants, and has been found in various environmental and biotic samples (Liu et al., 2016; Covaci et al., 2009; Qu et al., 2015). Because TBBPA has a similar chemical structure to THs, many researchers investigated its TH signaling disruption (Fini et al., 2012; Levy-Bimbot et al., 2012; Zhang et al., 2014, 2015). Hamers et al. (2006) reported that TBBPA inhibited binding of T3 and T4 to TR, with a stronger binding potency to TR than T4. Hofmann et al. (2009) observed agonist and antagonist activities of TBBPA (10^{-6} to 10^{-5} mol/L) in a transactivation assay system using mammalian cells (HepG2) and human TRs. Kitamura et al. (2005) demonstrated that TBBPA had an inhibitory effect on T3-induced tail shortening in *Rana rugosa* tadpoles, suggesting an anti-thyroidal activity of TBBPA. Recently, we found that in *X. laevis* tadpoles, TBBPA exhibited an antagonistic effect on TH actions in the presence of high TH levels, but an agonistic activity in the presence of low TH levels (Zhang et al., 2014). Based on enough evidence, TBBPA has been regarded as a typical TH signaling disruptor. In the present study, therefore, we chose TBBPA as a test chemical to validate the T3-induced *Xenopus* metamorphosis assay we developed.

The intestine of premetamorphic *X. laevis* is a sensitive organ to TH, which can up-regulate TH-response gene transcription and result in a dramatic remodeling of this organ (Heimeier et al., 2009; Zhang et al., 2014). In the T3-induced *Xenopus* metamorphosis we developed, the intestine was used to detect TH-response gene transcription. However, the intestines of stage 52 *Xenopus* really become too small following exposure to T3. There are some difficulties in sampling the small intestine and isolating enough RNA from single small intestine using the Automatic Nucleic Acid Extraction Apparatus, which needs bigger samples. Compared with the small intestine, the tail has a big size and is easy to be sampled. Also, the tail is a tissue commonly used to study T3-induced metamorphosis (Das et al., 2006; Searcy et al., 2012; Shi et al., 2001; Tamura et al., 2015; Veldhoen et al., 2002). In the present study, thus, we also aimed to address whether the tail can be used as an alternative tissue to the intestine for examining molecular endpoints in the T3-induced *Xenopus* metamorphosis assay.

1. Materials and methods

1.1. Chemicals

The chemicals used were obtained from the following companies: Tetrabromobisphenol A (2,2-bis-(3,5-dibromo-4-hydroxyphenyl) propane; TBBPA, 97%) and T3 (3,3′,5-Triiodo-L-thyronine, 95%) from Geel Belgium (New Jersey, USA), dimethyl sulfoxide (DMSO, 99.5%) and 3-aminobenzoic acid ethylester (MS-222, 98%) from Sigma-Aldrich (St. Louis, MO, USA), HCG (Human chorionic gonadotropin) from Yantai North Pharmaceutical Co. Ltd. (Shandong, China). RNase-free water, Quantscript RT Kit, Real Master Mix (SYBR Green) Kit were obtained from Tiangen (Beijing, China). Picric acid, formaldehyde, acetic acid, chloroform and ethanol were taken from Beijing Chemical Reagent Co., Ltd. (Pure analysis, Beijing, China). G-Red (Nucleic acid dye) was purchased from Bio Teke (Beijing, China). PCR primers were synthesized by Sangon Biotech (Beijing, China). The DMSO concentration in solvent control was 0.001%. The stock solutions of TBBPA (54.4 g/L) and T3 (7.10 mmol/L) were prepared by being dissolved in DMSO and distilled water, respectively, and then were sub-packaged and stored at −20°C.

1.2. Animals

X. laevis frogs, the offspring of adult frogs from Nasco (USA), were raised in charcoal-filtered tap water in our amphibian

house with a 12 hr light/12 hr dark cycle. Housing and breeding conditions were described in our previous study (Lou et al., 2013). On the fifth day post-fertilization, tadpoles were transferred into a flow through system (Esen, Beijing, China). Tadpoles were raised to stage 52 for exposure experiment.

1.3. Exposure to TBBPA in the presence of T3

According to the T3-induced *Xenopus* metamorphosis we developed, stage 52 tadpoles were exposed to a series of concentrations of TBBPA (10, 100, 500 nmol/L) in the presence of 1 nmol/L T3. Because our previous study has demonstrated that effects of single exposure to TBBPA within a short-term on stage 52 tadpoles are weak, we did not repeat single exposure to TBBPA in the present study. Three replicate test tanks (6 tadpoles per tank) were employed for each treatment group. The test water and chemical replacements were performed every 24 hr. Exposure was conducted at (22 ± 1)°C under a 12-hr light/12-hr dark cycle. The exposure experiment was repeated three times using tadpoles from different sets of adults.

1.4. Collection of samples

After 24 hr of exposure, three tadpoles from each tank were randomly selected and anesthetized in 100 mg/L MS-222, and then *Xenopus* tails from each treatment were immersed in RNA extraction kit separately for RNA extraction and subsequent transcription analysis. The other tadpoles in each tank were continuously exposed for measurement of body weight and morphology after 96 hr of exposure.

1.5. RNA extraction and RT

Total RNA was isolated from the *Xenopus* tails by Automatic Nucleic Acid Extraction Apparatus (Bio Teke, Beijing, China) according to the manufacturer's instructions. RNA quality was verified by electrophoresis (DYCP-31F, Six One Instrument Factory, Beijing, China) and by $A_{260\ nm}/A_{280\ nm}$ ratio (Nanodrop ND-1000, Nano-Drop, Wilmington, USA) ranging from 1.8 to 2.0. The first-strand cDNA was synthesized from 1 μg total RNA using the Fast Quant RT Kit from Tiangen (Beijing, China) following the manufacturer's instructions. Then the cDNA was stored at –20°C until further analysis.

1.6. TH-response gene transcription analysis by qPCR

According to the T3-induced *Xenopus* metamorphosis, we needed to examine the gene expressions of TH receptor beta (TRβ), TH-responsive basic leucine zipper transcription factor (TH/bZIP), amidohydrolase domain containing 1 (AMDHD1), stromelysin-3 (ST3), kruppel-like factor 9 (KLF9), sonic hedgehog (SHH), collagenase 3 (MMP13) and matrix metalloproteinase 2 (MMP2) (Yao et al., accepted for publication). In our pre-experiment, however, we found the transcriptional levels of AMDHD1, SHH, MMP13 and MMP2 were not well up-regulated by T3 in the *Xenopus* tadpole tail, whereas T3 up-regulated transcription of CCAAT/enhancer binding protein delta (CEBPD) and deiodinase III (DIO3), two important TH-response genes (Das et al., 2006; Fini et al., 2012). Therefore, we investigated expression of TRβ, TH-bzip, KLF9, ST3, DIO3 and CEBPD in the *X. laevis* tadpole tail in this study.

We conducted qPCR using SYBR Green I with the MX Real-time Polymerase Chain Reaction system (Light Cycler 480, Roche, Switzerland) for gene expression analysis. Ribosomal protein L8 (rpl8) was used as a reference gene to normalize mRNA expression. Specific primers and PCR conditions for these genes were shown in Table 1. PCR conditions were as follows: 95°C for 15 min, 40 cycles at 95°C for 10 sec, annealing at different temperatures for 20 sec, and 72°C for 20 sec. Following the manufacturer's instructions, melting curves were performed to determine the specific amplification of these genes (Fig. S1). Additionally, we determined the specificity of these primers by sequencing PCR products.

1.7. Analysis for gross morphology

After taking photos of the tadpoles following 96-hr exposure, we used the Image Processing Software of Chong Optec

Table 1 – Primers used for Real-time Quantitative polymerase chain reaction (RT-qPCR) and related information.

Gene	Primer sequences (5′–3′)	Annealing temperature (°C)	GeneBank ID
rpl8	F: CCGTGGTGTGGCTATGAATC R: TACGACGAGCAGCAATAAGAC	58	NM_001086996.1
TRβ	F: AGGACTCGGTGTGGTCTCAGA R: GATGACATAAGCAGCACAGCCT	58	NM_001087781.1
ST3	F: CCTCTGTCATACACTTACCTT R: TGAACCGTGAGCATTGAG	62	NM_001086342.1
KLF9	F: GTGGCCACTTGATTTCCCCT R: AAAGACACAAAACAGCGGCG	64	NM_001085597.1
TH/bZIP	F: CCACCTCCACAGAATCAGCAG R: AGAAGTGTTCCGACAGCCAAG	62	NM_001085805.1
CEBPD	F: AACATCGCCGTGAGGAAGAG R: TCAAGTCCCTGGTGAGAAGTTC	62	NM_001089609.1
DIO3	F: GATGCTGTGGCTGCTGGAT R: ATTCGGTTGGAGTCGGACAC	62	NM_001087863.1

F: forward; R: reverse; rpl8: ribosomal protein L8; TRβ: thyroid hormone receptor beta; ST3: stromelysin-3; KLF9: kruppel-like factor 9; TH/bZIP: TH-responsive basic leucine zipper transcription factor; CEBPD: CCAAT/enhancer binding protein (C/EBP), delta; DIO3: deiodinase iodothyronine type 3.

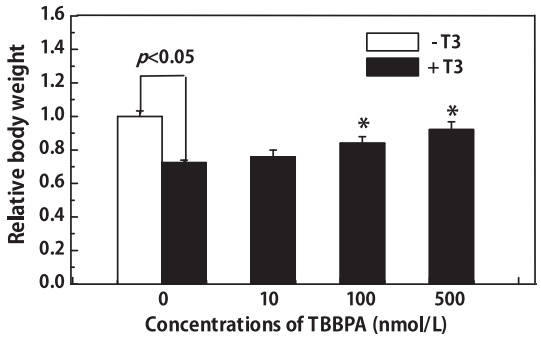

Fig. 1 – Relative body weight of stage 52 *Xenopus laevis* tadpoles following 96-hr exposure to a series of concentrations of TBBPA in the presence of 1 nmol/L T3. Each data was from 9 tadpoles from three replicate tanks for each treatment. Body weight of each tadpole was normalized by the mean body weight of the solvent control tadpoles at 96 hr. Data are shown as mean ± SEM. * indicate significant differences between TBBPA + T3 treatment and T3 treatment ($p < 0.05$). All experiments were repeated three times using tadpoles from different sets of adults with similar results. We show the data from one independent experiment.

Instrument Co. to analyze gross morphology of tadpoles. We measured HA, MW, ULBW/BL and HLL/SVL as gross morphology endpoints. Each parameter was normalized by the mean value for the solvent control tadpoles.

1.8. Data analysis

The fold changes of relative gene expression data was determined by the $2^{-\Delta\Delta Ct}$ methods. Quantitative data was shown as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS software version 16.0 (SPSS, USA). We use one-way analysis of variance (ANOVA) to test significant differences among treatment groups. Statistical significance was defined as $p < 0.05$.

2. Results

2.1. TBBPA inhibited T3-induced changes in body weight and gross morphology

Following the 96-hr exposure, T3 significantly resulted in a 1.5-fold decrease in the body weight compared to the solvent control, as shown in Fig. 1. The two higher concentrations (100, 500 nmol/L) of TBBPA significantly inhibited T3-induced declines in body weight in a concentration-dependent manner. Tadpoles exposed to 10 nmol/L TBBPA in the presence of T3 had slightly higher body weight than the T3 treatment group, despite the lack of statistical significance. The data show that TBBPA in the range of 10–500 nmol/L exhibited inhibitory effects on T3-induced decreases in body weight in a concentration-dependent manner.

Following the 96-hr exposure to 1 nmol/L T3, *X. laevis* tadpoles exhibited remarkable morphological changes, including head shrinkage, mouth sharpening, brain remodeling, and limb growth (Fig. 2). We used HA, MW, ULBW/BL, HLL/SVL to characterize head shrinkage, mouth sharpening, brain remodeling, and hindlimb growth, respectively. As shown in Fig. 3, in term of all the morphological parameters, 1 nmol/L T3 alone significantly induced metamorphic changes compared with the solvent control. In detail, T3 resulted in an about 2-fold decrease of HA and MW, and a 1.5-fold increase of ULBW/BL and HLL/SVL, compared with the solvent control. Moreover, compared with the T3 treatment group, TBBPA in the range of 10–500 nmol/L sharply improved the values of HA and MW and reduced the values of ULBW/BL and HLL/SVL in a concentration-dependent manner. The above results show that TBBPA in the range of 10–500 nmol/L displayed significant inhibitory effects on T3-induced metamorphosis at the morphological level in a concentration-dependent manner.

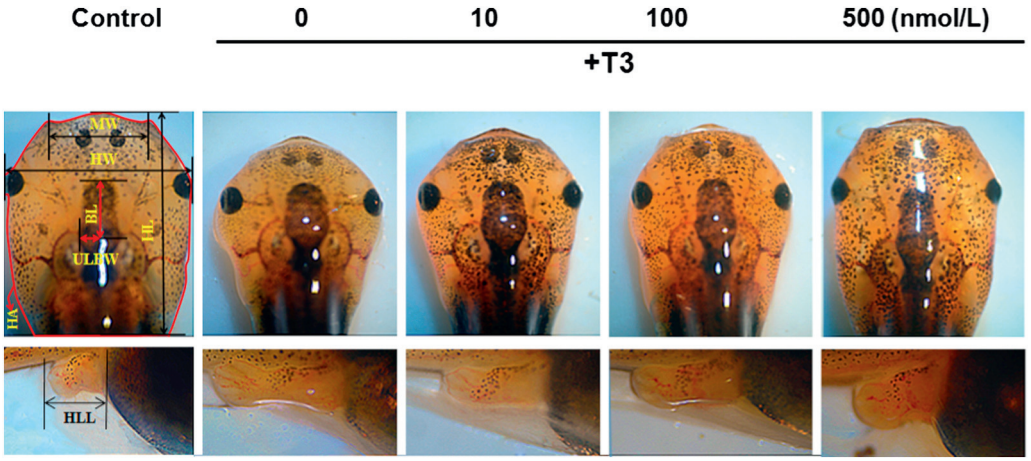

Fig. 2 – Grossly morphological changes of stage 52 *Xenopus laevis* tadpoles following 96-hr exposure to tetrabromobisphenol A (TBBPA) in the presence of 1 nmol/L T3. Nine tadpoles from three replicate tanks were observed for each treatment each day. TBBPA significantly inhibited metamorphosis compared with T3 treatment in a concentration-dependent manner. The experiment was repeated three times using tadpoles from different sets of adults with similar results, and the results from one independent experiment are shown here.

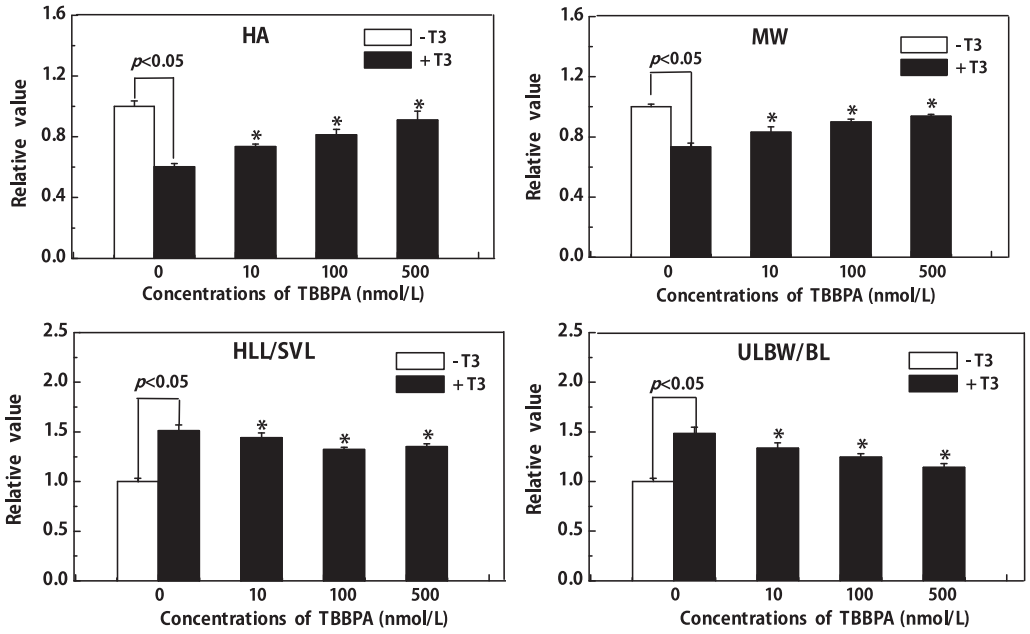

Fig. 3 – Morphologic changes of stage 52 *Xenopus laevis* tadpoles following 96-hr exposure to a series of concentrations of TBBPA in the presence of 1 nmol/L T3. Each data was from nine tadpoles from three replicate tanks for each treatment. Each parameter was normalized by the mean value of the solvent control tadpoles at 96 hr. Data are shown as mean ± SEM. * indicates significant differences between TBBPA + T3 treatment and T3 treatment ($p < 0.05$). The experiment was repeated three times using tadpoles from different sets of adults with similar results, and we show the data of 45 tadpoles in each treatment group from one independent experiment. HA: head area; MW: mouth width; ULBW: unilateral brain width/brain length; BL: brain length; HLL: hind-limb length; SVL: snout-vent length.

2.2. TBBPA affected T3-induced transcription of TH-response genes in Xenopus tails

As expected, T3 dramatically up-regulated transcriptional levels of TH-response genes of TRβ, ST3, KLF9, TH-bzip, CEBPD and DIO3 in tails after 24-hr exposure (Fig. 4). The peak levels of TRβ, ST3, KLF9, TH-bzip, CEBPD and DIO3 expressions in the T3 alone treatment group were about 2-, 1.5-, 6-, 18-, 2.5- and 1.3-folds the levels in the solvent control group, respectively. In the range of 100–500 nmol/L, TBBPA significantly reduced T3-induced up-regulation of TRβ, ST3, KLF9, CEBPD and DIO3 expression compared with the T3 treatment group. The lowest concentration (10 nmol/L) of TBBPA treatment dramatically decreased T3-induced expression of DIO3 with the lack of statistical significant changes of other genes tested. Specially, TBBPA decreased T3-induced KLF9 in a concentration-dependent manner in the range of 10–500 nmol/L.

3. Discussion

T3-induced amphibian metamorphosis is believed to be an ideal model to study TH signaling disruption of chemicals (Crump et al., 2002; Kitamura et al., 2005; Oka et al., 2009; Fini et al., 2012; Zhang et al., 2014). In a recent study (Yao et al., accepted for publication), we developed the T3-induced *Xenopus* metamorphosis assay, in which the endpoints included body weight and quantitative morphological parameters after 96 hr of exposure, and TH-response gene transcriptional levels and morphology of the intestine after 48 hr of exposure. In the present study, to validate the T3-induced *Xenopus* metamorphosis assay, we re-evaluated the TH signaling antagonism of TBBPA, a well-known TH signaling antagonist.

Expectedly, we found that 100–500 nmol/L TBBPA significantly inhibited T3-induced decreases in body weight in a concentration-dependent manner, and even 10 nmol/L also exhibited a slight inhibitory effect on T3 induction, despite no significant difference from T3 treatment. The data show that body weight can evaluate TH signaling antagonism of TBBPA, demonstrating that it is a sensitive and simple endpoint in the T3-induced metamorphosis assay. Because T3 induces dramatic morphological changes, such as head shrinkage, gill resportion, mouth sharpening, brain remodeling, and hindlimb growth, several studies qualitatively described the morphological changes for evaluating effects of a test chemical on T3-induced metamorphosis (Heimeier et al., 2009; Zhang et al., 2014). For example, Heimeier et al. (2009) presented gross morphology of tadpoles following exposure to BPA in the presence and absence of T3 to evaluate inhibitory effects of BPA on T3-induced *Xenopus* metamorphosis. We characterized the effects of TBBPA on T3-induced *Xenopus* metamorphosis by comparing morphological features of lower jaws, gills and limbs, and found the antagonism of TBBPA at 10 nmol/L following a 6-day exposure (Zhang et al., 2014). In the present study, we measured HA, MW, ULBW/BL, and HLL/SVL of tadpoles following 96-hr exposure to TBBPA in the presence of 1 nmol/L T3, according to the T3-induced *Xenopus* metamorphosis

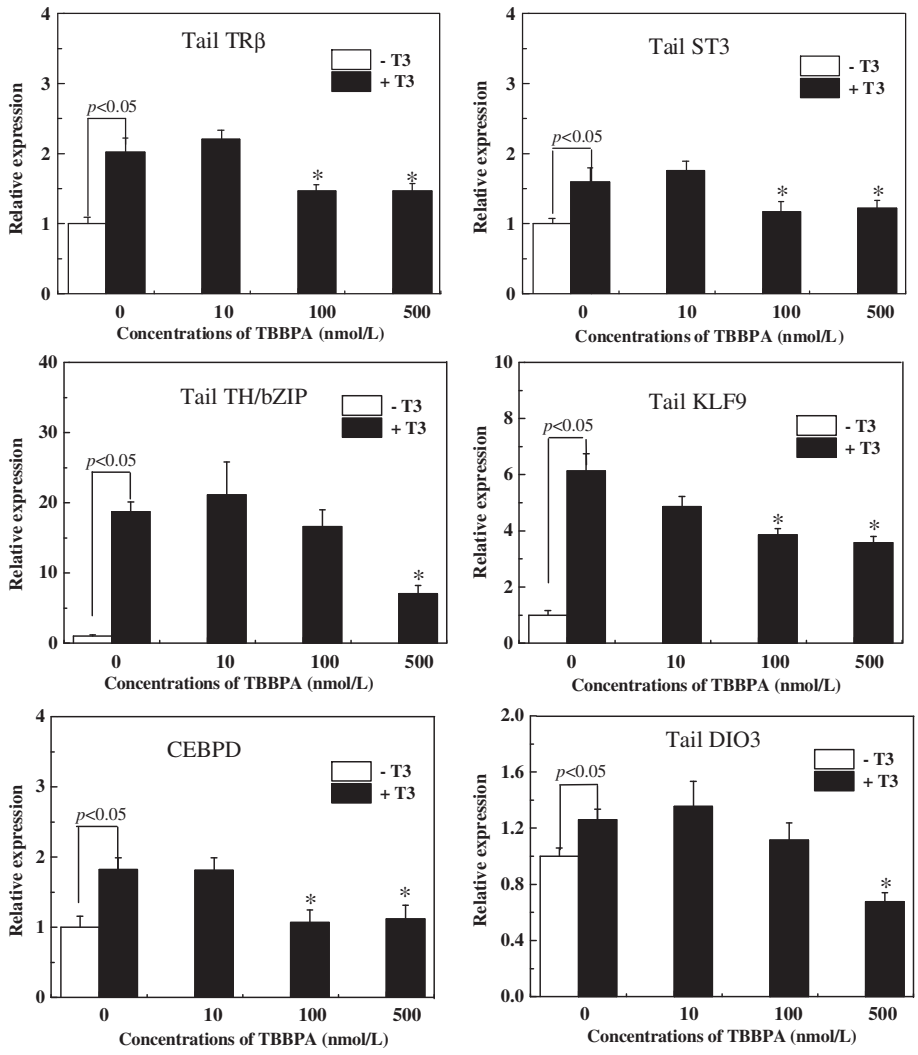

Fig. 4 – Relative expression of six thyroid hormone-response genes in the tails of stage 52 *Xenopus laevis* tadpoles following 24-hr exposure to a series of concentrations of tetrabromobisphenol A (TBBPA) in the presence of 1 nmol/LT3. Each data was from nine tadpoles from three replicate tanks for each treatment. Each data was normalized by the mean value of the solvent control tadpoles on day 1. Ribosomal protein L8 (rpl8) is used as a reference gene. Data are shown as mean ± SEM. * indicates significant differences between TBBPA + T3 treatment and T3 treatment ($p < 0.05$). The experiment was repeated three times using tadpoles from different sets of adults. The results were consistent among the three independent experiments. TRβ: thyroid hormone receptor beta; ST3: stromelysin-3; KLF9: kruppel-like factor 9; TH/bZIP: TH-responsive basic leucine zipper transcription factor; CEBPD: CCAAT/enhancer-binding protein delta; DIO3: type 3 iodothyronine deiodinase.

assay we developed. Nicely, TBBPA in the concentration range of 10–500 nmol/L significantly antagonized T3-induced changes of the four morphological endpoints in a concentration-dependent manner. The results strongly demonstrate that HA, MW, ULBW/BL, and HLL/SVL are ideal quantitative endpoints for detecting TH signaling disruption. In a previous study, we evaluated the inhibitory effects of 10 nmol/L TBBPA by qualitative description of tadpole morphology after 6 days of exposure (Zhang et al., 2014). In the present study, however, it is after 96 hr of exposure that we demonstrated the antagonistic effects of TBBPA using quantitative morphological endpoints we developed. The data show that the T3-induced *Xenopus* metamorphosis assay we developed is more rapid. Of particular note is 10 nmol/L as the lowest observed effective concentrations of TBBPA in the present study. Liu et al. (2016) reported the concentrations of TBBPA reached 1.5–9 nmol/L in water samples from an industry concentration site (Chouhu Lake, Anhui), meaning that 10 nmol/L is environmentally relevant. In other words, we have detected the antagonism of TBBPA at environmentally relevant concentration using the T3-induced *Xenopus* metamorphosis assay we developed, showing a high sensitivity of this *in vivo* assay.

Morphological endpoints in the T3-indcued *Xenopus* metamorphosis assay are simple and visual, but they only provide indirect evidence that a test chemical could disrupt TH signaling and result in TH-regulated biological changes. TH-response gene transcription regulated by TH is believed to be the first step for amphibian metamorphosis, although series of molecular cascade events involved in the developmental process are unclear (Shi et al., 2001). Therefore, TH-response gene

transcription is regarded as endpoints to be able to directly indicate the potential to disrupt TH signaling. In the T3-indcued *Xenopus* metamorphosis assay we developed, the intestine is proposed as the organ for detecting molecular endpoints (Yao et al., accepted for publication). In our previous study, TBBPA in the range of 100–500 nmol/L was found to concentration-dependently inhibit T3-induced up-regulation of TRβ, BTEB (KLF9), ST3, MMP2, and DIO2 transcription in *X. laevis* intestines after a 2-day exposure, with weak effects of 10 nmol/L TBBPA on T3 induction (Zhang et al., 2014). In the present study, similar results concerning molecular endpoints were observed in the *X. laevis* tail after 24 hr of exposure. Specifically, T3 up-regulated expression of TH-response genes (TRβ, TH-bzip, KLF9, DIO3, ST3, and CEBPD) in the tail. However, 100–500 nmol/L TBBPA inhibited the T3-induced transcriptional up-regulation of these genes, whereas the lowest concentration (10 nmol/L) of TBBPA treatment had a dramatically suppressive effect on the T3-induced expression of DIO3 with no significant changes of other tested genes. The results strongly show that the *Xenopus* tail is a suitable tissue for detecting TH-response gene expression in the T3-induced *Xenopus* metamorphosis assay. Moreover, the tail is easier to be sampled compared with the intestine of *Xenopus*.

Taken together, we demonstrate that 10–500 nmol/L TBBPA significantly inhibited T3-induced morphological changes of *Xenopus* tadpoles following a 96-hr exposure in a concentration-dependent manner, using body weight and four morphological endpoints (HA, MW, ULBW/BL, and HLL/SVL). The results show these endpoints are sensitive for detecting TH signaling disruption. The antagonistic effects of TBBPA on T3-induced TH-response gene expression in the tail were also observed following a 24-hr exposure, showing that the tail is appropriate as an alternative tissue to the intestine for examining molecular endpoints in the T3-induced *Xenopus* metamorphosis assay. In conclusion, our study demonstrates the T3-induced *Xenopus* metamorphosis assay we developed is an ideal *in vivo* assay for detecting TH signaling disruption. It is supposed to become a standard assay, like the AMA of OECD, for detecting TH signaling disrupting activity of chemicals, and further studies are needed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.09.021.

REFERENCES

Covaci, A., Voorspoels, S., Abdallah, M.A., Geens, T., Harrad, S., Law, R.J., 2009. Analytical and environmental aspects of the flame retardant tetrabromobisphenol-A and its derivatives. J. Chromatogr. A 1216 (3), 46–63.

Crump, D., Werry, K., Veldhoen, N., Van Aggelen, G., Helbing, C.C., 2002. Exposure to the herbicide acetochlor alters thyroid hormone-dependent gene expression and metamorphosis in *Xenopus laevis*. Environ. Health Perspect. 110 (12), 1199–1205.

Das, B., Cai, L., Carter, M.G., Piao, Y.L., Sharov, A.A., Ko, M.S.H., et al., 2006. Gene expression changes at metamorphosis induced by thyroid hormone in *Xenopus laevis* tadpoles. Dev. Biol. 291 (2), 42–55.

Degitz, S.J., Holcombe, G.W., Flynn, K.M., Kosian, P.A., Korte, J.J., Tietge, J.E., 2005. Progress towards development of an amphibian-based thyroid screening assay using *Xenopus laevis*. Organismal and thyroidal responses to the model compounds 6-propylthiouracil, methimazole, and thyroxine. Toxicol. Sci. 87 (2), 353–364.

Fini, J.B., Le Mevel, S., Palmier, K., Darras, V.M., Punzon, I., Richardson, S.J., et al., 2012. Thyroid hormone signaling in the *Xenopus laevis* embryo is functional and susceptible to endocrine disruption. Endocrinology 153 (10), 5068–5081.

Franco, B., Laura, F., Sara, N., Salvatore, G., 2013. Thyroid function in small for gestational age newborns: a review. J. Clin. Res. Pediatr. Endocrinol. 5 (Suppl. 1), 2–7.

Hamers, T., Kamstra, J.H., Sonneveld, E., Murk, A.J., Kester, M.H., Andersson, P.L., et al., 2006. *In vitro* profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol. Sci. 92 (1), 157–173.

Heimeier, R.A., Das, B., Buchholz, D.R., Shi, Y.B., 2009. The xenoestrogen bisphenol A inhibits postembryonic vertebrate development by antagonizing gene regulation by thyroid hormone. Endocrinology 150 (6), 2964–2973.

Kim, S., Sohn, J.H., Ha, S.Y., Kang, H., Yim, U.H., Shim, W.J., et al., 2016. Thyroid hormone disruption by water-accommodated fractions of crude oil and sediments affected by the Hebei Spirit oil spill in zebrafish and GH3 cells. Environ. Sci. Technol. 50 (11), 5972–5980.

Kitamura, S., Kato, T., Iida, M., Jinno, N., Suzuki, T., Ohta, S., et al., 2005. Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis. Life Sci. 76 (14), 1589–1601.

Levy-Bimbot, M., Major, G., Courilleau, D., Blondeau, J.P., Lévi, Y., 2012. Tetrabromobisphenol-A disrupts thyroid hormone receptor alpha function *in vitro*: Use of fluorescence polarization to assay corepressor and coactivator peptide binding. Chemosphere 87 (7), 782–788.

Liu, K., Li, J., Yan, S., Zhang, W., Li, Y., Han, D., 2016. A review of status of tetrabromobisphenol A (TBBPA) in China. Chemosphere 148, 8–20.

Lou, Q.Q., Zhang, Y.F., Zhou, Z., Shi, Y.L., Ge, Y.N., Ren, D.K., et al., 2013. Effects of perfluorooctanesulfonate and perfluorobutanesulfonate on the growth and sexual development of *Xenopus laevis*. Ecotoxicology 22 (7), 1133–1144.

Murk, A.J., Rijntjes, E., Blaauboer, B.J., Clewell, R., Crofton, K.M., Dingemans, M.M., et al., 2013. Mechanism-based testing strategy using *in vitro* approaches for identification of thyroid hormone disrupting chemicals. Toxicol. in Vitro 27 (4), 1320–1346.

OECD, 2009. Test No. 231: Amphibian Metamorphosis Assay. OECD Publishing, Paris. http://dx.doi.org/10.1787/9789264076242-en.

Oka, T., Miyahara, M., Yamamoto, J., Mitsui, N., Fujii, T., Tooi, O., et al., 2009. Application of metamorphosis assay to a native Japanese amphibian species, *Rana rugosa*, for assessing effects of thyroid system affecting chemicals. Ecotoxicol. Environ. Saf. 72 (5), 1400–1405.

Porterfield, S.P., 2000. Thyroidal dysfunction and environmental chemicals—potential impact on brain development. Environ. Health Perspect. 108 (Suppl. 3), 433–438.

Qu, R., Feng, M., Wang, X., Huang, Q., Lu, J., Wang, L., et al., 2015. Rapid removal of tetrabromobisphenol A by ozonation in water: Oxidation products, reaction pathways and toxicity assessment. PLoS One 10 (10), 1–17.

Hofmann, P.J., Schomburg, L., Kohrle, J., 2009. Interference of endocrine disrupters with thyroid hormone receptor-dependent transactivation. Toxicol. Sci. 110 (1), 125–137.

Searcy, B.T., Beckstrom-Sternberg, S.M., Beckstrom-Sternberg, J.S., Stafford, P., Schwendiman, A.L., Soto-Pena, J., et al., 2012. Thyroid hormone-dependent development in *Xenopus laevis*: a sensitive screen of thyroid hormone signaling disruption by municipal wastewater treatment plant effluent. Gen. Comp. Endocrinol. 176 (3), 481–492.

Senese, R., Cioffi, F., De, L.P., Goglia, F., Lanni, A., 2014. Thyroid: biological actions of 'nonclassical' thyroid hormones. J. Endocrinol. 221 (2), 1–12.

Shi, Y.B., Fu, L., Hsia, S.C., Tomita, A., Buchholz, D., 2001. Thyroid hormone regulation of apoptotic tissue remodeling during anuran metamorphosis. Cell Res. 11 (4), 245–252.

Tamura, K., Takayama, S., Ishii, T., Mawaribuchi, S., Takamatsu, N., Ito, M., 2015. Apoptosis and differentiation of *Xenopus* tail-derived myoblasts by thyroid hormone. J. Mol. Endocrinol. 54 (3), 185–192.

Tata, J.R., 2006. Amphibian metamorphosis as a model for the developmental actions of thyroid hormone. Mol. Cell. Endocrinol. 246 (1–2), 10–20.

Veldhoen, N., Crump, D., Werry, K., Helbing, C.C., 2002. Distinctive gene profiles occur at key points during natural metamorphosis in the *Xenopus laevis* tadpole tail. Dev. Dyn. 225 (4), 457–468.

Yao, X.F., Chen, X.Y., Zhang, Y.F., Li, Y.Y., Wang, Y., Zheng, Z.M., et al., 2016. Optimization of the T3-induced *Xenopus* metamorphosis assay for detecting thyroid hormone signaling disruption of chemicals. J. Environ. Sci. 52, 314–324.

Zhang, Y.F., Xu, W., Lou, Q.Q., Li, Y.Y., Zhao, Y.X., Wei, W.J., et al., 2014. Tetrabromobisphenol A disrupts vertebrate development via thyroid hormone signaling pathway in a developmental stage-dependent manner. Environ. Sci. Technol. 48 (14), 8227–8234.

Zhang, Y.F., Li, Y.Y., Qin, Z.F., Wang, H., Li, J., 2015. A screening assay for thyroid hormone signaling disruption based on thyroid hormone-response gene expression analysis in the frog *Pelophylax nigromaculatus*. J. Environ. Sci. 34, 143–154.

Zoeller, R.T., 2007. Environmental chemicals impacting the thyroid: Targets and consequences. Thyroid 17 (9), 811–817.